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TREATMENT OPTIMISATION OF MULTIDRUG-RESISTANT TUBERCULOSIS

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Treatment optimisation of multidrug-resistant tuberculosis

Thesis for Doctoral Degree (Ph.D.)

By

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To those affected by tuberculosis without access to effective and tolerable drug

Popular science summary of the thesis

Treatment of multidrug-resistant tuberculosis (MDR-TB) only cures 60% of those starting treatment in the world. The definition of MDR-TB is tuberculosis with resistance to the two most effective drugs used in treating TB and affects about 450 000 people every year. For many years, MDR-TB treatment consisted of a drug combination of four to five drugs for 18-20 months and led to many people experiencing side effects.

Improvements have been achieved in the last 10 years and two new drug combination treatments for MDR-TB are now recommended that last six to 12 months. Another improvement is that four new effective drugs are also recommended including linezolid and bedaquiline. Even if linezolid is an effective drug for TB, people treated with linezolid experience many side effects which can sometimes become chronic. When drug treatment is given for MDR-TB, the right dose of each drug is also important since a low dose could lead to a lower effect and fewer people achieving a cure. The optimal dose can be studied by measuring drug concentrations in the blood and comparing these concentrations to pre-specified targets. An unanswered question is if adjusting the drug dose after drug concentrations will lead to a higher cure and lower risk of side effects in MDR-TB. Despite improvements in treatment, people with MDR-TB are sometimes not diagnosed, as only 37% of people falling ill with MDR-TB worldwide have a test for drug resistance performed. If proper testing of resistance to drugs is not done, ineffective drugs could be given. Even if these new drugs and drug regimens are available, we need better ways of improving treatment for the 40% that are not cured with treatment.

This thesis will investigate how treatment can be optimised with studies on which drugs to combine in a treatment, how drug resistance can be detected, what level of drug concentrations in the blood are effective, and how to reduce side effects.

In the first study, we investigated treatment with the drug pyrazinamide among people who were treated for MDR-TB in Karakalpakstan, Uzbekistan. Our results showed that adding pyrazinamide to a drug combination did not improve the number of people being cured. The results were the same if pyrazinamide was given for a longer or shorter time. In the second study, we evaluated if a newer way of detecting resistance to pyrazinamide can be used, namely whole genome sequencing, and what effect that has on treatment response in people with MDR-TB in Sweden. Whole genome sequencing is a rapid way to detect drug resistance simultaneously to many drugs by analysing mutations in the genes. We found that if pyrazinamide resistance was detected using whole genome sequencing, persons receiving pyrazinamide treatment had a quicker response to MDR-TB treatment.

In the third study, we investigated if drug concentrations measured in blood compared to the level of resistance of the TB bacteria (the so-called, minimum inhibitory

concentration, MIC) was related to suggested targets for treatment effect. The two drugs evaluated were levofloxacin and moxifloxacin, which are both fluoroquinolones and key drugs in MDR-TB treatment. We found that only 60-73% of persons treated with moxifloxacin and none with levofloxacin reached these targets in persons treated for MDR-TB in Xiamen, China.

The last study investigated what risk factors are related to side effects due to the drug linezolid in persons treated for MDR-TB in Sweden. We found that persons treated with a higher dose of linezolid than 12 mg/kg developed more side effects. By measuring linezolid drug concentrations in blood, we also found that a higher level than 2 mg/L led to more side effects.

In this thesis we found that treatment with pyrazinamide could be important in MDR-TB, at least to improve the response to treatment. Performing whole genome sequencing to detect resistance to drugs is a promising technique and it could be one way forward to make resistance testing simpler and quicker. Increasing the dose of especially levofloxacin for persons treated in China is important since too low doses could result in fewer persons being cured. Treatment with linezolid might need to be adjusted based on a person's weight to reduce the risk of side effects. Another way to lower the risk of side effects is to adjust the linezolid dose according to drug concentrations.

In conclusion, we need better means of improving treatment to reach all people falling ill with MDR-TB. Personalising treatment by adapting treatment according to the person and to the TB bacteria could be one way to optimise treatment for MDR-TB.

Abstract

A successful treatment outcome is seen in only 60% of persons treated for multidrug-resistant tuberculosis (MDR-TB) worldwide, defined as resistance to both rifampicin and isoniazid. To improve these disturbingly low numbers, treatment optimisation is highly needed. Therefore, this thesis will evaluate how to optimise a treatment regimen using both older and repurposed drugs in studies on regimen composition, resistance detection, target attainment for efficacy, and reduction of adverse drug reactions.

In the first retrospective observational study (**study I**), we evaluated the effect of pyrazinamide treatment on end-of-treatment outcomes in a cohort (n=508) of persons affected by MDR-TB in Karakalpakstan, Uzbekistan. We found no evidence (aOR 0.86, 95% CI 0.51-1.44, p=0.6) that pyrazinamide treatment was associated with end-of-treatment outcomes. In **study II**, pyrazinamide treatment was evaluated using time to sputum culture conversion in a historical Swedish MDR-TB cohort (n=157). We found strong evidence that no pyrazinamide treatment compared to receiving pyrazinamide treatment was associated with a longer time to sputum culture conversion (aHR 0.49, 95% CI 0.29-0.82, p=0.007), when accounting for genotypic drug susceptibility testing (DST).

In **study III**, we assessed the total exposure of moxifloxacin and levofloxacin over the minimum inhibitory concentration of the infecting *Mycobacterium tuberculosis* strain in persons with MDR-TB in Xiamen (n=32), China. In this prospective observational study, we showed that no participants treated with levofloxacin, and 60-73% receiving moxifloxacin, reached the proposed efficacy targets when dosed according to the Chinese national guidelines. In the last retrospective observational study (**study IV**), we evaluated risk factors for adverse drug reactions associated with linezolid treatment (n=132) for MDR-TB in Sweden. We found strong evidence that a daily linezolid dose of ≥12 mg/kg was associated with a higher risk of peripheral neuropathy (aHR 2.92, 95% CI 1.09-7.84, p=0.033), anaemia, or leukopenia. Moreover, in an exploratory analysis, a linezolid trough concentration of ≥2 mg/L was associated with a higher risk of anaemia and thrombocytopenia.

In conclusion, treatment with pyrazinamide seems to have a role in MDR-TB, at least in terms of improving interim outcomes. The use of genotypic DST is highly promising and may simplify and shorten the time to resistance testing. Adequate dosing of fluoroquinolones is important as underdosing could reduce treatment effects. Linezolid dose adjustment based on weight, or a high trough level might avoid adverse drug reactions. Importantly, dose adjustment needs to consider both efficacy and risk of adverse drug reactions, therefore, therapeutic drug monitoring can be a useful tool in the quest to personalise treatment.

List of scientific papers

I. **Kuhlin J**, Smith C, Khaemraev A, Tigay Z, Parpieva N, Tillyashaykhov M, Achar J, Hajek J, Greig J, du Cros P, Moore D. Impact of pyrazinamide resistance on multidrug-resistant tuberculosis in Karakalpakstan, Uzbekistan. Int J Tuberc Lung Dis. 2018;22(5):544-50.

II. **Kuhlin J**, Davies Forsman L, Mansjo M, Jonsson Nordvall M, Wijkander M, Wagrell C, Jonsson J, Groenheit R, Werngren J, Schon T, Bruchfeld J. Genotypic Resistance of Pyrazinamide but Not Minimum Inhibitory Concentration Is Associated With Longer Time to Sputum Culture Conversion in Patients With Multidrug-resistant Tuberculosis. Clin Infect Dis. 2021;73(9):e3511-e7.

III. Davies Forsman L, Niward K, **Kuhlin J**, Zheng X, Zheng R, Ke R, Hong C, Werngren J, Paues J, Simonsson USH, Eliasson E, Hoffner S, Xu B, Alffenaar JW, Schon T, Hu Y, Bruchfeld J. Suboptimal moxifloxacin and levofloxacin drug exposure during treatment of patients with multidrug-resistant tuberculosis: results from a prospective study in China. Eur Respir J. 2021;57(3).

IV. **Kuhlin J**, Davies Forsman L, Osman A, Skagerberg M, Jonsson J, Groenheit R, Mansjö M, Werngren J, Alffenaar JW, Schon T, Bruchfeld J. Linezolid-associated adverse drug reactions in MDR-TB in Sweden over 20 years: a comprehensive analysis. In manuscript

Scientific papers not included in the thesis

I. Davies Forsman L, Niward K, Hu Y, Zheng R, Zheng X, Ke R, Cai W, Hong C, Li Y, Gao Y, Werngren J, Paues J, **Kuhlin J**, Simonsson USH, Eliasson E, Alffenaar JW, Mansjo M, Hoffner S, Xu B, Schon T, Bruchfeld J. Plasma concentrations of second-line antituberculosis drugs in relation to minimum inhibitory concentrations in multidrug-resistant tuberculosis patients in China: a study protocol of a prospective observational cohort study. BMJ open. 2018;8(9):e023899

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III. **Kuhlin J**, Sturkenboom MGG, Ghimire S, Margineanu I, van den Elsen SHJ, Simbar N, Akkerman OW, Jongedijk EM, Koster RA, Bruchfeld J, Touw DJ, Alffenaar JC. Mass spectrometry for therapeutic drug monitoring of anti-tuberculosis drugs. Clin Mass Spectrom. 2019;14 Pt A:34-45.

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V. **Kuhlin J**, Tammelin A, Petersson J, Chryssanthou E, Tideholm-Nylen A, Schon T, Bruchfeld J. [Is it time to use sputum induction as a complementary specimen collection procedure in adult patients with suspected pulmonary tuberculosis?]. Lakartidningen. 2020;117.

Contents

List of abbreviations

aHR/HR	Adjusted hazard ratio/hazard ratio
aOR/OR	Adjusted odds ratio/odds ratio
AUC	Area under the concentration-time curve
BMI	Body mass index
CI	Confidence interval
C_{max}	Peak (or maximum) concentration
C_{min}	Trough (or minimum) concentration before the next dose
CTCAE	Common Terminology Criteria for Adverse Events
DNA	Deoxyribonucleic acid
DST	Drug susceptibility testing
DS-TB	Drug-susceptible tuberculosis
ECOFF	Epidemiological cut-off value
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
HIV	Human immunodeficiency virus
M. tuberculosis	*Mycobacterium tuberculosis*
MDR-TB	Multidrug-resistant tuberculosis
MIC	Minimum inhibitory concentration
PD	Pharmacodynamic
PK	Pharmacokinetic
TB	Tuberculosis
WHO	World Health Organization

Preface

When I first arrived in Karakalpakstan, Uzbekistan, in 2011, working as a doctor for Médicines sans Frontières, I wasn't mentally prepared for what treatment for multidrug-resistant tuberculosis (MDR-TB) entailed. I had read up on all the available guidelines for treatment and discussed them with infectious diseases colleagues, but the reality was far worse than what those texts and words could describe. Behind those numbers of 32.1% experiencing nausea or 13.2% with psychiatric disorders[1], I met people throwing up every day or somebody who would later commit suicide, despite having the best available treatment at that time. Not only were the regimens toxic but despite 18 months of treatment, many persons affected by MDR-TB were still not cured. After almost two years of working in Karakalpakstan and seeing vast numbers of people struggling with MDR-TB treatment, I wanted to learn more. This thesis is the result of that first encounter that I will never forget.

To capture some of the hardships that persons falling ill with MDR-TB can face, I have included stories from meetings with persons affected by MDR-TB. All stories have been modified so that no person can be identified, and no real names are included.

A huge window faces the open fields around the house. The blue sky is framing the picturesque view of the yellow grass and we can see a cow that tied to the fence. Akram is lying under thick layers of duvets with multiple pillows behind his back so he can have a good view of the fields. "I'm coughing blood again," Akram says, and adjusts the duvet with his skinny arms. We leave painkillers and masks to his caregiver. This is probably our last visit to Akam's house. All the TB medicines have been stopped some months ago since no other drugs are available. There is no hope for a cure anymore. As we leave, Akram slowly turns his head towards the windows again, looking out onto the fields.

[1] Wu S, Zhang Y, Sun F, Chen M, Zhou L, Wang N*, et al.* Adverse Events Associated With the Treatment of Multidrug-Resistant Tuberculosis: A Systematic Review and Meta-analysis. Am J Ther. 2016;23(2):e521-30

1 Introduction

Tuberculosis (TB) is a global disease that disproportionally affects people in low- and middle-income countries (1). The End TB Strategy set out by the World Health Organization (WHO) is to end TB as a public health challenge by 2030 but the 10% decline in TB incidence between 2015 and 2020 is far from achieving this goal (2). People falling ill with multidrug-resistant tuberculosis (MDR-TB), defined as resistance to rifampicin and isoniazid, are highly affected by the lack of better means to reduce incidence and deaths, and improve TB treatment outcomes. Only 37% of the estimated 450 000 people with MDR-TB annually are tested for drug resistance, and if MDR-TB treatment is started, only 60% have a successful treatment outcome. Unsuccessful treatment outcomes are related to person specific factors such as comorbidities, treatment related factors, bacteriological factors such as drug resistance, and social and health care related factors, all of which need to be addressed (3–7). Furthermore, rapid and reliable ways of detecting drug resistance are needed to ensure effective drugs are prescribed and treatment regimens optimised (6).

In the last 10 years, highly promising results from several clinical trials and large-scale observational studies have become available providing evidence that treatment of MDR-TB for only six months is possible. However, these drugs and regimens are not yet available for most people with MDR-TB. Furthermore, different regimens are needed which can suit different populations and personal choices. Therefore, we need better ways of optimising the detection of drug resistance and improving the usage of all available drugs for MDR-TB to improve care for people affected by MDR-TB and treatment outcomes.

2 Literature review

2.1 Epidemiology

In 2021, the WHO estimated that 10.6 million people fell ill with TB of which 450 000 had MDR-TB (1). The incidence of both TB and MDR-TB increased compared to the year 2020, which is thought to be due to the Coronavirus disease (COVID-19) pandemic. In the same year 1.6 million people died of TB, second behind Coronavirus disease (COVID-19) but surpassing the deaths of both HIV and malaria together (1). This significant public health challenge largely overlaps with the HIV epidemic with a high incidence of TB in many sub-Saharan countries (>300 TB cases/100 000 people) as seen in Figure 1. Moreover, the HIV prevalence range between 1.4 and 48% in TB cases.

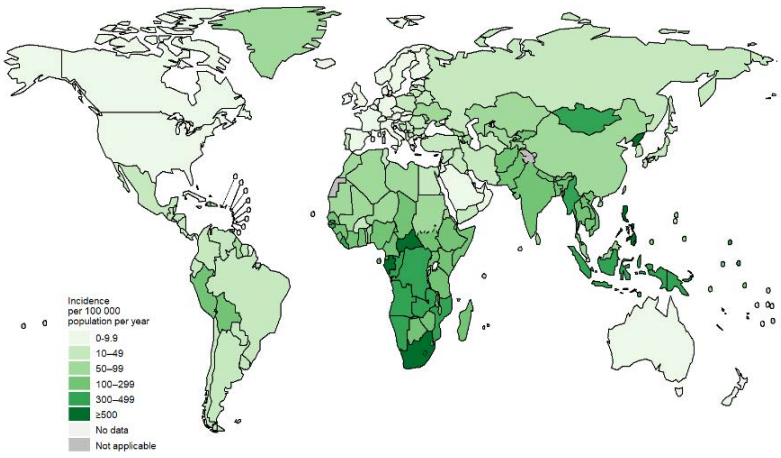

Figure 1 Estimates of the country-specific incidence of tuberculosis in 2021 by the World Health Organization (1). Reprinted with permission from the World Health Organization.

TB is a disease of social determinants of health such as poverty (8-10), undernutrition (11, 12), and crowded living conditions (9, 13) affecting vulnerable populations disproportionally (14, 15). In low-incidence countries like Sweden, migrants belong to an especially vulnerable group with a higher risk of developing TB disease (16-18). People moving within countries can also have a higher risk of TB, e.g., in China, partly due to differences in access to health care, lower socio-economic status, and crowded living conditions (19, 20).

The highest percentage of MDR-TB cases is seen in the Russian Federation, Central Asia, and countries in Eastern Europe (Figure 2). Globally, the percentage of MDR-TB among new TB cases was 3.9%, and in previously treated cases 20%, in 2021 (1).

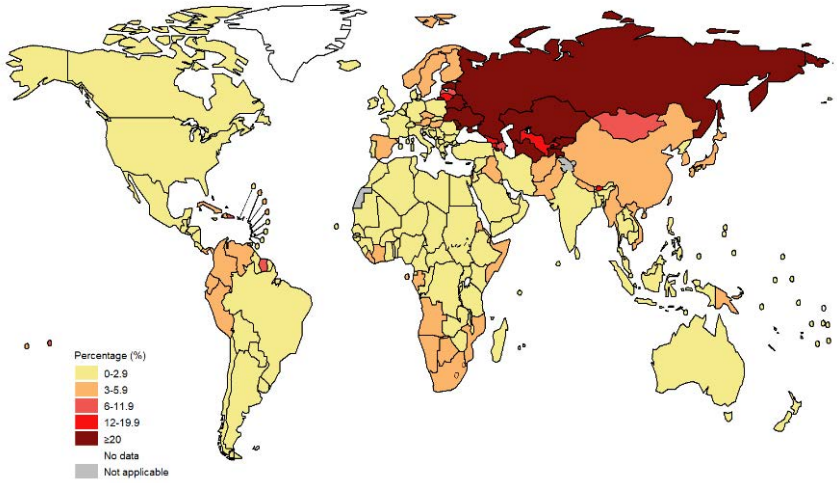

Figure 2 Percentage of new cases with multidrug-resistant tuberculosis and rifampicin-resistant tuberculosis in 2021 estimated by the World Health Organization (1). Reprinted with permission from the World Health Organization.

2.1.1 Epidemiology in Sweden, China, and Uzbekistan

Since this thesis includes studies from Sweden, China, and Uzbekistan, the epidemiology of TB in these countries will be described in more detail. The three countries differ in terms of the actual number of TB cases and the percentage of MDR-TB (Table 1), which will affect the countries differently since treating MDR-TB is resource intense. China has a dual burden of TB and MDR-TB with a high incidence of TB accounting for 7.4% of TB cases in the world but also a high number of MDR-TB cases (7% of MDR-TB cases in the world, Table 1) (1). Moreover, MDR-TB incidence varies between provinces in China (incidence between 4.2 and 29/100 0000), with a lower incidence along the coast (21). In countries in the former Soviet Union, like Uzbekistan, the TB epidemic is mainly due to MDR-TB. Many low-incidence countries including Sweden have a low burden of both the total number of cases and people with MDR-TB (MDR-TB incidence 0.17/100 000) with incidences seen in Table 1 (1).

Table 1 Incidence of tuberculosis and multi-drug tuberculosis in Sweden, China and Uzbekistan, 2021, according to the World Health Organization (1)

Rate or total (95% CI)	Sweden	China	Uzbekistan
Population (million)	10	1 426	34
TB rate per 100 000 population	3.8 (3.2-4.4)	55 (47-63)	62 (42-86)
Total TB cases	400 (340–460)	780 000 (665 000–905 000)	21 000 (14 000–29 000)
HIV positive TB incidence per 100 000	0.08 (0.05-0.13)	0.73 (0.62-0.85)	1.8 (1.2-2.5)
MDR-TB rate per 100 000 population	0.17 (0.11-0.23)	2.3 (1.9-2.8)	12 (7.9-17)
Total MDR-TB cases	17 (11–24)	33 000 (27 000–39 000)	4 200 (2 700–5 800)

TB = tuberculosis, CI = confidence interval, MDR-TB = multidrug-resistant TB

2.2 Mycobacterium tuberculosis

TB disease is caused by the *Mycobacterium tuberculosis* complex and disease in humans is predominantly caused by *Mycobacterium tuberculosis (M. tuberculosis*) (22). Mycobacteria are aerobic, acid-fast rod-shaped bacteria that grow slowly with an in vitro doubling time of about 20 hours (23, 24). They are facultative intracellular bacteria replicating predominantly within phagocytic cells such as macrophages where they can enter a dormant stage surviving for many years and even decades, leading to latent TB infection (previously called latent TB) (22, 25). In TB disease, three populations of bacteria have been described, namely extracellular rapidly growing bacteria, semi-dormant bacteria which either grow slowly or in an intermittent pattern, and intracellular slowly replicating bacteria mentioned above, so-called dormant bacteria (26, 27). For a long time, it was believed that semi-dormant bacteria are only intracellular, but lately, this has been disputed (28).

2.2.1 Development of drug resistance

On the molecular level, *M. tuberculosis* develops resistance mutations spontaneously (29) but in contrast to other bacteria such as *Escherichia coli*, there is no evidence that resistant genes are transferred between bacteria through plasmids (22). The growth of resistant clones occurs with positive selective drug pressure during treatment, while susceptible clones are suppressed or killed (30-32).

In clinical practice, the development of drug resistance during single-drug use was already noticed with streptomycin in the early TB studies in the 1950s (33). Therefore, multidrug regimens are key with at least three to four drugs in combination to prevent

resistant strains, preferably drugs that tackle different bacterial subpopulations such as rapidly growing and semi-dormant bacteria, mentioned above (31, 34).

Hence, drug resistance is a man-made phenomenon that is developed due to several reasons. One reason is a lack of appropriate drug intake due to; noncompliance by the person with TB (35, 36); prescription of inappropriate regimens; lack of quality drugs; or unreliable drug supply. Furthermore, pharmacokinetic (PK) variability of drug exposure can lead to drug resistance through several factors (see Section 2.8 for a general description of pharmacokinetics). Firstly, individual factors in the person such as malabsorption or genetic differences (37, 38) and, secondly, physiochemical properties of the drugs leading to variable drug distribution in different compartments, such as lung cavities or cerebrospinal fluid (39-42).

2.3 Diagnosis

Diagnosis of TB often includes several methods combined such as clinical, bacteriological, and radiological methods. A bacteriologically confirmed TB case is defined as TB with either a positive microscopy results, a WHO-recommended rapid molecular test, or a positive culture result, according to WHO (43). Having a bacteriological confirmation is preferred, to ensure the correct diagnosis and to detect possible drug resistance. Further discussion on bacteriological confirmation is provided below (Section 2.3.1., Detection of *M. tuberculosis*). A clinical diagnosis of TB disease is defined as a case that does not fulfil the criteria for bacteriological confirmation, but who has started treatment based on a decision by a physician (44). Typical symptoms of TB are a productive cough for more than two weeks, weight loss, and sweating at night. Characteristic radiological changes in pulmonary TB disease include apical opacifications, cavities, and enlarged intrapulmonary lymph nodes. In children, a bacteriologically confirmed TB diagnosis is often difficult to obtain, leaving symptoms, radiology, and a possible source for TB transmission such as a family member, the basis for diagnosis.

2.3.1 Detection of *Mycobacterium tuberculosis*

Bacteriological detection of *M. tuberculosis* in clinical practice is commonly performed by three methods, namely microscopy, rapid molecular assays, and culture.

2.3.1.1 Microscopy

Microscopy is a quick, relatively simple, and cheap method that is widely available but is also the least sensitive of the three methods, typically around 50% compared to culture. However, higher sensitivity around 68-73% has been described with concentration techniques using fluorescence microscopy compared to culture (45). Microscopy also predicts the risk of transmission as people with pulmonary TB having a positive microscopy result in sputum are the most infectious (15, 46).

2.3.1.2 *Rapid molecular tests*

Rapid molecular assays can be done directly using sputum giving a result within two hours to two days and have simplified and increased sensitivity of *M. tuberculosis* detection in many low- and middle-income countries (47-49). Using the commercially fully automated Xpert MTB/RIF Ultra (Cepheid Inc., Sunnyvale, USA) is one such example that is recommended by the WHO to replace microscopy as the initial test (47, 50).

2.3.1.3 *Culture*

Culture-based techniques to detect *M. tuberculosis* have the highest sensitivity but are slow. This is especially true for solid medium (Löwenstein-Jensen) on which *M. tuberculosis* usually grows within three to five weeks (51, 52). Liquid culture media is quicker and slightly more sensitive, typically with growth within two to three weeks, but requires more resources due to expensive equipment (e.g., BACTEC Mycobacterium Growth Indicator Tube [MGIT], Becton Dickson, Sparks, USA) (49, 51).

2.4 Detection of drug resistance

Since the included studies in this thesis are related to the drugs pyrazinamide, levofloxacin, moxifloxacin, and linezolid, these drugs will be used as examples in the following methods sections and later in forthcoming sections. A summary description of the four drugs including resistance breakpoints and minimum inhibitory concentrations (MICs) is provided in Table 2.

Table 2 Recommended doses for adults in multidrug-resistant tuberculosis and description of drug resistance testing including genes associated with resistance for pyrazinamide, levofloxacin, moxifloxacin, and linezolid

Drug	Recommended dose (44)	Critical concentration (53)	Tentative ECOFF (53)	MIC range (53)	Resistance genes (54)
Pyrazinamide	1500-2000 mg	100	64 (55)	≤8–64 (55)	*pncA*[1]
Levofloxacin	750-1125 mg	1.0	1.0	0.12–1	*gyrA, gyrB*
Moxifloxacin	400-800 mg	0.25[2]	0.25	≤0.06–0.25	*gyrA, gyrB*
Linezolid	600 mg	1.0	1.0	0.06–1.0	*rrl, rplC*

Critical concentrations, MICs and tentative ECOFFs are shown for liquid medium (MGIT, BACTEC Mycobacterium Growth Indicator Tube) since each MIC method have their own breakpoints. ECOFF = epidemiological cut-off value, MIC = minimum inhibitory concentration. 1 Other possible genes associated with pyrazinamide resistance are *panD* (56), *rpsA (57), and clpC* (58). 2 The clinical breakpoint is 1.0 mg/L using moxifloxacin 800 mg (53).

To detect drug resistance, different techniques can also be deployed for drug susceptibility testing (DST) of *M. tuberculosis*, namely phenotypic DST, MIC, rapid molecular tests, and whole genome sequencing.

2.4.1 Phenotypic drug susceptibility testing

Phenotypic DST using culture with a predefined breakpoint has traditionally been the reference method but this has slightly changed and i.e. genotypic DST is the proposed reference method for rifampicin and pyrazinamide (53). For phenotypic DST, both solid (commonly Löwenstein-Jensen) and liquid media (e.g., MGIT) can be used (53, 59, 60).

Additionally, phenotypic resistance (using phenotypic DST) is defined as the concentration that prevents the growth of 99% (90% for pyrazinamide) of an *M. tuberculosis* isolate and is called the indirect proportion method *(53)*.

Although phenotypic DST has been used since the first drugs for TB were developed in the 1950s, it can be challenging, which is especially true for pyrazinamide. Phenotypic DST for pyrazinamide is only performed using MGIT which limits the availability to high-resource settings and reference laboratories due to expensive equipment (55, 61). Furthermore, the methodology for pyrazinamide DST using MGIT differs from other drugs as an acidified culture medium is needed (62). Technical difficulties have been reported, especially related to reproducibility and false resistance (61, 62). This was highlighted in a Swedish study that reported a sensitivity of 97% and a specificity of 93% for pyrazinamide proficiency testing (61).

Phenotypic DST for fluoroquinolones and linezolid can both be performed using MGIT or solid media (53). Interpreting the results due to the definition of the critical concentrations by the WHO for fluoroquinolones, which has changed over the years, could be challenging which will be described below (1.4.5, Breakpoints to define resistance).

2.4.2 Minimum inhibitory concentration

Culture-based techniques are also used to quantify resistance using MIC, defined as the lowest concentration that inhibits the visible growth of bacteria. An advantage of MIC testing compared to phenotypic DST is that MIC gives a level of resistance that could guide physicians in dosing strategies. When performing MIC testing, an *M. tuberculosis* isolate is tested in two-fold serial dilutions of drug concentrations using solid or liquid culture (63). It is important to test the whole range of MICs since results can otherwise be truncated at the lower and upper end (64).

MIC testing is time-consuming and costly, therefore, performing MIC using broth *macro*dilution in MGIT or on solid media is rarely done in clinical routine except in high-resource settings for select drugs. For example, in Sweden, a limited range MIC for linezolid (0.25-1 mg/L) using MGIT in *M. tuberculosis* isolates has been performed since 2017 at the reference laboratory at the Public Health Agency. Due to cost, simplification and to increase the availability of MICs, broth *micro*dilution plates that simultaneously test several TB drugs have been developed (i.e. the commercially available Trek

Sensititre MYCOTB, [Trek Diagnostics, Cleveland, USA]) (65, 66). In 2019, the European Committee on Antimicrobial Susceptibility Testing (EUCAST), developed a carefully controlled broth microdilution method which was endorsed as the EUCAST reference method for MIC testing of *M. tuberculosis* (63).

2.4.3 Rapid genotypic drug susceptibility testing

Additional methods to detect drug resistance are rapid molecular tests, such as Xpert MTB/RIF assays which are simple and quick and can both detect *M. tuberculosis* as mentioned above, and resistance to rifampicin (95% pooled sensitivity and 98% specificity of both version 1 and Xpert MTB/RIF Ultra) (67, 68). Other WHO endorsed rapid molecular test are line-probe assays like Genotype MTBDR*plus* (Hain LifeScience GmbH, Nehren, Germany) and Genoscholar NTM + MDRTB II (Nipro, Tokyo, Japan), which have the advantage of both detecting rifampicin and isoniazid resistance (48, 69). In China, a, MeltPro MTB/RIF assay (Xiamen Zeesan Biotech Co Ltd, China) using a different technique based on a melting curve analysis, has been developed, however, it has not been endorsed by WHO (50, 70).

The advantages of the line-probe assays are their slightly higher sensitivity for rifampicin than Xpert MTB/RIF (pooled sensitivity and specificity using Genotype MTBDR*plus* and Genoscholar NTM + MDRTB II: 96.7% and 98.8% for rifampicin and 90.2% and 99.2% for isoniazid, respectively). The main disadvantage is their need for multiple manual steps, leading to limited use in low-resource settings except in regional or reference laboratories.

Rapid molecular tests to detect resistance to fluoroquinolones (and second-line injectable drugs) apart from rifampicin and isoniazid, have also been developed. The latest version of the line-probe assay provided by Hain LifeScience GmbH (Genotype MTBDR*sl*), has an 83-100% sensitivity and 93-100% specificity for fluoroquinolone resistance, when testing was done directly on sputum specimens (71). Similarly, in 2020, Xpert MTB/XDR was launched that could detect fluoroquinolone resistance (sensitivity, 88-96%, and specificity 91-100%) (72). Both tests are endorsed by WHO and could be used as the initial test in persons with bacteriologically confirmed pulmonary TB (50).

2.4.4 Whole genome sequencing and mutations involved in resistance

Although commercial rapid molecular tests are widely used and have improved detection of MDR-TB they are restricted to detecting a limited number of known mutations (64, 73, 74). Using whole genome sequencing is an alternative since many resistance genes are sequenced at once (75, 76). However, performing whole genome sequencing is often limited due to expensive equipment and trained staff (77). Furthermore, unknown genes conferring resistance and mutations with ambiguous resistance profiles are challenging, which could result in false results (64, 74, 75). Another

challenge is that whole genome sequencing is usually done from culture, limiting the benefit of a rapid turn-around time (77).

The different types of mutations in analysing whole genome sequencing for phenotypic drug resistance prediction are listed in Table 3. A single nucleotide polymorphism, SNP, leading to a change in amino acid (non-synonymous) could but does not always result in resistance. However, if no change in amino acid occurs (synonymous or silent mutation), the isolate will in the absolute majority of cases remain susceptible (75). Deletions and insertions are commonly associated with resistance, especially if a change in the reading frame arises.

Table 3 Types of mutations detected in whole genome sequencing

Genetic changes		Description
Single nucleotide polymorphism, SNP	Non-synonymous	A nucleotide substitution resulting in a change in amino acid.
	Synonymous	A nucleotide substitution that does not result in a change in amino acid.
Deletion		One or more nucleotides deleted. Could result in a frameshift.
Insertion		One or more nucleotides inserted. Could result in a frameshift.

The genes conferring fluoroquinolone resistance are *gyrA* and *gyrB* which code for the enzyme deoxyribonucleic acid (DNA) gyrase that is involved in the negative supercoiling of the DNA strand which is important for DNA replication (78) (Table 2). For linezolid, the genes *rrl,* and *rplC* are associated with resistance which are encoding mitochondrial ribosomal proteins involved in protein synthesis (79).

For pyrazinamide, the *pncA* gene has been established as being associated with resistance and a plethora of mutations have been found that are scattered throughout the *pncA* gene and its upstream promotor region (80, 81) (Table 2). The reason no hot-spot region is seen is likely related to the nonessential nature of the pyrazinamidase enzyme which is encoded by the *pncA* gene (82). The pyrazinamidase enzyme converts nicotinamide to niacin, which is important for cell metabolism, but also the pro-drug pyrazinamide to its active form pyrazinoic acid (83). Resistance in *pncA* was shown to lead to both lower abundance and lower enzyme activity of the enzyme pyrazinamidase (84). However, the mechanism of action is not fully understood. The longstanding theory has been that pyrazinoic acid led to acidification of the cytoplasm, resulting in the cytoplasmic enzymes becoming dysfunctional and disrupting the cell membrane, leading to bacterial death (83, 85). The acidification of the cytoplasm was believed to be due to pyrazinoic acid transporting extracellular H^+ ions into the cytoplasm. Hence, this was the suggested mechanism for why pyrazinamide was only active in an acidic environment (86). However, this has recently been challenged and pyrazinamide has

shown an effect on *M. tuberculosis* isolates growing in other conditions which result in environmental stress (apart from low pH), such as low oxygen tension (82, 87, 88).

