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Plasma concentrations of second-line anti-tuberculosis drugs in relation to minimum inhibitory concentrations in multidrug-resistant tuberculosis patients in China – a study protocol of a prospective observational cohort study

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ABSTRACT

Introduction

Individualized treatment through therapeutic drug monitoring (TDM) may improve tuberculosis (TB) treatment outcomes but is not routinely implemented. Prospective clinical studies of drug exposure and minimum inhibitory concentrations (MICs) in multidrug-resistant tuberculosis (MDR-TB) are scarce.

This translational study aims to characterize the area under the concentration-time curve (AUC) of individual MDR-TB drugs, divided by the MIC for *M tuberculosis* (Mtb) isolates, to explore associations with markers of treatment progress and to develop useful strategies for clinical implementation of TDM in MDR-TB.

Methods and analysis

Adult pulmonary MDR-TB patients treated in Xiamen, China, are included. Plasma samples for measure of drug exposure are obtained at 0, 1, 2, 4, 6, 8 and 10 hours after drug intake at week 2 and at 0, 4, and 6 hours during week 4 and 8. Sputum samples for evaluating time to culture positivity (TTP) and MIC determination, are collected at day 0, 2, 7 and at week 2, 4, 8 and 12 after treatment initiation. Disease severity are assessed with a clinical scoring tool (TBscore II) and quality of life evaluated using EQ-5D-5L.

Drug concentrations of pyrazinamide, ethambutol, levofloxacin, moxifloxacin, cycloserine, prothionamide and para-aminosalicylate are measured by liquid chromatography-mass tandem spectrometry (LC-MS/MS) and the levels of amikacin measured by immunoassay. Dried blood spot (DBS) on filter paper, to facilitate blood sampling for analysis of drug concentrations, is also evaluated.

The MICs of the drugs listed above are determined using custom-made broth microdilution plates and MYCOTB® plates with Middlebrook 7H9 media. MIC determination of pyrazinamide is performed in BACTEC MGIT™ 960.

Ethics and dissemination

This study has been approved by the Ethical Review Boards of Karolinska Institutet, Sweden and Fudan University, China. Informed written consent is given by participants. The study results will submitted to a peer-reviewed journal.

Study registration number: clintrials.gov NCT02816931

Article summary

Strengths and limitations of this study:

- To our knowledge, this is a novel study approach which fully investigates the distribution of drug exposure in relation to minimum inhibitory concentration (MIC) for *Mycobacterium tuberculosis* isolates from multidrug-resistant tuberculosis patients along with biomarkers (e.g. TTP), culture conversion and the clinical scoring tool TBscore II to assess treatment outcome.
- We used a translational approach with experts from research centres across the world to design a study protocol including both MIC-determinations, drug exposure estimation using novel technology, including dried blood spot (DBS), as well as microbiological and clinical surrogate markers for improvement, to enable strategies for therapeutic drug monitoring (TDM) use in TB treatment.
- The patients' drug exposure will be compared with individual Mtb MICs, exploring pharmacokinetics-pharmacodynamics (PK/PD) indices in MDR-TB treatment.
- A limitation of the study is the low target number of patients for inclusion, due to a laborious and costly study protocol, which might partly be compensated for by using

pharmacometric modelling.

Introduction

Despite programmatic management of tuberculosis (TB), the incidence of multidrug-resistant tuberculosis (MDR-TB), defined as *M. tuberculosis* (Mtb) resistant to rifampicin (RIF) and isoniazid (INH), is steadily increasing (1). Inconsistent treatment, due to poor treatment adherence, lack of drugs, as well as sub-therapeutic dosing are contributing factors. For many TB drugs the administered dose is not predictive of the drug exposure and clinical effect in the patient (2). A hollow-fibre study indicated that pharmacokinetic variability may be an underestimated cause of drug resistance development (3) and low drug concentrations in the treatment of drug susceptible TB have been associated with poor outcome in some prospective studies (4, 5).

Therapeutic drug monitoring (TDM) is a strategy to personalize treatment by measuring systemic drug levels in blood/plasma as a guide for individual dose adjustments (6). Specifically for infectious diseases, the drug efficacy not only depends on the drug exposure but also on the susceptibility level of the bacteria, the minimum inhibitory concentration (MIC) (7). The MIC is defined as the lowest concentration of a drug that inhibits visible growth of bacilli and needs to be exceeded to cure the infection (8). TDM has been recommended during MDR-TB treatment by several organisations, for example the Infectious Disease Society of America (9). The pharmacokinetic studies that have been performed have shown that sub-therapeutic drug levels in TB treatment are common, although with conflicting results regarding association between drug exposure and treatment outcome (10-12). However, studies on MDR-TB are limited and only a few studies have included drug concentrations as well as the individual MICs of the bacteria (13-15).

An optimal estimation of drug exposure (i.e. area under the concentration versus time curve; AUC) traditionally requires multiple venous blood samples, often followed by prompt centrifugation and sample storage at -80ºC. A simplified strategy for collection and

transportation of blood samples needed for TDM would aid its implementation in clinical practice. Dried blood spot (DBS) allows minimal blood sampling by capillary finger pricking on filter paper, which can be transported without a cold chain, simplifying transportation and storage (16). DBS is a well-established and validated method, but has only been evaluated for a few second-line TB drugs, for example moxifloxacin and linezolid (16-18). A clinical implementation of DBS could enable TDM for TB treatment in remote areas and reduce costs (16).

There is scarce data regarding drug exposure and treatment outcome in MDR-TB treatment. Assessing end-of treatment outcome in MDR-TB studies is cumbersome due to long treatment durations. Other strategies include using interim endpoints such as time to positivity (TTP) in liquid culture media, a surrogate of bactericidal activity (19), and sputum culture conversion (SCC) after two or three months of treatment (20), the latter commonly used in drug efficacy studies. A clinical composite scoring system, TBscore II, can be used as a surrogate marker for TB disease severity and to predict failure (21). Patients' quality of life can be objectified using the validated EQ-5D-5L tool assessing five different dimensions (mobility, self-care, typical activity, pain/discomfort and anxiety/depression), an often overlooked tool in clinical treatment studies.

China has the second highest burden of MDR-TB in the world and has existing resources to perform TDM, thus making it an ideal setting for pharmacokinetic/pharmacodynamic (PK/PD) studies. The overall incidence of TB in China was 895 000 TB cases in 2017, of which 8.2% were MDR-TB (22).

We describe a new comprehensive approach to TDM studies, assessing drug exposure, individual MICs as well as clinical outcome markers. The primary aim of the study is to investigate the distribution of AUC/MIC and C_{max}/MIC for MDR-TB drugs during MDR-TB treatment in China. Secondary aims are to analyse AUC/MIC in relationship to markers of

clinical improvement, such as SCC, TTP, TBscore II, body mass index (BMI) and qualitative measures of well-being (EQ-5D-5L). Signs of acquired resistance are assessed by investigating changes in MICs and genetic mutations, during the first three months of treatment. A clinical implementation of DBS as well as a method of simultaneous MIC determination are assessed to simplify the use of TDM in clinical practice.

Methods and analysis

Study design

We are conducting a prospective cohort study of TB drug exposure and MICs in MDR-TB patients in Xiamen, China. This is a joint project between the School of Public Health Fudan University Shanghai, Department of Medicine Karolinska Institutet, Department of Pharmaceutical Biosciences University of Uppsala and the Public Health Agency of Sweden, in collaboration with the Centre for Disease Control (CDC) in Xiamen. The study protocol conforms with the STROBE Statement for cohort studies (23).

Study setting

The study is carried out in Xiamen, Fujian region in Southeast China, where the incidence of TB in 2016 was 42.4 cases/100 000 inhabitants and of the 1661 confirmed cases that year, there were 28 MDR-TB patients (1.7%) (24).

