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Personalization Of Antitubercular Treatment: Evaluation Of Pharmacological Determinants Of Treatment Response

CANDIDATE Ilaria Motta

TUTOR Prof. Giovanni Di Perri

COORDINATOR Prof. Giuseppe Saglio

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To my sister

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1. Introduction

Tuberculosis (TB) with 10.4 million new cases/year is a worldwide leading infection, responsible for approximately 1.7 million deaths/year (2016 data)[1].

The goal of halting and reversing the TB epidemic, set within the context of the Millennium Development goals, has been reached globally, with progress in TB diagnosis (expanded use of Xpert MTB/RIF®), treatment, prevention (increased number of people living with HIV treated with isoniazid preventive therapy) and increase in funding and research. Currently, the WHO (World Health Organization) has built up a strategy to end the global epidemic by 2035 with targets to reduce TB deaths by 95%, cut new cases by 90% between 2015 and 2035, and to ensure that no family is burdened with catastrophic costs due to TB[2].

International guidelines recommend for drug-susceptible TB the association of rifampicin, isoniazid, pyrazinamide and ethambutol for two months followed by four months of rifampicin and isoniazid.

Combining several antitubercular drugs (ATDs) is a milestone of TB treatment[3].

This approach is due to the hypothesis of existing bacterial subpopulations with different drug susceptibility: a first subpopulation of extracellular and rapidly dividing mycobacteria early killed by therapy, a second subpopulation with an intermediate grade of replication residing in fagolisosomes and, possibly, a third subpopulation of dormant and persistent mycobacteria in monocytes/macrophages and in caseum lesions[4]. Conversely, a second hypothesis considers this latter population having the same drug susceptibility as the others, and being the cause of persistent disease, as mycobacteria, sequestered in thick-walled granuloma where ATDs hardly penetrate, are not exposed to the drugs. Therefore, the activity of a multidrug regimen with specific efficacy characteristics of each drug component ensures a fast bactericidal effect followed by a sterilizing effect to prevent relapse or selection of drug resistance.

Although the current TB regimen is effective with 95% of cure rate (in drug susceptible TB under optimal conditions), there are still many undefined issues such as the high variability of response, the likelihood of drugs underexposure, the high prevalence of drug-related toxicity and the selection of multi drug-resistant strains. The lack of early biomarkers for predicting treatment efficacy, cure and identification of patients requiring prolonged treatment increases complexity to this challenging condition[5]. Moreover, so far, the pipeline was merely sufficient to tackle the emergent issue of MDR/XDR TB (multi drug-resistant and extensively-drug resistant TB) [6]. After 40 years of no ATDs development, bedaquiline and delamanid have received accelerated regulatory approval bringing advances in the treatment of resistant strains, whilst other compounds are under Phase II studies (pretomanid).

Repurposing existing drugs and exploring new regimens (e.g. injections-free regimens), different doses, schedules and route of administration (e.g. inhalatory route) seem to be productive tools to possibly shorten the length of treatment in drug susceptible TB and improve outcome in MDR-TB[7]. Preliminary studies and evidence in selected extrapulmonary localizations have shown that higher rifampicin doses may be safe and increase treatment efficacy[8].

1.1 Pharmacokinetics Pharmacodynamics Approach

Studies of early bactericidal activity (EBA) have an important place in the experimental investigation of tuberculosis treatment.

EBA is defined as the ability to decrease the bacillary load in the first two days of therapy.

The assessment of EBA of antitubercular agents by measurement of viable colony-forming units (CFU) of *Mycobacterium tuberculosis* in sputum of patients with smear-positive pulmonary tuberculosis is now an established methodology for the early clinical evaluation of a new

antitubercular agent.[9] Simultaneous study of pharmacokinetics offers the opportunity to have a pharmacodynamics (PD) approach and evaluate the relationship between dosage, serum concentration, efficacy and specific host or mycobacterial factors relevant to the agent under investigation.

Several studies have shown that EBA is correlated with isoniazid[10] and rifampicin[11] dose, whereas ethambutol and pyrazinamide have a lower EBA.

Early bactericidal activity refers to the initial rapid kill of actively replicating mycobacteria (1).

Following this phase, the sterilizing activity is the ability of a drug to prevent relapse (after the completion of treatment) on long- term (≥18 months). The rates of decline in the *Mycobacterium tuberculosis* burden in sputum during the first 14 days and 2 months of therapy are also used as surrogate markers for sterilizing activity.

Serial sputum CFU counts on solid media are an established method to investigate bactericidal activities of antitubercular drugs.

CFU counting is expensive, labor-intensive, and technically challenging, with problems of bacterial clumping. In addition, this procedure is difficult to standardize across sites in multi-centre studies. Another problem with CFU counting is that it does not take into account the metabolic activity of the different types of bacteria growing on solid media (actively replicating, slowly replicating bacteria and persisters).

Recent experience has shown that time to detection of metabolic activity in liquid media reflects drug activity in a manner comparable to that of CFU counting and encourage further exploration. [12]

Liquid culture systems may better represent the overall population of bacteria (7), since there are some mycobacterial populations that do not grow on solid media but grow in liquid media. Mycobacterial Growth Indicator Tube (MGIT; Becton Dickinson, Sparks, MD) systems have

improved the detection of *Mycobacterium tuberculosis* in clinical samples and also shortened the time to obtain a positive result.

Chigutsa *et al.* used time to positivity (TTP) in liquid culture as a surrogate of bacillary burden[13] during the first 8 weeks of standard therapy in patients with pulmonary tuberculosis. With nonlinear mathematical models, they identified two bacterial subpopulations with different kill rates and the concentration thresholds at which the concentration-effect relationships occur in patients. Important predictor of sterilizing activity included rifampicin AUC and C_{max}, pyrazinamide AUC/MIC, ethambutol C_{max}/MIC and isoniazid C_{max}. Ethambutol and isoniazid were significant predictors of sterilizing activity only when rifampicin exposure was low.[13]

Bowness and colleagues created a mathematical model to understand the relationship between CFU and MGIT TTP. They observed that TTP showed a negative correlation with log10 CFU in the first 14 days. Moreover, there was an increasing gradient of the regression line and y-intercept as treatment progressed. There was also a trend towards an increasing gradient with higher doses of rifampicin.[14]

Several studies have shown the importance of antitubercular drug concentrations on the rate of kill of *Mycobacterium tuberculosis* in hollow fiber systems, animal models of tuberculosis and patients.

1.1.1 Plasma Pharmacokinetics

Precise targets for the maximum plasma concentrations over the MIC (C_{max}/MIC) or time over the MIC (T> MIC) from *in vivo* studies are not available. As reference, the concentrations obtained from the use of standard doses in healthy volunteers in controlled Phase I studies are generally accepted. It is well known that these doses are well tolerated, usually effective and therefore peak concentrations derived from them should also be effective. Although lower concentrations in some

patients have been associated with free-relapse cure, minimal effective concentrations are not known. In the last ten years, several studies about the application of TDM (Therapeutic Drug Monitoring) and its advantages in clinical practice have been published. The reference values published in 2002 by Peloquin *et al*. have been widely confirmed and are summarized in the table below.[15]

Drug	Adult dose	C_{max} (ng/mL)	T_{max} (h)	$t_{1/2}$ (h)
Rifampicin	600 mg/die	8000-24000	2	2-3
Isoniazid	300 mg/die	3000-6000	0.75-2	Acetylator status Fast: 1.5 Slow: 4
	900 mg/tw	9000-15000		
Pyrazinamide	25-35 mg/kg/die	20000-60000	1-2	9
Ethambutol	25 mg/kg/die	2000-6000	2-3	2-4

Table 1 Pharmacokinetic parameter of first-line anti-TB drugs[15]

Heysell *et al.* conducted a retrospective study to estimate the prevalence of low concentrations in patients in Virginia (USA) with slow clinical response to antitubercular therapy. Two hours post-dose samples were collected as peak concentration. The concentrations of rifampicin, isoniazid and ethambutol were below the expected ranges in 52%, 59%, 31% of patients, respectively. The concentrations of pyrazinamide were within normal ranges[16].

A recent study by Babalik *et al*. describes the results of TDM in 20 patients with slow response or comorbidity, such as HIV infection. Isoniazid, rifampicin, ethambutol and rifabutin were lower than the reference ranges in 87%, 67%, 15% and 80%, respectively. Furthermore, time to culture conversion was higher in patients with lower concentrations[17]

.

A study by McIlleron *et al.* conducted in HIV positive patients suggests the need to increase doses in this population for the observed lower concentrations[18]. Prahl *et al*, showed that low 2 hours post-dose of isoniazid and rifampicin were associated with therapy failure[19].

The timing of samples collection is also relevant: 2 hours post-dose could hide malabsorption or delayed peak concentrations; therefore a 6 hours post-dose sample is recommended to get information on drug absorption. Limited sampling strategies (LLS), aim to determine which samples are more predictive of AUC value.

Rifampicin, as we know, has concentration-dependent activity, the ratios C_{max}/MIC and AUC_{24}/MIC correlate with the killing ability. The current recommended rifampicin dose (10 mg/kg/day, maximum dose 600 mg per day), derives from economic reasons and from limited investigations of administered dose, exposure at the site of infection, therapeutic response and side effects. The wide success of the current therapeutic regimen may, over the years, have limited the development of rifampicin pharmacodynamic studies[20]. Recently, the current dose has been questioned due to the inability to achieve effective concentrations in the ELF (endothelial lining fluid), in the case of pulmonary disease localization. [21,22] The mutant prevention concentration (MPC) represents a threshold above which the selective proliferation of resistant mutants is expected to occur only rarely. This concept is used in antibiotic and antiviral therapy to allow therapy optimization. Antimicrobial compounds with higher AUC_{24}/MPC ratios have a lower risk of selecting resistant mutants. Some data are available for fluoroquinolones and linezolid and some for rifabutin, rifampicin, clarithromycin in non-tuberculous mycobacterial infections[23,24].

An increased dose of rifampicin might expose the mycobacterium to higher AUC_{24}/MPC values that would prevent the selection of resistant mutants. Phase 2 studies were conducted in Peru

and Brazil to evaluate the efficacy of regimens with high doses of rifampicin with encouraging results (HIRIF trial).[25,26]

Inter-individual variability in the pharmacokinetics (PK) of ATDs might be involved in explaining variability of response and it has been identified as a key factor for the sterilizing effect and for the selection of phenotypic resistance. Low maximum plasma concentrations have been associated with treatment failure, relapse and acquired drug resistance regardless of HIV status; dose-adjustments after drug monitoring has been related to better clinical outcomes.[17,27]

Oral or intravenous administration are considered equally effective and the guidelines indicate intravenous administration only for critically ill patients. Available data demonstrate that disseminated tuberculous infections or central nervous system localizations benefit, in terms of outcome, from parenteral administration.[8] Our previous report on 24 patients showed no statistically differences in drug exposure, except for isoniazid, according to route of administration.[28]

Some data are available regarding the relationship between PK and toxicity (see section 1.4 First-line drugs Characteristics). Combination of ATDs is associated with a significant incidence of side effects: preventing toxicity might be beneficial also in terms of treatment interruption (estimated approximately at 5%), failures and selection of resistance.[29]

The TDM is therefore a useful tool, entered into clinical practice, to monitor plasma drugs concentrations, to early detect therapeutic failure, to prevent toxicity and, eventually, to tailor the patient's regimen.

1.1.2 Intracellular Pharmacokinetics

Macrophages play an important role in the control of *Mycobacterium tuberculosis* growth, spread and granuloma formation and immunodeficient patients are at higher risk of developing disseminated and extrapulmonary infections. As antibacterial activity is concentration-dependent, ATDs must reach appropriate levels at the site of action, i.e. inside infected macrophages. However, since resident macrophages are usually derived from circulating monocytes and since they are far more easily accessed, it may be relevant to measure ATDs in peripheral blood mononuclear cells (PBMCs)[30].

During first days, a rapid killing of growing bacilli is observed in cavitary lesions, as mentioned above, and the predominant drug is isoniazid [4]. Thereafter, an early sterilizing activity with lower killing rate occurs and the predominant drug is rifampicin. The target of the drug regimen is then the bacilli inside the phagolysosomes, where an intermediate growing rate is observed; finally, the sterilization phase aims at the elimination of the very slow replicative compartment located inside monocytes and macrophages cells and within the caseous lesions (where mainly rifampicin and pyrazinamide are active).

Even if a correlation between ATDs plasma concentrations and outcome was described [31], the main site of action of the drug regimen is intracellular and to date no intracellular concentration efficacy ranges were established.

Mycobacteria can survive and grow within macrophages, evading the immune system and potentially antibiotics.

To be active inside the cell, the compound must penetrate through the membrane of macrophages, phagosomes and lately the mycobacterium wall; this process may be limited by the chemical-physical characteristics of the drugs and/or by efflux transporters present on the cell membrane of the host or the mycobacterium. Therefore, intracellular concentrations may

differ from extracellular ones and intracellular mycobacteria may have different sensitivity to the drugs used.

In pulmonary TB, alveolar macrophages are the primary site of infection and *in vivo* concentrations hardly can be determined. Thus, most of the studies with this objective used circulating mononuclear cells (PBMCs) as a surrogate of infected macrophages, for ease of sampling. PBMCs represent 2% of the entire mononuclear compartment, that includes lymph nodes and the reticuloendothelial system [MoMa (Monocyte-derived macrophages)].

The ability of drugs to cross membranes and reach adequate intracellular concentrations may be relevant for treatment outcome and for new shorter regimens, if we take into account the elimination phase of slow replicating mycobacteria within the macrophages.

Some data are available for rifampicin and isoniazid. The former has mycobactericidal activity both extracellular and intracellular and Hartkoorn *et al.* found that rifampicin accumulates around 1.7-fold within PBMCs *in vivo*, and 2.5-5-fold within PBMCs and cell line in vitro.[32]

Hartkoorn in another analysis showed that in vitro rifampicin and ethambutol are substrates of P-gp (glycoprotein P), observing a reduction of their concentrations in the presence of cells with over-expression of P-gp (CEMVBL). Moreover, rifampicin accumulation was 5.19-fold within cells and 0.28-fold for isoniazid.[30] Despite isoniazid has similar intra and extracellular activity, is known that isoniazid is not equally effective because its activity depends on the metabolic status of the microorganism: bactericidal activity with fast growing mycobacteria and bacteriostatic effect with quiescent microorganism.[33]

The intracellular role of pyraznamide at the moment is unclear, but the acidic pH-dependent activity (such as within the macrophages) toward slow replicating microorganisms make it potentially active inside the cells during the continuation phase of the treatment.[34] One study observed that pyrazinoic acid (POA), the active metabolite, accumulated inside the cells in acidic

environment, while at neutral or alkaline pH the compound remained extracellular.[35] An analysis from Mitchison showed that POA inhalation in association with oral intake of pyrazinamide could allow pH reduction with pyrazinamide activation and POA itself would avoid the first-pass effect reducing the concomitant pyrazinamide dose and lastly, considering that almost all the mutations are in *pncA* gene that codifies for the initial deamidation, would allow a bactericidal action even in the presence of resistance.[36]

1.2 Pharmacogenetics

Pharmacogenetics (PG) is the study of inter-individual variation in DNA sequences related to drug metabolic pathways. Inter-individual variability, tissue penetration and drug to drug interactions are partially explained by genetic variants in gene encoding for metabolizing or transporting proteins: knowing patients' genetic asset may pave the way to tailored treatment.

Historically, metabolizing phenotypes (later discovered to be associated with *NAT2* genetic variants) have been associated with INH metabolism and toxicity. Recently, several pieces of evidence have been published on the effect of single nucleotide polymorphisms (SNPs) in genes encoding for proteins involved in drug disposition (see section 1.5 First-line drugs characteristics).

1.3 Vitamin D

The role in TB treatment of vitamin D and its receptor (VDR) is a matter of intense debate amongst experts. A high frequency of vitamin D deficiency in TB patients has been reported, thus it is frequently administered.[37,38] Vitamin D is able to increase phagocytosis through activation of macrophages and subsequently to limit intracellular growth of *Mycobacterium tuberculosis*; in addition, SNPs on its receptor (vitamin D receptor, VDR) seem to play an important role in

treatment outcome.

Case-control studies investigated the association between *VDR* SNPs and the time to sputum culture conversion, with heterogeneous results according to studied populations[39].

A double-blind randomized controlled trial, published in 2011, showed that vitamin D supplementation reduced the time to sputum culture conversion in subjects with specific polymorphism in *TaqI VDR* gene[40].