Despite these new insights, several aspects of the mechanism of pyrazinoic acid remain uncertain. Between 70-100% of strains with phenotypic pyrazinamide resistance have been reported to have mutations in the *pncA* gene (89) which leaves room for other mechanisms. The strongest candidate gene is *panD* which is involved in fatty acid synthesis and cell metabolism, and pyrazinoic acid has been shown to degrade *panD* (56, 90). Furthermore, *rpsA* which is involved in protein degradation has also been related to pyrazinamide resistance (57, 91). However, the sensitivity of the detection of pyrazinamide resistance increased with a mere 2% if mutations in *rpsA* and *panD* were also included, apart from *pncA* (92). Recent work has also found *clpC* as a candidate gene, which is involved in the degradation of *PanD* (58, 90).

Since multiple mutations associated with pyrazinamide resistance are known in the *pncA* gene and its promotor region, and possible new mutations and target genes are being discussed, performing and interpreting whole genome sequencing is challenging (76, 80). To aid in the interpretation of whole genome sequencing, there are automated online resistance mutation catalogues such as the TB Profiler (93). Furthermore, international efforts to standardise and guide interpretation are also available such as the WHO mutation catalogue published in 2021 (54).

2.4.5 Breakpoints to define resistance

The main purpose of performing phenotypic DST for clinical use is to separate susceptible (or wild-type) strains from those with resistance to guide clinicians if a certain antimicrobial is effective and what dosing to use (94). A clinical breakpoint used for phenotypic DST takes into account the MIC distribution, genetic markers, the drug exposure in relation to drug resistance, and clinical outcomes (54, 63). The MIC distribution for wild-type bacteria (defined by EUCAST and WHO as "bacteria without phenotypically detectable resistance mechanisms") follows a normal distribution curve, typically with three to five two-fold dilution steps (53, 95). The highest concentration in this curve is called the epidemiological cut-off value (ECOFF) which is used in the definition of clinical breakpoints. In contrast to many other bacteria, only one drug (moxifloxacin, using a high dose of 800 mg) has a WHO-defined clinical breakpoint for *M. tuberculosis* since studies on drug exposure and clinical outcome in TB are sparse (53, 64, 96). Instead, critical concentrations are used, which were traditionally based on expert opinion and consensus (64, 97).

Using the critical concentration has been much debated and can cause problems with reproducibility if the MIC distribution of wild-type and non-wild-type strains lie near each other (64, 98). *M. tuberculosis* strains could then vary between susceptibility and resistance on repeated testing due to normal inter- and intra-laboratory variation (98,

99). This is highlighted with the use of the critical concentration definitions for levofloxacin and ethambutol, and WHO therefore does not recommend testing ethambutol using phenotypic DST (96, 100, 101).

Due to these reproducibility issues, WHO updated their guideline on resistance testing in 2018 and redefined the critical concentration to align with ECOFF as "the lowest concentration of an anti-TB agent in vitro that will inhibit the growth of 99% (90% for pyrazinamide) of phenotypically wild-type strains" (53). Moreover, several critical concentrations were withdrawn or changed in the guideline, including the critical concentrations and clinical breakpoint (moxifloxacin) for levofloxacin and moxifloxacin (Table 2).

Lastly, an aspect to consider in setting breakpoints for resistance testing is that the critical concentrations are different depending on which media and DST method is used (102). This is demonstrated in Table 4 where the critical concentrations for rifampicin and isoniazid are provided, which are used to define MDR-TB. Due to these various methods and breakpoints, EUCAST has developed and endorsed a broth microdilution method as the reference method (see Section 2.4.2 on MIC) to which all other methods could be calibrated and will be used for setting breakpoints for new TB drugs by the European Medicines Agency (63).

Table 4 Critical concentrations recommended for rifampicin and isoniazid for *Mycobacterium tuberculosis* using different culture media to define multidrug-resistant tuberculosis according to the World Health Organization (102)

	Solid media	**Liquid media**		
	Löwenstein-Jensen	Middlebrook 7H9 (used in MGIT)	Middlebrook 7H10	Middlebrook 7H11
Rifampicin	40 mg/L	0.5 mg/L[1]	1.0 mg/L	0.5 mg/L[1]
Isoniazid	0.2 mg/L	0.1 mg/L	0.2 mg/L	0.2 mg/L

1 Changed from 1.0 mg/L in 2021. MGIT = BACTEC Mycobacterium Growth Indicator Tube (Becton Dickson, Sparks, USA.

2.5 Clinical manifestation and transmission

TB has been called the great mimicker as the disease can present in numerous ways, sometimes baffling clinicians. Typical, but non-specific symptoms are weight loss, night sweats, and persistent fever. In pulmonary TB, initial dry cough followed by productive cough more than two weeks are common symptoms. In persons with extrapulmonary TB, such as lymph node TB, musculoskeletal TB, and TB affecting the central nervous system, symptoms depend on the location of TB disease.

Spread of *M. tuberculosis* bacteria occur through aerosols of droplets, emitted when a person coughs, sings, or talks. The smallest droplet nuclei (1–5 µm) can stay airborne for many hours and are able to infect a person when these droplet nuclei are inhaled and pass further to alveoli (103-105).

Established *M. tuberculosis* infection in a person has traditionally been divided into two distinct entities: Latent TB infection and TB disease. Persons with TB disease were those with clinical symptomatic TB who could be infectious and *M. tuberculosis* could be grown from specimens such as sputum. In contrast, persons with only latent TB infection had no symptoms and were neither infectious nor any *M. tuberculosis* could be grown. However, a spectrum of phases has instead been proposed ranging from latent TB infection to severe TB disease including subclinical disease (106). For pragmatic reasons, the two phases will be used in this thesis.

A person infected with *M. tuberculosis* has about a 5-10% risk of developing TB disease in their lifetime (107). Important and common clinical risk factors of developing TB disease are HIV infection (108), and undernutrition (14) as mentioned in Section 2.1 on epidemiology. Other factors are diabetes (109), treatment with anti-TNF drugs (110), excessive alcohol use (111), and smoking (112).

2.6 Treatment of tuberculosis

Treatment regimens for TB have classically been divided into two phases, first is the intensive phase which includes the highest number of drugs with the aim to rapidly reduce the number of viable bacteria and reduce the risk of acquired resistance (26, 27). Theoretically, this targets rapidly dividing *M. tuberculosis* bacteria. The continuation phase follows, where fewer drugs are used with the focus on targeting slowly replicating bacteria and reducing the risk of relapse (26). In some newer regimens developed (i.e. the new six-month regimen for MDR-TB including bedaquiline, linezolid, pretomanid, and moxifloxacin (113)), there is no such distinction between phases but instead, the same number of drugs are used throughout the whole treatment time.

Treatment of TB is also dependent on the resistance pattern of the infecting *M. tuberculosis* strain. These are divided into drug-susceptible TB (DS-TB), MDR-TB, pre-extensively drug-resistant TB (pre-XDR-TB), and extensively drug-resistant TB (XDR-TB), which are predictive of treatment success (Table 5). The definitions were updated in 2021 to reflect the new WHO-recommended regimens composition (114) (Table 6). Previously, extensively drug-resistant TB (XDR-TB) was defined as rifampicin resistance (with or without isoniazid resistance) and resistance to any fluoroquinolone, and at least one second-line injectable drug (capreomycin, kanamycin, or amikacin).

Table 5 Current disease categories based on resistant pattern and treatment outcomes according to the World Health Organization (1)

	Resistant pattern of the infecting *Mycobacterium tuberculosis* strain	Treatment success
DS-TB	Susceptible to all first line drug	86%[1] (1)
MDR-TB/RR-TB	Resistance to at least rifampicin and isoniazid/resistance to at least rifampicin (isoniazid could be susceptible or resistance)	60% (1)
Pre-XDR-TB	MDR-TB/RR-TB and resistance to a fluoroquinolone (moxifloxacin or levofloxacin)	57% (115)
XDR-TB	Pre-XDR-TB and resistance to at least bedaquiline or linezolid	Unknown

TB = tuberculosis, DS-TB = drug susceptible TB, MDR-TB = multidrug-resistant TB, RR-TB = rifampicin resistant TB, Pre-XDR-TB = pre-extensively drug-resistant TB, XDR-TB = extensively drug-resistant TB.
1 77% for those living with HIV.

2.6.1 Treatment of drug-susceptible tuberculosis

Until recently, the only treatment recommended for DS-TB was a six-month regimen consisting of rifampicin, isoniazid, pyrazinamide, and ethambutol for two months, followed by rifampicin and isoniazid for four months. The regimen was developed in the classic clinical trials led by the British Medical Research Council in 1946-1986 in mainly East and Central Africa, India, and Hong Kong (116).

A treatment length of six months was a dogma in TB, until 2021, when the long-awaited results of the trial TBTC Study 31/ACTG A5349 were published. The trial showed non-inferiority of a four-month regimen for adults and included high-dose rifapentine (a rifamycin similar to rifampicin), moxifloxacin, and pyrazinamide (117), and is now endorsed by the WHO (118). Moreover, a four-month regimen for children based on findings from the SHINE trial (119) aimed at those with non-severe disease, is also recommended by WHO and includes the four standard drugs for DS-TB (118).

During the development of the standard treatment for DS-TB between 1946 and 1986, important traits of pyrazinamide treatment were discovered that led the way to the DS-TB treatment regimen we have today (116). First, sputum culture conversion at two months was shown to increase with pyrazinamide treatment compared to ethambutol (116). Second, the sterilising effect of pyrazinamide was demonstrated by a lower relapse rate with pyrazinamide treatment (10-23% compared to 3-7%). Thirdly, the synergistic effect of pyrazinamide and rifampicin was shown when similar relapse rates were found in six- and nine-month regimens when the two drugs were used together. Lastly, the effect of pyrazinamide treatment was only seen in the first two months of a treatment since no difference in relapse was found if pyrazinamide was given for two, four or six months (116).

2.6.2 Treatment of multidrug-resistant tuberculosis

The WHO has continuously published guidelines for MDR-TB treatment and until 2018, there were only slight changes made to the recommended regimen (120-123). This previous longer MDR-TB regimen lasted 18-20 months and consisted of at least five drugs, including an injectable drug (kanamycin, amikacin, or capreomycin), a fluoroquinolone, and pyrazinamide. Typically, ethionamide/prothionamide and para-aminosalicylic acid or cycloserine were also included. However, this regimen resulted in a high risk of adverse drug reactions as will be described in Section 2.6.3 on adverse drug reactions.

A major improvement came in 2018 when an all-oral longer regimen was recommended. This regimen comprised four (to five) drugs that could be included from three WHO drugs groups (A, B, and C, Table 6) (53). The drug groups were based on efficacy and toxicity, with drugs in group A having the highest efficacy balanced with toxicity (43). The all-oral regimen also included the efficacious repurposed drug linezolid (an optional drug from 2006 to 2018), and the more recent TB drug bedaquiline (Table 6). Bedaquiline was approved by the United States Food and Drug Administration in 2012 and was the first new class of TB drug since the 1970s! (124). In 2014, delamanid, the second drug with a novel mechanism, was approved by the European Medical Agency (125). Delamanid (group C) can be included in the long all-oral regimen if other treatment options are not possible (43). The mode of action of these drugs will be described below in Section 2.6.6 on treatment with linezolid and in Section 2.6.7 on bedaquiline, delamanid, and pretomanid.

Pretomanid is the third new TB drug approved (first approved by United States Food and Drug Administration in 2019) and belongs to the same class of drug as delamanid (126). However, it is not recommended in the all-oral long regimen but in shorter drug combinations as will be discussed below (Section 2.6.7, Treatment with bedaquiline, delamanid, and pretomanid).

The evidence for the previous and current long regimens for MDR-TB (Table 6) is mostly based on observational data including meta-analyses (43, 115, 127, 128). The latest individual meta-analysis from 2018, included 12 030 people and evaluated the association between treatment with each drug in an MDR-TB regimen and treatment outcome (115). Treatment with linezolid, levofloxacin, meropenem/imipenem, moxifloxacin, and bedaquiline had the highest risk difference for treatment success compared to failure and relapse. Based on this meta-analysis and other evidence such as the STREAM Stage 1 trial mentioned below (129), the MDR-TB drugs were regrouped (groups A, B, and C, Table 6).

An advantage of a longer MDR-TB regimen compared to a standard regimen is the flexibility of regimen composition. Regimens can be designed based on a person's risk for adverse drug reactions, resistance pattern of the infecting *M. tuberculosis* isolate, and personal preferences. However, the current long MDR-TB regimen is limited by a long treatment duration of 18 to 20 months, potentially serious adverse drug reactions, especially due to linezolid, and poor treatment outcomes (60% globally) (1, 43).

Table 6 Current treatment regimens for multidrug-resistant tuberculosis recommended by the World Health Organization (43)

Regimens	Grouping of drugs or included drugs
6 months BPaLM, since 2022	
Three to four drugs in a standard combination. **Intensive phase**: No intensive phase **Total length:** Six months	Bedaquiline + pretomanid + linezolid +/- moxifloxacin (excluded when resistance to fluoroquinolones is present)
Shorter regimen, since 2016[1]	
Seven drugs in a standard combination. **Intensive phase:** Four to six months **Total length:** Nine to 12 months	Bedaquiline + levofloxacin/moxifloxacin + ethionamide/prothionamide + ethambutol + high-dose isoniazid + clofazimine + pyrazinamide
All-oral longer regimen, since 2018	
Four likely effective drugs in the intensive phase including all three drugs in Group A and at least one drug in group B. Drugs from Group C are added if a regimen cannot be composed of only Group A and B drugs. **Intensive phase:** Only if amikacin or streptomycin is included (given six to seven months) **Total length:** 18 to 20 months	**Group A:** Levofloxacin/moxifloxacin, bedaquiline, linezolid **Group B:** Clofazimine, cycloserine/terizodone **Group C:** Ethambutol, delamanid, pyrazinamide, imipenem/meropenem, amikacin/streptomycin, ethionamide/prothionamide, para-aminosalicylic acid

1 Linezolid for two months can replace ethionamide/prothionamide.

Due to these toxic MDR-TB regimens with poor outcomes for many years, new regimens have been in dire need. Therefore, in a little more than the last decade, several landmark studies have been conducted on MDR-TB which has markedly changed the treatment options and made shortened treatment for MDR-TB a reality. In 2010, the first landmark study was published which was a prospective observational study from Bangladesh showing high treatment success of a nine-to-12-month regimen (130). This standard regimen was composed of seven drugs including an injectable drug. The study from Bangladesh paved the way to the first randomised controlled MDR-TB trial in modern times evaluating a new regimen (STREAM Stage 1) (129). The STREAM trial assessed the shorter regimen including an injectable drug which showed non-inferiority of treatment outcome compared to the longer injection-based MDR-TB regimen.

In light of these studies, shorter MDR-TB regimens have been endorsed by WHO since 2016 (120). Initially, the regimen included an injectable drug which was replaced by bedaquiline in 2020. The shorter all-oral MDR-TB regimen is now recommended for fluoroquinolone susceptible MDR-TB (43). Apart from bedaquiline, this standard seven-drug regimen also includes a fluoroquinolone and pyrazinamide. Furthermore, ethionamide/prothionamide can be replaced by linezolid for two months (Table 6). The shorter regimens have been questioned as they could include potentially ineffective drugs (131), such as pyrazinamide, since global pyrazinamide resistance rates are estimated at 60.5% in MDR-TB strains (81). Advantages include preference by persons ill with TB, lower adverse drug reactions, similar success rates as the long MDR-TB regimen, and less strain on the health care system (43, 132).

The next step in shortening MDR-TB regimens came in 2020, when the first proof-of-concept study (the non-randomised single-arm Nix-TB trial) using a six-month regimen for MDR-TB was published (133). An entirely new regimen using a three-drug combination of bedaquiline, pretomanid, and linezolid (1200 mg daily) resulted in an impressively high success rate of 90%. However, the regimen was hampered by high rates of adverse drug reactions with 81% of participants experiencing peripheral neuropathy due to linezolid. Subsequently, two major randomised controlled trials were published in 2022, PRACTECAL and ZeNix, that evaluated a six-month regimen of bedaquiline, pretomanid, linezolid, and moxifloxacin (moxifloxacin only used in the PRACTECAL trial). The ZeNix trial (n=181) evaluated different doses and lengths of linezolid treatment (600 mg versus 1200 mg and 9 weeks versus 26 weeks) while PRACTECAL (n=145) compared the intervention regimen to the standard of care. Similar treatment success rates were seen in both trials (84-91%) when linezolid 600 mg was used for six months. The ZeNix trial team concluded that linezolid treatment at 600 mg for 26 weeks was preferred in terms of balancing success and toxicity (134). In the PRACTECAL trial, superiority of the trial regimen was observed over the standard of care (113). Interestingly, the difference in outcome between the standard of care and the trial regimen in the PRACTECAL trial was mainly driven by the early discontinuation of drugs due to adverse drug reactions in the standard of care arm (113). These six-month regimens are now recommended by WHO as the first option for persons with MDR-TB, followed by the shorter all-oral regimen, and lastly the longer all-oral regimen (43). Worth highlighting is that another randomised controlled clinical trial, MDR-END (n=168), has also been published in 2022 which showed non-inferiority using a nine-month regimen consisting of delamanid, linezolid, levofloxacin, and pyrazinamide over the standard of care (135). The authors report that rates of adverse drug reactions were similar between the intervention (75%) and standard of care (63%).

Several clinical trials are ongoing, evaluating new regimens so more treatment options for people affected by MDR-TB will likely be available in the next years (e.g., the PRESCIENT trial, ClinicalTrials.gov Identifier NCT05556746). Despite newly recommended all-oral longer and shorter MDR-TB regimens, the availability of especially the newer drugs bedaquiline, pretomanid, and delamanid, are limited in high-incidence countries due to costs and drug regulations, hampering the implementation of these regimens (136). Thus, many people are still treated with the older long and short MDR-TB regimens including injectable drugs.

2.6.3 Adverse drug reactions

Adverse drug reactions were common with the pre-2018 MDR-TB regimens with an estimated 32.1% suffering from gastrointestinal events, 14.6% ototoxicity, and 13.2% psychiatric disorders (137). A meta-analysis analysing each drug with the risk of adverse drug reactions showed that linezolid was associated with the highest risk of permanent discontinuation due to an adverse drug reaction (14.1%) (Table 7). Linezolid toxicity was followed by para-aminosalicylic acid, injectable drugs, and ethionamide/prothionamide, while the drugs with the lowest toxicity were clofazimine, bedaquiline, and levofloxacin (138).

Adverse drug reactions in MDR-TB due to linezolid are a limiting factor since the treatment duration is much longer than the 28 days it was approved for (139-142). The most serious adverse drug reactions are peripheral neuropathy (30%) and optic neuritis (8%) which could become irreversible, with the numbers in brackets referring to frequencies seen in meta-analyses (143, 144). Myelosuppression is also common (30%) which includes anaemia, thrombocytopenia, and neutropenia. Most adverse drug reactions are believed to be associated with human mitochondrial toxicity affecting the ribosomes (145, 146) (Figure 3). In the ribosome, linezolid has been shown to reduce protein synthesis of the respiratory chain complexes, thus, affects energy production in the human cell. An illustration of the proposed mode of action for toxicity (and mechanism of action in the bacteria) is seen in Figure 3. Another suggested mechanism for thrombocytopenia is immune-mediated (147).

Table 7 Adverse drug reactions[1] leading to permanent discontinuation of the drug in the treatment of persons with multidrug-resistant tuberculosis analysed in a meta-analysis (Adapted from Lan 2020, Table 5) (138)

Drug[2]	Incidence	Most frequent adverse drug reaction† (frequency)
Levofloxacin	1.3%	Musculoskeletal (64%), peripheral neuropathy (14%), rash (14%)
Clofazimine	1.6%	Hyperpigmentation (42%), cardiovascular (33%), rash (17%), gastrointestinal (8%)
Bedaquiline	1.7%	Cardiovascular (56%), hepatotoxicity (22%), CNS toxicity (11%), musculoskeletal (11%)
Ethambutol	1.8%	Visual impairment (70%), gastrointestinal (17%), musculoskeletal (3%), rash (3%)
Moxifloxacin	2.9%	Cardiovascular (21%), hepatotoxicity (17%), gastrointestinal (13%), peripheral neuropathy (13%), musculoskeletal (8%)
Imipenem, meropenem	4.9%	Hepatotoxicity (50%), rash (17%), fatigue (17%), pneumonia (7%)
Pyrazinamide	5.1%	Musculoskeletal (33%), gastrointestinal (23%), hepatotoxicity (20%), rash (13%), hyperuricaemia (6%)
Cycloserine, terizodone	5.7%	Psychiatric (66%), CNS toxicity (25%), gastrointestinal (4%)
Ethionamide, prothionamide	6.5%	Gastrointestinal (48%), hepatotoxicity (22%), psychiatric (6%), gynaecomastia (5%), musculoskeletal (5%)
Kanamycin	7.5%	Ototoxicity (75%), musculoskeletal (5%), CNS toxicity (4%), gastrointestinal (4%), hypotension (4%)
Capreomycin	8.2%	Nephrotoxicity (51%), ototoxicity (17%), rash (11%), gastrointestinal (7%), hypotension (3%)
Amikacin	10.2%	Ototoxicity (87%), nephrotoxicity (10%)
Para-amino-salicylic acid	11.6%	Gastrointestinal (79%), hypothyroidism (5%), hepatotoxicity (4%), rash (4%), nephrotoxicity (3%)
Linezolid	14.1%	Peripheral neuropathy (64%), myelosuppression (22%), optic neuritis (5%)

1 Only adverse drug reactions occurring ≥3% are specified. 2 Delamanid, pretomanid, and high-dose isoniazid were not included in the meta-analysis.

Pyrazinamide is a relatively safe drug in TB, however, approximately 5% of persons treated with pyrazinamide for MDR-TB experience adverse drug reactions leading to the withdrawal of the drug (Table 7). These adverse drug reactions are mostly due to musculoskeletal problems such as arthritis, gastrointestinal events, hepatotoxicity, and rash (138). Mild and moderate adverse drug reactions are more frequent, with 13% of people reporting toxicity likely due to pyrazinamide treatment, in one prospective study (148).

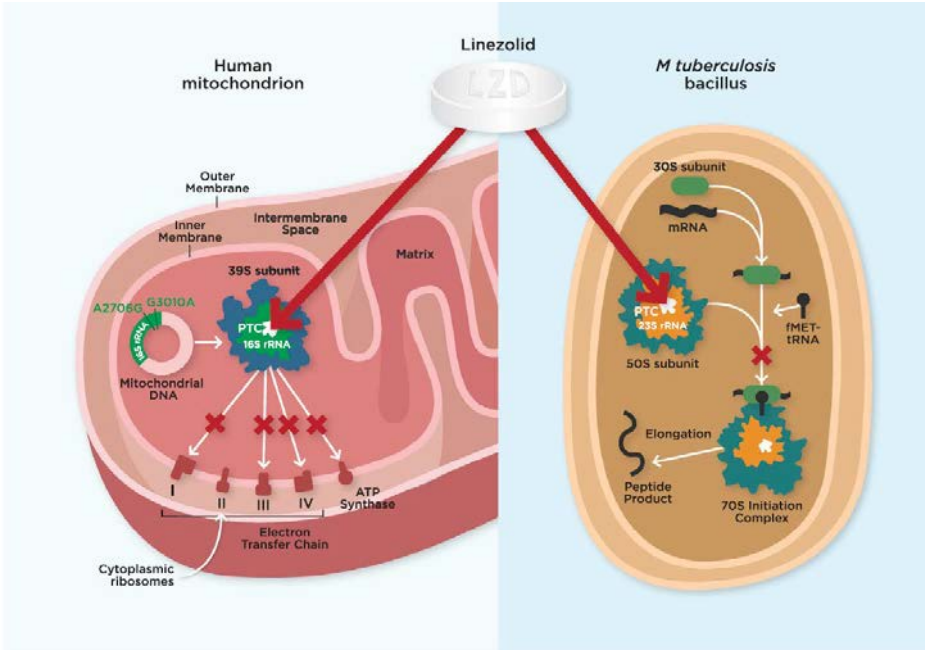

Figure 3 Linezolid mode of action in the bacterial mitochondria and the mechanism of toxicity in the human mitochondria (Wasserman, 2016, Figure 1) (142). Reprinted with permission from Taylor & Francis.

The antimicrobial effect of linezolid occurs due the binding of linezolid to the 50S ribosomal subunit (specifically 23S rRNA) in *M. tuberculosis* bacteria. This binding prevents the formation of the 70S initiation complex and, thus, inhibits protein synthesis. Toxicity of linezolid is due to the effect of linezolid in human mitochondria. Linezolid binds to the 16S rRNA of the ribosomal subunit and inhibits protein synthesis of the respiratory complex I, III, IV and ATP synthase which are encoded from mitochondrial DNA. Respiratory complex II is not affected since it is encoded from nuclear DNA. The respiratory complexes are involved in the oxidative phosphorylation process which synthesize ATP for energy production (145, 149).

Fluoroquinolones are considered to have the lowest toxicity compared with other drugs with levofloxacin likely being safer than moxifloxacin as seen in Table 7 (138). The most common severe adverse drug reactions include cardiotoxicity with QTc prolongation (dominated by moxifloxacin), musculoskeletal issues including tendon rupture (dominated by levofloxacin), and hepatotoxicity (138, 148, 150). Although fluoroquinolones are safer than other drugs in MDR-TB, a particular concern is related to cardiac toxicity including QTc prolongation since it can trigger torsade de point and lead to death. Other MDR-TB drugs such as bedaquiline, clofazimine, delamanid, and pretomanid also cause QTc prolongation (Table 7), and combining these drugs could pose an even greater problem (151). A meta-analysis mostly based on observational studies showed that people using fluoroquinolones had 85% higher odds of arrhythmias and 71% higher odds of cardiovascular mortality with the highest risk seen for arrhythmias for moxifloxacin, (152). The estimated absolute incidence of cardiac arrhythmias is though very low (0.2 per 1 million levofloxacin treatment episodes, while

no estimate was done for moxifloxacin in this study) (153). Moreover, an observational cohort study from Sweden (n=360 088) showed that the use of fluoroquinolones compared to amoxicillin was associated with a 66% higher risk of aortic aneurysm and dissection (absolute difference 88 cases/1 million treatment episodes), although most people received ciprofloxacin (154). Due to serious adverse drug reactions associated with fluoroquinolone treatment, including cardiac, musculoskeletal, and psychiatric issues, the European Medical Association cautioned against the use of fluoroquinolones in 2019, unless necessary (150). Some particular risk groups were specified, i.e., the elderly, those with renal disease, and those who use corticosteroids. Despite these adverse drug reactions associated with fluoroquinolone treatment, levofloxacin and moxifloxacin are considered tolerable and safe compare with other MDR-TB drugs and are frequently used throughout treatment in MDR-TB, as previously mentioned.

2.6.4 Treatment with pyrazinamide

In the long MDR-TB regimens previously recommended by WHO, pyrazinamide was recommended to be included in all regimens, unless resistance was confirmed (120). However, pyrazinamide was downgraded to a group C drug in 2018 (Table 6), and is no longer recommended to be included in longer MDR-TB regimens if drugs with higher efficacy are available (43). The mechanism of action of pyrazinamide has been discussed in Section 2.4.4 on whole genome sequencing and the development of current pyrazinamide treatment in Section 2.6.1 on DS-TB. Not mentioned previously is that pyrazinamide is thought to have a limited effect on actively growing bacteria, instead, the bactericidal effect is seen on *M. tuberculosis* bacteria that slowly replicate (semi-dormancy) (27, 83).

If pyrazinamide should be included in MDR-TB regimens, and if so, together with which drugs, is not clear. In observational studies based on the pre-2018 MDR-TB regimen with injectable drugs (120), the association between pyrazinamide treatment and treatment outcome is conflicting (123, 155-163). In the large individual meta-analysis from 2018 mentioned above, the authors concluded that pyrazinamide treatment was associated with a "slight improvement in outcomes" (115). Both a 30% lower risk of death in people with pyrazinamide-susceptible strains was reported and a 50% lower risk of success in people with pyrazinamide-resistant strains if pyrazinamide was prescribed (115). Moreover, in another individual meta-analysis, people with pyrazinamide resistant strains who were treated with the shorter MDR-TB regimen compared to the long regimen had a 10.7 (95% CI 1.8–64.5, n=619) higher risk of failure and relapse, although there was high heterogeneity for each outcome and wide confidence intervals (132).

The effect of pyrazinamide treatment on sputum culture conversion in MDR-TB has also been evaluated since more rapid sputum culture conversion was seen in DS-TB (116). In three observational studies, treatment with pyrazinamide was associated with increased

sputum culture conversion in people with pyrazinamide-susceptible isolates (or likely susceptible), including a study from Sweden from our group (155, 162, 164-167). However, two studies have failed to show a difference (155, 166), although no information on the methodology was provided in one study (166).

Interestingly, due to its importance in killing semi-dormant *M. tuberculosis* bacteria, and its relatively low toxicity, pyrazinamide is still included in many ongoing studies evaluating new regimens such as the PRESCIENT trial mentioned above (ClinicalTrials.gov Identifier NCT05556746) evaluating an eight-week regimen consisting of bedaquiline, clofazimine, pyrazinamide, and delamanid. Therefore, entangling the efficacy of pyrazinamide in combination with other drugs would be valuable to optimise treatment.

2.6.5 Treatment with fluoroquinolones

Fluoroquinolones are broad-spectrum antibiotics widely used for both Gram-positive and Gram-negative bacterial infections (168), although they have been known to be effective against mycobacteria since the 1980s (169, 170). They are sterilising bactericidal drugs for *M. tuberculosis* inhibiting DNA gyrase and thereby inhibiting DNA replication as well as incurring DNA breaks (78, 171, 172). Fluoroquinolones are considered key drugs in MDR-TB (group A) and are recommended to be included in all regimens recommended by WHO (Table 6) due to their high efficacy and low toxicity (43). Treatment of MDR-TB with a fluoroquinolone (moxifloxacin and levofloxacin) was associated with 2.8-5.4 higher odds of success in the individual meta-analysis mentioned previously (115). Furthermore, fluoroquinolone resistance in MDR-TB has also been associated with worse treatment outcomes (158, 173).

In 2020, the WHO updated the recommended doses for levofloxacin which are now 750–1150 mg (about 20 mg/kg) (44). For moxifloxacin, an option to increase the dose from 400 mg to 600-800 mg is also mentioned. Although there are safety concerns, optimising fluoroquinolone treatment in terms of dosing might lead to increased efficacy. Importantly, a dose-escalating study for levofloxacin (OPTI-Q) is ongoing (174).

2.6.6 Treatment with linezolid

Linezolid was developed in the 1980s for the treatment of Gram-positive bacteria (175, 176). Despite early in vitro and animal models showing excellent efficacy for mycobacteria (176, 177), it was not until 2000 that interest in linezolid was sparked (178, 179). Linezolid, an oxazolidine, has a unique mechanism of action by binding to the ribosome in the bacterial mitochondria, leading to the inhibition of early protein synthesis (176, 180, 181) (Figure 3). Furthermore, no cross-resistance is seen between other antibacterial drugs.

Treatment with linezolid in MDR-TB was shown to be associated with 3.5 higher odds of a successful outcome and 70% reduced mortality, in the recent individual meta-analysis

(115). Another study that formed the basis of the evidence of the efficacy of linezolid was a randomised controlled trial from South Korea (n=41) (182, 183). Despite linezolid was added as a single drug to a failing regimen, a successful treatment outcome was seen in 71% of participants. In light of its high efficacy, the current recommendation by WHO is that linezolid should be included in the six-month regimen, all long MDR-TB regimens (group A drug), and is recommended as an option for the shorter nine-to-12 month regimen (Table 6) (43). The main concern with linezolid treatment is toxicity which has already been described in Section 2.6.3 on adverse drug reactions.

Furthermore, the dosing of linezolid has rendered much debate due to its long treatment duration in MDR-TB. To balance efficacy and adverse drug reactions, once-daily dosing at half the standard dose for Gram-positive infections (600 mg once daily instead of 600 mg twice daily) is now the recommended dose in TB treatment (44, 115, 184, 185). Due to adverse drug reactions, dose reductions are often needed to 300 mg with time. This approach was part of the study protocol in the PRACTECAL trial and is also mentioned by WHO as an option (43). In one study, even 150 mg once daily was prescribed, when dosing was guided by drug concentrations (186). However, there are concerns about the risk of underdosing linezolid with lower doses, and optimising linezolid exposure using therapeutic drug monitoring to balance efficacy and adverse drug reactions is recommended in several overview articles (187, 188).

2.6.7 Treatment with bedaquiline, delamanid, and pretomanid

The three new drugs bedaquiline, delamanid, and pretomanid are new classes of drugs developed for TB. Bedaquiline is a diarylquinoline that inhibits ATP-synthetases and thus affects energy metabolism (189). The mechanism of action of delamanid and pretomanid which are both nitroimidazoles, involves the inhibition of the *M. tuberculosis* cell wall (190, 191).

The two new drugs, bedaquiline, and delamanid, have been investigated in phase II trials (192, 193). Excellent results on time to sputum culture conversion at two and six months were seen when either of the drugs were added to a background older long MDR-TB regimen. However, only delamanid has been evaluated in a phase III trial when added to a background-long MDR-TB regimen, with no association with treatment outcome (194). Due to the negative results of the clinical trial and lack of evidence from other studies, delamanid is currently recommended as a group C drug (Table 6). These results highlight that despite promising early results, evaluating the benefit of a single drug in a multidrug regimen can be challenging. The companion drugs can mask the effect of a new drug, or the effect may vary due to background regimens, which might lead to drug synergy or antagonism (195-197).

