Investigation into mycobacterial persistence and early markers of outcome in the treatment of pulmonary tuberculosis

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

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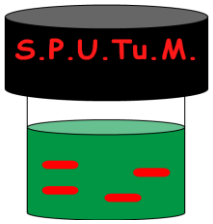
The 'S.P.U.Tu.M.' Study
Studying Persistence and Understanding Tuberculosis in Malawi



This thesis is the result of my own work. In some instances, work was done in conjunction with other colleagues and institutions. The roles of my co-workers and collaborators in this project are detailed below.

Activity	Responsibility
Conduct of patient screening, recruitment and follow-up visits *(Chapter 3)*	Chrissie Guwende*, Gertrude Banda*, Bryt Chisale* *(MLW Clinical Research Programme, Malawi)*
Quantitative bacteriology assays *(Chapters 4 and 6)*	Doris Shani* and Mercy Kamdolozi* *(College of Medicine, University of Malawi)*
Genotypic drug susceptibility testing *(Chapter 3)*	Geoffrey Kumwenda* *(College of Medicine, University of Malawi)*
Second reader on CXR interpretation *(Chapter 3)*	Elizabeth Joekes *(Royal Liverpool University Hospital, UK)*
Vitamin D bioassay *(Chapter 3)*	John Dutton *(Royal Liverpool University Hospital, UK)*
Electron microscopy *(Chapter 5)*	David Russell *(Cornell University, USA)*
LAM-ELISA *(Chapter 6)*	Mavis Menyere *(MLW Clinical Research Programme, Malawi)*
Statistical support with partial likelihood mixed effects models, second reader of ALTR microscopy images *(Chapter 6)*	Gerry Davies *(University of Liverpool, UK)*
Pharmacokinetic bioanalysis *(Chapter 7)*	David Waterhouse, Alison Ardery, Lisa Stone *(Liverpool School of Tropical Medicine)*

*These individuals were under my direct daily supervision

The material contained within the thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification.

This research was carried out in the following centres:

Institutes of Infection and Global Health and Translational Medicine, the University of Liverpool, UK

Liverpool School of Tropical Medicine, UK

Malawi Liverpool Wellcome Trust Clinical Research Programme, Blantyre, Malawi

Department of Microbiology, College of Medicine, University of Malawi, Blantyre Malawi

Dedication

This thesis is dedicated to my wife, Jennifer, whose selfless sacrifice, patient tolerance of my absence and unstinting support were more than I deserved and will not be forgotten.

Till a' the seas gang dry, my Dear,
And the rocks melt wi' the sun:
I will luve thee still, my Dear,
While the sands o'life shall run.

Robert Burns

Abstract

Background: Development of ultra-short chemotherapy for tuberculosis (TB) is thwarted by drug-tolerant bacillary persistence and a lack of surrogate endpoints to predict outcome from early clinical studies. Characterising bacillary elimination amongst TB patients may provide important new information. Bacilli harbouring intra-cytoplasmic lipid bodies (LBs) may represent a drug-tolerant phenotype, responsible for delayed bacterial clearance.

Methods: Malawian adults with pulmonary TB were treated with standard 6 month therapy. Two quantitative sputum culture methods were used to model bacillary elimination during the first 2 months; serial sputum colony counting (SSCC) and time to positivity (TTP) in BACTEC MGIT broth. Fluorescence microscopy was used to assess the proportion of LB positive bacilli on sputum smears. Plasma concentrations of anti-TB drugs were assayed at day 14 or 21. Patients were followed until one year post-treatment. Outcomes were defined as favourable (stable cure) or unfavourable (failure/relapse). The effect of microbiological and pharmacological factors on outcome was assessed.

Results: 169 patients (59% with HIV co-infection) were recruited. Of 133 final outcomes, 15 (11%) were unfavourable. Partial likelihood non-linear mixed effects (NLME) modelling of SSCC data from 86 (64%) patients showed biphasic bacillary elimination; slow late-phase eradication of persisters was associated with unfavourable outcome (p=0.001). Linear mixed effects (LME) modelling of TTP data from 124 (93%) patients showed that a slower MGIT Bacillary Elimination Rate (MBER) was associated with unfavourable outcome (p=0.007). 28% (range 0-79%) of acid-fast bacilli in baseline sputum samples were LB positive. During the first month there was a trend towards higher LB counts in patients who went on to have unfavourable vs. favourable outcomes (p=0.085). Low plasma rifampicin and isoniazid concentrations were reported in 87% and 50% patients respectively. A lower isoniazid $AUC_{0\text{-}6hr}$ exposure was associated with unfavourable outcome (p=0.035).

Conclusions: The two main findings were:

1. Modelling of bacillary elimination during the first 2 months of TB therapy predicted long-term outcome. The MBER, in particular, could be calculated for 93% of patients and should be considered as a surrogate marker for early clinical trials.
2. The observation of a higher proportion of LB positive bacilli in later sputum samples from patients with unfavourable outcomes suggests that these may be drug-tolerant persister cells, with negative implications for treatment efficacy.

Acknowledgements

This was a long and, at times, difficult project to complete. Thanking all of those who offered support and encouragement along the way is impossible but individual acknowledgements are required.

For technical support in TB laboratory work I am most indebted to Gavin Laing, Geoffrey Chipungu and Aaron Mdolo. For collaborative guidance on lipid body fluorescence microscopy I relied heavily on the contribution of time, ideas and expertise from Mike Barer and Natalie Garton. For laboratory support in the pharmacokinetics bio-assays I was dependent on Steve Ward.

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Peter Winstanley encouraged me to apply for a Wellcome Trust Fellowship in the first place. Liz Corbett, Anthony Butterworth, Rob Heyderman and David Lalloo provided regular advice and mentorship along the way.

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And the final word of gratitude is reserved for Dick Mizati, my Chichewa teacher and friend whose proverbs and aphorisms reminded me to persevere when obstacles seemed insurmountable and circumstances looked bleak.

"Tsokonombwe anatha mthunda ndi kulumpha"

The wingless grasshopper still covered the distance by hopping.

Publications and abstracts related to this work

Scientific publications

Sloan DJ, Davies GR, Khoo SH New drugs and treatment regimens. Current Respiratory Medicine Reviews: Recent Advances in Tuberculosis 2013 (in press)

Sloan DJ, Corbett EL, Butterworth AE, Mwandumba HC, Khoo SH, Mdolo A, Shani D, Kamdolozi M, Allen J, Mitchison DA, Coleman DJ, Davies GR Optimizing outpatient serial sputum colony counting for studies of tuberculosis treatment in resource-poor settings. J Clin Microbiol. 2012, 50(7):2315-20

Book chapter

Sloan DJ, Davies GR Anti-tuberculous chemotherapy: serial sputum colony counting in drug development, p 277–324. *In* Donald PR, Van Helden PD (ed), Progress in respiratory research, vol 40. Karger, Basel, Switzerland 2011

Oral and poster presentations

Sloan DJ, Mwandumba H, Guwende C, Chipungu G, Buttterworth A, Dutton J, Heyderman R, Corbett E, Khoo S, Davies G Intensive phase bacillary elimination rate predicts clinical outcome in HIV+ and HIV- Malawian adults with smear-positive pulmonary TB. Presented at 20th Conference on Retroviruses and Opportunistic Infections, Atlanta March 2013
(Young Investigator Award)

Sloan DJ, Mwandumba HC, Corbett EL, Butterworth AE, Chipungu G, Heyderman RS, Ardrey A, Waterhouse D, Ward SA, Khoo SH, Davies GR Pharmacokinetic-pharmacodynamic modelling of tuberculosis treatment response in Malawian adults with smear positive pulmonary tuberculosis. Presented at 5th International Workshop on Clinical Pharmacology of Tuberculosis Drugs, San Francisco, September 2012

Sloan DJ, Mwandumba HC, Corbett EL, Butterworth AE, Heyderman, RS, Garton N, Barer M, Khoo SH, Davies GR Bacillary elimination rates, persister phenotypes and clinical outcomes in a longitudinal study of TB treatment in Malawi. Presented at Keystone Symposium on Drug Resistance and Persistence, Kampala, May 2012
(US National Institute of Health and National Institute of Allergy and Infectious Diseases Keystone Symposium Scholarship Award)

Table of Contents

Table of Figures

Table of Tables

Abbreviations

Abbreviations are defined at the time of first use. The following list may also be useful

AFB	Acid Fast Bacilli
af-LB	Acid Fast Lipid Body
AIC	Akaike Information Criterion
ALT	Alanine Transaminase
ALTR	Auramine O/LipidTOX Red
AmB	Amphotericin B
ANOVA	Analysis of Variance
ANR	Auramine O/Nile red
AP	Auramine O Phenol
ART	Anti-retroviral Therapy
AUC	Area under the curve
BCG	*Bacillus Calmette–Guérin*
BL	Baseline
BMI	Body Mass Index
BMRC	British Medical Research Council
BSC	Biological Safety Cabinet
BSL-3	Biosafety Level 3
CFP-10	Culture Filtrate Protein-10
CFU	Colony Forming Units
CI	Confidence Intervals
C_{max}	Maximum drug concentration
CoM	College of Medicine
CRF	Case Record File
CRP	C-reactive protein
CT	Computed Tomography
CXR	Chest radiograph
DOTS	Directly Observed Short Course
DNA	Deoxyribonucleic Acid
DHS	Demographic Health Survey
DS	Drug Susceptible
E	Ethambutol
EBA	Early Bactericidal Activity
ELISA	Enzyme Linked Immunoabsorbant Assay
EM	Electron Microscopy
EOT	End of Treatment
EPTB	Extra Pulmonary Tuberculosis
ESAT-6	Early Secretory Antigen Target-6
FDA	Fluorescein-diacetate
FDC	Fixed Dose Combination
FITC	Fluorescein Isothiocyanate
FSC	Forward Scatter
H	Isoniazid
HCW	Health Care Workers
HIV	Human Immunodeficiency Virus

HPLC	High Performance Liquid Chromatography
IFN-γ	Interferon-γ
IGRA	Interferon- γ Release Assay
IRIS	Immune Reconstitution Inflammatory Syndrome
IUALTD	International Union Against Tuberculosis and Lung Disease
JHU	John Hopkins University
LAM	Lipoarabinomannan
LB	Lipid Bodies
LC/MS/MS	Liquid chromatographic/tandem mass spectrometry
LJ	Lowenstein-Jensen
LME	Linear Mixed Effects
LSTM	Liverpool School of Tropical Medicine
MBER	MGIT Bacillary Elimination Rate
MBL	Molecular Bacillary Load
MDG	Millennium Development Goal
MDR	Multi-drug resistant
MFI	Mean Fluorescence Intensity
MGIT	Mycobacteria Growth Indicator Tube
MIC	Minimum Inhibitory Concentration
MLW	Malawi Liverpool Wellcome Trust Clinical Research Programme
8-MQ	8-methoxyfluoroquinolones
MUT	Mutant
NALC	N-acetyl-L-cysteine
NaOH	Sodium hydroxide
NLME	Non Linear Mixed Effects
NRP	Non Replicating Persister
NTM	Non Tuberculous Mycobacteria
NTP	National TB Control Programme
OADC	Oleic Acid Albumin Catalase
OD	Optical Density
OR	Odds Ratio
PANTA	Polymyxin-Trimethoprim-Amphotericin B-Azlocillin-Nalidixic acid
PAR	Peak Area Ratio
PAS	Para-amino salicylic acid
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography
PI	Principal Investigator
PK-PD	Pharmacokinetic-Pharmacodynamic
PMA	Propidium monoazide
PNB	Para-nitrophenol benzoic acid
PTB	Pulmonary Tuberculosis
QECH	Queen Elizabeth Central Hospital
R	Rifampicin
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
Rpf	Resuscitation promoting factor
RR	Respiratory Rate
RRDR	Rifampicin Resistance Determining Region
rt-PCR	real-time PCR (a quantitative PCR assay)
S	Streptomycin

SCC	Sputum Culture Conversion
sICAM-1	soluble Intracellular Adhesion Molecule-1
SNP	Single Nucleotide Polymorphism
SSC	Side Scatter
SSCC	Serial Sputum Colony Counting
sTNFαR-1	soluble TNF-α receptor-1
suPAR	Soluble urokinase plasminogen activator receptor
T	Thiacetezone
TAG	Triacylglycerol
TB	Tuberculosis
TBTC	Tuberculosis Trials Consortium
TCA	Tricarboxylic Acid
TLB	Total Lipid Body
TRITC	Tetramethylrhodamine
TTP	Time to Positivity
WCC	White Cell Count
WHO	World Health Organisation
WT	Wild type
XDR	Extensively Drug Resistant
Z	Pyrazinamide
ZN	Ziehl-Neelsen

1. General Introduction

1.1 Tuberculosis (TB)

Tuberculosis (TB) is a disease which has plagued humankind for many millennia. Global dissemination of the causative pathogen, *Mycobacterium tuberculosis* probably began 15,000 years ago[1] but the organism was not identified until the work of Robert Koch in 1882[2]. At that time TB accounted for up to 25% of deaths in Europe[3].

Mortality from TB in industrialised countries declined dramatically in the first half of the 20th Century, largely due to improvements in living standards (housing, nutrition and income)[4]. A series of groundbreaking advances in anti-mycobacterial chemotherapy between the 1940s and the 1980s resulted in the development of drug regimens with proven efficacy in curing the disease and for a brief period it appeared that the major obstacles to global TB control had been overcome. However, two important events dispelled this optimism; the collapse of Public Health infrastructure in former Soviet states resulted in an epidemic of multidrug resistant (MDR) TB amongst marginalised populations in Eastern Europe[5] and the unrestrained Human Immunodeficiency Virus (HIV) pandemic fuelled a dramatic upsurge in morbidity and mortality from all forms of TB worldwide, particularly in southern Africa[6]. Up to one third of the world's population are latently infected with *M tuberculosis*[7] and in 1994 the World Health Organisation (WHO) declared that TB control was a "global emergency" [8]. In 2011, there were an estimated 8.7 million new TB cases and 1.4 million people died of the disease[9].

In 2000, the United Nations set a series of Millennium Development Goals (MDGs). Target 6c was to halt and begin to reverse the rising incidence of tuberculosis by 2015[10]. It currently appears that this target will be met. Additional goals set by the STOP-TB Partnership were that TB prevalence and mortality should be halved by 2015 compared with levels in 1990 and TB should be eliminated as a public health problem by 2050[11]. A 50% reduction in global mortality may be achieved by 2015 amongst HIV non-infected patients. Yet this progress conceals regional variations and ongoing difficulties: Africa is not on track to reach the mortality goal, incidence and death rates in HIV co-infected individuals remain unacceptably high and critical funding gaps may jeopardise recent advances[9].

Improving the global response to TB requires development of shorter and better treatments. This will be facilitated by improved understanding of factors which influence the clinical

response to therapy and optimisation of the methodology for clinical trials of new drugs. These topics are the focus of this thesis. As the research to be described was conducted in southern Africa, the remainder of this chapter will outline the specific epidemiology of the related TB and HIV epidemics in that region. It will then provide background information on the main research questions.

1.2 Recent epidemiology of TB: a focus on Africa

The disproportionate impact of the global TB disease burden on Africa is well known. 22 low- and middle-income countries account for more than 80% of active TB cases in the world and 9 of these are in Africa (DR Congo, Ethiopia, Kenya, Mozambique, Nigeria, South Africa, Uganda, Tanzania and Zimbabwe). Africa accounts for 79% of HIV-associated TB and 8 countries in the south and east of the continent report ≥50% HIV sero-prevalence amongst new TB patients (Lesotho, Malawi, Mozambique, Namibia, South Africa, Swaziland, Uganda and Zimbabwe)[9]. Figure 1.1 compares TB incidence trends in these 8 countries with other global territories to demonstrate the regional problem.

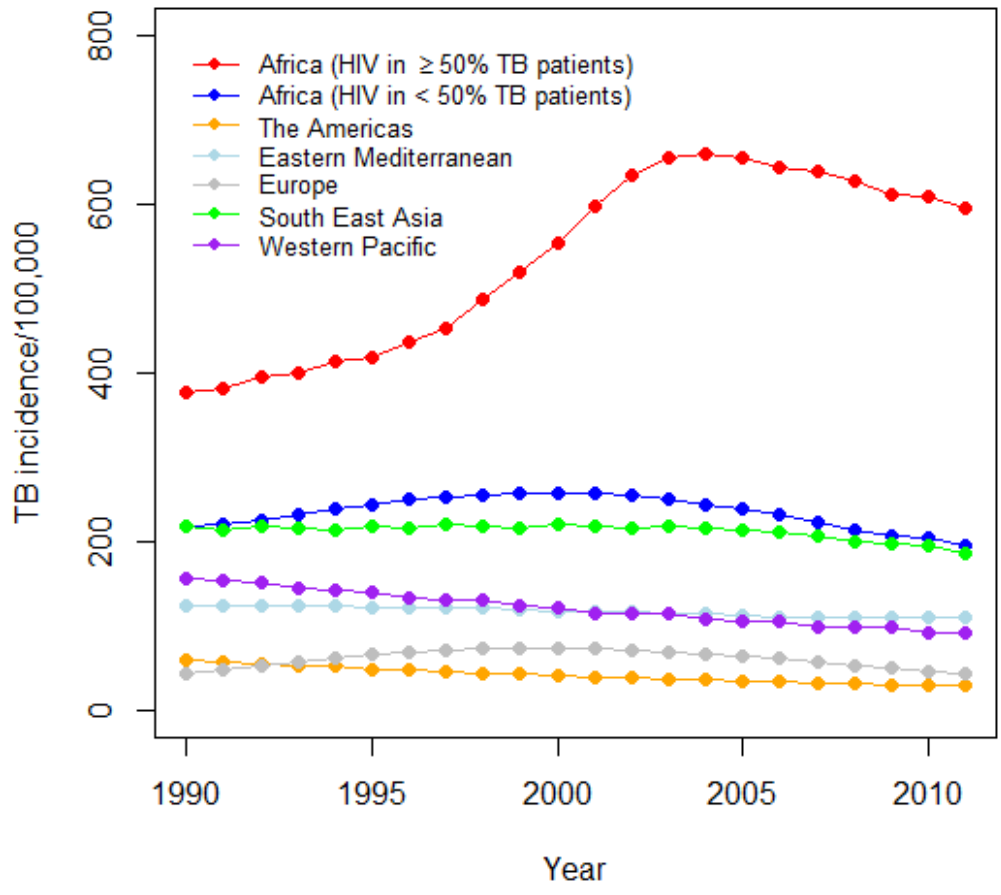
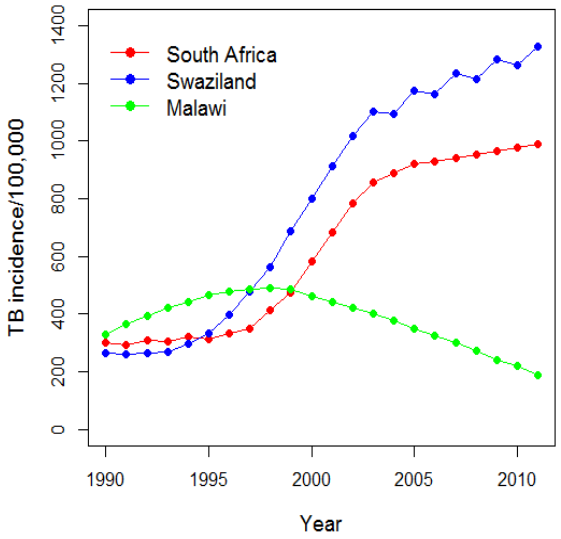


Figure 1.1 Trends in estimated TB incidence in global sub-regions from 1990-2011
Data extracted from WHO Global Tuberculosis Report 2012[9]

Even amongst the 8 African countries with the highest rates of HIV-TB co-infection epidemiological trends are diverse. South Africa and Swaziland have suffered a persistent rise in TB incidence during the last decade, whilst rates in other countries such as Malawi (where the work in this thesis was conducted) have begun to fall. MDR and extensively drug resistant (XDR) TB are well-described in South Africa[12-14] and Swaziland[15] but much less frequently reported in Malawi (Figure 1.2). The majority of data on the response to TB treatment in African patients currently come from South Africa. It is important that clinical, microbiological and pharmacological data are generated from other centres, both to validate findings which can be generalised and to highlight differences.



	Drug resistance amongst incident TB cases in 2011 (%)	
	New cases which are MDR	Cases in previously treated patients which are MDR
South Africa	1.8	6.7
Swaziland	7.7	34
Malawi	0.4	4.8

Figure 1.2 TB incidence and MDR-TB rates in South Africa, Swaziland and Malawi
Data extracted from WHO Global Tuberculosis Report 2012[9]

1.3 HIV: contribution to TB morbidity and mortality

Given their co-endemicity in Southern Africa, the close association between TB and HIV requires consideration. It is well known that the impairment in cellular immunity caused by HIV results in a 20-fold increase in the likelihood that latent TB infection will progress to active disease[16]. Whilst the lifetime risk of reactivating latent TB in HIV un-infected individuals is ≈10% the risk is nearer 10% per year in HIV-infected people[17]. The level of HIV-associated immunosuppression is also important; patients with CD4 counts <100 cells/μl have a 10 times higher risk of acquiring TB than patients with CD4 counts >500 cells/μl[18]. In patients with active TB, therapeutic principles and cure rates are not altered by HIV status[19]. However, HIV is associated with higher rates of relapse and re-infection after treatment particularly if an intermittent (non-daily) drug regimen is used or patients do not

initiate antiretroviral therapy (ART)[20-23]. These factors are important when analysing treatment response and outcome data.

HIV affects the nature of TB presentation. Active TB in HIV un-infected individuals typically presents with sputum smear positive pulmonary disease but in HIV-infected patients, particularly at lower CD4 counts, disease commonly presents beyond the lungs in other body sites including the pleural, pericardium, lymphatic system and central nervous system[3]. Whilst the work in this thesis will focus on sputum smear positive pulmonary TB (PTB) it is important to acknowledge that some individuals may also have disseminated or extra-pulmonary (EP) disease which cannot easily be measured.

1.4 Sputum smear microscopy for TB diagnosis and follow-up

The primary tool for diagnosis of PTB and monitoring of treatment response in low-income countries is sputum smear microscopy. Although limited by poor sensitivity, particularly amongst HIV-infected individuals[24], this technique is widely used because it is less expensive than newer methods (e.g. liquid culture[25] and the molecular Xpert MTB/RIF assay[26,27]). Baseline smears help determine which patients require anti-TB treatment whilst samples 2 and 5 months into therapy are used to identify individuals at high risk of treatment failure[28-31]. In this thesis, the role of a modified sputum smear microscopy technique to identify a bacillary phenotype which responds poorly to therapy is evaluated and the basis of smear microscopy should be outlined.

M tuberculosis organisms are acid-fast bacilli (AFB) meaning that they resist decolourisation by acid-alcohol during microbiological staining. As their thick cell wall is rich in mycolic acids (Figure 1.3) it absorbs standard microscopy stains very poorly. However, specific dyes bind directly to mycolic acid[32] or pass through the cell envelope to bind intracellular DNA/RNA[33]. When stained specimens are treated with acid alcohol, TB bacilli retain these dyes whilst other cells and inorganic debris are decolourised and adopt subsequently applied counter-stains. In the traditional Ziehl-Neelsen (ZN) AFB staining protocol (Appendix 10.4.1) red bacilli are seen against a blue background. This has been replaced in many centres by a more sensitive Auramine-O Phenol (AP) technique (Appendix 10.4.2) which shows bright fluorescent yellow-gold bacilli against a black background and allows faster reading of slides at lower magnification[34].

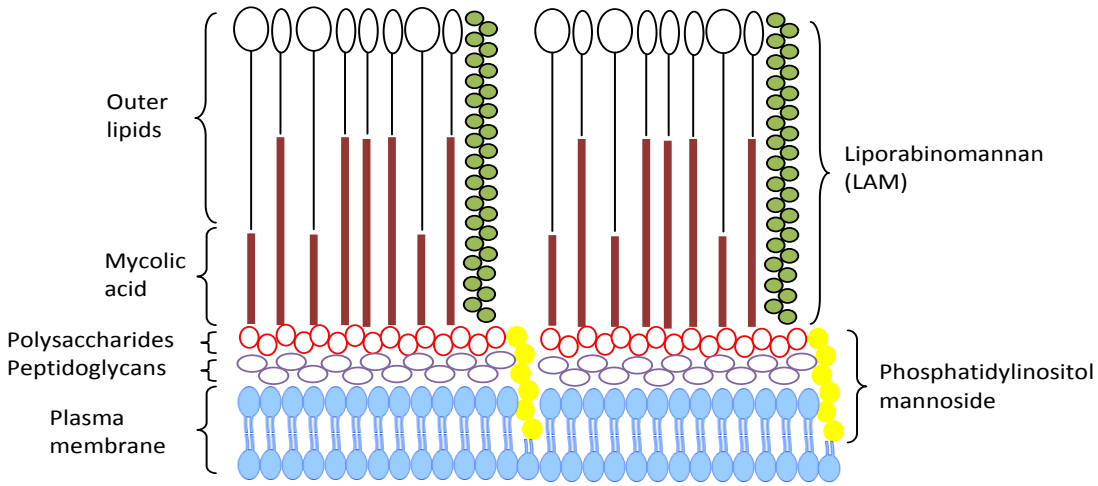


Figure 1.3 Structure of mycobacterial cell wall

The thick cell wall and mycolic acid content confer the staining properties of acid-fastness. Inhibition of synthesis of some cell wall components is exploited by some anti-TB drugs. Lipoarabinomannan (LAM) is excreted in the urine forming the basis of potential new diagnostic tests.

1.5 TB treatment: history and current therapy

An understanding of contemporary research goals in TB therapeutics requires appreciation of how existing treatments were developed, why they are now inadequate and the obstacles delaying their replacement.

1.5.1 Historical perspective

Prior to the 1940s, TB chemotherapy was unavailable. Robert Koch initially proposed a role for immunotherapy based on the "Koch phenomenon" of delayed skin hypersensitivity following subcutaneous injection of tuberculin[35], but this proved potentially harmful and no form of immunotherapy has since demonstrated efficacy in treatment of TB disease[36-39]. In the late 19th and early 20th Century environmental, nutritional and surgical measures were of limited benefit[40,41]. Surgery (including artificial pneumothorax, thoracoplasty and apicolysis) were also dangerous[42]. The case-fatality rate from pulmonary TB exceeded 50%.

Development of sulfanilamide[43] and penicillin[44] to treat other bacterial infections generated hope that TB may be cured by antibiotics but the early drugs were not useful against mycobacteria[45]. However, in 1944, para-aminosalicylic acid (PAS) was synthesised and showed efficacy against *M tuberculosis*[46]. In the same year, streptomycin (S), was isolated from the soil actinomycete *Streptomyces griseus* [47] and in 1952, following observations that nicotinamide had anti-mycobacterial activity in animal models [48,49] the nicotinamide analogue isoniazid (H) was discovered[43,50,51]. Within ten years, three new anti-TB agents had been found[52].

These drugs were rigorously evaluated in the first randomized controlled trials to be performed in clinical medicine[53-57] and it was quickly established that the initial response to chemotherapy especially in severe disease (e.g. meningitis) was life-saving. However, single drug therapy was compromised by the emergence of drug-resistant mutant organisms[58,59]. The natural frequency of mutants with resistance to each individual agent was much lower than the total bacillary load in a clinical infection[60], facilitating the adoption of combination therapy[61] as a means of ensuring durable cure[62,63]. In the 1960s, PAS was replaced by thioacetezone (T) to reduce the cost of treatment and Canetti proposed a biological model of two phase therapy which has endured to the present day[64]. The principle was that, in early treatment an "intensive phase" of three or four drugs is necessary to rapidly reduce the bacillary burden without generating resistance, whilst in later treatment a prolonged "continuation phase" of one or two drugs may be used to eradicate residual organisms. A regimen with a 2 month intensive phase of STH followed by a 16 month continuation phase of TH was introduced in several countries[65-67]. However, in Kenya this regimen performed less well under routine conditions than it had in clinical trials[68], providing an early indication of the difficulties associated with prolonged treatment.

1.5.2 Development of 'short course' anti-TB chemotherapy

Current "short course" chemotherapy has evolved from those early studies on the basis of a series of international multi-centre trials performed on sputum culture positive PTB patients from 1970 onwards by the Tuberculosis Research Unit at the British Medical Research Council (BMRC)[69] the US Public Health Service and others.

These trials depended on the re-introduction of an old drug, pyrazinamide (Z) and the discovery of a new one, rifampicin (R). Pyrazinamide, synthesized in 1952[70] and its derivative morphozinamide[71] are analogues of nicotinamide. Pyrazinamide has anti-mycobacterial activity at acid pH *in vitro*[72] and showed promise in animal models[73] but the initial doses used for humans were hepatotoxic[74,75] so, until the 1970s, it was excluded from trials of first-line treatment. Rifampicin is a semi-synthetic derivative[76] of the natural product Rifamycin S, a major secreted antibiotic of *Streptomyces mediterranei*[77]. Synthesized in 1966, *in vitro* data suggested that it was highly active against mycobacteria[78] and early human studies were promising[79,80].

The First BMRC Collaborative Study in East Africa, commenced in 1970, indicated that a 6 month combination of SHR was associated with effective cure and a post-treatment relapse

rate of only 3% at 2 years (equivalent to that of 2SHT/16HT). 6 months of SHZ was slightly less effective, but still resulted in only 8% relapses at 2 years. This historic result showed that rifampicin and pyrazinamide could facilitate dramatic treatment shortening[81-83]. The next two East African studies demonstrated that a 2 month, four drug intensive phase (SHRZ) achieved negative sputum cultures in more than 80% patients, and a subsequent continuation phase of daily TH or thrice weekly SHZ for 4 months resulted in a 2 year relapse rate of only 4-7%[84-87]. Later work revised the combination of continuation phase drugs and showed that rifampicin is beneficial when continued throughout therapy, whilst the effect of pyrazinamide is confined to the first 2 months (a result confirmed by a separate trial in Hong Kong[88]). In the Fifth and Sixth Collaborative Trials[89-91] it was determined that an HR continuation phase was superior to any other option, reducing the relapse rate by more than 50% compared to isoniazid alone. These findings were supported by the results of United States Public Health Service Studies 18 to 21[92-95] and a trial in the United Kingdom organized by the British Thoracic Association[96].

BMRC studies in Hong Kong and Algeria revealed that oral ethambutol (E) may replace parenteral streptomycin without compromising outcomes[97-101]. Although ethambutol is bacteriostatic rather than bactericidal, it is less toxic than streptomycin and its oral administration is more compatible with out-patient therapy. In 2004, the International Union Against Tuberculosis and Lung Disease (IUALTD) Study A validated an intensive phase of 2 months RHZE followed by a continuation phase of 4 months HR as the most efficacious and practical combination of currently available agents[102]. This regimen is currently the WHO approved first-line regimen for all forms of non-drug resistant TB[30]. Important characteristics of the constituents are summarised in Table 1.1.

In retrospect, one of the most important results of the BMRC research effort was the Fourth East African Collaborative Study which evaluated five four month regimens and showed that, despite favourable early data, no combination of streptomycin, isoniazid, rifampicin and pyrazinamide at this duration achieved a 2 year relapse rate of less than 10%. Furthermore, if rifampicin was omitted from the continuation phase relapses occurred in over 30% of patients (Figure 1.4)[103,104]. Several smaller and less methodologically rigorous studies have since suggested that shorter regimens comprising current first line drugs might achieve lower relapse rates[105] but, particularly in the era of HIV co-infection, the prevailing view until recently has been that treatment duration may not be shortened below six months[106].

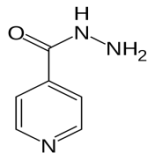
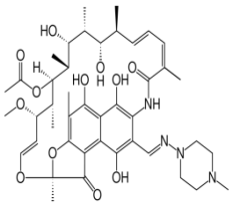
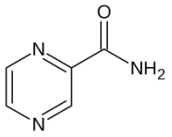
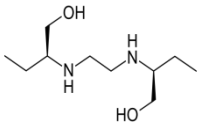
Drug	Chemical class and main mechanism of action	Key clinical studies supporting role in short course chemotherapy
Isoniazid (H) 	***Nicotinamide analogue*** Early bactericidal activity Pro-drug activated by KatG gene Generates nitric oxide and inhibits mycolic acid synthesis in the mycobacterial cell wall[107]	***Early studies (1950s & 60s):*** Culminated in 2SHT/16HT regimen with 3% relapses at 2 years[83] ***BMRC studies in 1970s & 1980s:*** Used in all trial regimens[69]
Rifampicin (R) 	***Rifamycin*** Early bactericidal and sterilising activity Inhibits bacterial DNA-dependent RNA polymerase to prevent protein synthesis[108]	***BMRC East Africa studies:*** 6SHR:3% relapses at 2 years[83] 2SHRZ: >80% SCC at 2 months[84,85] 2SHRZ/4HR: >80% SCC at 2 months and 3% relapses at 2 years[89,91] ***IUALTD Study A:*** 2RHZE/4RH: 5% 18 month relapses compared to 10% with 2RHZE/6HE[102]
Pyrazinamide (Z) 	***Nicotinamide analogue*** Sterilising activity Pro-drug of pyrazinoic acid Requires acid pH[109] Inhibits Fatty Acid Synthase I[110] Inhibits trans-translation in dormant bacilli[111]	***BMRC East Africa Studies:*** 6SHZ: 8% relapses at 2 years[83] 2SHRZ: >80% SCC at 2 months[81,82] 2SHRZ/4HR: >80% SCC and 3% relapses at 2 years[85,87] ***BMRC Hong Kong studies:*** Extending use of Z for >2 months did not improve relapse rates, particularly in regimens containing R[88]
Ethambutol (E) 	Parent to ***ethylenediamines*** Bacteriostatic activity Inhibits arabinosyl transferase to disrupt arabinogalactan synthesis in mycobacterial cell wall[112]	***BMRC Hong Kong studies:*** 6RHZE: 94% SCC at 2 months and 4% relapses at 5 years, no injections[98-100] ***BMRC Algeria studies:*** 2RHZE/4RH: 3% relapses at 2 years, no injections[97,101]

Table 1.1 Drugs used in current first-line anti-TB therapy

BMRC=British Medical Research Council, IUALTD=International Union Against Tuberculosis and Lung Disease, Numbers before drug regimens specify duration in months. SCC= Sputum culture conversion.

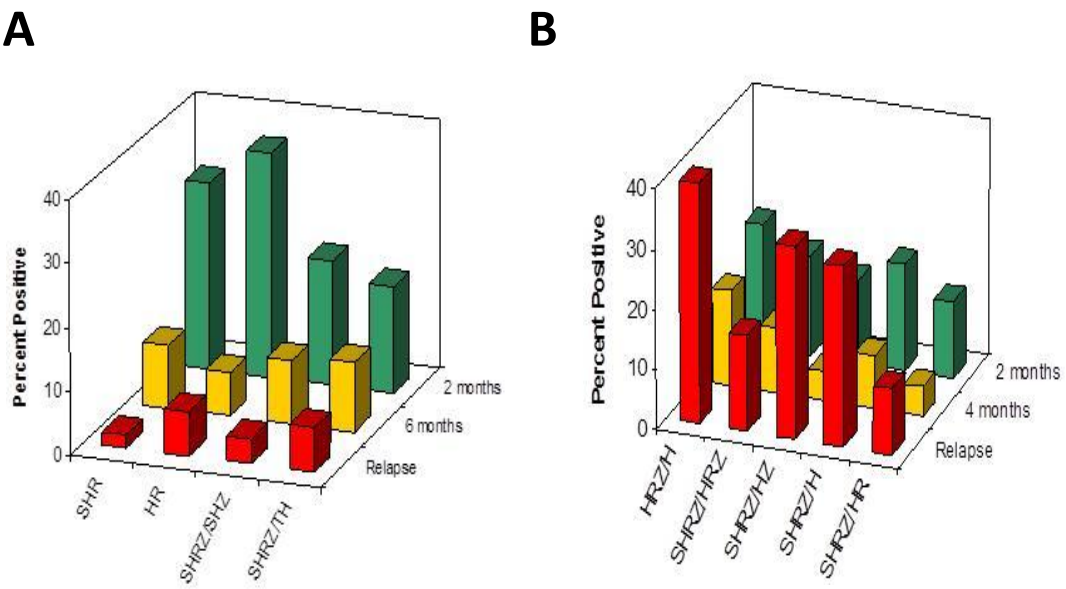


Figure 1.4 Bacteriological outcomes from BMRC East Africa Second and Fourth Studies

A: BMRC East Africa Second Study, Four 6 month regimens showing that 20-36% patients remained sputum culture positive at 2 months (**green** bars), 10-20% were culture positive at 6 months (**gold** bars), but <10% of cured patients relapsed by 2 years post-treatment (**red** bars)[84,85].

B: BMRC East Africa Fourth Study, Five 4 month regimens showing that whilst the proportion of positive sputum cultures was <20% at 2 and 4 months (**green** and **gold** bars), the 2 year relapse rate was unacceptably high (**red** bars). In particular, 30-40% patients who did not receive rifampicin (R) in the continuation phase suffered relapse[100,101].

1.6 The need for "ultra short" treatment

Progress from no effective drugs to highly efficacious fully oral treatment within 40 years was impressive. However, no new first line anti-TB drugs have been identified since rifampicin in 1967 and failure to achieve further treatment shortening is problematic. As the strain of the global TB-HIV pandemic on healthcare resources in low-income countries has increased the need for "ultra-short" regimens has become urgent[113] and predictions from mathematical models now suggest that therapy of less than 2 months duration could prevent 20% of new TB cases and 25% of TB deaths in some settings[114]. The major problems driving the need for "ultra-short therapy" will now be described.

1.6.1 Adherence and resistance

Until the 1960s, TB patients were managed in sanatoria. Thereafter, studies from south India proved that domiciliary therapy was equally effective to sanatoria based care and did not put household contacts at increased risk of infection[115,116]. The standard approach became out-patient treatment and it is now inconceivable that all TB patients should be hospitalised for the duration of therapy.

However, case-holding and supervision of out-patient treatment for 6 months requires a robust infrastructure and as the number of incident TB cases increased in the 1990s, National TB Control Programmes (NTPs) in many low-income countries struggled to cope. The immediate response of the WHO was to support NTPs by implementing a strategy adapted from successful grass-roots programmes in resource-poor settings[117-119]. This so-called DOTS (Directly Observed Therapy Short-Course) strategy achieved significant coverage in many parts of the world[120] but a continuing rise in disease incidence[121] and the growing emergence of M(X)DR-TB[121] strongly argue that operational improvements alone will not achieve adequate TB control in high-burden countries until therapy is shortened.

1.6.2 Drug-drug interactions with ART

A second incentive to shorten treatment is the requirement, amongst HIV-infected individuals to combine TB drugs with ART. Mortality in HIV-infected patients with pulmonary TB is lower when antiretroviral therapy (ART) is initiated during the first few weeks of TB treatment[122-124]. However, the heavy pill burden associated with co-administration of several concurrent medications can be confusing and poly-pharmacy increases the risk of drug-drug interactions. Rifampicin potently induces the activity of multiple hepatic cytochrome P450 isoforms including CYP3A4[125] which accelerates the metabolism of some anti-retrovirals (particularly protease inhibitors and non-nucleoside reverse transcriptase inhibitors). This may result in sub-therapeutic plasma concentrations of ART. TB regimens which can be completed more quickly to avoid interference with ART are desirable.

1.6.3 Drug toxicity

Although generally well-tolerated, all of the first line anti-TB drugs have side effects; rifampicin, isoniazid and pyrazinamide are potentially hepatotoxic[126], isoniazid may cause peripheral neuropathy[127], pyrazinamide is associated with raised uric acid levels and joint pain[128] and ethambutol can cause optic neuritis[129]. Some side-effects are mediated by discordant immune reactions making them more common in HIV-infected individuals[130]. Others overlap with ART toxicities or exacerbate co-existent pathologies (e.g. peripheral neuropathy can be caused by isoniazid, the anti-retroviral agent stavudine and a direct neuropathic effect of the HIV virus[131]). Amelioration of harmful effects would be easier if TB treatment was less protracted.

1.7 Bacterial persistence and the need for sterilisation

The need to reduce the duration of therapy prompts consideration of why this has not yet been achieved. *M tuberculosis* organisms recovered from relapsed patients almost always have the same antimicrobial susceptibilities as the original infection[132] indicating that mycobacterial survival does not depend on the acquisition of genetic resistance mutations. Drug-susceptible organisms may develop phenotypic tolerance allowing them to remain viable in the company of antimicrobial agents. This phenomenon, known as persistence[133,134], is only overcome by prolonged administration of anti-TB drugs[135].

Persistence is not new, nor is it unique to TB. In 1942, Hobby, Meyer and.Chaffee found that penicillin treatment of streptococci left 1% of susceptible organisms intact[136], and in 1944 Bigger described the problem more carefully[137], suggesting that antibiotic efficacy may be diminished by sub-populations of bacteria which enter a dormant state and are not killed. For many years, further study of persistence was deemed unnecessary because most pathogens which evade antibiotics are eliminated by host immunity. However, there are specific instances when this does not occur; in immunosuppressed patients, in pathogens which adapt to the immune response and in immunologically inaccessible anatomical niches[138]. It is clear from the natural history of latency and reactivation that all of these scenarios are applicable to TB, particularly in the era of HIV[139].

In the last decade, renewed study of several bacteria[140-143] including *M tuberculosis*[144] has shown that a range of molecular processes may promote persister formation via stochastic[145] and deterministic[146] mechanisms. Some key advances in this field will now be reviewed, focussing on those of specific relevance to the research questions posed by this thesis.

1.7.1 Mycobacterial persistence and metabolic quiescence in the laboratory

In the 1950s and 60s, McDermot developed the “Cornell mouse model” to provide the earliest description of persistence *in M tuberculosis*. Three months of treatment with RHZ resulted in apparent sterilisation of organ cultures from diseased mice but infection could be revived in survivors by immunosuppression with corticosteroids[147,148]. In 2000, this model was updated using molecular techniques to show ongoing *M tuberculosis* transcriptional activity by detection of mRNA in the lungs and spleens of culture negative mice 14 weeks into therapy[149]. These data, corroborated by similar findings in guinea pigs, support Bigger’s hypothesis that persistence is achieved by down-regulation of metabolic

activity in not replicating bacteria. Following this lead, numerous *in vitro* models have been developed in which Non Replicating Persister (NRP) bacilli are induced by exposure to environmental stressors including low oxygen concentration[150-153], high pH[154], and starvation[155]. Extensive study using the hypoxic Wayne model[151-153] has shown that rifampicin is 50% less effective against NRPs and isoniazid has no activity at all[156]. Additionally, drug exposure may promote metabolic quiescence and arrest of cellular replication. *In vitro* and *in vivo* models of mRNA transcription show that uptake of radio-labelled ^{3}H-uridine falls to 15% of that observed in log phase cultures when rifampicin is added and returns to normal levels when it is removed[149]. The experimental evidence for drug-tolerant persistence by metabolic shut-down seems strong.

However, extrapolation of these experimental findings to treatment responses in clinical TB infection is not straightforward. Pathological lesions[157] and drug metabolism[158,159] during animal and human TB are different and the power of *in vitro* models to predict bacterial behaviour or drug activity beyond the laboratory is unproven. Clinical perspectives are needed to relate this work to the problem of shortening anti-TB therapy.

1.7.2 Bacillary sub-populations and metabolic activity

An influential description of bacillary persistence in the context of clinical TB chemotherapy is the metabolic sub-populations hypothesis proposed by Mitchison in 1979[160,161] and schematically illustrated in Figure 1.5. Mitchison argued that metabolically distinct populations of bacilli occur naturally and are targeted differently by individual drugs, resulting in a biphasic model of bacillary elimination. An “Early Bactericidal Phase” occurs in the first 5-7 days of treatment when rapidly replicating, metabolically active organisms (Population A) are killed by isoniazid[162,163] at a fast elimination rate (α). After this, most surviving bacilli are in varying states of non-replicating persistence (depicted as Population B). As isoniazid is ineffective against NRPs they are eradicated slowly during a prolonged “Sterilisation Phase”. For up to two months, acute tissue inflammation creates a sufficiently acidic environment to support the potency of pyrazinamide against NRPs with intermittent spurts of metabolism (Population B_1)[162]. Thereafter rifampicin is the main sterilising drug against quiescent organisms (Population B_2). The overall “Sterilisation Phase” bacillary elimination rate (β) is slow, suggesting that improved activity against NRPs will be necessary for shorter therapy to be achieved.

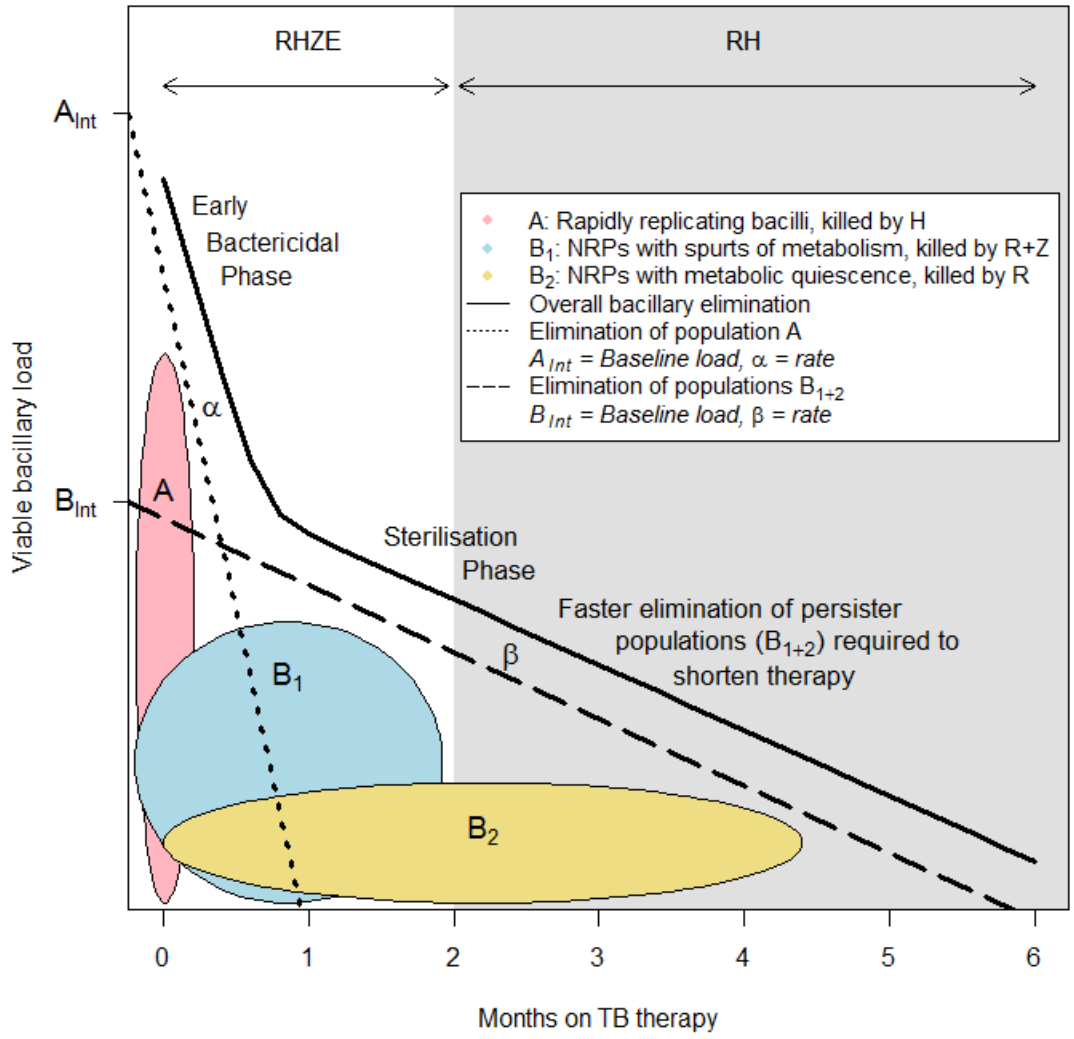


Figure 1.5 Mitchison's bacillary sub-populations hypothesis

Metabolically distinct sub-populations of TB bacilli are proposed to be responsible for bacillary persistence and biphasic bacillary elimination[160]. The Intensive and Continuation Phases of standard first line chemotherapy are shown by white and grey background shading. In this thesis, Populations B_1 and B_2 will be discussed collectively as 'persisters'. However, it is possible that further heterogeneity in the elimination of these cells adds further complexity to TB treatment response.

Although this is a compelling proposition, the factors driving bacillary heterogeneity remain incompletely understood. Knowledge of mycobacterial cell biology and TB pathogenesis may be used to generate a partial explanation (Figure1.6).

Pathogenic mycobacteria often use fatty acids rather than carbohydrate as carbon substrates during infection[163,164]. The classical histological lesion of human TB is the granuloma, where bacilli are surrounded by host macrophages. On contact with the *M*

tuberculosis cell wall, some host cells differentiate into 'foamy' macrophages containing large lipid droplets[165,166]. Electron Microscopy (EM), radio-isotope labelling and fluorescence studies have shown that bacilli internalised by macrophages migrate inside these lipid droplets to consume and degrade them as a fatty acid source[167,168]. Mycobacteria can then break down fatty acids by β-oxidation to Acetyl CoA which is combined with oxaloacetate by citrate synthase (Cit A) to form citrate, a metabolite of the tricarboxylic acid (TCA) cycle. This drives oxidative respiration and amino acid synthesis.

However, exposure of *M tuberculosis* to a variety of *in vitro* stresses (hypoxia, iron limitation, low pH and nitric oxide) have identified a 48 gene transcription factor called DosR which co-ordinates cell entry to a NRP state[169,170]. The specific role of this regulatory system in antibiotic tolerance is controversial[150,171] but it increases expression of the *tgs1* gene encoding an enzyme called TAG synthase, known to stimulate production and storage of intra cytoplasmic triacylglycerol (TAG)[154,172,173]. When *DosR/tgs1* mediated TAG synthesis is up-regulated there is competition for Acetyl CoA limiting its availability and creating a process akin to hibernation; TAG is retained as an intracellular energy store and cell growth is arrested[173]. During prolonged starvation an additional gene *lipY* is expressed[174], encoding an enzyme which hydrolyses intra-bacillary TAG stores back to Acetyl CoA for generation of energy. Overall, *DosR*, *tgs1*, and *lipY* may constitute a sophisticated mechanism of substrate storage and re-mobilisation compatible with persistence during periods of stress.

This model is supported by data showing that mutant bacilli with *tgs1* deletion or *citA* over-expression are less able to survive hypoxia and antibiotic exposure than wild-type organisms, perhaps because they are unable to arrest growth in adverse circumstances and die as a consequence of unsustainable metabolic activity. [^{14}C] radio labelling has shown that TCA and TAG pathways compete for the same carbon pool[173].

The possibility that TAG accumulation is a behavioural characteristic of persisters with reduced antibiotic susceptibility raises the possibility of intra-cellular TAG labelling as a phenotypic marker of Mitchison's metabolically quiescent sub-populations (B_1 and B_2 from Figure 1.5). Empirical evidence is lacking but three studies have used fluorescence microscopy to study the relationship between TAG lipid bodies, bacillary persistence and antibiotic tolerance[154,168,175]. This will be discussed further in Chapters 5 and 6.

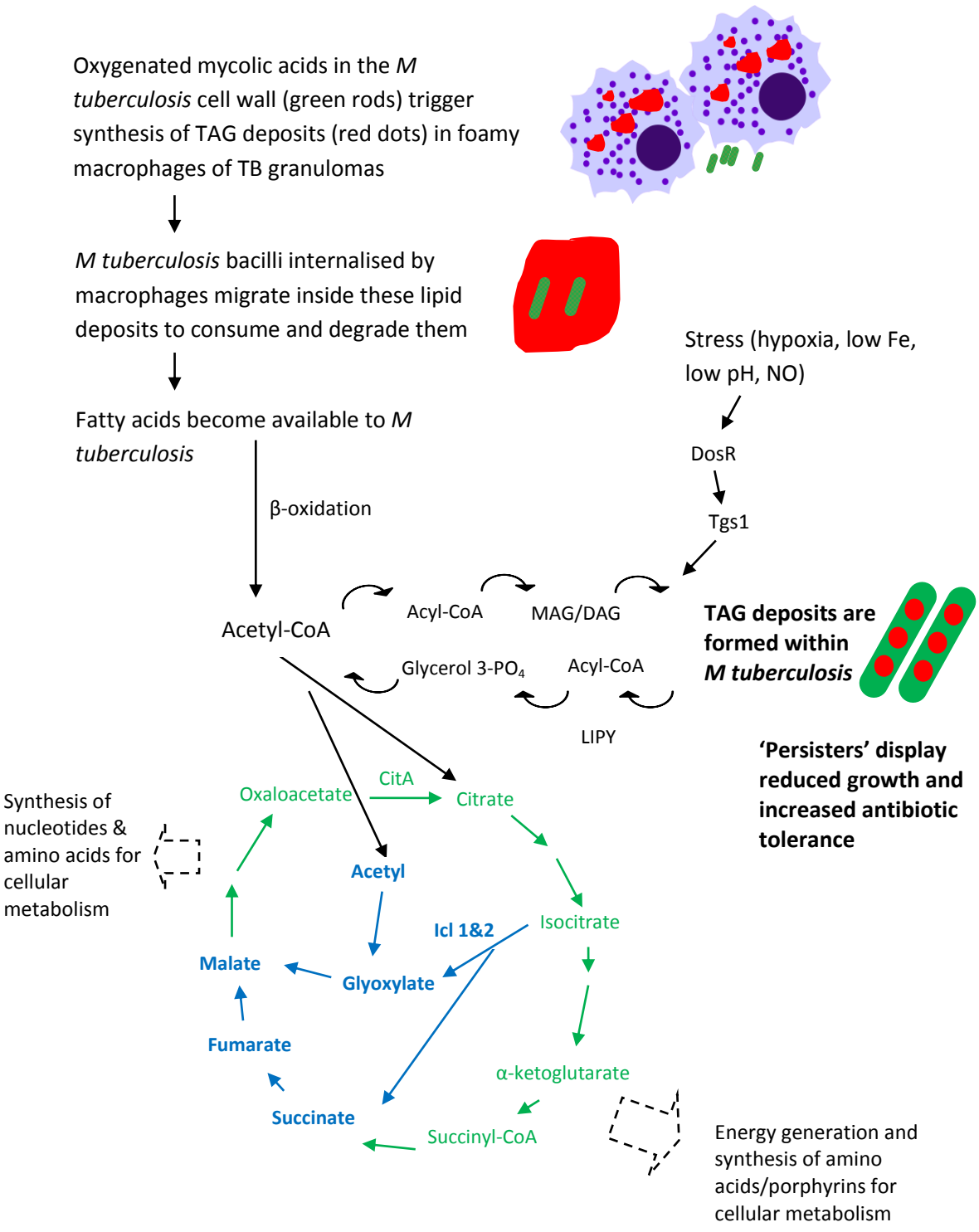


Figure 1.6 Model of lipid metabolism, TAG stores and persistence in *M tuberculosis*

M tuberculosis uses fatty acids from host macrophages to generate Acetyl-CoA. Under stress, Tgs1 drives formation of bacillary TAG stores from Acetyl-CoA, reducing its availability for the tricarboxylic acid cycle (shown in green) and glycoxylate shunt (shown in blue). This may result in metabolic quiescence and persistence. LIPY catabolises TAG during starvation, regenerating Acetyl-CoA as an energy source.

Other metabolic processes have also been implicated in persistence. Figure 1.6 shows an intracellular pathway known as the glyoxylate shunt used by bacteria to replenish some TCA cycle intermediates[176]. Entry to this shunt is gated by two isocitrate lyase enzymes and deletion of the *icl 1* and *icl2* genes encoding these prevents long-term bacillary survival[164,177]. Single gene associations in other energy generation systems (*sucB*[178], *menA*[179], *cydC*[180]) and the stringent response to starvation (*RelA*[181]) may also be important. Over 30 Toxin-antitoxin (TA) modules have been recognised in the *M tuberculosis* genome[182,183]. These encode mRNases that rapidly degrade mRNA, stopping translation of new proteins and slowing metabolism. TA-mediated persistence has been extensively researched in *E coli*[184].

Some recent data support metabolic mechanisms of persistence which are not related to arrest of replication. Experiments using time-lapse microscopy to record single cell replication of *M smegmatis* in a micro-fluidic device[185] have shown that cell death may be associated with pulsed stochastic expression of key proteins (e.g. cells which randomly produce the isoniazid activating enzyme catalase peroxidise [KatG] are killed whilst those which do not express this gene survive)[185]. It seems likely that persisters acquire drug tolerance in multiple ways and clinical treatment shortening may require improved understanding of all of them.

1.7.3 Bacterial impermeability: the cell wall, porins and efflux pumps

Aside from altered intracellular metabolism, the physical and biological properties of the *M tuberculosis* cell wall may contribute to persistence by manipulating permeability to anti-microbial agents. Lipid-rich cell wall components[186](Figure 1.3), are notoriously impenetrable to small hydrophilic molecules, including three of the current first line anti-TB drugs (isoniazid, pyrazinamide and ethambutol) which must negotiate cell entry via transmembrane porins (e.g. MspA) [187] [188]. Conversely, cell wall efflux transporters of the MFS, SMR, RND and ABC superfamilies[229,230] actively export drug metabolites from the cell[189]. Although constitutive expression of efflux pumps has been implicated in genotypic TB drug resistance[190-193] the role of variable porin and efflux pump expression as persistence mechanisms in DS-TB is unknown. These factors will not be explored further in this thesis but should be borne in mind.

1.7.4 Clinical environments for bacillary growth

The last persistence mechanism for current discussion is that poor tissue sterilisation may occur if organisms are sequestered in sanctuary sites where growth conditions favour persistence or penetration of drugs and immune responses are reduced.

M tuberculosis is known to be adaptable to an intracellular lifestyle. Although the specific location of bacilli during the course of clinical disease remains controversial[167,194,195], it is known that mycobacteria within macrophages have access to rich nutritional stores[166] and an extended armoury of intracellular immune and drug avoidance machinery including reduced acidification of the phagosome[196,197] and additional drug efflux pumps in host cell membranes[198]. Intra-cellular bacilli, therefore, may be well placed to become persisters.

Amongst pathological TB lesions, the tissue oxygen tension of open pulmonary cavities (60-100mmHg) is higher than that of closed granulomas (3mmHg)[199,200]. As bacilli in closed lesions grow more slowly[188,] these were historically proposed as a niche for persister survival. This hypothesis is now deemed an oversimplification because patients with open cavities on chest X-ray (CXR) and HIV-infected individuals without closed granulomas may be at high risk of relapse[135,201,202], but a possible association between hypoxia and persistence is maintained by the recent observation that a 20% reduction in oxygen saturation significantly increases mycobacterial antibiotic tolerance[203]. This may occur because many antibiotics cause cell death though generation of Reactive Oxygen Species (ROS)[140,204-207] and ROS generation diminishes in proportion to oxygen availability[208]. It is possible that metabolic and environmental persistence mechanisms are synergistic; TAG synthesis may deplete the TCA cycle of metabolites required for ROS production[173] in a process exacerbated by hypoxia at the site of infection[203].

Finally, lymphatic or haematogenous dissemination of bacilli may allow infection to settle in organs where bacterial eradication is difficult. *M tuberculosis* DNA has been found in adipose tissue of patients with latent TB infection or active disease[209] and viable bacilli have been grown from liquid culture of CD271+ bone marrow stem cells in patients who had apparently been cured of clinical TB by six month chemotherapy[210].

1.7.5 Bacillary persistence and drug development

Improved understanding of bacillary persistence is important to the development of new TB treatments. Effective ultra-short chemotherapy will require compounds which either kill persister organisms or prevent persister formation[173,203] and pre-clinical assessment of new

anti-TB compounds now routinely incorporates *in vitro* NRP models[151,211-213]. Animal studies are undertaken to test whether new drug regimens accelerate culture sterilisation and prevent relapse[214-216] because these end-points are deemed to reflect persister eradication.

However, no established laboratory assays directly label phenotypic persisters or specifically measure sterilising drug activity in clinical samples. This means that when early clinical studies of 8 weeks duration are performed in humans to predict the likely long-term efficacy of new regimens, the appropriate choice of study end-points remains unclear.

1.8 New drugs and treatment strategies

For the first time in 50 years, many new compounds are emerging from the drug development pipeline (Figure 1.7) for assessment in clinical trials against drug susceptible (DS) and M(X)DR TB. Ongoing or planned Phase IIb and III studies against DS-TB are summarised in Table 1.2. The apparent variation in the choice of efficacy end-points in the Phase IIb protocols directly reflects the lack of consensus on which measurement best reflect sterilisation. A closer analysis of recent data and ongoing trials illustrates this point.

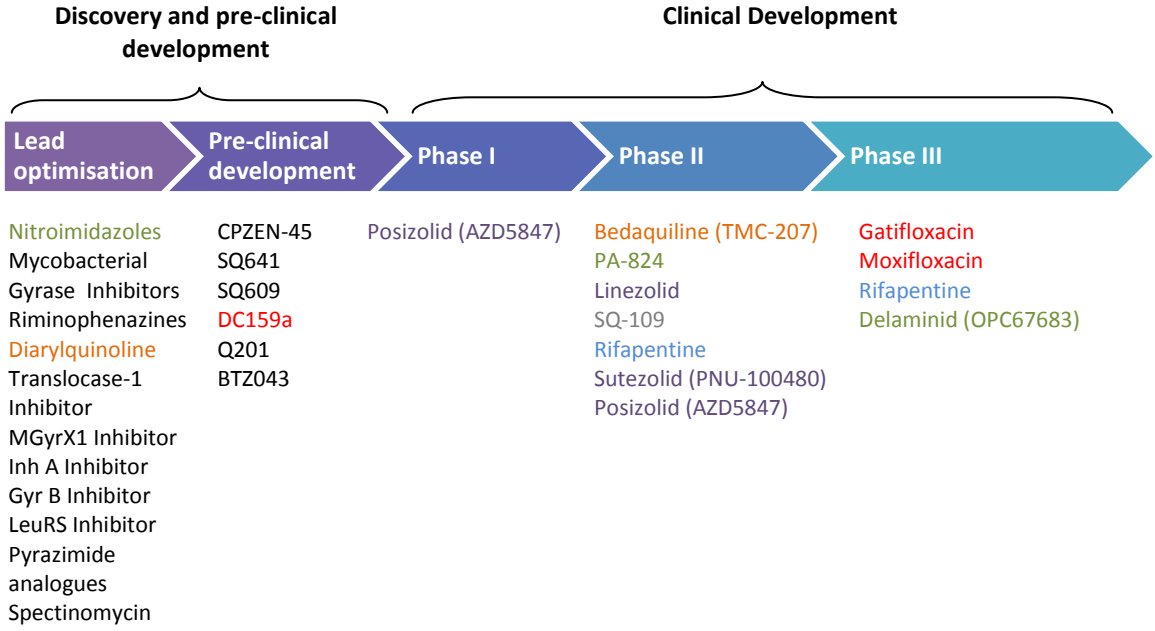


Figure 1.7 The global TB drug development pipeline

Schematic from the Working Group on New Drugs, Stop TB Partnership adapted from Leinhardt *et al*[217]. Chemical drug classes are shown in different colours: rifamycin (dark blue), fluoroquinolone (red), diarylquinoline (orange), nitroimidazopyrans (green), diethylamine (grey), oxazolidine (purple).

Drug classes	Study	Sites	Recruitment groups	Regimens	Primary end-point(s)
Phase III studies					
Q	OFLOTUB NCT00216385	Benin, Guinea, Kenya, Senegal, S Africa	DS-TB HIV-/+	2RHZG→2RHG 2RHZE→4RH	Evaluate at 30 months: Clinical failure/relapse
Q	REMoxTB NCT00864383	China, India, Kenya, Malaysia, Mexico, S Africa, Tanzania, Thailand, Zambia	DS-TB HIV-/+	2RHZM→2RHM 2RMZE→2 RM 2RHZE→4RH	Evaluate at 18 months: Clinical failure/relapse
Phase IIb studies with 8-12 week microbiological end-points					
Ry Q Ed	Uni of Munich NCT01785186	S Africa, Tanzanzia	DS-TB	$3R_{35mg/kg}HZE$, 3RHZQ $3R_{20mg/kg}HZQ$, $3R_{20mg/kg}HZM$ or RHZE	Proportion of patients with SCC Time to SCC
Ry	High RIF NCT00760149	Tanzania	DS-TB	$2R_{600/900/1200}HZE$	Proportion of patients with SCC Time to SCC Bacillary elimination rate
Ry	HIRIF NCT01408914	Peru ,Brazil	DS-TB HIV- /+ (CD4>350/µl)	$2R_{600/900/1200}HZE$	Proportion of patients with SCC Time to SCC Bacillary elimination rate
Ry	JHU Study NCT00814671	S Africa	DS-TB HIV-/+(CD4>200/µl)	2RHZE $2Rp_{450/600}HZE$	Proportion of patients with SCC
Ry Q	JHU Study NCT00728507	Brazil	DS-TB HIV-/+(CD4>200/µl)	$2Rp_{300/450}HZM$ 2RHZE	Proportion of patients with SCC
Q N	Global Alliance NCT01498419	Brazil, S Africa, Tanzania	DS and MDR-TB HIV-/+(CD4>200/µl)	DS-TB: $2MPa_{100/200}Z$ DS-TB: 2RHZE MDR-TB: $2MPa_{200}Z$	Bacillary elimination rate

Table 1.2 Current Phase IIb/III clinical efficacy trials against DS-TB

Drug classes colour coded as in Table 1, Ry=rifamycins, Q=fluoroquinolones, D=Diarlyquinoline, N=nitroimidazopyran, Ed=diethylamine. Numbers before drug regimens specify duration in months. Drugs: R=Rifampicin, Rp=Rifapentine, M=moxifloxacin, G=Gatifolxacin, O=Ofloxacin, Z=Pyrazinamide, E=Ethambutol, J=Bedaquiline, D=Delamanid, Pa=PA-824. SCC=Sputum culture conversion. Varying dosages are indicated by subscript.

1.8.1 Re-evaluating the rifamycins

Rifampicin is a key sterilising component of first-line therapy. It is lipophilic so penetrates the mycobacterial cell wall and retains efficacy throughout treatment but the current dose (10mg/kg) is low and was originally selected to minimise costs[218]. When the price dropped after the 1960s, higher dosing was not attempted due to concerns about toxicity. Recent experience of using up to 1200mg/day in non-mycobacterial infections now suggests that dose escalation is possible[219]. Animal and early clinical studies also indicate that rifampicin activity against *M tuberculosis* is concentration dependent and a 10mg/kg human dose may be at the very bottom end of a steep dose-response curve[220].

Two Phase IIb studies of "high dose" rifampicin TB treatment (High RIF [www.clinicaltrials.gov identifier NCT00760149] and HIRIF [NCT01408914]) are underway. Both compare regimens containing rifampicin 600-1200mg on sputum culture results during the first 8 weeks. An additional 12 week trial (PanACAEA MAMS Study, NCT01785186) of 4 experimental regimens including extended rifampicin dosing (10-35mg/kg) will start soon.

Table 1.2 shows that three efficacy end-points are being used in these studies; the proportion of patients who convert to negative sputum cultures by the end of the study, the time from initiating therapy to culture conversion and estimates of the bacillary elimination rate derived from statistical modelling. Multiple end-points are used because the best marker of sterilising activity is unknown, but if results are inconsistent (e.g. faster culture conversion at higher rifampicin doses but no difference in the overall proportion of culture negative patients at 8 weeks) interpretation of the differences between treatment regimens will be difficult.

Rifapentine is an alternative rifamycin with greater *in vitro* potency than rifampicin and mouse models suggest that daily rifapentine-based regimens may allow shorter treatment without increasing relapses[221]. One multi-centre Phase IIb clinical trial (Tuberculosis Treatment Trial Consortium [TBTC] study 29) has described equivalent 8 week sputum culture conversion rates between 2 months of rifapentine (10mg/kg)-isoniazid,-pyrazinamide-ethambutol and standard RHZE[222] and two further studies are ongoing (NCT00814671 and NCT00727507). A Phase III study (the RIFAQUIN trial) of novel four and six month regimens including rifapentine has recently reported results which will be discussed in Section 1.8.2.

1.8.2 Defining the role of 8-methoxyquinolones

The fluoroquinolones target DNA gyrase[223] with excellent activity against *M tuberculosis*. Levofloxacin and the 8-methoxyfluoroquinolones (8-MQ: moxifloxacin and gatifloxacin) are used in MDR-TB[218]. *In vitro* and murine studies have shown that 8-MQs are bactericidal against rifampicin-tolerant persisters[211] and might allow treatment shortening in drug susceptible TB[214,224] . Four important Phase IIb studies have been completed to assess the efficacy of 8-MQs in humans (Table 1.3).

Two multi-centre TBTC studies compared RHZE with regimens in which moxifloxacin replaced ethambutol (Study 27)[225] or isoniazid (Study 28)[226]. Both were based sputum culture conversion at 8 weeks and neither reported better outcomes with moxifloxacin. Conversely, investigators from John Hopkins University (JHU) assessed moxifloxacin-ethambutol substitution and reported better outcomes on the moxifloxacin arm. The TBTC-27 and the JHU study used different culture media (liquid broth and Lowenstein Jensen [LJ] slopes respectively) and this may have contributed to the conflicting outcomes[227]. Finally, the OFLOTUB consortium evaluated the effect of ethambutol replacement by moxifloxacin, gatifloxacin or ofloxacin by modelling bacillary elimination to show that regimens with moxifloxacin and gatifloxacin had greater sterilising activity than those with ethambutol or ofloxacin[228]. The statistical techniques used by OFLOTUB will be discussed in Section 1.10.2 but these results implied that 8-MQs accelerate bacillary clearance.

The overall trend towards faster sputum sterilisation with 8-MQ-based combinations prompted progression to several multi-centre Phase III trials. The first of these to report was the RIFAQUIN trial which compared standard six month 2RHZE/4RH with two new regimens; a 6 month course in which isoniazid was replaced by moxifloxacin during the intensive phase and once weekly rifapentine-moxifloxacin was given during the continuation phase, and a 4 month regimen comprising 2 months of daily rifampicin-moxifloxacin-pyrazinamide-ethambutol followed by 2 months of twice weekly rifapentine – moxifloxacin. The new 6 month regimen was equivalent to standard therapy, but the 4 month regimen was inferior (26% treatment failures/relapses vs. 5% on RHZE) suggesting that this approach to treatment shortening was unsuccessful[229]. Data from the OFLOTUB (NCT00216385) and REMoXTB (NCT00864383) studies are awaited to establish whether the 8-MQs might still facilitate ultra-short first-line therapy but the RIFQUIN results are a reminder that encouraging data from pre-clinical and Phase II studies may not translate into success when final clinical end-points are measured.

Study	Site	Regimens	Duration	Endpoint(s)	Results
TBTC-27[225]	North America Brazil South Africa Spain, Uganda	RHZM RHZE	8 weeks	Sputum culture conversion in liquid broth	SCC in 99/139 (71.0%) RHZM patients vs. 98/139 (71.0%) RHZE patients (p=0.97) Secondary analysis at 4 weeks showed SCC in 62/167 (37%) RHZM patients vs. 43/165 (26%) RHZE patients (p=0.05)
TBTC-28[226]	North America Brazil South Africa Spain, Uganda	RMZE RHZE	8 weeks	Sputum culture conversion in liquid broth	SCC in 90/164 (54.9%) RHZE patients vs. 99/164 (60.4%) RMZE patients (p=0.37); small but non-significant increase in culture conversion on RMZE
JHU[227]	Brazil	RHZM RHZE	8 weeks	Sputum culture conversion on LJ slopes	SCC in 59/74 (80%) RHZM patients vs. 45/72 (63%) RHZE patients (p=0.03) Secondary analysis showed shorter time to culture conversion in RHZM patients (p=0.005)
OFLOTUB[228]	South Africa	RHZM, RHZG, RHZO RHZE	8 weeks	Regression co-efficient of sterilisation phase bacillary elimination rate	Significantly greater regression co-efficients for RHZM and RHZG than RHZE (p=0.002 in both cases) No difference in regression co-efficients between RHZO and RHZE (p=0.14)

Table 1.3 Phase IIb treatment trials involving the 8-methoxyfluoroquinolones

R=Rifampicin, H=Isoniazid, Z=Pyrazinamide, E=Ethambutol, M=Moxifloxacin, G=Gatifloxacin, O=Ofloxacin, LJ=Lowenstein Jensen media, SCC=Sputum culture conversion

1.8.3 The emergence of bedaquiline (TMC-207)

Unlike the rifamycins and fluoroquinolones, the diarylquinoline, bedaquiline is an entirely new compound with a novel mechanism of action. It has attracted recent attention because the US Food and Drug Administration has granted it accelerated approval for use in MDR-TB regimens[230]. A provisional licence was issued in 2012 on the basis of emerging data from two Phase IIb studies in patients with drug-resistant disease[231-233] and a Phase III study is planned. It may also have sterilising and treatment-shortening activity in DS-TB.

In vitro and animal studies suggest that bedaquiline has desirable properties for a sterilising drug. It works by selective inhibition of mycobacterial ATP synthase, an enzyme required to maintain cell membrane potentials, even in metabolically quiescent organisms[156,234,235]. It is bactericidal in culture-based models of bacillary persistence and has been included in new drug combinations which achieve stable cure without relapse in mouse models[236,237].

In humans, a dose ranging study assessed the Early Bactericidal Activity (EBA) of bedaquiline by measuring the fall in sputum bacillary load in PTB patients over seven days of monotherapy. Interestingly, there was no EBA for the first four days but a daily dose of 400-800mg had equivalent efficacy to isoniazid and rifampicin from Day 5 onwards[238]. This delayed onset of action may reflect the time required for depletion of intra-bacillary ATP stores or accumulation of therapeutic drug levels. These results demonstrate that activity during the first few days may be a poor marker of a new drug's sterilising activity.

1.8.4 The nitroimidazopyrans and other drug classes

In addition to the agents that have been described in detail, two nitroimidazopyrans deserve mention. PA-824 [239] and delamanid (OPC-67683)[240] have bactericidal potency against replicating and non-replicating organisms. Murine studies have indicated treatment-shortening sterilizing activity and accelerated time to culture conversion when either drug is combined with rifampicin-pyrazinamide[240,241] or PA-824 is combined with moxifloxacin-pyrazinamide[226]. One clinical study has shown the efficacy of PA-824 monotherapy to be only slightly lower than that of RHZE during the first 14 days of treatment for DS-TB[242] but longer trials of the nitroimidazopyrans are currently focussed on MDR-TB rather than first line therapy.

Other drugs including new oxazolidinones (sutezolid [PNU-100480] and posizolid [AZD5847]) [243]and the ethylenediamine SQ109 [244,245] are at earlier stages of development. As they reach clinical evaluation the range of agents for new regimens will increase.

1.9 From sterilising drugs to new regimens: the challenge for clinical trials

Whilst the recent upsurge in research activity on novel TB treatments is encouraging, only rifapentine and the 8-MQs have been assessed in the type of rigorous Phase III clinical trial required before a new regimen can enter clinical practice for DS-TB.

Mobilising the global research capacity to undertake Phase III trials is a major undertaking[246] because the obligatory comparator regimen of current short course chemotherapy is highly efficacious[69] and the necessary clinical end-point of post-treatment relapse[247] requires prolonged follow-up. Introduction of methods for accurate strain differentiation using the IS6110 insertion sequence[248] and spoligotyping[249] reveal that an end-point of relapse may also be contaminated by a proportion of re-infections unrelated to treatment failure[250]. This problem may be amplified in HIV-infected cohorts.

Considering these factors, superiority designs for new Phase III trials are not feasible and trials based on non-inferiority require approximately 500 patients per arm on study for 18-30 months[251]. As a case in point, RIFAQUIN started in 2008 and recruited 872 participants before finally reporting a negative result for shortened therapy in 2013. It is clear that pre-trial screening of future candidate regimens should be meticulous in order to maximise the likelihood of success.

This reinforces the argument made in Sections 1.7 and 1.8 that there is need to optimise and standardise efficacy end-points for future Phase IIb treatment studies. These end-points should be selected to achieve two distinct but closely related goals:

1. From a microbiological perspective, they should measure the bactericidal effect of new regimens on “Sterilisation Phase” persister bacilli

2. From a clinical perspective, they should function as reliable surrogate biomarkers to predict the likelihood of post-treatment relapse from early studies of putative new regimens[216,252,253].

The second of these goals will now be discussed in more detail.

1.10 Surrogate end-points for TB treatment trials

1.10.1 Desirable properties for a surrogate end-point

Selecting the best biomarker for clinical TB studies requires an understanding of the principles of surrogacy[254]. A perfect marker would be measured early in treatment but capture all of the information available in a distant clinical reference endpoint. If the surrogacy were strong enough the reference end-point may even be completely replaced (Figure 1.8)[255] but this almost never occurs.

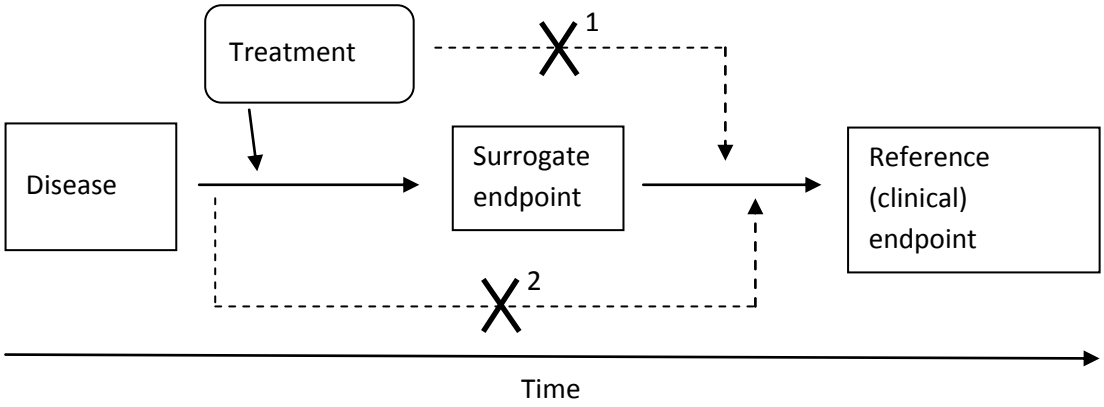


Figure 1.8 Surrogate endpoints in clinical trials

A perfect surrogate marker would be measured before the reference (clinical) endpoint, but contain all of the information contained within it. No treatment effect would bypass the surrogate (1) and no other causal disease pathway would influence the reference endpoint without similarly influencing the surrogate (2).

As virtually all biomarkers are imperfect, consideration of the varying extent of surrogacy is important[256]. In particular, it is clear that biomarker evaluation is a cumulative process proceeding at two levels; the individual patient's response and the aggregated response in the arms of a trial[257]. Very firm validation is necessary for surrogate markers intended as decision making tools for the patient management, whilst a lower level of precision may be acceptable for end-points in clinical studies designed to optimise new drugs and learn about dose-response [258] This is especially true if data from several Phase IIb studies will be meta-analysed[259] prior to regimen selection for a definitive Phase III trial based on the original reference end-point[256,257].

In general, a practically useful surrogate end-point should have a biologically plausible, preferably mechanism based, causal relationship with the reference endpoint. It should

also have useful statistical properties (i.e. be quantifiable and assessable) and successfully complete a process of validation against the reference endpoint[260]

1.10.2 Biologically plausible surrogate markers for TB

Elements of the TB treatment response with a biologically plausible relationship to outcome may be divided into three categories:

- **Pharmacodynamic (bacterial) response** – the relationship between anti-TB chemotherapy and bacillary elimination
- **Clinical/immunological response** – the relationship between host (patient) physiology and *M tuberculosis* organisms during TB disease
- **Pharmacokinetic response** – the relationship between host (patient) physiology and anti-TB drugs.

Interactions between these elements are illustrated in Figure 1.9, preceding a discussion of the potential role of each element in the development of new surrogate markers for Phase IIb trials of new treatment regimens.

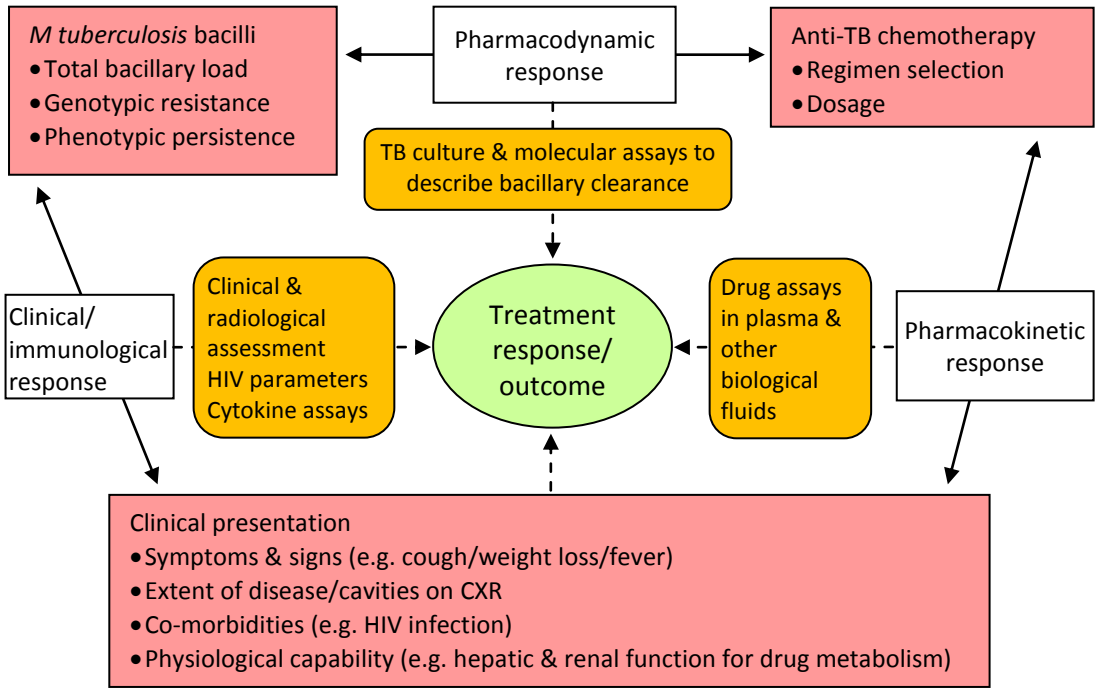


Figure 1.9 Biologically important elements of TB treatment response

Red boxes represent bacterial, pharmacological and clinical factors, white boxes represent elements of treatment response and gold boxes indicate methods of assessing these factors which may generate biomarkers of outcome.

1.10.3 Pharmacodynamic (bacterial) response

End-points based on sputum culture conversion

The trials underpinning current chemotherapy were designed with primary or secondary end-points based on sputum culture conversion. Sputum is easily accessible in PTB patients and there is evidence that pre-treatment bacillary burden in sputum is related to duration of therapy required for stable cure[135] Furthermore, there is a statistically significant relationship between sputum culture conversion and clinical TB end-points. A recent meta-analysis of 37 direct treatment comparisons across 49 study arms in 12 historical BMRC trials showed that regimens associated with higher likelihood of positive culture at the end of months 1-4 also carried a higher risk of post-treatment relapse (Figure 1.10, $p<0.005$ in all cases)[261].

However, validation of sputum culture conversion as a surrogate for relapse remains problematic. Individual patient data in the above meta-analysis showed that a positive culture at the end of the first month predicted relapse with 79% sensitivity but only 45% specificity. By the end of the second month, specificity was higher (82%) but sensitivity dropped to 48% (Table 1.4), meaning that over half of relapses would be missed. Even allowing that biomarkers for Phase II studies require less firm validation than individual surrogates this seems inadequate. At the level of treatment comparisons, Figure 1.10 shows that some trial regimens with lower likelihood of positive culture in months 1-4 were still more likely to result in relapse[261].

An additional difficulty is that sputum culture conversion is a binary measurement and its power as a clinical trials biomarker is dependent on the proportion of culture negative patients in the comparator arm. As standard 2 month RHZE therapy achieves negative cultures in over 80% patients[102], a large sample or effect size would be needed to show superior sterilising activity of a new regimen based on this end-point. Simulation studies confirm the expected theoretical result that prioritisation of binary end points incurs a significant penalty in terms of sample size[262]

Secondly, measurement of outcome at a fixed time point does not efficiently capture information about the effect of treatment over the entire period of culture positivity. For example, from Table 1.3, TBTC Study 27 reported no improvement in outcome when moxifloxacin replaced ethambutol, based on the proportion of negative cultures at 2 months (71% vs. 71%, $p=0.97$). However, at 4 weeks greater culture conversion was seen in

the moxifloxacin arm (37% vs. 26%, p=0.05). Analysis at an alternative time-point may have altered the conclusion about the sterilising potential of the new regimen[225].

