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TESI DI DOTTORATO DI RICERCA

Pharmacokinetic/pharmacodynamic relationship and simulation analysis for the evaluation of isoniazid hepatotoxicity risk development in patients with tuberculosis

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PRESENTATION

Tuberculosis is one of the oldest disease known to affect humanity and is likely to have existed in prehistory. The epidemiological impact of tuberculosis among the causes of death has been dramatically reduced over the XX century [1]. Nevertheless, it remains a major concern both for patients and for healthcare workers throughout the world, despite major progress in the development of new strategies for diagnosing and treating this disease [2].

Today, the major challenges with tuberculosis are 2-fold: the first is to deal with the growing epidemic around the world (and especially in 'low-income' countries) and the second is to ensure correct use of antitubercular medications in order both to protect these drugs for future use and to ensure an effective and safe treatment to affected patients [3].

The safety issue when administering antitubercular drugs is of foremost importance because standard first-line antitubercular drugs might unexpectedly cause life-threatening adverse drug events. In particular, isoniazid, that represents, along with rifampicin, the back-bone drug of almost any antitubercular regimen, has been associated with hepatotoxicity, a serious event that could vary from a mild increase of hepatic transaminases to fatal hepatitis. Clinicians usually struggle to cope with this complication, as decisions of continuing drug administration in order to provide effective treatment and preventing the emergence of resistance on the one hand, and reducing drug dose or even discontinue the causative agent to avoid toxicity on the other hand, are always fraught with consequences.

The aim of this research is to investigate the pharmacological determinants that are behind isoniazid-induced hepatotoxicity. This drug undergoes a peculiar hepatic elimination that might cause drug overexposure with consequent toxicity in certain subjects and not in others. The study

of the isoniazid PK/PD relationship, i.e. the convergence of isoniazid pharmacokinetics in terms of daily drug exposure with pharmacodynamics in terms of the impairment that drug causes on patient's hepatic function, for the first time unravel the link between drug exposure and the risk of developing hepatotoxicity during isoniazid treatment.

From a clinical perspective, these results are expected to be of interest for a safer administration of isoniazid. In fact, clinicians provided with patient isoniazid plasmatic exposure, could estimate *a priori* the intrinsic risk of developing hepatotoxicity, thus tailoring drug dose on each patient's pathophysiological characteristic, in the path of personalized medicine.

INTRODUCTION

1. Tuberculosis at a glance

1a. Global perspective and epidemiology of the disease

Tuberculosis (TB) has plagued humankind worldwide for thousands of years. In western countries, the highest mortality and morbidity rates of TB were observed at the end of the 18th and beginning of the 19th centuries as results of the unfavorable social situation for most people during and after the industrial revolution. Afterwards, the discovery of the etiologic agent of TB by German scientist Robert Koch on 1882, improvements in the diagnosis of TB (the x-ray was discovered in 1895 by Conrad Roentgen) and isolation of infectious cases in sanatoria along with improved socioeconomic conditions, resulted in a decrease in TB outbreak. Since the introduction of efficient antitubercular medications and control programs initiated after World War II, there has been an annual decline of approximately 5% in the number of TB cases observed in western countries between the 1950s and 1980s. Then, in the mid-1980s, this decline levelled off and a new increase in the number of cases was observed. This trend had continued until mid-2000s and peaked in 2004. Then a slight decline in incidence rates began to take place and still continues nowadays [4].

In this evolving scenario, new aspects of the disease have become of concern and three major challenges have advocated for a renewed, faster and more adequate approach to the diagnosis and treatment of this disease [5].

First of all the actual epidemiological burden of TB in the world is *per se* a matter of concern. In 2012, an estimated 8.6 million people developed TB and 1.3 million died from the disease, confirming TB as a major health problem. Even if incidence rates have been constantly decreasing since the peak reached in 2004 and also the prevalence of active disease has fallen by 37% globally since 1990, an estimated 2 billion persons worldwide have latent infection and are at risk for reactivation. This raises questions on economic investments for drug supply, especially in low-

income countries, and drug development, and more generally on access and effectiveness of care, as case detection accounts for about two-thirds of the estimated incident case of TB.

Secondly, treatment success is undermined by the emergence of microbiological strains with acquired resistant to the standard antitubercular drugs. Multidrug-resistant TB (MDR-TB), then extensively resistant TB (XDR-TB) and, most recently, strains that are resistant to all antitubercular drugs, have led the WHO to describe this scenario as a "crisis". Globally, in 2012, an estimated 450.000 people developed MDR-TB with estimated 170.000 deaths.

Thirdly, active TB is fostered by HIV co-infection as the two diseases potentiate each other. Although this problem is of utmost interest in countries with a high burden of HIV infections in which standard care is often inadequate for both conditions, its consequences contribute and foster the epidemic worldwide spread of the disease.

Population-based initiatives for TB control

More than 20 years have passed after the WHO declaration of TB as a global public health emergency in 1993 [6]. During this period several initiatives have been implemented to control and reduce the spread of TB (**Table 1**).

The directly observed treatment, short course (DOTS) strategy [7] is the first global approach to contain the spread of TB and was supported by Institutions such as the I*nternational Union against TB & Lung Disease*, the *Word Bank* and WHO. Its fundamentals are based on the recognition that the most cost-effective way to stop the spread of TB in communities with a high incidence is by curing it. DOTS is made up of five components, some advocating government commitments by means of implementing a centralized and prioritized system of TB monitoring, recording and training; others directed at the patient level, supporting the adoption of a strategy of drug administration under the direct supervision of an healthcare worker in order to optimize compliance of therapy. Between 1995 and 2008, 43 million people were treated under DOTS and 6 million deaths were potentially averted.

In 2006 WHO launched the Stop TB Strategy [8], which was linked to the Millenium Development Goal (MDG) 6.c target [9] of reversing the incidence, prevalence and death rates associated with TB by 2015. Although the rate of new TB cases has been falling worldwide for about a decade in all the six WHO regions (African Region – AFR; Eastern Mediterranean Region – EMR; European Region – EUR; Region of the Americas – AMR; South-East Asia Region – SEAR; Western Pacific Region – WPR) since 2004 thus achieving the MDG global targets, the Stop TB Partnership target of a 50% reduction of prevalence and deaths by 2015 compared with baseline levels of 1990, is not expected to be achieved. The epidemic of HIV-associated TB in Africa, resource constraints, conflicts and instability and the spread of MDR tuberculosis especially in eastern Europe are the main reasons that hamper this achievement.

The goal to eliminate TB as a public health problem has been set in 2050; nevertheless, its attainment seems already off-track, as TB incidence should decrease by an average of 16% yearly over the next 40 years, but the actual rates do not exceed 2%.

Table 1. Performance targets for tuberculosis control. Reproduced from [10].

World Health Assembly, 1991
Targets originally set for 2000, later postponed to 2005 and now deemed obsolete in view of the call for universal detection and cure:
• Achieve a worldwide case detection rate of 70%
• Achieve a worldwide cure rate of 85%
MDG 6
Target 6.c: halt and begin to reverse the incidence of tuberculosis by 2015
Targets linked to the MDGs and endorsed by the STOP TB Partnership
By 2015: reduce the prevalence of tuberculosis and deaths due to tuberculosis by 50% compared with the baseline of 1990
By 2050: to eliminate tuberculosis as a public health problem as defined by achieving a worldwide incidence of tuberculosis of less than 1 case per million population per year

Incidence

A global trend in the estimated incidence of TB from 1990 to 2011 among all patients is depicted in **Figure 1**. It is worth noting the continuing slight decrease from peak numbers in the mid-2000s.