The study hospital is the designated TB hospital in Xin Ling, Xiamen, a large teaching hospital with a specialised TB-ward with 105 beds as well as a negative pressure ward (12 beds) with specialised TB physicians and nurses. Recruitment of patients is performed by the Xiamen CDC, which also keeps a screening log. The study is registered at ClinicalTrials.gov (NCT02816931) and opened 17th of April 2016.

Study participants

A total number of 30 fully evaluable patients, according to the criteria below, will be included.

Inclusion criteria

- Consenting adults (≥18 years) with a verified diagnosis of pulmonary MDR-TB, by routine drug susceptibility testing (DST) admitted to the Xin Ling TB hospital, Xiamen.
- Eligible for and consent to MDR-TB treatment in Xiamen.

Exclusion criteria

- Pregnancy
- HIV infection
- Critically ill patients admitted to the ICU
- Confirmed extensively drug-resistant TB by DST
- Ongoing medication for MDR-TB (i.e. five active drugs or more for more than one day.)

Study outline

The overall study outline is shown in Figure 1. After informed consent, a completed inclusion questionnaire with demographic and clinical information, baseline blood and sputum samples are collected from the patient by a designated study nurse. The first day of MDR-TB treatment is defined as "day 0". Clinical data is collected at inclusion, day 2, week 1, 2, 4, 8 and week 12 after treatment initiation. Adverse events, routine blood tests and vital signs are closely monitored to ensure the safety of the study patients. The final treatment outcome is recorded at the end of MDR-TB treatment.

Drug concentrations of second-line TB drugs are measured at steady state at week 2, 4 and week 8 after treatment initiation. In order to estimate the AUC, multiple blood samples for

drug concentration analysis (i.e. rich sampling) are collected at week 2 (0, 1, 2, 4, 6, 8 and 10 hours after drug intake). A sparse-sampling strategy is applied at week 4 and 8 (0, 4 and 6 hours). Whole blood samples are simultaneously collected and pipetted directly onto DBS cards. Finger prick blood samples are collected on DBS cards at week 2 (0, 4 and 6 hours after drug intake) (Figure 2). The drug concentrations in plasma and DBS will be analysed using liquid-chromatography tandem mass spectrometry (LC-MS/MS) and immunoassay (25). In order to assess delayed absorption and possible interactions, information of concomitant drugs is noted in the medical records. Additionally, detailed food intake is noted by the patient in a diary on the days of blood sample collection. Pharmocometric modelling and simulation will be performed in the analysis phase.

Sputum is collected at day 0, 2 and week 1, 2, 4, 8 and 12 in order to evaluate changes in TTP. Whole genome sequencing (WGS) and MIC determination for TB drugs (pyrazinamide, ethambutol, levofloxacin, moxifloxacin, ofloxacin, cycloserine, ethionamide, PAS, amikacin, kanamycin, rifampicin and isoniazid) are performed at baseline and for any positive culture after one month or more of treatment, to assess development of acquired resistance. Time to sputum culture conversion is defined as the day from starting treatment until the day of the first of two consecutive negative sputum cultures, collected at least 30 days apart.

Disease severity is estimated and monitored using inflammatory markers such as CRP and ESR, presence of cavity on chest x-ray as well as the total score obtained in TB-scoreII, based on the following variables; cough, dyspnoea, chest pain, anaemia (pale lower conjunctivas), BMI and mid upper arm circumference (MUAC) (21). Quality of life during the first three months of treatment is estimated using the validated EQ-5D-5L-5L (Mandarin version) (26).

Laboratory methods

Drug concentration measurement

A combined assay for drug concentration analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS) is under development at the Xiamen CDC to measure the plasma concentrations of pyrazinamide, ethambutol, levofloxacin, moxifloxacin, cycloserine, prothionamide and PAS (27, 28). The second-line injectable drug amikacin will be analysed with a commercial immunoassay kit (Amikacin Assay kit, Beckman Coultier™). The collected venous blood samples will be centrifuged at 3500 rpm for 10 minutes within one hour from sampling. Aliquots of plasma are then frozen at -70ºC awaiting analysis.

A puncture from the DBS card will be immersed in extraction solution as previously described (18) and analysed through LC-MS/MS. Plasma concentrations will be compared with blood concentrations collected by DBS (18).

Microbiology – time to positivity (TTP), drug susceptibility testing (DST) and minimum inhibitory concentrations (MIC)

All microbiological tests are carried out at a biosafety laboratory level 3 (BSL-3) Biosafety Containment laboratory at the Xiamen CDC, apart from routine DST testing which is partly performed in local hospital laboratories and WGS analysis performed at the Public Health Agency of Sweden.

Time to culture positivity

Sputum samples are treated according to Chinese National standards based on a WHO recommended protocol (29). In short, NALC-NaOH is added to the sputum, then shaken using a vortex shaker until fully liquefied, followed by incubation for 15 minutes in room temperature. Phosphate buffer is added to reach a total volume of 45 ml after which the solution is centrifuged for 15 minutes at 3000g. The supernatant is removed and 1 ml phosphate buffer is added. Finally, 0,5 ml of the solution is transferred using a pipette to two labelled MGIT tubes, gently tilted for 1 minute and incubated in the BACTEC MGIT™ 960

machine at 37ºC. TTP is done in duplicate and is automatically recorded by the BACTEC MGIT™ 960.

Drug susceptibility testing (DST)

Routine DST is performed according to Chinese National guidelines with the proportion method on Lowenstein-Jensen (LJ) medium, according to WHO's recommendations (30).

Simultaneous MIC determination of Mtb using Sensititre broth microdilution plates TREK™

Since MIC testing in BACTEC MGIT™ is labour-intense and time-consuming, a high throughput broth microdilution plate has been developed to test up to 12 antibiotics simultaneously in Middlebrook 7H9 on a single MIC plate. We have designed a custom-made Sensititre plate for the drugs used at the study site, with concentration ranges including wild-type isolates, manufactured by Thermofisher™ (Figure 3). The reference isolate H37Rv ATCC 27294 is always included in each test run and compared to previously published quality control target ranges for each drug (31). The Thermofisher™ Sensititre MYCOTB plate is used for internal validation of ethionamide (range 0.5 mg/L-32 mg/L), which were not stable in the pre-trial validation of the customized plate.

After positive culture of Mtb in the BACTEC MGIT™ 960 and recording of TTP, the isolates are stored at -80 ºC awaiting MIC determination. After thawing and re-culturing on LJ media, bacterial suspensions are prepared from Mtb isolates which are no more than two weeks old. Bacterial suspension together with Middlebrook 7H9 stock solution are then added to each well, according to the manufacturer's instructions (32). The plates are sealed and left to incubate in 37 ºC. Manual reading is done after 10-21 days, depending on growth, assisted by an inverted mirror (Figure 4).

Due to specific pH requirements, pyrazinamide (PZA) susceptibility is determined using BACTEC MGIT™ 960 PZA Susceptibility Test®, with a pH of 5.9 as previously described (33). In short, colonies of Mtb no older than two weeks are suspended in Middlebrook 7H9 broth with phosphate-buffered saline (PBS). A bacterial suspension, corresponding to a McFarland turbidity of 0.5, is prepared. Following the test protocol provided by the manufacturer (Becton Dickinson Biosciences, Sparks, MD), the suspension is thereafter diluted 1:5 (inoculum A), from which a 1:10-diluted control is prepared (inoculum B). From inoculums A and B, 0.5 mL is then added to the MGIT 960 PZA tubes and the proportional growth control tube, respectively. The tubes are incubated in 37 ºC and read automatically by the BACTEC MGIT™.

Whole genome sequencing

All baseline study isolates, as well as any viable isolate after at least one month of treatment or more, will be analysed using WGS to detect new resistance mutations. In brief, DNA is extracted from Mtb LJ cultures using a chloroform/CTAB (N-cetyl-N,N,N-trimethyl ammonium bromide)-based protocol (34), transported to Sweden and sequenced using Illumina technology (Illumina Inc., San Diego, CA, USA).