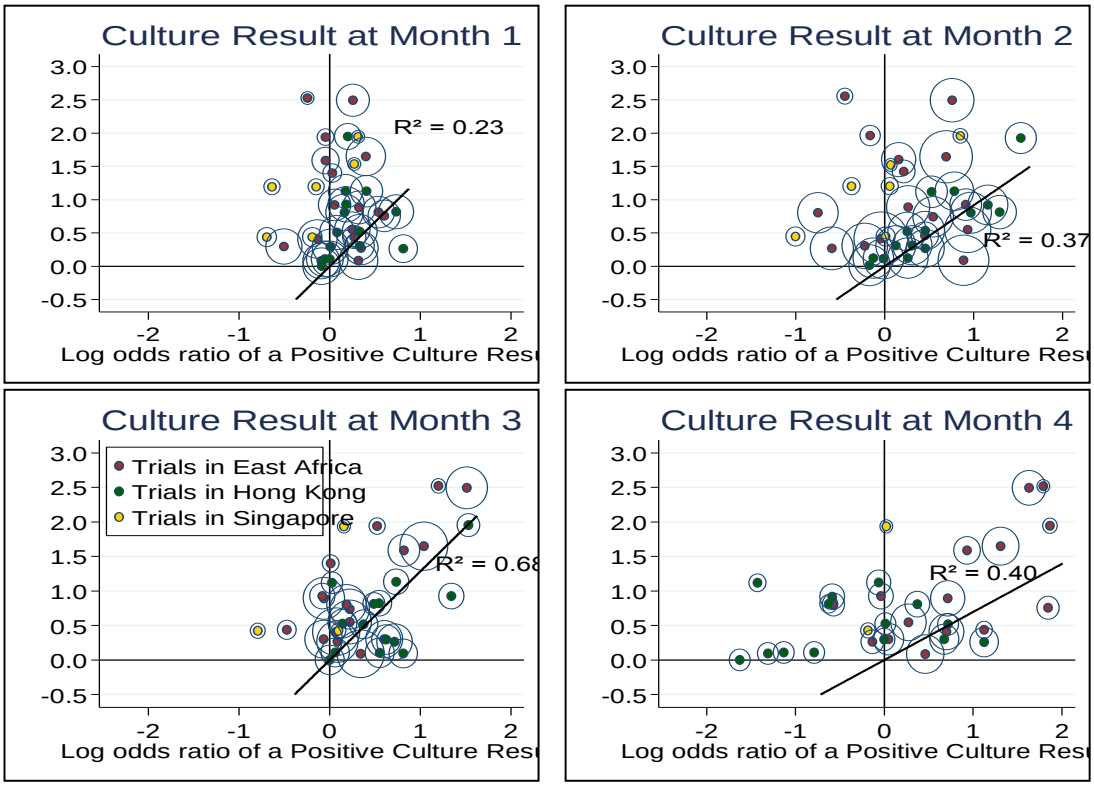


Figure 1.10 Treatment comparisons from 12 BMRC trials in Africa and East Asia

Each point corresponds to a single comparison of regimens within one trial. The radius of the circle represents the precision of the estimate for that comparison. At all months (1 to 4) there is a strong relationship between the treatment effect on a positive culture and the treatment effect on a poor outcome (p<0.005). In all plots some points are in the left upper quadrant, indicating that the treatment effect on a positive culture was opposite to that on a poor outcome. Poor outcome is defined as TB relapse 12-24 months after successfully completing therapy[261].

Month	Sputum culture result as a predictor of clinical outcome	
	Sensitivity	Specificity
1	79%	45%
2	48%	82%
3 or 4	<30%	>95%

Table 1.4 Individual level data from 12 BMRC trials in Africa and East Asia

A positive culture at Month 1 was 79% sensitive for poor outcome but only 45% specific. From Month 2 less than 50% of poor outcomes were predicted by the surrogate marker.

An alternative approach to sputum bacteriology is analysis of time to culture conversion using survival techniques[226]. This approach is not predicated on binary measurements at arbitrary time-points[260] but the sensitivity of survival analysis may be strongly influenced by the sampling schedule as the accuracy of any "time to conversion" measurement depends on the frequency of sample collection. Additionally, regimens with different bacillary elimination profiles could generate similar survival functions, obscuring important differences in drug action on persisters (Figure 1.11). To overcome these difficulties and show changes in treatment response throughout therapy, surrogate end-points based on continuous quantification of bacterial clearance are required[143].

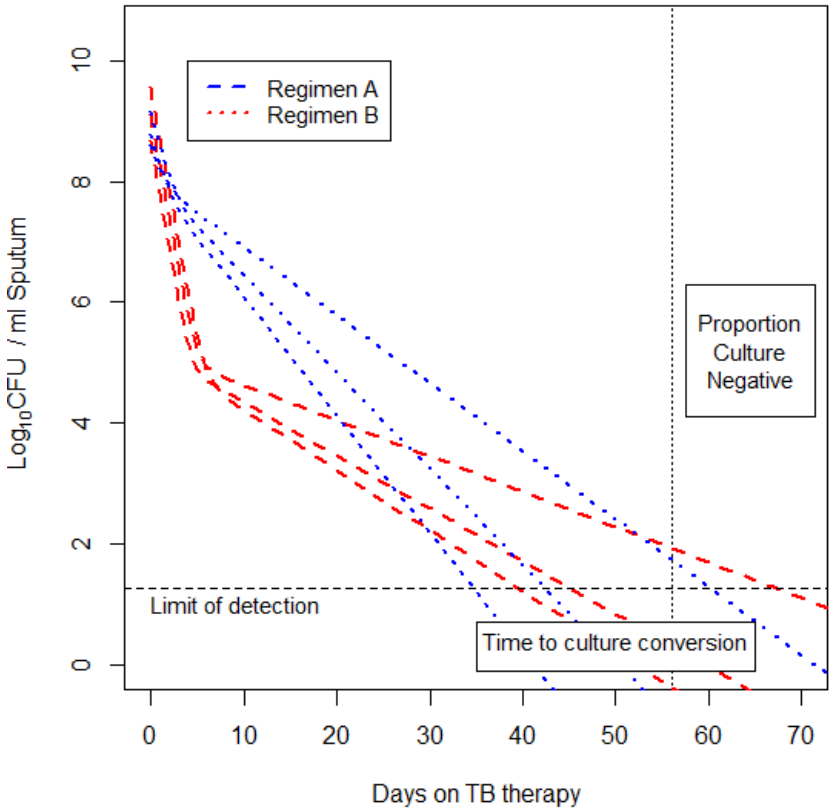


Figure 1.11 Methods of statistical analysis for sputum bacteriology data

2 hypothetical regimens are each given to 3 patients. At 2 months there is no difference in the proportion of patients who have culture converted (2/3 in each case) yet the mean times to conversion differ (A: 45.6 days, B: 51 days). The major difference between regimens is that A has greater sterilising activity after day 7. Only modelling of bacillary elimination throughout the study demonstrates this.

Serial Sputum Colony Counting (SSCC)

In the 1970s, a quantitative bacteriology method was developed for EBA studies[263,264]. Serial dilutions of homogenised overnight sputum collections are plated onto selective agar and single colony forming units (CFUs) were counted after incubation for 3-4 weeks and a back-titration from the number of CFUs visible at a countable dilution was used to quantify the bacillary load of the original sample, expressed as CFU/ml.

The first EBA studies showed that the fall in viable counts over days 0-2 varied between drug doses and regimens and helped to rationalise treatment combinations. However, the need for longer studies has subsequently emerged. A 5 day study was required to show that the potency of isoniazid waned more quickly than that of rifampicin[264], a 7 day study was needed to show activity of bedaquiline after day 4[238] and even extended EBA analysis to day 14 failed to demonstrate the sterilising capability of pyrazinamide[265]. No convincing statistical evaluation of the relationship between changes in bacillary counts over the first 2 weeks and final clinical trial endpoints has ever been undertaken and it seems unlikely that such a short period of study will be sufficient to provide a surrogate marker of long-term sterilisation. Serial Sputum Colony Counting (SSCC) has been proposed as a solution which extends the quantitative EBA approach throughout the first 8 weeks of therapy[228,260,266-268].

Three prior DS-TB studies have used an SSCC approach (summarised in Figure 1.12)[228,269,270] and lessons from them are important. Other studies have also used quantitative bacteriology during extended sputum sampling[271,272] but employed different laboratory methods (sputum was decontaminated with sodium hydroxide [NaOH] prior to culture, see Section 5.2.3). These will not be described in detail. However, it is notable that one such study compared a novel rifampicin-isoniazid-ciprofloxacin (RHC) regimen to standard RHZE and reported a smaller fall in $\log_{10}$CFU/ml counts on RHC during weeks 3-8 in addition to a higher rate of post-treatment relapse[272,273]. This correlation between early quantitative bacteriology and the reference clinical end-point is encouraging.

From 1989-90 the first SSCC study employing quantitative bacteriology methods on non-decontaminated sputum was performed in Nairobi. HIV-infected and un-infected patients received SHRZ or SHT during the first month of therapy and colony counts at 0, 2, 7, 14 and 28 days showed that HIV did not influence treatment response[274]. In 2001 the dataset was re-visited to assess differences between the regimens[269]. Analysis of variance showed that the decline in $\log_{10}$CFU/ml counts was faster from day 0-2 than day 2-28, making it

inappropriate to fit a single regression line through all of the time-points. This supports Mitchison's model of biphasic bacillary elimination due to differences in the antibiotic tolerance of bacillary sub-populations during treatment. When regression lines were fitted to counts from day 2-28, the slope for SHRZ patients was significantly steeper than for those on SHT (regression co-efficients: 0.163 vs. -0.095, p=0.004). This is consistent with prior knowledge of the enhanced sterilising activity of rifampicin and pyrazinamide.

The emergence of non-linear mixed effects (NLME) modelling[275] led to further review of the Nairobi data. In 2006 a bi-exponential model was fit with the following formula[266]:

$$Log_{10}CFU=log_{10}[(e^{\theta 1} \times e^{-day \times e\theta 2}) + (e^{\theta 3} \times e^{-day \times e\theta 4})]$$

This model applies a biphasic curve to the aggregate effect of treatment on Mitchison's putative sub-populations based on the following parameters expressed on a logarithmic scale; θ_1 and θ_3 represent baseline bacillary loads of metabolically active and quiescent organisms (corresponding to A_{Int} and B_{Int} from Figure 1.5) whilst θ_2 and θ_4 represent the two elimination rates (corresponding to α and β). When θ_4 from the NLME model was compared to the linear regression co-efficient from day 2-28 as a measure of sterilising activity the linear statistic was found to overestimate Sterilisation Phase bacillary elimination by 16-139%, probably because metabolically active organisms were not completely eradicated by day 2. θ_4 is more flexible and more likely to accurately reflect sterilising activity.

SSCC-NLME modelling has further advantages. The mixed effects model can explicitly account for parameter variability and accommodate missing observations. Mathematical simulations based on the Nairobi data suggested that a sampling schedule of 5-11 sputum collections based around a skeleton of day 0, 2, 7, 14 and 56 would optimise estimation of SSCC-NLME parameters for future studies[267] and sample size estimates for a Phase IIb SSCC superiority study using the θ_4 co-efficient as a surrogate for sterilising activity were less than a quarter of those required for definitive phase III non-inferiority trials using an end-point of relapse[267]. The OFLOTUB trial, conducted in Durban from 2004-5, tested some of these principles in the practical context of 8-MQ therapy and was described in Section 1.8.2[228].

The third clinical study to be analysed using an SSCC-NLME approach was conducted in Bangkok from 2006-7 to examine the pharmacokinetic-pharmacodynamic (PK-PD)

relationships of first-line agents during combination chemotherapy[270]. Parameter estimates were broadly similar to those from Nairobi and Durban (Figure 1.12) and dose-response relationships previously described in for isoniazid in EBA studies[276] were reproduced over a narrow dose range in the presence of companion drugs. This suggested that SSCC-NLME might be useful to assess the effect of dose-ranging on sterilisation. However, it was not possible to demonstrate dose-response relationships for rifampicin or pyrazinamide and the power of the study was reduced by a high rate of early culture conversion.

Ultimately, a combination of quantitative bacteriology and modern statistical modelling may enable study of bacillary persistence and generate surrogate markers of sterilisation for Phase IIb clinical studies. Similar model based approaches have been deployed successfully in other areas of drug development[277] However, the model parameters (especially θ_3 and θ_4 which are believed to relate to persister sub-populations) require appraisal against clinical end-points of TB treatment failure and relapse.

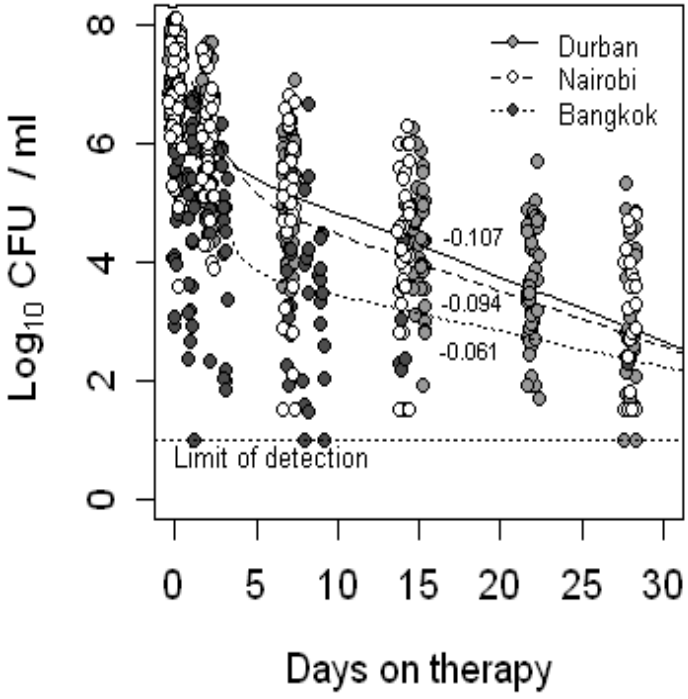


Figure 1.12 SSCC data analysed by NLME methods from three patient cohorts

NLME analysis of SSCC data demonstrated biphasic bacillary elimination, consistent with the model of persistence proposed by Mitchison. Adapted from Davies[260] and Sloan[278]

Time to Positivity (TTP) in liquid culture

Despite their potential benefits, SSCC methods on agar plates are labour intensive[279] and include a three week delay between inoculation of cultures and reading of results[280,281]. Liquid culture methods improve the speed and sensitivity of PTB detection[282] and since 2008 the WHO has advocated their introduction to laboratories in low- income countries[25]. When solid and liquid cultures are inoculated in parallel on serial sputum samples from patients on therapy, only 15% of SSCC plates may remain positive at 8 weeks[267] compared to 29-42% of samples in broth[225,226]. This is important as culture systems with enduring positivity may be more efficient at detecting persisters[265,283,284] and measuring sterilisation[216].

Liquid culture methods have evolved over time and may be used to quantify bacillary load. The initial BACTEC method was a radiometric assay which used ^{14}C-labelled fatty acid as a substrate and recorded mycobacterial growth when respiring organisms generated increased levels of radio-labelled CO_2[285]. This has been superseded by the BACTEC MGIT (Mycobacterial Growth Indicator Tube) system which uses an oxygen-quenched fluorescent indicator (Tris 4,7-diphenyl-1, 10-phenanthroline ruthenium chloride pentahydrate) embedded in silicone at the bottom of tubes containing selective broth. Bacillary growth results in oxygen consumption and the emergence of fluorescence[286,287]. With either method, larger inocula of respiring bacilli are likely to pass the threshold of detection more quickly and data from several sources demonstrate an inverse linear correlation between Time to Positivity (TTP) in liquid culture and $\log_{10}$CFU/ml counts[281,288-290].

Table 1.5 summarises studies which directly relate baseline TTP values from PTB patients to treatment outcome. These studies all focussed on HIV un-infected individuals and mainly used the older radiometric method of TTP measurement. Only two used a reference endpoint of relapse. Nevertheless, the association between shorter baseline TTP and poor outcome is striking. Clinical trials including HIV-infected patients corroborate these data, by reporting that shorter baseline MGIT-TTP is associated with an increased odds or hazard ratio for positive 2 month sputum cultures[226,291].

To use TTP in the comparison of treatment regimens, it will be important to relate dynamic changes during therapy to final outcome. Whilst no clinical trial has modelled serial TTPs as the primary biomarker of therapeutic response, several exploratory studies have argued

that TTP measurements after baseline are useful outcome predictors[271,281,292-294]. Others have described changes in TTP over time[281,288,289,295-301].

Investigators in New York in the 1990s were the first to use radiometric liquid cultures to report that TTP gradually rises in patients who are responding to therapy[295] but remains static in those who are doing badly[296]. Epstein studied a cohort of 26 patients (30% of whom had MDR-TB) and showed that those with little or no increase in TTP during the first 2 months were more likely to fail treatment[297]. Ten years later in South Africa, Pheiffer used a combination of radiometric and MGIT methods to show that the rate of increase in TTP during the first 14 days of first-line TB therapy was related to drug resistance(fastest in the sputum of patients with DS-TB, slower in those with isoniazid mono-resistance and slowest in those who had MDR-TB)[288]. These data demonstrated that TTP monitoring of bacillary elimination was associated with therapeutic repsonse, but it remained unclear whether drug tolerant persistence could be identified as readily as resistance

Two serial TTP studies were restricted to DS-TB patients. Carroll showed that changes in radiometric TTP over the first 14 days had moderate sensitivity and specificity to predict the likelihood of 2 or 3 month smear or culture conversion[298]. More importantly, Weiner applied a mixed model analysis of co-variance to extended follow-up data from 163 participants of the TBTC-27 trial and described an association between high MGIT-TTP readings during weeks 2, 4 6 and 8 and six patients with poor final outcome (five failures and one relapse) [300,301].

A meta-analysis of data from 5 studies involving bedaquiline, delamanid and PA-824 at a single South African centre was recently undertaken to compare estimation of EBA based on the rate of rise in TTP versus the traditional measurement of fall in $\log_{10}$CFU/ml counts. The results showed very high correlation for studies conducted over 7 or14 days[281]. Furthermore, the TTP method was a better discriminator of differential effects between drugs and dosages, perhaps because TTP is better able to assess the treatment effect on persister organisms[281,289].

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Lead author and year	Source of data	Patients	Type of TB	BACTEC Method	Outcome measure	Mean Liquid Culture TTP at baseline (days)		
						Good outcome	Poor outcome	p-value
Carroll, 2008[298]	Prospective: South Africa[302]	177, all HIV-	All DS-TB	Radiometric	2 month smear conversion	4.3	2.5	<0.001[a]
Pheiffer, 2008[288]		125, all HIV -	105 DS-TB 14 INH-R 6-MDR-TB	Radiometric & MGIT	2 month smear conversion	4.2	2.9	0.052[b]
Hesseling, 2010[299]	Prospective: South Africa	263, all HIV-	All DS-TB	Radiometric	2 month culture conversion	4.0	2.0	<0.001[a]
					Post-treatment relapse	3.0	1.0	<0.001[a]
Bark, 2011[290]	Retrospective: Uganda[303]	107, all HIV-	DS-TB	Radiometric	2 month culture conversion	3.0	2.0	0.042[b]
Bark, 2012[304]	Retrospective: Brazil, Phillipines, Uganda[305]	392, all HIV-	All DS-TB	Radiometric	Post-treatment relapse	9.6	5.0	0.01[b]

Table 1.5 Baseline TTP in liquid culture of sputum and response to therapy

All patients were HIV un-infected, and the majority of studies used the older radiometric ($^{14}CO_2$ based) method of TTP evaluation. Good outcome was defined as negative sputum smear or culture by two months or absence of post-treatment relapse. INH-R=isoniazid mono-resistance.

[a]Data analysed by two-sample t-test

[b]Data analaysed by Wilcoxon test

An important distinction between quantitative data from solid and liquid cultures is that TTP reflects not only the number of bacilli in a clinical specimen but their metabolic state at the time of sampling. Quiescent persisters may have a longer TTP than actively replicating organisms at a similar bacillary load. From this perspective, it is interesting that the inverse linear relationship between TTP and $\log_{10}$CFU/ml counts appears weaker for samples during the second month of therapy[290]. This might reflect changing metabolic characteristics of viable bacterial populations at later time-points. A recent study of TTP on treatment attempted to incorporate the behaviour of different bacillary sub-populations into an overall pharmacodynamic model[280] but the model parameters were not assessed against treatment outcome.

Cumulative experience of using TTP has generated optimism that it may augment or even replace SSCC for evaluation of new anti-TB regimens in Phase IIb clinical trials. This would be a considerable advance but, before it can occur, consensus is required on the optimal choice of statistical modelling techniques to analyse TTP data and systematic validation of those analytical methods should be undertaken against established clinical end-points.

Molecular biomarkers of bacillary load

Even liquid culture detection of *M tuberculosis* takes several days. Quantitative Polymerase Chain Reaction (rt-PCR) assays can identify mycobacterial DNA in sputum more quickly but, until now these have been unsuitable treatment biomarkers because they cannot distinguish between nucleic acid from viable or killed organisms[140,306-308]. In the last few years, the Xpert MTB/RIF assay has been developed[26,27]. This semi-automated technique incorporates a wash step to remove DNA from non-intact organisms before rt-PCR[26], and the cycle threshold (C_T)shows moderate correlation with culture-based estimates of viable bacillary load from *in vitro*[279] and clinical samples[309]. Pre-treating sputum with propidium monazide (PMA) inhibits amplification of DNA from dead bacteria[310] and a combined PMA Xpert MTB/RIF method has been used to follow bacillary elimination over the first 20 days of therapy in 10 patients[311]. However, the available data are very preliminary, rt-PCR based methods are not approved by the WHO for treatment monitoring in individual patients[312] and it is unclear whether they have a future role as trial level surrogates in evaluation of new anti-TB regimens.

Messenger RNA (mRNA) species have a shorter half-life than DNA, so detection of mRNA molecules may be a better discriminator of bacillary viability[313,314]. This approach has also

been investigated. Amongst 19 Brazilian patients on RHZE, rapid loss of sputum mRNA coding for *M tuberculosis* 85B alpha antigen during the first month of therapy correlated with faster culture conversion on solid media and the patient with the highest concentration of mRNA molecules on day 14 was the only one to relapse post-treatment[315]. In a separate cohort, EBA from seven days of therapy with isoniazid or several fluoroquinolones was the same when reported using measurement of $\log_{10}$CFU/ml counts or sputum concentrations of mRNA coding for isocitrate lyase[316]. Correlation has also been described between levels of isocitrate lyase mRNA and liquid culture estimation of bacillary load after 2 months of RHZE[316].

A molecular bacillary load (MBL) assay has recently been developed based on quantification of 16S rRNA, which has a short half-life, is expressed abundantly on mycobacterial ribosomes, and may respond rapidly to bacterial cell death[317-319]. This assay has been tested on serial sputum samples from 112 South African patients, showing the same biphasic profile of bacillary clearance as previously described for SSCC-NLME cohorts and a strong relationship between higher baseline MBL and increased risk of relapse[320].

In addition to detecting intracellular nucleic acids a small number of studies have sought to predict the outcome of TB treatment by identifying mycobacterial antigens. For example, production of *M tuberculosis* Antigen 85 (Ag85) is induced on exposure to isoniazid[294,321] and detection of Ag85 on day 14 may be implicated in prolonged culture positivity and relapse[322].

Overall, several molecular treatment biomarkers have been evaluated. Some, particularly the 16S rRNA MBL assay, show promise. These assays may have the advantage of measuring low-level metabolic activity in cells which are not replicating, allowing them to report on non-culturable cells which are still viable. However, they are all more expensive than existing technologies and some are technically complex. Adequate optimisation and validation for their use as surrogate markers in clinical trials will take time. These methods will not be assessed further in this thesis.

Urinary lipoarabinomannan (LAM) and extra-pulmonary disease

The biomarkers discussed so far are all measured in sputum. However, as described in Section 1.3, HIV-associated TB often disseminates beyond the lungs. Treatment response markers for studies in HIV-endemic settings may need to measure clearance of extra-pulmonary organisms.

Lipoarabinomannan (LAM) is a structurally important 19.5kD heat-stable glycolipid selectively found in the cell wall of mycobacteria and related actinomyces[323-325]. It accounts for up to 15% of total bacterial weight[323,326] and is released from metabolically active or degrading cells[327]. It is excreted in the urine where it can be detected by an enzyme-linked immunoabsorbant assay (ELISA)[325]. A commercial urinary LAM-ELISA has been developed (Clearview™ TB-ELISA, Alere Inc., Waltham, MA, USA) as a non-sputum based diagnostic test[328,329] and it has been proposed that urinary LAM excretion may be a semi-quantitative marker of whole body bacillary load[330]. Serial LAM-ELISA results during therapy could augment sputum data by providing information on extra-pulmonary bacillary elimination.

Evaluation of the LAM-ELISA for diagnosis of TB suspects at baseline has reported low sensitivity amongst HIV-uninfected individuals (6-21%)[331-334]. However, the test has progressively higher sensitivity in HIV-infected patients with diminishing CD4 counts (67-85% at CD4 <50 cells/μl)[335-337]. The ELISA is based on Optical Density (OD) readings from a plate reader and higher baseline ODs have been reported in patients with more advanced immunosuppression, more disseminated disease (e.g. mycobacteraemia) and higher AFB smear grading on sputum microscopy[338]. As these patients are likely to have a higher bacillary burden the data are consistent with the concept of urinary LAM as a quantitative marker.

Two studies confirm that the LAM ELISA remains positive after initiation of TB therapy[336,339] and one described that median OD readings remained unchanged during the first 2 weeks before declining sharply by week 8 in a cohort with 75% favourable outcomes[339]. Three further studies report a trend toward higher mortality in patients with a positive LAM ELISA at baseline[335,336,340]. However, a careful assessment of serial urinary LAM ELISAs to follow TB treatment response, assess sterilisation or predict clinical outcome has never been undertaken. If the test were to prove useful for this, it may become a valuable additional tool for patient monitoring and assessment of new therapies.

Selective detection of bacillary subpopulations

All of the methods for clinical treatment monitoring described so far make inferences about persistence based on the elimination kinetics of total bacillary load. No biomarker measures sterilising activity by selective examination of persister bacilli. This may be achieved by methods which phenotypically categorise individual cells, or by inoculation of clinical samples into media which is modified to preferentially revive non-replicating organisms.

Phenotypic characterisation of individual cells is often done on by fluorescence labelling[341]. This approach was described in Section 1.7.2 during discussion of stochastic Kat G pulsing of *M smegmatis* cells exposed to isoniazid *in vitro*[185]. Live imaging of single cells in micro-fluidic chambers has also shown that drug tolerant mycobacteria may be elongated and morphologically distinct from antibiotic susceptible cells[185,342]. Some studies have described heterogeneity in the acid-fast staining properties of persisters[343] and TAG storage by individual cells has been proposed as a metabolic persistence marker in the cytoplasm of *M tuberculosis* bacilli which can be seen on microscopy of clinical samples[175,344]. The possibility of using fluorescence microscopy or flow cytometry to develop single cell biomarkers of sterilisation will be discussed in Chapters 5 and 6.

Resuscitation promoting factors (Rpfs) are mycobacterial proteins which are known to reactivate chronic TB infections[270]. Supplementation of standard TB media with Rpfs increases the yield of organisms detected in clinical sputum samples by 80-99%, suggesting that a large population of Rpf-dependent bacilli in sputum may be invisible to conventional culture[345,346]. Preliminary data suggest that Rpf-dependent organisms are relatively preserved during TB treatment and may be rifampicin tolerant but larger studies are needed to establish whether Rpf-supplemented media revives a persister sub-population which could help with assessment of sterilising activity in clinical trials. At present, the process of purifying and storing Rpfs is too technically difficult[347,348] to conduct these studies in resource limited settings.

1.10.4 Clinical/immunological response

Whilst innovative new bacteriological and molecular techniques are important, evaluation of treatment efficacy in TB patients also requires consideration of host factors which influence or reflect the response to therapy. Clinical, radiological and immunological measurements at baseline provide information on extent of disease and underlying patient

health. These may have prognostic significance and are important covariates when interpreting data from clinical trials. Indices which show dynamic trends during therapy may also be investigated as biomarkers of outcome.

Clinical and radiological biomarkers

Low weight or Body Mass Index (BMI) have been associated with a higher risk of early TB mortality [349] and scoring systems based on vital signs or functional status have been advocated to assess baseline disease severity[349-351]. Characteristic CXR appearances have been associated with higher baseline bacillary burden[352,353], lower likelihood of two month sputum smear[140,354-356] or culture[291,357,358] conversion and greater risk of relapse[201,322,359,360]. Therefore, clinical variables and CXR appearances should be considered when designing treatment trials and analysing outcomes.

However, changes in clinical parameters (e.g. slow defervescence[361] or poor weight gain[362,363]) are inadequately sensitive or specific to be used as biomarkers of sterilisation. CXR appearances are also unsuitable because they change slowly and may fail to distinguish active TB disease from healed lesions or other causes of lung pathology[182].

More advanced imaging modalities such as computed tomography (CT)[201,364-366] and Positron Emission Tomography (PET)[367-370] have characteristic findings that may be more suitable for treatment monitoring but these methods are still imprecise, require expensive technology and are not feasible for studies recruiting large numbers of patients in low-income countries.

Immunological biomarkers

Potential immunological markers fall into two categories; those which measure non-specific inflammation or innate immunity and those which measure adaptive cell-mediated immune responses.

Many inflammatory markers in blood are elevated at TB diagnosis. Levels of some (e.g. soluble intracellular adhesion molecule [sICAM]-1[371-373] and the macrophage activation marker neopterin[374-376]) reflect disease severity whilst others (e.g. total white cell count [WCC], absolute monocyte and neutrophil count and soluble TNF-α receptor[sTNFαR-1]) predict the likelihood of 2 month smear conversion[302]. Inflammation declines during successful therapy[377-380] and there is a relationship between the fall in sICAM-1 concentration over the first week and 2 month culture conversion[372]. Persistent elevation

of serum C-reactive protein (CRP)[381] and soluble urokinase plasminogen activator receptor (suPAR)[382,383] are associated with increased mortality. High neopterin levels at the end of treatment predict relapse[297].

These markers appear attractive for biomarker development because they can be easily and inexpensively assayed but their reliability is reduced in HIV-infected individuals[376], they are not TB-specific and they provide no information about the effect of drugs on bacillary persistence. None have been systematically evaluated against reference clinical end-points and none are likely to become surrogate markers for clinical trials in the near future.

Recent study of the adaptive immune response to TB has centred on measurement of interferon(IFN)-γ production after T-lymphocytes stimulation by specific *M tuberculosis* antigens including Early Secretory Antigen Target (ESAT)-6 and Culture Filtrate Protein (CFP)-10[384]. Two commercial IFN-γ Release Assays (IGRAs) have been developed in an attempt to improve the diagnosis of latent TB infection[385] but have also been studied as treatment monitoring tools for active disease.

The rate of IGRA conversion from positive to negative during therapy is variable (5-71%) and does not correspond with clinical outcome[386]. Quantitative measurement of baseline IFN- γ responses correlate with sputum smear grading[387] but not with culture derived estimates of bacillary load[388]. Several studies have shown a gradual decline in the IFN-γ response over the course of treatment[389-391] but data inconsistency and variability[388,392,393] indicate that current IGRA methods are likely to be poor surrogates for cure or relapse.

The commercial IGRAs use overnight stimulation of T-lymphocytes to measure IFN-γ responses in early effector cells. 5 day stimulation assays demonstrate that longer term immunological memory cells behave differently, secreting higher levels of IFN-γ as treatment proceeds[394,395]. One study employing a memory cell assay found that stronger IFN-γ responses 2 months into therapy were associated with higher serum rifampicin concentrations at the same time-point and fewer relapses by 2 years[395]. This suggests that dynamic changes in the immune response to TB therapy are complex and further study may identify more useful markers of outcome. Multivariate models combining bacteriological and immunological biomarkers may generate better predictive models than individual parameters analysed in isolation[396].

1.10.5 Pharmacokinetic response

As indicated in Figure 1.9, a full assessment of TB treatment response requires data on drug exposure. PK-PD modelling is used for pre-clinical and early clinical development of new TB drugs[216] and Figure 1.13 describes commonly reported pharmacokinetic parameters including the maximum achieved plasma concentration (C_{max}) and the area under the concentration time curve (AUC). The minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial drug that inhibits bacterial growth *in vitro,* and the AUC/MIC ratio is related to the bactericidal efficacy of both rifampicin[397] and isoniazid[398,399].

Despite growing appreciation of the importance of PK-PD analysis, few clinical studies have analysed the relationship between pharmacokinetic indices and long-term outcomes[400]. The data which are available suggest that sub-optimal drug exposure have adverse consequences. In 2004-5, two trials of intermittent therapy in the U.S.A. evaluated combinations of isoniazid with rifapentine or rifabutin and described an association between low serum drug concentrations and treatment failure, relapse or acquisition of rifamycin resistance[401,402]. More recently, investigators in Botswana indicated that patients with low pyrazinamide C_{max} levels during standard chemotherapy were more likely to suffer failure or death[403]. Several less rigorous studies observed that patients with a poor clinical response at 1-2 months had low rifampicin C_{max} levels[404,405] and improved after dose escalation[405]. Finally, modelling from hollow fibre studies and computer aided trial simulations indicates that inter-individual pharmacokinetic variability plays a bigger role in the emergence of MDR-TB than poor treatment adherence[406] and a meta-analysis of 13 historical trials has speculated that rapid isoniazid metabolism may be associated with treatment failure, even amongst patients on daily multi-drug regimens[407,408].

Given that variable drug exposure may have a bearing on outcome, it is interesting that population studies, particularly from Africa have shown up to 10-fold inter-individual variability in the measurement of pharmacokinetic indices[408-412]. Pyrazinamide levels are the most stable[413-415] whilst rifampicin levels fluctuate most dramatically[416]. Differences in serum and plasma drug concentrations are seen between populations[417], with higher levels in healthy volunteers than patients with active disease, and higher levels in American than African cohorts[414]. Studies which compare drug levels (particularly C_{max}) against a pre-defined reference range invariably show that concentrations are low[140,400,403-405,414,418,419]. This is particularly concerning when it is remembered that that the currently recommended dose of rifampicin is at the bottom of a sharp dose response curve[397,420]

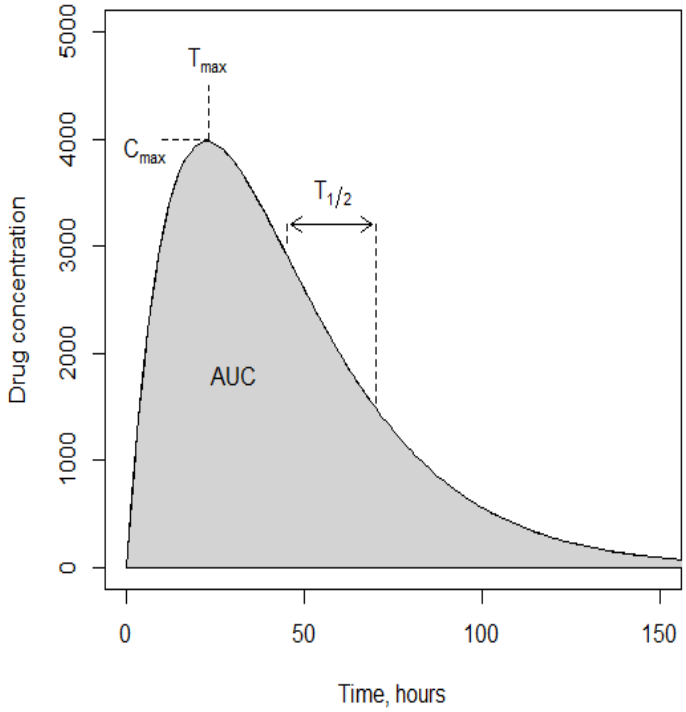


Figure 1.13 Basic pharmacokinetic parameters of drug exposure
C_{max}, T_{max} and the area under a plasma concentration-time curve (AUC) are common parameters of drug exposure. Half-life ($T_{1/2}$) is the time until the drug concentration reaches half of its original value. Not shown are volume of distribution (V), the apparent volume in which a drug is distributed and clearance, the volume of plasma cleared of the drug in unit time.

	Rifampicin	Isoniazid	Pyrazinamide	Ethambutol
Recommended dose	10mg/kg/day	5mg/kg/day	25mg/kg/day	15mg/kg/day
C_{max} (µg/ml)	8-24	3-6	35-50	2-6
T_{max} (h)	1.5-2	1-2	1-2	2-3
$AUC_{0\text{-Infinity}}$ (µg/ml.h)	5-150	4-30	300-550	20-40
$T_{1/2}$ (h)	2-5	0.5-2	9	2-4
Bioavailability (and effect of food)	68% C_{max} ↓36% AUC ↓6%	91% C_{max} ↓51% AUC ↓12%	>90% C_{max} ↓15% AUC ↑2%	80% C_{max} ↓17% AUC ↓4%
V (L/kg)	0.5	0.85-1.2	0.7	0.3-0.7
Protein binding (%)	85	20	50	40
Clearance (l/h)	4-40	9-80	3-5	50-150
Clinical factors associated with low C_{max} or $AUC_{0\text{-infinity}}$	Male sex HIV infection FDC formulation Low dose/kg Low serum bilirubin Malnutrition Alcohol consumption	Male sex Younger age FDC formulation Low dose/kg	HIV infection Low dose/kg High serum bilirubin	Female sex Younger age HIV infection Low dose/kg Higher serum albumin
Genetic factors associated with low C_{max} or $AUC_{0\text{-infinity}}$	SLCO1B1 genotype/ Anion-transporting polypeptide 1B1	NAT2 genotype/ rapid acetylor status		

Table 1.6 Pharmacokinetic properties of first-line anti-TB drugs
Data describing clinical factors associated with low plasma exposure are aggregated from a variety of references[400,408,411,412,414,421-424].
FDC formulations=Administration of fixed dose combination tablets containing all four drugs rather than individual preparations of each drug

Some of the observed variation in pharmacokinetic measurements may be attributable to technical and analytical inconsistencies between laboratories. However, a range of clinically relevant factors may also be implicated including patient age, gender[425], alcohol consumption[423], malnutrition[424] and the choice of drug formulation[400,423,426,427]. The role of HIV is controversial with some accounts of lower anti-TB drug concentrations in HIV-infected individuals[102,408,414,415,428-432] but other studies reporting no association[419,433,434]. Proposed reasons for a possible HIV effect are multi-factorial and include malabsorption[428,430] (due to HIV enteropathy or intercurrent episodes of infective gastroenteritis), hypoalbuminaemia (reducing rifampicin protein binding and increasing its hepatic clearance) and drug-drug interactions, Table 1.6 provides a summary of commonly reported pharmacokinetic parameters and factors which influence host exposure to the first-line anti-TB drugs.

Rifampicin has greatest pharmacokinetic variability because its absorption and elimination are influenced by several metabolic processes. It is subject to extensive first pass metabolism and auto-induces the enzymes responsible for its inactivation[422,435,436], causing a reduction in plasma levels at steady state[437]. Induction of cytochrome P450 isoforms (especially CYP3A4[438] but also CYP3A5, CYP2B6 and CYP2C9[439,440]) may result in clinically significant interactions with other drugs. Rifampicin is taken up by hepatocytes prior to biliary excretion. This process is mediated by organic anion-transporting polypeptide 1B1 coded for by the gene *SLCO1B1*[441,442]. Patients who are heterozygous and homozygous for a single nucleotide polymorphism (SNP) of this gene have 18% and 28% reductions in rifampicin AUC[443]. As this SNP is significantly more frequent in black African patients it may go some way to explaining the pharmacokinetic variability of rifampicin between populations[444].

The pharmacokinetic properties of isoniazid are highly dependent on its rate of elimination. This is controlled by the polymorphic *N*-acetyltransferase system of the liver and small intestine[276] which is regulated by the arylamine N-acetyl transferase (*NAT2*) gene[445]. Individuals can be partitioned according to NAT2 genotype. Slow acetylators generally achieve the greatest EBA with isoniazid due to higher drug exposure[446] and an independent *NAT2* effect on bacillary clearance[447]. They are also at increased risk of peripheral neuropathy[69]. The *NAT2* genotype-phenotype relationship may break down in advanced HIV infection because the overall acetylation rate begins to decline[448]. The distribution of alleles associated with acetylator phenotype varies with ethnicity[449]

Isoniazid and the rifamycins demonstrate marked post-antibiotic effects[450]. This has been used to make the scientific case for intermittent regimens based on these drugs. However, the shorter half-life of isoniazid causes pharmacokinetic mismatch, with the potential for effective rifamycin monotherapy and drug resistance. Fast acetylators may be at increased risk of this[401].

The pharmacokinetics of pyrazinamide are reliable and high plasma concentrations are usually achieved[408,413]. Incomplete understanding of pyrazindamide's intracellular mechanism of action makes it difficult to capture PK-PD relationships from plasma levels, although studies in the 1950s suggested a dose-response relationship for both efficacy and hepatotoxicity[451,452]. The drug exhibits no activity at all at neutral pH making it difficult to apply the MIC concept *in vivo*. It is the only one of the four first line drugs with no demonstrable EBA[265].

Ethambutol is primarily used during the first two months of TB chemotherapy to prevent the development of resistance to other drugs and some reports suggest that it may be discontinued when susceptibility to other components of the regimen is confirmed[421]. Linkages between the basic pharmacokinetic parameters of ethambutol and pharmacodynamic effect remain undefined but doses of <12mg/kg per day are no more effective than placebo so a minimum serum concentration seems to be important[453-455].

1.11 Study aims & hypotheses

The review of TB therapeutics research in this chapter has established bacillary persistence as a major obstacle to ultra-short TB chemotherapy, and explained why the lack of validated biomarkers of sterilising activity impedes end-point selection for Phase IIb clinical studies of new regimens.

The remainder of this thesis will report on a clinical study performed to assist in resolution of these issues. A cohort of Malawian adults were recruited at first presentation of sputum smear positive PTB and followed until one year after completion of treatment. Detailed clinical, bacteriological and pharmacokinetic data were collected during the intensive phase of therapy and related to final outcome. New candidate bacteriological biomarkers were assessed and novel laboratory methods to study persistence from clinical samples were evaluated. Specific study hypotheses are stated below.

1.11.1 Primary study hypothesis

The primary hypothesis of the study was that pharmacodynamic modelling of bacillary elimination using quantitative SSCC and MGIT culture data during the first 2 months of therapy will provide reliable surrogate markers of final clinical outcome suitable for use in Phase IIb trials of new chemotherapy regimens.

1.11.2 Secondary study hypotheses

Additional study hypothesis were that:

a) non sputum based assays (e.g. serial measurement of the urinary LAM-ELISA) will provide useful additional assessment on clearance of PTB
b) Single cell examination of bacilli in sputum (e.g. by fluorescence microscopy) will facilitate phenotypic characterisation and monitoring of drug tolerant persister organisms
c) Individual variation in the pharmacokinetic parameters of anti-TB drugs is an important additional determinant of treatment efficacy

2. Clinical Study Design

2.1 Overall Study Design

Patient recruitment, clinical management, sample collection, sputum bacteriology and long-term follow-up were all undertaken by a study team located at Queen Elizabeth Central Hospital (QECH), the Malawi Liverpool Wellcome Trust Clinical Research Programme (MLW) and the College of Medicine (CoM) in Blantyre, Malawi.

The overall design was of a prospective, observational clinical cohort study conducted under field conditions in a low resource setting in Southern Africa with a high TB burden in both HIV-infected and non-infected individuals.

Careful clinical profiling of all recruited patients was done at baseline and at four study visits during the first 8 weeks. Serial sputum, blood and urine samples were collected during these visits to perform the necessary bacteriology, immunology and pharmacokinetic assays for biomarker estimation. At the end of therapy, all patients were assessed for clinical or microbiological treatment failure. Thereafter, three-monthly follow-up was performed to detect TB relapse until one year post-treatment.

A schematic diagram of overall study design is provided in Figure 2.1 indicating how methodologies and data described throughout this thesis relate to the study hypotheses.

The remainder of this chapter outlines the clinical study protocol including background on the study site and participating laboratories and a detailed description of study visits and sampling strategy.

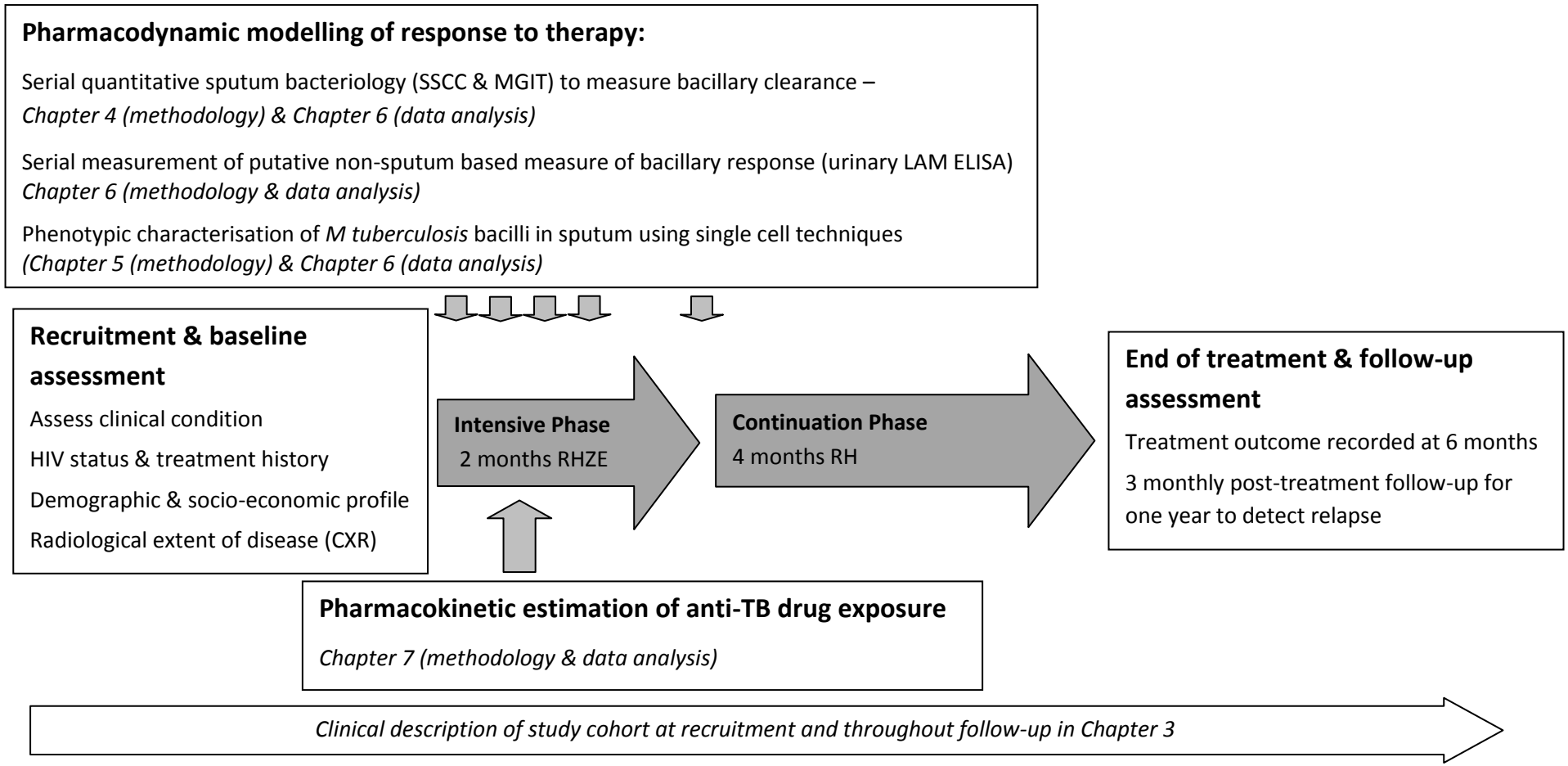


Figure.2.1 Schematic diagram of overall study design

2.2 Clinical Study Site

2.2.1 Demographic & health indicators in Malawi

Malawi is a small land-locked country in south-east Africa. The 2008 Population and Housing Census recorded the population as 13,077,160 and the population density as 139 persons per square kilometre, making Malawi one of the most densely populated countries in sub-Saharan Africa. 15% of households are in an urban setting whilst 85% are rural. 50% of men and 58% of women are employed in agriculture, many as *mlimi* (small scale subsistence farmers)[456]. Malawi is one of the world's least developed nations, ranking 171 out of 182 countries with comparable data for assessment in the 2011 Human Development Report. Gross National Income per capita is $753 and health indicators are poor; expenditure on health is 5.9% of Gross Domestic Product, the under-five mortality rate is 110 per 1000 live births and life expectancy at birth is 54.2 years[457].

Blantyre is a large city in the Southern Region of Malawi and accommodates 661,444 people. It accounts for 34.5% of the total urban population of the country. Approximately 62% of the economically active population is employed but 65% of households live below the poverty line and 70% of the city's residents live in unplanned settlements characterised by overcrowding and poor living conditions[458].

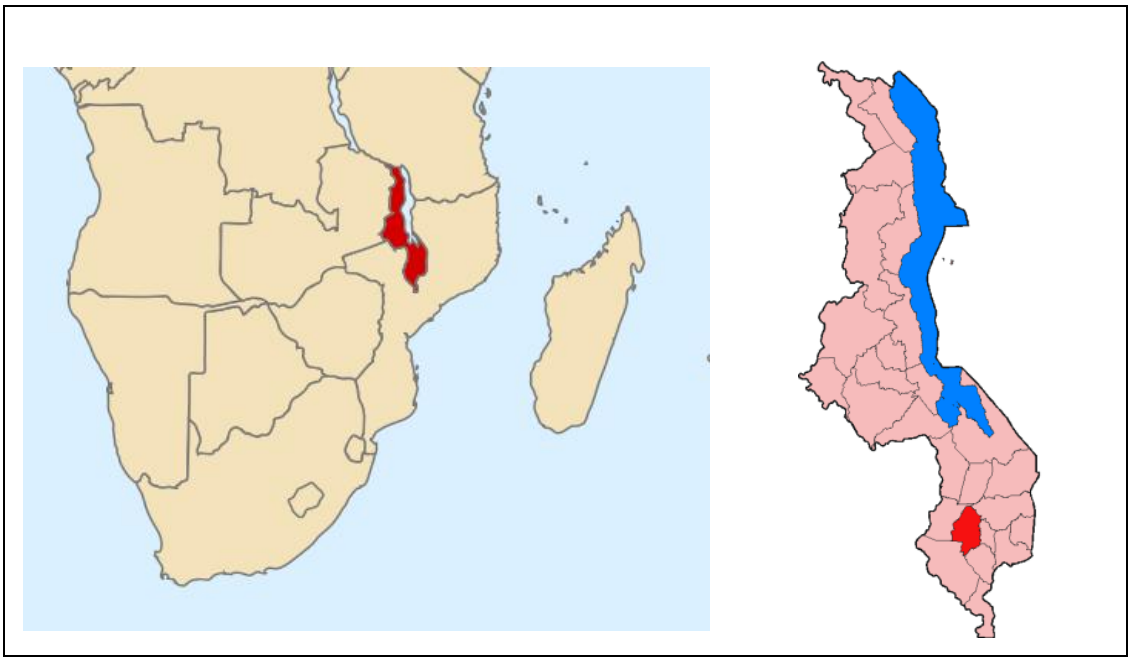


Figure 2.2 Maps indicating the position of Malawi and Blantyre

In the map on the left Malawi is shown in red on a map of southern Africa. In the map on the right, Blantyte is shown as red in a map of Malawi.

2.2.2 TB & HIV control in Malawi

In 2010, the estimated incidence of TB in Malawi was 219 cases per 100,000 persons. 90% are new cases in individuals who have never previously received TB treatment and 10% are re-treatments. 36% of new cases were sputum smear positive and 63% of TB patients tested for HIV were co-infected. 2.2% of new TB cases were estimated to be attributable to MDR-TB[459]. The low rate of primary resistance to first line anti-TB drugs indicates the strength of the NTP and made Malawi a suitable site for this study of response to TB therapy because factors influencing clinical end-points and the rate of bacillary elimination are unlikely to be confounded by unidentified MDR-TB cases.

The NTP recommends that all PTB suspects, particularly those who have been coughing for three weeks or more, submit three sputum specimens for direct smear microscopy. If at least two smears are positive for AFB, the patient is diagnosed with smear positive PTB and registered for treatment. If smears are negative, a chest CXR is performed and the patient is referred to a clinician to assess the possibility of smear negative PTB or EPTB[460].

For new TB cases, 6 month treatment is entirely administered on an out-patient basis via the Primary Health Care Centre nearest to the patient's home, unless clinical circumstances dictate a requirement for admission to hospital. Since 1984 a DOTS programme has been in place to co-ordinate administration and monitoring of therapy. A treatment supervisor is allocated to every patient to ensure that each dose of medication is appropriately swallowed and documented on a handheld TB treatment card[460]. The treatment success rate in Malawi is high: 88% for new smear-positive cases and 83% for new smear-negative or EPTB cases[459].

11% of Malawian adults aged 15-49 are HIV-infected. HIV prevalence is highest (15%) in Southern region (including Blantyre). Prevalence is also higher in urban (17%) than rural (9%) settings[456]. Since 2004, Malawian has successfully pioneered the public health approach to HIV treatment based on decentralisation of ART delivery and task-shifting from clinicians to nurses and counsellors in Primary Health Care clinics[461,462]. As a result, by mid-2011 382,953 Malawians had initiated ART through the National Programme and 276,987 (72%) of those were still alive on treatment[463]. Until recently, the standard first-line adult ART regimen for HIV-TB co-infected individuals consisted of stavudine, lamivudine and nevirapine[464]. However, concern about drug toxicity and interactions between ART and TB treatment has resulted in a recent change to national guidelines. Since September 2011, adults who are already taking ART when they commence TB treatment remain on their

exisiting regimen but ART naive TB patients are initiated on a combination of tenofovir, lamivudine and efavirenz[465]. Current guidelines also advocate ART initiation as soon as TB therapy is safely established. These changes explain some of the variation in ART regimes amongst study patients described in Chapter 3.

2.2.3 Clinical centres participating in the study

The main TB registration facility in Blantyre district is QECH, the country's largest government-owned health care facility. All hospital in-patients diagnosed with TB are notified there alongside smear positive cases and TB suspects from surrounding Primary Healthcare Clinics. A study office was set up in QECH adjacent to the TB registration office in order that all new smear positive patients could be screened and informed about the study. Two research nurses were specifically employed to run this office. They were trained in the study protocol, HIV Testing and Counselling and Good Clinical Practice guidelines prior to commencement of recruitment.

In 2011, decentralised TB registration was made possible at four Primary Healthcare Clinics with facilities to perform sputum smear microscopy. These included Ndirande, Bangwe, Zingwangwa and Chileka Health Centres. Nurses responsible for TB notifications at these clinics were educated on the study protocol and advised to refer eligible patients to the central study office at QECH in order that they could still be recruited.

2.2.4 Participating Research Laboratories

TB laboratory, College of Medicine, University of Malawi

International guidelines recommend that all TB specimen processing be conducted in a Class 1 or Class 2 Biological Safety Cabinet (BSC) in a dedicated mycobacteriology room[466] and that manipulation of TB cultures is confined to Biosafety Level 3 (BSL-3) laboratory[467]. In Malawi, all smear preparation, media inoculation, sub-culturing and isolate identification was performed under these conditions in the TB laboratory at the Department of Microbiology, CoM, University of Malawi.

This laboratory participates in suitable quality control programmes via the UK National External Quality Assessment Service, the National Health Lab Services in South Africa (International), and College of American Pathologists. It is located on the main CoM campus and is a 10-15 minute walk from QECH. Sputum samples were carried from the study office to this laboratory daily by a designated research assistant to ensure that they were processed within 24 hours of collection. Duplicate sample transportation logbooks were

kept in the study office and the laboratory and all specimens were transported in an appropriately labelled and secured biohazard container to minimise any transmission risks associated with TB-infected clinical material in transit.

Malawi-Liverpool-Wellcome Trust Clinical Research Programme

MLW was established in 1995 forming a partnership between the College of Medicine of the University of Malawi, the University of Liverpool, Liverpool School of Tropical Medicine (LSTM) and the Wellcome Trust. The MLW laboratory is situated within the grounds of QECH allowing rapid specimen transportation from clinical areas. All routine blood tests, plasma separation and storage and urinary LAM-ELISAs were done here. The fluorescence microscope used for lipid body fluorescence microscopy was also housed here.

Liverpool School of Tropical Medicine

LSTM contains BSL-3 facilities with two Class 2 BSCs. These were used for the lipid body fluorescence microscopy optimisation and flow cytometry experiments described in Chapter 5.

The Clinical Pharmacology laboratory at LSTM contains High Performance Liquid Chromatography (HPLC) and Mass Spectrometry equipment which is unavailable in Malawi. Plasma samples for the pharmacokinetic assays described in Chapter 7 were shipped to this laboratory for bio-analysis.

2.3 Clinical Study Structure and Staff

Whilst the Principal Investigator (PI) was responsible for the design, conduct and execution of the study, the multi-site nature of the project involved co-ordinated activity from a large number of individuals. The main flow of work and samples is indicated in Figure 2.3

2.4 Clinical Study Timeline

Laboratory preparation for the clinical study, particularly optimisation of the lipid body fluorescence microscopy technique was done at LSTM from August 2009-February 2010. On-site study preparation (e.g. recruitment and training of staff, refurbishment of the study office and piloting of study protocols) was done at MLW and QECH from March-June 2010.

Patient recruitment occurred from 26th June 2010 until 31st December 2011.

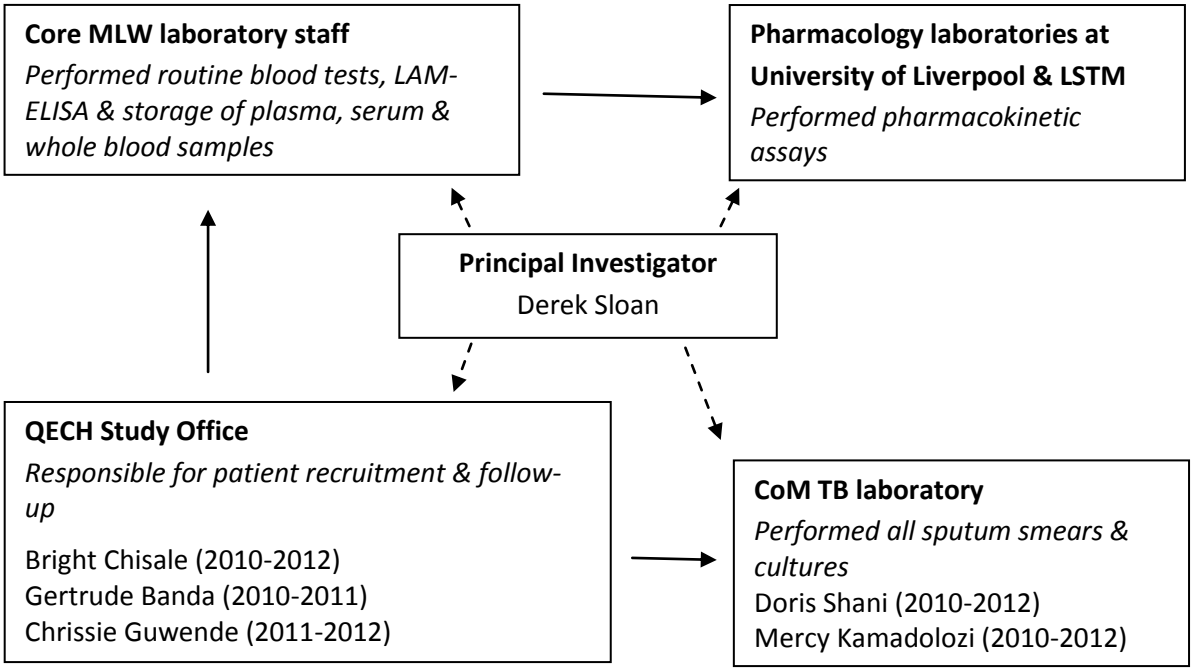


Figure 2.3 Flow of study work and samples

Solid arrows indicate flow of study samples. Dashed arrows indicate co-ordinating responsibilities of the PI

2.5 Patient Screening and Recruitment

TB registration officers at QECH and participating Primary Healthcare Clinics referred new smear positive TB patients to the study team prior to administration of the first dose of anti-TB chemotherapy. Study nurses immediately assessed each patient using a screening questionnaire based on the Inclusion and Exclusion Criteria detailed below. Those who fulfilled the eligibility criteria were provided with a Patient Information Document and had details of the study protocol outlined to them in detail. Patients who were willing to participate were asked to sign two consent forms: a general consent form covering data collection and sampling, and a DNA sampling consent form providing permission to ship whole blood samples overseas for extraction of human DNA in pharmacogenetic assays, which will be performed later in an extension of the work described in Chapter 7.

All Patient Information Documents and Consent Forms were provided in both English and Chichewa, the major language spoken in Southern and Central Malawi. To ensure accuracy back-translation was also done before the forms were authorised for use. 19% of Malawian men and 33% of Malawian women are illiterate[456]. In such cases, recommended principles regarding genuine informed consent [468]were adhered to and patient agreement was documented using a thumbprint.

Renewed consent for ongoing study participation was taken at each visit and recorded in the Case Record File (CRF).

2.5.1 Inclusion Criteria

To ensure enrolment of patients with confirmed smear positive tuberculosis at a sufficiently high bacterial load for bacillary elimination to be studied over time on therapy, only new smear positive patients with diagnostic clinical specimens graded "++" or "+++" for AFB in the QECH hospital laboratory were considered eligible for recruitment. This provided a patient cohort with a similar TB burden to that normally used for clinical trials of new TB treatment regimens.

IUALTD guidelines for assessment of ZN stained slides were used to assess sputum smears[469]. The grading scheme is summarised in Table 2.1.

AFB Counts	Grading
No AFB in at least 100 microscopy fields[a]	0 or 'negative'
1-9 AFB in 100 fields[b]	'Scanty'; record actual number of AFB counted
10-99 AFB in 100 fields	'+'
1-10 AFB per field in at least 50 fields[c]	'++'
>10 AFB per field in at least 20 fields[c]	'+++'

Table 2.1 IUALTD-recommended grading of ZN sputum smear microscopy results

[a]At least 5 minutes should be taken to read 100 fields before reporting the slide as negative
[b]A finding of 1-3 bacilli in 100 fields does not correlate well with culture positivity. It is recommended that a new smear be prepared from the same sputum specimen and be re-examined.
[c] In practice most microscopists read a few fields and confirm the findings by a quick visual scan of the remaining fields

Additional inclusion criteria were

- Patients should be aged 16-65
- Patients must consent to or already had a written result of serology for HIV
- Patients must provide genuine informed consent (via signature or thumbprint) to study participation

2.5.2 Exclusion Criteria

Patients meeting the following criteria at baseline were excluded from the study

- Significant anaemia (Hb <6g/dL)
- Significant renal dysfunction (Serum Creatinine >177µ mol/L [2 mg/dL])
- Significant hepatic dysfunction (Total Bilirubin >51 µmol (3 mg/dL), alanine transaminase (ALT) > 200 i.u./l)
- Very poor clinical performance status suggestive of imminent mortality (WHO Performance Score 4)
- Previous TB treatment in the last five years
- Administration of a TB treatment regime other than standard first line therapy (e.g. using intramuscular streptomycin) or adjunctive steroids
- Pregnancy
- Anticipated obvious difficulties with follow-up

2.5.3 Withdrawal Criteria

After recruitment, patients meeting the following criteria at any time were withdrawn

- Patients request
- Patient loss to follow-up and untraceable by study staff
- Patient transferred out of Blantyre
- All cultures negative for *M. tuberculosis*
- Baseline drug resistance identified to rifampicin and isoniazid
- Adverse drug reactions requiring interruption of treatment
- Poor adherence to therapy
- Complications arising from tuberculosis (e.g. requiring adjunctive steroids or surgery)
- Death unlikely to be attributable to active TB

2.6 Study Visits and Sampling Schedule

The laboratory work involved in the microbiology techniques for this study was labour intensive as there were serial specimens to be processed for each patient. To allow sample collection at a maximum number of time points whilst avoiding an unmanageable volume of laboratory work, the study cohort was divided into two staggered but balanced blocks. Patients were allocated to Block 1 or Block 2 sequentially. Each block had 5 study visits for sampling. Block 1 was sampled at Baseline (BL) and S1-4 visits on days 4, 14, 28 and 56 of therapy. Block 2 was sampled at BL and S1-4 visits on day 2, 7, 21 and 49. Computer simulated SSCC-NLME studies have suggested that a "balanced block" sampling strategy of this nature may be used to optimise datasets for Phase IIb clinical trials using end-points based on serial quantitative bacteriology[267].

An overview of all study visits and sampling is shown in Figure 2.4.

2.6.1 Baseline Visit

Demographic and biometric details were recorded for every patient on study entry. A clinical history was taken including details of presenting TB symptoms, HIV status and treatment history, co-morbidities and other current medications. To give an impression of overall condition, patients were asked to grade their own health as "excellent", "good", "fair" or "poor". To assess functional ability, WHO Performance status was assessed by the study nurses (Table 2.2)[470]. Prior BCG vaccination was assessed by inspection for a visible scar. Vital signs (pulse, blood pressure, temperature, respiratory rate (RR) and O_2 saturations) were documented to provide an objective picture of illness severity. A CXR was done to assess radiological extent of disease.

Grade	WHO Performance Status
0	Fully active, able to carry out all pre-disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature
2	Ambulatory and capable of all self-care but unable to carry out any work activities. Up an about ≥ 50% of waking hours
3	Capable of limited self-care, confined to bed/chair ≥ 50% of waking hours
4	Completely disabled. Cannot self-care. Totally confined to bed or chair[a]
5	Dead[a]

Table 2.2 WHO Performance Status gradings

[a]Patients with WHO status >3 were excluded from the study

Recruitment

Eligible patients allocated to Block 1 or 2 sequentially

	Sampling Visits	
	Block 1 (S1; day 4, S2; day 14, S3; day 28 S4; day 56)	**Block 2** (S1; day 2, S2; day 7, S3; day 21, S4; day 49)
BL	Blood for immediate tests (UE, FBC, LFT, HIV test ± CD4 count) Blood to store (serum [Vitamin D], whole blood [DNA extraction/pharmacogenomics]) Urine test for isoniazid & pregnancy (females only) Urine for LAM-ELISA CXR Overnight sputum sample collection for quantitative bacteriology (Serial Sputum Colony, Counting [SSCC)] & Liquid culture [MGIT])*	
S1	Overnight sputum for SSCC& MGIT*	Overnight sputum for SSCC & MGIT*
S2	Overnight sputum for SSCC & MGIT* Blood for pharmacokinetic analysis	Overnight sputum for SSCC & MGIT*
S3	Overnight sputum for SSCC & MGIT*	Overnight sputum for SSCC & MGIT* Blood for pharmacokinetic analysis
S4	Overnight sputum for SSCC & MGIT* Urine for LAM-ELISA	Overnight sputum for SSCC & MGIT* Urine for LAM-ELISA

Completion of 6 month therapy

Early morning sputum for MGIT & Lowenstein Jensen culture
Treatment outcome recorded
CD4+ count in HIV positive patients

Follow-up

All patients reviewed for TB relapse/reinfection at 3,6,9,12 months.
Sputum culture if recurrent productive cough

Figure 2.4 Clinical study sampling schedule

*1ml sputum is required for SSCC & MGIT respectively. Remainder of sputum stored at -20^{o}C for lipid body fluorescence microscopy (described in chapters 5 & 6)

Blood was sampled for serum creatinine, bilirubin and ALT and blood haemoglobin, WCC and platelet count. A urine sample was collected and tested for isoniazid to ensure no recent anti-TB drug exposure. A β-HCG pregnancy test was performed on urine from all female patients.

HIV testing was undertaken on all patients who did not know their HIV status, had previously tested negative or were not on ART. A CD4 count was performed on all HIV-infected individuals.

Serum for estimation of Vitamin D metabolites and whole blood for DNA extraction and pharmacogenomic assays were collected and stored at -70^{o}C. 2ml urine was collected, appropriately processed and stored at -20^{o}C for use in the LAM-ELISA described in Chapter 6.

An overnight sputum sample collection was performed. All patients were issued with a 100ml wide mouthed polypropylene collection pot and allowed home. After eating at 6pm they were instructed to rinse their mouth with water, collect all expectorated sputum until 6am the following morning and not eat again until the collection was complete. The sample pot was then returned to the study office. FRIO medication wallets (friouk.com) were used to keep sputum pots <15^{o}C during specimen collection and transportation. After delivery, the sputum samples were transferred to the TB laboratory. 2ml was processed immediately for quantitative cultures (1ml for SSCC plates and 1ml for liquid (MGIT) broth; specific methods described in Chapter 4). The remainder was stored at -20^{o}C for lipid body fluorescence microscopy.

Once sputum was submitted patients were commenced on TB therapy according to standard NTP guidelines[460]. A WHO approved weight-adjusted treatment regimen with daily Fixed Dose Combination (FDC) tablets containing rifampicin for all 6 months was used as shown in Table 2.3. HIV-infected individuals were also advised to commence co-trimoxazole 480mg PO twice daily and referred to QECH ART clinic for advice on ART initiation.

Body weight (kg)	**Initial Phase (2 month)** Number of RHZE tablets: (*rifampicin 150mg, isoniazid 75mg, pyrazinamide 400mg, ethambutol 275mg)*	**Continuation phase (4 months)** Number of RH tablets: *(rifampicin 150m, isoniazid 75mg)*
30-37	2	2
37-54	3	3
54-74	4	4
74 and over	5	5

Table 2.3 TB drug doses in relation to body weight using FDC tablets
Adapted from Ministry of Health, Malawi, NTP Manual, 6th Edition

2.6.2 Intensive Phase (S1-S4) sampling visits

At all Intensive Phase sampling visits a brief clinical assessment was performed. Severity of clinical illness, BMI and treatment adherence were all re-evaluated and an overnight sputum sample was collected for quantitative microbiology.

At S2, S3 and S4 visits 2ml urine samples were also collected stored and processed for LAM-ELISA.

Blood sampling for pharmacokinetic assays

To assess steady state plasma concentrations of anti-TB drugs, pharmacokinetic blood sampling was done at the S2 visit (day 14) in Block 1 patients and the S3 visit (day 21) in Block 2 patients.

On these days patients omitted their morning medications and attended the study clinic fasted at 7.30am. Time '0' venous blood samples were collected in 4ml lithium heparin monovettes, placed inside a polypropylene rubber-seal box, protected from light and transported immediately on ice to the MLW laboratory where plasma was separated by centrifugation (1000 x *g* for 10 minutes) and stored at -70^{o}C. Morning medications were then administered with a glass of water, but patients remained otherwise fasted until '2 hour' sampling was done in the same manner. Patients were given lunch in the study office prior to final '6 hour' sampling. As ambient temperature and light exposure may degrade rifampicin and adversely affect bio-analysis rapid transfer of blood samples from the clinic to the laboratory was essential.

2.6.3 End of Treatment (EOT) sampling visit

A further clinical assessment was performed on completion of 6 months TB therapy. An early morning sputum sample was collected for TB culture in MGIT broth and on

Lowenstein Jensen (LJ) slopes. If both cultures were positive the patient was deemed to have failed therapy and referred to the NTP for re-treatment with standard Regimen 2[460]. If only one culture was positive a second sputum sample was requested for confirmatory testing.

HIV-infected individuals also had a repeat CD4 count. Those who had not yet initiated ART were linked into HIV care by referral to their local ART clinic.

2.6.4 Post-treatment follow-up visits

Patients were asked to attend follow-up appointments with the QECH study team at 3, 6, 9 and 12 months after completion of therapy. Those who lived far away or who found hospital attendance difficult were permitted to conduct these visits by telephone so long as they were clinically well. Patients with recurrent cough were asked to submit sputum samples to test for TB relapse or re-infection. These samples were processed in the same way as EOT specimens. If recurrent TB was diagnosed, patients were referred to the NTP for treatment.

2.6.5 Unplanned visits

Throughout their participation in the study, patients were encouraged to contact the study team directly and visit the study office if they encountered any treatment complication, drug side-effects or inter-current illnesses. The PI was required to assess any patient attending for an unplanned visit as the problems precipitating them were often outside the scope of the study protocol and required the input of a clinician. A specific CRF was designed to record the number and nature of unplanned visits and document any clinical action taken.

2.6.6 Tracing of lost patients

In a cohort study, it is essential to retain as many patients as possible until a clear study-end-point is reached. Inevitably some patients forget to attend appointments, cannot come to hospital due to illness or miss study visits for other reasons.

To minimise loss to follow-up, detailed directions to each patient's house were recorded during the baseline assessment and kept in a 'map book' in a locked drawer in the study office. Mobile phone numbers for study patients and (where permitted) next of kin guardians were also recorded and checked at each study visit. Any patient who was more than 24 hours late for a study visit was traced by telephone and encouraged to attend as soon as possible. If a patient was late and could not be contacted for more than one week

after a scheduled appointment a study nurse visited them at home to identify the reason for non-attendance and facilitate the study visit. These home visits were undertaken in plain clothes to maintain patient confidentiality and cultural sensitivity. Traced patients who no longer wished to participate in the study were formally withdrawn.

2.7 Establishing Study Outcomes

WHO tuberculosis treatment guidelines outline six standard outcomes of therapy for TB control programmes; cure, treatment completion, treatment failure, death, default and transfer out[30]. However, these do not consider the possibility of post-treatment relapse, nor do they discriminate between TB-related deaths and death due to other causes. Additionally, the WHO definition of 'failure' relies only on persistent smear or culture positivity. Modern clinical trials protocols have structured their endpoints to acknowledge that, even in the absence of unambiguously positive bacteriology, some patients may be classified as failures/relapses on clinical ground whilst low numbers of colonies on a single EOT culture do not mandate re-treatment[471].

For this study outcomes were defined as shown in Table 2.4. In cases where treatment failure, relapse or cause of death had to be determined on clinical grounds because supportive bacteriological data was absent or inconsistent, a decision on outcome was made by two clinicians; the study PI in consultation with a QECH physician independent of the study team. Additionally, all patients with recurrent TB after completion of therapy were regarded, for the purpose of this thesis, as relapses on the basis that recurrence occurred within one year of treatment completion. Spoligotyping of *M tuberculosis* isolates recovered at the time of recurrence was not available and it is possible that some patients suffered re-infection rather than recurrence. Stored isolates may be used for verification of this at a later date.

Overall, cure or treatment completion was regarded as a "favourable outcome" and failure or relapse was regarded as an "unfavourable outcome". Patients who defaulted therapy, died of causes unrelated to TB or were transferred out of Blantyre with unknown outcomes were withdrawn from the study according to the criteria described in section 2.5.3.

Cure	A patient whose sputum culture was positive at baseline but who was culture negative at EOT and showed no clinical or bacteriological evidence of relapse during post-treatment follow-up
Treatment completion	A patient who completed treatment and showed no clinical or bacteriological evidence of relapse during post-treatment follow-up but who did not have a documented negative culture result at EOT[a]
Failure	A patient who had any of the following: (1) two positive TB sputum cultures at EOT; (2) one positive TB sputum culture at EOT and clinical features indicating a need for re-treatment[b]; (3) clinical features at EOT indicating a need for re-treatment in the absence of supporting bacteriology[b]; (4) death during treatment which was definitely or probably attributable to active TB[b]
Relapse	A patient who had a favourable outcome at EOT but one of the following during post-treatment follow-up: (1) two positive TB sputum cultures; (2) one positive TB sputum culture and clinical features indicating a need for re-treatment[b]; (3) clinical features indicating a need for re-treatment in the absence of supporting bacteriology[b]; (4) death which was definitely or probably attributable to active TB[b]

Table 2.4 Study outcome definitions

[a]Absence of an EOT culture result may be because a sample was not collected or cultures were contaminated
[b]Decisions on clinical indications for re-treatment or attributable cause of death were made by the PI in consultation with a QECH physician independent of the study team.

2.8 Sample Size Calculation

It is generally anticipated that an unfavourable outcome (i.e. treatment failure or relapse) will occur in approximately 5% of patients with drug susceptible TB[69]. To determine sample size for the study, this primary endpoint was related to one of the putative surrogate end-points in the primary hypothesis (rate of decline in CFU/ml counts on SSCC plates during the sterilisation phase of bacillary elimination in the first 8 weeks of therapy). An odds-ratio (OR) was used as the measure of effect in a linear regression model. Assuming that unfavourable outcomes occur at a rate of 4-8% and using conventional error values of two sided α=0.05 and β=0.20 the range of sample sizes required to detect an effect size in the range 2-3 are shown in Figure 2.5. These effects represent the increase in the odds of an unfavourable outcome at a value of the bacillary elimination rate one standard deviation

above the mean. At a primary outcome rate of 6% an OR of 2.5 can be detected with 80% power at a sample size of 165 patients. For this scenario, 12 poor outcomes are expected on average and allowance is made for up to 17% loss to follow-up. However, it was unclear exactly what the primary outcome rate would be in this study population, so the study team aimed to recruit a slightly larger sample of 200 patients.

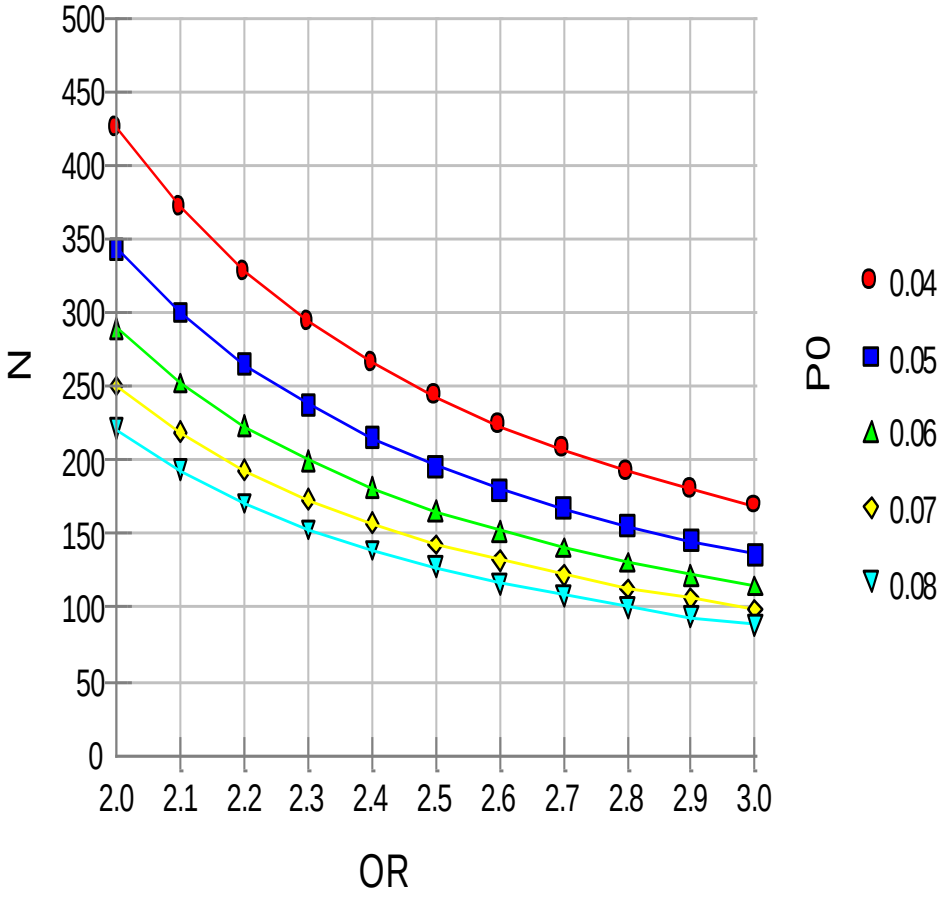


Figure 2.5 Power curves of relationship between sample size and detectable effect

Power curves are expressed as the odds ratio (OR) of primary outcome (combined treatment failure/relapse) using conventional error values of of two sided α=0.05 and β=0.20 for a range of underlying primary outcome (PO) end-points (4-8%) at the mean value of the SSCC slope

2.9 Ethical approval

Ethical approval for the study was granted by the College of Medicine Research Ethics Committee, University of Malawi (P.01/10/855) and the ethics committee of the LSTM (09.67). Copies of ethics approval letters are attached as Appendices 10.1 and 10.2.

2.10 Data Management

Paper copies of clinical and laboratory CRFs were designed in Microsoft Word. Although all forms were prepared in English, key clinical questions were translated into Chichewa and transcribed onto the CRFs to provide appropriate and consistent interview prompts during patient visits. Research nurses and laboratory staff were trained on data collection and entry by the Principal Investigator prior to the start of the study to ensure standardised documentation of study participants' medical history, clinical examination findings, and results of investigations. All paper CRFs are stored in the MLW archive and will be retained for 5 years after study completion.

All clinical and laboratory CRF data were entered into a master Microsoft Access database via an EpiInfo Version 3.5.3 user interface. Related information in different data tables were linked by unique patient and sample identifiers. All data was double entered and verified prior to analysis. The study database was stored on a password protected designated study computer and backed up on two password protected hard drives kept in separate locations.

Details of statistical techniques used to analyse the study data are provided in the relevant sections of chapters 3-7. Unless stated otherwise, all statistical analyses were done using 'R' version 2.15.2[472]. Throughout this thesis, statistical significance is reported at the level of $p<0.05$.

3. Clinical Study Description

3.1 Introduction

This chapter profiles baseline characteristics of the study cohort and events during TB treatment in order to identify clinical factors which independently predict treatment response or are important covariates for pharmacodynamic modelling. Final outcomes for study patients are reviewed and range of factors which may influence the effectiveness of TB therapy are assessed.

Firstly, socio-economic status is discussed. Globally, there is a well established link between TB prevalence and urban poverty[473-475] but it is plausible that socio-economic factors are also implicated in treatment outcomes. This is explored using information collected from patients at baseline interview.

Secondly, HIV parameters are outlined to provide information on patients' HIV status and describe access to ART during the study. Although HIV does not influence cure from PTB[476,477] more relapses are reported in HIV-infected individuals[478,479]. The specific effect of ART on TB outcome is unknown but emerging evidence suggests that it reduces total mortality[122,123] and Malawian national policy has recently changed from stating that naive patients should initiate ART during the continuation phase of TB therapy[464], to recommending initiation "within 14 days of TB diagnosis"[465]. As noted in Section 2.2.2, in September 2011 standard first-line ART regimen for TB patients was changed from stavudine, lamivudine and nevirapine to tenofovir, lamivudine and efavirenz. Assessing the effect of these changes when modelling bacillary persistence and elimination is not straightforward but a detailed description of HIV treatment amongst TB patients is provided to contextualise the analysis.

Thirdly, clinical factors are reviewed, several of which have previously been associated with treatment response including low baseline BMI, poor weight gain during treatment[363], ever smoking cigarettes[291] and BCG vaccination[480]. The strength of the evidence for these associations is mixed and prior studies are difficult to compare because they are of differing size, and use varying end-points of two month smear or culture conversion rather than final clinical cure or failure/relapse. Analysis of the current dataset is done to clarify the relative importance of these factors as covariates for the study cohort.

Fourthly, serum Vitamin D concentrations of study patients are assessed. Previous work has shown variable baseline Vitamin D deficiency in Malawian adult TB patients[481]. This may be relevant for two reasons. 1,25 (OH) D, the active metabolite, is an immunologically active hormone which induces anti-mycobacterial activity *in vitro*[482] and modulates the host response to mycobacterial infection by induction of reactive nitrogen and oxygen intermediates[483,484] and the antimicrobial peptide cathelicidin[485-487]. It has been proposed that these effects of Vitamin D may usefully augment TB treatment and whilst a recent clinical trial of did not show improved outcomes with Vitamin D supplementation, it demonstrated a trend towards faster bacillary clearance from sputum particularly in patients with a *tt* Vitamin D receptor genotype[488,489]. From a related perspective, it is necessary to evaluate whether varying Vitamin D levels in a generally deficient population have consequences for treatment response.

Additionally, some components of TB therapy and ART can pharmacologically lower serum concentrations of useful Vitamin D metabolites (Figure 3.1). Vitamin D is normally absorbed from the skin and diet and converted by sequential hydroxylation into 25 (OH) D (the routinely measured compound in serum) then 1,25 (OH) D (the active compound). Isoniazid inhibits both hydroxylation steps[490] and rifampicin induces activity of enzymes which degrade 25 (OH) D into an inactive waste product[491]. Combined rifampicin and isoniazid therapy may reduce serum levels of useful Vitamin D metabolites by 23-34%[491]. The anti-retroviral drug, efavirenz has also been associated with Vitamin D deficiency[492]. Although only recently introduced to first-line ART in Malawi, this drug is in the same class as nevirapine which is ubiquitously used, and may have similar metabolic effects. It is important to know whether Vitamin D levels are progressively compromised by drug therapy in a population at high risk of baseline deficiency.

Finally, radiological extent of TB disease is described. CXR abnormalities (especially cavitation) are associated with delayed therapeutic response[354,356,358] and clinical trials often use CXR appearance as a covariate in end-point analysis[225,228,232]. However, many CXRs assessment schemes[493,494] are complex, vulnerable to inter-reader variability[495] and poorly validated to predict outcome. In 2010, investigators in Papua, Indonesia reported that a simple scoring system based on percentage of lung affected and presence of cavitation predicted 2 month sputum smear conversion in a predominantly HIV-negative population[355]. This approach is used here to evaluate the significance of CXR abnormalities in a setting of higher HIV endemicity.

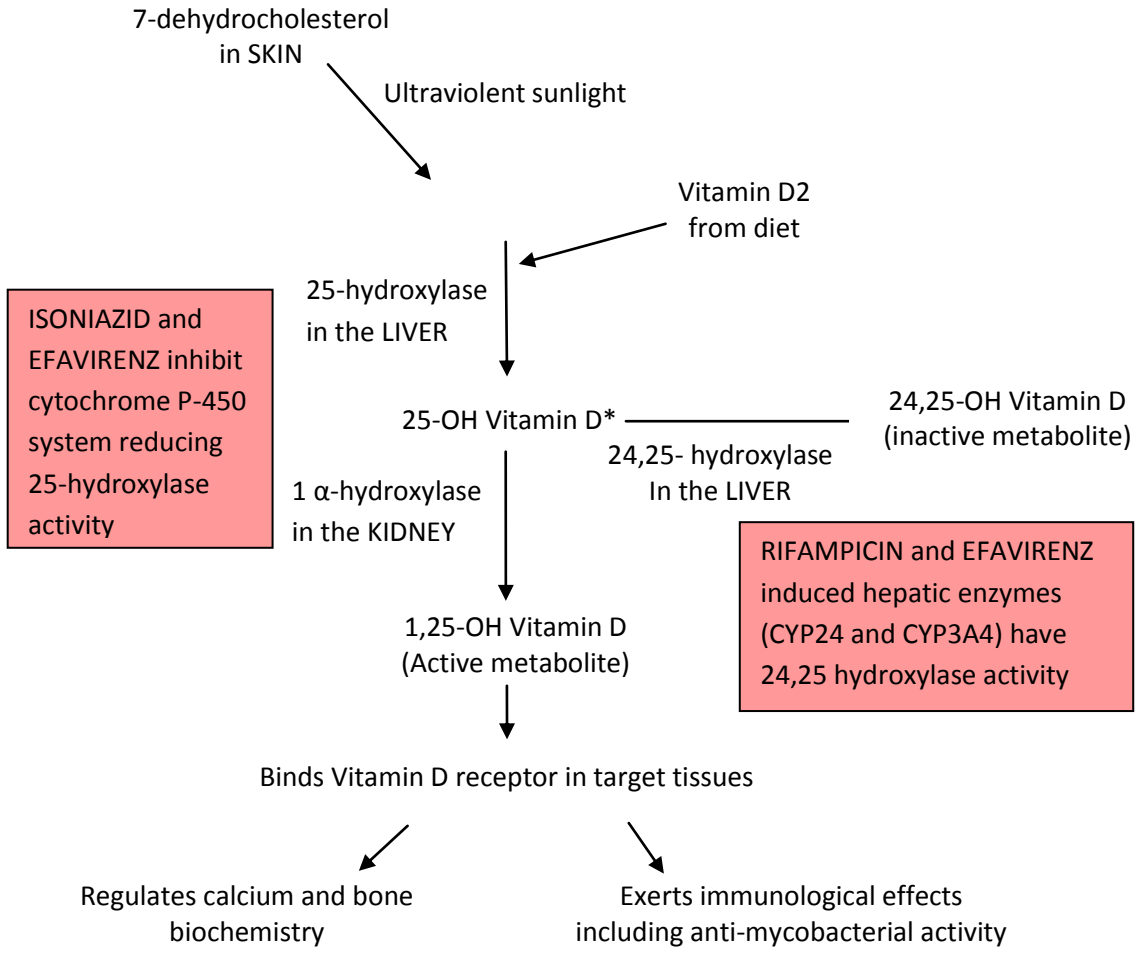


Figure 3.1 Vitamin D metabolism and the effect of TB and HIV drugs

*25 (OH) Vitamin D is the routinely measured metabolite, commonly used as a surrogate measure of the active compound 1,25 (OH) Vitamin D.

