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Investigating mortality risk in hospitalised patients in Africa with
HIV-associated tuberculosis and positive urine diagnostics: a
clinical, epidemiological and immunological study

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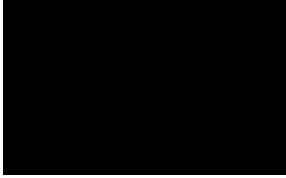
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Declaration

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



Ankur Gupta-Wright

25th March 2019

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Abstract

HIV-associated tuberculosis (HIV/TB) was responsible for an estimated 374,000 deaths globally in 2016. Much of this burden resides in hospitals in sub-Saharan Africa, where HIV/TB is usually disseminated and is the major cause of admission and death. Recently, urine-based detection of mycobacterial lipoarabinomannan (LAM) or nucleic acids (using the Xpert MTB/RIF assay) has improved diagnosis of HIV/TB in this population, with the potential to improve outcomes. However, disseminated 'urine-positive' TB may also be associated with a higher mortality risk and impaired immune responses compared to patients with HIV/TB disease who are urine TB test negative. This thesis addresses the hypothesis that positive urine-diagnostics in inpatients with HIV/TB disease are associated with mortality, can identify patients with impairment of immune responses, and can be used as useful prognostic markers for identifying patients with poor outcomes.

First, a systematic review and meta-analysis was undertaken to synthesise existing evidence of the association between urine-LAM detection and mortality risk in HIV/TB (*Gupta-Wright et al, BMC Med 2016*). Ten eligible studies were identified, and random-effects meta-analysis demonstrated urine-LAM positive patients had a 2.3-fold greater mortality risk, and 2.5-fold greater adjusted odds of death than urine-LAM negative HIV-TB patients.

Secondly, a prospective observational cohort study nested within a large randomised trial of urine-based TB screening was undertaken (STAMP trial in Malawi and South Africa, *Gupta-Wright et al BMC Inf Dis 2016 and Lancet 2018*). It included 322 patients with laboratory-confirmed HIV/TB disease and demonstrated a remarkably high (31%) 2-month mortality risk despite rapid initiation of TB treatment. It also demonstrated that advanced immunosuppression was common in HIV/TB disease despite high antiretroviral therapy (ART) coverage. Cohort data were used to identify clinical phenotypes associated with poor outcomes: urine-test positivity (LAM, Xpert or both) was independently associated with a 50% increase in 2 month case-fatality.

Thirdly, a study of immune responses was nested in the Malawi STAMP trial site. A functional whole blood assay of phagocytic activity was developed (*Gupta-Wright et al, Frontiers Immunology 2017*) and utilised in 65 HIV/TB patients and 16 matched HIV-positive TB-negative controls. Cellular and soluble markers of immune activation, ex-vivo monocyte and T-cell cytokine responses and multiplex plasma cytokine and chemokine levels were also measured. Poor outcomes and urine-positive HIV/TB disease were associated with broadly impaired

immune responses, including phagocytic oxidative burst function, monocyte activation and dominant innate immune responses. Finally, urine-LAM was included in a simple, pragmatic clinical score to identify patients at high risk of a poor outcome in resource-poor settings, which was externally validated on an individual-patient record dataset combining data from 2 different studies over 5 African countries (*Gupta-Wright et al, PLOS Medicine 2019*).

This PhD has demonstrated the high prevalence and case-fatality associated with disseminated HIV/TB disease in HIV-positive hospital admissions despite widespread access to ART, and the utility of urine-diagnostics in identifying patients at high risk of mortality, in addition to their diagnostic use. These linked studies could potentially inform on the choice and nature of interventions to reduce the high mortality of HIV/TB, including better identification and management of ART failure, therapies to address TB-related immune dysfunction, and improved supportive care and prevention and treatment of co-morbidities.

Table of Contents

Chapter 1: Introduction	16
Part 1: Review of HIV-associated Tuberculosis	17
1.1 Introduction	17
1.2 Epidemiology of HIV-associated TB	20
1.3 Public health interventions to address HIV-associated TB	21
1.4 Clinical features of HIV-associated TB.....	27
1.5 Diagnosis of HIV-associated TB (see [35]).....	28
1.6 Urine diagnostics for HIV-associated TB	31
1.7 Management of HIV-associated TB.....	38
1.8 Mortality in HIV-associated TB, and burden within hospitals	39
1.9 Risk factors for mortality.....	40
1.10 Association between urine diagnostics and outcomes.....	41
Part 2: Immune responses in HIV-associated TB	46
1.11 The immune response to TB	46
1.12 Impact of HIV on the immune response to TB.....	51
1.13 Relationship between immune responses and clinical features or outcomes in HIV-associated TB	52
1.14 Immune responses and reducing mortality in HIV-associated TB.....	57
1.15 References	61
Chapter 2: Overview of aims and study designs for thesis.....	80
2.1 Thesis rationale	81
2.2 Thesis aim and research questions	82
2.3 Study settings: Malawi	84
2.4 Study settings: South Africa	87
2.5 Design of studies contributing to thesis	90
2.6 Procedures for the STAMP trial and sub-studies contributing to the thesis	95
2.7 Data collection, management and analysis	97
2.8 Ethical considerations	99
2.9 References	100
Chapter 3: The STAMP trial study protocol (research paper).....	102
Chapter 4: Systematic review and meta-analysis of urine-LAM detection's association with mortality (research paper).....	117

Chapter 5: Cohort study of mortality, and risk factors for mortality (research paper)	137
Chapter 6: Cohort study of derivation and external validation of a clinical score predicting mortality risk (research paper)	166
Chapter 7: Cross-sectional study assessing the use of whole-blood functional assay of phagocyte activity (research paper)	196
Chapter 8. Soluble and cell surface markers of immune response in hospitalised patients with HIV-associated TB.....	209
8.1 Introduction (chapters 8 and 9)	210
8.2 Aims (chapters 8 and 9)	211
8.3 Methods.....	213
8.4 Results.....	220
8.5 Discussion.....	235
8.6 References	238
Chapter 9. Functional immune responses in hospitalised patients with HIV-associated TB	242
9.1 Methods.....	243
9.3 Discussion.....	266
9.4 References	270
Chapter 10: Discussion, implications and future directions	274
10.1 Summary of findings	274
10.2 Study limitations	278
10.3 Implications of findings.....	279
10.4 Future research.....	286
10.5 Conclusion.....	287
10.6 References	288
Appendix: The STAMP trial results paper.....	293

Figures and tables

Chapter 1

Figure 1.1 Estimated TB incidence rates.....	19
Figure 1.2 Deaths from TB and HIV-associated TB	20
Figure 1.3 Global HIV and TB statistics in 2016.	21
Figure 1.4 Global trends in TB incidence and mortality.....	22
Figure 1.5 The DOTS TB control strategy	23
Figure 1.6 The pillars and components of the End TB strategy	24
Table 1.1 HIV/TB collaborative activities	26
Figure 1.7 Current tuberculosis diagnostics pipeline.....	29
Figure 1.8 Determine TB-LAM Ag lateral flow assay with manufacturers reference card	32
Figure 1.9 Sensitivity of urine LAM assay in ART-naïve individuals	33
Table 1.2 Direct and indirect evidence that urine lipoarabinomannan (LAM) antigenuria is due to renal involvement with haematogenously disseminated TB	36
Figure 1.10 Diagnostic yield of various rapid TB diagnostic assays and combinations of assays.	38
Figure 1.11 Estimates of case fatality ratios for TB from 2016 data.....	40
Table 1.3 Summary of studies reporting mortality in hospitalised patients with HIV and TB co-infection	42
Figure 1.12 Cumulative probability of death of confirmed cases of HIV-associated TB.....	44
Figure 1.13 Range of outcomes from MTB exposure.	47
Figure 1.14 Summary of the immunological mechanisms by which LAM acts as a virulence factor and suppresses host immune responses to MTB.....	56
Figure 1.15 Diagrammatic summary of immune responses and outcomes based on clinical spectrum of HIV-associated TB disease	58
Table 1.4 Summary of immunological mechanisms that may be implicated in impaired immune responses in HIV-TB co-infected patients.....	60

Chapter 2

Figure 2.1 Map showing the location of the study sites.....	83
Figure 2.2 Photographs of Zomba Central Hospital.....	86
Figure 2.3 Facilities in the MLW immunology and tissue laboratory	87
Figure 2.4 Photographs of Edendale Hospital.....	90
Table 2.1 List of contributors to the STAMP trial and research presented within this thesis	92
Table 2.2 A range of sample size estimations.....	98
Figure 2.5 Sample size estimations.....	99

Chapter 3

Figure 1 Study design flow diagram.....	108
Table 1 Schedule of STAMP study activities.....	110
Figure 2 CONSORT flow diagram.....	111

Chapter 4

Figure 1 Flow chart showing study selection process.....	123
Table 1 Studies reporting urinary LAM detection and mortality included in the systematic review.....	124
Table 2 Studies reporting adjusted odds ratios for mortality.....	125
Figure 3 Forest plot showing adjusted odds ratio of mortality.....	127
Table 3 Potential mechanisms of the association between urinary LAM detection and increased mortality risk.....	127
Table S1 Search Strategy.....	131
Table S2 Quality assessment tool.....	132
Table S3 Additional information about studies included in the systematic review.....	133
Table S4 Effect estimates of mortality risk ratio.....	135
Figure S1 Funnel plot of log odds ratios.....	136

Chapter 5

Table 1 Baseline characteristics in laboratory confirmed HIV/TB patients.....	145
Table 2 TB investigations and results	147
Table 3 Factors associated with time to death over 56 days	149
Figure 1 Cluster analysis clinical phenotypes of HIV/TB patients and their mortality risk.....	151
Table 4 Implications and potential interventions to reduce HIV/TB mortality.....	155
Supplementary Figure 1 Timing of deaths, stratified by in-patient and out-patient.....	162
Supplementary Figure 2 ART status and mortality.....	163
Supplementary Table 1 Multivariable model for mortality by study site.....	164

Chapter 6

Figure 1 Study profile	175
Table 1 Baseline characteristics	176
Table 2 Univariable and multivariable logistic regression analysis.....	177

Figure 2 Risk score calculation to predict mortality	178
Figure 3 Distribution of risk scores and mortality in the derivation dataset.....	179
Figure 4 Observed mortality risk by risk score category in the derivation and validation cohorts	180
Figure 5 Survival curves stratified by clinical risk score category in the derivation dataset	182
Table S1 Univariable analysis of continuous variables and associations with mortality.....	189
Table S2 Risk score and mortality data	190
Table S3 C-statistic and Hosmer-Lemshow test for final model and risk scores	191
Figure S1 Performance of mortality prediction score in derivation cohort.....	192
Figure S2 Observed and predicted mortality risk for predictor score values	193
Figure S3 Risk score calculation (simplified score) to predict mortality	193
Figure S4 Urine TB-LAM grade and CD4 cell strata stratified by mortality risk score	193
Figure S5 Calibration plot for predictive model in external validation dataset.....	194

Chapter 7

Figure 1 Gating strategy for identification of phagocytes with zymosan reporter particles and quantitation of intraphagosomal oxidation.....	202
Figure 2 Phagocytosis of zymosan reporter particles by monocytes and neutrophils	203
Figure 3 Assessment of phagocytosis of zymosan reporter particles by electron microscopy.....	204
Figure 4 Cell concentrations and activity index (AI) of superoxide burst over time.....	204
Figure 5 Phagocytosis, superoxide burst, and monocyte phenotypes in peripheral blood phagocytes of HIV/tuberculosis (TB)-coinfecting patients	205

Chapter 8

Table 8.1 Summary of analysis of immune responses.....	212
Figure 8.1 Summary of immunological procedures.....	214
Figure 8.2 Fluorescence minus one control	215
Table 8.2 Antibodies used for flow cytometry.....	216
Figure 8.3 Flow cytometry gating strategy for monocyte and T-lymphocyte immunophenotyping	218
Figure 8.4 Study patient flow diagram.....	220
Table 8.3 Baseline characteristics	222
Figure 8.5 HIV/TB patients by sputum and urine diagnostic test status	223
Table 8.4 Clinical baseline variables and associations with mortality	224
Table 8.5 Outcome and TB diagnostic results.....	224
Table 8.6 White cell differentials	225

Table 8.7 Plasma cytokine and chemokine concentrations in HIV/TB patients with and without disseminated TB disease	226
Table 8.8 Plasma cytokine and chemokine concentrations in HIV/TB patients who died and survived	227
Figure 8.6 Hazard ratios for mortality of plasma chemokines and cytokines in HIV/TB patients	228
Figure 8.7 Heat-map of correlations between cytokines by patient TB status and outcome ..	229
Figure 8.8 Heparin concentrations by patient group and anaemia	230
Table 8.9 Monocyte phenotype and activation markers by patient group	232
Figure 8.9 Monocyte phenotype and soluble monocyte activation markers by patient group	233
Table 8.10 T-cell activation, exhaustion and senescence	234

Chapter 9

Figure 9.1 Flow cytometry gating strategy for intracellular cytokine staining assays	245
Figure 9.2 Phagocytosis of zymosan reporter particles by patient outcome in HIV/TB patients	248
Figure 9.3 Concentration of whole blood phagocyte and proportion phagocytosing zymosan reporter particles	249
Figure 9.4 phagocytosis and superoxide burst function by patient TB status and outcome ...	250
Figure 9.5 Classical monocyte phenotype (CD14+CD16-) and phagocytic function in HIV/TB patients	251
Table 9.1 Intracellular cytokine production by stimulated monocytes	252
Figure 9.6 Pro-inflammatory cytokine production by monocytes in HIV/TB patients by outcome	253
Figure 9.7 IL-10 production in HIV/TB patients and TB negative controls	254
Table 9.2 Intracellular cytokine production by stimulated CD4+ T-cells	255
Figure 9.8 Change in intracellular cytokine expression upon stimulation in CD4+ T-cells	256
Figure 9.9 Pro-inflammatory cytokine responses to stimulation by CD4+ T-cells	257
Figure 9.10 Polyfunctionality of CD4+ T-cells in production of pro-inflammatory cytokines by patient group	258
Table 9.3 Intracellular cytokine production by stimulated CD8+ T-cells	259
Figure 9.11 Change in intracellular cytokine expression upon stimulation in CD8+ T-cells	261
Figure 9.12 Pro-inflammatory cytokine responses to stimulation by CD8+ T-cells	262
Table 9.4 Cox regression model illustrating association between immune signature and mortality	263
Figure 9.13 Bar chart showing loadings of variables for principle component (PC) 1 and 2	264
Figure 9.14 Principal component analysis demonstrating differences in baseline immune response between HIV/TB patients by outcome and disseminated TB	265

Chapter 10

Table 10.1 Summary of key findings, implications, future research directions.....	281
Table 10.2 Comparison of STAMP and LAM RCT trials	283
Figure 10.1 Potential host directed therapy targets.....	285

Abbreviations

AI	Activity Index
AIDS	Acquired Immune Deficiency Syndrome
ART	Antiretroviral Therapy
AUC	Area Under the Curve
BCG	Bacillus Calmette-Guérin
BMI	Body Mass Index
CD	Cluster of Differentiation
CFP	Culture Filtrate Proteins
CFR	Case Fatality Rate
CI	Chief Investigator
CRF	Case Report Form
CRP	C-reactive Protein
CrAg	Cryptococcal Antigen
CT	Computed Tomography
DC	Dendritic Cell
DC-SIGN	DC-Specific Intracellular Adhesion Molecule-3 Grabbing Non-Integrin
DNA	Deoxyribonucleic Acid
DOTS	Directly Observed Therapy, Short Course
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Fluorescence-Activated Cell Sorting
FBC	Full Blood Count
GCS	Glasgow Coma Scale
GM-CSF	Granulocyte–Macrophage Colony-Stimulating Factor
HDL	High Density Lipoprotein
HIV	Human Immunodeficiency Virus
HIV/TB	HIV-associated TB
HR	Hazard Ratio
ICS	Intracellular Cytokine Staining
IFN	Interferon
IL	Interleukin
IP	Inducible Protein
IRIS	Immune Reconstitution Inflammatory Syndrome
IQR	Interquartile Range
LAM	Lipoarabinomannan
LFA	Lateral Flow Assay
LPS	Lipopolysaccharide
LSHTM	London School of Hygiene & Tropical Medicine
LTFU	Loss To Follow-Up
MCP	Monocyte Chemoattractant Protein

MDR	Multidrug Resistant TB
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MIP	macrophage inflammatory protein
MLW	Malawi-Liverpool-Wellcome Trust Centre Clinical Research Programme
<i>MTB</i>	<i>Mycobacterium tuberculosis complex</i>
NAAT	Nucleic Acid Amplification Test
OR	Odds Ratio
PBMC	Peripheral Blood Mononuclear Cells
PC	Principle Component
PD	Programmed Cell Death Protein
PHIA	Population-Based HIV Impact Assessments
PLHIV	People Living with HIV
PMA	Phorbol 12 Myristate 13-Acetate
RANTES	Regulated On Activation, Normal T Cell Expressed And Secreted
REC	Research Ethics Committee
RR	Relative Risk
<i>RpoB</i>	RNA polymerase β subunit gene
SAE	Severe Adverse Event
SD	Standard Deviation
SDF	Stromal Cell-Derived Factor
SDGs	Sustainable Development Goals
SOP	Standard Operating Procedure
SSA	Sub-Saharan Africa
STAMP	rapid urine-based Screening for TB to reduce AIDS Mortality in hospitalised Patients in Africa
T-regs	T-Regulatory Cells
TB	Tuberculosis
TB-LAM	Determine TB-LAM Ag assay
Th	T-helper
TLR	Toll Like Receptor
TNF	Tissue Necrosis Factor
Tregs	Regulatory T-Cells
TST	Tuberculin Skin Test
UNAIDS	Joint United Nations Programme on HIV/AIDS
WHO	World Health Organisation
Xpert	Xpert MTB/RIF assay
ZCH	Zomba Central Hospital

Chapter 1: Introduction

Part 1: Review of HIV-associated Tuberculosis

Part 2: Immune responses in HIV-associated TB

Chapter 1: Introduction

Part 1: Review of HIV-associated Tuberculosis

Summary

This section reviews the epidemiology of HIV-associated TB, highlighting the ongoing burden of this dual epidemic in sub-Saharan Africa in terms of mortality and morbidity. Despite public health interventions aimed at controlling HIV (and therefore reducing the degree of host susceptibility to TB), TB control efforts and collaborative HIV/TB activities, the incidence and mortality is not declining at the rate needed to meet global targets and avert millions of deaths. The diagnosis of HIV-associated TB remains a major barrier, especially in hospitals where HIV-associated immunosuppression is more advanced and case fatality is high. Rapid urine-based diagnostics provide some potential, as they have good diagnostic yield in this population and identify patients with disseminated TB at higher risk of poor outcomes and difficult to diagnose with means available, who may benefit from adjunctive interventions (in addition to TB therapy and appropriately timed antiretroviral therapy). However, relatively little is known about factors associated with mortality in this group, and therefore how to improve treatment outcomes.

1.1 Introduction

Tuberculosis (TB), caused by infection with *Mycobacterium tuberculosis* complex (*MTB*), has affected humans globally for millennia, with few infectious diseases probably having claimed as many lives as TB. The peak of TB occurred during the 17th century in Europe, when incidence rates were as high as 1000 per 100,000 (1%) per year, and accounted for up to one-quarter of deaths in Europe until the 19th century [1]. Both the incidence and mortality of TB began to fall during the 18th and 19th centuries due to improvements in housing, nutrition, reductions in poverty and recognition that TB was infectious. This is long before the discovery of the Tubercle bacillus by Robert Koch, Bacillus Calmette-Guérin (BCG) vaccination and anti-tuberculosis drugs in 1940s. Reductions in TB incidence continued after these discoveries, particularly in industrialised regions of the world.

However, TB emerged again as a public health threat in 1990s, with the World Health Organization (WHO) declaring TB a global emergency in 1993 [2]. Much of the sharp increase in TB incidence triggering that response was driven by the human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS) epidemic, particularly in southern Africa where TB incidence increased exponentially from 1990 (figure 1.1), despite being stable or

reducing in developed regions of the world [3]. Both in the individual host and at a population level, TB and HIV potentiate one another.

Soon after the first descriptions of AIDS in early 1980s, HIV rapidly emerged as the strongest risk factor for development of TB disease [4]. Although TB occurs at all levels of HIV-related immune suppression, it has become inextricably linked to the control of HIV immunosuppression in countries with generalised HIV epidemics, as well as countries with high HIV prevalence in substantial 'key populations' such as prisoners and injecting drug users. The risk of TB disease is up to 30-fold higher in the most immunosuppressed HIV positive patients, such as those with CD4+ T-lymphocyte (CD4 cell) counts less than 100 cells/ μ L [3]. Concerningly, even those HIV-positive patients who are stable on antiretroviral therapy (ART), with suppression of HIV viral replication and restoration of CD4 cell counts still have up to 5-fold increased risk of TB disease compared to their HIV-uninfected counterparts [5].

This inherent biological predisposition to TB caused by HIV was compounded by under resourced TB control programmes and health systems in sub-Saharan Africa, which impacted both HIV and TB. Vertical TB programmes (focussed on a single disease independent of the general health system) consisting of BCG vaccination and TB case management were not effectively transferred to resource-poor settings.

The DOTS strategy [6] was developed largely uninformed by HIV-associated TB patients. Although successful in improving treatment outcomes for HIV-negative patients, DOTS was ill-suited to high HIV-prevalence settings because of a reliance on sputum smear microscopy for diagnosis, lack of screening for HIV amongst TB patients, and no recognition of complications of HIV-associated TB, for example drug interactions and poorer survival.

This led to the integration of TB programmes into general health services, but this was not accompanied by an increase in resources, leading to the neglect of TB control outside resource-rich settings. Case detection of TB was often poor, and cure rates were low, fuelling both transmission of TB and high case fatality rates. Globally, investments in new technologies, research into TB diagnostics, drugs, treatment regimens and vaccines were also neglected for from the 1970s onwards, contributing to a return to TB incidence rates in pockets of sub-Saharan Africa resembling those of 19th century London.

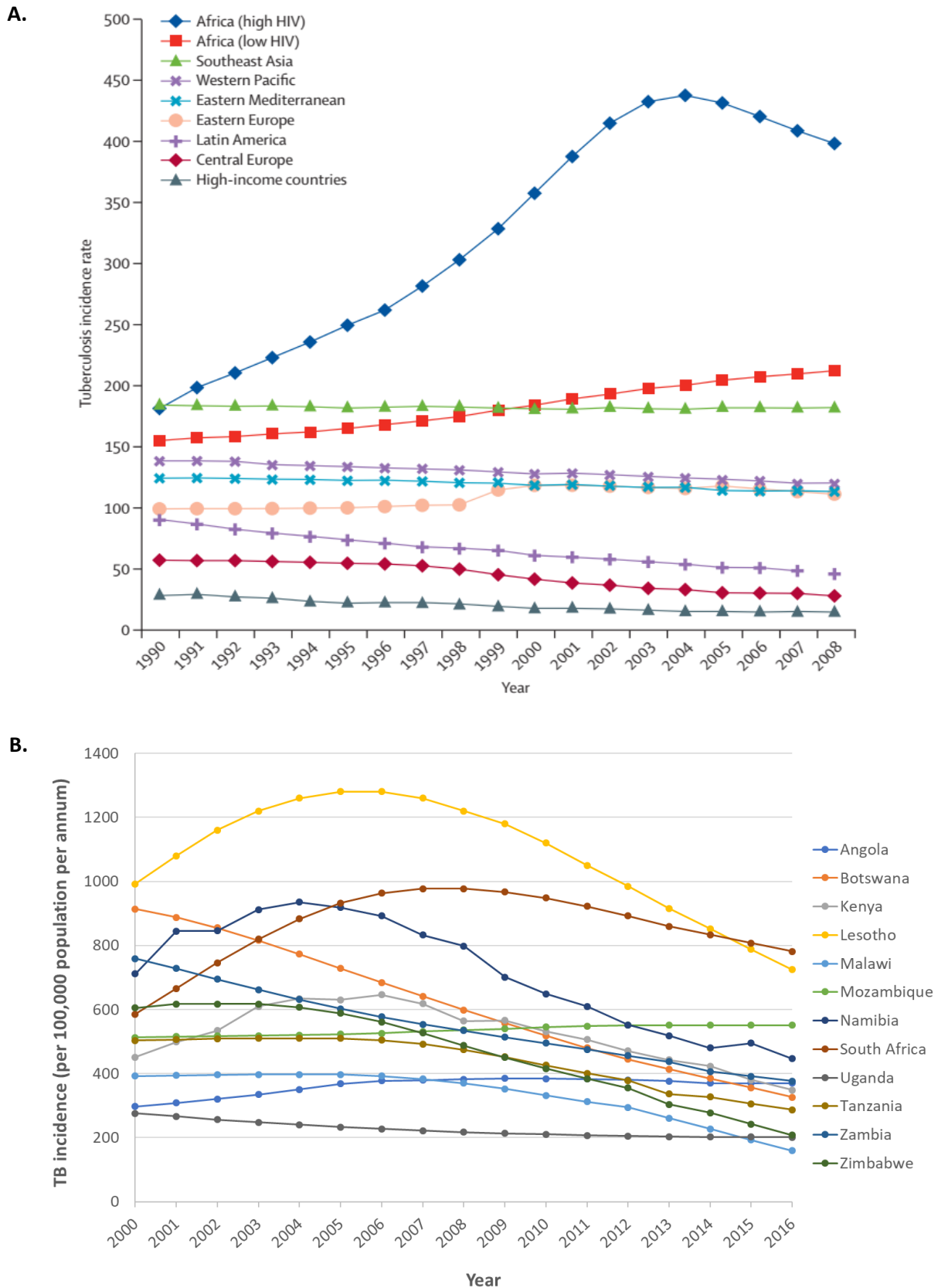


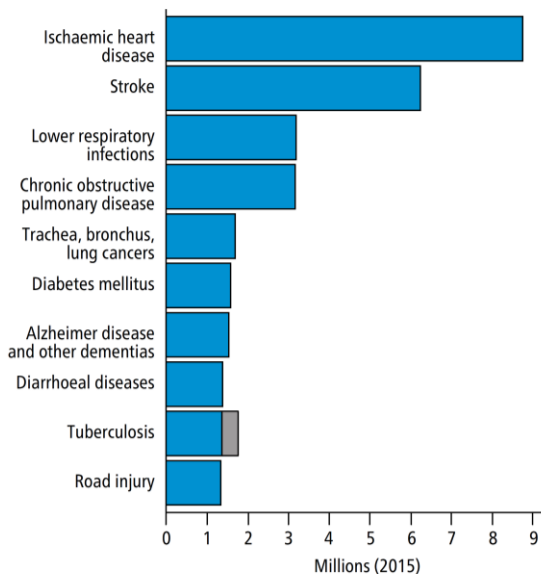
Figure 1.1 A. Estimated TB incidence rates in WHO regions globally between 1990 and 2008, showing the exponential increase in high HIV-prevalence settings in Africa. Data is from the Global Tuberculosis report 2009 [7], figure is reproduced from Lawn and Zumla [3]. B. WHO estimated TB incidence rates in 12 Southern and Eastern African countries from 2000 to 2016, showing only a slow decline in incidence in most countries. Data is from the Global Tuberculosis Report 2017 [8].

1.2 Epidemiology of HIV-associated TB

By 2017, there were estimated to be 36.7 million people living with HIV (PLHIV) worldwide, including 1.8 million new infections that year, a 46% decrease from peak annual incidence in 1996 [9]. Despite 21.7 million people accessing ART, there were almost 940,000 HIV related deaths in 2016, a 50% decrease from the peak in 2004 [9]. Although HIV is a global epidemic, the burden of prevalence, incidence and mortality lies in sub-Saharan Africa, and more specifically in Eastern and Southern Africa where 19.6 million (53%) of PLHIV and 380,000 (40%) of HIV deaths occur [9].

TB is still the leading single infectious cause of death and one of the top ten leading overall causes of death worldwide (figure 1.2) [8]. An estimated 10.4 million people had TB disease in 2016, of whom 25% lived in the WHO African region [8]. There were also an estimated 1.7 million deaths from TB in the same year, despite a 3% annual reduction in TB mortality and 2% reduction in incidence (figure 1.1).

Top causes of death worldwide in 2015.^{a,b,c} Deaths from TB among HIV-positive people are shown in grey.

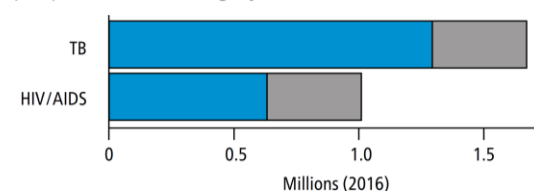


^a This is the latest year for which estimates for all causes are currently available. See WHO Global Health Observatory data repository, available at <http://apps.who.int/gho/data/node.main.GHECOD> (accessed 28 August 2017).

^b For HIV/AIDS, the latest estimates of the number of deaths in 2016 that have been published by UNAIDS are available at www.unaids.org/en/resources/documents/2017/HIV_estimates_with_uncertainty_bounds_1990-2016. For TB, the estimates for 2016 are those published in this report.

^c Deaths from TB among HIV-positive people are officially classified as deaths caused by HIV/AIDS in the International classification of diseases.

Estimated number of deaths from HIV/AIDS and TB in 2016. Deaths from TB among HIV-positive people are shown in grey.^{a,b}



^a For HIV/AIDS, the latest estimates of the number of deaths in 2016 that have been published by UNAIDS are available at www.unaids.org/en/resources/documents/2017/HIV_estimates_with_uncertainty_bounds_1990-2016. For TB, the estimates for 2016 are those published in this report.

^b Deaths from TB among HIV-positive people are officially classified as deaths caused by HIV/AIDS in the International classification of diseases.

Figure 1.2 Deaths from TB and HIV-associated TB. Taken from The Global Tuberculosis Report 2017 [8].

Both HIV and TB burden are highest in sub-Saharan Africa, and this is where the HIV-associated TB epidemic resides. Of the 30 countries on the WHO's 2016-2020 high-burden list for HIV/TB, 23 are in the sub-Saharan Africa region [8]. In 2016, globally there were an estimated 1 million

cases and 0.4 million TB deaths among PLHIV (see figure 1.3) [8]. TB is the leading cause of mortality amongst PLHIV, with 82% of these HIV related deaths occurring in sub-Saharan Africa.

Global trends in TB mortality are downwards, although reductions are predominantly in the HIV-negative population rather than PLHIV (figure 1.4) [8]. There are also substantial costs to the healthcare system of HIV-associated TB, and to individuals, for whom there is growing evidence of a high risk of catastrophic costs both pre- and post-diagnosis [10]. Given these high individual, financial and societal costs, there is a clear need to intensify and optimise control strategies as far as possible within available resources.

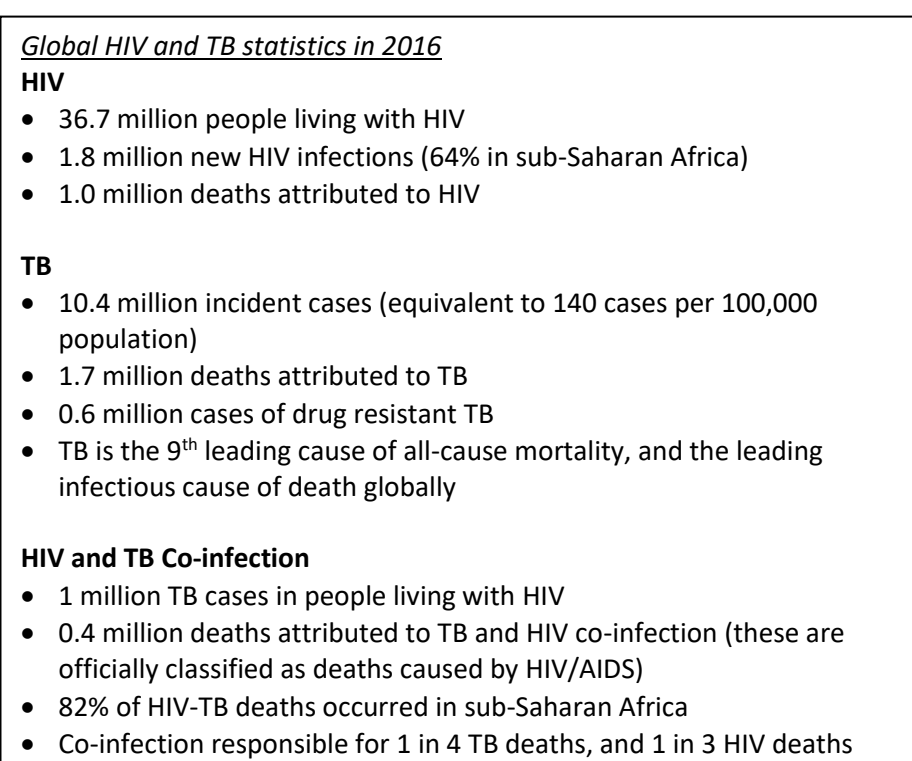


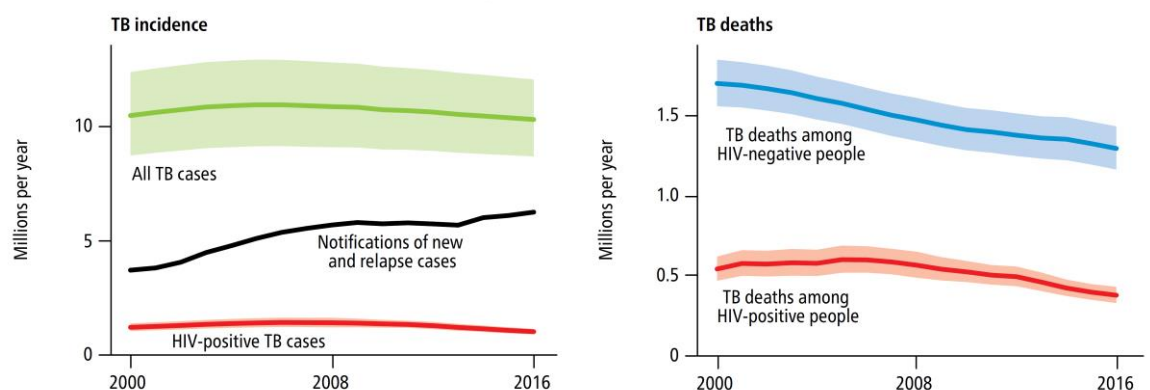
Figure 1.3 Global HIV and TB statistics in 2016. Data from the WHO Global tuberculosis report 2017 [8].

1.3 Public health interventions to address HIV-associated TB

Control of HIV-associated TB aims to reduce both incidence, and morbidity and mortality. Despite the link between TB and HIV having been recognised early in the HIV epidemic, collaborative HIV and TB public health activities began relatively late [11]. Thus, much of the early control of HIV-associated TB has resulted from control programs aimed at HIV or TB as single diseases entities.

Reducing incidence can be achieved through reducing TB transmission, for example within health care facilities and some congregate settings where transmission can be strongly driven by HIV. Within the community, prolonged infectious TB (more common in HIV-negative patients as HIV-positive patients progress to severe illness more quickly, therefore duration of infectiousness is thought to be less) presents a major threat to HIV-positive individuals [12]. In high TB prevalence settings, the majority of TB (including HIV-associated disease) is caused by recent infection rather than ‘reactivation’ of latent infection, suggesting transmission is a key factor in controlling HIV-associated TB [13,14].

Global trends in the estimated number of incident TB cases and the number of TB deaths (in millions), 2000–2016. Shaded areas represent uncertainty intervals.



Global trends in estimated TB incidence and mortality rates, 2000–2016. Shaded areas represent uncertainty intervals.

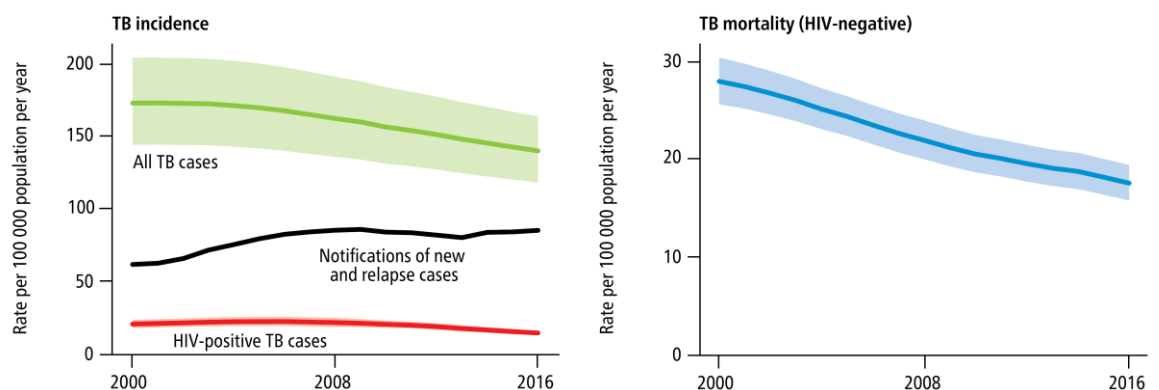


Figure 1.4 Global trends in TB incidence and mortality as numbers of cases and rates, stratified by HIV-status. Taken from The Global Tuberculosis Report 2017 [8].

TB incidence can also be addressed through reducing host susceptibility to TB (ie reducing time spent at lower CD4 cell counts when TB risk is greater) [5]. This is primarily through early diagnosis of HIV-infection and delivering accessible and effective HIV care (optimising the HIV

'treatment cascade'). The use of TB preventative therapy in HIV-positive patients can also reduce TB incidence [15].

TB Control

Following the alarming resurgence of TB in the early 1990s, including well publicised nosocomial and institutional epidemics of drug-resistant (DR)-TB with high mortality, and decades of neglect, global stakeholders developed a new approach to TB control which targeted all levels of the healthcare system, given the brand name 'DOTS' (not to be confused with directly observed therapy, DOT, which is a component of DOTS) [6]. The five elements of DOTS are summarised in figure 1.5, but the philosophical basis was prompt diagnosis and effective treatment of smear positive TB to interrupt transmission [16].

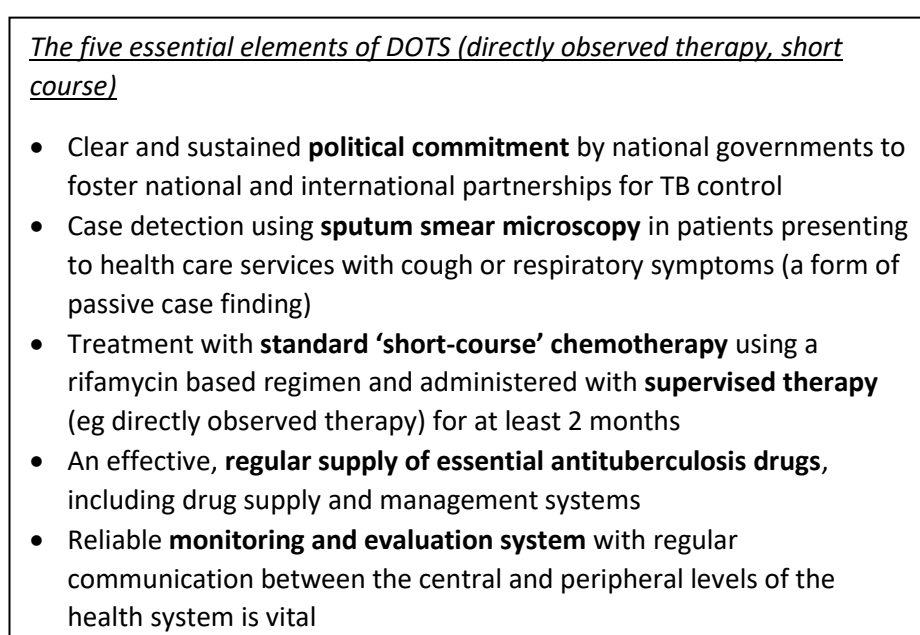


Figure 1.5 The DOTS TB control strategy

Reaching STOP TB Partnership targets for global TB, launched in 2006 and underpinning the Global plan from 2011 to 2015 (a 50% reduction in incidence and mortality compared to 1990 levels by 2015) had been the focus of national and international TB control efforts. In the post-2015 era of the sustainable development goals (SDGs), the international community has committed to ending the HIV/AIDS and TB epidemics [17]. Goals to be achieved include an 80% reduction in TB cases and a 90% reduction in TB deaths by 2030, with the ultimate aim of achieving a TB incidence of <10 cases per 100,000 people, considered sufficient to 'end' the global TB epidemic (figure 1.6) [17]. However, meeting these ambitious targets will require specific interventions targeted to PLHIV. Even though incidence and mortality have been declining over recent years (figure 1.4), this decline is much slower in HIV-positive compared to HIV-negative populations, and will be too slow to attain End TB targets by 2030.

Post-2015 End TB Strategy pillars and components

- The vision was a world free of TB with zero deaths, disease and suffering due to TB, and the goal to end the TB global epidemic
- Milestones for 2020 are (compared to 2015):
 - 35% reduction in TB deaths
 - 20% reduction in TB incidence rate
- Milestones for 2025 are (compared to 2015):
 - 75% reduction in TB deaths
 - 50% reduction in TB incidence rate
- Milestones for 2035 are (compared to 2015):
 - 95% reduction in TB deaths
 - 90% reduction in TB incidence rate
 - No affected families facing catastrophic costs due to TB
- The three 'pillars' of the End TB strategy are:
 - Integrated, patient centred care and prevention
 - Bold policies and supportive systems
 - Intensified research and innovation

Figure 1.6 The pillars and components of the End TB strategy.

Control of HIV

The advent of anti-retroviral drugs in the late 1980s and the use of combination therapy were enormous advances and altered the natural history of HIV. However, the impact on HIV-associated TB was not truly felt until the negotiated decline in the pricing of ART in low income countries to less than US\$150 per year (from over US\$15,000 per year) in the early 2000s, which allowed high HIV-prevalence countries to introduce ART through the public sector with donor funding support [16]. ART scale-up is one of the most important public health interventions in history in terms of the number of deaths averted, with 1.7 million adult lives saved in South Africa alone between 2000 and 2014 [18].

ART is one of the most potent tools for the public health control of HIV-associated TB via several mechanisms. Current CD4 cell count is the strongest risk factor for TB disease, and ART reduces the person-time spent at lower CD4 cell counts and therefore at higher risk of TB. Multiple cohort studies reported substantial risk reductions in TB incidence following ART initiation, with early studies estimating an overall risk reduction of two-thirds [19]. This risk reduction is irrespective of evidence of latent TB (eg tuberculin skin test positive), suggesting reduced TB infection as well as reactivation.

Entry into a life-long ART program also provides ongoing opportunity for intensified TB screening among HIV-positive individuals, potentially shortening TB disease duration and

transmission risk. ART also reduces individual level mortality in HIV-associated TB. Finally, effective ART may also prevent HIV through reduced transmission, and HIV testing could provide concomitant opportunities for TB screening in the general population.

Early global targets for ART scale-up included UNAIDS and WHO's '3 by 5' initiative, launched in 2003, which aimed to have 3 million HIV-positive people on ART by the end of 2005 [20]. Global scale-up of ART continues, with the more recent UNAIDS target of '90-90-90' - including having 90% of PLHIV who know their status on ART.

Many high HIV and TB burden settings are implementing universal ART ('test and treat') following updated WHO 2016 guidance and strong evidence of individual reductions in morbidity and mortality as well as the population benefit of treatment as prevention [21,22]. This increase in coverage, as demonstrated by population-based HIV impact assessments (PHIA) [20], has only been possible due to decentralisation of ART services and task shifting to lower level health care workers.

There is now compelling evidence at individual, cohort and national level of decreasing TB notifications at the population level during periods of extensive ART scale-up in settings such as South Africa and Malawi, with more marked declines in HIV-positive than HIV-negative populations supporting ART as a causal factor [23].

HIV and TB collaborative activities

The WHO published a strategic framework to decrease the burden of HIV-associated TB in 2002, followed by an interim policy on collaborative HIV/TB activities in 2004 [11,24]. The document recognised the morbidity and mortality from HIV-associated TB and aimed to assist countries on activities, whilst understanding the limited of evidence for collaborative activities available at that time. The policy was updated in 2012 using the same framework, but emphasising activities with the strongest evidence base [24]. Collaborative activities from both documents are summarised in table 1.1.

The WHO's HIV/AIDS and TB departments met in 2008, in collaboration with other stakeholders, to develop recommendations on the "Three I's", which were the key public health strategies to decrease the TB burden in PLHIV [25]. They included TB preventative therapy with isoniazid, intensified case finding to diagnose TB in PLHIV, and infection control to prevent TB in vulnerable PLHIV as well as health care workers and the community.

Framework for HIV/TB collaboration	Interim 2004 policy	2012 Policy changes	Evidence/Notes
A. Mechanisms for collaborative TB & HIV services	Set-up coordinating body for HIV/TB at all levels	Addition of strengthening coordinating bodies	Based on operational research showing such bodies ensure commitment and ownership
	Surveillance of HIV prevalence among TB patients	Addition of TB prevalence amongst PLHIV	Crucial to understanding the impact of interventions for HIV/TB
	Joint HIV/TB planning	Integration of HIV/TB services	Several models of care were identified, but referral between HIV and TB services was seen as risky. Integrated services at a single facility are considered the gold-standard, supported by observational data
	Monitoring and evaluation		Standardised monitoring and evaluation help determine the impact of activities and facilitate comparisons between services
B. Reduce burden of TB in PLHIV (Three I's for HIV/TB)	Intensified TB case-finding	Addition of ensuring high quality anti-TB treatment	High rates of undiagnosed TB in PLHIV and higher risk of incident TB, in part due to inadequate diagnostic tools. Improved outcomes with early initiation of ART in HIV/TB
	Initiate TB prevention with isoniazid preventative therapy	Addition of early antiretroviral therapy	Evidence that early initiation of ART substantially reduced TB incidence (even at higher CD4 counts), and the effect was additive to IPT.
	Infection control in healthcare and congregate settings		Tugela Ferry outbreak of DR TB (2006) in HIV-positive patients in hospital highlighted the importance of infection control for vulnerable PLHIV
c. Reduce burden of HIV in TB patients	Provide HIV testing and counselling to TB patients	Addition of presumed TB patients as well as diagnosed TB patients	Evidence emerged that HIV prevalence was higher in patients presenting with TB symptoms than the general population. This is another opportunity for HIV diagnoses and linkage to care
	HIV preventative interventions		
	Co-trimoxazole preventative therapy for PLHIV		Strong evidence to support reduced morbidity and mortality with CPT use in HIV/TB as well as PLHIV
	ART for TB patients living with HIV		

Table 1.1 HIV/TB collaborative activities, adapted from WHO's interim policy on collaborative HIV/TB activities 2004 [11] and WHO policy on collaborative TB/HIV activities 2012 [24]

1.4 Clinical features of HIV-associated TB

TB in adults has a number of clinical manifestations, although the lung is the predominant site of TB disease as infection almost always occurs from inhalation of *MTB* bacilli. Clinical features pertain to the site of the disease, as well as non-specific constitutional symptoms such as fevers, night sweats, and weight loss. Early during the pathogenesis of TB, patients may be asymptomatic due to subclinical disease [26]. Classical clinical features of pulmonary TB, defined as TB of the lung parenchyma and/or tracheobronchial tree, include cough (often lasting over two to three weeks), sputum production and/or haemoptysis [3].

Although physical signs of pulmonary TB are non-specific, radiographic features often show infiltrates which may be extensive and contain cavities, and are usually more pronounced in the upper lobes. Extra-pulmonary TB occurs in between 10 and 42% of patients, depending on several factors including age, ethnicity, co-morbidities, immunosuppression and *MTB* strain [26]. It can affect any organ, with a variety of signs and symptoms. Some more common extrapulmonary presentations include TB lymphadenitis, pleural disease, central nervous system disease or disseminated ('miliary') TB [27,28].

Impaired host immune responses against *MTB* not only make TB disease more common in PLHIV, advancing immunosuppression can markedly alter presentation. However, patients with higher CD4 cell counts often present with 'typical' HIV-negative clinical features [3]. Progressive immunosuppression, as reflected by a declining CD4 count, increases risk of extrapulmonary and disseminated disease, with increasingly non-specific presentations [29,30]. A Ugandan study showed no difference in the proportion of HIV-positive patients with CD4 cell counts >300 cells/ μ L with radiographic disease or cavitation in the upper lobes of the lungs than in HIV-negative patients [31]. However, as CD4 count declined, these classical radiographic features also became less common, and adenopathy, effusions, military patterns and lower lobe disease predominated.

This disease pattern in HIV-associated TB has also been confirmed in autopsy studies spanning the last 20-25 years, with a systematic review showing that 90% of fatal HIV-associated TB in health-facilities in sub-Saharan Africa were disseminated, with the lung, spleen, liver, lymph nodes and bone marrow being commonly affected, suggesting haematogenous spread of infection from the lungs [32,33]. Similar findings have been demonstrated ante-mortem using computed tomography (CT) [34].

1.5 Diagnosis of HIV-associated TB (see [35])

There remains a major gap between estimated TB incidence, and TB cases diagnosed and notified [8]. TB diagnostic tools are a major weakness in the TB care cascade, and in low- and middle-income settings where the major burden of TB resides, health systems still rely on outdated tools such as sputum smear microscopy, chest radiography or health care workers' clinical suspicion of TB disease [35]. These lack sufficient sensitivity and/or specificity [34]. The global 'gold standard' TB diagnostic, liquid culture, is expensive, requires considerable infrastructure, is too slow, not available in peripheral health care facilities close to patients, and still far from 100% sensitive. Immunological assays, such as the Tuberculin skin test (TST) or interferon-gamma release assays have limited role in the diagnosis of active TB disease, as they cannot differentiate active from 'latent' TB infection.

Diagnosis of HIV-associated TB remains a particular challenge owing to atypical host responses, which lead to atypical clinical presentations. Smear microscopy, even with concentration and fluorescence microscopy to increase sensitivity, has inadequate sensitivity in HIV [36]. Similarly, chest radiography has reduced diagnostic utility in HIV-positive compared to HIV-negative TB disease [37]. The underlying mechanism is advanced HIV-related immunosuppression causing reduced cavitation, resulting in lower mycobacterial burden in sputum specimens, compounded by rapid dissemination beyond the lungs [38–41].

If EndTB targets to reduce TB deaths by 95% and new cases by 90% by 2035 are to be met, major improvements in diagnostic strategies are amongst the most pressing needs [42–44]. TB has not kept pace with other infectious diseases such as HIV and malaria, where cheap and rapid point-of-care diagnostics have simplified diagnostic algorithms and been successfully scaled up throughout sub-Saharan Africa [35]. However, following decades of under-investment in research, development of TB diagnostics has progressed, and the TB diagnostics pipeline looks more promising over the past decade (figure 1.7) [35]. Advances include improvements in current diagnostic technologies such as smear microscopy and culture-based systems [45,46]. More importantly, some progress has been made with respect to development of new rapid diagnostic tests that are also applicable to HIV-associated TB [47].

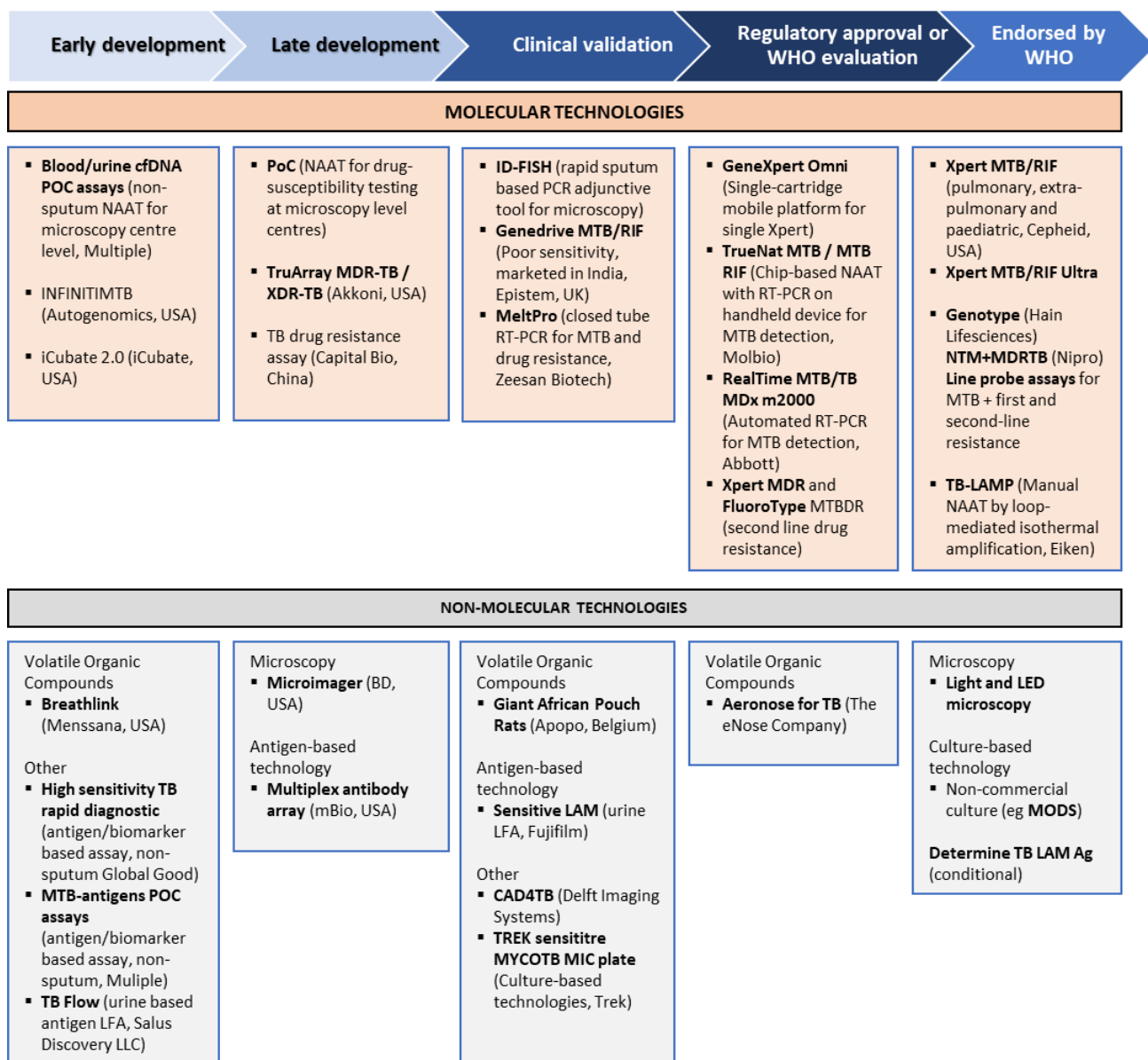


Figure 1.7 Current tuberculosis diagnostics pipeline listing examples of different types of diagnostics and their development phase (as of 2017). WHO: World Health Organization; LED: light-emitting diode; MODS: microscopic observation of drug susceptibility; LAM: lipoarabinomannan; NAAT: nucleic acid amplification test; TB: tuberculosis. Adapted from Gupta-Wright and Lawn [35], data from [48–50].

Xpert MTB/RIF assay

The development and introduction of nucleic acid amplification tests without the need for complex molecular laboratory infrastructure has been described as a ‘game changer’ [35]. The Xpert MTB/RIF assay (Xpert, Cepheid Inc, USA) is a self-contained, semi-automated and fully integrated nucleic acid amplification test developed for use by individuals without laboratory training [51,52]. Within a single use cartridge, it uses hemi-nested real-time polymerase chain reaction (PCR) and ‘molecular beacon’ technology to detect DNA sequences within *MTB*’s RNA polymerase β subunit gene (*RpoB*), allowing diagnosis of *MTB* and genotypic detection of approximately 95% of rifampicin resistant strains [53–55].

It was endorsed by WHO in 2010 due to superior diagnostic accuracy compared to sputum smear microscopy, in addition to ease of use, a substantially reduced biosafety risk and relatively short processing time (approximately two hours) [56,57]. Initial recommendations were for Xpert's use for rapid diagnosis of multidrug resistant (MDR)-TB, with its use as the primary diagnostic test for HIV-associated TB also being included [57]. However, logistical and health-system factors may make the use of Xpert outside the laboratory environment challenging [35]. Also, relatively low mycobacterial burden in sputum specimens and rapid dissemination beyond the lungs in PLHIV means the assay's sensitivity from testing sputum is reduced in HIV-positive TB compared to HIV-negative TB patients [58].

Since the initial multi-country assessment, many studies have been undertaken to assess the diagnostic accuracy of Xpert for HIV-associated TB [59]. The pooled sensitivity from meta-analyses of Xpert for diagnosis of culture-positive pulmonary TB in HIV-infected individuals was 79% (95% CI 70-86%), and specificity was 99% (95% CI 98-100%) compared to a culture reference standard [58]. Sensitivity was 61% (95% CI 40-81%) for sputum smear-negative TB, compared with 97% (95% CI 90-99%) for sputum smear-positive pulmonary disease [58].

Xpert's sensitivity appears to be related to mycobacterial load, thus is likely to be lower in populations with fewer symptoms and less smear-positive disease, for example when used for screening asymptomatic HIV-positive patients [60,61]. This may explain some of the variability between studies seen in sensitivity. The high proportion of patients with HIV-associated TB who have extrapulmonary TB provides another important use of this diagnostic assay [62]. Systematic reviews have reported very high specificity of Xpert when testing a very wide variety of non-respiratory clinical samples [63,64], despite the fact that culture is an imperfect reference standard for extrapulmonary TB which may lead to underestimation of specificity [65,66].

Overall sensitivity of Xpert was high for smear-positive extrapulmonary samples (97.4%, 95%CI 95.5-99.3%) [63]. However, sensitivity varied substantially with different specimen types when using a mycobacterial culture reference standard. The best sensitivities were observed with lymph node tissue (83.1%, 95% CI 71.4-90.7%) and cerebrospinal fluid (80.5%, 95% CI 59.0-92.2%), but poor sensitivity in pleural fluid (46.4%, 95% CI 26.3-67.8%) [63]. It is difficult to directly compare diagnostic accuracy of Xpert for extrapulmonary TB in HIV-positive and HIV-negative individuals due to paucity of data, but estimates did not differ substantially in studies with high and low proportions of HIV-positive patients [63]. WHO has also endorsed the use of Xpert for a variety (but not all) non-respiratory samples [67].

1.6 Urine diagnostics for HIV-associated TB

Urine has many advantages over sputum as a clinical sample for diagnostic testing, including the relative ease of collection and low biohazard risk when collecting specimens and during laboratory handling [61]. It is especially useful in severely unwell patients who are often too weak to produce sputum samples. Early morning urines were traditionally a common sample used to diagnose TB, despite low diagnostic yield [68], and urine culture was shown to provide a good diagnostic yield for TB in a cohort of patients with advanced HIV/AIDS in whom 77% tested urine culture positive [69]. However, the use of urine culture, as with sputum culture, and is not suitable for scaling up in high burden but resourced limited settings due to cost, infrastructure and turnaround time. However, two rapid diagnostic tests for urine that are potentially implementable have shown promise in diagnosis of HIV-associated TB: a lateral flow assay to detect lipoarabinomannan, and the Xpert assay.

Urinary detection of lipoarabinomannan (see [35] and [70])

Whereas assays of the immunological response to *MTB* are likely to be undermined in HIV-positive patients, direct detection of *MTB* antigens still has potential diagnostic utility in such patients [35]. Several different antigens of *MTB* have been detected from the urine of patients with microbiologically confirmed pulmonary TB [71,72]. Lipoarabinomannan (LAM), a mycobacterial cell wall lipopolysaccharide, is one such antigen and has emerged as a potential TB diagnostic assay target [73]. Several studies have investigated the diagnostic accuracy of enzyme-linked immunosorbent assays (ELISA) detecting LAM and, more recently, a low cost, point-of-care lateral flow assay (LFA)- the Determine TB-LAM Ag assay (TB-LAM, Alere, USA) [74]. This truly point-of-care bedside test can be performed with limited training and uses only 60µL of unprocessed urine, which is applied to the test strip. The result is then read after only 25 minutes by comparing visible bands with the manufacture's reference card (see figure 1.8A).

Although TB-LAM's overall sensitivity for diagnosing TB is sub-optimal (<25% HIV-uninfected populations), LAM assays are significantly more sensitive in HIV-positive patients [75]. An early meta-analysis of diagnostic accuracy in PLHIV reported pooled sensitivities of 56% (95% CI 40-71%) and specificities of 95% (95% CI 77-99%) [75]. A more recent systematic review reported overall sensitivities of 39-84% and specificities of 81-99% [76]. Specificities have been sub-optimal in many studies due to the use of mycobacterial culture on a only one sputum specimen as the reference standard, which will not reliably diagnose disseminated disease, and therefore may mis-classify some LAM-positive patients as 'false-positives' [66]. However, when reference standards include culture and nucleic acid amplification tests from several

extra-pulmonary sites, specificity is very high ($\geq 99\%$) [77]. Although there is some cross-reactivity with non-tuberculous mycobacteria [78,79], this is not thought to be a significant cause of false positive results in the context of patients with advanced HIV in sub-Saharan Africa [80].

A key observation for urine LAM testing was greater diagnostic accuracy with more advanced HIV-associated immunosuppression (figure 1.9) [74,75,81]. Studies stratifying urine LAM sensitivity by CD4 cell count demonstrated higher sensitivities (56-85%) among patients with CD4 cell counts <100 cell/ μL [82–88]. Observational studies have also shown significant incremental diagnostic yield when urine LAM assays were used to screen HIV-positive medical hospital admissions for TB, compared to sputum-based diagnostics alone [89]. This probably relates to the basis of urine LAM detection being haematogenously-disseminated TB with renal involvement, with dissemination more common in advanced immunosuppression (compared to immune-competent patients) [70,90]. Urine LAM detection also has strong associations with markers of increased mycobacterial load [91].

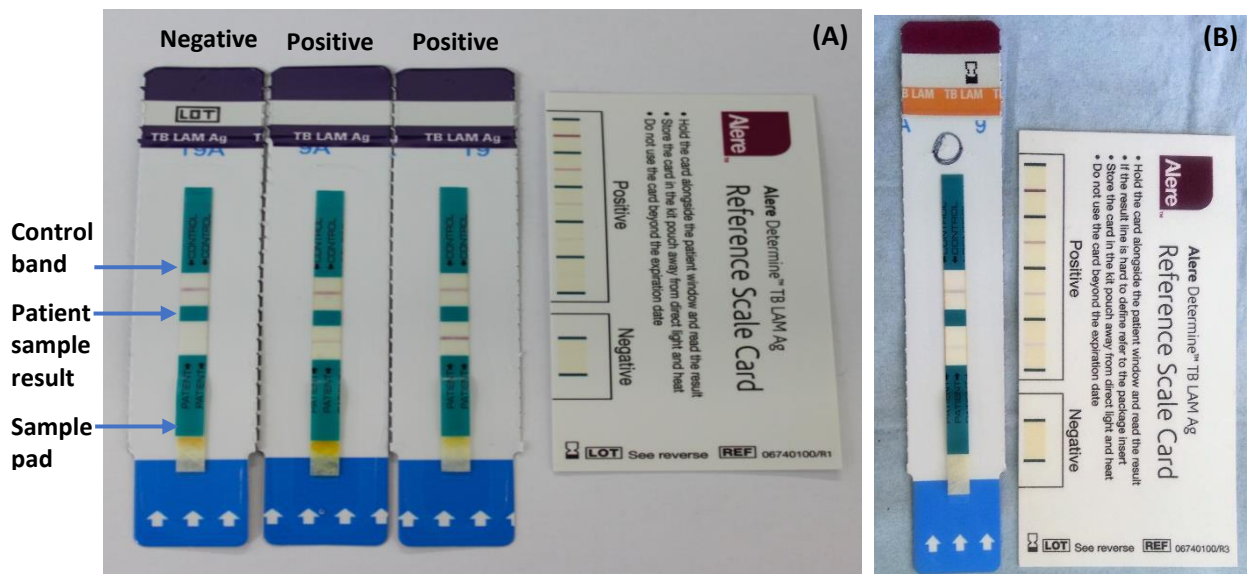


Figure 1.8 Determine TB-LAM Ag lateral flow assay with manufacturers reference card (A) Pre-2014 reference card. Photo provided by Dr Andrew Kerkhoff. (B) Manufacturers reference card after the weakest positive band was removed, leaving only four positive 'grades' (post 2014)

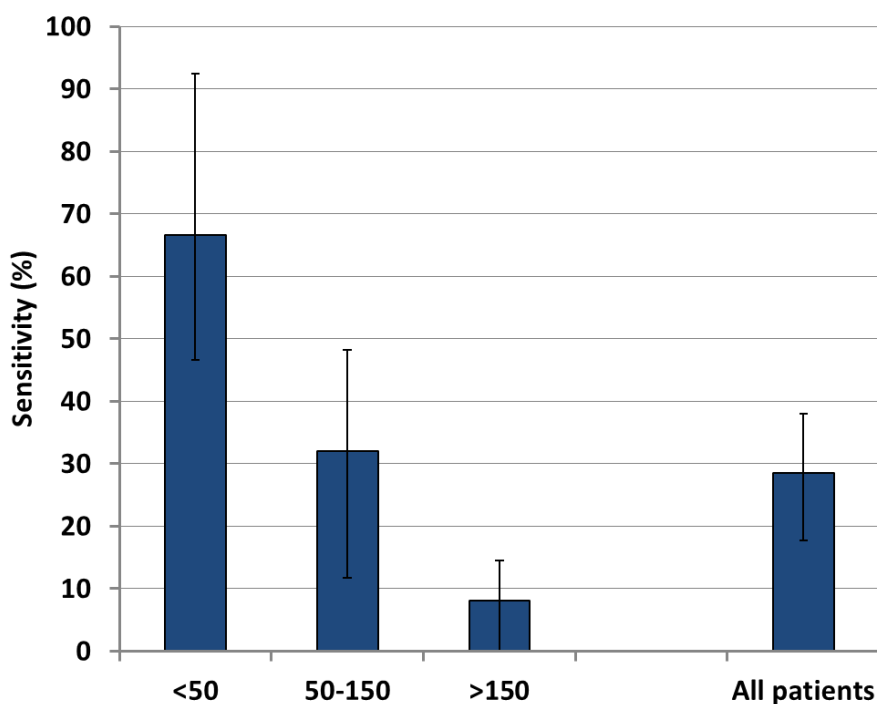


Figure 1.9 Sensitivity of urine LAM assay in ART-naïve individuals, stratified by CD4 cell count. Error bars represent 95% confidence intervals. Whilst overall sensitivity is inadequate with current assays, in advanced HIV urine LAM has good sensitivity. Adapted from Lawn et al [92].

The manufacture’s reference card provided with the TB-LAM assay contains bands of graded intensity within the ‘positive’ range, without clear instructions on interpretation [93]. The least intense band within the positive range was often referred to as ‘grade 1’ positive, with the band of next intensity being described as ‘grade 2’, up to ‘grade 5’ (see figure 1.8 A). In early studies of diagnostic accuracy of TB-LAM, researchers defined a positive result as either equal or greater intensity than the ‘grade 1’ or the ‘grade 2’ bands, with visible bands that are weaker in intensity being called ‘negative’.

Comparison of these two definitions of positivity from several studies concluded that the grade 1 cut-off was difficult to interpret (as it is quite faint and therefore less reproducible, especially in routine clinical practice), and less specific than the grade 2 cut-off [93]. Although using grade 2 to define a positive result led to a decrease in sensitivity, the area under the receiver operator curve (ROC) was greater. Therefore, researchers published a consensus that all future studies should use the grade 2 definition in 2013, to which the manufacture responded by updating their TB-LAM reference cards in 2014 so that the original ‘grade 1’ was no longer present (the new reference card only contains four bands of different intensity within the positive range, see figure 1.8B) [93]. The least intense band within the positive range on the new (post 2014) reference card is now also referred to as ‘grade 1’ (despite being different to the ‘old’ grade 1).

Urine Xpert *MTB*/RIF assay

Although Xpert has been endorsed for non-respiratory samples by WHO [67], its use on urine was not been recommended due to insufficient data. Three published studies have assessed Xpert's utility in diagnosing HIV-associated TB using urine samples. In outpatients with culture-positive TB, overall sensitivity of Xpert was poor (19%), but 44.4% in patients with CD4 counts <50 cells/ μ L [92]. In hospital inpatients with clinically suspected TB, sensitivity was 47.8% compared to the gold standard of liquid culture [94].

A recent study screening hospital admissions for TB in Cape Town demonstrated sensitivity of 59.0% when urine was concentrated by centrifugation prior to testing with Xpert (sensitivity was 42.4% using unconcentrated urine, a 40% relative increase) [95]. Specificity was >98% when reported [94,95]. Diagnostic accuracy, as with urine LAM, was greater with more advanced degrees of immunosuppression.

Evidence for positive urine diagnostic tests representing disseminated TB (see [70])

Early works presumed that LAM-antigenuria (and therefore positive urinary-LAM assays) resulted from renal filtration of free circulating LAM in the circulation [96,97]. This seemed plausible as it was known that replicating *MTB* produced LAM in large quantities at sites of TB disease, from which it could enter the bloodstream [98]. As a 17kDa glycolipid (similar in size to myoglobin) it should readily cross the renal glomerular basement membrane [74].

After production at sites of TB, it has been demonstrated that LAM is detectable in blood, albeit following extensive processing due to its incorporation within high density lipoprotein (HDL) particles [99], or a high degree of immune-complexing with anti-LAM antibodies [100]. Renal filtration of circulating LAM is also a plausible explanation for why LAM assays detected both pulmonary and extrapulmonary TB [70].

Neither immune-complexed nor HDL-associated LAM should pass from the systemic circulation through the intact basement membrane in a healthy renal glomerulus [101]. Whilst dysfunction of the glomerulus might theoretically allow LAM-containing-complexes to pass into the urine, studies have not found associations between urine LAM detection and glomerular damage or proteinuria [84,102], and there was no histological evidence of glomerular damage at autopsy in urine LAM positive TB patients [90].

LAM is also released by dying mycobacteria in large quantities [74]. If LAM freely filtered into urine, it would be expected that early during TB therapy, when substantial mycobacterial killing occurs, urine LAM concentrations would also rise. However, urine LAM levels do not

increase, and usually decrease, during early TB treatment, suggesting an alternative mechanism to simple renal filtration [102,103].

If LAM in the urine of HIV-infected patients were the result of renal TB, it may be expected that *MTB* would be culturable from LAM-positive urine samples. Unfortunately, data correlating urine LAM detection and urine mycobacterial culture are currently lacking. Nonetheless, other important evidence comes from studies testing urine with Xpert, which detects whole *MTB* bacilli through amplification of organism-associated DNA (rather than detection of free DNA which is filtered out early in the process) [51,104]. A large overlap exists between TB-LAM and Xpert positivity in urine from patients with HIV-associated TB, with $\geq 60\%$ of LAM-positive urine samples also testing positive by Xpert *MTB*/RIF [92,94,105]. The detection of whole *MTB* bacilli in urine of LAM-positive patients strongly corroborates the notion that renal TB as the source of urinary LAM.

Renal TB, usually in the form of microabscesses, arises in this patient population as a result of haematogenous dissemination, and is a common post-mortem finding in patients who have died from HIV-associated TB (50%-69%) [106–109]. In patients with advanced HIV (typically CD4 cell counts of <100 cells/ μL) a strong association has been observed between *MTB* bacteraemia and urine LAM-positivity, with 70-90% of bacteraemic patients also being urine LAM positive [110–113].

Renal TB readily provides a mechanism for this association, and Cox et al demonstrated (in post-mortem series) histological evidence of renal TB in most urine LAM positive patients [90]. In the same study, all patients with detectable urine LAM had evidence of either renal or disseminated TB. Renal TB also was also frequently found in cadavers of adults with disseminated TB in other post-mortem studies [106–109].

Discrepancy between urine LAM and urine Xpert *MTB*/RIF positivity

Few studies have compared urine LAM and urine Xpert positivity on urine samples from the same patients. Four studies have demonstrated urine LAM to have a higher diagnostic yield than urine Xpert [92,94,114,115]. In all these studies, approximately half of patients with positive urine LAM results were also urine Xpert positive. Although I hypothesise that the mechanism of positivity is similar for both assays in urine (haematogenously disseminated renal TB leading to *MTB* entering the urinary tract), the assays themselves detect *MTB* differently which may explain the discrepancy between LAM and Xpert positivity. The Xpert assay only detects whole *MTB* bacilli, and in spiking experiments was found to have a limit of detection of ~ 131 colony forming units (CFU)/ml. (automated liquid mycobacterial culture techniques have a lower limit of detection as low as ~ 10 CFU/ml) [54]. It is likely that assays

Observation	Evidence	References
LAM is unlikely to be able to pass from systemic circulation into the urine via the renal glomerulus	<ul style="list-style-type: none"> • LAM in the bloodstream is mostly immune-complexed or bound to high-density lipoprotein • These LAM containing molecules are too large to freely filter in the renal glomerulus 	Sada et al 1992[100] Sakamuri et al 2013[99] Haraldsson et al 2008[101]
LAM concentration in the urine does not increase during the first weeks of TB treatment	<ul style="list-style-type: none"> • Serum LAM concentration likely increases early after the massive mycobactericidal effect of anti-TB treatment. • If LAM was freely filtered in the kidneys, LAM concentration in the urine would also be expected to increase early after treatment 	Wood et al 2012[102] Bekker et al 1998[116]
Most LAM-positive urines test Xpert MTB/RIF-positive	<ul style="list-style-type: none"> • Xpert MTB/RIF detects <i>M. tuberculosis</i> DNA in whole bacilli (not free DNA), suggesting most LAM-positive urines contain whole <i>M. tuberculosis</i> bacilli 	Wood et al 2012[102] Lawn et al 2012[92] Blakemore et al 2010[104]
Frequent LAM-positive urine in patients with <i>M. tuberculosis</i> bacteraemia	<ul style="list-style-type: none"> • The strong association between <i>M. tuberculosis</i> bacteraemia and LAM-positive urine is very plausibly linked mechanistically by haematogenously disseminated renal TB 	Manabe et al 2014[110] Nakiyingi et al 2015[111] Nakiyingi et al 2014[112] Lawn & Kerkhoff 2015[113]
In patients with disseminated TB at autopsy, renal TB is common	<ul style="list-style-type: none"> • Prevalence of renal TB is similar to the sensitivity of LAM-positive urine in this population 	Lanjewar et al 2011[107] Ansari et al 2002[106] Cox et al 2015[90]
Post-mortem renal TB in HIV-positive patients is associated with LAM-positive urine	<ul style="list-style-type: none"> • Autopsy study revealed frequent renal TB in those whose urine also tested LAM-positive and all LAM-positive patients had haematogenously disseminated TB • No patients with renal TB were urine LAM-negative 	Cox et al 2015[90]

Table 1.2 Direct and indirect evidence that urine lipoarabinomannan (LAM) antigenuria is due to renal involvement with haematogenously disseminated TB, and not free filtration of LAM into the urine.
Taken from Lawn and Gupta-Wright [70]

detecting LAM antigen will be positive with a lower concentration of MTB than Xpert, explaining the superior sensitivity.

These studies have also reported a small proportion of patients who were urine Xpert positive but urine LAM negative. Reasons for this are unclear, but may be explained by LAM being variably bound to other proteins in the urine, preventing detection by ELISA or antibody-binding based assays in some patients [117]. A more recent study from South Africa found a larger proportion (47%, see figure 1.10) of urine Xpert positive patients to be urine LAM negative. This study concentrated urine through centrifugation prior to Xpert testing (but not prior to LAM testing), which may explain improved sensitivity of Xpert [77].

Diagnostic yield of rapid TB tests

Data show urine testing with both LAM and Xpert has inadequate overall sensitivity for HIV-associated TB, but moderate to good sensitivity in patients with advanced HIV, such as those admitted to hospital and/or those patients with low CD4 cell counts [70]. Sensitivity of sputum Xpert has been shown to be greater than those of urine TB diagnostics [58]. However, given that many of HIV-positive patients are unable to produce sputum (particularly those who have advanced disease), whereas almost all patients can produce urine samples [95], diagnostic yield is a better measure of utility than sensitivity (sensitivity of sputum-based assays can only be calculated in patients who were able to produce sputum).

A recent study by Lawn et al which screened all HIV-positive patients admitted to a hospital in Cape Town for TB, irrespective of symptoms or presentation, using rapid diagnostic assays was done to assess comparative diagnostic yields of sputum and urine [77,95,105]. The study used a robust reference standard which included culture and Xpert of any clinical samples (including sputum, blood, lymph node aspirate, cerebrospinal fluid, pleural fluid and urine), and collected a median of 5 samples per patient.

32% (n=139) of PLHIV admitted to hospital were diagnosed with microbiologically confirmed TB. Sputum samples were only produced by 37% of patients. Of all patients with TB, only 28% could be diagnosed by sputum Xpert (figure 1.10). However, 38% could be diagnosed by urine LAM, and 59% by urine Xpert. Combining urine LAM and Xpert would diagnose 67% of TB. Finally, combining sputum and urine Xpert with urine LAM could diagnose 82% of TB, and these assays could all be done within hours of admission, potentially expediting TB treatment. However, it is not known how many patients diagnosed using urine-based assays would have been started on TB treatment based on clinical grounds alone (empirical TB treatment).

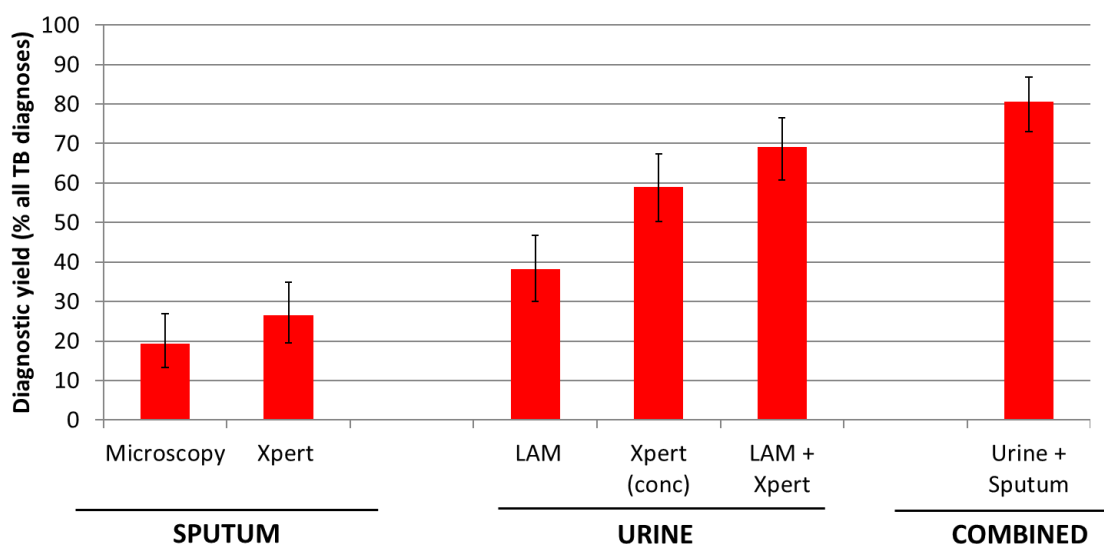


Figure 1.10 Diagnostic yield of various rapid TB diagnostic assays and combinations of assays. Error bars represent 95% confidence intervals. Total number of TB cases were 139, and were defined by any positive TB culture or Xpert result on any clinical sample. Data from Lawn et al [77,95,105]

1.7 Management of HIV-associated TB

The current management of patients with HIV-associated TB follows similar principles as HIV-negative patients, and drug-sensitive TB disease is usually treated with four standard drugs for 2 months (rifampicin, isoniazid, pyrazinamide and ethambutol, ‘intensive phase’), followed by 4 months of rifampicin and isoniazid (‘continuation phase’) [3,118]. There have been no advances in TB treatment over several decades, and patients with HIV are often excluded from trials of new TB drugs or drug regimens [3].

Firstly, early diagnosis is a prerequisite of optimal HIV-associated TB management, both to improve case ascertainment and to expedite anti-tuberculosis treatment. Non-specific clinical presentation, or sub-clinical disease means this must involve optimising diagnostic and screening strategies for PLHIV (including new diagnostic tools), and empirical TB treatment for certain high-risk patients when diagnostics are not available or fail [119]. Screening for HIV in patients diagnosed with TB or presumptive TB is also important [120].

In addition to effective anti-tuberculosis treatment, HIV-positive patients should receive an additional package of care to reduce their mortality and improve outcomes [119]. This includes early provision of ART – there are data from randomised controlled clinical trials informing the optimal timing to start ART, and evidence supports early initiation in ART naïve individuals as deferral is associated with higher mortality risk [121–123].

One potential drawback of early ART is increased risk of immune reconstitution inflammatory syndrome (IRIS). A clinical trial of prednisolone for the treatment of TB-IRIS demonstrated

reductions in the duration of symptoms and reduced hospitalisation compared to placebo [124]. However, IRIS is rarely fatal in HIV-associated TB [125]. Whilst concurrent first-line ART and TB treatment is relatively straightforward due to widespread availability of efavirenz based regimens and therefore relatively few pharmacokinetic drug interactions, second-line ART (which is mostly protease-inhibitor based) remains problematic [119]. Despite evidence that incident TB during ART is a predictor for poor virological control of HIV [126], few guidelines recommend screening for virological failure, and optimal management of ART switching has not been studied.

Other considerations include the prevention and treatment of HIV-related co-morbidities and co-infections. Co-trimoxazole preventative therapy has good observational and randomised controlled trial evidence of beneficial effect in patients with HIV-associated TB in a range of settings, and reduces early mortality [127–130]. A recent randomised control trial of a combination of preventative therapies in advanced HIV found mortality reductions, although many patients with active TB disease at admission were excluded [131]. There is little evidence for empirical prescription of antibacterial drugs, despite bacterial infections being common amongst TB patients [132].

At present, there remains a lack of evidence to support other adjunctive interventions in HIV-associated TB. However, there is growing interest in host-directed therapies in TB, and efficacy trials are already underway, including some recruiting patients with HIV-associated TB [133,134].

1.8 Mortality in HIV-associated TB, and burden within hospitals

TB patients undergoing treatment in sub-Saharan Africa still have a high risk of death, especially if HIV co-infected (figure 1.11) [135]. A meta-analysis relatively early in ART scale-up, and including data from 1991 to 2009, estimated that 18.8% (95% CI 14.8-22.8%) of HIV-infected patients die during treatment, compared to 3.5% of HIV uninfected patients [136]. This mortality is not spread evenly amongst different levels of health care. Another meta-analysis found patients with TB symptoms in high HIV-prevalence settings had a much higher mortality in hospital (22.6%), compared to primary care (3.1%) or community (1.6%) settings [120].

Both clinical and post-mortem studies from multiple settings have reported TB to be a major cause of hospitalisation among HIV-positive individuals: mortality among this population is high [32,137]. Meta-analysis of observational hospital cohorts found that 24% (95% CI 18-29%) of adult HIV-related hospital admissions in Africa and 27% (95% CI 21-33%) of hospital HIV-

related deaths were caused by TB, and in-hospital case fatality for HIV-associated TB was 29% (95% CI 20-38%), although these data are likely to be subject to misclassification [138]. This may be an underestimation, given that post mortem studies have found up to half of fatal HIV-associated TB in health facilities remains undiagnosed [32]. This high mortality risk appears to have persisted despite implementation of evidence-based interventions to reduce mortality (rifamycin containing regimen, co-trimoxazole prophylactic therapy and early initiation of antiretroviral therapy (ART) [119,139].

