Out of the

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Evaluation of potential surrogate markers to determine TB treatment response among TB patients in Mbeya, Tanzania

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Abstract

Background

Appropriate markers to reflect TB treatment responses are urgently needed mainly for patient care and their application in clinical trials. Additionally, more potent drug combinations in search for treatment shortening regimen are warranted. This work reports results of the TB treatment study PanACEA MAMS TB 01, with nested evaluation of the Molecular Bacterial Load Assay (MBLA) for its potential use as a marker to determine treatment success within a research setting.

Methods

This study was nested within the multiple-arm, multiple-stage (MAMS), phase 2 clinical trial that contributed data set to the PanACEA Biomarkers Expansion programme (PANBIOME) study. Eligible patients were randomised to either the control arm or one of four experimental arms. Culture and MBLA were performed at baseline through to treatment completion comparatively.

Results

High dose rifampicin at 35mg/kg ($RIF_{35}HZE$) was superior to control [Hazard ratio 1·46, 95% CI (1·02, 2·11)], p=0·04 for time to culture conversion to negative in MGIT at week 12, the primary endpoint. MBLA had the highest (19%) positive rates compared to MGIT (1.7%) and LJ (1.2%) media at week 26. The median time to negative culture was 35, 55 and 97 days on LJ, MGIT and MBLA respectively. Among the contaminated samples on MGIT and LJ media, MBLA reported 50.9% and 36.3 % as negative respectively. Furthermore, quantitative bacterial load measurements in MBLA and MGIT were significantly correlated ($p<0.001$).

Conclusion

A high dose of rifampicin showed superior efficacy in both MGIT and MBLA compared to the control regimen. MBLA as a marker to determine treatment success bears potentials that could contribute in routine patient care and trial setting. However, evaluation on its implementation in routine care and its usefulness as an end point in trials merit consideration.

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Abbreviations

AE	Adverse Event
AFB	Acid Fast Bacilli
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
BL	Bacterial Load
CD4	Cluster of Differentiation 4 (T Helper Cell)
CFU	Colony Forming Units
Ct;CT	Cycle threshold in GeneXpert MTB/RIF® test
CTCAE 4.0	Common Terminology Criteria for Adverse Events 4.0
DNA	Deoxyribonucleic acid
DOTS	Directly Observed Treatment Short course
DS TB	Drug sensitive TB
EBA	Early Bactericidal Activity
EMB	Ethambutol
GCLP	Good Clinical Laboratory Practice
GCP	Good Clinical Practice
GDP	Gross Domestic Product
GTC	Guanidine thiocyanate
H;INH	Isoniazid
HIV	Human Immunodeficiency Virus
INS	Insituto Nacional de Saude
ITT	Intentional To Treat
IUATLD	International Union against TB and Lung Disease
KCRI	Kilimanjaro Clinical Research Institute
kg	kilogram
LJ	Lowenstein-Jensen
LMU	Ludwig-Maximilians-Universitaet Muenchen
Mtb ; MTB	Mycobacterium tuberculosis
M; Moxi	Moxifloxacin
MAMS	Multiple-Arm, Multiple-Stage
MBL;MBLA	Molecular Bacterial Load Assay
MDR	Multidrug-Resistant

MGIT	Mycobacterium Growth Indicator Tube
MIC	Minimum Inhibitory Concentration
MoHSW	Ministry of Health and Social and Welfare
MRC	Medical Research Council
mRNA	Messenger RNA
NIMR-MMRC	National Institute for Medical Research – Mbeya Medical Research Centre
NTLP	National TB and Leprosy Programme
NTM	non-tuberculosis mycobacteria
OEBA	Observational of Early Bactericidal Activity
PanACEA	Pan African Consortium for the Evaluation of Antituberculosis Antibiotics
PANBIOME	PanACEA BioMarkers Extension
PCR	Polymerase Chain Reaction
PK	Pharmacokinetics
REMox TB	Rapid Evaluation of Moxifloxacin in Tuberculosis
RIF, R	Rifampicin
RNA	Ribonucleic acid,
rRNA	ribosomal RNA
SA	South Africa
SQ109	Sequella Medicinal Compound 109
TB	Tuberculosis
TFDA	Tanzania Food and Drug Authority
TTP	Time to Positivity in Liquid Media
UK	United Kingdom
UN	United Nations
UNSTAN	University of St. Andrews
WHO	World Health Organization
XDR	Extensively drug-resistant
Z; PZA	Pyrazinamide
ZN	Ziehl-Neelsen

1 Introduction

1.1 Global History of Tuberculosis

Despite recent successes in diagnosis and treatment of tuberculosis (TB), more than 130 years since its discovery by Robert Koch [2], [3, 4], the disease continues to have a serious impact on human health globally. In 2014, there were an approximated 9.6 million new cases of TB (12% co-infected with HIV) and nearly 1.5 million died from TB worldwide especially in settings dually affected by the HIV pandemic. It is now estimated that TB is blamed next to HIV as a leading cause of global mortality. A total of 9 out of 22 high TB burden countries come from the African region and the continent contributed about 28% of the new cases in 2014 while contributing only 14,8% of the world' s population (Figure 1)

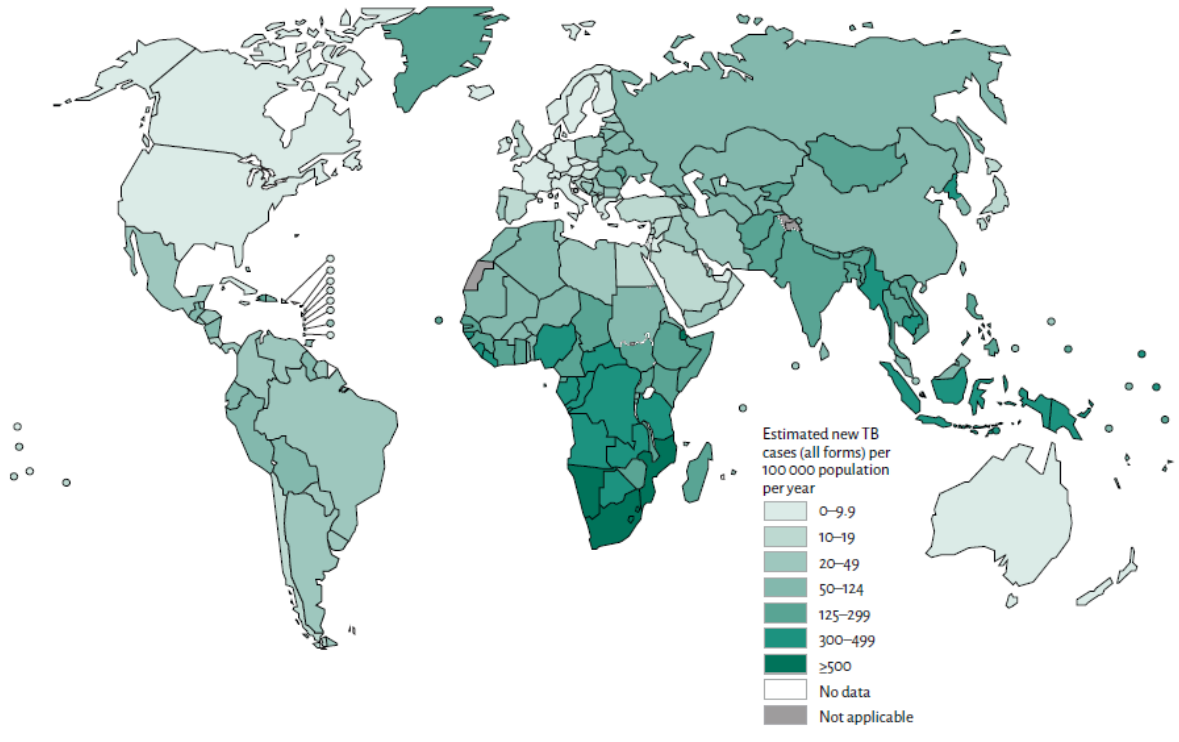

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Figure 1: Estimated incidence of TB in 2014 reproduced from WHO Global TB report 2015

TB control efforts in the African region have failed to reach the Millennium Development Goals by 2015, except for the reported falling of the incidence rate. On the contrary, despite having almost two third of the global burden, both South-East Asia and the Pacific regions managed to meet the stated targets [5]. The HIV epidemic has contributed to the TB problem and both infections have been given the status of global emergency by the WHO (TB) in 1993 and UN (HIV) in 2001. Clearly, highest TB-HIV co-infection

rates are encountered in African regions, and this complicates diagnostics, therapy and disease control (Figure 2).

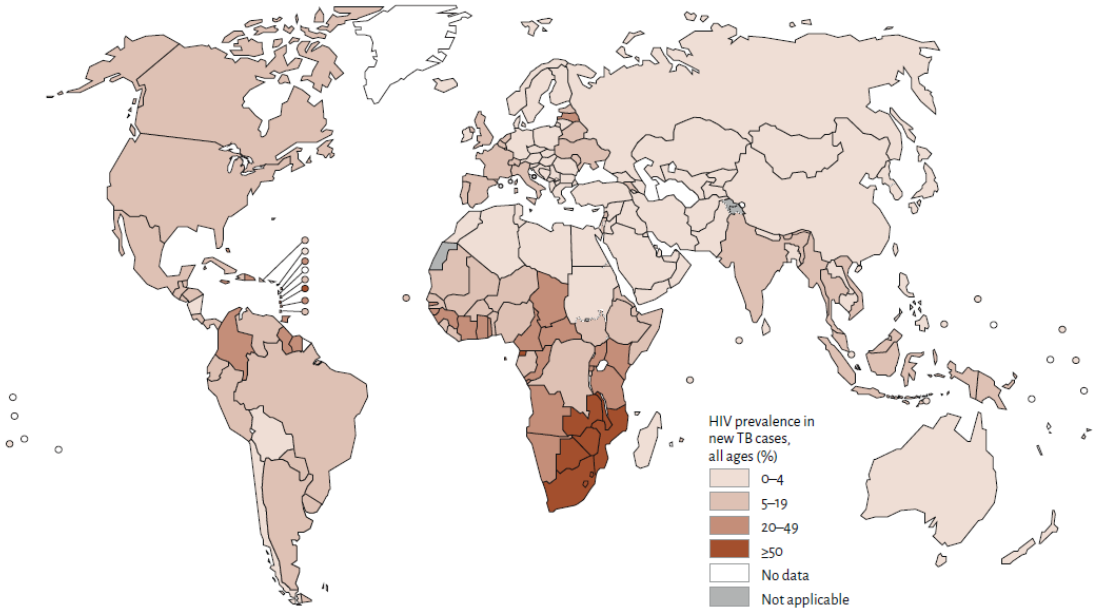


Figure 2: Estimated HIV prevalence in new and relapse cases in 2014 reproduced from the WHO Global TB report 2015

Apart from early diagnosis; early and effective TB treatment is considered to be a crucial cornerstone of TB-control. Understandably, the existing first line TB drugs entered into routine care more than two decades ago and are relatively ineffective in controlling TB as a public health problem over time. Treatment failure and relapse are reported to occur in 1-4% and 7%, respectively, of antibiotic-susceptible TB cases [6], despite the long duration of a "directly observed treatment short-course" (DOTS) treatment regimen which takes at least 6 months in the management of drug susceptible (DS TB) and up to 24 months in Mulltidrug Resistant (MDR) TB [7]. Clinical trial data of shorter regimens show that at least in DS TB, the vast majority of patients are cured within four months – relapse rates for different regimens after four months ranged from 11% to 40% depending on regimen [6]. Thus, the majority of DS TB patients are over treated with the current 6 months regimen since they would have achieved cure already earlier, but the current assessment techniques including culture do not allow reliably discriminating between those patient categories and adapting treatment [8].

Due to the long duration of therapy and associated side effects, poor adherence especially after week 8 of treatment has been known to occur worldwide [9, 10]. Additionally, the current rifampicin dose at 10mg/Kg has been reported to be suboptimal [11] favouring the development of resistance TB [12] thus warrants optimization of the current regimen considerably.

The full application of the DOTS strategy is posing more challenges particularly in the developing countries where most of the world' s TB burden is located (Figure 1)Figure 1: Estimated incidence of TB in 2014 reproduced from WHO Global TB report 2015 and which are dually battling to control the HIV epidemic (Figure 2) [13]. Consequently, poor treatment adherence is leading to the emergence of drug resistance TB in many parts of the world, with only half of MDR TB patients globally being successfully treated [7]. Next to MDR TB, virtually untreatable strains of TB have been reported [14, 15], and concerns of an epidemic of these strains of TB are being felt across the world.

1.1.1 The Burden of Tuberculosis in Tanzania

Tanzania, a country in the East African region WHO estimated a population of 51,823,000 [5], and a 2015 GDP per capita $864.9 [16] is among the 22 highest TB burden countries in the world. TB ranks third among causes of mortality in Tanzania, after malaria and HIV/AIDS. Estimates of TB incidence and prevalence originate from the first national prevalence survey of 2012/13, and are 240 and 295 per 100,000, respectively. However, following this survey, the World Health Organization (WHO) in its global TB report for 2015 has reviewed the incidence and prevalence at 324 and 528 per 100,000 respectively [5]. The numbers of notified TB cases has relatively been stable for the decade (Figure 3) [17]. In 2014, a total of 63,151 cases were noted and this was a decline of about 4% from the notification in 2013 [18]. More than two third of the notified patients in 2014 came from only 10 of the 30 Tanzanian regions (Figure 4). During the same year, about 4.1% were previously treated cases, which include among others treatment failure and relapse. These numbers also have been falling for over a decade now (Figure 5) alongside the overall incident rate, the overall treatment success for both new and relapse cases was over 90%[18] which is an increase from 73% in 1995 [5]. HIV prevalence for Tanzania mainland is estimated at 5.1% [19], however, the TB/HIV co-infection among TB patients in 2014 was reported around 36%[18].