After profiling each aspect of the study cohort, the relevance of each clinical and radiological covariate to treatment outcome is analysed in relation to binary measures of response. Two month sputum culture status is used as an early marker and final clinical outcome is used as a definitive end-point. The covariates described here will be re-visited in Chapter 6 and 7 to assess their relationship with variability in the parameters of pharmacodynamic and pharmacokinetic models.

3.2 Methods

The overall design of the clinical study was outlined in Chapter 2. Specific techniques for drug susceptibility testing of TB isolates, HIV testing of participants, CXR interpretation and measurement of 25 (OH) D levels are provided here. Statistical methods used in this chapter are also described.

3.2.1 Procedure for drug susceptibility testing

Drug susceptibility testing was performed on *M tuberculosis* isolates grown from baseline sputum samples of all patients to demonstrate that variability in treatment response was not attributable to undiagnosed drug resistance, including MDR TB.

Traditional phenotypic susceptibility testing involves growth of *M tuberculosis* in the presence of anti-TB drugs. Modern molecular techniques allow genetic mutations responsible for rifampicin and isoniazid resistance to be detected from bacterial DNA, giving faster results with minimal handling of viable bacilli. 96% of rifampicin resistant *M tuberculosis* strains possess alterations within a rifampicin resistance-determining region (RRDR) of the *rpoB* gene[496], and the majority of isoniazid resistant strains possess mutations on *katG* (70%) or *inhA* promoter (15-35%) genes[497]. The Genotype MTBDR*plus* 2.0 line probe assay (Hain Life Sciences, Nehren, Germany) detects mutations at these three sites[498]. This assay was introduced to the CoM TB laboratory in Blantyre and used for susceptibility testing of baseline isolates. The procedure was performed according to manufacturer's instructions[499] and WHO guidelines[500] as outlined below. Three separate rooms were used to prevent cross-contamination of DNA molecules.

In the CL-3 laboratory, the liquid culture (MGIT) method described in Section 4.3 was used to isolate *M tuberculosis* from sputum and DNA was extracted using a Genolyse manual extraction kit. Culture fluid was centrifuge concentrated at 3000 x *g* for 20 minutes then cell pellets were re-suspended in 100µl of Genolyse solution and incubated for 5 minutes at 95^{o}C. Neutralisation buffer was added, samples were spun again at 3000 x *g* for 5 minutes and the supernatants were used as DNA samples for the polymerase chain reaction (PCR) step.

In the second room, two commercially prepared 'amplification mix' solutions containing biotinylated primers and *Taq* polymerase were mixed together in preparation for PCR amplification of nucleic acid.

In the third room, the combined amplification mix was added to the DNA samples and PCR was performed according to a series of standard incubation cycles: 15 minutes at 95°C; 10 cycles of 30 seconds at 95°C and 2 minutes at 65°C; 20 cycles of 25 seconds at 95°C, 40 seconds at 50°C and 40 seconds at 70°C and a final 8 minutes at 70°C. Samples were mixed with hybridisation buffer in custom-designed wells of a Twincubator. A test-strip was added to each well and incubated for 15 minutes at 45°C to allow hybridisation of DNA from the sample to specific oligonucleotide probes on the strip. All solutions were completely aspirated, test-strips were washed twice with distilled water and colourimetic detection of hybridised amplicons was obtained by addition of a streptavidin alkaline phosphatase conjugate.

Interpretation of test-strip results was based on assessment of 27 oligonucleotide reaction zones (bands) including six controls, twelve *rpoB* loci (8 wild-type [WT] and 4 mutant [MUT] probes), three *katG* loci (1 WT and 2 MUT probes) and five *InhA* loci (1 WT and 4 mutant probes). Test-strips containing only WT bands were reported as sensitive, whilst resistance was defined as the presence of MUT bands with or without the simultaneous absence of the complementary WT. Only bands with at least equivalent intensity to the amplification control were considered positive.

3.2.2 HIV testing procedures

The HIV status of all study participants was confirmed at baseline. Patients who had written evidence of a positive HIV test from a recognised clinic and were already taking ART were regarded as infected. All other patients were asked to undergo an HIV test.

According to national guidelines a serial rapid testing algorithm was followed using point-of-care rapid HIV test kits on whole blood. After pre-test counselling, a finger prick blood sample was tested on a Determine HIV 1/2 kit (Abbot Diagnostic Divison, Hoofddorp, The Netherlands). If negative, the patient was given the result. If positive, the Determine result was confirmed using a Unigold HIV 1/2 kit (Trinity Biotech Inc, Wicklow, Ireland). Discordant Determine and Unigold results were resolved via a third finger prick test using a Bioline HIV 1/2 kit (Standard Diagnostics Inc., Kyonggi-do, South Korea). All test kits have previously published sensitivity and specificity of 99-100%[501-503].

Post-test counselling was provided to all patients. Those found to be HIV-infected were referred to ART clinic at QECH or their nearest health centre.

3.2.3 Vitamin D laboratory method

Serum samples from baseline, S4 and EOT visits were stored at -70^{o}C, shipped to the UK and analysed in the Clinical Biochemistry laboratory at the Royal Liverpool University Hospital according to their standard operating procedure. Briefly, 25 (OH) D_2 and D_3 were extracted from the samples using a zinc sulphate and acetonitrile containing 25 (OH) D_3 internal standard as a precipitant. The samples were mixed vigorously by vortexing and centrifuged to obtain supernatant. Analysis was done by reverse phase liquid chromatography coupled to a tandem mass spectrometer in electrospray ionization positive mode. Identification and quantification of 25 (OH) D_2 and D_3 metabolites was based on multiple reaction monitoring of the specific mass transition for each target analyte. The mass transitions were; 25 OH-D_3: 401m/z to 383m/z, 25 OH-D_2: 413 m/z to 383 m/z, internal standard: 407 m/z to 371 m/z. Total serum (OH) D levels were taken as the sum of (OH) D_2 and (OH) D_3 at each time-point of analysis.

Holick's definitions of Vitamin D status were used[504] (hypovitaminosis D if 25 (OH) D ≤75nmol/l, hypovitaminosis D; vitamin D deficiency if ≤50 nmol/l, and severe Vitamin D deficiency if ≤25 nmol/l).

3.2.4 Chest radiography

A standard full size postero-anterior CXR was performed at baseline in the radiology department of QECH. To grade the amount of affected lung, visual estimation of the extent of opacification, cavitation or other pathology was expressed as a percentage of visible lung. This assessment was based on the proportion of observed lung-field which looked abnormal and the density of opacification in abnormal areas. The presence of absence of specific features was also noted; namely cavities <4cm diameter, cavities ≥4cm diameter, consolidation, effusions, nodules, fibrosis, miliary shadowing and hilar lymphadenopathy.

All CXRs were blinded before analysis and interpreted independently by a clinician (the study PI) and an external radiologist. Concordance between each element of assessment was reviewed, discrepancies were resolved by consensus and only elements where there was substantial agreement were used for analysis of factors influencing treatment response.

3.2.5 Statistical analysis

Description of baseline characteristics was done for all eligible study participants. Methodology and detailed results of quantitative culture techniques will be described in Chapter 4 and Chapter 6 but all patients were sputum culture positive at baseline.

Persistently positive cultures for *M tuberculosis* using either technique at 2 months was used as an early marker of poor treatment response, and was assessed for all patients who finished intensive phase therapy. Treatment outcome definitions described Section 2.7 were used to classify final clinical outcomes for all patients who finished follow-up or reached a recognised end-point prior to this time.

Summary statistics were used to describe characteristics of study patients. Where proportions of patients were compared according to continuous variables, Wilcoxon's test was used and where assessment was done according to categorical variables a chi-squared test was performed, unless the number of patients in any category was <5, in which case Fisher's exact test was used. Differences between categorical variables with more than two categories were assessed by the Kruskal Wallis test.

For serially sampled continuous measurements, differences in values between time-points were analysed by 2-sample paired Wilcoxon tests. When analysing 25 (OH) D levels, total exposure to 25 (OH) D during TB treatment was taken to be the area under the serum concentration-time plot for each patient. This was calculated using the "auc" function from the Epicalc package in 'R' version 2.15.2.

Assessment of inter-reader agreement in CXR analysis was done using Lin's concordance co-efficient (ρ_c) and Bland and Altman 95% limits of agreement for the continuous variable (total amount (%) of lung affected). Agreement for dichotomous variables was assessed using the kappa statistic. Prevalence-adjusted, bias adjusted kappa values were calculated according to the method described by Byrt[505] and kappa values were interpreted according to the guidelines of Landis and Koch[506] (kappa ≤0.00, poor; 0.00-0.20, slight; 0.20-0.40, fair; 0.40-0.60, moderate; 0.60-0.80, substantial; 0.81-1.00, almost perfect).

Multiple linear regression was used to assess factors associated with 25 (OH) D levels and percentage of lung affected on CXR. Multiple logistic regression was used to assess factors associated with CXR cavitation, and to assess relationships between the variables described in this chapter and 2 month sputum culture status or final treatment outcome. For multivariate linear and logistic regression, all variables with $p<0.10$ on univariate analysis were included in the multivariate model. Results of logistic regression were expressed as OR with 95% Confidence Intervals (CI).

3.3 Results: patient recruitment, follow-up and outcomes

Patient participation in the study is summarised in Figure 3.2.

3.3.1 Enrolment and retention

Between June 2010 and December 2011, 287 patients were screened. 247 were eligible and 182 were recruited. 8 recruited patients were subsequently found to be ineligible and 5 withdrew consent prior to baseline sampling, leaving 169 participants.

85/247 (35%) eligible patients were female and women were more likely to decline enrolment. Of 32/85 (38%) women who declined participation, 10 felt unable to consent without consulting their spouse. By comparison, 38/154 (25%) men declined and none cited the need for family consultation. The relative risk of declining to participate in women was 1.53 (95% CI: 1.03-2.25). This resulted in recruitment of a gender imbalanced cohort of 116 (69%) men and 53 (31%) women.

Of the 169 participants, one failed therapy (died of TB[a]) and 22 were withdrawn during the first 8 weeks, leaving 146 active patients at the end of intensive phase TB therapy. Four withdrew during the continuation phase and 10 withdrew during post-treatment follow-up. Although total withdrawals accounted for 36/169 (21%) of participants after treatment initiation, only 15/169 (9%) were "lost to follow-up and untraceable", suggesting that better patient retention would be difficult to achieve. Economic difficulties in Malawi throughout the study including severe, protracted fuel shortages presented persistent obstacles to clinic attendance and patient tracing.

3.3.2 Baseline drug susceptibility testing

No rifampicin resistance was identified using the line probe assay on *M tuberculosis* isolates from study participants. No patient was withdrawn from the study because of MDR TB at baseline. Three samples demonstrated evidence of isoniazid resistance via S315T1 substitution mutations on the *katG gene* (representative test-strip in Figure 3.3A). No isoniazid mono-resistant patient failed therapy; one was transferred out of area during the intensive phase and the other two obtained favourable outcomes (one cure and one treatment completion) with no disease relapse during follow-up.

[a] According to outcome definitions from Section 2.4, deaths which were definitely or probably attributable to TB were regarded as failures, whilst deaths due to other causes were regarded as withdrawals. Judgement on causes of death was made by two doctors (the study PI and an independent physican.

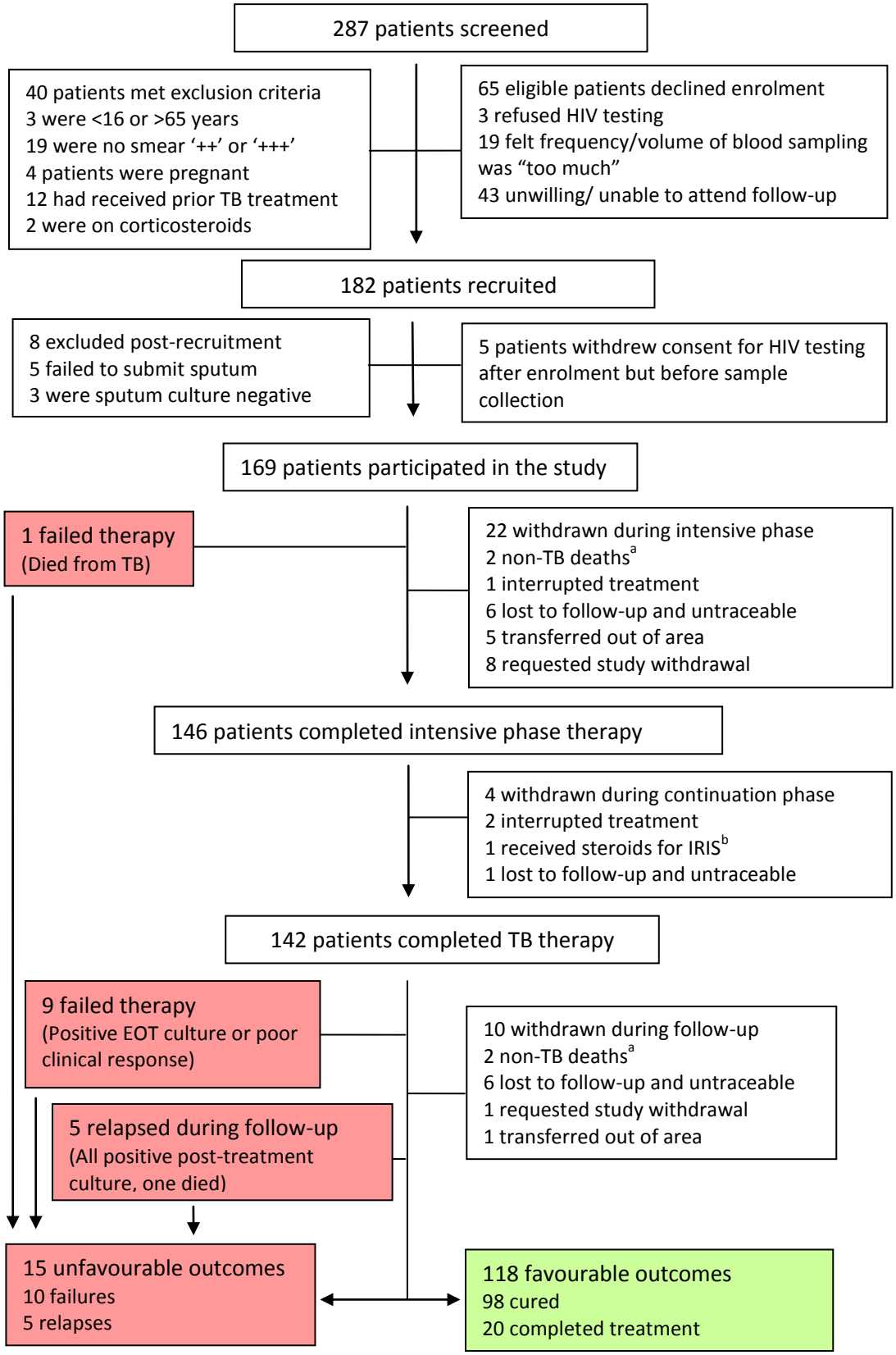


Figure 3.2 Patient screening, recruitment and follow-up

[a] Non-TB deaths discussed in Section 3.4.3: one from jaundice due to drug toxicity (Figure 3.3B)
[b]IRIS=Immune Reconstitution Inflammatory Syndrome affected 2 patients. One was treated with corticosteroid therapy and was withdrawn from the study (Figure 3.3.C)

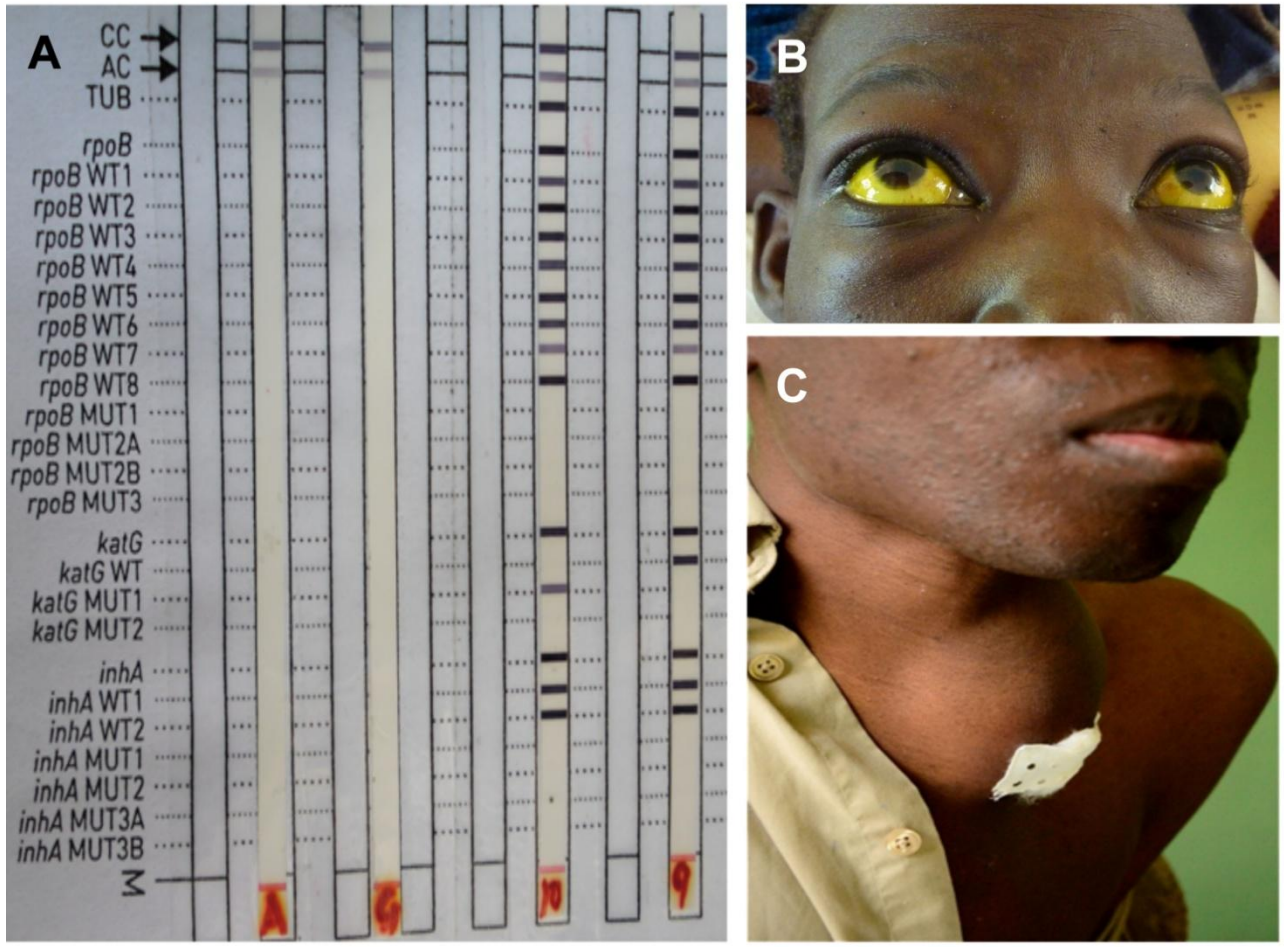


Figure 3.3 Baseline genotypic resistance testing and clinical events on TB treatment

A: Resistance patterns on Genotype MDRTB*plus* 2.0 test-strips. Samples were prepared and colourimetric bands on test-strips developed according to standard protocols from Hain Life Sciences, Germany.Lanes A&G show negative controls for the PCR Amplification mix and Genolyse respectively. Lane 9 shows *M tuberculosis* susceptible to both isoniazid and rifampicin. Lane 10 shows isoniazid mono-resistance due to a *katG* (S315T1) mutation.

B: Jaundice as a consequence of drug toxicity, necessitating study withdrawal.

C: Cervical lymphadenopathy and “cold” abscess in the neck due to Immune Reconstitution Inflammatory Syndrome; treated by repeated aspiration of pus from the neck and prescription of oral corticosteroids, necessitating study withdrawal.

3.3.3 Treatment Outcomes

All patients were sputum culture positive on either MGIT or SSCC at baseline. By two months 44/146 (30%) were culture negative whilst 86/146 (59%) remained culture positive by at least one technique. Two month cultures were unavailable for 16/146 (11%) patients because of contaminated S4 samples.

118/133 (89%) patients had favourable treatment outcomes (98 cured and 20 successfully completed follow-up but did not submit sputum for EOT or post-treatment culture). 15/113 (11%) had unfavourable outcomes (10 failures and 5 relapses, see Table 3.1).

For 13/15 (87%) unfavourable outcomes, the diagnosis of treatment failure or relapse was microbiologically confirmed by culture positive EOT or post-treatment sputum samples. Of the two unfavourable outcomes without confirmatory microbiology, one patient (ID: 182) died due to respiratory deterioration in the first week of therapy. No alternative diagnosis was made and he was deemed to have died of tuberculosis. The other (ID: 93) was adherent to TB treatment for 6 months. Although her purulent cough resolved she had persistent cachexia and lymphadenopathy. A lymph node biopsy did not reveal an alternative diagnosis and she was classified as a treatment failure by the study PI and an independent physician. She made a good initial response to a re-treatment TB regimen but her long-term outcome is unknown as she was withdrawn from the study.

All patients with unfavourable outcomes were originally infected with fully drug susceptible *M tuberculosis* according to the baseline line probe assay. Repeat genotypic susceptibility testing was done on the 13 positive EOT or post-treatment isolates. Fully drug susceptible *M tuberculosis* was still identified in 11 (85%), suggesting that unfavourable outcomes were attributable to persistence of drug-susceptible TB. One patient had developed isoniazid monoresistance and one had developed MDR TB. Although spoligotyping was unavailable, all bacteriologically confirmed TB recurrences occurred within 6 months of completing therapy suggesting that relapse was more likely that re-infection.

All failures and relapses were referred back to the NTP for further management. In most cases this comprised standard therapy with WHO approved regimen 2, an 8 month treatment regimen including injectable streptomycin for the first 8 weeks[460]. One relapsed patient (ID: 50) died before re-treatment could be initiated.

ID	Sex	Age (yrs)	HIV status	BL Line Probe Assay			2 month culture	Clinical indication of unfavourable outcome	EOT/PT culture	EOT/PT Line Probe Assay		
				Species	*RIF*	*INH*				*Species*	*RIF*	*INH*
10 patients failed therapy												
5	F	34	Infected	*Mtb*	*S*	*S*	Negative	Still producing purulent sputum at EOT	EOT positive	*Mtb*	*S*	*S*
15	M	47	Infected	*Mtb*	*S*	*S*	Negative	Still producing purulent sputum at EOT	EOT positive	*Mtb*	*S*	*S*
54	M	28	Infected	*Mtb*	*S*	*S*	Positive	Still producing purulent sputum at EOT	EOT positive	*Mtb*	*S*	*S*
84	M	28	Non-infected	*Mtb*	*S*	*S*	Positive	Still producing purulent sputum at EOT	EOT positive	*Mtb*	*S*	*S*
93	F	42	Infected	*Mtb*	*S*	*S*	N/A	Clinical deterioration and lymphadenopathy No EOT bacteriology, improved on TB regimen 2[a]	NA	*NA*	*NA*	*NA*
121	F	27	Infected	*Mtb*	*S*	*S*	Positive	Still producing purulent sputum at EOT	EOT positive	*Mtb*	*S*	*S*
145	F	27	Infected	*Mtb*	*S*	*S*	Negative	Still producing purulent sputum at EOT	EOT positive	*Mtb*	*S*	*S*
151	M	30	Infected	*Mtb*	*S*	*S*	Negative	Still producing purulent sputum at EOT	EOT positive	*Mtb*	*R*[b]	*R*[c]
155	M	30	Non-infected	*Mtb*	*S*	*S*	Negative	Still producing purulent sputum at EOT	EOT positive	*Mtb*	*S*	*S*
182	M	39	Infected	*Mtb*	*S*	*S*	N/A	Died during first week of therapy attributed to TB	NA	*NA*	*NA*	*NA*
5 patients relapsed after successful cure/treatment completion												
46	F	26	Infected	*Mtb*	*S*	*S*	Negative	Cured (EOT culture negative) but relapse of cough	PT positive	*Mtb*	*S*	*S*
50	F	61	Infected	*Mtb*	*S*	*S*	Negative	Cured (EOT culture negative) but recurrent fever, purulent cough, cachexia, death attributed to TB	PT positive	*Mtb*	*S*	*S*
58	F	30	Infected	*Mtb*	*S*	*S*	Positive	Cured (EOT culture negative) but recurrent cough and haemoptysis, improved on TB regimen 2[a]	PT positive	*Mtb*	*S*	*R*[c]
73	M	21	Non-infected	*Mtb*	*S*	*S*	Positive	Cured (EOT culture negative) but persistent cough	PT positive	*Mtb*	*S*	*S*
75	M	36	Infected	*Mtb*	*S*	*S*	Positive	Cured (EOT culture negative) but persistent cough	PT positive	*Mtb*	*S*	*S*

Table 3.1 'S.P.U.Tu.M.' study patients with unfavourable outcomes

Abbreviations; BL= baseline, EOT=End of treatment, PT=Post-treatment, *Mtb=Mycobacterium tuberculosis*, *RIF=Rifampicin, INH=Isoniazid, S=Sensitive, R=Resistant.*

[a]TB regimen 2 is the WHO approved protocol for re-treatment cases used in Malawi (8 months of therapy, including injectable streptomycin during the first 2 months[460]

[b]Mutation detected on *rpoB* conferring resistance to rifampicin

[c]Substitution mutation detected on *katG* (S315T1) conferring resistance to isoniazid

3.4 Results: Description of the study cohort

3.4.1 Socioeconomic profile

To contextualise socioeconomic features of the cohort, patient characteristics were compared with the general urban Malawian population using data from the 2010 Demographic Health Survey (DHS)[456]. Some characteristics were compared at the level of patient household, whilst others were compared at the individual level (Tables 3.2 and 3.3). To avoid confounding by gender, individual features were analysed separately for men and women.

There were no significant differences at household level between study patients and the general urban population except that study patients were less likely to use biomass fuel for cooking (70% vs. 90%, p<0.001). This is slightly surprising in view of previously described associations between biomass fuels and tuberculosis[507,508]. Of the 117 patients who used biomass at home 98 (84%) cooked inside the house and 19 (11%) cooked outside.

At the individual level, study participants were less likely to be able to read from a newspaper than the general population (Men: 72 vs. 91%, p<0.001, Women: 68 vs. 89%, p<0.001). Male study participants were also less likely to have ever attended school or received a secondary/tertiary education (p<0.001).

Obtaining enough food in the past month was a problem for 20% of men and 19% of women in the study but comparative data was unavailable from the DHS.

There were differences in employment patterns between study participants and the general population; study patients most commonly described their usual occupation as “unskilled manual labour/domestic service” (men) or “no employment for at least 12 months” (women). By contrast, “clerical/service/sales” work was most common in the general population for both genders (p<0.001).

Finally, study patients were more likely to have ever smoked tobacco than the general population (Men: 34 vs 10%, p<0.001, Women: 4% vs 0%, p=0.03) although absolute numbers are small.

Viewed collectively, these data suggest that smear positive TB patients enrolled to the study were less educated, more likely to have low/no salary and more likely to have ever smoked than urban Malawian adults in general.

	Study cohort N=169	Urban Malawian households N=4116[a]	p-value[b]
Number of people in household (median, range)	4 (1-10)	4 (1-9)	0.823
Female head of household (n, %)	32 (19)	852 (21)	0.647
Motorised vehicle owned by anyone in household (n, %)	16 (10)	354 (9)	0.800
In the past 2 weeks an adult at home missed a meal to ensure adequate food for children (n, %)	13 (8)	N/A	-
Electricity available at home (n, %)	65 (39)	1428 (35)	0.355
Cooks using biomass fuel (charcoal/wood) (n, %)	117 (70))	3683 (90)	<0.001*

Table 3.2 Household profile of study participants

	Study cohort	Urban Malawian adults[a]	p-value[b]
Male patients	**N=116**	**N=1440**	**-**
Able to read from a newspaper (n, %)	83 (72)	1326 (92)	<0.001*
Highest level of education (n, %)			<0.001*
Never attended school	8 (7)	24 (2)	
Primary school education only	45 (39)	556 (39)	
Secondary/tertiary education	63 (54)	859 (60)	
Difficulty obtaining enough food in past month (n, %)	23 (20)	NA	-
Usual occupation (n, %)[c]			<0.001*
Agriculture	11 (10)	124 (9)	
Clerical/services/sales	8 (8)	381 (27)	
Professional/technical	12 (11)	114 (8)	
Skilled manual	17 (16)	365 (25)	
Unskilled manual/domestic service	41 (38)	189 (13)	
No employment for at least 12 months	18 (17)	267 (19)	
Ever smoked tobacco (n, %)	39 (34)	144 (10)	<0.001*
Ever drinks alcohol (n, %)	63 (54)	NA	-
Female patients	**N=53**	**N=4302**	**-**
Able to read from a newspaper (n, %)	36 (68)	3566 (83)	0.007*
Highest level of education (n, %)			0.577
Never attended school	3 (6)	301 (7)	
Primary school only	22 (42)	2037 (47)	
Secondary/tertiary education	28 (53)	1964 (46)	
Difficulty obtaining enough food in past month (n, %)	10 (19)	NA	-
Usual occupation (n, %)			<0.001*
Agriculture	7 (13)	428 (10)	
Clerical/services/sales	5 (9)	1514 (57)	
Professional/technical	3 (6)	162 (6)	
Skilled manual	1 (2)	199 (8)	
Unskilled manual/domestic service	9 (18)	353 (13)	
No employment for at least 12 months	25 (50)	1645 (38)	
Ever smoked tobacco (n, %)	2 (4)	21 (0.0)	0.031*
Ever drinks alcohol (n, %)	3 (6)	NA	-

Table 3.3 Individual socio-economic profile of study participants

[a] Reference data on urban Malawian households/adults from Demographic Health Survey 2010
[b] Number of people in household compared by Wilcoxon's test, all other variables compared by chi-squared test unless <5 patients any category, in which case Fisher's Exact test was used
[c] For usual occupation, N=107 for males and N=50 for females

3.4.2 HIV profile

Baseline HIV parameters of study participants

Data on the HIV status of study participants are shown in Table 3.4.

Prior to enrolment, 112 (66%) participants had previously tested for HIV and 67 (40%) were known to be infected. During baseline assessment, 31 further individuals (26 with no previous test and 5 who had previously tested negative) were diagnosed with HIV infection for the first time. In total 98/169 (58 %) study participants were HIV infected and 71/169 (42%) were HIV negative.

Baseline CD4 count results were available for 84 HIV infected patients, the median result was 166 (range: 6-783) cells/µl. 8 (10%) patients had a CD4 count <50 cells/µl, 38 (45%) had 50-200 cells/µl, 20 (24%) had 200-350 cells/µl and 18 (21%) had >350 cells/µl.

Current Malawian guidelines recommend ART initiation for non-TB patients at CD4 counts <350 cells/µl[465]. Only 27 study participants were on ART prior to TB diagnosis; representing 28% of those who were HIV-infected and 31% of those with an eligible CD4 count before TB diagnosis. Sub-optimal levels of pre-study ART reflect the high frequency of severe immunosuppression in the cohort and the challenges to provision of HIV care confronting the national ART programme.

There were no differences in HIV parameters between male and female study participants.

	Male N=116	Female N=53	**Total N=169**
Pre-enrolment awareness of HIV status and baseline testing			
Ever HIV tested prior to study enrolment (n, %)	72 (62)	40 (76)	**112 (66)**
Known to be HIV infected prior to enrolment (n, %)	45 (39)	22 (42)	**67 (40)**
First positive HIV test at baseline assessment (n, %)	20 (17)	11 (21)	**31(18)** [a]
Confirmed HIV infection	65 (56)	33 (62)	**98 (59)**
Baseline CD4 count of HIV-infected individuals			
Baseline CD4 count, cells/µl (median, range)[b]	163 (6-688)	168 (31-783)	**166 (6-783)**
CD4 count <50 cells/µl (n, %)[b]	4 (7)	4 (15)	**8 (10)**
CD4 count 50-200 cells/µl (n, %)[b]	27 (47)	11 (41)	**38 (45)**
CD4 count 200-350 cells/µl (n, %)[b]	15 (26)	5 (16)	**20 (24)**
CD4 count >350 cells/µl (n, %)[b]	11 (19)	7 (26)	**18 (21)**
Pre-enrolment HIV treatment			
On ART at enrolment (n, %)[c]	16 (25)	11 (33)	**27 (28)**

Table 3.4 Baseline HIV parameters of 'S.P.U.Tu.M.' study participants

[a] 26 had never previously been tested, 5 had previously tested HIV negative
[b]Percentages use 84 (57 male, 27 female) patients with baseline CD4 results as the denominator
[c]Percentages use 99 (65 male, 33 female) HIV infected individuals as the denominator

ART initiation in HIV-infected patients

Table 3.5 shows timing of ART initiation and choice of regimen amongst all HIV-infected study recruits and the subset who reached a final study outcome.

71/98 (72%) HIV-infected recruits and 64/76 (84%) who reached an outcome were on ART by study exit, representing increases of 45% and 51% respectively from baseline.

The commonest timing of ART initiation for patients not on therapy at baseline was during continuation phase TB treatment. 90% of patients used stavudine, lamivudine and nevirapine as their sole regimen, reflecting that most patients were managed under the 2008 guidelines. One was recruited on zidovudine, lamivudine and nevirapine and continued this until end of follow-up. Three (all recruited after September 2011) were initiated on tenofovir, lamivudine and efavirenz. Three patients originally on stavudine, lamivudine and nevirapine were switched to tenofovir, lamivudine and efavirenz during the study because of severe peripheral neuropathy.

		All study recruits[a] N=169	Patients reaching final study end-point[b] N=133
HIV-infected (n, %)		98 (58)	76 (57)
Timing of ART intiation	Never (n, %)	27 (28)	12 (16)
	On ART at baseline (n, %)	27 (28)	25 (33)
	During intensive phase (n, %)	16 (16)	12 (16)
	During continuation phase (n, %)	19 (19)	18 (24)
	During follow-up (n, %)	8 (9)	9 (12)
ART regimen	d4T, 3TC, NVP (n, %)	64 (90)	57 (89)
	AZT, 3TC, NVP (n, %)	1 (1)	1 (2)
	TDF, 3TC, EFV (n, %)	3 (4)	3 (5)
	Started d4T, 3TC, NVP then switched to TDF, 3TC, EFV (n, %)	3 (4)	3 (5)

Table 3.5 ART initiation and regimens of study patients

d4T: stavudine, 3TC: lamivudine, NVP: nevirapine, EFV: efavirenz, TDF: tenofovir
[a]ART initiation data collected until the date of withdrawal or a study end-point was reached
[b]ART initiation data collected until a study end-point was reached

48 HIV-infected study patients had CD4 counts measured at baseline and EOT. CD4 counts amongst this group increased by a median of 71 (range:-232 to 456) cells/µl. Those who initiated during intensive phase TB therapy experienced a median CD4 count rise of 146 (40-508) cells/µl, whilst those who received no ART at all (or delayed until TB treatment was finished) experienced a median change of -12 (range: -232 to 432) cells/µl

3.4.3 Clinical patient assessment

Baseline assessment

Baseline clinical and laboratory characteristics of study patients are shown in Table 3.6. Results are sub-divided by HIV status to establish whether illness severity and baseline was influenced by immunosuppression.

HIV-infected individuals were older than those without HIV-infection (p<0.001) and more frequently had lymphadenopathy (p=0.007). However, the majority of study participants reported "fair" or "good" general health and assessment of functional status baseline BMI and vital signs suggested no difference in illness severity due to HIV infection. There were also no differences in duration of illness or healthcare seeking behaviour. Patients had been unwell for a median of 8 weeks (range 1-28) and 147/169 (87%) had received routine antibiotics. The number of prior antibiotic courses ranged from 1-8, suggesting that some earlier diagnoses may have been possible but exploration of this was beyond the scope of the study. Only 8 (5%) had consulted a traditional healer prior to presentation to allopathic health services.

8/169 (5%) study participants reported co-existing illnesses other than HIV and 2/99 (2.0%) HIV-infected patients had concurrent opportunistic infections (one case each of oesophageal candidiasis and cryptococcal meningitis). Non-communicable diseases were rare with only one case of diabetes mellitus and (11%) patients recording a blood pressure in the hypertensive range (>140/80mmHg).

Haemoglobin levels were low overall. 126/169 (75%) patients fulfilled the WHO definition of anaemia. HIV infection was associated with lower haemoglobin levels (p<0.001) and a higher incidence of anaemia (p=0.057). Serum ALT was also higher amongst HIV-infected individuals (median: 21 [range: 7-160] vs 17 [range: 5-190], p=0.037).

Attendance at study visits and treatment adherence

581 study visits (S1-S4) were arranged for the 146 participants who completed intensive phase TB treatment. 384 (66%) visits were conducted on the day specified by the study protocol, 189 (32%) were conducted on a different day (median deviation from intended date: 1 day late (range: 4 days early to 43 days late) and 10 (2%) were missed.

Patients were asked about adherence to TB treatment at each clinic visit. Amongst the 143 patients who finished therapy, 123 (87%) reported no missed doses, 12 (9%) reported 1-2 missed doses and 7/143 (5%) reported ≥3 missed doses.

	Total N=169	HIV-infected N=98	HIV-uninfected N=71	p-value[a]
Baseline visit clinical & demographic parameters				
Male sex (n, %)	116 (69)	65 (66)	51 (72)	0.553
Age (median, range)	30 (17-61)	33 (20-61)	27 (17-55)	<0.001*
Self-reported health (n, %)				0.255
Excellent	12 (7)	5 (5)	7 (10)	
Good	65 (39)	35 (35)	30 (44)	
Fair	89 (53)	57 (58)	32 (47)	
Poor	1 (1)	1 (1)	0 (0)	
WHO performance status (n, %)				0.356
0 – Fully active	157 (94)	89 (91)	68 (97)	
1 – Able to perform light work	9 (5)	7 (7)	2 (3)	
2 – Ambulatory but unable to work	2 (1)	2 (2)	0 (0)	
Neck swellings/lymphadenopathy	19 (11)	17 (17)	2 (3)	0.007*
Symptom duration (median, range)	8 (1-28)	8 (1-28)	6 (1-24)	0.105
BCG vaccinated (n, %)	135 (80)	77 (79)	58 (83)	0.622
Recent antibiotic used (n, %)	147 (87)	86 (88)	61 (86)	0.905
Consulted traditional healer (n, %)	8 (5)	6 (6)	2 (3)	0.479
Baseline co-morbidities (other than HIV)				
Any co-existing illness (n, %)[b]	8 (5)	5 (5)	3 (4)	1.000
Diabetes mellitus (n, %)[c]	1	0	1	NA
Fasting glucose (median, range)	4.8 (3.6-6.7)	4.8 (4.0-6.7)	4.9 (3.6-6.4)	0.285
Body mass index (BMI) & vital signs				
BMI, kg/m^2 (median, range)	18.4 (13.2-29.3)	18.4 (13.2-29.3)	18.4 (14.5-25.0)	0.633
Pulse, beats/min (median, range)	105 (39-148)	105 (39-148)	105 (58-147)	0.801
Systolic BP, mmHg (median, range)	113 (77-160)	111 (90-146)	117 (77-160)	0.015*
Patients with hypertension[d]	19 (11)	9 (13)	10 (10)	0.794
RR, breaths/min (median, range)	30 (12-81)	30 (12-81)	30 (12-44)	0.394
Temperature, ^{o}C (median, range)	36.5 (32.9-39.6)	36.5 (32.9-39.6)	36.6 (33.5 -39.5)	0.736
Baseline lab results				
Haemoglobin, g/dl (median, range)	10.9 (5.9-18.7)	10.1 (6.0-18.7)	11.5 (5.9-15.7)	<0.001*
Patients with anaemia (n, %)[e]	126 (75)	77 (89)	49 (73)	0.025*
WCC, x10^9 cells/l (median, range)	6.5 (1.4-21.4)	5.9 (1.4-21.4)	6.9 (3.2-14.0)	0.032
Platelets x10^9 cells/l (median, range)	340 (44-922)	330 (62-909)	365 (44-922)	0.224
Serum bilirubin, IU/l (median, range)	8 (1-50)	7.8 (1-50)	8 (3-39)	0.185
Patients with high bilirubin (n, %)[f]	7 (4)	3 (3)	4 (6)	0.448
Serum ALT, IU/l (median, range)	20 (5-190)	21 (7-160)	17 (5-190)	0.037*
Patients with high ALT (n, %)[g]	30 (18)	22 (22)	8 (11)	0.106
Serum creatinine, IU/l (median, range)	59 (29-117)	61 (29-117)	57 (31-100)	0.126
Patients with high creatine (n, %)[h]	59 (35)	6 (6)	1 (1)	0.242

Table 3.6 Baseline clinical assessment of 'S.P.U.Tu.M' study patients

[a]Wilcoxon's test for continuous variables, chi-squared or Fisher's Exact test for categorical variables
[b]Co-existing illnesses were: genital warts (1), diabetes mellitus (1), migraine (1), oesophageal candidiasis (1), asthma (1), cryptococcal meningitis (1), pneumonia (1) and urinary tract infection (1)
[c]WHO definition of diabetes mellitus includes fasting blood glucose >7.0µmol/l. No patients fulfilled this criterion; the sole diabetic was already on oral hypoglycaemic therapy.
[d]WHO definition of hypertension is systolic BP>140mmHg or diastolic BP>90mmHg.
[e]WHO definition of anaemia in adults is haemoglobin <13 g/dl (men) and <12 g/dl (women)
[f]High bilirubin is >25IU/l, [g]High ALT is >35 IU/l, [h]High creatinine is >98 µmol/l
*Denotes significance at p<0.05

Assessment during treatment

The general trend was towards clinical improvement at serial visits. Although only 12/169 (7%) patients reported "excellent" general health at baseline by S4 visit this figure had risen to 97/146 (66%) and by EOT it was 114/143 (80%). The BMI of study participants rose by a median of 0.7 (range: -3.5 to 4.9) kg/m^2 between baseline and S4 visits and by 1.6 (range: -3.7 to 6.0) kg/m^2 over the entire duration of therapy (Figure 3.4A). The median rise in BMI was greater amongst HIV-infected than non-infected individuals (1.8 vs. 1.3 kg/m^2, Wilcoxon test p-value=0.018), perhaps due to the additional effect of ART (Figure 3.4B).

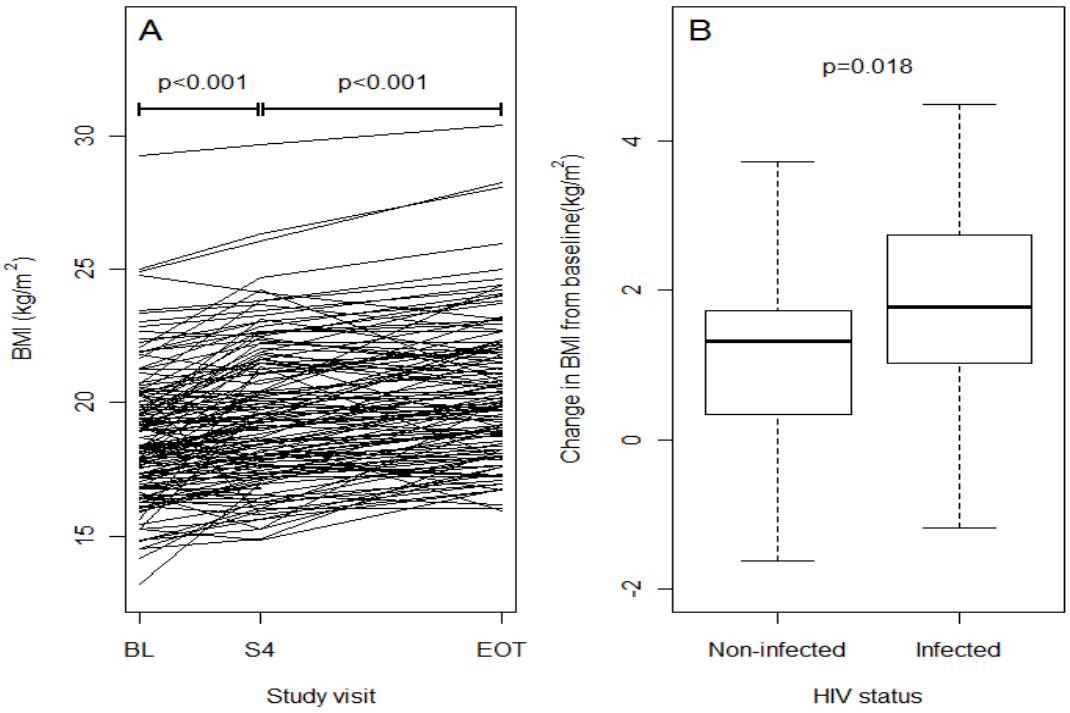


Figure 3.4 Change in BMI during TB therapy

A: BMI at baseline, S4 visit and EOT. Differences between time-points analysed by paired Wilcoxon test. B: Differences in change in BMI during TB therapy divided by HIV-status, analysed by two sample Wilcoxon test.

Inter-current illnesses

23/169 (14%) recruited patients had an inter-current illness during TB treatment (Table 3.7). 18 had super-added infections whilst 6 had non-infectious conditions. Infections were more common in HIV-infected individuals (15 [10%] vs. 3 [4%] p=0.040). 9 HIV-infected patients developed new WHO Stage III or IV conditions (4 severe bacterial infections [pneumonia or meningitis], 3 varicella zoster eruptions and one case respectively of *Pneumocystis jeroveci* pneumonitis [PCP] and cerebral toxoplasmosis).

	Total N=169	HIV-infected N=98	HIV-uninfected N=71	p-value[a]
Any inter-current illness (n, %)	23 (14)	17 (17)	6 (9)	0.151
Infections	18 (11)	15 (15)	3 (4)	0.040
Diarrhoea/Gastroenteritis[b]	5 (3)	3 (0)	2 (3)	-
Malaria	3 (2)	2 (2)	1 (1)	-
Pneumonia	3 (2)	3 (4)	0 (0)	-
Varicella Zoster Virus (shingles) eruption	3 (2)	3 (3)	0 (0)	-
Bacterial meningitis	1 (1)	1 (1)	0 (0)	-
Herpes Simplex Virus-2 genital ulcers	1 (1)	1 (1)	0 (0)	-
Pneumocystis jeroveci pneumonitis[c]	1 (1)	1 (1)	0 (0)	-
Cerebral toxoplasmosis[d]	1 (1)	1 (1)	0 (0)	-
Non-infectious illnesses	5 (3)	2 (2)	3 (4)	0.651
Haemorrhoids	1 (1)	1 (1)	0 (0)	-
Peptic Ulcer Disease	1 (1)	0 (0)	1 (1)	-
New hypertension	1 (1)	0 (0)	1 (1)	-
DVT	1 (1)	1 (1)	0 (0)	-
Pelvic Inflammatory Disease	1 (1)	0 (0)	1 (1)	-

Table 3.7 Inter-current illnesses during TB treatment

[a]Analysed by chi-squared test or Fisher's Exact test
[b]≥3 loose stools within a 24 hour period
[c]Typical CXR appearance and response to high dose co-trimoxazole therapy
[d]Hemiparesis, CD4 count: 6 cells/µl, typical Magnetic Resonance brain images and response to co-trimoxazole therapy

Drug toxicity and treatment complications

67 (40%) patients experienced at least one drug-associated adverse event (Table 3.8). 18 (11%) described multiple problems. Drug toxicity was more common in HIV-infected patients but the difference was not statistically significant. Joint pain and swelling was the most common, possibly associated with pyrazinamide. Second was peripheral neuropathy which is associated with isoniazid in the context of Vitamin B6 (pyridoxine) deficiency[509], a recognised feature of HIV-infection, and a side-effect of stavudine based ART[131].

All three patients who developed jaundice had negative blood tests for Hepatitis B surface Antigen and Hepatitis C antibody and a normal hepato-biliary ultrasound scan. One jaundiced patient died (discussed below and see Figure 3.3B). No other patient had treatment discontinued due to drug toxicity.

Two HIV-infected patients developed new cervical lymphadenopathy due to Immune Reconstitution Inflammatory Syndrome (IRIS) after ART initiation during the Continuation Phase of TB therapy. One of these was treated with corticosteroids and withdrawn from the study (see Figure 3.3C).

	Total N=169	HIV-infected N=98	HIV-uninfected N=71	p-value[a]
Any drug toxicity or complications	65 (39)	44 (45)	23 (32)	0.139
Skin rash	20 (12)	13 (13)	7 (10)	0.646
Nausea & vomiting	3 (2)	3 (3)	0 (0)	0.264
Jaundice	3 (2)	3 (3)	0 (0)	0.264
Joint pain and swelling	34 (20)	21 (22)	13 (18)	0.735
Peripheral neuropathy	28 (17)	19 (19)	9 (13)	0.343
New lymphadenopathy (IRIS)	2 (1)	2 (2)	0 (0)	0.510

Table 3.8 Drug toxicity in study patients during TB therapy

[a] Analysis by chi-squared test or Fisher's Exact test

Use of additional medications

52 (31%) patients took non-TB/non-HIV medications during TB treatment. 22 took more than one additional drug. In total, 14 patients took anti-microbials (antibiotics, anti-malarials, fluconazole or aciclovir), 33 took drugs for pain or neuropathy (Non Steroidal Anti-Inflammatory Drugs, amitryptilline or pyridoxine) and 17 took other medicines (chlopheniramine, promethazine, omeprazole, hydrochlorothiazide or sub-cutaneous heparin). One patient took an unidentified traditional remedy.

The only additional medication used with known anti-TB activity was ciprofloxacin, taken during intensive phase of TB therapy by one patient for 5 days. No interactions between additional medications and anti-TB drugs were identified.

Deaths not directly attributed to tuberculosis

6/169 (4%) recruits are known to have died. All were HIV-infected. Two deaths were directly attributed directly to TB and were discussed in Section 3.3.3.

The four non-TB deaths were withdrawn from the study and removed from analysis of final outcomes. Two occurred during Intensive Phase therapy despite sputum smear conversion by 14 days and a good initial response to TB treatment. The first had a baseline CD4 count of 325 cells/µl, did not commence ART and died unexpectedly at home between S3 and S4 visits. The cause of death is unknown. The second had a baseline CD4 count of 106 cells/µl and serum bilirubin of 5 µmol/l. After commencing ART (stavudine, lamivudine and nevirapine) at his S3 visit (28 days) he returned to hospital 3 weeks later with deep jaundice and serum bilirubin of 300 µmol/l. All drugs were stopped but he developed hepatic encephalopathy and died of liver failure, presumed secondary to drug toxicity.

The other two patients died during post-treatment follow-up. Both had low baseline CD4 counts (8 and 53 cells/µl respectively). Despite commencing ART during and achieving TB cure by EOT they died in the community thereafter. As neither patient returned to hospital

during the terminal illness, specific causes of death were unknown. However, both were smear and culture negative at last review and relatives did not describe recurrence of TB symptoms prior to death. It is possible that some unrecorded deaths occurred in patients who were withdrawn from the study, particularly amongst the 13 individuals who defaulted clinic visits and could not subsequently be traced.

3.4.4 Vitamin D levels

Baseline Vitamin D parameters

Baseline 25 (OH) D levels were low with a median of 57.3 (range: 11.8-113.3) nmol/l. 29 (18%) patients had 25 (OH) D levels in the normal range, 72 (43%) had hypovitaminosis D, 47 (28%) had Vitamin D deficiency and 18 (11%) had severe Vitamin D deficiency.

Factors associated with baseline Vitamin D levels

As sunlight is necessary for cutaneous accumulation of Vitamin D_3 it was proposed that seasonal variation in sunlight exposure may have influenced 25 (OH) D levels at baseline. Although Malawi consistently receives 12 hours of sunlight throughout the year, there is an annual "cold season" from May to July. During these months, people often remain indoors and wear additional layers of thicker clothing, reducing sunlight exposure. Figure 3.5 shows that baseline Vitamin D levels varied according to recruitment month and were lowest during and just after the cold season.

A linear regression model was also used identify factors associated with baseline 25 (OH) D levels. On multi-variate analysis recruitment in July/August (p=0.001) or September/October (p=0.001), male sex (p=0.040), difficulty obtaining enough food in the last month (p=0.035), cooking with biomass fuel inside the house (0.044) and low BMI (p=0.047) were also associated with lower levels (Table 3.9).

Viewed collectively, these data suggest that climatic changes and social factors were primarily responsible for variation baseline 25 (OH) D levels.

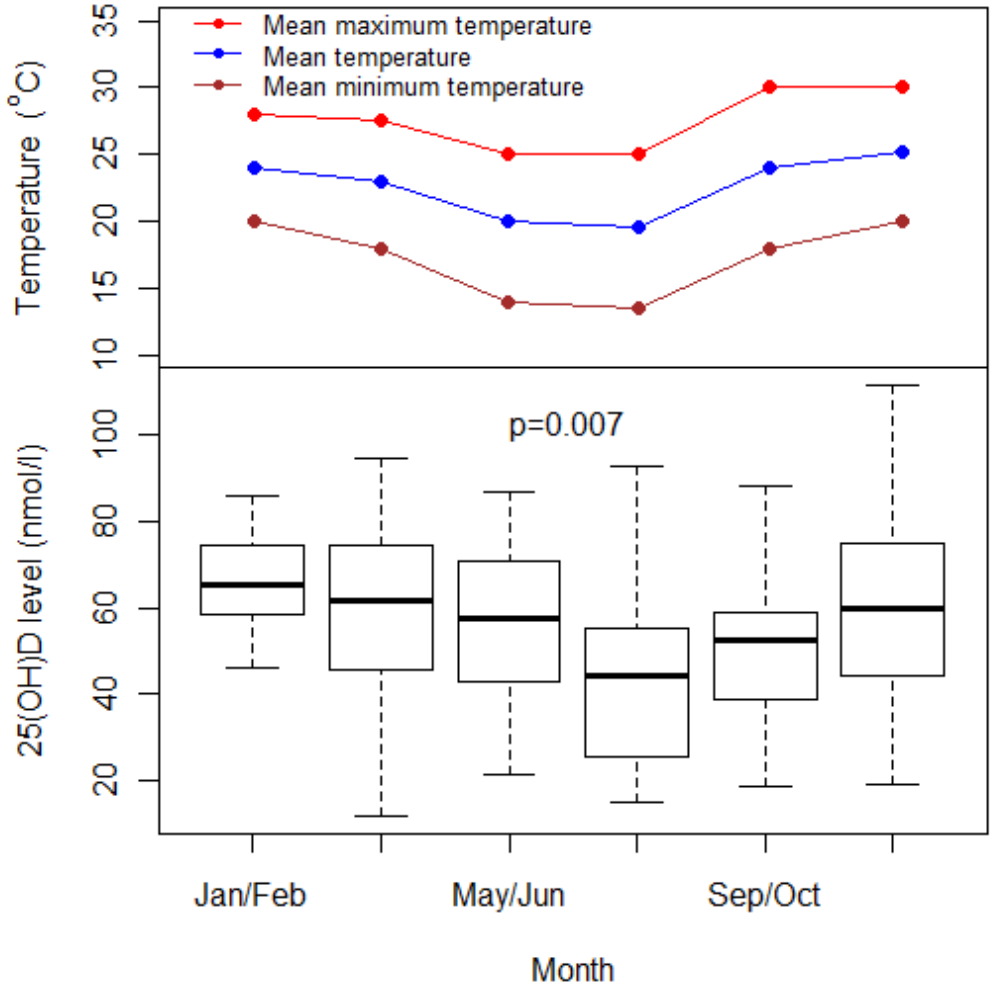


Figure 3.5 Seasonal variation and Vitamin D levels

Difference in baseline 25(OH) level by recruitment month analysed by Kruskal Wallis test (p=0.007)

Variable	Uni-variate analysis			Multi-variate analysis		
	Parameter estimate	Standard error	P-value	Parameter estimate	Standard error	P-value
Age	-0.192	0.199	0.336	-	-	-
Male sex	-6.778	3.729	0.071	-7.284	3.353	0.040*
Food insecurity	-8.415	4.380	0.056	-8.645	4.061	0.035*
Cooks with biomass fuel						
Inside the house	-14.022	3.974	0.020*	-11.677	5.560	0.044*
Outside	-1.131	3.859	0.770	-1.178	3.768	0.755
HIV-infected	4.296	3.560	0.229	-	-	-
CD4 count	0.029	0.016	0.081	-	-	-
On ART at baseline	6.852	5.920	0.250	-	-	-
Body mass index	1.349	0.715	0.061	1.381	0.690	0.047*
Month of recruitment[a]						
March/April	-5.831	6.910	0.399	-6.289	6.607	0.343
May/June	-12.610	6.710	0.062	-11.533	6.376	0.072
July/August	-24.085	6.910	<0.001*	-21.588	6.607	0.001*
September/October	-19.678	6.555	0.003*	-19.437	6.446	0.003*
November/December	-6.803	6.290	0.281	-5.664	6.070	0.035

Table 3.9 Factors influencing baseline 25 (OH) Vit D level

[a]Reference value for parameter estimates of recruitment month is January/February

Changes in Vitamin D level with time on therapy

As rifampicin, isoniazid and some ART constituents induce hepatic metabolism of Vitamin D metabolites, it has been proposed that low baseline levels of 25 (OH) D may drop further during TB therapy in southern African patients. However, Table 3.10 and Figure 3.6A demonstrate a trend towards rising 25 (OH) D serum concentrations during intensive phase therapy (p=0.058) and a statistical increase during the continuation phase (p=0.002). Additionally, the area under the serum 25 (OH) D-time plot for each patient was used as a measure of total Vitamin D exposure and Figure 3.6B shows no change in this value in relation to the timing of ART initiation (p=0.251). Collectively, these data suggest that prolonged exposure to TB and HIV drugs do not adversely affect 25 (OH) D levels during clinical treatment.

	Baseline	S4 visit	EOT visit
25 (OH) D nmol/l (median, range)	57.3 (11.8-113.3)	62.2 (22.4-133.7)	63.9 (14.9-121.1))
Normal 25 (OH) D (n, %)	29 (18)	31 (22)	40 (31)
Hypovitaminosis D (n, %)	72 (43)	67 (47)	65 (50)
Vitamin D deficiency (n, %)	47 (28)	40 (28)	24 (18)
Severe Vitamin D deficiency (n, %)	18 (11)	5 (4)	2 (2)

Table 3.10 Changes in 25 (OH) Vit D levels during TB therapy

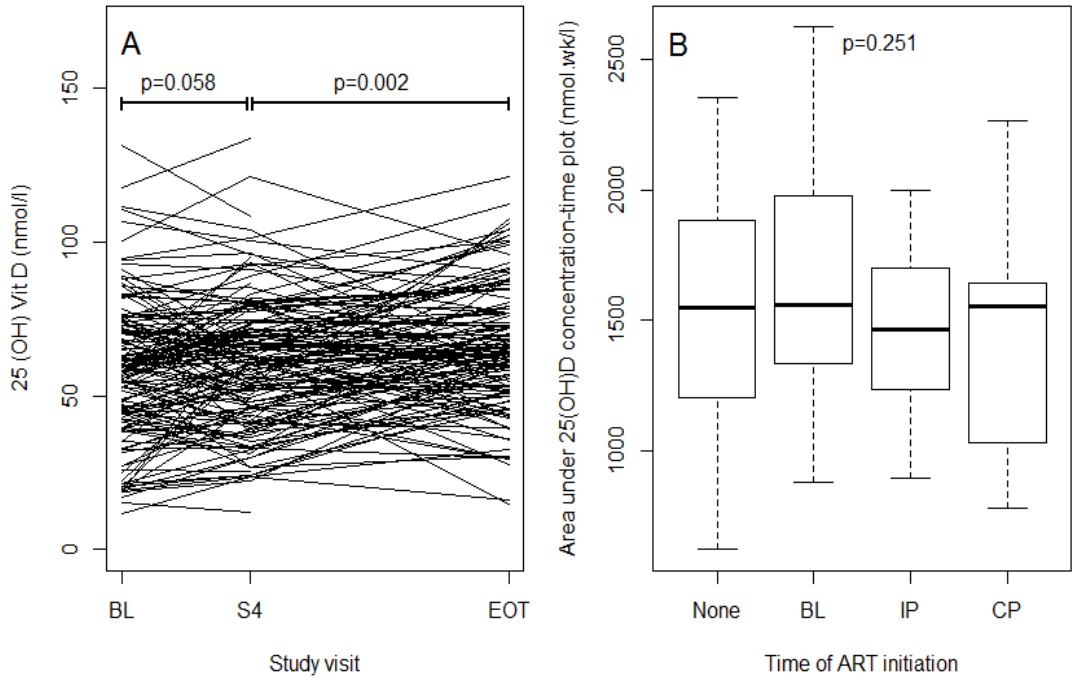


Figure 3.6 Changes in 25 (OH) Vit D levels during TB therapy

A: Changes in 25 (OH) D levels at different time-points analysed by paired Wilcoxon tests. B: Changes in 25 (OH) D levels during TB treatment divided by time of ART initiation, analysed by Kruskal Wallis test; No=No ART during TB therapy, BL=on ART pre-recruitment, IP=Initiated ART during Intensive Phase TB treatment, CP=Initiated ART during continuation phase TB treatment.

3.4.5 Baseline CXR assessment

Inter-reader agreement on CXR appearances

Baseline CXRs were available for 147/169 (87%) patients and interpreted by two independent readers according to the pre-determined scheme. A concordance co-efficient (ρ_c) of 0.71 suggested reasonable consensus in assessment of the percentage of lung affected at presentation. There was "almost perfect" agreement on the presence or absence of consolidation, pleural effusion and miliary shadowing and "substantial" agreement on the presence or absence of cavities ≥4cm in diameter. Agreement about smaller cavities, fibrosis, nodules or hilar lymphadenopathy was less satisfactory (Table 3.11). Only variables in which there was at least "substantial" inter-reader agreement were analysed further.

Inter-reader concordance in the continuous variable	**ρ_c**	**95% Limits of Agreement (Bland and Altman)**	
Total percentage of lung affected	0.71	-24.79 to 21.97%	
Inter-reader agreement among dichotomous variables	**Kappa**	**Prevalence-adjusted, bias-adjusted kappa**	**Interpretation of prevalence-adjusted bias-adjusted kappa[a]**
Presence of cavitation Small cavities, <4cm diameter Large cavities, ≥4cm	 0.62 0.73	 0.57 0.70	 Moderate Substantial
Any consolidation	0.76	0.82	Almost perfect
Any pleural effusion	0.82	0.97	Almost perfect
Nodules	0.43	0.31	Fair
Fibrosis	0.65	0.65	Moderate
Miliary shadowing	0.85	0.91	Almost perfect
Hilar lymphadenopathy	0.59	0.46	Moderate

Table 3.11 Agreement on CXR findings between 2 readers

[a]Landis and Koch guidelines were used to interpret kappa values for dichotomous variables; Poor=kappa ≤0.00, Slight=0.00-0.20, Fair=0.20-0.40, Moderate=0.40-0.60, Substantial=0.60-0.80, Almost perfect=0.81-1.00

Description of CXR appearances

A representative selection of CXRs is shown in Figure 3.7. Consensus assessment of all images indicated that a median of 25 (range: 0-100) % of lung was affected by TB. 136 (93%) patients had consolidation, 52(35%) had cavities ≥4cm in diameter and 15 (10%) had pleural effusions. One (1%) patient had miliary shadowing throughout both lung-fields (Figure 3.7D). However, this patient withdrew during the intensive phase of therapy and no outcome data was available.

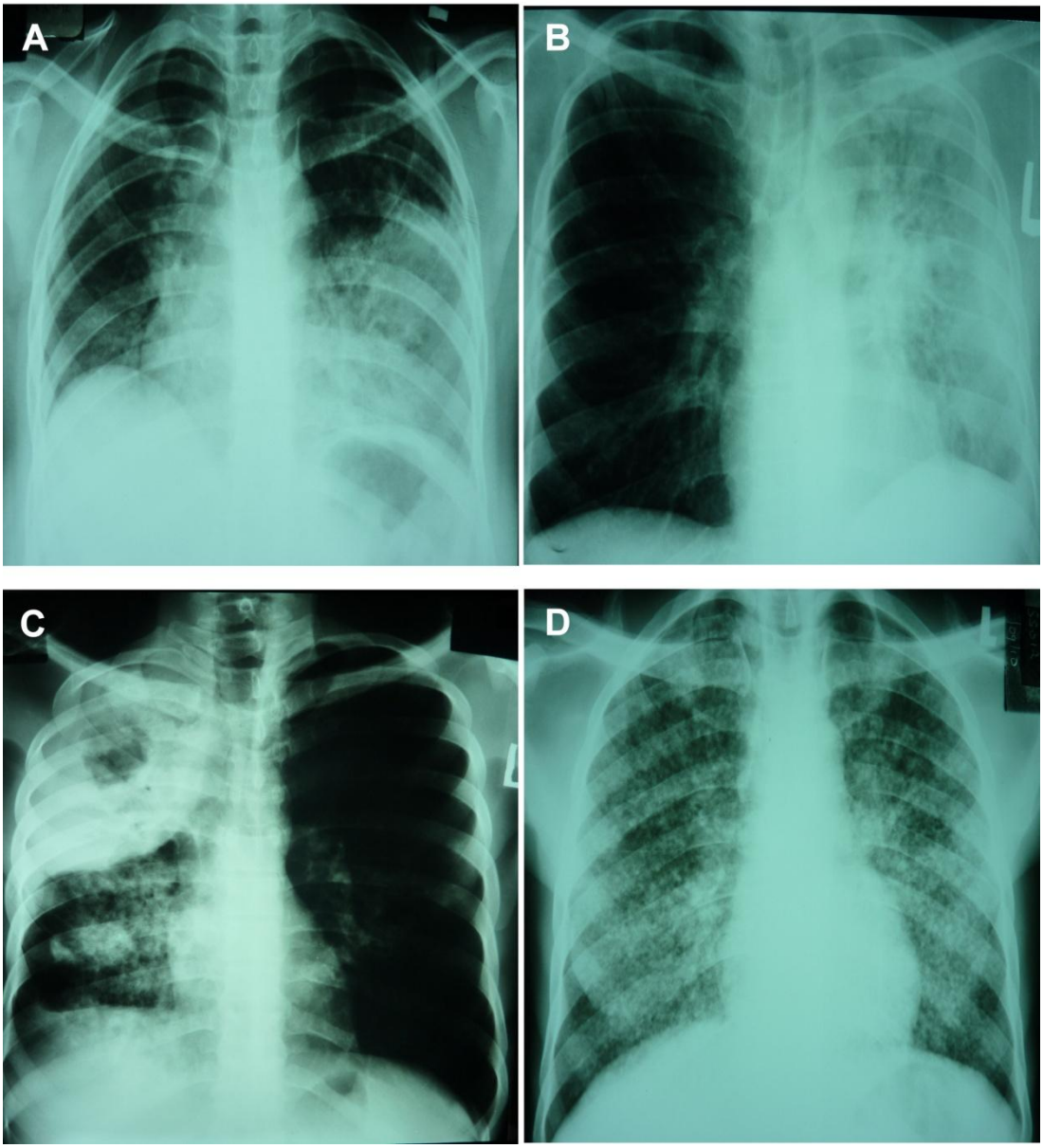


Figure 3.7 CXR appearances and interpretation

Representive CXRs show that the pattern and extent of radiological disease was diverse and complex. However, reasonable consensus could be reached on the major findings.

A: Lower left zone consolidation. Consensus: 30% total lung volume involved, no cavity and no effusion. B: Entire left lung consolidation. Consensus: 50% total lung volume involved, no cavity, and small effusion. C: Large thick walled cavity in right upper zone. Consensus: 45% total lung volume involved, >4cm cavity and no effusion. D: Miliary disease. Consensus: 100% lung volume involved, no cavity and no effusion.

Factors influencing percentage of lung affected

As the amount of lung affected varied from 0-100%, a linear regression model was used to assess clinical factors contributing to this measure of radiological disease (Table 3.12). On multivariate analysis male sex (p=0.009) and cooking with biomass fuel inside the house (0.035) were associated with more extensive radiological disease and there was a trend towards a greater amount of lung affected with higher WCC (p=0.064). Lower baseline BMI (p=0.020) and lower baseline 25 (OH) D level (p=0.008) were associated with more extensive radiological disease on univariate analysis only.

	Univariate analysis			Multivariate analysis		
Variable	Estimate	St Error	p-value	Estimate	St Error	p-value
Age, years	0.156	0.162	0.337	-	-	-
Male sex	10.780	2.980	<0.001*	8.804	3.339	0.009*
Cooks using biomass fuel						
Inside the house	10.588	4.973	0.035*	11.007	5.229	0.037*
Outside the house	2.618	3.130	0.404	2.979	3.241	0.340
Ever smoked tobacco	2.488	3.298	0.452	-	-	-
Ever drinks alcohol	2.727	2.926	0.353	-	-	-
HIV infected	-3.577	2.872	0.215	-	-	-
CD4 count, cells/µl	0.010	0.012	0.425	-	-	-
On ART at baseline	-25.983	4.303	0.109	-	-	-
BCG vaccinated	4.406	3.615	0.225	-	-	-
Baseline BMI, kg/m^2	-1.356	0.587	0.020*	-0.669	0.623	0.289
Baseline WCC, $x10^9$ cells/l	1.379	0.532	0.010*	0.992	0.533	0.064
Baseline 25(OH)D, nmol/l	-0.166	0.062	0.008*	-0.078	0.070	0.265

Table 3.12 Factors influencing percentage of lung affected by TB

Factors influencing the presence/absence of cavities ≥4cm diameter

A logistic regression model was used to assess factors contributing to development of cavities. On multivariate analysis HIV infection was associated with fewer cavities (OR: 0.03, 95% CI: 0.13-0.68, p=0.004) and higher WCC was associated with more cavities (OR: 1.17, 95% CI: 1.01-1.36, p=0.036). There was a strong trend towards cavitation in patients with previous BCG vaccination (OR: 3.32, 95% CI: 0.97-11.42, p=0.057). Male sex was associated with cavitation on univariate analysis only (OR: 3.72, 95% CI: 1.57-8.77, p=0.003).

There was a univariate association between cavity formation and ART prior to recruitment (3.08, 95% CI: 1.06-9.01, p=0.040) and a trend towards cavitation at higher CD4 counts (OR: 1.003, 95% CI: 0.999-1.007, p=0.057). These variables were excluded from multivariate analysis as they were not applicable to HIV non-infected individuals (Table 3.13).

	Univariate analysis			Multivariate analysis		
Variable	OR	95% CI	p-value	OR	95% CI	p-value
Age, years	0.97	0.94-1.01	0.203	-	-	-
Male sex	3.72	1.57-8.77	0.003*	2.00	0.68-5.87	0.208
Cooks using biomass fuel						
At all	1.12	0.35-3.66	0.845	-	-	-
Inside the house	1.10	0.52-2.32	0.810	-	-	-
Ever smoked tobacco	1.58	0.73-3.40	0.244	-	-	-
Ever drinks alcohol	1.93	0.97-3.84	0.063	1.77	0.71-4.40	0.221
HIV infected	0.30	0.15-0.61	<0.001*	0.30	0.13-0.68	0.004*
CD4 count	1.003	0.999-1.017	0.057	-	-	-
On ART at baseline	3.08	1.06-9.01	0.040*	-	-	-
BCG vaccinated	3.09	1.10-8.70	0.033*	3.32	0.97-11.42	0.057
Baseline BMI, kg/m^2	0.86	0.74-1.00	0.052	0.91	0.76-1.10	0.345
Baseline WCC, $x10^9$ cells/l	1.22	1.06-1.41	0.005*	1.17	1.01-1.36	0.036*
Baseline 25(OH)D, nmol/l	1.00	0.98-1.01	0.567	-	-	-

Table 3.13 Factors influencing the presence/absence of large cavities on CXR

3.5 Results: Factors associated with treatment response

Associations between the variables described in this chapter and end-points of 2 month sputum culture status or final clinical outcome were assessed by logistic regression.

Univariate analysis was done for all variables. Tables 3.14 and 3.15 show results of those for which an association was suggested by significance on univariate analysis at the p<0.10 level for at least one of the selected end-points, or for which prior publications suggest a likely relationship. Multivariate modelling was undertaken for variables with p <0.10 on univariate testing.

3.5.1 Variables associated with 2 month culture status

On multivariate analysis (Table 3.14), a positive 2 month sputum culture was associated with previous BCG vaccination (OR: 10.89, 95% CI: 2.01-58.82, p=0.006) and cavities ≥4cm on CXR (OR: 2.52, 95% CI: 1.00-6.31, p=0.049). There were trends towards positive cultures in patients who had consulted a traditional healer prior to attending hospital (OR: 8.85, 95% CI: 0.98-79.69, p=0.052), and who had a higher baseline respiratory rate (OR: 1.06, 95% CI: 1.00-1.13, p=0.059).

On univariate testing, difficulty obtaining enough food in the last month (OR: 3.19, 95%: 1.28-8.03, p=0.014) was associated with positive two month cultures and there were trends between positive cultures and male sex (OR: 2.19, 95% CI: 0.93-5.15, p=0.072), ever smoking tobacco (OR: 2.16, 95% CI: 0.97-4.85, p=0.061) and ever drinking alcohol (OR: 1.89, 95% CI: 0.90-3.98, p=0.093). All of these relationships were lost on multivariate modelling. There was a univariate association between positive cultures and percentage of lung

affected on CXR (OR: 1.03, 95% CI: 1.00-1.05, p=0.027) but this variable was not included in multivariate modelling because it displayed co-linearity with cavities ≥4cm.

There was no relationship between HIV status and 2 month culture status. However, amongst HIV-infected patients, ART prior to recruitment was associated with positive cultures (OR: 4.25, 95% CI: 1.53-11.85, p=0.005) and there was a trend towards positive cultures at higher CD4 counts (OR: 1.003, 95% CI: 1.000-1.006, p=0.054).

3.5.2 Variables associated with final treatment outcome

On multivariate analysis (Table 3.15) none of the variable associated with positive 2 month sputum cultures were associated with final clinical outcome. However, patients who could read from a newspaper had fewer unfavourable outcomes (OR: 0.25, 95% CI: 0.09-0.93, p=0.037) and there was a strong trend towards unfavourable outcomes in patients who had a high serum ALT (>35 IU/l) at baseline (OR: 3.30, 95%CI: 0.93-12.11, p=0.064).

There was a univariate trend towards unfavourable outcomes in HIV-infected individuals (OR: 3.49, 95% CI: 0.94-13.02, p=0.063) and, amongst HIV-infected patients, ART prior to recruitment displayed a univariate association with unfavourable outcome (OR: 3.79, 95% CI: 1.06-13.56, p=0.041).

Prior studies have described a relationship between rising BMI and a better response to TB therapy. This was not evident during the current study using early (2 month sputum culture) or late (final clinical outcome) end-points. Similarly there was no association at either end-point between any measure of 25 (OH) D and treatment response.

	Positive N=44	Negative N=86	Univariate analysis			Multivariate analysis		
			Odds Ratio	95% CI	p-value	Odds Ratio	95% CI	p-value
Socioeconomic factors								
Difficulty obtaining enough food in the last month (n, %)	13 (30)	10 (12)	3.19	1.26-8.03	0.014*	2.01	0.62-6.57	0.246
Able to read a newspaper (n, %)	29 (66)	64 (74)	0.66	0.30-1.46	0.310	-	-	-
Ever smoked tobacco (n, %)	16 (36)	18 (21)	2.16	0.97-4.83	0.061	1.20	0.35-4.13	0.768
Ever drinks alcohol (n, %)	21 (48)	28 (33)	1.89	0.90-3.98	0.093	1.60	0.52-4.93	0.411
HIV parameters								
Confirmed HIV infected (n, %)	29 (66)	50 (58)	1.39	0.65-2.97	0.391	-	-	-
Baseline CD4 count, cells/µl (median, range)[a]	270 (6-688)	138 (8-783)	1.003	1.000-1.006	0.054	-	-	-
On ART at enrolment (n, %)[a]	13 (52)	9 (21)	4.25	1.53-11.85	0.005*	-	-	-
Demographic, Clinical and laboratory parameters								
Male sex (n, %)	35 (80)	55 (64)	2.19	0.93-5.15	0.072	1.76	0.55-5.65	-
Attended traditional healer (n, %)	5 (11)	2 (2)	5.38	1.29-28.98	0.050	8.85	0.98-79.69	0.052
BCG vaccinated (n, %)	41 (93)	63 (73)	4.99	1.41-17.69	0.013*	10.89	2.01-58.82	0.006*
Baseline BMI, kg/m^2 (median, range)	17.9 (14.2-24.9)	19.0 (13.2-29.3)	0.89	0.76-1.05	0.166	-	-	-
Increase in BMI by S4 visit, kg/m^2 (median, range)	0.63 (-1.37-3.43)	0.76 (-1.56-5.78)	0.80	0.58-1.10	0.170	-	-	-
Baseline respiratory rate, breaths/min (median, range)	31 (12-81)	30 (16-42)	1.05	1.00-1.10	0.061	1.06	1.00-1.13	0.059
Patients with high baseline ALT (n, %)	8 (19)	13 (16)	1.23	0.47-3.25	0.676	-	-	-
Baseline CXR assessment								
Percentage of lung affected on CXR (median, range)[b]	33 (10-44)	25 (0-75)	1.03	1.00-1.05	0.027*	-	-	-
Presence of large cavity, ≥4cm diameter (n %)[b]	20 (51)	22 (29)	2.63	1.18-5.85	0.018*	2.52	1.00-6.31	0.049*
25 (OH) Vitamin D measurement								
Baseline 25(OH)D, nmol/l (median, range)	55.0 (16.9-111.5)	57.8 (11.8-117.5)	0.99	0.97-1.01	0.320	-	-	-
Increase in 25(OH)D by S4 visit, nmol/l (median, range)	3.1 (-30.9-40.9)	2.4 (-34.0-55.8)	0.99	0.97-1.02	0.484	-	-	-
Area under 25 (OH)D conc-time curve, nmol.wk/l (median, range)	429 (164.0-887)	490.2 (142-1000)	1.00	0.99-1.00	0.187	-	-	-

Table 3.14 Variables associated with 2 month culture status

[a]Baseline CD4 and ART at enrolment assessed on HIV-infected individuals only

[b]CXR available on 116 patients only (2 month culture positive, N= 39, 2 month culture negative, N=77)

	Unfavourable N=15	Favourable N=118	Univariate analysis			Multivariate analysis		
			Odds Ratio	95% CI	p-value	Odds Ratio	95% CI	p-value
Socioeconomic factors								
Difficulty obtaining enough food in the last month (n, %)	3 (20)	24 (20)	1.22	0.32-4.74	0.769	-	-	-
Able to read a newspaper (n, %)	7 (47)	89 (75)	0.29	0.10-0.85	0.025*	0.29	0.09-0.93	0.037*
Ever smoked cigarettes (n, %)	5 (33)	25 (21)	1.86	0.58-5.94	0.295	-	-	-
Ever drinks alcohol (n, %)	7 (47)	38 (32)	1.84	0.62-5.45	0.270	-	-	-
HIV parameters								
Confirmed HIV infected (n, %)	12 (80)	63 (53)	3.49	0.94-13.02	0.063	2.84	0.73-11.01	0.130
Baseline CD4 count, cells/µl (median, range)[a]	168 (6-539)	164 (10-783)	1.00	1.00-1.00	1.00	-	-	-
On ART at enrolment (n, %)[a]	7 (59)	17 (27)	3.79	1.06-13.56	0.041*	-	-	-
Demographic, Clinical and laboratory parameters								
Male sex (n, %)	10 (67)	79 (67)	0.99	0.32-3.09	0.983	-	-	-
Attended traditional healer (n, %)	0 (0)	5 (5)	0.00	0-Inf	0993	-	-	-
BCG vaccinated (n, %)	13 (87)	95 (81)	1.51	0.32-7.16	0.607	-	-	-
Baseline BMI, kg/m^2 (median, range)	19.5 (15.4-29.3)	18.3 (13.2-24.9)	1.18	0.96-1.45	0.108	-	-	-
BL to S4 change in BMI, kg/m^2 (median, range)	0.7 (-0.7-2.9)	0.7 (-4.6-5.7)	1.03	0.67-1.58	0.902	-	-	-
BL to EOT change in BMI, kg/m^2 (median, range)	1.6 (-1.4-4.3)	1.6 (-3.7-6.0)	0.85	0.56-1.29	0.447	-	-	-
Baseline respiratory rate, breaths/min (median, range)	30 (12-81)	30 (16-44)	1.00	0.94-1.07	0.895	-	-	-
Patients with high baseline ALT (n, %)	5 (33)	15 (13)	3.30	0.99-10.99	0.052	3.30	0.93-12.11	0.064
Baseline CXR assessment								
Percentage of lung affected on CXR (median, range)[b]	26 (0-48)	25 (0-75)	0.99	0.957-1.03	0.605	-	-	-
Presence of large cavity, ≥4cm diameter (n %)[b]	4 (31)	41 (38)	0.72	0.21-2.47	0.597	-	-	-
25 (OH) Vitamin D measurement								
Baseline 25(OH)D, nmol/l (median, range)	61.5 (18.6-106.4)	58.1 (16.9-111.5)	1.02	1.00-1.05	0.100	-	-	-
Increase in 25(OH)D by EOT, nmol/l (median, range)	4.1 (-31.1-55.2)	10.9 (-48.1-55.9)	0.99	0.97-1.02	0.490	-	-	-
Area under 25(OH)D conc-time curve, nmol.wk/l (median, range)	1574 (942-2626)	1510 (621-2560)	1.00	1.00-1.00	0.213	-	-	-

Table 3.15 Variables associated with final clinical outcome

[a]Baseline CD4 and ART at enrolment assessed on HIV-infected individuals only

[b]CXR available on 120 patients only (Unfavourable outcome, N= 13, Favourable outcome, N=107

3.6 Discussion

Recruitment was slower than anticipated, partially because of the decentralisation of TB services in 2011 described in Section 3.2.3. Once it became possible to register for TB therapy at Ndirande, Bangwe, Zingwangwa and Chileka Health Centres it was more difficult to persuade patients to enrol in a research project at QECH. Nevertheless, recruitment was within the range of the original power calculation. The gender imbalance of the cohort is unlikely to distort analysis of factors contributing to bacillary persistence or treatment response but may reflect differing social and domestic pressures on men and women during healthcare related decision-making in Malawi.

The absence of baseline rifampicin resistance is consistent with the general perception that Malawi has an effective NTP, low MDR-TB prevalence and minimal transmission of resistance to treatment-naive individuals. This is reassuring for ongoing TB control and contrasts with some other parts of southern Africa[12]. Although three patients had *KatG* mutations on the line probe assay, previous studies have reported good outcomes with standard TB therapy in isoniazid mono-resistant patients[510] and no mono-resistant patients in this study failed treatment or relapsed. Nevertheless, inadequately managed isoniazid resistance can progress to MDR-TB[511], highlighting the need for constant vigilance.

15/133 (11%) patients had unfavourable outcomes, which is higher than the 3-5% reported by other recent TB treatment studies[477]. This is explained by the study design; a stringent definition of unfavourable outcomes was used and inclusion criteria were constructed to selectively enrol individuals with high bacillary loads (i.e. pre-study diagnostic sputum smears '++' or '+++' for AFB).

13(87%) unfavourable outcomes were microbiologically confirmed, and 11 (85%) of those had fully drug sensitive disease at end of treatment or during subsequent follow-up. Therefore, the majority of failures and relapses were not due to emergence of drug resistance and it may be assumed that bacilli identified at later treatment time-points were predominantly drug-tolerant persisters.

Data on the HIV status of study participants are interesting for two reasons. Firstly, 31/169 (18%) smear positive TB patients were first diagnosed with HIV infection at enrolment, illustrating the ongoing role of TB facilities as an entry-point to HIV care in Malawi. Secondly, the high prevalence and careful characterisation of immunosuppression increases the value of the pharmacodynamic modelling data described in Chapter 6.

Previous SSCC studies have either been performed in settings of much lower HIV sero-prevalence[266,278] or selectively recruited individuals with high CD4 counts[228] and studies of treatment response based on time to positivity of liquid cultures have generally included HIV negative patients only[288,290,298]. The data in this thesis is the first pharmacodynamic analysis of bacteriological treatment response amongst TB patients irrespective of the degree of immunosuppression at recruitment.

Clinical characteristics at baseline indicate that patients were sub-acutely unwell and ambulatory. This is the type of patient normally recruited to studies of new anti-TB therapy, so bacillary persistence and treatment data from this cohort are relevant to the future design of clinical trials. The clinical data does not support a relationship described elsewhere between TB and Diabetes Mellitus[512,513] or lend weight to proposals that TB clinics may be a gateway to management of non-communicable diseases such as diabetes and hypertension[514]. Only one patient was diabetic and 19 (11%) were hypertensive at enrolment. Previous multi-centre clinical trials have also reported that southern African study sites recruit younger patients with lower rates of non-communicable disease[226,357]. Perhaps, as many southern African TB patients are young HIV-infected adults, the incidence of co-existent non-communicable diseases is small and the yield from screening programmes will be low. The situation is different in other populations; high rates of Diabetes Mellitus have been reported amongst TB patients in Asia and Central America[512,513,515]. Some studies have also observed more severe clinical disease, delayed sputum conversion and a higher probability of treatment failure/relapse amongst TB patients who were also diabetic[515,516].