Mapping to a set of resistant genes from the Mtb H37Rv reference genome (GeneBank accession nr NC_000962.3) and extraction of variants are performed in CLC Genomics Workbench 8 (Qiagen, Hilden, Germany) using the following filters: minimum coverage: 10x; minimum count of reads calling variants: 2, minimum frequency of reads calling variants: 10%; minimum frequency of reads calling variants in each direction: 5%. In addition, pyro-error variants in homopolymer regions with a minimum length of 3 and a frequency below 0.8, are removed. The remaining variants are then compared to our in-house database of resistance mutations.

Data analysis plan

Regular study monitoring is performed quarterly by the Swedish and Chinese researchers as well as biweekly reports from the study site. Study data from the case report forms are entered in EpiData with a range check by two independent researchers and results compared for coherence.

The distribution of AUC/MIC and C_{max}/AUC will be presented and visualised in graphs. The agreement between drug exposure in plasma and DBS will be assessed. An exploratory analysis of the PK/PD indices for key TB-drugs, such as fluoroquinolones, in relation to sputum culture conversion, TTP and changes in TBscore II during treatment will be performed. Pharmacometric modelling will assess the relationship between dose, concentrations and effect and population models will be applied, using the nonlinear mixed effects modelling software NONMEM (Icon Development Solutions, Ellicot city). Time to event data with censoring will be analysed using the Cox regression model, whereas binary outcomes will be analysed with logistic regression and continuous outcome with linear regression, if data is normally distributed. The validated Chinese value set of the quality of life tool EQ-5D-5L will be used and quality of life perception described.

For analysis of trends in drug exposure over time, the dependent nature of the data will be taken into account using mixed-effect models. Missing values will not be imputated. A p-value of <0.05 will be considered as statistically significant.

Information of potential confounders such as age, gender, BMI, concomitant treatment and comorbidities and disease severity assessed by TBscore II will be collected and evaluated during data analysis. As this is a feasibility and hypothesis-generating study, no power-calculation was performed.

Ethics and dissemination

The study is performed in accordance with Good Clinical Practice and the Declaration of Helsinki. Ethical approval was obtained from the regional Ethical Review Board of Stockholm (approval number EPN: 2015/646 31/1) and the Institutional Review Board of the School of Public Health, Fudan University, China (approval number IRB 2015-09-0565).

Prior to the study start, a designated study team of nurses, doctors and laboratory staff participated in training workshops of the study protocol and ethical considerations, led by the main study investigators from Fudan University and Karolinska Institutet. Patients are informed about the study orally and in writing, including information that neither study participation nor study termination will result in any changes in their treatment. An informed consent is signed or, in the case of illiteracy, a fingerprint given under observation by a witness. A travel grant to enable follow-up is offered to all the study participants. The sum was set as not to create financial motivation to accept study participation. All patients are treated according to standard of care at the designated MDR TB hospital and patients' safety ensured by regular monitoring.

Extensive blood sampling is a sensitive issue in China and should be avoided in severely ill patients. Therefore, the number of blood samples collected have been reduced to a minimum for the estimation of the AUC. Moreover, extensive blood sampling should be minimised in severely ill patients. An intravenous line inserted to minimise patient discomfort. The increased sputum sample collection is a potential hazardous risk for other patients, hospital and laboratory staff. Therefore, bio-safety and awareness training, as well as an upgrade of bio-safety equipment, have been implemented.

Dissemination

We aim to present our data in international conferences and to publish our results in a peer-reviewed journal, regardless of study results. Any significant protocol amendments will be reported to the respective ethical boards in Sweden and China.

Discussion

In this prospective observational cohort study, we present a comprehensive approach to TDM studies in MDR-TB and our translational approach is likely to be of benefit in future trials in the area. Multiple blood sampling and individual MIC determination will enable exploration of AUC/MIC for MDR-TB drugs, a poorly-investigated research area. In a key study, the level of peak drug concentrations and AUC of pyrazinamide, rifampicin and isoniazid strongly influenced treatment outcome, although no comparison with the Mtb MIC was performed (4). The bacterial MIC has also been found to influence treatment outcome of MDR-TB patients, with a six-fold increased odds of failure when comparing MIC of gatifloxacin of ≤0.25 mg/L to 1 mg/L, although both concentrations are still regarded as susceptible (35). There are very few tentative targets for most second-line TB drugs, although an AUC/MIC >100 for fluoroquinolones has been suggested (36). So far, the tentative targets have not been correlated with clinical outcome. To our knowledge, this is the first study to assess both AUCs and individual MICs for the most commonly used second-line drugs in MDR-TB regimen.

Not only have optimal PK/PD targets not been established, the critical concentrations used for DST are poorly validated (37). MIC determination provides more information of the level of the resistance, but it has the drawback of being time-consuming. Fortunately, commercial MIC plates are available, facilitating fast MIC determination and will be assessed in this study (38). When assessing individual MICs, the clinician should bear in mind the innate variability may be up to ± 1 two-fold MIC dilution step, but often less in a meticulous laboratory, which

impacts on PK/PD indices estimates. Furthermore, when results of MIC testing are reported, it is important to note that there is no reference method for MIC testing of Mtb.

A limitation of this study protocol is the limited target number of included patients, in common with other studies in the field, mainly due to MDR-TB incidence in Xiamen and costly and cumbersome sampling procedures. This may not allow us to perform extensive analysis of PK/PD indices in relation to treatment outcome, especially since all patients are treated with multiple drugs. However, pharmocometric mathematical modelling and simulation enables reduced sample sizes in clinical trials (39) and may partly compensate for the limited number of study patients in our study. Also, we use markers of early clinical improvement using microbiological surrogate measurements, such as TTP and SCC, due to the nature of long treatment periods and follow-up. Sputum culture conversion is an imperfect surrogate marker of the final treatment outcome but it is, nevertheless, commonly used in clinical trials and is a sign of clinical improvement (40).

This study will provide useful insights of the PK/PD relations in MDR-TB treatment and highlight the importance of individualised treatment, taking both drug concentrations and MICs and innovative surrogate markers of improvement into account. With a simultaneous method for drug concentration analysis and blood sample collection simplified through DBS, TDM would be more feasible in clinical practice, including low-resource, high-endemic settings. We hope that this study will inspire future randomised controlled studies for TDM for both drug susceptible and MDR-TB, including treatment groups such as children, pregnant women, diabetic and HIV infected patients who are prone to altered PK characteristics.

Author contributions: LDF, KN, JP, TS, YH and JB wrote the protocol for research ethics committees. All authors contributed to the study design and all authors revised and approved the final version of the manuscript.

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Competing interest

None to declare.