In 2012, there were an estimated 8.6 million incident cases of TB (range: 8.3 million–9.0 million) globally, equivalent to 122 cases per 100 000 population. Most of the estimated number of cases in 2012 occurred in Asia (58%) and the African Region (27%); smaller proportions of cases occurred in the Eastern Mediterranean Region (8%), the European Region (4%) and the Region of the Americas (3%).

The 8.6 million incident TB cases in 2012 included 1.1 million (13%) among people living with HIV. The proportion of TB cases co-infected with HIV was highest in countries in the African Region.

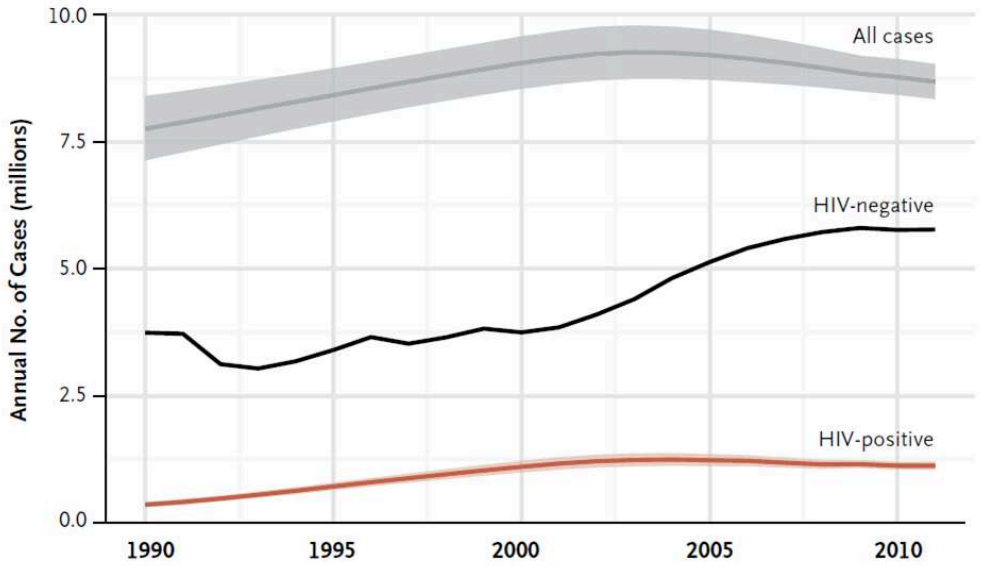

Figure 1. Global incidence trend of TB from 1990 to 2011. Reproduced from [11].

Prevalence

There were an estimated 12 million prevalent cases (range: 11 million–13 million) of TB in 2012, equivalent to 169 cases per 100.000 population. By 2012, the prevalence rate had fallen 37% in all the six WHO regions since 1990. Nevertheless, current forecasts suggest that the Stop TB

Partnership target of halving TB prevalence by 2015 compared with the baseline of 1990 will not be met worldwide.

Globally, in 2012 an estimated 450.000 people developed MDR-TB with estimated 170.000 deaths. Data from drug resistance surveys suggest that 3.6% of newly diagnosed TB cases and 20% of those previously treated for TB had MDR-TB. The highest levels of MDR-TB are found in Eastern Europe and central Asia where peaks from 20% to 50% of resistance have been recorded.

Mortality

Without treatment, TB mortality rates are high. In studies of the natural history of the disease among sputum smear positive/HIV-negative cases of pulmonary TB, around 70% died within 10 years; among culture-positive (but smear negative) cases, 20% died within 10 years.

There were an estimated 1.3 million TB deaths in 2012: 940 000 among HIV-negative people and 320 000 among HIV-positive people.

Approximately 75% of total TB deaths occurred in the African and South-East Asia Regions in 2012. India and South Africa accounted for about one-third of global TB deaths. Globally, mortality rates (excluding deaths among HIV-positive people) have fallen by 45% since 1990; the current forecast suggests that the Stop TB Partnership target of a 50% reduction in TB mortality by 2015 compared with a baseline of 1990 will be achieved.

1b. Pathogenesis and clinical features

Generally, TB is characterized by a well-defined series of phases. The first phase consists of *primary infection* which leads, in the majority of cases to *latent infection*. At this point, *M. tuberculosis* is contained by the immune system through the formation of granulomas where dormant bacilli may persist for years. Endogenous reactivation of distant latent infection gives rise to *secondary or post-primary or active tuberculosis,* in which, after reactivation, bacteria overcome host's mechanisms of defense and a progression to systemic TB occurs [12, 13] (**Figure 2**).

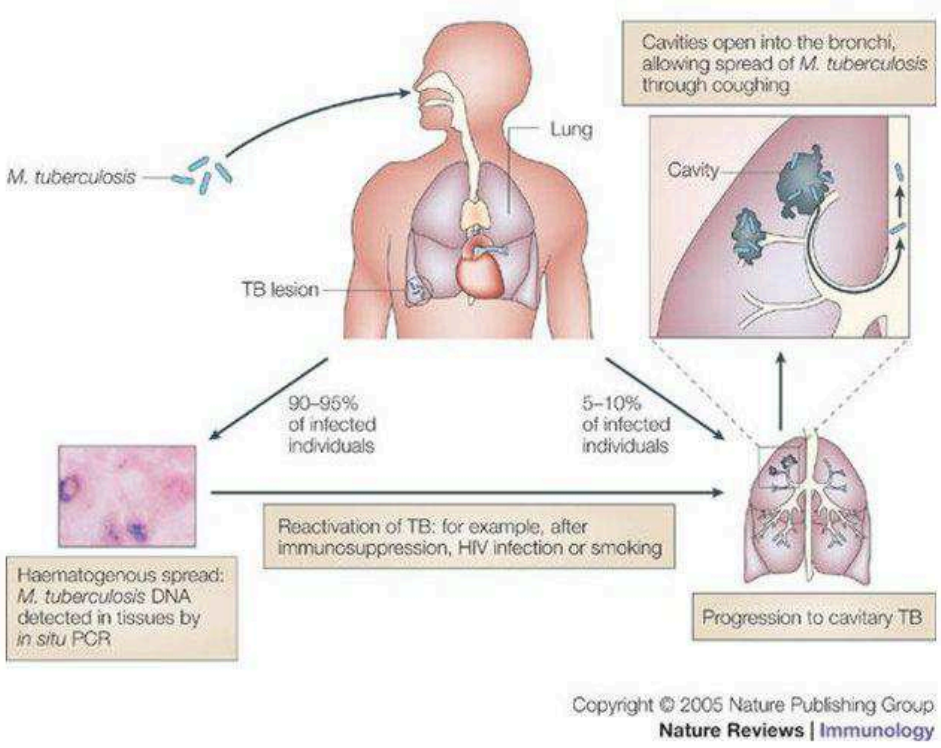

Figure 2. Pathophysiology of tuberculosis.

Latent Infection

Primary infection occurs when a person inhales droplet nuclei containing TB bacilli that reach the alveoli of the lungs. There, alveolar macrophages phagocytize the bacilli and a variety of cytokines are produced and secreted. Among these, mycobacterial protein ESAT-6 induces secretion of matrix metalloproteinase 9 (MMP9) by nearby epithelial cells that are in contact with infected

macrophages. MMP9 in turn stimulates recruitment of naïve macrophages, thus inducing granuloma maturation. TNF-α released from phagosome within macrophages, bacterial products released during the repeated rounds of cell lysis and infection and newly arriving macrophages, enable dendritic cells to access bacilli; these cells migrate to draining lymph nodes and present mycobacterial antigens to T lymphocytes, and the development of cell-mediated immunity begins.