Overall study protocol compliance was good and patients demonstrated gradual improvements in general health and BMI. ART uptake and CD4 cell reconstitution amongst HIV-infected study participants were also reassuring. Intercurrent illnesses and drug side-effects were common, highlighting the need for shorter TB therapy, and some patients took additional medications during TB treatment. However, very few individuals interrupted TB therapy and no additional medications had important interactions with TB drugs. It is unlikely that variable adherence to medication or drug interactions affected pharmacodynamic assessment of bacillary elimination (Chapter 6) or pharmacodynamic measurement of drug exposure (Chapter 7).

Baseline serum 25 (OH) D levels were similar to those described previously for adult Malawian TB patients[481] and demonstrated high rates of hypovitaminosis D and Vitamin D

deficiency. Factors contributing to low 25 (OH) D levels were month of recruitment (lower levels during and just after the cold season) and poor dietary intake. The seasonal variation in baseline serum 25 (OH) D concentrations has not previously been reported and is interesting. Yet it is reassuring that 25 (OH) D levels slowly increased during treatment and that TB drugs or ART did not increase hepatic breakdown of Vitamin D metabolites. Perhaps, as patients recover, improvements in factors associated with higher 25 (OH) Vit D levels (increased dietary intake, rising BMI and more sunlight exposure) counterbalance the negative pharmacological effects of administered medications.

The CXR scoring scheme was simple and provided good inter-reader concordance in assessment of precentage of lung affected and presence of cavities ≥4cm. A robust immune system and anti-inflammatory response was associated with cavitation; HIV infection reduced cavity formation, whilst pre-study ART, high WCC/CD4 count and BCG vaccination all made cavities more likely. These findings are consistent with established knowledge that the pathogenesis of cavity formation in pulmonary TB relies on an immunological delayed type hypersensitivity reaction[517,518].

On multivariate analysis of factors associated with positive 2 month sputum cultures, two variables were statistically significant; cavities ≥4cm on CXR and prior BCG vaccination. The first of these is consistent with recent literature[291,353,355] and it is probable that slower culture conversion in patients with CXR cavitation reflects higher baseline sputum bacillary load[353], and variable penetration of cavities by drug therapy[519] .

The association between positive 2 month sputum cultures and prior BCG vaccination was unexpected as prior work from Tanzania suggests that presence of a BCG scar increases the likelihood of rapid conversion to negative cultures[480]. However, that study recruited smear positive TB patients of any grade rather than selecting those with '++' or '+++' disease and did not assess for cavitation on CXR. It is possible that amongst the selectively higher bacillary loads recruited to the current study vaccination does not prompt earlier culture conversion but helps to contain infection in pulmonary cavities and prevent dissemination. Historical reports of the protective efficacy of BCG are variable[520-522], particularly against pulmonary disease in adults, and further studies would be required to support this conclusion. These may be superseded by the search for a better TB vaccine.

Neither of the factors associated with positive 2 month cultures were also associated with final outcome suggesting that some elements of eventual response are not captured by

early clinical or radiological measurements. This does not diminish the need to understand the issues affecting 2 month culture status as time to culture conversion remains an endpoint in several clinical trials and may be relevant to infectiousness and transmissibility of TB[523]. However, it does suggest that studies claiming to identify prognostic indicators or surrogate biomarkers based only on 2 month data should be interpreted with caution.

From multivariate analysis, the only factor associated with unfavourable final outcome was inability to read from a newspaper. Section 3.4.1 reported that illiteracy was more common in sputum smear positive TB patients than the general urban Malawian population. Inability to read may be a surrogate for low overall educational attainment and socio-economic status, and may have a multi-factorial relationship with poor health outcomes. Whilst the current study was designed to assess bacteriological and pharmacological aspects of TB treatment, the effect of underlying social factors should not be ignored.

It is also notable that there was a trend towards unfavourable outcomes in patients with a baseline ALT above the normal reference range (>35 IU/l). This is difficult to explain but an abnormal ALT may suggest more disseminated TB with increased risk of treatment failure or relapse. Should future data replicate this finding it will be worthy of further study.

Despite prior publications advocating that Vitamin D may supplement anti-TB chemotherapy by augmenting host immunity[489], 25 (OH) D concentrations did not affect early or late treatment end-points. Therefore, whilst Vitamin D deficiency may be linked to reactivation of latent disease[524,525] no evidence was found that variability in serum levels influences the outcome of therapy.

Finally, there is established evidence that ART reduces mortality in HIV-infected individuals with TB[122,123] and reduces the risk of TB treatment failure[526]. However, in this cohort all deaths occurred in HIV-infected individuals and 5/6 (83%) patients were on ART when they died. On univariate analysis pre-study ART was also associated with positive 2 month sputum cultures and unfavourable final outcomes. While the sample size was too small to draw clear conclusions on the role of HIV therapy during TB treatment, this indicates that, despite the proven benefits of ART, TB patients on HIV therapy remain at high risk.

There are several caveats to the findings described in this chapter. The socio-economic analysis compared the study cohort to the overall urban Malawian population from the DHS, rather than the more specific comparator of the high-density urban population in Blantyre. Outcome definitions may be imprecise as it was not possible to distinguish

between post-treatment re-infections and relapses at a molecular level. Multiple comparisons were done to evaluate factors contributing to unfavourable outcomes. This carries the risk of identifying incidental associations which may not be of clinical significance or reproduced in subsequent cohorts.

Nevertheless, the data show that unfavourable treatment outcomes in first presentations of smear positive TB in Malawi are often due to persistent fully drug susceptible infection with *M tuberculosis*. Patient variables including HIV status, baseline CXR appearance, serial Vitamin D levels and BMI measurements do not adequately explain inter-individual variations in final treatment outcome. Bacterial and pharmacological factors may be implicated. Assessment of these factors will be described in Chapters 4-6

4. Optimising Quantitative Sputum Culture

4.1 Introduction

To advance the analysis of bacillary persistence in TB treatment response, it is necessary to discuss methodological issues in the quantitative culture techniques used to measure the bacillary load in serial sputum samples.

In Chapter 1 the role of sputum culture in developing surrogate markers of clinical outcome was discussed. The biological plausibility of using the rate of decline in viable organisms as a pharmacodynamic measure of treatment activity is convincing and there is moderate correlation from historical studies between sputum culture conversion and clinical outcome[261]. This relationship may be strengthened by analysing quantitative culture data with NLME modelling techniques[266]. Although *M tuberculosis* culture is cumbersome and slow and other biomarkers are under development (including measurement of *M tuberculosis* mRNA[316] and estimation of a molecular bacillary load in sputum[320]) it will be some years before new methods are adequately evaluated for use in clinical trials. Therefore, optimising existing microbiology methods is of critical importance in accelerating progress towards new, shorter TB therapy now.

When on-site work for this study was commenced in Blantyre in March 2010, the TB laboratory at the College of Medicine routinely performed sputum smear microscopy and TB culture using egg-based LJ slopes. Whilst LJ media is cheap and effective for growth of *M tuberculosis* in resource poor settings it was deemed inappropriate for the current study for several reasons; growth of colonies on LJ slopes is slow[527], the surface area of the media was too small for colony counting and modern clinical trials prefer to monitor microbiological response to therapy with a combination of Middlebrook plates and liquid culture[227,228,232,488]. It was deemed necessary to introduce laboratory methods consistent with modern trials.

From March-June 2010, training on SSCC on Middlebrook plates and liquid culture in a MGIT system were provided for two dedicated TB laboratory technicians. Training was complete prior to commencement of patient recruitment. However, due to time constraints associated with the schedule for the clinical study, some improvements of SSCC media were investigated using patient samples after the study was underway.

Key steps in optimisation of the SSCC and MGIT culture techniques will now be described.

4.2 Serial Sputum Colony Counting

The principle of SSCC was outlined in Section 1.10.2. Serial dilutions of homogenised overnight sputum collections are incubated at 37°C for 3 weeks on selective agar and a back-titration calculation is used to quantify CFU/ml counts in the original sample[263,264]. Mean counts are taken from duplicate plates. Careful attention must be paid to the quality of sputum collection and transportation procedures.

4.2.1 Original SSCC media preparation (7H10-Amb10)

Initially, Middlebrook 7H10 media was used for SSCC plates. According to manufacturer's instructions, 19g 7H10 agar powder (Becton Dickinson) was added to 895ml of distilled water and 5ml of glycerol and autoclaved at 121°C for 15 minutes. After cooling to 45°C the media was supplemented with 100ml Oleic Acid-Albumin-Catalase (OADC, Becton Dickinson). Anti-microbial drugs were added in the form of two selectatabs (MAST24) to give final anti-microbial concentrations of polymyxin B (200u/ml), ticarcillin (100mg/l), trimethoprim (10mg/l) and amphotericin B (10mg/ml). As it was cooling, 8ml of media was transferred by sterile pipette into each compartment of 100mm diameter triple segment petri dishes. Plates were inverted, dried overnight at 37°C, stored at 4°C and used within 3 weeks.

This preparation was the standard SSCC media used at the start of the study and will be referred to as 7H10-AmB10.

4.2.2 Plate set-up and reading of results

On arrival in the laboratory, overnight sputum samples were homogenised by vortex agitation with glass beads to break down clumps and ensure equal dispersal of *M tuberculosis* bacilli. The specimen was split as described in Section 2.6.1 with 1ml allocated for each of SSCC and MGIT. The remainder was stored at -20°C. The SSCC aliquot was incubated with an equal volume of dithiothreitol 1g/l (Oxoid) for 1 hour in order to liquefy the sample by dissociation of sulfide bonds in mucoid sputum[528]. 5 serial ten-fold dilutions of homogenised and liquefied sputum were then prepared in sterile phosphate buffered saline (PBS). 50µl of neat sputum and all five dilutions from each sample were inoculated onto duplicate 7H10-AmB10 plate sets. Culture plates were placed in zip-lock polythene bags and incubated in the dark at 37°C. Sample preparation and plate set-up is shown in Figure 4.1.

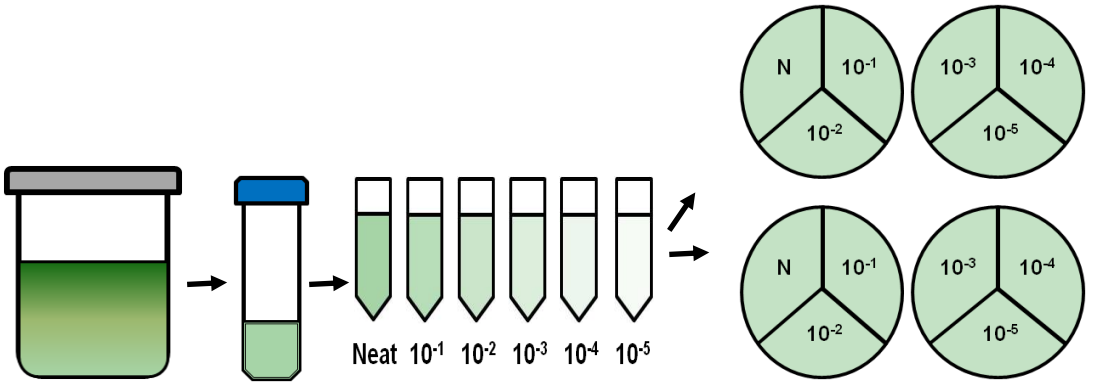


Figure 4.1 Sputum processing and SSCC plate set-up

Plates were read at 3 weeks. If characteristic *M tuberculosis* colonies (Figure 4.2, A-D) were visible ZN smears were done to confirm AFB growth and the sample was reported as positive. If no growth was seen on either plate set it was reported as negative.

For positive plate sets, a dilution yielding 10-100 TB colonies was selected for colony counting. CFU/ml of sputum was calculated by;

$$CFU/ml = \textit{No of colonies counted in plate segment} \times \textit{2(dithiothreitol dilution)} \times \textit{20 (50µl were inoculated per segment} \times \textit{Dilution factor}$$

Counts from both plate sets were used to calculate mean CFU/ml and $\log_{10}$ CFU/ml counts for all positive samples.

Weekly contamination checks were done during incubation. Contaminating organisms were classified as fungal or bacterial based on macroscopic appearance and gram stain. Plates were regarded as contaminated if there were sufficient contaminating organisms to prevent TB colony counting.

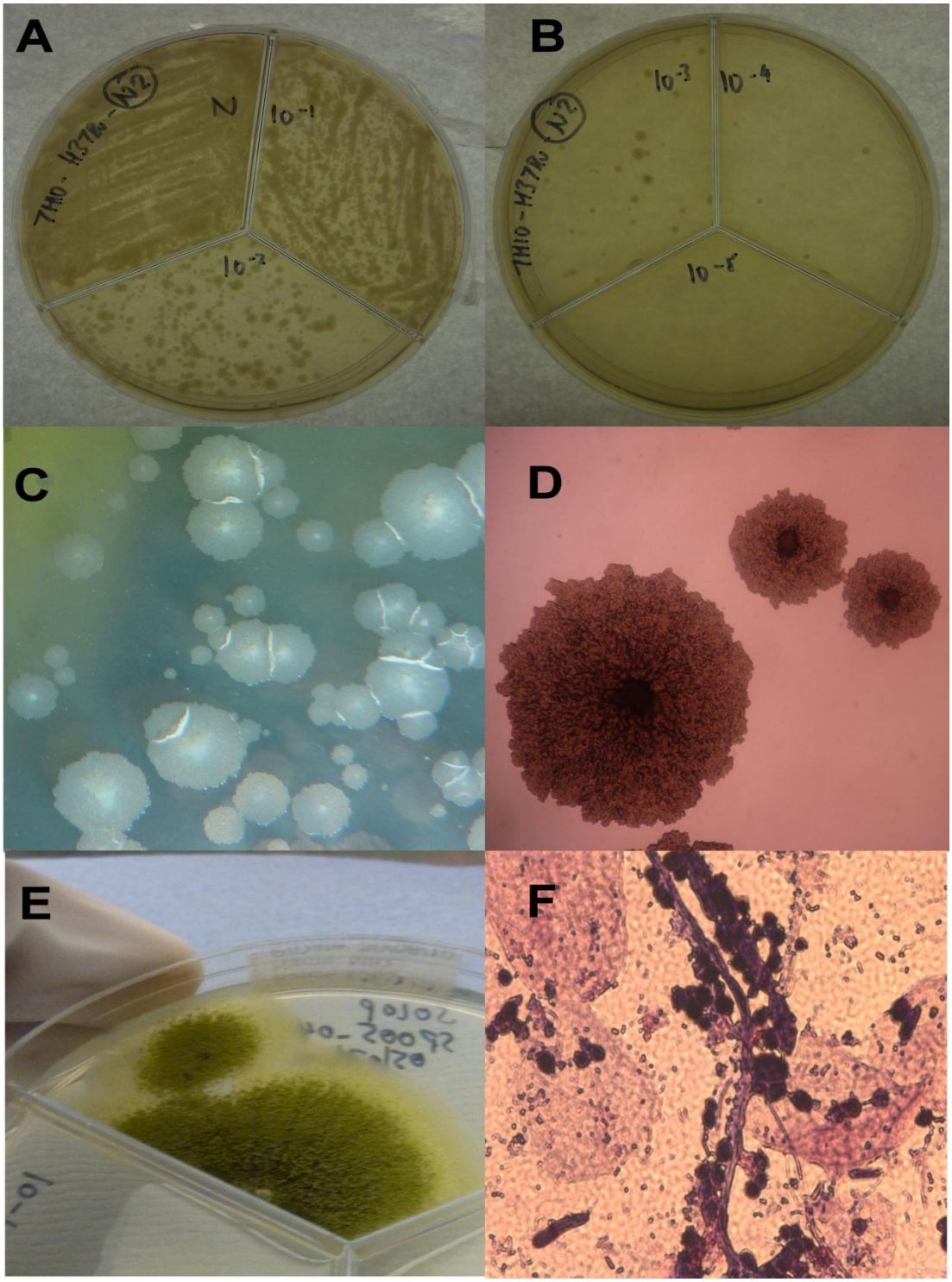


Figure 4.2 SSCC plates and colonies

A and B: A typical plate set after 3 weeks incubation. The 10^{-2} dilution (bottom segment of top left image) contains a suitable number of colonies for counting (94 colonies were counted which gives an estimate of 376,000 [$\log_{10}$ 5.57] CFU/ml sputum in the original sample). C: Characteristic *M tuberculosis* colonies; white, slightly raised and rugose (further identification was done by ZN microscopy) D: Characteristic *M tuberculosis* colonies magnified x100. E: Neat plate segment from a clinical sample showing fungal contamination. F: Gram stain of contaminated plate segment showing hyphae and blastospores typical of fungal growth.

On each day of sample set-up, 'negative control' plates of diluted SSCC reagents without sputum were also prepared to ensure that any observed contamination originated from the sputum specimens rather than a laboratory problem.

4.2.3 Contamination of early study specimens

Sample contamination presents difficulties in clinical mycobacteriology because sputum samples often contain a variety of micro-organisms from the oral cavity and upper respiratory tract which quickly overgrow *M tuberculosis* cultures[529]. Therefore, contamination rates on standard SSCC plates were monitored throughout the study. Results from June 2010 until the last patient was recruited in December 2011 are shown in Figure 4.3 and Table 4.1.

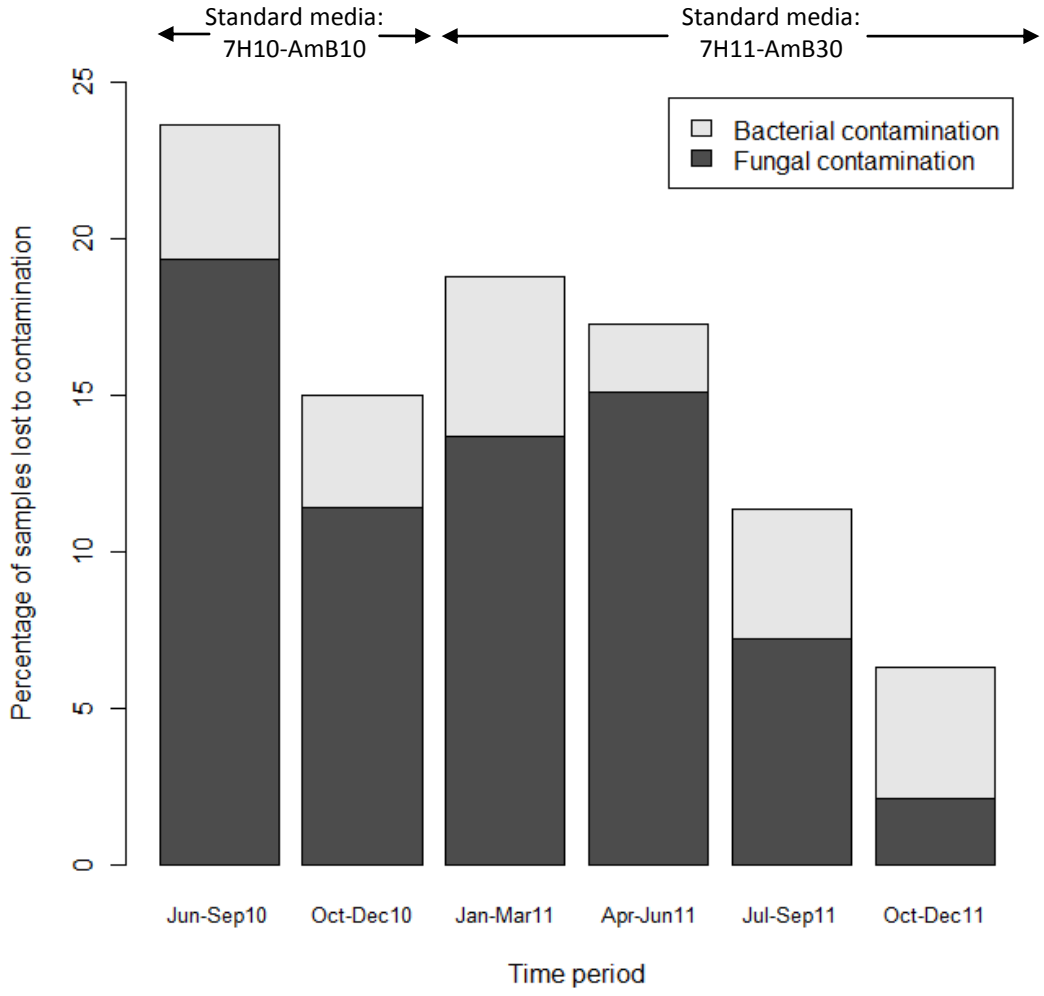


Figure 4.3 Contamination of standard SSCC plates from June 2010-December 2011

Standard SSCC plates were on 7H10-AmB10 media from June-December 2010 and 7H11-AmB30 media thereafter. Data from experimental media (7H10-AmB30 and 7H11-AmBC) during the plate comparisons described in sections 5.2.4 and 5.2.5 is not represented here.

	Standard media: 7H10-AmB10		Standard media: 7H11-AmB30			
	Jun-Sep 2010	Oct-Dec 2010	Jan-Mar 2011	Apr-Jun 2011	Jul-Sep 2011	Oct-Dec 2011
Total number of samples	93	135	117	138	96	95
Contaminated samples Total (n,%) Fungal (n, %) Bacterial (n ,%)	 22 (24) 18 (19) 4 (4)	 21 (15) 16 (11) 6 (4)	 22 (19) 16 (14) 6 (5)	 24 (17) 21 (15) 3 (2)	 11 (11) 7 (7) 4 (4)	 6 (6) 2 (2) 4 (4)
Samples collected after ≥14 days of treatment	34	60	48	62	46	41
≥14 day sample contamination (n, %)	11 (32)	12 (20)	10 (21)	22 (36)	8 (17)	3 (7)

Table 4.1 Contamination of standard SSCC plates from June 2010-December 2011

From June-September 2010, 24% of SSCC plates were contaminated and 82% of contamination was attributed to fungi (see Figure 4.2, images E-F). Amongst samples collected from patients after day 14 of TB therapy, the contamination rate was 32%. This was an unacceptable loss of data, particularly at later sampling time-points when valid data are needed to track persister organisms, evaluate time to culture conversion and calculate the Sterilisation Phase bacillary elimination rate[267]. There was no contamination of negative control plates indicating that non-mycobacterial organism from sputum were adversely affecting the results.

The explanation for this was clear. In many TB culture techniques (including the MGIT method described later in this chapter) non-mycobacterial organisms in sputum are selectively destroyed by sample decontamination with NaOH prior to inoculation of media. However, this process is inappropriate for SSCC because, even when done carefully, it adversely affects recovery of *M tuberculosis*[530] and lowers the bacillary load[531,532]. Colony counting studies following sputum decontamination have previously reported a monophasic rather than biphasic pattern of bacillary elimination[272,273] suggesting an undesirable distortion of treatment response. Therefore, this SSCC study was performed on non-decontaminated sputum and the risk of plate contamination was higher.

Older studies with non-decontaminated sputum reported contamination rates of only 2.7-3.5%[533,534] but these were not conducted in resource-poor tropical settings. Only 30% of our patients had electricity to refrigerate samples at home, consistent with data from the Malawian DHS (2004-5)[458], and patients travelled for 2 hours or more to deliver specimens to hospital. Prolonged exposure of unrefrigerated samples to high ambient temperatures (>30^{o}C) during transportation may encourage contaminant overgrowth.

A recent study using non-decontaminated sputum in Peru reported a culture contamination rate of 18%[530], similar to the current data. Over 80% of their contaminants were bacterial whilst >80% in Malawi were fungal, perhaps reflecting underlying differences in oral or respiratory flora and the effect of HIV infection (HIV prevalence in Peruvian TB patients is 3% compared to 67% in our study cohort). Nevertheless, there was an obvious need for improved selective media when dealing with non-decontaminated sputum in resource-poor settings.

4.2.4 Improving SSCC media: the "AmB10 v AmB30" comparison

To reduce plate contamination, the anti-microbial composition of SSCC media was reviewed. As the predominant contaminants in our study were fungal, media preparations containing different anti-fungal drug formulations were tested to establish whether contamination could be reduced without compromising CFU/ml counts of *M tuberculosis*.

The original SSCC formulation described in Section 4.2.1 included AmB (10mg/l). However, AmB is light and temperature sensitive, loses activity 3-15 days after reconstitution[535] and may have diminishing efficacy during media storage or prolonged incubation. Personal correspondence with the laboratory team who generated SSCC data for the OFLOTUB study[228] suggested that increasing the AmB concentration to 30mg/ml may be beneficial.

From August-December 2010, sputum samples from study patients were incubated in parallel on standard study media (7H10-AmB10) and an experimental media (7H10-AmB30) containing AmB (30mg/ml). Plate sets were not blinded. The effect of different media preparations on viable bacillary load, observed patterns of bacillary elimination and culture contamination rates were assessed.

Preparation of "7H10-AmB30" media

The experimental media, 7H10-AmB30, was prepared in the same way as 7H10-AmB10 but an additional 20mg/ml solution of AmB (Sigma Aldrich), was prepared in dimethyl sulfoxide (DMSO) and 1ml was added along with the selectatabs to give a final AmB concentration of 30mg/l.

Statistical analysis

Differences between bacillary counts on different media were analysed by paired two sample t-tests. Modelling of bacillary elimination was done by NLME methods outlined in Chapters 1 and 6[266]. Differences in the frequency of 'clean' or 'contaminated' sample results were compared by relative risk ratios and 95% CI.

Results of "AmB10 vAmB30" comparison

Due to the serial sampling protocol, some patients provided multiple samples on different days of treatment. 127 sputum samples from a total of 52 patients were used in this comparison. The mean number of samples per patient was 2.5.

Table 4.2 shows mean $\log_{10}$CFU/ml counts of samples collected at different time-points and inoculated on both media types.

7H10-AmB10 sample counts were 0.19 $\log_{10}$ CFU/ml higher (95% CI: 0.10-0.27) than 7H10-AmB30 counts. Although statistically significant, this difference was small. Biphasic bacillary elimination curves were observed (Figure 4.4) suggesting that the change in bacillary counts did not distort treatment response.

34/127 (27%) 7H10-AmB10 plates were contaminated: 28 by fungi and 6 by bacteria. 15/127 (12%) 7H10-AmB30 plates were contaminated: 11 by fungi and 4 by bacteria (Table 4.3). The relative risk of sample contamination with AmB10 was 2.10 (95% CI: 1.25-3.55).Individual plate segment contamination on 7H10-AmB10 was 18%. This was reduced to 8% on 7H10-AmB30. The main reduction was in fungal growth (from 15% to 6% of segments). Taken together, these data suggest that increasing the AmB dose to 30mg/ml resulted in better selective media for growth of *M tuberculosis*.

As with the early study specimens, samples collected later in therapy were at higher risk of contamination. 24(71%) of the contaminated samples on 7H10-Amb10 were collected after day 14. However, 11(46%) of these were not contaminated on 7H10-AmB30, suggesting that better media minimised data loss at later, more vulnerable study time-points.

Negative control plates persistently showed no growth. In addition, Table 4.3 shows that plate segment contamination was highest in neat sputum and sequentially reduced in serial dilutions. This is consistent with organisms from sputum being gradually diluted below the growth threshold on selective plates and reinforces the view that SSCC contamination originated from the samples rather than the laboratory.

Sampling time	Number of samples	Mean log_{10} CFU/ml		Mean of differences in log_{10} CFU/ml counts between media[a] (95% Confidence Interval)	p-value
"AmB10 v 30"		7H10-Amb10	7H10-Amb30		
Baseline	32	6.815	6.527	0.267 (0.061-0.473)	0.014*
Day 1-14	44	5.318	5.072	0.163 (0.071-0.255)	0.001*
Day15-28	26	3.914	3.744	0.217 (0.002-0.432)	0.048*
Day 28-56	25	4.078	4.373	-0.143 (-1.025-0.737)	0.557
Total	127	5.616	5.370	0.189 (0.103-0.275)	<0.001*
"AmB30 v AC"		7H11-AmB30	7H11-AC		
Baseline	22	6.552	6.340	0.108 (0.010-0.207)	0.031*
Day 1-14	46	5.606	5.355	0.113 (-0.027-0.253)	0.110
Day 15-28	18	3.917	4.076	0.263 (-0.546-1.072)	0.293
Day 28-56	16	3.150	3.400	-0.250 (-0.631-0.131)	0.076
Total	102	5.757	5.506	0.106 (0.020-0.193)	0.017*

Table 4.2 Colony counts on Middlebrook media containing different antifungal drugs

[a]For samples in the AmB10 v 30 comparison, the difference in log_{10} CFU/ml counts between media was calculated by subtracting the log_{10}CFU/ml on 7H10-AmB30 from the log_{10}CFU/ml on 7H10-AmB10. The mean of the differences for all positive samples at each time interval is shown in the table.
For samples in the AmB30 v AC comparison the difference in log_{10} CFU/ml counts between media was calculated by subtracting the log_{10}CFU/ml on 7H11-AC from the log_{10}CFU/ml on 7H11-AmB30. The mean of the differences for all positive samples at each time interval is shown in the table.

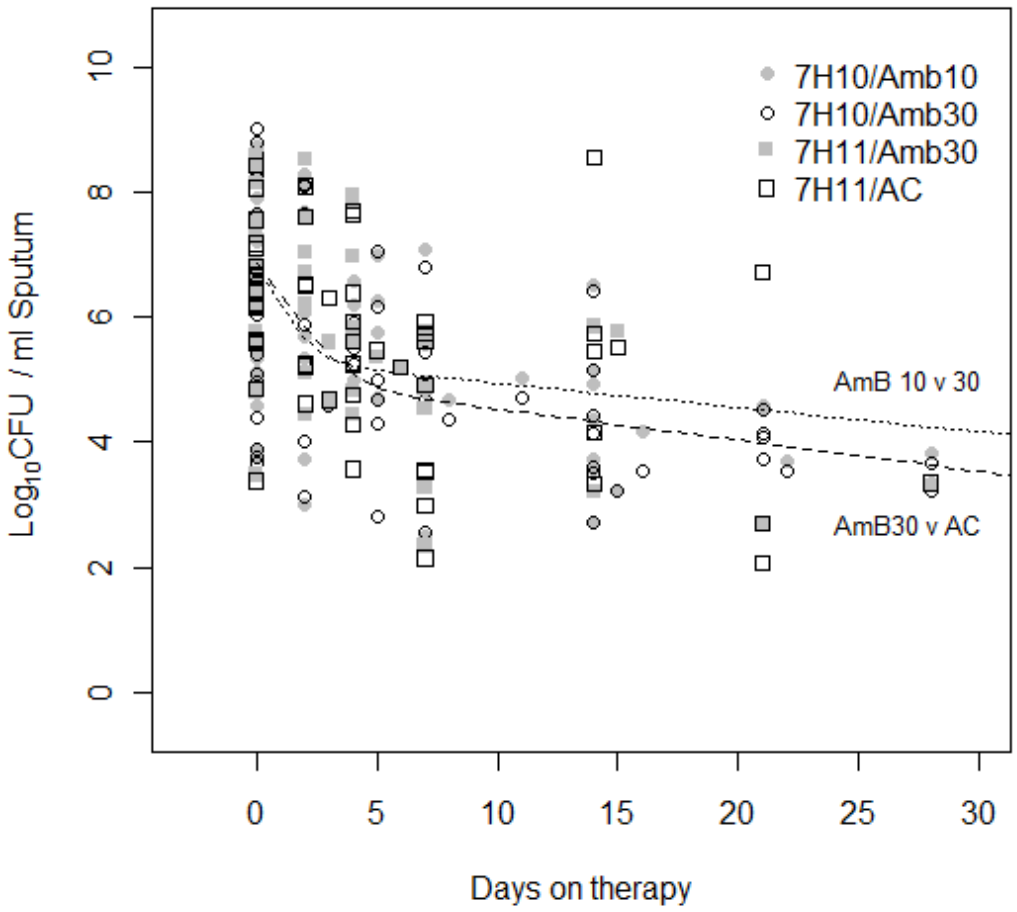


Figure 4.4 SSCC modelling of bacillary elimination using different media preparations

Changes in bacillary load (in log_{10}CFU/ml sputum) over time on therapy are plotted for both media comparisons in the study. NMLE methods were used to demonstrate that a biphasic bacillary elimination model fits the data for both comparisons, suggesting that the effect of different anti-fungal drug concentrations in selective plate media does not adversely affect analysis of bacillary elimination in SSCC studies.

	Overall plate result	Contamination of plate segments inoculated with 10-fold sputum dilutions						
		Neat	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Totals
"AmB 10 v 30" : 7H10 (AmB 10mg/ml)								
No contamination (n, %)	93 (73)	163 (64)	190 (75)	199 (79)	228 (90)	232 (91)	232 (91)	1244 (81)
Contamination (n, %)	34 (27)	91 (36)	64 (25)	55 (21)	26 (10)	22 (9)	22 (9)	280 (18)
Fungal contaminants (n, %)	28 (22)	71 (28)	46 (18)	41 (16)	24 (9)	22 (9)	22 (9)	226 (15)
Bacterial contamination (n, %)	6 (5)	20 (8)	18 (7)	14 (6)	2 (1)	0	0	54 (4)
"AmB 10 v 30": 7H10 (AmB 30mg/ml)								
No contamination (n, %)	112 (88)	203 (84)	213 (88)	218 (90)	232 (96)	232 (96)	234 (97)	1331 (92)
Contamination (n, %)	15 (12)	38 (16)	27 (12)	23 (10)	9 (4)	9 (4)	7 (3)	113 (8)
Fungal contamination (n, %)	11 (8)	27 (11)	19 (8)	16 (7)	9 (4)	9 (4)	7 (3)	87 (6)
Bacterial contamination (n, %)	4 (4)	11 (5)	9 (4)	7(3)	0	0	0	27 (2)
"AmB30 v AC": 7H11 (AmB 30mg/ml)								
No contamination (n, %)	76 (75)	128 (62)	137 (66)	151 (73)	172 (83)	174 (84)	177 (86)	939 (76)
Contamination (n, %)	26 (25)	79 (38)	70 (34)	56 (27)	35 (16)	33 (15)	30 (14)	303 (24)
Fungal contamination (n, %)	21 (20)	54 (26)	46 (22)	45 (22)	32 (15)	30 (14)	29 (14)	236 (19)
Bacterial contamination (n, %)	5 (5)	25 (12)	24 (12)	11 (5)	3 (1)	3 (1)	1 (0)	67 (5)
"AmB30 v AC": 7H11 (AmB 10mg/ml + Carbendazim 50mg/ml)								
No contamination (n, %)	87 (85)	157 (77)	168 (82)	181 (89)	195 (96)	196 (96)	197 (97)	1094 (89)
Contamination (n, %)	15 (10)	47 (23)	36 (18)	23 (11)	9 (4)	8 (3)	7 (3)	130 (11)
Fungal contamination (n, %)	11 (11)	21 (10)	12 (6)	9 (4)	6 (3)	5 (2)	6(3)	59 (5)
Bacterial contamination (n, %)	4 (4)	26 (13)	24 (12)	14 (7)	3 (1)	3 (1)	1 (0)	71 (6)

Table 4.3 Contamination of Middlebrook media containing different anti-fungal drug

Conclusions of "AmB10 vAmB30" comparison

Increasing the AmB concentration to 30mg/l demonstrated a 17% reduction in contaminated samples. Better anti-fungal activity was accompanied by a small drop in bacillary load but this had no effect on modelling of bacillary elimination and the magnitude of the drop in $\log_{10}$CFU/ml was felt to be clinically unimportant.

Additionally, it was reassuring that in samples collected from patients after day 14 of TB treatment, AmB (30 mg/ml) allowed recovery of data from 46% of specimens which were contaminated on the weaker media. As loss through contamination of later samples impairs data analysis, the case for switching to a more potent anti-fungal media was strong.

After this comparison, the benefit of higher dose AmB was deemed to be such that the standard study media was switched to an AmB (30mg/ml) preparation from January 2011. Figure 4.1 demonstrates a sustained reduction in overall SSCC contamination from this point onwards.

4.2.5 Improving SSCC media: the "AmB30 v AmBC" comparison

To establish whether further SSCC media improvements could be achieved, from April-July 2011 a second comparison was undertaken in which sputum samples were incubated in parallel onto AmB (30mg/ml) media and a preparation containing AmB (10mg/ml) supplemented with carbendazim, a benzimidazole anti-fungal treatment used in crops which inhibits fungal (eukaryotic) mitotic microtubulin formation to prevent sporulation and germination of spores[536]. Carbendazim was chosen for this comparison as it has several potential advantages over AmB; alongside a wide range of anti-fungal activity, it is more stable, cheaper and can be stored and shipped at ambient temperature.

Preparation of "7H11-AmB30" and "7H11-AmBC" media

Prior to the "AmB30 v AmBC" comparison, due to a change in available supplies, the agar base for SSCC media was changed from Middlebrook 7H10 to 7H11. The main differences between these two media is that 7H11 contains an enzymatic digestion of casein which is thought to enhance growth of fastidious *M tuberculosis* strains but comparisons in our laboratory (data not shown) indicated that changing the media base alone had no difference on bacillary counts or contamination.

7H11-AmB30 media was made in an identical manner to 7H10-AmB30 except that 21g of 7H11 powder was substituted for 19g of 7H10. For 7H11-AmBC media, a 2% (w/v) suspension of carbendazim (Sigma Aldrich) in 70% ethanol was prepared and added to the

media before autoclaving. It is insoluble in water but at an acid pH in the presence of phosphate and heat it converts to a soluble compound with no loss of anti-fungal activity (personal communication with David Coleman, St George's Medical School, London). OADC and selectatabs were added as usual. Final anti-fungal concentrations in 7H11-AmBC were AmB 10mg/l and carbendazim 50mg/l.

Results of "AmB30 vAmBC" comparison

102 sputum samples were used in this comparison, collected from 44 patients. The mean number of samples per patient was 2.3.

7H11-AC bacillary counts were 0.11 $\log_{10}$ CFU/ml lower (95% CI: 0.02-0.19) than 7H11-AmB30 counts (Table 4.2). As with the "AmB 10 v 30" comparison the difference was statistically significant but small and did not affect the biphasic bacillary elimination curve after NLME modelling of the data (Figure 4.4).

26/102 (25%) 7H11-AmB30 plates were contaminated: 21 by fungi and 5 by bacteria. 15/102 (15%) 7H11-AC plates were contaminated: 11 by fungi and 4 by bacteria (Table 4.3). The relative risk of sample contamination with 7H11-AmB30 was 1.79 (95% CI: 1.01-3.17). Individual plate segment contamination fell from 24% on 7H11-AmB30 to 11% on 7H11-AC. Again, the major reduction was in fungal growth (from 19% to 5%). Overall, 7H11-AmBC was better selective medium for *M tuberculosis*.

20 (77%) samples which were contaminated on 7H11-AmB30 were collected after day 14. As 9 (45%) of these were not contaminated on 7H11-AC, it again appeared that improved selective media reduced data loss at later sampling time-points.

Conclusions of "AmB10 vAmB30" comparison

When compared with AmB30 media, plates containing AmB (10mg/ml) and carbendazim (50mg/ml) displayed a 10% reduction in contamination. Media containing both AmB and carbendazim was best overall. This data did not emerge until late in the study and it was deemed undesirable to change the standard media again, so 7H11-AmBC media was not used after completion of the work described above. However, this preparation is recommended for future SSCC projects in settings in with high rates of fungal contamination.

4.2.6 Patient factors and sample contamination

In addition to the anti-fungal content of SSCC media, patient-related factors may contribute to sample contamination. To assess this, demographic and clinic data from the patients who contributed specimens to the "AmB10 vs Amb30" and "Amb30 vs AmBC" comparisons were combined with microbiology results from those comparisons. For this analysis, samples were regarded as contaminated if there was contamination on either media preparation.

Data from 229 sputum specimens from 96 patients was included. The demographic and clinical profiles of this patient subset were similar to the overall study cohort description provided in Chapter 3. Assessment of patient factors contributing to sample contamination was done by logistic regression, with incorporation of hierarchical random effect modelling in the multivariate analysis to account for repeated sampling from some patients. Results of the logistic regression analysis are shown in Table 4.4.

There was a trend towards less contamination in HIV positive individuals. Malawian HIV treatment guidelines recommend daily co-trimoxazole prophylaxis for all infected persons and fluconazole therapy for patients with oral or oesophageal candidiasis[465] so some HIV-infected individuals may have received prior therapy which reduced oral and respiratory commensual flora. There were also trends toward higher contamination with increasing age and use of wood/charcoal for cooking at home. The latter factor may be related to a general increase in respiratory infections and pulmonary disease associated with indoor air pollution from biomass fuels[537,538].

However, the only significant factor on both univariate and multivariate analysis was that samples collected on later days of therapy were more likely to be contaminated (OR: 1.06, 95% CI: 1.03-1.08, p<0.001). This corroborates earlier observations that contamination was disproportionately high in samples collected after day 14. Later samples may be more prone to contamination as elimination of *M tuberculosis* during therapy facilitates easier growth of other organisms. Generally, it appears that specimens collected on different days in serial sampling studies behave differently in culture. In a longitudinal study, the laboratory methods used must be able to cope with this.

Patient factor associated with the sample	Contaminated on any media[a] N=62	Clean on all media[b] N=167	Univariate analysis			Multivariate analysis		
			Odds Ratio	95% CI	p-value	Odds Ratio	95% CI	p-value
Age (years), median (IQR[c])	34 (27-40)	31 (26-37)	1.04	0.99-1.08	0.060	1.04	0.99-1.04	0.086
Male sex, n (%)	37 (60%)	119 (71%)	0.61	0.30-1.26	0.185	0.72	0.32-1.63	0.430
HIV positive, n (%)	41 (66%)	129 (80%)	0.52	0.24-1.10	0.088	0.43	0.18-0.44	0.071
Baseline CD4 count(cells/µl), median (IQR[c])	163 (72-339)	159 (101-407)	0.99	0.99-1.00	0.442	-	-	-
Patient on ART, n (% of HIV positive)	15 (37%)	40 (31%)	1.50	0.62-3.66	0.368	-	-	-
Smoker, n (%)	14 (22%)	47 (28%)	0.78	0.35-1.72	0.532	-	-	-
Use of biomass fuel (wood/charcoal) for cooking and heating at home (n, %)	55 (88%)	134 (80%)	2.25	0.54-9.32	0.259	1.90	0.64-5.61	0.247
Recent antibiotic use[d], n (%)	57 (92%)	151 (90%)	1.05	0.27-4.00	0.946	-	-	-
Time since initiation of TB treatment, days (IQR[c])	22 (14-49)	4 (0-14)	1.05	1.03-1.06	<0.001*	1.05	1.03-1.07	0.002*

Table 4.4 Patient factors influencing sputum sample contamination

[a]Contaminated on any media = the sample was contaminated in at least one arm of the comparison in which it was studied

[b]Clean on all media = the sample gave a positive' or negative result in both arms of the comparison in which it was studied (i.e. this sample never got contaminated)

[c]IQR=Inter-quartile range

[d]Not including co-trimoxazole prophylactic therapy given routinely to HIV positive individuals

4.2.7 SSCC discussions and conclusions

It was clear during the first three months of the study that SSCC plate contamination was a problem. Sputum from patients after day 14 of TB therapy appeared to be at particular risk, and this was confirmed later by multivariate analysis of patient factors associated with culture contamination. It was feared that loss of study data would compromise modelling of bacillary elimination and jeopardise assessment of microbiological biomarkers of treatment response.

However, two comparisons of SSCC media preparations with different anti-fungal content, demonstrated that contamination could be significantly reduced without compromising measurement of bacillary load and cultures from later time-points of treatment could be salvaged for analysis. These data are the first to show that selective media can be optimised for use in resource-poor settings with high rates of fungal contamination. This is important for the future design of Phase IIb studies using SSCC to evaluate new anti-TB treatment regimes. If better recovery of valid results, particularly in the second month of treatment, is a replicable finding the power of SSCC studies will improve, enhancing capacity to perform treatment studies in African countries where the burden of TB is highest.

There were several limitations to the SSCC optimisation experiments. The media comparisons were not blinded or performed simultaneously. Baseline contamination rates were high and the benefit of altering media may have been smaller in settings with less fungal contamination. Due to resource limitations, precise speciation and drug susceptibility testing of contaminants were not performed. As AmB is generally unavailable in Malawi it is unlikely that antimicrobial resistance contributed to plate contamination.

Overall, increasing the AmB dose in SSCC media to 30mg/ml assisted in the conduct of this study and high concentrations of anti-fungal drugs can be recommended for future work either in the form of AmB 30mg/ml or the carbendazim method.

4.3 MGIT liquid culture

The second microbiological method used to monitor bacillary elimination was the MGIT liquid culture system described in Section 1.10.2. Sputum was inoculated into commercially prepared microMGIT tubes containing 4ml Middlebrook 7H9 broth and an oxygen-quenched fluorescent indicator. A manual microMGIT reader was used to repeatedly measure fluorescence intensity in the culture tubes. When fluorescence reached a pre-determined threshold the tube was read as "positive". TTP was used as an inverse marker of bacillary load.

4.3.1 Sputum sample decontamination

Although liquid culture supports bacillary growth more effectively than solid media[282] growth of non-tuberculous mycobacteria (NTMs) and other bacteria/fungi is also enhanced and high contamination rates (3.7-8% in high-income settings[539,540] and 29.3% in Zambia[541]) are often a limitation of MGIT culture systems. Therefore, it was necessary to routinely decontaminate all sputum samples for this part of the study.

As described in section 2.6.1, after sputum homogenisation, 1ml aliquots of each sample were used for MGIT culture. These were placed in 50ml Falcon tubes. 10µl was transferred with a sterile loop onto microscope slides to make initial smears for ZN and AP staining (methods in Appendices 10.4.1 and 10.4.2).

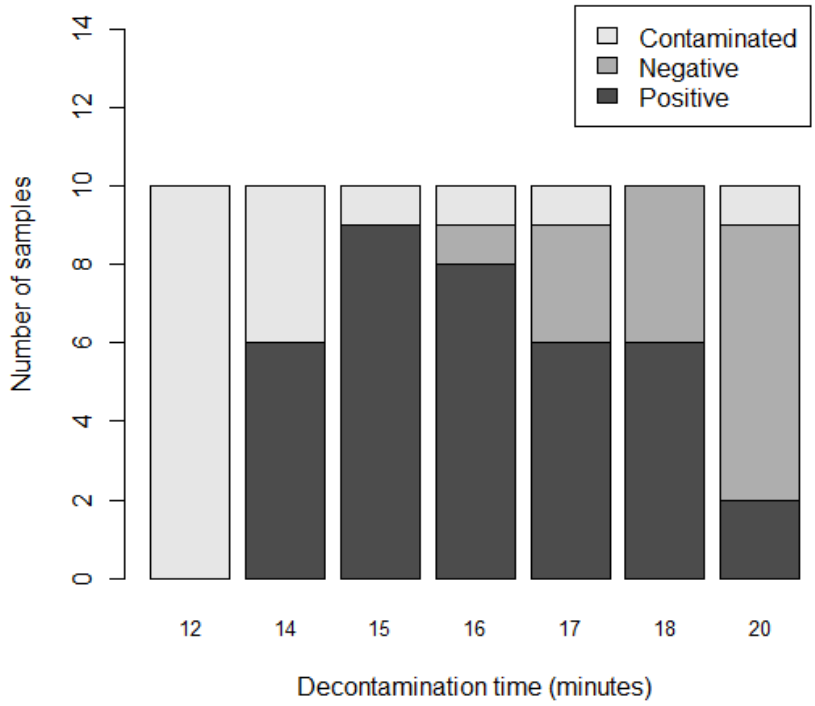
The NAC-PAC EA3 system (AlphaTec Systems, Vancouver) was used for sample decontamination. 300mg N-Acetyl-L-Cysteine (NALC) was added to 60ml of NAC-PAC Red TB Base™ solution containing 3% NaOH. 1ml of NALC/NAC-PAC Red was added to each sputum sample, turning the contents of the Falcon tubes pink/red. Samples were allowed to stand for a decontamination time of 15 minutes, with vortexing 2-3 times during this period.

NPC-67™ AFB neutralising buffer was then slowly added to each tube until effective neutralisation of NaOH was indicated by a colour change from pink/red to colourless. Samples were spun at 3000 x *g* for 15 minutes. After discarding of supernatant the remaining pellets were re-suspended in 1ml of NACPAC Pellet Resuspension Buffer. 10µl of the re-suspended pellets were used to make concentrated smears for ZN and AP staining.

Ensuring optimal sample decontamination time was important; excessive NaOH exposure kills *M tuberculosis* bacilli resulting in false negative results and under-estimation of

bacillary load whilst inadequate exposure results in insufficient destruction of non-mycobacterial organisms and a high contamination rate. Prior to the clinical study, 10 smear '+++' sputum samples were divided into 7x1ml aliquots and prepared for liquid culture with decontamination times ranging from 12-20 minutes (Figure 4.5). A decontamination time of 15 minutes was selected as this resulted in the highest yield of positive results, and only one sample was lost to contamination.

Median TTP varied with decontamination time. After commencement of patient recruitment, the decontamination time remained fixed to prevent variation in NaOH exposure from affecting the consistency of TTP readings.



TTP in days, median (range)	NA	4 (3-7)	4.5 (3-8)	4 (3-5)	6 (4.5-8)	4 (6-10)	10 (10-10)

Figure 4.5 Selecting a decontamination time for MGIT cultures

Methods to confirm that organisms grown in MGIT culture are *M tuberculosis* rather than contaminants are outlined in Section 5.3.3.

4.3.2 Inoculation and incubation of MGIT bottles

For each specimen, 0.5ml MGIT-OADC enrichment supplement was added to a 4ml microMGIT bottle. Freeze-dried MGIT-PANTA antibiotic supplement was reconstituted in sterile water (each vial containing polymyxin B 6000 units, trimethoprim 600µg, amphotericin B 600µg, azlocillin 600µg, nalidixic acid 2400µg) and 0.1ml was also added. 0.5ml of the decontaminated and re-suspended sputum pellet was transferred to the tube, which was tightly capped, placed in a rack and incubated at 37^{o}C.

4.3.3 Confirming MGIT culture positivity

MicroMGIT tubes were read for fluorescence twice per day for 49 days. The MicroMGIT reader was calibrated prior to each use. Sample tubes were read as positive or negative. Bottles which were positive on two consecutive occasions were removed from the incubator and TTP was recorded in intervals of 0.5 days. Bottles which were not positive by 49 days were removed and reported as negative.

Macroscopic colony appearance cannot be used to confirm that organisms grown in liquid broth are *M tuberculosis*[24]. Therefore, an algorithm was developed to identify the contents of positive bottles (Figure 4.6).

The contents of each positive bottle were concentrated by centrifugation and a ZN smear was prepared from the re-suspended cell pellet. If AFB were identified, two rapid mycobacterial speciation tests were performed.

Firstly, slides was assessed for microscopic cording (a property of *M tuberculosis* in which bacilli form serpentine cords with the orientation of the long axis of each cell running parallel to the long axis of the cord [Figure 4.7, Image A]). As cording is a virulence-related characteristic absent from most NTMs, cord formation on ZN microscopy is regarded as a discriminating feature of *M tuberculosis*[542].

Secondly, MPT64 Antigen testing was performed on 100µl of each re-suspended cell pellet using an MGIT TBc Identification Test kit[543]. MPT64 is a mycobacterial protein specific to *M tuberculosis* complex cells which is secreted during culture. The test kit is a chromatographic lateral flow immunoassay which uses monoclonal antibodies to detect this protein (Figure 4.7, Images B and D). Cultures which contained AFB in cords and were positive on the MPT64Ag test were confirmed as *M tuberculosis.*

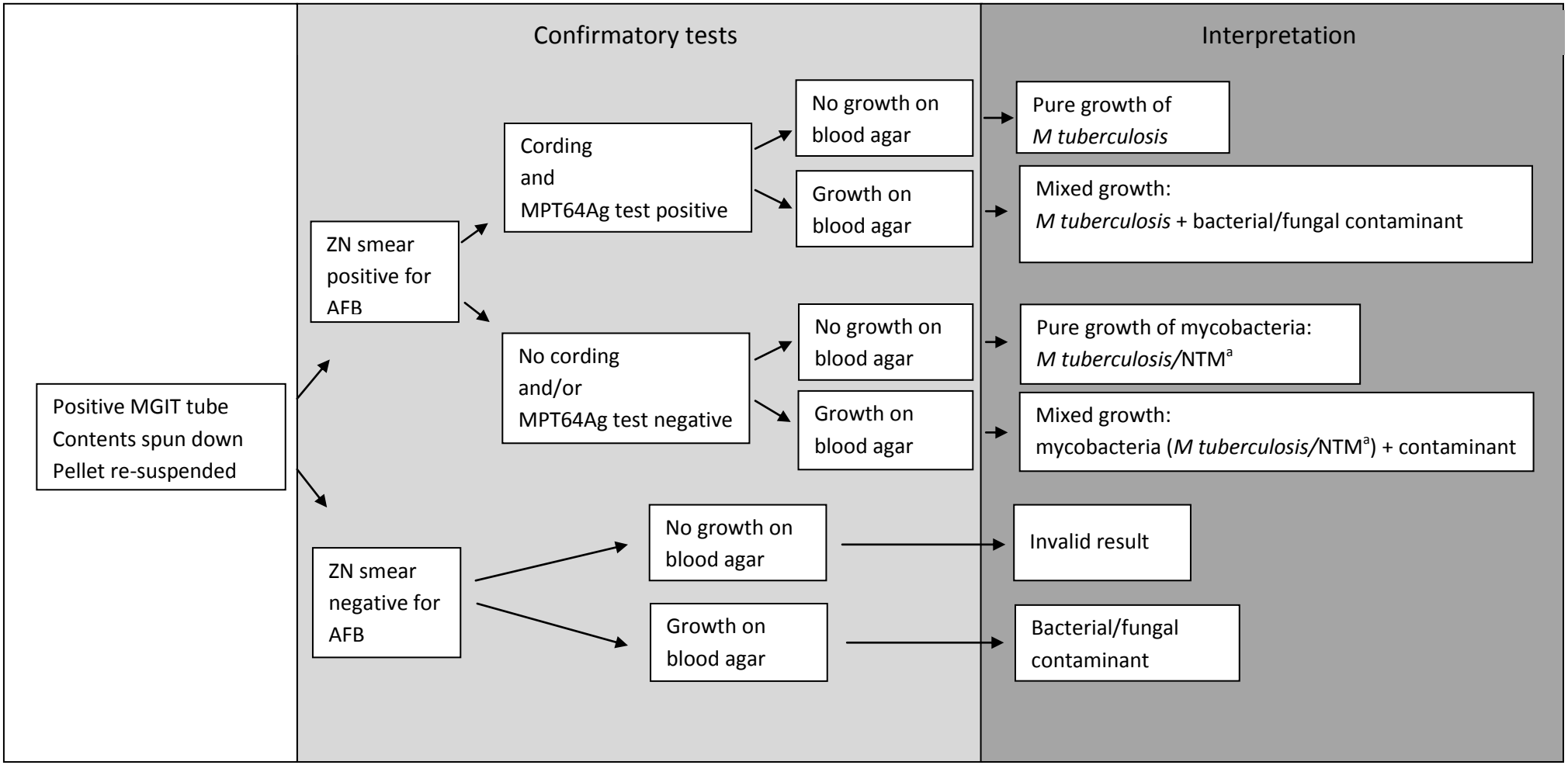


Figure 4.6 Confirmatory tests for positive MGIT cultures

[a]To clarify mycobacterial species, isolates were inoculated onto a set of 4 LJ slopes (37°C, 25°C, 45°C and supplemented with Paranitrobenzoic acid). Growth of AFBs at 37°C only indicated *M tuberculosis.* Growth on any other slope indicated non-tuberculous mycobacteria (NTM).

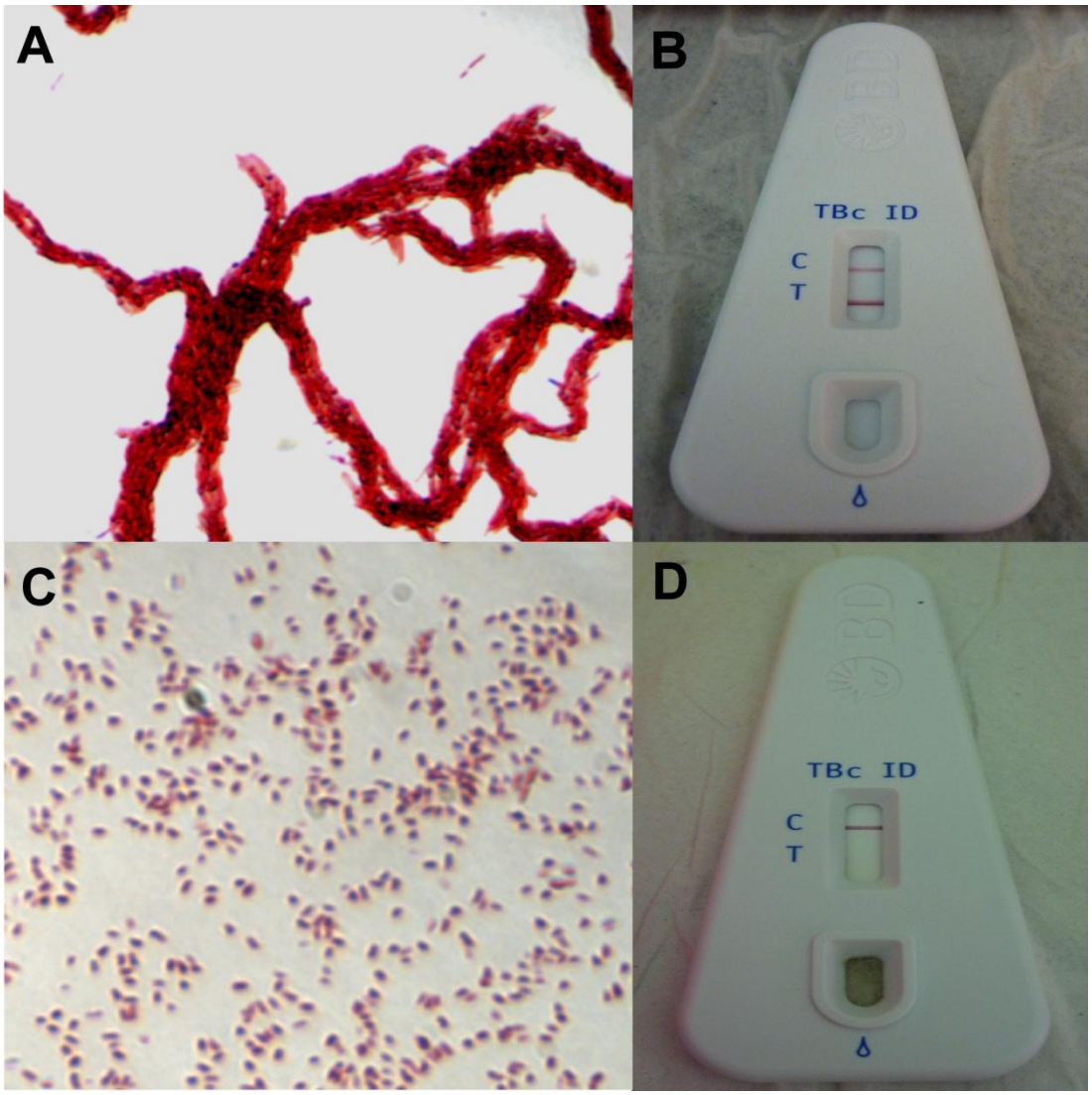


Figure 4.7 Microscopic cording and MPT64 Ag identification tests

A: An AFB positive ZN smear from a positive MGIT bottle which demonstrates cording. B: A positive MPT64Ag test (there is clear pink/red line in both Control (C) and (T) test windows). C: An AFB positive ZN smear in which cells have different morphology and there is no cording. D: A negative MPT64Ag test (there is a clear line in the Control (C) but not in the test (T) window). Together, A&B indicate growth of *M tuberculosis*. C&D indicate non-tuberculous mycobacterial.

AFB positive MGIT isolates which were negative for either cording or MPT64 underwent further assessment using LJ slopes as a reference test (Figure 4.8, LJ culture method details in Appendix 10.5). Although *M tuberculosis* grows well on LJ slopes at 35-37°C it does not grow at room temperature (25°C), at 45°C, or when media is supplemented with paranitrophenol benzoic acid (PNB). NTM species show variable growth under all of these conditions[544]. Therefore, samples were sub-cultured onto four LJ slopes as shown in Figure 4.8. Growth occurring only at 37°C was confirmed as *M tuberculosis*. Growth under any other conditions indicated an NTM. More detailed speciation of NTMs was not undertaken.

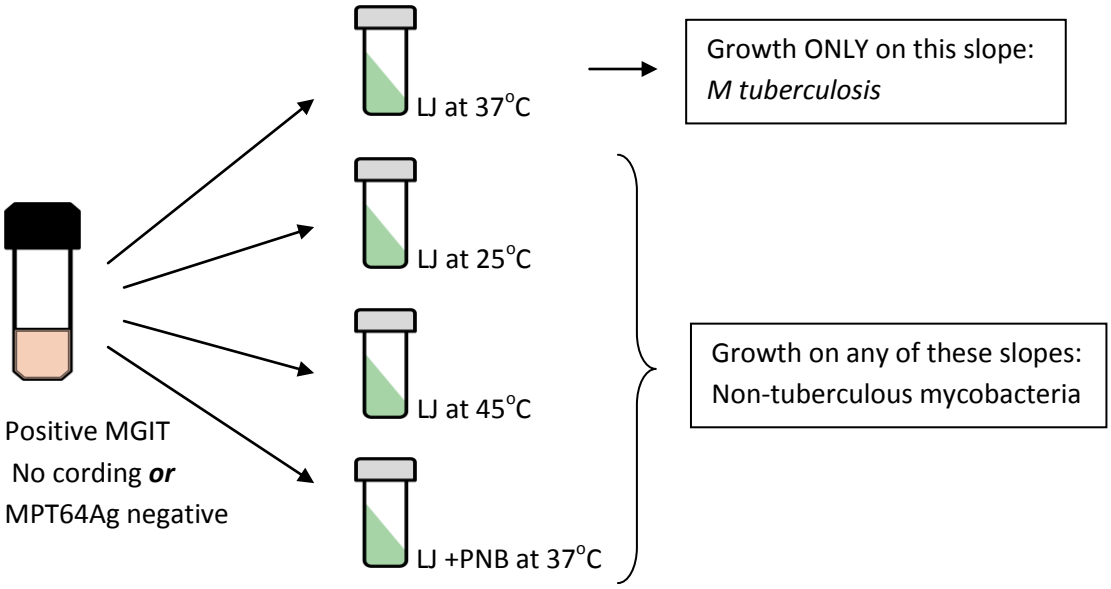


Figure 4.8 Use of LJ slopes to discriminate between *M tuberculosis* and NTMs

Finally, 50µl of each pellet was inoculated onto a blood agar plate for 48 hours to identify contamination by non-mycobacterial organisms (bacteria/fungi).

783 samples from baseline and S1-4 patient visits were set up for MGIT culture during the clinical study (Table 4.5). 584 demonstrated mycobacterial growth. In 566 (73%) cases this was due to *M tuberculosis* and in 18 (2%) cases it was due to an NTM. 138 (18%) samples were negative and 37 (5%) were contaminated by bacteria/fungi[a]. 19 (2%) samples signalled positive on the microMGIT reader but were regarded as invalid because all identification tests were negative. All NTMs, contaminated and invalid samples were excluded from pharmacodynamic data analysis.

[a] 24 of these samples also grew *M tuberculosis* but were regarded as contaminated because growth was polymicrobial

Culture result/ organism id	Day of sample collection				Total N=783
	Baseline N=170	Day 1-14 N=303	Day15-28 N=155	Day29-56 N=150	
Mycobacterial growth *M tuberculosis* (n,%) NTMs (n, %)	 146 (86) 3 (2)	 261 (86) 7 (2)	 117 (76) 3 (2)	 42 (28) 5 (3)	 566 (73) 18 (2)
Contaminated (n, %)	15 (9)	11 (4)	8 (5)	3 (2)	37 (5)
Negative (n, %)	6 (4)	22 (7)	24 (16)	86 (57)	138 (18)
Invalid (n, %)	0 (0)	2 (1)	3 (2)	14 (9)	19 (2)

Table 4.5 Results of MGIT culture for clinical study

The overall rate of contamination was similar or lower to other recent reports from Africa[527], indicating successful introduction of MGIT culture to the laboratory. Recovery of NTMs was lower than other African studies (21.1%[527] in South Africa and 33.3% in Sudan[545]). This reassuringly suggests that most strongly sputum smear positive patients presenting to QECH for TB treatment have *M tuberculosis* rather than a misdiagnosed NTM. It also suggests that modelling of bacillary elimination using TTP data from this cohort is unlikely to be confounded by accidental inclusion of NTMs.

In contrast to SSCC, there was a slightly lower risk of contamination in MGIT cultures from sputum collected on later treatment days (OR: 0.98, 95% CI: 0.97-0.99, p<0.0001)[a]. This may be an advantage for serial sampling studies seeking to grow 'persister' organisms.

Table 4.6 shows identification tests results for pure positive *M tuberculosis* cultures.

Identification tests	Day of sample collection				Total N=566
	Baseline N=146	Day 1-14 N=261	Day14-28 N=117	Day 28-56 N=42	
Cording & MPT64Ag positive LJ not done (n, %)	144 (99)	242 (93)	98 (85)	34 (83)	516 (91)
Cording positive, MPT64Ag negative *M tuberculosis* on LJ (n, %)	0 (0)	7 (3)	6 (5)	4 (10)	17 (3)
MPT64Ag positive, cording negative *M tuberculosis* on LJ (n, %)	2 (1)	12 (5)	11 (9)	3 (7)	28 (5)
Cording and MPT64Ag negative *M tuberculosis* on LJ (n, %)	1 (1)	0 (0)	2 (1)	2 (5)	5 (1)

Table 4.6 Rapid mycobacterial identification tests from positive MGIT cultures

[a] As in Section 4.2.6 the odds ratio for contamination risk per successive day of sample collection was done by logistic regression with incorporation of hierarchical random effect modelling in the multivariate analysis to account for repeated sampling from each patients.

91% of pure positive cultures had concordant positive results for both microscopic cording and MPT64Ag. Isolates which were positive on only one of the rapid mycobacterial identification tests were set up on LJ slopes. All of these demonstrated typical *M tuberculosis* growth characteristics, suggesting that both tests were highly specific.

The cost of preparing ZN slides for microscopy is approximately $0.1 per slide, whilst the cost of a single MPT64Ag test kit is $2.90. Given the high concordance between the results, the additional cost of MPT64Ag testing may be considered unnecessary in a resource poor setting. However, 45 *M tuberculosis* isolates would have been misclassified as NTMs if both tests had not been performed (28 cording negative, MPT64Ag positive cultures and 17 cording positive, MPT64Ag negative cultures all of which had *M tuberculosis* growth subsequently confirmed on LJ slopes). Additionally, in 5 AFB positive cultures both rapid tests were negative making the sample invalid until LJs slopes grew *M tuberculosis*. Overall, the use of an algorithm incorporating multiple mycobacterial identification methods prevented loss of 50 (9%) positive samples.

It is observed from Table 4.6 that the risk of a false negative result from at least one of the rapid mycobacterial tests was progressively higher in samples collected on later treatment days (OR : 1.05, 95% CI: 1.04-1.07, p<0.0001)[a]. Although the reason for this is unclear, it is possible that viable *M tuberculosis* bacilli lose some of their ability to form cords or secrete MPT64Ag when exposed to chemotherapy. Whatever the explanation, it appears once again that specimens collected on different days behave differently and multiple identification techniques are particularly indicated to study growth of late persister organisms.

4.3.4 Manual v automated MGIT readings

As MicroMGIT bottles were only read manually twice per day, there was concern that TTP readings expressed in intervals of 0.5 days may be inaccurate. During the course of the study an automated BACTEC MGIT 960 machine became available in the laboratory. This is a self-contained incubation unit in which MGIT culture bottles are continuously tested for fluorescence to give a TTP reading for positive in intervals of 0.1 days. Sample decontamination and inoculation steps are identical to the manual system.

[a] As in Section 4.2.6 the odds ratio for contamination risk per successive day of sample collection was done by logistic regression with incorporation of hierarchical random effect modelling in the multivariate analysis to account for repeated sampling from each patients.

After installation of the BACTEC MGIT system, a sequential set of 74 samples were incubated in parallel for manual MicroMGIT and for automated fluorescence reading in the machine. 51 (69%) were positive, 15 (18%) were negative and 8 (11%) were contaminated on manual reading, whilst 50 (68%) were positive, 13 (18%) were negative and 11 (15%) were contaminated on the automated system. Correlation between TTP readings for samples which were positive via both methods was high (Figure 4.9)

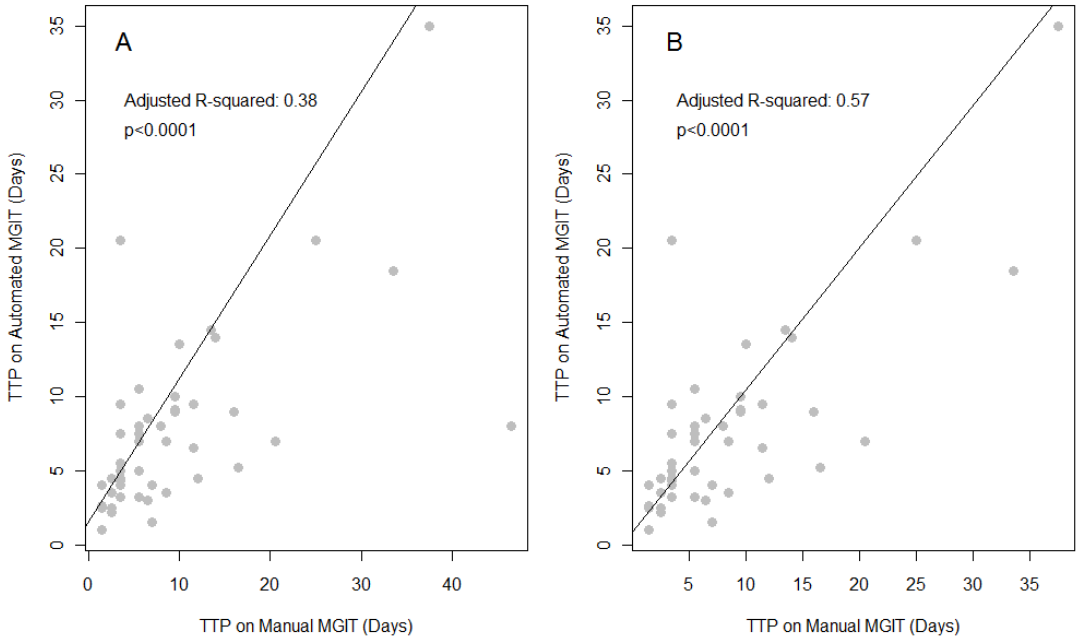


Figure 4.9 TTP of positive samples on manual and automated MGIT cultures

50 samples were positive on both culture systems, with good correlation in TTP readings. Panel A includes all samples including one outlier (Manual TTP: 46.5 days, Automated TTP: 8 days). In Panel B, this data-point is removed and the adjusted R^2 correlation co-efficient rises to 0.57.

Although automated culture has considerable advantages of convenience over twice daily manual tube reading, a decision was made to complete the clinical study using manual reading to ensure methodological consistency. The strong correlation between manual and automated results provided confidence in the reliability of TTP readings achieved by both methods.

4.4 In vitro relationship between log_{10} CFU/ml and MGIT TTP

The use of MGIT TTP readings on serial patient samples to monitor bacillary elimination relies on the principle that TTP is inversely related to bacillary load. Prior data supports this assumption and the relationship between SSCC log_{10} CFU/ml counts and MGIT TTP from study samples will be analysed in detail in Chapter 6. An *in vitro* test of concordance between the two quantitative bacteriology methods was also performed to confirm the relationship between the techniques.

TB reference strain H37Rv (NCTC number 3616), obtained from the National Culture Type Collection, was grown in a MGIT bottle until three days after a positive signal was obtained on the microMGIT reader. Five 10-fold dilutions were prepared in sterile PBS and inoculated in parallel in two microMGIT tubes and on two sets of 7H10-AmB10 plates. Reading of fluorescence and plate counting were performed as described above. Figure 4.10 demonstrates a strong relationship between the mean TTP from liquid culture bottles and mean log_{10}CFU/ml counts from agar plates (adjusted R^2=0.92, p=0.006).

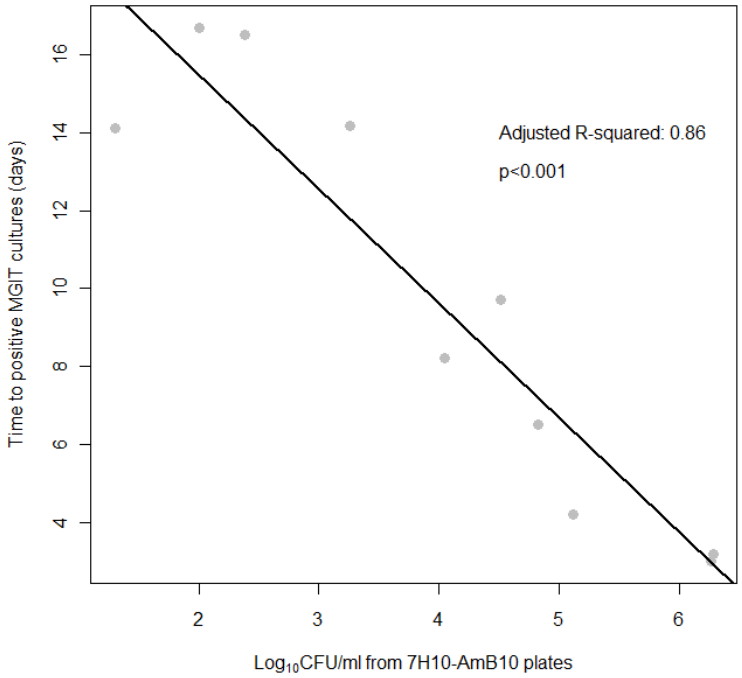


Figure 4.10 In vitro bacillary load by SSCC and MGIT using H37Rv

4.5 MGIT culture discussion and conclusions

As with SSCC, the MGIT liquid culture system was successfully introduced to the CoM TB laboratory. Sample decontamination was optimised and standardised prior to commencement of patient recruitment. A series of tests were performed to identify organisms growing in MGIT cultures. As there was increased risk of false negative results from individual rapid tests for *M tuberculosis* at later time points, an algorithm incorporating multiple tests was necessary to ensure that no positive cultures were missed.

A manual microMGIT reader was used to measure fluorescence. TTP readings from this method correlated well with results from the automated BACTEC MGIT 960 system when a sub-set of samples were run in parallel. Whilst the automated system is considerably more time-efficient and convenient and the cost of reagents for the two systems is comparable, the initial financial outlay to install an automated MGIT system is $39,950 compared to $850 for a manual MGIT reader. Although provision of a 37^{o}C incubator and additional staff costs for weekend readings of tube fluorescence have to be included for the manual system it worked in this study and is a useful option in resource-poor settings.

An *in vitro* comparison of bacillary load measurement with using $\log_{10}$CFU/ml counts from SSCC plates and TTP from MGIT bottles showed close correlation. Analysis of the relationship between the results of the two culture systems using clinical samples will be described in Chapter 6.

Overall, introduction of both quantitative culture techniques to the TB laboratory was a considerable capacity building investment. Careful attention to training, technical optimisation and quality control procedures was vital to generate reliable microbiological data from the clinical cohort and laid the groundwork for future TB therapeutics studies in Malawi, including trials of new candidate anti-TB regimens.

5. Optimising Single Cell Techniques

5.1 Introduction

Although quantitative sputum culture is important in pharmacodynamic monitoring of TB therapy there are two major limitations to this approach. Quantitative cultures only measure the treatment effect of total bacillary load, so identifying and directly monitoring the effect of persister sub-populations of drug-tolerant organisms is impossible. Additionally, persisters may acquire phenotypic drug tolerance by entering a non-replicating, metabolically quiescent state. Non-replicating organisms are difficult to revive in standard culture[546], making their analysis more challenging.

Two non-culture based techniques which assess cells on an individual basis may allow selective study of non-replicating bacillary sub-populations; fluorescence lipid body microscopy and flow cytometry. A possible role for these techniques was evaluated in Liverpool prior to the clinical study. Both techniques required use of equipment housed outside CL3 containment facilities. Biosafety experiments were done to ensure that samples for these methods were safe for manipulation outside the BSC.

5.2 Handling samples outside the Bio-safety Level 3 laboratory

5.2.1 TB infection control and the laboratory

The risk of TB to Health Care Workers (HCWs) is high. Studies from low and middle income countries report an annual incidence of 69-5780 clinical cases of TB disease per 100,000 HCWs[547,548]. In Malawi, the risk of TB disease amongst HCWs is 1446-5361 cases per 100,000 person-years[549-552]. Peruvian data reports that the incidence risk ratio for TB disease between laboratory workers and the general population is 78.6[553] suggesting that laboratory workers are particularly vulnerable.

Throughout this project, in accordance with international guidelines, all smear preparation, media inoculation and isolate identification was performed in a BSL-3 laboratory. However, in Liverpool and Malawi the fluorescence microscope and flow cytometer were in BSL-2 facilities. Bacilli on microscopy smears remain viable even after heat-fixation at 75^{o}C for 2 hours[554] and several steps in flow cytometry procedures including sample aspiration and ejection allow dissemination of aerosolised droplet nuclei containing *M tuberculosis*[555].

Prior reports state that staining of sputum smears or treatment of flow cytometry samples with 0.5-1% formaldehyde renders them non-infectious but this data is sparse and there have been few publications on the bio-safety of sample processing for 15-20 years[555,556]. It was, therefore, necessary to show that there were no viable bacilli in laboratory TB isolates or clinical specimens before removal from the BSL-3 laboratory for analysis.

5.2.2 Method to assess bio-safety of smears and cell suspensions

Three samples were used for bio-safety experiments; an *in vitro* isolate of H37Rv[a], a "spiked sputum" sample (1ml of the H37Rv isolate added to 1ml of smear negative sputum) and a clinical smear '+++' sputum sample. Sputum was homogenised with dithiothreitol before the procedures described below. 20µl aliquots of each sample were smeared onto 12 microscopy slides. Slides 1-2 were air-dried in the BSC for 30 minutes. Slides 3-12 were fixed on a hotplate at 85^{o}C until smears were dry and the slides were hot to touch. Slides 5-6 were then immersed in 1% formaldehyde for 30 minutes and rinsed in sterile PBS. Slides 7-8, 9-10 and 11-12 were stained according to AP, ZN and Gram protocols. Using sterile forceps all slides were then placed inside 50ml Falcon tubes containing selective Middlebrook 7H9 broth and incubated at 37^{o}C for 7 days. The broth was then centrifuge-concentrated and 0.5ml of each concentrate was set up for in MGIT tubes. Organisms in positive MGIT bottles were identified using the methods in Section 4.3.3. Negative bottles at 42 days were deemed not to contain viable bacilli.

Flow cytometry is done on cell suspensions, not microscopy smears. Therefore, after slide preparation, the three test samples were suspended in sterile PBS and divided equally between five 15 ml Falcon tubes for further experiments.

For each sample, Tube 1 was left untreated. 2ml 0.5% formaldehyde were added to tubes 2 and 3 and 2ml 1% formaldehyde were added to tubes 4 and 5. Tubes 2 and 4 were left at room temperature for 30 minutes. Tubes 3 and 5 were left for one hour. Thereafter, cells in all tubes were washed with 10ml sterile PBS, centrifuged and re-suspended. 125µl of concentrate from each tube were inoculated onto two 7H10-AmB10 plate sets. 250µl were inoculated into two liquid culture bottles. Cultures were incubated and read as usual.

[a] H37Rv was grown in liquid culture, centrifuged at 3000xg for 10 minutes and re-suspended in sterile PBS. ZN smears and colony counting of the re-suspended cell pellet showed *M tuberculosis* at a concentration of 4.6 x10^{9} CFU/ml, similar to the bacillary load in strongly smear positive sputum.

5.2.3 Results of bio-safety experiments

M tuberculosis was grown from all air-dried or heat-fixed smears and untreated cell suspensions. After AP, ZN or Gram's staining or formaldehyde treatment, culture of all smears (Table 5.1) and cell suspensions (Table 5.2) were negative.

Slides	Sample processing	Results of TB liquid culture after processing		
		H37Rv *in vitro* isolate	H37Rv spiked sputum	Smear '+++' sputum
		Positive bottles *Mean TTP*	Positive bottles *Mean TTP*	Positive bottles *Mean TTP*
1 + 2	Air dried	2/2 (100%) *5.5 days*	2/2 (100%) *3.5 days*	2/2 (100%) *6.5 days*
3 + 4	Heat-fixed (85°C)	2/2 (100%) *7 days*	2/2 (100%) *7.5 days*	2/2 (100%) *11.0 days*
5+ 6	1% formaldehyde	0/2 (0%)	0/2 (0%)	0/2 (0%)
7 + 8	AP stained	0/2 (0%)	0/2 (0%)	0/2 (0%)
9 +10	ZN stained	0/2 (0%)	0/2 (0%)	0/2 (0%)
11 +12	Gram's stain	0/2 (0%)	0/2(0%)	0/2 (0%)

Table 5.1Biosafety of smears on slides prepared for TB microscopy

Formaldehyde treatment		H37Rv *in vitro* isolate		H37Rv spiked sputum		Smear '+++' sputum	
		7H10 plates *Mean CFU/ml*	Liquid culture *Mean TTP*	7H10 plates *Mean CFU/ml*	Liquid culture *Mean TTP*	7H10 plates *Mean CFU/ml*	Liquid culture *Mean TTP*
Untreated		Positive *3.1 x10^8*	Positive *7.0 days*	Positive *7.5 x10^7*	Positive *5.5 days*	Positive *5 x 10^4*	Positive *12 days*
0.5%	30 mins	Negative	Negative	Negative	Negative	Negative	Negative
	60 mins	Negative	Negative	Negative	Negative	Negative	Negative
1%	30 mins	Negative	Negative	Negative	Negative	Negative	Negative
	60 mins	Negative	Negative	Negative	Negative	Negative	Negative

Table 5.2 Growth of *M tuberculosis* after formaldehyde treatment

5.2.4 Conclusions from bio-safety experiments

AP, ZN or Gram's staining of microscopy smears kills all bacteria. For this project, only stained smears were examined outside the CL3 laboratory.

These data are relevant for clinics and hospitals in Malawi where smear microscopy is performed daily without CL3 facilities. When possible, smears should be stained immediately. Unstained slides for storage or transportation can be rendered non-infectious by 1% formaldehyde immersion for 30 minutes.

Bacilli in cell suspensions are also inactivated by 1% formaldehyde treatment for 1 hour. Flow cytometry samples were treated in this way before removal from the BSC.

5.3 Fluorescence lipid body microscopy

5.3.1 Mycobacterial lipids and bacillary persistence

M tuberculosis contains a diverse and complex collection of lipids. These are broadly divisible into amphiphilic phospho- and glyco- lipids in the external cell envelope and hydrophobic TAG in the cytoplasm[557]. Historically, study of mycobacterial lipid biochemistry has focussed on the external "waxy coat". However, since the 1950s it has been proposed that intracellular lipids are important to long-term bacillary survival[163]. The growing body of contemporary evidence was discussed in Section 1.7.2. In Figure 1.6 it was proposed that, except during prolonged starvation, persister bacilli may be phenotypically identifiable by the presence of microscopically visible TAG inclusion bodies

5.3.2 Intra-cytoplasmic lipid bodies: identification and importance

Intra-cytoplasmic lipid bodies were first reported in 1946[558,559] but have attracted renewed interest in the last decade. In 2002, Garton and colleagues used the fluorescence probe Nile red (9-diethylamino-5H-benzo[α]phoenoxazine-5-one) to demonstrate TAG lipid bodies (LBs) in *M smegmatis* grown to stationary phase or cultured in media supplemented with fatty acids. A combined Auramine O/Nile red (ANR) assay was also developed to demonstrate LBs in *M tuberculosis* [344]. Using this technique, LB counts (the proportion of TB bacilli containing LBs) could be calculated in microscopy smears.

Three studies have subsequently employed ANR microscopy and LB counts to study lipid metabolism in experimental models of bacillary persistence[154,168,175]. In each, transcriptomic analysis was performed to identify changes in gene regulation associated with different conditions of growth and lipid metabolism. Key findings are summarised in Table 5.3.

In all three studies LB counts increased during stress and transcriptomic patterns were similar; lipid metabolism genes including *tgs1*, *lipY* and *icl-1* were up-regulated, and TCA cycle genes coding for aerobic respiration were down-regulated. In Garton's study, higher LB counts amongst 15 smear '+++' clinical samples were correlated with longer TTP in liquid culture. Viewed collectively, this supports an association between LB positivity, metabolic quiescence and slow bacterial growth.

Lead author(s)	Year	Source of *M tuberculosis*	Model of persistence	Description of observed bacillary phenotype	Transcriptomics[a]
Garton NJ & Waddell SJ[175]	2008	H37Rv & clinical isolate	'Wayne & Hayes' hypoxic NRP model	1. High LB counts (29-65%) in NRP cultures	**Up-regulated:** DosR genes (*tgs1, hspX, narK2*) and glyoxylate shunt genes (*icl-1)* **Down-regulated:** aerobic respiration chain genes (*nuoB, qcrC,ctaD*)
		Baseline smear positive sputum samples	-	1. Mean LB count: 45% (range 3-86%) 2. Strong correlation between LB counts in smear '+++' sputum and TTP of liquid culture	
Deb C[154]	2009	H37Rv	Multiple stress model (5% O_2, 10% CO_2, nutrient starvation, acidic [pH5])	1. Increased LB counts (70% by day 18)[b,c] 2. Cessation of bacillary replication 2. Loss of acid-fastness 3. Phenotypic tolerance to antibiotics (isoniazid and rifampicin)[c]	**Up-regulated:** DosR genes (*tgs1,hspX*) and glyoxylate shunt genes (*icl1, citA*) **Down-regulated:** Aerobic respiration chain genes (*mdh*)
Daniel J[168]	2011	H37Rv	Hypoxic (1% O_2) intra- macrophage cultures	1. Increased LB counts (81% by day 5)[b] 2. Loss of acid-fastness 3. Phenotypic tolerance to antibiotics (isoniazid and rifampicin)	**Up-regulated:** DosR genes (*tgs1, hspX),* glyoxylate shunt genes (*icl1)* and TAG hydrolysis genes (*LipY)*

Table 5.3 Studies of *M tuberculosis* persistence incorporating LB counts

[a]Changes in transcript levels vs. standard aerobic cultures only reported for selected genes in each study.