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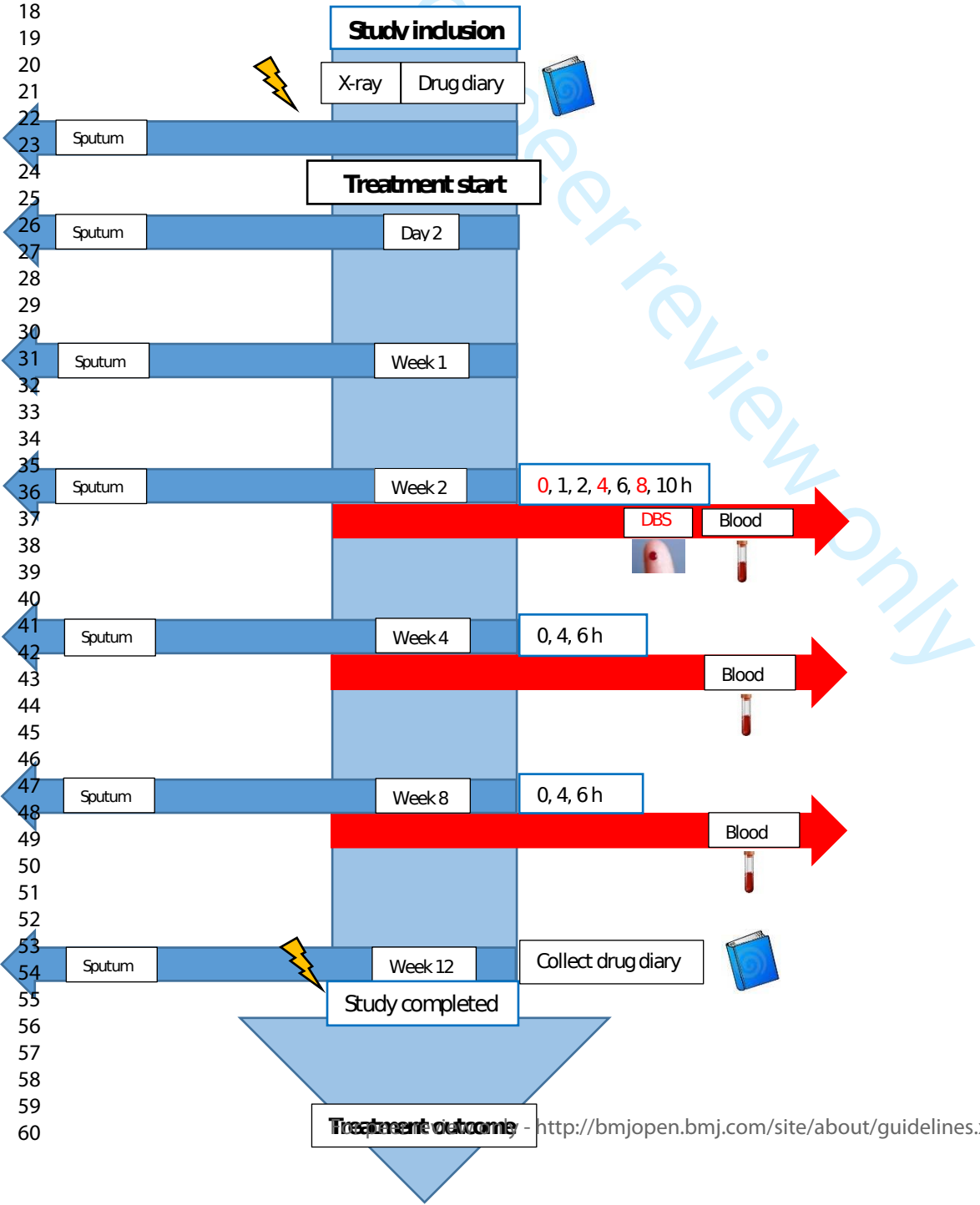

Figure 2. Blood collected through finger prick onto dried blood spot (DBS) filter paper.

101x68mm (240 x 240 DPI)

	1	2	3	4	5	6	7	8	9	10	11	12
A	OFL 16	MOXI 4	RIF 8	AMI 16	CAP 16	LEVO 8	PAS 16	ETH 16	EMB 16	CYK 64	KAN 16	INH 4
B	OFL 8	MOXI 2	RIF 4	AMI 8	CAP 8	LEVO 4	PAS 8	ETH 8	EMB 8	CYK 32	KAN 8	INH 2
C	OFL 4	MOXI 1	RIF 2	AMI 4	CAP 4	LEVO 2	PAS 4	ETH 5	EMB 5	CYK 16	KAN 4	INH 1
D	OFL 2	MOXI 0,5	RIF 1	AMI 2	CAP 2,5	LEVO 1	PAS 2	ETH 4	EMB 4	CYK 8	KAN 2	INH 0,5
E	OFL 1	MOXI 0,25	RIF 0,5	AMI 1	CAP 2	LEVO 0,5	PAS 1	ETH 2	EMB 2	CYK 4	KAN 1	INH 0,25
F	OFL 0,5	MOXI 0,12	PTH 0,5	AMI 0,5	CAP 1	LEVO 0,25	PAS 0,5	ETH 1	EMB 1	PTH 1	KAN 0,5	INH 0,12
	OFL 0,25	MOXI 0,06	PTH 0,25	AMI 0,25	CAP 0,5	LEVO 0,12	PAS 0,25	ETH 0,5	EMB 0,5	PTH 2	KAN 0,25	PTH 2,5
	OFL 0,12	MOXI 0,03	PTH 0,12	AMI 0,12	CAP 0,25	LEVO 0,06	PAS 0,12	ETH 0,25	EMB 0,25	PTH 4	POS	POS

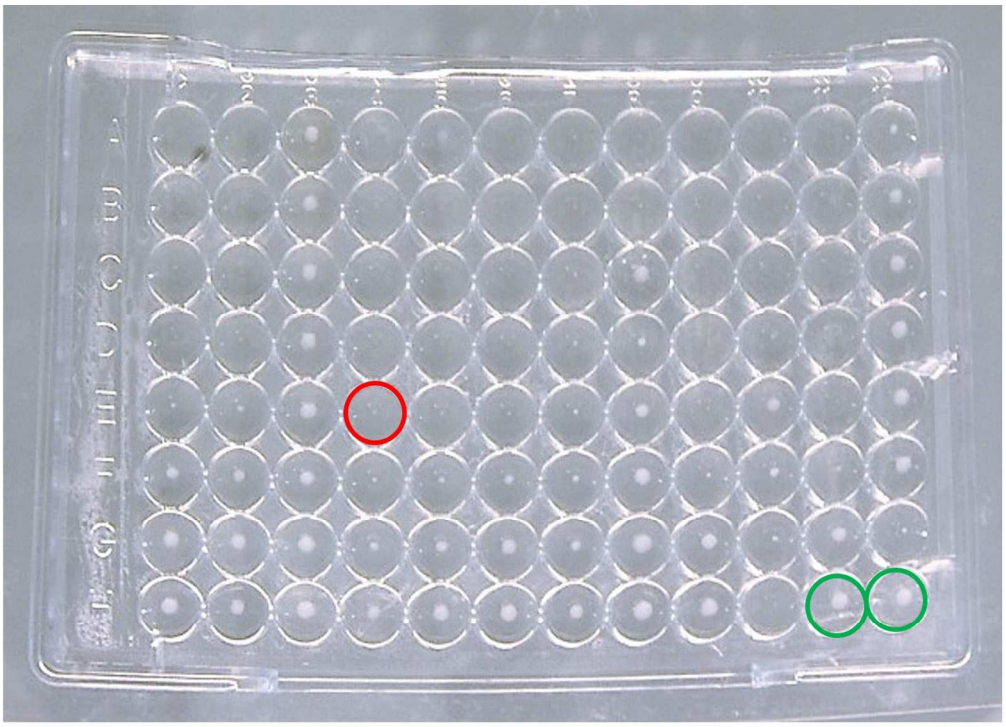

Figure 4. Example of minimum inhibitory concentration (MIC) determination using microdilution plate. The red ring denotes the first well of Amikacin (AMI) will no visible growth, i.e. the MIC of AMI of this Mycobacterium tuberculosis isolates is 1 mg/L. The two green rings represent growth controls.

401x289mm (96 x 96 DPI)

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Plasma concentrations of second-line anti-tuberculosis drugs in relation to minimum inhibitory concentrations in multidrug-resistant tuberculosis patients in China – a study protocol of a prospective observational cohort study

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Plasma concentrations of second-line anti-tuberculosis drugs in relation to minimum inhibitory concentrations in multidrug-resistant tuberculosis patients in China – a study protocol of a prospective observational cohort study

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Abstract

Introduction

Individualized treatment through therapeutic drug monitoring (TDM) may improve tuberculosis (TB) treatment outcomes but is not routinely implemented. Prospective clinical studies of drug exposure and minimum inhibitory concentrations (MICs) in multidrug-resistant tuberculosis (MDR-TB) are scarce.

This translational study aims to characterize the area under the concentration-time curve (AUC) of individual MDR-TB drugs, divided by the MIC for M tuberculosis (Mtb) isolates, to explore associations with markers of treatment progress and to develop useful strategies for clinical implementation of TDM in MDR-TB.