Vitamin D3 (cholecalciferol) is taken in diet or it is synthesized in skin from 7-dehydrocholesterol by ultraviolet irradiation, which varies with season and latitude.[41] It is transported in blood by vitamin D-binding protein (VDBP) to liver. Here, it is hydroxylated at C:25 by one or more cytochrome P450 vitamin D 25-hydroxylases (CYP2R1, CYP2D11 and CYP2D25), resulting in the formation of 25-hydroxyvitamin D3 (25(OH) D3). Cheng *et al.* suggest that CYP2R1 is the key enzyme for 25-hydroxylation of vitamin D and homozygous mutation of CYP2R1 gene was found in a patient with low circulating levels of 25(OH)D3 and classic symptoms of vitamin D deficiency.[42] 25(OH)D3 is transported by VDBP to kidney, where magalin, a member of the low-density lipoprotein receptor superfamily, allows the endocytic internalization of the vitamin. In the proximal renal tubule 25(OH)D3 is hydroxylated in C-1 of the A ring, resulting in the hormonally active form of vitamin D, 1,25-dihydroxyvitamin D3 (1,25(OH)2D3). The CYP450 CYP27B1, which metabolizes 25(OH)D3 to 1,25(OH)2D3, is present predominantly in kidney, but also in placenta, monocytes and macrophages.[43] Xu *et al.* reported that CYP3A4 also catalyzes 23-/24-hydroxylations of 1,25(OH)2D3, which provides an alternative pathway for the inactivation of vitamin D3.[44] Isoniazid inhibits CYP27B1 activity, whereas rifampicin is a strong CYP3A4 inducer; combined use of these two drugs reduces 25(OH)D and 1,25(OH)2D levels both in healthy volunteers and TB patients, but no evidence on effect has been reported.[40,45] In mice, it was recently observed that rifampicin and isoniazid co-administration inhibits CYP27B1

expression and it induces CYP24A1 one. These data first showed that rifampicin and rifampicin/isoniazid co-treatment can both enhance 25-hydroxylation and 24-hydroxylation of vitamin D.[46]

Our previous analysis on 24 patients with pulmonary TB showed the involvement of vitamin D pathway gene polymorphisms in rifampicin pharmacokinetics.[47]

Active form of vitamin D binds to VDR. VDR–vitamin D complex modulates many immune, endocrinal and neural activities. *VDR* gene, located on chromosome 12 (12q13.11), is expressed in at least 37 tissues, grouped into seven biological systems – calcium homeostasis, immune, pancreatic β cells, muscle, cardiovascular, brain and lung.[48,49] VDR is a member of the family of steroid receptors that mediates the effects of vitamin D in regulating the transcription of multiple genes.

The main functional loci of *VDR* gene are *FokI* (rs2228570), *BsmI* (rs1544410), *ApaI* (rs7975232) and *TaqI* (rs731236) and all of them are located between exons 8 and 9 apart from *FokI* is in exon. Mutations in this gene are associated with type II vitamin D-resistant rickets. It forms a heterodimer with the retinoid-X-receptor and interacts with a specific promoter region (vitamin D response element). Microarray analyses with different human cells reveal that more than 100 genes have vitamin D response elements in their promoter regions.[50] Furthermore, VDR is also located in the plasma membrane caveolae, where, binding active vitamin D, can exert rapid response, by second messenger production.[51]

1.4 First-line drugs characteristics

We here report a brief description of the first-line drugs. Mechanism of action, administration, distribution, metabolism and elimination processes (ADME), PK characteristics (PK/PD parameters of efficacy, toxicity and TDM implications) and available PG data of each compound

are described (Table 2). The text is partially included in the review by Motta *et al.* "Pharmacokinetics and pharmacogenetics of anti-tubercular drugs: a tool for treatment optimization?"[52]

	RIF	INH	PZA	ETB
Dose	600 mg	300 mg	25-30 mg/kg	20-25 mg/kg
Binding protein [%]	88.6	10-15	10-40	10-30
Metabolism	Hepatic deacetylation Autoinduction	Acetylation by NAT2	Hepatic, 3% excreted unchanged	10-15% hepatic, 50-55% eliminated unchanged
Plasma half-life [hours]	3-4	1.5 fast acetylators 4 slow acetylators	10	2-4
Parameter best predictive of activity[53]	AUC/MIC C_{max}/MIC	AUC/MIC> C_{max}/MIC	AUC/MIC	AUC/MIC
Therapeutic range μg/mL	8-24	3-6	20-60	2-6
Threshold associated with poor outcome[54]	AUC≤13 mg*h/l	AUC≤52 mg*h/l	AUC≤363 mg*h/l	N/A

TDM for efficacy	++	+	+	+
TDM for toxicity	0	+	++	++
PG data	*SLCO1B1* *CES2*	*NAT2*	N/A	N/A
I/E ratio	2-5	1	<1	10-20

Table 2 Pharmacokinetic and pharmacodynamic properties of first-line antitubercular drugs

(Rif, rifampicin; Inh, isoniazid; Pza, pyrazinamide; Etb, ethambutol; AUC, Area Under the Curve; N/A, not assessed; TDM Therapeutic Drug Monitoring; PG data Pharmacogenetic data; I/E Intracellular/Extracellular Ratio; +++, TDM is indicated to ensure therapeutic dose and ensure that accumulation is not occurring; ++, TDM is indicated when known or suspected malabsorption, poor treatment response or suspected toxicity; +, TDM is not routinely performed; 0, PK studies found no association).

1.4.1 Rifampicin (RIF)

RIF is a key sterilizing drug in the treatment of TB with relatively low early bactericidal activity usually administered at 8-12 mg/kg.

Mechanism of action

RIF blocks the transcription inhibiting the bacterial DNA-dependent RNA polymerase. Mutations in the *rpoB* gene that codes for the beta-subunit of the RNA polymerase are responsible for resistance to RIF.

ADME

It is metabolized by a liver esterase (arylacetamide deacetylase) to 25-desacetyl rifampicin and it is excreted via biliary and renal route.

The best size predictor for RIF clearance is fat-free mass instead of total body weight and it should be used to optimize dose. Antiacids do not affect the absorption of RIF. Simultaneous

intake of high-fat food decreases RIF C_{max} by 36% (AUC is less affected) and increase T_{max} by 103%[55], thus RIF should be given on a empty stomach.

PK target

In most patients, the target RIF C_{max} is 8 to 24 μg/mL after 600 mg of oral dose and an increased dose is recommended if C_{max} is less than 6 μg/mL.

Two and 6-hour sampling strategy captures most C_{max} values and potentially delayed absorption.

PK characteristics

The MIC of RIF is 0.15-0.5 μg/mL. Several evidences point out that RIF dose is at the lower limit of optimal efficacy and that the maximum effective dose has yet to be found[20]. The consensus among experts on the use of higher doses is increasing dramatically, as shown by growing data. A dose range trail from Boeree and colleagues showed safety of two weeks of RIF up to 35 mg/kg[56]. A higher dose (13 mg/kg intravenously) improved treatment outcome in Indonesian TB meningitis patients as reported by Ruslami[8], results from HIRIF trial with 3 study arms of 10, 15, 20 mg/kg showed interesting results.[25,26] Last year, preliminary results of PanACEA MAMS-TB-01 trial were presented and two weeks high-dose (up to 35 mg/kg) RIF in combination with INH, PZA and ETB showed a significant shortening of time to culture conversion over 12 weeks. Final results have still to be published.[57]

Some other clinical experiences have been conducted and TDM-based increased RIF dosage led to improved outcome[27,58,59].

Diacon and colleagues[22] found almost the double 2-day early bactericidal activity (EBA) and higher AUC_{24} (171 versus 100 μg* h/ml) at 20 mg/kg comparing to 600 mg/die but C_{max} did not differ in this study.

Moreover, a non linear increase in exposure might be observed because of saturation of hepatic extraction, but a model-based evaluation showed saturation of RIF clearance already at doses of about ≥ 450 mg, confirming previous studies. This could explain the non-linearity of RIF concentration with dose, underexposure of lower-weight patients and the correlation between faster RIF absorption and higher bioavailability.[60]

RIF is a potent inducer of pregnane X receptor mediated expression of CYP3A4 in liver and in intestine and plasma concentration of several CYP3A4 substrates are reduced (e.g. HIV protease inhibitors, oral contraceptives, azoles, statins, methadone and quinidine). Auto-induction has been reported to take about a week and full induction takes about three to four weeks.[61] This causes a decline in the AUC and terminal half-life over the first weeks of administration. It induces several other CYPs (CYP1A2, CYP2D6), phase II enzymes and efflux transporters. It inhibits OATP1B1, an organic transporter protein expressed by hepatocytes responsible for the uptake of many drugs into portal circulation.[53]

Toxicity

RIF-induced hepatitis occurs in up to 2.5% and it does not seem to be dose related even with higher doses.[62] Intermittent regimens with higher RIF doses (1200 mg or more) seem to be rather related with higher risk of flulike syndrome.[63][64][65]

Pharmacogenetic data

RIF is a substrate of P-glycoprotein and OATP1B1; the latter is an influx transporter mainly expressed in basolateral membrane of hepatocytes and that facilitates RIF uptake into hepatocytes. Genetic variants in *SLCO1B1* (encoding for OATP1B1) have been shown to affect the protein expression and activity.

In particular, Chigutsa and colleagues[66] undertook a study in South African patients with TB and found that *SLCO1B1* genetic polymorphism rs4149032 (which occurs at a high frequency

in the black African population) is associated with reduced RIF concentrations. Patients presenting heterozygous and homozygous polymorphism had reductions in the RIF bioavailability (and, thus, AUC) of 18% and 28%, respectively. This study suggests that an increase in RIF dose would be desirable for carriers of the *SLCO1B1* polymorphism and a simulation showed that increasing the daily RIF dose by 150 mg in patients with the polymorphism would result in plasma concentrations similar to those of wild-type individuals and would reduce the percentage of patients with C_{max} below 8 mg/L (from 63% to 31%). Two other common non-synonymous *SLCO1B1* variants have been studied: rs2306283 (previously referred to as 388A>G) and rs4149056 (commonly referred to as 521T>C). These two variants are in partial linkage disequilibrium. Consequently, there are four important haplotypes: *SLCO1B1*1A*, containing neither variant, *SLCO1B1*1B* (rs2306238), *SLCO1B1*5* (rs4149056) and *SLCO1B1*15* (both). The *SLCO1B1*15* haplotype have been found to be associated with rifampicin-induced liver injury in a Chinese population and may have a role in cholestatic/mixed injury although other studies in similar ethnic groups did not confirm such observation.[67,68]

Weiner and colleagues[69] studied the effect of genetic polymorphisms of *ABCB1* (encoding for P-glycoprotein), *SLCO1B1* and *SLCO1B3* in patients with TB from different regions (North America, Spain, Africa) and in healthy subjects. The results showed that patients with *SLCO1B1* 463C>A variants (rs11045819) had a 36% lower RIF AUC compared to CC genotypes. Hennig *et al*. found that this polymorphism was associated with 30% higher bioavailability of RBT in heterozygous carriers.[70]

Another investigated gene was the carboxylesterase 2 (*CES2*) because have been shown to significantly affect the plasma concentrations of RIF. *CES2* is thought to be responsible for the formation of the main metabolite of RIF by deacetylation.[71]

Moreover, mixed results have been published on the effect of *ABCB1* SNPs on RIF exposure. *ABCB1* encodes for P-gp that is both a substrate of RIF and could be induced by RIF. More studies are needed to investigate the potential to influence drugs transport and intracellular accumulation.[72]

1.4.2 Isoniazid (INH)

Mechanism of action

INH is a prodrug, converted by mycobacterial enzyme katG to its active form. The mechanism of action is to inhibit the mycolic acid synthesis, disrupting the bacterial cell wall.[73] Mutations in *katG* and *inhA* genes are responsible for main mechanisms of INH resistance.[74]

ADME

It is generally absorbed quickly from gastro-intestinal tract.

INH is metabolized in the liver to acetyl-isoniazid via N-acetyltransferase (NAT2) enzyme, then, acetyl-isoniazid is hydrolysed to acetylhydrazine that is further hydrolysed to hepatotoxic compounds by cytochrome P4502E1.

It is recommended to give INH on empty stomach because high-fat meals reduce INH C_{max} (high-fat meal causes a drop of 51% of C_{max}) and delays the absorption[55].

PK target

Peak concentration after 300 mg dose is 3 to 5 μg/mL. Experts recommend a dose increase if the peak is less than 2 μg/mL.

PK characteristics

INH has the highest early bactericidal activity (EBA) and acts against replicant extracellular mycobacteria. Its effect rapidly decreases after first days and its activity is associated with AUC and acetylator status. MIC ranges from 0.01 to 0.25 μg/mL. Studies investigating EBA activity have shown that maximum achievable EBA with clinically tolerable doses[10] was at

plasma concentrations of 2-3 μg/mL. C_{max} occurs 1 to 2 hours post-dose when given on an empty stomach.

INH is used in the regimen of multiresistant TB at higher dose (15-20 mg/kg) unless high-level resistance is known to exist. Isolates from INH-resistant cases often include a mixture of susceptible and resistant strains and INH at high dose eliminates strains that are susceptible or have low-level resistance.[75] Usually low-level INH-resistant organisms (often strains bear mutations in the promoter region of the *inhA* gene[76]) are resistant to ethionamide (ETO), and high-level resistant organisms (usually harbouring *KatG* mutations) are susceptible to ETO. Therefore the recommendation for MDR-TB regimen (in a standardized therapeutic approach scenario) is to include both high-dose INH and ETO to be active against both low- and high-level INH resistant populations[77][78].

It has been shown that MIC of INH-resistant strains is generally below plasma concentrations achieved after high-dose of INH.[79] From a clinical point of view the recommendation for the inclusion of high-dose INH in adult MDR-TB regimens is largely based on evidence from the analysis of paediatric data. In adults a randomised controlled trial of high-dose INH found beneficial effects in terms of sputum culture conversion at 6 months, no increased risk of hepatotoxicity and a higher incidence of peripheral neuropathy.[80]

High-dose INH is one of the core components of the shorter MDR-TB treatment regimen (9-12 months) for patients who have not been previously treated with second-line drugs and in whom resistance to FQs and second-line injectable agents has been excluded.[78]

Toxicity

Periphery neuropathy, through the increase of the excretion of pyridoxine, is a rare INH dose-dependent adverse event. Patients at increased risk are those with HIV infection, diabetes, renal failure, alcoholism, malnutrition and pregnant/lactating women. Supplemental

pyridoxine (vitamin B_6) is recommended. In lactating women taking INH, supplementation of pyridoxine has to be administered to new-borns as well for the passage of INH through breast-feeding.

Pharmacogenetic data

INH is primarily metabolized by acetylation via N-acetyl transferase 2 (NAT2). The rate of elimination of INH depends on NAT2 metabolic activity and the activity is controlled by active alleles. According to *NAT2* genotype patients can be characterized as slow (without any active alleles), intermediate (heterozygous for *NAT2*4*) and rapid acetylators (homozygous for *NAT2*4*, wild type). Rapid acetylators are at higher risk of treatment failure whereas slow acetylators may develop hepatotoxicity. Gene frequency for the slow allele varies in different ethnic groups and geographical areas being 10% in Japaneses and Eskimos, 60% in Caucasians and subjects of African Ancestors and 90% in subjects from the Middle East.

A genotype-guided randomized and controlled trial investigated the rate of treatment failure and INH related liver injury (INH-DILI) in 172 Japanese patients with pulmonary TB[81]. Patients in the pharmacogenetics-guided arm (PGx arm) received 2.5 mg/kg, 5 and 7.5 mg/kg INH according to their slow, intermediate or rapid acetylator status, respectively while those in the standard dose arm received approximately 5 mg/kg. INH-DILI occurred in 78% of the slow acetylators in the standard-treatment (STD), while none of the slow acetylators in the PGx-treatment experienced either INH-DILI or early treatment failure. Among the rapid acetylators, early treatment failure was observed with a significantly lower incidence rate in the PGx-treatment than in the STD-treatment (15% vs. 38%).

1.4.3 Pyrazinamide (PZA)

Mechanism of action

PZA's mechanism of action is still not well defined and the drug appears to act, at least partially, by acidifying the cytoplasm of the cell. It is a prodrug that requires conversion to pyrazinoic acid (POA) by an amidase encoded by *pncA*.

ADME

Well absorbed orally, PZA is widely distributed throughout the body, achieving concentrations above that needed to inhibit tubercle bacilli. PZA crosses inflamed meninges. It is metabolized by the liver and metabolic products are excreted by the kidneys, requiring dosing modification in renal failure.[82]

PK target

Recommended therapeutic range according to data on healthy volunteers is 20-60 μg/mL after 25 mg/kg daily dose. Experts recommend a dose increase if the peak is less than 75% of the desired range.[15]

PK characteristics

PZA usually reaches C_{max} 1 to 2 hours post-dose. It has a long half-life. Therefore the 2- and 6-hour sampling strategy for RIF and INH is adequate also for PZA. PZA has the potential effect of shortening treatment duration due to its great sterilizing activity from 9-12 months to 6 months. Recent hollow fiber PK/PD study demonstrated that PZA's sterilizing effect was best explained by the AUC/MIC ratio, whereas resistance suppression was linked to T>MIC. Monte Carlo simulations revealed that doses higher than the currently recommended 2 g/day would have a better likelihood of achieving the AUC/MIC ratio associated with 90% of maximal effect, but safety concerns arise.