FIG. 3.17
Estimates of the case fatality ratio (CFR), (including HIV-negative and HIV-positive people), 2016

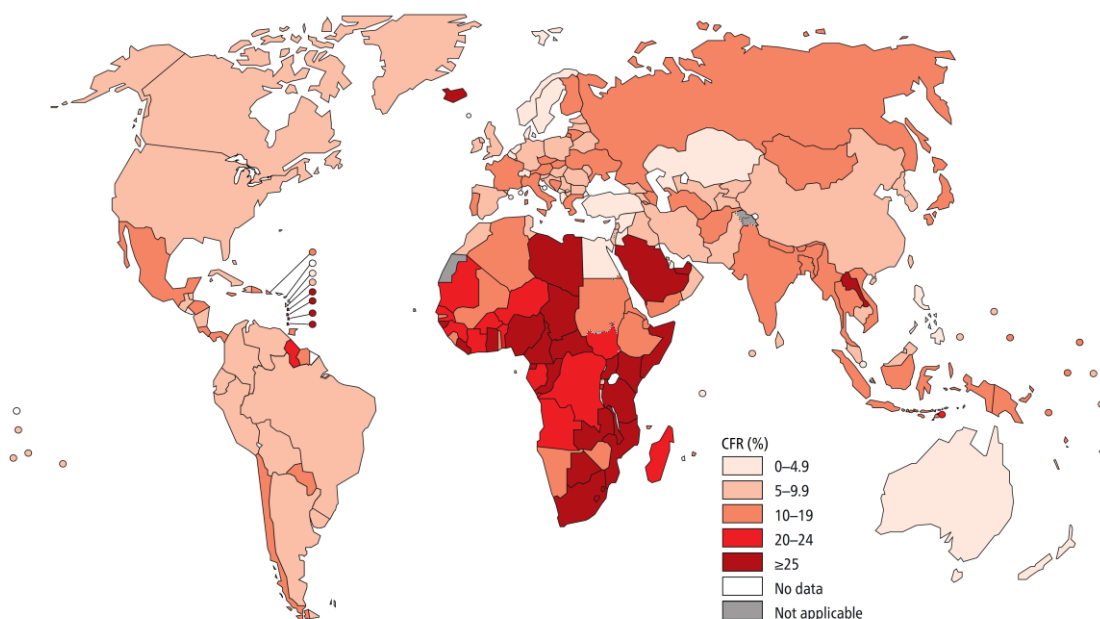


Figure 1.11 Estimates of case fatality ratios for TB from 2016 data. Taken from The Global Tuberculosis Report 2017 [8].

1.9 Risk factors for mortality

Few studies have described in detail the clinical and/or epidemiological risk factors for mortality among hospitalised patients with HIV-associated TB after diagnosis and commencement of treatment (see table 1.3). HIV-positivity is a strong risk factor for death among TB patients [135]. However, among HIV and TB co-infected inpatients, current CD4 cell count (measured at the time of diagnosis or admission) was the overriding predictor of mortality in both crude and adjusted analyses [110,140–142].

Whereas greater mortality has been found in smear-negative pulmonary TB patients in high HIV-prevalent settings [135], no studies in HIV-positive in-patients have reported sputum smear-negativity as a risk factor, and only one study found extrapulmonary TB to be risk

associated with death [143]. *MTB* bacteraemia has been identified as a common cause of severe sepsis syndrome in hospitalised HIV-infected adults in Uganda [144]. However, few studies have examined clinical signs on presentation in relationship to early mortality. Increased respiratory rate and lower peripheral capillary oxygen saturation were associated with early mortality in one study [140]. A clinical prediction score has been developed to predict mortality in HIV-positive patients with pneumonia (including pulmonary TB), but has not been validated in extrapulmonary HIV-associated TB or in different settings [145].

Both indices of malnutrition (e.g. body mass index <17kg/m² and mid-upper arm circumference <220mm) and anaemia are risk factors for death in TB patients in ambulant TB patients [135], but no studies have demonstrated this association in hospitalised HIV-TB patients. TB treatment delay was shown to predict mortality in one study [146]. Being ART naïve or having a more recent HIV diagnosis were also predictors for mortality [140,141,143]. However, these data mainly relate to ART naïve individuals, and include patients without bacteriologically-confirmed TB disease. Risk factors for mortality in hospitalised patients with HIV-associated TB may have also changed over time given the implementation of public health interventions to address HIV-TB, such as isoniazid preventative therapy, co-trimoxazole and improved ART coverage.

1.10 Association between urine diagnostics and outcomes

In addition to its diagnostic yield, observational studies from SSA have shown increased mortality in patients with HIV-associated TB who test positive for LAM in urine compared to those who are *urine LAM* negative [74]. A cohort in South Africa demonstrated 25% mortality in hospitalised patients with HIV-TB who were urine LAM-positive compared to 7% in those who were urine LAM-negative (figure 1.12) [77,89]. After adjustment for other risk factors, urine LAM was associated with an adjusted hazard ratio for death of 4.2 (95%CI 1.5-11.8). Urine LAM status was a stronger predictor of mortality than CD4 cell count.

The association between urine Xpert positivity and mortality has not been reported in the literature, but given the similar pathological mechanisms to LAM positivity, urine Xpert is likely to be associated with increased mortality risk.

Citation	TB definition	Population	Total HIV-TB patients	Deaths (%)	Follow-up duration	Factors associated with mortality (unadjusted analysis)	Factors associated with mortality (in adjusted analysis)	Proportion taking ART at baseline	Comments and limitations
Kyeyune et al 2010 [140]	Culture +ve TB	TB suspects (cough >2 weeks, 353)	190	59 (31)	2 months	Lower CD4 cell count; Higher Respiratory Rate, Lower oxygen saturation, Known HIV infection	Not reported	16%	Factors associated with mortality in all TB suspects, not confirmed TB. 13% LTFU.
Diendere et al 2015 [146]	Smear -ve or EPTB (presumptive)	Not reported	116	48 (41)	2 months	TB treatment delay (>30 days), hospital admission duration >15 days	Not reported	54%	Data pre ART scale-up (2007-2008), only smear -ve
Bigna et al 2015 [143]	Clinical and/or microbiological	Retrospective TB cohort	337	89 (26)	2 months	Extrapulmonary TB, non-AIDS comorbidity	Increased duration of known HIV infection	5%	Data pre ART scale-up (2006-2013), retrospective data collection
Holtz et al 2011 [142]	Presumptive (smear -ve)	Seriously ill ad suspected TB	344	87 (25)	2 months	Not reported	Lower CD4 cell count	15%	Old data (2008-2009), excluded patients unable to provide sputum
Manabe et al 2014 [110]	Clinical and/or microbiological	Suspected TB	145	32 (22)	2 months	Not reported	CD4 <50 cells/ μ L	38%	High LTFU, Factors associated with mortality in all TB suspects, not confirmed TB.

Continued on the next page

Kirenga et al 2014 [141]	Microbiological (culture-based)	Suspected TB	64	25 (39)	6 months	Not reported	CD4 <50 cells/ μ L, Karnofsky performance score <60	23%	Old data (2003-2006)
Crump et al 2014 [147]	Mycobacterial blood culture positive	Febrile with weight loss	29	15 (52)	36 days	Lower CD4 cell count or total lymphocyte count	Not reported	Not reported	Non-generalisable cohort with only mycobacteraemia
Marcy et al 2014 [148]	Smear +ve pulmonary TB	CD4<200, ART naïve	661	149 (23)	50 weeks	Age >40 years, BMI <16, CD4 <25 cells/ μ L, Haemoglobin <7 g/dL, Disseminated TB, MDR-TB	Disseminated, Age >40 years, BMI <16, inadequate TB therapy, Haemoglobin <7 g/dL	All ART naïve	All patients ART naïve, non-African setting
Subbarao et al 2015 [149]	Microbiological or clinico-radiologically	Consecutive hospitalised TB patients	99	32 (32)	8 weeks	Age>50, CD4 <50 cells/ μ L, poor performance status, GCS <14, Albumin <24g/L, Lactate >4mmol/L, CRP >165mg/L	Age>50, CD4 <50 cells/, GCS <14, Lactate >4mmol/L,	22%	Small cohort, small number of deaths
Talbot et al 2012 [82]	Microbiological (culture-based)	Suspected TB	69	22 (58)	2 months	Not reported	Not taking ART	Not reported	Data pre ART scale-up (2007-2008), very high LTFU

Table 1.3 Summary of studies reporting mortality in hospitalised patients with HIV and TB co-infection.

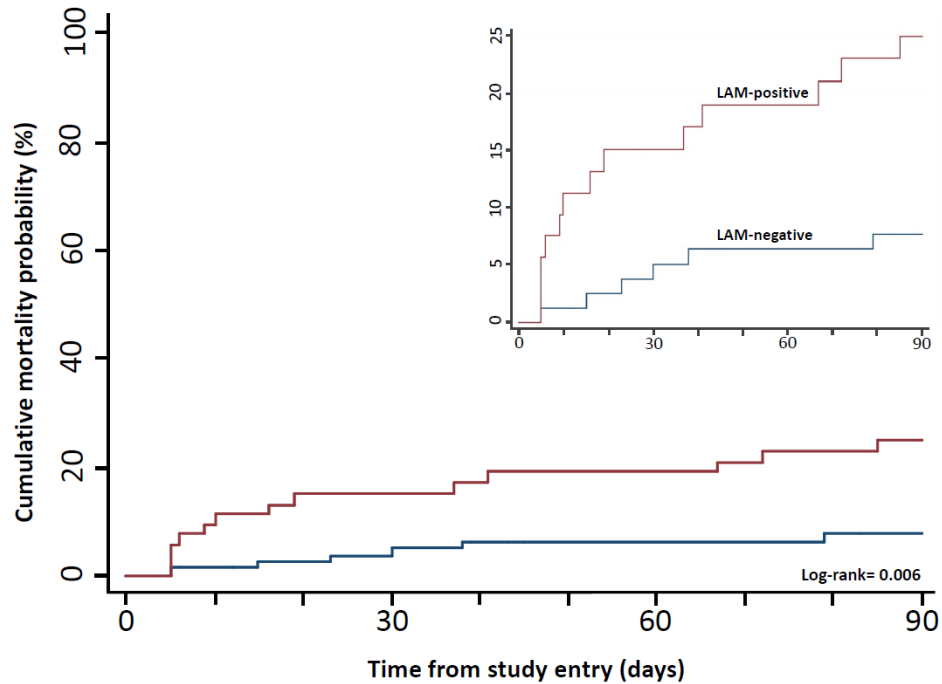


Figure 1.12 Cumulative probability of death of confirmed cases of HIV-associated TB over 90 days from study enrolment, stratified by *urine* LAM-status. Urine LAM positive is the red solid line, and urine LAM negative is the blue solid line. Magnified view shown in upper right [89].

Clinical utility of urine diagnostics for HIV-associated TB

Based on the diagnostic accuracy and yield, urine LAM lateral flow assays (specifically the Determine TB-LAM assay which was the only commercial assay available) were conditionally approved by WHO for use in HIV-positive patients with CD4 cell counts <100 cells/ μ L, or those who are seriously ill in 2015 [150]. Furthermore, in 2016, an individually randomised controlled trial showed a 4% absolute reduction in early mortality when the TB-LAM assay was used to test HIV-positive hospital admissions suspected of having TB, and positive results triggered TB treatment [151].

Urine-testing identifies a different population of patients with HIV-associated TB than to sputum-based diagnostics assays, raising the possibility of combining them to screen for or diagnose HIV-TB [70,74]. Similarly, urine LAM identified a different population to those treated empirically for HIV-associated TB [94]. In addition to increasing diagnostic yield, urine LAM appears to identify those patients who have worse prognostic indicators or higher mortality risk [86,91,110]. These patients maybe the most likely to benefit from interventions such as early commencement of TB-treatment, supportive care or adjunctive therapies. By virtue of their low-cost, technically simple operation, existing infrastructure (in the case of urine Xpert)

and rapid results, urine diagnostics appears to be well suited for use in low-resource settings among patients with advanced HIV, for example in hospitalised patients.

Implications of increased mortality risk with positive urine diagnostics

The mechanisms underlying the association between urine LAM-positivity and increased mortality risk after diagnosis and commencement of anti-tuberculosis treatment remain unclear, although there are multiple potential mechanisms. Urine LAM detection has been associated with mycobacteraemia and other markers of higher mycobacterial burden [91,112,113,152,153]. Therefore, urine LAM detection may simply be a marker of more severe, disseminated TB, explaining its association with mortality. However, the increased mortality risk may be related to TB-disease, to other co-morbidities, for example increased susceptibility to other infections, or a combination of both. Urine LAM assays are also more sensitive in patients with low CD4 cell counts (typically <50-100 cells/ μ l) [74], and these patients are thought to have higher mortality risk. Urine LAM detection may simply be an epiphenomenon detecting patients with more advanced immunosuppression.

The host immune responses to *MTB* has long been considered an important factor in the outcome of TB infection, progression to disease and outcome of treatment. *MTB* is an intracellular pathogen, and its survival depends on its ability to suppress host immune responses. Much *in vitro* research has been done examining LAM's role as a key virulence factor for *MTB*. LAM interacts with several receptors of the immune-system and has direct effects which impair host immune defences [154]. Therefore, urine diagnostic test positive patients may have a greater degree of immune suppression resulting from a higher mycobacterial and/or LAM burden, directly contribute to increased mortality risk among urine-positive patients.

In short, urine diagnostics (particularly urine LAM assays) are a promising tool that could contribute as point of care tests able to detect TB disease in the sickest HIV-positive patients. They are aimed at disseminated TB, and are therefore particularly suited to hospital inpatients. Interventions are still needed for HIV-associated TB, as mortality in HIV-positive inpatients with suspected or confirmed TB remains unacceptably high (despite ART scale-up) and TB is the major cause of death. Furthermore, point of care diagnostics for critically ill patients will open up many research avenues beyond simple diagnostic tests, such as predicting outcomes or benefit from additional interventions.

Part 2: Immune responses in HIV-associated TB

Summary

This section reviews the human host immune response to *Mycobacterium tuberculosis* (*MTB*) infection. It summarises the multifaceted immune responses that control tuberculosis (TB), including innate and adaptive immunity, and cytokine and cellular functions. The impact of HIV infection on host susceptibility to *MTB* and control of infection, beyond the simple depletion of CD4⁺ T-lymphocytes is also discussed, as HIV causes widespread immune deficits that can have an impact on the pathogenesis of TB. I summarise the evidence linking immune responses with clinical features and outcomes in HIV-associated TB, including studies of mechanisms and/or pathways as well as more functional aspects of the immune system. Finally, I review specific evidence that implicates disseminated HIV-associated TB (diagnosable by urine diagnostic assays including lipoarabinomannan detection) with impaired immunological function.

1.11 The immune response to TB

Introduction

One-quarter of the world's population is thought to be infected with *Mycobacterium tuberculosis* complex (*MTB*), yet only 5-10% will develop active tuberculosis (TB) disease during their lifetime, indicating the complexity and variability of the human immune response to TB [155,156]. There are a range of outcomes following exposure to and infection with *MTB* (figure 2.1) [38]. Development of active TB depends upon several factors, including the environment, the pathogen and suppression of host defences by HIV co-infection and other immune-deficiencies [157].

It is clear from studying human TB disease and animal models that cluster of differentiation (CD) 4⁺ T-cells, macrophages, interferon-gamma (IFN- γ) and tumour necrosis factor-alpha (TNF- α) are crucial to the control of *MTB*, yet our understanding of the wide heterogeneity of clinical disease and immune control of *MTB* remains incomplete [158,159]. An important feature of *MTB* is its ability to undermine and therefore survive the host's immune responses, yet also use immune-mediated tissue destruction to further its transmission [38].

Protective immune responses against *MTB* are multifaceted, and involve an interaction between innate immune responses, granuloma development and adaptive immunity. Innate responses are fundamental to antigen processing and presentation required by adaptive immunity, as well as early bacterial control [157]. *MTB* usually infects humans through

inhalation of aerosolised bacilli within small (1-5µm) droplets [160]. Internalisation of *MTB* by macrophages and dendritic cells (DCs) is an important initial step following infection, and typically occurs in the lower respiratory tract alveolar macrophages [161]. DCs containing phagocytosed *MTB* can then migrate to regional lymph nodes where they activate T-cells, which will subsequently migrate back to the site of infection, attracted by inflammatory signals such as chemokines [162]. Neutrophils also predominate in this early phase.

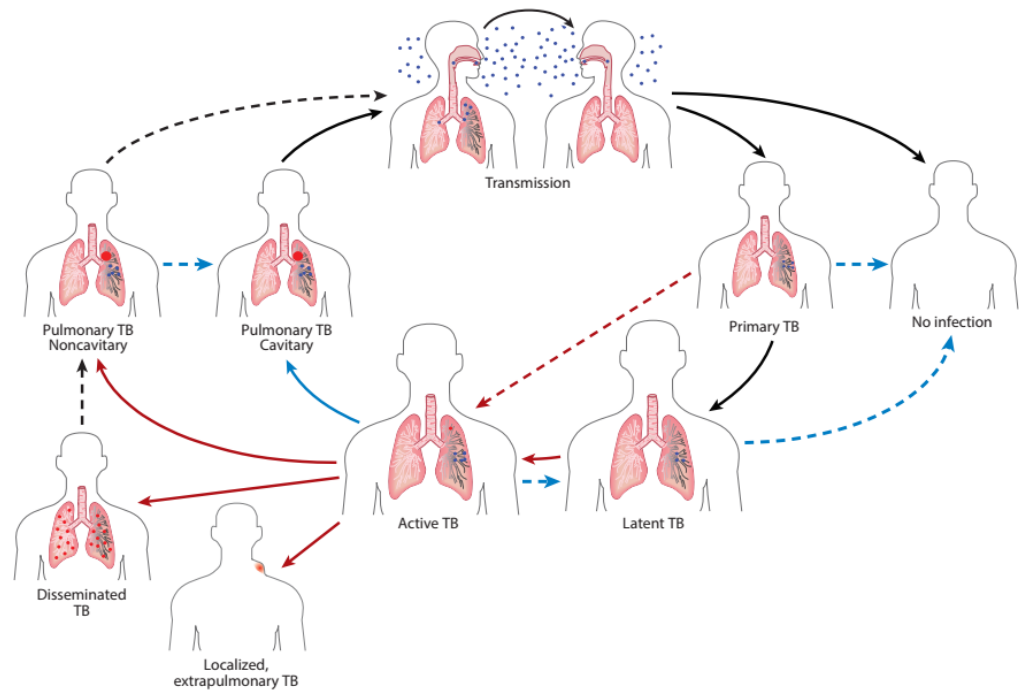


Figure 1.13 Range of outcomes from *MTB* exposure. HIV infection alters the risk of active TB, and the spectrum of clinical disease (red arrows), especially in advanced HIV disease when immune responses are inadequate to control *MTB* replication and infection, causing progressive dissemination and reduced immune mediated tissue damage (which causes pulmonary cavitation). Figure reproduced from Esmail et al [38]

A later stage in the immune response is granuloma formation, which coincides with development of delayed hypersensitivity (a positive tuberculin skin test in some people) and emergence of activated T-cell subsets [163]. Granulomata serve to contain *MTB* infection by acting as an immunological platform to limit replication, and involve activated macrophages, DCs, T-cells, fibroblasts and B-cells. There is also a humoral response producing a wide array of antibodies to *MTB* which are likely to be important in protective immunity, although their exact role remains less clear [164]. For instance, specific antibodies to *MTB* in patients with latent disease were better at controlling *MTB* growth than those from patients with active TB disease [165]. The host response to the failure of granuloma to contain *MTB* infection is also important and likely to influence outcomes, especially in HIV when this is more common [38].

Phagocytosis, macrophages and antigen presentation

Phagocytosis is mediated by an array of receptors on the surface of macrophages, including complement receptors (especially CR3), scavenger receptors, toll-like receptors (TLR) and the mannose receptor (MR) [166,167]. Mycobacterial cell wall glycoproteins, such as mannosylated lipoarabinomannan (LAM) and 19 kDa protein, also play a key role mediating entry into the macrophage independently of complement [168].

Once *MTB* is internalised by macrophages, processes begin that lead to degradation and killing of the organism and presentation of *MTB* antigens to T-cells via antigen-presenting molecules, most notably major histocompatibility complex (MHC) class I and II [169]. DCs are responsible for processing and presenting *MTB* antigens with co-stimulatory molecules to activate CD4⁺ T-cells (via MHC II) and CD8⁺ cytotoxic T-cells (via MHC I) [170,171]. DC-specific intracellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) and C-type lectin are important receptors for *MTB*'s entry into DCs, binding strongly to LAM and other *MTB* surface glycolipids [172].

The major mechanism of killing is phagocytosis followed by maturation of phagosomes through fusion with endosomes and lysosomes and gradual acidification, which is thought to occur predominantly in activated macrophages [173]. However, in contrast to non-pathogenic organisms such as non-tuberculous mycobacteria, *MTB* can prevent these processes and adapt to the intracellular macrophage environment through several mechanisms [174]. Other mechanisms of killing *MTB* include macrophage necrotic death (which may allow *MTB* to escape during cell-lysis) and apoptotic death (after which the cell membrane remains intact preventing escape of *MTB*). Prevention of macrophage apoptosis is a further virulence feature of *MTB*, and *MTB* may also use macrophages as vectors for translocation [175–177].

Monocytes, which develop from myeloid progenitors and retain the ability to differentiate into macrophages and DCs, are also involved in the response to *MTB*, as demonstrated by the importance of monocyte chemoattractant protein 1 (MCP-1) in TB patients [178], and increased susceptibility to TB in patients with de-sensitisation of C-C chemokine receptor type 2 (CCR2) to MCP-1 [179]. However, their exact role and underlying mechanisms are not well characterised. Monocytes recognise mycobacterial antigens through pathogen recognition receptors (PRRs), including Toll like receptor (TLR) 2 and TLR4, and are capable of phagocytosis, degradation and presentation of *MTB* antigens via MHC II [180]. Monocytes are multi-functional, and directly produce pro-inflammatory cytokines (such as interleukin [IL]-1, IL-6, TNF- α) as well anti-inflammatory cytokine production (for example IL-10), and are important in activating T-cells [181].

Neutrophils are also important in the early defence against TB, contributing to innate immune responses through phagocytosis and killing, as well the release of antimicrobial peptides [182,183]. Their importance is a relatively recently recognised phenomenon – neutrophils are the predominant intra-pulmonary cell type infected with *MTB*, were the main driver in a transcriptional signature which differentiated active and latent TB infection, and are also sources of pro-inflammatory cytokines [184–187]. As well as clearing *MTB* bacilli, they may also contribute to dissemination of bacilli and exacerbate pathology [188]. Virulent *MTB* can also inhibit apoptosis of neutrophils, which delays antigen presentation and activation of CD4⁺ T-cells, and escape oxidative killing by neutrophils [189,190].

T-lymphocytes and the adaptive immune response

Controlling bacterial growth during *MTB* infection is reliant on CD4⁺ T-cells, as demonstrated by failure to control bacterial replication in CD4 deficient mice and high TB susceptibility amongst HIV-infected patients with low CD4⁺ T-cell counts [5,191]. Human and animal models have suggested a considerable delay in *MTB* specific T-cell responses, probably due in part to *MTB* antigen presentation and activation occurring in regional lymph nodes [192]. It can take up to 10 days after infection before naïve T-cells are activated, and around 20 days for effector immune cells to mediate protection at the site of infection [158].

This delay may allow for dissemination of *MTB* beyond the lungs, is markedly different to other lung infections and may be prolonged by *MTB*'s modulation of the immune response [193,194]. Both activated CD4⁺ T-cells and, to a lesser extent, CD8⁺ T-cells contribute to the control of *MTB* through proliferation, differentiation, and secretion of IFN- γ which activates macrophages (important for their control of *MTB*), upregulates cell-surface MHC expression and induces production of cytokines and microbicidal products such as inducible nitric oxide synthase (iNOS) [171].

However, many effector mechanisms of IFN- γ are still being elucidated [157]. Although *MTB*, being an intracellular pathogen, is preferentially recognised by the MHC II pathway, secreted *MTB* antigens are also presented through MHC I pathways and therefore elicit CD8⁺ T-cell responses [195]. CD8⁺ T-cells can kill *MTB* through lysis of *MTB* infected macrophages by granule exocytosis (using perforin, a pore-forming protein), caspase-dependent apoptosis and the microbicidal molecule granulysin, as well as producing proinflammatory cytokines [196,197].

Cytokines and chemokines

The role of TNF- α in the immune response to TB is confirmed by the substantial increase in risk of active TB disease faced by individuals on anti-TNF therapies [198,199]. TNF- α is a pro-inflammatory cytokine produced by macrophages, neutrophils, DCs and T-cells. It is fundamental to early responses to *MTB* through its role in activating macrophages, inducing cytokine and chemokine production, maturation of DCs and migration of cells to regional lymph nodes, as well as being fundamental to effective granuloma formation [200]. Murine and primate models have suggested TNF- α boosts the intracellular killing of *MTB* [201]. Interleukin (IL)-6 drives a similar pro-inflammatory response, and is produced predominantly by activated macrophages [163].

IFN- γ is secreted by activated CD4⁺ T-helper type 1 (Th1)-cells, CD8⁺ T-cells and natural killer (NK) cells [171]. A lack of IFN- γ in mice and humans is associated with failure to control *MTB* and more severe mycobacterial disease [159,202]. IFN γ is one of the most commonly detected cytokines at sites of *MTB* infection in humans, and is used to measure responses of peripheral blood mononuclear cells (PBMCs) to mycobacterial antigens [157]. Levels of IFN- γ have been shown to correlate with disease severity [203]. However, IFN- γ production is dependent on stimulation by interleukin-12 (IL-12) which is produced by *MTB*-activated macrophages and DCs [204]. IL-12 is necessary for the initial activation of IFN- γ T-Cell responses and the ongoing *MTB*-specific Th1 responses required to control chronic TB

Furthermore IL-12 upregulates CD8⁺ T-cell killing function through increasing expression of its cytotoxic products [205]. Studies have demonstrated that a lack of IL-12 increases bacterial growth, reduces IFN- γ T-cell responses and increases disease severity [206,207]. Mutations in the IL-12/IFN- γ axis increase susceptibility to and severity of mycobacterial disease [208]. There are several other cytokines with IL-12 like function that are produced by antigen-presenting cells (e.g. IL-18, IL-23 and IL-27) [163].

IL-17 is thought to be protective against *MTB* by enhancing Th1 responses, recruiting monocytes and contributing to neutrophil mediated inflammation, although much of this is inferred from mouse models [209,210]. There are suggestions that overexpression of IL-17 could be detrimental also, as it has been associated with neutrophil-recruitment and exacerbated lung inflammation in mice, and TB-immune reconstitution inflammatory syndrome (IRIS) in patients with meningitis [211,212]. IL-22, which like IL-17 is expressed by Th17 CD4 cells, has been associated with severe TB in animal models, and elevated levels have been found at the site of TB disease and in the plasma of TB patients [213,214].

As uncontrolled TNF- α responses can cause significant damage to human tissues, regulation mechanisms are required, which are provided by regulatory T-cells (Tregs), T-helper type 2 (Th2) and IL-10 in *MTB* infection [193]. The balance between these inflammatory and regulatory systems is crucial to the outcome of *MTB* infection, with excesses of either system being broadly detrimental to the host [157]. If IL-10, an immunosuppressive cytokine made by macrophages, DCs and lymphocytes, is overproduced it can contribute to chronic infection [215]. IL-10 works through inhibiting macrophage killing of *MTB*, DC function and production of pro-inflammatory cytokines such as TNF- α [216].

1.12 Impact of HIV on the immune response to TB

Several clinical and epidemiological studies have shown a significantly increased risk of active TB disease associated with HIV-infection, with depletion of CD4⁺ T-cells (the hallmark of HIV) being the strongest risk factor [217,218]. The annual risk of reactivation of latent TB infection in HIV-infected individuals is estimated to be 5-15% annually, and this population is also at increased susceptibility to new *MTB* infections [219]. Evidence from DNA fingerprinting studies suggest that, in high-incidence settings, TB is predominantly due to acquisition of new infection rather than reactivation of 'latent' infection [13,14,220].

HIV replication has been shown to be increased at sites of *MTB* infection [221,222]. Whilst this may have an adverse impact on the immune response to HIV, it can also impact the immune response to TB [223]. Mechanisms through which HIV interacts with *MTB* include up-regulating *MTB* entry receptors on macrophages [224], interfering with macrophage killing of *MTB* [225–227] and macrophage apoptosis [228,229], inhibiting chemotaxis pathways, and decreasing Th1 responses/increasing Th2 responses [230]. These effects may be particularly marked in the lungs [231].

T-cells

As described above, CD8⁺ T-cells also play a role in the immune response to TB and can be impaired by HIV infection, although this is little studied in the context of TB [38,232]. Chronic activation is characteristic of advanced HIV, and impacts CD8⁺ T-cells more than CD4⁺ T-cells, therefore it is plausible that their activity in *MTB* control is impaired in HIV-coinfected individuals [233]. Granulomas require CD4⁺ T-cells and TNF α for maintenance, and are crucial for containing *MTB* infections. HIV and *MTB*-coinfected individuals have atypical granuloma structure, which probably makes them less effective at containing *MTB* infection [223]. Clinically, this manifests as fewer cavitory lesions on chest x-ray and more disseminated TB disease with multiple organ involvement outside the lungs [40,234].

MTB-specific memory CD4⁺ T-cells have been shown to be decreased in HIV-infection [235,236] with lower IFN- γ , TNF α and IL-2 production [237–239]. Very early impairment of *MTB*-specific immune responses and increased risk of TB disease before extensive CD4⁺ T-cell depletion in peripheral blood suggests that HIV also impairs CD4⁺ T-cell function (in addition to depletion of numbers) [240,241]. Potential mechanisms include reducing capacity to secrete chemokines and cytokines (e.g. macrophage inflammatory protein 1 β [MIP-1 β]), impairing recruitment of monocytes and granulocytes and activation of macrophages [236,242,243].

Innate immunity

HIV also adversely impacts the function of neutrophils- impairing activation, phagocytosis, respiratory burst and subsequent killing capacity [188]. This appears to be correlated with HIV-associated immunosuppression, as patients with higher HIV plasma viral loads and lower CD4 cell counts have greater impairment of neutrophil function. Neutrophils from HIV-positive patients were less effective at restricting *MTB* growth *ex vivo* than HIV-negative whole blood neutrophils [244], and HIV can impair *MTB* phagocytosis by neutrophils [245].

Traditionally, monocytes were not considered to be a major target of HIV infection, despite increased activation, with their differentiation into macrophages considered more important [246]. However, monocytes can be infected by HIV, and are potential viral reservoirs [247,248]. HIV impairs the phagocytic ability and cytokine production of monocytes through similar mechanisms as in macrophages [246]. This increases host susceptibility to broad range of pathogens including *MTB*. However, monocyte function in HIV-associated TB has not been well studied beyond finding increased levels of activation [249,250].

The aim of antiretroviral therapy (ART) is to suppress HIV viral replication, and subsequently restore circulating CD4⁺ T-cell numbers and protective immune function. Although CD4⁺ T-cell depletion is an important factor in the increased risk of TB, recovery of CD4⁺ T-cells associated with ART does not entirely mitigate increased TB risk compared to HIV-uninfected individuals [5]. Suppression of HIV viral replication does not fully restore immune responses to *MTB*, including alveolar macrophage function and pulmonary cytokine networks [251,252].

1.13 Relationship between immune responses and clinical features or outcomes in HIV-associated TB

Although a vast literature exists describing the human immune response to *MTB* [253], few studies have sought to correlate immune responses with clinical features of disease or outcomes in patients. Host immune responses are central to the control of TB, as

demonstrated by the marked increase in TB incidence among HIV-positive patients with impaired cell-mediated immunity in the form of reduced CD4 cell counts [5].

Most immunological research on *MTB* has been in the context of animal models, comparing TB patients with healthy controls (without TB), or developing diagnostics and/or biomarkers for TB infection and disease. Most HIV-associated TB research has aimed to understand the phenomenon of TB-IRIS. Furthermore, in the few studies that have examined relationships with clinical outcomes, those with HIV-infection are often excluded (usually to reduce the 'confounding' caused by HIV's impact on the immune system). Studies examining the immune mechanisms of TB-IRIS in HIV-infected patients frequently exclude those who die early. Those studies that have examined immune responses and clinical features usually recruit HIV and/or TB patients from outpatient clinics, rarely studying immune responses in those with advanced or severe infections.

Cytokine responses in HIV-associated TB and 'immune exhaustion'

Cytokines have constituted the main line of investigation for characterising the immune responses of patients with HIV-associated TB. This is justified given the importance of cytokines in orchestrating protective immunity against *MTB* [254]. Pro-inflammatory cytokines such as IL-6, IL-12, IFN- γ and TNF- α , as well as other markers of inflammation such as C-reactive protein (CRP), have been repeatedly found to be higher at baseline in TB and HIV-associated TB patients compared to healthy controls in unstimulated plasma or serum samples [255–257]. Several studies have compared cytokine levels in HIV-positive and HIV-negative TB patients, however, results have differed. Some studies found higher levels of pro-inflammatory cytokines including IFN γ , TNF α , IL-12 and IP-10 [258–260], whilst others found lower levels or no differences [255,261–263]. These differing findings may be due to TB disease severity being more important in influencing immune responses than HIV co-infection alone.

TNF- α and other pro-inflammatory cytokines have been associated with more advanced TB disease and constitutional symptoms [203,264], and clinical deterioration during treatment [256,265]. The few studies stratifying cytokine responses in TB patients by survival have found higher plasma or serum levels of IL-6, CRP and TNF- α in those that died [266–268]. However, studies examining inflammatory cytokine responses to *ex vivo* stimulation with bacterial or mycobacterial antigens have found decreased cytokine responses following stimulation among patients who die, compared to those who survive [269,270].

These decreased cytokine responses are even more marked in HIV-TB co-infected patients compared to TB alone [255,271], although this maybe confounded by the finding of suppressed inflammatory cytokine responses in HIV-infected patients who die irrespective of

cause [272–274]. Suppressed cytokine responses to antigenic stimulation seem contradictory to the raised unstimulated inflammatory cytokine levels, but may be part of an overall picture of ‘immune exhaustion’ or anergy, caused by chronic stimulation or active immune suppression by endogenous or exogenous substances.

This phenomenon is well described for T-cells in association with the cell surface marker programmed cell death protein 1 (PD-1), and is hypothesised to be caused by chronic and/or overwhelming antigen stimulation in chronic infections such as HIV, but has not been well described in the context of TB [275–280]. There appears to be an overlap with ‘anergy’ (non-responsiveness to *in vitro* or *ex vivo* stimulation) and ‘senescence’ (terminal differentiation and loss of proliferative capacity), although molecular-signatures of the processes have been shown to differ.[281]

Some studies found high serum levels of the anti-inflammatory cytokine IL-10 and increased IL-10 responses to stimulation, especially in advanced disease, which may partially explain observed anergy [203,256,282,283]. No studies have clearly demonstrated high baseline inflammatory cytokine profiles and reduced responses to stimulation in patients with advanced HIV-TB coinfection in the post-ART era, nor studied the association between immune exhaustion and clinical outcomes in this patient population. The containment of *MTB* likely requires a tightly regulated cytokine response, as both inflammation and activation are required but an excess can be detrimental to control of *MTB* and outcome [38]. However, relationships between different cytokines are rarely studied in relation to outcomes.

In addition to absolute cytokine levels, functional deficits in CD4⁺ T-cells, which are important in the production of cytokines in HIV-TB, have been demonstrated in response to *MTB* antigens [284]. Polyfunctional CD4⁺ T-cells are capable of simultaneous release of multiple inflammatory cytokines (with multiple effector functions), and have been associated with improved TB control in HIV-uninfected patients, as well as better control of several other pathogens [285–287]. Increasing HIV-1 viral load has been shown to reduce numbers of polyfunctional CD4⁺ T-cells, and lower proportions have been found at sites of TB disease compared to blood in HIV-TB [288,289]. However, their role in advanced disease or with relation to clinical outcomes has not been described.

Functional immune responses in HIV-associated TB

One criticism of studies examining cytokine responses alone is interpretation of the results. Cytokine networks are complex, and making distinctions between the innate and adaptive immune responses or underlying pathogenic mechanisms based on cytokines alone is difficult as they are intricately related and regulate one another. Detected associations may arise

simply by chance when multiple comparisons are made without appropriate statistical adjustments. 'Appropriate' or 'effective' immune responses are likely to require a balance between anti-inflammatory and pro-inflammatory responses, and involve both innate and adaptive immune systems, driven by many host and pathogen factors.

Studies that examined 'functional' immune responses in patients with HIV-TB, especially with relation to clinical outcomes, are limited. One recent study showed reduced PPD-specific ELISpot responses in HIV-TB patients who died compared to those who survived [266]. Tuberculin-skin-test (TST) anergy was an independent risk factor for death among HIV-infected patients in a high TB prevalence settings [290], but this relationship has not been examined or confirmed in HIV-associated TB.

ELISpot and TST outputs relate to TB-specific immunity alone, and broader immune competence is likely to be important in outcomes as co-infections are important causes of mortality [32,291,292]. Whilst whole-blood killing assays are well-established [293], their use tends to be to assess responses to vaccinations or pathogen-specific questions rather than being applied to broader questions regarding the general state of immune competence of individuals [294]. These methods have not been widely applied to individuals with HIV-associated TB.

Immunological effects of *MTB*, the example of LAM

LAM is a 19kDa glycolipid, a major constituent of *MTB* and one of several interrelated lipopolysaccharides within the mycobacterial cell wall [295]. LAM is made up of three structural domains: the glycopospholipid anchor, the mannan core and arabinan domain [296]. Variable capping of side-chains of the arabinan domain lead to several different types of LAM, with a mannosylated cap giving rise to the specific type of LAM (ManLAM) associated with pathogenic mycobacterial species such as *M. tuberculosis* complex (*MTB*), *M. leprae* and *M. bovis* [297].

ManLAM is considered a major virulence factor of *MTB* through its *in vitro* immunomodulatory effects (figure 2.2), promoting the survival of *MTB* in the human host [297]. In contrast, non-pathogenic mycobacterial lipoarabinomannans that are capped with phosphate-inositol (PILAM) or not capped at all (AraLAM) promote strong pro-inflammatory responses [297,298]. ManLAM binds to several receptors on the surface of macrophages [299]. Notably, LAM has been shown to inhibit apoptosis [300] and phagosome-lysosome fusion within macrophages [301,302]. ManLAM (from here on referred to as LAM) reduces secretion of pro-inflammatory cytokines including IL-12, IFN- γ and TNF- α [154,303,304], while increasing anti-inflammatory cytokines such as IL-10 [303,305,306]. It has also been demonstrated to negatively modulate

nitric oxide and free radical production within cells, and to downregulate T-cell signalling [299]. Although these *in vitro* immunomodulatory effects are likely to promote survival of *MTB* in the host and impair host immune response to other pathogens, this has not been tested or demonstrated in TB patients.

In addition to being found in the urine of patients with pulmonary TB, LAM has been detected from sites of TB infection, including sputum [307,308], pleural fluid [309], and cerebrospinal fluid (CSF) [310]. LAM has also been detected in the blood of patients with pulmonary TB, albeit following extensive sample processing due to being complexed with anti-LAM antibodies and/or high density lipoprotein (HDL) particles [99,100]. LAM can be recovered in large quantities from cultures of *MTB* (up to 15mg per gram of *MTB*) [307,311], suggesting there may be sufficiently high systemic concentrations of LAM in patients with disseminated TB to be immunologically active. However, due to LAM being largely intracellular and/or protein- or lipid-bound, the relationship between urine LAM detection and serum LAM concentrations has not been established.

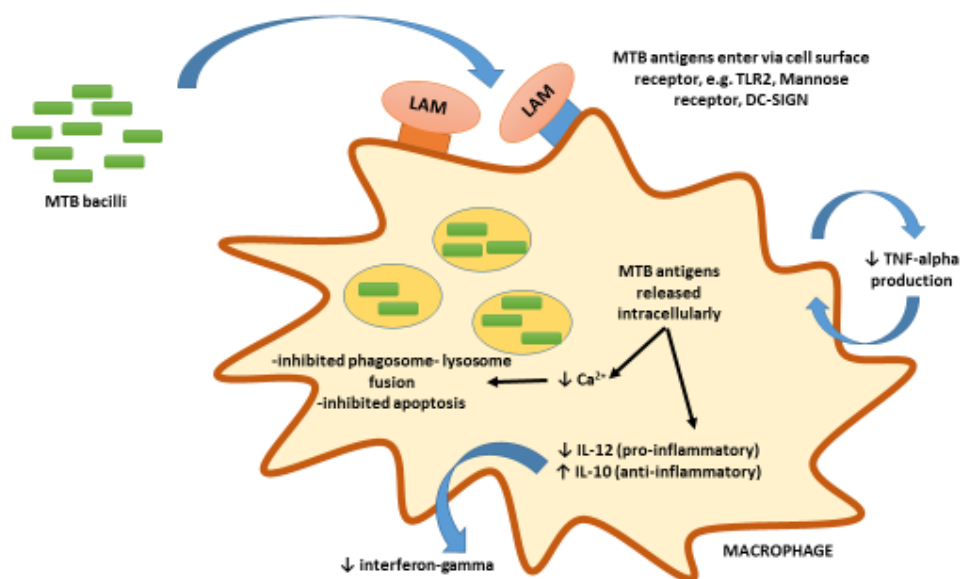


Figure 1.14 Summary of the immunological mechanisms by which LAM acts as a virulence factor and suppresses host immune responses to *MTB*.

Other potential immunological mechanisms of poor clinical outcomes

LAM's direct suppressive effects on macrophages and cytokine responses could be a potential mechanism for the poor clinical outcomes in HIV-associated TB patients with positive urine-

LAM assays. Another potential mechanism for poor outcomes in advanced HIV-associated TB includes T-regulatory cells (T-regs), which reduce effector T-cell activation and response as part of an immune homeostatic mechanism in response to inflammation [312]. This may favour the survival of *MTB*. Several studies have also demonstrated increased T-regs in disseminated and extra-pulmonary TB [313,314], but none in relation to outcomes.

Monocytes are a key component of the immune response to HIV and TB [180]. Soluble markers of monocyte activation (sCD14) have been described in HIV-associated TB [249,250,315].

Human monocyte populations can also be described in terms of their surface markers (CD14 and CD16), with very different functional roles. CD14⁻CD16⁺ (non-classical) monocytes are usually referred to as 'pro-inflammatory' as they produce higher quantities of TNF- α , IL-6 and IL-1, but low quantities of IL-10, and are upregulated in most infections, including TB [316,317].

However, CD14⁺CD16⁻ (classical) monocytes have been associated with TB-IRIS [318].

Monocyte phenotype or function have not been systematically investigated with respect to clinical outcomes in advanced HIV-associated TB, although deactivation, monocyte dysfunction and increased production of IL-10 have been described in bacterial sepsis, including correlation with poor outcomes [319–321].

Anaemia is strongly associated with mortality in HIV-associated TB, and is a strong predictor of urine LAM-detection in HIV-TB patients [322]. Hepcidin, an acute phase reactant that is stimulated by pro-inflammatory cytokines (e.g. IL-6) and antigens from *MTB*, is a central regulator of iron homeostasis [323]. In vitro studies have demonstrated that hepcidin has an important role in the immune response against *MTB*, as demonstrated *in vitro*, and recent clinical data has shown that hepcidin was independently associated with increased mortality risk in HIV-TB co-infected patients [322,324].

1.14 Immune responses and reducing mortality in HIV-associated TB

Despite public health interventions including improved access to HIV testing, coverage of ART and isoniazid preventative therapy, HIV-TB continues to have a high mortality, even after appropriate initiation of both anti-TB therapy and ART [121,122]. There is observational evidence to suggest expediting treatment through improved diagnostics can reduce mortality [142], and trials have been undertaken to address this question [325–327]. However, even after expedited diagnosis, mortality in this population remains substantial, and reductions in mortality are currently too small to reach global End TB targets.

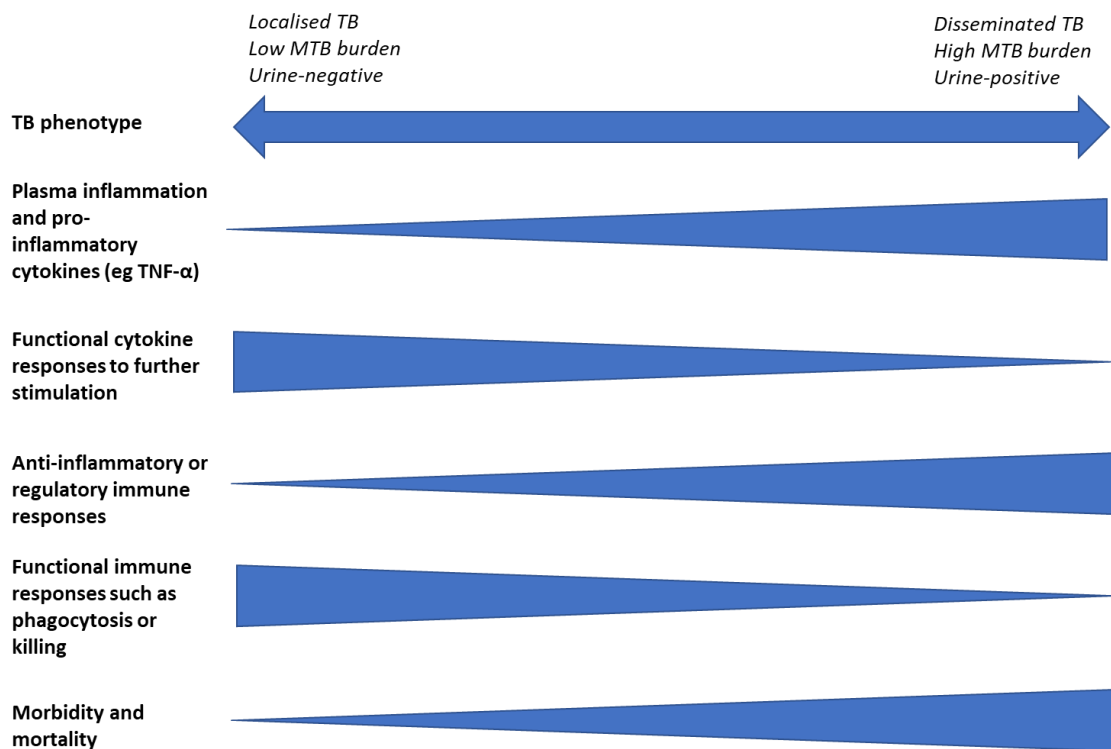


Figure 1.15 Diagrammatic summary of immune responses and outcomes based on clinical spectrum of HIV-associated TB disease. Extent of the immunological phenomenon denoted on the left is summarised by the height of the wedge.

The mechanisms of death in this population remain unclear. Post-mortem studies have established disseminated TB as commonplace in advanced HIV, and TB was thought to be the primary cause of death in 91% of patients [32]. However, HIV-infection impairs host-immune responses not only to TB but to bacterial and other pathogens, which are also common in advanced HIV [291,292]. However, the spectrum and burden of TB is also an important factor in effective immune responses, control of TB and/or HIV, and outcomes (figure 1.15). Immunological impairment is likely to contribute towards high mortality rates, although the specific mechanisms remain incompletely defined.

TB-IRIS is a condition where immune recovery associated with ART leads to pathological inflammatory responses directed at microbial antigens, and is an example of how a loss of balance between protective immunity and immunopathology can impact clinical outcomes [328]. Studies in HIV-negative TB patients also demonstrated death from TB due to extensive tissue damage even despite effective control of TB infection [329]. Immunological failure is also an interruption in the immunological balance, with inadequate pathogen-specific immune responses and control of infection. It can lead to early mortality in advance HIV-associated TB, and seems more common than TB-IRIS and has worse outcomes [329]. Addressing

'immunological failure' and IRIS would require differing approaches, with IRIS requiring inhibition and immune failure requiring enhancement of immune responses.

It is likely that interventions adjunctive to anti-TB therapy and ART will be needed to significantly reduce mortality in this patient population, for example adjunctive anti-bacterial drugs or host-directed immune-therapies [329]. In the context of a limited TB-drug pipeline, growing drug-resistance and continuing high-mortality in HIV-TB co-infection, host-directed therapies for TB are climbing up the research agenda [133]. A better understanding of immune responses and mechanisms underlying impaired immunological responses (see table 1.1) will inform further research into such adjunctive and host-directed interventions, and help guide which type of interventions to evaluate (for instance, if anergic or exhausted immune responses predominate, anti-inflammatory treatments such as steroids are unlikely to be effective). Furthermore, urine-based diagnostics, such as LAM detection or Xpert, in addition to their diagnostic utility, may prove a useful method of identifying patients at increased mortality risk who could benefit from adjunctive interventions.

Observation	What remains unknown	Possible implications
<p>HIV-associated TB patients have high baseline plasma levels of inflammatory cytokines such as TNF-α, IFN-γ and IL-6, but ex-vivo stimulated responses appear suppressed compared to HIV+ patients without TB [255], and HIV-negative TB patients [269,270]</p>	<ul style="list-style-type: none"> • Inflammatory cytokine responses following antigenic stimulation in hospitalised patients with HIV-associated TB and associations with mortality have not been well studied in the post-ART era • Cytokine data have not been linked with cellular function in this population, therefore cellular factors in this process remain unknown • If suppressed immune responses are a manifestation of ‘immune exhaustion’ 	<p>Reduced pro-inflammatory immune responses may reduce host ability to deal with <i>MTB</i> or other bacterial pathogen infections, impacting morbidity and mortality</p>
<p>More advanced clinical HIV-positive TB disease is associated with TST anergy [290] and reduced PPD-specific ELISpot responses [266]</p>	<ul style="list-style-type: none"> • Broader functional immune responses in patients with HIV-associated TB have not been well investigated, especially in relation to clinical features and outcomes 	<p>Impaired broader functional immune responses may impair host defences to <i>MTB</i> and other co-pathogens</p>
<p><i>MTB</i> antigens (eg LAM) are associated with reduced macrophage killing of <i>MTB</i> [301,302], reduced production of inflammatory cytokines (IL-12, TNF-α and IFN-γ) and greater production of anti-inflammatory cytokines (IL-10) <i>in vitro</i> [154,303,304]</p>	<ul style="list-style-type: none"> • If patients with disseminated TB and high mycobacterial burden (signified by positive urine diagnostics) have similarly impaired phagocytosis, killing and inflammatory cytokine production 	<p>Impaired immunological responses in urine-positive patients may implicate impaired immune responses in the causal pathway to increased mortality</p>
<p>Monocyte activation and function is a key immune response in HIV-associated TB [249,250,315]</p>	<ul style="list-style-type: none"> • Monocyte phenotype, activation and function in HIV-associated TB are not well described, nor are their association with outcomes 	<p>Impaired monocyte function may be responsible for impaired immune responses in HIV-TB patients, and may be a potential therapeutic target.</p>

Table 1.4 Summary of immunological mechanisms that may be implicated in impaired immune responses in HIV-TB co-infected patients

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Chapter 2: Overview of aims and study designs for thesis

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2.1 Thesis rationale

HIV-associated tuberculosis (TB) is currently one of the leading health challenges facing sub-Saharan Africa, with substantial mortality and morbidity, especially amongst people living with HIV (PLHIV) who are admitted to hospitals [1]. TB is still the major cause of death in this population, and mortality remains unacceptably high despite sustained public health interventions over the past two decades [2].

Urine-based TB diagnostics - lipoarabinomannan (LAM) detection using the Determine TB-LAM lateral flow assay (TB-LAM) and nucleic acid detection using Xpert MTB/RIF (Xpert) - are rapid, affordable, point of care (or near patient in the case of Xpert), potentially implementable and can detect disseminated TB in the sickest PLHIV, for example those admitted to hospital. Their use to diagnose HIV-associated TB, thereby reducing the number of patients with missed TB diagnoses and expediting TB treatment, may reduce early mortality [3,4]. However, mortality remains unacceptably high despite better diagnostics, TB treatment and optimally timed antiretroviral therapy (ART). Therefore, adjunctive interventions will be needed to substantially reduce mortality to meet ambitious global TB targets.

In addition to their diagnostic function, urine-based TB diagnostic assays seem to detect the sickest HIV-positive patients at highest risk of mortality. The basis of this association is not fully defined, but may be related to one, or a combination, of the following:

- disease dissemination (haematogenously disseminated disease)
- higher mycobacterial burden
- more advanced HIV-related immunosuppression
- impaired immunological host defences against *Mycobacterium tuberculosis* (*MTB*) and/or other opportunistic infections

A better understanding of the mechanisms underlying the association between positive urine diagnostic tests and mortality in HIV-associated TB could help to optimize the use of urine-based TB assays, using their prognostic as well as diagnostic value to inform the design of simple interventions applicable even in low resource health systems that are aimed at identifying and reducing the risk of death in the most severely ill PLHIV. For this to be realised, there would have to be reversible pathology as well as affordable therapeutic options but, for instance, demonstrating that the presence of *MTB* DNA and/or LAM in urine is associated with an anergic or immunosuppressed state would encourage evaluation of a different set interventions than would be the case if the association was instead with hyperinflammation.

2.2 Thesis aim and research questions

Aim

The overarching aim of this PhD was to investigate mortality and urine-based diagnostics among hospitalised patients with HIV-associated TB in sub-Saharan Africa, with specific focus on characterising the epidemiological and clinical factors associated with increased mortality risk in patients who test positive with rapid urine-based diagnostic assays, and characterising immune responses in this patient population.

Specific research questions

1. Are positive urine-based diagnostic tests associated with increased mortality in HIV-associated TB?
2. What is the absolute risk and timing of mortality in hospitalised patients with HIV-associated TB?
3. What are the risk factors for early mortality, and the clinical phenotype of patients with high mortality risk, among hospitalised patients with HIV-associated TB?
4. Can rapid urine diagnostic tests, in addition to other clinical features at hospital admission, be used to develop a pragmatic clinical score to predict mortality risk (and can such a score be externally validated)?
5. Can an *ex vivo* immunological assay be used to classify and provide insight into the immune state and functional responses of patients with HIV-associated TB in high burden settings?
6. How do *ex vivo* immune responses differ according to TB disease dissemination (based on urine diagnostic tests) and in relation to mortality?

Hypotheses

1. Urine diagnostic tests are associated with increased mortality risk in HIV-associated TB.
2. Mortality risk is high in hospitalised patients with HIV-associated mortality, and deaths occur early during hospital admission.
3. Positive urine-diagnostic assays (as a marker of disseminated TB disease), and features of advanced immunosuppression will predominate as risk factors for mortality in hospitalised patients with HIV-associated TB.
4. Urine diagnostic tests, along with other markers of advanced disease can form a pragmatic clinical score that predicts which patient have the highest mortality risk.
5. Functional immunological assays can be developed to assess clinically relevant immune states in HIV-associated TB, even in high burden settings.
6. *Ex vivo* analysis of immune responses demonstrates impairment and/or anergy in patients with HIV-associated TB who have positive diagnostic tests and in those with poor outcomes.

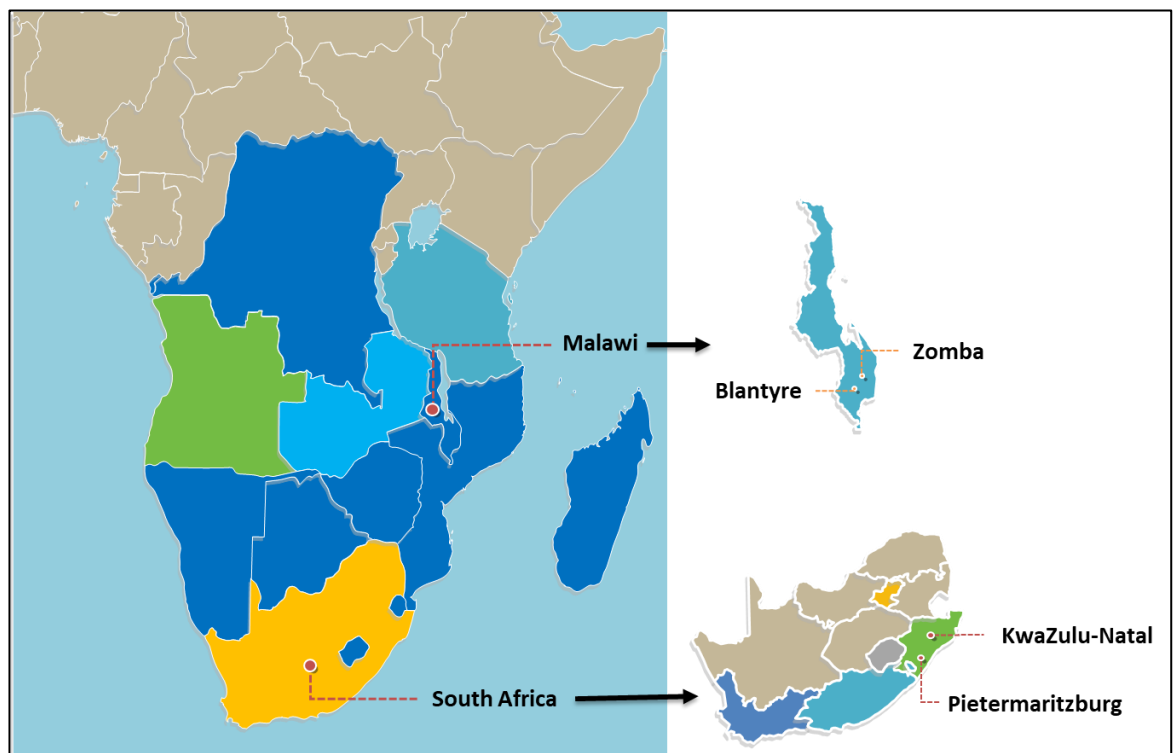


Figure 2.1 Map showing the location of the study sites

2.3 Study settings: Malawi

Malawi is one of the poorest countries globally, ranking 170/188 in the 2016 human development index [5]. Malawi is economically vulnerable with a largely agriculture based economy. Recent extreme weather events and ongoing energy shortages (<10% of the population have access to electricity) continue to hamper economic growth [6]. Meanwhile, population growth continues to place more strain on limited resources, with a population of 17.2 million growing at a predicted 3.2-3.4% rate for the next 5 years [7]. Total fertility rate is 4.4 children per women. Under-five mortality has fallen 4-fold to 64/1000 births over the last 20 years, contributing to population growth [5,6]. Malawi remains mostly rural, with 80% of the population living rurally. Only 36% of men and 26% of women have some secondary education [6].

HIV

Malawi has a generalised HIV-epidemic, with an estimated 11.2% (95% CI 10.5 to 11.9%) prevalence in adult (aged 18-49) women and 7.1% (95% CI 6.7 to 7.5%) in men [8]. However, Malawi has long had a forward-looking strategy for control of HIV, and new infections and HIV-related mortality continue to fall and ART coverage continue to grow [8]. Anti-retroviral drugs are provided free to all patients. Malawi has implemented universal HIV 'test and treat' since mid-2016, prior to that the threshold for ART was a CD4 cell count of 500 cells/ μ L. HIV care is usually delivered through local health centres, and monitoring visits are monthly for patients newly initiated for 6 months, then 2 monthly for 6 months, and thereafter 3 monthly [9]. Viral load testing is recommended to monitor response to ART, but CD4 cell count testing is not routinely available. The first line ART regimen is tenofovir, lamivudine and efavirenz as a fixed-dose combination.

TB

The 2014 Malawian TB prevalence survey included 31,579 adults screened using TB symptoms, chest radiographs and 2 sputum samples for microscopy and liquid culture if symptomatic or suggestive chest radiograph. Data from this survey suggest a national prevalence of microbiologically confirmed pulmonary TB of 452 per 100,000 (95% CI 312 to 593 per 100,000), and sputum smear positive TB of 220 per 100,000 (95% CI 142 to 297 per 100,000) [10]. 16,959 patients with microbiologically confirmed TB were notified in 2016 [11]. Drug-resistant (DR) TB is uncommon in Malawi, with 0.75% of new cases and 6.4% of 'retreatment' cases estimated to have DR-TB [10].

Malawian TB services are decentralized with dedicated Environmental Health cadre health care workers ('TB officers') within community health centres providing treatment registration

and monitoring, so that TB management is usually provided separately to other health services. Diagnostics within the national TB programme are largely limited to sputum smear microscopy in community settings, with sputum Xpert and chest radiography limited to secondary care facilities.

In Malawi, TB is treated using the standard WHO recommended 4-drug regimen for 6 months (a 2-month intensive phase with isoniazid, rifampicin, pyrazinamide, and ethambutol, followed by a 4-month continuation phase with isoniazid and rifampicin) [12]. Patients being treated for a second time, or failing their first course of treatment, are managed with streptomycin added to the intensive phase, usually requiring hospitalisation and daily intramuscular injections for 60-days. TB drugs are dispensed every 2 weeks during the first 2-months and monthly thereafter, provided free of charge. Directly observed therapy (DOT) is implemented via an appointed family member or guardian, and undertaken in the home setting. In the event of a patient not attending for follow up, health centres are required to follow-up individuals in the community.

During 2015, national treatment success rates (classified as treatment completion or cure) were estimated at 81%, comparable to global treatment success (83% in 2015) despite much higher HIV prevalence in Malawian TB patients than the global average [13].

Zomba Central Hospital

Zomba is a largely rural district in Southern Malawi (figure 2.1) with an estimated population of 799,000 in 2015, of whom approximately 138,000 live in urban Zomba. HIV prevalence is estimated to be 16.8% among 15-49 year olds in 2015 [14]. Zomba is one of the districts with the highest TB incidence in Malawi, with estimated notification of 150 per 100,000 population per annum in 2015. Over half of the TB notifications occur at Zomba Central Hospital (unpublished data, Malawi National TB Programme).

Zomba Central Hospital serves as a district hospital within Zomba district and a tertiary referral centre to 4 other district hospitals. The hospital has 500 beds and 4 main clinical departments: internal medicine, surgery, paediatrics and obstetrics & gynaecology. There is no emergency department, with admissions instead made through outpatient clinics. The hospital is mostly staffed by clinical officers (a 3-year Diploma training in Clinical Medicine). All services are provided free at the point-of-care.

The medical department has 160 beds spread over 3 wards (figure 2.2). Bed occupancy can, however, be >100% with use of mattresses on the floor during busy periods. The inpatient care is delivered by 6 clinical officers supervised by 3 medical doctors. The nurse to patient ratio is usually between 1:20 and 1:30. Only basic haematology (full blood count, cross-matching, CD4

count), biochemistry (renal and liver function), microbiology (microscopy), serology (hepatitis and syphilis) and HIV-1 viral load testing are available, although there are frequent disruptions when these services are not available. The radiology department offers radiography and ultrasound during office working hours. Complex patients can be referred to the regional referral centre (Queen Elizabeth Central Hospital) in Blantyre (approximately 60km away).

Routine TB diagnostics available on-site include sputum smear microscopy and Xpert MTB/RIF assays (during office working hours only). Only respiratory samples are processed for Xpert, and no mycobacterial culture facilities are available. Patients with suspected MDR-TB (eg Xpert rifampicin resistant samples) are managed by the National TB Programme, and samples are sent to Lilongwe for culture and first line drug sensitivity testing (second line drugs are centralized at national level in Malawi). Sputum induction is not available. Cerebrospinal fluid (CSF) can be processed for microscopy, cryptococcal antigen testing and India ink staining only. No other routine TB diagnostics are available.



Figure 2.2 Photographs of Zomba Central Hospital general medical ward (top) and the corridor outside the TB ward (bottom). Photographs were taken as part of the STAMP trial film [15], and consent was obtained from patients pictured.

HIV-associated TB is managed by TB clinics, except for at Zomba Central Hospital where there is an integrated HIV and TB clinic. ART is commenced within 2 weeks if ART naïve using the first line regimens. After discharge, HIV-associated TB patients are seen monthly at Zomba Central Hospital

for clinical review and drug refills, unless they live nearer a peripheral health facility in which case they will be referred there for TB care.

Malawi-Liverpool-Wellcome Trust Clinical Research Programme (MLW)

MLW was established over 20 years ago and is a research centre build around high quality laboratory infrastructure and strategically located in the largest hospital in the country – Queen Elizabeth Central Hospital. MLW is affiliated with the University of Malawi’s College of Medicine, and is funded by a core grant from the Wellcome Trust. I was based at MLW whilst undertaking the work reported in this thesis, and the immunology laboratory work was undertaken within laboratories at MLW (figure 2.3).



Figure 2.3 Facilities in the MLW immunology and tissue laboratory.

2.4 Study settings: South Africa

Despite South Africa being classed as a middle income country, and within medium human development category (ranked 119/188 in 2016), there remain huge inequalities [16]. The majority (65%) of 54.5 million population live in urban settings [17]. Health indicators are generally better than the rest of the Southern African region, with under-five mortality rate 40.5 per 1,000 live births. However, life expectancy remains only 57.7 years, with the HIV and TB epidemics being leading causes of reduced life expectancy [16]. KwaZulu-Natal is the province located in the Southeast of the country, with Pietermaritzburg being the

governmental capital and Durban its most populated city. The province has a population of 11.4 million and is the second most populous in South Africa.

HIV

South Africa has the highest number of HIV-positive residents of any country in the world, with South Africans accounting for almost one-fifth of the global burden of people living with HIV (PLHIV), as well as 15% of all new HIV infections and 11% of all HIV-related deaths [18]. Among adults aged 15-49 years, HIV prevalence is estimated to be 18.8% (95% CI 16.2 to 20.9%). HIV incidence was an estimated 9.6 per 1,000 adults aged 15-49 in 2016, and there are 110,000 deaths annually attributed to HIV. There have been some successes in HIV control – new infections have reduced by 31% and deaths by 43% since 2010. South Africa also has the largest treatment programme in the world, providing 20% of ART use globally, with 80% funded directly by the government.

In January 2015, the ART treatment threshold was 500 cells/ μ L, and from September 2016 universal ‘test and treat’ was introduced, prioritising patients with CD4 cell counts <350 cells/ μ L [19]. First line ART currently consists of Tenofovir, Emtricitabine and Efavirenz as a fixed-dose combination pill. HIV care is managed at the primary healthcare clinics and is primarily nurse led, with doctor input when required. Viral load monitoring is recommended at month 6, month 12 and then every 12 months while on ART. CD4 monitoring is only recommended annually and if ‘clinically indicated’.

TB

South Africa is one of the highest burden countries for TB, HIV-associated TB and drug resistant TB [13]. It is also one of seven countries that account for almost two-thirds of new TB cases, globally. TB incidence is estimated at 781 per 100,000 (95% CI 543 to 1,065 per 100,000), meaning approximately 0.8% of the population develop active TB disease each year [20,21]. Over 244,000 new TB cases were notified in 2016, with TB treatment success of 81% in new and relapsed cases. It is estimated 3.4% of new cases and 7.1% of retreatment cases have DR-TB. KwaZulu-Natal, and the Eastern and Western Cape Provinces have the highest incidence rates of TB (and highest prevalence of HIV).

TB is treated using the standard rifamycin-based 4-drug regimen for 6 months [22]. The continuation phase is extended to 7 months for TB meningitis. HIV-associated TB care is managed mainly by primary healthcare clinics, including provision of ART. Treatment naïve patients are usually prescribed ART within 2-8 weeks of TB diagnosis, often during hospital admission, along with cotrimoxazole prophylaxis.

Edendale hospital, KwaZulu-Natal

Edendale Hospital is a regional level public-sector institution, situated in the peri-urban outskirts of the uMgungundlovu District in Pietermaritzburg, KwaZulu-Natal, South Africa (figure 2.1). There is a high burden of both HIV and TB: the uMgungundlovu District has a TB notification rate of 678 cases per 100,000 population p.a. and an antenatal HIV prevalence of 44% [20].

With a 900 bed capacity, Edendale is South Africa's 4th largest hospital serving about 1 million individuals. A level 2 healthcare centre, Edendale provides specialist support to Level 1 facilities. These include four referring district hospitals, two TB hospitals, and an additional 18 Primary and Community Health Care Centres. Specialist services include emergency medicine, general internal medicine, paediatrics, general and specialist surgery, obstetrics and gynaecology, ophthalmology and psychiatry. Services are charged for on an income-based sliding scale and are essentially free to patients who are unable to pay.

The medical department consists of 234 inpatient beds across 7 wards (figure 2.4). Admissions are from the emergency department, ambulatory care or outpatient department and referrals from other hospitals. Internal medicine is staffed by 40 doctors, including eight specialist physicians, fourteen medical officers, and eighteen medical interns. Intern doctors are each responsible for approximately 15 to 20 patients. Nursing care is provided by approximately 140 nurses, with a nurse-to-patient ratio of approximately 1:8. Inpatient HIV prevalence is estimated to be 35-50%, with new TB diagnoses among inpatients estimated to be 15-20% [23]. Laboratory testing includes full biochemistry, haematology and microbiology services. Inpatient point of care HIV testing is performed by ward staff.

Routine TB diagnostics at Edendale include onsite fluorescence microscopy for acid fast bacilli and Xpert MTB/RIF (respiratory and non-respiratory samples) during working hours, with results usually available within 48 hours. Mycobacterial culture is available (off-site) for respiratory and non-respiratory samples, and drug-resistance testing for suspected drug resistant TB (eg Xpert rifampicin resistance samples). Sputum induction is not routinely performed but is available if requested. Biochemical and cell analysis are performed on extra-pulmonary specimens, including cerebrospinal, pleural and ascitic fluid. Cytology and histology services are also available. Chest radiography is available 24 hours per day and other radiology services include ultrasound and CT scanning, available during working hours. Laboratory testing includes CD4 cell count and viral load testing.



Figure 2.4 Photographs of Edendale Hospital (top) a general medical ward (bottom), demonstrating differences in resources between the two study settings. Photographs were taken as part of the STAMP trial film [15], and consent was obtained from patients pictured.

2.5 Design of studies contributing to thesis

Overview

The thesis aims and research questions were answered using prospective observational cohort designs, nested within a randomised clinical trial of rapid urine-based diagnostic assays (the STAMP trial).

The thesis consists of the following five main components which addressed the overall aim and specific research questions:

1. Systematic review and meta-analysis of the association between urine-LAM positivity and short-term (3-6 months) risk of death in patients with HIV-associated TB (research question 1)

2. Cohort study of the absolute risk of death by 56 days, and clinical and epidemiological risk factors for mortality in patients admitted to hospital with HIV-associated TB (research questions 1, 2 and 3)
3. Cohort studies for derivation (STAMP study) and external validation of a clinical score predicting short-term mortality risk in patients admitted to hospital with HIV-associated TB (research question 4)
4. Cross-sectional study assessing a whole-blood assay measuring *ex vivo* phagocyte function in HIV/TB patients and healthy volunteers (research question 5)
5. A cohort study of immunological responses in patients admitted to hospital with HIV-associated TB and their relationships to short-term mortality and urine Xpert/LAM positivity (research question 6)

An overview of each component is given below, with detailed methodologies reported in the respective research papers or chapters reporting the results.

The STAMP trial

The STAMP trial (rapid urine-based Screening for TB to reduce AIDS Mortality in hospitalised Patients in Africa) was a multi-centre trial investigating if the addition of rapid urine-based diagnostic screening for TB (using urine TB-LAM and Xpert assays) in unselected HIV-positive medical admissions can reduce mortality compared to standard screening based on sputum-testing. The trial recruited 2,600 patients in Malawi and South Africa between October 2015 and September 2017.

The STAMP trial provided the opportunity to further study mechanisms relating to mortality, factors associated with outcomes and immune responses in patients with HIV associated-TB, by recruiting and clinically characterising HIV-positive patients with microbiologically confirmed TB, including ascertaining outcomes at 2 months. The design and methodologies for STAMP trial are outlined in the study design and protocol paper that follows in chapter 3 [24], and in the main trial publication which is presented in the appendix [25], respectively.

My role within the STAMP trial was running the trial as the trial co-ordinator. Specifically, I wrote the trial protocol and site specific standard operating procedures for the conduct of the trial. I wrote and submitted the applications for ethical and regulatory approvals in the UK and at both trial sites. I designed the data collection tools and I trained the local trial teams in all trial procedures (including laboratory procedures). During the trial, I oversaw the running of both trial sites, including leading the quality assurance processes, reviewing the data collected and overseeing data query management. I oversaw data management procedures during the trial in collaboration with trial data manager and trial statisticians.

The pre-specified primary and secondary analyses for the trial (see chapter 3) were done by the trial statistician and were not part of this thesis (see appendix for main trial results). The trial design and securing funding was led by Prof Lawn. My role within the trial was supervised and supported by my supervisors (and STAMP trial co-investigators) Prof Lawn, Prof Corbett and Prof Fielding, as well as the site principal investigators (PIs) Prof Joep van Oosterhout and Dr Doug Wilson. There were a large team of research physicians, nurses and support staff who were vital to the STAMP clinical trial, and whose roles related to this thesis are summarised in table 2.1.

Name	Position	Role
Stephen Lawn	Professor of Infectious Diseases, LSHTM	PhD supervisor; Chief investigator of STAMP trial; conceived STAMP trial and secured funding (died Sept 2016)
Elizabeth Corbett	Professor of Tropical Epidemiology, LSHTM	PhD supervisor; co-investigator STAMP trial
Katherine Fielding	Professor of Medical Statistics & Epidemiology, LSHTM	PhD supervisor; co-Chief investigator STAMP trial
Henry Mwandumba	Reader, Liverpool School of Tropical	PhD supervisor (immunology component)
David Russell	Professor of Infection Biology, Cornell University	Collaborator, input in to phagocytosis assays and immunology studies
Kondwani Jambo	Post-doctoral fellow, MLW	Collaborator, input in to phagocytosis assays and immunology studies
Doug Wilson	Head of Medicine, Edendale Hospital	Co-investigator STAMP trial, responsible for the Edendale trial site
Joep van Oosterhout	Head of Research, Dignitas international	Co-investigator STAMP trial, responsible for the Zomba trial site
Clare Flach (until Oct 2016)	Lecturer in Statistics, LSHTM	STAMP trial statistician, PhD advisory committee member, input into data analysis methods
Daniel Grint (post Oct 2016)	Assistant Professor in Statistics, LSHTM	STAMP trial statistician, PhD advisory committee member, input into data analysis methods
Lingstone Chiume	Data Manager, MLW	Essential role in data management of STAMP trial
Elizabeth Chimbayo	Laboratory research assistant, MLW	Support for performing immunology assays in MLW lab in Malawi
Leonard Mvaya	Laboratory research assistant, MLW	Support for performing immunology assays in MLW lab in Malawi
Melanie Alufandika-Moyo	Physician, Dignitas International	Trial physician, input into recruitment of patients at the Zomba trial site
Jurgens Peters	Clinical Research Fellow, LSHTM	Trial physician, input into recruitment of patients at the Edendale trial site

Table 2.1 List of contributors to the STAMP trial and research presented within this thesis.

Systematic review and meta-analysis of urine-LAM detection's association with mortality (Chapter 4)

Assays detecting mycobacterial LAM in urine are promising new tools for the diagnosis of HIV-associated TB. Whilst some prospective cohort studies primarily aimed at assessing diagnostic accuracy or clinical utility have noted associations between LAM detection and increased mortality risk, the strength of this relationship had not been clearly defined. I therefore conducted a systematic review and meta-analysis summarising the current evidence for increased mortality risk in HIV-positive patients with microbiologically confirmed TB who are urine-LAM positive compared to urine-LAM negative.

The search identified studies reporting patient characteristics and urine LAM-status in adult patients with HIV-TB co-infection, and mortality (the primary outcome). The primary outcome was mortality risk. The other outcome of interest was adjusted odds ratio of mortality for urine LAM-positive versus LAM-negative TB-cases based on a multi-variable analysis including other predictors of mortality. Pooled estimates were calculated using a random-effects modelling, summarising the assay's potential prognostic value. The findings are discussed along with potential mechanisms underlying any associations, and the implications for the implementation of urinary LAM testing in this vulnerable population.

Cohort study of mortality, and risk factors for mortality (Chapter 5)

There is a scarcity of data in settings of widespread access to ART and improved diagnostics such as the Xpert assay describing the mortality and factors associated with mortality in hospitalised patients with HIV-associated TB. Understanding such factors could help develop strategies to identify high-risk patients, and interventions to reduce their mortality. To address these questions, I constructed a prospective cohort study nested within the STAMP trial, restricted to patients from the STAMP trial diagnosed with microbiologically confirmed TB.

The prevalence of disseminated TB, as diagnosed by positive urine rapid diagnostic tests, and associations with mortality are reported. Baseline clinical phenotypes are described using hierarchical cluster analysis, and Cox regression analysis was used to identify associations with early mortality. The findings are discussed, as are the implications for reducing mortality in this patient population.

Cohort study of derivation and external validation of a clinical score predicting mortality risk (Chapter 6)

Clinical decision tools and risk scores are used widely in clinical practice to simplify the identification of patients at highest risk for poor health outcomes. Although scores have been developed to predict risk of TB disease in various, they cannot be used to predict outcomes of TB disease. Being able to identify patients at the highest risk of mortality could inform the development and assessment of new interventions, and also identify which patients would benefit most from these. I therefore undertook a study to assess if urinary LAM detection, along with other clinical variables readily available in high-burden settings, could be used to predict early mortality in HIV-positive patients admitted to hospital and diagnosed with TB, and to externally validate this predictive tool.

The study used data from the same cohort nested within the STAMP trial as described above (Chapter 5) for the clinical predictor score derivation using multivariable regression modelling. The score was internally evaluated against observed mortality risk at 2-months, and externally validated using data collected independently from two studies: a multicentre diagnostic clinical trial of adjunctive urine TB-LAM testing in HIV-positive patients with TB symptoms who were admitted to hospital [3]; and a prospective cohort study assessing the diagnostic yield of TB-LAM in HIV-positive patients in Kenya [26].

Cross-sectional study assessing the use of whole-blood functional assay of phagocyte activity (Chapter 7)

The accurate assessment of immune competence through *ex vivo* analysis is paramount to our understanding of those immune mechanisms that lead to protection or susceptibility against a broad range of human pathogens. Such an assay opens opportunities for the rapid assessment of immune responses from whole blood, allowing comparisons of immune responses patients with HIV-associated TB according to outcome and/or positive urine diagnostic tests.

I therefore developed a flow cytometry based, whole blood phagocyte functional assay that utilizes the inflammatory inducer zymosan, coupled to OxyBURST-SE, which measures both phagocytic uptake and the superoxide burst in the phagocyte populations in whole blood [27]. The assay was first applied to whole blood from Malawian healthy volunteers to demonstrate kinetics, reproducibility and validity. It was then used in demonstrating the perturbation of phagocyte function in the blood from patients with HIV-associated TB in Malawi compared to healthy volunteers.

A cohort study of immunological responses in HIV-associated TB (Chapters 8 and 9)

Disseminated TB disease in HIV-positive patients can be identified by detecting mycobacterial nucleic acids (using the Xpert MTB/RIF assay) or LAM (using TB-LAM lateral flow assays) in urine, a sign of haematogenously disseminated renal TB [28,29]. Given these patients have a higher burden of TB, and poorer outcomes [30], I undertook a study in patients with microbiologically confirmed TB to compare baseline immune responses among those with and without positive urine-diagnostics (indicating disseminated disease). I also compared baseline immune responses in microbiologically confirmed TB patients who died with those who survived to 2-months, to help understand if patients with poor outcomes have altered immune responses and inform strategies to reduce the high mortality from HIV-associated TB.

This study is based on a cohort of patients with microbiologically confirmed TB disease nested within the STAMP trial Zomba (Malawi) site. The study was limited to Malawi as this was the site within close proximity to an immunology research laboratory with the capacity to undertake the assays. The cohort was recruited between June 2016 (after commencement of the trial) until September 2017. The inclusion criteria were the same as for the STAMP trial, and patients were approached for enrolment into this sub-study after their microbiologically confirmed TB diagnosis. An additional group of HIV-positive 'control' patients without TB who were enrolled in the STAMP trial and matched by baseline CD4 cell count to enrolled TB patients were also recruited. These patients were approached if their STAMP TB screening test results were negative.

Immunological mechanisms characterised included cytokine, chemokine and inflammation biomarker concentrations, markers of immune activation and exhaustion, and functional responses including cytokine production following stimulation and phagocyte function (using the assay described in chapter 6).

2.6 Procedures for the STAMP trial and sub-studies contributing to the thesis

Study procedures were undertaken according to the protocol for the parent STAMP trial, which is described in Chapter 3. Additional details or procedures relevant to the thesis are detailed below or described in the relevant research paper or chapter reporting the results.

TB screening

In the STAMP trial, patients randomised to the intervention arm had urine tested for TB using TB-LAM and Xpert assays in real time. Patients randomised to the SOC had concentrated and unconcentrated urine stored at -80°C for retrospective testing with TB-LAM and Xpert assays.

Testing of stored urine using TB-LAM and Xpert was done for all patients diagnosed with TB in the SOC arm to determine if they had 'urine-positive' TB disease (indicative of haematogenously disseminated TB). This was done in batches and followed the same procedures as fresh urine samples.

The TB-LAM assay was performed as per the manufacturer's instructions. 60µL of unconcentrated urine was pipetted onto the sample pad of new TB-LAM strip. A timer was set for 25-minutes, after which the validity of the test was assessed by checking for a band in the control window. The test result was then assessed by comparing the intensity of the band in the sample window (if present) with the manufacturer's reference card. If the band was as or more intense than the lightest positive band (grade 1 on the post 2014 reference card), the test was deemed positive. The positive results were graded (grade 1-4) according to the band which the sample window was as intense as (see chapter 1). Non-valid tests were repeated. TB-LAM test strips were stored and a random sample independently double-read, masked to the original result, for quality assurance. TB-LAM tests were read prior to the results of the Xpert MTB/RIF and without knowing any clinical details of the patient to reduce bias in how the assays were read.

Approximately 40-50mLs of urine was concentrated for Xpert testing urine by centrifugation at 3000g for 15 minutes. The supernatant was discarded, and the urine pellet resuspended in approximately 2ml of residual urine/supernatant. 0.75mL of the resuspended pellet was added to 1.25mL of Xpert MTB/RIF sample reagent and incubated for 15 minutes, then added to the cartridge and processed as per the manufacturer's instructions [25]. Cycle time values for positivity for each of the Xpert probes was also recorded.

Other procedures

Upon enrolment to the STAMP trial at admission to hospital, all patients had blood taken for haemoglobin (HemoCue) and full blood count (Horiba), CD4 cell count (PIMA or BD FacsCount) and 4mLs of plasma for storage at -80°C, which was processed by STAMP trial laboratory technician immediately after collection of samples. Eligible consenting patients (eligibility criteria are described in chapter 8) in the immunology sub-study (in Malawi only) underwent additional venepuncture for 10-12mLs of heparinised whole blood, which was taken before TB treatment was commenced. This sample was immediately transferred to the laboratory at the Malawi-Liverpool-Wellcome Trust Clinical Research Programme for processing.

Masking

All STAMP trial team members were masked to the study arm, TB diagnostic results (ie if urine diagnostic tests were positive or negative), and patient outcomes until December 2017, apart

from the STAMP trial laboratory technicians who performed the TB screening (based at Zomba Central or Edendale Hospitals). Thus, all immunological assays were performed and analysed masked to the urine TB diagnostic status of the patient, clinical features, CD4 cell count and outcome.

2.7 Data collection, management and analysis

Patients were identified using a unique patient identifier (5 digits and a check digit), which was used to identify all records and samples. Clinical and demographic data for all patients was collected as part of the STAMP trial onto case report forms (CRFs). Both data and sample collection for the above cohort studies was the same as for the parent STAMP trial, no additional clinical data were collected.

Clinical data were collected by study nurses, clinical officers or doctors either directly from the patient, from medical notes or a combination of both. At hospital admission, a detailed clinical history was taken including duration of illness, TB symptoms, past history of TB, HIV care, WHO clinical stage and history of ART. Vital signs, performance status (measured as Karnofsky score) and nutrition status (body mass index and mid-upper arm circumference) were also assessed. During the inpatient admission, all medical events were captured including TB diagnoses (including basis of diagnosis, nature of TB disease), TB treatment (including time from admission to TB treatment), initiation of ART, concomitant infections and prescriptions of antimicrobials, length of hospital stay and death.