The result is the activation and proliferation of $CD4^+$ T cells, which are crucial to the host's defense against *M. tuberculosis*. TH1 cells produce IFN-γ – an activator of macrophages and monocytes and inducer of reactive nitrogen intermediates – and IL-2. Moreover, $CD8^+$ cells have been associated with protective effects via cytotoxic responses and direct lysis of infected cells [14].

Coincident with the appearance of immunity, a delayed-type hypersensitivity reaction to *M. tuberculosis* develops and could be revealed by the tuberculin skin test (TST), which is used primarily for the detection of *M. tuberculosis* infection in persons without symptoms. More practical and convenient tests for the diagnosis of latent infection are represented by IFN-γ release assays (IGRAs) tests, such as the QuantiFERON-TB Gold® , that are in vitro assays that measure T cell release of IFN-γ with higher specificity than the TST.

At this point, *M. tuberculosis* is contained in more than 90% of persons as asymptomatic latent infection. Recent evidence raised the possibility that some persons acquire and then eliminate acute infection with *M. tuberculosis*. On the contrary, the risk of active disease at this stage is estimated to be of 9% in the 18 months after initial infection and then approximately 5% for the remaining lifetime.

It is worth noting that persons with latent TB have *M. tuberculosis* in their bodies, but do not have TB disease, are asymptomatic and cannot spread the infection to other people. Therefore a person with latent TB is not regarded as having a case of TB.

Overall, an estimated 2 billion people worldwide (i.e. one person out of three) have latent infection and are at risk of reactivation.

Active TB

Reactivation of the latent *M. tuberculosis* infection often occurs in apparently healthy people, and very frequently in people presenting one or more risk factors as those reported in **Table 2**. In particular, infection with HIV is the most potent of these risk factors, the risk being 20-times greater than that in HIV-negative people. Other important risk factors include smoking, diabetes and the assumption of immunosuppressive drugs.

Reactivation of TB most commonly occurs in the lung, but can involve any organ. The disease is a chronic wasting illness characterized by fever, weight loss, night sweats, and in the case of pulmonary reactivation, cough. Extrapulmonary TB occurs in 10 to 42% of patients, depending on race or ethnic background, age, presence or absence of underlying disease, genotype of the *M. tuberculosis* strain, and immune status [15]. Extrapulmonary TB can affect any organ in the body, has varied and protean clinical manifestations, and therefore requires a high index of clinical suspicion [11]. In order of frequency the extrapulmonary sites most commonly involved are the lymph nodes (>40% of cases), pleura (20% of cases), genitourinary system (10-15% of cases), bones and joints or Pott's disease (10% of cases), central nervous system (5% of cases) and gastrointestinal apparatus (3.5%). As a result of hematogenous dissemination in HIV-infected individuals, extrapulmonary TB is seen more commonly today than in the past.

The initial suspicion of pulmonary TB is often based on abnormal chest radiographic findings in a patient with respiratory symptoms. Virtually any picture could be seen, although an upper-lobe disease with infiltrates and cavities is the classic pattern.

The diagnosis is traditionally based on the finding of acid-fast bacilli on microscopic examination of a diagnostic specimen such as a smear of expectorated sputum or of tissue. Definitive diagnosis depends on the isolation of *M. tuberculosis* from cultures (results available in 4-8 weeks due to the slow bacterial growth) or after identification of specific sequences of DNA in a nucleic amplification test (results in hours).

Table 2. Risk factors associated with tuberculosis. Reproduced and adapted from [10].

HIV	Greatly increased susceptibility to infection, primary progressive disease, reactivation, and recurrence; disease incidence rate ratio of between 20 and 37 for people infected with HIV depending on country HIV prevalence
Diabetes	About three-times increased risk of tuberculosis (especially in insulin-dependent disease); higher mortality
Undernutrition and vitamin deficiencies	Undernutrition, low body-mass index, and vitamin D deficiency are each associated with increased risk of tuberculosis disease
Overcrowded living conditions	Increased exposure to infectious cases
Smoking	About two-times increased risk of infection, progression to tuberculosis disease and death
Indoor air pollution	About two-times increased risk of disease (weak evidence)
Silicosis	About three-times greater risk in South African gold miners with silicosis
Alcohol	About three-times increased risk of disease associated with consumption >40 g per day
Sex	The ratio of incident tuberculosis disease in men:women is about 2:1 in adults but not children
Age	Major effect on risks of acquisition, disease progression, form of disease, and mortality risk
End-stage renal failure	More than ten-times increased risk
Malignancy	Both solid organ and hematological malignancies associated with increased risk
Genetic susceptibility	There is a growing list of genes associated with risk of tuberculosis, including genes for natural resistance-associated macrophage protein 1, interferon γ, nitric oxide synthase 2A, mannan-binding lectin, vitamin D receptor, and some Toll-like receptors
TNF-antagonist therapy	Risk of tuberculosis disease increased about one and a half times in rheumatology patients in North America; risk greater with TNF antibodies than with soluble TNF receptor
Corticosteroid therapy	Risk of tuberculosis disease increased about two times in rheumatology patients in North America

1c. Treatment principles

The overall goals for treatment of TB are (*1*) to cure the individual patient, and (*2*) to minimize the transmission of *Mycobacterium tuberculosis* to other persons.

Chemotherapy for TB became possible with the discovery of streptomycin in 1943. Several cases of successful treatment were documented, but a substantial proportion of patients had a relapse with subsequent resistance to streptomycin. That same year, two new antitubercular agents, thiacetazone and para-aminosalicylic acid (PAS) came on the market followed by isoniazid, tested at Sea View Hospital in New York in 1951. It soon became clear that cure of TB required the concomitant administration of at least two agents to which the organism was susceptible, for a long period of time (12-24 months). The introduction of rifampicin in the early 1970s heralded the era of effective short-course chemotherapy, with a treatment duration of less than 12 months. The discovery that pyrazinamide augmented the potency of isoniazid/rifampicin regimens led to the use of a 6-month course of this triple drug regimen as standard therapy [16].

TB treatment is therefore characterized by a long-term, multiple-drug combination regimen. This chemotherapeutic regimen is undertaken in order to neutralize both a heterogeneous bacterial populations, made up of active as well as dormant bacilli, and the high tendency to develop resistance, especially if one or two drugs are used.

It has been speculated that there are three different subpopulations of bacilli in the host, each with its own characteristics in relation to growth rate and susceptibility to antitubercular agents, depending on the type of lesion [17]. The largest of this subpopulation consists of bacilli residing in the cavity walls located extracellularly and actively multiplying in the liquefied caseous material covering the cavity wall. Isoniazid (and to a lesser degree also streptomycin) have been shown to possess very strong early bactericidal activity and to have the most potent ability to kill these rapidly multiplying bacilli during the initial phase. A second subpopulation consists of bacilli inhabiting solid caseous material and considered to be semidormant because they exhibit only intermittent bursts of metabolic activeness. These organisms are killed preferentially by rifampicin.

Another rather small population of bacilli is believed to reside in an acidic environment either intracellularly (within macrophages) or extracellularly (in areas of active inflammation or recent necrosis). These bacilli also exhibit a semidormant rate of activity and are particularly susceptible to pyrazinamide, which acts only in acidic conditions.

Therefore, the use of drugs that have good sterilizing properties is essential for regimens as short as 6 months. Rifampicin and pyrazinamide have the greatest sterilizing activity followed by isoniazid and streptomycin [18, 19].