As already mentioned in Section 2.6.2 on the treatment of MDR-TB, pretomanid and bedaquiline were included in the PRACTECAL and ZeNix trials (113, 134), showing high success rates (84-91%). However, pretomanid is not listed in the WHO groupings of drugs used in the long MDR-TB regimen, likely due to a lack of data (43).

2.7 Treatment outcomes

A challenge when performing prospective MDR-TB studies is the treatment time, rendering drug trials very costly and long. Therefore, a huge effort is spent on finding surrogate endpoints that can predict treatment outcomes.

2.7.1 Sputum culture conversion

A common surrogate endpoint is sputum culture conversion, which has traditionally been defined as the date of the first of two negative sputum cultures taken at least 30 days apart, in a person with pulmonary TB (127). However, in the latest WHO guideline from 2022, the definition has changed and the 30 days are replaced by seven days (now also called bacteriological conversion) (43).

The evidence for using delayed sputum culture conversion at two months to predict relapse is conflicting and at best moderate in DS-TB (198). A positive predictive value of 18% and a negative predictive value of 95% has been described (199-202) (Table 8). Similar predictive values at two months are seen in MDR-TB for failure (203). This would mean that out of all persons with a positive sputum culture at two months, only one in five would fail treatment. Although a delayed sputum culture conversion at two months has been used in MDR-TB trials (193), studies have suggested that a delayed sputum culture conversion at six months or nine months (203) might be better in predicting failure or relapse (204) (Table 8). Another drawback is that using sputum culture conversion as a surrogate endpoint excludes persons who cannot produce sputum such as small children and those with extrapulmonary TB. In addition, people having pulmonary TB who recover and stop coughing cannot produce sputum with ease at two months.

Table 8 Positive and negative predictive values of sputum culture conversion in predicting relapse[1] and failure[1,2] in tuberculosis studies (199, 203)

Sputum culture conversion	Positive predictive value	Negative predictive value
2 months	18%[1,2,3]	95%[1,3] and 96-98%[2,4]
6 months	53-58%[2,4]	96-98%[2,4]
9 months	61-79%[2,4]	96-98%[2,4]

1 Drug-susceptible tuberculosis 2 Multidrug-resistant tuberculosis. 3 To predict relapse or failure.
4 To predict failure.

Delayed time to when a culture becomes positive in MGIT is another surrogate endpoint (205) which has been used in clinical trials (134). Limitations of this approach include the long time needed until culture results are available and the limited access of MGIT in low-resource settings. Therefore, biological markers predictive of treatment outcomes that can be used for all people treated for TB and can be utilised in both high and low-resource settings are needed. These markers should preferably be possible to use during the whole treatment time, such as viral load in treatment for HIV (206).

2.7.2 End-of-treatment outcomes

When treatment outcome is evaluated as defined by WHO (Table 9), both treatment success (cure and treatment completed), poor treatment outcomes (treatment failed, died, and lost to follow-up), and ideally relapse should be included. Relapse is thought to occur due to the failure of drugs to kill semi-dormant bacilli (26) and is more common in the first 24 months after treatment, which is why studies in TB follow-up participants during many months after the end of treatment (Table 9) (116).

A major change was introduced in 2013 regarding failure in treatment outcome definitions (207). This new failure definition also included failure due to an adverse drug reaction leading to the change of at least two drugs. Previously, only microbiological failure was included as per the 2005 definitions (208). If applying these new 2013 definitions, failure could increase from 11 to 38% in the same cohort, as highlighted in one study (209). The change of definitions has been questioned since a change of two drugs due to an adverse drug reaction could be considered part of adequate health care (210). However, others have highlighted that the 2013 failure definition evaluates a regimen that fails, which is an important aspect to record and report (209). In the update of WHO outcome definitions in 2022, the change of two drugs to declare failure was less stringently defined, instead only a change of treatment strategy or treatment regimen change were mentioned (Table 9). Another change in the latest 2022 WHO guideline was the harmonisation of the DS- and MDR-TB outcomes as well as simplifications, but these changes were minor (44).

Table 9 Treatment outcomes for people with drug-susceptible and multidrug-resistant tuberculosis according to the World Health Organization 2022 definitions (Adapted from WHO 2022, Table 10.1) (44)

Treatment outcomes	Definition
Cured	A patient with pulmonary TB with bacteriologically confirmed TB at the beginning of treatment who completed treatment as recommended by the national policy, with evidence of bacteriological conversion[1] (without reversion) and no evidence of failure.
Treatment completed	A patient who completed treatment as recommended by the national policy but whose outcome does not meet the definition for cure or treatment failure.
Treatment failed	A patient whose treatment regimen needed to be terminated or permanently changed to a new regimen or treatment strategy due to; - no clinical response or no bacteriological response - adverse drug reaction - evidence of additional drug-resistance to medicines in the regimen
Died	A patient who died before starting treatment or during the course of treatment.
Lost to follow-up	A patient who did not start treatment or whose treatment was interrupted for two consecutive months or more.
Not evaluated	A patient for whom no treatment outcome was assigned (includes cases "transferred out" to another treatment unit).
Treatment success	Cured and Treatment completed

1 Bacteriological conversion = two consecutive negative cultures (or smear in drug-susceptible tuberculosis), taken at least seven days apart.

2.8 Pharmacokinetics and Pharmacodynamics

Concepts used in describing the effects of a drug are PK (pharmacokinetics) which refers to what the body does to the drug over time and pharmacodynamics (PD) which is what the drug does to the body, or in infectious diseases, the microorganism.

2.8.1 Pharmacokinetics and pharmacodynamic parameters

The study of pharmacokinetics describes how a drug is taken up into the body (absorbed), distributed to peripheral tissues including the site of action (distribution), and eliminated from the body (elimination). Elimination can be through conversion of the drug to a metabolite (metabolism), or excretion from the body in the unchanged form (excretion) (Table 10) (211).

Table 10 Description of the key pharmacokinetic parameters of absorption, distribution, and elimination

	Parameters and description	Important factors
Absorption	Oral bioavailability (F) The proportion of a drug that reaches the system circulation unchanged (e.g., plasma), 0-100%.	For an oral drug, this could be decreased by i.e., first-pass metabolism in the liver, slow gastric emptying, or metabolism in the gut wall.
Distribution	Volume of distribution (V_d) An apparent volume in which the drug is dissolved. Calculated through the drug amount in the body over the drug concentration in the system circulation. A wide range between <5-50 000 L.	Lipophilic drugs usually have a higher volume of distribution as they are distributed in fat tissue. A high plasma protein binding typically leads to a low volume of distribution. Both renal and liver disease usually leads to lower protein binding.
Elimination	Clearance (CL) The volume of plasma/blood that is cleared from the drug per time unit (e.g., L/h). Sum of different routes for clearance, i.e., renal clearance and hepatic. Elimination consists of both metabolism and excretion. Metabolism is the conversion of a drug to a metabolite and excretion is the loss of the unchanged drug.	The major pathway for metabolism is the liver, and the kidneys for excretion. In renal disease, the renal excretion is reduced. Hepatic metabolism can increase by upregulation of hepatic enzymes, i.e., cytochrome P450 enzymes, by certain drugs. Clearance is a constant in linear kinetics but can vary in saturable kinetics.

Absorption of a drug depends on oral bioavailability (F), which is the fraction of unchanged drug that reaches the systemic circulation (Table 10) (211). For a drug with high bioavailability, similar drug concentrations are seen independently if the drug is given orally or intravenously. The distribution describes how a drug is transported between the central compartment and peripheral tissues in the body and is measured through the volume of distribution (V_d). This is an apparent volume as it is calculated using the total drug given over the plasma/blood concentration measured. Water solubility and plasma protein binding of the drug can affect the volume of distribution (Table 10). The elimination of a drug is measured by the clearance (CL), thus, what volume of plasma/blood is cleared from the drug per time unit. Elimination is usually occurring both through the renal and hepatic elimination routes. One example is the elimination of linezolid, that has a 30% renal excretion while 65% is metabolised through the liver (212). The metabolism of linezolid gives rise to two major inactive metabolites which are eliminated renally (50%) and through the faeces (10%) in an unchanged form. Two factors that reduces renal elimination are a higher age and renal disease. The liver metabolism of a drug can increase if certain liver enzymes are upregulated (e.g., the cytochrome P450 system), leading to increased drug clearance (211).

Common PK parameters to estimate are the maximum concentration (C_{max}), the total exposure of a drug over time measured by the area under the concentration-time curve (AUC), and the trough concentration (C_{min}) which is the lowest concentration estimated before the next dose as displayed in Figure 4. The maximum concentration is the highest concentration during the dosing interval. The AUC is often calculated using non-compartmental analysis using the log-linear trapezoidal rule since this gives more accurate estimations for both the absorption and elimination phase (211). A frequently used and simpler method in non-compartmental analysis in clinical studies is the linear trapezoidal rule (see Section 4.4.1 on AUC calculations). AUC can also be expressed in the formula of the dose received over the clearance. If clearance decreases as in e.g., renal failure, less volume with the drug can be eliminated per time, and AUC increases. An important factor to consider is that it is only the free drug that exerts an effect. Therefore, for a drug like moxifloxacin that has a protein binding up to 50%, only 50% of the drug is available for distribution into the site of infection to kill the pathogen (188).

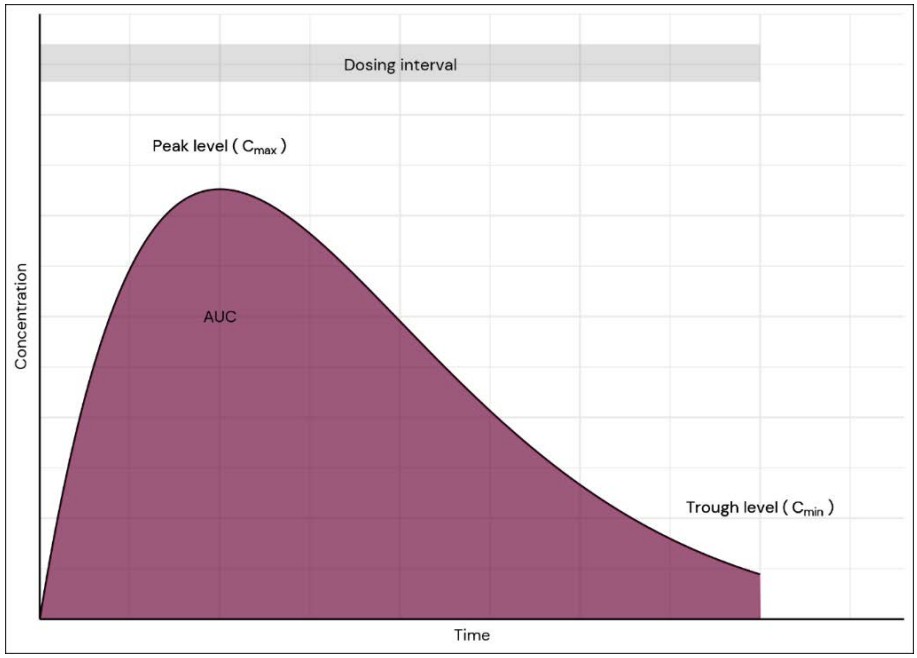

Figure 4 Pharmacokinetic-pharmacodynamic parameters of a drug displaying the area under the concentration-time cure (AUC), the peak level (C_{max}), and the trough level (C_{min})

Now, turning to the PD-parameters which in infectious disease is the effect the drug concentration over time has on the microorganism and can be measured by MIC. The drug effect on the microorganism can lead to killing (bactericidal drugs) or growth suppression (bacteriostatic drugs) (26). Moreover, the drug effect of certain drugs can

differ depending on drug concentration, i.e., linezolid displays bacteriostatic effect at lower concentrations but bactericidal effect at higher concentrations in in vitro models (184, 213). Combining both PK and PD parameters to estimate different targets for effect such as AUC/MIC, C_{max}/AUC, or %T>MIC, is preferable since the MIC can vary between bacterial isolates. Evaluation of the best predictive PK-PD target for effect can be done using in vitro models, animal models, and human studies. An example of a complex dynamic in vitro model is the hollow-fibre model which simulates human pharmacokinetics (214, 215). Hollow-fibre models deploy *M. tuberculosis* bacteria which grow in hollow fibre tubes. The bacteria are exposed to various nutrients and drug-concentrations which mimic the desired concentration-time curve in humans. Apart from efficacy for the optimal kill, the estimated PK-PD parameter for suppression of resistance can be estimated (215). By combining the results of the hollow-fibre model with mathematical modelling studies, utilising human PK data and dosing information, the predicted dose needed to achieve the PK-PD target can be estimated. The hollow-fibre model has been approved by the European Medicines Agency and the United States Food and Drug Administration as being one part of drug development for TB (214, 216).

The PK-PD parameters associated with efficacy, suppression of resistance, or toxicity are different depending on which antimicrobial is used. Antimicrobials can exert a concentration-dependent effect like the fluoroquinolones with the AUC/MIC being the best parameter associated with efficacy (217). For other antimicrobials, a time-dependent effect is seen, i.e., penicillin, described by the percentage time the concentration is over MIC (%T>MIC). Toxicity can be related to concentrations exceeding a critical threshold, i.e., a concentration toxic to mitochondria for aminoglycosides (188). Alternatively, toxicity is related to accumulated exposure (211).

Another aspect to consider when measuring drug concentrations is the concentration at the site of infection. This site could be epithelial lining fluid or within lesions in the lungs (41, 218) or cerebrospinal fluid (40, 219) including the intracellular concentration (218, 220). However, the access to measure concentrations in these compartments is limited to research settings. Partition coefficients have been calculated which are specific for each drug and compartment, i.e., the ratio of plasma drug concentration to the cerebrospinal fluid concentration (40, 219). Therefore, drug concentrations in plasma or serum could be used as a surrogate for the site of infection.

2.8.2 Liquid chromatography-tandem mass spectrometry

Drug concentrations can be analysed using liquid chromatography-tandem mass spectrometry (LC-MS/MS), which is a common method that utilized the mass-to-charge difference between different compounds (221). Briefly, the first step involves a protein precipitation set followed by separating drug components using liquid

chromatography (LC). Separation occurs since the hydrophilic components of a sample have less affinity for the column through which the sample is injected and, therefore, a shorter retention time. In contrast, the retention time is longer for hydrophobic components. After separation, the drug components are ionized by electron spray before they enter the final step of passing through the tandem mass spectrometry phase (MS/MS). In the tandem mass spectrometry phase, further separation occurs using electric-magnetic fields. The different components can be distinguished based on the ratio between ionization and mass, and the subsequent number of ions detected is related to the drug concentration. To ensure reliable and accurate results, stable isotope (deuterated) internal standards are important (221).

2.9 Therapeutic drug monitoring

Therapeutic drug monitoring is a method in which drug concentrations are measured and the drug dose adjusted. The aim is to reduce toxicity while optimising efficacy and the risk of acquired drug resistance (188). Therapeutic drug monitoring is a well-established method in clinical medicine used for, e.g., antiepileptic drugs and some antimicrobials like vancomycin and aminoglycosides (222-224). Although, for TB drugs, the concept has been recommended for over 30 years it was not until the latest WHO guideline that therapeutic drug monitoring is mentioned as an option to balance efficacy and risk for toxicity (44). Even though there is a plausibility of effect (225), linking therapeutic drug monitoring as an intervention to clinical outcomes such as sputum culture conversion or preferably treatment outcome in MDR-TB in a well-designed randomised controlled trial would be ideal (226). However, having low exposure to a drug is probably not beneficial even with or without a randomised controlled trial.

In DS-TB, several studies have been conducted evaluating drug concentration against different targets with various results and a recent meta-analysis found that low pyrazinamide concentrations and maybe low rifampicin concentrations were associated with unsuccessful treatment outcomes (225). Furthermore, in a randomised controlled trial in DS-TB (n=172) which assessed the intervention of dosing isoniazid according to slow or fast acetylation status (i.e. by how fast isoniazid was metabolised), a lower risk of failure at eight weeks was seen (227).

The evidence for using therapeutic drug monitoring in MDR-TB to predict treatment outcome, has long been based on in vitro models (hollow-fibre models described above, Section 2.8.1 on PK-PK parameters) where low dosages of standard drug doses are predicted (215, 228, 229). Studies analysing drug concentrations with treatment outcomes in MDR-TB have been scarce (40, 230). However, in the last year, two larger observational prospective studies have been published (231, 232). In the study from China by Zheng *et al*. (n=197), participants were treated according to Chinese national guidelines with an all-oral long MDR-TB regimen (231). In participants with a higher AUC_{0-}

$_{24h}$/MIC for fluoroquinolones, linezolid, and pyrazinamide, a positive association was seen with 2-month sputum culture conversion and treatment success. Likewise, in the multi-centre study by Heysell and colleagues (n=290), having an AUC_{0-24h}/MIC above certain thresholds for moxifloxacin, levofloxacin, clofazimine, and pyrazinamide (AUC_{0-24}), an association was observed with a faster sputum culture conversion, and for moxifloxacin also end-of-treatment outcome (232). In the study, most participants received an injectable-based long MDR-TB regimen but since linezolid was only prescribed to a few participants it could not be analysed.

2.9.1 Pharmacokinetic-pharmacodynamic targets

To optimise treatment, the PK-PD parameter AUC/MIC is likely the best predictive marker of effect and preventing resistance development for the fluoroquinolones, pyrazinamide, and linezolid as seen in in vitro studies (184, 213, 228, 229) and mouse-models (233). Peak concentration has also been described for pyrazinamide (234), and %T>MIC for fluoroquinolones (233) and linezolid (235). For adverse drug reactions, the trough level for linezolid is the most cited PK-PD parameter (236-238) which will be described below. In contrast, toxicity thresholds for fluoroquinolones (also discussed below) and pyrazinamide are not known.

For fluoroquinolones, different targets for efficacy on *M. tuberculosis* have been found in mouse models suggesting an AUC_{0-24}/MIC >100 (233, 239), similar to other pathogens (240-242). However, both higher targets have been suggested in hollow-fibre models for levofloxacin (free fraction AUC_{0-24}/MIC >146-360) and lower for moxifloxacin (AUC_{0-24}/MIC 42-133) (213, 215, 228) as seen in Table 11. There is substantial variation in the efficacy targets for moxifloxacin, with two studies showing a more than double AUC_{0-24}/MIC target of 96, 133, and 231 (213, 231), see Table 11. The hollow-fibre study predicted a free fraction AUC_{0-24}/MIC of 96 for efficacy (213). Interestingly, the targets would be similar if the total AUC_{0-24}/MIC of 231 found in the prospective study from China (231) was calculated with an estimated protein binding of 50% (around 100). Further studies evaluating these higher targets for moxifloxacin efficacy would be needed.

To achieve the tentative PK-PD targets for fluoroquinolones, the current doses of levofloxacin (750-1150 mg) and moxifloxacin (400 mg) are predicted of being too low (213, 215, 228, 243). Observational studies in MDR-TB have shown that only 45-87% of people using moxifloxacin and 70-85% of people using levofloxacin reach exposure targets, although different targets were evaluated (231, 244-247). Therefore, higher doses have been proposed, typically moxifloxacin 800 mg and levofloxacin up to 25 mg/kg (215, 228, 243, 248). Indeed, a moxifloxacin dose of 600-800 mg is already an option in the last WHO MDR-TB guideline (44). According to the guideline, a higher moxifloxacin dose could be prescribed if low-level resistance is detected, or if there is a chance of lower exposure due to drug-drug interaction or malabsorption, assuming

there is no increased risk for toxicity. In animal models, toxicity has been related to QTc prolongation and associated with increased moxifloxacin concentrations (249, 250). However, since the relationship between PK-PD parameters is different in animals compared to humans, with higher C_{max} seen at the same AUC, this could be an explanation. High-dose moxifloxacin (600–800 mg) has been evaluated in two randomised open-label studies (n=272) and appeared safe, although 5% (n=12) of people had QTc prolongation that led to the withdrawal of moxifloxacin in one of the studies (129, 251). For levofloxacin, safety results are awaited in an MDR-TB dose-ranging study (up to 20 mg/kg) in the OPTI-Q study (174).

For linezolid, suggested targets associated with effect are around AUC_{0-24h}/MIC >120 in both hollow-fibre models (184, 213), and from clinical studies (232, 252) (Table 11). A considerably lower free fraction AUC_{0-24h}/MIC of 36 was seen in one hollow-fibre model study (213). However, it is important to consider that targets derived from the in vitro models estimate the free fraction while the clinical studies usually measure the total concentration. Dosing of linezolid at 600 mg has been predicted to achieve the suggested target of AUC_{0-24h}/MIC >120 in most cases but when the dose is reduced to 300 mg once daily there are concerns about adequate exposure (253). Since adverse drug reactions is a particular issue with linezolid, several observational studies and one randomised controlled trial have been conducted to evaluate adverse drug reactions (and efficacy) in TB (236-238). A trough level >2-2.5 mg/L was associated with linezolid toxicity, when using a composite outcome of linezolid toxicity (or only anaemia in one study) (Table 11). Other parameters such as cumulative dose, treatment duration, and AUC have also been discussed to be related to adverse drug reactions (254, 255). However, the exact timing, dosing, and trough levels associated with each adverse drug reaction of linezolid is not fully elucidated, especially related to clinical characteristics and PK-parameters.

For pyrazinamide, the most cited PK-PD target derived from clinical studies is an AUC_{0-24h}/MIC of >11 although this target is derived from a DS-TB study (188, 234) (Table 11). Interestingly, in modelling studies of both DS- and MDR-TB, pyrazinamide was the main predictor for sputum culture conversion (159) and an unsuccessful treatment outcome (234). As mentioned above, a low pyrazinamide concentration (mainly C_{max}), was associated with an unsuccessful treatment outcome in a meta-analysis (225). In the same meta-analysis, 5-39% of participants had low exposure to pyrazinamide.

Table 11 Pharmacokinetic-pharmacodynamic parameters associated with optimal microbial kill (A), acquired drug resistance (B), sputum culture conversion (C), end-of-treatment-outcome (D), and toxicity (E) for pyrazinamide, levofloxacin, moxifloxacin, and linezolid

Drug	Target C_{max} (mg/L)	Target AUC_{0-24h} (mg×h/L)	Targets for efficacy*, total AUC_{0-24h}/MIC	Risk for toxicity	Protein binding
Pyrazinamide	20–60, >35	**Clinical** C) total >379 (232) D) total >363 (234)	**Clinical** C) total 2.79 (159) D) total 11.3 (234)	NA	0-7%
Levofloxacin	8–13	150	**In vitro** A) free >146[1] (228) B) free >360[1] (228) **Clinical** C) total 287 (231) C) total 118 (232)	NA	24-38%
Moxifloxacin	3–5	55	**In vitro** A) free >42[1,2] (215, 228) B) free >53[1] (215) A, B) free >96[1,3] and >133[1,3] (213) **Clinical** C) total 231 (231) D) total 58 (232)	NA	30-50%
Linezolid	12–26	total >100 (256)	**In vitro** A) free 119[1] (184) A, B) free >35.6[1,3] and >89[1,3] (213) **Clinical** C) total 125 (252) C) total 287 (231)	**Clinical, C_{min}** E) <2-2.5 (236-238)	31%

AUC = area under the concentration-time curve, Cmax = peak concentration, NA = not available. All figures without a reference are from Märtson *et al.* 2021 (188). A: Target for antimicrobial kill from in vitro model. B Target for suppression of resistance from in vitro model C: Target from clinical study on sputum culture conversion. D Target from clinical study on end-of-treatment outcome. E target for toxicity from clinical study 1 Targets are based on monotherapy with the drug 2 JW Alffenaar and D Deshpande 2020, personal communication 10 Oct. This target is cited as 56 in the manuscript, but after communicating with the authors it was corrected to 42 (228). 3 Two targets were evaluated in the study under various test conditions (neutral pH and under acidic condition). The difference between the two values needs to be further evaluated and carefully interpreted.

2.10 Treatment optimisation

To reach the End TB Strategy and end TB as a public health challenge by 2030 as set out by the WHO (2), further improvements are needed in all areas from prevention, diagnosing, detecting drug resistance, starting treatment, and optimising treatment regimens. Improvements in treatment for MDR-TB are highly needed since only 60% of people starting treatment had a successful outcome in the latest cohort under evaluation globally (1).

Of those people who did not have a successful treatment outcome, 12% died, 10% failed, and 14% were lost to follow up, highlighting the ineffective regimens (1). Possible reasons for an increased risk of unsuccessful treatment outcomes are multifactorial and have been evaluated in several studies. Among them are person-related factors such as comorbidities including diabetes (7) and living with HIV (7), increasing age (3, 5), and being underweight (3). Regimen-based reasons are adverse drug reactions (4) and long treatment duration (3). Bacteriological factors include previous treatment (could also be a social factor) (3, 5, 7), ineffective drug regimens (6), and resistance patterns in the infecting *M. tuberculosis* isolate (5, 7). Lastly, social factors such as education level (7), low trust and support from the health care provider (4) have also been described as increasing the risk of an unsuccessful outcome. Interestingly, the effectiveness and adverse drug reactions are only one aspect that can lead to unsuccessful outcomes.

Although bedaquiline was introduced for MDR-TB in 2012 and linezolid has been a possible drug to include in regimens since 2006, treatment outcomes have only slightly improved from 50% in 2012 to 60% today, globally (1, 43). Since access to these newer and repurposed drugs is limited in many settings (136), optimising all drugs in terms of dosing, adverse drug reactions, and regimen composition seems important to improve care. Furthermore, using therapeutic drug monitoring to adjust dosing based on efficacy and risk for adverse drug reactions could be one part of delivering quality health care. Therefore, addressing comorbidities, ensuring effective drugs are prescribed, reducing adverse drug reactions, and having a person-centred approach are key aspects as outlined in the End TB Strategy (2).

To achieve effective treatment upfront, improved resistance testing is needed (6). With the roll-out of rapid molecular tests like Xpert MTB assays which at least detect resistance to rifampicin, diagnostic improvement has been achieved with increased case detection and resistance testing (47, 49, 257). However, access to resistance testing of other drugs is limited in high-incidence settings, except perhaps isoniazid, fluoroquinolones and second-line injectables (Xpert MTB/XDR (72)). Therefore, implementing newer technologies such as whole genome sequencing could be one strategy to achieve the goal of universal DST in line with the End TB strategy (2).

Accelerated improvements are needed in treatment optimisation to achieve the goal of the End TB Strategy by 2030 (2). This thesis will address some different aspects of how treatment for MDR-TB can be improved as shown in Figure 5. The studies include resistance testing using whole genome sequencing, evaluating the inclusion of pyrazinamide in a regimen, measuring drug concentrations for fluoroquinolones, and reducing adverse drug reactions for linezolid.

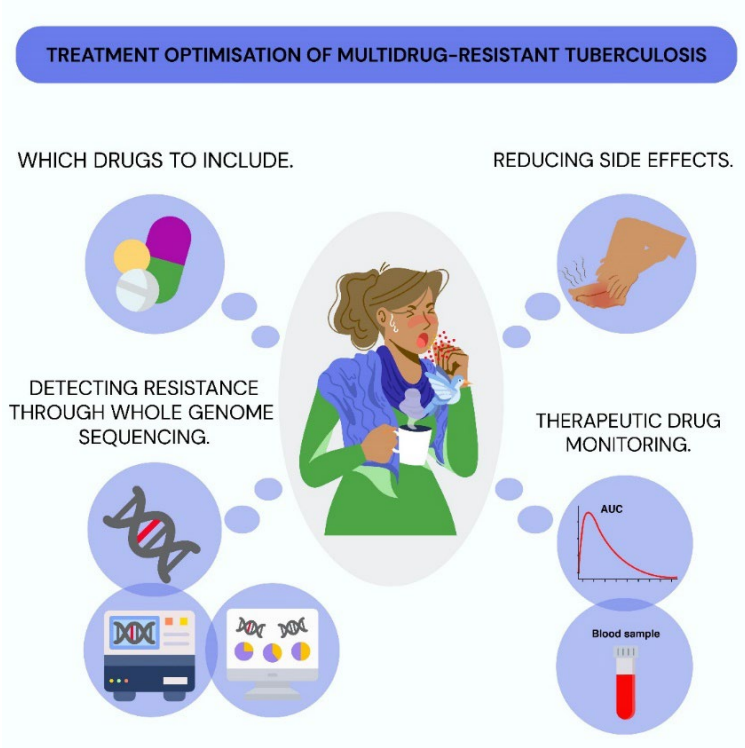

Figure 5 Different ways in how a treatment regimen for multidrug-resistant tuberculosis can be optimised

Illustration by Ahmad Usman Noor

Anora is only 12 years old. When we enter the house, she is sitting on a bright red cushion next to her grandmother and waiting for us. The nurse takes out the syringe and draws up the two injections, one to take in each buttock. "I don't want to," Anora says. As the nurse slowly injects the kanamycin, the young girl sobs loudly and big tears drop down on the cushion. Her grandmother hugs her tightly, comforts her, and strokes her back while the nurse gives the second injection. Finally, the pain disappears. She is so brave today, but the procedure will repeat itself every morning, every day of the week, for at least another six months. And she is only 12 years old.

3 Research aims and objectives

The overall aim of this thesis was to evaluate the effect of pyrazinamide and fluoroquinolone treatment in MDR-TB including resistance testing and drug concentrations, and to assess linezolid-related adverse drug reactions in persons treated for MDR-TB.

3.1 Objectives

To assess the association between pyrazinamide susceptibility measured by phenotypic DST and end-of-treatment outcome among persons treated with a pyrazinamide-containing MDR-TB regimen in Uzbekistan (**Study I**).

To assess the effect of different lengths of pyrazinamide treatment on end-of-treatment-outcome among persons having an *M. tuberculosis* MDR-TB isolate with unknown pyrazinamide DST or a pyrazinamide resistant isolate measured by phenotypic DST in Uzbekistan (**Study I**).

To analyse the effect of pyrazinamide treatment with a pyrazinamide resistant or susceptible *M. tuberculosis* isolate measured by genotypic, phenotypic, or composite pyrazinamide DST and MIC on sputum culture conversion and unsuccessful treatment outcome in persons treated for MDR-TB in Sweden (**Study II**).

To describe total drug exposure of moxifloxacin and levofloxacin over MICs of *M. tuberculosis* isolates and explore target attainment of previously suggested indices for efficacy in persons treated for MDR-TB in China **(Study III**).

To assess the effect of linezolid treatment measured by dose in mg and mg/kg on linezolid-associated adverse drug reactions in persons treated with a linezolid-containing MDR-TB regimen in Sweden (**Study IV**).

To explore linezolid drug concentrations and their association with adverse drug reactions due to linezolid in persons treated with a linezolid-containing MDR-TB regimen in Sweden (**Study IV**).

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4 Materials, methods, and methodological considerations

In this section, a description of the materials and methods used in all four studies is provided and a discussion about methodological considerations. Section 4.6 on study design, 3.7 on epidemiological concepts, an 3.8 on statistical methods offer a more in-depth discussion since only a brief description was included in the manuscripts.

4.1 Setting

4.1.1 Karakalpakstan, Uzbekistan

Study I is conducted in Karakalpakstan (Table 12) which is a sparsely populated semi-autonomous republic in the western part of Uzbekistan with a population of about two million. Médecins sans Frontières and the Ministry of Health started working together in 2003 to treat people with MDR-TB in Karakalpakstan. TB care was led by specialised TB physicians working in hospitals and outpatient clinics. All microbiological samples for people who were investigated or diagnosed with TB were sent to the quality assured (5) regional biosafety-3 laboratory in the capital, Nukus. However, in the initial years of the programme, 2003-2006, phenotypic DST was also conducted at the supranational reference laboratory in Borstel using liquid media (258).

Médecins sans Frontières and the Ministry of Health jointly developed treatment guidelines, based on WHO guidelines. Recommendations regarding pyrazinamide treatment changed slightly over the years; in 2003-2009 pyrazinamide could be stopped due to pyrazinamide resistance if phenotypic DST was available; while in 2010-2015 pyrazinamide was stopped after the intensive phase if resistance was detected and if susceptible continued the whole treatment length. Furthermore, the reason for hospitalisation changed over the years, and from 2003, people with pulmonary MDR-TB were hospitalised until culture conversion. However, from 2007 people were hospitalised until negative microscopy in sputum, and from 2011 most people with MDR-TB were treated in an ambulatory setting. TB care was considered free of charge, however, as is the practice in many settings, small fees were often included. All persons in the study had a treatment regimen according to the WHO 2016 guidelines (120), including at least four drugs in the intensive phase with an injectable drug and a fluoroquinolone, excluding pyrazinamide.