Methods and analysis

Adult pulmonary MDR-TB patients treated in Xiamen, China, are included. Plasma samples for measure of drug exposure are obtained at 0, 1, 2, 4, 6, 8 and 10 hours after drug intake at week 2 and at 0, 4, and 6 hours during week 4 and 8. Sputum samples for evaluating time to culture positivity (TTP) and MIC determination, are collected at day 0, 2, 7 and at week 2, 4, 8 and 12 after treatment initiation. Disease severity are assessed with a clinical scoring tool (TBscore II) and quality of life evaluated using EQ-5D-5L.

Drug concentrations of pyrazinamide, ethambutol, levofloxacin, moxifloxacin, cycloserine, prothionamide and para-aminosalicylate are measured by liquid chromatography-mass tandem spectrometry (LC-MS/MS) and the levels of amikacin measured by immunoassay. Dried blood spot (DBS) on filter paper, to facilitate blood sampling for analysis of drug concentrations, is also evaluated.

The MICs of the drugs listed above are determined using custom-made broth microdilution plates and MYCOTB® plates with Middlebrook 7H9 media. MIC determination of pyrazinamide is performed in BACTEC MGIT™ 960.

Ethics and dissemination

This study has been approved by the Ethical Review Boards of Karolinska Institutet, Sweden and Fudan University, China. Informed written consent is given by participants. The study results will submitted to a peer-reviewed journal.

Study registration number: clintrials.gov NCT02816931

Strengths and limitations of this study:

- To our knowledge, this is a novel study approach which fully investigates the distribution of drug exposure in relation to minimum inhibitory concentration (MIC) for *Mycobacterium tuberculosis* isolates from multidrug-resistant tuberculosis patients along with biomarkers (e.g. time to positivity; TTP), culture conversion and the clinical scoring tool TBscore II to assess treatment outcome.
- We used a translational approach with experts from research centres across the world to design a study protocol including both MIC-determinations, drug exposure estimation using novel technology, as well as microbiological and clinical surrogate markers for improvement, to enable strategies for therapeutic drug monitoring (TDM) use in TB treatment.
- The patients' drug exposure will be compared with individual Mtb MICs, exploring pharmacokinetics-pharmacodynamics (PK/PD) indices in MDR-TB treatment.
- Dried blood spot (DBS) as a method to simplify blood sampling by finger prick instead of venous sampling will be investigated.
- A limitation of the study is the low target number of patients for inclusion, due to a laborious and costly study protocol, which might partly be compensated for by using pharmacometric modelling.

Introduction

Despite programmatic management of tuberculosis (TB), the incidence of multidrug-resistant tuberculosis (MDR-TB), defined as *M. tuberculosis* (Mtb) resistant to rifampicin and isoniazid, is steadily increasing (1). Inconsistent treatment, due to poor treatment adherence, lack of drugs, as well as sub-therapeutic dosing are contributing factors. For many TB drugs the administered dose is not predictive of the drug exposure and clinical effect in the patient (2). A hollow-fibre study indicated that pharmacokinetic variability may be an underestimated cause of drug resistance development (3) and low drug concentrations in the treatment of drug susceptible TB have been associated with poor outcome in some prospective studies (4, 5).

Therapeutic drug monitoring (TDM) is a strategy to personalize treatment by measuring systemic drug levels in blood/plasma as a guide for individual dose adjustments (6). Specifically for infectious diseases, the drug efficacy not only depends on the drug exposure but also on the susceptibility level of the bacteria, the minimum inhibitory concentration (MIC) (7). The MIC is defined as the lowest concentration of a drug that inhibits visible growth of bacilli and should be exceeded to cure the infection (8). TDM has been recommended during MDR-TB treatment by several organisations, for example the Infectious Disease Society of America (9). The pharmacokinetic studies that have been performed have shown that sub-therapeutic drug levels in TB treatment are common, although with conflicting results regarding association between drug exposure and treatment outcome (10-12). However, studies on MDR-TB are limited and only a few studies have included drug concentrations as well as the individual MICs of the bacteria (13-15).

An optimal estimation of drug exposure (i.e. area under the concentration versus time curve; AUC) traditionally requires multiple venous blood samples, often followed by prompt centrifugation and sample storage at -80 ºC. A simplified strategy for collection and transportation of blood samples needed for TDM would aid its implementation in clinical

practice. Dried blood spot (DBS) allows minimal blood sampling by capillary finger pricking on filter paper, which can be transported without a cold chain, simplifying transportation and storage (16). DBS is a well-established and validated method, but has only been evaluated for a few second-line TB drugs, for example moxifloxacin and linezolid (16-18). A clinical implementation of DBS could enable TDM for TB treatment in remote areas and reduce costs (16).

There is scarce data regarding drug exposure and treatment outcome in MDR-TB treatment. Assessing end-of treatment outcome in MDR-TB studies is cumbersome due to long treatment durations. Other strategies include using interim endpoints such as time to positivity (TTP) in liquid culture media, a surrogate of bactericidal activity (19), and sputum culture conversion after two or three months of treatment (20), the latter commonly used in drug efficacy studies. A clinical composite scoring system, TBscore II, can be used as a surrogate marker for TB disease severity and to predict failure (21). Patients' quality of life can be objectified using the validated EQ-5D-5L tool assessing five different dimensions (mobility, self-care, typical activity, pain/discomfort and anxiety/depression), an often overlooked tool in clinical treatment studies.

China has the second highest burden of MDR-TB in the world and has existing resources to perform TDM, thus making it an ideal setting for pharmacokinetic/pharmacodynamic (PK/PD) studies. The overall incidence of TB in China was 895 000 TB cases in 2017, of which 8.2% were MDR-TB (22).

We describe a new comprehensive approach to TDM studies, assessing drug exposure, individual MICs as well as clinical outcome markers. The primary aim of the study is to investigate the distribution of AUC/MIC and C_{max}/MIC for MDR-TB drugs during MDR-TB treatment in China. Secondary aims are to analyse AUC/MIC in relationship to markers of clinical improvement, such as sputum culture conversion, TTP, TBscore II, body mass index

(BMI) and qualitative measures of well-being (EQ-5D-5L). Signs of acquired resistance are assessed by investigating changes in MICs and genetic mutations, during the first three months of treatment. A clinical implementation of DBS as well as a method of simultaneous MIC determination are assessed to simplify the use of TDM in clinical practice.

Methods and analysis

Study design

We are conducting a prospective cohort study of TB drug exposure and MICs in MDR-TB patients in Xiamen, China. This is a joint project between the School of Public Health Fudan University Shanghai, Department of Medicine Karolinska Institutet, Department of Pharmaceutical Biosciences University of Uppsala and the Public Health Agency of Sweden, in collaboration with the Centre for Disease Control (CDC) in Xiamen. The study protocol conforms with the STROBE Statement for cohort studies (23).

Patient and public involvement

The original study protocol by the co-authors was changed by reducing the number of blood samples after feed-back from patients included in a pilot study. The result of the study can be obtained in Mandarin upon request at the Xiamen CDC. Patients were not involved in the recruitment and the conduct of the study.

Study setting

The study is carried out in Xiamen, Fujian region in Southeast China, where the incidence of TB in 2016 was 42.4 cases/100 000 inhabitants and of the 1661 confirmed cases that year, there were 28 MDR-TB patients (1.7%) (24).

The study hospital is the designated TB hospital in Xin Ling, Xiamen, a large teaching hospital with a specialised TB-ward with 105 beds as well as a negative pressure ward (12

beds) with specialised TB physicians and nurses. Recruitment of patients is performed by the Xiamen CDC, which also keeps a screening log. Patients are routinely admitted for two months of in-patient treatment. The study is registered at ClinicalTrials.gov (NCT02816931) and opened 17th of April 2016.

Study participants

A total number of 30 fully evaluable patients, according to the criteria below, will be included.

Inclusion criteria

- Consenting adults (≥18 years) with a verified diagnosis of pulmonary MDR-TB, by routine drug susceptibility testing (DST) admitted to the Xin Ling TB hospital, Xiamen.
- Eligible for and consent to MDR-TB treatment in Xiamen.

