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Efficacy and Safety of Anti-Tuberculosis Therapy: Pharmacokinetic and Pharmacogenetic Studies in Latvian Patient Population

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Abstract

The first studies supporting the use of rifampicin (RIF), pyrazinamide (PZA), isoniazid (INH), and ethambutol (ETB) for tuberculosis (TB) treatment date back to the 1950s and 1960s. Today, this four-drug combination is one of the first-line treatment regimens used for drug-susceptible tuberculosis (DS-TB). Yet, the many aspects of their exposure-response relationship remain unclear, likely hindering efforts to control this persistent global public health problem.

Regarding treatment efficacy, pharmacokinetic (PK) studies have reported substantial interpatient variability in drug plasma (or serum) exposure using standard doses, with lower values often linked to suboptimal treatment response and even treatment failure. At the same time, despite increasing interest in using C-reactive protein (CRP) as a biomarker of treatment response, data on the influence of anti-TB drug exposure on TB-associated inflammation, including the dynamics of serum CRP levels, are limited. As for safety, an important concern related to the combined RIF, PZA, INH, and ETB regimen is drug-induced liver injury (DILI), an adverse drug reaction with a complex and poorly understood nature, carrying the potential for fatal outcomes. To date, various patient- and disease-related factors have been described to affect the PK of anti-TB drugs and increase the risk of DILI. By comparison, pharmacogenetic (PGx) studies focusing on RIF-metabolising enzymes, transporters, and associated regulatory proteins have not yet reached a consensus on the impact of genetic variability in this context. Therefore, this Thesis aimed to investigate the effect of variants in RIF pharmacogenes on its plasma exposure and the development of anti-tuberculosis drug-induced liver injury (anti-TB DILI), as well as to characterise the early changes in serum CRP levels and their influencing factors in Latvian patients with DS-TB.

Towards this aim, two methodological studies and two subsequent prospective observational studies were conducted. The first methodological study focused on developing a liquid chromatography-tandem mass spectrometry method for quantifying RIF, PZA, INH, and ETB in human plasma, whereas the second involved creating a targeted next-generation sequencing protocol for PGx analysis. Both approaches were successfully applied to generate data, which were then interpreted in conjunction with clinically relevant information. Thus, the third study examined whether variants detected in the genes encoding enzymes (arylacetamide deacetylase gene), transporters (solute carrier organic anion transporter family member 1B1 and 1B3 genes, and adenosine triphosphate-binding cassette subfamily B member 1 gene), and nuclear receptors (nuclear receptor subfamily 1 group I member 2 [*NR1I2*] gene) involved in RIF disposition are related to its plasma exposure and the development of anti-TB DILI. The fourth study investigated the impact of patient- and disease-related factors, including

anti-TB drug plasma exposure, on the decline in serum CRP levels during the first 10–12 days of therapy and assessed the predictive value of this biomarker for bacteriological response.

In brief, analysis of the variants detected in the set of genes revealed the relationship between one intronic *NR1I2* gene variant (rs3732357) and variation in RIF plasma exposure. None of the examined variants were related to anti-TB DILI. When exploring early changes in serum CRP levels after the initiation of anti-TB treatment, several patient characteristics and disease severity at diagnosis were identified as factors affecting the reduction in levels of this biomarker. Despite considerable underexposure rates of RIF, PZA, INH, and ETB among enrolled patients, the plasma exposure of these drugs did not significantly influence the serum CRP levels within the given timeframe. Furthermore, levels of this biomarker on the 10^{th} to 12^{th} day of therapy, along with their change from baseline, were not predictive of bacteriological response.

Summarising all the above, this Thesis contributes to the current understanding of PK and PGx variability and their implications for the efficacy and safety of anti-TB treatment. It provides new data on the genotype-phenotype relationships for variants in the RIF pharmacogenes, offers a detailed characterisation of early changes in serum CRP levels, and underscores the clinical significance of the obtained findings. The methodologies developed for PK and PGx data acquisition hold practical value for future TB research and clinical management.

Keywords: tuberculosis, pharmacokinetics, pharmacogenetics, drug-induced liver injury, C-reactive protein, treatment response, liquid chromatography-tandem mass spectrometry, next-generation sequencing.

Anotācija

Prettuberkulozes terapijas efektivitāte un drošums: farmakokinētiskie un farmakoģenētiskie pētījumi Latvijas pacientu populācijā

Pirmie pētījumi, kuros tika gūts apstiprinājums rifampicīna (angl. *rifampicin,* RIF), pirazīnamīda (angl. *pyrazinamide,* PZA), izoniazīda (angl. *izoniazid*, INH) un etambutola (angl. *etambutol,* ETB) izmantošanai tuberkulozes (TB) ārstēšanai, datējami ar 20. gadsimta 50.–60. gadiem. Šobrīd šī četru zāļu kombinācija ir viena no pirmās izvēles terapijām zāļu jutīgas tuberkulozes (angl. *drug-susceptible tuberculosis*, DS-TB) ārstēšanai. Tomēr jāatzīmē, ka joprojām daudzi šo zāļu iedarbības aspekti nav pilnībā izprasti, kas, iespējams, kavē centienus ierobežot TB – ieilgušu globāla mēroga sabiedrības veselības problēmu.

Raksturojot ar terapijas efektivitāti saistīto problemātiku, farmakokinētisko (FK) pētījumu rezultāti liecina, ka, lietojot standarta devas, pacientu vidū pastāv ievērojamas šo zāļu plazmas (un seruma) koncentrācijas atšķirības. Būtiski, ka pazemināta koncentrācija nereti tiek saistīta ar nepietiekamu ārstēšanas efektu un pat terapijas neveiksmi. Tikmēr, neskatoties uz pieaugošo interesi par C reaktīvā proteīna (CRP) izmantošanu, lai uzraudzītu atbildes reakciju uz terapiju, dati par prettuberkulozes zāļu koncentrācijas ietekmi uz TB izraisīto iekaisumu, tai skaitā seruma CRP koncentrācijas izmaiņām dinamikā, ir ierobežoti. Attiecībā uz RIF, PZA, INH un ETB kombinētās terapijas drošumu, viena no nozīmīgākajām problēmām ir zāļu izraisīti aknu bojājumi (angl. *drug-induced liver injury*, DILI), kuri dažkārt var būt letāli un kuru pamatā ir sarežģīti un līdz galam neizpētīti mehānismi. Patlaban ir identificēti vairāki gan ar pacientu, gan ar pašu slimību saistīti faktori, kuri ietekmē prettuberkulozes zāļu FK un paaugstina DILI attīstības risku. Vienlaikus, farmakoģenētiskajos (angl. *pharmacogenetics*, PGx) pētījumos, kuros aplūkoti RIF metabolizējošie enzīmi, transportvielas un ar to saistītie regulatorie proteīni, nav gūti pārliecinoši pierādījumi par ģenētiskās mainības nozīmīgumu minētajā kontekstā. Tādējādi, šī promocijas darba mērķis bija izpētīt RIF farmakogēnos esošo variantu saistību ar tā koncentrāciju plazmā un prettuberkulozes zāļu izraisītu aknu bojājumu (angl. *anti-tuberculosis drug-induced liver injury*, anti-TB DILI) attīstību, kā arī raksturot agrīnās seruma CRP koncentrācijas izmaiņas un tās ietekmējošos faktorus Latvijas pacientiem ar DS-TB.

Izvirzītā mērķa sasniegšanai secīgi tika veikti divi metodoloģiski un divi prospektīvi novērojuma pētījumi. Pirmā metodoloģiskā pētījumā laikā tika izstrādāta šķidruma homatogrāfijas–tandēma masspektrometrijas metode RIF, PZA, INH un ETB kvantificēšanai cilvēka plazmā, bet otrs pētījums bija veltīts PGx izpētes veikšanai nepieciešamā mērķētās

nākamās paaudzes sekvencēšanas protokola izveidei. Abas pieejas tika veiksmīgi pielietotas, lai iegūtu datus, kas pēc tam tika interpretēti kopā ar citu klīniski nozīmīgu informāciju. Trešajā pētījumā tika pārbaudīts, vai varianti gēnos, kuri kodē RIF FK procesos iesaistītos enzīmus (gēns: arilacetamīda deacetilāze, *AADAC*), transportvielas (gēni: izšķīdušo vielu nesēju virssaimes organisko anjonu transportvielu saimes 1B1 un 1B3 loceklis [angl. *solute carrier organic anion transporter family member 1B1 and 1B3*, *SLCO1B1* un *SLCO1B3*], adenozīna trifosfātu saistošās kasetes B apakšsaimes 1. loceklis [angl. *adenosine triphosphate-binding cassette subfamily B member 1*, *ABCB1*]) un kodola receptorus (gēns: kodola receptoru 1. apakšsaimes I grupas 2. loceklis [angl. *nuclear receptor subfamily 1 group I member 2*, *NR1I2*]), ietekmē RIF koncentrāciju plazmā un ir saistīti ar anti-TB DILI. Ceturtajā pētījumā tika analizēts, kā ar pacientu un slimību saistītie faktori, tai skaitā prettuberkulozes zāļu koncentrācija plazmā, ietekmē seruma CRP koncentrācijas samazināšanos pirmo 10–12 terapijas dienu laikā, un tika vērtēts, vai šis biomarķieris varētu prognozēt bakterioloģisko atbildes reakciju.

Īsumā, pētāmo gēnu kopā identificēto variantu analīzes laikā tika atklāta saistība starp vienu intronisko *NR1I2* gēna variantu (rs3732357) un RIF koncentrāciju plazmā. Turpretī neviens no analizētajiem variantiem nebija saistīts ar anti-TB DILI attīstību. Vērtējot agrīnās seruma CRP koncentrācijas izmaiņas pēc prettuberkulozes terapijas uzsākšanas, tika noteikti vairāki ar pacientu saistīti parametri un slimības smaguma pakāpe sākumstāvoklī kā faktori, kas ietekmēja šī biomarķiera koncentrācijas samazināšanos. Neskatoties uz to, ka ievērojamai pacientu daļai tika konstatēta nepietiekama RIF, PZA, INH un ETB koncentrācija plazmā, tas būtiski neietekmēja seruma CRP koncentrāciju analizētajā laika periodā. Visbeidzot, ne šī biomarķiera koncentrācijai 10.–12. terapijas dienā, ne tās izmaiņām, salīdzinot ar sākumstāvokli, nebija saistības ar bakterioloģisko atbildes reakciju.

Kopumā, šis darbs paplašina izpratni par FK un PGx mainību un tās ietekmi uz prettuberkulozes terapijas efektivitāti un drošumu. Tas sniedz jaunus datus par RIF farmakogēnos identificēto variantu genotipa un fenotipa saistību un padziļinātu ieskatu par agrīnām seruma CRP koncentrācijas izmaiņām, kā arī iezīmē iegūto rezultātu klīnisko nozīmi. Izstrādātajām FK un PGx datu ieguves metodēm ir praktiska nozīme turpmākajā TB izpētē un tās klīniskajā pārvaldībā.

Atslēgvārdi: tuberkuloze, farmakokinētika, farmakoģenētika, zāļu izraisīti aknu bojājumi, C reaktīvais proteīns, atbildes reakcija uz terapiju, šķidruma homatogrāfija–tandēma masspektrometrija, nākamās paaudzes sekvencēšana.

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Abbreviations used in the Thesis

AADAC	arylacetamide deacetylase
ABCB1	adenosine triphosphate-binding cassette subfamily B member 1
ADR	adverse drug reaction
Anti-TB DILI	anti-tuberculosis drug-induced liver injury
ATP	adenosine triphosphate
AUC	area under the time-concentration curve
BMI	body mass index
C_{max}	peak concentration 2 hours post-dose
CRP	C-reactive protein
CRP_b	C-reactive protein levels measured before initiating anti-tuberculosis therapy
$CRP_{10–12d}$	C-reactive protein levels measured 10–12 days after initiating anti-tuberculosis therapy
C_{trough}	trough concentration
CYP3A	cytochrome P450 family 3 subfamily A
CYP3A4	cytochrome P450 family 3 subfamily A member 4
DILI	drug-induced liver injury
DNA	deoxyribonucleic acid
DS-TB	drug-susceptible tuberculosis
ETB	ethambutol
HIV	human immunodeficiency virus
IL-1β	interleukin-1 beta
IL-6	interleukin-6
INH	isoniazid
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MIC	minimal inhibitory concentration
M. tuberculosis	*Mycobacterium tuberculosis*
N	number of patients
NAT2	N-acetyltransferase 2
NGS	next-generation sequencing
NR1I2	nuclear receptor subfamily 1 group I member 2
OATP1B1	organic anion transporting polypeptide 1B1
OATP1B3	organic anion transporting polypeptide 1B3
Pgp	P-glycoprotein
PGx	pharmacogenetics

PK	pharmacokinetics
PXR	pregnane X receptor
PZA	pyrazinamide
RIF	rifampicin
SLCO1B1	solute carrier organic anion transporter family member 1B1
SLCO1B3	solute carrier organic anion transporter family member 1B3
TB	tuberculosis
TDM	therapeutic drug monitoring
TNF-α	tumour necrosis factor alpha
tSCC	time to sputum culture conversion
WHO	World Health Organization

Introduction

With a global incidence of 10–12 million people and a mortality of 1–2 million people annually over the past decade, tuberculosis (TB), a seemingly preventable and curable infectious disease, is an alarming public health problem even in the era of advanced medical technologies (World Health Organization [WHO], 2024). The majority of patients initially present with drug-susceptible tuberculosis (DS-TB) and accordingly receive the WHO-recommended four- or six-month treatment regimen (WHO, 2022a, 2024). In most countries within the WHO European Region, including Latvia, the latter regimen with a four-drug combination – namely rifampicin (RIF), pyrazinamide (PZA), isoniazid (INH), and ethambutol (ETB) – appears to be superior in terms of accessibility and provides a cure rate of up to 88 % (Masini et al., 2022; Günther et al., 2023; WHO, 2024).

To date, many investigators have reported substantial variability in the pharmacokinetics (PK) of the four aforementioned anti-TB drugs when administered at standard doses (Fahimi et al., 2013; Pasipanodya et al., 2013; Prahl et al., 2014; Kloprogge et al., 2020; Ramachandran et al., 2020). The importance of maintaining anti-TB drug exposure within therapeutic ranges lies in the fact that each drug exhibits a distinct mechanism of action and penetrates heterogeneous tuberculous lesions to varying extents. This enables the drugs to target *Mycobacterium tuberculosis* (*M. tuberculosis*) at different stages of replication (Sirgel et al., 2000; Prideaux et al., 2015; Strydom et al., 2019; Ignatius & Dooley, 2023). Consequently, reduced exposure to anti-TB drugs has been recognised as a reason for delayed treatment response and treatment failures, including the development of drug-resistant forms of the disease that are difficult to treat, requiring an immediate transition to extended regimens with second-line drugs (Pasipanodya et al., 2013; Prahl et al., 2014; Kloprogge et al., 2020; Ramachandran et al., 2020).

Prahl et al. (2014) studied the effect of anti-TB drug plasma exposure from a different perspective; they established a relationship between INH exposure and the degree of TB-associated inflammation during the first two months of anti-TB therapy, i. e. the intensive treatment phase. Typically, serum C-reactive protein (CRP) levels complement the disease severity at diagnosis and begin to decline after the first weeks of treatment, reflecting the gradual clearance of *M. tuberculosis* (Djoba et al., 2008; Miranda et al., 2017; Wilson et al., 2018; Azam et al., 2022). Several authors have highlighted it as a promising biomarker for monitoring the bacteriological response and predicting adverse treatment outcomes (Djoba et al., 2008; Miranda et al., 2017; Wilson et al., 2018; Azam et al., 2022). Nevertheless, the impact of anti-TB drug exposure on the patterns of serum CRP level reduction and the clinical relevance of these findings has not been further explored.

Concerning the safety of a six-month regimen for treatment of DS-TB, nearly a third of treated patients experience drug-induced liver injury (DILI), manifesting as an asymptomatic, transient elevation of liver enzymes in mild cases or acute liver failure and death in the worst-case scenarios (Devarbhavi et al., 2013; Chen et al., 2015a; Zhuang et al., 2022). This adverse drug reaction (ADR) has been attributed to the hepatotoxic properties of RIF, PZA, and INH, whether used alone or in combination (Chen et al., 2015a; Zhuang et al., 2022). Although doses at the upper end of the recommended range and higher exposure are known to trigger anti-tuberculosis drug-induced liver injury (anti-TB DILI), the complex nature of this ADR is still poorly understood (Satyaraddi et al., 2014; Chen et al., 2015a; Zheng et al., 2021; Zhuang et al., 2022).

Apart from patient-dependent factors such as age, biological sex, and comorbidities, which are often mentioned when discussing differences in anti-TB drug exposure and the development of DILI, an important consideration is gene variants altering the function of drug-metabolising enzymes and transporters (McIlleron et al., 2006; Nijland et al., 2006; Yimer et al., 2011; Chen et al., 2015a; Zhuang et al., 2022). The impact of pharmacogenetic (PGx) variability on drug efficacy and safety has been well demonstrated in other therapeutic areas. For example, Manolopoulos et al. (2010) have estimated that variants in the genes encoding cytochrome P450 family 2 subfamily C member 9 and vitamin K epoxide reductase complex subunit 1 may account for 35–50 % of the variation in the warfarin dose. Similarly, more than 60 % of statin-induced myopathy cases are linked to variants in the solute carrier organic anion transporter family member 1B1 (*SLOC1B1*) gene encoding organic anion transporting polypeptide 1B1 (OATP1B1) (SEARCH Collaborative Group et al., 2008). In the context of the present work, only the N-acetyltransferase 2 (NAT2) encoding gene is considered of significant PGx importance due to extensive evidence of a genotype-phenotype relationship, at least in part, explaining INH-related hepatotoxicity (McDonagh et al., 2014; Chen et al., 2015a; Zhuang et al., 2022; Ulanova et al., 2024). Other findings regarding the effect of patient genetic background on variability in anti-TB drug exposure and treatment-related hepatotoxicity remain controversial (Chigutsa et al., 2011; Wang et al., 2019; Zhang et al., 2019; Weiner et al., 2021).

Aim of the Thesis

To investigate the effect of variants in RIF pharmacogenes on its plasma exposure and the development of anti-TB DILI, as well as to characterise the early changes in serum CRP levels and their influencing factors in Latvian patients with DS-TB.

Objectives of the Thesis

The following objectives were set to reach the aim of the doctoral Thesis:

1. Develop and validate a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for quantification of RIF, PZA, INH, and ETB in human plasma.
2. Design a targeted next-generation sequencing (NGS) protocol for analysing genomic regions of interest.
3. Investigate whether variants detected in genes encoding enzymes (arylacetamide deacetylase [*AADAC*] gene), transporters (*SLCO1B1* and solute carrier organic anion transporter family member 1B3 [*SLCO1B3*] genes, and adenosine triphosphate-binding cassette subfamily B member 1 [*ABCB1*] gene), and nuclear receptors (nuclear receptor subfamily 1 group I member 2 [*NR1I2*] gene) involved in RIF disposition are related to RIF plasma exposure and anti-TB treatment-related hepatotoxicity.
4. Examine the role of patient- and disease-related factors, including plasma exposure of four anti-TB drugs, in the reduction in serum CRP levels after the first 10–12 days of therapy and evaluate the potential of these early changes to predict bacteriological response.

Hypotheses of the Thesis

1. One or more variants detected across the five investigated genes encoding RIF-metabolising enzymes, transporters, and associated regulatory proteins are related to variability in RIF plasma exposure and to the development of anti-TB DILI.
2. Higher serum CRP levels 10–12 days after anti-TB treatment onset, along with less than two-fold reduction compared to baseline, are related to lower plasma exposure of one or several anti-TB drugs.

Novelty of the Thesis

This Thesis analyses anti-TB drug exposure and RIF-related genetic determinants from various perspectives and outlines their role in the efficacy and safety of DS-TB therapy.

The newly developed LC-MS/MS method is the first to offer simultaneous quantification of RIF, PZA, INH, and ETB alongside their six primary metabolites in human plasma, enabling a more comprehensive PK characterisation. This assay can be adapted for therapeutic drug monitoring (TDM) to support the implementation of personalised TB treatment strategies in clinical practice. Meanwhile, the designed NGS-based protocol for targeted genetic analysis proved suitable and readily adjustable for detecting variants dispersed

across multiple genes or gene fragments of interest. Both methodologies were applied in the two subsequent studies.

The first study characterised the impact of variants in the genes encoding enzymes (*AADAC* gene), transporters (*SLCO1B1*, *SLCO1B3*, and *ABCB1* genes), and nuclear receptors (*NR1I2* gene) involved in RIF disposition (collectively referred to as RIF pharmacogenes) on its plasma exposure and anti-TB treatment-related hepatotoxicity, providing valuable data on genotype-phenotype associations in patients of European ancestry (Caucasians). Even though the obtained results largely support the view that alterations in the function of the explored proteins caused by variants in the analysed genes play a limited role, one *NR1I2* gene variant (rs3732357) was found to be related to RIF plasma exposure. Notably, its effects have not been previously documented in patients with TB.

The second study adds new evidence on the impact of patient- and disease-related factors on the early reduction in serum CRP levels following initiation of anti-TB therapy. Importantly, it demonstrates that the assessment of this biomarker levels after 10–12 days of anti-TB drug administration appears to lack clinical relevance, as the observed changes were not predictive of bacteriological response.

Ethical aspects

Each of the studies described in this Thesis was carried out according to the guidelines of the Declaration of Helsinki. Where applicable, the study protocol was approved by the Central Medical Ethics Committee of Latvia (Approval Nos 01-29.1/1 and 01-29.1.2/1736), the Research Ethics Committee of Rīga Stradiņš University (Approval No 6-3/1/6), the Ethics Committee of the Riga East Clinical University Hospital (Approval No 24-A/15), and the Scientific Department of the Riga East Clinical University Hospital (Approval No ZD/08-06/01-21/187).

Discussion

Addressing the knowledge gap outlined above and the raised scientific questions, the first part of this Thesis presents an analysis of genetic factors potentially contributing to variability in RIF plasma exposure and anti-TB DILI, focusing on five RIF pharmacogenes: *AADAC*, *SLCO1B1*, *SLCO1B3*, *ABCB1*, and *NR1I2*. The second part summarises key findings from a study on early changes in inflammation caused by anti-TB treatment, with particular emphasis on the impact of anti-TB drug plasma exposure. In addition, this Thesis encompasses the development of LC-MS/MS and NGS-based methods, which were required to support both areas of research. A schematic representation of the study designs is given in Figure 1.

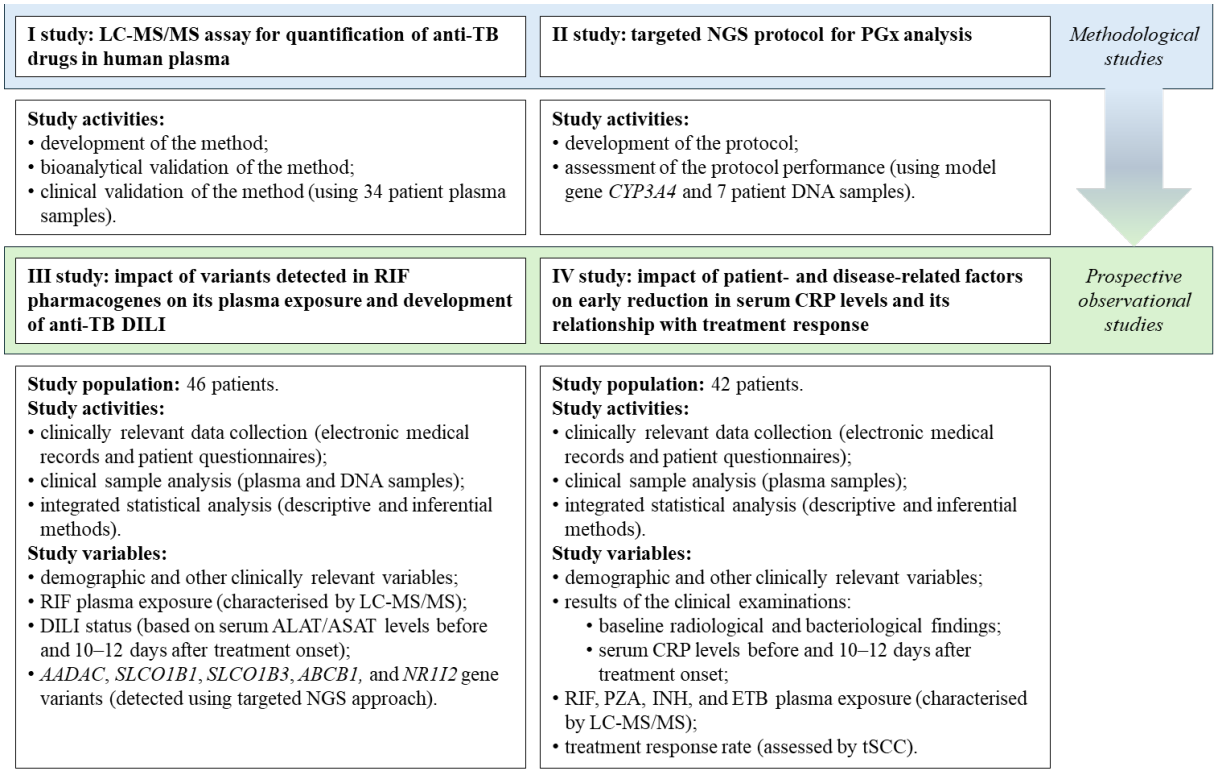

Figure 1. **Schematic representation of the study designs***

*Across all studies, a total of 64 otherwise healthy adult patients diagnosed with pulmonary DS-TB and admitted to the Riga East University Hospital, Centre of Tuberculosis and Lung Diseases (Latvia) were enrolled between April 2017 and May 2023. All patients were of European ancestry (Caucasians). At the time of clinical sample collection, the patients were in the intensive phase of the six-month treatment course, receiving RIF (8–12 mg/kg), PZA (20–30 mg/kg), INH (4–6 mg/kg), and ETB (15–25 mg/kg) for 10–12 days in accordance with the WHO guidelines for treatment of DS-TB (2022a).

Abbreviations: TB, tuberculosis; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NGS, next-generation sequencing; PGx, pharmacogenetics; DNA, deoxyribonucleic acid; *CYP3A4*, cytochrome P450 family 3 subfamily A member 4 gene; RIF, rifampicin; anti-TB DILI, anti-tuberculosis drug-induced liver injury; DILI, drug-induced liver injury; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; *AADAC*, arylacetamide deacetylase gene; *SLCO1B1*, solute carrier organic anion transporter family member 1B1 gene; *SLCO1B3*, solute carrier organic anion transporter family member 1B3 gene; *ABCB1*, adenosine triphosphate-binding cassette subfamily B member 1 gene; *NR1I2*, nuclear receptor subfamily 1 group I member 2 gene; CRP, C-reactive protein; PZA, pyrazinamide; INH, isoniazid; ETB, ethambutol; tSCC, time to sputum culture conversion.

Method development: LC-MS/MS assay for quantification of anti-TB drugs in human plasma

Overall, LC-MS/MS has become the gold standard for drug quantification in various biological matrices for research purposes and TDM in a clinical setting. As highlighted in the literature, the main challenge in the simultaneous analysis of multiple anti-TB drugs and their metabolites lies in the diversity of their physicochemical properties, which complicates the selection of a suitable sample preparation method and the optimisation of the chromatographic conditions (Zhou et al., 2013; Kim et al., 2015; Shah et al., 2016; Wang et al., 2020). During the initial experiments conducted in this work, attempts to employ existing protocols, such as those offered by Kim et al. (2015), Luyen et al. (2018), and Sundell et al. (2019), resulted in unacceptable analyte retention, poor peak shapes, and ionisation issues. Furthermore, in some cases, the quantification ranges of the reported methods did not cover the middle and upper parts of the therapeutic range, necessitating an additional dilution step during sample pretreatment to fit the calibration curves (Zhou et al., 2013; Gao et al., 2018; Wu et al., 2020). The use of advanced sample preparation techniques and separate analytical method for each analyte was not considered for practical reasons, as it would be time-consuming, require large sample volumes, and involve complex technical solutions.

These issues were resolved by developing a new method for the simultaneous quantification of RIF, PZA, INH, and ETB in human plasma, which, compared to previous assays, has the advantage of measuring their six primary metabolites – 25-desacetylrifampicin, acetylisoniazid, isonicotinic acid, pyrazine-2-carboxylic acid, 5-hydroxypyrazinamide, 5-hydroxypyrazine-2-carboxylic acid – within a single run. This approach incorporates simple single-step protein precipitation with methanol for the sample pretreatment, followed by chromatographic separation on a reverse-phase C8 column using a mobile phase gradient (0.1 % formic acid solution in water (A) and methanol (B); A from 99 % to 2 %), with a total run time of 10 minutes per sample. Analytes were detected and quantified using the multiple reaction monitoring mode. Additional sample pretreatment steps were unnecessary, as the quantification ranges for RIF, PZA, INH, and ETB were selected based on expected peak plasma concentrations 2 hours post-dose (C_{max}) (Alsultan & Peloquin, 2014).

The validation procedure was carried out in accordance with the European Medicines Agency's guidelines on bioanalytical method validation (2012), and the obtained results confirmed satisfactory performance of the developed method, thereby guaranteeing the quality and reliability of the acquired data. As part of the validation, stability tests under various storage conditions revealed limited stability of RIF, INH, and their metabolites, in agreement with findings by Sturkenboom et al. (2015), Luyen et al. (2018), and Wu et al. (2020). Therefore, to

maintain the declared performance, plasma samples should be processed within 4 hours of collection, with temporary storage at −20 °C permitted for up to 24 hours.

The clinical applicability of the method was verified by analysing plasma samples from 34 patients receiving anti-TB therapy. This preliminary assessment of anti-TB drug exposure showed that the mean C_{max} was within the therapeutic range for PZA (38.4 μg/mL *versus* 20–60 μg/mL) and ETB (2.7 μg/mL *versus* 2–6 μg/mL), but for RIF and, to a lesser extent, INH – below the expected range (2.3 μg/mL *versus* 8–24 μg/mL for RIF and 2.8 μg/mL *versus* 3–6 μg/mL for INH) (Alsultan & Peloquin, 2014). Generally, these findings are consistent with those reported in other populations and underscore the need for an in-depth analysis of factors causing variation in anti-TB drug plasma exposure and its potential clinical consequences (Fahimi et al., 2013; Prahl et al., 2014; Niward et al., 2018; Kloprogge et al., 2020; Ramachandran et al., 2020).

Although the metabolite profiling of patient plasma samples was beyond the scope of the studies included in this Thesis, data on INH and its metabolites generated during the clinical validation step are reported separately in Ulanova et al. (2024), where they were used to characterise INH PK and to assess the impact of genetic factors. Accordingly, this extended capability broadens the applicability of the developed method for future PK studies.

Method development: targeted NGS protocol for PGx analysis

Compared to conventional first-generation sequencing techniques such as Sanger sequencing, second-generation or NGS technologies have revolutionised genomic research by offering rapid, cost-effective, and high-throughput analysis of genetic variability. Given the objectives of the planned PGx research, the targeted NGS approach appeared to be more convenient than whole genome or exome sequencing, which are typically used for comprehensive genetic characterisation. At the time of designing the sequencing experiment, commercially available targeted sequencing panels covered only a limited number of variants in genes encoding enzymes, transporters, and regulatory proteins involved in the disposition of RIF and other anti-TB drugs.

