**Sputum derived biomarkers of anti-tuberculosis drug activity in early bactericidal activity (EBA) studies.**

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# Declaration

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# Summary

Sputum sample is a crucial material to diagnose tuberculosis (TB), the resistance to different drugs and the assessment of new drug effectiveness in clinical trials. The first step to evaluate the efficacy of a new anti-TB drug is the determination of its early bactericidal activity (EBA) which is the decline of bacterial load per milliliter of sputum per day on solid agar plates during the first two weeks of treatment. The count of colony forming units (CFU) of *Mycobacterium tuberculosis* (Mtb) on agar plates and the time to positivity (TTP) in liquid media are two parameters currently used in EBA studies. These two methods are time consuming, require highly skilled staff, an expensive infrastructure and are prone to contamination. Therefore, new methods which are more sensitive, specific, fast and automated are urgently needed. In this study, we compared the EBA determined by CFU and TTP to the EBA determined by Xpert MTB/RIF assay (Xpert). Culture methods proved to be superior to Xpert to assess the two weeks EBA of different compounds tested.

Sputum samples collected from TB patients on treatment contain a mixture of dead, injured and viable cells of Mtb. We hypothesized that the poor performance of Xpert in the determination of the EBA was due to the presence of DNA from bacteria unable to grow which was amplified together with DNA from viable ones. To overcome this problem, before performing Xpert, we have pre-treated pan-susceptible and extensively drug resistance isolates subjected to prior standard drug susceptibility testing with propidium monoazide (PMA), a reagent that penetrates only non viable cell, binds to its DNA and prevents its amplification. Then, we applied PMA pre-treatment protocol to clinical isolates from TB patients under standard TB treatment. The combination of Xpert and PMA improved the specificity to detect viable Mtb compared to Xpert alone. This improvement was statistically significant in pan-susceptible isolates incubated with isoniazid (INH) and ethambutol (EMB) and extensively drug resistant isolates incubated with EMB. Unfortunately, the effect of PMA was not statistically significant in clinical isolates from TB patients on standard treatment. Due to these conflicting results, we could not recommend the use of Xpert-PMA combination to quantify viable Mtb in preference of culture media.

Dormant mycobacteria are believed to be the result of internal and external stress including the effects of anti-TB drugs on viable mycobacteria and they are assumed to be the reason for the prolongation of TB treatment up to 6 months. In this work, the activity of SQ109, an investigational drug, Rifampicin (RMP) an already established anti-TB drug and their combination (SQ109/RMP) was assessed on both, replicating and non-replicating forms of Mtb, by using a combination of Auramine O/Nile Red staining and confocal microscopy. We found that SQ109 and RMP monotherapy increase the number of non-replicating Mtb while

SQ109/RMP combined prevents this increase. These findings show that the pressure of SQ109 alone causes Mtb to switch to dormancy and once combined with RMP, SQ109 enhances the sterilizing activity of RMP.

Monotherapy is the underlying cause of the emergence of drug resistance. We evaluated the change in proportion of RMP mutants in patients under RMP monotherapy for two weeks from baseline to day 14. We have applied statistical modelling to estimate when a patient kept on RMP monotherapy beyond two weeks would become clinically resistant. We found that RMP monotherapy beyond two weeks will induce clinical relevant resistance only after 30 days of treatment. This indicates that during TB treatment RMP resistance develops gradually due to pharmacodynamic and pharmacokinetic factors as it was previously reported.

In this work, we showed that Xpert, the combination of Xpert with PMA and staining of sputum smears with Nile Red/Auramine O are promising biomarker candidates to determine the EBA of novel anti-TB drugs. Furthermore, we demonstrated that if RMP was the only drug used for TB treatment, a resistance against RMP would become clinically relevant after 30 days.

# Opsomming

Sputum monsters is belangrike materiaal vir die diagnose van TB, om die weerstandigheid van sekere anti-TB middels te bepaal en om die effektiwiteit van nuwe middels te bepaal in geneesmiddel evaluering. Die eerste stap in die evalueringsproses van 'n nuwe anti-TB middel is om die vroeë bakerisidiese aktiwiteit (EBA) te bepaal. Dit is 'n bepaling van die bakteriële lading per milliliter sputum per dag op soliede agar plate gedurende die eerste twee weke van TB behandeling. Die bepaling van kolonie-vormende eenhede (CFU) van *M.tb* op agar plate en die tyd-tot-positiwiteit (TTP) in vloeibare groeimedia is twee metodes wat tans wyd in gebruik is by EBA studies. Hierdie twee metodes is tydrowend, vereis 'n groot mate van tegniese vernuf, benodig duur infrastruktuur en is geneig tot kontaminering. Daarom word nuwe metodes wat sensitief, spesifiek, vinnig en geoutomatiseerd is benodig. In hierdie studie vergelyk ons die EBA bepalings deur middel van CFU en TTP met EBA bepalings deur middel van Xpert MTB/RIF ontledings. Metodes deur kultuur tegnieke word bewys as superieur teenoor Xpert vir die bepaling van die twee-week EBA vir verskillende middels.

Sputum monsters wat versamel is vanaf TB pasiënte wat op behandeling is, bevat 'n mengsel van dooie, beskadigde en lewensvatbare *M.tb* selle. Ons hipotiseer dat die swak resultate van Xpert gedurende EBA bepalings as gevolg van die teenwoordigheid van DNA vanaf bakterië is wat nie lewensvatbaar is nie en wat dan saam met lewensvatbare bakterië geamplifiseer word. Om hierdie probleem te oorkom, en voordat die Xpert tegniek gebruik word, het ons pan-vatbare en uiters middelweerstandige *M.tb* isolate vooraf behandel met propidium monoazied (PMA) wat nie-lewensvatbare selle binnedring en aan hul DNA bind om sodoende amplifikasie te verhoed. Ons het dan die PMA voorafbehandelingsprotokol toegepas op kliniese isolate van TB pasiënte wat op standaard anti-TB behandeling is. Die kombinasie van Xpert en PMA het die spesifisiteit verhoog om lewensvatbare bakterieë waar te neem teenoor die Xpert metode alleen. Hierdie verbetering was statisties betekenisvol in pan-vatbare isolate wat behandel is met isoniazied (INH) en ethambutol (EMB) en uiters middelweerstandige isolate wat behandel is met EMB. Ogelukkig was die effek van PMA nie statisties betekenisvol vir kliniese isolate vanaf TB pasiënte op standaard behandeling nie. As gevolg van hierdie teenstrydige resultate kon ons nie die gebruik van die Xpert-PMA kombinasie vir die kwantifisering van lewensvatbare *M.tb* bo die gebruik van kultuurmedia bepalings aanbeveel nie.

Daar word aanvaar dat dormante mycobacteria die gevolg is van interne en eksterne stres, insluitend die effek van anti-TB middels, op lewensvatbare mycobacteria is en dat hierdie effekte verantwoordelik is vir die verlengde behandelings periode van 6 maande vir TB infeksie. In hierdie navorsingswerk word die aktiwiteit van SQ109, 'n middel nog onder evaluering,

Rifampisien (RMP), 'n gevestigde anti-TB middel, en hul kombinasie (SQ109/RMP) geëvalueer teen repliserende en nie-repliserende vorme van *M.tb* deur gebruik te maak van 'n kombinasie van Auramine O/Nylrooi kleuring en konfokale mikroskopie. Ons het gevind dat SQ109 en RMP monoterapie verhoog die aantal nie-repliserende *M.tb* terwyl SQ109/RMP kombinasie hierdie toename verhoed. Hierdie bevindings toon dat SQ109 stres alleen lei daartoe dat *M.tb* oorskakel na 'n sluimertoestand en, gekombineer met RMP, verhoog dit die steriliserende effek van RMP.

Monoterapie is die onderliggende oorsaak van middelweerstandigheid. Ons het die verandering in die verhouding van RMP mutante in pasiënte op RMP terapie vir twee weke geëvalueer. Ons het 'n statistieke model gebruik om te bepaal of 'n pasiënt wat vir twee weke op RMP monoterapie gehou word, kliniese weerstandigheid ontwikkel. Ons het gevind dat RMP monoterapie vir meer as twee weke lei tot klinies relevante weerstandigheid eers na 30 dae behandeling. Dit dui daarop dat gedurende TB behandeling met RMP weerstandigheid stelselmatig ontwikkel as gevolg van farmadinamiese en farmakinetiese faktore soos voorheen aangedui.

Hierdie navorsingk bewys dat Xpert, die kombinasie van Xpert en PMA en die kleuring van sputumsmere met Nylrooi/Auramine O belowende biomerker kandidate is om die EBA van nuwe anti-TB middels te bepaal. Ons wys ook dat indien RMP die enigste anti-TB middel is wat gebruik is vir behandeling, weerstandigheid teen RMP klinies relevant word na 30 dae behandeling.

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# Dedication

This thesis is dedicated to both my parents passed away 20 years ago. My dad and mom your departure was so premature, so that you did not get a chance to see us your children growing up, we love you and we will never forget you. Rest in eternal peace.

# Contents

# List of abbreviations and acronyms

| | |
|---|---|
| A: | Adenine |
| AFB: | Acid fast bacilli |
| Ag85A/B: | Antigen 85A and B |
| AIDS: | Acquired immunodeficiency syndrome |
| AMK: | Amikacin |
| AMX/CLV: | Amoxicilin plus clavulanate |
| ATP: | Adenosine triphosphate |
| ART: | Antiretroviral therapy |
| BC: | Before Christ |
| BCG: | Bacillus Calmette Guerin |
| C: | Cytosine |
| CAF: | Centre for analytical facilities |
| CCT: | Chest computed tomography |
| CCTR: | Centre for clinical tuberculosis research |
| CDC: | Centre for disease control |
| CFP-10: | Culture Filtrate Protein 10 |
| CFU: | Colony forming unit |
| CFX: | Ciprofloxacin |
| CFZ: | Clofazimine |
| CI: | Confidence interval |
| CLR: | Clarithromycin |
| CM: | Capreomycin |
| CON: | Drug free control |
| $C_T$: | Cycle threshold |
| D: | Aspartic acid |
| DCS: | Cycloserine |

| | |
|---|---|
| DNA: | Deoxyribonucleic acid |
| DOTS: | Directly observed short course therapy |
| DST: | Drug susceptibility testing |
| EBA: | Early bactericidal activity |
| EDCTP: | European and developing countries clinical trials partnership |
| EMA: | European Medicines Agency |
| EMB: | Ethambutol |
| ESAT-6: | 6 kDa early secretory antigen target |
| ETH or ETO: | Ethionamide |
| GFX: | Gatifloxacin |
| G: | Guanine or Glycine |
| H: | Histidine |
| HIV: | Human immunodeficiency virus |
| IGRA: | Interferon gamma release assay |
| INH: | Isoniazid |
| IPM/CLN: | Imipenem plus cilastatin |
| IUATLD: | International union against tuberculosis and lung diseases |
| kg: | Kilogram |
| KM: | Kanamycin |
| l: | Liter |
| Leu: | Leucine |
| LFX: | Levofloxacin |
| Log/Log$_{10}$: | Logarithm base 10 |
| LPA: | Line probe assay |
| LTBI: | Latent tuberculosis infection |
| LSM: | Laser scanner microscopy |
| LZD: | Linezolid |

| | |
|---|---|
| MCC: | Medicines Control Council |
| MDR: | Multidrug resistant |
| MDR-TB: | Multidrug resistant tuberculosis |
| mg: | Milligram |
| MGIT: | Mycobacteria Growth Indicator Tube |
| MIC: | Minimum inhibitory concentration |
| μl: | Microliter |
| ml: | Milliter |
| MOX or MXF: | Moxifloxacin |
| Mtb: | *Mycobacterium tuberculosis* |
| MTBC: | *Mycobacterium tuberculosis* complex |
| MVA85A: | Modified Vaccinia Ankara 85A |
| NAAT: | Nucleic acid amplification test |
| NALC: | N-acetyl-L- cysteine |
| NaOH: | Sodium hydroxide |
| NHLS: | National Health Laboratory Services |
| Nt: | Total number |
| OADC: | Oleic acid, albumin, dextrose, catalase |
| OFX: | Ofloxacin |
| OPC-676883: | Delamanid |
| PZA: | Pyrazinamide |
| PA-824: | Pretomanid |
| PANTA: | Polymixin B, amphothercin B, nalidixic acid, trimethoprim and azlocilin |
| PanACEA: | Pan-African consortium for evaluation of anti-tuberculosis drugs |
| PAS: | Para-aminosalicilic acid |
| PBS: | Phosphate buffered saline |
| PCR: | Polymerase chain reaction |

xiv

| | |
|---|---|
| PMA: | Propidium monoazide |
| PNU-100480: | Sutezolid |
| PSM: | Pan-susceptible *Mycobacterium tuberculosis* |
| PTB: | Pulmonary tuberculosis |
| PTH or PTO: | Prothionamide |
| rBCG30: | Recombinant Bacillus Calmette-Guerin 30 |
| RFB: | Rifabutin |
| RMP or RIF: | Rifampicin |
| RNA: | Ribonucleic acid |
| RPT: | Rifapentine |
| RRDR: | Rifampicin resistance determining region |
| RRTB: | Rifampicin resistant tuberculosis |
| S: | Serine |
| SA: | South Africa |
| SD: | Standard deviation |
| SM or STM: | Streptomycin |
| SPC: | Specimen processing control |
| SQ: | SQ109 |
| T: | Thymine |
| TB: | Tuberculosis |
| TDR: | Totally drug resistant |
| TDR-TB: | Total drug resistant tuberculosis |
| THZ: | Thioacetazone |
| TMC207: | Bedaquiline |
| TNFα: | Tumor necrosis factor alpha |
| TRD: | Terizidone |
| TST: | Tuberculin skin test |
| TTP: | Time to positivity |

UK:                          United Kingdom

USA:                         United States of America

VIM:                         Viomycin

WHO:                         World Health Organization

XDR:                         Extensively drug resistant

XDR-TB:                      Extensively drug resistant tuberculosis

Y:                           Tyrosine

ZN:                          Ziehl-Neelsen

# List of figures

# List of tables

# Chapter one

# General introduction and thesis outline

## 1.1.   The history of tuberculosis

Tuberculosis (TB) is an infectious disease which is thought to be endemic for over 20,000 years. Human TB was identified by molecular techniques in human remains of the Neolithic era dated 9,000 years ago (1). TB is a worldwide disease which is reflected by names given to it in different languages (Greek: phthisis, Latin: consumptio, India: yaksma, Incan: chaky oncay, English: white plague, china: Xulao bing and French: le mal du roi). All these names describe the cachectic status of a TB patient (2).

Hippocrates the father of Medicine, during his time described TB as a disease characterized by coughing of thick sputa, fever, colourless urine and loss of appetite. He observed also that TB was more prevalent in young people between the age of eighteen and thirty-five years (3). He believed that TB is hereditary but others like Aristotle and Galen disagreed with this theory and insisted that TB is contagious. This was shown later in 1865 by Jean Antoine Villemin, a French surgeon, by inoculating rabbits with purulent liquid from lung cavities of cadavers with TB (4).

TB was documented in ancient Egypt and characteristic evidence of TB was found in mummies namely typical skeletal abnormalities known as Pott's deformities and DNA from *Mycobacterium tuberculosis* (Mtb) detected by a molecular biologic method named polymerase chain reaction (PCR) (5,6). Several old scripts from China (2300 BC) and India (3300 BC) describe TB in which the physicians prescribed sunlight, rest, wholesome diet and moving to high altitudes as treatment (7). In Europe, evidence of TB was found from the 5th century after the fall of the Roman Empire; there TB became epidemic in the 17th century (8). One TB treatment in Europe was the Royal touch, especially in France and England. This ceremony was held by the King once a week leading to the name "King's evil" for mycobacterial cervical lymphadenitis (9). Epidemic TB developed in Northern America in the 18th to 19th century where it was called the "Captain among the men of death" (10). There is considerable evidence, also from bones of skeletons that, TB was present in North and South America before European explorers reached these continents. The epidemic of TB however could be stated to have developed with urban centres resulting from European settlements.

The pathogenesis of TB was elucidated by Rene Hyacinthe Theophile Laennec a French physician in 1819. He invented the Stethoscope and the lung findings described by him are still used in medicine to diagnose TB (11).

The history of TB changed in 1882 when Robert Koch demonstrated the aetiology of the disease and extracted a protein from tubercule bacilli which he named tuberculin. It is a substance he thought should render Mtb harmless (4). The tuberculin skin test (TST) was developed by Clemens Freiherr von Pirquet which he used to diagnose TB infection in children (12). After that, Charles Mantoux developed the current intradermal method to inject tuberculin to diagnose TB.

In 1908, Albert Calmette and Camille Guerin developed the first TB vaccine (BCG) from attenuated *Mycobacterium bovis* which has lost its virulence to human. The first anti-TB drug, streptomycin (SM), was discovered in 1943 by Abert Schatz at the Rutgers University under the supervision of Selwyn Waksman and Elizabeth Buggie (13-15) and other drugs followed later namely para-aminosalisylic acid (PAS) in 1946, isoniazid (INH) in 1951, pyrazinamide (PZA) in 1952, rifampicin (RMP) in 1957 (16) and ethambutol (EMB) in 1961. In December 2012, TMC207 or bedaquiline was partially registered by the Food and Drug Administration (FDA) to treat multidrug resistant tuberculosis (MDR-TB) being the first new anti-TB drug since the discovery of EMB (17).

The first TB sanatorium was opened in 1859 by Herman Brehmer in the Silesian mountain village of Göbersdorf in Poland (Figure 1.1) and others followed in Falkenstein, Germany, Asheville in North Carolina, USA, and the one in Davos, Switzerland which was named "Magic Mountain" by the novel writer Thomas Mann (18). The TB patients were treated by bed rest, a rich diet and supervised exercise. Furthermore, the disease was treated by pneumothorax and thoracoplasty, surgical techniques to close cavities (19, 20). Since it started to infect people more than 10,000 years ago, TB may have killed a greater number of humans than any other infectious disease (4).

**Figure 1.1**: The first TB Sanatorium in the Silesian mountain village of Göbersdorf in Poland. TB patients were exposed to fresh air of high altitude, given rich diet, bed rest and physical exercise. Adapted from: https://en.wikipedia.org/wiki/Sokolowsko.

The discovery of anti-TB drugs brought hope to cure and eradicate TB. But the scenario was changed by the emergence of multi-drug resistant (MDR), extensively drug resistant (XDR) and totally drug resistant (TDR) strains of Mtb. The problem was worsened by the association of TB with HIV/AIDS. Latent TB infection (LTBI), the chronic and asymptomatic form, characterized by the presence of dormant mycobacteria in the lung granuloma after being engulfed by alveolar macrophages, is liable to reactivate wherever it may be present under the influence of HIV/AIDS. In presence of HIV, reactivation might occur in the lungs but also more frequently than usual in other organs.

All these problems left health institutions and decision-makers worldwide with no other option than to invest money and resources into the discovery of new anti-TB drugs effective against both susceptible and drug resistant Mtb, easy to administer together with antiretroviral (ARV) drugs and able to shorten the long duration of six months of TB therapy by also killing the dormant forms of Mtb which remain in the lungs after treatment is completed.

## 1.2.   Tuberculosis as a global threat to humanity

TB is spread by the inhalation of small droplets approximately 5 µm in size and containing 1-3 bacilli from people with pulmonary TB when they cough and sneeze (21). While approximately 5% of infected individuals will develop TB, the lifetime risk is closer to 10% and the risk becomes 10% per annum if an individual is HIV-infected. It is estimated that one third of the global population is latently infected with TB (22). The reactivation of latent TB many years after the initial infection can cause disease to develop e.g. in immune-supressed patients with HIV/AIDS, patients on antitumor necrosis factor α or patients with diabetes mellitus (23). The control of TB is complicated due to its association with HIV/AIDS and by the emergence of MDR strains (24). For many years, TB was considered the second cause of death from infectious diseases after HIV/AIDS (25, 26) and currently new report shows that TB is the number one killer from infectious diseases (27).

In 2015, 10.4 million new TB cases and 1.8 million deaths from TB were reported to the World Health Organisation (WHO); among these causalities, 400,000 were HIV positive. Eleven percent of all people who developed TB worldwide were co-infected with HIV. This proportion was highest in African region (31%) and exceeded 50% in southern Africa (27). Among new TB cases occurred in 2015, 5.9 million were men, 3.5 million women and 1 million children (27).

South Africa is ranked third after India and China among countries with a high burden of active TB with almost 0.5 million new TB cases annually; its incidence rate is estimated at 834 in every 100,000 citizens each year (Figure 1.2) (28).
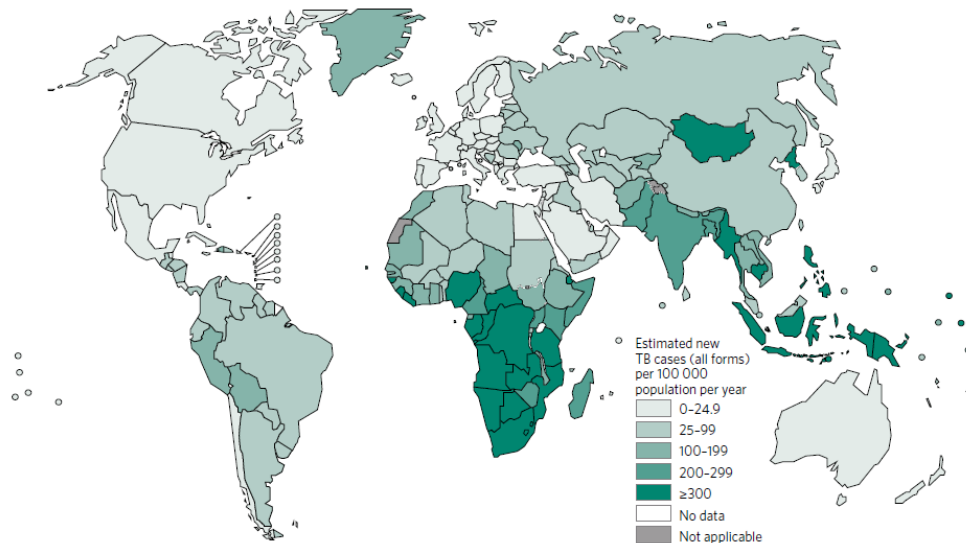


**Figure 1.2**: The estimated global TB incidence rate in 2015 (27).

MDR-TB is resistant to both isoniazid (INH) and rifampicin (RMP) and is found worldwide but mostly in South Africa, China, India and the former Soviet Union (Figure 1.3) (29). In May 2016, WHO have released a new MDR-TB treatment guideline in which RMP resistant TB (RR-TB) is treated as MDR-TB regardless to whether it is resistant to other drugs or not. In 2015, the number of RR-TB cases was estimated at 100,000 increasing the number of MDR-TB cases by this count compared to the WHO report from 2014 (30). The number of deaths due to MDR-TB was estimated at 250,000 patients per year (27). Once a MDR strain extends its resistance to fluoroquinolones and to at least one of the injectable drugs (kanamycin, capreomycin or amikacin), it leads to an XDR strain. The first XDR-TB outbreak happened in the Kwazulu-Natal province, South Africa in the year 2006 (31). Mtb resistant to all existing anti-TB drugs are called TDR strains and were recently discovered in India, Iran and South Africa (32-35). Among all MDR-TB cases reported to the WHO in 2015, 9.5% had XDR-TB (27).
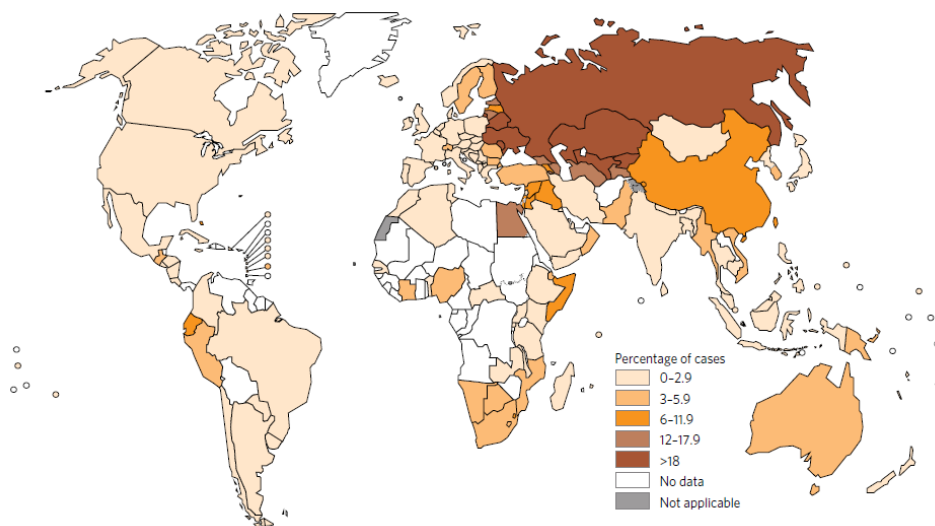
**Figure 1.3**: The percentage of new TB cases with MDR/RR-TB in 2015 (27).

The management of drug-resistant TB is difficult due to its long duration of treatment (9 months to 2 years) and the results from drug susceptibility testing methods can take four to eight weeks to be available (36). Therefore, new anti-TB drugs effective against MDR-TB, XDR-TB or TDR-TB and able to shorten the duration of treatment are highly needed (37, 38). Furthermore, new and faster tests are needed to determine the efficacy of these new anti-TB drug candidates before they can be approved and utilized.

## 1.3.    Tuberculosis treatment and Early Bactericidal Activity

### 1.3.1   Cell wall of *Mycobacterium tuberculosis*

Mtb together with *Mycobacterium africanum, Mycobacterium bovis, Mycobacterium microtii* and *Mycobacterium cannetti* form the *Mycobacterium tuberculosis* complex (MTBC), i.e. species of mycobacteria capable of causing TB in humans. About 60% of the cell wall of Mtb is made of lipids and its main compound is mycolic acid. The backbone of Mtb's cell wall consists of peptidoglycan covalently bound to arabinogalactan and mycolic acid (39,40). The outside capsule consists of proteins and polysaccharides (Figure 1.4). Mtb is a facultative anaerobic bacterium and therefore needs oxygen to grow. It is able to resist weak disinfectants and to survive in a dry state without exposure to light for weeks. The replication rate of Mtb is very low compared to other bacteria with one division requiring 15-20 hours. The abundance of lipids in its cell wall is the key element for its resistance to antibiotics, its virulence and its low division rate. Mtb can be stained only with acid-fast dyes like Ziehl-Neelsen which is being the reason for Mtb to be referred to as acid-fast bacilli (AFB) (41).