Co-administration of allopurinol with PZA decreases pyrazinoic acid clearance and causes acid uric accumulation due to inhibition of acid uric excretion.[83]

PZA serum clearance has been shown to increase with increases in body weight.[84]

A study in mice found that systemic delivery of POA was not sufficient to reduce bacillary burden, even if POA concentration in plasma, ELF (epithelial lining fluid) and lung lesion were similar to those produced by effective doses of PZA. New technics exploring delivering of POA at the site of infection[85] (into macrophages or intrapulmonary with adjunct inhalation therapy[36]) are on-going.

PZA agent is included in multi-resistant TB treatment and is the only first-line ATDs most likely to be maintained in all new regimens to shorten the duration. Exploring its use in novel combination regimens, both in multi-sensitive and in multi-resistant cases, is giving promising findings.

In 2015 a phase 2b trial showed efficacy at 8 weeks of regimen containing PZA, pretomanid and moxifloxacin, even in MDR-TB strains.[86] The same regimen was investigated in the phase 3 STAND trial[87] but the superiority of the regimen containing bedaquiline, pretomanid, moxifloxacin and PZA in NC-005 trial by TB Alliance led to withhold of STAND trial in favour of this new regimen.[88]

The scenario of drug-resistant TB is further being complicated by the emergence of strains resistant to PZA. This represents a clinical and global health issue. The PZA resistance prevalence has been estimated 16.2% among all TB cases and 60.5% among MDR-TB cases.[89]

Mutation of *pncA* gene is the more common cause of PZA resistance but the variants of SNPs complicates the development of rapid molecular diagnostics.

Phenotypic DST using the Mycobacterial Growth Indicator Tube (MGIT) 960 system is so far considered the gold standard, but is difficult to perform and false-resistance errors occur. Some authors proposed a screening for mutations in the *pncA* gene in MDR-TB patients to test susceptibility to PZA and tailor the regimen accordingly.[90][91]

Toxicity

The most common adverse events are nausea and vomiting. Hepatotoxicity occurred in nearly 15% of patients receiving doses of 40-50 mg/kg for prolonged periods. Current recommended doses (25 mg/kg daily) are safer. Subjects with pre-existing liver disease should have closed monitoring of liver function. Asymptomatic urate retention occurs in 50% of patients receiving PZA.

Pharmacogenetic data

As far as our knowledge, no published data are available regarding the impact of genetic polymorphisms on pharmacokinetic of PZA.

1.4.4 Ethambutol (ETB)

ETB is a semisynthetic antibiotic that is bacteriostatic against *Mycobacterium tuberculosis.*

Mechanism of action

ETB acts by inhibiting arabinosyl transferase enzyme, thus blocking the synthesis of arabinogalactan, which forms the mycobacterial cell wall.[92] Mutations in *embB* gene that codes for arabinosyl transferase enzyme are related with ETB resistance (MIC above 5 μg/mL). Resistance to ETB is higher in INH-resistant strains due to the correlation with mutation at *katG* Ser315 and *inhA*, which encodes an efflux pump transporter.

ADME

ETB administered orally is 75% to 80% absorbed. It is distributed throughout the body. Concentrations measured in lung tissue, ascites and pleural fluids are far higher than ones reached in plasma. ETB does not penetrate intact meninges, but in patients with TB meningitis the penetration increases by 10-50%. After conversion of approximately 25% of absorbed ETB

to inactive metabolites, 80% of the parent together with metabolites is excreted in urine. Therefore, dose adjustment is advisable in significant renal failure.

PK target

Recommended plasma concentrations are between 2 and 6 μg/mL after a 15 to 25 mg/kg daily dose. Experts recommend a dose increase if the peak is less than 2 μg/mL. Some reports suggest that increasing the dose to 25 mg/kg/daily would be the preferred regimen for most patients, monitoring for toxic effects.[93]

PK characteristics

ETB reaches C_{max} 2 to 3 hours post-dose, so sample strategy at 2 and 6 hours works well to detect delays or malabsorption. Aluminium-containing antiacids decrease the C_{max} by 28% and AUC by 10%. Although mechanisms of sex-related differences in drug concentrations are poorly understood lower ETB concentrations were found in female patients. In the same study albumin levels were inversely correlated with concentrations and the reason for this is unclear but is possibly related to altered pharmacokinetics in more severe diseases or malnutrition.[94]

Toxicity

The major toxicity is neuropathy. Peripheral neuropathy is infrequent; retrobulbar optic neuritis is more common. Frequent documentation of visual acuity and red-green colour discrimination is recommended. Blurry vision is usually the early symptom that patients complain. Prolonged administration and high dose (50 mg/kg) are more associated with retrobulbar neuritis than with low dose (15 mg/kg) and intermitting administration (25 mg/kg thrice weekly).

In patients with renal failure, is recommended TDM because ETB is eliminated by renal route.[82]

Pharmacogenetics data

A recent pharmacogenetic study from our group found associations between SNPs in *ABCB1*, *CYP24A1*, Vitamin D Receptor (*VDR*) gene and plasma/intracellular ETB concentrations. Further researches are needed to understand the clinical relevance of these findings.[95]

1.4.5 Hepatotoxicity and PG

The incidence of hepatotoxicity during antitubercular treatment varies from 2% to 28%.[96] The exact mechanism is unknown but toxic metabolites have a role in the development of it. Investigations of genetic polymorphisms relating to drug induced hepatitis have been conducted and *CYP2E1* and *GST* genes resulted to influence the incidence of it.

CYP2E1 activity depends on INH blood concentrations. INH or its metabolite could both induce and inhibit CYP2E1. The variant *CYP2E1* genotype is more susceptible to the inhibition than the common genotype. The enhanced activity causes increased production of hepatotoxins and consequently increased risk of hepatotoxicity.[97]

Furthermore *CYP2E1* polymorphisms were found to be related with the severity of antitubercular drug-induced hepatotoxicity.[98]

GSTs enzymes are involved in detoxification of drugs and other chemical substances. Of the five encoding loci *GSTM1* and *GSTT1* were reported to be associated with hepatotoxicity in Western Indian population. Homozygous deletion at *GSTM1* and *GSTT1* loci could influence susceptibility to INH-induced liver toxicity.[99]

2. Aim of the study

Considering the above presented data, we investigated the role of plasma, PBMCs concentrations and SNPs of genes coding for specific transporters and enzymes potentially

involved in ATDs absorption, distribution, metabolism, elimination and Vitamin D pathway. Pharmacogenetic and pharmacokinetic results were, then, evaluated for their relationships with microbiological markers of response (TTP) and data on hepatotoxicity.

Therefore, primary aim of this research was to describe plasma and PBMCs concentrations of first line TB drugs in patients with *Mycobacterium tuberculosis* infection.

Secondary aims were:

i. the correlation between pharmacokinetic parameters and the sputum increase at 7 and 14 days in patients with pulmonary infections;
ii. the correlation between pharmacokinetic parameters and the risk of development hepatotoxicity;
iii. the impact of different allelic variants on pharmacokinetic parameters;
iv. the impact of different allelic variants on sputum Time To Positivity (TTP) increase at 7 and 14 days in patients with pulmonary infection;
v. the impact of different allelic variants on the risk of development of hepatotoxicity.

3. Materials and Methods

A prospective observational study was conducted between January 2015 and January 2018 in patients with proven *Mycobacterium tuberculosis* infection at Infectious Diseases In-patients and Out-patients Unit (University Clinic and Divisione A, ASLTO2) at Amedeo di Savoia Hospital and Department of Respiratory Medicine at Poliambulatorio Lungo Dora Savona (ASLTO2), Turin, Italy. Amedeo di Savoia Hospital is a regional referral centre for diagnosis and treatment of infectious and tropical diseases with around 90-100 hospitalizations for tuberculosis per year. Department

of Respiratory Medicine in Lungo Dora Savona is the main Out-Patient clinic for care of patients with tubercular infections with 142 incident cases in 2017 and 133 incident cases in 2016.

The study was conducted in accordance with the guidelines of the Declaration of Helsinki and ethics committee approval (Comitato Etico Interaziendale, Ospedale San Luigi Gonzaga, Orbassano, Torino and from 2017 onwards, Comitato Etico Città della Scienza e della Salute, Ospedale Molinette, Torino) was obtained for study protocol (TB_INTRA study) and protocol amendments. All the included subjects provided written informed consent (with translator and counsellor assistance if needed).

3.1 Patients

All the patients hospitalized for proven pulmonary/extrapulmonary tuberculosis or for highly suspected tubercular infection (pending microbiological results) who started multi-sensitive TB treatment were identified for the participation in the study.

Inclusion criteria were:

- ✓ adult age (≥18 years old),
- ✓ proven tubercular infection defined as:
    - positive sputum/pleural fluid GenExpert® (Cepheid) or positive sputum microscopy and radiological findings;
    - culture positive from any site for *Mycobacterium tuberculosis*;
    - positive biopsy at microscopy and clinical/radiological findings from any site;
    - positive GenExpert® (Cepheid) and clinical/radiological findings compatible with tubercular infection (extrapulmonary disease);
- ✓ normal liver and renal function.

Exclusion criteria were:

- ✓ severe malnutrition;

- ✓ HIV infection;
- ✓ concomitant administration of drugs with known interactions.

Drop out criteria were:

- ✓ Any resistance to first-line drugs at DST for microbiological proven infections;
- ✓ Need of stopping within 2 weeks any of TB drugs for clinical reasons (see section 3.5 Scheduled visits);
- ✓ Patient nonadherence (<90%);
- ✓ Withdrawal of consent at any time;
- ✓ Death.

Enrolled patients received standard once-daily weight-adjusted ATDs according to international guidelines: RIF (10 mg/kg/day, maximum 600 mg/day), INH (5 mg/kg/day, maximum 300 mg/day), PZA (25 mg/kg/day, maximum 2500 mg) and ETB (25 mg/kg/day, maximum 2000 mg).

For patient who received intravenous treatment (PZA available only as oral compound), drugs were administered in the following order and dilution rate:

- RIF in 250 ml Sodium Chloride 0.9% in 90 minutes,
- INH in 100 ml Sodium Chloride 0.9% in 30 minutes,
- ETB in 100 Sodium Chloride 0.9% in 30 minutes,
- PZA was administered at the start of RIF infusion.

Patients with prescription of oral treatment received all drugs on an empty stomach, once daily. Breakfast was given after 1 hour.

All the oral administrations were directly observed by nurses or doctors.

Patients with baseline 25-OH vitamin D levels below 30 ng/ml received supplementation with colecalciferol 10000 U/ml 28 droplets on bread, weekly.

3.2 Biological sampling and timing of collection

Venous blood samples were collected at the end of the dosing interval (C_{trough}) and 2 h post-dose (C_{max}). From October 2016 onwards, an intensive sampling at 0, 2, 4 and 6 hours post-dose was proposed to hospitalized patients.

The blood sampling for pharmacokinetic analysis was performed at 7 and 14 days from the start of antitubercular treatment.

3.2.1 Plasma pharmacokinetic analysis

Our laboratory developed and validated a new chromatographic method to simultaneously quantify first-line antituberculars in plasma.[100] Our method of extraction from plasma, compared with the previously published method, has the advantage of a faster extraction procedure using a single precipitation step with acetonitrile and direct injection into the UPLC system. The chromatographic system used was an AcquityTM Ultraperformance Liquid Chromatography system (UPLC) (Waters, Milford, MA, USA) coupled with a Quadrupole Detector (TQD). An AcquityTM UPLC HSS T3 1.8 µm (2.1x150 mm) column (Waters), protected by an Acquity UPLC Column In-Line Filter (Waters), was used. An internal standard working solution (IS) was made by diluting thymidine and QX in water/methanol (50:50, v/v) at a concentration of 20 and 1 mg/mL, respectively.

One 7 ml tube coated with lithium heparin was collected at each time point (0-2-4-6 hours post-dose) at week 1 and week 2.

Blood samples were centrifuged (900 g for 15 min at 4°C) to separate the plasma.

Two hundred µL of standard, quality control and patient samples were added to 50 µL of IS and 400 µL of acetonitrile. After centrifugation at 20000 g for 10 min at 4°C, supernatant was diluted 1:10 with water before injection into the UPLC system.

3.2.2 Intracellular pharmacokinetic analysis

To measure intraPBMCs concentrations a method was developed and published by our laboratory from Baietto *et al*. in 2015.[100] PBMCs were isolated from blood (28 mL) using BD Vacutainer® CPTTM tubes. Two tubes (7 ml each) were collected at each time point (0-2-4-6 hours post-dose) at week 1 and week 2.

Tubes were centrifuged at 800 g for 15 min at 20°C. PBMCs were then washed twice in ice-cold 0.9% NaCl solution. Cell number and mean cellular volume (MCV) were determined using an automated cell counter (Z2™ Coulter Counter®, Beckman Coulter, Brea, CA, USA). Standards, QCs and samples from patients consisting in 1 mL of PBMCs at a concentration of $10x10^6$ cells/mL were spiked with 50 µL of IS. Each sample was vortexed for 15 s and then sonicated in a water bath for 15 min at room temperature. Samples were centrifuged at 20000 g for 10 min at 4°C, and supernatant was transferred to glass tubes. Supernatant was evaporated in a vacuum centrifuge at 60°C, reconstituted with 300 µL of water/acetonitrile (95:5, v/v) and injected into the UPLC system. Blank PBMCs used for the preparation of standards and QCs were extracted from buffy-coats from healthy donors, kindly supplied by the Blood Bank of the Maria Vittoria Hospital (Turin, Italy).

PBMCs-associated concentrations of antituberculars, expressed in ng/mL, were obtained using the following formula:

$$\frac{\text{antitubercular amount (ng)}}{\text{number of PBMCs}} \times \text{MCV (fL)} \times 10^{-12}$$

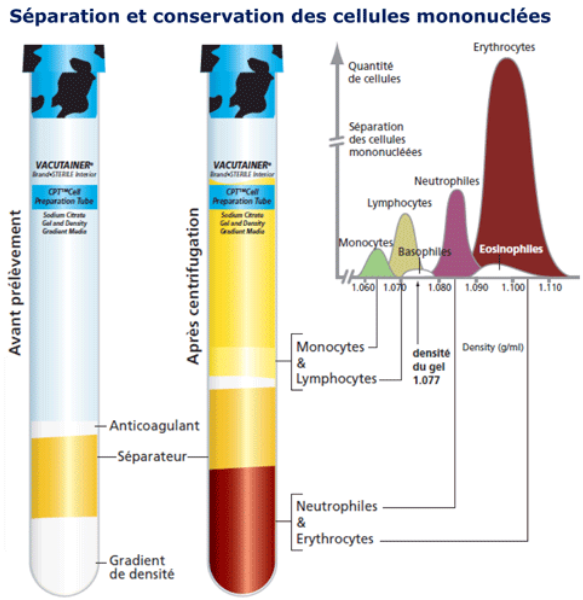


Figure 1 BD Vacutainer® Mononuclear Cell preparation Tube (CPT). A closed-system tube for blood collection, mononuclear cell separation, and trasportation

We measured MCV instead of assuming a fixed MCV of 400 fL, like the previous published method; this may be more accurate when estimating intracellular concentrations, reducing the probability of underestimating or overestimating drug levels in PBMCs.[101]

If the analysis was not performed the same day of the collection, the plasma samples and the resulting pellets of PBMCs, dissolved in 800 µL of water/methanol solution (30:70, v/v), were stored at -80^{o}C. The interval from blood sampling to PBMC storage was less than 1 h.

All the PK analysis were performed at Laboratory of Clinical Pharmacology and Pharmacogenetics, Department of Medical Sciences, University of Turin, ASLTO2, Amedeo di Savoia Hospital.