Use of drugs with potent early bactericidal activity, such as isoniazid and rifampicin, reduces the risk of developing resistance within bacillary populations. There is a natural risk of random mutations with a frequency of about 1 in 10^6 for isoniazid and streptomycin, 1 in 10^8 for rifampicin and 1 in 10^5 for ethambutol, but mechanism of resistance are independent when drugs are given in combination. The cumulative risk for resistance to both isoniazid and rifampicin is estimated to be 1 in 10^{14} [20].

Antitubercular drugs

Four drugs are considered the first-line agents for the treatment of TB: isoniazid, rifampicin, pyrazinamide – which have a bactericidal action – and ethambutol, which has a bacteriostatic activity. In terms of antibactericidal potency, isoniazid is followed by rifampicin and ethambutol, whereas pyrazinamide shows only very weak bactericidal activity.

A number of second-line agents, less potent and with major toxicity, include amynoglicosides (streptomycin, kanamycin and gentamicin); the injectable polypeptide capreomycin; the oral agents ethionamide, cycloserine and PAS; third generations quinolones (levofloxacin and moxifloxacin). Other drugs that have been used in the treatment of patients with resistance to most of first- and second-line agents include clofazimine, amoxicillin/clavulanic acid, clarithromycin, imipenem and linezolid.

Ten new or repurposed TB drugs are in late phase of clinical development. In late 2012, bedaquiline became the first novel TB drug approved in 40 years. In June 2013, WHO issued interim guidance for its use in treatment of MDR-TB. Moreover, there are also 10 vaccines for TB prevention and two immunotherapeutic vaccines in the pipeline [21].

Antitubercular regimens

Standard short-course regimens are divided into an initial, or bactericidal, phase and a continuation, or sterilizing, phase. The treatment regimen of choice for virtually all forms of TB in adults consists of a 2-month initial phase of isoniazid, rifampicin, pyrazinamide and ethambutol followed by a 4-month continuation phase of isoniazid and rifampicin (**Table 3**).

Table 3. Current recommandations for tuberculosis treatment. Reproduced from [11].

Type of Infection	Recommended Regimen	Comments
Active disease		
Newly diagnosed cases that are not multidrug-resistant	Isoniazid, rifampin, ethambutol, and pyrazinamide for 2 mo (intensive phase), followed by isoniazid and rifampin for 4 mo (continuation phase)	Pyridoxine supplementation recommended to prevent isoniazid-induced neuropathy
Multidrug-resistant disease	Four second-line antituberculosis drugs (as well as pyrazinamide), including a fluoroquinolone, a parenteral agent, ethionamide or prothionamide, and either cycloserine or para-aminosalicylic acid if cycloserine cannot be used	Initial treatment based on local disease patterns and pending drug-susceptibility results; later-generation fluoroquinolones (e.g., moxifloxacin or levofloxacin) preferred
Latent infection	Isoniazid at a dose of 300 mg daily for at least 6 mo and preferably for 9 mo	Recommended for 9 mo or more in HIV-infected persons; daily administration for 6 mo also an option but with lower efficacy; extension to 36 mo further reduces risk among HIV-positive patients in regions in which tuberculosis is endemic
	Isoniazid at a dose of 900 mg plus rifapentine at a dose of 900 mg weekly for 3 mo (directly observed therapy)	Studied with directly observed therapy in predominantly HIV-uninfected persons; higher completion rates and equal efficacy, as compared with isoniazid for 9 mo
	Rifampin at a dose of 600 mg daily for 4 mo	Shown to be effective in persons with silicosis
	Isoniazid at a dose of 300 mg plus rifampin at a dose of 600 mg daily for 3 mo	Effective alternative for HIV-infected persons
	Isoniazid at a dose of 900 mg plus rifampin at a dose of 600 mg twice weekly for 3 mo	Another effective alternative for HIV-infected persons

With the use of the above-mentioned strategy, the rapidly growing population of bacilli is eliminated quickly, resulting in clearing of live bacilli from the sputum within 2 months in 80% of

patients. The remaining groups of slow multiplying bacilli or intermittently dividing bacilli can be responsible for relapse if the duration of therapy is inadequate.

Patients with cavitary pulmonary TB and delayed sputum culture conversion (i.e. those who remain culture-positive at two months) should have the continuation phase extended by 3 months, for a total course of 9 months.

To prevent isoniazid-related neuropathy, pyridoxine (10-25 mg/die) should be added to regimens given to persons at high risk of vitamin B6 deficiency (e.g., alcoholics; malnourished persons; pregnant and lacting women; persons with chronic renal failure, diabetes, HIV). In children, most forms can be safely treated without ethambutol in the intensive phase [22].

The treatment algorithm of reference [23], published by a joint collaboration between the American Thoracic Society (ATS), the Infectious Disease Society of America (IDSA) and the Center for Disease Control and Prevention (CDC) is reported in **Figure 3**.

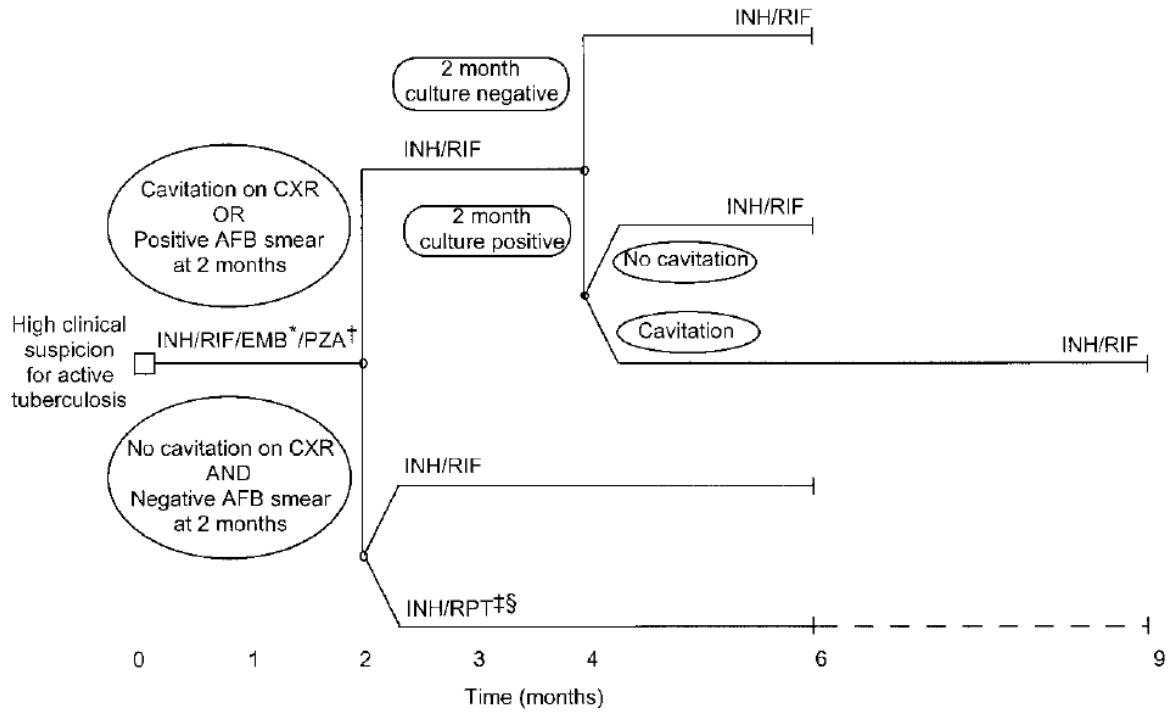

Figure 3. Treatment algorithm for tuberculosis. Reproduced from [23].