Estimated MDR TB cases among newly diagnosed and previously treated patients is still low at about 1.1% and 3.1 respectively and treatment success among the relapsed cases was 73% in 2014 [5]. Bearing this in mind, the impact of TB is of great social and financial concerns to developed countries and a cause of untold morbidity and mortality in developing countries including Tanzania.

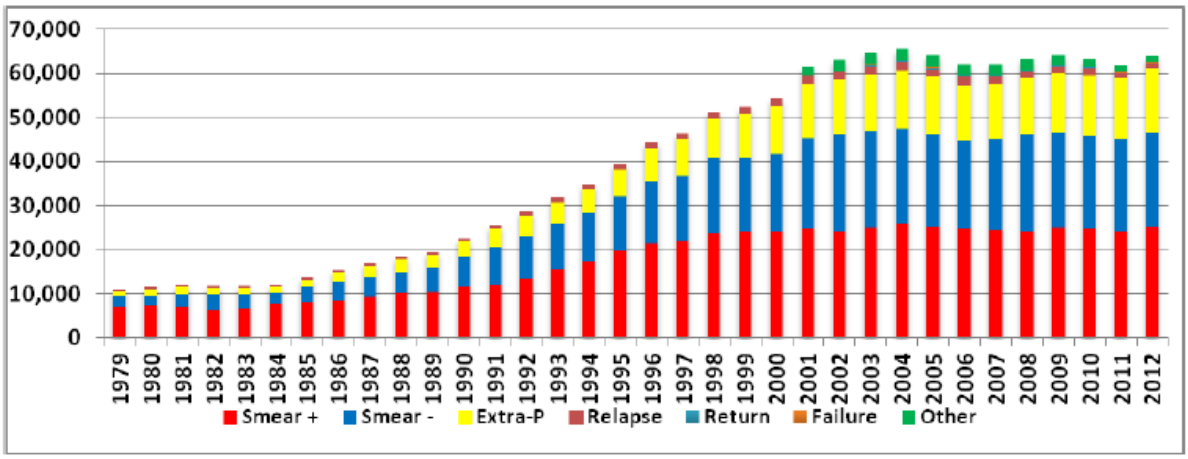


Figure 3: Trends in case notification rates of all forms 1979 to 2012 [17]

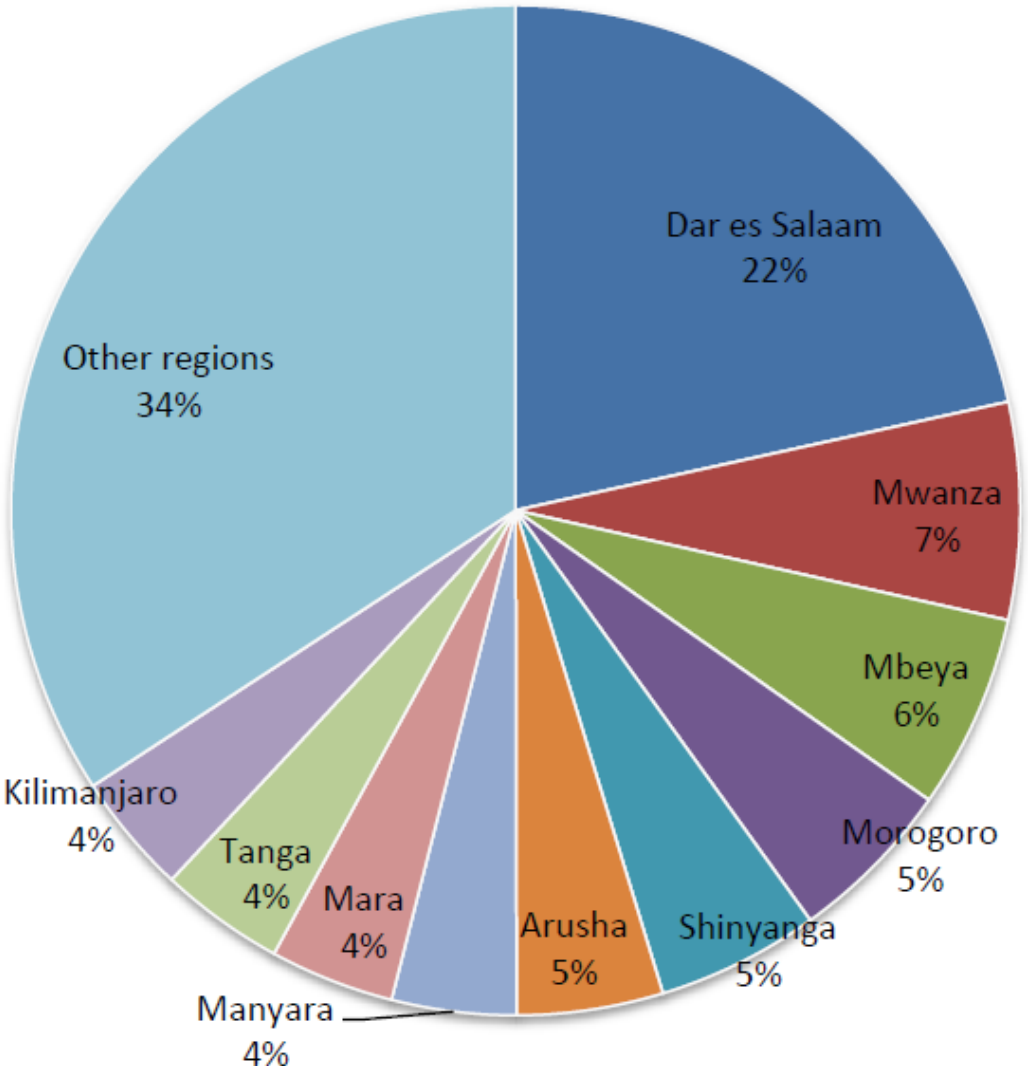


Figure 4: Distribution of TB cases notified by region in 2014 as reproduced from the NTLP Annual report 2014 [18]

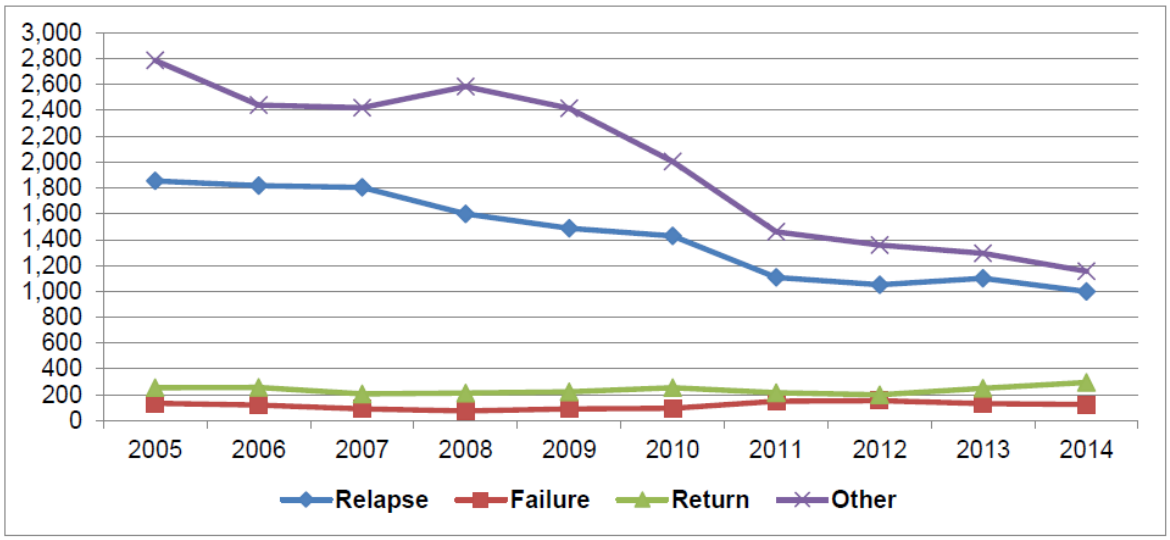


Figure 5: Trends of previously treated TB cases notified from 2005 to 2014 as reproduced from the NTLP Annual Report 2014 [18]

1.1.2 Monitoring of treatment success

As much as new drugs and shorter regimens are needed to interrupt transmission earlier and relieve the health systems, appropriate markers to reflect response to TB treatment and/or predict treatment success are urgently needed. Firstly, to tailor TB therapy according to the "needs" of individual patients to achieve cure, which probably depend on bacterial load in the lungs or other site of disease, the infecting TB strain, and the response of the host to therapy [20]. Such tailored individualized therapy could benefit in two ways: shorten un-necessary long treatment in most patients and reduce side effects due to drug toxicities; and on the other hand, reduce treatment failure and/or relapse through extending treatment in patients when required.

Further, such markers used in clinical studies of TB treatment would allow to define new study endpoints, which could be assessed more quickly than conventional culture with its long incubation period, or the clinical endpoint of lasting cure which take long observation time after treatment is completed. This would enhance the efficacy of new study designs which trigger recruitment stop to insufficiently active arms based on microbiological results, which are only available after seven (MGIT liquid media) or eight (LJ solid media) weeks of incubation. Such trials would be cheaper and allow faster evaluation of all the new TB drugs which are currently in the evaluation pipeline [21].

In summary, any attempt to effectively monitor treatment response will assist the health system to better understand patients' response to TB medication. Eventually, identification of those poorly

responding to TB therapy and therefore at risk of failing treatment and or developing drug resistance gives attending health care works an opportunity to assign the appropriate treatment [22, 23] and duration.

In the last few years, several new diagnostic assays, including those targeting the pathogen and the host have been developed and evaluated for their capacity in TB diagnosis within different clinical settings. An important candidate assay under this study is based on detection of ribosomal RNA, and it is called Molecular Bacterial Load Assay (MBLA).

Historically, the Observational of Early Bactericidal Activity (OEBA) TB was the first study in which MBLA was compared to a conventional culture endpoint. This study occurred before PANBIOME. Methods and results of this study are briefly displayed in this work for the purpose of comparison to the larger, much more comprehensive PANBIOME study.

OEBA TB was a single treatment group, unblinded, observational clinical study conducted in Tanzania at NIMR- MMR and Kilimanjaro Clinical Research Institute (KCRI), which was done in time before MAMS started in these centres. The study recruited adult males and females with newly diagnosed, uncomplicated, smear-positive, pulmonary TB, who were then hospitalized for the duration of at least 17 days and were given standard TB treatment as per the Tanzanian national guidelines. Culture and MBLA were tested from overnight sputa from NIMR-MMRC patients.

Data analysis, Culture and MBLA tests were performed as described in the publication [24] which explored the MBL Assay as a measure of quantitative bacterial load. OEBA-TB was registered in the Pan African Clinical Trials Registry (pactr.org) under PACTR201209000394102

1.2 Literature review – high-dose rifampicin, SQ109 and Moxifloxacin as new principles in treatment of tuberculosis

Effective treatment and early diagnosis of TB remain the cornerstone of TB control – these are important to prevent adverse disease outcomes to the patient, but also to interrupt disease transmission to others. Both rely on a functional TB control programme as part of the health system.

The long duration of the current standard regimen is a burden to patients and to the health system, and thus is an obstacle to effective TB control [9, 10]. A search for different new drugs and/or regimens is one of the priority aims in the STOP TB Global Plan to End TB [25]. Of great interest, is the evaluation of these candidates and/or regimen in the pipeline for their potential as treatment shortening drugs and regimen. Shortening and optimizing the treatment of DS TB is generally considered as an important

strategy for halting the continued spread of TB, since this is expected to increase adherence [26], rapid reduction in infectiousness hence transmissibility potential, and decreasing the development of new drug resistance [12].

However, clinical trials to develop new drugs or regimen for TB treatments remain complex. Preclinical animal models do not always translate well to efficacy in humans [27]. Furthermore, the example of moxifloxacin showed a discordance between encouraging results in some phase 2 studies including fluoroquinolones, with a high percentage of patients who converted to negative culture status at month 2 and shorter time to culture conversion; so advancement to phase III trials was recommended [28]. Using this evidence a trial for evaluation of Moxifloxacin in Tuberculosis (REMoxTB), the largest contemporary phase III trial, was therefore designed to shorten the standard TB therapy to four months, but this proved to be unsuccessful [26]. As shown in this pathway, the said phase 2 trial had good results on developing a phase III using a marker of treatment success, of which did not translate into the intended aim of treatment shortening. Better markers for treatment success for earlier phases of TB drug trials are therefore needed.

High – dose rifampicin

Rifampicin (RIF) inhibits the β-subunit of the DNA-dependent RNA polymerase which is a multi-subunit enzyme in *Mtb* synthesis [29, 30]. From this mechanism of action, RIF has bactericidal activity and furthermore it is reported as a sterilizing drug that continues to kill persisting bacilli during the entire treatment period [31, 32]. Under the current standard regimen, RIF is used at a dose of 10 mg/kg, which corresponds to 450-600 mg per day, has been reported in murine studies of TB treatment to be on the lower end of the active dose range [33, 34], and achieves suboptimal exposures in a large fraction of patients [11], increasing their risk to acquire drug resistance. Pharmacodynamics studies in both mice and humans with pulmonary TB disease suggested that higher RIF doses will increase efficacy [32]. An increase in RIF dose is expected to increase the exposure and peak concentration in plasma and, in turn, at other sites of action. It is hoped that such an increase in drug concentrations will enhance efficacy more than toxicity, corresponding to a concentration-response curve as depicted in Figure 6. Eventually, such an increased RIF dose could lead into an enhanced sterilizing activity and a decrease in treatment duration for pulmonary TB.