Diagnostic laboratory assay results (eg all TB diagnostics, CD4 cell counts, haemoglobin, CRP, HIV viral load, CrAg) were also entered onto CRFs. All CRFs were entered into a database via optical character recognition software (TeleForm, Hewlett Packard), with entered data verified with case report forms by a data coordinator. Data were extracted from the database as CSV files and imported into Stata version 14.0 (StataCorp). All flow cytometry, ELISA and multiplex bead-based assays had results exported as CSV files, which were also imported into Stata for data cleaning and analysis.

Sample size justification

The cohort study aimed to describe the clinical and epidemiological risk factors for mortality in HIV-associated TB is based on the hypotheses that disseminated TB is an independent risk factor for mortality. Sample size justification was based upon the following assumptions, which were extrapolated from previous studies reporting urine LAM status and mortality: 50% of patients with HIV-associated TB were urine positive, the increased risk ratio of mortality associated with detectable urine-LAM was 2-3 fold, the overall mortality at 2-months was

between 15-40% and the 2-sided type I error was 5%. A range of sample sizes are presented in table 2.2. Little data were available upon expected relative risk of mortality based on other variables. This study was nested within the STAMP trial, which had an expected overall sample size of 2,600 patients, and with TB prevalence expected to be about 20%, this would provide a total sample of 520 patients with HIV-associated TB for this study. Thus, based on a range of possible mortality risk ratios, this study had >80% power to detect a mortality difference between urine positive and urine negative patients, even accounting for a 10% loss to follow-up.

Overall mortality	Urine-positive 2-month mortality	Urine-negative 2-month mortality	Power	Sample size (including 10% LTFU)
13.5%	20%	7%	80%	230
15%	20%	10%	80%	432
20%	30%	10%	80%	130
22.5%	30%	15%	80%	259
26.5%	40%	13%	80%	84
30%	40%	20%	80%	173
33.5%	50%	17%	80%	62
37.5%	50%	25%	80%	121
40%	50%	30%	80%	200

Table 2.2 A range of sample size estimations based upon 50% of HIV-TB patients will test LAM-positive on urine and a 2-sided type I error is 5%

Sample size calculations for the immunology study were challenging as few studies had compared immune responses in hospitalised patients with HIV-associated TB who died compare to those who survived, and no studied had compared patients with and without positive urine diagnostic tests. Therefore, sample size calculations were based primarily upon data on differences in stimulated TNF- α levels in patients with good and poor outcomes reported from a similar patient cohort in Blantyre, Malawi [31]. This study demonstrated significantly lower TNF- α levels (median 47pg/mL) in patients with poor outcomes (death or acute deterioration requiring hospitalisation) compared to those with good outcomes (median 290 pg/mL). Based on Wilcoxon non-parametric tests on two independent groups, with the assumption that mortality among hospitalised TB-HIV patients would be 30%, a total sample size of 56 patients provided 80% power to detect a difference in TNF α levels between patients who died and who survived with a two-sided type 1 error (α) probability of 0.03 (adjusted for multiple tests) (see figure 2.5). The sample size was also limited by practical limiting factors of sample volume, laboratory capacity and study budget. Assuming a mortality of 25% increased the sample size to 66.

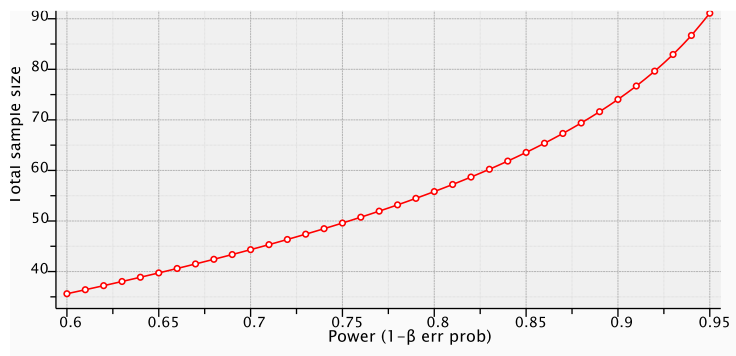


Figure 2.5 Sample size estimations. Sample size estimates plotted against power based on detecting a difference in TNF- α levels in patients with good and poor outcomes, assuming 30% have a poor outcome, and a two-sided type 1 error (α) probability of 0.03

2.8 Ethical considerations

The STAMP trial and associated sub-studies were carried out to good clinical practice (GCP) standards and according to the 2008 Declaration of Helsinki, and all study staff trained in GCP. Ethical approval for the STAMP trial was been granted by three research ethics committees: London School of Hygiene & Tropical Medicine Research Ethics Committee (LSHTM REC), the College of Medicine Research Ethics Committee in Malawi (COMREC), and the University of KwaZulu Natal Biomedical Research Ethics Committee (UKZN BREC). Ethical approval for the sub-studies were also granted by the LSHTM REC and relevant local ethics committee.

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Chapter 3: The STAMP trial study protocol **(research paper)**

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RESEARCH PAPER COVER SHEET

PLEASE NOTE THAT A COVER SHEET MUST BE COMPLETED FOR EACH RESEARCH PAPER INCLUDED IN A THESIS.

SECTION A – Student Details

Student	Ankur Gupta-Wright
Principal Supervisor	Prof Liz Corbett
Thesis Title	Investigating mortality risk in hospitalised patients in Africa with HIV-associated tuberculosis and positive urine diagnostics: a clinical, epidemiological and immunological study

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	BMC Infectious Diseases		
When was the work published?	22nd September 2016		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion			
Have you retained the copyright for the work?*	Yes	Was the work subject to academic peer review?	Yes

**If yes, please attach evidence of retention. If no, or if the work is being included in its published format, please attach evidence of permission from the copyright holder (publisher or other author) to include this work.*

SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	
Please list the paper's authors in the intended authorship order:	
Stage of publication	Choose an item.

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	My role was writing the study protocol upon which the manuscript is based, and writing the manuscript. The other authors then contributed to the review and refinement of the manuscript.
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Student Signature:

Date:

31st August 2018

Supervisor Signature:

Date:


31st August 2018

STUDY PROTOCOL

Open Access



Rapid urine-based screening for tuberculosis to reduce AIDS-related mortality in hospitalized patients in Africa (the STAMP trial): study protocol for a randomised controlled trial

Ankur Gupta-Wright^{1,2*} , Katherine L. Fielding^{3,4}, Joep J. van Oosterhout^{5,6}, Douglas K. Wilson⁷, Elizabeth L. Corbett^{1,2}, Clare Flach³, Krishna P. Reddy^{8,11}, Rochelle P. Walensky^{9,10,11}, Jurgens A. Peters¹, Melanie Alufandika-Moyo⁵ and Stephen D. Lawn^{1,12}

Abstract

Background: HIV-associated tuberculosis (TB) co-infection remains an enormous burden to international public health. Post-mortem studies have highlighted the high proportion of HIV-positive adults admitted to hospital with TB. Determine TB-LAM and Xpert MTB/RIF assays can substantially increase diagnostic yield of TB within one day of hospital admission. However, it remains unclear if this approach can impact clinical outcomes. The STAMP trial aims to test the hypothesis that the implementation a urine-based screening strategy for TB can reduce all cause-mortality among HIV-positive patients admitted to hospital when compared to current, sputum-based screening.

Methods: The trial is a pragmatic, individually randomised, multi-country (Malawi and South Africa) clinical trial with two study arms (1:1 recruitment). Unselected HIV-positive patients admitted to medical wards, irrespective of presentation, meeting the inclusion criteria and giving consent will be randomized to screening for TB using either: (i) 'standard of care'- testing of sputum using the Xpert MTB/RIF assay (Xpert) or (ii) 'intervention'- testing of sputum using Xpert and testing of urine using (a) Determine TB-LAM lateral-flow assay and (b) Xpert following concentration of urine by centrifugation. Patients will be excluded if they have received TB treatment in the previous 12 months, if they have received isoniazid preventive therapy in the last 6 months, if they are aged <18 years or they live outside the pre-specified geographical area. Results will be provided to the responsible medical team as soon as available to inform decisions regarding TB treatment. Both the study and routine medical team will be masked to study arm allocation. 1300 patients will be enrolled per arm (equal numbers at the two trial sites). The primary endpoint is all-cause mortality at 56 days. An economic analysis will be conducted to project long-term outcomes for shorter-term trial data, including cost-effectiveness.

(Continued on next page)

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Discussion: This pragmatic trial assesses an intervention to reduce the high mortality caused by HIV-associated TB, which could feasibly be scaled up in high-burden settings if shown to be efficacious and cost-effective. We discuss the challenges of designing a trial to assess the impact on mortality of laboratory-based TB screening interventions given frequent initiation of empirical treatment and a failure of several previous clinical trials to demonstrate an impact on clinical outcomes. We also elaborate on the practical and ethical issues of 'testing a test' in general.

Trial registration: ISRCTN Registry (ISRCTN71603869) prospectively registered 08 May 2015; the South African National Controlled Trials Registry (DOH-27-1015-5185) prospectively registered October 2015.

Keywords: TB, HIV, HIV-associated TB, Screening, LAM, Xpert

Background

HIV-associated TB remains an enormous burden to international public health, even in regions with high coverage of antiretroviral therapy (ART). Globally, in 2014, there were an estimated 0.4 million TB related deaths in people living with HIV, which accounts for approximately one-quarter of TB deaths and one-third of HIV deaths [1]. This burden disproportionately affects sub-Saharan Africa where TB is a common cause of hospital admission and mortality among HIV-positive patients admitted to hospital [2].

Diagnosis of TB in people living with HIV remains challenging due to non-specific clinical features, early dissemination beyond the lungs and relatively low mycobacterial burden within sputum samples [3–5]. A meta-analysis of post-mortem studies in adult HIV-positive patients dying in hospitals in sub-Saharan Africa reported that between 32 and 67 % (pooled summary estimate 43 %) had evidence of TB at post-mortem [6]. TB was disseminated in almost 90 % of patients, and remained undiagnosed at the time of death in almost one-half of TB cases, reflecting a failure of current sputum and clinical based diagnosis of TB, and presenting a strong rationale for routine systematic screening of HIV-positive hospital admissions.

New diagnostic tools have been high on the TB research agenda for the past decade, and are recognised as crucial to the World Health Organization's (WHO) End TB Strategy [7]. The Xpert MTB/RIF rapid molecular assay (Xpert, Cepheid, Sunnyvale, CA, USA) has a pooled sensitivity for diagnosis of pulmonary TB in HIV-positive adults of 79 % (95 % CI 70–86 %), with 99 % specificity. The test has been approved by WHO and widely implemented in high burden settings [8]. Systematic reviews have also reported very high specificities for Xpert when testing a wide-variety of non-respiratory clinical samples, despite culture being an imperfect reference standard for extra-pulmonary TB [9, 10]. Although data were insufficient for the WHO guidelines to endorse the use of Xpert for TB diagnosis from urine, studies have demonstrated useful diagnostic yield and high specificity in urine among hospitalised HIV-positive patients [11–14].

Urine also has several advantages as a diagnostic sample for hospitalised HIV-positive patients, including relative ease of collection and lower biohazard risk during specimen handling during collection and in the laboratory. The Determine TB-LAM (TB-LAM, Alere, Waltham, MA, USA) lateral flow assay is a simple, point-of-care test for detecting the mycobacterial cell wall antigen lipoarabinomannan (LAM) in urine. It requires 60 µL of unprocessed urine, giving a result in 25 min at a relatively low cost (approximately US\$2.50). Whilst sensitivity of this assay is poor in general populations, it is improved in advanced HIV-related immunosuppression, and studies in HIV-positive patients admitted to hospital have demonstrated sensitivities between 40 and 70 % [15–20]. Specificity is exceptionally high when the reference standards include culture of non-respiratory samples [19, 21]. TB-LAM was conditionally approved in November 2015 by WHO for use in diagnosing TB in hospitalised HIV-positive patients [22].

Evidence suggests that high mortality amongst hospitalised patients with HIV/TB co-infection is fuelled by under-diagnosis of TB and delays in diagnosis due to overreliance on sputum based diagnostics, imaging and/or clinical features, and an inability to diagnose disseminated and extra pulmonary TB disease. We therefore sought to evaluate the impact on mortality of a high-yield, rapid-urine based screening approach for TB in HIV-positive medical admissions to hospital in South Africa and Malawi.

Rationale for studies of clinical impact

A study from South Africa intensively screened unselected HIV-positive hospital admissions to medical wards with comprehensive clinical sampling (sputum, blood, urine and other clinically relevant samples) [14]. Using mycobacterial liquid culture and/or Xpert, TB was diagnosed in 139/427 patients (33 % TB prevalence, 95 % CI 28–37 %). However, only 28 % of microbiologically confirmed TB in this study could have been diagnosed by Xpert testing of sputum alone. In contrast, 81 % of TB cases could have been diagnosed by Xpert testing of both sputum and urine, with additional TB-

LAM testing of urine, and results are available within the first 24 h of hospital admission [14, 23]. Xpert is the best diagnostic test available in most high-burden settings and the WHO's recommended initial TB test for HIV-positive patients. The use of this rapid, relatively low cost screening approach increased diagnostic yield by almost three-fold.

Despite this increase in diagnostic yield, there is no evidence that this urine-based screening strategy will impact mortality or clinical outcomes; it is recommended that impact be demonstrated before such interventions can be endorsed or implemented [24]. This is especially true, as numerous studies have noted that the replacement of sputum smear-microscopy with the more sensitive Xpert has failed to demonstrate any impact on clinical outcomes [25]. A recently published randomised controlled trial of adjunctive urine testing with the TB-LAM assay (the LAM RCT) in a selected population of hospitalised HIV-positive patients being investigated for TB demonstrated an absolute mortality reduction of 4 % (95%CI 1–7 %) and a relative reduction in mortality of 17 % (95%CI 4–27 %). These data support the potential for a urine-based screening approach to reduce mortality among unselected HIV-positive in-patients [26].

Rationale for a Randomized Controlled Trial (RCT)

Whilst a high yield, urine-based screening strategy might benefit this patient population by increasing the number of TB diagnoses and decreasing time to TB treatment, it may also be associated with a range of adverse consequences. A rapid TB diagnosis within the first 1–2 days of hospital admission may divert clinical attention from co-pathologies, and may reduce the likelihood of empirical prescription of antibiotics for occult bacterial infections. Further, despite excellent specificity, this screening approach may lead to some false-positive TB diagnoses and false-positive rifampicin resistance results from Xpert assays, leading to inappropriate use of potentially harmful medication. Although relatively low cost, the implementation of such a screening strategy will require investment of limited health service resources, and may divert resources from other interventions with potentially greater impact on health outcomes.

Although diagnostic tests are routinely implemented on the basis of diagnostic accuracy or effectiveness without assessment of impact on clinical outcomes (e.g. Xpert and rapid malaria diagnostics), their impact on population health is part of a wider cascade of processes including care seeking and initiation of appropriate treatment [27]. Thus, they should be evaluated under such circumstances too, and evidence of diagnostic accuracy alone should not be taken as evidence of impact on patient-appropriate outcomes such as reducing morbidity or mortality [27]. This is especially true of TB

diagnostics, as even mycobacterial culture (the most sensitive diagnostic) can have minimal impact on clinical decision to initiate TB treatment [28].

Methods/Design

Aim

The principal aim of this trial is to test the hypothesis that the implementation of a rapid, sensitive urine-based screening strategy for TB can reduce all cause-mortality among HIV-positive patients admitted to hospitals in sub-Saharan Africa when compared to current, sputum-based screening.

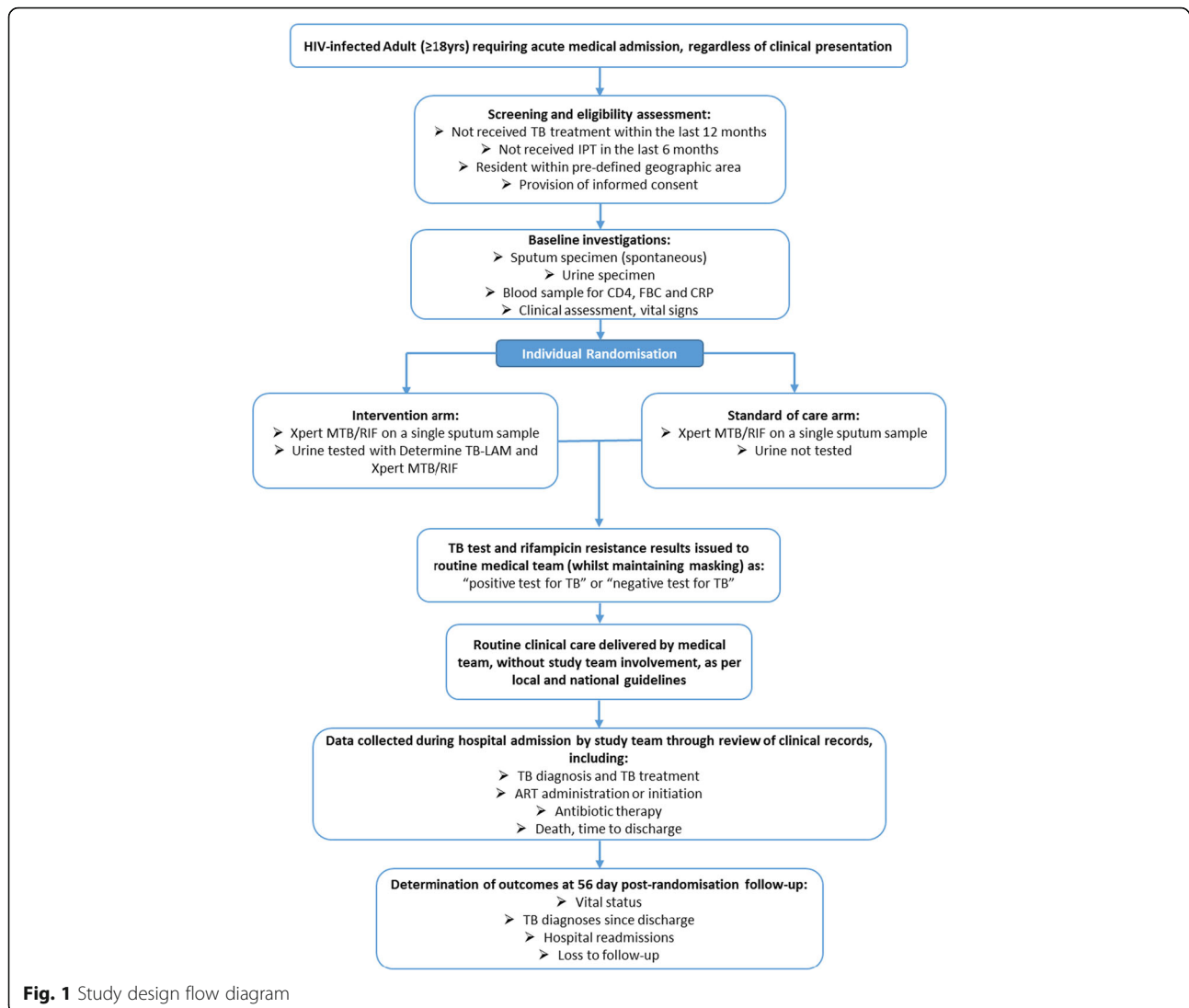
Study design

The trial is a pragmatic, individually randomised, multi-country clinical trial with two study arms with 1:1 allocation between arms (Fig. 1). HIV-positive patients randomised to the 'standard of care' arm will be screened for TB by testing sputum (if produced by spontaneous expectoration) using the Xpert assay. HIV-positive patients randomised to the intervention arm will, in addition to the standard of care, have a urine sample (if produced) tested for TB using the TB-LAM assay and, following concentration of 40–50mls of urine by centrifugation, the Xpert assay. Patients, the responsible medical team and study team will be masked to the study arm allocation (except for the study statistician, data managers and laboratory technicians). TB screening test results will be communicated to the medical teams responsible for clinical care of the patients (whilst maintaining masking to study arm allocation), but the clinical management decisions informed by the test results, including whether to commence TB treatment, will not be altered by the study team. Beyond collection of TB screening samples, running of assays and issuing of results, the study team will have no involvement in the clinical care of participants.

Study population

The trial will take place at two sites: Zomba Central Hospital, Southern Malawi, and Edendale Hospital, KwaZulu-Natal, South Africa. Both hospitals serve populations with high HIV prevalence and TB incidence.

All new admissions to medical wards who have confirmed HIV-infection (either an existing or new diagnosis), irrespective of antiretroviral therapy (ART) status, will be screened for eligibility. HIV testing of all hospital admissions with unknown HIV-status is recommended as part of national guidelines at both trial sites, and will be supported by study staff where necessary. Eligibility criteria are designed to be as broad as possible. Patients will be eligible for inclusion regardless of reason for medical admission or presence of TB symptoms (unselected), although patients <18 years old, those who have



received TB treatment in the preceding 12 months or isoniazid preventive therapy in the preceding 6 months, those unable to provide informed consent or living outside a pre-defined geographical area will be excluded. It is envisaged that recruitment will take place over a period of 24 months, with equal numbers recruited at each study site.

Study procedures

After obtaining written informed consent from eligible patients, a study nurse will collect data at baseline to determine clinical history, including TB symptoms, past history of TB and HIV care, vital signs and nutritional and performance status. Venepuncture for haemoglobin concentration and CD4 cell count will be conducted and single sputum sample and up to 50mls of urine will be collected from all participants, irrespective of study arm. If sputum and/or urine samples cannot be spontaneously

produced, participants will remain in the study but no further samples for TB investigation will be arranged by the study team. However, the responsible medical team will remain at liberty to arrange further TB investigations that are available as standard of care at the study hospital.

A randomisation list stratified by study site using computer generated random block size will be generated. Randomisation will occur at enrolment, but the study team will be masked to study arm allocation. When TB samples (sputum and/or urine) from participants arrive in the laboratory, the study laboratory technician will identify the allocated study arm by opening a sealed envelope marked with the unique participant ID, and determine which samples will be screened for TB. Urine from participants randomised to the standard of care arm will not be tested for TB and will be safely discarded. TB-LAM assays will be read by the laboratory

technician and results will be deemed as positive using the grade 1 cut-off on the manufacturer's reference card. 20 % of TB-LAM assays will read by a second, blinded reader for quality control.

TB screening tests will only be processed during office working hours on weekdays. Once the TB screening tests have been completed (anticipated to be within 24-48 h of sample collection), the results will be issued whilst maintaining masking of the study arm and responsible medical teams (reported as either 'TB screening test positive', 'TB screening test negative' or 'TB screening test not done'). Rifampicin resistance results, when available, will also be communicated to the medical teams.

The responsible medical team will receive training on how to interpret the screening tests, including estimated positive and negative predictive values of the screening algorithms in each study arm, and that a negative screening test does not 'rule out' TB. The responsible medical team will be at liberty to organise further TB investigations and commence TB treatment as clinically appropriate. Management of HIV will be as per local and national guidelines, including timing of ART initiation. Data on TB investigations, diagnoses (including if microbiologically confirmed or 'empirical') and treatment will be collected during follow-up.

Outcomes

The primary outcome is risk of all-cause mortality at 56-days following enrolment. Secondary outcomes are: time to all-cause mortality; proportions of patients with (i) microbiologically confirmed and (ii) clinically diagnosed TB disease; time to TB diagnosis and commencement of TB treatment; proportions of patients receiving antimicrobials and ART; and (i) duration of hospital admission, (ii) cumulative incidence of hospital readmission, and (iii) cumulative incidence of loss to follow-up. Outcomes will be ascertained by patient interview and review of medical notes during in-patient admission, and patient interview for vital status at 56-days post enrolment. This will be supplemented with information from patients' next of kin for those lost to follow-up.

Microbiologically confirmed TB is defined as at least one positive smear-microscopy, Xpert, or culture positive result on any specimen or a positive urine TB-LAM result. Clinically diagnosed TB is defined as having a compatible clinical illness or radiological disease and/or the decision of the responsible clinical team to commence TB treatment in the absence of any positive microbiological tests for TB.

Economic analysis

An economic analysis from a societal perspective will be undertaken to demonstrate the longer-term clinical and budgetary impact as well as the cost-effectiveness of the

intervention; demonstrating economic feasibility and cost-effectiveness will be essential prior to implementation, should this screening intervention prove effective. Given the complexities of diagnosis and treatment of HIV/TB coinfection for patients requiring hospital admission, we will estimate health service costs based upon resource utilization of trial participants, inclusive of laboratory reagents and services, inpatient hospitalization days and outpatient visits, as well as drug costs. Trial-site specific costs will be collected on a limited cohort of 100 participants per site. We anticipate that, in the short term, costs of hospital admission are likely to account for a large proportion of health service provider expenditure; over the longer term, we expect increased survival related to the intervention will result in greater cumulative antiretroviral drug costs. Longer term cost-effectiveness will be estimated based on the Cost-Effectiveness of Preventing AIDS Complications-International (CEPAC-I) computer simulation model [29]. Model-based outcomes will include a short-term validation of trial results; that is, we will ensure that model results after 2 months of simulation accurately reflect trial outcomes of 56 days. Longer-term model results will include: 1-year and 5-year survival, TB-deaths averted, overall deaths averted and per person costs as well as life expectancy (LE) and lifetime costs. At each time horizon (1-year, 5-year and lifetime), the incremental cost-effectiveness of the intervention compared with the standard of care ($\Delta\$/\Delta LE$, both discounted by 3 % per year) will be calculated. Model results will be examined in one-way and multi-way deterministic sensitivity analyses, examining influential parameters of interest (e.g. mean CD4 at presentation, TB screening test sensitivity/specificity and cost, active TB prevalence on admission). In addition to cost-effectiveness analyses, the budgetary impact of implementation and scale-up at 1- and 5- years of this intervention will be reported.

Sample size justification

Sample size calculations were based upon unpublished data from the trial sites, which showed a mortality of HIV-positive medical in-patients of 21-23 % during hospital admission and are supported by subsequently published meta-analyses on hospitalised HIV-positive patients in Africa [2, 30]. We assumed that 56-day mortality would be 25-30 % in the standard of care arm and, based upon post-mortem prevalence of TB, we estimated 40-50 % of deaths in the standard of care arm would be TB-related.

The sensitivity of the intervention for diagnosing TB disease was assumed to be 80 % (a 3-fold increase in diagnostic yield compared to sputum alone) based upon a background study [14]. An observational study of early-initiation of TB treatment among HIV-positive

smear-negative hospital in-patients observed a reduction in mortality at 2-months of 47 % in patients whose TB treatment was expedited by use of WHO-recommended diagnostic algorithm compared to standard practice [31]. Our study was powered to detect a 40–50 % reduction in TB-related mortality, and assuming half of deaths are TB related, this would equate to a 20–25 % reduction in all-cause mortality. Our sample size of 1300 patients per arm would provide 90 % power to detect a 25 % reduction in mortality and 80 % power to detect a 20 % reduction in mortality, assuming an all-cause mortality of 25 % in the ‘standard of care’ arm and a 2-sided type 1 error of 5 % and 10–15 % loss to follow-up. If the 56-day mortality was unexpectedly lower (20 %) in the standard of care arm, our sample size would still provide 80 % power to detect a 25 % reduction in all-cause mortality.

Data collection and management

Data will be collected at four main times: enrolment, during the hospital admission, at hospital discharge and at the 56-day follow-up visit (Table 1). Specially designed case report forms are completed by study staff at each time point, and scanned, verified and committed to a local site database within 48 h of completion using the optical-character-recognition software TeleForm (Hewlett Packard Software, CA, USA). All data in critical fields are verified upon scanning. Completed forms are stored as the source documentation in a locked cabinet, with access restricted to specified study team members. Locator

information is stored separately to other case report forms which are identified by unique participant ID number and do not contain any patient identifiable information. Queries based on data in the database are generated weekly, including date, range and logic checks, and sent to sites for resolution.

Follow-up at 56-days is undertaken through outpatient appointment attendance at the study site. If participants are unable to attend the outpatient appointment, they are traced by telephone and/or home visit if they have provided prior consent for this to occur. Participants are defined as lost to follow-up if they are unable to be contacted after three tracing attempts after 56-days from enrolment. Quality assurance processes are in place to check all consent forms, screening case report forms, laboratory case report forms and a random sub-sample of all other case report forms.

Statistical analysis

The trial profile will be summarised using a CONSORT flow chart, including reasons for non-eligibility and non-enrolment (Fig. 2) [32]. All analyses will be conducted by initially assigned study arm in an intention-to-treat analysis, and adjusted for randomisation site. Baseline variables will be presented by study arm.

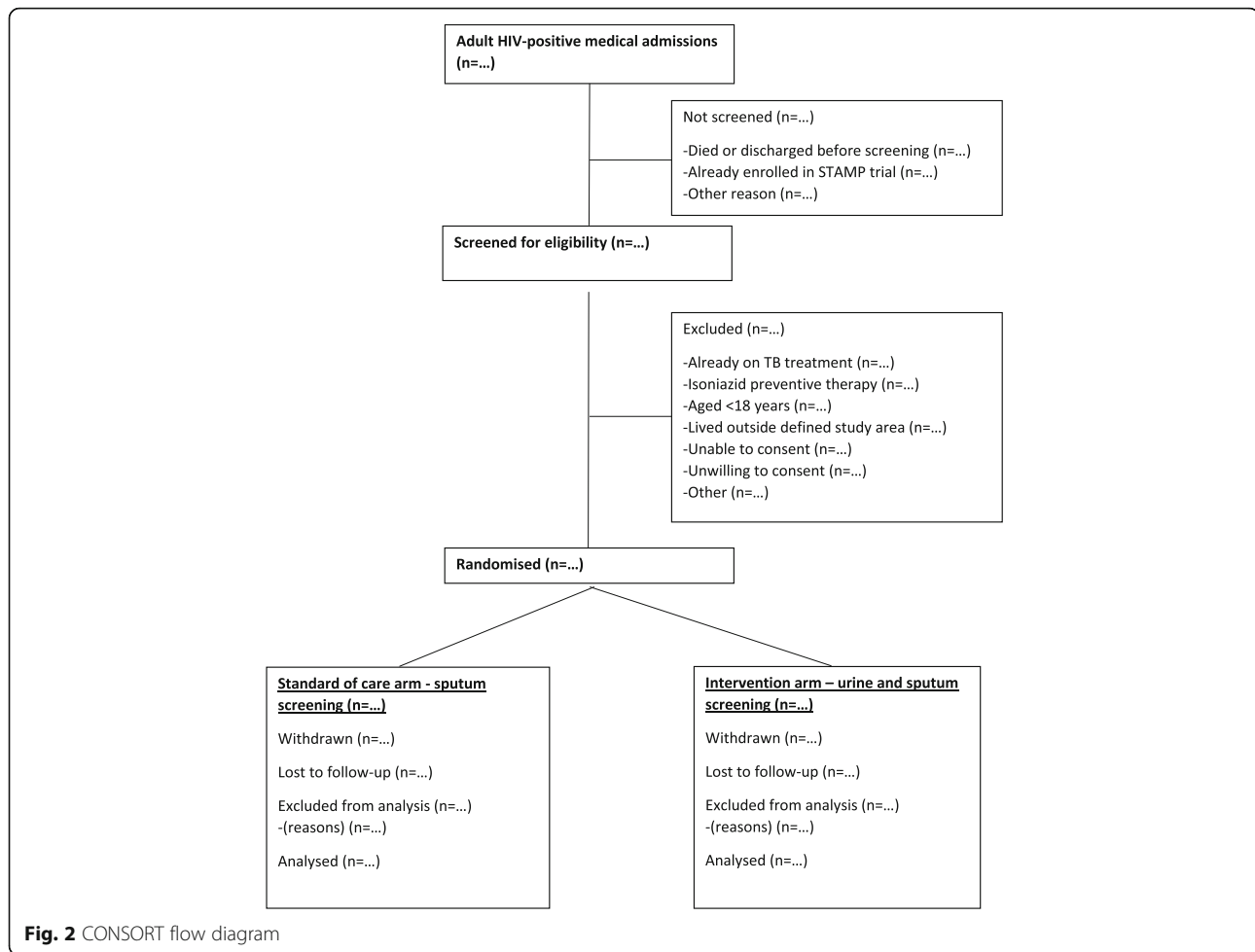
For the primary outcome the risk difference and odds ratio, and their associated 95 % confidence intervals for the effect of study arm on mortality risk will be calculated. The primary analysis of this endpoint will assume

Table 1 Schedule of STAMP study activities

	Time-point:	Enrolment	Baseline assessment	TB testing	In-patient stay	Hospital exit	Outpatient Review
	Time On-Study:	Day 0	Day 0	Day 0–1	Day 0+	Day 0+	Day 56
Enrolment:							
Eligibility Screen		X					
Informed consent		X					
HIV confirmation		X					
Study arm allocation		X					
Interventions:							
Collect admission specimens (blood, sputum and urine)			X				
TB screening & issuing results				X			
Assessments:							
Baseline demographic and clinical information			X				
EQ5D			X				X
TB investigations, diagnosis and/or treatment					X	X	X
Vital status					X	X	X
HIV care/ART					X	X	X
Health service use					X	X	X

EQ5D quality of life questionnaire [38]

ART antiretroviral therapy



participants who are lost to follow-up before 56 days have not died. Sensitivity analyses will be conducted to assess assumptions made regarding missing data and patients lost to follow-up. A sub-group analysis of the primary outcome will be conducted for the following variables: study site, calendar time, baseline CD4 cell count (<100 or >100 cells/ μ L), presence of severe anaemia (Hb <80 g/L) and clinically suspected TB at admission. The interaction effects by subgroup will be investigated and p-values reported.

Secondary outcomes (as defined above) will be compared between study arms using logistic regression for binary outcome and survival analysis with Kaplan-Meier curves and Cox proportional hazards for time to event. All analyses will be adjusted for randomisation site.

A statistical analysis plan documents the analysis of all trial outcomes in detail, and was reviewed by the Data Safety and Monitoring Board (DSMB).

Trial governance and approvals

The trial will be governed by a Trial Steering Committee (TSC), including an independent chairperson and at

least three other independent members. The TSC will oversee the trial and advise the investigator team, including monitoring progress, receiving reports from the Data and Safety Monitoring Board (DSMB), and assessing the impact of new scientific evidence. The DSMB's role is to protect the safety of trial participants, they will meet every six months to review data from the trial, as set out in the DSMB charter, and are independent to the trial sponsor (the London School of Hygiene & Tropical Medicine). Monitoring visits will be undertaken at least three-monthly for quality assurance and to ensure adherence to the trial protocol, and auditing of the trial conduct will be undertaken by the trial sponsor.

The trial has also received approval from the Research Ethics Committees of the London School of Hygiene & Tropical Medicine (ref: 9630), College of Medicine University of Malawi (ref: P.06/15/1743) and the University of KwaZulu-Natal Biomedical Research Ethics Committee (ref: BFC215/15). STAMP is registered with the ISRCTN Registry (ref: ISRCTN71603869) and the South African National Controlled Trials Registry (ref: DOH-27-1015-5185). The trial is funded by the Joint

Global Health Trials scheme (a collaboration between the UK Department for International Development, the UK Medical Research Council and the Wellcome Trust), and was peer reviewed as part of the funding application process.

Discussion

The STAMP trial aims to assess whether a novel, rapid urine-based screening strategy for TB with a high diagnostic yield can reduce early mortality in HIV-positive patients admitted to hospital in sub-Saharan Africa. The need for this trial is clear given the high mortality among HIV-positive patients admitted to hospital in these settings, and the high proportion of patients that die with undiagnosed TB disease [6, 30]. Positive urine-based TB diagnostic tests in advanced HIV are a marker of haematogenously disseminated renal TB, and these patients are at higher risk of mortality, supporting the rationale that earlier diagnosis and treatment may reduce mortality [33, 34].

Other trials have assessed empirical TB treatment in HIV-positive patients at high risk of TB, although mostly in outpatient settings. The REMEMBER trial failed to demonstrate a mortality benefit of empirical TB treatment compared to isoniazid preventive therapy [35]. The TB Fast Track trial assessed an algorithm using body mass index, presence of anaemia and urine TB-LAM testing to identify HIV-positive ambulatory patients with CD4 cell counts ≤ 150 cells/ μL at high risk of TB in whom empirical TB treatment was started immediately, but found no reduction in 6-month mortality compared to the standard of care [34, 35]. The STAMP investigators believe that utilising new TB diagnostics can provide better specificity than empirical TB treatment and prevent the potential harm from prescription of TB treatment to HIV-positive patients without TB disease among hospital inpatients.

A trial of adjunctive urine testing with the TB-LAM assay in a selected population of hospitalised HIV-positive patients being investigated for TB (the LAM RCT) recently demonstrated a reduction in mortality at 2-months [26]. The STAMP trial differs from this RCT in respect to several factors. Firstly, the inclusion criteria differ in that STAMP is a screening intervention targeting unselected HIV-positive patients admitted to hospital regardless of clinical presentation, and the LAM RCT investigated the TB-LAM diagnostic in hospitalised HIV-positive patients who were presumed to have TB. As such, STAMP has the potential to detect TB among those without any clinical suspicion for TB on hospital entry. Secondly, the STAMP intervention includes the Xpert MTB/RIF assay performed on urine in addition to TB-LAM, whereas LAM RCT evaluated urine testing with TB-LAM alone. This increases the diagnostic yield

of the intervention and adds the potential to diagnose rifampicin resistance. Thirdly, the standard of care in the STAMP trial includes Xpert testing of sputum (if produced) at all study sites, whilst the standard of care in LAM RCT varied from easy access to Xpert in South Africa to no access to Xpert in Zimbabwe. Finally, in the STAMP trial but not in the LAM RCT, both study teams and routine medical teams managing patients are masked to the study intervention arm (i.e. which TB screening tests are done).

The rationale for a clinical trial to demonstrate impact prior to implementation is supported by the lack of demonstrable benefit upon patient outcomes of sputum screening using Xpert in HIV-positive patients, despite increases in diagnostic yield compared to sputum smear microscopy [25]. Reasons for the failure of such trials of Xpert sputum testing to demonstrate impact may include lower occurrence of mortality endpoints in populations studies (largely outpatient based studies), high rates of empirical TB treatment and the failure of some positive Xpert results to be translated to TB treatment due to delays between submission of samples and issuing of results. The decision to start TB treatment is complex, and in such trials is likely to be influenced by the premise of the study to reduce undiagnosed, or 'missed' TB cases, potentially increasing empirical TB treatment [36].

These issues have been considered when designing the STAMP trial. For example, in-patient settings have a high mortality, and initiating TB treatment following positive results is more likely to occur when patients are admitted to hospital compared to community settings. Masking of clinicians, investigators and patients to the study intervention arm should reduce any 'Hawthorne' effect which could increase empirical TB treatment in the standard of care arm due to clinicians observing higher diagnostic yield in the intervention arm [37]. The use of 'double-blinding' in this manner is unusual in assessments of diagnostics, and, to our knowledge, this is the first time it has been used in TB diagnostics. However, a disadvantage of masking is that it might impair the ability of clinicians to make decisions about TB treatment in the absence of a positive screening result.

The STAMP trial sites have also been chosen to represent differing facilities, cadre of healthcare workers and resource utilisation in the region. Two studies to date, including one RCT, have demonstrated decreases in early mortality from expedited TB treatment in hospitalised HIV-positive patients, supporting the STAMP trial hypothesis that urine-based screening could reduce mortality [26, 31].

The unique challenges of 'testing a test' have also been considered in the STAMP study design, in particular, regarding the ethics of conducting such a trial. If considering the diagnostic accuracy or yield of the STAMP study

intervention compared to sputum screening or clinical diagnosis alone, [14] it could be argued there is a lack of equipoise (i.e. disagreement about the relative merits of the intervention). Whilst equipoise may not exist for diagnostic accuracy of the intervention for detecting HIV-TB, equipoise clearly exists for the impact of the study intervention on early mortality in unselected HIV-positive hospital admissions when compared to the sputum and clinical screening strategies that are current standard of care [27]. We have also considered the potential negative consequences of a rapid microbiological TB diagnosis, such as delay in initiating ART, a reduction in the prescription of empirical antibiotics which may treat covert sepsis in this severely immunosuppressed population and false-positive rifampicin resistance results. This study will be able to describe and document the impact of these potential harms and compare these secondary endpoints between study arms.

Furthermore, the pragmatic nature of the STAMP trial, with clinical care including TB diagnostics outside the study intervention, TB treatment and ART under routine conditions for each study setting, make the findings more generalisable and applicable to scale up. The wide inclusion criteria and not restricting TB testing to patients with 'presumptive' TB (for which the definition often varies between settings) also makes the results more applicable. The recently published WHO guidance on the use of LAM lateral flow assays advises against its use for screening, based upon low quality evidence [22]. The STAMP study can provide randomised trial quality evidence to support or refute these recommendations for HIV-positive, hospitalised patients. The study intervention should be possible to replicate in resource-limiting settings given the widespread availability of the Xpert assay and lack of equipment required for the TB-LAM testing, in keeping with STAMP being a pragmatic trial. The concentration of urine prior to testing with the Xpert assay (which has been shown to increase sensitivity [14]) is done using a bucket centrifuge. Laboratory based research is also being undertaken to look for alternative, lower resource methods to concentrate urine prior to testing with Xpert.

The STAMP trial acknowledges the potential disadvantages of urine-based TB screening for this population, especially the cost implications. Although the intervention is relatively low-cost, health budgets in high TB and HIV settings are limited, and therefore widespread implementation of the STAMP study intervention may have substantial budgetary impact. The STAMP trial incorporates an economic analysis which will assess cost-effectiveness of the study intervention from a societal perspective and will include an analysis of budgetary impact of scale-up of this intervention. Should the intervention prove both effective and cost-

effective, these analyses will assist in motivating the financial resources to promote its implementation.

In summary, the STAMP trial is assessing a novel, urine-based screening strategy utilising the TB-LAM and Xpert assays in HIV-positive patients admitted to hospital in sub-Saharan Africa. We hypothesise that use of this screening algorithm will reduce all-cause mortality at 2 months compared to sputum and clinical diagnostics (using the Xpert assay) which is the standard of care as currently recommended by the WHO. The trial commenced recruitment of patients in October 2015, and is projected to complete recruitment by September 2017 and follow-up by November 2017.

Abbreviations

ART: Antiretroviral therapy; CEPAC-I: Cost-Effectiveness of Preventing AIDS Complications-International; DSMB: Data Safety and Monitoring Board; HIV: Human immunodeficiency virus; LAM: Lipoarabinomannan; LE: Life expectancy; RCT: Randomized Controlled Trial; TB: Tuberculosis; WHO: World Health Organization

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DSMB members: Andrew Nunn*, Anton Pozniak*, Tom Harrison*, Katherine Fielding, Clare Flach, Stephen Lawn, Ankur Gupta-Wright
*Independent members

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Availability of data and materials

Final study datasets will be stored locally and securely at the London School of Hygiene & Tropical Medicine for long-term storage and access. Anonymised participant level data will be made available by request on a case-by-case basis.

Authors' contributions

SDL (chief-investigator) conceived the study and acquired funding with ELC, KF, JvO and DW. AGW wrote the first draft of the manuscript. RW and KR revised and augmented the methodological detail of the economic analysis section. KF, DW and JvO reviewed and refined the draft. All co-authors contributed to review and refinement of the manuscript, and read and approved the final version.

Competing interests

The authors declare that they have no competing interests

Consent for publication

Not applicable

Ethics approval and consent to participate

This trial protocol has been approved by the Research Ethics Committees of the London School of Hygiene & Tropical Medicine (ref: 9630), College of Medicine University of Malawi (ref: P.06/15/1743) and the University of KwaZulu-Natal Biomedical Research Ethics Committee (ref: BFC215/15). All patients in the trial will provide informed written consent to participate.

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**Chapter 4: Systematic review and meta-analysis
of urine-LAM detection's association with
mortality (research paper)**



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RESEARCH PAPER COVER SHEET

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SECTION A – Student Details

Student	Ankur Gupta-Wright
Principal Supervisor	Prof Liz Corbett
Thesis Title	Investigating mortality risk in hospitalised patients in Africa with HIV-associated tuberculosis and positive urine diagnostics: a clinical, epidemiological and immunological study

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	BMC medicine		
When was the work published?	23rd March 2016		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	The work was submitted for publication prior to registration for the research degree. Registration for my research degree was delayed by 4 months due to administrative issues.		
Have you retained the copyright for the work?*	Yes	Was the work subject to academic peer review?	Yes

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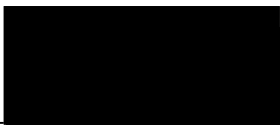
SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	
Please list the paper's authors in the intended authorship order:	
Stage of publication	

SECTION D – Multi-authored work

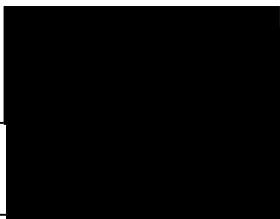
For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	My role was coming up with the concept of the review article, designing the study and search methodology, conducting the search, selecting the articles, extracting the data, analysing the data, and writing the manuscript.
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Student Signature:

_____  _____

Date: 31st July 2018

Supervisor Signature:

_____  _____

Date: 31st July 2018



World TB Day

RESEARCH ARTICLE

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Detection of lipoarabinomannan (LAM) in urine is an independent predictor of mortality risk in patients receiving treatment for HIV-associated tuberculosis in sub-Saharan Africa: a systematic review and meta-analysis

Ankur Gupta-Wright^{1,2*} , Jurgens A. Peters¹, Clare Flach³ and Stephen D. Lawn^{1,4}

Abstract

Background: Simple immune capture assays that detect mycobacterial lipoarabinomannan (LAM) antigen in urine are promising new tools for the diagnosis of HIV-associated tuberculosis (HIV-TB). In addition, however, recent prospective cohort studies of patients with HIV-TB have demonstrated associations between LAM in the urine and increased mortality risk during TB treatment, indicating an additional utility of urinary LAM as a prognostic marker. We conducted a systematic review and meta-analysis to summarise the evidence concerning the strength of this relationship in adults with HIV-TB in sub-Saharan Africa, thereby quantifying the assay's prognostic value.

Methods: We searched MEDLINE and Embase databases using comprehensive search terms for 'HIV', 'TB', 'LAM' and 'sub-Saharan Africa'. Identified studies were reviewed and selected according to predefined criteria.

Results: We identified 10 studies eligible for inclusion in this systematic review, reporting on a total of 1172 HIV-TB cases. Of these, 512 patients (44 %) tested positive for urinary LAM. After a variable duration of follow-up of between 2 and 6 months, overall case fatality rates among HIV-TB cases varied between 7 % and 53 %. Pooled summary estimates generated by random-effects meta-analysis showed a two-fold increased risk of mortality for urinary LAM-positive HIV-TB cases compared to urinary LAM-negative HIV-TB cases (relative risk 2.3, 95 % confidence interval 1.6–3.1). Some heterogeneity was explained by study setting and patient population in sub-group analyses. Five studies also reported multivariable analyses of risk factors for mortality, and pooled summary estimates demonstrated over two-fold increased mortality risk (odds ratio 2.5, 95 % confidence interval 1.4–4.5) among urinary LAM-positive HIV-TB cases, even after adjustment for other risk factors for mortality, including CD4 cell count.

(Continued on next page)

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(Continued from previous page)

Conclusions: We have demonstrated that detectable LAM in urine is associated with increased risk of mortality during TB treatment, and that this relationship remains after adjusting for other risk factors for mortality. This may simply be due to a positive test for urinary LAM serving as a marker of higher mycobacterial load and greater disease dissemination and severity. Alternatively, LAM antigen may directly compromise host immune responses through its known immunomodulatory effects. Detectable LAM in the urine is an independent risk factor for mortality among patients receiving treatment for HIV-TB. Further research is warranted to elucidate the underlying mechanisms and to determine whether this vulnerable patient population may benefit from adjunctive interventions.

Keywords: HIV, Tuberculosis, Lipoarabinomannan, LAM, Mortality, Systematic review

Background

Tuberculosis (TB) remains the most frequent cause of HIV/AIDS-related deaths globally, accounting for 0.4 million deaths in 2014 alone [1]. Diagnosis of HIV-associated TB (HIV-TB) remains challenging due to non-specific clinical features, early dissemination beyond the lungs, the relatively low mycobacterial burden within sputum samples, and clinical over-reliance on sputum-based diagnostic tests [2–4]. Mycobacterial culture is still regarded as the ‘gold standard’ diagnostic test; however, in practice its use and utility are greatly limited by prolonged turnaround times and lack of widespread availability due to the need for expensive infrastructure and skilled laboratory personnel. Diagnostic tools that are rapid, have good diagnostic accuracy and can be used at all levels of the healthcare system will be required to meet ambitious World Health Organization (WHO) goals of reducing TB deaths by 95 % and new cases by 90 % by 2035 [5–7].

Recent years have seen increased investment and research into rapid, ‘point-of-care’ diagnostics. Given the challenges of obtaining sputum samples and limited yield in extrapulmonary TB, urine has been identified as a favourable alternative biological sample due to the ease of obtaining samples from patients, the ease of laboratory handling and processing, and the lower risk of nosocomial transmission to healthcare and laboratory workers. Several mycobacterial antigens have been identified in the urine of patients with active TB [8, 9]. Promising diagnostic assays to emerge are those that detect the mycobacterial cell wall lipopolysaccharide lipoarabinomannan (LAM) using simple immune capture assays [10]. Testing for LAM in the urine has proved particularly useful in those with HIV-TB, with incrementally greater sensitivity with the progression of immunodeficiency [11–13]. In addition to commercially available enzyme-linked immunosorbent assays (ELISA), a simple, low-cost and rapid lateral flow point-of-care assay has also been developed (Determine TB-LAM; Alere Inc., Waltham, MA, USA). This is undergoing impact evaluation as part of the diagnostic algorithms

for HIV-TB in clinical trials in sub-Saharan Africa [14–16], and WHO have conditionally recommended its use to assist in TB diagnosis in hospitalised patients with low (≤ 100 cells/ μ l) CD4 cell counts or patients who are seriously ill [17].

The diagnostic accuracy of urinary LAM detection for HIV-TB has been extensively studied and is the subject of a comprehensive Cochrane systematic review and meta-analysis [18]. However, recent prospective studies of diagnostic accuracy have also highlighted its prognostic value, demonstrating strong associations between the detection of urinary LAM and mortality risk during follow-up on TB treatment; this association persists even after adjustment for key confounding factors such as blood CD4 count, blood haemoglobin level and age [19]. Testing for LAM in the urine may therefore be of additional clinical benefit, over and above the diagnosis of TB, by identifying patients with the highest mortality risk who may potentially benefit from closer follow-up or adjunctive interventions used in combination with TB treatment, anti-retroviral therapy (ART) and co-trimoxazole prophylaxis.

We have performed a systematic review and meta-analysis to summarise the strength of the relationship between urinary LAM and mortality in adults with HIV-TB. We also discuss potential mechanisms underlying these associations and discuss the need for future research and implications for the implementation of testing for urinary LAM in this vulnerable population.

Methods

Search strategy

We searched MEDLINE and Embase databases for studies reporting urinary LAM status in HIV-infected adults, and published up until 1 November 2015. The search strategy involved combining four search ‘sets’ with the Boolean operator ‘AND’. The search sets included comprehensive terms for ‘tuberculosis’, ‘HIV/AIDS’ and ‘lipoarabinomannan’, and ‘sub-Saharan Africa’. References of relevant studies and review articles were

also searched, and experts in the field contacted to suggest additional references. The search strategy was pre-specified in the review protocol (Additional file 1: Table S1). In addition, abstract books from the International Union Against Tuberculosis and Lung Disease were manually searched, and abstracts from the Conference of Retroviruses and Opportunistic Infections were electronically searched (both from 2007 to 2015). The studies identified were compiled into a database and screened on title and/or abstract, with duplicates removed. Full texts of those potentially eligible articles were reviewed further. This review was conducted and reported in accordance with the PRISMA checklist [20]. Research ethics permission was not sought because this was a secondary analysis of published anonymised data.

Study selection

Identified studies were included if they reported mortality and urinary LAM status in adult patients with HIV-TB co-infection, if they had at least 10 TB cases with urinary LAM results and at least five deaths, and if they were undertaken in sub-Saharan Africa. Studies were excluded if they did not report mortality outcomes (our primary outcome of interest), if they related only to paediatric populations, or if they were studies of particular sub-populations that were not easily generalisable (e.g., miners or prisoners). Studies including both HIV-positive and HIV-negative patients with TB were only included if they presented disaggregated data based upon HIV status or if <10 % of the patients with TB were HIV negative. Non-English-language studies were only included if adequate data could be extracted from an English abstract.

Data extraction and analysis

Data were extracted directly into a database by two reviewers, including study citation, year of publication, setting (country/healthcare level), number of patients and TB diagnoses, baseline characteristics, TB reference standard, method of testing for urinary LAM, number of LAM-positive and LAM-negative TB cases, mortality in patients diagnosed with TB (overall and stratified by urinary LAM status), and risk factors for mortality. The primary outcome was the risk of mortality in urinary LAM-positive TB cases compared to LAM-negative TB cases. Case fatality rates were calculated based on total number of TB cases with follow-up data and total number of deaths. The other outcome of interest was adjusted odds ratio (OR) of mortality for urinary LAM-positive TB cases (based on a multivariable analysis including other predictors of mortality). Studies were included in the analysis if these data were presented. If adjusted OR of death was not presented, the author was contacted to ask if those data were available. Study

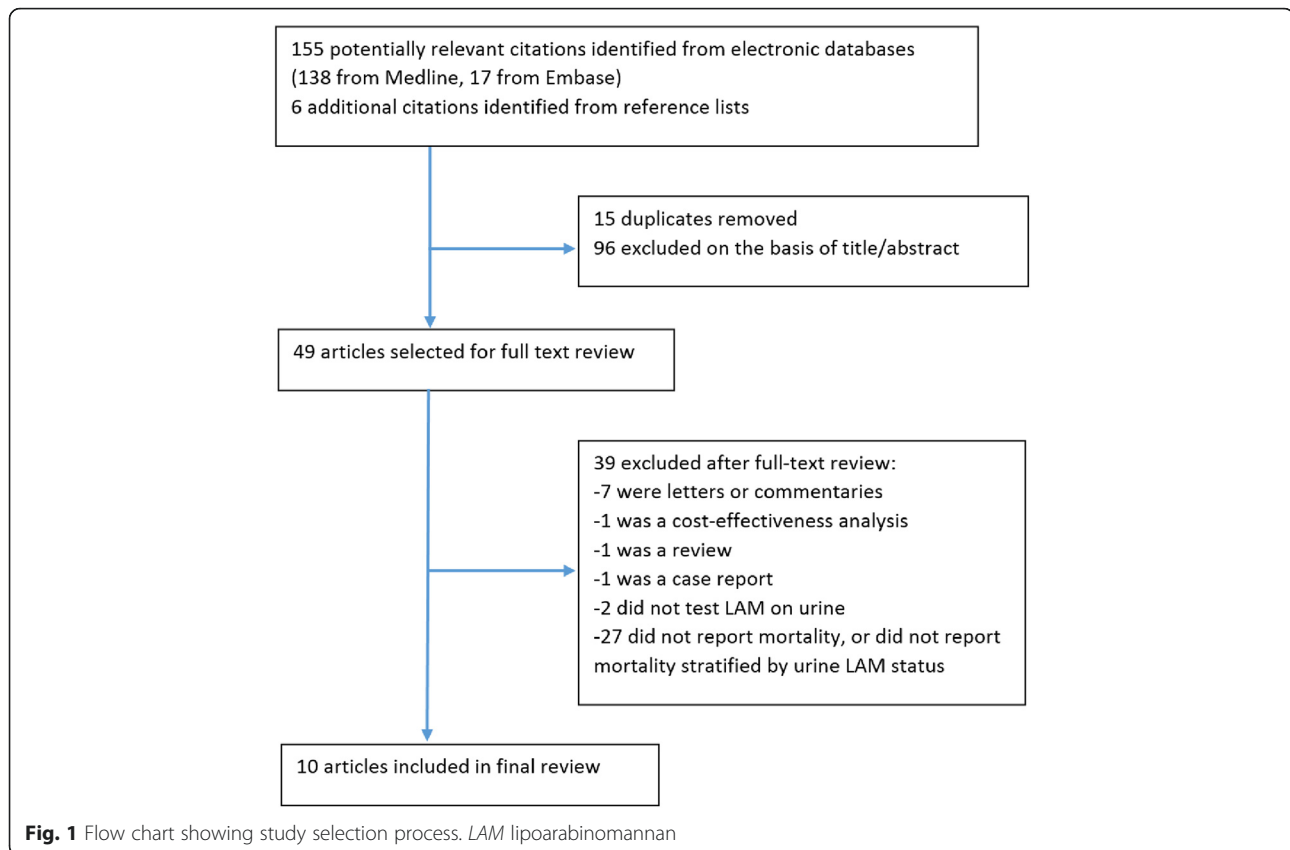
quality was graded according to a pre-specified checklist, which was adapted from the QUADAS-2 tool (see Additional file 1: Table S2) [21].

All analyses were done using Stata 11.0 (StataCorp, College Station, TX, USA) and were done on study-level data. Forest plots were generated for mortality risk ratio. The heterogeneity of study outcomes were calculated using the I^2 statistic. Pooled estimates were calculated using random-effects modelling, with study weights assigned based on inverse variance. The source of heterogeneity was explored using sub-group analyses (study setting, median CD4 cell count, overall TB mortality, time at which mortality outcome was measured). A fixed continuity correction of 0.5 was used for studies with 0 or 100 % mortality in any group. The adjusted ORs for mortality from multivariate regression analyses were also presented in a forest plot and summary statistics calculated as above. Funnel plots of log ORs against their standard error and Egger's test were used to aid assessment of bias.

Results

A total of 161 citations were identified, 49 studies selected for full-text review, and 10 studies eligible for inclusion (Fig. 1). Included studies are summarized in Table 1. All studies were cohort in design, conducted in sub-Saharan Africa, enrolled adults ≥ 18 years of age, and reported between 2009 and 2015. Three studies were based in an outpatient setting, six were of hospital inpatients, and one enrolled patients from both settings. Most studies enrolled patients in whom TB was the suspected diagnosis based on clinical presentation, although three studies were done in patients due to start ART, and one study was restricted to patients with a TB diagnosis (Table 1). Two studies reported results in HIV-positive and HIV-negative patients. These were eligible for inclusion in this review because the number of HIV-negative cases comprised <10 % of the total TB cases. Eight studies used the Determine TB-LAM lateral flow assay to test for urinary LAM, and two studies used the Clearview TB ELISA (Inverness Medical Innovations, UK). All studies used standard first-line TB treatment and initiated ART in accordance with national guidelines at the time. This review includes data from 1172 TB cases and 512 (44 %) urinary LAM-positive cases in total. Five studies were deemed as being of good quality, and five of moderate quality (Table 1).

The proportion of urinary LAM-positive TB cases varied from 22 % to 65 %, and was higher in studies of hospital inpatients than those of outpatients (mean 49 % and 30 % respectively, $P = 0.04$). Nine of the studies used microbiological definitions of TB cases, which included mycobacterial culture and/or the Xpert MTB/RIF assay



(Cepheid, Sunnyvale, CA, USA). Three studies included only sputum samples in the reference standard, and six included at least one non-respiratory sample within the reference standard for TB diagnosis. Of the eight studies using the TB-LAM assay, four used the grade 2 cut-off as a 'positive' result, two used a grade 1 or higher cut-off, and two did not report which cut-off was used (Additional file 1: Table S3). Median loss to follow-up was 13.4 % (range 0–43 %, Additional file 1: Table S3).

Mortality outcomes in TB cases were assessed at between 2 and 3 months of follow-up for six studies, at 6 months in three studies, and at 36 months in one study. Overall case fatality rates among HIV-TB cases varied between 7 % and 53 %. Median CD4 cell counts varied between 57 and 210 cells/mm³ (overall median 114 cells/mm³), indicating severely immunocompromised patient populations. Loss to follow-up rates varied greatly between studies (Additional file 1: Table S3).

All studies demonstrated an increased risk of mortality amongst urinary LAM-positive TB cases compared to LAM-negative cases, with the relative risk (RR) of mortality varying from 1.2 to 7.5 (median 2.5, interquartile rate 1.8–3.4; Table 1, Fig. 2). A pooled

summary estimate generated using a random-effects meta-analysis showed a two-fold increased risk of mortality (RR 2.3, 95 % confidence interval [CI] 1.6–3.1; Fig. 2), but demonstrated moderate heterogeneity ($I^2 = 37.0 %$, $P = 0.113$).

Sub-group analyses were undertaken to try to explain the heterogeneity in mortality risk across studies (Additional file 1: Table S4). Stratifying studies by healthcare setting removed heterogeneity amongst studies conducted on hospitalised patients, giving a pooled summary RR for mortality of 1.9 (95 % CI 1.5–2.5; Fig. 2). The summary RR of mortality was higher at 3.4 (95 % CI 1.2–9.5, $n = 4$) for outpatient studies, but showed greater variation ($I^2 = 69.2 %$). A similar effect was seen in sub-group analysis by overall TB mortality, with an almost two-fold increase in mortality risk associated with LAM-positivity when overall mortality was >20 % (RR 1.8, 95 % CI 1.4–2.2, $n = 5$). Studies with overall mortality risk ≤20 % showed an even greater RR of mortality for LAM-positive patients (RR 3.7, 95 % CI 2.2–6.2, $n = 5$). Studies with a median CD4 cell count >100 cells/μl had a higher RR for mortality in LAM-positive patients than those with medians ≤100 cells/μl (RR 2.7, 95 % CI 1.5–4.7, $n = 5$ and RR 1.9, 95 % CI 1.4–2.6, $n = 5$ respectively). The

Table 1 Studies reporting urinary LAM detection and mortality included in the systematic review

Study	LAM assay used (type of urine sample)	Study setting and population (country)	Median CD4 cell count (by LAM status; if presented) (cells/ μ l)	Number of TB cases/total number in study (prevalence %)	Number of TB LAM-positive cases (%)	Duration of follow-up (months)	Overall mortality in TB cases (%)	Number of TB deaths/number of TB cases (%)	Number of TB deaths		RR of mortality ^a (95 % CI)	Quality assessment score ^b
									LAM positive	LAM negative		
Shah et al. (2009) [47]	Clearview TB ELISA (frozen urine)	Hospitalised patients; TB suspected (South Africa)	79	193/499 (38.7)	114 (59.1)	2	22.3	31/114 (27.2)	12/79 (15.2)	1.8 (1.0–3.3)	60	
Lawn et al. (2012) [48]	Determine TB-LAM lateral flow assay (frozen urine)	Outpatient clinic; patients initiating ART (South Africa)	100 (LAM+positive 37; LAM-negative 115)	59/325 (18.2)	23 (39.0)	3	8.5	5/23 (21.7)	0/36 (0.0)	NA	70	
Talbot et al. (2012) [49]	Clearview TB ELISA (fresh and frozen urine)	Hospitalised patients; TB suspected (Tanzania)	86	69/212 (32.5)	45 (65.2)	2	52.9	25/38 (65.8)	33/83 (39.8)	1.7 (1.2–2.3)	75	
Peter et al. (2013) [50]	Determine TB-LAM lateral flow assay (frozen urine)	Hospitalised patients; TB suspected (South Africa)	89 (LAM+positive 62; LAM-negative 180)	116/281 (4.2)	58 (50.0)	2	13.9	6/25 (24.0) ^c	1/23 (8.5) ^c	5.5 (0.7–42.4)	80	
Balcha et al. (2014) [51]	Determine TB-LAM lateral flow assay (frozen urine)	Outpatient clinic; ART naive; sputum producers (Ethiopia)	176 (LAM+positive 94; LAM-negative 187)	128/757 (16.9)	35 (27.3)	6	6.8	7/35 (20.0)	3/113 (2.7)	7.5 (2.1–27.6)	60	
Manabe et al. (2014) [28]	Determine TB-LAM lateral flow assay (fresh urine)	Hospitalised patients; TB suspects (Uganda)	57	145/351 (41.3)	90 (62.1)	2	22.1	25/90 (27.8)	7/37 (12.7)	2.2 (1.0–4.7)	75	
Drain et al. (2015) [52]	Determine TB-LAM lateral flow assay (frozen urine)	Outpatient clinic; patients initiating TB treatment (South Africa)	168 (LAM+positive 106; LAM-negative 198)	90/90 (100.0)	29 (22.2)	36	27.8	9/29 (31.0)	16/61 (26.2)	1.2 (0.6–2.4)	70	
Peter et al. (2015) [53]	Determine TB-LAM lateral flow assay (frozen urine)	Hospitalised patients; TB suspected (South Africa)	210	181/583 (31.0)	41 (22.7)	6	13.0	6/17 (35.2)	15/106 (14.2)	2.5 (1.1–5.5)	85	
Lawn et al. (2015) [54]	Determine TB-LAM lateral flow assay (frozen urine)	Hospitalised patients; all HIV+ patients (South Africa)	148	136/427 (31.2)	53 (39.0)	3	13.7	13/5 (24.5)	6/83 (7.2)	3.4 (1.4–8.4)	75	
Bjerrum et al. (2015) [55]	Determine TB-LAM lateral flow assay (fresh urine)	Hospital inpatient and outpatient; TB suspected (Ghana)	127	55/469 (11.7)	24 (43.6)	6	32.7	13/24 (54.2)	5/31 (16.1)	3.4 (1.4–8.1)	70	

ART antiretroviral therapy. CI confidence interval. ELISA enzyme-linked immunosorbent assay. LAM liparabinomannan, NA not applicable, RR relative risk, TB tuberculosis. ^aUrinary LAM-positive TB cases compared to urinary LAM-negative TB cases. ^bQuality assessment score graded as follows: <50 poor, 50–74 moderate, >74 good. ^cMortality in TB cases with urinary LAM results only reported in patients who did not receive 'early empirical TB therapy'

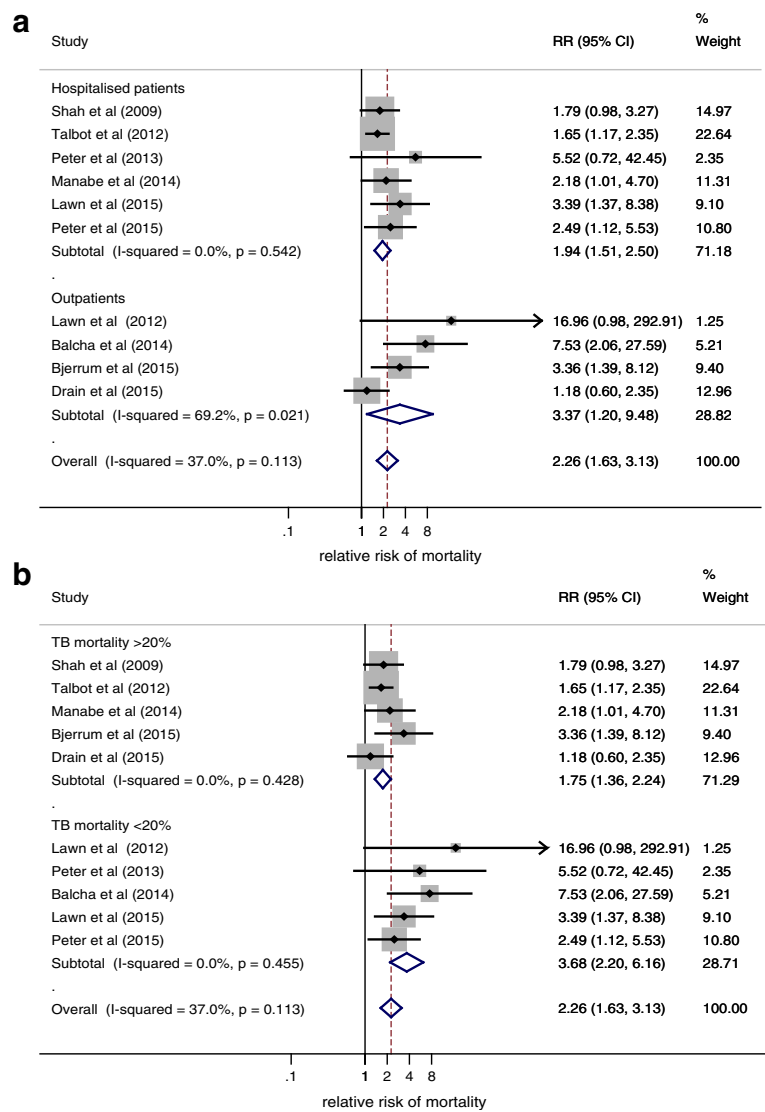


Fig. 2 Forest plot showing relative risk (RR) of mortality in urinary lipoarabinomannan (LAM)-positive tuberculosis (TB) cases compared to urinary LAM-negative TB cases, stratified by (a) study setting and (b) overall mortality in patients with TB

summary RRs were similar when studies were stratified by time at which mortality was measured (≤ 3 months RR 2.1, 95 % CI 1.5–2.9, $n = 6$; > 3 months RR 2.6, 95 % CI 1.3–5.2).

Sensitivity analyses showed that excluding the two studies reporting a small number of HIV-negative cases (< 1 % of total TB cases included in the meta-analysis) did not alter the overall effect size. Further sensitivity analyses excluding studies using a (pre-January 2014) grade 1 cut-off for TB-LAM or not reporting cut-off grade, studies using the Clearview TB-ELISA and not the TB-LAM assay, studies with only respiratory samples in the TB reference standard, studies of low or moderate quality, and studies with > 20 % loss to follow-up also

resulted in no substantial change in the overall effect size. Analyses with little heterogeneity were also repeated using fixed-effects meta-analysis, which did not alter the effect size (data not shown). A funnel plot showed few studies with small or no effect size, which may suggest publication bias (Egger’s test $P = 0.025$; Additional file 1: Figure S1).

Five studies reported results of multivariable regression analyses for mortality, including urinary LAM detection as a variable. No studies were powered to detect a difference in mortality by urinary LAM status, but three studies demonstrated urinary LAM was an independent predictor for mortality (adjusted OR varied from 2.2 to 4.7). Risk factors included in multivariable

models are outlined in Table 2 and included CD4 cell count (as a marker of HIV-associated immunosuppression) in four studies. A pooled summary estimate calculated using meta-analysis showed an over two-fold increased odds of mortality (OR 2.5, 95 % CI 1.4–4.5; Fig. 3) among HIV-TB cases with positive urinary LAM tests, even after adjustment for other risk factors for mortality. However, these studies did show a large degree of heterogeneity in their effect sizes. Studies in which overall mortality was $\leq 20\%$ had a greater summary RR for mortality in LAM-positive patients (RR 4.4, 95 % CI 2.1–9.5) compared to studies with a mortality $>20\%$ (1.9, 95 % CI 1.1–3.3; Fig. 3), although there were few studies in each sub-group and CIs overlapped.

Discussion

This is the first systematic review and meta-analysis to assess the association between urinary LAM detection and mortality during TB treatment in patients with HIV-TB. The meta-analysis demonstrated a two-fold greater mortality risk among those patients with urinary LAM-positive compared to urinary LAM-negative TB. Five studies reported adjusted OR of mortality in LAM-positive TB cases, and demonstrated that urinary LAM is an independent predictor of mortality in TB cases, even after adjustment for other important risk factors for mortality, such as degree of immunosuppression. There are multiple potential mechanisms that may underlie this association (Table 3).

The strengths of this systematic review and meta-analysis include the synthesis of data from a large number of patients enrolled in studies in diverse settings across sub-Saharan Africa, with data from 1172 patients with TB, of whom 44 % had LAM detected in their urine. The selection of patients was well described in all studies, and was mostly representative of the population for which urinary LAM testing has been conditionally recommended in recent WHO policy guidance (patients

suspected of having TB, HIV-positive patients with low CD4 cell counts, or seriously unwell patients such as those requiring hospitalisation) [17]. Studies were not powered to demonstrate a mortality difference based on urinary LAM detection, but the meta-analysis has demonstrated a robust association.

Although their effect size varied across studies, heterogeneity appeared to be moderate at most ($I^2 = 39\%$). The most likely sources of variation were in the settings and patient populations. Studies conducted in hospital settings, which had higher overall TB mortality and lower median CD4 cell counts, showed approximately double the mortality risk in LAM-positive compared to LAM-negative TB patients. Surprisingly, the association between LAM-positivity and mortality was even stronger in outpatient settings, with lower overall TB mortality and higher median CD4 cell counts, although effect sizes were more variable. Urinary LAM testing may be an even better marker of poor prognosis in these settings, although recent WHO recommendations do not specify its use as diagnostic for TB in these patient populations [17].

Limitations of this systematic review include the over-representation of South Africa amongst the study settings, which is true for HIV-TB research as a whole. Different methods of detecting LAM in urine were used, as were different cut-offs for the Determine TB-LAM lateral flow assay. In addition, although most studies used mycobacterial culture from sputum as the reference standard for diagnosing TB, there was some variation in case definitions for HIV-TB. Although two eligible studies included a very small number of HIV-negative cases, their exclusion in a sensitivity analysis did not impact the overall effect size. All studies were deemed of moderate or good quality. Sensitivity analyses demonstrated that none of the above issues substantially altered the overall effect size.

There were some potential sources of bias in this study. Three studies reported high rates of loss to follow-up

Table 2 Studies reporting adjusted odds ratios for mortality in urinary LAM-positive compared to urinary LAM-negative TB cases, after adjustment for other predictors of mortality

Study	Study setting and population	Adjusted odds ratio for mortality in urinary LAM-positive compared to urinary LAM-negative TB cases (95 % CI)	Variables included in the multivariable analysis with urinary LAM status
Talbot et al. (2012) [49]	Hospital inpatients; TB suspected	1.3 (0.9–1.8)	CD4 cell count, ART
Drain et al. (2015) [52]	Outpatients; confirmed TB patients	5 (1.1–23.9)	CD4 cell count, age, gender, Karnofsky score
Peter et al. (2015) [53]	Hospital inpatients; TB suspected	4.7 (1.6–15.9)	Study site, gender, age, CD4 cell count
Lawn et al. (2015) [54]	Hospital inpatients; All HIV+ patients	4.2 (1.5–11.8)	Age, CD4 cell count
Bjerrum et al. (2015) [55]	Hospital inpatients and outpatients; TB suspected	2.2 (1.1–3.5)	Gender, hospitalisation, CD4 cell count, Medical Early Warning Score

ART antiretroviral therapy, CI confidence interval, LAM lipoarabinomannan, TB tuberculosis

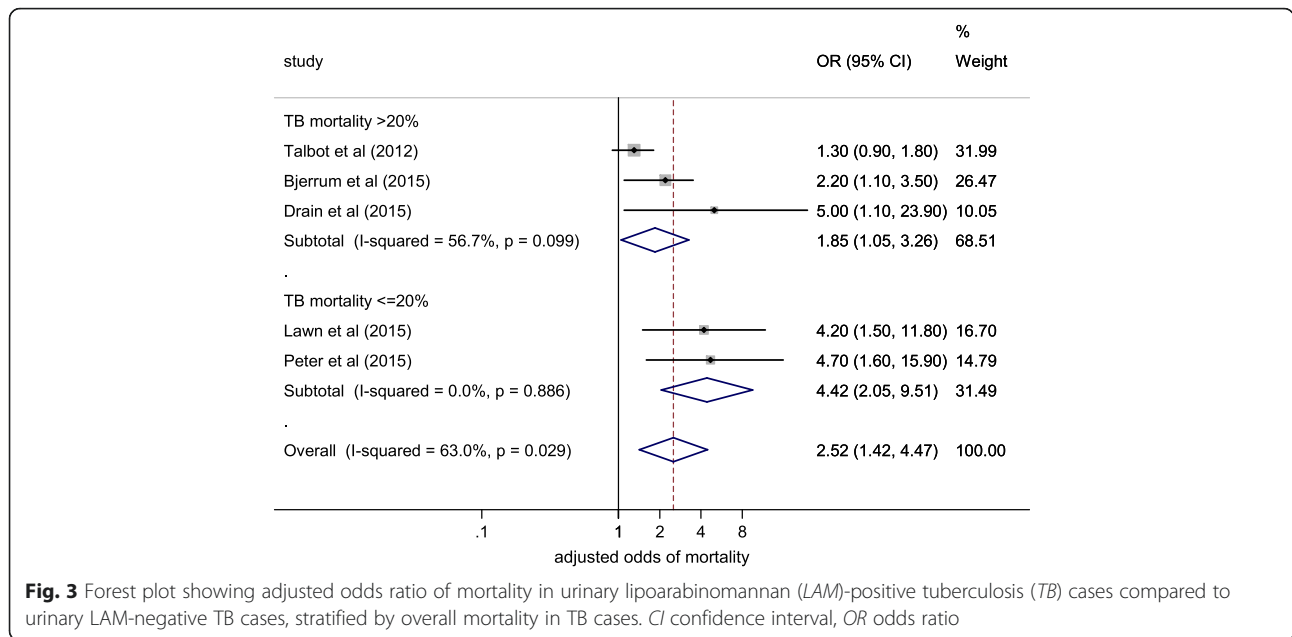


Fig. 3 Forest plot showing adjusted odds ratio of mortality in urinary lipoarabinomannan (LAM)-positive tuberculosis (TB) cases compared to urinary LAM-negative TB cases, stratified by overall mortality in TB cases. CI confidence interval, OR odds ratio

(>20 %), which may have included a disproportionate number of unascertained deaths, especially if patients that died later were more likely to be LAM-negative. A sensitivity analysis showed that excluding studies with high loss to follow-up did not alter effect size. Many studies were excluded from the systematic review because they did not report mortality rates, and assessment for publication bias revealed a lack of smaller studies demonstrating small, weak associations or no association at all. Most studies reporting urinary LAM testing in HIV-TB co-infection were studies on diagnostic accuracy. It is likely that the majority of these did not gather mortality data, and studies

included in this review mostly reported mortality as a secondary outcome. Finally, only five studies reported adjusted multivariable models for mortality that included urinary LAM detection as a variable, making exploration of heterogeneity by sub-group analysis challenging.

The mechanism by which LAM enters the urine has been an issue of contention, with early literature assuming LAM antigenuria resulted from free renal glomerular filtration of circulating LAM from the bloodstream [22, 23]. However, some researchers had also speculated that haematogenous dissemination of TB resulted in renal involvement and direct shedding into the urine, and this

Table 3 Potential mechanisms of the association between urinary LAM detection and increased mortality risk

Potential mechanism	Evidence	References
Urinary LAM is a marker of disseminated TB and higher mycobacterial burden, which is associated with a worse prognosis	• Urinary LAM is due to haematogenously disseminated renal TB	Cox et al. 2015 [26]
	• HIV-TB patients with mycobacteraemia have a higher mortality	Cummings et al. 2015 [56]
	• Higher concentrations of urinary LAM are associated with higher mycobacterial burden	Kerkhoff et al. 2014 [19]
Urinary LAM is a proxy for a low CD4 cell count	• HIV-TB patients with positive urinary LAM tests have lower CD4 cell counts	Minion et al. 2011 [11]
	• Mortality is higher in patients with lower CD4 cell counts	Gupta et al. 2015 [34]
LAM itself contributes to immunosuppression, impairing host defences against MTB and other opportunistic infections	• LAM is a virulence factor for MTB	Strohmeier et al. 1999 [35]
	• LAM inhibits immune responses, with direct inhibitory effects on macrophage activation and function	Mishra et al. 2011 [38] Neyrolles et al. 2011 [41]
	• LAM inhibits pro-inflammatory cytokines, e.g. IL-12 and TNF- α	
	• LAM enhances the secretion of anti-inflammatory cytokines, e.g. IL-10	

LAM lipoarabinomannan, MTB *Mycobacterium tuberculosis*, TB tuberculosis

mechanism has now been demonstrated beyond doubt by multiple lines of evidence, including post-mortem data [24–27]. Moreover, urinary LAM has also been associated with mycobacteraemia and other markers of higher mycobacterial burden [19, 28–31]. Therefore, urinary LAM may simply be a marker of more severe, disseminated TB, explaining its association with mortality. Early studies of urinary LAM detection in patients with HIV-TB co-infection also demonstrated an association with TB immune reconstitution inflammatory syndrome (IRIS) [32], although this is unlikely to be an important cause of increased mortality because TB-IRIS is rarely fatal [33].

Urinary LAM assays are also more sensitive in patients with lower CD4 cell counts (typically <100 cells/ μ l) [11, 25], because these patients have the highest risk of disseminated and renal TB. However, these are the very patients with the highest mortality risk [34], and therefore the higher mortality risk associated with urinary LAM detection may be confounded by CD4 cell count. However, of particular note, we have demonstrated in this meta-analysis that, even after adjusting for CD4 cell count as well as other predictors of mortality, urinary LAM remains an independent marker of mortality. Although mortality in LAM-negative TB cases is lower than that in LAM-positive cases, it was still up to 40 % in some studies. Without post-mortem data, it is difficult to attribute cause of death to TB or other co-infection or pathologies.

LAM itself may be on the causal pathway leading to increased mortality risk. Much in vitro research has examined the role of LAM as a virulence factor for *Mycobacterium tuberculosis* (*MTB*) [35]. LAM is a 19 kDa glycolipid and is a major constituent of *MTB* and the mycobacterial cell wall. It binds to several cell surface receptors of the immune system, especially macrophages [36, 37]. Through its immunomodulatory effects, LAM is thought to promote the survival of *MTB* in the human host [38] by directly impairing host immune defences. Its immunological effects include inhibition of cytokines that are key in the host immune response to *MTB*, such as interleukin (IL)-12, tissue necrosis factor alpha (TNF- α) and other inflammatory mediators [39–41]. Furthermore, LAM has been shown to enhance the secretion of anti-inflammatory cytokines such as IL-10 [42, 43]. LAM contributes to increased survival of *MTB* in macrophages, employing mechanisms that include prevention of macrophage phagosome maturation [44, 45]. These immunosuppressive effects of LAM could impair host responses to *MTB* and also to other opportunistic infections, and thereby contribute to increased mortality risk amongst patients who test positive for urinary LAM. In contrast, non-pathogenic mycobacteria contain structurally different

LAM molecules to *MTB* that promote strong pro-inflammatory responses [38, 46].

The diagnostic utility of urinary LAM assays appears to be limited to HIV-positive patients with advanced immunosuppression. It is important to note that whilst we have found that patients with LAM detected in their urine had a higher mortality risk from the point of diagnosis and commencing anti-TB treatment, it is plausible that the use of urinary LAM assays to expedite the diagnosis and treatment of TB can also reduce mortality. This hypothesis and the impact of urinary LAM assays as a diagnostic tool in HIV-TB are currently being evaluated [14–16].

However, these assays identify patients at significantly increased risk of mortality during follow-up on TB treatment, above and beyond that accounted for by advanced immunosuppression and TB diagnosis. This appears to be true even in populations in which urinary LAM testing is not recommended for diagnostic purposes. This finding should prompt further research into whether interventions, in addition to the timely initiation of anti-TB therapy and ART, might benefit these patients and reduce their high mortality risk. Given the potential immunosuppressive nature of LAM itself, research is warranted to explore whether patients who are LAM-positive have altered immune responses compared to those who are LAM negative. Potential adjunctive interventions for these patients might be needed.

Conclusions

This systematic review and meta-analysis has shown that patients with HIV-TB and detectable urinary LAM have increased mortality risk compared to those patients with TB without detectable urinary LAM. Urinary LAM is an independent risk factor for mortality, suggesting this finding is not simply an epiphenomenon detecting patients with more advanced immunosuppression. We have presented several plausible biological explanations for this association, including LAM's immunosuppressive effects in vitro. Urinary LAM detection appears to be a feasible tool to highlight patients at high risk of mortality as well as identifying potential targets for adjunctive therapeutic interventions for reducing TB deaths over the next 20 years.

Additional file

Additional file 1: Table S1. Search Strategy. **Table S2.** Quality assessment tool. **Table S3.** Additional information about studies included in the systematic review. **Table S4.** Effect estimates of mortality risk ratio for urine LAM-positive TB patients compared to urinary LAM-negative TB patients from sub-group analyses. **Figure S1.** Funnel plot of log odds ratios plotted against the standard error of the log odds ratio. (DOCX 25 kb)

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SDL and AGW came up with the concept of the review article; AGW and JAP conducted the search, selected articles and extracted the data; AGW and CF did the data analysis, which was reviewed by JAP; AGW and SDL drafted the article; and all authors contributed to the final published article and approved the final version.