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The majority of established anti-TB drugs and the ones still under clinical investigations target different cell wall constituents or enzymes involved in the cell wall synthesis (42).
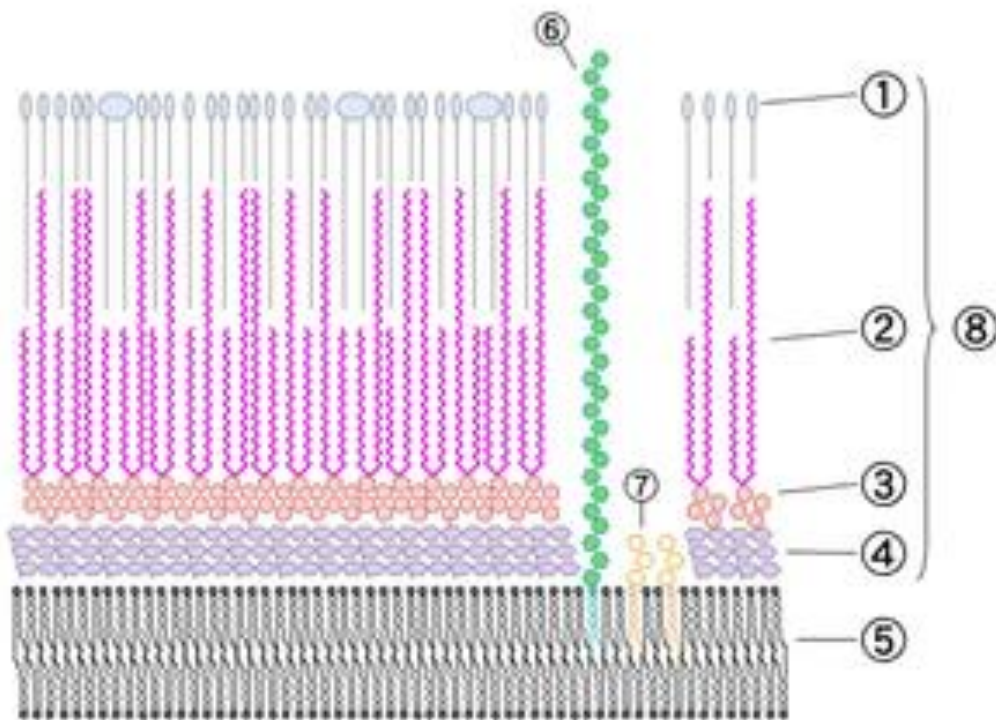


**Figure 1.4:** Illustration of *Mycobacterium tuberculosis* cell wall components: outer lipids (1), mycolic acid (2), polysaccharides (arabinogalactan) (3), peptidoglycan (4), plasma membrane (5), lipoarabinomanann (LAM) (6), phosphatidylinositol mannoside (7), cell wall skeleton (8). Adapted from https://en.wikipedia.org.wiki/Mycobacterium.

## 1.3.2. Tuberculosis treatment

The discovery of SM was a breakthrough in the history of TB treatment and it was the beginning of an era using antibiotics. Later, many more drugs have been discovered and registered as anti-TB drugs. The anti-TB medications are classified as first-line, second line and third line drugs according to their efficacy and potency to treat TB (42). Table 1.1 and Figure 1.5 summarize all anti-TB drugs currently used in their respective groups and classes.

**Table 1.1:** The WHO Classification of anti-TB drugs according to their efficacy and potency (43).

| Group | First line anti-TB drugs: For drug susceptible TB | Second line anti-TB drugs: For drug resistant TB | Third line anti-TB drugs: Unclear efficacy |
|---|---|---|---|
| Group one (oral) | Isoniazid (INH)<br>Rifampicin (RMP)<br>Pyrazinamide (PZA)<br>Ethambutol (EMB)<br>Rifapentine (RPT) or<br>Rifabutin (RFB) | | |
| Group two (injectable) | | **Aminoglycosides:**<br>Streptomycin (SM)<br>Kanamycin (KM)<br>Amikacin (Amk)<br>**Polypeptides:**<br>Capreomycin (CM)<br>Viomycin (VIM) | |
| Group three (Oral and injectable) | | **Fluoroquinolones:**<br>Ciprofloxacin (CFX)<br>Levofloxacin (LFX)<br>Moxifloxacin (MFX)<br>Ofloxacin (OFX)<br>Gatifloxacin (GFX) | |
| Group four (Oral) | | Para-aminosalicylic acid (PAS)<br>Cycloserine (DCS)<br>Terizidone (TRD)<br>Ethionamide (ETO)<br>Prothionamide (PTO)<br>Thioacetazone (THZ)<br>Linezolid (LZD) | |
| Group five | | | Clofazimine (CFZ)<br>Linezolid (LZD)<br>Amoxicillin plus Clavulanate (AMX/CLV)<br>Imipenem plus Cilastatin (IPM/CLN)<br>Clarithromycin (CLR) |

A real change in TB treatment came with the incorporation of RMP into the TB treatment regimen in 1972 (44) and it has halved the treatment duration from 18 to 9 months. The addition of pyrazinamide (PZA) to the TB regimen containing both RMP and INH has further reduced the treatment duration from 9 to 6 months (45-47).

With the emergence of MDR-TB, XDR-TB and TDR-TB, the drugs available are not effective anymore and new compounds are urgently needed to fight these new strains. There are various new drugs proven to be effective *in vitro* against susceptible and drug resistant Mtb which are currently under clinical investigation.

The diamine SQ109 is an analogue of EMB (48) and was assessed in a phase 2 clinical trial. SQ109 targets the cell wall biosynthesis, particularly the *mmpL3* gene that encodes the mycolic acid protein transporter (49) but its precise mechanism of action is not yet clarified. *In vitro* studies showed that SQ109 has a synergistic activity with INH, RMP, PNU-100480 and TMC207 (50-52). This new drug has demonstrated activity against drug susceptible, drug resistant and extensively drug resistant Mtb in mice (53). However, findings of recently conducted phase 2 clinical trial which compared SQ109 to RMP did not show a significant early bactericidal activity (EBA) in TB patients with the dosages given (54).

Bedaquiline (BDQ) or TMC207 is a diarylquinoline inhibiting the ATP synthase of Mtb and interfering with the energy supply of the cell. It is effective against both susceptible and drug resistant Mtb (55). A randomized phase 2 clinical trial showed that TMC207 has a late bactericidal activity compared to RMP and INH (56). In a following trial, TMC207 was added to a five-drug second line regimen (KM, OFX, ETO, PZA and DCS or TRD) and has significantly increased the proportion of MDR-TB patients converting to smear-negative after two months of treatment (57). TMC207 has received partial approval by the FDA to be used against MDR-TB since December 2012 (17, 58).

Benzothiazinone (BTZ043) is a compound active against susceptible and MDR strains of Mtb. It inhibits the synthesis of decaprenylphosphoarabinose which is the precursor of arabinan in the mycobacterial cell wall (59). It has shown an additive activity with RMP, INH, EMB, MOX, PA-824, meropenem with or without clavulanate and SQ109. BTZ043 has a synergistic effect with TMC207 (59) and is currently being investigated in clinical trials.

Sutezolid or PNU-100480 is an oxazolidinone derivative still under investigation. It prevents the initiation of protein synthesis by binding to the 23S RNA in the 50S ribosomal subunit of bacteria. It was shown to be active against Mtb in murine models (60), and is well tolerated and safe in humans (61).

Pretomanid or PA-824 is a nitroimidazole derivative, which inhibits protein and lipid synthesis and is effective on both susceptible and MDR strains (62).

Used in a two weeks EBA clinical drug trial, PA-824 combined with moxifloxacin (MOX) and PZA showed an EBA comparable to the standard TB treatment regimen (INH, RMP, PZA and EMB) (63, 64).

The antibiotic compound Delamanid or OPC-676883 is a nitro-dihydro-imidazooxazole derivative. This drug inhibits the mycolic acid synthesis (65) and showed a significant two weeks EBA in adult patients with TB (66). It was partially approved by the European Medicines Agency (EMA) in 2014 to treat MDR-TB in adults (67). Additional studies are on-going.

Three more promising drug candidates are LL-3858 (a pyrolle derivative), AZD 5847 (an oxazolidinone) and FAS 20013 (an ATP synthase inhibitor). All of them have demonstrated an *in vitro* activity against drug susceptible and drug resistant strains of Mtb. They are still under investigation in clinical trials at different phases (68,69).

Additionally, old and approved drugs like β-lactams are being tested in clinical trials to evaluate their efficacy to kill Mtb. Recently, a two week EBA drug trial showed that the combination of meropenem and amoxicillin-clavulanic have potential to treat TB in comparison to the standard TB regimen (70).
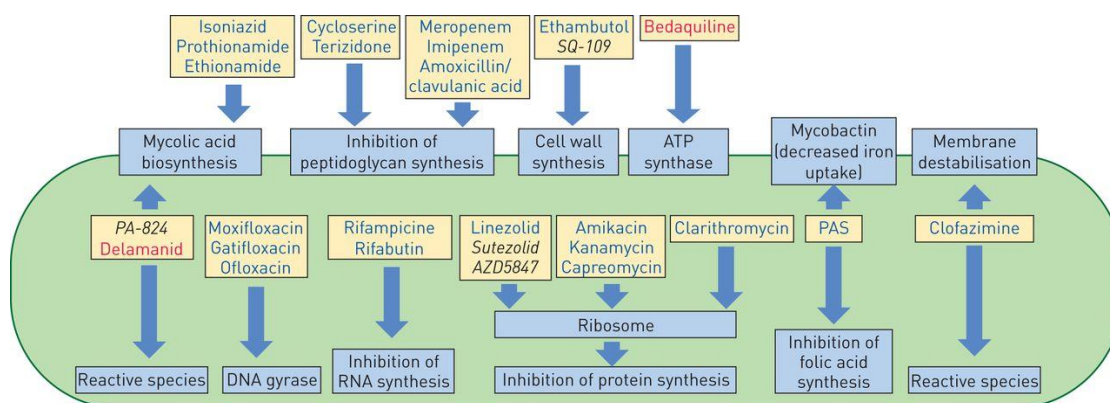


**Figure 1.5**: The mechanisms of action of already used, newly approved and novel anti-TB drug candidates. The anti-TB drugs already in use are in blue, newly approved in red and novel anti-TB drug candidates in black (71).

## 1.3.3. Early Bactericidal Activity of anti-TB drugs

EBA is defined as the decrease in counts of colony forming units (CFU) per millilitre of expectorated sputum per day (72) and it reflects the ability of an antibiotic compound to kill mycobacteria in pulmonary cavities during the first days of treatment. The first comprehensive EBA study was conducted in Nairobi, Kenya and published in 1980. This study yielded

significant new evidence for early bactericidal activity of anti-TB drugs used either alone or in combination. Since then, the EBA studies have provided a safe and relatively inexpensive method to assess the early activity of novel or approved antibiotic drugs (72).

The determination of EBA using sputum samples allows a comparison of the capability of different compound regarding their bactericidal activity and the appropriate dosage to be used in subsequent clinical studies (72-74).

Currently, CFU counts on solid media and the determination of time to positivity (TTP) in liquid media are the only two methods available to determine an EBA (75). For this, CFU and TTP values of sputum samples collected before treatment are compared to CFU and TTP values from sputum samples daily collected from day 1 to day 14 (maximally). In an early EBA study using solid media for culture and referred to above the greatest differences between the various drugs were found during the first two days of treatment (72). The methods to determine CFU counts and TTP are labour intensive require skilled staff and are time consuming (CFU up to 8 weeks, TTP up to 42 days). They are prone to contamination and demand an expensive infrastructure (Biosafety level 3 laboratory). Therefore, new tests are needed which are more sensitive, more specific, faster, easier to use, less expensive and accessible to all health institutions, especially in low and middle income countries. This will help to shorten the drug development process which is currently estimated to take around 20 years from drug design to registration and can cost billions of dollars (76, 77).

## 1.4.    Drug resistant tuberculosis

Drug resistant TB is defined as active TB not responding to standard TB treatment consisting of a cocktail of RMP, INH, EMB and PZA given daily for two months followed by a combination of RMP and INH for four months, three times a week (78). The resistance can be induced by chemotherapy as a consequence of non-adherence to treatment therefore it is called a "man-made phenomenon". The severity of drug resistant TB ranges from mono-resistant (resistant to a single drug) to multi-drug resistant or MDR (resistant to at least RMP and INH) to extensively drug resistant or XDR (MDR additionally resistant to fluoroquinolones and to at least one injectable drug) ending at totally drug resistant or TDR (resistant to all existing anti-TB drugs). In Mtb, like in other bacteria, drug resistance can occur through spontaneous chromosomal mutations which are the consequences of errors arising during DNA replication (79, 80). The mutation can be a deletion when a nucleotide sequence is erased, an insertion when an extra-nucleotide sequence is added or a substitution when a particular nucleotide sequence is replaced by another one.

A silent mutation does not result in an amino-acid substitution thereby the phenotype of the organism is not changed (81). The probability of a mutation occurring in a bacterial population is called the mutation rate and it is evaluated per bacterium per generation (82), whereas the mutation frequency is the proportion of mutants in a bacterial population. Both, mutation rate and mutation frequency, are determined on culture media containing the critical concentration of an antibiotic. The critical concentration of an antibiotic compound is defined as being able to inhibit 95% of the growth of wild type cells. A pioneering study to determine the bacterial mutation rate of *E. coli in vitro,* was done by Luria and Delbruck in 1943 using a fluctuation assay (83) and is still used today. The mutator strain is defined as a strain with high mutation rate compared to its progenitor.

Beijing strains are believed to be more virulent than other strains of Mtb and to be associated with the emergence of drug resistance (84, 85). However, this has not been proven *in vitro* by comparing the mutation rate of Beijing and non-Beijing strains using the fluctuation assay (86). Beijing strains were found to be predominant in Eastern and Southeast Asia, in Russia and in South Africa; the sublineage 7 is the most prevalent (87-91).

The evolution of mutations conferring resistance to certain drugs could be caused by mutagens encountered by Mtb such as oxidative stress (92), antibiotics (93), antiretroviral drugs (94), host environment (95), smoking and pollution (96, 97). Mtb mutates *in vitro* at a low rate compared to other bacteria like *E. coli* (98) and several factors are implicated in the selection of drug resistant cells *in vivo* namely the fitness of the individual mutant (99), the compliance with treatment (100), the size of the infecting bacterial population (101), the pharmacokinetic variability amongst the patients (102) and the heterogeneity of drug distribution (103).

Soon after the discovery of SM, the first treated patients began developing resistance against SM monotherapy (104) and a lot of funds have been invested to develop other new and effective anti-TB drugs. This was the beginning of multidrug combination anti-tuberculosis treatment with the rational that the emergence of drug resistance can be averted using a combination. Unfortunately, there is an increasing number of patients diagnosed with drug resistant TB each year (3.9% of new cases and 21% of previously treated cases) (27). The patients are particularly resistant to RMP which is a powerful anti-TB drug and currently utilized as a surrogate biomarker of MDR-TB (105). Therefore, it is of utmost importance to investigate how this resistance against RMP develops during TB treatment to better manage this disease and to prevent the on-going spread of resistant strains in the community.

## 1.4.1.     Rifampicin resistance

RMP is an anti-TB drug considered to be the backbone of the standard TB treatment consisting of four first line anti-TB drugs (INH, RMP, PZA and EMB). Mono-resistance against RMP is rare, once a strain is resistant to RMP, it is resistant to at least another agent (106). RMP inhibits the RNA polymerase, an enzyme necessary for the transcription of RNA from DNA, thereby blocking the elongation of the RNA chain. Resistance to RMP is the result of an amino-acid exchange in the *rpo*B gene which encodes the B-subunit of the RNA polymerase. Ninety-seven percent of all mutations conferring resistance to RMP occur in an 81 bp region (codons 507-533) of the *rpo*B gene, called the Rifampicin Resistance Determining Region (RRDR) (Figure 1.6) (107,108). A high level of resistance against RMP is caused by mutations in codons 526 and 531 leading to a minimum inhibitory concentration (MIC) of more than 64 µg/ml (109). Changes in codons 511, 516, 519 and 522 confer a low level of resistance with a MIC of less than 64 µg/ml (109, 110). There is one mutation described in codon 149 (outside the RRDR) conferring a low level of resistance to RMP (111). As an oral drug, a mono-resistance against RMP is mostly found in HIV infected patients due to their malabsorption of this drug in the intestines resulting in a low blood serum level and allowing the selection and the overgrowth of resistant mutants (112,113). Furthermore, the level of the intracellular RMP concentration was found to be dependent on the efflux pump activity (114). Resistance to RMP spontaneously appears at a rate of $10^{-9}$ to $10^{-8}$ (115,116) and there is still a lack of understanding on how it develops during treatment. A recent study demonstrated that during standard TB treatment only RMP can enter the lung lesions creating a monotherapeutic situation (117); this could easily promote the selection of RMP resistant strains. Therefore, more studies are needed on the development of RMP resistance during TB treatment to improve TB management since a TB regimen without RMP has limited efficacy.
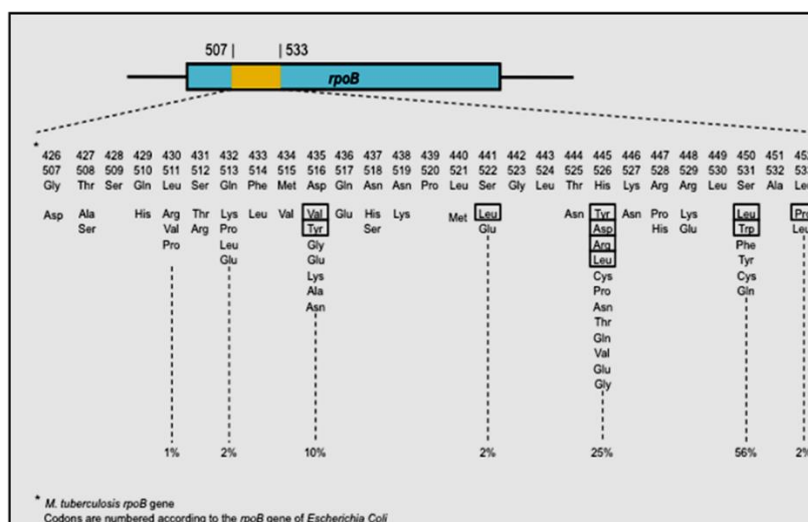
**Figure 1.6**: Rifampicin resistance determining region of the *rpo*B gene of *M.tuberculosis*. It is an 81 bp region with 27 codons numbered from codon 507 to 533 according to the *rpo*B gene of *Escherchia coli* (118).

## 1.5.    Latent tuberculosis

LTBI is defined as an individual living with Mtb without showing any symptoms of active disease (119). The bacilli enter the human lung through inhalation of aerosols expelled by patients with active TB when they cough, sneeze, shout, sing and laugh. Then, the viable bacteria are contained by cells of the immune system particularly alveolar macrophages and remain in the body (120). During latency, Mtb is believed to be dormant but is able to maintain its integrity and ability to grow on culture media when resuscitation promoting factors are present (121). After an infection, only 5-10% of immune-competent individuals have a life time risk to develop the disease (122). A deficiency of the immune system can remarkably increase the risk of developing active TB (Figure 1.7) (123,124), like an infection with HIV/AIDS (125), a haematological malignancy, immune suppressive drugs such as TNFα antagonist agents, cigarette smoking, the use of  biomass fuels, alcohol consumption, diabetes mellitus, a vitamin D deficiency and indoor cooking (126-129). A third of the global population is latently infected with TB (22,130) which represents the future source of active disease as the pathogen is dormant and is able reactivate anytime.
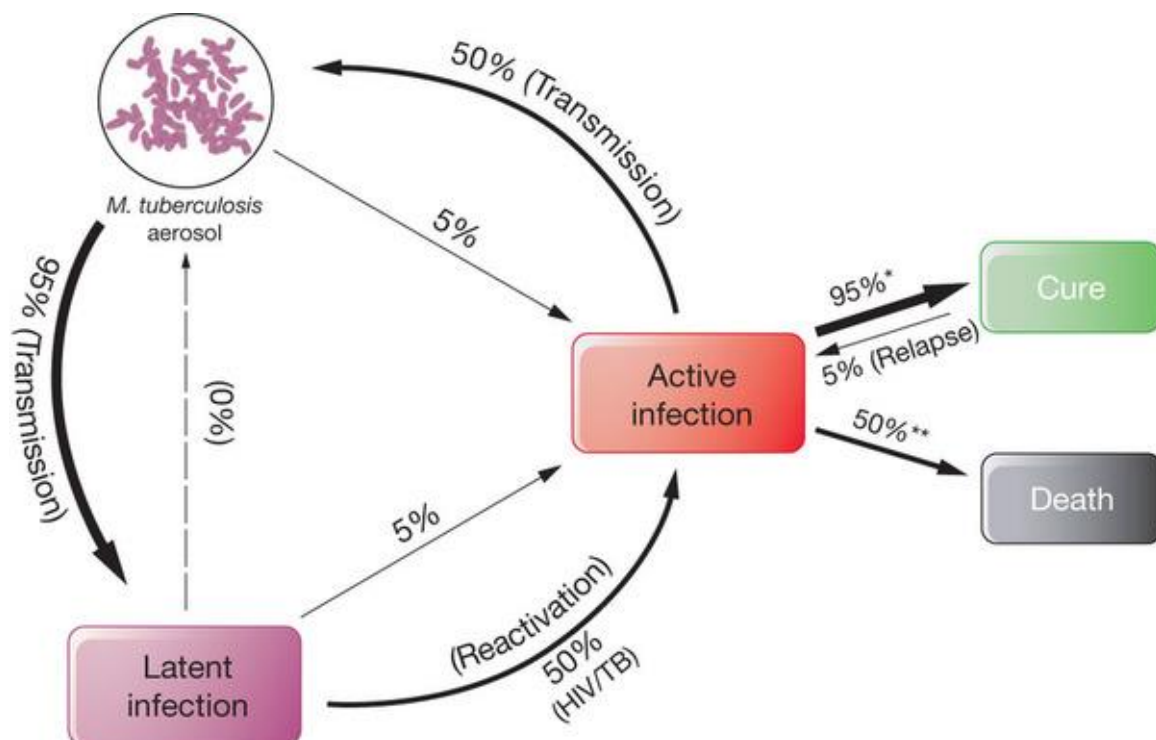
**Figure 1.7:** Possible outcomes of an infection with Mtb. Once infected, 5% of patients progress to an active disease whereas for the others the infection remains latent. Once treated, active TB is cured in 95% of all patients with few cases of relapse. If not treated, active TB kills 50% of its patients and continues to infect other humans. The risk of a reactivation of latent TB is high in immunocompromised patients (50%) like those living with HIV/AIDS (131).

The metabolic processes leading to the state of dormancy and reactivation are still unclear; however, several *in vitro* and *in vivo* studies have shown that the mycobacteria accumulate lipid bodies as source of energy under multiple stresses (132-135). The stress factors tested so far are hypoxia, nitric oxide, starvation and acidic media. These factors are similar to what is encountered by Mtb in tuberculous host lesions (136-139). Using the lipophilic stain Nile Red combined with Auramine O and fluorescent microscopy it is possible to visualize Mtb with intracellular lipid bodies on smears from bacterial cultures under stress and from sputum samples (Figure 1.8) (134,140-143). It was shown that these lipid bodies are utilized when dormant bacilli are placed into conditions of nutrient deprivation (144) thereby indicating their role in mycobacterial cell growth during reactivation after provision of energy and carbon (145).
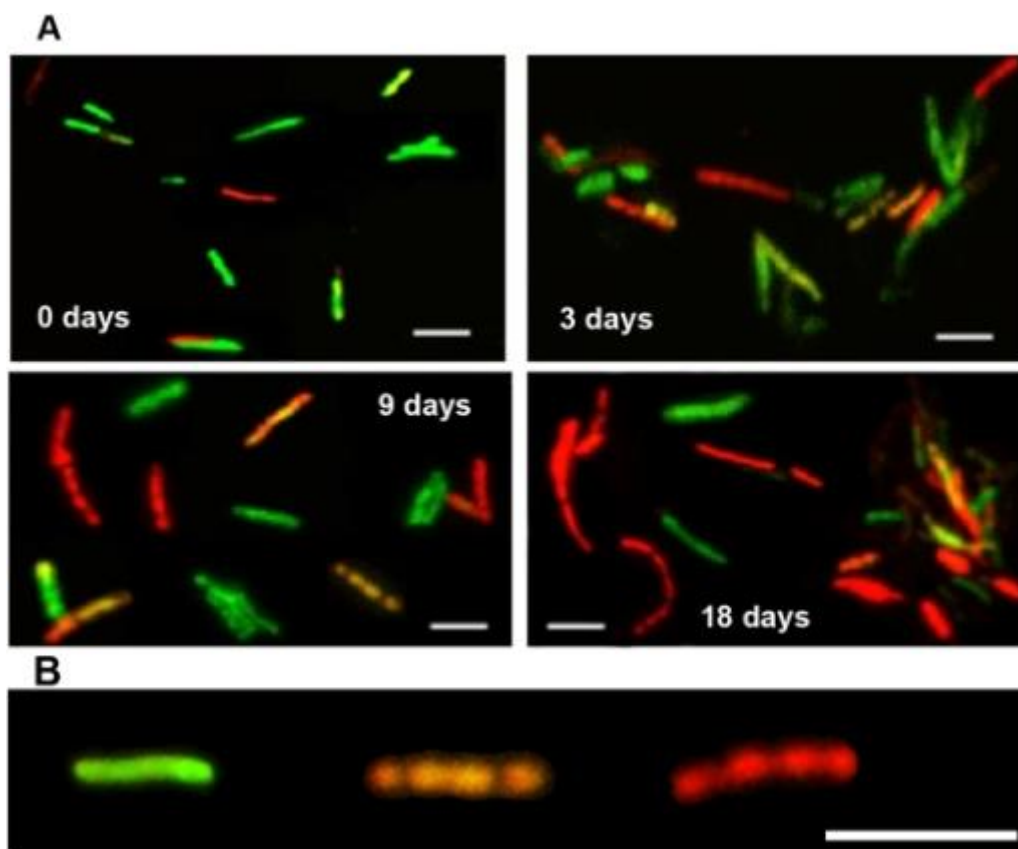
**Figure 1.8**: *M. tuberculosis* accumulates lipid bodies and loses its acid fastness under stress. Panel A shows that multiple stresses can cause a gradual decrease of cells over time stained green by Auramine O (acid fast stain) and an increase of cells stained red by Nile Red (lipid stain), bar: 4 µm. Panel B depicts three distinct magnified phenotypes of *M.tuberculosis*: acid fast positive (green), both acid fast and lipid stain positive (orange/yellow) and lipid stain positive (red), bar: 5 µm (141).