Monitoring treatment response and drug toxicity

Patients suspected of having TB should have appropriate specimens collected for microscopic examination and mycobacterial culture. Susceptibility testing for isoniazid, rifampicin and ethambutol should be performed on an initial positive culture, regardless of the source.

In addition, at the time treatment is initiated, it is recommended that all patients with TB have counseling and testing for HIV infection [24]. Patients with epidemiologic factors suggesting a risk for hepatitis B or C, for example injection drug use, birth in Asia or Africa, or HIV infection, should have serologic tests for these viruses. Measurements of AST, bilirubin, alkaline phosphatase, serum creatinine and a platelet count should be obtained for all adults as baseline functionality parameters.

Bacteriological evaluation is essential in monitoring the response to treatment for TB. Patients with pulmonary disease should have their sputum examined monthly until cultures become negative. As mentioned above, by the end of the second month more than 80% of patients will have negative sputum cultures. When a patient's sputum cultures remain positive at ≥ 3 months, treatment failure and drug resistance or poor adherence to the regimen should be suspected. A sputum specimen should be collected at the end of treatment to document cure.

In addition to the microbiological evaluations, it is essential that patients have clinical evaluations at least monthly to identify possible adverse effects of the antitubercular medications and to assess adherence [23]. The most common adverse reaction is hepatitis. Guidelines suggest that, after baseline assessment of liver function, older patients, those with concomitant diseases, those with a history of hepatic diseases and those using alcohol daily should have repeated measurement of aminotransferases during the initial phase of treatment and then monthly for the rest of the treatment period. Appropriate management of therapy in relation to the level of hepatotoxicity should be undertaken as discussed in the next session.

Multidrug-resistant TB

When treatment failure is suspected (i.e. patient's sputum smear and/or cultures positive after 3 months of therapy), it is imperative that the current isolate be tested for susceptibility to second-line agents. Multidrug-resistant TB is defined as disease caused by strains of *Mycobacterium tuberculosis* that are at least resistant to treatment with isoniazid and rifampicin; extensively drug-resistant (XDR) TB refers to disease caused by multidrug-resistant strains that are also resistant to treatment with any fluoroquinolone and any of the injectable drugs used in treatment with second-line anti-tubercular drugs (amikacin, capreomycin, and kanamycin) [25].

The treatment of multidrug-resistant TB is based on expert opinion and requires the adoption of combination drug regimens chosen in a step-wise selection process [26] through five groups of drugs on the basis of efficacy, safety, and cost. Among the first group (the oral antitubercular first-line drugs) high-dose isoniazid, pyrazinamide, and ethambutol are thought of as an adjunct for the treatment of MDR and XDR TB. The second group is the fluoroquinolones, of which the first choice is high-dose levofloxacin. The third group is the injectable drugs, which should be used in the following order: capreomycin, kanamycin, then amikacin. The fourth group is called the second-line drugs and should be used in the following order: thionamides, cycloserine, then aminosalicylic acid. The fifth group includes drugs that are not very effective or for which there are sparse clinical data. Drugs in group five should be used in the following order: clofazimine, amoxicillin with clavulanate, linezolid, carbapenems, thioacetazone, then clarithromycin [27].

Such therapy is associated with a high risk of intolerance and serious toxic effects, and should be administered for at least 20 months in patients who have not received previous treatment for multidrug-resistant TB and for up to 30 months in those who have received previous treatment.

Extensively drug-resistant TB is extremely difficult to diagnose and treat in countries in which the disease is endemic. The condition has been associated with death rates as high as 98% among HIV-infected persons [28-30].

Latent TB infection

Treatment of selected persons with latent TB infection aims at preventing active disease. This intervention (also called *preventive chemotherapy* or *chemoprophylaxis*) is based on the results of a large number of randomized, placebo-controlled clinical trials, demonstrating that a 6- to 12-month course of isoniazid reduces the risk of active TB in infected people by up to 90% [14].

The preferred regimen is isoniazid alone for 9 months, or for a longer duration in HIV-infected persons (**Table 3**).

2. Pharmacological basis in the treatment of tuberculosis

2a. Clinical pharmacology of first-line antitubercular drugs

Isoniazid

In 1945 research in Europe discovered that the vitamin nicotinamide (PP or B3) had activity against TB. In 1952, in attempts to enhance the activity of nicotinamide, an intermediate molecule, the hydrazide of isonicotinic acid (INH), appeared to hold an extraordinary efficacy against *M. tuberculosis* growth, highly superior to any other compound in use at that time. Nevertheless, it would take more than 50 years to uncover its mechanism of action.

Pharmacodynamics

Isoniazid is the hydrazide of isonicotinic acid, and its structural formula is depicted in **Figure 4**. The isopropyl derivative of isoniazid, iproniazid (1-isonicotinyl-2-isopropylhydrazide), also inhibits the multiplication of the tubercle bacillus. This compound, which is a potent inhibitor of monoamine oxidase, is too toxic for use in human beings.

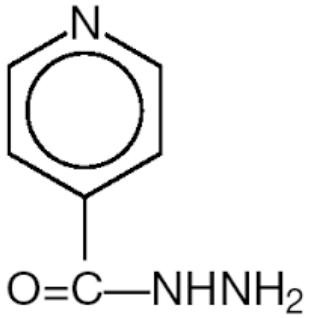

Figure 4. Isoniazid molecular structure.

Isoniazid is active exclusively against mycobacteria, especially slowly growing mycobacteria. Its action is reported to be bacteriostatic for "resting" bacilli but is bactericidal for rapidly dividing microorganisms. The minimum inhibitory concentration (MIC) is 0.025 to 0.5 mg/L. The bacteria undergo one or two divisions before multiplication is arrested. A 3- to 4- log drop in colony-forming units is usually observed after 4 days of therapy. Isoniazid also induces changes in

mycobacteria, such as the loss of internal structure or the appearance of surface wrinkles and bulging. Among the various non-tubercular (atypical) mycobacteria, only *M. kansasii* is usually susceptible to isoniazid.

A range of mechanisms of action have been recently uncovered that may act additively or synergistically.

Isoniazid enters the mycobacterial cell through passive diffusion. Isoniazid itself is not toxic but acts as a pro-drug and is activated by the mycobacterial enzyme KatG, a multifunctional catalase-peroxidase with peroxynitritase and NADH oxidase activity. KatG oxidatively activates isoniazid via the production of a range of carbon-, oxygen- and nitrogen-centered free radical species such as the isonicotinic hydrazyl radical and the isonicotinic acyl radical, this latter being the key intermediate that adds to intracellular NAD^+ and $NADP^+$ to produce a range of powerful inhibitors. The resulting INH-NAD adduct inhibits the FASII enoyl-ACP reductase InhA, the main target of isoniazid. InhA is a carrier protein reductase involved in the synthesis of mycolic acids, unique and important mycobacterial cell wall lipids, and so its inhibition is in accord with the unique sensitivity of mycobacteria to isoniazid. This inhibition leads to accumulation of long-chain fatty acids, inhibition of mycolic acid biosynthesis, and ultimately cell death [31, 32] (**Figure 5**).

Bacterial resistance

Resistance to isoniazid in *M. tuberculosis* clinical isolates is most commonly associated with mutations in KatG, encoding the isoniazid activator, that result in a decrease in or loss of catalase-peroxidase activity. So far, at least 130 mutations have been reported, with MIC ranging from 0.2 to 256 mg/L. The second most frequent mutation is related to a missense mutation within the mycobacterial InhA gene, reducing the affinity of isoniazid for its cofactor NADH.