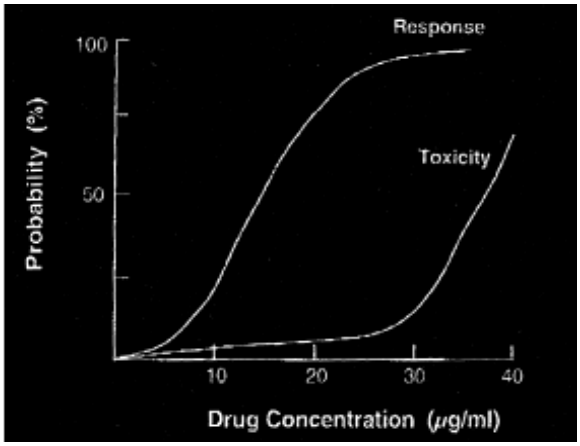


Figure 6: Pharmacodynamics: ideal concentration-response-toxicity curve, from [35]..

At least 14 clinical trials have explored the idea of rifampicin high doses with different dosages. A meta-analysis indicated a favourable outcome during culture conversion especially at doses more than 900mg per day, and it was observed to be safe and therefore, high dose rifampicin was recommended in this context [36, 37]. One trial of the PanACEA consortium had evaluated the highest RIF dosage given to date, at 35 mg/kg during 14 days - encompassing monotherapy and in combination with standard TB drugs, to evaluate the safety and tolerability, pharmacokinetics and bacterial load response. Surprisingly, even the dosage of 35mg/kg was well tolerated, all patients finished the study treatment, and there was a trend for increasing bactericidal activity across increasing doses in that study [38].

SQ109

Sequella medicinal compound 109 (SQ109) is a new agent being developed for TB treatment and thus far its efficacy in animal models have shown synergistic effect with RIF to eliminate *Mtb* alongside a decline in mycobacterial colony-forming units (CFU) in lungs and spleen of mice and prevented progression to lethal TB disease [39]. Furthermore, results form a DOTS-like treatment study substituting ethambutol with SQ109 in a mouse model indicated a more rapid decrease in mycobacterial load at the end of week eight of TB treatment. Findings from healthy volunteers showed no safety concerns [40]. Further analysis from a two-week EBA study (LMU-IPMH-SQ109-01) indicated that SQ109 alone, or in combination with RIF lacked bactericidal effect during 14 days of treatment among drug susceptible pulmonary TB patients at oral doses of up to 300 mg [41], which was not unexpected since efficacy in the mouse was obvious only at later stages of therapy, after 30 days [42]. However, alone or in combination with RIF, SQ109 was safe and well tolerated [41]. Evaluation on prolonged drug exposures beyond 2 weeks was deemed necessary in the treatment of DS pulmonary TB to assess the drug' s efficacy and possibly the treatment shortening potential.

Moxifloxacin

Most of fluoroquinolones are currently used as second line in treating MDR TB. However Moxifloxacin (MOX) which is approved for various indications worldwide, was considered sufficiently active to perform large-scale clinical trials for its potential in shortening DS TB treatment. Over time Moxifloxacin has shown to be safe [43] including in recently completed and ongoing TB drug trials at different phases. Moxifloxacin has a demonstrable bactericidal effect in animal models, early and long time exposures in humans [44].

Substituting ethambutol (EMB) with moxifloxacin yielded higher sputum culture conversion rates at week 8 [28, 45] in some phase II studies, equivocally in another trial [46]. However, the substitution of isoniazid (INH, rather than EMB) with moxifloxacin did not show significant changes in bacteriological response after 8 weeks of TB treatment [27]. In all these trials, the Moxifloxacin- containing regimen was found to be well-tolerated.

Such findings have contributed to starting the largest contemporary phase III trial in TB, the REMox TB study which evaluated two treatment-shortening regimens whereby moxifloxacin replaced EMB and INH in different arms during a four-month treatment course. The findings from the REMox trial has confirmed that the two shorter regimens with moxifloxacin led to faster culture conversion to negative. Nevertheless, these shorter arms had significantly more relapses of TB during follow-up [26], showing that this increased bacterial killing obviously was not enough to shorten therapy to four months. However, the safety of moxifloxacin and its activity against TB have paved ways for continued testing as a component of other novel TB regimens with a number of trials including the PanACEA MAMS TB 01 trial.

1.2.1 The PanACEA MAMS TB 01 trial

These three novel treatment options were available to the PanACEA consortium whereby they were combined into one study known as PanACEA MAMS TB 01 trial, to be evaluated simultaneously, while saving cost through a shared control arm, and an innovative study design. This study was designed to include several experimental arms, minimising patient numbers whilst retaining power and a level of significance commonly expected for phase 2 studies. It employed the use of the Multiple Arm, Multiple Stage (MAMS) trial design, a methodological approach previously used mostly in cancer drug trials, which allows to stop experimental arms from recruitment during the study, if they do not achieve pre-

defined efficacy thresholds. In collaboration with MRC UK, Clinical Trials Unit, the PanACEA consortium adapted upon recognizing the promise of this trial design.

1.3 Literature review – measuring tuberculosis treatment success

The current standard: sputum culture and smear

Currently, reliable biomarkers for monitoring TB treatment or predicting successful treatment of pulmonary tuberculosis do not exist.

The markers which were mostly evaluated in the past include a) sputum culture status after 2 months of treatment b) measurement of sputum bacterial load and its change over time by using Time To Positivity (TTP) in the most widely spread liquid culture system, Mycobacterial Indicator Tube (MGIT), a semi-automated incubator system. MGIT detects organism growth by a colour change in an indicator followed by oxygen consumption, and a shorter time to positivity would be associated to a higher bacillary load in the inoculum.

In summary, these markers are not able to predict the treatment outcome on an individual level with enough certainty, but are useful as efficacy endpoints in cohorts of TB drug trials [47, 48]. Sputum culture conversion after 2 months of treatment, time to sputum culture conversion to negative, and the speed of decline of bacterial load as measured by TTP, are surrogate markers of sterilizing activity [49]. This were validated against the more meaningful clinical endpoint of cure or relapse in British MRC studies, and in the REMox TB study[26, 47, 50, 51]. Follow-up of TB patients for relapse has revealed that a two-month sputum culture positivity is correlated with treatment failure or relapse, but that predictive accuracy on an individual bases is low[48, 52] with reported geographical and population variation , only at 50% sensitivity to detect a later relapse [49, 53].

Methodologically, culture is beset by overgrowth from opportunist microorganisms present in the sputum, which multiply during culture and can invalidate the results failing to ascertain study end points or treatment outcome. Further, culture does also fail to capture a population of bacteria that do not grow in culture but are viable, and most likely are associated to later relapse [32, 47, 54].

Recent studies have evaluated the time to positivity (TTP) in the MGIT culture displayed by automated culture like MGIT, which has also shown promise as a predictor of relapse [55]. TTP is a good measure of the quantity of viable *Mtb* in sputum, however this method is complicated by contamination with

other organisms. The long duration experienced by MGIT to confirm results causes additional concerns on clinical decision both in routine and trial settings..

Sputum smear microscopy is widely used at health centre levels in the first line to establish TB diagnosis but also to monitor treatment success among sputum smear positive patients in developing countries including Tanzania. The test is inexpensive, simple to perform and detects the most infectious group of patients[56, 57]. However, results are investigator dependent and well trained technicians are scarce in resource-constrained countries. Microscopy is unable to differentiate between live and dead bacilli, drug resistant strains, and can not accurately discriminate non-tuberculosis mycobacteria (NTM) from *Mtb* . Further, the rising HIV epidemic, leads to increasingly smear-negative disease patterns with about 50% smear negative patients treated for TB. As reported, its sensitivity remain low in children and HIV patients[58] a critical subpopulation of patients that merit a concern, so these patients could not be monitored by microscopy in any case.

In smear positive disease, even with an experienced microscopist, it has a low sensitivity with poor predictive value regarding treatment outcome, and thus WHO abandoned recommendation to extend treatment based on a positive two-month smear as it led to massive overtreatment [47, 59, 60] [58, 61].Therefore, these limitations potentially impede the quality and extent of its use in treatment monitoring and eventually its impact on TB control.

Molecular methods

Monitoring of Mycobacterial DNA in sputum

Endorsed by WHO in December 2010 as a new test, Xpert MTB/RIF is a rapid novel molecular test for TB diagnosis detecting DNA in sputum specimens and Rifampicin resistance[62]. As a diagnostic assay, it attains higher sensitivity than smear microscopy, thus detecting both smear-positive and negative cases[63]. The reported ability of Xpert assay to detect TB in smear negative patients further encourages exploration of this rapid test for monitoring of treatment success in this subpopulation. A multicenter study evaluated the performance of Xpert MTB/RIF as a potential test for monitoring of TB treatment outcome among sputum smear and culture positives. It was found that Xpert MTB/RIF positive rates lagged behind the conventional methods and remain high (27%) at the end of treatment (6months) compared to 3% and 4% for Solid LJ and liquid MGIT culture respectively. Therefore excludes the assay as a tool for monitoring TB treatment success [64]. Additionally, the assay has been shown to be inferior to culture on its application for monitoring early bactericidal effects of TB therapy in sputum [65].

Failure to differentiate between viable, dead and free DNA released from degraded organisms remains as the major limitation of mycobacterial DNA amplification in monitoring TB treatment, including the Xpert MTB/RIF assay.

Mycobacterial mRNA and rRNA

An alternative surrogate biomarker is mycobacterial messenger RNA (mRNA) and ribosomal RNA (rRNA), present in sputum and other body samples. Previous studies have identified *Mtb* RNA species in sputum and it is possible to quantify these [66, 67]. Of nucleic acid species, mRNA or rRNA appear to be the most promising surrogate markers of treatment response as it has been validated as a measure of viable bacteria[68],. Unlike *Mtb* DNA, RNA in sputum is likely to be cleared rapidly after initiation of tuberculosis therapy and this could provide an accurate assessment of TB treatment success on real time as a measure *Mtb* viability. The mRNA is present in small quantity, prone to degradation and unstable [67] offering challenges in its limit of detection as opposed to rRNA which is plenty and stable.

Based on this background, the Molecular Bacterial Load (MBL) Assay was developed - a real time PCR, culture free biomarker that measures TB bacterial load (BL) in patient sputum by detecting the decline of *Mtb* specific *16S rRNA* during anti-tuberculosis therapy [24, 69]. These studies have provided baseline data on *in vitro* test performance of the assay and the recent study further compared the MBL assay with solid culture during the OEBA study which was also done at the NIMR-Mbeya Medical Research Centre. During this preliminary exploration the assay showed a decline of bacterial load during initial treatment [24]. Noticeably, the assay uses cfu-based standard curve to quantify bacterial load translated as described in the methods below.

2 Rationale and Objectives

As described above, accurate, rapid, inexpensive and convenient TB markers to predict TB cure and relapse are needed for patient management and disease control particularly in high TB burden settings including Tanzania. Next to a reliable tool for monitoring TB treatment response, more potent drug combinations using current and/or new drugs with high bactericidal and sterilizing abilities are importantly needed. With this background the PanACEA MAMS TB 01 trial (explained above and below) evaluated various new drugs and regimens for their potential to shorten the current TB treatment duration.

On one hand, several new assays are currently available with variable reported potential diagnostic capabilities. However, in this study we aimed at evaluating a Molecular Bacterial Load (MBL) assay which is an RNA based assay as a marker for monitoring treatment responses among TB patients treated with different drug combinations. The PanACEA Biomarkers Expansion Programme (PANBIOME) in which the MBL Assay was nested, evaluated patients which were taken from the aforementioned MAMS trial. In this PhD work, we compared the MBL assay against the conventionally known liquid and solid culture media.

3 Methodology

This PhD was nested in multiple studies overtime and therefore various methodologies were employed respectively.

3.1 The PanACEA MAMS TB 01 study

This study contributed data that were used under the context of the PANBIOME study explained below.

3.1.1 Study design

This was a multiple-arm, multiple-stage (MAMS), phase 2, open label, randomized, controlled clinical trial in seven African sites (three from Tanzania, four from South Africa). The study compared the efficacy and safety of four experimental drug regimens with a standard control regimen in patients with smear positive, pulmonary tuberculosis (TB). The control arm included isoniazid, rifampicin standard, pyrazinamide, ethambutol (HRZE). There were four experimental arms; isoniazid (INH), RIF 35 mg/kg, pyrazinamide (PZA or Z), EMB ($HR_{35}ZE$) in which high RIF at 35mg/kg body weight was used; INH, RIF at standard dose, PZA, SQ109 300 mg (HRZQ) in which SQ109 replaced EMB; INH, RIF 20 mg/kg, PZA, SQ109 300mg ($HR_{20}ZQ$) in which SQ109 replaced EMB and RIF was increased to 20mg/kg and INH, RIF 20 mg/kg, PZA, Moxifloxacin 400 mg ($HR_{20}ZM$) whereby high RIF at 20mg/kg was used with Moxifloxacin replacing EMB.

Experimental treatment was given for 12 weeks (3 months), followed by the standard RIF and INH for up to 26 weeks (6 months). Standard treatment consisted of HRZE given for eight weeks, followed by RH to complete 26 weeks of treatment.

The primary endpoint of the MAMs trial was time to stable culture conversion to negative in liquid media. This was defined as the time from baseline to the first of two negative weekly sputum cultures (up to week 12, which was the end of experimental treatment) without an intervening positive culture in liquid media. There were several secondary end points including Time to stable culture conversion to negative on solid media (defined as two negative cultures without an intervening positive culture), and time to culture conversion (converting from positive to negative) in liquid and on solid culture media at each time in the course of treatment. Nevertheless, Mycobacteriology endpoints explored at acquisition of resistance against rifampicin, pyrazinamide, Isoniazid, ethambutol and Moxifloxacin during the treatment. Genome sequencing as a strain typing method allowing to differentiate true relapse from reinfection is planned. However these secondary endpoints are currently being examined and shall be reported beyond the scope of this work.