Table 12 Overview of included studies

Study features	Study I	Study II	Study III	Study IV
Design	Retrospective cohort study	Retrospective cohort study	Prospective cohort study	Retrospective cohort study
Setting	All health care providers in Karakalpakstan, Uzbekistan	All health care providers in Sweden	Xin Ling hospital, Xiamen, China	All health care providers in Sweden
Population	Persons with bacteriologically confirmed[1,2] pulmonary MDR-TB	Persons with bacterio-logically confirmed[1,3] MDR-TB	Adults with bacteriologically confirmed[1,3] pulmonary MDR-TB	Persons with bacteriologically confirmed[1,3] MDR-TB treated with at least one day of linezolid
Number	2446	157	32	132
Time period	2003-2013	1992-2014	2016-2018	1999-2018
Data sources	Records in routinely collected data	Medical records, TB laboratories	Case Report Forms	Medical records, Pharmacological laboratory
Main statistical method	Logistic regression	Cox regression	Descriptive	Cox regression
Main exposures	1. PZA susceptibility (in persons treated with PZA) 2. Length of PZA treatment in the intensive phase	No effective PZA treatment[4] 1. gDST 2. pDST 3. MIC 4. Composite DST	Levofloxacin and moxifloxacin free fraction AUC_{0-24h}/MIC	Linezolid treatment 1. mg/day 2. mg/kg/day
Main outcomes	Successful treatment outcome[5]	1. Sputum culture conversion 2. Unsuccessful treatment outcome[6]	% target attainment of the free fraction AUC_{0-24h}/MIC for 1. Optimal microbial kill 2. Prevention of acquired resistance (215, 228)	First occurrence of each adverse drug reaction 1. Peripheral neuropathy 2. Anaemia 3. Leukopenia 4. Thrombo-cytopenia

MDR-TB = multidrug-resistant tuberculosis, PZA = pyrazinamide, DST = drug susceptibility testing, gDST = genotypic DST, pDST = phenotypic DST, AUC/MIC(0-24h) = area under the concentration-time curve over minimum inhibitory concentration between 0 and 24 hours. 1 Defined as having a positive culture for *M. tuberculosis* with resistance to at least rifampicin and isoniazid using phenotypic DST. 2 A microbiological sample was submitted before treatment start and up to seven days after starting treatment. 3 A microbiological sample submitted before treatment started. 4 Treatment less than 30 days or having an isolate resistant to pyrazinamide. 5 Treatment cured and completed according to WHO 2005 definitions (208). 6 Treatment failure, death, loss to follow-up, and relapse according to WHO 2005 definitions.

4.1.2 Sweden

Both **study II and IV** included people with MDR-TB in Sweden (Table 12). TB care in Sweden is currently primarily organised by infectious diseases physicians, although historically, respiratory physicians have been responsible in some regions. Additionally, paediatricians also care for children with TB in most regions. Since Sweden is a low-incidence country, most persons with MDR-TB were diagnosed and treated in three large University hospitals in the large cities of Stockholm, Gothenburg, and Malmö/Lund. For diagnosis, all strains with resistance to rifampicin were sent to the supranational TB reference laboratory at the Public Health Agency of Sweden to perform additional DST to other drugs.

Treatment guidelines were mostly unavailable for MDR-TB in Sweden during the study and instead the prevailing WHO guideline was followed. However, in 2022 a national guideline including treatment for MDR-TB was published (259). Moreover, a national Concilium chaired by the Public Health Agency to support physicians treating people with MDR-TB was founded in 2001 and all cases with MDR-TB were discussed with at least one member of this expert group. Since this study included participants starting treatment until 2018, an individualised long MDR-TB regimen was given to all participants which for the vast majority was based on an injectable drug and a fluoroquinolone, while cycloserine and prothionamide were frequently prescribed. People with pulmonary TB were generally hospitalised until sputum culture conversion for public health reasons. All care leading up to, treatment and follow-up regarding TB is free of charge in Sweden.

4.1.3 Xin Ling hospital, Xiamen, China

In **study III**, all participants were recruited from the Xin Ling hospital just outside the city of Xiamen, Fuijan province, which is in the south-eastern part of China (Table 12). Three other hospitals could care for people with MDR-TB in the Fuijan province, but the Xin Ling university hospital was the largest with a capacity of 105 beds and where specialised TB doctors and nurses worked. The Xin Ling hospital served a population of 4.0 million people at the time of the study including people that had moved from other areas of China (the so-called floating population (20)). All people diagnosed with MDR-TB were initially hospitalised for two months. Treatment for MDR-TB was given according to Chinese national guidelines which a long MDR-TB regimen based on an injectable drug, a fluoroquinolone and most commonly at least prothionamide and pyrazinamide. In Xin Ling hospital, like in the rest of China, the cost of treatment was free for people who received treatment in their area of residence, e.g., people residing in Xiamen. However, temporary residents, such as the floating population, had to pay for their treatment. Furthermore, all people had to pay for certain drugs for MDR-TB, namely cycloserine and linezolid. The study protocol with additional details has been published (260) and the trial is registered at ClinicalTrials.gov (NCT02816931).

4.2 Data sources and data management

4.2.1 Data sources

For **study I**, a dataset starting in 2003 for people with MDR-TB was used in the joint TB programme run by Médecins sans Frontières and the Ministry of Health of Karakalpakstan, Uzbekistan (Table 12). This dataset consisted of baseline socio-demographic, clinical, treatment, adherence, and outcome data for all persons treated within the programme (5, 261). Data was routinely and continuously collected as part of monitoring and follow-up. A trained epidemiology team was responsible for ensuring data collection, double entry, and verifying source data if discrepant results. In addition, a laboratory dataset was available with data on microscopy, rapid diagnostic tests, culture, and DST results.

For **study II and IV**, clinical records from routine care from each region in Sweden were used (Table 12). In addition, microbiological data from the five specialised TB laboratories in Sweden was collected for **study II** to ensure adequate data on follow-up cultures (Table 12). Pharmacological data for linezolid for **study IV** was collected through participants' clinical records and from the Department of Pharmacology at Karolinska University Hospital, Stockholm, which is the only laboratory in Sweden that analyses linezolid drug concentrations.

Data for **study III** was prospectively collected using Case Record Forms filled in by healthcare staff at the Xin Ling hospital in China (260) (Table 12).

4.2.2 Identifying participants with multidrug-resistant tuberculosis

In study I, the routinely collected dataset was used to find all cases fulfilling the inclusion and exclusion criteria (156). To cross-link the datasets, the unique person identification number assigned to each person in the TB programme was used.

Participants with MDR-TB in **study II and IV** were identified through the Public Health Agency of Sweden which holds a national record of persons diagnosed and treated for TB in Sweden since TB is a notifiable disease according to the Communicable Diseases Act (SFS 2004:168 with the amendments of SFS 2022:217). Notification of cases is doubly reported by both laboratories and physicians who are obliged to report cases. To match cases from different data sources, the Swedish Personal Identification Number was used, which is a number assigned to each person born or resident in Sweden and used in contact with health care and government bodies. For participants without a Personal Identification Number, e.g., asylum seekers, each person is given a unique temporary number in contact with health care in each region. The disadvantage of using the latter is that multiple temporary numbers could be available. If these records are not linked there is an increased risk of missing data. Although TB is a reportable disease in Sweden, it is possible that a few cases have not been reported to the Public Health

Agency, especially those that are not microbiologically confirmed. As seen in a recent thesis on malaria, more malaria cases were found when malaria registers were linked with the death register in Sweden (262).

For **study III**, each person hospitalised for MDR-TB in the designated TB hospital in Xin Ling, Xiamen was screened for inclusion and exclusion in the study by the treating physician or study nurse. Moreover, the regional Centre for Disease Control in Xiamen was notified of any new cases with MDR-TB and kept a screening log (260).

4.2.3 Data entry and analysis

Data entry was done using EpiInfo (EpiInfo, Centers for Disease Control and Prevention, Atlanta, GA, USA) (**study I)**, Excel (Microsoft, Redmond, WA, USA) (**study II)**, and EpiData (EpiData, Odense, Denmark) (**study III and IV)**. Advantages of using a professional data entry software programme such as EpiInfo and EpiData include possibilities for built-in checks for ranges and restrictions in values entered to increase data quality. To further ensure data quality, double data entry (263) was done for **study I and IV** (in **study IV** double entry was done for participants starting treatment 2015-2018), and if discrepancies were found source data was consulted. In **study II and IV** (for participants starting treatment 1992-2014), a 20% data collection overlap was used to ensure adequate quality in data entry.

Data cleaning and analysis were conducted in STATA software version 14.1 and 16.1 (StataCorp LCC, College Station, Texas, USA) while Excel was used for the non-compartmental analysis using the linear trapezoidal rule for **study III**.

4.3 Microbiological methods

4.3.1 Diagnosis of multidrug-resistant tuberculosis

All participants had a diagnosis of MDR-TB using phenotypic DST according to, at that time, prevailing WHO recommendations with confirmed resistance to both rifampicin and isoniazid (Table 4 and Table 12). Although all settings also utilized various rapid diagnostic tests such as Xpert MTB/RIF (**study I, II, and IV**), line-probe assays (**study I, II, and IV**), and MeltPro TB assay (**study III**), we only included participants who also had resistance to rifampicin and isoniazid using phenotypic DST. Two reasons for this exist, firstly, to ensure that participants had TB disease since a rapid molecular test can remain positive a long time after a TB episode. Secondly, to ensure strains had confirmed isoniazid resistance since the sensitivity for detecting isoniazid resistance is lower (about 15% of isoniazid resistance is missed if only using line-probe assays) using rapid molecular test than phenotypic DST. This is also the reason I have used the term MDR-TB throughout this thesis since only participants with resistance to both rifampicin and isoniazid are included in the studies.

4.3.2 Culture methods of *Mycobacterium tuberculosis*

For detection of *M. tuberculosis*, both solid media (Löwenstein-Jensen) and liquid media (MGIT) were used in **study I, II, and IV.** However, only MGIT was used for the detection in **study III** since this was standard according to local guidelines in Xiamen**.**

Pyrazinamide phenotypic DST in **study I** was performed routinely using MGIT between 2003-2006 in the supranational laboratory in Borstel, Germany. In 2010, pyrazinamide phenotypic DST started in Karakalpakstan, Uzbekistan, and since 2012 it was routinely performed. In **study II and IV**, routine pyrazinamide phenotypic DST was performed in the regional TB laboratories around Sweden and at the supranational reference laboratory at the Public Health Agency of Sweden. The critical concentration used to define pyrazinamide resistance was 100 mg/L in all studies (96, 258).

Phenotypic DST for levofloxacin in **study III** was performed on solid media (Löwenstein-Jensen) using the proportion method according to WHO at the time of the study, with a critical concentration of 2 mg/L (96). Any isolate resistant to levofloxacin by phenotypic DST was also considered resistant to moxifloxacin in **study III**.

Linezolid phenotypic DST in **study IV** was performed using liquid media (BACTEC 460 TB system until 2008, then MGIT 960) at the laboratory at the Public Health Agency of Sweden. The critical concentration used to define resistance was 1.0 mg/L in accordance with WHO at the time of the study (96).

Phenotypic DST for other drugs (rifampicin, isoniazid, ethambutol, ofloxacin, streptomycin, kanamycin, and capreomycin) was conducted in parallel in **study I** using both solid (Löwenstein-Jensen) and liquid media (BACTEC 460 TB system and MGIT 960) (5, 261). Although this has the advantage to increase sensitivity, it also poses a problem with discrepant results due to false resistance or susceptibility (264). A particular challenge was phenotypic DST for kanamycin and capreomycin, therefore, in **study I,** we constructed a rule-based decision tool to decide if resistance was present or not. Firstly, a phenotypic DST from the supranational reference laboratory in Borstel, Germany, was considered, secondly phenotypic DST from solid medium using the proportion method since the programme in Karakalpakstan had more experience with using this method, and lastly MGIT 960. In **study II and IV**, only phenotypic DST in liquid media (BACTEC 460 TB system until 2008, then MGIT 960) was performed for most drugs. However, phenotypic DST for cycloserine and para-aminosalicylic acid was performed on solid media. Routine phenotypic DST on solid media (Löwenstein-Jensen) was performed in **study III** for the following drugs: rifampicin, isoniazid, ethambutol, levofloxacin, streptomycin, kanamycin, and para-aminosalicylic acid. All testing of resistance in both solid and liquid media was performed according to the manufacturer's instructions and according to WHO recommendations (96).

4.3.3 Minimum inhibitory concentrations

Pyrazinamide MIC using a broth macrodilution method was performed in serial two-fold dilution steps in MGIT using a test concentration between 8-128 mg/L for PZA susceptible strains and 32-512 mg/L for PZA resistant strains at the supranational reference laboratory in Sweden (**study II**). The *M. tuberculosis* reference strain H37Rv ATCC 27294 was included in all runs for quality control. Since PZA MIC is cumbersome and resource intense, we used different concentration ranges for susceptible and resistant isolates with concentrations that we estimated would be within the test range.

Details of the pyrazinamide MIC broth macrodilution method in MGIT are described briefly and were performed according to the manufacturer's instructions (55). Isolates of *M. tuberculosis* from persons with MDR-TB stored at the Public Health Agency were thawed and sub-cultured on solid media. Bacterial colonies not more than two to three weeks old were used. One additional step of homogenising colonies with glass beads was added before a suspension of McFarland turbidity of 0.5 was prepared (55). Then, bacterial dilutions were made using PBS (phosphate-buffered saline). From the dilution, 100 µL was added to each MGIT tube together with OADC (oleic acid, albumin, dextrose, and catalase) with the growth control in a 1:10 dilution. Serial pyrazinamide stock solution concentrations (Sigma-Aldrich, Germany) were added to each test tube. When the antibiotic-free growth control reached a growth unit of 400, the test tube containing pyrazinamide where the growth unit was lower than 100 was read as the MIC concentration.

Similarly, linezolid MICs using a broth macrodilution method for *M. tuberculosis* strains were performed in serial two-fold dilutions using MGIT with a limited range test concentration between 0.25-1.0 mg/L (**study IV**). In clinical practice, the limited-range MIC was introduced routinely in 2017 for all MDR-TB isolates analysed at the supranational TB reference laboratory at the Public Health Agency but could be performed earlier on request. The methodology for linezolid MIC was similar to the method for pyrazinamide MIC mentioned above. Exceptions were that fresh samples were used since it was part of clinical practice, and the antibiotic-free growth control was prepared in a 1:100 dilution (265). No reference strain was included as control since this was part of routine clinical practice.

4.3.3.1 Broth microdilution plates for minimum inhibitory concentrations

In **study III and IV**, commercially available broth microdilution plates were used to assess MIC for moxifloxacin and linezolid as well as for other drugs (Table 13). The MYCOTB plate (65) (Thermo Fisher Scientific Inc., USA) used in **study III** includes moxifloxacin with a test range of 0.06-8.0 mg/L. We decided to use the MYCOTB plate in **study III** since it would cover most drugs used for MDR-TB treatment in China. However, in **study IV**, we utilized the UKMYC5 plate (266) (Thermo Fisher Scientific Inc.,

USA) as this plate included more drugs used in the treatment of MDR-TB in later years in Sweden, including linezolid, with a linezolid test range of 0.03-2.0 mg/L.

Table 13 Commercially available broth microtiter plates for minimum inhibitory concentration included in studies III and IV

Microtiter plate	Liquid media	Included drugs
MYCOTB	Middlebrook 7H9	Rifampicin, rifabutin, isoniazid, ofloxacin, moxifloxacin, cycloserine, ethionamide, ethambutol, amikacin, kanamycin, streptomycin and para-aminosalicylic acid
UKMYC5	Middlebrook 7H9	Rifampicin, rifabutin, isoniazid, bedaquiline, linezolid, levofloxacin, moxifloxacin, clofazimine, ethionamide, ethambutol, delamanid, kanamycin, amikacin and para-aminosalicylic acid

MYCOTB (65) and UKMYC5 (266) plates are both manufactured by Thermo Fisher Scientific Inc., USA.

Broth microdilution MIC testing from both plates were performed according to the manufacturer's instructions (65, 266). Briefly, not more than 14-day-old *M. tuberculosis* colonies from solid media were suspended to a turbidity of 0.5 McFarland. The suspension was diluted to 1:100 in Middlebrook 7H9 media with OADC (oleic acid albumin dextrose catalase). From this suspension, 100 µL was distributed in each well and incubated and read after 10-21 days when the positive growth-control was visible. MIC testing by broth microdilution was performed from frozen *M. tuberculosis* samples in both studies since MIC was done in batches in **study III** and was not in clinical practice in Sweden in **study IV** but was performed for another study (164). The reference strain H37Rv ATCC 27294 was included in each run in **study III and study IV**.

4.3.4 Whole genome sequencing

Whole genome sequencing was performed for all strains in **study II** according to the manufacturer's instructions. The principles for whole genome sequencing involve three main steps; 1) DNA extraction; 2) sequencing; and 3) analysing the data.

The first step of DNA extraction was conducted from *M. tuberculosis* cultures that were thawed and then sub-cultured before being used. Either QiAmp Mini DNA Kit (Qiagen, Hilden, Germany) (267) or a chloroform/cetrimonium bromide-based protocol were used (268). The main steps of DNA extraction involve first killing the *M. tuberculosis* bacteria using a high temperature before lysing them to release the DNA into a solution. To purify the DNA, the solution is run through a membrane where the DNA bind while contaminants are washed away (QIAmp protocol) (267). Alternatively, alcohol is added to precipitate DNA which is then frozen while contaminants can be washed away (Chloroform/cetrimonium bromide) (268). Lastly, the pure DNA is eluted and can be used for the next step of sequencing.

In the second step of sequencing, two platforms were used, either Illumina HiSeq 2000 (Illumina, San Diego, CA, USA) as part of the TB-ARC project (269) (n=103 strains) or Ion torrent S5 XL instrument (Thermo Fisher Scientific Inc., USA) (n=45 strains). Both Illumina and Ion Torrent are referred to as next generation sequencing due to their high efficiency and ability to detect single nucleotides with higher precision compared to Sanger sequencing (270). The principles for sequencing involve dividing the DNA into shorter fragments, then attaching adapters to the ends of the DNA fragments for enabling identification and binding, before multiple amplifications of each DNA fragment are done. Lastly, each nucleotide in the DNA fragments is detected using either fluorescent light (Illumina) or a change in pH using ions (Ion Torrent). Each part of the DNA is sequenced multiple times (so-called read depth) which enables higher precision. A disadvantage with both platforms is that the read length is short (length of the DNA fragments), usually around 300 base pairs, which could pose a problem in regions with repetitive nucleotides which is present in TB (271).

The third step involves analysing the sequenced reads. The assembled sequence is then mapped (compared) to a reference genome (H37Rv NC_000962.3 was used in **study II**) where genetic differences are noted such as single nucleotide polymorphisms (SNPs), insertions or deletions (see Section 2.4.4 on whole genome sequencing and Table 3). In **study II**, the *pncA*-gene and its promotor region as well as the two other genes *rpsA* and *panD* that have been associated with pyrazinamide resistance were analysed. To correctly identify DNA variants, we used criteria with a minimum frequency of 25% to identify single nucleotide polymorphisms (SNPs), and 80% for insertions and deletions. The identified variants were then compared to the in-house database at the Public Health Agency of Sweden which was continuously updated. At the time of the study, the WHO classifications list of mutations had not been published (published in 2021), therefore, it was not included (54). However, mutations associated with pyrazinamide that had been published from other sources were used to define resistance (75, 80, 89, 272).

4.4 Drug concentrations

Analysis of drug concentrations was conducted for moxifloxacin and levofloxacin in **study III** and linezolid in **study IV**. A rich sampling scheme was used in **study III** to estimate $AUC_{0\text{-}24h}$ concentration from plasma that was sampled at pre-dose and 1, 2, 4, 6, 8, and 10 hours after drug intake. In **study IV**, we used the available routinely conducted linezolid concentrations, which were mainly sampled pre-dose (estimate of trough concentration) and 2 hours post-dose (estimate of peak concentration). Since actual sampling around drug intake was sometimes not precisely documented or sampled at the planned time in **study IV**, a permissive rule was applied to also estimate both trough and 2-hour concentrations. Trough concentrations sampled three hours before or after a planned trough concentration (i.e., 21-27 hours after drug intake) but

before the next dose were also included. Furthermore, 2-hour concentrations that were sampled one hour before or after (i.e., sampled at one to three hours after drug intake) were also included.

Drug concentrations in **study III** were analysed in batches from frozen plasma using a validated five-drug assay adapted to common drugs used in MDR-TB treatment guidelines in China (moxifloxacin, levofloxacin, pyrazinamide, ethambutol, and prothionamide). The assay was developed for this study and was performed on a liquid chromatography–tandem mass spectrometry platform with deuterated internal standards (273). Since it was an observational PK-study, the concentrations were not used to guide MDR-TB treatment as the samples were analysed batchwise after the person had completed the treatment. As **study IV** was a retrospective observational cohort study, linezolid concentrations were continuously analysed during the study period in Sweden. Since concentrations were part of routine practice, results could also have guided treatment decisions. Samples in **study IV** were analysed at the quality-assured laboratory at the Department of Pharmacology at Karolinska University Hospital in Sweden. This laboratory is the only laboratory in Sweden routinely analysing linezolid concentrations and the assay was made available from June 2015.

4.4.1 Estimates of area under the concentration-time curve

Two different methods to analyse $AUC_{0\text{-}24h}$ were used; in **study III** the linear trapezoidal method; and in **study IV** an already published linear model (274).

The linear trapezoidal method is a simple way for non-compartmental analysis of mathematically calculating the AUC (Equation 1). After plotting the concentrations and the time each sample was drawn on a graph, the graph is divided into sections between each time point (Figure 6). The area of each section is calculated using the trapezoidal rule (Equation 1) and then taking the sum of section areas to calculate the total AUC over the whole time period.

$$\text{Area} = \left(\frac{\text{concentration}[t+1] + \text{concentration}[t]}{2}\right) \times (\text{time}[t+1] - \text{time}[t]) \qquad \textbf{Equation 1}$$

t = time

Since only concentrations up to 10 hours were sampled in **study III** (Figure 6), the AUC between 10 and 24 hours was estimated using the same formula and assumed a linear decline with the same concentration at 24 hours as the pre-dose concentration.

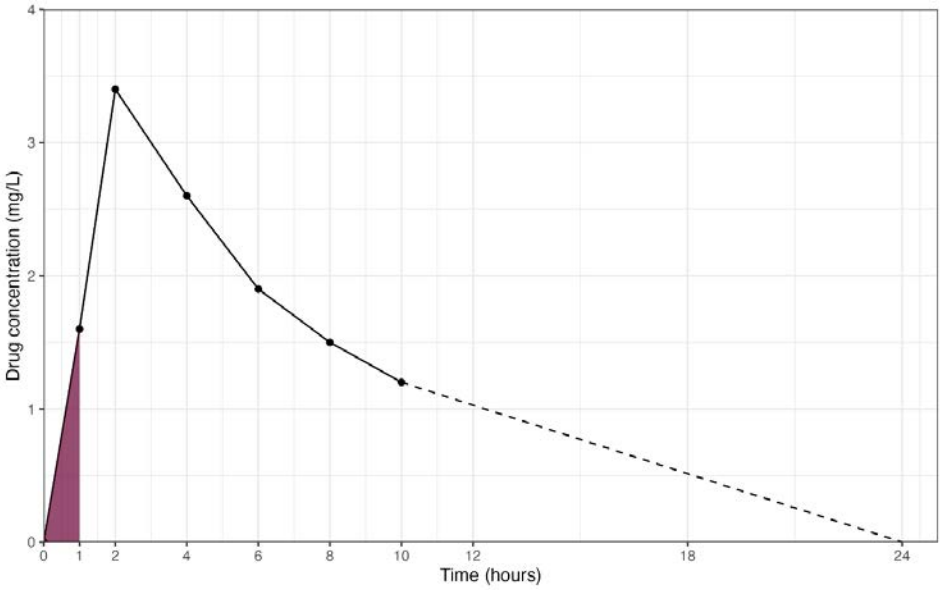

Figure 6 Plasma concentration over time between zero and 10 hours for one person treated with moxifloxacin 400 mg once daily in study III

The purple area is the average concentration between 0 and 1 hour, AUC(0–1h). The dotted line indicates assumed decline in concentration.

In **study IV**, the published linear model estimating linezolid AUC_{0-24h} (Equation 2) was used (274). The linear model is an estimate of a population PK model developed from linezolid concentrations sampled from people treated for MDR-TB (n=38) in the Netherlands and validated in a cohort of people treated for MDR-TB in Italy (274). The linear model is simple and only includes the sum of two linezolid concentrations sampled at pre-dose (0 hours) and 2 hours, except for constants.

$$AUC\ (0\text{–}24h) = 2 \times (\ 6.13x - 2.2636) \qquad \textbf{Equation 2}$$

x = sum of concentration at pre-dose and 2 hours

4.5 Definitions of exposures and outcomes

The main exposures and outcomes are described in Table 12 but further descriptions and definitions are provided here.

4.5.1 Treatment with pyrazinamide

A pyrazinamide-containing regimen as the exposure, defined as at least 80% treatment with pyrazinamide in the intensive phase, was used for the analysis of pyrazinamide resistance in **study** I. This equates to a person taking pyrazinamide treatment for five out of six months in a six-month intensive phase. In two other models (**study I**), different length of pyrazinamide treatment was categorised according to Table 14 to reflect relevant clinical time periods used in treatment. A partial intensive phase pyrazinamide regimen would reflect a person receiving pyrazinamide between one (>16% of the intensive phase) and five months (<80% in the intensive phase) while no pyrazinamide treatment would equate to pyrazinamide treatment <30 days in the intensive phase (Table 14).

Table 14 Definitions of different lengths of pyrazinamide treatment in study I

PZA Treatment	Definition
Treatment more or less than 80% in the intensive phase	
Partial PZA treatment	Less than 80% of the days in the intensive phase
Full intensive phase treatment	At least 80% of the days in the intensive phase
Different length of treatment in the intensive phase	
No PZA treatment	Less than 16% of the days in the intensive phase
Incomplete intensive phase	More than 16% but less than 80% of the days in the intensive phase
Full intensive phase treatment	At least 80% of the days in the intensive phase

PZA = pyrazinamide.

We wanted to include an analysis of the number of days of pyrazinamide used since the intensive phase could be either six or eight months long (**study I)**. However, due to a bi-directional association between the number of days and outcome, since the outcome was highly associated with the length of pyrazinamide treatment, this would mean adjusting for the outcome itself. Therefore, we used different percentages of pyrazinamide treatment.

Pyrazinamide treatment in **study II** was simplified and only defined as receiving 30 days or more.

4.5.2 Treatment with linezolid

The definition of linezolid treatment as the exposure in **study IV** in mg/day was calculated using cumulative dose in mg until the occurrence of either the adverse drug reaction (e.g., anaemia) or censoring, whichever came first, divided by the number of days until the event. When calculating linezolid dose in mg/kg/day, the same calculation was done as above and divided by the initial weight. Weight during treatment could not

be used due to missing data. Furthermore, the exposure variable mg/day was categorized as <600 mg and ≥600 mg. The dose in mg/kg/day was divided into three clinically relevant dosing categories <8, ≥8 to 12, and ≥12 mg/kg/day. These categories were chosen to correspond to 450 mg, 600 mg, and 750 mg daily for a person weighing 60 kg, respectively. Similarly, as for pyrazinamide treatment, days of linezolid treatment or the cumulative dose could not be used due to a bi-directional association with the outcome (e.g., having an adverse drug reaction, which often meant an early cessation of linezolid).

4.5.3 Pyrazinamide resistance

Different definitions of pyrazinamide resistance were used in **study II** as the exposure variable. Genotypic resistance to pyrazinamide was defined as having an established resistance mutation where there was high or very high confidence that it was associated with resistance in relation to phenotypic DST (75, 80). A high and very high confidence mutation was defined as those mutations found in more than 70% (high) or 100% (very high) pyrazinamide-resistant strains, respectively, when compared with phenotypic DST. The in-house catalogue of mutations in the *pncA*, *rpsA*, and *panD*-genes at the Public Health Agency of Sweden was used which was mostly based on two publications by Miotto and colleagues (75, 80, 89, 275). The WHO mutation catalogue was not used since it was not published at the time the study was acceptable for publication (54).

Our composite definition of pyrazinamide resistance was created in an effort to establish an expert opinion using all three methods (phenotypic DST, genotypic DST, and MIC) to which we could compare each method and evaluate the overall research question (Table 15).

Table 15 Definition of composite pyrazinamide resistance used in study II
(Adapted from Kuhlin 2021, Table 1) (165)

No	Whole genome sequencing for *pncA*, *panD,* and *rpsA*	pDST	Composite pyrazinamide classification of resistance or susceptibility
1	*pncA* WT	S	S
2	*pncA* high confidence mutation for resistance	R	R
3	*pncA* low confidence mutation for resistance	S	S
4	*pncA* low confidence mutation/WT	R	Investigate MIC If MIC ≤100 mg/L isolate regarded as S[1]. If MIC >100 mg/L isolate regarded as R. Consider investigating heteroresistance.
5	*pncA* high confidence mutation for resistance	S	Investigate MIC If MIC ≤100 mg/L isolate regarded as S[1]. If MIC >100 mg/L isolate regarded as R.
6	*pncA* WT, *rpsA*/*panD* mutation	S	S
7	*pncA* WT, *rpsA*/*panD* mutation	R	R
8	WGS available but not classified according to table[2]	S	R
9	WGS not available	R	R
10	WGS not available	S	S

No = number, pDST = phenotypic drug susceptibility testing in BACTEC 960 MGIT, WT = wildtype, S = susceptible, R = resistant, MIC = minimum inhibitory concentration, WGS = whole genome sequencing. If only WGS or pDST or MIC was done, the composite pyrazinamide classification was according to the available method. 1 In clinical practice a note should be given that the isolate shows discrepant results which should be considered when selecting the final drug treatment regimen. 2 One isolate did not fit the composite definition, and when retested an MIC>128 mg/L was found, and the isolate was classified as pyrazinamide resistant.

4.5.4 Minimum inhibitory concentration

MICs were performed for pyrazinamide (**study II**), moxifloxacin (**study III**), and linezolid (**study IV**) and used as the exposure variable. However, a limited range of concentrations was tested which could pose problems as the MIC could fall outside of the tested range (MIC truncation) (63). Therefore, we categorised strains with the lowest MIC tested as the lowest boundary (e.g., pyrazinamide MIC ≤8.0 mg/L was classified as 8.0 mg/L), and strains with the highest MIC tested were classified as one step higher MIC (e.g., pyrazinamide MIC >512 mg/L was classified as 1024 mg/L).

4.5.5 Sputum culture conversion

Sputum culture conversion as the outcome in **study II** was defined as the date of the first negative respiratory sample submitted if the person also improved clinically or radiologically, in a person with an initial positive respiratory sample. Furthermore, a respiratory sample could either be submitted by coughing, sputum induction, gastric aspiration, or from bronchoalveolar lavage/bronchial secretion. Therefore, we did not use

the classic WHO definition of having two negative sputum samples at least 30 days apart (44). An alternative definition was used since many participants could no longer submit a spontaneous sputum sample after starting treatment and persons treated in the earlier years of the cohort submitted sputum samples less regularly according to the clinical routine at that time point.

4.5.6 End-of-treatment outcomes

End-of-treatment outcome in **study I** was defined according to the 2013 criteria and in **study II and IV** according to the 2005 criteria (see Section 2.7.2 on treatment outcomes). The reason for not using the 2013 outcome definitions in the latter two studies was based on a higher proportion of failure and lower proportion of success compared to 2005 definitions. In a high-resource setting when individualised treatment is given, there is often a low threshold for changing two drugs due to adverse drug reactions and this rarely means a failing regimen. Therefore, the 2005 definitions, especially regarding the definition of failure, was regarded to reflect more on what is clinically relevant. However, in **study IV**, we have additionally included end-of-treatment outcomes according to 2013 definitions in the supplement.

4.5.7 Adverse drug reactions

Adverse drug reactions as the outcome in **study IV** were defined according to the Common terminology criteria for adverse events (CTCAE), version 5.0 (United States Department of Health and Human Services, 27 November 2017) which includes severity grading between one to five for each adverse event (276). Grade one events are usually asymptomatic and only noted in blood tests or examinations, grade two events are symptomatic or need treatment but do not limit normal activities in daily life. However, grade three events would limit normal activities of daily life and severely compromise the person. A grade four event is life-threatening, while grade five means death. A grade three or higher event is considered severe. Various scales are available to define adverse events (e.g. Division of AIDS and CTCAE) which in general are similar in grading, but the CTCAE was chosen since it is widely adopted in clinical trials (135, 276, 277). The specific adverse drug reactions of peripheral neuropathy, anaemia, leukopenia, thrombocytopenia, optic neuritis, and elevated lactate were considered for evaluating adverse drug reactions during linezolid treatment. Furthermore, peripheral neuropathy was combined into one category including polyneuropathy with paraesthesia, motor neuropathy, and sensory neuropathy (276).

To estimate causality, the WHO-Uppsala Monitoring Centre definitions (278) were used to assess how likely linezolid caused the adverse event, and only those that fulfilled the criteria for probable or certain were included (Table 16). Since we tried to establish causality between linezolid treatment and an adverse event, we used the term adverse

drug reaction (we added "drug" to the word adverse reaction for clarity) according to the European Medicinal Agency's definitions (279).

Table 16 Definitions used in establishing likely causality between linezolid and an adverse event in study IV (adapted from WHO-Uppsala Monitoring Centre) (278)

Causality term	Assessment criteria
Certain	• Event or laboratory test abnormality, with plausible time relationship to drug intake • Cannot be explained by disease or other drugs • Response to withdrawal plausible (pharmacologically, pathologically) • Event definitive pharmacologically or phenomenologically (i.e., an objective and specific medical disorder or a recognised pharmacological phenomenon) • Rechallenge satisfactory, if necessary
Probable	• Event or laboratory test abnormality, with reasonable time relationship to drug intake • Unlikely to be attributed to disease or other drugs • Response to withdrawal clinically reasonable • Rechallenge not required

WHO-Uppsala monitoring centre (278).