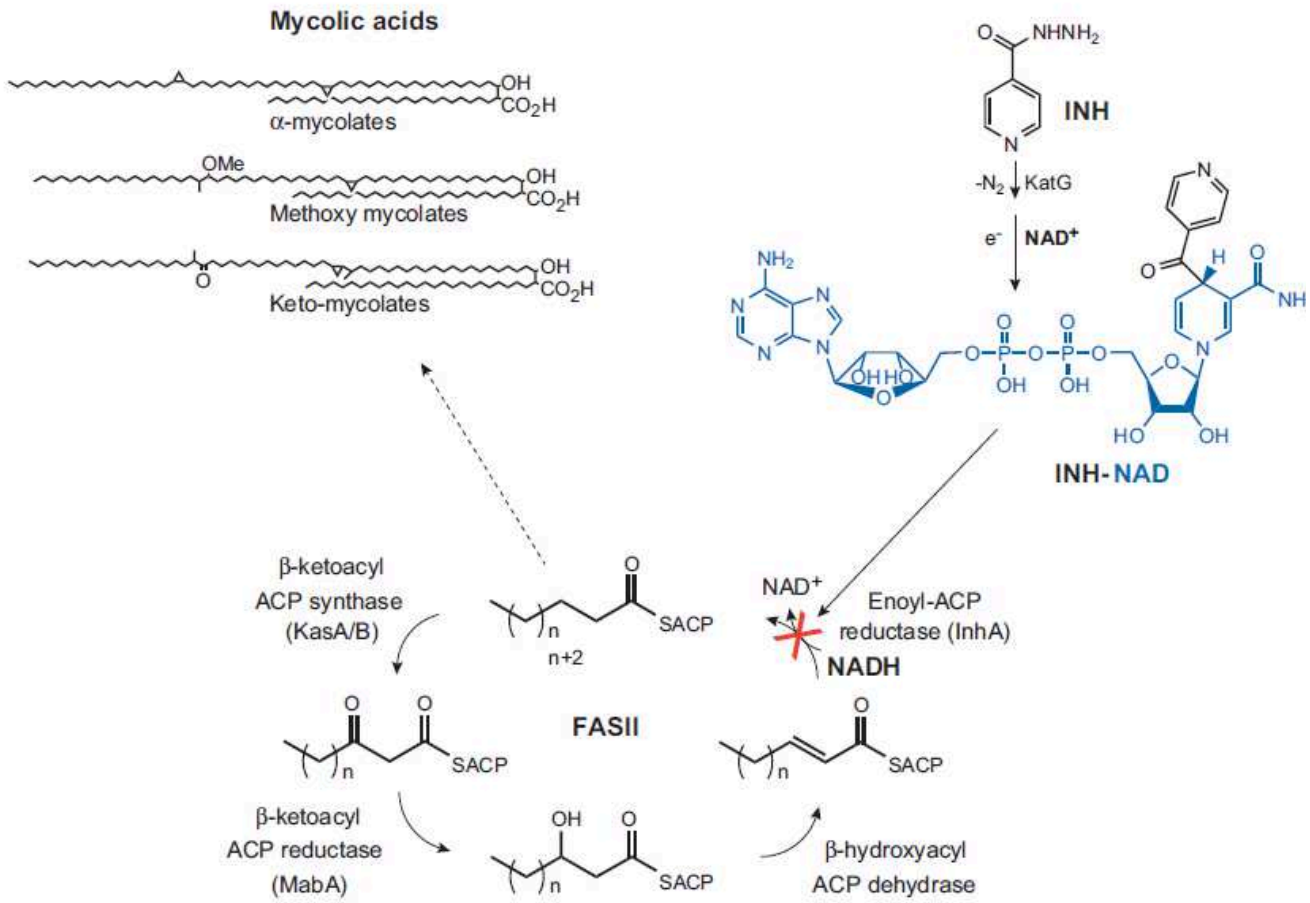

Figure 5. Mechanism of action of isoniazid in *M. tuberculosis.* Reproduced from [31].

Pharmacokinetics

Isoniazid has a very good oral bioavailability (F_{os} > 90%) and it is usually stated that it is completely absorbed after oral administration. However, absorption is significantly decreased by food and aluminum-magnesium antiacids [33]. Thanks to its high liposolubility, isoniazid rapidly diffuses into all body fluids and cells. It penetrates well into the central nervous system, pleural and ascitic fluids. Although isoniazid crosses the placenta and is distributed into breast milk, the global exposure to the infant does not represent a safety concern [34]. In plasma, isoniazid is not bound to plasma proteins (P_b ~ 0%) and it distribution volume (V_d) is 0.67 L/kg.

From 75% to 95% of a dose is excreted in the urine within 24 hours, mostly as metabolites. The predominant metabolic pathway of isoniazid is acetylation by the phase II hepatic enzyme N-acetyltransferase 2 (NAT2) that produces acetylisoniazid which is subsequently hydrolyzed into acetylhydrazine, a toxic metabolite, and isonicotinic acid. Acetylhydrazine is either hydrolyzed in

hydrazine, another toxic metabolite, or acetylated into diacetylhydrazine. A small part of isoniazid is directly hydrolyzed into isonicotinic acid and hydrazine and this pathway is of greater quantitative significance in subjects having a slow rate of acetylation, as in this case a greater amount of hydrazine is produced due to a shift between the two metabolic pathways [35] (**Figure 6**).

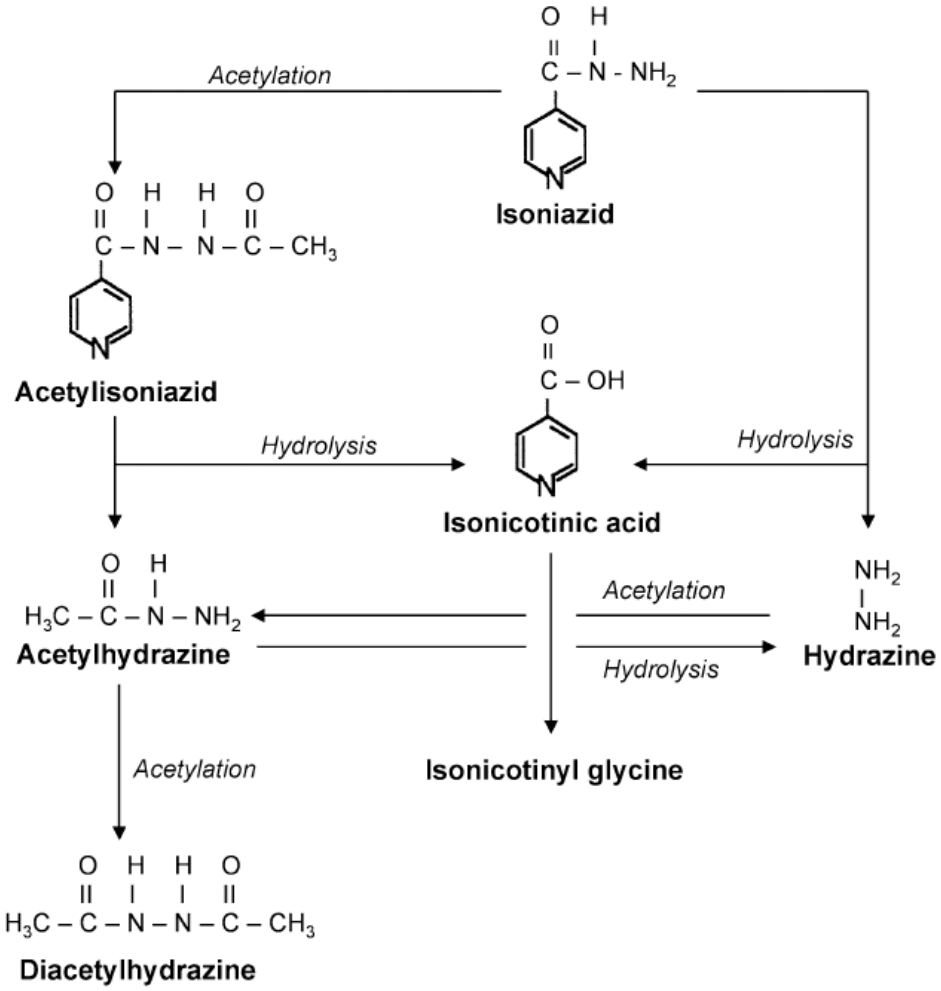

Figure 6. Isoniazid metabolism. Reproduced from [35].