Dormant forms of Mtb are not culturable on solid media but were found to be able to grow in liquid media (146-150). Likewise, Mtb inside alveolar macrophages are loaded with lipid bodies and only grow in liquid media (151,152). In LTBI, the dormant bacilli are not totally inactive, they are able to divide from time to time during latency and the proof of this is that treatment with INH prevents the reactivation of LTBI; INH is only effective on replicating Mtb (153,154). As the conventional culture method on solid agar is inaccurate to estimate the load of dormant bacteria, it is important to develop new tests enabling the determination of the entire bacterial load including dormant Mtb. This would help to evaluate the response of both dormant and replicating bacilli to anti-TB drugs and to evaluate which drugs are able to kill dormant forms of Mtb and by this to shorten TB therapy.

## 1.6.    Vaccines against tuberculosis

Albert Calmette and his colleague Camille Guerin have started to develop the first TB vaccine known as Bacillus Calmette-Guerin (BCG) in 1908 at the Institute Pasteur in Lille, France (155). It is prepared from *Mycobacterium bovis* which has lost its virulence to humans by being repeatedly subcultured into Middlebrook 7H9. This vaccine was used for the first time in humans in 1921 (156) and was approved by the Health Committee of the League of Nations (former WHO) in 1928. Its efficacy depends on the BCG strains used, changes in environment, interference with other bacterial infections (non-tuberculous mycobacteria or concurrent parasitic infections) and conditions in the laboratory where the bacteria were grown (e.g. exposure to ultraviolet light) (157,158). BCG has its greatest effect in preventing miliary TB and TB meningitis in children (159) but 20 years after vaccination its grade of protection against pulmonary TB is variable from 60 to 0%. BCG as a live vaccine requires the replication in the host and cannot be used as a booster vaccine due to its cross-reaction with pre-existing immune responses.

New vaccines are currently tested in clinical trials at different stages. These new vaccines use proteins from culture filtrates secreted by actively growing mycobacteria. Examples are the Early Secretary Antigen Target-6 (ESAT-6), the Modified Vaccinia Ankara 85A (MVA85A), the recombinant Bacillus Calmette–Guerin 30 or rBCG30, the 72F Fusion Protein Vaccine (a combination of protein Rv0125 and Rv1196 from the bacterial Antigen 85A and B (Ag85A/B)) and the TB 10.4 vaccine. All these vaccines showed a good protective efficacy against an infection with Mtb *in vitro*, whereas MVA85A seems to be the best booster candidate (160).

## 1.7.    Biomarkers to diagnose tuberculosis

### 1.7.1.  Chest X-ray

The radiographic examination of the chest using X-ray (chest X-ray) is a standard method to evaluate patients suspected to have pulmonary TB. The cavitary opacity in the apical and posterior segments of the upper lobes or in the superior segments of the lower lobes is the characteristic feature found on radiologic images of pulmonary TB (Figure 1.9) (161-164). The chest X-ray is a vital test to examine any complications due to TB. It is not specific for TB, though, as it can appear normal while the lung is infected. Therefore, each chest X-ray has to be confirmed by sputum smear microscopy to confirm the presence of Mtb; an ordinary chest X-ray done after 6 months of treatment together with the conversion of sputum from culture positive to negative confirm that TB is cured (165).

The Chest Computed Tomography (CCT) is a more advanced technology and is able to identify small lung lesions which are not detected by chest X-ray (161,163).
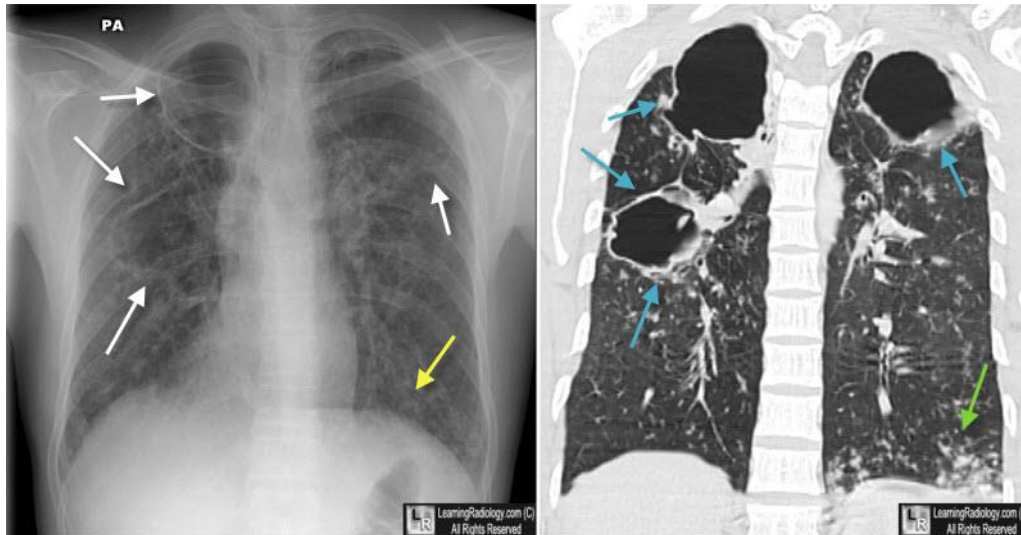


**Figure 1.9**: Typical image of lung cavities on chest X-ray (left image) and CCT (right image). The pictures show large cavities in both lung apices (white arrows on X-ray and blue arrows on CCT). The yellow arrow on the chest X-ray indicates an airspace disease at the left base, whereas the green arrow on the CCT image indicates a consolidation of lung tissue at the left base.

Adapted from http://www.learningradiology.com/notes/chestnotes/tbpage.html.

## 1.7.2.  Sputum smear microscopy

Sputum is material coughed up by TB patients from their lower lung airways. The confirmation of pulmonary TB relies on the presence of Mtb bacilli also known as Acid Fast Bacilli (AFB) on sputum smear. Ziehl-Neelsen (ZN) staining of sputum smears followed by light microscopy reading is the most used and accessible diagnostic method for TB in low and middle income countries. The technique is fast, inexpensive, reproducible, effective and highly specific to detect Mtb (166,167). On the other hand, ZN microscopy requires well trained technologists, is not very sensitive (168) and does not detect resistances to antibiotic drugs. A more sensitive method is fluorescence microscopy on sputum smears stained with Auramine O (169) which is used as an alternative to ZN microscopy (Figure 1.10). Sputum smear microscopy is used to monitor the treatment of TB and the conversion of smears after two months of therapy from positive to negative is considered as an indication of cure (170-172).

But since the sensitivity of sputum smear microscopy is poor, the conversion of culture is considered the most reliable biomarker for treatment success (173). Because of this, new tests are urgently needed that are more sensitive, more specific and better able to identify drug-resistant Mtb in order to better monitor treatment outcome and to ensure that the correct regimen is rapidly initiated to avoid the spread of MDR-TB.
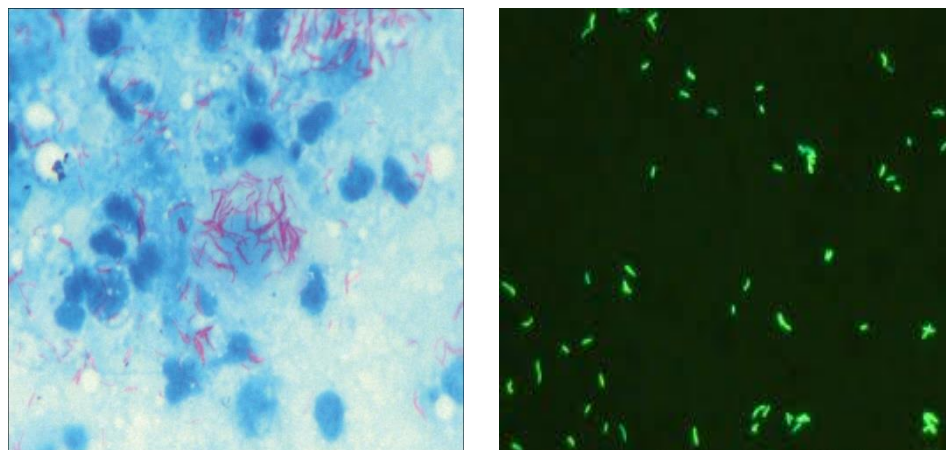


**Figure 1.10**: Images of *Mycobacterium tuberculosis* stained by Ziehl-Neelsen (ZN) and Auramine O. Mtb stained by ZN appear in pink colour on blue background (left image) (174) whereas they appear in green colour on dark background once stained by Auramine O (right image) (Adapted from http://www.int.laborundmore.com/archive/343509/when-tuberculosis-faces-an-even-stronger-opponent.html).

## 1.7.3. Colony forming unit on solid agar media

CFU count on solid agar plates is a quantitative method used to enumerate viable mycobacteria and is the conventional method for quantifying mycobacteria (175). It was first used by Fenner and co-authors to quantify mycobacteria in biological specimens (176). Since the discovery of SM, the determination of CFU is utilized to assess the effectiveness of standard and novel drugs to treat TB (177,178). However, this method has drawbacks namely the long incubation time (3 weeks), the occurrence of contaminations, the overcrowding of colonies and the requirement of expensive infrastructure like a Biosafety level 3 laboratory. The long incubation time is caused by the slow growth of Mtb (15-20 hours for one replication) whereas the overcrowding of colonies or contaminations complicate the determination of the correct number of colonies (179). The methodology of CFU count is used in South Africa, Thailand and Kenya (180).

In clinical trials, particularly in EBA studies, the killing activity of each drug can be calculated by the decrease in CFU counts and drugs can be compared to each other. However, an overcrowding of colonies may generate false positive results (181) and an accurate counting of CFU needs the

full attention of the performer as it is subject to errors (182). Sputum samples contain Mtb with different metabolic activity (dormant, slow growing and fast growing), and dormant and slow growing bacteria are unable to grow colonies on solid media. The counting of CFU is not accurate but it is selective and does not represent all forms of *M. tuberculosis* present in sputum. This indicates that CFU counting represents only the actively dividing forms of Mtb and that other tests are needed that can quantify both replicating and dormant forms at the same time.

## 1.7.4. Time to positivity of liquid media

The assessment of TTP of liquid media is a diagnostic method designed as an indicator of metabolic activity but not quantitative mycobacterial load (183,184). This test uses the Mycobacteria Growth Indicator Tube (MGIT) containing 7H9 liquid medium which is incubated in an automated system (BACTEC MGIT960) using fluorescence sensors to detect growing bacteria by the amount of oxygen consumed in the tube (185,186). Decontaminated sputum samples are normally used as inoculum for MGIT and incubated until the tube is determined positive for growth by the system. Specimens from smear-positive TB patients need an average of 6-10 days incubation to detect bacterial growth. TTP determination was shown to be the best replacement for CFU counting, the conventional culture method for quantification of Mtb (75).

TTP may not detect all forms of Mtb in sputum but it will likely detect some forms that are not represented by CFU counting. It was shown that bacilli from chronic infections in mouse grow in liquid culture but not on solid media (148), showing that liquid culture may be a better tool in evaluating drugs active against dormant Mtb (74). The MGIT system enables most of mycobacterial species to grow (187) and detects them in short time (188).

In EBA studies, TTP was found to correlate well with CFU count during the first two weeks of treatment (75,189,190), with the severity of the disease and with the treatment outcome in TB patients. Liquid media has been found to be more sensitive than solid media to detect Mtb in sputum samples (190,191).

## 1.7.5. Xpert MTB/RIF assay

The Xpert MTB/RIF assay (Xpert) is a real time PCR based test to diagnose the presence of Mtb and resistance to RMP at the same time in processed or unprocessed sputum samples (192-194). It was named a TB diagnosis game changer (195,196) and it was endorsed by the WHO in 2010 as a new TB diagnostic method for HIV-TB co-infected patients and for patients with susceptible or drug resistant TB (197). Unlike the conventional culture methods, it provides fast results in less than 2 hours (Figure 1.11).

It is highly sensitive with a low limit of detection equivalent to 131 CFU/ml sputum sample and has a high specificity of 95% (192,194). Like other PCR based techniques, it detects DNA from dead and viable mycobacteria therefore its usefulness for monitoring TB treatment is limited since it uses sputum which contains a mixture of live, dead and injured cells of Mtb.

The reagent Propidium monoazide (PMA) is able to penetrate dead or injured bacteria and to bind to intracellular DNA after activation by light preventing its amplification by PCR (198). Using PMA together with Xpert, Miotto and co-authors showed that this combination can be used as a new method to monitor TB treatment by excluding non-viable cells (199). Considering Xpert's advantages of a speedy and specific diagnosis, its combination with PMA could be a candidate biomarker to be used in EBA studies.
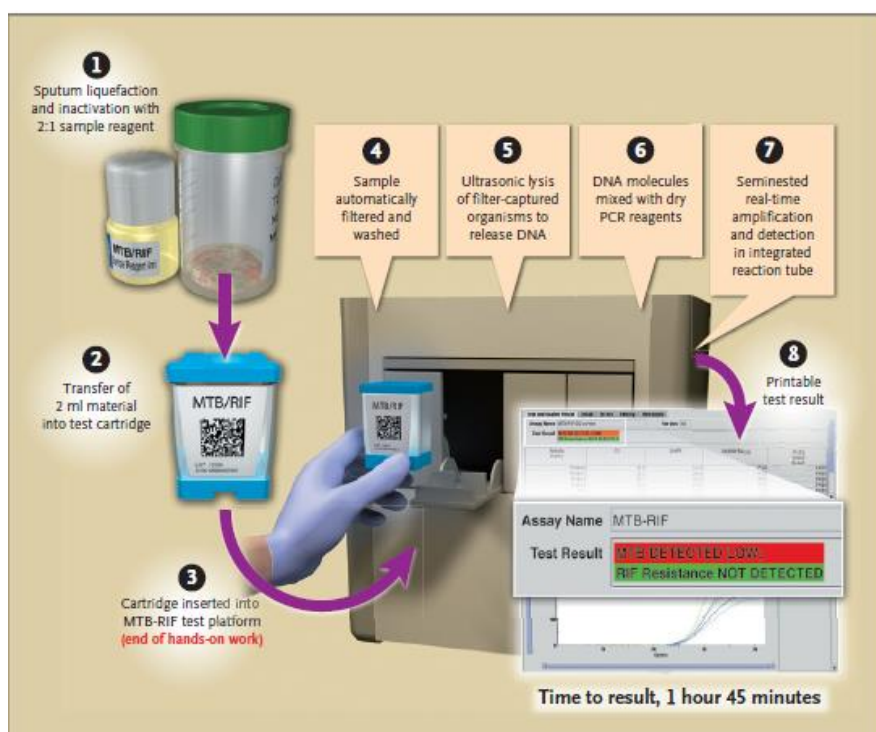


**Figure 1.11**: A summary of all processing steps of the Xpert MTB/RIF assay for sputum samples. The assay has the advantage of being useable for both processed and unprocessed sputum samples. The results are available in less than two hours (192).

## 1.7.6. Line probe Assay

Another PCR based technique to detect Mtb and its resistance against RMP (*rpo*B gene) and/or INH (*kat*G and *inh*A genes) in microscopy-positive sputum samples is the Line Probe Assay (LPA) (200). In 2008, LPA was endorsed by the WHO as a diagnostic test for the detection of drug resistance in TB patients being at high risk of developing MDR-TB (201). Currently, there are three commercial LPAs available on the market: INNO-LiPA Rif.TB test, GenoType

MTBDR*plus* and Genotype MTBDR*sl* (202). These LPAs test for Mtb's susceptibility to first and second line anti-TB drugs and are highly sensitive and specific methods, giving results in 5 hours. However, any LPA test cannot replace the conventional method with drug containing liquid culture (DST) as it is only useable on smear positive sputum samples. Smear negative sputum samples always need a culture to rule out any resistances of Mtb (201,203).

## 1.7.7. Immunological biomarkers to diagnose tuberculosis

Once immunocompetent people are infected with Mtb, the bacilli are either killed and eliminated from the body or contained in lung granulomas by the immune system. There the bacilli can remain in a dormant state known as LTBI (119). A positive diagnosis of LTBI is very important in immunosuppressed people as they are at higher risk of developing active TB. The Interferon-γ Release Assay (IGRA) measures interferon-γ in blood secreted by T cells in response to Mtb specific antigens (ESAT-6 and CFP-10) (204,205). This test was found to be more sensitive than the Tuberculin Skin Test (TST) which is in use for more than a century. Unfortunately, none of these two tests are able to distinguish LTBI from active disease. Furthermore, a positive TST result is not specific for Mtb due to the tuberculin purified protein derivative used which contains antigens also found in BCG and in Non-Tuberculous Mycobacteria species (206,207).

## Thesis outline

The aim of this work was to investigate and provide answers to different questions as described in the subsequent chapters of this book. Firstly, we evaluated the performance of Xpert to monitor the outcome of TB treatment, secondly we assessed the effects of SQ109, RMP and SQ109/RMP combined on different mycobacterial subpopulations present in sputum samples and lastly we determined the kinetic of RMP resistance under monotherapy.

**In chapter two** we compared the EBA of SQ109, RMP and SQ109/RMP measured by CFU count, TTP and Xpert. Xpert showed an inferior performance in assessing an EBA in comparison to CFU or TTP during the first two weeks of treatment. The most likely explanation for this is that the standard Xpert protocol does not distinguish DNA from dead and viable cells.

To address this shortcoming, we evaluated the beneficial role of PMA used together with Xpert to prevent the amplification of DNA from dead bacilli present in the samples under treatment **in chapter three.** Surprisingly, a beneficial effect of PMA added to both sputum samples and cultures grown in the presence of bactericidal compounds was not as distinct as described in

previous studies. Therefore, we cannot recommend the use of PMA with Xpert in routine diagnostics or clinical drug trials.

It was shown that Mtb accumulates lipid bodies in its cytoplasm when exposed to certain stress. **Chapter four** details the dynamic of dormant (lipid body loaded Mtb), slow growing and fast growing mycobacteria over two weeks of experimental treatment. Sputum samples from TB patients were stained by a combination of Auramine O and Nile Red and analysed by confocal fluorescence microscopy. The investigational drug SQ109 has a poor EBA and increases the number of dormant mycobacteria probably due to a bacteriostatic activity. Contrary to this, RMP showed a good EBA with a decrease in replicating (fast growing) forms of Mtb and an increase in its dormant forms at the same time.

RMP is considered the cornerstone of TB therapy and the resistance to it as biomarker of MDR-TB leading to treatment failure and death. **Chapter five** describes investigations conducted on the development of resistance against RMP in Mtb isolated from TB patients under monotherapy for two weeks. We determined CFU counts and the proportion of RMP resistant mutants (mutation frequency) in both baselines and day 14 samples. The results received were used to statistically model the time point when a clinically relevant resistance would be reached in case of RMP monotherapy. The outcome was that RMP monotherapy were to be pursued for at least one month for RMP resistance to reach a significant level.

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# Chapter two

# Direct comparison of Xpert MTB/RIF with liquid and solid mycobacterial culture for the quantification of early bactericidal activity.

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## Abstract

The early bactericidal activity of antituberculosis agents is usually determined by measuring the reduction of the sputum mycobacterial load over time on solid agar media or in liquid culture. This study investigated the value of a quantitative PCR for early bactericidal activity determination. Groups of 15 patients were treated with 6 different antituberculosis agents or regimens. Patients collected sputum for 16 hours overnight at baseline and at day 7 and 14 after treatment initiation. We determined the sputum bacterial load by colony forming unit counting (CFU, logCFU/ml sputum ±SD), time to culture positivity (TTP, hours ±SD) in liquid culture and Xpert MTB/RIF cycle thresholds ($C_T$, $n$ ±SD). The ability to discriminate treatment effects between groups was analyzed with One-Way-ANOVA. All measurements showed a decrease in bacterial load from mean baseline (logCFU: 5.72 ±1.00; TTP: 116.0 ±47.6; $C_T$: 19.3 ±3.88) to day 7 (logCFU: -0.26 ±1.23, $P$ = 0.2112; TTP: 35.5 ±59.3, $P$ = 0.0002; $C_T$: 0.55 ±3.07, $P$ = 0.603) and day 14 (logCFU: -0.55 ±1.24, $P$ = 0.0006; TTP: 54.8 ±86.8, $P$ < 0.0001; $C_T$: 2.06 ±4.37, $P$ = 0.002). The best discrimination between group effects was found with TTP at day 7 and day 14 (F = 9.012, $P$ < 0.0001 and F = 11.58, P < 0.0001), followed by logCFU (F = 4.135, $P$ = 0.0024 and F = 7.277, $P$ < 0.0001). $C_T$ was not significantly discriminative (F = 1.995, $P$ = 0.091 and F = 1.203, $P$ = 0.316, respectively). Culture based methods are superior to PCR for the quantification of early anti-tuberculosis treatments effects in sputum.

**My contribution: Planning experiment**

    **Laboratory experiment**

    **Writing the manuscript**

## 2.1. Introduction

Several novel anti-tuberculosis drugs and regimens are currently under clinical investigation. The first step in their evaluation is the measurement of early bactericidal activity (EBA) in sputum over up to 2 weeks of treatment in smear-positive, treatment-naïve pulmonary tuberculosis patients. The EBA is commonly defined as the mean daily fall of colony forming units counted on agar plates (log CFU) per ml of expectorated sputum (1,2). Alternatively, the change in time to culture positivity (TTP) in broth culture can be measured in the Mycobacterial Growth Indicator Tube system (MGIT; Becton Dickinson, Sparks, USA) (3). This is based on the inverse relationship of the time a culture requires to develop a critical measure of metabolic activity to the number of viable bacteria initially inoculated into the system. TTP in semi-automated liquid culture has been shown to correlate well with CFU counting in the first two weeks of treatment (3-5).

The Xpert MTB/RIF assay (Xpert; Cepheid, Sunnyvale, CA) is a new nucleic acid amplification test (NAAT) detecting *M. tuberculosis* complex on sputum specimens with real time PCR. Xpert is rapid (less than one hour hands on time), standardized, and easy to use (6,7). Its sensitivity to detect *M. tuberculosis* in sputum of untreated tuberculosis suspects in high burden countries is 98% for AFB positive samples and 72% for AFB negative specimens with an overall specificity of >99% (8). Xpert is able to detect as few as 100 organisms per ml *in vitro* suspension (9). It is less well known that Xpert also provides a semi-quantitative measurement based on the number of PCR cycles required for detection of a critical amount of DNA (cycle threshold, $C_T$). It was repeatedly demonstrated on sputum samples collected from untreated tuberculosis patients that the quantitative $C_T$ read-outs of Xpert correlate with (semi-) quantitative results of conventional microbiological tests such as smear microscopy grade, liquid culture TTP, and solid culture CFU counts (10-13).

As a platform for quantifying *M. tuberculosis* in sputum Xpert could make EBA studies less costly, more reproducible, and technically more accessible if the change in $C_T$ was a reliable measure of treatment effects. However, it is unclear how DNA load correlates with culture based quantification of viable bacteria in patients being treated for tuberculosis. To investigate this question we performed a prospective study to compare the ability of logCFU, TTP, and Xpert $C_T$ to monitor and distinguish between treatment effects in a 14-day EBA study with 6 parallel groups receiving different antituberculosis treatments.

## 2.2.    Material and methods

### 2.2.1. Sample collection and ethics

Sputum samples were collected during a 14-day early bactericidal activity study from untreated, adult, pulmonary tuberculosis patients. Participants were required to be smear-positive (≥1+; WHO/IUATLD scale) (14) and free of severe co-morbidities. HIV positive patients with a CD4-count of <250/µl and those on antiretroviral therapy were excluded. From December 2010 until August 2011 90 patients (59% male, 10% HIV-positive, median age: 30.4 years) were hospitalized at one of two centres in Cape Town, South Africa (University of Cape Town Lung Institute, Mowbray; Task Applied Science, Intercare, Durbanville) and randomized to one of six equally sized groups that were treated with different dosages and/or combinations of two anti-tuberculosis agents (RMP alone 10 mg/kg/day, SQ109 300 mg/day + RMP 10 mg/kg/day, SQ109 alone 150 mg/day, SQ109 alone 300 mg/day, SQ109 alone 150 mg/day + Rifampicin 10 mg/kg/day and SQ109 alone 75 mg/day). All patients' isolates were susceptible to the treatment given. Spontaneously expectorated sputum was collected for 16 hours overnight, refrigerated after collection and transported to the central study laboratory (Centre of Clinical Tuberculosis Research, Department of Biomedical Sciences, Stellenbosch University). Specimens were allowed to warm up to room temperature before homogenization for 30 minutes with a magnetic stirrer and processing. The institutional ethical committees and the Medicines Control Council (MCC) granted approval for the main study and this sub study with ethics number: N11/09/294.

### 2.2.2. Determination of logCFU

A maximum of 10 ml of homogenized sputum was digested by addition of an equal volume of 0.1% dithiothreitol (Sputasol; Oxoid, Cambridge, UK) for 20 minutes. Sterile saline (0.85%) containing 0.01% Tween was used to generate 10-fold serial dilutions. A volume of 100 µl of every dilution was incubated on each half of two selective agar plates containing Middlebrook 7H11 agar enriched with Middlebrook OADC (oleic acid-albumin-dextrose-catalase, Becton Dickinson) and Selectatab (Mast; Bootle, Merseyside, UK) containing polymyxin B sulphate (200 units/ml), amphotericin B (10 mg/l), carbenicillin (50 mg/l) and trimethoprim (20 mg/l). The plates were incubated at 37ºC for 3 to 4 weeks before colonies were counted on the dilution that yielded between 20 and 200 colonies. These data were log transformed to provide logCFU.