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Table S1: Search Strategy

Search concept	Search Terms
1. HIV/AIDS	HIV-1* OR HIV-2* OR HIV* or HIV Infections* OR HIV Seropositivity* OR “human immunodeficiency“ OR “human immune deficiency virus” OR human immune-deficiency syndrome OR human immune-deficiency syndrome AIDS OR “Acquired Immunodeficiency Syndrome”* OR acquired immune deficiency syndrome.mp.
2. LAM	Lipoarabinomannan* OR LAM*
3. Tuberculosis	TB* OR Tuberculosis* OR Mycobacterium tuberculosis* TUBERCULOSIS*
4. Sub-Saharan Africa*	“Africa South of the Sahara”* OR Central Africa* OR Western Africa* OR Eastern Africa* OR Southern Africa* OR Benin* OR Benin* OR Burkina Faso* OR Burundi* OR Central African Republic* OR Chad* OR Comoros* OR “Democratic Republic of the Congo”* OR Eritrea* OR Ethiopia* OR Gambia* OR Guinea* OR Guinea-Bissau* OR Kenya* OR Liberia* OR Madagascar* OR Malawi* OR Mali* OR Mozambique* OR Niger* OR Rwanda* OR Sierra Leone* OR Somalia* OR Tanzania* OR Togo* OR Uganda* OR Zimbabwe* OR Cameroon* OR Cape Verde* OR Congo* OR Cote d'Ivoire* OR Ghana* OR Lesotho* OR Mauritania* OR Nigeria* OR Atlantic Islands* OR Senegal* OR Sudan* OR South Sudan* OR Swaziland* OR Zambia* OR Angola* OR Botswana* OR Gabon* OR Mauritius* OR Namibia* OR Seychelles* OR South Africa* OR Equatorial Guinea*
	All the above sets (1-4) were combined with “AND”

All terms were searched as keywords, * denotes also searched as subject heading word and MeSH, \$ denotes truncation, * based on OVID expert search strategy for ‘countries of sub-Saharan Africa’.

Table S2: Quality assessment tool

QUALITY ASSESSMENT TOOL			
Selection of study participants	Score		
Was the spectrum of patients representative of the patients who will undergo urine LAM testing?	0	0.5	1
Were selection criteria clearly described?	0	0.5	1
What was the HIV prevalence amongst TB-case (90 to <100%=0.5, 100%=1)	0	0.5	1
Total			
Testing	Score		
Is the reference standard for tuberculosis adequate?	0	0.5	1
Was the methodology for performing LAM testing adequate?	0	0.5	1
What cut-off was used for positive LAM test (not described/grade 1= 0.5, grade 2=1, ELISA=1)?	0	0.5	1
Total			
Mortality ascertainment	Score		
Was method of ascertaining mortality clearly described?	0	0.5	1
Was overall mortality rate appropriate for the clinical setting?	0	0.5	1
Was the loss-to-follow rate appropriate?	0	0.5	1
Was a multivariate risk factor analysis performed?	0	0.5	1
Total			
OVERALL TOTAL (out of 10)			
<p><5= poor</p> <p>5-7.4= moderate</p> <p>>7.4=good</p>			

Table S3: additional information about studies included in the systematic review

Study	LAM assay used (type of urine sample)	LAM cut-off (based on pre-2014 TB-LAM reference card)	TB reference standard	Method of ascertaining mortality	Overall loss to follow-up (%)	Median CD4 cell count (cells/mm ³)	Quality assessment score (<50 poor, 50-74 moderate, >74 good)
Balcha et al (2014)	Determine TB-LAM lateral flow assay (frozen urine)	not reported	Positive TB culture or Xpert from sputum or lymph node aspirates	not reported	3.6	176 (LAM +ve 94; LAM -ve 187)	60
Lawn et al (2012)	Determine TB-LAM lateral flow assay (frozen urine)	not reported	Positive TB culture from sputum	record review	13.4	100 (LAM+ve 37; LAM-ve 115)	70
Manabe et al (2014)	Determine TB-LAM lateral flow assay (fresh urine)	any band	Positive TB culture from any specimen	record review and follow-up appointment	21.1	57	75
Talbot et al (2012)	Clearview TB ELISA (fresh and frozen urine)	OD >450nm	Positive TB culture from sputum or blood culture	follow-up, tracing	43	86	75

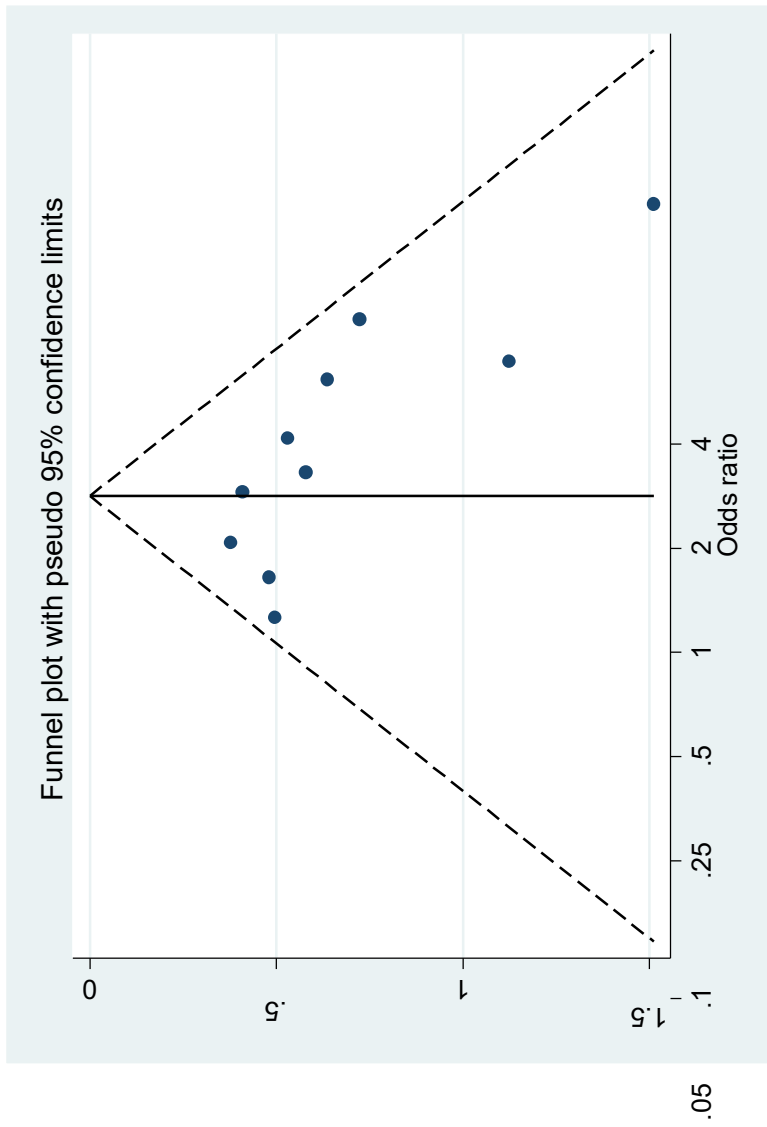
Drain et al (2015)	Determine TB-LAM lateral flow assay (frozen urine)	grade 1	TB symptoms (63% culture-confirmed)	follow-up	0	168 (LAM+ve 106; LAM-ve 198)	70
Shah et al (2009)	Clearview TB ELISA (frozen urine)	OD >450nm	Positive TB culture from any specimen, or acid fast bacilli/granuloma	follow-up	0	79	60
Peter et al (2013)	Determine TB-LAM lateral flow assay (frozen urine)	grade 2	Positive TB culture from any specimen	not reported	15.8	89 (LAM +ve 62; LAM -ve 180)	80
Peter et al (2015)	Determine TB-LAM lateral flow assay (frozen urine)	grade 2	Positive TB culture from sputum	follow-up	32.6	210	85
Lawn et al (2015)	Determine TB-LAM lateral flow assay (frozen urine)	grade 2	Positive TB culture or Xpert from any specimen	not reported	NR	148	75
Bjerrum et al (2015)	Determine TB-LAM lateral flow assay (fresh urine)	grade 2	Positive TB culture or Xpert from sputum	record review, tracing	13.4	127	70

Table S4: Effect estimates of mortality risk ratio for urine LAM-positive TB patients compared to urine LAM-negative TB patients from sub-group analyses

Sub-group	Number of studies included in analyses	Summary mortality risk ratio (95% CI)	Heterogeneity
Overall summary estimate	10	2.3 (1.6-3.1)	I²=37.0%
Hospitalised patients	6	1.9 (1.5-2.5)	I ² = 0%
Outpatients	4	3.4 (1.2-9.5)	I ² = 69%
Overall TB mortality >20%	5	1.8 (1.4-2.2)	I ² = 0%
Overall TB mortality ≤20%	5	3.7 (2.2-6.2)	I ² = 0%
Median CD4 cell count ≤100 cells/μl	5	1.9 (1.4-2.6)	I ² = 12%
Median CD4 cell count >100 cells/μl	5	2.7 (1.5-4.7)	I ² = 52%
Mortality measured ≤3months	6	2.1 (1.5-2.9)	I ² = 21%
Mortality measured >3months	4	2.6 (1.3-5.2)	I ² = 61%

All analyses using random-effects meta-analysis

Figure S1: funnel plot of log odds ratios plotted against the standard error of the log odds ratio.



Chapter 5: Cohort study of mortality, and risk factors for mortality (research paper)

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RESEARCH PAPER COVER SHEET

PLEASE NOTE THAT A COVER SHEET MUST BE COMPLETED FOR EACH RESEARCH PAPER INCLUDED IN A THESIS.

SECTION A – Student Details

Student	Ankur Gupta-Wright
Principal Supervisor	Prof Liz Corbett
Thesis Title	Investigating mortality risk in hospitalised patients in Africa with HIV-associated tuberculosis and positive urine diagnostics: a clinical, epidemiological and immunological study

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?			
When was the work published?			
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion			
Have you retained the copyright for the work?*	Choose an item.	Was the work subject to academic peer review?	Choose an item.

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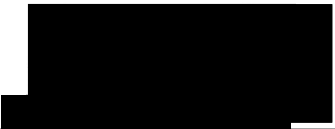
SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Clinical Infectious Diseases
Please list the paper's authors in the intended authorship order:	Ankur Gupta-Wright, Katherine Fielding, Douglas Wilson, Joep J van Oosterhout, Daniel Grint, Henry C Mwandumba, Melanie Alufandika-Moyo, Jurgens A Peters, Lingstone Chiume, Stephen D Lawn, Elizabeth L Corbett
Stage of publication	Submitted

SECTION D – Multi-authored work

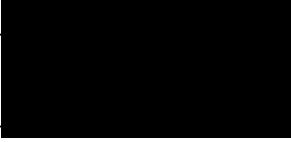
For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I came up with the concept, designed the study, was involved in data collection and data cleaning, I did the data analysis and wrote the manuscript.
--	--

Student Signature:



Date: 22 Sept 2018

Supervisor Signature:



Date: 22 Sept 2018

Clinical Infectious Diseases: Major Article

Title:

Tuberculosis in hospitalised patients with HIV: clinical characteristics, mortality, and implications from the STAMP trial

Authors:

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- †Died 23rd September 2016

Keywords: HIV, TB, hospital inpatients, mortality, urine LAM, Xpert

Running Title: HIV/TB mortality in hospital inpatients

Abstract:

Background: TB is the major killer of people living with HIV globally, with suboptimal diagnostics and management contributing to high case-fatality rates.

Methods: A prospective cohort of confirmed (Xpert MTB/RIF and/or Determine TB-LAM Ag positive) TB patients identified through screening HIV-positive inpatients with sputum and urine diagnostics in Malawi and South Africa (STAMP trial). Urine was tested prospectively (intervention) or retrospectively (standard of care arm). We defined baseline clinical phenotypes using hierarchical cluster analysis, and also used Cox regression analysis to identify associations with early mortality (≤ 56 days).

Results: Of 322 patients with TB confirmed between October 2015 and September 2018, 78.0% had ≥ 1 positive urine test. Antiretroviral therapy (ART) coverage was 80.2% among those not newly diagnosed, but with median CD4 count 75 cells/ μL and high HIV viral loads. Early mortality was 30.7% (99/322), despite near-universal prompt TB treatment. Older age, male sex, ART before admission, poor nutritional status, lower haemoglobin, and positive urine tests (TB-LAM and/or Xpert MTB/RIF) were associated with increased mortality in multivariate analyses. Cluster analysis (on baseline variables) defined 4 patient subgroups with early mortality ranging from 9.8% to 52.5%. Although unadjusted mortality was lower 9.3% lower in South Africa than Malawi, in adjusted models mortality was similar in both countries (HR 0.9, $p=0.729$).

Conclusions: Survival following prompt inpatient diagnosis of HIV-associated TB remained unacceptably high, even in South Africa. Intensified management strategies are urgently needed, for which prognostic indicators could potentially guide both development and subsequent use.

Introduction

HIV-associated tuberculosis disease (HIV/TB) is a leading cause of mortality globally, accounting for approximately 370,000 deaths globally in 2016 [1]. Much of this burden resides in patients admitted to hospitals in high HIV-prevalence settings in Africa [2]. A systematic review and meta-analysis of hospital cohort studies estimated that TB caused 24% of hospital admissions and 27% of deaths amongst HIV-positive patients, with 30% case fatality from HIV/TB [3]. Post-mortem data suggest an even greater burden, as up to half of fatal TB remains undiagnosed [4]. However, these data predate widespread access to antiretroviral therapy (ART) and improved diagnostics such as the Xpert MTB/RIF assay.

There is still a scarcity of data on factors associated with mortality in HIV/TB. Post-mortem studies suggest TB as the predominant cause of death [4], but substantial co-morbidity from other opportunistic infections and non-infectious conditions means that death *with* confirmed TB does not necessarily imply death *from* TB. Observational cohorts report low baseline CD4 cell count and older age to be associated with mortality, but these data mainly relate to ART naïve individuals, and include patients without bacteriologically-confirmed TB disease [5–7]. Disseminated HIV/TB, as indicated by MTB detection in blood or urine, is also common and is an independent predictor of mortality [8–10].

Understanding factors associated with mortality in hospitalised patients with HIV/TB in the context of high ART coverage, rapid TB diagnostics with better diagnostic yield, and prompt TB treatment could help develop strategies to identify high-risk patients, and interventions to reduce their mortality. Here we describe characteristics of patients diagnosed with bacteriologically-confirmed TB from the STAMP (Screening for TB to reduce AIDS related Mortality in hospitalised Patients) randomised controlled trial of urine-based TB screening in HIV-positive inpatients in Malawi and South Africa.[11]

Our specific aims were to describe the clinical phenotypes, mortality and risk factors for mortality in hospitalised HIV/TB patients; the prevalence and mortality of disseminated TB; and the impact of ART and study site on mortality.

Methods

This prospective cohort study was nested within the STAMP trial [11,12], which recruited adult HIV-positive patients, irrespective of TB symptoms or clinical presentation, at admission to medical wards in two hospitals in Malawi and South Africa. Upon enrolment, patients were randomised to TB screening using sputum testing alone (standard of care, SOC), or sputum and urine testing (intervention). The primary outcome was all-cause mortality at 2-months, and secondary outcomes included TB diagnosis and treatment. Patients were excluded if they were taking TB treatment at the time of screening or within the last 12 months, or isoniazid preventative therapy (IPT) in the last 6 months, unable to provide informed consent, or had been admitted for >48 hours at the time of screening.

Trial procedures have been detailed previously and are outlined in the Supplementary Methods [11,12]. After enrolment, urine and spontaneously expectorated sputum samples were obtained and tested for TB according to trial arm. Patients allocated to the intervention arm underwent sputum testing (if sputum was produced) with Xpert MTB/RIF assay (Xpert), unconcentrated urine was tested with Determine TB-LAM Ag assay (TB-LAM), and concentrated urine was tested with Xpert [13]. Patients allocated to the SOC arm had sputum Xpert testing only: with unconcentrated and concentrated urine immediately frozen. Clinical events during hospital admission were recorded, and patients were followed-up at 56-days post enrolment through in-person interview. Patients not attending their follow-up appointment were contacted by telephone and/or home visit, with interview of next of kin to establish vital status if necessary.

If patients had been diagnosed with TB in the SOC trial arm (i.e. not had real time urine TB testing), TB-LAM and Xpert testing was performed on stored urine (Supplementary Methods). Patients were enrolled in this sub-study if they were diagnosed with TB and had a positive laboratory test (≥ 1 positive specimen on microscopy for AFB, Xpert, culture or TB-LAM) on any sample. Patients diagnosed or treated with TB without positive diagnostic tests were not included. The study was approved by the research ethics committee of the London School of Hygiene & Tropical Medicine, and local research ethics committees in Malawi and South Africa.

Definitions

Patients were defined as 'clinically suspected TB' if TB was recorded in the admitting differential diagnosis. Disseminated urinary TB was defined by any positive urine TB assay (TB-LAM or Xpert), and non-disseminated TB was a positive sputum TB assay with negative urine TB assays.

A urine 'TB score' was calculated based on the number of positive urine TB tests (TB-LAM or Xpert, possible values 0-2) [14]. The Supplementary Methods outlines other definitions.

Statistical analysis

Mortality risk was calculated 56-days from admission. All baseline demographics, clinical variables, laboratory results and physiological measurements were considered for association with mortality using Cox proportional hazards models. An explanatory model was built excluding the most proximal factors on the causal pathway to mortality (notably, functional impairment). Separate models were built to assess associations of recruitment site with mortality. Models used step-wise backward elimination (variables with $p > 0.1$ were excluded) and were restricted to complete cases. Non-linearity of continuous variables was assessed using fractional polynomials.

Clinical phenotypes of HIV/TB patients were identified using unsupervised (i.e. without the mortality outcome) hierarchical cluster analysis, with the number of clusters determined by stopping rules. Clusters were then described by comparing means and proportions to the overall population, and associations with mortality assessed using Kaplan-Meier curves, and Cox regression. Supplementary methods describe the analysis in further detail.

Results

Patient characteristics

Between October 2015 and September 2017, 506 HIV-positive patients were diagnosed with TB, of whom 322 were laboratory-confirmed and included in this analysis. Two-thirds (63.7%) were clinically suspected to have TB and similar numbers were from Malawi (155, 48.1%) and South Africa (167, 51.9%) (Table 1).

At admission, median CD4 cell count was 75 cells/ μ L, and 93% had advanced HIV (as defined by WHO). 139 (43.2%) patients had at ≥ 1 WHO danger sign, and 77 (23.9%) had sepsis. Patients had substantial functional impairment, with 165 (51.2%) being unable to perform usual activities and 74 (23.0%) assessed as severely disabled (Karnofsky score ≤ 40). Patients also had poor nutritional status with low median BMI (18.2). Anaemia was very common, only 30 (9.3%) patients had normal haemoglobin and 62 (19.3%) had life-threatening anaemia (haemoglobin < 6.5 g/dL).

Table 1: Baseline characteristics in laboratory confirmed HIV/TB patients, overall and stratified by disseminated urinary TB.

		All HIV/TB (n=322)		Disseminated TB				p-value ^b
		n	% ^a	Yes (n=251)		No (n=71)		
		n	% ^a	n	% ^a	n	% ^a	
Age	Mean	37.2	10.5	36.8	10.3	38.7	11.3	0.175
Gender	Male	175	54.3%	131	52.2%	44	62.0%	0.144
	Female	147	45.7%	120	47.8%	27	38.0%	
Site	Malawi	155	48.1%	131	52.2%	24	33.8%	0.006
	South Africa	167	51.9%	120	47.8%	47	66.2%	
Smoking	Current	35	10.9%	26	10.4%	9	12.7%	0.009
	Former	77	23.9%	51	20.3%	26	36.6%	
Co-morbidity	Yes	65	20.2%	49	19.5%	16	22.5%	0.577
New diagnosis of HIV	Yes	55	17.1%	43	17.1%	12	16.9%	0.964
ART status^d	Naïve	34	12.7%	25	12.0%	9	15.3%	0.167
	Current	214	80.1%	165	79.3%	49	83.1%	
	Interrupted	19	7.1%	18	8.7%	1	1.7%	
Time on ART (years)^e	Median	1.5	5.0	0.9	4.2	1.6	5.2	0.962
Second-line ART^e	Yes	6	2.8%	2	2.5%	4	4.1%	0.547
Cough	Yes	234	72.7%	175	69.7%	59	83.1%	0.026
Fever	Yes	228	70.8%	182	72.5%	46	64.8%	0.206
Night Sweats	Yes	170	52.8%	136	54.2%	34	47.9%	0.348
Weight Loss	Yes	293	91.0%	227	9.4%	66	93.0%	0.513
≥1 WHO TB symptom	positive	317	98.4%	247	98.4%	70	98.6%	0.911
Duration of illness (days)	Median	14	21	14	21	14	21	0.873
Previous TB Treatment	Yes	73	22.7%	53	21.1%	20	28.2%	0.21
EQ5D mobility	Some problems	163	50.6%	129	51.4%	34	47.9%	0.067
	Confined to bed	77	23.9%	65	25.9%	12	16.9%	
EQ5D self-care	Some problems	129	40.1%	107	42.6%	22	31.0%	<0.001
	Unable to wash/ dress	86	26.7%	75	29.9%	11	15.5%	
EQ5D usual activities	Some problems	91	28.3%	72	28.7%	19	26.8%	0.002
	Unable to perform	165	51.2%	138	55.0%	27	38.0%	
EQ5D health score	Mean	52.1	13.6	48.0	14.2	54.0	11.4	<0.001
BMI	Median	18.2	5.0	18.0	4.6	19.4	6.2	0.049
MUAC	Median	20	5.5	20	4.5	21	6.5	0.039
Karnofsky score	Median	50	10	50	20	60	20	0.001
	≤40	74	23.0%	65	25.9%	9	12.7%	0.019
Blood pressure	Median SBP	102		101		106		0.009
	Median DBP	67		66		70		0.060
Heart Rate	Mean	104.7	20.8	105.5	21.1	101.8	19.5	0.189

Respiratory rate	Mean	23.3		22.8		23.4		0.265
Oxygen saturations (%)	Median	96	4	96	4	96	4	0.991
WHO danger sign	Yes	139	43.2%	117	46.6%	22	31.0%	0.019
Sepsis	Yes	77	23.9%	69	27.5%	8	11.3%	0.005
Haemoglobin (g/dl)	Mean	8.8	27.7	8.4	26.1	10.2	28.9	<0.001
Anaemia	Severe	133	41.3%	115	45.8%	18	25.4%	
	Moderate	117	36.5%	91	77.8%	26	22.2%	0.001
	Mild	42	13.1%	27	64.3%	15	35.7%	
CD4 count	Median	74.5	182	61	157	129	295	0.002
	<100	188	58.4%	155	61.8%	33	46.5%	0.017
C- reactive protein	Median	138	112	141.5	106	117	136	0.619
WHO stage	1 or 2	35	10.9%	28	11.2%	7	9.9%	
	3	111	34.5%	78	31.1%	33	46.5%	0.052
	4	176	54.7%	145	57.8%	31	43.7%	
Clinically suspected TB	Yes	205	63.7%	147	58.6%	58	81.7%	<0.001

Data are numbers and % unless otherwise stated. ^a If variable is median represents interquartile range, if variable is mean represents standard deviation. ^b Comparing disseminated TB yes/no, calculated using Chi-squared for proportions, t-test for means and Wilcoxon rank sum for medians. ^d restricted to patients with known HIV diagnosis. ^e restricted to patients reporting current ART use. Missing data: 1 missing haemoglobin, 2 missing second-line ART regimen, 93 missing C-reactive protein. ART is antiretroviral therapy, WHO World Health Organization, EQ5D EurQol 5 Dimension, BMI Body Mass Index, MUAC mid-upper arm circumference, SBP systolic blood pressure, DBP diastolic blood pressure. Heart rate and respiratory rate are measured per minute.

267 (82.9%) HIV/TB patients knew their HIV status before admission, of whom 214 (80.1%) were currently taking ART and 19 (7.1%) had interrupted their ART. Median duration taking ART was 1.0 year (interquartile range [IQR] 0.3-4.2) and 68 (21.1%) were on ART for <3 months. Most (58.4% [125/214]) patients reporting current usage had been taking ART for ≥6 months, although their median CD4 cell count was only 96 cells/μL (IQR 32-319). HIV viral load results were available for 97/125 patients on ART for ≥6 months; 48 (49.5%) had >1000 copies/mL (median 557,000 copies/mL) highly suggestive of ART failure.

TB characteristics

TB diagnostic results are outlined in Table 2. Most (242/322, 75.2%) patients could provide sputum for Xpert testing. Disseminated urinary TB was common (78.0%, 251/322). When restricted to patients in the trial intervention arm, 181/212 (85.4%) patients had disseminated TB. One-third (34.2%, 86/251) of disseminated urinary TB patients were positive on both urine TB-LAM and Xpert assays.

Of 197 patients undergoing chest radiography, 107 (54.3%) were interpreted by clinicians as consistent with TB. Only four patients (8.9% of those with CSF results) were diagnosed with TB meningitis, of whom all had positive urine TB tests (3 Xpert positive, 1 TB-LAM positive).

Table 2: TB investigations and results

TB investigation	n=322	%
Sputum		
Sample sent for TB testing	242	75.2
Xpert positive	168	52.2
Urine		
Sample sent for TB testing	321	99.7
TB-LAM positive	209	66.1
Xpert positive	128	40.5
Any urine TB test positive	251	78.0
Urine TB score		
0	71	22.0
1	165	51.2
2	86	26.7
Chest radiography		
Underwent chest radiography	197	61.2
Clinicians report as “consistent with TB”	107	33.2
Cerebrospinal Fluid (CSF)^a		
Tested	45	14.0
Consistent with TB	4	1.2
Rifampicin resistant TB		
Xpert rifampicin result available (sputum or urine)	223	69.3
Rifampicin resistance detected	11	4.9

% are based on all patients (n=322) as the denominator. Sputum includes study TB screening and routine clinical samples. ^aCSF testing consistent with TB includes lymphocytes with raised protein or positive Xpert. One patient missing data for CSF testing. CSF is cerebrospinal fluid, Xpert is Xpert MTB/RIF assay, TB-LAM is Determine TB-LAM Ag assay. TB score calculated based on the number of positive urine TB tests (TB-LAM or Xpert, possible values 0-2).

Patients with disseminated TB were less likely to report a cough or to be clinically suspected of having TB. They were predominantly male and had more functional impairment (bedbound, unable to perform usual activities or wash/dress, and lower Karnofsky score); worse nutritional status (lower BMI and MUAC); were more likely to have WHO danger signs, sepsis and severe anaemia at presentation; and were more profoundly immunosuppressed (lower CD4 cell count, Table 1).

Eleven patients died before TB treatment initiation (including two in the SOC arm whose TB was diagnosed on chest radiograph and retrospectively confirmed through testing stored urine), while for those treated, median time from admission to treatment was 2 days (IQR 1 day). Four (1%) patients stopped TB treatment during hospitalisation due to treatment side-effects. Eleven patients were diagnosed with rifampicin-resistant TB: six from sputum Xpert only, four from urine Xpert only, and one on both urine and sputum Xpert.

Mortality

Overall, 99 (30.7%) patients died by 56-days. Six patients were lost to follow-up after discharge. Mortality in HIV/TB patients did not differ by STAMP trial arm ($p=0.30$), consistent with mortality benefit in the trial being restricted to patients with missed TB diagnoses (and therefore not included in this analysis) [11]. Median time to death was 12 days (IQR 5-27), and 71.7% (71/99) occurred during admission, with 9 (9.1%) within 48 hours and 32 (32.3%) within 7 days of admission (Supplementary Figure 1).

Mortality was lowest in patients with negative urine TB tests (19.7% by 56-day), compared to disseminated urinary TB with one (30.5%) or two (40.7%) positive urine tests ($p=0.018$). Reduced functional ability (lower Karnofsky score, self-reported reductions in mobility, self-care and usual activities) and poor nutritional status (MUAC and BMI) were strongly associated with mortality (Table 3). Mortality was also associated with lower CD4, haemoglobin and renal impairment, although not with WHO danger signs.

In the multivariable model ($n=320$, Table 3), mortality was independently associated with advancing age, male gender, lower MUAC, lower haemoglobin and higher urine TB test score. CD4 cell count was not independently associated with mortality.

Not currently taking ART was associated with a lower mortality than being on ART (HR 0.6, $p=0.035$, Supplementary Figure 2), despite having lower median CD4 counts than those taking ART (39 cells/ μL [IQR 13-122] compared to 84 cells/ μL [IQR 29-244]). Although unadjusted mortality was 9.3% lower in patients from the South African site, in an adjusted model mortality was similar to those patients from Malawi (adjusted hazard ratio [HR] 0.9, $p=0.729$, supplementary table).

Clinical Phenotype

Cluster analysis identified four distinct groups of patients based on correlation of clinical features at admission. Although not informed by outcome data, mortality differed substantially between these groups (Figure 1, HR 2.4 for group 2, 4.5 for group 3 and 6.7 for group 4 compared to group 1, $p<0.001$). Group 1 (lowest mortality risk, 9.8% [5/51]) were more likely to be ART naïve, have better functional status and nutrition (higher MUAC and BMI), less severe anaemia and higher CD4 cell count. Patients in group 2 (moderate mortality risk, 22.6% [23/102]) were characterised by a longer time on ART and almost half reporting normal physical function.

Groups 3 and 4 (highest mortality risks, 37.1% [39/105] and 52.5% [31/59] respectively) patients all reported problems with usual activities, most had WHO danger signs and severe anaemia, and men predominated. Median CD4 counts were 78 and 38 cells/ μL respectively.

Clinical phenotype was also strongly associated with disseminated TB: groups 3 and 4 had the highest proportion of urine-positive patients (83% and 86% respectively compared to 55% in group 1, $p < 0.001$).

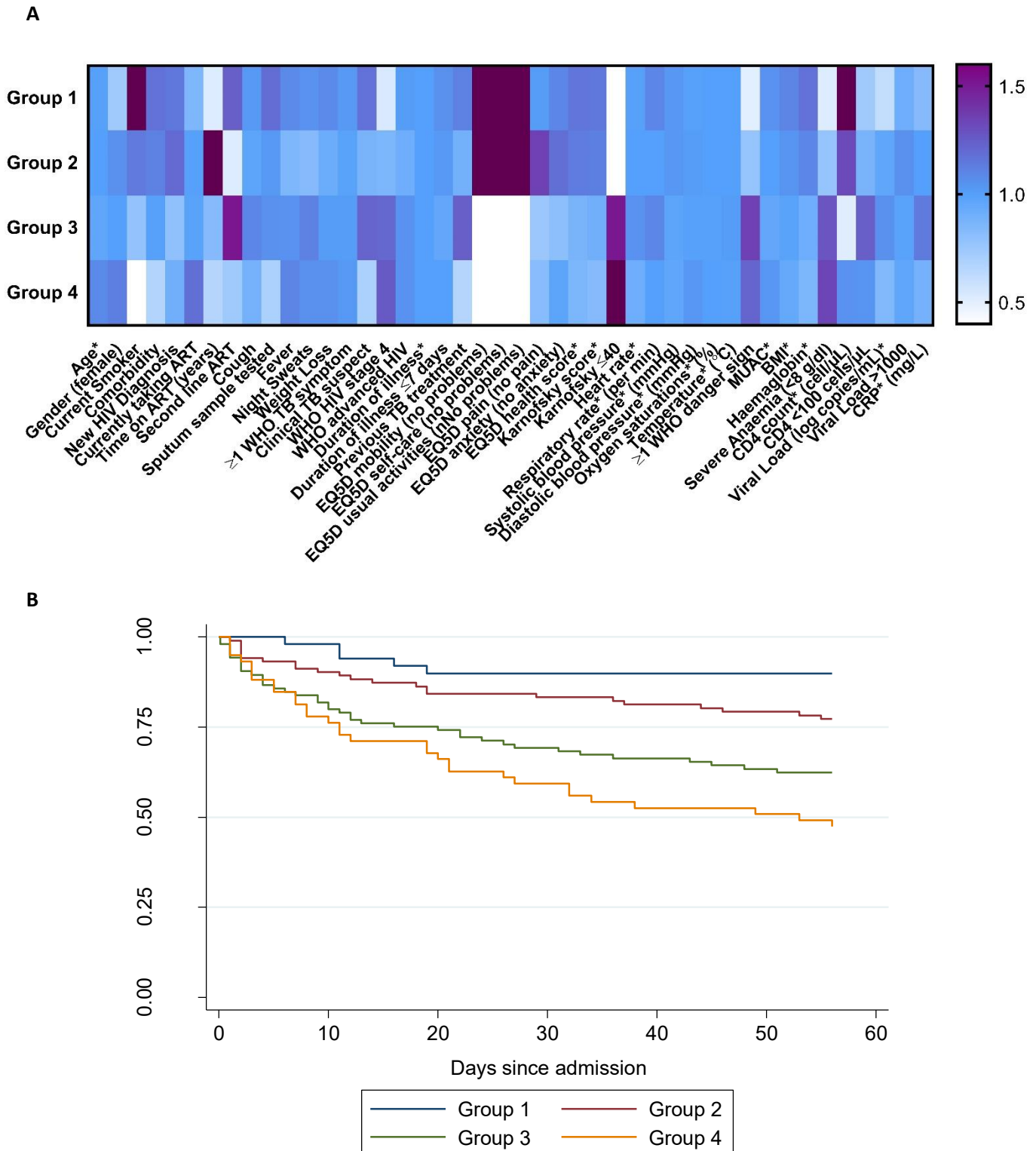
Table 3: Factors associated with time to death over 56 days

Characteristic	Deaths (n=99)	Mortality risk (%)	Univariable				Multivariable			
			HR	Lower CI	Upper CI	p-value	HR	Lower CI	Upper CI	p-value
Age			1.02	1.00	1.03	0.073	1.03	1.01	1.05	0.003
Sex										
Female	35	23.8	1				1			
Male	64	36.8	1.74	1.15	2.63	0.007	1.77	1.01	2.70	0.008
Site										
Malawi	54	34.8	1				-			
South Africa	44	25.5	0.78	0.53	1.16	0.217				
ART										
Currently taking	80	34.3	1				*			
Not currently taking	19	21.6	0.60	0.36	0.99	0.035				
Duration of illness										
<7 days	28	22.6	1				*			
>7days	71	36.2	1.77	1.14	2.74	0.008				
EQ5D mobility[#]										
No problems	9	11.0	1				-			
Some problems	51	31.5	3.21	1.58	6.52	<0.001				
Confined to bed	99	50.7	6.26	3.03	12.93					
EQ5D self-care[#]										
No problems	10	9.4	1				-			
Some problems	47	36.4	4.63	2.34	9.17	<0.001				
Unable to wash/dress	42	48.8	6.86	3.44	13.68					
EQ5D usual activities[#]										
No problems	5	7.6	1				-			
Some problems	29	32.2	5.05	1.96	13.06	<0.001				
Unable to perform	65	39.4	6.30	2.54	15.65					
EQ5D health score[#]			0.97	0.96	0.99	<0.001	-			
BMI			0.96	0.91	1.01	0.086	-			
MUAC			0.87	0.82	0.92	<0.001	0.89	0.83	0.94	<0.001
Karnofsky[#]			0.96	0.95	0.98	<0.001	-			
WHO danger sign										
No	51	27.9	1				-			
Yes	48	34.8	1.34	0.90	1.99	0.146				
Haemoglobin[§] (g/dL)			0.86	0.80	0.93	<0.001	0.88	0.81	0.95	0.002
CD4 count (cells/μL)			0.93	0.88	0.99	0.022	*			
eGFR[§] \geq60ml/min	17	25.0	1				-			
<60ml/min	15	51.7	2.45	1.23	4.93	0.013				
C-reactive protein[§] (mg/L)			1.01	1.00	1.01	0.043	-			
WHO clinical stage										
1 or 2	7	20.0	1				-			

3	28	25.5	1.36	0.60	3.12	0.031				
4	64	36.4	2.14	0.98	4.66					
Able to produce sputum										
No	32	40.0	1							
Yes	67	27.8	0.66	0.43	1.0	0.061	*			
TB-LAM										
Negative	29	25.9	1							-
Positive	70	33.5	1.72	1.00	2.95	0.044				
Urine Xpert positive										
Negative	49	25.3								-
Positive	50	39.4	1.91	1.28	2.87	0.002				
Urine TB score			1.84	1.05	3.24	0.024	1.41	1.03	1.91	0.025

Hazard ratios (HR) calculated using Cox proportional hazards models. Continuous variables were all modelled as linear after checking for departures from linearity. For continuous variables, HR is for every unit increase in the variable, except for CD4 count where HR is for every 50 cells/ μ L increase. Urine TB score was modelled as a continuous linear variable. All p-values calculated using likelihood ratio testing. [§]Missing data: 1 missing haemoglobin, 93 missing C-reactive protein, 225 missing eGFR. [#]Variable excluded from multivariable model as distal on causal pathway to death. CRP and eGFR were excluded from the multivariable model due to >25% missing data. The following variables were excluded due to collinearity: BMI was colinear with weight; WHO stage was colinear with CD4 count; TB-LAM and urine Xpert were colinear with urine TB score. All other variables with $p > 0.1$ in univariable analysis were entered into the multivariable model using backwards stepwise elimination (variables exited the model if $P > 0.1$), $n = 321$. *Variables eliminated from the multivariable model. ART is antiretroviral therapy, BMI Body Mass Index, CI confidence interval, eGFR estimated glomerular filtration rate, EQ5D EurQoL 5 Dimension, HR Hazard Ratio, MUAC mid-upper arm circumference, WHO World Health Organization.

Figure 1: Cluster analysis clinical phenotypes of HIV/TB patients and their mortality risk



A is a heat map comparing characteristics of clinical phenotype groups (from cluster analysis) to the overall population. Overall $n=317$, group 1 $n=51$, group 2 $n=102$, group 3 $n=105$, group 4 $n=59$. For continuous variables (marked with *), colours represent a ratio of mean or median values for the group compared to the overall mean or median. For categorical variables, the ratio is the group proportion compared to the overall proportion. Dark purple (■) represents a ratio >1.6 , and white (□) represents a ratio <0.4 . Missing data: 1 missing haemoglobin, 2 missing second-line ART regimen, 93 missing C-reactive protein, 222 missing HIV viral load. EQ5D variables are the proportion reporting 'no problem'. Time on ART and second-line ART are restricted to patients reporting current ART use.

B Kaplan-Meier plot of time to death by clinical phenotype group. Grey dashed vertical line represents median length of hospital stay (10 days). Hazard ratio (HR) compared to group 1 is 2.4 (95%CI 0.9-6.3) for group 2, 4.5 (95%CI 1.8-11.4) for group 3 and 6.7 (95%CI 2.6-17.3) for groups, $p < 0.001$.

Non-TB management

Broad-spectrum antibiotics were given to 86.0% (277/322) of patients (at least one dose,) with median duration 6 days (IQR 4-7 days), and 45.1% (125/277) receiving two or more different drugs. 10.6% (10/94) of patients tested were cryptococcal antigen positive. Median duration of hospital stay was 10 days (IQR 5-15 days). Of those surviving to 56-days, 65.2% (45/69) of ART naïve patients had commenced ART, median time to starting ART was 19 days (IQR 9-29). 66.7% (14/21) of those who had discontinued ART had been restarted.

14.8% (37/250) of patients discharged were readmitted to hospital during the 56-day follow-up, with readmission associated with higher mortality (24% vs 9%, $p = 0.006$). Outpatient attendance was also common, with patients discharged having a median of 2 (IQR 1-4) clinic attendances by 56 days.

Discussion

The main findings of this study were that two-month mortality in patients with HIV-associated TB was substantial (31%) despite good ART coverage and TB screening with prompt TB treatment. Urine diagnostic tests (defining disseminated TB) were positive in 78.0% of TB patients, and were associated with higher mortality. Despite most patients being knowing their HIV status and established on ART, advanced immunosuppression and poor virological control were common. Counterintuitively, we report lower mortality in patients not taking ART at TB diagnosis. Hierarchical cluster analysis defined four distinct clinical phenotypes with highly variable mortality (9.8% to 52.5%), suggesting that baseline risk-profiles could be used to prioritise patients for intensified care. The potential to target patients at highest risk of death was underscored by risk factors identified, including older age, male sex, ART before admission, severity of anaemia, poor performance and poor nutritional status.

Our observed early mortality is close to that estimated for patients with HIV-associated TB from a recent meta-analysis [3], and was high in both South Africa (25.5%) and Malawi (34.8%). The 9.3% risk difference between the two countries indicates the magnitude of effect that could be attributed to the better resources in South Africa (middle income) compared to Malawi (one of the poorest countries globally).

Irrespective of cause of admission, HIV-positive patients in hospital do badly, with early mortality risks of 20-30% reported consistently for the African region [15]. However, patients with TB do consistently worse than other admission categories. In the STAMP trial, for instance, HIV-positive inpatients without confirmed TB had early mortality of 16.5%, below that of patients with urine-positive TB, although similar to TB diagnosed only through sputum [11]. Improving survival of TB patients may, then, require a combination of interventions aimed both at TB, for instance high dose rifampicin and host-directed therapies [16,17], and more generally at supporting HIV-positive inpatients, such as multiple empirical anti-infective agents [18,19] and aggressive management of presumptive virological failure [20].

In this context, TB-LAM or urine Xpert positivity, indicative of haematogenous dissemination, was associated with a severely ill clinical phenotype, and was independently associated with mortality. If we assume that positive urine results provide more rapid, less costly, and more sensitive equivalent of mycobacteraemia [21,22], then this simple prognostic marker could be used to target the need for intensified TB therapy, as well as for TB screening.

Moreover, the strong relationship between disseminated TB and immunosuppression also suggests that urine-positive TB could serve as an indicator of ART treatment failure. Some countries, including Malawi, approach the UNAIDS “90-90-90 targets”, whereby 90% of those on ART should be virologically suppressed [23]. With successful global scale-up of HIV-diagnosis and ART, the case-mix of inpatients has changed from predominantly ART-naïve [24] to that reported here: 80% with a known HIV-diagnosis had been on ART for a median of 1.5 years. Interesting, this appears to correspond with a reversal in the prognostic value of ART prior to TB diagnosis, from beneficial [25,26] to a risk-factor for mortality. Urine-positive TB patients taking ART ≥ 6 months had median CD4 count of only 96 cells/ μL , with half having high viral loads (median 557,000 copies/mL). However, recommendations for viral load testing in ART-experienced patients and expedited adherence support and/or switching ART have proved prohibitively expensive and slow [18,27].

Using both regression and cluster analysis, we found clinical phenotypes with more severe functional impairment, worse nutritional status and severe anaemia being associated with both higher mortality and disseminated HIV/TB. The remarkably high mortality (50%) in those reporting being confined to bed highlights the importance of upstream interventions to prevent TB, supporting early diagnosis, and improving recognition of critically ill patients in community and clinic settings. Most patients in STAMP reached their critically-ill state despite attending HIV care services: improving TB screening at ART initiation and follow-up, expanding use of TB preventive therapy, and empowering patients to seek care promptly could each contribute to better inpatient outcomes.

Intervening to avert mortality may be challenging in critically ill HIV/TB patients (table 4). However, only one-third of deaths occurred within 1 week, suggesting a potential window of opportunity. Anaemia has previously been associated with poor outcomes in HIV/TB [28], and we also found low haemoglobin to be associated with mortality, almost one-fifth had life-threatening anaemia. There are currently no normative guidelines on blood transfusion in HIV/TB, and further research is needed before recommendations can be made [29].

The best strategy for supportive care in African hospitals with high HIV-prevalence remains unclear, with a recent trial of early fluid resuscitation paradoxically increasing mortality in patients with sepsis [30]. Provision of broad-spectrum antibiotics in our cohort was almost universal, but resistance to first-line antibiotics is now substantial, especially in Gram-negative bacterial isolates [31]. Cryptococcal meningitis is also a common cause of death in advanced HIV, and may be associated with HIV/TB [32,33]. Despite exclusion of patients with altered conscious level, CrAg positivity was over 10%, supporting screening in this population.

Almost one-third of deaths occurred after discharge, suggesting need to provide more intensive follow-up and support at discharge, especially for those with disseminated TB likely to imply failing ART [34,35]. Men had almost double the risk of mortality than women, despite adjusting for other predictors of death. This gender difference has been reported previously, and although potentially an epiphenomenon related to barriers accessing care or confounders, it could plausibly be a combination of social and biological differences [36,37].

The strengths of this study include its being nested within a TB screening trial, that management was undertaken by routine health services, and that these data reflect the high ART coverage for the Southern Africa region. Limitations include suboptimal sensitivity of the TB screening algorithms, notably for the sputum-only arm of the parent trial. Our definition of confirmed TB included some patients who were only TB-LAM positive, and we cannot exclude a small number of false positives [38]. Finally, we do not have post-mortem data.

In summary, we have shown high mortality in hospitalised patients with HIV-associated TB despite current public health interventions. Urine diagnostics provide useful prognostic and well as diagnostic information, and could be used to guide the development and targeting of intensified inpatient management strategies. HIV/TB inpatients in this region now predominantly affects patients established on ART with advanced immunosuppression likely to indicate ART failure. This population will be important to meet both End TB and UNAIDS 2020 and 2025 targets for reducing TB deaths. Early diagnosis and management of ART failure is one of several potential interventions that could improve survival of patients hospitalized with TB.

Implementation is needed in parallel with further research and upstream public health interventions.

Table 4: Implications and potential interventions to reduce HIV/TB mortality

Issue	Evidence for association with HIV/TB mortality	Possible interventions	Further research/ unanswered questions
ART failure	<ul style="list-style-type: none"> • Higher mortality in ART experienced patients • Low median CD4 counts and high viral loads in patients taking ART >6 months 	<ul style="list-style-type: none"> • Rapid screening for virological failure at admission (eg using point-of-care HIV viral load assay) • Adherence interventions or switch to second-line ART • Integrase inhibitors (few drug-drug interactions with TB medication) 	<ul style="list-style-type: none"> • Prevalence of HIV drug resistance • Timing of ART switch in HIV/TB patients failing ART • Optimal regimen for switching
TB during early ART	<ul style="list-style-type: none"> • One-fifth of HIV/TB patients were within 3 months of ART initiation • High mortality 	<ul style="list-style-type: none"> • Improved TB screening at ART initiation • Better implementation of TB preventative therapy 	<ul style="list-style-type: none"> • Best approach for TB screening in ART naïve patients (eg TB-LAM, Xpert and/or chest radiography) • Implementation research for TB preventative therapy
Supportive care and co-morbidities	<ul style="list-style-type: none"> • WHO danger signs and sepsis are common • 10% cryptococcal antigenemia • Life-threatening anaemia has high mortality • 28% of deaths after discharge 	<ul style="list-style-type: none"> • Screening, treatment and/or prophylaxis for co-infection • Improved supportive care • More intensive follow-up post discharge from hospital 	<ul style="list-style-type: none"> • Prevalence of bacterial co-infection • Evidence for safety and efficacy of supportive care (eg IV fluids and/or blood transfusion) • Can enhanced follow-up improve outcomes
Identification of high risk patients	<ul style="list-style-type: none"> • Clinical phenotype associated with high mortality • Urine diagnostics associated with higher mortality 	<ul style="list-style-type: none"> • Predictive tools to identify patients at higher risk of mortality who may benefit from interventions (eg clinical risk score) 	<ul style="list-style-type: none"> • Derivation and validation of prognostic score • Use of score(s) for implementation of interventions aimed at mortality

Funding

This STAMP trial was funded by the Joint Global Health Trials Scheme of the Medical Research Council, the UK Department for International Development, and the Wellcome Trust [MR/M007375/1]. AG-W has received a Royal College of Physicians London JMGP Fellowship, ELC has received a Wellcome Trust Fellowship [WT200901/Z/16/Z].

Conflicts of Interest

The authors declare no conflicts of interest

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Supplementary methods

Study procedures

The study was undertaken at Zomba Central Hospital, Southern Malawi, and Edendale Hospital, KwaZulu-Natal, South Africa). Data were collected on clinical presentation (including TB symptoms), past medical history including HIV status and previous TB, physical examination (including vital signs, WHO HIV staging, body mass index (BMI), mid-upper arm circumference (MUAC) and Karnofsky functional score) and the EuroQol 5 Dimension (EQ5D) quality of life questionnaire. ART status was verified by checking prescriptions or health records. Clinical events during admission were recorded, and blood was also collected for CD4 cell count, haemoglobin and plasma storage.

TB screening test results were reported to the attending clinical team (reported as positive, negative or not done to maintain masking to trial arm) who made all decisions regarding TB treatment and other management. Clinicians could request further investigations (for example Xpert testing of sputum, chest radiography or cerebrospinal fluid examination) or prescribe empirical TB treatment. The study team were not involved in the management of patients, which was done as per local practice and guidelines. First-line TB treatment was the standard four-drug regimen and first line antiretroviral therapy (ART) was efavirenz-based using a fixed-dose combination pill at both sites. Patients were followed-up at 56-days post enrolment through in person interview. Patients who did not attend their follow-up appointment contacted by telephone and/or home visit, with interview of next of kin to establish vital status if necessary.

All TB assays were performed as per manufacturer's instructions. A grade-one cut-off defined positive TB-LAM tests (using the manufacturers post-2014 reference card) and all TB-LAM results were read by a second, blinded, reader for quality assurance. Stored urine was tested with TB-LAM by thawing an aliquot of unconcentrated urine at room temperature, and with Xpert by thawing an aliquot of frozen concentrated urine (produced by concentrating approximately 40-50mls of urine by centrifugation). Stored urine was tested using the same procedures for testing urine in real-time, and followed manufacturer's instructions.

HIV viral load testing was done retrospectively from frozen plasma using the Xpert quantitative HIV viral load assay on a consecutive sub-sample of HIV/TB as part of another sub-study. Cryptococcal antigen testing was done using IMMY CrAg LFA (Cryptococcal Antigen Lateral Flow Assay) on a random sample of patients in the same sub-study. Serum creatinine was measured by the South African National Health Laboratory Service (NHLS).

Definitions

Anaemia was defined using the WHO criteria: no anaemia (haemoglobin [Hb] concentration ≥ 13.0 g/dL for males, ≥ 12.0 g/dL for females), mild anaemia (11.0–12.9 g/dL for males, 11.0–11.9 g/dL for females), moderate anaemia (8.0–10.9 g/dL for males and females), severe anaemia (< 8.0 g/dL for males and females), with Hb < 6.5 g/dl classified as life threatening ^{1,2}. Co-morbidity was defined as self-reporting of diabetes or renal, liver or cardiovascular disease. WHO danger signs were any one of heart rate > 120 beats per minute (bpm), respiratory rate > 30 per minute, temperature > 39 degrees Celsius or being unable to walk unaided. Sepsis was defined as systolic blood pressure < 90 mmHg, oxygen saturations $< 90\%$, respiratory rate > 30 per minute or GCS < 15 (adapted from Sepsis-3 as some measurements were unavailable) ³. WHO TB symptom screen is one of current cough, fever, night sweats or weight loss. Advanced HIV is defined as CD4 cell count < 200 cells/ μ l or WHO stage 3 or 4 disease ⁴. Rifampicin resistant TB was defined as the presence of *rpoB* mutations on Xpert that were confirmed on repeat Xpert testing of the same or a repeated sample ⁵. Estimated glomerular filtration rate (eGFR) was calculated using the 4 variable Modification of Diet in Renal Disease (MDRD) formula in the subset of patients with serum creatinine levels measured by clinicians ⁶.

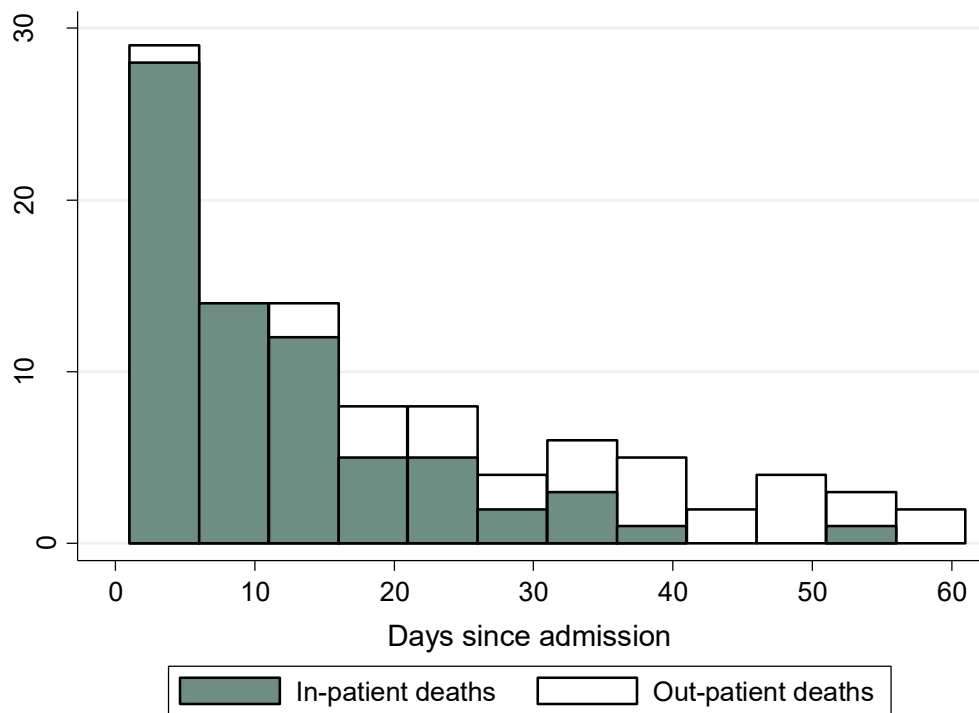
Statistical analysis

Patients were characterised using simple descriptive statistics. Proportions were compared using Chi-squared, Fisher's exact and McNemar's tests as appropriate, medians were compared using Wilcoxon rank-sum tests and means using unpaired t-tests.

Time-to-mortality was calculated using survival analysis and Kaplan-Meier curves. Time was censored at 56-days post enrolment, or at the time last seen alive for those lost to follow-up. For mortality risk, patients lost to follow-up were assumed to be alive at 56-days. Schoenfeld residual plots and were used to identify departure from the proportional hazards assumption. If variables were colinear, only one was included in the model. Variables with $> 25\%$ missing data were excluded from multivariable models and no imputation of missing data was done (C-reactive protein and estimated glomerular filtration rate [eGFR]). Variables considered too proximal to death which were excluded in the multivariable models were: EQ5D mobility, self-care and usual activities and Karnofsky functional score. Likelihood ratio testing was used to compare models and assess for interactions. In the final models, there was no evidence for any significant interaction between variables.

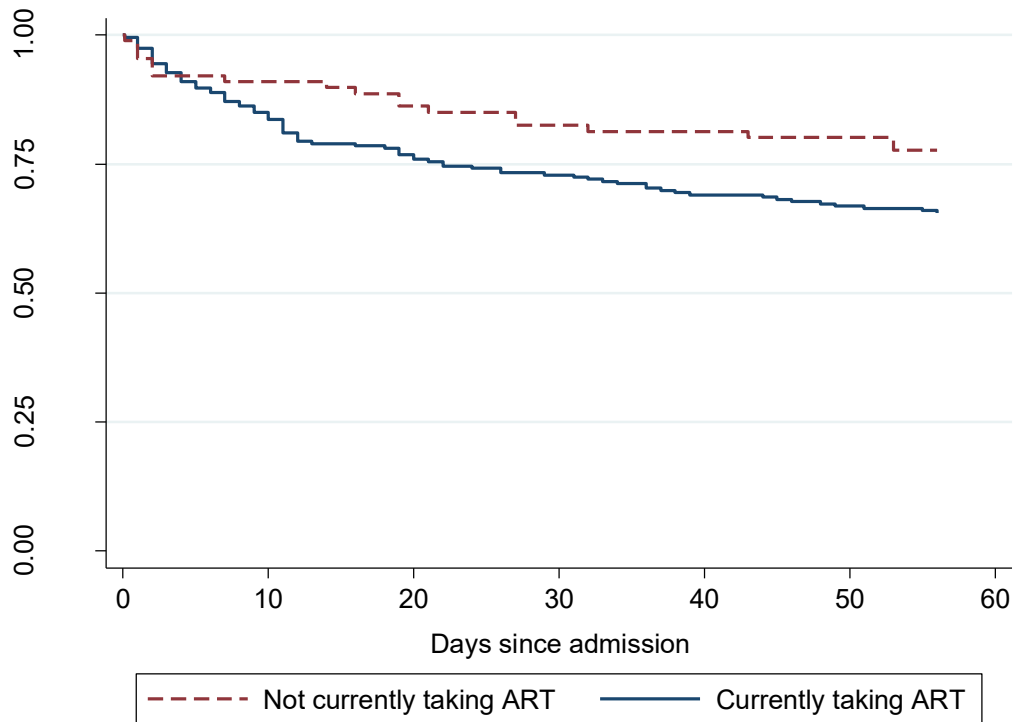
For the unsupervised analysis of clinical phenotype, all continuous variables were reduced using principal components analysis to the smallest number of components which have an eigenvalue ≥ 1 and explain $>50\%$ of the variance. Hierarchical cluster analysis was then used on the reduced principal components and remaining categorical factors, using Ward's average linkage and selecting the number of clusters based on Duda and Calinski stopping rules (stopping at the number of clusters with largest pseudo-F and pseudo-T-squared values)⁷. The identified clusters were validated using kmeans cluster analysis. To describe the phenotypes of the cluster groups, a ratio of mean or median values for the group compared to the overall mean or median was calculated for continuous variables, and for categorical variables a ratio of the group proportion compared to the overall proportion.

Supplementary Figure 1: Timing of deaths, stratified by in-patient and out-patient



Stacked bar chart showing timing of deaths in days after admission, stratified by if death occurred as inpatient (green) or as outpatient (white). Total number of deaths is 99.

Supplementary Figure 2: ART status and mortality



Kaplan-Meier plot of survival by ART status. Patients currently taking ART (blue, solid) includes all patients reporting current ART use. Hazard ratio 0.6 for mortality in ART naïve compared to ART experienced patients (95% confidence interval 0.4-1.0), $p=0.035$.

Supplementary Table 1: Multivariable model for mortality by study site

Characteristic	Univariable			
	HR	Lower CI	Upper CI	p-value
Site				
Malawi	1			
South Africa	0.92	0.56	1.50	0.729
Age	1.02	1.00	1.04	0.075
Sex				
Female	1			
Male	1.98	1.25	3.11	0.003
ART				
Currently taking	1			
Not currently taking	0.64	0.38	1.08	0.097
Body Mass Index (BMI)	1.01	0.95	1.07	0.806
Cough				
No	1			
Yes	1.46	0.91	2.34	0.115
WHO danger sign				
No	1			
Yes	0.67	0.40	1.14	0.144
Haemoglobin (g/dL)	0.99	0.98	1.00	0.009
Urine TB score	1.23	0.90	1.71	0.195
Karnofsky	0.96	0.94	0.98	0.001
CD4 count (cells/μL)	1.00	1.00	1.00	0.324
Clinical TB suspect				
No	1			
Yes	0.83	0.49	1.38	0.468

Model includes variables that differed between study sites and other confounders for mortality. N=321. ART is antiretroviral therapy, BMI Body Mass Index, CI confidence interval, HR hazard ratio, WHO World Health Organization.

Supplement References

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Chapter 6: Cohort study of derivation and external validation of a clinical score predicting mortality risk (research paper)

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SECTION A – Student Details

Student	Ankur Gupta-Wright
Principal Supervisor	Prof Liz Corbett
Thesis Title	Investigating mortality risk in hospitalised patients in Africa with HIV-associated tuberculosis and positive urine diagnostics: a clinical, epidemiological and immunological study

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	PLoS Medicine		
When was the work published?	April 5th 2019		
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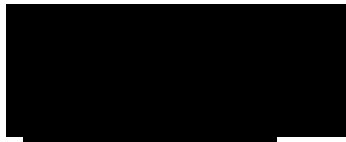
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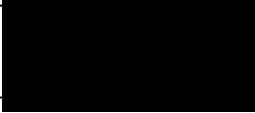
For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I came up with the concept, designed the study, was involved in data collection and data cleaning, I did the data analysis and wrote the manuscript.
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Student Signature:



Date: 22 March 2019

Supervisor Signature:

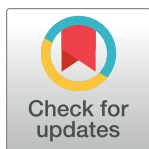


Date: 22 March 2019

RESEARCH ARTICLE

Risk score for predicting mortality including urine lipoarabinomannan detection in hospital inpatients with HIV-associated tuberculosis in sub-Saharan Africa: Derivation and external validation cohort study

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Data Availability Statement: Data are publicly available through London School of Hygiene & Tropical Medicine Data Compass repository. <https://doi.org/10.17037/DATA.00001026>

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Abstract

Background

The prevalence of and mortality from HIV-associated tuberculosis (HIV/TB) in hospital inpatients in Africa remains unacceptably high. Currently, there is a lack of tools to identify those at high risk of early mortality who may benefit from adjunctive interventions. We therefore aimed to develop and validate a simple clinical risk score to predict mortality in high-burden, low-resource settings.

Methods and findings

A cohort of HIV-positive adults with laboratory-confirmed TB from the STAMP TB screening trial (Malawi and South Africa) was used to derive a clinical risk score using multivariable predictive modelling, considering factors at hospital admission (including urine lipoarabinomannan [LAM] detection) thought to be associated with 2-month mortality. Performance was evaluated internally and then externally validated using independent cohorts from 2 other studies (LAM-RCT and a Médecins Sans Frontières [MSF] cohort) from South Africa, Zambia, Zimbabwe, Tanzania, and Kenya. The derivation cohort included 315 patients

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Competing interests: I have read the journal's policy and the authors of this manuscript have the following competing interests: KD has obtained speaker fees at industry-sponsored symposia and non-financial support from Alere in the form of kits and test strips, outside the submitted work. No other authors declare competing interests.

Abbreviations: ART, antiretroviral therapy; C-index, concordance index; HIV/TB, HIV-associated tuberculosis; LAM, lipoarabinomannan; MSF, Médecins Sans Frontières; OR, odds ratio; TB, tuberculosis; TB-LAM, Determine TB LAM Ag; Xpert, Xpert MTB/RIF.

enrolled from October 2015 and September 2017. Their median age was 36 years (IQR 30–43), 45.4% were female, median CD4 cell count at admission was 76 cells/ μ l (IQR 23–206), and 80.2% (210/262) of those who knew they were HIV-positive at hospital admission were taking antiretroviral therapy (ART). Two-month mortality was 30% (94/315), and mortality was associated with the following factors included in the score: age 55 years or older, male sex, being ART experienced, having severe anaemia (haemoglobin < 80 g/l), being unable to walk unaided, and having a positive urinary Determine TB LAM Ag test (Alere). The score identified patients with a 46.4% (95% CI 37.8%–55.2%) mortality risk in the high-risk group compared to 12.5% (95% CI 5.7%–25.4%) in the low-risk group ($p < 0.001$). The odds ratio (OR) for mortality was 6.1 (95% CI 2.4–15.2) in high-risk patients compared to low-risk patients ($p < 0.001$). Discrimination (c-statistic 0.70, 95% CI 0.63–0.76) and calibration (Hosmer-Lemeshow statistic, $p = 0.78$) were good in the derivation cohort, and similar in the external validation cohort (complete cases $n = 372$, c-statistic 0.68 [95% CI 0.61–0.74]). The validation cohort included 644 patients between January 2013 and August 2015. Median age was 36 years, 48.9% were female, and median CD4 count at admission was 61 (IQR 21–145). OR for mortality was 5.3 (95% CI 2.2–9.5) for high compared to low-risk patients (complete cases $n = 372$, $p < 0.001$). The score also predicted patients at higher risk of death both pre- and post-discharge. A simplified score (any 3 or more of the predictors) performed equally well. The main limitations of the scores were their imperfect accuracy, the need for access to urine LAM testing, modest study size, and not measuring all potential predictors of mortality (e.g., tuberculosis drug resistance).

Conclusions

This risk score is capable of identifying patients who could benefit from enhanced clinical care, follow-up, and/or adjunctive interventions, although further prospective validation studies are necessary. Given the scale of HIV/TB morbidity and mortality in African hospitals, better prognostic tools along with interventions could contribute towards global targets to reduce tuberculosis mortality.

Author summary

Why was this study done?

- HIV-associated tuberculosis (TB) is very common in hospitals in sub-Saharan Africa, and is a major cause of morbidity and mortality.
- There is a lack of tools to identify which patients are more likely to die early; therefore, these patients cannot be targeted for more intensive clinical care or other treatments in addition to TB antibiotics and antiretroviral drugs.
- A new urine TB diagnostic test (detecting a substance called lipoarabinomannan [LAM]) can identify patients at higher risk of dying and, along with other simply measured clinical signs or symptoms, may be useful for predicting which patients are most likely to do poorly.

What did the researchers do and find?

- We used data from HIV-positive adults admitted to hospital in Malawi and South Africa and diagnosed with TB to develop a simple clinical risk score to identify patients with a 50% chance of dying within 2 months.
- The score included 6 factors that can be measured at admission to hospital, including the results of the urine TB test, presence of anaemia, and some demographic factors.
- We then tested the performance of the risk score using data from studies of different patients from sub-Saharan Africa, and it performed similarly.
- The score was able to identify patients at higher risk of dying during admission to hospital, and after discharge from hospital.

What do these findings mean?

- This score could be used to identify patients admitted to hospital and diagnosed with HIV-associated TB who may benefit most from more intensive clinical care, additional treatments, and/or closer follow-up after discharge.
- It could also be used as a research tool to study new drugs or strategies aimed at reducing mortality from with HIV-associated TB.
- This is the first such tool to our knowledge in this patient population, and future studies could optimise such predictive tools, in particular if appropriate new interventions and/or diagnostics become available.

Introduction

Tuberculosis (TB) is the leading infectious disease killer globally, causing an estimated 1.7 million deaths globally in 2017 [1]. This burden lies disproportionately in people living with HIV, who account for approximately 1 in 4 TB deaths. The case fatality rate of HIV-associated TB (HIV/TB) is particularly high in hospitals, estimated at 29% in a recent meta-analysis [2]. This may be an underestimate, given that post-mortem studies from sub-Saharan Africa have demonstrated that a high proportion of HIV-positive deaths in facilities have evidence of undiagnosed TB [3].

Interventional studies aiming to reduce mortality in this patient population have demonstrated mortality reductions with improved TB diagnostics [4,5] and appropriately timed initiation of antiretroviral therapy (ART) [6,7]. However, mortality remains substantial despite these interventions, and adjunctive interventions are likely to be needed to further impact mortality. Currently, predictors for mortality are poorly defined. Being able to identify patients at the highest risk of mortality could inform the development and assessment of new interventions, and also identify which patients would benefit most from interventions beyond TB therapy and appropriately timed ART [8].

Clinical decision tools and risk scores are used widely in clinical practice to simplify the identification of patients at highest risk for poor health outcomes. Predictor scores for

mortality have been developed for HIV-associated cryptococcal meningitis and pneumonia, and are used to guide management in *Pneumocystis jiroveci* pneumonia [9–11]. Although scores have been developed to predict risk of TB disease in various populations, including TB bacteraemia in hospitalised patients [12], to our knowledge no externally validated scores exist to predict outcomes of TB disease among hospitalised patients with HIV [13,14]. Scores developed to predict TB mortality in settings with low HIV prevalence are also of limited use in people living with HIV due to differences in clinical presentation, pathogenesis, and outcomes [15–18]. A recent study from the US developed and internally validated a score to predict mortality in HIV/TB in low-prevalence settings (US), but this would not be applicable to hospitalised patients in Africa given that many of the variables are not routinely available [19].

We have previously shown that detection of lipoarabinomannan (LAM) in the urine of HIV/TB patients using a cheap (approximately US\$3) and quick (testing takes 25 minutes) lateral flow assay is independently associated with a 2- to 3-fold increased risk of mortality [20]. We therefore aimed to investigate if urinary LAM detection, along with other clinical variables readily available in high-burden settings, could be used to predict which HIV-positive patients admitted to hospital and diagnosed with TB were at high risk of early mortality, and to externally validate the predictive tool.

Methods

Study design and participants for prediction tool development

We used data from the STAMP (‘rapid urine-based screening for tuberculosis in HIV-positive patients admitted to hospital in Africa’) trial for the clinical risk score derivation [5,21]. The STAMP trial recruited HIV-positive adults (aged 18 years or more), irrespective of symptoms or clinical presentation, who were admitted to medical wards of 2 hospitals in Malawi and South Africa between 26 October 2015 and 19 September 2017. On admission, patients were screened for TB using Xpert MTB/RIF (Xpert; Cepheid) on sputum in both study arms, and Xpert and Determine TB LAM Ag (TB-LAM; Alere) assays on urine in the intervention arm.

Exclusion criteria in the trial were already taking TB treatment and inability to give consent. The clinical teams managing the patients were masked to which TB tests were positive; therefore, management of TB patients should not have differed between arms. The management of HIV/TB in the study hospitals was representative of their local settings and followed local and national guidelines, with no input from the study team (beyond TB diagnostic tests).

Patients diagnosed with TB in the standard-of-care arm had stored urine tested with Xpert and TB-LAM retrospectively. Data were collected at baseline (at or close to admission) on demographics and clinical characteristics, and subsequently on TB investigations and treatment, and clinical events, including death or discharge from hospital. Patients discharged alive were followed up at 2 months by outpatient attendance, home visit, or telephone for vital status. The derivation cohort included all patients (from both trial arms) with laboratory-confirmed TB. The outcome was mortality risk at 2 months after admission. Patients lost to follow-up were assumed alive at 56 days.

Definitions

Laboratory-confirmed TB was defined as any 1 of a positive smear microscopy, mycobacterial culture, Xpert from any site, or urinary TB-LAM. TB-LAM assay was positive if recorded as ‘grade 1’ or higher on the manufacturer’s (post-2014) reference card. Ability to walk unaided was assessed by healthcare workers (not self-reported by patients), and was equivalent to a Karnofsky functional score below 40 points [22]. WHO danger signs were heart rate > 120 beats per minute, respiratory rate > 30 per minute, temperature > 39°C, and being unable to

walk unaided. 'ART experienced' was defined as receiving ART at the time of enrolment to the study.

Score derivation

Candidate predictor variables were identified for inclusion in the predictive model based on a priori clinical knowledge, previous literature, and the need for variables to be objective, reproducible, and available in resource-constrained settings [23]. We considered variables known to be associated with mortality in HIV/TB, including age, sex, ART experience, physiological measurements at admission, weight and/or body mass index, CD4 cell count, functional status (being unable to walk unaided), and haemoglobin [24–28]. Time on ART was not considered as not all patients take ART, and because of challenges in accurately ascertaining duration. Where 2 or more predictors were highly correlated, only 1 was selected, to simplify the prognostic model, as inclusion of all would contribute little additional predictive information [23]. Analyses were planned prospectively (see [S1 Appendix](#)) except where indicated as post hoc.

Continuous variables were assessed for non-linearity using fractional polynomials, and categorised based on previously established cutoffs (e.g., CD4 cell count and haemoglobin) or associations with mortality (e.g., age and weight, using the *fp plot* command in Stata). Complete case analysis was chosen for the derivation score as few data (<5%) were missing. We first performed univariable analyses assessing the association of each variable with mortality risk using logistic regression. We then used a backward elimination, stepwise approach to create a multivariable predictive model, starting with all candidate variables, and excluding variables sequentially if $p > 0.1$ using likelihood ratio tests and the Akaike information criterion. Given that there were 94 deaths, we did not want to estimate more than 9 candidate predictors (various studies have shown each candidate predictor studied requires a minimum of 10 events) [29]. Interactions were also assessed using likelihood ratio testing. All analyses were done using Stata version 14, and all p -values were 2-sided.

Regression coefficients from the final multivariable model were multiplied by the smallest possible constant and then rounded to the nearest integer, and then assigned as 'points' to each variable. The clinical risk score was derived by combining the points based on each patient's characteristics. High-, medium-, and low-risk groups for mortality were then arbitrarily defined after plotting risk score against observed mortality such that the high-risk group accounted for most (>50%) deaths and the low-risk group accounted for as few deaths as possible.

Risk score evaluation and internal performance

Mortality risk at 2 months and 95% confidence intervals (CIs) were calculated for each risk group, as were odds ratios (ORs) and 95% CI for mortality. In exploratory analyses, inpatient and outpatient (post-discharge) deaths were also compared between risk groups by restricting analyses to deaths occurring during hospital admission or to deaths occurring after discharge in the subset of patients who were discharged alive from hospital. CD4 cell count and TB-LAM grade were also compared between risk groups. Mortality risk was compared between groups using chi-squared tests.

We assessed the model discrimination (ability to differentiate patients who would die within 2 months and those who would survive) by calculating the concordance index (c-statistic) (also known as the area under the receiver operator curve), assuming a c-statistic < 0.6 showed poor discrimination [30]. Model calibration was assessed by plotting the probability of mortality predicted by the model against observed mortality in the derivation dataset using a calibration plot and the Hosmer-Lemeshow test, assuming a $p < 0.05$ indicated poor

calibration. In post hoc analysis, in response to reviewer request and to better understand the utility of the score, the sensitivity and positive predictive value of the score were calculated.

External validation

To externally validate the clinical risk score, we used data collected independently from 2 studies: (1) a multicentre diagnostic clinical trial of adjunctive urine TB-LAM testing in HIV-positive patients with TB symptoms who were admitted to hospitals in 4 sub-Saharan African countries (South Africa, Zambia, Zimbabwe, and Tanzania) (LAM-RCT) [31] and (2) a prospective cohort study assessing the diagnostic yield of TB-LAM in HIV-positive patients with TB symptoms in Kenya (Médecins Sans Frontières [MSF] cohort study) [32]. Patients were included in the validation cohort if they were adults and had laboratory-confirmed TB (as previously defined). Patients from the LAM-RCT in the 'no TB-LAM' arm were excluded, as were outpatients (i.e., patients not admitted to hospital) from the MSF cohort study.

The validation cohort sites were all in settings in sub-Saharan Africa with high HIV prevalence and TB incidence, but differed from the derivation cohort in that all patients had at least 1 TB symptom (cough, fever, weight loss, or night sweats). The LAM-RCT recruitment occurred between 1 January 2013 and 2 October 2014, and the MSF cohort recruitment between 22 October 2013 and 20 August 2015. Mortality outcomes were assessed at 2 months in both studies.

The clinical risk score for mortality was calculated by assigning the same 'points' to variables as for the derivation cohort, and the same cutoffs were used to define high-, medium-, and low-risk groups for mortality. Patients with missing observations were excluded, for a complete case analysis. However, sensitivity analyses were done for score performance using multivariate multiple imputation with chained equations for missing data as 42% of patients had missing data in the validation cohort. Data were assumed to be missing at random, and were imputed for missing candidate predictor variables using mortality risk, other candidate predictor variables, and other baseline demographic variables, with 100 imputations.

Evaluation of the score in the validation dataset was done using the same statistical methods as the internal evaluation, with calculation of mortality risk at 2 months, ORs for mortality, and survival curves. Discrimination was assessed using the c-statistic, and calibration with a calibration plot and the Hosmer-Lemeshow test.

The study is reported in concordance with TRIPOD guidance for multivariable prediction models (see [S2 Appendix](#)) [33]. Ethical approval for each of the source studies was obtained from the relevant ethics committees in the country of data collection and from the trial sponsors (see [S3 Appendix](#) for list of ethics committees). All patients provided informed written consent.

Results

Baseline characteristics

Of 506 HIV-positive patients diagnosed with TB in the STAMP trial derivation cohort, 322 had laboratory-confirmed TB. Seven patients were excluded from the complete case analysis for missing data ([Fig 1](#)). The median age of TB patients included in the derivation cohort was 36 years (interquartile range [IQR] 30–43), 172 (55%) were men, 53 (17%) were newly diagnosed with HIV, and the median CD4 cell count was 76 cells/ μ l (IQR 23–206; [Table 1](#)). In all, 209 (65%) patients were positive on urine TB-LAM testing, indicating probable disseminated TB disease. Anaemia was common and median haemoglobin was 86 g/l (IQR 67–108). Patients presented with advanced disease: 133 (42%) had 1 or more WHO danger signs, and 71 (23%) were severely disabled or unable to walk unaided.

In the derivation cohort, 94 (30%) patients died within 2 months, with 66 (70%) dying during their hospital admission; 29 (31% of deaths) patients died by 1 week, and 52 (55%) by 2

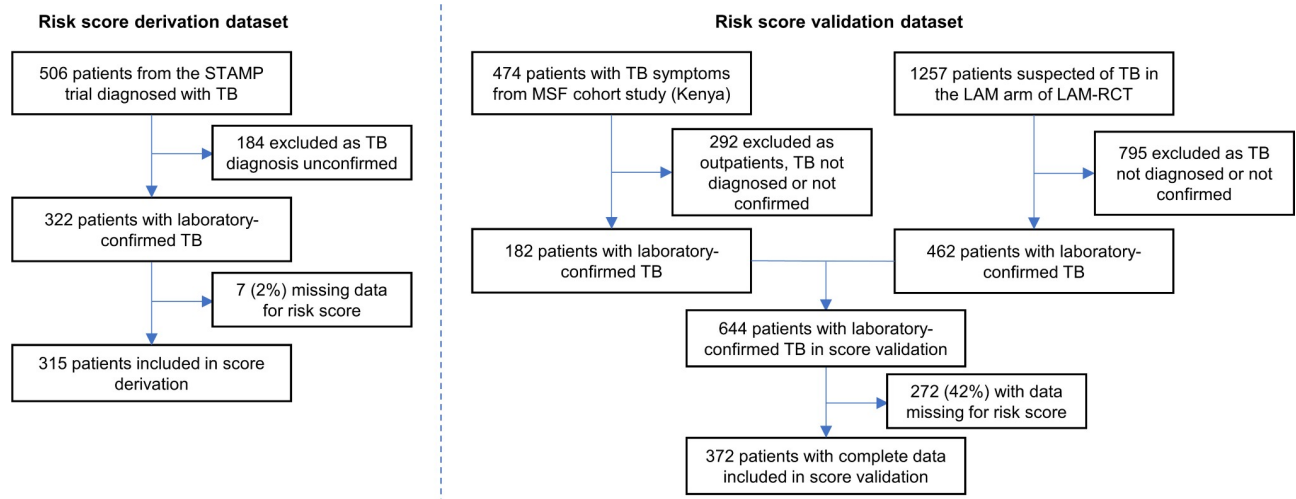


Fig 1. Study profile. LAM, lipoarabinomannan; MSF, Médecins Sans Frontières; TB, tuberculosis.

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weeks, after admission. In unadjusted analyses, mortality risk was higher in patients aged 55 years or older, men, ART-experienced patients, those unable to walk, patients with severe anaemia (haemoglobin < 80 g/l), patients with CD4 cell count < 100 cells/ μ l, and those with positive urine TB-LAM tests (Table 2). Six out of 322 (2%) patients were lost to follow-up after hospital discharge.

Multivariable model and clinical risk score

The final multivariable logistic regression model for mortality at 2 months included age, sex, ART experience, haemoglobin, functional status (being unable to walk unaided), and urine TB-LAM result (Table 2). For associations of linear continuous variables with mortality see S1 Table. CD4 count and weight were dropped from the final predictor score model as their relationship with mortality was mediated by functional status and urine TB-LAM result. We found no significant interactions between variables in the final model. The c-statistic for the predictive model in the derivation dataset was 0.70 (95% CI 0.63–0.76), showing moderate discrimination. Calibration of the predictive model was good, as shown by the calibration plot (see S1 Fig) and a Hosmer-Lemeshow statistic $p = 0.78$.

The clinical risk score for mortality, based on the regression coefficients, is outlined in Fig 2. Observed and predicted mortality risks for the risk score are reported in S2 Fig. Mortality risk groups were defined as low risk (10 points or fewer), medium risk (11 to 20 points), or high risk (more than 20 points) (Fig 3). Therefore, in the derivation cohort, 48 (15%) patients were deemed low risk, 142 (45%) were deemed medium risk, and 125 (40%) were deemed high risk. Median risk score was 19 (IQR 13–22, range 0–42). Observed mortality risk by 2 months was 12.5% (95% CI 5.7%–25.4%), 21.1% (95% CI 15.1%–28.7%), and 46.4% (95% CI 37.8%–55.2%) in the low-, medium-, and high-risk groups, respectively ($p < 0.001$). ORs for mortality were 6.1 (95% CI 2.4–15.2) in the high-risk group and 1.9 (95% CI 0.7–4.8) in the medium-risk group compared to low-risk patients ($p < 0.001$).

Simplified clinical risk score for mortality

As the regression coefficients and points in the clinical risk score were similar for all 6 variables, we created a simplified version of the score by assigning each variable within the score 1

Table 1. Baseline characteristics.

Characteristic	Category	Median (IQR) or N (%)	
		Derivation dataset (n = 315)	Validation dataset (n = 644)
Demographics			
Age (years)		36 (30–43)	35 (30–42)
Sex	Female	143 (45.4)	315 (48.9)
Country of enrolment	South Africa	162 (51.4)	102 (15.8)
	Tanzania	—	70 (10.9)
	Zambia	—	151 (23.5)
	Zimbabwe	—	139 (21.6)
	Kenya	—	182 (28.3)
	Malawi	153 (48.6)	—
HIV history			
New HIV diagnosis	Yes	53 (16.8)	—
Currently taking ART	Yes	210 (80.2)	290 (45.0)
Time on ART (years) ^a		1.0 (0.2–4.4)	0.7 (0.1–3.2)
CD4 cell count (cells/ μ l) ^b		76 (23–206)	61 (21–145)
TB history			
Cough	Yes	228 (72.4)	601 (93.5)
Fever	Yes	223 (70.8)	562 (87.3)
Weight loss	Yes	286 (90.8)	595 (95.8)
Night sweats	Yes	165 (52.4)	531 (82.6)
WHO TB symptom screen	Yes	310 (98.4)	644 (100)
Previous history of TB	Yes	72 (22.9)	123 (19.1)
Clinical presentation			
Weight (kg) ^c		50 (42–57)	49 (43–55)
BMI ^d		19.1 (16.2–21.0)	17.6 (15.9–20.3)
Heart rate (bpm)		104 (90–118)	102 (90–119)
Respiratory rate (per minute)		22 (20–26)	24 (22–28)
Systolic blood pressure (mm Hg)		102 (92–116)	104 (95–116)
Temperature ($^{\circ}$ C)		36.5 (36.1–37.2)	37.0 (36.6–38.0)
Haemoglobin (g/l) ^e		86 (67–108)	85 (68–100)
WHO danger sign ^f	Yes	133 (42.2)	399 (62.0)
Unable to walk unaided ^g	Yes	71 (22.5)	262 (40.7)
TB diagnosis			
Sputum Xpert positive	Yes	168 (52.2)	217 (33.7)
Sputum smear microscopy positive	Yes	—	211 (32.8)
TB culture positive (any site)	Yes	—	388 (60.3)
Urine LAM positive	Yes	209 (64.9)	424 (65.8)
Chest X-ray suggestive of TB	Yes	107 (33.2)	336 (52.2)
Outcome			
Died by 2 months	Yes	94 (29.8)	147 (22.8)

Sputum smear and TB culture were not routinely performed in the STAMP trial (derivation dataset). Missing data are for the validation dataset only.

^aTime on ART missing for 19 (3%) patients.

^bCD4 cell count missing for 27 (4%) patients.

^cWeight missing for 75 (12%) patients.

^dBMI missing for 90 (14%) patients.

^eHaemoglobin missing for 272 (42%) patients.

^fOne of heart rate > 120 bpm, respiratory rate > 30 per minute, temperature > 39 $^{\circ}$ C, or unable to walk unaided.

^gAbility to walk unaided was assessed by healthcare worker and not self-reported.

ART, antiretroviral therapy; BMI, body mass index; bpm, beats per minute; IQR, interquartile range; LAM, lipoarabinomannan; TB, tuberculosis; WHO, World Health Organization; Xpert, Xpert MTB/RIF.