The newly designed targeted NGS protocol encompasses steps from amplification and sequencing library preparation to bioinformatic analysis of the raw sequencing data. Its greatest advantage is that, technically, it can be adapted for analysing multiple genes or gene fragments of interest by modifying the amplification and, accordingly, the bioinformatic analysis step.

First, using the cytochrome P450 family 3 subfamily A member 4 (*CYP3A4*) gene as a model, it was demonstrated that the developed protocol for paired-end sequencing on the Illumina platform yielded high-quality data (e. g. the mean base quality score Q > 30, mean

read depth > 100) for the analysed patient desoxyribonucleic acid (DNA) samples (number of patients, N = 7). In parallel, 10 randomly selected *CYP3A4* gene variants were re-sequenced using the reference method, Sanger sequencing. The results obtained were consistent between the two methods, confirming the reliability of the proposed protocol.

However, in two of these samples, up to 16 % of the target sequence lacked read coverage, which was attributed to issues with the quality of individual amplicons, as no substantial variation in read depth was seen in other sequence regions. Seth-Smith et al. (2019) noted that the performance of sequencing library preparation reagents can be affected by low (< 50 %) guanine/cytosine content in the target sequence. Both factors should be considered when adapting the protocol for analysing other genes or gene fragments, thereby ensuring its robustness across different targets.

Among five patients with full target sequence coverage, one carrier of the *CYP3A4*22* (rs35599367) allele and one carrier of the *CYP3A4*1G* (rs2242480) allele were identified; the latter allele has been retired according to the recently revised *CYP3A4* allele classification (Gaedigk et al., 2021). These *CYP3A4* gene variants have been associated, for example, with variability in tacrolimus PK; nevertheless, a detailed analysis of this pharmacogene was beyond the objectives of the present work (Elens et al., 2013; Pallet et al., 2015; Liu et al., 2017; Dong et al., 2022).

Impact of variants detected in RIF pharmacogenes on its plasma exposure and development of anti-TB DILI

RIF is considered one of the most potent components of the six-month regimen for the treatment of DS-TB due to its ability to penetrate various types of tuberculous lesions and act on both metabolically active and dormant forms of *M. tuberculosis* (Dickinson & Mitchison, 1981; Prideaux et al., 2015; Rifat et al., 2018).

At the same time, it demonstrates the highest PK variability among the four anti-TB drugs at standard daily doses, with reported underexposure rates ranging from 42 % to 93 % (McIlleron et al., 2006; Fahimi et al., 2013; Prahl et al., 2014; Niward et al., 2018; Ramachandran et al., 2020). In line with these findings, LC-MS/MS analysis in the present study (N = 46) confirmed a similar trend – 91 % of patients had RIF plasma concentrations at C_{max} below the recommended range (Alsultan & Peloquin, 2014). Compared to patients with optimal RIF plasma exposure, those with underexposure had higher body weight and, thus, a lower body weight-adjusted RIF dose. The impact of other factors described elsewhere was not evident, likely due to the study population's structure, i. e. otherwise healthy adult patients, 76 % of whom were male, and only 11 % were aged 60 years or older (Walubo et al., 1991; McIlleron et al., 2006; Nijland et al., 2006; Weiner et al., 2010; Milán Segovia et al., 2013).

As previously mentioned, DILI is a well-documented ADR in patients treated for DS-TB (Chen et al., 2015a; Zhuang et al., 2022). Abbara et al. (2017) estimated that symptoms of hepatotoxicity emerge in approximately half of cases within the first two weeks of anti-TB drug administration. Notably, symptoms of RIF-related hepatotoxicity may appear earlier than those linked to PZA and INH toxicity (Durand et al., 1996; Abbara et al., 2017). In this study, 13 % of patients developed DILI within 10–12 days of anti-TB therapy, aligning with other reports where incidence rates can reach up to 28 % (Chen et al., 2015a). Regarding patient characteristics, no significant differences were found between the DILI and non-DILI groups, except for elevated serum alanine aminotransferase and aspartate aminotransferase levels on the 10^{th} to 12^{th} day of treatment, reaffirming the homogeneity of the study population discussed above.

The patient's genetic background is another factor that can potentially compromise the efficacy and safety of therapy. Thus, this study further explored the contribution of 10 variants, selected from those detected in the four RIF pharmacogenes (*SLCO1B1*: rs2306283, rs11045819, and rs4149056; *SLCO1B3*: rs60140950; *ABCB1*: rs9282564 and rs1045642; and *NR1I2*: rs3814055, rs3732357, rs2276707, and rs3732359), to the variability in RIF plasma exposure, characterised by C_{max} and area under the time-concentration curve ($AUC_{0–6h}$), as well as to the development of anti-TB DILI.

Initially, *AADAC*, a gene encoding a liver microsomal enzyme responsible for hydrolysing rifamycins into non-toxic 25-desacetyl derivatives, was also considered for analysis, as AADAC enzymatic activity has been reported to be influenced by variants in the respective gene (Nakajima et al., 2011; Shimizu et al., 2012; Francis et al., 2019; Weiner et al., 2021; Ignatius & Dooley, 2023). However, evaluation of *AADAC* gene variants was not feasible due to insufficient statistical power, stemming from their inadequate frequency in the study population.

Continuing with *SLCO1B1* and *SLCO1B3*, these genes encode liver-specific membrane influx transporters, OATP1B1 and organic anion transporting polypeptide 1B3 (OATP1B3), respectively, expressed on the basolateral membrane of hepatocytes (Nie et al., 2020). OATP1B1 and OATP1B3 mediate the hepatic uptake of various endogenous and exogenous substances, including RIF, from the bloodstream (Nie et al., 2020). *In vitro* studies have shown that the investigated *SLCO1B1* exonic variant rs4149056 (T>C) impairs the transport of RIF, atorvastatin, gliclazide, and many other substrates, while the effects of exonic variants rs11045819 (C>A) and rs2306283 (A>G) vary depending on the substrate (Nie et al., 2020). In clinical practice, rs4149056 was identified as a predictor of RIF plasma trough concentration (C_{trough}) and C_{max} in the study conducted by Allegra et al. (2017). Meanwhile, the rs11045819

CA genotype, in conjunction with the African origin and male sex, was reported to be associated with lowered RIF plasma exposure (Weiner et al., 2010; Kwara et al., 2014). On the contrary, in a study involving Ghanaian children, individuals carrying the rs2306283 GG genotype demonstrated higher RIF plasma exposure estimated by $AUC_{0-\infty h}$, but the differences in other PK parameters approached significance (Dompreh et al., 2018). Despite these findings, neither this study nor other authors have confirmed the effect of these *SLCO1B1* gene variants on RIF exposure (Chigutsa et al., 2011; Huerta-García et al., 2019; Naidoo et al., 2019; Medellin-Garibay et al., 2020; Weiner et al., 2021). Similarly, the *SLCO1B3* exonic variant rs60140950 (G>C), which has been reported to adversely impact OATP1B3 expression without altering its activity *in vitro* and to influence telmisartan PK in a cohort of healthy individuals, had no effect in the present study (Schwarz et al., 2011; Hirvensalo et al., 2020; Nie et al., 2020).

Clinically, three DILI patterns are distinguished: hepatocellular, cholestatic, and mixed, all of which have been recorded in patients receiving treatment for DS-TB (David & Hamilton, 2010; Chen et al., 2015a). One proposed mechanism of cholestasis is the competitive inhibition of OATP1B1 and OATP1B3 by RIF, leading to reduced hepatic uptake of another substrate – bilirubin (Campbell et al., 2004; Nie et al., 2020). Although Li et al. (2012) and Chen et al. (2015b) described associations between the *SLCO1B1*15* (rs2306283 + rs4149056) haplotype and anti-TB treatment-related hepatotoxicity, findings from analogous works and this particular study did not confirm the effect of both variants (Yimer et al., 2011; Kim et al., 2012). Also, the third *SLCO1B1* gene variant examined in this study, rs11045819, was not found to be a significant predictor of this ADR. Interestingly, in healthy volunteers, the *SLCO1B1*15* haplotype was related to higher bilirubin levels, which even more increased after RIF initiation, albeit to a similar extent as in individuals without this haplotype (Zhang et al., 2007). This observation suggests that RIF may exacerbate pre-existing hepatobiliary disorders but is unlikely to be their primary cause. In contrast, previous studies assessing the clinical relevance of *SLCO1B3* gene variants have primarily focused on taxane toxicity without convincing evidence supporting any drug-variant combinations (Jabir et al., 2012; Mbatchi et al., 2015). Here, in patients undergoing anti-TB treatment, rs60140950 was not related to the development of hepatotoxicity; however, to the author's knowledge, this is the first report on *SLCO1B3* in this context.

Another transporter-coding gene included in the analysis, *ABCB1*, encodes adenosine triphosphate (ATP)-dependent membrane transporter P-glycoprotein (Pgp). Pgp is expressed in excretory organs and the blood-brain barrier, where it, as a natural defence mechanism, alters the disposition of xenobiotics like RIF by mediating their efflux from the intracellular space (Cascorbi, 2011). Among the *ABCB1* gene variants assessed, the exonic variant rs1045642

(A>G) has exhibited inconsistent effects on messenger ribonucleic acid and Pgp expression and the PK of its substrates (Cascorbi, 2011). In a study with Mexican patients, the rs1045642 genotype, along with biological sex and RIF dose received, was reported as a genetic determinant of RIF plasma exposure (Huerta-García et al., 2019). Regrettably, this finding was not replicated in the present study, and similar negative results have been reported by others (Weiner et al., 2010; Chigutsa et al., 2011; Allegra et al., 2017; Naidoo et al., 2019; Medellin-Garibay et al., 2020). Likewise, *ABCB1* gene variant rs2032582, which is in linkage disequilibrium with rs1045642, was shown to impair Pgp function *in vitro*, but its effect on RIF oral clearance was of marginal significance in a clinical setting (Cascorbi, 2011; Chigutsa et al., 2011). The impact on the evaluated RIF PK parameters also was not evident in the case of another *ABCB1* exonic variant within the scope of this work, rs9282564 (T>C), despite the decrease in tacrolimus plasma C_{trough} reported by Hu et al. (2018).

In the context of DILI, Yimer et al. (2011) showed that the *ABCB1* rs1045642 GG genotype increased susceptibility to this ADR in patients receiving RIF- and efavirenz-containing regimens for the treatment of TB and human immunodeficiency virus (HIV) coinfection. Conversely, a protective effect was found in patients with HIV mono-infection, whereas it had no association with INH-related hepatotoxicity in otherwise healthy patients with TB (Ritchie et al., 2006; Chan et al., 2017). In line with these earlier reports, a relationship between the *ABCB1* gene variants of interest, rs1045642 and rs9282564, and anti-TB treatment-related hepatotoxicity in patients without comorbidities was not established here.

The last of the analysed genes, *NR1I2,* encodes pregnane X receptor (PXR), a ligand-dependent transcription factor whose effects extend to numerous phases I and II drug-metabolising enzymes and transporters (Ma et al., 2008). RIF, one of the most potent PXR ligands, upregulates the transcription of PXR downstream targets and, therefore, is thought to induce its own metabolism, observed as an approximately 40 % decrease in its plasma exposure during the first weeks of anti-TB therapy (Ma et al., 2008; Smythe et al., 2012). As the *NR1I2* exonic variants were underrepresented in the enrolled patient population, this study assessed the effects of flanking intronic variants (rs2276707 (C>T) and rs3732357 (G>A)) and those located in the 5' and 3' untranslated regions (rs3814055 (C>T) and rs3732359 (G>A), respectively). Specifically, carriers of rs3732357 were found to have lower RIF plasma $AUC_{0–6h}$ under the dominant genetic model (GG *versus* GA+AA genotypes), while the difference in plasma C_{max} approached significance. A similar trend was noted for rs3732359, though it was insignificant. The potential clinical relevance of both variants has been indicated before; using midazolam as a model compound, He et al. (2006) and Oleson et al. (2010)

reported that these variants increased cytochrome P450 family 3 subfamily A (CYP3A) enzyme activity in African Americans. Nevertheless, the lack of comprehensive functional data on rs3732357 and rs3732359, particularly the impact on the enzymes and transporters directly implicated in RIF PK, precludes a mechanistic rationale for the described relationship. The two other *NR1I2* gene variants examined – earlier shown to be associated with increased *NR1I2* promoter activity *in vitro* (rs3814055) or elevated intestinal CYP3A expression and contrasting evidence about its impact on tacrolimus PK (rs2276707) – did not demonstrate a relationship with the investigated RIF PK parameters in the performed analyses (Zhang et al., 2001; Barraclough et al., 2012; Rana et al., 2017; Lu et al., 2021).

There has been considerable interest in whether *NR1I2* gene variants modulate individuals' predisposition to anti-TB treatment-related hepatotoxicity, as changes in the basal transcriptional activity of PXR upon RIF exposure are believed to contribute to increased fatty acid absorption and subsequent accumulation in the liver, aggravation of INH-induced oxidative stress due to excessive production of hepatotoxic INH metabolites, and cholestasis caused by increased accumulation of the heme biosynthesis intermediate protoporphyrin IX, all of which are implicated in the development of this ADR (Metushi et al., 2011; Zhuang et al., 2022). Regarding the four *NR1I2* gene variants analysed in this study (rs3814055, rs3732357, rs2276707, and rs3732359), none was significantly related to anti-TB DILI. Analogous studies conducted in populations of Asian ancestry have documented conflicting findings on rs3814055. Zazuli et al. (2015) described an increased susceptibility to this ADR in patients carrying the rs3814055 TT genotype, while Wang et al. (2022), in a more recent study, clarified that a combination of the rs3814055 T allele and NAT2 non-slow acetylator status might be a predisposing factor. In contrast, Zhang et al. (2019) reported a protective effect of this variant. In the case of rs2276707, Yang et al. (2020) concluded that, under the recessive genetic model, it was associated with a reduced risk of anti-TB treatment-related hepatotoxicity, though this finding has not yet been confirmed in different settings.

A considerable strength of this study is its design, which enabled the evaluation of the clinical effects of selected gene variants without interference from other health conditions, as the resulting physiological changes and concomitantly used drugs could influence the anti-TB drug PK and the occurrence of treatment-related ADRs. In parallel, given that the study was conducted in a low-endemic setting (with a provisional TB incidence of 15 cases per 100 000 population in 2024, for a total population of 1.8 million), the applied patient exclusion criteria substantially reduced the number of eligible patients (Centre for Disease Prevention and Control of Latvia, 2025). Consequently, the size of the study population, in

conjunction with the utilised NGS protocol, affected the number of gene variants studied. For instance, it was impossible to verify the finding by Chigutsa et al. (2011) that the *SLCO1B1* deep intronic variant rs4149032, located > 2 kb from the exon-intron junction, was associated with reduced RIF plasma exposure. Notably, this limitation does not extend to low-frequency gene variants such as *AADAC* stop-loss variant rs61733692, with a minor allele frequency of < 1 % in the European population. Expanding the study cohort is unlikely to increase statistical power for testing whether the decreased enzymatic activity determined by the *AADAC*3* (rs1803155 + rs61733692)/*AADAC*3* diplotype, as described in the *in vitro* study by Shimizu et al. (2012), holds clinical significance. Despite these limitations and emphasising the novelty of this work, some of the evaluated gene variants have not been previously explored in patients with TB. Finally, assessing blood biochemical parameters at a single time point within the first few weeks after initiating anti-TB therapy carries the risk of missing DILI cases that develop later. However, the primary interest was RIF-related hepatotoxicity, with its signs and symptoms typically manifesting during this period (Durand et al., 1996; Abbara et al., 2017).

Taken together, current evidence indicates that the analysed variants in the four RIF pharmacogenes (*SLCO1B1*, *SLCO1B3*, *ABCB1*, and *NR1I2*) do not exert a fundamental impact on RIF plasma exposure or the development of anti-TB treatment-related hepatotoxicity. The observed discrepancies between *in vitro* and *in vivo* findings suggest that the biological effects of these gene variants may be mitigated by compensatory mechanisms or influenced by other patient-dependent factors. Furthermore, inconsistencies in *in vivo* findings across studies are likely attributable to variation in study design and patient characteristics. At the same time, this study identified a previously unreported relationship between the *NR1I2* gene variant rs3732357 and RIF plasma exposure. As such, the first hypothesis of this Thesis is considered partly confirmed.

Impact of patient- and disease-related factors on early reduction in serum CRP levels and its relationship with treatment response

Given the complexity and duration of anti-TB therapy required to completely eradicate the infection, monitoring of treatment response and early identification of patients at risk of adverse treatment outcomes are crucial for achieving therapeutic goals. According to WHO (2022b), patients treated for DS-TB should demonstrate clinical improvement and achieve sputum-smear and sputum-culture conversion by the end of the second month of therapy. Calderwood et al. (2021) estimated that approximately 20 % of patients fail to meet this short-term goal, experiencing a delayed bacteriological response, indicative of limited antibacterial effect and an increased risk of treatment failure or relapse in subsequent months.

Measuring serum CRP levels is a well-established approach for assessing and monitoring inflammation in various acute and chronic conditions (Sproston & Ashworth, 2018). Mechanistically, the increased production of CRP is an inherent response to infection or tissue damage mediated by macrophages (Sproston & Ashworth, 2018). Upon activation, macrophages release proinflammatory cytokines interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumour necrosis factor alpha (TNF-α), which, in turn, stimulate the production of this acute-phase protein, primarily in human hepatocytes (Sproston & Ashworth, 2018). In the context of TB, this biomarker has recently been approved for screening of active TB in individuals living with HIV, and there is increasing interest in its potential utility for monitoring treatment response in patients receiving anti-TB therapy (Djoba et al., 2008; Miranda et al., 2017; Sigal et al., 2017; Musteikienė et al., 2021; WHO, 2021). However, given the non-specific nature of CRP expression, its levels may vary considerably over the six-month treatment period, making it more suitable for predicting short-term rather than long-term effects (Sproston & Ashworth, 2018).

This study first assessed the impact of patient characteristics on the serum CRP levels measured before initiating anti-TB therapy (CRP_b) (Wener et al., 2000; Khera et al., 2005; Ohsawa et al., 2005; Wyczalkowska-Tomasik et al., 2016). While biological sex, age, and smoking status had negligible impact, body mass index (BMI) exhibited a moderately strong negative correlation with the levels of this biomarker in the study population (N = 42). Despite marginal significance, there was a 10-fold difference in CRP_b levels between underweight patients and those categorised as overweight. It contrasts with observations in patients without TB infection, where serum CRP levels typically increase with BMI (Onwubalili, 1988; Visser et al., 1999; Lönnroth et al., 2010). Nutritional status influences both innate and adaptive immunity and, in the case of TB, modulates susceptibility to infection and the ability to clear an existing one (Gupta et al., 2009; Chandrasekaran et al., 2017; Cho et al., 2022). Among patients with TB, lack of appetite and weight loss are frequently reported symptoms, with severe wasting linked to advanced disease, delayed treatment response, and adverse outcomes (Hoyt et al., 2019; Diktanas et al., 2018; Kornfeld et al., 2020; Sinha et al., 2023).

Characterising the relationship between TB clinical features and serum CRP_b levels, Djoba et al. (2008), Furuhashi et al. (2012), and other authors have shown that levels of this biomarker reflect disease severity, mirroring the radiological and bacteriological findings at the time of diagnosis (Brown et al., 2016; Sigal et al., 2017; Azam et al., 2022). Aligning with that, radiologically confirmed cavitary disease and positive sputum-smear microscopy result, suggestive of severe lung damage with presumably higher bacillary loads, were related to higher serum CRP_b levels in this study. Although Djoba et al. (2008), Furuhashi et al. (2012), and

Musteikienė et al. (2021) reported that the levels of this biomarker may vary depending on the extent of lung involvement, this was not confirmed here, and other groups did not report such a relationship (Mendy et al., 2016; Kumar et al., 2019). The plausible explanation for this discordance is the methodological differences in estimating the TB-affected lung area.

Next, this study examined changes in serum CRP levels 10–12 days after anti-TB treatment onset and evaluated their utility in predicting bacteriological response. This timeframe was deemed appropriate, as a prior study determined that the failure to achieve a 55 % reduction in the levels of this biomarker within the first two weeks of therapy was associated with hospitalisation and death in patients with TB-HIV coinfection (Wilson et al., 2018). Moreover, it is the earliest point at which anti-TB drug exposure can be objectively assessed, ensuring steady-state plasma concentration is reached (Tostmann et al., 2013).

After 10–12 days of treatment, the median serum CRP level in the enrolled patient population decreased from 21.9 to 6.4 mg/L, reaching the reference range of < 8 mg/L utilised at the study site. These results are consistent with earlier studies, reporting a significant drop in serum CRP levels between the first and fifth weeks of anti-TB therapy (Djoba et al., 2008; Miranda et al., 2017; Wilson et al., 2018; Musteikienė et al., 2021). A more detailed analysis demonstrated that underweight patients, smokers, and those presenting with lung cavitations and positive sputum-smear microscopy result at the initial examination exhibited a significant decline compared to the baseline, yet their serum CRP levels 10–12 days after initiating anti-TB therapy ($CRP_{10–12d}$) remained above the target range. As previously discussed, low body weight and smoking potentiate TB-associated inflammation, while the aforementioned radiological and bacteriological findings, characteristic of the advanced TB, are typically accompanied by a high degree of inflammation (Djoba et al., 2008; Furuhashi et al., 2012; Chandrasekaran et al., 2017; Opolot et al., 2017; Sigal et al., 2017). These factors indeed have been associated with slower sputum bacillary clearance in the studies by Nijenbandring de Boer et al. (2014), Kanda et al. (2015), Diktanas et al. (2018), Hernandez-Romieu et al. (2019), and Kornfeld et al. (2020). Consequently, it is unsurprising that patients with a combination of these factors may require a longer time to recover from the infection and normalise their serum CRP levels. Meanwhile, the composition of the enrolled patient population, with 81 % aged < 60 years, may have limited the ability to observe the relationship between age and serum CRP levels. However, it was noticed that patients ≥ 60 years of age failed to achieve a significant decline in the levels of this biomarker within the specified period. This distinct pattern of serum CRP level reduction in older patients could be attributed to the deleterious impact of ageing on the immune system (immunosenescence) and inflammation (inflammaging) (Li et al., 2023).

With age, inflammatory stimuli induce more intense and prolonged secretion of IL-1β, IL-6, and TNF-α, leading to enhanced CRP production (Li et al., 2023). Ageing also impairs the function of immune cells, such as macrophages and CD4+ T lymphocytes, which are key components of immune defence against *M. tuberculosis*, thereby diminishing the ability to rapidly clear the infection (O'Garra et al., 2013; Li et al., 2023).

In the absence of clinical guidelines defining the reduction pattern or timeframe for serum CRP normalisation in response to anti-TB therapy, a slightly modified approach proposed by Wilson et al. (2018) was implemented here to stratify patients and categorise changes in the levels of this biomarker for subgroup analyses. Specifically, 31.7 % of patients had serum CRP levels within the reference range at both time points (Group A), 34.1 % experienced a reduction in serum CRP levels ≥ 2 times from baseline or reached the reference range (Group B), and the remaining 34.1 % did not achieve the stated goal (Group C). Multiple-group comparisons revealed that patients in Group C more frequently presented with lung cavitations or positive sputum-smear microscopy result at diagnosis compared to those in Groups A and B, underscoring the role of disease severity in the early reduction of inflammation highlighted in the primary analyses.

Another factor explored in relation to TB-associated inflammation was anti-TB drug plasma exposure, characterised by C_{max} and $AUC_{0–6h}$. The LC-MS/MS analysis indicated that a considerable proportion of patients had suboptimal anti-TB drug plasma exposure at C_{max}, with the highest underexposure rate recorded for RIF (92.5 %), followed by INH (54.8 %), ETB (26.2 %), and PZA (9.5 %). These results are comparable to those reported by others and support the notion that the currently employed therapeutic ranges proposed by Alsultan & Peloquin (2014) differ substantially from the drug plasma concentrations documented in clinical practice (Fahimi et al., 2013; Pasipanodya et al., 2013; Prahl et al., 2014; Kloprogge et al., 2020; Ramachandran et al., 2020). Interestingly, in the present study, drug exposure at the site of infection appeared sufficient to provide an early antibacterial effect, as evidenced by the significant reduction in serum CRP levels. This observation might be explained by the variation in anti-TB drug distribution across different body compartments, as described by Ziglam et al. (2002) and McCallum et al. (2021, 2022), who reported drug accumulation in epithelial lining fluid and alveolar cells to varying extents, but plasma and serum exposure, determined in parallel, tended to be lower or even below the therapeutic range.

Subsequent analyses did not confirm a relationship between the plasma exposure of any of the four anti-TB drugs and serum $CRP_{10–12d}$ levels. Additionally, when patients were stratified based on a 2-fold reduction in serum CRP levels, the assessed PK parameters were similar among Groups A, B, and C. On the contrary, Prahl et al. (2014) reported an inverse

correlation between INH C_{max} and serum CRP levels. The considerable differences in study design and patient characteristics may have led to discordant findings. Here, the period between treatment onset and PK sampling was constant, and the study population was homogeneous in terms of ethnicity, race, form of TB, comorbidities, care setting, and drug formulation used. Several of these factors have been reported to influence the PK of anti-TB drugs (Walubo et al., 1991; McIlleron et al., 2006; Nijland et al., 2006; Weiner et al., 2010; Milán Segovia et al., 2013).

Lastly, this study evaluated the utility of the serum $CRP_{10–12d}$ levels and the patterns of early changes in the serum CRP levels for predicting the bacteriological response to anti-TB treatment, assessed by time to sputum culture conversion (tSCC). The structure of the study population and absence of concomitant diseases likely determined relatively fast sputum culture conversion (median tSCC: 56 days), even among patients who did not achieve a rapid reduction in serum CRP levels within the specified period (median tSCC: 66 days in Group C *versus* 46 days in Groups A and B). While patients with delayed bacteriological response (tSCC $\geq$ 60 days) had higher serum $CRP_{10–12d}$ levels compared to rapid responders (tSCC < 60 days), the difference was not statistically significant, and none of the examined parameters emerged as independent predictors of tSCC in the Cox regression analysis. This suggests that changes in serum CRP levels, although reflecting the effect of treatment, may be excessively dynamic due to the non-specific nature of CRP expression, and are therefore unable to reliably predict the bacteriological response to anti-TB therapy (Sproston & Ashworth, 2018). Similarly, Musteikienė et al. (2017, 2021) reported that serum CRP_b levels were higher in patients with tSCC > 30 days, yet this biomarker exhibited limited utility in predicting sputum culture status or treatment outcomes. Another group of authors found that the ratio of serum CRP levels at Week 8 to Week 0 was more strongly associated with sputum culture status at Week 8 than at Week 12, but testing of multiple biomarker combinations containing serum CRP levels failed to yield a signature with high predictive performance (Sigal et al., 2017). By comparison, Djoba et al. (2008) identified a biomarker signature encompassing serum CRP levels at baseline and Week 1 that could predict sputum culture status at Month 2 with more than 80 % accuracy. Notably, Ferrian et al. (2017) proposed a CRP-based biomarker combination for predicting delayed bacteriological response in patients with drug-resistant TB, though this finding requires confirmation in other studies.

A strength of this study is the use of various data sources to comprehensively characterise the relationship between numerous patient- and disease-related factors and TB-associated inflammation. Additionally, enrolling patients without concomitant diseases

enabled a clearer assessment of serum CRP kinetics and emphasised the potential influence of other health conditions.

However, the present work shares limitations previously discussed concerning the sample size when exploring the role of PGx in RIF PK and anti-TB DILI. The impact of sample size was evident in the high data skewness and unequal distribution across subcategories. Complex statistical approaches were employed to overcome this limitation. Another point to mention is that there were no cases of treatment failure or serious adverse events detected in the study population, so it was impossible to test the predictive value of the examined CRP parameters in such contexts. After all, this study lacked data on the patients' *M. tuberculosis* isolates, including minimal inhibitory concentration (MIC), which would have enabled consideration of strain-specific effects and isolate drug susceptibility. Other authors have demonstrated the utility of combined indices, such as C_{max}/MIC, in predicting bacteriological response and treatment outcomes in patients with DS-TB (Chigutsa et al., 2011; Fahimi et al., 2013; Zheng et al., 2021).

To summarise, this study identified patient- and disease-related factors influencing the reduction in serum CRP levels shortly after the initiation of anti-TB therapy. The absence of a measurable effect of suboptimal anti-TB drug plasma exposure within the given timeframe did not exclude the possibility of negative clinical implications emerging at later stages of therapy. Finally, in this setting, neither serum $CRP_{10–12d}$ levels nor the fold change between serum CRP_b and $CRP_{10–12d}$ levels could predict bacteriological response to treatment, questioning the clinical relevance of the early assessment of the levels of this biomarker, at least in otherwise healthy adult patients with TB. Consequently, the second hypothesis of this Thesis was not confirmed.

Conclusions

1. According to the analytical and clinical validation results, the developed LC-MS/MS method is suitable for the simultaneous analysis of RIF, PZA, INH, and ETB in human plasma samples, with the added capability to quantify six primary metabolites within the same run, thereby enabling expanded PK profiling.
2. The designed NGS-based protocol for targeted genetic analysis produced reliable, high-quality data suitable for PGx applications, as demonstrated by analyses of the *CYP3A4* gene and five RIF pharmacogenes.
3. Among the 10 evaluated variants detected across four RIF pharmacogenes (*SLCO1B1*, *SLCO1B3*, *ABCB1*, and *NR1I2*), the intronic variant rs3732357 in the *NR1I2* gene was found to influence RIF plasma exposure. In contrast, the study results did not support the contribution of any of these variants to the development of anti-TB DILI in the enrolled population.
4. Several patient characteristics and disease severity at diagnosis, but not the anti-TB drug plasma exposure, were identified as affecting the pattern of serum CRP level reduction during the first 10–12 days of therapy. Early changes in the serum CRP levels could not predict the bacteriological response to anti-TB treatment.

Proposals

1. Based on the findings from the study exploring the role of genetic determinants in RIF plasma exposure and anti-TB DILI, further studies should consider:
   a) *in vitro* functional characterisation of the *NR1I2* intronic variant rs3732357 to provide a mechanistic rationale for the observed impact on RIF PK;
   b) evaluation of additional liver-specific biomarkers, such as alkaline phosphatase, to better characterise the pattern of liver injury caused by the concomitant use of RIF, PZA, and INH, and clarify whether genetic factors contribute to liver injury primarily induced by RIF.
2. The study characterising early changes in serum CRP levels after anti-TB treatment initiation indicated that, while measuring levels of this biomarker after 10–12 days of anti-TB drug administration may be useful for assessing dynamics of TB-associated inflammation, these early reduction patterns cannot predict bacteriological response to treatment. Further research should aim to identify robust biomarker or multimodal biomarker signatures linked to relevant clinical, radiological, and/or bacteriological endpoints, enabling the timely identification of patients at risk of treatment failure.
3. The considerable rates of anti-TB drug underexposure observed across the conducted studies, together with the absence of a clear relationship between plasma concentrations and early changes in TB-associated inflammation, underline the importance of investigating how plasma exposure reflects drug concentrations at the site of infection. Addressing this aspect could support the refinement of therapeutic ranges, which currently appear inadequately high. Moreover, defining clinically relevant thresholds would enhance the utility of TDM in assessing the efficacy and safety of anti-TB therapy.

