

DETERMINANTS OF TUBERCULOSIS TREATMENT OUTCOMES

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I dedicate this thesis to the Daniels out there- who persevere with a spirit of excellence.

Philippians 3:13-14

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DECLARATION

Apart from the assistance I have outlined in the acknowledgement section, the work described in this thesis is my own, with everything else being appropriately referenced. The work in this thesis has not been previously submitted for a degree or qualification at this or any other university.

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ABSTRACT

Background: Despite 6 months of standardised tuberculosis treatment, unsuccessful treatment outcomes (acquired drug resistance, failure, relapse) may occur in a subset of patients with drug-susceptible tuberculosis. The aim of this thesis was to study determinants of treatment outcomes in rifampicin susceptible pulmonary tuberculosis in a setting with a high prevalence of HIV-1 co-infection.

Methods: A prospective cohort study was carried out to determine the frequency and determinants of acquired drug resistance. The minimum inhibitory concentration (MIC) of sputum TB isolates was determined in a sub-set (BACTEC 960 system). The clinical utility of MTBDR*plus* performed directly on 2-month sputum for treatment monitoring was assessed. Expression of 12 MTB-specific messenger (m) and small (s) RNAs was quantified in RNA extracted from sputum before tuberculosis treatment and compared to expression in exponential and stationary phase H37Rv cultures.

Intensive pharmacokinetic studies were carried out for rifampicin, isoniazid and pyrazinamide and non-linear mixed effects modeling was used to derive individual pharmacokinetic parameters. Multivariate logistic regression and multivariate adaptive regression splines (MARS) analyses were used to evaluate utility of pharmacokinetic/pharmacodynamics measures ($AUC_{0-24} : MIC$, $C_{max} : MIC$, % time above MIC) to predict 2-month culture conversion.

A panel of analytes measured by ELISA and Luminex in plasma/serum taken pre-treatment and at 2 and 5-6 months on treatment was evaluated to determine correlates of HIV-1 co-infection, disease severity and unsuccessful outcomes.

Results: 0.3- 1% of patients treated for rifampicin susceptible-tuberculosis (n=306) acquired drug resistance during or subsequent to treatment. None of the 17 cases of isoniazid monoresistance had unsuccessful outcomes. There were no differences in baseline MIC profiles (n=109) when analysed by retreatment, 2-month culture conversion or HIV-1 status and there was no evidence of increase in MIC during treatment comparing baseline and 2-month isolates (n=20).

Direct MTBDR*plus* (n=289) on 2-month sputa had a sensitivity of 78%(95%CI 65-87) and specificity of 80%(95%CI 74-84) for predicting culture conversion with a negative

predictive value of 93%(95%CI 89-96). This was in comparison to a gold standard of MTBDR $plus$ on positive 2-month cultures.

The transcriptomic signature (here limited to 11 selected mRNAs/sRNAs) of sputum (n=19) more closely resembled H37Rv in exponential phase culture than stationary phase culture. There was evidence of biological variation and a trend towards an increased *hspX*^{hi} *atpA*^{lo} *nuoG*^{lo} signal in the sub-group who were culture positive at 2 months.

Ambulant HIV-1-tuberculosis co-infected patients (n=100), the majority of whom were co-prescribed ART, did not have reduced anti-tuberculosis drug concentrations compared with HIV-1 uninfected tuberculosis patients. No relationship was found between pharmacokinetic exposures and 2-month culture conversion using logistic regression, after adjusting for MIC and clinical factors. However, MARS identified negative interactions between isoniazid C_{max} and rifampicin C_{max}/MIC ratio on 2-month culture conversion. If isoniazid C_{max} was below 4.6 mg/L and rifampicin C_{max} /MIC below 28, the isoniazid concentration had an antagonistic effect on culture conversion. For patients with isoniazid C_{max}>4.6 mg/L, higher isoniazid exposures were associated with improved rates of culture conversion.

We found significant differences in the majority of blood analytes over the course of treatment (n=133). Within the HIV-1 co-infected sub-group, there were marked differences comparing subjects with and without advanced immunosuppression and with and without virological suppression. We found an association between Type 1 interferon response, neutrophil-associated cytokines (IL-17 and IL-8), neutrophil-associated MMPs (MMP-8 and MMP-9) and the presence of cavitary disease. On multivariate analyses, receiver operator curve-derived thresholds at baseline for IL-8, IL-6, IL-1RA, MMP-3 and ferritin and thresholds for IL-8, IL-6, IL-1RA, MMP-3, MMP-8, TIMP-1, TIMP-3, GM-CSF and VEGF at 2 months were able to predict unfavourable outcomes with reasonable performance characteristics.

Conclusion: Although rare, in this high burden setting, acquired drug resistance does contribute to the growing drug-resistant tuberculosis epidemic. Emphasis should be on prevention of transmission to others. Ambulant HIV-1 co-infected patients, the majority of whom were co-prescribed antiretroviral therapy, did not have reduced anti-tuberculosis drug concentrations compared with HIV-1 uninfected patients. The majority of patients had plasma drug exposures well below accepted thresholds, but nevertheless had good treatment outcomes. Further clinical studies are required to investigate the potential role

of nonlinear pharmacokinetic/pharmacodynamic relationships such as concentration-dependent antagonism. A deeper understanding of host and mycobacterial factors influencing treatment outcomes is required in the search for biomarkers and surrogate endpoints predicting long-term outcomes.

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List of Abbreviations

8OH-dG	8-hydroxy-2'-deoxyguanosine
ADR	Acquired drug resistance
ANOVA	Analysis of variance
ART	Antiretroviral therapy
ATP	Adenosine triphosphate
ATT	Antituberculosis treatment
AUC ₀₋₂₄	Area under the curve over 24 hours
BF	Basis functions
BLQ	Below level of quantification
CART	Classification and regression trees
CI	Confidence interval
CL	Clearance
C _{max}	Maximum concentration
CRP	C reactive protein
Ct	Cycle threshold
CTCAE	Common Terminology Criteria for Adverse Events
DNA	Deoxyribonucleic acid
DOT	Directly observed therapy
DR TB	Drug resistant tuberculosis
DS TB	Drug susceptible tuberculosis
DST	Drug susceptibility testing
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbant assay
EMB	Ethambutol
F	Bioavailability
FDC	Fixed dose combination
FFM	Fat free mass
GM-CSF	Granulocyte macrophage colony stimulating factor
GOI	Gene of interest
HO-1	Haemoxygenase-1
HIV-1	Human immunodeficiency virus-1

IL	Interleukin
IL-1RA	Interleukin-1 receptor antagonist
IMR	Isoniazid monoresistance
IFN	Interferon
IQR	Interquartile range
INH	Isoniazid
Ka	Absorption constant
LC-MS	Liquid chromatography mass spectrometry
LJ	Lowenstein Jensen
LPA	Line probe assay
MCC	Methyl citrate cycle
MDR	Multi-drug resistant
MGIT	Mycobacterial indicator growth tube
MIC	Minimum inhibitory concentration
MMP	Matrix metalloproteinase
mRNA	messenger ribonucleic acid
MTB	<i>Mycobacterium tuberculosis</i>
MDTE _x	Mean deviation from exponential phase culture
MDTStat	Mean deviation from stationary phase culture
NAT	N-acetyltransferase-2
OFV	Objective function value
OR	Odds ratio
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Pharmacodynamic
PK	Pharmacokinetic
PZA	Pyrazinamide
qPCR	quantitative polymerase chain reaction
RCT	Randomized controlled trial
RIF	Rifampicin
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
rRNA	Ribosomal ribonucleic acid
RR	Risk ratio

RS	Rifampicin susceptible
SAP	Serum amyloid P
SAT	Self administered therapy
sCD14	Soluble CD14
SNP	Single nucleotide polymorphism
sRNA	small ribonucleic acid
TB	Tuberculosis
TIMP	Tissue inhibitor of metalloproteinase
%T _{MIC}	% time above MIC in a dosing interval
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
TTP	Time to culture positivity
T2DM	Type 2 Diabetes Mellitus
V	Volume of distribution
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
XDR	Extensively drug resistant

1. CHAPTER 1- Introduction

1.1. Overview of tuberculosis as a global health problem

Mycobacterium tuberculosis (MTB), the causative agent of tuberculosis (TB), is responsible for causing disease in humans for thousands of years (1) and remains a leading cause of morbidity and mortality today. As late as the early 1940s, treatment for TB was limited to optimized hygiene and nutrition, convalescence in sanatoria and in some cases, surgical lung collapse (2). The advent of single and then multi-drug therapy in the 1940s heralded new hope but was quickly followed by evolution of drug resistant MTB strains (3). Multi drug resistant (MDR)-TB has been described since the late 1980s. MDR TB is defined by the WHO as resistance to the first line drugs rifampicin and isoniazid. These drugs comprise the cornerstone of the first line TB regimen due to their potent bacteriocidal activity against MTB (4). Hence, loss of efficacy of these drugs significantly compromises a regimen and necessitates addition of multiple other second line antituberculous drugs. The global human immunodeficiency virus-1 (HIV-1) pandemic has increased both TB incidence and TB related deaths, particularly in Africa (5). In 2014, the World Health Organization (WHO) estimated there were 9.6 million incident TB cases (12% HIV-1 co-infected) and 1.5 million TB deaths (27% HIV-1 co-infected)(6). Sub-Saharan Africa accounts for 80% of the global burden of HIV-1-TB co-infection and the majority of resulting deaths.

In 2014, there was an estimated 480,000 MDR TB cases, of whom approximately 9.7% had additional resistance to fluoroquinolones and aminoglycosides (extensively drug resistant (XDR)-TB) (6). More than half the global burden of drug resistant TB is in China, India and the Russian Federation. Over 50% of all drug resistant TB are in new diagnoses who have never previously received TB treatment i.e. primary drug resistance. Globally, 5% of TB cases are estimated to have MDR TB (3.5% of new cases and 20.5% of previously treated TB cases) (7). Rates of any isoniazid resistance vary widely between countries from 2.6% to 35.2% (the latter is in former Russian Federation states) and is subject to sampling bias and varying proportions of surveillance of new and retreatment cases (8).

Drug-resistant TB (inclusive of baseline isoniazid resistance, MDR TB and XDR TB), is associated with lower cure and completion rates than for drug susceptible TB (DS) TB, with higher rates of acquired drug resistance, relapse and mortality (9, 10) and there is a huge cost to health systems (11). The WHO has prioritized addressing of the global drug resistant (DR) TB crisis via a 5 action plan:

- 1) Prevention of development of drug resistance via quality treatment of drug-susceptible TB.
- 2) Rapid testing and detection of cases of drug resistance.
- 3) Timely access to effective treatment and proper care.
- 4) Prevent transmitted drug resistance through infection control. Particular focus should be on settings such as prisons and in health care facilities.
- 5) Increased political commitment and financing.

The EXPAND-TB project has relied heavily on the roll-out of the rapid diagnostic test , GeneXpert® MTB/RIF, to simultaneously detect TB and rifampicin resistance. By 2014, a total of 3269 GeneXpert machines had been procured in the public sector in 108 countries eligible of concessional pricing (7). However, GeneXpert® MTB/RIF does not diagnose baseline isoniazid resistance.

There has also been a great focus on the amalgamation and streamlining of HIV-1 and TB services at a programmatic level, leading to intensified case finding and earlier diagnosis, earlier commencement of TB and antiretroviral therapy (ART) treatment and subsequent improvement in treatment outcomes and future TB prevention (12) .

1.2. Overview of acquired drug resistance in rifampicin susceptible tuberculosis

Whilst transmitted drug resistance has been highlighted as important in fuelling the spread of the MDR TB epidemic (13), a better understanding of what factors contribute to the initial emergence of resistance is needed to inform policy. Acquired drug resistance (ADR) is the development, fixation and amplification of mutations conferring resistance under drug pressure during treatment. The rate of stochastic ADR has been calculated to be in the order of 1 in 2.25×10^{-10} mutations per bacterium per generation for rifampicin and 1 in 2.56×10^{-8} mutations per bacterium per generation for isoniazid (14) within the human host.

Upon the background of this natural evolution of resistance, there are host and pathogen-based and programmatic factors which contribute to ADR and are summarized in figure 1.1. The relative significance of these determinants of ADR are debatable as the literature to date is contrasting. Studies in the literature reporting factors associated with ADR vary by geographical population and MTB lineage, HIV-1 co-infection rates, the proportion being retreated for TB, TB treatment regimens given (dosing frequency, formulation, length of treatment, number of drugs in intensive and continuation phase) and programmatic factors such as directly observed therapy versus self-administered therapy, dispensing of non-fixed dose combinations and the effect of incarceration.

Challenges in maintaining drug supplies and ensuring patient adherence and treatment completion have contributed to the global MDR epidemic, possibly through creating the selective pressure necessary for ADR to emerge. There is no standardized means of measuring adherence and the measure chosen (review of treatment cards, pill counts, medication event monitoring system (MEMS), pharmacy returns and measurement of drug urinary metabolites) depends on the setting, burden of disease, infrastructure and resources available. A recent controversial study of ADR in a hollow fibre model system has questioned the conventional notion that poor adherence accounts for the majority of ADR (15). The authors found that a threshold of up to 60% of missed treatment doses was compatible with treatment success. However, these findings need to be verified in clinical cohorts.

Studies to date, appear discrepant when comparing HIV-1 co-infection as a risk factor for ADR, stratified by high income and low income countries. There is an increased risk of ADR (both in drug-susceptible and MDR TB) in HIV-1 co-infected patients reported in

USA populations (16, 17). This does not appear to be generalizable to nations with a much higher proportion of HIV-1 co-infection in the general population (18). Several plausible explanations as to how HIV-1 could predispose to ADR have been proposed including a higher overall baseline bacterial load, malabsorption of antitubercular drugs (19) and host immunosuppression leading to tolerance of strain-specific polymorphisms in the pathway to drug resistance (20). However, whether HIV-1 co-infection is indeed a risk factor for ADR remains to be clarified. This is particularly pertinent in the context of current guidelines which support the earlier commencement of antiretroviral therapy in HIV-1 co-infected patients, irrespective of CD4 lymphocyte count.

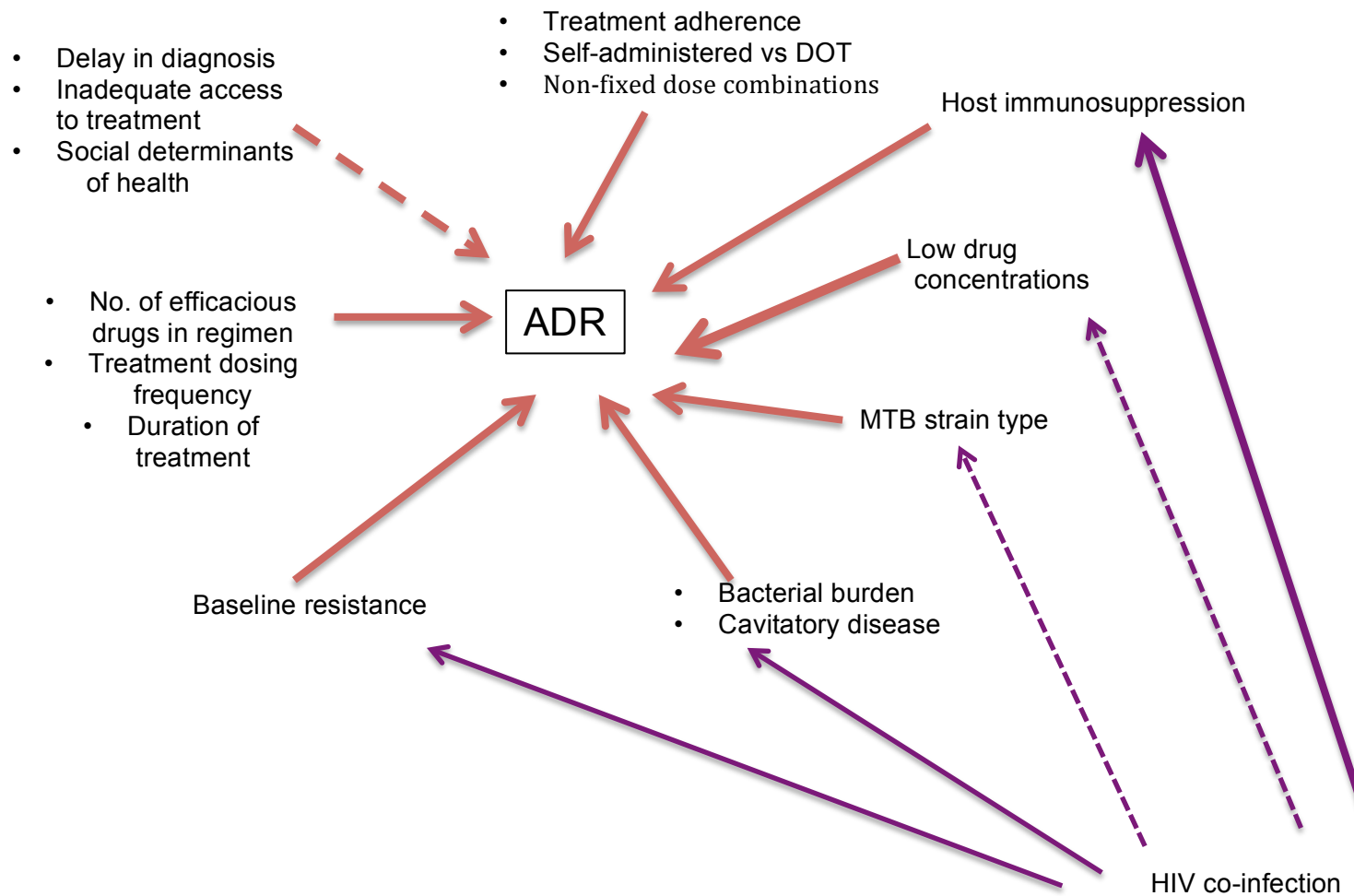
Verification of true ADR requires ruling out initial dual mixed infection or subsequent exogenous re-infection with a drug resistant strain of MTB. Many of the studies to date are retrospective and have not collected this information.

For all the reasons outlined above, estimates of the frequency of ADR vary widely, exacerbated by missing follow up culture and drug susceptibility testing (DST) results, and incomplete MTB strain genotyping to ascertain true ADR (21).

Diagnosis of ADR is an evolving field. Although culture-based phenotypic drug susceptibility is the current gold standard, molecular diagnostic assays such as the polymerase chain reaction-based line probe assay GenoType MTBDR*plus* may have utility in expediting early diagnosis of ADR.

The most significant risk factor for amplification of drug resistance in patients with baseline MDR TB and also the determinant of treatment outcomes in these patients, is the number of efficacious drugs included the combination treatment regimen (22, 23). As standard treatment (2 months of daily quadruple rifampicin/isoniazid/pyrazinamide/ethambutol followed by 4 months of daily dual rifampicin/isoniazid) for rifampicin susceptible (RS) TB is associated with good treatment outcomes in the vast majority, the key risk factors for ADR in RS TB need to be considered independently to those for DR TB.

Figure 1.1 Determinants of acquired drug resistance



The dashed lines represent a potential relationship

DOT Directly observed therapy

1.3. Monitoring and determinants of treatment response

Currently endorsed treatment regimens vary in duration between 6 months in DS TB to 24 months in drug resistant (DR)-TB.

In DS TB, reported successful treatment outcomes (culture/smear negative in the last month of treatment or completion of treatment without evidence of failure) have been as high as 95%, with an overall global reported success rate of 86% in new TB cases (24). Rates of relapse in DS TB have been reported to be approximately 5% (25). Hence, studies incorporating inclusion of large numbers of participants at high costs would be required to adequately assess predictors of unsuccessful treatment outcome [defined as a) smear or culture positivity in the last month of treatment (failure), b) relapse independent of re-infection post successful treatment or c) acquisition of drug resistance during treatment]. Consequently, interim treatment outcomes such as culture conversion by 2 months of treatment and time to culture conversion (26-28) have been used as surrogates of treatment outcomes. However, the data from randomized controlled studies (RCTs) trialing fluoroquinolone-inclusive regimens caution that these may be imprecise biomarkers of long term treatment outcomes as illustrated by the high rates of relapse in fluoroquinolone-inclusive treatment shortening regimens which were brought forward to phase 3 clinical studies based upon promising 2-month culture conversion data (29).

Currently, treatment response in a clinical setting is predominantly monitored by assessment of clinical parameters, radiological and bacteriological measures. Drug side effects from anti-TB treatment, along with overlapping toxicity from ART +/- septrin prophylaxis (in the context of HIV-1 co-infection) may influence adherence and lead to discontinuation of treatment. Blood tests are performed in some settings to monitor normalization of inflammatory markers and to monitor drug-induced toxicity and paradoxical reactions.

TB treatment is believed to follow a biphasic response to chemotherapy with the majority of bacilli being rapidly killed early on in the bactericidal phase whilst the sterilising phase slowly results in eradication of persisting MTB that reduces risk of relapse. Hence, in the treatment of TB, there is likely over-treatment of some patients to prevent failure/relapse secondary to bacterial persisters in a minority. Further understanding of this mechanism

of persistence, despite prolonged multi-drug therapy is key to development of alternative and potentially treatment shortening regimens.

Prior to and during treatment, interaction with host and microenvironment, may generate bacillary subpopulations with genotypic and phenotypic diversity (30, 31). There are multiple sources of genotoxic stress between infection, replication in host and transmission including nutrient deprivation, nitrosative, oxidative and hypoxic stress, phagosomal acidification and antimycobacterial chemotherapy (32). These subpopulations may be anatomically sequestered and thereby escape immune surveillance and drug penetration (33). Expecterated sputum, samples bacilli from the alveoli and bronchi. These bacilli are in a metabolic state which potentially then infect new hosts. Hence, there has been interest in using MTB ribosomal (r), messenger (m) and small (s) ribonucleic acid (RNA) from sputum expecterated prior to and during chemotherapy to shed light on mycobacterial adaptation to its environment. In a clinically well-characterised cohort, by assessing differences in transcriptional signature in individuals at baseline, it may be possible to identify pre-ponderance of bacillary subpopulations and hence potentially identify early biomarkers of treatment response (34).

Advancing immunosuppression in HIV-1 disease leads to functional disruption and decreased responsiveness within the microenvironment of the granuloma that can be accompanied by increased bacterial replication (35). Mediators of tissue extracellular matrix destruction, fibrosis and remodeling such as matrix metalloproteinases are also down regulated leading to less tissue necrosis and cavitary disease(36). Hence, it is important to appreciate trends and correlations of potential blood biomarkers, stratified by HIV-1 serostatus, across a spectrum disease severity, during the course of anti-TB treatment and to recognize changes specific to advanced immunosuppression in the HIV-1 co-infected host. We need blood biomarkers that reflect these tissue level phenomena and thereby also reflect disease susceptibility, severity and treatment response.

In some settings, therapeutic drug monitoring is used (37), particularly where malabsorption and slow therapeutic response is suspected. Although advanced immunosuppression has been identified as a risk factor for inadequate drug concentrations (19), now that expedited commencement of antiretroviral therapy is recommended in patients with HIV1-associated TB, it remains to be established if HIV-1

co-infected patients on ART are at risk of significantly lower drug concentrations than HIV-1 uninfected TB patients. Whilst the induction of cytochrome P450 enzymes and P-glycoprotein by rifampin results in reduced concentrations of nonnucleoside reverse-transcriptase inhibitors and in some protease inhibitors, it is conventionally accepted the ART has negligible effect on the pharmacokinetic profile of first line anti-TB drugs (38). Pharmacogenomics may also have a bearing on population pharmacokinetics and ensuing treatment efficacy and potentially toxicity. An example would be the proportion of the population having a slow N-acetylase 2 status, which could significantly affect isoniazid drug concentrations.

The relationship between bacterial growth and different antibiotic concentrations can be obtained from a single pharmacodynamic (PD) parameter, the minimum inhibitory concentration (MIC). In the context of MTB, this is the lowest of a series of drug dilutions which will prevent growth of over 1% (over 10% for pyrazinamide) of bacilli after incubation with a standardised inoculum *in vitro*. The pharmacokinetic (PK)-PD parameter which best predicts microbial kill in hollow fibre models for isoniazid, rifampicin and pyrazinamide is the ratio of area under the PK curve over 24 hours ($AUC_{0-24hrs}$) to minimum inhibitory concentration (MIC) of the strain (39, 40). The use of normal ranges of peak concentrations (C_{max}) that have been determined in health volunteers to guide response to treatment is a subject of controversy with conflicting data in the literature (41, 42).

The use of therapeutic drug monitoring to predict treatment outcomes is contentious. The literature to date on pharmacokinetic-pharmacodynamic parameters and treatment outcomes varies in the proportion of HIV-1 co-infection in study populations, the drug concentration sampling schedules used, the access to MIC data of the infecting strain and the access to pharmacokinetic data in an appropriate comparator group.

In summary, there is a need for research assessing the frequency of ADR in a HIV-1 co-prevalent population where patients are commenced on treatment for DS TB based upon a rifampicin susceptible GeneXpert® MTB/RIF result and in whom baseline isoniazid monoresistance would be undiagnosed in the majority of cases. We carried out a prospective cohort study to determine this and to ascertain whether poor adherence, HIV-1 co-infection and baseline drug resistance, including isoniazid monoresistance were significant risk factors.

We also sought to use assess whether currently widely utilized molecular diagnostics could be used to screen for ADR and to monitor treatment response.

Within the framework of our prospective cohort study, we aimed to study host and pathogen-based determinants of treatment outcomes.

In order to study host determinants of the interim outcome of 2-month culture conversion and long term outcomes of failure/relapse in this cohort, we carried out intensive PK studies and tested a panel of potential plasma/serum bioanalytes in nested sub-studies.

To examine pathogen determinants of 2-month culture conversion, we examined MIC data for the infecting MTB strains and a selected panel of small/messenger RNAs extracted from sputum in a sub-cohort of patients.

All analyses performed, factored in clinical meta-data collected prospectively from the cohort, to stratify by potential markers of disease severity and explore potential confounding.

1.4. Thesis aims

1.4.1. Overall thesis aims:

To evaluate bacteriological, pharmacological and host determinants of treatment outcomes (failure, relapse, acquired drug resistance) in rifampicin susceptible (RS) TB in a programmatic setting with a high burden of HIV-1 co-infection.

Specific aims:

1. Conduct a systematic review on the risk factors for acquired drug resistance in RS TB (chapter 2).
2. Determine the frequency and risk factors/mechanisms for acquired drug resistance in RS TB in a programmatic setting with a high prevalence of HIV-1 co-infection (chapter 4).
3. To assess the clinical utility of *MTBDRplus* directly on 2-month sputum, a hitherto unreported test/potential biomarker, in monitoring treatment response (this included 2-month culture conversion, the composite of failure/recurrence/death during treatment and acquired drug resistance) (chapter 5).
4. To look for evidence of biological variation in expression of 12 selected MTB-specific messenger (m) and small (s) RNA in sputum pre-treatment and to compare expression stratified by 2-month culture converter status, thereby exploring potential bacillary adaptation mechanisms responsible for delayed sputum clearance (chapter 6).
5. To assess if HIV-1 co-infected ambulatory patients, predominantly on ART and being dosed according to WHO weight bands, have lower plasma drug concentrations of rifampicin, isoniazid and pyrazinamide than HIV-1 uninfected TB patients (chapter 7).
6. To assess if PK/PD parameters, in combination with relevant clinical covariates, are important predictors of 2-month culture conversion and to assess clinical utility of currently recommended thresholds (chapter 8).
7. To assess if selected analytes in plasma/serum prior to or during chemotherapy correlate with HIV-1 co-infection, level of immunosuppression, virological suppression on ART and radiological disease severity and whether these can be used as potential biomarkers to predict treatment outcomes (chapter 9).

1.4.2. Specific chapter aims:

Chapter 1 aim:

- To give an overview of TB as a global health problem and a background to the bacteriological, host and programmatic determinants of treatment outcomes and current challenges in treatment monitoring.

Chapter 2 aim:

- To carry out a systematic review and meta-analysis on the risk factors for acquired rifamycin and /or isoniazid resistance.

Chapter 3 aim:

- To describe recruitment and follow up of participants in the prospective cohort study and the materials and methods used in the thesis.

Chapter 4 aim:

- To evaluate the frequency and determinants of ADR in RS TB in a programmatic setting with a high prevalence of HIV-1 co-infection.

Chapter 5 aim:

- To determine the clinical utility of MTBDR*plus* carried out directly on a 2-month sputum specimen in treatment monitoring.

Chapter 6 aim:

- To study expression patterns of selected mRNAs and sRNAs in sputum pre-treatment, to compare with expression patterns in exponential and stationary phase H37Rv cultures and to stratify by culture converter status and strain type.

Chapter 7 aim:

- To use non-linear mixed effects modeling to determine C_{max} in plasma drug concentrations of rifampicin, isoniazid and pyrazinamide are lower in ambulant HIV-1 co-infected subjects, predominantly on ART.

Chapter 8 aim:

- To determine whether PK/PD indices C_{max}/MIC , AUC/MIC , $\%T_{MIC}$ predict 2-month culture conversion.

Chapter 9 aim:

- To identify changes in longitudinal expression of selected blood analytes during chemotherapy, to assess blood analytes as correlates of disease severity and their utility in predicting long term outcome (cure/completion vs. failure/relapse).

Chapter 10 aim:

- To draw overall conclusions, define the contribution of thesis to the field of tuberculosis research and identify future research priorities.

2. CHAPTER 2- Risk factors for acquired rifamycin and /or isoniazid resistance- a systematic review and meta-analysis

There is no review in the literature which systematically consolidates the multifactorial risk factors for acquired rifamycin and/or isoniazid resistance. Hence, the level of heterogeneity and generalisability of the data has not been assessed. For example, the literature to date includes varying study types, geographical populations, proportions HIV-1 co-infection and retreatment status, dosing frequency and treatment duration. There are also non-uniform methodologies, including varying frequency and mode of drug sensitivity testing, ascertainment of true ADR and differences in the denominator used for calculation of ADR incidence. The objective of this review was to consolidate and critique evidence from studies that examined any risk factors for acquired rifamycin and/or isoniazid resistance in patients undergoing antitubercular therapy containing a rifamycin at least during the intensive phase.

2.1. Methods

We followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. We registered the review in PROSPERO (crd.york.ac.uk CRD42014003856).

2.1.1. Selection criteria

We included case-control and cohort studies and randomised controlled trials (RCTs) reporting ADR as either a primary or secondary adverse outcome. To be included, studies had to examine the association of at least 1 risk factor with ADR. Also, patients of any age needed to be on regimens of at least 6 months' duration which contained a rifamycin at least in the intensive phase. We excluded studies which defined ADR as cases of baseline resistance in patients undergoing retreatment for TB. Studies which reported no cases of ADR were excluded. We did not limit our case definition of ADR to studies that had ruled out exogenous re-infection or initial dual mixed infection with different strains using genotypic methods. However, where the data was available, we excluded cases identified as exogenous re-infection via genotyping. Although we collected data on baseline drug sensitivities, the performance of drug susceptibility testing (DST) at baseline in the entire cohort was not required for inclusion. This allowed for inclusion of studies from settings where baseline DST was not routinely performed, but our analyses focused on those

patients in the cohort who did have baseline DST. ADR was defined as identification of new resistance (compared with a baseline isolate of known DST) to rifamycin and/or isoniazid which was made after a minimum of 2 weeks on TB treatment or after completion of TB treatment.

2.1.2. Search strategy

Searches were run in Pubmed/MEDLINE, EMBASE, Cochrane Library, Web of Science, Biosis previews and the Trip Database from 1950 to January 2014. In Pubmed, filters were used to select the following languages: Chinese; English; Italian; Russian; Spanish; French. Our keywords were 'tuberculosis' or '*Mycobacterium tuberculosis*' AND 'ADR' OR 'amplified drug resistance.' We hand-searched reference lists of reviews and eligible papers for other relevant articles in English.

2.1.3. Study selection, data extraction and quality assessment

Two reviewers (Neesha Rockwood, Graeme Meintjes) independently assessed the titles and abstracts of studies from the searches based on pre-specified eligibility criteria. If it was unclear from the abstract whether inclusion were met, the full article was reviewed. Any uncertainty or disagreement about eligibility was resolved through discussion.

The two reviewers then independently extracted data using a structured data extraction form. Any disagreements were discussed. Critical appraisal tools, developed in the Critical Appraisal Skills Programme for judging methodological quality of RCTs, cohort and case control studies, were amalgamated and used to judge methodological quality (43).

2.1.4. Data synthesis

Risk factors for ADR were tabulated for all studies. If univariate or multivariate analyses were performed, then only if there was a significant positive association with ADR was the factor categorised as 'risk factor for ADR'. If no statistical analysis was performed but a risk factor for ADR was described in the study, it was reported as per trend noted in the study (i.e. increased risk vs decreased risk vs no difference). After conducting the systematic review, a post-hoc decision was taken to carry out 2 separate meta-analyses focused on: 1) HIV-1 co-infection 2) baseline drug resistance as risk factors for the binary outcome of ADR. In conducting the meta-analyses, where specific denominator data could not be deciphered from the paper in stratifying proportion of ADR vs no ADR according to 1) HIV-1 status 2)

baseline drug resistance status, authors were contacted for this missing data. Random effects meta-analyses with Mantel-Haenszel weighting were performed for the covariates baseline drug resistance and HIV-1 co-infection for the binary outcome of ADR using the Cochrane Collaboration Review Manager Version 5.3 statistical software. We calculated risk ratios (RR) and their corresponding 95% confidence intervals (CI) and p-values. Heterogeneity between studies was assessed by calculating the I^2 statistic and its corresponding 95% CI using Stata version 13.1. As a secondary outcome, the cumulative incidence of acquired isoniazid, rifamycin and MDR was reported for individual studies. Patients with known baseline MDR were excluded from calculations. When feasible, the incidence of ADR was calculated using the following denominators: 1) as a proportion of the whole cohort, 2) as a proportion of those with follow up DST, 3) as a proportion of those with baseline pansusceptibility, 4) as a proportion of those with baseline monoresistance and 5) as a proportion of those with baseline polyresistance [resistance to >1 drug (this included streptomycin, ethambutol and pyrazinamide) but with no resistance to both isoniazid and rifampicin). The distribution of cumulative ADR incidence was non-parametric and hence the median cumulative acquired isoniazid, acquired rifamycin and acquired MDR incidence (with 5th to 95th percentile) across all studies that reported these was also calculated, weighted by the overall sample size of each study.

2.2. Results

2.2.1. Study selection and assessment

We identified 798 citations through the electronic database searches: 703 were excluded after abstract review. Another 26 studies were identified through reference review. One hundred full text articles were examined and 32 deemed eligible (6 RCTs, 8 prospective cohort, 15 retrospective cohort and 3 case control studies) (Figure 2.1).

Figure 2.1 Summary of literature search and study selection

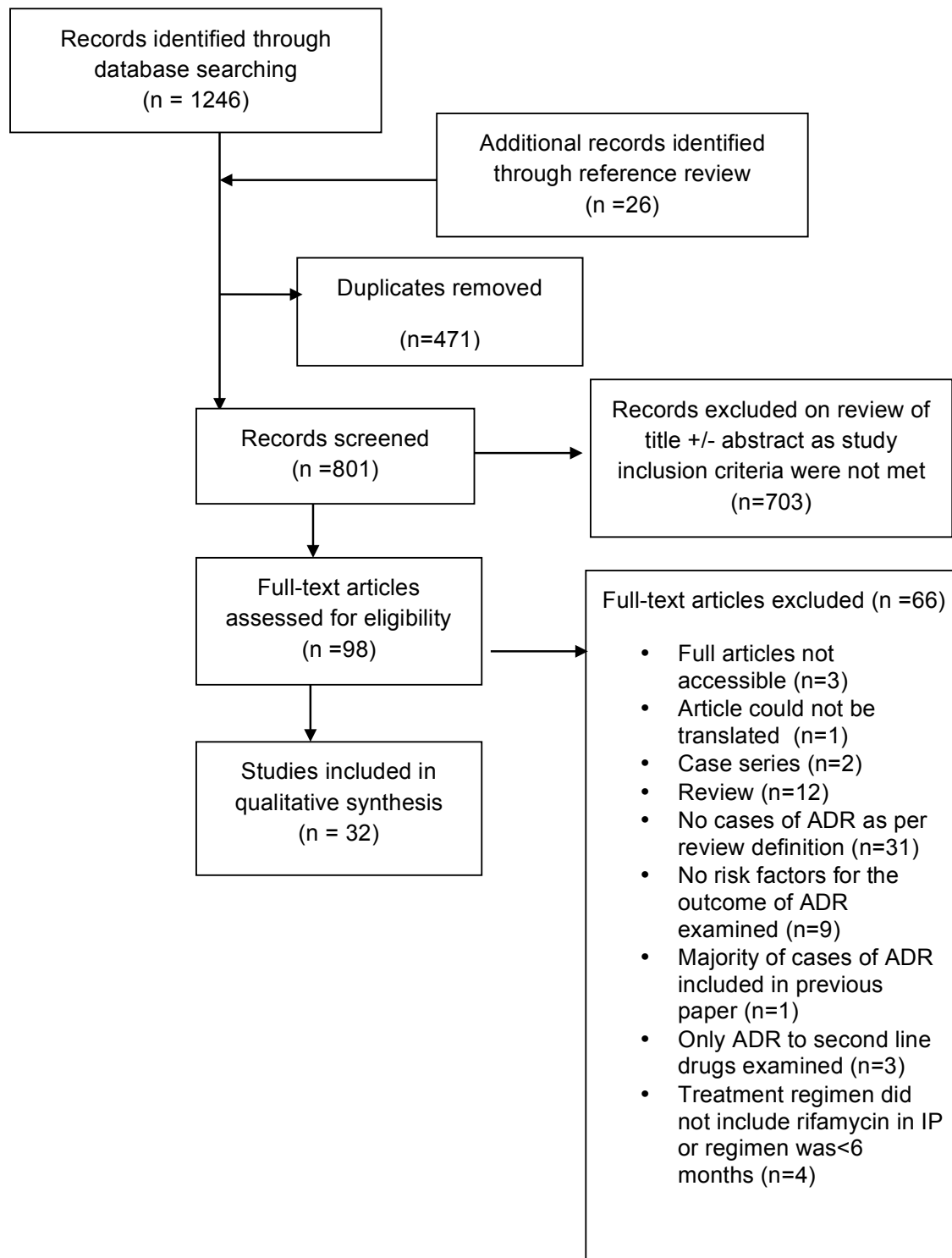


Table 2.1 provides a detailed break down and table 2.2 shows the aggregate data of studies included in the review. Certain studies restricted inclusion to specific populations: those with HIV-1 co-infection (n=5)(44-46), those incarcerated (n=1)(47), those with silicotuberculosis (n=1)(48), those with isoniazid monoresistance (n=1)(49) and retreatment patients (n=2)(18, 50) and hence results are not generalisable. Table 2.3 provides an appraisal of study quality (43). Loss to follow up was not noted to be significant (pre-defined threshold 20%) in any study. We assessed that in all selected RCTs, treatment effect was measured precisely. However, in Although powering was deemed inadequate in 2/6 RCTs and 4/23 cohort studies, this was in reference to another primary outcome i.e. not referring to the outcome of ADR. We assessed in 20/23 cohort studies, that exposure was accurately measured to minimise bias. As illustrated in Table 2.1, only a proportion of individuals included as the 'whole cohort' at baseline had follow-up DST as per criteria detailed in table 2.2. These criteria ranged from being performed at a regular monthly interval in all culture positive isolates; to those who were smear/culture positive at 2 and 5-6 months; to being only performed in cases of suspected failure/relapse. In some cases, this may have compromised accuracy of measurement of outcome. Two studies were excluded for the meta-analysis of HIV-1 as a risk factor for ADR as the authors did not respond. Two studies were excluded for the meta-analysis of baseline drug resistance as a risk factor for ADR because the authors did not respond.

Table 2.1 Characteristics of included studies in systematic review

Reference		HIV-1 prevalence	Retreatment (ReRx)	Regimen	DOT	Genotyping carried out in a proportion of available isolates
RCTs						
Algerian Working Group/British MRC 1991 Tubercle (51)	Algeria Oct 1981-Dec1983	0%	Not specified	IP: Regimen 1) 2(HRSZ ₇) Regimen 2) 2(HREZ ₇) CP: Regimen 1) 4(HR ₇) Regimen 2) 2(HR ₇)4(H ₇)	DOT in IP (whilst on streptomycin)	No
Hong Kong TB Research Centre Madras/BMRC Am Rev Resp Disease 1991(48)	Hong Kong Dec 1980-Dec 1985	Not specified	Not specified	Regimen 1) 6(RHSZ) ₇ Regimen 2) 8(RHSZ) ₃ (E was added for first 3 months if retreatment patient)	100%	No
Lienhardt JAMA 2011 (52)	Algeria, Colombia, Guinea, Vietnam, Peru, Mozambique, Tanzania, Bolivia 2003-2008	6.6%	0%	IP: Regimen 1) 2(RHEZ ₇) as FDC Regimen 2) 2(RHEZ ₇) as single drugs CP: 4(RH ₃)	100%	Yes Spoligo and MIRU-VNTR
Swaminathan AJRCCM 2010 (46)	Chennai, India Feb '01-Sep '05	100%	0%	IP: Regimen 1) 2(RHEZ ₃) Regimen 2) 2(RHEZ ₃) CP: Regimen 1) 4(RH ₃) Regimen 2) 7(RH ₃)	DOT was given during IP. 1/3 doses was given as DOT during CP	Yes IS6110, MIRU-VNTR, Spoligo
TB Research Centre IJTLD 1997(53)	Chennai not specified	Not specified	Not specified	IP: Regimen 1) 2(HREZ ₇) Regimen 2) 2(HREZ ₂) Regimen 3) 2(HRZ ₂) CP: Regimen 1) 6(HE ₇) Regimen 2) 4(HRE ₂) Regimen 3) 4(HR ₂)	Regimen 1 was fully unsupervised. Regimen 2 and 3 were either fully or partially supervised.	No
Vernon Lancet 1999(44)	USA Apr 1995-early 1997	100%	47.5%	IP: 2(RHEZ _{7/3/2}) CP: Regimen 1) 4(rifapentine/H ₁) Regimen 2) 4(RH ₂)	100%	Yes IS6110
Prospective cohorts						
Aung, IJTLD 2012(54) *operational study with randomisation	Bangladesh Jan '06-Jun '07	Not specified	0%	IP: 2(3) RHEZ ₇ CP: 4(HR ₃)	100%	Yes Sequencing of core region of <i>rpoB</i> gene
Burman AJRCCM 2006(45)	New York City Dec 1998-Mar 2002	100%	Not specified	IP: First 2 weeks: (RHEZ ₇) Next 6 weeks: (RHEZ ₅) or (RHEZ ₃) or (RHEZ ₂) (78% received rifampin in IP) CP: 4-7(RH ₂) R=rifabutin	100%	Yes Sequencing of core region of <i>rpoB</i> gene
Cox, Clin Infect Dis 2007(55)	Karakalpakstan, Uzbekistan and Dashoguz, Turkmenistan Jul 2001-Mar 2002	Not specified	45%	IP: New 2(HREZ ₇) ReRx 2(SRHEZ ₇), 1(RHEZ ₇) CP: New: 4(HR ₃) ReRx: 5(HRE ₃)	DOT during IP	Yes RFLP of IS6110 and spoligo
El Sahly, J of Infect, 2006 (56)	Houston, USA 1995-2001	18.1%	6.3%	*Not specified	Not specified	Yes RFLP of IS6110 and spoligo
Murray SAMJ 2000 (57)	Goldmines in Gauteng,	49%	27%	IP: 2RHZE CP: 4RH	DOT if smear+	No

	South Africa, 1995					
Nettles, Clin Infect Dis 2004 (58)	Baltimore, USA Jan '93-Dec '01	27%	Not specified	IP: 2wks (RHEZ ₇) 6wks (RHEZ ₂) Rifampicin or rifabutin CP: (RHEZ ₂) Rifampicin or rifabutin, duration individualised	100%	Yes RFLP of IS6110
Pasipanodya, J Inf Dis 2013 (41)	Western Cape, South Africa	10%	64%	IP: New 2(HREZ ₇) ReRx 2(SRHEZ ₇), 1(RHEZ ₇) CP: New: 4(HR ₃) ReRx: 5(HRE ₃)	DOT during IP	No
Temple Clin Infect Dis 2008(18)	Kampala, Uganda Jul 2003-Nov 2006	48%	100%	IP: 1(SRHEZ ₇) 2(RHEZ ₇) CP: 5(RHE ₇)	DOT in IP (hospitalised)	Yes RFLP of IS6110
Retrospective cohorts						
Chien, JAC 2013 (59)	Taiwan 2005 to 2011	0%	Not specified	WHO recommendations IP: New 2(HREZ ₇) ReRx 2(SRHEZ ₇), 1(RHEZ ₇) CP: New 4(HR ₃) ReRx 5(HRE ₃)	57% received DOTS	No
Driver, Clin Infect Dis, 2001 (60)	New York City Jan 1993-Jun 1996	33% , (unknown 36%)	0%	IP: Regimen 1) Regimen 2(RHZ ₇) Regimen 2) 2(RHZ ₇) Regimen 3) IP with < 8weeks of Z CP: Regimen 1) 4(RH ₇) Regimen 2) 6(HE ₇) Regimen 3) 7(RH ₇)	DOT median 21 weeks	Yes RFLP of IS6110
Gelmanova, Bull WHO, 2007 (61)	Tomsk, Siberia Jan 2001-Dec2001	1%	Not specified	WHO recommendations IP: New 2(HREZ ₇) ReRx: 2(SRHEZ ₇), 1(RHEZ ₇) CP: New: 4(HR ₃) ReRx: 5(HRE ₃)	DOT in inpatient, outpatient and home care setting. Small proportion self-administered therapy	No
Jasmer, AJRCCM, 2004 (62)	San Francisco, United States 1998 to 2000	13%	9%	*Not specified	DOT (n=149) and SAT (n=223)	No
Kim BMC ID 2008(49)	Seoul, Korea Jul 2001-Jun 2005.	36%	Not specified	IP: 2(RHEZ ₇) CP: 33% 4(REZ ₇) 54% 10(RE ₇) 13% 7(RE ₇)	Not specified	No
Li CID 2005 (63)	New York City Jan 1997-Dec 2000	Not specified	28%	IP: Variable rifampin or rifabutin-based regimen, daily or intermittent dose (2/wk or 3/wk) for 2 months CP: rifampin or rifabutin regimen given x2 or 3/wk for 4-6, 7-10 or >10 months	Not specified	Yes RFLP of IS6110 and spoligo
Matthys, PLoS ONE, 2009 (47)	Mariinsk, Siberia, Russia 1997 to 1998	None at entry into prison	65%	IP: 2(SRHEZ ₇), 1(RHEZ ₇) CP: 5(RHE ₇)	100%	Yes RFLP of IS6110
Moulding IJTLD 2004 (64)	Los Angeles, US Jun 1985-Jul 1992	Cohort known or presumed to be HIV-1 negative	Not specified	IP: HR and Z or E or ZE (duration and frequency not specified) CP: HR (duration and frequency not specified)	Not specified	No
Porco CID 2012(16)	California, USA Jan 1994-Dec 2006	7.5%	Not specified	*Not specified	100%	No

Quy IJTLD 2003 (65)	Ho Chi Minh City, Vietnam Aug 1996- Jul 1998	Not specified	0%	IP: New: 2(SHRZ ₇) ReRx 2(SRHEZ ₇),1(RHEZ ₇) CP: New: 6(HE ₇) ReRx:5(HRE ₃)	100%	Yes RFLP of IS6110
Seung CID 2004 (66)	Tomsk, Siberia Nov 1996- Dec 2000	Not specified	0%	IP: 2(HREZ ₇) In some cases S was given instead of E CP: 4(HR ₇)	DOT programme IP- hospitalised CP- outpatient	No
Spellman 1988 AIDS (67)	Miami and New York, USA Jan '88- Dec '95	12.8	5.2%	*Not specified	100%	No
Weis, NEJM 1994(68) * Although presented under cohort studies, this may also be classified as an ecological study	United States 1980 to 1992	58 amongst 485 those tested from 1987 (12%)	Not specified	IP: 1980 to 1986 included HRE. 1986 to 1992 included HRZ +/- E or injectable CP: Not specified	Until 1986 not DOT, from 1986 90.5% received DOT	No
Yoshiyama IJTLD 2004 (50)	Chiang Rai, Thailand May 1996- Dec 2000	31%	100% of re-registered cohort	IP: 2(SRHEZ ₇),1(RHEZ ₇) CP: 5(RHE ₇)	DOT introduced in 1996	Yes RFLP of IS6110
Yuen, PLoS ONE 2013 (69)	United States 2004 to 2011	Positive 7% Negative 67.5% Unknown 25.5%	0%	*Not specified	DOT only 61%, DOT + SAT 37%, SAT only 2%	No
Case controls						
Bradford Lancet 1996 (70)	San Francisco, USA Jan '85-Dec '94	Cases 79% Controls 27%	Cases 14% Controls 14%	*Not specified	Not specified	Yes RFLP of IS6110
Munsiff, Clin Infect Dis 1997 (71)	New York City, USA 93-94	100%	Not specified	IP: Regimen contained RHZ (+/-E), dosing regimen not specified *CP: Not specified	Cases: 24% received DOT Controls: 31% received DOT	No
Weiner CID 2005(72)	New York City Dec 1998- Mar 2002	100%	Not specified	IP: First 2 weeks: (RHEZ ₇) Next 6 weeks: (RHEZ ₅) or (RHEZ ₃) or (RHEZ ₂) CP: 9(RH ₂)	100%	Yes Sequencing of core region of <i>rpoB</i> gene

Abbreviations: IP intensive phase CP continuation phase R rifampin H isoniazid E ethambutol Z pyrazinamide S streptomycin Rx treatment wk week DOT directly observed therapy SAT self-administered therapy X(RHEZ_y) X= number of months on regimen y= number of days/week on regimen ARR acquired rifamycin resistance Spoligo Spoligotyping MIRU-VNTR (mycobacterial interspersed repetitive unit-variable- number tandem repeat) typing RFLP of IS6110 restriction fragment length polymorphism of the IS6110 insertion element

*individualised treatment as per Centre of Disease Control, USA guidelines <http://www.cdc.gov/mmwr/pdf/rr/rr5211.pdf>

Whilst treatment regimens were not explicitly stated in 6 (19%) of studies, these all included treatment with a rifamycin during intensive phase and were of minimum 6 months duration.

Table 2.2 Aggregate data of studies included in the review

Characteristics of studies (N=32)	Number of studies (%)
Year study completed	
1980- 2000	15 (47)
After 2000	17 (53)
Geographical location*	
North America	15(47)
South America	1 (3)
North Africa	2 (6)
South and East Africa	4 (12.5)
Indian subcontinent	4 (12.5)
South East Asia	5 (16)
Former Soviet Union states	4 (12.5)
HIV-1 co-infection	
HIV-1 co-infection data reported for cohort	25 (78)
All patients in cohort had HIV-1	5 (16)
Presumed 0% HIV-1 co-infection in cohort	4 (12.5)
Retreatment	
Proportion patients receiving retreatment for TB reported for cohort	19 (59)
Retreatment patients excluded	6 (19)
Criteria for repeat DST	
Unfavourable treatment response such as smear or culture positivity at the end of treatment or clinical relapse	15 (47)
Cultures were done at regular intervals throughout study and follow up and DST carried out for positive culture	10 (31)
Not specified	7 (22)
Genotype carried out to confirm ADR	15 (47)

DST drug sensitivity testing ADR acquired drug resistance *some studies were carried out in more than 1 continent

Table 2.3 Study quality based on criteria developed in the Critical Appraisal Skills Programme

Study ID	Study design	Was LTFU significant ?	Was study appropriately powered?	Was blinding appropriate ?	Was randomisation appropriate?	Apart from intervention were groups treated equally?	Was treatment effect measured precisely?	Was study population representative ?	Was exposure accurately measured to minimise bias?	Have confounders been accounted for?	Were cases and controls defined precisely ?	Were controls appropriate ?
Randomized controlled trials												
Algerian Working Group/British MRC 1991 Tubercle (51)	RCT	No	Yes	N/A	N/A	Yes	Yes	N/A	N/A	N/A	N/A	N/A
Hong Kong TB Research Centre Madras/BMRC Am Rev Resp Disease 1991(48)	RCT	Yes	No	Yes	Yes	Yes	Yes	N/A	N/A	N/A	N/A	N/A
Lienhardt JAMA 2011 (52)	RCT	No	Yes	N/A	Yes	Yes	Yes	N/A	N/A	N/A	N/A	N/A
Swaminathan AJRCCM 2010 (46)	RCT	No	Yes	N/A	Yes	Yes	Yes	N/A	N/A	N/A	N/A	N/A
TB Research Centre IJTLD 1997(53)	RCT	No	Yes	N/A	UC	No	Yes	N/A	N/A	N/A	N/A	N/A
Vernon Lancet 1999(44)	RCT	No	No	N/A	Yes	Yes	Yes	N/A	N/A	N/A	N/A	N/A
Prospective cohorts												
Aung, IJTLD 2012(54) *operational study with randomisation	Cohort	No	Yes	NA	NA	NA	NA	Yes	No	No	N/A	N/A
Burman AJRCCM 2006(45)	Cohort	No	Yes	NA	NA	NA	NA	No	Yes	Yes	N/A	N/A

Cox, Clin Infect Dis 2007(55)	Cohort	No	Yes	NA	NA	NA	NA	Yes	Yes	No	N/A	N/A
El Sahly, J of Infect, 2006 (56)	Cohort	No	Yes	NA	NA	NA	NA	Yes	Yes	Yes	N/A	N/A
Murray SAMJ 2000 (57)	Cohort	No	No	NA	NA	NA	NA	No	Yes	No	N/A	N/A
Nettles, Clin Infect Dis 2004 (58)	Cohort	No	Yes	NA	NA	NA	NA	Yes	Yes	Yes	N/A	N/A
Pasipanodya , J Inf Dis 2013 (41)	Cohort	No	Yes	NA	NA	NA	NA	No	Yes	Yes	N/A	N/A
Temple Clin Infect Dis 2008(18)	Cohort	No	Yes	NA	NA	NA	NA	Yes	Yes	Yes	N/A	N/A
Retrospective cohorts												
Chien, JAC 2013 (59)	Cohort	No	Yes	NA	NA	NA	NA	Yes	Yes	Yes	N/A	N/A
Driver, Clin Infect Dis, 2001 (60)	Cohort	Not stated	Yes	NA	NA	NA	NA	Yes	Yes	Yes	N/A	N/A
Gelmanova, Bull WHO, 2007 (61)	Cohort	No	Yes	NA	NA	NA	NA	Yes	Yes	Yes	N/A	N/A
Jasmer, AJRCCM, 2004 (62)	Cohort	No	Yes	NA	NA	NA	NA	Yes	Yes	Yes	N/A	N/A
Kim BMC ID 2008(49)	Cohort	No	No	NA	NA	NA	NA	No	Yes	Yes	N/A	N/A
Li CID 2005 (63)	Cohort	No	Yes	NA	NA	NA	NA	Yes	Yes	Yes	N/A	N/A
Matthys, PLoS ONE, 2009 (47)	Cohort	No	No	NA	NA	NA	NA	No	Yes	Yes	N/A	N/A
Moulding IJTLD 2004 (64)	Cohort	No	Yes	NA	NA	NA	NA	No	No	No	N/A	N/A
Porco CID 2012(16)	Cohort	No	Yes	NA	NA	NA	NA	Yes	Yes	Yes	N/A	N/A
Quy IJTLD 2003 (65)	Cohort	No	Yes	NA	NA	NA	NA	Yes	Yes	No	N/A	N/A

Seung CID 2004 (66)	Cohort	No	Yes	NA	NA	NA	NA	Yes	Yes	No	N/A	N/A
Spellman 1988 AIDS (67)	Cohort	No	Yes	NA	NA	NA	NA	Yes	Yes	Yes	N/A	N/A
Weis, NEJM 1994(68) * Although presented under cohort studies, this may also be classified as an ecological study	Cohort			NA	NA	NA	NA				N/A	N/A
Yoshiyama IJTLD 2004 (50)	Cohort	No	No	NA	NA	NA	NA	No	Yes	No	N/A	N/A
Yuen, PLoS ONE 2013 (69)	Cohort	No	Yes	NA	NA	NA	NA	Yes	Yes	Yes	N/A	N/A
Case controls												
Bradford Lancet 1996 (70)	Case-control	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Yes	Yes	Yes
Munsiff, Clin Infect Dis 1997 (71)	Case-control	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Yes	Yes	Yes
Weiner CID 2005(72)	Case-control	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Yes	Yes	Yes

N/A Not applicable

2.2.2. Risk factors associated with ADR

Tables 2.4-2.7 summarise significant associations and trends for ADR. Table 2.8 summarises all covariates which were examined as potential risk factors. Studies varied considerably in the potential risk factors examined. The disease burden and pathogen factors most frequently examined were baseline mono and polyresistance (16/32), smear positivity (8/32) and cavitatory disease (7/32). Host immune factors most frequently examined were HIV-1 co-infection (10/32) and CD4 lymphocyte count in HIV-1-infected patients (8/32). The most frequent sociodemographic covariate examined was age (11/32). The most frequently examined programmatic factor was self-administered therapy versus directly observed therapy (8/32).