<https://doi.org/10.1371/journal.pmed.1002776.t001>

Table 2. Univariable and multivariable logistic regression analysis of factors associated with mortality in the derivation cohort (n = 315).

Characteristic	Category	Died ^a (n = 94)	Univariable		Multivariable		Regression (β) coefficient
			OR (95% CI)	p-Value	OR (95% CI)	p-Value	
Demographics							
Age	<55 years	82 (28.4)	1 (ref)	0.067	1 (ref)		0.710
	≥55 years	12 (46.2)	2.2 (1.0–4.9)		2.0 (0.9–4.9)	0.10	
Sex	Female	32 (22.4)	1 (ref)	0.012	1 (ref)		0.923
	Male	62 (36.0)	2.0 (1.2–3.2)		2.5 (1.5–4.3)	0.001	
HIV infection							
ART experienced	No	18 (20.9)	1 (ref)	0.024	1 (ref)		0.621
	Yes	76 (33.2)	1.9 (1.1–3.4)		1.9 (1.0–3.5)	0.048	
CD4 cell count [#]	≥100 cells/μl	31 (23.9)	1 (ref)		—	—	—
	<100 cells/μl	62 (33.9)	1.7 (1.0–2.8)	0.040	—	—	—
Clinical presentation							
WHO danger sign	No	50 (27.5)	1 (ref)	0.185	—	—	—
	Yes	48 (34.8)	1.4 (0.9–2.2)		—	—	—
Weight	<35 kg	10 (43.5)	3.3 (1.2–8.9)	0.054	—	—	—
	35–60 kg	73 (31.5)	1.8 (0.9–3.5)		—	—	—
	>60 kg	11 (18.3)	1 (ref)		—	—	—
Haemoglobin (g/l)	≥80 g/l	44 (23.7)	1 (ref)	0.003	1 (ref)		0.703
	<80 g/l	50 (38.8)	2.0 (1.3–3.3)		2.0 (1.2–3.4)	0.008	
Unable to walk unaided	No	64 (26.2)	1 (ref)	0.004	1 (ref)		0.689
	Yes	30 (42.3)	2.2 (1.3–3.8)		2.0 (1.1–3.6)	0.022	
TB diagnosis							
Urine LAM positive	No	24 (22.6)	1 (ref)	0.044	1 (ref)		0.603
	Yes	70 (33.5)	1.7 (1.0–2.9)		1.8 (1.0–3.2)	0.040	

The constant (intercept) was -2.8 . *p*-Values were calculated by likelihood ratio tests. There was no evidence of interaction between urine LAM positivity, being unable to walk, and haemoglobin < 80 g/l in the multivariable model (likelihood ratio test *p*-values all > 0.1). Weight and being unable to walk were strongly associated.

^aData are number of patients in category who died (%).

ART, antiretroviral therapy; LAM, lipoarabinomannan; OR, odds ratio; TB, tuberculosis.

<https://doi.org/10.1371/journal.pmed.1002776.t002>

point if present (age 55 years or over, male sex, ART experienced, severe anaemia, being unable to walk unaided, or urine TB-LAM positive; see [S3 Fig](#)). A high mortality risk was defined as 3 or more points, medium risk as 2 points, and low risk as 0 or 1 point.

In the derivation cohort, patients with 3 or more points (high risk) had a mortality of 40.0% (70/175, 95% CI 33.0%–47.5%), compared to 19.6% (18/92, 95% CI 12.6%–29.0%) mortality in those with 2 points and 12.5% (6/48, 95% CI 5.7%–25.4%) in those with 0 or 1 point ($p < 0.001$) (see [Fig 3](#) and [S2 Table](#)). The sensitivity of the risk score for mortality in was 0.75 (the score correctly identified 70/94 deaths), and the positive predictive value was 0.4.

The clinical risk score was useful in predicting deaths that occurred during inpatient admission (50 [28.6%, 95% CI 22.3%–35.8%] in the high-risk group compared to 6 [10.9%, 95% CI 5.9%–19.1%] in the low-risk group, $p = 0.001$) as well as deaths occurring after discharge (20 [16.0%, 95% CI 10.5%–23.6%] in the high-risk group compared to 0 [0%] in the low-risk group, $p = 0.015$; [Fig 4](#)). More patients in the high-risk group were TB-LAM positive and had higher grades of positive result, but CD4 cell count did not differ by risk group (see [S4 Fig](#)). Survival curves by risk group are presented in [Fig 5](#).

Demographic factors:

- 1. Is the patient male? Yes: add 9 points
- 2. Is the patient aged 55 years or older? Yes: add 7 points

HIV factors:

- 3. Is the patient currently taking antiretroviral therapy? Yes: add 6 points

Clinical presentation and TB diagnosis:

- 4. Is the patient unable to walk unaided? Yes: add 7 points
- 5. Does the patient have severe anaemia (haemoglobin <80g/l)? Yes: add 7 points
- 6. Is the patient positive on urine TB-LAM testing? Yes: add 6 points

Total points (min 0, max 42):

Fig 2. Risk score calculation to predict mortality. TB, tuberculosis; TB-LAM, Determine TB LAM Ag.

<https://doi.org/10.1371/journal.pmed.1002776.g002>

External validation

The external validation cohort included 644 HIV-positive patients with laboratory-confirmed TB, of whom 372 (58%) patients had no missing data for the risk score and were therefore included in the complete case analysis (Fig 1). Baseline characteristics were similar between cohorts, although fewer patients reported taking ART and more patients presented with severe functional impairment and 1 or more WHO danger signs in the validation cohort (Table 1). A similar proportion of patients were positive on urine TB-LAM testing (65% in the derivation cohort compared to 66% in the validation cohort). Mortality at 2 months was lower in the validation cohort (22.8%) compared to the derivation cohort (29.8%). Loss to follow-up was 4% in the validation cohort (15/372).

In complete case analysis ($n = 372$), the observed mortality risks in the validation cohort were 8.7% (95% CI 4.6%–16.0%) in the low-risk group, 19.1% (95% CI 13.2%–26.8%) in the medium-risk group, and 35.5% (95% CI 27.9%–43.9%) in the high-risk group (see Fig 4). Median risk score was 16 (IQR 10–22, range 0–42). The ORs for mortality by risk group were similar to those in the derivation cohort (5.8 [95% CI 2.7–12.3] for the high-risk group and 2.5 [95% CI 1.1–5.5] for the medium-risk group compared to the low-risk group). The risk score was also useful in predicting both inpatient and post-discharge deaths (the high-risk group had a 20% risk of post-discharge death compared to 5% in the low-risk group). The simplified risk score performed similarly to the full score in the validation cohort.

The predictive model had similar calibration and discrimination in the validation cohort as in the derivation cohort: the c-statistic was 0.68 (95% CI 0.61–0.74; see S3 Table), and the Hosmer-Lemeshow statistic had $p = 0.13$ (see S5 Fig for the calibration plot). In a sensitivity analysis using multiple imputation for missing data in the validation dataset ($n = 644$), the c-statistic for the predictive model was 0.64 (95% CI 0.60–0.69), and the Hosmer-Lemeshow statistic had $p = 0.67$. ORs for mortality were 5.3 (95% CI 2.2–9.5) for the high-risk group and 2.1 (95% CI 1.0–4.6) for the medium-risk group compared to low-risk patients ($p < 0.001$).

Discussion

In this study, we developed and externally validated a pragmatic clinical risk score to predict early mortality in HIV-positive patients admitted to hospital and diagnosed with laboratory-confirmed TB. Our score used 6 clinical and laboratory factors that could be readily collected at admission to hospital in settings with high HIV and TB burden. The score was able to

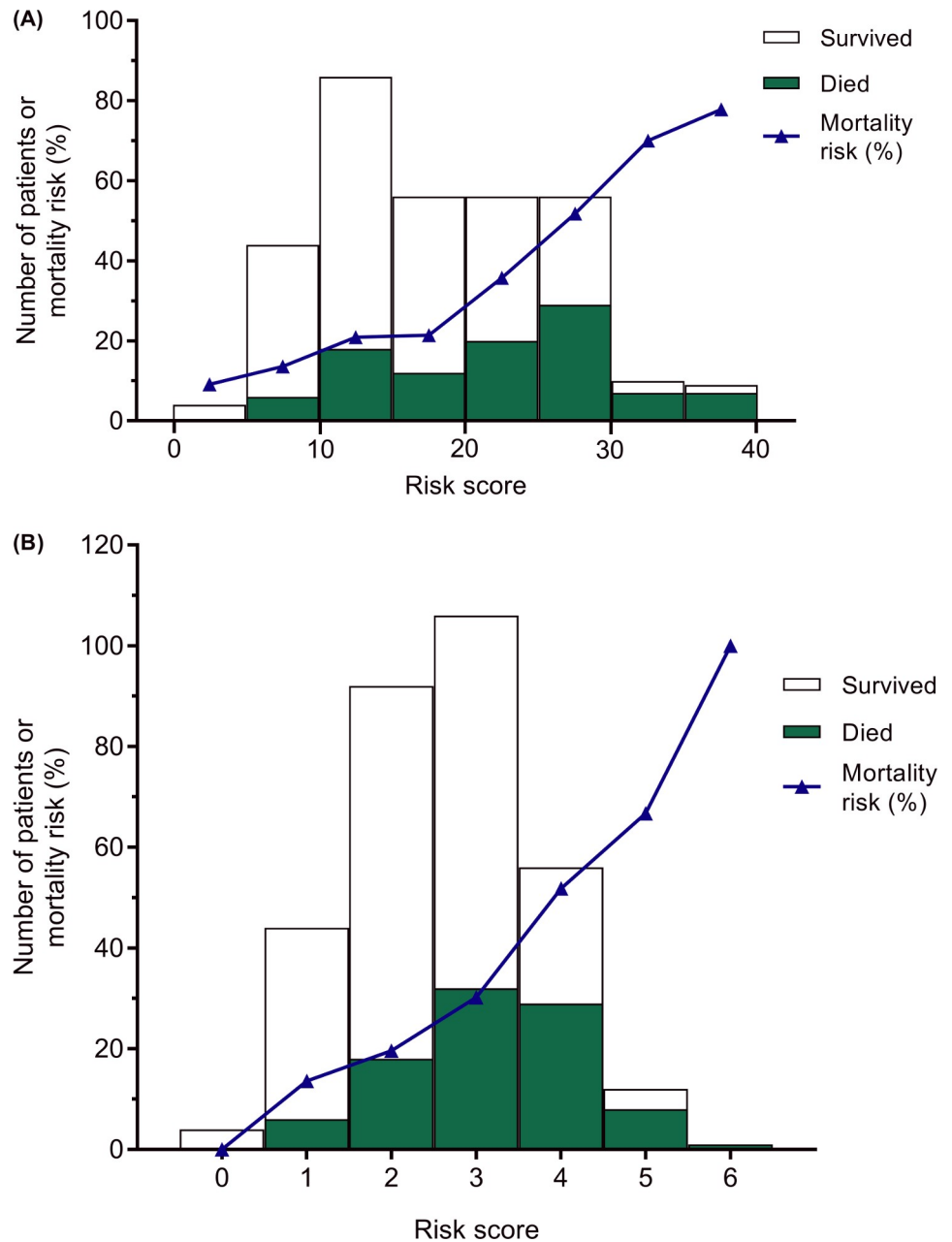


Fig 3. Distribution of risk scores and mortality in the derivation dataset. Distribution of risk scores for mortality stratified by outcome at 2 months (stacked bar chart) and mortality risk (percent, shown by blue line) for (A) the full risk score (based on the regression coefficients) and (B) the simplified risk score. Mortality risks and absolute numbers in each category are presented in [S2 Table](#).

<https://doi.org/10.1371/journal.pmed.1002776.g003>

categorise patients into 3 risk groups. One-third of the high-risk group died during hospital admission, and almost 50% had died by 2 months. A simplified ‘3 of 6 predictors’ version of the score performed similarly. This is the first study to our knowledge to derive and externally validate a risk score to predict mortality in this patient population.

We found older age, being male, being ART experienced, having severe anaemia, being severely disabled or unable to walk unaided, and being urine TB-LAM positive were all risk

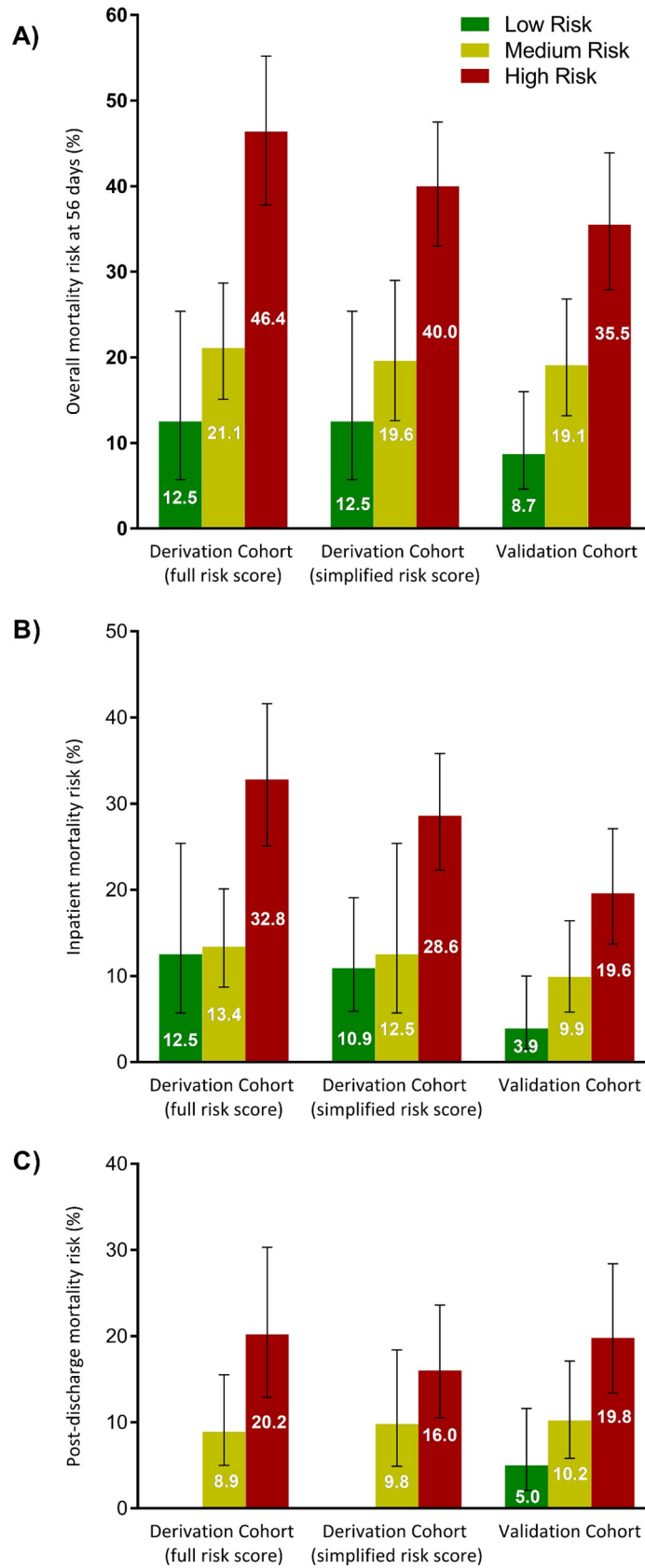


Fig 4. Observed mortality risk by risk score category in the derivation and validation cohorts. Observed mortality risk (A) at 56 days, (B) during inpatient stay, and (C) post-discharge in the derivation and validation cohorts, stratified by risk score category (derivation and validation cohorts) and simplified risk score category (derivation cohort). Numbers on bars represent absolute mortality risk; error bars represent 95% confidence intervals. For the full risk score, low risk was defined as 10 points or fewer, medium risk as 11 to 20 points, and high risk as more than 20 points. For the simplified risk score, the low-risk group had a predictor score of 0 or 1 point, the medium-risk group had a predictor score of 2 points, and the high-risk group had predictor score of ≥ 3 points. *p*-Values based on chi-squared tests between groups for derivation and validation cohorts, respectively, are (A) $p < 0.001$ and $p < 0.001$, (B) $p = 0.001$ and $p = 0.001$, and (C) $p = 0.015$ and $p = 0.003$.

<https://doi.org/10.1371/journal.pmed.1002776.g004>

factors for mortality. These factors have been established as being associated with outcome in HIV/TB in previous studies [24–28], and most likely reflect more advanced HIV-related immunosuppression, late presentation to healthcare services, and/or poorer underlying physiological reserve. Positive urine diagnostic tests (including LAM detection and *Mycobacterium tuberculosis* nucleic acid detection) in the context of HIV infection are thought to represent haematogenously disseminated renal TB with high mycobacterial burden, which may explain why it is associated with a worse prognosis [34]. Interestingly, clinical signs and symptoms (such as WHO danger signs) were not predictive of mortality.

In contrast to previously published data, ART-experienced patients had a higher mortality risk in our study [28,35]. This likely reflects a high burden of unrecognised ART failure, due to either poor adherence or drug resistance among patients admitted to hospital. Another potential cause is immune reconstitution inflammatory syndrome (IRIS) in patients who have recently started ART. The relationship between ART and mortality is likely to be more complex, representing different groups of patients with different mortality risks, but for this pragmatic tool we have not been able to explore this further. CD4 cell count, which has been previously shown to be associated with mortality in HIV-positive patients, dropped out of our final multivariable predictive model due to mediation by other variables. Furthermore, in the era of test and treat for HIV and use of quantitative HIV viral load for monitoring, CD4 testing services are being scaled back, and are often not available in resource-limited settings.

Mechanisms and causes of mortality in advanced HIV/TB are still not well understood. Copathologies, including other opportunistic infections and bacterial pneumonia or sepsis, are commonly detected post-mortem [36,37]. High-risk patients could be prioritised for screening for co-infections, for example using cryptococcal antigen point-of-care tests, or empirical prophylactic treatment with antibacterial agents, an approach that has been shown to reduce mortality in advanced HIV infection [38].

Whilst this clinical risk score can identify patients with the highest risk of mortality, there remains an absence of proven interventions (beyond TB therapy and appropriately timed ART) to reduce mortality in this population. Therefore, we propose this score could be used as a clinical tool to alert clinicians to patients at high risk of mortality who should be reviewed before discharge and/or flagged for early clinical follow-up in settings where urine TB-LAM scale-up is occurring. The score could also be used as a research tool to aid evaluation of intensified or optimised TB treatment regimens or adjunctive interventions aimed at reducing high mortality in this population.

Possible interventions include rapid viral load testing with ART adherence support and early switching for those with virological failure. Host-directed therapies, which target host immune responses, are in clinical trials for TB, including some specifically for HIV/TB [39,40]. Patients identified as being at highest risk for mortality could also be offered more intensive monitoring or supportive care, for example better management of severe anaemia [41], although optimal strategies of supportive care are not clear [42]. Enhanced treatment and prophylaxis for co-infections have been shown to reduce early mortality in patients with

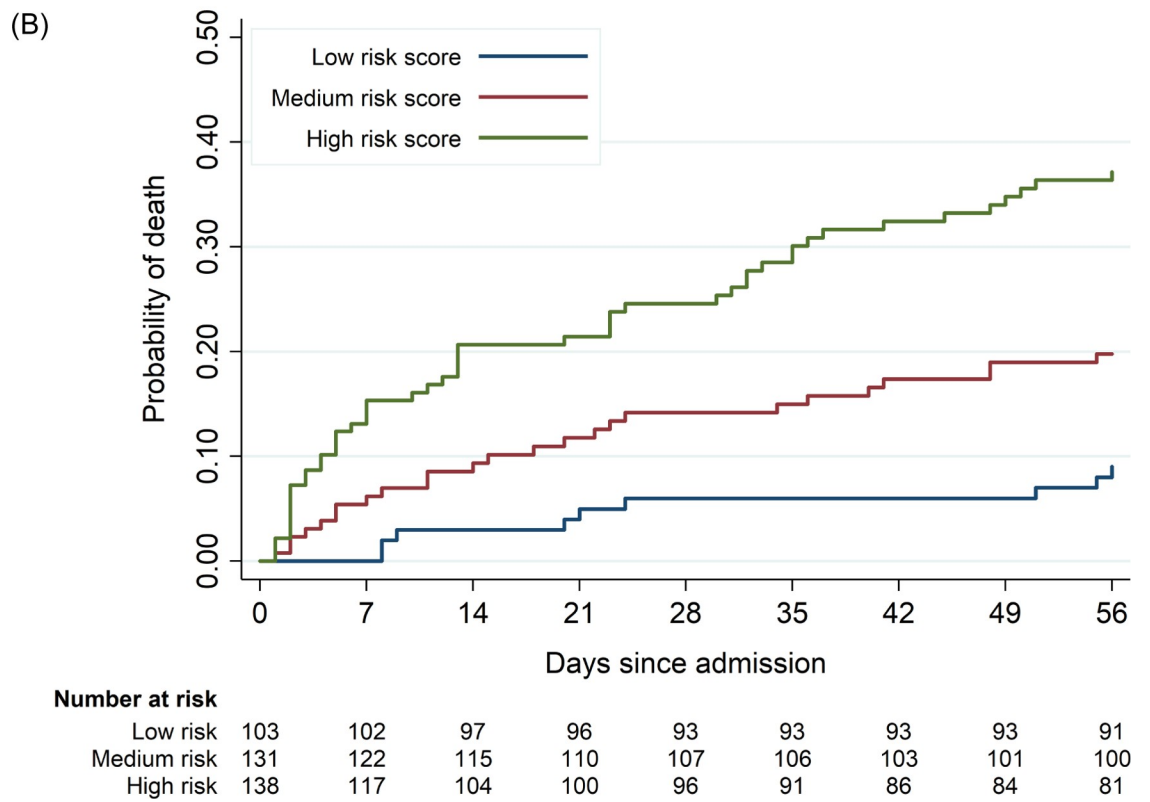
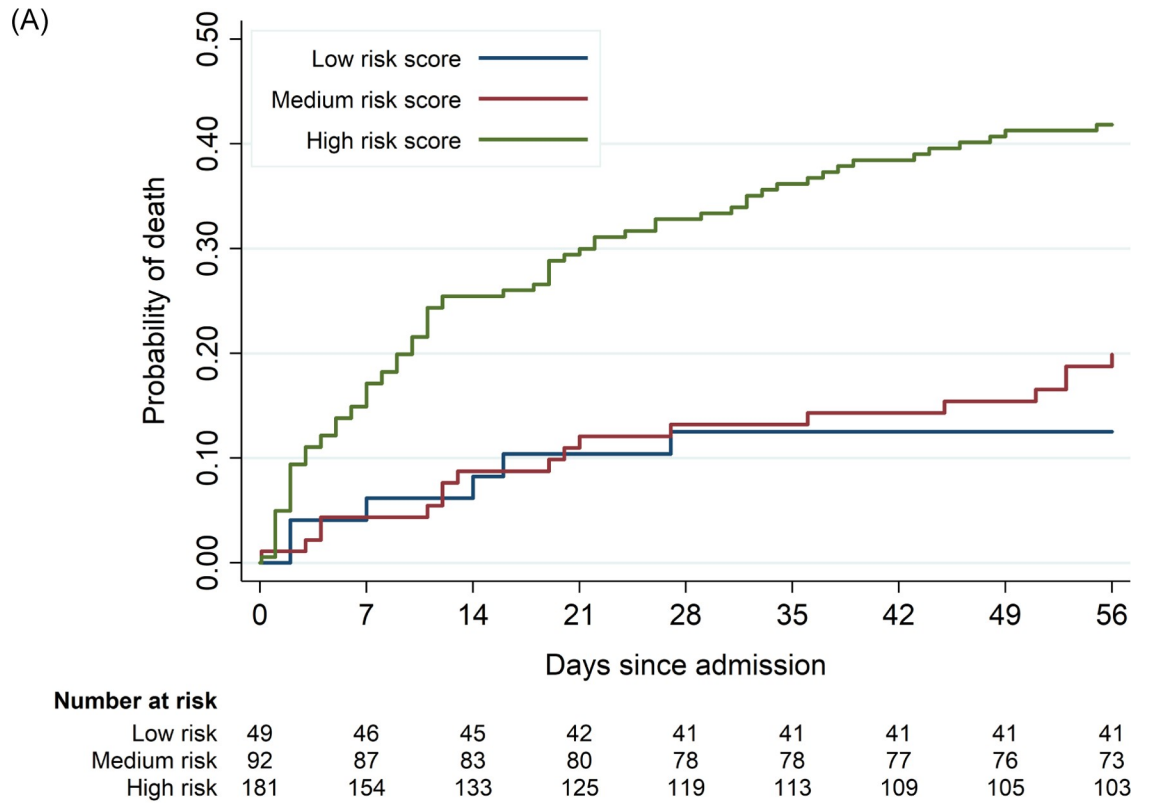


Fig 5. Survival curves stratified by clinical risk score category in the derivation dataset. Survival curves and risk tables with number at risk for the (A) derivation cohort and (B) validation cohort stratified by risk group using the simplified clinical risk score. The low-risk group (blue line) had a risk score of 0 or 1 point, the medium-risk group (red line) had a risk score of 2 points, and the high-risk group (green line) had a risk score of ≥ 3 points. Log-rank test $p < 0.001$.

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advanced HIV initiating ART [38], and may also benefit those with HIV/TB disease. Interventions will likely need to be instituted rapidly after TB diagnosis to alter outcomes.

The risk score was able to highlight patients at highest risk of death post-discharge, in addition to those at high risk of death during hospitalisation, and could be used to prevent too early discharges. Enhanced community support, including home visits, has been shown to reduce mortality after starting ART in advanced HIV [43], and could have a similar impact for HIV/TB patients. Current services in high-burden settings take a public health approach to service delivery, whereas prognostic risk scores can identify patients suitable for differentiated care [8].

The main aim of this risk score was to detect patients at high risk of early mortality who may benefit from interventions in addition to TB treatment. Although the discrimination of the model was not perfect, the sensitivity of the simplified score was 75%; the score did not identify 25% of patients who died within 2 months, and such patients would still receive standard-of-care management of HIV/TB. Proposed interventions to reduce mortality would have limited adverse events, so those deemed as 'high risk' by the score but surviving to 2 months are unlikely to come to significant harm from such interventions. However, if adjunctive interventions are found to reduce early mortality, better predictive biomarkers or more accurate predictive tools would allow more efficient use of resources through targeting of patients.

Limitations of our study include the potential for selection bias. In the STAMP trial standard-of-care arm, only patients started on TB treatment for clinical/radiological criteria or following a positive sputum Xpert result had stored urine retrieved for TB testing. Patients with otherwise undiagnosed TB who would have been urine test positive if they had been tested were not included in this study. Patients unable to provide consent, mostly due to being severely unwell and having altered consciousness, were also excluded. Although our risk score did not have optimal discrimination and calibration, performance was adequate and similar to that of other prognostic scores widely used in clinical practice (e.g., the Framingham cardiovascular risk score) [15,44]. Performance may have been reduced by categorising continuous variables for simplicity. TB drug resistance was not a predictor of mortality in this cohort; however, prevalence of rifampicin resistance was low in these settings. Not all established risk factors for mortality were characterised, leaving potential to improve on performance. Future studies could assess more detailed markers of physiology, as well as social and more distal risk factors.

Whilst the score is pragmatic and its constituent factors are widely available in hospitals in African regions with high HIV and TB burdens, it does rely on access to the TB-LAM lateral flow assay. There is now good evidence to support mortality reductions with the use of TB-LAM in HIV-positive patients admitted to hospital [4,5], and its use as a screening test has been incorporated into the latest guidelines in Malawi and South Africa. The assay has also been scaled up nationally in eSwatini, Kenya, and Uganda [45]. Missing data were common in the validation cohort. However, sensitivity analyses using multiple imputation gave similar results as the complete case analysis. We assumed patients lost to follow-up were alive at 2 months, although only 2% in the derivation cohort and 4% in the validation cohort were not followed up after hospital discharge. Our cohort did not include patients treated for TB without a positive diagnostic test, which remains common in HIV-positive patients admitted to hospital, and this patient group may be an important group for whom to apply risk

stratification and predictive scores. The biomarkers studied are imperfect predictors of mortality, and further research is needed to focus on better biomarkers to predict outcome.

Strengths of this study include that the derivation cohort and the LAM-RCT external validation cohort were nested within randomised controlled trials. Our predictive model had similar discrimination and calibration in the validation cohort, and was able to identify groups of patients with similarly increased odds of mortality. This was despite the validation cohort being from geographically distinct locations, collected at different times by different investigators, and with a lower overall mortality risk at 2 months. The factors required for the score can be obtained rapidly after admission.

In conclusion, we have developed and externally validated a clinical risk score capable of identifying, among patients admitted to hospital in settings with high HIV/TB burden, those with the highest risk of early mortality. This score could be a useful clinical and research tool, and could prove beneficial in identifying patients who would gain most from adjunctive interventions to reduce mortality. Further work to assess the impact of such risk scores, and to identify which interventions could potentially reduce mortality, is urgently needed if ambitious global targets to reduce TB mortality are to be met by 2025.

Supporting information

S1 Appendix. Statistical analysis plan. Prospective statistical analysis plan (version 1.2, 4 June 2017).

(PDF)

S2 Appendix. TRIPOD checklist.

(PDF)

S3 Appendix. List of ethics committees that provided approval.

(PDF)

S1 Fig. Performance of clinical risk score in derivation cohort. (A) Receiver operator curve of the predictive model: area under the curve = 0.70 (95% CI 0.63–0.76). (B) Calibration plot of observed probability of mortality plotted against predicted probability of mortality by the risk score multivariable regression model, with variables grouped into deciles based on predicted probability, and 95% CIs. Black dashed line shows perfect prediction. Hosmer-Lemeshow statistic $p = 0.78$.

(TIF)

S2 Fig. Observed and predicted mortality for risk score values in the derivation cohort.

The size of the blue circles representing observed mortality risk is proportional to the number of patients with that score. Predicted mortality risk is represented by the green line/triangles.

(TIF)

S3 Fig. Risk score calculation for the simplified risk score to predict mortality.

(TIF)

S4 Fig. Urine TB-LAM grade and CD4 cell strata stratified by clinical risk score for the derivation cohort ($n = 315$).

(TIF)

S5 Fig. Calibration plot for the predictive model in the external validation dataset ($n = 372$). Plot shows the observed compared to expected probability of risk for the external validation cohort as deciles based on risk score, with 95% CIs. Hosmer-Lemeshow statistic $p = 0.13$. c-Statistic (or area under the receiver operator curve) was 0.68 (95% CI 0.61–0.74). Dotted line

represents perfect prediction.
(TIF)

S1 Table. Univariable analysis of continuous variables and associations with mortality.
(PDF)

S2 Table. Risk score and mortality data. Number of patients surviving and patients dying, and observed and predicted mortality risk for (A) the full clinical risk score (based on the regression coefficients) and (B) the simplified risk score.
(PDF)

S3 Table. C-statistic, 95% confidence intervals, and Hosmer-Lemeshow test for final model and risk scores in derivation and validation cohorts.
(PDF)

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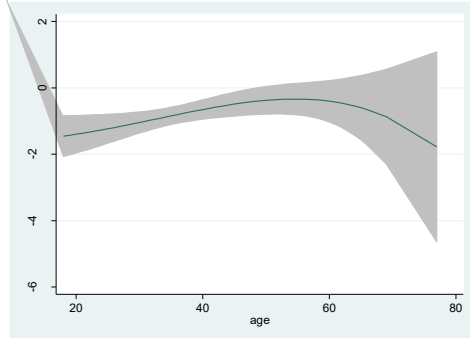
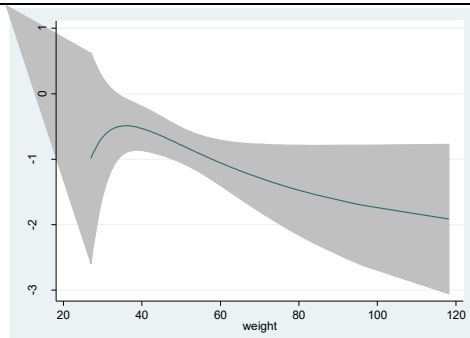
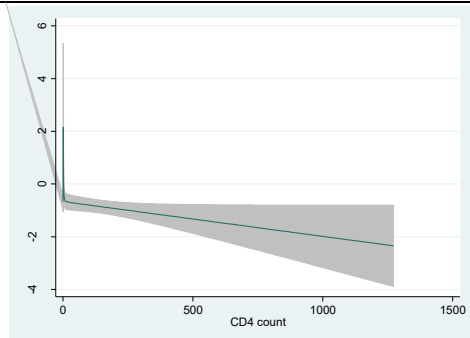
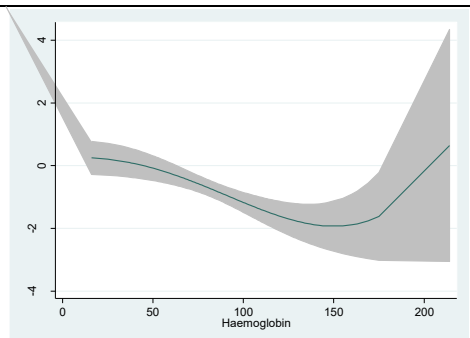
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S1 Table. Univariable analysis of continuous variables and associations with mortality.

	Fitted fractional polynomial function	Odds ratio (OR), 95% confidence interval	p-value
Age (years)^a			
Linear		1.02 (1.00 – 1.05)	0.0485
Age ³ Age ³		1.00 (1.00 – 1.00) 1.00 (1.00 – 1.00)	0.0526
Weight (kgs)^b			
Linear		0.98 (0.96 – 1.00)	0.0486
Weight ⁻² Weight ⁻²		0 0	0.0897
CD4 count^c			
Linear		1.00 (1.00 – 1.00)	0.0158
CD4 count ⁻² CD4 count		16.7 (0.65 – 430.71) 1.00 (1.00 – 1.00)	0.0055
Haemoglobin (g/L)^d			
Linear		0.98 (0.97 – 0.99)	0.0002
Haemoglobin ³ Haemoglobin ³		1.00 (1.00 – 1.00) 1.00 (1.00 – 1.00)	0.0001

^a This function has a small increase to 55 years, then a steeper increase

^b This gives a “U-shaped” function and has an increase until 35kg, then a sharper decrease until 60kg, and then a less steep increase

^c This function has a sharp peak in the low values (<20 cell/microL), followed by a linear decrease

^d This is a “U-shaped” function and has a decrease until about 150 g/L, followed by an increase.

S2 Table. Risk score and mortality data

Number of survivors, patients dying, observed and predicted mortality risk for **A)** 'full' mortality predictor score (based on the regression coefficients) and **B)** simplified mortality risk score.

A)

Risk score category	Proportion of patients	Survived	Died	Observed mortality	Predicted Mortality
0-5	1.3	4	0	0.0	7.7
6-10	14.0	38	6	13.6	12.1
11-15	27.3	68	18	20.9	18.4
16-20	17.8	44	12	21.4	27.0
21-25	17.8	36	20	35.7	37.8
26-30	17.8	27	29	51.8	49.9
31-35	3.2	3	7	70.0	62.0
>35	1.0	1	2	66.7	72.8
Low	15.2	42	6	12.5	9.7
Medium	45.1	112	30	21.1	23.0
High	39.7	67	58	46.4	45.3

Mortality risk groups were defined as low risk (10 points or fewer), medium risk (11 to 20 points) or high risk (more than 20 points)

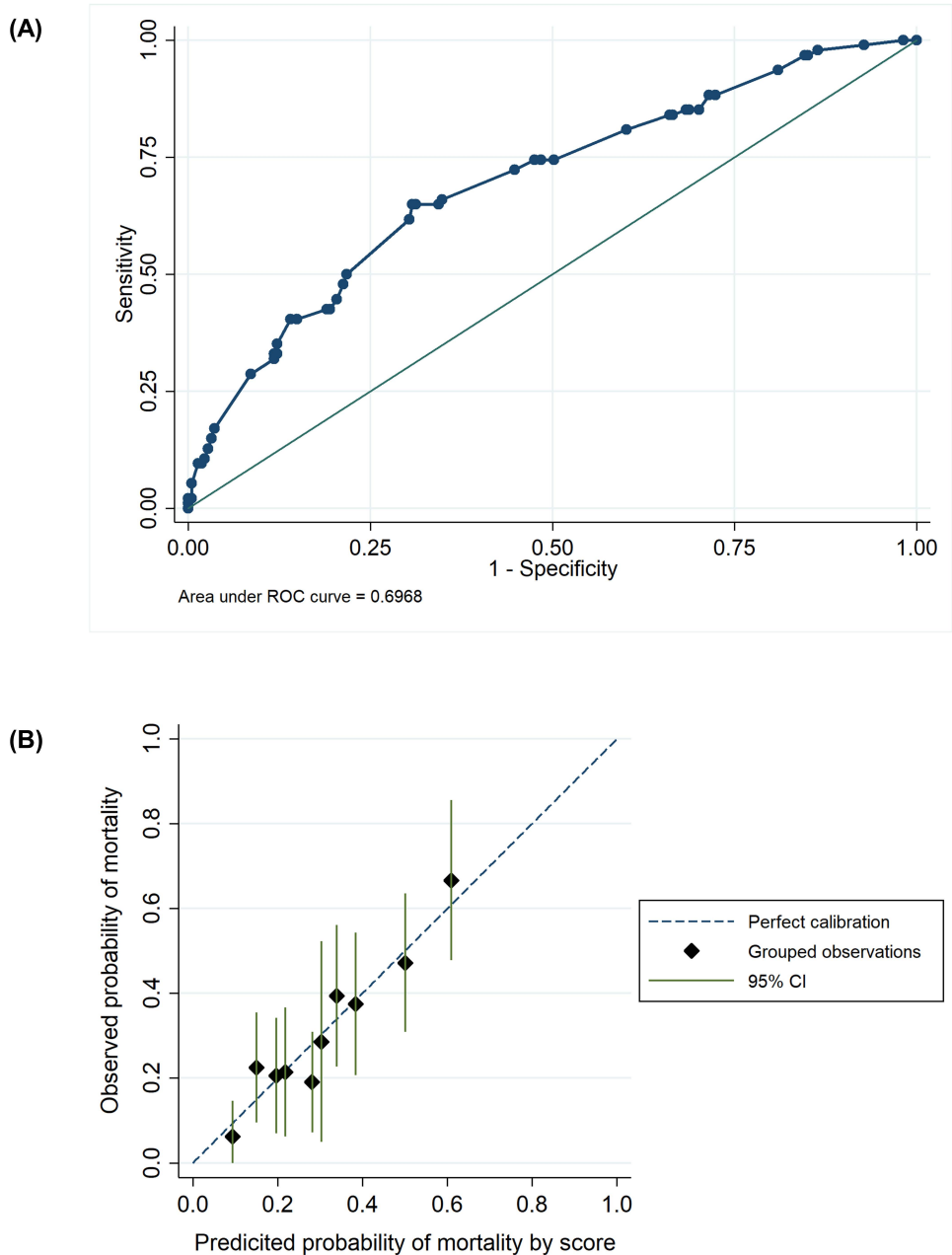
B)

Risk score or risk score category	% of patients	Survived	Died	Observed mortality	Predicted Mortality
0	1.3	4	0	0.0	5.6
1	14.0	38	6	13.6	10.7
2	29.2	74	18	19.6	19.5
3	33.7	74	32	30.2	32.7
4	17.8	27	29	51.8	49.5
5	3.8	4	8	66.7	66.3
6	0.3	0	1	100.0	79.9
Low	15.2	42	6	12.5	10.5
Medium	29.2	74	18	19.6	21.7
High	55.6	105	70	40.0	39.5

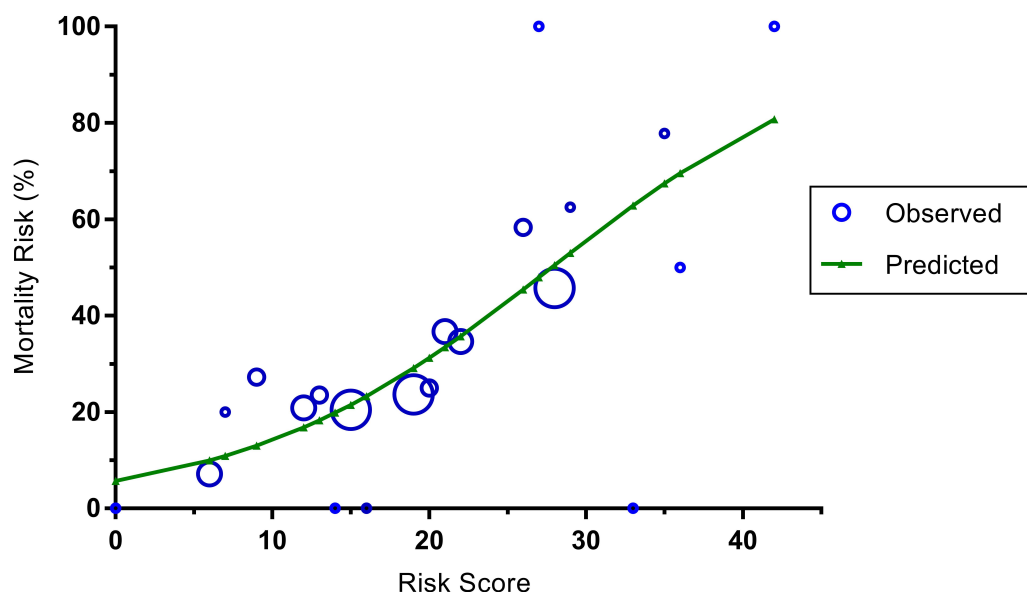
Mortality risk groups were defined as low risk (1 point or fewer), medium risk (2 points) or high risk (more than 2 points)

S3 Table. C-statistic, 95% confidence intervals and Hosmer-Lemshow test for final model and risk scores in derivation and validation cohorts.

Cohort/model	C-statistic	95% Conf. Interval	Hosmer-Lemshow test p-value
Derivation cohort full model	0.7299	0.66957 - 0.79015	0.4527
Derivation cohort full score	0.6968	0.63280 - 0.76077	0.7813
Derivation cohort simple score	0.6471	0.59337 - 0.70092	0.4956
Validation cohort full model	0.6659	0.60062 - 0.73123	0.2334
Validation cohort full score	0.6770	0.61307 - 0.74083	0.1315
Validation cohort simple score	0.6605	0.59756 - 0.72336	0.2269



S1 Fig. Performance of mortality prediction score in derivation cohort. (A) Receiver operator curve of the predictive model, area under the curve= 0.70 (95% CI 0.63 – 0.76). (B) Calibration plot of observed probability of mortality plotted against predicted probability of mortality by the mortality score multivariable regression model, with variables grouped into deciles based on predicted probability, and 95% CIs. Black dashed line shows perfect prediction. Hosmer-Lemeshow statistic $p=0.78$



S2 Fig. Observed and predicted mortality risk for predictor score values in the derivation cohort. The size of the blue circles representing observed mortality risk are proportional to the number of patients with that score. Predicted mortality risk is represented by the green line/triangles.

Demographics factors:

- 1. Is the patient male? Yes: add 1 point
- 2. Is the patient aged 55 years or older? Yes: add 1 point

HIV factors:

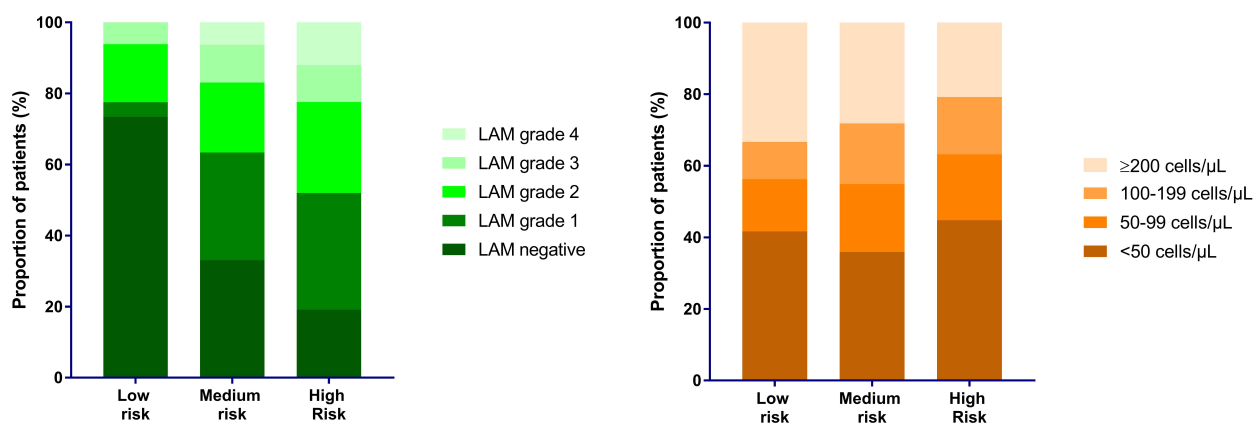
- 3. Is the patient currently taking antiretroviral therapy? Yes: add 1 point

Clinical presentation and TB diagnosis:

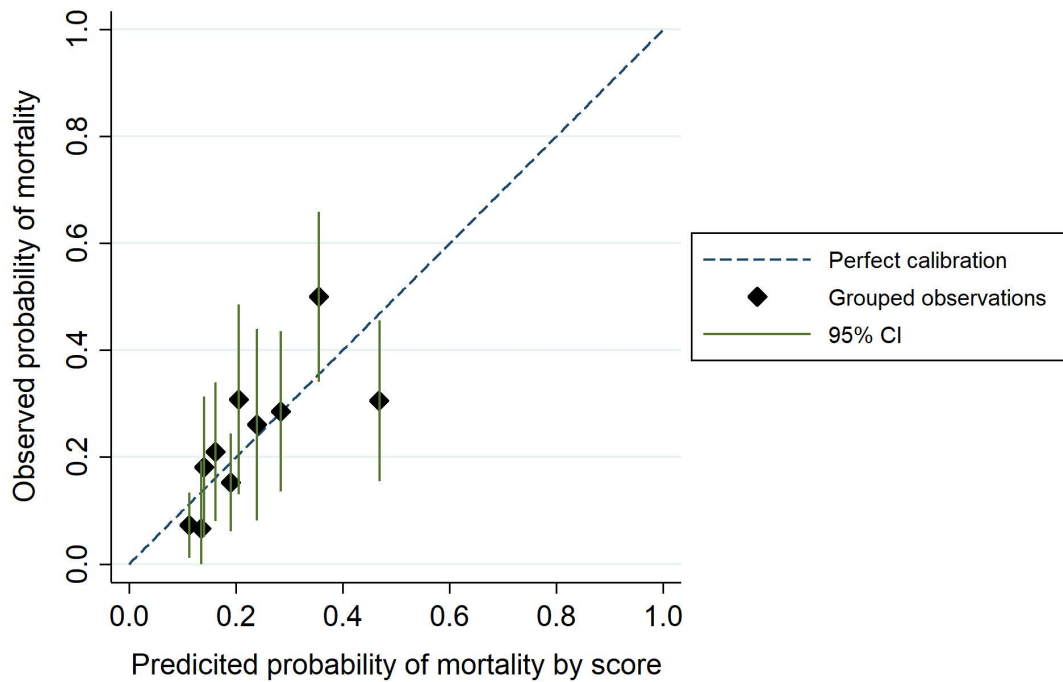
- 4. Is the patient unable to walk unaided? Yes: add 1 point
- 5. Does the patient have severe anaemia (haemoglobin <8g/dL)? Yes: add 1 point
- 6. Is the patient positive on urine TB-LAM testing? Yes: add 1 point

Total points (min 0, max 6):

S3 Fig. Risk score calculation (simplified score) to predict mortality



S4 Fig. Urine TB-LAM grade and CD4 cell strata stratified by mortality risk score for the derivation cohort (n=315)



S5 Fig. Calibration plot for predictive model in external validation dataset (n=372). Plot shows observed compared to expected probability of risk for external validation cohort as deciles based on predictor score, with 95% CIs. Hosmer-Lemeshow statistic p=0.13. C-index (or area under receiver-operator curve) was 0.677 (95% CI 0.61–0.74). Dotted line represents perfect prediction.

Revision to discussion section

Although the mortality score predicted mortality during hospital admission and after discharge, the underlying pathophysiological mechanisms leading to death may differ in those who die very early (eg within the first few days), compared to those who die later (eg after several weeks, or after discharge). Thus, risk factors for mortality may also differ between these groups. This warrants further research to determine if a more accurate predictor score can be derived for very early deaths, and if factors measured at discharge may be more accurate at predicting early out-patient mortality. These groups are likely to benefit from different interventions to prevent mortality.

Chapter 7: Cross-sectional study assessing the use of whole-blood functional assay of phagocyte activity (research paper)



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SECTION A – Student Details

Student	Ankur Gupta-Wright
Principal Supervisor	Prof Liz Corbett
Thesis Title	Investigating mortality risk in hospitalised patients in Africa with HIV-associated tuberculosis and positive urine diagnostics: a clinical, epidemiological and immunological study

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	Frontiers in Immunology		
When was the work published?	28th September 2017		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion			
Have you retained the copyright for the work?*	Yes	Was the work subject to academic peer review?	Yes

**If yes, please attach evidence of retention. If no, or if the work is being included in its published format, please attach evidence of permission from the copyright holder (publisher or other author) to include this work.*

SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	
Please list the paper's authors in the intended authorship order:	
Stage of publication	Choose an item.

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	My role was designing the experiments for optimising the assay and assessing its performance in healthy volunteers and HIV/TB patients, undertaking the experiments, analysing the results and data, and writing the report. Early work on assay
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	development (eg developing and optimising the reporter particles themselves) was done by DT, KCJ and DGR.
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Student Signature: _____

Date: 31st July 2018 _____

Supervisor Signature: _____

Date: 31st July 2018 _____



Functional Analysis of Phagocyte Activity in Whole Blood from HIV/Tuberculosis-Infected Individuals Using a Novel Flow Cytometry-Based Assay

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The accurate assessment of immune competence through *ex vivo* analysis is paramount to our understanding of those immune mechanisms that lead to protection or susceptibility against a broad range of human pathogens. We have developed a flow cytometry-based, whole blood phagocyte functional assay that utilizes the inflammatory inducer zymosan, coupled to OxyBURST-SE, a fluorescent reporter of phagosomal oxidase activity. The assay measures both phagocytic uptake and the superoxide burst in the phagocyte populations in whole blood. We utilized this assay to demonstrate impaired superoxide burst activity in the phagocytes of hospitalized HIV-positive patients with laboratory-confirmed tuberculosis. These data validate the use of the assay to assess the immune competence of patients in a clinical setting. The method is highly reproducible with minimal intraindividual variation and opens opportunities for the rapid assessment of cellular immune competence in peripheral blood in a disease setting.

Keywords: phagocytosis, zymosan, inflammation, monocytes, neutrophils, HIV, tuberculosis, whole blood assay

INTRODUCTION

Bacterial killing assays in whole blood are well established and allow *ex vivo* assessment of immune function in patients, particularly in the context of assessing response to vaccines or evaluating new bactericidal therapies (1–4). The main read out of these assays is microbial killing measured *via* culture and colony counting, or fluorescence if reporter strain organisms are used.

Potential problems of these microbiological killing assays include difficulties in standardizing the number of microbes and their multiplication rate. The tendency of the microbes to aggregate inconsistently during assays may also result in misrepresentation of the actual numbers of microbes measured at the end of the assay. In addition, there are other factors that can result in microbial loss that are not dependent on the host immune response or antimicrobial therapy (5). Finally, because the read out is simply bacterial survival, these assays lack the ability to differentiate mechanisms of killing and the relative contributions of the different phagocyte lineages present in the blood.

Phagocytosis is an important mechanism in the microbial killing pathway of phagocytes. Deficiencies in phagocyte function likely predispose individuals to acquire or succumb to infectious diseases. An extensive range of dynamic assays of phagosome function have been developed that are capable of providing a broad range of physiological readouts from the phagosome (6, 7).

These assays have mostly utilized inert beads derivatized with different fluorescent reporters and focused on human alveolar macrophages or murine bone marrow-derived macrophages in culture (8–10). By removing cells from whole blood or their usual tissue fluid, we are unable to assess the potentially important influence of soluble proteins such as cytokines, chemokines, or antibodies on phagocytosis and phagosomal behavior. We therefore sought to develop an assay using a reporter particle more suitable for probing phagocyte biology in whole blood. The assay is designed to provide reproducible, unbiased, real-time analysis of phagosomal function of immune cells and potentially identify patients with impaired immune responses.

We utilized zymosan derivatized with the oxidation-sensitive fluorescent reporter, OxyBURST-SE, to quantify phagosomal oxidase activity in peripheral blood phagocytes *in situ*. Zymosan is a preparation of a cell wall glucan from *Saccharomyces cerevisiae* that has been used as a model microbial particle in immune assays for over half a century (11). Zymosan is highly mannosylated and linked to β -glucan, making it susceptible to phagocytosis by monocytes, polymorphonuclear leukocytes, and macrophages through various receptors, including C-type lectin receptors such as dectin-1 and mannose receptors (12, 13). Phagocytosis of zymosan can occur independent of opsonization, of which complement factor 3 (C3) predominates with immunoglobulin G (IgG) being of minor importance (14). Zymosan also stimulates an inflammatory cytokine response *via* toll-like receptors (TLR) 2 and 6, although activation of these receptors is not required for internalization by phagocytes (12). We had demonstrated previously how inert particles coupled to OxyBURST-SE can be used to quantify the superoxide burst of murine macrophages *in vitro* (15).

Superoxide burst is one of the key enzymatic activities involved in killing microbes during the process of phagocytosis. The generation of oxygen radicals *via* nicotinamide adenine dinucleotide phosphate (NADPH) oxidase leads to the production of noxious compounds such as hydrogen peroxide with potent antimicrobial activity (16, 17). Superoxide burst's importance is clearly demonstrated by the greatly increased risk of bacterial, fungal, and mycobacterial infection in patient with chronic granulomatous disease due to mutations in NADPH oxidase (18). It has also been shown to be suppressed in individuals with HIV infection (19) and by *Mycobacterium tuberculosis* (TB) infection *in vitro* (20).

In this study, we report the application of this novel reporter platform to quantify the phagocytic and superoxide burst functions of phagocytes in whole blood obtained from individuals in a clinical setting. First, we detail the information generated by application of the assay in whole blood from healthy controls. We then present data showing the utility of this assay in demonstrating the perturbation of phagocyte function in the blood from HIV- and TB-coinfected patients in Malawi.

MATERIALS AND METHODS

Study Population

Adult patients with HIV and tuberculosis coinfection (HIV-TB) were recruited as part of a sub-study examining immune responses

in the Malawi arm of the rapid urine-based screening for TB to reduce AIDS-related mortality in hospitalized patients in Africa (STAMP) (21). Healthy HIV-negative adults with no evidence of active TB were also recruited as controls. 5 ml of blood was collected from both patients and controls in sodium heparin tubes. All samples were processed and analyzed by flow cytometry at the Malawi-Liverpool-Wellcome Trust Clinical Research Programme in Blantyre, Malawi within 2 h of blood draw. The study has been approved by the London School of Hygiene & Tropical Medicine Research Ethics Committee and the College of Medicine Research Ethics Committee, Malawi.

Zymosan Reporter Particles

To quantify both phagocytic activity and the magnitude of the superoxide burst we utilized zymosan particles coupled to both a calibration fluorochrome (Alexa Fluor 405-SE, Invitrogen) and an oxidation-sensitive fluorescent reporter (OxyBURST® Green H₂DCFDA-SE, Invitrogen). Zymosan reporter particles were prepared by washing 6 mg of zymosan (Sigma-Aldrich) three times in 1× phosphate-buffered saline (PBS) by centrifugation at 10,000 rpm for 1 min. Particles were resuspended in 950 μ l coupling buffer (0.1 M boric acid to pH 8.0 with NaOH) containing 10 μ l of 25 mg/ml OxyBURST-SE/DMSO stock solution and 5 μ l of 5 mg/ml Alexa Fluor 405-SE/DMSO solution. The particles were mixed well and incubated on a tube rocker in the dark for 1 h at room temperature and washed with 1 ml of coupling buffer. The 1 h coupling with OxyBURST-SE and calibration fluorochrome was repeated twice. Finally, particles were washed three times with PBS and stored in 1 ml of PBS containing 0.01% sodium azide in the dark at 4°C generating a final stock concentration of approximately 5×10^6 particles/ml.

Whole Blood Assay

Zymosan reporter particles were prepared for the whole blood assay by washing 50 μ l of stock zymosan particle suspensions three times with 1 ml of RPMI-1640 to remove sodium azide and resuspended in 250 μ l RPMI-1640 to give a 1:6 dilution and a final concentration of approximately 8×10^5 particles/ml.

Whole blood was diluted 1:1 with warm RPMI-1640. 20 μ l of washed and diluted reporter particles (containing approximately 2×10^4 particles) were added to 1 ml of diluted blood and incubated at 37°C with rocking to ensure particles and cells remain in suspension. Diluted blood without zymosan reporter particles was also processed in parallel as control. Phagocytosis of zymosan reporter particles and superoxide burst was assessed at 10, 30, 60, 90, and 180 min after the addition of reporter particles.

100 μ l of diluted blood was harvested from the zymosan reporter and biological control tubes 10 min before each time point for cell surface staining (as phagocytosis continues during cell surface staining of live cells). Once harvested, the diluted blood was stained with appropriately titrated concentrations of antibodies (anti-CD45 PerCP 1:33, anti-CD66b APC 1:50, and anti-CD14 PE-Cy7 1:100; all from BioLegend) for 10 min. Biological activity was arrested, red blood cells were lysed, and leukocytes fixed by adding 3 ml of BD FACS lysing solution (BD Biosciences), containing formaldehyde and diethylene glycol, to each tube and incubating at room temperature for 10 min.

The cells were washed once with 1× PBS by centrifugation at 500 g for 10 min then resuspended in 500 µl 1× PBS. Counting beads (CountBright, Life Technologies) were added per the manufacturer's instruction before acquisition on a CyAn ADP flow cytometer (Beckman Coulter, USA). The phagocytosis assay was performed in triplicate on the whole blood samples from healthy, HIV-negative adults. Data were analyzed using FlowJo version 10 (Treestar, USA).

In addition to the zymosan reporter assay, for HIV/TB-coinfected patients, immunophenotyping of monocytes in fresh whole blood was undertaken to investigate the association between monocyte phenotype and phagocytosis. In brief, 100 µl of fresh whole blood was stained with anti-CD45 Pacific Orange (Invitrogen), anti-HLA-DR PE-Cy7, anti-CD14 PE, and anti-CD16 FITC (all from BioLegend) for 10 min. Red blood cells were lysed, and leukocytes fixed with BD FACS lysing solution, washed once with 1× PBS by centrifugation at 500 g for 10 min then resuspended in 300 µl PBS for flow cytometry acquisition.

Electron Microscopy (EM)

In parallel, 2 ml of whole blood from a healthy HIV-negative control was incubated with approximately 8×10^4 zymosan reporter particles to confirm the zymosan particles were internalized by whole blood phagocytes. White blood cells were harvested after 10, 60, and 180 min by centrifugation at 500 g for 10 min and carefully pipetting out the buffy coat layer in buffered glutaraldehyde fixative solution (2.5% glutaraldehyde in 0.1 M sodium cacodylate, 5 mM CaCl₂, 5 mM MgCl₂, 0.1 M sucrose, pH 7.2). The samples were processed and stained for EM as described previously (22).

Calculations and Statistical Analysis

The proportion of cells that had phagocytosed reporter particles was calculated based on expression of calibration fluorochrome, and absolute cell numbers calculated using counting beads. An "activity index" of phagocytosis and superoxide burst was calculated by subtracting the median fluorescence intensity of the negative cells from the positive cells, and dividing this by two times the robust SD of the negative cells (23). This method accounted for variations in auto fluorescence between cells from different individuals.

Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, USA) and Stata 11 (StataCorp, USA). Peak activity index (AI) was calculated and mean AI was compared between groups. The AI at each time point was also used to calculate the area under the curve. Means were compared using paired *t*-tests and median using Wilcoxon rank-sum.

RESULTS/DISCUSSION

Zymosan Uptake by Whole Blood Phagocytes

We used whole blood from four healthy HIV-negative controls to measure phagocytosis and superoxide burst of phagocytes *ex vivo* using zymosan-reporter particles. We first sought to determine the kinetics of zymosan uptake by whole blood phagocytes.

The flow cytometry gating strategy to identify neutrophils and monocytes is outlined in **Figure 1**. Cells that had phagocytosed zymosan-reporter particles were identified and quantified through measurement of the calibration fluor, Alexa Fluor 405.

Zymosan particles were avidly internalized by both neutrophils and monocytes in blood from healthy controls. Uptake was rapid, with a mean of 26% of neutrophils phagocytosing the particles compared with 12% of monocytes by 30 min (**Figure 2A**). The proportion of neutrophils phagocytosing zymosan did not increase substantially between 30 and 180 min, whereas the percentage of monocytes associated with zymosan-reporter particles increased gradually during the assay. This pattern of uptake was consistent across all healthy controls.

The uptake of zymosan reporter particles by both monocytes and neutrophils is dose dependent as shown in the dose-response curve generated for 0.5×10^4 – 8×10^4 zymosan particles/ml (**Figure 2B**). The abundance of the phagocytic cells in whole blood also influences the overall proportion of cells phagocytosing zymosan particles (**Figure 2C**). The higher the concentration of cells, the lower the proportion of cells carrying the zymosan-reporter signal, shown for both neutrophils and monocytes (**Figures 2D,E**). This relationship persists throughout the assay and demonstrates the importance of the phagocyte to particle ratio in the kinetics of phagocytosis. Relying solely on internalization of particles to assess phagocytic function is a potential limitation of the assay, as the magnitude of phagocytosis may be influenced by a function of cell concentration and/or cell to particle ratio, rather than cellular deficiencies in phagocytic capacity.

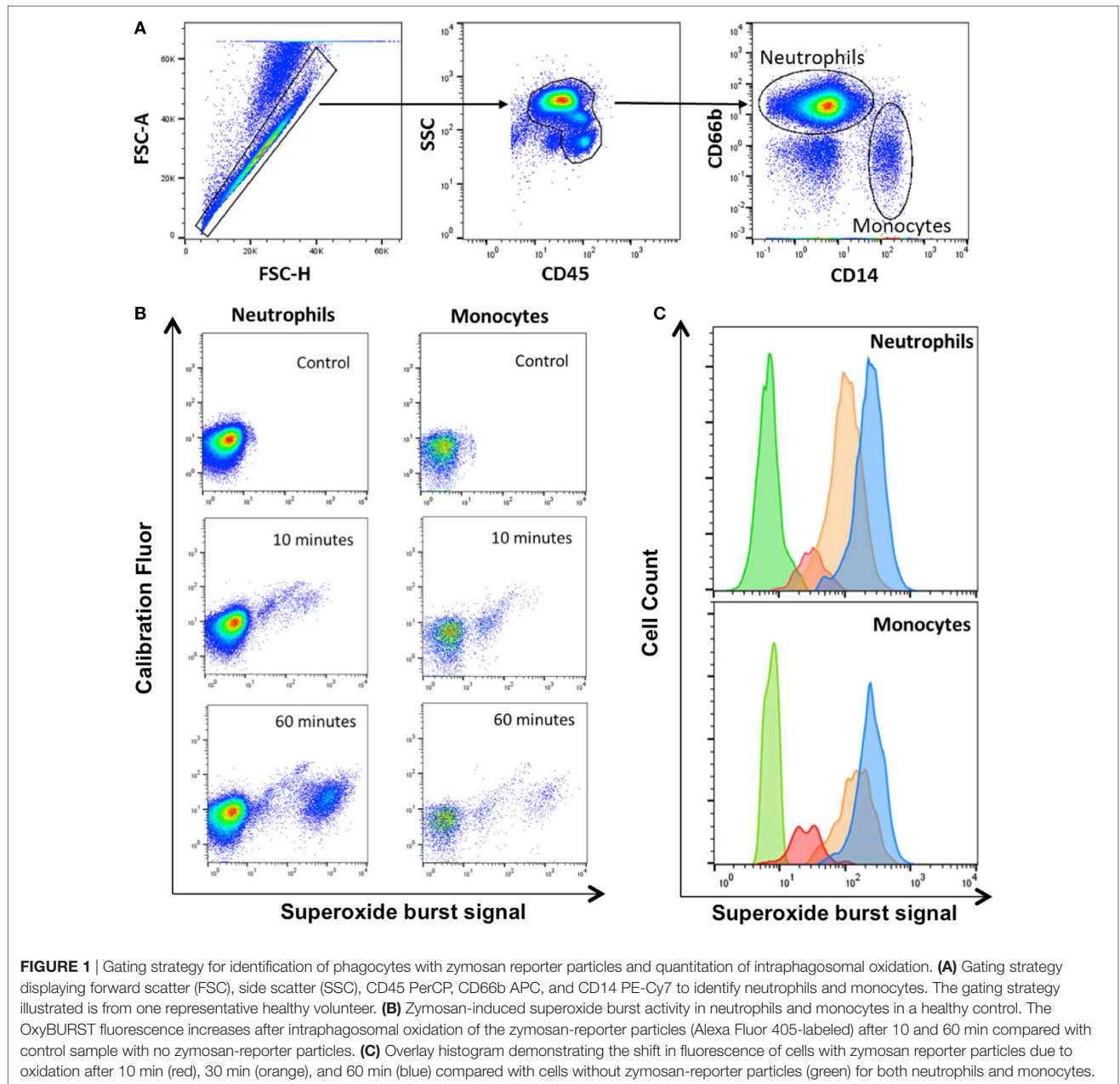
Electron microscopy of white blood cells from a healthy control whole blood incubated with zymosan particles demonstrates phagocytosis of zymosan particles by peripheral blood phagocytes (**Figure 3**). The EM images support the assumption that the zymosan reporter signal detected by flow cytometry originates from phagocytosis rather than the association of zymosan particles with the phagocyte surface. Almost without exception, the zymosan particles were observed inside the phagocyte.

Cell Loss Associated With Zymosan

To examine the effect of the zymosan particles on cell loss, we compared the samples containing zymosan reporter particles and control samples from the same healthy individuals. The mean concentration of neutrophils and monocytes declined during the assay more rapidly in the presence of zymosan than in control samples, with the largest decline occurring between 90 and 180 min (**Figures 4A,B**).

Furthermore, the concentration of neutrophils associated with zymosan-reporter signal peaked at 60 min, followed by a decline (**Figure 4A**). By contrast, the concentration of zymosan-associated monocytes plateaus at 30 min (**Figure 4B**). However, in both cell types the peak in zymosan uptake coincided with cell loss, suggesting that zymosan plays a role in inducing cell death. This is also supported by increased cell loss at higher concentrations of zymosan in the assay (**Figure 2B**).

These observations are consistent with neutrophil and monocyte biology. Neutrophils are known to have a short half-life *in vitro*, estimated to be 6–12 h, and do not proliferate (24). Programmed cell death of neutrophils occurs rapidly following

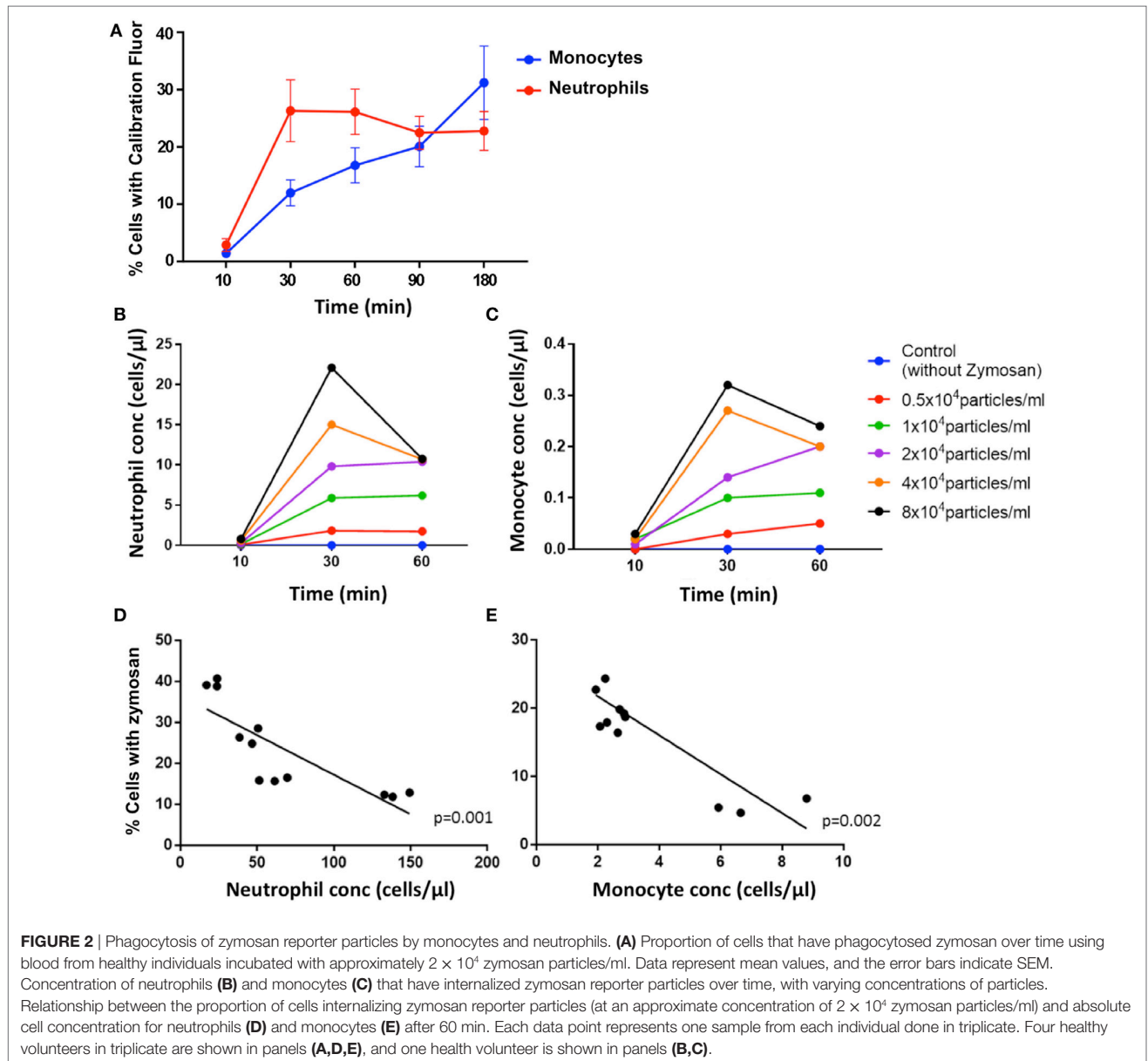


phagocytosis of inflammatory particles, and reactive oxygen species may be important triggers for induction of apoptosis (20). In neutrophils that have not phagocytosed zymosan particles, activation *via* direct binding of zymosan to TLR2 and TLR6 or in response to inflammatory cytokine and chemokine production may also contribute to cell death (12). By contrast, monocytes have an estimated half-life of <20 h *in vivo*, although this may be shorter *ex vivo* (25). Monocytes also undergo programmed cell death, unless they migrate to tissues and undergo differentiation into tissue macrophages (26). However, in contrast to neutrophils, inflammatory cytokine production and stimulation *via* TLR2 can promote survival by blocking programmed cell death (27). This

may explain why monocytes that had phagocytosed zymosan reporter particles did not substantially decrease in number during the assay.

Phagocytosis and Superoxide Burst

Superoxide burst activity was measured at 10, 30, 60, 90, and 180 min by comparing fluorescence of cells that had internalized zymosan reporter particles (calibration fluor-positive cells) with the cell population without zymosan (calibration fluor-negative cells) through measurement of the OxyBURST, superoxide sensor signal. The proportion of cells and intensity of superoxide reporter fluorescence increased over the time course of the assay in both



monocytes and neutrophils (Figures 1B,C). The intensity of the calibration fluorochrome signal did not increase over time, suggesting the increase in superoxide reporter signal was not due to cells internalizing greater numbers of zymosan-reporter particles but was specific to the oxidase activity (Figure 1B).

Both peripheral blood monocytes and neutrophils showed rapid oxidation within 30–60 min (Figures 4C,D). The kinetics of oxidation in neutrophils and monocytes were similar to macrophages in other studies, with rapid oxidation before an equilibrium being reached, which likely represents cessation of NADPH oxidase activity (6, 15).

When the concentration of zymosan reporter particles was varied, the AI remained constant despite the concentration and proportion of cells taking up zymosan changing. This indicates

the assay is able to measure physiological changes in the intensity and duration of phagocytosis and superoxide burst within the phagosome at an individual cell level. This is a significant advance over existing assays, which measure the extracellular accumulation of products of oxidation that is dependent on the summation of phagocytosis and superoxide burst (28). Moreover, because this assay has cellular resolution, the relative contribution of the different phagocyte subsets can be accurately measured. We have also demonstrated that the assay is reproducible with minimal intraindividual variation.

Assays using OxyBURST coupled to IgG coated beads have previously been used to investigate oxidation within macrophage phagosomes (6, 15), and more recently in whole blood (29). The current assay exploiting zymosan as a reporter particle is an

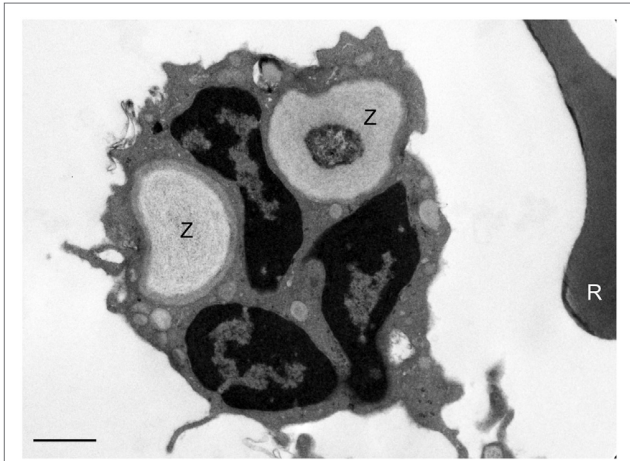
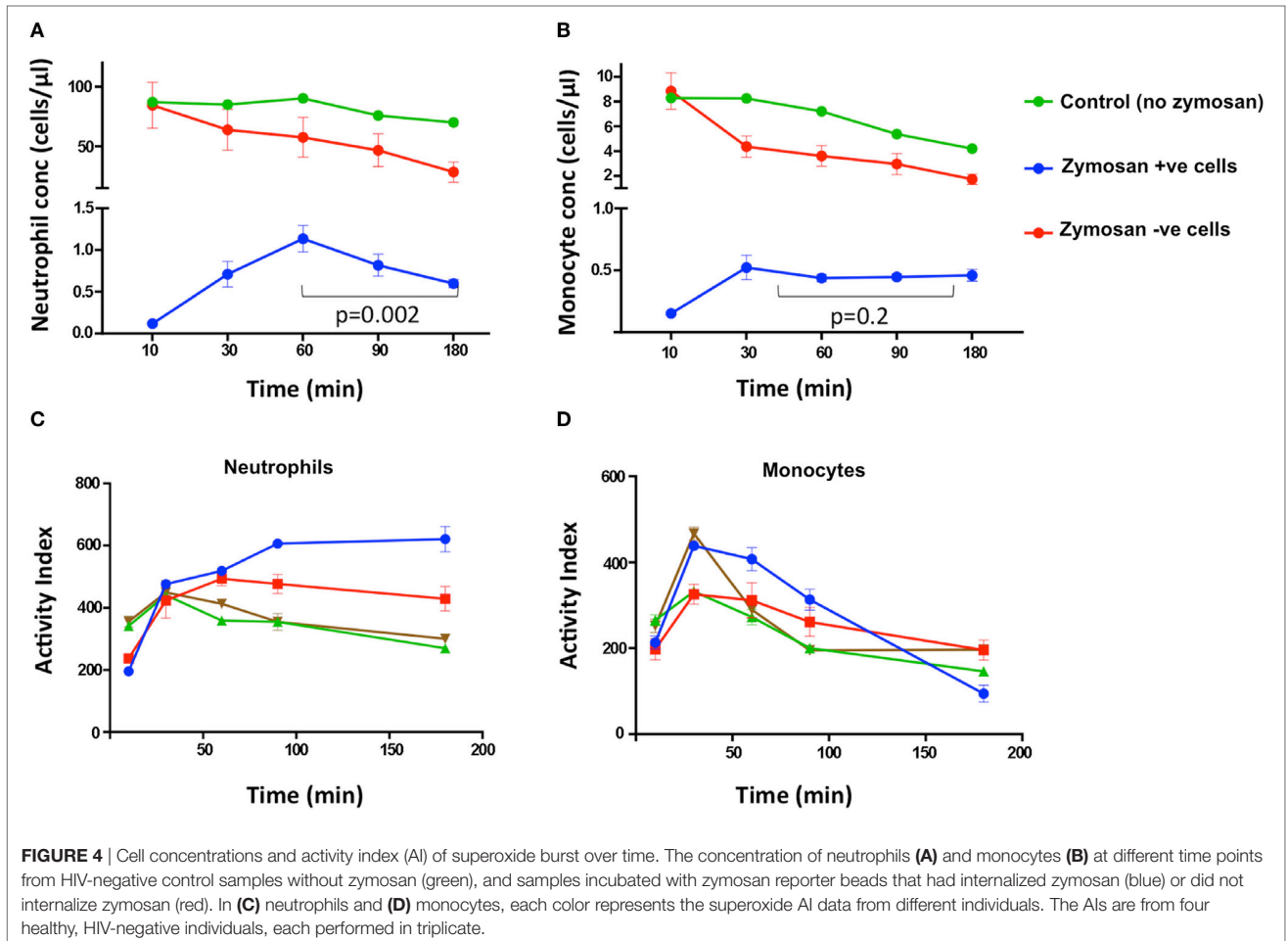


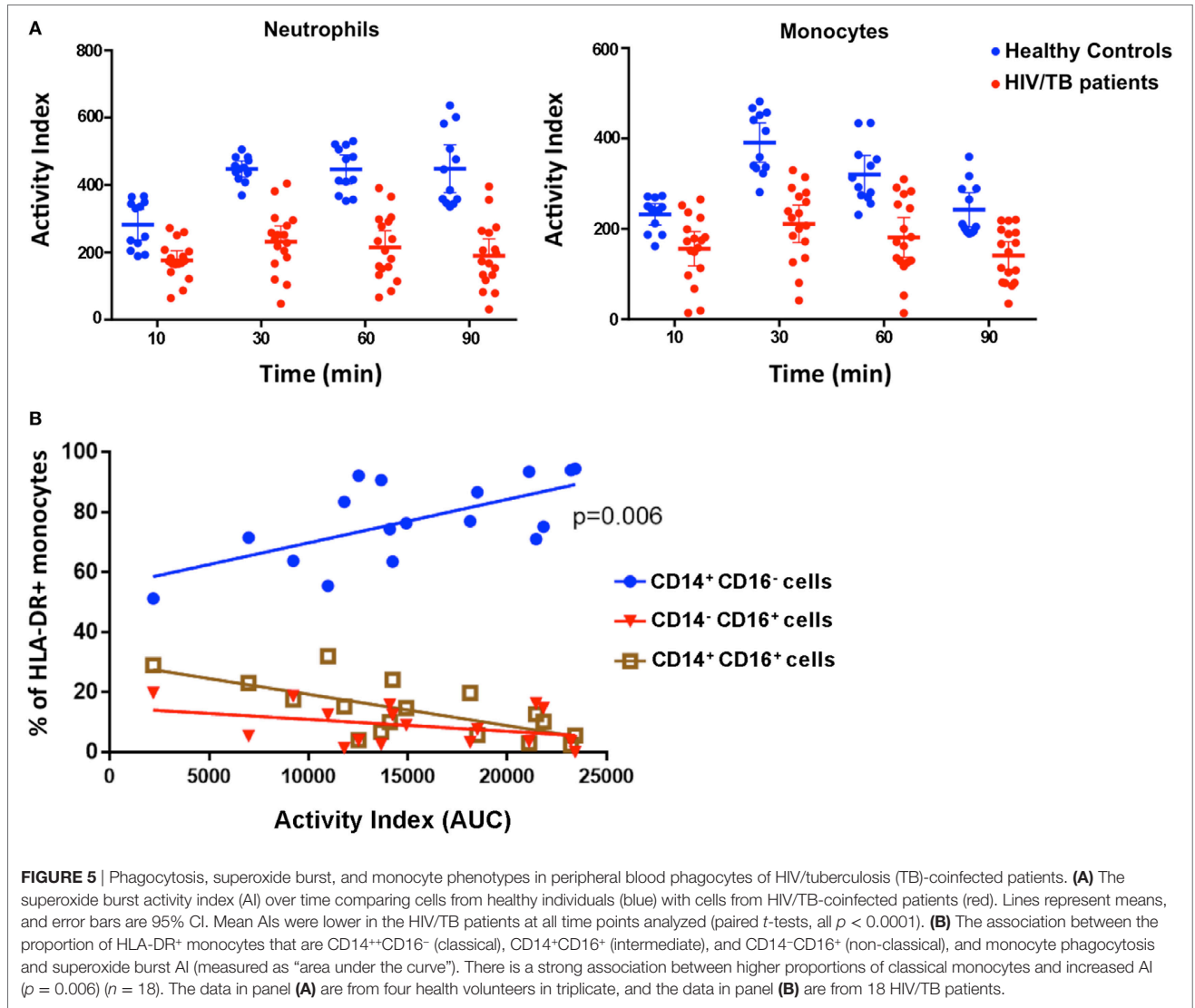
FIGURE 3 | Assessment of phagocytosis of zymosan reporter particles by electron microscopy. An electron micrograph illustrating zymosan particles (Z) inside a neutrophil 60 min post incubation of the reporter particles with whole blood. A red blood cell (R) can be seen to the right of the neutrophil. This image is representative and indicates that the zymosan particles are effectively internalized by cells in suspension. The scale bar = 1 μ m.

important addition to the range of phagocyte functional assays and offers considerable practical and technical advantages in the functional interrogation of whole blood direct from human subjects of interest.

Assessment of Whole Blood Phagocyte Function in Patients with HIV/TB Coinfection

The zymosan reporter assay was performed on blood samples obtained from 18 hospitalized HIV-positive patients with laboratory confirmed TB disease to compare phagocytic and superoxide respiratory burst activity in the phagosome between patients and healthy, HIV-negative, controls. The HIV/TB patients had a mean age of 41.4 years, a median CD4 cell count of 108.5 cells/mm³ and 13/18 were taking antiretroviral therapy at the point of hospital admission. The HIV/TB-coinfected patients demonstrated marked variation in phagosomal oxidation activity compared with healthy controls with peak activity occurring at 30 min, although overall mean intensity of superoxide burst was significantly reduced throughout the assay (paired *t*-test, all *p* < 0.0001) (Figure 5A).





There was also a strong association between increased monocyte superoxide burst activity and the presence of a higher proportion of “classical” CD14⁺CD16⁻ monocytes (Figure 5B, linear regression coefficient 0.0014, 95% CI 0.0005–0.0024, *p* = 0.006). This association is consistent with the suggestion that classical monocytes are thought to specialize in phagocytosis compared with other monocyte subsets (30). However, the superoxide activity in monocytes was not related to the overall concentration of monocytes in patient’s blood (linear regression slope 0.0002, 95% CI –0.0002 to 0.0005, *p* = 0.19), supporting the contention that the superoxide AI was not simply a function of phagocyte abundance.

These data demonstrate that the whole blood assay with zymosan reporter particles is a robust tool for assessing phagocyte function in a clinical setting. The time required to run the assay once the reporter particles have been made is minimal, with the processing of the sample through to acquisition by flow

cytometry taking less than 4 h. We also demonstrated this assay can show marked differences between individuals and groups of patients based on clinical phenotype. It is interesting to note that the reduced superoxide burst in the phagocytes from HIV/TB-coinfecting individuals observed in this study is consistent with a recent report of impaired innate immune function of monocytes from HIV/TB-coinfecting patient cohort in South Africa (31).