Publications and reports on topics of Doctoral Thesis

Publications:

1. **Kivrane, A.**, Grinberga, S., Sevostjanovs, E., Igumnova, V., Pole, I., Viksna, A., Bandere, D., Krams, A., Cirule, A., Pugovics, O., & Ranka, R. (2021). LC-MS/MS method for simultaneous quantification of the first-line anti-tuberculosis drugs and six primary metabolites in patient plasma: Implications for therapeutic drug monitoring. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, *1185*, 122986. https://doi.org/10.1016/j.jchromb.2021.122986

2. **Kivrane, A.**, Igumnova, V., Kimsis, J., Freimane, L., Sadovska, D., Viksna, A., Pole, I., & Ranka, R. (2021). Implementation of a next-generation sequencing-based targeted approach for full-length CYP3A4 gene sequencing. *Pharmacogenomics*, *22*(9), 519–527. https://doi.org/10.2217/pgs-2020-0128

3. **Kivrane, A.**, Ulanova, V., Grinberga, S., Sevostjanovs, E., Viksna, A., Ozere, I., Bogdanova, I., Zolovs, M., & Ranka, R. (2024). Exploring Variability in Rifampicin Plasma Exposure and Development of Anti-Tuberculosis Drug-Induced Liver Injury among Patients with Pulmonary Tuberculosis from the Pharmacogenetic Perspective. *Pharmaceutics*, *16*(3), 388. https://doi.org/10.3390/pharmaceutics16030388

4. **Kivrane, A.**, Ulanova, V., Grinberga, S., Sevostjanovs, E., Viksna, A., Ozere, I., Bogdanova, I., Simanovica, I., Norvaisa, I., Pahirko, L., Bandere, D., & Ranka, R. (2024). Identification of Factors Determining Patterns of Serum C-Reactive Protein Level Reduction in Response to Treatment Initiation in Patients with Drug-Susceptible Pulmonary Tuberculosis. *Antibiotics*, *13*(12), 1216. https://doi.org/10.3390/antibiotics13121216

Reports and theses at international congresses and conferences:

1. **Kivrane, A.**, Igumnova, V., Kimsis, J., Freimane, L., Sadovska, D., Viksna, A., Pole, I., & Ranka, R. (2021, March 24–26). *Next-generation sequencing-based targeted-sequencing approach for the full-length CYP3A4 gene sequencing* [Poster abstract]. RSU International Conference on Medical and Health Care Sciences: Knowledge for Use in Practice, Riga, Latvia. Hybrid event. Abstract book, 442.

2. **Kivrane, A.**, Igumnova, I., Grinberga, S., Sevostjanovs, E., Viksna, A., Ozere, I., Bandere, D., & Ranka, R. (2021, October 19–22). *First-line anti-tuberculosis drug exposure in newly diagnosed TB patients: Latvian perspective* [Poster abstract]. 52nd World Conference on Lung Health of the International Union Against Tuberculosis and Lung Diseases: TBScience 2021. Virtual event. In: *Int J Tuberc Lung Dis*, *25*(S2), S425.

3. **Kivrane, A.**, Igumnova, V., Viksna, A., Simanovica, I., Ozere, I., Pole, I., Bandere, D., & Ranka, R. (2022, March 25–26). *Assessment of complete blood count-derived marker association with pulmonary tuberculosis severity and treatment response* [Oral presentation]. International Scientific Conference on Medicine organized within the frame of the 80th International Scientific Conference of the University of Latvia. Virtual event. In: *Medicina (Kaunas)*, *58*(S1), 48.

4. **Kivrane, A.**, Igumnova, V., Viksna, A., Simanovica, I., Ozere, I., Pole, I., Bandere, D., & Ranka, R. (2022, July 9–14). *The utility of complete blood count-derived markers for characterising pulmonary tuberculosis severity and prediction of treatment response* [Poster abstract]. The Biochemistry Global Summit Lisbon, The 46th FEBS congress, Lisbon, Portugal. In: *FEBS Open Bio*, *12*(S1), 160.

5. **Kivrane, A.**, Ulanova, V., Grinberga, S., Sevostjanovs, E., Viksna, A., Pole, I., Ozere, I., Bogdanova, I., Zolovs, M., Bandere, D., & Ranka R. (2023, March 27–31). *Assessment of rifampicin exposure in Latvian pulmonary tuberculosis patients in the context with the AADAC genetic polymorphisms* [Poster abstract]. RSU International Conference on Medical and Health Care Sciences: Knowledge for Use in Practice, Riga, Latvia. In: *Medicina (Kaunas)*, *59*(S2), 351.

6. **Kivrane, A.**, Ulanova, V., Grinberga, S., Sevostjanovs, E., Viksna, A., Pole, I., Ozere, I., Bogdanova, I., Zolovs, M., Bandere, D., & Ranka, R. (2023, June 14–16). *Assessment of four first-line anti-tuberculosis drug exposure in pulmonary tuberculosis patients with drug-induced hepatotoxicity* [Poster abstract]. Second Nordic Conference Personalized Medicine, Turku, Finland. In: *Basic and Clinical Pharmacology and Toxicology*, *132*(S1), 19–20.

7. **Kivrane, A.**, Ulanova, V., Grinberga, S., Sevostjanovs, E., Viksna, A., Bogdanova, I., Pahirko, L., Bandere, D., & Ranka, R. (2024, June 8–11). *Identification of factors determining patterns of serum CRP level reduction in response to anti-tuberculosis treatment initiation* [Poster abstract].16th congress of the European Association for Clinical Pharmacology and Therapeutics: WHO Step 6. Monitoring outcome and compliance, Rotterdam, Netherlands. Abstract book, 273–274.

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LC-MS/MS method for simultaneous quantification of the first-line anti-tuberculosis drugs and six primary metabolites in patient plasma: Implications for therapeutic drug monitoring

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ABSTRACT

The pharmacokinetic profiling of drug substances and corresponding metabolites in the biological matrix is one of the most informative tools for the treatment efficacy assessment. Therefore, to satisfy the need for comprehensive monitoring of anti-tuberculosis drugs in human plasma, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated for simultaneous quantification of first-line anti-tuberculosis drugs (ethambutol, isoniazid, pyrazinamide, and rifampicin) along with their six primary metabolites. Simple single-step protein precipitation with methanol was chosen as the most convenient sample pre-treatment method. Chromatographic separation of the ten analyte mixture was achieved within 10 minutes on a reverse-phase C8 column using mobile phase gradient mode. The multiple reaction monitoring mode (MRM) was used for analyte detection and quantification in patient samples. The chosen quantification ranges fully covered expected plasma concentrations. The method exhibited acceptable selectivity; the within- and between-run accuracy ranged from 87.2 to 113.6%, but within- and between-run precision was between 1.6 and 14.9% (at the LLOQ level CV < 20%). Although the response of the isonicotinic acid varied depending on the matrix source (CV 21.8%), validation results proved that such inconsistency does not affect the accuracy and precision of results. If stored at room temperature plasma samples should be processed within 4 h after collection, temporary storage at −20 °C up to 24 h is acceptable due to stability issues of analytes. The developed method was applied for the patient sample analysis (n = 34) receiving anti-tuberculosis treatment with the first-line drugs.

1. Introduction

Tuberculosis (TB) is an airborne infectious disease caused by *Mycobacterium tuberculosis*. Despite the current medical development, an estimation of 10 million new cases and 1.4 million deaths in 2019 proves that TB remains a global health problem [1]. The cure rate of 85% is reached using a 6-month standardized treatment regimen consisting of four first-line anti-tuberculosis drugs (ethambutol (ETB), pyrazinamide (PZA), rifampicin (RIF) and, isoniazid (IZN)) [1,2]. However, 15% of patients experience a relapse or develop a drug-resistant form of the disease and require treatment prolongation with costly and more toxic second-line drugs. Poor treatment adherence, nutrition, comorbidities, drug-drug interactions, and even the patient's genotype may contribute to inter-individual differences in drug exposure among TB patients [3–10]. The pharmacokinetic profiling of drug substances in various biological matrices is an efficient approach to investigate

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pharmacokinetic variability and to evaluate drug efficiency and the outcome of the therapy. At the individual level, interpretation of a patient's pharmacokinetic profile as a part of therapeutic drug monitoring (TDM) program allows to assess the dosing regime and decide whether a transition from standardized to personalized treatment strategy could improve treatment outcome [3,10–13].

The liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the most utilised analytical technique for the TDM in research laboratories and has become the method of choice in the clinical setting. In the past years, numerous reverse-phase (RP) and hydrophilic interaction liquid chromatography (HILIC) based methodologies have been developed and validated to analyse different combinations of the first-line anti-tuberculosis drugs in clinical samples (Table 1) [14–24]. The selection of appropriate separation technique highly depends on the available technical solutions and the ability to adjust chromatographic conditions. For example, *Gao et al.* [16] performed chromatographic separation on the Zorbax-SB C18 column, but *Zhou et al.* [17] used Intersil HILIC to separate the same combination of four first-line anti-tuberculosis drugs. In contrast, *Wang et al.* [24] reported that ETB was weakly retained on C18 chromatographic columns, but the use of Acquity BEH HILIC column resulted in issues with RIF and its derivate peak shapes. The best results were achieved with CAPCELL PAK-ADME – a chemically modified RP column [24]. *Hee et al.* [15] and *Song et al.* [23] also both employed modified C18 chromatographic columns specifically designed to retain hydrophilic compounds such as acetylisoniazid and isoniazid.

Regarding sample preparation techniques, the majority of the authors had chosen single-step protein precipitation with organic solvents (e.g., acetonitrile, methanol) for plasma sample preparation due to the balance between easiness of sample handling and sufficient quality of plasma extracts that are compatible with LC-MS/MS system.

As the metabolite profiling is essential for the correct establishment of pharmacokinetic profile and has to be viewed in context with the parent drug, a single method is needed to quantify both the first-line anti-tuberculosis drugs and their primary metabolites. Only a few of the published methods incorporate primary metabolites (Table 1). Although the method reported by *Sundell et al.* [19] comprises the broadest spectra of analytes within a single LC-MS/MS method, it lacks such important primary metabolites as pyrazine-2-carboxylic acid (the active form of PZA) and hepatotoxic 5-hydroxypyrazine-2-carboxylic acid. On the other hand, as illustrated in Table 1, the quantification ranges used by various methods broadly cover the subtherapeutic concentration range of the drug substances but, in some cases, lack the middle and upper range of clinically expected plasma concentrations described in the literature [3]. Routine sample dilution to fit the calibration curves and a combination of multiple LC-MS/MS methods to obtain pharmacokinetic data for all compounds of interest is time-consuming, requires large sample volumes and complex technical solutions.

Therefore, this study aimed to develop and validate a single LC-MS/MS method for simultaneous determination of all four first-line anti-tuberculosis drugs and their six primary metabolites in human plasma. The relevance of the developed method was confirmed by clinical sample analysis of TB patients undergoing treatment with first-line anti-tuberculosis drugs.

2. Materials and methods

2.1. Chemicals and materials

5-hydroxypyrazine-2-carboxylic acid (5OHPZ2A; purity: 98%), acetylisoniazid (ACIZN; purity: 98%), and 25-desacetylrifampicin (25DRIF; purity: 94%) were supplied from Carbosynth (Berkshire, UK). Pyrazinamide (PZA; purity: 98%), ethambutol (ETB; purity: 98%), isoniazid (IZN; purity: 98%), isonicotinic acid (IZNAC; purity: 99%), pyrazine-2-carboxylic acid (PZ2A; purity: 99%), rifampicin (RIF; purity: 99%) were from Alfa Aesar (Kandel, Germany). 5-hydroxypyrazinamide (5OHPZA; purity: 97%) was purchased from Cymit (Barcelona, Spain), phenformin hydrochloride (IS; analytical standard, purity: 98%) was from Sigma-Aldrich (Schnelldorf, Germany). Formic acid (FA; MS grade) and dimetylsulfoxide (DMSO; HPLC grade) were from Sigma-Aldrich (Schnelldorf, Germany). Methanol (MeOH; HPLC grade) was purchased from Merck (Darmstadt, Germany). Water was purified by Milli-Q Plus water purification system (Millipore, Molsheim, France). Commercial human plasma (anticoagulant: ethylenediaminetetraacetic acid (EDTA), Innovative Research, Novi, MI, USA) and blank human plasma samples from national biobank (section 2.6.) were used for the development and validation of the assay.

2.2. UPLC-MS/MS instrumentation and conditions

An Acquity UPLC H-Class chromatographic system (Waters, Milford, MA, USA) coupled to a XEVO TQ-S tandem mass spectrometer (Waters, Milford, MA, USA) was used for analysis. The chromatographic separation was achieved on the Waters Acquity UPLC BEH C8 column (2.1 × 75 mm; 1.7 µm). The other columns tested were: Waters Acquity UPLC BEH HILIC (2.1 × 50 and 100 mm; 1.7 µm), Acquity UPLC BEH Amide (2.1 × 50 and 100 mm; 1.7 µm), and Acquity UPLC BEH C18 (2.1 × 50 mm; 1.7 µm). The analyte mixture consisting of ten compounds was separated using a gradient elution at a flow rate of 0.2 mL/min. The mobile phase consisted of 0.1% FA solution (A) and methanol (B). The gradient mode was as follows: 0–1 min, 99% A; 1–2.5 min, 99 → 2% A; 2.5–4.5 min, 2% A, then equilibration to the initial conditions for 5.5 min. The total run time was 10 min. The volume of injection was 1 µL. The autosampler and the column temperature was maintained at 5 °C and 30 °C, respectively. The calibration standards were run in triplicate but all samples in duplicate.

The mass spectrometer was operated in positive electrospray ionisation mode (ESI+) with the capillary voltage of 3.0 kV. The source temperature was set to 140 °C, the desolvation gas (N_2) temperature was kept at 600 °C at a flow rate of 1000 L/h, and the cone gas (N_2) flow rate was 200 L/h. The multiple reaction monitoring mode (MRM) was used for detection and quantification of the analytes. The precursor-to-product ion transitions and MRM parameters specified for each compound and IS are listed in Table 2. Data acquisition and analysis were performed using MassLynx software and TargetLynx module (version 4.1., Waters, Milford, MA, USA).

2.3. Preparation of stock solutions, calibration standards, and quality control samples

The stock solutions of the analytes were prepared in DMSO to a final concentration of 10 mg/mL (RIF, 25DRIF, IZN, ACIZN, IZNAC, and ETB) or 2 mg/mL (PZA, PZ2A, 5OHPZA, 5OHPZ2A). The internal standard was dissolved in the mobile phase (0.1% FA solution) to reach a final concentration of 1 mg/mL. The stock solutions were stored at −20 °C.

The highest calibration standard was prepared by spiking an appropriate volume of stock solutions of the analytes into blank plasma. The prepared calibration standard was serially diluted with blank plasma to obtain six more standards for the construction of the calibration curves. The calibration ranges for all analytes are given in Table 3. Quality control (QC) samples were prepared at the lower limit of quantification (LLOQ), low (LQC), medium (MQC), and high (HQC) concentrations.

2.4. Sample preparation

The following single-step protein precipitation with methanol was used to prepare calibration standards, QC samples, and clinical samples. An aliquot of plasma sample (50 µL) was mixed with 450 µL of freshly prepared internal standard solution in methanol (10 µg/mL) and centrifuged at 10000 rpm for 10 min at 5 °C. Then, 100 µL of the

Table 1
Overview of main characteristics of the published methods offering simultaneous analysis of the first-line anti-tuberculosis drugs with or without primary metabolites and comparison to the method developed in this study.

Reference	Analytes	Quantification range (μg/mL)	Sample volume (V, μL)	Sample preparation technique	LC column	Analysis time, min
[14]	PZA	0.10–30.0	200	LLE	Agilent	4.0
	PZ2A	0.03–9.00			Zorbax Eclipse XBD-C18	
	5OHPZ2A	0.002–0.60			(4.6 × 100 mm; 3.5 μm)	
[15]	RIF	0.025–50.0	20	SPE	Agilent	6.0
	25DRIF	0.0025–5.00			Zorbax SB-aq	
	IZN	0.005–10.0			(4.6 × 50 mm; 5 μm)	
	IZNAC	0.0125–5.00				
	ACIZN	0.0125–5.00				
[16]	PZA	0.20–4.00	100	PP	Agilent	8.5
	IZN	0.08–2.00			Zorbax SB-C18	
	ETB	0.0002–1.00			(2.1 × 100 mm; 3.5 μm)	
	STM	2.00–200				
	RIF	0.20–4.00				
[17]	PZA	0.004–4.00	100	PP	GL Sciences	<2.0
	IZN	0.004–4.00			Intersil HILIC	
	ETB	0.0005–0.50			(2.1 × 75 mm; 3 μm)	
	STM	0.01–16.0				
	RIF	0.004–4.00				
[18]	PZA	0.31–39.2	100	PP	GL Sciences	3.5
	IZN	0.077–9.80			Intersil HILIC	
	ETB	0.0015–1.96			(2.1 × 150 mm; 3 μm)	
	RIB	0.0015–1.96				
	RIF	0.0038–4.90				
[19]	PZA	0.32–40.0	200	LLE at neutral and acidic conditions	GL Sciences	4.0
	IZN	0.08–10.0			Intersil HILIC	
	ETB	0.04–5.00			(2.1 × 75 mm; 3 μm)	
	RIF	0.20–25.0				
	25DRIF	0.04–5.00				
	IZNAC	0.08–10.0				
	ACIZN	0.04–5.00				
	5OHPZA	0.06–7.50				
[20]* Group 1	PZA	2.00–100	100	PP	Waters	9.0
					Atlantis HILIC	
					(2.1 × 150 mm; 3 μm)	
[20]* Group 2	IZN	0.10–5.00	50	PP	Waters	13.0
	ETB	0.10–5.00			Atlantis dC18	
	RIF	0.20–10.0			(2.0 × 150 mm; 3 μm)	
[21]	PZA	1.00–100	500	PP	Phenomenex	8.0
	IZN	0.10–10.0			Gemnini C18	
	ETB	0.02–5.00			(4.6 × 150 mm; 4.6 μm)	
	RIF	0.20–20.0				
	ACIZN	0.10–10.0				
[22]	PZA	2.00–80.0	10	Ultrafiltration	Waters	2.5
	ETB	0.20–8.00			Atlantis T3 C18	
	IZN	0.20–8.00			(2.0 × 100 mm; 3 μm)	
[23]	PZA	5.00–80.0	50	Two-step PP	YMC Co.	3.0
	IZN	0.50–8.00			Hydrosphere C18	
	ETB	0.50–8.00			(2.0 × 50 mm; 3 μm)	
	RIF	5.00–80.0				
	25DRIF	N/D**				
	ACIZN	N/D				
[24]*	PZA	0.005–50.0	100	PP	Shiseido	7.0
	IZN	0.005–7.50			CAPCELL PAK-ADME	
	ETB	0.001–5.00			(2.1 × 50 mm; 3 μm)	
	RIF	0.005–7.50				
This study***	PZA	1.17–75.0	50	PP	Waters	10.0
	IZN	0.16–10.0			Acquity UPLC BEH C8	
	ETB	0.16–10.0			(2.1 × 75 mm; 1.7 μm)	
	RIF	0.47–30.0				
	25DRIF	0.47–30.0				
	IZNAC	0.16–10.0				
	ACIZN	0.16–10.0				
	5OHPZA	1.17–75.0				
	5OHPZ2A	2.34–75.0				
	PZ2A	1.17–75.0				

STM – streptomycin; RIB – rifabutin; IZN – isoniazid; IZNAC – isonicotinic acid; ACIZN – acetylisoniazid, RIF – rifampicin; 25DRIF – 25-desacetylrifampicin; PZA – pyrazinamide; PZ2A – pyrazine-2-carboxylic acid; 5OHPZA – 5-hydroxypyrazinamide; 5OHPZ2A – 5-hydroxypyrazine-2-carboxylic acid; ETB – ethambutol; LLE – liquid-liquid extraction; SPE – solid phase extraction; PP – protein precipitation; *quantification ranges are showed only for the first-line anti-tuberculosis drugs; **N/D – not determined; ***method developed in this study.

Table 2
Optimized mass spectrometric parameters used for detection of the analytes and IS in the MRM mode and corresponding retention times of the analytes and IS.

Compound	Precursor ion (m/z)	Fragment ion (m/z)	Cone voltage (V)	Collision energy (eV)	Retention time (min)
IZN	138.3	121.0	40	10	1.09
IZNAC	124.3	79.9	30	16	1.20
ACIZN	180.3	121.1	60	18	1.32
RIF	821.4	789.3	30	14	4.57
	823.4	791.3	30	14	4.71
25DRIF	781.4	399.2	50	15	4.62
PZA	124.3	80.9	20	12	3.62
PZ2A	125.1	80.9	20	6	2.94
5OHPZA	140.1	122.9	30	8	2.06
5OHPZ2A	141.1	54.8	50	15	1.71
ETB	205.4	116.0	40	18	0.83
IS	206.2	104.9	30	20	4.10

IZN – isoniazid; IZNAC – isonicotinic acid; ACIZN – acetylisoniazid, RIF – rifampicin; 25DRIF – 25-desacetylrifampicin; PZA – pyrazinamide; PZ2A – pyrazine-2-carboxylic acid; 5OHPZA – 5-hydroxypyrazinamide; 5OHPZ2A – 5-hydroxypyrazine-2-carboxylic acid; ETB – ethambutol; IS – phenformin (internal standard).

Table 3
Calibration range and LLOQ for the first-line anti-tuberculosis drugs and their primary metabolites.

Compound	Calibration range (μg/mL)	LLOQ (μg/mL)	r^2
IZN	0.16–10.0	0.16	0.995
IZNAC	0.16–10.0	0.16	0.991
ACIZN	0.16–10.0	0.16	0.9992
ETB	0.16–10.0	0.16	0.9998
RIF	0.47–30.0	0.47	0.996
25DRIF	0.47–30.0	0.47	0.989
PZA	1.17–75.0	1.17	0.998
PZ2A	1.17–75.0	1.17	0.997
5OHPZA	1.17–75.0	1.17	0.993
5OHPZ2A	1.17–75.0	2.34	0.996

supernatant was transferred to a glass vial with 900 μL of mobile phase A (0.1% FA solution), briefly vortexed, and used for analysis.

The IS solution in methanol (10 μg/mL) and the mobile phase A used for the sample dilution were stored at 5 °C before use.

2.5. Method validation

The validation procedure of the developed method was performed according to the guidelines of European Medicines Agency on bioanalytical method validation [25].

2.5.1. Selectivity

The selectivity of the method was evaluated by comparing the signal intensity in the blank sample with that in the sample containing IS and analytes at the LLOQ level, both prepared in duplicate from six individual sources of the matrix. The analyte signal intensity in the blank sample should be less than 20% of the analyte signal at the LLOQ level. The IS signal intensity in blank sample should not exceed 5% of that in analytical sample.

2.5.2. Calibration curve and lower limit of quantification

The calibration ranges for PZA, RIF, IZN, ETB, and corresponding metabolites (Table 3) were chosen, based on the therapeutic range of the particular drug [3]. Generally, seven nonzero calibration standards were prepared in blank plasma and analysed in triplicate in three separate analytical runs. The seven-point calibration curve for each analyte was constructed by plotting the peak area against the analyte concentration in the calibration standard and applying an appropriate weighting factor ($1/x$ or $1/x^2$). Since the IS was used for system stability monitoring during an analytical run, absolute calibration was applied to calculate the analyte concentration. The back-calculated concentrations of the calibration standards should be within 15% of the nominal concentration (at the LLOQ level – 20%).

The LLOQ was considered the lowest analyte concentration in the sample that can be measured with a certain degree of accuracy (within 20% of the nominal concentration) and precision (coefficient of variation, CV $\leq$ 20%). At the LLOQ level, the signal-to-noise ratio should be at least 5.

2.5.3. Accuracy and precision

The within-run accuracy and precision were demonstrated by analysis of five replicates of QC samples per concentration level at four concentration levels (LLOQ, low, medium, and high). Data from three analytical runs performed in three consecutive days were used to ascertain between-run accuracy and precision.

The accuracy was expressed as the difference between the measured and nominal concentrations of the QC samples. Assessing within- and between-run accuracy, the concentration of QC samples should be within 15% of the nominal concentration, except for the LLOQ level, where 20% is acceptable.

The precision was defined as the variance between replicate samples and expressed as the coefficient of variation (CV, %). Evaluating within- and between-run precision, the CV should not exceed 15% (at the LLOQ level CV $\leq$ 20%).

2.5.4. Matrix effect

The matrix effect was determined at two concentration levels (LLOQ and high) using matrix factor (MF). The MF was the ratio between the analyte peak area in the post-extraction spiked samples and that of reference sample prepared in 0.1% FA solution. The post-extraction spiked samples were prepared using blank plasma from six different individuals. The samples were prepared in duplicate. The CV of MF calculated from six different plasma sources should be within 15%.

2.5.5. Stability

The analyte stability in human plasma was investigated at different storage conditions by analysing QC samples at the LQC and HQC levels. For short-term stability, samples were stored on a benchtop at room temperature for 1 and 4 h before processed. The long-term stability was assessed after QC sample storage at −20 °C for 24 h, 7 days, and 3 months, respectively. The freeze-thaw stability was evaluated after subjecting QC samples to 3 freeze-thaw cycles from the freezer (−20 °C) to room temperature in a seven-day period. All QC samples were prepared in duplicate. For the autosampler stability, calibration standards were reanalysed after 24 h of storage in the autosampler (5 °C). The stability of analyte stock solutions was evaluated after stock storage at −20 °C for 2 months, while the stability of the IS stock solution was assessed after storage at −20 °C for 14 days. All analytes were quantified against the freshly prepared calibration standards. The stability was calculated from the reference sample concentration and expressed as a percentage of the nominal concentration. Stability should be within 15% of the nominal concentration.

2.5.6. Carry-over

Sample-to-sample carry-over was assessed by injecting the blank sample immediately after three consecutive injections of the highest calibration standard. Following the highest calibrator, the signal of the blank sample should be less than 20% of the analyte signal at LLOQ level and 5% of the IS signal.

2.5.7. Dilution integrity

The dilution integrity was demonstrated using samples containing analytes at a concentration two times the highest calibration standard. The samples were diluted before analysis with blank plasma in the ratio

1:5 and 1:10. In total, five replicates per dilution factor were prepared and analysed. The dilution of the study samples should not affect the accuracy and precision of the results; the accuracy and precision should be within 15%.

2.6. Collection of clinical samples

Blank human plasma samples (anticoagulant: EDTA) from healthy volunteers were received from the national biobank Genome Database of Latvian population [26] and used to validate the developed method. The clinical applicability of the reported method was assessed by analysing human plasma samples from newly diagnosed otherwise healthy pulmonary TB patients (n = 34) admitted to the Riga East University Hospital, Centre of Tuberculosis and Lung Diseases. All patients were undergoing WHO-recommended TB treatment regimen [2] and were receiving ETB, PZA, RIF, and IZN for ten days before sample collection. Blood samples were collected pre-dose (0 h) and 2 h, 6 h after drug administration into vacutainers with EDTA. The samples were then immediately centrifuged at 4000 rpm (3488*g*) for 15 min at 4 °C to separate the plasma. The separated plasma was collected and stored at −70 °C until analysis.

The study was approved by the Central Medical Ethics committee of Latvia (approval No: 01-29.1/1), the Ethics Committee of Riga East University Hospital (approval No: 24-A/15), and the Ethics Committee of Riga Stradins University (approval No: 6-3/1/6).

3. Results and discussion

3.1. Optimisation of UPLC-MS/MS conditions

Both HILIC and RP modes were tested for separation of the analytes. In the experiments conducted, the mobile phase organic constituent was acetonitrile, methanol, or isopropanol. Mobile phase additives (i.e., FA, ammonium acetate, ammonia hydroxide, acetic acid) were used to improve the peak shape and retention times for better separation of the analytes. The representative chromatograms are shown in (Supplementary Fig. 1).

HILIC separation mode was tried on Acquity UPLC BEH HILIC and Acquity UPLC BEH Amide columns. The analytes of interest were weakly retained on the BEH HILIC column and eluted within 3 min, even using 100 mm column. *Sundell et al.* [19] achieved optimal results under similar conditions, but, in our hands, observed peak overlapping due to early analyte elution was unacceptable. The use of the BEH Amide column allowed to achieve sufficient retention for all analytes. The prolongation of retention times for polar analytes on amide columns, in comparison to chemically unmodified HILIC stationary phases, was confirmed earlier and might be explained by differences in dominating type of interactions between the stationary phase and analytes [27]. However, RIF, 25DRIF, and ETB yielded poor peak shapes and attempts to improve that by addition of ammonium acetate (10–50 mM) to the mobile phase as suggested by the other authors [17,18] caused severe ionisation suppression of PZ2A and 5OHPZ2A. *Priyanka et al.* [14] experimentally proved that the ionisation of acidic PZA metabolites is either sensitive to mobile phase pH changes. In our hands, the analysis on the BEH Amide column was possible only in two mobile phase systems with opposite pH. Therefore, the optimisation of the chromatographic conditions on this column was discontinued.

Acquity UPLC BEH C18 and BEH C8 columns were applied in RP mode. The most polar analytes (ETB, IZN, and IZNAC) eluted in void volume on the BEH C18 column. Application of highly aqueous initial mobile phase composition (up to 99% water) did not result in sufficient retention of these analytes. The published data [15,23,24] suggested that the use of chemically modified C18 columns could facilitate chromatographic separation of the analytes. However, considerably better results were achieved on the unmodified BEH C8 column. The shorter alkyl chains are less hydrophobic and thus enhanced retention of polar constitutes at similar conditions. The further adjustment of chromatographic conditions (e.g., mobile phase composition, flow rate) was directed towards the separating overlapping peaks – IZN and metabolites, 5OHPZ2A and 5OHPZA. An increase in column temperature would again result in shortening of retention times and for this reason, was not considered. The mobile phase gradient (0.1% FA solution (A) and methanol (B); 99 → 2% A) at a flow rate of 0.2 mL/min was suitable for the separation of the analyte mixture (Fig. 1). At the given conditions, ionisation issues or peak splitting were not observed. The sensitivity of the developed method allowed us to reduce the injection volume to 1 μL. If the injection volume was increased, peaks tended to broaden, and column overload was expected. Although the analytes were separated in less than 5 min, the total run time was extended to 10 min for system equilibration.

ESI+ mass spectra was acquired for all analytes. The most abundant fragments were chosen from product ion spectra to set appropriate MRM transitions. Precursor-product ion pairs and optimised MRM parameters for ten analytes and IS are shown in Table 2.

Optimised chromatographic conditions prevented peak overlapping and enabled accurate quantification of the analytes. Due to the formation of rifampicin quinone, two RIF peaks were observed, as reported in the literature [28]. Thereby, two ion-transitions were used for accurate rifampicin quantification.