The study sample size was calculated using a target hazard ratio for culture conversion over control of 1.8; in order to improve upon the hazard ratio seen for Moxifloxacin in the Oflotub phase II study, which was 1.7 [28]. 124 patients in the control arm, and 62 in each experimental arm were required to detect the target hazard ratio at a power of 90% and 5% type I error likelihood. With four experimental arms, this would have resulted in 372 patients.

One to two interim analyses were planned, when 28 and 50 patients in the control arm would have reached the primary endpoint. Experimental arms would be stopped from recruitment with a hazard ratio <1.09 (first) or <1.23 (second analysis).

Patients were randomly allocated to receive the control regimen and one of the four experimental arms in the ratio of 2:1:1:1:1 respectively. All eligible patients were centrally randomized using a web-based computerized algorithm system that was developed and maintained by the clinical trials unit at the Medical Research Council (MRC). Minimization with a random element of 80% was used, stratifying on study site, baseline bacterial load reported by Xpert MTB/RIF (high vs. low), and HIV status. The minimization approach was used to balance the composition of the stated treatment arms with regards to prognostic factors.

In the course of the study, a telephonic follow up call was introduced to patients at 3 and 6 months within a time window of ±2 weeks after study completion, but at least once, in their best interest. Designated staff with an ability to ascertain on the wellbeing of the stud participants through oral information were designated to make this telephonic follow up. Participants found not clearly doing well were invited to the study site for further investigations on a possibility of experiencing TB relapse or treatment failure. Investigations made, included clinical, chest radiography and sputum culture if a sample was obtained.

3.1.2 Study population

Within the MAMs trial, subjects were enrolled once met all inclusion criteria and none of the exclusion criteria. In summary, eligibility entailed a written informed consent, being adult with at least 18 years of age, having a body weight ranging from 35 to 90 kg. Furthermore, having newly diagnosed, not previously treated pulmonary TB confirmed to be rifampicin sensitive by Xpert MTB/RIF, and having positive sputum smear microscopy of at least 1+ on the IUATLD/WHO scale ascertained in the research laboratory. HIV-infected patients with a CD4 count of more than 200 cells/mm^3 where eligible if local ethics committees accepted antiretroviral treatment being withheld until month 3 (after intensive phase) which was necessary to avoid potential of interactions with SQ109. However none of them were recruited in Tanzania, due to the national guideline recommending initiation of ART within two weeks

for HIV infected TB patients regardless of their CD4 counts. Female patients were not included in the trial if they were confirmed to be pregnant or breastfeeding.

3.1.3 Study procedures

Both early morning and spot sputum samples were collected from enrolment at baseline on a weekly basis up to 12 weeks (intensive), and monthly during the continuation phase up to week 26. In this trial, early morning samples were preferably tested by mycobacterial culture in liquid and on solid media, with the spot specimen being used as a back up. Safety assessments included safety laboratory testing at weeks 1, 2, 4, 6, 9, 12, 14 after start of therapy; and physical examinations at every visit.

3.1.4 Culture

Eligible patients attended the clinic on a weekly basis from baseline up to week 12, and there after at weeks 14, 17, 22 and 26. Sputum samples for smear and culture tests were taken on two days prior to treatment as well as during all visits in the course of treatment. The collected sputa samples were decontaminated with 2% NaLC/NaOH for 15min at room temperature and concentrated using a refrigerated centrifuge at 4°C and RCF of 3000g.500μL and 200μL aliquots of the concentrated pellet were inoculated into liquid culture, the Mycobacterial Growth Indicator Tube (MGIT) and incubated in the Bactec MGIT 960 system and on Lowenstein-Jensen (LJ) solid medium as previously reported [26]. Additionally, assessment of sensitivity profiles of Isoniazid, rifampicin and ethambutol (IRE) in a liquid culture susceptibility testing for baseline and *Mtb* positive isolates after treatment week 12 was performed following the same procedures described earlier [26] *Mtb* culture confirmation were performed through microscopic examination of the acid fast bacilli (AFB) cording after Ziehl-Neelsen (ZN) staining followed by identification of the *Mtb* antigen (MPT64) by a rapid immune chromatograph test (TB Ic). Additionally, we used blood agar plates to detect false positive cultures for *Mtb* with suspicious of bacteria and fungi contaminants. Therefore, sputum samples were considered contaminated if flagged positive on MGIT culture and showed one or more of the following: growth on blood agar plates, fungal hyphi on the confirmatory ZN stain or AFB positive on the confirmatory ZN stain, but a negative MPT64 Ag test (for all sites in Tanzania) and/or samples visibly contaminated for example with fungi. For samples without contamination, time to positivity in liquid culture (TTP) was documented in days. A culture negative result was declared if the liquid culture media remained negative up to 42 days or no growth detected on solid media agar up to 56 days of incubation at 37°C. Other study procedures and details are presented in the main MAMS paper [1]which is in press for

publication at the time of thesis submission. The PanACEA MAMS TB 01 was registered at ClinicalTrials.gov with an identifier number NCT01785186

3.1.5 Data analysis

Data from the MAMS study were analysed by the trial statisticians at the Medical Research Council (MRC) Clinical Trials Unit (CTU), using Stata 13.1 (Statacorp, College Station, Texas). A Cox proportional hazards model was used to analyse time-to-event data, resulting from time to achievement of the primary endpoint of two successive negative cultures. Results were used unadjusted for interim analysis. Results in this work and the publication are presented unadjusted, and adjusted for minimization variables (HIV status, Xpert MTB/RIF cycle threshold, centre). In addition, baseline time to positivity in liquid culture as a measure of bacterial load was used for adjustment. The proportional hazards assumption was tested using Schoelfeld residuals, with $p < 0.5$ considered evidence for non-proportionality. For patients who did not achieve the primary endpoint within 12 weeks, their time to culture conversion was censored at week 12, or at the time of study withdrawal. All analyses had been laid down in the statistical analysis plan before database lock.

Safety was analysed by displaying the proportion of adverse events by arm without statistical testing.

Patients analysed in this work are defined by a modified intention-to-treat population (mITT). This includes patients who received at least one dose of study treatment and who had evidence of RIF susceptibility on phenotypic test.

3.1.6 Ethical consideration

The PanACEA MAMS TB 01 protocol and other associated research documents were approved by the Ethics Committee of the University of Munich (LMU), Mbeya Medical Research and Ethics Committee (MMREC), IRB at the Kilimanjaro Clinical Research Institute (KCRI), Moshi, National Research and Ethics Committee at the NIMR HQ as well the Tanzania Food and Drugs Authority (TFDA), and all South African site Ethics committees and the Medicines Control Commission (MCC) as regulatory agency of South Africa. All ethical principles guiding the conduct of human research were adhered to in accordance to the Declaration of Helsinki. Written informed consent was obtained from all participants or in the case of illiterate participants, in the presence of a literate witness.

3.2 PANBIOME

3.2.1 Study design

The PanACEA Biomarkers Expansion programme (PANBIOME) study was developed as a strategy where African centres work closely with designated European academic partners (The University of St. Andrews (USTAN) in UK, University of Munich (LMU). The sites where patient recruitment took place included; NIMR-Mbeya Medical Research Centre (NIMR-MMRC), Kilimanjaro Clinical Research Institute (KCRI) in Tanzania; Insituto Nacional de Saude (INS) Maputo, Mozambique and College of Medicine University of Malawi, Blantyre Malawi. Among other objectives, PANBIOME explored the potential of the Molecular Bacterial Load (MBLA to replace conventional culture and for concordance with culture to predict differences between treatment regimens.

3.2.2 Study population

Analysis for PANBIOME was based on a total of 100 patients who were enrolled in the MAMS trial at two Tanzanian sites namely NIMR-MMRC and KCRI. These patients had either a sputum smear or Xpert MTB/RIF positive with a HIV negative result (refer the explanation above on MAMs trial in Tanzania). Patients were randomised to receive either the standard or one of the four experimental arms as described above. While usually early morning sputum samples were used for MAMS efficacy measurements using liquid culture in MGIT, spot samples were used for molecular assessment using the MBL assay. Spot sputa samples were homogenized using a magnetic stirrer for 30 minutes at room temperature and mixed with 4M Guanidine thiocyanate (GTC) using the 1:5 ratio of sputum GTC volume. The GTC used was prior been mixed with 1% of β-mercaptoethanol to preserve RNA. Resulting sputum-GTC suspension were aliquoted into 2mL aliquots and stored at -80°C until the day of RNA extraction for MBL assay

3.2.3 Culture

Only samples from the two Tanzanian sites (NIMR-MMRC and KCRI) was used and the procedures employed are explained in section 3.1.4

3.2.4 Molecular Bacterial Load Assay (MBLA) performance

Preserved sputum samples were allowed to thaw at room temperature for 1hr and aliquots pooled together into a sterile and RNA free falcon tube to obtain the 5mL sputum volume in GTC. Same internal

control used for PANBIOME study of 100µl of *Mycobacterium marinum* at 10^4 CFU/m was spiked to each falcon tube containing sputum-GTC samples prior to RNA extraction. The mixture was centrifuged at 3000g for 30 min and the cell sediment were re-suspended in lysis buffer, the RNA pro blue solution (MP Biomedicals IIIkrich, France) and bead homogenized for 40sec at 6000rpm using the FASTPrep instrument. RNA was extracted using FASTprep RNA kit (MP Biomedicals, IIIkrich, France) according to the manufacturer's instructions. DNAase treatment at 37°C for 1hr was performed to remove the Genomic DNA from RNA extracts using the Ambion Turbo DNase kit (Life Technologies, Lithuana).

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was performed using a RotorGene 5plex platform (Qiagen) using primers and dual labelled probes (Taqman) targeting 16S rRNA specific for Mtb and transfer messenger RNA (tmRNA) for the internal control of *M. marinum*. All primers and probes were procured from MWG Eurofins, Munich, Germany and lyophilized following the manufacturer's instructions. The optimal RT-PCR cycling conditions were 30 min at 50°C for reverse transcription, 15min at 95°C for denaturation, and 40x cycling at 94°C for 45sec and finalized with 60°C for 60sec. The cycle threshold (CT) was translated into bacterial load (BL) using cfu based standard curve calculated from samples performed in the same run or separately but on the same machine and imported into the analysis after the reaction. Therefore, it uses the cfu based standard curve to translate CT generated by PCR into bacterial load. In this case, The study adapted similar positive control BCG standards (BCG NCTC 5692) used in the PANBIOME project for the high positive (10^7 cfu/mL) and low positive (10^3 cfu/mL) which were spiked in artificial sputum following similar procedure used for patient samples prior to RNA extraction. RNase free molecular grade water was used as negative control and included in each assay run.

3.2.5 Data Analysis

Data from PanBIOME was analysed using Stata 14.1 (Statacorp, College Station, Texas). For each visit, a maximum of one MBLA result was available, while there may have been more than one culture results available due to repeated inoculation after finding a culture contaminated, or multiple cultures ordered by the investigators.

For comparisons of positive and negative results, a negative MBLA result was assumed when the bacterial load result from MBLA was zero, and a positive result if it was higher than zero.

For quantitative comparisons between MGIT TTP and MBLA as measures of bacterial load, TTP was log(10) – transformed, since log(10)TTP had previously been found to be normally distributed, while TTP

was not [41]. For these comparisons, TTPs found in contaminated cultures defined as having a positive blood agar plate after 48h incubation were censored since these were assumed to have been influenced by the contaminant. Also, TTPs of negative MGIT cultures (42 days) and negative MBL results were censored. The degree of correlation was assessed using the Spearman rank test.

Cox proportional hazards analysis was used for the time to culture conversion, or MBL conversion to negative, respectively. The culture or MBL conversion endpoint was defined in analogy to the MAMS study as the time to the first of two successive negative results from two weekly visits without an intervening positive or contaminated result. Unlike the MAMS main analysis where patients were censored at 12 weeks if they did not achieve the primary endpoint since this was the end of experimental treatment, in this analysis we censored patients at 26 weeks, and a value of 26 weeks was assumed.

4 Results

4.1 The PanACEA MAMS TB 01 study

4.1.1 Study population

From May 2013 to March 2014, seven African sites screened a total of 632 patients, from which 365 were allocated to different arms through randomization. The CONSORT flowchart for recruitment and retention is included as Figure 7.

The two Tanzanian sites namely; NIMR-Mbeya Medical Research Centre and Kilimanjaro Clinical Research Institute, which later produced MBL data, contributed in the MAMS 52 and 51 patients respectively [1].

The first interim analysis was conducted in February 2014, and resulted in stop of recruitment to both arms containing SQ109, reducing the total sample size.

Patients across all arms had similar baseline characteristics for prognostic factors as seen in *Table 1*, and baseline drug resistance was not much different across groups (Table 2).

Two patients were excluded from the modified ITT analysis population due to phenotypic RIF resistance, which was diagnosed after treatment had started.