CONCLUDING REMARKS

We present a new method for studying whole blood phagocyte functional capacity *ex vivo*. This technique uses fluorescent-tagged zymosan-reporter particles and whole blood, preserving, at least in part, the physiological *in vivo* conditions. It offers several advantages over standard microbiological killing assays because of its speed and simplicity, and its increased resolution whereby

cellular responses such as phagocytic capacity and superoxide burst, can be quantified at the level of the individual cell.

We have demonstrated that the assay can be used to characterize immune function and to detect perturbation of cellular function in patients with severe immunological impairment (in HIV/TB-coinfected individuals). This assay is easily adaptable to standard immunological assays based on cell surface marker expression measured by flow cytometry and has the capacity to provide direct functional readouts of immune cell activities. Previously, we have used inert reporter particles to measure rates of phagosomal acidification, intraphagosomal proteolytic and lipolytic activities, as well as superoxide burst in tissue macrophages in culture. These activities are differentially modulated by immune status and infection (7–9). The use of zymosan as an alternative, biologically active carrier particle for whole blood-based assays brings these complex biological readouts into a clinical setting for functional interrogation of patient-derived samples linked to disease status.

ETHICS STATEMENT

The study was carried out in accordance with the recommendations from the London School of Hygiene & Tropical Medicine

Research Ethics Committee and the College of Medicine Research Ethics Committee, Malawi, with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

All the authors contributed to the analysis and interpretation of data and preparation of the manuscript. All the authors have approved the final article.

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

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Revision to research paper

The analysis comparing the concentration of phagocytic cells with the proportion of cells taking up zymosan (Figure 2D, E) did not account for clustering of data due to observations being repeated in triplicate for individuals. Therefore, the linear regression analysis was repeated accounting for clustering amongst the same individuals. The association between concentration of phagocytic cells and proportion of cells taking up zymosan reporter particles remained, albeit the associations were less robust ($p=0.049$ for monocytes and $p=0.046$ for neutrophils).

**Chapter 8. Soluble and cell surface markers of
immune response in hospitalised patients with
HIV-associated TB**

Chapter 8. Soluble and cell surface markers of immune response in hospitalised patients with HIV-associated TB

Summary

Understanding whether patients with poor outcomes have altered immune responses can help inform urgently needed strategies to reduce the high mortality from HIV/TB. Therefore I sought to measure cytokines, chemokines and markers of inflammation in plasma, as well as cell immunophenotype and activation, in patients with HIV-associated TB (HIV/TB) and compare patients who died with those who survived. I also compared HIV/TB patients with and without positive urine-diagnostic tests (a marker of disseminated TB). Patients with poor outcomes had higher concentrations of cytokines responsible for innate and pro-inflammatory signalling, and markers of inflammation such as CRP and hepcidin. They also had evidence of more marked monocyte activation and non-classical monocyte phenotype. There were no differences between HIV/TB patients with positive and negative urine diagnostic tests.

8.1 Introduction (chapters 8 and 9)

Despite sustained public health efforts, HIV-associated TB (HIV/TB) remains a leading cause of morbidity and mortality in sub-Saharan Africa. HIV/TB is the most common reason for admission to hospital, and is estimated to cause around one in four HIV-associated deaths [1,2]. This high mortality persists despite starting appropriate therapy for both HIV and TB, and the mechanisms of death are not clearly defined. Post-mortem studies demonstrate that disseminated TB is common [3], as are other bacterial and/or opportunistic infections [4,5], all of which are likely to contribute to poor outcomes, and may reflect a failure of host immune responses to control infection.

Few studies have characterised and compared immune responses in HIV/TB patients with differing clinical outcomes. There is some evidence of innate immune activation with inflammation characterised by raised inflammatory biomarkers and plasma or serum cytokines involved in the innate immune response (eg IL-6 and TNF α) in patients who died compared to survivors [6]. However, studies examining immune responses to stimulation have also found hypo-responsiveness and dysfunction, particularly of monocytes [7,8].

This type of intense immune activation accompanied by dysfunction, dysregulation and subsequent 'immune suppression' has been described in acute bacterial sepsis, and associated with mortality [9]. In HIV/TB co-infection, immune responses may be also be further perturbed

by the presence of HIV, which is only in part restored by antiretroviral therapy (ART) [10]. I therefore hypothesised that HIV/TB patients with poor outcomes have high levels of inflammatory cytokines and biomarkers (chapter 8), yet impaired functional responses indicative of immunosuppression (presented in chapter 9).

I was able to identify patients with disseminated disease by detecting nucleic acids of *Mycobacterium tuberculosis* complex (MTB, using the Xpert MTB/RIF assay), or lipoarabinomannan (LAM, using TB-LAM lateral flow assays) in urine, a sign of haematogenously disseminated renal TB [11,12]. Given that these patients have a higher mycobacterial burden, and poorer outcomes [13,14], I also sought to compare immune responses in patients with and without disseminated disease, hypothesising that dissemination would also be associated with similar immunological profiles to sepsis.

Understanding whether patients with poor outcomes have altered immune responses can help inform urgently needed strategies to reduce the high mortality from HIV/TB, for example host-directed or immune-modulatory treatments. Furthermore, simple rapid point-of-care urinary tests may help identify patients who may benefit from such adjunctive interventions.

8.2 Aims (chapters 8 and 9)

The overall aim of these investigations was to characterise immune responses in patients with microbiologically confirmed HIV/TB, and assess associations with mortality and/or disseminated TB.

Specific aims were to characterise the following aspects of the peripheral (whole blood) immune response and compare them between HIV/TB patients by outcome (died or survived), and evidence of urine diagnostic test-positive disseminated disease (see table 8.1):

1. Cytokine, chemokine and inflammation soluble biomarker concentrations (both pro- and anti-inflammatory) and networks (chapter 8)
2. Immune activation markers, and markers of exhaustion or senescence (chapter 8)
3. Functional responses of the innate (phagocytosis and superoxide burst) and adaptive (T-cell cytokine production following stimulation) immune systems (chapter 9)

Secondarily, I aimed to compare HIV/TB immune responses to those of hospitalised HIV-positive (control) patients without evidence of TB to understand the contribution of TB to immune dysfunction. Finally, I sought to use the above immunological parameters to determine whether particular immune signatures were independently associated with mortality and/or disseminated disease (chapter 9).

Immune response	Analysis of cell population and/or features	Assay methods	Results
Pro-inflammatory signalling			
Innate	Plasma IL-1, IL-6, IL-8, IL-12p70, IFN- γ , TNF- α , MIP-1, MCP-1, IP-10	Multiplex-bead assays	<i>Table 8.7 and 8.8; Figure 8.6 and 8.7</i>
Adaptive	Plasma IL-2, IL-4, IL-13, IL-17A, IL-22		
Anti-inflammatory signalling	Plasma IL-10, IL1-RA	Multiplex-bead assays	<i>Table 8.7 and 8.8; Figure 8.6</i>
Inflammation markers	C-reactive protein, hepcidin	ELISA	<i>Table 8.3; Figure 8.8</i>
Immune activation			
Innate	Monocyte HLA-DR, monocyte phenotype, sCD14, sCD163, Neutrophil CD66b and Cd11b expression	Flow cytometry immunophenotyping	<i>Table 8.9; Figure 8.8</i>
Adaptive	T-cell HLA-DR and CD38 expression		<i>Table 8.10</i>
Immune exhaustion	T-cell PD-1 and CD57 expression	Flow cytometry immunophenotyping	<i>Table 8.10</i>
Immune cell function			
Innate	Phagocytosis and superoxide burst	Whole blood zymosan phagocytosis assay	<i>Figure 9.2 to 9.4; Table 9.1</i>
	Monocyte cytokine response to stimulation	Intracellular cytokine stimulation assays	
Adaptive	T-cell response to stimulation	Intracellular cytokine stimulation assays	<i>Table 9.2 and 9.3; Figure 9.8-9.12</i>

Table 8.1 Summary of analysis of immune responses. Abbreviations: IL interleukin, IFN interferon, TNF tissue necrosis factor, MIP macrophage inflammatory protein, MCP monocyte chemoattractant protein, IP interferon-gamma induced protein, ELISA enzyme-linked immunosorbent assay, CD cluster of differentiation, HLA-DR human leucocyte antigen – antigen D related, PD programmed cell-death receptor

8.3 Methods

Study design and participants

The study was a prospective cohort study of patients with HIV-associated TB nested within the STAMP trial at the Zomba Central Hospital site in Malawi. The hospital setting, procedures and eligibility criteria for the STAMP trial have been described previously (see chapters 2 and 3) [15,16].

Participants were eligible for the immunology sub-study if they were enrolled in the STAMP trial and had a laboratory confirmed TB diagnosis (positive with a STAMP study Xpert *MTB*/RIF or TB-LAM test, or a non-study Xpert *MTB*/RIF result or positive mycobacterial culture). Patients who started TB treatment in the absence of a confirmatory diagnostic test were not included. There were no additional inclusion or exclusion criteria. Patients were approached about the immunology sub-study and asked to provide written informed consent to participate. All eligible patients and reasons for not enrolling were documented.

An additional group of 'control' patients without TB disease who were enrolled in the STAMP trial and matched by baseline CD4 cell count to enrolled HIV/TB patients were also recruited. These patients were approached if their STAMP TB screening test results were negative. 'TB-negative' control patients were excluded if they were diagnosed with TB during the study period, or had clinical signs or symptoms of TB at the 56-day follow-up visit.

Immunological laboratory assays

Laboratory procedures have been summarised in figure 8.1.

Flow cytometry

Immunophenotyping assays were undertaken using a CyAn ADP flow cytometer (Beckman Coulter). This flow cytometer has three lasers which excite fluorochromes at the following wavelengths: 488nm (blue laser), 405nm (violet laser) and 640nm (red laser) and has nine detection filter channels. Intracellular cytokine staining assays (chapter 9) were acquired on an LSR Fortessa (BD Biosciences) flow cytometer. This also has three lasers but has eleven detection filter channels.

Antibody staining panels were constructed so that brighter fluorochromes were paired with antibodies to less commonly expressed markers, and markers on the same cell were selected from different lasers to minimise spectral overlap and spill over. Titration of antibody concentrations was first done

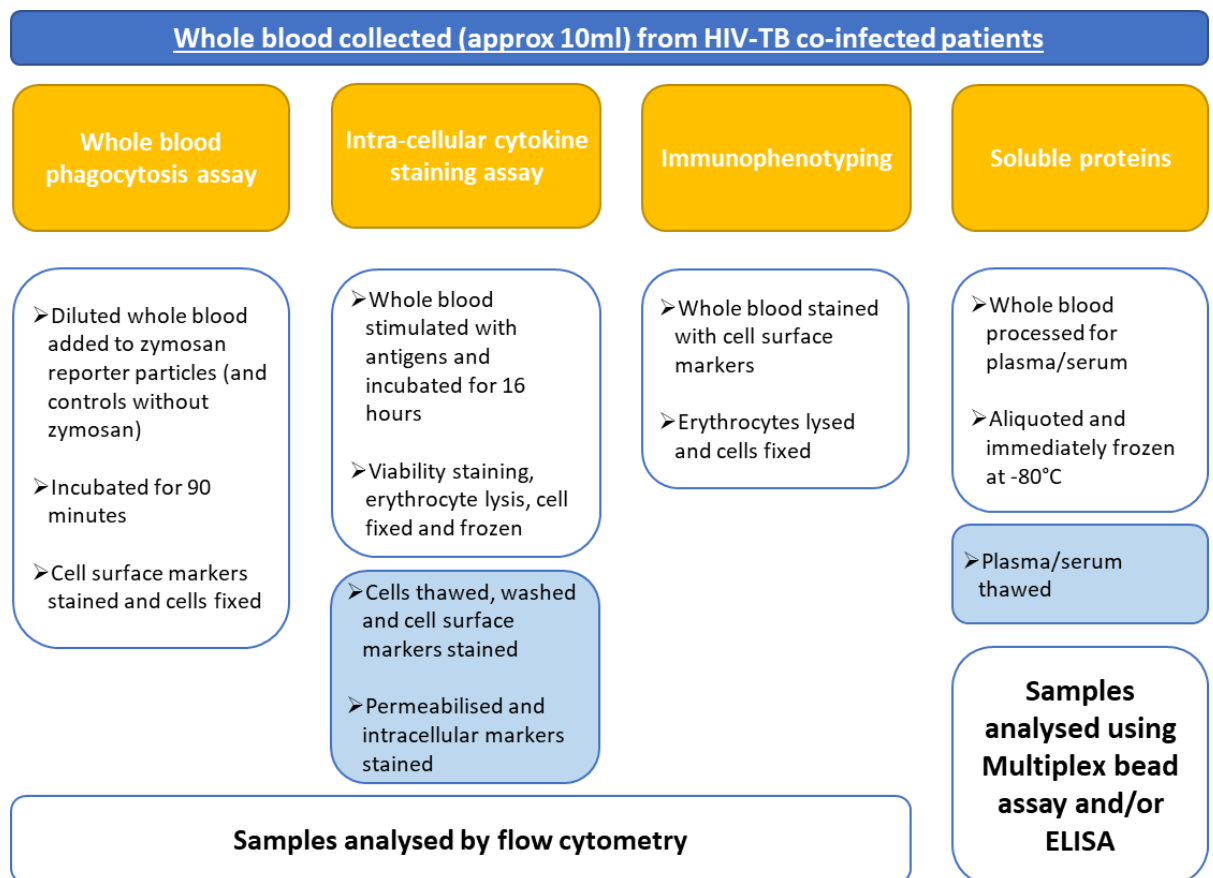


Figure 8.1 Summary of immunological procedures. Procedures in white boxes were done in real time, procedures in blue boxes were done in batches on stored frozen samples.

individually and then tested as part of the panel. The lowest antibody concentrations that gave maximum separation of positive and negative populations were chosen.

Flow cytometers were maintained as per manufacturer's recommendations, and as per local protocols, and including regular cleaning and calibration. Standard protocols for each panel were constructed and used for all experiments. Each day the flow cytometer was used, standardised beads with known fluorescence (8 peak Rainbow Calibration Beads, Sphero for the CyAn and Cytometer Setup & Tracking Beads, BD biosciences, for the LSR Fortessa) were used to adjust and standardise photomultiplier tube (PMT) voltages.

Compensation for spectral overlap was done by acquiring unstained and single-stained samples. Single-stained samples used compensation beads (AbC Total Antibody Compensation Bead Kit, Thermo Fisher Scientific) bound to antibody and with negative controls.

Compensation matrices were automatically generated and applied for each panel by flow cytometry acquisition software (Summit for the CyAn, Beckam Coutler, and FACS Diva for the LSR Fortessa, BD Biosciences). All flow cytometry data were analysed using FlowJo version 10 (TreeStar). For analysis, gating strategies for markers without clear positive and negative

populations were validated using fluorescence minus one (FMOs) experiments, meaning that cells were stained with antibodies except for the one of interest (figure 8.2).

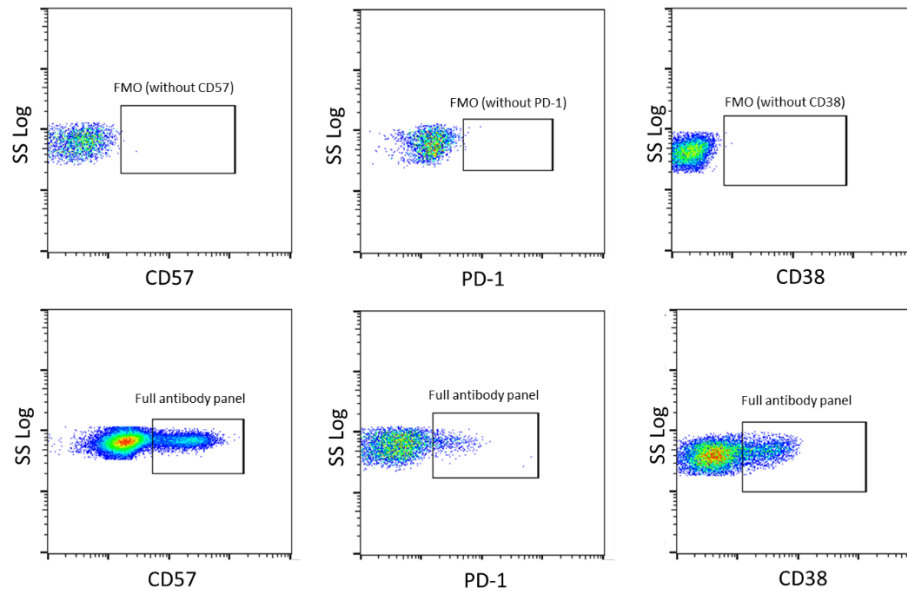


Figure 8.2 Fluorescence minus one control. Top row represents controls without the antibody to the marker of interest, which was used to identify the negative population and draw the gate for the positive population. The bottom row is the full antibody panel. The same results were achieved using internal negative controls. Abbreviations: CD cluster of differentiation, PD programmed cell death, SS side-scatter, FMO fluorescence minus one.

Immunophenotyping assays

Immunophenotyping of monocytes and T-lymphocytes was undertaken using antibody cell surface staining followed by flow cytometry. 200 μ L of fresh heparinised whole blood was incubated with 1:200 concentration of fixable violet viability stain (Thermo Fisher Scientific) for 30 minutes at room temperature, followed by staining with one of two antibody panels (see table 8.2, one panel predominantly for monocytes, the other for T-cells) for 10 minutes. Red blood cells were lysed, and leukocytes fixed with BD FACS lysing solution, washed once with 1 \times PBS by centrifugation at 500 g for 10 min then resuspended in 300 μ l PBS for flow cytometry acquisition.

A separate panel was used to assess exhaustion and senescence of T-lymphocytes. After excluding doublets, dead cells and debris, lymphocytes were identified based on CD45 expression and side-scatter. CD8 and CD4 expressing T-lymphocytes were then assessed for expression of PD-1 and CD57 (figure 8.3C).

Antibody	Fluorochrome	Panel	Concentration	Clone	Company
Viability	405/450	1,2,3,4	1:100		Thermo Fisher Scientific
CD66b	APC	1	1:100	G10F5 IgM	Biolegend
CD14	PE-Cy7	1	2:100	M5E2 IgG2a	Biolegend
CD16	FITC	2	1:100	3G8 IgG1	Biolegend
CD14	PE	2	1:100	M5E2 IgG2a	Biolegend
CD38	PE-Dazzle 594	2	1:100	HIT2 IgG1	Biolegend
HLA-DR	PECy7	2	1:100	L243 IgG1	Biolegend
CD45	Pacific Orange	2,4	1:100	HI30 IgG2a	Invitrogen
CD3	APC-H7	2,3,4	1:100	SK7 IgG1	Biolegend
CD45	PerCP	1,3	3:100	HI30 IgG1	Biolegend
CD8	PE-Dazzle	3,4	1:100	SK1 IgG1	Biolegend
CD4	BV421	3	1:100	SK3 IgG1	Biolegend
CD57	APC	3	2:100	HNK-1 IgM	Biolegend
PD-1/ CD279	PECy7	3	2:100	EH12.2H7 IgG1	Biolegend
CD69	BV510	3	1:100	FN50 IgG1	Biolegend
CD66b	PE	3	1:100	G10F5 IgM	Biolegend
CD11b	AF700	3	1:100	M1/70 IgG2b	Biolegend
CD14	BV605	4	5:100	M5E2 IgG2a	Biolegend
CD4	PerCPCy5.5	4	1:100	SK3 IgG1	Biolegend
IL-22	PE-Cy7	4	5:100	4S.B3 IgG1	eBioscience
IFN- γ	PE	4	5:100	Mab11 IgG1	Biolegend
TNF- α	FITC	4	5:100	MQ2-13A5 IgG1	Biolegend
IL-6	AF700	4	5:100	JES3-9D7 IgG1	eBioscience
IL-10	APC	4	5:100	22URTI IgG1	Biolegend

Table 8.2 Antibodies used for flow cytometry. Panels: 1 was used for phagocytosis assay (chapter 9), 2 was immunophenotyping of monocytes, 3 was immunophenotyping of T-cells, 4 was for intracellular cytokine staining. Abbreviations: CD cluster of differentiation, HLA-DR human leucocyte antigen – antigen D related, IL interleukin, IFN interferon, TNF tissue necrosis factor, PD programmed cell death, APC Allophycocyanin, PE Phycoerythrin, FITC fluorescein isothiocyanate, Cy cyanine, AF Alexa fluor, PerCP peridinin-chlorophyll.

Standardised gating strategies were used for all panels (illustrated in figure 8.3). After excluding doublets, dead cells and debris, CD45⁺ leukocytes were selected. Monocytes were gated based on side-scatter and HLA-DR staining, and then divided into three subsets based on expression of CD14 and CD16 (figure 8.3A)- classical (CD14⁺CD16⁻), intermediate (CD14⁺CD16⁺) and non-classical monocytes (CD14⁻CD16⁺) [17].

Lymphocytes were identified based on CD45 and side-scatter, and then T-lymphocytes gated based on CD3 expression. CD3⁺ T-cells were then assessed for activation based on expression of HLA-DR and CD38 (figure 8.3B), with activated cells defined as expressing HLA-DR or CD38. HLA-DR and CD38 expression can be continuous (rather than binary), therefore gating was

done using CD3⁺ T-lymphocytes (which show distinct positive or negative populations) as internal negative controls.

Plasma cytokines and chemokines

Plasma was harvested from whole blood and immediately frozen at -80°C. I undertook enzyme linked immunosorbent assays (ELISAs) in batches for measurement of plasma concentrations of soluble CD14 (sCD14), soluble CD163 (sCD163, both Quantikine kits, R&D systems) and hepcidin (DRG International) as per manufacturers' instructions. In brief, titration experiments were undertaken to determine the optimal dilution of plasma for each ELISA assay. An automated microplate washer (Biochrom) was used to optimise processing, and plates were read using a microplate reader at 450nm. All samples were processed in duplicate, and standard curves were constructed by generating a four-parameter logistic curve fit.

The levels of 34 cytokines and chemokines (IL-12, IL-23, IL-27, monocyte chemoattractant protein (MCP)-1 (CCL2), regulated on activation, normal T cell expressed and secreted (RANTES, or CCL5), GRO- α (CXCL1), stromal cell-derived factor (SDF)-1 α (CXCL12), interferon- γ -inducible protein (IP)-10 (CXCL10), Eotaxin, granulocyte–macrophage colony-stimulating factor (GM-CSF), IFN- α , IFN- γ , IL-1 α , IL-1 β , IL-1RA, IL-10, IL-13, IL-15, IL-17A, IL-18, IL-2, IL-21, IL-22, IL-31, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, macrophage inflammatory protein (MIP)-1 α , MIP-1 β , TNF- α and TNF- β) were also measured from stored plasma using a magnetic-bead multiplex assay (ProcartaPlex 34-plex Human Cytokine and Chemokine Panel 1A, eBioscience). Samples were tested in duplicate according to the manufacturer's protocols, and reported as concentrations per mL based on standard curves generated by Procarta Analyst v1.0 software (eBioscience). Concentrations below the limit of detection were recorded as half of the lower limit. Analytes were excluded from analysis if not detected in over 50% of the samples tested.

Other laboratory assays

C-reactive protein (CRP) was measured from frozen plasma using the Beckman Coulter AU Analysers by staff at the MLW diagnostics laboratory. HIV-1 viral load was also measured from frozen plasma using HIV-1 Quantitative assay on the GeneXpert platform (Cepheid), with a lower limit of detection of 40 copies/mL. Cryptococcal antigen (CrAg) was measured using the qualitative CrAg lateral flow assay (Immy) as per manufacturer protocol, and LAM in frozen plasma was measured using the Determine TB-LAM assay (Alere, plasma is a non-validated specimen for the TB-LAM assay).

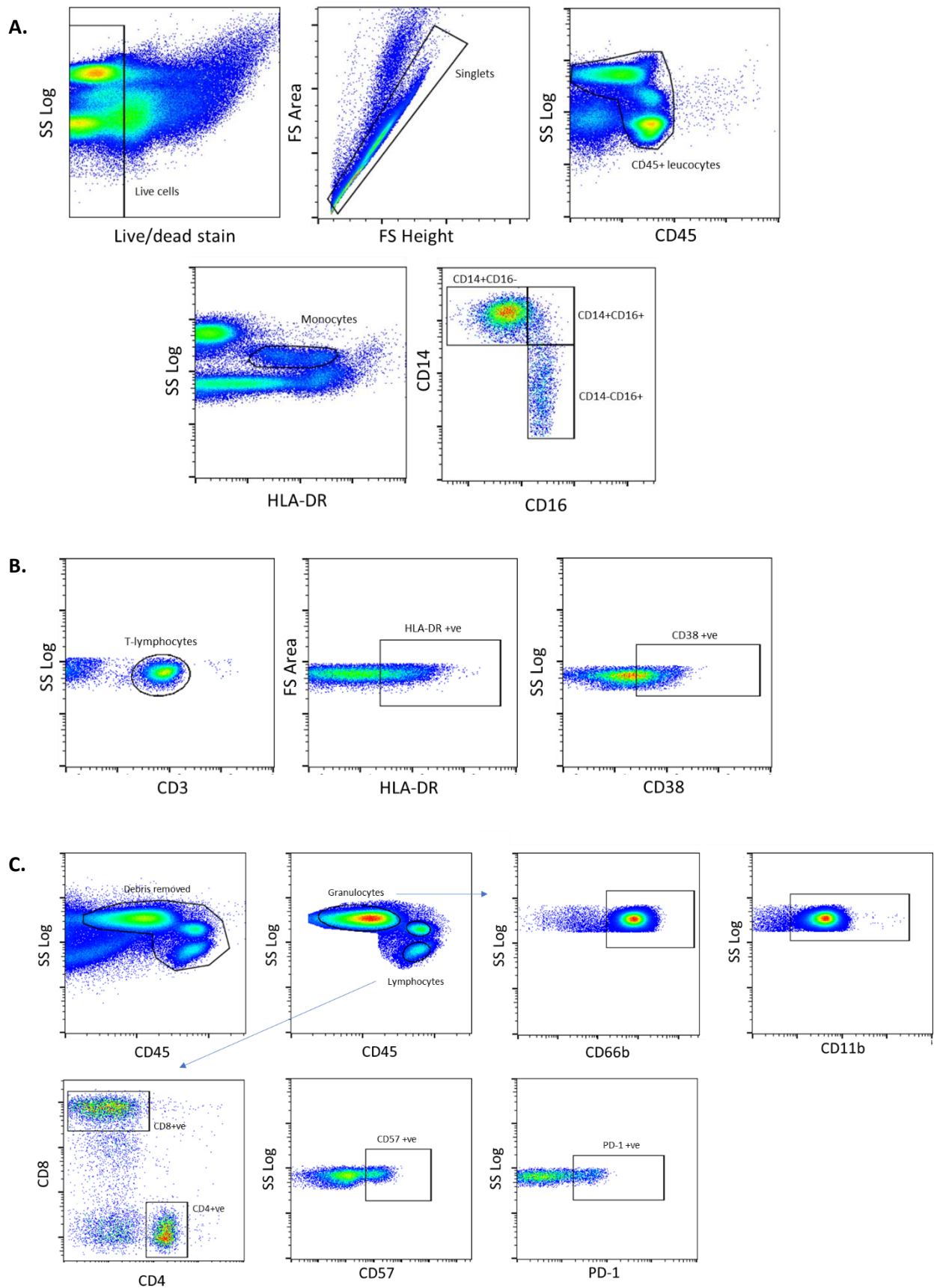


Figure 8.3 Flow cytometry gating strategy for monocyte and T-lymphocyte immunophenotyping. A. Monocytes were identified after exclusion of dead cells, doublets and debris, and after identifying leucocytes based on side-scatter (SS) and CD45 expression. Monocytes were defined as moderate SS and expressing HLA-DR, and then subdivided based on expression of CD14 and CD16. **B.** In the same panel as A, lymphocytes were identified based on low SSC and high CD45 expression, then T-lymphocytes identified based on CD3 expression. Gating for activated T-cells was based on HLA-DR and

CD38. C. In a separate panel, after exclusion of dead cells, doublets and debris, granulocytes were gated on high SSC and lower CD45 expression, and assessed for CD66b and Cd11b. Lymphocytes were identified based on low SSC and high CD45 expression, then CD4 and Cd8 T-cells gated and assessed for senescence based on CD57 and exhaustion based on PD-1 expression. Abbreviations: CD cluster of differentiation, HLA-DR human leucocyte antigen – antigen D related, PD programmed cell death, SS side-scatter, FS forward scatter.

Statistical methods

Preliminary data review was done for all variables with basic descriptive statistics, including graphical displays to identify distributions, spread and maximum and minimum values. Outliers were reviewed for evidence of contamination, operator error or mechanical failure. All analyses were performed based on existing data; it was assumed that any missing data was missing at random and no data imputation was done.

Simple descriptive statistics were done for continuous variables by calculating means or medians, standard deviations and interquartile ranges, and comparing groups using parametric tests (t-test) or non-parametric tests (Wilcoxon rank sum or Kruskal Wallis tests) depending on the distribution. Categorical data were summarised with proportions (and 95% confidence intervals if appropriate), and compared using χ^2 or Fisher's exact tests. All statistical tests were two-sided at α value of 0.05.

For plasma cytokine and soluble protein analysis, mean (and standard deviation, SD) or median (and interquartile range, IQR) concentrations were compared between patients who died and survived, and those with disseminated and non-disseminated disease. P-values were corrected using the Benjamini-Hochberg procedure for multiple-testing, assuming a 10% false-discovery rate. Data were log transformed and Cox regression modelling was also used to calculate hazard ratio (HR) for mortality of plasma cytokines and chemokines, with the HR representing a doubling of concentration. Any variables with >25% missing data were excluded.

Cytokine networks were assessed by constructing pairwise correlation matrices for all cytokines and chemokines using Spearman's rank correlation. The number of correlations for each patient group (controls and HIV/TB patients, HIV/TB patients who died or survived and disseminated and non-disseminated HIV/TB) were reported, and Rho values and p-values for pairwise correlations were displayed using heatmaps for each group.

Analyses were done using Stata version 14 and GraphPad version 7.0.

8.4 Results

Between 22 June 2016 and 28 August 2017, we recruited 66 consecutive laboratory-confirmed HIV/TB patients for the immunology cohort study (figure 8.4). One patient died before blood samples could be taken and was therefore excluded. No other patients were excluded or declined participation. Sixteen HIV-positive control patients enrolled in the STAMP trial with negative TB screening tests and not subsequently diagnosed with TB were also recruited during the same period. None of the control patients had signs or symptoms consistent with TB, nor were diagnosed with TB during the study follow-up.

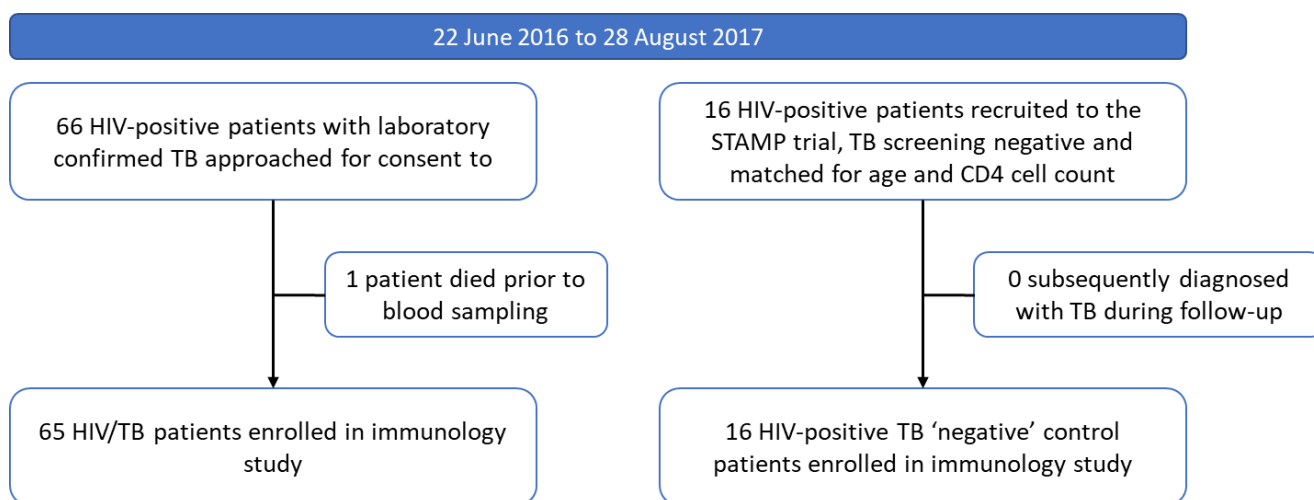


Figure 8.4 Study patient flow diagram

Baseline characteristics

The mean age of HIV/TB patients was 38.0 years and 57% of patients were female (table 8.3). 14% of patients were newly diagnosed with HIV at admission. Of those who knew their HIV status, 88% were currently taking ART for a median duration of 1.1 years (IQR 0.1 to 4.6), 5% were ART-naïve and 7% had interrupted their ART. In those on ART for at least six months (n=27), 44% had viral loads greater than 1000 copies/mL (median 281,500 copies/mL, IQR 31,195 – 590,000). The overall median CD4 cell count was 79 cells/ μ L (IQR 35-194), showing advanced immunosuppression.

One-third (33%) presented with one or more WHO danger signs (heart rate >120 beats per minute, respiratory rate >30 breaths per minute, temperature >39°C or unable to walk unaided), and 26% had sepsis criteria. There was evidence of poor nutritional state: median BMI was 18.0 (57% were underweight), and median MUAC was 20.6 cm. Median CRP was 117 and median haemoglobin was

Characteristic	TB negative control patients n=16	HIV/TB patients				
		All n=65	Disseminated urinary TB		Died n=23	Survived n=42
			Yes n=54	No n=11		
Age , mean, years (SD)	43.6 (15.7)	38.0 (9.4)	38.4 (8.9)	36.4 (11.6)	39.2 (9.8)	37.4 (9.2)
Sex , % female	62.5	56.9	61.1	36.4	43.5	64.3
Cough , % Yes	62.5	69.9	64.8	90.9	65.2	71.4
Length of illness , median weeks (IQR)	1 (0-4)	2 (1-4)	2 (1-4)	2 (1-2)	2 (1-4)	2.0 (0-4)
ART status , %						
New HIV Dx	18.8	13.9	16.7	0.0	17.4	11.9
ART naïve	0.0	4.6	1.9	18.2	0.0	7.1
Currently taking	81.3	75.4	75.9	72.7	78.3	73.8
Interrupted	0.0	6.1	5.6	9.1	4.4	7.1
Time on ART , median, years (IQR)	6.1 (4.9-8.1)	1.1 (0.1-4.7)	1.9 (0.1-6.7)	0.4 (0.0-1.0)	2.2 (0.1-9.1)	1.0 (0.1-4.2)
Weight , median, kg (IQR)	50.0 (41-52)	47.3 (40-52)	47.2 (40-53)	51.0 (38-52)	45.0 (40-50)	46.6 (39-53)
BMI , mean (SD)	19.3 (2.8)	18.0 (3.2)	18.0 (3.2)	17.7 (3.3)	17.8 (2.8)	18.1 (3.4)
MUAC , mean, cm (SD)	21.5 (2.9)	20.6 (3.2)	20.6 (3.1)	20.4 (3.5)	19.8 (2.6)	21.0 (3.4)
Blood Pressure , mean						
SBP	114.8	103.4	102.6	107.6	102.2	104.1
DBP	78.1	70.8	71.0	70.0	69.7	71.3
Heart Rate , mean, bpm	95.0	100.0	99.1	104.0	98.5	100.8
Oxygen saturation , mean, %	95.5	95.0	95.3	94.0	95.2	95.0
Temperature , mean, °C	36.6	36.1	36.6	36.5	36.5	36.7
Respiratory rate , mean, bpm	22.7	23.3	23.2	23.5	23.7	23.0
Karnofsky score , mean	56.0	50.0	50.0	51.0	44.8	52.6
WHO danger signs , % Yes	18.8	33.9	35.2	27.3	39.1	31.0
Sepsis criteria , % Yes	12.5	26.2	27.8	18.2	21.7	28.6
CD4 count , (median, cells/ μ L (IQR))	92.5 (39-207)	79.0 (35-194)	76.5 (29-180)	112.0 (67-243)	37 (22-115)	113 (54-245)
HIV VL , log copies/ml	5.2	3.0	3.0	3.5	3.8	3.1

HIV VL >1000 copies/ml, % Yes	62.5	53.9	51.9	63.6	60.9	50.0
Haemoglobin, mean, g/dL (SD)	8.4 (3.5)	7.9 (2.4)	7.7 (2.5)	9.0 (1.5)	7.6 (2.1)	8.0 (2.6)
CRP, median, g/mL*	33.8	117.1	122.1	74.1	132.7	56.7
CrAg positive, % Yes	0.0	14.8	15.7	10.0	23.8	10

Table 8.3 Baseline characteristics. Numbers are either means, medians or proportions. Median ART duration is only presented for patients currently reporting ART use. *missing CRP values for 12 patients. Abbreviations: ART antiretroviral therapy, BMI body mass index, MUAC mean upper arm circumference, VL viral load, CRP C-reactive protein, CrAg cryptococcal antigen.

7.9 g/dL, with 40% having severe anaemia (haemoglobin <8 g/dL). 28% (18/65) had life-threatening anaemia (haemoglobin <6.5 g/dL). 14.8% of patients were CrAg positive on plasma. Baseline characteristics of the control patients were similar to the HIV/TB patients, although the mean age was slightly older, median time on ART was longer and they were less likely to have WHO danger signs or sepsis at presentation.

TB diagnosis

All patients were able to provide urine, and 60% of patients produced a sputum sample for TB testing. 45% of patients were sputum Xpert positive, 48% were urine Xpert positive and 65% were urine LAM positive (figure 8.3). Disseminated TB was very common, with 83% (54/65) of patients having evidence of urinary TB on either LAM or Xpert testing, and only 17% having non-disseminated TB. Median time from admission to TB treatment was 2 days (IQR 2-3 days).

18 (28%) of 65 patients with TB were positive on urine and sputum diagnostics, 10 (15%) of patients were positive on all three tests and 26 (40%) patients were only positive on urine tests (figure 8.5). 34 (52%) of patients were positive on one urine diagnostic test (urine TB score = 1), and 20 patients (31%) were positive on both urine test (urine TB score =2). Of those who were urine LAM positive, 51% were grade 1 positive, 16% were grade 2 positive and 33% were grade 3 or more positive. Patients with disseminated TB were more likely to be female, have a lower median CD4 cell count and higher CRP than those without dissemination.

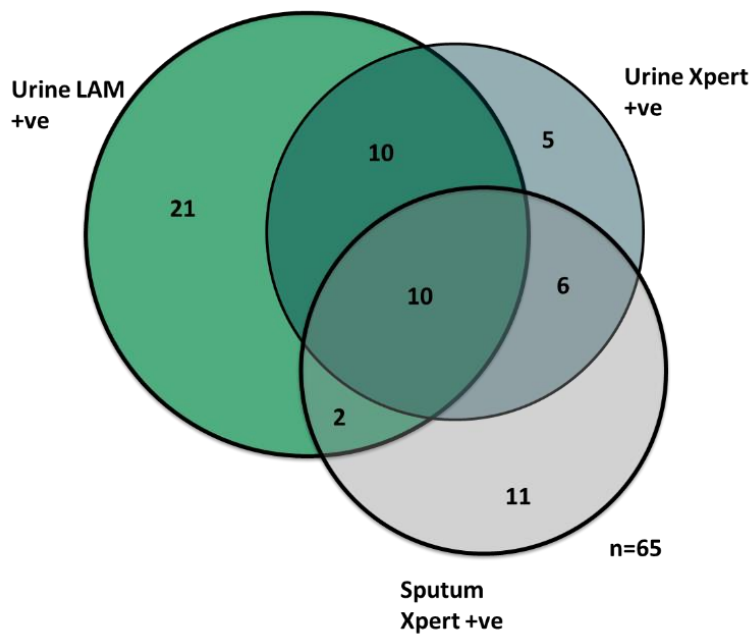


Figure 8.5 HIV/TB patients by sputum and urine diagnostic test status. All patients were positive using at least one assay. LAM is Determine TB-LAM assay, urine Xpert is Xpert *MTB*/RIF on concentrated urine, sputum Xpert is Xpert *MTB*/RIF. n=65

Outcomes

Despite TB screening and prompt TB treatment, mortality in the HIV/TB patients was high. 35.4% (23/65) patients died within 2 months, with no patients lost to follow-up. 74% (17/23) of deaths were during inpatient admission, and median time to death was 13 days (IQR 5-35 days). 26% (6/23) of deaths were within 1 week of admission.

HIV/TB patients who died were more likely to be male, have a lower Karnofsky score ($p=0.002$), lower CD4 cell counts ($p=0.025$), and higher CRP ($p=0.043$, table 8.4). TB diagnostic test results strongly predicted outcome (table 8.4 and 8.5). Patients with disseminated TB (any positive urine test) had a four-fold increase in mortality compared to non-disseminated TB ($p=0.029$). Mortality was also strongly associated with urine TB score (table 8.4, $p=0.005$).

HIV/TB patients were admitted to hospital for a median of 10 days (IQR 5-20 days) overall, and 16 days (IQR 15-27 days) if discharged alive. Control patients without TB had a lower mortality (18.8% at 56-days) than HIV/TB patients. Their median length of stay was also much shorter (5 days).

Characteristic	Hazard ratio (unadjusted)	Lower 95% CI	Upper 95% CI	p-value	
Age (years)	1.02	0.97	1.07	0.435	
Gender (female)	0.51	0.22	1.16	0.106	
Cough	1.34	0.57	3.17	0.507	
Length of illness (weeks)	0.99	0.87	1.12	0.843	
ART naïve	0.82	0.28	2.42	0.717	
Time on ART (year)	1.11	0.99	1.25	0.098	
weight (kg)	0.99	0.95	1.03	0.594	
BMI	0.98	0.87	1.11	0.762	
MUAC (cm)	0.90	0.80	1.03	0.125	
Karnofsky score	0.93	0.90	0.97	0.002*	
WHO danger signs	1.43	0.50	4.15	0.507	
Sepsis	0.69	0.21	2.30	0.545	
CD4 count (cells/ μ L)	1.00	0.99	1.00	0.025	
HIV VL (log)	1.09	0.90	1.32	0.361	
HIV VL >1000 copies/ml	1.34	0.58	3.10	0.489	
Haemoglobin (g/dL)	0.99	0.98	1.01	0.466	
CrAg positive	1.92	0.70	5.26	0.231	
TB diagnostics					
Sputum Xpert +ve (%)	2.11	0.37	11.86	0.432	
Urine Xpert	5.67	1.83	17.55	0.002*	
Urine LAM +ve (%)	1.93	0.72	5.20	0.170	
Urine TB score					
	1	3.36	0.43	26.56	0.005*
	2	9.72	1.27	74.52	

Table 8.4 Clinical baseline variables and associations with mortality. Hazard ratios are for every on unit increase in continuous variables. P-values are calculated by likelihood ratio testing. *indicated p-values that remained significant after correction with the Benjamini-Hochberg procedure for multiple comparisons. Abbreviations: CI confidence interval

	N	Survived	Died	Mortality (%)	Mortality 95% CI
Sputum Xpert +ve	29	19	10	34.5	18.9 - 54.3
Urine Xpert +ve	31	14	17	54.8	36.4 - 72.0
Urine LAM +ve	43	25	18	41.9	27.6 - 57.5
Pulmonary TB only*	11	10	1	9.1	0.9 - 53.7
Disseminated urinary TB	54	32	22	40.7	28.1 - 54.6
Urine TB score – 1	34	25	9	26.5	14.1 – 44.2
Urine TB score – 2	20	7	13	65.0	40.4 - 83.6

Table 8.5 Outcome and TB diagnostic results. Data are number of patients. Urine score is calculated by the sum of positive urine TB assays. *only sputum assay(s) were positive for TB, urine assays were negative, equivalent to urine TB score=0.

Cell types

There were no differences in leucocyte differentials (based on flow cytometry data) between patient groups, other than HIV/TB patients who died having a lower proportion of lymphocytes than survivors ($p=0.036$), which is consistent with the lower CD4 cell count and more advanced HIV in this group. CD4:CD8 ratios were universally decreased consistent with low absolute and relative CD4 cell counts in this population. Similarly, HIV/TB patients with disseminated TB had a higher proportion of CD8⁺ lymphocytes and lower proportion of CD4⁺ lymphocytes than those without disseminated disease (54.3% compared to 30.9% CD8⁺, 8.6% compared to 13.8% CD4⁺, $p=0.03$) (table 8.6).

characteristic	TB negative control patients n=16	HIV/TB patients				
		All n=65	Disseminated urinary TB		Died n=23	Survived n=42
			Yes n=54	No n=11		
Monocytes (% of leucocytes)	5.0 (3 - 8)	5.3 (3.2 - 11.7)	4.8 (2.7 - 11.7)	8.6 (5.3 - 19.1)	3.8 (2.6 - 11.7)	5.5 (3.5 - 11.7)
Neutrophils (% of leucocytes)	52.7 (39.2 - 69.2)	55.6 (37.5 - 72.2)	55.1 (36.8 - 74.4)	58.3 (37.5 - 68.3)	64.6 (42.9 - 74.4)	54.7 (37 - 69.5)
Neutrophils ($\times 10^9/L$)	8.5 (4.3 - 14.2)	4.5 (1.6 - 11.2)	4.5 (1.8 - 10.8)	5.1 (1.6 - 11.7)	3.5 (1.3 - 25.2)	6.7 (2.1 - 11.2)
Monocytes ($\times 10^9/L$)	0.6 (0.2 - 1)	0.3 (0.1 - 0.7)	0.3 (0.1 - 0.7)	0.3 (0.2 - 0.8)	0.2 (0.1 - 0.9)	0.3 (0.2 - 0.7)
Lymphocytes (% of leucocytes)	21 (10.3 - 30.8)	17.4 (7.6 - 29.2)	16.0 (7.6 - 29.2)	17.7 (7.1 - 41.5)	11.9 (6.7 - 18.6)	20.7 (8.4 - 40.4)
T-cells (% of all lymphocytes)	74.1 (58.1 - 83.9)	67.5 (59 - 76.1)	67.7 (60.4 - 78.0)	63.9 (58.5 - 73.3)	64.3 (55.5 - 72.2)	68.4 (61.9 - 78.75)
CD4 ⁺ T- cells (% of T-lymphocytes)	4.7 (3.1 - 10.2)	10 (3.6 - 17.2)	8.6 (3.6 - 13.2)	10.3 (3.6 - 19.7)	8.6 (3 - 14.5)	13.8 (10 - 23.8)
CD8 ⁺ T- cells (% of T-lymphocytes)	33.6 (15.1 - 46.4)	52 (29.8 - 63.9)	51.4 (32.7 - 60)	52.2 (29.3 - 65)	54.3 (31.9 - 65.7)	30.9 (14.4 - 52.3)
CD4:CD8 ratio	0.2 (0.1 - 0.4)	0.2 (0.1 - 0.5)	0.2 (0.1 - 0.3)	0.5 (0.2 - 0.9)	0.1 (0.1 - 0.3)	0.2 (0.1 - 0.6)

Table 8.6 White cell differentials. Abbreviations: CD cluster of differentiation.

Plasma cytokines and chemokines

We assessed concentrations of cytokines and chemokines in plasma, and compared patients by disseminated disease status (table 8.7) and outcome (table 8.8). Levels of all proteins were in the detectable range other than IL-27, IL-9, IFN- α and TNF- β , which were therefore excluded. IFN- γ levels were higher in patients who died than those who survived, however TNF- α levels were not.

Cytokine or chemokine	Disseminated HIV/TB		p-value
	Yes (n=10)	No (n=51)	
IFN- γ	369.8 (173.4 - 867.8)	188.8 (188.8 - 148.7)	0.173
IL-12p70	21.6 (10.9 - 56.7)	30.2 (30.2 - 6.5)	0.938
IL-13	39.3 (11.4 - 64.9)	37.9 (37.9 - 24.1)	0.711
IL-1beta	35 (19.9 - 67.4)	50.2 (50.2 - 11.6)	0.370
IL-2	62.3 (29.1 - 104.5)	87.6 (87.6 - 53.5)	0.360
IL-4	667 (287 - 1317)	1001.9 (1001.9 - 524.1)	0.173
IL-5	128.8 (34.5 - 246.7)	262.2 (262.2 - 89.7)	0.096
IL-6	314.5 (182.2 - 540.5)	318.9 (318.9 - 290.2)	0.340
TNF- α	93.9 (44.1 - 154.5)	90.7 (90.7 - 60.2)	0.907
GM-CSF	91 (32.9 - 179.7)	148 (148 - 76.5)	0.170
IL-18	407.6 (102.4 - 803.2)	183.5 (183.5 - 79.9)	0.381
IL-10	29 (10.1 - 67.9)	35.1 (35.1 - 6.9)	0.969
IL-17A	72.9 (35.5 - 116.9)	55.5 (55.5 - 34.1)	0.800
IL-21	200.4 (80.2 - 290.6)	172.2 (172.2 - 81.9)	0.846
IL-22	378.5 (219.2 - 542.4)	512.4 (512.4 - 237.8)	0.311
IL-23	121.9 (72.5 - 227.2)	233.5 (233.5 - 136.6)	0.025
IL-15	74.2 (43.4 - 107.3)	94.7 (94.7 - 67.4)	0.284
IL1- α	13.7 (4.2 - 54.2)	19.9 (19.9 - 2.7)	0.869
IL1-RA	3751 (991.2 - 7215.3)	2290.1 (2290.1 - 1234.3)	0.293
IL-7	18 (4.3 - 45.6)	36.5 (36.5 - 16)	0.250
Eotaxin	32.2 (8.4 - 58.2)	44.9 (44.9 - 17.3)	0.533
GRO- α	49.9 (14.2 - 99.5)	124.8 (124.8 - 58.1)	0.012
IL-8	54.8 (31.5 - 102.9)	72.5 (72.5 - 48.5)	0.424
IP-10	99.1 (41.8 - 317.5)	171.8 (171.8 - 41.3)	0.599
MCP-1	156.8 (68.1 - 352.1)	91.2 (91.2 - 57.1)	0.320
MIP-1 α	67 (39.1 - 111.5)	85 (85 - 65.4)	0.302
MIP-1 β	238.4 (139.6 - 324.2)	141.2 (141.2 - 106.4)	0.025
SDF-1 α	491.8 (277 - 665.2)	489.1 (489.1 - 263.2)	0.907
RANTES	45.5 (20 - 75.1)	77.3 (77.3 - 46.5)	0.070

Table 8.7 Plasma cytokine and chemokine concentrations in HIV/TB patients with and without disseminated TB disease. P-values calculated by Wilcoxon rank sum test. *indicates p-values that remained significant after correction with the Benjamini-Hochberg procedure for multiple comparisons. Data missing for 4 individuals. Abbreviations: CD cluster of differentiation, IQR interquartile range, CI confidence interval, IL interleukin, IFN interferon, TNF tissue necrosis factor, MIP macrophage inflammatory protein, MCP monocyte chemoattractant protein, IP interferon-gamma induced protein, SDF stromal cell-derived factor, RANTES regulated on activation, normal T cell expressed and secreted.

Cytokine or chemokine	Died (n=21) median pg/mL (IQR)	Survived (n=40) median pg/mL (IQR)	Hazard Ratio (95%CI)	p-value
IFN- γ	400.7 (209 - 1255)	291.4 (291.4 - 148)	1.28 (0.99 - 1.66)	0.047
IL-12p70	21.6 (10.9 - 42.5)	23.8 (23.8 - 10)	0.87 (0.68 - 1.12)	0.291
IL-13	36.2 (13.4 - 59.3)	43.4 (43.4 - 17.4)	0.93 (0.73 - 1.2)	0.598
IL-1 β	50.9 (26.1 - 68.2)	36.1 (36.1 - 16.5)	1.23 (0.93 - 1.65)	0.132
IL-2	55 (33.4 - 104.5)	71.9 (71.9 - 31.7)	0.86 (0.68 - 1.08)	0.212
IL-4	406.1 (224 - 791.3)	970.4 (970.4 - 522.4)	0.69 (0.53 - 0.91)	0.01*
IL-5	114.8 (37 - 550.7)	149 (149 - 47.3)	0.93 (0.78 - 1.1)	0.397
IL-6	540.5 (283.1 - 656.3)	294.9 (294.9 - 189.2)	1.77 (1.23 - 2.54)	0.002*
TNF- α	124.2 (58.5 - 189.6)	81.1 (81.1 - 42.7)	1.13 (0.88 - 1.45)	0.335
GM-CSF	100.7 (32.9 - 194.1)	94.9 (94.9 - 39.9)	0.9 (0.74 - 1.09)	0.31
IL-18	299.7 (121.6 - 803.2)	368.5 (368.5 - 89.1)	1.12 (0.92 - 1.36)	0.247
IL-10	25.2 (15.4 - 71.5)	36.2 (36.2 - 8.9)	1.11 (0.9 - 1.36)	0.325
IL-17A	51.5 (15.5 - 95.6)	78.8 (78.8 - 45.4)	0.93 (0.8 - 1.09)	0.364
IL-21	225.7 (72.7 - 290.6)	166.8 (166.8 - 82.2)	1.09 (0.87 - 1.35)	0.458
IL-22	381.3 (258.2 - 461.5)	386.8 (386.8 - 196.8)	0.93 (0.69 - 1.24)	0.608
IL-23	91.6 (63.6 - 165.9)	177.7 (177.7 - 102.8)	0.88 (0.72 - 1.07)	0.199
IL-15	82.5 (51.5 - 107.3)	77.3 (77.3 - 52.1)	0.99 (0.77 - 1.28)	0.967
IL1- α	13.2 (2.9 - 62.9)	14.6 (14.6 - 4.8)	0.97 (0.84 - 1.13)	0.704
IL1-RA	4730.4 (3751 - 8510.3)	2277.9 (2277.9 - 879.6)	1.35 (1.05 - 1.72)	0.009*
IL-7	18.2 (8.1 - 57.1)	20.9 (20.9 - 4.7)	1 (0.86 - 1.16)	0.99
Eotaxin	33.6 (8.4 - 67.5)	29.7 (29.7 - 14.3)	0.96 (0.79 - 1.16)	0.672
GRO α	51.3 (16.8 - 104.7)	63.8 (63.8 - 22.8)	1 (0.85 - 1.17)	0.961
IL-8	84.7 (56 - 126.6)	48.3 (48.3 - 33.4)	1.22 (0.95 - 1.56)	0.099
IP-10	158.3 (70.9 - 381.2)	66 (66 - 35.9)	1.06 (0.98 - 1.14)	0.192
MCP-1	300.3 (178.6 - 632.8)	95.8 (95.8 - 47.4)	1.68 (1.28 - 2.19)	<0.001*
MIP-1 α	95.1 (54.6 - 125.9)	65.6 (65.6 - 42.3)	2.14 (1.24 - 3.7)	0.002*
MIP-1 β	304.5 (183.7 - 366.3)	199.4 (199.4 - 130.9)	2.03 (1.19 - 3.47)	0.002*
SDF-1 α	611.8 (297.1 - 718.6)	426.5 (426.5 - 269.1)	1.21 (0.88 - 1.66)	0.22
RANTES	50.3 (21.2 - 75.1)	59.1 (59.1 - 30.8)	1.1 (0.86 - 1.41)	0.443

Table 8.8 Plasma cytokine and chemokine concentrations in HIV/TB patients who died and survived.

Hazard ratios are for a doubling of concentration. P-values calculated by likelihood ratio testing.

*indicates p-values that remained significant after correction with the Benjamini-Hochberg procedure for multiple comparisons. Data missing for 4 individuals. Abbreviations: CD cluster of differentiation, IQR interquartile range, CI confidence interval, IL interleukin, IFN interferon, TNF tissue necrosis factor, MIP macrophage inflammatory protein, MCP monocyte chemoattractant protein, IP interferon-gamma induced protein, SDF stromal cell-derived factor, RANTES regulated on activation, normal T cell expressed and secreted.

After accounting for multiple comparisons, disseminated HIV/TB disease did not have any significant associations with plasma cytokine and chemokine concentrations (table 8.7). Six soluble proteins were found to be associated with mortality, with hazard ratios >1.0 (table 8.8 and figure 8.6). These included chemokines important in signalling in the innate immune response such as MIP-1 α , MIP-1 β and MCP-1, and pro-inflammatory cytokines that are produced by or promote innate immune responses (IL-6). Two anti-inflammatory proteins

were also associated with mortality, although IL-1RA levels were increased whilst IL-4 levels were decreased.

Cytokine networks were also assessed by testing for correlations (defined as $Rho \geq 0.6$ and p -value < 0.05) between different cytokines and chemokines (figure 8.7). HIV-positive patients without TB had more correlations between cytokines ($n=92$) than HIV/TB patients ($n=32$, figure 8.7). However, amongst HIV/TB patients, those who died had more cytokine correlations than those who survived, and these seem to be mostly driven by innate cytokines and chemokines.

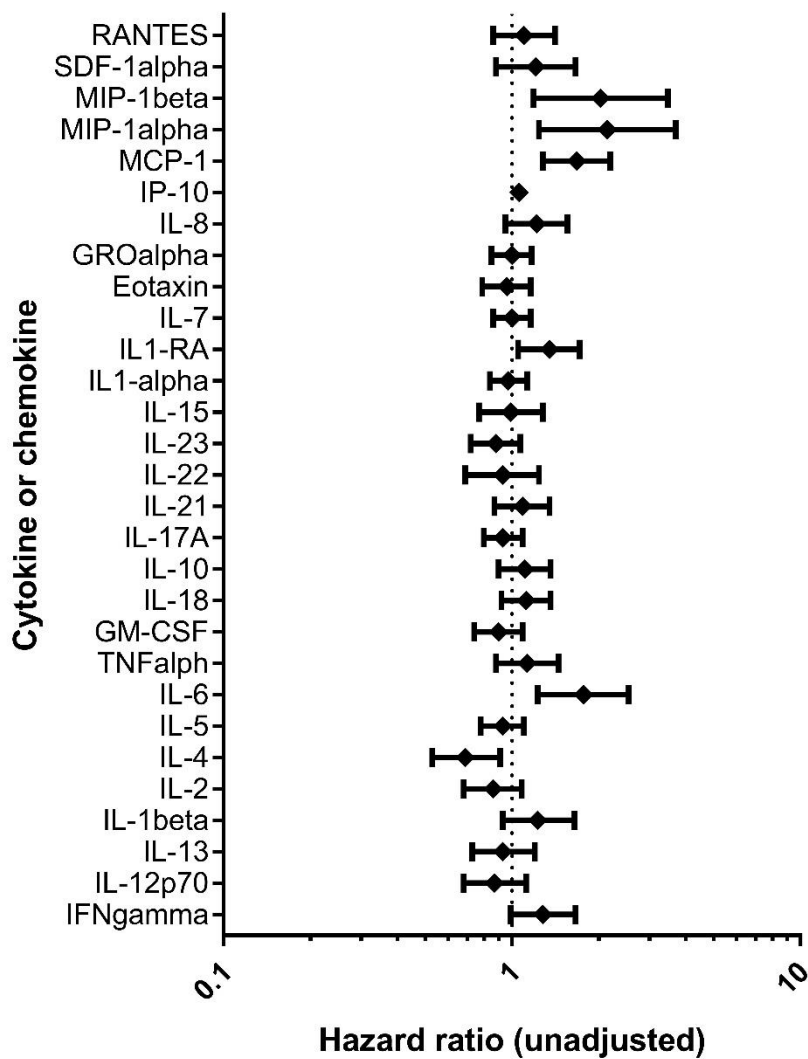


Figure 8.6 Hazard ratios for mortality of plasma chemokines and cytokines in HIV/TB patients. Hazard ratios are for a doubling of concentration. $n=61$. Abbreviations: CD cluster of differentiation, IQR interquartile range, CI confidence interval, IL interleukin, IFN interferon, TNF tissue necrosis factor, MIP macrophage inflammatory protein, MCP monocyte chemoattractant protein, IP interferon-gamma induced protein, SDF stromal cell-derived factor, RANTES regulated on activation, normal T cell expressed and secreted.

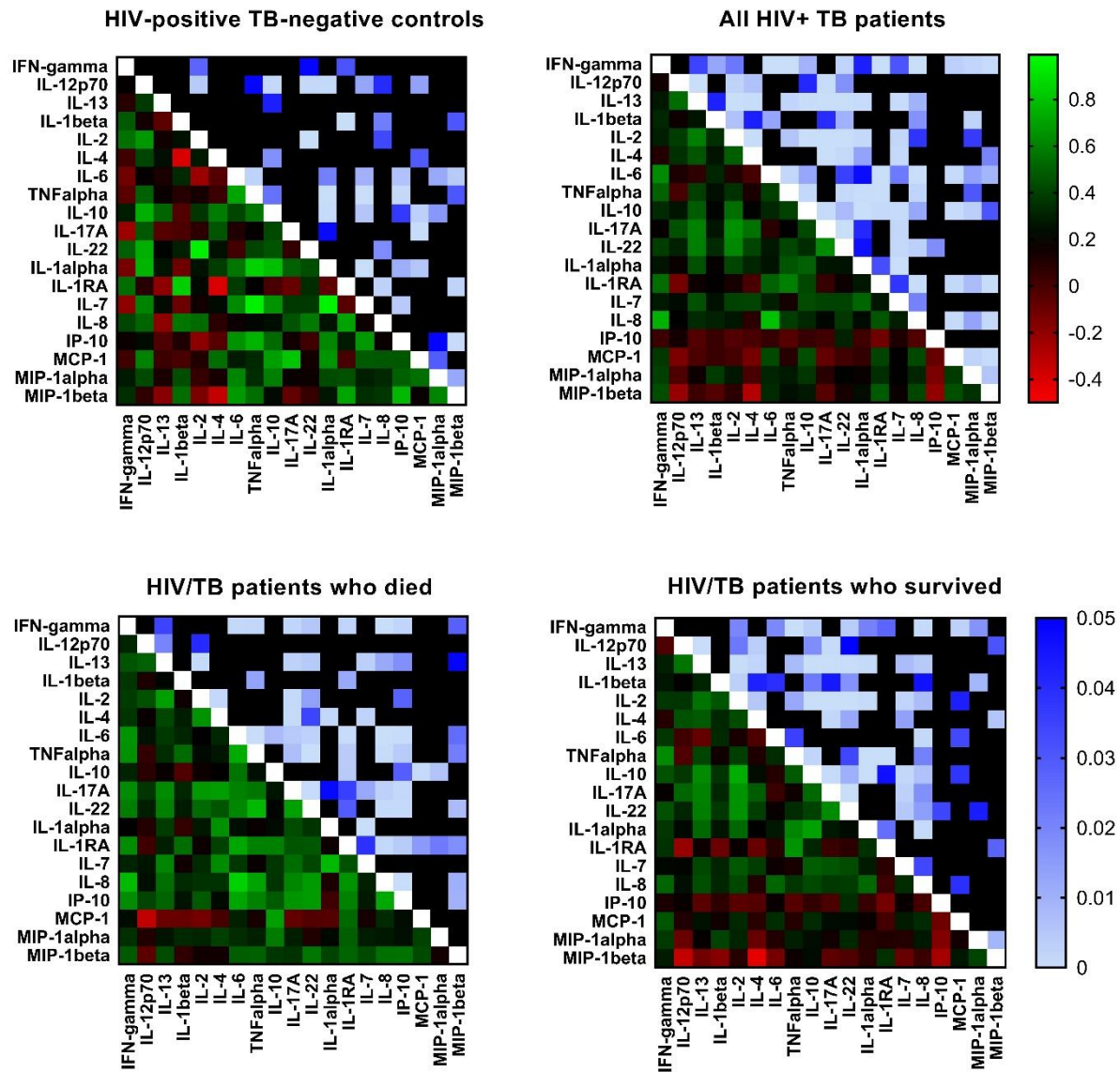


Figure 8.7 Heat-map of correlations between cytokines by patient TB status and outcome. Bottom left side of the heat maps indicate rho values for correlations (green are positive correlations, and red are negative correlations). The upper right side of the heat-map is the p-value for the same correlations. HIV-positive TB negative control patients had 92 correlations (defined as $\rho > 0.6$ and $p\text{-value} < 0.05$), and overall HIV/TB co-infected patients had 32 correlations. Amongst the HIV/TB patients, those who died had 94 correlations, whereas those who survived had 50. $n=61$ (21 patients died).

Hepcidin

Plasma hepcidin concentrations were raised in all patient groups. Hepcidin concentrations were higher in HIV/TB patients who died compared to survivors (117ng/ml versus 69ng/ml, hazard ratio 1.5 for a doubling of hepcidin concentration, $p=0.021$, figure 8.8). However, hepcidin concentrations were similar in HIV/TB patients and controls without TB, and in HIV/TB patients with disseminated and non-disseminated disease. In exploratory analyses, hepcidin levels were lower in patients with mild anaemia or normal haemoglobin compared to

those with moderate or severe anaemia ($p=0.001$, figure 8.8), and a strong correlation with CRP ($p<0.0001$).

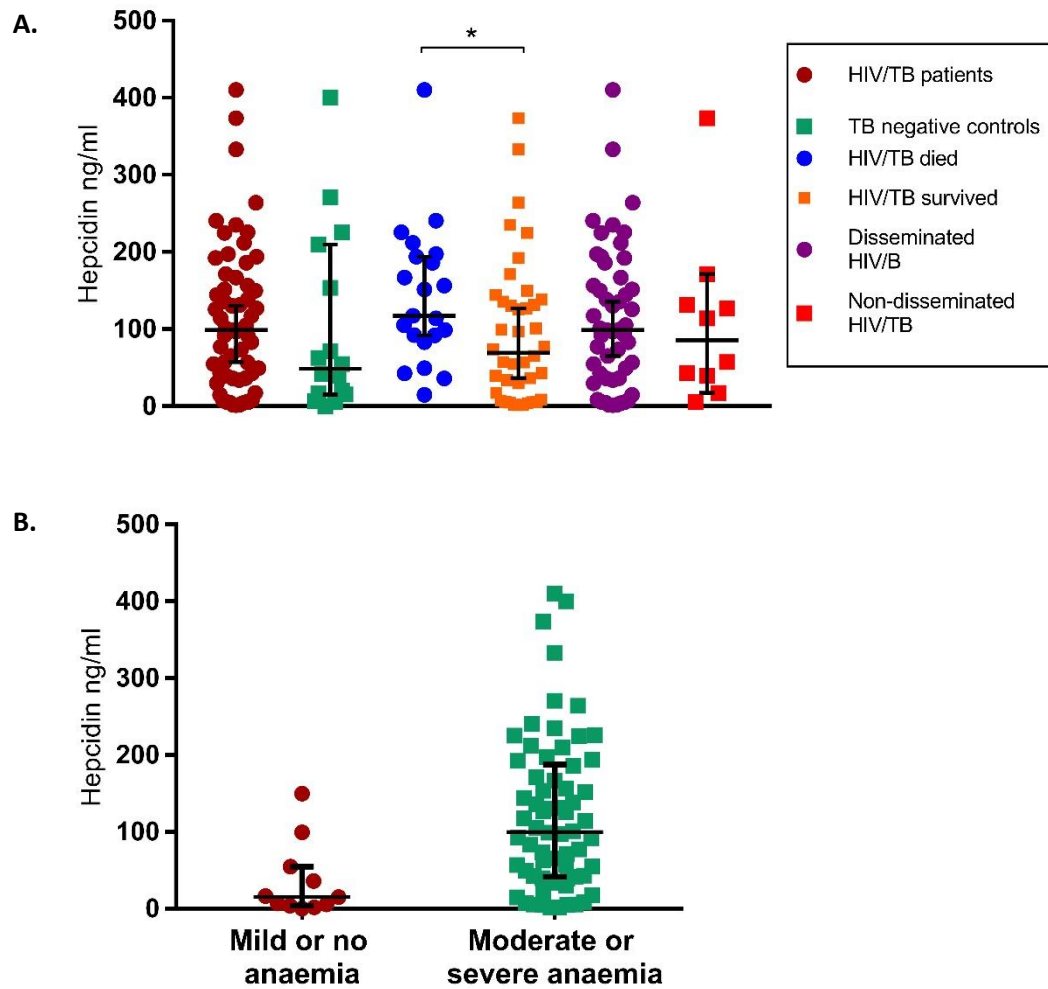


Figure 8.8 Hepcidin concentrations by patient group and anaemia. A. Hepcidin concentration by patient group. B. median hepcidin concentrations for patients with mild or no anaemia (dark red) and those with moderate to severe anaemia (green). Data points represent concentration for each patient, horizontal bar represents median concentration for that group and error bars represent the interquartile range. * indicates p -value < 0.05

Immune activation and exhaustion

Monocyte phenotype and activation

In all patient groups the proportion of classical monocytes was lower than in 'healthy' individuals. HIV/TB patients who died had a lower proportion of classical monocytes ($p=0.0018$) and a higher proportion of non-classical monocytes ($p=0.0051$) compared to HIV/TB patients who survived (figure 8.9). There was also evidence of lower proportions of classical monocytes and higher proportions of non-classical monocytes in disseminated compared to non-disseminated HIV/TB ($p=0.028$ and 0.015 respectively). However, monocyte phenotype did not differ substantially between HIV/TB patients and the HIV-positive controls (table 8.9). There was high surface expression of HLA-DR on monocytes throughout, but no differences between patient groups. Similarly, MFI of HLA-DR and CD11b surface markers did not differ between groups.

Soluble markers of monocyte activation were strongly associated with patient groups. sCD163 concentrations were substantially higher in HIV/TB patients compared to control patients ($p=0.0002$), and there was also evidence of higher concentrations in HIV/TB patients who died and those with disseminated disease ($p=0.013$ and $p=0.028$ respectively). sCD14 concentrations were also higher in HIV/TB patients ($p<0.0001$), and HIV/TB patients who died compared to survivors ($p<0.0001$) (figure 8.9).

Monocyte characteristic	TB negative control patients (n=16)	All (n=65)	HIV/TB patients			
			Died (n=23)	Survived (n=42)	Disseminated Yes (n=11)	Disseminated No (n=54)
CD14 ⁺ CD16 ⁻ (%)	77.6 (69.9 - 85.1)	82 (75.8 - 87.95)	73.8 (57.5 - 79.6)	79.65 (75.4 - 85.5)	76.2 (68.2 - 83.5)	85.2 (76.2 - 89.4)
CD14 ⁺ CD16 ⁺ (%)	10.49 (6.02 - 16.5)	11.75 (8.555 - 14.25)	12.3 (6.26 - 24.8)	9.545 (6 - 14.6)	10.595 (6.02 - 16.5)	8.38 (3.45 - 18.4)
CD14 ⁻ CD16 ⁺ (%)	9.85 (4.6 - 15.2)	5.245 (3.775 - 10.45)	15 (9.87 - 18.06)	7.895 (3.74 - 14.3)	10.8 (6.14 - 17.7)	7.36 (2.92 - 8.2)
HLA-DR expression (%)	95.1 (86.6 - 98.1)	93.3 (87.3 - 97.1)	91.2 (81.1 - 97.3)	96.8 (89.8 - 98.3)	92.5 (85.5 - 98.1)	96.45 (90 - 98.7)
HLA-DR (median MFI)	51.4 (30.3 - 88.7)	55.15 (41.55 - 72.2)	49.9 (30.3 - 116)	51.55 (32.5 - 86.95)	50.8 (29.4 - 88.7)	60.7 (43 - 178)
CD11b (median MFI)	46.7 (27.1 - 61.3)	41.3 (25.9 - 51.65)	39.2 (27.1 - 52.1)	53.95 (26.75 - 65.75)	45.3 (27.1 - 60.4)	51.55 (17.3 - 65.1)
sCD14 (median pg/mL X10 ⁶)	2.1 (1.8 - 3.1)	1.2 (1.0 - 1.6)	3.2 (2.7 - 4.0)	1.8 (1.5 - 2.4)	2.5 (1.8 - 3.2)	1.8 (1.6 - 2.1)
sCD163 (median ng/mL X10 ³)	1.2 (0.9 - 1.8)	0.7 (0.6 - 1.1)	1.8 (0.9 - 2.9)	1.1 (0.9 - 1.5)	1.3 (0.9 - 1.9)	0.9 (0.3 - 1.1)

Table 8.9 Monocyte phenotype and activation markers by patient group. Monocyte phenotype and HLA-DR expression and is the median % and interquartile ranges of all monocytes. MFI is median fluorescence intensity (on a log₁₀ scale). sCD14 and sCD163 are median concentrations and interquartile ranges. sCD is soluble cluster of differentiation.

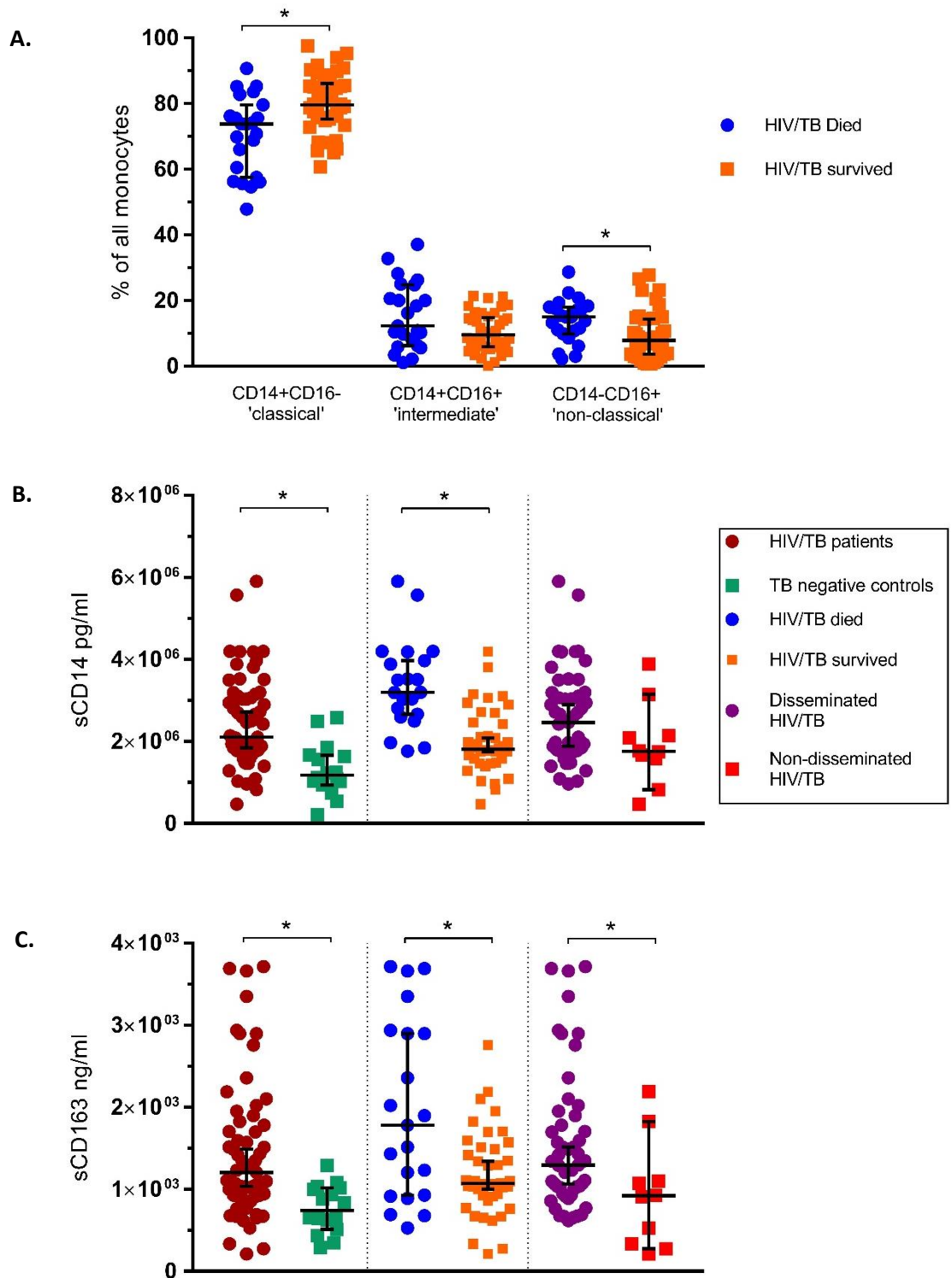


Figure 8.9 Monocyte phenotype and soluble monocyte activation markers by patient group

A. % of monocytes in each phenotypic group by outcome in HIV/TB patients. **B.** Soluble CD14 concentration and **C.** median soluble CD163 concentration by patient group. Data points represent concentrations or percentages for each patient, horizontal bar represents medians for that group and error bars represent the interquartile range. * indicates p-value < 0.05

T-cell immunophenotyping

Lymphocyte activation was high in all patient groups and was strongly correlated with HIV viral load ($p=0.0001$) but not TB status or outcomes (table 8.10 and figure 8.10). T-cell exhaustion (defined as PD1 expression) and senescence (defined as CD57 expression) also did not differ substantially between patient groups (table 8.10). Senescence of CD8⁺ T-cells was weakly correlated with age ($p=0.06$).

T-cell characteristic	TB negative control patients (n=16)	HIV/TB patients				
		All (n=65)	Died (n=23)	Survived (n=42)	Disseminated	
					Yes (n=11)	No (n=42)
CD38⁺ (% CD3 ⁺ T-cells)	31.6 (20.5 - 44.1)	34.6 (19.4 - 42)	31.2 (19.4 - 37.8)	34.8 (20.7 - 47.3)	35 (18.5 - 44.1)	26.7 (22.5 - 36.8)
HLA-DR⁺ (% CD3 ⁺ T-cells)	11.2 (6.1 - 26.3)	9.2 (4.7 - 15.1)	8.6 (6.4 - 14.6)	9.2 (4.1 - 15.4)	8.6 (4.6 - 14.6)	10.4 (5.7 - 16.9)
% activated CD38⁺ OR HLA-DR⁺ T-cells (% CD3 ⁺ T-cells)	39.2 (26 - 51.7)	34.5 (17.2 - 48.9)	35.5 (24.2 - 43.3)	33.3 (12.4 - 50.3)	35.5 (14 - 48.9)	27.6 (17.2 - 65.5)
CD57⁺ CD4⁺ cell exhaustion (% CD4 ⁺ T-cells)	2.4 (0.6 - 9.6)	3.3 (1.3 - 9.8)	2.4 (0.9 - 7.4)	4.6 (2.3 - 14.9)	2.8 (1 - 9.8)	4.1 (2.8 - 14.1)
CD57⁺ CD8⁺ cell exhaustion (% CD8 ⁺ T-cells)	3.1 (1.7 - 8.2)	14.5 (5.6 - 25.2)	10.5 (6.1 - 23.3)	15.3 (5.5 - 26.4)	15 (5.4 - 24.5)	9.3 (5.6 - 37.1)
PD-1⁺ CD4⁺ cell senescence (% CD4 ⁺ T-cells)	3.7 (1 - 8.5)	9 (3 - 14.4)	3.1 (0.9 - 7.8)	3.9 (1.8 - 9.5)	3.9 (1.7 - 10.5)	2.5 (0.2 - 6.4)
PD-1⁺ CD8⁺ cell senescence (% CD8 ⁺ T-cells)	4.1 (3.4 - 6.6)	4.8 (1.7 - 8.5)	5.9 (3.1 - 9.6)	3.1 (1.7 - 7.8)	4.8 (1.9 - 7.9)	4.8 (0.5 - 9.5)

Table 8.10 T-cell activation, exhaustion and senescence. All data are median % and interquartile ranges of the parent T-cell population (denoted in brackets).

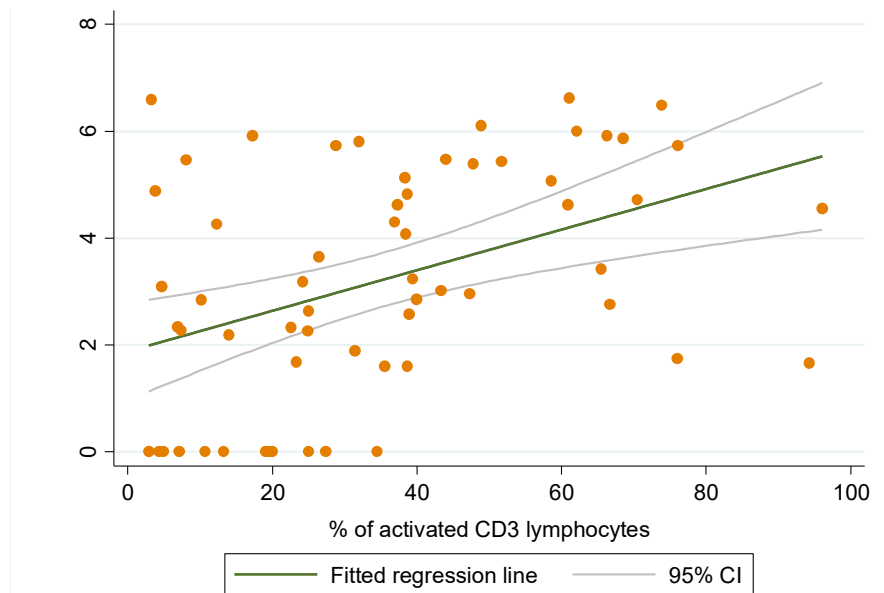


Figure 8.10 HIV viral load and T-cell activation. Graph shows correlation between HIV viral load and proportion of T-cell activated (defined as expression of HLA-DR or CD38), with regression line (green) and 95% CIs (grey lines). $P=0.0001$. $n=65$

8.5 Discussion

Despite widespread coverage of ART, patients admitted to hospital and diagnosed with HIV-associated TB mostly had advanced HIV, with a very high prevalence of disseminated urinary TB and high early mortality risk (35% by two months). Mortality risk was substantially higher than in HIV-positive patients without evidence of TB and matched for CD4 count. The case fatality of HIV/TB in this cohort was similar to that reported in meta-analyses, although higher than expected given the high ART coverage, TB screening and prompt initiation of TB therapy [2]. The prevalence of disseminated disease was also very high, and may have been underestimated given that some patients with only positive urine tests would have been missed from the SOC arm of the trial. Mortality was also strongly associated with urinary disease- those with 2 positive urine TB tests had a 65% risk of death.

The main findings from this study of soluble immunological biomarkers was that patients with poor outcomes had higher concentrations of cytokines responsible for innate and pro-inflammatory signalling, and markers of inflammation such as CRP and hepcidin. They also had evidence of more marked monocyte activation and non-classical monocyte phenotype. I found no differences between HIV/TB patients with positive and negative urine diagnostic tests.

I demonstrate increased plasma concentrations of innate and inflammatory cytokines (MCP-1, MIP-1 and IL-6), supporting findings from recent studies in HIV-positive outpatients and early

studies from the pre-ART era [18,19]. These cytokines are mainly produced by monocytes and macrophages, highlighting the importance of these cells in *MTB* control [20]. Previously, similar cytokine profiles have also been noted at sites of infection, but not in the systemic circulation [21]. The presence of such changes in plasma support the finding that most patients had disseminated disease, likely involving mycobacteraemia and haematogenous spread [11].

I also found very high levels of IL-1RA (especially in patients with early mortality), a soluble inhibitor of IL-1 which is important in tightly regulating and preventing excessive tissue-damaging inflammation [22]. However, I did not find increased concentrations of other anti-inflammatory or T-helper 2 cytokines (eg IL-4, IL-10 or IL-13) in patients who died or those with disseminated disease as hypothesised. Successful containment of TB most likely requires tightly balanced pro- and anti-inflammatory responses. It is clear that, whilst activation and inflammation are necessary in the host response, disruption of either TNF- α and IFN- γ pathways leads to overwhelming TB disease [23,24]. Excessive inflammation is also detrimental, for instance in TB immune reconstitution inflammatory syndrome [25]. Similarly, removing inhibitory pathways in mice exacerbates TB disease [26], whilst excessive anti-inflammatory responses can promote latency and dissemination [25].