3.2.3 Pharmacogenetic analysis

QIAamp DNA minikit was purchased from Qiagen (Valencia, CA) for DNA extraction. TaqMan assay was used to allelic discrimination and all "Master Mix" and primers-probes were

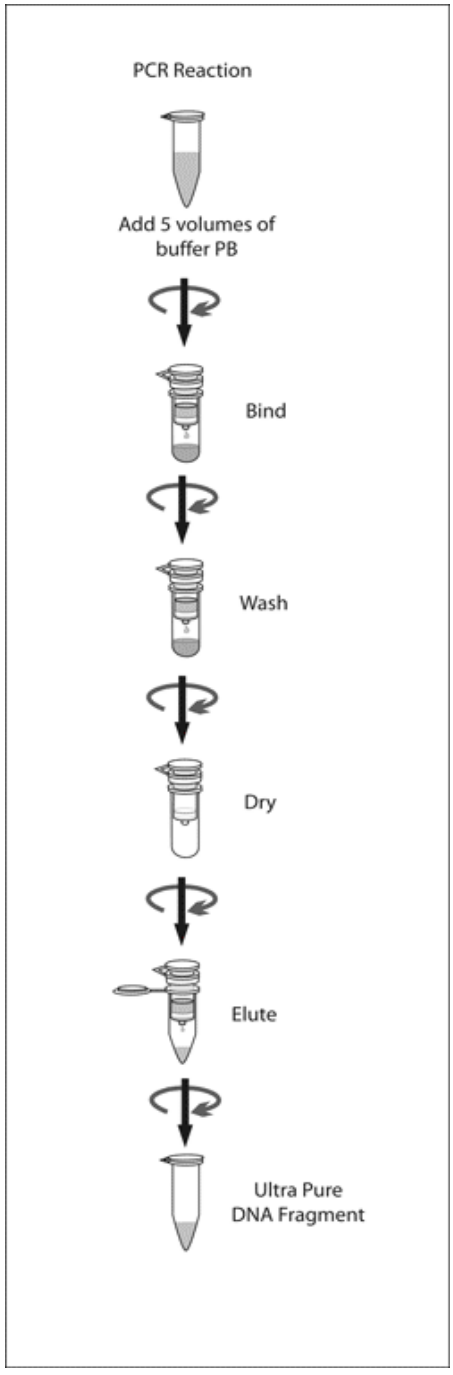
purchased from Applied Biosystems (Foster City, CA). Ultrapure water was produced by Milli-Di coupled to Synergy 185, Millipore (Milan, Italy).

One EDTA tube (4 ml) was used for collecting blood at 7 days. Each sample was stored in criovials at -80 °C for pharmacogenetics analysis.

Basing on results obtained from previously described studies focused on transporters, receptors and enzymes involved in the ADME and/or clinical effects of ATDs and vitamin D, the choice of investigated genetic polymorphisms are resumed in Table 3.

Gene	SNP name	rs	Position	Alleles
ABCB1	ABCB1 3435	rs1045642	25002550	C>T
SLCO1B1	SLCO1B1 521	rs4149056	14093965	T>C
NR1I2	PXR 63396	rs2472677	26093996	C>T
NAT2	NAT2 G590A	rs1799930	18400593	G>A
VDR	*TaqI*	rs731236	48238757	T>C
VDR	*ApaI*	rs7975232	48238837	C>A
VDR	*BsmI*	rs1544410	48239835	G>A
VDR	*FokI*	rs10735810	46559162	T>C
VDR	*Cdx2*	rs11568820	48302545	A>G
CYP24A1	*CYP24A1 +22776*	rs927650	52772741	C>T
CYP24A1	*CYP24A1 +3999*	rs2248359	52791518	T>C
CYP24A1	*CYP24A1 +8620*	rs2585428	52786897	A>G
CYP27B1	*CYP27B1 +2838*	rs4646536	58157988	C>T
CYP27B1	*CYP27B1 -1260*	rs10877012	58162085	G>T

Table 3 List of investigated SNPs. Genes, rs, positions and alleles are reported



Extraction protocol was performed as here described:

1. For each sample, firstly 20 µl of Proteinase K was spiked in 1,5 ml PFTE tubes, subsequently 200 µl of blood sample and 200 µl of Lysis Buffer were introduced. Afterwards, these samples have been vortexed for 10 seconds, leaved at 56 °C for 10 minutes to activate proteinase K and then centrifuged for 1 minute at 6080Xg.
2. For each PFTE tube 200 µl of Ethanol 99% were added in order to preserve samples for long-term and being ensure that every trace of the virus was eliminated. Thus, samples were vortexed for 10 seconds and centrifuged for 1 minute at 6080Xg.
3. Each sample was transferred in the appropriate column and centrifuged (1 minute at 6080Xg). In this way, DNA binds beads present in the silical filter and the waste was eliminated. The column was recovered, then underwent two washing steps, which the first one is followed by centrifugation (1 minute at 6080Xg).
4. Afterwards, a centrifuge step (3 minutes at 18630 g) eliminated all scrap residues. Finally, each column was transferred in the appropriate 1.5 ml PFTE tubes and underwent elution step with Elution Buffer. It was necessary waiting for 1 minute, leaving the PFTE tubes in vertical position at room temperature and then make a final centrifuge step (1 minute at 6080 g). Thus, it was possible trough away the column and preserving each PFTE tube at -80 °C.

Allelic discrimination

Allelic discrimination consists in determining the two possible variants on a DNA sequence by "5 nuclease fluorogenic assay". In detail, this technique exploits the 5' to 3' esonuclease activity of Taq polymerase: when the enzyme meets an oligonucleotide perfectly matched with the DNA sequence, this one is used by Taq polymerase as plate for continuing elongation during the reaction.

In this way, it is possible to investigate single nucleotide polymorphisms (SNPs). As well as a valid flexibility and efficiency, it is realizable to genotype a wide number of samples simultaneously by a single PCR (Polymerase Chain Reaction) race, without using other additional techniques. TaqMan® Allelic Discrimination was adopted to analyze samples. The process is based on a Real Time PCR (RT-PCR), namely an amplification and an evaluation of DNA samples in real time, following each amplification step phase to phase.

The RT-PCR needs three important elements:

- ✓ primer "sense" or 5',
- ✓ primer "anti-sense" or 3',
- ✓ "probe".

The latter is an oligonucleotide formed of two parts linked to opposite extremities:

- ✓ "Quencer" (TAMRA fluorochrome), which is a particular fluorescence silencer. In a deeper analysis, the silencing occurs through energy transfer between both fluorochromes, but only when these ones are near.
- ✓ "Reporter" (FAM or VIC fluorochrome), which is the real responsible of fluorescence signal

The presence of this probe allows to make both a quantitative analysis, because of emission signal is proportional to how much DNA derives from each sample, and a qualitative analysis evaluating the energy emission of one of two allelic-specific probes linking different kind of fluorochromes

(FAM or VIC fluorochrome). During the extension, esonuclease activity of Taq polymerase occurs only when there is a perfect match with the template, improving the analysis specificity: thus, whether Taq polymerase moves away the probe matched with the "wild-type" SNP will be possible to observe a type of signal (i.e. FAM); conversely if the probe links the "mutant variant", the other fluorochrome (i.e. VIC) will release and be detectable from the instrument. For these reasons, RT-PCR appears as a simple and rapid approach to genotype a great number of samples for different SNPs.

The fluorometer is located inside the instrument and reveals the emission signals at a wave length included between 500 and 600 nm ($500 < \lambda < 600$). Subsequently, a software coupled with the instrument elaborates data and develops a clusterization of fluorescent signals in groups corresponding to different genotypes.

Sample preparation

Concerning samples preparation, a protocol previously validated for allelic discrimination was used. Once thawed DNA samples, positive controls ("wild-type homozygous", "heterozygous" and "mutated homozygous") and Master Mix, allelic-specific primers-probes were kept in absence of light.

Mix preparation: Master Mix had already $MgCl_2$, dNTPs and Taq Polymerase collected together in an unique solution. Thus, it needed only a dilution (with ultra-pure water that was changed each week) step and the addition of allelic specific primer-probes. Each SNP had an own appropriate protocol.

Once each mix was ready to use, 96-wells plates were used as base for spiking in every well the mix previously obtained. All of three passages had to occur in sterility conditions.

Then, 2 µl from DNA samples were added to the plate containing every SNP mix.

Afterwards, the plate was covered with an adhesive transparent film and underwent a centrifugation step ("spin") for some seconds.

Finally, the plate was loaded on the instrument for the analysis.

All the SNPs were tested for Hardy-Weinberg equilibrium by the χ^2 test with the OEGE software (Online Encyclopedia for Genetic Epidemiology studies)[102], to determine the observed genotype frequencies: χ^2 indicates the difference among expected and observed values for genotype counts and a $\chi^2 < 3.84$ was considered in Hardy-Weinberg equilibrium.

Linkage Disequilibrium (LD) was evaluated with Haploview 4.2 software (Cambrige, Massachusetts, USA). We measured LD among a pair of SNPs by the statistic D': |D'| of 1 indicates complete LD and 0 corresponds to no LD.

All the PG analysis were performed at Laboratory of Clinical Pharmacology and Pharmacogenetics, Department of Medical Sciences, University of Turin, ASLTO2, Amedeo di Savoia Hospital.

3.3 Microbiological investigations

Patients with pulmonary tuberculosis defined by positive microscopy were included.

Sputum sample were collected at baseline (for microscopy, PCR, culture and DST), at 7 days and 14 days for microscopy, and culture.

TTP on sputum cultures at baseline, 7 and 14 days from the start of antitubercular treatment was calculated using software BD Epicenter integrated in BACTEC MGIT System (Mycobacteria Growth Indication Tube) 960. Default time was 42 days (1032 hours).

For IGRA test, Quantiferon TB Gold® and new version TB Gold plus® were used, following manufacturer's instructions.

Serology for HIV, HBV and HCV was performed in accordance to internal protocols using chemiluminescent immunoassay technology.

All the microbiological investigations (culture on liquid media, DST, TTP, IGRA test, serology testing) were performed at the Laboratory of Microbiology and Virology, ASLTO2, Amedeo di Savoia Hospital, Turin, Italy.

3.4 Blood chemistry tests

At baseline samples for creatinine, urea, liver function (AST, ALT, total bilirubin), TSH_{reflex}, 25-OH vitamin D, albumin, glycated haemoglobin (only for diabetic patients) were collected.

At day 7 and 14 a blood sample for creatinine and ALT was repeated.

All the tests were performed at Laboratory Analysis of Maria Vittoria Hospital and Giovanni Bosco Hospital, ASLTO2, Turin, Italy.

3.5 Scheduled visits

At baseline, a full past and current medical history was collected, weight, height and concomitant treatments were recorded; ECG, chest X ray (or other radiological images, if available) were reviewed.

A full clinical examination was performed and vital signs recorded.

Scheduled visits at day 7 and 14 from the start of TB treatment were performed. Current medical history, full clinical examination and vital signs were recorded. Side-effects, adverse events, tolerability and treatment compliance (hospitalized patients received DOT) were monitored and recorded. In case of withhold of a drug the reason and date were recorded and the patient was dropped-out from the study.

3.6 Statistical analysis

Data were assessed for normality with Shapiro-Wilk test. Descriptive data are presented as median with interquartile range [IQR] for continuous variables, and as numbers and percentages for categorical variables.

AUCs were calculated using Kinetica version 5.1 SP1 (Thermo Fisher Scientific Inc. 2014).

To evaluate possible correlations between continuous data (concentrations, AUCs and TTP), Pearson correlation test was adopted.

Kruskal Wallis test by ranks, the non-parametric version of analysis of variance, was used to test equality of AUC and concentrations medians among polymorphisms of investigated SNPs, while binomial groups were compared exploiting a Mann-Whitney test.

Wilcoxon matched pairs signed rank test was used to test intra-patient variability of results.

Logistic regression was used to assess possible causal effect on the hepatotoxicity or categorized TTP by other independent variables. Effect of each single variable was tested using univariate and multivariable regression models. Multilevel model analysis was used to evaluate the conditional causal effect of some categorical, ordinal and continuous covariates. Bonferroni-adjusted significance level was calculated to account for the increased possibility of type-I error.

All the analyses were performed with STATA software (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP).

4. Results

Patients' characteristics

Between January 2015 and January 2018, 75 subjects were enrolled in TB_intra study.

Fifty-four were males (72%) with median age of 36.1 (25.8-46.3) years old.

Europe was the country of birth for 44 (58.7%) subjects [Romania (24, 54.5%), Italy (18, 41%) and Moldavia (2, 4.5%)], Africa for 23 (30.7%) [Morocco (11, 47.8%), Senegal (4, 17.4%) Mali (1, 4.3%), Cameroun (2, 8.7%), Eritrea (2, 8.7%), Somalia (2, 8.7%), Gambia (1, 4.4%)], Asia for 6 (8%) [Bangladesh (2, 33.3%), Afghanistan (1, 16.7%), Pakistan (1, 16.7%), China (2, 33.3%)] and South America for 2 (2.7%, Peru) subjects. Median baseline BMI was 20.9 (19.1-23.5) available for 74 subjects, median baseline plasma 25-OH vitamin D [normal value (n.v.) >30 ng/mL)] was 12.2 (7.6-

21.4) ng/mL, available for 61 subjects, median baseline albumin (n.v. 35-50 g/L) was 35 g/L (31-40), available for 62 subjects, baseline ALT (n.v. <35 U/L for females and <50 U/L for males) was 16 U/L (11-23), available for 68 patients.

Known comorbidities were present in 15 patients (20%): cardio-vascular diseases (secondary arterial hypertension 3, 4%), Diabetes mellitus type 2 (6, 8%), HCV (1, 1.33%), HBV (2, 2.66%), previous diagnosis of hepatic cirrhosis (3, 4%), chronic pancreatitis (1, 1.33%), haematological malignancies (2, 2.66%), solid malignancies [lung and gastric cancer, 2 (2.66%)]. All the patients at the time of enrolment have normal renal and liver function tests, as *per protocol* inclusion criteria.

Regarding TB disease: IGRA test (see section 3.3 Microbiological investigations) was available in 72 patients, positive in 57 (76%), negative in 13 (17.3%) and indeterminate result in 2 patients (2.67%).

Pulmonary TB was diagnosed in 74 patients (in 8 patients, 10.7%, a concomitant extra-pulmonary localization was found), renal TB in 1 patient (1.33%).

Extra-pulmonary localizations were: lymph nodes (4), gastro-intestinal system (2), larynx (1), major psoas muscle (1), kidney (1), pericardium (1) and central nervous system (1). Two patients had more than 1 extra-pulmonary localization.

Among pulmonary TB patients: 48 (64%) were microscopy sputum positive, 56 (74.7%) were GenExpert® sputum positive and 10 (13.3%) on broncho-alveolar fluid, 58 (77.3) were culture positive for *Mycobacterium tuberculosis* on liquid media. One patient was treated *ad juvantibus* due to pulmonary lesions, enlargement of hilar lymph nodes and presence of pericardial effusion and positive IGRA test.

Sixty-nine (92%) patients were newly diagnosed, 4 patients completed a previous treatment course, 1 patient received isoniazid preventive treatment and 1 patient defaulted on previous treatment (treatment interruption for more than two months).

Patients' demographic characteristics are summarized in Table 4.

Baseline characteristics (N=75)			
Gender [male]		N(%)	54 (72)
Age [years]		Median (IQR)	36.1 (25.8-46.3)
Ethnicity (see text for details)	Caucasian	N(%)	44 (58.7)
	African		23 (30.7)
	Asian		6 (8)
	South-American		2 (2.7)
BMI [Kg/m^2]		Median (IQR)	20.9 (19.1-23.5)
Vitamin D [ng/mL]		Median (IQR)	12.2 (7.6-21.4)
Albumin [g/L]		Median (IQR)	35 (31-40)
ALT [U/L]		Median (IQR)	16 (11-23)
Co-morbidities (see text for details)	Diabetes Mellitus	N(%)	6 (8)
	Others		14 (18.6)
IGRA test [positive]		N(%)	57 (76)
Pulmonary TB		N(%)	74 (98.7)
Sputum microscopy [positive]		N(%)	48 (64)
GenExpert® sputum [positive]		N(%)	56 (74.7)
Sputum culture [positive]		N(%)	58 (77.3)

Table 4 Enrolled patients' characteristics

Three patients were excluded for further analysis due to:

- Confirmation at DST of resistance to INH (1 case) and to INH+RIF (1 case),
- Isolation of non-tuberculous mycobacteria at sputum culture (1 case, *Mycobacterium schimoidei*).

The following analysis were run on 72 patients.

Patients received weight-based doses and median milligrams per Kilogram (mg/kg) received were: 10 (9.5-10.7) mg/kg and 10 (9-10.5) mg/kg for RIF, 4.8 (4.2-5.1) mg/kg and 4.7 (4-5) mg/kg for INH, 24 (21.2-25.3) mg/kg and 22.9 (20.8-25) mg/kg for PZA and 19.8 (17.6-21.7) mg/kg and 19.5 (16.7-20.9) mg/kg for ETB, respectively at week 1 and 2. Non-significant differences were found between doses per Kilogram at the two time-points ($p>0.05$ for all compounds).

Pharmacokinetics results

Plasma and intraPBMCs concentrations and AUC_{24} for every compound at week 1 and week 2 are summarized in Tables 5-8.

AUC_{24} was calculated for patients with more than 3 sampling times (25-29 subjects depending on compound, see Tables 5-8).

Median days from the start of treatment, at week 1 and 2, were 7 (7-9) and 14 (14-16) days, respectively.

In three patients, the clinicians did not include pyrazinamide in the regimen: in two cases the choice was due to concomitant liver cirrhosis (no episodes of decompensation and normal liver function tests at the time of enrolment), in one case the patient was critically ill and unsuitable to receive oral administration. The patients were included in the pharmacokinetic and pharmacogenetic descriptive analysis and excluded from microbiological evaluation.