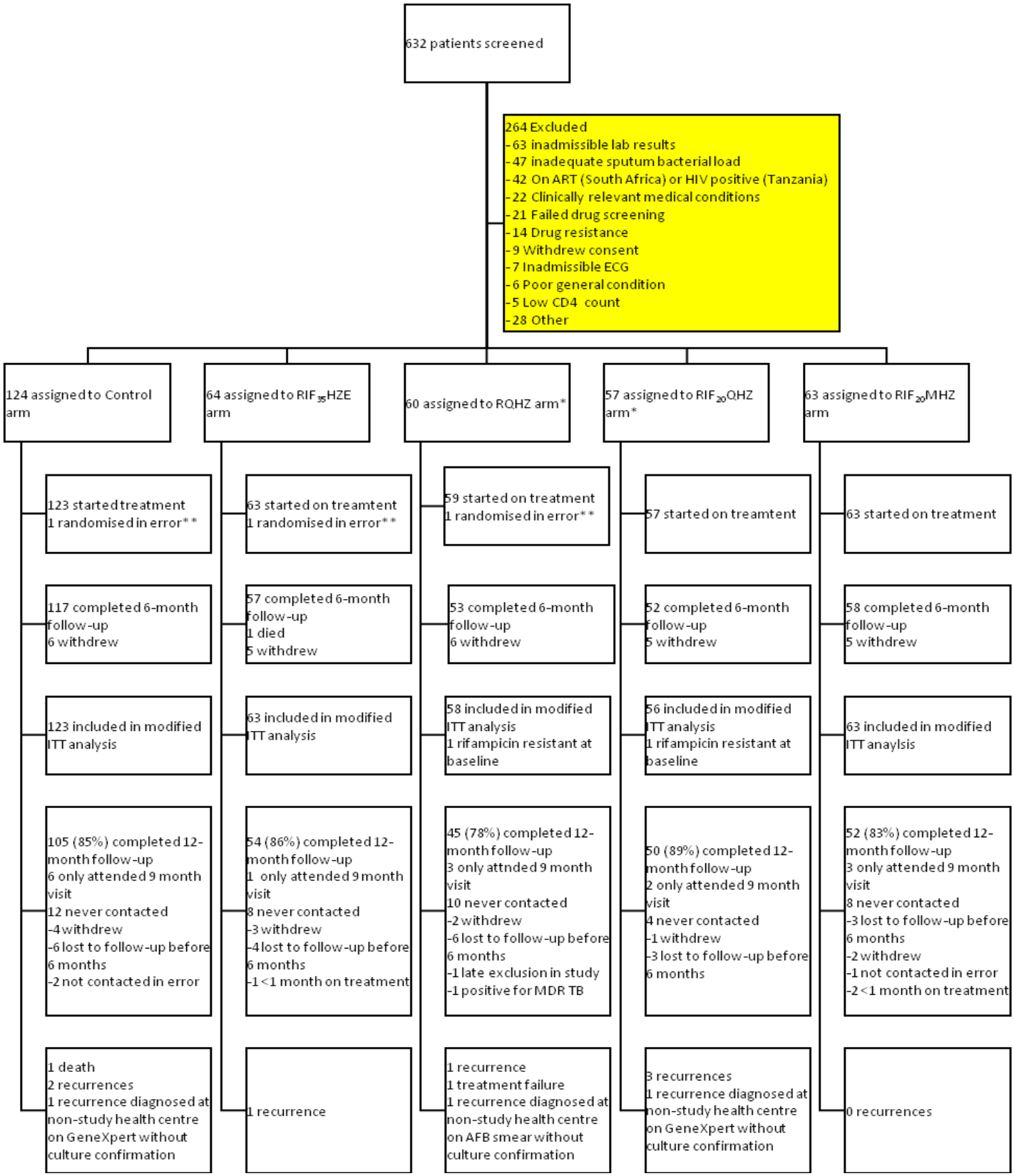


Figure 7: the CONSORT flowchart of screening, follow-up and patient retention in the study reproduced from [1]

Characteristics	Control	$RIF_{35}HZE$	RIFQHZ	$RIF_{20}QHZ$	$RIF_{20}MHZ$	Total
Total randomised*	**123**	**63**	**59**	**57**	**63**	**365**
Age (yrs) - median (IQR)	34 (26 to 41)	33 (23 to 40)	32 (25 to 40)	34 (27 to 41)	31 (24 to 38)	33 (26 to 40)
Male	94 (76%)	42 (67%)	38 (64%)	45 (79%)	39 (62%)	258 (71%)
Weight (Kg) - median (IQR)	54 (49 to 59)	52 (47 to 58)	53 (47 to 57)	53 (49 to 56)	52 (48 to 61)	53 (49-58)
HIV Positive	9 (7%)	4 (6%)	5 (8%)	3 (5%)	3 (5%)	24 (7%)
Ethnicity						
Black	101 (82%)	51 (81%)	50 (85%)	50 (88%)	48 (76%)	300 (82%)
White	0 (0%)	1 (2%)	0 (0%)	1 (2%)	0 (0%)	2 (1%)
Mixed	19 (15%)	11 (17%)	9 (15%)	6 (11%)	15 (24%)	60 (16%)
Other	3 (2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (1%)
Xpert MTB/RIF cycle threshold - median (IQR)	16 (14 to 19)	17 (14 to 20)	17 (14 to 19)	16 (14 to 18)	16 (14 to 19)	16 (14 to 19)

*Table 1: Baseline characteristics of MAMS patients. * An additional 3 patients were randomised in error and did not start treatment or remain in follow-up and are therefore not included in this table.*

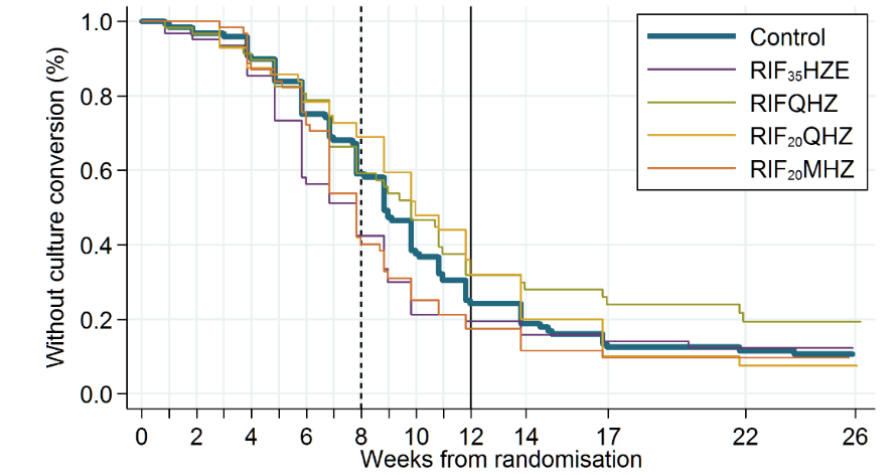
Sensitivity testing	Control	$RIF_{35}HZE$	RIFQHZ	$RIF_{20}QHZ$	$RIF_{20}MHZ$	Total
Phenotypic resistance to rifampicin						
Resistant	0 (0%)	0 (0%)	1 (2%)	1 (2%)	0 (0%)	2 (1%)
Sensitive	112 (91%)	59 (94%)	54 (92%)	53 (93%)	56 (89%)	334 (92%)
Missing	11 (9%)	4 (6%)	4 (7%)	3 (5%)	7 (11%)	29 (8%)
Phenotypic resistance to INH						
Resistant	3 (2%)	0 (0%)	3 (5%)	1 (2%)	1 (2%)	8 (2%)
Sensitive	109 (89%)	59 (94%)	52 (88%)	53 (93%)	55 (87%)	328 (90%)
Missing	11 (9%)	4 (6%)	4 (7%)	3 (5%)	7 (11%)	29 (8%)
Phenotypic resistance to Moxifloxacin						
Resistant	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Sensitive	115 (93%)	59 (94%)	55 (93%)	54 (95%)	56 (89%)	339 (93%)
Missing	8 (7%)	4 (6%)	4 (7%)	3 (5%)	7 (11%)	26 (7%)
Phenotypic resistance to ethambutol						
Resistant	0 (0%)	0 (0%)	0 (0%)	1 (2%)	0 (0%)	1 (0%)
Sensitive	112 (91%)	59 (94%)	55 (93%)	53 (93%)	56 (89%)	335 (92%)
Missing	11 (9%)	4 (6%)	4 (7%)	3 (5%)	7 (11%)	29 (8%)
Phenotypic resistance to pyrazinamide						
Resistant	4 (3%)	2 (3%)	2 (3%)	0 (0%)	4 (6%)	12 (3%)
Sensitive	110 (89%)	57 (90%)	53 (90%)	55 (96%)	53 (84%)	328 (90%)
Missing	9 (7%)	4 (6%)	4 (7%)	2 (4%)	6 (10%)	25 (7%)

Table 2: Baseline drug resistance of MAMS patients

4.2 Efficacy of experimental arms

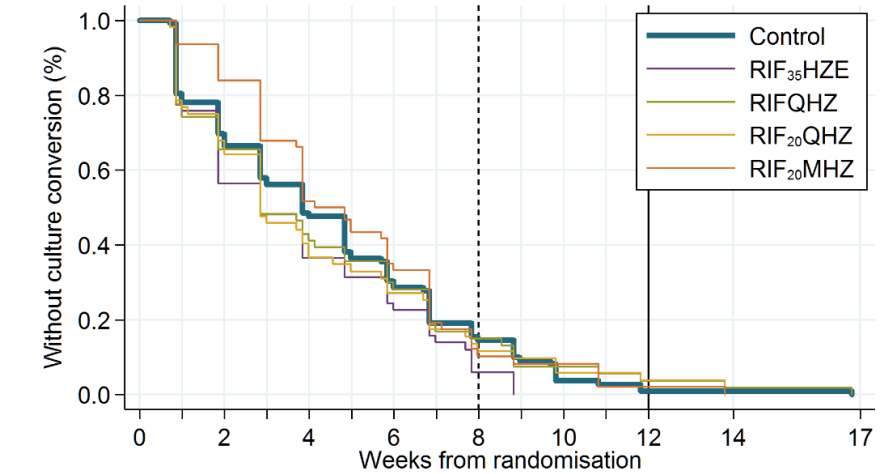
4.2.1 Monitoring of treatment response through culture based methods

A



At risk						
Control	123	105	66	27	14	13
$RIF_{35}HZE$	63	50	24	11	8	7
RIFQHZ	58	51	33	17	12	8
$RIF_{20}QHZ$	56	49	36	16	5	3
$RIF_{20}MHZ$	63	54	22	9	5	5

B



At risk							
Control	123	79	56	33	16	1	0
$RIF_{35}HZE$	63	35	21	13	3	0	0
RIFQHZ	58	38	23	15	8	2	0
$RIF_{20}QHZ$	56	35	20	14	6	2	0
$RIF_{20}MHZ$	63	52	32	19	5	1	0

Figure 8: Kaplan Meier curve for time to culture conversion in liquid MGIT media (A) and on solid Lowenstein–Jensen (LJ) media (B) as per mITT. Reproduced from [1]

In Kaplan Meier analysis, patients in arms $RIF_{35}HZE$ and $RIF_{20}MHZ$ achieved more rapid culture conversion when MGIT liquid media was used as the endpoint (Figure 8A). On solid media, differences between arms were less pronounced. (Figure 8B)

	Control	**$RIF_{35}HZE$**	**RIFQHZ**	**$RIF_{20}QHZ$**	**$RIF_{20}MHZ$**	**Total**
Total in analysis (mITT)	123	63	58	56	63	363
Number of patients achieving culture conversion definition by end of study (26 weeks)						
MGIT liquid media	101 (82%)	51 (81%)	44 (76%)	48 (86%)	52 (83%)	296 (82%)
LJ solid media	117 (95%)	59 (94%)	59 (97%)	54 (96%)	59 (94%)	345 (95%)
Primary analysis to 12 weeks (MGIT culture)						
Cumulative probability of culture conversion	70.1%	79.9%	65.2%	58.6%	78.7%	
Time to conversion: median (IQR)	62 (41-83)	48 (34-69)	63 (48-83)	66 (41-83)	55 (41-69)	
Adjusted hazard ratio (95% CI)*, p for difference from control		1.78 (1.22, 2.58) p=0.003	0.85 (0.57, 1.27) p=0.42	0.76 (0.50, 1.17) p=0.21	1.42 (0.98, 2.05) p=0.07	
Hazard ratio (95%), unadjusted		1.46 (1.02, 2.11)	0.90 (0.60, 1.34)	0.76 (0.50, 1.16)	1.34 (0.93, 1.93)	
		p=0.04	p=0.60	p=0.21	p=0.12	
Solid LJ culture to 12 weeks (secondary)						
Cumulative probability of culture conversion by 12 weeks	97.3%	100.0%	94.4%	94.2%	98.0%	
Median time to culture conversion (25^{th}-75^{th} centiles)	27 (13-48)	20 (7-41)	20 (7-48)	20 (11-44)	29 (20-48)	
Adjusted hazard ratio (95%)*		1.23 (0.89, 1.69)	0.91 (0.66, 1.27)	0.98 (0.70, 1.38)	0.77 (0.56, 1.06)	
		p=0.21	p=0.58	p=0.93	p=0.11	
Hazard ratio (95%), unadjusted		1.28 (0.93, 1.75)	1.02 (0.73, 1.41)	1.06 (0.76, 1.47)	0.90 (0.65, 1.23)	
		p=0.13	p=0.92	p=0.74	p=0.50	

Patients in arm $RIF_{35}HZE$ converted to negative culture earlier compared to those in the control arm in liquid MGIT media when analysed up to week 12, which was the primary study endpoint. A median time

to stable culture conversion of 48 (34-69) days was recorded in $RIF_{35}HZE$ as opposed to 62 days for the control (41-83).

In Cox proportional hazards analysis, the unadjusted hazard ratio for $RIF_{35}HZE$ compared to control was 1·46, 95% CI (1·02, 2·11), p=0·04 (***Error! Reference source not found.***). Adjustment was done for site, baseline bacterial load, GeneXpert cycle threshold and HIV status, which were thought to be important prognostic markers. With the exception of baseline bacterial load which was not available at randomization, these were used for balancing groups at randomization in the minimization algorithm employed. This yielded an adjusted hazard ratio of 1·78, 95% CI (1·22, 2·58), p=0·003. The other experimental arms RIFQHZ, $RIF_{20}QHZ$ and $RIF_{20}MHZ$ did not show significant difference in time to culture conversion in liquid media until week 12. However, the $RIF_{20}MHZ$ arm nearly reached significance with the largest effect (adjusted hazard ratio 1·42, 95% CI (0·98, 2·05), p=0·07). In the $RIF_{35}HZE$ arm, the highest proportion (79.9%) of patients achieved a conversion to negative culture of any arm, with arm $RIF_{20}QHZ$ having the smallest proportion (58·6%), and control registering third with 70.1% of patients achieving the primary endpoint.