Table 2.4 RCTs- ADR + associated risk factors

Reference	Cohort description and numbers	Acquired isoniazid resistance (%) [*]	Acquired rifamycin resistance (%) [*]	Acquired MDR TB (%) [*]	Risk factors associated with ADR (ADR)
Algerian Working Group/British MRC 1991 Tubercle (51)	Whole cohort n=2218 Known baseline drug sensitivity n=2071 Follow up drug sensitivity n=1415	WC= 1/2071 (0.05) WCFU= 1/1415 (0.07) PS= 1/1376 (0.07) MR _(S) = 0/40 (0)	WC= 4/2071 (0.19) WCFU= 4/1415 (0.28) PS= 1/1376 (0.07) MR _(H/S) =0/61 (0) PR _(H+S) = 3/34 (8.8)	WC= 4/2071 (0.19) WCFU= 4/1415 (0.28) PS=1/1376 (0.07) MR _(H/S) =0/50 PR _(H+S) = 3/33 (9)	-Rifampicin in regimen only during intensive phase -Baseline resistance to INH and STREP
Hong Kong TB Research Centre Madras/BMRC Am Rev Resp Disease 1991 (48)	Whole cohort n=145 Culture proven TB with known baseline sensitivity n=127	WCFU 2/127 (1.6) PS= 2/91 (2.2) MR _(S/R) = 0/13 (0)	WCFU 5/127 (3.9) PS 1/91 (1.1) MR _(H/S) = 1/22 (4.5) PR= 3/9 (33)	WC= 4/127 (3.1) PS= 1/91 (1.1) MR _(H/S/R) = 1/25 (4) PR _(H+S) = 3/9 (33)	-Baseline drug resistance
Lienhardt JAMA 2011(52)	Culture confirmed smear +ve new TB patients either pan-susceptible or INH monoresistant n=1170	WCFU 1/1170 (0.09) PS 1/1005 (0.1)	WCFU 1/1170 (0.09) PS 0/1005 (0) MR _(H) 1/127 (0.79)	WCFU 1/1170 (0.09) PS 0/1005 (0) MR _(H) 1/127 (0.79)	None of the factors analysed were associated (see table 2.8)
Swaminathan AJRCCM 2010 (46)	New TB cases (baseline MDR excluded) n=327 Culture confirmed with DST and results at end of treatment n=212	WC 7/327 (2) WCFU 7/212 (3.3) PS 7/194 (3.6) *	WC 20/327 (6.1) WCFU 20/212 (9.4) PS 11/194 (5.7) *	WC 17/327 (5.2) WCFU 17/212(8.0) PS 11/194 (5.7) *	-Lower median CD4 lymphocyte count (p 0.054) - Higher median HIV-1 VL (p 0.009) Non-adherence (adherence <90%) (p 0.000) -Baseline isoniazid resistance (OR 8.43, p 0.002)
TB Research Centre IJTLD 1997 (53)	Smear +ve TB n=1203, Followed up post end of treatment and included in	WC 22/1053 (2.1) PS 22/825 (2.7) MR _(R) 1/1 (100)	WC 26/1053 (2.4) PS 3/825 (0.3) MR _(H) 23/227 (10)	WC 26/1053 (2.4) PS 1/825 (0.1) MR _(H/R) 23/228(10)	-Lack of ethambutol in a twice weekly regimen - Baseline drug resistance

	infected n=61		*		-Age (p 0.04) -Extrapulmonary + pulmonary disease (p 0.03) - Use of antifungal azoles (p 0.006)
Abbreviations: WC whole cohort denominator known DST ; WCFU denominator f/u DST ; PS denominator initial pan-sensitivity ; MR denominator initial monoresistance ;PR denominator initial polyresistance. H isoniazid S Streptomycin R rifampicin *ADR data presented is not stratified by baseline monoresistance and polyresistance as this information cannot be ascertained from the paper DOT directly observed therapy NTM non-tuberculous mycobacteria MDR multidrug resistant ART antiretroviral therapy BMI body mass index INH isoniazid PZA pyrazinamide					

Table 2.5 Prospective cohort studies- ADR and risk factors

Reference	Cohort description and numbers	Acquired isoniazid resistance (%) [*]	Acquired rifamycin resistance (%) [*]	Acquired MDR TB (%) [*]	Risk factors associated with ADR (ADR)
Aung, IJTLD 2012 (54) *operational study with randomisation	Patients who were smear -ve at 2mth and whose IP was not extended n=12967 Patients who were smear +ve at 2mth and whose IP was extended by 1 mth n=1871 Patients who were smear +ve at 2mth and whose IP was not extended n=1870 Smear defined relapses/failures n=595	Not specified	WC 16/16708 (0.09) WCFU 16/595 (2.7)	WC 12/16708 (0.07) WCFU 12/595 (2)	None of the factors analysed were associated (see table 2.8)
Burman AJRCCM 2006 (45)	Culture confirmed TB, HIV-1 co-infection and on intermittent rifabutin* based regimen n=169 *78% initially received a median of 33.5 days of rifampin based therapy	Not specified	WC=8/169 (4.7)	WC=1/169 (0.59)	-Lack of use of ART in first 2 months TB treatment (p=0.05) - Lower CD4 lymphocyte count at diagnosis (p=0.001)

	during IP				
Cox, Clin Infect Dis 2007 (55)	Smear +ve TB (baseline MDR patients and mixed infection excluded) n=314, smear +ve with identical spoligotype as baseline at end of IP or 2 months into CP n=62	WCFU 1/314 (0.3) PS 1/177 (0.6) MR _(R/S/Z/E) 0/51 (0)	WCFU 11/314 (3.5) PS 1/177 (0.6) MR _(I/S/Z/E) 0/71 (0) PR _(H+S or I+S+E or I+S+E+Z) 10/65 (15.3)	WCFU 11/314 (3.5) PS 1/177 (0.6) MR _(I/S/R/Z/E) 0/72 (0) PR _(I+S or I+S+E or I+S+E+Z) 10/65 (15.3)	-Baseline polyresistance (p<0.05) - Beijing genotype in polyresistant strain (11 out of 28 polyresistant Beijing strains amplified their resistance, compared with none of the 27 non-Beijing strains)
El Sahly, J Infect, 2006 (56)	Pan-susceptible TB n=1977	PS 9/1977(0.45)	PS 7/1977 (0.3)	PS 1/1977 (0.05)	-HIV-1 positivity (aOR 5.52 95% CI 1.55-19.68) - Asian ethnicity (aOR 16.74 95% CI 3.8-73.72) - smear positive (aOR 4.76 95% CI 1.42-15.96) - Disseminated TB (with pleural effusion) (aOR 9.22 95% CI 2.82-30.17) aOR adjusted odds ratio
Murray SAMJ 2000 (57)	Culture confirmed drug sensitive/monoresistant TB cases (MDR cases excluded) n=400	WCFU 6/400 (0.15) PS 6/350 (0.17) ¥	WCFU 6/400 (0.15) PS 3/350 (0.9) ¥	WCFU 6/400 (0.5) PS 3/350 (0.9) ¥	-Baseline drug resistance
Nettles, Clin Infect Dis 2004 (58)	Culture confirmed TB (excluding drug resistance, no DOT, alternative regimen, loss to follow up, death) n=407	Not specified	WCFU 3/407 (0.7)	Not specified	-HIV-1 co-infection(p=0.02) - Lower baseline median CD4 lymphocyte count (p= 0.02)
Pasipanodya , J Inf Dis 2013 (41)	Smear or culture confirmed drug sensitive TB n=142	WCFU 3/142 (2%)	WCFU 1/142 (0.7%)	WCFU (0.7%)	-PK variability including PZA AUC ₂₄ ≤363 mg*h/L, RIF AUC ₂₄ ≤13 mg*h/L, and INH AUC ₂₄ ≤52mg*h/L
Temple Clin Inf Dis 2008 (18)	Smear +ve, culture +ve retreatment TB cases with known DST at baseline admitted to hospital (excluding MDR) n=269, 5 month follow up sputa n=237	WC 2/269 (0.7) WCFU 2/237 (0.8) PS 2/226 (0.9) MR _(S/R) 0/11 (0) PR _(S+Z/S+E/S+Z+E) 0/9 (0)	WC 6/269 (2.2) WCFU 6/237 (2.5) PS 3/226 (1.3) MR _(I/S/Z/E) 3/38 (10.3) *PR _(S+Z/S+E/S+Z+E) 0/9 (0)	WC 5/269 (1.9) WCFU 5/237 (2.1) PS 2/226 (0.9) MR 3/31 (9.6) PR 0/9 (0)	-Baseline resistance (HR 10, p=0.003)

Abbreviations: WC whole cohort denominator known DST ; WCFU denominator f/u DST ; PS denominator initial pan-sensitivity ; MR denominator initial monoresistance ; PR denominator initial polyresistance. H isoniazid S Streptomycin R rifampicin E ethambutol Z pyrazinamide *ADR data presented is not stratified by baseline monoresistance and polyresistance as this information cannot be ascertained from the paper, DOT directly observed therapy IP intensive phase CP continuation phase Hb haemoglobin ART antiretroviral therapy BMI body mass index INH isoniazid PZA pyrazinamide RIF rifampin AUC₂₄ 24 hr area under the concentration-time curve

Table 2.6 Retrospective cohort studies- ADR and risk factors

Reference	Cohort description and numbers	Acquired isoniazid resistance (%) [*]	Acquired rifamycin resistance (%) [*]	Acquired MDR TB (%) [*]	Risk factors associated with ADR (ADR)
Chien, JAC 2013 (59)	Culture confirmed pulmonary TB without HIV-1 co-infection (baseline MDR/XDR excluded) n=2080	WCFU 108/2080 (5.2)	WCFU 160/2080 (4.7)	WCFU 178/2080 (8.6)	-Age group 45-64 (OR 2.07 , p=0.01) -Smear positivity (OR 2.09 , p=0.01) - Self-administration of treatment/lack of DOT (OR 2.94, p=0.01)
Driver, Clin Infect Dis, 2001 (60)	Drug sensitive at baseline n=4571, Known relapses/recurrences at end of at least 6mths Rx n=123, Known DST at relapse or recurrence n=95	WCFU 14/4571 (0.3) PS 14/4571 (0.3)	WCFU 21/4571 (0.5) PS 21/4571 (0.5)	WCFU 9/4571 (0.2) PS 9/4571 (0.2)	-HIV-1 co-infection (risk factor for acquired RIF monoresistance)
Gelmanova, Bull WHO, 2007 (61)	Enrolled in DOT n=260 , Culture +ve, with known DST (baseline MDR at excluded) n=207	Not specified	Not specified	WCFU 15/207 (7.3)	-Substance abuse (HR 2.88, p=0.04) -Treatment commenced in hospital setting (HR=6.34, p=0.02) - Hospitalisation later in treatment (HR=6.26 ,p=0.047) -Self-administration of Rx/lack of DOT (HR 0.25, p=0.03)
Jasmer, AJRCCM, 2004 (62)	Drug sensitive cases of TB who started treatment with allocated DOT status n=372, Had f/u cultures as part of post-treatment evaluation n=330	WC 2/372 (0.5) WCFU 2/330 (0.6) Details of which drugs resistance is acquired to not specified			None of the factors analysed were associated (see table 2.8)
Kim BMC ID 2008 (49)	INH resistant at baseline n=39	N/A	WCFU 2/39 (5.1) MR _(H) 2/39 (5.1%)	WCFU 2/39 (5.1) MR _(H) 2/39 (5.1)	-2 vs 3 drugs in continuation phase -Extensive radiological disease

					-Smear positivity
Li CID 2005 (63)	Confirmed TB with known DST, n=2861	Not specified	WCFU 10/2861 (0.3)	Not specified	-HIV-1 infection aOR, 5.5; 95% CI, 1.4–21.5) Analysis restricted to CD4 count <100: Rifampicin-based (as opposed to rifabutin) regimens aHR 8.5; 95% CI, 1.03–70.9) Analysis restricted to HIV-1 patients on rifampicin-based regimens: patients received intermittent dosing during IP HR 6.4; 95% CI, 1.1–38.44) aOR adjusted odds ratio aHR adjusted hazards ratio
Matthys, PLoS ONE, 2009 (47)	Admitted and remained inpatient in penitentiary hospital during treatment (baseline MDR excluded) n=189	WCFU 0/189 (0) PS 0/81 (0) MR _(S/R/E) 0/20	WCFU 6/189 (3.2) PS 0/81 (0) MR _(H/S/E) 0/46 (0) PR _(H+E/H+S/H+S+E) 6/61 (9.8)	WCFU 6/189 (3.2) PS 0/81 (0) MR _(H/S/R/E) 0/47 (0) PR _(H+E/H+S/H+S+E) 6/61 (9.8)	-Amplification of resistance occurred in 10.7% of those with polyresistance at baseline (vs 3.4% in the whole cohort)
Moulding IJTLD 2004(64)	Drug sensitive at baseline n=5337	WC 25/5337 (0.47) Details of which drugs resistance is acquired to not specified			- Separate drug formulation as opposed to fixed dose combination -Private sector management
Porco CID 2012(16)	Drug sensitive at baseline n=33725 Repeat DST at follow up n=1792	WC 52/33725 (0.15) WCFU 52/1792 (2.9) PS 46/30 548 (0.15) MR _(R) 6/138 (4.3)	WC 64/33725 (0.19) WCFU 64/1792 (3.5) PS 37/30 548 (0.1) MR _(I) 27/3039 (0.9)	WC 49/33725 (0.1) WCFU 49/1792 (2.7) PS 16/30 548 (0.05) MR _(H/R) 33/3177 (1)	<u>Acquired INH resistance</u> - Initial rifampicin resistance (aOR 10.3 p<0.001] -HIV-1 infection (aOR=3.36, p =0.01) <u>Acquired rifampicin resistance</u> - Age per year (aOR 0.96, p=0.002) -HIV-1 infection (aOR=9.39 , p<0.001) -Initial resistance to isoniazid (aOR=11.2, p<0.001) -Cavitatory disease in the absence of DOT (OR= 2.65, p 0.03) <u>Acquired MDR</u> - Initial isoniazid resistance (aOR=19.2p<0.001) - Initial rifampicin resistance (aOR=35.9, p<0.001) -HIV-1 infection (aOR=5.07, p=0.003)

					-Cavitatory disease in the absence of DOT (aOR=2.65,p=0.04) aOR=adjusted odds ratio
Quy IJTLD 2003 (65)	New smear +ve cases starting TB Rx n=2901, Culture positive failure and relapse cases with repeat DST and identical ISS610 patterns compared with baseline (baseline MDR excluded)n= 62	WC 3/2901 (0.1) WCFU 3/62 (4.8) ¥	WC 18/2901 (0.6) WCFU 17/62 (2.7) ¥	WC 18/2901 (0.6) WCFU 17/62 (2.7) ¥	-Baseline drug resistance (OR 6.6,95%CI 1.4–32)
Seung CID 2004 (66)	Enrolled in category 1 treatment regimen n=2194, Culture positive cases with known DST (baseline MDR cases excluded) n=1610	WC 19/1610 (1.2) PS 9/1212 (0.7) MR _(R/E/S) 6/191 (3.1) PR _(R+E/R+S/R+E+S) 2/27 (7.4)	WC 31/1610 (1.9) PS 9/1212 (0.7) MR _(H/E/S) 6/212 (2.8) PR _(H+S/H+E/H+S+E) 16/153 (10.4)	WC 28/1610 (1.7) PS 8/1212 (0.7) MR 6/225 (2.7) PR 18/180 (10)	-Baseline drug resistance
Spellman 1988 AIDS (67)	TB patients with known DST (baseline MDR cases excluded) n=739	WC 2/739 (0.3) PS 2/682 (0.3) MR _(S/R/Et) 0/23 (0) PR _(S+E/S+Et) 0/5 (0)	WC 4/739 (0.5) PS 2/682 (0.3) MR _(H/S/Et) 1/42 (2.4) PR _(H+S/H+E/H+Et/H+Et+S+E/S+Et) 1/14 (7.1)	WC 2/739 (0.3) PS 2/682 (0.3) MR _(H/S/R/Et) 1/65 (1.5) PR H+S/H+E/H+Et/H+Et+S+E/S+Et 1 /14 (7.1)	-Baseline drug resistance
Weis, NEJM 1994 (68)	Culture confirmed TB (baseline MDR cases excluded), n=957	Not specified	Not specified	WC 47/957 (4.9)	-Self-administration of treatment/lack of DOT (p<0.001)
Yoshiyama IJTLD 2004 (50)	Culture positive cases (baseline MDR cases excluded) n=1871, patients with repeat DST n=704 Re-registered cases with DST n=59	WC 5/1871 (0.3) WCFU 5/704 (0.7) PS 1/1634 (0.06) MR _(R) 4/43 (0.09)	WC 11/1871 ((0.6) WCFU 11/704 (1.6) PS 4/1634 ((0.2) MR _(H) 7/107 (6.5)	WC 12/1871 (0.6) WCFU 12/704 ((1.7) PS 1/1634 (0.06) MR _(H/R) 11/150 (7.3)	-Previous treatment failure -Baseline resistance - HIV-1 co-infection
Yuen, PLoS ONE 2013 (69)	Culture confirmed new cases with initial DST n= 51,223, Known genotype and follow up DST (n= 3696 for isoniazid, and n=4005 for	WC 61/51223 (0.1) WCFU 61/3696 (1.7)	WC 50/51223 (0.1) WCFU 50/4005 (1.2)	Not specified	<u>Acquired INH resistance</u> - <i>M. bovis</i> (aPR==8.46, 95% CI 2.96–24.14)

	rifamycin)				<u>Acquired rifamycin resistance</u> - <i>M. bovis</i> (aPR = 4.53, 95% CI 1.29–15.90) -Homeless (aPR=2.21, 95% CI 1.08–4.52) -HIV-1 co-infection(aPR 8.89, 95% CI 4.43–17.85) -Initial isoniazid resistance (aPR 10.37, 95% CI 5.65–19.00) - Extrapulmonary only disease (aPR 2.31, 95% CI 1.17–4.58) -Self-administered therapy/lack of DOT (aPR 2.52, 95% CI (1.01–6.30) -Initial ethambutol resistance (aPR 4.22, 95% CI (1.06–16.76) -Injecting drug use (aPR 4.09, 95% CI 1.66–10.10) -Age 45-64 (aPR 0.46, 95% CI 0.25–0.85), -Age>65 (aPR 0.14 , 95% CI 0.03–0.58) aPR=adjusted prevalence ratio
Abbreviations: WC whole cohort denominator known DST; WCFU denominator f/u DST; PS denominator initial pan-sensitivity; MR denominator initial monoresistance; PR denominator initial polyresistance .H isoniazid S Streptomycin R rifampicin E ethambutol Et ethioniamide *ADR data presented is not stratified by baseline monoresistance and polyresistance as this information cannot be ascertained from the paper DOT directly observed therapy IP intensive phase CP continuation phase Hb haemoglobin ART antiretroviral therapy BMI body mass index INH isoniazid PZA pyrazinamide R rifampin E ethambutol					

Table 2.7 Case control studies- ADR and risk factors

	Cohort description and numbers	Acquired isoniazid resistance (%) [*]	Acquired rifamycin resistance (%) [*]	Acquired MDR TB (%) [*]	Risk factors associated with ADR (ADR)
Bradford Lancet 1996 (70)	Total TB cases reported with known DST n=2612 Cases: acquired resistance to R,H or E with baseline pan-susceptibility n= 14 Control: baseline pan-susceptibility, no ADR, matched to time of diagnosis as cases n=56	WC 7/2612 (0.3)	WC 10/2612 (0.3)	WC 3/2612 (0.1)	- White ethnicity (p=0.015) -Foreign birth (p=0.007) -Unemployment (p=0.017) - Self-administration of treatment/lack of DOT (p=0.045) -ART use (p=0.014) -Azole use (p<0.001) -GI symptoms (aOR=11.5, 95%CI=1.23-107) -Non-adherence (aOR=19.7, 95%CI=1.66-234.4) -Baseline AIDS (aOR=20.2, 95%CI=1.12-363.6) aOR adjusted odds ratio
Munsiff, Clin Infect Dis 1997(71)	Cases: HIV-1-TB co-infected patients with confirmed acquired rifamycin monoresistance n=29 Control: HIV-1-TB co-infected patients with drug sensitive TB n=58	N/A	N/A	N/A	-Non-adherence (OR 11.0, p<0.001) -Baseline AIDS (OR 5.6, p=0.005) -Baseline smear positivity (OR= 4.1, p=0.02)
Weiner Clin Infect Dis 2005(72)	Total in TBTC Study 22 n=169 Culture confirmed TB, HIV-1 co-infected on intermittent dosing rifabutin regimen who participated in PK sub-study n=102. Cases of ADR, n=7. Controls n=95	Not specified	WC 7/102 (6.9)	Not specified	-Lower baseline CD4 lymphocyte count (p=0.001) -Rifabutin area under curve (AUC ₀₋₂₄ , p=0.01) and maximal concentration (p=0.03)* *This association remained, having adjusted for CD4 lymphocyte count
Abbreviations: WC whole cohort denominator known DST; WCFU denominator f/u DST; PS denominator initial pan-sensitivity; MR denominator initial monoresistance; PR denominator initial polyresistance DOT directly observed therapy IP intensive phase CP continuation phase Hb haemoglobin ART antiretroviral therapy BMI body mass index INH isoniazid PZA pyrazinamide					

Table 2.8 Risk factors for acquired drug resistance examined

Randomised controlled trials	
Reference	Potential risk factors for acquired drug resistance analysed
Algerian Working Group/British MRC 1991 Tubercle (51)	-Rifampicin in regimen only during intensive phase * -Baseline resistance to INH and STREP *
Hong Kong TB Research Centre Madras/BMRC Am Rev Resp Disease 1991(48)	-6 vs 8 months treatment including RHSZ throughout * -Baseline drug resistance *
Lienhardt JAMA 2011 (52)	-Fixed dose combination in IP vs separate drug formulation *
Swaminathan AJRCCM 2010 (46)	-Baseline median CD4 count -Baseline median HIV viral load -Nonadherence -Baseline isoniazid resistance -4 vs 7 month continuation phase -Baseline weight -Baseline Haemoglobin -Sputum smear grade
TB Research Centre IJTLD 1997(53)	-Ethambutol in a twice weekly regimen * -Frequency of dosing (twiceweekly or oncedaily) * -Baseline drug resistance *
Vernon Lancet 1999(44)	-Once weekly isoniazid/rifapentine -Baseline CD4 -Age -Extrapulmonary + pulmonary disease -Use of antifungal azoles
Prospective cohort studies	
Reference	Potential risk factors for acquired drug resistance analysed
Aung, IJTLD 2012(54) *operational study with randomisation	-Extension of intensive phase of treatment by 1 month for patients who are smear positive after 2 months *
Burman AJRCCM 2006(45)	-Age -Sex -Ethnicity -Foreign birth (immigrant) -Baseline BMI -Baseline resistance to INH/PZA -Extrapulmonary TB -Cavitary disease -Extensive (bilateral) radiological disease -Receiving of both rifampin and rifabutin during intensive phase -Culture positivity at 2months -Concurrent drugs which reduce rifabutin levels -Concurrent drugs which increase rifabutin levels -Baseline HIV viral load -Lack of use of ART during TB treatment -Lack of use of ART in first 2 months of TB treatment -Baseline CD4 lymphocyte count
Cox, Clin Infect Dis 2007(55)	-Baseline drug resistance (polyresistance) -Strain type
El Sahly, J of Infect, 2006 (56)	-HIV coinfection -Ethnicity -Smear positive -Disseminated TB (with pleural effusion)
Murray SAMJ 2000 (57)	-Baseline drug resistance * -HIV coinfection *
Nettles, Clin Infect Dis 2004 (58)	-HIV coinfection -Baseline median CD4 lymphocyte count -Type of rifamycin used (rifampicin vs rifabutin)

Pasipanodya , J Inf Dis 2013 (41)	-PK variability including peak and 24 hr area under the concentration time curve for drugs R,H,PZA *statistical analyses only performed for a composite outcome of death, treatment failure and relapse, not for acquired drug resistance
Temple Clin Infect Dis 2008 (18)	-Baseline resistance -Age -Sex -Baseline BMI -Nonadherence HIV coinfection -Baseline CD4 -ART use -Baseline extensive radiological disease -Baseline cavitary disease
Retrospective cohort studies	
Reference	Potential risk factors for acquired drug resistance analysed
Chien, JAC 2013 (59)	-Age group 45-64 -Smear positivity -Self-administration of treatment/lack of DOT -Cavitary disease
Driver, Clin Infect Dis, 2001 (60)	-HIV coinfection * -Self-administration of treatment/lack of DOT *
Gelmanova, Bull WHO, 2007 (61)	-Age -Gender -Non-adherence -Side effects -Substance abuse -Cavitary disease -Previously incarcerated -Smear positivity -Treatment commenced in hospital setting -Hospitalisation later in treatment Self-administration of treatment/lack of DOT
Jasmer, AJRCCM, 2004 (62)	-Self-administration of treatment/lack of DOT
Kim BMC ID 2008(49)	-Age * -Sex * -Comorbidity * -Previous TB treatment * -Length of RE in continuation phase * -Extensive radiological disease * -Smear positivity *
Li CID 2005 (63)	-HIV coinfection -CD4 count in the HIV coinfecting cohort -Baseline drug resistance in the HIV infected cohort -Change in type of rifamycin (rifampin to rifabutin or vice versa) during treatment -Intermittent dosing of rifampin or rifabutin during intensive phase
Matthys, PLoS ONE, 2009 (47)	-Baseline drug resistance *
Moulding IJTL 2004 (64)	-Separate drug formulation (as opposed to fixed dose combination) * -Private sector management *
Porco CID 2012(16)	-Age -Ethnicity -Gender -Foreign birth -Previous TB -Extrapulmonary TB -Smear positivity -Private sector care -Baseline drug resistance -HIV coinfection -Cavitary disease

Quy IJTLD 2003 (65)	-Age -Sex -Baseline drug resistance
Seung CID 2004 (66)	-Baseline drug resistance *
Spellman 1988 AIDS (67)	-HIV coinfection * -Baseline drug resistance *
Weis, NEJM 1994(68) * Although presented under cohort studies, this may also be classified as an ecological study	-Self-administration of treatment (lack of DOT)
Yoshiyama IJTLD 2004 (50)	-Previous treatment failure * -Baseline resistance * -HIV co-infection * -Strain type *
Yuen, PLoS ONE 2013 (69)	-Age -Ethnicity -Country of birth -Region of birth -In correctional facility at time of diagnosis -Injecting drug use -Non-injecting drug use -Alcohol -Strain type -HIV co-infection -Homelessness -Baseline drug resistance -Smear positivity -Cavitary disease -Extrapulmonary only disease -Self-administration of treatment/lack of DOT
Case control studies	
Reference	Potential risk factors for acquired drug resistance analysed
Bradford Lancet 1996 (70)	-Ethnicity -Foreign birth -Unemployment -Homelessness -Alcohol excess -Baseline AIDS -Self-administration of treatment/lack of DOT -ART use -Previous TB treatment -Initial 4 drug regimen -GI symptoms -Azole use -Non-adherence -CD4 lymphocyte count -Fixed dose RH tablet
Munsiff, Clin Infect Dis 1997 (71)	-Age -Gender -Country of birth -Recreational drugs -Homelessness -Self-administration of treatment/lack of DOT -Non-adherence -Baseline AIDS -Baseline smear positivity

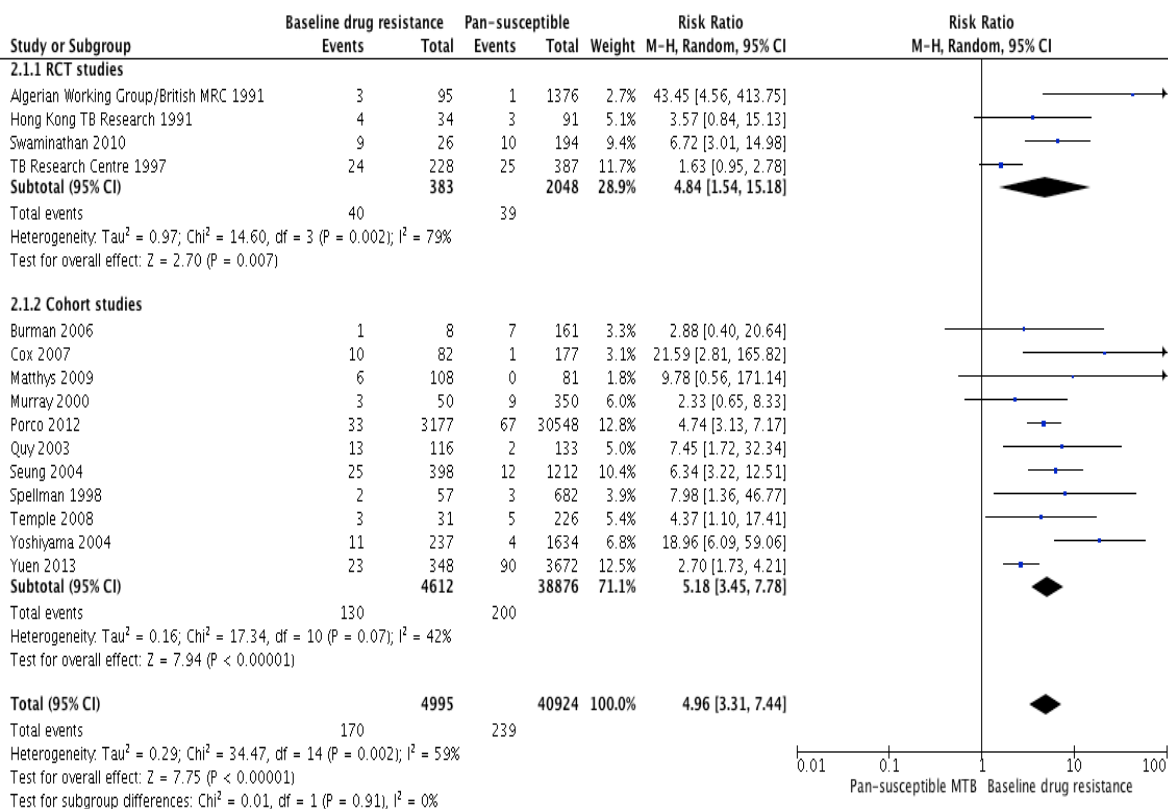
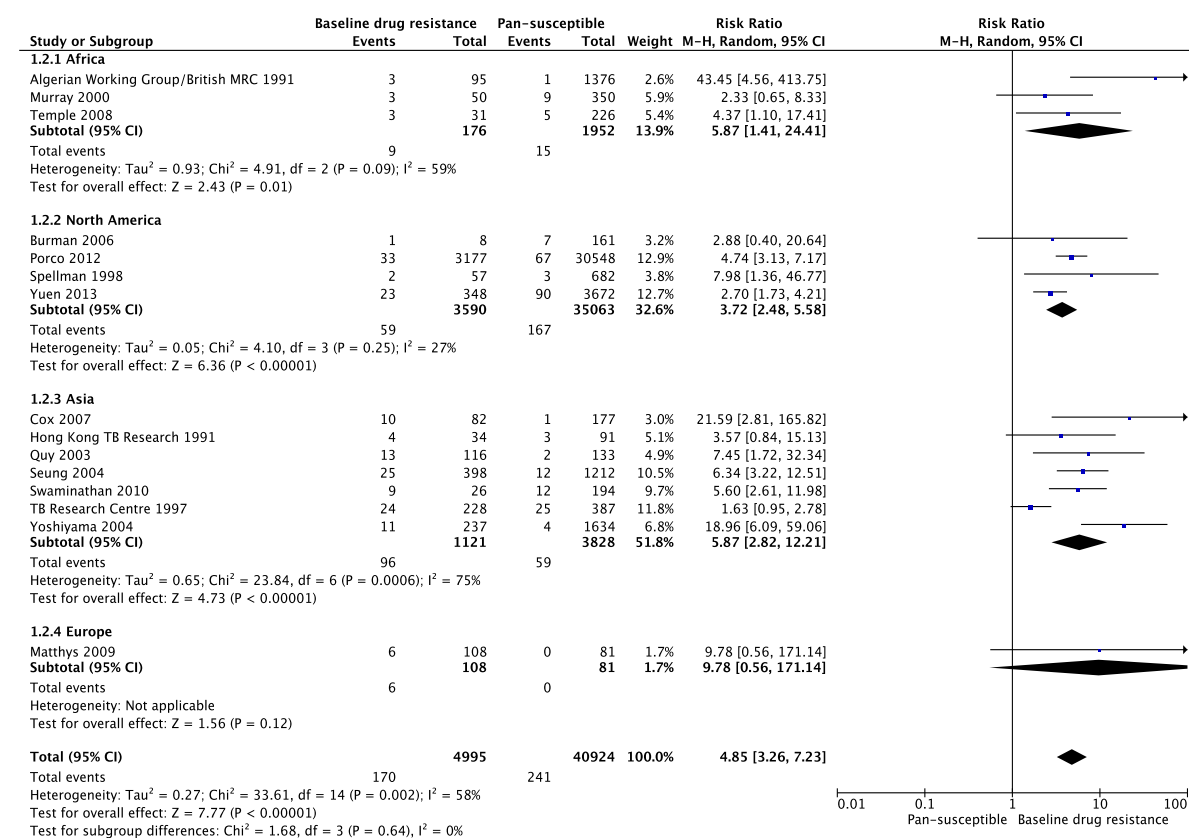
Weiner CID 2005(72)	-Age -Gender -Ethnicity -Baseline BMI -Site of TB involvement -Extensive (bilateral) radiological disease -Cavitatory disease -Rifabutin area under curve (AUC_{0-24hrs}) and maximal concentration -Deacetylirifabutin under curve (AUC_{0-24hrs}) and maximal concentration -Isoniazid AUC and maximal concentration -Birth in Mexico -Baseline CD4 lymphocyte count -Baseline HIV viral load -Deacetylirifabutin AUC and maximal concentration -Nacetyltransferase type 2 genotype
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Abbreviations: DOT directly observed therapy NTM nontuberculous mycobacteria MDR multidrug resistant ART antiretroviral therapy BMI body mass index INH/H isoniazid PZA pyrazinamide R rifampin E thambutol IP intensive phase CP continuation phase Hb haemoglobin ART antiretroviral therapy BMI body mass index . * No statistical analysis was performed in the study and therefore the risk factor for ADR was reported as per trend note

Disease burden and pathogen factors

Baseline drug resistance was positively associated with ADR in 15/16 studies which examined its association. In our meta-analysis of 15 studies (including 45,919 patients), baseline drug resistance (monoresistance or polyresistance) was found to be a significant risk factor for ADR (RR 4.85, 95% CI 3.26 to 7.23), when compared with patients with baseline pan-susceptible MTB (Figure 2.2). There was moderate heterogeneity of the data as evidenced by $I^2=58\%$ (95% CI 26 to 76%), the same positive trend was seen in all 15 studies included.

Figure 2.2 Forest plot of comparison: baseline drug resistance vs pansusceptible MTB, outcome of ADR: 1) by region 2) by study design



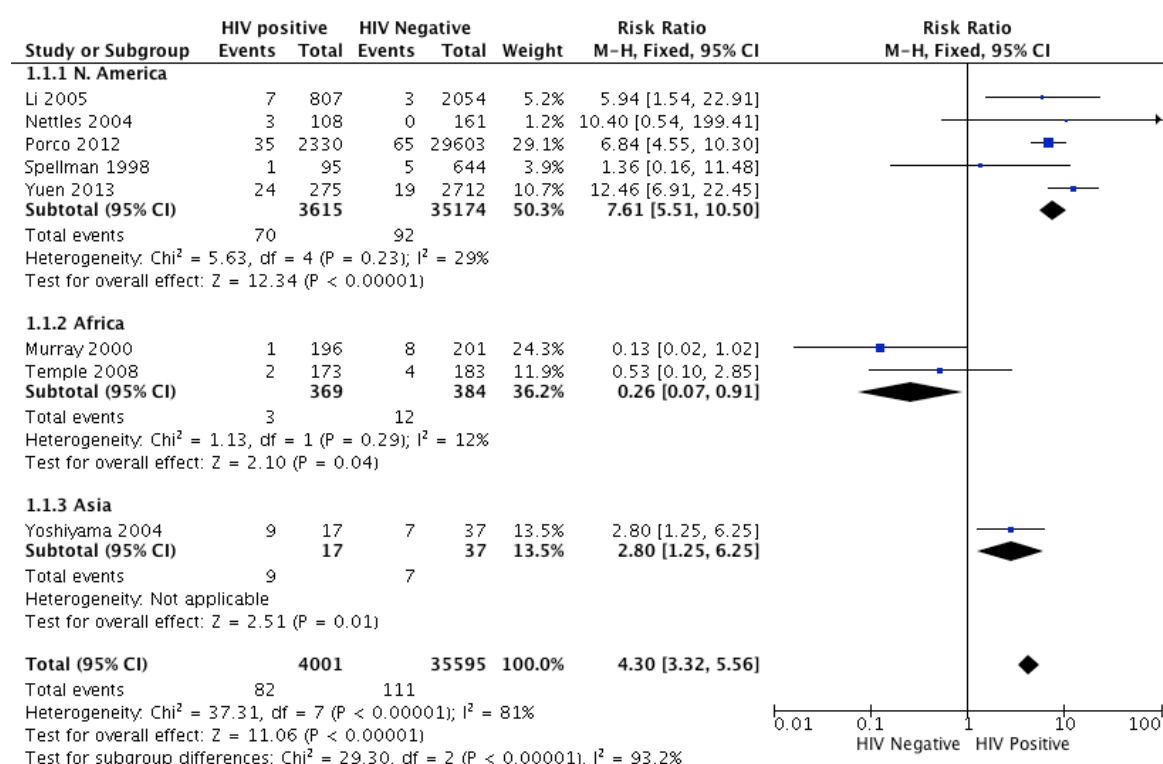
* 1 study was excluded as we were unable to obtain the exact proportion of patients in the study with non-MDR baseline drug resistance and baseline pan-susceptibility either from the paper or by contacting the authors. The endpoint used for the plot for 12 studies was acquisition of isoniazid/rifamycin/multidrug resistance and the end point for 3 studies was acquisition of rifamycin resistance, based on data available.

ADR was significantly associated with extrapulmonary/disseminated TB in 3/5 (60%) studies; with smear positivity in 4/8 (50%) studies; and with extensive radiological disease and cavitary disease in 1/4 (25%) and 1/7 (14%) studies respectively. *M. tuberculosis* complex strain was a risk factor for ADR in 2/4 (50%) studies that examined its role: one study found increased risk with Beijing strains and another with *M. bovis*.

Host immunity and PK variability

HIV-1 co-infection was a risk factor for ADR in 8/10 (80%) studies which assessed it. In a meta-analysis of 8/10 studies (35,595 patients), HIV-1 was a significant risk factor for ADR (RR 4.3, 95% CI 3.32 to 5.56) with overall high heterogeneity I^2 81% (95% CI 64 to 90%) (Figure 2.3). Sub-group analysis by continent for ADR showed a RR of 7.61 (95% CI 5.51 to 10.50) with HIV-1 co-infection in 5 North American studies (heterogeneity I^2 29%, 95% CI 0 to 72%) whilst there was a negative association in 2 African studies (RR 0.26, 95% CI 0.07 to 0.91) with heterogeneity I^2 12%.

Figure 2.3 Forest plot of comparison: HIV-1 status, outcome of ADR by region.



*Only 8/10 studies which examined HIV-1 as a risk factor for ADR were included as we were unable to obtain the exact proportion of those HIV-1 seropositive among the patients that developed ADR from either the paper or by contacting the authors in the other 2 studies. The end point for 5 studies was acquisition of isoniazid/rifampicin/multidrug resistance and the end point for 3 studies was acquisition of rifampicin resistance based on data available.

A low CD4 lymphocyte count at diagnosis in 5/8 studies (63%) and an AIDS diagnosis in 2/2 studies were significant risk factors for ADR amongst HIV-1-infected patients (70, 71). Gastrointestinal symptoms at baseline were associated with ADR in 1/1 study (70) and concurrent use of antifungal azoles in 2/2 studies (44,70). PK variability was found to be a risk factor for ADR in both studies examining its role. Weiner *et al* found that a lower area under the curve (AUC_{0-24hr}) and lower peak concentration (C_{max}) for rifabutin was associated with increased risk of ADR. This was in a sub-cohort of patients who were sampled during continuation phase therapy. There was no significant difference in isoniazid C_{max} or AUC_{0-24hr} in cases of ADR, compared with controls (72). Pasipanodya *et al* found that low rifampicin and isoniazid peak concentrations and AUC_{0-24hr} preceded ADR in 3 patients (41).

Sociodemographic factors

Older age [4/11(36%)], foreign birth [1/3 (33%)], ethnicity [2/5 (40%)], unemployment [1/1], substance abuse [2/4 (50%)] and homelessness [1/3 (33%)] were found to be risk factors for ADR in certain studies.

TB regimen and adherence

Non-adherence was assessed as a risk factor for ADR in 5/32 studies and was associated with ADR in 3/5 (60%) of studies. Directly observed therapy was a risk factor for ADR in 1/8 (12.5%) studies that compared the practice of SAT with DOT. In contrast, SAT was found to be a risk factor for ADR in 4/8 (50%) studies. There was no association between DOT or SAT and ADR in 3 studies. Separate drug formulation, as opposed to fixed dose combination (FDC), was found to be a risk factor for ADR in 1/3 (33%) of studies. Use of rifampicin in the regimen only during intensive phase(51) and lack of ethambutol(53) in a twice/once weekly dosing regimen were associated with cases of ADR in individual RCTs carried out in the 1990s. In one retrospective cohort study, in a sub-analysis of HIV-1 co-infected patients, intermittent dosing of rifampicin during the intensive phase and use of rifampicin instead of rifabutin was associated with ADR(63). This was in contrast to a sub-analysis of HIV-1-infected patients in another study where

there was no significant difference in ADR comparing rifampicin and rifabutin-based regimens(58). In a RCT, a once weekly rifapentine based regimen in continuation phase was associated with ADR in HIV-1 co-infected individuals(45).

2.2.3. Cumulative incidence of ADR

Tables 2.4 to 2.7 report DST data and cumulative incidence of acquired isoniazid, rifamycin and MDR for individual studies stratified by whole cohort, whole cohort with follow up DST, baseline pan-susceptibility, baseline mono-resistance and baseline poly-resistance. In 25 studies which reported acquired MDR, when considering the overall cohort as denominator, the weighted median incidence of acquired MDR was 0.1% (5th to 95th percentile 0.07 to 3.2%). In 20 studies reporting acquired isoniazid resistance, when considering the overall cohort as denominator, the weighted median incidence of acquired isoniazid resistance was 0.1% (5th to 95th percentile 0.1 to 0.7%). In the 27 studies reporting acquired rifamycin resistance, when considering the overall cohort as denominator, the weighted median incidence of acquired rifamycin resistance was 0.1% (5th to 95th percentile 0.09 to 0.7%). In patients with baseline pan-susceptibility (data available in 15 studies) the weighted median incidence of acquired MDR was 0.2% (5th to 95th percentile 0 to 0.9%). In those with baseline pan-susceptibility, acquired isoniazid resistance (weighted median incidence 0.3%, 5th to 95th percentile 0.06 to 2.7%) did not appear to be more frequent than acquired rifamycin resistance (weighted median incidence 0.3%, 5th to 95th percentile 0 to 0.9%). The weighted median incidence of acquired MDR in patients with baseline monoresistance (data available in 12 studies) was 1% (5th to 95th percentile 0.79 to 10%). The weighted median incidence of acquired MDR in patients with baseline polyresistance (data available in 7 studies) was 10% (5th to 95th percentile 7.1 to 15.5%). It must be noted, that the above estimates of incidence of ADR refer only to studies included in this review and with our search strategy, we excluded studies in which no cases of ADR occurred. It should also be noted that the cumulative incidence of ADR is heavily weighted by a single North American study (n=33,725) (16).

2.3 Discussion

Although acquired MDR was rare overall [weighted median frequency 0.1%], it was more frequent in certain risk groups such as those with baseline mono or polyresistance. A

meta-analysis of 15 studies with a moderately heterogeneous data set showed a RR for ADR of 4.85 (95% CI 3.26-7.23) in patients with baseline drug resistance compared with baseline pan-susceptible profiles. Studies reporting ADR as a treatment outcome varied in geographical location, HIV-1 co-infection, retreatment proportions and treatment regimens administered during intensive and continuation phase as summarised in Table 1 and 2. Weighted pooled analysis of a highly heterogeneous data set showed an increased risk of ADR (RR 4.3, 95% CI 3.32-5.56) with HIV-1 co-infection. The data presented disaggregated by continent showed a significant association with HIV-1 co-infection in 5 North American studies whilst there was a trend towards a negative association in 2 African studies. This negative association of HIV-1 with ADR in Africa, may partly be explained by a relatively higher proportion of HIV-1 infected patients who develop ADR dying prior to the detection of ADR. The proportion of patients who died/defaulted in these studies was 11% and 10% respectively. Unfortunately, the 2 African-based studies do not state how many of the patients who died/defaulted were HIV-1 co-infected and hence, it is not possible to verify this hypothesis. Therefore, there is a need for further prospective cohort studies in settings with a high burden of HIV-1, to address whether HIV-1 co-infection is associated with ADR. Advanced immunosuppression as reflected by a lower baseline CD4 lymphocyte count or AIDS at diagnosis was a risk factor in HIV-1 co-infected patients. Poor adherence and extrapulmonary/disseminated disease were risk factors for ADR in 60% of studies. There was less conclusive evidence regarding the role of PK variability, strain type, DOT versus self-administered therapy, fixed dose combinations and choice of rifamycin as risk factors.

The wide range in reported incidence of ADR may be partially explained by lack of standardization in reporting. For example, where follow up culture and DST results are missing, either the denominator can be altered to reflect this, or the denominator remains as the original cohort number; the assumption being that those with missing DST did not develop ADR. In this review the cumulative incidence of ADR in individual studies was presented for both the whole cohort and limited to those with follow up DST data.

Baseline mono or polyresistance has previously been recognised as a significant risk factor for ADR. Lew *et al* (73) carried out a meta-analysis looking at the role of initial drug resistance on TB treatment outcomes. Of note, many studies carried out in the

1970s only used rifamycins during a 2 month intensive phase. Lew *et al* found that the cumulative incidence of ADR increased from 0.8% (95% CI 0.5 to 1%) in baseline pansusceptible cases to 6% (95% CI 4 to 8%) in baseline monoresistant cases and 14% (95% CI 9 to 20%) in baseline polyresistant cases(73). A review by Menzies *et al* (10) found that in patients with baseline isoniazid monoresistance, a longer duration of rifampicin, use of streptomycin, daily therapy initially, and treatment with a greater number of effective drugs were associated with reduced risk of ADR. Jacobsen *et al* reported 9% progression to MDR TB in a cohort with baseline isoniazid monoresistance who received 12 months of quadruple therapy(74). In many resource limited settings, GeneXpert® MTB/RIF is used to test for baseline rifampicin resistance and baseline isoniazid resistance will go undetected. During the continuation phase, those with isoniazid monoresistance (particularly high level) who are still culture positive, will be effectively receiving rifampicin monotherapy. Hence, there is potential for amplification of drug resistance.

The role of HIV-1 co-infection in the acquisition of TB drug resistance has been a topic of debate. In an immunocompromised host, there is an increased risk of disseminated TB; the latter being an independent risk factor for ADR. Hence, there may be an increased bacterial burden leading to an increased probability of bacteria undergoing spontaneous mutation. It is also hypothesized that less fit drug resistant strains survive longer in the context of poor immunity, allowing for development of compensatory mutations to restore fitness(75-77). Some MTB strain types are particularly prevalent in immunocompromised hosts(77). HIV-1 co-infection may cause changes in gut permeability leading to malabsorption of antituberculous drugs(19, 78). As ART becomes increasingly available and guidelines advocate early commencement of ART, it remains to be seen if HIV-1 co-infection will continue to be associated with ADR.

In vitro work in hollow fibre models has suggested that PK variability and inadequate dosing of TB drugs may be an important risk factor for ADR(39, 79). This is supported by findings from cohort studies(41, 72). However, these results need to be confirmed in studies with robust determination of PK indices and appropriate controls. Pasipanodya *et al* (80) reviewed the role of N-acetyl-transferase type 2 genotype in acquired isoniazid resistance. The link they found between fast acetylator status and increased ADR may

be less significant in the context of currently utilised rifampicin-containing multidrug regimens.

Two studies showed an association with MTB strain. Cox *et al* found an association between Beijing strain and ADR(55). In a database of 3696 MTB complex strains, 72% of which were Euro-American lineage, only *M. bovis* was associated with ADR(69). Luria-Delbrück fluctuation analyses have suggested that MTB lineage 2 (Beijing) strains are associated with increased mutation rates and acquisition of drug resistance(81). This may potentially be through sign epistasis where there is favourable interaction between drug resistance mutations and genetic background of the strain(82).

Whilst some studies have used DOT (as opposed to SAT), as a surrogate measure of adherence, this assumption was not made as the outcome of DOT may be confounded by its indication. Non-adherence was measured as a separate risk factor to DOT versus SAT. Non-adherence was a significant risk factor in 3/5 (9, 36,37) of the studies which examined its association with ADR. For the 2 studies which showed no significant association between non-adherence and ADR, there was a trend of positive association for 1 of the studies (26) but in the other, all 5 cases of ADR were noted to be adherent with therapy. The impact of DOT versus SAT on ADR was less clear with a protective effect of DOT seen in 50% of studies. A meta-analysis by Pasipanodya *et al* showed no increased risk of microbiologic failure, relapse, or ADR with SAT compared with DOT(83). Intermittent dosing frequency has been linked with adverse outcomes, including ADR, when administered during intensive phase(44, 63), particularly in the context of HIV-1 co-infection.

There are several limitations to this review. The primary focus of the review was evaluating risk factors for ADR. It is not possible to gather any meaningful data regarding risk factors for an event from a study in which no events are reported and consequently, studies that either reported ADR but no risk factors or 0% ADR were excluded and this potentially affected the estimates of ADR cumulative incidence which was a secondary analysis. These estimates were also heavily weighted by a single large North American study. There was incomplete MTB strain genotyping to rule out the possibility of dual mixed infection or exogenous re-infection. Only 47% of studies confirmed ADR with identical MTB genotype at baseline and follow-up. Even where

genotyping was part of the study design, in some, a proportion of suspected ADR isolates were not available for genotyping. Many studies were retrospective and had small sample sizes and missing DST. Hence, some studies were likely to have been underpowered and there may have been misclassification bias. There were no statistical analyses of risk factors for ADR in 13/32 studies because the primary outcome was not ADR. We were limited to noting trends of risk for ADR in the studies. We only conducted meta-analyses of HIV-1 co-infection and baseline drug resistance as risk factors. For the meta-analyses undertaken, the weighted estimates of effect size, must be taken in context of moderate to high heterogeneity in the random effects model which may be due to clinical and/or methodological diversity.

Previous reviews have focussed on a specific risk factor such as FDC vs. separate drug formulation(84), duration and dosing frequency of rifamycin (85) and baseline isoniazid monoresistance (73). The strength of this review is that it consolidates the multifactorial aetiology of ADR within a single systematic review.

This systematic review concluded that baseline drug resistance and HIV-1 co-infection were significant risk factors for ADR. Overall, there were several limitations of the current evidence and difficulties in evaluating possible contributors to ADR. Although the data are variable, disseminated disease and non-adherence had positive trends of association for ADR. There are likely many other variables contributing to acquired rifamycin and/or isoniazid resistance and studies to date have not adequately evaluated factors such as PK variability and MTB strain type as risk factors for ADR. The multifactorial aetiology of ADR in a programmatic setting should be further evaluated via appropriately designed studies. A prospective cohort is the optimal study design, with nested sub-studies as appropriate. Microbiological outcomes (culture +/- DST) should be ascertained at an interim time point and at the end of treatment in all patients. In cases of a DST profile showing a new resistance pattern, confirmation of true ADR via appropriate MTB strain genotyping is required. Prospective collection of data pertaining to host (socio-demographics, previous TB treatment, co-morbidities, drug side effects, HIV-1 sero-status, CD4 lymphocyte count, ART and viral load and pharmacokinetic data), pathogen and bacillary burden (MIC, smear grading, time to culture conversion, radiological grading of severity and cavitary disease) and programmatic factors

(adherence monitoring, side effects, drug dosing and frequency) will facilitate further understanding of risk factors for ADR. In light of ADR being a relatively rare occurrence, multi-site consortia prospectively building up TB specimen and data repository associated with clinical studies will increase the power of analyses.

3. CHAPTER 3-the cohort, materials and methods

3.1. The cohort and participant-related activities

3.1.1. Setting

Khayelitsha is a predominantly black African township, approximately 30 kilometers from Cape Town in Western Cape, South Africa. Approximately 10% of Cape Town's overall population and 27% of the city's African population live in Khayelitsha. It is one of the largest and fastest growing townships with a population of approximately 500,000. The majority of residents have migrated from rural Eastern Cape in search of better work and education opportunities. Xhosa is the predominantly spoken language. Levels of socioeconomic deprivation and overcrowding are high, 38% of the adult population are unemployed and 74% of households have a monthly income of less than R3200 (£250). Only 36% have completed high school education, although literacy levels are high at 94%. 55% of household live in informal shack accommodation, 62% have access to piped water, 81% electricity and 72% access to a flush toilet (86). Crime rates are very high. Recent improvements in living conditions have been seen secondary to the building of new brick housing, new schools, a new district general hospital and creation of a central business district in the township.

The burden of HIV-1 in Khayelitsha is extremely high (37% antenatal prevalence).

There were 4695 registered TB cases in 2014 (54% microbiologically confirmed) and 227 cases of rifampicin monoresistant or MDR TB (City Health, routine TB data).

Participant recruitment was at Site B Ubuntu Clinic, a primary care integrated HIV/TB clinic. On the same site, there are several healthcare facilities including a day hospital providing outpatient services for stable chronic conditions and an emergency and trauma unit for the stabilization of acutely unwell patients prior to onward referral to the district hospital. This site has a longstanding relationship with Médecins Sans Frontières (MSF) who opened the first antiretroviral therapy clinic here in 2001. Several clinical studies focusing on HIV-1 and TB have been conducted at this site since 2004, with an ensuing growth in research capacity and infrastructure. This includes consultation rooms for the purpose of recruitment and follow up of research patients, office space, phlebotomy area and pharmacy and a separate container housing a digital CXR machine with an adjoining sputum induction booth.

The pharmacokinetic study days were conducted both at Khayelitsha District Hospital and latterly at Site B clinic, in a building previously utilized for the MVA85A vaccine trial.

3.1.2. Study procedures

The study received ethical approval from the University of Cape Town Human Research Ethics Committee (HREC Ref 568/2012). Approval to carry out this study at Khayelitsha Site B Ubuntu Clinic was received from the Western Cape Government, Department of health (RP0006/2013). Informed consent was obtained from all patients prior to enrolment.

Patient recruitment was from March 2013-July 2014. Study recruitment was halted for the majority of November and December 2013 due to a combination of civil unrest in Khayelitsha and on account of annual patient immigration to the Eastern Cape over Christmas.

Patients who had been screened for TB by the clinic and who had been given a diagnosis of rifampicin susceptible pulmonary TB on the basis of a positive GeneXpert® MTB/RIF result, were recruited to the study prior to commencement of TB therapy or during Day 1-3 of TB therapy. Exclusion criteria included age <18 years, receipt of treatment for a previous episode of TB within the last 6 months, a positive pregnancy test, declined HIV testing, inability to expectorate sputum or ≥3 doses of TB treatment taken prior to study screening. Participants received routine programmatic management via the Ubuntu Clinic. They received 2 months of daily rifampicin/isoniazid/pyrazinamide/ethambutol followed by 4 months of daily rifampicin/isoniazid. Antituberculosis therapy (ATT) was delivered in a 4-drug FDC supplied by the National Tuberculosis Control Programme (Rifafour® e-275, Sanofi-Aventis or Ritib®, Aspen, South Africa). Each tablet contained rifampin 150 mg, isoniazid 75 mg, pyrazinamide 400 mg, and ethambutol 275 mg. Weight band-based dosing was used in line with WHO guidelines (87). Patients weighing 38 to 55 kg, >55 to 70 kg, and >70 kg were given doses of 3, 4, and 5 tablets daily, respectively. ATT was administered 7 days/week, along with 25mg pyridoxine daily.

Data was collected on sociodemographics, MDR TB contacts, previous TB treatment and co-morbidities. Co-enrollment of a proportion of participants also involved in an ongoing cross-sectional type 2 diabetes mellitus (T2DM) study allowed identification of both previously known and newly diagnosed T2DM patients as determined by either self-

report/medications review, fasting plasma glucose above 7 mmol/l, random plasma glucose above 11.1mmol/l, glucose tolerance test or HbA1c above 6.5%. At baseline, 2 and 5-6 months, participants had clinical and adherence review, blood was also collected for plasma (sodium heparin tube) and serum (serum separating tube) and patients were asked to expectorate 3 sputa at each study time point. Sputum was expectorated via sputum induction using nebulized 3% saline. At baseline, if expectorating large volumes spontaneously, patients were allowed, to produce a spontaneous sputum sample. At the 2 and 5-6 time points, to maximize quality of sample, sputum induction was performed in all cases, unless the patient specifically refused. The largest volume sputum was sent for culture in MGIT and Lowenstein-Jensen (LJ) slopes with the other 2 frozen and subsequently used for re-culture in case of culture contamination. In a sub-cohort additional sputa were taken pre-chemotherapy and at weeks 1-2 of chemotherapy for RNA extraction and culture (see section 3.7). TRIzol ® LS Reagent (Invitrogen, Carlsbad, CA, USA) in 3:1 volume was immediately added to this sputum, the sample was then vortexed vigorously until the mixture was homogenous and stored on ice, pending freezing at -20°C, and later used for RNA extraction. Baseline bacterial load was estimated via smear grading and days to culture positivity in liquid mycobacterial growth indicator tubes (MGIT). The presence of cavitation >1cm maximum diameter was noted. Extensive disease on chest x-ray was noted as either involvement of >1 lung or involvement of ≥ 1 out of 3 (upper, mid or lower) zones per lung. Participants underwent HIV testing (Abbott Architect HIV Ag/Ab Combo test) and if positive, CD4 lymphocyte count and HIV-1 viral load quantification.