### 2.2.3. Determination of TTP

Five ml of digested sputum was mixed with an equal volume of 2% NaOH (BBL Mycoprep; Becton Dickinson) and decontaminated for 20 minutes at room temperature. This mixture was

neutralized by addition of sterile phosphate buffered saline (PBS, pH 6.8; Becton Dickinson) to a final volume of 45 ml and centrifuged for 15 minutes at 3,000 X g and 4°C. The supernatant was discarded and the pellet re-suspended with PBS to a final volume of 2 ml. Two MGIT for each specimen were prepared by addition of 0.8 ml PANTA containing polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin mixed with OADC (Becton Dickinson). Each MGIT was inoculated with 0.5 ml of re-suspended pellet and incubated at 37ºC in a BACTEC MGIT960 instrument. Cultures flagged positive were tested for contamination by placing one drop on a blood agar plate (NHLS, Cape Town, South Africa) and incubating at 37°C for 48 hours. The presence of acid-fast bacilli (AFB) in positive liquid culture was confirmed by Ziehl-Neelsen staining and microscopy; only TTPs from AFB positive and non-contaminated cultures were used for analysis.

## 2.2.4. Xpert MTB/RIF assay

An aliquot of digested sputum sample (1 - 2 ml) was frozen at -80°C at the time of culture processing. After thawing, the specimen was re-suspended by vortexing and 1 ml used according to the instructions of the manufacturer. Briefly, the diluted sample was mixed with 2 ml of Xpert sample reagent, 10 times inverted, and incubated for 15 minutes at room temperature; inversion was repeated after the first 8 minutes. This mixture was then transferred into an Xpert cartridge and loaded into the GeneXpert instrument, which conducts all necessary steps automatically using GeneXpert Dx Software (version 4.0, Cepheid). The software reports results as $C_T$s which represent the number of PCR cycles needed to reach a detection threshold; $C_T$ of probe B was used for all analyses according to previous published data (6).

## 2.2.5. Analysis plan

From the sputum samples available from the EBA study we prospectively selected the two baseline samples collected on the days directly preceding the treatment initiation, and those collected after 7 and 14 days of treatment when we expected treatment effects to emerge. We analyzed the data in stepwise fashion. Firstly, we studied the baseline samples to gain an impression of the variation of the measurements without treatment effects. Secondly, individual values were plotted against one another and inspected for correlation. Next, we examined the correlation of individual treatment activities for all measurements. Finally, we quantified the group effects and the respective ability of the measurements to differentiate between group effects by comparing variation within the groups to the variation between the groups for the periods 0-7 days and 0-14 days.

## 2.2.6. Statistical methods

The group sample size of 15 patients and study duration of 14 days were pre-determined by the parent EBA study. Correlation between the methods of detection was quantified with Spearman rank sum test. Treatment activity was calculated as the arithmetic difference between the means of the baseline values (also termed day 0) and the values obtained after 7 or 14 days, first for each individual and then averaged to obtain a group result. The significance of treatment effects within groups from day 0 to 7 or day 0 to 14 was determined by t-tests. Variance within groups and between groups was compared with F-statistics (One-Way-ANOVA). The larger the F-statistic the better the chance that a measurement can differentiate group effects. A *P* value < 0.05 was considered statistically significant.

## 2.3.    Results

### 2.3.1. LogCFU, TTP, and $C_T$

Out of a possible 360 values 304 logCFU (84.4%), 341 TTP (94.7%), and 317 $C_T$ (88.1%) results were obtained. The missing values were due to contamination, no growth, no sample received, or invalid results. There was no significant difference between groups regarding the proportions of available data or the magnitude of the mean baseline values. The day-to-day variance estimated using the baseline values within subjects was compared with the total baseline variance to give ratios of 0.1621/0.9853 = 0.165 for logCFU, 748.7/3448.5 = 0.220 for TTP and 12.25/6.94 = 0.567 for $C_T$. While 16.5% and 22% are similar the 56.7% of $C_T$ suggests a larger day-to-day variability and smaller between subject variability for $C_T$ than for the culture based methods logCFU and TTP. Individual logCFU, TTP, and $C_T$ values were moderately correlated at baseline, day 7, and day 14 and are illustrated in Figure 2.1A, B&C.

**C**
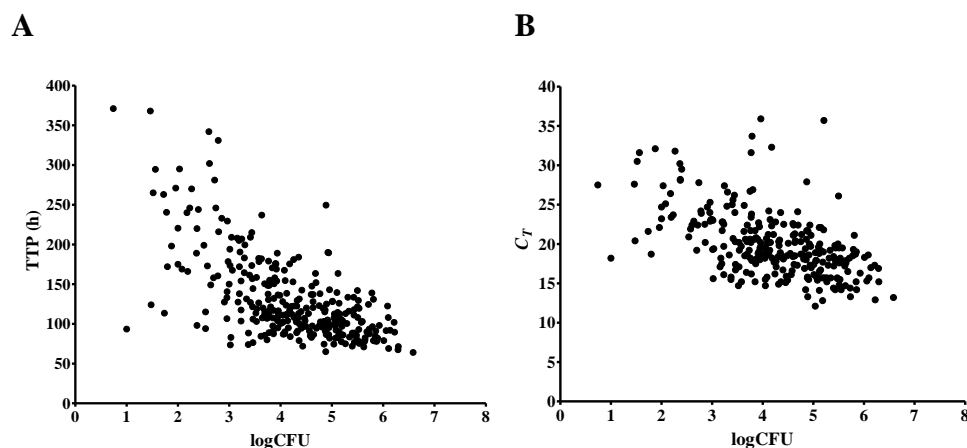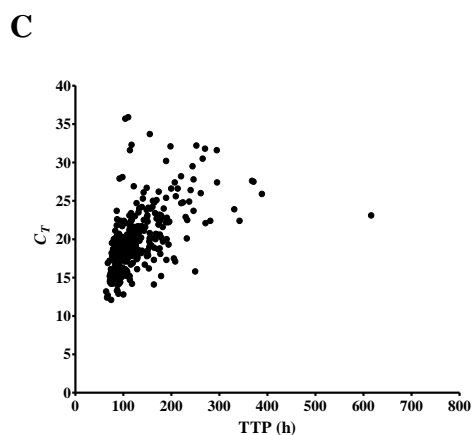


**Figure 2.1:** Individual logCFU, TTP, and $C_T$ values are shown for all groups and time points combined. Rho-values were calculated by Spearman rank sum test for TTP vs logCFU (rho = -0.613; $P < 0.0001$) (2.1A), logCFU vs $C_T$ (rho = -0.547; $P < 0.0001$) (2.1B) and TTP vs $C_T$ (rho = 0.632; $P < 0.0001$) (2.1C).

## 2.3.2. Treatment effects measured with logCFU, TTP and $C_T$

Individual treatment effects were strongly correlated between logCFU and TTP; logCFU and $C_T$ and TTP and $C_T$ were moderately correlated (Figure 2.2).

All three measurements showed an overall decrease from baseline, which was significant for TTP at day 7 and for all three measurements at day 14 (Table 2.1, Figure 2.2). A significant difference between baseline and day 7 and/or day 14 was found in 4 groups with logCFU, in 5 groups with TTP and in 2 groups with $C_T$. The real test for discrimination between treatment effects at day 7 and day 14 is shown in Table 2.2. One-Way-ANOVA delivered the greatest F-values for TTP indicating that liquid culture had the most favourable ratio of within group versus between group variation to discriminate between groups for treatment effects at day 7 and day 14. LogCFU from solid culture medium displayed acceptable discriminatory ability while quantitative PCR represented by Xpert $C_T$ was relatively poor.

**A**



**B**

**C**
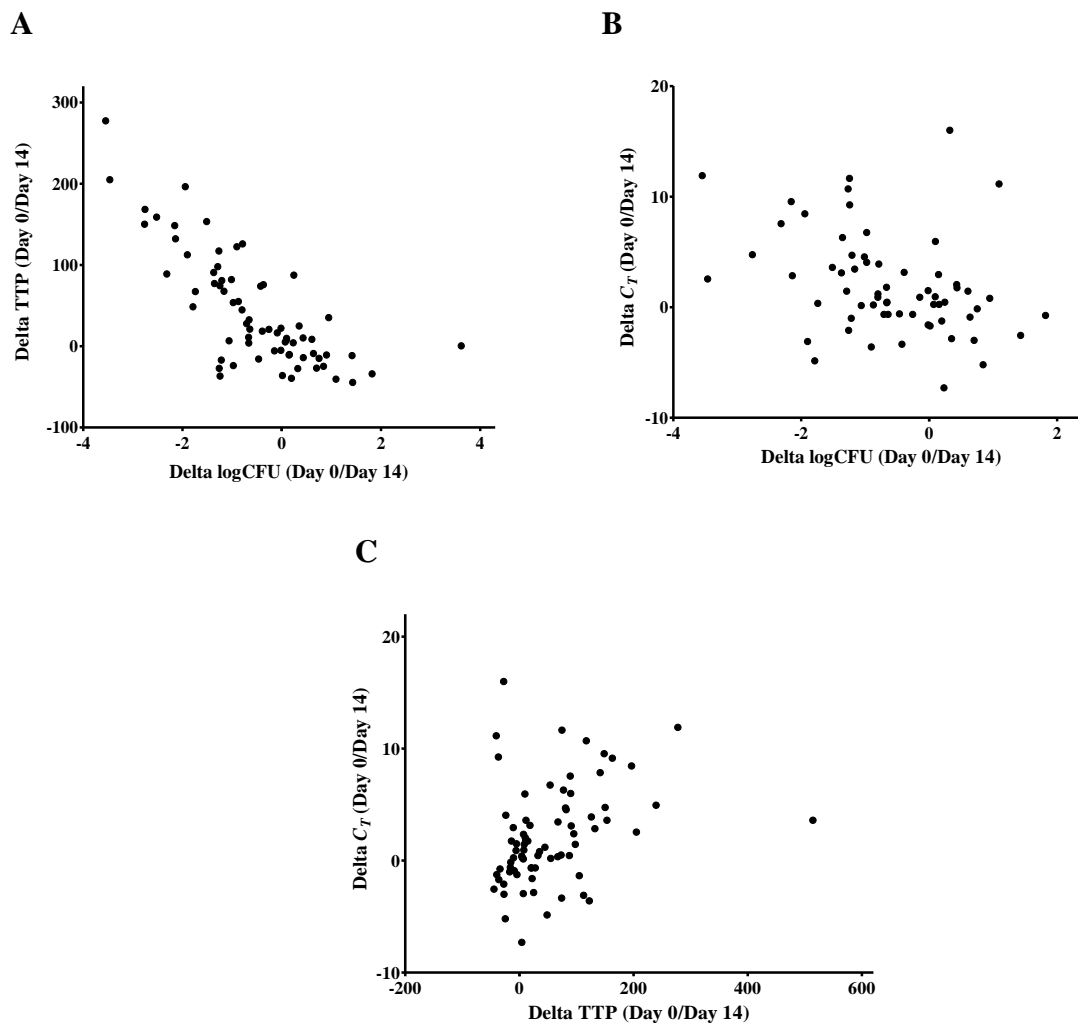
**Figure 2.2:** Individual activities of logCFU, TTP, and $C_T$ from day 0 to day 14 are plotted against each other. A strong correlation was found between logCFU and TTP (rho = -0.726; $P < 0.0001$) (2.2A), and a moderate correlation between logCFU and $C_T$ (rho = -0.385; $P = 0.0017$) (2.2B) and TTP and $C_T$ (rho = 0.400; $P = 0.0004$) (2.2C).
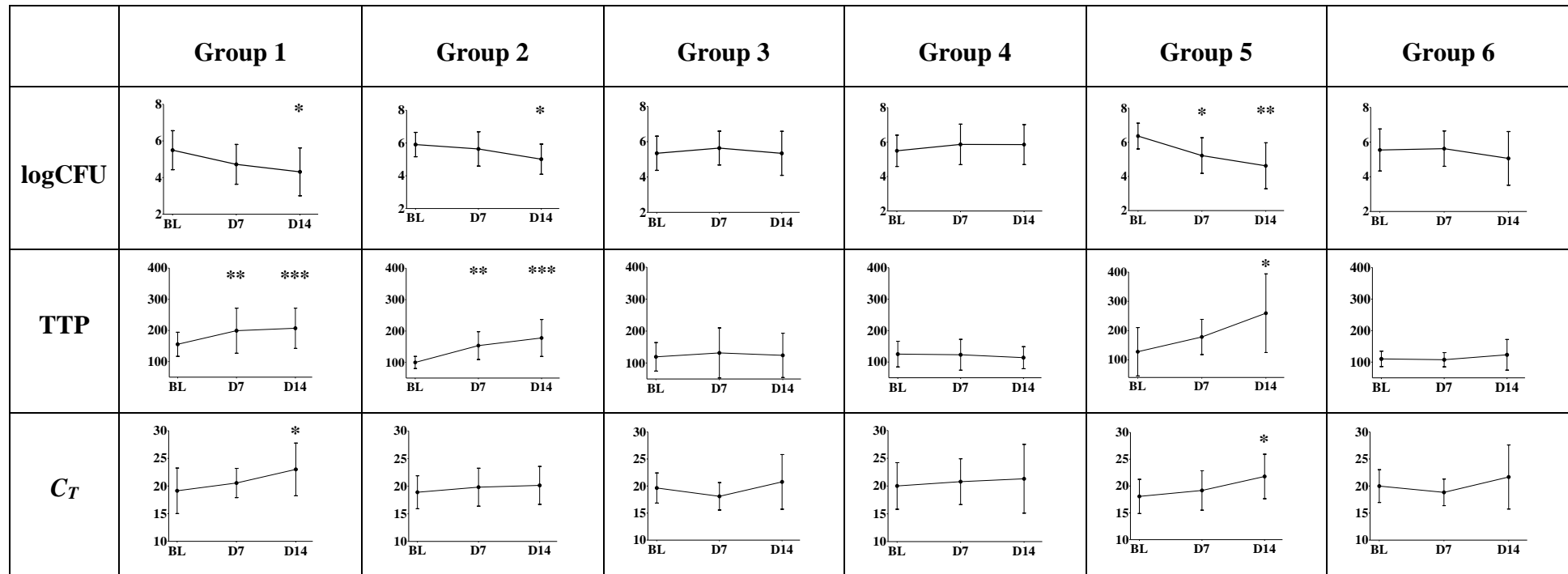
**Figure 2.3:** The mean change from baseline (±SD as vertical bars) of logCFU, TTP, and $C_T$ after 7 and 14 days of treatment is shown for all six groups. Significantly different measurements for days 0-7 or days 0-14 were found for TTP in 2 and 3, for logCFU in 1 and 3 and for $C_T$ in 0 and 2 groups, respectively. Statistically significant differences between time points are indicated by stars (* = $P < 0.05$, ** = $P < 0.001$, *** = $P < 0.0001$).

|  |  | *n* | Baseline | *n* | Day 0-7 | *P* | *n* | Day 0-14 | *P* |
|---|---|---|---|---|---|---|---|---|---|
| **logCFU** | Group 1 | 14 | 5.51 (1.15) | 11 | -0.94 (1.76) | 0.096 | 12 | -0.97 (1.35) | 0.018 |
|  | Group 2 | 15 | 5.91 (0.77) | 14 | -0.32 (1.18) | 0.429 | 13 | -0.97 (0.72) | 0.009 |
|  | Group 3 | 14 | 5.42 (0.97) | 13 | +0.47 (1.41) | 0.434 | 12 | +0.17 (1.41) | 0.994 |
|  | Group 4 | 14 | 5.48 (0.91) | 12 | +0.19 (0.56) | 0.367 | 12 | 0.39 (0.76) | 0.385 |
|  | Group 5 | 13 | 6.34 (0.79) | 12 | -1.17 (0.82) | 0.004 | 10 | -1.84 (1.03) | 0.0006 |
|  | Group 6 | 13 | 5.67 (1.17) | 13 | +0.08 (0.47) | 0.860 | 12 | -0.28 (0.71) | 0.384 |
|  | Total | 83 | 5.72 (1.00) | 75 | -0.26 (1.23) | 0.2112 | 71 | -0.55 (1.24) | 0.0006 |
| **TTP (hours)** | Group 1 | 15 | 115.6 (39.4) | 15 | +83.7 (73.7) | 0.0005 | 15 | +91.5 (77.5) | <0.0001 |
|  | Group 2 | 15 | 100.0 (20.6) | 15 | +53.7 (40.6) | 0.0002 | 15 | +78.1 (54.5) | <0.0001 |
|  | Group 3 | 15 | 120.4 (56.0) | 14 | +10.6 (62.2) | 0.607 | 12 | +6.68 (36.7) | 0.835 |
|  | Group 4 | 15 | 125.7 (42.4) | 15 | -2.13 (23.2) | 0.897 | 15 | -11.3 (24.9) | 0.423 |
|  | Group 5 | 15 | 124.3 (77.4) | 14 | +70.5 (50.9) | 0.073 | 13 | +151.1 (125.1) | 0.004 |
|  | Group 6 | 15 | 110.1 (24.5) | 15 | -2.52 (21.9) | 0.770 | 14 | +13.2 (41.0) | 0.365 |
|  | Total | 90 | 116.0 (47.6) | 88 | +35.5 (59.3) | 0.0002 | 84 | +54.8 (86.8) | <0.0001 |
| $C_T$ | Group 1 | 14 | 19.1 (4.57) | 11 | +2.33 (2.80) | 0.331 | 14 | +3.87 (5.75) | 0.030 |
|  | Group 2 | 14 | 18.9 (3.81) | 12 | +1.06 (3.18) | 0.476 | 15 | +1.27 (3.32) | 0.311 |
|  | Group 3 | 14 | 19.6 (3.54) | 10 | -0.92 (2.71) | 0.181 | 12 | +0.61 (3.86) | 0.478 |
|  | Group 4 | 14 | 20.1 (4.45) | 14 | +0.77 (2.18) | 0.628 | 14 | +1.29 (3.60) | 0.524 |
|  | Group 5 | 13 | 17.9 (3.27) | 12 | +0.91 (3.72) | 0.425 | 11 | +3.56 (2.63) | 0.021 |
|  | Group 6 | 14 | 20.0 (3.34) | 14 | -0.87 (3.05) | 0.277 | 15 | +1.85 (5.58) | 0.350 |
|  | Total | 83 | 19.3 (3.88) | 73 | +0.55 (3.07) | 0.603 | 81 | +2.06 (4.37) | 0.002 |

**Table 2.1:** Mean values (±SD) of logCFU, TTP, and $C_T$ for treatment groups at baseline and change from baseline observed at day 7 and day 14. All *P* values were calculated using unpaired Student's t-test (*n*: number of patients with measurements).

| Activity period | $n$ | logCFU | | TTP | | $C_T$ | |
|---|---|---|---|---|---|---|---|
| | | F | $P$ | F | $P$ | F | $P$ |
| Day 0-7 | 6 | 4.135 | 0.0024 | 9.012 | < 0.0001 | 1.995 | 0.091 |
| Day 0-14 | 6 | 7.277 | < 0.0001 | 11.58 | < 0.0001 | 1.203 | 0.316 |

**Table 2.2**: One-Way-ANOVA of treatment activities over 7 days and 14 days measured with logCFU, TTP, and $C_T$. Greater F values represent better ability to discriminate between groups for treatment effects ($n$: number of treatment groups).

## 2.4. Discussion

This prospective study directly compared three different methods for the quantification of the mycobacterial load in sputum specimens collected 7 and 14 days after initiation of anti-tuberculosis treatment. All three methods were able to demonstrate an average decrease of the mycobacterial sputum load. In comparison, TTP in liquid culture detected treatment effects earlier and more frequently than the other methods, and it discriminated best between treatment groups. CFU counted on agar plates was comparable to TTP. Both culture-based methods had clearly superior discriminatory power to real-time PCR.

Why does PCR in the form of standardized Xpert $C_T$ perform so poorly in the detection of early treatment effects? One can explain this with the ability of Xpert to detect DNA from whole but metabolically inactive or dead bacteria or even free DNA causing false positive signals. For establishing the diagnosis of tuberculosis a high sensitivity is an advantage because the presence of mycobacterial DNA in sputum is associated with disease, and it does not matter whether the DNA detected comes from viable or dead bacteria. For monitoring of treatment effects, however, the distinction between DNA contained in viable mycobacteria and DNA originating from other sources becomes critical because bacteria killed by anti-tuberculosis treatment are subsequently expectorated in sputum and it is known that mycobacterial DNA survives for an extended period (15). This is not encouraging hopes that Xpert could emerge as a treatment monitoring tool for the HIV-positive, smear-negative tuberculosis frequently encountered in countries such as South Africa. A modified protocol for Xpert intended to exclude DNA from non-viable mycobacteria from the reaction could remedy this problem and research in this area is on-going (16).

The analysis reported here is based on a single study of only 14 days duration with only 4 time points assayed. We are confident, however, that the results are valid since TTP and $C_T$ were

similarly well correlated in a previous study at our laboratory with 74 sputum samples collected before treatment (rho = 0.539, present study rho = 0.632) (13). Also, merged data from five different EBA studies revealed very similar correlations between 7-day treatment activities determined by TTP and logCFU (rho = 0.649, present study rho = -0.673) (4).

In conclusion our results demonstrate that culture based methods remain the standard to measure the decline of sputum bacterial load during the early phase of anti-tuberculosis treatment. The Xpert's easy handling and quick results do not compensate for its low discriminatory power between groups with different treatments. Measures that reduce contamination with DNA from non-viable bacteria might transform the value of this technique.

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## Conflict of interests

No contributing author has a conflict of interest to state.

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# Chapter three

# Propidium monoazide and Xpert MTB/RIF to quantify *Mycobacterium tuberculosis* cells

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## Abstract

Propidium monoazide (PMA) penetrates non-viable cells with compromised membranes. PMA has been proposed to improve the specificity of Xpert MTB/RIF (Xpert) for the detection of viable *Mycobacterium tuberculosis*. This study assessed the effect of PMA on Xpert cycle thresholds ($C_T$) of *M. tuberculosis* made non-viable under antibiotic pressure. *In vitro*, we measured the difference between $C_T$ with and without PMA ($\Delta C_T$) in liquid cultures treated with one of six anti-tuberculosis drugs (isoniazid, rifampin, pyrazinamide, ethambutol, streptomycin, moxifloxacin) and found significant $\Delta C_T$ only with isoniazid and ethambutol for pan-susceptible *M. tuberculosis* and only with ethambutol for extensively drug-resistant *M. tuberculosis*. In the clinic we assessed $\Delta C_T$ in sputum samples collected from patients with pulmonary tuberculosis before and at regular intervals over 12 weeks after initiation of treatment. Before treatment start, estimated $C_T$ were 19.3 (95% CI: 17.1-21.4) and 19.8 (95% CI: 17.6-22.1) without and with PMA, respectively. Under treatment $C_T$ increased by 2.54 per $\sqrt{\sqrt{day}}$ (95% CI: 1.38-3.69) without PMA and an additional 0.55 per $\sqrt{\sqrt{day}}$ (95% CI: 0.37–0.74); $p < 0.0001$) with PMA. We conclude that PMA increases the specificity of Xpert for viable *M. tuberculosis* but the effect is small and dependent on the antibiotics used.

**My contribution: Planning experiment**

     **Laboratory experiment**

     **Writing the manuscript**

# 3.1.  Introduction

The Xpert MTB/RIF assay (Xpert; Cepheid, Sunnyvale, CA) is an automated rapid polymerase chain reaction (PCR) based method for assessing the presence or absence of *Mycobacterium tuberculosis* complex by fluorescent probes that interact with PCR products of its DNA (1,2). A multicenter study in patients with suspected pulmonary tuberculosis (TB) returned an overall sensitivity of 98.2% in smear microscopy positive and 72.5% in smear microscopy negative patients compared to fluid culture as reference standard; the total specificity of the assay was 99.2% (3). This low rate of false positive results might be due to a technical detail in the assay as the sample is filtered to wash out DNA not contained within intact cells (4). We recently evaluated Xpert on sputum samples from patients receiving TB treatment which is likely to increase DNA from non-viable bacteria in sputum thereby giving a false positive result on Xpert. We found that the specificity of Xpert compared to a combined reference standard of sputum smear microscopy and liquid culture averaged over 26 weeks of TB treatment was only 48.6% (5). In a separate study our group found quantitative Xpert measurements to have less precision than culture-based methods for determining the decline in mycobacterial sputum load over the first 2 weeks of treatment (6). These results strongly suggest an imperfect selection for viable bacteria if Xpert is used in samples harvested under antibiotic treatment.

A promising method to overcome this problem is the addition of propidium monoazide (PMA) to the sample. PMA is a molecule unable to penetrate the membrane of viable cells. If a cell is unable to exclude PMA from its cytoplasm, it can be considered non-viable; PMA can bind to accessible intracellular DNA and after activation inhibits it as a template for PCR (7). The usefulness of PMA to exclude DNA of dead bacilli from conventional real-time PCR was shown for clinical *M. tuberculosis* isolates and both smear-positive and negative sputum specimens from TB patients (8,9). Furthermore, the addition of PMA to Xpert was found advantageous in a clinical study that included a small number of TB patients and collected sputum samples before and up to 20 days into TB treatment (10,11).

To investigate whether PMA can improve the specificity of Xpert for the detection of viable *M. tuberculosis* we studied clinical bacterial populations made non-viable under the influence of antibiotics *in vitro* and sputum samples collected from 20 pulmonary TB patients over 12 weeks of TB treatment.

## 3.2. Material and methods

### 3.2.1. Patients and materials

Twenty patients participating in a larger observational study cohort contributed sputum samples for this study. Eligible patients had Xpert-positive sputum, HIV co-infection, were 18 years or older, drug-sensitive, treatment-naïve and free of clinically relevant co-morbidities. Each participant gave written informed consent and received standard first-line TB treatment per national guidelines (weight-adjusted combination of isoniazid, rifampin, pyrazinamide, ethambutol for 8 weeks, followed by isoniazid, rifampin for 16 weeks). Ethical approval was received by the local ethics committee (Stellenbosch University, IRB no. N13/07/098).

### 3.2.2. Specimens, transport and processing

Sputum specimens were delivered from two clinics (TASK Delft CHC and Mfuleni) to the central laboratory (Department of Biomedical Sciences, Stellenbosch University, Tygerberg) at 4 to 8 °C. After arrival, the samples were processed or stored refrigerated at 4 to 8 °C for a maximum of two days. The specimen was liquefied for processing by magnetic stirring for 30 minutes at room temperature. An equivalent amount of Sputasol (Oxoid, Cambridge, UK) containing 0.1% dithiothreitol was added and the mixture incubated for 20 minutes to digest. An aliquot of 1 ml was transferred into a sterile tube, decontaminated with an incubation time of 20 minutes and neutralized using the NAC-PAC *Red* system (AlphaTec, Washington, DC, USA) according to the instructions of the manufacturer. The sample was centrifuged (3,000 X g, 15 minutes, 4 °C), the supernatant removed and the remaining pellet resuspended in 2 ml pellet resuspension buffer (AlphaTec).