3.2. Sample preparation

Literature reports the application of liquid-liquid extraction (LLE), solid-phase extraction (SPE), and protein precipitation (PP) techniques for sample pre-treatment (Table 1). Preliminary experiments of LLE with ethyl acetate resulted in low recovery for PZ2A and 5OHPZ2A, and interferences with unseparated plasma components for the other analytes were observed. The introduction of polar acidic pyrazinamide metabolites in the analyte mixture required sample fractionation to achieve optimal recovery for acidic and basic compounds, as suggested by the other authors [14,19]. Similarly, the HybridSPE^{TM}-Phospholipid Ultra cartridge (Supelco) retained both polar analytes due possible Lewis acid-base interaction on the surface of the stationary phase under chosen conditions. Then, single-step PP with methanol and acetonitrile (with or without FA or ammonium hydroxide additive) were investigated. The PP with methanol without the use of any additives successfully reduced the excessive amount of endogenous matrix components in the final sample and provided consistent results, as shown in Section 3.3.4. The applied PP method was simple, fast, and cost-effective, allowing high-throughput analysis of study samples.

3.3. Method validation

3.3.1. Selectivity

In the MRM chromatograms shown in Fig. 2 representing the blank plasma sample and the first calibration standard containing IS and analytes at LLOQ level, it is seen that no potentially interfering signals at the expected retention time of the analytes and IS appear in the individual MRM channels of the blank plasma sample. If comparing the analytical signal of blank plasma samples to their equivalents with IS and analytes at LLOQ level, it was found that the signal intensity of 5OHPZ2A at LLOQ level is lower than expected, and the signal of blank matrix in this MRM channel comprised 25% of the analyte signal.

3.3.2. Calibration curve and lower limit of quantification

The seven-point calibration curves were obtained by plotting the peak area versus analyte concentration in the corresponding calibration standard. The results confirmed a linear analytical response in calibration ranges given in Table 2 with the correlation coefficients (r^2) in the range of 0.989–0.9998 when weighting factors 1/x or $1/x^2$ applied (Table 3). The back-calculated concentrations for the calibration standards were 86.3–108.7% (at the LLOQ level 83.7–106.1%)

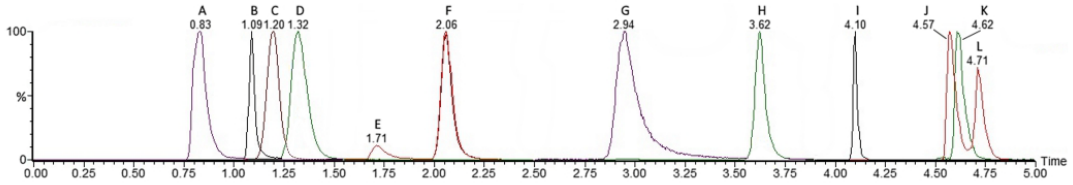

Fig. 1. Overlay plot of MRM chromatograms (seventh calibration standard, STD7). The chromatographic separation of the analyte mixture was achieved on BEH C8 column within 5 min. The total run time was extended to 10 min for system equilibration. A – ETB (0.83); B – IZN (1.09); C – IZNAC (1.20); D – ACIZN (1.32); E – 5OHPZ2A (1.71); F – 5OHPZA (2.06); G – PZ2A (2.94); H – PZA (3.62); I – IS (4.10); J, L – RIF (4.57, 4.71); K – 25DRIF (4.62).

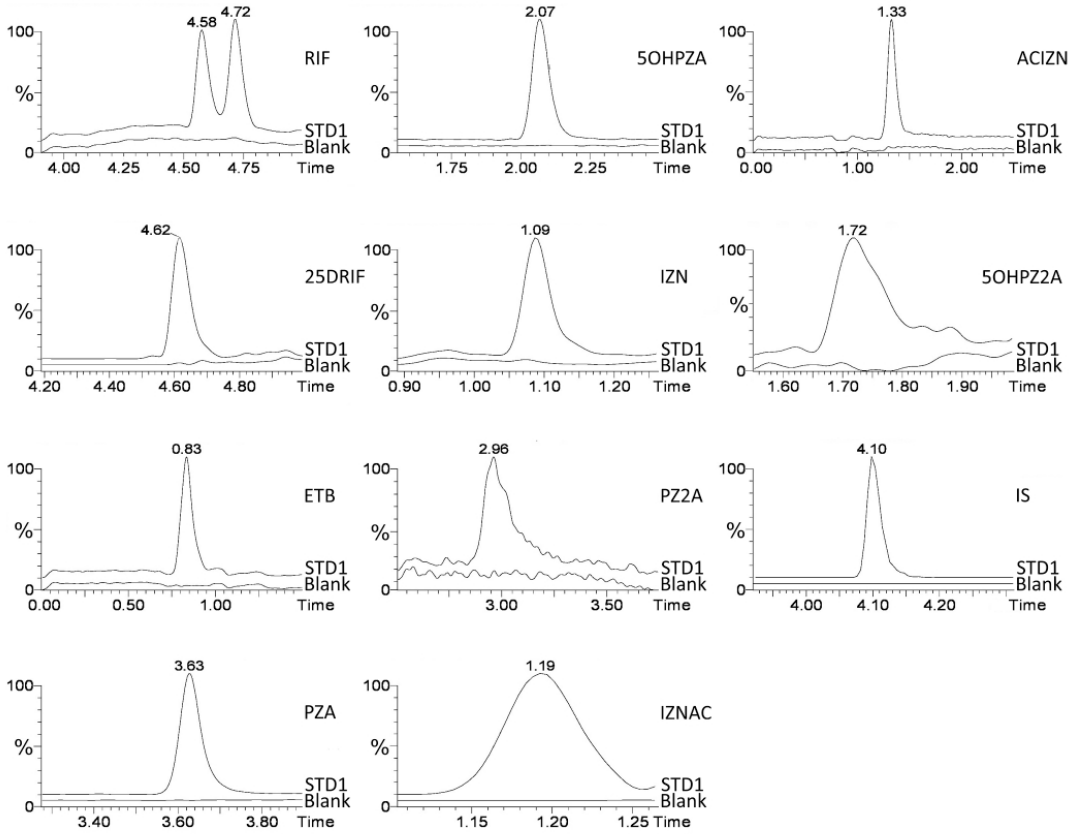

Fig. 2. Representative MRM chromatograms of the blank plasma sample (Blank) and the first calibration standard (STD1), containing analytes at LLOQ level and IS.

(Supplementary Table 1).

The LLOQ for all analytes was set at a concentration of the lowest calibration standard where the CV between replicate samples was in the range 0.3–12.9% but signal-to-noise ratio $\geq$ 5. Considering the low signal intensity of 5OHPZ2A at the LLOQ level, it was decided to set the second level calibration standard as LLOQ, where the signal-to-noise ratio was >5.

The chosen quantification ranges for all first-line anti-tuberculosis drugs correspond to the theoretically expected plasma concentrations [3] and are broader than those reported in the majority of previous studies (Table 1). The quantification ranges for metabolites were derived from the respective quantification range of the parent drug. As a consequence of expanding quantification ranges, the LLOQ values tend to be slightly higher. However, quantitation below the therapeutic window still is possible with a sufficient degree of accuracy and precision.

3.3.3. Accuracy and precision

The within- and between-run accuracy and precision were evaluated at four concentration levels (LLOQ, LQC, MQC, and HQC). The results are given in Table 4. The within-run accuracy in all concentration levels was between 87.2 and 113.6%. The CV between replicate samples (n = 5) at all concentration levels was 1.6–14.9%, except for LLOQ level, where 16.6% for IZNAC and 17.3% for 25DRIF complied with the

Table 4
The within- and between-run accuracy and precision for the first-line anti-tuberculosis drugs and their primary metabolites in human plasma.

Compound	QC level	Nominal concentration (μg/mL)	Within-run		Between-run	
			Accuracy (%)	Precision (CV, %)	Accuracy (%)	Precision (CV, %)
IZN	LLOQ	0.16	108.0	12.8	101.9	8.7
	LQC	0.40	98.7	11.5	97.0	7.4
	MQC	4.00	92.9	6.8	94.5	7.4
	HQC	8.00	99.0	9.2	95.5	9.6
IZNAC	LLOQ	0.16	95.2	16.6	97.2	12.1
	LQC	0.40	105.4	8.6	101.7	8.3
	MQC	4.00	94.9	8.4	97.7	6.8
	HQC	8.00	97.5	5.4	99.6	4.7
ACIZN	LLOQ	0.16	94.1	5.3	91.1	6.8
	LQC	0.40	93.7	4.0	97.4	5.2
	MQC	4.00	91.1	3.9	96.3	4.3
	HQC	8.00	90.2	3.7	92.5	5.3
ETB	LLOQ	0.16	109.3	4.8	99.3	7.6
	LQC	0.40	104.3	5.4	97.0	5.9
	MQC	4.00	97.1	5.8	04.0	5.2
	HQC	8.00	96.2	4.7	94.4	5.2
RIF	LLOQ	0.48	113.6	6.1	109.3	5.6
	LQC	1.20	104.7	3.2	103.5	5.4
	MQC	12.0	107.1	3.1	104.6	3.5
	HQC	24.0	101.4	3.3	100.8	4.7
25DRIF	LLOQ	0.48	100.7	10.0	98.7	13.3
	LQC	1.20	91.1	12.5	97.5	10.0
	MQC	12.0	92.5	6.0	98.0	6.0
	HQC	24.0	102.5	4.5	97.1	6.4
PZA	LLOQ	1.20	96.4	11.2	93.7	8.4
	LQC	3.00	99.0	9.3	100.2	6.6
	MQC	30.0	93.3	9.4	96.7	5.6
	HQC	60.0	89.5	9.2	91.7	6.4
PZ2A	LLOQ	1.20	95.5	9.5	93.8	11.0
	LQC	3.00	103.1	7.1	102.4	7.3
	MQC	30.0	96.9	3.8	98.3	3.8
	HQC	60.0	94.0	3.3	93.5	4.7
5OHPZA	LLOQ	1.20	99.1	7.9	93.4	7.9
	LQC	3.00	107.3	5.9	103.9	5.3
	MQC	30.0	97.2	4.4	97.8	3.8
	HQC	60.0	89.4	4.5	91.4	5.2
5OHPZ2A	LLOQ	1.20	88.0	14.9	92.2	11.5
	LQC	3.00	105.4	9.9	102.2	7.6
	MQC	30.0	102.9	5.8	99.3	4.4
	HQC	60.0	96.3	5.1	93.3	4.8

requirements. The between-run accuracy and precision were calculated as the average of three analytical runs performed on three consecutive days and ranged 91.1–109.3% and 3.5–13.3%, respectively.

3.3.4. Matrix effect

Matrix effect was assessed at LQC and HQC concentration levels using MF calculated for six different matrix sources. The mean MF for most of the analytes was in the range of 0.9–1.1, thus exhibiting a minimal contribution of the matrix to ion suppression or enhancement (Table 5). The MF < 0.5 for ETB and IZN indicated significant ion suppression by unseparated matrix components. The response of IZNAC at the LQC level varied depending on the matrix source; the CV between six different matrix sources was 21.8%. However, the results of linearity, accuracy, and precision confirmed that observed inconsistency did not affect the quantification of the IZNAC.

3.3.5. Stability

The obtained results of analyte stability in human plasma at various storage conditions are summarized in Table 6. Accordingly, all tested compounds were found to be stable in the human plasma samples at room temperature up to 1 h, but after 4 h, a significant reduction in 25DRIF, IZN, and IZNAC concentrations was observed. The samples were stable when stored at −20 °C for 24 h; storage time extension to 7 days resulted in the IZN and IZNAC concentration decrease by more than 15%. Moreover, the plasma sample exposure to three freeze-thaw cycles in 7 days contributed to the degradation of RIF and 5OHPZ2A. The long-

Table 5
The calculated matrix factors (MF) for the analytes and IS at the low and high QC levels.

Compound	QC level	MF (mean ± SD; n = 6)	CV (%)
IZN	LQC	0.4 ± 0.03	6.3
	HQC	1.3 ± 0.1	6.6
IZNAC	LQC	0.6 ± 0.1	21.8
	HQC	0.8 ± 0.1	11.9
ACIZN	LQC	1.1 ± 0.1	5.1
	HQC	1.1 ± 0.04	3.5
ETB	LQC	0.4 ± 0.02	3.8
	HQC	0.5 ± 0.02	3.9
RIF	LQC	1.1 ± 0.1	6.6
	HQC	0.8 ± 0.04	5.0
25DRIF	LQC	1.3 ± 0.2	14.2
	HQC	1.1 ± 0.2	17.5
PZA	LQC	1.0 ± 0.02	1.7
	HQC	1.0 ± 0.01	0.9
PZ2A	LQC	1.1 ± 0.1	6.0
	HQC	1.0 ± 0.03	2.5
5OHPZA	LQC	0.9 ± 0.1	9.2
	HQC	1.0 ± 0.1	8.2
5OHPZ2A	LQC	1.0 ± 0.04	3.7
	HQC	1.0 ± 0.02	2.2
IS	LQC	0.7 ± 0.1	7.3
	HQC	0.8 ± 0.05	6.6

Table 6
Stability of the analytes in human plasma at various storage conditions.

Compound	QC level	Stability (%)					
		Stored at RT* for:		Stored at −20 °C for:			Freeze-thaw (3 cycles, −20 °C, 7 days)
		1 h	4 h	24 h	7 days	3 months	
IZN	LQC	102.9	82.2	87.2	82.8	56.4	73.1
	HQC	89.9	93.0	100.3	84.0	64.7	93.1
IZNAC	LQC	93.1	78.1	94.3	91.0	95.4	89.1
	HQC	90.3	72.3	98.9	75.0	80.3	91.8
ACIZN	LQC	95.7	88.8	84.7	98.5	80.6	86.6
	HQC	95.2	95.0	98.6	94.3	91.3	101.7
RIF	LQC	95.4	89.9	90.9	97.0	94.4	75.8
	HQC	94.5	94.3	92.7	94.8	90.3	87.5
25DRIF	LQC	95.6	89.3	89.5	94.5	81.3	92.4
	HQC	89.4	76.7	93.6	86.3	77.7	94.8
PZA	LQC	97.8	94.2	86.9	90.8	112.1	90.3
	HQC	95.4	92.9	99.4	96.8	101.7	98.3
PZ2A	LQC	92.0	94.5	88.2	93.7	116.5	86.6
	HQC	91.5	91.6	98.3	93.0	103.6	97.3
5OHPZA	LQC	98.8	92.8	84.9	97.3	148.6	87.6
	HQC	94.5	90.9	96.4	92.5	138.5	93.7
5OHPZ2A	LQC	94.2	86.1	89.7	98.4	83.7	76.5
	HQC	93.5	90.6	99.3	92.9	80.6	87.0
ETB	LQC	98.1	97.3	88.5	105.0	103.9	94.3
	HQC	100.0	96.2	98.7	98.2	100.9	100.8

*RT – room temperature

term stability test at −20 °C for 3 months caused substantial degradation of the analytes. Similar findings regarding the limited plasma stability of IZN, RIF and their metabolites were previously mentioned by the other authors [18,21,22]. Based on these results, it is recommended that plasma samples should be processed within 4 h after collection. If necessary, temporary storage at −20 °C up to 24 h is acceptable.

The IZNAC and 25DRIF were found to be unstable in the autosampler (stability 71.6–81.3%), as the stability of the other analytes ranged from 86.1 to 100.1% and complied with the requirements. The IS stock solution remained unchanged for 14 days at −20 °C, whereas the 5OHPZA stock solution was the only exhibiting a concentration reduction of more than 15% when stored at −20 °C for 2 months.

3.3.6. Carry-over

The signal of the blank plasma sample following the highest calibration standard ranged from 0.9 to 20.3%. The IS signal in the corresponding MRM channel was not detected at all. As a result, it was assumed that significant carry-over between samples did not occur.

3.3.7. Dilution integrity

Dilution integrity was demonstrated by analysing the samples containing analytes above HQC before 5- and 10-fold dilution. When performing analysis of processed samples, it was found that the CV between replicate samples (n = 6) was between 3.6 and 10.3%. In contrast, the accuracy for ETB and IZN was between 78.4 and 83.7%, but for the remaining analytes was within 15% and met the acceptance criteria. The obtained results suggest that study samples with analyte concentration above HQC can be diluted before sample preparation, apart from cases when ETB and IZN exceed the upper limit of quantification.

3.4. Clinical application

The validated method was applied to the human plasma samples from otherwise healthy pulmonary TB patients (n = 34) subjected to the TB treatment regimen with four first-line anti-tuberculosis drugs 10 days before sample collection. Concentrations of four drug substances and six metabolites were measured before drug administration (0 h), 2 and 6 h after receiving a single dose of anti-tuberculosis drugs. The mean concentrations are given in Table 7. Overall, the measured plasma

Table 7
Concentration of the first-line anti-tuberculosis drugs and six primary metabolites in TB patient plasma (n = 34) pre-dose (0 h), 2 and 6 h after drug administration.

Compound	Measured concentration (μg/mL, mean ± SD; n = 34)			Therapeutic range 2 h post-dose [3] (μg/mL)
	Pre-dose (0 h)	2 h post-dose	6 h post-dose	
IZN	0.14 ± 0.11	2.79 ± 1.61	1.39 ± 0.88	3–6
IZNAC	0.08 ± 0.09	0.38 ± 0.20	0.39 ± 0.15	N/A*
ACIZN	0.23 ± 0.22	1.38 ± 1.05	1.58 ± 0.88	N/A
RIF	0.17 ± 0.42	2.31 ± 2.59	2.63 ± 1.70	8–24
25DRIF	0.00 ± 0.00	0.05 ± 0.13	0.07 ± 0.12	N/A
PZA	9.52 ± 7.37	38.4 ± 16.8	36.1 ± 12.3	20–60
PZ2A	4.36 ± 3.82	8.77 ± 4.89	11.4 ± 4.85	N/A
5OHPZA	1.07 ± 0.88	2.38 ± 1.09	3.22 ± 1.06	N/A
5OHPZ2A	3.09 ± 2.85	3.29 ± 2.45	5.40 ± 3.00	N/A
ETB	0.44 ± 0.28	2.68 ± 1.60	1.94 ± 0.86	2–6

N/A* – not applicable.

concentrations were within the analytical range of the proposed method. It was possible to determine baseline concentrations before drug intake and estimate whether plasma concentrations confirm the therapeutic window reported in the literature [3]. The observed subtherapeutic plasma concentrations of the RIF (<8 μg/mL) and IZN (<3 μg/mL) 2 h after drug intake in the study population were consistent with the previous findings and can be explained with pharmacokinetic variability among TB patients [28–31]. The auto-induction capacity of RIF over the period of treatment tends to reduce its exposure, whereas IZN plasma concentration mostly depends on the N-acetyltransferase 2 (NAT2) enzymatic activity [10,32,33]. The full interpretation of the study results will be published in a separate paper.

To date, this is the first report on the LC-MS/MS method that allows determining the full primary metabolite profile of anti-tuberculosis drugs in human plasma within a single run. Therefore, the developed method can be employed in population-scale studies to enhance the knowledge of the pharmacokinetics of anti-tuberculosis drugs and for the therapeutic drug monitoring purposes. The offered method can be either employed in clinical practice, as the obtained pharmacokinetic data would justify the need for transition from standardized to individualized TB therapy.

4. Conclusions

The validation results confirmed the performance of the reported method, thus guaranteeing the quality of the results. The main advantages are the requirement of small sample volume for analysis, simple sample pre-treatment procedure, and the ability to analyse all four first-line anti-tuberculosis drugs and all six primary metabolites within a single run to enhance the utility of pharmacokinetic studies. Besides, the chosen quantification ranges fully cover clinically expected plasma concentrations, thereby reducing the need for sample dilution before the pre-treatment procedure. The clinical sample analysis gave an insight into the method's clinical applicability, where the aim was to determine TB drug exposure in the study population. The obtained results highlight the importance of future studies addressing issues related to the efficacy of TB treatment.

CRediT authorship contribution statement

Agnija Kivrane: Writing – original draft, Validation. **Solveiga Grinberga:** Methodology, Project administration, Supervision. **Eduards Sevostjanovs:** Methodology, Validation. **Viktorija Igumnova:** Conceptualization, Resources. **Ilva Pole:** Clinical sample collection. **Anda Viksna:** Clinical sample collection. **Dace Bandere:** Project administration, Supervision, Funding acquisition. **Alvils Krams:** Project administration. **Andra Cirule:** Project administration. **Osvalds Pugovics:** Project administration, Supervision. **Renate Ranka:** Project administration, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2021.122986.

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Pharmacogenomics

Implementation of a next-generation sequencing-based targeted approach for full-length *CYP3A4* gene sequencing

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Aim: To evaluate the application of next-generation sequencing-based targeted protocol for full-length *CYP3A4* gene sequencing analysis. **Materials & methods:** The developed sequencing protocol was applied to analyze human DNA samples (n = 7) obtained from tuberculosis patients admitted to the Riga East University Hospital, Center of Tuberculosis and Lung diseases. **Results:** The sequencing data quality was sufficient for the detection of already known genetic variants, as well as for identifying rare and novel variants dispersed throughout the *CYP3A4* gene with a high degree of confidence. **Conclusion:** Developed protocol can be applied in subpopulation level association studies to determine whether specific genetic variants or variant combinations from multiple regions of the *CYP3A4* gene are of clinical significance.

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Numerous studies conducted over the past decade on pharmacogenetic variability in different populations suggest that inter-individual variations in drug response caused by genetic factors can affect treatment response and should be taken into account in making prescribing decisions [1–3]. Based on these studies, the Clinical Pharmacogenetics Implementation Consortium (CPIC) has developed 25 evidence-based and peer-reviewed clinical guidelines that link together information from genetic testing with phenotype and provide therapeutic recommendations to clinicians with the purpose of therapy optimization by genotype-guided dosing [4].

CYP3A4 is a member of the well-studied CYP450 enzyme superfamily, which catalyzes the Phase I biotransformation reactions of exogenous compounds (xenobiotics). It is estimated that CYP3A4 alone is involved in the metabolism of 30% of commonly used medications [5]. The genetic variability of the *CYP3A4* gene has been reported in different populations. However, unlike the other *CYP450* family members, association studies investigating *CYP3A4* genetic variability in context with drug response have not resulted in strong evidence supporting the relationship between specific allele or single nucleotide variant (SNV), impaired drug metabolism and/or treatment response [6–8]. Nevertheless, several *CYP3A4* alleles have been identified that alter the enzymatic activity; for example, *CYP3A4*22* (rs35599367, C>T) allele carriers will likely have decreased enzymatic activity and require lower statin doses for lipid control [9]. Also, in addition to *CYP3A5* genotyping for adjustment of the tacrolimus dose before kidney, heart and lung transplantation, it is advised to test for *CYP3A4*22* alleles to confirm the metabolic status of the patient [10].

The ability to identify specific genetic variants that may have phenotypic implications on human health is of great importance. It would be necessary to obtain the most comprehensive full-length gene sequencing data to systematically investigate the genetic variability of the *CYP3A4* gene in different populations. Whole-exome sequencing and whole-genome sequencing techniques are widely used in human genetics to identify novel genetic variants and to conduct large-scale association studies. Nevertheless, the targeted next-generation sequencing (NGS)-based method is a more cost-effective, time-saving and convenient approach that enables detection of

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Table 1. Designed primer pairs for the full length *CYP3A4* gene amplification.

Primer name	Sequence (5′-3′)	Position in the reference genome†	Product size (bp)
3A4_1_Fw	AGTCACAATCAGGGGGTCCT	99779622-99779641	4588
3A4_1_Rw	AACAATCCAACAGCCTCACTG	99784189-99784209	
3A4_2_Fw	GCTCCCAGCCTAGTTCAGAC	99777349-99777368	2836
3A4_2_Rw	CGTTCTCTTGAGCATTCCAGT	99780164-99780184	
3A4_3_Fw	AGCCAGACTTTGATCCTGACTT	99772758-99772779	5955
3A4_3_Rw	TGTCCGATGTGTAGAGGGGA	99778693-99778712	
3A4_4_Fw	TGAAGCCCTCAAATCCCTAAG	99764093-99764113	5936
3A4_4_Rw	AGTCCACTGAATTTGAGCTG	99770009-99770028	
3A4_6_Fw	AAGAAGTGCCAGGTTGGGAG	99759013-99759032	5141
3A4_6_Rw	CACCCCCAGTGTACCTCTGAA	99764134-99764154	
3A4_7_Fw	TAGCCAAACCTCACCCAACC	99756631-99756650	4372
3A4_7_Rw	AATCTACCAACGTGGAACCAGA	99760981-99761002	
3A4_5_Fw	CCACATATCCCTGGTCTGCC	99769158-99769177	3758
3A4_9_Rw	TCTTCCTTGTTTGGCCCACAT	99772895-99772915	
3A4_10_Fw	GTGGGGAGACATTCGAGACG	99763245-99763264	4147
3A4_10_Rw	GGAATGGCTTCCAGTTGAGAAC	99767370-99767391	

†Human reference genome (GRCh38.p13, GCF_000001405.39).

variants or exploring specific genomic regions at the subpopulation level while maintaining the same quality of sequencing depth and coverage [11,12]. *CYP3A4* gene also has been introduced in several sequencing panels targeting only specific genetic regions to screen for SNVs of interest [13,14] or haplotype determination [15]. Specifically, this approach could be beneficial for rapid variant screening for clinical purposes.

In this study, we evaluated whether the targeted NGS approach could be used to develop a simple protocol for the full-length *CYP3A4* gene sequencing, including all 13 exons with interleaving introns and untranslated (UTR) regions. This protocol combined sequencing breadth and data quality of whole-exome sequencing and whole-genome sequencing with the relatively low costs and the relatively simple technical execution, enabling the identification of novel or already known genetic variants for a better understanding of *CYP3A4* gene-related factors that has clinically relevant consequences.

Materials & methods

Clinical samples

Human DNA samples (n = 3) from the national biobank Genome Database of Latvian population [16] were used to optimize the PCR conditions. Performance of the developed protocol for the target amplification and sequencing was assessed using human DNA samples (n = 7) obtained from tuberculosis patients admitted to the Riga East University Hospital, Centre of Tuberculosis and Lung Diseases. The study was approved by the Central Medical Ethics Committee of Latvia (approval no. 01-29.1/1) and the Ethics Committee of Riga East University Hospital (approval no. 24-A/15).

Full-length gene amplification & sequencing

For the full-length *CYP3A4* gene amplification, eight primer pairs (Table 1) targeting overlapping gene fragments were designed using an online-based Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Figure 1). Amplification was performed with All Taq PCR Core Kit (QIAGEN, Germany) following the manufacturer's protocol for large-sized fragment amplification; the PCR amplicon size ranged from 2836 to 5955 bp (Table 1). PCR products were analyzed by agarose gel electrophoresis and bioanalyzer (2100 Bioanalyzer instrument, Agilent Technologies, CA, USA).

PCR amplicons were pretreated with NucleoMag NGS Clean-up and Size Select (MACHEREY-NAGEL, Germany) magnetic beads, pooled and normalized to a final concentration of 1 ng/µl. NGS paired-end libraries were prepared using Nextera XT DNA Library perp kit and Nextera Index kit (Illumina, CA, USA) according to the manufacturer's instructions. NucleoMag NGS Clean-up and Size Select magnetic beads were used for the library purification and double size-select to achieve an optimal library size of 300-500 bp. Subsequently, libraries were sequenced using the MiSeq platform (Illumina) to generate paired-end reads with a maximum length of 250 bp.

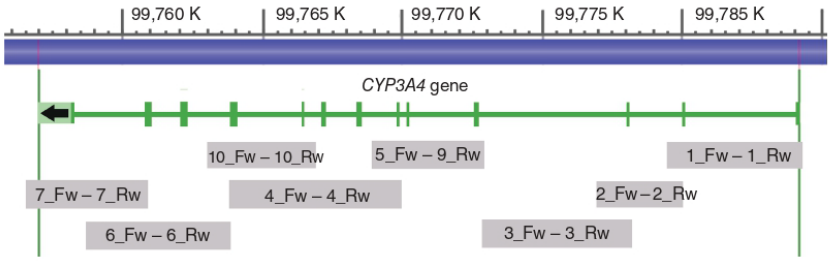

Figure 1. Graphical representation of the PCR fragment position in the reference genome.

Data analysis

Sequencing data analysis was performed on the Galaxy online-based platform using the public server at https://usegalaxy.org [17]. Trimmomatic (v0.38) was used to trim adapter sequences and low quality read ends (Phred quality score <20). Reads were mapped against the human reference genome (GRCh38.p13, GCF_000001405.39) using Map with BWA-MEM (v0.7.17.1). BAM filter (v0.5.9) was applied to keep only mapped reads, remove PCR duplicates and discard reads shorter than 50 bp, but Markduplicates (v2.18.2.2) was used to finalize deduplication. Alignment was converted with Samtools mpileup (v2.1.4) for SNV calling with VarScan (v2.4.2). Detected variants were filtered using the following criteria: minimum supporting reads: six (SNV must be represented on both positive and negative strands), read depth ≥10, base quality ≥20 and minimum read frequency for homozygous positions: 75%. All detected variants were visually inspected using Integrative Genome Viewer, v2.8.9. Functional annotation of the identified SNVs was performed using online-based wANNOVAR tool (http://wannovar.wglab.org/) [18]; reference SNP (rs) reports accumulated in dbSNP database (https://www.ncbi.nlm.nih.gov/snp/) and PharmGKB (https://www.pharmgkb.org/) were used for the detected variant identification and annotation [10,19].

Confirmation of detected variants

For the confirmation of randomly selected genetic variants (n = 10) Sanger sequencing was used. Briefly, the obtained amplicons were subjected for the standard Sanger sequencing procedure using either forward or reverse amplification primers (Table 1). BrilliantDye Terminator Cycle Sequencing kit v1.1 (NimaGen, Nijmegen, The Netherlands) was used. Sequencing was carried out according to a standard protocol using an ABI Prism 3100 Genetic Analyzer (Perkin-Elmer, MA, USA). The MEGA and FinchTV programs were used for sequence alignment and analysis; Basic Logical Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for sequence comparison to previously published data in GenBank.

Results

Amplification of the full length *CYP3A4* gene with most of the designed primer pairs for the test samples resulted in specific products corresponding to the expected fragment length (Table 1). The specificity of the primer pairs CYP3A4_1_Fw/1_Rw, CYP3A4_6_Fw/6_Rw, CYP3A4_7_Fw/7_Rw and CYP3A4_5_Fw/9_Rw was considered to be lower because additional bands of fragments were visible on the agarose gel (Figure 2). The nonspecific amplification observed could not be avoided by optimizing the PCR conditions (data not shown). However, the amount of nonspecific fragments can be reduced by the amplicon pretreatment step (single side size-select).

Sequencing data quality for the test samples (n = 7) after the applied quality filters is summarized in Table 2. The mean read base quality score of >30 was achieved, thus guaranteeing high base call accuracy. Even though 500-fold coverage appeared to be sufficient to achieve the optimal read depth, two of the seven samples lacked coverage for 14.7 and 15.5%, respectively, of the target fragment. No significant differences in the coverage distribution between exons and introns were observed. Overall, mean mapped read depth of >100 indicated that base calls have a high degree of confidence in fully covered regions.