3.1.3. Assessment of adherence to TB medications

At 2 and at 5-6 months, adherence was reviewed via 2 different methods. The first was a missing ≥5 doses as per pill counts and/or self-report in the previous month on interview. The second was performance of the Arkansas test (detection of isoniazid and its metabolites) on archived 2-month urine stored at -80°C (88). Briefly, 4 drops of thawed 2-month urine was added to test tubes containing 30mg of barbituric acid. Two drops of chloramine-T solution (14g/dl) was added, followed by 2 drops of potassium cyanide (5g/dl). All chemicals were purchased from Sigma Aldrich, South Africa. The tube was shaken for 10 seconds. A positive test was recorded if a purple, blue or green colour was seen. A negative test was recorded if a peach, grey or yellow colour was seen. In

case of mixture of colours (indeterminate), the test was repeated. All negative and indeterminate tests were further validated by a qualitative test for acetylisoniazid via liquid chromatography mass spectrometry (LC-MS) performed by the Department of Clinical Pharmacology, University of Cape Town. Previous studies have shown reliable detection (94%) of acetylisoniazid at 24 hours using LC-MS (89). All negative Arkansas test results which were positive (i.e. above the lower level of quantification) by LC-MS, were re-designated as 'positive' for acetylisoniazid.

3.1.4. Characterization of side effects

Participants were interviewed at the 2-month clinical review using a systems-based symptom questionnaire that included the categories central nervous system/neuropsychiatric, peripheral nervous system, gastrointestinal system, musculoskeletal system, skin and other. As all side effects were Common Terminology Criteria for Adverse Events (CTCAE) grade 1-2 (90) (see Appendix 1) and did not require drug withdrawal, attribution of causality to the anti-TB regimen was made in the 'probable'/'possible' categories as per WHO-Uppsala Monitoring Center system(91).

3.1.5 Intensive sampling pharmacokinetic studies

All day intensive PK studies were carried out over a period of 10 months (August 2013-June 2014) in batches of 1 to 5. Participants were fasted 8 hours prior to the commencement of the PK study and consumed standardized meals at 2 hours and 4-5 hours post dose. Blood samples were collected in K2 EDTA bottles immediately before and 1, 2, 3, 4, 6, and 8 hours after observed drug ingestion. They were immediately placed on ice and plasma separated by centrifugation (1500 x g for 10 minutes) within 30 min, before storage at -80°C until analysis. The storage tubes containing the plasma samples were transferred to the analytical laboratory in dry ice.

3.1.6. Definition of outcomes and database search for recurrence

Participants were classified as 2-month culture converters if their 2-month culture was negative for MTB in MGIT and LJ culture. At 5-6 months of treatment, those who were culture negative were defined as cured. Those who were culture positive at 5-6 months

were defined as treatment failures. These patients were recalled for clinical review and further samples for culture and drug susceptibility testing, prior to retreatment/optimization of their regimen as appropriate. Those who completed treatment and were symptom free (as ascertained by the study team or clinic nurses/doctors), without microbiological ascertainment of cure, were classified as treatment completers. We liaised with the clinic doctors and nurses to obtain all end of treatment smear results. Home visits were made to participants who failed to attend their final follow up visits and if we were unable to interview the patient, we left letters at their residence and followed this up with phone calls to them or to their next of kin to ascertain if they were currently in good health. Those who had treatment cure/completion with no microbiologically-confirmed TB recurrence (by censor date 01/11/15) were classified as 'successful outcome'. Electronic database searches were conducted of 1) Western Cape Department of Health Data Repository 2) National Health Laboratory Service database to ascertain reported deaths and TB recurrences during the study follow up period. 'True recurrence' was defined as culture positivity and/or smear 2+/3+ positivity. 'Possible recurrence' was defined as positive GeneXpert® MTB/RIF result of a differing resistance profile to baseline and/or a smear grading of scanty/1+ positivity in the absence of culture confirmation. The rationale here, was even in the persistence of MTB DNA, a differing resistance profile would be indicative of acquired drug resistance. A positive GeneXpert® MTB/RIF result of the same resistance with smear negativity was not regarded as a recurrence due to potential false results in patients with recent previous TB Treatment (92). Failures and recurrences with new patterns of drug resistance were evaluated further for confirmation of homogeneity via spoligotyping (see sections 3.5). All treatment failures and true/possible recurrences were classified as 'unsuccessful outcomes.'

Death subsequent to treatment completion/cure which was not attributable to true/possible TB recurrence was not defined as an unsuccessful outcome.

3.2. Processing of sputum for microscopy and culture

An aliquot of sputum ≤5ml was utilized. 6% NaOH was added in an equal estimated volume to sputum. Phosphate buffered saline (PBS) was added (up to 50 ml mark) in a falcon tube and allowed to stand for 20 minutes. The mixture was vortexed and then centrifuged for 15 minutes at 3000 xg (4 °C). The supernatant was discarded and then

the pellet was resuspended in the supernatant remaining. 1-2 drops directly from the pellet was used for microscopy and then the pellet was re-suspended in 0.5ml of PBS prior to inoculation in MGIT cultures for 42 days as per manufacturers instructions. This smear grading and culture work were carried out by the National Health Laboratory Service, Groote Schuur Hospital.

3.3. MTBDR*plus* 2-month sputa and on 2 and 5/6-month positive cultures (chapter 4 and 5)

MTBDR*plus* line probe assay (LPA), version 2.0 (Nehren, Germany: Hain Lifescience) (hereafter referred to as MTBDR*plus*) was retrospectively carried out on all baseline culture isolates as per manufacturer's instructions to screen for baseline isoniazid monoresistance. Screening for ADR to rifampicin and/or isoniazid was via MTBDR*plus* performed directly on sputum at 2 months. The largest volume sputum was used for both the direct MTBDR*plus* test and MGIT culture. At 2 months, the pellet (derived as described in 3.2), was re-suspended in 1ml PBS with 0.5ml aliquot being used for direct MTBDR*plus* and 0.5ml aliquot inoculated into MGIT for culture. The manufacturer recommended MTBDR*plus* protocol included an additional 20 amplification cycles when performed directly on clinical samples, compared with cultured samples. In cases of new isoniazid/resistance resistance detected on direct MTBDR*plus*, a repeat confirmatory direct MTBDR*plus* test was performed on an archived 2-month sputum. All suspected cases of ADR were also confirmed via MTBDR*plus* carried out on positive cultures of the paired 2- month sputum. MTBDR*plus* was also performed on positive culture isolates at 5-6 months of follow up. This work was carried out by the National Health Laboratory Service, Groote Schuur Hospital.

3.4. Minimum inhibitory concentration (MIC) determination (chapter 4 and 9)

Minimum inhibitory concentrations (MIC) work was performed by Dr. Frederick Sirgel, Stellenbosch University using the BACTEC MGIT 960 system with the EpiCenter software and TBeXist application according the 1% proportion method (93). MIC determinations were run in triplicate. As a difference of 1 log₂ between isolates could be

attributed to technical error, a significant change in MIC in paired baseline/2-month cultures was pre-defined as ≥ 4 fold increase/decrease.

Bacterial suspensions were made from frozen stock (-80°C) in MGIT 960 medium grown at 37°C . Inocula were then prepared from MGIT subcultures 2 days after the tubes flagged positive. Isoniazid and rifampicin were purchased from Sigma Aldrich, South Africa while the BACTEC MGIT 960 PZA Kit (Becton Dickinson Biosciences, Sparks, USA) was used for pyrazinamide. Isoniazid and pyrazinamide was dissolved in sterile distilled water (SDW) and RIF in dimethyl sulfoxide (DMSO) with subsequent dilution in SDW. Stock solutions of the drugs were prepared at concentrations 84 times higher than the highest test concentrations required. These solutions were filter sterilized and stored at -80°C in small aliquots for not longer than 6 months. Serial 2-fold dilutions were made in SDW to obtain working solutions ranging from 21–168 mg/L; 2.54–84 mg/L and 2118 – 8400 mg/L for isoniazid, rifampicin and pyrazinamide, respectively. From each drug dilution, 0.1 ml quantities of isoniazid and rifampicin were transferred to standard MGIT tubes containing 7.0 ml modified Middlebrook 7H9 broth base (pH 6.8) supplemented with 0.8 ml oleic acid-albumin-dextrose-catalase (OADC). The procedure for pyrazinamide was similar, apart from using MGIT 960 pyrazinamide medium (Becton Dickinson Biosciences, Sparks) at a reduced pH of 5.9. The tubes were then inoculated with 0.5 ml of the test organisms to give final 2-fold drug concentrations ranging from 0.25 – 2 mg/L, 0.03 – 1.0 mg/L and 25 – 100 mg/L for isoniazid, rifampicin and pyrazinamide, respectively. A drug-free 1:100 diluted inoculum (1:10 for pyrazinamide) was included to represent the 1% critical proportion which is used to differentiate between susceptible and resistant bacilli. The interpretation of the results were based on a threshold growth unit (GU) reading of 400 by the drug-free control. Drug containing tubes with GU readings of ≥ 100 at the time when the drug-free control reached a value of 400 were considered resistant and those with values < 100 as susceptible. The MIC was therefore defined as the lowest drug concentration that inhibits growth of more than 99% of the bacterial population. Critical concentrations of 0.1 mg/L, 1.0 mg/L and 100 mg/L were used to define resistance to isoniazid, rifampicin and pyrazinamide, respectively.

3.4.1. Extended drug susceptibility testing using Sensititre MycoTB plates (chapter 4)

For participants with suspected ADR, extended phenotypic DST was performed in collaboration with Dr. Frederick Sirgel, Stellenbosch University using Sensititre MycoTB plates as previously described (94) (see table 3.1).

Table 3.1 Extended phenotypic drug sensitivity testing

Critical concentration (µg/ml) used in sensititre MycoTB plates											
Drugs											
INH	RMP	OFL	MXF	AM	SM	RBU	PAS	ETH	CYC	KAN	EMB
0.2/1	1	2	2	5	2/10	0.5	2	5	25	5	5/10

Abbreviations:

INH isoniazid, RIF rifampicin, OFL ofloxacin, MXF moxifloxacin, AM amikacin, SM streptomycin, RBU rifabutin PAS para-aminosalicylic acid, ETH ethionamide, CYC cyclserine, KAN kanamycin, EMB ethambutol

3.5. Spoligotyping (chapter 4 and 6)

Spoligotyping (Gentaur, Kampenhout, Belgium) was carried out by Dr.Lizma Streicher and Ms Ronnett Seldon as per manufacturer's instructions (95). Briefly, 500µl aliquots of MGIT culture were heat killed via heating at 80°C for 1 hour and 5µl of lysate used for PCR. Chromosomal DNA of *M. tuberculosis* H37Rv and *M. bovis* BCG P3 were used as positive controls and water as a negative control. Primers DRa and DRb were used to amplify spacers in the DR region. Biotin labeled PCR products were then hybridized to immobilized spacer-oligos that represent spacers of known sequence. The presence of spacers is visualized on film as black squares after incubation with streptavidin-peroxidase and ECL-detection. Hybridisation patterns obtained were used to assign the isolates to genotype families using the SPOTCLUST SpoIDB3 database (96).

3.6. Liquid chromatography tandem mass spectrometry (LC-MS) for determination of plasma drug concentration (chapter 7 and 8)

Drug concentrations were determined at the Pharmacology Laboratory at the University of Cape Town by Dr. Lubbe Wiesner using validated LC-MS methods. The methods were validated over the concentration ranges 0.0977 to 26.0 µg/ml for isoniazid, 0.117 to 30.0 µg/ml for rifampicin and 0.200 to 80.0 µg/ml for pyrazinamide. (97). The precision (% CV) was less than 3% at low, medium, and high quality control levels. Rifampicin, isoniazid, and pyrazinamide concentrations reported as below the limit of quantification (BLQ) were less than 0.117 µg/ml, 0.1 µg/ml and 0.2 µg/ml respectively.

3.7. Sputum RNA extraction, cDNA synthesis, primer design and qPCR (chapter 6)

One millilitre aliquots of thawed TRIzol-LS-sputa samples were ribolyzed at the setting of 6.5 for 45 seconds and this was repeated after 5 minutes. 200 µl of chloroform (CHCl₃) was added, the mixture vortexed vigorously and centrifuged at 3000rpm for 10mins. The supernatant was transferred to a new tube, 600µl CHCl₃/ isoamyl alcohol (IAA) (24:1) was added and centrifuged at 13000 rpm for 1 minute. The top aqueous layer was transferred to a new tube and mixed well with an equal volume of 100% ethanol. RNA from the RNA-ethanol mixture was then extracted using the RNA Clean and Concentrator kit (Zymo, Irvine, CA, USA).

First strand cDNA was synthesized from 1 µg RNA from each sputum sample using the Superscript VILO system (Invitrogen Carlsbad, CA, USA) and random hexamers according to manufacturer's instructions. This process was also carried out for H37Rv exponential and stationary phase cultures. Serial dilutions were carried out on H37Rv cDNA until the average cycle threshold (Ct) for 16S was comparable for 16S of sputa. This concentration was carried through for qPCR to allow optimal comparability. Primers and probes complementary to 8 messenger (m)RNAs (atpA, SigA, SigB, nuoG, ndh, icl1, prpD, rpsN1, rpsN2, HspX), 2 small (s)RNAs (MTS2823 and MTS1338) and ribosomal RNA 16s were designed using Primer Quest Tool (Integrated DNA Technologies, UK).

Table 3.2 Primer and probe sequences

Gene	Name	Probe (dye-sequence)	Forward primer	Reverse primer
atpA	SP_Rv1308	FAM-CTGGATGTACGAGGGCAAGCATGT	AGTCCGCCGGTTTCAAAT	CTTAGTCAGGTCGTCGAAGATG
sigA	SP_Rv2703	FAM-ACGCAAGGACGCCGAAGTCA	GTGATTTCTGCTGGGATGAAGA	TACCTTGCCGATCTGTTTGAG
sigB	SP_Rv2710	FAM-TATCTGAACGGCATCGGCAAGACG	AGCGATCTGGATGCTCAAAG	TTGGCCAGTTCGACTTCAC
prpD	SP_Rv1130	FAM-TTGACGAGCACAAGATCGACCAT	CTATGAGATCCACATCGACCTAAC	GCGTGGTAGATGGTCTCTTG
icl1	SP_Rv0467	FAM-TCTGCTCGGCTCGC	CAGCACATCCGCACTTTGAC	ATCACCACCGTGGAACATC
nuoG	SP_Rv3151	FAM-CCTTGTCGCACATCGGGCAATC	GTGATGGAAGTCTGCTGAT	CCGTTAGACATTGCCTGGTT
ndh	SP_Rv1854c	FAM-AAGATCGGTCCCGTTGAGTTCAGC	ATTCCAGTACTTCGACAAGGG	CAAATCAGCCAGGCGATAAAG
rpsN1	SP_Rv0717	FAM-TCTACCGCAAGTTCGGGCTGTG	CGAAGAAGGCACTGGTCAA	ATCTCGCGCAGGCAAAT
rpsN2	SP_Rv2056c	FAM-CCACCGTCGCCCCGT	AAGAAGTCCAAGATCGTCAAGAATC	GGGATCGGATGATGTCTTTGAG
MTS2823	SP_MTB000078	FAM-CAGATGGCATCGACGG	GGTCTGCGTAATTGCGAACA	CTGTAGCCCCACCCAAAGG
hspX	SP_Rv2031c	FAM-CGGCTGGAAGACGAG	CCTTCGACACCCGGTTGAT	CGTAGCGCCCCTCTTTCA
MTS1338	SP_MTB000077	FAM-CAGTATTGGTGATACCGGG	CCGCCTGCTGTGTTTGGT	GGGCAGATCACCGGGTTT
16s	SP_MTB000019	FAM-TGAAATCTCACGGCTTAA	GGTGGTTTGTGCGTGTGTTT	GCCCGCACGCTCACA

Primers and probes with strong potential to form hairpin structure were excluded and potential cross-reactivity was checked by BLASTN tool. qPCR was carried out on customized TaqMan array plates (Applied Biosystems, UK) in triplicate on an ABI Prism 7000 (Applied Biosystems, UK). Conditions were 95°C/10 minutes, followed by 40 cycles of 95°C/15 seconds and 60°C/60 seconds.

3.8. Axenic culture of reference *M. tuberculosis* (chapter 6)

H37Rv was cultured by Dr. Rachel Lai in standard 7H9 media containing 0.2%v/v glycerol, 0.05%v/v Tween 80 and 10% ADC in roller bottles. Exponential phase culture was harvested when OD600 measured 0.71-1.0 and stationary phase culture was harvested 4 weeks after the OD600 reached a plateau of 1.0. 10mL of culture was pelleted by centrifugation and washed once with PBS before addition of 2ml TRIzol® LS Reagent (Invitrogen, Carlsbad, CA, USA). RNA was extracted using the same protocol as for sputum (3.7).

3.9. ELISA assays (chapter 10)

A quantitative sandwich enzyme-linked immunosorbant assay (ELISA) technique was used to determine serum concentrations of sCD14 (R&D systems, Minneapolis, USA) and plasma concentrations of haemoxygenase-1 (HO-1) (EKS-800, Stressgen/ Assay Designs, Ann Arbor, MI, USA) according to manufacturers' instructions. Three metabolites of oxidative damage to nucleic acids (8-hydroxy-2'deoxyguanosine, 8-hydroxyguanine and 8-hydroxyguanosine), hereafter referred to as 8-OHdG, were quantified in plasma using a competitive ELISA technique (Cayman Chemical, Ann Arbor, MI, USA) as per manufacturer's instructions. The inter-plate co-efficient of variation for all assays was less than 1%. All plates were read on a Bio-Rad iMark microplate reader (Bio-Rad; Hercules, CA, USA) with final concentrations calculated from a standard curve using MPM6 software (Bio-Rad; Hercules, CA, USA). All samples had been biobanked at -80°C prior to the experiments. The running of these assays was performed by the candidate, assisted by Dr. Bruno Andrade.

3.10. Luminex/xMAP® technology-based multiplex assays (chapter 10)

Multiplex assays are particularly useful in allowing the simultaneous evaluation of multiple analytes within a single specimen using a small volume of sample.

Principles of assay: The technology uses 5.6µm polystyrene beads. Analyte-specific antibodies are pre-coated onto colour-coded magnetic microparticles. Microparticles, standards and samples are pipetted into wells and the immobilized antibodies bind the analytes of interest. After washing away any unbound substances, a biotinylated antibody cocktail specific to the analytes of interest is added to each well. Following a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate, which binds to the biotinylated antibody is added to each well. A further wash removes unbound streptavidin-phycoerythrin, the microparticles are re-suspended in buffer and read using a Luminex analyser. A magnet in the analyser captures and holds the microparticles in a monolayer whilst 2 spectrally distinct light emitting diodes identify the analyte and quantifies the phycoerythrin-derived signal (proportional to the amount of analyte bound). All standards are run in duplicate on each plate. For each analyte, the software plots a best-fit (linear or curvilinear) regression line through the standard points, interpolates the test samples on that regression line, and reports the calculated concentrations.

Samples were diluted to varying degrees so that the analyte of interest was in the dynamic range of the standard curve. The MILLIPLEX® kit human cytokine/chemokine magnetic bead panel (Millipore, Billerica, MA, USA) was used to detect interleukin (IL)-12p40, IL-12p70, interferon (IFN)α2, tumour necrosis factor (TNF)α, IFNγ, CXCL-10, IL-1α, IL-1β, IL-8, IL-6, IL-17, granulocyte macrophage colony stimulating factor (GM-CSF) and vascular endothelial growth factor (VEGF) in serum as per manufacturer's instructions. Human matrix metalloproteinase (MMPs) 1,3,7,8,9,10,12 and human tissue inhibitors of metalloproteinases (TIMPS) 1,2,3,4 were measured in serum using magnetic luminex® performance assay (R&D Minneapolis, MN, USA) as per manufacturer's instructions. Acute phase proteins C-reactive protein (CRP), serum amyloid protein P (SAP), tissue plasminogen activator (tPA), ferritin, α-2 macroglobulin and haptoglobin in serum were determined using the Bioplex PRO™ acute phase

multiplex assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. All plates were read on a Bio-plex 200 reader (Bio-Rad; Hercules, CA, USA). All samples had been biobanked at -80°C prior to the experiments. These assays were performed assisted by Dr. Bruno Andrade.

CHAPTER 4- Frequency and determinants of acquired drug resistance in a programmatic setting with a high prevalence of HIV-1 co-infection

Tuberculosis remains the foremost cause of death in HIV-1-infected individuals in Africa. An increasing problem is the acquisition and transmission of drug resistant (DR) strains of *Mycobacterium tuberculosis* (MTB). In 2014, in South Africa there were 18,734 laboratory-diagnosed cases of rifampicin (RIF) resistance among an estimated 450,000 incident TB cases (98).

Acquired drug resistance (ADR) is the amplification and fixation of both new mutations within a clonal population and minority pre-existing mutants leading to phenotypic resistance. This process occurs during drug treatment. The systematic review conducted in Chapter 2 showed a significantly increased risk of acquired RIF and/or isoniazid (INH) resistance with baseline mono/polyresistance and with HIV-1-co-infection. However, HIV-1 was not a increased risk factor for ADR in African countries with a high burden of HIV-1 co-infection. With earlier commencement of antiretroviral therapy (ART), as per programmatic guidelines, it remains to be seen if HIV-1 co-infection will continue to be associated with ADR. One study conducted in India showed that in patients taking a thrice-weekly TB regimen, ART reduced but did not eliminate risk of ADR (99).

Genotyping studies suggest multidrug-resistant (MDR)-TB in South Africa is perpetuated chiefly through transmitted drug resistance (13, 100, 101). Incident ADR cases are hypothesized to fuel and sustain transmission of resistance. In many resource-limited settings, including the South African TB programme, GeneXpert® MTB/RIF is used to test for baseline RIF resistance. Baseline INH monoresistance (IMR) will be undetected. According to programmatic guidelines, those who are smear negative at 2 months should be switched to a continuation phase with RIF/INH. Hence those with IMR, will effectively receive RIF monotherapy and there is potential for amplification of drug resistance.

We carried out a prospective cohort study to determine the incidence of and risk factors for ADR in Khayelitsha, Western Cape South Africa.

4.1. Chapter hypothesis and aims

Overall hypothesis

In a high burden TB setting with a high prevalence of HIV-1 co-infection, acquired drug resistance in RS RIF susceptible (RS) TB contributes to the MDR TB epidemic.

Specific chapter aims

1. To determine the cumulative incidence of ADR in cases which are RS at baseline.
2. To compare 2 methods for assessing treatment adherence.
3. To assess determinants of 2-month culture conversion.
4. To ascertain if there is any increase in MIC acquired during chemotherapy, comparing baseline and 2-month MTB strains for RIF, INH and pyrazinamide (PZA).
5. To determine the prevalence of IMR at baseline and to evaluate its significance in clinical outcomes in this setting.

4.2. Methods

Study cohort recruitment (3.1.1.), procedures (3.1.2.) and adherence assessment (3.1.3.) are previously described. At 2 and 5-6 month follow up, participants had sputum induction and screening for ADR to RIF +/-INH via use of MTBDR*plus* line probe assay performed on positive cultures (3.2. and 3.3.). Spoligotyping was carried out on the sequential isolates from cases with new resistance detected to investigate dual mixed infection at baseline and exogenous re-infection during treatment (3.5.). Stored baseline isolates were retrospectively tested for IMR (n=279) through use of MTBDR*plus* (3.3.) and/or minimum inhibitory concentration (MIC) testing and notes review was undertaken to determine duration of treatment in cases of IMR. MICs were determined in a sub-cohort at baseline (n=109) and 2 months (n=20) using BACTEC MGIT 960 system with the EpiCenter software and TBExist application (3.4.). Treatment outcomes were

determined as per 3.1.6. For participants with ADR, extended phenotypic drug susceptibility testing (DST) was performed using Sensititre MycoTB plates (3.4.1.).

Statistical analyses

We sought to ascertain the probability of occurrence of ADR as our primary endpoint. We assumed a poisson distribution with background rate. Assuming an incidence of ADR as 1%, in order to see at least 1 case of ADR with 95% probability, 299 patients needed to be followed up during chemotherapy.

Differences in categorical variables were compared using chi-squared tests. clinical covariates were considered in univariate logistic regression analyses to determine predictors of 2-month culture conversion. P-values from the univariate analyses were used to guide variable selection for the multivariate model.

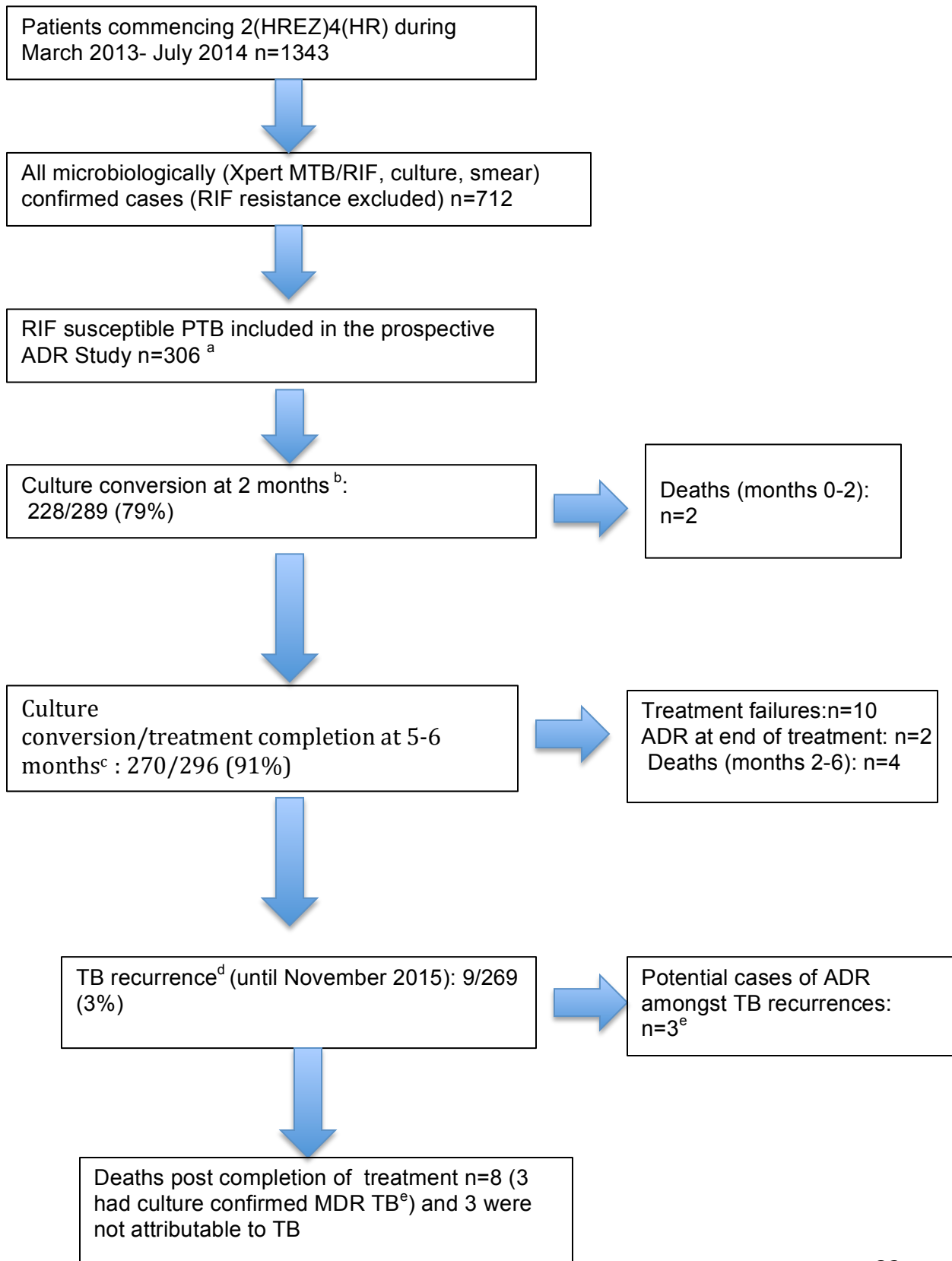
All figures and statistical analyses were carried out in GraphPad (La Jolla,CA) Prism (6.0).

4.3. Results

4.3.1. Clinical characteristics of cohort

306 microbiologically confirmed RS pulmonary TB patients were recruited. Figure 4.1 outlines study recruitment in the context of the overall clinic TB treatment starters. Patients in the cohort had clinical follow up for the duration of TB treatment and a median of 22 months (IQR 18-28) follow up to ascertain disease recurrence via electronic database searches.

Figure 4.1 Study recruitment and follow up



^a Reasons why patients were not recruited to ADR study included: 1) they had taken ≥ 3 doses of TB treatment (transfers in from other clinics/hospitals and those who started treatment on days the study team was not recruiting) 2) declined participation/unable to provide informed consent 3) unwilling to undergo HIV testing 4) unable to expectorate sputum

^b 17 did not produce sputa at 2 months

^c 10/296 patients were treatment defaulters and assumed not to culture convert at 5-6 months. There were 10 participants with unknown treatment outcomes (not included in denominator).

^d 8 of these recurrences were either culture confirmed and/or smear 2+/3+ positive (definite recurrence). One of these recurrences was symptomatic, scanty smear positive and had a confirmatory rifampicin susceptible Xpert MTB/Rif test (possible recurrence).

^e All 3 potential cases of ADR amongst TB recurrences died.

Abbreviations: PTB; pulmonary tuberculosis; RIF rifampicin; ADR acquired drug resistance

Demographic and clinical characteristics were comparable between the study cohort and the overall clinic (table 4.1). None of the study participants had known MDR TB contacts or had received INH monotherapy for latent TB infection. The cohort was 62% HIV-1 co-infected. The median CD4 count was 231 cells/mm³ (IQR 101-376): 25% had a CD4 lymphocyte count <100 cells/mm³. 39% were on ART at baseline and 25% were virologically suppressed (VL<40copies/ml). 20/59 (34%) patients on ART >6 months at baseline had viral load >300 copies/ml. 8/20 (40%) demonstrated poor virological control (viral resistance testing not done to distinguish between resistance and poor adherence) whilst on a second line (protease inhibitor based) ART regimen. By 2 months, 71% of HIV-1-infected patients had been commenced on ART. Table 4.1 details clinical characteristics of the study cohort, stratified by HIV-1 serostatus. Thirty two percent of participants had been previously treated for TB. The prevalence of T2DM in this cohort was 6%. Thirty seven percent of the cohort reported at least weekly consumption of alcohol. Thirty two percent reported drug side effects. Of note was the observation that 57/180 (32%) of all male study participants had previously been in prison.

Table 4.1 Characteristics of study cohort and overall clinic population

	ADR cohort n=306 (68% new cases)		Microbiologically confirmed TB in overall clinic n=712* (69% new cases)	
Cohort characteristic	HIV +ve n=191 (62%)	HIV -ve n=115 (38%)	HIV +ve* n=448 (64%)	HIV -ve * n=252 (36%)
% Male	48	77	47	70
Median age/years (IQR)	35 (30-42)	34 (27-49)	36 (31-43)	37 (29-52)
% xhosa ethnicity	95%	99	NA	NA
Smoker status:			NA	NA
% Never	57	43		
% Ex	22	20		
% Current	21	37		
% Alcohol use	33	43	NA	NA
% Recreational drug use	5	7	NA	NA
% Ex-prisoner	20	20	NA	NA
% Ex-miner	4	10	NA	NA
% Diabetic	5	7	NA	NA
% Drug side effects during treatment	36	25	NA	NA
Median BMI	22 (20-24)	20 (19-23)	NA	NA
% Extensive radiological disease	55	83	NA	NA
% Cavities	35	53	NA	NA
Median CD4/cells/mm ³ (IQR)	231 (101-376)		210 (96-357)	
% VL<40 copies/ml at baseline	25		NA	
% ART at baseline	39		34	
% Smear +ve (1+ to 3+)	43	63	52	59
Median TTD/days (IQR)	12 (8-17)	9 (7-13)	NA	NA

* 12 cases with unknown status excluded from overall clinic statistics

NA data not available

Abbreviations: BMI body mass index, VL viral load, TTD time to detection (of culture positivity)

4.3.2. Adherence

Adherence was assessed by self-report/pill count in the previous month (method 1) and spot check for urinary metabolites of acetylisoniazid at 2/5-6-month review (method 2). All samples which were negative/indeterminate via Arkansas test (peach/yellow/grey), were also tested for acetylINH using LC-MS (see 3.1.2). All negative Arkansas test results which were positive (i.e. above the lower level of quantification) by LC-MS, were re-designated as 'positive' for acetylINH (table 4.2).

Table 4.2 Arkansas/LC-MS data

	≤24hrs since last dose	>24hrs since last dose
Arkansas positive n=218 'time since last dose' data available for n=203	150/203 (74%)	53/203 (26%)
Changed to positive as per mass spec (Arkansas and Mass Spec discordant) n=32	23/32 (81%)	9/32 (28%)
Negative by Arkansas and Mass Spec n=26 'time since last dose' data available for n=21	14/21 (67%)	7/21 (33%)
Near LLQ as per mass spec and negative by Arkansas n=10	5/10 (50%)	5/10 (50%)

Abbreviations: LLQ low level of quantification

Potential confounders of the Arkansas test included whether or not urine collected was the first voided urine for the day, variation in total fluid intake in the preceding 24 hours, variation in timing of previous dose and the participant's acetylator status. All these could potentially have affected results. Both the Arkansas test and LC-MS have not been validated to reliably detect presence of urinary INH metabolites beyond 24 hours (89). Hence, it is not a reliable test of longer term adherence and it is possible that participants were more likely to take their medication on the day of scheduled study visit. This may partially explain the poor concordance between identified non-adherent participants between the 2 methods.

8 and 12% of the cohort were considered to be non-adherent at 2/5-6-month review by methods 1 and 2 respectively (table 4.3). There was no significant difference in the proportion of retreatment vs new patients who were non-adherent by method 1,(6/93 (6%) vs 23/192 (12%)), nor was there any significant difference in the proportion of retreatment vs new patients who were non-adherent by method 2 (6/98 (6%) vs 18/191 (9%).

Table 4.3 Measure of adherence and culture conversion outcomes at 2 months with both methods

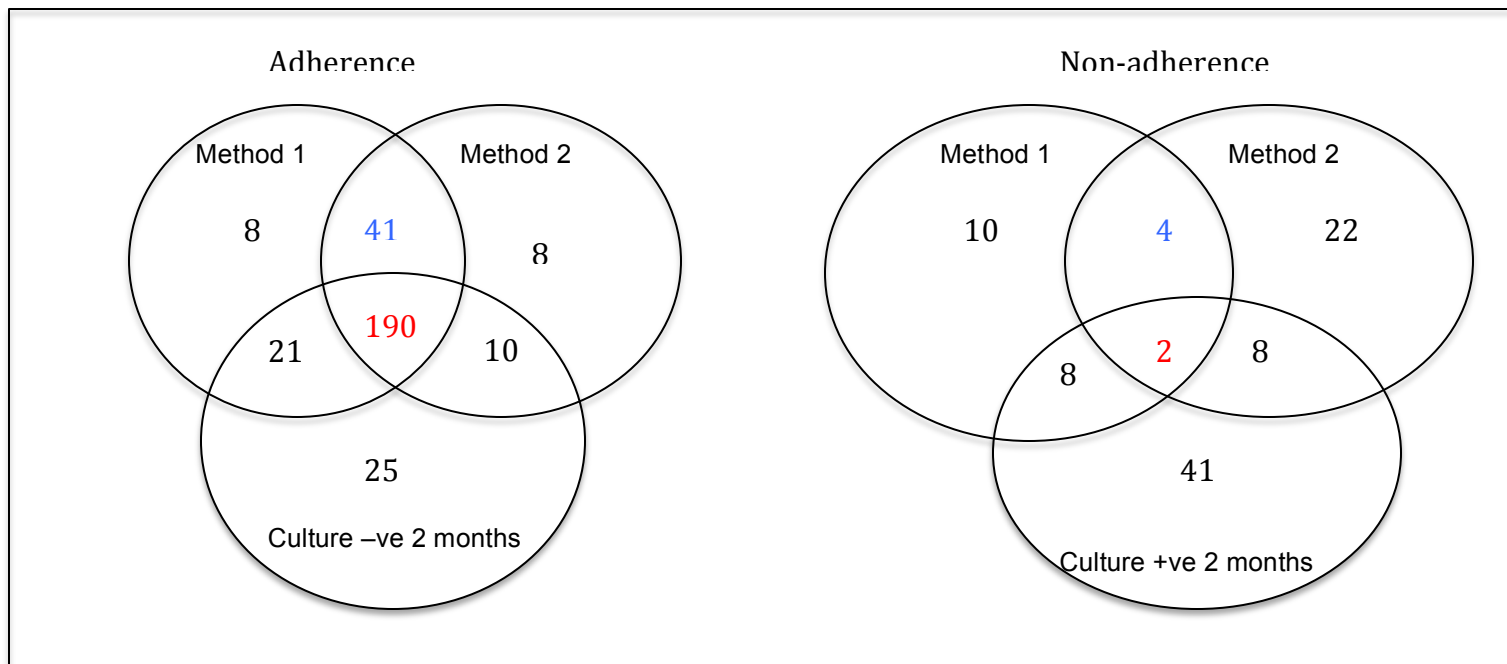
Method of adherence measure at 2 month clinic visit	Non-adherent	Proportion culture conversion in adherent (% , 95% CI)	Proportion culture conversion in non-adherent, (% , 95% CI)	P value
Method 1: Missing $\geq$ 5 doses TB medication in past month as per any of 2 assessments i) self-reports ii) pill counts	24/284 (8%)	211/260 (81, 76-86)	14/24 (58, 37-78)	0.01
Method 2: 'spot check' for non-adherence in past 24 hrs as per a -ve Arkansas test/LC-MS performed to detect urinary INH metabolites	36/285 (12%)	200/249 (80, 75-85)	26/36 (72, 54-86))	0.2

Abbreviations: INH isoniazid, LC-MS liquid chromatography- mass spectrometry.

Adherence assessment by self-report /pill count (method 1), compared with a spot check for urinary metabolites of acetylisoniazid (method 2) had good concordance for identifying 'adherent' cases (see figure 4.2). In the 24 cases of 'non-adherence', as per method 1, there was concordance with method 2 in 25% and in the 36 cases of 'non-adherence' as per method 2, there was concordance with method 1 in 17%. Hence, the

2 methods had low concordance for 'non-adherent' cases (see figure 4.2). The overall inter-assay agreement was summarized by kappa statistic 0.11, $p=0.02$.

Figure 4.2 Concordance of 2 measures of adherence and prediction of culture conversion



Method 1 Self report/pill counts, Method 2 Urinary isoniazid metabolite

In method 1 only, there was a significant difference in rate of culture conversion in 'adherent' vs 'non-adherent' cases, suggesting that method 1 was a more discerning method of judging adherence relative to its effect on 2-month culture conversion (see table 4.4). There was a significant association with previous incarceration and non-adherence by method 1. Non-adherence rates were 11/55 (20%) in ex-prisoners vs 13/229 (6%) in those who had never previously been incarcerated ($p < 0.001$).

Assessment of adherence in a high burden low resource setting is challenging. In this programmatic setting many patients were switched from directly observed therapy (DOT) to self-administered therapy (SAT) after a few days post commencement of treatment. Community support workers were designated to visit patients at home to do unscheduled adherence assessments. However, the community is mobile with participants travelling to and from their home provinces, often living in informal settlements and contact details changing frequently. Hence, measurement of adherence needs to be logistically feasible and context specific. In this study, missing ≥ 5 doses TB medication in the past month as per any of 2 assessments i) self-reports ii) pill counts was found to be a better discriminator of culture conversion at 2 months than a 'spot check' for non-adherence in past 24 hours as per a negative Arkansas test/LC-MS performed to detect urinary INH metabolites.

4.3.3. Treatment outcomes

2-month culture conversion

The culture conversion rate at 2 months was 79% (95% CI 74-83%). On univariate analysis, retreatment status (OR 0.44, 95% CI 0.26-0.84), non-adherence by method 1 (see table 4.3) (OR 0.32, 95% CI 0.13-0.75), T2DM (OR 0.34, 95% CI 0.12-0.93), smear 1+ positivity (OR 0.39, 95% CI 0.17-0.92), smear 3+ positivity (OR 0.21, 95% CI 0.07-0.61), baseline time to culture positivity (TTP) (OR 1.12, 95% CI 1.05-1.19), extensive radiological disease (OR 0.24, 95% CI 0.11-0.52) and cavitatory disease (OR 0.45, 95% CI 0.25-0.80) were significantly associated with 2-month culture conversion. Multivariate logistic regression analyses for the whole study cohort showed non-adherence (by method 1) (OR 0.29, 95% CI 0.11-0.80), T2DM (OR 0.24, 95% CI 0.07-0.83), smear grade 3+ (OR 0.21, 95% CI 0.07-0.61), baseline TTP (OR 1.1, 95% CI 1.02-1.18) and extensive disease on chest x-ray (OR 0.32, 95% CI 0.14-0.75) remained significant determinants of culture conversion (table 4.4). Multivariate logistic regression analysis

for HIV-infected participants only showed that smear grading 3+ (OR 0.13, 95% CI 0.04-0.43) and cavitatory disease (OR 0.33, 95% CI 0.13-0.82) on chest x-ray were significant determinants of 2-month culture conversion at 2 months (table 4.4).

These significant predictors of the interim outcome of 2-month culture conversion have translational implications. Patients who are diabetic, non-adherent, those with high smear grading/short time to culture positivity, and those with extensive disease on chest radiograph should have more intensive monitoring and may require a longer duration of intensive phase treatment.

Table 4.4 Predictors of culture conversion at 2 months

Variable	Whole cohort ^a OR univariate (95% CI)	p- value	OR multivariate (95% CI)	p- value	HIV cohort ^b OR univariate	p- value	OR multivariate (95% CI)	p- value
Male sex	0.65 (0.35-1.20)	0.17			1.05 (0.51-2.52)	0.89		
10 yr increase in age	0.78 (0.61-1.01)	0.06	0.75 (0.56-1.01)	0.06	0.90 (0.59-1.38)	0.63		
BMI	1.04 (0.98-1.12)	0.21			1.05 (0.95-1.16)	0.31		
Retreatment	0.46 (0.26-0.84)	0.01	0.54 (0.30-1.04)	0.07	0.55 (0.25-1.23)	0.15	0.54 (0.22-1.32)	0.18
Smoker status: Never Ex Current	1 1.14 (0.53-2.56) 0.82 (0.42-1.59)	0.77 0.54			1 1.21 (0.44-3.32) 1.14 (0.42-3.13)	0.71 0.56		
Alcohol use	0.58 (0.32-1.03)	0.06	0.54 (0.28-1.07)	0.08	0.60 (0.27-1.32)	0.21	0.43 (0.16-1.11)	0.08
Ex-prisoner	0.55 (0.28-1.08)	0.08			0.49 (0.20-1.20)	0.12		
Ex-miner	0.52 (0.19-1.44)	0.21			1.21 (0.14-10.4)	0.86		
Diabetes	0.34 (0.12-0.93)	0.04	0.18 (0.06-0.55)	<0.01	0.28 (0.07-1.07)	0.06	0.13 (0.03-0.62)	0.01
Drug side effects	0.95 (0.51-1.75)	0.86			1.17 (0.51-2.69)	0.78		
Non-adherence method 1 ^c	0.32 (0.13-0.75)	0.01	0.29 (0.11-0.80)	0.02	0.26 (0.08-0.80)	0.02	0.24 (0.07-0.90)	0.03
Non-adherence method 2 ^c	0.62 (0.28-1.37)	0.24			0.43 (0.16-1.14)	0.08		

INH resistance ^d	0.88 (0.27-2.8)	0.83			1.32 (0.15-11.42)	0.82		
Smear grading: Neg/scanty	1		1		1		1	
1+	0.39 (0.17-0.92)	0.04	0.54 (0.22-1.30)	0.17	0.31 (0.11-0.92)	0.08	0.33 (0.11-0.99)	0.05
2+	0.53 (0.16-1.68)	0.28			0.52 (0.165-1.69)	0.28		
3+	0.21 (0.07-0.61)	<0.01	0.18 (0.08-0.41)	<0.01	0.21 (0.07-0.61)	<0.01	0.13 (0.06-0.64)	0.01
Time to culture positivity	1.12 (1.05-1.19)	0.001	1.1 (1.02-1.18)	0.01	1.10 (1.01-1.18)	0.03	1.06 (0.98-1.16)	0.16
HIV status	1.77 (0.99-3.17)	0.05			N/A	N/A		
Log ₁₀ CD4 count	N/A	N/A			0.54 (0.22-1.33)	0.18		
Log ₁₀ Viral Load	N/A	N/A			0.99 (0.99-1.00)	0.92		
ART at baseline	N/A	N/A			0.84 (0.40-1.36)	0.23		
Extensive radiological disease	0.24 (0.11-0.52)	<0.01	0.32 (0.14-0.75)	0.01	0.31 (0.13-0.78)	0.01		
Cavitatory disease	0.45 (0.25-0.80)	<0.01	0.55 (0.27-1.10)	0.09	0.29 (0.13-0.64)	<0.01	0.33 (0.13-0.82)	0.02

^a n= 287

^b n=180

^c method 1 was missing ≥ 5 doses TB medication in past month as per any of 2 assessments i) self-reports ii) pill counts
method 2 was a 'spot check' for non-adherence in past 24 hrs as per a -ve Arkansas test performed to detect urinary INH metabolites

^d n=261

Long-term outcomes

91% (95% CI 87-94%) of patients had an outcome of 5/6-month culture conversion/treatment completion (230 were culture converters, 40 were treatment completers). There were 10 treatment failures and 9 recurrences over the study follow up period. There were 6 deaths during treatment and 8 subsequent to treatment completion, of which 3 were confirmed cases of MDR TB (figure 4.1). There were 2 cases of ADR identified during treatment. Their clinical characteristics and sequential phenotypic DST profiles and spoligotypes are outlined in table 4.5. Both had cavitary disease on chest x-ray and documented poor adherence. Case 1 (HIV-1-uninfected) was a smear negative retreatment case and had PZA monoresistance at baseline. Case 2 was a smear negative new TB case and was newly diagnosed with both HIV-1 infection and T2DM. Case 2 (table 2) was subsequently reclassified as exogenous re-infection following whole genome sequencing (data not shown).

Following treatment cure, there were 3 cases of culture-confirmed drug resistant (MDR in 2 cases and RIF monoresistance in 1 case) TB within 14 months of treatment cessation. All 3 cases died (figure 4.1) and had advanced HIV (CD4 lymphocyte count <100 cells/mm³) at the time of death. The spoligotype of sequential isolates was different (LAM3/Beijing) in 1 of these 3 cases. The drug resistant strain could not be recovered for spoligotyping in the other 2 cases.

Table 4.5 Cases of acquired drug resistance on treatment

Time point	Regimen	Spoligo	MICs µg/ml						
			Sensititre Mycotab Plate Method [critical concentration]			BACTEC 960 Method [critical concentration]			
			INH [0.2/1]	RIF [1]	EMB [5/10]	PZA [100]	INH [0.1]	RIF [1]	EMB [5]
Case 1									
Baseline	RIF/INH/PZA/EMB	LAM3	0.06–0.12 (S)	0.12 (S)	2 (S)	>100 (R)	ND	ND	ND
Month 2 ^a	RIF/INH/PZA/EMB	LAM3	2 (R)*	0.12(S)	1 (S)	>100(R)	0.05 (S)*	ND	ND
Month 5	RIF/INH	LAM3	2 (R)	0.12 (S)	1 (S)	>100 (R)	ND	ND	ND
Month 7	RIF/INH	LAM3	2(R)	0.12 (S)	1 (S)	>100 (R)	ND	ND	ND
Case 2									
Baseline	RIF/INH/PZA/EMB	Beijing	0.03 (S)	0.12 (S)	1.0 (S)	≤100 (S)	0.05(S)	0.125 (S)	≤2.5 (S)
Month 2	RIF/INH/PZA/EMB	Beijing	0.03 (S)	0.12 (S)	1.0 (S)	≤100 (S)	0.05(S)	0.125 (S)	≤2.5(S)
Month 5 ^b	RIF/INH	Beijing	0.06 (S)*	>16 (R)	2.0 (S)*	>100 (R)	>1≤10(R)*	>20 (R)	10 (R)*
Month 7	RIF/INH	Beijing	2(R)	>16(R)	4-8 (S)*	>100 (R)	>1≤10 (R)	>20 (R)	10(R)*

^a KatG mutation S315T only detected at 5 and 7 months on MTBDR_{plus}

^b rpoB mutation S531L detected at 5 and 7 months on MTBDR_{plus}. However, no KatG or inhA mutation detected.

*Discrepancy in drug susceptibility test result between sensititre trek and MGIT 960 method

4.3.4. MIC profiles

As illustrated in figure 4.2, the MIC profile at baseline, stratified by HIV-1 serostatus, retreatment status and culture converter status were comparable. The median MIC, for RIF, INH and PZA was 0.06µg/ml, 0.05 µg/ml and 25 µg/ml. In a small cohort of 20 patients, the baseline MIC was compared with the MIC at 2 months. As illustrated by the paired plots in Figure 4.2, no significant increase in MIC occurred during treatment.

4.3.5. Baseline isoniazid mono-resistance

In this cohort, the prevalence of IMR by either MIC and/or MTBDR_{plus} was 6.1% (95% CI 3.6-9.6). The prevalence of IMR as determined only by MIC in 119 isolates was higher at 8.4% (95% CI 4.1-14.9). 41% were HIV-1 co-infected, 18% were retreatment patients and 12% had been previously in prison. Out of 13 cases determined to have IMR by MTBDR_{plus}, all had *inhA* mutations. 13/17 (76%) of cases of IMR culture converted at 2 months and there were no cases of treatment failure, relapse or ADR (see table 4.6). Although INH DST results were not available to programme doctors and nurses in real time, the median duration of intensive phase was >8 weeks (10.3 weeks IQR 8.4-12.6). Hence, clinically +/- radiologically, improvement may have been assessed as slower, necessitating longer than the typical 2 month duration of intensive phase treatment.

Figure 4.3 Minimum inhibitory concentration distribution for rifampicin, isoniazid and pyrazinamide

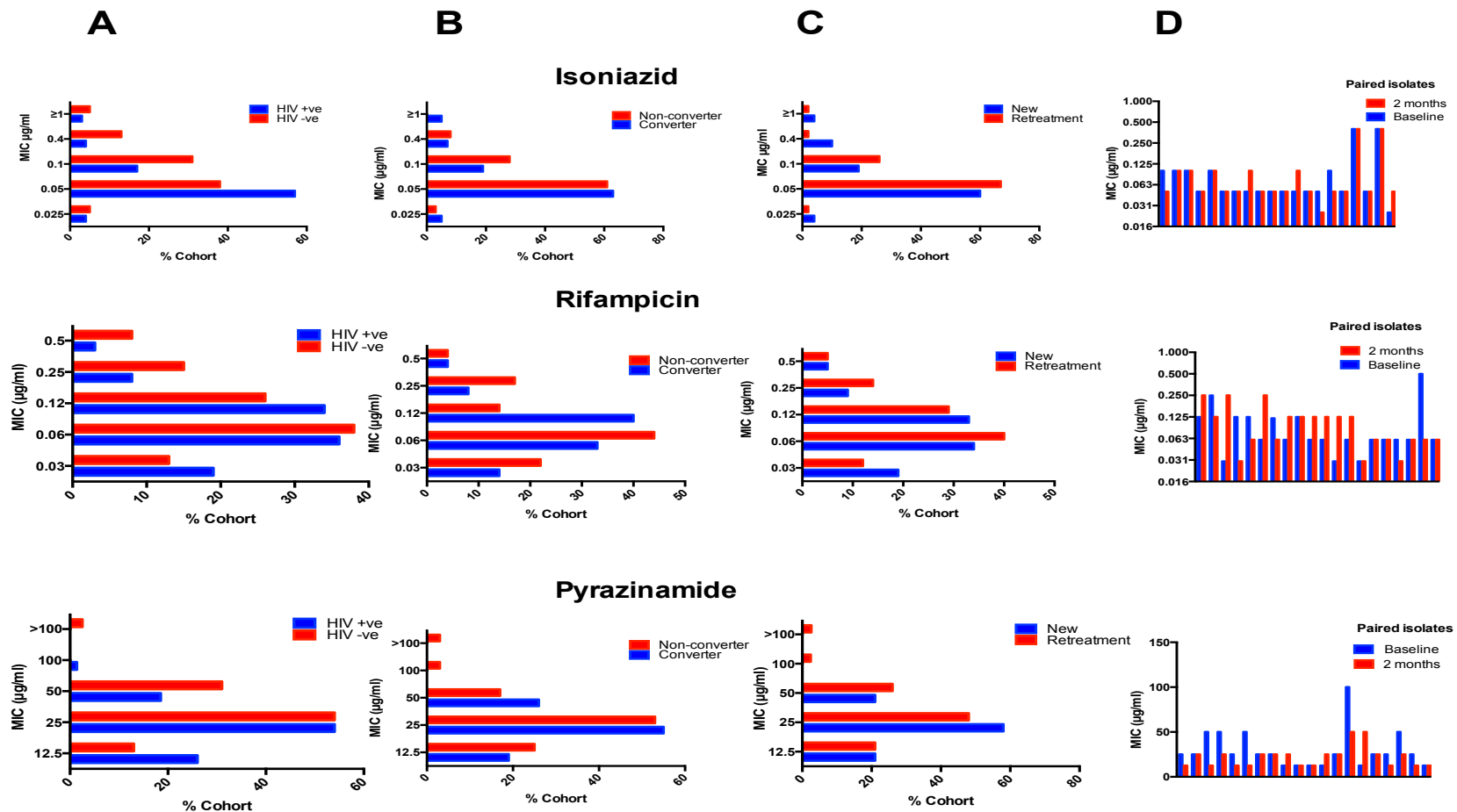


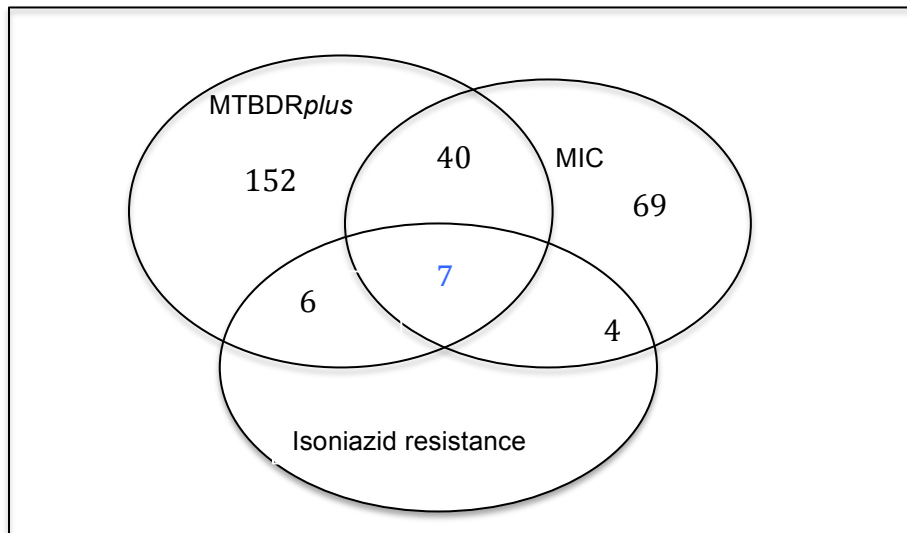
Table 4.6 Impact of isoniazid mono-resistance at baseline on treatment outcomes

	No. cases of resistance/ total isolates tested (%, 95% CI)	Median duration of intensive phase Rx /weeks (IQR)	Culture conversion at 2 mths (%)	Culture conversion at 5 mths
INH resistance determined by MIC OR MTBDR _{plus}	17/279 (6.1, 3.6-9.6)	10.3 (8.4-12.6)	13/17 (76)	15 culture converters 1 Rx completer 1 LTFU ^a
INH resistance determined by MIC (>0.2mg/l)	10/119 (8.4, 4.1-14.9)	10.3 (9.7-11.6)	8/10 (80)	8 culture converters 1 Rx completer 1 LTFU ^a
INH resistance determined by MTBDR _{plus}	13/206 (6.3, 3.4-10.5) All inhA mutations	10.3 (8.2-12.9)	8/13 (61)	13 culture converters
Discordance where INH DST determined by both methods	3/49 (6.1, 1.3-16.9)		3/3 (100)	1 culture converter 1 Rx completer 1 LTFU

^a Participant alive but refused to attend clinic for end of treatment visit

Abbreviations: INH isoniazid RIF rifampicin DST drug sensitivity testing Rx treatment LTFU lost to follow up

Figure 4.4 Isoniazid resistance detected by MTBDRplus and MIC testing



MIC minimum inhibitory concentration

4.4. Discussion

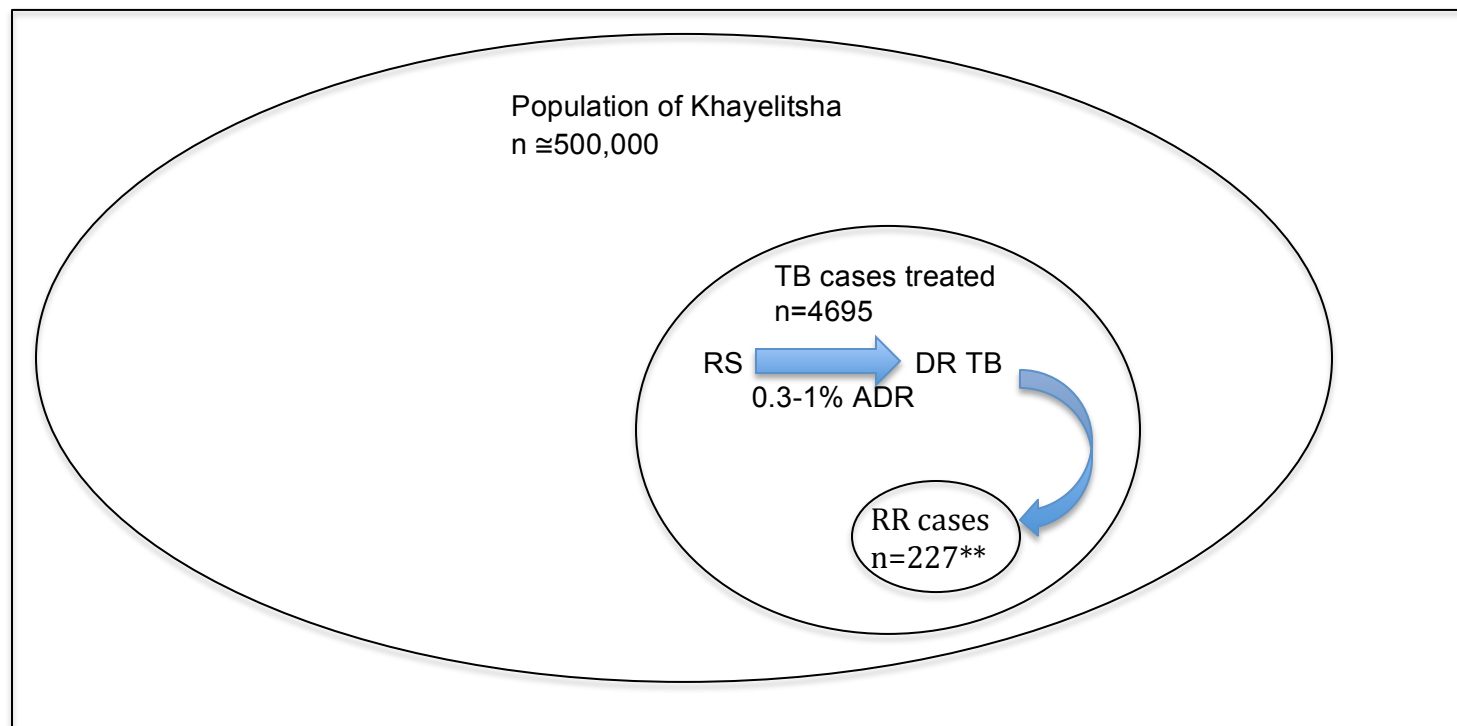
4.4.1. ADR in RIF susceptible TB in a programmatic setting of high prevalence HIV-TB co-infection

As illustrated by our systematic review in Chapter 2, in the current era of both ART and a daily TB treatment regimen consisting of 2 months of RIF/INH/ethambutol (EMB)/PZA and 4 months of RIF/INH, there are limited data available on frequency and associated factors in the development of ADR in RS TB. In the context of 6.1% baseline IMR and a significant proportion with advanced immunosuppression (25% HIV-TB CD4<100 cells/mm³), ADR was rare, with only 2 cases being identified during treatment, 1 of which was subsequently re-classified as exogenous re-infection based upon sub-lineage typing via whole genome sequencing (102). There were a further 3 cases of RIF +/- INH resistant culture-confirmed TB recurrences, within 14 months of treatment cure, 1 of which was confirmed to be exogenous re-infection. Confirmation of true ADR was not ascertainable in 2 recurrence cases. Therefore, assuming the 2 recurrence cases were true ADR, the maximum cumulative incidence of ADR in Khayelitsha was 3/306 (1%). Assuming the 2 recurrence cases were exogenous re-infection, the minimum incidence was 1/306 (0.3%). These findings are x3-10 higher than the cumulative incidence of ADR in pan-susceptible TB in our systematic review but in keeping with the incidence of

ADR in mono-resistant TB. A proportion of these ADR cases may transmit their drug resistant (DR) TB strain, hence, perpetuating the DR TB epidemic as illustrated in figure 4.3).