There was no significant difference in time to stable culture conversion on solid LJ media between any of the experimental arms and the control group when data were censored at any time points including week 8 and 12 and this was the secondary end point within the MAMs trial.

	Control	**$RIF_{35}HZE$**	**RIFQHZ**	**$RIF_{20}QHZ$**	**$RIF_{20}MHZ$**	Total
Total in safety analysis	123	63	59	57	63	365
Patients with at least one AE	92 (75%)	53 (84%)	49 (83%)	42 (74%)	49 (78%)	285 (78%)
Patients with at least one Grade 3,4 or 5 AE	13 (11%)	9 (14%)	7 (12%)	7 (12%)	9 (14%)	45 (12%)
Patients with at least one Grade 3,4 or 5 AE considered probably related or related	1 (1%)	3 (5%)	0	0	4 (6%)	8 (2%)
Patients with at least one Serious AE	6 (5%)	4 (6%)	4 (7%)	5 (9%)	4 (6%)	23 (6%)
Deaths	0	1	0	0	0	1
Total number of patients with treatment changed due to hepatic AE	2 (2%)	5 (8%)	0 (0%)	3 (5%)	0	10 (3%)
Number of patients with treatment changed due to hepatic AE –symptomatic or meeting protocol criteria*	2 (2%)	3 (5%)	0 (0%)	3 (5%)	0	8 (2%)
Treatment changed due to hepatic AE – not fulfilling protocol criteria and not being symptomatic*	0	2 (3%)	0	0	0	2 (1%)

*Table 3: Summary of safety profiles of participants in relation to adverse events as reproduced from [1] *Protocol criteria for treatment interruption due to hepatic AE: elevation of AST and/or ALT >3x, but less than 5x the upper limit of normal WITH associated symptoms or elevation of AST and/or ALT >5x the upper limit of normal irrespective of the presence of symptoms.*

A total of 365 patients who took investigational medicinal drugs were included in the safety analysis from which only 12% (45/365) experienced adverse event (AE) of a severity grade of at least grade 3, 4 or 5, graded in CTCAE 4.0 . Patients in the control and experimental arms had relatively similar proportions of reported adverse events (Table 3). One patient died 14 weeks after completion of experimental treatment ($RIF_{35}HZE$), shortly before continuation treatment was completed, with unexpected onset of chest pain just before death. A second patient successfully completed the standard

TB medication however died during the follow up period after developing a relapse. The patient had an underlying pneumoconiosis that was related to the reported mining activities in Tanzania. Protocol criteria for treatment interruption due to hepatic AE was reached by eight patients during the experimental period (Table 3).

4.3 Validation of the MBLA against classical culture methods in PANBIOME, a substudy of PanACEA MAMS TB 01

4.3.1 Population included in the PANBIOME study

This study utilized data from a subset of patients who were enrolled into the MAMS trial, at the National Institute of Medical Research-Mbeya Medical Research Centre (NIMR-MMRC) and Kilimanjaro Clinical Research Institute (KCRI) in Tanzania from May 2013 to March 2014. NIMR-MMRC and KCRI contributed 52 and 51 patients respectively into the MAMS trial.

In addition to the early morning sputum sample submitted for the MAMS primary endpoint assessment, a spot sputum sample was preserved for later performance of MBLA. Results of this test were then compared with culture results from the MAMS database.

Altogether, there were results of 1,597 MBLA tests, 2,101 MGIT cultures and 2,082 LJ cultures, available.

4.3.2 Comparisons of positive and negative results among different tests

The three methods were compared for their categorized positive, negative, and contaminated results on each visit. Visits with a positive LJ had negative MBL results only in 9.1%. Conversely, In visits with a negative LJ result however, MBL was positive in 47.4 % , a much higher percentage (Table 5) . This means that more patients are declared free of TB disease if LJ is used as tool for monitoring TB treatment success.

		MGIT Liquid medium				
		Negative	**Positive**	**Contamination**	**Missing**	**Total**
MBLA	**Negative**	260	63	423	30	776
	Positive	174	690	350	59	1,273
	Missing	46	38	57	2	143
	Total	480	791	830	91	2,192

Table 4: Comparison between MBLA and MGIT media: qualitative negative/positive results. Since there may be more than one culture result per visit, the MBLA result may have used for comparison to culture more than once.

		LJ Solid medium				
		Negative	**Positive**	**Contamination**	**Missing**	**Total**
MBLA	Negative	668	48	33	0	749
	Positive	695	454	52	2	1,203
	Missing	102	24	6	0	132
	Total	1,465	526	91	2	2084

Table 5: Comparison between MBLA and LJ media: qualitative negative/positive results. Since there may be more than one culture result per visit, the MBLA result may have used for comparison to culture more than once.

In 7.9 % of visits with positive MGIT cultures, a negative MBLA was seen; as opposed to 36.2 % of visits with negative MGIT cultures, which had a positive MBLA result (Table 4).

Of the 91 contaminated samples on LJ solid media with no evaluable end points, the MBLA reported 33/85 (36.3 %) as being negative. Whereas MBLA showed 50.9 % negative results among the contaminated samples using liquid culture medium (Table 4, Table 5).

4.3.3 Positive/negative results of MGIT, LJ and MBL over time

MGIT final results	**Visit (weeks of treatment)**						
	0	**2**	**4**	**8**	**12**	**17**	**26**
Negative (%)	0 (0)	5 (4.0)	9 (7.2)	37 (28.6)	42 (32.8)	50 (36.7)	75 (43.1)
Positive (%)	103 (97.1)	96 (76.8)	76 (60.8)	39 (30.2)	15 (11.7)	7 (5.1)	3 (1.7)
Contamination (%%)	2 (1.8)	21 (16.8)	33 (26.4)	43 (33.3)	67 (52.3)	79 (61.7)	93 (53.4)
Missing (%)	1 (0.9)	3 (2.4)	7 (5.6)	10 (7.7)	4 (3.1)	0 (0)	3 (7.4)
Total	106	125	125	129	128	136	174

Table 6: Qualitative liquid MGIT media final results per visit from baseline to the end of treatment. Note: contaminated samples may have been re-treated and re-inoculated; final results may be not contaminated. Due to this, there may be more than one result per sample, patient and visit.

LJ final results	Visits (weeks of treatment)						
	0	**2**	**4**	**8**	**12**	**17**	**26**
Negative (%)	27 (25.9)	38 (32.2)	54 (45.0)	99 (84.6)	105 (88.9)	105 (90.5)	152 (92.6)
Positive (%)	71 (68.2)	79 (66.9)	62 (51.6)	13 (11.1)	4 (3.3)	4 (3.6)	2 (1.2)
Contamination (%)	6 (5.8)	1 (0.8)	4 (3.3)	5 (4.3)	9 (7.6)	7 (6.0)	10 (6.1)
Total	104	118	120	117	118	116	164

Table 7: Qualitative solid LJ media results from baseline to end of treatment. Note: contaminated samples may have been re-treated and re-inoculated; final results may be not contaminated. Due to this, there may be more than one result per sample, patient and visit.

MBLA Result	Visits (weeks)						
	0	**2**	**4**	**8**	**12**	**17**	**26**
Negative (%)	0	4 (4.2)	13 (13.3)	32 (33.7)	61 (64.2)	54 (62.1)	68 (80.9)
Positive (%)	94 (100)	91 (95.8)	85 (86.7)	63 (66.3)	34 (35.8)	33 (37.9)	16 (19.1)
Total	94	95	98	95	95	87	84

Table 8: Qualitative MBLA final results from baseline to the end of treatment. Missing results were not due to assay failure but due to missing sample.

Overall negative readouts increased with time of treatment and across the three evaluated methods (Table 6, Table 7 and Table 8, Figure 9).

The MGIT contamination rates at both sites rose from 1.9% at baseline to more than a half (53.4%) at week 26 (Table 6). Contamination rate in LJ was lower and did not exceed 10% of all results at any given time.

Notably, the molecular bacterial load assay (MBLA) was not affected by contamination, and no assay inhibition was noted; there was no any invalid/error results reported in the MBLA (

Table 8), hence no missing data due to assay problems. Missing MBLA results were solely due to missing sample.

LJ culture turned negative much earlier in treatment than MGIT culture. Even at baseline, 25.9% of LJ cultures were negative, while no negative MGIT cultures were seen at that time point, indicating a relatively low bacterial load in the study population which was picked up by the more sensitive MGIT culture only.

It was noted that especially late in treatment, MBLA was more frequently positive, compared to the culture methods as treatment progressed (examplified by 19.0% for MBLA, 1.7% for MGIT media and 1.2% for LJ at the end of treatment (Table 6, Table 8, Figure 9).

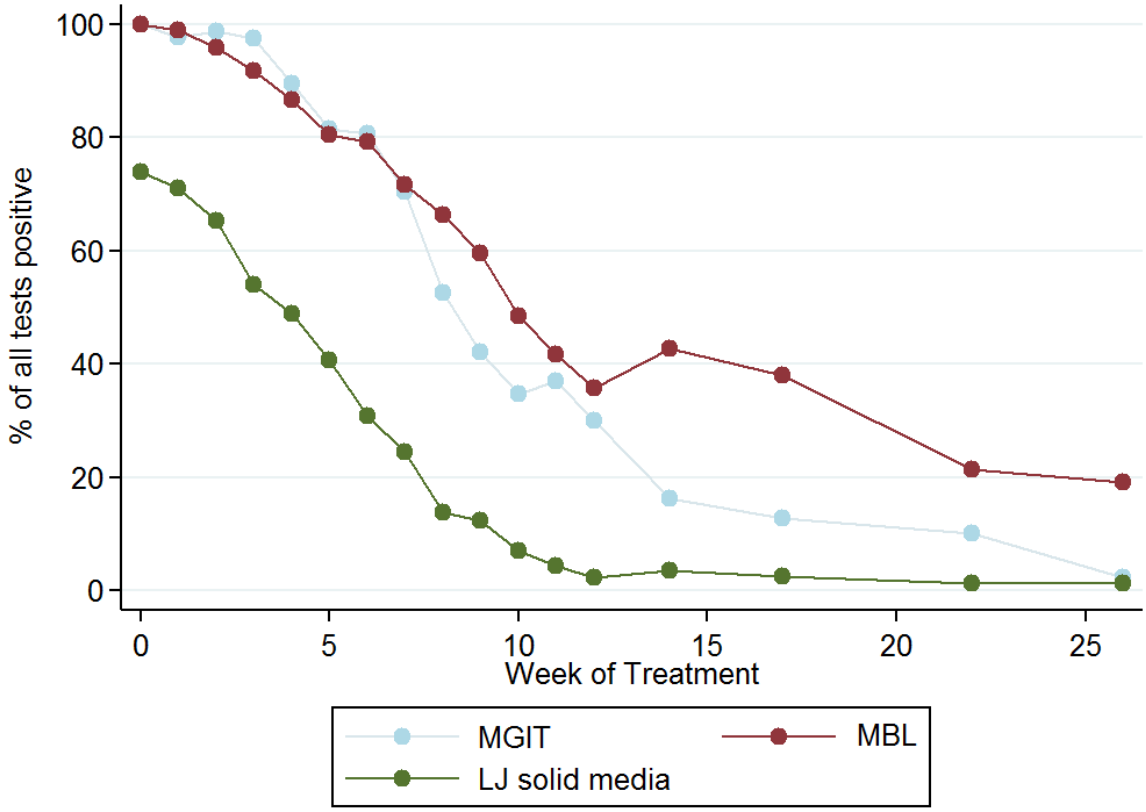


Figure 9: percent of positive tests of all tests with valid results over treatment. Contaminated cultures and missing results are excluded.

4.3.4 Comparison of quantitative bacterial load measured by MBLA and MGIT culture

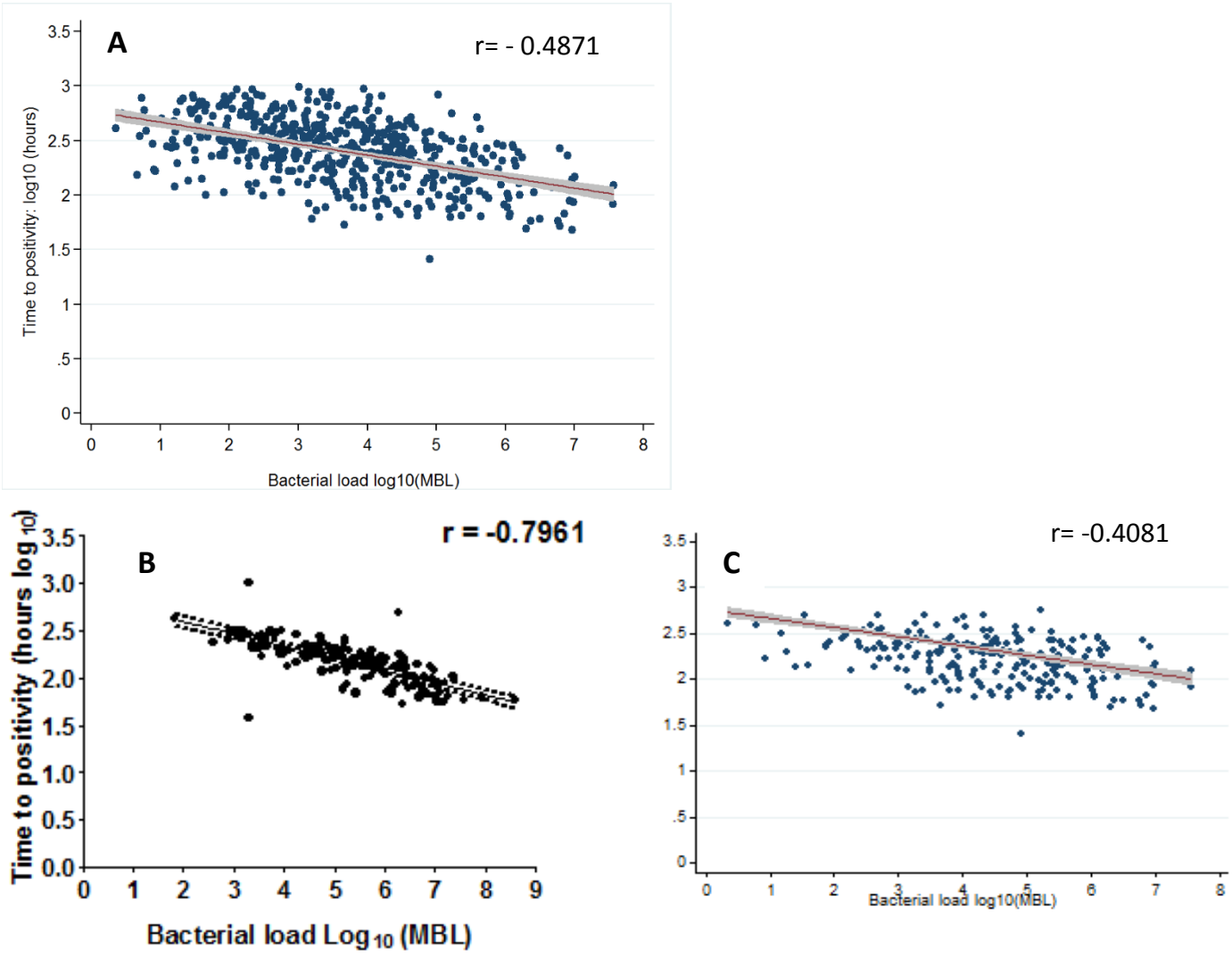


Figure 10: Scatterplot of correlation between MGIT log10 time to positivity (TTP) and MBL.
A: PANBIOME study, entire study period.
B: OEBA, reproduced from Honeyborne et al, 2014;
C: PANBIOME study, data from up to day 14 for comparison to OEBA dataset. R= Spearman rho to describe the degree of correlation.