### 3.2.3. Sample preparation

For the *in-vitro* experiment 16 clinical isolates expected to be pan-susceptible and two known extensively drug resistant (XDR) strains of *M. tuberculosis* were subjected to standard drug susceptibility testing (DST) utilizing the Bactec MGIT kit (Becton Dickinson, Sparks, MD, USA). For this, 0.5 ml of the pellet was inoculated into a Mycobacteria Growth Indicator Tube (MGIT; Becton Dickinson) and incubated at 37 °C in a Bactec MGIT 960 instrument until flagged positive (12). The XDR strains were picked from separately grown Löwenstein-Jensen cultures, transferred into one MGIT each and incubated as above. Equal aliquots of each positive culture were then inoculated into different tubes with antibiotics at the following concentrations: rifampin (RMP, 1.0 µg/ml), isoniazid (INH, 0.1 µg/ml), ethambutol (EMB, 5.0 µg/ml), streptomycin (STM, 1.0 µg/ml), pyrazinamide (PZA, 100 µg/ml) or moxifloxacin (MXF, 0.250 µg/ml) and a drug free growth control diluted 100 times. The tubes were loaded into the

Bactec MGIT960 instrument, automatically monitored for growth comparing the control with drug containing cultures and the tubes removed shortly after the assay was completed. All clinical strains were confirmed susceptible to the respective drug by the instrument software indicating absence of or minimal bacterial growth in all drug containing MGITs (13). The cultures from both XDR strains showed growth in all tubes and were therefore determined as resistant against all six drugs. Every culture was used for Xpert measurement with and without addition of PMA (Biotium Inc., Hayward, CA, USA). For the clinical experiment 20 patients produced sputum at eight time points in total (before treatment, on treatment days 3, 7, 14, 28, 35, 56 and 84). Leftover material of the pellets, when available, was utilized for Xpert measurement with and without addition of PMA.

## 3.2.4.  Xpert MTB/RIF with and without addition of PMA

The protocol for the usage of Xpert and PMA was as per manufacturer's instructions and adapted from Miotto *et al.* [10]. Briefly, a volume of 0.5 ml of culture medium or resuspended pellet was transferred into a translucent 1.5 ml Eppendorf tube and carefully protected from light at all times, mixed with 10 µl PMA solution (20 mM) or 10 µl distilled water, incubated for 30 minutes at 4 °C and mixed by vortexing every five minutes to optimize the penetration of PMA into the cells. The tubes were illuminated for 15 minutes at room temperature (PHAST Blue, GenIUL, Terrassa, Spain) to initiate the reaction of PMA with accessible bacterial DNA. The illuminated samples were transferred into 50 ml centrifuge tubes and each mixed with 1.5 ml Xpert sample reagent (Cepheid). This mixture was inverted 20 times and incubated for eight minutes at room temperature. A second inversion of 10 times was done and the incubation continued for a total of 15 minutes. Then, the mixture was pipetted into one Xpert cartridge (Cepheid) and loaded into the GeneXpert instrument (Cepheid). The instrument and the software provided by the manufacturer (GeneXpert Dx Version 4.4, Cepheid) performed all further processing, measurement and analysis steps automatically. The results from each of five fluorescent probes (A to E) binding to a discrete target region of the DNA of *M. tuberculosis* complex and from one probe binding to the DNA of *Bacillus globigii* present in the cartridge (sample processing control, SPC) were reported as cycle thresholds ($C_T$). A signal for SPC indicates that the PCR was conducted successfully and was not impaired by inhibitors introduced with the sample. The magnitude of the $C_T$ value of each probe depends on the amount of target DNA present in the sample. A low $C_T$ value corresponds to a high number of target DNA copies and vice versa.

## 3.2.5.  Statistical analysis

Mixed-effect modeling was used to express $C_T$ as a function of PMA use and each experiment's other appropriate variables as fixed or random effects as described in the results. The model was adjusted for the specific probe used and to correct for differences between probes. We adjusted for the correlation between measurements on the same patient by including a patient identifier as random effect. Difference in $C_T$ ($\Delta C_T$) was modelled as function of drug and resistance status. All effect sizes, 95% confidence intervals (95% CI) and $p$ values were derived from the same two models. Mathematical functions from R (freely available from www.r-project.org) and the R packages nlme and effects were used for all analyses and graphics (14,15).

## 3.3.  Results

### 3.3.1.  Cultures from pan-susceptible and XDR strains

A total of 66 susceptibility tests and 16 growth controls were available for RMP ($n = 11$), INH ($n = 11$), EMB ($n = 11$), STM ($n = 11$), PZA ($n = 11$), and MXF ($n = 11$). Both XDR isolates were successfully grown in control or drug containing medium and the cultures measured. SPC observations were omitted after confirming that they did not influence the results and that PMA did not interfere with the PCR. A linear mixed-effects model was constructed for probe data containing the interaction between drug and PMA, and the underlying main effects, as fixed effects. There was a highly significant interaction between drug and strain meaning that the $\Delta C_T$ was different in pan-susceptible and resistant strains ($p = 0.0016$). The resulting estimated $\Delta C_T$ is shown in Figure 3.1. Only incubation with INH and EMB caused a significant elevation in estimated $\Delta C_T$ compared to control (effect size: 1.8) by 2.78 (95% CI: 1.96 – 3.58) for INH and by 3.85 (95% CI: 3.04 – 4.66) for EMB ($p < 0.0001$ for both). This shows that PMA was able to penetrate and to bind bacterial DNA better in the presence of one of these two drugs while the other four drugs did not improve penetration and binding. A significant difference in $C_T$ after PMA treatment was not detectable for both XDR strains except for EMB where the estimated $\Delta C_T$ increased by 3.95 (95% CI: 1.94 – 5.96; $p = 0.0002$). This indicates that despite the instrument classifying the strain as resistant to EMB a portion of bacteria must have lost their membrane integrity enabling PMA to penetrate. The difference seen with INH in susceptible strains was no longer present in the XDR strains.
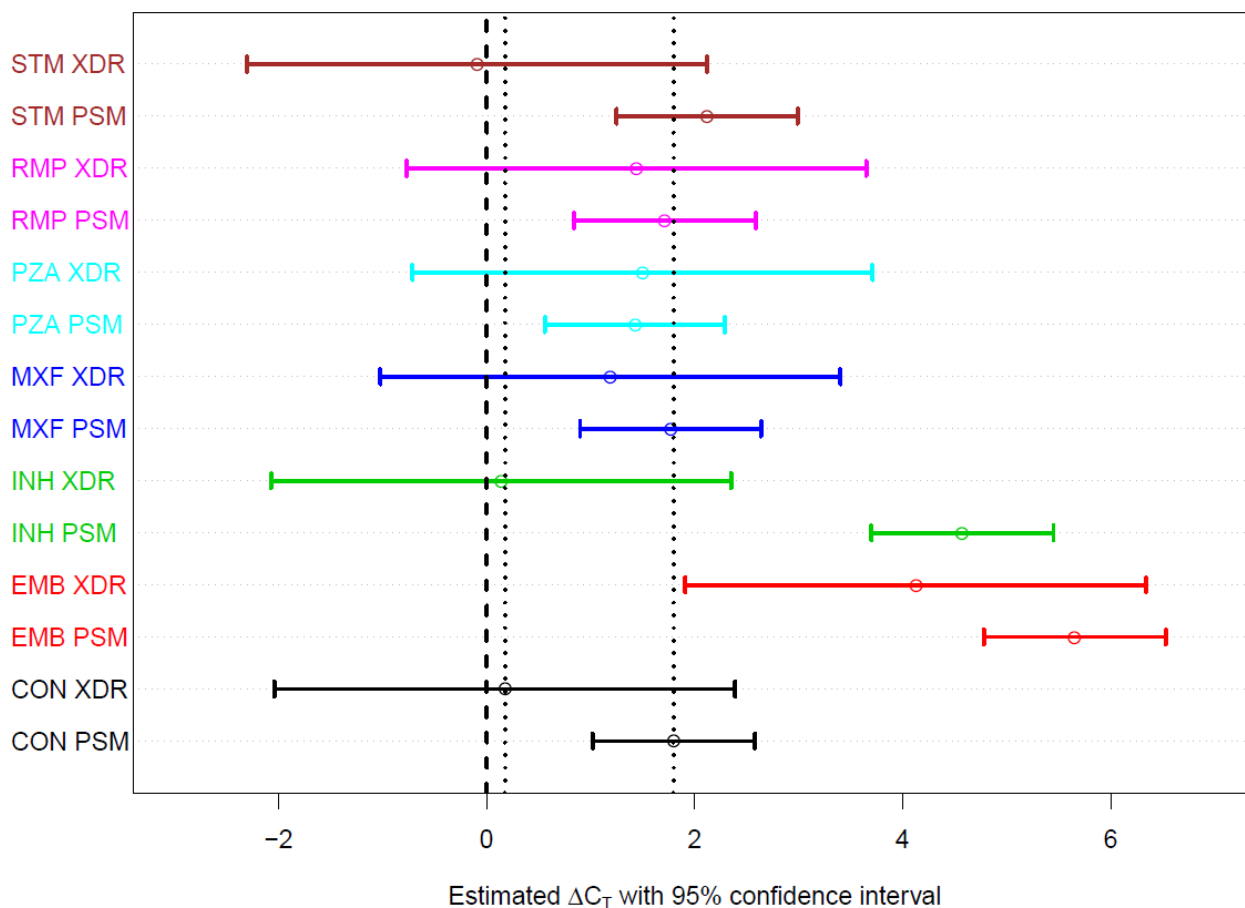
**Figure 3.1:** Estimated $\Delta C_T$ of *M. tuberculosis* cultures growing in drug free medium or in medium containing antibiotics, measured by Xpert with and without PMA.

The figure shows the modeled $C_T$ difference from cultures of pan-susceptible and drug resistant *M. tuberculosis* incubated with six anti-TB drugs (bars are 95% CI) and measured by Xpert with and without addition of PMA. A greater $\Delta C_T$ value means an elevated $C_T$ value of samples incubated with PMA compared to samples without PMA. The $\Delta C_T$ was significantly different only in cultures from susceptible bacilli containing INH or EMB and in cultures from resistant bacilli containing EMB (overall $p = 0.0016$). The dotted lines represent the levels of drug free control cultures from pan-susceptible and drug resistant *M. tuberculosis*.

CON = drug free control; $C_T$ = cycle threshold; EMB = ethambutol; INH = isoniazid; MXF = moxifloxacin; PZA = pyrazinamide; RMP = rifampin; STM = streptomycin; PMA = propidium monoazide; PSM = pan-susceptible *M. tuberculosis*; XDR = extensively drug resistant *M. tuberculosis*.

## 3.3.2. Clinical sputum samples

Two of the 20 patients withdrew during the study. All 151 samples received were measured with liquid culture and with standard Xpert and 74 (49.0%) with the addition of PMA. Seventy-seven

(51%) PMA-Xpert measurements were unavailable because of invalid readings ($n = 8$), missing reagent ($n = 24$) or too little remaining sputum sample after processing for the parent study ($n = 45$) mostly at later treatment time points. Comparing Xpert to liquid culture as the reference standard to detect a culture positive for *M. tuberculosis*, the sensitivity and specificity of Xpert was determined to be 93.3 and 41.8% ($n = 151$) and 94.8 and 62.5% for PMA-Xpert ($n = 74$), respectively.

A linear mixed-effects model was used to express $C_T$ as a function of time (8 time points), PMA (yes/no) and probe or SPC. As with the *in-vitro* cultures the model showed no difference whether SPC was included or not confirming that PMA did not interfere with the PCR. SPC was thus excluded from further analyses. The time factor was transformed to improve model fit by Akaike's criterion (16). The transformation providing the best fit to the data was the fourth root of day of treatment yielding the modeled curves shown in Figure 3.2A and B. Before treatment start the estimated $C_T$ values were similar with PMA than without PMA ($C_T$: 19.8 (95% CI: 17.6 – 22.1) and 19.3 (95% CI: 17.1 – 21.4) respectively). The modeled curves increased steeply at first and more gradually at later time points. The estimated increase was significant ($C_T$: 2.54 (95% CI: 1.38 – 3.69) per $\sqrt{\sqrt{\text{day}}}$; $p < 0.0001$) and the difference between estimated curves with and without PMA increased as well ($\Delta C_T$: 0.55 (95% CI: 0.37 – 0.74) per $\sqrt{\sqrt{\text{day}}}$). Figure 3.2B shows wider error bars at later time points owing to the relative paucity of measurements when the sputum samples were not voluminous enough for all experimental procedures to be performed.
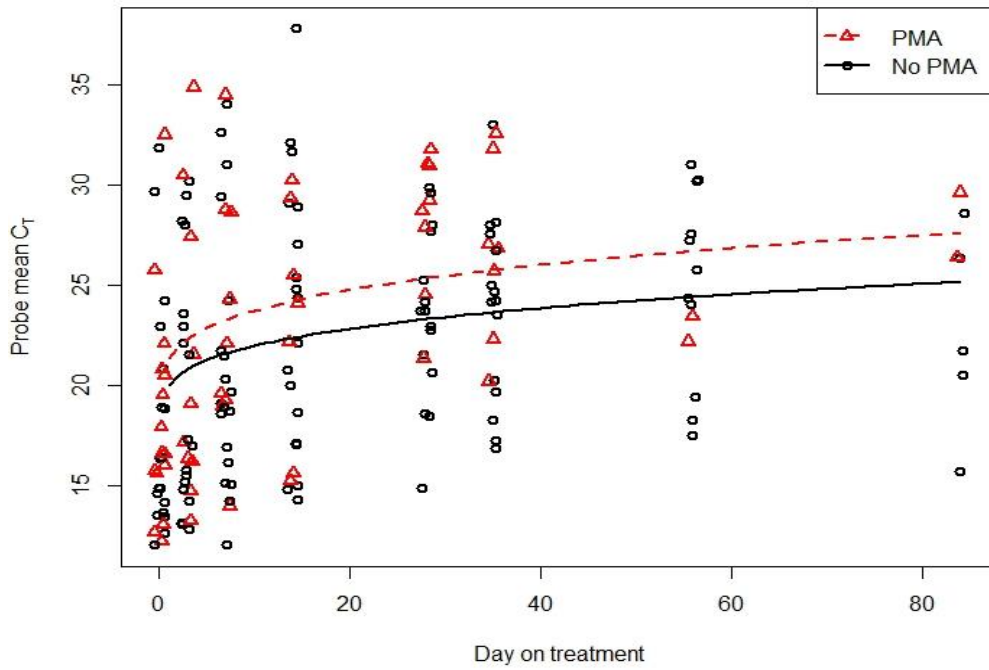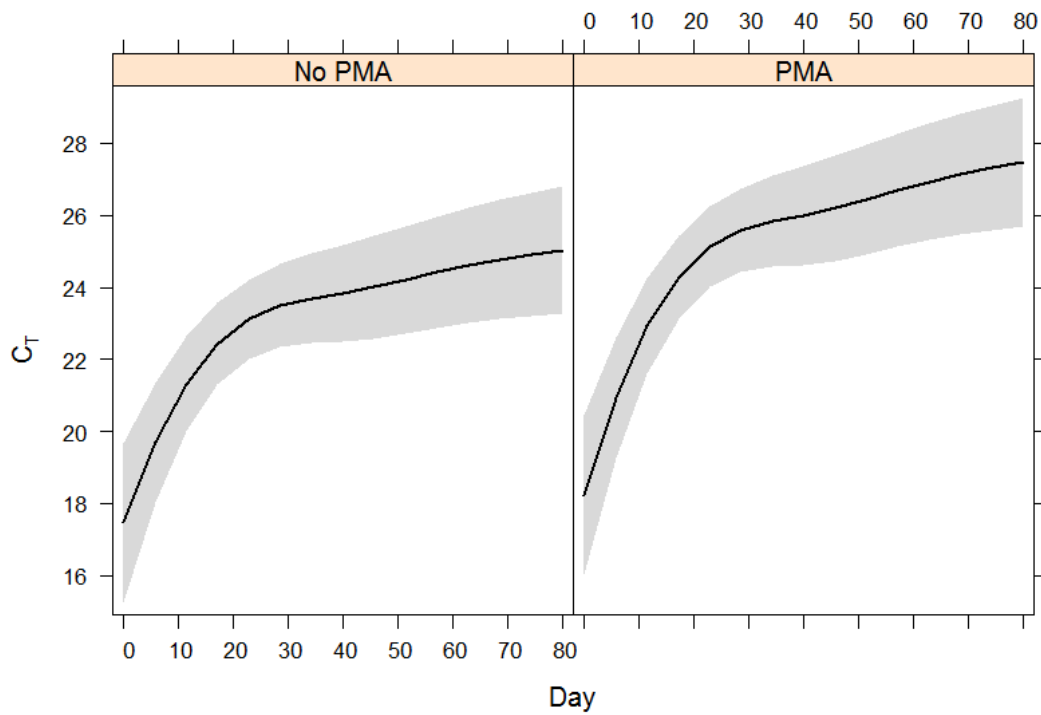
**A**



**B**



**Figure 3.2A&B:** Observed Xpert $C_T$ and modeled Xpert $C_T$ curves from samples collected at specific time points during treatment and measured with or without PMA

Figure 3.2A shows observed $C_T$ means for each time point with (triangles) and without (dots) PMA pre-incubation in 151 sputum samples. The curves demonstrate the modeled effect of PMA on $C_T$ over time. Both measurements increase over time but the increase becomes progressively slower. The estimated $C_T$ of samples pretreated with PMA is always higher than without PMA. Figure 3.2B shows that the estimated difference increases over time but error bands (95% CI) overlap at later time points.

$C_T$ = cycle threshold; PMA = propidium monoazide.

## 3.4.    Discussion

Our study shows that Xpert $C_T$ measurements with and without PMA pre-incubation differ significantly in pan-susceptible cultures inhibited *in vitro* by INH and EMB whereas cultures from extensively resistant bacteria only show significant differences if incubated with EMB. Xpert $C_T$ measurements in clinical samples collected before initiation and during combined TB treatment (including INH and EMB) demonstrated a small difference between PMA and non-PMA supported Xpert $C_T$ measurements at baseline, which increased modestly over 12 weeks following initiation of treatment.

It is interesting that several anti-TB drugs showed different effects depending on the compound inhibiting their growth in culture. In pan-susceptible bacteria, incubation with INH and EMB resulted in an increase in $C_T$ compared to the corresponding growth control indicating that more DNA molecules were detected without PMA (Figure 3.1). These two drugs act on essential biochemical processes for cell wall synthesis probably affecting its integrity (17,18). Conversely, XDR strains grown in culture medium containing INH showed no effect of PMA on Xpert $C_T$ as it was expected, but PMA and EMB had a similar effect on Xpert $C_T$ as for susceptible bacteria. A partial suppression of growth with the concentration of EMB utilized in the culture could explain this finding as it has been described that multiple mutations lead to various degrees of EMB resistance (19). No significant influence of PMA on $C_T$ was observed with MXF, RMP and STM. An explaination for this is that those substances are inhibiting bacterial biosynthesis but do not directly affect the integrity of the cell wall thus not offering PMA a substrate to bind (20-23). Alternatively, a major cellular disruption could lead to fragmented dead bacilli not detected at all by Xpert. PZA's main mechanism of action is still unclear nevertheless unlikely to be cell wall related (24).

We cannot fully explain why the impressively increased PMA-Xpert $C_T$ induced by INH and EMB *in vitro* was not equally replicated in sputum samples from patients treated with a combination of agents including INH and EMB. Our experiments did not extend to exposing cultures to the combination of INH, RIF, PZA and EMB to which bacteria are subjected to in

human lungs. However, it would be difficult to directly compare such results as the single drug cultures are grown under controlled conditions and with exact concentrations of drugs that might not correspond to those that expectorated *M. tuberculosis* are exposed to, and the *in-vitro* conditions exclude the pressure of the immune system.

PMA-Xpert measurements on samples collected from patients with pulmonary TB at baseline and under TB treatment resulted in an expected increase in $C_T$ compared to measurements without PMA. The average difference between PMA and no PMA in probe $C_T$ for samples up to treatment day 14 ($\Delta C_T$: 1.1) was much smaller than that described by Miotto *et al.* ($\Delta C_T$: 8.2) on specimens from patients up to 20 days on treatment (10). Nikolayevskyy *et al.* recently found a similar magnitude of $\Delta C_T$ in 181 samples from 68 patients that were one to two months on standard anti-TB medication (11). Albeit a reasonable correlation of PMA-Xpert $C_T$ with time to liquid culture positivity was seen in that study (r: 0.61; 95% CI: 0.54 to 0.67), 76 of 378 PMA-Xpert positive samples (20.1%) remained culture negative (11). In our study only 6 out of 74 PMA-Xpert positive samples (8.1%) remained culture negative. Though direct comparison is difficult this indicates that our method of PMA-treatment fulfilled its purpose of identifying samples containing viable bacteria at least as well as that used by Nikolayevskyy *et al* and that the method of decontamination used in our study did not critically decrease the proportion of viable bacilli while it increased the effect of PMA on bacilli rendered non-viable (11).

In summary, we demonstrated that PMA-enhanced Xpert is able to give an indication about the presence of dead bacilli in artificially created or clinical samples containing *M. tuberculosis* exposed to anti-TB agents. However, the specificity of PMA-enhanced Xpert for viable *M. tuberculosis* remains imperfect, the *in-vitro* effect of PMA is drug-specific and the clinical difference to non-enhanced Xpert is small. These shortcomings should be addressed before PMA-enhanced Xpert can replace conventional methods for the monitoring of patients on TB treatment.

## Acknowledgements

## Conflicts of interests

The authors have no conflict of interest to declare.

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# Chapter four

# Simultaneous staining of sputum smears for acid-fast and lipid-containing *Myobacterium tuberculosis* can enhance the clinical evaluation of antituberculosis treatments.

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## Abstract

Dormant, slow-growing, antibiotic-tolerant *Mycobacterium tuberculosis* undermine the shortening of tuberculosis treatment to less than 6 months and are thought to be characterised by intracellular lipid bodies. Antibiotic effects on such persisting bacilli escape evaluation as they cannot be readily cultured. We identified cells containing lipid bodies in sputum smears from 86 newly diagnosed pulmonary tuberculosis patients and monitored these cells daily in 42 patients over the first 14 days of treatment with rifampicin, the experimental compound SQ-109, or both agents combined. Counts of Nile-Red-positive lipid-body containing cells were correlated with those of Auramine-O-positive cells and colony forming units of viable *Mycobacterium tuberculosis* on agar plates. Rifampicin but not SQ-109 significantly reduced colony forming units but all treatments distinctively and significantly changed the proportions of lipid body-containing bacilli and viable *Mycobacterium tuberculosis*. Monitoring lipid bodies containing bacilli in sputum during treatment with experimental anti-tuberculosis regimens may identify putative treatment-shortening regimens.

**My contribution: Planning experiment**

        **Laboratory experiment**

        **Writing the manuscript**

## 4.1. Introduction

In 1944 Joseph Bigger observed a small number of survivors among cultures of *Staphylococcus pyogenes* exposed to lethal doses of penicillin, and coined the term "persisters" to describe bacteria surviving antibiotic attack by adopting a reversibly altered physiologic state that is still incompletely understood (1). The phenomenon of persistence and phenotypic antibiotic tolerance has since been documented for a range of bacterial species and antibiotics. Bacteria persisting through phenotypic tolerance for one antibiotic will probably also be tolerant to the bactericidal effects of other antibiotics (2).

Though its clinical relevance is still unclear persistence was noted early in *Mycobacterium tuberculosis* (*M. tuberculosis*) cultures in vitro (3). In murine experiments infected animals treated with anti-tuberculosis agents recovered fully and were free of culturable *M. tuberculosis* only to fall ill again after a prolonged interval or following treatment with immunosuppressive steroid drugs (4); notably, all mycobacteria recovered from the second episode were fully susceptible to the anti-tuberculosis agents initially used. It is well described *in vitro* that *M. tuberculosis* develops a reversible transition to a persistent state of general metabolic down-regulation and activation of alternative pathways that stop replication, decrease ATP production and shift energy sources from carbohydrates to fatty acids (5-12). These persisters are tolerant to commonly used anti-tuberculosis drugs (6,13,14) and unable to form colonies on agar plates but may grow in liquid media (15), or when treated with recombinant or supernatant-derived resuscitation-promoting factors (16,17). Such cells lack a proper cell membrane, are no longer acid-fast and fail to retain enough Auramine-O stain for detection with routine fluorescent microscopy, whereas accumulated intracellular lipids can be stained with fluorescent Nile-Red stain (6,13,18,19). Nile-Red-positive, "fat and lazy" *M. tuberculosis* cells have been found in sputum collected from untreated tuberculosis patients, but it is not clear if these bacteria are identical to those artificially generated by exposure to suboptimal conditions *in vitro* (6,20,21).

Anti-tuberculosis treatments are currently assessed in pulmonary tuberculosis (PTB) patients by sputum culture assays that recover viable bacteria and measure treatment effects either quantitatively by the reduction of the viable mycobacterial load in sputum cultures (22-24) or qualitatively by sputum culture conversion from positive to negative (25,26). It has been observed since the introduction of anti-tuberculosis chemotherapy that, in order to cure PTB, treatment must be far longer than the period during which bacteria can be grown from sputum with such assays. The validity of sputum culture for predicting a treatment's potential to cure patients or to shorten treatment duration has been questioned recently with several large clinical trials failing to confirm promising results based on sputum culture conversion in PTB patients

and murine studies (27-29). Studies that measure treatment effects only on viable bacteria naturally fail to determine how persister-type mycobacteria with associated phenotypic tolerance, either pre-existent or induced by chemotherapy, influence their outcome.

A method to quantify viable and persisting *M. tuberculosis* cells in sputum samples during early treatment might help to identify treatments that target both metabolically active and persisting mycobacteria. We hypothesized that counts and proportions of Auramine -O-positive and Nile-Red positive cells in sputum might represent persisting mycobacteria and thus aimed to confirm the presence of these cells in clinical sputum samples, investigate them during early treatment with different agents and evaluate the potential of combined Auramine-O and Nile-Red fluorescence microscopy to measure treatment effects on persister type *M. tuberculosis.* In the course of the first clinical evaluation of the investigational compound SQ-109 (SQ) we collected daily sputum samples from newly diagnosed PTB patients treated for 14 days with either SQ, rifampicin (RIF), or SQ and RIF (SQ+RIF) combined and performed colony forming unit (CFU) counting on solid agar and fluorescence microscopy of double-stained smears. Through correlation of viable CFU counts and proportions of Auramine-O-positive and Nile-Red-positive cells we aimed to extrapolate counts of putative drug-tolerant persisters unable to grow on culture media, and study their dynamics during drug treatment. Although treatments would likely reduce viable *M. tuberculosis* CFU counts the lipid bodies containing persister-type *M. tuberculosis* counts might show a different dynamic, i.e. decrease more slowly, remain constant or even increase over time depending on the treatment given.