In this cohort, there was evidence suggestive of incomplete adherence to both ART (34% not virologically suppressed at baseline) and TB treatment (8-12%). No patient with good adherence developed ADR during treatment. Whilst 3 studies have shown non-adherence as a risk factor for ADR (45, 69, 70), there is still a need for further clinical studies examining this risk factor(21). We also identified that there was a higher proportion of non-adherence in patients who had previously been in prison and hence, this is a group likely to benefit from interventions such as DOT.

Figure 4.5 Acquired drug resistance in baseline rifampicin susceptible tuberculosis cases in Khayelitsha



*The circles are not drawn to scale

The above schematic diagram illustrates the proportion of cases which were RS at baseline which go on to acquire drug resistance. A proportion of these cases of ADR may transmit their drug resistant strain, hence contributing the total number of rifampicin resistant cases

** This is the sum of both acquired and transmitted drug resistance, where acquired drug resistance can be up to $0.01 \times 4695 = 47$ cases

Abbreviations: TB tuberculosis, RS rifampicin susceptible, DR drug resistant, RR rifampicin resistant

4.4.2. TB and prison

There is a scarcity of data estimating TB in the general population attributable to transmission within prisons in African countries with a high prevalence of HIV-1 (103). In this study, 20% of the overall cohort (and 32% of men) had previously spent time in prison where they potentially may have encountered inmates with both DS TB and DR TB. Poor health systems and fear of stigma are likely to contribute to unplanned treatment interruptions of both ART and TB treatment during incarceration(104). Overcrowding, poor nutrition and poor infection control may also be putative factors associated with high rates of spread of both TB in sub-saharan African prisons (105). On returning from prison into the community, these patients may contribute to the TB epidemic in Khayelitsha.

4.4.3. Interpretation of MIC profiles

The MIC profiles of MTB strains from a population enriched for HIV- infected participants were similar to MIC distributions from different geographical populations and lineages (106, 107). Khayelitsha has a preponderance of lineage 2 and 4(108). Given the extremely high burden of TB in Khayelitsha, it is likely many of these strains have been previously exposed to drug treatment. The observation is that retreatment status was not associated with higher MICs at baseline is an important one. Potential mechanisms of mycobacterial persistence and phenotypic tolerance are via altered transcriptional (+/- pre/post-transcriptional modification) which allow sub-populations to adapt metabolically (109) to sterilising drugs like RIF and PZA, often within different microenvironments (110). However, there was no evidence of increase in MIC accumulated over the first 2 months of TB treatment, comparing paired baseline and 2-month isolates. Nor was there evidence of higher MICs of the baseline strains of those who were still culture positive at 2 months. These results are reassuring and support efficacy of currently utilised regimens, if taken with good adherence.

When sputum is expectorated, the sampling of disease sites is random and inoculum size may vary. In the laboratory, decontamination practices, use of media and supplements can affect surrogate endpoints such as 2-month culture conversion. In determination of MIC profiles, there were multiple passages from sputum expectorated by participant to the exponential growth phase cultures that was used in the BACTEC

MGIT 960 system/ Sensititre MycoTB plates. Hence, minority drug resistant strains may have been lost through preferential selection of drug susceptible strains of enhanced fitness. Adaptive metabolic changes such as upregulation of efflux pumps, altered stress signaling, energy metabolism and DNA repair mechanisms (34) could have been altered through serial *ex vivo* cultures. *Ex vivo* culture of sputum and susceptibility testing does not provide an understanding of or quantify the complex interaction between the host, drug and bacteria that occurs *in vivo* (which is influenced by drug protein binding, tissue distribution and antimicrobial regulation by host innate and adaptive immune system). Despite running all MIC determinations in triplicate, a difference of 1 log₂ between isolates could easily be attributed to technical error. MIC does not give information on the rate of bactericidal activity which can only be obtained from time-kill curves. Also, the post antibiotic effect is not quantified and this could be therapeutically relevant (111).

4.4.4. Implications of baseline drug resistance

In this study, pyrazinamide mono-resistance is likely to have been a risk factor for acquired isoniazid resistance in Case 1 (table 2). In a systematic review of risk factors for acquired rifampicin/isoniazid resistance, baseline monoresistance was associated with cumulative incidence of ADR of 1% (5th to 95th percentile 0.79 to 10%) (21). However, after acquiring resistance to isoniazid, in the presence of functional rifampicin monotherapy, Case 1 did not further amplify drug resistance to develop MDR TB. The prevalence of baseline IMR in the cohort was 6.1%. Baseline IMR was not associated with ADR and there were no failure or relapse cases. The majority of IMR cases were new cases of TB, had low level *inhA* mutations, had good adherence and all received a daily regimen, which may have influenced the favourable treatment outcome. Hence, these data suggest use of GeneXpert® MTB/RIF in this programmatic setting to commence rifampicin susceptible patients on a standard regimen of IP 2(HREZ₇) CP 4(HR₇) does not significantly amplify drug resistance, even in patients with baseline isoniazid monoresistance. There are contrasting reports in the literature regarding the impact of IMR on treatment outcomes, including ADR. Of note, these studies (74, 99, 112, 113) vary significantly in proportion of retreatment patients, treatment regimens and duration of treatment given, prevalence of HIV-1 co-infection and proportion of high resistance (*katG*) vs low resistance (*inhA*) mutations. A review by Menzies *et al*, showed

incidence of ADR in retreatment patients with baseline IMR to vary between 0-28% (10). Narendran *et al*/ found that in a thrice weekly TB regimen, baseline IMR was associated with an odds of ADR of 9.97 (95% CI 3.43-28.37) and this was even higher when limited to HIV-1 infected participants with IMR [OR 13.28 (95% CI 4.25-41.37)]. In a study in retreatment patients with baseline IMR in Western Cape, South Africa (74), there were poor outcomes in 16% and acquired MDR in 14/155 patients with baseline IMR (9%, 95% CI 5.02-14.7). This was despite treatment in the majority with a 12 month regimen, inclusive of high dose isoniazid in 70%, Poor outcomes (failure or death) were unrelated to type of isoniazid mutation (katG or inhA) in this and other studies (74, 114, 115). Other studies have found significantly improved outcomes for IMR with increased duration of rifampicin of over 6 months (10, 113, 115). The limitations of this study included low numbers of cases with baseline isoniazid monoresistance.

4.4.5. Detection and confirmation of ADR

Detection of ADR can be challenging. Molecular techniques like GeneXpert® MTB/RIF and MTBDR*plus* require resistant mutant DNA to be present at 65%(116) and 5% (117)respectively, before resistance is detected. The presence of DNA from dead wild type MTB may give a false negative result with PCR based tests. Pre-treatment with propidium monoazide to bind DNA from membrane-damaged organisms to prevent PCR amplification, may offer a solution to this issue in future (118).

Even where detection of resistance is being performed on a positive culture, DST results for first line TB drugs can be discrepant. PCR and probe-based molecular genotyping assays do not include all rare or 'disputed' mutations associated with intermediate MICs (119). This was seen in Case 2 (table 3), where MTBDR*plus* did not identify INH resistance, although INH resistance was phenotypically detected at both 5 and 7 months.

In case 2 there was a discrepancy in EMB phenotypic susceptibility between the BACTEC 960 method and Sensititre plate method at months 5 and 7. Certain phenotypic DST methods have also been shown to miss clinically relevant drug resistant mutations for both EMB and RIF (120-123). Hence, assigning a 'gold standard' for detection of ADR is challenging and is a current field of research. The proportions method of phenotypic DST detects growth of $\geq 1\%$ drug resistant mutants at a predefined critical concentration (124). Expectoredated sputum stochastically samples the upper and lower

airways. Post sputum decontamination, there will be sub-culture stages and subsequent standardization of inoculum size as per MIC testing protocols. Hence, the bacilli in culture may not fully represent drug resistant sub-populations in the original sputum. Recent advances using whole genome sequencing directly from sputum samples may assist in these circumstances (125).

Confirmation that drug resistance was acquired/amplified during treatment requires ruling out infection at baseline with 2 strains of discordant drug susceptibility (dual mixed infection) and subsequent exogenous re-infection. Spoligotyping was carried out on sequential isolates to screen for the above. Conventional molecular typing which utilizes mobile or repetitive elements [IS6110 RFLP, spoligotyping and mycobacterial interspersed repetitive unit-variable tandem number repeat (MIRU-VNTR)] lack discriminatory power (126, 127). Through high coverage whole genome sequencing, strains with apparently identical fingerprints but discordant DST patterns have been shown to have significant diversity with up to 130 SNPs (126). Sub-lineage typing through WGS has enabled greater discriminatory power (102). A limitation of this study included incomplete genotyping in 2 suspected cases of ADR.

4.5 Conclusion

In this programmatic setting with a high burden of HIV-TB co-infection, an estimated 0.3-1% of cases with baseline RS TB acquired drug resistance during or subsequent to therapy. Exogenous re-infection with DR strains did occur and in the circumstance of not being able to verify true ADR in some cases, this potentially inflated the calculated cumulative incidence. Although ADR is relatively rare, it plays a role in fuelling MDR TB transmission in this setting. Greater programme effort is required to prevent the transmission of DR TB. Despite the relatively few cases, there was a lack of association between low level isoniazid resistance and ADR.

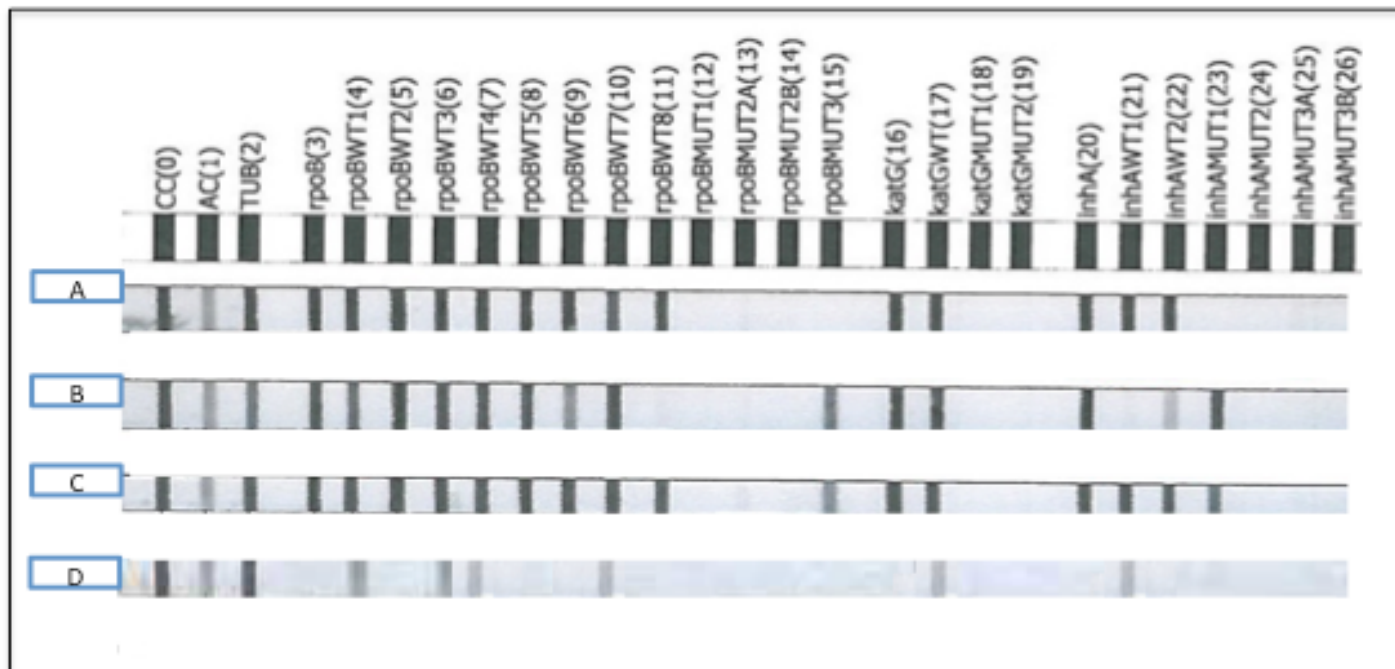
5. CHAPTER 5- clinical utility of MTBDR*plus* carried out directly on a 2-month sputum specimen in treatment monitoring

Interim outcome measures such as 2-month culture conversion have some utility in rapidly evaluating efficacy of novel regimens in a clinical trial setting(26, 27, 128). Programmatically, patients are traditionally switched from a quadruple drug regimen to dual therapy at 2 months and culture negativity at 2 months has a good negative predictive value for long term treatment outcomes of failure/relapse (28). A proportion of patients may acquire and amplify mutations conferring resistance under drug pressure. This process can occur at different time points during treatment, ranging from within the first 2 months when bacterial load is highest, to later on in treatment and this can be screened for using molecular or culture-based drug susceptibility tests on sputum obtained during treatment. Such screening is typically conducted at 2 months and at month 5-6 of treatment (at which point the patient is considered to have failed treatment if still culture positive)(129). Acquired drug resistance (ADR) is influenced baseline drug susceptibility patterns, HIV-1 co-infection, baseline extent of disease and treatment adherence (21).

GenoType MTBDR*plus* line probe assay (LPA), version 2.0 (Hain Lifescience, Nehren, Germany) is a molecular diagnostic assay that has been widely used to identify resistance to first line drugs rifampicin and isoniazid amongst TB cases. It uses polymerase chain reaction (PCR) to amplify regions specific to *M. tuberculosis* (MTB) complex and areas of the *rpoB* gene associated with rifampicin resistance and *katG* and *inhA* genes associated with isoniazid resistance from smear positive or smear negative clinical samples (130) (131) or culture isolates. Hybridization of amplified sequences to membrane-bound probes on a strip (both wild type and mutant sequences) identifies specific mutant bands or absence of wild type bands. Heteroresistance, defined as the simultaneous presence of DS and DR populations in the same patient (see figure 5.1), is perceived to be an early step in the pathway of resistance amplification (132). ADR can only be reliably detected, when drug resistant mutants are present at greater than 65% and 5% of overall bacterial population for Xpert® MTB/RIF(116) and MTBDR*plus*

respectively(117, 132). Limitations of both tests in treatment monitoring include amplification of DNA from non-viable bacteria. Figure 5.1 illustrates interpretation of the MTBDR*plus* line probe assay. Recording of 'inconclusive' rifampicin or isoniazid genotypic susceptibility from the MTBDR*plus* membrane strip is made on the basis of the presence of the 'TUB' band confirming *M. tuberculosis* (MTB) complex with failure of hybridization of DNA to the wild type probes leading to multiple missing/faint wild type bands (compared with amplification control). This usually occurs concurrently with missing/faint locus control bands.(see figure 5.1).

Figure 5.1 Interpretation of MTBDRplus



A) positive MTB complex (presence of 'TUB' band*), RIF/INH susceptible (presence of all wild type bands, absence of all mutation bands)

B) positive MTB complex (presence of 'TUB' band*), RIF/INH resistant (absence of wild-type band *rpoBWT8*, presence of a mutation band *rpoBMUT3*; absence of wild-type band *inhAWT1* and presence of mutation band *inhAMUT1*)

C) positive MTB complex (presence of 'TUB' band*), RIF heteroresistant, INH heteroresistant (Presence of all wild-type bands; presence of mutation band *rpoBMUT3*; presence of mutation band *inhAMUT1*)

D) positive MTB complex (presence of 'TUB' band*, inconclusive RIF and INH inconclusive (inconclusive as missing *rpoB*, *inhA* and *KatG* locus control regions (compared with amplification control) and numerous faint /missing wild-type bands)

* whilst in the presence of both a conjugate control (CC) and amplification control (AC)

4.3. Chapter hypothesis and aims

Overall hypothesis

MTBDR*plus* carried out directly on a 2-month sputum specimen can expedite prediction of 2-month culture converter status.

Specific chapter aims

1. To determine the capacity of MTBDR*plus* to predict culture conversion, when carried out directly on a 2-month sputum specimen.
2. to assess the potential of a directly performed MTBDR*plus* to detect early ADR/heteroresistance on a 2-month sputum specimen, compared to a reference of MTBDR*plus* on positive cultures..
3. To determine the association between having a positive 2-month MTBDR*plus* and the composite long-term unsuccessful outcome of failure/death during treatment/recurrence.

4.4. Methods

Patient recruitment and study procedures are described in 3.1.1. and 3.1.2. Processing of sputum and carrying out of MTBDR*plus* testing both on a direct specimen at 2 months and on positive cultures at 2 and 5-6 months is described in 3.2. and 3.3. Of note, direct MTBDR*plus* was compared with a reference of MTBDR*plus* on positive cultures. All potential cases of ADR were confirmed by phenotypic drug susceptibility testing (DST). The determination of treatment outcomes was as described in 3.1.6.

Statistical analyses

The two-tailed Chi-square test and multivariate logistic regression analysis was carried out in Stata/SE (13.1). To determine the association between a positive 2-month MTBDR*plus* and the composite long-term unsuccessful outcome of failure/death during treatment/recurrence, it was decided *a priori* to adjust for the potential confounders HIV-1 serostatus and extensive radiological disease at baseline. Individuals who defaulted

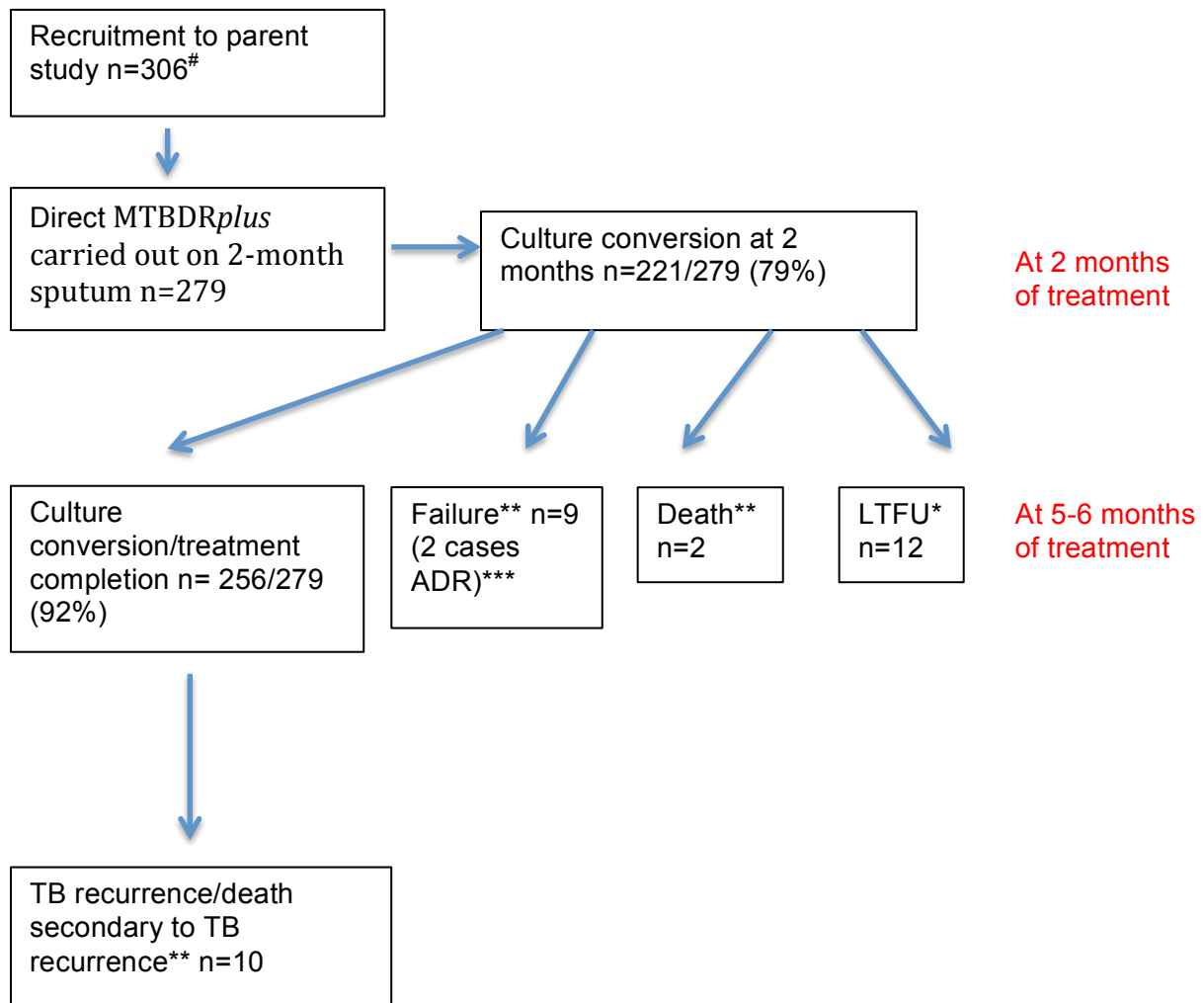
treatment or were lost to follow up/transferred care with unknown long-term treatment outcome were excluded from long term treatment outcome analyses.

5.3. Results

5.3.1. Outcomes

Out of the larger study of 306 participants, an overall 279 participants (62% HIV co-infected, 51% smear grade 1+ to 3+ at baseline, 32% retreatment status), who had a direct MTBDR $plus$ carried out on 2-month sputum, were included in the 2-month culture conversion analyses. The smear and culture conversion rate at 2 months was 88% and 79% respectively. There were no culture confirmed cases of ADR at 2 months. At 5-6 months, the rate of culture conversion (denominator including only those who produced sputum at 5-6 months) was 223/233 (95%). Culture conversion /treatment completion was 256/279 (92%). There were 267/279 participants who had both a 2-month direct MTBDR $plus$ result and a known long-term treatment outcome. There were 8 individuals with a negative 2-month MTBDR $plus$ result who defaulted treatment and 3 who were lost to follow up. There was 1 individual with a positive 2-month direct MTBDR $plus$ result who had an unknown long term treatment outcome. Two cases of ADR (1 with acquired isoniazid resistance and 1 with acquired rifampicin resistance) were identified on MTBDR $plus$ and DST of positive 5-6 month cultures. Overall, there were 21/267 (8%) individuals with unsuccessful outcomes (11 failures/deaths during treatment and 10 TB recurrences (see figure 5.2).

Figure 5.2 Study outcomes



[#] 17 did not produce sputa at 2 months, 2 died before follow up, 8 produced only 1 sputum and it was erroneously used only for culture.

^{*} 1 LTFU participant had a positive MTBDR_{plus} result and 11 LTFU participants had a negative MTBDR_{plus} at 2 months

^{**} Unsuccessful outcomes n=21

^{***} 1 case acquired isoniazid resistance, 1 case acquired

LTFU Lost to follow up

5.3.2. Direct MTBDR*plus* for assessment of culture conversion

A positive direct MTBDR*plus* on a 2-month sputum sample (presence of TUB band, amplification and locus control) had a sensitivity of 78% (95% CI 65-87) and specificity of 80% (95% CI 74-85) to predict culture conversion in liquid (MGIT) cultures. The negative predictive value of MTBDR*plus* directly on 2-month sputum at predicting culture conversion was high at 93% (95% CI 89-96) (table 5.1). The proportion of positive direct MTBDR*plus* results at 2 months was increased in HIV-1 uninfected patients, compared with HIV-1 infected patients ($p=0.04$) and in patients with smear grades 1+ to 3+, compared with smear scanty/negative ($p<0.01$) (table 5.1). However, when stratified by HIV-1 serostatus, there were no significant differences in sensitivity, specificity, positive and negative predictive value of the direct MTBDR*plus* to predict 2-month culture conversion (table 5.1). There were no associated clinical characteristics noted in the 13 individuals who were direct MTBDR*plus* negative and culture positive in whom a negative result did not correctly predict culture conversion at 2 months.

5.3.3. Direct MTBDR*plus* for assessment for long-term treatment outcomes

The 2-month direct MTBDR*plus* test was more likely to be positive in those with unsuccessful 12/21 (57%) vs successful 76/246 (31%) outcomes ($p=0.01$). The 2-month smear positivity rates were also significantly increased comparing those with unsuccessful 6/21 (29%) vs successful 27/246 (11%) outcomes ($p=0.02$). (Table 5.2). When adjusted for baseline extensive radiological disease and HIV-1 serostatus, the adjusted odds ratio for unsuccessful treatment outcome of a positive direct MTBDR*plus* at 2 months was 2.88 (95% CI 1.11- 7.42). When adjusted for baseline extensive radiological disease and HIV-1 serostatus, the adjusted odds ratio for unsuccessful treatment outcome of a positive culture at 2 months was 2.90 (95% CI 1.11- 7.56). Both 2-month culture (94%) and 2-month direct MTBDR*plus* (94%) had a good negative predictive value for long term unsuccessful treatment outcome.

Table 5.1 Utility of MTBDRplus directly on 2-month sputum to detect 2-month culture conversion

Detection of culturable MTB in liquid culture (MGIT) (Reference method)	MTBDRplus direct on 2 month sputa (%, 95% CI)		
	All N=279	HIV-1 infected N=172	HIV-1 uninfected N=107
Sensitivity	45/58 (78, 65-87)	23/30 (78, 58-90)	22/28 (79,59-92)
Specificity	177/221 (80, 74-85)	118/142 (83, 76-89)	59/79 (75, 64-84)
Positive predictive value	45/89 (51, 40-61)	23/47 (49,34-64)	22/42 (52, 36-58)
Negative predictive value	177/190 (93, 89-96)	118/125 (94, 89-98)	59/65 (90, 81-97)

This table compares the sensitivity, specificity, positive predictive value and negative predictive value of a direct MTBDR*plus* on a 2-month sputum for predicting 2-month culture conversion in liquid culture (MGIT). The cohort is also stratified by HIV-1 serostatus to assess effect of HIV-1 serostatus on the utility of the test.

MGIT mycobacterial growth indicator tube

Table 5.2 Direct MTBDRplus result at 2 months stratified by HIV-1 serostatus, smear grade at baseline

	HIV -1 infected (N=172) n (%)	HIV -1 uninfected (N=107) n (%)	p-value	Baseline smear grade negative/scanty (N=136) n (%)	Baseline smear grade 1+ to 3+ (N=143) n (%)	p-value
Direct MTBDRplus positive for MTB complex (N=88)	47 (27)	42 (39)	0.04	27 (20)	62 (43)	<0.01

Abbreviation: MTB *Mycobacterium tuberculosis*

5.3.4. Direct MTBDR*plus* for assessment of ADR

The use of MTBDR*plus* directly on 2-month sputa did not identify any culture proven cases of ADR. There were no cases where both wild type and mutant bands were simultaneously represented on the MTBDR*plus* strip demonstrating heteroresistance. There were inconclusive rifampicin and isoniazid genotypic susceptibility results in 26% of positive direct MTBDR*plus* tests. When this analysis was restricted to the 2-month samples which subsequently had paired positive cultures at 2 months, the proportion of inconclusive rifampicin and isoniazid genotypic susceptibility results reduced to 16% and 7% respectively (table 5.3). There were false rifampicin resistance calls in 4% of cases and false isoniazid resistance calls in 2% of cases (table 5.4). None of these cases showed heteroresistance on review of the MTBDR*plus* membrane strip. On repeat direct MTBDR*plus* performed on archived sputa, the result was either negative for MTB complex or rifampicin/isoniazid susceptible. Only 3 sputa (out of 11 with false positive rifampicin +/- isoniazid resistance) were subsequently culture positive and DST on the culture was rifampicin/isoniazid susceptible. All patients with false positive resistance results completed standard anti-TB treatment and had successful treatment outcomes.

Table 5.3 Utility of MTBDRplus directly on 2-month sputum to detect acquired drug resistance- inconclusive results

MTBDRplus result direct on 2 month sputa	All 'positive' results N=89 n (%)	Restricted to confirmatory positive culture* N=58 n (%)
Inconclusive isoniazid genotypic susceptibility result	23 (26%)	4 (7%)
Inconclusive rifampicin genotypic susceptibility result	22 (25%)	9 (16%)

* All positive cultures had confirmed rifampicin and isoniazid susceptibilities

INH isoniazid, RIF rifampicin

Table 5.4 Utility of MTBDRplus directly on 2-month sputum to detect acquired drug resistance- false positive results

MTBDRplus result direct on 2 month sputa	All samples N=279 n(%)	Repeat direct MTBDRplus testing on archived sputa in cases of suspected false positive resistance
False positive isoniazid resistance*	6 (2)	Result was negative for MTB complex in 4/6 cases and isoniazid susceptible in 2/6 cases
False positive rifampicin resistance*	11 (4)	Result was negative for MTB complex in 7/11 cases and rifampicin susceptible in 4/11 cases

* Compared with a MTBDRplus and phenotypic drug susceptibility testing performed on culture if flagged positive. Only 3 patients (out of 11 with false positive rifampicin +/- isoniazid resistance) were subsequently culture positive.

5.4. Discussion

This is the first study to report the utility of MTBDR*plus* directly on sputum to predict 2-month culture conversion in liquid culture. The sub-optimal sensitivity and positive predictive value do not support replacement of the conventional culture gold standard to monitor this interim treatment response. Friedrich *et al* assessed the utility of Xpert®/MTB RIF (also carried out directly on 2-month sputum) to monitor treatment response against conventional smear and culture positivity in 177 HIV-1 uninfected patients. They reported a sensitivity and specificity of 95% (95% CI 87-98) and 23% (95% CI 15-32) respectively of predicting 2-month culture positivity with a positive predictive value of 47% (95% CI 39-56) and a negative predictive value of 85% (95% CI 66-96) (133). Hence, the specificity of the direct MTBDR*plus* assay (80%) was much better than results reported for the qualitative Xpert® MTB/RIF test. This was not explained by potential differences in sputum bacillary load in HIV-1 infected and uninfected individuals (134) (Friedrich *et al* excluded HIV co-infected individuals whereas 62% of individuals in this study were HIV-1 co-infected). A negative MTBDR*plus* test had a high negative predictive value for 2-month culture conversion in both HIV-1-infected and HIV-1 uninfected individuals. There was a significantly lower sensitivity of direct MTBDR*plus* compared with GeneXpert® MTB/RIF at 2 months (78% vs 95%). Potentially, the former is less likely to be falsely positive (secondary to DNA from non-viable bacilli) several months post successful treatment of TB, than has been reported with Xpert® MTB/RIF (135). However, this hypothesis should be confirmed in larger studies.

Pre-emptive detection of heteroresistance which may give rise to ADR, can be challenging. Whilst phenotypic or genotypic DST on a positive culture is currently the gold standard, it can take several weeks to culture MTB from expectorated sputum, delaying commencement of individualised treatment regimens in cases of ADR. In this study, neither of the cases of ADR detected by MTBDR*plus* and phenotypic DST at 5-6 months, were detected as heteroresistance on direct MTBDR*plus* at 2 months. The majority of sputa at 2 months are smear negative and this study demonstrated unacceptably high rates of inconclusive and false positive resistance results using MTBDR*plus* directly on sputum when compared to culture as gold standard.

The inconclusive results may be secondary to insufficient DNA in the context of increased amplification cycles included in the direct MTBDR*plus* test protocol. This interpretation is supported by the proportion of inconclusive direct MTBDR*plus* genotypic susceptibility results that significantly decreased when restricted to only those samples, that were subsequently also culture positive. The false positive resistance results may be secondary to handling PCR amplicons in open molecular diagnostic laboratories dealing with high volumes of routine clinical samples. Repeat direct MTBDR*plus* on archived sputa were either negative or showed RIF/INH susceptibility. Only 3 cultures from patients who had false positive resistance results at 2 months were subsequently positive and MTBDR*plus* on the cultures also showed RIF/INH susceptibility. Although not the primary aim of the study, in this cohort a positive MTBDR*plus* at 2 months was associated with a 2.87 increased odds of the composite unsuccessful outcome of death/failure/relapse, having adjusted for baseline extensive disease on chest radiograph and HIV-1 serostatus. We see a similar predictive utility as a biomarker comparing MTBDR*plus* and culture status at 2 months.

A strength of this study was, considering varying cough efforts, stochastic sampling of upper and lower respiratory tract and varying inoculum size, the same 2-month sputum specimen was utilized for both direct MTBDR*plus* and liquid culture. A limitation of this study is that direct MTBDR*plus* test was only carried out at a single time interim time point, thus limiting its assessment as a biomarker earlier or later in treatment. It was only repeated on a second sputum specimen in cases where false positive resistance was suspected. Hence, we may have underestimated sensitivity of the test. There was no clear explanation for the 13 individuals who were direct MTBDR*plus* negative and culture positive. It is possible that the bacillary load was simply too low. In this cohort there were only 2 culture-confirmed cases of ADR, neither of which were detected at 2 months. Hence, we were limited in our capacity to gauge the ability of direct MTBDR*plus* as a screening tool for ADR. However, the inconclusive and false positive genotypic resistance results are clearly of concern.

5.5. Conclusion

This test may have moderate value in expediting prediction of 2-month culture conversion, its major value being that if negative, there is a 93% probability that the 2-month culture will be negative. However, use of this test for treatment monitoring in routine patient management is not indicated. Its potential as a biomarker of long term

treatment outcomes warrants further research. In the context of a busy routine diagnostic laboratory, a direct MTBDR*plus* on a 2 month sputum does not appear to contribute to management or assessment of resistance. It may wrongly result in diagnosis of acquired rifampicin (+/- isoniazid) resistance with considerable therapeutic implications for the patients and with secondary implications for health care resource allocation, infection control and contact tracing. Overall, this test should be with caution and its diagnostic utility and limitations interpreted in the appropriate clinical context.

6. CHAPTER 6- Expression patterns of selected RNA in sputum pre and during treatment

Antitubercular chemotherapy results in a biphasic decline in bacillary burden, which is thought to reflect rapid early killing of the major actively-metabolizing population, followed by a prolonged sterilizing phase of slowly-metabolizing persisters (136). Our aim was to screen for this second subpopulation in sputum and to test the hypothesis that response to treatment is influenced by the presence of metabolically less-active bacterial subpopulations.

A well-defined feature of MTB phenotypic adaptation involves up-regulation of the dormancy survival regulon (DosR) in response to reduced oxygen availability, or exposure to nitric oxide or carbon monoxide (137, 138). This regulon consists of 48 genes under the control of transcription factor DosR, including *hspX* coding for alpha-crystallin stress protein. Previous studies have reported abundant DosR-regulated transcripts in sputum pre-chemotherapy (34, 139). It was of interest to examine the possible influence of MTB genotype on transcriptional profiles observed in sputum. In particular, it has been reported that a mutation in strains from the Beijing family introduces a new transcriptional start site that results in constitutive expression of the *dosR* gene (and hence the associated regulon) independent of stress induction (140). The DosR regulon includes a small RNA (sRNA) transcript MTS1388 (141). This was reported to accumulate in infected tissues and we anticipated that its enhanced stability might make it a particularly sensitive target for reverse transcriptase polymerase chain reaction (RT-PCR).

MTB phenotypes with reduced replication and metabolism, particularly in the context of hypoxia, are characterized by a low level of ATP and a switch from proton-pumping NADH dehydrogenase-1 to non-proton-pumping NADH dehydrogenase-2 (142, 143). We therefore included *atpA*, *nuoG* and *ndh* in our panel of target genes. In addition, we included genes encoding the major sigma factors *sigA* and *sigB*, together with *icl1* and *prpD* as markers of the methylcitrate cycle that has been shown to be induced as a consequence of cholesterol catabolism during infection(144). A second abundant sRNA,

MTS2823, has been linked to the methylcitrate cycle (145) and was also included in the set of target genes.

Finally, we included two paralogous ribosomal protein genes, *rpsN1* and *rpsN2*, on the basis of previous studies of the MTB sputum phenotype (Dr. Rachel Lai, unpublished data). MTB has four ribosomal proteins that can be expressed as alternative zinc-binding or zinc-free forms dependent on the availability of zinc in the growth medium (146); *rpsN1* encodes a zinc-binding S14 protein and *rpsN2* the corresponding zinc-free form.

We anticipated that sputum obtained pre-chemotherapy would contain a mixture of MTB subpopulations and hypothesized that differences in the prevalence of a pre-existing drug-tolerant subpopulation of MTB may be partially responsible for differences in the rate of sputum clearance during treatment. To test this, we used qRT-PCR to measure the abundance of the selected transcripts in sputum samples from a panel of patients infected with different MTB genotypes and with differing responses to subsequent antitubercular chemotherapy.

6.1. Chapter hypothesis and aims

Overall hypothesis

Pre-chemotherapy, a minority pre-existing drug tolerant subpopulation of MTB in sputum is partially responsible for delayed sputum clearance and that analysis of the expression pattern of selected genes of interest can identify presence of such a drug tolerant subpopulation in individuals who are still culture positive at 2 months compared with individuals who have culture converted.

Specific chapter aims

1. To quantify the abundance of selected MTB mRNA/sRNA in sputum from patients pretreatment relative to exponential and stationary phase H37Rv culture
2. To look at expression of selected genes stratified by the 2-month culture converter status of patients and by strain characteristic (Beijing vs non-Beijing).

6.2. Methods

Patients with GenXpert ® MTB/Rif confirmed rifampicin susceptible pulmonary TB, produced spontaneous sputum samples pre commencement of quadruple therapy with rifampicin/isoniazid/ethambutol/pyrazinamide. All patients had undergone combined HIV serology testing (Abbott Architect HIV Ag/Ab Combo test) and only HIV-negative TB patients were included.

Separate sputum samples taken simultaneously at baseline were grown in MGIT liquid cultures (3.2) and genotyped using spoligotyping (3.5). TRIzol ® LS reagent (Invitrogen, Carlsbad, CA, USA) in 3:1 volume was immediately added to sputum, vortexed vigorously until the mixture was homogenous and stored on ice, pending freezing at -20°C, pending RNA extraction as described in 3.7. H37Rv exponential and stationary cultures were grown as described in 3.8. Primer design, cDNA synthesis and qPCR is described in 3.7. Rationale for selection of mRNA/sRNA is summarized in table 6.1.

Table 6.1 Rationale for selection of mRNA/sRNA

Gene ID	mRNA/sRNA* target	Role in MTB metabolism
Rv1308	<i>atpA</i>	ATP synthesis by the membrane bound F ₁ F ₀ ATP synthase is essential for growing and non growing mycobacteria and the enzyme is able to function over a wide range of PMF values (aerobic to hypoxic) during respiration and oxidative phosphorylation (142)
Rv2703	<i>sigA</i>	Codes for a protein enabling specific binding of RNA polymerase to gene promoters and initiation of transcription. SigA is a 'housekeeping' sigma factor and is constitutively expressed, has an unusually long half life. In clinical strains, its expression increases after phagocytosis which may be responsible for enhanced intracellular growth and increased resistance to superoxides. This suggests SigA modulates expression of virulence genes. SigA goes down 2 fold in response to starvation and oxidative stress (147).
Rv2710	<i>sigB</i>	Goes up 5-10 fold in starvation and response to NO providing a marker of stress/non-replicating persisters. Upregulation also leads to increased production of surface hyperglycosylated polar glycopeptidolipids that are produced in response to carbon starvation (147)
Rv1130	<i>prpD</i>	Propionyl-CoA generated by β -oxidation of odd chain

		fatty acids is metabolized via the methyl citrate cycle. prpD encodes methyl citrate dehydratase and is specific to the methyl citrate cycle. Propionate metabolism provides a source of energy and carbon and also prevents build up to toxic antibacterial levels (148)
Rv0467	<i>icl1</i>	Enzymes ICL1 and ICL2 are required for growth and persistence of MTB in macrophages and mice. Icl1 plays an essential role in virulence, functioning both as a methyl citrate lyase in the metabolism of propionylCoA in the methyl citrate cycle and a role in the glyoxylate shunt where it catalyses cleavage of isocitrate into glyoxylate and succinate. The glyoxylate cycle appears to be critical for bacterial growth and survival <i>in vivo</i> , consistent with the idea that <i>M. tuberculosis</i> chiefly subsists on fatty acid substrates during infection (149).
Rv3151	<i>nuoG</i>	Part of 14 sub-unit nuo operon which codes for proton pumping type I NADH dehydrogenase. NDH1 uses flavin mononucleotide and iron sulfur clusters to transport electrons from NADH to the quinone pool (menaquinone). The release of the two electrons during the NADH oxidation produces enough energy to pump four protons across the membrane to generate a proton motor force. Also an anti-apoptosis factor (142).
Rv1854c	<i>ndh</i>	Non proton translocating type II NADH dehydrogenase that does not conserve energy and recycles NADH both during aerobic and hypoxic, non-replicating respiration (142).
Rv0717	<i>rpsN1</i>	Encoded ribosomal protein S14-1 is cysteine rich and requires zinc binding for its structural integrity within the ribosome. In the presence of zinc e.g. intracellular infection within phagosomes, S14-1 is bound to the ribosome and is constitutively expressed. S14-1 which along with ribosomal proteins L28, L33, S18 also function as a zinc storage compartment (150).
Rv2056c	<i>rpsN2</i>	Ribosomal protein S14-2 is regulated by transcription factor zinc uptake regulation (Zur). In the presence of zinc, Zur binds to zinc responsive element of the Zur regulon (zinc ribbon) which includes 4 genes encoding for ribosomal proteins rpsN2, rpmB2, rpsR2 and rpmG1 negatively controlling their expression. rpsN2 is the paralog of rpsN1. rpsN2 is expressed during zinc deprivation. It displaces rpsN1 from the ribosome allowing mobilization of the stored metal (150).
Rv2031c	<i>hspX (acr1)</i>	This mRNA codes for a stress protein alpha crystallin induced by anoxia and nitric oxide and has a proposed role in maintenance of long-term viability during latent, asymptomatic infections. It is one of 48 genes activated by <i>dosR</i> . The presence of inflammatory granulomas in clinical infection, reduced oxygen availability, together with expression of nitric oxide and carbon monoxide is associated

		with ubiquitous expression of the DosR regulon. (137, 138).
MTB000077	MTS2823	This is a highly expressed sRNA. In exponential phase cultures MTS2823:16S rRNA is approximately 1:100 and is further induced up to ten fold during the transition to stationary phase. In mouse granulomas, this ratio approaches 1:1. Overexpression of MTS2823 during the exponential phase results in down-regulation of energy metabolism genes associated with the methyl citrate cycle (141).
MTB000078	MTS1338	This sRNA is significantly up-regulated during transition from exponential to the stationary phase. Deletion of dormancy regulator <i>dosR</i> , virtually prevents up-regulation of MTS1338 during the stationary phase suggesting a potential role for this sRNA in the early hypoxic stress response mediated by the DosR regulon (141).

ATP Adenosine triphosphate, NO nitric oxide, PMF proton motor force, mRNA messenger ribonucleic acid, rRNA ribosomal ribonucleic acid, sRNA small ribonucleic acid, Zur zinc uptake regulation

Data normalization and statistical analyses

To normalize for overall bacterial load, all cycle thresholds (Ct) of the genes of interest (GOI) were divided by cycle threshold (Ct) of the housekeeping gene 16S (Ct_{GOI} / Ct_{16S} ; hereby denoted as $SPUT_{GOI}$). Where Ct 16S was not quantified due to inadequate RNA for cDNA synthesis, available pairs of genes and reference 16S data were used to develop a regression model. The regression model was used to estimate 16S for those samples with missing 16S values. The weighted mean (using adjusted R^2) of the predicted 16S was then used as final estimate. For individual GOI (*atpA*, *SigA*, *SigB*, *nuoG*, *ndh*, *icl1*, *prpD*, *rpsN2*, *HspX*, *MST2823*, and *MTS1338*), the difference between exponential phase culture (Ct_{GOI} / Ct_{16S} , hereby denoted as EXP_{GOI}) and $SPUT_{GOI}$ was calculated for each patient along with the median for all patients (ΔGOI_{EXP}).

For individual GOI (*atpA*, *SigA*, *SigB*, *nuoG*, *ndh*, *icl1*, *prpD*, *rpsN2*, *HspX*, *MST2823*, and *MTS1338*), the difference between stationary phase culture (Ct_{GOI}/Ct_{16S} , hereby denoted as $STAT_{GOI}$) and $SPUT_{GOI}$ was calculated for each patient, along with the median for all patients (ΔGOI_{STAT}).

This process was carried out for the pre-chemotherapy time point and was stratified by converter and non-converter status at 2 months.

The difference in abundance of the selected mRNA/sRNA transcripts, in sputum

compared with the reference H37Rv cultures, was summarized as an overall ‘mean deviation’ from exponential/stationary culture. The ‘overall mean deviation from exponential phase culture’ (MDE_x) was calculated as the mean of all values of [EXP_{GOI} – SPUT_{GOI}] inclusive of 11 GOIs (*atpA*, *SigA*, *SigB*, *nuoG*, *ndh*, *icl1*, *prpD*, *rpsN2*, *HspX*, *MST2823*, *MTS1338*) and inclusive of all patients (N=19).

$$MDE_x = \frac{\sum j (\sum_{i=1}^N (EXP_{GOI} - (SPUT_{iGOI})))}{j \cdot N}$$

where j represents each GOI and N=total number of patients

The ‘mean deviation from stationary phase culture’ (MDStat) was calculated as the mean of [STAT_{GOI} – SPUT_{GOI}] inclusive of all GOIs (*atpA*, *SigA*, *SigB*, *nuoG*, *ndh*, *icl1*, *prpD*, *rpsN2*, *HspX*, *MST2823*, *MTS1338*) and inclusive of all patients.

$$MDStat = \frac{\sum j (\sum_{i=1}^N (STAT_{GOI} - (SPUT_{iGOI})))}{j \cdot N}$$

where j represents each GOI and N=total number of patients

This process was carried out for the pre-chemotherapy time point (N=19) and was stratified by converter and non-converter status.

The Wilcoxon rank-sum test and Fisher’s exact test was used compare statistical significance between converter vs non-converter groups. Correlation between continuous variables was assessed by the Spearman correlation co-efficient.

All analyses were performed using Stata (13.1) and Graph Pad (La Jolla CA) Prism (6.0).

6.3. Results

Nineteen participants were included in the study; eight were non-converters who remained MTB culture-positive at 2 months and eleven were converters who became culture-negative by 2 months. All patients were culture-negative after 5 months of therapy. Seven patients were infected by a strain from the MTB Beijing family, one with a Lineage 3 strain, and eleven with strains belonging to MTB Lineage 4 table 6.2.

The bacterial load in sputum was assessed by time to positivity (TTP) in MGIT culture and by the Ct for 16S ribosomal RNA. For four patients the Ct for 16S was estimated via the regression model described in Methods. Median TTP at baseline for non-converters who remained culture-positive at 2 months was 7.5 days (IQR 6-9) and was 7 days (IQR 6-11.5) for converters who were culture-negative at 2 months. 16S Ct values were significantly correlated with TTP at baseline ($R= 0.70$, $p<0.0001$, Figure 6.1).

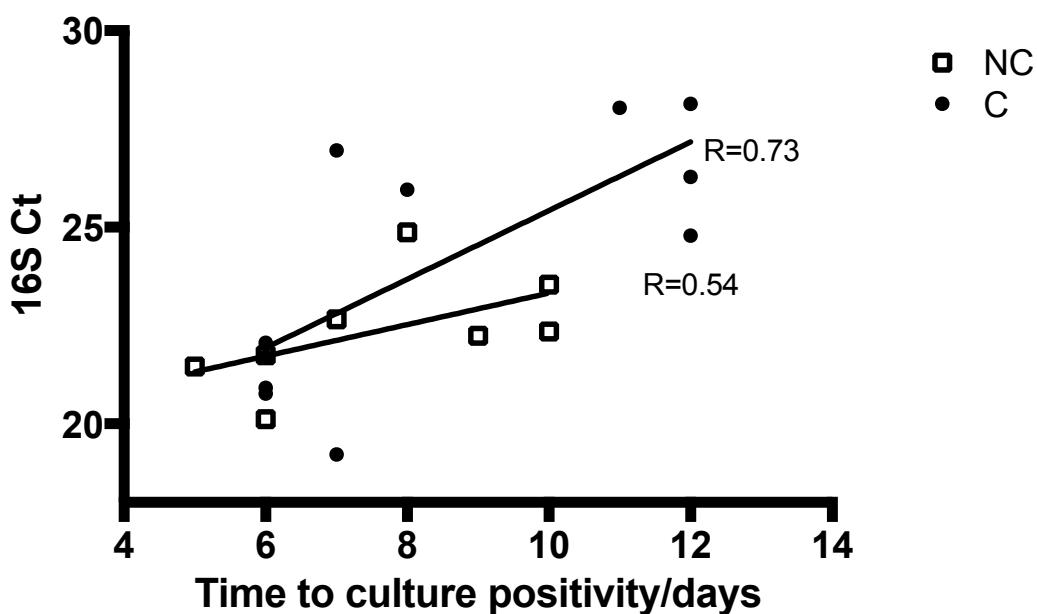
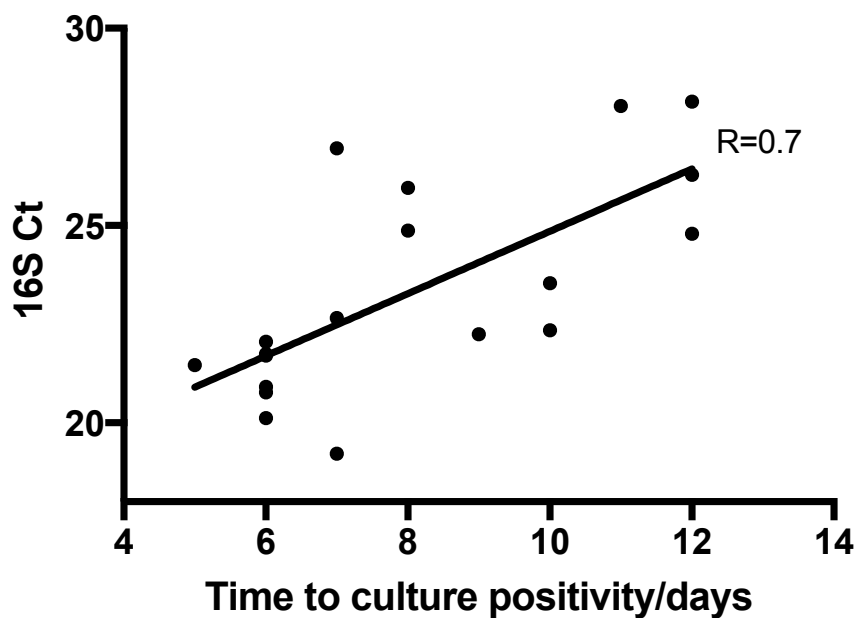
Table 6.2 Characteristics of included participants

	All participants (n=19)	Non-converters (n=8)	Converters (n=11)
% male	15 (79)	8 (100)	7 (64)
Median age (IQR)	34 (27-45)	39 (28-50)	29 (26-39)
%Smear grading			
Neg/scanty	2 (11)	0 (0)	2 (18)
1+	4 (21)	1 (12.5)	3 (27)
2+	7 (37)	3 (37.5)	4 (37)
3+	6 (31)	4 (50)	2 (18)
Median time to culture positivity at baseline (IQR)	7 (6-10)	7.5 (6-9)	7 (6-11.5)
% extensive radiological disease*	18 (95)	100	10 (91)
% cavities on CXR *	12 (63)	4 (50)	8 (73)
%SPOTCLUST Classification			
Beijing (lineage 2)	7 (37)	4 (50)	3 (27)
CAS (lineage 3)	1 (5)	1 (12.5)	0
LAM8 (lineage 4)	1 (5)	1 (12.5)	0
X3 (lineage 4)	3 (16)	1 (12.5)	2 (18)
T1 (lineage 4)	4 (21)	1 (12.5)	3 (27)
LAM3 (lineage 4)	2 (11)	0	2 (18)
T4 (lineage 4)	1 (5)	0	1 (10)

All participants were HIV-1 uninfected

* The presence of cavitation >1 cm maximum diameter was noted. Extensive disease on chest radiograph was noted as either involvement of >1 lung or involvement of ≥ 1 out of 3 (upper, mid or lower) zones per lung.