Exclusion criteria

- Pregnancy
- HIV infection
- Patients admitted to the ICU
- Confirmed extensively drug-resistant TB by DST
- Ongoing medication for MDR-TB (i.e. five active drugs or more for more than one day.)

Study outline

The overall study outline is shown in Figure 1. After informed consent, a completed inclusion questionnaire with demographic and clinical information, baseline blood and sputum samples are collected from the patient by a designated study nurse. Treatment regimens adhere to

WHO guidelines and are adjusted following DST results. The first day of MDR-TB treatment is defined as "day 0". Clinical data is collected at inclusion, day 2, week 1, 2, 4, 8 and week 12 after treatment initiation. Adverse events, routine blood tests and vital signs are closely monitored to ensure the safety of the study patients. The final treatment outcome is recorded at the end of MDR-TB treatment.

Drug concentrations of second-line TB drugs are measured at steady state at week 2, 4 and week 8 after treatment initiation. In order to estimate the AUC, multiple blood samples for drug concentration analysis (i.e. rich sampling) are collected at week 2 (0, 1, 2, 4, 6, 8 and 10 hours after drug intake). A sparse-sampling strategy is applied at week 4 and 8 (0, 4 and 6 hours). Whole blood samples are simultaneously collected and pipetted directly onto DBS cards. Finger prick blood samples are collected on DBS cards at week 2 (0, 4 and 6 hours after drug intake) (Figure 2). The drug concentrations in plasma and DBS will be analysed using liquid-chromatography tandem mass spectrometry (LC-MS/MS) and immunoassay (25). In order to assess delayed absorption and possible interactions, information of concomitant drugs is noted in the medical records. Additionally, detailed food intake is noted by the patient in a diary on the days of blood sample collection. Pharmacometric modelling and simulation will be performed in the analysis phase.

Sputum is collected at day 0, 2 and week 1, 2, 4, 8 and 12 in order to evaluate changes in TTP. Whole genome sequencing (WGS) and MIC determination for TB drugs (pyrazinamide (PZA), ethambutol, levofloxacin, moxifloxacin, ofloxacin, cycloserine, ethionamide, para-aminosalicylic acid (PAS), amikacin, kanamycin, rifampicin and isoniazid) are performed at baseline and for any positive culture after one month or more of treatment, to assess development of acquired resistance. Time to sputum culture conversion is defined as the day from starting treatment until the day of the first of two consecutive negative sputum cultures, collected at least 30 days apart.

Disease severity is estimated and monitored using inflammatory markers such as CRP and ESR, presence of cavity on chest x-ray as well as the total score obtained in TB-score II, based on the following variables; cough, dyspnoea, chest pain, anaemia (pale lower conjunctivas), BMI and mid upper arm circumference (MUAC) (21). Quality of life during the first three months of treatment is estimated using the validated EQ-5D-5L-5L (Mandarin version) (26). The patients are followed up until treatment completion or loss to follow-up, whichever occurs first, through information accessible from the TB-registry, Xiamen CDC.

Laboratory methods

Drug concentration measurement

A combined assay for drug concentration analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS) is under development at the Xiamen CDC to measure the plasma concentrations of PZA, ethambutol, levofloxacin, moxifloxacin, cycloserine, prothionamide and PAS (27, 28). The second-line injectable drug amikacin will be analysed with a commercial immunoassay kit (Amikacin Assay kit, Beckman Coultier™). The collected venous blood samples will be centrifuged at 3500 rpm for 10 minutes within one hour from sampling. Aliquots of plasma are then frozen at -70ºC awaiting analysis.

A puncture from the DBS card will be immersed in extraction solution as previously described (18) and analysed through LC-MS/MS. Plasma concentrations will be compared with blood concentrations collected by DBS (18).

Microbiology – time to positivity (TTP), drug susceptibility testing (DST) and minimum inhibitory concentrations (MIC)

All microbiological tests are carried out at a biosafety laboratory level 3 (BSL-3) at the Xiamen CDC, apart from routine DST testing which is partly performed in local hospital laboratories and WGS analysis performed at the Public Health Agency of Sweden.

Time to culture positivity

Sputum samples are treated according to Chinese National standards based on a WHO recommended protocol (29). In short, NALC-NaOH is added to the sputum, then shaken using a vortex shaker until fully liquefied, followed by incubation for 15 minutes in room temperature. Phosphate buffer is added to reach a total volume of 45 ml after which the solution is centrifuged for 15 minutes at 3000g. The supernatant is removed and 1 ml phosphate buffer is added. Finally, 0.5 ml of the solution is transferred using a pipette to two labelled MGIT tubes, gently tilted for 1 minute and incubated in the BACTEC MGIT™ 960 machine at 37ºC. TTP is done in duplicate and is automatically recorded by the BACTEC MGIT™ 960.

Drug susceptibility testing (DST)

Routine DST is performed according to Chinese National guidelines with the proportion method on Lowenstein-Jensen (LJ) medium, according to WHO's recommendations (30).

Simultaneous MIC determination of Mtb using Sensititre broth microdilution plates TREK™

Since MIC testing in BACTEC MGIT™ is labour-intense and time-consuming, a high throughput broth microdilution plate has been developed to test up to 12 antibiotics simultaneously in Middlebrook 7H9 on a single MIC plate. We have designed a custom-made Sensititre plate for the drugs used at the study site, with concentration ranges including wild-type isolates, manufactured by Thermofisher™ (Figure 3). The reference isolate H37Rv ATCC 27294 is always included in each test run and compared to previously published quality control target ranges for each drug (31). The Thermofisher™ Sensititre MYCOTB plate is used for internal validation of ethionamide (range 0.5 mg/L-32 mg/L), which were not stable in the pre-trial validation of the customized plate.

After positive culture of Mtb in the BACTEC MGIT™ 960 and recording of TTP, the isolates are stored at -80 ºC awaiting MIC determination. After thawing and re-culturing on LJ media, bacterial suspensions are prepared from Mtb isolates which are no more than two weeks old. Bacterial suspension together with Middlebrook 7H9 stock solution are then added to each well, according to the manufacturer's instructions (32). The plates are sealed and left to incubate in 37 ºC. Manual reading is done after 10-21 days, depending on growth, assisted by an inverted mirror (Figure 4).

Due to specific pH requirements, PZA susceptibility is determined using BACTEC MGIT™ 960 PZA Susceptibility Test®, with a pH of 5.9 as previously described (33). In short, colonies of Mtb no older than two weeks are suspended in Middlebrook 7H9 broth with phosphate-buffered saline (PBS). A bacterial suspension, corresponding to a McFarland turbidity of 0.5, is prepared. Following the test protocol provided by the manufacturer (Becton Dickinson Biosciences, Sparks, MD), the suspension is thereafter diluted 1:5 (inoculum A), from which a 1:10-diluted control is prepared (inoculum B). From inoculums A and B, 0.5 mL is then added to the MGIT 960 PZA tubes and the proportional growth control tube, respectively. The tubes are incubated in 37 ºC and read automatically by the BACTEC MGIT™.

Whole genome sequencing

All baseline study isolates, as well as any viable isolate after at least one month of treatment or more, will be analysed using WGS to detect new resistance mutations. In brief, DNA is extracted from Mtb LJ cultures using a chloroform/CTAB (N-cetyl-N,N,N-trimethyl ammonium bromide)-based protocol (34), transported to Sweden and sequenced using Illumina technology (Illumina Inc., San Diego, CA, USA).

Mapping to a set of resistant genes from the Mtb H37Rv reference genome (GeneBank accession nr NC_000962.3) and extraction of variants are performed in CLC Genomics

Workbench 8 (Qiagen, Hilden, Germany) using the following filters: minimum coverage: 10x; minimum count of reads calling variants: 2, minimum frequency of reads calling variants: 10%; minimum frequency of reads calling variants in each direction: 5%. In addition, pyro-error variants in homopolymer regions with a minimum length of 3 and a frequency below 0.8, are removed. The remaining variants are then compared to our in-house database of resistance mutations.