Detected SNVs were grouped according to the location and functional classification (Table 3). In total, each sample contained 5-24 variants. Most of the detected variants were intronic, one sample had two nonsynonymous exonic SNVs and three samples had variants located in a 5′ untranslated region (5′UTR). Overall, 35 of detected variants were dbSNP database-referenced and the one (rs2242480) also was included in the Catalogue of Somatic

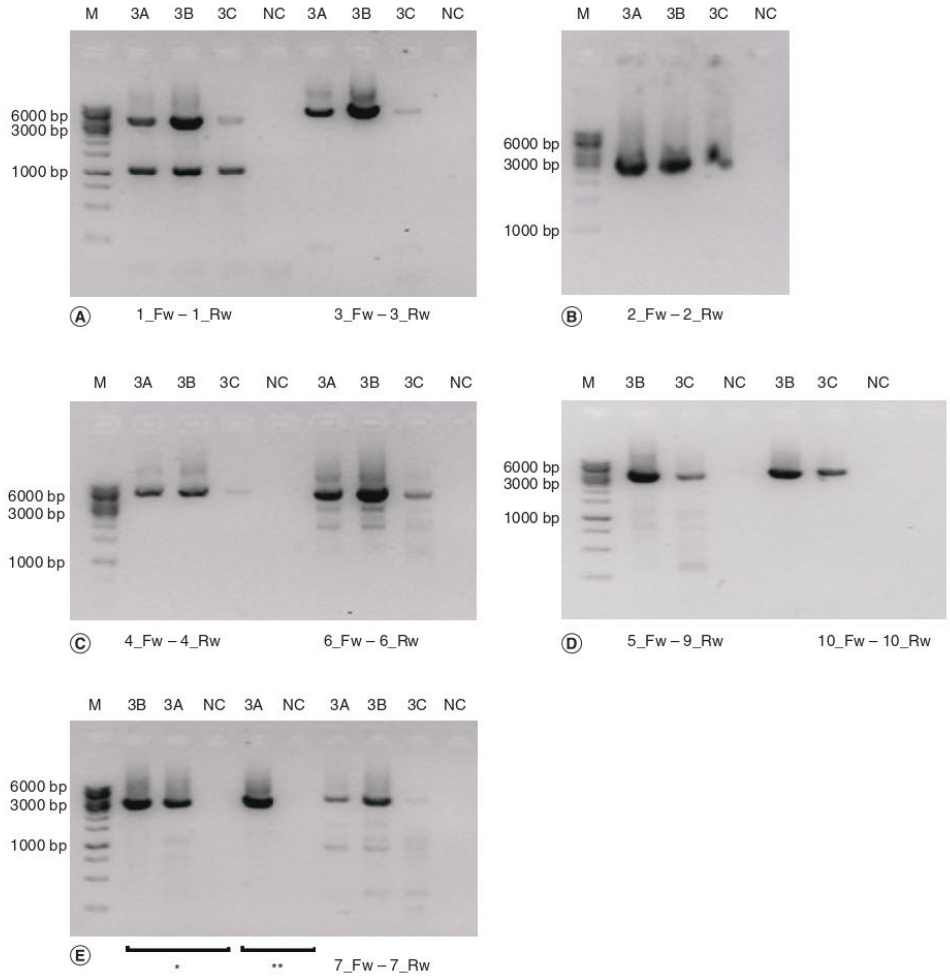

Figure 2. *CYP3A4* gene PCR product analysis for the test samples (n = 3) by agarose gel electrophoresis. Primer pairs are indicated for each panel. 3A, 3B, 3C – DNA samples; NC – negative control; *praimer pair 5_Fw – 9_Rw; **praimer pair 10_Fw – 10_Rw. Fragment sizes of the brighter bands of the GeneRuler 1 kb DNA ladder are indicated.

Table 2. Sequencing data quality for the test samples (n = 7).

	Sample ID						
	1RJ	2LN	4AP	5RL	6JK	7SG	8VL
Number of reads mapped	67131	52549	50679	51515	51370	52983	57028
Average read length (bp)	183	166	161	162	160	169	168
Mean base quality score	37.5	37.5	37.6	37.6	37.6	37.5	37.5
Mean read depth	230	229	187	187	184	198	211
≥Tenfold coverage† (%)	83.6	85.3	100.0	100.0	100.0	99.9	100.0
No coverage‡ (%)	15.5	14.7	0.0	0.0	0.0	<0.1	0.0

†Percentage of the target fragment (27,218 bp) with at least tenfold coverage, genomic coordinates chr7:99756967-99784184.
‡Percentage of the target fragment (27,218 bp) with zero coverage, genomic coordinates chr7:99756967-99784184.

Table 3. Classification of the identified variants.

	Sample ID						
	1RJ	2LN	4AP	5RL	6JK	7SG	8VL
Intronic variants	5	13	13	17	15	22	15
Exonic variants	0	0	0	0	0	0	2†
5′UTR variants	0	0	0	0	2	2	1
Total	5	13	13	17	17	24	18

†Exonic nonsynonymous variants.

Table 4. Variant identification results using reference SNP (rs) reports accumulated in dbSNP database (https://www.ncbi.nlm.nih.gov/snp/).

	SNV ID
Exonic nonsynonymous variants	rs371360704
Intronic variants	rs12670850 rs144203939 rs145664747 rs2242480† rs2246709 rs2404767 rs2687105 rs2687107 rs2687110 rs2687116 rs2687117 rs2738258 rs2738262 rs2740580 rs28988583 rs28988600 rs35599367 rs3735451 rs4281055 rs4646437 rs6956344 rs759566058 rs908169177 rs887674423 rs1236894720 rs1376594393 rs1442309874 rs894688127 rs1482389008 rs929493496 rs970353690 rs1002555313
5′UTR variants	rs1285769624 rs1261008170

†rs2242480 was found in the Catalogue of Somatic Mutations In Cancer (COSMIC) (https://cancer.sanger.ac.uk/cosmic) ID: COSN17135646.

Mutations in Cancer (Table 4) [20]. In our sample set, two allelic variants *CYP3A4*1G* (rs2242480, C>T) and *CYP3A4*22* (rs35599367, G>A) were identified. Among the test samples, three carried wild-type *CYP3A4* gene (*CYP3A4*1/*1*), whereas the other two had *CYP3A4*1/*1G* and *CYP3A4*1/*22* genotypes, respectively. However, resequencing should be performed to establish genotype for the two samples that were lacking coverage. Besides, six previously unreported variants were detected: 5′UTR SNVs (n = 1), exonic nonsynonymous SNVs (n = 1) and intronic SNVs (n = 4) (data not shown). The Sanger sequencing results for randomly selected variants (n = 10) were consistent with the results of NGS sequencing, thus confirming the accuracy of the proposed protocol. Representative chromatograms of the Sanger sequencing results are shown in Figure 3.

In this study, the main objects of interest were exonic nonsynonymous and intronic variants with potential impact on CYP3A4 enzymatic activity and with at least moderately supported association with drug-drug interactions, impaired drug metabolism or manifestations of adverse drug reactions (at least 2A and 2B clinical annotation level

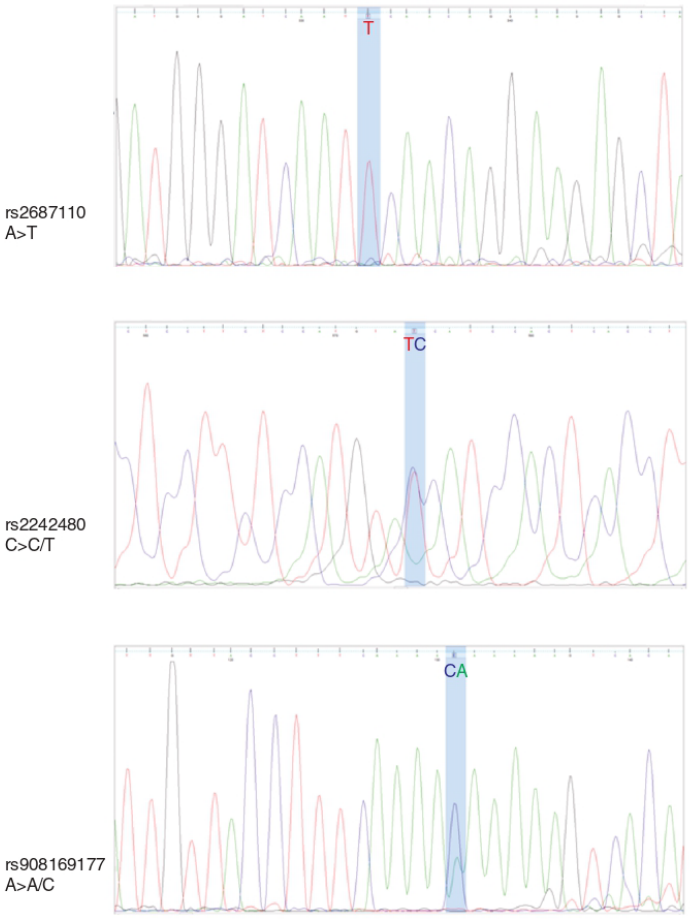

Figure 3. Representation of confirmatory chromatograms of the selected *CYP3A4* genetic variants obtained by Sanger sequencing.

of the evidence). Of all the variants identified, both *CYP3A4*1G* and *CYP3A4*22* allelic variants were of clinical significance [10].

Discussion

The study results demonstrated that the targeted NGS sequencing technique is superior for the full-length gene sequencing, as it is not limited by the screening of specific SNVs or sequencing of coding regions. Overall, this NGS-based protocol allowed the identification of polymorphic sites dispersed throughout the entire *CYP3A4* gene.

The presence of nonspecific PCR fragments reduces the number of unique reads aligned to the reference and; therefore, lower coverage of the target fragment could be expected. As nonspecific PCR amplification sometimes cannot be avoided, the introduction of amplicon quality control and PCR fragment size selection prior to sequencing library preparation minimized the amount of unintended amplification products. The coverage loss for two samples for 15% could be attributed to sample-specific amplicon quality, as the coverage for the rest of the samples was satisfying. The other authors suggest that G and C nucleotide content (GC content) less than 50% of the target fragment can also play a crucial role in the achievement of the desired coverage, and, on this basis, depending on chosen library preparation protocol, coverage adjustment might be required [21].

Studies on the pharmacogenetic variability of the other *CYP450* family members, such as *CYP2D6*, *CYP2C9*, *CYP3A5*, have resulted in evidence-supported clinical annotations of specific genetic variants that affect treatment response and should be identified before therapy onset [4]. For *CYP3A4*, there is still uncertainty about the possible linkage between genetic variants and corresponding phenotypes, as well as the role of genetic polymorphisms in regulatory pathways [22]. Moreover, a recent discovery of seven new *CYP3A4* allelic variants suggests that the current understanding of the *CYP3A4* genetic variability is incomplete and further studies must be conducted [23].

In this study, two common alleles in the European population among study samples were identified (allele frequency: 4.6 and 9.5% for the *CYP3A4*22* and *CYP3A4*1G* allele, respectively) [19]. Referring to the effects of the *CYP3A4* gene polymorphism, *CYP3A4*1/*1G* and *CYP3A4*1/*22* genotypes are associated with altered metabolism of tacrolimus (1B level of the evidence) [10]. To our knowledge, this is the first report on the identification of *CYP3A4* alleles in the Baltic States. Additional studies are required to determine the functional consequences of detected novel variants and to assess their frequency in the population.

To sum up, detection of database-referenced variants, as well as the discovery of numerous previously unreported exonic and intronic variants, confirms that the developed sequencing protocol could be applied in subpopulation-level studies focusing on the genetic variability of the *CYP3A4* gene and the clinical manifestations of the detected variants. The future perspective holds an evaluation of the developed targeted sequencing protocol for the potential of multiplexing for simultaneous analysis of multiple genomic regions in pharmacogenetic studies.

Conclusion

This study offers a simple and cost-effective NGS-based targeted sequencing approach for the full-length *CYP3A4* gene sequencing with sequencing data quality supporting deep-sequencing concept that is valuable for identification of rare and novel variants and crucial for further studies. The assessment of functional consequences and population-level prevalence of the novel *CYP3A4* variants detected in this study should be performed.

Summary points

- CYP3A4 is one of the major drug-metabolizing enzymes, and currently available studies suggest that *CYP3A4* genetic variations may alter enzymatic activity and point out potential associations with certain drug metabolic pathways.
- The ability to identify specific genetic variants that may have phenotypic implications on human health is of great importance.
- The purpose of this study was to develop next-generation sequencing-based targeted sequencing protocol for the full-length *CYP3A4* gene sequencing. Performance of the developed protocol was assessed by analysis of human DNA samples (n = 7).
- The developed protocol allowed to generate sequencing data with sufficient quality (mean read base quality score of >30; mean mapped read depth of >100) to detect both previously reported and novel variants with a high degree of confidence.
- In total, 35 database-referenced genetic variants were identified; two variants corresponded to *CYP3A4*1G* (rs2242480, C>T) and *CYP3A4*22* (rs35599367, G>A) alleles.
- The six previously unreported variants (5′UTR SNVs (n = 1), exonic nonsynonymous SNVs (n = 1), intronic SNVs (n = 4)) identified in this study should be further investigated.
- This study demonstrates that the next-generation sequencing-based targeted sequencing approach can be successfully used for the full-length *CYP3A4* gene sequencing, whereas the developed protocol has potential applications in future studies to improve knowledge of *CYP3A4* variability.

Author contributions

A Kivrane wrote a manuscript draft and together with V Igumnova, designed and performed experimental work and NGS sequencing data analysis. J Kimsis, L Freimane and D Sadovska contributed to the experiment design and the interpretation of NGS sequencing data. A Viksna was responsible for the clinical care of the patients and, together with I Pole, contributed to the collection of the test samples. R Ranka supervised the research work, critically revised and approved the final version of the manuscript. All authors approved the final manuscript.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review from Central Medical Ethics Committee of Latvia and Ethics Committee of Riga East University Hospital for the research described. In addition, informed consent has been obtained from the patients involved.

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Third publication

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Article

Exploring Variability in Rifampicin Plasma Exposure and Development of Anti-Tuberculosis Drug-Induced Liver Injury among Patients with Pulmonary Tuberculosis from the Pharmacogenetic Perspective

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Abstract: Genetic polymorphisms can exert a considerable impact on drug pharmacokinetics (PK) and the development of adverse drug reactions (ADR). However, the effect of genetic polymorphisms on the anti-tuberculosis (anti-TB) drug, and particularly rifampicin (RIF), exposure or anti-TB drug-induced liver injury (DILI) remains uncertain. Here, we evaluated the relationship between single nucleotide polymorphisms (SNPs) detected in the RIF pharmacogenes (*AADAC*, *SLCO1B1*, *SLCO1B3*, *ABCB1*, and *NR1I2*) and RIF PK parameters, as well as anti-TB treatment-associated DILI. In total, the study enrolled 46 patients with drug-susceptible pulmonary TB. The RIF plasma concentration was measured using the LC-MS/MS method in the blood samples collected pre-dose and 2 and 6 h post-dose, whilst the DILI status was established using the results from blood biochemical analysis performed before and 10–12 days after treatment onset. The genotyping was conducted using a targeted NGS approach. After adjustment for confounders, the patients carrying the rs3732357 GA/AA genotype of the *NR1I2* gene were found to have significantly lower RIF plasma $AUC_{0-6\,h}$ in comparison to those with GG genotype, while the difference in RIF plasma C_{max} was insignificant. None of the analyzed SNPs was related to DILI. Hence, we are the first to report *NR1I2* intronic SNP rs3732357 as the genetic component of variability in RIF exposure. Regarding anti-TB treatment-associated DILI, the other preexisting factors promoting this ADR should be considered.

Keywords: pharmacogenetics; tuberculosis; pharmacokinetics; drug-induced liver injury; next-generation sequencing; liquid chromatography–tandem mass spectrometry

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1. Introduction

Cost-effectiveness, availability, and high cure rate (around 86%) are the major considerations for the standard 6-month regimen consisting of rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA), and ethambutol (ETB) to remain the first choice for the treatment of drug-susceptible tuberculosis (DS-TB) [1,2]. RIF remains one of the most important drugs in TB treatment; it is able to penetrate granulomatous lesions and acts on both metabolically active and, most importantly, dormant *Mycobacterium tuberculosis* bacteria by selectively inhibiting bacterial DNA-dependent RNA polymerase [3–6]. The RIF bactericidal activity is concentration-dependent; therefore, it is essential to maintain RIF plasma

concentration within the recommended range throughout the treatment course [7]. Many studies investigating anti-TB drug exposure have highlighted a problem of substantial interindividual variability in the RIF pharmacokinetics (PK) at a standard daily dose of 8–12 mg/kg [2,8–14]. Indeed, the real-world data demonstrated that the RIF plasma concentration tends to be lower than the currently recommended cutoff (8–24 μg/mL) [15], resulting in underexposure rates from 42% to 93% in different settings [9,13,16–18]. This RIF PK variability has been attributed to the patient's biological sex, body weight, nutrition, comorbidities (e.g., HIV infection and *diabetes mellitus*), and drug formulation used [8–14]. Clinically, underexposure to one or more anti-TB drugs is associated with poor treatment response, relapse, or the development of drug-resistant TB forms requiring a transition to more toxic and less potent second-line drugs and prolongation of treatment [18–22].

Another point for consideration in relation to anti-TB drug exposure is drug-induced liver injury (DILI)—a frequent adverse drug reaction affecting up to 28% of patients with DS-TB receiving the standard four-drug combination regimen recommended by the World Health Organization (WHO) [2,23]. The DILI severity may vary, ranging from an asymptomatic elevation of liver enzymes to acute liver failure and death [23,24]. Among the identified factors predisposing to anti-TB treatment-related hepatotoxicity are older age, female sex, malnutrition, previous history of hepatobiliary disorders, and comorbidities (e.g., viral hepatitis B and C, and HIV infection), as well as anti-TB drug dose at the upper end of therapeutic range and higher exposure [22–26]. Nevertheless, the mechanism of DILI, including RIF-related hepatotoxicity, has a complex and poorly understood nature.

According to the estimates, genetic polymorphisms causing alterations in the function of drug-metabolizing enzymes and transporters may account for 95% of PK variability for certain drugs [27]. As one example, the carriers of the particular set of mutant alleles encoding N-acethyltransferase-2 (NAT2) are proven to have reduced enzymatic activity, which subsequently leads to the excessive accumulation of acetylhydrazine and hydrazine, which are hepatotoxic metabolites, thus contributing to INH-related hepatotoxicity [23,27–29]. Association studies of RIF PK and anti-TB treatment-related hepatotoxicity have exposed numerous targets within the RIF metabolic pathways trying to identify clinically relevant single nucleotide polymorphisms (SNPs); those were genes encoding metabolizing enzymes (arylacetamide deacetylase (*AADAC*)), drug transporters (solute carrier organic anion transporter family member 1B1 and 1B3 (*SLCO1B1* and *SLCO1B3*, respectively)), and efflux pumps (ATP binding cassette subfamily B member 1 (*ABCB1*)), and regulatory proteins (nuclear receptor subfamily 1 group I member 2 (*NR1I2*)) [23,30–47]. Still, the current evidence regarding the effect of patient genetic background on RIF exposure variability is inconclusive. The observed discrepancy between studies could be explained, at least partly, by considerable differences in the study design and patient characteristics. In addition, studies were conducted in geographically distinct populations, predominantly in the WHO TB-endemic regions, i.e., Sub-Saharan Africa and South-East Asia, whereas information on the other populations is scarce.

Therefore, to fill this gap and elucidate the role of pharmacogenetics in RIF exposure and the potential effect on the development of anti-TB treatment-related hepatotoxicity, this study aimed to investigate (a) the relationship between SNPs detected in the set of the genes (*AADAC*, *SLCO1B1*, *SLCO1B3*, *ABCB1*, and *NR1I2*) and RIF PK parameters; and (b) the relationship between the detected SNPs and the development of anti-TB treatment-associated DILI in Latvian patients with pulmonary tuberculosis (PTB).

2. Materials and Methods

2.1. Study Design

This retrospective observational study was conducted from April 2017 to May 2023, involving adult patients (18 years and older) diagnosed with PTB who were admitted to the Riga East University Hospital, Centre of Tuberculosis and Lung Diseases (CTLD). All study participants were of European descent.

In order to minimize the impact of other health conditions on the study results, the following exclusion criteria were applied: pregnancy or lactation, history of cancer, other infectious diseases (e.g., HCV, HBV, HIV, and AIDS), and acute or chronic diseases that could alter liver or renal function. All the clinically relevant patient data were obtained from medical records and patient questionnaires and included demographic and anthropometric information; comorbidities and concomitantly used drugs; smoking and alcohol intake habits; and laboratory findings before TB treatment onset and between the 10th and 12th day of the treatment (alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), total bilirubin, and conjugated bilirubin levels).

The study was approved by the Central Medical Ethics Committee of Latvia (Approval No. 01-29.1/1 and No. 01-29.1.2/1736), the Ethics Committee of the Riga East Clinical University Hospital (Approval No. 24-A/15), and the Scientific Department of the Riga East Clinical University Hospital (Approval No. ZD/08-06/01-21/187). Before enrolment, all patients were familiarized with the study protocol and then asked to provide written informed consent, thus allowing the use of their genetic and phenotypic information for study purposes.

2.2. Clinical Sample Collection and Pretreatment Procedure

All patients were subjected to a WHO-recommended treatment regimen for DS-TB [2] and, at the time of clinical sample collection, were receiving ETB (12–25 mg/kg), PZA (20–30 mg/kg), RIF (8–12 mg/kg), and INH (4–6 mg) once a day for 10–12 days. Blood samples for PK analysis were collected pre-dose (0 h) and 2 and 6 h post-dose into vacutainers with EDTA (BD, Plymouth, UK) and immediately centrifuged at 4000 rpm (3488× *g*) for 15 min at 4 °C to separate the plasma. Plasma aliquots were stored at −70 °C until further analysis. During the PK sampling, additional blood aliquots were drawn, and DNA isolation from whole blood samples was performed using the standard phenol–chloroform method [48]. DNA samples were stored at −20 °C until analysis.

2.3. Determination of RIF Pharmacokinetic Parameters

A validated liquid chromatography–tandem mass spectrometry (LC-MS/MS) method [49] was employed to determine RIF concentration in the plasma samples. According to the literature [15], the time point 2 h post-dose is appropriate to assess RIF peak plasma concentration (C_{max}), while the area under the time–concentration curve from 0 to 6 h ($AUC_{0–6\,h}$) was calculated using the linear trapezoidal rule based on data obtained in the three time points: pre-dose (0 h) and 2 and 6 h post-dose.

2.4. SNP Detection and Analysis

The targeted next-generation sequencing (NGS) approach was chosen as the most convenient for the large-scale genetic analysis; our previously published protocol [50] was applied with modifications in the amplification step targeting the *AADAC*, *SLCO1B1*, *SLCO1B3*, *ABCB1*, and *NR1I2* genes. Specifically, in total, 40 new primer pairs were constructed using an online-based Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/; accessed on 8 May 2023) to amplify exons and untranslated regions (UTR) with flanking sequences (≥100 bp and ≥500 bp, respectively) (Supplementary Table S1). All other steps, such as library preparation and sequencing, remained unchanged. Sequencing data bioinformatic analysis was completed on the Galaxy online-based platform (https://usegalaxy.org; accessed on 8 May 2023) [51]. The workflow included the following steps: (a) trimming adapter sequences and low-quality reads (sliding window size 4; quality threshold Q < 20; Trimmomatic, v 0.38); (b) mapping reads to the reference sequence GRCh38.p13, GCF 000001405.39 (Map with BWA-MEM, v 0.7.17.1); (c) filtering out unmapped reads and reads shorter than 50 bp (BAM filter, v 0.5.9); (d) removing PCR duplicates (MarkDuplicates, v 2.18.2.2); (e) variant calling in the genome region of interest (quality metrics: variant depth ≥ 10; mapping quality ≥ 10; base quality ≥ 20; fraction of reads supporting the alternative allele to consider position—0.2; number of reads support-

ing the alternative allele to consider position ≥ 6; FreeBayes, v 1.3.1); and (f) variant filtering (quality metrics: QUAL > 5; VCFfilter, v 1.0.0). All detected SNPs passed visual inspection using the Integrative Genome Viewer web application (https://igv.org/app/; accessed on 8 May 2023) [52] and subsequently were annotated and identified using the wANNOVAR online-based tool (http://wannovar.wglab.org/; accessed on 8 May 2023) [53] and the NCBI dbSNP database (https://www.ncbi.nlm.nih.gov/snp/; accessed on 8 May 2023). Linkage disequilibrium (LD) was calculated for the detected polymorphisms using the Ensembl Linkage Disequilibrium Calculator [54], which utilizes genotyping data from the 1000 Genomes Project database for SNP pairwise comparisons. Polymorphisms were considered in high LD if $r^2 \geq 0.8$. When selecting tagging SNPs, a priority was given to those with minor allele frequency (MAF) > 5% in the NCBI dbSNP database (SNP frequency in the study population 10–90%), located within coding exons, and/or with previous evidence of functional or regulatory consequences reported in the other studies [23,30–47].

2.5. Assessment of DILI

Liver function was assessed at the baseline and 10–12 days after anti-TB treatment initiation by determining serum levels of ALAT, ASAT, total bilirubin, and conjugated bilirubin. According to the criteria provided by the CTLD clinical laboratory, DILI was defined as mild if ALAT/ASAT levels were 1.5–5 times the upper limit of normal (ULN) (ALAT 60–205 U/L for males and 45–155 U/L for females; ASAT 55–185 U/L for males and 45–155 U/L for females), moderate if ALAT/ASAT levels 5–10 times the ULN (ALAT 205–410 U/L for males and 155–310 U/L for females; ASAT 185–370 U/L for males and 155–310 U/L for females), and severe if ALAT/ASAT levels >10 times ULN (ALAT > 410 U/L for males and > 310 U/L for females; ASAT > 370 U/L for males and > 310 U/L for females). The increased levels of total and conjugated bilirubin (> 19.0 µmol/L and > 3.4 µmol/L, respectively) were only indicative and were not considered in determining DILI severity.

2.6. Statistical Data Analysis

The data distribution of quantitative variables was evaluated by the Shapiro–Wilk test and exploring normal Q-Q plots. Accordingly, quantitative variables were presented as the median and interquartile range (IQR), whereas qualitative variables were expressed as a number of observations (percentage). All detected SNPs were assessed for compliance with the Hardy–Weinberg Equilibrium (HWE) by means of the Chi-Square goodness-of-fit test, using genotype frequencies observed in the study. The size of the study population was the reason for collapsing detected genotypes and employing the dominant genetic model (major allele homozygotes versus heterozygotes plus minor allele homozygotes) in the further analyses. The Pearson's correlation, Mann–Whitney U test, Kruskal–Wallis test, and Fisher's exact test were used to describe the study population stratified by RIF plasma exposure and DILI status.

The multivariate linear regression was conducted to test the relationship between RIF exposure and the genotyping data after controlling for confounders: biological sex, age, body weight, and RIF dose. Similarly, the logistic regression was used to assess whether the detected genotypes were related to DILI and estimate odds ratios considering biological sex, age, smoking and alcohol consumption status, and ALAT and ASAT levels at the baseline as confounders. The statistical data analysis was performed using the Jamovi software (v 2.3) [55], and the result was considered statistically significant if the *p*-value was less than 0.05.

3. Results

3.1. Patient Characteristics and RIF Exposure

A detailed characterization of the study population is given in Table 1. Briefly, 46 otherwise healthy patients with PTB were included in the present study; the majority were males (76.1%) and smokers (76.1%), but the median age was 46 years (IQR: 38–55 years). Albeit, the RIF dose received by the patients conformed to the WHO recommendations (median 9.4 mg/kg; IQR: 8.3–10.9 mg/kg) [2]; the PK analysis showed that the median RIF plasma concentration C_{max} at 2 h was 2.09 µg/mL (IQR: 0.39–5.63 µg/mL); and, based on the reference range defined by Alsultan et al. [15], all but four patients (91.3%) were identified as RIF underexposed (C_{max} at 2 h < 8 µg/mL) (Table 1).

Table 1. Characterization of the study population (n = 46).

		No./Total (%)	Median (IQR)
Demographic and anthropometric characteristics			
Biological sex	Male	35/46 (76.1)	
	Female	11/46 (23.9)	
Age, years			46 (38–55)
	<60 years	41/46 (89.1)	
	≥60 years	5/46 (10.9)	
Body weight, kg			64 (55–73)
BMI [a]	Underweight	11/46 (23.9)	
	Normal weight	27/46 (58.7)	
	Overweight	8/46 (17.4)	
Smoking status	Smoker	35/46 (76.1)	
	Non-smoker	11/46 (23.9)	
Increased alcohol consumption	Yes	15/46 (32.6)	
	No	31/46 (67.4)	
Baseline blood biochemical parameters			
ALAT, U/L			17 (13–25)
ASAT, U/L [b]			18 (15–27)
Total bilirubin, µmol/L [c]			6.3 (5.2–8.7)
Conjugated bilirubin, µmol/L [b]			3.2 (2.4–4.0)
Rifampicin-related data			
RIF dose, mg/kg			9.4 (8.3–10.9)
RIF C_{max}, µg/mL			2.09 (0.39–5.63)
	≥8 µg/mL	4/46 (8.7)	
	<8 µg/mL	42/46 (91.3)	
RIF $AUC_{0–6\ h}$, µg × h/mL			12.75 (6.29–22.34)

[a] According to the World Health Organization recommendations [56], a patient was classified as underweight if the BMI was < 18.5 kg/m^2 and overweight if the BMI was ≥ 25.0 kg/m^2. [b] Data were available for 44 patients. [c] Data were available for 45 patients. Abbreviations: RIF—rifampicin; BMI—body mass index; ALAT—alanine transaminase; ASAT—aspartate aminotransferase.

Patient age, biological sex, BMI, smoking status, and self-reported increased alcohol consumption were not significantly associated with the variability in either RIF C_{max} at 2 h or RIF $AUC_{0–6\ h}$ (Figure 1). However, among the patient-related factors studied, in comparison to the patients with normal RIF plasma exposure, the underexposed patients had greater body weight ($U = 142$, $p = 0.021$) and thus lower body weight-derived RIF dose ($U = 26$, $p = 0.021$) (Supplementary Table S2).