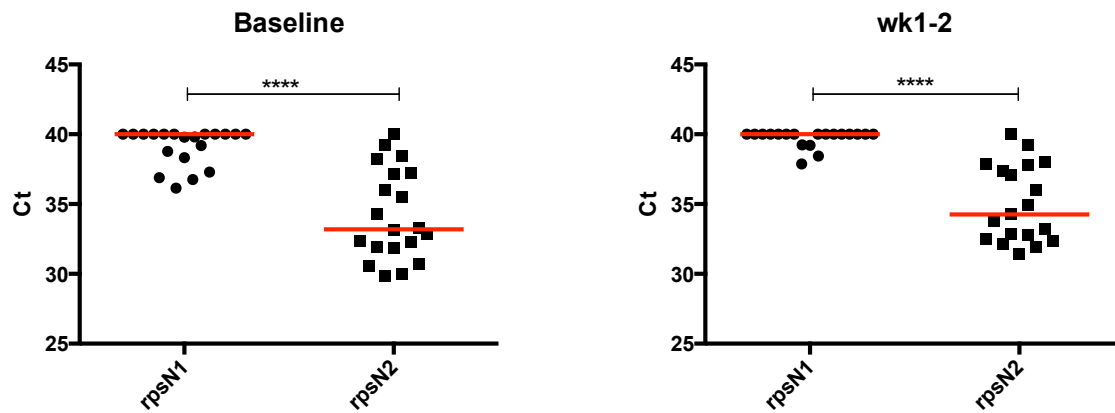
Figure 6.1 Relationship between 16S cycle threshold and time to culture positivity



NC non-converter C converter

Figure 6.2 illustrates primary Ct data for *rpsN1* and *rpsN2* transcripts. In exponential cultures of MTB H37Rv, the Ct for *rpsN1* was lower than *rpsN2* – i.e. expression of the *rpsN2* transcript is repressed, consistent with growth in zinc-replete 7H9 medium (146)- while the reverse was observed in stationary phase. Ct values were much higher in sputum, reflecting the lower number of bacteria, with the ratio of *rpsN1* to *rpsN2* indicative of growth in a low-zinc environment. To compare transcript abundance between samples, Ct values for target genes were normalized with respect to 16S Ct.

Figure 6.2 Comparison of cycle threshold (Ct) of *rpsN1* and *rpsN2* in sputum.



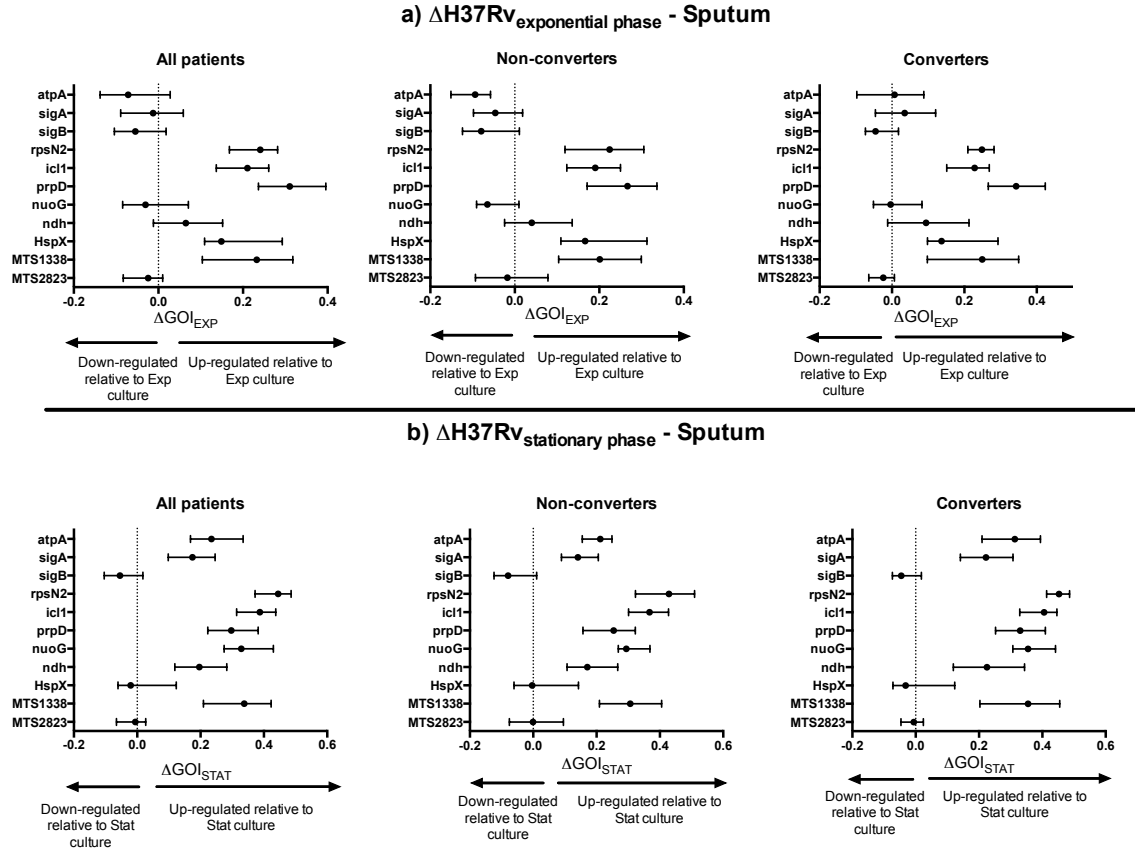
Where readings were beyond the level of detection, they were allocated Ct value of 40

Comparison of 16S-normalized transcript abundance between exponential and stationary phase cultures revealed a significant decrease in expression of *atpA*, *sigA* and *nuoG* and a significant increase in expression of *hspX* in the metabolically less-active stationary phase bacteria. We did not observe an anticipated increase in stationary phase expression of *sigB* or *ndh*, and surprisingly observed no difference in the abundance of sRNA transcripts MTS1338 and MTS2823.

Figure 6.3 illustrates 16S-normalized transcript abundance in sputum compared to exponential and stationary phase culture. Median levels of *atpA*, *sigA* and *nuoG* in sputum were slightly lower than exponential culture (Figure 6.3a) but significantly higher than stationary phase (Figure 6.3b). There was a non-significant trend towards reduced expression of *atpA*, *sigA* and *nuoG* in non-converters compared with converters.

Conversely, *hspX* in sputum was higher than in exponential culture but lower than stationary phase. The 16S-normalized abundance of *rpsN2*, *icl1* and *prpD* transcripts in sputum was significantly higher than under either phase of laboratory culture. The overall mean deviation of the selected set of transcripts in sputum from H37Rv growth status at baseline (MDEx and MDStat) showed that the overall sputum profile resembled exponential culture more closely than stationary phase (Figure 6.4).

Figure 6.3 Distance from exponential phase and stationary phase cultures



- a) $\Delta\text{GOI}_{\text{EXP}}$ Difference in expression of gene of interest in exponential phase culture compared with gene of interest in sputum at baseline (median $\pm$ IQR). A positive number indicates increased relative to exponential phase culture and negative number indicates decreased relative to exponential phase culture.
- b) $\Delta\text{GOI}_{\text{STAT}}$ Difference in expression of gene of interest in stationary phase culture compared with gene of interest in sputum at baseline (median $\pm$ IQR). A positive number indicates increased relative to stationary phase culture and negative number indicates decreased relative to stationary phase culture.

Exp: exponential phase; Stat: stationary phase GOI gene of interest

* Expression of all genes was normalized to 16s

Figure 6.4 Mean deviation from H37Rv growth status

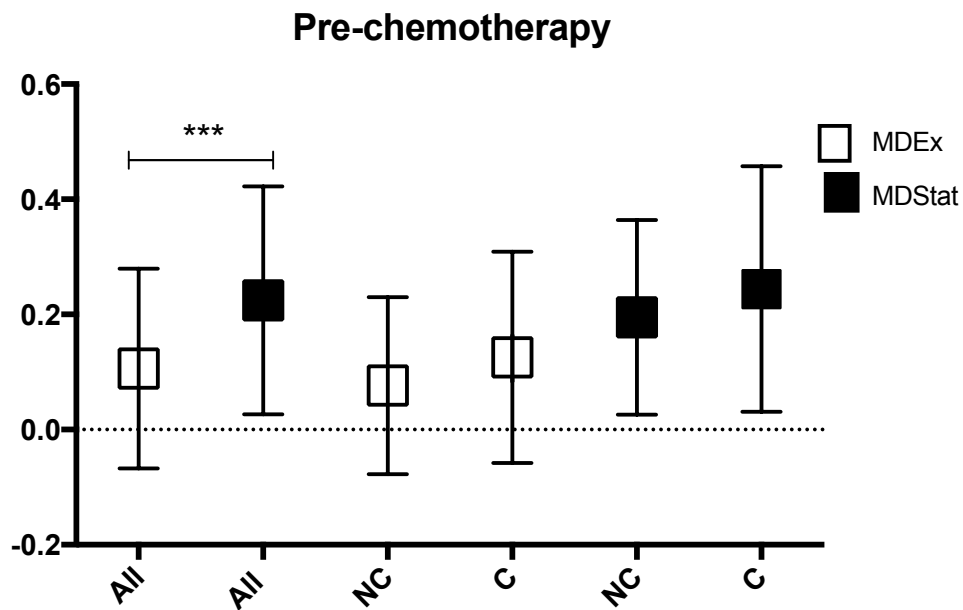


Figure 3 Overall mean deviation (+/-SD) from H37Rv growth status at baseline

The further away from 'zero' line, the greater the difference in patterns of abundance. At both time points, sputum was closer in pattern of abundance to exponential phase H37Rv cultures.

MDEx Mean deviation from Exponential phase

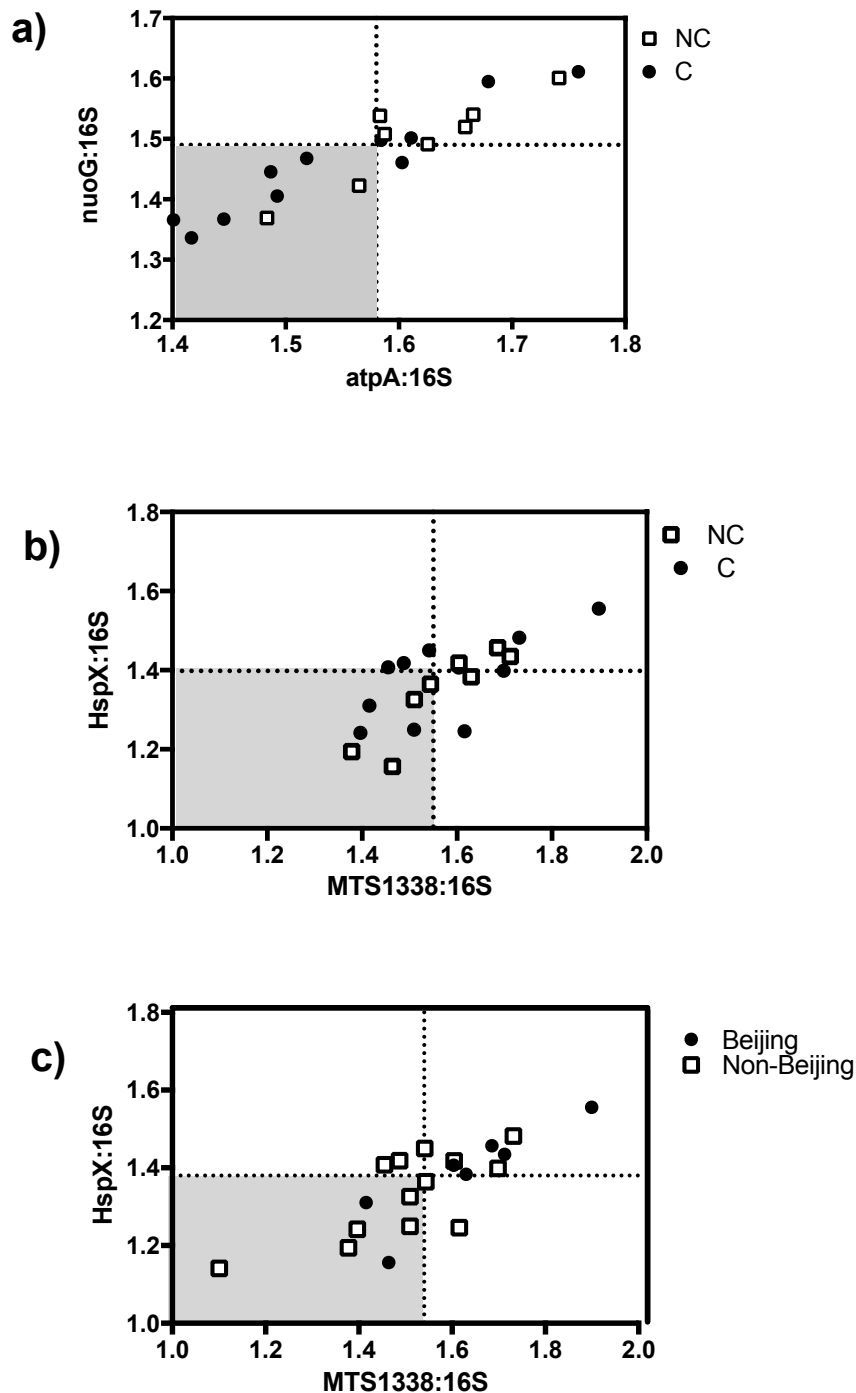
MDStat Mean deviation from Stationary phase

NC non-converter

C converter

To further define a potential reduced-metabolism phenotype in sputum, we searched for a $hspX^{hi}atpA^{lo}nuoG^{lo}$ signal and its variability between individuals. A strong correlation was observed between 16S-normalized abundance of *atpA* and *nuoG* ($R=0.92$, $p<0.001$). There was a non-significant trend of reduced expression of these markers of oxidative phosphorylation in non-converters. 6/8 (75%) had below median levels for both *nuoG* and *atpA*. This was compared with 4/11 (36%) and 5/11 (45%) of converters, (figure 6.5a). There was a non-significant trend of increased expression of the DosR regulated transcript (*hspX*) above the median of the entire group, in non-converters 5/8 (63%) compared with converters 4/11 (36%) (Figure 6.5b). The 16S-normalized transcript abundance of *hspX* showed no significant difference between the 7 Beijing and 12 non-Beijing samples included in the study (figure 4c), consistent with the conclusion that the DosR phenotype detected in sputum reflects an induced response to hypoxia, nitric oxide or carbon monoxide.

Figure 6.5 Pre-chemotherapy expression of transcripts influenced by oxygen gradients in sputum



The ratio of cycle threshold of genes of interest (nuoG , atpA , HspX , MTS1338) normalized to 16S ($\text{Ct}_{\text{GOI}} / \text{Ct}_{16\text{S}}$) as expressed in sputa is shown. Lower values for ($\text{Ct}_{\text{GOI}} / \text{Ct}_{16\text{S}}$) represent higher levels of expression. The median abundance as shown by the dotted lines, were used to distinguish the highest expressors (shaded grey).

a) and b) Stratified by converter status c) Stratified by Beijing/non-Beijing status.

6.4. Discussion

This study adds to a growing literature describing the sputum phenotype of *M. tuberculosis* by demonstrating the potential application of qRT-PCR to analyse differences in well-characterized patient cohorts. By avoiding the pre-amplification step characteristic of previous protocols, the present study avoids potential technical issues involving non-linear amplification and preferential enrichment of abundant transcripts. In spite of this, direct qRT-PCR had sufficient sensitivity to detect transcripts in sputum samples that were negative or scanty by smear microscopy.

Based on the selected set of target genes, the overall transcription profile detected for MTB in sputum more closely resembled H37Rv in exponential culture than in stationary phase culture. It has previously been proposed that MTB assumes a non-replicating phenotype in sputum (139), but comparison of our own with published data (151, 152) indicates that the sputum profile falls between replicating and non-replicating states, and that differing interpretations may partially reflect inter-laboratory variation in reference cultures. The extent to which MTB continues to replicate within the respiratory tract could have an important influence on disease transmission.

Consistent with previous descriptions of the transcriptional phenotype of MTB during infection (34, 139, 152), *icl1* and *prpD* transcripts were significantly higher in sputum than in laboratory culture. The products of these two genes participate in the methylcitrate cycle that is required to process potentially toxic propionate generated during metabolism of cholesterol and odd branch length fatty acids. This suggests that MTB in sputum resembles intracellular MTB in utilizing cholesterol as a carbon source (153). In contrast, abundant representation of *rpsN2* transcript in sputum is characteristic of growth in a low-zinc environment, which is inverse to the zinc-intoxication associated with MTB in activated macrophages (154).

Increased abundance of *hspX* and decreased abundance of *atpA* and *nuoG* transcripts relative to exponential culture is consistent with the presence of a metabolically less-active MTB phenotype in sputum. Our starting hypothesis was that this subpopulation would be enriched in patients who required prolonged treatment to achieve culture conversion. While a trend towards this outcome was observed, it did not reach statistical significance. By knowing the genotype of the infecting strain in each case, we were able to exclude the possibility that genotypic variation – for example, constitutive *dosR*

expression in Beijing strains – was responsible for the observed variable representation of the low-metabolism phenotype.

Limitations

Findings were limited by the relatively small sample size and suggest the need for expanded studies focusing on phenotypically-informative transcripts. An important limitation relates to the use of 16S rRNA to normalize samples with respect to bacterial load. The relative stabilities of rRNA and mRNA in the respiratory tract are unknown (155) and preferential enrichment for one or the other in non-replicating or dead bacteria could skew the data. Such an effect could be particularly important when monitoring the transcriptional phenotype during the course of anti-mycobacterial therapy.

7. Pharmacokinetics of rifampicin, isoniazid, and pyrazinamide in South African tuberculosis outpatients

Despite global initiatives prioritising reduction of incidence and mortality attributable to tuberculosis (TB), in 2014 there were an estimated 9.6 million new TB cases (12% were HIV-TB co-infected) and 1.5 million deaths (27% were HIV-TB co-infected)(24). In the case of rifampicin susceptible pulmonary tuberculosis, World Health Organization (WHO) guidelines advocate a daily regimen inclusive of 2 months of intensive phase therapy with first line drugs rifampicin, isoniazid, pyrazinamide, and ethambutol followed by 4 months of continuation phase therapy with rifampicin and isoniazid. Fixed dose combination formulation (FDC) tablets are widely used to deliver standardized doses according to weight (87).

There may be important physiological variation of pharmacokinetics (PK) in different populations and at different time points during the course of treatment. An example of the latter results from rifampicin auto-induction, which can lead to delay in reaching steady state of up to 40 days (156). There are multiple causes leading to significant inter-individual PK variability including pharmacogenomics (157, 158), sex (159, 160), weight(160), and co-morbidities like diabetes mellitus (159, 161-163) There have been contrasting findings published regarding the effect of HIV-1 co-infection on anti-TB drug PK with some studies showing reduced drug exposures (78, 164, 165) and others no significant difference between HIV-1 co-infected and uninfected patients (159, 166-168). Of note, cohorts studied had varying degrees of nutritional deprivation, immunosuppression, and varying proportions concurrently taking antiretroviral therapy (ART).

The relationship between drug concentrations of standard dose first line anti-TB drugs and drug side-effect profiles is unclear. Serious adverse drug reactions (Common Terminology Criteria for Adverse Events (CTCAE)(90) grade 3 and above) such as drug-induced hepatotoxicity are well documented and can sometimes be attributed to a specific anti-TB drug based upon temporality, cessation and sequential drug re-challenge(91). Mild to moderate drug side effects of CTCAE grades 1-2 are common (169-171). Even though they may be difficult to attribute to a specific anti-TB drug with any certainty, they, they are likely to contribute to sub-optimal adherence (172) and may

adversely affect treatment outcomes (169).

7.1. Chapter hypothesis and aims

Overall hypothesis

HIV-1 co-infected TB patients have reduced plasma drug concentrations of rifampicin, isoniazid and pyrazinamide compared with HIV-1 uninfected patients.

Specific chapter aims

1. Assess PK parameters for rifampicin, isoniazid and pyrazinamide in a cohort of both HIV-1 uninfected and HIV-1 co-infected TB patients.
2. To assess the effect of ART on PK exposures.
3. To assess the effect of dosing as per WHO guided weight bands on PK exposures
4. To determine whether plasma drug concentrations were associated with reported drug side effects at 2 months.

7.2. Methods

Patient recruitment, antitubercular treatment (ATT) regimen and dosage as per WHO weight bands are outlined 3.1.1. Side effects were characterized as per Common Terminology Criteria for Adverse Events (CTCAE) grading and attribution of causality to the anti-TB regimen made in the 'probable'/'possible' categories as per WHO-Uppsala Monitoring Center system (3.1.4). Intensive sampling PK studies and liquid chromatography tandem mass spectrometry (LC-MS) are described in 3.1.5. Plasma concentration-time data from all subjects were simultaneously analysed by a non-linear mixed-effects model utilising Monolix (version 4.3.3). Previously published structural models were selected and optimized on the current data (173-175). The structural models tested included one- and two- compartment disposition with first-order elimination and first-order absorption, testing the presence of an absorption lag time or a delay modeled via a chain of transit compartments. Random inter-individual variability (IIV) and inter-occasional variability (IOV) were assumed log-normally distributed, and correlations between the random effects was tested. Inter-occasional variability was

included by treating the data prior to the observed dose administration as a separate PK occasion. During treatment, IOV could have been as a result of unmeasured factors e.g. timing of meals in relation to drug dose. Hence, IOV was included as a random effect within the stochastic model for parameters like bioavailability and absorption constant. IOV was included by treating the drug concentration measured prior to the observed dose administration (predose concentration) as a separate pharmacokinetic occasion (occasion 1). Of note, occasion 1 included only 1 measurement (pre-dose concentration) with a subsequent 6 data points for occasion 2. Hence, we did not aim to accurately quantify IOV, rather to allow the model to account for this variability, whilst allowing the model to more accurately estimate the parameters and BSV.

A mixture model was evaluated to explore the multi-modal distribution of isoniazid CL, due to the polymorphic N-acetyltransferase-2 (NAT2) gene. Residual unexplained variability arising from unspecified within-subject variability, model misspecification, and experimental error, was described using a combined additive and proportional model. Data points which were BLQ were treated as censored and handled with the Monolix implementation of the M3 method (176). Allometric scaling with either total body weight, or fat-free mass (FFM)(177) was applied to clearance (CL) and volume of distribution (V), as suggested by Anderson and Holford (178). Fat-free mass was calculated using the empirical model developed by Janmahasatian *et al* (179)

$$FFM = \frac{WHS_{max} \cdot Ht^2 \cdot Wt}{Ht^2 \cdot WHS_{50} \cdot Wt}$$

where Wt denotes body weight in kg, Ht is height in m, and the constants WHS_{max} and WHS_{50} have the value of 42.92 and 30.93 in men, and 37.99 and 35.98 in women, respectively.

Other covariates tested included the effect of sex, age, serum albumin, total protein, HIV serostatus, CD4 lymphocyte count (as a binary variable above and below 200 cells/mm³), type of ART (none vs non-nucleoside reverse transcriptase (NNRTI) -based vs protease inhibitor (PI)-based), total dose, and dose in mg/kg. Model development and selection was based on optimisation of the objective function value (OFV), inspection of goodness of fit plots including visual predictive checks, and biological plausibility. Stepwise covariate selection was performed using a drop in OFV of >3.84 (corresponding to a significance level of 5%) as the cut-off for inclusion and an increase of >6.63 OFV points as a cut-off for the backward elimination step.

Finally, model-based individual PK parameter values referring to the PK profile after the observed dose were used in the R package Simulx, by Dr. Paolo Denti, to simulate steady-state individual profiles and calculate peak concentration (C_{max}) and area under the concentration-time profile curve from 0 to 24 hours (AUC_{0-24}).

Statistical analyses

The Wilcoxon-Mann-Whitney test was used to compare PK exposures between those with side effects and those without. Logistic regression analyses were used to calculate odds ratios for side effects at different drug exposure quartiles and adjusted for potential confounders. Stata (version 13.1) and GraphPad (La Jolla,CA) Prism (6.0) were used for all analyses.

7.3. Results

7.3.1. Patient demographics

Of the 100 study participants, 57% were male and 65% were HIV-1 infected with a median CD4 lymphocyte count of 233 cells/mm³ (IQR 106-386). The proportion on ART increased from 27/65 (42%) at baseline to 50/65 (77%) at the time of PK study with 45/50 (90%) on NNRTI-based (96% on efavirenz, 5% on nevirapine) regimens and 5/50 (10%) on protease inhibitor (PI) based (Lopinavir/ritonavir) regimens. The median body mass index (BMI) were 21 kg/m² (IQR 19-23). The median age was 33 years (IQR 29-40). Table 1 provides the clinical characteristics of the PK cohort, stratified by HIV-1 serostatus. This PK cohort did not differ substantially from the larger cohort of 306 patients (see chapter 4, table 4.1).

Table 7.1 Clinical characteristics of the cohort stratified by HIV serostatus

Clinical characteristic*	Whole PK cohort (N=100)	HIV-1 infected (N=65)	HIV-1 uninfected (N=35)
Male n (%)	57 (57)	30 (46)	27 (77)
Xhosa ethnicity n (%)	98 (98)	63 (97)	35 (100)
Median age/years (IQR)	33 (29-40)	34 (30-40)	32 (27-38)
Smoking history n (%):			
Current	24 (24)	9 (14)	15 (43)
Previous	27 (27)	19 (29)	8 (23)
Never	49 (49)	37 (57)	12 (34)
Alcohol consumption n (%)	37 (37)	16 (25)	17 (49)
Recreational drug use n (%)	5 (5)	3 (5)	2 (6)
Previously in prison n (%)	14 (14)	10 (15)	4 (11)
Previous mining history n (%)	5 (5)	1 (1)	4 (11)
Retreatment n (%)	39 (39)	29 (45)	10 (29)
Type 2 Diabetes Mellitus n (%)	4 (4)	3 (5)	1 (3)
Median BMI at baseline (IQR), kg/m ²	21 (19-23)	21 (20-23)	20 (19-23)
Median BMI at PK study (IQR), kg/m ²	21.5 (20-23)	22 (20-23)	20.5 (19-23)
Median FFM at PK study (IQR), kg	45 (38-49)	40.5 (36-47)	49 (46-51)
Median CD4 count (IQR), cells/mm ³		233 (106-386)	
VL<40 copies/mL at baseline n (%)		26	
Median albumin at PK study (IQR), g/l	38 (34-40)	36 (34-39)	38 (40-43)
Median total protein at PK study (IQR), g/l	86 (79-92)	88 (82-93)	82 (75-86)
Months on ART at time of PK (IQR)		1.32 (0-15.5)	
Smear grading at baseline n (%):			
3+	24 (24)	14 (21.5)	10 (29)
2+	22 (22)	11 (17)	11 (31)
1+	20 (20)	14 (21.5)	6 (17)
Scanty/negative	34 (34)	26 (40)	8 (23)
Median baseline time to culture positivity /days (IQR)	10 (7-14)	12 (7-15)	8 (6.5-12.5)
Extensive radiological disease at baseline n (%)	71 (71)	41 (63)	30 (86)
Cavities at baseline n (%)	52 (52)	32 (49)	20 (57)
mg/kg dose at PK study (range):			
Rifampicin	10 (7-11.5)	10 (7-11.5)	10 (7-11.5)
Isoniazid	5 (3.5-6)	5 (4-6)	5 (3.5-6)
Pyrazinamide	26 (19-31)	26 (20-31)	25.5 (19-31)
Number of patients with side-effects of tuberculosis treatment (%)	35 (35)	25 (38)	10 (29)
Non-adherence as per pill counts/self-report n (%)	10 (10)	8 (12)	2 (6)

*Characteristics are reported at time of diagnosis (baseline) unless otherwise specified (at PK visit or 2-month visit)

Abbreviations: BMI body mass index, FFM fat free mass, ART antiretroviral therapy, TTD time to culture positivity at baseline.

7.3.2. Patient pharmacokinetic parameters

The population PK final parameter estimates for rifampicin, isoniazid, and pyrazinamide are shown in tables 7.2-7.4 and visual predictive checks are provided in Figures 7.1-7.3. The optimised structural model for rifampicin was a one-compartment model with first-order elimination and first-order absorption with an absorption lag time. A two-compartment model with first-order elimination and absorption through a series of transit compartments was optimal for isoniazid. Finally, a one-compartment model with first-order elimination, and transit compartment absorption was used for pyrazinamide. Allometric scaling for CL and V using FFM as body size descriptor improved the OFV of the models by 20, 34, and 64 points respectively for rifampicin, isoniazid, and pyrazinamide. We found that inclusion of inter-occasional variability (IOV) in the structural model on bioavailability, mean transit time and absorption constant led to a large improvement in OFV of 44 points.

During model development, the multi-modal distribution of isoniazid CL attributed to the polymorphic nature of the NAT2 genotype (157) was described using a mixture-model which improved the model fit. However, the current version of Monolix does not support both mixture modelling and estimation of inter-occasional variability, so the latter was included because it was more significant in terms of model fit.

Table 7.2 Parameter values estimated by the final pharmacokinetic model for Rifampicin

Parameter	Estimated typical value (95% CI)	Inter-individual* or inter-occasional** variability (95% CI)
Bioavailability (F)	1 (fixed)	29.1% (24.2% to 34.0%)**
Absorption lag time (h)	0.691 (0.59 to 0.791)	76.2% (62% to 89.9%)**
Absorption constant (h^{-1})	1.21 (1.03 to 1.38)	63.2% (49% to 77.5%)**
Clearance/F – HIV- (L/h)***	25.1 (21.8 to 28.4)	34.3% (28.8% to 39.8%)*
Clearance/F – HIV+ not on LPV/r (L/h)***	19.9 (17.8 to 21.8)	
Clearance/F – HIV+ on LPV/r (L/h)***	10.8 (7.08 to 14.5)	
Volume of distribution (L)***	56.4 (53.7 to 59.1)	
Additive error (mg/L)	0.196 (0.174 to 0.218)	
Proportional error (%)	15.0 (13.2 to 16.8)	

Abbreviations: F bioavailability, LPV/r lopinavir/ritonavir

*** CL and V have been allometrically scaled using individual values of fat-free mass (FFM), so the typical values reported here refer to the median value of FFM in the cohort, 45 kg (e.g. a 1.7 m tall man weighing 51 kg)

Table 7.3 Parameter values estimated by the final pharmacokinetic model for Isoniazid

Parameter	Estimated mean population value (95% CI)	Inter-individual* or inter-occasional** variability (95% CI)
Bioavailability (F)	1 (fixed)	32.3% (27.2% to 37.4%)**
Mean transit time (h)	0.32 (0.12 to 0.51)	92.7% (65.3% to 120%)**
Number of absorption transit compartments	2.04 (1.55 to 2.53)	
Absorption constant (h^{-1})	1.20 (1.03 to 1.36)	17.7% (11.8% to 23.6%)**
Clearance/F – HIV- (L/h)***	26.0 (21.1 to 30.9)	54.8% (46.9% to 62.6%)*
Clearance/F – HIV+ (L/h)***	20.02 (12.9 to 25.2)	
Volume of distribution of central compartment (L)***	31.9 (30.8 to 36.2)	
Inter-compartmental clearance/F (L/h)***	12.6 (6.13 to 19.07)	
Volume of distribution of peripheral compartment (L)***	21.4 (18.5 to 24.4)	
Additive error (mg/L)	0.0146 (0.003 to 0.03)	
Proportional error (%)	13.1 (11.9-14.3)	

Abbreviations: F bioavailability

*** CL and V have been allometrically scaled using individual values of fat-free mass (FFM), so the typical values reported here refer to the median value of FFM in the cohort, 45 kg (e.g. a 1.7 m tall man weighing 51 kg)

Table 7.4 Parameter values estimated by the final pharmacokinetic model for pyrazinamide

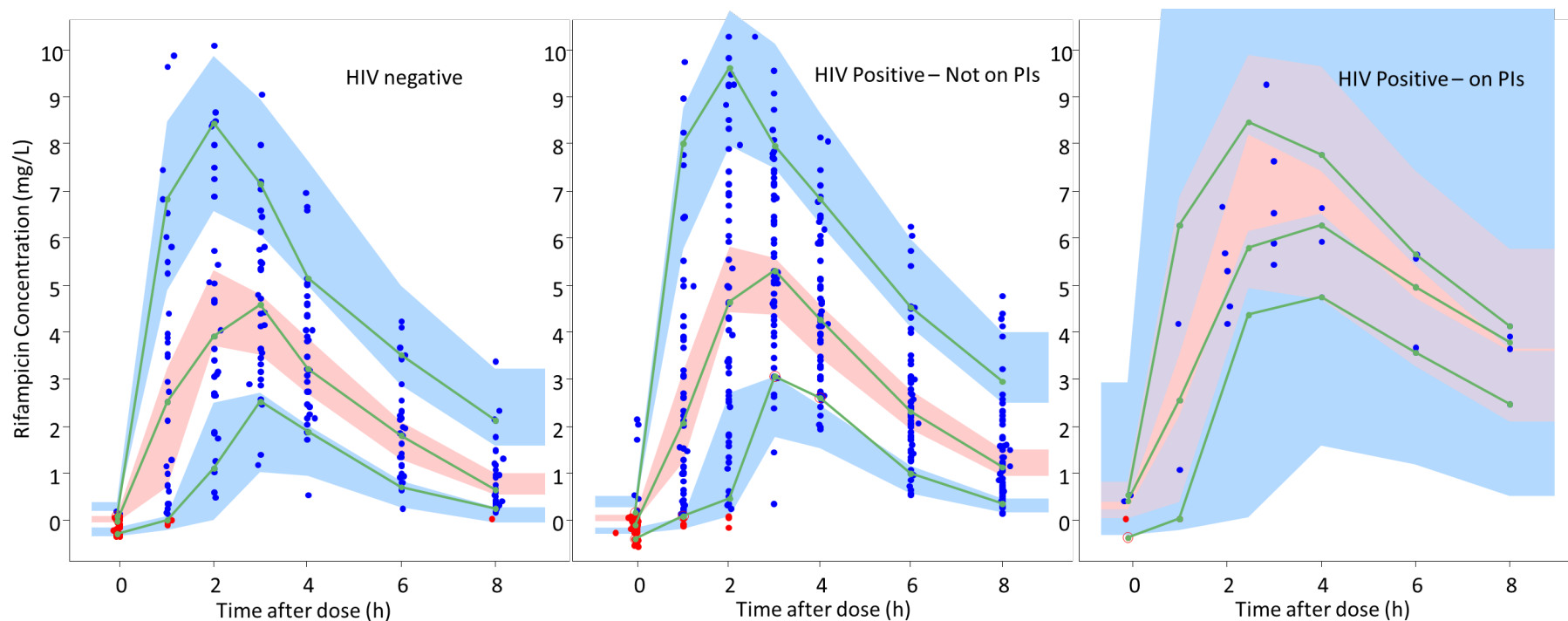
Parameter	Estimated population value (95% CI)	Inter-individual* or inter-occasional** variability (95% CI)
Bioavailability (F)	1 (fixed)	13.1% (10.2% to 16%)**
Mean transit time (h)	0.74 (0.65 to 0.84)	54.5% (45.1% to 63.9%)**
Number of absorption transit compartments	2.06 (1.59 to 2.53)	
Absorption rate constant (h^{-1})	50.0 (fixed)****	
Volume of distribution (L)**	41.9 (40.4 to 43.4)	
Clearance/F (L/h)**	4.17 (3.90 to 4.44)	29.6% (24.7% to 34.5%)*
Additive error (mg/L)	1.95 (1.77 to 2.13)	
Proportional error (%)	10.7 (9.60 to 11.80)	

Abbreviations: F bioavailability

*** CL and V have been allometrically scaled using individual values of fat-free mass (FFM), so the typical values reported here refer to the median value of FFM in the cohort, 45 kg (e.g. a 1.7 m tall man weighing 51 kg)

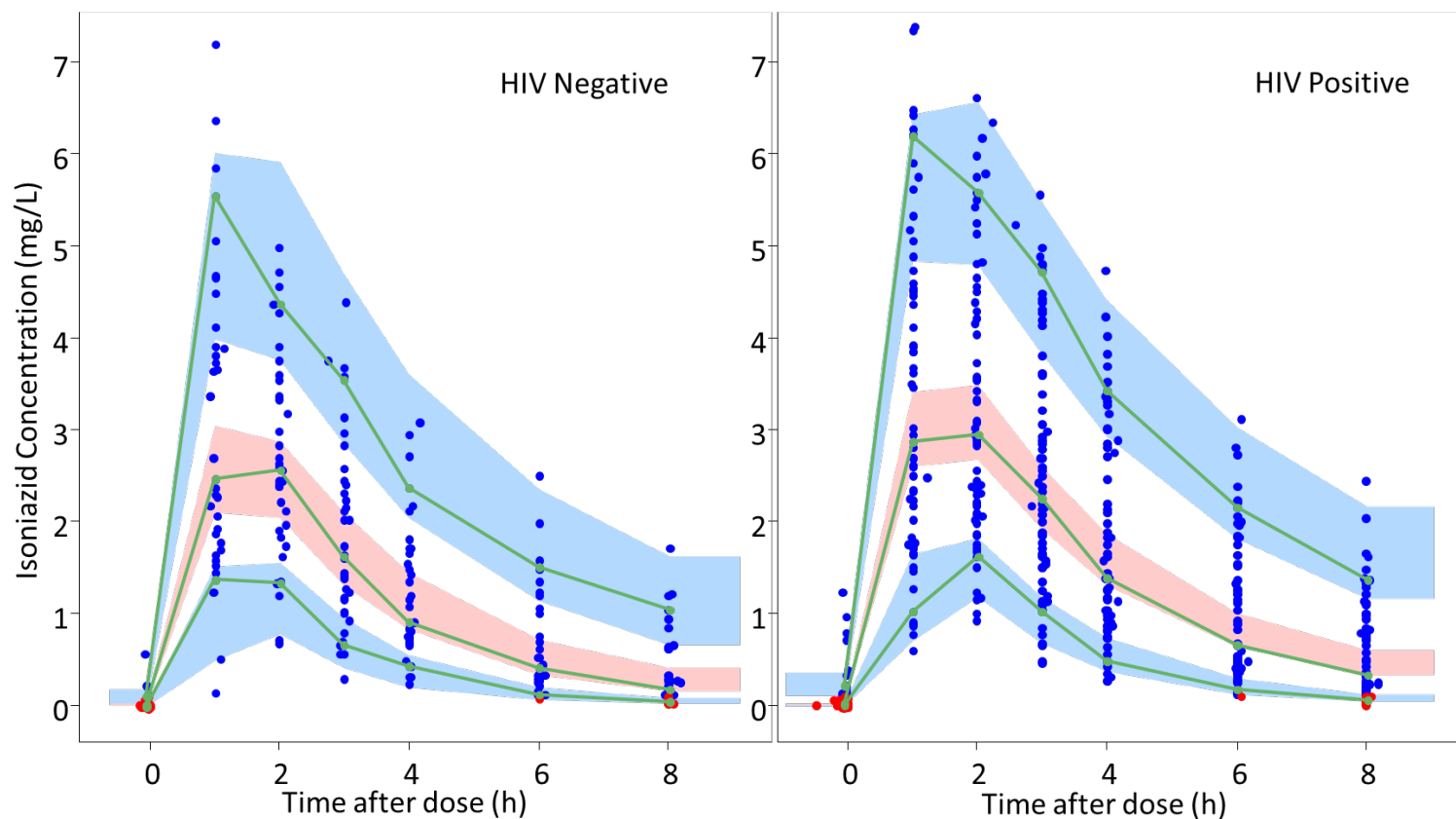
****The model estimated a very large value of K_a , so it was fixed to 50 to stabilise the model without significantly affecting the fit.

Figure 7.1 Visual predictive check for rifampicin concentration versus time, stratified by HIV status and co-administration of PIs



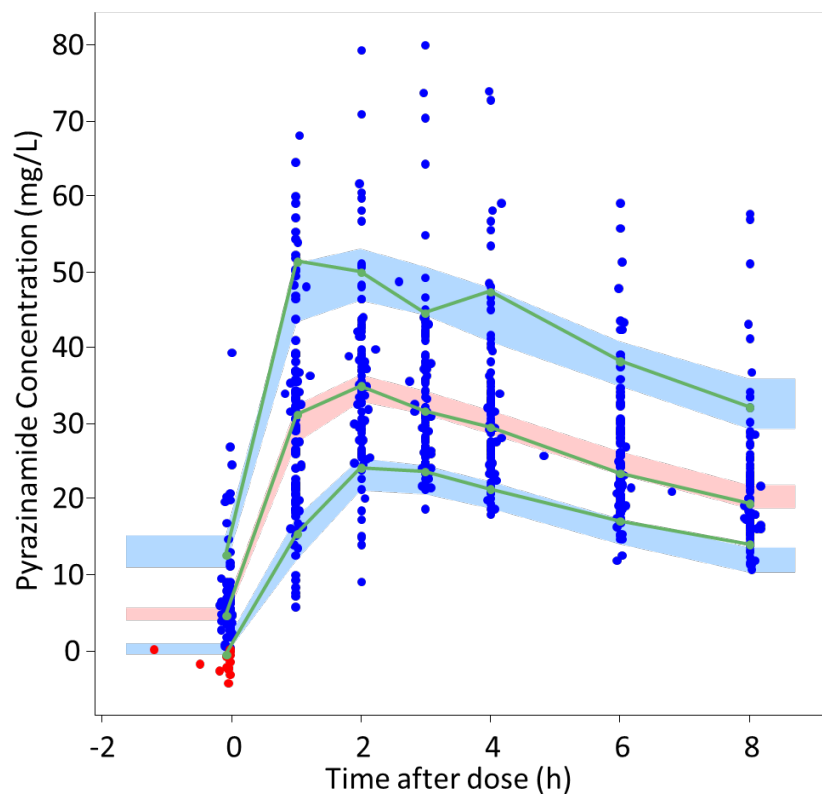
The blue dots are observed concentrations, while the red dots are simulation-based values below the limit of quantification (BLQ). Lines are 10th, 50th, and 90th percentile of observed data, while the shaded areas represent the 90% confidence intervals for the same percentiles, as predicted by the model.

Figure 7.2 Visual predictive check for isoniazid concentration versus time, stratified by HIV-1 status



The blue dots are observed concentrations, while the red dots are simulation-based values below the limit of quantification (BLQ). Lines are 10th, 50th, and 90th percentile of observed data, while the shaded areas represent the 90% confidence intervals for the same percentiles, as predicted by the model.

Figure 7.3 Visual predictive check for pyrazinamide concentration versus time



The blue dots are observed concentrations, while the red dots are simulation-based values below the limit of quantification (BLQ). Lines are 10th, 50th, and 90th percentile of observed data, while the shaded areas represent the 90% confidence intervals for the same percentiles, as predicted by the model.

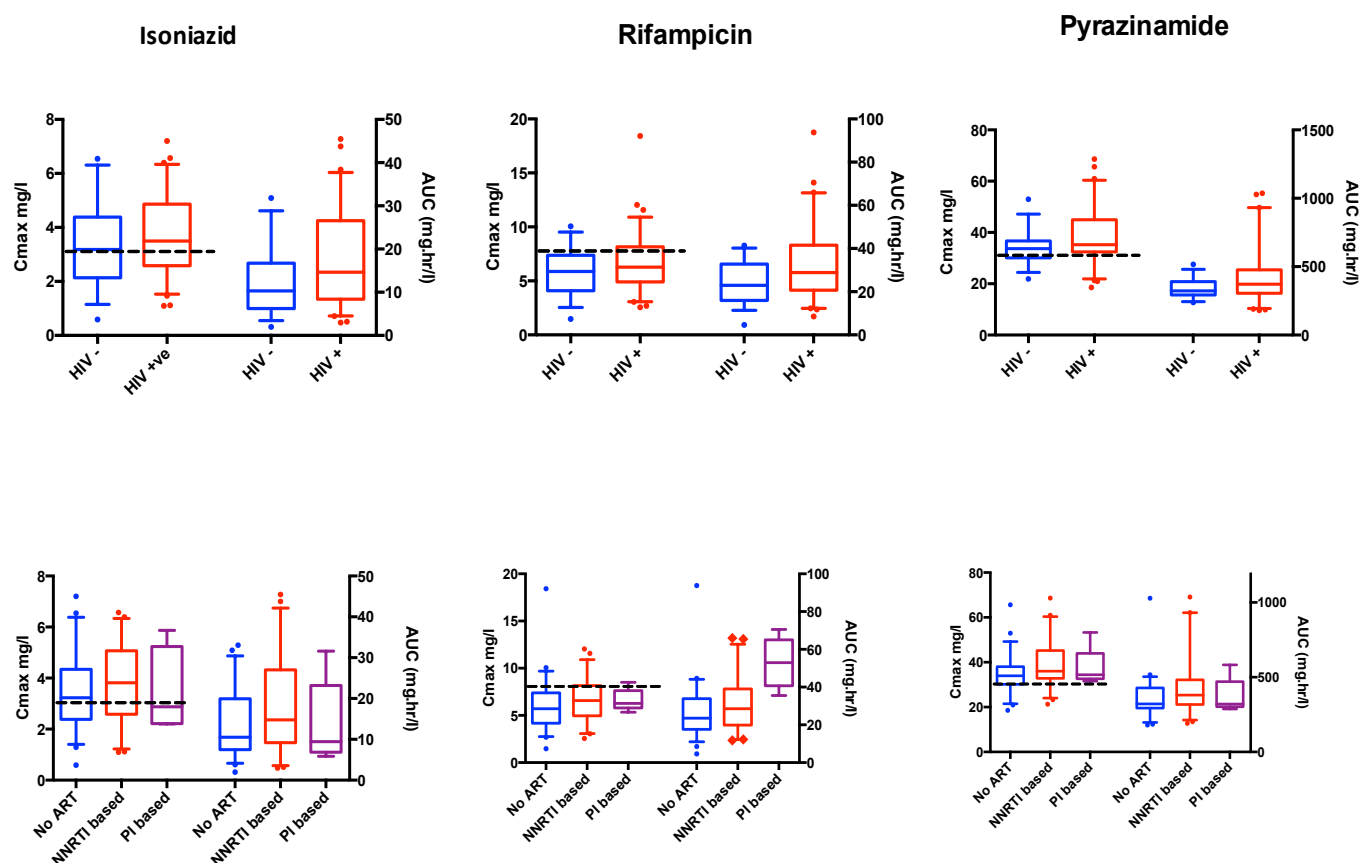
7.3.2.1. HIV-1 co-infection and ART

Since HIV-1 serostatus (infected vs. not infected) and ART status (on ART vs not on ART) are collinear, the covariate effect “HIV-1 status” and “ART status” on the pharmacokinetic parameters were tested separately. ‘HIV-1 status’ as a covariate caused improvement in the model to greater extent than ‘ART status’ (7 point drop in OFV, as compared to only 4) and diagnostics plots were better for the model including HIV, hence this was selected in the final model rather than ART. The models found that HIV-1 co-infection significantly decreased CL of rifampicin (21% decrease, OFV drop 7.00, $p < 0.01$, see Table 7.2) and isoniazid (23% decrease, OFV drop 8.63, $p < 0.01$, see Table 7.3). Inclusion of HIV-1 co-infection in the covariate model, did not significantly improve the model fit for pyrazinamide (see Table 7.4). Although HIV-1 infected participants had lower FFM than HIV-1 uninfected participants (see Table 7.1), the effect of HIV-1 on CL was independent of differences in FFM.

We split ‘ART status’ into ‘type of ART’ (i.e. no ART, NNRTI-based ART, LPV/r-based ART) and tested it as a further covariate (in addition to the HIV effect). We separately tested the effect of both NNRTI-based regimen (yes/no) and LPV/r-based regimen (yes/no) as a covariate on bioavailability, V and CL in an optimised model which was already adjusting for the effect of ‘HIV-1 status’ on CL. On top of the effect of HIV-1 on CL, patients on double-dose LPV/r-based ART regimen (dosed at 800mg/200mg twice daily in all 5 cases) had a further significant decrease in rifampicin CL of 46% (OFV drop 7.00, $p < 0.01$) and hence, increased AUC (Table 7.2). When the model was re-run excluding the 5 participants on PI-based regimens, the effect of HIV-1 on CL was still significant.

Figure 7.4 shows C_{max} and AUC_{0-24} stratified by HIV-1 serostatus. For all 3 drugs, PK exposures were either increased in those HIV-1 infected or no difference was detected. There were no differences in exposures, comparing HIV-1 infected patients with CD4 lymphocyte counts above or below 200 cells/mm³. In HIV-1 infected participants, 41% had low isoniazid C_{max} (<3mg/L), 75% had low rifampicin C_{max} (<8mg/L) and 31% had low pyrazinamide C_{max} (<35mg/L) (180). In HIV-1 uninfected participants, 46% had low isoniazid C_{max} , 88% had low rifampicin C_{max} and 63% had low pyrazinamide C_{max} .

Figure 7.4 Pharmacokinetic measures C_{max} and AUC_{0-24} stratified by HIV-1 serostatus and ART regimen



The box and whisker plots show model derived PK measures. C_{max} and AUC_{0-24} are plotted on the left and right y-axes. The boxes show median and interquartile range. The whiskers represent the 5th to 95th percentiles and illustrate variability in both HIV infected (+) and HIV uninfected (-) patients and different antiretroviral therapy categories. Patients on inhibitors appear to have higher rifampicin AUC_{0-24} compared with 'No ART' and NNRTI based' categories

The dotted black line indicates current recommended thresholds for C_{max} of 3 mg/L, 8 mg/L and 30 mg/L for isoniazid, rifampicin and pyrazinamide respectively.

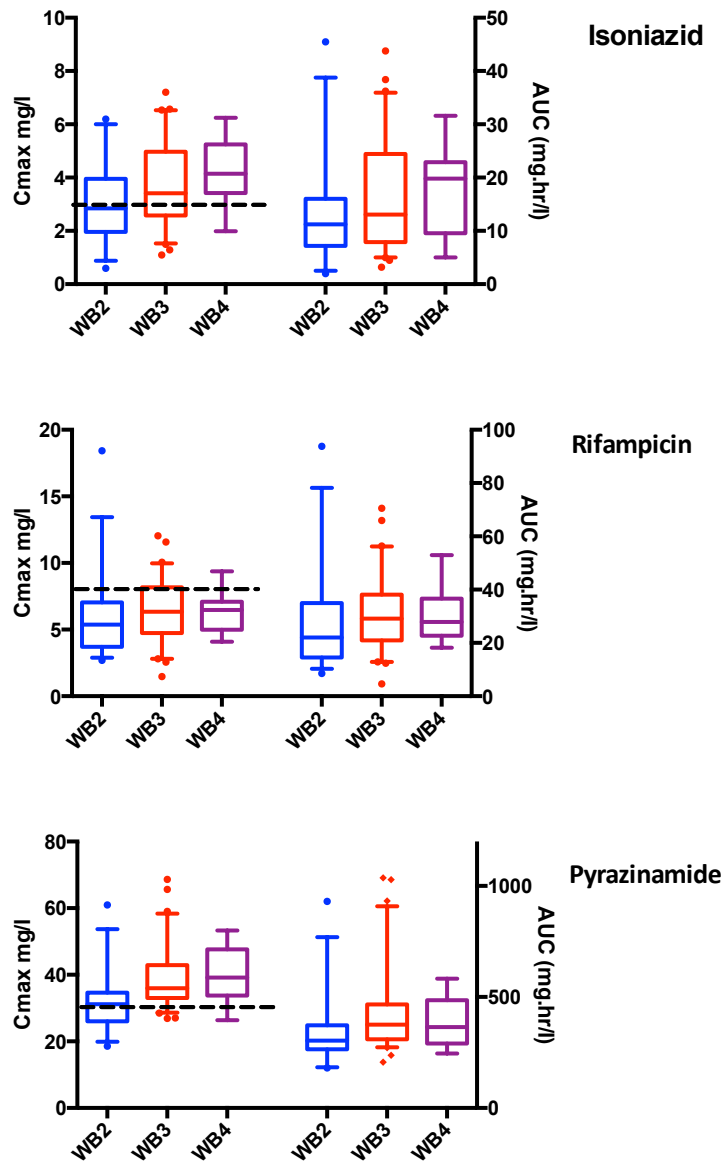
Statistical tests to generate p-values were not run, as reported individual values are based on the population PK models and are hence inter-dependent. Significance of covariates were tested within the model (table 2-4).

Abbreviations: ART antiretroviral therapy, NNRTI non-nucleoside reverse transcriptase inhibitor, PI protease inhibitor, C_{max} maximum concentration, AUC area under curve from 0 to 24 hours

7.3.2.2 WHO weight bands

There was a range of dosing in mg/kg for all 3 drugs (table 7.1). Eight participants required change in weight band during treatment. Three had their dose adjusted appropriately, one was put in a weight band higher than his weight and 4 were put in a weight band lower than their weight. Hence, on the day of PK sampling, 95 participants were being dosed correctly according to current weight and weight band. Patients in the lowest weight band had a trend of lower drug exposures and this was explained by a relatively higher CL in smaller individuals. Figure 7.5 shows differences in PK exposures stratified by the WHO weight band doses assigned by the programme. The predictions shown included allometric scaling with FFM which accounted for the increased clearance per kilogram of body weight in smaller individuals, and resulted in a significant improvement of the model.

Figure 7.5 Pharmacokinetic measures $C_{\max}$ and AUC_{0-24} , respectively stratified by WHO weight band



The box and whisker plots show model derived PK measures stratified by WHO weight band. $C_{\max}$ and AUC_{0-24} are plotted on the left and right y-axes. The boxes show median and interquartile range. The whiskers represent the 5th to 95th percentiles. The predictions include allometric scaling which is necessary to account for the nonlinear differences by weight/size. This explains differences in PK measures - in spite of same mg/kg dosing by weight band.

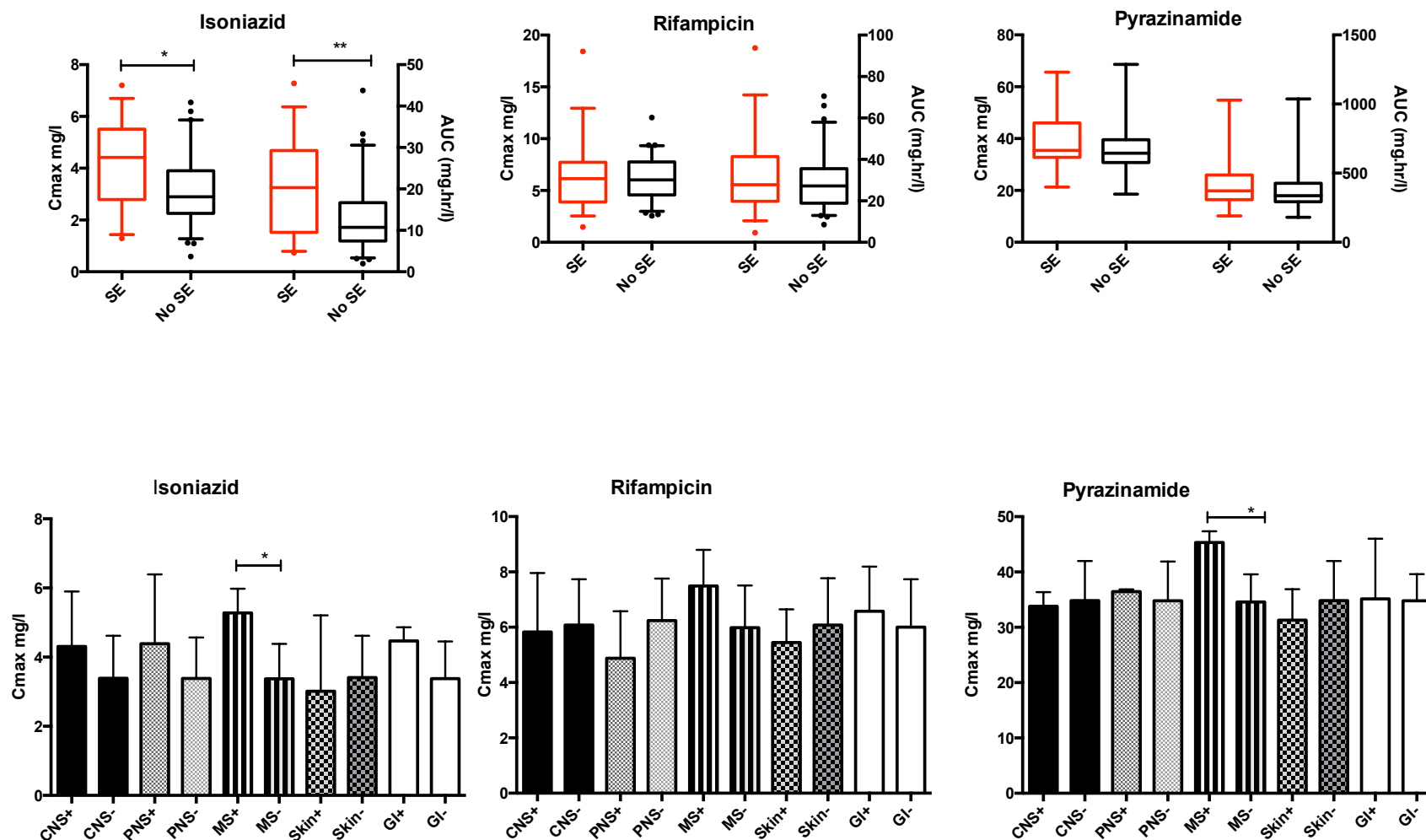
The dotted black line indicates current recommended thresholds for $C_{\max}$ of 3 mg/L, 8 mg/L and 30 mg/L for isoniazid, rifampicin and pyrazinamide respectively

Abbreviations: $C_{\max}$ maximum concentration, AUC_{0-24} area under curve from 0 to 24 hours, WB weight band

7.3.2.3 Side effects

All side-effects were CTCAE grade 1-2 and did not require drug withdrawal. Thirty-five participants (35%) reported CTCAE grade 1-2 side-effects which were possibly/probably attributed to anti-TB drugs including central nervous system/neuro-psychiatric (4/35), peripheral neuropathy (7/35), nausea/gastro-intestinal (11/35), musculoskeletal (11/35), skin (8/35), other (2/35). Patients presenting side-effects had a significantly higher median $C_{\max}$ for isoniazid [4.42 mg/L (IQR 2.79-5.51) compared with those who did not 2.89 mg/L (IQR 2.28-3.87, $p=0.001$). Table 7.2 shows the risk factors for reported side effects at 2-month review. Having adjusted for age, HIV-1 serostatus, diabetes mellitus status, alcohol intake, age and previous isoniazid treatment, the highest quartile of isoniazid AUC_{0-24} was still associated with increased side effects [OR 7.11 (95% CI 1.99- 25.47, $p=0.003$)] compared with the lowest quartile of isoniazid AUC_{0-24} (see table 7.4). There were no significant differences for rifampicin or pyrazinamide (Figure 7.6). Although there was a trend of high isoniazid $C_{\max}$ in those with central nervous system, peripheral nervous system, gastro-intestinal and musculoskeletal side-effects, this was only statistically significant for musculoskeletal side effects. A significantly higher pyrazinamide $C_{\max}$ was also seen in patients with musculoskeletal side-effects (Figure 7.6).