Indeed, cytokines exist and function in complex integrated networks, and we have uniquely demonstrated differences in correlations between cytokines in HIV/TB patients compared to TB-negative HIV-positive patients, and according to outcome. Cytokine environment and networks are crucial in the immune response and cellular function, and HIV has been associated with their disruption [27]. Cytokine profiles in this patient population resembled those found in sepsis, which is not surprising given the prevalence of sepsis features and danger signs, and the overlap of disseminated *MTB* and sepsis [28–30]. Similar cytokine profiles have also been associated with severity of organ dysfunction and outcome in sepsis [31,32].

Immune activation and inflammation may also drive immunoregulatory mechanisms, for example production of T-helper 2 type cytokines (which I did not see in this cohort), or expansion of regulatory cells (eg T-regulatory cells) [33–35]. Activation and inflammation may directly lead to exhaustion of the immune response. I did not find differences in exhaustion (PD-1) or senescence (CD57) markers on CD4⁺ or CD8⁺ T-cells. However the mechanism of exhaustion or anergy in HIV/TB may not lead to expression of these markers, but may still impair host defences against *MTB* and other pathogens. Tolerance to endotoxins has been demonstrated in the context of challenge with lipopolysaccharide (LPS), which can affect T-cells in addition to monocytes and may play a role in HIV/TB [36,37]. *MTB* antigens themselves can also downregulate the host immune response [38–40].

The acute-phase marker of inflammation, hepcidin, which also has antimicrobial properties and a role in the innate response to *MTB*, was also associated with mortality in this study. Heparin also has an important role in driving anaemia of chronic disease through serum iron restriction and iron retention in macrophages and lymphocyte, often in response to IL-6 [41–43]. I show that patients with moderate or severe anaemia also had substantially higher plasma concentrations of hepcidin. It has previously been associated with high mycobacterial load, dissemination and mortality in HIV/TB patients [44], although it is unclear whether hepcidin plays a part in or is a consequence of severe HIV/TB disease.

I found an expanded population of non-classical (CD16⁺CD14⁻) monocytes, which have previously been associated with reduced phagocytosis compared to classical monocyte, although studies conflict on the exact function and role of classical, intermediate and non-classical monocyte subsets [17,45,46]. The association between reduced phagocytic responses and monocyte phenotype in our study supports the hypothesis that classical monocytes have a more inflammatory and phagocytic role, which may be driven by *MTB* itself [47].

In summary, I report increased plasma concentrations of inflammatory cytokines and markers of inflammation in HIV/TB patients who died compared to those who survived, but few differences by TB urine-diagnostic status. Strengths and weaknesses of this study, as well as further implications are discussed in chapter 9 alongside the data on functional immune responses.

8.6 References

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Chapter 9. Functional immune responses in hospitalised patients with HIV-associated TB

Chapter 9. Functional immune responses in hospitalised patients with HIV-associated TB

Summary

This chapter assesses functional responses of the innate and adaptive immune systems, including whole blood phagocyte function and T-lymphocyte and monocyte cytokine responses to stimulation. These responses are then compared between patients according to outcome (death or survival by two months), and disease dissemination. The main findings are that patients with poor outcomes had impaired *ex vivo* functional responses, including monocyte and neutrophil phagocyte superoxide function, and T-cell and monocyte cytokine production in response to antigenic stimulation. Data reduction techniques identified distinct immunological phenotypes/signatures that were strongly associated with both disseminated TB score based on urine diagnostics and outcome.

9.1 Methods

Study design, flow cytometry, immunophenotyping and other laboratory assays are described in chapter 8.

Immunological laboratory assays

Phagocytosis and superoxide burst assay

The preparation of zymosan phagocytosis reporter particles and the phagocytosis whole blood assay have been described previously (chapter 7). In brief, 0.5mL of whole blood (kept warm after venepuncture) was diluted 1:1 with warmed (37°C) RPMI to which 20µL of washed and diluted particles (1:6 in RPMI, approximately 8×10^5 /ml) were added. A biological control of 1mL of diluted whole blood without zymosan particles was also prepared. Samples were incubated at 37°C with rocking. Phagocytosis and superoxide burst were assessed at 10, 30, 60 and 90 minutes after the addition of reporter particles.

100µL of blood from zymosan reporter and control tubes were harvested 10 minutes before each time point for antibody staining of cell surface markers (as phagocytosis will continue during staining). Antibody concentrations were previously titrated for maximum separation between positive and negative populations, and staining was for 10 minutes at room temperature. Antibodies used were anti-CD45 PerCP 3µL, anti-CD66b APC 1µL and anti-CD14 PE-Cy7 2µL (all Biolegend, see table 8.2 for full panel).

Following 10 minutes incubation at room temperature with antibodies, biological activity was arrested, and red cells lysed by incubating for 10 minutes with BD FACS lysing solution (BD

Biosciences, containing formaldehyde and diethylene glycol). Cells were washed once with 1 x PBS solution by centrifugation at 500g for 10 minutes then resuspended in 500µL of PBS.

Counting beads (Countbright, Life Technologies) were added per the manufacturer's instruction to allow determination of cell concentration before acquisition on a CyAn ADP flow cytometer (Beckman Coulter, USA). The gating strategies to identify neutrophils, monocytes, and cells phagocytosing zymosan-reporter particles and undergoing superoxide burst are outlined in chapter 7 (figure 1) and in figures 8.2 to 8.3.

Intracellular cytokine staining assays

Intracellular cytokine staining (ICS) was done using whole blood that was cryopreserved and fixed after stimulation. This method was chosen over peripheral blood mononuclear cell (PBMC) based assays due to smaller blood volume requirements, less processing leading to less cell death and ability to immediately stimulate cells. Cryopreservation was chosen over real time analysis as it was more efficient (staining and analysis was done in batches) and reduced day-to-day variations in the flow cytometer. Long-term cryopreservation has been well validated for whole blood ICS [1].

Whole blood was diluted 1:1 with warmed (37°C) RPMI. 1mL of diluted whole blood was stimulated in FACS tubes with culture filtrate proteins (CFP) of H37Rv *Mycobacterium tuberculosis* (10µg/ml, BEI resources), phorbol 12-myristate 13-acetate (PMA, 10ng/ml, Sigma) plus ionomycin (1µg/ml, Sigma-Aldrich), or left unstimulated for 2 hours. Concentrations of antigens were based on previous studies and piloted prior to use [2–4]. Brefeldin A (5µg/ml, BD Bioscience) was added after 2 hours, and the cells were incubated for a further 16 hours at 37°C in 5% CO₂.

Cells were then stained with violet viability dye and then red cells lysed and leucocytes fixed using BD FACS lysing solution. Cells were washed once with BD FACS lysing solution followed by centrifugation at 500 g for 10 min then resuspended in BD FACS lysing solution and frozen at -80°C for later staining and acquisition in batches [1,5].

Samples were later thawed in a 37°C water bath and stained for surface markers by incubation with anti-CD45 PO (Invitrogen), anti-CD14 BV605, anti-CD3 APC-H7, anti-CD4 PerCPCy5.5 and anti-CD8 PE/Dazzle 594 (all Biolegend) for 15 minutes (table 8.2). Next, cells were permeabilised using 2mLs of FACS Perm/Wash buffer (diluted 1:10 with distilled water, BD Biosciences) for 15 minutes as per manufacturer's instructions [6], and intracellular proteins

stained by incubating with anti-IFN- γ PE, anti-TNF- α FITC, anti-IL-10 APC (all Biolegend), anti-IL-6 AF700 and IL-22 PECy7 (both eBiosciences) for 30 minutes.

Cells were then washed with 2mL Perm/Wash buffer, resuspended in 500 μ L PBS and acquired by flow cytometry. For analysis, dead cells, doublets and debris were gated out (figure 9.1). Granulocytes and lymphocytes were identified on CD45 expression and side-scatter, and monocytes gated based on CD14 expression. T-lymphocytes were identified based on CD3 expression, and subdivided into CD8 and CD4 T-lymphocytes. Intracellular cytokine expression was then done by gating on the cells of interest from unstimulated samples (monocytes, CD8 and CD4 T-lymphocytes), using Boolean gates to identify cells producing single or multiple cytokines.

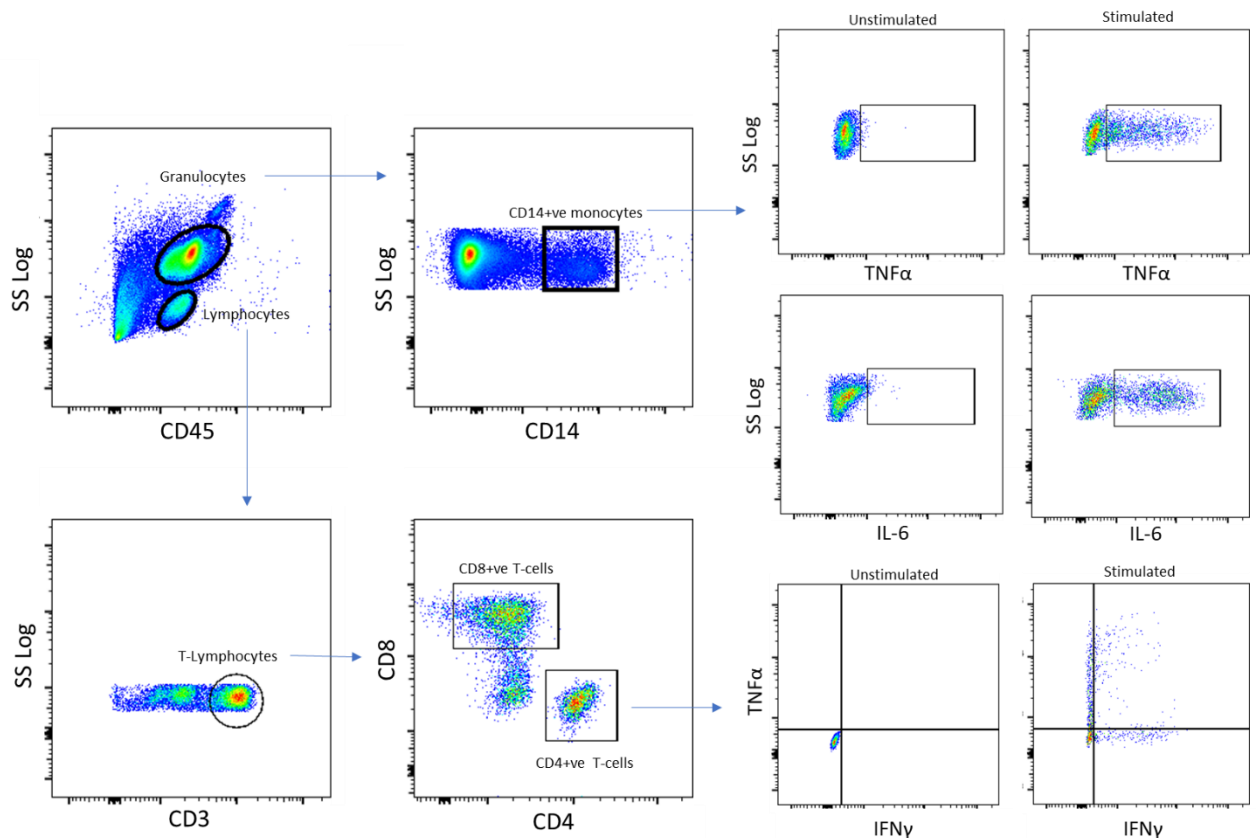


Figure 9.1 Flow cytometry gating strategy for intracellular cytokine staining assays.

Granulocytes were identified after exclusion of dead cells, doublets and debris based on high SS. Monocytes were then identified based on CD14 expression. Lymphocytes were gated on SS, and T-cell based on CD3 expression. T-cells were then subdivided based on CD4 and CD8 expression. Cells were identified as expressing the intracellular marker of interest, with gates drawn based on the unstimulated samples. Abbreviations: CD cluster of differentiation, PD programmed cell death, SS side-scatter, IL interleukin, IFN interferon, TNF tissue necrosis factor.

Statistical methods

In the phagocytic function assay, the proportion of cells that had phagocytosed reporter particles was calculated. All analyses were stratified by phagocyte type- monocyte or neutrophil. This was based on expression of calibration fluorochrome (AF405). Absolute cell numbers and cell concentrations were calculated using counting beads (based on a known concentration of counting beads in the assay). An 'activity index' (AI) of phagocytosis and superoxide burst was calculated by subtracting the median fluorescence intensity (MFI) of the cells not taking up reporter particles from the cells that did take up the zymosan reporter particles (thereby expressing AF405) and undergoing superoxidation (thereby positive for FITC), and dividing this by two times the robust standard deviation of the negative cells (this was based on the 'staining index' as method of assessing separation between two populations) [7].

This method also accounted for variations in auto fluorescence between cells from different individuals. AI at each assay timepoint and peak AI was compared between groups. Furthermore, pharmacokinetic analysis was used to calculate the 'area under the curve' (AUC) for the time-versus-activity index analysis for each patient (using Stata's *pkexamine* command and the trapezoidal rule). Mean or median AUCs were then compared for monocytes and neutrophils between patient groups.

For ICS assays, the proportion of cells producing each cytokine under each stimulation condition was calculated for monocytes, CD4⁺ T-cells and CD8⁺ T-cells. Increases in cytokine production on stimulation were calculated by subtracting the proportion of cells producing cytokine in the unstimulated sample. Polyfunctionality (defined as production of more than one cytokine) for TNF- α and IFN- γ was calculated for T-cells based on the proportion of cells produced each cytokine alone, or in combination. The median proportion of polyfunctional cells and IQR were calculated for each patient group and compared using Wilcoxon rank sum tests.

All immunological variables were standardised (such that mean represented zero and the SD was 1) after appropriate transformation, and reduced using principal components analysis (PCA). The number of principle components (PC) selected was based on Eigen values (>1.0), scree plots and the degree of variance explained. The PC(s) explaining the greatest degrees of variation were characterised by looking at the variables that contributed most to that PC using variable loading waterfall plots.

Scoring plots for PCs 1 and 2 were constructed and assessed for clustering based on mortality outcome or urine TB score. PCs were assessed for associations with mortality using Cox

regression, and PCs associated with mortality were entered into a multivariable model to adjust for confounders (including HIV viral load, CD4 cell count and age *a priori*). PCs were also assessed for association with urine TB score using linear regression modelling. Analyses were done using Stata version 14 and GraphPad version 7.0.

9.2 Results

Functional immune responses

Phagocytosis and superoxide burst

Zymosan particles were rapidly taken up by whole blood phagocytes, and kinetics over 90 minutes were similar to healthy controls (see Chapter 7) and did not differ between HIV/TB patients and TB negative controls, or by disseminated TB status or outcome in HIV/TB patients (figure 9.2). The proportion of whole blood phagocytes taking up zymosan particles varied greatly and was associated with the abundance of the phagocytic cell in peripheral whole blood- a higher concentration of phagocytes was associated with a smaller proportion taking up reporter particles ($p=0.037$ for monocytes and $p=0.002$ for neutrophils), as demonstrated for healthy volunteers previously (see [8], Chapter 7 and figure 9.3).

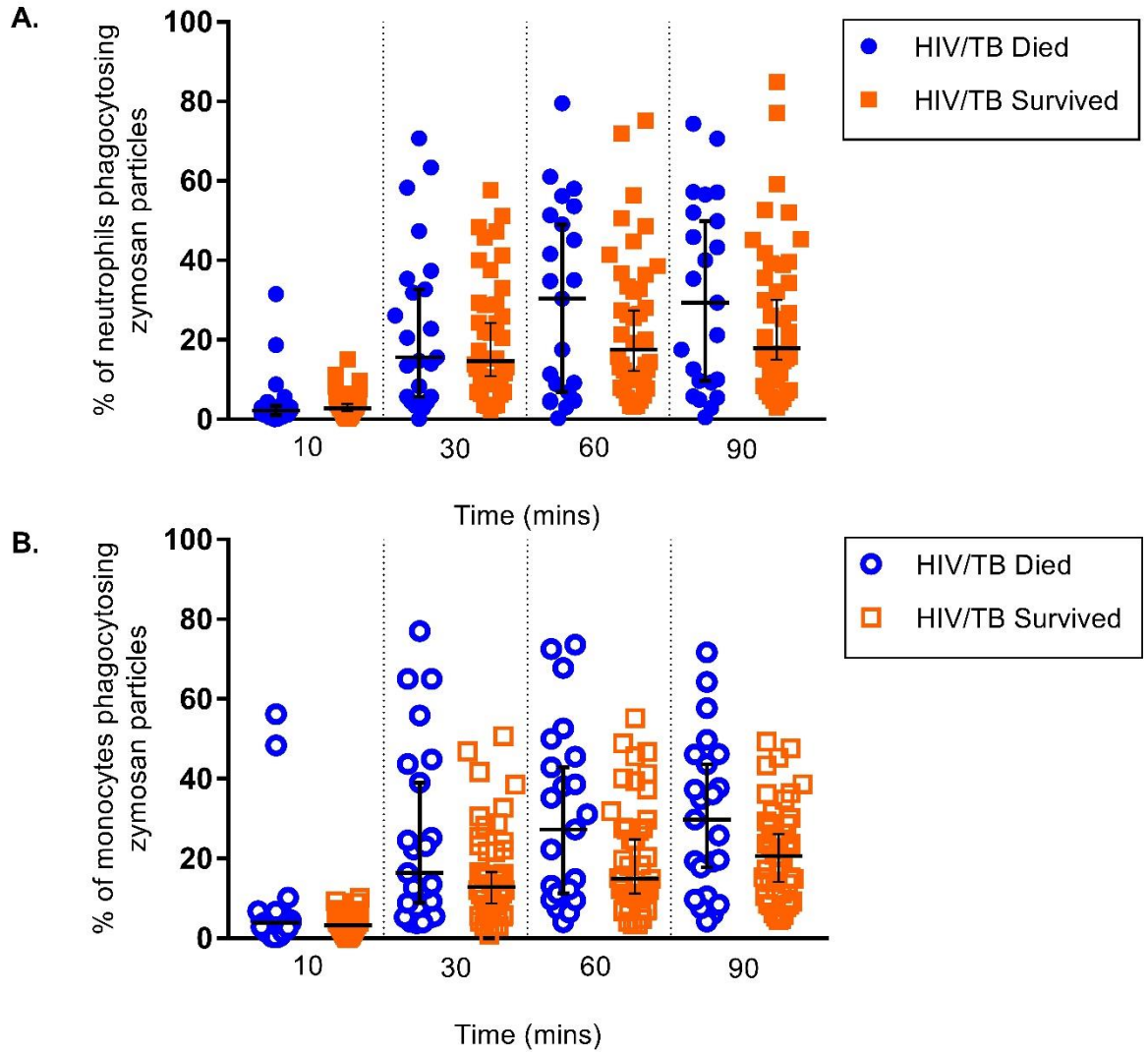


Figure 9.2 Phagocytosis of zymosan reporter particles by patient outcome in HIV/TB patients. Data represent the proportion of all neutrophils (A, solid markers) or monocytes (B, hollow markers) that have phagocytosed zymosan reporter particles at each timepoint of the assay by patient outcome. Data points are values for individual patients, horizontal lines are medians for that group, and error bars are 95% CIs for that group. There was no significant difference in neutrophil or monocyte phagocytosis between patients who died or survived at any timepoint. n=65

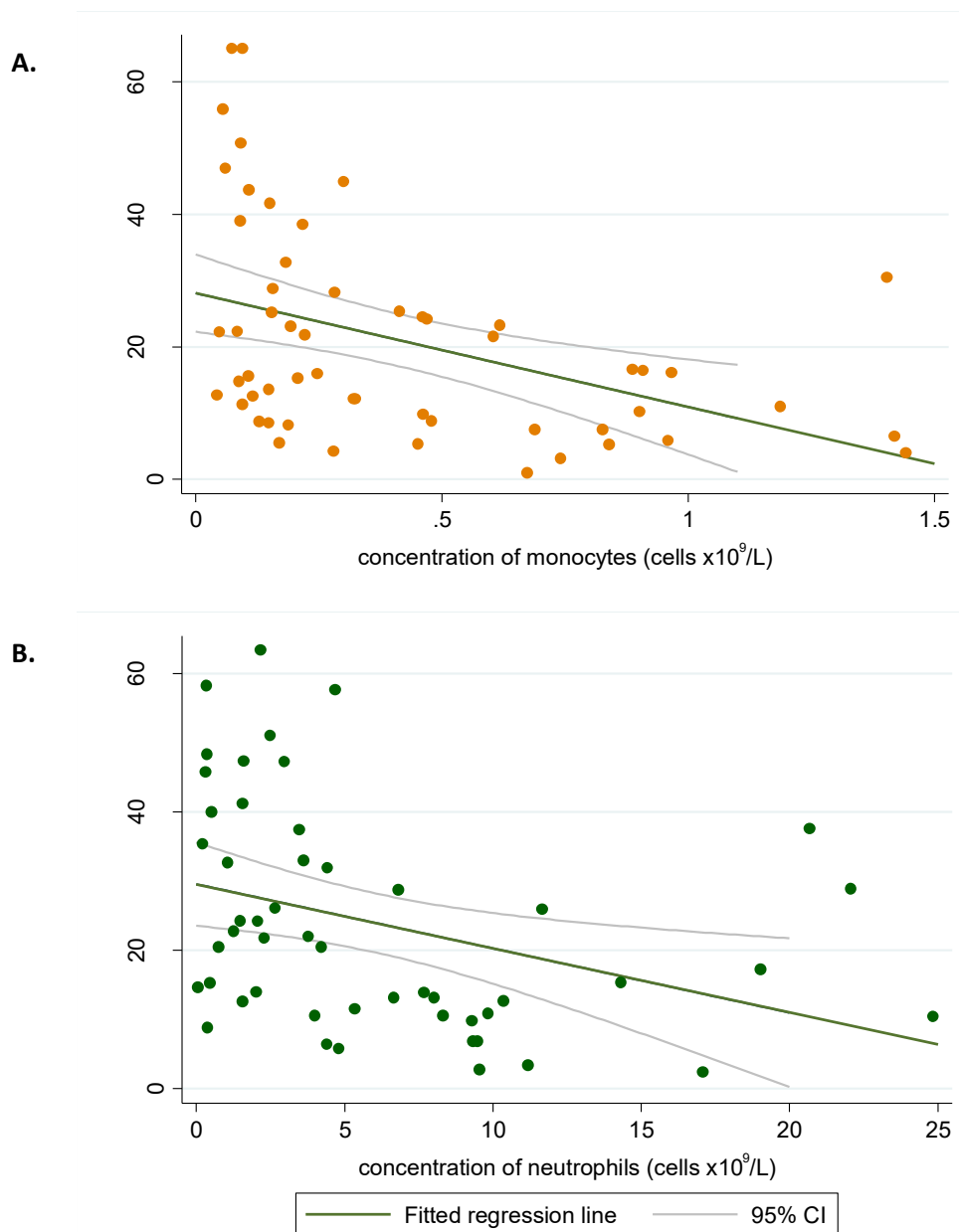


Figure 9.3 Concentration of whole blood phagocyte and proportion phagocytosing zymosan reporter particles. Phagocytosis, as measured by % of cells taking up zymosan particles, was strongly correlated with the concentration of **A.** monocytes and **B.** neutrophils. Each data point represents a single patient. Lines represent fitted regression line (green) and 95% confidence interval (grey). Regression coefficients were **A.** -4.14 (95%CI -8.02 to -0.26), $p=0.037$ and **B.** -0.63 (95%CI -1.02 to -0.25), $p=0.002$.

Phagocyte superoxide burst activity was reduced for monocytes in both HIV/TB patients compared to controls and in HIV/TB patients who died compared to survivors (figure 9.4). Disseminated HIV/TB was not associated with impaired superoxide burst function (although this comparison was underpowered due to small numbers of non-disseminated TB cases). Phagocyte superoxide function remained associated with mortality after adjusting for age, gender, baseline CD4 cell count and HIV viral load (HR 0.24 for every 1×10^4 increase in AI for monocytes, $p=0.006$; HR 0.34 for every 1×10^4 increase in AI for neutrophils, $p=0.004$).

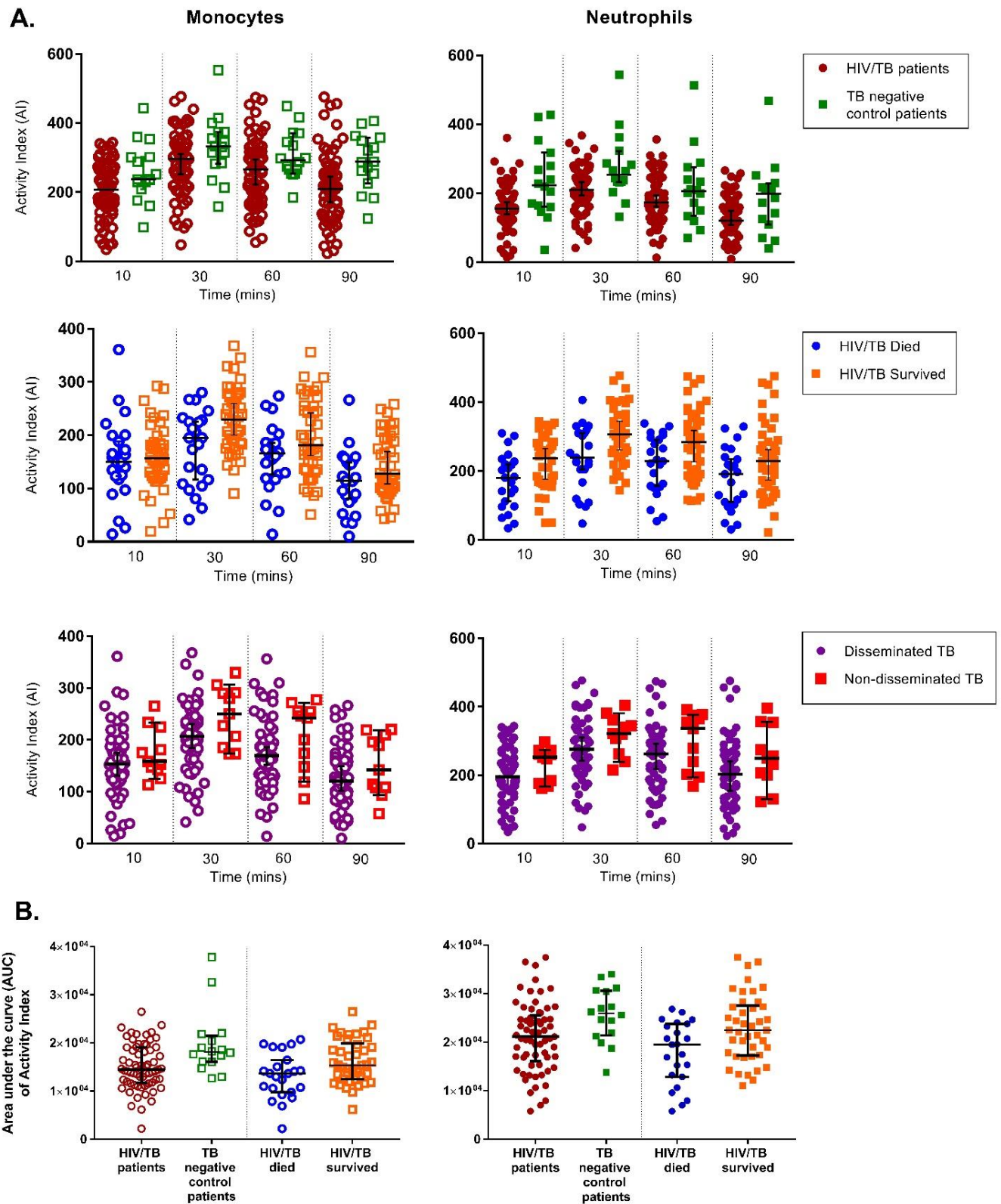


Figure 9.4 phagocytosis and superoxide burst function by patient TB status and outcome.

A. Data represent the activity index for neutrophils (solid markers) or monocytes (hollow markers) at four timepoints in different groups of patients. Data points are values for individual patients, horizontal lines are medians for that group, and error bars are interquartile ranges for that group. **B.** Area under the curve of phagocyte superoxide activity index for monocytes (hollow symbols) and neutrophils (solid symbols). Superoxide activity was reduced in HIV/TB patients compared to TB-negative controls ($p=0.002$ for monocytes and $p=0.02$ for neutrophils), and in HIV/TB patients who died compared to HIV/TB patients who survived ($p=0.01$ for monocytes and $p=0.004$ for neutrophils).

In monocytes, phagocyte superoxide burst function was correlated with monocyte phenotype ($r=0.32$, $p=0.0084$). Patients with higher proportion of monocytes expressing the 'classical' phenotype ($CD14^+CD16^-$) had increased superoxide burst function (figure 9.5).

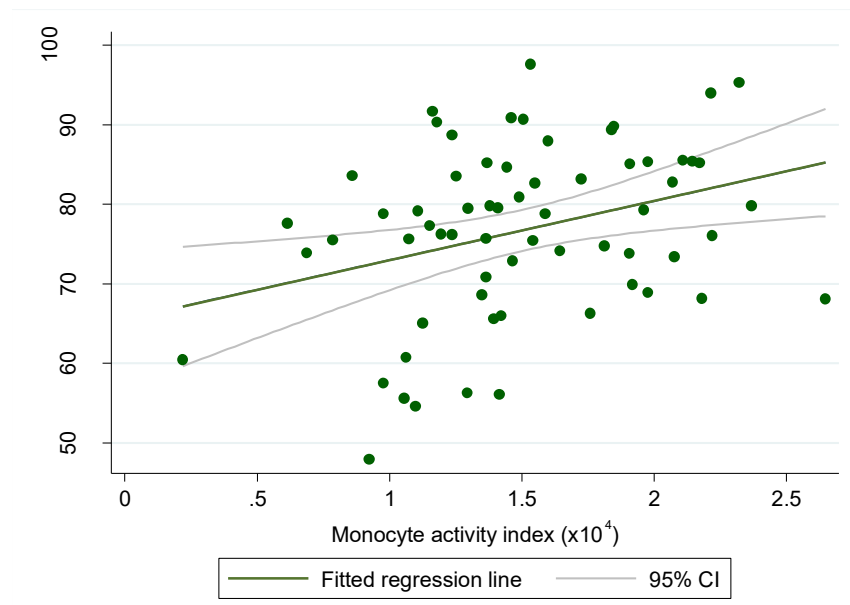


Figure 9.5 Classical monocyte phenotype ($CD14^+CD16^-$) and phagocytic function in HIV/TB patients. Proportion of classical monocytes and monocyte phagocytosis and superoxide burst activity. Each data point represents a single patient. Lines represent fitted regression line and 95% confidence interval.

Monocyte cytokine production in response to stimulation

Intracellular cytokine staining showed that lower percentages of whole blood monocytes produced IL-6 and TNF- α in HIV/TB patients who died compared to survivors in response to TB antigens (*Mycobacterium tuberculosis* culture filtrate protein) ($p=0.001$ and $p=0.01$ respectively) and PMA/ionomycin ($p=0.025$ and $p=0.005$ respectively, table 9.1 and Figure 9.6). Similarly, a greater proportion of monocytes produced no cytokines after stimulation with TB antigens for patients who died compared to survivors ($p=0.006$), and disseminated HIV/TB compared to non-disseminated TB (unstimulated sample, $p=0.01$).

Higher proportions of monocytes produced IL-10 in HIV/TB patients compared to controls after stimulation with TB antigens ($p=0.008$), but there were no differences in IL-10 production by outcome or disseminated TB status (table 9.1). Pro-inflammatory cytokine production (IL-6 and TNF- α) did not differ significantly between HIV/TB patients and controls (Figure 9.7).

Cytokine	Stimulation	TB negative control patients (n=16)	HIV/TB patients				
			All (n=61)	Died (n=21)	Survived (n=40)	Disseminated	
						Yes (n=10)	No (n=51)
IL-6	Unstimulated	10.1 (5.5 - 18.2)	19.4 (10.2 - 25.2)	15.8 (7.4 - 23.6)	20.4 (10.4 - 27.7)	19.4 (10.2 - 25.2)	19.4 (8.4 - 26.8)
	TB antigens	38.7 (28.1 - 48.1)	34.1 (17.5 - 65.5)	18.4 (12.6 - 27.4)	50.9 (30 - 69.1)	33.1 (15.9 - 65.5)	52.8 (25.7 - 66.1)
	PMA/ionomycin	12.8 (5.2 - 17.8)	9.8 (6.6 - 17)	7.8 (5.6 - 12.7)	11.3 (7.4 - 20.2)	9.5 (6.5 - 17.3)	13.3 (8.6 - 17)
TNF- α	Unstimulated	3.7 (1.7 - 6.3)	4.9 (3.1 - 9.2)	4.2 (3 - 8.2)	4.9 (3.4 - 9.8)	4.4 (2.7 - 9.2)	5.5 (4 - 30.3)
	TB antigens	32.9 (25.8 - 43.5)	20.4 (10.9 - 44.3)	11.9 (6 - 26)	26.3 (15.3 - 50.1)	19.4 (10.6 - 44.3)	26.5 (12.1 - 54.2)
	PMA/ionomycin	36.9 (23 - 43.7)	17.8 (12 - 35.4)	13.6 (7 - 17.4)	27.9 (14.1 - 44.6)	17.5 (9.9 - 35.4)	23.1 (14.9 - 50.8)
IFN- γ	Unstimulated	5 (3.6 - 7.3)	6.4 (3.6 - 10.9)	6.5 (3.5 - 10.4)	6.2 (3.7 - 11.1)	6.5 (3.6 - 10.4)	6.2 (3.9 - 21.2)
	TB antigens	8.3 (4.6 - 15.1)	12.2 (7.2 - 16.6)	8.7 (6.2 - 13.9)	13.9 (7.8 - 19.9)	12.1 (7.1 - 17.2)	13.6 (9.1 - 16.6)
	PMA/ionomycin	17.8 (12.7 - 38)	18.1 (11.3 - 26.3)	15.5 (7.7 - 18.2)	20.8 (13.7 - 30.2)	18.1 (10.3 - 27.1)	18.9 (14.1 - 25.3)
IL-10	Unstimulated	8 (6.1 - 11)	13.4 (8.1 - 22.6)	13.4 (8.1 - 23.3)	13.5 (8.4 - 21.9)	13.1 (8 - 21.1)	18.2 (9.6 - 37.4)
	TB antigens	13 (5.6 - 19.6)	22.2 (11.7 - 40.8)	20 (14.4 - 32.2)	27.7 (8.6 - 41.3)	20.7 (9.9 - 41.2)	30.4 (12.7 - 39.6)
	PMA/ionomycin	6 (3.1 - 10.2)	7.9 (4.9 - 12.5)	6.6 (3.6 - 9.3)	8.3 (5.3 - 14.3)	7.3 (4.5 - 10.3)	19.8 (8.3 - 26.1)
No cytokines	Unstimulated	72.4 (66.3 - 78.7)	62.4 (53.1 - 69.6)	66 (60.9 - 73.6)	58 (45.1 - 69.1)	64 (54.7 - 70.7)	48 (34.1 - 62)
	TB antigens	46.8 (35.2 - 59.8)	36.9 (19.1 - 57.9)	54 (32 - 65.3)	28.7 (17.2 - 49.4)	40 (20.3 - 59.6)	21.1 (17.8 - 47.6)
	PMA/ionomycin	43.3 (34.5 - 50)	55.5 (42.7 - 67.9)	66.2 (59.9 - 75.8)	48.3 (37.6 - 58.7)	57.7 (44.4 - 71.8)	49.2 (29 - 52.6)

Table 9.1 Intracellular cytokine production by stimulated monocytes. Data are median % and interquartile range of cells expressing each cytokine intracellularly by patient group. Abbreviations: IL interleukin, IFN interferon, TNF tissue necrosis factor.

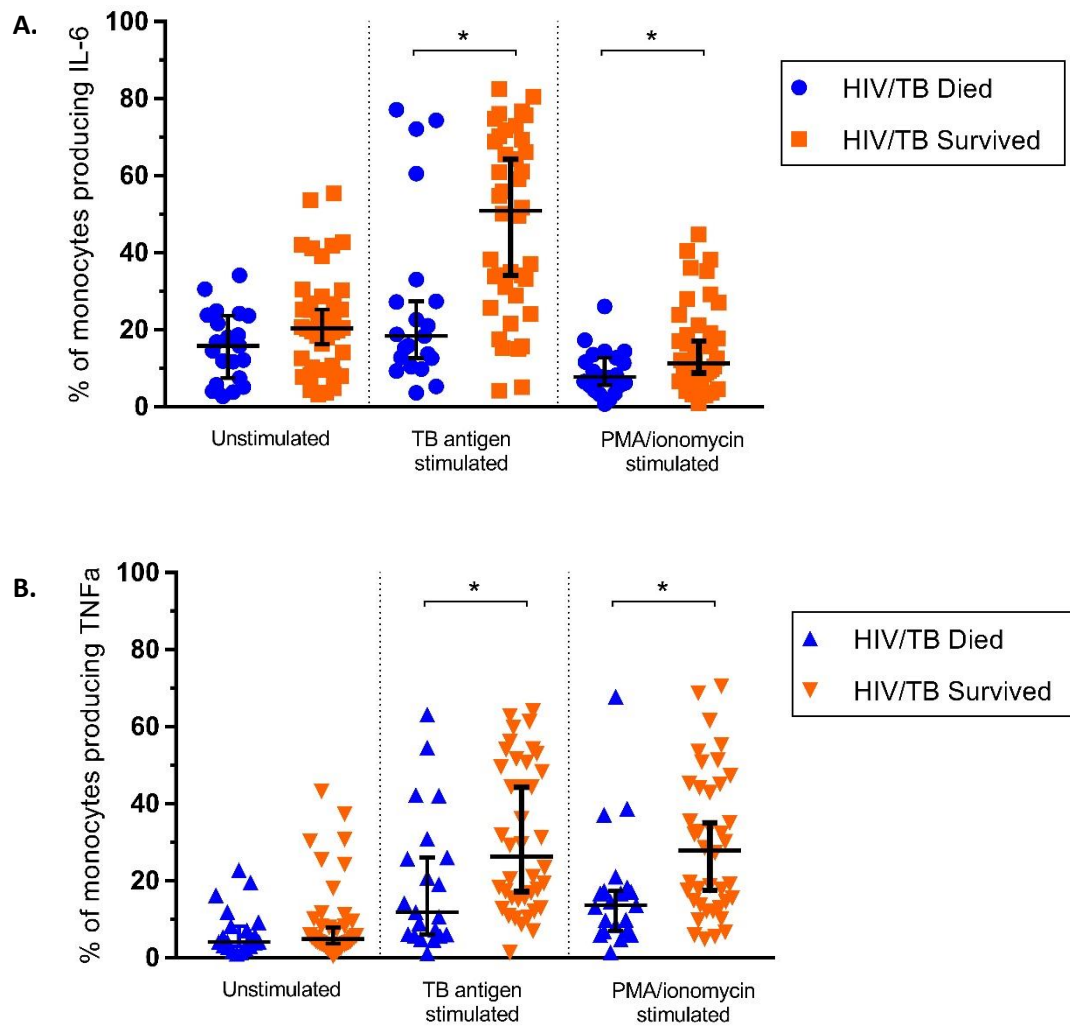


Figure 9.6 Pro-inflammatory cytokine production by monocytes in HIV/TB patients by outcome. Data represent the proportion of monocytes expressing IL-6 (A, circles or squares) or TNF- α (B, triangles) intracellularly for patients who died or survived. Horizontal lines are medians for that group, and error bars are interquartile ranges for that group. * represents $p < 0.05$.

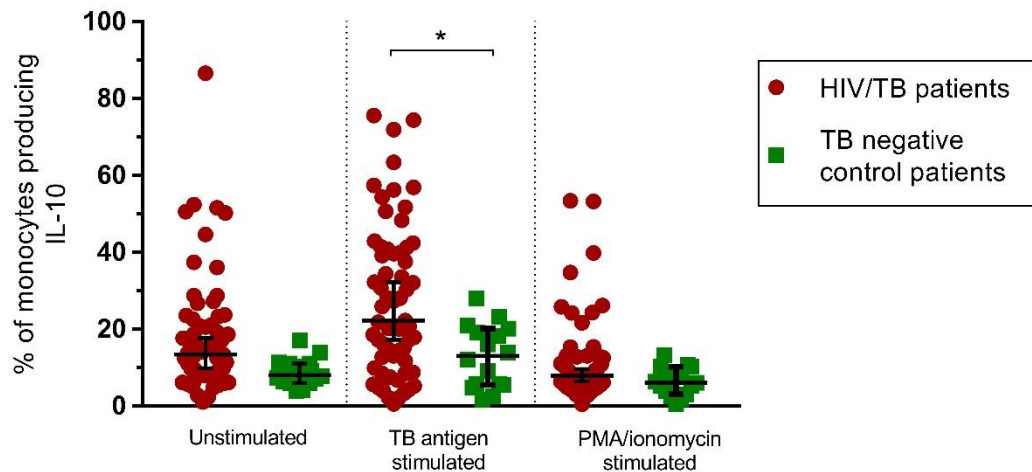


Figure 9.7 IL-10 production in HIV/TB patients and TB negative controls. Data represent the proportion of monocytes expressing IL-10 intracellularly for patients who were HIV-positive TB negative controls or HIV/TB patients. Horizontal lines are medians for that group, and error bars are interquartile ranges for that group. * represents $p < 0.05$.

T-cell cytokine production in response to stimulation

We assessed antigen-specific CD4⁺ T cell responses to TB antigens and to the mitogens PMA/ionomycin. Amongst HIV/TB patients, those who died had a lower proportion of cells producing TNF- α (19.9% compared to 38.5%, $p = 0.0002$) and IFN- γ (20.0% compared to 41.2%, $p = 0.0003$) than survivors in response to PMA/ionomycin (table 9.2, Figure 9.8). Similarly, patients who died had a lower intracellular pro-inflammatory cytokine response to TB antigens ($p = 0.032$ for TNF- α and $p = 0.0065$ for IFN- γ , table 5.12, Figures 5.19 and 5.20). When comparing cytokine responses in disseminated and non-disseminated TB, those with disseminated disease had lower proportions of CD4⁺ T cells producing TNF- α ($p = 0.006$) and IFN- γ ($p = 0.02$) in response to TB antigens.

Control patients had a substantially higher frequency of CD4⁺ T cells produce both TNF- α and IFN- γ than HIV/TB co-infected patients in response to PMA/ionomycin (table 9.2, Figure 9.9). Responses to TB antigens did not differ. IL-22 production on stimulation did not differ by patient group or outcome. In exploratory analysis, CD4⁺ T cell responses were not associated with HIV viral load, absolute CD4 cell concentration or presence of danger signs at admission.

The proportion of CD4⁺ T-cells producing IFN- γ or TNF- α alone, or both cytokines in response to PMA/ionomycin differed between HIV/TB patients and controls (29.1% compared to 44.9% respectively, although not statistically significant, $p = 0.059$) and HIV/TB patients who died and survived (20.1% compared to 37.5%, $p = 0.005$, figure 9.10). Polyfunctionality (production of more than one cytokine) after TB antigen stimulation did not differ by patient group.

Cytokine	Stimulation	TB negative control patients (n=16)	HIV/TB patients				
			All (n=61)	Died (n=21)	Survived (n=40)	Disseminated	
						Yes (n=10)	No (n=51)
TNF- α	Un-stimulated	0.7 (0.1 - 1.6)	0.1 (0 - 1.2)	0.3 (0 - 1.2)	0.1 (0 - 0.8)	0.1 (0 - 1.2)	0.3 (0 - 4.6)
	TB antigens	1.1 (0.1 - 2.5)	1.1 (0.2 - 3.4)	0.5 (0 - 1.5)	1.6 (0.4 - 4.1)	0.7 (0.2 - 2.8)	4 (1.8 - 7.9)
	PMA/ ionomycin	59.5 (47.6 - 64.1)	32.5 (17.7 - 46.2)	19.9 (12.1 - 29.6)	38.5 (27 - 52.8)	32 (17.3 - 44.1)	46.5 (22.2 - 66.6)
IFN- γ	Un-stimulated	1 (0.5 - 2.7)	0.5 (0.2 - 3)	2.1 (0.2 - 4.4)	0.4 (0.1 - 2.2)	0.4 (0 - 3)	1.8 (0.3 - 4.9)
	TB antigens	1.5 (0.7 - 3)	1.9 (0.8 - 4.6)	1.3 (0.7 - 3.3)	2.6 (0.9 - 6.6)	1.7 (0.7 - 4.1)	4.9 (3.9 - 9)
	PMA/ ionomycin	60.4 (49.7 - 68.3)	28.6 (19.2 - 47.8)	20 (10.1 - 26.3)	41.2 (23.5 - 54.1)	28.5 (17.9 - 46.8)	43 (21.4 - 72.4)
IL-22	Un-stimulated	0.2 (0.1 - 0.5)	0.3 (0 - 0.7)	0.4 (0.1 - 0.6)	0.3 (0 - 1.2)	0.2 (0 - 0.6)	0.7 (0.2 - 1.4)
	TB antigens	0.2 (0.1 - 0.7)	0.5 (0.2 - 1)	0.5 (0.1 - 1)	0.5 (0.2 - 1.1)	0.5 (0.2 - 1)	0.6 (0.3 - 0.8)
	PMA/ ionomycin	1.8 (1.4 - 3.2)	1.5 (0.8 - 2.4)	1.2 (0.8 - 1.9)	1.6 (0.8 - 2.7)	1.4 (0.7 - 2.7)	1.7 (1.5 - 2.1)

Table 9.2 Intracellular cytokine production by stimulated CD4+ T-cells. Data are median % and interquartile range of cells expressing each cytokine intracellularly by patient group. Abbreviations: IL interleukin, IFN interferon, TNF tissue necrosis factor.

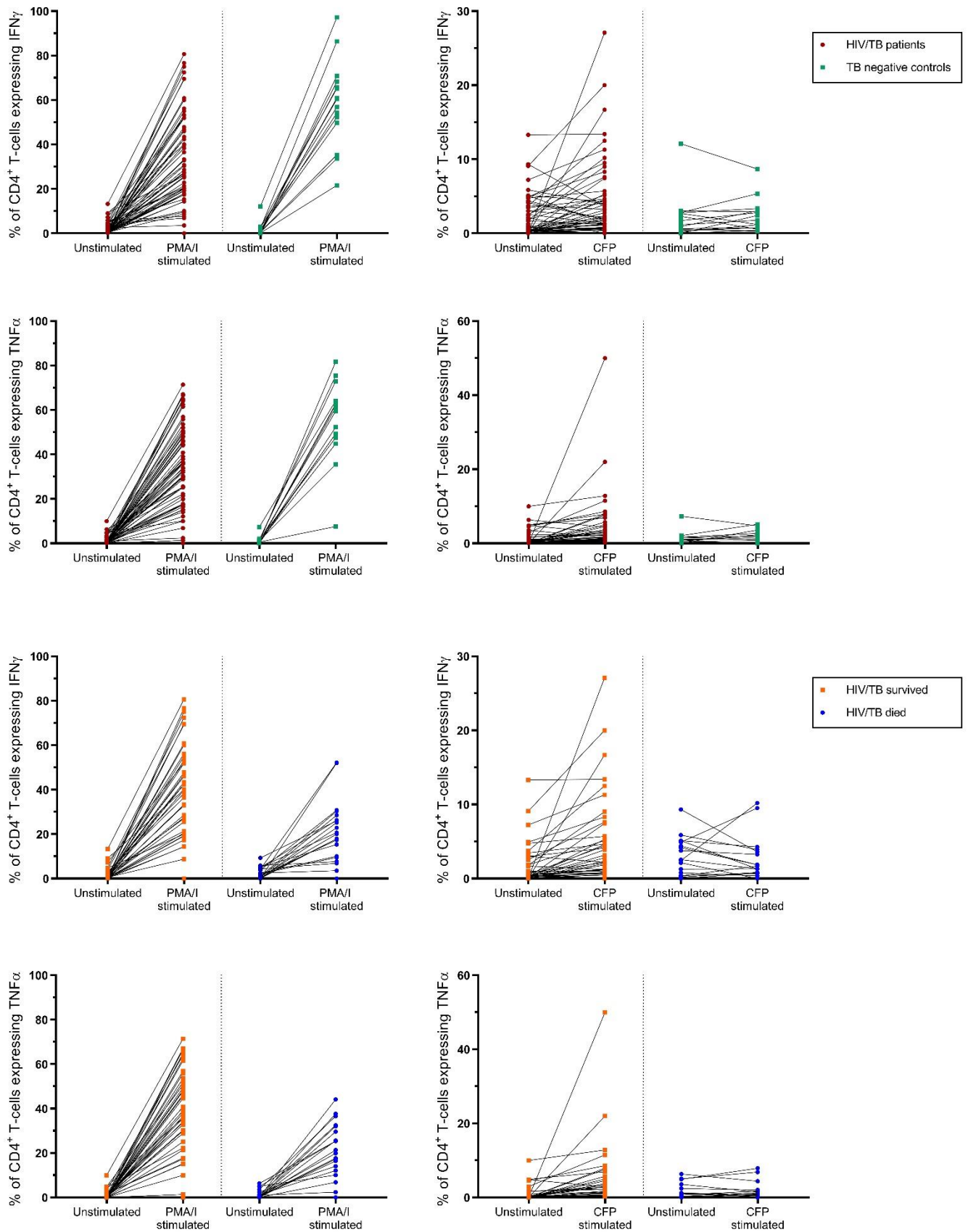


Figure 9.8 Change in intracellular cytokine expression upon stimulation in CD4+ T-cells. Data show % of cells expressing IFN- γ or TNF- α in unstimulated or stimulated conditions by outcome or disseminated TB in HIV/TB patients. Lines connect data points for the same individual.

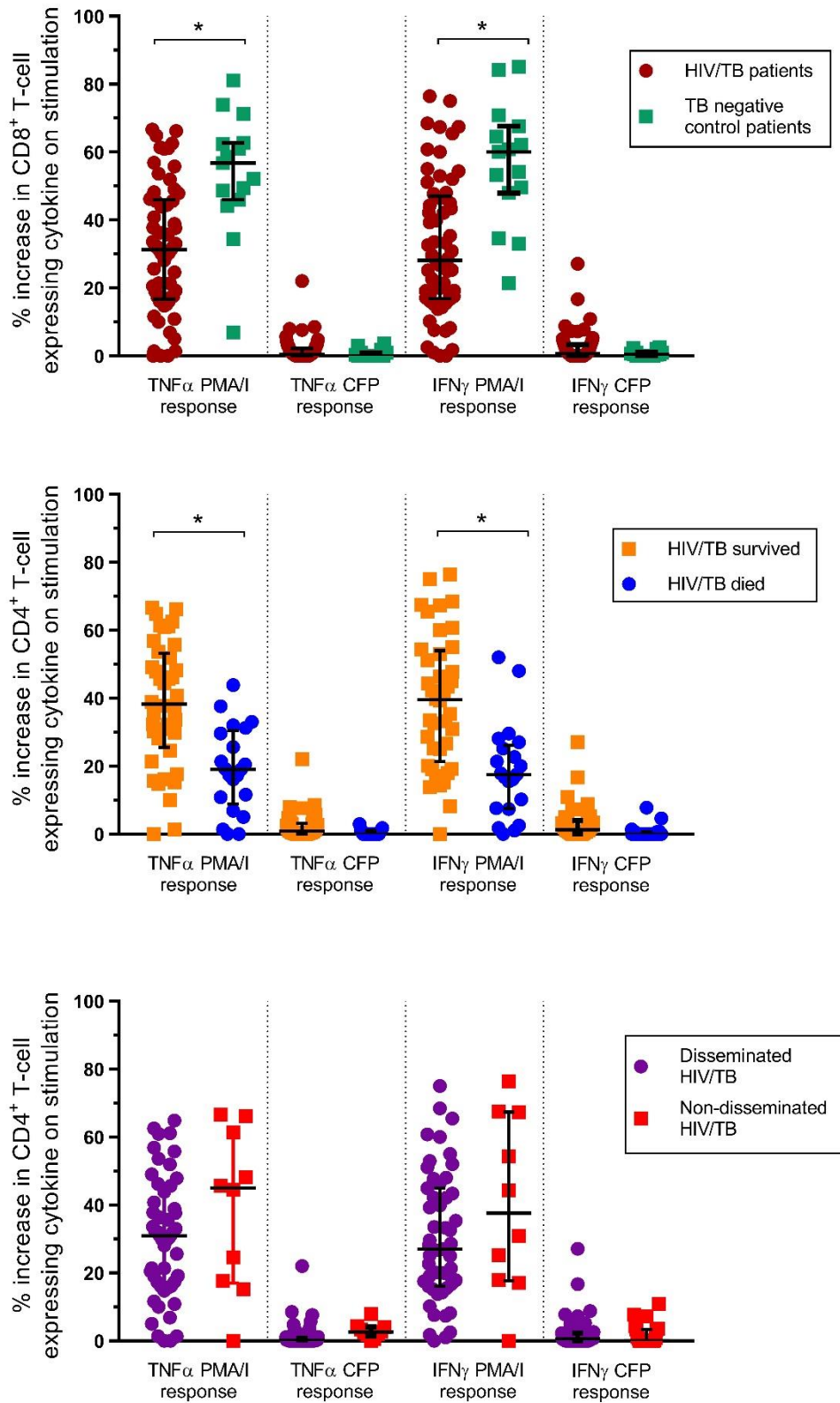


Figure 9.9 Pro-inflammatory cytokine responses to stimulation by CD4⁺ T-cells.

Data represent the proportion increase of CD4⁺ T-cells expressing TNF α (left) or IFN γ (right) intracellularly upon stimulation compared to unstimulated samples by patient group. Horizontal lines are medians for that group, and error bars are interquartile ranges for that group. * represents p < 0.05.

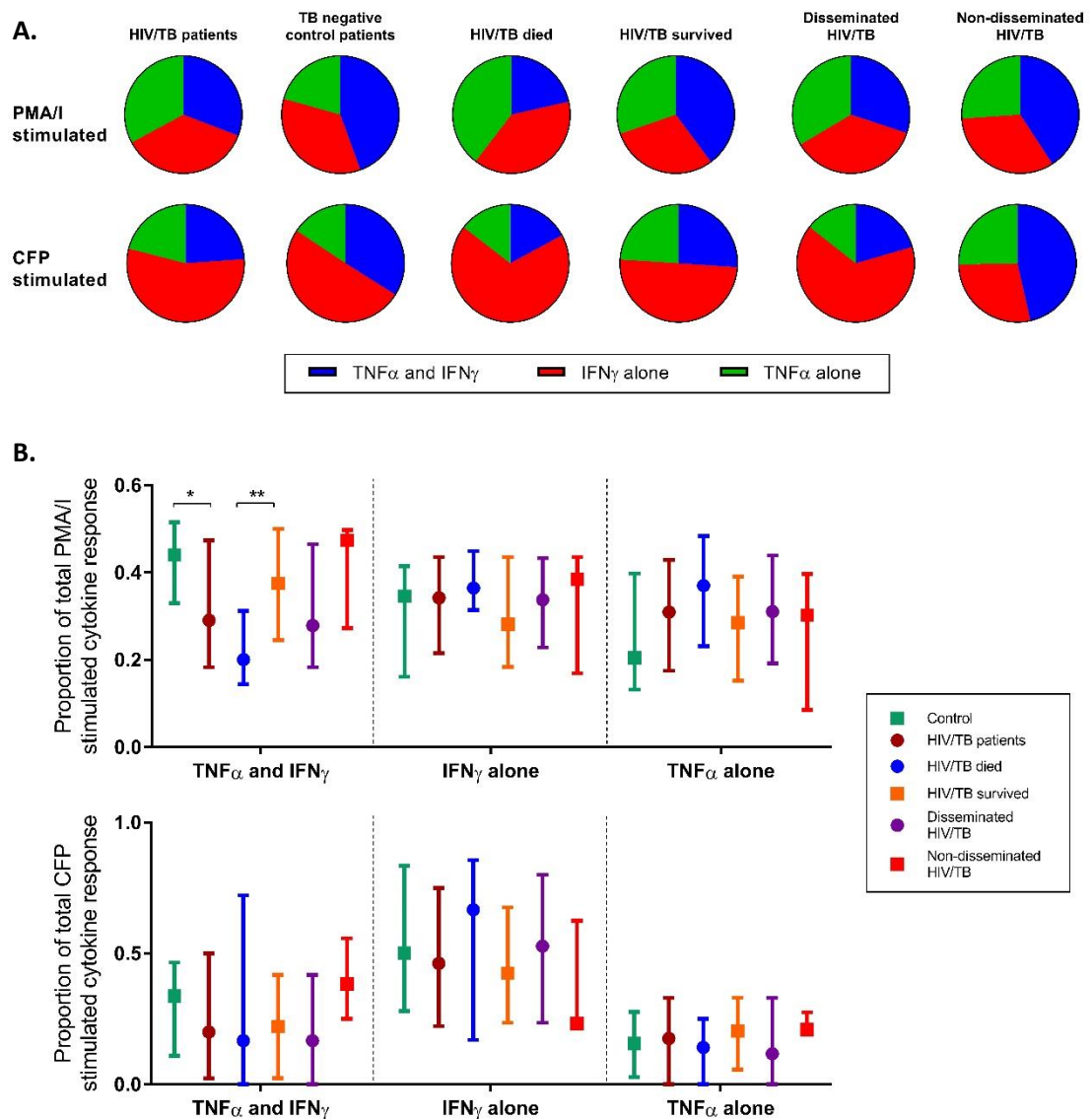


Figure 9.10 Polyfunctionality of CD4⁺ T-cells in production of pro-inflammatory cytokines by patient group. **A.** Pie charts presents the median proportion of total stimulated cytokine response that was composed of cells producing both TNF- α and IFN- γ (blue, polyfunctional cells), or cells producing IFN- γ alone (red) or TNF- α alone (green). **B.** Data points represent the median proportion of total stimulated cytokine response and errors bars are the interquartile range. * denotes $p=0.06$, ** denotes $p<0.05$.

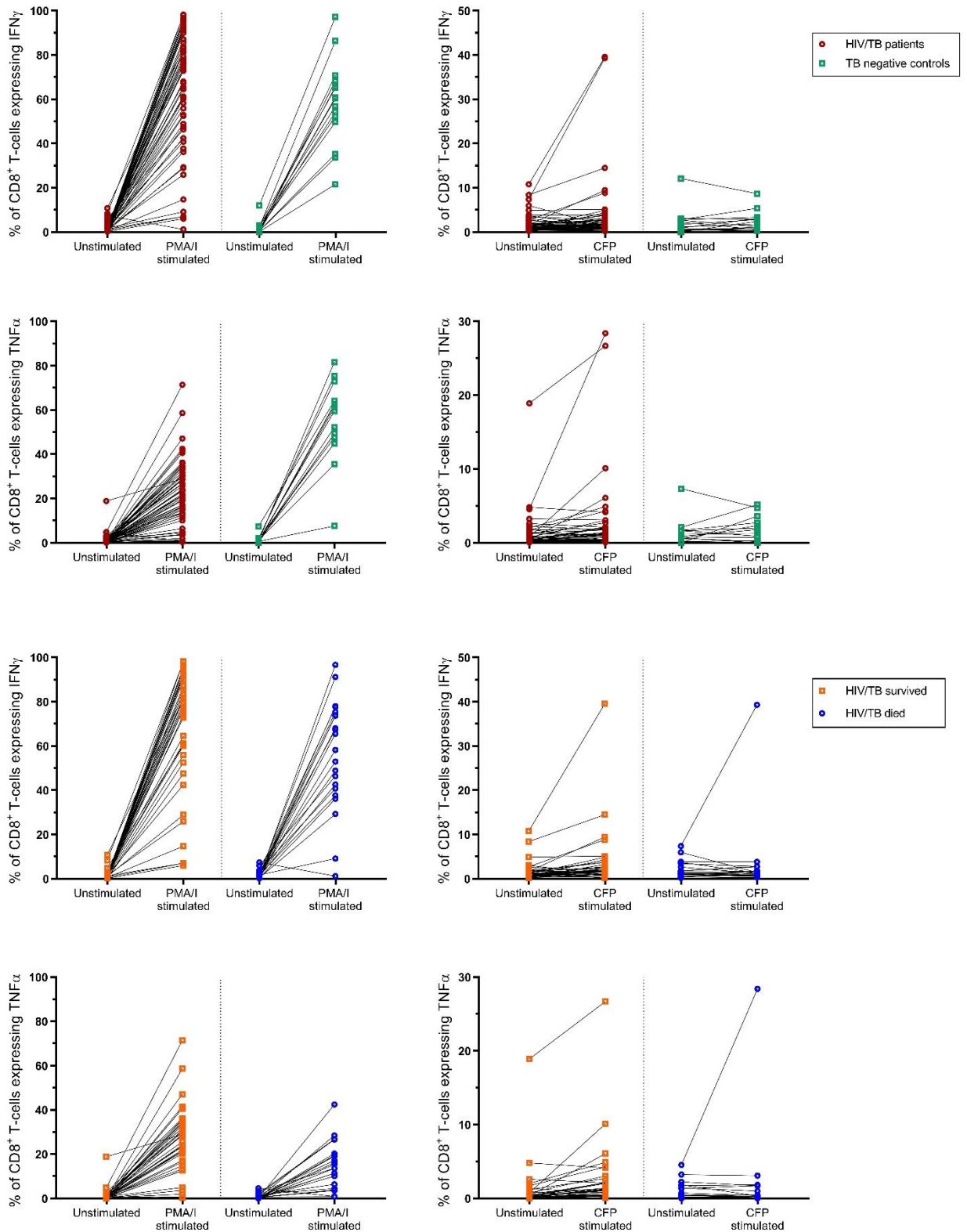
Next, we compared CD8⁺ T-cell pro-inflammatory cytokine production in response to TB antigens and PMA/ionomycin (table 9.3). CD8⁺ T-cells from HIV/TB patients who died differed from survivors in their ability to produce cytokines on stimulation. A lower proportion of cells from HIV/TB patients who died compared to survivors produced TNF- α in response to both TB antigens (0.1% versus 0.7%, $p=0.003$) and PMA/ionomycin (13.6% versus 23.8%, $p=0.005$). Similarly, IFN- γ was produced by a lower proportion of cells from HIV/TB patients who died

compared to survivors in response to TB antigens (0% versus 0.5%, $p=0.002$) and PMA/ionomycin (57.3% versus 76.4%, $p=0.024$, figure 9.11). Patients with disseminated HIV/TB also had a lower increase in the proportion of CD8⁺ cells producing TNF- α (0.1% versus 2.1%, $p=0.002$) and IFN- γ (0.3% versus 2.8%, $p=0.0003$) than patients with non-disseminated TB (figure 9.12).

HIV/TB patients had a lower proportion of CD8⁺ cells producing TNF- α in response to PMA/ionomycin compared to HIV-positive but TB-negative controls (12.5% versus 21.2%, $p<0.0001$), but not IFN- γ or either cytokine in response to TB antigens.

Cytokine	Stimulation	TB negative control patients (n=16)	HIV/TB patients				
			All (n=61)	Died (n=21)	Survived (n=40)	Disseminated	
						Yes (n=10)	No (n=51)
TNF- α	Un-stimulated	0.7 (0.1 - 1.6)	0.3 (0.2 - 0.8)	0.3 (0.2 - 0.8)	0.3 (0.1 - 0.8)	0.3 (0.1 - 0.8)	0.4 (0.3 - 2)
	TB antigens	1.1 (0.1 - 2.5)	0.8 (0.3 - 1.8)	0.3 (0.1 - 1)	1.1 (0.4 - 2.1)	0.5 (0.2 - 1.4)	3.2 (0.5 - 6.1)
	PMA/ionomycin	59.5 (47.6 - 64.1)	21.2 (12.5 - 30.5)	13.6 (6.4 - 19.4)	24.9 (15.6 - 33.6)	20.3 (12.5 - 30)	28.9 (1.9 - 31.8)
IFN- γ	Un-stimulated	1 (0.5 - 2.7)	1.1 (0.6 - 1.8)	1.3 (0.9 - 2.7)	1 (0.5 - 1.8)	1.1 (0.5 - 1.8)	1.3 (0.8 - 2.7)
	TB antigens	1.5 (0.7 - 3)	1.6 (1 - 2.7)	1.3 (0.9 - 1.8)	1.8 (1 - 3)	1.4 (0.9 - 2)	3.5 (2.3 - 8.8)
	PMA/ionomycin	60.4 (49.7 - 68.3)	73.9 (47.5 - 85.6)	58.2 (40.8 - 73.9)	78.6 (58 - 90.1)	73.7 (46.4 - 85.6)	80.5 (47.5 - 87)

Table 9.3 Intracellular cytokine production by stimulated CD8⁺ T-cells. Data are median % and interquartile range of cells expressing each cytokine intracellularly by patient group. Abbreviations: IL interleukin, IFN interferon, TNF tissue necrosis factor.



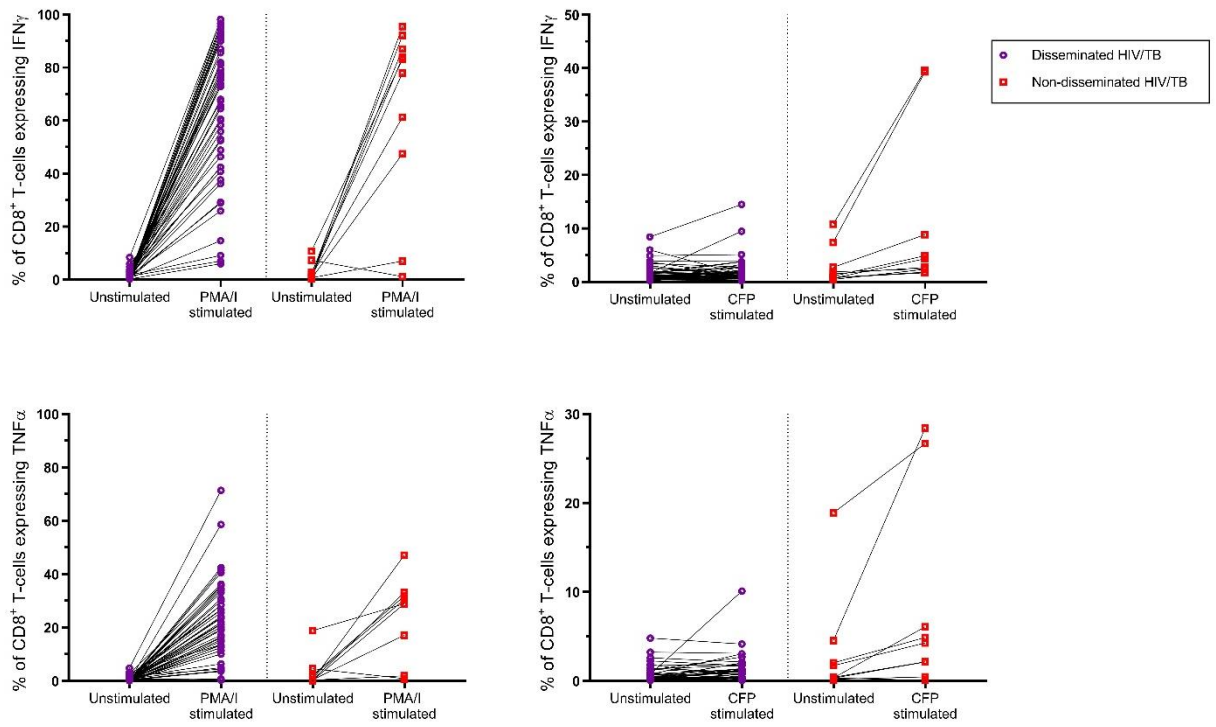


Figure 9.11 Change in intracellular cytokine expression upon stimulation in CD8⁺ T-cells. Data show % of cells expressing IFN- γ or TNF- α in unstimulated or stimulated conditions by outcome or disseminated TB in HIV/TB patients. Lines connect data points for the same individual.

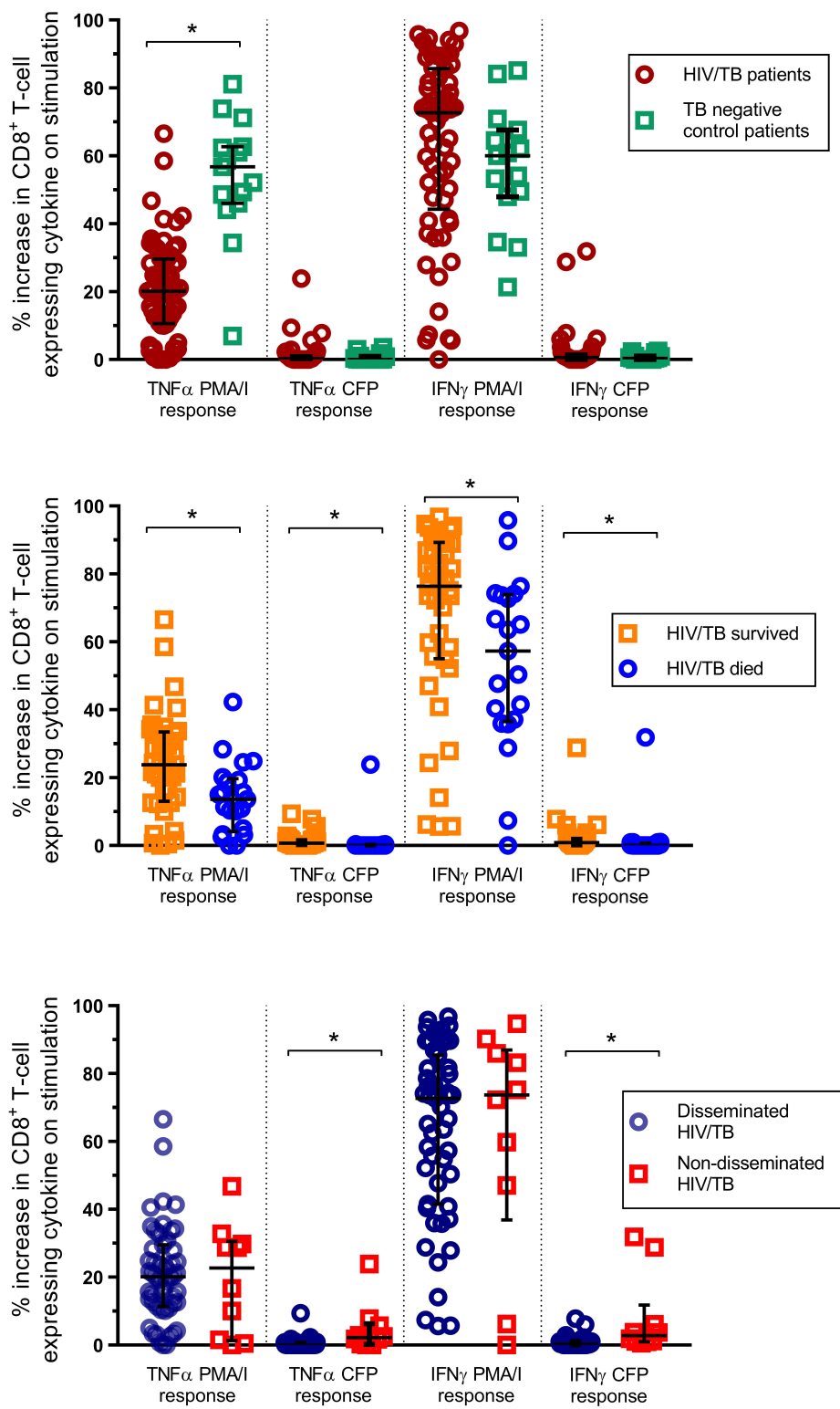


Figure 9.12 Pro-inflammatory cytokine responses to stimulation by CD8⁺ T-cells.

Data represent the proportion increase of CD8⁺ T-cells expressing TNF- α (left) or IFN- γ (right) intracellularly upon stimulation compared to unstimulated samples by patient group. Horizontal lines are medians for that group, and error bars are interquartile ranges for that group. * represents p < 0.05.

Immunological signature

PCA reduced 39 immunological parameters to ten components which explained 71% of the variance. Principal component (PC) 1 was characterised by unstimulated pro-inflammatory cytokine concentrations such as IFN- γ , TNF- α , IL-8, IL-6, MIP-1 and MCP-1 (figure 9.13). PC2 was characterised predominantly by anti-inflammatory cytokines (IL-4, IL-10 and IL-13) as well as inflammation markers (sCD163 and hepcidin) and T-cell and monocyte TNF- α and IFN- γ responses to TB antigen and mitogen stimulation. Patients with HIV/TB who died clustered together when PC1 and PC2 values were plotted (mortality was associated with higher value PC1 and lower value PC2, figure 9.14).

PC1 and PC2 were also strongly associated with mortality in HIV/TB in adjusted and unadjusted models (table 9.4). PC5 (characterised by CD4⁺ T-cell responses to stimulation) was associated with mortality in a model adjusted for PC1 and PC2, however, no other components were associated with mortality. PC1 (p=0.0001) and PC2 (p=0.038) were also associated with TB urine score, with patients with urine score 2 grouped together on the score plot of PC1 and PC2 (figure 9.14).

	Hazard ratio	Lower 95% CI	Upper 95% CI	p-value
Unadjusted/univariable				
PC1	1.3	1.1	1.6	0.0029
PC2	0.6	0.4	0.7	<0.0001
Multivariable				
PC1	2.3	1.5	3.4	<0.0001
PC2	0.4	0.3	0.5	<0.0001
PC5	1.4	0.9	2.0	0.141
HIV viral load	0.8	0.6	1.4	0.160
CD4 cell count	1.0	0.7	1.4	0.862
Urine TB Score	0.75	0.3	1.7	0.488
Age	1.1	1.0	1.20	0.004

Table 9.4 Cox regression model illustrating association between immune signature and mortality. Hazard ratios are for every one unit increase in continuous variables, except HIV and CD4 count where it is for a doubling (\log_2). P-values are calculated by likelihood ratio testing. Abbreviations: CI confidence interval. n=61

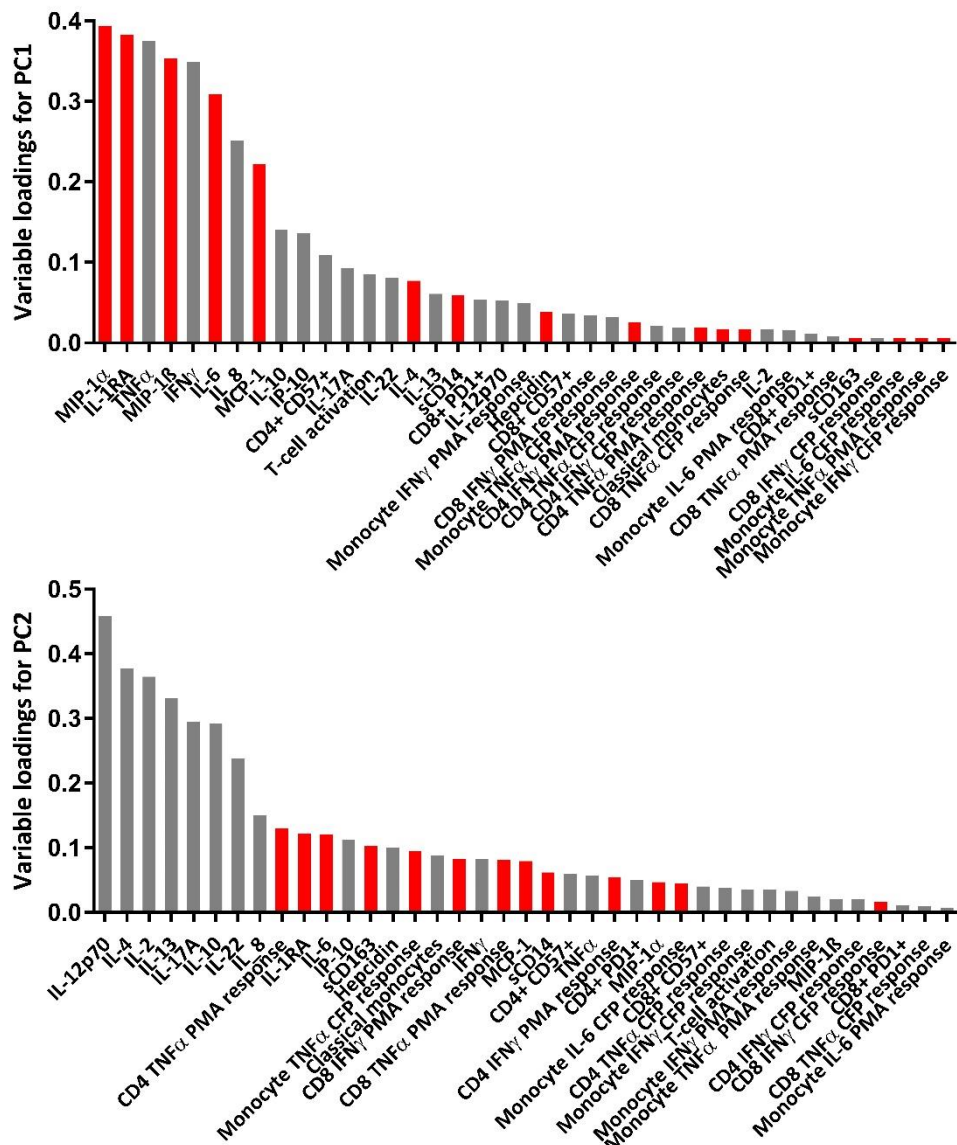


Figure 9.13 Bar chart showing loadings of variables for principle component (PC) 1 and 2. Variables in red were associated with mortality, whereas those in grey were not found to be significantly associated with mortality.

In exploratory analysis, immune responses in HIV/TB patients were not associated with HIV viral load (no correlation with PC1 or PC2, $r = 0.21$, $p = 0.11$ and $r = -0.11$ and $p = 0.40$ respectively). PC1 was correlated with baseline CD4 cell count ($r = -0.48$, $p = 0.001$), but not PC2 ($r = 0.16$, $p = 0.23$). Immune responses were also weakly associated with urine TB-LAM grade ($r = 0.25$, $p = 0.032$ for PC1 and $r = -0.26$, $p = 0.024$ for PC2). There was no association with cycle time threshold for urine or sputum Xpert.

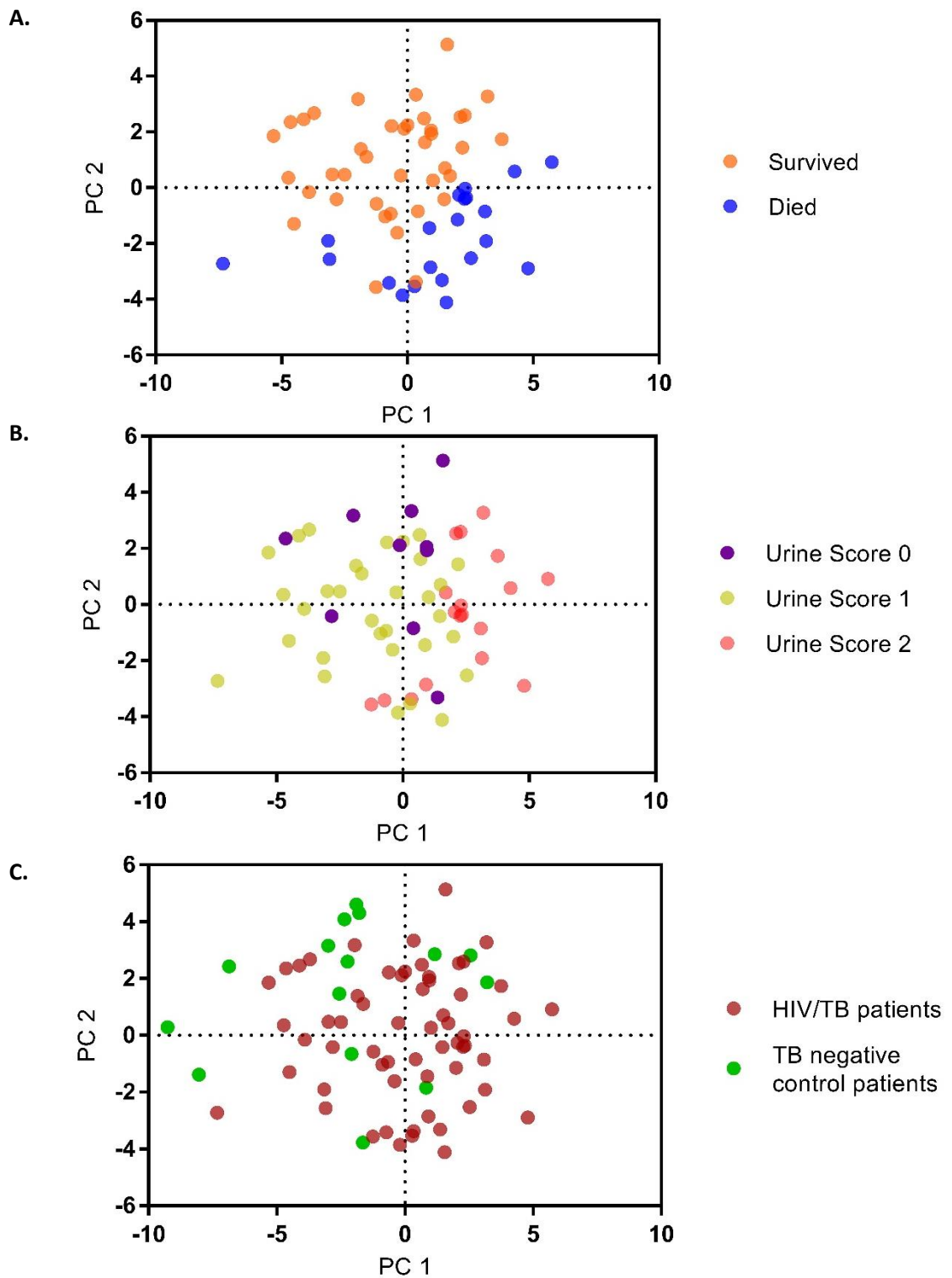


Figure 9.14 Principal component analysis demonstrating differences in baseline immune response between HIV/TB patients by outcome and disseminated TB. Data show principal component (PC)1 and PC2 for baseline immune response plotted for HIV/TB patients by A. their outcome (orange represents patients who survived, and blue patients who died), B. urine TB score representing a degree of dissemination based on number of positive urine tests. C. shows PC1 and PC2 values for HIV/TB patients and TB negative control patients. Clustering is seen most clearly for patients who died (blue) and those with urine TB score 2 (pink). n=61

9.3 Discussion

The main findings of this study were significantly altered immune responses in HIV/TB patients who died compared to those surviving. Patients with poor outcomes had impaired *ex vivo* functional responses, including monocyte and neutrophil phagocyte superoxide function, and T-cell and monocyte cytokine production in response to antigenic stimulation. Data reduction techniques identified distinct immunological phenotypes/signatures that were strongly associated with both disseminated TB score based on urine diagnostics and outcome.

This is one of the first studies assessing immune responses in relation to point-of-care diagnostic tests (most studies to date have focussed on TB-IRIS or differentiating latent and active TB disease). This was also one of few studies to assess plasma and intra-cellular cytokine production. Previous studies have suggested monocytes to be the major source of TNF- α [9], however, we show CD8⁺ T-cells are also an important source (especially given the low absolute numbers of CD4⁺ T-cells in advanced HIV) [10].

The role of CD8⁺ T-cells in HIV/TB co-infection is poorly studied. Whilst, in active TB disease, CD4⁺ T-cells are preferentially involved in antigen presentation and immune activation, CD8 responses are also elicited [11]. This includes cytotoxic roles through granzyme and perforin expression [12,13], but also production of pro-inflammatory cytokines (eg IFN- γ , IL-2 and TNF- α) which orchestrate host responses against *MTB* [14]. This may be even more important in the context of HIV-associated CD4⁺ T-cell depletion, where CD8⁺ T-cell populations are expanded. HIV may also promote the exhaustion of the CD8⁺ T-cell response to *MTB* [15].