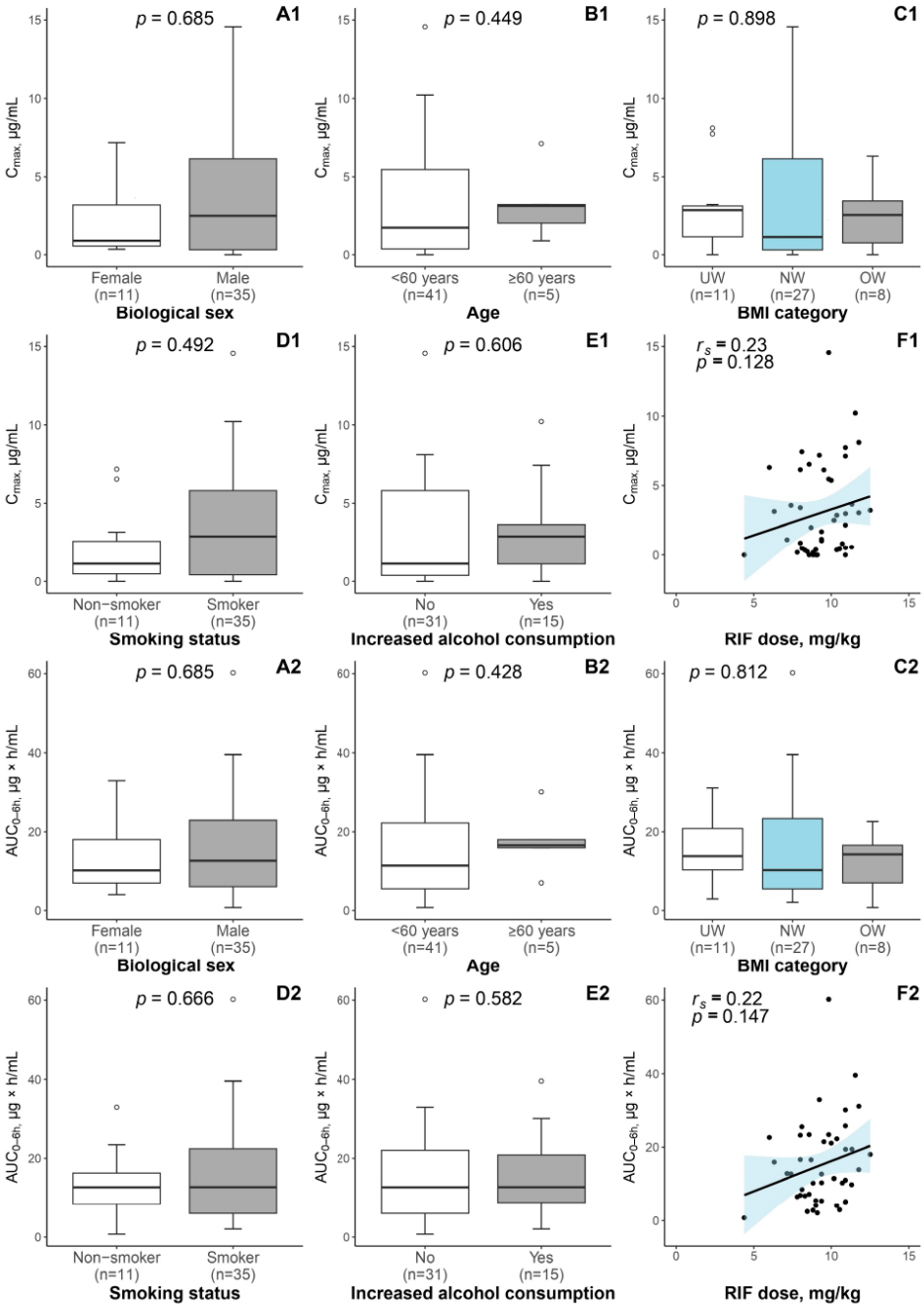

Figure 1. Evaluation of the relationship between RIF PK parameters (1—C_{max}; 2—$AUC_{0-6\,h}$) and patient characteristics (**A1–E1**; **A2–E2**). The analysis was performed using the Mann–Whitney U test

or Kruskal–Wallis test, where applicable. The margins of the boxes illustrate the first (Q1) and third (Q3) quartiles, whereas the median is indicated with a horizontal line within the box. The top and bottom whiskers represent the highest and lowest values within 1.5 times the IQR. Outliers are plotted as empty dots. The relationship between RIF PK parameters and RIF dose (**F1**; **F2**) was investigated using the Spearman's correlation; based on a linear model, a trendline with a blue shaded 95% CI was added. The black filled dots are individual measurements. A p-value < 0.05 was considered statistically significant. Abbreviations: IQR—interquartile range; BMI—body mass index; RIF—rifampicin; C_{max}—peak plasma concentration at 2 h; $AUC_{0\text{-}6\,h}$—area under the time–concentration curve from 0 to 6 h post-dose; UW—underweight (BMI < 18.5 kg/m^2); NW—normal weight (18.5 kg/m^2 < BMI ≤ 25.0 kg/m^2); OW—overweight (BMI ≥ 25.0 kg/m^2); r_s = Spearman's correlation coefficient.

3.2. Results of DILI Assessment

In total, six patients (13.0%) developed mild-to-severe DILI, and in two of them, a slight increase in ALAT/ASAT (up to three times ULN) and conjugated bilirubin levels were observed at the baseline. Clinical symptoms attributable to hepatotoxicity were reported in four patients; three patients had nausea, and two had abdominal pain, while diarrhea, dizziness, and rash were each recorded once. By the end of the study, there were no reports of acute liver failure or fatal cases. When comparing patients in the DILI and non-DILI groups in terms of patient characteristics, RIF dose, and determined PK parameters, significant differences were found solely in the median ALAT and ASAT levels (U = 240, p < 0.001; U = 240, p < 0.001) measured in the time frame from 10th to 12th day of anti-TB treatment, whereas the difference in conjugated bilirubin levels was approaching the significance threshold (U = 179, p = 0.055) (Supplementary Table S3). Regrettably, performing a subgroup analysis after the stratification of patients by the DILI grade was impossible due to a lack of statistical power.

3.3. RIF-Associated Pharmacogene SNP Detection

Overall, sequencing data were generated for five RIF-associated pharmacogenes, i.e., *AADAC*, *SLCO1B1*, *SLCO1B3*, *ABCB1*, and *NR1I2*, for 46 patients. Of all polymorphisms detected, 10 SNPs were selected for further analyses based on the criteria outlined in the Materials and Methods section (*SLCO1B1* gene: rs2306283, rs11045819, and rs4149056; *SLCO1B3* gene: rs60140950; *ABCB1* gene: rs9282564 and rs1045642; and *NR1I2* gene: rs3814055, rs3732357, rs2276707, and rs3732359). Table 2 summarizes information on the genomic coordinates, type of nucleotide substitution, and calculated allele frequencies for each SNP. The SNPs were located within coding exons, except for the *NR1I2* gene: a rare exonic SNP was detected in a single patient (rs61755051, C > T; MAF < 1%), so that, for the *NR1I2* gene, intronic and UTR polymorphisms were included in the analysis. In contrast, none of the detected polymorphisms located upstream and downstream of the *AADAC* transcription initiation site, including one exonic SNP carried by 45 out of 46 patients (rs1803155, G > A; MAF > 5%), was included in the analyses primarily due to excessively high (in more than 90% of patients) or low (in less than 10% of patients) variant frequency found in our study population. For all 10 selected SNPs, the observed genotype frequencies conformed to those predicted by the HWE (Table 2).

Table 2. Characterization of the single-nucleotide polymorphisms detected in the RIF pharmacogenes (*SLCO1B1*, *SLCO1B3*, *NR1I2*, and *ABCB1*) and observed allele frequencies in the study population (n = 46).

Gene	SNP ID [a] (Nucleotide Change)	Genomic Coordinates [b]	Region [c]	Type of Substitution	Allele Frequency	HWE *p*-Value [d]
SLCO1B1	rs2306283 (A > G)	chr12:21176804	Exon 5	nonsyn.	A (0.65) G (0.35)	0.713
	rs4149056 (T > C)	chr12:21178615	Exon 6	nonsyn.	T (0.84) C (0.16)	0.810
	rs11045819 (C > A)	chr12:21176879	Exon 5	nonsyn.	C (0.92) A (0.08)	0.577
SLCO1B3	rs60140950 (G > C)	chr12:20875274	Exon 9	nonsyn.	G (0.92) C (0.08)	0.577
ABCB1	rs1045642 (A > G)	chr7:87509329	Exon 28	syn.	A (0.57) G (0.43)	0.676
	rs9282564 (T > C)	chr7:87600124	Exon 4	nonsyn.	T (0.88) C (0.12)	0.357
NR1I2	rs3814055 (C > T)	chr3:119781188	UTR5	N/A	C (0.62) T (0.38)	0.401
	rs2276707 (C > T)	chr3:119815306	Intron 6	N/A	C (0.78) T (0.22)	0.474
	rs3732357 (G > A)	chr3:119812011	Intron 4	N/A	G (0.35) A (0.65)	0.351
	rs3732359 (G > A)	chr3:119817582	UTR3	N/A	G (0.33) A (0.67)	0.457

[a] SNP ID based on the NCBI dbSNP database (https://www.ncbi.nlm.nih.gov/snp/; accessed on 8 May 2023). [b] Reference genome: GRCh38.p13, GCF 000001405.39. [c] Following transcripts were used to locate region containing SNP: SLCO1B1: NM_006446.5; SLCO1B3: NM_019844.4; NR1I2: NM_003889.4; ABCB1: NM_001348944.4. [d] HWE was tested using the Chi-Square goodness-of-fit test based on the genotype frequency detected in the study. A *p*-value of < 0.05 was considered statistically significant. Abbreviations: RIF—rifampicin; *SLCO1B1*—solute carrier organic anion transporter family member 1B1; *SLCO1B3*—solute carrier organic anion transporter family member 1B3; *ABCB1*—ATP binding cassette subfamily B member 1; *NR1I2*—nuclear receptor subfamily 1 group I member 2; SNP—single nucleotide polymorphism; syn.—synonymous nucleotide substitution; nonsyn.—synonymous nucleotide substitution; N/A—not applicable; UTR—untranslated region; HWE—Hardy–Weinberg Equilibrium.

3.4. Relationship between the Detected SNPs and the RIF Pharmacokinetic Parameters

The potential impact of the selected polymorphisms on the RIF PK parameters was investigated under the dominant genetic model, and the obtained results are shown in Table 3. Overall, none of the SNPs located within genes encoding drug transporters (i.e., *SLCO1B1*, *SLCO1B3*, and *ABCB1*) was related to the C_{max} and $AUC_{0–6\,h}$ when considering biological sex, age, body weight, and RIF dose as confounders ($p > 0.05$). However, a relationship was found between one of the *NR1I2* gene intronic polymorphisms, i.e., rs3732357, and RIF plasma exposure: the results showed that patients with the GA/AA genotype had lower RIF $AUC_{0–6\,h}$ in comparison to the GG genotype ($p = 0.026$). The trend towards significance was also identified between the rs3732357 genotype and RIF concentration in blood plasma 2 h post-dose ($p = 0.077$).

Table 3. Genotype frequencies of the selected SNPs and comparison of the RIF PK parameters between genotype groups.

Gene	SNP ID [a]	Genotype [b]	Genotype Frequency, n (%)	Pharmacokinetic Parameters			
				C_{max}, µg/mL [c]	*p*-Value [d]	$AUC_{0–6\ h}$, µg × h/mL [c]	*p*-Value [d]
SLCO1B1	rs2306283	AA	19 (41.3)	1.13 (0.37–6.13)	0.145	10.39 (5.21–22.25)	0.148
		AG + GG	27 (58.7)	3.06 (0.39–5.47)		15.98 (6.95–22.62)	
	rs4149056	TT	32 (69.6)	1.15 (0.40–6.26)	0.689	10.72 (5.43–23.07)	0.635
		TC + CC	14 (30.4)	3.20 (0.19–4.13)		14.95 (9.36–19.97)	
	rs11045819	CC	39 (84.8)	2.18 (0.37–6.13)	0.850	12.88 (6.55–22.62)	0.999
		CA+ AA	7 (15.2)	0.89 (0.39–5.37)		10.32 (5.49–21.07)	
SLCO1B3	rs60140950	GG	39 (84.8)	2.18 (0.37–6.13)	0.850	12.88 (6.55–22.62)	0.999
		GC + CC	7 (15.2)	0.89 (0.39–5.37)		10.32 (5.49–21.07)	
ABCB1	rs1045642	AA	14 (30.4)	3.30 (0.47–6.40)	0.302	16.29 (9.87–23.86)	0.346
		AG + GG	32 (69.6)	1.44 (0.35–5.02)		11.29 (5.43–22.05)	
	rs9282564	TT	35 (76.1)	1.17 (0.35–5.47)	0.466	11.04 (5.49–22.62)	0.435
		CT + CC	11 (23.9)	3.01 (0.48–6.13)		15.98 (8.56–21.45)	
NR1I2	rs3814055	CC	19 (41.3)	2.18 (0.89–6.53)	0.746	15.98 (5.41–23.39)	0.917
		CT + TT	27 (58.7)	1.13 (0.19–5.47)		11.53 (6.55–22.25)	
	rs2276707	CC	29 (63.0)	3.01 (0.31–6.42)	0.354	15.98 (5.88–23.39)	0.510
		CT + TT	17 (37.0)	1.71 (0.43–3.33)		11.04 (6.15–18.04)	
	rs3732357	GG	7 (15.2)	3.16 (0.48–8.08)	0.077	22.25 (8.56–31.04)	**0.026**
		GA + AA	39 (84.8)	1.71 (0.35–5.37)		11.53 (5.41–21.07)	
	rs3732359	GG	6 (13.0)	3.02 (0.46–9.70)	0.211	19.12 (7.79–38.33)	0.058
		GA + AA	40 (87.0)	1.85 (0.35–5.44)		12.13 (5.70–21.36)	

[a] SNP ID was extracted from the NCBI dbSNP database (https://www.ncbi.nlm.nih.gov/snp/; accessed on 8 May 2023). [b] The dominant genetic model was used to explore the association between the RIF pharmacokinetic parameters and selected SNPs. [c] Variables are presented as median (interquartile range). [d] Group comparison was performed using linear regression after controlling for biological sex, age, body weight, and rifampicin dose. A *p*-value of < 0.05 was considered statistically significant. Abbreviations: RIF—rifampicin; PK—pharmacokinetics; *SLCO1B1*—solute carrier organic anion transporter family member 1B1; *SLCO1B3*—solute carrier organic anion transporter family member 1B3; *ABCB1*—ATP binding cassette subfamily B member 1; *NR1I2*—nuclear receptor subfamily 1 group I member 2; SNP—single nucleotide polymorphism; C_{max}—peak plasma concentration measured 2 h post-dose; $AUC_{0–6\ h}$—area under the time–concentration curve from 0 to 6 h post-dose.

3.5. Relationship between the Selected SNPs and the Development of DILI

The genotype frequencies for the selected *SLCO1B1*, *SLCO1B3*, *ABCB1*, and *NR1I2* polymorphisms were compared between the patients with and without DILI, considering the demographic characteristics, self-reported lifestyle factors, and baseline ALAT and ASAT levels as confounders. In general, the regression analysis did not reveal any relationship between the studied SNPs and the development of DILI when assessed under the dominant genetic model (Table 4).

Table 4. Comparison of genotype frequencies between patients in the DILI and non-DILI groups for selected SNPs.

Gene	SNP ID [a]	Genotype [b]	Genotype Frequency, n (%)		*p*-Value [d]	OR (95% CI)
			DILI Group (n = 6) [c]	Non-DILI Group (n = 40)		
SLCO1B1	rs2306283	AA	2 (33.3)	17 (42.5)	0.963	1.07 (0.05–22.80
		AG + GG	4 (66.6)	23 (57.5)		
	rs4149056	TT	3 (50.0)	29 (72.5)	0.571	2.49 (0.11–58.54)
		TC + CC	3 (50.0)	11 (27.5)		
	rs11045819	CC	5 (83.3)	34 (85.0)	0.230	8.27 (0.26–260.17)
		CA+ AA	1 (16.6)	6 (15.0)		

Table 4. *Cont.*

Gene	SNP ID [a]	Genotype [b]	Genotype Frequency, n (%)		*p*-Value [d]	OR (95% CI)
			DILI Group (n = 6) [c]	Non-DILI Group (n = 40)		
SLCO1B3	rs60140950	GG	5 (83.3)	34 (85.0)	0.230	8.27 (0.26–260.17)
		GC + CC	1 (16.6)	6 (15.0)		
ABCB1	rs1045642	AA	2 (33.3)	12 (30.0)	0.706	0.55 (0.02–12.68)
		AG + GG	4 (66.6)	28 (70.0)		
	rs9282564	TT	5 (83.3)	30 (75.0)	0.299	0.11 (0.002–7.00)
		CT + CC	1 (16.6)	10 (25.0)		
NR1I2	rs3814055	CC	3 (50.0)	16 (40.0)	0.447	0.28 (0.01–7.46)
		CT + TT	3 (50.0)	24 (60.0)		
	rs2276707	CC	4 (66.6)	25 (62.5)	0.681	0.55 (0.03–9.59)
		CT + TT	2 (33.3)	15 (37.5)		
	rs3732357	GG	0 (0.0)	7 (17.5)	0.997	N/A
		GA + AA	6 (100.0)	33 (82.5)		
	rs3732359	GG	0 (0.0)	6 (15.0)	0.998	N/A
		GA + AA	6 (100.0)	34 (85.0)		

[a] SNP ID was extracted from the NCBI dbSNP database (https://www.ncbi.nlm.nih.gov/snp/; accessed on 8 May 2023). [b] The dominant genetic model was used to explore the association between anti-TB drug-induced liver injury and selected SNPs. [c] According to the CTLD clinical laboratory, DILI was defined as follows: ALAT > 60 U/L for males and > 45 U/L for females and ASAT > 55U/L for males and > 45 U/L for females and/or total bilirubin > 19.0 µmol/L and/or conjugated bilirubin > 3.4 µmol/L. [d] Group comparison was performed using logistic regression after controlling for biological sex, age, smoking and alcohol consumption status, and ALAT and ASAT level at the baseline. A *p*-value of < 0.05 was considered statistically significant. Abbreviations: *SLCO1B1*—solute carrier organic anion transporter family member 1B1; *SLCO1B3*—solute carrier organic anion transporter family member 1B3; *ABCB1*—ATP binding cassette subfamily B member 1; *NR1I2*—nuclear receptor subfamily 1 group I member 2; DILI—drug-induced liver injury; SNP—single nucleotide polymorphism; OR—Odds Ratio; 95% CI—95% Confidence Interval; N/A—not applicable; CTLD – Riga East University Hospital, Centre of Tuberculosis and Lung Diseases; ALAT—alanine aminotransferase; ASAT—aspartate aminotransferase.

4. Discussion

At first, we evaluated RIF exposure in our study population of Latvian patients with PTB. The median RIF plasma concentration 2 h post-dose (C_{max}) was 2 µg/mL, which is nearly four times below the lower end of the target range (8–24 µg/mL), using the currently recommended RIF dose [2,15]. An underexposure rate of 91% confirms an alarming tendency of subtherapeutic RIF plasma concentration reported elsewhere and highlights the well-documented but obscure PK variability of anti-TB drugs [9,13,16–18]. The underexposed patients had higher a body weight and, consequently, lower body weight-derived RIF dose. However, we did not detect the impact of other frequently discussed determinants of anti-TB drug plasma exposure, as the study population comprised otherwise healthy adult patients, 11% of whom were aged 60 years or older, and the majority were males (76%) [8–14].

Three out of four drugs in the regimen for treatment of DS-TB, namely RIF, PZA, and INH, induce hepatotoxicity via multiple different mechanisms as early as in the first weeks of the treatment, with half of the cases observed within the first 14 days [57,58]. Additionally, symptoms referable to RIF-related hepatotoxicity may appear sooner than in the case of the other anti-TB drugs [57]. In our study population, 13% of the patients presented with DILI after 10–12 days of anti-TB therapy, which is theoretically consistent with the incidence reported worldwide [23]. Nevertheless, this result should be interpreted carefully, as the definition of DILI may vary between studies. The homogeneity of the study population mentioned earlier was also evident when comparing patient characteristics in the DILI and non-DILI groups, thus raising the question of the underlying molecular mechanisms promoting hepatotoxicity.

In our study, we evaluated 10 SNPs located in the *SLCO1B1*, *SLCO1B3*, *ABCB1*, and *NR1I2* genes as genetic determinants of RIF PK, as well as investigated the possible role of

these polymorphisms in the development of anti-TB treatment-related hepatotoxicity since these genes and their encoded products are recognized as being of pharmacogenetic importance. Although the information on *AADAC* genetic polymorphisms is limited, initially, it was considered for analysis because the encoded microsomal enzyme is responsible for the formation of non-toxic 25-desacetyl derivatives of rifamycins, which can be later excreted by bile and eliminated via feces [6,59]. The two groups of investigators have reported on the lower clearance and higher plasma concentration of RIF analogue rifapentine in patients with rs1803155 AA genotype, which may be related to patient race [42,45]. The results of the in vitro study conducted by Shimizu and colleagues [60] suggested that *AADAC**3/*3 carriers may have decreased enzymatic activity compared to other diplotypes (*AADAC***1/***1* (wild-type—wt), *AADAC**1/*2, and *AADAC**2/*2). Unfortunately, all detected polymorphisms, including exonic SNP rs1803155, were excluded from the further analysis in our study due to insufficient statistical power resulting from the inadequate variant frequency in the study population. Moreover, the *AADAC**3 haplotype consists of two SNPs, the common rs1803155 (MAF > 5%) and the rare rs61733692 (MAF < 1%); so, in a low-endemic setting, there would be a relatively small chance of detecting a sufficient number of patients with *AADAC**3/*3 diplotype required to test the hypothesis.

The *SLCO1B1* and *SLCO1B3* transporters are expressed on the sinusoidal membrane of hepatocytes and contribute to the hepatic uptake of RIF from the bloodstream [61]. During in vitro experiments, some *SLCO1B1* genetic polymorphisms have shown promising results, i.e., diminished transporter activity altering RIF exposure [61]. In clinical practice, Allegra et al. [37] reported the *SLCO1B1* rs4149056 TT genotype (wt) to be a predictor of the increased RIF peak plasma concentration. The opposite effect, with lowered plasma concentration, was found in the Ghanaian children bearing two mutant alleles of rs2306283 when performing a subgroup analysis [38]. In the two other studies, rs11045819 was associated with RIF plasma exposure; the other covariates identified were the patient's geographical origin and biological sex [30,34]. Nevertheless, we did not observe the impact of these three exonic *SLCO1B1* SNPs, as did other authors [31,39,40,43]. In the case of *SLCO1B3*, the exonic polymorphism rs60140950, which was previously described to lower *SLCO1B3* protein expression without any change in transporter function and increase telmisartan plasma concentration, also did not affect the RIF plasma concentration in our patients with PTB [61–63].

In the context of DILI, one of the proposed mechanisms of the cholestasis is the competitive inhibition of *SLCO1B1* and *SLCO1B3* transporters by RIF, thereby impairing hepatic uptake of bilirubin—their endogenous substrate [61,64,65]. To our knowledge, there are many studies which have assessed *SLCO1B1* gene polymorphisms as potential contributors in the development of anti-TB treatment-related hepatotoxicity, with conflicting results [32,33,35]. In our work, we failed to replicate the relationship between SNPs comprising the *SLCO1B1*15* haplotype (rs2306283 + rs4149056) and RIF-related hepatotoxicity and likewise observe the effect of rs11045819. Interestingly, Zhang et al. [66] reported that serum bilirubin level is influenced by the *SLCO1B1* diplotype, whilst an increase in the serum bilirubin level after low-dose RIF use did not depend on genetic background, suggesting that RIF administration may aggravate pre-existing hyperbilirubinemia and liver impairment but not to be their primary cause. Instead, the *SLCO1B3* polymorphisms have mostly been studied within the frame of taxane toxicity, while the level of evidence supporting particular drug–variant combinations remains low [67,68]. In the present study, the polymorphism rs60140950 was not related to anti-TB treatment-associated DILI.

The *ABCB1* gene encodes the ATP-dependent efflux transporter P-glycoprotein, which mediates the unidirectional transport of xenobiotics, including RIF, from intra- to extracellular space in various tissues and accordingly limits cellular uptake and the distribution of foreign compounds [69]. Regarding the effect of our two investigated *ABCB1* polymorphisms on RIF PK, Huerta-García et al. [39] reported that the rs1045642 AA genotype (wt), along with other patient-dependent factors, accounts for lowered RIF plasma exposure. The functional assays did not provide clear evidence of the effect of this polymorphism on

mRNA and protein expression, while the reported association was not re-established in our study or in the other studies [30,31,37,40,43,69]. Similarly, P-glycoprotein harboring the polymorphism rs2032582 from the same haplotype block has shown altered transport activity in vitro, but in the clinical setting, carriers of this polymorphism presented with insignificantly lower RIF plasma clearance [31,69]. The other *ABCB1* polymorphism we investigated, rs9282564, was previously documented to increase tacrolimus plasma concentration [70], but no effect was observed in the case of RIF. When assessing the potential impact of *ABCB1* polymorphisms on the development of DILI in TB-HIV co-infected patients receiving efavirenz and RIF-containing regimens, Yimer and colleagues showed that the rs1045642 GG genotype increased susceptibility to hepatotoxicity independently of concomitant RIF use [32]. On the contrary, in HIV patients without TB co-infection, the rs1045642 mutant allele demonstrated a protective effect, thus proving the complexity of DILI mechanisms, especially in patients with comorbidities [71]. Nevertheless, conflicting results on rs1045642 have been shown in patients with TB and suspected INH-related hepatotoxicity but without other chronic conditions reported [72,73]. Also, our findings do not support the relationship between *ABCB1* polymorphisms, rs1045642 and rs9282564, and anti-TB drug-related hepatotoxicity.

The product of the *NR1I2* gene (also known as *PXR*) belongs to the nuclear receptor family and holds transcriptional regulator functions extending to numerous phases I and II drug-metabolizing enzymes and transporters [74]. In addition, RIF is one of the most potent ligands upregulating the transcription of *NR1I2* downstream genes, and this is thought to partially explain RIF autoinduction phenomena, which is estimated to cause up to 40% of the reduction in RIF plasma concentration in the first weeks of anti-TB treatment [74,75]. In the present study, we assessed the effect of the *NR1I2* intronic and UTR SNPs on RIF PK and found that patients with rs3732357 GA and AA genotypes had significantly lower RIF $AUC_{0–6\ h}$ compared to GG genotype (wt) carriers. Using midazolam as a model substrate, He et al. [76] detected changes in CYP3A activity in the presence of this polymorphism, but the impact on RIF plasma exposure had not been described previously. The remaining polymorphisms, which were associated with higher CYP3A activity in African Americans (rs3732359 AA genotype), with higher *NR1I2* promoter activity in vitro (rs3814055 T allele), or substantially increased CYP3A expression and, in turn, altered tacrolimus plasma concentration (rs2276707 T allele), did not exhibit a significant relationship in our study [77–80].

Calcagno et al. [81] reported that intronic rs2472677 affects INH but not RIF plasma exposure in TB-HIV co-infected patients, while a recently published paper demonstrated its association with a 25% reduction in RIF plasma concentration in patients with TB using a moxifloxacin-containing regimen [46]. This SNP is located in the binding site of hepatic nuclear factor 3β (HNF3β) and is characterized by increased *NR1I2* mRNA levels and CYP3A4 activity; unfortunately, due to limitations of the employed NGS protocol, the effect of rs2472677 was not evaluated in our study [82]. The current evidence indicates the possible involvement of *NR1I2* in the pathogenesis of RIF and INH co-treatment-related hepatotoxicity via RIF-induced upregulation of CYP2E1 and aminolevulinic synthase-1 (ALAS1) expression, leading to excessive production of hepatotoxic INH intermediates, which exacerbates INH-induced oxidative stress, and accumulation of the heme precursor protoporphyrin IX, responsible for cholestatic liver injury [28,83]. Concerning the impact of *NR1I2* genetic polymorphisms on the development of DILI, the four investigated SNPs (rs3814055, rs3732357, rs2276707, and rs3732359) did not yield any relationship in the present study. Meanwhile, the studies conducted with patients of Asian ancestry reported controversial conclusions. Zazuli et al. [36] and Wang et al. [47] showed that patients carrying the rs3814055 TT genotype or T allele in conjunction with NAT2 non-slow acetylator status, respectively, are more susceptible to anti-TB treatment-associated DILI, but another group of investigators described a protective effect [41]. The protective effect recently reported for rs2276707 under the recessive genetic model has not been replicated yet [44]. Again, two *NR1I2* gene SNPs that were beyond the scope of our study, namely rs7643645

and rs2461823, are believed to affect HNF binding sites and modulate the risk of anti-TB treatment-related hepatotoxicity and severity of non-alcoholic fatty liver disease [41,44,84].

Considering that the anti-TB treatment relies on the administration of drug combinations, implying a wide range of factors affecting drug exposure and occurrence of adverse events, the advantage of our study is the engagement of patients without other severe health conditions, thus limiting any resulting bias, e.g., from comorbidities. Moreover, controlling for relevant cofounders during the statistical data analysis allowed us to reduce variability within the study population. As already mentioned, the pool of studied polymorphisms was limited by the scope of our NGS protocol and sample size. Increasing the study cohort is unlikely to improve the statistical power for those polymorphisms with low frequency in the population of European ancestry in conjunction with low TB incidence within the WHO European Region. Nevertheless, we were able to assess the impact of polymorphisms previously unreported in the present context. Lastly, there is a possibility that an evaluation of blood biochemical parameters in the first weeks of anti-TB treatment bears the risk of missing DILI cases developed lately; however, according to the literature data, signs and symptoms of RIF-related hepatotoxicity usually manifest within this short period.

To summarize all the abovementioned information, we found that the intronic polymorphism rs3732357 in the *NR1I2* gene is related to RIF plasma exposure, whereas none of the studied SNPs in the RIF-associated pharmacogenes, i.e., *SLCO1B1*, *SLCO1B3*, *ABCB1*, and *NR1I2*, was related to the anti-TB drug-induced liver injury in Latvian patients with PTB.

5. Conclusions

Our findings, together with previous reports, suggest that the biological effects of the analyzed SNPs are rather insignificant or minor and, thus, do not have a pivotal role in RIF disposition and the mechanism of anti-TB treatment-related hepatotoxicity, which appears to be more likely affected by other patient-dependent factors discussed elsewhere.

Further studies are warranted to include (a) in vitro assays for SNPs lacking functional data to clarify their impact on RIF PK and DILI mechanisms; (b) an analysis of other biomarkers to discriminate liver injury patterns and to speculate on liver injury primarily caused by RIF, as it may cause intrahepatic cholestasis in some patients; and (c) confirmation of the observed relationship between *NR1I2* intronic polymorphism rs3732357 and RIF PK parameters in the other populations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pharmaceutics16030388/s1, Table S1: Sequences of the primers specifically designed for this study and used in the amplification step in the NGS workflow; Table S2: Characterization of the study population stratified by RIF plasma concentration 2 h post-dose, and comparison of patient characteristics between the patients with normal exposure (≥8 μg/mL) and underexposure (<8 μg/mL) 2 h post-dose; Table S3: Characterization of the study population stratified by DILI status and comparison of patient characteristics between DILI and non-DILI groups.

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Institutional Review Board Statement: The study protocol was approved by the Central Medical Ethics Committee of Latvia (Approval No. 01-29.1/1 and No. 01-29.1.2/1736), the Ethics Committee of the Riga East Clinical University Hospital (Approval No. 24-A/15), and the Scientific Department of the Riga East Clinical University Hospital (Approval No. ZD/08-06/01-21/187), and the study was conducted following the principles of the Helsinki Declaration.

Informed Consent Statement: The written informed consent was obtained from all patients before enrolment in the study.

Data Availability Statement: The datasets supporting the findings of this study are not publicly available due to ethical restrictions but are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare no conflict of interest.