Figure 7.6 Pharmacokinetic measures $C_{\max}$ and AUC_{0-24} stratified by side-effect profile



The upper 3 graphs show $C_{\max}$ on the left axis and AUC_{0-24} on the right y-axis, stratified by presence or absence of drug side-effects. The bottom 3 graphs detail drug peak concentrations in those with (+) and without (-) CNS, PNS, MS, skin and GI side-effects

Abbreviations: C_{max} peak concentration, AUC₀₋₂₄, SE side-effects, CNS central nervous system, PNS peripheral nervous system, MS musculoskeletal system, GI gastrointestinal

Table 7.5 Risk factors for reported side effects at 2-month review- univariate model

Variable	OR (95% CI) for reported side effects
INH AUC ₀₋₂₄ Quartile 1	1
INH AUC ₀₋₂₄ Quartile 2	1.26 (0.34-4.84)
INH AUC ₀₋₂₄ Quartile 3	1.88 (0.52-6.84)
INH AUC ₀₋₂₄ Quartile 4	7.11 (1.99-25.47)
10 year increase in age	1.09 (0.71-1.67)
Previous isoniazid treatment	1.54 (0.67-3.55)
HIV-1 serostatus	1.56 (0.64-3.80)
Alcohol intake	1.46 (0.63-3.41)
Type 2 diabetes mellitus	1.99 (0.26-14.17)

7.4 Discussion

Peak concentrations and AUC_{0-24} of rifampicin, isoniazid and pyrazinamide were low and highly variable, and findings were comparable with other cohorts in both similar (Southern African) (41, 78, 160, 166, 181) and different (182-184) study populations.

There have been previous studies examining anti-TB PK in HIV-1 co-infected patients with a HIV-1 uninfected comparator group. However, none of these studies included HIV-1 co-infected patients concomitantly taking ART. Reduced rifampicin concentrations have been recorded in HIV-1 co-infected patients with diarrhoea and this has been associated with malabsorption and advanced immunosuppression (164, 185, 186).

Further studies have also shown evidence for reduced rifampicin concentration in HIV co-infected patients (78, 165), whilst others show no significant difference (159, 166).

There have also been contrasting results in the literature regarding the effect of HIV-1 co-infection on pyrazinamide concentrations (78, 187).

In this cohort of ambulatory patients, with 77% of HIV-1 co-infected patients on ART, there was no evidence of lower plasma concentrations of rifampicin, isoniazid, or pyrazinamide in HIV-1 infected patients. Conversely, the population PK model, which accounted for the effect of FFM, showed evidence of reduced rifampicin and isoniazid CL in HIV-1 infected compared to HIV-1 uninfected patients leading to increased AUC_{0-24} .

Although use of ART or CD4 stratification as a covariate did not significantly improve the model, there was an independent effect of “LPV/r” vs “no LPV/r” on rifampicin AUC_{0-24} , again explained by a reduction in CL. Although only 5 patients in the study were on an ART regimen inclusive of LPV/r, this effect of augmented dose LPV/r on rifampicin exposures was statistically significant. This has not been reported previously. Rifampicin is a substrate of p-glycoprotein and organic anion-transporting polypeptide (OATP) 1B1 and OATP 1B3. Lopinavir and ritonavir are inhibitors of both p-glycoprotein and OATP1/3(188). OATP1/3 can be involved in the influx of rifampicin from blood into the hepatocytes, the precursor to biliary clearance. Hence, its inhibition may be a potential mechanism for decreased clearance of rifampicin. Inhibition of gastrointestinal p-glycoprotein could also increase systemic rifampicin concentrations through increased bioavailability. There are potential implications for dosing and toxicity profiles, particularly in future regimens incorporating higher dosing of rifampicin and these findings should be explored in further PK studies. These results provide evidence that, at the time of switch

to continuation phase, ambulant HIV-1-TB co-infected patients who are reconstituting their immune system on ART, do not have reduced anti-TB drug concentrations compared with HIV-1 uninfected patients. These findings are not necessarily generalizable to HIV-1-TB co-infected patients in an inpatient setting, or those with advanced immunosuppression (median CD4 of this cohort was 233 cells/mm³). As previously reported (160), weight and in particular FFM, influenced CL in a non-linear fashion and hence uniform dosing in mg/kg across the WHO weight bands, was associated with the lowest weight band having a trend of lower drug concentrations compared with the highest weight band. Therefore, dosing could be optimised according to FFM and in particular, dosing for the lower weight band should be reviewed. Future work includes use of population PK modeling, in tandem with simulated dose adjustments for all 3 drugs, to calculate optimised dosing for lower weight bands. Having adjusted for potential confounders, there was still a significantly increased proportion of side-effects in patients with the highest isoniazid AUC₀₋₂₄ quartile. Overall incidence of drug side-effects secondary to isoniazid reported in the literature ranges from 1-3% for dermatological, gastrointestinal and neurological side-effects, to 1-17% for hypersensitivity reactions (170). Thirteen of the 16 patients who had side-effects and were in the highest isoniazid AUC₀₋₂₄ quartile were HIV-1 co-infected. Studies have shown that 8-20% of patients taking isoniazid can develop anti-nuclear antibodies. This is increased in slow acetylators (189) and may be potentiated in HIV-1 co-infection. One randomized controlled clinical trial conducted in Japan, showed that isoniazid related liver injury in the first 8 weeks of anti-TB treatment occurred in 78% of slow acetylators given standard 5mg/kg dose compared to 0% in slow acetylators given 2.5mg/kg dose(190). Hence, significant PK variability for isoniazid, even at standard dosing, may be contributing to toxicity.

There were several limitations in this study. Pharmacogenomic data such as NAT-2 and SLCO1B1 genotype was not available for incorporation into the population PK models. Although IOV was included as a random effect in the stochastic model, use of a single time point would not have accurately incorporated uncertainty secondary to timing of dose, adherence, meals and changes in weight and immune reconstitution secondary to ART over the course of TB treatment. A previous study in HIV-infected patients did not find an independent effect of first dose ART or steady state ART (at 2 weeks) on TB PK compared with day 1 of ATT(160). There was no routine monitoring of blood tests such

as liver and renal function. Hence, asymptomatic adverse drug reactions would not have been ascertained. Reported side effects may have been subject to ascertainment and observer bias. Many participants were also HIV-1 co-infected and were taking concomitant medications which may have also contributed to the reported side effect profiles. There were only 5 patients in this cohort on lopinavir/ritonavir based ART and hence, further studies are required to assess impact of protease inhibitor based regimens on rifampicin exposures.

In this outpatient setting with a high burden of HIV-1 co-infected patients, the majority of whom were immune reconstituting on ART, there was no evidence that HIV-1 co-infection led to lower anti-TB drug concentrations. In this specific cohort, HIV-1 coinfecting patients had a trend of increased drug concentrations. The results of this study are not necessarily generalisable to HIV-1 co-infected patients with advanced immunosuppression who are hospitalised and/or have co-morbidities causing malabsorption.

8. Pharmacokinetic/pharmacodynamic determinants of two-month culture conversion in rifampicin susceptible tuberculosis

The rationale for multi-drug anti-tuberculosis (TB) therapy administered over 6 months is to ensure sterilization of both actively and slow/non-replicating bacilli, and to prevent selection of resistant mutants. A treatment success rate of 86% has been reported in new TB cases (24). The rates of relapse in drug susceptible TB have been reported to be approximately 5% (25). Hence, studies including large numbers of participants at high cost are required to adequately assess predictors of unsuccessful treatment outcomes (relapse/failure). Consequently, interim treatment outcomes such as culture conversion by 2 months of treatment and time to culture conversion (26, 27) have been used as surrogates of outcome. Arguably, these are sub-optimal measures of sterilising activity against drug tolerant persisting bacillary sub-populations and subsequent relapse (29, 191).

There have been hypothesis-generating *in vitro* studies (79, 192, 193), animal models (194, 195) and Monte Carlo simulation analyses (15, 196) that predict that variability of drug concentrations both in plasma and at the site of disease (197, 198) significantly affect treatment outcomes. The relationship between bacterial growth and different antibiotic concentrations can be obtained from the pharmacodynamic (PD) parameter, the minimum inhibitory concentration (MIC). In the context of *M. tuberculosis* (MTB), this is the lowest of a series of drug dilutions which will limit growth of less than 1% (<10% for pyrazinamide) of the bacterial population under defined *in vitro* conditions. The pharmacokinetic (PK)-PD parameter which best predicts microbial kill in hollow fibre models for isoniazid, rifampicin and pyrazinamide is the ratio of area under the curve over 24 hours ($AUC_{0-24hrs}$) to minimum inhibitory concentration (MIC) of *M. tuberculosis* (MTB) strain (39, 40). Predictors of resistance suppression are ratio of peak concentration (C_{max}) to MIC and $AUC_{0-24hrs}/MIC$ for isoniazid, C_{max}/MIC for rifampicin and proportion of time between doses where drug concentrations are above MIC (% T_{MIC}) for pyrazinamide (40). Studies evaluating PK-PD predictors of 2-month culture conversion and unsuccessful treatment outcomes are conflicting (table 8.1) with heterogeneity in geographical populations studied, % HIV-1 co-infection, dosing in mg/kg, dosing frequency, PK sampling methodology (i.e. intensive vs sparse) and methods of PK-PD

analysis. Few studies have MIC data on the infecting *M. tuberculosis* (MTB) strain. Due to the retrospective nature of many studies, not all have comparator PK data available from control patients with successful outcomes. Also, the target ranges traditionally used were derived from healthy volunteers in phase 1 studies (199).

8.1. Chapter hypothesis and aims

Overall hypothesis

Plasma concentrations of rifampicin, isoniazid and pyrazinamide are lower in patients who are culture positive at 2 months (non-converters) compared with those who have culture negative (converters) at 2 months and the PK measures C_{max} and AUC_{0-24} , as well as the PK/PD measures $C_{max}:MIC$, AUC_{0-24} and $\%T_{MIC}$ can be utilized to predict the probability of 2-month culture conversion.

Specific chapter aims

1. To summarize literature of existing studies examining the association of PK and PK/PD parameters on treatment outcomes for pulmonary TB treated with first line quadruple therapy (table 8.1).
2. To utilize C_{max} , AUC_{0-24} , $C_{max}:MIC$, AUC_{0-24} and $\%T_{MIC}$ in multivariate logistic regression and multivariate adaptive regression splines (MARS) to predict 2-month culture conversion.
3. To use MARS model to assess for any drug interactions (synergism/antagonism) pertaining to the binary outcome of 2-month culture conversion.

Table 8.1 Existing literature on PK/PD parameters and treatment outcomes for pulmonary TB treated with first line quadruple therapy

Study	Location, sample size (% HIV co-infected)	TB regimen	PK sampling	MIC data available	Analytical method and relationship with treatment outcome
^a Pasipanodya <i>et al</i> 2013 <i>J inf Dis</i> (41)	S Africa n=142 (11)	4RHEZ(S) ₇ 2(HR) ₅	Intensive	No	CART analyses identified PZA AUC≤363 mg*h/l, RIF AUC≤13 mg*h/l and INH AUC≤52 mg*h/L as predictors of the composite outcome of death/relapse /failure, and RIF C _{max} >6.6 mg/l, INH C _{max} >8.8 mg/l and PZA C _{max} >58.3 mg/l as thresholds predicting 2 month culture conversion.
^a McIlleron <i>et al</i> 2007, <i>Tuberculosis</i> (200)	S Africa n=142 (11)	4RHEZ(S) ₇ 2(HR) ₅	Intensive	Yes	Using Cox proportional hazards regression, drug concentrations were not associated with failure/death/relapse
Gumbo <i>et al</i> 2014 <i>Antimicrob Agents Chemother</i> (201)	S Africa n=36(8)	4RHEZ(S) ₇ 2(HR) ₅	Intensive	Yes	CART analyses suggested MIC breakpoints for prediction of treatment failure: 0.0312 mg/L for INH and 0.0625 mg/L for RIF
Chigutsa <i>et al</i> 2014, <i>Antimicrob Agents Chemother</i> (181)	S Africa, n=54 (13)	4RHEZ ₅ 2(HR) ₅	4-8 time points	Yes	MARS analyses identified RIF C _{max} >8.2 mg/l and PZA AUC/MIC>11.3 were predictors of the β slope and interacted synergistically. At RIF AUC<35.4

					mg*h/l, increase in PZA AUC:MIC and ETHAM C _{max} :MIC increased β slope whilst increase in INH C _{max} was antagonistic
Heysell <i>et al</i> 2010, <i>Emerg Infect Dis</i> (202)	USA, n=45 (0)	RHEZ was dosed ≥ 5 /wk or x2/wk in IP	2h	No	Multivariate logistic regression identified low RIF and INH concentrations at 2h (but not PZA) noted in 52-59% of slow responders (defined as persistent symptoms, lack of radiological improvement) post 1mth of treatment ^b
Burhan <i>et al</i> 2013, <i>Antimicrob Agents Chemother</i> (182)	Indonesia, n=181 (1.1)	4RHEZ(S) ₇ 2(HR) ₅	2h	No	Multivariate logistic regression identified no RIF, INH and PZA levels at 2h had no effect on culture conversion at 2mths
Van Crevel <i>et al</i> 2002, <i>Int J Tuberc Lung Dis</i> (203)	Indonesia n=62 (1.6)	4RHEZ(S) ₇ 2(HR) ₃	2h	In 43/62 cases	No association was found between RIF concentration at 2h (or estimated C _{max} :MIC) and delayed sputum conversion or treatment failure.
Van Tongeren <i>et al</i> , 2013 <i>Int J Tuberc Lung Dis</i> (204)	Canada, n=52 (23)	As per national guidelines	2h	No	Patients with at least 1 drug level below normal range: - 21/47 experienced failure - 5/47 experienced relapse. - 3/4 patients with baseline drug resistance amplified drug resistance.
Weiner <i>et al</i> , 2003, <i>Am J Respir Crit Care Med</i> (205)	USA and Canada n=133 (0)	Either x1/wk or x2/wk rifapentine and isoniazid in	0,1,2,5,24h	No	Low INH AUC _{0-12h} (cox proportional hazards regression) and acetylator genotype (chi-squared test) was

		CP			associated with failure/relapse in the once weekly regimen
Weiner <i>et al</i> 2005, <i>Clin Infect Dis</i> (72)	USA n=102 (100)	Rifabutin/H x2/wk in CP	Intensive	No	Multivariate logistic regression revealed low rifabutin AUC (but not INH AUC) were associated with failure/relapse
Chideya <i>et al</i> 2009, <i>Clin Infect Dis</i> (78)	Botswana n=225 (69)	4RHEZ(S) ₇ 2(HR) ₅	1,2,6h	No	Mantel-Haenszel weighted risk ratio revealed PZA C _{max} <35 mg/l was associated with treatment failure or death
Chang <i>et al</i> 2008, <i>Eur J Clin Micro Inf Dis</i> (206)	Hong Kong, n=72 (0)	Standard PZA containing regimens	2 and 4h	No	Multivariate logistic regression showed no effect of RIF C _{max} on culture conversion at 2mths
Mehta <i>et al</i> , 2001, <i>Chest</i> (207)	USA n=6 (17)	1RHEZ ₇ 1RHEZ ₂ 2HR ₅	1 sample at 1.5-2.5h	No	Sub-therapeutic RIF level at 1.5-2.5h was associated with poor response at 3 months ^b
Babalik <i>et al</i> , 2011, <i>Can Resp Journal</i> (208)	Canada n=20 (40)	RHEZ daily at least during intensive phase	Average 2.25 drug level measurement per patient	No	Chi-squared/independent t tests showed no significant difference in time to culture conversion between those with low INH/RIF/RB C _{max} and those with concentrations within normal range. Low PZA concentrations were associated with greater time to culture conversion .
Kimmerling <i>et al</i> , 1998, <i>Chest</i> (209)	USA n=22	x2/wk RH in 62.5%, daily in 37.5%	2h	No	INH concentrations at 2h were sub-therapeutic in 15/22 and RIF concentrations at 2h were sub-therapeutic in 14/22 patients with adverse clinical outcomes ^b

Narita <i>et al</i> , 2001, <i>Clin Infect Dis</i> (42)	USA n=188 (37)	RHZ for IP	2 and 6h	No	Risk of tuberculosis recurrence was not associated with RIF and INH C _{max}
Prahl <i>et al</i> , 2014, <i>J Antimicrob Agents Chemother</i> (210)	Denmark n=35 (6)	4RHEZ(S) ₇ 2(HR) ₇	2h	No	As per chi-squared test, significantly more patients with both low rifampicin and isoniazid 2h Concentrations and individually low concentrations of RIF and INH experienced therapy failure
Park <i>et al</i> 2015, <i>Antimicrob. Agents Chemother</i> (211)	Korea n=413 (0)	Daily RHZ for IP	2h	No	Multivariate logistic regression revealed no significant relationship between drug concentration at 2h and 2-month culture conversion or disease recurrence
Ruslami, 2007, <i>Antimicrob Agents Chemother</i> (212)	Indonesia n=47 (2)	4RHEZ ₇ 2(HR) ₃	Intensive	No	Chi-squared test showed no significant difference in culture conversion rate between the higher (600mg)- and standard-dose (450mg) RIF groups

^a Same clinical cohort, data analysed using different methodological approaches

^b Drug concentrations only measured in patients with adverse treatment outcome (no comparator PK group)

Case reports not included in this table

Abbreviations: H/INH isoniazid, R/RIF rifampicin, Ethambutol, Z /PZA pyrazinamide, Rx treatment, h hour, mths months, f/u follow up, IP intensive phase, CP continuation phase, wk week, C_{max} peak concentration, AUC area under curve

8.2. Methods

Patient recruitment, antitubercular treatment (ATT) regimen and dosage as per WHO weight bands are outlined in (3.1.1). Study procedures (3.1.2), side effects characterization (3.1.4), sputum processing (3.2), MIC determination (3.4) and determination of treatment outcomes is described in (3.1.6). Intensive sampling PK studies (3.1.5) and liquid chromatography tandem mass spectrometry (LC-MS) (8.2) are previously described (8.2). Plasma concentration-time data from all subjects were simultaneously analysed by a non-linear mixed-effects model utilising Monolix (version 4.3.3) as described in 8.2. Finally, model based individual PK parameter values after the observed dose were used in the R package Simulx by Dr. Paolo Denti to simulate individual profiles. Adherence of 100% every 24 hours and a free unbound fraction (f) for rifampicin, isoniazid and pyrazinamide of 0.2, 0.9 and 0.9 were assumed. PK/PD parameters $C_{\max}$, $C_{\max}$ (free), trough concentration ($C_{\min}$), $fC_{\min}$, % T_{MIC} and % fT_{MIC} , AUC_{0-24} and $fAUC_{0-24}$ were calculated.

Statistical analyses

Sample size for this study assumed a coefficient of variation for rifampicin $C_{\max}$ (drug with greatest PK variability) of 40% (McIlleron *et al* unpublished). Assuming an estimated culture conversion rate at 2 months of 70%, a sample size of 92 was required to detect a 25% difference in $C_{\max}$ between converters and non-converters, with 80% power and an alpha of 0.05.

The median PK values for AUC_{0-24} and $C_{\max}$ for pyrazinamide were imputed for 2 patients who took only rifampicin/isoniazid on the day of the study. The Kruskal Wallis test was used to compare groups of independent variables. The Spearman's rank correlation coefficient test was used to assess correlation. Unadjusted PK measures, PK measures adjusted by MIC and clinical covariates were considered in univariate logistic regression analyses to determine predictors of 2-month culture conversion. P-values from the univariate analyses were used to guide variable selection for the multivariate model and should be interpreted with caution in light of the potential for multiple comparisons. Clinical covariates were tested for pairwise interactions with PK parameters and the outcome of 2-month culture conversion. It was decided *a priori*, to include AUC_{0-24}/MIC for rifampicin, isoniazid and pyrazinamide in the multivariate model, along with the clinical covariates which were significant ($p \leq 0.2$) in the univariate analysis. Stata version

13.1 (College Station, Texas) was used for these analyses.

Multivariate analysis regression splines (MARS) in Salford Predictive Modeler[®] was used in collaboration with Dr. Jotam Pasipanodiya to identify predictors of the binary outcome of culture conversion as a continuous outcome between 0 and 1 (i.e. probability of culture conversion). Unlike logistic regression, MARS breaks up complex nonlinear relationships in distinct ranges or regions of the dataset to perform piecewise regression with automatic examination of high-order (i.e. both one-way and two-way) interactions. The data ranges are delineated by hinges/knots, and the relationships are given as slopes in basis functions (BFs) that specify the hinges or data range reported (213). The receiver operating characteristics (ROC) values of the learn and test models after 10-fold cross-validation was used to select best model. All demographic, clinical, laboratory, and PK/PD exposure variables (C_{max} , $f_u \cdot C_{max}$, $\%T_{MIC}$, $\%T_{MIC (free)}$, C_{max}/MIC , $f_u \cdot C_{max}/MIC$, AUC_{0-24} , $f_u \cdot AUC_{0-24}$ and AUC_{0-24}/MIC) were included as potential predictors in initial stepwise modeling exercises and parameters were arbitrarily set at a maximum of 15 basis functions. Thereafter, parameters in basis function format were pruned back to increase prediction on test sample as well as to improve interpretability and parsimony. Salford Predictive Modeler[®] version 7.0 was used for MARS (213). Finally, we used logistic regression to compute an adjusted odds ratio with a 95% confidence interval using thresholds identified by the MARS model.

8.3. Results

8.3.1. Clinical characteristics and treatment outcomes

Table 8.2 provides the clinical characteristics of the PK cohort, stratified by HIV-1 serostatus. There were two patients in whom pyrazinamide values were missing and in whom the median AUC_{0-24} and C_{max} values for pyrazinamide were imputed in the PK analysis. Of the 100 study participants, 65% were HIV-1 infected with a median CD4 lymphocyte count of 233 cells/mm³ (IQR 106-386). The proportion on ART increased from 27/65(42%) at baseline to 50/65(77%) at the time of the PK study. 52% had lung cavities present at baseline and 66% were smear positive (grading 1 to 3+), of whom 24/66 (36%) were graded 3+. Culture conversion at 2 months was 77%. The rate of culture conversion was higher than seen in some studies of smear positive HIV-1 uninfected participants(214, 215). However, in the context of baseline smear

negative/scanty rates of 34%, the study ascertained rates of conversion in MGIT cultures are unlikely to be over-estimated and are comparable to populations with similar HIV-1 co-infection rates(216). At the end of study follow up there was 1 death in the PK cohort and 7 failure/relapses (table 8.2).

Table 8.2 Treatment outcomes for cohort

Outcomes	Whole PK cohort (N=100)	HIV-1 infected (N=65)	HIV-1 uninfected (N=35)
2 month culture conversion n (%)	77 (77)	53 (81)	24 (69)
5 month culture conversion (out of 83 patients) (%) *7 defaulters, 1 transfer of care, 9 treatment completers (no sputum produced at 5 months)	80/83 (96)	50/53 (94)	30/30 (100)
Treatment failures over study duration n (%)	3 (3)	3 (5)	0
Treatment relapse	4 (4)	3 (5) * 1 relapse died and had acquired drug resistance	1 (3)
Overall successful outcome (treatment cure/completion without relapse) *defaulters assumed to have unsuccessful outcome. Transfer of care with unknown outcome not included in denominator)	86/99 (87)	54/64 (84)	32/35 (91)

8.3.2. PK and PK/PD parameters

Figure 8.1 shows that, for all three drugs, there was no significant difference in medians and distribution of C_{max} , %T_{MIC} and AUC₀₋₂₄, between patients who converted and those who did not convert. In addition, for both converters and non-converters, a significant proportion of patients had a C_{max} lower than the currently recommended guidelines for all drugs (199). For isoniazid 43% patients had a low C_{max} (<3mg/L) and 6% had very low peak concentrations (<1.5mg/L). For rifampicin 80% had a low C_{max} (<8mg/L) and 17% had a very low C_{max} (<4mg/L). None of these C_{max} cut-off values for isoniazid or rifampicin predicted 2-month culture conversion and/or failure/relapse. For pyrazinamide 53% patients had C_{max} <35mg/L (78) and 1% had C_{max} <20mg/L. The cut-off of pyrazinamide <35mg/L was not predictive of 2-month culture conversion, however, but predicted failure/relapse (OR=0.16, p=0.03). Twenty-six of 31 patients (84%) with low concentrations of all 3 drugs had culture converted at 2 months, and 4/31 (13%) had treatment failure/relapse compared with 3/69 (4%) who did not have low concentrations

of all 3 drugs ($p=0.12$). The proportion of all patients with estimated free drug in plasma $\%T_{MIC (free)}$ above the MIC for at least 12 hours (i.e. 50% of dosing interval) was 49%, 21% and 4% for rifampicin, isoniazid and pyrazinamide respectively. C_{max}/MIC and AUC_{0-24}/MIC and $\%T_{MIC}$ for the same drug co-varied ($r>0.8$; $p<0.01$), but not between different drugs ($r\leq 0.32$; $p<0.01$).

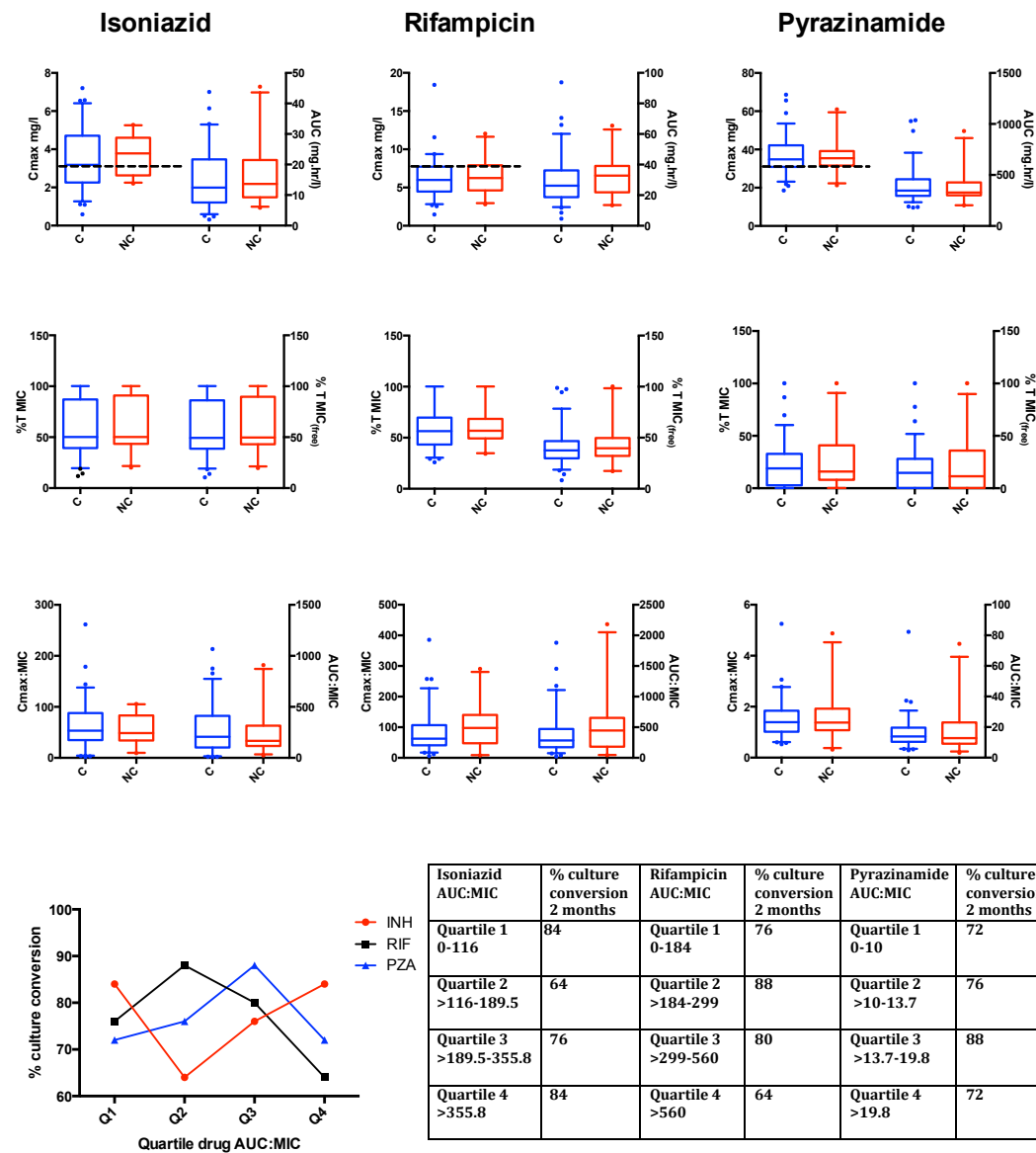
8.3.3. Determinants of 2-month culture conversion

As detailed in figure 8.1, on adjusting PK exposures for the MIC of infecting MTB strain, there was considerable inter-individual variability of AUC/MIC , C_{max}/MIC and $\% T_{MIC}$ with no linear relationship between PK/PD parameters and the proportion culture converting at 2 months.

On univariate logistic regression analysis, age (OR 0.56 for each 10 year increment, 95%CI 0.35-0.91, $p=0.02$), smear 3+ positivity (OR 0.2, 95%CI 0.06-0.71, $p=0.01$), $\log_{10}$ HIV-1 viral load (OR 1.5, 95%CI 1.02-2.19, $p=0.04$) and days to culture positivity (TTD) at baseline (OR 1.15, 95%CI 1.01-1.28, $p=0.04$) were significant predictors of culture conversion at 2 months (see Table 8.3). On multivariate analyses executed inclusive of C_{max}/MIC or AUC/MIC for rifampicin, isoniazid and pyrazinamide as covariates, 10 year increment in age (OR 0.44, 95%CI 0.24-0.81, $p=0.01$), TTD at baseline (OR 1.16, 95% CI 1.02-1.33, $p=0.02$), smear 3+ positivity (OR 0.09, 95%CI 0.02-0.35, $p<0.001$) and drug side effects (OR 0.17, 95%CI 0.05-0.63, $p=0.01$) were the only significant predictors of 2-month culture conversion.

The proportion of 2-month culture conversion for rifampicin and pyrazinamide were highest in the second and third AUC/MIC quartile (see figure 8.1). Conversely, culture conversion rates were lowest in the second and third AUC/MIC quartile for isoniazid. Table 8.4 shows a break down of potential clinical predictors of conversion within the different strata of AUC/MIC quartiles for isoniazid and pyrazinamide. The only significant difference was higher % reported side effects at the highest quartile of isoniazid AUC/MIC . No statistically significant interaction was found between isoniazid exposures, drug side effects and the outcome of culture conversion. There was also a non-significant trend towards increased % smear 3+ at the highest rifampicin AUC/MIC quartile, suggesting that confounding by severity of the pulmonary disease may help explain reduced culture conversion at this top quartile of rifampicin exposure.

Figure 8.1 PK and PK/PD measures stratified by culture converter status



The box and whisker plots show model derived PK measures. C_{max} and AUC_{0-24} (with and without adjustment for MIC) are plotted on the left and right Y-axes. The boxes show median PK and PK/PD measures (and interquartile range) and the whiskers show 5th to 95th percentile and illustrate considerable variability within converter and non-converter groups. The proportion of 2-month culture conversion is also shown stratified by AUC:MIC quartile for INH, RIF and PZA.

The dotted black line indicates current recommended thresholds for C_{max} of 3mg/l, 8mg/l and 30mg/l for INH, RIF and PZA respectively

Abbreviations: C converter, NC non-converter, C_{max} maximum concentration, AUC_{0-24} area under curve from 0 to 24 hours, MIC minimum inhibitory concentration, % T_{MIC} proportion of time between dosing intervals that drug concentration is above MIC, % $T_{MIC(free)}$ proportion of time between dosing intervals that unbound drug concentration is above MIC, INH isoniazid, RIF rifampicin, PZA pyrazinamide

Table 8.3 Univariate analysis of risk factors for culture conversion at 2 months

Variable [#]	Whole cohort (n=100) OR	95% CI	p-value
Male sex	0.81	0.31-2.10	0.67
10 yr increment in age	0.56	0.35-0.91	0.02
BMI	0.97	0.89-1.06	0.48
Retreatment status	0.39	0.15-1.01	0.05
Smoker status:			
Never	1		
Ex	1.86	0.54-6.48	0.33
Current	0.79	0.26-2.35	0.67
Alcohol use	0.70	0.27-1.81	0.46
Ex-prisoner	0.71	0.20-2.52	0.60
Ex-miner	0.18	0.03-1.13	0.07
Diabetes	0.28	0.04-2.11	0.22
Drug side effects at 2-month review	0.39	0.15-1.01	0.05
Poor adherence at 2-month review as per pill counts/self-report n (%)	0.67	0.16-2.81	0.58
Isoniazid resistance	0.89	0.17-4.73	0.89
Smear grading:			
Neg/scanty			
1+	0.69	0.16-2.93	0.61
2+	1.09	0.23-5.11	0.91
3+	0.20	0.06-0.71	0.012
Time to culture positivity at baseline	1.14	1.01-1.28	0.035
HIV-1 status	2.02	0.78-5.23	0.15
Log ₁₀ CD4*	0.70	0.20-2.42	0.57
Log ₁₀ VL*	1.50	1.02-2.19	0.04
ART at baseline*	0.5	0.14-1.67	0.25
Extensive radiological disease	0.61	0.20-1.85	0.39
Cavitary disease	0.62	0.24-1.62	0.33

[#] At TB diagnosis (i.e. baseline) unless otherwise specified as day of PK study or 2-month review

* analyses only included 65 HIV-1 infected patients

8.3.4. MARS model

Next MARS, a machine-learning and assumption free algorithm, was used to rank predictors and identify interactions between variables. The most important variable ranked as follows: smear grading (100%), followed by isoniazid C_{max} (87%), the CD4 lymphocyte count (75%) and rifampicin C_{max}/MIC (73%).

Next we used MARS, to identify and rank potential predictors of 2-month conversion. The findings are shown as basis functions (BFs), and the meaning of each BF is explained in Table 8.5. The probability of culture conversion increased with an increase in isoniazid C_{max} above 4.6mg/L (as indicated by the positive co-efficient of BF₁). At an isoniazid C_{max} of 4.6mg/L, this effect was reversed, with the probability of culture conversion increasing as isoniazid C_{max} decreased (as per positive co-efficient of BF₂). These are termed mirror BFs, the reason for which can be seen in Figure 3, where the BFs are characterised by a hinge on the value of C_{max} 4.6 mg/L and show a V-shaped relationship of isoniazid C_{max} versus the probability of culture conversion. BF₆ shows the interaction on condition of an isoniazid C_{max} <4.6 mg/L and rifampicin C_{max}/MIC <28. Below the rifampicin C_{max}/MIC ratio of 28, the probability of culture conversion decreased as rifampicin C_{max}/MIC decreased from 28 to 0. This effect was modified by an interaction whereby the effect of increasing rifampicin C_{max}/MIC was increased as isoniazid C_{max} decreased from 4.6 to 0 mg/L. Hence, in this subset of patients, with isoniazid $C_{max} \leq 4.6$ mg/L and rifampicin $C_{max}/MIC < 28$, the antagonistic effect of isoniazid on culture conversion was counteracted by increasing rifampicin C_{max}/MIC . The robustness of this finding was further verified via multivariate logistic regression analysis. Amongst patients with isoniazid $C_{max} \leq 4.6$ mg/L, isoniazid C_{max} was associated with reduced culture conversion (aOR 0.35 for each 1 mg/L, 95%CI 0.15-0.80, p=0.01) and patients with rifampicin $C_{max}/MIC > 28$ had adjusted odds of 6.44(95%CI 1.02-40.54, p=0.04) for culture conversion at 2 months (table 8.6).

The optimised MARS model after stepwise elimination represented the probability of culture conversion by the equation:

$$Y = 0.65 + 0.24 * BF_1 + 0.17 * BF_2 - 0.001 * BF_4 - 0.33 * BF_5 - 0.016 * BF_6$$

Overall, the ROC for the selected model was 87% in the learn model and 66% on cross-validation, while the misclassification rates were 14% and 28%, respectively. These model performance figures are reassuring, suggesting that similar estimates could be expected in an independent sample of patients.

Table 8.4. Distribution of independent variables in patients within different quartiles of AUC/MIC

Covariate	Quartile 1	Quartile 2	Quartile 3	Quartile 4	p value
ISONIAZID					
Median age	32.6	31.1	32.3	33.7	0.95
% Retreatment	36	44	40	36	0.92
% Side effects	24	32	20	64	0.004
% Extensive radiological disease	64	88	64	68	0.19
% Cavities	44	60	44	60	0.46
% Smear 3+	28	24	20	24	0.93
Median TTD	10	9	11	11	0.94
% Poor adherence	4	16	4	16	0.26
Median log₁₀VL	4.8	4.5	3.4	5.16	0.22
Median log₁₀CD4	2.1	2.3	2.5	2.4	0.68
RIFAMPICIN					
Median age	32.6	32.6	32.4	33.7	0.68
% Retreatment	40	44	32	40	0.85
% Side effects	32	28	32	48	0.46
% Extensive radiological disease	64	60	84	76	0.22
% Cavities	44	48	60	56	0.66
% Smear 3+	16	12	28	40	0.09
Median TTD	11	13	10	9	0.20
% Poor adherence	8	4	12	16	0.53
Median log₁₀VL	4.6	4.7	4.5	5.2	0.22
Median log₁₀CD4	2.1	2.3	2.5	2.4	0.32
PYRAZINAMIDE					
Median age	31.15	32.47	34.8	33.1	0.75
% Retreatment	48	36	28	44	0.48
% Side effects	28	44	48	20	0.12
% Extensive radiological disease	72	88	68	56	0.09
% Cavities	40	68	60	40	0.11
% Smear 3+	20	24	24	28	0.93
Median TTD	11	8	9	11	0.87
% Poor adherence	12	8	12	8	0.93

Table 8.5 Explanation of Basis Functions identified in the final MARS model

Basis Function	Function	Co-efficient in model	Interpretation and number of patients BF applies to
BF0	Constant/intercept	0.652	
BF1	$\max(0, \text{INH } C_{\max} - 4.6)$	0.24	Where $\text{INH } C_{\max} > 4.6 \text{ mg/l}$ ($n=26$), the probability of culture conversion was increased as an additive effect ($+0.24 \cdot \text{BF1}$). However, upon reaching 4.6 and below, the effect of the BF1 became zero.
BF2	$\max(0, 4.6 - \text{INH } C_{\max})$	0.17	Basis function 2, the mirror image of BF1, had a lower bound of 0 and was only retained when $\text{INH } C_{\max} < 4.6 \text{ mg/l}$ ($n=74$). As the value of $\text{INH } C_{\max}$ decreased, the value of the function included increased as an additive effect ($+0.17 \cdot \text{BF2}$). There were also some interactions with BF3, BF6 (i.e. HIV-1 status and $\text{RIF } C_{\max} \cdot \text{MIC}$
BF3	$\max(\text{subset}=\text{HIV infected}) \cdot \text{BF2}$	Nil	Basis function 3 was a dummy variable for HIV infected patients and solely existed to interact with BF2 and only applied to HIV-1 infected patients with $\text{INH } C_{\max} < 4.6 \text{ mg/l}$ ($n=45$).
BF4	$\max(0, \text{CD4} - 190) \cdot \text{BF3}$	- 0.001	BF4 retained its function only in HIV infected patients with CD4 lymphocyte count > 190 ($n=38$). For these patients, probability of culture conversion was reduced by a small factor ($-0.001 \cdot \text{BF4}$) as CD4 lymphocyte count increased from 190 upwards. This function was modified by an interaction with BF2 where the effect size increased as $\text{INH } C_{\max}$ decreased from 4.6 to 0 mg/l.
BF5	$\text{Max}(\text{subset}=\text{smear grade } 3+)$	- 0.33	In those with an initial sputum smear grade of 3+, the average probability of culture conversion was decreased ($-0.33 \cdot \text{BF5}$) ($n=24$)
BF6	$\max(0, 28.00 - \text{RIF } C_{\max} / \text{MIC}) \cdot \text{BF2}$	- 0.016	For patients with $\text{RIF } C_{\max} \cdot \text{MIC} < 28$, probability of culture conversion decreased as per negative coefficient (-0.016) as $\text{RIF } C_{\max} / \text{MIC}$ decreased from 28 to 0 ($-0.016 \cdot \text{BF6}$). This function was modified by an interaction with BF2, where the negative effect increased as INH

			$C_{\max}$ decreased from 4.6 to 0 mg/l. Hence, in this subset of patients (n=9), there was an antagonistic interaction between RIF and INH.
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INH Isoniazid RIF rifampicin PZA pyrazinamide

Table 8.6 Multivariate logistic regression analysis in subset of patients with Isoniazid $C_{\max}$ <4.6mg/L

Variable	Multivariate analysis [#] OR (95% CI, p-value)
Isoniazid $C_{\max}$.	(aOR 0.35, 95%CI 0.15-0.80, p= 0.01)
Rifampicin $C_{\max}$ /MIC ≤28 $C_{\max}$ /MIC ≤28	Referent (aOR 6.44, 95%CI 1.02-40.54, p= 0.04)

[#]Adjusted for all significant determinants of 2-month culture conversion in table 2

8.4. Discussion

This is the largest study to date reporting the effect of first line anti-TB drug exposures, measured by intensive sampling and inclusive of adjustment for MIC of infecting MTB strain, on the interim outcome of 2-month culture conversion in a predominantly HIV-1 co-infected cohort. MIC distributions were representative of populations reported elsewhere (106, 107).

With only 3 treatment failures and 4 relapses, the study was underpowered to look at clinical and PK/PD predictors of long-term treatment outcomes. However, we did find that a pyrazinamide $C_{\max}$ <35mg/L was predictive of unfavourable treatment outcome, consistent with findings from other groups (78, 217).

Despite significant variability of AUC/MIC for rifampicin, isoniazid and pyrazinamide, the range of culture conversion over the AUC/MIC quartiles was only 64-88%, 64-84% and 72-88% for rifampicin, isoniazid and pyrazinamide respectively. Logistic regression did not identify a relationship between $C_{\max}$ /MIC, AUC/MIC or %T_{MIC} and the probability of culture conversion. This is likely because this approach compares measures of central tendency and distributions with no predefined thresholds for what are the high and low drug concentrations, further complicated by U-shaped relationships. When we examined the effect of traditionally used $C_{\max}$ breakpoints, themselves derived using normal distributions in healthy volunteers, concentration was still not significantly associated with outcome. A recent dose-escalation study showed a non-significant increase in early bactericidal activity (EBA) on colony forming units (CFU) when rifampicin doses were trebled to 30-

35mg/kg. There was also only a weak association between AUC_{0-24} at 14 days and EBA on CFU ($R^2 = 0.12$) (218).

The MARS model identified a potential interaction of concentration-dependent antagonism between isoniazid and rifampicin affecting outcome at the 2-month time point. This finding of concentration-dependent antagonism at the lower concentration range of rifampicin and isoniazid in the current study are consistent with murine and hollow fibre pre-clinical model data and antagonism in sterilizing effect rates in patients.

Chigutsa *et al* (181) showed in an adult South African population that an increase in isoniazid C_{max} was antagonistic when rifampicin $AUC < 35.4$ mg*h/L for rates of sterilizing effect based on TTD, which supported our finding of potential isoniazid antagonism in the MARS model below thresholds of rifampicin $C_{max}/MIC < 28$ and isoniazid $C_{max} < 4.6$ mg/L. Similarly, Swaminathan *et al* (217) showed in Indian children with pyrazinamide $C_{max} \leq 38.10$ and rifampin $C_{max} \leq 6.20$ mg/L, isoniazid $AUC_{0-24} > 31.80$ mg.h/L led to higher proportions of children with poor outcomes. Supplementary Figure 1 shows the % culture conversion stratified by AUC_{0-24} / MIC quartile for isoniazid, rifampicin and pyrazinamide. This is consistent, with findings of Almeida *et al*, in a mouse model of tuberculosis that showed dose-dependent antagonistic response of isoniazid on rifampicin/pyrazinamide activity, measured by colony forming units in mouse lung (219). The antagonistic relationship was narrowed down to the dual combination of isoniazid and pyrazinamide which are both structural analogues of nicotinamide (219). Grosset *et al* found that discontinuation of isoniazid after the first 2 days improved bactericidal activity over days 3-14 of anti-tuberculosis treatment in mice (220). In the hollow fiber system, co-administration of isoniazid and rifampicin at both drugs' highest $C_{max}/MICs$ was associated with inferior microbial kill compared to administration of rifampicin after a delay of 6 hours, 12 hours, and 24 hours, (coinciding with progressive fall in isoniazid concentrations), consistent with concentration-dependent antagonism (221).

Although this data set does not lend itself to further in depth analysis of drug-drug antagonism and synergism, the finding of drug-drug antagonism at the lower range of isoniazid and rifampicin may be a contributory factor to treatment outcomes and must be studied in further clinical studies and simulation analyses which encompass further dosing ranges of both isoniazid and rifampicin. The efficacy of isoniazid beyond its initial early bactericidal activity, should be evaluated further in the context of randomized controlled studies with appropriate follow up and long term treatment outcomes. Further research questions include determination of efficacy and tolerability of increased isoniazid concentrations in patients with slow N-acetyltransferase 2 status and the potential for

staggered dosing e.g. 12 hour difference in dosing time between rifampicin/pyrazinamide and isoniazid in light of potential drug-drug antagonism.

Despite significant variability of AUC/MIC for rifampicin, isoniazid and pyrazinamide, the range of %culture conversion over different AUC/MIC quartiles was limited; 64-88% for rifampicin, 64-84% for isoniazid and 72-88% for pyrazinamide. Logistic regression failed to identify a relationship between C_{max}/MIC , AUC/MIC or %T_{MIC} and the probability of culture conversion. As an example, these would average out outcomes on either side of the “V-shaped” relationship we identified, so that measures of central tendency would not differ for the range of exposures. A similar problem is encountered with quartiles, unless the distribution happens to fall neatly on either side of the V-shape. We also note clinical covariates which may contribute to the PK-PD trends observed. For example, there was a non-significant trend for patients with rifampicin AUC₀₋₂₄/MIC above the 75th percentile to have a baseline sputum smear grading of 3+, suggesting that confounding by severity of the pulmonary disease may explain reduced culture conversion at this top quartile of rifampicin exposure. Despite having the highest proportion of drug side effects in the top quartile of isoniazid C_{max} (which one would have predicted was associated with poorer adherence), there was an increase in proportion culture conversion which would have been in keeping with the overcoming concentration-dependent antagonism above isoniazid $C_{max}>4.6\text{mg/L}$.

The concentration thresholds derived by Chigutsa *et al*(181) (rifampicin $C_{max}>8.2\text{ mg/L}$ and pyrazinamide AUC/MIC>11.3) and Pasipanodya *et al*(41) (pyrazinamide $C_{max}>58.3\text{mg/L}$) were not predictive of greater % culture conversion in the current study. This could be because low numbers of participants had exposures above the specified thresholds. In other words, the concentration ranges identified in the pharmacokinetic variability encountered in the current cohort unexpectedly grouped most patients on one side of the concentration thresholds. Part of this may be due to doses employed by the program. For example, in the Pasipanodya *et al* study(41); the average pyrazinamide dose was 36mg/kg, roughly 40% higher than was used by the program in our current study.

There were several limitations in this study. Ethambutol exposures, which could have contributed both to the sterilising activity of the quadruple drug regimen and also to drug-drug antagonism/synergism, were not measured. The outcome measure was a binary one (2-month culture conversion). Time to culture conversion would have allowed richer exploration of potential drug-drug interactions. The binary outcome of culture conversion was via a single optimised volume induced sputum expectorated at week 7-8 of treatment. Lack of multiple cultures may have decreased sensitivity in determining culture conversion. Measurement of plasma drug concentrations could not accurately predict spatio-temporal

drug distribution and drug activity against bacillary sub-populations at anatomically sequestered sites e.g. in cavitary disease.(198). The disadvantage of using of MIC as a single PD parameter, compared with time-kill curves, is that it provides no information on rate of bactericidal activity or persistent antimicrobial activity post exposure (post antibiotic effect). This is particularly relevant for pyrazinamide where the %T_{MIC} was low (111). There may have been unmeasured confounders in this observational study and we may have under-estimated inter-occasional PK variability secondary to drug side effects and fluctuating adherence. Lastly, whilst machine-learning models are very good for generating precise hypotheses, the derived interactions need to be tested in larger prospective studies with appropriate designs.

In summary, in this outpatient setting with a high burden of HIV-1-TB co-infected patients, the majority who had plasma drug exposures below accepted thresholds nevertheless had good treatment outcomes. This was not explained by any measured clinical or programmatic factors, nor by adjusting for MIC of infecting MTB strain. We found concentration-dependent antagonism of isoniazid at the lower range of rifampicin affecting the interim outcome of 2-month culture conversion. Large studies with better biomarker models of disease response, detailed accounting for day to day PK variability e.g. due to adherence patterns, optimized adjustment for organism susceptibility via use of time-kill curves and further pre-clinical and clinical studies evaluating the nonlinear effects of drugs in combination may further the evidence base for treatment monitoring using PK/PD measures.

9.0. Blood analytes as biomarkers of disease severity and potential biomarkers of treatment response

In TB, adequate powering of studies, and lengthy follow up times are required to reliably predict long term unfavourable outcomes, which is expensive and logistically challenging. Biomarkers of disease severity and surrogate endpoints predicting long-term outcomes are desirable.

Tuberculosis infection is believed to be established post engulfment of MTB by alveolar macrophages. Persistence of MTB is aided by incomplete fusion of the phagosome and acidic lysosome. The macrophages (which may transform into epithelioid and multi-nucleate giant cells) recruit a local inflammatory response and death of inflammatory cells form the center of the characteristic caseous granuloma which is then surrounded by lymphocytes, neutrophils, natural killer and fibroblast cells (222). Pathogenesis in pulmonary TB is characterized by destruction and remodeling of the extracellular matrix (ECM) mediated by matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) (223). They are believed to play a crucial role in cavity formation and fibrosis (224, 225). Cavities are associated with high bacillary loads and the persistence of cavities at the end of treatment has been associated with increased risk of relapse (226). MMP-1 (collagenase-1) and 7 (matrilysin) are collagenases expressed by macrophages in granulomas and MMP-8 (collagenase-2) and 9 (gelatinase B) are associated with neutrophil markers (225). Numerous MMPs, most notably MMP-1 and MMP-3, have been found to be increased in patients with active pulmonary TB(227), independently associated with higher TB severity scores (36). MMP-1, -3 and -8 concentrations in sputum decline in response to successful ATT(228). Delayed sputum culture conversion has been associated with increased MMP-1 levels (229). Elevated MMP-9 levels in cerebrospinal fluid have been associated with TB meningitis, with treatment related decline in concentrations (230). Thus, MMPs represent attractive candidates as TB correlates of disease severity and biomarkers of treatment response.

Although not specific to TB, a number of acute-phase proteins synthesized by the liver and triggered by inflammation, infection or tissue injury have been proposed as useful in monitoring the decline in inflammation typically observed in response to antimicrobials (231). Markers of oxidative stress may have potential in monitoring response to treatment (232). Haemoxygenase 1 (HO-1) is an important antioxidant in the lungs and has been shown to be useful in discriminating between active, latent TB infection and uninfected controls (233). Antibiotic-mediated increases in reactive oxygen species can lead to DNA, lipid and protein

damage and subsequent cell death. This is a potential mechanism for eradicating of non-replicating persister MTB organisms (234). Both clinical and experimental animal studies in TB have described oxidative cellular and tissue damage leading to lipid peroxidation and cell death (235, 236). 8-hydroxy-2'-deoxyguanosine (8OH-dG), is a marker of damaged guanine nucleotides accumulated in the oxidative environment of host macrophages (237).

TB is characterized by an interferon (IFN)-inducible myeloid derived monocyte/neutrophil-driven peripheral blood transcriptional signature that has been correlated with radiological measures of disease severity (238). Expression of type I IFNs induces interleukin-1 receptor antagonist (IL-1RA) which in turn induces the immunoregulatory cytokine IL-10 and inhibition of the production of protective pro-inflammatory cytokines IL-12, IFN γ and IL-1 β (239). Conversely, IL-1 α and IL-1 β inhibit type I IFN induction in mouse and human macrophages and *vice versa* (240, 241).

9.1. Chapter hypothesis and aims

Overall hypothesis

There are significant differences in concentrations of blood analytes involved in TB pathogenesis when stratified by HIV-1 serostatus and cavitary disease at baseline. These analytes can be used individually and in combination to predict long-term treatment outcomes.

Specific chapter aims

1. To identify differences in concentrations of selected blood analytes longitudinally during chemotherapy.
2. To assess the association or correlation between biomarkers of HIV-1 serostatus, advanced immunosuppression, virological suppression and extensive disease on chest x-ray +/- cavities.
3. To assess the utility of analytes at baseline and 2 months in predicting long term outcome (cure/completion vs. failure/recurrence).

9.2. Methods

A sub-cohort of study participants were randomly selected for measurement of blood analytes. Study recruitment (3.1.1.), study procedures (including disease severity as per chest x-ray grading (3.1.2)) and determination of outcomes (3.1.6.) are previously described. ELISA and Luminex/xMAP ® technology-based multiplex assays are described in 3.9 and 3.10.

Statistical analyses

Normality of data was assessed using the Shapiro wilk test. The Kruskal-Wallis test with Dunn's multiple comparisons test was used to assess change in concentrations of analytes during treatment (comparing baseline, at 2 months and 5-6 months of treatment).

Differences in analyte concentrations comparing 2 independent groups at a single time point e.g. successful vs failure/recurrence, cavities vs no cavities, HIV-1 co-infected vs HIV-1 uninfected were assessed using Wilcoxon signed rank test. For analytes where over 50% of participants had concentrations below the minimum level of detection of an analyte (IL-12p40 and IL-12p70), the proportion with measured concentrations above the detection level was presented. Spearman's rank correlation was calculated between analytes and between analytes and continuous clinical covariates. Multivariate logistic regression was used to assess factors independently associated with cavitary disease at baseline.

Derivation of thresholds to predict treatment outcomes

All analytes with a difference between successful vs unsuccessful groups ($p \leq 0.2$) were further assessed using receiver operating characteristic (ROC) curves to determine thresholds predicting a) failure b) recurrence c) composite of failure/recurrence (i.e. unsuccessful outcome). A multivariable logistic regression model was developed using analytes which were associated with a significantly ($p < 0.05$) increased odds of unsuccessful treatment outcome, and adjusting for potential confounders including age, time to culture positivity (TTD) sex, BMI, HIV-1 serostatus, T2DM, adherence and cavities. On multivariate analysis, analytes which were significantly associated with increased odds of unsuccessful outcome ($p < 0.05$) were assessed in combinations of up to 3 analytes for their performance in predicting unsuccessful outcomes by optimized area under the ROC curves.

9.3. Results

133 participants from the original cohort were included in these analyses. 77/133 (58%) were HIV-1 co-infected, of whom 29/77 (38%) had a baseline CD4 lymphocyte count <100 cells/mm³. 29 HIV-1 co-infected patients were on ART at baseline, of whom only 19/29 were virologically suppressed (VL<40 copies). 32/48 who were not on ART at baseline, commenced ARV by the 2 month visit. 69/133 (52%) had cavitary disease on chest x-ray at baseline. 115 participants were treatment cure/completers (successful outcome), 7 were treatment recurrences and 11 were treatment failures (18 unsuccessful outcomes). Data for analyte concentrations at 5 months was only available for 54 participants (10 unsuccessful outcomes and 44 successful outcomes). 50/54 (93%) who had 5-6 month concentrations assessed were culture positive at 2 months compared with 54/133 (41%) who did not have 5-6 month concentrations assessed.

Plasma was not available for 1 participant (recurrence patient) and hence HO-1 and 8-OH-dG readings were available for 132 participants. Serum was not available for 4 participants (all successful outcomes) and hence all other analytes (IL-12p40, IL-12p70, IFN α , TNF α , IFN γ , CXCL-10, IL-1 α , IL-1 β , IL-8, IL-6, IL-17, GM-CSF, VEGF, MMP-1,3,7,8,9,10,12, TIMP 1-4, CRP, SAP, tPA, ferritin, α -2 macroglobulin and haptoglobin) had measurements available for 129 participants.