## 4.2.    Material and methods

### 4.2.1.  Patients, treatments and samples

Samples for this study were from 90 adults, newly diagnosed, untreated, fully drug susceptible, smear-positive (at least 1+ on the IUATLD/WHO scale) PTB patients who were recruited from community clinics in Cape Town, South Africa (TB incidence >1,000/year) and agreed to take part in an early bactericidal activity (EBA) trial. Eligible subjects were hospitalised for the duration of the study and randomized to receive one of 6 experimental treatments (RIF alone 10 mg/kg/day, SQ109 300 mg/day + RIF 10 mg/kg/day, SQ109 alone 150 mg/day, SQ109 alone 300 mg/day, SQ109 alone 150 mg/day + RIF 10 mg/kg/day and SQ109 alone 75 mg/day). Sputum was collected over 16 hours overnight for two days before treatment start (baseline) and for 14 days under treatment. Samples were transported to a single laboratory (Department of Biomedical Sciences, Stellenbosch University, Tygerberg, South Africa) at 4 to 8 °C. Baseline samples from all patients were used to assess the day-to-day variation of measurements without

treatment. Baseline and on-treatment samples from patients in 3 groups were used to assess treatment effects. Groups were treated either with rifampicin (RIF; daily dose 10 mg/kg body weight), with SQ-109 (SQ; daily dose 150 mg), or with RIF and SQ-109 combined (RIF+SQ) in the same dosages. RIF is an established anti-tuberculosis agent whose bactericidal activity over 14 days has been measured at 0.113 $\log_{10}$colony forming units/day*mL sputum previously (22). SQ-109 is a novel anti-tuberculosis drug candidate with promising results in murine studies showing synergistic activity with RIF. At the time of this analysis SQ had not been evaluated in humans. The full results of the clinical study have been published recently; RIF 10 mg/kg showed the expected 14-day bactericidal activity while SQ 150 mg did not show activity and was not synergistic with RIF (30).

## 4.2.2. Quantitative sputum culture

The samples were homogenised by magnetic stirring for 30 minutes and digested for 20 minutes with an equal volume of 0.1% dithiothreitol (Sputasol; Oxoid, Cambridge, UK) at room temperature. Of this mixture 100 µL were inoculated in serial dilutions (0 to $10^{-4}$) onto each side of two Middlebrook 7H11 agar biplates enriched with OADC (oleic acid, albumin, dextrose, catalase; Becton Dickinson, Sparks, MD, USA) and made selective with antibiotics (Selectatab, Mast, Merseyside, UK), containing polymyxin B sulfate (200,000 U/L), amphothercin B (10 mg/L), ticarcillin (100 mg/L) and trimethoprim (10 mg/L). The plates were incubated at 37 °C for 3 weeks. CFU were counted at the dilution that yielded between 20 to 200 colonies. Each half plate was counted separately. Contaminated plates were excluded from analyses.

## 4.2.3. Sputum smear preparation

We used a modified method described by Garton *et al* (21). Three to 5 mL of homogenised and digested sputum as described above were mixed with an equal volume of 2% NaOH (BBL, Mycoprep; Becton Dickinson) and decontaminated for 15 minutes at room temperature by inverting the tube gently but not vortexing. The mixture was neutralized by addition of phosphate buffered saline (PBS, pH 6.8, Becton Dickinson) to a final volume of 45 mL and centrifuged for 20 minutes at 1400 × g and 4 °C. The supernatant was discarded and the pellet resuspended with 0.5 mL of PBS. Two smears were prepared by spreading 10 µL suspension, fixed at 70 °C overnight on a heating block, and kept in the dark until staining.

Staining: The day before microscopy was scheduled an appropriate number of slides were stained for 15 minutes with Auramine-O (National Health Laboratory Services, Johannesburg, South Africa), decolorized for 15 minutes with 0.5% acid alcohol (National Health Laboratory Services) counter stained for 10 minutes with Nile Red (10 µg/mL in PBS; Sigma-Aldrich, Cape Town,

South Africa), and oxidized for one minute with 0.1% potassium permanganate (National Health Laboratory Services). Slides were kept in the dark, dried, mounted overnight with granules of propyl-gallate dissolved in Mowiol 4-88 as antifading medium (Sigma-Aldrich) and transported to the Central Analytical Facility, Stellenbosch University, the following morning for microscopy.

### 4.2.4. Microscopy

Smears were examined using a LSM 780 confocal laser scanning microscope on an Elyra S1 super resolution platform with an objective of 100×/1.46 and oil (Carl Zeiss, Jena, Germany). In accordance with previously published work (21) the wave lengths used for excitation/emission of green fluorescence were 488 nm/493-544 nm and of red fluorescence 514 nm/567-753 nm, respectively. Because of rapid fading of stain under scanning complete counting of a slide was not attempted. Instead, we took at least five digital images of areas that appeared to contain the highest density of stained mycobacteria (ZEN 2011, resolution 96 dpi, image size $1024 \times 1024$ pixels; Carl Zeiss) and saved the images for counting off-site.

### 4.2.5. Cell counting

Despite the presence of a considerable amount of extraneous debris not unexpectedly found in sputum smears we were able to distinguish clearly 3 cell types: green cells were usually rod-shaped, homogenously stained and larger than red cells, which were more curved and contained red cytoplasmic bodies. Cream cells had a size and appearance between the green and red cells (31,32); examples are shown in Figure 4.6A. We counted cells on consecutive images until all were counted or the total number of cells counted was 100 or more. A single person (XAK) counted all cases on the same computer screen with the assistance of a digital counter (ImageJ 1.46, National Institute of Health, Bethesda, MD, USA) in a non-blinded fashion. After counting was completed the same person repeated the counts on 50 randomly selected and anonymized images with excellent concurrence for all colour types.

### 4.2.6. Statistics

Cell counts were available from a total of 729 samples (90% out of a theoretical maximum of 810 samples from 86 patients). Counts were not available if no sample was submitted or if not enough material was left after processing for the main study. Absolute cell counts on slides were reported as numbers per slide and converted into proportions of colours per patient per day. CFU counts were available from 653 samples with cell counts (90%). Missing CFU were due to no growth or contamination. CFU counts were averaged from all available counts per sputum, corrected for the dilution factor and reported as CFU/mL of sputum. Pre-treatment samples were analysed for

natural day-to-day variation. After confirming that they did not differ each pre-treatment sample was denoted day 0.

From the premise that the green or the green and cream subset of cells counted on smears would correlate with CFU counts derived from viable cells we aimed to estimate a number of elusive CFU from the remaining cell subsets that would represent persister CFU that cannot grow on the culture medium. We were interested in the response of these elusive CFU to the treatments, particularly whether their number would remain stable, indicating drug tolerance, or would grow, indicating induction of persisters, or decrease, indicating a bactericidal effect on persisters.

To display data distributions over time on treatment in figures we used notched box plots (33). A notched box plot is an enhanced box plot where the line in the centre of each box is the median and the upper and lower hinges of the box are the first and third quartiles, respectively. The centre 50% of all measurements lie within the box. The whiskers extend to the minimum and maximum of the data. The notches are approximate 95% confidence intervals for the median. If the notches of two plots do not overlap, it is strong evidence that the two medians differ, especially in our figures where the individuals represented in the boxplots are not from independent groups, but the same groups of patients were measured over time (34).

We developed statistical linear mixed-effects models of several variables including visible CFU and estimated elusive CFU as different functions (main effects and/or interactions) of time on treatment, treatment and/or type of cell as fixed effects. Specific fixed effects are described in the sections where results are reported. In all models, patients were included as random effect to adjust for the fact that measurements on a specific patient will be more strongly correlated than those on different patients. Where required, variables were log-transformed to base 10, denoted $\log_{10}$(variable), towards symmetry, prior to modelling and plotting. An advantage of mixed-effects modelling is that all valid observations are included in the analysis; a missing value does not result in all observations from a patient having to be excluded. All reported effect estimates, such as daily changes (slopes), and differences between them, their 95% confidence intervals and p-values were derived from these models. Note that a negative daily change is a decrease. If a 95% confidence interval includes zero, the underlying effect is not statistically significant. The colours used in the box plots and bar charts are the following; turquois denotes CFU, blue denotes total number of cells counted, green, yellow and red represent proportions of green, cream and red cells, respectively.

All graphics and statistical modelling were done in R, freely available from www.r-project.org and functions from R package nlme (35).

## 4.3. Results

### 4.3.1. Sputum measurements at baseline

To examine day-to-day variation we used a linear mixed-effects model with only the intercept as fixed effect. The mean baseline bacterial sputum load of 5.77 (95% CI: 5.60 – 5.93) was as expected in 86 untreated, sputum smear-positive PTB patients. Viable CFU counts, total cell counts and proportions of stained cells did not significantly differ over the two baseline days (Figure 4.1). Green cells were dominant with cream-staining cells and red stained cells making up only a small proportion of the baseline counts.
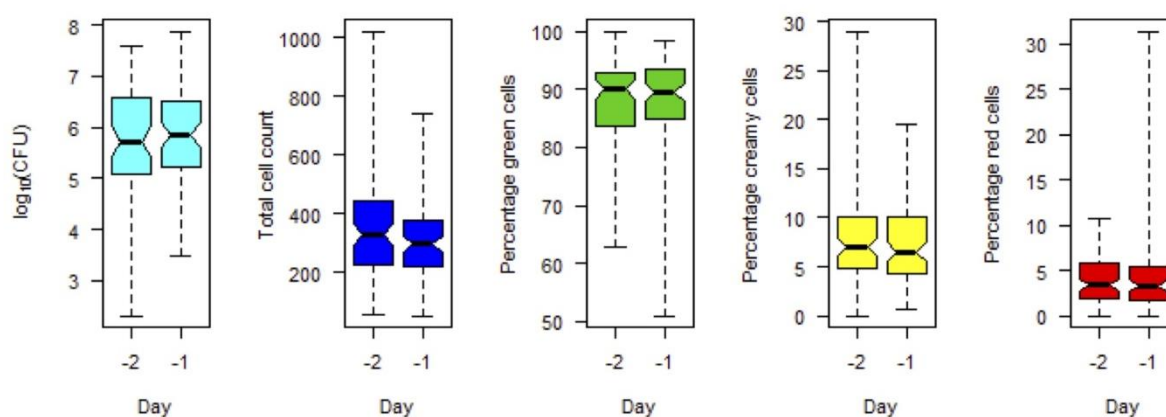


**Figure 4.1:** Notched boxplots of distributions of baseline $\log_{10}$ (CFU), cell counts and proportions of stained cells.

Linear mixed-effects models of $\log_{10}$(CFU) counts per mL sputum, cell counts per field, and proportions of green, cream, and Nile red stained cells as function of day as fixed effect, showed that measurements on pre-treatment days are stable (all $P > 0.05$).

CFU = colony forming units

### 4.3.2. Measurements on treatment

On-treatment CFU, total cell count and proportions of different stained cells from 42 patients were modelled as functions of day as fixed effect irrespective of the kind of treatment. Overall we observed a significant daily reduction of viable CFU counts, a steady number of cells, and a significant change in proportions of stained cells to fewer green cells, more red and cream cells (Figure 4.2).
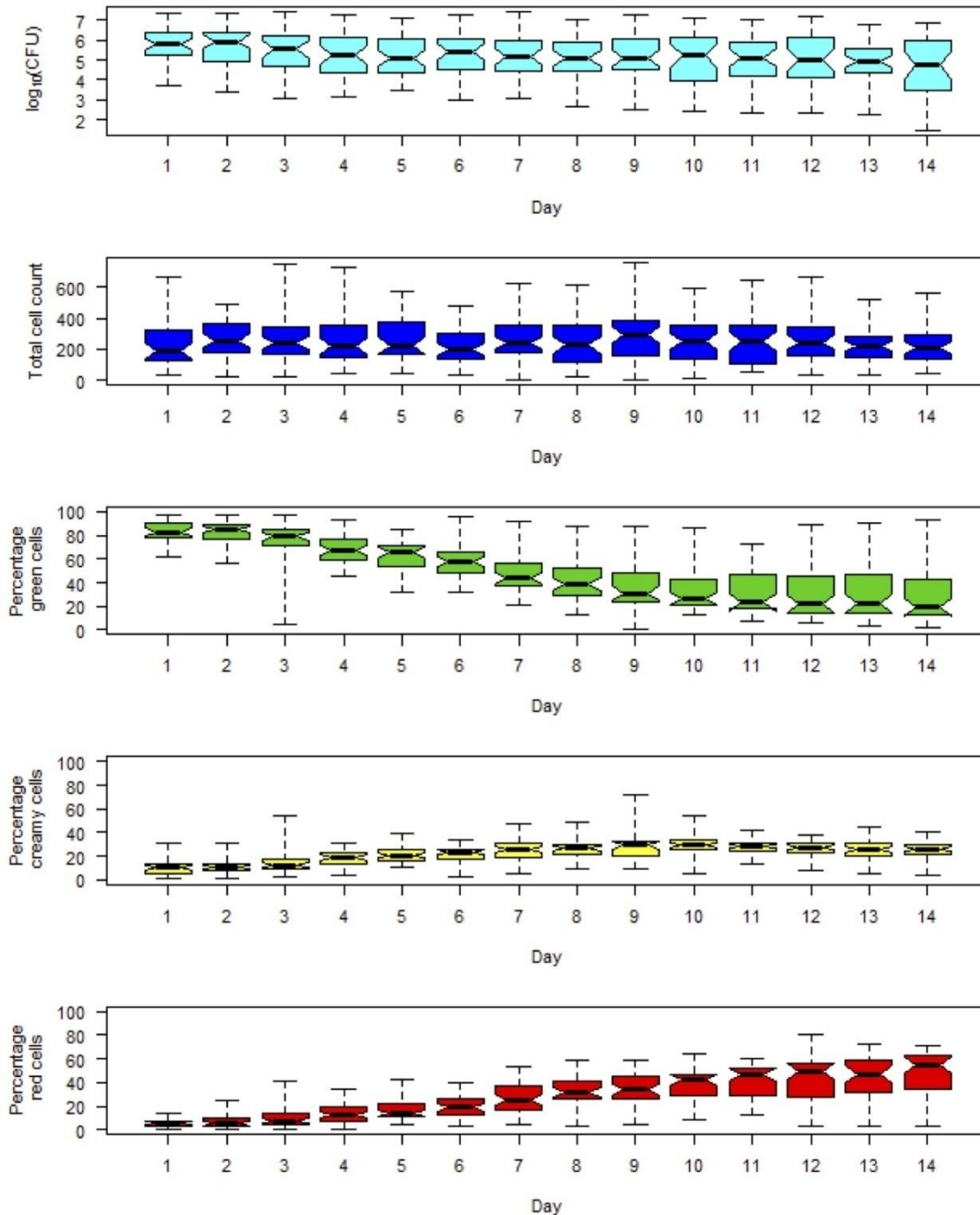
**Figure 4.2:** Notched boxplots of the distributions of on-treatment CFU, cell counts and proportions of stained cells.

From top to bottom: Under treatment we observed a reduction of CFU counts (daily change of -0.07 $\log_{10}$(CFU)/mL sputum per day; 95% CI: -0.09 to -0.06), a steady total number of cells (daily change = -0.65; 95% CI: -3.09 to 1.79), and a clear trend in the proportions of stained cells to fewer green cells (daily % change = -4.92; 95% CI: -5.17 to -4.67), more cream cells (daily % change = 1.33; 95% CI: 1.19 to 1.47) and more red cells (daily % change = 3.59; 95% CI: 3.40 to 3.77).

### 4.3.3. Drug treatment effects

For the estimation of treatment effects both baseline values were put at day 0. Fifteen, 13 and 14 patients received treatment with RIF, SQ and RIF+SQ, respectively, over 14-days. In order to estimate daily treatment effects, $\log_{10}$ (CFU) was modelled in a single mixed-effects linear model with the interaction between treatment and day of treatment as fixed effects. RIF and RIF+SQ treatments significantly decreased the mean $\log_{10}$ (CFU) during the first 14 days, but SQ treatment did not (Figure 4.3).
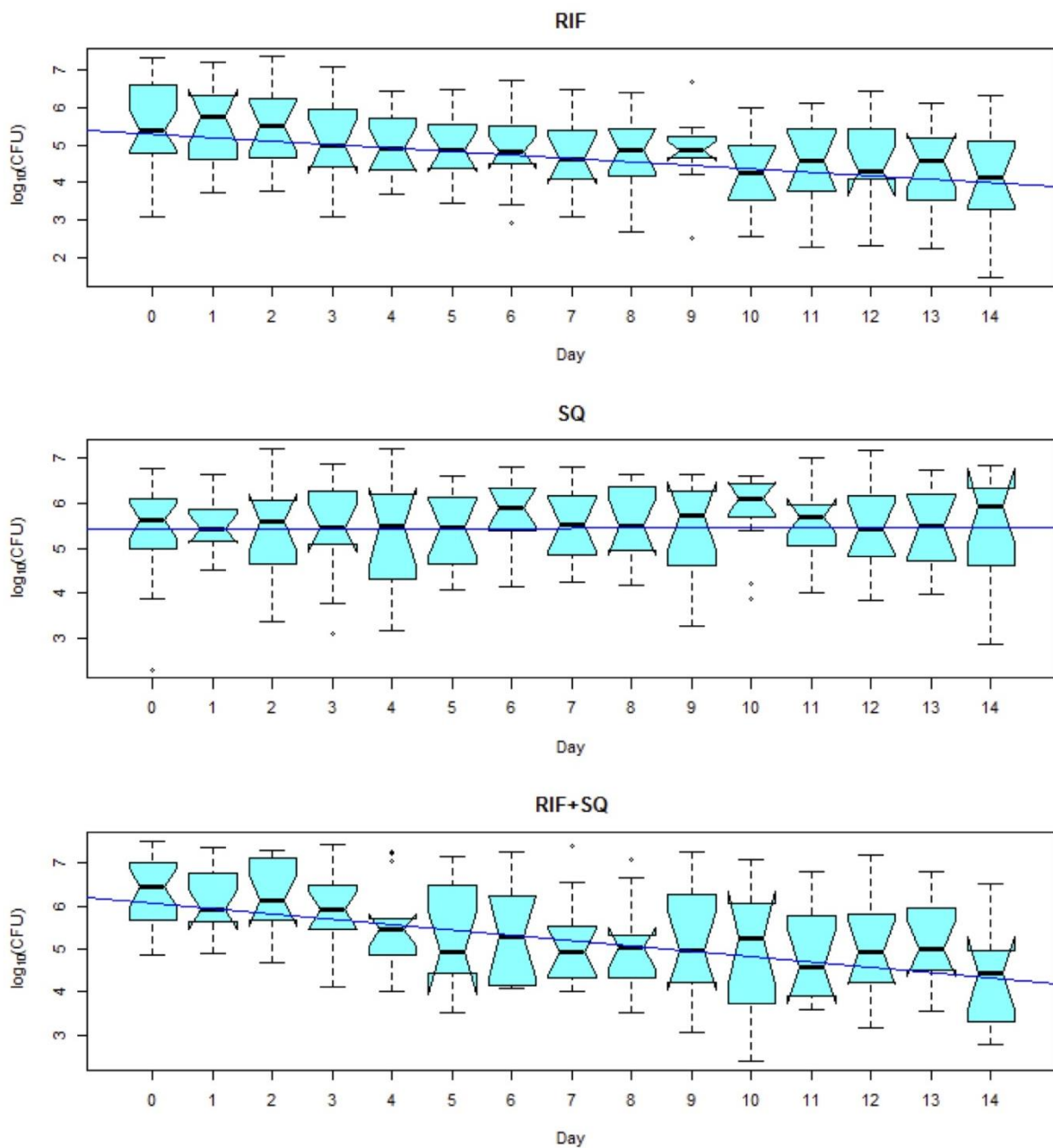
**Figure** **4.3**: Notched boxplots of $\log_{10}$ (CFU) counts over 14 days of treatment with SQ, RIF, RIF+SQ.

SQ treatment did not change the CFU count (daily change = 0.00; 95% CI: -0.02 to 0.02). RIF +SQ and RIF treatments reduced the daily sputum bacterial load count (daily change = -0.12; 95% CI: -0.14 to -0.10, and -0.09; 95% CI: -0.11 to -0.07, respectively), per day of treatment. The *P* was < 0.0001 for SQ versus both RIF containing treatments. The slopes of the lines indicate the average change per day.

CFU = colony forming units. RIF = rifampicin. SQ = SQ-109.

Total counts of green, cream and red staining cells per field did not change significantly over time on treatment in any of the treatment groups (data not shown) but the proportion of cells showed a clear trend to fewer green cells and more cream and red cells over time of treatment. This was more prominent with RIF-containing treatments that reduced the green cell proportion to less than 20% and increased the red cell proportion to more than 50%. With SQ, the green cell proportion remained larger than 50% throughout the treatment period (Figure 4.4).
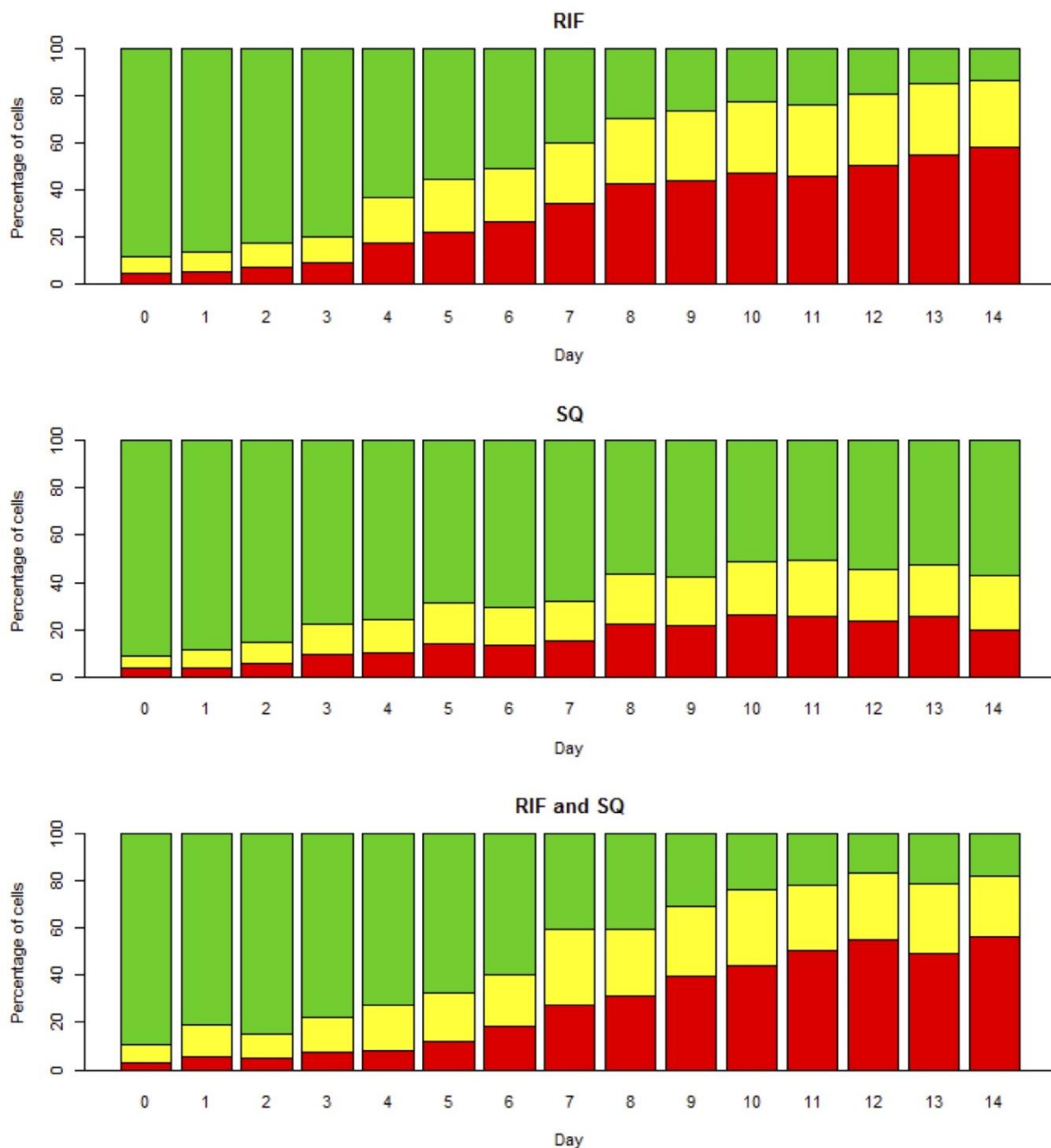
**Figure 4.4:** Stacked bar charts of mean percentages of cells over 14 days of treatment with RIF, SQ, RIF+SQ.

The percentages are from top to bottom of each bar green cells, cream cells and red cells. There is a steady reduction of green cells and an increase of red and cream cells. This is more prominent with RIF-containing treatments.

RIF = rifampicin. SQ = SQ-109.