4.5.8 Targets of optimal microbial kill and prevention of acquired resistance

The targets of the free fraction AUC_{0-24h}/MIC for optimal microbial kill and prevention of acquired resistance used for outcome in **study III** and **study IV** were derived from hollow-fibre models (184, 213, 215, 228) (see Section 2.8.1 on PK-PD parameters). We used the most cited targets for moxifloxacin, levofloxacin, and linezolid in overview articles (188). The fraction refers to the protein unbound concentration of moxifloxacin and levofloxacin as it is only the unbound part of the drug that exerts an anti-bacterial effect. Protein binding was estimated at 50% for moxifloxacin (280), 30% for levofloxacin (281), and 31% for linezolid (140) according to the package insert at the time of registration since we did not measure the free fraction directly in **study III** and **study IV**. Moreover, since the targets are derived from the hollow-fibre models where no human plasma is included, these targets are free concentrations. A disadvantage of using estimates of the fraction of unbound drug is that protein levels in plasma can change with e.g., severity of TB. People who are severely ill can have lower albumin levels (the most common protein that binds drugs), and the level of unbound fraction could increase up to 85% according to one study (282). Therefore, people that were severely ill (i.e., admitted to the intensive care unit) were excluded from **study III**, thus, we assumed that the commonly used protein-binding fractions of both drugs would be sufficient. However, all people including severely ill persons were included in **study IV**, thus, this is a limitation of the estimated linezolid free fraction AUC_{0-24h}/MIC.

4.6 Study design

All studies (**study I-IV**) used a cohort study design with the advantage that multiple outcomes and multiple exposures can be studied (283) (Table 12). Furthermore, the time relationship between the exposure and the outcome can be studied, e.g., using a Cox regression analysis (**study II and IV**). A disadvantage of a cohort study design is loss to follow-up of participants, especially if the follow-up time is long as it can be in studies in MDR-TB (283).

We used both a prospective (**study III**) and retrospective (**study I, II, and IV**) study design (Table 12). A prospective study has the advantage of purposefully collecting data in a planned manner, regarding timing, frequency, and type of data. However, a prospective study also requires more resources, therefore, only 32 participants were included in **study III**. A known limitation of retrospective studies is that data on exposures or confounders could be incomplete or missing. Despite known limitations with retrospective studies, an advantage is that a large number of participants can be included, this is especially true for MDR-TB where treatment duration is long. Therefore, we could include all participants with MDR-TB in Sweden from 1992-2014 in **study II** and 1992-2018 in **study IV** (Table 12).

All studies (**study I-IV**) were observational in nature, meaning that no intervention was conducted (Table 12). Although a randomised controlled trial would be ideal in trying to establish causality between an exposure and an outcome, observational studies are often the initial step in establishing possible associations between an exposure and an outcome which justifies our studies. Furthermore, a randomised controlled trial is highly time-consuming and costly, which was beyond the time and scope of this thesis.

Another disadvantage with cohort studies could arise if there is a large proportion of participants who are lost to follow-up as seen in **study I** (22.9% lost to follow-up). If participants who were lost to follow-up would be different in terms of treatment success (i.e., participants who received pyrazinamide had more adverse drug reactions and as a result would stop the whole treatment early on their own and be lost to follow-up) compared to participants who were still in the study, this could lead to false conclusions. Therefore, we performed a sensitivity analysis **in study I** when participants lost to follow-up were included, which showed similar results.

4.7 Epidemiological concepts and considerations

When conducting an analysis, five possible reasons for any association found should be considered; random error; bias; confounding; reverse causation; and causation. Random error occurs at random due to sampling and cannot be adjusted for but is present in all studies and is accounted for when data is presented, e.g., using 95% confidence intervals. Bias is a systematic error occurring in epidemiological studies and can be

generally divided into information and selection bias, which will be further described below (Section 4.7.1 on bias). Some people also consider confounding a type of bias and it will also be described (see Section 4.7.2 on confounding). Reverse causation means that the outcome itself leads to the exposure, and it is usually a risk in case-control studies, but it has a lower risk in cohort studies when the exposure is recorded before the outcome in time. Causation is the optimal reason for an association when the exposure is the cause of the outcome. An important aspect to consider in thinking about causation is that the exposure is often one of several factors leading to the outcome.

4.7.1 Bias

Selection bias refers to the bias occurring when participants are selected to take part in a study. Case-control studies are particularly prone to selection bias due to difficulties in selecting the controls that are representative of the cases. However, in prospective cohort studies, selection bias is less problematic since the exposure is measured before the outcome, therefore, the outcome could not affect exposure (283). However, retrospective cohort studies could be prone to selection bias, if knowledge of the outcome would affect the classification of exposure. We mitigated selection bias in all three retrospective studies by including all participants treated for MDR-TB in Sweden or Uzbekistan (**study I, II,** and **IV**) and having a clear definition of the exposure. A form of selection bias in cohort studies is the so-called "healthy worker effect" (284). E.g., if mortality is studied in a cohort of workers, it is usually lower than in the general population since workers are usually healthier than non-workers. Similarly, one could hypothesise that participants residing in the region around Xiamen in **study III** would be healthier than persons from the so-called floating population which has been reported in other studies (285). To mitigate, a healthy worker effect, both persons that were permanently residing in the region and from the floating population were included. Moreover, a small incentive was included to enable all persons to participate in the study, in case of income loss associated with extra study visits (260).

Information bias could also be called measurement error (continuous variables) or misclassification (categorical variables). For example, the measurement of a participant's age is wrongly reported, or a participant is classified as having an adverse drug reaction of anaemia due to linezolid when it is actually due to a pregnancy. To mitigate the misclassification of pyrazinamide resistance in **study II,** we compared each way of measuring pyrazinamide resistance to a composite definition of resistance. In the prospective **study III**, data collection was planned to reduce missing data. Furthermore, a known limitation of retrospective studies is that data could be incomplete or missing, leading to a risk of information bias. Therefore, in **study II** and **study IV** we contacted each region in Sweden where the participants were treated for MDR-TB at least twice to collect all data. Moreover, misclassification in data entry was addressed in **study II** and

study IV by a 20% overlap in data collection (participants treated 1992-2014) or by using double data entry in **study I** and **study IV** (participants treated 2015-2018).

Another aspect of misclassification relates to non-differential and differential misclassification. Non-differential misclassification occurs when participants are misclassified but it is not dependent on the exposure nor the outcome. In general, non-differential misclassification often leads to dilution of the results while differential misclassification could either lead to over-estimation or underestimation of the results.

Although retrospective observational studies have inherent limitations depicted above, evidence from multiple different study designs over time together form the basis of the current knowledge, motivating all kinds of studies.

4.7.2 Confounding

Confounding occurs when a factor is associated with the exposure and a risk factor for the outcome, but the factor is not on the causal pathway or a consequence of the outcome. A way of handling confounding at the analysis stage is adjusting for the confounder, so it does not distort the relationship between the exposure and the outcome. An example of a confounder when analysing the association between TB treatment and treatment outcome is age (286). Confounding occurs if persons receiving TB treatment who have a high age also have higher mortality compared to those with a lower age and if participants with a higher age are unevenly distributed in the treatment groups. Common confounders assessed in studies in analysis on TB treatment and outcome are age, sex, disease severity (e.g. microscopy results, cavities on chest X-ray), and previous use of TB drugs (286), which were also considered in **study I and II**.

Another way of handling confounding at the analysis stage is stratification or restriction which means that the analysis is divided based on the confounder. We used restriction in **study I** when the analysis was only performed in a subset of participants who received pyrazinamide in the whole intensive phase.

An important type of confounding is "confounding by indication" which means that the knowledge of the exposure would results in different ways of handling those exposed and those not exposed. An example could be in **study I** if the knowledge of the pyrazinamide DST of a person's *M. tuberculosis* strain would affect if pyrazinamide was prescribed or not. Therefore, we used the fact that different treatment protocols for pyrazinamide were used. Furthermore, we did a sub-group analysis in those with unknown pyrazinamide DST and performed a sensitivity analysis which was restricted to those who were treated in the later years when one protocol was in place.

4.8 Statistical methods and considerations

Both logistic (**study I**) and Cox regression models (**study II and IV**) were used for the main analysis in the studies, which are both regression models used for binary outcomes (287). Logistic regression models estimate the odds ratio which could be interpreted as the risk ratio if the outcome is rare. One advantage with logistic regression is that it can be utilized in both case-control and cohort study designs (287). Furthermore, no time variable is included which simplifies modelling.

In Cox regression, hazard ratios are fitted, which could be thought of as rate ratios. One advantage of Cox regression modelling is that it assumes no distribution and is a non-parametric test, which increases its usage. Other features of Cox regression include the concept of censoring which is important as participants are followed-up over time (287). Censoring occurs when a participant is no longer at risk for experiencing the outcome (e.g., time of stopping linezolid or death in **study IV**), meaning that they no longer contribute to follow-up time for the study. Another aspect of Cox regression modelling is taking into account the underlying time scale (e.g., time since starting treatment [**study II**] or linezolid treatment [**study IV**]) which is always included and adjusted for. This is of particular advantage when incidence changes rapidly over time (i.e. anaemia had a much higher incidence in the first month compared to the rest of the treatment as in **study IV**) (287). However, an important limitation is that the ratio of the exposed and unexposed group is assumed to be constant over time, the so-called proportional hazard assumption. This assumption needs to be assessed, which could be done using Schoenfeld's residuals (**study II and IV**) or visually viewing the data (**study IV**) (287).

When interpreting p values, a p value of <0.05 was considered evidence, $p<0.01$ strong evidence, and $p<0.001$ very strong evidence against the null hypothesis for the study.

Kaplan-Meier curves were fitted in **study II and IV** and describe the probability that a participant is event free at each time point (287).

4.8.1 Missing data

We used various methods of dealing with missing data. When a small fraction of data was missing (less than 10% (288)), we excluded missing cases in the final analysis since it is usually considered a low risk of information bias. Hence, we performed a complete-case analysis (**study I, II, and IV**). However, in **study I and IV**, two possible confounders had 50% (diabetes in **study I**) and 36% (BMI, only height was missing, **study IV**) of missing data. Therefore, we included the missing group as an own category. Another option could have been imputation of missing data. However, since the imputation of height to calculate BMI requires more advanced assumptions leading to uncertainness in the results and is rarely done in clinical TB studies, we decided against imputing the

variable height. The disadvantage of using the category of missing for BMI is a risk of residual confounding. Despite uncertainty in data imputation, we did impute data for fraction of protein binding for moxifloxacin and levofloxacin in **study III** and for linezolid in **study IV** since we considered these estimates likely reliable (140, 280, 281). Moreover, the tentative ECOFF (moxifloxacin 0.5 mg/L and levofloxacin 1.0 mg/L) was imputed for fluoroquinolone susceptible strains without an available MIC for moxifloxacin and levofloxacin (100, 275).

4.9 Ethical considerations

Four moral principles could be considered when conducting ethically sound research; doing good; avoiding harm; autonomy; and justice. The principle of doing good entails reducing suffering, avoiding ill-health, or increasing knowledge about a disease. Avoiding harm includes avoiding conducting research that harms participants physically och psychologically but also not breaching confidentiality. Other ways could be to avoid harmful research practices, such as deliberately publishing fabricated results. The principle of respecting autonomy includes respecting each person's right to decide upon participation in research as well as the importance of informed consent. Lastly, the principle of justice is about conducting research in populations that benefits from the research themselves, thus making sure vulnerable populations are not taken advantage of.

4.9.1 Study I

This study uses routinely collected data from the TB programme in Karakalpakstan, Uzbekistan. Due to the retrospective design, no extra involvement of included participants was needed which reduces the risk of possible harm. To not breach confidentiality and risking identifying participants, we only had access to pseudonymised data which was safely stored on a password-protected computer in a password-protected file. Furthermore, data was and will only be presented in an aggregated form to avoid the risk of an individual being identified. People falling ill with TB belong to a vulnerable group in general due to illness and stigma, and specifically related to often having a poorer economic background or migration status. Other vulnerable groups included in this study were children and pregnant women. Therefore, extra care is needed to avoid harm and protect persons being identified through our research, which we have mitigated through the aforementioned reasons. However, in line with the justice principle, it is also important that research is conducted on people with TB and especially in countries with a high incidence of MDR-TB like Uzbekistan. The results would be more relevant to specific populations in improving care and treatment. The benefit of the study is increased knowledge, specifically useful for the TB programme itself but also to other researchers since the results have been published in a peer-review journal.

4.9.2 Study II and IV

These two studies use routinely collected data from medical files in Sweden, thus, reducing harm by not using additional time and resources from participants or caregivers. Furthermore, confidentiality was considered by limiting the number of people conducting the data collection and keeping data secure in a fire-safe password-protected locked cabinet in a locked room. The need for informed consent was waived in this study (as well as in **study I**) due to the nature of routinely collected retrospective data. There are also practical difficulties in providing informed consent in a retrospective study over many years since people may be impossible to contact as they could have moved or died. Thus, the number of participants in the study would be reduced leading to uncertainty regarding the results. The benefits of conducting the studies were to increase knowledge and understanding of MDR-TB in general and in particular related to pyrazinamide and linezolid to improve health care for people with MDR-TB in Sweden and elsewhere.

4.9.3 Study III

This study uses prospectively collected data from people admitted to a hospital in China. Data collection was done by researchers in China and only pseudonymised data was available to our research group in Sweden, which was kept on password-protected computers. Informed consent was required to participate in the study, thus considering the principle of justice. Ensuring that people understand the concept of informed consent is important in all settings since people might feel pressured to participate. Therefore, we had a training session with study personnel before the initiation of the study, which included discussions on ethical principles and informed consent. A potential harm with the study was the additional time, blood tests, and sputum the participants needed to submit. However, we considered the time as low risk since it was part of routine care to be in the hospital during the first two months of treatment when most samples were submitted. Although we considered the practical part of drawing blood of low risk, there is a belief in China as in many other places, that drawing your blood is harmful. Hence, we conducted a small pilot study and after feedback from the participants, we adjusted the study protocol with fewer time points for blood sampling. A small monetary subsidy was also given to people to cover extra costs incurred by our study. Similarly, to the other studies, data was and will only be presented in aggregated form to reduce the risk of any person being identified. The benefit of conducting the study involves increasing knowledge, especially for this group of people with MDR-TB in China.

4.9.4 Ethical permits

Study I

- Letter by Médecins sans Frontières, Amsterdam, the Netherlands (original).
- Letter by Medical Director of Médecins sans Frontières, Amsterdam, the Netherlands (addendum).
- MSc Research Ethics Committee reference 10847, London School of Hygiene & Tropical Medicine, United Kingdom (original).

Study II and IV

- Study II and IV: Reference 2012/197-31 (original), reference 2013/174-32 (addendum) and reference 2016/417-32 (addendum), Regional Ethical Review Board in Linköping, Sweden.
- Study IV: Reference 2019-00320 (addendum) and refererence 2022-02861-02 (addendum), Swedish Ethical Review Authority, Sweden.

Study III

- Reference 2015/6464-31/1, Regional Ethical Review Board in Stockholm, Sweden (original).
- The Institutional Review Board number 2015-09-0565, School of Public Health, Fudan University, Shanghai, China (original).

Daruna is sitting totally still on the floor with her back tight against the wall. Her knees are tightly bent and reach all the way to her chin. She does not react when we enter, instead, her empty gaze is fixed on the opposite wall. Daruna's daughter tells us that she has barely talked or moved in the last few days. "And she does not want to eat or drink". I am well aware of that the drug cycloserine, which she started not long ago can give psychiatric side effects. I slowly approach Daruna to examine her. I can see fear in her eyes. Still, there is no reaction, she doesn't even turn her head. We decide to immediately stop all drugs. A few days later we talk to her daughter, Daruna is finally feeling better and is eating again.

5 Results

5.1 Study I

In this large retrospective cohort study from Karakalpakstan, Uzbekistan, 2446 people treated for pulmonary MDR-TB between 2003 and 2013 were included of which 34.0% (n=832) had an available phenotypic DST result for pyrazinamide. Overall, 51.4% (n=1257) of the study participants were female with a median age of 30.5 (IQR 24-42) and 87.4% (n=2 137) had previously received treatment with first-line TB drugs. A cavity on chest X-ray was seen in 81.4% (n=1835) and 16.0% (n=392) of participants were infected with an *M. tuberculosis* strain defined as pre-XDR TB at the time of the study (MDR-TB and additional resistance to either a second-line injectable drug or a fluoroquinolone). About a third of participants were included from three time periods where different treatment guidelines were used; 34.8% (n=852) from 2003-2008; 34.5% (n=844) from 2009-2011; and 30.7% (n=750) from 2012-2013.

The median treatment length was 20 months (range 0-38), while the median pyrazinamide treatment was 12 months (IQR 4-4). A pyrazinamide-containing regimen was prescribed to 90.8% (n=197/217), 76.6% (n=469/612), and 90.1% (n=1450/1610) of participants with pyrazinamide susceptible strains, pyrazinamide resistant strains or where pyrazinamide phenotypic DST was unavailable at diagnosis. Phenotypic pyrazinamide resistance was confirmed in 73.6% of strains (n=612/832) where pyrazinamide phenotypic DST was performed.

Treatment for MDR-TB resulted in 59.4% (n=1453) of people having a successful treatment outcome according to WHO definitions (208), 5.8% died (n=141), 11.9% failed treatment (n=291), and 22.9% (n=561) were lost to follow-up. When comparing baseline characteristics between participants having a pyrazinamide-resistant or susceptible strain, the groups were similar in general, except for more participants with a pyrazinamide-resistant strain were included in the 2009 programme and were never tested for diabetes (unknown status).

In the adjusted analysis of pyrazinamide susceptibility, we found no evidence of an association between having a pyrazinamide susceptible strain and treatment success (aOR 0.86, 95% CI 0.51-1.44, p=0.6) among participants who were treated with a pyrazinamide-containing MDR-TB regimen (Table 17). Adjustments were made for sex, age, previous use of first-line drugs, having cavities on chest X-ray, number of resistant drugs at baseline, and programme year. Furthermore, there was strong evidence (aOR 0.64, 95% CI 0.50-0.81, p<0.001) that for each drug the infecting *M. tuberculosis* strain was resistant to at diagnosis, there was a 34% lower odds of treatment success when adjusted for the other variables (Table 17).

Table 17 Crude and adjusted analysis of pyrazinamide susceptibility associated with treatment success in people treated with a pyrazinamide-containing regimen for multidrug-resistant tuberculosis in Karakalpakstan, Uzbekistan (n=508) using logistic regression

Variable	Crude OR (95% CI)	p value	Adjusted OR (95% CI)	p value
PZA susceptibility	1.04 (0.65-1.65)	0.9	0.86 (0.51-1.44)	0.6
Male	1.07 (0.70-1.63)	0.7	1.04 (0.67-1.61)	0.9
Median age[1], years (IQR)	0.99 (0.97-1.00)	0.06	0.99 (0.97-1.00)	0.07
TB programme 2003 (reference)	1.0		1.0	
2009	0.69 (0.33-1.42)	0.3	0.58 (0.26-1.29)	0.2
2012	1.04 (0.52-2.08)	0.9	0.91 (0.41-1.99)	0.8
Previous use of first-line drugs	0.54 (0.31-0.93)	0.03	0.55 (0.31-0.99)	0.05
Cavities on chest X-ray	0.75 (0.46-1.22)	0.3	0.83 (0.49-1.39)	0.5
Median baseline resistant drugs (IQR)[1]	0.64 (0.51-0.81)	<0.001	0.64 (0.50-0.81)	<0.001

OR = odds ratio, CI = confidence interval, PZA = pyrazinamide, TB = tuberculosis, IQR = inter quartile range.
1 continuous variable.

In the adjusted analysis of the length of pyrazinamide treatment, we found no evidence of an association between pyrazinamide treatment when it was given 80% or more of the time in the intensive phase and treatment success neither among people with unavailable pyrazinamide phenotypic DST (aOR 0.86, 95% 0.91-1.51, p=0.6) nor among people with pyrazinamide resistant *M. tuberculosis* strains at diagnosis (aOR 1.38, 95% CI 0.71-2.68, p=0.3).

5.2 Study II

In this nation-wide study, all persons treated with an MDR-TB regimen in Sweden from 1992 to 2014 were included (n=157). Out of all participants, 42.0% were female (n=66) and 26.8% (n=42) were above 40 years of age. Pulmonary TB was seen in 75.2% (n=118) of participants, 41.5% (n=49/118) had a cavity on chest X-ray, and 55.1% (n=65/118) were positive in sputum microscopy. Extensively drug-resistant TB (XDR-TB) as defined at the time of diagnosis was observed in 5.7% (n=9) of participants. A later generation fluoroquinolone (ofloxacin, levofloxacin, or moxifloxacin) was given to 84.6% (n=132/156) while prothionamide or ethionamide were given to 63.6% (n=98/154). A pyrazinamide-resistant strain at baseline was diagnosed in 56.1% (n=88) of participants.

Of all participants, 49.7% (n=78/157) received 30 days or more of pyrazinamide treatment, while the median duration of pyrazinamide treatment was 466 days (IQR 101-598), for participants with pyrazinamide-susceptible *M. tuberculosis* strains at baseline. An effective pyrazinamide treatment (participants with pyrazinamide susceptible *M. tuberculosis* isolates who received a pyrazinamide-containing MDR-TB regimen) was given to 38.9% (n=61), while 61.2% (n=96) were without an effective pyrazinamide treatment (participants with pyrazinamide resistant isolates at baseline or those having a pyrazinamide susceptible isolate but received <30 days of pyrazinamide treatment). The median time to sputum culture conversion was 24 days (IQR 8-85) and 63 days (IQR 35-91) in participants with effective pyrazinamide treatment and in those without effective pyrazinamide treatment, respectively. In total, a successful treatment outcome was observed in 80.9% (n=127), 1.3% (n=2) failed, 4.4% (n=7) died, 3.2% (n=5) relapsed, and 5.7% (n=9) were lost to follow-up.

When assessing the performance of the different ways of measuring pyrazinamide resistance, pyrazinamide genotypic and phenotypic resistance had a 97.6% and 95.5% sensitivity and 97.0% and 98.6% specificity compared to the composite definition of pyrazinamide resistance. The mode of pyrazinamide MIC for susceptible isolates was 12 mg/L (IQR ≤8-32, n=64).

In the adjusted analysis, there was strong evidence that no effective pyrazinamide treatment was associated with longer time to sputum culture conversion (aHR 0.52, 95% CI 0.32-0.85, p=0.009) using the composite definition (Table 18). The same results were observed, taking into account pyrazinamide resistance testing measured by genotype (aHR 0.49, 95% CI 0.29-0.82, p=0.007), or phenotype (aHR 0.52, 95% CI 0.32-0.84, p=0.008). However, no evidence was seen for an association between a lower MIC and time to sputum culture conversion (Table 18).

In the analysis of effective pyrazinamide treatment and time to an unsuccessful outcome, only crude analysis could be performed due to sparse data since only 19 participants had an unsuccessful treatment outcome. We found no evidence of an

association between ineffective pyrazinamide treatment considering our composite pyrazinamide resistance definition (HR 0.84, 95% CI 0.34-2.11, p=0.72), genotypic (HR 1.08, 95% CI 0.42-2.77, p=0.87) or phenotypic resistance (HR 0.91, 95% CI 0.34-2.42, p=0.85), and time to an unsuccessful treatment outcome.

Table 18 Adjusted analysis of ineffective[1] pyrazinamide treatment using different ways of defining pyrazinamide resistance and time to sputum culture conversion in people with multidrug-resistant tuberculosis in Sweden 1992-2014 using Cox regression

Models with different ways of measuring pyrazinamide resistance	Sputum culture conversion/total number	aHR (95% CI)	p value
PZA composite resistance testing[2]	90/98	0.52[2] (0.32-0.85)	0.009
PZA genotypic resistance testing[2]	90/98	0.49[2] (0.29-0.82)	0.007
PZA phenotypic resistance testing[2]	83/91	0.52[2] (0.32-0.84)	0.008
Lower PZA MIC[3]	41/44	0.52[3] (0.32-0.85)	0.14
Lower PZA MIC in PZA susceptible strains[3]	31/34	0.98[3] (0.73-1.31)	0.89

aHR = adjusted hazard ratio, CI = confidence interval, PZA = pyrazinamide, MIC = minimum inhibitory concentration. 1 Without effective pyrazinamide treatment = participants having a pyrazinamide resistant strain at diagnosis regardless of pyrazinamide treatment and participants having a pyrazinamide susceptible strain and receiving <30 days of pyrazinamide treatment. Effective pyrazinamide treatment = participants having a pyrazinamide susceptible strain at diagnosis and receiving 30 days or more of pyrazinamide treatment. 2 adjusted for age, sex, year of diagnosis, microscopy positivity in sputum and treatment with prothionamide or ethionamide. 3 adjusted for year of diagnosis and positive microscopy result in sputum.

5.3 Study III

In this prospective cohort study, 32 participants who were admitted to the TB-designated hospital in Xiamen, China, and received moxifloxacin or levofloxacin treatment for pulmonary MDR-TB were included. The median age was 33 years (IQR 22.8-43.3), 53.1% (n=17) were female and 6.3% (n=2) had a comorbidity of diabetes mellitus type II. Treatment was given according to Chinese national guidelines, 2016-2018. Out of all participants, 87.5% (n=28) had a successful treatment outcome, 3.1% (n=1) failed treatment and 9.4% (n=3) were lost to follow-up. No participants had a serious adverse event, however, two participants experienced arthralgia. Resistance to a fluoroquinolone was seen in 15.6% (n=5/32) of isolates at baseline.

Evaluable drug concentrations for moxifloxacin and levofloxacin were available from 27 participants, of which 20 received moxifloxacin 400 mg once daily (median 7.8 mg/kg) and seven levofloxacin 500 mg once daily (median 10 mg/kg). The median MIC for moxifloxacin was 0.25 mg/L (range 0.125-1 mg/L) when including *M. tuberculosis* isolates with susceptibility to fluoroquinolones (n=15). The ECOFF of 0.5 mg/L (n=5/20) (275) for moxifloxacin and 1.0 mg/L (n=7/7) (100) for levofloxacin were used for fluoroquinolone susceptible *M. tuberculosis* isolates by phenotypic DST when MIC was unavailable.

The median estimated AUC_{0-24h} was 36.1 mg×h/L (range 19.3-60.3) for moxifloxacin and 63.7 mg×h/L (range 47.8-75.3) for levofloxacin. Median AUC_{0-24h}/MIC and free fraction AUC_{0-24h}/MIC for moxifloxacin were 104 (range 14-430) and 52 (range 8-215) and the corresponding values excluding the five fluoroquinolone-resistant *M. tuberculosis* isolates were 145 (19-430) and 73 (10-215), respectively, assuming a protein binding of 50%. For levofloxacin, estimates of median AUC_{0-24h}/MIC and free fraction AUC_{0-24h}/MIC were 64 (range 48-75) and 45 (range 33-53), when protein binding was estimated at 30%.

In total, 73% (n=11/15) and 60% (n=9/15) of participants with moxifloxacin treatment reached suggested targets for antimicrobial kill (≥42) and suppression of development of resistance (≥53) (215, 228) using the free fraction AUC_{0-24h}/MIC, respectively (Figure 7). For participants receiving levofloxacin, none reached the proposed targets of antimicrobial kill (≥146) and suppression of development of resistance (≥360) (228) using the free fraction AUC_{0-24h}/MIC (Figure 7).

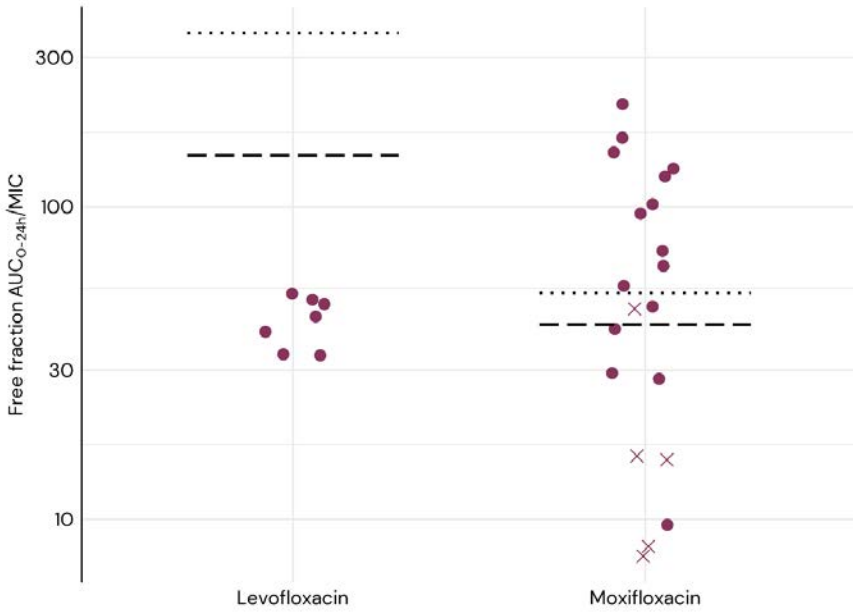

Figure 7 Levofloxacin and moxifloxacin free fraction of the total exposure over minimum inhibitory concentration in participants with multidrug-resistant tuberculosis in Xiamen, China, 2016-2018

AUC_{0-24h} = area under the concentration time-curve. MIC = minimum inhibitory concentration. Free fraction is the non-protein bound AUC_{0-24h}. The dashed and dotted lines refer to published suggested targets for optimal microbial kill (dashed line) and suppression of development of resistance (dotted line) (215, 228). The purple filled circles are participants harbouring a fluoroquinolone susceptible *Mycobacterium tuberculosis* isolate while the crosses are fluoroquinolone resistant isolates. When actual MICs for isolates were unavailable for fluoroquinolone susceptible isolates, the estimated epidemiological cut-off of 0.5 mg/L (n=5/20) (275) and 1.0 mg/L (n=7/7) were used for moxifloxacin and levofloxacin, respectively. The y-axis of the graph is on a logarithmic scale.

In the analysis of target attainment of tentative thresholds, if treatment was prescribed according to the doses in our study, only participants harbouring a strain with an MIC ≤0.25 mg/L for moxifloxacin and ≤0.125 for levofloxacin would ensure that 90% of participants reached the target of the free fraction AUC_{0-24h}/MIC for antimicrobial kill.

5.4 Study IV

In this nationwide study which further expands the cohort from **study II**, we included all 132 participants who received at least one day of linezolid treatment for MDR-TB in Sweden between 1992 and 2018. The median age was 28 years (range 12-75) with 43.2% (n=57) being female and 77.3% (n=102) having pulmonary TB. Out of all participants, extensively drug-resistant TB (XDR-TB) was observed in 11.2% (n=13/116) when using the pre-2021 WHO definition of having an *M. tuberculosis* strain with MDR plus any resistance to a later-generation fluoroquinolone and any second-line injectable drug. No strain was found to have linezolid resistance (n=0/120), while the mode of linezolid MIC was 0.5 mg/L (IQR 0.25-0.5). The median treatment length was 18.3 months (IQR 15.5-20.8). A successful treatment outcome was seen in 90.2% (n=119) of participants, while 0.8% (n=1) failed, 1.5% (n=2) died, and 5.3% (n=7) were lost to follow-up.

The median linezolid treatment was 6.5 months (IQR 3.0-12.7) with 76.3% (n=100) receiving a daily median linezolid dose of 600 mg or more, corresponding to a daily dose of 9.6 mg/kg. The median linezolid trough concentration was 1.33 mg/L (IQR 0.62-2.8, n=40), the median 2-hour concentration was 12 mg/L (IQR 8.5-14.9, n=44), and AUC_{0-24h} was 159 mg×h/L (IQR 113-196, n=39). In total, 85.7% (n=24/28) of participants had a free fraction AUC_{24h}/MIC of more than 119 which is a suggested efficacy target (184) (median free AUC_{24h}/MIC 210 [IQR 152-251]), assuming a 31% protein binding (176).

In the descriptive analysis, a total of 55.3% (n=73) of participants experienced at least one adverse drug reaction of peripheral neuropathy, anaemia, leukopenia, thrombocytopenia, optic neuritis, or elevated lactate. Peripheral neuropathy was seen in 35.6% (n=47) and anaemia in 27.3% (n=36), which were the two most common adverse drug reactions. In contrast, optic neuritis was objectively verified in 6.1% (n=8) of participants. Moreover, a grade three or more adverse drug reaction was observed in 14.4% of participants, with anaemia being the most common (8.3%, n=11). Linezolid was withdrawn in 47.7% (n=63) of all participants due to any adverse drug reactions. Optic neuritis (87.5%, n=7/8) and peripheral neuropathy (68.1%, n=32/47) were the adverse drug reactions that led to the highest proportion of linezolid withdrawn. Anaemia led to the permanent discontinuation of linezolid in 27.8% (n=10/36) of participants.

The overall incidence of each adverse drug reaction per person-year was 0.58 (95% CI 0.44-0.77), 0.47 (95% CI 0.34-0.65), and 0.09 (95% CI 0.04-0.18) for peripheral neuropathy, anaemia, and optic neuritis, respectively. The incidence of anaemia per person-year was highest in the first month (incidence 2.54, 95% CI 1.70-3.79), similar to leukopenia and thrombocytopenia (Figure 8).