[b]Increased in LB counts over time were mirrored by increased TAG detection via Thin Layer Chromatography

[c]*tgs1*deletion mutant organism demonstrated impaired ability to accumulate TAG and less antibiotic tolerance. *tgs1*re-complementation reversed these effects

In two of the studies (Deb and Daniel), LB positive, *tgs-1* expressing bacilli demonstrated phenotypic antibiotic tolerance. Tolerance of isoniazid greatly exceeded that of rifampicin, a finding compatible with rifampicin's established role as a better sterilising drug. Combined with a separate report of LB induction by isoniazid[560] this suggests that LB positive bacilli possess appropriate phenotypic features for persistence during treatment.

Garton's study reported wide variation of LB counts amongst 83 baseline sputum samples (3-86%) but the relationship between LB positivity and persistence during clinical infection has never been studied. It is important to address this. Therefore, during the clinical study two specific questions were posed by fluorescence LB microscopy;

1. Does the LB count in the baseline sputum samples of smear positive TB patients affect treatment response?
2. Does the LB count in serially collected sputum samples during therapy change as a result of drug exposure?

Analysis of LB counts from study samples in relation to these questions will be described in Section 6.5. The next section of this chapter will describe how the microscopy method was optimised to ensure robust and standardised data collection.

5.3.3 Samples used to optimise LB microscopy

Experiments to improve and standardise LB microscopy methods were done in LSTM from August 2009-February 2010. LBs are more easily visualised within non-pathogenic species of mycobacteria than *M tuberculosis* (NJ Garton, personal communication) so initial experiments were done using Nile red staining of *in vitro* cultures of fast-growing *M smegmatis* (MC^2-155). Thereafter, baseline sputum samples from two patients with smear positive pulmonary TB diagnosed at Royal Liverpool University Hospital were used. Both patients provided large volumes (>30ml) sputum which grew *M tuberculosis* without contamination on SSCC plates and in MGIT culture. These samples were separated into 1ml aliquots and frozen at -20^{o}C to allow repeated use without exposure to multiple freeze-thaw cycles. Ethical approval for use of clinical samples in was obtained from Bolton NHS Research Ethics Committee (09/H1009/27, approval letter attached as Appendix 10.3). On arrival in Malawi, the final microscopy protocol was re-validated on a set of local sputum samples prior to assessment of specimens from the clinical study.

5.3.4 Initial fluorescence stains, filters and image analysis methods

M smegmatis was incubated on 7H10-AmB10 plates at 37°C. On days 2, 4, 6 and 8 a few colonies were harvested using a disposable loop, re-suspended in sterile PBS and passed through a 70µm filter to break up clumps. 10µl of each sample were heat-fixed onto a pre-demarcated 25mm x 15mm elliptical area of a microscopy slide.

Smears were labelled with Nile red solution (10µg/ml in ethanol) for 10 minutes, washed with mycobacteria-free distilled water and counterstained with $KMNO_4$ (0.5% w/v) for 1 minute. After a further wash with distilled water, slides were mounted in PBS, transported in a closed box to the microscopy dark room and read within 24 hours using a Leica DMLB microscope equipped with an EL6000 alignment-free metal halide bulb for epifluorescence illumination at high power (x1000) magnification[a].

Maximum excitation of Nile red fluorescence occurs at a wavelength of 549nm, and maximum emission occurs at 628nm so a long-pass Wide Green (WG) filter was used for LB assessment[163,166]. A Lecia DFC300FX R2 digital camera attached to the microscope was linked to a desktop computer. Each smear was assessed by three systematic sweeps (Figure 5.1), and 100 organisms were photographed. Images were viewed later using Corel Paintshop Photo Pro x3 and the proportion of cells containing LBs was recorded.

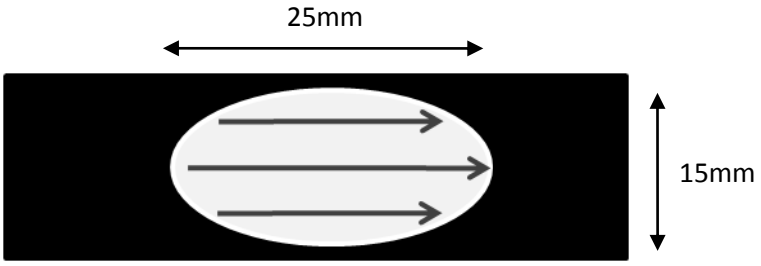


Figure 5.1 Standard approach to smear reading on microscopy slide
Cellbond slides contain a standard elliptical surface area to ensure a uniform size and thickness of microscopy smears (maximum diameter 25mm x15mm). During microscopy three horizontal sweeps (direction shown by arrows) were made of each smear.

Sputum has a more complex background matrix that *in vitro* cultures of so, as with Garton's method[344], *M tuberculosis* bacilli from clinical samples were identified using Auramine O prior to co-staining with Nile red. 1ml of each sample was dithiothreitol treated and centrifuge-concentrated. 10µl smears were heat-fixed onto slides and labelled with Auramine O/phenol for 15 minutes. The stain was differentiated in 0.5% acid-alcohol for 2

[a] Oil immersion lens: x100 and eyepiece lens x10= total x100 magnification

minutes and slides were washed briefly with mycobacteria-free distilled water. Nile red labelling and $KMNO_4$ counter-staining was done as described above.

Auramine O excites at a maximum wavelength of 475nm, and emits maximum fluorescence at 540nm, making it compatible with a Wide Blue (WB) filter (Figure 5.2). Sputum smears were assessed by three sweeps on this filter and 100 auramine positive bacilli were imaged. Corresponding images of the each relevant field were then taken under WG. To calculate LB counts, images were read in pairs and the proportion of organisms containing LBs was expressed as a percentage.

At least two ANR smears were prepared from every sputum sample and mean LB counts were recorded. Slides prepared under different conditions were blinded prior to analysis; sample identifiers were covered with thick non-transparent tape immediately after staining and slides were re-labelled with a "blinding code" which remained unbroken until analysis of all images from the relevant experiment was complete.

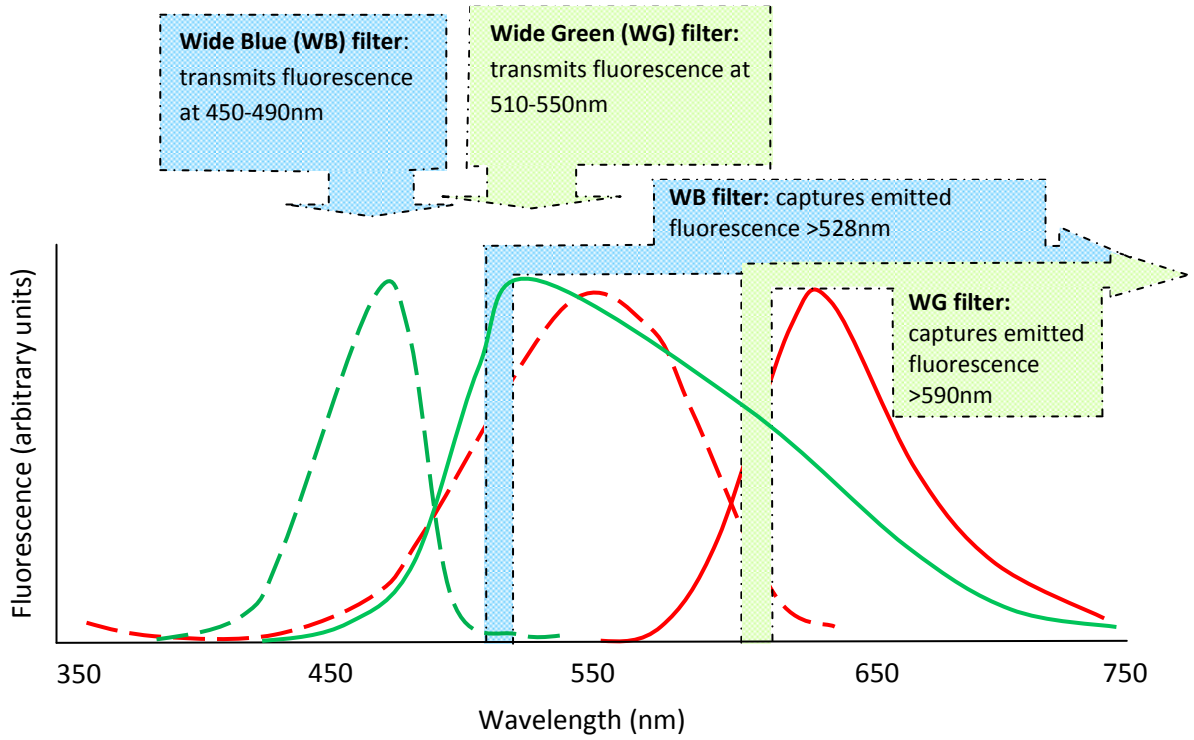


Figure 5.2 Initial probes and filters for fluorescence microscopy
Excitation (dotted line) and emission (solid line) spectra are shown in green and red for auramine O and Nile red respectively. The WB filter transmits fluorescence at appropriate wavelengths to excite auramine O bound to the smear. Emitted fluorescence is then captured by the same filter. The WG filter has similar properties for Nile red.

5.3.5 *In vitro* Nile red staining of intracellular lipid in *M smegmatis*

Intracellular LBs were easily visualised within *M smegmatis* (Figure 5.4A). LB counts increased from a mean of 18% on day 2 to 63% on day 4 and remained constant thereafter (Figure 5.3). This is consistent with observations from previous studies[344,561]. As LB counts of *M smegmatis* cultures were consistently >50% from cultures on day 4-8, these were used as positive controls to confirm effective LB staining during the clinical study.

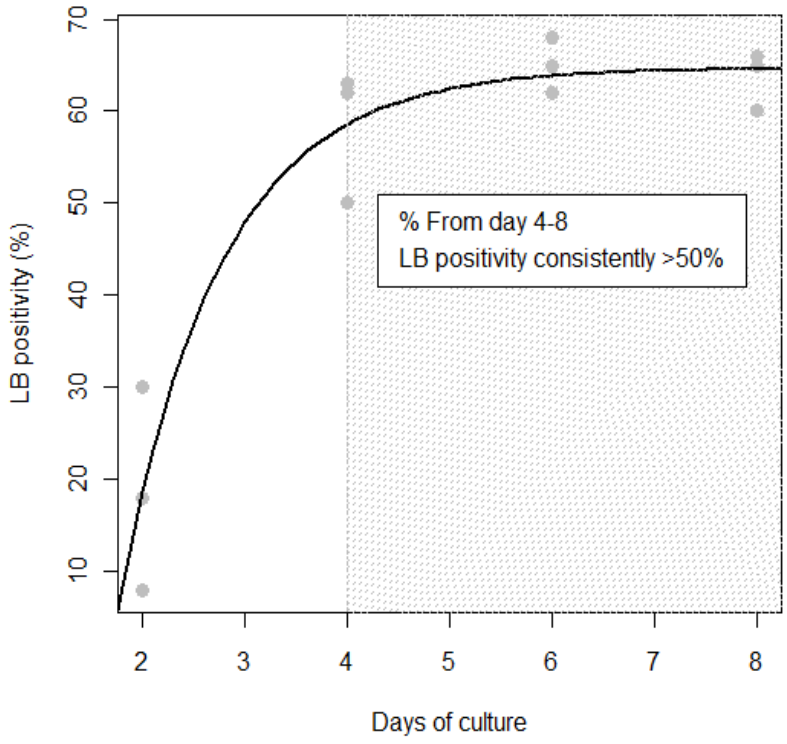


Figure 5.3 Changes in LB positivity during *in vitro* growth of *M smegmatis*

5.3.6 Auramine/Nile red staining of *M tuberculosis* in sputum

Merged WB and WG images for LB positive organisms in clinical samples are shown in Figure 5.4B. Preparation of sputum for microscopy was problematic as the background matrix contains a mixture of glycoproteins and cellular debris including nucleic acid and lipids released from lysed and expectorated foamy macrophages[167,562]. This material fluoresces brightly with Nile red, obscuring identification of LBs (Figure 5.4 C and D).

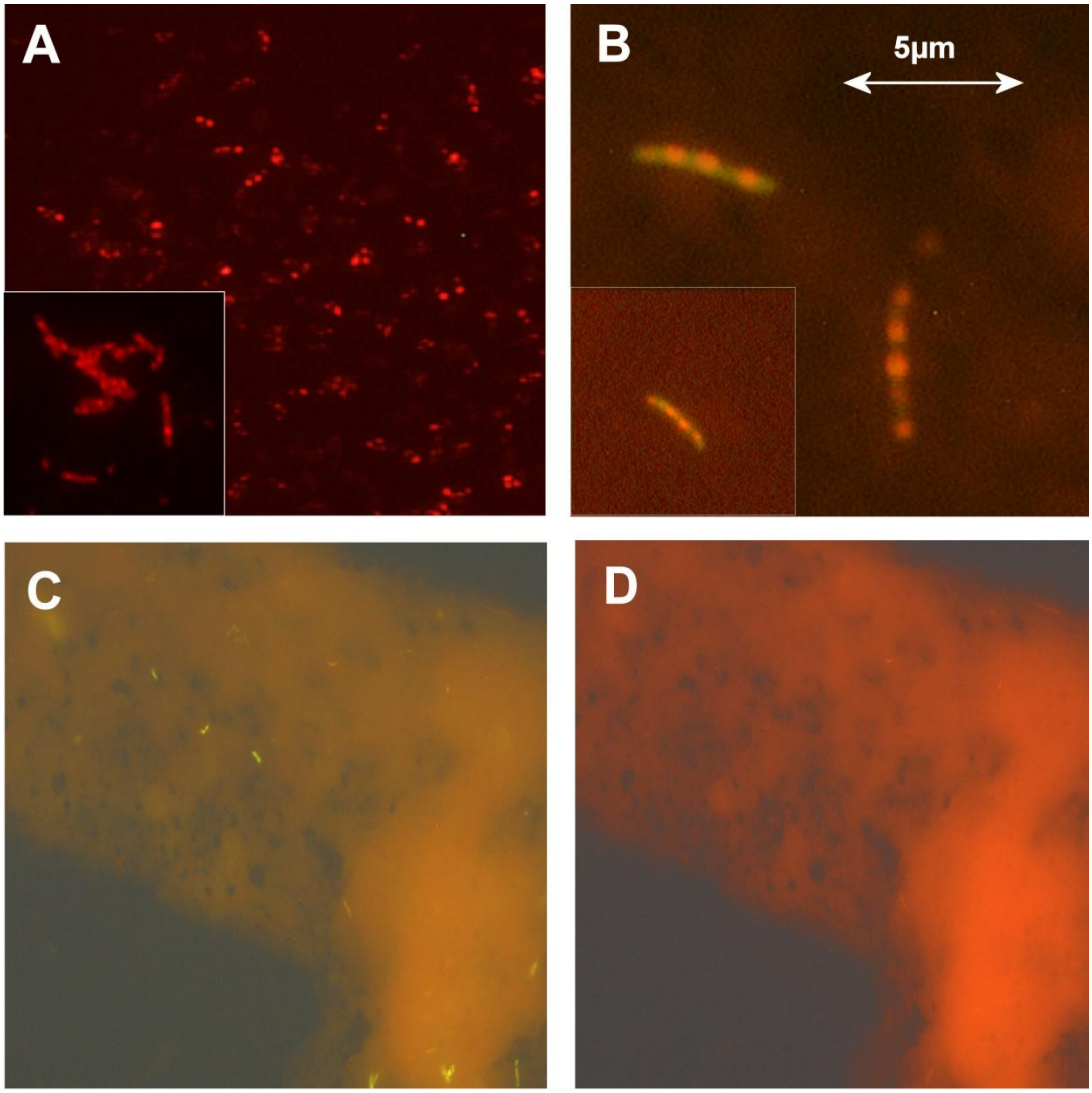


Figure 5.4 Early LB microscopy images

A: Main panel - *M smegmatis* stained with Nile red. Inset – close up of LB positive organisms within the sample. B: Main panel – Merged images of bacilli using wide blue and wide green filters after ANR staining. Two LB positive *M tuberculosis* bacilli are clearly seen. The left (horizontal) organism stained well with auramine (green) and Nile red (red dots). The right (vertical) organism has stained poorly with auramine but still contains LBs. Inset – A LB positive bacillus from a different patient. C: An ANR stained sputum sample with the WB filter. Auramine stained bacilli are visible through the thick background matrix. D: The same microscopy field with the WG filter. Nile red staining of the background prevents assessment of intracellular LBs.

Substituting dithiothreitol for an alternative sputum digestant (N-Acetyl-L-Cysteine) during sample processing did not improve the background. Therefore, a lipase treatment procedure was developed to remove extra-cellular lipid from the sputum matrix.

The aim of lipase treatment was to remove lipids from the background matrix without degrading intra-cellular LBs. To confirm that LBs were not digested by lipase, a few colonies from a six day old *M smegmatis* culture plate were suspended in PBS, and divided into four 1ml aliquots. Dithiothreitol-treated sputum samples from both clinical samples were split in the same way. A lyophilised powder preparation of lipase from *Candida Rugosa* (Sigma-Aldrich) was dissolved in PBS to produce concentrations of 1mg/ml, 5mg/ml and 10mg/ml. An aliquot of each of the mycobacterial samples (*M smegmatis,* Patient 1 sputum and Patient 2 sputum) was incubated at 37^{o}C with an equal volume of sterile water (negative control) and each of the lipase solutions for one hour then washed in sterile PBS.

Smears were made in triplicate from all samples at all lipase concentrations and stained with Nile red (*M smegmatis*) or ANR (sputum). Slides were blinded and read. Changes in the percentage of LB positive bacilli in each sample at different lipase concentrations were assessed by Analysis of Variance (ANOVA). The difference in LB positivity between Patient 1 and 2 sputum samples was assessed by a two-sample t-test.

Figure 5.5 shows that LB counts in all three samples were unaffected by lipase treatment (*M smegmatis,* p=0.22; Patient 1, p=0.78; Patient 2, p=0.28). There was a difference in % LB positive counts between the two patients (Patient 1 mean LB count: 27.6% [95%CI: 15.7-39.6], Patient 2 mean LB count: 47.3% [95%CI: 43.9-50.7], p=0.003), consistent with Garton's observation that baseline LB positivity varies between patients with clinical disease[175].

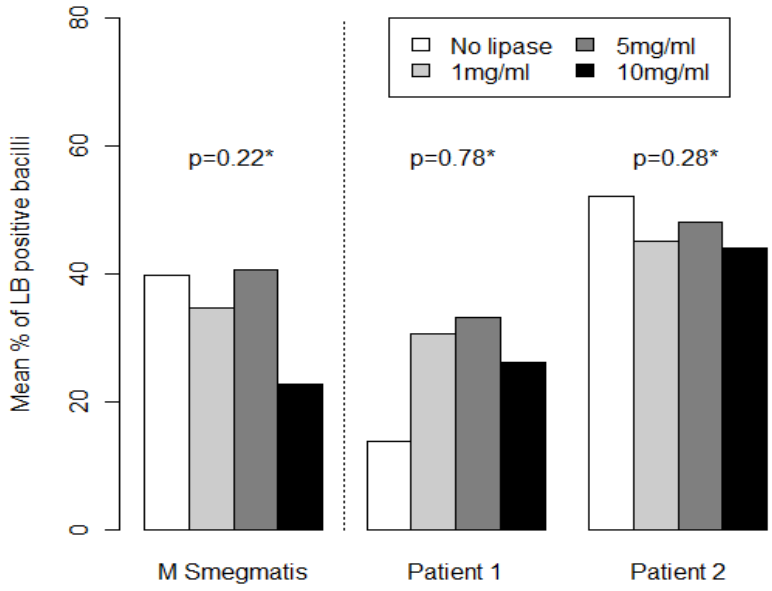


Figure 5.5 Effect of lipase treatment on LB counts
There was no change in LB counts in any sample after treatment with different lipase concentrations
*p values shown are for statistical analysis by ANOVA

To test whether lipase treatment reduced Nile red staining of the sputum matrix, 1ml aliquots of sputum from Patient 2 were treated with dithiothreitol alone, dithiothreitol and lipase (1mg/ml) or dithiothreitol and lipase (10mg/ml). Ten smears from each preparation were blinded, stained, imaged and counted. Background Nile red staining of each slide was graded as mild (assessment of bacilli unaffected by background matrix), moderate (LB assessment difficult in <50% of bacilli) or severe (LB assessment difficult in >50% of bacilli). Results are shown in Figure 5.6A-E and Table 5.4. The effect of different lipase concentrations on LB counts was assessed by ANOVA whilst the effect on the background matrix was assessed by Fisher's exact test.

	No lipase	Lipase (1mg/ml)	Lipase (10mg/ml)	p-value
LB count (%), mean (SD)	67.1 (8.44)	66.1 (9.42)	60.9 (11.5)	0.33[a]
Background matrix				<0.001[b]
Mild	0	8	5	
Moderate	8	2	2	
Severe	2	0	3	

Table 5.4 Effect of lipase on LB counts and background sputum matrix
[a]Statistical analysis by ANOVA
[b]Statistical analysis by Fishers exact test

Once again, lipase concentration was not associated with a change in LB counts (p=0.33) but there was an association between lipase use and background severity (p<0.002). Despite the small sample set, the background matrix was significantly reduced by lipase (1mg/ml). There was no additional benefit from lipase (10mg/ml).

For all subsequent lipid body microscopy, lipase 1mg/ml was added to sputum processing. A similar method was previously used by Daniel to effectively remove radiolabelled TAG from the extracellular surface *M tuberculosis* bacilli during the work on bacillary persistence described in Table 5.3[168].

A single step dithiothreitol-lipase digestion method was developed to streamline the procedure[a]. Changes to dithiothreitol-lipase (1mg/ml) incubation temperature (4°C or room temperature) and duration (one hour or overnight) did not alter image quality or LB counts.

5.3.7 Improving image quality: alternative stains and filters

Additional problems with ANR microscopy were attributable to the spectrofluorometric properties of the dyes.

Established texts state Auramine O labels mycolic acids in the mycobacterial cell wall[32] but direct evidence for this is lacking. Fluorescence is enhanced on binding to DNA and RNA and some acid-fast staining may be due to nucleic acid labelling[33]. The total emission spectrum of auramine O is wide (500-700nm) and overlaps with Nile red. Labelling of nucleic acids may create the impression of 'beads' within the organism which are difficult to discriminate from LBs emitting fluorescence at similar wavelengths.

Furthermore, Nile red is solvatochromatic and binds different lipid structures with shifting excitation and emission spectra according to the position, shape and intensity of the solvent. When hydrophilic membrane phospholipids are stained, entire cells emit a diffuse red fluorescence (λ>610nm) and intracellular LBs may be obscured. Alternatively, staining of hydrophobic intra-cytoplasmic TAG results in emission of yellow-gold fluorescence (λ≈528nm)[563] with increased spectral overlap with Auramine O (Figure 5.7). Nile red is also prone to photo-bleaching (fade of fluorescence on exposure to light) which can cause loss of labelling during microscopy and imaging[564].

[a] For 50ml of dithiothreitol-lipase (1mg/ml), 3.75ml dithiothreitol concentrate (Oxoid) was added to 50mg lipase from Candida rugosa (Sigma) and 46.25ml mycobacterial free distilled water. 1ml of this preparation could be incubated with an equal volume of sputum for 1 hour.

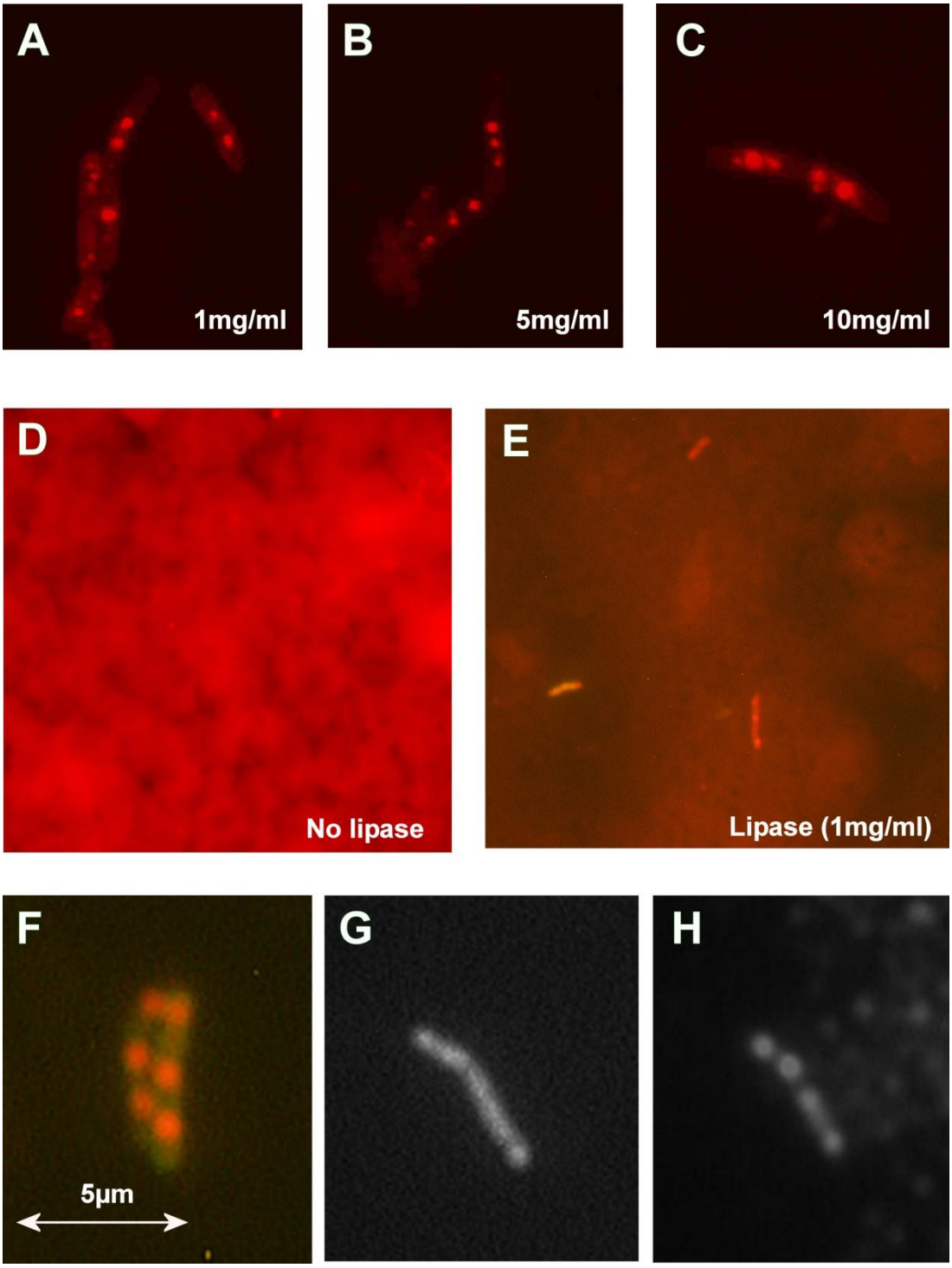


Figure 5.6 Improvements to staining protocol for LB microscopy

A-C: *M smegmatis* stained with NR after lipase treatment at different concentrations. LBs are visible in all samples. D and E: A sputum sample from a smear positive TB patient. Without lipase treatment, the background matrix on the WG filter is severe and no bacilli can be seen. After lipase (1mg/ml) treatment, the background is mild and LB positive bacilli are seen through it. F: Merged ALTR (1:200) image of two LB positive bacilli viewed with FITC and TRITC filters. G and H: Black and white images of an ALTR (1:200) LB positive bacillus viewed with FITC and TRITC. Different staining patterns indicate labelling of different cellular components by auramine (G) and LTR (H).

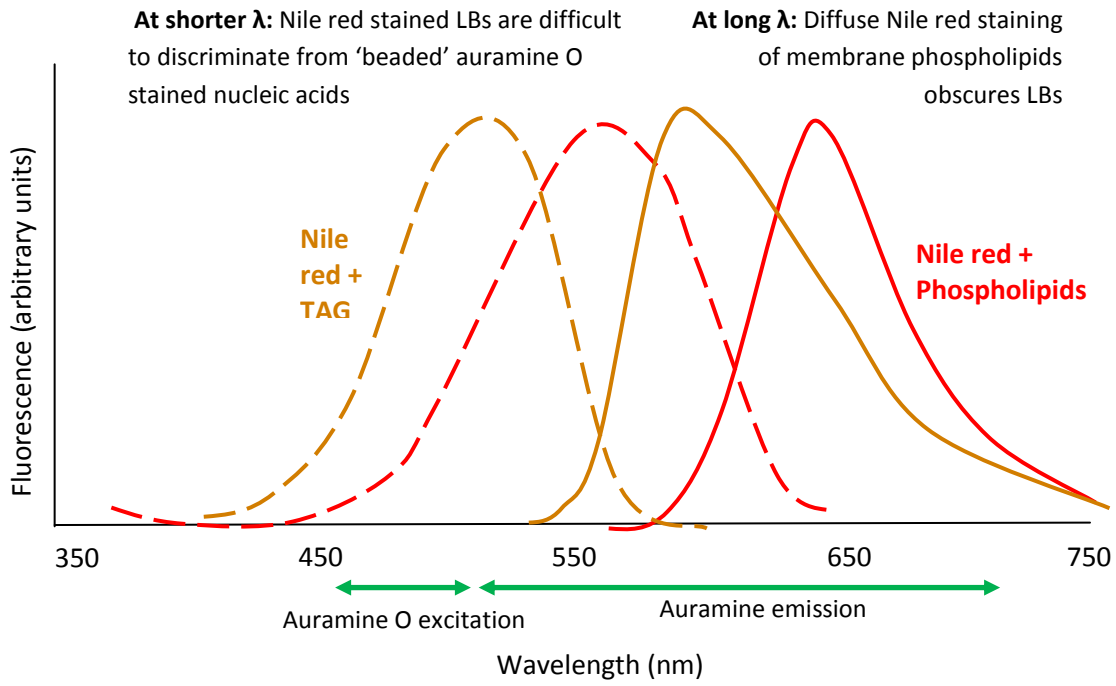


Figure 5.7 Difficulties associated with spectrochromatic properties of Nile red

Excitation (dotted line) and emission (solid line) spectra for Nile red are at shorter wavelengths for hydrophobic (gold) than hydrophilic (red) environments. Auramine O spectra are shown below the x-axis.

The LipidTOX™ neutral stains (Invitrogen) are a new set of fluorescence probes with more specific binding to neutral lipids and greater photostability than Nile Red. LipidTOX™ Red neutral has good spectral characteristics ($\lambda_{excitation}$>577m, $\lambda_{emission}$>609nm) for combination with auramine O (Figure 5.8), so an AuramineO/LipidTOX Red neutral (ALTR) technique was optimised and compared with ANR.

1:50, 1:200 and 1:1000 dilutions of LTR were prepared in PBS. A sputum sample from patient 2 was processed with dithiothreitol-lipase (1mg/ml) and four batches of three heat-fixed 10µl smears were prepared. The first batch was ANR stained in the usual manner. Batches 2, 3 and 4 were stained with Auramine O and differentiated with 0.5% acid-alcohol, then labelled respectively with one of the three LTR dilutions for 20 minutes before washing and counterstaining with $KMNO_4$. Stained slides were blinded and taken from the BSL-3 laboratory to the darkroom for reading within 24 hours. LB counts and the severity of background Nile red/LTR staining were recorded. Results are shown in Table 5.5. Differences in LB counts between ANR staining and each of the ALTR preparations were analysed by two-sample tests and differences in background staining were assessed by Fisher's exact test.

Stain	LB count (%), mean(SD)	Change in mean LB count from ANR[a], % (p-value[a])	Background			Change in background from ANR, p-value[b]
			Mild (n, %)	Moderate (n, %)	Severe (n, %)	
ANR	62.4 (9.3)	-	5	6	1	-
ALTR (1:50)	92.4 (7.9)	30.0 (<0.001)	5	6	1	1
ALTR (1:200)	81.3 (10.8)	18.9 (<0.001)	6	3	3	0.32
ALTR (1:1000)	40.8 (16.5)	-21.6 (<0.001)	9	3	0	0.21

Table 5.5 LB background sputum matrix staining with ANR or ALTR microscopy

[a]Statistical analysis by two-sample t-test

[b]Statistical analysis by Fisher's exact test

Images from ALTR slides prepared using 1:50 and 1:200 dilutions of LTR were similar to ANR images (Figure 5.6F, G and H) but had higher LB counts ($p<0.001$ in both cases). This reflects less photo-bleaching with LTR. LB counts were lower on slides prepared using the 1:1000 dilution of LTR, suggesting that this preparation was too weak. No significant difference in staining of the background matrix was detected between ANR and ALTR.

On the basis of these experiments, the ALTR method using a 1:200 dilution of LTR replaced ANR microscopy for the clinical study.

After altering the fluorescence stains for LB microscopy, the choice of microscope filters was reviewed. Whilst the 'long-pass' filters employed until this point produced reasonable images, narrower 'band-pass' filters are more selective in the wavelengths of light they allow to pass, improving image detail, reducing the risk of bleed-through between dyes and minimising fluorescence of the background matrix[33]. A fluorescein isothiocyanate (FITC) and tetramethylrhodamine (TRITC) filter-set (Figure 5.8) was found to be optimal for ALTR staining and was used in the clinical study.

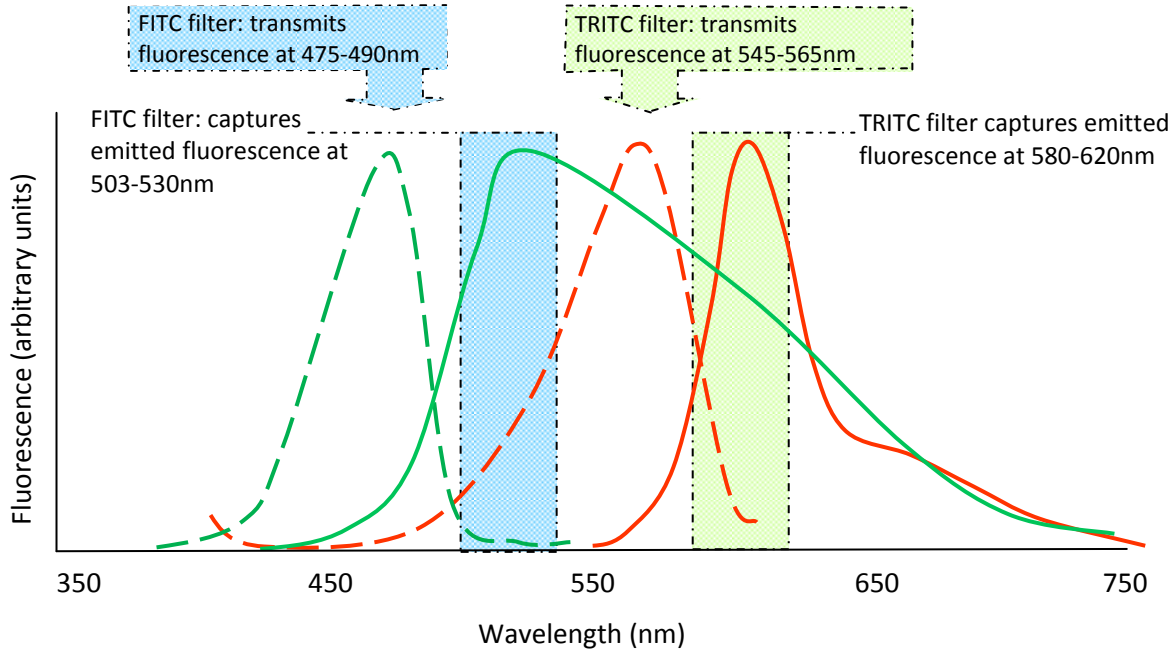


Figure 5.8 ALTR labelling and FITC-TRITC filters for the clinical study

Excitation (dotted line) and emission (solid line) spectra are shown in green and red for auramine O and LTR respectively. A good fit is demonstrated between these dyes and the FITC-TRITC filter set, and the use of bandpass filters reduces the effect of spectral overlap previously seen with ANR and long-pass filters.

5.3.8 Improving image quality: magnetic beads

An additional means of 'cleaning' sputum samples after lipase digestion would have been physical removal of *M tuberculosis* bacilli from the background matrix. Paramagnetic beads have been developed which are coated with a polymeric ligand with high selective binding affinity for LAM and mycolic acids on the surface of mycobacteria[565]. The intended purpose of these 'TB Beads' (Microsens, UK) is to concentrate bacilli from sputum samples in laboratories without a centrifuge but the same method was assessed as a tool to improve ALTR images by separating bacilli from other lipophilic sputum content.

3-4ml sputum samples from 25 new TB patients with different smear grades of AFB positivity were digested for 1 hour in dithiothreitol-lipase (1mg/ml) and split into two equal aliquots. The first was concentrated by centrifugation at 3000 x *g* for 15 minutes and the second was treated with 'TB Beads' according to the manufacturer's instructions. Briefly, an equal volume of a pre-prepared solution of TB Beads was added to dithiothreitol-digested sputum in a Falcon tube for two minutes to bind bacilli. The tube was then placed in a magnetic rack for one minute until all the beads had collected at the side near the magnet. Keeping the tube in the rack, liquid was removed with a Pasteur pipette, without

disturbing the beads. 4ml of a 0.04% NaOH wash solution was added to the tube, the beads were re-suspended by vortexing, re-captured magnetically and the wash solution was pipetted off. Finally 500µl of elution buffer was added to detach the bacilli from the beads and the beads were magnetically re-captured, leaving the bacilli suspended in a small volume of supernatant.

Smears from centrifuge concentrated and bead-treated preparations of all samples were blinded, ALTR stained and read for AFB smear status, LB counts and severity of LTR background.

Smears prepared by either methods showed no differences in LB counts (mean LB count post-centrifugation: 47% [SD: 26.0] vs. post-beads: 44% [SD: 18.9], paired two-sample t-test p=0.58). There were also no differences in severity of background staining; 18/25 (72%) samples had 'mild' background irrespective of the processing method used.

Table 5.6 shows that in 14 (56%) samples there was concordance in AFB smear status post-centrifugation or post-beads. In 9 (36%) discordant samples, the smear status was higher after centrifugation whilst in only 2 (8%) samples the smear status was higher after beads. This suggests that centrifugation was more effective at concentrating bacilli than 'TB Beads', a finding corroborated by other studies[566]. Furthermore, centrifugation was a single step procedure, whilst bead treatment required a labour intensive three step protocol.

		Smear status after concentration by centrifugation (n, %)			
		Negative	Scanty/1+	2+	3+
AFB status after 'TB beads' (n, %)	Negative	5 (20.0)	0	0	0
	Scanty/1+	0	2 (8.3)	4 (16.7)	3 (12.5)
	2+	0	1 (4.2)	2 (8.3)	2 (8.33)
	3+	0	0	1 (4.2)	5 (20.83)

Concordant smear status Beads> Centrifugation Centrifugation> Beads

Table 5.6 AFB smear status in sputum concentrated by centrifugation or TB beads

Overall, with less effective sample concentration and no change in the severity of LTR background, TB bead treatment was not added to the sputum processing method. The final microscopy protocol was as indicated in Appendix 10.4.3.

5.3.9 Validation of LB observation by Electron Microscopy

Fluorescence microscopy to study LBs in *M tuberculosis* is new. EM was used to validate the technique as it can also identify intracellular lipids.

After successful transfer of the optimised ALTR technique to Malawi, baseline sputum samples from five Malawian patients with smear '+++' TB were treated with dithiothreitol-lipase (1mg/ml) and split in half. One half was processed for ALTR microscopy. The other was stored at 4^{o}C in EM fixate (2.5% gluteraldehyde in 0.1M sodium cacodylate, 5mM calcium chloride, 5mM magnesium chloride and 0.1M sucrose [pH 7.2]) and shipped at room temperature to Professor David Russell (Cornell University) who performed EM. A representative image is displayed in Figure 5.9E.

Although quantitative LB counts on EM were not possible, LB observation inside *M tuberculosis* using this technique corroborated their identification by fluorescence microscopy. Except in Garton's publications[175,344] this is the first description of bacillary LBs from clinical samples. The data in Chapter 6 represents the only patient cohort ever to have LB counts related to treatment response.

5.3.10 Assessment of non-acid fast, LB positive bacilli

Some early LB microscopy images apparently demonstrated LBs inside TB organisms with little or no auramine staining, raising the possibility of non-acid fast LB positive bacilli (Figure 5.9C and D). The potential consequences of this for image analysis required consideration.

As current diagnostic stains for TB in clinical samples rely on acid fastness, it is tempting to argue that non-acid fast LB aggregations were not intra-bacillary: they may have been within contaminant organisms, or simply composed of co-incidental stain deposits. However, only a few other organisms (mainly actinomycetes[567] and rhodococcus[568] species) store TAG and the clinical samples used to optimise LB microscopy were not contaminated on SSCC or MGIT culture. The LTR staining patterns were morphologically convincing for *M tuberculosis* and persisted after lipase digestion of the samples making them unlikely to be extracellular artefacts.

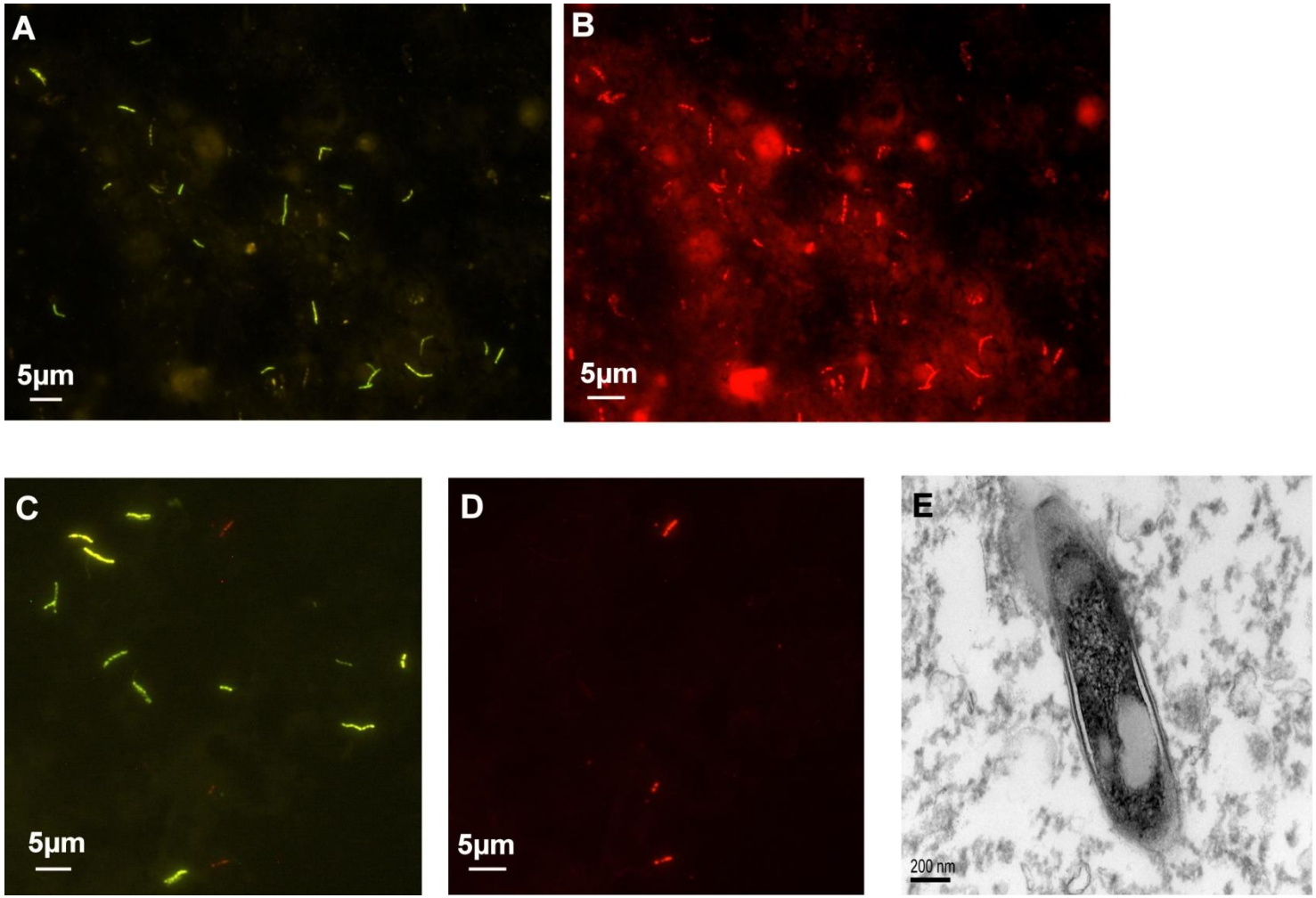


Figure 5.9 ALTR and EM images of sputum samples

A and B: FITC and TRITC filter images of ALTR stained sputum, most organisms are auramine and LB positive.

C and D: Images from a different sample. 3 LB positive bacilli (visible in plate D) appear to be auramine negative (invisible in Plate C).

E: EM photograph of an *M tuberculosis* bacillus from a Malawian patient. LBs are visible in the lower pole of the organism.

Furthermore, there has been a small but evolving literature on non-acid fast *M tuberculosis* organisms since the 1930s[569]. In the 1960s viable chromophobic bacilli were associated with reactivation of pulmonary TB disease[570-572] and in 2007 non-acid fast cells were generated by mutation of the *kasB* gene, involved in mycolic acid synthesis[573,574]. The phenotypic importance of non-acid fast organisms is unknown but they have been linked to latency[575] and survival during chemotherapy[576]. The studies by Deb[154] and Daniel[168] reported in Table 5.3 described declining acid-fastness as bacilli acquire LBs and antibiotic tolerance, providing contemporary evidence for a possible role in persistence.

Overall, the observation of auramine-negative LB-positive bacilli seems genuine, but accurately count such bacteria without a second definitive mycobacterial label was subjective and problematic. Therefore, in the clinical study, imaging remained targeted towards acid-fast auramine-labelled organisms and the main analysis was done on these. However, a supplementary analysis was done including putative counts of non-acid fast, LB positive cells to estimate their phenotypic prevalence and offer insights into their behaviour under drug pressure.

5.3.11 Standardised methods for ALTR microscopy in the clinical study

Based on the experimental experience detailed above, a standard approach to ALTR microscopy and image analysis was developed and used for LB counting in the clinical study. Key points are outlined below.

Sample preparation and blinding of slides

ALTR microscopy was too labour intensive to be done in real time alongside SSCC and MGIT cultures. During the clinical study, sputum was stored from every sample at -20^{o}C. At a later date, batches of 6-8 samples were sequentially thawed, treated with dithiothreitol-lipase (1mg/ml) and stained for ALTR microscopy.

Two 10µl smears were stained for each sample and dried in the dark. Slides from each batch were shuffled and blinded by a laboratory worker un-related to the study. Information linking the "blinding code" to clinical sample identifiers was inaccessible during LB counting. All ALTR slides were read within 24 hours of staining.

Microscopy, photography and image analysis

To prevent biasing of LB counts by selective photography every slide in the clinical study was viewed as described in section 5.3.4. All microscopy fields containing individually

assessable, auramine-stained bacilli were photographed using both FITC and TRITC filters and analysed. The number of photographed auramine-stained bacilli was counted using a hand-held tally-counter. Each slide was viewed until ≥100 auramine positive organisms were imaged or the slide was viewed for 15 minutes. Clumps of organisms from which individual bacilli could not be discriminated were disregarded. Scouring slides on the TRITC filter for LB positive organisms was forbidden.

Altering camera exposure times changed the images. Longer exposure made faint fluorescence labelling brighter and increased the risk of bleed-through of dyes, creating the impression of more LB positive organisms. To reduce the effect of variable exposure times, two control slides with known LB counts >50% were stained alongside each batch of clinical samples; a day 4-8 *in vitro* culture of *M smegmatis* and 1ml of sputum from the Liverpool Patient 2 sample used in optimisation experiments. These specimens were used to check the efficacy of ALTR staining and set photography exposure times before each microscopy session. Although fine focus alteration was subsequently permitted between clinical samples, exposure times were fixed for the entire session.

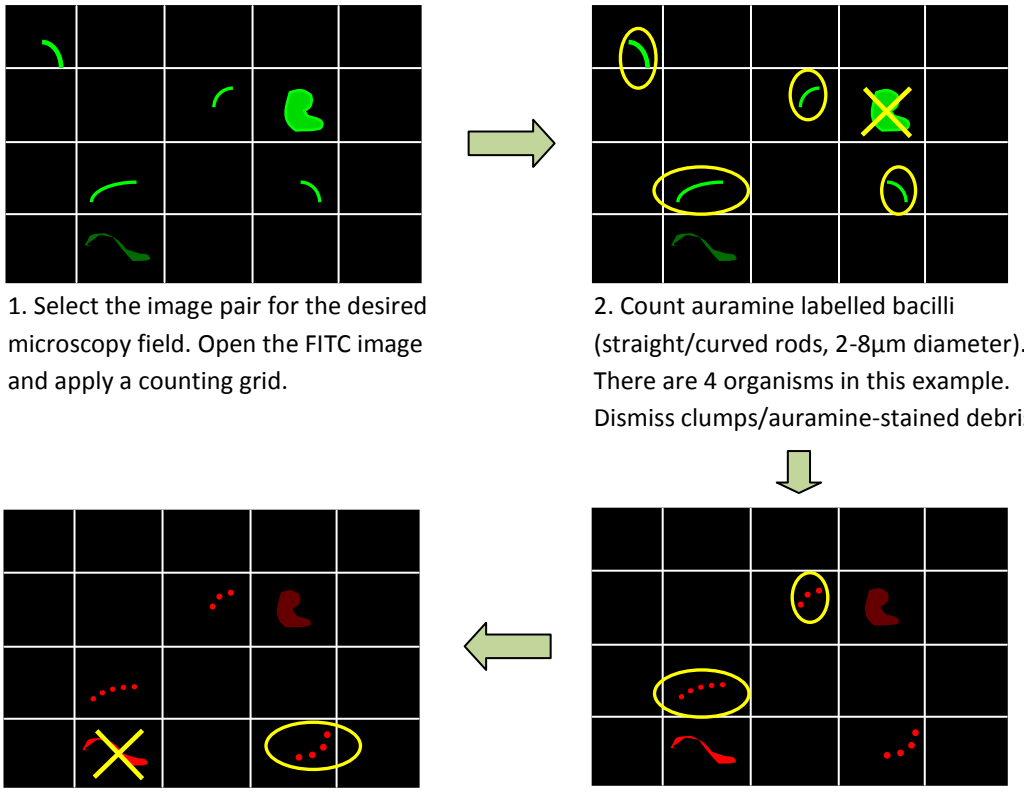
All digital images were stored as high resolution 'tif' files with a back-up copy in a separate location. Images were indexed according to the study blinding code. The Microsoft Excel file necessary to break the "blinding code" was retained on a separate computer until completion of image analysis.

Finally, a pre-agreed written protocol was used for image analysis (Figure 5.10) using standard definitions of different bacillary sub-types (Figure 5.11). LB counts for each slide were calculated by:

$$\textit{Auramine LB (af-LB) count (\%)} = 100 \; X \; \frac{\textit{Total LB positive AFB on all images}}{\textit{Total AFB on all images}}$$

$$\textit{Total LB (TLB) count (\%)} = 100 \; X \; \frac{\textit{Total LB positive AFB on all images}}{\textit{Total acid fast and non acid fast bacilli on all images}}$$

The mean af-LB and LTB counts from both slides of each sample were taken as the results for that sample.



1. Select the image pair for the desired microscopy field. Open the FITC image and apply a counting grid.

2. Count auramine labelled bacilli (straight/curved rods, 2-8µm diameter). There are 4 organisms in this example. Dismiss clumps/auramine-stained debris.

3. Open the TRITC image. Decide which auramine labelled bacilli contain LBs (2 organisms in this example).

4. Assess the TRITC image for LTR staining suggestive of auramine negative (non-acid fast) LB positive bacilli (1 organism in this example). Dismiss LTR-stained debris.

Figure 5.10 ALTR image reading protocol

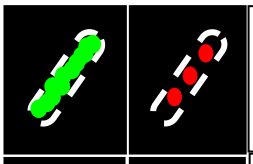
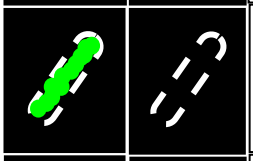
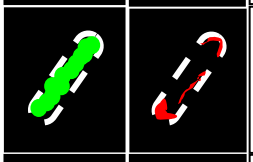
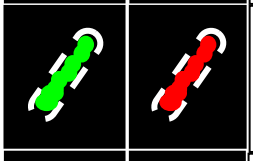
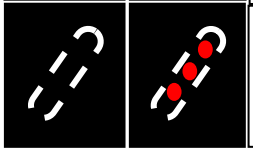
	Auramine labelled bacillus on FITC filter. LBs identified with different LTR staining pattern on TRITC filter	Acid fast, LB positive
	Auramine labelled bacillus on FITC filter. No LBs identified (the organism is invisible) on TRITC filter	Acid fast, LB negative
	Auramine labelled bacillus on FITC filter. LTR staining on TRITC filter at margins of bacilli only; may represent binding to polar membrane phospholipid NOT LBs.	Acid fast, LB negative
	Auramine labelled bacillus on FITC filter. Identical staining pattern on TRITC filter may represent “spectral overlap” of dyes between filters; LBs unassessable.	Acid fast, LB unknown
	No auramine labelled bacillus on FITC filter. LTR staining pattern on TRITC filter strongly suggestive of LBs in a non-acid fast organism	Non-acid fast, LB positive

Figure 5.11 Definitions of bacillary sub-types for LB counting

5.3.12 Fluorescent lipid body microscopy conclusions

The ALTR microscopy technique provided the second ever successful demonstration of LBs within bacilli from clinical sputum samples containing *M tuberculosis*. Considerable progress was made in improving image quality, particularly in digesting the extracellular sputum matrix and selecting the appropriate fluorescent dyes and filters. Although some problems remained with consistency of image quality, reliability of slide photography and subjectivity of image analysis, this technique was suitable for analysis of clinical study samples.

5.4 Flow Cytometry

5.4.1 Flow cytometry and microbiology

Flow cytometry is an alternative means of assessing single cell characteristics. A laser beam is directed onto a hydrodynamically focussed stream of cells (Figure 5.12). After striking each cell, scattered light in the same direction as the incident beam (Forward Scatter [FSC]) provides information on cell size, whilst scattered light at an angle of 90^{o} (Side Scatter [SSC]) provides information on granularity. Fluorophore labelling of cellular components results in emission of fluorescence which is filtered onto a range of detectors. Information on light scatter and fluorescence is stored on a computer. Overall sample results represent cumulative data on the individual cells within it. The advantages of flow cytometry over fluorescence microscopy are that thousands of cells are assessed in a few seconds and analysis is automated according to pre-set criteria, eliminating the potential for bias or subjectivity during data collection.

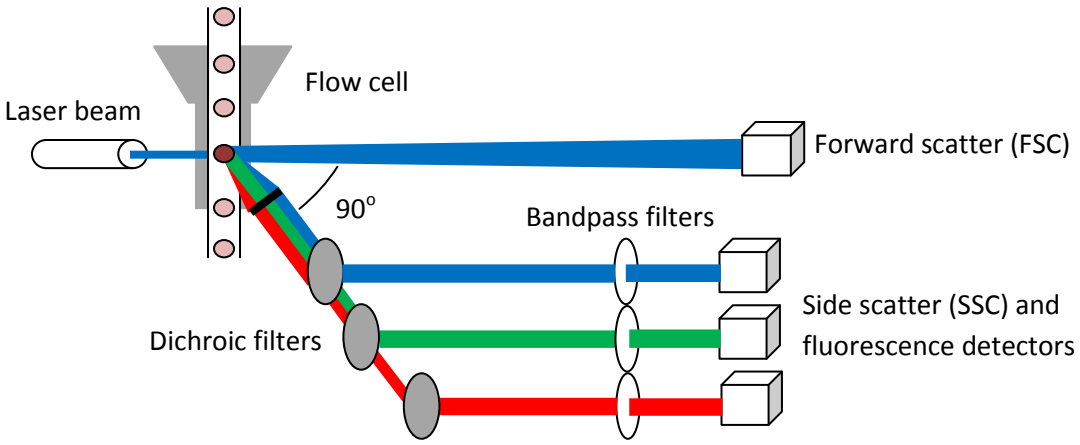


Figure 5.12 Analysis of single cells by flow cytometry

Fluorophores are classifiable according to their mechanism of action; some bind cell surface proteins, some bind nucleic acid (providing information on cell replication) and some have fluorescence dependent on physiological parameters such as membrane potential or enzymatic activity (providing information on viability)[577,578]. Studies on *E coli*[579] and some mycobacteria (*M avium*[580] and *M smegmatis*[581]*)* have exploited these fluorophores to describe metabolically heterogeneous groups of organisms. Therefore, there is a potential role for the technique in studying viable persister *M tuberculosis* bacilli during chemotherapy, even if the cells are non-culturable.

5.4.2 Flow cytometry and tuberculosis

Although flow cytometry has been used to investigate immunological host cell responses during TB infection, it has rarely been directly used to study the pathogen. However, several groups have used formaldehyde or heat inactivated organisms to assess TB drug susceptibility by flow cytometry without the need for prolonged culture[582,583]. This work shows that the intracellular viability dye fluorescein diacetate (FDA) discriminates between live and dead TB *M tuberculosis* organisms by only emitting green fluorescence when hydrolysed by enzymes within living cells[555,584] and some nucleic acid labels (e.g. the SYTO probes from Invitrogen) successfully stain intra-bacillary DNA[585]. Authors including Shapiro have argued for increased use of flow and imaging cytometry in TB research and diagnostic practice for several years[586].

In Liverpool, experiments were performed to establish whether a fluorescence label for TB would permit single cell analysis of *M tuberculosis* by flow cytometry of sputum samples.

5.4.3 Finding a fluorescence label for *M tuberculosis*

Prior flow cytometry studies of TB drug susceptibility used organisms isolated from pure culture. Clinical samples are more complex because they contain other organisms, host cells and non-organic matter, all of which register as analysable events when they pass through the flow cytometer. A clinically useful assay would require a selective label to discriminate *M tuberculosis* from background events. Three potential labels were considered *in vitro* prior to experiments on sputum; two monoclonal antibodies and Auramine O.

Monoclonal antibody labels

The 38kDa antigen is a ubiquitously expressed, phosphate-transport protein expressed by *M tuberculosis* particularly in multi-bacillary disease. Although widely secreted in culture fluids, it is also bound to the surface of mycobacterial cells[587]. Antigen 85 is a complex of three genetically related proteins (Ag85A, B and C) which act as mycolyltransferases in mycobacterial cell wall assembly[588]. Ag 85 may be up-regulated in response to isoniazid exposure[321] and increased Ag 85 expression after 14 days of therapy may denote persister organisms in patients who are more likely to fail therapy[294,322].

Primary antibodies to the 38kDa Ag (0100-0520, Serotec) and Ag85 (ab36731, Abcam) were assessed as labels for *M tuberculosis*. Neither antibody was bound to a fluorescence probe so they were studied via a two-step staining protocol with the secondary FITC-conjugated antibodies shown in Table 5.7. Isotype controls were used to confirm that labelled events in fluorescence experiments were due to interactions between Fab epitopes of the primary antibody and target cell proteins rather than non-specific binding to cellular Fc receptors or inorganic debris.

Primary antibody	Isotype control	Secondary (FITC-conjugated) fluorescence antibody
α-38kDa antibody (Serotec) 0100-0520 (0.3125-0.1mg/ml) *(Mouse anti-mycobacterium tuberculosis 38kDa antibody)*	MCA928 (0.3125-0.1mg/ml) *(Mouse IgG1 negative control)*	STAR117F (0.05 & 0.5mg/ml) *(Goat anti-Mouse IgG (H/L): FITC, multi species absorbed)*
α-Ag85 antibody (Abcam) ab36731 (0.3125-0.1mg/ml) (Mouse *anti-mycobacterium tuberculosis Ag85 antibody [HYT 27])*	Ab18448(0.3125-0.1mg/ml) *(Mouse IgG1, kappa monoclonal [MG1-45])*	ab6785 (0.02 & 0.2mg/ml) *(Goat polyclonal to Mouse IgG (H/L): FITC)*

Table 5.7 Antibodies used for flow cytometry

Preparation of M tuberculosis cultures for flow cytometry experiments

Middlebrook 7H9 media was prepared, with the addition of Tween-80 to counter clumping of *M tuberculosis* cells and increase the number of organisms passing through the flow cytometer in single cell suspension. H37Rv was incubated until there was obvious turbidity in the media and growth was confirmed by ZN microscopy. The positive culture was diluted in sterile PBS to provide a suspension at equivalent turbidity to a MacFarland 1.0 standard on an OD reader. CFU/ml counts confirmed a bacillary load of 2.5×10^7 CFU/ml in this suspension.

To ensure consistency in bacillary load, H37Rv cells grown in 7H9 media were diluted to MacFarland 1.0 standard turbidity and treated with 2ml of 1% formaldehyde prior to fluorophore labelling for all flow cytometry experiments.

Preparation of non-mycobacterial organisms as negative controls

Streptococcus pneumoniae, Haemophilus influenzae, Klebsiella pneumonia, Moraxella catarrhalis and yeasts are commonly found in sputum. Non-typhoidal salmonellae (e.g. *Salmonella enteritidis* or *typhimurium*) are a major cause of severe bacterial infection in HIV-positive Malawian adults[589]. Isolates of these organisms were used to confirm that labelled events during flow cytometry were H37Rv-specific, and not attributable to other pathogens.

Non-mycobacterial organisms were grown for 48 hours in Muller-Hinton broth, diluted to MacFarland 1.0 standard turbidity and formaldehyde inactivated. When unstained cells were run on the flow cytometer, large yeast particles were identifiable by FSC and SSC alone (Figure 5.13). However, all bacteria were of similar size and granularity (Figure 5.13) so *M tuberculosis* required selection by fluorescence probes.

Monoclonal antibody titrations

For both monoclonal antibodies, 100µl aliquots of H37Rv cells were incubated in plain flow cytometry tubes with the primary antibody or matched isotype control at serial two-fold dilutions from 0.1mg/ml to 0.3125mg/ml. The cells were washed twice, incubated with the secondary FITC-antibody at the concentrations shown in Table 5.7 and washed again. All incubations were at 4^{o}C for 1 hour. Dilutions and washes were done in sterile PBS. Antibody-labelled cells were suspended in 1ml of 1% formaldehyde and run on an LSR-2 (Becton Dickinson) flow cytometer using a laser emitting light at 488nm.

Data was analysed using FloJo (Treestar, Ashland, USA) software. Unstained H37Rv cells were used to gate cells with typical properties for *M tuberculosis* on a scatter plot of FSC and SSC with logarithmically scaled axes. 10,000 events were recorded for each sample. Mean Fluorescence Intensity (MFI) at different antibody concentrations were compared (Figure 5.14).

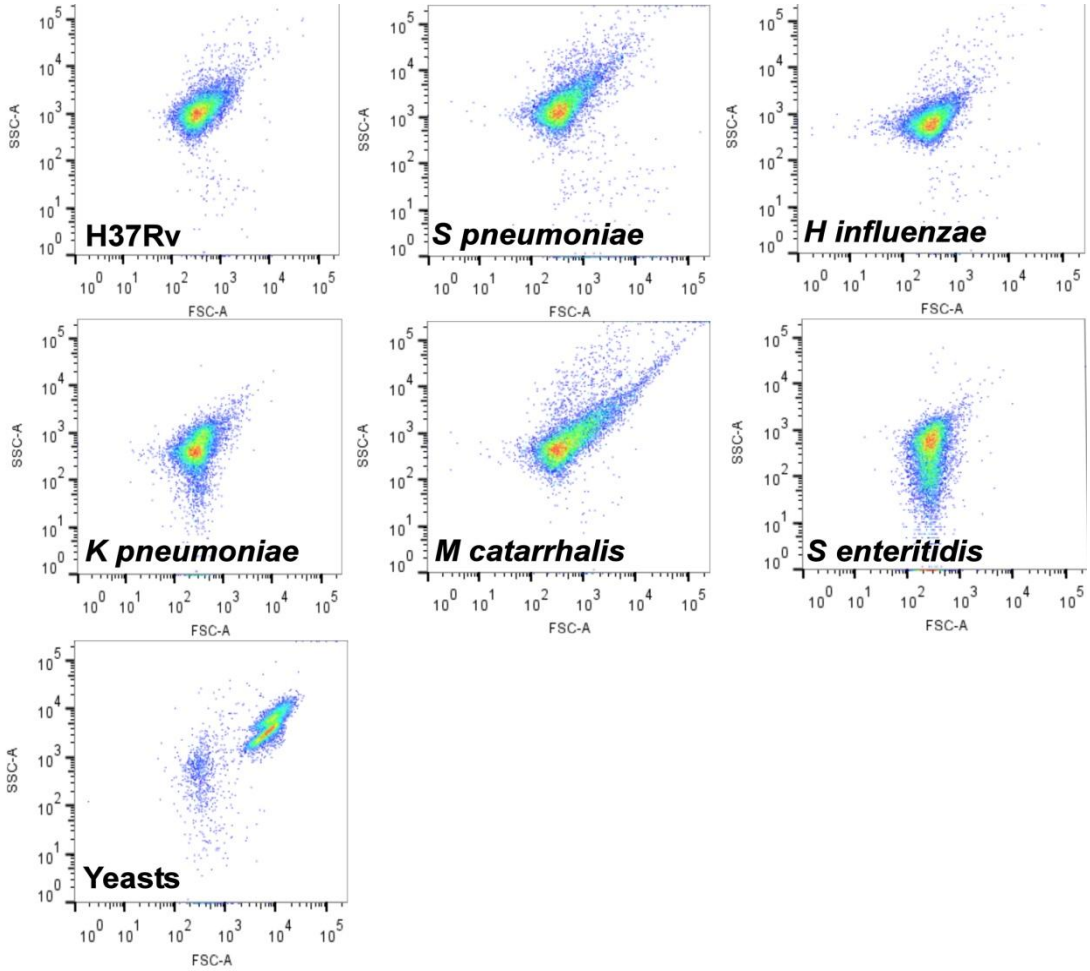


Figure 5.13 FSC and SSC of H37Rv and other respiratory micro-organisms

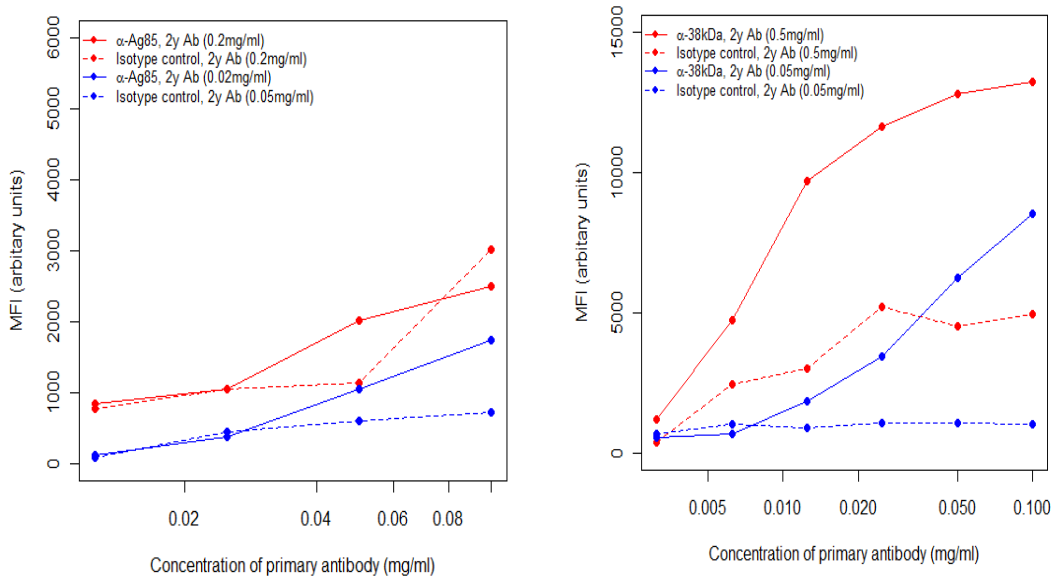


Figure 5.14 Titration of α-Ag85/α-38kDa and FITC-conjugated secondary antibodies

For the α-Ag85 antibody, there was poor MFI separation between cells labelled with the primary antibody or isotype control. This antibody was not studied further. For the α-38kDa antibody, a primary antibody concentration of 0.0125mg/ml and a secondary antibody concentration of 0.5mg/ml showed better selective labelling.

The selected α-38kDa antibody combination was incubated with the negative control organisms. Despite some auto-fluorescence from yeasts, the greatest MFI signal was from labelled H37Rv cells (figure 5.15), confirming specific bacterial staining of *M tuberculosis*.

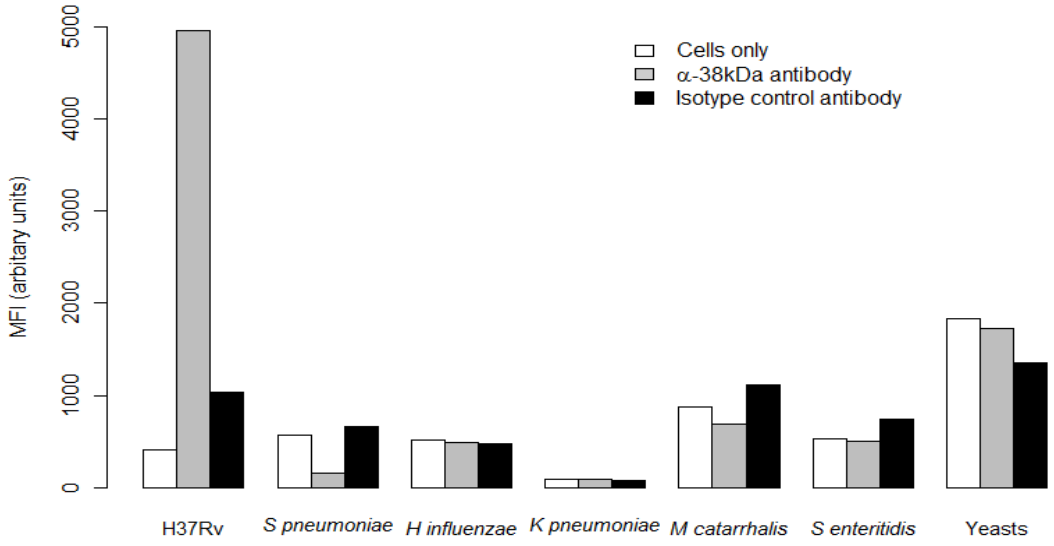


Figure 5.15 α-38kDa antibody labelling of H37Rv or other respiratory organisms

Assessment of Auramine O/phenol

As previously discussed, the binding properties of Auramine O to *M tuberculosis* are incompletely understood and its excitation/emission spectra are wide. Nevertheless, Auramine O/phenol was assessed as a flow cytometry label because it is cheap, widely available and the most widely used fluorescence TB stain in clinical practice.

Microscopy strength Auramine O/phenol solution was prepared as described in Appendix 10.4.2 and passed through a 0.22µm filter. A series of two-fold dilutions (neat solution to 1/32) were prepared. 100µl of each dilution were added to 100µl of H37Rv cells and each of the negative control organisms for 10 minutes at 4°C or 20°C and washed twice in PBS. 100µl 1% acid-alcohol was added to each tube for a further 10 minutes, prior to a final wash and re-suspension in 1% formaldehyde. When run on the flow cytometer good selective labelling of *M tuberculosis* cells was shown down to a dilution of 1/4. This dilution of Auramine O/phenol was used for further experiments (Figure 5.16).

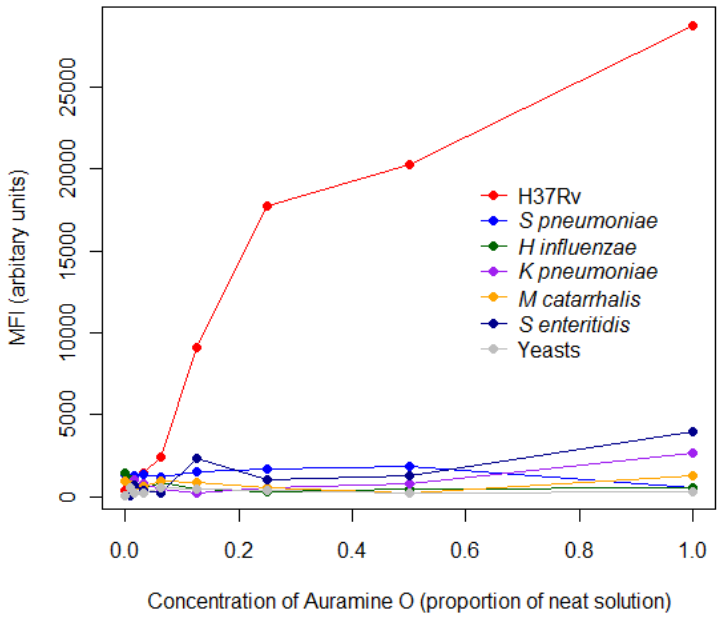


Figure 5.16 Titration of Auramine O against H37Rv

5.4.4 Identifying *M tuberculosis* in mixed cultures

Mixed suspensions of H37Rv and the negative control organisms were used to establish whether specific labelling of TB cells could be reproduced in polymicrobial samples. A bacterial counting kit (Invitrogen) was used to calculate the proportion of each cell type added to each suspension. Briefly, 1µl of kit Component A (SYTO BC nucleic acid stain) was added to individual 1ml samples of each microorganism at a MacFarland 1.0 turbidity standard and incubated at room temperature for 5 minutes. 10µl of kit Component B (microsphere beads) were then added, samples were mixed thoroughly and run on the flow cytometer. On a FSC vs. green (FITC) fluorescence scatter plot, events due to microsphere beads had higher FSC and events due to SYTO BC stained organisms had greater fluorescence. As 10µl of kit Component B contained 10^6 microsphere beads, each bead represented 10^{-6}ml of a 1ml sample. The concentration of organisms in each sample was calculated by;

$$\textit{Bacteria/ml of sample} = 10^6 \; X \; \frac{\textit{No of SYTO BC stained events due to microorganisms}}{\textit{No of events due to microsphere beads}}$$

When the bacterial concentration of each cell type was known, two polymicrobial suspensions were prepared; Suspension A was a cocktail of non-mycobacterial organisms in equal proportions and Suspension B was a 50:50 mixture of *M tuberculosis* and the non-mycobacterial cocktail. These samples were labelled with Auramine O/Phenol or α-38kDa

antibody and run on the flow cytometer (Table 5.8). Amongst cells gated for FSC/SSC properties of *M* tuberculosis, Auramine O/phenol picked out a distinct population of high-fluorescence events in Suspension B which accounted for 46% of total events in the sample and was absent in Suspension A. This was believed to represent successful identification of *M tuberculosis.* There was also a change in the fluorescence histogram between Suspension A and Suspension B after labelling with α-38kDa antibody but it was difficult to discriminate a clear population of *M tuberculosis* cells.

A further set of mixed bacterial suspensions were prepared containing H37Rv and the non-mycobacterial cocktail in the following ratios; 0:100, 25:75, 50:50, 75:25 and 100:0. These suspensions were stained in quadruplicate with Auramine O/phenol and run on the flow cytometer to test whether this label could identify changes in the mycobacterial burden of polymicrobial samples. Figure 5.17 shows some inter-sample variability in the proportion of events reported as H37Rv for each suspension. However, the overall correlation between the proportion of events counted as H37Rv on flow cytometry and the proportion expected from the known composition of cell suspensions was strong (Adjusted R^2: 0.84, $p<0.005$).

Although imperfect, on the basis of these experiments, a 1:4 dilution of Auramine O/phenol was the best of the fluorescence labels tested *in vitro* for staining of *M tuberculosis*. It was assessed further in flow cytometry of sputum samples.

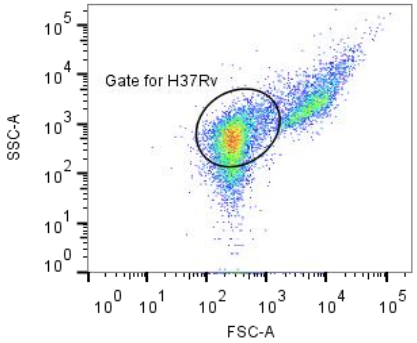
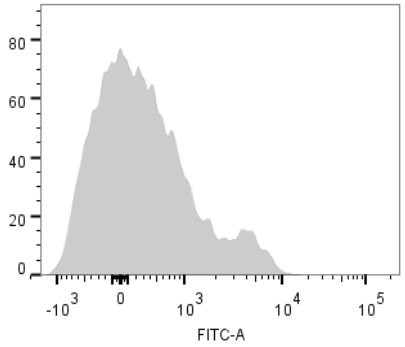
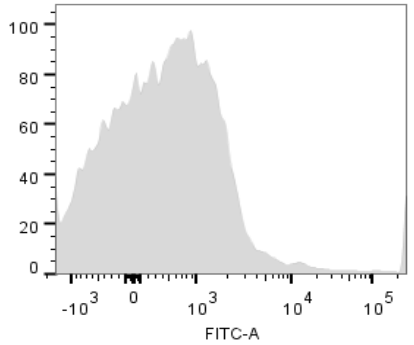
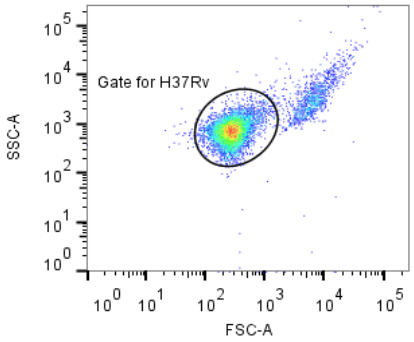
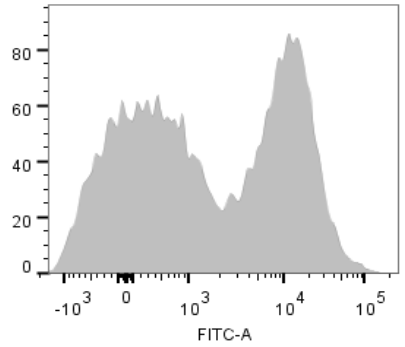
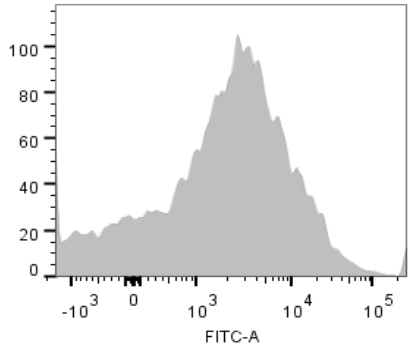
	FFS/SSC scatter plot	Fluorescence histogram for H37Rv-gated events labelled with Auramine O/phenol	Fluorescence histogram for H37Rv-gated events labelled with α-38kDa antibody
Suspension A Non-mycobacterial cocktail			
Suspension B *M tuberculosis* + non-mycobacterial organisms			

Table 5.8 Flow cytometry labelling of mixed suspensions of micro-organisms

Cells with FSC/SSC properties typical of H37Rv were gated. After Auramine O/phenol labelling, the fluorescence histogram showed a population of cells with high FITC-signal in Suspension B but not Suspension A. This accounted for 46% of total events in the sample and was believed to represent successful identification of *M tuberculosis*. After α-38kDa antibody labelling, the fluorescence histogram showed a shift towards higher fluorescence in Suspension B but a clear population of labelled *M tuberculosis* cells was difficult to discriminate.

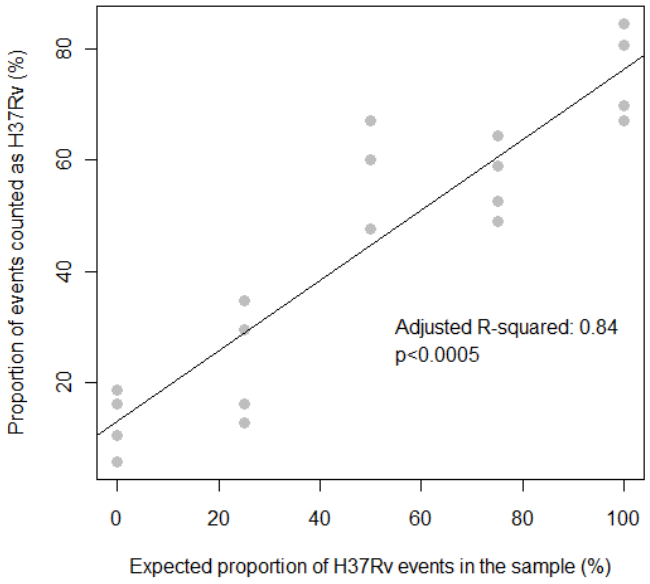


Figure 5.17 Auramine O labelling of H37Rv in mixed suspensions of organisms

5.4.5 Flow cytometry of TB-spiked sputum

Experiments were performed to test whether AuramineO/phenol staining could pick out *M tuberculosis* events from the background sputum matrix. A 2ml smear negative sputum specimen was divided in two; one half was retained as an "un-spiked" control and the other was "spiked" with 1ml of an H37Rv cell suspension diluted to MacFarland 1.0 turbidity standard. Both samples were digested in dithiothreitol for one hour and labelled according to the Auramine O/phenol staining protocol. The samples were then passed through a sterile 70µm filter to remove large clumps and run on the flow cytometer.

Both spiked and un-spiked samples contained a large amount of particulate debris resulting in a very high event rate dominated by background noise. This made it almost impossible to discriminate between them. Previous flow cytometry studies have been able to find host lymphocytes cells in the sputum of asthmatic patients[590], but eukaryotic cells are much larger (≈12µm diameter) than non-cellular debris. Small TB cells (length≈5µm), are difficult to distinguish on the basis of FSC/SSC scatter and some particulate matter auto-fluoresces, blurring the degree of separation from other sputum constituents. One prior study of TB-spiked sputum to assess sample decontamination methods overcame this problem by performing flow cytometry on diluted supernatant after sample centrifugation[591] but this

approach reduces the sensitivity of the assay and it seems unlikely that it would work on clinical specimens of low bacillary burden.

Attempts to clean the sputum by passing it through a range of smaller filters (10µm, 20µm and 45µm) did not bring improvement, because some micro-particles were not removed. NALC-NaOH decontamination did not solve the problem as non-mycobacterial cells were lysed without removal of debris. Attempting to break down the sample by sonication in a water-bath was also unsuccessful and carried the additional risk of damaging *M tuberculosis* cells.

Careful analysis of a FSC vs. FITC scatter plot did show a population of probable H37Rv cells in spiked sputum which was absent from the un-spiked specimen (Figure 5.18). However, this population represented <5% of total events in the sample and could only be identified when more than 10^7 H37Rv cells/ml were added. Data from Chapter 4 and Chapter 6 show that, except at baseline, the sputum bacillary load is normally lower than this during clinical infection. Therefore, the flow cytometry assay developed so far would be unlikely to find persister organisms from later samples collected during chemotherapy.

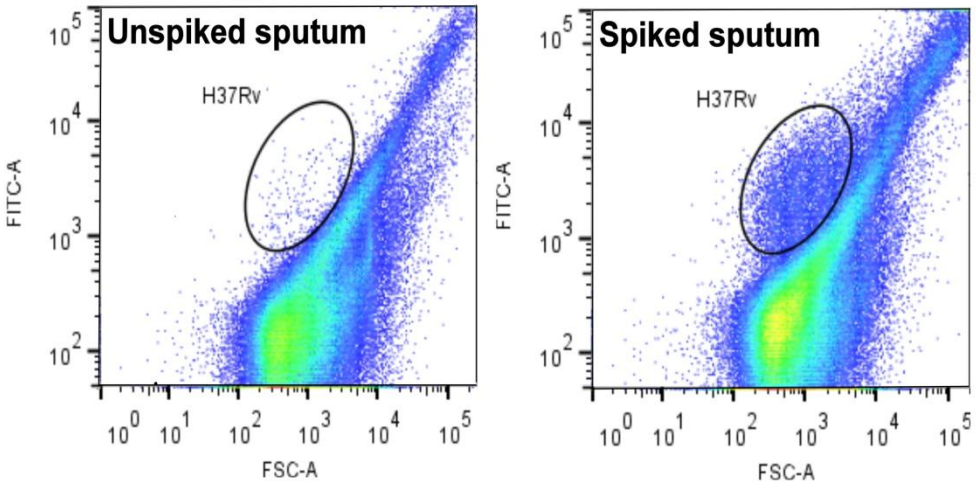


Figure 5.18 FITC fluorescence vs FSC scatter plot for Auramine O stained sputum

After spiking smear negative sputum with a ≥10^7 cells/ml suspension of H37Rv, a small population of cells (<5% of total events) is identified by flow cytometry.

5.4.6 Flow cytometry: conclusions

A satisfactory flow cytometry assay to facilitate study of persister sub-populations of *M tuberculosis* was not available in time for the clinical study. Because optimisation of ALTR microscopy was more successful, a decision was made to use that technique in Malawi.

However, the data described above is encouraging. Microbiological flow cytometry is a relatively new discipline and the potential of the technique to improve understanding of the response of *M tuberculosis* to treatment has not been explored. The experiments here confirm that flow cytometry of TB is safe and that TB cells can be discriminated *in vitro* from other respiratory microorganisms. In principle, selective identification of TB bacilli in sputum is possible. This lays a useful foundation for future work. Further refinement of the sputum processing method (e.g. labelling TB cells with antibody-bound magnetic nano-particles and removing them from the sputum matrix via a Magnetic Activated Cell Sorting column) may facilitate more satisfactory study of clinical samples, and DNA labelling or cell viability dyes may yield new information on cell cycle and survival of non-culturable bacteria during drug exposure, either via *in vitro* models of persistence or study of samples collected during clinical infection. Plans for additional work in this area are in progress.