9.3.1. Change in analytes during treatment

Table 9.1 Analytes pre, 2 and 5-6 months of treatment

Analyte	Baseline median (IQR) n=129 ^a	2 months median (IQR) n=129 ^a	5-6 months median (IQR) n=54 ^b	Kwallis p-value	Dunns post test		
					0 v 2mth	0 v 5-6mth	2 v 5-6mth
CRP mg/l	48 (27-80)	11 (4-20)	5 (2-13)	<0.0001	<0.0001	<0.0001	<0.05
Ferritin ng/ml	250 (121-506)	119 (42-252)	61 (36-188)	<0.0001	<0.0001	<0.0001	ns
HO-1 ng/ml	17 (12-22)	12 (9-18)	10 (6-16)	<0.0001	<0.001	<0.0001	<0.05
8-OhdG ng/ml	11 (9-14)	14 (11-17)	12 (9-14)	<0.0001	<0.0001	ns	<0.05
SAP µg/ml	44 (33-59.)	38 (30-46)	35 (26-43)	<0.001	<0.01	<0.001	ns
tPA µg/ml	7 (6-9.)	6 (5-9)	5 (4-8)	<0.01	<0.05	<0.01	ns
α-2 macroglobulin µg/ml	993 (1213-1475)	1346 (1057-1678)	1091 (1294-1626)	ns	ns	ns	ns
Haptoglobin µg/ml	401 (237-1368)	924 (219-3276)	123 (447-1172)	ns	ns	ns	ns
MMP-1 ng/ml	17 (10-28)	9 (4-18)	6 (3-10)	<0.0001	<0.0001	<0.0001	<0.05
MMP-3 ng/ml	20 (12-30)	15 (11-23)	20 (11-30)	<0.01	<0.01	ns	ns
MMP-7 ng/ml	4. (3-6)	4 (3-6)	4 (3-5)	ns	ns	ns	ns
MMP-8 ng/ml	12 (5-36)	5 (2-9.)	4 (2-9)	<0.0001	<0.0001	<0.0001	ns
MMP-9 ng/ml	221 (129-354)	136 (73-242)	127 (75.6-214)	<0.01	<0.05	<0.05	ns
MMP-10 ng/ml	2. (2-3)	2 (1-2)	2 (1-2)	<0.0001	<0.0001	<0.0001	ns
MMP-12 pg/ml	168 (103-241)	122 (53-196)	140 (74-203)	<0.01	<0.01	ns	ns
TIMP-1 ng/ml	299 (216-409)	196 (160-269)	158 (127-225)	ns	ns	ns	ns
TIMP-2 ng/ml	104 (95-118)	123 (106.-143)	129 (104-150)	<0.0001	<0.0001	<0.0001	ns
TIMP-3 ng/ml	29 (26-29)	26 (22-31)	22 (18-28)	<0.0001	<0.0001	<0.0001	ns
TIMP-4 ng/ml	2 (1.5-2.3)	2 (1-2)	2 (1-2)	<0.01	<0.01	ns	ns
sCD14 µg/ml	3 (3-5)	4 (3-5)	3 (2-4)	<0.01	ns	<0.01	<0.01
Il-1α pg/ml	1 (1-2)	1 (1-3)	1 (1-2)	ns	ns	ns	ns
Il-1β pg/ml	4 (3-6)	3 (2-4)	3 (2-4)	<0.01	<0.05	<0.05	ns
IL-1Ra pg/ml	16 (2-55)	4 (1-12)	3 (1-7)	<0.0001	<0.0001	<0.0001	ns
IL-6 pg/ml	11 (5-25)	8 (4-14)	8 (4-12)	<0.01	<0.01	<0.01	ns
IL-8 pg/ml	9. (15-25)	9. (6-18)	8 (6-12)	<0.0001	<0.0001	<0.0001	ns
IL-10 pg/ml	7 (5-11)	6.(4-10)	7 (5-11)	ns	ns	ns	ns
IL-12p40 detectable (%) DL ≥3pg/ml	16/129 (12)	21/129 (16)	5/54 (9)	ns*			
IL-12p70 detectable (%) DL ≥ 3pg/ml	23/129 (18)	17/129 (13)	6/54 (11)	ns*			
Il-17 pg/ml	3 (2-4)	2 (2-3)	2 (1-2)	<0.0001	<0.01	<0.0001	ns
TNFα pg/ml	13 (8-21)	11 (6-18)	7 (3-11)	<0.0001	ns	<0.0001	<0.01

IFN α pg/ml	12 (7-19)	9 (5-14)	8 (5-12)	<0.001	<0.01	<0.05	ns
IFN γ pg/ml	19 (7-39)	7 (1-19)	2 (1-9)	<0.0001	<0.0001	<0.0001	<0.05
GM-CSF pg/ml	13 (9-16))	10 (8-13)	10 (8-12)	<0.001	<0.001	<0.01	ns
VEGF pg/ml	133 (2-381)	5 (1-174)	9 (1-121)	<0.01	<0.01	<0.05	ns
DL $\geq$ 1.4 pg/ml Detectable (%)	88/129 (68)	102/129 (79)	41/54 (76)				
CXCL-10 pg/ml	2. (2-4)	1 (1-3)	1 (0-1)	<0.0001	<0.0001	<0.0001	<0.0001
DL $\geq$ 0.1 pg/ml							
IFN γ /IL-10	3 (1-5)	1(0.3)	0 (0-1)	<0.0001	<0.0001	<0.0001	<0.01

^an=132 for HO-1 and 8-OHdG ^b n=52 for HO-1 and 8-OHdG *chi-squared test

DL minimum level of detection of assay Kwallis Kruskal-Wallis

Table 9.2 Analytes at baseline and 2 months stratified by treatment outcome

Analyte	Baseline median (IQR)		p-value	2 months median (IQR)		p-value
	Successful (n=111) ^a	Unsuccessful (n=18) ^b		Successful (n=111) ^a	Unsuccessful (n=18) ^b	
CRP mg/l	42 (26-77)	69.(40-112)	<0.05	10 (4-20)	16 (6-51)	ns
Ferritin ng/ml	236 (117-441)	534 (126-1071)	<0.05	102 (39-229)	176 (65-646)	ns
HO-1 ng/ml	17 (12-22)	16.(12-21)	ns	12 (9-18)	16 (12-19)	ns
8-OhdG ng/ml	12 (9-14)	10 (6-14)	ns	14 (11-17)	11 (10-14)	<0.05
SAP µg/ml	46(35-60)	39 (29-50)	ns	38 (30-49)	35 (25-41)	ns
tPA µg/ml	7 (6-9)	8 (5-10)	ns	6 (5-9)	6 (4-9)	ns
α-2 macroglobulin µg/ml	1242 (990- 1550)	1143 (1034- 1357)	ns	1373.(1092-1755)	1166.(1042.-1434)	ns
Haptoglobin µg/ml	476 (237-1898)	317 (234-435)	ns	876 (215-3317)	954 (297-3273)	ns
MMP-1 ng/ml	17 (10-28)	14 (9-25)	ns	9 (4-17)	9.(4-19)	ns
MMP-3 ng/ml	19 (12.-28.)	28.(16.37)	ns	13 (10.-22)	22 (12-37)	ns
MMP-7 ng/ml	4. (3-6)	4 (2-6)	ns	4 (3-6)	4 (3-6)	ns
MMP-8 ng/ml	11 (5-31)	18 (5-41)	ns	5 (2-9)	7 (2-21)	ns
MMP-9 ng/ml	205 (121-352)	241 (137-383)	ns	137 (77-239)	130 (53-364)	ns
MMP-10 ng/ml DL≥ 3.2pg/ml	2 (2-3)	2 (2-3)	ns	2 (1-2)	2 (1-3)	ns
MMP-12 pg/ml	168 (119-238)	168 (73-269)	ns	126 (61-200)	106 (45-196)	ns
TIMP-1 ng/ml	285 (215-401)	327 (227-468)	ns	193 (161-245)	389 (145-389)	ns
TIMP-2 ng/ml	104 (94-114)	110 (98-130)	ns	122 (106-143)	128 (106-144)	ns
TIMP-3 ng/ml	28 (26-34)	33 (26-38)	ns	25 (22-30.2)	30 (25-37)	<0.05
TIMP-4 ng/ml	2 (1.5-2.3)	2 (1.5-2.2)	ns	2 (1.3-2)	2 (1.6-2.4)	ns
sCD14 µg/ml	3 (3-5)	5 (3-6.4)	<0.05	4 (3-5)	4 (3-5)	ns
IL-1α pg/ml	1 (0-2)	1 (1-3)	ns	1 (1-2)	2 (1-4)	ns
IL-1β pg/ml	4 (3-5)	5 (3-7)	ns	3 (2-4)	3 (2-8)	ns
IL-1Ra pg/ml	14 (2-49)	46 (2-93)	ns	4 (2-11)	9 (2-83)	ns
IL-6 pg/ml	11 (5-23)	23 (9-66)	<0.05	8 (3-14)	12 (6-22)	ns
IL-8 pg/ml	15 (9-25)	21 (13-36)	<0.05	9 (6-17)	14 (9-27)	<0.05
IL-10 pg/ml	7 (5-11)	8 (5-10)	ns	6 (4-10)	6 (4-9)	ns
IL-12p40 detectable (%)	22/111 (20)	6/18 (33)	ns*	21/111 (19)	5/18 (28)	ns*
IL-12p70 detectable (%)	23/111 (21)	5/18 (28)	ns*	11/111 (10)	5/18 (28)	<0.05 *
IL-17 pg/ml	3 (2-4)	3 (2-6)	ns	2 (2-3)	3 (2-6)	<0.05
TNFα pg/ml	12 (8-21)	17 (12-29)	ns	10.(5-18)	14.3 (8-21)	ns
IFNα pg/ml	11 (7-19)	13.(8-21)	ns	9 (5-14)	9 (5-22)	ns
IFNγ pg/ml	19 (6-35)	33 (8-108)	ns	7 (1-18)	11 (2-38)	ns
GM-CSF pg/ml	12.8 (9.2-15.6)	13.1 (11-15.8)	ns	10 (7.8-12.2)	11.9 (9-16.9)	ns

VEGF pg/ml Detectable (%)	138. (2-374) 88/111 (79)	86 (3-477) 14/18 (78)	ns	4(1-157) 74/111 (67)	128 (3-329.) 14/18 (78)	<0.05
CXCL-10 pg/ml	2 (2-4)	3 (2-6)	ns	1 (1-2)	2(1-3)	ns
IFN γ /IL-10	2.5 (1-5)	5 (1-9)	ns	1 (0-3)	2 (1-4)	Ns

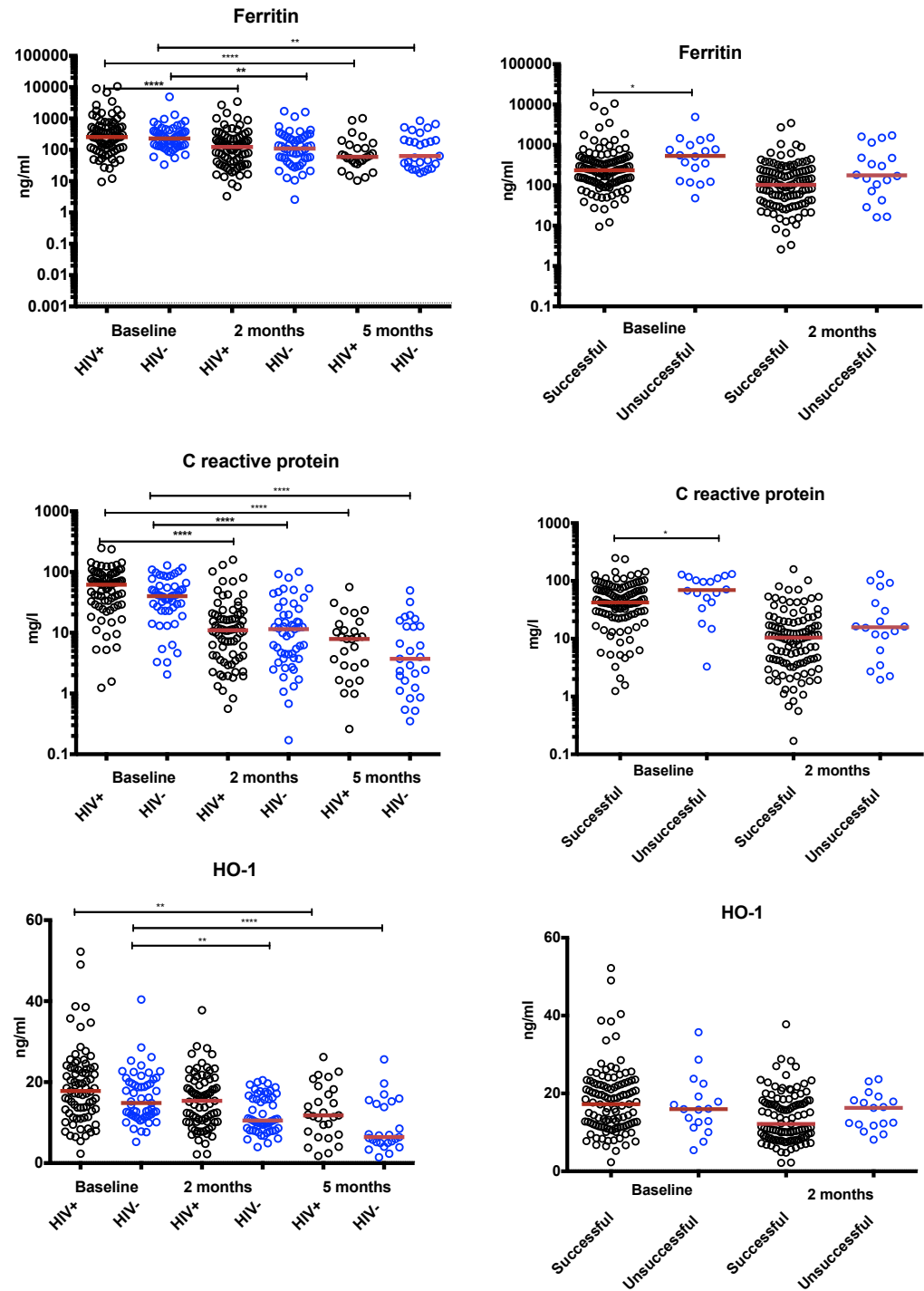
^an=115 ^b n=52 for HO-1 and 8-OHdG *chi squared test

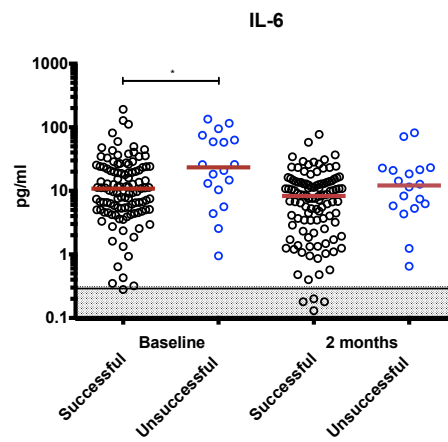
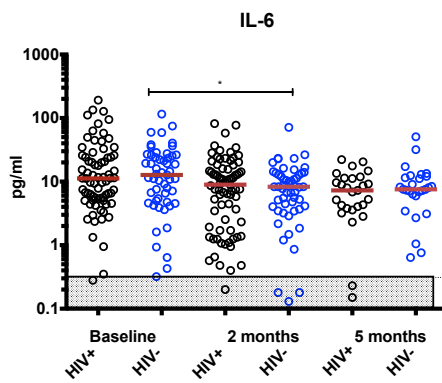
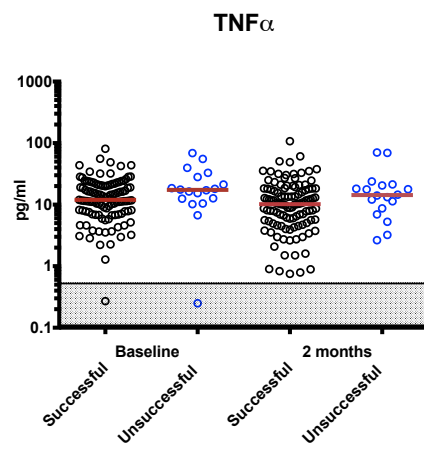
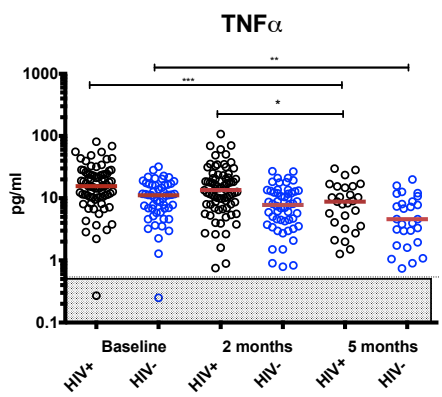
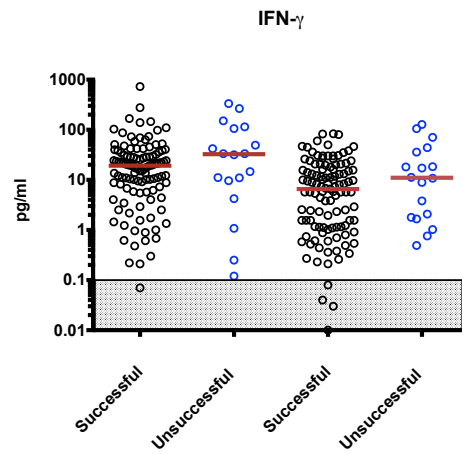
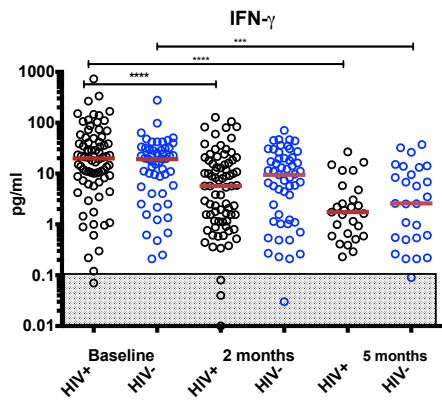
DL minimum level of detection of assay

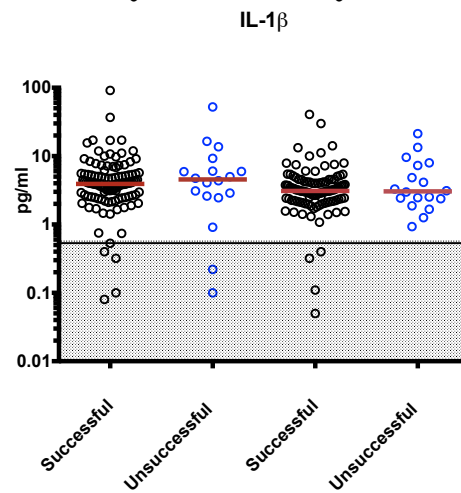
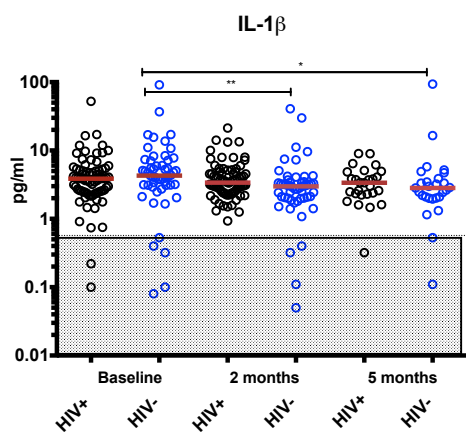
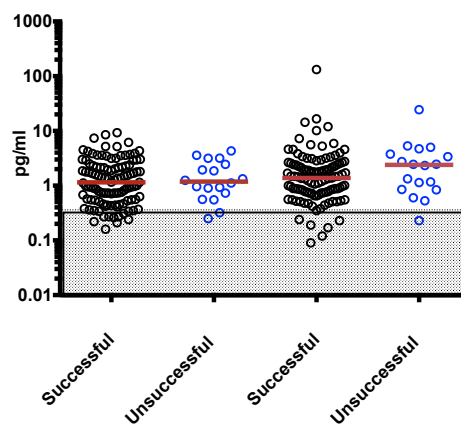
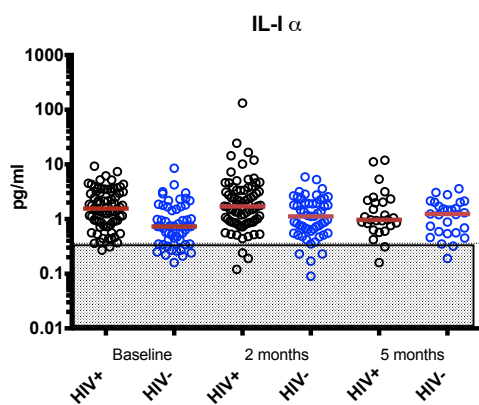
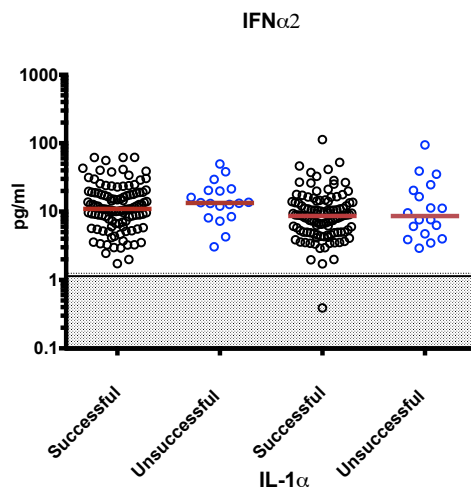
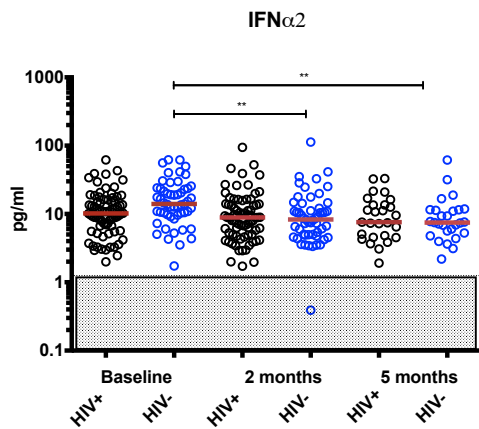
CRP, ferritin, HO-1, 8OHdG, SAP, tPa, MMP-1, MMP-3, MMP-8, MMP-9, MMP-10, MMP-12, TIMP-3, TIMP-4, sCD14, IL-1 β , IL-1RA, IL-6, IL-8, IL-17, TNF α , IFN α 2, IFN γ , GM-CSF, VEGF and CXCL-10 decreased significantly during chemotherapy and TIMP-2 increased significantly during chemotherapy. MMP-7, TIMP-1, IL-1 α , IL-10, IL-12p40 and IL-12p70, did not change significantly over the course of chemotherapy. 8-OHdG (figure 9.1) and α -2 macroglobulin (not shown graphically) increased only in HIV-1 co-infected participants between baseline and 2 months ($p < 0.05$). sCD14 (figure 9.1) and tPA (not shown graphically) decreased significantly only in HIV-1 co-infected participants. IL-6, MMP-9, IFN α 2, IL-1 β (figure 1), SAP, MMP-12, MMP-9, VEGF, decreased only in HIV-1 uninfected participants and haptoglobin increased between baseline and 2 months in HIV-1 uninfected participants. The ratio of IFN γ /IL-10 decreased significantly, both in HIV-1 co-infected and HIV-1 uninfected participants, over the course of chemotherapy. All results were not significantly changed, when stratified by virologically suppressed on ART status.

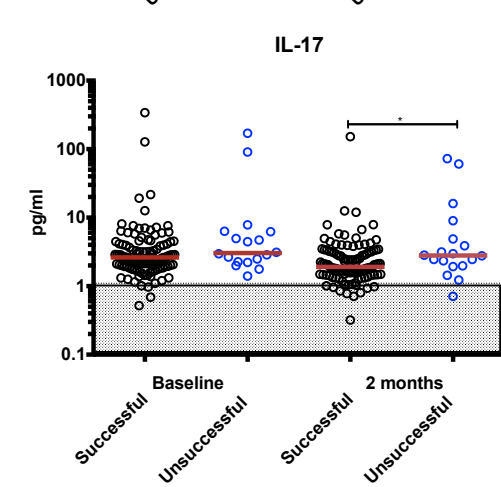
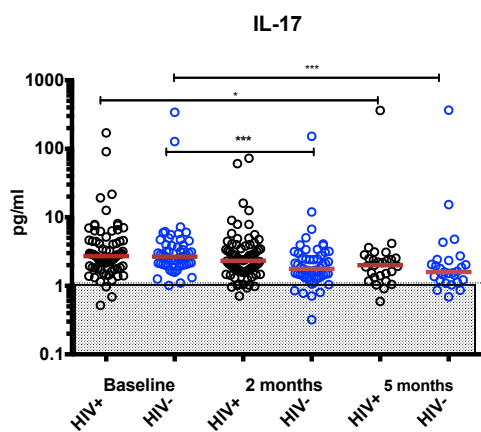
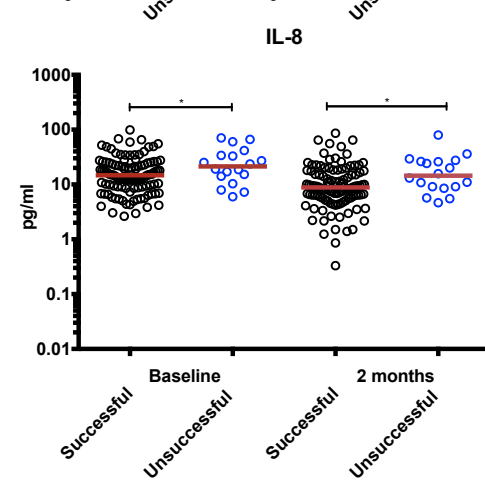
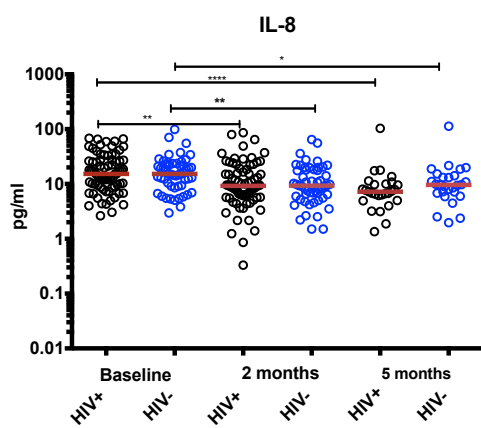
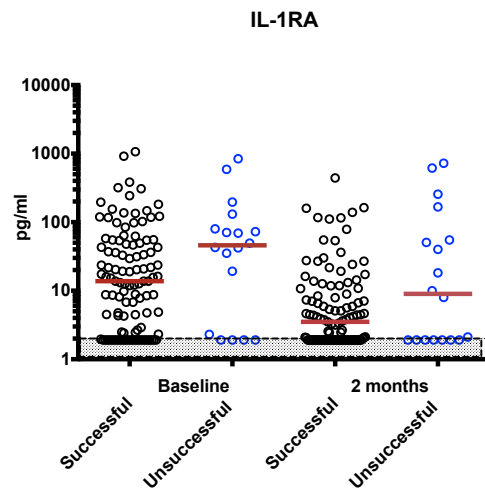
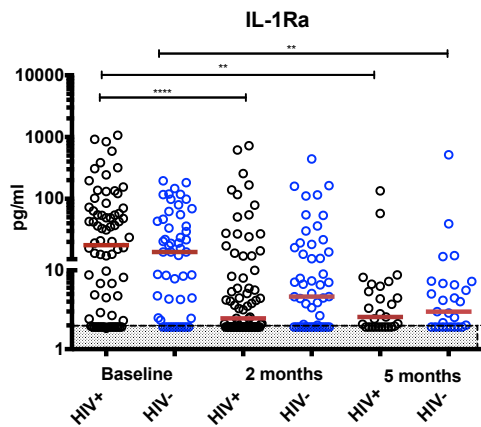
CRP, ferritin, sCD14, IL-6 and IL-8 were significantly higher at baseline in participants with unsuccessful outcomes. 8-OHdG at 2 months was significantly lower in participants with unsuccessful outcomes. TIMP-3, IL-8, IL-12p70, IL-17 and VEGF were significantly higher at 2 months in participants with unsuccessful outcomes (table 9.2).

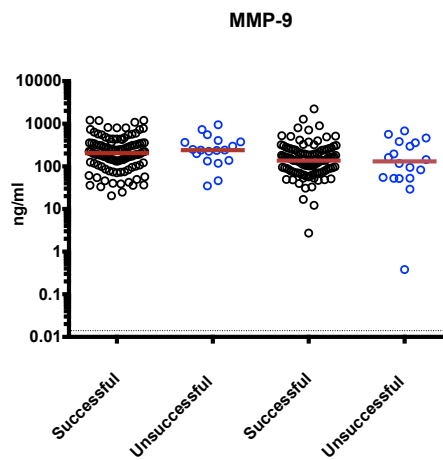
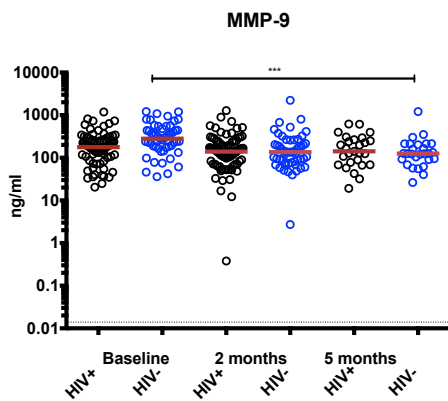
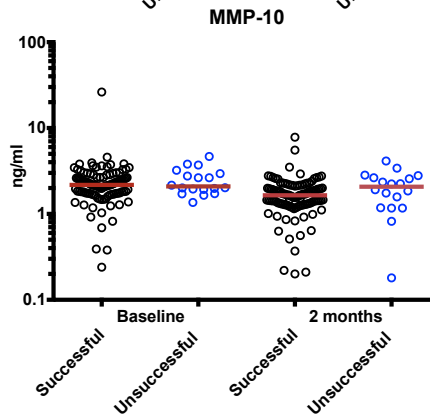
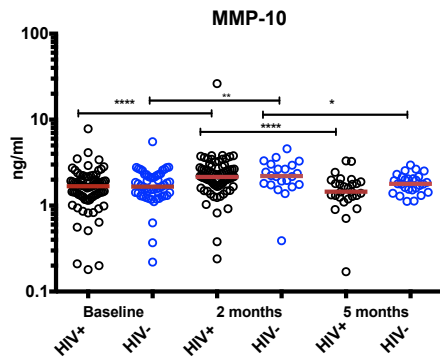
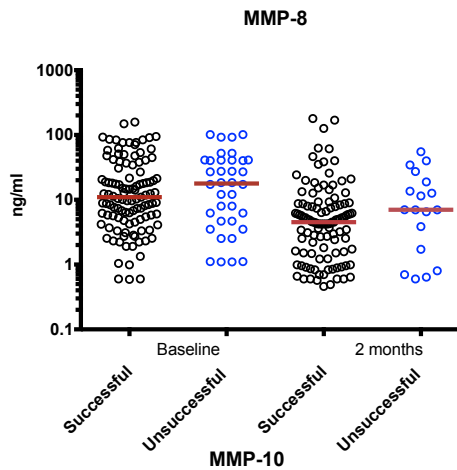
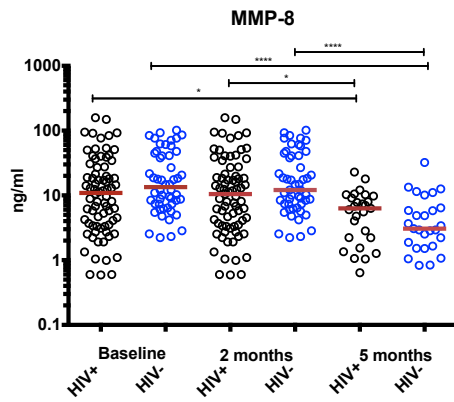
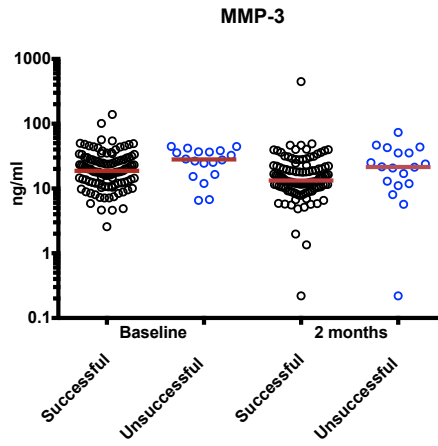
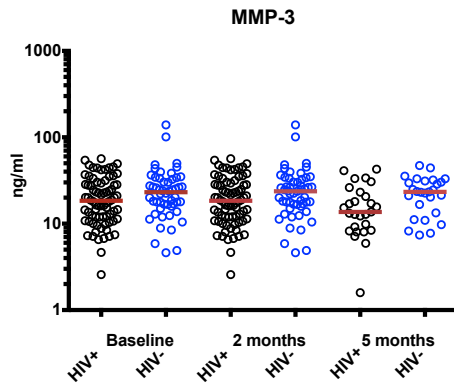
Figure 9.1 Selected analytes pre-chemotherapy, 2 and 5-6 months stratified by HIV-1 serostatus and at baseline and 2 months stratified by treatment outcomes

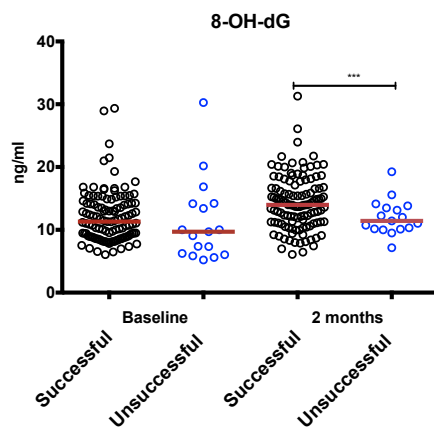
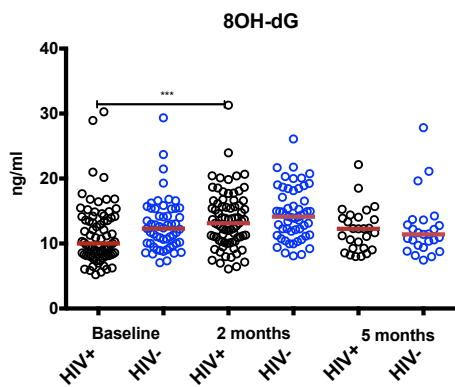
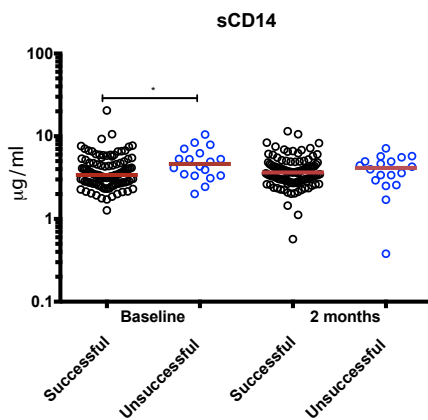
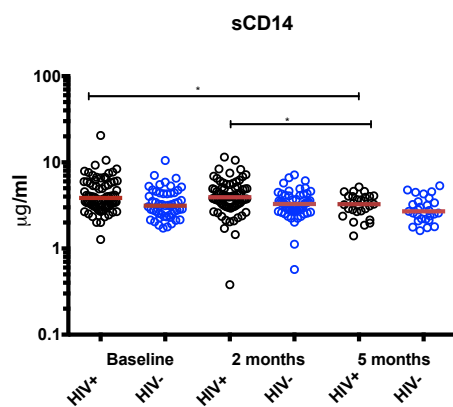
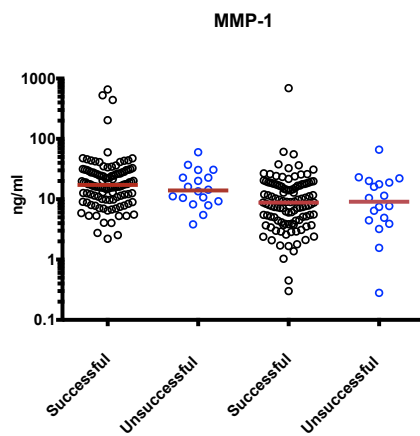
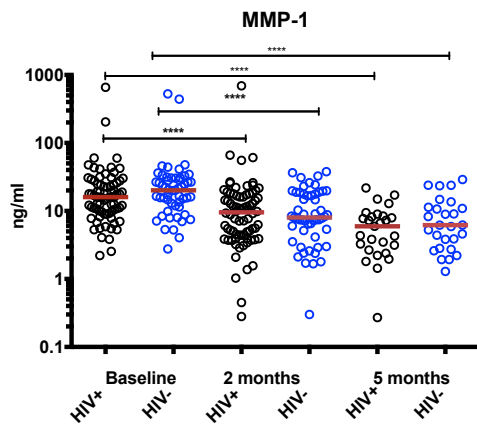


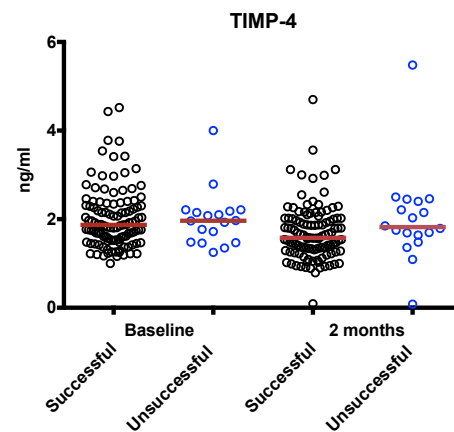
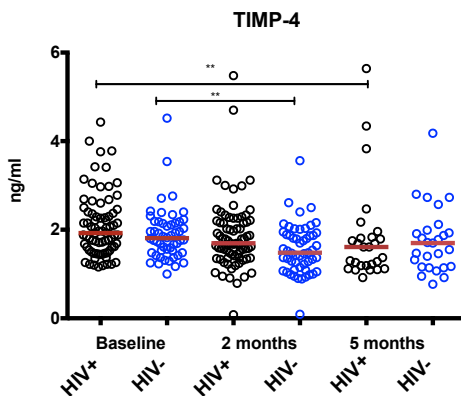
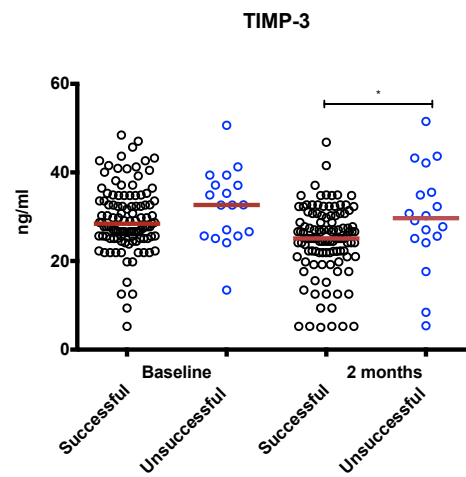
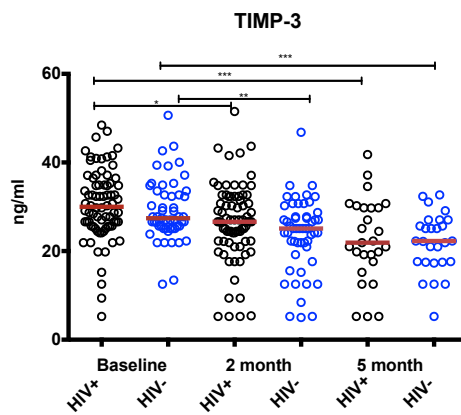
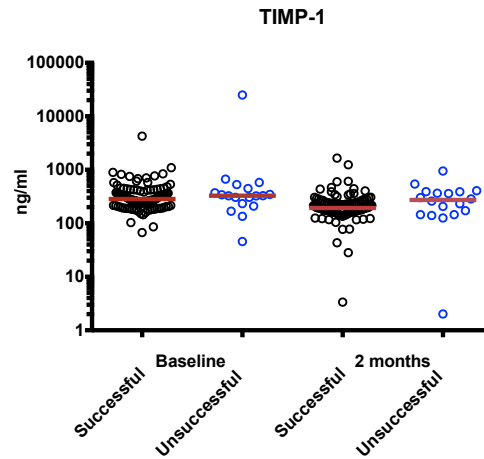
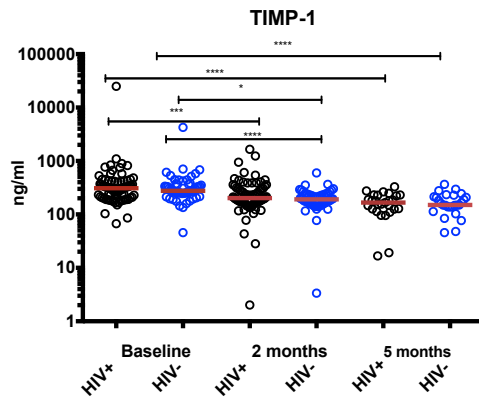












Changes in analytes over time were compared using Kruskal-wallis test with Dunn's multiple comparisons test. Differences between successful and unsuccessful outcome groups at each time point was assessed using Wilcoxon signed rank test
 <0.05 ** <0.01 *** <0.001 **** <0.0001

The grey shaded zones indicate values below the level of detection

9.3.2. Analytes stratified by HIV-1 serostatus and cavitation on chest x-ray at baseline

Table 9.3 shows analytes (8-OHdG, CRP, haptoglobin, sCD14, MMP-9, TIMP-2, TNF α , IL-1 α , IFN α 2) which were significantly different pre-chemotherapy, according to HIV-1 serostatus.. In this cohort 35/77 (45%) of HIV-1 co-infected participants had cavities ≥ 1 cm on chest x-ray compared with 34/56 (61%) HIV-1 uninfected participants ($p=0.08$). 4/29 (14%) of HIV-1 co-infected participants with CD4 lymphocyte count <100 had cavities compared with 31/48 (65%) of HIV-1 participants with CD4 lymphocyte count ≥ 100 ($p<0.0001$). Table 9.4 shows analytes (ferritin, HO-1, TNF α , MMP-3, IL-17, IL-8, IL-12p40, IL-1 α , IL-1Ra, IFN γ) were significantly different at baseline comparing HIV-1 co-infected patients with CD4 lymphocyte counts above and below 100 cells/mm³.

Table 9.3 Analytes significantly different between HIV-1 co-infected and HIV-1 uninfected participants at baseline

Analyte	HIV-1 co-infected (n=76) Median (IQR)	HIV-1 uninfected (n=53) Median (IQR)	p-value
8-OHdG ng/ml*	10 (8-14)	12 (10-15)	0.01
CRP mg/l	62 (29-93)	40 (23-58)	0.02
Haptoglobin μ g/ml	533 (317-2149)	290 (209-595)	<0.01
sCD14 μ g/ml	3 (2-4)	4 (3-6)	<0.01
MMP-9 ng/ml	178 (107-279)	281 (183-444)	<0.001
TIMP-2 ng/ml	101 (93-112)	111 (100-123)	<0.01
TNF α pg/ml	16 (10-24)	11 (7-16)	<0.001
IL1- α pg/ml	2 (1-3)	1 (0-2)	<0.0001
IFN α 2 pg/ml	10 (7-16)	14 (10-23)	<0.01

* 77 HIV-1 co-infected participants and 56 HIV-1 uninfected individuals included

Table 9.4 Analytes significantly different between HIV-1 co-infected participants with CD4<100 and CD≥100cells/mm³ at baseline

Analyte	CD4<100 cells/mm ³ (n=28) Median (IQR)	CD4≥100 cells/mm ³ (n=48) Median (IQR)	p-value
Ferritin ng/ml	626 (320-1631)	151 (84-370)	<0.0001
HO-1 ng/ml *	24 (20-27)	15 (11-19)	<0.0001
TNFα pg/ml	21 (12-38)	14 (8-21)	0.01
MMP-3 ng/ml	28(14-40)	16 (11-23)	<0.01
IL-17 pg/ml	3 (2-6)	2 (2-3)	0.03
IL-8 pg/ml	26 (15-43)	12 (7-19)	<0.001
CXCL-10 pg/ml	5 (3-8)	2 (1-3)	<0.001
IL-1α pg/ml	2 (1-4)	1 (1-2)	0.01
IL-1RA pg/ml	49 (11-134)	10 (2-40)	0.02
IFNγ pg/ml	23 (11-104)	14 (4-35)	0.03

* 29 participants with CD4<100 and 48 participants with CD4>100 cells/mm³ included.

Haptoglobin, ferritin, TNFα, IL-1α, CXCL-10, IL-12p40 were significantly lower in participants with extensive radiological disease on chest x-ray at baseline and IFNα and MMP-9 was significantly higher. MMP-9/TIMP-1, MMP-9/TIMP-2, MMP-9/TIMP-3 and MMP-9/TIMP-4 were also significantly higher (table 9.5). Hence, MMP-9 was raised in patients with baseline extensive radiological disease regardless of levels of the paired physiological antagonists, the tissue inhibitors of matrix metalloproteinases (TIMPS 1-4). HO-1, ferritin, TNFα, IL-1α, IL-8, IL-12p40 and CXCL-10 at baseline were significantly lower in participants with cavities, compared with participants without cavities. MMP-3/TIMP-2 ratio was significantly lower in participants with cavities and MMP-9/TIMP-1 was significantly higher (table 9.6). The ratio of MMP-9/TIMP-3 was significantly higher at 2-months in those with cavities at baseline (figure 9.2). Figure 9.2 shows analytes both at baseline and 2 months, stratified by cavity status at baseline. The same trends are seen both at baseline and 2 months. Figure 9.3 shows HO-1, ferritin, TNFα, IL-1α, IL-8, and CXCL-10 at baseline stratified by both HIV-1 serostatus and presence/absence of cavities. There was no significant correlation between any analyte and time to culture positivity (TTD) nor was there any significant differences when analytes were stratified by smear grade positive compared with smear negative/scanty. After adjusting for HIV-1 co-infection and TTD as potential confounders, higher concentrations of IL-8, IL-1 α and ferritin at baseline were significantly associated with a reduced odds of cavities on chest x-ray at baseline (table 9.6). HIV-1 viral load was also a predictor of cavities with 41% decreased odds of cavities with every 1 log₁₀ increase in HIV-1 viral load (OR 0.59,

95%CI 0.44-0.80, $p < 0.01$). Virological suppression on ART (HIV-1 viral load < 40 copies/ml) at baseline was associated with 5.55x fold increased odds of cavitary disease (OR 5.55, 95% CI 1.76-17.5, $p < 0.01$). Figure 9.4 shows the spearman rank correlation of HIV-1 viral load at baseline with these analytes.

Table 9.5 Analytes which were significantly different between participants with and without extensive disease at baseline

Analyte	Extensive disease on CXR* (n=91) Median (IQR)	No extensive disease on CXR (n=38) Median (IQR)	p-value
Haptoglobin $\mu\text{g/ml}$	346 (210-1029)	639 (376-5009)	< 0.01
Ferritin ng/ml	236 (117-429)	357 (152-1180)	0.05
MMP-9 ng/ml	241 (141-405)	170 (107-249)	0.01
TNF α pg/ml	12 (7-18)	18 (11-28)	< 0.01
IFN $\alpha 2$ pg/ml	13 (8-20)	10 (7-14)	0.05
CXCL10 pg/ml	2 (1-3)	4 (2-6)	< 0.01
IL12p40 pg/ml detectable ($> 3\text{pg/ml}$) n (%)	15/91 (16)	13/38 (34)	0.03
IL-1 α pg/ml	1 (0.5-2)	2 (1-3)	< 0.01
MMP-9/TIMP-1	0.8 (0.4-1.4)	0.4 (0.3-1.1)	0.01
MMP-9/TIMP-2	2.3 (1.3-3.8)	1.7 (1-2.4)	0.04
MMP-9/TIMP-3	8.9 (5.5-14.7)	5.8 (3.6-7.3)	< 0.01
MMP-9/TIMP-4	134 (75-229)	76 (53-133)	0.02

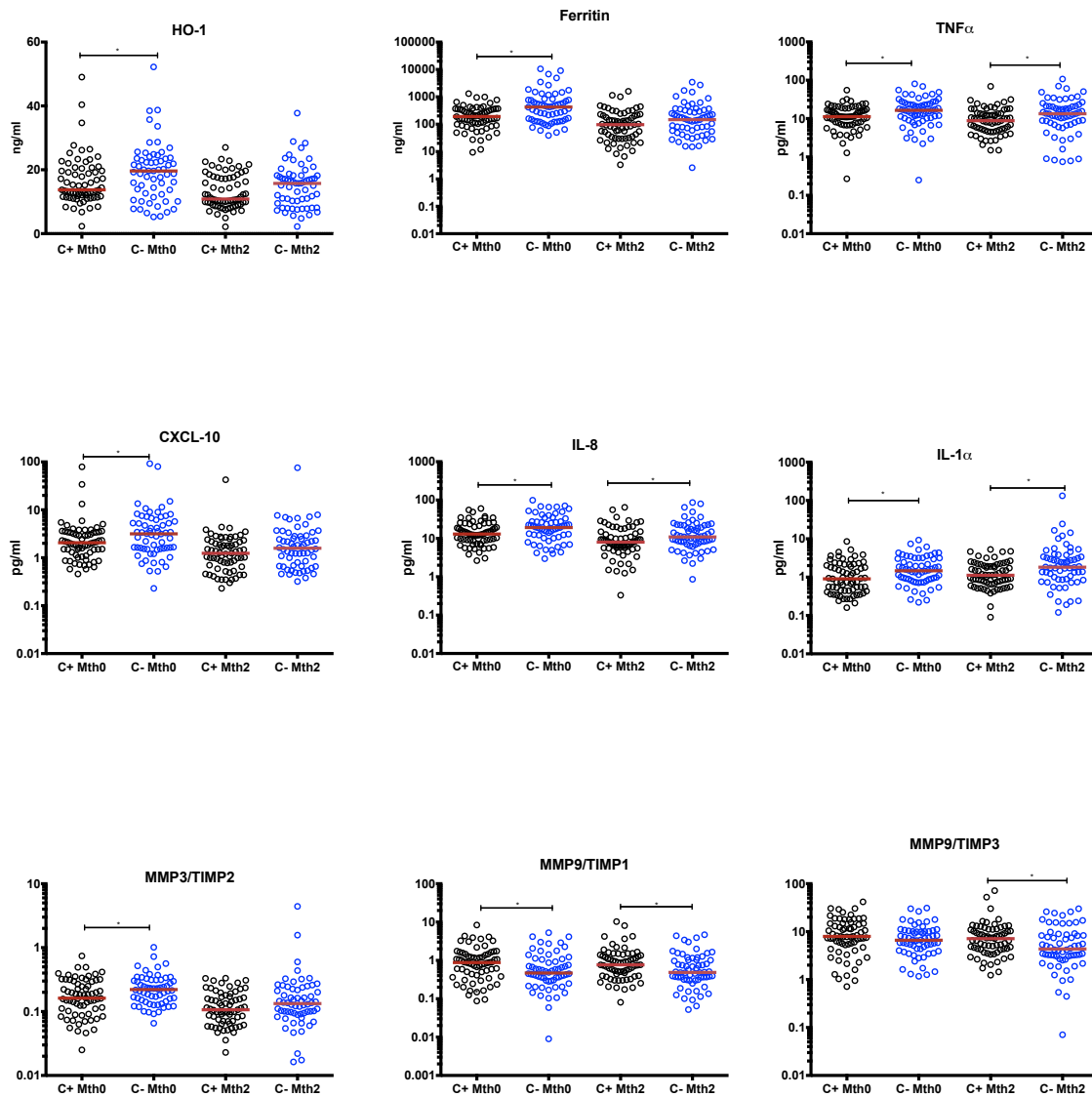
*involvement of both lung fields or greater than 1 out of 3 zones per lung

Table 9.6 Analytes which were significantly between participants with and without cavities at baseline

Analyte	Cavities on CXR (n=67) Median (IQR)	No cavities on CXR (n=62) Median (IQR)	p-value
HO-1 ng/ml *	14 (12-20)	19 (13-24)	0.04
Ferritin mg/l	192 (100- 354)	425 (133-773)	<0.01
TNF α pg/ml	11 (7-18)	16 (10-24)	0.01
CXCL-10 pg/ml	2(1-3)	3 (2-6)	0.02
IL-12p40 detectable (>3pg/ml) n (%)	8 (12%)	19 (31)	<0.01
IL-8 mg/ml	13 (9-21)	19 (11-28)	0.02
IL-1 α pg/ml	1 (0-2)	1 (1-3)	0.01
MMP-3/TIMP-2	0.1 (0.1-0.3)	0.2 (0.1-0.3)	0.03
MMP-9/TIMP-1	0.9 (0.4-1.5)	0.5 (0.3-1)	0.03

* 69 participants with cavities and 64 participants without cavities included

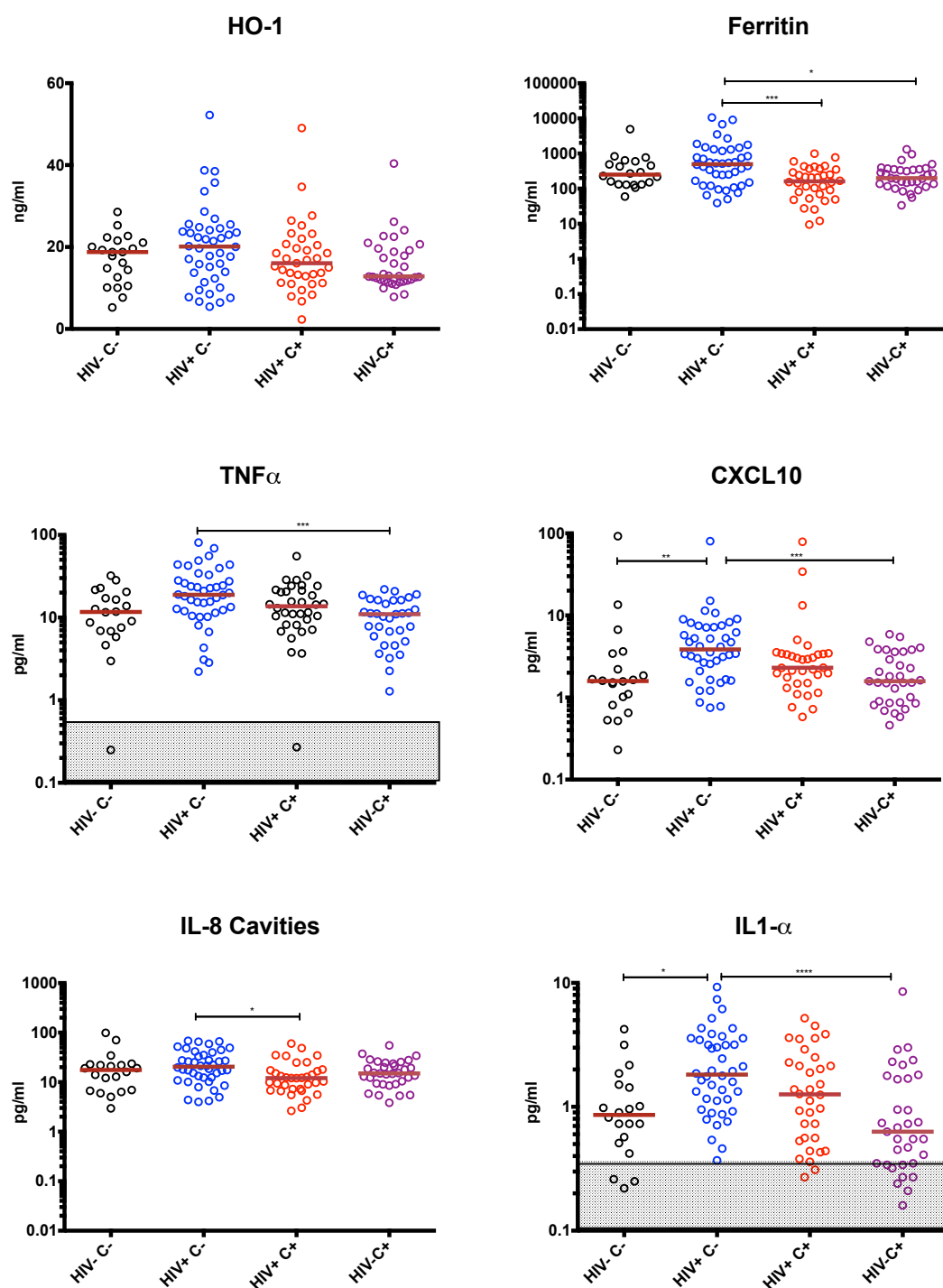
Figure 9.2 Analytes at baseline and 2 months stratified by cavity status at baseline



Differences between the cavities vs no cavities at each time point were compared using Wilcoxon signed rank test

C+ cavities present ,C- Cavities absent, Mth 0 baseline, Mth 2 2 months

Figure 9.3 Analytes at baseline stratified by HIV-1 and cavities status



Comparisons between the groups were made using Kruskal-wallis test with Dunn's multiple comparisons test. HIV+ HIV-1 infected, HIV- HIV-1 uninfected, C+ cavities present, C- cavities absent