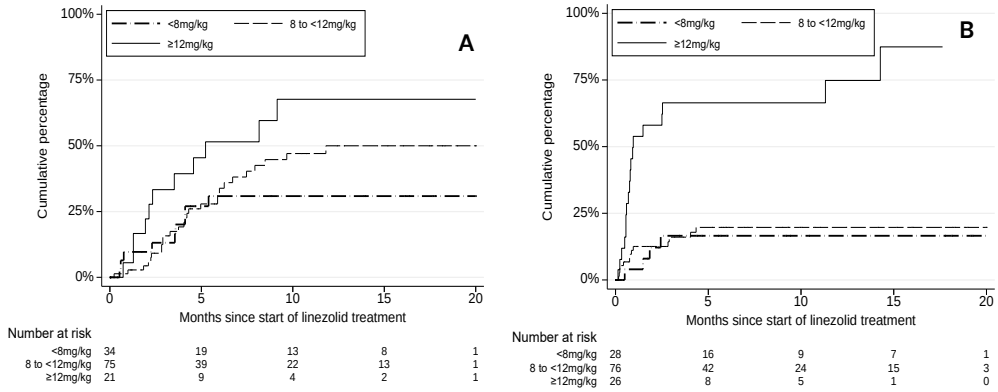

Figure 8 Kaplan-Meier graphs of daily linezolid dose in mg/kg and cumulative percent of peripheral neuropathy and anaemia among people treated with linezolid for multidrug-resistant tuberculosis in Sweden 1999-2018

A = Peripheral neuropathy, B= anaemia. The graphs are censored at 20 months after starting linezolid treatment.

In the adjusted analysis of daily linezolid dose in mg, we found evidence that a daily dose of ≥600 mg was associated with a 2.4 times higher hazard of peripheral neuropathy (aHR 2.36, 95% CI 1.04-5.35, p=0.039), however, we found insufficient evidence for an association with anaemia, leukopenia and thrombocytopenia when adjusted for sex.

In the multivariable analysis of daily linezolid dose using mg/kg, we found evidence that a dose of ≥12 mg/kg/day was associated with a higher risk of peripheral neuropathy (aHR 2.92, 95% CI 1.09-7.84, p=0.033), anaemia (aHR 7.13, 95% CI 2.17-23.5, p=.0001), and leukopenia (aHR 5.12, 95% CI 1.44-18.1, p=0.011) when adjusted for baseline creatinine or sex (Table 19).

We then performed an exploratory analysis of drug concentrations, where only a crude analysis could be done due to sparse data. We found evidence that a concentration of ≥2 mg/L was associated with a higher risk of anaemia (HR 3.60, 95% CI 1.0-12.0, p=0.0367) and thrombocytopenia (p=0.009, using Fischer's exact test), however, insufficient evidence for an association was seen for peripheral neuropathy and leukopenia.

Table 19 Adjusted analysis between daily linezolid treatment in mg/kg and an adverse drug reactions of peripheral neuropathy, anaemia, and leukopenia in people with multidrug-resistant tuberculosis in Sweden 1999-2018 using Cox regression

Variable	Peripheral neuropathy (n=131)		Anaemia (n=131)		Leukopenia (n=131)	
	aHR (95% CI)	p value	aHR (95% CI)	p value	aHR (95% CI)	p value
Female sex	-	-	-	-	2.55 (1.16-5.58)	0.019
Creatinine[1]	0.99 (0.66-1.49)	0.96	0.95 (0.58-1.58)	0.86	-	-
Linezolid treatment (mg/kg/day) <8 (ref)	1.0		1.0		1.0	
8 to 12	1.44 (0.65-3.20)	0.37	0.99 (0.31-3.15)	0.99	1.79 (0.51-6.28)	0.36
≥12	2.92 (1.09-7.84)	0.033	7.13 (2.17-23.5)	0.001	5.12 (1.44-18.1)	0.011

HR = hazard ratio, CI = confidence interval, Ref = reference. The adverse drug reactions thrombocytopenia, optic neuritis and elevated lactate could not be analysed due to sparse data. 1 Creatinine was measured at baseline as a continuous variable in steps of 20 µmol/L.

"Can I start treatment again?" Rustam asks me at our first meeting. He had been treated for MDR-TB one and a half years ago, but had stopped taking his TB medicines after six months as he began to feel better. The doctor, nurse, and counsellor that was treating him at the time had tried their best to convince him to continue treatment. "I need to earn money and support my family," was Rustam's response. "I don't have time to stay here and just get treatment, it's too long." Then he was gone. Rustam was now back at the clinic. "How have you been?", I ask. "I worked in another country, but about four months ago, I started to cough again, and became weak, then I couldn't work anymore. I know I need treatment – now. I will take it the whole time, I promise!" We talk about starting treatment and as he leaves the room to pick up his new medicines, I feel grateful that I don't have to choose between my own and my family's life.

6 Discussion

The major findings of this thesis are that we showed no added benefit of including pyrazinamide on treatment outcome when using an injectable-based standardised background-long MDR-TB regimen (**study I)**. Furthermore, we found insufficient evidence that different lengths of pyrazinamide treatment in the intensive phase affected treatment outcomes (**study I**). In **study II,** we expanded the analysis to include an interim outcome and we showed strong evidence that pyrazinamide treatment, considering resistance testing by genotypic DST, was associated with a shorter time to sputum culture conversion. In contrast, we showed no evidence that a lower MIC of pyrazinamide was associated with a shorter time to sputum culture conversion (**study II**). The major finding of **study III** was that no participant treated with levofloxacin and only 73% treated with moxifloxacin according to Chinese national guidelines reached the suggested PK-PD targets (215, 228). In **study IV,** we evaluated adverse drug reactions and showed strong evidence that daily linezolid treatment of 12 mg/kg or more was associated with an increased risk of an adverse drug reaction of peripheral neuropathy, anaemia, or leukopenia.

6.1 Optimising pyrazinamide – useful or useless?

Pyrazinamide treatment for TB has rendered much interest due to its important sterilising effect to prevent relapse despite that the mode of action is not fully understood. Another "curios characteristic", to use the words from the much-cited paper on pyrazinamide by Zhang and Mitchinson (83) is that an effect is only seen in the first two months of treatment in DS-TB (116). Thanks to its sterilising effect, pyrazinamide is the only first-line drug that is included in several new drug regimens and trials such as MDR-END trial (135).

6.1.1 The effect of pyrazinamide on end-of-treatment outcomes

We found no evidence of an effect of pyrazinamide treatment on treatment success (aOR 0.86, 95% CI 0.51-1.44, p=0.6, n=508) in persons treated with a standard MDR-TB regimen from our study in Uzbekistan (**study I**). Although only an unadjusted analysis could be made, similar results were seen in the Swedish cohort (**study II**) using the composite pyrazinamide-resistant definition (HR 0.84, 95% CI 0.34-2.11, p=0.72, n=153).

It is assumed that an *M. tuberculosis* strain with susceptibility or resistance to pyrazinamide does not affect treatment outcomes on its own. Instead, it is the pyrazinamide treatment given to a person that exerts its effect on an *M. tuberculosis* strain that is resistant or susceptible. Therefore, throughout this discussion, I am referring to pyrazinamide treatment or effective pyrazinamide treatment while the analyses in the studies are made of the effect on pyrazinamide susceptibility (or

resistance or of a combination thereof) in persons treated with (or without) pyrazinamide in an MDR-TB regimen.

Our findings in **study I** were not very encouraging as we expected pyrazinamide treatment to positively affect MDR-TB outcomes due to its important role in the regimen for DS-TB (116). As pointed out in the manuscript, the power to show a difference in our analysis was low (40%). However, these results are in line with four other original studies analysing the effect between pyrazinamide treatment in a long MDR-TB regimen (Table 20) and treatment outcome (155, 157, 158, 160), when pyrazinamide DST was known. Worth highlighting is that the PETTS study published by Cegielski *et al*., which is a large observational prospective multi-centre study (n=727), showed no evidence of a difference between pyrazinamide treatment and treatment success (158). In contrast, there were two smaller studies from Peru (n=75) and China (n=74) that reported evidence of an effect of pyrazinamide treatment on treatment success (161, 162). The limitation of the Peruvian study is that pyrazinamide and ethambutol treatment were analysed together, therefore, it is difficult to entangle the individual effect of pyrazinamide treatment in the analysis (161). However, ethambutol might have a limited role in MDR-TB regimens (115). Moreover, the study from China measured pyrazinamide resistance using genotypic DST, although their results would likely be similar if phenotypic DST was used as will be discussed below (Section 6.2 on optimising pyrazinamide and whole genome sequencing) (162).

Despite these results, the latest individual meta-analysis published in 2018 by Ahmad *et al*. (which included the cohort from the study by Cegielski *et al*. (158) but not the other studies in Table 20. showed that pyrazinamide treatment was associated with 30% lower odds of death, in participants with pyrazinamide-susceptible strains (115). If pyrazinamide treatment was given despite a person having a resistant strain, 50% lower odds of success was seen compared to if pyrazinamide was not given. In contrast to the above mentioned studies, another study from Peru (2821 person-months) (163) based on a similar but larger cohort compared to the study above from Peru (161), showed strong evidence that treatment with four drugs plus pyrazinamide compared to five effective drugs (without pyrazinamide) was associated with a higher hazard of death. Although this study included a large sample, the result is based on only 12 deaths, and it is uncertain if pyrazinamide was effective. Pyrazinamide DST was not routinely tested and of those with previous exposure to pyrazinamide, 51% had a pyrazinamide-resistant isolate.

Furthermore, pyrazinamide treatment was evaluated in the 18-month control regimen (n=73) in the recently published randomised open-label controlled trial MDR-END from South Korea (the intervention arm was a nine-month regimen consisting of delamanid, linezolid, levofloxacin, and pyrazinamide) (135). A 78.8% success rate was seen in participants having a phenotypic pyrazinamide-susceptible strain receiving the control

regimen compared to 61.9% in those with pyrazinamide-resistant strains. Although an effect measure or p value was not provided as this was not a planned analysis, a 16.9% absolute difference seems large enough to suggest a difference in outcome between the two groups. However, treatment in the control regimen was guided based on DST and treatment might have been adjusted by the physicians differently in participants having pyrazinamide susceptible or resistant strains (135).

Table 20. Studies evaluating the association between pyrazinamide treatment and treatment success when pyrazinamide drug susceptibility testing is known using the long regimen for multidrug-resistant tuberculosis

First author, Year	Country	n	Point estimate (95% CI)	p value
Mitnick, 2003	Peru	75	HR 3.33 (1.20-9.09)[1,2]	Not available
Chang, 2012	Hong-Kong	94	RR 1.38 (0.88-2.17)[3]	Not available
Budzik, 2014	USA	42	OR 3.23 (0.47-25)[3]	Not available
Cegielski, 2016	Multi-country	727	RR 1.28 (0.80-2.04)[1,4,5]	0.31
Zheng, 2017	China	74	OR 4.76 (1.33-16.7)[3,6]	0.02
Kuhlin, 2018	Uzbekistan	508	OR 0.86 (0.51-1.44)[3]	0.6
Park, 2020	Korea	216	OR 1.45 (0.59-3.58)[3]	0.7
Kuhlin, 2020	Sweden	153	Unadjusted HR 1.08 (0.42-2.38)[1,4]	0.87

CI = confidence interval, HR = hazard ratio, RR = risk ratio, OR = odds ratio. A long regimen for multidrug-resistant tuberculosis mainly refers to second-line injectable based regimens with the duration of 18-20 months according to WHO guidelines before 2018 (120). 1 Pyrazinamide treatment in persons with a pyrazinamide-susceptible isolate compared to persons with a pyrazinamide-resistant isolate and no pyrazinamide treatment in persons with a pyrazinamide-susceptible isolate. 2 Pyrazinamide and ethambutol treatment analysed together. 3 Pyrazinamide treatment received by all participants and comparing those having a pyrazinamide-resistant to susceptible isolate. 4 The analysis was done in a subset of participants, in those receiving treatment for 1854 person-months compared to 2821 person-months for the whole cohort. 5 Participants who were lost to follow-up were excluded. 6 Pyrazinamide resistance measured by genotypic DST.

Until now, all studies discussed have evaluated the long MDR-TB regimen, but pyrazinamide treatment has also been evaluated in shorter MDR-TB regimens. In the nine-month investigational regimen (n=67) in the MDR-END trial, no difference in treatment success was seen in participants with pyrazinamide susceptible or resistant strains (76.0% versus 76.2%) (135). In contrast, in the WHO-recommended short regimen for MDR-TB based on an injectable, pyrazinamide treatment in persons with phenotypic pyrazinamide-resistant strains has been associated with higher odds of relapse and bacteriological failure (132).

While previous research has evaluated the effect of pyrazinamide treatment, to our knowledge, no clinical studies were found, analysing the effect of different lengths of pyrazinamide treatment in MDR-TB on treatment outcome, making our findings novel (**study I**). We showed insufficient evidence that different lengths of pyrazinamide treatment in the intensive phase were associated with treatment success. This analysis was done, both in persons having no available pyrazinamide DST (aOR 0.86, 95% 0.91-

1.51, p=0.6) and among persons with a pyrazinamide-resistant isolate (aOR 1.38, 95% CI 0.71-2.68, p=0.3) (**study I**). The reason the analysis was done in persons with different pyrazinamide DST results separately, was possibly confounding by indication. Our results would have been more convincing if the length of pyrazinamide treatment was analysed in persons with only pyrazinamide-susceptible isolates, however, this analysis was not included due to the few participants who did not receive pyrazinamide treatment in this group.

When interpreting our results of the length of pyrazinamide treatment in persons with an unknown pyrazinamide DST (**study I**), one must consider that 73.6% of persons with a known pyrazinamide DST result in Karakalpakstan, had an isolate resistant to pyrazinamide. Therefore, only about 25% of people in the analysis would benefit from pyrazinamide treatment (assuming pyrazinamide does not have an effect in those with resistant isolates), which is probably too few to possibly show an effect. The analysis was performed since WHO at the time recommended pyrazinamide to be included in all longer MDR-TB regimens unless DST was known (120). However, the prevalence of pyrazinamide resistance in our study was higher than the global estimated prevalence of 60.5% (range 52.3-68.6%) in MDR-TB cases as shown in a meta-analysis (81).

To summarize, our results together with available data on the use of pyrazinamide for the treatment of MDR-TB, show a likely beneficial effect of pyrazinamide on end-of-treatment outcome if pyrazinamide DST is available and pyrazinamide is devoted to the treatment of susceptible isolates. This is likely due to the unique sterilising capacity of pyrazinamide enabling the treatment shortening for DS-TB treatment from nine to six months (116). The timing and availability of pyrazinamide DST and the variability of MDR-TB treatment regimens are well-known challenges for the interpretation of the efficacy of individual TB drugs.

6.1.2 The effect of pyrazinamide on sputum culture conversion

In **study II**, we showed strong evidence that effective pyrazinamide treatment was associated with a shorter time to sputum culture conversion, both using our composite DST definition (aHR 1.19, 95% CI 1.18-3.13, p=0.009), phenotypic DST (HR 1.19, 95% CI 1.19-3.13, p=0.008), and genotypic DST (HR 2.04, 95% CI 1.22-3.45, p=0.007). These results are similar to a study from China by Zheng and colleagues (n=74) who evaluated sputum culture conversion at two months using genotypic DST (162) and the previous study from Sweden from a similar cohort (n=77), but which only analysed pyrazinamide phenotypic DST (164) (Table 21). One study from Hong Kong (n=94) and one from Bangladesh (n=124) reported no difference between effective pyrazinamide treatment and sputum culture conversion (155, 166) (Table 21). However, in the Bangladesh study, it is unclear if an adjusted analysis was conducted, and a detailed description of the analysis was lacking.

Furthermore, the detailed analysis of pyrazinamide treatment from the PETTS study (167) showed that pyrazinamide treatment was associated with a higher rate of sputum culture conversion (HR 2.00, 95% CI 1.65–2.41, n=1137), despite unavailable pyrazinamide DST at the time of the study. Moreover, unsurprisingly, the number of potentially effective drugs in the regimen seemed to be important for sputum culture conversion, similar to the analysis of end-of-treatment outcomes (158). The authors concluded that pyrazinamide should be counted as an effective drug (167). Later, when the full details of the cohort study were published in 2016 (158), pyrazinamide-resistance was seen in 13.7% of tested isolates (n=904), which is much lower than the cohorts from Sweden in **Study I** (56.1%) and Uzbekistan from **study II** (73.6%). The low number of pyrazinamide-resistant isolates would likely explain why pyrazinamide treatment was effective in this cohort, even if pyrazinamide treatment was not guided by DST. Furthermore, the results highlight the importance of performing pyrazinamide DST upfront.

Table 21. Studies analysing the effect of pyrazinamide treatment upon sputum culture conversion in people treated for multidrug-resistant tuberculosis taking into account drug susceptibility testing of pyrazinamide in *Mycobacterium tuberculosis* isolates

First author, Year	Country	n	DST method	Point estimate (95% CI)	p value
Chang, 2012	Hong-Kong	94	Phenotypic	RR 1.38 (0.89-2.12)[1]	Not available
Zheng, 2017	China	74	Genotypic	HR 2.86 (1.33-6.25)[2]	0.01
Rahman, 2017	Bangladesh	124	Genotypic	No effect measure	No difference[3]
Davies Forsman, 2019	Sweden	77	Phenotypic	HR 2.38 (1.39-4.06)[4]	0.02
Kuhlin, 2020	Sweden	98	Composite[5]	HR 1.92 (1.18-3.13)[4]	0.009
Kuhlin, 2020	Sweden	98	Genotypic	HR 2.04 (1.22-3.45)[4]	0.007

DST = drug susceptibility testing, CI = confidence interval, n= number of participants in the study, RR = risk ratio, HR = hazard ratio. 1 Sputum culture conversion within 90 days. Pyrazinamide treatment in persons with pyrazinamide-susceptible strains compared to pyrazinamide treatment among persons having resistant strains. 2 Sputum culture conversion within two months. 3 No effect measure and information about what test was used is provided in the manuscript, however, it is only mentioned that that no significant difference was found. 4 Pyrazinamide treatment in persons having pyrazinamide-susceptible strains compared to no pyrazinamide treatment in persons having pyrazinamide-susceptible strains or in people with pyrazinamide-resistant strains, regardless of pyrazinamide treatment. 5 Composite pyrazinamide DST considering phenotypic, genotypic, and minimum inhibitory concentration.

6.1.3 When could pyrazinamide be useful?

As mentioned in the introduction, the effect of including pyrazinamide in the treatment of DS-TB was previously shown in the early TB trials with an increase in the proportion of sputum culture conversion at two months, a lower rate of relapse, and a possibility to shorten treatment to six months (116). Hence, these results relating to sputum culture conversion seem to hold also in regimens for MDR-TB, although the optimal duration of pyrazinamide treatment is not clear and the effect on treatment outcome in MDR-TB could be more convincing.

Compared to the early studies, the evaluation of relapse as the only outcome has not been done for studies on MDR-TB including **study I**. Instead, unsuccessful treatment outcome is typically used which includes death, failure, and sometimes relapse, and loss to follow-up. The reason that relapse is not evaluated on its own in MDR-TB is probably due to that the outcome is uncommon (around 1-3% in clinical trials (134, 135) which was also seen in **study II**) and the long treatment duration and follow-up required.

Another aspect regarding pyrazinamide treatment is the concept that it could shorten a regimen, as was shown by the synergistic effects together with rifampicin for DS-TB (116). This could be one of the reasons that the seven-drug regimen developed for MDR-TB in Bangladesh including pyrazinamide has similar success rates when given for nine months compared to the 18-month regimen (129, 130).

When comparing results across studies (Table 20 and Table 21) the prevailing background regimen might differ, which could affect the results. If fewer effective drugs are included in the background regimen, treatment with pyrazinamide might have a positive effect. The studies using the long regimen in the analysis of treatment outcomes (Table 20) all included five likely effective drugs, a fluoroquinolone, and a second-line injectable drug. Only linezolid of the newer drugs was given sparsely in the Korean (157) and PETTS study (158). In contrast, the newer drugs bedaquiline, delamanid, and linezolid could be included in the 18-month control regimen in MDR-END trial if resistance was found to injectables or intolerance. In **study II**, linezolid treatment differed between the groups and was included for 30.0% and 51.6% of participants who were with and without an effective pyrazinamide-containing regimen (data not shown in the manuscript), respectively. However, treatment with linezolid was not associated with the outcome of sputum culture conversion in our analysis and hence, it was not retained as a confounder in the multivariable analysis. In the larger study from Peru analysing death as the outcome, four likely drugs (pyrazinamide likely not effective) were compared with five likely drugs showing a positive effect (163). Therefore, fewer drugs than five might be the underlying reason for their results.

One difference when older regimens are analysed like both Peruvian studies (161, 163), was that only ciprofloxacin and ofloxacin were prescribed and not the later generation fluoroquinolones levofloxacin and moxifloxacin which have shown a higher efficacy (115, 243). This could be one reason why pyrazinamide with or without ethambutol had a positive effect on treatment outcomes. Regarding the study from China by Zheng *et al.* (162), participants were given levofloxacin, and although dosing was not reported we know from **study III** that the dosing of levofloxacin according to national Chinese protocols is low (typically 500 mg daily). The low levofloxacin dose might explain the positive association between pyrazinamide treatment and both a more rapid sputum culture conversion, and a successful treatment outcome. Furthermore, the participants received para-aminosalicylic acid as one of five drugs, a drug which is considered to

have a low effect (43, 115). In the Karakalpakstan study (**study I**), ofloxacin was also prescribed to 64.4% (n=549/852) of participants in the 2003-2009 programme. However, none received ofloxacin from 2009 and onwards but were instead given levofloxacin or moxifloxacin. In **study II**, only 5.2% percent of participants received ofloxacin which did likely not affect treatment outcome (164). Thus, it is important to study accompanying drugs in detail when evaluating the efficacy of pyrazinamide on its own.

The notion that the number of drugs in a regimen is important for treatment outcome has been evaluated in the individual meta-analysis by Ahmad and co-authors (115), which showed that five drugs in the intensive phase and four in the continuation phase were optimal using the long MDR-TB regimen for lower risk of death and higher success rates. The current recommendation by WHO for a long MDR-TB regimen is a minimum of four effective drugs initially (43) (Table 6), however, before 2018 WHO also recommended five drugs in the intensive phase. In **study I**, 83.0% of participants had five to six likely effective drugs in their regimen, which might make pyrazinamide redundant, especially if high rates of background resistance are present. However, in the two studies from China (162) and Peru (161) as well as in the control regimen in MDR-END (135) where a positive association between pyrazinamide susceptibility and success was seen, the included participants had five drugs in the intensive phase. In **study II**, the median number of effective drugs was 4 (IQR 4-5) over the whole length of treatment (164) but the maximum number of drugs was 5 (IQR 4-5) (data not shown in manuscript) which was similar in participants with and without an effective pyrazinamide treatment. Although the number of drugs seems to be important when designing a long MDR-TB regimen, the efficacy of individual drugs should also be considered since the ZeNix trial achieved high rates of success using only three drugs (134).

The limitations of our analysis include confounding by indication which was already described in the methods section for **study I** (Section 4.7.2 on confounding) and above in the discussion. However, confounding by indication might be a larger issue in the Swedish cohort (**study II**). Since treatment with pyrazinamide was individualised, pyrazinamide was given for longer periods to people with pyrazinamide-susceptible MDR-TB isolates (median 466 days (IQR 101-598) compared to zero (range 0-22.5 days [data for those with resistant isolates not shown in manuscript]). Hence, we divided the groups differently compared to **study I**. Instead, we compared participants without effective pyrazinamide treatment (having a resistant isolate or having a susceptible isolate and receiving <30 days of pyrazinamide treatment) compared to those with effective pyrazinamide treatment (pyrazinamide treatment ≥30 days in persons with a susceptible isolate). These groups were in general overall similar as seen in the supplement of the manuscript of **study II** (165). Another limitation in **study I and II** was

that we only analysed actual drug treatment and no account was made for drug exposure since pyrazinamide concentrations were not measured. In one modelling study based on data from South Africa (n=61), up to 32% of persons treated with pyrazinamide 35 mg/kg, which is similar to current WHO recommendations in MDR-TB (44), failed to achieve the suggested target of AUC_{0-24h} of 343 mg×h/L, especially in persons weighing <50kg (289). Furthermore, in a meta-analysis, low pyrazinamide concentrations were seen in 5-39% of participants (mainly measured by C_{max}) with low pyrazinamide exposure (C_{max}) associated with an unsuccessful treatment outcome (225).

Another limitation in **study I** is how pyrazinamide resistance was measured, as only phenotypic DST was used. Phenotypic DST for pyrazinamide could result in false positive resistance (62, 83) which has been described in <5% of cases (62). However, the supranational TB laboratory in Borstel initially performed pyrazinamide DST in **study I**, and it was only introduced routinely in 2012 when the programme had already had time to get familiar with using MGIT (started in 2007) (for further details see Section 4.3.2 on culture methods). If misclassification of pyrazinamide resistance occurred, it might dilute our results and underestimate the OR, but the effect is likely small.

The synergy between different drugs is another aspect to consider when analysing the effect of pyrazinamide treatment in **study I and II**, since the effect might depend on which other drugs are included in the regimen. Pyrazinamide has shown synergistic effects with rifampicin (116) in the early clinical trials and animal models with bedaquiline (290). Since only bedaquiline was given to a minority of participants in the Swedish cohort in **study II** (1.9% (n=3/157) received bedaquiline, data not shown in manuscript) and none in **study I,** it is unlikely that any synergistic effect influenced our results, unless other drugs have an unknown synergistic effect. The reason bedaquiline was not given to more participants in **study I** and **study II** was that participants were included earlier than the drug was introduced in Sweden and Uzbekistan. If pyrazinamide would be added to regimens including bedaquiline it may be advantageous and lead to more than an additive effect, which remains to be explored.

The impact of pyrazinamide treatment might also be affected by the mechanism of action of the other included drugs, e.g., which bacterial population they target. If several highly sterilising drugs are added such as a later generation fluoroquinolone, bedaquiline, delamanid, or clofazimine (291), pyrazinamide might be redundant. Indeed, as already mentioned above, the trial regimen in MDR-END consisting of delamanid, linezolid, levofloxacin, and pyrazinamide had similar rates of success independently of pyrazinamide DST (135). Therefore, pyrazinamide treatment needs to be evaluated further when newer drugs are given in combinations such as in the current recommended long MDR-TB regimen which includes bedaquiline, linezolid, and a fluoroquinolone (43).

Some authors have speculated if pyrazinamide has an effect even if DST shows resistance due to its synergy with other drugs or due to false positive pyrazinamide phenotypic DST (163, 167). Therefore, we performed a sensitivity analysis in **study I** of the effect of different lengths of pyrazinamide treatment in an MDR-TB regimen in persons having a pyrazinamide-resistant *M. tuberculosis* isolate, however, no evidence for a difference in effect was seen. Other possible reasons for an effect of pyrazinamide despite phenotypic resistance are hetero-resistance or low-level in vitro resistance which might be overcome by high-level drug concentrations at the infecting site (167).

Although pyrazinamide treatment has relatively low toxicity compared to other drugs, pyrazinamide was withdrawn in about one in 10 persons when treated for MDR-TB due to toxicity in a meta-analysis (138). Furthermore, in the STAND trial, which was halted early due to possible higher toxicity and lower efficacy, participants receiving the experimental regimen with pretomanid, moxifloxacin, and pyrazinamide during four to six months led to 33.3% having a grade three or higher adverse drug reaction (292). Therefore, considering the high rate of resistance in MDR-TB isolates (60.5% globally (81)) when pyrazinamide likely has no effect and additionally, when it must be withdrawn due to toxicity, other drugs with high sterilising effect are needed.

It is not known if the effect of pyrazinamide treatment is mainly early in treatment regimens in MDR-TB, in analogue to DS-TB, to achieve a more rapid sputum culture conversion but also to reduce relapse and death. If this is true, one option could be to withdraw pyrazinamide after sputum cultures have become negative to decrease pill burden and lower the risk of toxicity. Considering that time to sputum culture conversion is longer in MDR-TB compared to DS-TB, pyrazinamide treatment would also be longer than the two months in DS-TB. In the Swedish cohort (**study II),** the median time to sputum culture conversion was 1.5 months (IQR 0.4-2.8, n=99), while the median time was 3.0 months (IQR 2.0-5.0) in a multi-centre study analysing predictors of sputum culture conversion (293). Therefore, giving pyrazinamide for at least two to six months could be one way forward (294), unless other drugs with highly sterilising activity are included such as high-dose rifampicin or bedaquiline (291). Interestingly, in a mouse model, pyrazinamide showed a sterilising effect beyond two months when given together in a four-drug regimen including moxifloxacin or levofloxacin (295). Furthermore, in the MDR-TB studies mentioned above that showed evidence for an effect of pyrazinamide treatment and treatment outcome in MDR-TB (135, 161-163), pyrazinamide was given throughout treatment. Considering these results, further evaluation is needed of the optimum length of pyrazinamide treatment.

Together, these results appear to show that effective pyrazinamide treatment has a role in MDR-TB treatment to shorten the time to sputum culture conversion and is likely to reduce death and increase treatment success, and potentially reduce relapse. Worth noticing is that most studies discussed evaluated the effect of pyrazinamide treatment

without the newer drugs except for the MDR-END trial (delamanid, linezolid) (135), the study from China (bedaquiline, linezolid) (162), and in **study II** where linezolid was included. Despite a high rate of resistance in drug-resistant *M. tuberculosis* isolates and more to elucidate about the mechanism of action (see Section 2.4.4 on whole genome sequencing), pyrazinamide seems to have a special role in treatment despite the development of new and effective drugs. Furthermore, in several trials, pyrazinamide is the drug that is most strongly associated with interim or end-of-treatment outcomes (231, 232, 234) and low drug exposure to pyrazinamide was associated with an unsuccessful treatment outcome in DS-TB (225). It is also chosen to be part of an optimal regimen developed by artificial intelligence (296). Therefore, it is not surprising that pyrazinamide is contained in several novel regimens under evaluation such as the SimpliciTB trial investigating the combination of bedaquiline, pretomanid, moxifloxacin, and pyrazinamide for both DS-TB (4 months) and MDR-TB (6 months; ClinicalTrials.gov Identifier NCT03338621).

6.2 Optimising pyrazinamide – whole genome sequencing as a rapid marker of pyrazinamide resistance?

Detecting pyrazinamide resistance by genotypic DST could be one way forward to increase access to resistance testing in low-resource settings as current liquid culture-based methods are frequently unavailable (275). However, before implementing a new method that is costly, evidence from studies showing that pyrazinamide genotypic DST has a clinical impact on treatment outcomes is important.

In **study II,** when pyrazinamide resistance was based on genotypic DST, we found strong evidence (HR 2.04, 95% CI 1.22-3.45, p=0.007) that effective pyrazinamide treatment was associated with a shorter time to sputum culture conversion among persons treated for MDR-TB in Sweden. These results are similar to previous studies from China (162) while the study from Bangladesh showed no difference (166) (see Table 21 for both studies), although there was no information in the latter study regarding what analysis was conducted and if adjustments were made for potential confounders.

The challenges in interpreting the results of genotypic DST for pyrazinamide are that mutations with unclear association to resistance exist and it is not fully elucidated which genes confer resistance (54, 82). We included mutations in all three genes that are possibly associated (at the time of the study) with resistance in **study II**, namely the *pncA*, *rpsA*, and *panD* genes (56, 57, 89, 297), although the latter two are believed to be rarely detected (<5%) (92). In comparison, the study from China (162) and Bangladesh (166), only included mutations in the *pncA* gene. However, in **study II** no isolate had a mutation in *rpsA* or *panD* without a *pncA*-mutation that defined that isolate resistant (Supplement Table 8 in the manuscript of **study II**). The risk of also including mutations in genes with less established certainty of resistance could be false resistance and thus

reduce specificity. To mitigate false resistance, we only included mutations that were highly likely or very highly likely associated with resistance as described in studies at the time of our study (75, 80, 89, 272) (see Section 4.5.3. on pyrazinamide resistance).

There are different ways of interpreting pyrazinamide resistance mutations and in **study II** we used an in-house dataset curated by the supranational reference laboratory in Sweden at the Public Health Agency which was based on established mutations at the time of the study (75, 80, 89, 272). In contrast, the Chinese study (162) used the online platform DNAStar Lasergene (DNASTAR, Inc., Madison, WI) (162), and the Bangladesh study did not specify how they interpreted possible pyrazinamide resistance mutations (166). The same online platform (DNAStar Lasergene) as in the Chinese study (162) was evaluated in a study from West Africa showing a 70.0% sensitivity and 96.6% specificity to detect pyrazinamide resistance compared to phenotypic DST, which was low (298). However, when other online platforms have been evaluated (299), the sensitivity for automatic pyrazinamide resistance detection for TB profiler (93) was 60.1% (specificity 100%) and TGS-TB 97.2% (300) (specificity 98.8%). Although these platforms are designed to be used without extensive knowledge in bioinformatics, the sensitivity for TB profiler improved to 94.4% by manually viewing the included output data and interpreting all non-synonymous mutations, insertions, and deletions as conferring pyrazinamide resistance (299). However, the limitations of this approach are that the benefit of an automated system is not met and that not all non-synonymous *pncA* mutations are categorised as resistant compared to phenotypic DST to resistance (84, 299). Therefore, the usefulness of online platforms seems to be varied and more development is needed.