These findings demonstrate broad functional immune impairments in HIV/TB patients with poor outcomes. Whilst two recent studies have shown monocyte dysfunction and hyporesponsiveness in TB or HIV/TB co-infected patients who died early [16,17], I also demonstrate dysfunction of T-cells and impairment of monocyte and neutrophil phagocyte function. The pro-inflammatory cytokine profile and raised markers of innate activation (soluble CD14 and CD163) strongly suggest a more activated immunological state, somewhat paradoxically to the impaired functional responses. Possible explanations include anergy, exhaustion or overactivated immunoregulatory pathways, and this finding is consistent with other studies of TB and/or HIV [16,17], and other severe infections such as sepsis and or cryptococcal meningitis [18–20].

Phagocytosis and superoxide production is an important mechanism of killing *MTB* and other pathogens [21]. Whole blood phagocyte superoxide burst was impaired in HIV/TB patients who died. Impaired phagocytic responses have been reported in lung macrophages of HIV-infected patients in the presence and absence of TB co-infection [22,23], and in whole blood of

patients with severe sepsis [24], but not in relation to outcome or disease severity in HIV/TB. In blood, this impairment was seen in neutrophils as well as monocytes, which has not been previously demonstrated in this population. *MTB* antigens, including mannosylated LAM, have been associated with impaired phagocytosis *in vitro*, thought to be an important in the survival of *MTB* within macrophages [25,26]. I was unable to show association between urine-positivity and impaired phagocyte function *ex vivo*, possibly due to too few HIV/TB patients without disseminated disease in our cohort (thereby our comparisons were underpowered).

Neutrophils have only recently been recognised as important in the host responses in HIV/TB co-infection, mostly through their ability to phagocytose *MTB* bacilli and other defence mechanisms (including oxidative burst, apoptosis, antimicrobial peptides and cytokine production) [27,28]. Neutrophils predominate in gene expression signatures of *MTB*, are crucial in defence against bacterial superinfection or sepsis, and are putative targets for host-directed therapies [29]. HIV-infection has been shown to impair activation, phagocytosis, oxidative burst and microbial killing ability of neutrophils, although few data exist on the impact of TB on neutrophil function. The consequence of impaired neutrophil killing of *MTB* maybe the induction of anti-inflammatory responses and subsequent persistence and/or propagation of infection [27,30].

Principle component analysis demonstrated a blood immune response at hospital admission that was associated with urine TB score (and therefore disseminated TB), and differed between patients who survived or died. Patients who died clustered based on their values for PC1 and PC2, which were characterised by higher levels of pro-inflammatory cytokines IFN- γ , TNF- α , IL-8, IL-6, MIP-1 and MCP-1 (PC1), and reduced anti-inflammatory (IL-4, IL-10, IL-13) and T-cell and monocyte responses to stimulation (PC2). This immune profile remained strongly associated with outcome even after adjustment for other predictors of mortality, including HIV viral load and CD4 cell count.

PC1 was more strongly associated with disseminated TB, suggesting a more activated and inflammatory immune response in this patient group. A recent study in hospitalised HIV/TB co-infected patients from South Africa found a similar immunological signature associated with mortality, characterised by impaired cytokine responses to stimulation despite high unstimulated proinflammatory cytokine production [17]. Similar signatures have also been reported in severe HIV-associated cryptococcal meningitis [31]. This immunological dysfunction could be due to advanced HIV-infection. However, I found that baseline HIV viral load and absolute CD4 cell count were not strongly associated with the immune signature whilst TB dissemination was, suggesting that TB may be a more important driver of this immune dysfunction than HIV.

In addition to impairing anti-tuberculosis host defences, this immune profile may impair responses to bacterial or other co-infections in HIV/TB patients, which may be important in the pathway to mortality. Although we did not look for bacterial co-infections in this cohort it is possible that they were present since 15% of the patients also had circulating cryptococcal antigen. Furthermore, previous post-mortem studies suggest that bacterial superinfection is common [32,33].

The strengths of this study include being nested within a TB screening trial, therefore this cohort was not restricted to patients with TB symptoms or suspected of TB by clinical teams. In addition, we assessed a broad range of immune responses beyond simply profiling cytokines levels, incorporating several functional responses of multiple cell types. Our phagocytosis and superoxide burst assay measured function at an individual cellular level, and was not biased by cell counts or concentrations [8]. The high mortality risk in this cohort meant that the study was well powered to compare immune responses between patients with good and poor outcomes. The cohort was well characterised clinically, and had high ART coverage reflecting contemporary HIV/TB patient cohorts in sub-Saharan Africa. There were few missing data from this study.

There are, however, several limitations to the study. Due to the high prevalence of disseminated TB disease, the study was underpowered to detect differences between patients with disseminated and non-disseminated TB. Measuring multiple immunological mediators and pathways led to multiple testing, which increases risks of false positive hypothesis tests. Methods used to reduce the risks of false positive associations may also increase the risk of missing associations. In addition, due to the cross-sectional design and measuring immune responses at only one timepoint, we are unable to say if the immune profiles associated with mortality are causal, or they are markers of more severe illness, which could be associated with a worse prognosis through other mechanisms. Similarly, it is not possible to say if innate activation, inflammation and anergy promote dissemination of *MTB* or are a consequence of disseminated disease.

I have described exhaustion or anergy causing impaired production of the inflammatory cytokines TNF- α and INF- γ from whole blood T-cells and monocytes recovered and stimulated *ex vivo*. This does not explain which cells are responsible for high *in vivo* concentrations in the same patients. *Ex vivo* experiments may also not reflect *in vivo* conditions, although I chose to use whole blood instead of peripheral blood mononuclear cells (PBMCs) to better reflect *in vivo* conditions and reduce alterations in cell function caused by processing and isolating PBMCs [3]. Fixing, freezing and storage of cells prior to intracellular cytokine staining may have impacted the results, although this method likely improves reliability by reducing day-to-day

variations in flow cytometry. T-cell responses to CFP overall were poor. However, monocyte responses and healthy volunteer responses (data not shown) were good suggesting this was not due to a problem with the antigen.

The differences in immune parameters between TB-negative controls and HIV/TB patients, and the lack of associations between altered immune responses and HIV viral load and CD4 cell count suggest that *MTB* may be a stronger contributor than persistent/chronic HIV. However, we did not elucidate the mechanisms underlying altered immune responses in HIV/TB co-infection. Specifically, it is not clear if altered phagocyte functions are due to cellular defects or alterations in soluble immune mediators. I only measured one aspect of phagocyte function (super oxide burst) – the same assay could be adapted to measure lysosomal pH and/or proteolysis. Other aspects of killing could be assessed, for example granzyme B and perforin in CD8⁺ T-cells and neutrophils [34].

Since some HIV/TB patients were diagnosed based on TB-LAM alone, we cannot exclude a small number of false positive results, although the specificity of TB-LAM is >99% in hospitalised cohorts when appropriate reference standards are used [35]. I did not ascertain the cause of death in patients who died, although cause of death is difficult to interpret in the absence of post-mortem examination. Finally, we could not exclude coexistent bacterial infection influencing the immune profile. Blood cultures were not performed. However, given that almost all patients received broad-spectrum anti-bacterial agents at or very soon after admission, cultures will have had greatly reduced sensitivity.

Despite these limitations, this study provides important insights into the immune profile of hospitalised patients with HIV-associated TB, a subset of patients with high mortality risk who need to be prioritised for adjunctive interventions to reduce mortality. Our findings show extensive inflammation and immune activation in disseminated HIV/TB, but simultaneously associated with altered and dysregulated cellular and soluble immune responses. This may lead to impaired defence against *MTB*, but also a predisposition or impaired ability to clear co-infections, and is similar to those seen in bacterial sepsis and severe cryptococcal disease. They also suggest that urine diagnostics may be a useful method of identifying patients with higher mortality risk, and immune dysfunction. Although further work investigating mechanisms and pathways will be needed, these findings support ongoing studies of immunomodulatory interventions, in addition to TB treatment and ART, to reduce mortality.

9.4 References

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Chapter 10: Discussion, implications and future directions

Chapter 10: Discussion, implications and future directions

10.1 Summary of findings

This section summarises the findings presented in chapters 3 to 9, in relation to the thesis aim and specific research questions.

Research question 1: Are positive urine-based diagnostics associated with increased mortality?

The systematic review and meta-analysis presented in chapter 3 found a pooled 2.3 (95% CI 1.6-3.1) fold increase in mortality risk for urine LAM positive compared to urine LAM negative patients with HIV-associated TB across 10 studies with 1,172 participants. Importantly, there was also a pooled 2.5 (95% CI 1.4-4.5) fold increase in odds ratio for mortality in LAM positive compared to LAM negative in multivariable analyses adjusted for other risk factors.

Prospectively collected data presented in chapter 5 showed that a positive urine LAM assay was associated with a 1.7 (95% CI 1.0-3.0) fold increase in mortality risk at 2-months in patients with confirmed TB disease. Two-thirds of patients had a positive LAM test. Data from two cohorts of hospitalised patients with HIV-associated TB published after the systematic review was conducted (but presented in chapter 6 and used to validate the clinical score) also demonstrated increased mortality risk in LAM-positive patients [1,2]. Chapter 5 also reported an increased mortality risk associated with positive urine Xpert (OR 1.9, 95% CI 1.3-2.9 compared to urine Xpert negative TB).

Together, these findings provide strong evidence that positive urine diagnostic tests are associated with increased mortality risk in HIV-associated TB. This suggests urine testing can provide valuable prognostic as well as diagnostic uses, and may identify vulnerable patients who could benefit from adjunctive interventions to reduce mortality.

Research question 2: What is the mortality risk and timing of mortality in HIV-associated TB?

Chapter 5 reported a 31% mortality risk by 2 months among 322 inpatients with microbiologically-confirmed TB, despite high antiretroviral therapy (ART) coverage, TB screening and near-universal prompt TB treatment. Mortality during hospital admission was 23%. The mortality risk from TB in HIV-positive patients admitted to hospital in sub-Saharan Africa remains similar to those reported in the review of the literature (see section 1.10, chapter 1), and in a published systematic review and meta-analysis (Ford et al 2015) [3]. Earlier studies predated high population coverage of ART and access to improved diagnostics such as Xpert, suggesting these interventions have not had substantial impact on mortality in the

hospitalised population with confirmed TB-disease. However, the numbers of patients admitted, and therefore diagnosed with TB, may have reduced over time.

Only 9% of deaths occurred within 48 hours of admission and 32% within one week of admission. 28% occurred after discharge from hospital. These findings show the substantial burden of mortality in hospitals attributable to HIV-associated TB, and that mortality in HIV-positive patients with TB is higher than in patients without TB (chapter 8 and [4]). They also imply a potential window to intervene and improve outcomes in the majority of patients, and a need for better evaluation of patients prior to discharge with more intensive follow-up for the sickest patients.

Research question 3: What are the risk factors for mortality and clinical phenotypes of patients with high mortality risk?

Systematic review and meta-analysis in chapter 3 described five studies reporting predictors of mortality, including CD4 cell counts, age, ART status, gender and functional ability. Analysis of risk factors for mortality presented in chapter 5 found older age, male sex, being ART-experienced prior to admission, having poor nutritional status (measured by weight), severe anaemia and positive urine diagnostic tests as factors associated with mortality after adjusting for confounders in a multivariable model. Being positive on both urine LAM and Xpert assays was associated with the highest mortality. Cluster analysis, conducted without reference to the outcome of interest, was also able to identify distinct clinical phenotypes associated with a high (>50%) mortality risk, and included reporting functional problems with activity/mobility, severe anaemia, male gender and positive urine-diagnostics.

High mortality was underscored by disseminated TB, advanced HIV-associated immunosuppression, and/or poor virological control of HIV. These findings present several possible areas for interventions to reduce mortality, including intensified inpatient management strategies, and earlier diagnosis and management of ART failure. Effective upstream public health interventions such as earlier diagnosis of both HIV and TB (using targeted screening as well as passive case finding) and TB prevention with preventative treatment will reduce the burden of patients with advanced HIV-associated TB.

Research question 4: Can a pragmatic clinical score (including urine diagnostic results) be developed and validated?

Chapter 6 reports a clinical score derived from the cohort of microbiologically confirmed TB patients from the STAMP trial using multivariable predictive modelling. Six variables were identified to be included in the score (age 55 years or older; male gender; being ART experienced; severe anaemia (haemoglobin <8g/dL); being unable to walk unaided; and having a positive urine LAM test). The model had good discrimination (c-statistic = 0.70) and calibration (Hosmer-Lemeshow statistic p=0.78).

The score was able to identify a group of patients with a 46% mortality risk, and 6.1 (95% CI 2.4-15.2) fold increase in odds compared to the low mortality risk group. External validation in an independent cohort of patients from two other studies found similar performance of the score. A simplified score using 'any three of six' factors also performed well enough to provide a simple tool that can be implemented in settings moving forward with urine LAM scale-up (eg Malawi and South Africa).

The score shows potential to identify patients who could benefit from enhanced clinical care and follow-up, and as a research tool for assessing new interventional strategies. For example, the score could be delivered at admission for patients diagnosed with TB. It could also be used alongside urine LAM to recruit high risk patients into clinical trials of adjunctive therapies for HIV-associated TB. This score is unlikely to be useful in outpatient or community settings, although future research could look to derive and validate such a score for less acute settings.

Research question 5: Can an immunological assay to classify/assess functional immune responses be developed for use in high HIV/TB burden settings?

Work developing a functional whole blood phagocytosis assay using a fluorescent zymosan reporter particle and flow cytometry is reported in chapter 7. The assay assesses superoxide burst function of monocytes and neutrophils. Kinetics and reproducibility were determined in a cohort of healthy (HIV-negative) volunteers. The readout from the superoxide burst was independent of changes in cell or particle concentration, indicating that the assay was measuring physiological changes in the phagocyte at an individual cell level: this is a significant methodological advance over existing assays, and allows interrogation of this important cellular immune pathway in a range of disease settings.

Assessment of peripheral blood phagocyte function using this assay showed that phagosomal superoxide burst activity of monocytes and neutrophils was substantially and significantly

depressed in a cohort of patients with HIV-associated TB compared to HIV-negative 'healthy' volunteers, and that this was also in relation to monocyte phenotype. The assay showed marked differences between groups based on TB disease phenotype, was highly reproducible and allowed the rapid assessment of functional immune competence of phagocytes in peripheral blood.

Research question 6: Do immune responses differ according to TB disease dissemination (based on urine diagnostic tests) and in relation to mortality?

Immune responses, including soluble plasma immune mediators, peripheral blood cell phenotypes and functional innate and adaptive responses were measured in a cohort of Malawian patients with confirmed HIV-associated TB, and are reported in chapters 8 and 9. Patients dying by 2-months had higher concentrations of cytokines responsible for innate and pro-inflammatory signalling, and markers of inflammation such as C-reactive protein and hepcidin. They also had more marked monocyte activation, but impaired *ex vivo* functional responses, including cytokine production and phagocyte function. CD8⁺ T-cell function was also impaired.

Although the cytokine profiles or functional immune responses were not significantly different in patients with urine-positive disseminated TB compared to urine negative TB patients when comparing individual assay results, the statistical power was limited by the small number of patients with urine-negative TB disease.

However, principle component analysis incorporating 39 immunological parameters identified a blood immune signature at hospital admission that differed by urine TB score (based on urine LAM and urine Xpert) as well as mortality.

The results suggest not only impairment of anti-TB host defences in patients with poor outcomes and/or disseminated disease, but potentially also those against bacteria or other co-infections in patients with HIV-associated TB. Similar profiles have been reported in bacterial sepsis and severe cryptococcal disease [5,6]. The findings also support research into immunomodulatory and adjunctive interventions focusing on reversing immune cell anergy or exhaustion. Urine diagnostics are then a promising method to identify patients with abnormal immune responses who could be targeted for host directed immunotherapies.

10.2 Study limitations

Limitations are discussed in the individual research papers and results chapters (chapters 3-9). Here, overall limitations of the study are discussed.

The studies of mortality were cohort studies nested within a randomised clinical trial of a TB screening strategy [4]. Overall TB prevalence and mortality was lower than anticipated, likely reflecting ART scale-up. Therefore, there were fewer patients in the clinical mortality studies than planned, although sample sizes still had adequate statistical power.

The urine-based (intervention) arm of the study diagnosed significantly more patients with TB. Some TB patients in the sputum only (standard of care, SOC) screening arm will have remained undiagnosed (ie those with negative sputum tests and not diagnosed clinically or radiologically), which will have introduced some selection bias into these studies. In Malawi, only one-third of patients produced sputum samples for TB testing, much lower than in South Africa [4], and making the SOC cohort in Malawi especially vulnerable to under-diagnosis of TB.

The TB diagnostic strategy of the trial did not include a culture reference standard. Therefore, whilst studies with adequate reference standards have shown very high specificity of urine LAM, 'false-positive' TB diagnoses cannot be excluded. The strategy did not include extensive extra-pulmonary sampling (beyond testing urine), and therefore may have missed some TB disease that could have been 'microbiologically confirmed': we estimate that the diagnostic yield of the STAMP screening strategy will have identified approximately 80% of all culture-confirmed TB [7–9]. If false-positive TB patients were included in the sub-studies which form part of this thesis, I will have overestimated the proportion of TB patients detectable by urine diagnostics ('urine-positive') and potentially underestimated group differences between HIV-positive inpatients with and without confirmed TB. Patients diagnosed with TB without any microbiological confirmation were not included.

The study was nested in a clinical trial. Whilst the management of patients was undertaken by routine health systems, the turnaround time for TB diagnosis may have been better than under routine hospital conditions, which may have impacted outcomes.

When assessing factors associated with mortality, I was unable to account for all factors, and there may be residual confounding that was unaccounted for in the association between mortality and disseminated urine-positive TB disease. In particular, I did not collect data on the presence of bacterial co-infection, and data on renal function were collected only from South Africa. TB immune reconstitution syndrome (IRIS) was also not considered as a factor associated with mortality in this study. Given the low proportion of ART naïve patients, low mortality associated with IRIS and difficulty with diagnosis, IRIS was thought not to be a

significant risk factor for mortality [10,11]. Ultimately, detailed post-mortem and ante-mortem data are required to accurately determine causes of death.

Although missing data was minimal for the STAMP trial participants and sub-studies, there was considerable loss to follow-up and missing explanatory data in the cohorts used to externally validate the clinical score for predicting mortality. My sensitivity analyses using multiple imputation suggests, however, that this is unlikely to have had a major impact on the overall findings.

The study of immune responses was underpowered to detect differences between urine-positive and urine-negative TB patients, due to the small number of patients with urine-negative disease. Whilst the immunology studies detected differences in immune responses between patients with differing mortality outcomes, the exact mechanisms cannot be elucidated from this study. I cannot assign causality to the association between immune responses and mortality. There are many important aspects of the immune response that were not assessed, which may be important. Immune responses were assessed *ex vivo*. Whilst using whole blood is more representative of *in vivo* conditions than isolating peripheral blood mononuclear cells, which can have a substantial impact on phenotype and function, it may not represent how cells behave in the host.

10.3 Implications of findings

Ending TB

The findings from the studies presented in this thesis (see table 10.1) are relevant to the World Health Organization's End TB Strategy, which aims to eliminate TB as a public health issue by 2035 [12]. Although most TB is thought to be acquired at the community level, HIV-related TB diagnoses and deaths occur disproportionately at secondary care level [13], making prevention and better management of HIV-associated TB in hospitals is a prerequisite for reaching targets for TB mortality reductions.

Disseminated disease is a relatively common manifestation of HIV-related TB, making up an increasing proportion of all TB disease as immunosuppression increases [14,15]. Importantly, the clinical manifestations and rate of progression of disseminated HIV-related TB are very different from localised pulmonary or extrapulmonary TB, with disseminated disease less easy to diagnose as outpatients and tending to have a relatively fulminant presentation with progressively severe systematic illness leading to prompt hospitalisation or death.

Key finding(s) / identified problem	Implications for HIV-associated TB	Future research directions	Relevant other research
Urine-diagnostics (especially urine-LAM LFA) increase TB diagnosis/ treatment in HIV+ inpatients, and reduce mortality in 'high risk' subgroups (low CD4 count, severe anaemia, clinically suspected TB)	Screening of all HIV+ inpatients for TB with urine LAM and sputum Xpert (in high prevalence settings)	-Operational research as urine screening is implemented in Malawi +/- South Africa -Interventional studies using new/more accurate LAM assays -Studies of screening strategies in outpatient settings (STAMP results cannot be extrapolated to outpatient settings)	STAMP trial [4]; LAM RCT [2]
HIV-associated TB is still common in hospitals in SSA, and presents with advanced disease with a high mortality risk	Better implementation of public health interventions, including: -prevention and early diagnosis of HIV -early initiation of effective ART -screening for TB at ART initiation and regularly thereafter TB prevention in PLHIV (eg IPT)	-Research/modelling to describe the proportion of all HIV-associated TB that is diagnosed in hospitals, and the proportion of all TB mortality that occurs in hospitals -Clinical trials of interventions to reduce mortality in HIV/TB (eg prophylactic treatment for co-infections, antibiotics, high dose rifampicin, supportive care)	Studies of TB screening at ART initiation [16]; IPT [17,18]; early ART [19–21]; other public health interventions [22]; REALITY study of enhanced prophylaxis in HIV [23]
Urine diagnostics have prognostic as well as diagnostic utility	Use to identify patients with highest mortality risk, to be targeted for additional care: -supportive care -enhanced follow-up (eg lay HCW, telephone follow-up, early clinic appointments)	-Evaluation of implementation of clinical prognostic score for mortality	REMSTART (enhanced follow-up in advanced HIV) [24]
High prevalence of ART failure, with worse outcomes for those on ART (compared to ART naïve)	-Screening for virological failure, and/or empirically switching HIV/TB patients on ART to new/better regimens -Better quality of ART services	-Studies of HIV drug resistance to establish aetiology of ART failure -Interventional trials of POC viral load testing and/or empirical switching	Meta-analyses of HIV drug resistance [25,26]; Dolutegravir PK/PD studies in HIV/TB [27,28];

		-studies of newer ART regimens in HIV/TB	studies of timing of ART initiation in HIV/TB [19–21]
Impaired immune protective immune mechanisms in HIV/TB patients with poor outcomes	Patients at high risk of poor outcomes may benefit from adjunctive interventions to address co-infections and/or immunomodulatory therapies	-Clinical trials of high-dose rifampicin, empirical antibiotics and preventative therapies for infections -Development of HDTs for HIV/TB	High dose rifampicin is safe and has better outcomes [29,30]

Table 10.1 Summary of key findings, implications, future research directions

As such, disseminated disease is the dominant form of TB among HIV-positive inpatients, and by far the most common form of fatal TB diagnosed at post-mortem in patients dying as inpatients [31]. This also makes the findings from this thesis (and the STAMP trial) difficult to extrapolate outside the hospital context.

Furthermore, no contemporary data describe the proportion of HIV-positive TB notifications that are diagnosed in hospitals. Data from the STAMP trial and Malawian National TB Programme indicates in 2016, 66% (95% CI 60% – 71%) of all TB notifications in HIV-positive patients in Zomba District were from inpatients on medical wards.

Screening for TB in hospitals

The WHO four symptom screen for HIV-associated TB (any one of cough, fever, night sweats or weight loss) has high sensitivity, although only moderate specificity in outpatient settings [15]. However, data from STAMP and other inpatient hospital cohorts show imperfect sensitivity for bacteriologically confirmed HIV-associated TB in hospitals, as well as poor specificity (90% of all HIV-positive patients admitted were positive) [4,7,32]. Thus, this screen has no role within TB testing algorithms in hospitals. A simple clinical diagnostic score with good accuracy in hospital has proven challenging to establish.

Empirical TB treatment is an alternative approach to screening. A recent trial of extensive screening compared to systematic empirical treatment in ART naïve patients with HIV/TB and advanced immunosuppression found no decrease in mortality and more adverse events with empirical treatment, suggesting intensive TB screening should be the preferred approach [33].

Use of urine-diagnostic tests

Findings from the STAMP trial [4], in addition to the diagnostic clinical trial of LAM use (LAM RCT) [2] support the implementation of urine-based TB diagnostics to test all HIV-positive admissions to medical wards for TB in high HIV and TB prevalent settings (see table 10.2) [34]. This approach would likely substantially improve diagnosis of HIV-associated TB. It could also reduce mortality, at least in patients with more advanced disease such as low CD4 cell counts. Cost-effectiveness analyses from the STAMP trial indicate the approach is cost-effective, although using urine LAM alone (without urine Xpert) was more cost-effective and affordable with only a small reduction in diagnostic yield [34]. These data have led to incorporation of urine LAM screening for HIV-positive patients in hospitals as part of the latest guidelines for Malawi (personal communication Malawi National TB Programme) and in South Africa (plenary presentation at the SA TB conference, Durban 2018).

This thesis delivers evidence that urine diagnostics provide prognostic as well as diagnostic benefit. As urine LAM is implemented for screening/diagnosis in hospitals, patients with detectable urine LAM (and/or other high risk factors as highlighted in the clinical score) can be targeted for additional care in view of their increased risk of mortality. Whilst STAMP data did not support routine screening with urine Xpert, it is a useful additional diagnostic in patients who are sputum Xpert negative (or sputum scarce), and urine LAM negative, and can also provide prognostic information.

Reducing mortality in advanced HIV-associated TB

I have shown that, despite early TB diagnosis and TB treatment initiation based on screening HIV-positive inpatients, mortality in HIV-associated TB remains substantial (31% by 2 months). Therefore, interventions adjunctive to TB treatment and appropriately timed ART will be needed to further reduce mortality. Findings presented in this thesis support various interventions aimed at reducing mortality, although further studies may be required to confirm findings and prior to implementation.

Firstly, patients with the worst outcomes present with advanced disease, therefore 'upstream' public health interventions (ie prevention and early diagnosis of both HIV and TB) need to be better implemented. Such interventions (see table 10.1) include those described in Chapter 1 of the thesis, especially prevention of TB in PLHIV (eg isoniazid preventative therapy), early diagnosis of HIV-associated TB (eg TB screening at ART initiation and follow-up), early HIV diagnosis and ART initiation and better HIV and TB service integration.

	LAM RCT (Peters et al, Lancet 2016) [2]	STAMP Trial [4]
Setting/Sample Size	2,569 randomised in South Africa, Tanzania, Zambia, Zimbabwe. 2013-2014	2,600 randomised in South Africa, Malawi. 2015-2017
Intervention	TB-LAM in addition to routine diagnostics	TB-LAM & Urine Xpert & Sputum Xpert (no sputum induction)
Comparator group	Routine diagnostics (not defined, varied by site, included sputum induction and culture)	Sputum Xpert + routine diagnostics (no sputum induction or routine culture)
Population	Hospitalised HIV+ patients 'presumed to have TB'	Hospitalised HIV+ patients <u>irrespective of presentation or TB symptoms</u>
Outcomes	1. Mortality at 56-days 2. TB score/Karnofsky performance score	1. Mortality at 56-days 2. TB diagnosis and treatment
Exclusions	-1074 unable to consent -2569 started TB treatment between admission and screening	-654 unable to consent -150 started TB treatment between admission and screening
Patient Characteristics	Median CD4 cell count: 84 (IQR 26-208) Taking ART at baseline: 48%	Median CD4 cell count: 230 (IQR 78-438) Taking ART at baseline: 72.3%
56-day mortality (INT vs SOC)	24.9% vs 20.8%, p=0.012 Absolute ↓4% (1-7), Relative ↓17% (4-28)	21.1% vs 18.3% p=0.073 Absolute ↓2.8% (-0.3 to 5.8), Relative ↓13% (0-27)
TB diagnosis & treatment (INT vs SOC)	52% vs 47% (treatment), p=0.024 Absolute ↑5%, Relative ↑10%	21.9% vs 14.9% (diagnosis) 20.8% vs 14.1% (treatment) Absolute ↑7%, Relative ↑50%
LAM yield in microbiologically confirmed TB*	250/460 (60%)	158/210 (75%)
Only LAM positive	94/1254	87/1287

Table 10.2 Comparison of STAMP and LAM RCT trials. *Microbiologically confirmed TB was described as Xpert, mycobacterial culture or urine LAM positive. INT intervention arm (urine screening in STAMP, LAM testing in LAM RCT); SOC standard of care/non-intervention arm; IQR interquartile range.

Mortality was higher in patients on ART compared to ART naïve patients, which is a novel finding, and patients on ART had high prevalence of ART failure. Rapid diagnosis of ART failure (for example, using point of care HIV viral load assays) followed by adherence interventions and/or adherence or switching of regimens could improve outcomes. Dolutegravir, an integrase inhibitor, is a promising antiretroviral for such settings as it has a high barrier to resistance and has few interactions with TB drugs [27,28]. Switching TB patients to dolutegravir based regimens without viral load testing is one option, however recent concerns about teratogenicity (specifically associations with neural tube defects) may limit the applicability in women of childbearing age [35].

In addition to interventions aimed directly at TB and HIV, TB is highly immunosuppressive and can disrupt innate pulmonary defences leaving patients at increased risk of concurrent opportunistic infections, notably Cryptococcus and bacterial infection. Furthermore, these patients also have advanced HIV-related immunosuppression which also increases risks of concurrent infections. High prevalence of cryptococcal antigenaemia supports screening of TB patients for cryptococcal disease followed by treatment or prophylaxis. Current guidelines recommend CD4 count based cryptococcal screening, and do not account for programmes where CD4 testing has been phased out (eg Malawi, where spending on CD4 counts was instead used for virological testing), and thus many TB patients would not currently be tested for cryptococcus.

Bacterial infections are common in HIV-positive patients admitted to hospital, as shown by post-mortem data [31,36,37], and studies doing blood culture [38–40] and more sensitive PCR based methods [41]. Diagnosis in routine settings is challenging, and empirical prophylaxis and/or treatment has been shown to reduce mortality in advanced HIV by 25% in the REALITY trial [23]. Therefore, empirical anti-infectives should be considered in patients with HIV-associated TB, keeping in mind the increasing prevalence of antimicrobial resistance, especially in gram-negative organisms [42].

Supportive care in high HIV-prevalence and resource limited settings remains challenging. Interventions for patients with sepsis tend to generalise poorly from high-resource to low-resource settings: for example, ‘early-goal directed therapy’ (intravenous fluid boluses with monitoring of physiological response, vasopressors for refractory hypotension, and blood transfusions for life-threatening anaemia), paradoxically led to increased mortality in sub-Saharan Africa [43]. Given the high prevalence of severe anaemia and renal dysfunction in HIV-associated TB, interventions to improve supportive care such as blood transfusion and intravenous fluid are of high importance but also high risk. Finally, over one-quarter of deaths

occurred after discharge, therefore enhanced follow-up and support strategies that can reduce mortality in advanced HIV [24][44] should also be considered for HIV-associated TB.

Host-directed therapies

Adjunctive host-directed therapies (HDTs) are in development primarily for shortening TB treatment duration, targeting lung immunopathology, and treating drug resistant TB disease [45]. They work by promoting effector mechanisms against MTB, for instance cytokine production, antimicrobial peptide production (eg reactive oxygen species), phagolysosome fusion and autophagy. I found impairment in several protective immune functions in patients with poor outcomes, including production of inflammatory cytokines to stimulation, monocyte function and phagocytic killing through oxidative burst. These findings support the development of HDTs for HIV-associated TB, as they have the potential to improve outcomes. Potential host therapeutic targets supported by the findings of this thesis are outlined in figure 10.1.

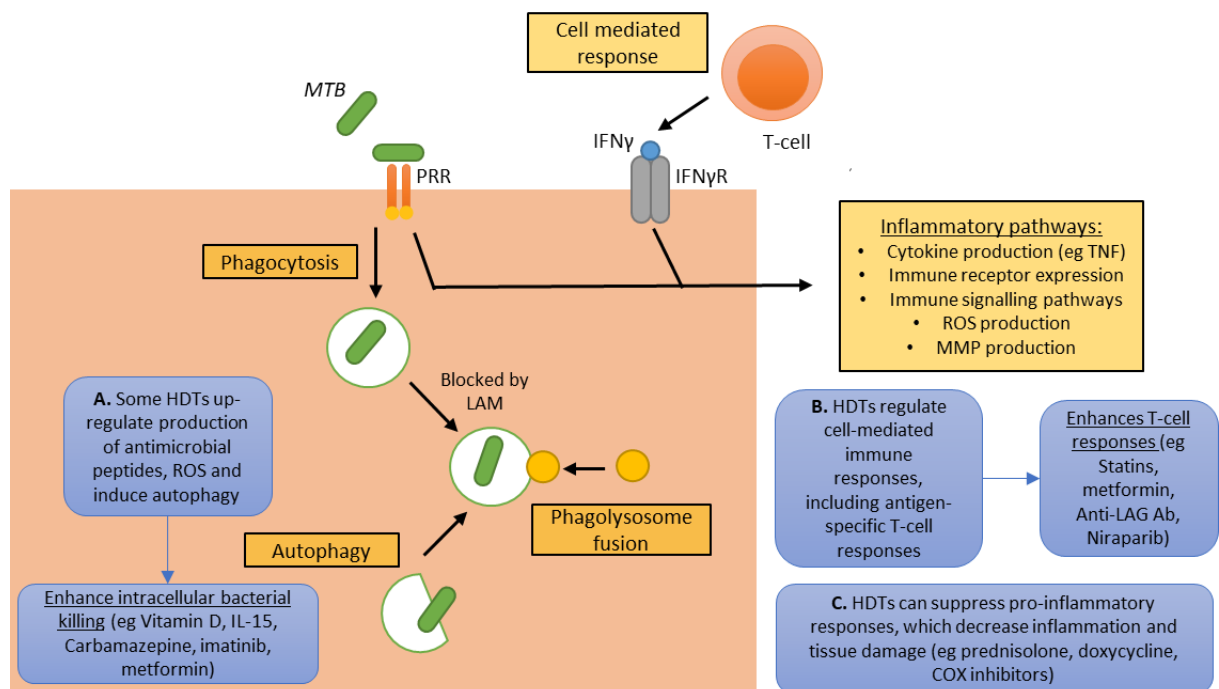


Figure 10.1 Potential host directed therapy targets. Pathological processes are in yellow boxes, host directed therapies (HDTs) are in blue boxes. Lipoarabinomannan (LAM) inhibits phagolysosome fusion and other aspects of phagocytosis to promote *MTB* intracellular survival. Some agents can overcome this, or circumvent it through promoting autophagy. TNF tissue necrosis factor, MTB Mycobacterium tuberculosis, ROS reactive oxygen species, IFN interferon, IFNR interferon receptor, PRR pattern recognition receptor. Adapted from Wallis et al [45] and Kolloloi et al [46].

Several agents are in clinical trial for TB treatment [46]. However, few include patients with HIV-associated TB. Prednisolone is being considered for TB and TB-IRIS [46]. Although short courses of prednisolone increased the rate of sputum *MTB* clearance (thought to be through reduction in immune activation and increasing CD4+ T-cell responses), the adverse effects of steroids outweighed the benefits [47,48]. TNF- α blockers such as etanercept are being considered for HIV-associated TB [49], as are cytokine supplementation (eg INF- γ) [46]. LAM itself is a potential target of anti-*MTB* antibodies [50,51].

10.4 Future research

The findings presented in this thesis, and the STAMP trial within which it was nested, can inform future research targeting mortality in HIV-associated TB and/or urine-diagnostics (table 10.1).

The prevalence of TB and associated mortality in hospitalised HIV-positive patients has now been well described. However, the absolute numbers of TB diagnoses and proportion of total incident TB that are diagnosed in hospital in high prevalence settings has not been described or modelled. These data, in addition to data on proportions of total TB deaths occurring in hospitals, are vital to direct interventions and resources to this population in efforts to 'end TB'. Scaling up of urine LAM screening in PLHIV in hospitals is already occurring: implementation and operational research should accompany scale-up to measure 'real-life' impact and outcomes, as well as strategies to improve uptake. More accurate LAM detection techniques are in the pipeline [52,53], including a more accurate LAM lateral flow assay [54]. Diagnostic accuracy and validation studies are needed before interventional studies.

ART failure was shown for a high proportion of TB patients, and in HIV-positive patients in the STAMP trial more broadly. The aetiology is not clear (poor adherence versus HIV drug resistance), and studies on stored plasma from the STAMP trial are being undertaken to describe the prevalence of drug resistance mutations. Potential strategies to tackle ART failure include viral load testing with early switching to second line therapy, or switching high risk patients without measuring viral load. Further research is needed to establish the virological, TB and mortality outcomes of these strategies.

Higher dose rifampicin (up to 35mg/kg compared to current dosing of 10mg/kg) has shown promising results in early clinical trials, being safe and leading to more rapid culture conversion [29,30] and better outcomes for TB meningitis [55]. The impaired host responses demonstrated in this thesis and association between mortality and urine-positivity strongly support trial of high dose rifampicin for urine-positive HIV-associated TB. Other potential

interventions that should be trialled include: empirical broad-spectrum antimicrobials, packages of supportive care such as intravenous fluids and/or blood transfusion if these can be provided with sufficiently intensive monitoring, as well as enhanced follow-up (for example, early clinic appointments, lay health care worker visits or telephone calls) for patients discharged alive but with ongoing high mortality risk.

The confirmation that outcomes and clinical features are associated with immune responses also warrants further mechanistic studies. It was unclear if defects in phagocyte function were driven by cellular factors or soluble proteins. Further experiments assessing phagocyte responses in healthy control cells incubated with plasma from HIV-associated TB patients with impaired phagocytic responses could help delineate this. Assessment of other antimicrobial pathways, for example perforin and granzyme expression are also being undertaken from stored samples in this cohort.

10.5 Conclusion

The studies presented in this thesis demonstrate the unacceptably high mortality of HIV-patients admitted to hospital with TB, and that positive urine-diagnostic tests (indicative of disseminated TB disease) are associated with increased mortality. Urine-diagnostics, particularly urine LAM lateral flow assays, provide a simple and easily implementable method to predict which patients are at highest risk of poor outcomes, either alone or when used in a simple score with other easily obtained variables.

Reliance on TB therapy alone, even in the context of screening and rapid initiation of treatment is unlikely to be enough to meet global targets for mortality reduction. Adjunctive interventions targeting advanced HIV-immunosuppression, advanced TB disease and impaired immune responses will be needed to further reduce mortality, in addition to better implementation of existing upstream public health interventions. Research and implementation should consider both these approaches together, and address the TB disease burden in hospitals in sub-Saharan Africa as part of the 'End TB' movement.

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Appendix: The STAMP trial results paper



Rapid urine-based screening for tuberculosis in HIV-positive patients admitted to hospital in Africa (STAMP): a pragmatic, multicentre, parallel-group, double-blind, randomised controlled trial



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See [Comment](#) page 256

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Summary

Background Current diagnostics for HIV-associated tuberculosis are suboptimal, with missed diagnoses contributing to high hospital mortality and approximately 374 000 annual HIV-positive deaths globally. Urine-based assays have a good diagnostic yield; therefore, we aimed to assess whether urine-based screening in HIV-positive inpatients for tuberculosis improved outcomes.

Methods We did a pragmatic, multicentre, double-blind, randomised controlled trial in two hospitals in Malawi and South Africa. We included HIV-positive medical inpatients aged 18 years or more who were not taking tuberculosis treatment. We randomly assigned patients (1:1), using a computer-generated list of random block size stratified by site, to either the standard-of-care or the intervention screening group, irrespective of symptoms or clinical presentation. Attending clinicians made decisions about care; and patients, clinicians, and the study team were masked to the group allocation. In both groups, sputum was tested using the Xpert MTB/RIF assay (Xpert; Cepheid, Sunnyvale, CA, USA). In the standard-of-care group, urine samples were not tested for tuberculosis. In the intervention group, urine was tested with the Alere Determine TB-LAM Ag (TB-LAM; Alere, Waltham, MA, USA), and Xpert assays. The primary outcome was all-cause 56-day mortality. Subgroup analyses for the primary outcome were prespecified based on baseline CD4 count, haemoglobin, clinical suspicion for tuberculosis; and by study site and calendar time. We used an intention-to-treat principle for our analyses. This trial is registered with the ISRCTN registry, number ISRCTN71603869.

Findings Between Oct 26, 2015, and Sept 19, 2017, we screened 4788 HIV-positive adults, of which 2600 (54%) were randomly assigned to the study groups (n=1300 for each group). 13 patients were excluded after randomisation from analysis in each group, leaving 2574 in the final intention-to-treat analysis (n=1287 in each group). At admission, 1861 patients were taking antiretroviral therapy and median CD4 count was 227 cells per μL (IQR 79–436). Mortality at 56 days was reported for 272 (21%) of 1287 patients in the standard-of-care group and 235 (18%) of 1287 in the intervention group (adjusted risk reduction [aRD] -2.8% , 95% CI -5.8 to 0.3 ; $p=0.074$). In three of the 12 prespecified, but underpowered subgroups, mortality was lower in the intervention group than in the standard-of-care group for CD4 counts less than 100 cells per μL (aRD -7.1% , 95% CI -13.7 to -0.4 ; $p=0.036$), severe anaemia (-9.0% , -16.6 to -1.3 ; $p=0.021$), and patients with clinically suspected tuberculosis (-5.7% , -10.9 to -0.5 ; $p=0.033$); with no difference by site or calendar period. Adverse events were similar in both groups.

Interpretation Urine-based tuberculosis screening did not reduce overall mortality in all HIV-positive inpatients, but might benefit some high-risk subgroups. Implementation could contribute towards global targets to reduce tuberculosis mortality.

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Introduction

Tuberculosis remains the single major cause of mortality in patients with HIV globally, accounting for an estimated 374 000 deaths in 2016.¹ In many parts of sub-Saharan Africa, most admitted medical inpatients are HIV-positive and tuberculosis is the major cause of both admission

(18–29%) and in-hospital death (21–33% in cohort studies and 32–67% in autopsy studies).^{2,3}

Suboptimal diagnostics are an important contributor to poor outcomes from HIV-associated tuberculosis. Tuberculosis is commonly disseminated, presents with non-specific clinical features, and is only diagnosed

Research in context

Evidence before the study

We searched MEDLINE for studies that investigated the effect of urine lipoarabinomannan assay (LAM) or Xpert MTB/RIF assay (Xpert) on mortality or tuberculosis diagnosis in HIV-positive patients published from Jan 1, 2000, to Sept 30, 2016. We combined search terms for LAM ("lipoarabinomannan", "LAM", "TB LAM", or "urine LAM") or Xpert ("urine Xpert" or "urinary Xpert") with HIV ("HIV", "HIV-1", "AIDS", or "human immunodeficiency virus") and mortality ("mortality", "adult mortality", or "death"), or tuberculosis diagnosis or screening ("diagnosis", "diagnostic", or "screening"). We identified 14 observational studies, mostly done in antiretroviral therapy naive outpatients or hospital inpatients, which assessed the diagnostic accuracy of urine LAM or Xpert for tuberculosis or their association with mortality, or both. These studies showed moderate-to-good diagnostic yield of urinary assays in patients with advanced immunosuppression and in hospital inpatients, and an association with higher disease severity, poor prognosis, and mortality. Since undertaking our trial, one randomised trial has assessed adjunctive urine LAM testing in HIV-positive inpatients suspected of tuberculosis and found a reduction in 8-week mortality. However, whether systematic urine-based screening for tuberculosis (using urine LAM and Xpert) for all HIV-positive hospital inpatients (irrespective of tuberculosis symptoms) could reduce mortality compared with sputum tuberculosis testing remained unclear.

Added value of this study

The findings from this randomised trial suggest that urine-based tuberculosis screening of HIV-positive hospital

inpatients might reduce 56-day mortality in defined clinical subgroups (low CD4 count, severe anaemia, or clinically suspected tuberculosis). Moreover, wider application (screening all HIV-positive inpatients) could substantially reduce the risk of being discharged from hospital with undiagnosed tuberculosis in all patient groups. The major incremental diagnostic benefit was from urine LAM.

Implications of all the available evidence

These data support implementation of urine LAM-based screening of all HIV-positive inpatients for tuberculosis in hospitals in high HIV and tuberculosis burden settings, because the reliance on a combination of sputum-based diagnosis and clinically guided empirical treatment left patients at unacceptably high risk of discharge and death from undiagnosed tuberculosis. Collectively, current evidence supports international policy change to scale-up and broaden the use of urine-LAM testing in patients admitted to hospital (currently only recommended as an additional diagnostic in inpatients with symptoms of tuberculosis and CD4 counts <100 cells per μL or danger signs). Incremental gain was too limited to support urine Xpert. Because screening efficiency is dependent on prevalence, these results cannot be extrapolated to outpatients. Urine LAM screening could contribute towards reducing mortality and morbidity from HIV-associated tuberculosis and meeting global targets for tuberculosis mortality reduction.

before death in half of cases with a fatal outcome.^{3,4} Mycobacterial culture, the current gold standard, is too centralised and slow to be clinically useful. Both culture and chest radiography are often unavailable in many African settings. The Xpert MTB/RIF assay provides robust and rapid detection of *Mycobacterium tuberculosis* nucleic acids from sputum and has been widely scaled-up and decentralised, but patients with HIV-associated tuberculosis tend to have relatively low mycobacterial concentrations in pulmonary secretions and difficulty expectorating.⁵ Despite improved sensitivity (79% in patients with HIV),⁶ randomised trials comparing clinical outcomes between sputum Xpert MTB/RIF and microscopy have largely shown scant effect because of empiric tuberculosis therapy, other than systematic screening in HIV-positive outpatients with advanced disease.^{7,8}

Urine can be readily obtained from patients admitted to hospital and is suitable for rapid tuberculosis diagnosis using either a lateral flow assay for lipoarabinomannan (LAM; a mycobacterial cell wall glycolipid) or Xpert MTB/RIF. Although urine is not a sample recommended by WHO for Xpert, studies report high specificity for tuberculosis in HIV-positive patients.^{9,10}

Diagnosis using urinary LAM, reflecting frequent renal involvement from disseminated HIV-associated tuberculosis, is complementary to sputum testing, and identifies a subgroup of patients with poor prognosis.^{11,12} Current commercial LAM kits have a specificity of 98% or more and sensitivity of 40–70% in HIV–tuberculosis–coinfecting patients with CD4 counts less than 100 cells per μL .^{13–15} Combined testing with urine LAM, plus urine and sputum Xpert, can rapidly diagnose about 80% of all culture-positive tuberculosis in unselected HIV-positive admissions to medical wards in high HIV burden settings.^{15,16}

Urine-based screening might provide more complete, timely, and potentially life-saving diagnosis of tuberculosis among HIV-positive hospital inpatients.⁷ We, therefore, aimed to investigate the effect of adding urine to sputum tuberculosis screening on early mortality, and its effect on diagnosis and treatment of tuberculosis in unselected HIV-positive hospital admissions.

Methods

Study design and patients

We did a pragmatic, multicentre, parallel-group, double-blind, randomised controlled trial. We enrolled patients

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who were admitted to medical wards at Zomba Central Hospital in Malawi (a district and referral hospital covering urban and rural populations) and Edendale Hospital in South Africa (a large referral hospital covering a mostly urban population), irrespective of tuberculosis symptoms or admitting presentation. The study design has been previously described in detail,¹⁷ and additional methods are provided in the appendix. We obtained ethical approval from the relevant committees in Malawi, South Africa, and from the trial sponsor in the UK, and the study was approved by the relevant national regulatory bodies (appendix). Deviations from the study protocol are described in the appendix.

See Online for appendix

All admissions to the medical wards were screened for eligibility by study nurses or clinicians. Screening occurred during office hours on weekdays, with patients enrolled as close to admission as possible. All patients with an unknown HIV status were offered point-of-care rapid HIV testing as per local guidelines (appendix). We included patients that were HIV positive and aged 18 years or older. We excluded those that were currently taking tuberculosis treatment, had been treated for tuberculosis in the preceding 12 months, taken isoniazid preventive therapy in the preceding 6 months, were unable or unwilling to provide informed consent, had been admitted to hospital for longer than 48 h at the time of screening, or lived outside the predefined hospital catchment area (appendix). We obtained written informed consent from all eligible patients.

Randomisation and masking

We randomly assigned eligible patients on enrolment (1:1) to either the standard-of-care tuberculosis screening group or the intervention screening group. Randomisation was stratified by site and a randomisation list of unique patient identifiers was generated by the study statistician using a computer-generated random block size. On enrolment, study nurses or clinicians took a consecutive sealed opaque envelope containing the unique patient identifier but not the study group, to which they remained masked. A paired set of sealed envelopes were kept in a locked cabinet in the study laboratory, labelled with the unique patient identifier and containing the study group allocation. These were opened by the laboratory technician on receipt of study tuberculosis screening specimens. Investigators, all study staff (other than the laboratory technician and statistician), hospital attending clinical teams, and patients were masked to the study group allocation.

Procedures

Following enrolment, 50 mL of urine and a single, spontaneously expectorated sputum sample were collected by the study team for tuberculosis screening. Failure to produce a specimen was not an exclusion criterion. The patient's attending clinical team had the

option of sending additional samples for routine tuberculosis investigations available at the study hospital; the appendix provides further details of the tests available at each hospital.

Tuberculosis screening samples (ie, sputum or urine, or both) were processed according to study group allocation by the study laboratory technician, and assays were run during office hours and processed as soon as possible after arrival of a specimen in the laboratory. In both groups, sputum was tested using the Xpert MTB/RIF assay (Xpert; Cepheid, Sunnyvale, CA, USA). In the standard-of-care group, urine samples were not tested for tuberculosis. In the intervention group, 60 µL of unconcentrated urine was tested with the Alere Determine TB-LAM Ag assay (TB-LAM; Alere, Waltham, MA, USA) as per the manufacturer's instructions, and 40–50 mL of urine was concentrated by centrifugation for testing with Xpert. Urine Xpert and TB-LAM were processed simultaneously. We deemed TB-LAM positive using the grade 1 cutoff on the manufacturer's post-2014 reference card, which was referred to as the grade 2 cutoff before 2014. The appendix provides further details of the laboratory methods used in this study.

Once all the tuberculosis specimens received had been processed, tuberculosis screening results were reported to the attending clinical team as positive, negative, or not done to maintain masking, with neither study group nor individual test results communicated to attending clinical or study teams. Rifampicin resistance results, if available, were also reported (appendix). Clinical management, including tuberculosis treatment decisions and management of antiretroviral therapy (ART), relied on the attending clinical team according to local and national guidelines and was independent of study nurses, clinicians, or investigators.

The study team documented patients' clinical events during hospital admission, including but not limited to tuberculosis investigations and diagnosis, commencement of tuberculosis treatment and any side-effects, management of HIV (including stopping or starting ART), and discharge or death. Follow-up at 56 days for those discharged from hospital alive was done in person by outpatient attendance. Those who did not attend were contacted by telephone or a home visit, or both, with interview of next of kin to establish vital status if required.

Outcomes

The primary outcome was the cumulative risk of all-cause mortality at 56 days from enrolment. Subgroup analysis for the primary outcome was prespecified in populations with higher risk of tuberculosis, mortality, or both (ie, low baseline CD4 cell count, low haemoglobin, or clinical suspicion for tuberculosis); and by study site and calendar time. Secondary outcomes were time to mortality, proportions of patients with microbiologically confirmed tuberculosis and clinically diagnosed tuberculosis, time

from randomisation to tuberculosis diagnosis and to start of tuberculosis treatment, time from tuberculosis diagnosis to treatment initiation, prescription of antimicrobials, ART initiation (if ART naive at hospital admission), duration of hospitalisation, and hospital readmission events.

Microbiologically confirmed tuberculosis was defined as one or more positive specimens for acid fast bacilli, Xpert, mycobacterial culture, or TB-LAM. Clinically diagnosed tuberculosis was defined by the decision to treat for tuberculosis in the absence of microbiological confirmation. Patients with any tuberculosis diagnosis (microbiologically confirmed or clinically diagnosed) were also reported. We recorded for all patients whether tuberculosis was included in the admitting differential diagnoses by attending clinicians, referred to as clinically suspected tuberculosis.

Statistical analysis

The sample size calculation was based on the assumption that 56-day mortality risk would be 25% in the standard-of-care group, and loss to follow-up would be 10% or less. We therefore calculated that enrolling 1300 patients per group would provide at least 80% power to detect a 5% absolute mortality reduction in the intervention group, with a two-sided type 1 error of 5% (appendix).

We used an intention-to-treat principle for all our analyses, including all eligible patients that were randomly assigned. For the primary outcome, we calculated a risk difference with 95% CIs for mortality at 56 days comparing the standard-of-care group with the intervention group with the following: using a generalised linear model with identity link function and binomial family, adjusting for study site, using a fixed effect, and assuming participants lost to follow-up had not died. An odds ratio adjusted for site with 95% CIs was also calculated using logistic regression. Prespecified subgroup analyses were done for the primary outcome. These subgroups were study site (Malawi or South Africa), baseline CD4 counts (<100 cells per μL or ≥ 100 cells per μL), severe anaemia (haemoglobin <8 g/dL or ≥ 8 g/dL), whether tuberculosis was clinically suspected at admission, and calendar time (by 6 monthly intervals from Oct 1, 2015, to Sep 30, 2017). A sensitivity analysis was also done assuming all losses to follow-up had died.

Secondary outcomes were compared between the study groups using adjusted risk difference and adjusted odds ratio (aOR) for binary outcomes, Cox proportional hazards regression for time-to-event outcomes, and Kaplan-Meier curves for time to mortality. 95% CI were calculated for all analyses. In exploratory post-hoc analyses, tuberculosis diagnoses were also compared between study groups using the same subgroups as the primary outcome (study site, baseline CD4 cell count, severe anaemia, and clinically suspected tuberculosis) to investigate whether the absence of mortality benefit was accompanied by a lack of difference in tuberculosis diagnosis. Diagnostic

yields of urine-based tuberculosis tests were calculated post hoc as a proportion of all microbiologically confirmed tuberculosis to better understand the respective contributions of TB-LAM and urine Xpert.

We did all the analyses using SAS (version 9.4). This study is registered with the ISRCTN registry, number ISRCTN71603869.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of

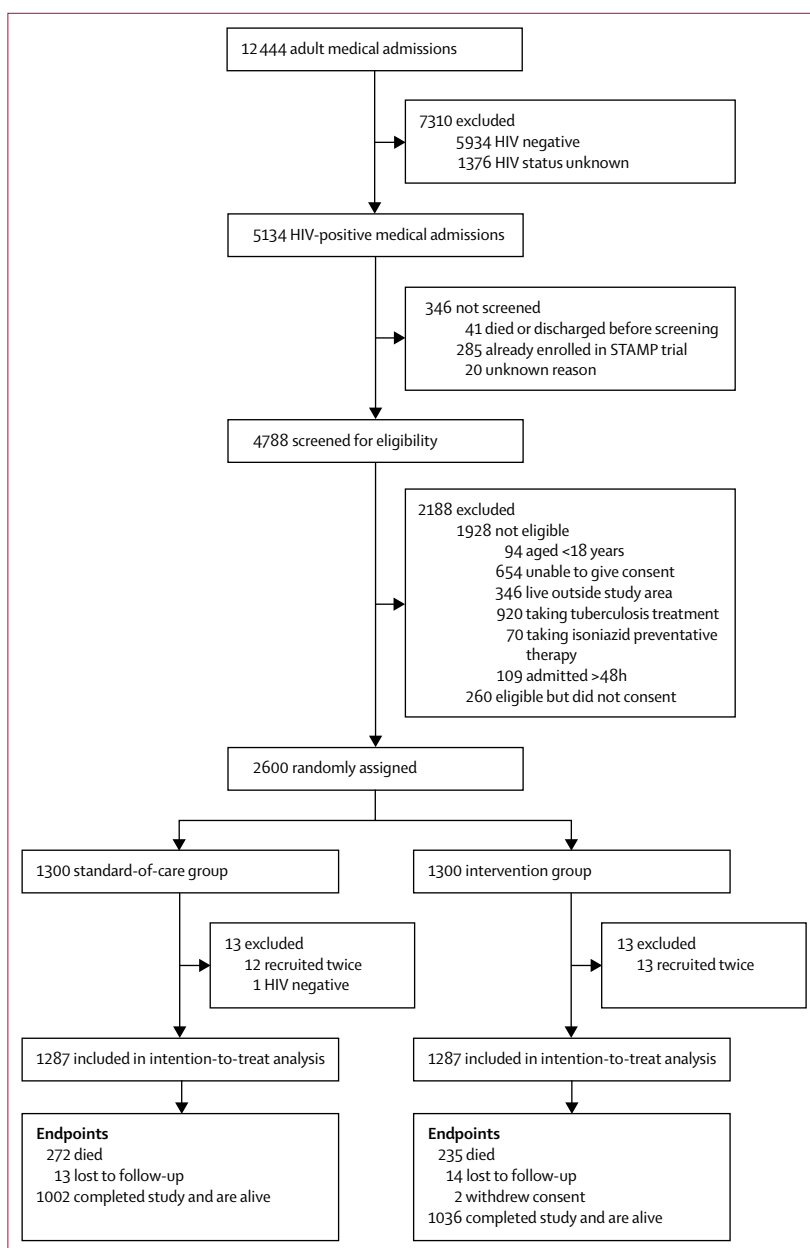


Figure 1: Trial profile

Patients could have more than one reason for exclusion. Data stratified by site are shown in the appendix.

	Standard-of-care group (n=1287)	Intervention group (n=1287)	Malawi (n=1316)	South Africa (n=1258)
Age (years)	39.6 (11.9)	39.7 (11.6)	40.1 (11.7)	39.1 (11.7)
Sex				
Women	734 (57%)	727 (56%)	829 (63%)	632 (50%)
Men	553 (43%)	560 (44%)	487 (37%)	626 (50%)
New HIV diagnosis	212 (16%)	194 (15%)	208 (16%)	198 (16%)
ART status*				
Never	93 (9%)	121 (11%)	57 (5%)	157 (15%)
Currently taking	935 (87%)	926 (85%)	1021 (92%)	840 (79%)
Interrupted	47 (4%)	46 (4%)	30 (3%)	63 (6%)
Time on ART (years)†	3.0 (0.7–6.7)	3.0 (0.8–6.7)	3.4 (0.8–7.4)	2.6 (0.6–5.8)
TB symptoms reported				
Cough	681 (53%)	651 (51%)	611 (46%)	721 (57%)
Fever	747 (58%)	753 (59%)	761 (58%)	739 (59%)
Night sweats‡	540 (42%)	497 (39%)	488 (37%)	549 (44%)
Weight loss‡	875 (68%)	906 (70%)	863 (66%)	918 (73%)
Any WHO TB symptom	1164 (90%)	1152 (90%)	1187 (90%)	1129 (90%)
Clinically suspected TB§	495 (38%)	501 (39%)	353 (27%)	643 (51%)
Previous TB treatment	309 (24%)	335 (26%)	202 (15%)	442 (35%)
Body-mass index (kg/m ²)	21.7 (5.8)	21.6 (5.8)	20.0 (4.1)	23.3 (6.7)
Morbidity at admission				
WHO danger sign¶	275 (21%)	277 (22%)	337 (26%)	215 (17%)
Karnofsky score	60 (50–70)	60 (50–70)	60 (50–70)	60 (50–70)
CD4 cell count§				
Median (cells per µL)	222 (80–436)	231 (78–438)	219 (86–431)	236 (70–445)
<100 cells per µL	377 (29%)	371 (29%)	365 (28%)	383 (30%)
Haemoglobin				
Median (g/dL)	10.4 (8.1–12.9)	10.8 (8.3–12.7)	10.4 (7.8–12.4)	113 (8.8–13.1)
<8 g/dL	298 (23%)	289 (22%)	355 (27%)	232 (18%)

Data are mean (SD), n (%), or median (IQR). TB symptoms are reported if present for any duration. ART=antiretroviral therapy. TB=tuberculosis. *ART status denominator is the number of patients with a known HIV diagnosis (n=1075 for standard-of-care group and n=1093 for intervention group). †Missing data for 26 patients in Malawi and 26 in South Africa. ‡Missing data for one patient in South Africa. §Missing data for three patients in Malawi and six in South Africa. ¶WHO danger signs are one or more of the following: heart rate more than 120 beats per minute, respiratory rate more than 30 breaths per minute, temperature more than 39°C, or being unable to walk unaided. ||Missing data for five patients in South Africa.

Table: Patient characteristics at enrolment by study group and country

the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Between Oct 26, 2015, and Sept 19, 2017, we screened 4788 HIV-positive adult medical admissions. Of these admissions, 1928 (40%) patients were ineligible, 260 (5%) did not provide consent to participate, and 2600 (54%) were randomly assigned to the study groups (n=1300 for each group; figure 1). The appendix shows those who were excluded by site. 26 patients were excluded after randomisation because of ineligibility, leaving 2574 in the final intention-to-treat analysis (n=1287 in each group). 27 (1%) of 2574 patients were lost to follow-up at 56 days after hospital discharge (figure 1).

Baseline characteristics were balanced between the study groups (table; appendix). Mean age was 39.6 years (SD 11.7 years) and 1461 (57%) of 2574 participants were women. 2168 (84%) patients already knew their HIV diagnosis before admission, of whom 1861 (86%) were currently taking ART. Median CD4 count was 227 cells per µL (IQR 79–436), 748 (29%) of 2574 patients had a CD4 count of less than 100 cells per µL, and 587 (23%) had severe anaemia (haemoglobin <8 g/dL). 1332 (52%) patients reported a cough, 2316 (90%) had one or more WHO tuberculosis symptoms (ie, cough, fever, weight loss, or night sweats), and 996 (39%) were clinically suspected of tuberculosis at admission. Differences between sites included higher ART coverage, fewer patients reporting cough, fewer able to expectorate sputum, and fewer having clinically suspected tuberculosis at admission in Malawi than in South Africa (table).

By 56 days, 507 (20%) of 2574 patients had died: 272 (21%) of 1287 in the standard-of-care group and 235 (18%) of 1287 in the intervention group, giving an adjusted risk difference of –2.8% (95% CI –5.8 to 0.3; p=0.074; figure 2). The aOR for mortality in the intervention group compared with the standard-of-care group was 0.83 (95% CI 0.69–1.01; p=0.068; appendix). Mortality in the intervention group was significantly lower than in the standard-of-care group for the three prespecified high-risk subgroups: adjusted risk difference –7.1% (95% CI –13.7 to –0.4) in patients with baseline CD4 counts less than 100 cells per µL, –9.0% (–16.6 to –1.3) in patients with severe anaemia, and –5.7% (–10.9 to –0.5) in patients with clinically suspected tuberculosis at admission (figure 2). p values for interaction between the subgroup and study group are reported in figure 2. 1567 (61%) of 2574 patients were in one or more high-risk subgroups (low CD4 count, severe anaemia, or clinically suspected tuberculosis). Sensitivity analysis assuming losses to follow-up had died did not alter overall or subgroup mortality risk differences (appendix).

Overall, 36 patients would need to be screened with the study intervention (ie, TB-LAM and urine Xpert) to prevent one death (appendix). Median duration of hospital stay was 6 days (IQR 2–11), and did not differ between the two groups (appendix). Although 273 (54%) of 507 deaths occurred during hospital admission, overall and high-risk subgroup survival curves did not substantially diverge until after day 21 (figure 3). Among patients discharged alive from hospital, 134 (12%) of 1146 patients died in the standard-of-care group and 100 (9%) of 1150 died in the intervention group. In time-to-mortality analysis, the adjusted hazard ratio (aHR) for intervention compared with standard of care was 0.86 (95% CI 0.72–1.02; p=0.086; figure 3A).

Of the study's tuberculosis screening samples at baseline, urine was provided by 2548 (99%) of 2574 patients, whereas only 1464 (57%) produced sputum (518 [39%] of 1316 in Malawi and 946 [75%] of 1258 in South Africa). Chest radiographs as part of

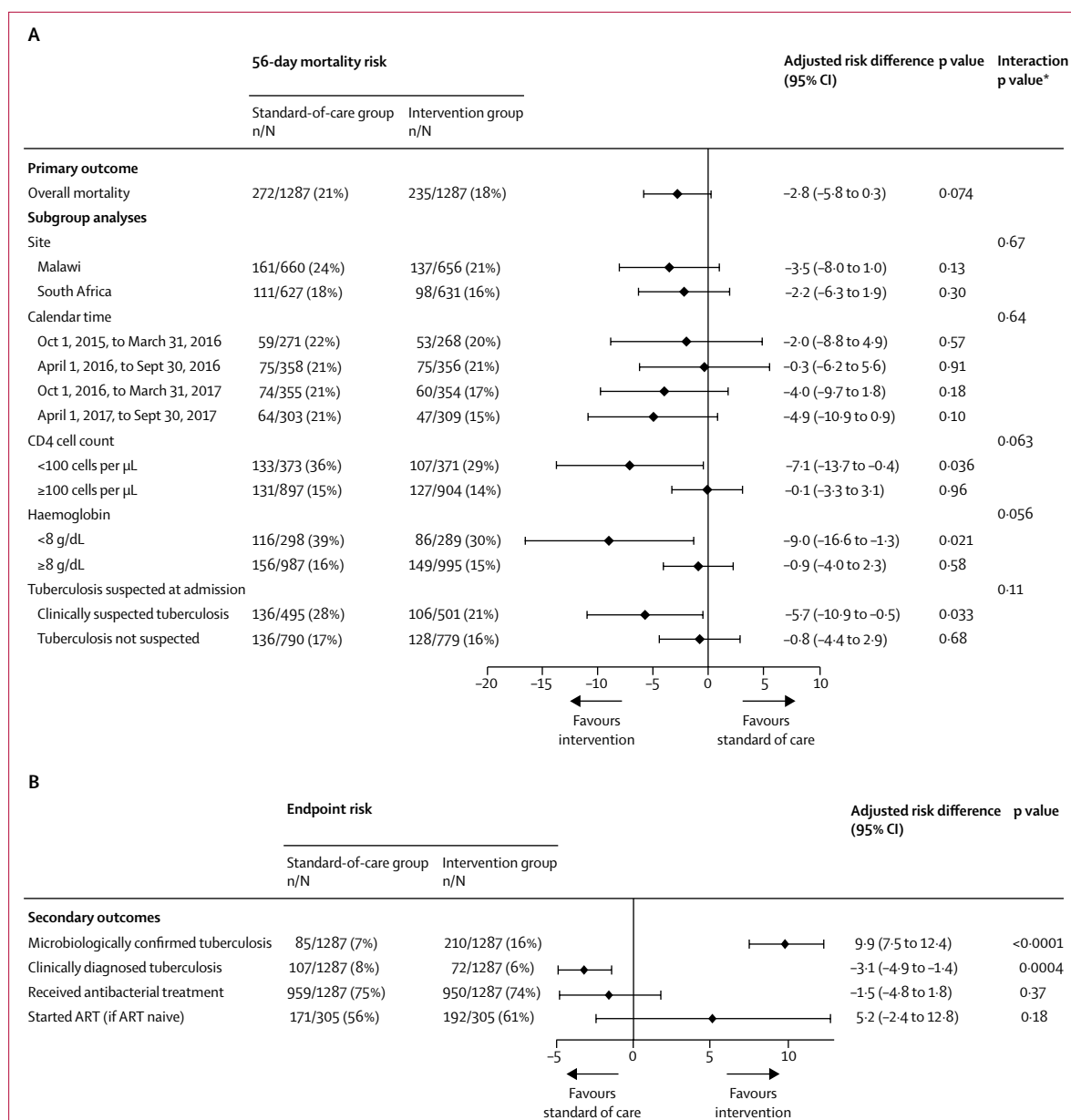


Figure 2: Primary outcome and predefined subgroup analyses (A), and secondary outcomes (B)
 All analyses are adjusted for study site. (A) The primary outcome is mortality at 56 days after randomisation. Risk differences are the risk in the intervention group minus the risk in the standard-of-care group. (B) Secondary outcomes are measured at the end of hospital admission except for those who started ART, which is measured at 56 days. Antibacterial treatment excludes anti-TB medications. ART=antiretroviral therapy. *Interaction between study group and subgroup.

routine care were taken in 1231 (48%) of 2574 patients during inpatient stay (300 [23%] of 1316 in Malawi and 931 [74%] of 1258 in South Africa; appendix). Overall, tuberculosis was diagnosed during hospital admission in 474 (18%) of 2574 patients, with 282 (22%) diagnoses in the intervention group and 192 (15%) in the standard-of-care group (appendix). The adjusted risk difference for tuberculosis diagnosis between the two groups was 7.3% (95% CI 4.4–10.2; $p < 0.0001$). The intervention group also had more microbiologically confirmed tuber-

culosis diagnoses than the standard-of-care group (210 [16%] of 1287 vs 85 [7%] of 1287; adjusted risk difference 9.9% [95% CI 7.5–12.4]; $p < 0.0001$) and fewer clinically diagnosed tuberculosis (77 [6%] vs 114 [9%]; adjusted risk difference -3.1% [-4.9 to -1.4]; $p = 0.0004$; figure 2). 14 patients would need to be screened with the study intervention to prevent one missed tuberculosis diagnosis (appendix).

Time from randomisation to tuberculosis diagnosis was marginally shorter in the intervention group than in the

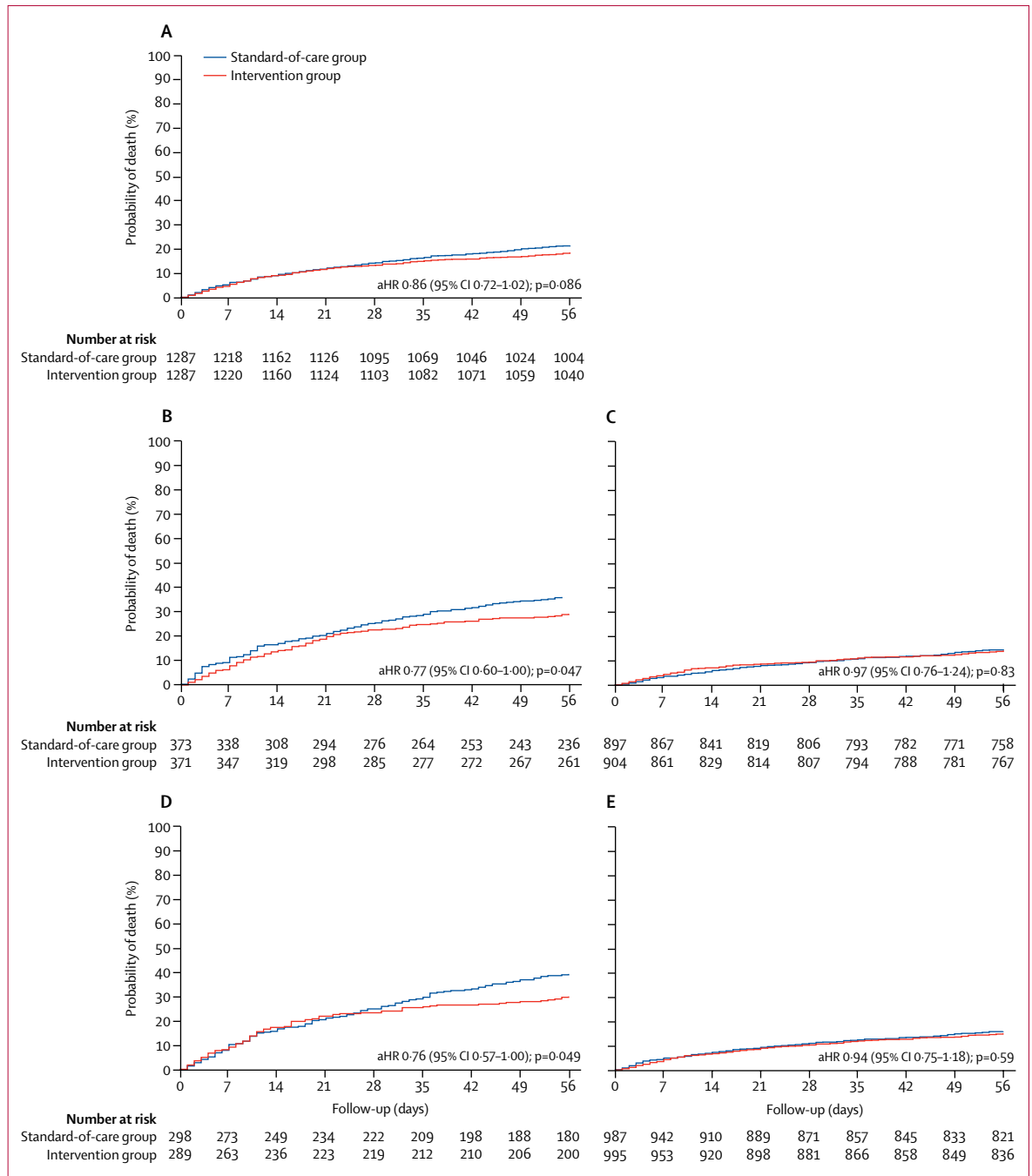


Figure 3: Time to 56-day mortality overall and stratified by high-risk subgroups
 All aHRs were adjusted for study site. (A) Survival analysis over 56 days in the standard-of-care group and intervention group. (B) Survival analysis stratified by CD4 counts less than 100 cells per μL in both groups. (C) Survival analysis stratified by CD4 counts of 100 cells per μL or more in both groups. (D) Survival analysis stratified by haemoglobin of less than 8 g/dL in both groups. (E) Survival analysis stratified by haemoglobin of 8 g/dL or more in both groups. aHR=adjusted hazard ratio.

standard-of-care group (median 0 days [IQR 0–1] vs 1 day [0–6]; appendix). 450 patients were started on tuberculosis treatment during admission, 268 in the intervention group and 182 in the standard-of-care group (aHR 1.56, 95% CI 1.29–1.88; $p < 0.0001$; appendix). Time from diagnosis to tuberculosis treatment was universally short (median of 1 day, IQR 0–1) and was similar in both

groups. Adverse events related to tuberculosis treatment were similar in both groups; the appendix summarises these adverse events. Antibacterial treatment and ART initiation did not differ by group (figure 2), although time to ART initiation was shorter in the intervention group than in the standard-of-care group (appendix). Of the 24 patients diagnosed with tuberculosis but not started on

treatment during hospital admission, ten (42%) had died and 14 (58%) had been prematurely discharged. Only 27 other patients were started on tuberculosis treatment between discharge and day 56, with no difference between groups (appendix). Hospital readmission, losses to follow-up, adverse events (tuberculosis treatment discontinuation and side-effects), and rifampicin resistance detection did not differ between groups (appendix).

In post-hoc exploratory analyses, the increases in tuberculosis diagnoses in the intervention group versus the standard-of-care group were not confined to high-risk subgroups, unlike mortality, with an adjusted absolute risk increase of 7.0% (95% CI 4.1–10.0) in tuberculosis diagnoses in patients with CD4 counts of 100 cells per μL or more, and 8.0% (5.0–11.1) in those not clinically suspected of tuberculosis at admission (appendix). Increased tuberculosis diagnoses were more pronounced in Malawi than South Africa, although there was no evidence for an interaction between group and country ($p=0.19$). The largest increase in tuberculosis diagnoses in the intervention group was in the severe anaemia group, with an adjusted risk increase of 18.6% (95% CI 11.5–25.6; interaction $p=0.0002$). There were 93 extra patients on treatment for confirmed tuberculosis who were discharged alive and 34 fewer post-discharge deaths in the intervention group than in the standard-of-care group. Sputum Xpert diagnosed a similar number of patients with tuberculosis in both groups (appendix). In the intervention group, TB-LAM provided the highest diagnostic yield (158 [75%] of 210 patients with microbiologically confirmed tuberculosis), compared with 74 (35%) patients positive with urine Xpert and 85 (40%) positive with sputum Xpert (appendix). The incremental diagnostic yield from urine Xpert as the only positive assay was only 13 (6%) patients, compared with 87 (41%) patients from TB-LAM and 30 (14%) patients from sputum Xpert.

Discussion

Although the 56-day all-cause mortality showed no significant differences between the standard-of-care and intervention groups, the addition of urine-based tuberculosis screening using TB-LAM and Xpert to sputum-based screening in all HIV-positive medical inpatients significantly reduced mortality at 56 days in prespecified high-risk subgroups, and substantially increased tuberculosis diagnoses and treatment across all patients. Fewer patients were on tuberculosis treatment at discharge in the standard-of-care group than in the intervention group, and more patients died after discharge, suggesting discharge with undiagnosed and untreated active tuberculosis as the main underlying mechanism. For every ten extra patients with confirmed tuberculosis discharged on treatment in the intervention group, there were 3.7 fewer deaths after discharge, supporting high individual risk of rapid progression to death in undiagnosed HIV-associated tuberculosis that could have been detected and treated through urine-based screening.

Morbidity and mortality from HIV-associated tuberculosis are slowly decreasing in Africa, mainly reflecting the expansion of HIV diagnosis and ART programmes rather than tuberculosis-specific diagnostic and prevention interventions.^{18,19} Although these trends are encouraging, we found disturbingly high risk of death or microbiologically confirmed tuberculosis, or both, within 56 days of admission, despite high ART coverage and median CD4 cell count. We report substantial mortality reductions from urine-based tuberculosis screening in prespecified high-risk subgroups, consistent with current recommendations for diagnostic LAM testing, but no significant effect on overall mortality at 56 days between groups (adjusted risk difference -2.8% , 95% CI -5.8 to 0.3). However, our study was underpowered to detect small ($<5\%$) absolute reductions in mortality at 56 days.

Our findings are consistent with the 4% (95% CI 1–7) mortality reduction in HIV-positive inpatients with clinically suspected tuberculosis reported from a diagnostic (not screening) randomised trial of urine LAM testing.²⁰ The participant profile in the diagnostic trial differed notably from this study, with lower ART coverage and CD4 counts (median 84 cells per μL vs 227 cells per μL), and a greater proportion of participants had tuberculosis (intervention groups: 51.6% vs 21.8%), reflecting different inclusion criteria (clinical suspicion of tuberculosis compared with an unselected population in our STAMP trial), as well as underlying population trends in ART coverage. Early survival benefit from these two trials underscores the fulminant course of undiagnosed tuberculosis in highly immunosuppressed patients, and the higher yield of urinary diagnostics and difficulty diagnosing tuberculosis among groups of hospitalised HIV-positive patients by other means.^{13,15}

Uniquely, STAMP recruited considerable numbers of less immunosuppressed or critically ill patients who fall outside current recommendations for urinary tuberculosis diagnostic assays.²¹ We show differences between groups in tuberculosis diagnosis, although with a corresponding mortality benefit only for predefined high-risk groups (ie, low CD4 cell counts, low haemoglobin, or clinically suspected tuberculosis). The absence of detectable survival benefit in patients with less profound immunosuppression might then simply reflect a slower time-course if median survival following discharge with undiagnosed tuberculosis is considerably longer than 56 days. If so, increases in tuberculosis treatment through early urine-based diagnosis will still have averted months of morbidity and contributed to reduced transmission, particularly in health-care settings.²² An alternative explanation is a higher proportion of false-positive urinary screening results in patients with CD4 counts of 100 cells per μL or more, which we consider unlikely given high specificity ($\geq 99\%$) shown elsewhere.^{14,15,23}

Better clinical acumen and alternative investigations such as radiology leading to early empirical tuberculosis

treatment can mitigate the effect of new diagnostic tests, as observed in relatively well resourced inpatient and outpatient settings, for example in South Africa.²⁴ We saw little evidence of this effect for urine-based screening in either Malawi or South Africa in our STAMP trial, and also showed no difference in routine management between groups, for instance in the use of broad-spectrum antibiotics to treat presumed bacterial infections. Both urine LAM (point-of-care lateral flow assay) and Xpert (approximately 2 h after urine centrifugation) are rapid, as was initiation of tuberculosis treatment in this trial, which are crucial to affect mortality and potentially transmission.

The least costly and easiest urine test (TB-LAM) had major incremental diagnostic benefit in this trial, with urine Xpert (which is more complex because of urine centrifugation) contributing few additional diagnoses. This finding argues for the use of LAM alone as the urinary diagnostic for screening, an approach supported by STAMP's cost-effectiveness projections being reported separately.²⁵ Urine Xpert might still have a place alongside other diagnostic modalities for urine LAM-negative patients with high clinical suspicion for tuberculosis.¹⁶ Sputum Xpert is already a recommended standard of care for HIV-positive individuals with tuberculosis symptoms (although not uniformly implemented), and was included for all patients able to expectorate in both trial groups.⁹ Our data support this approach, because sputum provided the only microbiological diagnosis for 14% of patients with tuberculosis in the intervention group.

Despite the qualified mortality benefits, we consider our results to be supportive of routine implementation of systematic screening with urinary LAM, in addition to sputum Xpert, for all HIV-positive inpatients, given that LAM screening provides a substantial increase in diagnosis of a treatable but frequently fatal condition (disseminated tuberculosis). Systematic screening on admission to hospital is a simple strategy that avoids expense and delay from identifying high-risk groups (including by CD4 count testing, which might not be routinely available).²⁶ Tuberculosis symptoms were present in 90% of patients yet only 39% were considered to have tuberculosis by clinicians, who would have missed a substantial number of diagnoses. Notably, 61% of inpatients in STAMP were in one or more high-risk groups with a mortality benefit. Pronounced differences in short-term mortality and underlying prevalence of disseminated tuberculosis between HIV-positive inpatients and outpatients also means that current WHO policy (specific recommendation against use for screening, with use of TB-LAM indicated only for diagnostic purposes in patients with tuberculosis symptoms and CD4 counts <100 cells per μ L or signs of severe illness) should remain in use for outpatients.²¹

There are limitations to our study. In sample size calculations, we assumed a higher mortality and burden

of tuberculosis than we observed, possibly because of the success of ART scale-up. We did not include a culture reference standard, as this reference is neither standard of care nor routinely available in Malawi, and would have presented ethical dilemmas or affected generalisability. Relatively few participants in Malawi produced sputum. This difficulty in expectorating sputum is, however, typical of unselected HIV-positive outpatient and inpatient cohorts in resource-constrained settings, and is a major barrier to implementation of sputum-based tuberculosis screening.^{15,27} Generalising the true effect of this intervention might be compromised by uncharacteristically prompt specimen collection and results reporting in the study setting, and masking of routine clinicians to exactly which tests had been done might have altered their clinical decision making.²⁸ Conversely, because both urine assays were run in a laboratory, we might have underestimated the effect on outcomes from TB-LAM if used at the bedside with faster turnaround times. Patients excluded because of their inability to consent will have introduced bias to the study towards the less critically ill, potentially affecting generalisability.

In conclusion, we report a survival benefit from urine-based tuberculosis screening of HIV-positive hospital admissions in high-risk subgroups, and a broader benefit through substantially increased predischARGE tuberculosis diagnosis and treatment in all patients. Tuberculosis screening with urine-LAM lateral flow assays is inexpensive and easily implementable, requiring minimal infrastructure and training. Tuberculosis diagnosed through urine-based screening was complementary to tuberculosis diagnosed through standard clinical investigations in both countries, with inferred higher risk of discharge with undiagnosed tuberculosis in the standard-of-care group than in the intervention group. Anticipated improvements in analytical performance of next-generation LAM assays might add to the diagnostic yield from systematic urine screening.²⁹ Current WHO guidelines on the diagnostic use of TB-LAM in HIV-positive inpatients have been insufficient to motivate widespread implementation within African hospitals.³⁰ These results support changes to current policy and guidelines for routine inclusion of urine-based tuberculosis screening using TB-LAM in a package of interventions for HIV-positive patients admitted to hospital in high-burden settings, aiming to reduce short-term morbidity and mortality.³¹⁻³³ Such new strategies need to be urgently implemented to achieve WHO's End TB Strategy targets of a 75% reduction in tuberculosis mortality by 2025.

Contributors

ELC, JjvO, DW, SDL, and KF were involved in the study conception. AG-W, ELC, JjvO, DW, SDL, and KF were involved in the study design. SDL obtained the funding. AG-W, ELC, JjvO, DW, MA-M, JAP, LC, SDL, and KF implemented the study. DG and CF were the study statisticians. AG-W, DG, CF and KF were involved in the analysis. AG-W, ELC, JjvO, DW, DG, and KF were involved in interpreting the data. AG-W, ELC, and KF wrote the first draft. All authors, except SDL (deceased), read and approved the manuscript.

Declaration of interests

We declare no competing interests.

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