In 9 patients was not possible to collect PBMCs, due to technical, logistic issues or refused by the patient. In one subject, with concomitant lymphoproliferative disease, the estimation of cells was out of range and was excluded. Two patients withdraw the consent after week 1. Two patients were

enrolled after week 1. One patient due to concomitant investigation was not able to complete the PK sampling. In 43 patients only samples for C_{trough} and 2 hours post-dose were collected because enrolled before October 2016, out-patients or because refusal of the full PK sampling.

	Week 1				
RIF	C_{trough}	C_{2h}	C_{4h}	C_{6h}	AUC_{24}
	ng/mL				ng*h/mL
Plasma	N=66 <LOD	N=70 6873 (4791-8779)	N=25 4131 (3194-7250)	N=28 2789 (1751-4790)	N=28 23992 (20986-3628)
PBMCs	N=61 <LOD (<LOD-147)	N=60 9193 (4245-12997)	N=25 6714 (5427-11601)	N=26 4836 (2088-6973)	N=26 33564 (23059-2407)
$Ratio_{l/P}$	N/A	1.5(0.7-2.2)	1.5 (1.1-2.4)	1.4 (0.8-2.1)	1.3 (0.9-2.2)

Table 5A

	Week 2				
RIF	C_{trough}	C_{2h}	C_{4h}	C_{6h}	AUC_{24}
	ng/mL				ng*h/mL
Plasma	N=60 <LOD	N=63 6859 (4677-9059)	N=24 4891 (3349-7348)	N=29 2556 (1777-4872)	N=27 23645 (19403- 28991)
PBMCs	N=55 <LOD	N=55 9709 (5018-12625)	N=24 9183 (4829-11487)	N=25 3575 (2084-5683)	N=26 34282 (24784-46410)
$Ratio_{l/P}$	N/A	1.3 (0.8-1.8)	1.4 (1.2-2.2)	1.3 (0.8-1.8)	1.3 (0.9-1.8)

Table 5B

	Week 1				
INH	C_{trough}	C_{2h}	C_{4h}	C_{6h}	AUC_{24}
	ng/mL				ng*h/mL
Plasma	N=66 <LOD	N=70 2779 (1553-3953)	N=25 1632 (608-2277)	N=28 1016 (315-1456)	N=27 10212 (4897-12763)
PBMCs	N=61 <LOD	N=60 439 (<LOD-867)	N=25 641 (254-1161)	N=26 266 (<LOD-598)	N=23 4227 (2241-5942)

$Ratio_{l/P}$	N/A	0.2 (<LOD-0.3)	0.4 (0.3-0.9)	0.3 (0.1-0.6)	0.3 (0.2-0.5)

Table 6A

	Week 2				
INH	C_{trough}	C_{2h}	C_{4h}	C_{6h}	AUC_{24}
	ng/mL				ng*h/mL
Plasma	N=60 <LOD	N=63 2952 (1993-3727)	N=24 1604 (1058-2320)	N=29 1024 (650-1398)	N=27 24529 (18269- 33127)
PBMCs	N=55 <LOD	N=55 481 (<LOD-768)	N=24 490 (318-981)	N=25 264 (103-458)	N=24 3025 (1833-4318)
$Ratio_{l/P}$	N/A	0.2 (<LOD-0.3)	0.3 (0.2-0.8)	0.3 (0.05-0.5)	0.3 (0.2-0.5)

Table 6B

	Week 1				
PZA	C_{trough}	C_{2h}	C_{4h}	C_{6h}	AUC_{24}
	ng/mL				ng*h/mL
Plasma	N=64 5813 (4012-7719)	N=67 33316 (26173-42399)	N=23 29870 (25932-34583)	N=26 26477 (24599-29600)	N= 27 161816 (143222-179044)
PBMCs	N=59 498 (178-1011)	N=58 2492 (1197-4876)	N=23 4225 (2965-7276)	N=24 2509 (1669-3882)	N=25 15212 (12501-28897)
$Ratio_{l/P}$	0.08 (0.05-0.15)	0.09 (0.05-0.15)	0.16 (0.09-0.23)	0.09 (0.06-0.17)	0.13 (0.09-0.19)

Table 7A

	Week 2				
PZA	C_{trough}	C_{2h}	C_{4h}	C_{6h}	AUC_{24}
	ng/mL				ng*h/mL
Plasma	N=59 3673 (2236-5389)	N=61 33214 (24432-37183)	N=22 28186 (23173-30152)	N=27 23645 (19403- 28991)	N=26 145097 (121634-160427)
PBMCs	N=53 362 (149-625)	N=53 2470 (1247-3954)	N=22 3692 (2515-4727)	N=23 2266 (1615-3533)	N=24 15829 (11174-23156)
$Ratio_{l/P}$	0.09 (0.05-0.2)	0.07 (0.04-0.12)	0.14 (0.1-0.2)	0.12 (0.09-0.14)	0.13 (0.09-0.15)

Table 7B

	Week 1				
ETB	C_{trough}	C_{2h}	C_{4h}	C_{6h}	AUC_{24}
	ng/mL				ng*h/mL

Plasma	N=66 227 (165-401)	N=70 3859 (2084-6382)	N=25 1340 (1105-1494)	N=28 763 (666-1096)	N=29 8497 (7083-15202)
PBMCs	N=61 6882 (2840-17017)	N=60 38714 (17212-98198)	N=25 25507 (19178-37646)	N=26 17940 (10518-29109)	N=27 123042 (75877-196269)
$Ratio_{I/P}$	26.1 (15.7-55.2)	10.6 (5.9-26.7)	17.6 (13.6-28.6)	24.1 (16.6-40.6)	13.5 (7.8-17.6)

Table 8A

	Week 2				
ETB	C_{trough}	C_{2h}	C_{4h}	C_{6h}	AUC_{24}
	ng/mL				ng*h/mL
Plasma	N=60 271 (178-428)	N=63 3124 (1868-6311)	N=24 1568 (1193-2133)	N=29 985 (703- 1238)	N=27 8465 (6940- 16771)
PBMCs	N=55 6291 (2479-17997)	N=55 31736 (18841-64145)	N=24 27380 (17920-38394)	N=25 17068 (11600-26177)	N=25 112502 (90751-183538)
$Ratio_{I/P}$	30.6 (12.6-54.5)	9.9 (6.1-19.8)	19.1 (13.4-23.8)	19.8 (12.1-30.1)	13.1 (8-17.4)

Table 8B

Tables 5-8 Median concentrations and AUC_{24} (IQRs) in plasma and intraPBMCs at week 1 (A) and 2 (B)
"LOD" limit of detection. LODs for plasma and PBMCs were 117 and 0.976 ng/mL (RIF), 58 and 0.391 ng/mL (INH), 68 and 0.391 ng/mL (PZA), 58 and 2.93 ng/mL (ETB), respectively.

Test of matched pairs for intra-patient analyses at week 1 and 2 showed statistical significant differences of intraPBMCs concentrations for RIF and PZA trough concentrations after oral administration (p=0.03 and p<0.01, respectively), with lower concentrations at week 2; for ETB 6 hours post-dose (iv administration at week 1 and oral administration at week 2, p=0.04), with higher concentrations at week 2; for INH AUC_{0-24} for oral administration (p=0.01) with higher concentrations at week 2. Regarding plasma concentrations, the only differences were found for PZA with lower concentrations at week 2 [AUC_{0-24} (p=0.01), trough, 2, 4 (p<0.01) and 6 hours post-dose concentrations (p=0.02)].

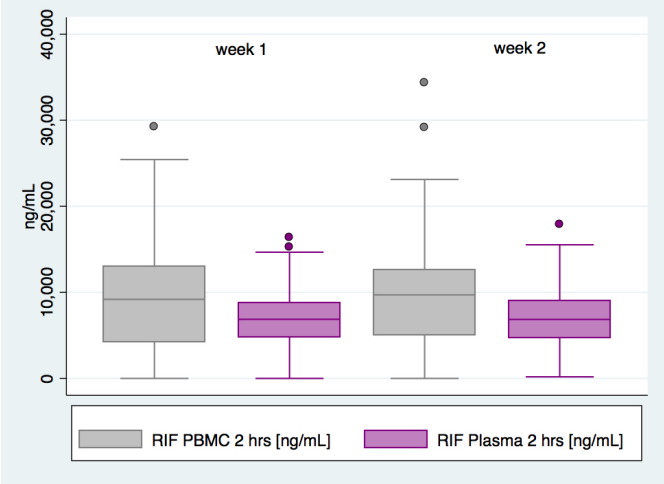


Figure 2 RIF 2hrs intraPBMCs and plasma concentrations at week 1 and week 2

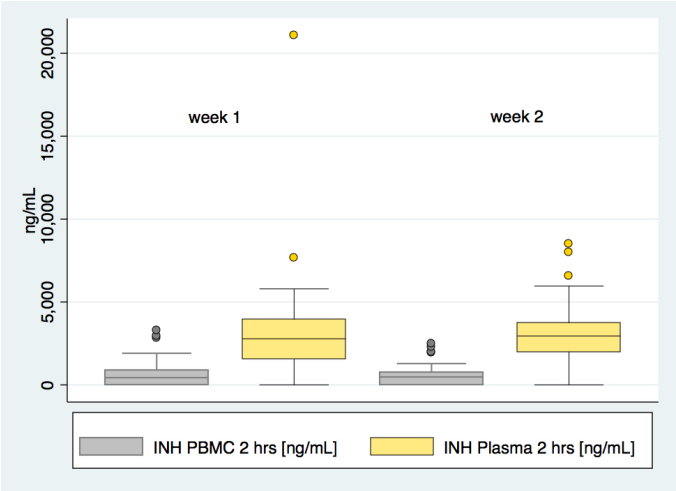


Figure 3 INH 2hrs intraPBMCs and plasma concentrations at week 1 and week 2

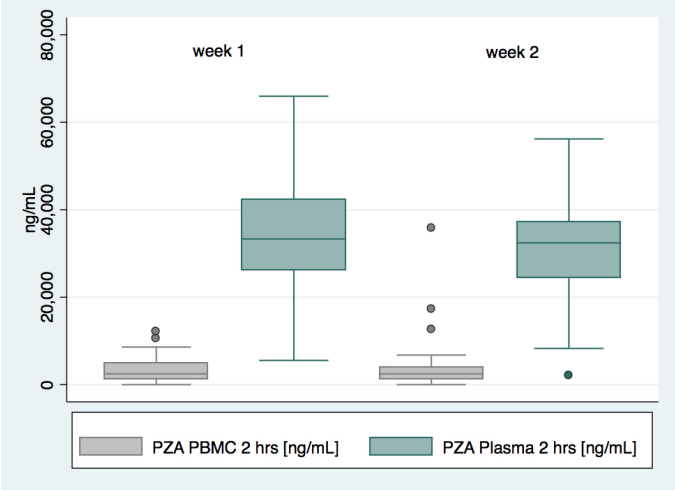


Figure 4 PZA 2hrs intraPBMCs and plasma concentrations at week 1 and week 2

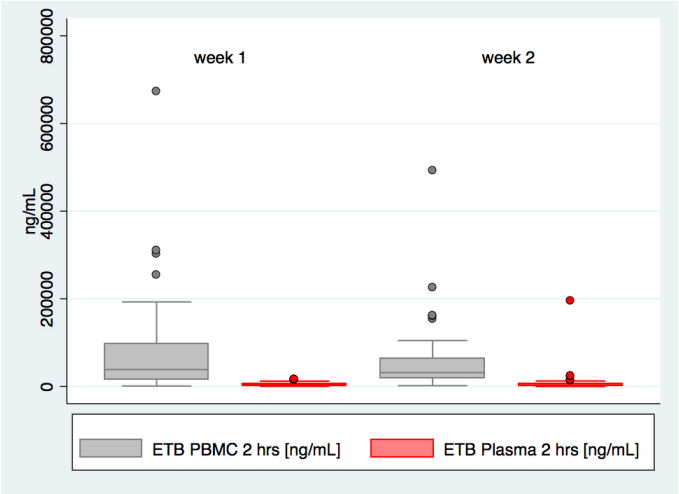


Figure 5 ETB 2hrs intraPBMCs and plasma concentrations at week 1 and week 2

At week 1 correlations were found between intraPBMCs and plasma trough concentrations for ETB, (r=0.6, p<0.01) and PZA (r=0.4, p>0.01); at 2 hours post-dose for RIF (r=0.5, p<0.01), INH (r=0.3, p<0.01) and ETB (r=0.5, p<0.01); at 4 hours post-dose for INH (r=0.7, p<0.01) and PZA (r=0.5, p=0.02); at 6 hours post-dose for RIF (r=0.4, p=0.03) and ETB (r=0.5, p<0.01); moreover correlations between intraPBMCs and plasma AUC_{24} were found for RIF (r=0.5, p=0.01), INH (r=0.5, p=0.02), ETB (r=0.5, p<0.01) and PZA (r=0.4, p=0.05).

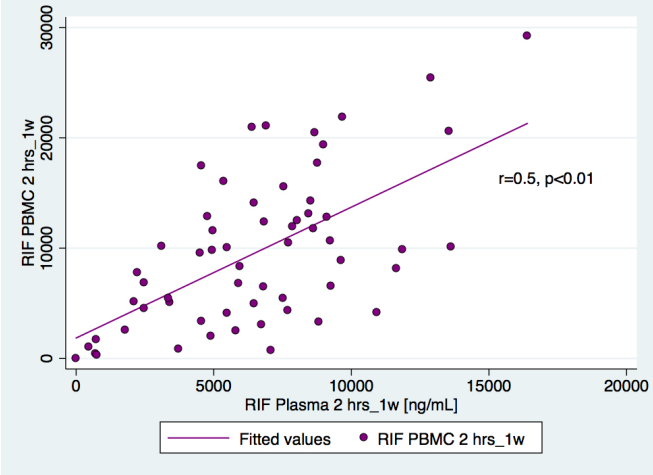


Figure 6 Correlation between plasma and intraPBMCs RIF 2hrs concentrations at week 1

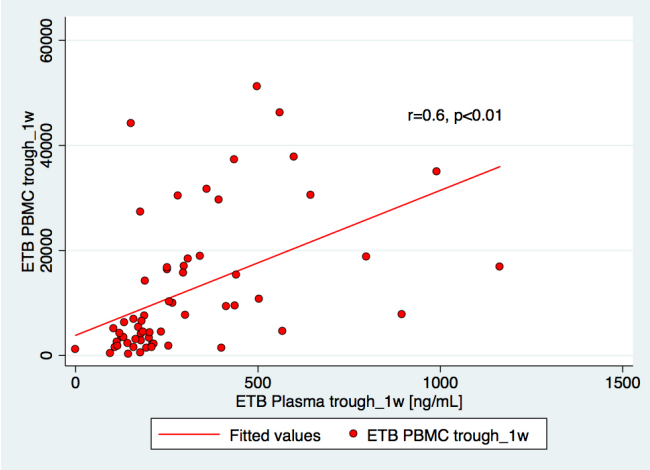


Figure 7 Correlation between plasma and intraPBMCs ETB trough concentrations at week 1

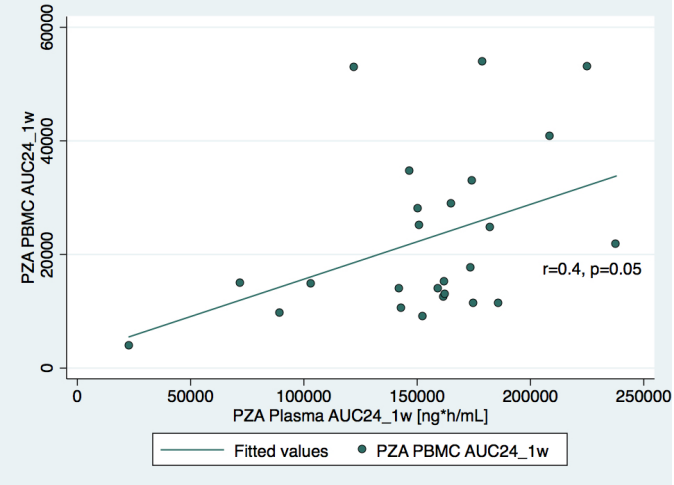


Figure 8 Correlation between plasma and intraPBMCs PZA AUC_{24} at week 1

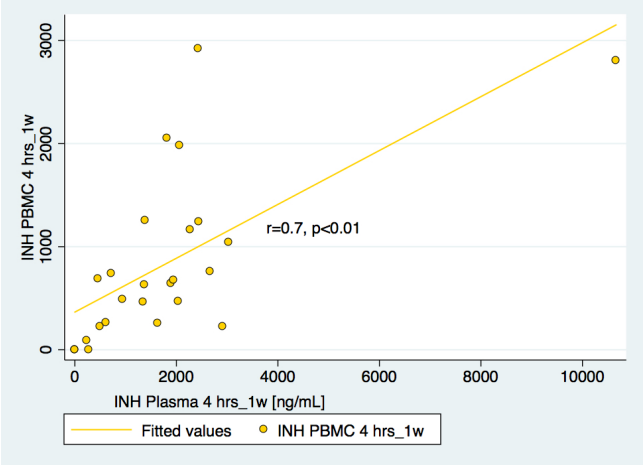


Figure 9 Correlation between plasma and intraPBMCs INH 4hrs concentrations at week 1

At week 2 correlations were found between intraPBMCs and plasma trough concentrations for RIF (*r*=0.4, p<0.01), ETB (*r*=0.6, p<0.01) and PZA (*r*=0.5, p<0.01); at 2 hours post-dose for RIF (*r*=0.6, p<0.01) and ETB (*r*=0.6, p<0.01); at 4 hours post-dose for RIF (*r*=0.5, p=0.02) and PZA (*r*=0.5, p=0.02); at 6 hours post-dose for RIF (*r*=0.7, p<0.01), ETB (*r*=0.5, p=0.02) and PZA (*r*=0.4, p=0.04).