A problem with evaluating genotypic DST for pyrazinamide is the lack of a robust gold standard to evaluate it against since false resistance could occur using phenotypic DST (55, 62). Therefore, we developed a consensus definition of pyrazinamide resistance in **study II**. When evaluating genotypic and phenotypic DST to this method we found that 7.6% (n=12/157) of isolates needed further investigations. One example is the five isolates that were defined as genotypic resistant with a high or very high confidence *pncA* mutation but that had a susceptible phenotypic DST. When MIC was performed, three isolates were found to be resistant (MIC 128 to >128 mg/L) while two were classified as overall susceptible (MIC 100 mg/L). A possible explanation is that low level resistance was present, and the discrepant results were related to a cut-off problem (see Section 2.4.5 on breakpoints to define resistance). Since the isolates had MICs near the critical concentration for pyrazinamide of 100 mg/L, the critical concentration might not divide the resistant and susceptible populations in a clear manner, i.e., a cut-off problem. The current critical concentration for pyrazinamide has been question, instead a cut-off of 64 mg/L has been suggested for pyrazinamide susceptible isolates using MGIT (301). Other possible explanations for the discordant results may be hetero resistance or that

the mutations are challenging to classify as resistant or susceptible since data on each mutation is scarce (54). However, overall, we found 2.9% (n=4/148) misclassifications using genotypic DST compared to our composite standard (**study II**). Despite these challenges of performing genotypic and phenotypic DST for pyrazinamide for MDR-TB isolates, our results showed a low level of misclassification and genotypic DST has a potential to resolve some of the challenges around performing and interpreting phenotypic DST, at least if established resistance mutations are used.

The sensitivity and specificity of our genotypic DST to detect pyrazinamide resistance in **study II** compared to the composite definition were 97.6% and 97.0%, respectively. When evaluating the genotypic DST against phenotypic DST, sensitivity was 97.5% and specificity 92.7% (data not shown in the manuscript). In other studies, the sensitivity of genotypic DST to detect pyrazinamide resistance compared to phenotypic DST varies and in the Bangladesh study, 90.3% sensitivity and 76.7% specificity were reported (166). Although not specified in the Bangladesh study, it seems that they interpreted all non-synonymous single nucleotide polymorphisms (SNPs), in the *pncA* gene as resistant (see Section 4.5.3 on pyrazinamide resistance), which might explain their result of low specificity. However, the authors suggested that low level resistance and hetero resistance could be one explanation (166). The Chinese study by Zheng et al (162), did not provide sensitivity and specificity. Up to 30% of strains without any mutation associated with pyrazinamide resistance have been found to test phenotypically resistant, as mentioned in the introduction (297). Variability of the sensitivity and specificity for genotypic DST compared to phenotypic DST could be due to several aspects such as; local transmission of different variants, sequencing techniques; definitions used in interpreting mutations; and proficiency in performing phenotypic DST for pyrazinamide (54, 55, 297, 302).

The problem with the *pncA* gene is that it contains multiple mutations and is also associated with lack of solid validation data to phenotypic DST. This poses a challenge to keep any online platform or in-house resistance definition up to date. WHO has published a catalogue of mutations which could aid in interpretations (54). In the WHO catalogue, the sensitivity of genotypic pyrazinamide resistance using their definition was 72.3%, if the two groups with the highest association with resistance were considered, which is low compared to 93.8% sensitivity for rifampicin. Since new mutations in the *pncA* gene are often found, WHO proposes a pragmatic approach to define pyrazinamide resistance (54). Interestingly, they suggest that pyrazinamide resistance should be assumed for any new non-synonymous mutation found in the *pncA* gene not classified in their catalogue in clinical practice, for strains with rifampicin resistance. This is since the positive predictive value of the pyrazinamide resistance mutations depends on the prevalence which is higher for rifampicin resistance (272). Another approach to classifying pyrazinamide resistance in clinical practice was proposed by Köser and

colleagues (303). They divided mutations into five groups and for mutations associated with pyrazinamide resistance (according to their classification), no further tests were needed. However, for the other four groups, e.g., mutations likely associated with pyrazinamide resistance, phenotypic DST should be performed (303).

Since the sensitivity of pyrazinamide resistance based on the WHO catalogue was low at 72.3% (54) and the method provided by Köser *et al.* (303) suggested phenotypic DST in the absence of a mutation associated with pyrazinamide, it seems likely that phenotypic DST will still have a role in the diagnosis of pyrazinamide resistance. Moreover, the pyrazinamide mutations currently reported need further characterisations since the critical concentration used in phenotypic DST for pyrazinamide might falsely classify mutations as susceptible or resistance (55). New mutations found in the *pncA*-gene or other genes also need evaluation against a golden standard. Ideally, each mutation should be characterised by its MIC, since some mutations might lead to low-level resistance. This is seen with mutations in the *inhA*-gene for isoniazid where low-level resistance could be overcome by increasing doses (304). One option is to use the newly implemented S-I-R system implemented by EUCAST for other bacteria in which the "I" stands for susceptible, increased dosing (95). The same system could be implemented for each mutation in *M. tuberculosis*, however, WHO does not recommend testing at a higher intermediate concentration (53). Furthermore, there is a challenge in setting clinical breakpoints (S-I-R system) in TB since WHO base the clinical breakpoints on expert opinion in combination with MIC distributions (53). In the future, clinical outcome, PK-PD data, MIC-distributions should be considered for TB, similar as for all other bacteria. There is no data on a clinical breakpoint for pyrazinamide (95) and WHO only provides critical concentrations which are equal to ECOFFs (53), therefore, much work is still needed.

The benefit of using genotypic DST compared to phenotypic DST is simultaneous testing of all drugs and a lower biosafety risk as no biohazard 3 laboratory is needed. Time is also an important aspect and if genotypic DST based on whole genome sequencing is conducted directly on microscopy positive samples, results can be obtained within 1-2 weeks (305, 306). Additionally, more genes can be tested with whole genome sequencing compared to using currently available rapid molecular tests, such as the line-probe assays (GenoType MTBDRsl assay (71)) which at most test rifampicin, isoniazid, fluoroquinolones, and injectable drugs. Although phenotypic DST is cheaper, the methodology of genotypic DST is evolving and will likely become cheaper and more feasible in the future (306). Furthermore, the recommendation by WHO is that the detection of resistance mutations in the *pncA* gene should be the reference method for pyrazinamide resistance testing (53).

Performing whole genome sequencing directly on microscopy-positive sputum is possible, although the current methodology is labour intense with challenges, especially

related to DNA extraction (306, 307). A study from India using an enrichment technique to improve DNA extraction showed an overall sensitivity of 90.9% for resistance testing for seven drugs (pyrazinamide not included) compared to phenotypic DST (306). Another option is targeted next generation sequencing (i.e., Deeplex Myc-TB [Genoscreen, Lille, France]) directly from microscopy-positive sputum. The promising results of a study using the Deeplex Myc-TB that amplifies 18 selected genes associated with resistance including *pncA* (308), reported results within three days and an overall 93.6% concordance with phenotypic DST, including pyrazinamide (308).

It seems clear that genotypic methods for detecting resistance for all drugs including pyrazinamide in *M. tuberculosis* are here to stay as they are more rapid and show signs of improvement in performance with time. Our results in **study II** which showed that effective pyrazinamide treatment is associated with sputum culture conversion, considering genotypic pyrazinamide DST, further support this statement. An important aspect is the development of techniques to analyse genotypic DST including pyrazinamide from sputum that is microscopy negative. This would increase the utility of genotypic DST and enable a more rapid decision on effective regimen composition including pyrazinamide, for more people falling ill with TB. Therefore, it is hopeful to see that new techniques are developed such as the Deeplex Myc-TB, which could make genotypic DST simpler, faster and more accessible in high-incidence settings (308).

6.3 Optimising fluoroquinolones – should we increase doses?

Fluoroquinolones are key drugs in MDR-TB regimens and are included in all regimens recommended by WHO (43), and resistance to fluoroquinolones have been associated with worse treatment outcomes (115). Therefore, using fluoroquinolones for MDR-TB wisely regarding dosing, accompanying drugs, and reducing toxicity is impertinent. In this section, the dosing of fluoroquinolones will mainly be discussed.

In **study III**, we evaluated the free fraction of levofloxacin and moxifloxacin ($AUC_{0\text{-}24h}$) over the level of resistance (MIC) in MDR-TB against suggested targets for antimicrobial kill and development of resistance. We found that none and only 60–73% of participants in our study in China achieved these targets when treated with levofloxacin and moxifloxacin, respectively. Participants were treated with a dose of 500 mg levofloxacin once daily which is lower than the currently recommended dose (since 2020) by WHO of 750–1125 mg (about 20 mg/kg) once daily based on weight (44). Previously, 10–15 mg/kg (750–1000 mg in weight bands) were recommended from 2014 (121), and 7.5–10 mg/kg (750–1000 mg) from 2008 for persons weighing >33 kg. For moxifloxacin, the standard dosing was given with 400 mg once daily in **study III,** according to the WHO recommendation since 2008 (122). However, in 2020, the WHO included an option to increase moxifloxacin dosing to 600–800 mg in certain cases as already described (309).

In an additional study by our research collaborators in China by Zheng *et al*. (n=197) described in the introduction (Section 2.9 on TDM) which evaluated each drug target against sputum culture conversion and treatment success, a similar proportion of participants reaching the targets for levofloxacin was seen (231). In participants treated with levofloxacin, only 14.1% achieved the suggested free fraction target AUC_{0-24}/MIC for antimicrobial kill of 160 derived from hollow-fibre models (228) using levofloxacin 500 mg once daily (see Section 2.9.1 on PK-PD targets). However, 87.3% of participants reached the free fraction AUC_{0-24}/MIC target (228) of 56 for moxifloxacin (231). Furthermore, our results were similar to a recent South African study (n=131, 60.3% of participants were living with HIV) were 64% of participants who were treated with moxifloxacin 400 mg once daily had a free fraction AUC_{0-24}/MIC above the suggested target of 53 (310). Furthermore, in the South African study, among those who received efavirenz as part of their HIV treatment, only 24% reached the suggested target for moxifloxacin (310).

Although the targets for moxifloxacin of AUC_{0-24}/MIC 42, 52, and 56 are different (Table 11), considering the methodological variability in performing MIC of plus or minus one dilution step (100), the targets are comparable. Moreover, the most commonly cited targets for levofloxacin which were also used in **study III** (AUC_{0-24}/MIC of 160 and 360) (188) seems markedly different, however, they are just above the methodological variability of MIC testing.

Dosing of levofloxacin in other studies is usually higher than the 500 mg given in **study III**, therefore, higher AUC_{0-24}/MIC has been reported. In a study from Nepal (n=12), 67% of participants reached the free fraction target of 146, using levofloxacin 750-1000 mg daily (244). Despite higher doses, a population PK modelling study from Ethiopia using actual MICs and sampling of a full PK curve reported that only 29% (using 750 mg) and 62% (using 1000 mg) of participants reached the free fraction AUC_{0-24}/MIC target of 146 (311). Therefore, a wide variability of target attainment is seen and the relevance of targets attainment in different settings and populations with various comorbidities needs further evaluation. Moreover, although the moxifloxacin targets are comparable, evaluating both targets (AUC_{0-24}/MIC of 160 and 360) for levofloxacin might be important.

A limitation of **study III** was its small size (n=32) which was due to that it was planned as a pilot study and the high resources needed with a prospective study design. However, similar results with low target attainment were seen for levofloxacin in the larger prospective Chinese study mentioned above (231).

How to handle protein binding in PK-PD studies could also be important. Two options are either to directly measure the free fraction of a drug concentration or to derive the free concentrations from the total drug concentration. We used the latter and estimated

protein binding at 50% for moxifloxacin and 30% for levofloxacin in **study III** to calculate the free fraction of AUC_{0-24}/MIC. The derived targets from the hollow-fibre models which estimate the concentration at the active site in the lungs (epithelial lining fluid) (215, 228) are for the unbound drug since protein binding in the epithelial lining fluid is negligent (312). The estimated protein unbound fractions were from the drug insert package for each drug at registration (280, 281) which are similar to other studies (310). However, the larger recent study from China by Zheng *et al*. analysed the total AUC_{0-24}/MIC (personal communication with Xubin Zheng 19 April 2023) against targets of the free fraction, which could have affected their results, especially for moxifloxacin if a 50% protein binding was used. Similarly, a recent multicentre study (n=290) by Heysell and colleagues described in the introduction (Section 2.9 on TDM) that evaluated PK-PD targets against treatment outcomes, compared the total AUC_{0-24}/MIC to targets of the free fraction AUC_{0-24}/MIC (232).

If we recalculated our results in **study III** and instead used the lower bound of the range of levofloxacin protein binding (range 24-38%) (281) (24% instead of 30%), the median free fraction would be AUC_{0-24}/MIC 48.4 (data not shown in manuscript), compared to 45. However, still, no participant would reach the suggested free targets of 146 or 360 for antimicrobial kill and suppression of resistance (228). In contrast, if we changed the protein binding of moxifloxacin to 30% (range 30-50% (280)), a median free fraction AUC_{0-24}/MIC of 101.5 would be found (data not shown in manuscript in **study III**), instead of 73. Using this new value, 80% of participants would reach the target of the free fraction AUC_{0-24}/MIC of 42 and 53 (215, 228) for antimicrobial kill and suppression of resistance, respectively. This highlights the importance of considering protein binding in PK studies, at least for moderately or highly protein-bound drugs. Moreover, protein binding can vary in populations since it can decrease in persons who are severely ill or in those with renal disease (211) (Table 10). Furthermore, it is important to consider which study, e.g., hollow-fibre study, animal model or from a clinical study, the targets were derived from since protein binding is not present in hollow-fibre studies (unless human plasma is used) but relevant in animal and clinical studies. However, if enough evidence from in vitro and clinical studies shows an association between a specific target and efficacy or a clinically relevant outcome, it might be acceptable to use these targets in clinical care. Thus, the variation of studies with different populations, *M. tuberculosis* isolates, protein binding, drug-drug interactions, and background regimens might be of less importance.

There seems to be confusion or at least a lack of reporting in some studies and overview articles concerning the specification if a suggested target is the total or the free fraction AUC_{0-24}/MIC (311, 313). Other studies compare the total AUC_{0-24h}/MIC to the free fraction AUC_{0-24h}/MIC which was mentioned above (231, 232). It would be useful if a more detailed description of whether the total or free fraction concentrations are reported

since these values could vary substantially and, therefore, preclude comparisons between studies.

A limitation of **study III** is that we imputed the MICs at the ECOFF (1.0 mg/L) for all participants who had a fluoroquinolone susceptible *M. tuberculosis* strain and received levofloxacin. The imputation was done since levofloxacin was not included in the broth microtiter plate used (MYCOTB (65)) (See Section 4.3.3.1 on broth microdilution plates). We used a conservative approach when imputing the MICs since the ECOFF is the highest MIC in a Gaussian curve of fully phenotypically susceptible *M. tuberculosis* strains for levofloxacin (53). Furthermore, the normal variability of MICs is at most one dilution step up and down due to the methodology, which might have affected our results (100). However, only if all strains in **study III** had a levofloxacin MIC of ≤0.125 mg/L would 90% of participants reach the target of the free fraction AUC_{0-24}/MIC ≥146. Detecting such low levofloxacin MIC of ≤0.125 mg/L in all strains is unlikely since the MIC mode was 0.5 mg/L in the Chinese study by Zheng *et al.* (231) and it was found in <2% of wildtype *M. tuberculosis* strains in the WHO report (53). Furthermore, we reported a low free fraction AUC_{0-24} of 44.6 mg×h/L for levofloxacin in **study III** compared to the suggested AUC_{0-24} of 100–150 mg×h/L (188). This suggests that the more likely explanation for the low target attainment for levofloxacin in **study III** is low exposure due to a subtherapeutic dose of 500 mg instead of the imputed MICs.

Although these suggested free fraction AUC_{0-24}/MIC targets towards which we evaluated our results in **study III** are derived from qualified hollow-fibre models (214), further validation of the targets is needed in clinical studies. Importantly, this has been conducted in two larger prospective studies in the last year already mentioned, although slightly different targets were used and the total AUC_{0-24}/MICs were reported (231, 232). In the Chinese prospective multi-centre study by Zheng *et al.* (n=197) (231), a total AUC_{0-24}/MIC above 160 for levofloxacin and 56 for moxifloxacin was associated with sputum culture conversion at two months as well as treatment success. The prospective multi-centre study (n=290) by Heysell and colleagues (232), showed that there was evidence for an association between participants attaining a total AUC_{0-24}/MIC of the target 58 for moxifloxacin and favourable treatment outcome. Furthermore, for participants reaching a levofloxacin AUC_{0-24}/MIC target of 118, an association was seen with a shorter time to sputum culture conversion. Interestingly, in the larger study from China by Zheng *et al*. (231), treatment was given with an injection-free five-drug standard regimen using both bedaquiline and linezolid, highlighting the continuously important role of fluoroquinolones together with newer drugs. In comparison, most participants were treated with an injectable-based regimen in the study by Heysell *et al*. (232).

The dosing of levofloxacin at 500 mg daily was low in **study III** compared to the prevailing (314) and previous WHO guidelines (122) since this was the recommended

dose according to the National Chinese guidelines at the time of the study in 2016-2018 (260). In contrast, moxifloxacin dosing followed previous and current WHO guidelines in **study III** using the standard long regimen of 400 mg once daily. One reason for the lower dosing of levofloxacin was the anticipated risk of adverse drug reactions (231). However, serious adverse drug reactions of fluoroquinolone treatment compared to other TB drugs are infrequent as depicted in a meta-analysis (138). Only 2.9% of persons receiving moxifloxacin and 1.3% receiving levofloxacin had to stop the drug due to adverse drug reactions. However, the European Medicinal Agency has warned against the unnecessary use of fluoroquinolones as described in the introduction, due to adverse drug reactions related to musculoskeletal events such as tendon rupture, and cardiac and psychiatric issues (150). Cardiac events including arrhythmias and aortic aneurysms have been described in large population-based studies, although a very low absolute risk increase was seen (e.g., an increase of cardiac arrhythmias was 0.2 per 1 million levofloxacin treatment episodes) (153, 154, 315).

The participants in **study III** received a levofloxacin dose with a median of 10 mg/kg, which is substantially lower than the WHO currently recommended dose that equates to about 20 mg/kg for adults (range 14-25 mg/kg depending on weight band) (44). To further evaluate efficacy and safety using different levofloxacin doses, the results from the OPTI-Q study are much awaited comparing increasing doses from 11 to 20 mg/kg of levofloxacin (174). For moxifloxacin, WHO gives the option to give a higher moxifloxacin dose of 600 mg (from 30kg) or 800 mg (from 46 kg) if low exposure might be anticipated, e.g., due to interaction with other drugs, or if low-level resistance is detected (44). The toxicity of moxifloxacin was evaluated in TB treatment in a meta-analysis from 2020 which showed a similar risk for adverse events to levofloxacin, however, no evaluation of >400 mg doses of moxifloxacin was conducted (316). Furthermore, cardiac and musculoskeletal toxicity were not evaluated. Two randomised controlled trials using moxifloxacin 600-800 mg (of which one used the shorter MDR-TB regimen and one treated TB meningitis) reported that moxifloxacin was withdrawn in 5% of participants due to QTc prolongation (129, 251). Moreover, in a retrospective observational study from India (n=354), moxifloxacin 600 mg daily compared to 400 mg was associated with an increased risk of joint pain (317). However, drug concentrations or doses in mg/kg were not reported (only BMI) so it is uncertain if drug exposure or dose based on weight was associated with toxicity.

The hollow-fibre studies which developed the suggested targets for levofloxacin and moxifloxacin (215, 228), predicted that a dose of levofloxacin 1500 mg or 25 mg/kg and a moxifloxacin dose between 600 and 800 mg were needed for 90% of persons to reach the targets. Similar results were seen in the Ethiopian population PK study already mentioned (311), which suggested that at least 1500 mg levofloxacin and 600 mg moxifloxacin were needed for about 95% of participants to reach the PK-PD targets.

Although this study evaluated total concentration to the free fraction targets of AUC_{0-24}/MIC, which would have underestimated their results. Even if the predicted effective doses of moxifloxacin and levofloxacin from the hollow-fibre models are based on single drug use of the fluoroquinolones, similar predicted doses were found in the modelling study from Ethiopia (311) which was based on clinical samples. However, one cannot rule out that the concomitant use of other drugs throughout treatment would affect the importance of these targets, i.e., use of the new 6-month treatment regimen with bedaquiline, linezolid, pretomanid, and moxifloxacin (113). Furthermore, few studies report acquired fluoroquinolone resistance using the recommended WHO doses (158, 318), instead, it has been proposed that the overall use of fluoroquinolones for other bacterial infections might have a larger impact on resistance development to fluoroquinolones in *M. tuberculosis* as seen with other bacteria such as Escherichia coli (319, 320).

From a clinical perspective, not only dose but also choosing which fluoroquinolone to use could be important for toxicity. As seen in the meta-analysis, adverse drug reactions associated with moxifloxacin are dominated by cardiac toxicity while for levofloxacin musculoskeletal toxicity is more frequent (138). Furthermore, in contrast to levofloxacin (281), moxifloxacin does not need to be adjusted in renal insufficiency (280) which is why it is preferred in those with renal disease.

The results of **study III** showed that a low proportion of participants reached the suggested PK-PD targets, especially for levofloxacin dosed at 500 mg daily. These results are in line with other recent studies using similar doses (231, 310). Importantly, WHO is recommending a higher levofloxacin dose of 750-1150 mg and moxifloxacin could be increased from 400 mg to 600-800 mg for persons with a risk of underdosing or in those with low-level fluoroquinolone resistance (44). Interestingly, higher dosing of levofloxacin (1500 mg) and the now WHO-recommended dose of moxifloxacin (600-800 mg) have been suggested in modelling studies. To evaluate these higher moxifloxacin doses, studies evaluating the efficacy and adverse drug reactions in combination with PK parameters in TB would be useful.

6.4 Optimising linezolid – can we minimise toxicity?

Already before 2018, when linezolid was included as an optional repurposed drug to be used in MDR-TB, it was known that treatment with linezolid was associated with toxicity (144, 321). It was clear that treatment longer than the approved 28 days (140) increased the risk of toxicities (146, 236, 321), however, effective drugs for MDR-TB were desperately needed. Linezolid as a drug in MDR-TB treatment has shown high efficacy in multiple studies including the individual patient data meta-analysis (115) and is included in the new six-month full oral regimens now endorsed by WHO (43). However, adverse

drug reactions still remain a major concern and the question is how we can mitigate adverse drug reactions while still including the efficacious drug in MDR-TB regimens.

In **study IV,** we showed that 55.3% of people treated with linezolid in a low incidence setting in Sweden had an adverse drug reaction related to linezolid treatment, of which peripheral neuropathy (35.6%) was mostly common followed by anaemia (27.3%), leukopenia (22.0%), thrombocytopenia (14.4%), and optic neuropathy (6.1%). Furthermore, in 47.7% of cases, linezolid was withdrawn due to any adverse drug reaction. The main finding of **study IV** was that a dose of ≥12 mg/kg/day was associated with peripheral neuropathy, anaemia, and leukopenia. Moreover, a dose of ≥600 mg was associated with peripheral neuropathy. In an exploratory univariable analysis, a trough concentration of ≥2.0 mg/L was associated with anaemia, and thrombocytopenia.

Although previous studies have evaluated adverse drug reactions with linezolid treatment, it is often analysed as one entity instead of each adverse drug reaction separately. Mitochondrial toxicity is thought to be the main mechanism behind all adverse drug reactions we analysed (peripheral neuropathy, optic neuropathy, anaemia, thrombocytopenia, leukopenia, and elevated lactic acid) (see Section 2.6.3 on adverse drug reactions and Figure 3) (145, 146). However, the timing until the occurrence of each adverse drug reaction has been different in previous studies which warranted a separate analysis of both time and risk factors with each adverse drug reaction (141, 236).

6.4.1 Frequency of adverse drug reactions

Our results of the frequency of adverse drug reactions in **study IV** were similar compared to two meta-analyses (144, 322) which included studies from mostly South-East Asia, Europe, and the USA, while no studies were from Africa. Likewise, comparable frequencies of all linezolid-associated adverse drug reactions were seen in a recent study from France by Eimer *et al.*, except that myelotoxicity (anaemia, leukopenia, and thrombocytopenia) was lower (11% compared to 38.6% in **study IV** [data not shown in manuscript]) (238). Moreover, comparable frequencies were seen in a prospective study from South Africa by Wasserman *et al.* (237) and a recent study from South Korea by Kwon and colleagues (323), except for peripheral neuropathy which was higher at 49.3% (35.6% in **study IV**) in the latter study.

Since the evaluation of, if, and when, an adverse drug reaction occurs could be subjective and depend on multiple reasons such as the study population, definitions, and frequency of monitoring, this requires a more in-depth discussion. Another reason is healthcare provider factors which I will mention briefly here. Healthcare provider's training and experiences could be different depending on the setting which might lead to higher attention to detecting adverse drug reactions, resulting in a higher frequency

(324). In contrast, a weak support structure or healthcare provider fatigue due to work overload would likely result in a lower notification of adverse drug reactions.

The notion that the study populations are different is likely mostly seen when comparing the results of **study IV** with clinical trials. A lower frequency of adverse drug reactions was seen among participants in the ZeNix trial arm receiving linezolid at 600 mg daily for 26 weeks (n=45) (similar duration as compared to our study) (134). Among participants in the ZeNix trial, 2% experienced myelosuppression, none had optic neuropathy, and 24% experienced peripheral neuropathy. Although a clinical trial has a rigorous follow-up schedule and standardised monitoring protocols for detecting adverse drug reactions, the possibility that the study population is healthier due to strict inclusion and exclusion criteria could lead to lower frequencies of adverse drug reactions. For example, in the ZeNix trial participants were excluded based on albumin <30 g/L and BMI <17 kg/m^2, or unstable diabetes mellitus, while we included all persons with linezolid treatment in **study IV**. The fact that linezolid was withdrawn in only one (2.2%) out of 45 persons receiving six months of treatment in the ZeNix trial (134) is strikingly different from our study where linezolid was withdrawn from 47.7% of participants. Therefore, **study IV** (as well as the other observational studies) most likely better reflects a real-world population which frequencies one would expect in clinical practice.

Apart from a healthier study population, using different inclusion or exclusion criteria could also exclude participants who already had the outcome. In the French study by Eimer and colleagues, an inclusion criterion was that linezolid treatment had to be given for at least four weeks (238). The authors justify their decision by stating that adverse drug reactions are unlikely in the first month of treatment. However, the results of **study IV** show the opposite, with about 50% of persons experiencing myelosuppression within the first month. If participants who had myelosuppression in the first month had linezolid withdrawn, they would not have been included in the French study. Neither in **study IV**, the South African study (237) nor in the South Korean study (323) was a certain treatment length of linezolid an inclusion criterion. Likely, the frequency of peripheral neuropathy would be less impacted by such an inclusion criterion since the median time to its occurrence is longer (median 1.5-4 months) than myelosuppression (median 0.4-4 months) (141, 325-328). Thus, using the inclusion criterion of at least one month of linezolid treatment could introduce selection bias and be one explanation for that only 11% of participants had myelosuppression in the French study.

Various definitions of an adverse drug reaction can also affect the different frequencies seen in studies. In the study from France by Eimer *et al*. (238) a serious adverse drug reaction was used as the primary outcome. Only using serious events would lead to a lower frequency of events (39% of participants had any serious adverse drug reaction compared to 56.2% with any linezolid-attributable adverse drug reaction in the study by Eimer *et al.*).

To define peripheral neuropathy, we (**study IV**) used a clinical definition, similar to the French study (238) and the South Korean study by Kwon *et al.* (323), which could have underestimated our results. A clinical definition could entail multiple ways of diagnosing neurological symptoms such as performing a neurological examination, actively asking about adverse drug reactions in general or specifically about neurological symptoms, or passively letting a person report symptoms. If passive reporting or only asking about adverse drug reactions in general is practiced, persons treated for MDR-TB might not report neurological symptoms since the connection to treatment might not be clear, leading to an underestimation. Interestingly, a lower frequency of peripheral neuropathy (compared to 35.6% in **study IV**) was seen in the observational prospective cohort study from South Africa (20%) (237) and the ZeNix trial (24%, as already mentioned) when they instead used a validated clinical score protocol for peripheral neuropathy (BPNS, Brief Peripheral Neuropathy Screen (329)).

Regarding optic neuritis, we only reported confirmed cases by an ophthalmologist in **study IV** since 25% of participants receiving linezolid complained of visual disturbances at least once (blurred vision) compared to 6.1% that had confirmed optic neuritis. These results are similar to an Indian study (n=85) where 27.9% of participants had visual complaints (mainly blurred vision) while 5.8% had confirmed optic neuritis by an ophthalmologist (330). Furthermore, the frequency of optic neuritis is similar to the 9% found in the prospective study from South Africa (237) which instead used visual screening tests (e.g. logMAR score test (331)). It is noteworthy that none of the participants in the ZeNix trial (134) receiving 600 mg had optic neuritis (9% optic neuritis when 1200 mg was given for 26 weeks) which also used visual screening tests as the South African study. However, dose adjustments or withdrawal of linezolid were frequent (30-50%) when using the 1200 mg dose (compared to 13% using the 600 mg dose) which could have impacted the frequency of optic neuritis in the trial. These differences further highlight the different populations. Although different definitions could limit the possibility to compare studies, it seems that overall similar frequencies are reported.

Another aspect is the difficulty in how to reduce the risk of bias when defining an event. In **study IV**, we used the first occurrence of each adverse drug reaction which would lead to mostly grade one events (at least for myelosuppression). One could argue that it would be more interesting to evaluate serious adverse drug reactions (grade three) since this is what we would like to prevent as was analysed in the French study by Eimer *et al.* (238). However, there are two main limitations to using a serious adverse drug reaction as the outcome. Firstly, physicians might have stopped linezolid earlier due to a grade one or grade two event, which would underestimate the number of grade three events. To mitigate this first limitation, it seems the authors in the study by Eimer and colleagues (238), instead used a composite definition of severe linezolid-associated

adverse drug reactions. This composite definition also included any event leading to the withdrawal of linezolid by a physician (238). Since physicians at the time of the study had access to trough levels, although there was no clear guideline on how to interpret trough levels according to the manuscript, these concentrations could have guided their decision to stop linezolid (238). Therefore, secondly, another risk of bias of a bi-directional association arises when using their composite definition. Thus, using the first occurrence of an adverse drug reaction seems to be associated with less bias. However, another limitation with measuring the time to each adverse drug reaction as in **study IV** is a bias of competing risks. Once a person has experienced an adverse drug reaction and linezolid is stopped, they are no longer at risk for other events. Although most physicians did not stop linezolid due to myelosuppression (27.5%, n=14/51, data not shown in **study IV**), there is still a risk of bias which would likely underestimate the risk of peripheral neuropathy and optic neuritis which occurred later than myelosuppression. Despite these differences and limitations in defining outcomes and time to an event, all studies included in this discussion show similar results which strengthens the conclusions from **study IV.**

Dosing of linezolid is a major factor that affects the frequency of adverse drug reactions in studies since meta-analyses have shown a higher risk with higher doses (144, 322). A remarkably high proportion of participants experiencing peripheral neuropathy (81%) was seen in the prospective observational Nix-TB study (n=109), while myelosuppression was lower at 45% when linezolid was dosed at 1200 mg. In other studies, 600 mg was the most prescribed dose (141, 237, 238, 323). In **study III,** we showed that a linezolid dose >600 mg daily was associated with peripheral neuropathy. This result is not surprising considering the results of the Nix-TB study and several meta-analyses (144, 322). However, we had insufficient evidence to show an association between linezolid treatment >600 mg and anaemia, leukopenia, and thrombocytopenia. Likely, this was due to fewer events when each adverse drug reaction was analysed separately. Reducing the dose to 300 mg is often used as a strategy to reduce the risk of adverse drug reactions which was done for all in the PRACTECAL trial after 16 weeks (113) and is a strategy recommended by WHO in case of adverse drug reactions (44). In **study IV**, lowering the dose was also done with 96.1% having ≥600 mg as their first dose, while the last dose was ≥600 mg for 79.8% of participants (data not shown in manuscript). The limitations of reducing the dose will be discussed in Section 6.4.4. on dosing linezolid based on body weight.

HIV positivity has also been suggested as a risk factor, especially for neuropathy and anaemia. Toxicity could be due to toxicity of HIV itself or overlapping toxicities between linezolid and the HIV treatment or prophylactic treatment used in HIV care, i.e., zidovudine, stavudine and co-trimoxazole (44, 332). Although this seems plausible, studies have failed to show such a difference, i.e., in the South African study (237),

despite 63% of participants were living with HIV. Furthermore, no evidence was seen for a difference in adverse drug reactions due to linezolid by HIV status in the ZeNix trial (134), while the PRACTECAL trial did not report this subgroup analysis (113). Interestingly, a higher proportion of peripheral neuropathy (33.3%) was seen in the South African population in the ZeNix trial compared to the other trials sites in Russia, Moldova, and Georgia (16.7%), in participants receiving 600 mg for 26 weeks. We had a low proportion of people living with HIV in **study IV** of 6.1% (n=8/132). Noteworthy, the proportion of people with MDR-TB living with HIV was >80 times higher than the general Swedish population (estimate of 0.07% of the total population of Sweden (333)), which highlights the dual HIV-TB epidemic globally since the vast majority of people in **study IV** were not born in Sweden (1). Due to the low frequency of HIV, no subgroup analysis could be done.

Genetic differences between populations are also possible reasons for different risks of linezolid-associated toxicities. Genetic differences could be mutations (single nucleotide polymorphisms, SNPs) in the human ribosomal 16s rRNA genes in mitochondria leading to a predisposition for toxicity, which have been described in case reports of lactic acidosis in linezolid treatment (334). However, a randomised control trial from South Korea did not find an association between 16s rRNA polymorphism and a higher risk for toxicity (none had elevated lactic acid) (236). Similar results were seen in the South African study despite the high frequency of hyperlactatemia at 31%, for which there was no clear reason (237). Importantly, these genetic polymorphisms in the 16s rRNA genes are found in up to 80% of the general population, which would mean at least other mechanisms also need to be present since elevated lactic acid is rare in most studies (335). In the Nix-TB trial, elevated lactic acid was seen in 5.5% of participants when treated with linezolid 1200 mg daily (133), while we recorded elevated lactate in 1.5% (n=2/132) of participants in **study IV**. A limitation of the low frequency in **study IV** is that lactic acid was not regularly monitored, but only tested at the discretion of the treating physician, which would likely underestimate our results.