In summary, the study of non-culture based single cell techniques for assessment of *M tuberculosis* prior to commencement of the clinical study was useful. A novel fluorescence microscopy technique was optimised and exported to Malawi, and preliminary work on flow cytometry generated data to guide ongoing research.

6. Pharmacodynamics

6.1 Introduction

The clinical cohort description in Chapter 3 reported that 15/133 (11%) patients had unfavourable outcomes (treatment failure or relapse), predominantly caused by DS-TB. However, analysis of clinical and radiological factors did not explain inter-individual differences in treatment response. Pharmacodynamic analyses will now be undertaken to establish whether bacteriological data provide additional information.

The simplest early measures of pharmacodynamic response are sputum smear and culture conversion. The Malawian NTP uses smear conversion at 2 months to assess individual patients and culture conversion is often used as a surrogate end-point in Phase IIb clinical trials. The relationship between these measurements and clinical outcome will be assessed for study participants.

Serial $\log_{10}$CFU/ml counts and TTP data obtained using the culture techniques from Chapter 4 will used to model bacillary elimination. Associations between model parameters and final outcome will be examined to establish whether this approach generates novel biomarkers to replace traditional assessment of smear/culture conversion at a single time-point.

As described in Chapter 2, urine samples were stored from each patient at baseline and S2-S4 visits for the urinary LAM-ELISA. The potential role of repeated urinary LAM measurement as a measure of "whole body bacillary load" to augment information on bacillary clearance from sputum will be considered.

The novel ALTR microscopy technique from Chapter 5 was used to evaluate associations between LB counts, TB therapy and clinical outcomes. The first clinical exploration of this method as a tool to identify putative drug tolerant sub-populations of persister bacilli will be described.

As these pharmacodynamic analyses aimed to improve understanding of the clinical significance of persistence and validate surrogate markers for use in Phase IIb clinical trials, the datasets for this Chapter were restricted to samples from the 133 patients who reached a pre-defined study end-point.

6.2 Methods

Procedures used to collect clinical and laboratory data were outlined in Chapters 2-5. Further operational aspects are provided here, alongside the LAM-ELISA method and the statistical approach to pharmacodynamic data analysis.

6.2.1 Additional aspects of the quantitative bacteriology methods

As described in Figure 4.3, contamination of SSCC plates in the clinical study necessitated a change in media formulation. Standard media from June-December 2010 was 7H10-Amb10, whilst standard media thereafter was 7H11-AmB30. However, plate comparison experiments showed that media alterations did not significantly alter colony counts. For pharmacodynamic analysis, SSCC data from different media preparations were analysed together.

As described in Section 4.3.3, organisms from positive MGIT bottles were speciated. Only TTP values from samples containing pure growth of *M tuberculosis* were analysed.

6.2.2 LAM-ELISA method

10ml urine was collected from patients in a sterile universal container at the specified visits. In the MLW laboratory 2ml aliquots were transferred into tightly sealed micro-centrifuge tubes and heated to 95^{o}C for 30 minutes. After cooling to room temperature the samples were centrifuged at 10,000rpm for 15 minutes. Supernatant was stored at -20^{o}C.

At a later date, sample batches were defrosted and analysed using Clearview^{TM} TB-ELISA kits according to the manufacturer's instructions. 100µl of Positive control, Negative control and patient samples were pipetted into duplicate wells of anti-LAM antibody coated microtitre plates which were sealed with adhesive film and incubated at room temperature for 25 minutes. Contents were aspirated and plates were inverted and firmly tapped over a paper towel to remove residual fluid. Plates were washed with a pre-prepared solution (PBS with 0.05% Tween-20) four times. Following the last wash, 100µl of HRP-conjugate solution (a ready to use preparation of rabbit anti-LAM antibodies conjugated to horseradish peroxidase) were added to each well. Plates were re-sealed and incubated at room temperature for a further 60 minutes prior to removal of contents and repeat washing as described above. 100µl of chromogenic substrate solution (tetramethylbenzidine) were added to each well and incubated at room temperature for 15 minutes before the final addition of 100µl of Stop Solution (1M sulfuric acid). Plates were gently shaken and read immediately in a plate reader at a wavelength of 450nm.

The mean OD from duplicate readings was taken as the result for all samples. Results of each plate were valid if the mean OD of the negative Control was 0.1-0.3 and the mean OD of the positive control OD was 0.3-0.5 units above the negative control. The positive/negative cut-off value for the assay was the mean negative control value +0.1. The LAM OD of each sample was calculated by:

LAM OD = *Mean of ODs measured from duplicate wells* - *Mean OD of negative control*

If this gave a negative value, the sample LAM OD reading was regarded as zero.

6.2.3 Additional aspects of ALTR microscopy method

Selected samples were thawed for analysis. Baseline samples were used to assess factors contributing to variability in LB counts at PTB diagnosis. To ensure that smears contained sufficient organisms to calculate af-LB counts, only patients whose sputum had been graded '+++' on initial AP staining were used.

Analysis of serial samples was undertaken to study changes in LB counts during therapy. This was done for patients whose sputum had been graded '+++' at baseline and at least '++' on one or more subsequent occasion. All patients who reached an unfavourable final end-point and had submitted adequate samples were included. Each was matched to three patients who reached a favourable end-point and had submitted an eligible sample set.

No more than 8 samples were read on duplicate slides in a single batch. Microscopy sessions lasted 3-4 hours. Samples were thawed and smears heat-fixed the afternoon before microscopy, staining was done in the early morning and most slides were read the same day. Any slides left over were stored at 4^{o}C in the dark and read the next morning.

During image analysis, the severity of background matrix staining for each slide was graded using criteria from Section 5.3.6. The quality of bacillary labelling with auramine and LB labelling with LTR was graded as clear or blurred/faint. Mean af-LB and TLB counts were calculated as described in Section 5.3.11.

6.2.4 Data analysis and statistical methods

General methods

Non-parametric summary statistics were used. Inter-group comparisons of continuous measurements were done using Wilcoxon or Kruskal-Wallis tests and inter-group comparisons of categorical measurements were done using the chi-squared test. Assessment of relationships between continuous variables was done by linear regression. Inter-reader variability in af-LB counts was evaluated using Lin's concordance co-efficient (ρ_c). Linear and logistic regression were used to assess factors contributing to variability in baseline colony counts, MGIT-TTP, LAM-ELISA results and proportions of LB positive cells. For multivariate modelling, all variables with $p<0.10$ on univariate analysis were included. Logistic regression was used to assess relationships between early pharmacodynamic measurements and treatment outcome.

Analysis of sputum culture conversion

Time to sputum smear and culture conversion were evaluated by survival analysis. Smear and culture conversion dates were taken as the midpoint between the last positive and first negative result for each patient. "Time to conversion" was described by Kaplan Meier plots. Differences between different smear (ZN vs. AP) and culture (SSCC vs. MGIT) methods were reported by Cox proportional hazards ratios.

NLME modelling of SSCC data

The SSCC-NLME model fitting procedure required the 'nlme' package in 'R' and was adapted from previous similar studies[228,266,270]. To provide information on bacillary elimination, individual patients were required to contribute a minimum of two bacillary load measurements at different sampling time-points. Patients with only one positive sample were excluded. Individual and non-parametric summary plots were used to assess trends in bacillary clearance and identify outliers. Mono- and bi-exponential functions were evaluated as non-linear models for the pooled SSCC data (Figure 6.1).

Mono-exponential – one phase bacillary elimination

$$\log_{10}CFU=\log_{10}(e^{\theta 1} \times e^{-day \times e\theta 2})$$

Bi-exponential – two phase bacillary elimination

$$\log_{10}CFU=\log_{10}[(e^{\theta 1} \times e^{-day \times e\theta 2}) + (e^{\theta 3} \times e^{-day \times e\theta 4})$$

Figure 6.1 Non-linear exponential functions fit to SSCC dataset

In the mono-exponential function, all *M tuberculosis* organisms in sputum are viewed as a single population, with intercept θ_1 representing the baseline bacillary load and rate constant θ_2 describing the elimination rate. The bi-exponential function is analogous to the biphasic model of two distinct bacterial populations (A and B) described in Figure 1.5. θ_1 and θ_3 intercepts represent baseline bacillary loads A_{Int} and B_{Int} whilst rate constants θ_2 and θ_4 represent elimination rates α and β.

The equation for each exponential function expresses θ parameters on the natural log scale. These may be transformed to the $\log_{10}$ scale normally used for colony counting as follows:

$$\text{Intercept on } \log_{10} \text{ scale} = \theta_{1,3 \text{ or } 5}/2.303$$

$$\text{Rate constant on } \log_{10} \text{ scale} = e^{\theta 2,4 \text{ or } 6}/2.303$$

The exponential functions were fit to the pooled data by non-linear least squares. The bi-exponential function was then fit using a NLME maximum likelihood method in which each parameter was assigned a distribution (random effect) to account for non-independence of serial observations and inter-individual variability. For each function, starting values were obtained by graphical methods and varied to check the stability of solutions.

Models were compared using the Akaike Information Criterion (AIC), likelihood ratio and model-based F-tests. Goodness of fit was examined by graphical analysis of residuals. The effect of clinical and radiological covariates on NLME bi-exponential model parameters $\theta_{1\text{-}4}$ was explored.

Best unbiased estimates of each parameter were extracted from the NLME bi-exponential model for every patient and transformed to the $\log_{10}$ scale in order that relationships between treatment outcome and baseline bacillary load or elimination rate for bacterial populations A and B could be studied by logistic regression.

A limitation of SSCC-NLME modelling is that culture results below the limit of detection (1.27 $\log_{10}$ CFU/ml) are censored. Detailed consideration of methods to account for this missing data is beyond the scope of this thesis but a preliminary analysis was undertaken to explore the effect of integrating likely data below the limit of detection into the model. For this, a partial likelihood approach was implemented using the M3 Method in NOMMEM VVII v2 with Pirana and R. Model checking and diagnostics were similar to those described for other mixed effects models.

Linear mixed effects modelling of MGIT data

MGIT-TTP analysis was restricted to patients with at least 2 TTP measurements. After review of individual and non-parametric summary plots a linear mixed effects (LME) model of the form

$$TTP = a + b \times [\text{time on therapy}]$$

was fit to the data. The most important fixed effects parameter of this model is the rate constant (*b)* which may be viewed as the MGIT bacillary elimination rate (MBER). Quadratic and SPLINE functions were used to test for curvature and compared to the LME approach.

The effect of clinical and radiological covariates on the MBER was explored. Unbiased parameter estimates for each patient were extracted from the model to assess the relationship between MBER and final treatment outcome.

Negative cultures without a TTP value were censored. This is more problematic for MGIT than SSCC, as illustrated in Figure 6.2. Two approaches to negative data were considered; a simple rule-based TTP imputation of 50 days (one day after the limit of detection) was allocated to the first negative result for each patient, and NONMEM software was used to explore the effect of integrating the likelihood of data beyond the limit of detection.

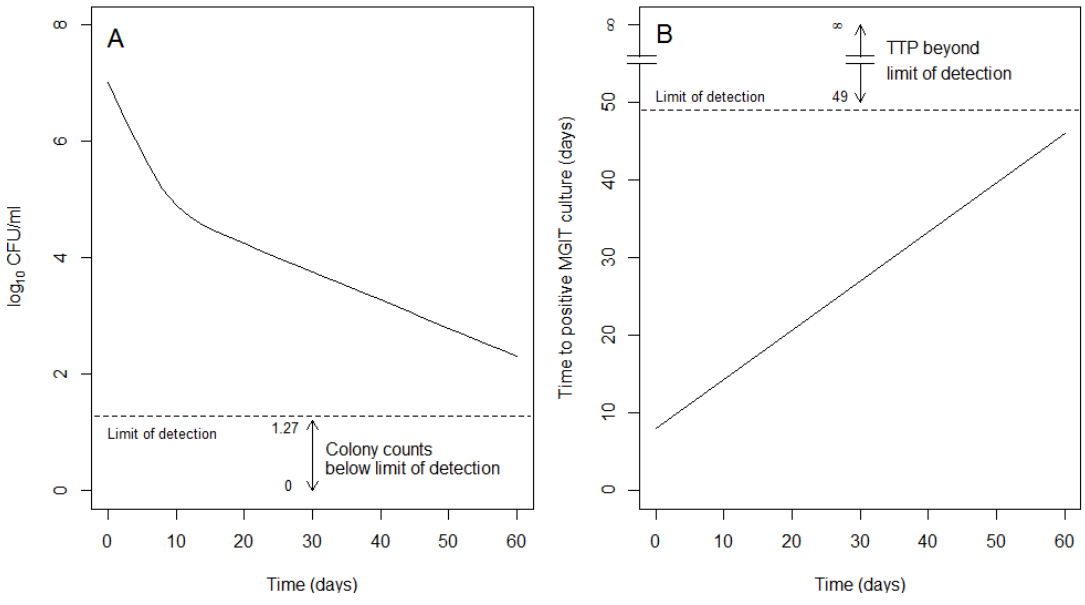


Figure 6.2 Data-points beyond the limit of detection in SSCC AND MGIT modelling

A: Data-points below the limit of detection in SSCC-NLME modelling range from 0-1.27 $\log_{10}$CFU/ml. B: Data-points beyond the limit of detection in MGIT-TTP modelling are more variable, ranging from 49.5 days to infinity.

6.3 Results

6.3.1 Sputum smear and culture conversion

Table 6.1 shows the proportion of study patients who remained smear (ZN or AP) or culture (SSCC or MGIT) positive at S1-S4 visits and Figure 6.3 shows Kaplan-Meier plots of Cox proportional hazards models for time to smear/culture conversion. Table 6.2 shows relationships between smear and culture status at 2 months and final clinical outcome.

17-21 (13-16%) patients remained sputum smear positive at the S4 visit and there was no significant difference in time to smear conversion between ZN and AP methods (Hazard ratio for AP smear conversion for each day of treatment: 0.90, 95% CI: 0.69-1.20, p=0.482). However, it was impossible to verify whether bacilli visualised on microscopy of samples collected during therapy were alive or dead and there was no association between smear conversion and final clinical outcome.

Culture conversion on SSCC plates was much quicker than in MGIT bottles. Only 7 (6%) patients remained positive on Middlebrook agar plates by the S4 visit, compared to 33 (25%) patients in liquid broth. The difference in time to culture conversion between methods was significant (Hazard Ratio for MGIT culture conversion for each day of treatment: 0.27 (95% CI: 0.19-0.37, p<0.001), perhaps because the more nutritious growth environment provided by liquid broth more efficiently revives fastidious organisms and increases the likelihood of identifying persisters at later time-points.

2 month culture status on SSCC plates was strongly predictive of final clinical outcome (p=0.005) whilst the result in MGIT bottles was not (p=0.128). This suggests that, for prediction of outcome with a binary end-point the less sensitive technique was a more useful surrogate marker. A proposed explanation for this is that patients with heavily positive cultures (detectable by SSCC) at the end of the Intensive Phase are most likely to suffer treatment failure or relapse, lending prognostic value to the S4 result. Some patients with scantily positive cultures (detectable only by MGIT) will achieve stable cure during the Continuation Phase; their inclusion as S4 positives dilutes the association with unfavourable outcome.

	Number of patients (n, %)				
	Valid BL[a]	S1 (Day 2 or 4)	S2 (Day 7 or 14)	S3 (Day 21 or 28)	S4 (Day 49 or 56)
2 month smear positive					
ZN smear	133	119 (93)	109 (85)	70 (54)	17 (13)
AP smear	126	120 (96)	110 (87)	73 (58)	20 (16)
Any smear (ZN or AP)	133	121 (95)	112 (88)	77 (60)	21 (16)
2 month culture positive					
Middlebrook SSCC plates	121	89 (74)	67 (55)	25 (21)	6 (6)
MGIT liquid culture	130	124 (95)	119 (92)	102 (79)	31 (23)
Any culture (SSCC or MGIT)	133	126 (95)	121 (91)	103 (77)	34 (25)

Table 6.1 Smear and culture conversion during treatment

[a]Baseline AP smears were not done in the CoM research laboratory on 7 patients. Baseline SSCC plates were contaminated or negative in 12 patients and baseline MGIT bottles were contaminated in 3 patients. Patients without a positive result at baseline were removed from this analysis

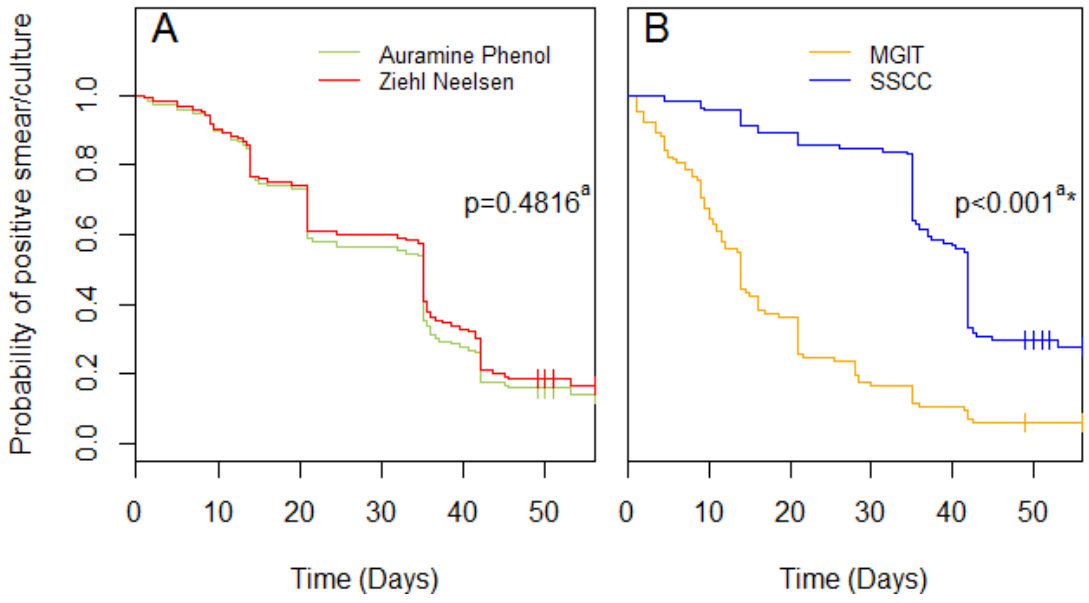


Figure 6.3 Kaplan Meier plots of time to smear and culture conversion

[a]Survival analysis by Cox proportional hazards models. Hazard ratio for AP vs. ZN smear conversion per day: 0.90 (95% CI: 0.69-1.20, p=0.482) and for MGIT vs. SSCC culture conversion per day: 0.27 (95% CI: 0.19-0.37, p<0.001).

	Unfavourable N=15	Favourable N=133	Odds Ratio	95% CI	p-value
2 month smear positive					
ZN smear, n (%)	1 (8)	17 (16)	0.44	0.05-3.58	0.440
AP smear, n (%)	1 (8)	21 (20)	0.34	0.04-2.77	0.314
Any smear (ZN or AP), n (%)	1 (8)	21 (20)	0.35	0.04-2.84	0.325
2 month culture positive					
Middlebrook SSCC plates, n (%)[a]	4 (31)	2 (2)	19.33	3.10-120.63	0.005*
MGIT liquid culture, n (%)[b]	6 (47)	25 (25)	2.50	0.77-8.16	0.128
Any culture (SSCC or MGIT), n (%)	7 (50)	27 (26)	2.85	0.92-8.88	0.071

Table 6.2 Associations between smear and culture conversion and clinical outcome

[a]Smear positivity at baseline is required to assess conversion. N=126 for AP smear.
[b]Culture positivity at baseline is required to assess conversion. N=121 for SSCC, N=130 for MGIT

6.3.2 Quantitative sputum culture: baseline bacillary load

As the baseline bacillary load may influence subsequent bacterial elimination and clinical outcome, factors influencing baseline SSCC and MGIT results were assessed prior to pharmacodynamic modelling.

SSCC does not include a decontamination step, so distortion of data during sample processing was unlikely but some specimens were lost due to bacterial or fungal overgrowth and a valid baseline colony count was available for 101/133 (76%) patients. The median baseline bacillary load was 6.19 (range 2.20-9.30) $\log_{10}$ CFU/ml. Conversely, the MGIT TTP results may be vulnerable to an unpredictable NaOH effect, but contamination was less. A valid baseline TTP was available for 113/133 (85%) patients. Median TTP at baseline was 4.0 (range 0.5-27.0) days.

Linear regression was used to investigate factors influencing results from both methods (Table 6.3). On multivariate analysis, greater percentage of lung affected on CXR (p=0.035) was the only variable associated with both higher colony count and shorter TTP (p=0.035 and p=0.002 respectively). A univariate association was also described between cavities ≥4cm (p=0.011) and shorter TTP. Overall, the relationship between CXR appearance and quantitative bacteriology was strong.

Although higher baseline 25 (OH) D level was associated with shorter TTP (p<0.001), the effect size was very small and the corresponding colony counting analysis was non-significant (p=0.737). Further assessment is required in other clinical cohorts.

Univariate analysis suggested that male sex was associated with higher colony count (p=0.021) and shorter TTP (p=0.027). There were also univariate relationships between higher colony count and inability to read (p=0.009), lack of electricity at home (p=0.018) and higher baseline temperature (p=0.007). Poorly educated or socially disadvantaged men may present with more severe disease at higher bacillary loads but the study was not designed to explore healthcare seeking behaviour and over-interpretation of the data would be inappropriate.

ART prior to recruitment was associated with shorter TTP (p=0.031). This unexpected result requires assessment in other cohorts.

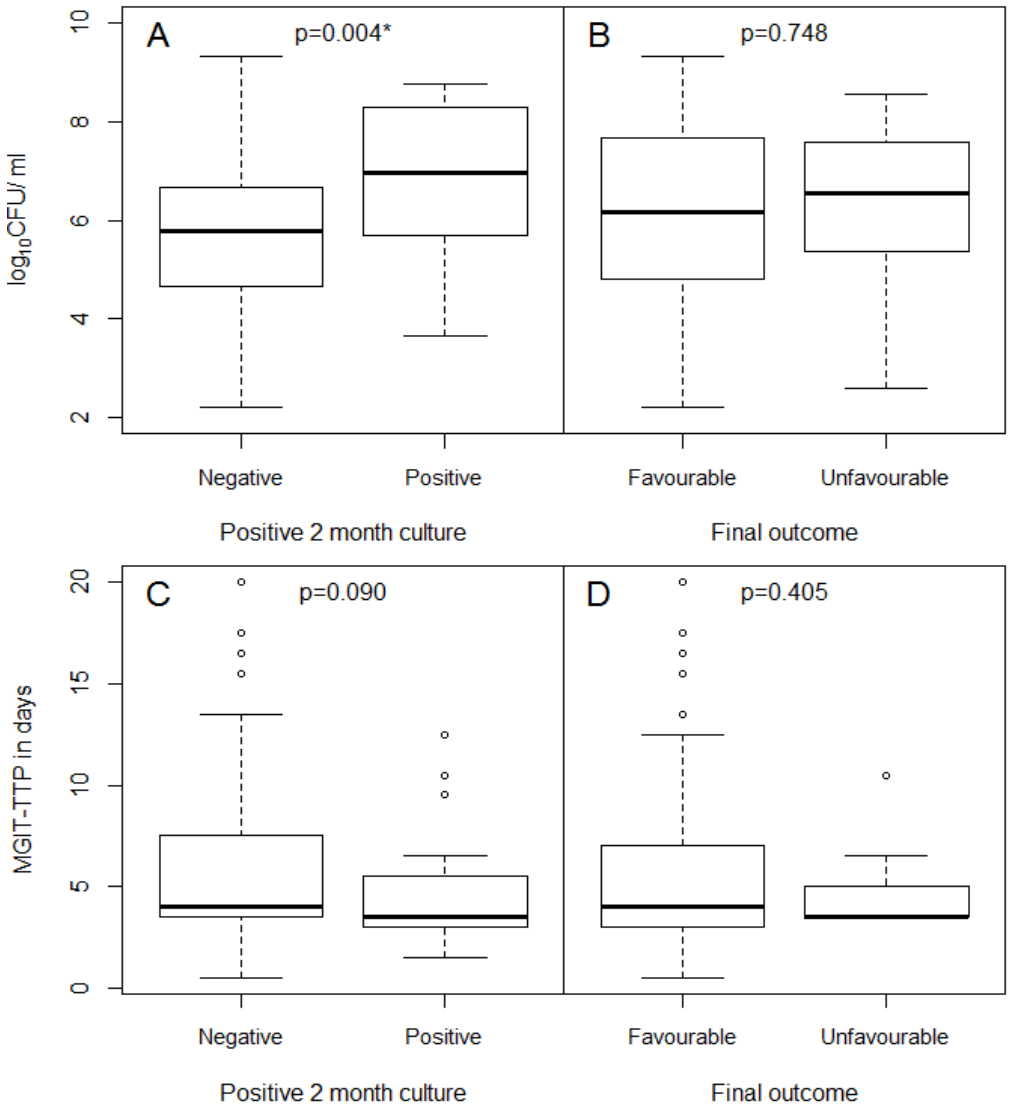
	Baseline log_{10}CFU/ml colony count						Baseline MGIT TTP					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
Variable	Estimate	Std. Error	p-value	Estimate	Std. Error	p-value	Estimate	Std. Error	p-value	Estimate	Std. Error	p-value
Age, years	0.017	0.020	0.422	-	-	-	-0.012	0.049	0.806	-	-	-
Male sex	0.899	0.384	0.021*	0.532	0.340	0.187	-2.087	0.929	0.027*	-1.820	1.075	0.092
Able to read a newspaper	-0.659	0.389	0.009*	0.190	0.439	0.666	0.523	0.991	0.599	-	-	-
Electricity at home	-0.839	0.360	0.018*	-0.567	0.376	0.136	0.771	0.895	0.391	-	-	-
Ever drinks alcohol	0.4884	0.3970	0.222	-	-	-	-1.585	0.913	0.086	-0.803	1.008	0.428
HIV infected	0.043	0.355	0.904	-	-	-	-0.389	0.895	0.664	-	-	-
CD4 count, cells/µl[a]	0.002	0.002	0.429	-	-	-	-0.004	0.004	0.290	-	-	-
On ART at baseline[a]	0.273	0.508	0.592	-	-	-	-2.571	1.166	0.031*	-	-	-
Recent antibiotic used	0.932	0.559	0.010	0.896	0.557	0.111	0.248	1.387	0.859	-	-	-
Consulted traditional healer	2.180	1.249	0.084	1.958	1.149	1.092	-0.556	2.396	0.817	-	-	-
Baseline temperature, ^{o}C	0.434	0.157	0.007*	0.233	0.175	0.187	-0.351	0.413	0.398	-	-	-
Baseline WCC, $x10^{9}$ cells/l	0.006	0.009	0.458	-	-	-	-0.287	0.161	0.077	-0.233	0.153	0.131
Baseline 25(OH)D, nmol/l	-0.003	0.009	0.737	-	-	-	-0.035	0.021	0.092	-0.075	0.022	<0.001*
CXR Appearance[b]												
Percentage of lung affected	0.032	0.010	0.002*	0.024	0.011	0.035*	-0.083	0.026	0.002*	-0.089	0.279	0.002*
Cavities ≥4cm	0.608	0.372	0.106	-	-	-	-2.409	0.934	0.011*	-	-	-

Table 6.3 Factors influencing baseline colony count and TTP results

[a] Baseline CD4 and ART at enrolment assessed on HIV-infected individuals only

[b] CXR available for 93 patients with valid baseline log_{10} CFU/ml and 103 patients with valid baseline TTP

Figure 6.4 summarises the effect of baseline colony count and TTP on bacteriological response at 2 months and clinical end-points. Although there was an association with positive 2 month cultures in patients with higher baseline colony counts (p=0.004) and a similar trend with shorter TTP (p=0.090) there was no relationship between either measure of baseline bacillary load and unfavourable final outcome.



Method	Response measure	OR	95% CI	p-value
Baseline colony count	Positive 2 month culture	1.55	1.15-2.08	0.004*
	Unfavourable outcome	1.06	0.75-1.52	0.748
MGIT TTP	Positive 2 month culture	0.87	0.81-1.02	0.090
	Unfavourable outcome	0.94	0.80-1.09	0.405

Figure 6.4 Baseline bacillary load and treatment response

Relationships are shown between baseline $\log_{10}$ CFU/ml or TTP and treatment response.
A and C: Patients were positive if S4 visit samples were positive by either SSCC plates or MGIT.
B and D: Clinical outcomes were as described in Chapter 2. Analysis was by logistic regression.

6.3.3 NLME modelling of SSCC data

43/133 (33%) patients had only one available log_{10}CFU/ml count and were excluded from SSCC-NLME modelling. Figure 6.5 displays individual profiles of the change in log_{10} CFU/ml counts over time for the remaining 90 patients. Plots are ordered from bottom left to top right according to the baseline colony count. Patients with a higher pre-treatment bacillary burden contributed more positive serial samples and the decline in bacillary load followed a curvilinear pattern over time. Serial data from patients with lower baseline colony counts was limited by early culture conversion.

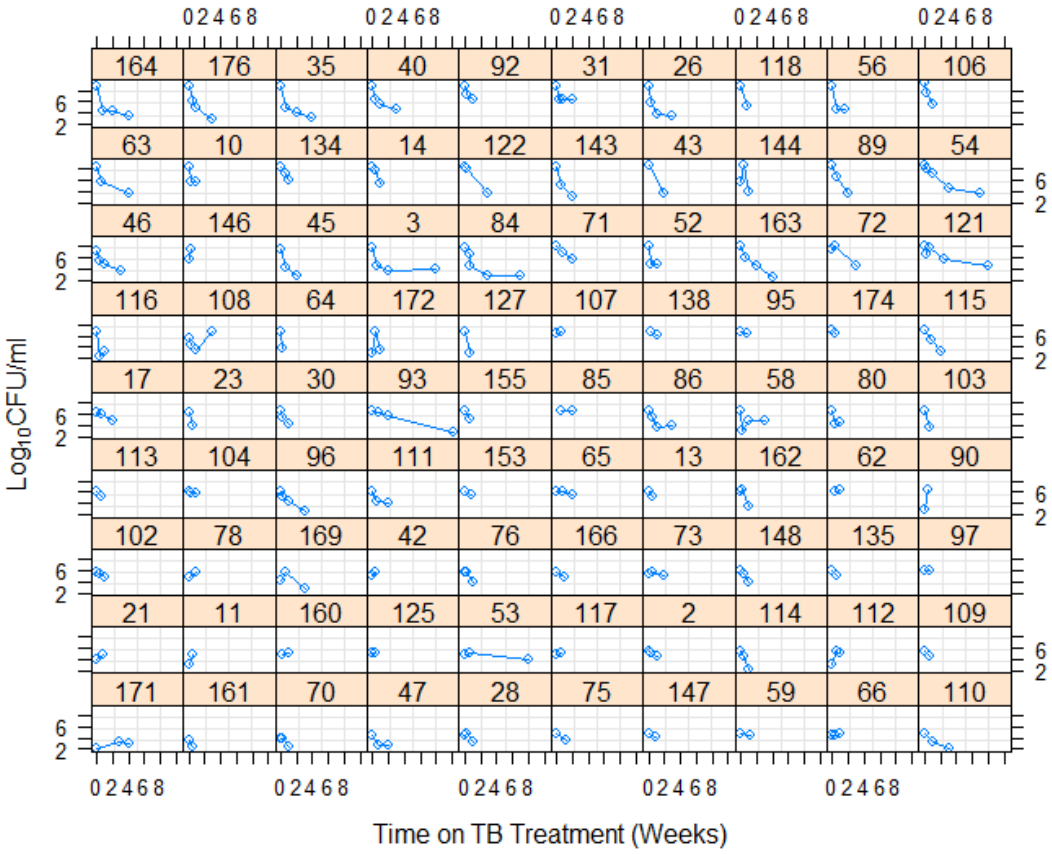


Figure 6.5 Individual patient profiles for log_{10}CFU/ml counts over time on therapy

8 patients (171, 21, 11, 169, 90, 172, 146 and 144) had individual profiles which were qualitatively different from the remainder of the cohort because the baseline colony count was ≥1 log_{10}CFU/ml lower than at the S1 visit. These increases in bacillary load during therapy are implausible as none of these patients had drug resistance. Laboratory worksheets were inspected for baseline samples from the atypical patients and revealed unusual features; smear positivity of baseline study specimens was "scanty" despite "++" or "+++" screening samples from the same day, or baseline study specimens were "salivary" or "heavily blood-stained". Incorporation of atypical baseline data into modelling functions

resulted in non-convergence or statistically unstable outcomes so these counts were removed from the dataset. For 4 individuals this left only one valid log_{10}CFU/ml result and the patient was excluded.

Consequentially, the final SSCC-NLME dataset was restricted to 244 data-points from 86/133 (64%) patients. Figure 6.6A is a spaghetti plot of these data. Figure 6.6B provides non-parametric summary measures (median and inter-quartile range) of colony counts from positive samples on the main sputum collection days. These plots support the impression of curvilinear bacillary elimination.

Choice of structural model

Details of the SSCC-NLME model building process are shown in Table 6.4 and Figure 6.6C. From non-linear least squares analysis of the pooled data, the bi-exponential function provided a significantly better stable fit than the mono-exponential function. It was not possible to fit a more complex tri-exponential function, perhaps due to the paucity of positive data after 28 days.

As the bi-exponential function had the lowest stable AIC an NLME model was fitted using this equation. The addition of mixed effects further improved the fit so this model was examined in relation to covariates and outcomes.

Mixed effects model	Parameters of model equations[a]				AIC	Likelihood ratio Test	p-value
	A_{Int}	A	B_{Int}	B			
Mono-exponential pooled data(1)	5.978	0.075	-	-	865.204	-	-
Bi-exponential pooled data (2)	6.638	0.323	4.416	0.020	814.225	(2) vs.(1)	<0.001
Bi-exponential NLME effects (3)	6.779	0.618	4.910	0.039	777.103	(2) vs.(3)	<0.001

Table 6.4 Fit of exponential models to the SSCC data

[a] θ_1- θ_4 values were transformed as described in Section 6.2.4 to provide A_{int}, α, B_{int} and β on the log_{10} scale. Units for baseline bacillary loads A_{Int} and B_{Int} are log_{10}CFU/ml, units for elimination rates α and β are change in log_{10}CFU/ml per day.

Table 6.5 provides full bi-exponential SSCC-NLME model parameters. The standard deviations of random effects demonstrate considerably more inter-individual variation in the θ_1 (A_{Int}) and θ_3 (B_{Int}) intercepts than θ_2 (α) and θ_4 (β) elimination rates. Underlying assumptions of a valid NLME model are that variation in residual values does not change at different fitted value results, and residuals are normally distributed. Figures 6.7A and B confirm that, with two outlying values from patients 58 and 112, this is so.

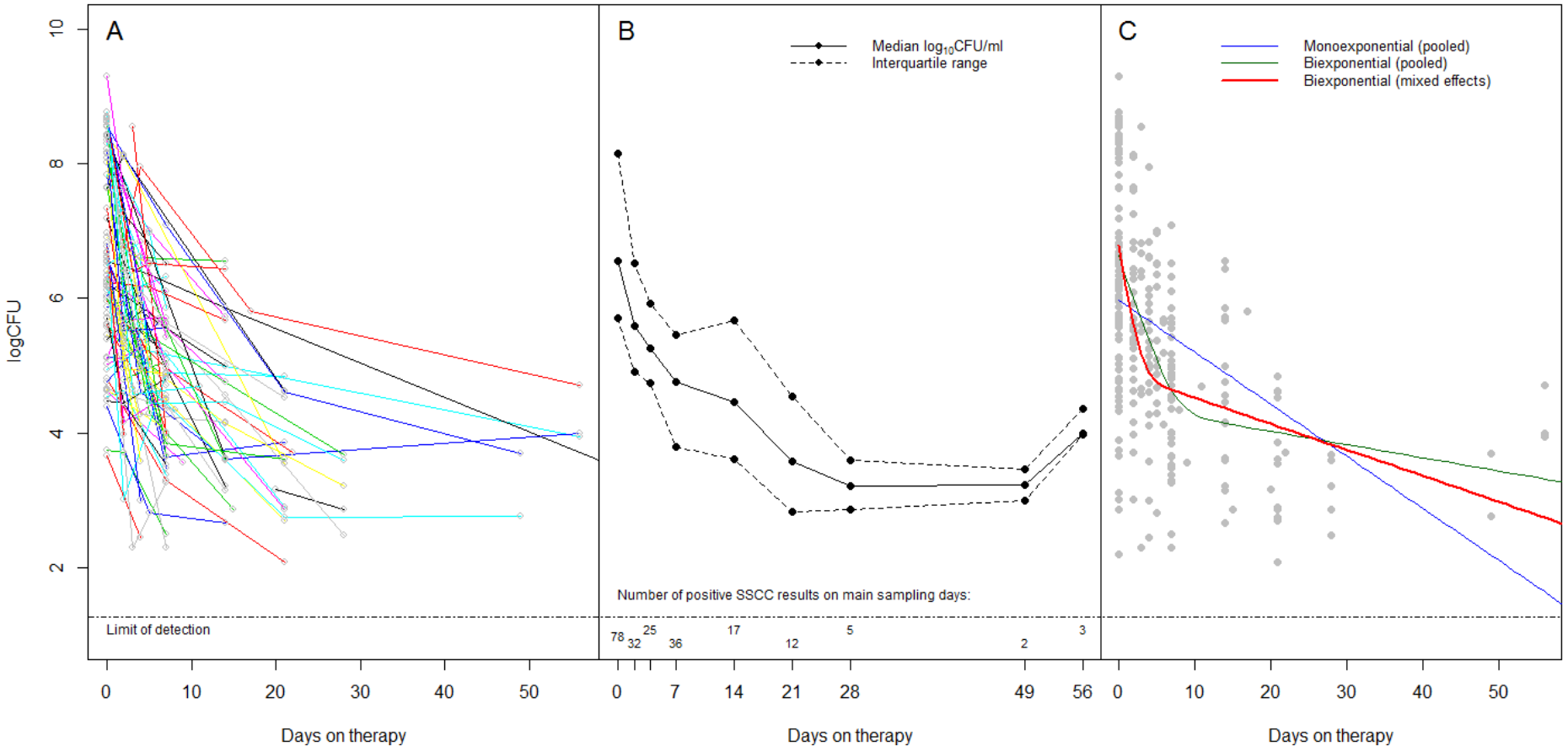


Figure 6.6 Plots for construction of SSCC-NLME model

A: Spaghetti plot of positive counts over time for each individual patient. B: Pooled non-parametric summary measures of log_{10}CFU/ml counts on each of the main sampling days. C: Fixed effects of SSCC-NLME modelling. Blue and green lines show mono- and bi-exponential models functions fit to the pooled data. The red line shows the mixed effects bi-exponential function which displayed the best fit (lowest AIC with convergent stable solution).

Non-linear mixed effects model
fit by maximum likelihood
AIC : 3203.985 logLik: 777.103

Fixed effects: logCFU~Days on treatment

	Value	Standard Error	p-value	*Value converted to log_{10} scale*
Metabolically active bacillary population				
θ_1	15.611	0.392	<0.001	$A_{Int} = 6.779$
θ_2	0.350	0.098	<0.001	$\alpha = 0.616$
Persister population				
θ_3	11.308	0.354	<0.001	$B_{Int} = 4.910$
θ_4	-2.420	0.187	<0.001	$\beta = 0.039$

Random effects (by patient)

	Std. Deviation
θ_1	2.905
θ_2	4.62×10^{-5}
θ_3	2.048
θ_4	9.03×10^{-5}
Residual	0.769

Table 6.5 Full parameters of SSCC-NLME model

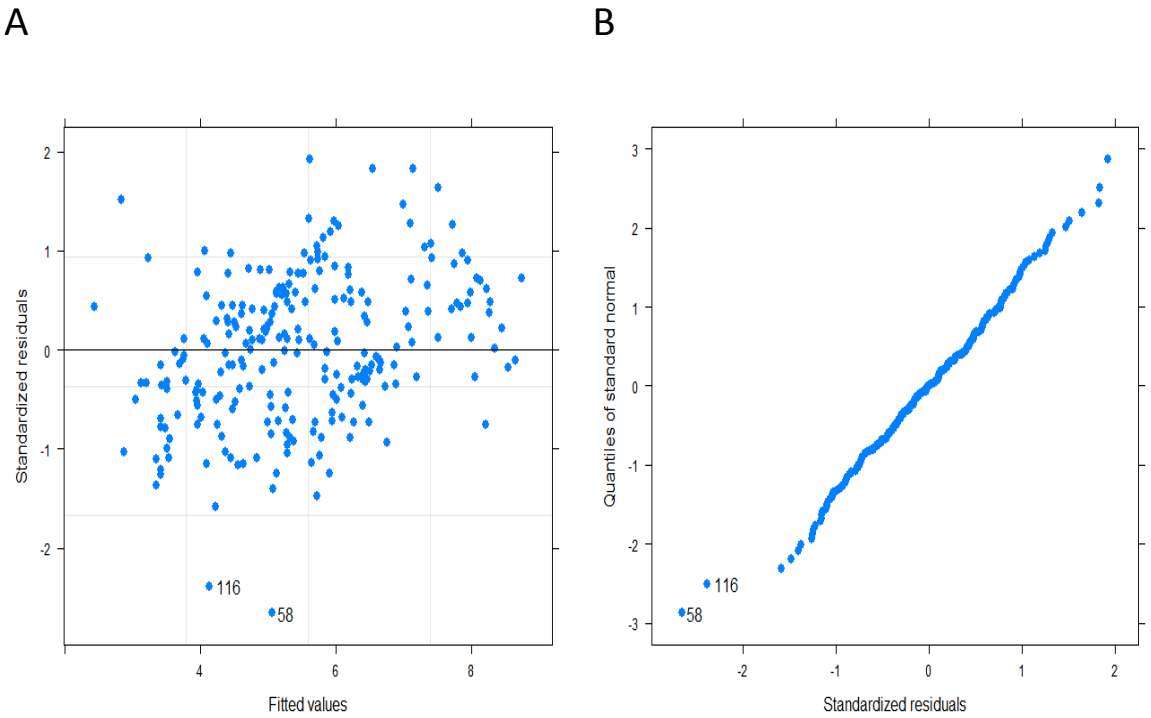


Figure 6.7 Testing SSCC-NLME model assumptions
A: Distribution of standardised residuals against fitted log_{10}CFU/ml values. B: The near linearity and symmetry around zero of points on the QQ-plot illustrates that residual values are normally distributed.

Covariates influencing the SSCC-NLME model parameters

The effect of clinical and radiological covariates from Chapter 3 on SSCC-NLME model parameters was assessed by graphical display of each covariate against best unbiased estimates of $\theta_{1\text{-}4}$ (representative plots in Figure 6.8). Sequential univariate model fitting was also done for each covariate. No factor altered any parameter in a manner which was clinically and statistically significant, suggesting that inter-individual variability attributable to these factors was accounted for by the model.

Associations between SSCC-NLME parameters and clinical outcome

Figure 6.9 and Table 6.6 show results of univariate logistic regression analysis performed to examine relationships between best unbiased estimates of model parameters for each patient and clinical outcomes. Day 0 intercepts and rate constants for putative metabolically active (A_{Int} and α) and persister (B_{Int} and β) sub-populations were expressed on the $\log_{10}$ scale. There was a slight trend towards unfavourable outcome in patients with a greater baseline bacillary load of persisters (OR for each $\log_{10}$CFU/ml increase in B_{Int}: 2.32, 95% CI: 0.84-6.40, p=0.104). The degree of inter-individual variation in the rate constants was too small for a meaningful interpretation of their effect on outcome.

Using partial likelihood to handling SSCC data below the limit of detection

Figure 6.10 compares the maximal likelihood model described above with a partial likelihood model incorporating possible values of bacillary counts below the limit of SSCC detection. The overall form of the partial likelihood model was unchanged, but both early and late phase bacillary elimination slopes steepened to reflect the probability of very low colony counts at late time-points.

To optimise the fit of the partial likelihood model, random effects on the θ_2 (α) parameter were removed. Analysis of the effect of variability in the other three parameters on clinical outcome is shown in Figure 6.11 and Table 6.7. Unfavourable outcomes were associated with a greater baseline concentration of persisters (OR for each $\log_{10}$CFU/ml increase in B_{Int}: 80.45, 95% CI: 4.97-1302.02, p=0.002) and slower sterilisation phase bacillary elimination (OR for each 0.01 increase in the β rate constant: 0.39, 95% CI: 0.22-0.70, p=0.001).

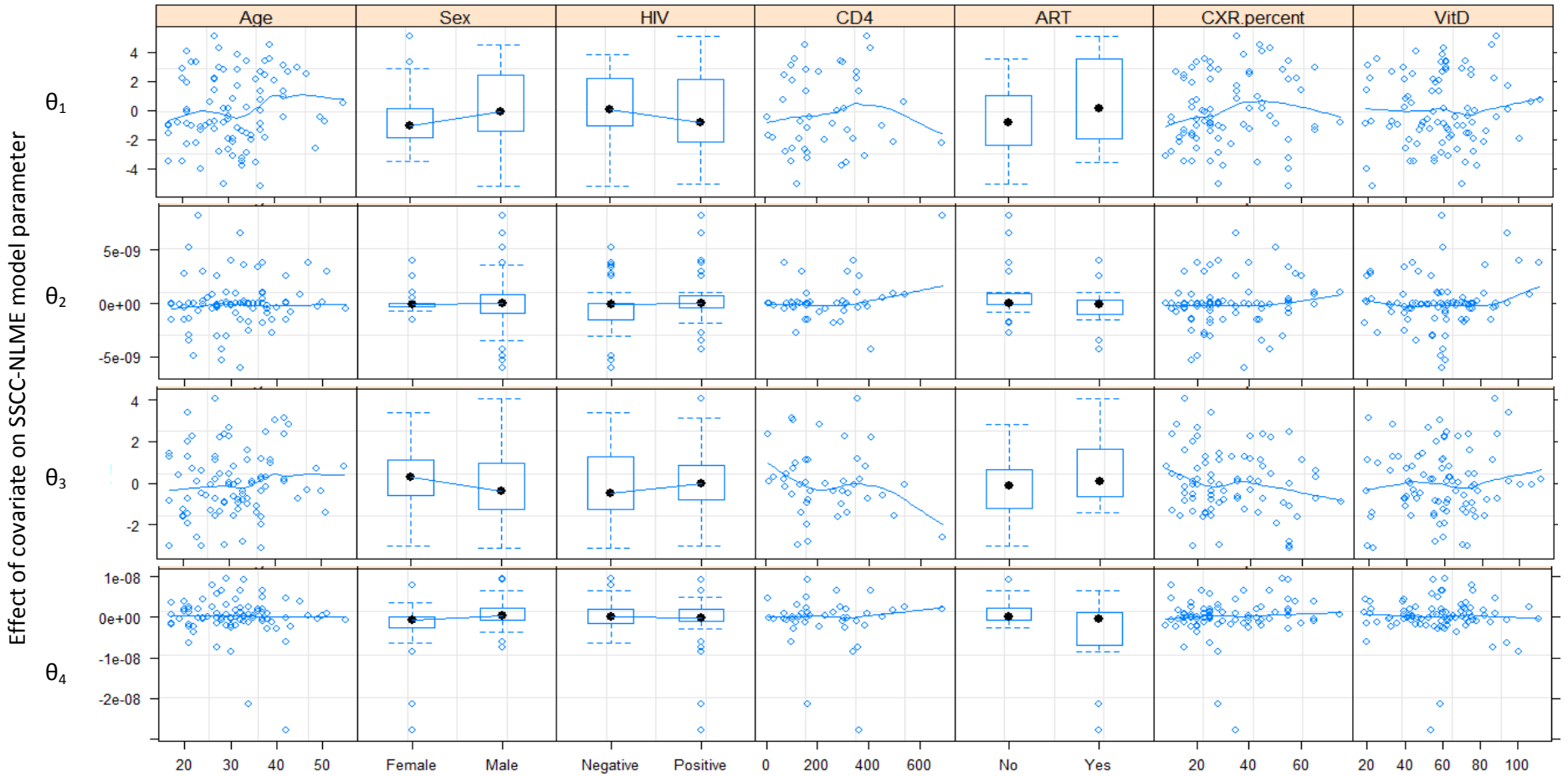


Figure 6.8 Effect of clinical covariates on SSCC-NLME model parameters

The effect of clinical and radiological covariates on SSCC-NLME model parameters was small; especially with regard to rate constants θ_2 and θ_4. CD4 and prior ART were only assessed for HIV positive patients. Incorporating covariates did not significantly alter the overall fit of the model.

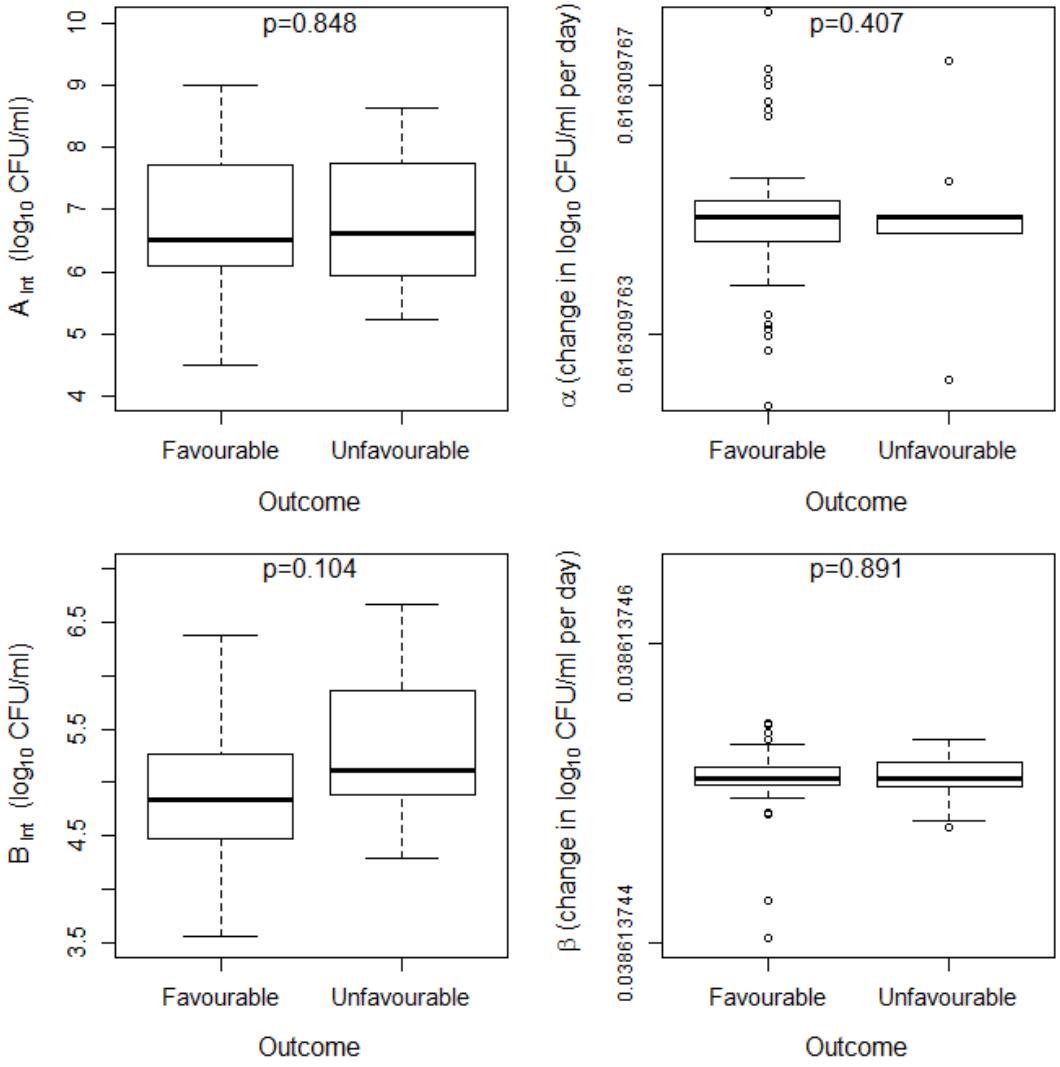


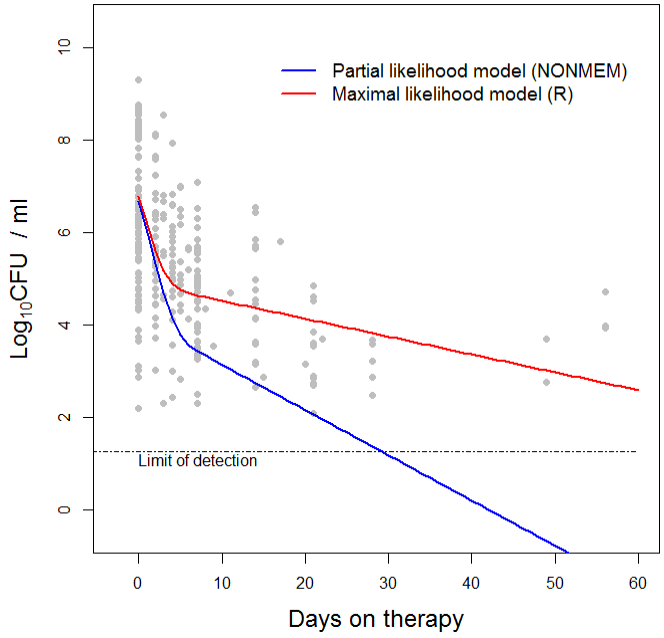
Figure 6.9 SSCC-NLME parameters and clinical outcomes

Best unbiased estimates of A, B, α and β were extracted from the maximal likelihood model for all patients. Outcome definitions were as previously described. Analysis was by logistic regression (details in Table 6.5).

	OR of unfavourable outcome[a]	95% CI	p-value
Metabolically active population			
A_{Int}: Baseline load (log_{10}CFU/ml)	1.07	0.56-2.04	0.848
α: Elimination rate constant (reduction in log_{10}CFU/ml per day)	0.8	0.47-1.35	0.407
Persister population			
B_{Int}: Baseline load (log_{10}CFU/ml)	2.32	0.84-6.04	0.104
β: Elimination rate constant (reduction in log_{10}CFU/ml per day)	0.79	0.03-23.95	0.891

Table 6.6 Associations between SSCC-NLME parameters and outcome

[a]For baseline bacillary loads ORs are reported as the effect size per log_{10} CFU/ml increase in sputum bacterial concentration. For elimination rate constants the degree of inter-patient variability was very small so ORs were reported as effect size per 10^{-9} log_{10}CFU/ml reduction in sputum bacterial concentration per day.



Partial likelihood model fixed effects	
Metabolically active population	
θ_1 = 15.40	(A_{Int} = 6.48)
θ_2 = -0.46	(α = 0.68)
Persister population	
θ_3= 9.50	(B_{Int} = 4.12)
θ_4= -1.49	(β = 0.095)

Figure 6.10 Partial and maximal likelihood methods in SSCC-NLME modelling

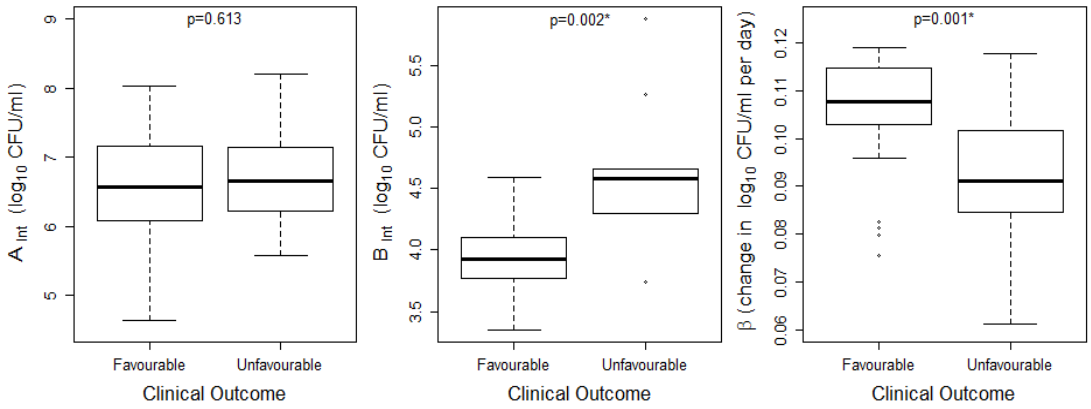


Figure 6.11 Partial likelihood SSCC-NLME parameters and outcome

Best unbiased estimates of A, B and β were extracted from the partial likelihood model for all patients. Outcome definitions were as previously described. Analysis was by logistic regression (details in Table 6.7).

	OR of unfavourable outcome[a]	95% CI	p-value
Metabolically active population			
A_{Int}: Baseline load (log_{10}CFU/ml)	1.26	0.51-3.13	0.848
α: Elimination rate constant (reduction in log_{10}CFU/ml per day)	NA	NA	NA
Persister population			
B_{Int}: Baseline load (log_{10}CFU/ml)	80.45	4.97-1302.02	0.002*
β: Elimination rate constant (reduction in log_{10}CFU/ml per day)	0.39	0.22-0.70	0.001*

Table 6.7 Partial likelihood SSCC-NLME parameters and outcome

[a]ORs for baseline bacillary loads are reported as the effect size per log_{10} CFU/ml increase in sputum bacterial concentration. ORs for elimination rate constant were reported as effect size per 0.01 log_{10}CFU/ml reduction in sputum bacterial concentration per day.

6.3.4 LME modelling of MGIT TTP data

124/133 (93%) patients who reached a final study end-point had least two positive TTP results. 467 TTP measurements from these patients were included in the analysis. Fewer contamination problems and later culture conversion with liquid culture resulted in more complete data profiles for bacillary elimination modelling. Figure 6.12 presents individual patient profiles of change in TTP over time on treatment. Plots are ordered from bottom left to top right according to baseline TTP and only positive samples are included. Despite considerable variability in plots, the general trend shows increased TTP with longer time on therapy.

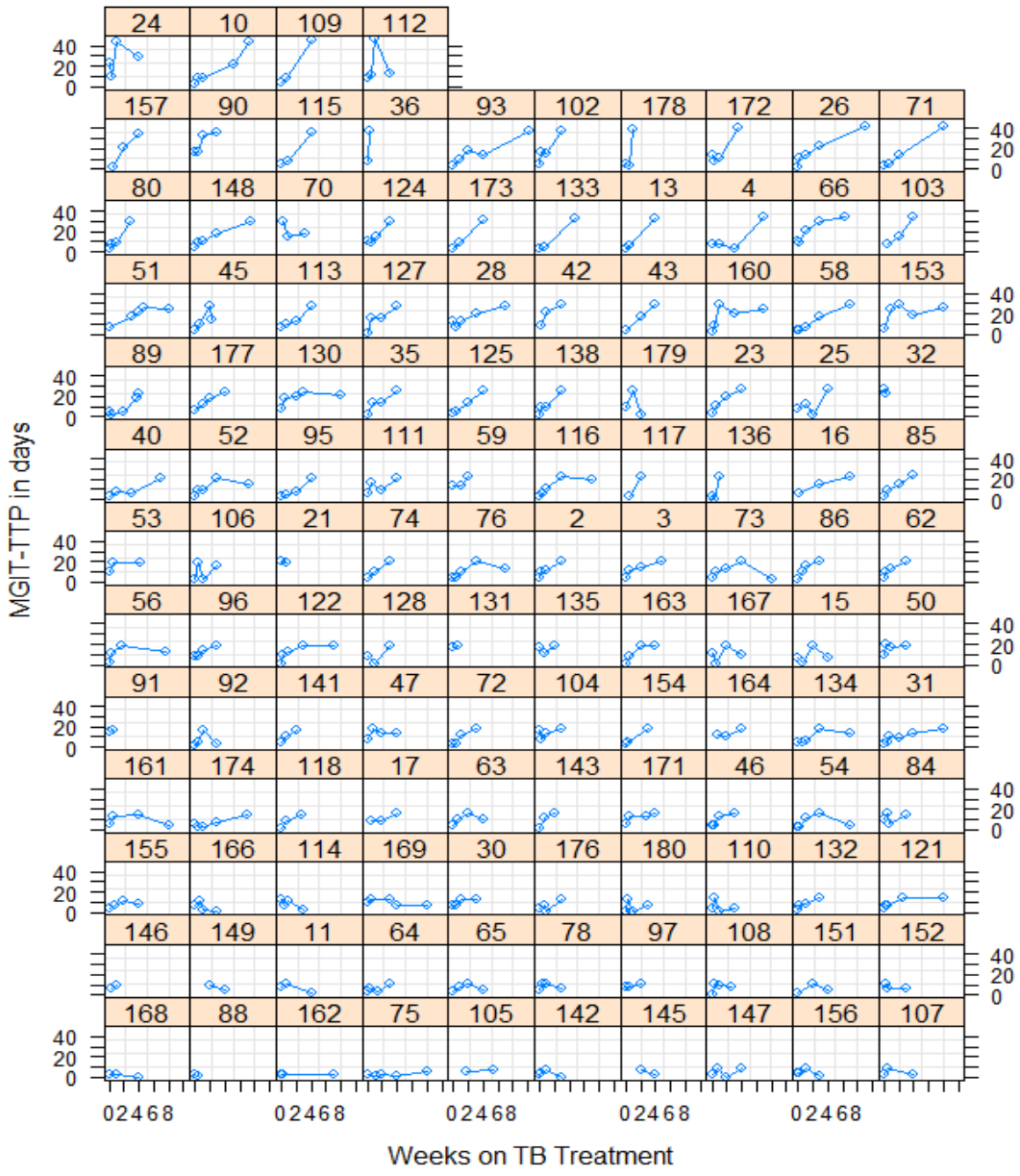


Figure 6.12 Individual patients profiles for TTP over time on therapy

Figure 6.13A is a spaghetti plot of positive TTP values for the study population and Figure 6.13B shows non-parametric summary measures of positive samples from the main sputum collection days. Although the rate of increase in TTP appears to declines during the second month of therapy this may be attributable to censoring of negative data when the true TTP exceeds the limit of detection at 49 days. As the potential effect of censoring negative data is greater for MGIT than SSCC cultures (Figure 6.2) an alternative MGIT dataset was constructed in which an imputed TTP value of 50 days was allocated to the first negative culture for each patient. Non-parametric summary measures of TTP for the alternative dataset are shown in Figure 6.13C.

Choice of structural model

Considering that much of the late curvature in the non-parametric summary plots was due to censoring of negative data, a maximum likelihood method was used to fit LME models to the original (positive values only) and alternative (positive and imputed values) MGIT-TTP datasets. Figure 6.14 illustrates fixed effects of these models and Table 6.8 provides full details of model parameters. Quadratic and SPLINE functions were also fitted to the TTP data to assess for curvature but there was no improvement of fit (data not shown) and the biological relevance of parameters from these model functions was difficult to interpret. The LME models were most suitable for further analysis.

The slope (*b*) of the fitted lines will be used as to represent the MBER. The LME model from the original dataset indicates that TTP increased by 2.643 (Std. Error: 0.242) days per week of TB treatment, whilst the model from the alternative dataset indicates that TTP increased by 4.73 (Std. Error: 0.228) days per week of TB treatment. The steeper MBER from the alterative dataset reflects the effect of additional information on culture conversion which is only available after inclusion of imputed “first negative” data-points.

As with the SSCC-NLME model, the validity of a TTP-LME model is predicated on assumptions that variation in residual values does not change at different fitted value results, and that residual values and random effects from the model are normally distributed. Figure 6.15 confirms that, with a few outliers, these assumptions are met for the original TTP dataset.

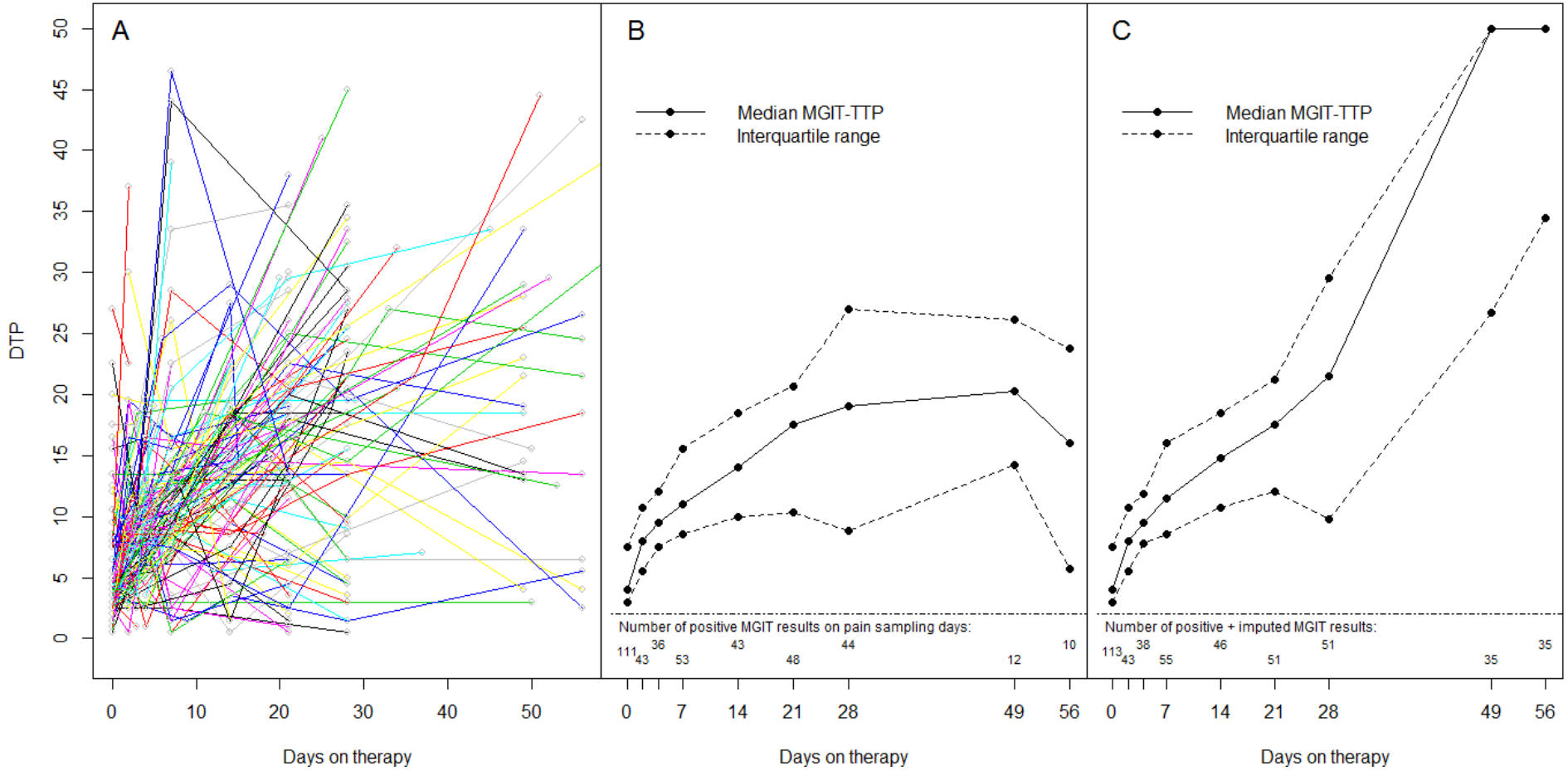


Figure 6.13 Plots for construction of TTP-LME model

A: Spaghetti plot of TTP over time for each individual patient B: Pooled non-parametric summary measures of TTP for the original dataset (positive values only) on the main sampling days. C: Pooled non-parametric summary measures of TTP for the alternative dataset (positive and imputed values only) on the main sampling days.

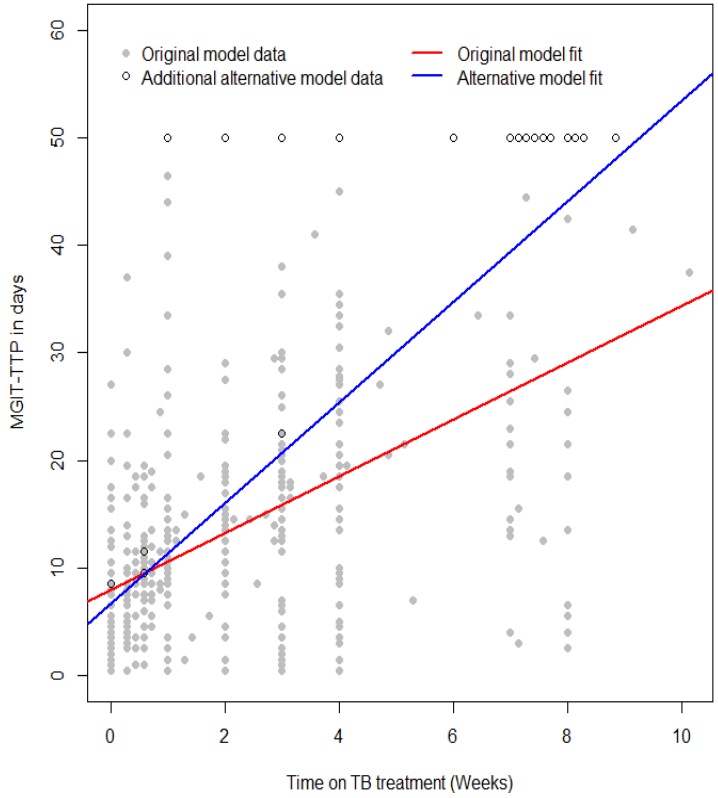


Figure 6.14 Fixed effects of TTP-LME models

LME models fit by maximum likelihood

Original model fit

AIC : 3201.583 logLik: -1594.792

Fixed effects: DTP~Weeks on treatment

	Value	Std. Error	p-value
Intercept (*a*)	7.973	0.431	<0.001
Slope (*b*)	2.643	0.242	<0.001

Random effects: ~Week|Patient

	Std. Dev.
Intercept (*a*)	1.933
Slope (*b*)	1.743
Residual	6.191

Alternative model fit (inc. imputed data)

AIC: 4043.874 logLik: -2015.937

Fixed effects: DTP~Weeks on treatment

	Value	Std. Error	p-value
Intercept (*a*)	6.693	0.532	<0.001
Slope (*b*)	4.673	0.228	<0.001

Random effects: ~Week|Patient

	Std. Dev.
Intercept (*a*)	3.070
Slope (*b*)	1.971
Residual	7.334

Table 6.8 Parameters for TTP-LME models

In Figure 6.15 and Table 6.7, the original model includes positive TTP values only. The alternative model imputes a TTP value of 50 days for the first negative result from each patient.

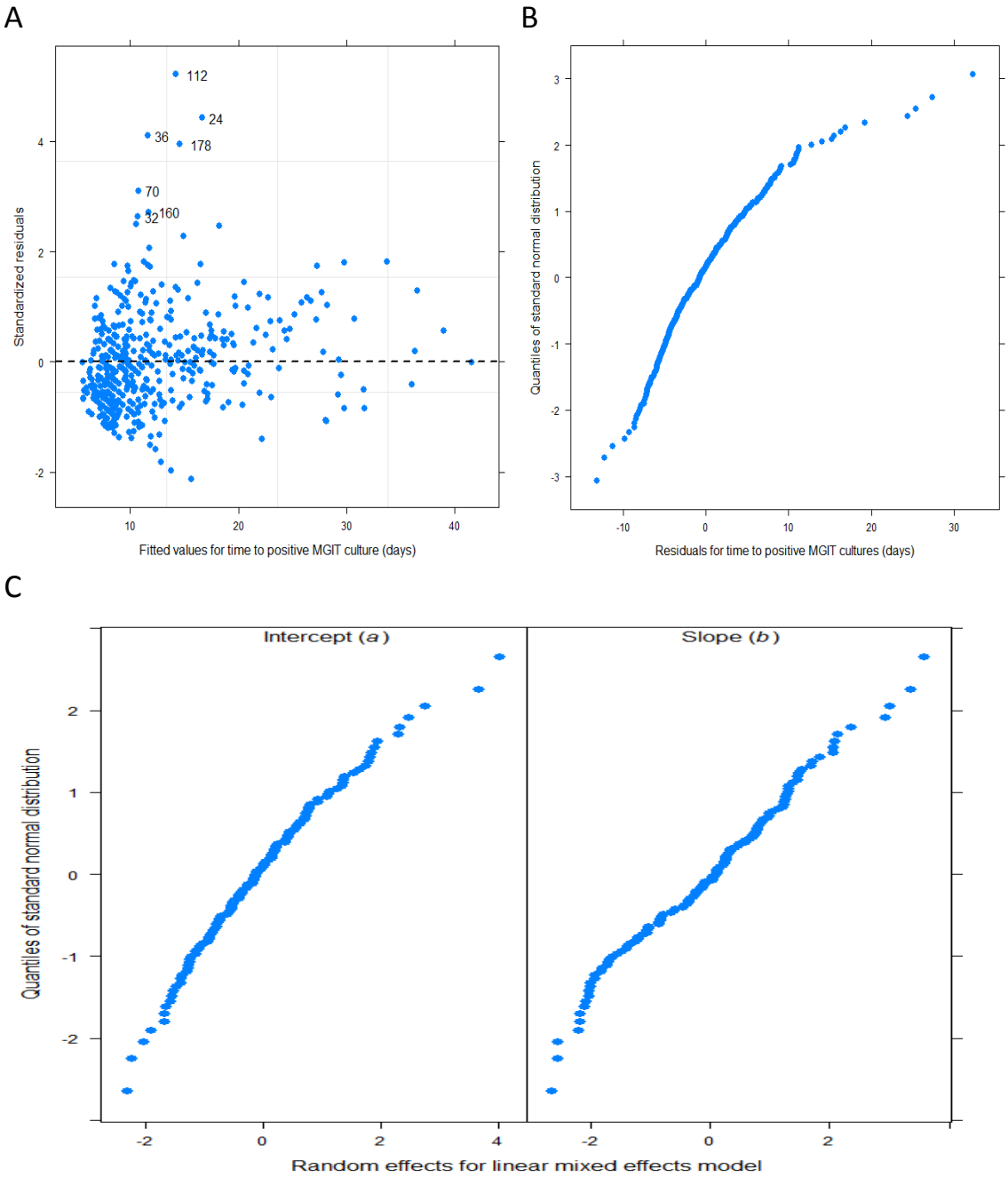


Figure 6.15 Testing TTP-LME model assumptions (original model)

A: Distribution of standardised residuals against fitted MGIT TTP values. The largest residuals were observed for individual data-points from patients 24, 32, 36, 70, 112, 160 and 178 but there was no systematic trend associated with individual patients or the size of fitted values. B: A Q-Q plot of residuals against quantiles of a normal distribution. Data-points with large residuals are seen at the top-right corner, but otherwise the plot is near-linear and symmetrical around zero. C: Q-Q plots of random effects against quantiles of a standard normal distribution. Excepting a small number of outliers, both plots are near linear and symmetrical around zero.

Covariates influencing the TTP-LME slope (MBER)

The effect of clinical and radiological covariates on the MBER was assessed by multivariate linear regression for both original and alternative models (Table 6.9).

In the original model no covariate significantly altered the MBER but there was a trend towards a shallower slope (slower bacillary elimination) in males (p=0.065). In the alternative model, a steeper slope (faster bacillary elimination) was associated with ability to read from a newspaper (p=0.049) whilst shallower slopes were associated with difficulty obtaining food (p=0.026), ever drinking alcohol (p=0.023) and male sex (p<0.001). The alternative model also showed trends towards shallower slopes with percentage of lung affected on CXR (p=0.088) and cavities ≥4cm (p=0.068).

That no factor had a significant effect on the original model whilst several had an effect on the alternative model suggests that imputing a measure of culture conversion increased the capacity of the model to detect the influence of clinical covariates on the MBER.

The majority of HIV-associated, clinical and radiological factors were not associated with variation in the MBER, as measured by the slope of either model. This suggests that variability due to these factors is largely accounted by the model and that clinical trials using TTP-LME modelling to assess treatment response are unlikely to be confounded by clinical or radiological covariates.

Associations between TTP-LME slope and clinical outcome

Figure 6.16 shows that, for both TTP-LME models, there was a strong relationship between best unbiased estimates of the MBER for each patient and clinical outcome (OR of unfavourable outcome for each 1 day increase in TTP per week of therapy in original model: 0.5, 95% CI: 0.30-0.83, p=0.007; OR in alternative model: 0.63, 95%CI: 0.44-0.91, p=0.014). This suggests that using slope of the TTP-model (with or without imputed data) as a measure of the MBER is a strong pharmacodynamic predictor of final outcome in a heterogeneous cohort of HIV-infected and uninfected individuals.

	Slope (*b*) of original LME model			Slope (*b*) of alternative LME model		
	Covariate effect on slope	Standard error of covariate effect	p-value	Covariate effect on slope	Standard error of covariate effect	p-value
Socio-economic factors						
Difficulty obtaining enough food in the last month	-0.806	0.600	0.180	-1.336	0.597	0.026*
Able to read a newspaper	0.176	0.523	0.737	0.990	0.502	0.049*
Ever smoked tobacco	-0.004	0.544	0.994	-0.754	0.525	0.152
Ever drinks alcohol	-0.397	0.492	0.420	-1.061	0.465	0.023*
HIV parameters						
Confirmed HIV infected	0.084	0.492	0.864	-01.46	0.463	0.752
Baseline CD4 count, cells/µl	-0.002	0.002	0.327	-0.001	0.002	0.521
On ART at enrolment	0.505	0.592	0.395	-1.801	0.604	0.003*
Demographic and clinical parameters						
Male sex	-1.011	0.546	0.065	-1.916	0.466	<0.001*
BCG vaccinated	-0.273	0.656	0.677	-0.586	0.578	0.311
Baseline BMI, kg/m^2	0.086	0.118	0.461	0.033	0.103	0.743
Baseline CXR assessment						
Percentage of lung affected on CXR	0.009	0.017	0.599	-0.025	0.015	0.088
Presence of large cavity, ≥4cm diameter	-0.381	0.520	0.464	-0.878	0.479	0.068
25 (OH) Vitamin D measurement						
Baseline 25(OH)D, nmol/l	-0.003	0.011	0.822	0.008	0.011	0.493

Table 6.9 Factors influencing the MGIT bacillary elimination rate

Original and alternative lme models of changes in MGIT TTP over time on therapy were run using the clinical and radiological covariates. A positive effect indicates that a covariate was associated with a steeper slope (faster bacillary elimination). A negative effect indicates that a covariate was associated with a shallower slope (slower bacillary elimination). The effect size shows the extent to which each covariate influences the slope of the model. All clinical and radiological factors were analysed, the table shows those with p<0.100 or for which a prior publications suggest a likely effect on treatment response.

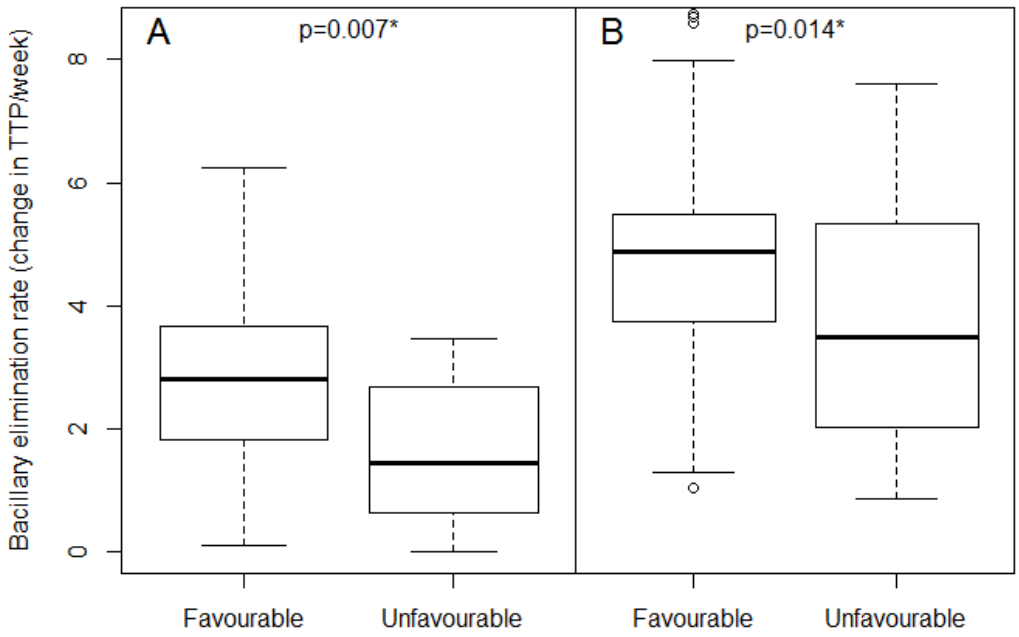


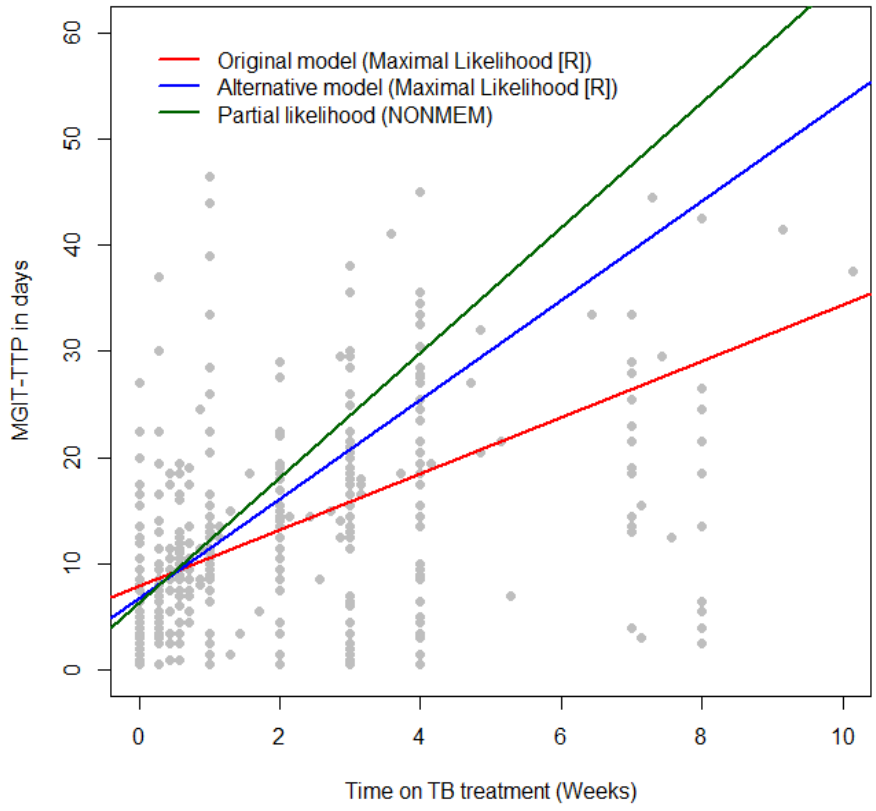
Figure 6.16 Associations between MBER and clinical outcome

A: Relationship between MBER and clinical outcome for original model B: Relationship between MBER and clinical outcome for alternative model. Analyses were performed by univariate logistic regression.

Using partial likelihood to handle TTP data beyond the limit of detection

Although the significant relationship between the MBER and clinical outcome for both LME models is interesting, data handling methods for negative data remain problematic; the original model ignored culture conversion completely whilst the TTPs value allocated to first negative cultures in the alternative model were probably inaccurate.

Figure 6.17 demonstrates that use of a partial likelihood method to incorporate possible data beyond the limit of detection generates a steeper slope than either earlier model (TTP increases by 5.894 days per week on therapy). This is unsurprising as the imputed value of 50 days attributed to first negative samples in the alternative model represented the lowest possible true reading. Partial likelihood method estimates of TTPs for some samples will have been higher, resulting in a steeper MBER.



Fixed effects of partial likelihood model	
Intercept (*a*)	6.28 days
Slope[MBER] (*b*)	5.894 days per week of TB treatment

Figure 6.17 Partial and maximal likelihood methods in TTP-LME modelling

As with the other MGIT-TTP models, there was a strong relationship between best unbiased estimates of MBER from the partial likelihood model and clinical outcomes (OR of unfavourable outcome for each 1 day increase in TTP per week of therapy: 0.71, 95% CI: 0.55-0.94, p=0.015).

The robustness of pharmacodynamic estimation of MBER across three different TTP-LME models using a variety of approaches to handle negative data post culture conversion suggests that MBER measurement may be a useful marker of persistence and warrants further evaluation as a surrogate endpoint for Phase IIb clinical trials of new TB therapies.

6.3.5 Relating colony counts to TTP in clinical samples

In section 5.4 a strong inverse relationship was described between colony counts and MGIT TTP from *in vitro* H37Rv cultures. Having modelled the clinical SSCC and MGIT datasets separately, a combined analysis allowed relationships between the results of quantitative culture methods to be examined in the context of a treatment study.

Figure 6.18 illustrates the association between colony counts and TTP for all clinical samples with a positive result for both techniques. Although the previously described inverse relationship remained ($p<0.001$), the strength of the association at the level of individual data-points was weaker for clinical than *in vitro* samples (Figure 6.18A, adjusted R^2=0.19 vs Figure 4.10, adjusted R^2=0.86). There are several possible explanations for this, including that dispersal of organisms is less likely to be homogenous in sputum (even after processing) than liquid broth, so inoculation of SSCC plates and MGIT tubes with aliquots of the same sputum sample are more likely to contain different bacillary concentrations than inoculation with aliquots of *in vitro* grown H37Rv.

An alternative explanation is based on differences in bacillary load quantification between SSCC and MGIT. Each SSCC colony grows from a single bacterium, making CFU counting analogous to directly counting bacilli in the sample. Rapidly replicating bacilli form large colonies whilst slow replicators form small ones but this does not affect the $\log_{10}$ CFU/ml count provided that colonies of all sizes are counted equally. By contrast, in the MGIT assay TTP simultaneously reflects the concentration of bacteria in the sample and the rate of oxygen consumption by those organisms; rapid replicators have a shorter TTP than those which are metabolically quiescent at identical initial bacillary load (Figure 6.19). As clinical specimens are likely to contain organisms at a wider range of metabolic states than *in vitro* samples grown under uniform conditions, it is possible that metabolic interference with TTP results confounds the relationship with colony counts in patient samples.