Correlations between intraPBMCs and plasma AUC_{24} were found for RIF (*r*=0.5, p<0.01).

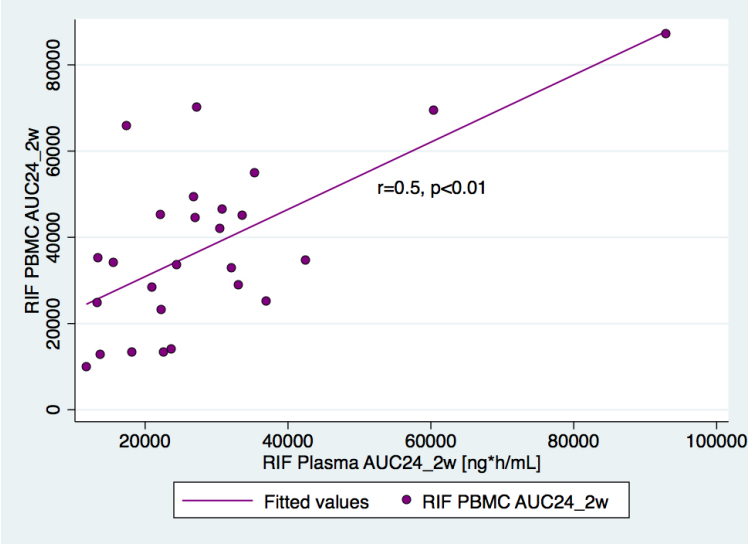


Figure 10 Correlation between plasma and intraPBMCs RIF AUC_{24} at week 2

At week 1, 4 hours post-dose ETB intraPBMCs concentrations correlated with INH (r=0.6, p<0.01) and PZA (r=0.65, p<0.01) intraPBMCs concentrations; ETB plasma concentrations correlated as well with RIF plasma concentration (r=0.53, p<0.01). ETB intraPBMCs AUC_{24} was found to correlate with INH (r=0.64, p<0.01) and PZA (r=0.63, p<0.01) intraPBMCs AUC_{24}. The latter two were found to correlate, as well (r=0.51, p=0.02).

At week 2 ETB intraPBMCs AUC_{24} correlated with plasma INH (r=0.5, p=0.02). RIF plasma AUC_{24} was correlated with PZA intraPBMCs AUC_{24} (r=0.5, p=0.02).

Other correlations were found with positive or negative coefficient of correlation (r) below 0.5 and, thus, not reported.

According to recommend plasma ranges for maximal concentrations: RIF was below 8000 ng/mL in 44 (63%) and 39 (62%) patients; INH was below 3000 ng/mL in 39 (56%) and 35 (55%) patients; PZA was below 20000 ng/mL in 7 (10.5%) and 6 (9.8%) patients; ETB was below 2000 ng/mL in 15 (21.4%) and 15 (24%) patients, at week 1 and 2 respectively.

Overall 83 (62.4%), 50 (37.6), 0 and 74 (55.7%), 54 (40.6), 5 (3.7) of samples were below, in range and above the range for RIF and INH, respectively (see Figure 11 for all compounds).

Thirty-five (51%) and 33 (53%) patients had at least two drugs under the recommended plasma range at week 1 and 2, respectively.

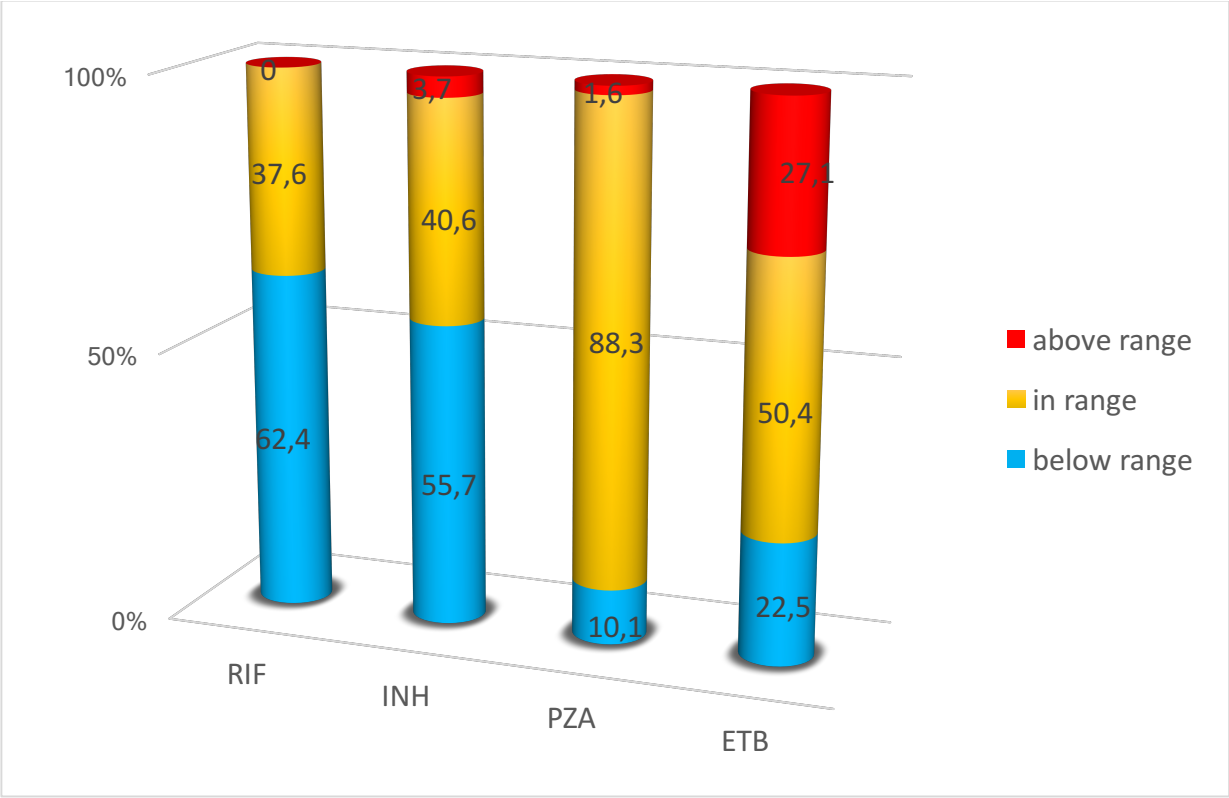


Figure 11 Maximal plasma concentrations according to reference ranges (Peloquin *et al*)[15]

Pharmacokinetics and Time To Positivity

TTP from sputum liquid culture was available at baseline and at week 1 for 48 patients, the median time was 191 (149-245) hours and 329 (264-567) hours, respectively. At week 1, 10 patients culture converted (1032 hours as default time).

The difference between TTP at week 1 and baseline was defined as DeltaTTP, available for 41 patients, was 161 (87-312) hours.

At week 2, TTP was available in 47 patients and the median time was 362 (272-619) hours, taking into account that 8 patients culture converted (1032 hours as default time). DeltaTTP between week 2 and baseline, available for 41 patients, was 187 (113-289) hours and 27.5 (0-68) hours between week 2 and week 1, available for 42 patients.

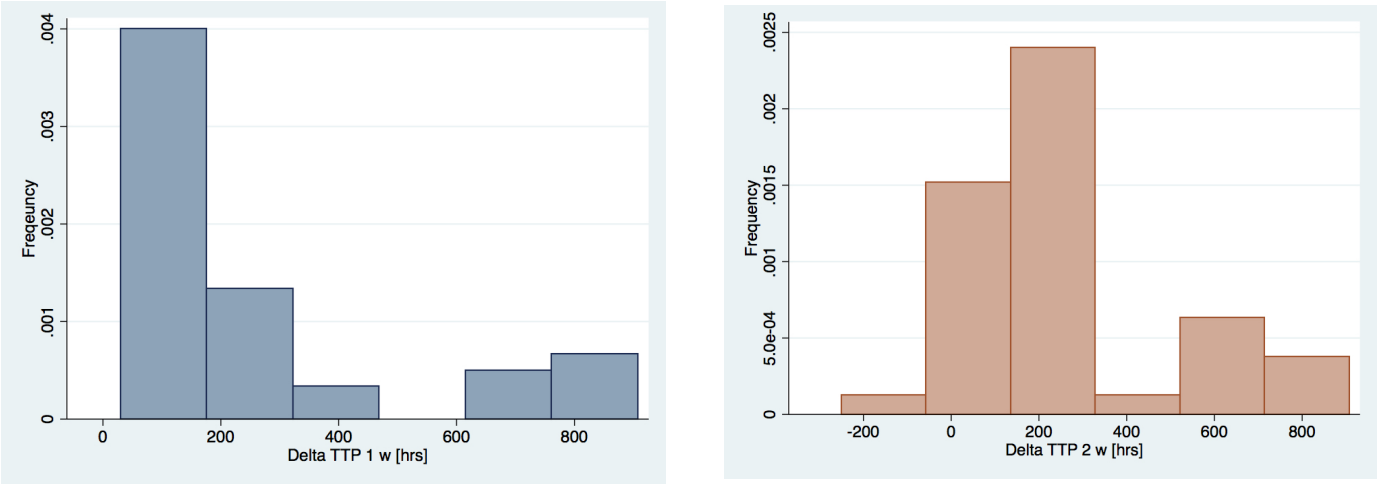


Figure 12 Distribution of frequencies of DeltaTTP at week 1 (left) and week 2 (right)

The differences between TTP at baseline, week 1 and week 2 were statistically significant (p<0.01).

At week 1, 2 hours post-dose PZA intraPBMCs concentrations correlated with DeltaTTP (*r*=0.35, p=0.03).

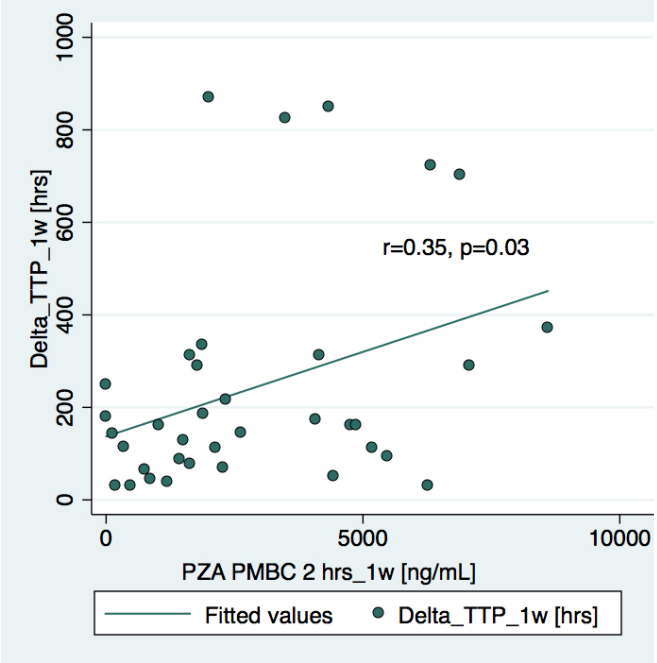


Figure 13 Correlation between Delta TTP at week 1 and PZA 2 hours post-dose intraPBMCs concentrations

No other correlations were found between concentrations, including AUCs (available data for 14 and 12 patients at week 1 and 2, respectively), and Time To Positivity at week 1 or week 2.

A trend was noticed for age and baseline 25-OH vitamin D level with DeltaTTP at week 1: younger patients and higher plasma 25-OH vitamin D levels were correlated with DeltaTTP above 500 hours (p=0.07 and p=0.1, respectively). Moreover, patients receiving vitamin D supplementation at week 1 were correlated with DeltaTTP below 500 hours (p=0.08).

A score (0 to 4) was generated according to the number of drugs above the recommended plasma ranges at week 1 and 2. At week 2, a significant higher DeltaTTP was found in patients with 2 or 3 drugs above the range (p=0.03). Patients with score of 4 (all 4 drugs above the ranges) were 3 and merging this group with patients with 2/3 drugs above the range (≥2 drugs above the range), the trend persisted but not statistical significant (p=0.8).

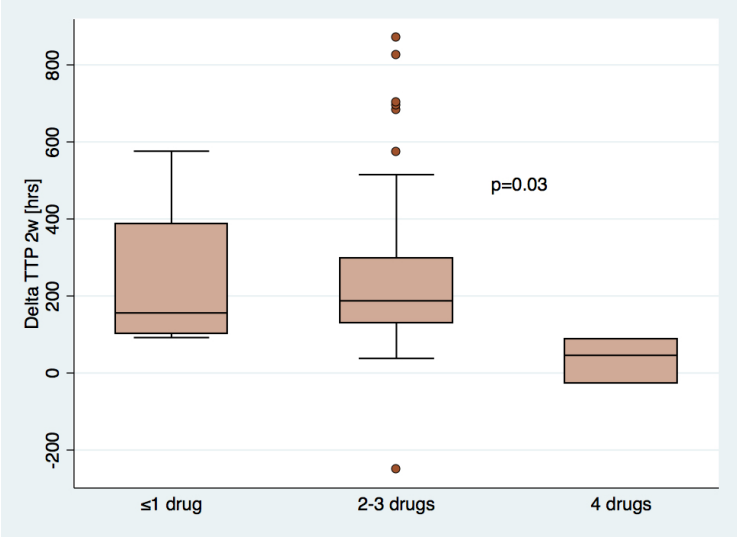


Figure 14 DeltaTTP according to drugs above the range at week 2.

Patients with both RIF and PZA above the recommend ranges showed a trend to higher DeltaTTP at week 1 (217 vs 152 hours, p=0.06).

Pharmacokinetics and Hepatotoxicity

During the first two weeks of treatment no serious event of hepatotoxicity was recorded.

Generally, the treatment was well tolerated apart from transient, self-limiting nausea in few cases (6 cases).

We, then, investigated the increase of transaminases above the normal value (n.v <35 U/L for females, <50 U/L for males).

At week 1, ALT increase was more frequent in patients with 2 hours post-dose plasma INH concentrations above 5000 ng/mL (p<0.01).

The logistic regression model showed INH concentrations above 5000 ng/mL as causal factor (p=0.02) for an increase in ALT value at week 1, adjusting for age and for *NAT2* genotype (Table 9).

Logistic regression — Number of obs = 71; LR chi2(6) = 11.59; Prob > chi2 = 0.0719; Log likelihood = -38.923579; Pseudo R2 = 0.1295

ALT_above_1w	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
P_inh1w_over5000	7.077221	5.776288	2.40	0.017	1.429318	35.0426
agecat						
1	1.593407	1.185757	0.63	0.531	.3705892	6.85110
2	.5500977	.487683	-0.67	0.500	.0967869	3.12653
3	.6566487	.5462811	-0.51	0.613	.1285876	3.3532
NAT2						
1	.8063025	.5175129	-0.34	0.737	.2291751	2.83679
2	2.053967	2.422091	0.61	0.542	.2036266	20.7182
99	1	(empty)				
_cons	.1196464	.1060381	-2.40	0.017	.0210626	.679653

Table 9 Logistic regression results of hepatotoxicity and INH concentrations adjusting for age (categorized) and *NAT2*

Pharmacogenetic results

The prevalence of genetic variants is shown in Table 10. In one patient, the sample was not available and the analyses were conducted on 71 patients.