Human population shows genetic heterogeneity with regard to the rate of acetylation of isoniazid. This trait is inherited in an autosomal dominant fashion.

Traditionally, a bimodal distribution of acetylation appears within a population, enabling a subject to be classified as a slow or a fast acetylator [36] (**Figure 7**). The frequency of the slow acetylator

phenotype is reported to varying between 50% and 60% among white americans and caucasian and 17% in japaneses.

The rate of acetylation significantly alters drug concentrations achieved in plasma and drug elimination half-life. In fact, the average plasma concentration of isoniazid in fast acetylators is about 30% to 50% of that present in persons who acetylate the drug slowly. In the whole population, the half-life ($t_{1/2}\beta$) of isoniazid varies from less than 1 to more than 4 hours. The mean half-life in fast acetylators is approximately 70-130 minutes, whereas 2,2 to 5 hours are reported for of slow acetylators [37].

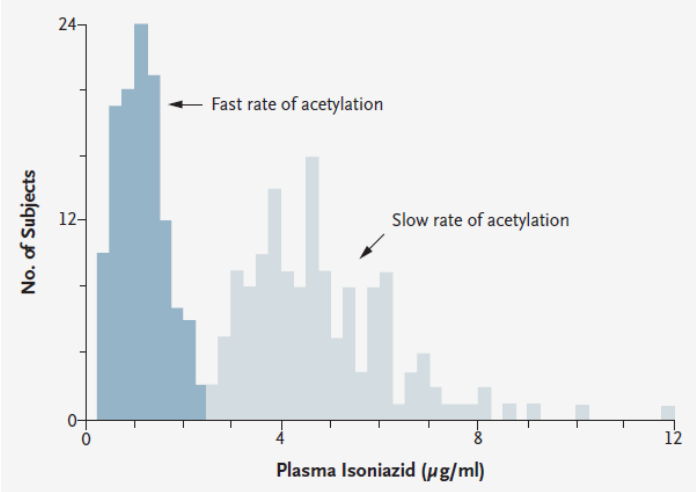

Figure 7. Pharmacogenetics of acetylation. Reproduced from [36].

Some authors, more correctly, prefer to describe the phenotypic status of acetylation among populations according to the number of active inherited NAT2 alleles (NAT2*4) that are present in each individual. Therefore subjects with two active alleles (homozygous fast) are described as rapid acetylators, those with one active allele (heterozygous fast) are described as intermediate acetylators and those with no active alleles (homozygous slow) are described as slow acetylators [38].

However, the traditional classification is still in use because it rapidly distinguishes the slow acetylator phenotype from the others, collectively though improperly refered to "rapid" acetylators.

From a clinical point of view, in order to identify those subjects potentially experiencing drug overexposure, i.e. slow acetylators, two methods are currently available. The first method consists in the genetic sequencing of the NAT2 gene. It unequivocally allows to the correct identification of the specific phenotype, and it could be considered as the "gold standard" method. In context in which pharmacogenetic analysis could not be performed, the pharmacokinetic study of the elimination phase of isoniazid leads to the determination of the elimination constant *k* and finally the elimination half-life of the drug. Accordingly, all subjects with an elimination half-life grater that 130 minutes are considered slow acetylators. The elimination half-life threshold that distinguishes between intermediate and rapid acetylators is 72 minutes [38].

Untoward Effects

The incidence of adverse drug reactions (ADRs) to INH is estimated to be 5.4% [39].

The major toxic adverse reaction to isoniazid is hepatotoxicity, that includes a wide spectrum of pathological conditions from mild jaundice to fatal hepatitis. Patients aged 35 years or older are three times more likely to develop drug-induced hepatitis than those under 35. Progressive liver damage may be seen in up to 2.3% of patients over age 50. Overall, hepatitis occurs in about 2.1% of drug recipients and has been reported as fatal in 4.6% of these cases as will be discussed in the following section.

Peripheral neuritis (most commonly paresthesia of feet and hands) is also amongst the most common reactions to isoniazid but it is usually prevented by the concomitant administration of 15 to 50 mg/die of pyridoxine. In the absence of prophylactic administration of pyridoxine, the incidence of this reaction would be 2%. Much less frequently reported neurotoxic effects include toxic encephalopathy and optic neuritis.

Gastrointestinal side effects, such as diarrhea, abdominal pain, and nausea/vomiting, have been reported with oral use of isoniazid.

Hematologic reactions that occur infrequently with isoniazid include agranulocytosis, hemolysis with anemia, sideroblastic anemia, aplastic anemia, pancytopenia, and thrombocytopenia.

Dermatologic reactions that occur infrequently with isoniazid include maculopapular rash, acneiform rash, or exfoliative dermatitis. Isoniazid has also been associated with acute generalized exanthematous pustulosis (AGEP).

Pharmacological interactions

Isoniazid is a potent CYP3A4 inhibitor and may reduce the hepatic metabolism of CYP3A4 substrates. Therefore isoniazid can increase carbamazepine concentrations causing carbamazepine toxicity. Isoniazid has been reported to inhibit the hepatic metabolism of diazepam and that of oxidized benzodiazepines including alprazolam, clonazepam, estazolam, flurazepam, midazolam, prazepam and triazolam. Isoniazid also inhibits the metabolism of valproate and the parahydroxylation of phenytoin, and sign and symptoms of toxicity occur in approximately 27% of patients given both drugs. Monitoring is recommended for signs of toxicity such as ataxia, nystagmus, mental impairment, involuntary muscular movements, and seizures.

Although isoniazid does not inhibit mitochondrial MAO, it does appear to inhibit plasma MAO. INH may possess enough MAO inhibiting activity to produce clinical symptoms consistent with serotoninergic excess. Thus, co-administration with selective serotonin reuptake inhibitors, COMT-inhibitors like entacapone or tolcapone and all the MAO-inhibitors, should be avoided.

Isoniazid is known to inhibit the hepatic metabolism of drugs that undergo oxidation including warfarin. Daily consumption of ethanol increases the risk of isoniazid-induced hepatitis and also can increase the clearance of isoniazid.

Dose

Isoniazid is available for oral and parenteral administration. The commonly used total daily dose is 5 mg/kg in single administration, independently of the patient's specific acetylator status, and with a maximum of 300 mg/die.

Rifampicin

The discovery of rifampicin dates back to 1957, when a new antibiotic named rifamycin was obtained from fermentation cultures of *Streptomyces mediterranei*. Rifamycin was subsequently shown to be a group of five substances, then named rifamycin A-E. Further researches in 1965 proved that a hydrazone of a rifamycin B derivative retained the highest bactericidal action and was well absorbed orally. This led to the FDA approval of rifampicin (**Figure 8**) in 1971. More recently, in the 1990s, two additional rifamycin antibacterial have been licensed, rifabutin and rifapentine.