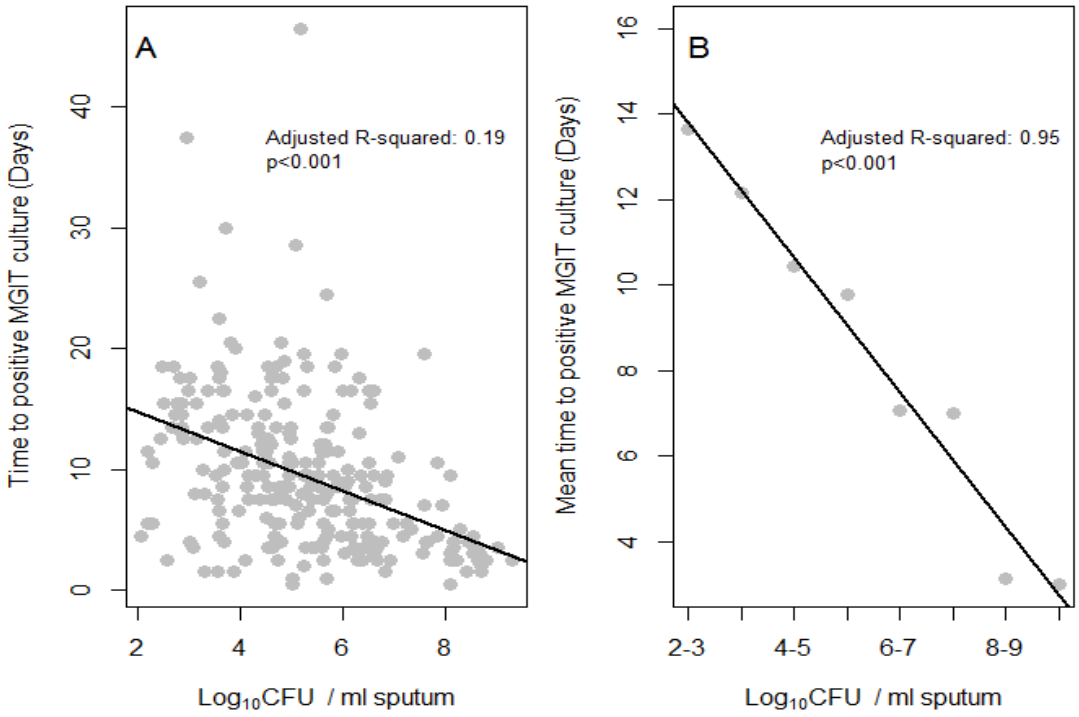
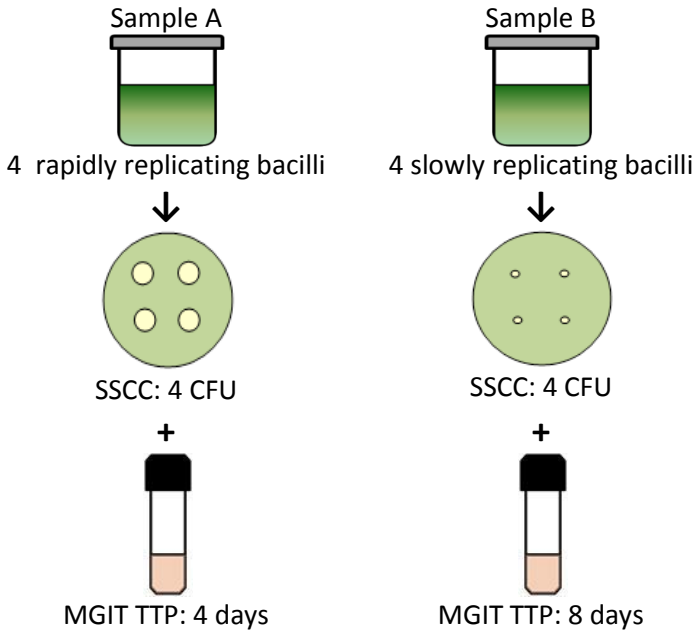


Figure 6.18 Relationship between log_{10}CFU/ml and MGIT TTP for clinical samples

A: Plot of log_{10} CFU/ml against MGIT TTP for all samples with a positive qudantitative result by both techniques. B: Plot of mean MGIT TTP for each logarithmic subdivision of bacillary load from 2-10 log_{10} CFU/ml.



SSCC: Sample A & B bacillary loads are equal; different colony sizes reflect different baseline metabolic conditions.

MGIT: Sample TTP are different; rapidly replicating organisms from A consume oxygen more quickly than quiescent organisms from B

Figure 6.19 Bacillary load quantification by MGIT and SSCC

It has been proposed that baseline sputum samples from TB patients contain rapidly replicating organisms, whilst later specimens predominantly contain metabolically quiescent persisters which may have a longer TTP at a given bacillary load. Table 6.10 shows that the relationship between colony counts and TTP for clinical samples is strongest for baseline samples (R^2=0.27, p<0.001) and completely lost amongst samples collected after Day 14 (R^2=-0.027, p<0.583). Perhaps, at baseline, the bacillary load of replicating organisms is the main driver of TTP but at later time-points reduced metabolic activity of persisters slows TTP and it reflects bacillary load less precisely.

Sample collection	Number of samples	Adjusted R^2	p-value
Total	265	0.19	<0.001
Baseline	93	0.27	<0.001
Day 1-14	145	0.04	0.014
>Day 14	27	-0.027	0.583

Table 6.10 Changing relationship between log_{10}CFU/ml and TTP with day of sampling

Further evidence of the changing relationship between log_{10} CFU/ml counts and MGIT TTP over time on treatment is provided by Figure 6.20. Positive SSCC data were divided into low (2-4 log_{10} CFU/ml), medium (4-6 log_{10} CFU/ml) and high (>6 log_{10} CFU/ml) counts. TTP in each of these categories was assessed according to time of sample collection (Baseline, Day 1-14 or Day>14) by the Kruskal Wallis test.

There was a trend towards longer TTP at later sampling times for low log_{10} CFU/ml (p=0.092) and a clear association between longer TTP and later sampling at medium (p<0.001) and high (p<0.001) counts. Longer TTP at later time-points after stratification for bacillary load is consistent with a higher proportion of metabolically quiescent persisters in later samples, which take longer to grow.

Overall, therefore, whilst quantitative culture techniques cannot directly identify different sub-populations of bacilli within sputum samples during TB treatment, analysis of the relationship between colony counts and TTP supports the proposed phenomenon of reduced metabolic activity on exposure to anti-TB drugs, and the predominance of persisters at later sampling times.

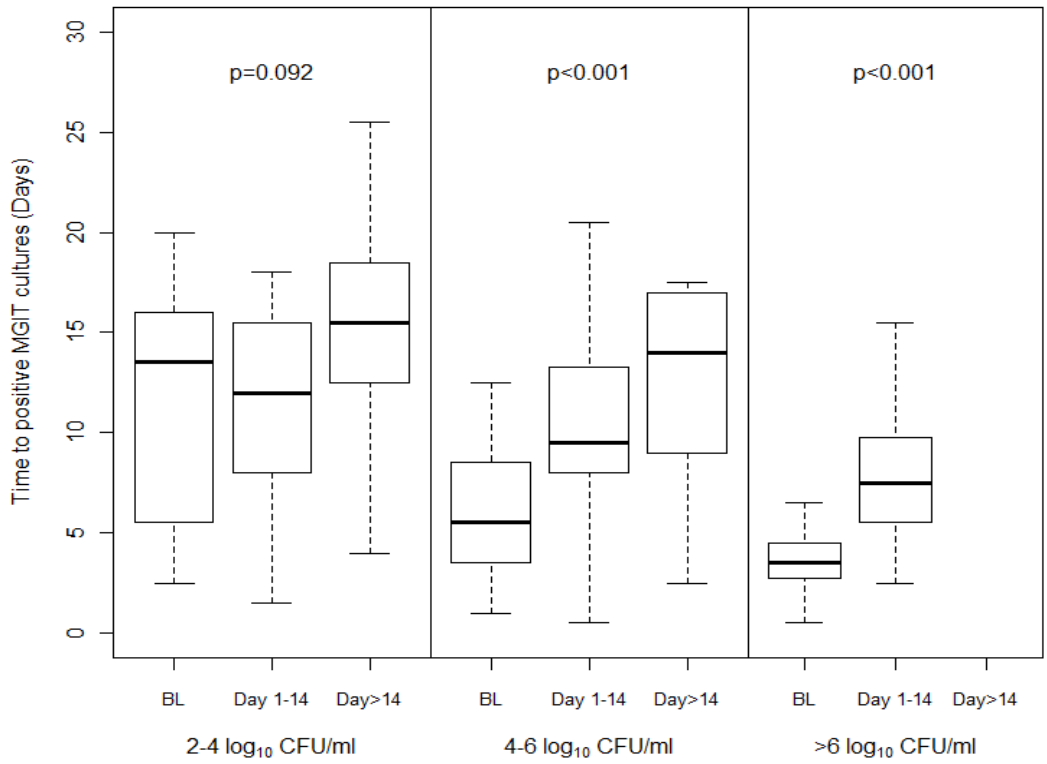


Figure 6.20 MGIT TTP with time on therapy, stratified by log_{10} CFU/ml

Differences in TTP on different sampling days analysed by Kruskal Wallis test for samples of low (2-4 log_{10} CFU/ml), medium (4-6 log_{10} CFU/ml) and high (>6 log_{10} CFU/ml) colony counts.

6.4 LAM-ELISA

6.4.1 LAM-ELISA and HIV status

The LAM-ELISA was explored as a technique to measure non-sputum bacillary load. Difficulties with kit availability meant that this assessment was restricted to 148 samples from a sub-set of 51 patients who reached a final study end-point. Samples from other patients remain stored at -20°C. 23 (45%) LAM-ELISA sub-study patients had detectable urinary LAM from at least one sample submitted on BL, S2, S3 or S4.

Table 6.11 shows that the incidence of LAM-positivity in baseline urine samples was significantly higher amongst HIV-infected (20/36 [56%]) than non-infected (3/15 [20%]) individuals (p=0.04). LAM OD readings were used as a quantitative measure of LAM detection. The results were markedly skewed to the left, so data were logarithmically transformed prior to further analysis. Results of LAM OD (p=0.003) and LAM log_{10}OD (p=0.011) were significantly higher amongst HIV-infected individuals (p=0.003 and p=0.011 respectively).

	HIV status		p-value
	Infected n=36	Non-infected N=15	
LAM detected (n, %)	20 (55.6)	3 (20.0)	0.044[a]*
LAM OD (median, IQR)	0.138 (0.071-0.388)	0.020 (0.003-0.096)	0.003[b]*
LAM log_{10}OD (mean, SD)	-0.780 (-1.125 to -0.345)	-0.118 (-1.707 to -0.876)	0.010[c]*

Table 6.11 LAM-ELISA results and HIV status

[a]Analysed by Chi-squared test; [b]Analysed by Wilcoxon test; [c]Analysed by two sample t-test

Extremely low LAM-ELISA sensitivity amongst HIV non-infected patients was consistent with prior reports that the assay is only useful in the context of HIV infection. Subsequent analysis was limited to samples from the 36 HIV-infected sub-study patients.

6.4.2 Factors affecting baseline LAM-ELISA results

Univariate logistic and regression analyses were performed to investigate associations between clinical or radiological factors and LAM positivity or higher LAM logOD readings. Multivariate analysis was not performed because of the small sample size.

Table 6.12 shows that a positive LAM-ELISA was associated with male sex (p=0.014) and lower baseline BMI (p=0.043). Table 6.13 demonstrates trends towards higher LAM log_{10}OD readings with lower baseline BMI (p=0.088) and haemoglobin (p=0.076) levels.

As lower BMI and haemoglobin results may occur in patients with more disseminated TB the observed associations and trends between these variables and higher LAM positivity support the possibility that LAM is a whole body disease marker. The relationship between male gender and positive LAM-ELISA is consistent with previous univariate associations between gender and baseline bacillary load in sputum; men also had higher colony counts and lower TTP (Table 6.3).

Previous studies have reported a higher proportion of LAM positive samples at lower CD4 counts[333,335,337] and higher LAM OD measurements as CD4 counts drop[338] but the current data shows no relationship between LAM and CD4 results. A potential explanation is that only 3/31 (10%) patients with baseline LAM-ELISA results in this study had CD4 counts <50 cells/µl. Prior descriptions of a CD4-LAM association were from South African cohorts in which up to 36% patients had CD4 counts in this range[338]. Extreme immunosuppression may be required for this relationship to become apparent.

	LAM result		OR	95% CI	p-value
	Positive (n=20)	Negative (n=16)			
HIV parameters					
CD4 count, cells/µl (median, range)[a]	163 (6-539)	161 (31-497)	1.00	1.00-1.01	0.348
Demographic & clinical parameters					
Male sex (n, %)	18 (90)	8 (50)	9.00	1.55-52.27	0.014*
BMI, kg/m^2 (median, range)	18.3 (14.5 20.0)	19.8 (14.8-24.9)	0.68	0.46-0.99	0.043*
Haemoglobin, g/dl (median, range)[b]	9.7 (60-12.8)	10.4 (7.2-13.4)	0.89	0.61-1.29	0.533

Table 6.12 Factors associated with baseline LAM positivity

	Parameter estimate	Std. Error	p-value
HIV parameters			
CD4 count, cells/µl[a]	-0.001	0.002	0.757
Demographic and clinical parameters			
Male sex	0.235	0.253	0.361
BMI, kg/m^2	-0.022	0.126	0.088
Haemoglobin, g/dl (median, range)[b]	-0.240	0.130	0.076

Table 6.13 Factors associated with baseline LAM logOD readings

[a]CD4 results available for 31 patients: <50 cells/µl in 3 (10%) patients, 50-200 cells/µl in 15 (48%), 200-350 cells/l in 6 (19%) and <350 cells/µl in 7 (23%).
[b]Haemoglobin results available for 30 patients.

30 patients included in analysis of baseline LAM-ELISA results had favourable final outcomes and 6 had unfavourable outcomes. However, no relationship was evident between baseline LAM-ELISA result and treatment response. There were no associations with 2 month sputum culture status or MBER.

6.4.3 LAM-ELISA during TB treatment

All of the HIV-infected patients with baseline LAM-ELISA results had at least one additional result from a later time-point, permitting examination of changes in urinary LAM measurements with time on therapy.

Of the 20 patients who were LAM-positive at baseline, 11 (55%) had converted to negative by the S2 visit, 14 (70%) by S3 and 16 (80%) by S4. Figure 6.21A shows time to LAM-ELISA conversion. LAM $\log_{10}$OD readings fell during therapy, particularly during the first month. Figure 7.21B shows non-parametric summary measures of pooled $\log_{10}$OD readings at each study visit, Figure 6.21C contains individual patient profiles of $\log_{10}$OD readings over time and Figure 6.21D is a spaghetti plot of the same information. There were no associations between time to LAM-ELISA conversion or the rate of change in $\log_{10}$OD readings and clinical outcomes but the dataset was small.

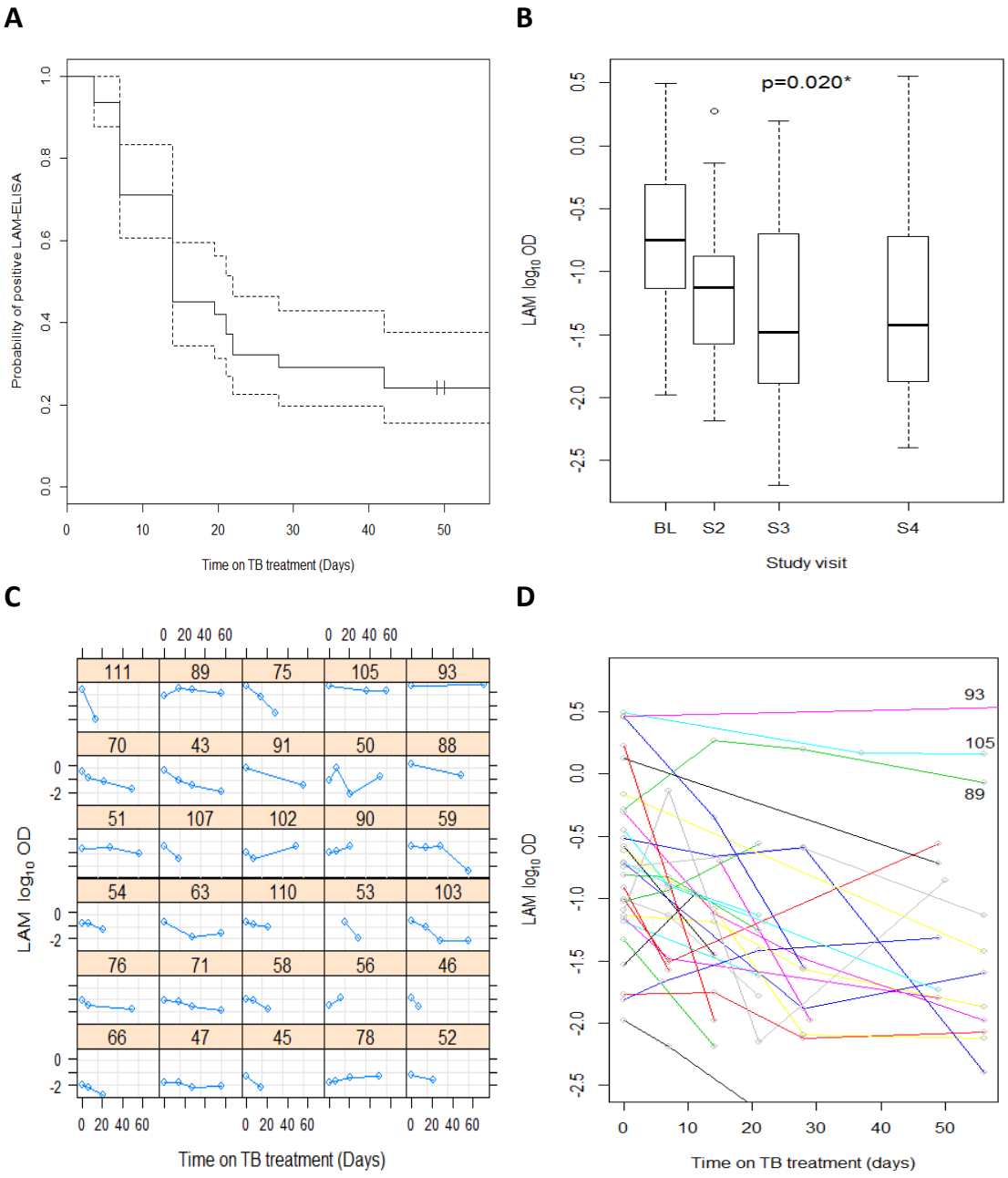


Figure 6.21 Changes in LAM-ELISA results during therapy

A: Kaplan Meier plot of LAM-ELISA conversion during treatment. B: Pooled LAM log_{10}OD at BL and S2-4 study visits assessed by analysis of variance. C: Individual patient profiles of change in LAM log_{10}OD during therapy. D: Spaghetti plot of change of LAM log10OD during therapy for all patients. Note the persistently high readings for 3 individuals (patients 89, 93 and 105).

Relating LAM-ELISA results to sputum bacillary load

Contemporaneous MGIT TTP and urinary LAM-ELISA data were available for 80 patient visits from BL-S4. Contemporaneous colony counts and LAM-ELISA data were available for 50 visits. Pooled analysis was undertaken to relate the LAM-ELISA results to sputum bacillary load. Table 6.14 shows that when the sputum TTP and log_{10}CFU/ml were analysed as continuous variables there was no association with LAM-ELISA result. However, patients with TTP <7 days or colony counts > 6 log_{10} CFU/ml were more likely to be positive by LAM-ELISA (Table 6.14, p=0.020 and 0.008 respectively). These tentative associations imply that higher LAM excretion has some quantitative significance but is an imprecise measurement in smear positive pulmonary disease.

	LAM-ELISA result		OR	95% CI	p-value
	Positive (n=30)	Negative (n=50)			
MGIT TTP					
MGIT TTP, days (median, range)	5.25 (1.50-37.50)	11 (1.00-45.00)	0.98	0.93-1.02	0.327
Samples with TTP> 7 days (n, %)	13 (43.3)	35 (70)	0.33	0.13-0.84	0.020*
SSCC log_{10} CFU/ml					
Log_{10} CFU/ml (median, range)	6.49 (2.50-9.30)	4.84 (2.09-9.02)	1.27	0.95-1.71	0.107
Samples with > 6 log_{10} CFU/ml (n, %)	14 (56.0)	5 (16.1)	6.62	(1.91-22.89)	0.003*

Table 6.14 Relationship between the LAM-ELISA and quantitative sputum cultures

Persistently high LAM logOD readings at the S4 visit

From Figure 7.21.C and D, three HIV-infected individuals (patients 89, 93 and 105) had much higher LAM log_{10}OD readings at the S4 visit than the rest of the cohort. Review of the clinical data for these patients indicates that they all had fever >38.5^{o}C and marked lymphadenopathy at baseline. These non-specific features would be compatible with greater bacillary dissemination. Patient 93 went on to have an unfavourable outcome (treatment failure). The other two were successfully cured.

Overall, the dataset was too small for full evaluation of the LAM-ELISA as a marker of non-sputum bacillary load and there was no evidence that it could be used as a surrogate marker of treatment outcome. However, the data are a novel description of serial monitoring of LAM excretion in TB patients on therapy and several features support the notion that the test has quantitative dimension in disseminated disease and converts to negative with gradually reducing OD over time.

6.5 ALTR fluorescence microscopy

6.5.1 Image quality and standardisation

A total of 152 samples were stained and photographed for LB counting during 20 microscopy sessions. Camera exposure times were fixed for each session using control slides from pre-specified samples. Median exposure times were 1.10s (range: 0.76—1.40s) on the FITC filter and 1.00s (range 0.60-1.40) on the TRITC filter.

As each sample was assessed on duplicate smears, a total of 304 slides were prepared. LB counting was performed on up to 20 images from each slide. The images obtained from both smears of 8 (5%) samples were un-interpretable due to excessive LTR staining of the background sputum matrix. These samples were excluded from analysis. LB counts were obtained from 144 samples. The image quality from valid samples is shown in Table 6.15.

	Number of samples (%) N=144
Auramine labelling of AFB[a]	
Clear	128 (89)
Blurred/faint	16 (11)
LTR labelling of intracellular LBs[a]	
Clear	58 (40)
Blurred/faint	86 (60)
LTR staining of background sputum matrix[a]	
Mild (assessment of bacilli unaffected by background matrix)	79 (55)
Moderate (LB assessment difficult in <50% of bacilli)	51 (35)
Severe (LB assessment difficult in >50% of bacilli)	14 (10)

Table 6.15 Image quality indicators during ALTR microscopy

[a]Each slide was graded according to the collective appearance of acquired images. Samples were graded by the worst score on either slide (i.e. if one slide had "mild" background and the other had "severe", the sample was deemed to be "severe").

Additional factors which hindered but did not prevent image analysis were thick smears, clumping of organisms, spectral overlap between dyes and filters and difficulty discriminating *M tuberculosis* organisms from inorganic artefact. These problems were described in at least one slide from 6 (4%), 2 (1%), 4 (3%) and 2 (1%) samples respectively.

The study PI read all images and reported af-LB and TLB counts for each sample. These data will be used for the remainder of this chapter. 29 samples were independently reported by a second reader to assess observer agreement. For af-LB counts the concordance co-efficient (ρ_c) was 0.84 (95% Limits of Agreement: -29.45 to 21.83%). For TLB, ρ_c was 0.85 (95% Limits of Agreement: -29.45 to 21.83%). These values demonstrate slightly higher agreement for LB counting than reading of CXRs (Section 3.4.5).

6.5.2 Baseline ALTR counts

Quantification of LB counts in baseline samples

Baseline samples were assessed from 69 patients. In the analysis of auramine-labelled cells the median af-LB count was 28% (range 0-79%). Figure 6.22A shows the distribution of baseline af-LB counts. Figure 6.22C shows the proportions of AFB which were LB+ and LB- in the sputum of each patient. 13 patients had baseline af-LB counts ≥50%. Four patients (35, 46, 88 and 122) submitted baseline samples with an af-LB count of 0%.

In the analysis of all bacilli (including non-acid fast LB+ organisms) the median TLB count was 35% (range: 2-98%). Figure 6.22B shows the distribution of TLB counts. Figure 6.22D shows the proportions of acid fast LB+, acid fast LB- and non-acid fast LB+ cells in the sputum of each patient. 18 patients had baseline TLB counts ≥50%. No patients had a TLB count of 0%.

The TLB dataset provides information on the contribution of non-acid fast bacilli to the total bacillary load. The median proportion of non-acid fast LB+ cells in baseline samples was 3% (range: 0-68%). All four samples with an af-LB count of 0% contained some non-acid fast LB+ cells and two patients (113 and 124) submitted samples in which the non-acid fast LB+ phenotype accounted for >50% of all bacilli. Although it is possible that some non-acid fast LB+ cells were non-mycobacterial contaminants, these cells were morphologically identical to their auramine-labelled counterparts and the circumstantial evidence that they were *M tuberculosis* is strong. The SSCC plates all grew *M tuberculosis* without contamination. MGIT cultures were all positive. TTP and liquid culture speciation test were all consistent with pure TB growth.

Patient factors influencing LB counts in baseline samples

Linear regression models were used to investigate patient factors contributing to baseline heterogeneity in LB counts (Table 6.16). For baseline af-LB counts, univariate analysis showed a strong trend towards a higher proportion of LB+ cells in patients who were tachypnoeic (p=0.055). No association was seen with any other factor so multivariate analysis was not performed. For baseline TLB counts, univariate analysis showed that counts were higher in patients who could not read a newspaper (p=0.011) or had never received BCG vaccination (p=0.046). Inability to read remained significant on multivariate analysis (p=0.026).

Tables 6.17 shows logistic regression analyses of factors associated with "high" LB counts (≥50%). In univariate models, high af-LB and TLB counts were more likely in patients who cooked indoors with biomass fuel (p=0.041 and p=0.017 respectively). There were also relationships between TLB counts ≥50% and female sex (p=0.020), inability to read a newspaper (p=0.009) and absence of prior BCG vaccination (p=0.047). On multivariate modelling, female sex (p=0.012) and inability to read (p=0.007) remained strongly associated with high TLB counts.

No other clinical parameters (including HIV-infection) were related to baseline af-LB or TLB counts. Interpreting the relationships between patient factors and LB counts in sputum is not easy but some explanations may be considered. Associations with tachypnoea and biomass cooking suggest induction or natural selection of LB+ bacilli in an environment of indoor air pollution and chronic lung disease. Women perform a greater share of domestic chores (including cooking), and may present with more LB+ organisms as a result of biomass exposure. However, LB counts were not linked to tobacco smoking or CXR abnormalities, arguing against an association with underlying lung disease. Higher counts in non-BCG-vaccinated patients suggest that investigating the effect of host immunity on LB formation may be worthwhile. The relationship between illiteracy and higher LB counts is similar to the earlier association of illiteracy with poor TB outcomes.

Bacteriological factors influencing LB counts in baseline samples

Table 6.18 summarises linear regression analyses of the relationships between bacillary load measurement and the proportion of LB+ cells in sputum, showing that af-LB and TLB counts are higher in samples with lower $\log_{10}$CFU/ml counts and longer TTP. When a TTP/$\log_{10}$CFU ratio is calculated to provide an estimate of bacillary metabolism corrected by bacillary load for each sample, a moderately strong relationship is seen between higher proportions of LB+ cells and lower metabolic activity (Figure 6.23). Whilst TTP/$\log_{10}$CFU is a relatively crude measure of bacterial metabolism, these data are consistent with the hypothesis that LB+ bacilli display the qualities of non-replicating persistence believed to be beneficial for drug tolerance.

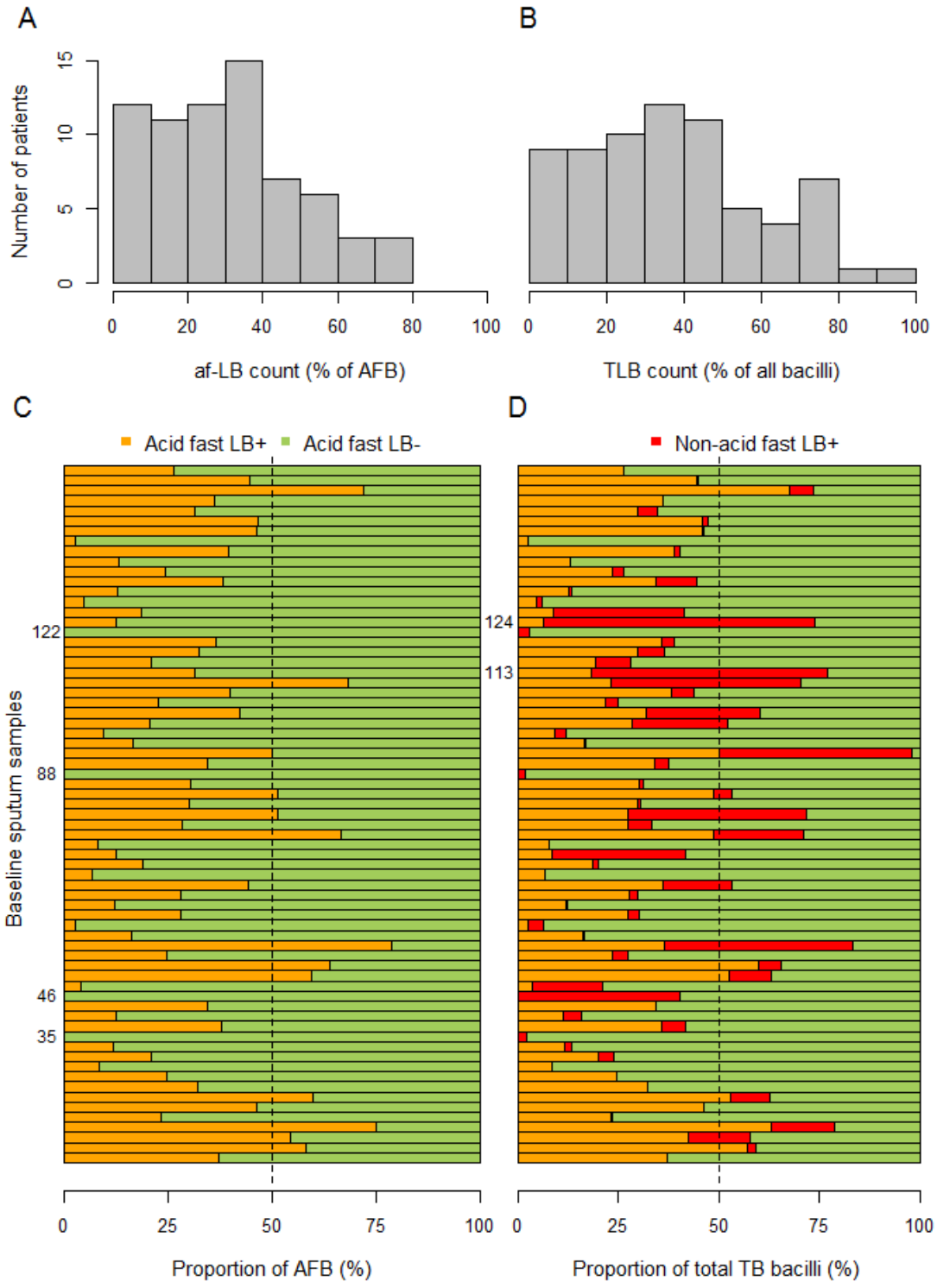


Figure 6.22 af-LB and TLB counts in AFB '+++' baseline sputum samples

A and B: Histograms demonstrating the distribution of af-LB and TLB counts amongst study patients at baseline. C: Bar-chart showing the proportion of auramine labelled (acid fast) cells which were LB+ or LB- for each patient. Four patients (35, 46, 88 and 122) submitted baseline sputum samples with af-LB counts of 0%. D: Bar-chart showing the proportion of total TB bacilli, including putative non-acid fast organisms, which were LB+ and LB- for each patient. Two patients (113 and 124) submitted baseline samples in which ≥50% of bacilli had a non-acid fast LB+ phenotype.

	af-LB counts						TLB counts					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
Variable	Estimate	St Error	p-value	Estimate	St Error	p-value	Estimate	St Error	p-value	Estimate	St Error	p-value
Male sex	-3.223	5.064	0.527	-	-	-	-7.093	5.664	0.215	-	-	-
Able to read a newspaper	-4.504	5.139	0.384	-	-	-	-14.565	5.551	0.011*	-12.615	5.552	0.026*
Cooks using biomass fuel												
At all	10.969	7.931	0.171	-	-	-	12.832	8.908	0154	-	-	-
Inside the house	4.934	5.656	0.386	-	-	-	8.264	6.353	0.198	-	-	-
BCG vaccinated	-7.457	5.898	0.210	-	-	-	-13.256	6.533	0.046*	-11.638	6.323	0.070
Tachypnoea[a]	14.705	7.527	0.055	-	-	-	15.885	8.515	0.067	11.365	8.252	0.173

Table 6.16 Factors influencing af-LB and TLB counts

	af-LB counts ≥50%						TLB counts ≥50%					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
Variable	OR	95% CI	p-value	OR	95% CI	p-value	OR	95% CI	p-value	OR	95% CI	p-value
Male sex	0.36	0.10-1.28	0.113	-	-	-	0.27	0.09-0.81	0.020*	0.13	0.03-0.64	0.012*
Able to read a newspaper	0.46	0.13-1.63	0.230	-	-	-	0.22	0.07-0.68	0.009*	0.15	0.03-0.70	0.007*
Cooks using biomass fuel												
Inside the house	12	1.11-129.41	0.041*	10.19	0.91-113.55	0.059	18	1.69-191.51	0.017*	4.63	0.34-63.51	0.252
Outside the house	3.82	0.43-33.52	0.227	3.24	3.24-29.24	0.294	7.71	0.92-64.53	0.059	4.38	0.48-40.23	0.192
BCG vaccinated	0.30	0.08-1.13	0.075	0.35	0.09-1.42	0.142	0.29	0.09-0.98	0.047*	0.24	0.05-1.20	0.082
Tachypnoea[a]	1.57	0.18-14.11	0.687	-	-	-	1.09	0.20-5.97	0.920	-	-	-

Table 6.17 Factors influencing "high" vs "low" af-LB count

	af-LB counts				TLB counts			
Variable	Estimate	St Error	Adjusted R^2	p-value	Estimate	St Error	Adjusted R^2	p-value
log_{10}CFU/ml count	-3.744	1.708	0.060	0.032*	-4.209	1.949	0.058	0.035*
TTP (Days)	2.484	1.105	0.063	0.028*	4.421	1.165	0.183	<0.001*
TTP/log_{10}CFU ratio	15.402	4.895	0.143	0.003*	18.698	5.513	0.165	0.001*

Table 6.18 Baseline bacillary load and af-LB/TLB counts

[a] Tachypnoea=Respiratory rate>30 breaths per minute during baseline assessment

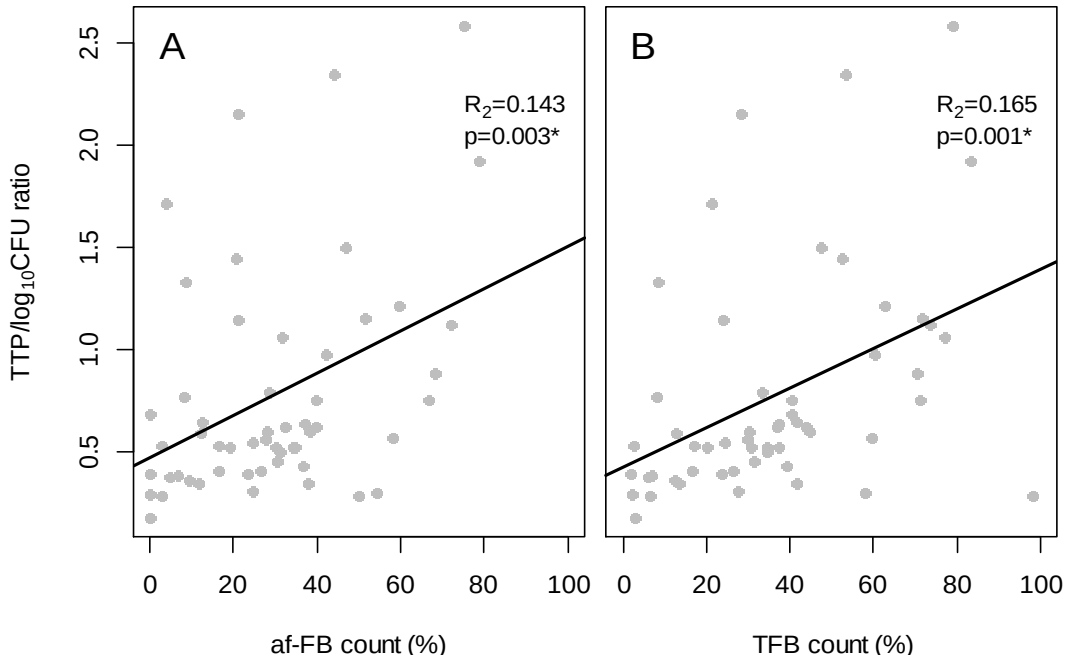


Figure 6.23 Relationship between DTP/log_{10}CFU ratio and LB counts

TTP/log_{10}CFU ratios for each sample may give an indication of *M tuberculosis* metabolic activity corrected by bacillary load. Higher ratios indicate reduced oxygen consumption by bacteria in the sample. The relationship between af-LB or TLB counts and TTP/log_{10}CFU ratios suggests that LB positivity correlates with metabolic quiescence.

Association between baseline LB counts and treatment response

10 patients whose baseline sputum samples were used for LB counting went on have unfavourable clinical outcomes. 59 had favourable outcomes. Logistic regression showed no relationship between final outcome or two month culture status and baseline af-LB or TLB counts (Table 6.19). Similarly, there was no association between baseline LB counts and any of the bacillary elimination rate parameters derived from mixed effects modelling of MGIT or SSCC data (analysis not shown).

	Treatment Response		OR	95% CI	p-value
Final clinical outcome	**Unfavourable N=10**	**Favourable N=59**			
Af-LB count (median,range)	22 (0-67)	30 (0-79)	1.00	0.97-1.03	0.928
TLB, % (median, range)	40 (3-71)	34 (2-98)	1.00	0.97-1.03	0.828
2 month culture status[a]	**Positive N=23**	**Negative N=39**			
Af-LB count (median,range)	36 (0-78)	24 (0-75)	1.01	0.99-1.04	0.306
TLB, % (median, range)	39 (3-83)	30 (2-98)	1.01	0.98-1.03	0.515

Table 6.19 Relationship between treatment outcome and baseline LB counts

[a]7 patients whose samples were used in the ALTR sub-study had contaminated sputum cultures at 2 months, so only 62 2 month culture outcomes were available (23 positive and.39 negative).

6.5.3 Serial ALTR counts

As baseline LB counts did not correlate with treatment response, it was important to study changes in the proportion of LB+ organisms in serial samples collected during therapy. 109 samples from 38 patients (mean number of samples per patient: 2.86) were assessed. Clinical and demographic features of patients in this sub-study were similar to those of the overall cohort. The choice of samples for serial analysis is shown in Figure 6.24.

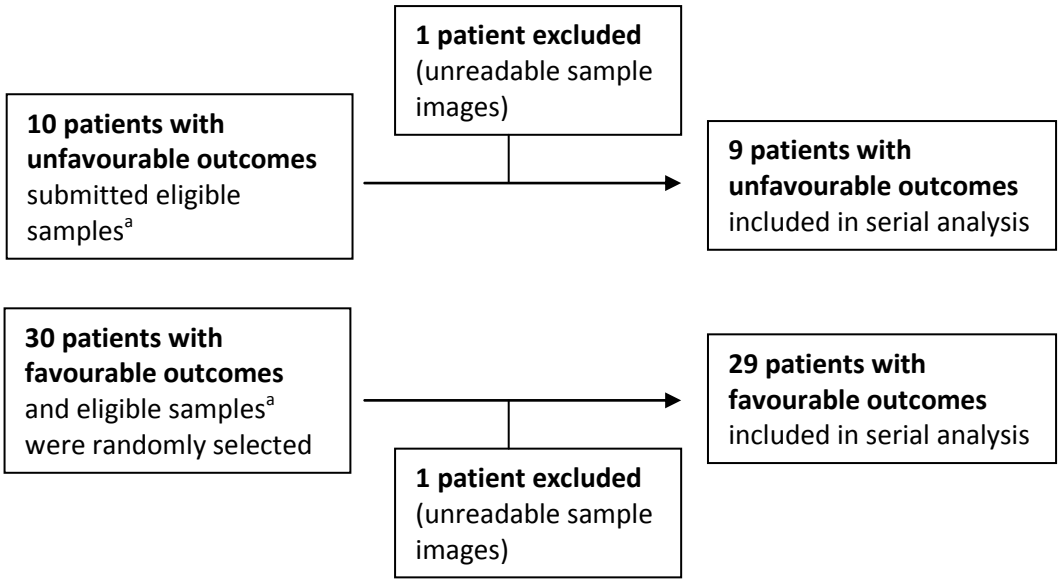


Figure 6.24 Selection of samples for serial ALTR microscopy analysis

[a]AFB smear '++' and'+++' samples collected after baseline were eligible for inclusion

Because smear positivity declines quickly after the first two weeks (Figure 6.3A), all eligible samples after baseline were collected between S1 and S3 visits (day 2, 4, 14, 21 and 28, depending on sampling block).

Figure 6.25A and Table 6.20 summarise af-LB counts at each visit. The median baseline af-LB count amongst patients included in serial sample analysis was 27% (range: 0-75%) and the median af-LB count at S3 was 25% (range: 4-64%) implying a small drop in the proportion of LB+ AFB in expectorated sputum after drug exposure. However, when the data are evaluated according to treatment response patients who went on to have unfavourable outcomes developed progressively higher af-LB counts compared to patients who went on to have favourable outcomes. On logistic regression, the odds ratio for an unfavourable outcome on the basis of S3 af-LB count is not statistically significant for this small cohort but the trend is striking (OR: 1.21, 95%: 0.97-1.50, p=0.088). The TLB count data follow a similar pattern (6.25B and Table 6.21).

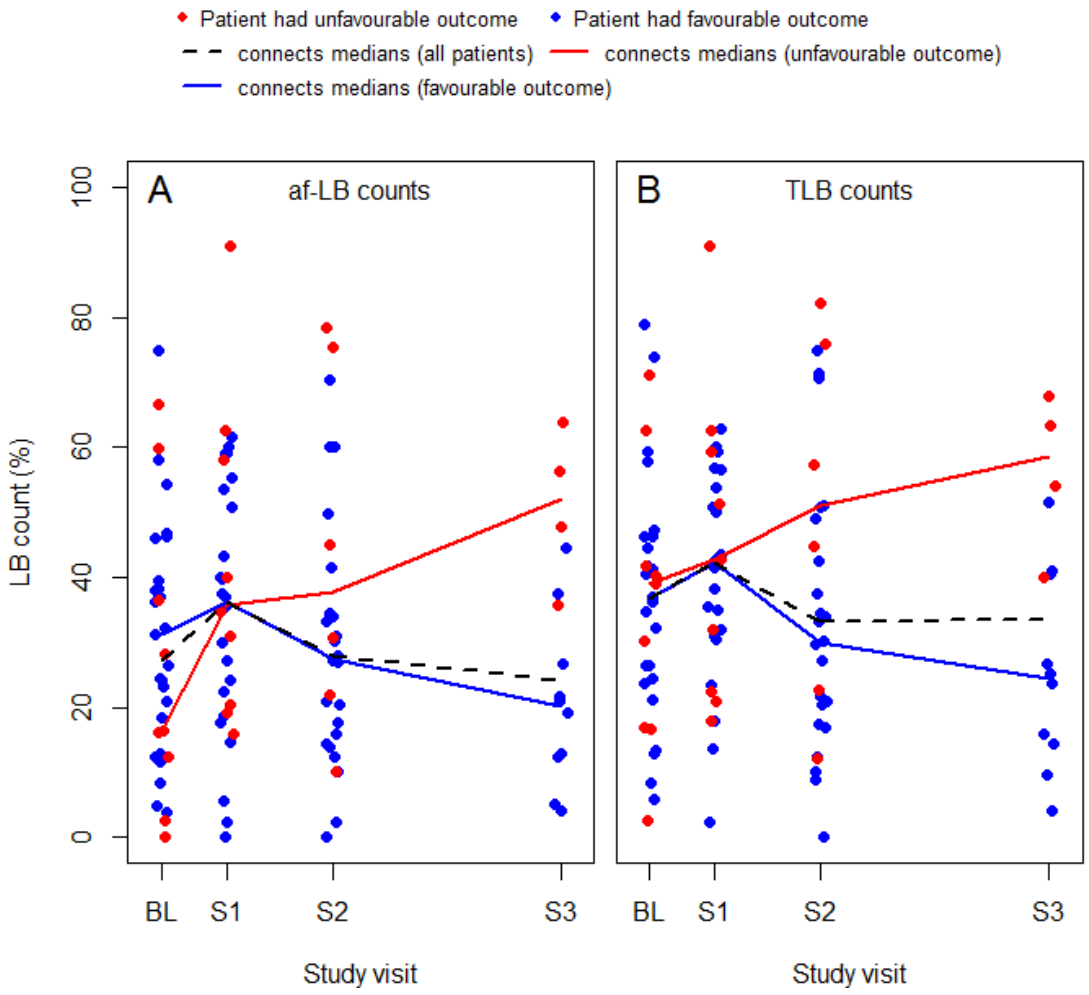


Figure 6.25 Changes in af-LB and TLB counts during therapy

	af-LB counts, % (median, range)			OR	95% CI	p-value
	All patients N=29	Patients with unfavourable outcomes N=9	Patients with favourable outcomes N=29			
BL visit	27 (0-75)	16 (0-67)	31 (4-75)	0.99	0.95-1.03	0.562
S1	36 (0-91)	35 (16-91)	37 (0-61)	1.02	0.98-1.06	0.397
S2	28 (0-78)	38 (10-78)	28 (0-62)	1.03	0.99-1.08	0.130
S3	25 (4-64)	52 (35-64)	20 (4-44)	1.21	0.97-1.50	0.088

Table 6.20 af-LB counts at BL-S3 visits

	Median T-LB count (%)			OR	95% CI	p-value
	All patients N=29	Patients with unfavourable outcomes N=9	Patients with favourable outcomes N=29			
BL visit	37 (3-79)	39 (3-71)	36 (6-79)	0.99	0.96-1.04	0.946
S1	42 (2-91)	43 (18-91)	41 (2-63)	1.01	0.97-1.06	0.614
S2	33 (0-82)	51 (12-82)	30 (0-75)	1.03	0.99-1.08	0.137
S3	33 (4-68)	59 (40-68)	25 (4-52)	1.18	0.98-1.42	0.079

Table 6.21 TLB counts at BL-S3 visits

A mixed effects model may be fit to the serial af-LB and TLB data to evaluate dynamic changes in LB counts for each patient. Figure 6.26 is a spaghetti plot of serial af-LB counts from day 0-28 and Table 6.22 summarises LME model parameters for these data. BLUEs of the slope can be extracted for each patient and used to represent the change in proportion of LB+ AFB in expectorated sputum during early therapy. Figure 6.27 indicates that af-LB counts increased by a median of 0.21% (range: -3.17% to 4.81%) per week in patients who had unfavourable final outcomes and decreased by a median of -0.52% (range: -3.72% to 3.60% per week) in patients who achieved stable cure. The OR for an unfavourable outcome for each percentage increase in af-LB count per week was 1.44 (95% CI: 0.95-2.18, p=0.085). The same modelling strategy with TLB counts yielded a similar result (data not shown).

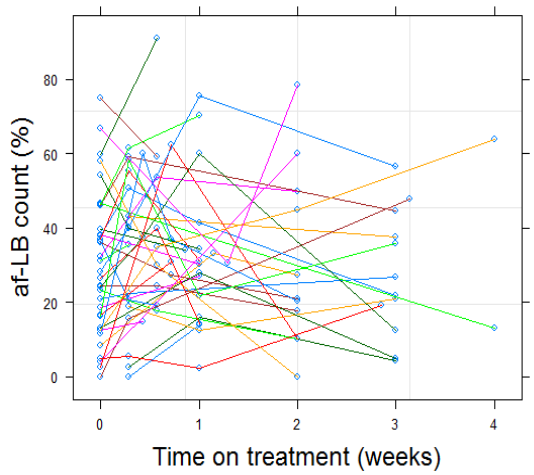


Figure 6.26 Spaghetti plot of change in af-LB (%) count

LME model fit by maximum likelihood
Model fit
AIC : 963.5112

Fixed effects: af-LB~Weeks on treatment

	Value	Std. Error	p-value
Intercept	32.196	2.973	<0.001
Slope	0.13	1.726	0.9417

Random effects: ~Week|Patient

Intercept	13
Slope	4.351
Residual	15.584

Table 6.22 af-LB LME model parameters

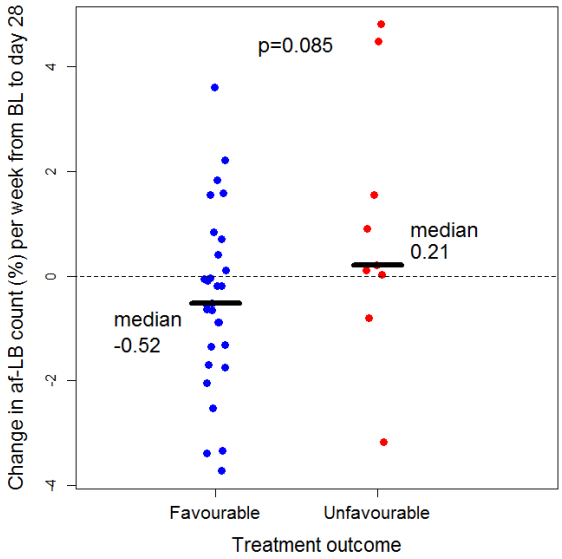


Figure 6.27 Association between change in af-LB count and treatment outcome

As with the earlier analysis, these trends towards poor long-term treatment response in patients with increasing LB counts are interesting, even if they did not reach statistical significance in this small sub-study. The findings are consistent with LBs as a phenotypic marker of bacillary persistence during anti-TB chemotherapy and warrant further investigation in larger cohorts.

6.6 Discussion

For management of individual patients, sputum smear conversion is the simplest and cheapest laboratory method of treatment monitoring but standard microscopy methods cannot distinguish between viable and killed organisms[592]. In this study, 16-20% of patients with positive 2 month ZN or AP smears had favourable final outcomes, showing that persistent smear positivity over-estimates the risk of treatment failure or relapse. Prior studies have reported similar results[29,358,593]. Alternative microscopy stains (e.g. FDA[594]) may more selectively discriminate between live and dead AFB but have not entered routine practice. The possibility that new molecular technologies (e.g. the Xpert MTB/RIF assay) have a role in assessment of sputum samples during treatment requires detailed assessment.

Sputum culture status at 2 months is the commonest surrogate marker for Phase IIb clinical trials but reports of its validity and efficacy are mixed, and may depend on the choice of bacteriological method. In this study, 2 month positivity on SSCC plates was a better predictor of outcome than positivity in MGIT liquid culture, perhaps because later culture conversion with the MGIT method generates more positive S4 results and reduces specificity to detect failure or relapse. Previous data have shown that less sensitive media (LJ slopes[227] or SSCC plates[228]) are more likely than liquid culture[225,226] to differentiate between treatment regimens in 8 week trials based on binary end-points. This suggests that a high bacillary load cut-off is preferable when binary end-points are used, but such an approach still precludes pharmacodynamic assessment of treatment response over the entire study period.

Description of baseline bacillary load by SSCC and MGIT was undertaken prior to modelling of bacterial elimination over time. A median of 6.19 $\log_{10}$ CFU/ml of sputum and a median TTP of 4 days indicated a high pre-treatment burden of *M tuberculosis* organisms in the study cohort. This is consistent with other descriptions that the bacterial load at diagnosis

is higher in African patients than those from other regions[357]. Higher bacillary loads were associated with more extensive radiological disease.

There was no relationship between baseline colony count or TTP and final clinical outcome, contrasting published reports that high pre-treatment bacillary load is a risk factor for unfavourable outcomes. However, enrolment to the study required smear '++' or '+++' sputum samples, limiting bacteriological heterogeneity at baseline. If smear '+' or 'scanty' patients had been included, associations between baseline colony count/TTP and outcome might have emerged.

SSCC-NLME modelling was complicated by a high proportion of missing data due to plate contamination and early culture conversion. However, a bi-exponential model was successfully fitted to the dataset, supporting Mitchison's long-standing hypothesis that metabolically active (Population A) and persister (Population B) organisms are cleared at different rates during combination chemotherapy. Extraction of best unbiased estimates of model parameters for each patient failed to show relationships with study end-points when only positive data-points were used, but incorporation of a partial likelihood method to estimate colony counts for samples below the limit of laboratory detection showed that patients with larger baseline persister populations (higher B_{Int}) and faster persister elimination (β) were more likely to have unfavourable outcomes. This demonstrates the benefit of including information of bacteriological clearance after the threshold of culture conversion, but further work is needed to ascertain the most satisfactory means of representing the missing data.

TTP-LME modelling was more straightforward; the laboratory method was easier with fewer contaminated specimens, culture conversion occurred later creating a larger dataset and a linear function could be fit to the data which was simple to interpret. The most striking feature of the LME analysis was that the slope (*b*) of all three tested models generated a MBER that strongly predicted clinical outcome. If this convincing result is replicated in other cohorts then the MBER from TTP-LME modelling may become a validated early surrogate of TB treatment response with the potential to improve the power of Phase IIb clinical trials of new anti-TB regimens.

Two additional observations are important regarding the TTP-LME method. Firstly, neither HIV parameters nor radiological extent of disease influenced the MBER, suggesting that any variability in the pharmacodynamic response attributable to these factors is incorporated

by the mixed effects structure of the analysis. On this basis, these covariates are unlikely to confound the results of clinical trials analysed by LME methods, permitting unselected recruitment of HIV-infected and un-infected individuals with or without CXR cavities.

Secondly, this study describes a much stronger predictive effect of serial TTP measurement than previously published reports. Review of the prior studies (summarised in Section 1.10.3) reveals that one was limited to drug resistant TB[297], one used TTP measurements from the first 2 weeks only[298], and the only one to include follow-up beyond 2 months was a retrospective evaluation with incomplete long-term data. TTP was also assessed at individual time-points rather than as a continuously modelled variable[300,301]. Careful combined selection of extended sampling schedule, post-treatment follow-up and appropriate statistical methods may be required for effective use of MGIT-TTP as a surrogate marker of final outcome.

The current study facilitated direct comparison of SSCC and MGIT data from many samples collected at an extended range of visits. Earlier culture conversion on solid media than in liquid broth is well documented[228,232], particularly in African cohorts[357]. However it is unclear whether extended viability of bacilli in liquid cultures occurs because broth revives a distinct population of persister bacilli which is unsupported on plate agar. Several authors have argued that media formulations vary in their ability to revive non-replicating organisms[283,343,346] and further information on persisters may be obtained by studies which inoculate the same specimens in different media.

It has recently been shown that there is a strong inverse correlation in clinical samples between $\log_{10}$CFU/ml counts and TTP[281,289,290] but that the relationship weakens in the second month of therapy[290]. The data in this chapter agree with these findings, and propose that diminishing correlation over time occurs because later samples contain a higher proportion of persisters whose oxygen consumption depends more on the metabolic activity of each cell than the total number of organisms. This hypothesis supports the concept of metabolically quiescent persisters and illustrates that combined use of different quantitative culture methods allows novel insights into bacillary behaviour under drug pressure.

The work on the LAM-ELISA demonstrated limitations of this technique; its utility is limited to a sub-set of HIV-infected patients (56% of those with a positive HIV test in this cohort). Log_{10}OD readings fell with time on therapy and some data could be interpreted to suggest

that LAM excretion is quantitatively linked to bacillary burden but the association with sputum bacterial load was inconsistent and it was ultimately unsurprising that there was no relationship between LAM-ELISA result and treatment outcome for smear positive PTB.

Although pulmonary containment of organisms reduces the value of urinary LAM measurement in patients without severe immunosuppression, the importance of disseminated disease in those with advanced HIV infection (e.g. CD4 count ≤50 cells/μl) should not be disregarded as there is evidence that the LAM-ELISA is useful for detection of EPTB (including mycobacteriuria[339]) in these individuals[335-338,595,596], who are commonly sputum smear negative. As post-treatment relapse may be higher in HIV-infected individuals[20-23] and extra-pulmonary sanctuary sites may contribute to long-term bacillary persistence[209,210], development and optimisation of assays to monitor the treatment response of extra-pulmonary organisms is still required.

The most novel, technique described in this chapter is ALTR microscopy which was also evaluated as a sub-study. Variable LB positivity and the probable detection of non-acid fast bacilli in baseline sputum samples confirmed that phenotypically distinct populations of *M tuberculosis* organisms exist during clinical infection. Higher TTP/$\log_{10}$CFU ratios in samples with higher af-LB and TLB counts indicated that the LB positive phenotype is metabolically quiescent, and trends towards unfavourable outcomes in patients with increasing proportions of af-LB and TLB cells during therapy supported the hypothesis that LB positive organisms are implicated in the phenomenon of drug tolerant persistence which currently thwarts development of ultra-short anti-TB chemotherapy.

There were a number of limitations of ALTR microscopy. The number of patients assessed was small and results from serial samples were not quite statistically significant. The assay was performed on stored, frozen sputum and it is not known whether intracellular lipid metabolism is affected by the freeze-thaw cycle. Slide reading was subjective and predominantly done by the study PI. All images were blinded prior to analysis but assessment of larger patient cohorts with independent image interpretation is required to validate conclusions on the importance of LB positivity. Even under optimal conditions, ALTR microscopy requires smear positive slides and as sputum smears generally turn negative at bacillary loads $<10^3$ organisms/ml[597,598] the capacity of this method to identify small populations of persisters after the first 4 weeks of therapy may always be compromised.

Overall, the data presented in this chapter are important. A role has been proposed for TTP-LME modelling in the generation of new surrogate markers for Phase IIb studies and validation of the MBER as a predictor of treatment outcome would be a major advance in clinical trials methodology. Additionally, the ALTR microscopy work has provided preliminary evidence that single techniques may identify phenotypically distinct bacillary sub-populations with suitable characteristics for drug-tolerant persistence.

7. Pharmacokinetics

7.1 Introduction

Complete assessment of responses to TB therapy requires pharmacokinetic data. Inter-patient variability in drug exposure has previously been described for anti-TB drugs and may have implications for bacillary clearance and treatment outcome.

In line with WHO recommendations, the Malawian NTP provides anti-TB therapy for adults as FDC tablets[599], with dosing according to weight bands (Table 2.3). However, individual patients still receive different mg/kg of doses of each drug. Furthermore, there are inconsistencies in the bioequivalence of anti-microbial compounds (especially rifampicin) between FDC tablets[600]. Excipients in some formulations may non-specifically adsorb the active compounds and some FDC tablets may be of sub-optimal quality[400,426]. Measurement of drug concentration was required to confirm that study patients (particularly those with unfavourable outcomes) were dosed appropriately and had detectable plasma drug levels.

Despite several decades of clinical experience with the current first-line drugs, target therapeutic concentrations for treatment of clinical TB disease are undefined, as large studies providing pharmacokinetic data combined with treatment outcome are lacking. Nevertheless, low concentrations of some drugs have been described in African populations[400,411,412] and associations have been between observed between inadequate exposure to the sterilising effects of rifamycins or pyrazinamide and unsuccessful treatment[401-403].

This chapter provides a non-compartmental analysis of key pharmacokinetic parameters (C_{max} and AUC) for all four first line anti-TB drugs in Malawian adults. Clinical factors contributing to variability in drug exposure are evaluated, and the effect of this variability on pharmacodynamic measures of bacillary elimination and final clinical outcome is assessed.

7.2 Methods

Intensive pharmacokinetic studies require blood sampling on several days at multiple time-points from 0-24 hours after drug administration. This was impractical for the purposes of the current study so patients were sampled at 0, 2 and 6 hours during a single (S2 or S3) visit as described in Section 2.6.2. Sparse sampling strategies have previously been advocated for population pharmacokinetic studies[421] and therapeutic drug monitoring[601,602] of TB patients.

7.2.1 Drug assays

Plasma samples were stored at -70^{o}C in the MLW laboratory, shipped to the Clinical Pharmacology laboratory at LSTM and heat-inactivated before bio-analysis. Sample preparations were conducted in a darkened room. Rifampicin concentrations were measured by a liquid chromatographic/tandem mass spectrometry (LC/MS/MS) method[603]. Pyrazinamide concentrations were measured by HPLC using an Ultraviolet visible (UV-Vis) absorption detector. Isoniazid and ethambutol concentrations were measured using a two drug LC/MS/MS technique[604].

For all methods, drug dilutions in blank plasma were used to generate standard curves for peak area ratios (PARs) of drug/internal standard on the chromatogram over an appropriate concentration range. Concentrations of anti-microbial agents in clinical samples were calculated from their PARs against the calibration line. Drug levels below the limit of detection were omitted. Sample runs included quality control specimens with high, medium and low drug concentrations to ensure consistency of operating conditions.

The assays used to quantify plasma concentrations of each anti-TB drug, including calibration curve ranges and analyte concentrations for quality control specimens are summarised in Table 7.1 and full details are provided below.

Analyte	Method	Internal standard	Calibration curve range (µg/ml)	Analyte concentrations for quality control specimens (µg/ml)		
				Low	Medium	High
Rifampicin	LC/MS/MS	250ng/ml rifapentine in acetonitrile/methanol [50:50, v/v])	0.025-6.40	0.075	0.6	4.8
Pyrazinamide	HPLC-UV-Vis	acetazolamide 10µg/ml in acetyl nitrate	2.5-80	8	38	64
Isoniazid	LC/MS/MS	methanol containing 200ng/ml metformin	0.001-5	0.04	2	4
Ethambutol			0.001-5	0.06	2	4

Table 7.1 Standard curve and quality control specimens for TB drug assays

LC/MS/MS method to determine rifampicin concentration

For drug extraction, 100µl of standard curve, quality control and clinical samples were added to 300µl of the internal standard and vortex-mixed for 20 seconds. Samples were spun at 16,200 x *g* for 25 minutes and supernatants were transferred to glass autosampler vials. 50µl aliquots were injected onto the HPLC column and eluted with a mobile phase of acetonitrile containing formic acid. Quantification was achieved by MS-MS detection in positive ionisation mode for both rifampicin and the internal standard. MS operating conditions were optimized as follows: the spray voltage was 4500V with a tube lens voltage of 124V and skimmer offset of 0V. The capillary temperature was set to 275^{o}C. Nitrogen was the sheath gas (40psi) and auxiliary gas (25psi). Argon was the collision gas at a pressure of 1.5 mTorr (1 Torr = 133.3Pa). The optimized collision energies for rifampicin and the internal standard were 10 and 30eV, respectively. Detection of the ions was performed in the multiple reaction monitoring mode using transitions of m/z 823.4 to 791.4 for RIF and m/z 877.4 to 150.8 for the internal control. Data acquisition was performed using Xcalibur 1.3 software (Thermo Electron Corporation, Hemel Hepstead, UK). Peak integration and calibrations were performed using LC QuanTM software (Version 2.5.6, Thermo Electron Corporation, Hemel Hempstead, UK).

HPLC method to determine pyrazinamide concentration

For drug extraction, 100µl of each sample were added to 200 µl of the internal standard, vortexed for 10 seconds and spun for 10 minutes at 17,000 x *g*. 250µl of supernatant were pipetted into a clear dry tube and evaporated to dryness under a stream of nitrogen at 30^{o}C. 200µl of 95/5 water (0.06% TFA)/acetonitrile was then added to each dried sample and vortex mixed for 10 seconds to dissolve the extract. For quantification, 60µl of reconstituted specimens were injected onto a Shimadzu LC 2010 HT HPLC system with wavelength detection at 268nm. The compounds were separated on a HyPURITY C18 250 x 4.6mm, 5µm column (Thermohypersil) protected by a LiChrospher 100 RP-18 (5µm) column with a mobile phase of 95/5 water (0.06% TFA)/acetonitrile. Data on peak chromatogram areas for pyrazinamide and the internal standard were acquired using Chromeleon (Dionex) software.

LC/MS/MS method to determine isoniazid and ethambutol concentrations

For drug extraction, 400µl of internal standard-precipitation solvent were added to 100µl of each sample, vortexed for 2 minutes and spun at 2000 x *g* for 5 minutes to remove the protein precipitate. The supernatant was transferred into a glass tube containing 200µl of

water, vortex-mixed with 2ml of dichloromethane for 1 minute and centrifuged at 2000 x *g* for 5 minutes. A 100µl aliquot of this supernatant was transferred to another tube and evaporated to dryness under nitrogen at 45°C. The extract was dissolved in 200µl of the mobile phase (methanol/water/formic acid [10:90:0.3, v/v/v]). For quantification, a 10µl aliquot of the final solution was injected for LC/MS/MS. Operating conditions were optimized as following: the spray voltage was 4500 V with a tube lens voltage of 65V skimmer offset of 0V. The capillary temperature was set to 250°C. Nitrogen was the sheath gas (15psi) and auxiliary gas (20psi). Argon was the collision gas at a pressure of 1.5mTorr. Ion Detection was performed in single reaction monitoring mode with optimized collision energies and transitions as shown in Table 7.2. Data acquisition was performed with the software described for the rifampicin LC/MS/MS assay.

Analyte	m/z Parent Ion	m/z Product Ion	Collision Energy (eV)
Metformin (IS)	130.190	60.410	13
Metformin (IS)	130.190	71.340	22
Isoniazid	138.160	79.293	32
Isoniazid	138.160	121.049	13
Ethambutol	205.190	116.100	14

Table 7.2 SRM conditions of isoniazid and ethambutol measurement by LC/MS/MS

7.2.2 Statistical analysis

Weight-adjusted doses were calculated for each patient by dividing the milligrams of drug administered in FDC tablets by weight in kg. These were compared with WHO recommended dose ranges (rifampicin: 8-12mg/kg, isoniazid: 4-6mg/kg, pyrazinamide: 20-30mg/kg and ethambutol: 15-20mg/kg) [30] to assess whether patients were dosed correctly.

Plasma drug concentrations at two (C_{2hr}) and six (C_{6hr}) hours post-dose were determined directly from concentration-time data. From Table 1.6, the expected T_{max} is 1-2 hours for rifampicin, isoniazid and pyrazinamide and 2-3 hours for ethambutol so normal absorption of all drugs is reflected by $C_{2hr} > C_{6hr}$. The number of patients with this pharmacokinetic profile was reported for each drug. The C_{2hr} of these profiles was the best available estimate of C_{max} and the $AUC_{0\text{-}6hr}$ was calculated using the linear trapezoid rule. Profiles indicating delayed drug absorption ($C6_{hr} > C2_{hr}$) were not analysed because an approximate measure of C_{max} could not be obtained, and interpretation of the $AUC_{0\text{-}6hr}$ was impossible.

Given the absence of data on target therapeutic concentrations for anti-TB therapy it was difficult to select reference standards for interpretation of C_{2hr} and $AUC_{0\text{-}6hr}$ results. However, published definitions of low and very low C_{max} have been derived from the

distribution of measurements amongst healthy Western volunteers[414,603] and are shown in Table 7.3. These values were used to study C_{2hr} results as categorical variables. For AUC_{0-6hr} there are no published low or normal values. Based on the distribution of the current dataset, categorical analysis of relationships between AUC_{0-6hr} results and treatment response was tested at several arbitrary thresholds for each drug. The following thresholds were selected for data presentation; rifampicin <25 µg/ml.hr, isoniazid <15 µg/ml.hr, pyrazinamide <200 µg/ml.hr and ethambutol <15 µg/ml.hr.

Drug	Low C_{max} (µg/ml)	Very low C_{max} (µg/ml)
Rifampicin	<8	<4
Isoniazid	<3	<2
Pyrazinamide	<35	<20
Ethambutol	<2	<1

Table 7.3 Published low and very low Cmax values for anti-TB drugs

Additionally, C_{2hr} and C_{6hr} concentrations from Malawian patients were compared directly with values previously obtained from population pharmacokinetic studies by M^{c}Illeron in South Africa[400] and Tappero in Botswana[400,414]. This approach was imperfect as patient recruitment, dosing regimens and bio-analytical techniques were not standardised across the cohorts but it allowed the pharmacokinetic parameters of the current project to be contextualised against TB patients in similar clinical environments.

The effect of clinical and radiological covariates on C_{2hr} and AUC_{0-6hr} values for each drug was assessed by multivariate linear regression according to methods previously described.

PK-PD relationships between the pharmacokinetic parameters of each drug and several measures of treatment response were examined. Logistic regression was used to relate drug exposure to categorical treatment end-points (final clinical outcome and or the traditional surrogate marker of 2 month culture status). Linear regression was used for analyses using the newly proposed surrogate efficacy markers of rate co-efficients from SSCC-NLME and TTP-LME modelling.

Best estimates of the rate of change in af-LB counts over time on therapy were extracted for each patient included in the LME model constructed during the serial ALTR microscopy sub-study (Section 6.5.3, Table 6.22). Wilcoxon and Kruskal-Wallis tests were used to establish whether low or very low levels of one or more anti-TB drugs affected temporal trends in af-LB counts.

Analyses were done in R Version 2.15.2 using “PK”, “epicalc” and “nlme” packages.

7.3 Results

7.3.1 Drug dosing and pharmacokinetic sample collection

126/133 (95%) patients who reached a final study end-point provided plasma for pharmacokinetic analysis. Weight-adjusted doses met or exceeded the WHO recommended range for 122 (97%) patients with rifampicin and isoniazid, 125 (99%) patients with pyrazinamide and 119 (92%) patients, with ethambutol.

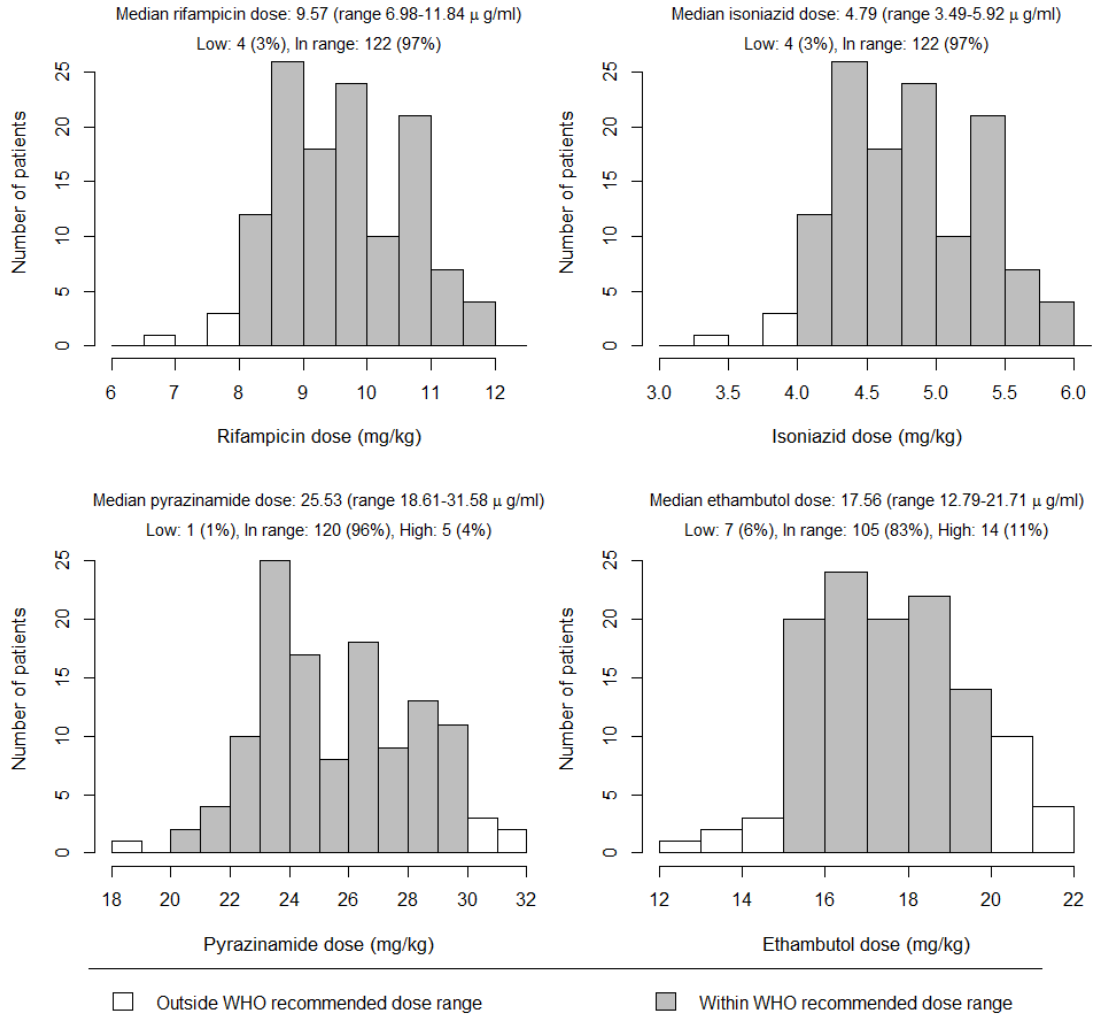


Figure 7.1 Weight adjusted doses of anti-TB drugs achieved with FDC tablets

As absorption, particularly of rifampicin, is reduced by food consumption, patients were instructed to fast from the evening before the pharmacokinetic study visit until blood sampling at 2 hours post-doses. The median duration of fast prior to drug administration was 13.17 (range 11.25-16.12) hours.

7.3.2 Pharmacokinetic parameters of anti-TB drugs

Rifampicin

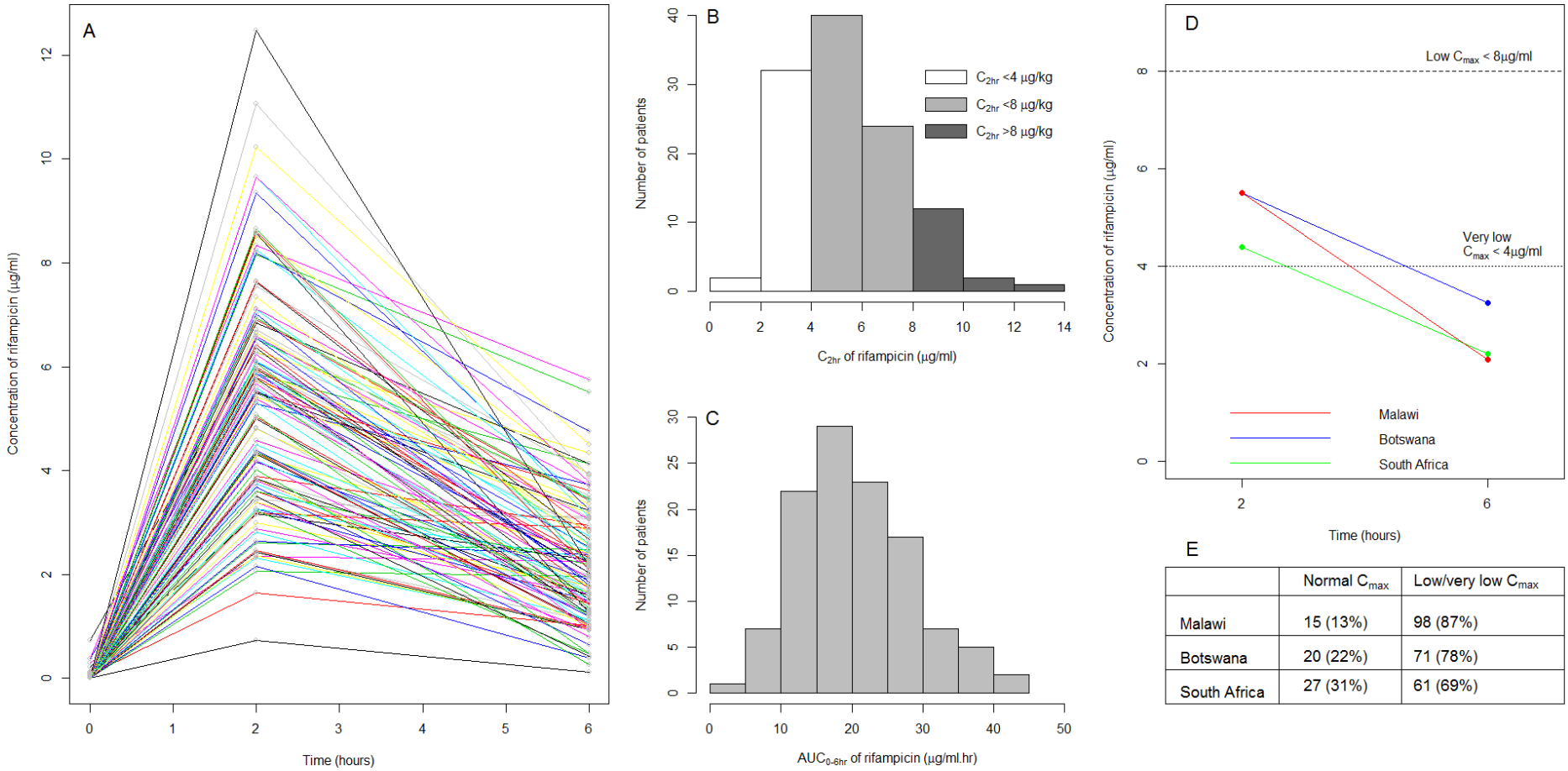
113/126 (89%) patients had normal rifampicin absorption profiles and were used for pharmacokinetic analysis. Individual patient profiles are shown in Figure 7.2A. The median C_{2hr} was 5.50 (range: 0.73-12.48) µg/ml. The distribution of results is shown in Figure 7.2B. 64 (56%) patients had low C_{2hr} rifampicin concentrations and 34 (30%) had very low concentrations, compared to reference C_{max} levels from healthy volunteers. The median AUC_{0-6hr} was 19.80 (range: 2.43-41.75) µg/ml.hr and 82 (73%) patients had an AUC_{0-6hr} <25 µg/ml.hr. The distribution of results is shown in Figure 7.2C.

Figure 7.2D compares median C_{2hr} and C_{6hr} plasma rifampicin concentrations from the current study on Malawian adults with the comparator populations from Botswana and South Africa. Rifampicin concentrations measured in Malawi were similar to those from other African cohorts. The median C_{2hrs} from all cohorts was lower than the published reference C_{max} level suggesting that individuals with active TB absorb or metabolise rifampicin differently from healthy controls.

Isoniazid

118/126 (94%) patients with normal isoniazid absorption profiles were used for pharmacokinetic analysis. Individual profiles are shown in Figure 7.3A. The median C_{2hr} for isoniazid was 2.94 (range: 0.85-5.99) µg/ml. The distribution of results is shown in Figure 7.3B. 35(30%) patients had low $C2_{hr}$ isoniazid concentrations and 24 (20%) had very low concentrations, compared to reference C_{max} levels. The median AUC_{0-6hr} was 11.50 (range: 2.69-25.02) µg/ml.hr and 82 (71%) patients had an AUC_{0-6hr} <15 µg/ml.hr. The distribution of results is shown in Figure 7.3C.

Figure 7.3D compares median C_{2hr} and C_{6hr} plasma isoniazid concentrations from the current study on Malawian adults with the prior population pharmacokinetic data. Isoniazid concentrations measured in Malawi were similar to those measured in Botswana, but lower than those in South African. Although the median C_{2hr} from three of the four populations was lower than the published reference C_{max} level, the frequency and extent of low concentrations was less for isoniazid than rifampicin.

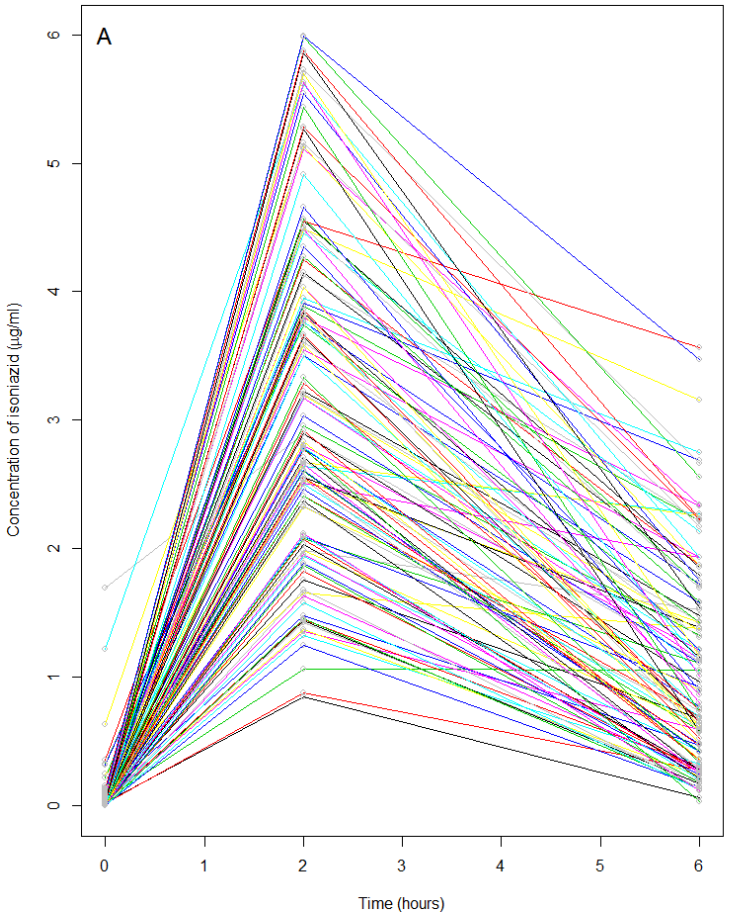


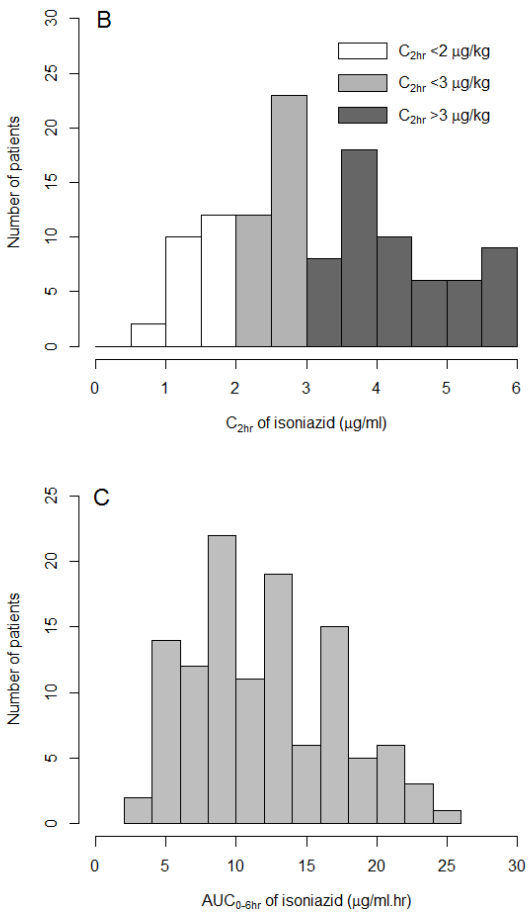
E

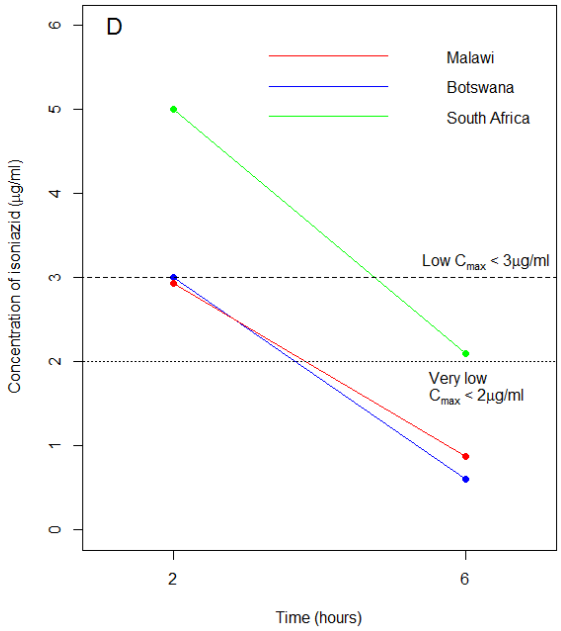
	Normal C_{max}	Low/very low C_{max}
Malawi	15 (13%)	98 (87%)
Botswana	20 (22%)	71 (78%)
South Africa	27 (31%)	61 (69%)

Figure 7.2 Pharmacokinetic parameters for rifampicin

A: Profiles of patients with normal rifampicin absorption. B: Distribution of C_{2hr} concentrations, indicating patients with very low (white bars), low (grey) and normal (dark grey) levels according to published C_{max} reference values. C: Distribution of $AUC_{0\text{-}6hr}$ concentrations. D: Median C_{2hr} and C_{6hr} concentrations from the current study, compared to studies in similar settings. E: Number (%) of patients with low/very low C_{max} in African studies. For the current cohort C_{2hr} is the best available estimate of C_{max}.







E

	Normal C_{max}	Low/very low C_{max}
Malawi	59 (50%)	59 (50%)
Botswana	63 (70%)	27 (30%)
South Africa	139 (98%)	3 (2%)

Figure 7.3 Pharmacokinetic parameters for isoniazid

A: Profiles of patients with normal isoniazid absorption. B: Distribution of C_{2hr} concentrations, indicating patients with very low (white bars), low (grey) and normal (dark grey) levels according to published C_{max} reference values. C: Distribution of AUC_{0-6hr} concentrations. D: Median C_{2hr} and C_{6hr} concentrations from the current study, compared to studies in similar settings. E: Number (%) of patients with low/very low C_{max} in African studies. For the current cohort C_{2hr} is the best available estimate of C_{max}.