## 4.3.4. Exploration of Nile-Red-positive cell dynamics under treatment

Based on the observation that both green cell proportions and viable CFU counts decrease over time we postulated that viable CFU might be reflected by the green or by the green and cream cells combined, whereas the red or red and cream cells combined represented putative persisters. We used different linear mixed-effects models of $\log_{10}$(CFU) counts as function of colour, adjusting for day as a fixed effect, and determined that the relationship between CFU and green and cream proportions combined was stronger ($P < 0.001$) than between CFU and the proportion of any individual colour or other combination (all $P > 0.002$). From the premise that viable CFU are formed by the green and cream cells, but red cells would not grow a CFU, we estimated a total number of CFU by dividing viable $\log_{10}$(CFU) by the proportion of green+cream cells for each patient at each time point. The proportion of this total number of CFU represented by red cells was the count of elusive CFU reflecting persisting or dormant cells. Figure 4.5 (left panels) shows these estimated elusive CFU for each treatment. There was a strong, significant increase of elusive CFU with SQ, a weaker but still significant increase of elusive CFU with RMP and no significant change with RIF+SQ combined. The increase of elusive CFU with SQ was significantly higher than with both RIF ($P = 0.0012$) and RIF+SQ ($P < 0.0001$) but there was no significant difference between the RIF and RIF+SQ combined ($P = 0.2399$). In perspective with viable CFU the modelling indicated that SQ treatment had no significant effect on viable CFU but significantly increased the number of persister CFU over time, RIF significantly reduced the viable CFU counts and mildly increased persister CFU, and the addition of SQ to RIF significantly increased the reduction in viable CFU and prevented an increase in persister CFU (Figure 4.5, right panels).
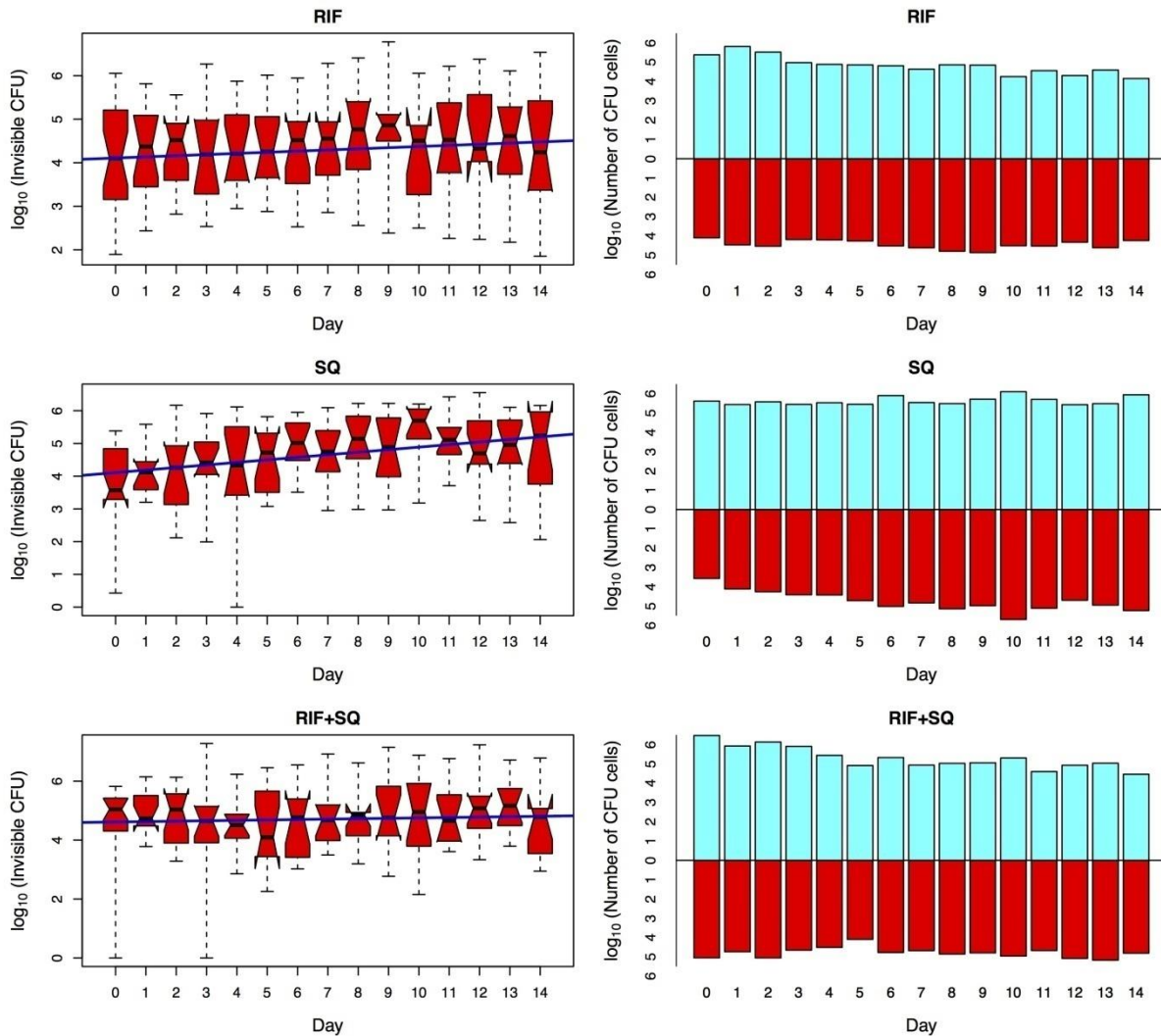
**Figure 4.5**: Viable, elusive and total modelled CFU over 14 days of treatment with RIF, SQ, RIF+SQ.

On the left are notched boxplots of estimated elusive CFU counts over 14 days of treatment with RIF, SQ, RIF+SQ. There is a strong and significant increase of red cells with SQ (daily increase = 0.08 $\log_{10}$ (CFU); 95% CI: 0.06 to 0.10; $P < 0.0001$), a milder but significant increase with RIF (daily increase = 0.03 $\log_{10}$(CFU); 95% CI: 0.00 to 0.05; $P = 0.0163$), and no significant increase with SQ+RIF (daily change = 0.01 $\log_{10}$(CFU); 95% CI: -0.01 to 0.03; $P = 0.4751$). The slope of the line indicates the average change per day derived from the model. The right panel of the figure shows bar charts of estimated median $\log_{10}$ (total CFU), represented by the total bar length, with the viable CFU in blue above the line and the elusive CFU in red below the line. This graphic is comparable to the proverbial "iceberg". Above the line is what is naturally visible, and under the line is what normally remains below the surface.

CFU = colony forming units. RIF = rifampicin. SQ = SQ-109.

82

Figure 4.6A



Figure 4.6B

Figure 4.6C



Figure 4.6D



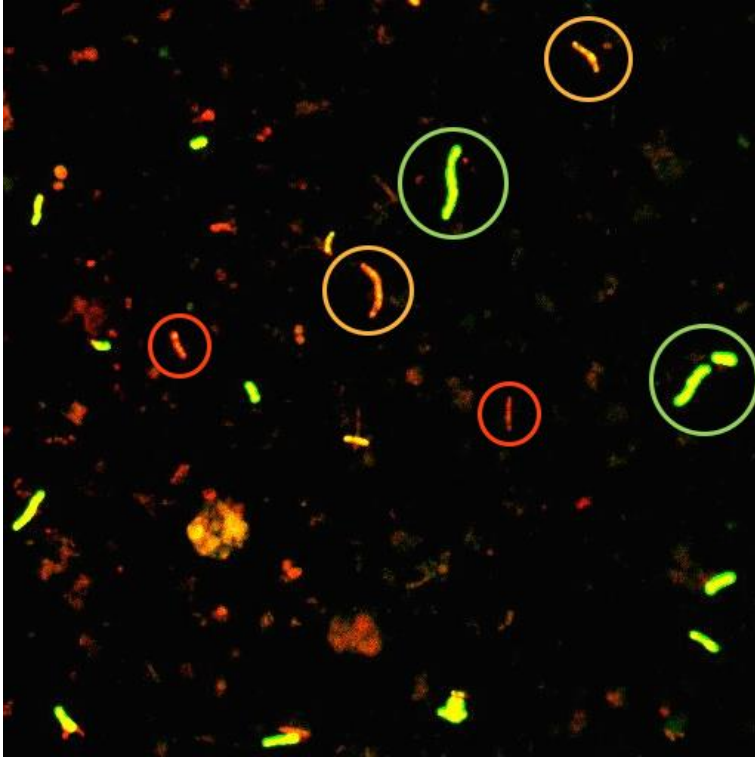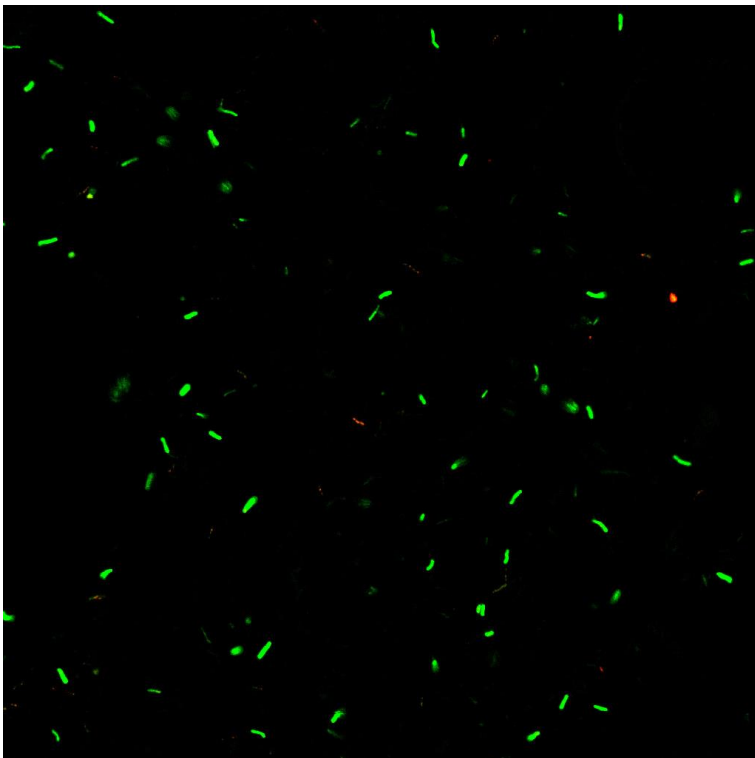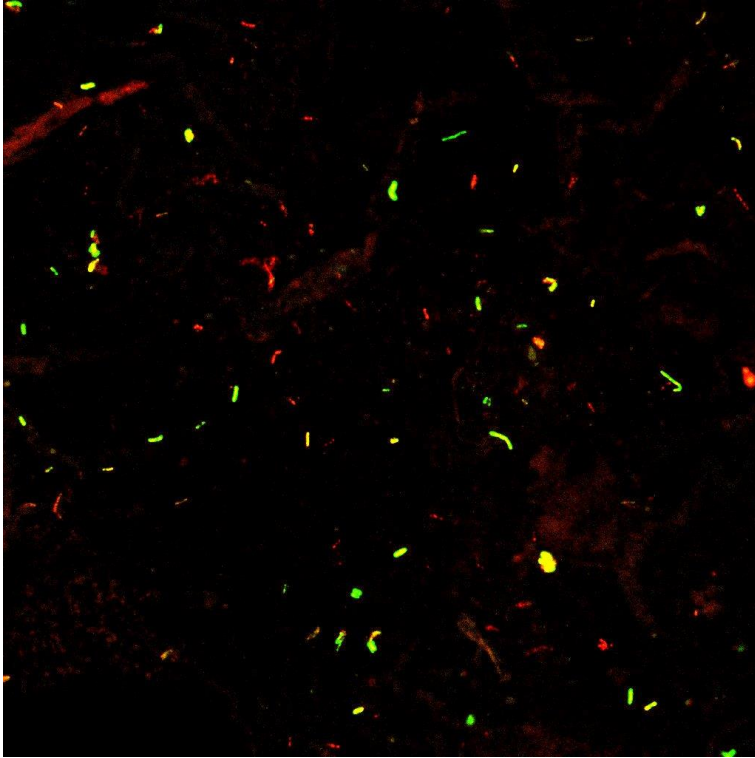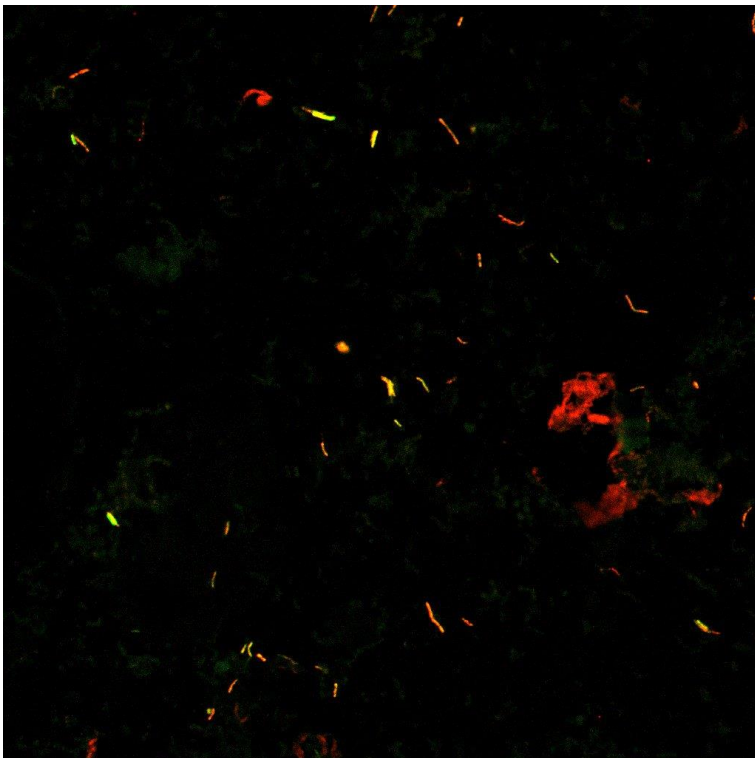**Figure 4.6:** Examples of green, cream and red stained cells in sputum samples observed by confocal microscopy after Nile-Red and Auramine-O staining with 1000 × magnification and 96 dpi resolution

Examples of typical cells (A, enlarged for illustration): Green cells are bright green, rod shaped and large (green circles). Cream cells are of intermediate colour with occasional green or red spots, rod shaped or curved and of smaller size (orange circles). Red cells are homogenous or dotted red, rod shaped or curved and smaller than green and cream cells (red circles). Before start of treatment green cells are predominant (B) while the cream and red cells are more frequent at day 7 of treatment (C) and dominate at day 14 of treatment (D).

## 4.4.  Discussion

Our observations confirm that a small population of Nile Red-positive, persister-type *M. tuberculosis* exists next to a much larger population of Auramine-O-positive and culture-viable mycobacteria in sputum of untreated tuberculosis patients. The size of each population was stable before treatment. After initiation of one of three different drug treatments the size of each population changed gradually and significantly over 14 days in a manner specific for the treatment. These findings suggest that microscopic enumeration of Auramine-O-positive and Nile Red-positive *M. tuberculosis* cells combined with conventional CFU counting in serial sputum collections could assist in simultaneously assessing the activity of anti-tuberculosis agents against both persister and viable populations of *M. tuberculosis*.

The efficacy of new anti-tuberculosis drugs and regimens is conventionally assessed by the decline of the sputum mycobacterial load in culture based assays such as CFU counting on solid agar plates during the first fortnight of treatment (36). Further evaluation of treatments found promising in short-term assessment over a longer period poses a greater challenge. Sputum culture conversion from positive to negative is the only currently validated trial end point, but this occurs many weeks before patients can be considered cured (37). Consequently, patients having successfully completed a supposedly curative treatment must be observed for many more months to ensure that recurrence does not occur and cure has indeed been achieved. This adds greatly to trial costs, delays the availability of results and slows the progress of antituberculosis drug development. The problem is not only the lack of a reliable alternative biomarker of disease activity but also the very nature of current sputum culture methods. A considerable body of evidence indicates that not all bacteria found in sputum grow in culture with currently available media. It has been postulated, but never proven, that a relatively drug-tolerant, persister-type sub-population of *M. tuberculosis* is responsible for the necessity of prolonged treatment and the propensity of tuberculosis patients to relapse if treatment for fully drug-susceptible PTB is not taken over the full duration of 6 months. Such *M. tuberculosis* persisters are hard to detect and quantify in sputum samples because they have lost acid fastness, making them invisible to conventional staining, and they do not grow in conventional culture media (15,18,19,38).

Consequently, antibiotic treatments aimed at the entire bacterial population are evaluated only on the population that can grow in culture, in the hope that activity measured on bacteria viable in culture will translate into activity against all populations. Recently completed clinical trials have failed to reach expected cure rates based on these conventional paradigms. The time seems right to challenge them.

A laboratory assay applicable early in treatment that reveals both viable and persister-type *M. tuberculosis* populations could greatly aid the early evaluation of potentially curative treatments. But are the Nile Red-positive cells, identified by our assay, in fact persisters? Zhang recently described persisters as: "a small fraction of quiescent bacterial cells that survive lethal antibiotics or stresses but can regrow under appropriate conditions" (38). Our method precludes attempts to regrow the stained cells but there are arguments in support of the other elements of this definition. Firstly, our persisters meet the description of Garton *et al* as "fat and lazy" cells lacking a proper cell wall and containing lipid bodies, which is common in quiescent cells (20,21). Secondly, we found a small proportion of persisters in all 86 patients before treatment, possibly induced by the hostile environment in host cavities (39) or present as an inherent phenotypic heterogeneity found in all microbial populations (40,41). Thirdly, in the absence of molecular resistance, the putative persisters survived bactericidal treatment better than the viable population, which was killed at the expected rate by RIF. The clinical relevance of emerging RIF-tolerant mycobacteria is supported by Sloan *et al.*who recently investigated a series of 38 selected pulmonary tuberculosis patients treated with a rifampicin-based regimen (42). They found that a high proportion of lipid body-positive cells present at day 21 or day 28 of treatment was associated with unfavourable treatment outcomes at 6 months (42).

The different response of the populations of stained cells to the different treatments was intriguing. For SQ, our results indicated no significant effect on viable cells but a significant increase in the number of Nile Red-positive cells. It has been shown *in vivo* that antibiotic therapy lasting from hours to a few days can generate a population of persisters that prevent the sterilization of infected mice, zebra fish and macrophages (43,44). Assuming that the red stained cells are indeed persisting *M.tuberculosis*, SQ may thus not be as "ineffective" as the lack of killing activity on viable cells suggests (30) but might be present at the site of infection at concentrations that force bacteria towards a persister state as a way to escape killing. Bactericidal activity of SQ was observed in murine models only after 3-4 weeks (45,46) and the 14 days of treatment in our study might have insufficient to reach bactericidal SQ concentrations (45,47). RIF treatment significantly reduced the viable cell count and caused a mild increase of Nile Red positive bacteria. This concurs with *in vitro* observations (48) and is at least not at odds with the fact that RIF provides cure from clinical tuberculosis only in combination with pyrazinamide for

the first 2 months and isoniazid throughout the 6-month treatment. These drug combinations might be critical in order to reduce persisters over time. Lastly, addition of SQ to RIF significantly increased the reduction in CFU counts and prevented an increase in Nile Red-positive cells. Such synergy has been observed for persisters *in vitro* and in murine experiments (49,50).

This adds to a growing body of evidence that a previously invisible persister population of *M. tuberculosis* might be of clinical relevance. Three treatments were used which, if somewhat serendipitously as SQ's properties were unknown before the study, allowed observation of different activities on viable and persister populations. However, for this assay to be part of future anti-tuberculosis drug evaluations manual counting must be automated to assure higher throughput and reproducibility. The method of double-stained smears and confocal laser scanner microscopy identified only three different colours of mycobacteria, which we then reduced to two populations with statistical modelling. This simplification is in the interest of practical application but there might be a future assay that can measure persistence in a more gradual way. Finally, critics will argue that since Bigger's description of persister bacteria in 1944 there has been no demonstration of their implication in critical clinical events. The response of those in support will be that our inability to grow persisters in experiments does not mean that the same applies to their natural environment. We believe that our results are the first look beneath a surface above which only the tip of the tuberculosis iceberg is visible.

In conclusion, we provide evidence that non-culturable *M. tuberculosis* persisters can be identified in sputum of tuberculosis patients before and during the first two weeks of treatment. These putative persisters show different responses to antibiotic treatment compared to those of culturable *M. tuberculosis*. The parallel quantification of both populations would be extremely useful in the clinical evaluation of novel antituberculosis treatments and advance the more rapid identification of curative antituberculosis regimens.

## Acknowledgements

## Conflict of interests

No conflict of interests stated by authors.

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# Chapter five

# Acquisition of rifampicin resistance in pulmonary tuberculosis.

Xavier A Kayigire, Sven O Friedrich, Lize van der Merwe and Andreas H Diacon.

# Abstract

*Mycobacterium tuberculosis* with spontaneous mutations conferring resistance to rifampin (RIF) are exceedingly rare and fixed drug combinations typically prevent augmentation of resistance. Fourteen newly diagnosed tuberculosis patients were treated with RIF only for 14 days and bacterial loads including mutation frequencies were determined. A statistical model estimated that 1% of the remaining viable mycobacteria could be resistant after 30 days of monotherapy, indicating that pharmacodynamic variation could contribute to the acquisition of resistance against RIF.

**My contribution: Planning experiment**

                        **Laboratory experiment**

                        **Writing the manuscript**

# Main text

Drug resistance is a peculiarity of *Mycobacterium tuberculosis* in which resistance to antibiotics is genetically encoded. Resistant *M. tuberculosis* can be transmitted from one person to another, but resistance cannot be transmitted from one bacterium to another (1). Mycobacteria with spontaneous resistance mutations are naturally present in every population of *M. tuberculosis* (2,3). Mycobacteria with spontaneous resistance mutations are naturally present in every population of *M. tuberculosis* (4). However, Beijing strains, which are more prevalent in the Eastern and Western Cape provinces, South Africa, were found to acquire drug resistance *in vitro* faster than other strains (5-7).

It is generally accepted that clinical drug resistance, i.e. the failure of antibiotic treatment to control tuberculosis (TB) in a patient, can emerge through augmentation of a small initial population of resistant mycobacteria that become dominant when susceptible bacteria are eliminated by antibiotic treatment (1,8). Thus, antibiotic treatment combining different agents can prevent that a mutant which is resistant to a single antibiotic is able to become clinically relevant and an effective combination has shown to provide cure within 6 to 8 months, also in an outpatient setting (9-11). Lacking a better explanation, patient non-compliance with the prescribed treatment has been blamed for drug resistance but other lines of evidence, such as the hollow fiber model, have confirmed that non-compliance alone is unlikely to create drug resistance when a fixed-dose combination is used (12). A more convincing theory is pharmacokinetic and pharmacodynamic variability between patients and within patients referring to differences in drug absorption and metabolism, uneven drug distribution and inflammation containing bacterial subpopulations that are not equally susceptible. All these factors could conspire to create pockets or temporal windows of monotherapy within patients' lungs where resistance develops (13). Recently published work by Prideaux and co-authors appear to confirm this theory with RIF being present at higher concentrations in lung lesions including pleural empyema and necrotic caseum mimicking RIF monotherapy (14, 15). The pivotal question at this point is whether, and how quickly, RIF monotherapy could lead to clinically relevant RIF resistance with more than 1% RIF resistant bacteria found in a sputum sample.

To approximate this clinical scenario, we treated 14 newly diagnosed, RIF-susceptible TB patients with RIF at the standard dose of 10 mg/kg/day for 14 days, the longest time considered safe for monotherapy, followed by a full course of standard combination treatment to ensure cure of these subjects (16). We harvested sputum before treatment and daily for the first 2 weeks to measure the total sputum bacterial load over the 14-day period as colony forming unit (CFU) counts and the mutation frequency for RIF before and at 14 days (for detailed methods see online supplemental material). From these data we constructed a statistical model and extrapolated,

assuming linear continuation to the rates of change measured over the first 14 days, how quickly RIF resistance could become clinically relevant.

At baseline, we found a median of 5.63 (range: 4.21 to 7.12) $\log_{10}$CFU/ml of sputum and a mutation frequency of 4.9 (range: 0.8 to 151.8) $\times 10^{-9}$. Over 14 days, the CFU counts dropped by an average of 0.093 $\log_{10}$CFU/day, the expected effect of the antibiotic, and the mean mutation frequency increased 1,144-fold (Table 5.1). Twenty-four out of 28 phenotypically resistant cultures recovered from the mutation frequency experiments were found resistant with the GenoType MTBDR*plus* line probe assay (Hain Lifescience, Nehren, Germany) that covers the region with the most common resistance-conferring mutations on the *rpo*B gene (17). The genome of one baseline and three day 14 cultures phenotypically resistant but determined as susceptible by the line probe assay was sequenced, which revealed one less commonly found mutation on codon 537 located outside the region of the *rpo*B gene (Table 5.2).

Reflecting the epidemiological situation in Cape Town we found Beijing strains (43%) to be predominant. Figure 1 shows modelled (first 2 weeks) and interpolated lines for the total sputum CFU (descending black line) and mutation frequency (ascending black line) with confidence intervals. Also depicted are the predicted resistant sputum CFU (blue) as the product of mutation frequency and total CFU, and the level of clinical resistance at a 1% of total CFU (red) (18). The illustration shows that, in the absence of resistance, culture-negative sputum could be expected at around 2 months of treatment with RIF alone. However, resistant CFU would become measurable at the critical proportion of 1% of the remaining CFU at about 1 month of RIF monotherapy and would subsequently dominate in sputum, preventing culture conversion to negative.

This scenario, though hypothetical and assuming linear continuation of the trends observed in the first two weeks, ties in very well with clinical observations made in some patients treated with RIF-based regimens. In these individuals, initial clinical improvement stagnates after a few weeks and is followed by clinical deterioration and persisting positive sputum or reversion of negative to positive sputum. Clinical guidelines that have stood the test of time recommend that patients who fail to clinically improve or convert sputum smears to negative at two months of treatment are at risk of having acquired resistance and should undergo sputum culture and resistance testing (9). Depending on the proportion of resistant bacteria present in this sputum sample this could be diagnosed as hetero-resistance or resistance to RIF necessitating a switch of treatment in these patients (19).

Our findings can add to the growing evidence that pharmacodynamic and pharmacokinetic factors are potentially to blame for rising rates of clinical TB drug resistance despite strong support for consequent combination treatment of TB by the public health sector. Our approach is limited by the fact that we interpolate rather than directly measure the augmentation of RIF

resistance clinically, but it would be unethical to expose patients to the risk of acquisition of resistance to RIF to validate this in the field. Further research should focus on methods to measure drug concentrations, drug effects and drug resistance at the site of disease. This will aid to develop treatment regimens that protect patients from the acquisition of drug resistance during treatment.

## Acknowledgements

## Conflict of interest

The authors declare no conflict of interest.