A direct comparison between log10 time to positivity from the MGIT system, and quantification of the bacterial load by MBLA was performed. For this, all visits were censored that either had negative or missing MBL results, or negative, missing or contaminated MGIT results, since in the latter case the time to positivity in the MGIT culture would not have been a reflection of MTB multiplication and metabolism, but much abbreviated by the contaminating organism and its consumption of oxygen.

This analysis shows a significant correlation ($p<0.001$) between both quantitative readouts, although the correlation is not very strong (**Error! Reference source not found.** A).

Next, a direct comparison between the published dataset from the OEBA study [24], which showed a stronger correlation between the two quantitative readouts (**Error! Reference source not found.** B), and the dataset from this study (PANBIOME) was performed. For this, all PANBIOME visits that occurred after day 14 were censored, to match OEBA sampling which had been done only up to day 14 of treatment (**Error! Reference source not found.** C). This confirmed that the correlation between both tests' quantitative readouts as measured by Spearman rank test was significant in both studies, but less strong in PANBIOME (r = -0.4081 compared to r=-0.7961 in OEBA).

The decrease of bacterial load measured by MBLA is similar to that in liquid MGIT culture presented by the noted increase in time to positivity (TTP). Over the period of six months of treatment, the mean bacterial load declined from 5.12 $\pm$ 1.3$\log_{10}$CFU/ml (mean ± standard deviation) at baseline, to 2.65 $\pm$ 1.6$\log_{10}$CFU/ml at week 8 (months 2) and 0.80 $\pm$ 1.2$\log_{10}$CFU/ml at week 12 and 0.34 $\pm$ 0.74 $\log_{10}$CFU/ml (mean ± standard deviation) at week 26 (month six). Similarly, TTP as bacterial load measurement of liquid MGIT culture increased from 5 $\pm$ 3 days at baseline to 17 $\pm$ 11 days, 28 $\pm$ 16 days and 32 $\pm$ 15 by week 8, 12 and 26 of the corresponding duration of treatment, respectively – in this analysis, a negative MGIT culture had a TTP of 42 days assigned.

4.3.5 Comparison between culture-based and MBLA-based time to negativity endpoints

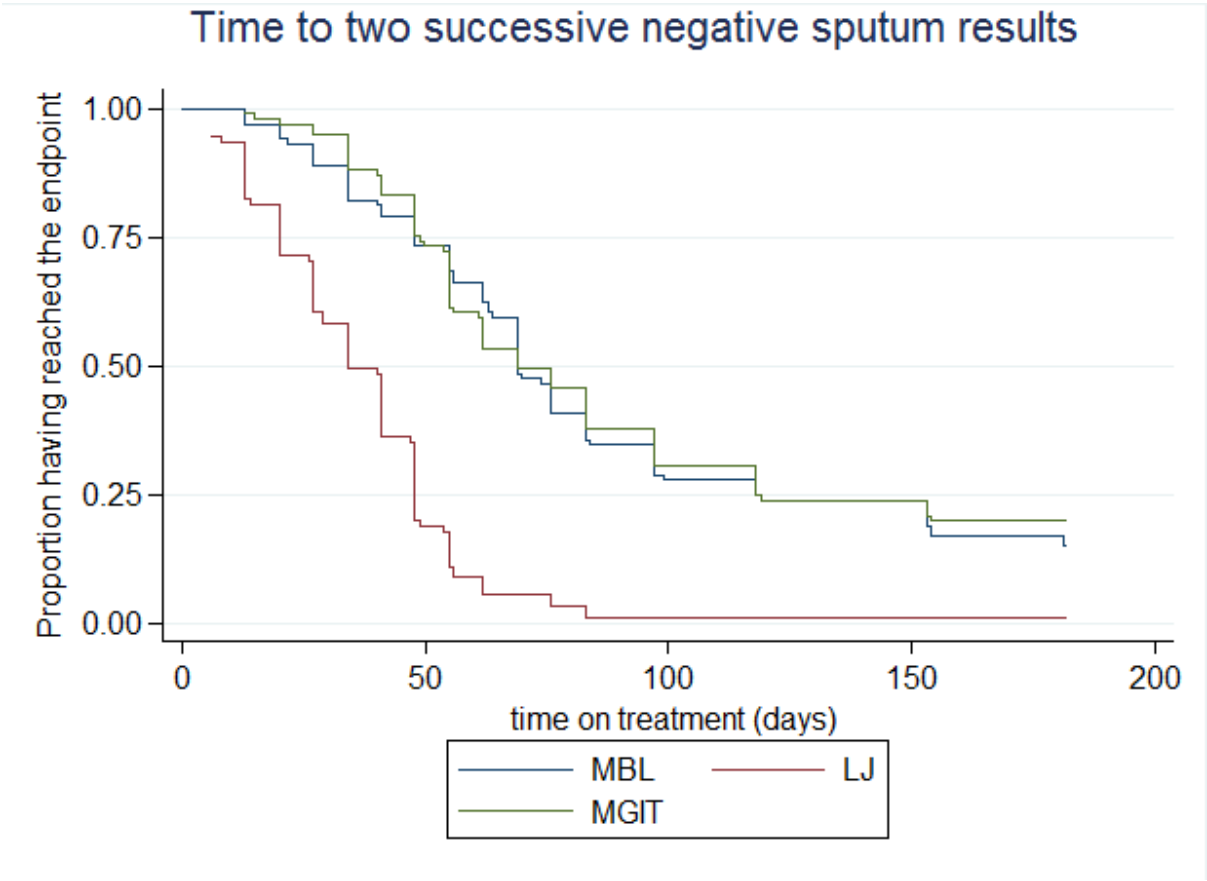


Figure 11: Kaplan-Meier curve comparing time to conversion to negative, defined as two successive negative results without an intervening positive or missing result, between all three methods, patients of all treatment arms combined.

	Control	$RIF_{35}HZE$	RIFQHZ	$RIF_{20}QHZ$	$RIF_{20}MHZ$
A: Hazard ratios using MGIT culture at week 12					
Unadjusted hazard ratio		1.90	1.24	1.61	1.71
(95% CI)		(1.00 - 3.61)	(0.63 - 2.45)	(0.84 - 3.10)	(0.89 - 3.29)
p for difference from control		0.049	0.538	0.154	0.106
B: Hazard ratios using MBLA at week 12					
Unadjusted hazard ratio		1.30	1.19	1.23	2.24
(95% CI)		(0.69, 2.45)	(0.61,2.32)	(0.63, 2.39)	(1.23, 4.10)
p for difference from control		p=0.415	p=0.601	p=0.538	p=0.008

Table 9: Hazard ratios for culture conversion of different experimental treatment arms over control; A: using MGIT; B: using MBLA

A comparison between the hazard ratios for culture conversion over control of different treatment arms was done between the MGIT primary study endpoint, and the experimental MBLA-based conversion endpoint. In both instances, the definition of culture conversion of two successive negative sputum samples without an intervening missing or positive results was used, concordant with the primary MAMS study analysis.

In the PANBIOME patient dataset, which is a subset of the MAMS study, the $RIF_{35H}ZE$ arm showed a hazard ratio which was higher than 1; with borderline significance in MGIT (Table 9: Hazard ratios for culture conversion of different experimental treatment arms over control; A: using MGIT; B: using MBLA). Conversely, the $RIF_{20}ZM$ arm, which was not significantly superior to control using the MGIT endpoint, showed significantly superior performance in the MBLA endpoint.

Altogether, there were moderate differences in hazard ratios using MGIT or MBLA as detection assays. However, in all cases confidence intervals for arms overlapped between use of both methods.

	p25	p50	p75
LJ	21	35	63
MGIT	41	55	83
MBLA	62	97	153

Table 10: Comparison between culture methods and MBLA for time to last positive in days

To counter-check the results on negative samples for an influence of potentially missing results, a comparison of time to last positive result was done (Table 10). This showed that the median time to negative culture was 35, 55 and 97 days on LJ, MGIT and MBLA platforms respectively. A time to the last positive result was recorded correspondingly 49, 67 and 101 days. MBLA, therefore gave more time to follow patients under treatment as opposed to the classical culture media. Culture based methods would declare more patients to have ceased excreting bacteria sooner than the molecular bacterial load assay.

5 Discussion

Monitoring of TB treatment success amid the search for new drugs and /or regimen is of paramount importance. This not only in the routine setting where individualized care is mainly practiced but in therapeutic trials as well for the control TB worldwide to be realized.

5.1 PanACEA MAMS TB 01

Our study indicates that sputum culture conversion in MGIT liquid medium was accelerated in the arm with high rifampicin at 35mg/kg, compared to the control regimen. However, the rapid reported sputum conversion in the $RIF_{35}HZE$ arm was less pronounced in the LJ solid medium. This is contrary from the findings of the REMox trial that evaluated two Moxifloxacin-containing regimens and did not find a difference between both media with regards to culture conversion [26]. Rifampicin is effective against actively dividing and non-replicating persisting bacteria that are drug tolerant [70]. Higher rifampicin doses are likely to kill this subpopulation effectively as shown both in models [32, 70] and in humans [38, 71, 72]. As opposed to solid LJ culture, liquid culture has been documented to detect non replicating persisters [73] and therefore the reported higher $RIF_{35}HZE$ efficacy in liquid media suggests an enhanced bactericidal activity of this regimen to these bacilli. Comparatively the four experimental regimen had relatively similar safety profiles to the control regime with lower number of hepatic adverse events being reported.

In summary, the MAMS study showed that high doses of RIF are safe and can accelerate culture conversion. At a dose of 35mg/kg, the effect of high RIF was more pronounced than at 20mg/kg, in line with other studies that assessed this dose where increased efficacy could only be described in a meta-analysis of several studies [37]. Pharmacokinetic and pharmacodynamics assessments are underway and will help in understanding this phenomenon further.

SQ109 did not show any additional efficacy over EMB, which it replaced in this study. It is possible that the drug itself has activity, but this was not enough to be superior to that of EMB in the control arm. Further, SQ109 metabolism is induced by RIF as demonstrated in the phase IIa – study. It is possible that this effect in MAMS was stronger than seen during the first 14 days, which will be known when pharmacokinetics data are analysed.

In taking high RIF further, it has to be stated that the current dose of 35mg/kg does not seem to be the highest dose that can be safely employed, and further increase may be possible. It therefore may be reasonable to test even higher doses of RIF for enhanced efficacy and for their toxicity.

In the landscape of new TB treatment interventions, high RIF is attractive since it is a licensed compound for decades now and would not have to undergo submission at regulatory agencies and long review delays.

5.2 MBLA validation results

Laboratory methods to measure treatment success using nucleic acids have been studied for almost two decades ago [24, 64, 67-69, 74]. However, there is no any molecular tool that has been deployed as a marker for monitoring treatment success. It is currently understood that molecular assays aiming at DNA like Xpert MTB/RIF are hampered by prolonged DNA detection even up to six month post TB treatment [64]. Conversely, mRNA is known for its availability in small quantity, prone to degradation and unstable [67] therefore a notable short half-life that has its eventual challenges on the limit of detection. In this study we report findings that encompasses the use of the MBLA which determines bacterial load (BL) for up to six months in sputum of TB patient by detecting *Mtb* specific *rRNA* during the course of TB treatment. It has been shown that RNA degrades faster than DNA when *Mtb* are killed by anti-TB drugs and therefore offers a platform for both TB diagnosis and real-time monitoring of treatment success [24, 69].

We have shown that culture methods like MGIT liquid medium would declare patients free of disease faster than the MBLA. Interestingly, the treatment arm $RIF_{20}HZM$ containing moxifloxacin showed significantly superior performance when the MBLA was used as an endpoint. This could be the combined bactericidal effect caused by high RIF and moxifloxacin on the bacterial populations detected by MBLA, but not by MGIT, as previously reported [26, 32, 38]. However, fewer patients were included in the MBLA sub-study within the PANBIOME study as opposed to the parent MAMS trial [1], lifting the power of such statements.