Pyrazinamide

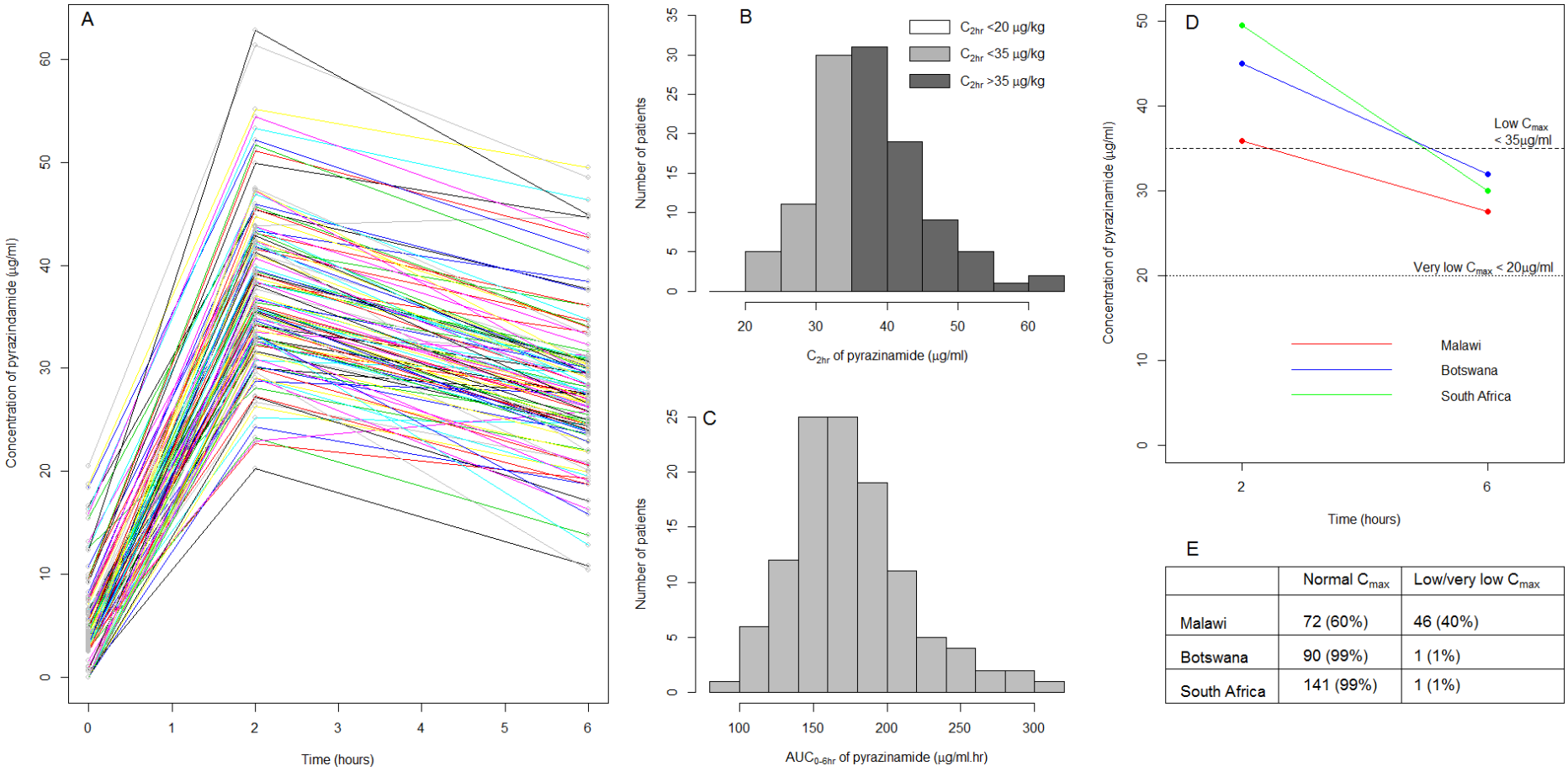
118/126 (94%) patients with normal pyrazinamide absorption profiles were used for pharmacokinetic analysis. Individual profiles are shown in Figure 7.4A. The median C_{2hr} for pyrazinamide was 37.37 (range: 20.24-62.79) µg/ml. The distribution of results is shown in Figure 7.4B. 46 (40%) patients had low $C2_{hr}$ pyrazinamide concentrations compared to the reference C_{max} but none had very low concentrations. The median AUC_{0-6hr} was 167.70 (range: 82.94-301.80) µg/ml.hr and 88 (78%) patients had an AUC_{0-6hr} <200 µg/ml.hr. The distribution of results is shown in Figure 7.4C.

Figure 7.4D compares median C_{2hr} and C_{6hr} plasma pyrazinamide concentrations from the current study on Malawian adults with the comparator African data. Pyrazinamide concentrations measured in Malawi were lower than those measured in both other cohorts, but in all of the studies the frequency and extent of low concentrations was less for pyrazinamide than either rifampicin or isoniazid.

Ethambutol

104/126 (83%) patients with normal ethambutol absorption profiles were used for pharmacokinetic analysis. Individual profiles are shown in Figure 7.5A. The median C_{2hr} for ethambutol was 2.54 (range: 1.07-9.75) µg/ml. The distribution of results is shown in Figure 7.5B. 24 (23%) patients had low ethambutol $C2_{hr}$ results compared to the reference C_{max} but none had very low concentrations. The median AUC_{0-6hr} was 10.68 (range: 4.76-35.68) µg/ml.hr and 87 (84%) patients had an AUC_{0-6hr} <15 µg/ml.hr. The distribution of results is shown in Figure 7.5C.

Figure 7.5D compares median C_{2hr} and C_{6hr} plasma ethambutol concentrations from the current study on Malawian adults with the comparator African data. Ethambutol concentrations measured in Malawi were similar to those measured in other cohorts. A low median C_{2hr} concentration was reported from Botswana, but overall, like pyrazinamide, the frequency and extent of low concentrations was less for ethambutol than either rifampicin or isoniazid.

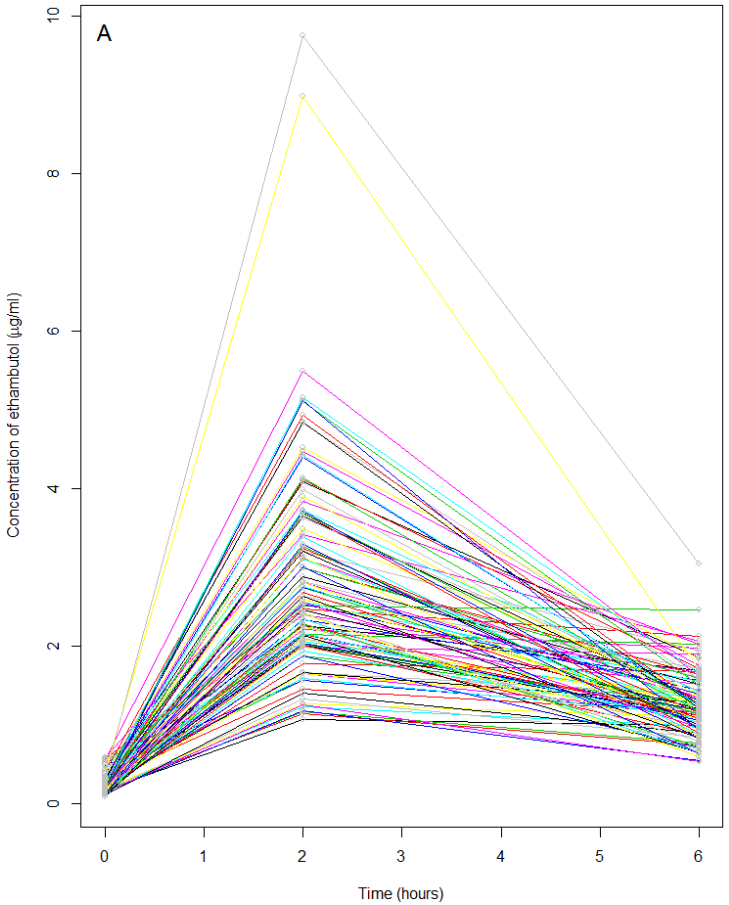


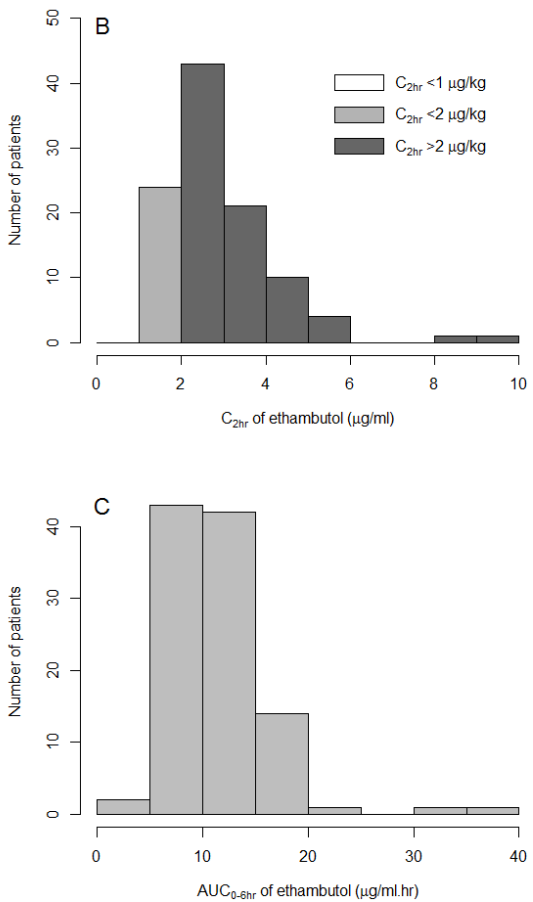
E

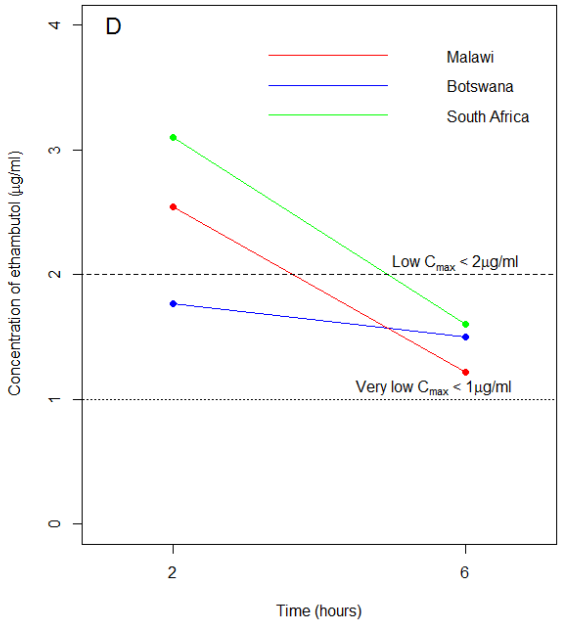
	Normal C_{max}	Low/very low C_{max}
Malawi	72 (60%)	46 (40%)
Botswana	90 (99%)	1 (1%)
South Africa	141 (99%)	1 (1%)

Figure 7.4 Pharmacokinetic parameters for pyrazinamide

A: Profiles of patients with normal pyrazinamide absorption. B: Distribution of C_{2hr} concentrations, indicating patients with very low (white bars), low (grey) and normal (dark grey) levels according to published C_{max} reference values. C: Distribution of AUC_{0-6hr} concentrations. D: Median C_{2hr} and C_{6hr} concentrations from the current study, compared to studies in similar settings. E: Number (%) of patients with low/very low C_{max} in African studies. For the current cohort C_{2hr} is the best available estimate of C_{max}.







E

	Normal C_{max}	Low/very low C_{max}
Malawi	80 (77%)	24 (23%)
Botswana	54 (59%)	37 (41%)
South Africa	126 (98%)	3 (2%)

Figure 7.5 Pharmacokinetic parameters for ethambutol

A: Profiles of patients with normal ethambutol absorption. B: Distribution of C_{2hr} concentrations, indicating patients with very low (white bars), low (grey) and normal (dark grey) levels according to published C_{max} reference values. C: Distribution of AUC_{0-6hr} concentrations. D: Median C_{2hr} and C_{6hr} concentrations from the current study, compared to studies in similar settings. E: Number (%) of patients with low/very low C_{max} in African studies. For the current cohort C_{2hr} is the best available estimate of C_{max}.

Concurrent low and very low drug concentrations

As the C_{2hr} of each first-line anti-TB drug was below the published reference range in some individuals, the incidence of concurrently low concentrations of multiple agents was assessed. 92/126 (73%) patients had normal absorption profiles for all four drugs and were used for this analysis. Table 7.4 shows the number of patients with low or very low C_{2hr} of multiple drugs and Figure 7.6 shows frequencies of drug combinations which were concurrently low.

59 (64%) patients had low $C2_{hr}$ concentrations of >1 drug and 8 (9%) patients had low $C2_{hr}$ concentrations of all drugs. Concurrent very low $C2_{hr}$ concentrations of more than one drug were observed in 6 (7%) patients and in each of these cases rifampicin and isoniazid were the implicated agents.

Number of drugs	Low C_{2hr} concentration n=92	Very low C_{2hr} concentration n=92
None, n (%)	8 (9)	51 (55)
One, n (%)	25 (27)	35 (38)
Two, n (%)	30 (32)	6 (7)
Three, n (%)	21 (23)	0
Four, n (%)	8 (9)	0

Table 7.4 Patients with concurrent low and very low drug concentrations

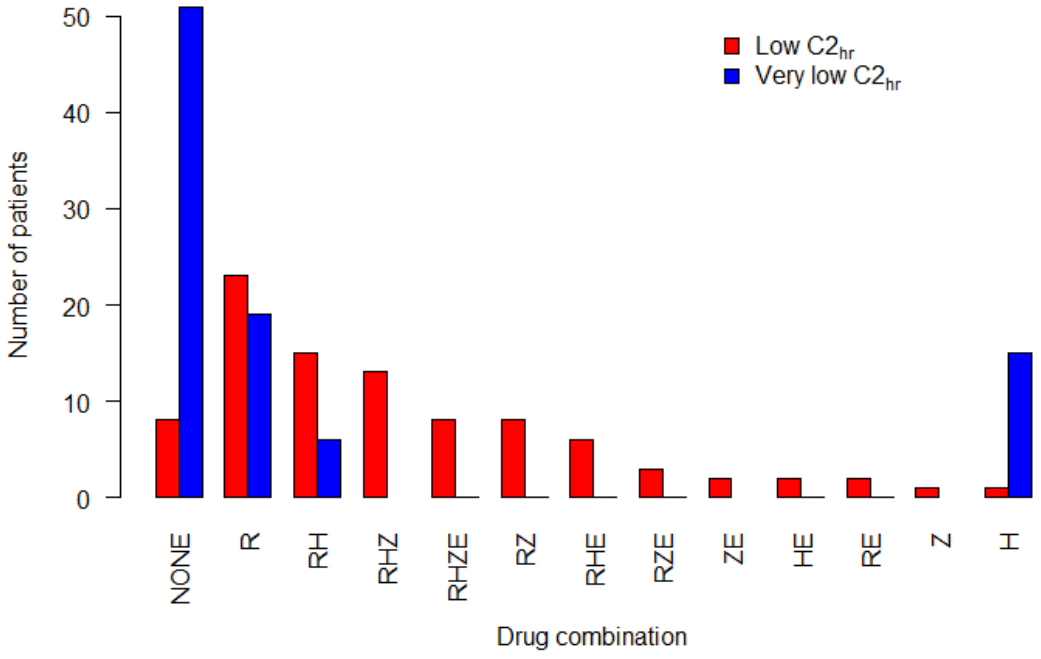


Figure 7.6 Combinations of concurrent low and very low drug concentrations

7.3.3 Factors influencing pharmacokinetic parameters of anti-TB drugs

The effects of clinical and radiological covariates on the C_{2hr} and $AUC_{0\text{-}6hr}$ of each anti-TB drug are shown in Tables 7.5 and 7.6. All factors which exerted a significant effect on the C_{2hr} or $AUC_{0\text{-}6hr}$ of any agent during univariate or multivariate analysis are displayed.

On multivariate analysis, male patients had a lower C_{2hr} (p=0.026) and $AUC_{0\text{-}6hr}$ (p=0.030) of rifampicin. There was a trend towards lower rifampicin C_{2hr} (p=0.086) in HIV-infected patients but HIV was not linked to altered pharmacokinetic parameters for isoniazid, pyrazinamide or ethambutol. Baseline CD4 counts or co-administration of ART did not significantly influence exposure to anti-TB drugs.

Patients with higher baseline temperature had a lower rifampicin C_{2hr} (p=0.045). Lymphadenopathy was associated with lower rifampicin $AUC_{0\text{-}6hr}$ (p=0.044) and a strong trend towards lower C_{2hr} (p=0.050). Tachypnoea at baseline was associated with lower ethambutol C_{2hr} (p=0.023) and $AUC_{0\text{-}6hr}$ (p=0.020). There were strong trends towards lower ethambutol C_{2hr} (p=0.052) and $AUC_{0\text{-}6hr}$ (p=0.058) in patients with greater percentage of lung affected on CXR. Collectively, these findings point towards reduced drug exposure in patients with more severe clinical disease, although the danger of over-interpreting the results of multiple comparisons should be borne in mind.

No data are tabulated for isoniazid because no covariate had an identifiable effect on exposure to this drug. The only factor associated with reduced pyrazinamide exposure was lower weight-adjusted dose achieved by FDC tablets.

No relationships were observed between the pharmacokinetic parameters of any drug and inter-individual variability in quantitative baseline bacillary load (by log_{10}CFU/ml, DTP and LAM-ELISA) or LB counts (data not shown).

Parameter	Regression co-efficients describing covariate effects on $C2_{hr}$ of rifampicin, pyrazinamide and ethambutol											
	Rifampicin Median C_{2hr}: 5.5 (range: 0.7 to 12.5) µg/ml n=113				Pyrazinamide Median C_{2hr}: 37.4 (range: 20.2 to 62.8) µg/ml n=118				Ethambutol Median C_{2hr}: 2.5 (1.1 to 9.8) µg/ml n=104			
	Univariate		Multivariate		Univariate		Multivariate		Univariate		Multivariate	
	Estimate (95% CI)	p-value	Estimate (95% CI)	p-value	Estimate (95% CI)	p-value	Estimate (95% CI)	p-value	Estimate (95% CI)	p-value	Estimate (95% CI)	p-value
Male sex	-1.06 (-1.91, -0.21)	0.02*	-0.98 (-1.84,-0.12)	0.03*	-2.75 (-5.86, 0.37)	0.08	-2.17 (-5.26, 0.93)	0.17	-0.08 (-0.68, 0.51)	0.78	-	-
Uses biomass fuel												
Inside the house	-1.31 (-2.69, 0.06)	0.06	-1.01(-2.38, 0.36)	0.15	1.74 (-3.23, 6.72)	0.49	-	-	-	-	-	-
Outside	-0.36 (-1.24, 0.53)	0.43	-0.13 (-1.00, 0.74)	0.77	1.29 (-2.03, 4.61)	0.44	-	-	-	-	-	-
Ever smoked cigarettes	0.61 (-0.36, 1.58)	0.22	-	-	-2.66 (-6.25, 0.92)	0.14	-	-	-0.70 (-1.34, -0.05)	0.03*	-0.39 (-0.97, 0.20)	0.19
HIV infection	-0.74 (-1.55, 0.06)	0.07	-0.69 (-1.49, -0.10)	0.09	0.81 (-2.19, 3.80)	0.60	-	-	0.05 (-0.50, 0.60)	0.87	-	-
CD4 count (cells/µl)[a]	0.00 (0.00-0.00)	0.513	-	-	-0.01 (-0.03, 0.01)	0.21	-	-	0.00 (-0.01, 0.00)	0.09	-	-
On ART at PK visit												
All patients	0.4 (-0.7, 1.4)	0.50	-	-	-1.19 (-4.92, 2.54)	0.53	-	-	0.34 (-0.33, 1.01)	0.31	-	-
HIV-infected[b]	1.00 (-0.10, 2.10)	0.08	-	-	-1.99 (-6.26, 2.32)	0.36	-	-	0.41 (-0.41, 1.23)	0.32	-	-
Weight adjusted dose (mg/kg)	-0.01 (-0.44, 0.41)	0.96	-	-	0.69 (0.14, 1.24)	0.01*	0.65 (0.11, 1.2)	0.02*	0.11 (-0.04, 0.27)	0.16	-	-
Lymphadenopathy	-1.92 (-3.57, -0.28)	0.02*	-1.58 (-3.15-0.00)	0.05	1.71 (-3.75,7.16)	0.54	-	-	-0.66 (-1.82, 0.50)	0.26	-	-
Baseline temp (^{o}C)	-0.53 (-0.89, -0.16)	0.01*	-0.38 (-0.76, -0.01)	0.05*	1.02 (-0.36, 2.39)	0.15	-	-	0.10 (-0.16, 0.35)	0.45	-	-
Tachypnoea[c]	-1.09 (-2.21, 0.03)	0.06	-0.61 (-1.71, 0.48)	0.23	1.63 (-2.38, 5.64)	0.42	-	-	-0.69 (-1.46, 0.08)	0.08	-0.77 (-1.44, -0.11)	0.02*
% lung affected on CXR[d]	0.00 (-0.02, 0.03)	0.81	-	-	0.00 (-0.09, 0.09)	0.97	-	-	-0.02 (-0.03, 0.00)	0.05	-0.02 (-0.03, 0.00)	0.05

Table 7.5 Factors associated with variability in C_{2hr} of anti-TB drugs

[a] Number of patients with CD4 count in C_{2hr} analysis; for rifampicin n=54, for pyrazinamide and ethambutol n=55
[b] Number of HIV infected patients in C_{2hr} analysis; for rifampicin n=62, for pyrazinamide n=64, for ethambutol n=55
[c] Tachypnoea=Respiratory rate>30 breaths per minute during baseline assessment
[d] Number of patients with CXR in C_{2hr} analysis; for rifampicin n=102, for pyrazinamide n=103, for ethambutol n=95

Parameter	Regression co-efficients describing covariate effects on $AUC_{0\text{-}6hr}$ concentrations of rifampicin, pyrazinamide and ethambutol											
	Rifampicin Median $AUC_{0\text{-}6hr}$: 19.8 (range: 2.4 to 41.7) µg/ml.hr n=113				Pyrazinamide Median $AUC_{0\text{-}6hr}$: 37.4 (range: 20.2 to 62.8) µg/ml.hr n=118				Ethambutol Median $AUC_{0\text{-}6hr}$: 10.6 (4.8 to 35.7) µg/ml.hr n=104			
	Univariate		Multivariate		Univariate		Multivariate		Univariate		Multivariate	
	Estimate (95% CI)	p-value	Estimate (95% CI)	p-value	Estimate (95% CI)	p-value	Estimate (95% CI)	p-value	Estimate (95% CI)	p-value	Estimate (95% CI)	p-value
Male sex	-3.56 (-6.74, -0.39)	0.03*	-3.58 (-6.80, -0.36)	0.03*	-9.09 (-25.24, 7.05)	0.27	-	-	-0.24 (-2.23, 1.74)	0.81	-	-
Uses biomass fuel												
Inside the house	-5.98 (-11.0, -0.91)	0.02*	-5.13 (-10.26, -0.00)	0.05	1.10 (-24.56, 26.76)	0.93	-	-	0.51 (-2.49, 3.51)	0.74	-	-
Outside	-2.17 (-5.43, 1.1)	0.19	-1.66 (-4.69, 1.79)	0.38	2.70 (-14.41, 19.80)	0.76	-	-	-1.09 (-3.09, 0.91)	0.28	-	-
Ever smoked cigarettes	1.93 (-1.69, 5.55)	0.29	-	-	-10.89 (-29.39, 7.60)	0.25	-	-	-2.38 (-4.53, -0.22)	0.03*	-1.40 (-3.33, 0.53)	0.15
HIV infection	-2.56 (-5.57, 0.45)	0.09	-2.20 (-5.17, 0.78)	0.15	1.40 (-14.03, 16.83)	0.86	-	-	0.30 (-1.54, 2.14)	0.75	-	-
CD4 count (cells/µl)[a]	0.00 (-0.02, 0.01)	0.677	-	-	-0.07 (-0.15, 0.01)	0.10	-	-	-0.01 (-0.02, 0.00)	0.08	-	-
On ART at PK visit												
All patients	0.82 (-2.93, 4.57)	0.66	-	-	-12.18 (-31.26, 6.65)	0.21	-	-	1.17 (-1.05, 3.40)	0.30	-	-
HIV-infected[a]	2.86 (-1.32, 7.04)	0.18	-	-	-15.82 (-38.30, 6.65)	0.17	-	-	1.31 (-1.51,4.13)	0.36	-	-
Weight adjusted dose (mg/kg)	0.34 (-1.24, 1.92)	0.67	-	-	3.27 (0.43, 6.10)	0.02*	-	-	0.44 (-0.09. 0.97)	0.11	-	-
Lymphadenopathy	-7.44 (-13.54,-1.33)	0.02*	-6.06 (-11.96, -0.16)	0.04*	5.81 (-22.26, -33.87)	0.68	-	-	-2.50 (-6.39, 1.39)	-0.21	-	-
Baseline temp (^{o}C)	-1.68 (-3.05, -0.30)	0.02*	-1.10 (-2.50, 0.30)	0.12	4.90 (-2.22, 12.02)	0.18	-	-	0.31 (-0.53, 1.16)	0.46	-	-
Tachypnoea[b]	-4.04 (-8.2, 0.11)	0.06	-2.20 (-6.10, 2.10)	0.34	5.97 (-14.78, 26.72)	0.57	-	-	-2.36 (-4.93, 0.22)	0.07	-2.63 (-4.82, -0.43)	0.02*
% lung affected on CXR[c]	0.01 (-0.09, 0.11)	0.80	-	-	-0.03 (-0.48, 0.43)	0.90	-	-	-0.05 (-0.10, 0.00)	0.05*	-0.05 (-0.10, 0.00)	0.06

Table 7.6 Factors associated with variability in $AUC_{0\text{-}6hr}$ of anti-TB drugs

[a] Number of patients with CD4 count in $AUC_{0\text{-}6hr}$ analysis; for rifampicin n=54, for pyrazinamide and ethambutol n=55
[b] Number of HIV infected patients in $AUC_{0\text{-}6hr}$ analysis; for rifampicin n=62, for pyrazinamide n=64, for ethambutol n=55
[c] Tachypnoea=Respiratory rate>30 breaths per minute during baseline assessment
[d] Number of patients with CXR in $AUC_{0\text{-}6hr}$ analysis; for rifampicin n=102, for pyrazinamide n=103, for ethambutol n=95

7.3.4 Effect of pharmacokinetic variability on treatment response

Table 7.7 and 7.8 describe relationships between the C_{2hr} and $AUC_{0\text{-}6hr}$ of each drug and treatment end-points of 2 month sputum culture status and final clinical outcome. In logistic regression analyses, with pharmacokinetic parameters assessed as continuous variables, rifampicin exposure did not predict either end-point. Unfavourable final outcomes were less likely in patients with a higher isoniazid C_{2hr} or $AUC_{0\text{-}6}$ (p=0.041 and p=0.035 respectively). Positive 2 month sputum cultures were less likely in patients with higher $AUC_{0\text{-}6hr}$ of isoniazid (p=0.045), pyrazinamide (p=0.048) or ethambutol (p=0.046).

When the pharmacokinetic parameters were analysed as categorical variables, low or very low C_{2hr} and low $AUC_{0\text{-}6hr}$ were not related to 2 month culture status or final outcome for any drug. Additionally, the number of drugs for which concurrently low or very low C_{2hr} were measured was not significant (data not shown).

Linear regression was used to evaluate the influence of plasma drug concentrations on bacillary elimination rates extracted from the maximal and partial likelihood SSCC-NLME models constructed in Chapter 6. No relationships with bacterial clearance were identified for any drug when C_{2hr} and $AUC_{0\text{-}6hr}$ values were assessed as continuous or categorical variables, except in the analysis of isoniazid $AUC_{0\text{-}6hr}$. In this case, the effect of a low $AUC_{0\text{-}6hrs}$ (<15µg/ml.hr) on sterilisation phase (β) rate constants is shown in Figure 7.7A and B. Whilst inter-individual variability in β estimates from the maximal likelihood model was too small to identify any drug effect, analysis of the partial likelihood model suggests that lower isoniazid exposure was associated with slower elimination of persister organisms (Figure 7.7B, p=0.038).

Table 7.9 and 7.10 show the effect of C_{2hr} and $AUC_{0\text{-}6}$ measurements on MBER estimates from all three TTP-MGIT models. The pharmacokinetic parameters were assessed as continuous or categorical variables respectively. Once again, the $AUC_{0\text{-}6hr}$ of isoniazid was the only important parameter. On the continuous scale, a higher $AUC_{0\text{-}6hr}$ value was linked with a trend towards a faster MBER in the alternative model (p=0.076). In the categorical analysis (illustrated in detail in Figure 7.7C-E), an isoniazid $AUC_{0\text{-}6hrs}$ <15µg/ml.hr was associated with a slower MBER in the alternative model (p=0.040) and there was a trend towards the same finding in the partial likelihood model (p=0.068).

Drug		Positive 2 month culture median (range)	Negative 2 month culture median (range)	OR of positive 2 month culture	95% CI	p-value
Rifampicin	C_{2hr}, µg/ml	5.45 (2.32-11.06)	5.49 (1.64-12.48)	0.99	0.82-1.21	0.978
n positive=31, n negative=72	AUC_{0-6hr} µg/ml.hr	19.63 (8.94-40.98)	19.84 (7.00-41.75)	0.99	0.94-1.04	0.818
Isoniazid	C_{2hr} µg/ml	2.64 (0.88-5.43)	3.29 (0.84-5.99)	0.76	0.54-1.07	0.120
n positive=29, n negative=73	AUC_{0-6hr} µg/ml.hr	10.67 (3.23-17.81)	12.09 (2.69-25.02)	0.91	0.83-1.00	0.045*
Pyrazinamide	C_{2hr} µg/ml	35.41 (20.24-53.27)	37.26 (22.66-61.38)	0.94	0.88-1.00	0.062
n positive =30, n negative=70	AUC_{0-6hr} µg/ml.hr	160.51 (82.94-268.86)	177 (109.89-301.80)	0.99	0.97-1.00	0.048*
Ethambutol	C_{2hr} µg/ml	2.28 (1.07-5.11)	2.71 (1.16-9.75)	0.69	0.45-1.05	0.080
n positive = 29, n negative= 64	AUC_{0-6hr}µg/ml.hr	8.92 (4.93-17.39)	11.12 (5.19-35.78)	0.87	0.77-1.00	0.046*

Table 7.7 Pharmacokinetic parameters and 2 month culture status

Only patients with normal absorption profiles and a valid 2 month sputum culture result could be assessed for each drug. The number of patients and the breakdown of positive or negative sputum culture results for each PK-PD analysis are shown in italics beneath the drug name. P-values were generated by logistic regression.

Drug		Unfavourable outcome median (range)	Favourable outcome median (range)	OR of unfavourable outcome	95% CI	p-value
Rifampicin	C_{2hr}, µg/ml	5.77 (2.46-11.06)	5.45 (0.73-12.48)	1.12	0.87-1.44	0.366
n unfavourable=14, n favourable =98	AUC_{0-6hr} µg/ml.hr	20.36 (9.34-40.98)	19.80 (2.43-41.75)	1.03	0.96-1.10	0.397
Isoniazid	C_{2hr} µg/ml	2.55 (0.88-3.84)	3.18 (0.85-5.99)	0.58	0.35-0.98	0.041*
n unfavourable=14, n favourable=101	AUC_{0-6hr} µg/ml.hr	8.78 (3.22-16.19)	12.03 (2.69-25.02)	0.87	0.76-0.99	0.035*
Pyrazinamide	C_{2hr} µg/ml	36.79 (28.07-51.13)	36.00 (20.24-62.79)	1.02	0.95-1.09	0.675
n unfavourable=14, n favourable=98	AUC_{0-6hr} µg/ml.hr	168.02 (125.53-248.30)	168.28 (82.84-301.80)	1.00	0.99-1.01	0.962
Ethambutol	C_{2hr} µg/ml	3.06 (1.24-5.11)	2.53 (1.07-9.75)	0.98	0.63-1.53	0.933
n unfavourable=12, n favourable=91	AUC_{0-6hr} µg/ml.hr	11.30 (4.93-18.56)	10.69 (4.76-35.68)	0.99	0.87-1.13	0.902

Table 7.8 Pharmacokinetic parameters and clinical outcome

Only patients with normal absorption profiles and a documented final outcome could be assessed for each drug. The number of patients and the breakdown of clinical end-points for each PK-PD analysis are shown in italics beneath the drug name. P-values were generated by logistic regression.

Collectively, these data imply that variability in isoniazid exposure may impact on the sterilising activity of TB treatment and final outcome. This is somewhat surprising as isoniazid is not generally regarded as a sterilising drug. As some PK-PD relationships were dependent on specific approaches to the handling of missing data, examination of other cohorts is required to explore this issue further.

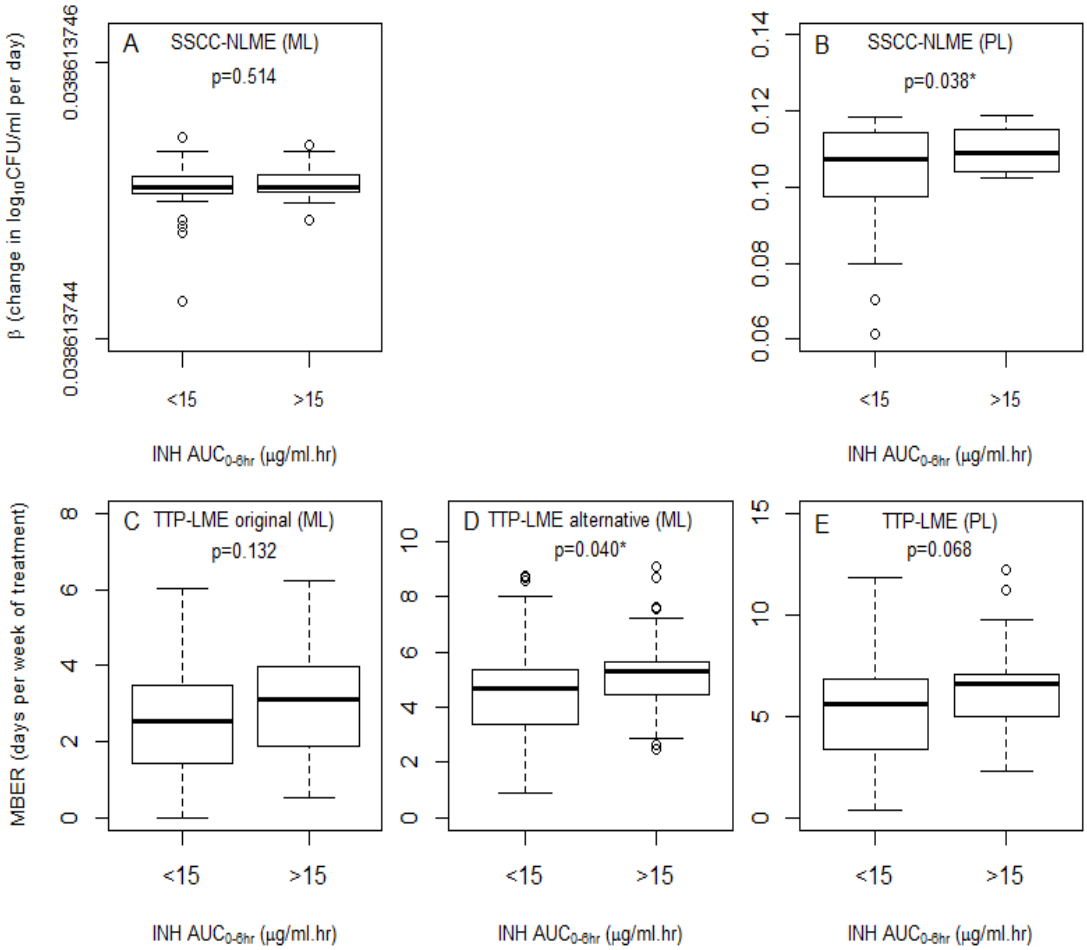


Figure 7.7 Effect of low isonazid $AUC_{0\text{-}6hr}$ on bacillary elimination rates

Boxplots summarise the effect of a low isoniazid $AUC_{0\text{-}6hr}$ (<15µg/ml.hr) on pharmacodynamic measures of treatment response derived from quantitative bacteriology. P-values were generated by linear regression. A: Effect on the sterilisation phase bacillary elimination rate (β) from the maximal likelihood (ML) SSCC-NLME model. As previously shown in Figure 6.9, there was little inter-individual variability in the β rate constant from this model, making the effect of covariates difficult to detect. B: Effect on β from the partial likelihood (PL) SSCC-NLME model. Patients with a low isoniazid $AUC_{0\text{-}6hr}$ had a slower sterilisation phase elimination rate (p=0.038). C-E: Effect on the MBER from original, alternative and partial likelihood TTP-LME models. In each case, patients with a low isoniazid $AUC_{0\text{-}6hr}$ had a slower MBER. The difference was statistically significant for the alternative model (p=0.040) and a strong trend was observed in the partial likelihood model (p=0.068).

The effect of a low $AUC_{0\text{-}6hr}$ on bacillary elimination is not displayed for rifampicin, pyrazinamide or ethambutol; no important trends or significant relationships were observed for these drugs.

Drug		Regression co-efficient describing effect of pharmacokinetic parameters on MBER					
		Original model (Max Likelihood[R])		Alternative model (Max Likelihood [R])		Partial likelihood model (NONMEM)	
		Estimate (95% CI)	p-value	Estimate (95% CI)	p-value	Estimate (95% CI)	p-value
Rifampicin	C_{2hr}, µg/ml	-0.04 (0.17, 0.10)	0.591	-0.05 (-0.21, 0.12)	0.556	-0.14 (-0.39, 0.11)	0.269
	AUC_{0-6} µg/ml.hr	-0.01 (-0.05, 0.03)	0.588	-0.01 (-0.06, 0.03)	0.523	-0.04 (-0.10, 0.03)	0.286
Isoniazid	C_{2hr} µg/ml	0.11 (-0.10, 0.32)	0.318	0.21 (-0.05, 0.46)	0.112	0.20 (-0.18, 0.57)	0.304
	AUC_{0-6} µg/ml.hr	0.03 (-0.03, 0.08)	0.361	0.06 (-0.01, 0.13)	0.076	0.06 (-0.04, 0.16)	0.225
Pyrazinamide	C_{2hr} µg/ml	0.00 (-0.04, 0.03)	0.929	0.01 (-0.03, 0.06)	0.547	0.00 (-0.06, 0.07)	0.910
	AUC_{0-6} µg/ml.hr	0.00 (-0.01, 0.01)	0.903	0.00 (-0.01, 0.01)	0.475	0.00 (-0.01, 0.01)	0.784
Ethambutol	C_{2hr} µg/ml	0.03 (-0.20, 0.26)	0.782	0.12 (-0.16, 0.40)	0.389	0.21 (-0.20, 0.62)	0.306
	AUC_{0-6} µg/ml.hr	0.01 (-0.05, 0.08)	0.670	0.05 (-0.03, 0.13)	0.229	0.08 (-0.04, 0.20)	0.178

Table 7.9 Continuous pharmacokinetic parameters and MBER from MGIT-TTP models

Detail on the construction of all three MGIT-TTP models are provided in Section 6.3.4. C_{2hr} and AUC_{0-6hr} measurements for all drugs were assessed as continuous variables. P-values were generated by linear regression.

Drug		Regression co-efficient describing effect of pharmacokinetic parameters on MBER					
		Original model (Max Likelihood[R])		Alternative model (Max Likelihood [R])		Partial likelihood model (NONMEM)	
		Estimate (95% CI)	p-value	Estimate (95% CI)	p-value	Estimate (95% CI)	p-value
Rifampicin	C_{2hr} <8µg/ml	0.31 (-0.53, 1.15)	0.468	0.1 (-0.97, 1.17)	0.849	0.25 (-1.33, 1.84)	0.752
	AUC_{0-6} <25µg/ml.hr	-0.03 (-0.65, 0.59)	0.919	-0.17 (-0.96, 0.62)	0.674	0.16 (-1.01, 1.32)	0.789
Isoniazid	C_{2hr} <3µg/ml	-0.06 (-0.64,0.52)	0.837	-0.54 (-1.24, 0.17)	0.137	-0.84 (-1.86, 0.18)	0.107
	AUC_{0-6} <15µg/ml.hr	-0.46 (-1.07, 0.14)	0.132	-0.77 (-1.50,-0.03)	0.040*	-0.99 (-2.06, 0.08)	0.068
Pyrazinamide	C_{2hr} <35µg/ml	0.12 (-0.40, 0.64)	0.649	-0.08 (0.76, 0.60)	0.818	0.11 (-0.88, 1.11)	0.820
	AUC_{0-6} <200µg/ml.hr	-0.06 (-0.72, 0.60)	0.850	0.03 (-0.79, 0.85)	0.947	0.26 (-0.98, 1.49)	0.682
Ethambutol	C_{2hr} <2µg/ml	-0.25 (-0.92, 0.41)	0.454	-0.31 (-1.12, 0.50)	0.451	-0.66 (-1.82, 0.51)	0.268
	AUC_{0-6} <15µg/ml.hr	0.04 (-0.80, 0.89)	0.918	-0.25 (-1.27, 0.78)	0.636	-0.45 (-1.94, 1.03)	0.548

Table 7.10 Categorical pharmacokinetic parameters and MBER from MGIT-TTP models

C_{2hr} and AUC_{0-6hr} measures assessed as categorical variables according to "low" values described on Section 7.2.2. P-values were generated by logistic regression.

7.3.5 Effect of pharmacokinetic variability on af-LB counts

In Section 6.5.3 a LME model was constructed to describe changes in af-LB counts of serial sputum samples during TB therapy and it was postulated that alterations to intra-cellular lipid metabolism under drug pressure contribute to antibiotic tolerance. If this is true, slower emergence of LB positive organisms may occur in patients with lower $C2_{hr}$ drug concentrations because the pharmacological pressure favouring persister phenotypes is less.

By integrating pharmacokinetic and serial ALTR microscopy data, Figure 7.8A demonstrates a drop during therapy in the af-LB count of patients with very low isoniazid $C2_{hr}$ compared to those with low or normal measurements (p=0.026). Figure 7.8B shows that the af-LB count appeared to drop more in patients with very low $C2_{hr}$ for multiple drugs than in patients with less extensive deficiencies in drug exposure (p=0.091). The number of patients involved in these exploratory analyses was small, so further exploration of the relationship between drug pressure and af-LB counts is needed.

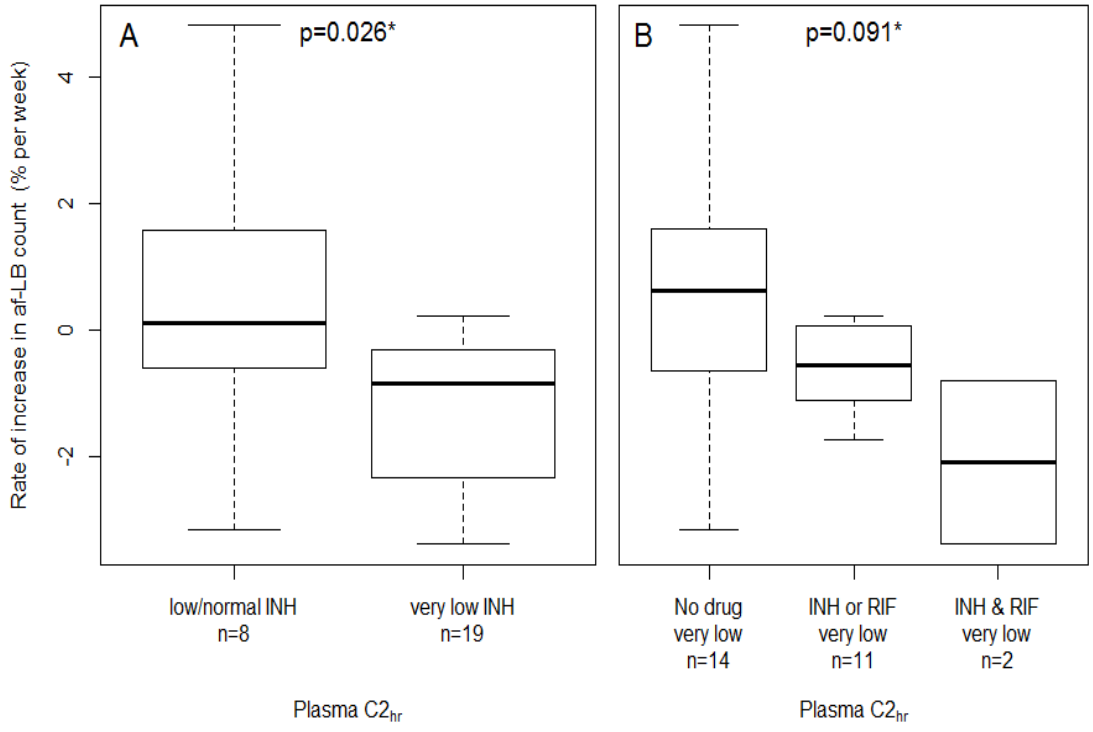


Figure 7.8 Drug exposure and change in af-LB count

A: Change in af-LB counts during the first 4 weeks from LME model for patients with very low or low/normal isoniazid $C2_{hr}$. Data analysed by Wilcoxon test. B: Change in af-LB counts from LME model for patients with very low $C2_{hr}$ of no, one (rifampicin or isoniazid) or two (rifampicin and isoniazid) drugs. Data analysed by Kruskal-Wallis test.

7.4 Discussion

The data in this chapter are important because there is currently limited information on pharmacokinetic parameters in African populations, and few existing studies relate measurement of drug exposure to therapeutic response and treatment outcome.

A striking feature is that, despite compliance with approved dosing protocols, rifampicin C_{2hr} results were low in 87% of patients according to a published C_{max} reference range. C_{2hr} results were also low in 50% of patients for isoniazid, 40% for pyrazinamide and 19% for ethambutol. 59 (64%) patients had low C_{2hr} of ≥1 drug and 8 (7%) patients had low C_{2hr} of all drugs. Bloods sampling was co-ordinated around supervised dose administration so poor adherence does not explain these findings. From Table 3.7, only 5 study recruits had diarrhoea or gastroenteritis at any time reducing the likelihood that malabsorption was the cause. No patients were concurrently taking medicines which are known to interact with anti-TB drugs (Section 3.4.3) eliminating drug-drug interactions as a possible explanation.

The observed rifampicin concentrations were consistent with other recent studies[403,412,419,427] including M[c]Illeron in South Africa[400] and Tappero in Botswana[414]. The relationship between male sex and low plasma rifampicin exposure has previously been attributed to gender-based differences in volume of drug distribution[419,605]. The trend towards low rifampicin C_{2hr} and $AUC_{0\text{-}6hr}$ in HIV-infected patients was compatible with prior reports from South Africa[400] but the overall effect of HIV on the pharmacokinetics of anti-TB chemotherapy remains controversial and in the current study there was no HIV effect on any parameters of isoniazid, pyrazinamide or ethambutol.

SLCO1B1 gene polymorphisms are common in black South Africans and may contribute to low rifampicin concentrations[441-444]. However, the incidence of these polymorphisms in Malawi is unknown and requires exploration. Whole blood samples containing DNA of all study samples are stored and pharmacogenomic analysis is planned.

Low or very low isoniazid concentrations were more commonly found in the current study than in recent comparable African cohorts but no clinical or radiological covariates were linked with low C_{2hr} or $AUC_{0\text{-}6hr}$. Pharmacogenomic analysis of stored DNA is also necessary here as NAT2 genotype and acetylator status may determine up to 88% of isoniazid variability[606] and the epidemiology of these factors in Malawi is unknown.

Pyrazinamide pharmacokinetics are the most stable of the first-line drugs, and in the current study the only covariate associated with inter-individual variability was the weight-adjusted dose. Lower pyrazinamide concentrations in Malawi than in comparator African studies may be explained by differences in dosing between centres. In Botswana, the daily pyrazinamide schedule[403,414] results in higher mg/kg dose than the Malawian protocol. Although South African and Malawian NTPs use the same weight bands and FDC approach, the median weight-adjusted pyrazinamide dose reported by M^{c}Illeron was 35.7 (range: 25.2-47.3) mg/kg[400] compared to 25.5 (range: 16.6-31.6) mg/kg in Malawi.

Some data suggested that severe or disseminated clinical disease may be associated with lower rifampicin or ethambutol exposure. Inferior concentrations of these drugs have previously been noted in TB patients than healthy controls[414] and it is possible that this discrepancy is exaggerated in the sickest patients whose altered physiology affects drug metabolism and excretion.

As a major aim of this thesis is to examine factors associated with bacillary persistence and treatment outcome, the most important part of the pharmacokinetic analysis is the PK-PD study of relationships between patient measurements and therapeutic response.

Rifampicin is the principal sterilising drug used throughout TB therapy and historical studies suggest that intermittent rifamycin dosing or low concentrations are associated with worse outcomes[94,395,401,402]. It was initially surprising that rifampicin variability was not related to any outcome measure. However, prior studies have described similar results[427] and it is possible that low rifampicin concentrations during treatment of clinical infection are insufficient to detect the effect of variable drug exposure on bacillary elimination. MICs for rifampicin amongst *M tuberculosis* isolates from Malawi are unknown but if a value of 1mg/l (from published *in vitro* data[397]) is assumed and rifampicin concentration-time plots are extrapolated, the estimated $AUC_{0\text{-}Infinity}$/MIC ratio of the study population was 29.7 (95% CI: 27.3-32.1) µg/ml.hr. This is several-fold lower than the steepest portion of AUC/MIC vs. bactericidal effect curves from macrophage and animal models[397] supporting the case for trials of higher rifampicin doses to accelerate bacillary elimination and possibly shorten treatment duration.

In contrast, isoniazid variability was associated with several measures of treatment response; a lower C_{2hr} and $AUC_{0\text{-}6hr}$ increased the likelihood of positive 2 month sputum cultures, a higher $AUC_{0\text{-}6hr}$ was associated with faster bacillary elimination in some models

and a lower C_{2hr} was related to unfavourable final outcome. Isoniazid is potently bactericidal against replicating bacilli, and some authors have linked rapid drug elimination by fast acetylators to bacteriological failure[407] so greater efficacy at higher exposure is plausible. As with rifampicin, if an MIC of 0.05mg/l (from *in vitro* data[399]) is assumed and isoniazid concentration-time plots are extrapolated the estimated $AUC_{0\text{-infinity}}$/MIC of the study population was 301.4 (95% CI: 276.8-326.0) µg/ml.hr. This value is within the steepest portion of AUC/MIC vs. bactericidal effect curves from macrophage and animal models[399], potentially explaining why isoniazid pharmacokinetics were more strongly associated with therapeutic response than those of rifampicin. Nevertheless, isoniazid is generally considered a poor steriliser of metabolically quiescent persister and the relationship with long-term outcome was unexpected.

Variability in the $AUC_{0\text{-}6hr}$ of pyrazinamide or ethambutol was associated with 2 month culture status but not final outcome. As these drugs are only used during the intensive phase of therapy, it is consistent that the effect of their pharmacokinetic variability is reflected in early bacteriological markers. In Botswana, lower pyrazinamide C_{max} was previously associated with higher risk of treatment failure at 6 months[403] but this was not observed in Malawi. The higher pyrazinamide dosing regimen in Botswana may have a role in explaining the discordant results.

When C_{2hr} values were analysed as categorical variables, only isoniazid displayed a relationships between drug concentration and 2 month culture status or clinical outcome. This challenges the suitability of defining low and very low drug levels solely on the basis of results from healthy volunteers. For pharmacokinetic parameters to be useful, reference ranges should be relate to relevant bacteriological and clinical end-points rather than normal values from ethnically and physiologically dissimilar populations.

The final analysis in this chapter explored relationships between pharmacokinetic parameters and serial trends in af-LB counts. The ALTR microscopy sub-study was too small to draw strong conclusions but it is interesting that af-LB counts dropped more quickly in the sputum of patients with very low C_{2hr} of isoniazid and/or rifampicin than in those with greater drug exposure. This suggests that there may be more than one explanation for bacillary persistence. Some patients do not achieve sputum sterilisation as their plasma drug concentrations are inadequate. In this scenario, drug-tolerant persister bacilli have no

selective advantage. However, in patients with high drug concentrations, bacterial survival may be driven by organisms with a lipid-laden persister phenotype.

The work in this chapter had several limitations. The sparse sampling schedule compromised measurement of pharmacokinetic parameters; drug profiles were removed if $C_{6hr} > C_{2hr}$ and the approximation of C_{2hr} for C_{max} is likely to have been imprecise, particularly in the case of ethambutol where T_{max} may be nearer 3 hours. Additionally, AUC estimates based on 3 data-points and not extrapolated to infinity may under-estimate drug exposure. Plasma concentrations were only measured on a single day and it is possible that treatment adherence and exposure to therapy were not constant at other times. Drugs were assayed in plasma but during clinical infection *M tuberculosis* bacilli are predominantly found in extra-vascular sites where anti-microbial penetration is variable and poorly understood[409]. Some assayed compounds (isoniazid and pyrazinamide) are pro-drugs which require conversion to an active compound. Inability to measure the active compound may confound PK-PD assessment of treatment response.

Yet these limitations did not obscure some important findings. Rifampicin concentrations were low and dose escalation may be required to improve TB control in Malawi. Variability in isoniazid exposure was associated with early and late end-points of treatment response. Anti-microbial drug exposure appeared to influence the metabolic characteristics of persister organisms and future studies of bacillary phenotype (including af-LB counts) should explore this further.

8. General Discussion

8.1 Introduction

This thesis began by describing the urgent need for ultra short first-line chemotherapy in HIV-endemic African countries with a high burden of TB. Advances in drug development over the last decade have generated optimism that a combination of anti-microbial agents may be found to achieve durable TB cure in less than 6 months.

However, two long-standing obstacles continue to thwart rapid clinical assessment of new regimens; the mechanisms underpinning bacillary persistence are incompletely understood, and no surrogate markers of sterilising activity and long-term outcome are validated to predict the long-term efficacy of new treatments from Phase IIb clinical trials.

The project described in Chapters 2-7 was designed to confront these obstacles by modelling bacterial elimination over the first two months of therapy, evaluating methods for the study of persister organisms and assessing surrogate markers of clinical outcome. The study hypotheses are re-stated in Figure 8.1 in advance of a final discussion of how effectively they have been addressed.

Primary Hypothesis:

Pharmacodynamic modelling of bacillary elimination using quantitative SSCC and MGIT culture data will provide reliable surrogate markers of clinical outcome suitable for use in Phase IIb trials of new chemotherapy regimens.

Secondary hypotheses:

a) Non-sputum based assays (e.g. serial measurement of the urinary LAM-ELISA) will provide additional information on clearance of EPTB
b) Single cell examination of bacilli in sputum (e.g. by fluorescence microscopy) will facilitate phenotypic characterisation and monitoring of drug tolerant persister organisms
c) Individual variation in the pharmacokinetic parameters of anti-TB drugs is an important additional determinant of treatment efficacy

Figure 8.1 Review of study hypotheses

8.2 Treatment outcomes and clinical covariates

Testing of all study hypotheses was dependent on prospective observation of a patient cohort from smear positive PTB diagnosis until one year after completion of therapy. A detailed cohort description was provided in Chapter 3. Several aspects of that chapter merit specific emphasis.

Long-term patient follow-up in low-income clinical environments is difficult and there is a lack of longitudinal studies with intensive early sampling and clinical end-points of post-treatment relapse. Studies evaluating quantitative bacteriology techniques as treatment monitoring tools have generally excluded patients with advanced HIV infection[228,266,278,288,290,298], and several have focused on drug resistant disease[297,299]. Thus, the careful assembly of a long-term cohort containing HIV-infected individuals with low baseline CD4 counts, genotypically confirmed DS-TB and detailed early bacteriological data was an important step in the study of bacillary persistence.

The stable cure rate amongst study patients who reached a final end-point was 118/133 (89%), compared to other reports of stable cure in 95% of patients treated with modern drug combinations[102,477]. The large number of unfavourable outcomes was attributed to selective recruitment of individuals with high bacillary loads. However, it was necessary to consider whether treatment failure was influenced by acquisition of resistance mutations during therapy or the end-point of relapse was contaminated by re-infection. Only one patient had MDR TB at EOT, and all relapses occurred within 6 months of initial therapy. Whilst spoligotyping of stored isolates is awaited, it is reasonable to assume that unfavourable outcomes were predominantly due to drug-tolerant bacillary persistence rather than genotypic resistance.

The finding that cavities on baseline CXR were associated with two month sputum culture status is consistent with existing literature[291,357,358], and the association between inability to read and unfavourable outcomes is a reminder that socio-economic issues impact on the results of clinical research. Otherwise, clinical and radiological factors were not predictive of long-term outcome on multivariate analysis. The principal determinants of bacillary persistence are likely to be microbiological and bacteriological measurements were used to measure sterilising drug activity and generate biomarkers of treatment response.

8.3 Pharmacodynamic modelling of SSCC and MGIT data

The primary study hypothesis was framed around quantitative sputum cultures because bacteriological methods currently possess the strongest evidence base of any existing candidate biomarkers[260]. Optimisation of the techniques was outlined in Chapter 4 and the results of pharmacodynamic modelling were described in Chapter 6.

The laboratory method for SSCC was challenging and has only been successfully described on three previous occasions. Difficulties included loss of data when bacillary loads fall below the limit of detection during the first treatment month and excessive contamination of later specimens.

Modification of selective SSCC media reduced late sample contamination but the problem of early culture conversion was hard to overcome. In prior studies only 6% of subjects had detectable colony counts at 60 days[271] and computer simulations indicate that mean colony counts should fall below zero at around 56 days[266]. Intuitively, a technique which is poor at reviving persisters after the first few weeks may seem inadequate to study bacillary persistence and sterilisation phase drug activity.

Nevertheless, when serial positive counts were assessed with NLME statistical techniques a biphasic model of bacillary clearance was successfully fit to the data. The SSCC-NLME model was then refined using a partial likelihood method to estimate colony counts for samples below the limit of detection. Extraction of best unbiased parameter estimates for individual patients from this model revealed that a larger baseline population of persister organisms and a slower sterilisation phase elimination rate were associated with unfavourable outcome. These results are encouraging because they provide empirical data to support Mitchison's theory that distinct bacterial populations are cleared at different rates[160,161] and implicate sterilisation phase bacillary elimination as a predictor of long-term outcome. However, it remains uncertain whether the partial likelihood method is the best statistical technique for handling data below the limit of detection.

A feature of SSCC plate counting not exploited in the current study is that each colony grows from a single *M tuberculosis* organism. This could allow identification of individual CFUs as rapid replicators or slow persisters based on their time of appearance and rate of growth. Investigators working on bacterial persistence in *E coli* have developed ScanLag technology to take multiple photographs of the same culture plates over time and grade

bacterial persistence based on the pattern of colony growth[607]. A similar approach may yield new insights into drug-tolerant persister populations during TB treatment.

Overall, the fragility of the laboratory technique and complexity of methods required for data analysis means that SSCC-NLME methods require further evaluation. Two ongoing Phase IIb studies (NCT 00760149 and NCT01498419) have incorporated SSCC-NLME parameters as their primary end-points and meta-analysis of results from these alongside existing studies will be important to clarify the role of SSCC in future clinical trials.

Serial measurement of MGIT-TTP was the second quantitative bacteriology technique to be assessed. Although there is minimal prior data on validation of this method against long-term end-points, the MGIT-TTP results are the most important findings presented in this thesis. Despite some laboratory issues with serial MGIT sampling (e.g. loss of cording and acid fastness during confirmatory isolate identification) this method achieved better revival of persisters, fewer contaminated samples and a more complete dataset than SSCC. LME modelling of MGIT-TTP results generated the MBER as a parameter with considerable ability to predict long-term outcome.

The MBER possessed useful properties as a surrogate marker of sterilisation. Using positive TTP data only, the odds ratio of an unfavourable outcome for each unit increase in MBER was 0.5 (95% CI: 0.30-0.83, p=0.007) representing a large effect size and strong statistical significance. MBER estimation was not confounded by common clinical or radiological covariates, which will be an advantage for Phase IIb studies with heterogeneous patient cohorts. As with SSCC-NLME modelling, there were issues regarding how best to handle data beyond the limit of detection but three distinct LME approaches (positive data only, imputation of TTP=50 days for the first negative results, and the partial likelihood method) yielded similar results. Use of a linear model meant that the MBER was a simple parameter to interpret.

In keeping with other recent reports[281,288-290] a strong inverse correlation was seen between MGIT-TTP measurements and colony counts from SSCC plates. However, the strength of this relationship weakened over time, because samples with broadly similar $\log_{10}$CFU/ml counts had longer TTP at later study visits. This was taken to imply that the metabolic oxygen consumption of viable bacilli decreases during treatment, supporting the existence of a relationship between metabolic quiescence and drug-tolerant persistence.

With additional resources, the liquid culture protocol used for this study might be improved. Only one MGIT bottle was inoculated per sputum specimen, increasing vulnerability to several sources of error (e.g. inadequate sample homogenisation, unequal NaOH decontamination or variable calibration of the fluorescence reader). Inoculating multiple bottles and taking an average for each sample would have protected against these potential confounders. A manual microMGIT reader was used to assess fluorescence from incubating bottles twice per day. Although parallel incubation of 74 samples showed equivalent TTP results between this method and continuous fluorescence reading in an automated BACTEC MGIT 960 machine, routine use of the automated system may still have improved accuracy. It is unlikely that these protocol modifications would have substantively altered the study conclusions.

Full validation of the MBER as a surrogate outcome marker will require the findings described here to be replicated in high-burden TB countries outside Africa. It cannot be assumed that the results will be the same. Comparison of data from different locations in multi-centre clinical trials reveals that African sites often report greater disparity in time to solid and liquid culture conversion[357], higher 2 month sputum culture positivity[357] and higher post-treatment relapse rates[305] than sites in Asia or South America. It is known that infecting *M tuberculosis* strains differ between populations and strain variation may cause heterogeneity in persistence mechanisms and treatment response. In particular, the Beijing strain has been associated with constitutive up-regulation of the DosR regulon[608], slower culture conversion[291] and early relapse[609]. The epidemiology of infecting TB strains in Malawi is not yet known and, although genetic strain differentiation is planned, studies in diverse settings are needed for the MBER to be globally assessed.

Notwithstanding these reservations, there is good evidence that the primary study hypothesis is true. Whilst aspects of SSCC modelling require refinement, the MBER is a robust parameter which measures bacillary elimination throughout intensive phase TB therapy, predicts long-term outcome and should be considered as a surrogate end-point for Phase IIb studies of new treatment regimens.

8.4 The LAM-ELISA and the problem of EPTB

A proportion of the total bacillary burden in TB infection, particularly in HIV-infected patients, is likely to be extra-pulmonary[3] and not reflected in bacteriological assays on sputum. A sub-study was performed to assess the urinary LAM-ELISA as a means of non-invasively monitoring clearance of EPTB and results were presented in Chapter 6.

This part of the project was limited by sample size; delays in kit availability meant that the LAM-ELISA was only performed on urine from 51 patients. Poor sensitivity of the test in HIV un-infected individuals restricted the analysis to 36 patients with HIV-infection. 20 (55%) of those had a positive baseline LAM-ELISA and were used for serial study.

The LAM-ELISA generally converted from positive to negative between baseline and S4 visits and there was a gradual fall in serial log_{10}OD readings on the plate reader. Insufficient samples were examined to determine whether changes in LAM-ELISA results over time predicted outcome. Stored specimens from the remaining HIV-infected patients are currently being tested but it seems unlikely that, in its current form, this assay will have adequate sensitivity to serve as biomarker of outcome in patients with PTB.

A mitigating factor for the LAM-ELISA may be its ability to detect bacillary populations which are otherwise inaccessible. Diagnostic studies of HIV-infected TB suspects with CD4 <100 cells/µl have shown that the urinary LAM-ELISA is often positive in patients who are sputum smear negative[337,610]. Therefore, whilst the work presented in this thesis has assessed the assay as a tool to find disseminated disease in patients with a predominantly pulmonary illness, it primary function may be to identify patients whose TB is mainly or entirely located outside the lungs. It is currently very difficult to perform treatment trials in patients who cannot expectorate (including children), or who suffer exclusively from EPTB so a more targeted appraisal of the LAM-ELISA in carefully selected patient groups may yield useful results. It should be recalled from Chapter 1 that a proposed mechanisms for bacillary persistence, aside from metabolic heterogeneity, was sequestration of bacilli in sanctuary sites with poor drug penetration[209,210]. From this perspective, ongoing efforts to develop and improve techniques which report on hidden foci of EPTB are extremely important.

At present the secondary study hypothesis that non-sputum based assays can provide additional information on clearance of EPTB remains unproven.

8.5 A potential role for single cell techniques

The most ambitious component of this project was the attempt to develop single cell fluorescence techniques to specifically label persister *M tuberculosis* organisms in sputum. This work was presented in Chapters 5 and 6.

Although Mitchison's proposal of drug tolerant persisters as a phenotypically distinct bacterial population is supported by the fit of the biphasic SSCC-NLME model, no established assay directly identifies persisters or tracks their behaviour during therapy. The benefits of a method for this are clear; important insights into mechanisms of mycobacterial persistence would be revealed and the sterilising effects of new drug regimens could be directly assessed. Fluorescence microscopy and flow cytometry are regularly used to discriminate between properties of individual cells but are difficult to deploy on sputum due to the complexity of extracting bacilli from the biological matrix.

Most success was achieved with ALTR fluorescence microscopy. Metabolic pathways were outlined which may relate accumulation of TAG LBs to bacterial persistence[154,172,173]. Published microscopy and image analysis protocols[175,344] were then modified to quantify the proportion of LB+ organisms in sputum samples from study patients. ALTR microscopy revealed acid-fast and non-acid LB+ cells, highlighting that some TB bacteria in sputum may not be detected by conventional ZN or AP staining techniques. Prior *in vitro* data supports the notion than LB+ bacilli may be drug tolerant[154,168].

Amongst baseline sputum samples, there was an association between higher af-LB and TLB counts and higher TTP/$\log_{10}$CFU ratios from quantitative cultures. This was interpreted as demonstrating that the LB+ phenotype may be metabolically quiescent. Amongst a sub-study of serial sputum samples collected during the first month of TB therapy, there was an slight downward trend in af-LB and TLB counts in patients who went on to have favourable outcomes but an upward trend in patients who went on to have failure or relapse. Patient numbers were too small for this result to achieve statistical significance, but these results collectively support the argument that LB+ organisms are implicated in drug-tolerant persistence.

Further work is required to refine the ALTR techniques and evaluate them in larger patient cohorts. A rate-limiting step for the current study was that LB counts from all images of every microscopy slide were done manually. Collaboration is ongoing with the Department of Physics at the University of Liverpool to customise computer software for automated

image analysis. It is hoped that this will improve the time-efficiency and objective standardisation of data collection[611]. Existing protocols for LB counting dichotomise bacteria into LB+ and LB-, but in reality the number and size of LBs within each cell may vary. With an automated algorithm it may be possible to perform more detailed sub-divisions of phenotype. Extensive exploration of the importance of non-acid fast bacilli will require an alternative means of definitively labelling these organisms as *M tuberculosis*.

Despite the potential benefits of fluorescence microscopy to examine LB+ bacilli during therapy this method is unlikely to ever permit study of putative persisters during the second month of TB therapy because microscopy rarely identifies bacilli in sputum at an organism density less than 10^3 cells/ml[597,598]. It remains to be seen whether a non-culture based method can be found to efficiently label viable bacteria at lower sample concentrations. In the current study, preliminary experiments were done with flow cytometry, which can assess a large volume of fluorescence-labelled specimens very quickly. *M tuberculosis* was successfully differentiated *in vitro* from cocktails of other respiratory micro-organisms but a suitable assay for clinical samples has not yet been developed.

Overall, there is preliminary evidence to support the secondary study hypothesis that single cell examination of bacilli in sputum using ALTR microscopy facilitates phenotypic characterisation and monitoring of drug tolerant persister organisms. However, the ALTR method requires further optimisation and independent evaluation.

8.6 Pharmacokinetic parameters and treatment response

Relationships between clinical covariates, treatment response and pharmacokinetic parameters are examined in Chapter 7 and three points should be highlighted.

Firstly, plasma rifampicin concentrations amongst study patients were low. This was consistent with a growing body of prior pharmacokinetic data[140,400,403,412,414,427] and strengthens the argument that rifamycin dosing should be revised. A fourteen day clinical study from Cape Town in which 98% of patients were HIV-uninfected has recently described safe administration of rifampicin doses up to 35mg/kg with an incremental dose effect on bactericidal activity[612]. To be relevant for TB control in Malawi, it is important that future dose-ranging studies of longer duration also report on HIV-infected individuals.

Secondly, variability in the pharmacokinetics of isoniazid was related to treatment response. Unfavourable final outcomes and positive 2 month sputum cultures were less likely in patients with higher $AUC_{0\text{-}6hr}$. Unfavourable outcomes were also less likely in patients with a higher C_{2hr} and there were relationships between the $AUC_{0\text{-}6hr}$ and bacillary elimination rates from some SSCC and MGIT models. As isoniazid is widely regarded as an ineffective sterilising drug these findings were slightly surprising. However, prior data do describe less successful treatment in patients with lower isoniazid exposure[407] and it is clear that new information can be learned about established drugs from clinical PK-PD studies.

Thirdly, patients with a higher C_{2hr} of isoniazid and/or rifampicin appeared to retain higher proportions of LB+ organisms more slowly on therapy than patients with very low drug concentrations. Although this analysis was exploratory, the fact that af-LB organisms had a greater survival advantage under more strenuous anti-microbial pressure is compatible with the view that LBs are a phenotypic marker of drug tolerance.

Further interrogation of the pharmacokinetic dataset is planned. Isolates of all *M tuberculosis* isolate from the study are stored in Malawi and the MICs of these will be determined to allow incorporation of AUC/MIC ratios into the analysis of each drug. Stored DNA from study patients will be tested for relevant mutations in *NAT2* and *SLCO1B1* genes to establish whether genetic factors explain some of the variability in pharmacokinetic parameters or treatment response.

In anticipation of this additional work, the data presented here provisionally support the secondary hypothesis that individual variation in the pharmacokinetic parameters of anti-TB drugs (particularly isoniazid) is an additional determinant of treatment efficacy.

8.7 Final conclusions

The body of work collected in this thesis has established that mixed effects modelling of quantitative bacteriology data will benefit the clinical development of new chemotherapy regimens against TB In particular, the MBER from LME analysis of MGIT-TTP data was statistical measure of sputum sterilisation mean which could be calculated on the majority of study patients and was strongly predictive of long-term outcome. Further prospective evaluation of this parameter should be undertaken as it may be a useful surrogate end-point for future Phase IIb clinical trials.

Additionally, ALTR microscopy provided novel information on LB accumulation in *M tuberculosis* cells during clinical infection. Phenotypically distinct populations of LB+ and LB- bacilli were demonstrated and exploratory data were presented to support the theory that LB+ bacilli are implicated in drug-tolerant persistence. Whilst the mechanisms driving persistence during TB treatment are likely to be multi-factorial and the ALTR technique requires further optimisation the emergence of a new tool to study metabolic heterogeneity in clinical samples is encouraging.

The last decade has been associated with the greatest advances in TB therapeutics since the 1960s, but ultra-short chemotherapy has not yet been achieved and scientific breakthroughs have not yet impacted on the stubbornly high global burden of disease. The work presented here advances our existing knowledge by identifying candidate bacteriological biomarkers which could genuinely impact on the conduct of future clinical trials, and describing a novel approach to the direct study of bacillary persistence in TB patients.

9. References

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10. Appendices

10.1 COMREC ethical approval letter (P.01/10.855)

UNIVERSITY OF MALAWI

Principal

Prof. R.L. Broadhead, MBBS, FRCP, FRCPCH, DCH

Our Ref.:

Your Ref.: P.01/10/855

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28th April 2010

Dr. Derek Sloan
Wellcome Trust
Blantyre 3

Dear Dr. D. Sloan,

P.01/10/855 – Understanding Mycobacterium Persistence in Sputum during Drug Treatment of Tuberculosis

I write to inform you that COMREC reviewed your proposal mentioned above which you resubmitted. I am pleased to inform you that your proposal was approved after considering that you addressed all the queries which were raised in an earlier review.

As you proceed with the implementation of your study we would like you to take note that all requirements by the college are followed as indicated on the attached page.

Yours Sincerely,

Approved by
College of Medicine
28 APR 2010
(COMREC)
Research and Ethics Committee

Dr. S.Kamiza
For: CHAIRMAN - COMREC

SK/ck

10.2 LSTM ethical approval letter (09.67)

Dr D Sloan
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Wednesday, 14 October 2009

Dear Dr D Sloan

Re: Research Protocol (09.67) Understanding mycobacterial persistence in sputum during treatment for tuberculosis

Thank you for your letter dated 29th September 2009 responding to the points raised by the Research Ethics Committee. The protocol now has formal ethical approval from the Chair of LSTM Research Ethics Committee.

The approval is for a fixed period of three years or for the duration of the grant, renewable annually thereafter. The committee may suspend or withdraw ethical approval at any time if appropriate.

Approval is conditional upon:

- Submission of ethical approval from other ethics committees.
- Notification of all amendments to the protocol for approval before implementation.
- Notification of when the project actually starts.
- Provision of an annual update to the Committee. Failure to do so could result in suspension of the study without further notice.
- Reporting of all severe unexpected Adverse Events to the Committee
- Reporting of new information relevant to patient safety to the Committee
- Provision of Data Monitoring Committee reports (if applicable) to the Committee

Failure to comply with these requirements will result in withdrawal of approval. The Committee would also like to receive copies of the final report once the study is completed.

Yours sincerely

Stephen Gord

Dr Stephen Gordon
Acting Chair, Research Ethics Committee

10.3 NHS Bolton Research Ethics Committee approval letter

Bolton Research Ethics Committee
Room 181, Gateway House
Piccadilly South
Manchester
M60 7LP
Telephone: 0161 237 2585
Facsimile: 0161 237 2

1 June 2009

Private & Confidential

Dr S Khoo,
Reader
University of Liverpool/ Royal Liverpool University Hospital
70 Pembroke Place
Liverpool
L69 3GF

Dear Dr Khoo

Study Title: **Understanding mycobacterial persistence in sputum during treatment for tuberculosis**
REC reference number: **09/H1009/27**
Protocol number: **3**

Thank you for the information and revised documentation provided by Dr Sloan in response to the Committee's request. This has been considered on behalf of the Committee by the Chair and Dr Ong.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

The favourable opinion applies to the following research site(s):

Research Site	Principal Investigator / Local Collaborator
Royal Liverpool and Broadgreen University Hospitals Trust	Dr Derek Sloan

Condition of the favourable opinion

The favourable opinion is subject to the following condition being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Response to Request for Further Information	1	07 May 2009
Participant Consent Form	2	05 May 2009
Participant Information Sheet: Non -TB patients	2	05 May 2009
Participant Information Sheet: TB patients	2	05 May 2009
Participant Consent Form	1	11 March 2009
Letter from Sponsor	1	12 March 2009
Investigator CV		19 March 2009
Application	2.0	19 March 2009
Response to Request for Further Information	2	
Protocol	3	19 May 2009

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

Dr Frank Bowman
Chair

10.4 TB microscopy staining techniques

10.4.1 Routine Ziehl Neelsen (ZN) staining method

Preparing staining reagents

Carbol fuschin: Make solution 1 by dissolving 3g basic fuschin in 100ml 95% ethanol. Make solution 2 by dissolving 50g phenol crystals in 900ml distilled water (gentle heat may be required). Combine 100ml solution 1 with 900ml solution 2. Filter into an amber bottle and store for a maximum of 12 months.

Decolourising agent (3% acid alcohol): Carefully add 3ml concentrated hydrochloric acid to 97ml 70% alcohol (always add acid to alcohol, not vice versa). The mixture will heat up. Transfer to an amber bottle and store for a maximum of 6 months.

Counter-stain (methylene blue): Dissolve 3g methylene blue in 1000ml distilled water. Transfer to an amber bottle and store for a maximum of 12 months.

Staining procedure

1. Prepare smears on microscopy slides with 10µl sputum
2. Fix smears by passing slides, with the smear uppermost, through a flame 3-4 times.
3. Place slides on the staining rack in batches. Do not let slides touch one another.
4. Include a known positive smear slide on every batch or whenever a freshly prepared stain is used.
5. Filter the carbol fuschin solution and flood slides with the stain for 15 minutes.
6. During carbol fuschin staining, heat the slides slowly until they are steaming at 5 minute intervals but do not let the slides boil dry.
7. Rinse each slide individually in a gentle stream of running water until all free stain is washed away.
8. Flood the slides with 3% acid alcohol decolouriser for a maximum of 3 minutes. If carbol fuchsin is retained in the smear, it is considered under-decolourised. Repeat the decolourisation, if necessary for a further 1 minute.
9. Rinse slides thoroughly with distilled water.
10. Flood slides with methylene blue for 30 seconds.
11. Rinse slides thoroughly with distilled water.
12. Allow to air-dry. Do not blot.

Interpretation of ZN microscopy result

1. All slides should be assessed by two independent readers, using a 100x oil immersion lens.
2. Make a series of systematic sweeps across the smear. Move the slide longitudinally after examining a field so that the field to the right can be examined.
3. Examine a minimum of 100 fields before reporting a slide as negative.
4. Wipe the oil immersion objective with lens tissue after examining a positive slide.
5. Store the slides in indexed slide boxes for future reference.

On each slide look for fine red rods, slightly curved, approximately 1-10µm long, more or less granular, isolated, in pairs or groups, standing out clearly against the blue background. Interpret the results as follows:

Number of acid-fast bacilli	Fields	Report
0	Per 100 oil immersion fields	No bacilli observed
1-9	Per 100 oil immersion fields	Scanty (record exact number)
10-99	Per 100 oil immersion fields	1+
1-10	Per field	2+
>10	Per field	3+

Table 10.1 Interpretation of ZN microscopy smears

10.4.2 Routine Auramine Phenol (AP) staining method

Preparing staining reagents

Auramine phenol solution: Make solution 1 by dissolving 1g auramine in 100ml 96% ethanol. Make solution 2 by dissolving 30g phenol crystals in 900ml distilled water. Filter solution 1 into solution 2 and store in a tightly stopped amber bottle away from light and heat for a maximum for 3 months.

Decolourising solution (0.5% acid alcohol): Add 5ml hydrochloric acid to 1000ml ethanol. Transfer to an amber bottle and store for a maximum of 3 months.

Counter-stain (Potassium permanganate [$KMNO_4$]): Dissolve 5g $KMNO_4$ in 1000ml distilled water. Transfer to an amber bottle and store for a maximum of 3 months.

Staining procedure

1. Fix and arrange smears as for ZN microscopy
2. Flood slides with auramine phenol for 15 minutes.

3. Rinse with distilled water (tap water contains chlorine which may interfere with fluorescence.
4. Decolourise with 0.5% acid alcohol for 3 minutes.
5. Rinse with distilled water.
6. Flood with $KMnO_4$ and allow counter-staining for 1 minute. Timing is critical; prolonged counter-staining may quench the fluorescence of acid-fast bacilli.
7. Rinse with distilled water.
8. Allow to air-dry. Do not blot. Read as soon as possible (under x40 objective lens) after staining or store in the dark at 4^{o}C.

Interpretation of AP microscopy results

Examine as for ZN slides with the following exceptions:

a. Examine AP stained smears with a 40x objective lens through a filter for yellow-green fluorescence
b. Examine a minimum of 70 fields before reporting as negative

On the slides; look for rod-shaped or slightly curved bright yellow fluorescence emitting bacilli against a dark background. Interpret the results as follows:

Number of acid-fast bacilli	Fields	Report
0	70	Negative
1-2	70	Doubtful
1-19	70	Scanty (record exact number)
2-18	50	1+
4-36	10	2+
10-90	1	3+

Table 10.2 Intepretation of AP microscopy smears

10.4.3 Optimised Auramine LipidTOX Red (ALTR) staining method

Preparing staining reagents

1mg/ml lipase-dithiothreitol mix: measure 50mg of lipase from *Candid rugosa* (Sigma L1754) into a 50ml Falcon tube, add 3.75ml neat DTT to it and then adding 46.25ml distilled water to make up to 50ml.

Auramine phenol and decolourising (0.5% acid alcohol) solutions: Prepare according to routine AP staining protocol.

LipidTOX Red neutral – defrost a vial from -20^{o}C storage. Make a 1:200 solution in PBS

Staining procedure

1. Fix and arrange smears as for ZN microscopy, using Cellbond slides to standardise the smear area.
2. Flood slides with auramine phenol solution for 15 minutes. Wash slides gently with mycobacteria-free distilled water.
3. Decolourise with 0.5% acid alcohol for 2 minutes and wash gently with mycobacteria-free distilled water
4. Cover smear with 1:200 dilution of LipidTOX red neutral stain (usually need 0.4ml working strength solution per slide) for 20 minutes. Wash gently with mycobacteria-free distilled water
5. Flood slide with $KMNO_4$ and allow counterstaining for 1 minuteiming is critical.
6. Store slides in the dark and read within 24 hours.

Interpretation of ALTR microscopy results

This is outlined in detail in Section 5.3.11. In brief,

1. Examine slides with a x100 oil immersion lens on a fluorescence microscope with a digital camera attachment
2. Before each microscopy session, set camera focus and photography exposure times using pre-specified control samples
3. Blind slides prior to reading
4. Scan slides systematically through a yellow-green (FITC) filter. Photograph each discretely visible auramine labelled bacillus twice – once through the FITC filter and once through a read (TRITC) filter.
5. Continue scanning slides until 100 auramine-labelled bacilli have been photographed or the slide has been read for 15 minutes.
6. Assess bacilli on stored images the presence of lipid bodies (red dots within auramine-labelled bacilli. The af-LB count for each slide is calculated as:

$$\textit{af-LB count (\%)} = 100 \; X \frac{\textit{Total acid-fast LB positive bacilli on all images}}{\textit{Total acid-fast bacilli on all images}}$$

Identification of auramine negative LB+ bacilli is discussed in Chapter 5.

10.5 Preparation and use of Lowenstein Jensen (LJ) media

LJ media preparation

1. Weigh the following ingredients *in order* in a sterile reagent bottle containing the distilled water; Potassium dihydrogen phosphate anhydrous (KH_2PO_4) - 1.2g, Magnesium sulphate ($MgSO_4.7H_2O$) -0.12g, Magnesium citrate – 0.3g, Asparagine – 1.8g, Glycerol – 6ml, Malachite green – 0.3g, Distilled water – 300ml
2. Autoclave at 121^{o}C at 15lbs pressure for 15 minutes
3. Cool to room temperature. This mixture can be kept overnight in the fridge but is best used on the day of preparation.
4. Clean 10 fresh hens' eggs (<7 days old) by scrubbing thoroughly with a hand brush in warm water and a plain alkaline soap
5. Rinse eggs thoroughly in running water and soak them in 70% ethanol or methylated spirits for 15 minutes.
6. Before handling the clean eggs, scrub the hands with methylated spirit,, wash with tap water and rinse with methylated spirit.
7. Crack the eggs with a sterile knife into a sterile beaker and beat them with a sterile glass rod for 5 minutes until they are watery.
8. Place the salt solution into the egg solution to homogenise.
9. Wait for 30 minutes with the mixture covered with sterile foil paper to prevent contamnation. This time allows the malachite green to absorb to the albumin.
10. Check pH (6.8-7.2) by taking a small sample for the mixture into a separate universal bottle and measure the pH using a pH meter. If too acid add sodium hydroxide. If too alkaline add hydrochloric acid.
11. Dispense the complete medium in 8ml volumes into 28ml universal containers.
12. Inspissate the medium within 15 mins of distribution to prevent sedimentation of the heavier ingredients.
13. Place the bottles in a slanted position on the inspissator and coagulate the medium for 60 minutes at 85^{o}C.
14. . After 60 minutes, switch off the inspissator and leave bottles to cool before taking them off the inspissator.
15. Label the LJ bottles with the day of preparation.
16. Store at 2-8^{o}C. For optimal isolation from specimens, LJ medium should not be more than 4 weeks old.

To make specialist LJ media containing Paranitrophenol Benzoic Acid (PNB) for identification of MGIT isolates (as described in Section 4.3.3) make LJ as above but add 500mg PNB dissolved in Dimethylsulphoxide (DMSO) to the salt solution.

LJ media quality control

Media quality deteriorates if coagulation is done at excessive temperature or for too long. Assess as follows:

1. Once media cools to room temperature, check media quality. Discolouration of coagulated media or the appearance of tiny bubbles on the surface of the media indicates a faulty coagulation procedure. Discard poor quality media.
2. Prepare 10-fold dilutions from a 0.5 McFarland standard suspension of H37Rv (neat, 1:10, 1:100, 1:1000 using phosphate buffer pH 6.8). Inoculate 4 drops from each dilution onto a fresh slope from the prepared batch of LJ media and incubate for 2-3 weeks. Confirm growth by weekly checks.
3. After inspissations, incubate a representative sample of culture media at 35-37^{o}C for 24 hours to check sterility. Discard any media growing bacterial colonies.

Inoculation and incubation

1. Discard condensed moisture at the bottom of LJ slopes.
2. With sterile pipette, inoculate using sterile pipettes, 4 drops of sample/isolate onto each slope.
3. Incubate at 37^{o}C in a slanted position with loosened caps for at least 24 hours to ensure even distribution of inoculums and ventilation. Place bottles thereafter in an upright position with caps tightened to avoid contamination.
4. Incubate for 8 weeks, checking for growth every week.

Examination of LJ slopes

1. Examine 24 hours after inoculation to check that liquid has completely evaporated and detect contaminants. Tighten caps to prevent drying out of media.
2. Examine cultures weekly thereafter.
3. Look for rough, crumby, waxy, non-pigmented (cream-coloured) or yellow pigmented colonies appearing 3-8 weeks after inoculation.
4. Confirm growth macroscopically and by microscopic examination of colonies with ZN stain
5. Include a known positive sample on every new culture batch.

10.6 Storing *M tuberculosis* isolates in 30% glycerol

Preparing storage media

1. Add 30ml glycerol to 70ml distilled water

2. Autoclave at 121^{o}C for 15 minutes

3. Store at 2-8^{o}C for maximum 6 months

NB. Isolates can also use tryptone soya broth: add. 3g tryptone soya broth (Oxoid, Basingstoke, UK) to 20ml glycerol and 80ml distilled water

Quality control checks for storage media:

1. Distribute 1ml of each type of storage media into 2ml cryotubes.

2. Suspend a colony of *M tuberculosis* from a re-subbed slope less than 3 weeks old.

3. Refrigerate cryotubes at -20^{o}C for 3 weeks.

4. Re-inoculate the preserved cultures onto LJ slopes and incubate for up to 3 week.

5. Check for growth every week – growth of TB colonies shows media of good quality. Discard any media showing sterility.