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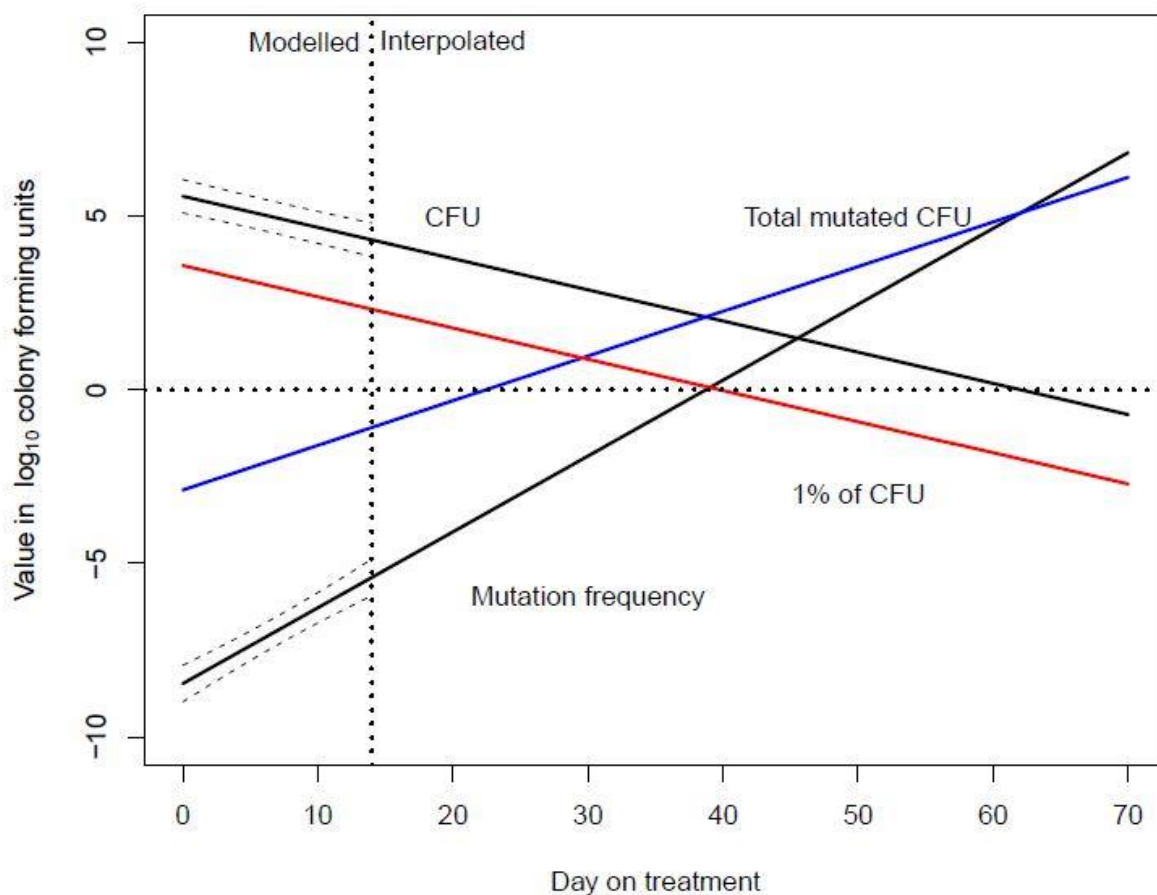
**Figure 5.1:** Statistical modelling estimating the time required for the emergence of RIF resistance in the *M. tuberculosis* population when TB patients are kept on RIF monotherapy beyond 2 weeks.

Two weeks of RIF monotherapy induced a significant decrease of $\log_{10}$(CFU) (black line with dotted confidence interval) ($P < 0.0001$) and a significant increase of the mutation frequency (black line with dotted confidence interval) ($P < 0.0001$). The blue line is the proportion of RIF mutants and the red line is the estimated daily remaining 1% CFU. The model estimates that the proportion of 1% CFU considered as breakpoint to define clinical resistance can be reached after 30 days of RIF monotherapy (blue line crosses the red) and around 40 days (blue line crosses the black) all remaining CFU will be resistant.

**Table 5.1:** Colony forming unit counts and mutation frequency at baseline and at day 14 of treatment with RIF.

| Strain type | CFU counts ($\log_{10}$(CFU)) | | Mutation frequency ($\times 10^{-7}$) | |
|---|---|---|---|---|
| | Baseline | Day 14 | Baseline | Day 14 |
| Beijing | 6.84 | 5.09 | 0.503 | 45.405 |
| T1 | N/A | N/A | 0.026 | 301.793 |
| Beijing | 5.20 | 2.82 | 0.008 | 64.044 |
| X2 | 4.92 | 3.67 | 0.587 | 103.569 |
| CAS1_KIL | 5.09 | 3.3 | 0.719 | 97.667 |
| Beijing | 5.11 | 3.67 | 0.060 | 151.128 |
| Unknown | 5.06 | 4.16 | 0.555 | 159.136 |
| Beijing | 6.93 | 6.24 | 0.035 | 9.012 |
| S | 6.64 | 4.94 | 0.009 | 11.994 |
| Beijing | 4.21 | 4.93 | 0.017 | 6.512 |
| LAM3 | 7.12 | 5.49 | 0.038 | 60.964 |
| Beijing | 6.00 | 6.31 | 0.442 | 90.420 |
| LAM3 | 6.59 | 5.21 | 1.518 | 64.763 |
| S | 5.63 | 2.40 | 0.028 | 210.312 |
| Median | 5.63 | 4.93 | 0.049 | 77.60 |
| Minimum | 4.21 | 2.04 | 0.008 | 6.512 |
| Maximum | 7.12 | 6.31 | 1.518 | 301.80 |

*Abbreviations:* CFU, colony forming unit; T1, T1 family; X2, X2 family; CAS1_KIL, Central Asia 1 Kilimanjaro family; S, S family; LAM3, Latin American and Mediterranean 3 family; N/A, value not available at this time point due to contamination.

**Table 5.2:** Codons and changes in *rpo*B of four isolates of *M. tuberculosis* determined as susceptible to RIF by line probe assay.

| Isolate | Codon no | Wild type | Mutation | Change |
|---------|----------|-----------|----------|--------|
| 1 | 526 | CAC | TAC | H → Y |
| 2 | 531 | TCG | TTG | S → L |
| 3 | 526 | CAC | TAC | H → Y |
|   | 537 | GGT | AGT | G → S |
| 4 | 526 | CAC | GAC | H → D |

*Abbreviations:* A, adenine; C, cytosine; G, guanine; T, thymine, D, aspartic acid; G, glycine; H, histidine; L, leucine; Y, tyrosine and S, serine.

# Online supplemental material

## Participants, parent study, samples and ethical approval

Fifteen smear positive and drug sensitive pulmonary tuberculosis patients ($\geq$ 1+ IUATLD and WHO scale) were recruited as control group for an early bactericidal activity (EBA) study receiving daily rifampin (RIF; 10 mg/kg/day) for 2 weeks. Details of the parent drug trial (clinicaltrials.gov NCT01218217) are published elsewhere [1]. In total, sixteen overnight sputum samples were collected from each patient, two before the start of treatment and on treatment from day 1 to day 14. After collection, specimens were transported to the laboratory (Department of Biomedical Sciences, University of Stellenbosch, Tygerberg, South Africa) at 2 to 8ºC where they were processed. Ethical approval for this study was granted by the local ethics committee and the Medicines Control Council of South Africa.

## Colony forming unit count

Upon arrival at the laboratory, samples were homogenized for 30 minutes by magnetic stirring and a volume of 10 ml homogenized sputum sample were digested with an equal volume of 0.1% dithiothreitol (Sputasol, Oxoid, Cambridge, UK) for 20 minutes. Serial dilutions of digested sputum were prepared ($10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$) and 100 µl of these were inoculated onto each half of a Middlebrook 7H11 agar bi-plate supplemented with oleic acid, albumin, dextrose, catalase (OADC; Becton Dickinson, Sparks, MD, USA) and Selectatab (Mast; Merseyside, UK). The plates were then incubated for 3 weeks at 37ºC, the colony forming unit (CFU) counts determined, averaged from a maximum of four possible counts and converted into $\log_{10}$(CFU) for statistical analyses; contaminated plates were excluded from analysis.

## Determination of mutation frequency

Frozen baseline and day 14 isolates from 14 patients were thawed and cultured in Middlebrook 7H9 liquid media supplemented with 10% OADC, 0.5% glycerol (Sigma Aldrich, Cape Town, South Africa) and 0.05% Tween 80 (Sigma Aldrich) and incubated for 3 weeks at 37°C. The grown cultures were adjusted to 1 McFarland and used for a fluctuation assay according to a modified protocol published elsewhere [2]. In brief, 1.2 µl of adjusted culture was inoculated into 120 ml of 7H9 media supplemented with 10% OADC, 0.5% glycerol, and 0.05% Tween 80 to prepare a low density culture with a bacterial load of 3,000 cells/ml[3]. The volume was divided into 24 cultures of 5 ml each and incubated for 4 weeks at 37ºC. After this, 1 ml of volume from 21 cultures was each transferred into a 1.5 ml reaction tube and centrifuged for 3 minutes at $10,000 \times g$. The supernatant was discarded, the pellet of about 500 µl was vortexed and 100 µl of

these were used to inoculate each compartment of a 7H11 agar bi-plate containing 1 µg/ml RIF (critical concentration); each plate was incubated for 4 weeks at 37ºC. The three remaining cultures of each isolate were diluted up to $10^{-3}$ in Tween Saline and further diluted to $10^{-4}$, $10^{-5}$ and $10^{-6}$ in 7H9. A volume of 100 µl of these three last dilutions were each inoculated onto both compartments of a drug free 7H11 agar bi-plate and incubated as above. After incubation, the 21 RIF-containing plates with resistant colonies were individually counted, summed up and averages were calculated for baseline and day 14. The same procedure was followed for the drug free 7H11 plates to estimate the CFU count which is equivalent to the total number of viable bacteria in each culture. Finally, the mutation frequency was calculated for each isolate by dividing the average number of resistant colonies counted on all 21 RIF-containing agar plates by the total number of bacteria in culture. This experiment was done in duplicate and the results averaged.

## Determination of mutation types in resistance bacteria

Colonies of baseline and day 14 samples resistant to 1 µg/ml were grown again for 3 weeks on 7H11 agar plates containing 10 µg/ml RIF. Cultures were inactivated for 2.5 hours at 80ºC and DNA was extracted using the phenol-chloroform-isoamyl alcohol method (4). The colonies were harvested using a sterile loop and re-suspended into 6 ml DNA extraction buffer (5% sodium glutamate, 50 mM Tris-HCl [pH 7.4] and 25 mM EDTA) containing beads (4 mm diameter). The mixture was vortexed for 2 minutes, 500 µl of sterile water with 50 mg lysozyme (Sigma-Aldrich) were added and incubated for 2 hours at 37ºC while being vortexed every 30 minutes. After this, 600 µl Proteinase K buffer (5% sodium dodecyl sulfate, 100 mM Tris-HCl [pH 7.8], 50 mM EDTA) and 150 µl of Proteinase K enzyme solution (10 mg/ml, Sigma-Aldrich) were added to the mixture and incubated for 16 hours at 45ºC. Then 5 ml of phenol-chloroform-isoamyl alcohol (25/24/1, v/v) were added and incubated for 2 hours with inversion every 30 minutes. The tube was spun down at 3,000 rpm for 20 minutes at 21ºC, the aqueous phase removed and transferred into a new tube containing 5 ml chloroform-isoamyl alcohol (24/1, v/v). A second centrifugation step followed as before, the supernatant was removed and transferred into a new tube with 600 µl of 3 M sodium acetate [pH 5.2]. The tube was gently inverted for 5 minutes, 7 ml of ice-cold isopropanol was added, the tube was inverted again and the DNA fished using a glass rod. The tube was left for 3 hours at room temperature to air dry, 150 µl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) was added and left for 1 hour before it was stored at -20ºC. The concentration of DNA in the tube was determined using the nanodrop machine (Inqaba Biotec, Pretoria, South Africa) and only samples with DNA of good quality (250 ng/µl, 260/280 = 2 and 260/230 = 1.8) were used for PCR amplification. The necessary

primers were designed using Primer 3 software version 0.4.0 and 4 sets of primers were designed as follows (5):

Forward 1: 5' accgaggactgatgaaggtg 3', Reverse 1: 5' agcggaccagatattcgatg 3',

Forward 2: 5' cctggaagaggtgctctacg 3', Reverse 2: 5' cgttgtcgtgcatcacagt 3',

Forward 3: 5' agtacgtgccctcgtctgag 3', Reverse 3: 5' atcgcctcgtacaccttgac 3',

Forward 4: 5' gagctggtgcgtgtgtatgt 3', Reverse 4: 5' acggatctggcgcatctc 3'.

The HotStarTaq Master Mix Kit (Qiagen, Hilden, Germany) was used to generate the PCR mix and to conduct the amplification following the instructions of the manufacturer. Each reaction tube contained 25 µl of HotStar Master Mix, 5 µl of primers, 19 µl of RNase free water and 1 µl of DNA template. The following cycling program was applied using a 2720 thermocycler machine (Applied Biosystems, Foster City, CA, USA): Initial heat activation for 15 minutes at 95ºC, denaturation for 1 minute at 94ºC, annealing for 1 minute at 56ºC, extension for 4 minutes at 72ºC and final extension for 10 minutes at 72ºC. After amplification, the PCR products from four RIF resistant isolates determined susceptible by line probe assay and a DNA standard (Universal DNA ladder, Kappa Biosystems, Cape Town, South Africa) were loaded on a 1% agarose gel (w/v) (SeaKem LE Agarose, Lonza, Rockland, ME, USA) in a box containing 2 l of TBE (Tris–borate-EDTA, [pH 8.3]) buffer mixed with 50 µl of ethidium bromide (Sigma-Aldrich). The gel was run for 2 hours at 150 volts. Separated DNA bands were visualized using the Gel Doc Imaging system (Bio-Rad Laboratories, Johannesburg, South Africa) and compared to the DNA standard. The PCR products were isolated and prepared for DNA sequencing.

## DNA sequencing and characterization of mutations

Firstly, the DNA amplified by PCR was extracted using the Nucleospin DNA extraction kit from Macherey Nagel (Macherey Nagel GmbH & Co KG, Duren, Germany) on a Tecan Genesis robotic station (Tecan Group Ltd, Mannedorf, Switzerland). Then a post PCR clean-up was performed using the Nucleofast 96-well PCR plate from Macherey Nagel on a Tecan EVO150 robotic station and DNA sequencing was conducted using a Big Dye Terminator V3.1 sequencing kit (Applied Biosystems). The sequencing product was treated with SDS before being transferred to a Sephadex column using the Tecan EVO150 robotic station and centrifuged. The cleaned sequencing reaction product was dried using a heated vacuum drier and re-suspended in Hi-Di formamide (Applied Biosystems). The sample was denatured for 2 minutes at 95ºC and placed on ice for 5 minutes. DNA sequencing electrophoresis was performed on either an ABI3130xl or an ABI3730xl genetic analyzer using a 50 cm capillary array and POP-7 polymer (Applied Biosystems). Lastly, 2 µl of cleaned PCR product were mixed with appropriate internal size standard (Applied Biosystems) and Hi-Di prior to denaturing for 5 minutes at 95ºC and placed on

ice for 5 minutes directly after this. The fragment analysis electrophoresis was done on either an ABI3130xl or an ABI3730xl genetic analyzer using a 50 cm capillary array and POP-7 polymer (Applied Biosystems). All protocols were supplied by the manufacturers.

The sequences received were aligned using Sequencher 5.1 software (Gene codes corporation, Ann Arbor, MI, USA) and then compared to the *rpo*B gene from the H37Rv reference strain of *M. tuberculosis* (Tuberculist database) to identify mutations.

# Spoligotyping

Strain types were determined according to the method described by Kamerbeek *et al* (6*)*. Briefly, the Direct Repeat (DR) region of *Mycobacterium tuberculosis* was amplified using two primers; DRa: 5'-ggttttgggtctgacgac-3' and DRb: 5'-ccgagaggggacggaaac-3'. After amplification, PCR products were hybridized to a membrane containing a set of 43 oligonucleotides corresponding to each spacer of the DR region. DNA from H37Rv and BCG were used as controls. The hybridized PCR products were incubated together with streptavidin-peroxidase conjugate, then the membrane was exposed to a chemiluminescent system followed by the exposure to an X-ray film according to the manufacturer's instructions. The X-ray film was developed, the spoligotype analyzed and the strain types determined using the SITVIT database (Institut Pasteur de la Guadeloupe, Abymes, Guadeloupe).

# Statistical analysis

It was assumed that the calculated mean mutation frequencies, estimated on baseline and day 14, were from an exponential base 10 distribution, so that the logs are linear over time. A linear mixed-effects model was fitted to $\log_{10}$(CFU) daily measurements over the 14-day period. These lines and the 95% confidence intervals for the points on the lines are shown in black on Figure 1, extended and interpolated beyond the 14 days. This interpolation made it possible to predict the time point where 1% of the remaining CFU will be RIF-resistant mutants. Additionally, the line of total mutated CFU was inferred in order to estimate the time point when all CFU will be mutated. All modelling and graphics were done using functions from R (www.r-project.org) (7,8).

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# Chapter six

# General discussion and future perspectives

The development process of novel drugs can take more than 20 years and passes through different phases of clinical trials (1-3). This long duration combined with other factors like costs and lack of investors in TB research are the reasons why the anti-TB drugs available on the markets are more than 40 years old. New anti-TB drugs are urgently needed to tackle the problem of drug resistance which is now taking TB treatment back to the era of pre-antibiotic therapy confirmed by the recent discovery of TDR strains (4-7). These new compounds should be ideally effective against LTBI so that treatment could be shortened and TB possibly eradicated. The long duration of TB treatment is one of the underlying causes of the emergence of drug resistance as some patients quit early before the completion of treatment (8,9); if this duration was shortened the emergence of drug resistance could be prevented.

Among the different phases of clinical trials, the EBA study of two weeks is the first step to assess the early efficacy of new anti-TB drug candidates. CFU count and TTP determination are two culture methods currently used to evaluate an EBA (10). They are time consuming and need sophisticated and expensive infrastructure. This can lead to delays in the drug development process thereby making this very costly. New and fast methods particularly automated and affordable are needed to speed up the drug development process. One new PCR based diagnostic method is the Xpert also called "game changer". It uses both processed and unprocessed sputum samples, gives results in less than two hours and detects Mtb and its resistance to RMP simultaneously (11-13). Considering this advance, the WHO has endorsed Xpert as a new diagnostic test for TB in December 2010 (14).

Chapter two of this thesis elaborates the comparison of EBA determined by CFU or TTP to the assessment by Xpert. The EBA of an anti-TB drug is defined as its capacity to decrease the mycobacterial load in lung lesions during the first two weeks of treatment (15). It is currently evaluated using sputum samples and culture of the mycobacteria present on solid and in liquid media. Here, the culture methods were found to discriminate better between the treatment groups than Xpert. The highly sensitive Xpert is not able to distinguish DNA from dead and viable cells. As dead bacilli remain longer in lung lesions, one can easily explain these discrepant results in mycobacterial load since these dead bacilli do not grow anymore but their DNA is detected. In order to make Xpert a method of choice for the assessment of drug EBA and TB treatment monitoring, it is important to be able to separate dead and injured bacilli from samples before using them for Xpert.

In chapter three, we described the pre-incubation of sputum samples with PMA before doing Xpert. PMA is a reagent which is able to penetrate bacteria with compromised integrity, to bind to their DNA after activation by light and to prevent its amplification by PCR (16). *In vitro*, an effect of PMA was observed in susceptible strains of Mtb grown in liquid culture containing INH or EMB. These two drugs are described to target different components of Mtb's cell wall (17,18). In XDR strains, an effect of PMA was only seen in strains incubated with EMB probably due to the requirement of several mutations for a full resistance against this drug (19). On the contrary, in clinical sputum samples collected from TB patients under standard treatment (i.e. combination of INH, RMP, EMB and PZA), the effect of PMA was not statistically significant. One wonders why the effect of PMA observed in INH or EMB single treated bacteria could not be repeated in clinical samples from patients receiving standard treatment including EMB and INH. A possible explanation for this observation could be *in vivo* factors such as the pressure of the immune system, pharmacokinetics/pharmacodynamics and drug absorption default at intestinal level. *In vitro* experiments are done in absolute controlled environment where the strains are exposed to an exact concentration of drug whereas the conditions *in vivo* cannot be controlled entirely. Our findings increase the doubts regarding the usage of PMA combined with Xpert to monitor TB treatment or to evaluate the EBA of novel anti-TB drug candidates.

Future research could focus on the discovery of more suitable reagents to be combined with Xpert to enable an effective separation of viable and dead bacteria. Then, Xpert could be integrated into both EBA studies and TB treatment monitoring.

TB eradication is made difficult due to the ability of Mtb to enter a state of latency once it is in a hostile environment and to stay there until reactivation. Many *in vitro* models have been established to study dormant forms of Mtb (20-23) but these models do not mimic exactly what is happening inside the lungs. Different mechanisms such as the immune system, hypoxia, starvation and low pH interact together to induce dormancy. This makes it challenging to study how dormant cells of Mtb respond to chemotherapy as they are the ones that should be eliminated to achieve total cure. In chapter four, we showed that dormant Mtb exists in sputum samples from TB patients before the start of treatment that this type of bacteria increases by treatment of patients with RMP or SQ109 but not by treatment with a combination of RMP/SQ109. Evaluating the proportion of dormant Mtb during therapy could help to identify drugs that are effective against dormant forms of Mtb since most anti-TB drugs currently used target replicating bacilli; standard TB treatment is extended to duration of six months in order to clear the lungs of dormant cells (24). A drug acting specifically on dormant bacteria would be a big step towards shortening of TB treatment and eradication of this disease.

The subpopulation of dormant Mtb is present in lungs and is not detectable by normal diagnostic methods such as ZN microscopy and solid culture. We compared the two main population of Mtb in the lesions to an iceberg (see Figure 4.5) where the top represents the replicating bacteria which can be detected and killed by anti-TB drugs and where the bottom represents the dormant bacteria, always invisible but present. The "melting" of the top does not mean that the entire iceberg disappears: Once the replicating forms of Mtb are no longer detected in sputum samples for example by ZN microscopy, this does not imply that all bacteria were killed. The dormant ones are still present and are waiting for reactivation.

The treatment of TB patients with a combination of RMP and SQ109 did not significantly increase the proportion of dormant Mtb. One can speculate that this was due to the fact that these two drugs have shown a synergy *in vitro* (25) with RMP being mainly active against fast replicating bacteria but also against dormant forms of Mtb on a lower level (26-28). Therefore, SQ109 may be able to enhance the killing activity of RMP on dormant Mtb. Future research could look at other anti-TB drugs, as single or combined treatment, to assess how these dormant Mtb respond to them. Moreover, the development of methods for automated counting and analysis like flow cytometry or similar methods could increase the speed of determination and its specificity. At the moment, sputum smears with bacteria of different phenotypes are counted manually. An isolation of dormant Mtb in sputum samples from the entire bacterial population based on their respective size could allow a separate study without any influence of replicating cells. In this perspective, dormant bacteria could be resuscitated using resuscitation promoting factors and their subsequent exposure to antibiotics would be another way to study if reactivated Mtb responded differently to antibiotics compared to replicating cells.

The emergence of drug resistant Mtb occurred in the early days of TB treatment using SM only (29) and since then, resistances arose against all antibiotics currently used. Unlike other bacteria, Mtb does not possess the ability to transfer bacterial DNA conferring any drug resistance from one bacterium to another. Therefore, mutations in Mtb's genome have to occur spontaneously by errors during DNA replication (30). It was found that non-compliance to treatment and pharmacokinetic/pharmacodynamic factors are the main causes of development of drug resistance in Mtb (31,32). RMP being an important anti-TB drug has the lowest spontaneous mutation rate (33,34), therefore it is not clear how the resistance against RMP develops under chemotherapy. An assessment of the RMP mutation frequency in chapter five demonstrates that in a susceptible bacterial population, monotherapy of TB patients with RMP for two weeks increases the mutation frequency leading to an increase in the RMP resistant mutant subpopulation and to a decrease in the RMP susceptible subpopulation. Fortunately, the induced level of detectable resistance was

not clinically relevant. In order to predict when a clinical relevant resistance will develop in case the RMP monotherapy is continued beyond these two weeks, we modeled mutation frequency and daily CFU counts to determine the time point with a proportion of 1% mutants. We found that it can be reached after one month of monotherapy and can say that drug resistance is a process which develops gradually until it becomes clinically relevant. This probably happens due to an increasing pressure by the drug on the bacterial population escaping the killing activity of RMP by mutating.

Pharmacokinetic and pharmacodynamic factors are assumed to be the reasons for the emergence of drug resistance under chemotherapy. A system monitoring drug delivery in lung lesions using devices currently applied in nanotechnology to treat different diseases could help to avoid sub-inhibitory concentrations there (35,36). This could be a possible solution to any default in drug absorption at the intestine level particulary in HIV/AIDS infected people where RMP monoresistance is often found due to the low concentrations of INH, a protective companion drug to RMP during the continuation phase and to any decrease in permeability of fibrotic cavities and pleural empyema considered also to be the main cause of sub-inhibitory drug concentrations reaching lung lesions (37-39). Once the bactericidal concentration of drug is immediately deposited in lung lesions, Mtb will encounter the appropriate dosage and there will be no escape route. An implementation of such system could improve the treatment outcome and could decrease the emergence of drug resistance.

RMP and INH are used in combination to treat TB throughout the entire duration of standard TB treatment of 6 months. INH is metabolized in the liver by an enzyme called N-acetyltransferase 2 (NAT2) and among the patients, some are rapid acetylators and others are slow acetylators. A rapid INH acetylation would result in a low plasma concentration of INH therefore further investigations should be conducted to determine if there is any association between a low concentration of INH and acquired RMP resistance.

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# Main findings of this work

- Xpert performs poorly compared to culture methods (CFU and TTP) to evaluate the two weeks EBA of anti-TB drugs; therefore, it is not advisable to use it for this nor as TB treatment monitoring method (Chapter two).

- The incubation of samples with PMA before being measured by Xpert improves the assay's ability to quantify only viable cells from susceptible Mtb cultured in the presence of INH or EMB or from XDR strains cultured in the presence of EMB. Nonetheless this effect of PMA was not detectable in clinical samples collected from TB patients on standard treatment containing INH and EMB in combination with RMP and PZA (Chapter three).

- Staining of sputum smears with a combination of Nile Red and Auramine O analysed by confocal fluorescence microscopy is a useful tool to study alterations of the replicating and non-replicating subpopulations of Mtb in response to TB treatment (Chapter four).

- Monotherapy of TB patients with RMP for two weeks on TB patients induces an increase in the RMP resistant subpopulation of Mtb. However, a clinically relevant proportion of resistant Mtb will only occur if this monotherapy is continued for one month or longer (Chapter five).