Data analysis plan

Regular study monitoring is performed quarterly by the Swedish and Chinese researchers as well as biweekly reports from the study site. Study data from the case report forms are entered in EpiData with a range check by two independent researchers and results compared for coherence.

The distribution of AUC/MIC and C_{max}/AUC will be presented and visualised in graphs. The agreement between drug exposure in plasma and DBS will be assessed. An exploratory analysis of the PK/PD indices for key TB-drugs, such as fluoroquinolones, in relation to sputum culture conversion, TTP and changes in TBscore II during treatment will be performed. Pharmacometric modelling will assess the relationship between dose, concentrations and effect and population models will be applied, using the nonlinear mixed effects modelling software NONMEM (Icon Development Solutions, Ellicot city). Time to event data with censoring will be analysed using the Cox regression model, whereas binary outcomes will be analysed with logistic regression and continuous outcome with linear regression, if data is normally distributed. The validated Chinese value set of the quality of life tool EQ-5D-5L will be used and quality of life perception described.

For analysis of trends in drug exposure over time, the dependent nature of the data will be taken into account using mixed-effect models. Missing values will not be imputated. A p-value of <0.05 will be considered as statistically significant.

Information of potential confounders such as age, gender, BMI, concomitant treatment and comorbidities and disease severity assessed by TBscore II will be collected and evaluated during data analysis. As this is a feasibility and hypothesis-generating study, no power-calculation was performed.

Ethics and dissemination

The study is performed in accordance with Good Clinical Practice and the Declaration of Helsinki. Ethical approval was obtained from the regional Ethical Review Board of Stockholm (approval number EPN: 2015/646 31/1) and the Institutional Review Board of the School of Public Health, Fudan University, China (approval number IRB 2015-09-0565).

Prior to the study start, a designated study team of nurses, doctors and laboratory staff participated in training workshops of the study protocol and ethical considerations, led by the main study investigators from Fudan University and Karolinska Institutet. Patients are informed about the study orally and in writing, including information that neither study participation nor study termination will result in any changes in their treatment. An informed consent is signed or, in the case of illiteracy, a fingerprint given under observation by a witness. A travel grant to enable follow-up is offered to all the study participants. The sum was set as not to create financial motivation to accept study participation. All patients are treated according to standard of care at the designated MDR-TB hospital and patients' safety ensured by regular monitoring.

Extensive blood sampling is a sensitive issue in China and should be avoided in severely ill patients. Therefore, the number of blood samples collected have been reduced to a minimum

for the estimation of the AUC. Moreover, extensive blood sampling should be minimised in severely ill patients. An intravenous line is inserted to minimise patient discomfort. The increased sputum sample collection is a potential hazardous risk for other patients, hospital and laboratory staff. Therefore, bio-safety and awareness training, as well as an upgrade of bio-safety equipment, have been implemented.

Dissemination

We aim to present our data in international conferences and to publish our results in a peer-reviewed journal, regardless of study results. Any significant protocol amendments will be reported to the respective ethical boards in Sweden and China.

Discussion

In this prospective observational cohort study, we present a comprehensive, translational approach to TDM studies in MDR-TB, likely to be of benefit in future trials in the area. Multiple blood sampling and individual MIC determination will enable exploration of AUC/MIC for MDR-TB drugs, a poorly-investigated research area. In a key study, the level of peak drug concentrations and AUC of PZA, rifampicin and isoniazid strongly influenced treatment outcome, although no comparison with the Mtb MICs was performed (4). Bacterial MIC has also been found to influence treatment outcome of MDR-TB patients, with a six-fold increased odds of failure when comparing MIC of gatifloxacin of ≤0.25 mg/L to 1 mg/L, although both concentrations are still regarded as susceptible (35). There are very few tentative targets for most second-line TB drugs, although an AUC/MIC >100 for fluoroquinolones has been suggested (36). So far, the tentative targets have not been correlated with clinical outcome. To our knowledge, this is the first study to assess both AUCs and individual MICs for the most commonly used second-line drugs in MDR-TB regimen.

Not only have optimal PK/PD targets not been established, the critical concentrations used for DST are poorly validated (37). MIC determination provides more information of the level of the resistance, but it has the drawback of being time-consuming. Fortunately, commercial MIC plates are available, facilitating fast MIC determination and will be assessed in this study (38). When interpreting individual MICs, the clinician should bear in mind the innate variability may be up to ± 1 two-fold MIC dilution step, but often less in a meticulous laboratory, which impacts on PK/PD indices estimates. Furthermore, when results of MIC testing are reported, it is important to note that there is no reference method for MIC testing of Mtb.

A limitation of this study protocol is the limited target number of included patients, in common with other studies in the field, mainly due to MDR-TB incidence in Xiamen and costly and cumbersome sampling procedures. This may not allow us to perform extensive analysis of PK/PD indices in relation to treatment outcome, especially since all patients are treated with multiple drugs. However, pharmacometric mathematical modelling and simulation enables reduced sample sizes in clinical trials (39) and may partly compensate for the limited number of study patients in our study. Also, we use markers of early clinical improvement using microbiological surrogate measurements, such as TTP and sputum culture conversion, due to the nature of long treatment periods and follow-up. Sputum culture conversion is an imperfect surrogate marker of the final treatment outcome but it is, nevertheless, commonly used in clinical trials and is a sign of clinical improvement (40).

This study will provide useful insights of the PK/PD relations in MDR-TB treatment and highlight the importance of individualised treatment, taking both drug concentrations and MICs and innovative surrogate markers of improvement into account. With a simultaneous method for drug concentration analysis and blood sample collection simplified through DBS, TDM would be more feasible in clinical practice, including low-resource and high-endemic

settings. We hope that this study will inspire future randomised controlled studies for TDM for both drug susceptible and MDR-TB, including treatment groups such as children, pregnant women, diabetic and HIV infected patients who are prone to altered PK characteristics.

Data sharing statement: No data sharing is planned but can be discussed if requested.

Author contributions: LDF, KN, YH, RZ, XZ, JW, JP, US, EE, JWA, SH, XB, TS and JB designed the study. RK, WC, YH, CH, YL, YG, JW, XZ, TS, LDF, KN, MM developed the plan for the microbiological part of the study. US, EE, TS, XZ, YH, LDF, KN, JB, JK, WC, JWA developed the pharmacokinetic part of the study. LDF wrote the first draft of the manuscript together with KN and YH. All authors contributed and approved the final version.

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Competing interest

None to declare.

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Figure legends

Figure 1. Study overview. Study patients are given a drug diary to record concomitant drugs and food intake during the first 12 weeks. Sputum samples are collected regularly during the study to assess time to positivity (TTP) in Bactec MGIT. Rich blood sampling is collected after two weeks of treatment and sparse blood sampling at week six and eight. Venous blood samples are collected as well as finger pricks on dried blood spot (DBS). The final treatment outcome is registered after treatment completion.