All the SNPs were in Hardy-Weinberg equilibrium. Linkage disequilibrium (LD) analyses are shown in Figure 15. The extent of LD decreases in proportion to the number of generations since the LD-generating event; an alternative explanation is that the recombination rates in the studied regions might be markedly less than the genome-wide average.[103]

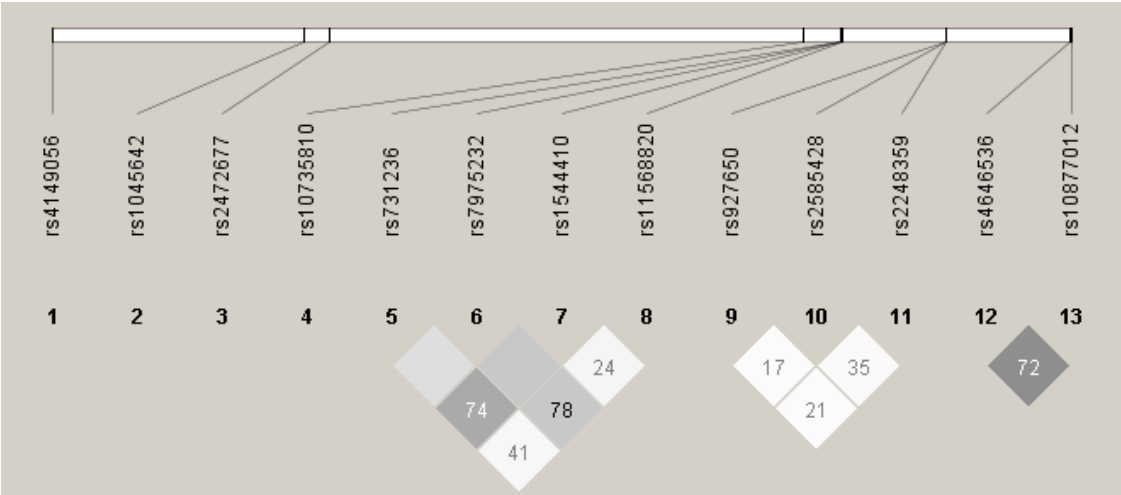


Figure 15. Pairwise linkage disequilibrium (LD) calculated with HaploView 4.2 software using the D' statistic for SLCO1B1 521 rs4149056 T>C, ABCB1 3435 rs1045642 C>T, PXR 63396 rs2472677 C>T, FokI rs10735810 T>C, TaqI rs731236 T>C, ApaI rs7975232 C>A, BsmI rs1544410 G>A, Cdx2 rs11568820 A>G, CYP24A1 +22776 rs927650 A>G, CYP24A1 +8620 rs2585428 T>C, CYP24A1 +3999 rs2248359 C>T, CYP27B1 +2838 rs4646536 C>T, CYP27B1 -1260 rs10877012 G>T gene SNPs

The panel shows pairwise LD of the studied population among the eleven studied SNPs. Each value in the box represents r^2. Dark grey, regions with high r^2 values; light grey, regions with low r^2 values. Intensity of shading indicates the degree of confidence in the r^2 value and numbers in blocks denote r^2 values. Dark filled squares indicate a r^2 value of 1 with LOD (logarithm of odds) < 2.0. r^2 was calculated as follows: r^2 = r scales D by the standard deviation of the allele frequencies at two loci, where statistic D is the range of values regardless of the frequencies of the SNPs compared. LOD was defined as $\log_{10}$(L1/L0), where L1 = likelihood of the data under LD, and L0 = likelihood of the data under LD. The physical position of each SNP is shown in the figure. *NAT2* rs1799930 G>A is not shown.

Gene	*ABCB1*	*SLCO1B1*	*NR1I2*	*NAT2*
	ABCB1 3435 C>T	*SLCO1B1* 521 T>C	*NR1I2* 63396 C>T	*NAT2* G>A
	rs1045642	rs4149056	rs2472677	rs1799930
	P-gp	OATP1B1	PXR	NAT2
WT N(%)	23 (31.9)	53 (73.6)	14 (19.4)	41 (56.9)
HET N(%)	35 (48.6)	14 (19.4)	37 (51.4)	26 (36.1)
MUT N(%)	13 (18)	4 (5.6)	20 (27.8)	4 (5.6)
N/A	1 (1.4)	1 (1.4)	1 (1.4)	1 (1.4)
Tot	72 (100)	72 (100)	72 (100)	72 (100)

Gene	*VDR*	*VDR*	*VDR*	*VDR*
	TaqI T>C	*ApaI* C>A	*BsmI* G>A	*FokI* T>C
	rs731236	rs7975232	rs1544410	rs10735810
	VDR	VDR	VDR	VDR
WT N(%)	23 (31.9)	13 (18)	30 (41.7)	7 (9.7)
HET N(%)	35 (48.6)	28 (38.9)	41 (56.9)	27 (37.5)
MUT N(%)	13 (18)	30 (41.7)	0	37 (51.4)
N/A	1 (1.4)	1 (1.4)	1 (1.4)	1 (1.4)
Tot	72 (100)	72 (100)	72 (100)	72 (100)

Gene	*VDR*	*CYP24A1*	*CYP24A1*	*CYP24A1*
	VDR Cdx2 A>G	*CYP24A1* +22776 C>T	*CYP24A1* +3999 T>C	*CYP24A1* +8620 A>G
	rs11568820	rs927650	rs2248359	rs2585428
	VDR	CYP24A1	CYP24A1	CYP24A1
WT N(%)	11 (15.3)	21(29.2)	15 (20.8)	12 (16.7)
HET N(%)	31 (43)	35 (48.6)	35 (48.6)	21 (29.1)
MUT N(%)	29 (40.3)	15 (20.8)	21 (29.2)	38 (52.8)
N/A	1 (1.4)	1 (1.4)	1 (1.4)	1 (1.4)
Tot	72 (100)	72 (100)	72 (100)	72 (100)

Gene	*CYP27B1*	*CYP27B1*
	CYP27B1 +2838 C>T	*CYP27B1* -1260 G>T
	rs4646536	rs10877012
	CYP536	CYP012
WT N(%)	16 (22.2)	36 (50)
HET N(%)	19 (26.4)	24 (33.3)
MUT N(%)	36 (50)	11 (15.3)
N/A	1 (1.4)	1 (1.4)
Tot	72 (100)	72 (100)

Table 10 Analysed SNPs characteristics: gene, variant name (SNP name), alleles, *rs* number, coded protein, observed frequencies; WT, wild type, HET heterozygous, MUT homozygous for the mutation

ABCB1 3435 C>T

At week 1 significant association was found between homozygous patients for mutant variant of *ABCB1* and RIF intraPBMCs AUC_{24} with lower AUC_{24} (25148 vs 55820 ng*h/mL, p=0.04). Same association was found with 2 hours post-dose plasma RIF concentrations (4764 vs 8722 ng/mL p=0.02).

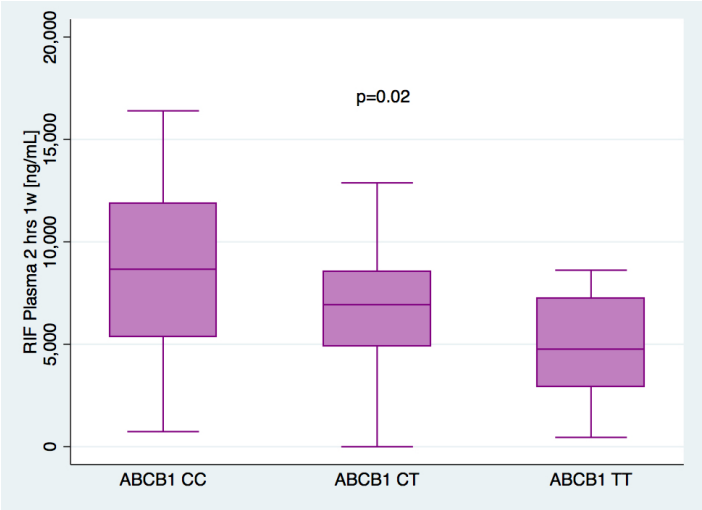


Figure 16 RIF 2hrs plasma concentrations according to ABCB1 3435 genotype at week 1

At week 2, PZA plasma AUC_{24} was lower in patients harbouring the mutant variant (136232 vs 156844 ng*h/mL, p=0.04). This was shown also by the lower punctual concentrations at 4 hours post-dose in patients homozygous for the mutant variant (28774 vs 37073 ng/mL, p=0.04) and in higher intraPBMCs concentrations (4637 vs 3372 ng/mL, p=0.05).

At week 2, ETB intraPBMCs AUC_{24} were higher in patients harbouring the mutant variant (128857 vs 69905 ng*h/mL, p=0.02), at week 1 the same trend was noted (138541 vs 81581 ng*h/mL, p=0.059).

For INH no correlations were found.

SLCO1B1 521 T>C

At week 2, RIF intraPBMCs 2 hours post-dose concentrations were lower in patients with the investigated mutant SNP (6998 vs 10396 ng/mL, p=0.01).

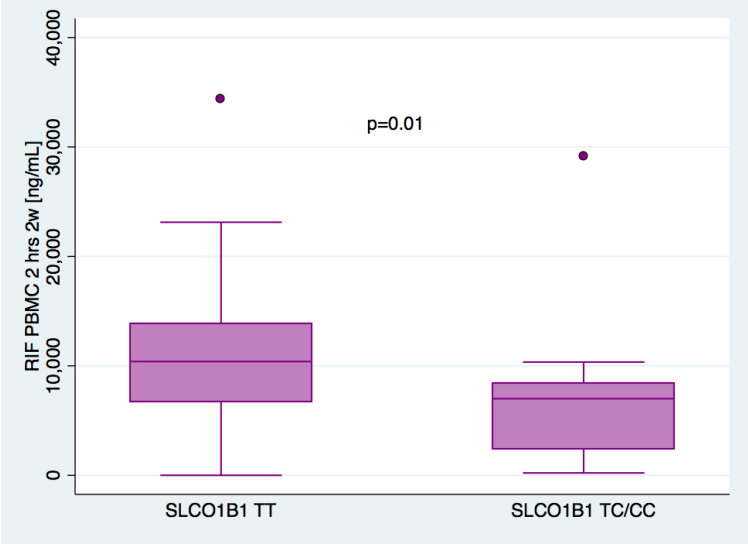


Figure 17 RIF 2hrs PBMCs concentrations according to SLCO1B1 521 genotype at week 2

For INH, ETB and PZA no correlations were found.

NR1I2 63396 C>T

At week 1, RIF intraPBMCs AUC_{24} were lower in homozygous patients for the mutant variant (49435 vs 27024 ng*h/mL, p=0.02). For INH, PZA and ETB no significant correlations were found.

NAT2 G>A

Plasma and intraPBMCs INH AUC_{24} were higher in patients harbouring the mutant variant (11864 vs 8613 ng*h/mL, p=0.056, not statistically significant; 5942 vs 2732 ng*h/mL, p=0.04) at week 1.

No other correlations were found with PK of other compounds.

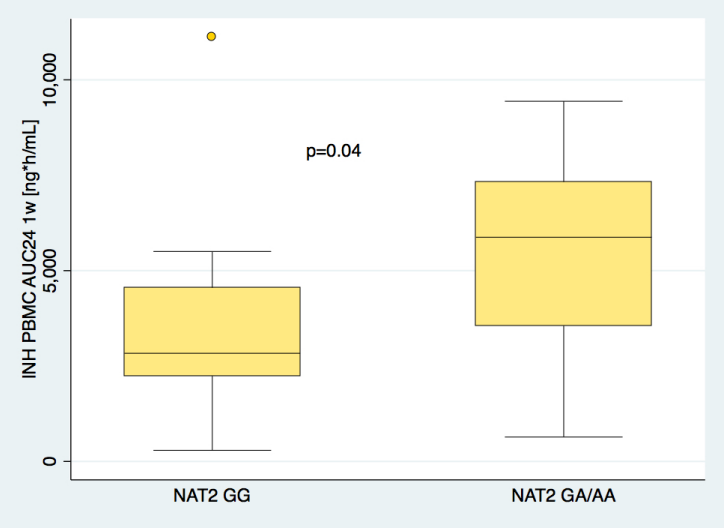


Figure 18 INH PBMCs AUC_{24} concentrations according to *NAT2* genotype at week 1

VDR (TaqI T>C, ApaI C>A, BsmI G>A, FokI T>C and Cdx2 A>G)

No correlations were found between VDR SNPs and concentrations of four drugs.

CYP24A1 (CYP24A1 +22776 C>T, CYP24A1 +3999 T>C and CYP24A1 +8620 A>G)

Higher RIF 2 hours post-dose concentrations were recorded in patients with mutant variant for SNP *CYP24A1 +22776* both in plasma and intraPBMCs (7194 vs 5949 ng/mL, p=0.03 and 9900 vs 4601 ng/mL, p=0.01, respectively) at week 2.

RIF intraPBMCs AUC_{24} and 4 hours post-dose concentrations resulted lower in patients with homozygous mutation for SNP *CYP24A1 +3999* (20294 vs 51446 ng*h/mL, p=0.01 and 3234 vs 11047 ng/mL, p=0.03) at week 1.

Plasma PZA AUC_{24} (at week 2), 4 hours post-dose concentrations (at week 1 and 2) and intraPBMCs AUC_{24} (at week 1, in homozygosis) were lower in presence of the mutant variant *CYP24A1 +3999* (138344 vs 182755 ng*h/mL, p<0.01; 29480 vs 41286 ng/mL, p=0.02; 26914 vs 34494 ng/mL, p<0.01 and 10958 vs 31268 ng*h/mL, p=0.03, respectively).

Plasma ETB 4 hours post-dose concentrations at week 1 were lower in patients with homozygous mutant variant *CYP24A1 +3999* (1069 vs 2475 ng/mL, p=0.04).

For INH no correlations were found.

CYP27B1 (CYP27B1 +2838 C>T and CYP27B1 -1260 G>T)

At week 2, plasma INH 2 hours post-dose concentrations were higher in patients harbouring the mutant variant of *CYP27B1 +2838* (3983 vs 2168 ng/mL, p<0.01).

At week 2, plasma ETB AUC_{24}, 4 and 6 hours post-dose concentrations were higher in patients with the mutant variant *CYP27B1 -1260* (16357 vs 7626 ng*h/mL, p=0.01; 1706 vs 1243 ng/mL, p=0.03 and 1326 vs 732 ng/mL, p<0.01).

For RIF and PZA no correlations were found.

Pharmacogenetics and Time To Positivity

At week 2, a shorter DeltaTTP of sputum culture was calculated in patients with mutant variant of SNP *VDR Cdx2* (151 vs 599 hours, p<0.01), the result was confirmed at multivariate logistic regression (DeltaTTP above or below 500 hours) adjusting for 25 OH vitamin D levels (p=0.01).

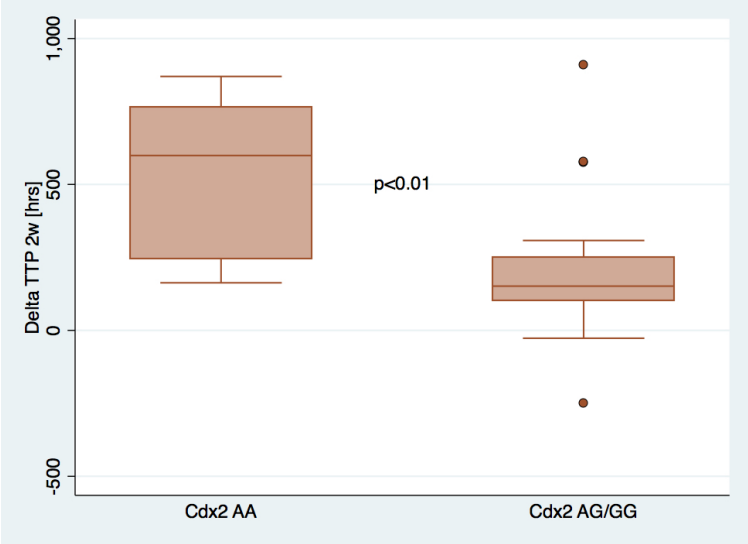


Figure 19 Delta TTP according to *VDR Cdx2* genotype at week 2

Patients harbouring mutant SNP *VDR ApaI* showed a shorter Delta TTP at week 2 (p=0.04); *CYP24A1 +22776* had higher probability to have shorter TTP at week 2 (over 500 hours vs below 500 hours,

p<0.01), a causal relationship was confirmed at logistic regression for both associations (p=0.01 and 0.02, respectively).

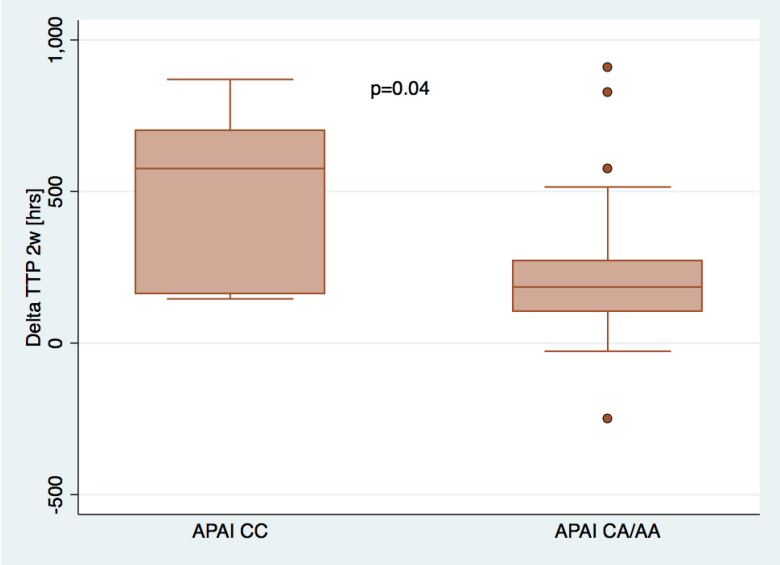


Figure 20 Delta TTP according to *VDR ApaI* genotype at week 2

Using a multilevel model (Table 11), having three drugs above the recommended ranges and mutant variant of SNP *SLCO1B1 521* showed an increase of DeltaTTP at week 2 (p=0.01 and p=0.03) statistically higher than having the other cofactors, whereas the presence of mutant variant for *NR1I2 63396* was associated with a decrease in DeltaTTP (p=0.02).