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Identification of Factors Determining Patterns of Serum C-Reactive Protein Level Reduction in Response to Treatment Initiation in Patients with Drug-Susceptible Pulmonary Tuberculosis

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Abstract: Background: Serum C-reactive protein (CRP) levels vary depending on radiological and bacteriological findings at the time of tuberculosis (TB) diagnosis. However, the utility of this biomarker in monitoring response to anti-TB treatment and identifying patients at risk of treatment failure is not well established. **Objectives:** This study evaluated the impact of patients' baseline characteristics and anti-TB drug plasma exposure on the early reduction in serum CRP levels and its relationship with treatment response. **Methods:** We enrolled 42 patients with drug-susceptible pulmonary TB, who received a standard six-month regimen. The plasma concentrations of four anti-TB drugs were analysed using LC-MS/MS. Clinically relevant data, including serum CRP levels before and 10–12 days after treatment initiation ($CRP_{10–12d}$), were obtained from electronic medical records and patient questionnaires. **Results:** In 10–12 days, the median serum CRP level decreased from 21.9 to 6.4 mg/L. Lower body mass index, positive sputum-smear microscopy results, and lung cavitations at diagnosis were related to higher biomarker levels at both time points; smoking had a more pronounced effect on serum $CRP_{10–12d}$ levels. Variability in anti-TB drug plasma exposure did not significantly affect the reduction in serum CRP levels. The serum $CRP_{10–12d}$ levels, or fold change from the baseline, did not predict the time to sputum culture conversion. **Conclusions:** Disease severity and patient characteristics may influence the pattern of early CRP reduction, while anti-TB drug plasma exposure had no significant effect at this stage. These early changes in serum CRP levels were not a predictor of response to anti-TB therapy.

Keywords: tuberculosis; pharmacokinetics; anti-tuberculosis drugs; C-reactive protein; treatment response

1. Introduction

With a global incidence of 10–12 million cases per year, tuberculosis (TB) remains an alarming public health problem, even in the era of advanced medical technologies [1]. Despite the potential benefits of the four-month rifapentine-based regimen recently introduced

in the World Health Organization (WHO) guidelines on the treatment of drug-susceptible tuberculosis (DS-TB), issues with rifapentine availability prevent the incorporation of this regimen into national TB programmes worldwide [2–4]. Therefore, the standard of care for DS-TB in most countries within the WHO European Region, including Latvia, remains the six-month regimen consisting of rifampicin (RIF), pyrazinamide (PZA), ethambutol (ETB), and isoniazid (INH).

In patients undergoing the lengthy anti-TB treatment course, monitoring of treatment response to identify those at risk of adverse treatment outcomes is the cornerstone of effective disease management. Once treatment is started, the alleviation of clinical symptoms, radiological improvement, and a reduction in the sputum bacillary load are generally expected after two to four weeks [5–10]. According to the WHO, the matter of concern is the patients who lack clinical response and fail to convert their sputum-smear and sputum culture by the end of the intensive phase of treatment, i.e., within the first two months of anti-TB drug administration [11]. Calderwood et al. [12] revealed that around 20% of patients fall into the category of delayed treatment response—an unfavourable short-term outcome indicating a limited bactericidal effect, which may translate into treatment failure or relapse.

C-reactive protein (CRP), an acute-phase protein, is widely employed to assess and monitor inflammation in acute and chronic conditions [13]. Mechanistically, inflammatory stimuli resulting from exposure to infectious agents or tissue damage activate the macrophages, which then release proinflammatory cytokines, namely, IL-1, IL-6, and TNF-α, driving the expression of CRP, primarily in the hepatocytes [13]. In patients with TB, serum CRP levels were reported to correlate with disease severity and bacillary load at diagnosis [9,14–19]. Several studies exploring changes in serum CRP levels throughout the anti-TB treatment course have confirmed a substantial decline in the levels of this biomarker during the first weeks of treatment, reflecting the attenuation of the inflammation related to *Mycobacterium tuberculosis* (Mtb) clearance from the lungs and extrapulmonary sites [9,14,17–20]. Furthermore, Wilson et al. [20] showed that the failure to achieve a 55% reduction in serum CRP levels within the first two weeks of anti-treatment was a predictor of hospitalisation and death in patients with TB-HIV coinfection. Currently, point-of-care CRP testing is approved for screening of active TB in people living with HIV, but there is little role of this biomarker in monitoring treatment response due to inconclusive evidence regarding its association with bacteriological endpoints [14,17–19,21–23]. Considering the non-specific nature of CRP expression and the fact that its serum levels can considerably vary over the six-month treatment period, this biomarker could be more suitable for predicting short-term rather than long-term effects [13].

A variety of lifestyle-, health-, and disease-related factors bear the potential to slow down the progress of anti-TB therapy and, in some cases, endanger treatment success [21,23–31]. At the same time, pharmacokinetic/pharmacodynamic (PK/PD) studies have drawn attention to the variability in plasma concentrations of anti-TB drugs, along with considerable underexposure rates observed with recommended daily doses [23,32–39]. Maintaining PK/PD targets is highly important, especially given the complexity of eradicating Mtb [40]. Each of the four anti-TB drugs has a distinct mechanism of action and penetration rates into heterogenous tuberculous lesions, enabling them to target Mtb at different stages of replication [8,10,40–43]. In line with that, patients experiencing delayed treatment response and adverse treatment outcomes often exhibit lower plasma exposure (or related PK/PD indices) of one or more anti-TB drugs [23,33–37,39]. On the contrary, the role of anti-TB drug exposure in the reduction in TB-associated inflammation has not been extensively discussed; however, Prahl and colleagues [23] have described a negative correlation between serum CRP levels and INH plasma concentration during the intensive phase of treatment.

Thus, this study sought to (a) further investigate the influence of patients' baseline characteristics and plasma exposure of four anti-TB drugs on the reduction in the serum CRP levels in response to treatment onset and (b) determine whether these patterns of

serum CRP reduction are linked to the treatment response in Latvian patients with drug-susceptible pulmonary tuberculosis (DS-PTB).

2. Results

2.1. Baseline Characteristics of the Study Population

In total, 42 otherwise healthy patients with radiologically and bacteriologically confirmed DS-PTB were enrolled in the study; 81.0% (34/42) of the patients were males, and the mean age was 47 years (±14 years). A detailed patient demographic and anthropometric structure, along with lifestyle and clinical characteristics, are provided in Table 1. At admission, all but five patients presented with clinical symptoms suggestive of TB, such as persistent cough, night sweats, and unexplained body weight loss. The baseline radiological assessment revealed that 73.8% (31/42) of the patients had tuberculous lesions in both lungs, and 61.9% (26/42) had cavitary disease. Also, two patients had developed pleuritis secondary to PTB. Nearly two-thirds of all investigated patients (27/42, 64.3%) had positive sputum-smear microscopy results at the baseline (Table 1). When characterising the degree of inflammation before starting anti-TB treatment, the median baseline serum CRP level (CRP_b) in the study population was 21.9 mg/L (interquartile range [IQR]: 3.3–51.5 mg/L); significant differences were observed between patients with and without cavitary lesions (median 36.3 mg/L [IQR: 19.8–66.6 mg/L] vs. 3.0 mg/L [IQR: 1.1–7.6 mg/L], $U = 379$, $p < 0.001$) and between patients with positive and negative sputum-smear microscopy results (median 29.2 mg/L [IQR: 8.2–60.6 mg/L] vs. 3.4 mg/L [IQR: 1.1–26.6 mg/L], $U = 299$, $p = 0.011$) (Table 2). In contrast, the localisation of lung lesions was not related to the levels of this biomarker ($U = 203$, $p = 0.365$). The levels of serum CRP_b inversely correlated with body mass index (BMI) (Spearman's rho = −0.39, $p = 0.011$) and varied across BMI categories ($H_{(2)} = 6.46$, $p = 0.040$). After correction for multiple comparisons, a difference of marginal significance was found between underweight and overweight patients (adjusted $p = 0.050$). Concerning other baseline characteristics, serum CRP_b levels did not vary depending on the patient's biological sex, age, or smoking status.

Table 1. Characteristics of the study population ($N = 42$).

Demographic, anthropometric, and lifestyle characteristics		
Biological sex	Male, *n* (%)	34 (81.0)
	Female, *n* (%)	8 (19.0)
Age	Overall, years (mean [±SD])	47 (±14)
	<60 years, *n* (%)	34 (81.0)
	≥60 years, *n* (%)	8 (18.0)
BMI	Overall, kg/m^2 (median [IQR]) [a]	20.9 (18.4–22.7)
	Underweight, *n* (%)	11 (26.2)
	Normal weight, *n* (%)	25 (59.5)
	Overweight, *n* (%)	6 (14.3)
Smoking status	Smoker, *n* (%)	31 (73.8)
	Non-smoker, *n* (%)	11 (26.2)
Baseline radiological findings		
Localisation of lung lesions	Unilateral, *n* (%)	11 (26.2)
	Bilateral, *n* (%)	31 (73.8)
Cavitations	Present, *n* (%)	26 (61.9)
	Absent, *n* (%)	16 (38.1)

Table 1. *Cont.*

Baseline sputum-smear microscopy results		
	Positive, *n* (%)	27 (64.3)
	Negative, *n* (%)	15 (35.7)
Bacteriological response to treatment		
tSCC	Overall, days (median [IQR])	56 (27–79)

Qualitative variables are expressed as counts (percentage). The quantitative, normally distributed variables are presented as mean and standard deviation (±SD), while the non-normally distributed variables are expressed as median and interquartile range (IQR). [a] In conformity with WHO recommendations [44], a patient was classified as underweight if the BMI was <18.5 kg/m^2 and overweight if the BMI was ≥25.0 kg/m^2. Abbreviations: BMI—body mass index; tSCC—time to sputum culture conversion.

Table 2. Distribution of serum CRP levels in the study population and comparison within subcategories.

	CRP_b, mg/L [a]	***p* Value**	**$CRP_{10–12d}$, mg/L [a,b]**	***p* Value**
Overall	21.9 (3.3–51.5)	N/A	6.4 (1.4–34.2)	N/A
Demographic, anthropometric, and lifestyle characteristics				
Biological sex				
Male	21.9 (5.4–56.2)	0.421	6.8 (2.6–34.2)	0.235
Female	15.0 (1.4–47.7)		1.5 (0.9–42.4)	
Age, years	–	0.882	–	0.161
<60 years	21.9 (3.1–56.2)	0.718	4.8 (1.4–24.8)	0.248
≥60 years	21.8 (9.3–47.7)		18.0 (3.2–64.7)	
BMI, kg/m^2 [c]	–	0.011	–	0.035
Underweight	55.5 (24.4–79.0)	0.040	34.2 (10.9–65.3)	0.116
Normal weight	16.0 (3.3–41.4)		4.0 (1.4–19.5)	
Overweight	5.2 (1.3–24.4)		4.1 (1.1–15.7)	
Smoking status				
Smoker	22.8 (5.7–58.3)	0.163	12.1 (3.2–45.0)	0.046
Non-smoker	8.2 (1.1–32.2)		1.8 (0.8–14.1)	
Radiological findings				
Localisation of lung lesions				
Unilateral	19.1 (1.1–40.3)	0.365	2.2 (1.2–14.2)	0.249
Bilateral	24.4 (4.4–55.5)		6.6 (2.7–48.5)	
Cavitations				
Present	36.3 (19.8–66.6)	<0.001	14.2 (6.2–60.9)	<0.001
Absent	3.0 (1.1–7.6)		1.4 (0.7–3.2)	
Baseline sputum-smear microscopy results				
Positive	29.2 (8.2–60.6)	0.011	13.5 (4.0–52.0)	<0.001
Negative	3.4 (1.1–26.6)		1.3 (0.6–6.2)	

All variables are expressed as median and interquartile range (IQR). The groups were compared using the Mann–Whitney U test or Kruskall–Wallis H test. The correlation analysis was performed using Spearman's rank correlation. A *p* value of <0.05 was considered statistically significant. [a] The serum CRP reference range established by the clinical laboratory of the Centre of Tuberculosis and Lung Diseases at Riga East University Hospital is <8 mg/L. [b] Data were available for 41 patients. [c] In conformity with WHO recommendations [44], a patient was classified as underweight if the BMI was <18.5 kg/m^2 and overweight if the BMI was ≥25.0 kg/m^2. Abbreviations: CRP—C-reactive protein; CRP_b—serum C-reactive protein level at the baseline; $CRP_{10–12d}$—serum C-reactive protein level 10–12 days after anti-tuberculosis treatment onset; BMI—body mass index; N/A—not applicable.

2.2. Assessment of Anti-TB Drug Plasma Exposure

The data on the resulting plasma exposure of the four anti-TB drugs after single-dose administration are summarised in Table 3. In conformity with the criteria proposed by Alsultan et al. [45], the peak plasma concentration (C_{max}) evaluated 2 h post-dose was below the therapeutic range for RIF and INH (median 1.9 µg/mL [IQR: 0.3–5.5 µg/mL] vs. 8.0 µg/mL and mean 2.6 µg/mL [±1.38 µg/mL] vs. 3.0 µg/mL, respectively), whereas for the remaining drugs, it was within the target range. As such, the highest underexposure rate of 92.9% (underexposed 39 out of 42 patients) was detected for RIF, followed by INH, ETB, and PZA, with insufficient plasma concentration accordingly in 54.8% (23/42), 26.2% (11/42), and 9.5% (4/42) of patients. The prevalence of simultaneous four-drug underexposure was 7.1%.

Table 3. Assessment of anti-TB drug plasma exposure and relationship with serum CRP levels determined 10–12 days after treatment onset.

	Anti-TB Drug Doses and Pharmacokinetic Parameters [a]		**Linear Regression—Relationship with Serum $CRP_{10–12d}$ Levels** [c]		
		Mean (±SD)	β	95% CI	*p* value
RIF	Dose, mg/kg	9.5 (±1.5)	–	–	–
	C_{max}, µg/mL	1.89 (0.32–5.50) [b]	−0.012	−0.069, 0.064	0.934
	$AUC_{0–6h}$, µg × h/mL	12.82 (6.75–22.54) [b]	0.092	−0.012, 0.024	0.520
PZA	Dose, mg/kg	31.2 (±4.7)	–	–	–
	C_{max}, µg/mL	36.74 (±11.85)	−0.074	−0.023, 0.013	0.597
	$AUC_{0–6h}$, µg × h/mL	187.18 (±49.65)	−0.094	−0.006, 0.003	0.505
ETB	Dose, mg/kg	21.0 (18.1–23.2) [b]	–	–	–
	C_{max}, µg/mL	2.22 (1.48–3.91) [b]	−0.155	−0.216, 0.065	0.282
	$AUC_{0–6h}$, µg × h/mL	13.00 (±4.97)	−0.148	−0.065, 0.020	0.295
INH	Dose, mg/kg	4.8 (±0.8)	–	–	–
	C_{max}, µg/mL	2.64 (±1.38)	0.128	−0.086, 0.227	0.365
	$AUC_{0–6h}$, µg × h/mL	10.02 (6.38–14.58) [b]	0.140	−0.019, 0.057	0.321

The relationship between PK parameters and log-transformed $CRP_{10–12d}$ levels was tested using linear regression adjusted for the patient's biological sex, age, and BMI. A *p* value of <0.05 was considered statistically significant. [a] Data were available for 42 patients. [b] The variable is presented as the median and interquartile range (IQR). [c] Data were available for 41 patients. Abbreviations: TB—tuberculosis; $CRP_{10–12d}$—serum C-reactive protein level 10–12 days after anti-tuberculosis treatment onset; SD—standard deviation; β—standardised beta coefficient; CI—confidence interval; RIF—rifampicin; PZA—pyrazinamide; ETB—ethambutol; INH—isoniazid; C_{max}—peak plasma concentration measured 2 h post-dose; $AUC_{0–6h}$—area under the time–concentration curve from 0 to 6 h post-dose.

2.3. The Reduction in Serum CRP Levels in Response to Anti-TB Treatment and the Impact of Anti-TB Drug Plasma Exposure

Overall, in the study population, the median serum CRP level after 10–12 days of anti-TB treatment ($CRP_{10–12d}$) decreased to 6.4 mg/L (IQR: 1.4–34.2 mg/L, $W = 142$, $p = 0.001$), thereby meeting the reference range of <8 mg/L established by the clinical laboratory of Centre of Tuberculosis and Lung Diseases at Riga East University Hospital. Even in the subcategories with median serum $CRP_{10–12d}$ levels above this threshold, except in patients aged ≥60 years, the decline was significant (Figure 1 and Table 2). As seen with CRP_b, serum $CRP_{10–12d}$ levels varied depending on the baseline sputum-smear microscopy results ($U = 324$, $p < 0.001$) and the presence of cavitary lesions at the radiological examinations ($U = 356$, $p < 0.001$), and correlated with BMI (Spearman's rho = −0.33, $p = 0.035$) (Table 2). It is noteworthy that the difference in the median level of this biomarker between smokers and non-smokers was more pronounced than with CRP_b and reached significance (median 12.1 mg/L [IQR: 3.2–45.0 mg/L] vs. 1.8 mg/L [IQR: 0.8–14.1 mg/L], $U = 233$, $p = 0.046$).

Despite the aforementioned variability in anti-TB drug exposure, the linear regression analysis did not demonstrate a significant relationship between serum $CRP_{10–12d}$ levels and the PK parameters of the four anti-TB drugs (Table 3).

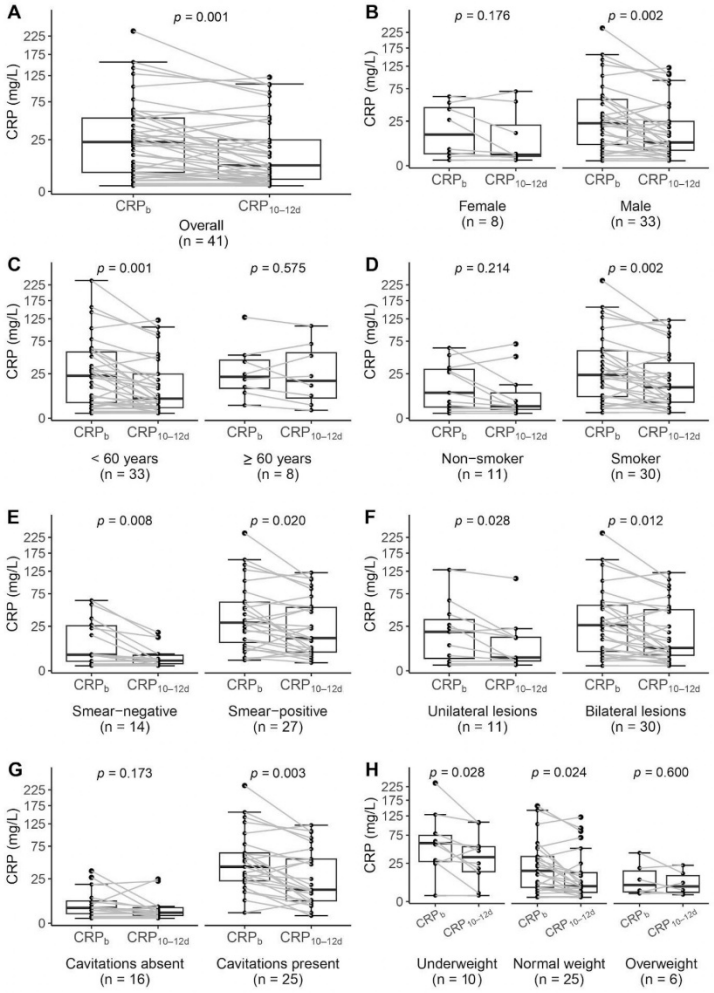

Figure 1. Changes in serum CRP levels measured before anti-tuberculosis treatment initiation (CRP_b) and 10–12 days afterwards ($CRP_{10–12d}$) (**A**) and comparison across different subcategories ((**B**)—biological sex; (**C**)—age; (**D**)—smoking status; (**E**)—baseline sputum-smear microscopy results; (**F**)—localisation of lung lesions; (**G**)—presence of cavitary lesions; (**H**)—BMI category) using the Wilcoxon signed-rank test. BMI categories were assigned according to WHO classification [44]: underweight—BMI < 18.5 kg/m^2; normal weight—18.5 $kg/m^2 \leq$ BMI < 25.0 kg/m^2; overweight—BMI $\geq$ 25.0 kg/m^2. The upper and lower margins of the boxes indicate the first and third quartiles, respectively, with the horizontal line within the box indicating the median. The whiskers show the highest and lowest values within 1.5 times the interquartile range from the first and third quartile. The grey lines connect the paired data points for each patient. A p value of <0.05 was considered statistically significant. Abbreviations: CRP—C-reactive protein.

In the subgroup analyses, nearly one-third of patients (13/41; 31.7%) had serum CRP levels within the reference range at both time points (Group A). In 34.1% (14/41) of patients, serum CRP levels decreased ≥2 times from the baseline or reached the reference range (Group B), while the same proportion of patients did not meet this criterion (Group C). Referring to Supplementary Table S1, the baseline characteristics were consistent across the groups, except for radiological and bacteriological findings. After applying Bonferroni correction, it was observed that patients in Group A less frequently presented with cavitary disease compared to those in Group B (adjusted $p = 0.003$) and Group C (adjusted $p < 0.001$), as well as with positive sputum-smear microscopy results compared to Group C (adjusted $p = 0.012$). The estimated marginal means of PK parameters slightly varied across the three groups, but statistical significance was not achieved (Supplementary Table S2). Further analysis to assess the effect of previously discussed factors on the odds of reaching at least a 2-fold reduction in serum CRP levels was limited by statistical power.

2.4. Treatment Response and Its Relationship with Early Changes in Serum CRP Levels

Thirty-eight patients (90.5%) fully recovered from the Mtb infection and met the WHO definition for cured [11]; three patients completed the six-month anti-TB course, but their treatment outcome could not be classified as cured (7.1%); one patient was lost to follow-up (2.4%). In patients whose treatment outcome was "cured" (n = 38), the median time to sputum culture conversion (tSCC) was 56 days (IQR: 27–79 days), and 55.3% (21/38) had their sputum culture converted within the first two months, while at the end of the sixth month, the culture conversion rate reached 97.4% (37/38). Although data distributions were overlapping, patients with delayed treatment response (tSCC ≥ 60 days) tended to have higher serum $CRP_{10–12d}$ levels compared to those with tSCC < 60 days (median 13.0 mg/L [IQR: 3.1–66.9 mg/L] vs. median 5.4 mg/L [IQR: 1.5–20.4 mg/L], $U = 211$, $p = 0.220$).

According to the subgroup analysis, the median tSCC in Groups A and B—representing patients with serum CRP levels within the reference range at both time points, and those who achieved at least a 2-fold reduction in the levels of this biomarker (or reached the reference range) after 10–12 days of therapy—was 46 days (IQR: 27–77 days for Group A and IQR: 25–73 days for Group B, respectively). Meanwhile, in Group C, which included patients who did not fulfil the criteria for CRP reduction, the median tSCC was 66 days (IQR: 36–86 days). The difference in tSCC between all three groups was insignificant ($H_{(2)} = 0.63$, $p = 0.626$).

In the univariate Cox regression models, neither serum $CRP_{10–12d}$ levels nor the subgroups assigned based on changes in serum CRP levels over the specified period were significant predictors for tSCC (Supplementary Table S3). The best-performing multivariate models, which include clinically relevant factors commonly associated with delayed treatment response, are given in Table 4. However, including these covariates did not improve the predictive value of the investigated CRP parameters.

Table 4. Multivariate Cox proportional hazard models of patient characteristics predicting time to sputum culture conversion.

	Model 1		**Model 2**		**Model 3**		**Model 4**		**Model 5**	
Predictors	**HR (95% CI)**	***p* Value**	**HR (95% CI)**	***p* Value**	**HR (95% CI)**	***p* Value**	**HR (95% CI)**	***p* Value**	**HR (95% CI)**	***p* Value**
Male sex									0.82 (0.29–2.30)	0.696
Age, years					1.00 (0.66–1.50)	0.947				
≥60 years	0.94 (0.37–2.40)	0.902	0.92 (0.36–2.40)	0.862						
BMI, kg/m^2	1.39 (0.91–2.10)	0.130	1.35 (0.90–2.00)	0.153	1.30 (0.84–2.00)	0.248	1.53 (0.99–2.40)	0.056	1.43 (0.97–2.10)	0.072
Smoker	1.58 (0.69–3.60)	0.276					1.83 (0.73–4.60)	0.194	1.82 (0.69–4.80)	0.228
Lung cavitations			1.35 (0.60–3.00)	0.465						
Positive baseline sputum-smear microscopy results							0.75 (0.29–1.90)	0.556		
RIF $AUC_{0–6h}$					1.30 (0.95–1.80)	0.105				
$CRP_{10–12d}$ level	0.96 (0.69–1.30)	0.792	0.93 (0.64–1.40)	0.704	1.00 (0.73–1.50)	0.883				
Serum CRP level changes from the baseline										
Group A							1.37 (0.56–3.40)	0.497	1.37 (0.55–3.40)	0.498
Group B							Reference	N/A	Reference	N/A
Group C							1.65 (0.66–4.10)	0.285	1.49 (0.65–3.40)	0.350
Global *p* value		0.444		0.556		0.307		0.425		0.448
Concordance index		0.56		0.57		0.63		0.56		0.55

All continuous predictors were scaled before inclusion in the regression model. Group A—serum CRP levels were within the reference range at both time points; Group B—serum CRP levels decreased by ≥2 times from the first to the second time point or reached the reference range at the second time point; Group C—serum CRP levels decreased by <2 times from the first to second time point. A *p* value of <0.05 was considered statistically significant. Abbreviations: TB—tuberculosis; RIF—rifampicin; $AUC_{0–6h}$—area under the time–concentration curve from 0 to 6 h post-dose, CRP—C-reactive protein; $CRP_{10–12d}$—serum C-reactive protein level 10–12 days after anti-tuberculosis treatment onset; HR—hazard ratio; CI—confidence interval; N/A—not applicable.

3. Discussion

At first, we conducted a series of correlation analyses and group comparisons to explore variations in serum CRP levels based on patient characteristics. Among the factors naturally contributing to the variability in the serum CRP levels, we observed differences in the median values when analysing the impact of biological sex, age, and smoking status, though these differences did not reach significance [46–51]. Only BMI showed a moderately strong negative correlation with serum CRP_b levels. The difference between underweight patients and those categorised as overweight was of marginal significance, yet the levels of this biomarker differed 10-fold between these BMI subcategories. While serum CRP levels typically increase with BMI in healthy adults, the opposite trend is reported in patients with TB [52–54]. In this context, malnutrition is a well-recognised risk factor for developing TB, whereas wasting is a common symptom associated with the advanced forms of the disease, delayed treatment response, and adverse treatment outcomes [27,29,52–57].

Relating to characteristics specific to TB, several studies have shown that serum CRP levels complement radiological and bacteriological findings obtained at the time of diagnosis [9,14–19]. Similarly, our patients with radiologically confirmed cavitary disease and positive sputum-smear microscopy results had higher serum CRP_b levels than their counterparts. We did not observe the impact of the extent of lung involvement; meanwhile, other studies report conflicting findings [14,15,18,58,59]. The discrepancy likely arises from the differences in the type of radiological examination and methodology utilised to estimate the TB-affected lung area.

In 10–12 days, the median serum CRP level in our study population decreased from 21.9 to 6.4 mg/L, falling within the reference range (<8 mg/L). This complies with the results from the studies investigating CRP kinetics in patients with TB, where a drop in the levels of this biomarker occurred between the first and fifth weeks of treatment [14,18–20]. Subsequent analyses revealed that underweight patients, smokers, those with lung cavitations, and positive sputum microscopy results before anti-TB treatment onset experienced a significant decrease in serum CRP levels compared to the baseline, but their serum $CRP_{10–12d}$ levels were still distinct from the target. As described elsewhere, low body weight and smoking have additive effects on inflammation and serum CRP levels in patients with TB, but the highlighted radiological and bacteriological findings, characteristic of the advanced disease, are independently associated with high-degree inflammation [14–18,49–51,53,54]. In addition, these factors determined slower sputum bacillary clearance in the studies by Nijenbandring de Boer et al., Kanda et al., and other groups [24,25,27–29]. It is, therefore, not surprising that patients with a combination of these factors may require a longer time to sterilise the infection and reach the established serum CRP reference range. Even though, in our work, patient age did not significantly correlate with the serum CRP levels at any time point, possibly due to the high proportion of patients aged <60 years (81%), the impact of age manifested as the inability of patients aged 60 years and older to achieve a significant decrease in the level of this biomarker within the specified period. Ageing is a degenerative process with effects extending to the immune system (immunosenescence) and inflammation (inflammaging) [60]. Hence, the slow decline in serum CRP levels, at least in part, could be attributed to intensified and prolonged proinflammatory cytokine production, e.g., TNF-α, IL-6, and IL-1β, as well as to the altered function of immune cells, including macrophages and CD4+ T lymphocytes—the crucial components of immune defence against Mtb [60,61].

Given the observed variability in the reduction in serum CRP levels and the absence of clinical recommendations on expected reduction patterns or timeframes, we applied the approach described by Wilson et al. [20], with minor modifications, to categorise changes in the levels of this biomarker for subgroup analyses.

After stratifying patients based on a 2-fold reduction in serum CRP levels from the baseline and attainment of the reference range after 10–12 days of anti-TB therapy, it was found that 31.4% did not achieve the expected decrease (Group C). They more frequently exhibited lung cavitations or positive sputum-smear microscopy results at the initial clinical

evaluation compared to patients with serum CRP levels within the reference range at both time points (Group A) and those who achieved the stated goal (Group B). These observations align with the results obtained in our primary analyses and reaffirm the role of disease severity in the early reduction in inflammation.

Next, we investigated whether anti-TB drug plasma exposure accounts for the variability in serum CRP levels on the 10th–12th day of treatment. The liquid chromatography–tandem mass spectrometry (LC-MS/MS) data showed suboptimal RIF plasma concentrations in 92.9% of patients at 2 h post-dose, while underexposure rates for other drugs ranged from 9.5% to 54.8%. Other studies have reported comparable rates and identified factors contributing to the observed underexposure [23,32–37]. From our study's perspective, since the initiation of anti-TB treatment caused a significant decline in serum CRP levels, it appears that drug exposure at the site of infection was sufficient for effective early Mtb killing. Indeed, several groups of authors have documented the accumulation of anti-TB drugs in epithelial lining fluid and alveolar cells to varying extents, with plasma or serum concentrations being considerably lower or even below the therapeutic range [62–64]. Notably, a review published a few years ago underlined the discordance between widely used therapeutic ranges (suggested by Alsultan et al. [45]) and real-world data [65]. Nevertheless, an in-depth analysis of PK variability was beyond the scope of this study.

Compared to an earlier study [23], our results did not confirm a relationship between the PK parameters of any of the four anti-TB drugs and serum $CRP_{10–12d}$ levels. In the subgroup analyses, patients whose CRP levels reduced <2 times (Group C) had similar anti-TB drug plasma exposure compared to those belonging to Groups A and B. Thus, in our setting, the variations in anti-TB drug plasma exposure did not result in clinically relevant consequences, although a long-term effect cannot be ruled out. It is plausible that differences in study design and patient characteristics may explain the conflicting results to some extent. In our study, the time between treatment onset and PK sampling was fixed, and the study population was homogeneous in terms of ethnicity, form of TB, comorbidities, care setting, and drug formulation used. Emphasising the significance of these discrepancies, PK/PD studies have reported that some of these factors may contribute to the PK variability of anti-TB drugs [37,66–68].

Finally, we evaluated serum $CRP_{10–12d}$ levels and the patterns of early changes in serum CRP levels as independent predictors of tSCC. The median tSCC in our patients was 56 days, which could be considered a "good treatment response" [11]. We assume that the population structure and the absence of concomitant diseases determined the relatively fast sputum culture conversion, even in patients who did not achieve a rapid reduction in serum CRP levels after the initiation of anti-TB treatment (46 days in Group A and B vs. 66 days in Group C).