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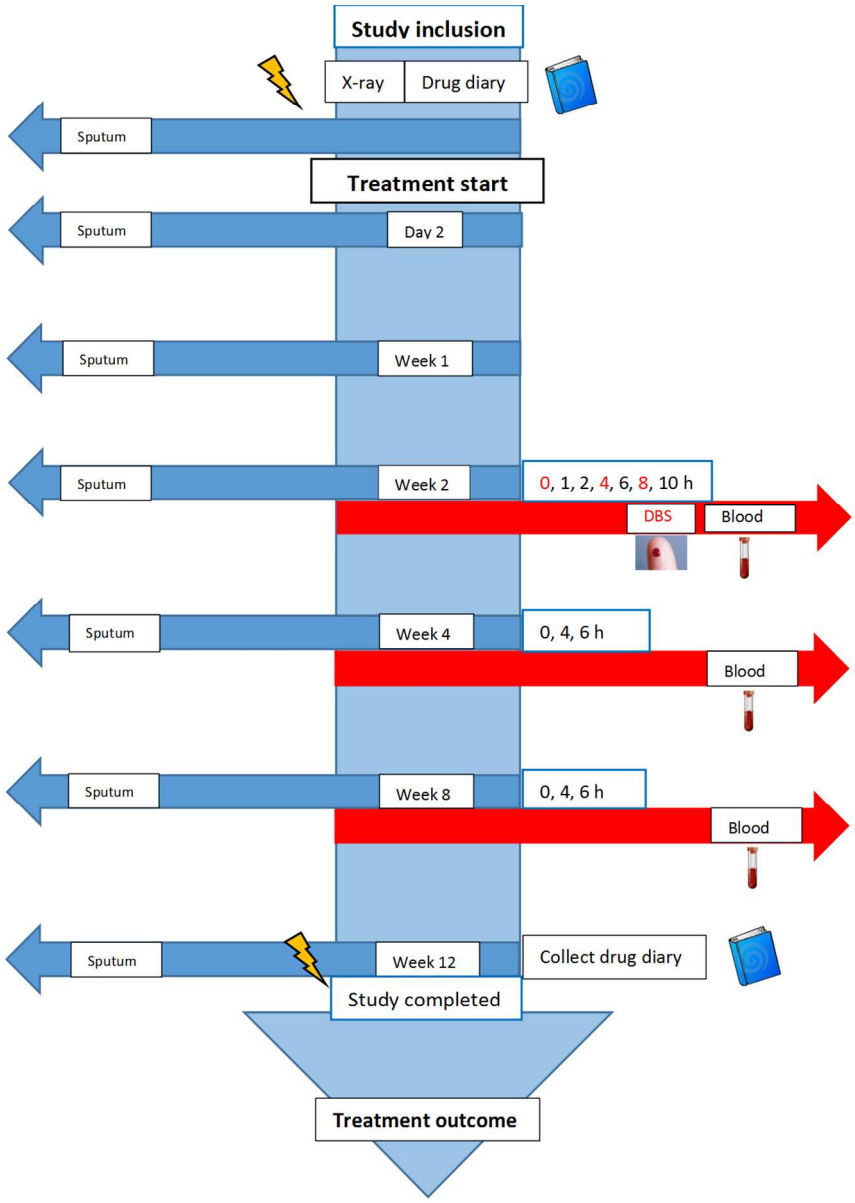

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122x146mm (300 x 300 DPI)

Figure 2. Blood collected through finger prick onto dried blood spot (DBS) filter paper.

81x54mm (300 x 300 DPI)

	1	2	3	4	5	6	7	8	9	10	11	12
A	OFL 16	MOXI 4	RIF 8	AMI 16	CAP 16	LEVO 8	PAS 16	ETH 16	EMB 16	CYK 64	KAN 16	INH 4
B	OFL 8	MOXI 2	RIF 4	AMI 8	CAP 8	LEVO 4	PAS 8	ETH 8	EMB 8	CYK 32	KAN 8	INH 2
C	OFL 4	MOXI 1	RIF 2	AMI 4	CAP 4	LEVO 2	PAS 4	ETH 5	EMB 5	CYK 16	KAN 4	INH 1
D	OFL 2	MOXI 0,5	RIF 1	AMI 2	CAP 2,5	LEVO 1	PAS 2	ETH 4	EMB 4	CYK 8	KAN 2	INH 0,5
E	OFL 1	MOXI 0,25	RIF 0,5	AMI 1	CAP 2	LEVO 0,5	PAS 1	ETH 2	EMB 2	CYK 4	KAN 1	INH 0,25
F	OFL 0,5	MOXI 0,12	PTH 0,5	AMI 0,5	CAP 1	LEVO 0,25	PAS 0,5	ETH 1	EMB 1	PTH 1	KAN 0,5	INH 0,12
G	OFL 0,25	MOXI 0,06	PTH 0,25	AMI 0,25	CAP 0,5	LEVO 0,12	PAS 0,25	ETH 0,5	EMB 0,5	PTH 2	KAN 0,25	PTH 2,5
H	OFL 0,12	MOXI 0,03	PTH 0,12	AMI 0,12	CAP 0,25	LEVO 0,06	PAS 0,12	ETH 0,25	EMB 0,25	PTH 4	POS	POS

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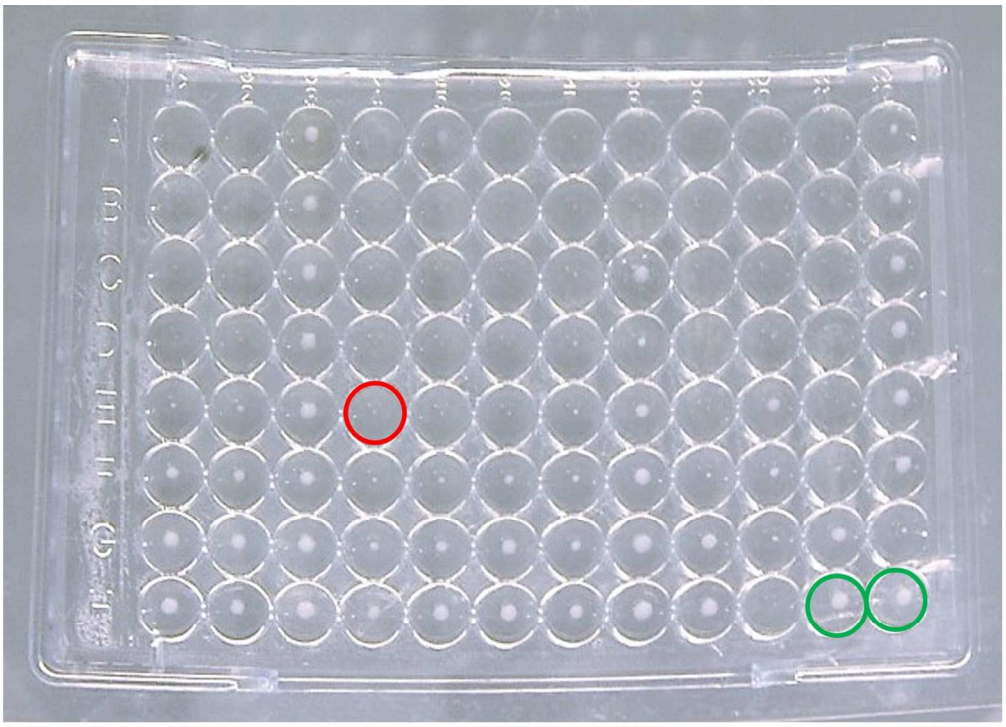
125x87mm (300 x 300 DPI)



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128x92mm (300 x 300 DPI)

STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale *Lines 60-104*	2	Explain the scientific background and rationale for the investigation being reported
Objectives *Lines 105-133*	3	State specific objectives, including any prespecified hypotheses
Methods		
Study design *Lines 116-121*	4	Present key elements of study design early in the paper
Setting *Lines 127-136*	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants *Lines 140-149* *Lines 183-184 follow-up*	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed
Variables *Lines 159-184*	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement *Lines 159-184*	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias *Prospective study, no recall-bias* *Line 133 Screening log, reduction of selection bias* *Line141 Verified MDR-TB cases reducing risk of misclassification bias.* *Lines 167-168* *Lines 257-279*	9	Describe any efforts to address potential sources of bias
Study size *Lines 278-279*	10	Explain how the study size was arrived at
Quantitative variables *Lines 262-271*	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods *Lines 257-279*	12	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) If applicable, explain how loss to follow-up was addressed
		(*e*) Describe any sensitivity analyses
Results		
Study protocol- not applicable		

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
		(c) Summarise follow-up time (eg, average and total amount)
Outcome data	15*	Report numbers of outcome events or summary measures over time
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion *Lines 306-347*		
Key results	18	Summarise key results with reference to study objectives
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding *Lines 355-358*	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.