DeltaTTP2_bl	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
drugs_above_2w						
1	300.2278	197.3649	1.52	0.128	-86.60027	687.0559
2	246.8286	153.7572	1.61	0.108	-54.52994	548.1871
3	386.113	158.3074	2.44	0.015	75.83621	696.3897
4	164.1092	200.3307	0.82	0.413	-228.5318	556.7502
age	-.996621	2.235094	-0.45	0.656	-5.377326	3.384083
OATP_01	182.8621	83.13722	2.20	0.028	19.91619	345.8081
PXR_01	-217.809	92.95366	-2.34	0.019	-399.9949	-35.62322
_cons	143.6357	153.6948	0.93	0.350	-157.6006	444.872
/sigma_u	0	(omitted)				
/sigma_e	194.4015	22.29545			155.2663	243.4007
rho	0	(omitted)				

Likelihood-ratio test of sigma_u=0: chibar2(01)= 0.00 Prob>=chibar2 = 1.000

Table 11 Multilevel model results for Delta TTP and drugs above the range at week 2, age, *SLCO1B1 521* and *NR1I2 63396* genotypes

Pharmacogenetics and hepatotoxicity

At week 1, the mutant variant *ABCB1 3435* was more frequent in patients with normal ALT comparing to patients with ALT increase (p=0.01).

Bonferroni-adjusted significance level of 0.003 was calculated to account for the increased possibility of type-I error. With this correction, the following results were observed: plasma PZA AUC_{24} and 4 hours post-dose concentrations at week 2 resulted lower in presence of the mutant variant *CYP24A1 +3999;* at week 2, plasma INH 2 hours post-dose concentrations were higher in patients harbouring the mutant variant of *CYP27B1 +2838*; at week 2, plasma ETB 6 hours post-dose concentrations were higher in patients with the mutant variant *CYP27B1 -1260;* a shorter DeltaTTP of sputum culture was recorded at week 2 in patients with mutant variant of SNP *VDR Cdx2* and *CYP24A1 +22776* had higher probability to have shorter TTP at week 2.

5. Discussion

We here describe first-line antitubercular drugs concentrations achieved inside PBMCs in patients with *Mycobacterium tuberculosis* infection.

As far as our knowledge, this is the largest cohort of patients with description of intracellular concentrations and AUC_{24} with related microbiological, pharmacogenetic and safety data.

We confirm high concentrations achieved by ethambutol and rifampicin as opposed to isoniazid and pyrazinamide, consistent with our previous published report.[28]

Rifampicin accumulation inside PBMCs was slightly lower comparing to previous observation [intracellular:plasma ratio of 1.5 (1 week) and 1.3 (week 2) versus 1.8)] by Hartkoorn *et al.*[32]

The potential role of very high intracellular ethambutol accumulation warrants evaluation in prospective studies. Data on ethambutol intracellular penetration in animal models were published in the past, but the role in clinical setting has not yet been investigated.[104]

In our previous report from Baietto *et al.*[100] we described the development and validation of a new chromatographic method to simultaneously quantify first-line antituberculars in plasma and PBMCs. This method was used for this cohort of patients, it performed well and is confirmed to be a practical tool for monitoring drugs' exposure.

The belief that first-line drugs have relatively predictable pharmacokinetics is likely unjustified. Indeed, high between-patient pharmacokinetic variability has been a consistent finding in prior studies.[66,93,105]

The reasons for this variability are many but, according to available data, could include pharmacogenetic factors, drug formulation, quality of tablets, patient weight, age, gender, adherence patterns, and comorbid conditions such as AIDS.

In our patients, wide inter-patient variability was observed, both for plasma and for intraPBMCs concentrations, and this finding highlights the need of drug monitoring during the TB treatment course for patients, because achieved concentrations are hardly predicted by dose received.

Moreover, intra-patient pharmacokinetic data showed significant differences between week 1 and 2 with lower pyrazinamide (plasma and intraPBMCs) and rifampicin (intraPBMCs) concentrations and higher isoniazid and ethambutol intraPBMCs concentrations at week 2. The effect of induction and autoinduction of rifampicin on these results is unclear, as we didn't measure the induction of cytochrome P450, but this is a worthwhile topic to be addressed in future.[106]

Even if a high inter- and intra-patient variability was noted, the observed correlations between PBMCs and plasma concentrations (among others, AUC_{24} of rifampicin, isoniazid, ethambutol and pyrazinamide at week 1 and rifampicin at week 2, see section 4. Results) may support the use of

plasma as predictive compartment for assessing drug concentrations adequacy. Furthermore, the method of extraction from plasma results less expensive, and less time-consuming than intraPBMCs one, and this needs to be taken into account when considering implementation.

As several analyses reported, a high proportion of patients showed maximal plasma concentrations below the proposed targets[16]. More than half cases presented rifampicin and isoniazid 2 hours post-dose concentrations below 8000 ng/mL and 3000 ng/ml (62.4% and 55.7%), respectively. Generally, 2 hours post-dose are considered peak concentration [19] and this result is consistent with our previous published.[107]

Several case report series[16,108,109], using predetermined peak concentration drug cut-off values classified patients as having either low or high drug concentrations. The peak concentrations recommended are: 8000–24000 ng/mL for rifampicin, 3000–5000 ng/mL for isoniazid, and 20000–50000 ng/mL for pyrazinamide.[15] These concentrations ranges were used to adjust regimen in special populations, as diabetics, delayed responders and HIV positive patients[17,108,110], with good results in terms of clinical outcomes; other authors used AUC_{24} as drug AUCs are strongly associated with efficacy of first-line anti-tuberculosis agents in preclinical models.[31,111] A more intensive multi-sample schedule that allows AUC identification may be more informative. Pasipanodya found that low drug AUCs were predictive of clinical outcome. The top predictor of poor long-term outcome (defined by the author as failure, relapse or death up to 2 years), by rank of importance, were a pyrazinamide AUC_{24}≤363mg*h/L, rifampicin AUC_{24}≤13mg*h/L, and isoniazid AUC_{24}≤52 mg*h/L.[112]

In our study, we collected samples to measure trough and peak concentrations from January 2015 to October 2016, then, considering our preliminary data, we performed intensive samplings to calculate AUC_{24}.

The main result from a PK/PD point of view was that having two or three drugs above the recommended ranges at week 2 was a predictive factor of higher DeltaTTP (p=0.03), this data was better defined with a multilevel model including pharmacogenetics data. No associations were found with AUC_{24}, probably due to the small sample size. In addition, a trend of synergistic effect between rifampicin and pyrazinamide at week 1 was noted. Patients with both rifampicin and pyrazinamide over the recommended ranges presented a higher DeltaTTP (217 vs 152 hours, p=0.06). This highlights the central role of these drugs and their concentration-dependent interactions in determining the activity of the regimen. The finding is consistent with Chigutsa and colleagues, they observed that rifampicin maximal concentration above 8.2 mg/L and pyrazinamide AUC/MIC above 11.3 were key predictors of the sterilizing effect of drugs over the decline in sputum bacillary burden, and interacted positively. The authors found that this effect decreased when increasing isoniazid maximal concentration, in patients with low rifampicin concentrations. In our cohort, this antagonist effect of isoniazid was not noted, nonetheless the mutual interaction among drugs needs further evaluations.[13]

The interesting associations between Time To Positivity and drugs concentrations we observed, are encouraging results to support the use of therapeutic drug monitoring for optimizing the antitubercular drug pharmacokinetic profile.

Moving from drug exposure to individual-level other factors can have a role in predicting microbiological response.

Heterogeneous results were published in the last years regarding vitamin D supplementation and *VDR* gene polymorphisms.

Radiological improvement and sputum conversion were more frequent in patients receiving vitamin D according to Wijaya Nursyam *et al.*[38] Babb *et al.* showed that *ApaI* AA genotype and

TaqI T containing genotype were predictive of faster response to TB therapy.[39] In a double-blind randomised controlled trial, adjunctive vitamin D significantly hasten sputum culture conversion in patients with *Taqi* TT genotype.[40]

In our population, shorter DeltaTTP was observed at week 2 in subjects harbouring the mutant variant for *Cdx2* and *ApaI*, the latter in disagreement with the results from Babb *et al.*[39]

Furthermore, we noted a trend of higher DeltaTTP in younger patient with higher vitamin D levels, independent from *VDR* genotype at week 1. Patients receiving vitamin supplementation for one week had lower DeltaTTP, this observation probably is due to the fact that the effect of vitamin D supplementation is not yet complete and lower levels are correlated with lower DeltaTTP, further confirmation is needed on larger samples.

Other genetic assets in relation both to drug exposure and microbiological response were investigated in our analysis.

ABCB1 gene is expressed in many tissues and encodes P-glycoprotein transporter, which removes chemical toxins and metabolites from cells into bile, urine and intestinal lumen. Alterations in P-glycoprotein function may affect bioavailability, distribution and clearance of many drugs. *ABCB1* 3435, a synonymous SNP in exon 26 has been associated with reduced functionality.[113,114] This silent SNP could alter substrate specificity of the transporter, influencing protein folding and function.[115] We observed a positive trend in CT/TT genotype patients, both at week 1 and 2 in intraPBMC AUC_{24} for ethambutol and a negative trend for rifampicin (both in plasma and intraPBMCs). The former result confirms the reduced activity of the efflux pump already reported [113,114], and consistent with our previous report.[95] The latter diverges from Weiner *et al.* findings, they could not find effects of *ABCB1* polymorphisms on rifampicin exposure in TB patients from different regions.[116] His results on *ABCB1* and *SLCO1B1* polymorphisms (rs4149032, highly prevalent in South African population) referred to plasma concentrations. Our

investigated SNP for *SLCO1B1 521* (rs4149056), at week 2, was predictive of lower rifampicin intraPBMCs concentrations by 33%. In a study from Li the haplotype constructed by this SNP and rs2306283 was associated with rifampicin-induced liver injury.[68] In a South-Indian population investigation of rs11045819, rs4149033 and rs4149033 polymorphisms in *SLCO1B1* gene did not influence rifampicin concentrations.[117]

NAT2 (rs1799930) is part of a genotyping panel of 4 SNPs for predicting acetylator phenotype. [40–42] This variant, either on its own or when in the NAT2*6A haplotype (with variant rs1041983 282T that alone does not confer reduced activity), results in a slow acetylator phenotype. This variant has been associated with risk of anti TB drug-induced hepatitis in several studies, due to the role of *NAT2* in isoniazid metabolism. Korean patients with TB and genotype AA or GA displayed significantly reduced acetylation and clearance of isoniazid compared to those with genotype GG. Allele A was associated with increased risk of drug-induced hepatitis also in Turkish and Tunisian TB patients.[118–120] This association was also observed in Chinese patients, but was not statistically significant after controlling for gender.[121] In our cohort at week 1, plasma ($p=0.056$) and intraPBMCs isoniazid AUC_{24} were higher in patients with GA/AA genotypes confirming previous results.

In the multilevel model analysis having three drugs above the recommended plasma ranges confirmed to be a predictive factor of better pharmacodinamic outcome combined with positive effect of TC/CC variants for *SLCO1B1 521* polymorphism and negative effect of *NR1I2* variant.

Patients with three drugs above the range and *SLCO1B1* TC/CC genotype had a higher increase in Delta TTP at week2, whereas the presence of NR1I2 was associated with a higher decrease of DeltaTTP.

The positive effect of *SLCO1B1* polymorphism is probably explained by the fact that the C allele gives rise to an amino acid change which has reduced uptake/transport activity. Therefore, drugs metabolized by OATP1B1 tend to have higher plasma levels than they would otherwise.

Regarding hepatotoxicity, no major adverse events that required withhold of drugs occurred.

Two interesting findings emerged from our analysis: first, patients with isoniazid peak concentrations over 5000 ng/mL had increased transaminases, independently from *NAT2* genotype; secondly *ABCB1* CC (wild type) genotype was associated with ALT increase.

It has been reported that no single gene variant of *NAT2* or *CYP2E1* is significantly associated with isoniazid-driven hepatotoxicity.[97] Hence, a combination of polymorphisms could impact the pharmacokinetic of isoniazid. For the majority of the antitubercular drugs, efficacy and toxicity are most likely determined by multiple factors, with both genetic and non-genetic factors playing a role. Large clinical trials will be needed to evaluate prospectively the merits of using pharmacogenetics and pharmacokinetics and adjustment of doses during treatment.

Several limitations must be highlighted in this study: number of available samples, the lack of complete AUCs for all the patients and the parameter AUC/MIC to permit further pharmacodynamic considerations. A non-compartmental pharmacokinetic analysis was used, however pharmacokinetics of some antitubercular drugs might be best described using multiple compartments. The small sample size and the lack of clinical and microbiological follow up limit further the observations. The correlations found with different SNPs need further discussion on the biological plausibility. Additionally, as every PG/PK study with multiple dependent and independent variables observed correlations should be confirmed in independent cohorts.

Moreover, the correlation between PBMCs and alveolar macrophages still need to be fully confirmed. The study is still ongoing and the safety data on liver toxicity will be extend beyond 2

weeks and will include neuropathy and ophthalmological assessment. A substudy on intraPBMCs concentrations isolated from broncho-alveolar samples is planned.

6. Conclusions

This study investigates the possible determinants of treatment response at two weeks of treatment in patients with tuberculosis. This analysis prepares the ground for a personalization of antitubercular treatment in order to maximise efficacy to avoid selection of resistance.

Several attempts to relate drug concentrations to tuberculosis outcome have been made with conflicting results. Other factors that play a role are genetically determined, related to the extent of the disease, to the bacterial drug resistance or to the presence of comorbidities.

A personalized approach is the future in many fields of the medicine and the present analysis tries to address some of the important questions on this topic.

Antitubercular drugs' plasma concentrations are associated with treatment efficacy but clear cut-off has not been defined and the majority of patients are cured despite very low plasma levels; yet significant variability and underexposure of antitubercular drugs are common features and the relationship with concentrations at the site of action (intra-macrophage and intra-lesion) are poorly understood.

TDM may be suggested in groups at risk of failure, toxicity or lower exposure such as children and adolescents, HIV-positive individuals, diabetics, patients with renal or liver impairment or subjects taking potentially interacting drugs. Its use in those with delayed response to treatment has been tested and we strongly recommend its application. Underexposure, especially if involving more than one compound, requires adequate dose adjustments and further controls. The lack of PK laboratories (performing HPLC/UPLC or GC techniques) calls for capacity building in centralized facilities and for

the use of dried blood/plasma spots for safe and cheap samples delivery. Sequential samples or application of Limited Sample Strategy (LSS) to calculate AUC are preferred rather than a 2-hour post-dose sample to correctly estimate drugs' peak concentrations.[122]

Furthermore, the optimization of currently available first-line drugs involves their tailored per kilogram dose, their once-daily administration, the correct relationship to food intake (fasted rifampicin, isoniazid, ethambutol) and the avoidance of potentially interacting drugs.

The individualization of antitubercular drugs' dose according to subjects' genetic variants requires more prospective data as well as controlled trials.

Finally, cellular and tissue pharmacology need to be assessed and new formulations explored (such the promising nanoformulations allowing for slow and/or targeted release of drugs) in order to tailor antitubercular treatment dose and duration to patients' and disease characteristics.

We believe this may be the future of antiTB pharmacology: the goal would be to have an imaging technique allowing us to estimate drug penetration *in vivo*. An integration of drugs' plasma information with tissue specific PK/PD parameters could lead to optimization of existing drugs: physiologically based pharmacokinetic modelling is a promising field that may help designing informative trials.[123,124]

One of the challenging areas in understanding PK/PD relationships of antitubercular drugs is the lack of early biomarkers for defining drug efficacy. While EBA is expensive and not related to sterilizing activity other markers are under investigation such as sputum or urine molecular studies and whole blood bactericidal activity (WBA) or plasma drug activity assay.[125,126]

TB is a challenging infection for the long-needed treatment, multidrug regimen and potential toxicities and drug interactions. Ending the TB epidemic is one of the key goals of the WHO post-2015 strategy. Only with global initiative and collaborations between researchers the open issues may be

resolved: in the future, we will be able to administer to all cases the right drug at the right dose for the right patient.

7. Acknowledgements

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