Another genetic polymorphism (single nucleotide polymorphism, SNP) that could be related to linezolid toxicity or at least apparent toxicity is mutations in the Duffy antigen receptor. This polymorphism is commonly called benign ethnic neutropenia leading to a lower absolute neutrophil count, <1.5 10^9/L in adults, and it is not associated with an increased risk of infections (336). Benign ethnic neutropenia has been associated with protection against malaria and is seen in 98-100% of people from western, middle, and south eastern regions of Africa (337), 7% in a South African population (338) but is rare in most other parts of the world (337). In **study IV**, we reported that 22.0% of participants experienced leukopenia. Interestingly, 51.5% of persons were born in Africa while 32.6% were born in the WHO Europe region (data not shown in the manuscript). In comparison, a much lower rate of myelotoxicity was seen in the French study at 11%

(leukopenia or neutropenia not specified) (238). In their study, 56.2% of participants originated from what the authors described as the Eastern WHO Europe region (238), which could be one explanation for the different frequencies of myelosuppression. We found weak evidence for an association between leukopenia (we did not measure neutropenia) and origin in Africa in **study IV** which might be due to the polymorphism seen in benign ethnic neutropenia. Another suggested name by Merz *et al.* is typical neutrophil count with Fy(a-b-) status as this is not an abnormal finding (339). Here, Fy is an antigen in the Duffy blood group, and a- and b- refer to negativity for the Fy*A and Fy*B alleles (337). However, this polymorphism was not regularly tested in **study IV**, therefore, it requires further investigation. If linezolid is withdrawn based on a low neutrophil count, when in fact it is a normal variant, this could have implications for treatment effect. Few studies have described leukopenia (or neutropenia) associated with linezolid treatment in sub-Saharan Africa (except South Africa where the polymorphism in the Duffy antigen receptor is rare, as described above). In one study from Niger (n=33), severe myelosuppression was seen in 18% of persons treated with linezolid, but it is unclear how many had leukopenia (326). Thus, further characterisation of linezolid treatment in these populations is highly needed.

Nutritional deficiencies, such as insufficient vitamin B12 levels due to diet or malnutrition could also differ between populations and lead to disparate risk for adverse drug reactions during linezolid treatment (340). Vitamin B12 status was not regularly monitored in **study IV**, although it is helpful that in the new Swedish clinical guideline for TB, it is recommended to screen for vitamin B12 and folate deficiencies at the treatment start if linezolid is given (259). Furthermore, alcohol-related neuropathy could be seen in up to 45% of persons, and additionally, there are overlapping toxicities between alcohol-related neuropathy and nutritional deficiencies (340). In **study IV**, alcohol overuse was described in four participants of which none developed peripheral neuropathy.

Another important limitation in **study IV** is drug-drug interaction as a potential confounder, which we did not assess due to a lack of reliable data. Rifampicin can lower the exposure of linezolid with an effect of up to three weeks after withdrawing rifampicin, as seen in a case report (341). In **study IV**, some persons had rifampicin in the initial regimen while waiting for the results of rapid molecular tests, therefore, this could have lowered the initial linezolid effect and toxicity. In contrast, clarithromycin can increase linezolid exposure (around 40-50%) which is thought to be through inhibition of P-glycoprotein, a membrane efflux pump (342). However, this interaction is currently less relevant since clarithromycin is not recommended in the treatment of MDR-TB anymore (43). Other potent inhibitors of P-glycoprotein, such as omeprazole and the antihypertensive drug amlodipine have also been suggested to increase linezolid exposure, although this has not been confirmed (343). Especially omeprazole is a very common drug used to reduce adverse drug reactions of gastritis and nausea in MDR-TB.

Thus, this possible interaction would have been useful to assess. Moreover, linezolid is a weak reversible inhibitor of monoamine oxidase and a slightly higher risk of serotonin syndrome has been described when co-administered with drugs such as selective serotonin reuptake inhibitors (SSRI) for depression. (140). However, this has been challenged in recent studies showing no difference in serotonin syndrome with co-treatment (overall incidence 0.58%) (344). Although the aim of **study IV** was not to assess serotonin syndrome, we did not find any person experiencing this event (2.3%, n=3/132, had a psychiatric disease including depression) with the limitation that it could be difficult to diagnose (344).

In summary, the most important difference between our results and the ZeNix and PRACTECAL trials is likely different study populations, with the trial populations being healthier due to exclusion criteria (113, 134). However, why a higher frequency of myelosuppression was seen in **study IV** compared to the study from France (238) which has a similar setting to ours is less clear, especially since similar results were seen regarding peripheral neuropathy. A possible genetic difference due to the origin of the study participants could be a reason as mentioned above. Another reason could be that participants who experienced myelosuppression within the first four weeks leading to the withdrawal of linezolid were excluded from the study, since an inclusion criterion was at least four weeks of linezolid treatment. Moreover, a higher frequency of peripheral neuropathy was seen in the South Korean study (323) compared to **study IV,** which will be discussed below (Section 6.4.5 on measuring linezolid drug concentrations)

6.4.2 Peripheral neuropathy and symptom duration

The notion that peripheral neuropathy can increase with time, even if linezolid is stopped was seen in **study IV**, where 12.7% of participants with peripheral neuropathy had onset or worsening symptoms after linezolid was withdrawn. The same phenomenon was seen in the Nix-TB trial (dosing 1200 mg daily), when the severity of peripheral neuropathy increased between 12- and 24 months post-treatment for four participants (345). Although this phenomenon is already known in cancer treatment as coasting and associated with several agents such as platinum-based drugs (e.g., cisplatin och carboplatin) (346), it is important that also infectious diseases physicians are aware of the phenomenon to be able to give due support.

Furthermore, 51.2% of participants with peripheral neuropathy in **study IV** had symptoms more than 12 months after the first occurrence which indicates the severity and long-lasting toxicity of neurological adverse drug reactions. An even higher risk of long-lasting toxicity was seen in a study from France by Jaspard and colleagues (n=78) where 78% of persons with confirmed peripheral neuropathy (n=18) had symptoms at 12 months (141). How long it takes until peripheral neuropathy disappears, if it does disappear at all for all persons, is unclear. In a modelling study from the Nix-TB trial, it

was estimated that it would take 15 months to reverse peripheral neuropathy (345). However, in a study that followed up participants treated with the anti-cancer drug cisplatin for a median time of 15 years, 28% of participants still had symptomatic peripheral neuropathy, and for 6% it was disabling (347). Further characterisation of symptoms and duration is needed in persons with linezolid-associated peripheral neuropathy as this is an important and disabling post-TB disease.

Our study (**study IV**) was limited by the lack of medical records for all participants up to 18-24 months post treatment (medical records were found until treatment ended for all), which could have been further characterised if symptoms resolved. Despite these limitations, our results highlight the important aspect of that follow-up, and further support is needed for this group of persons having long-term sequala of linezolid treatment.

6.4.3 Timing of adverse drug reactions

We found (**study IV**) similar times to each adverse drug reaction as other studies when comparable definitions were used (141, 325-328). Myelosuppression typically occurred first (median 0.5-4 months), followed by peripheral neuropathy (median 1.5-4 months), and lastly optic neuritis (median 5-9 months). A longer median time to peripheral neuropathy of 8.6 months was seen in the study from France by Jaspard and colleagues, however, they used another definition (141). In the study, all cases with peripheral neuropathy were confirmed using nerve conduction studies which would likely result in a delayed diagnosis due to the additional time required for the investigation as suggested by the authors (141). Since few studies have reported on time until leukopenia, it is important to point out that the median time of 1.1 months until the first event of leukopenia was similar to anaemia and thrombocytopenia in **study IV**.

A limitation of **study IV** is how we measured the time to each adverse drug reaction since participants were monitored at the physicians' discretion due to the observational study design. In comparison, the retrospective observational French study by Eimer *et al*. monitored myelotoxicity weekly throughout treatment but they did not specify how often peripheral neuropathy was assessed (238). The frequency of monitoring could affect the occurrence of an adverse drug reaction. For example, if blood is drawn weekly, the time to an adverse drug reaction would likely be shorter compared to if blood is drawn every three months. However, participants were typically admitted to the hospital for the first months and later seen at least every three months in **study IV**. Moreover, the frequency of myelosuppression was highest in the first months of treatment when participants were typically in the hospital and phlebotomy was conducted regularly (usually two to three times per week). Therefore, this was regarded as a lower risk of misclassification of the outcome. Regarding optic neuritis and peripheral neuropathy, we recorded the time of onset as the participants described it in the medical records.

Therefore, a less frequent monitoring schedule in the follow-up period would have had a lower risk of misclassification.

Considering the timing of adverse drug reactions in **study IV**, different monitoring schedules for detection could be employed. Since 50% of participants developing anaemia, thrombocytopenia, and leukopenia would have done so within the first month in **study IV**, frequent monitoring during this time is also needed. WHO recommends monitoring with a full blood count every two weeks in the first month of treatment and then monthly (44). However, data from **study IV** suggest more frequent monitoring, preferably weekly, at least during the first month. Regarding peripheral neuropathy, continuous monitoring seems prudent since the incidence in **study IV** was fairly similar throughout treatment (Figure 4). This is in line with recommendations from WHO (44) and the Swedish Tuberculosis guidelines (259). However, informing persons that peripheral neuropathy can start even after linezolid withdrawal is important for enabling due support.

6.4.4 Dosing linezolid according to body weight?

We found that a linezolid dose of 12 mg/kg/day or above was associated with each of the adverse drug reactions peripheral neuropathy, leukopenia, and anaemia in **study IV**. Only one study in TB conducted in Indonesia (n=93) previously reported an association between a higher linezolid dose of 11 mg/kg or more and anaemia when linezolid 600 mg daily was given (348). This dose would correspond to a weight less than 54 kg if 600 mg linezolid daily was given as mentioned in the study. Interestingly, a similar cut-off using weight has been associated with linezolid toxicity in non-TB studies from Japan and China, although a higher linezolid dose of 1200 mg daily was given (349-351). The studies reported that a lower body weight than 55 kg or a dose ≥19-22 mg/kg was positively associated with thrombocytopenia. A limitation when comparing our results with non-TB studies (apart from the higher dose) is that the duration of linezolid treatment is much shorter with a mean treatment duration of 10-14 days in the above studies (349-351).

Considering these results, it is worth discussing if linezolid should be dosed according to weight which has also been suggested in overview articles (352). Currently, flat dosing of linezolid at 600 mg daily is recommended by WHO in adults weighing 46-70 kg (44). Interestingly, in this recommendation, there is no dose assigned for persons weighing more than 70 kg, which might be due to that a higher dose could be prescribed. Currently, many TB drugs are dosed based on body weight, such as levofloxacin, pyrazinamide, and prothionamide (44), so it is not far-fetched to consider dosing linezolid based on body weight. Apart from the evidence described above (349-351), including **study IV,** there is other evidence supporting this suggestion. Firstly, there was a linear relationship between a higher linezolid dose in mg/kg (starting from a category of <17 mg/kg to ≥27 mg/kg) and a higher risk of thrombocytopenia when linezolid was

dosed 1200 mg daily, in the study from Japan by Natsumoto *et al*. Secondly, a lower weight <50 kg was associated with higher linezolid exposure using AUC_{0-24h} in a modelling study based on data from persons (n=455) from both Japan and Europe (mean AUC_{0-24h} 730 mg*h/L in persons ≥80 years and weight <50 kg compared 207 mg*h/L in person <60 years and weight ≥50 kg). Similarly, a higher body weight showed an association with a lower free fraction linezolid AUC_{0-24h} in a modelling study from China (353). However, in the same study, linezolid trough levels were not associated with body weight. Moreover, a modelling study using data from the Nix-TB study found neither that body weight nor BMI were co-factors in the association between trough-level exposure and peripheral neuropathy, anaemia, or thrombocytopenia (345).

Different mechanisms have been proposed as to why a higher linezolid exposure (AUC) could be seen in a person with a lower body weight. A higher body weight might increase metabolism (increased clearance) through the production of reactive oxygen species in fat which might be involved in the metabolism of linezolid (354). Moreover, in the recent study by Zhou *et al*. from China, the authors conclude that body weight was associated with AUC through the correlation with creatinine clearance and haemoglobin levels (353). One limitation of these studies is that AUC is discussed while clinical studies have mostly evaluated trough levels in relation to adverse drug reactions (236-238). However, AUC might be a parameter involved in adverse drug reactions as seen in a hollow-fibre model (185). In the study linezolid AUC_{0-24h} (and trough concentrations) correlated well with toxicity measured by reduced mitochondrial protein levels in human cells. Moreover, a Georgian study (n=74) found an association between AUC_{0-24h}>160 mg×h/L and myelosuppression, although it was based on only six events (325). Since linezolid AUC is rarely measured in clinical studies (355), likely due to limited resources, it is uncertain if a higher AUC is associated with linezolid adverse drug reactions, although trough levels have been linearly correlated with AUC_{0-24h} in studies (274, 343). Considering these different suggestions for a mechanism, it is not clear how body weight is associated with a higher risk of toxicity.

Another unanswered question is if it is mostly persons that are underweight or persons with a normal BMI having a low body weight that might have a higher risk for adverse drug reactions of linezolid. The above studies which were predominantly conducted in Chinese and Japanese populations did not analyse BMI as a risk factor but only weight (349-351, 353), and weight is often lower in these populations compared to European populations. However, in the study from South Korea by Kwon *et al*., a low BMI was associated with peripheral neuropathy (323), which could be one explanation since being underweight is a risk factor for peripheral neuropathy (340). Although underweight does not explain the higher risk seen between a low body weight and thrombocytopenia, since underweight is not a risk factor for thrombocytopenia. To further investigate underweight as a risk factor for linezolid adverse drug reactions, a

study investigating linezolid PK parameters separately in underweight persons is needed.

One mechanism to why persons that are underweight could have a higher drug exposure is due to a lower metabolism or an overestimated renal function due to low body mass (if measured by creatinine clearance) (356). Furthermore, a lower volume of distribution seen in underweight persons could increase the risk of higher variability of drug exposure, especially related to peak concentrations (356). This could be particularly troublesome with linezolid since it is known to have high variability. One study reported up to a 20-fold variability of trough concentrations overall (both between and within subjects) and >60% variability between subjects (352). Another mechanism in persons that are severely ill and underweight is lower albumin levels leading to a higher free linezolid concentration which could increase toxicity (357). When comparing the weight of persons in the categories of mg/kg/day for all participants in **study IV**, the median weight was 48.9, 58.0, and 76.6 kg in the categories of <8, 8 to <12, and ≥12 mg/kg/day, respectively. These results are not far from the studies discussed above showing that a weight less than 50 or 55 kg is associated increased risk for an adverse drug reaction (348-351).

One suggestion from the modelling study from China based on prospectively collected data (353) found that linezolid 450 mg daily for persons weighing <50kg and 600 mg daily in those ≥50 kg would be optimal in terms of efficacy, however, as already pointed out, no difference in toxicity was seen in this study based on body weight. Data is inconclusive, but it seems prudent to further investigate linezolid dosing based on body weight, especially for persons that are underweight and in populations apart from those in Japan and China since body weight and genetics might differ.

6.4.5 Measuring concentrations of linezolid?

We did an exploratory univariable analysis in **study IV** of linezolid concentrations and their association with each adverse drug reaction since a limited number of concentrations were available. Similar to the prospective observational study from South Africa (n=151), we saw evidence of an association between a trough level of ≥2 mg/L and anaemia (they found a cutoff of ≥2.5 mg/L) (237). Furthermore, a trough level ≥2 mg/L was associated with thrombocytopenia. Other studies, including the randomised control trial from South Korea, found an association between a trough level >2 mg/L and general linezolid toxicity (peripheral neuropathy, anaemia, thrombocytopenia, and leukopenia analysed together) (236, 238, 325).

In the clinical trial from South Korea, among those with a trough concentration above 2 mg/L, 100% of participants experienced a linezolid-associated adverse drug reaction while in those with a trough concentration <2 mg/L, 58% had an adverse drug reaction (236). In **study IV**, 50.0% compared to 16.7% of those with anaemia had trough

concentrations ≥2 mg/L and <2 mg/L, respectively, while the same comparison for thrombocytopenia was 29.4% and 0%. Although our results are limited since we only performed a univariable analysis, the results still show the same trend as previous studies.

Interestingly, in non-TB studies, the suggested trough target associated with a higher risk of adverse drug reactions due to linezolid (mainly thrombocytopenia) is much higher and commonly described as around 8 mg/L (358). The probable reason that a higher trough threshold is found is likely the shorter duration of linezolid treatment given (around 14 days) (350, 359). Furthermore, a higher exposure is needed compared to the treatment of *M. tuberculosis* since the linezolid MIC of Staphylococcus aureus is higher with a clinical breakpoint of 4 mg/l (compared to the ECOFF for *M. tuberculosis* of 1 mg/L) which warrants a higher dose (95).

We found no evidence in **study IV** of an association between linezolid 2-hour and AUC_{0-24h} and adverse drug reactions, except for a positive association between 2-hour concentration and thrombocytopenia. As mentioned above, linezolid AUC_{0-24h} >160mg×h/L has been reported to be associated with myelosuppression in a prospective Georgian study, which needs further exploration (325).

One way to mitigate toxicity and continue with linezolid treatment is lowering the dose of linezolid to 300 mg daily which was described above. The main concern about lowering the dose is insufficient efficacy (253). In the hollow-fibre study which developed the suggested efficacy target of a free linezolid fraction >119 AUC_{0-24h}/MIC for optimal microbial kill (184), it was estimated that 87% of people receiving 300 mg daily would achieve this target. The recent modelling study from China mentioned above (353) showed that the achieved target could be reached by using 450 mg in persons <50 kg and 600 mg daily in those 50 kg and above. In **study IV**, participants treated with a dose of 600 mg and <600 mg (mostly 300 mg) reached the suggested target for efficacy (184) in 92.6% and 79.0% (overall 85.7% target attainment, data for doses not shown in manuscript). The estimates are based on the assumption of a protein binding fraction of 31% (140) which has its limitations as already discussed (Section 4.5.8 on targets). These results using 600 mg are reassuring, however, using a dose of 450 mg could be further explored.

We estimated AUC_{0-24h} in **study IV** based on a previously published linear model published by Kamp and colleagues (274) since this is a simplified way of estimating AUC_{0-24h}. Although we found no other publication using this approach, one other study mentioned above from China (353) used a limited sample strategy based on evidence from the study by Kamp *et al.*, to develop a population PK model.

We found that a higher linezolid concentration (≥2 mg/L) was associated with anaemia and thrombocytopenia in MDR-TB which is similar to other studies as discussed above.

Therapeutic drug monitoring for linezolid is already recommended by various experts such as the Infection Unit of the European Society of Intensive Care Medicine among others in a consensus document (360) and the recently published Clinical standards for TB written by experts in the field (187) considering the high variability and narrow therapeutic index of linezolid. A threshold of 2 mg/L is suggested for an increased risk of adverse drug reactions in TB with long treatment duration, but clearly, this will not prevent all events of adverse drug reactions. The linezolid dose was lowered to 300 mg in **study IV** to mitigate toxicity which is also seen in other studies. However, the balance between effect and toxicity needs to be considered. The next step could be to perform an intervention study using these suggested thresholds, or at least an observational study where therapeutic drug monitoring has been conducted.

6.5 Concluding remarks

MDR-TB is a global disease affecting disproportionally people in low- and middle-income countries. To end this long-term silent public health threat, we need better means to improve treatment and reduce morbidity and mortality for people suffering from MDR-TB disease. Although downplayed by WHO, pyrazinamide may have a role in the treatment of people with MDR-TB if given to the right person with the right combination of drugs. Detecting drug-resistance using whole genome sequencing has potential to detect resistance more rapidly to multiple drugs, however, technical challenges exist and cost is a current limitation. Fluoroquinolones are the backbone of MDR-TB treatment together with bedaquiline and linezolid, but recent research suggests that higher doses could even further improve their efficacy, if safety studies allow. The continuous use of linezolid in MDR-TB is promising but the risk of adverse drug reactions with long-term use is still a limiting factor that might be overcome with therapeutic drug monitoring. Optimising the current drugs used in MDR-TB treatment by including the right drug at the right dose at the right time is one way forward to improve outcomes.

Timur always comes early. He arrives when the streets are still empty. The nurse has unlocked the door to the clinic for him to enter but it is still dark in the hallway and the posters on the concrete walls with information about TB are barely visible. Timur quickly swallows the 15 tablets with a glass of water and leaves. We almost bump into each other as I enter the clinic. Timur looks down and quickly walks away on his way to work. "We've now made a special arrangement," the nurse says. "He doesn't want to be seen by anybody in the TB clinic, so I come early - just for him".

7 Conclusions

Pyrazinamide treatment and the length of pyrazinamide treatment in the intensive phase were not associated with end-of-treatment outcomes in people treated with an MDR-TB regimen in Uzbekistan.

When considering genotypic DST for pyrazinamide, treatment with pyrazinamide was associated with a shorter time to sputum culture conversion among persons treated for MDR-TB. However, no association between a lower pyrazinamide MIC level and time to sputum culture conversion was seen.

The total drug exposure over MIC of moxifloxacin but especially levofloxacin was insufficient in reaching the suggested targets for efficacy when using standard doses according to Chinese national guidelines in persons with MDR-TB.

Treatment with a linezolid dose of 12 mg/kg or more was associated with an increased risk of the adverse drug reactions peripheral neuropathy, anaemia, and leukopenia among people with MDR-TB.

A linezolid trough concentration of 2 mg/L or more was associated with anaemia and thrombocytopenia in an exploratory analysis in persons treated for MDR-TB.

Treatment outcomes might be improved by carefully selecting which persons should receive pyrazinamide, ensuring adequate exposure to fluoroquinolones, using whole genome sequencing for DST, and adjusting the dose of linezolid by weight and trough concentration levels.

8 Points of perspective

8.1 Treatment outcomes and treatment response

To be able to fully evaluate the effect of pyrazinamide and other drugs with a highly sterilising activity in clinical studies, appropriate, validated, and widely accepted outcomes and surrogate markers are needed, especially related to relapse (361). Therefore, it is a step in the right direction that WHO has included an additional outcome measure to be used under operational research conditions of sustained treatment success in their latest guidelines (in drug-resistant TB, defined as a person who is alive and free of TB, 12 months after successful treatment) (44). Another suggestion to predict relapse is to use sputum culture conversion at six months, since 2-month culture conversion is less predictive than what was previously thought (198, 204). Even if relapse is uncommon in individual studies of MDR-TB (relapse seen in 1-3% in clinical trials (134, 135, 204)) pooling studies and conducting a meta-analysis could be one way forward to evaluate drugs with sterilising ability.

Moreover, better ways to measure treatment response are needed as sputum culture conversion at two months is an imperfect surrogate endpoint of treatment outcome, as outlined in the introduction. Improved biomarkers to evaluate treatment response, is also a WHO prioritised research (43). Ideally, such a test should not be based on sputum since not all persons ill with TB can produce sputum, e.g., small children, those living with HIV, and persons with extrapulmonary TB. A range of non-sputum-based biomarkers such as transcriptomics (i.e. RNA in whole blood), cytokine profiles, positron emission tomography combined with computer tomography (PET/CT), and different clinical-based TB scores, have been suggested in overview articles (206). However, advanced, and expensive techniques such as positron emission tomography combined with computer tomography (PET/CT) seem impractical and less feasible in especially high-incidence countries in routine care. One promising example is the specific proteomic signature (consisting of the immunological markers TNF-β, sIL-6R, IL-12p40, and IP-10) which was evaluated in different clinical cohorts and could predict relapse with a sensitivity of 83% and specificity of 61% if measured in serum at baseline (362).

It is not strange that developing a predictive biomarker in TB is challenging. TB disease is very heterogenous ranging from a person with lymph node TB only noticing a lump on their neck to severe TB disease in a person admitted to the intensive care unit due to TB meningitis. In clinical practice in infectious diseases, multiple ways of predicting response to treatment are used, also in more acute infections such as sepsis. In the latest Sepsis-3 criteria at least two parameters of worsened respiratory rate, altered mental status, or a lower systolic blood pressure are needed to predict a higher mortality (363). Therefore, in predicting treatment response for such a wide spectrum

of TB disease, a combination of biomarkers for TB is likely needed, used in different stages of the treatment (206).

8.2 Optimising current and new drugs

Although four new or repurposed drugs (bedaquiline, delamanid, linezolid, and pretomanid) have been included in the treatment of MDR-TB in little more than the last 15 years, there is room for further improvements of the current drugs and new drug development, particularly due to adverse drug reactions and long duration of treatment.

Pyrazinamide does likely have a role in MDR-TB treatment, however, since up to 30% of pyrazinamide-resistant isolates lack mutations in the *pncA* gene (89), and the additionally suggested genes *panD* (56, 58), rpsA (57, 91) and *clpC* (58, 90) are rare in clinical isolates (92, 364), there might be other target genes. By finding a better understanding of the mechanism of action of pyrazinamide, additional drugs could be developed that target the possible mechanism of surviving semi-dormancy. Semi-dormancy is not only important for *M. tuberculosis* but is also seen with other bacteria such as e.g., *Staphylococcus aureus* in prosthetic joint infections, where rifampicin is used to target this bacterial subpopulation (365). Perhaps the TB community would benefit from collaborations with other researchers in finding further targets for treating semi-dormant and dormant bacteria, as this is a joint problem. Furthermore, an unsettled query is a long-standing belief that pyrazinamide has the highest effect of reducing relapse early in the first two months of treatment, which has been challenged by researchers in the field already 40 years ago (294) and now (366, 367). Bacterial *M. tuberculosis* populations would highly likely switch from semi-dormancy and actively dividing, therefore, even in the later stages of treatment semi-dormant bacteria would be present. Thus, it would be intriguing to evaluate the efficacy of pyrazinamide at the end of treatment (367).

Since the available fluoroquinolones, moxifloxacin and levofloxacin, are sometimes limited by adverse drug reactions (i.e., cardiotoxicity usually due to moxifloxacin (138), having the option of switching to another fluoroquinolone would be preferable. Currently, there seems to be no plan to re-approve gatifloxacin, a fluoroquinolone used in TB (130), which was withdrawn by the United States Food and Drug Administration in 2006 due to a higher risk of dysglycaemia (368). However, suggestions have been made to reintroduce gatifloxacin (369) but at this moment it seems extremely unlikely. Moreover, no other fluoroquinolone is investigated in pre-clinical or phase 1-3 trials for TB (370). Although other drug classes might be more prioritised than a new fluoroquinolone, one option could be to investigate sitafloxacin for TB which is already used in clinical medicine (371).

Treatment options instead of linezolid are also needed despite its high efficacy since this drug has become the “new aminoglycoside” in terms of adverse drug reactions and

withdrawing linezolid is frequently done as seen in **study IV**. Sutezolid and delpazolid are two oxazolidones under investigation among others under evaluation for TB (370). Currently, a phase 2 trial investigating sutezolid (SUDUCO, ClinicalTrials.gov Identifier: NCT03959566) is ongoing, evaluating sutezolid at 0-800 mg, when added to a background regimen of bedaquiline, delamanid, and moxifloxacin. Delpazolid is also included in a phase 2 trial (ClinicalTrials.gov Identifier: NCT04550832). These studies are highly interesting and especially if the toxicity profile is lower or different from linezolid which has been suggested for at least sutezolid in an in vitro study (372).

8.3 Further use of whole genome sequencing

It is highly likely that we have only seen the beginning of what whole genome sequencing could add to establishing DST and making clinical decisions. As already highlighted in the discussion on pyrazinamide genotypic DST (Section 6.2 on optimising pyrazinamide and whole genome sequencing), studies have shown that whole genome sequencing to detect resistance can be performed directly on a sputum sample if the bacterial load is high enough (306). However, it is still challenging in persons who have microscopy negative sputum. Furthermore, a more sensitive option is targeted next generation sequencing (i.e., Deeplex Myc-TB) which amplifies specific genes associated with resistance (308). Another method that could improve access and move sequencing nearer the person who is ill with TB is the portable sequencing platform MinION (Oxford Nanopore Technologies, Oxford, UK) (373). Moreover, a possibility for rapid detection of at least fluoroquinolone resistance (apart from rifampicin and isoniazid) is already here in the form of Xpert MTB/XDR (Cepheid Inc., Sunnyvale, USA) (72). If more resistance markers for i.e., linezolid, bedaquiline, and ideally pretomanid/delamanid and pyrazinamide, are added to the Xpert MTB assay it could be used to guide treatment for all recommended WHO regimens (374). However, currently, the cost is still a major limiting factor (375). Using rapid and simpler methods to detect resistance is also in line with the WHO-recommended features of newly developed tests for DST (target product profiles) that recommends access at the peripheral level with results available in less than six hours (374). Furthermore, comprehensive DST is mentioned in the End TB Strategy, which favours further development of whole genome sequencing and targeted next generation sequencing (2).

Another advantage of whole genome sequencing that has recently emerged is the importance of the *M. tuberculosis* lineage (376). The type of lineage has been associated with the type of disease (e.g. higher frequency of lymph node TB in Lineage 3) (377), prediction of resistance (e.g. delamanid and pretomanid resistance in Lineage 4) (378, 379), and elevated MIC (e.g. Lineage 1 associated with higher MIC for pyrazinamide) (364). Furthermore, using data from whole genome sequencing from *M. tuberculosis* combined with artificial intelligence to aid in the prediction of new resistance mutations, drug development, and treatment outcomes as seen for

Coronavirus disease (COVID-19) (380) is likely not far away. However, the implementation of new techniques and improving access in high-incidence settings is imperative to achieving the END TB strategy (2).

8.4 Therapeutic drug monitoring

It seems clear that therapeutic drug monitoring for TB is here to stay but unresolved issues remain. Although targets derived from hollow-fibre models are highly valuable as a start (215, 228), these suggested targets need evaluation in ideally randomised trials in persons treated for TB associated with relevant outcomes (381). Few prospective studies have been conducted (231, 232, 234) but further studies evaluating these targets using shorter regimens in combination with the newer drugs are needed as well as evaluating these targets using therapeutic drug monitoring as an intervention. Another issue is how to simplify sampling and analysis as access to phlebotomy and liquid chromatography-tandem mass spectrometry could be a barrier to implementation in low-resource settings (382). Important work on developing other sampling techniques such as using capillary blood on dried blood spots (382), saliva (383), and urine (384) have been developed. These techniques could be combined with i.e., spectrophotometers (383) or colorimetry (384), to simplify analysis. Thus, some solutions to increase access and reduce cost have already been proposed but further work is needed as well as studies on implementation.

8.5 Person-centred care

As technologies improve and regimens become more effective, we must not lose the perspective of what the person affected by TB wants and how they experience care. A person (or people)-centred approach should include empowerment and building a trusting relationship between the caregiver and the person affected by TB (385). Certain person-centred interventions have been evaluated such as a package of cash transfers, psychosocial support, and nutritional supplements which led to improved outcomes (386). However, far too little attention has been given to evaluating the experiences and perceptions among persons receiving such interventions (387) which are important for the perceived quality of care and satisfaction (385). Certainly, more evaluations of person-centred approach are needed, including the usage of language that empowers and treats people with dignity (388) and personal preferences in terms of treatment regimens (2).

Lastly, the implementation and roll-out of improved technologies and effective drugs cannot be emphasised enough as their usage is key to improving outcomes and reducing adverse reactions. Access should be made available to all people affected by TB regardless of age, gender, origin, and setting, and cannot be hidden behind costs with double standards for people residing in low- or high-resource settings (389).

In conclusion, better means to optimise the current treatment for MDR-TB is urgently needed to achieve the ambitious goals of the End TB Strategy (2). This thesis has included studies on some aspects on how treatment can be optimised related to detecting drug resistance, inclusion of drugs, measuring drug concentrations, and reducing adverse drug reactions. To achieve improved outcomes, personalised treatment is proposed when different drug regimens could be tailored to the infecting strain, therapeutic drug monitoring be performed for each drug, and genetic analysis of the person ill with TB conducted to predict drug effect (390). Although personalised treatment is tantalising, – using the simplest way would be much more desirable in low-resource settings. If the drugs we used for MDR-TB are already optimised with low toxicity, have a wide therapeutic window, and are effective so we could just give a similar dose to all people and ensure high cure rates without the risk of acquired drug resistance and toxicity, this would be ideal. Although this seems utopic, it is somewhere to strive. Beyond personalised medicine is when we can wisely decide when and with what tools we need to personalise and optimise a treatment regimen but also when we can abstain and give a simple regimen that we know will cure the person who has fallen ill with TB.

"Hi," Gulisa says – "do you know I'm cured?, I'm well now!" Gulisa's face lights up with a big smile as she looks at me. Her skin is still red-brown as if she had been out in the sun too long – a well-known side-effect of the drug clofazimine which normally disappears after many months. Everybody around Gulisa would know that she has been treated for MDR-TB. I see in her face that she doesn't care, she is cured. She has managed all those hard months, swallowed all those tablets, and received all those painful injections. She hugs me and we said bye. As I turn around, Gulisa's short-sleeved multicoloured dress blows around her red-brown arms. On my way home, her straight back and her head held high are all I think about.

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