We have demonstrated that MBLA allows a longer and consistent follow up profiles for TB patients as highlighted by higher proportions of positive, compared to the MGIT and LJ media as treatment progressed. This is important as it allows enough time to ascertain the study end points and treatment successes. Additionally, there are more positive MBLA readings at any given time point than MGIT results could. This might be due to a subpopulation of bacteria that cannot be cultured in MGIT[69] but could be picked up by the MBLA. A mouse model showed several functional forms of bacilli including sub-dormant, semi-dormant and rifampicin-tolerant persisters which are likely to survive anti-tuberculous activity much longer during the treatment period than actively replicating bacilli [75].

Our study shows that there is no much difference with regards to positivity rates between the MBLA and MGIT medium during the first half of the treatment period, thereafter the former detected more TB than the latter. Conversely, the analysis of the MAMS endpoint using either MGIT or MBLA did not show a difference during the entire treatment period. This may be due to missing data from MGIT due to contamination, which would have led to reaching the endpoint later.

As recently reported from the OEBA study, MBLA correlates with MGIT TTP while patients are on treatment due to the decline in the bacterial load [24, 69]. Though less pronounced, our study still demonstrates the correlation between MBLA and MGIT TTP as treatment progresses. It should be emphasized that in the OEBA study, all tests were done from the same, homogenized sputum sample [24]. However, in the PANBIOME study, cultures were done mostly from the early morning sample, and the spot sputum samples were being preserved for later testing with MBLA. This is expected to significantly add to between test variation, caused by a high variation in bacterial load between the two analysed sputum samples.

In both situations, time to get results in MGIT medium increases with decrease in bacterial load compared to the MBLA which gives out results within 4 hours regardless of the bacterial load and at any given time point. It is well understood that MGIT takes up to 42 days of culture time to declare the final result and this delay information that could assist prompt clinical judgement. Therefore, this delay further limits its wide application as a method for monitoring TB treatment response.

As previously documented [24, 69], our study has continued to confirm that throughout the treatment period, the MBLA is very unlikely to produce missing data. Another advantage is MBLA specificity for MTB complex organisms - primers are specific for *M. tuberculosis complex* 16S rRNA. In the Tanzanian setting, many cultures turn positive with non-tuberculous mycobacteria which need to be distinguished from MTB complex by further testing; while an MBLA result does not need further verification.

In this study, we have shown that MBLA is promising and bears several advantages over the conventional culture methods as the means of monitoring TB treatment success which include being fast, sensitive and contamination free. Currently, a rapid, cheap and improved biomarker for monitoring success to TB treatment is urgently needed in the TB field particularly in developing countries like Tanzania. It would be of great help in patient management by predicting early on in the course of treatment any emergence of ineffectiveness of therapy or relapse of the disease. A predictor of treatment response would possibly help to reduce duration and costs of drug trials based on its use as a

surrogate marker [21, 53]. Nevertheless, it would as well define which TB patients can best benefit from the current move towards treatment shortening regimen[76].

Our study has several limitations. Currently, the MBLA can only be compared to standard culture based methods which are known for being inaccurate in predicting treatment outcome. As we have shown, more culture results were not evaluable at the end of the treatment due to contamination thus failing to elucidate the sensitivity and specificity of MBL Assay. Additionally, this study captured only two relapse cases due to time constraints (length of follow up; at least12months), which were not enough to validate the ability of the MBLA to predict such important and meaningful clinical outcomes among TB patients. However, during the course of the study, patients were followed up telephonically up to 6 months post treatment. This was assumed as a proxy measure to explore any treatment failure or relapse as stated in the study design above.

Furthermore, the recruitment of participants receiving different TB medication (standard and/or experimental) in this study causes cohort heterogeneity. Therefore, the detection of these surrogate markers could be affected by the type of drug combinations the patient is receiving. We did not aim to explore differing mechanism of action within experimental arms rather in comparison with the control regimen.

Bearing this in mind, the reported dynamics may not present the reality in routine care where only one regime is in use. On the other hand, this heterogeneity is also a plus for the study, since we have been be able to assess whether the studied surrogate markers reflect those differences adequately. Lastly, absence of HIV infected patients in the Tanzanian sites as explained above further precludes the generalisation of these findings in real setting as stated above, TB and HIV overlap geographically.

6 Conclusion

We have presented that high rifampicin at a dosage of 35mg/kg body weight decreases the time to negative culture conversion in MGIT medium faster than the control. The utilization of MBLA as a rapid means to monitor TB treatment response bears a great potential to contribute in TB patient management eventually control. Unlike culture, there are no additional tests to confirm any MBLA results, it provides response to TB treatment in real time without being affected contamination challenges while offering long time follow up of TB patients. However, studies including implementation in routine care, exploring MBLA as a study end point in trials and evaluation in childhood TB merit further consideration, as well as studies validating MBLA against the true clinical endpoint, relapse or failure. Eventually, application of MBLA could offer TB diagnosis and monitoring opportunities to make same day clinical judgements at an individual patient and clinical trial level.

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8 Annexes

8.1 Curriculum Vitae (CV)

Personal/Employers details		
Nyanda E. Ntinginya	NIMR-Mbeya Medical Research Centre (MMRC), P.O. Box 2410, Mbeya Tanzania	
Tel: +225252503364	Fax: +255-25-2503134	Mobile:+255713243836 and 255766243836
E-mail: nelias@nimr-mmrc.org or nelias@mmrp.org		Nationality: Tanzanian
Place and Date of Birth: Maswa, 20/05/1978		Gender: Male
Work experience		
27/07/2016 to date:	Ag. Centre Director at NIMR-MMRC	
10/2011-26/10/2016	HOD; TB and Emerging Diseases Research Programs at NIMR-MMRC	
05/2015 to date	Senior Research Scientist at NIMR-MMRC	
08/2008 -05/2015	TB Clinical Research Coordinator/Research Scientist at NIMR-MMRC	
12/2007-07/2008	Medical Registrar at the Mbeya Consultant Hospital under the Tanzanian MoH	
07/2006-11/2006	Volunteering to train about HIV at workplace and community support with Human Development Trust, Tanzania	
Education and training		
10/ 2013 to date	PhD candidate, Medical Research-International Health at the Centre for International Health, Ludwig-Maximilians-Universität, (CIH^{LMU})Munich; Germany	
09/ 2010 -09/2011	MSc in Tropical and Infectious Diseases, Liverpool School of Tropical Medicine/University of Liverpool, United Kingdom	
11/2006-11/2007	Certificate of Internship at Mbeya Consultant Hospital under the Tanzanian MoH	
09/ 2001-07/ 2006	Doctor of Medicine (MD) at the University of Dar Es Salaam, Tanzania	
08/ 1998-05/ 2000	Advanced Secondary Education at Kibaha Secondary School Coast Region in Tanzania	
01/1994-11/1997	Secondary School Education at Ihungo Secondary School Kagera; Tanzania	
Jan 1987- 1993	Primary Education at Kabila Primary School Magu, Mwanza; Tanzania	

Technical and Personal Skills
I have more than 8 years of experience in managing TB studies ranging from diagnostic, clinical trials to operational research as a Clinician, Coordinator, Principal Investigator, Clinical Trial manager at institutional and national levels in cross-functional and multicultural and international environment. Having a track record of carrying out studies TB and other diseases compliant to ICH GCP guidelines successfully. Excellent listener and communicator who effectively share the information in writing and verbally with outstanding presentation skills . Dedicated self starter, reliable, a team leader with great ability to network, proven relationship-builder due to unique interpersonal and public relation skills.
Membership/Affiliation
1. Medical Council of Tanganyika 2. International Union of Tuberculosis and Lung Disease 3. Pan African Consortium For the Evaluation of the Anti-tuberculosis Antibiotic (PanACEA) 4. National TB Research Committee in Tanzania 5. Expert meeting on harmonization of regulatory frameworks for control of clinical trials on medical products, vaccines and health technologies among member states of the East African Community, organised by the East African Community'

8.2 List of publications

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8.3 Statement on Pre-release and contribution

The results from the aforementioned studies are the original work that contributed data to the finalization of this PhD and it is from these studies the current PhD was nested. I worked at the capacity of the National and the Site Principal Investigator for the MAMS trial in Tanzania, whereby I was responsible for adequate training of study teams. These included but not limited to study procedures, timely recruitment and follow-up of patients, ensuring protocol-compliant study conduct in all Tanzanian sites according to GCP/GCLP guidelines, and national authorities., I was the Site Principal Investigator for PANBIOME, a study which was nested within the MAMS trial at NIMR-MMRC and Kilimanjaro Clinical Research Institute. I was involved in interpretation of the data and participated in writing the main manuscript of the MAMS trial [1]. I further performed data analysis of the Molecular Bacterial Load assay data described in chapter 4.3, in collaboration with my supervisors. In the OEBA study, I was involved as the Clinical Research Coordinator/Site Investigator who carried out patient recruitment, patient assessments, and data collection. I supervised the clinical team and participated in analysis and the write up of the manuscript[24].

8.4 Acknowledgement

Blessed be the name of our Lord God Almighty as in him '*we live, go and have our being*' (Acts 17:28). His mercies are new every day, every season, year in and out in my life including the commencement and completion of my PhD studies at the Centre for International Health, Ludwig-Maximilians-Universität, Munich (LMU), Germany.

My heartfelt appreciations to Professor Dr. Michael Hoelscher, who is my habilitated supervisor and co-estalished NIMR-MMRC, [former Mbeya Medical Research Programme (MMRP)]. From you Prof Hoelscher, i have learnt so much in the field of scientific TB research, management, and leadership, spanning locally from the Mbeya site to diverse international platforms. It was a great learning undertaking and its impacts are far reaching beyond the scope of this PhD.

Very special thanks to Dr. Norbert Heinrich, my direct supervisor, for your undivided commitment and support. You have been there during all seasons and places ranging from offices, classrooms, airports, homes and recently at the Lake Nyasa shores in Matema since the study design, conduct, data analysis to completion of this PhD work. You have always guided me to build my independent ideas while closely reviewing the processes in real time.

I am very much indebted to Dr. Leonard Maboko, my local supervisor and former Director at NIMR-MMRC currently the Executive director at the Tanzania Commissions for AIDS (TACAIDS). You have been so instrumental and supportive during this exciting PhD journey. Working on the same floor with you at NIMR-MMRC, i had such an opportunity to reach out to you for any needed assistance for over time. Nevertheless, you have always been a leader, model and mentor to me since then to date. Your balanced critical feedback and guidance remain one of an important asset i continue to admire even now that your miles away from Mbeya. Thus far, you and Prof Dr. Hoelscher have always been there nurturing my career and professional development at all capacities.

Many thanks to Dr. Andrea Rachow, former head of the TB department at NIMR-MMRC for your endless support since 2009 and eventually the conception to submission of this PhD proposal. You have continued following up on my scientific progress providing such a professional guidance on this interesting concept (TB treatment markers) that you did establish while in Mbeya.

Dr. Anke Kohlenberg, i owe you many thanks as a the first local supervisor, our partnership in the TB dept at NIMR-MMRC was highly recognized due to the highest level of professionalism and scientific feedback you did communicate including during the proposal write up to submission in March 2013.

Special thanks to;

- Bariki Mtafya, Fred Njeleka, John Joseph and other TB laboratory colleagues at NIMR-MMRC for carrying out all myco laboratory procedures during the entire study conduct.
- The TB clinical team of the NIMR-MMRC lead by Dr. Christina Manyama, Dr. Issa Sabi, Dr. Chacha Mangu, Dr. Wiston William, Dr. Henry Msila, Ms Ruth Bakuza,Ms Margareth Sembo, Ms Josephine Mwaijande and Ms Meckitidis Mwalongo for your valuable commitment during clinical procedures that lead to these data set and output.
- The TB clinical and Laboratory team of the KCRI lead by Professor Gibson Kibiki for your dedicated clinical and laboratory procedures during the MAMs trial that lead to additional patient data set into this work .
- Professor Stephen Gillespie and Dr. Wilber Sabiit of the University of St Andrews, UK for training and supervising the TB laboratory staff at NIMR-MMRC in performing MBLA procedures.
- The CIH coordination and all lecturers for your unwavering and valuable support within the academic cycle and beyond. The practical learning curves experienced in all fronts under your guidance since Oct 2013 have remained practical, solid and useful in my daily undertakings.

My sincere thanks to all TB patients who willingly participated in various studies from which this PhD work is nested, without you this output would not be achievable..

My heartfelt appreciation goes to the entire assemblies of the Total Healing Ministries (THM) and Rehoboth International Christian Centre (RICC) in Mbeya-Tanzania for their prayerful and moral support during the whole period of my PhD study.

My parents, Mzee Elias Ntinginya and Mayu Leticia Clement, thank you so much for laying such a foundation of commitment and responsibility remembering the rainy seasons with the cattle flocks in the field.

Finally, I am highly indebted to my dearly wife Carolyne Mwaipaja and our five wonderful children for such an ever present and unquestionable support all the way long. I started this journey while only Sandra-Bridget, Joy-Abigail and Shekinah-Glory were present in our family. Now Jesse Joshua (famous JJ) and his brother Jeremiah Jotham Gwandumi are all over energizing the family joyfully. I had to leave you in many occasions for my studies and related undertakings yet our lovely family remained united for

the common agenda. Thank you so very much Carolyne alongside my daughters and sons for your love, support, encouragement and tolerance.

Before ending this acknowledgement, I would like to express my gratitude to my classmates at the CIH LM and whomsoever provided any assistance to me through out the scope of this PhD journey.

God bless us all and Asanteni Sana.

8.5 Affidavit

Nyanda Elias Ntinginya
Name

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I hereby declare, that the submitted thesis entitled

Evaluation of potential surrogate markers to determine TB treatment response among TB patients in Mbeya, Tanzania

is the result of my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

The submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

I further declare that the electronic version of the submitted thesis is congruent with the printed version both in content and format.

Mbeya, 30 Sept, 2016
Place, Date

Signature of PhD Candidate