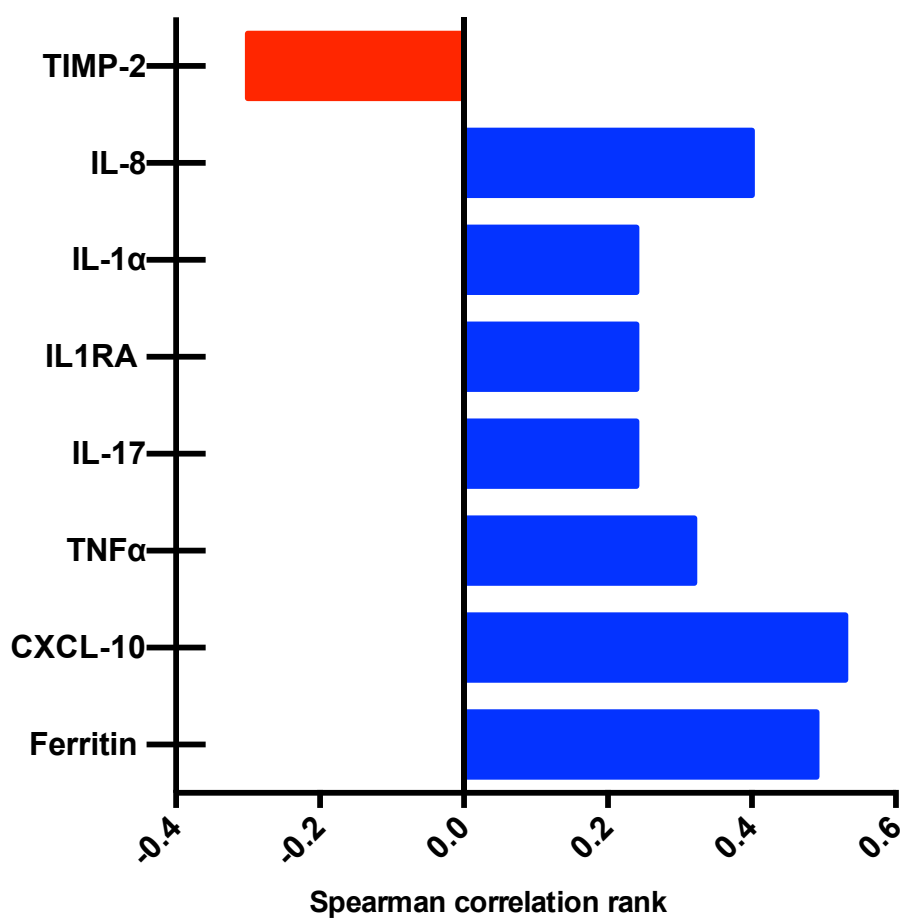
The grey shaded zones indicate values below the level of detection

Table 9.7 Model for predicting cavities, adjusted for HIV-1 serostatus and time to culture positivity at baseline

Variable	Adjusted Odds ratio (95% CI) [#]	P value
Log ₁₀ (IL-1-α)	0.62 (0.38-0.99)	0.05
Log ₁₀ (TNF-α)	0.65 (0.40-1.05)	0.08
Log ₁₀ (IL-8)	0.54 (0.31-0.93)	0.03
Log ₁₀ (Ferritin)	0.51 (0.34-0.76)	<0.01
Log ₁₀ (HO-1)	0.58 (0.25- 1.35)	0.2
Log ₁₀ (CXCL-10)	0.98 (0.41-2.36)	0.96

[#]Increase in analyte x was associated with decreased odds of baseline cavities in the multivariate model

Figure 9.3 Correlation of analytes with HIV-1 viral load at baseline



[#] All presented Spearman correlation rank coefficients were statistically significant (p<0.05)

Table 9.8 Analytes which were significantly different between virologically suppressed and non-virologically suppressed participants at baseline

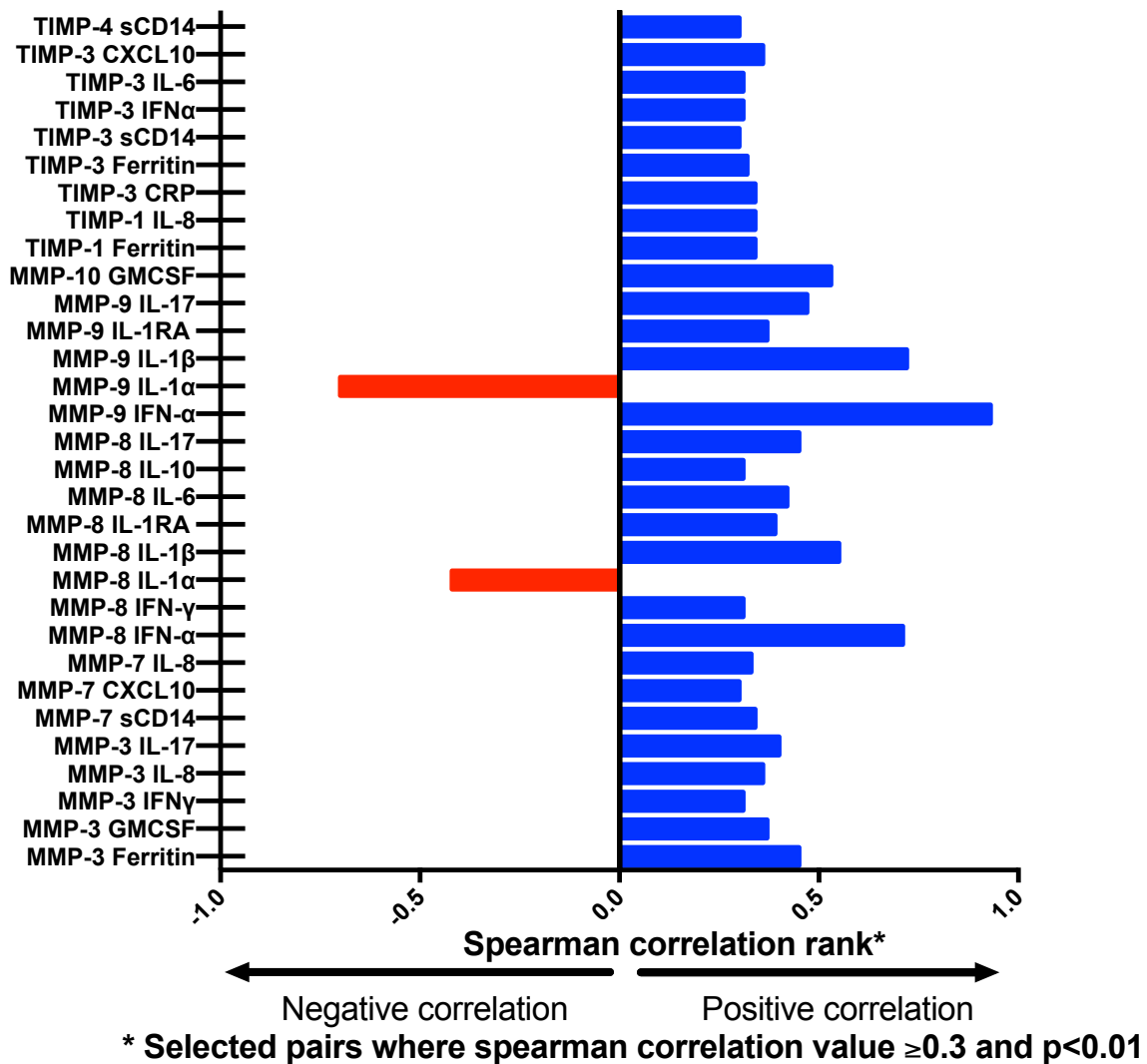
Analyte	Virologically suppressed ^a (n=20) Median (IQR)	Not virologically suppressed (n=57) ^b Median (IQR)	p-value
Ferritin mg/l	99 (49-224)	371 (153-806)	<0.001
Serum amyloid P	59 (27-64)	42 (29-53)	<0.001
TNFα pg/ml	11 (7-15)	20 (12-28)	<0.001
CXCL-10 ng/ml	1(1-2)	3 (3-7)	<0.0001
TIMP-1 ng/ml	239 (191-302)	329 (225-426)	<0.05
IL-8 mg/ml	7 (5-17)	18 (11-33)	<0.01
IL-17	2 (1-3)	3 (2-5)	<0.05

^a HIV-1 VL<40 copies/ml, median CD4 lymphocyte count 330 (IQR 226-389)

^b HIV-1 VL≥ 40 copies/ml, median CD4 lymphocyte count of 99 (IQR 53-287), median HIV-1 VL 169,667 copies/ml

Figure 9.5 shows moderate to high spearman's rank correlation co-efficient ($Rho \geq 0.3$) between MMPs, TIMPs and other analytes at baseline. Of note, IL-1α was negatively correlated with IL-1β ($Rho = -0.47$, $p < 0.0001$) and IFNα2 ($Rho = -0.77$, $p < 0.0001$). IL-1β was positively correlated with IFNα2 ($Rho = 0.75$, $p < 0.0001$). IL-8 was positively correlated with IL-17 ($Rho = 0.75$, $p < 0.0001$). MMP-8 was positively correlated with MMP-9 ($Rho = 0.76$, $p < 0.0001$). At baseline, neutrophil counts in whole blood were ascertained for a random subset of 23 participants. In these participants, the Spearman's Rho between blood neutrophil count and IL-8, IL-17, MMP-8 and MMP-9 was 0.42, 0.72, 0.6 and 0.41 ($p < 0.0001$) respectively.

Figure 9.4 Correlation between MMPs, TIMPs and other analytes



9.3.3. Prediction of outcomes using baseline and 2-month thresholds

On univariate analysis, baseline analytes ferritin (≥ 590 ng/ml), MMP-3 (≥ 24.6 ng/ml), TIMP-1 (≥ 303 ng/ml), TIMP-3 (≥ 34.84 ng/ml), IL-6 (≥ 25.63 pg/ml), IL-8 (≥ 23.63 pg/ml), IL-1RA (≥ 34.34 pg/ml) and sCD14 (≥ 4.12 µg/ml) were associated with significantly increased odds of unsuccessful outcomes (table 9.7). A final model for predicting failure and for predicting failure/relapse (i.e. unsuccessful outcome) was optimised using 2 and 3 baseline predictor variables respectively. Combination of IL-1RA and MMP-3 thresholds in the model predicted the outcome of failure with area under ROC curve of 0.79. Combination of MMP3, IL1Ra and ferritin thresholds in the model predicted the composite outcome of failure/recurrences with an area under ROC curve of 0.77.

Table 9.9 Unadjusted odds ratios for predicting unfavourable outcomes using ROC-derived thresholds of analytes at baseline.

Analyte (threshold)	OR for predicting Failures only (95% CI) [area under ROC curve] n=11	OR for predicting recurrence only (95% CI,) [area under ROC curve] n=7	OR for predicting composite failure/recurrence (95% CI) [area under ROC curve] n=18
Ferritin (≥590ng/ml)	3.37 (0.94-12.06)	5.39 (1.12-25.87) [0.69]	4.04 (1.44-11.39) [0.65]
MMP-3 (≥25ng/ml)	5.12 (1.28-20.4) [0.69]	4.80 (0.89-25.92)	4.99 (1.66-15.05) [0.69]
TIMP-1 (≥303ng/ml)	5.49 (1.13-26.58) [0.68]	1.63 (0.35-7.61)	3.17 (1.06-9.50) [0.64]
TIMP-3 (≥35ng/ml)	3.19 (0.89-11.38) [0.62]	2.87 (0.60-13.73)	3.06 (1.09-8.63) [0.62]
IL-6 (≥26pg/ml)	4.85 (1.36-17.38) [0.67]	3.03 (0.63-14.55)	4.04 (1.43-11.39) [0.65]
IL-8 (≥24pg/ml)	3.56 (1.01-12.56) [0.65]	2.22 (0.47-10.54)	2.96 (1.07-8.21) [0.62]
IL-1RA (≥34pg/ml)	5.79 (1.45-23.15) [0.71]	2.89 (0.61-13.63)	4.34 (1.51-12.52) [0.67]
sCD14 (≥4µg/ml)	2.61 (0.74-9.11)	5.43 (1.00-29.36) [0.70]	3.06 (1.10-8.50) [0.64]

Failures n=11, Recurrences n=7, Composite of failure/recurrence n=18
The highlighted figures are statistically significant, p<0.05

On univariate analysis, 2-month analytes MMP-3 ($\geq 20.98\text{ng/ml}$), MMP-8 ($\geq 6.43\text{ng/ml}$), TIMP-1 ($\geq 244.5\text{ng/ml}$), TIMP-3 ($\geq 35\text{ng/ml}$), IL-6 ($\geq 18.4\text{pg/ml}$), IL-8 ($\geq 20\text{pg/ml}$), IL-17 ($\geq 2.44\text{pg/ml}$), IL1-RA ($\geq 17.7\text{pg/ml}$), VEGF ($\geq 220\text{pg/ml}$), GM-CSF ($\geq 13\text{pg/ml}$) were associated with a significantly increased odds of unsuccessful outcomes (table 9.8). A final model for predicting failure and for predicting failure/relapse (i.e. unsuccessful outcome) was optimised using 2 and 4 two month predictor variables respectively. Combination of MMP-8 and GM-CSF thresholds in the model predicted failures with an area under ROC curve of 0.77. Combination of TIMP1 and IL-8 in the model predicted recurrences with an area under ROC curve of 0.76. Combination of MMP-3, MMP-8, TIMP-1 and IL-17 in the model predicted the composite outcome of failure/recurrence with an area under ROC curve of 0.80.

Table 9.10 Unadjusted odds ratios for predicting unfavourable outcomes using ROC-derived thresholds of analytes at 2 months

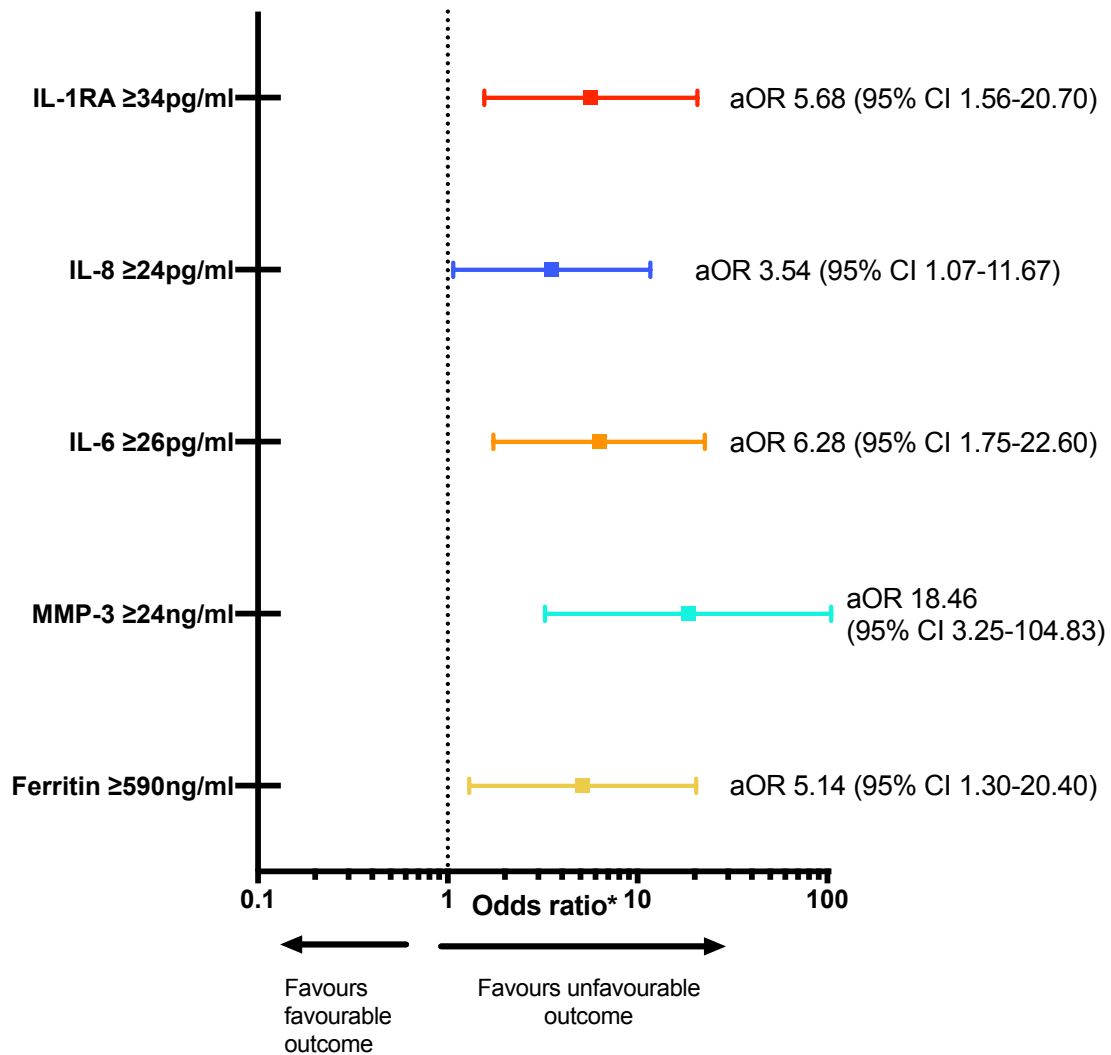
Analyte (threshold)	OR for predicting Failures only (95% CI) [area under ROC curve]	OR for predicting recurrence only (95% CI) [area under ROC curve]	OR for predicting composite failure/recurrence (95% CI) [area under ROC curve]
MMP-3 (≥ 21 ng/ml)	4.32 (1.18-15.77) [0.67]	3.29 (0.70-15.54)	3.88 (1.38-10.90) [0.66]
MMP-8 (≥ 6 ng/ml)	5.56 (1.39-22.20) [0.70]	2.78 (0.59-13.07)	4.17 (1.45-12.00) [0.67]
TIMP-1 (≥ 245 ng/ml)	2.47 (0.70-8.72)	7.41 (1.36-40.36) [0.73]	3.70 (1.33-10.31) [0.65]
TIMP-3 (≥ 35 ng/ml)	12.11 (2.64-55.45) [0.66]	8.48 (1.31-54.9) [0.62]	10.6 (2.81-40.02) [0.64]
IL-6 (≥ 18 pg/ml)	4.95 (1.35-18.15) [0.66]	2.38 (0.42-13.31)	3.78 (1.28-11.19) [0.62]
IL-8 (≥ 20 pg/ml)	2.77 (0.73-10.40)	6.46 (1.33-31.23) [0.70]	3.88 (1.35-11.10) [0.64]
IL-17 (≥ 2 pg/ml)	3.11 (0.86-11.26) [0.64]	4.44 (0.82-23.92) [0.68]	3.55 (1.24-10.18) [0.65]
IL1-RA (≥ 18 pg/ml)	6.2 (1.71-22.52) [0.69]	2.07 (0.37-11.49)	4.13 (1.43-11.90) [0.64]
VEGF (≥ 220 pg/ml)	4.30 (1.19-15.64) [0.65]	2.07 (0.37-11.49)	3.29 (1.12-9.62) [0.61]
GM-CSF (≥ 13 pg/ml)	5.46 (1.52-19.67) [0.68]	3.41 (0.71-16.46)	4.55 (1.60-12.91) [0.66]

Failures n=11, Recurrences n=7, Composite of failure/recurrence n=18

The highlighted figures are statistically significant, $p < 0.05$

Figure 9.6 and 9.7 show the baseline and 2-month predictors of the composite outcome of failure/recurrence, which remained significant having adjusted, in a multivariable model for potential confounding clinical covariates including sex, BMI, TTD, age, HIV-1 serostatus, cavities, T2DM and poor adherence (missing ≥ 5 doses as per pill counts and/or self-report in the previous month on interview).

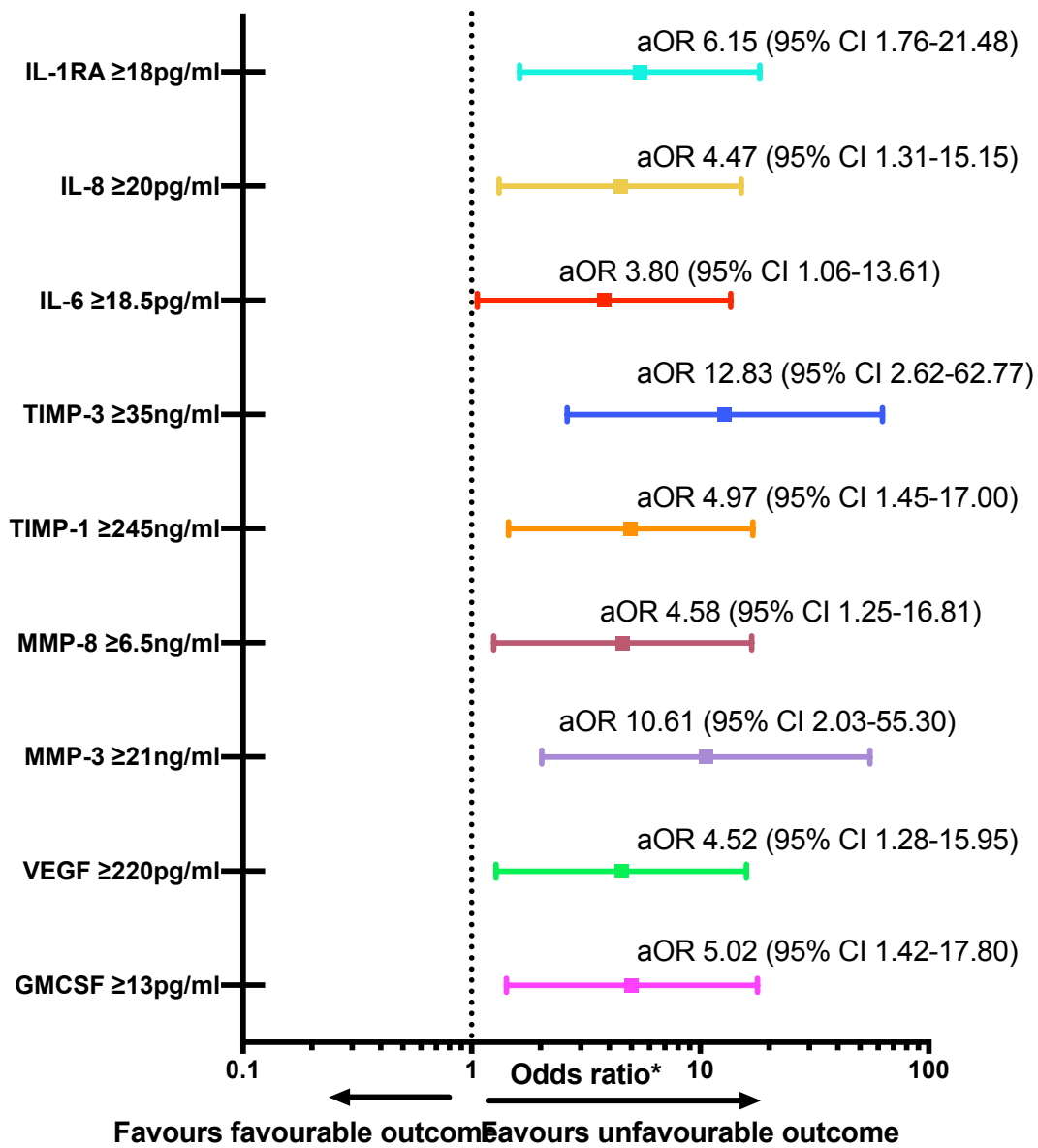
Figure 9.5 Baseline predictors of unsuccessful outcomes



* Adjusted for sex, BMI, time to culture positivity, age, HIV serostatus, cavities, T2DM, poor adherence

ROC curves were plotted and threshold selected with optimized likelihood ratio, sensitivity and specificity

Figure 9.6 2 month predictors of unsuccessful outcome



* Adjusted for sex, BMI, time to culture positivity age, HIV serostatus, cavities, T2DM, poor adherence

ROC curves were plotted and threshold selected with optimized likelihood ratio, sensitivity and specificity

9.4. Discussion

9.4.1. Immune correlates during chemotherapy

CRP, ferritin, HO-1, 8OHdG, SAP, tPa, MMP-1, MMP-3, MMP-8, MMP-9, MMP-10, MMP-12, TIMP-3, TIMP-4, sCD14, IL-1 β , IL-1RA, IL-6, IL-8, IL-17, TNF α , IFN α 2, IFN γ , GM-CSF, VEGF and CXCL-10 decreased significantly during chemotherapy and TIMP-2 increased significantly during chemotherapy. Analytes such as tPA and sCD14 decreased significantly only in HIV-1 co-infected participants. Conversely, IL-6, MMP-9, MMP-12, VEGF, SAP, IFN α 2 and IL-1 β decreased significantly only in HIV-1 uninfected participants and hence, may be proposed as a more specific measure of anti-mycobacterial response to chemotherapy. These results were unaffected when stratified by HIV-1 virological suppression on ART at baseline. IL-10 and IL-12 did not significantly change during treatment. The ratio of IFN γ /IL-10 decreased significantly, both in HIV-1 co-infected and HIV-1 uninfected participants, over the course of chemotherapy suggesting a shift toward a less inflammatory cytokine profile by the end of treatment.

31/48 HIV-1 infected participants who were not on ART at baseline, commenced ARV by the 2 month visit. Hence, in these participants, change in immune correlates reflects both the effect of anti-tuberculous chemotherapy and ART.

9.4.2. Immune correlates with HIV-1 co-infection and radiological disease severity

Cavities were significantly less frequent in those with CD4 lymphocyte count <100cells/mm³. The analytes which were significantly lower in participants with cavities (HO-1, ferritin, TNF α , CXCL-10, IL-8, IL-1 α) were also significantly higher in participants with CD4 lymphocyte count <100 cells/mm³ and hence, CD4 lymphocyte count may have been a confounder. HIV-1 viral load was also a predictor of cavities with a 41% decreased odds of cavities with every 1 log₁₀ increase in HIV-1 viral load. Conversely, virological suppression on ART was associated with 5.5x fold increased odds of cavitary disease.

MMP-9 and TIMP-2, both of which can be neutrophil-derived (242), were significantly lower in HIV-1 co-infected participants, compared with HIV-1 uninfected participants.

MMP-3/TIMP-2 ratio was significantly lower at baseline in participants with cavities at baseline. MMP-9/TIMP-1 ratio was significantly higher, both at baseline and 2 months in participants with cavities at baseline. MMP-9/TIMP-3 ratio was significantly higher at 2 months in participants with cavities at baseline. IFN α 2, MMP-9, MMP-9/TIMP-1, MMP-9/TIMP-2, MMP-9/TIMP-3 and MMP-9/TIMP-4 were significantly higher in participants with extensive radiological disease on chest x-ray at baseline. Hence, MMP-9 showed a consistent trend even when endogenous inhibitors were factored. MMP/TIMP imbalance has been suggested to be important in the progression of cavitary disease in a rabbit model of tuberculosis (224).

MMP-9 was strongly positively correlated with IFN α 2 (Rho= 0.93), IL-1 β (Rho =0.72) and IL-17 (Rho= 0.45) and strongly negatively correlated with IL-1 α (Rho= -0.7). MMP-8 was strongly positively correlated with IFN α 2 (Rho= 0.72), IL-1 β (Rho= 0.55) and negatively correlated with IL-1 α (Rho= -0.42). IL-17 released by T-helper 17 lymphocytes recruits neutrophils to site of inflammation and was positively correlated with blood neutrophil count (Rho=0.72). MMP-8 is released by neutrophils and was positively correlated with blood neutrophil count (Rho=0.61). There was a positive correlation between IL-17 and MMP-8 (Rho= 0.43) suggesting a putative IL-17-potentiated release of MMP-8. There was also a strong positive correlation between IL-17 and neutrophil chemoattractant IL-8 (Rho= 0.8). A transcriptional signature for an IFN-inducible myeloid derived monocyte/neutrophil-driven response has been linked with radiologically assessed TB disease severity (238). This response may in part be mediated via up-regulation of gelatinase B (MMP-9) and collagenase-2 (MMP-8), both of which are associated with neutrophil markers (225, 243). IL-1 α appears protective in this pathway as it is strongly negatively correlated with MMP-8 and MMP-9, whilst IL-1 β is not. Studies have shown that the absence of IL-1 α propagates progressive chronic inflammation (244).

Conversely, prolonged IL-1 β production promotes recruitment of neutrophils to the disease site, inducing release of prostaglandins and MMPs (245) resulting in the progression of chronic tissue damage with associated radiographic manifestations e.g. cavities and nodules (246). This IL-1 β -mediated tissue-damaging inflammatory response is blocked by IFN γ – induced nitric oxide inhibition of the NLRP3 inflammasome (247).

Participants with virological suppression (HIV-1 viral load <40 copies/ml) at baseline (median CD4 lymphocyte count 330 cells/mm³ in this sub-cohort) had increased odds of cavitary disease and significantly decreased ferritin, CXCL-10, TNF α , IL-17 and IL-8 and significantly increased TIMP-2 compared with those with detectable HIV-1 viraemia (median CD4 lymphocyte count 128 cells/mm³ in this group). These results suggest that increase in cavitary radiological changes can occur in response to virological suppression, a phenomenon most recognized in immune reconstitution inflammatory syndrome. Other studies have only found a significant association between CXCL-10 and HIV-1 viral load (248).

9.4.3. Potential predictors of unsuccessful outcomes

At present there is little evidence for any single or combination of acute phase proteins, MMP/TIMPs or cytokines/chemokines that can reliably serve as a surrogate marker of TB treatment response (249).

On adjusting for potential confounding clinical covariates, ROC curve-derived thresholds IL-8, IL-6, IL-1RA, MMP-3 and ferritin at baseline (figure 10.6) and IL-8, IL-6, IL-1RA, MMP-3, MMP-8, TIMP-1, TIMP-3, GM-CSF and VEGF at 2 months were used to predict long term unfavourable outcomes (figure 9.7). A previous study also found an association between VEGF levels and therapeutic response (250). The combination of analytes achieved an optimized area under ROC curve for predicting outcomes of between 0.76 and 0.80. There were differences in calculated odds ratios when the outcome variable was stratified by failure vs recurrence vs the composite of failure/recurrence. This reflects potential differences in immunobiology of the host in treatment failures compared with those who would subsequently suffer a recurrence.

9.4.4. Limitations

Lack of an HIV-1 control group with no TB co-infection limited our ability to attribute analyte secretion patterns as specific to HIV-1 infection.

There were low numbers of participants with unsuccessful outcomes and lack of a validation cohort. Hence, there was a possibility of over-fitting the data when combining ROC curve derived thresholds to predict unsuccessful outcomes. For multivariate analyses (inclusive of clinical covariates), 'failure' and 'recurrence' were combined. This

may be inappropriate as the host immune environment could differ significantly for these 2 outcomes, particularly as treatment failures have a much higher bacterial load compared with those who will subsequently relapse. Genotyping was not carried out on the recurrence cases included in this analysis and hence, exogenous re-infection cannot be excluded. Comparison of paired data at baseline vs 5-6 months and 2 months vs 5-6 months was not possible as samples at 5-6 months were only collected for 54/133 participants, the majority of whom were culture positive at 2 months. Hence, there was selection bias towards those who were still culture positive at 2 months.

Lack of sampling at time points between baseline and 8 weeks of treatment meant that potential biomarkers for predicting treatment outcomes at an earlier stage in treatment (i.e. between 0 and 8 weeks) could not be explored.

The duration of infection is an unknown at baseline and it is not possible to conclusively distinguish temporality in the regulation acute phase protein/MMP/TIMP/cytokine secretion and associated clinical phenotypes. These associated clinical phenotypes are secondary to complex interactions and reflect both the effect of and response to the disease, anti-tuberculous chemotherapy and ART. The balance between pro and anti-inflammatory cytokines which favours the host is different in early vs chronic established infection(246). In chronic infection, modulation of tissue-damaging inflammatory response is a determinant of disease progression. There is also the limitation of assuming cytokine responses in peripheral blood reflects what occurs locally at the site of disease.

In summary, we found significant differences in the majority of blood analytes over the course of treatment. Some analytes were different comparing subjects with or with HIV-1. Within the HIV-1 co-infected sub-group, there were marked differences comparing subjects with and without advanced immunosuppression and with and without virological suppression. There were further differences, stratifying subjects by baseline cavitory status, some of which may have been influenced by degree of immunosuppression and viral load of the HIV-1 infected subjects. There was a strong positive correlation between the analytes MMP 8, MMP 9, IL-17 and IL-8; all of which are involved in sequestration of neutrophils. MMP8 and 9 had strong positive correlation with IFN α 2 and IL-1 β and a strong negative correlation with IL-1 α . Hence, the IFN-inducible myeloid derived

monocyte/neutrophil-driven response may be driven in part by MMP 8 and 9. IL-1 α is likely to be protective in this pathway.

IL-8, IL-6, MMP-3 and IL-1RA showed promise as biomarkers of unsuccessful treatment response both at baseline and 2-months. These findings need to be verified in further validation cohorts, including both HIV-1 co-infected and HIV-1 uninfected subjects.

10. Scope of thesis, contribution to the field and research priorities

In the context of the WHO recommended DS TB treatment regimen (2 months of daily quadruple rifampicin/isoniazid/pyrazinamide/ethambutol followed by 4 months of daily dual rifampicin/isoniazid 2(HRZE₇)4(HR₇)), a key research challenge is to identify and understand the complex host, bacillary and environmental/programmatic factors which lead to successful treatment outcomes in the majority, and unsuccessful treatment outcomes in a minority of cases of rifampicin susceptible-TB. In TB, large studies with adequate powering and lengthy follow up times are required to reliably determine long term unfavourable outcomes, which is expensive and logistically challenging. For this reason, biomarkers and correlate endpoints predicting long-term outcomes are desirable.

The scope of this thesis was to:

1. Conduct a systematic review on the risk factors for acquired drug resistance in rifampicin susceptible (RS) TB (chapter 2).
2. Determine the frequency and risk factors/mechanisms for ADR in a programmatic setting with a high prevalence of HIV-1 co-infection (chapter 4).
3. To assess the clinical utility of MTBDR*plus* directly on 2-month sputum, a hitherto unreported test/potential biomarker, in monitoring treatment response (this included 2-month culture conversion, the composite of failure/recurrence/death during treatment and acquired drug resistance) (chapter 5).
4. To look for evidence of biological variation in expression of 12 selected MTB-specific messenger (m) and small (s) RNA in sputum pre-treatment and to compare expression stratified by 2-month culture converter status, thereby exploring potential bacillary adaptation mechanisms responsible for delayed sputum clearance (chapter 6).
5. . To assess if HIV-1 co-infected ambulatory patients, predominantly on ART and being dosed according to WHO weight bands, have lower plasma drug concentrations of rifampicin, isoniazid and pyrazinamide than HIV-1 uninfected TB patients (chapter 7).
6. To assess if PK/PD parameters, in combination with relevant clinical covariates, are important predictors of 2-month culture conversion and to assess clinical utility of currently recommended thresholds (chapter 8).

7. To assess if selected analytes in plasma/serum prior to or during chemotherapy correlate with HIV-1 co-infection, level of immunosuppression, virological suppression on ART and radiological disease severity and whether these can be used as potential predictors of treatment outcome (chapter 9).

10.1. Summary of main findings

Chapter 2- Systematic review of risk factors for ADR in RS TB

A meta-analysis of 15 studies showed an increased risk of ADR with baseline mono- or polyresistance (RR 4.85 95% CI 3.26 to 7.23, heterogeneity I^2 58%, 95% CI 26 to 76%). Meta-analysis of 8 studies showed that HIV-1 co-infection was associated with increased risk of ADR ((RR 4.3, 95% CI 3.32 to 5.56) , heterogeneity I^2 81%, 95% CI 64 to 90%). There was significant methodological and clinical diversity in included studies as evidenced by the high I^2 value. Importantly, HIV-1 co-infection was not associated with ADR in African populations. Non-adherence, extrapulmonary/disseminated disease and advanced immunosuppression in HIV co-infection were other risk factors noted. The weighted median cumulative incidence of acquired multi drug resistance calculated in 24 studies (assuming whole cohort as denominator, regardless of follow up DST) was 0.1% (5th to 95th percentile 0.07 to 3.2%) of note, this value was significantly weighted by 1 large North American study.

This review clearly demonstrated the need for further prospective cohort studies examining the incidence and risk of ADR in the context of the currently used 2(HRZE₇)4(HR₇) regimen, particularly in the era of ART being administered early in treatment,

Chapter 4- Prospective cohort study of ADR in a programmatic setting with high burden of HIV-1 co-infection

There was 1 confirmed individual who acquired drug resistance during TB treatment. There were 3 subsequent cases who had culture-confirmed recurrent TB with rifampicin resistance within 14 months of being discharged cured. One case, was confirmed re-infection with a MDR TB strain. We were unable to ascertain the spoligotype of the recurrent strain in 2 of the cases. Hence, they may or may not have been re-infection or true ADR. Hence, 1/306 (0.3%) to 3/306 (1%) of the entire cohort treated for rifampicin susceptible TB acquired drug resistance during or

subsequent to treatment. Although infrequent, in this extremely high burden setting, ADR does contribute to the growing drug-resistant TB epidemic and emphasis should be on identification of ADR cases and prevention of transmission to others.

We assessed for a change in MIC in the infecting MTB strain and showed that there was no evidence for increase in MIC during treatment, when comparing paired baseline and 2 month isolates. We showed, in this high prevalence setting, there was no difference in MIC profiles of infecting strain when stratified according to HIV-1 serostatus, retreatment and 2-month culture conversion status.

6% of the cohort had baseline isoniazid resistance (13/17 had *inhA* mutations). In this setting of low level isoniazid resistance, no participants amplified resistance despite receiving a standard anti-TB regimen. None of these patients presented with recurrent TB during the study follow up.

Two methods of adherence assessment were compared. Classification as 'poor adherence' based upon the composite measure of ≥ 5 missed doses in the previous month as per self-report/pill count was significantly associated with non-converter unlike 'poor adherence' defined by using a negative Arkansas test on 2-month urine samples. The latter is a 'snap-shot' of recent adherence and needs to be interpreted in the context of whether or not urine collected was the first voided urine for the day, variation in total fluid intake in the preceding 24 hours, variation in timing of previous dose and the participant's acetylator status. There was a significant association with previous incarceration and non-adherence.. Hence, this is a group likely to benefit from interventions such as DOT.

Chapter 5- Direct MTBDR*plus* on 2-month sputum to monitor treatment response

Direct MTBDR*plus* at 2 months had a sensitivity of 78%(95%CI 65-87) and specificity of 80%(95%CI 74-84) to predict culture positivity at 2 months with a high negative predictive value of 93%(95%CI 89-96). Inconclusive genotypic susceptibility for both rifampicin and isoniazid were seen in 26% of MTDDR*plus* tests performed directly on sputum. When compared to a reference of MTBDR*plus* performed on positive cultures, the false positive resistance rate for direct testing of MTBDR*plus* on sputa was 4% for rifampicin and 2% for isoniazid. Whilst a positive 2-month smear was not significantly associated with an unsuccessful treatment

outcome, (aOR 2.69, 95% CI 0.88-8.21), a positive direct MTBDR_{plus} at 2 months was associated with an unsuccessful outcome (aOR 2.87, 95%CI 1.11-7.42). We were limited in our capacity to gauge the diagnostic utility of direct MTBDR_{plus} as a screening tool for early ADR/heteroresistance as there were only 2 cases of ADR diagnosed using MTBDR_{plus} on culture isolates.

Chapter 6- Expression patterns of selected m/sRNA in sputum pre and during chemotherapy

Consistent with previous descriptions of MTB phenotype during infection, we observed a marked increase in *icl1* and *prpD* transcripts in sputum compared to culture, characteristic of cholesterol utilization. The ratio of *rpsN2* to paralogous *rpsN1* transcript was significantly higher in sputum compared to culture, indicative of a low-zinc environment. Increased abundance of *hspX* together with decreased abundance of *atpA* and *nuoG* relative to exponential culture indicated a metabolically less-active subpopulation in sputum, though the overall sputum profile had a significantly lower mean deviation from exponential phase culture than from stationary phase culture. While the magnitude of the *hspX*^{hi}*atpA*^{lo}*nuoG*^{lo} signal varied between individuals, we did not observe statistically significant enrichment of this phenotype in the non-converter population nor a significant association with the genotype of the infecting strain. These findings were limited by a small sample size and imprecise quantification of MTB-specific RNA in sputum. Also, as analyses were only carried out in HIV-1 uninfected patients, results may not be generalizable to HIV-1 co-infected patients.

Chapter 7- The effect of HIV-1 co-infection on the pharmacokinetics of rifampicin, isoniazid and pyrazinamide in ambulatory South African patients

The chief finding was that ambulant HIV-1-TB co-infected patients, the majority who were co-prescribed ART, did not have reduced anti-TB drug concentrations compared with HIV-1 uninfected patients.

HIV-1-TB co-infected patients had a lower clearance of rifampicin and isoniazid, with resulting higher AUC_{0-24hr} and C_{max}. Antiretroviral therapy that included double dose lopinavir/ritonavir further lowered rifampicin CL and increased AUC_{0-24hr}. Current uniform dosing per kilogram of

body weight across WHO weight bands was associated with a trend of decreased PK exposures in the lowest weight-band. Use of fat-free mass for allometric scaling of CL, as opposed to total body weight, significantly improved the model fit. Having adjusted for potential confounders, there was an increased odds of side-effects in the highest vs. the lowest quartile of isoniazid C_{max} (OR 7.11, 95%CI 1.99- 25.47, $p=0.003$).

Chapter 8- Pharmacokinetic/pharmacodynamic determinants of two-month culture conversion in rifampicin susceptible tuberculosis in a high HIV burden setting

31% of patients had low levels for all 3 drugs, defined as C_{max} for rifampicin, isoniazid and pyrazinamide less than 8, 3 and 35mg/l respectively (as per thresholds based on healthy volunteer studies). No relationship was found between PK exposures and 2-month culture conversion using logistic regression, after adjusting for MIC. This was unrelated to clinical factors. However, MARS identified concentration-dependent antagonism between isoniazid and rifampicin on sputum conversion. Below rifampicin C_{max} /MIC of 28 and below isoniazid C_{max} of 4.6mg/l, isoniazid concentration had an antagonistic effect on culture conversion. Above an isoniazid C_{max} of 4.6 mg/l, this relationship was negated and higher isoniazid exposures were associated with improved rates of culture conversion.

Chapter 9- Blood analytes as correlates of HIV-1 co-infection, disease severity and potential biomarkers of treatment response

There were significant differences in the immune profile of HIV-1 co-infected participants versus HIV-1 uninfected participants both pre-chemotherapy and in response to chemotherapy.

Ferritin, HO-1, $TNF\alpha$, MMP-3, IL-17, IL-8, IL-12p40, IL-1 α , IL-1RA and IFN γ were significantly higher in HIV-1 co-infected participants with CD4 lymphocyte counts <100 cells/mm³ at baseline compared with those with CD4 lymphocyte counts $\geq$ 100 cells/mm³.

HO-1, ferritin, $TNF\alpha$, CXCL-10, IL-8, IL-1 α , MMP-3/TIMP-2 were significantly decreased in participants with cavities at baseline.

Participants with virological suppression had increased odds of cavitary disease and significantly lower ferritin, CXCL-10, $TNF\alpha$, IL-17 and IL-8 and significantly increased TIMP-2 compared to those with detectable viraemia.

MMP-9 and MMP-8 was strongly positively correlated with IFN α 2, IL-1 β and IL-17 and strongly negatively correlated with IL-1 α . On multivariate analysis, having adjusted for HIV-1 serostatus and TTD, higher IL-1 α , ferritin and IL-8 were associated with significantly decreased odds of cavitary disease. These immune correlates suggest the IFN-inducible myeloid derived monocyte/neutrophil-driven response which has been linked with radiological disease severity (238) may be mediated via up-regulation of gelatinase B (MMP-9) and collagenase-2 (MMP-8), both of which are associated with neutrophil markers. IL-1 α appears protective in this pathway whilst IL-1 β is not.

ROC derived thresholds for IL-8, IL-6, MMP-3 and IL-1RA showed were used to predict unsuccessful treatment response both at baseline and 2-months.

10.2. Impact of thesis and future direction for the field

In the context of a daily rifampicin-based 4-drug anti-TB regimen and in the era of increasingly earlier commencement of ART, we have shown that the incidence of ADR is around 0.3-1%. In a high burden setting like Khayelitsha township, this is still up to 47 ($0.01 \times 4700 = 47$) additional cases of DR TB per year (refer figure 4.5), a proportion of whom may also transmit their DR TB strain, hence perpetuating the DR TB epidemic. In tackling the MDR TB epidemic, health systems should prioritise prevention of transmission of drug resistance through making rapid diagnostics widely available, expediting commencement of efficacious regimens and optimising infection control.

The risk factors which we found to be significant predictors of the interim outcome of 2-month culture conversion were type 2 diabetes mellitus, non-adherence, high smear grading/short time to culture positivity, and extensive disease on chest radiograph. These have translational implications including consideration of directly observed therapy, more intensive monitoring and prolongation of the intensive phase of treatment. Both cases of ADR had documented poor adherence and cavitary disease at baseline. Our results do not support the hollow fibre model/monte carlo simulation data which suggested that treatment failure was only encountered at nonadherence levels $\geq 60\%$ and even in the scenario of 100% adherence, there would be incidence of ADR of 1%, based upon PK variability alone (15).

Twenty percent of the overall cohort (and 32% of men) had previously spent time in prison. We found a significant association with previous incarceration and non-adherence. The reasons for this are multifold including other concurrent psychosocial risk factors such as unemployment and substance abuse. There is a need for prison systems to address and prevent unplanned treatment interruptions, with optimised access and sharing of medical records between penitentiary and community health care facilities.

Assessment of adherence in a high burden low resource setting is challenging. We show that a combination of self-reporting, coupled with pill counts over the previous month is more predictive of 2-month culture conversion than a single assessment of urinary metabolites of isoniazid via the Arkansas Test. The reason for this is that the

latter is only a measure of adherence over the past 24 hours and could also have been affected by the timing of the previous dose and fluid intake over the past 24 hours.

Since the widespread dissemination of GeneXpert® MTB/RIF in TB programmes, there has been a hypothetical concern regarding the diagnosis of baseline isoniazid resistance being missed, leading to amplification of drug resistance in these patients commenced on standard 2(HRZE₇)4(HR₇), based upon a GeneXpert® MTB/RIF result. We show data disputing this concern, as long as patients are fully adherent with treatment. These results contradict previous studies (74, 99) which looked at the effect of isoniazid resistance on treatment outcomes. The apparent differences in outcomes may reflect differing treatment regimens e.g. daily vs thrice weekly, differing levels of adherence and different proportions of low vs high level isoniazid resistance. Whilst baseline drug resistance is a risk factor for ADR, the findings of this thesis do not support HIV-1 co-infection as a driver of ADR. Our study results are generalizable to HIV-1 co-infected patients with varying degrees of immunosuppression, the majority of whom were immune reconstituting on ART by 2 months. There was no evidence of differences in distribution of MICs, stratified by HIV-1 serostatus.. We did not find that retreatment patients had higher MIC of the baseline infecting strain, nor did we find that MIC of the MTB strain increased during anti-TB treatment.

The detection of ADR needs to be optimized. The use of MTBDR*plus* directly on sputum for this purpose is not advocated due to high number of inconclusive tests and false resistance calls. Our data do not support the use of a direct MTBDR*plus* in routine treatment monitoring. There is ongoing research to improve specificity of DNA amplification from live bacteria through treatment of sputa with agents such as propidium monazide, a DNA-binding dye that penetrates through damaged cell walls and inhibits PCR amplification via DNA modification (118). The availability of high coverage genome sequencing directly from clinical samples may assist optimal characterization of minority bacterial subpopulations (125) and heteroresistance but would not assess phenotypically tolerant sub-populations.

We showed no evidence that ambulant HIV-1 co-infected subjects have reduced anti-TB drug concentrations compared with TB mono-infected subjects. In a small number of patients concurrently taking double dose lopinavir/ritonavir based ART, we found

evidence of increased rifampicin concentrations. As more patients are commenced on second-line protease inhibitor-based ART regimens, and in the context of on-going trials advocating increased dosing of rifampicin of up to 35mg/kg, future studies should assess the safety and tolerability of co-administration of increased dose rifampicin with double dose lopinavir/ritonavir. We also find evidence of increased Grade 2 toxicity in the highest quartile of isoniazid C_{max} which could have implications for increased toxicity and poorer adherence in certain patients, particularly in populations with a high proportion of slow acetylators status and in patients concurrently taking co-medications associated with toxicity.

As advocated by previous studies (160), there is a need for further population PK modeling and dose simulation to optimize dosing in the lower weight bands. The findings of this thesis do not support therapeutic drug monitoring of rifampicin, isoniazid and pyrazinamide as part of routine treatment monitoring. In addition, for both converters and non-converters, a significant proportion of patients had a C_{max} lower than the currently recommended guidelines for all drugs. We found that the thresholds for isoniazid and rifampicin were not predictive of 2-month culture conversion and/or failure/relapse. The cut-off of pyrazinamide <35mg/L was not predictive of 2-month culture conversion, but did predict failure/relapse (OR=0.16, $p=0.03$) as previously seen in another cohort(78). Twenty-six of 31 patients (84%) with low concentrations of all 3 drugs had culture converted at 2 months, and 4/31 (13%) had treatment failure/relapse compared with 3/69 (4%) who did not have low concentrations of all 3 drugs. Hence, only the threshold for pyrazinamide, had utility for predicting treatment outcomes.

Logistic regression failed to identify a relationship between C_{max}/MIC , AUC/MIC or $\%T_{MIC}$ and the probability of culture conversion. As an example, these would average out outcomes on either side of the “u-shaped” relationship we identified, so that measures of central tendency would not differ for the range of exposures. As we have illustrated, both measured and un-measured clinical covariates e.g. high proportion of baseline smear 3+ grade in the top quartile of rifampicin may have influenced the PK-PD relationships seen.

The MARS model allowed exploration of non-linear relationships within the data-set, the chief one being concentration-dependent antagonism between isoniazid and rifampicin at the lower range of rifampicin C_{max}/MIC (<28) which is also negated after isoniazid C_{max}

exceeds 4.6mg/ml. The same was seen for a paediatric cohort (including for mortality) (217) , in murine (219) and hollow fiber studies (221), so that the relationships are unlikely not an artifact but are in fact consistent.

This finding has therapeutic implications. One potential option would be to drop isoniazid after the first week of TB treatment i.e. at the point after its chief early bactericidal activity. Another approach would be to tailor dosage of isoniazid, based on acetylator status. The feasibility and cost-effectiveness of such an approach need to be considered. Another strategy would be to stagger dosing of rifampicin and isoniazid to exploit PK mismatch and avoid drug-drug antagonism. There are currently on-going studies evaluating the safety and efficacy of higher dosing of rifampicin and isoniazid which would also potentially avoid concentration-dependent antagonism.

Further pre-clinical and clinical studies are required to evaluate the potential PD interactions between these drugs and their implications for dosing regimes. Novel methods of characterizing the time-kill effect of chemotherapy on bacterial sub-populations over the dosing interval could be developed through pharmacodynamic modeling. As demonstrated by Prideaux *et al*, differential spatial distribution and kinetics of accumulation in diseased tissue can help predict sterilizing activity of existing and new drugs (198). Enhanced understanding of the tissue micro-environment will guide rationale for optimised dosing and duration of treatment with specific drugs based upon the kinetics of drug action (251). A multi-systems approach has been used by Pienaar *et al*. to integrate spatio-temporal dynamics of granuloma formation and immune function, PK in plasma and tissue and time to sterilization of intracellular, extracellular replicating and extracellular non-replicating MTB populations (252).

Characterisation of the transcription phenotype of MTB by direct qRT-PCR in sputum from well-defined patient cohorts can be used to address biological and clinical questions relevant to treatment and transmission of tuberculosis. It is proposed that lack of pre-amplification step, allowed optimised detection of inter-individual variation of gene expression. This is contrary to other studies which have relied on pre-amplification +/- pooled samples from different patients (152). This work shows preliminary evidence that MTB in sputum pre-chemotherapy is potentially zinc deprived and uses cholesterol as a carbon source. There was increased abundance of *hspX* together with decreased

abundance of *atpA* and *nuoG* relative to exponential culture indicating a metabolically less-active subpopulation in sputum and there was a non-significant trend towards enrichment of *hspX*^{hi}*atpA*^{lo}*nuoG*^{lo} in non-converters, independent of concomitant Beijing lineage. Future research objectives include advancing knowledge of transcriptomic change further on in treatment (up to 2 months) and ascertaining the maximum mRNA, sRNA and rRNA half lives in sputum under different temperatures, oxygen gradients and sputum viscosities. This will shed light upon how much of the transcriptomic signature comes from dead/dying bacilli during treatment and how much can be attributed to non-replicating persisters. Other future research objectives include study of how the lung and sputum microbiome affects MTB response during chemotherapy.

During treatment monitoring, there are thought to be limitations in our ability to monitor viable MTB sub-populations including minority non-replicating persisters using current culture-based approaches. Studies are required to ascertain if detection of rRNA and/or mRNA in the sputum of clinically asymptomatic culture negative patients at the latter stages of treatment (i.e. potentially viable but non-culturable MTB) correlates with relapse. The PanBiome Study by the PanACEA and PreDiCT-TB consortia (<http://www.predict-tb.eu/panbiome-project>) are studying novel molecular and culture approaches as biomarkers of treatment response, as compared with traditional solid and liquid culture techniques. *In vitro* models of dormancy have shown accumulation of host triacylglycerol as lipid bodies under control of triacyl glycerol synthase 1 (153). These lipid bodies can be detected in sputum via staining with auramine-labelled Nile-Red stain both pre- and during treatment. Change in detectable lipid bodies during treatment, is hypothesized to reflect an increase in viable but non-culturable persisters. In a preliminary study, Sloan *et al.* showed that although baseline counts of % lipid body positive acid-fast bacilli did not predict treatment response, at week 3-4 of treatment, there was 21% increased odds of unfavourable outcome (failure/relapse) for each 1% rise in lipid body positive acid-fast bacilli (216).

There is considerable heterogeneity observed in the immune response to tuberculosis with a myriad of host and microbial related factors playing a role in inter-individual and geographic variation in the measured immune response to MTB (229, 246). This work suggests, in both HIV-1 co-infected and HIV-1 uninfected hosts, there is a relationship between type 1 interferon, neutrophil-associated cytokines (IL-17 and IL-8) and

neutrophil-associated MMPs (MMP-8 and MMP-9) and the development of cavitary disease. IL-1 α is likely to have a protective role, whilst IL-1 β does not. Further animal models and *in vitro* studies will enhance understanding of spatio-temporal interactions at the site of disease, will help determine the temporality of blood analytes in infection and associated clinical phenotypes and help elucidate downstream molecular mechanisms.

As specimen and data repositories are established and novel technologies are developed, it is hoped that better biomarkers for predicting TB treatment outcomes will be discovered, validated and have application in improving clinical trials, developing better treatments and treatment monitoring in the clinic. Whilst biomarkers should ideally be generalizable across ethnically diverse populations, they also need to be tailored to specific patient groups e.g. children and drug resistant TB. Ultimately, a deeper understanding of pathogenesis, including both host and mycobacterial factors, is needed. This is alongside programmatic determinants of treatment outcomes such as adherence assessment and support. This cohort illustrates the multifactorial determinants of treatment outcomes. Although the majority of patients had good treatment outcomes, it is important to establish and optimize composite disease severity scores to screen individuals at high risk of unsuccessful outcome at baseline or relatively early during treatment. These individuals can then be prioritized for intensive treatment monitoring and potential treatment intensification or prolongation.

11. Publications

- Rockwood N, Abdullahi LH, Wilkinson RJ, Meintjes G. Risk Factors for Acquired Rifamycin and Isoniazid Resistance: A Systematic Review and Meta-Analysis. PLoS One. 2015;10(9):e0139017.
- Rockwood N, Meintjes G, Chirehwa M, Wiesner L, McIlleron H, Wilkinson RJ, et al. HIV-1 Coinfection Does Not Reduce Exposure to Rifampin, Isoniazid, and Pyrazinamide in South African Tuberculosis Outpatients. Antimicrob Agents Chemother. 2016;60(10):6050-9.
- Rockwood N, Pasipanodya JG, Denti P, Sirgel F, Lesosky M, Gumbo T, Meintjes G, McIlleron H, Wilkinson RJ. Concentration-dependent antagonism and culture conversion in pulmonary tuberculosis. Clin Infect Dis. 2017 (*in press*)
- Rockwood N, Wojno J, Ghebrekristos Y, Nicol M, Meintjes G, Wilkinson RJ. Utility of second generation line probe assay (Hain MTBDR*plus*) directly on 2-month sputa specimens to monitor tuberculosis treatment response. J Clin Microbiol. 2017 (*in press*)

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Appendix

Symptom review at 2 months (Y/N)	Details (time relationship, duration, change in dose/management, relevant blood tests)	Adverse drug reaction Yes/No CTCAE Grading	WHO-Uppsala monitoring Center system Causality assessment (Certain/Probable/Possible/Unlikely/Unclassifiable)
Likely disease-related (not drug-related)			
Fever			
sweats			
weight loss			
Enlarged lymph nodes			
Cough			
haemoptysis			
Pleuritic chest pain			
Shortness of breath			
		Central nervous system	
Headache			
Dizziness/unsteadiness			
Visual disturbance			
Hearing disturbance			
Depression			
Psychosis			
Seizures			
Peripheral nervous system			
Pins and needles/numbness			
Painful neuropathy			
Weakness in muscle group			
Musculoskeletal system			

Arthritis large joints			
Back pain			
Cramps			
Gastrointestinal system			
Nausea and vomiting			
Reflux			
Abdominal pain			
Diarrhoea			
Constipation			
Abdominal distension			
skin			
Rash			
Itching			
Oedema			
Other			