In general, our patients with delayed sputum culture conversion (tSCC $\geq$ 60 days) had higher, albeit not significantly, serum CRP levels 10–12 days after starting anti-TB treatment. Consequently, in Cox regression analysis, none of the studied factors predicted tSCC. Musteikienė et al. [18,21] recorded significantly higher baseline serum CRP levels for patients with SCC after Month 1 in both of their studies but also failed to observe the effect on sputum culture status in their later analyses. Djoba et al. [14] predicted sputum culture status by the end of the intensive phase of anti-TB treatment with more than 80% accuracy using a biomarker signature incorporating serum CRP levels at baseline and Week 1. Another study found that serum CRP levels were more strongly associated with sputum culture status at Week 8 vs. Week 12. Yet, employing biomarker combinations did not improve the predictive performance [17]. Interestingly, the relationship between serum CRP levels and treatment response in the subpopulation of patients with drug-resistant TB has not been replicated so far [69]. Taken together, serum CRP has outperformed symptom-based screening tools for active TB in people living with HIV, but its role in predicting treatment response seems to be limited [22].

This study has several strengths. We used various data sources to comprehensively explore factors potentially related to inflammation and address the raised scientific ques-

tions. The impact of other health conditions and natural variability on the study results was mitigated by applying exclusion criteria at the patient enrolment and, whenever required, considering common confounders as covariates in statistical analyses of the data.

There are also several study limitations. When conducting research in a low-endemic setting like Latvia (where the TB incidence in 2022 was 17 cases per 100,000 population, with a total population of 1.8 million [70]), one of the main shortcomings is the limited sample size, further narrowed by the exclusion criteria. Consequently, an unequal distribution of patients across multiple subcategories was observed. The impact of sample size was also evident in the variability of serum CRP levels, resulting in extremely positively skewed data distribution and thus requiring complex statistical approaches. Although the obtained results conform to the existing literature, they should be interpreted cautiously. Additionally, we did not record any cases of treatment failure or severe adverse events in our study population, precluding speculation on the role of inflammation. Lastly, another limitation is the lack of data on minimal inhibitory concentration (MIC), which would have allowed us to account for the susceptibility of the infecting Mtb isolates. Moreover, the combined PK/PD indices, such as C_{max}/MIC and area under the time–concentration curve (AUC)/MIC, have been reported by others as useful in predicting treatment response and outcomes in patients with DS-TB [32,38,39].

4. Materials and Methods

4.1. Study Design and Population

From April 2017 to May 2023, we conducted a retrospective observational study of patients hospitalised in the Centre of Tuberculosis and Lung Diseases at Riga East University Hospital, who underwent the WHO-recommended treatment [2].

The diagnosis of DS-PTB was established based on the comprehensive initial clinical evaluation that included the following: (a) interpretation of clinical symptoms reported by the patient and the results of peripheral blood testing; (b) assessment of the radiological findings (chest X-ray supplemented with data from computer tomography in case of inconclusive findings); and (c) the data from bacteriological testing (sputum-smear microscopy [auramine–rhodamine staining], mycobacterial culture [Löwenstein–Jensen [LJ] media and mycobacteria growth incubator tubes [BACTEC MGIT 960 system, Becton Dickinson, Heidelberg, Germany]], and molecular assay [GeneXpert MTB/RIF Ultra, Cepheid, Sunnyvale, CA, USA]).

Patients were excluded from the study if the following criteria were applicable: (a) extrapulmonary TB; (b) culture-confirmed drug-resistant TB; (c) age < 18 years; (d) pregnancy or lactation; (e) severe acute or chronic conditions that may interfere with blood test results; (f) concomitant infectious diseases (e.g., HVB, HVC, and HIV); and (g) previous history of malignancy.

4.2. Clinical Data

The clinical information retrospectively retrieved from electronic medical records and patient questionnaires included the following: (a) serum CRP level determined prior to anti-TB treatment onset (CRP_b) and, aligned with PK sampling, 10–12 days afterwards ($CRP_{10–12d}$) to characterise the degree of inflammation at baseline and an early effect of therapy; (b) radiologically determined localisation of the TB-associated lung abnormalities such as consolidations and nodules (unilateral or bilateral lesions) and the presence or absence of the cavitations to determine the extent of lung involvement; (c) sputum-smear microscopy results (simplified to dichotomous variable—"positive" or "negative") to distinguish infectious patients with a presumably higher bacillary load. In addition, the demographic and anthropometric information, comorbidities, concomitantly used drugs, and self-reported smoking status were also considered clinically relevant information. In conformity with WHO recommendations [44], a patient was classified as underweight if the BMI was <18.5 kg/m^2 and overweight if the BMI was ≥25.0 kg/m^2. The reference range

for serum CRP levels, established by the clinical laboratory of the Centre of Tuberculosis and Lung Diseases at Riga East University Hospital, is <8 mg/L.

Since there are no clinical recommendations on the pattern or timeframe within which serum CRP levels should normalise in response to anti-TB treatment, we stratified patients for subgroup analyses based on a modified criterion proposed by Wilson et al. [20]. To further characterise the changes in the levels of this biomarker, patients were stratified into the following groups: Group A comprised patients whose serum CRP levels were within the reference range before starting anti-TB treatment and 10–12 days later; Group B—patients whose serum CRP levels reduced ≥2 times or reached the reference range; and Group C—patients whose serum CRP levels reduced <2 times, including cases when serum CRP levels remained unchanged or even increased.

4.3. Determination of the Anti-TB Drug Plasma Exposure Using LC-MS/MS

The measurements of anti-TB drug plasma concentration were performed when patients were in the intensive phase of treatment and routinely receiving ETB, PZA, RIF, and INH at daily doses of 12–25 mg/kg, 20–30 mg/kg, 8–12 mg/kg, and 4–6 mg/kg, respectively, as per the WHO guidelines [2]. PK sampling was performed at three consecutive time points—pre-dose (0 h), 2 and 6 h post-dose—between the 10th and 12th days of therapy, the earliest point at which anti-TB drug exposure could be assessed to ensure steady-state plasma concentration has been reached [71].

Venous blood was collected into BD Vacutainer tubes with EDTA coating (Becton Dickinson, Plymouth, UK) and promptly centrifuged at 4000 rpm (3488 g) at +4 °C for 15 min to separate and harvest the plasma. The obtained samples were stored frozen at −70 °C until analysis. Then, plasma samples were processed and analysed adhering to the LC-MS/MS protocol described earlier [72]. The patients were classified as underexposed if the plasma concentration of the respective anti-TB drug 2 h post-dose (at C_{max}) was below the expected range: 2–6 µg/mL for ETB, 20–60 µg/mL for PZA, 8–24 µg/mL for RIF, and 3–6 µg/mL for INH [45]. The total plasma exposure was evaluated using the AUC from 0 to 6 h ($AUC_{0–6h}$) constructed from the data over the three time points mentioned above. Missing values, if any, were replaced with values corresponding to ½ of the lower limit of quantification of the respective drug [72].

4.4. Assessment of Treatment Response Employing a Culture-Based Approach

The response to anti-TB treatment was assessed using the results from sputum culture on LJ media, aimed at detecting viable Mtb. The testing frequency depended on the sputum-smear status at the baseline—once a month for smear-negative patients and twice a month for smear-positive patients. The primary endpoint was tSCC, and it was defined as the number of days from the start of anti-TB treatment to the sample collection date (including) of the first from two sputum cultures consecutively reported to be negative. Cases were censored to the last available sample collection date if a patient was lost to follow-up or completed treatment but whose treatment outcome did not comply with the WHO definition of cure [11].

4.5. Statistical Data Analysis

All the quantitative variables were assessed for data conformity to a normal distribution by performing the Shapiro–Wilk test and visually inspecting data frequency distribution histograms. Whenever required, non-normally distributed variables (BMI, tSCC, ETB dose and C_{max}, RIF C_{max} and $AUC_{0–6h}$, INH $AUC_{0–6h}$, CRP_b, and $CRP_{10–12d}$) were log-transformed before the analyses to reduce data skewness and conform statistical assumptions. Qualitative variables were summarised using counts and percentages and analysed using the Chi-square or Fisher's exact test, where applicable. Quantitative variables were given as the mean and standard deviation (±SD) for normally distributed data and the median with interquartile range (IQR) for non-normally distributed data; group comparisons were performed using Student's *t*-test or one-way ANOVA, along with their

non-parametric equivalents as the Mann–Whitney U test or the Kruskal–Wallis H test for non-normally distributed data. Additionally, Spearman's rank correlation was required for the correlation analysis of non-normally distributed variables. The CRP_b and $CRP_{10–12d}$ levels were compared using the Wilcoxon signed-rank test. Linear regression was applied to examine the relationship between $CRP_{10–12d}$ levels and PK parameters of each anti-TB drug, adjusted for sex, age, and BMI. Next, ANCOVA was conducted to compare the anti-TB drug exposure in the groups based on the fold change in the CRP levels over the specified time, considering biological sex, age, and drug dose as covariates.

In the end, time-to-event analysis incorporating Kaplan–Meier curves and Cox proportional hazards regression were employed to study the association between the serum CRP level reduction and tSCC.

The statistical data analysis was performed using the software IBM SPSS Statistics (version 29.0.0.0, IBM Corp: Armonk, NY, USA) and R (version 4.2.1, R Core Team (2022): Vienna, Austria, R Foundation for Statistical Computing, https://r-project.org, accessed on 10 September 2024). The statistically significant findings were indicated by a two-sided p value of less than 0.05.

During the preparation of this manuscript, AI-based tools ChatGPT 3.5 (version October 2023, OpenAI, https://chat.openai.com/chat, accessed on 10 September 2024) and Grammarly (version 14.1086.0, Grammarly, https://app.grammarly.com/, accessed on 10 September 2024) were used for English language editing.

5. Conclusions

Summarising all the above, we provided novel insights into TB-associated inflammation by identifying patient characteristics and findings from the initial clinical evaluation, which were related to delayed reduction in serum CRP levels shortly after anti-TB treatment onset. In the present setting, the variability in anti-TB drug plasma exposure did not directly impact the early changes in the levels of this biomarker. Furthermore, the serum CRP levels measured after 10–12 days of anti-TB drug administration, or the fold change from the baseline, did not predict the treatment response. Thus, in our view, the assessment of the serum CRP levels at this time point lacks clinical relevance, at least in patients without comorbidities.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics13121216/s1, Table S1: Comparison of patient characteristics after stratification based on changes in serum CRP levels 10–12 days after treatment onset; Table S2: Comparison of anti-TB drug plasma exposure after patient stratification based on changes in serum CRP levels 10–12 days after anti-tuberculosis treatment onset; Table S3: Univariate Cox proportional hazard models of patient characteristics predicting time to sputum culture conversion.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki. The study protocol was approved by the Central Medical Ethics Committee of Latvia (Approval No. 01-29.1/1 and No. 01-29.1.2/1736), the Ethics Committee of the Riga East Clinical University Hospital (Approval No. 24-A/15), and the Scientific Department of the Riga East Clinical University Hospital (Approval No. ZD/08-06/01-21/187).

Informed Consent Statement: All patients were acquainted with the study protocol and subsequently signed informed consent, thus agreeing to their biological samples and data from electronic medical records and patient questionnaires being collected and processed solely for research purposes.

Data Availability Statement: The datasets supporting the findings of this study are not publicly available due to ethical restrictions but are available from the corresponding author upon reasonable request.

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Approvals of the Central Medical Ethics Committee of Latvia

Centrālā medicīnas ētikas komiteja

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Rīgā

21.05.2015. Nr.01-29.1/1

Latvijas Biomedicīnas
pētījumu un studiju centram
projekta vadītājai
Dr.biol. Renātei Rankai

Atzinums Nr.01-29.1/1 par pieteikuma projektu "Ar prettuberkulores zāļu lietošanu saistīto blakusparādību molekulāro mehānismu izpēte"

Centrālā medicīnas ētikas komiteja 2015.gada 21.maijā ir izskatījusi Latvijas Biomedicīnas pētījumu un studiju centra iesniegto pieteikuma projektu *„Ar prettuberkulores zāļu lietošanu saistīto blakusparādību molekulāro mehānismu izpēte"*.

Pamatojoties uz Centrālās medicīnas ētikas komitejas 2015.gada 21.maija sēdes protokola Nr.5 punktu Nr.3 – tiek izsniegts atzinums, ka Latvijas Biomedicīnas pētījumu un studiju centra pieteikuma projekts *„Ar prettuberkulores zāļu lietošanu saistīto blakusparādību molekulāro mehānismu izpēte"* nav pretrunā ar bioētikas normām.

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Rīgā

Datums skatāms laika zīmogā Nr. 01-29.1.2/1736
Uz 25.01.2021. Nr. 1.1-2/7

APP "Latvijas Biomedicīnas
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Par atļaujas saņemšanu pētījumam "AR PRETTUBERKULOZES ZĀĻU LIETOŠANU SAISTĪTO BLAKUSPARĀDĪBU UN TUBERKULOZES PATOĢENĒZES MOLEKULĀRO MEHĀNISMU IZPĒTE"

Centrālā medicīnas ētikas komiteja izskatīja APP "*Latvijas Biomedicīnas pētījumu un studiju centrs*" 2021.gada 25.janvāra iesniegumu Nr.1.1-2/7 "*Par labojumu iesniegšanu*" par pētījumu "*AR PRETTUBERKULOZES ZĀĻU LIETOŠANU SAISTĪTO BLAKUSPARĀDĪBU UN TUBERKULOZES PATOĢENĒZES MOLEKULĀRO MEHĀNISMU IZPĒTE*" (reģistrēts Veselības ministrijā 2021.gada 25.janvārī Nr.1594).

Atbilstoši Centrālā medicīnas ētikas komitejas 2021.gada 18.marta sēdes protokola Nr.2021-10 II.daļas "*PRECIZĒTIE IESNIEGUMI*" 1.punktam tiek sniegts atzinums, ka pētījums nav pretrunā bioētikas normām.

Centrālās medicīnas ētikas
komitejas priekšsēdētājs (paraksts*) V. Sīlis

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*Dokuments ir parakstīts ar drošu elektronisko parakstu un satur laika zīmogu

Patient written informed consent form

3.PIELIKUMS. Informētas piekrišanas veidlapa dalībai biomedicīnas pētījumā latviešu valodā.

Cienītā kundze!
Godātais kungs!

Mēs uzaicinām Jūs piedalīties pētījumā „AR PRETTUBERKULOZES ZĀĻU LIETOŠANU SAISTĪTO BLAKUSPARĀDĪBU MOLEKULĀRO MEHĀNISMU IZPĒTE", ko veic Latvijas Biomedicīnas Pētījumu un Studiju Centrs un Rīgas Stradiņa Universitāte, pētnieki: Viktorija Igumnova, Renāte Ranka, Dace Bandere, Inta Jansone, Liāna Pliss, Jānis Ķimsis, Ilva Pole, Valentīna Čapligina, Iveta Ozere, Anda Nodieva, Ģirts Šķenders, Pavels Sudmalis, Inga Urtāne. Vēlamies Jūs iepazīstināt ar pētījuma mērķi, norisi un saturu. Pirms šī dokumenta parakstīšanas rūpīgi izlasiet visu informāciju! Pirms dokumenta parakstīšanas Jums ir tiesības uzdot jautājumus par pētījumu un saņemt uz tiem atbildes.

Pētījuma mērķis:

Tuberkuloze (TB) ir ārstējama infekcijas slimība. Tuberkulozes ierosinātāju – tuberkulozes bacili – apkārtējā vidē izdala ar plaušu tuberkulozi slims cilvēks elpojot, runājot, šķaudot vai klepojot. Pēc tuberkulozes baciļu ieelpošanas saslimst 5-10 % cilvēku, pārējie 90 % nesaslimst un tiem nav tuberkulozes simptomu – to sauc par latentu tuberkulozes infekciju. Šobrīd joprojām nav skaidrs, kāpēc daži cilvēki saslimst, bet citi nē. Visticamāk, saslimšanu veicina dažādu faktoru, tai skaitā ģenētisko, mijiedarbība.

Tuberkulozes ārstēšanai izmanto dažādus medikamentus, zāles jālieto regulāri un tik ilgi, kamēr ārsts apstiprina izveseļošanos (parasti vairākus mēnešus). Diemžēl dažkārt zāles var izraisīt dažādas blakusparādības, kas var ietekmēt tuberkulozes slimnieka pašsajūtu. Tāpēc būtu ļoti svarīgi uzzināt, kad un kāpēc šādas blakusparādības var rasties, lai nākotnē varētu izstrādāt ātrās metodes blakusparādību laicīgai prognozēšanai. Tas savukārt palīdzētu ārstiem izvēlēties tādas terapijas shēmas, kas ir efektīvas un ar vismazāko blakusparādību risku katram konkrētam pacientam.

Pētījuma norise:
Projekts norisināsies 4 gadu garumā. Pētījumā tiks lietotas molekulārās bioloģiskas metodes.

Nepilngadīgiem pacientiem:
Apmācīts medicīnas darbinieks bērnam lūgs nodot urīna paraugu gan pirms zāļu lietošanas, gan 2 un 6 stundas pēc zāļu lietošanas. Šajos paraugos tiks noteikts bērna lietoto prettuberkulozes zāļu daudzums. Tāpat apmācīts medicīnas darbinieks bērnam paņems siekalu paraugu ģenētiskās informācijas analīzei, palūdzot iespļaut sterilā trauciņā.
Pēc tam kopā ar ārstu vecāki vai aizbildnis aizpildīs veselības un iedzimtības anketu. Gan bērna veselības un iedzimtības datiem, gan arī siekalu un urīna paraugam tiks piešķirts unikāls kods, kas nodrošinās šīs informācijas konfidencialitāti.

Kodētie paraugi tiks īpaši apstrādāti, lai tajos esošo ģenētisko informāciju varētu analizēt un ilgstoši uzglabāt drošā vietā ar ierobežotu pieejamību.

Pilngadīgiem pacientiem:

Apmācīts medicīnas darbinieks pētījuma dalībniekam no vēnas paņems 10 ml asiņu pirms zāļu lietošanas, kā arī 2 un 6 stundas pēc zāļu lietošanas. Tāpat pētījuma dalībniekam palūgs nodot urīna paraugu gan pirms zāļu lietošanas, gan 2 un 6 stundas pēc zāļu lietošanas. Šajos paraugos tiks noteikts lietoto prettuberkulozes zāļu daudzums. Pēc tam kopā ar ārstu pētījuma dalībnieks aizpildīs veselības un iedzimtības anketu. Gan veselības un iedzimtības datiem, gan arī asiņu un urīna paraugam tiks piešķirts unikāls kods, kas nodrošinās šīs informācijas konfidencialitāti.

Kodētie asiņu paraugi tiks īpaši apstrādāti, lai tajos esošo ģenētisko informāciju varētu analizēt un ilgstoši uzglabāt drošā vietā ar ierobežotu pieejamību.

Ja pētījuma dalībnieks piekrīt piedalīties pētījumā, viņš var atļaut veikt savu genoma izpēti tikai šī konkrētā pētījuma ietvaros vai arī neierobežot.

Ieguvumi:
Kopumā šī pētījumā gūtie rezultāti dos svarīgu zinātnisku informāciju par prettuberkulozes zāļu blakusparādību procesos iesaistītiem molekulāriem mehānismiem, kā arī var būt ļoti nozīmīga tuberkulozes pacientu slimības prognozes un racionālas farmakoterapijas izstrādāšanā katram pacientam individuāli. Līdz ar to šī pētījuma rezultātiem būs ne tikai zinātniska, bet arī praktiska nozīme Latvijas iedzīvotāju veselības aizsardzībā.

Pētījumā iegūtie dati ļaus novērtēt prettuberkulozes zāļu metabolizējošo enzīmu gēnu polimorfismu izplatību Latvijas populācijā, kas savukārt parādīs relatīvo blakusparādību risku Latvijas tuberkulozes pacientiem un norādīs uz prettuberkulozes terapijas iespējamās pārtraukšanas riskiem zāļu nepanesamības dēļ.

Iespējamie riski:
Piedalīšanās pētījumā dalībnieka veselībai nenodarīs nekādu ļaunumu. Pētījuma dalībniekam tiks paņemts asins un urīna paraugs kā parastai rutīnas asins analīzei un urīna analīzei. Siekalu parauga noņemšana ir nekaitīga, nesāpīga procedūra. Asins ņemšanas vietā retos gadījumos var rasties neliels asins izplūdums, vai retāk – neliels lokāls ādas iekaisums.

Konfidencialitāte:
Pacienta dati, kas nepieciešami viņa personas identifikācijai, trešai personai nebūs pieejami, kā to pieprasa LV esošā likumdošana.

Brīvprātīga piedalīšanās:
Piedalīšanās šajā pētījumā ir brīvprātīga. Jums ir tiesības atteikties piedalīties pētījumā vai pārtraukt dalību pētījumā jebkurā laikā. Jūsu atteikšanās piedalīties pētījumā vai dalības pārtraukšana neradīs nekādu nevēlamu ietekmi uz Jums sniegtās veselības aprūpes kvalitāti.

Ja jums ir jebkādi jautājumi par šo pētījumu, lūdzu, sazinieties ar Viktoriju Igumnovu, adrese: Rātsupītes ielā 1, Rīgā, LV-1067, tālrunis: 67808200, e-pasts:viktorija.igumnova@gmail.com vai Ivetu Ozeri, adrese: stacionāra "Tuberkulozes un plaušu slimību centrs" Stopiņu novadā, Upeslejās, LV-2118, tālrunis: 67285003, e-pasts: iveta.ozere@aslimnica.lv.

Aizpilda pacients

1. Esmu saņēmis un iepazinies ar informāciju par projekta mērķi un iespējamiem riskiem. Uz visiem maniem jautājumiem esmu saņēmis saprotamas un izsmeļošas atbildes. Man bija pietiekami daudz laika, lai pārdomātu savu lēmumu piekrist kļūt par bioloģiskā materiāla (audu) donoru.

2. Apzinos, ka man nebūs tiesību pieprasīt maksu par audu paraugu nodošanu, mana veselības stāvokļa apraksta (mana bērna veselības stāvokļa apraksta, ja pētījumā piedalās Jūsu bērns) vai ģenealoģisko datu sastādīšanu vai izpēti, ka arī par izpētes rezultātu izmantošanu.

3. Piekrītu piedalīties (piekrītu, ka mans bērns piedalās) šajā pētījumā brīvprātīgi, bez maksas. Piekrītu, ka izpētei tiek ņemts **asins** un **urīna paraugs**. Piekrītu, ka ģenētiskai izpētei tiek ņemts mans **asins paraugs** (mana bērna **siekalu paraugs**, ja pētījumā piedalās mans bērns), un sastādīts veselības stāvokļa apraksts:
   □ atļauju
   □ azliedzu

4. Manu audu paraugu un veselības stāvokļa aprakstu nosūtīsanu ģenetiskajai izpetei ārpus Latvijas:
   Mana bērna audu paraugu un veselības stāvokļa aprakstu nosūtīsanu ģenetiskajai izpetei ārpus Latvijas:
   □ atļauju
   □ azliedzu

5. Mana veselibas stavokļa apraksta papildināsanu, atjaunosanu (Mana bērna veselības stāvokļa apraksta papildināšanu, atjaunošanu:
   □ atļauju
   □ azliedzu

Šis dokuments ir sastādīts divos eksemplāros, no kuriem viens atrodas pie pētījuma veicēja, bet otrs – pie pētāmās personas.

Es ar savu parakstu apliecinu, ka esmu iepazinies/usies ar šī dokumenta saturu. Es saprotu, ka mana dalība šajā pētījumā ir brīvprātīga, un atteikšanās piedalīties neizraisīs nekādas nelabvēlīgas sekas. Man ir tiesības pārtraukt dalību pētījumā jebkurā laikā. _____ _____ Vārds, uzvārds Datums Paraksts

Pētnieks: _____ _____ Vārds, uzvārds Datums Paraksts

Approval of the Research Ethics Committee of Rīga Stradiņš University

Veidlapa Nr. E-9(3)
APSTIPRINĀTA
ar Rīgas Stradiņa universitātes rektora
2018. gada 26. septembra rīkojumu Nr. 5-1.238/2018

Rīgas Stradiņa universitātes
Pētījumu ētikas komitejas
LĒMUMS
Rīgā

31.01.2019. Nr.6-3/1/ 6

	Komitejas sastāvs	Kvalifikācija	Nodarbošanās
1.	Profesors Olafs Brūvers	Dr.theo.	teologs
2.	Asoc.prof. Santa Purviņa	Dr.med.	farmakologs
3.	Asoc.prof. Voldemārs Arnis	Dr.biol.	rehabilitologs
4.	Profesore Regīna Kleina	Dr.med.	patalogs
5.	Profesors Guntars Pupelis	Dr.med.	ķirurgs
6.	Asoc.prof. Viesturs Liguts	Dr.med.	toksikologs
7.	Docente Iveta Jankovska	Dr.med.	ortodonts
8.	Docents Kristaps Circenis	Dr.med.	docētājs
9.	Lektore Ilvija Razgale	Mg.soc.d.	docētājs

Pieteikuma iesniedzējs/i: **Asoc. prof. Renāte Ranka**
Farmācijas fakultāte

Pētījuma / pētnieciskā darba nosaukums: "Prettuberkulozes zāļu metabolītu noteikšana asins plazmā"

Iesniegšanas datums: 24.01.2019.

Pētījuma protokols: Izskatot augstāk minētā pētījuma pieteikuma materiālus (protokolu) ir redzams, ka pētījuma mērķis tiek sasniegts veicot pētījumu ar jau ievāktu materiālu no Valsts iedzīvotāju genoma datu bāzes, to izpēti, iegūto datu apstrādi un analīzi, kā arī izsakot priekšlikumus. Personu (pacientu, dalībnieku) iegūto datu (asins paraugu ņemšana) apstrāde, to pielietošana, glabāšana un konfidencialitāte ir ievērota un nodrošināta. Līdz ar to pieteikums atbilst pētījuma ētikas prasībām.

Komitejas lēmums: **piekrist** pētījumam

Komitejas priekšsēdētājs Olafs Brūvers Tituls: Dr. miss., prof.

Paraksts

RĪGAS STRADIŅA UNIVERSITĀTE ĒTIKAS KOMITEJA

I.Bēniņa
67061596

Approvals of the Ethics Committee and the Scientific Department of the Riga East Clinical University Hospital

afonds.ētika
MEDICĪNISKO UN BIOMEDICĪNISKO PĒTĪJUMU ĒTIKAS KOMITEJA

Darbojas saskaņā ar SHK LKP noteikumiem

Nr. 24-A/15
03.09.2015.
Rīgā

Rīgas Austrumu klīniskās universitātes slimnīcas atbalsta fonda
Medicīnisko un biomedicīnisko pētījumu Ētikas komitejas

ATZINUMS

Pētījuma nosaukums : Ar prettuberkulozes zāļu lietošanu saistīto blakusparādību molekulāro mehānismu izpēte

Pētījuma pieteikuma iesniedzējs: Viktorija Igumnova

Pētījuma pieteikuma iesniedzēja darba vieta: Latvijas Biomedicīnas Pētījumu un Studiju centrs, zin.asist.

SIA "Rīgas Austrumu klīniskās universitātes slimnīcas" atbalsta fonda Medicīnisko un biomedicīnisko pētījumu Ētikas komiteja(sēdes prot.10/15., 03.09.15.) ir izvērtējusi plānotā zinātniskā pētījuma nozīmi un mērķi, iesniedzēja sniegto paredzamā ieguvuma un riska novērtējumu un tā pamatotību. Balstoties uz iesniegto dokumentu izvērtējumu, komiteja nolēma izteikt:

- ■ pozitīvu atzinumu
- ☐ negatīvu atzinumu, ar iespēju veikt izmaiņas un iesniegt pieteikumu atkārtoti
- ☐ negatīvu atzinumu

par pieteikuma atbilstību zinātnisko pētījumu ētikas prasībām.

Rīgas Austrumu klīniskās universitātes slimnīcas atbalsta fonda
Medicīnisko un biomedicīnisko pētījumu
Ētikas komitejas priekšsēdētājs Roberts Stašinskis

Rīga, Hipokrāta iela 2, LV1038
t. 20281174

1/1

aslimnīca
RĪGAS AUSTRUMU KLĪNISKĀ UNIVERSITĀTES SLIMNĪCA

SIA Rīgas Austrumu klīniskā universitātes slimnīca
Reģistrācijas Nr.: 40003951628
Hipokrāta iela 2, Rīga, LV-1038, Latvija
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E-pasts: aslimnica@aslimnica.lv, www.aslimnica.lv

ZINĀTNES DAĻA
Hipokrāta iela 2, Rīga, LV-1038, Latvija
Tālr.: 67 303 180
E-pasts: zinatne@aslimnica.lv

APSTIPRINĀTS
ar SIA „Rīgas Austrumu klīniskā universitātes slimnīca" valdes 2019. gada 22. janvāra lēmumu
Nr. V1/01-01/19/31

Rīgā

2021.gada 29.septembrī
Nr. ZD/08-06/01-21/187

Renātei Rankai

ATĻAUJA AKADĒMISKĀ PĒTĪJUMA VEIKŠANAI

Zinātnes daļa ir izskatījusi Jūsu iesniegto akadēmiskā pētījuma „***Tuberkulozes ārstēšana: personalizētās terapijas perspektīvas izpēte (projekta numurs: lzp-2020/1-0050)***" *do*kumentāciju, kas reģistrēta Zinātnes daļā ar numuru **AP-104/21**, kas apstiprina akadēmiskā pētījuma veikšanu SIA „Rīgas Austrumu klīniskā universitātes slimnīca" (turpmāk – Iestāde) stacionārā "Tuberkulozes un plaušu slimību centrs", galvenā ārste Andra Cīrule.

Atbildīgais par pētniecības norisi Iestādē ir Andra Cīrule.

Zinātnes daļā iesniegti un izskatīti:

1. Pieteikums par akadēmiskā pētījuma AP-104/21 veikšanu,
2. Pētījuma protokols ar pielikumu,
3. R.Rankas, A.Vīksnas, A.Krama, A.Cīrules, I.Ozeres, A.Šmites, L.Barkānes, A.Kazarinas, V.Igumnovas, D.sadovskas,I.Norvaišas, A.Kivrānes, L.Freimanes, J.Ķimsa, E.Sevastjanova un S.Grīnbergas konfidencialitātes apliecinājumi.
4. Centrālās medicīnas ētikas komitejas atzinums, izsniegts 2021.gada 25.janvārī.

Prospektīvā pētījumā, iegūstot pacientu rakstisku piekrišanu, tiks iegūts 200 pacientu ar tuberkulozi bioloģiskais materiāls un dati.

Pētnieku pienākums ir izpildīt 2020.gada 9.jūlija "Infekciju izplatības ierobežošanas pasākumu kārtība SIA "Rīgas Austrumu klīniskajā universitātes slimnīca" prasības.

Pētījums tiek finansēts no LZP-2020/1-0050 projekta līdzekļiem un uzsākams pēc līguma parakstīšanas ar iestādi.

Atļauja derīga līdz 2023.gada 30. jūnijam.

Dr.med. Daiga Šantare

(paraksts)

Speciāliste akadēmisko pētījumu jautājumos
Zinātnes daļa
Šantare, 67303179