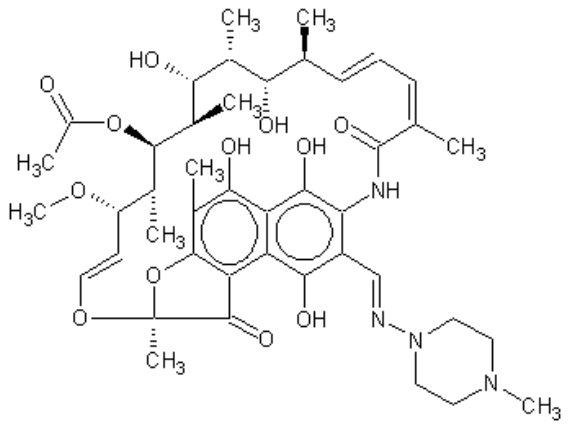

Figure 8. Rifampicin molecular structure.

Pharmacodynamics

Rifampin inhibits bacterial and mycobacterial RNA synthesis. It binds to the beta-subunit of DNA-dependent RNA polymerase, thereby inhibiting the binding of the enzyme to DNA and blocking RNA transcription. Rifampin does not bind to RNA polymerase in eukaryotic cells, so RNA synthesis in human cells is not affected.

Rifampin inhibits the growth of most Gram-positive bacteria as well as many Gram-negative microorganisms such as *Escherichia coli*, *Pseudomonas* spp., *Proteus* and *Klebsiella*. Rifampicin is very active against *Staphylococcus aureus*, coagulase-negative staphylococci, *Mycobacterium tuberculosis* and also against some nontubercular mycobacteria (e.g. *Mycobacterium kansasii*). Rifampin is effective against bacilli that are rapidly dividing in extracellular cavitary lesions, as

well as against slowly or intermittently dividing organisms such as those found in closed caseous lesions and macrophages. Rifampin is bactericidal both for intracellular and extracellular microorganisms [39].

Because of the ease with which many pathogens develop resistance to rifampicin monotherapy, this drug is almost used in combination with other antibacterials. Microbial resistance to rifampicin is due to one-step process alteration of the target of this drug, the DNA-dependent RNA polymerase.

The clinical settings in which rifampicin is currently used include the treatment of tuberculosis, staphylococcal infections, such as osteomyelitis and endocarditis, and as an adjunctive drug in multiple combination regimens for the treatment of gram-negative multidrug resistant infections.

Pharmacokinetics

Rifampin is rapidly absorbed from the gastrointestinal tract following oral administration, with an oral bioavailability of approximately 68%. Food alters the rate and extent of absorption, therefore it is recommended that the drug be given on an empty stomach. It is distributed into most body tissues and fluids including lungs, liver, bone, saliva, and peritoneal and pleural fluids and its volume of distribution is 0,97 l/kg. It penetrates inflamed meninges, and CSF concentrations are roughly 10-20% of the plasmatic ones.

Rifampin is metabolized in the liver to an active metabolite, desacetyl-rifampicin, via deacetylation. Rifampin undergoes enterohepatic circulation with significant reabsorption. The elimination half-life is 3-5 hours, but it increases in the presence of hepatic dysfunction. The parent compound and its metabolite are primarily excreted (60%) in feces via biliary elimination, while up to 30% of an administered dose is excreted in the urine [39].

Untoward Effects

Generally rifampin is well tolerated. Gastrointestinal disturbances such as dyspepsia, nausea/vomiting, flatulence, cramps, diarrhea, abdominal pain and pyrosis have been reported in 1-2% of patients receiving this drug.

The treatment-limiting adverse effects of rifampicin are drug-induced hepatic disorders and a "flu-like" syndrome. Specifically, rifampin may occasionally cause dose-dependent interference with bilirubin uptake, resulting in subclinical hyperbilirubinemia or jaundice without hepatocellular damage. Nevertheless, rifampicin is reported to potentiate the hepatotoxicity of the other antitubercular medications.

A hypersensitivity reaction manifesting as a flu-like syndrome (fever, chills, headache, and fatigue) has been reported in as many as 50% of patients. The flu-like syndrome occurs more commonly with higher than standard doses [40].

Finally, due to its wide distribution into almost all body tissue and fluids, rifampicin may impart an orange-red color to the urine, feces, saliva, sputum, tears and sweat.

Pharmacological interactions

Rifampicin is a potent inducer of hepatic microsomal enzymes and efflux transporters such as the P-glycoprotein. Therefore, drugs metabolized by CYP450 cytochromes (CYP 2C9, 2C19, 3A4) when co-administered with rifampicin, undergo to an enhanced metabolism that leads to subtherapeutic concentrations with consequent underexposure. This has been demonstrated for many drugs, including HIV protease inhibitors, digoxin, corticosteroids oral anticoagulants, sulfonylureas and oral contraceptives.

Dose

The dose of rifampicin for treatment of tuberculosis is 10 mg/kg daily, either 1 h before or 2 hours after a meal. In the clinical practice, the usually administered daily dose for adults has been 600 mg (eventually titrated to 900 mg daily in special population such as the obese patients). Recently, some authors advocated the adoption of higher doses mainly to overcome drug resistance and to ensure more adequate plasmatic exposure by optimizing the dose-response pharmacokinetic curve [41].

Pyrazinamide

Pyrazinamide is the syntethic pyrazine analog of nicotinamide. Shortly after its discovery in the late 1940s, it was immediately tested in the treatment of tuberculous patients and found to be effective, even though it didn't retain any appreciable activity against *Mycobacterium tuberculosis* under usual culture. The reasons for this paradoxical behavior have been only recently unraveled, and led to the initial comprehension of the mechanism of action of this drug. Pyrazinamide structural formula is reported in **Figure 9**.

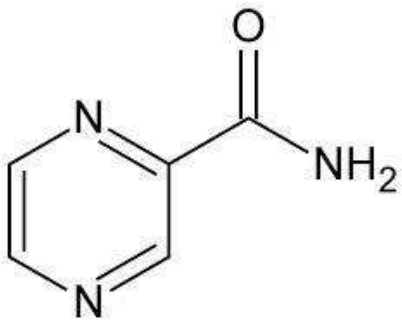

Figure 9. Pyrazinamide molecular structure.

Pharmacodynamics

Pyrazinamide is a pro-drug that necessitates conversion to an active derivative to exert its antibacterial action. As a pro-drug, pyrazinamide enters the bacilli by passive diffusion and possibly active transport, then is converted by the enzyme nicotinamidase (PZase) into pyrazinoic acid (POA). POA exits from the cell by passive diffusion. If the extracellular pH is acidic, a small proportion of the POA outside the bacterial cell membrane will take an uncharged conjugate acid form, HPOA, which back permeates through the membrane easily (**Figure 10**). The theory of HPOA re-entering the bacilli best explains the role of acid pH in pyrazinamide action, and is based the observations that POA is accumulated inside the bacilli at acidic pH but not neutral pH and that Pyrazinamide-resistant *M. tuberculosis* lacking PZase does not take up pyrazinamide, but takes up POA at acid pH. The acid-facilitated POA influx is apparently stronger than the weak or deficient

POA efflux, so that there is an accumulation of POA in *M. tuberculosis* cells. The protonated POA brings protons into the cell, which could eventually cause cytoplasmic acidification such that vital enzymes can be inhibited. In addition, protonated POA could potentially de-energise the membrane by collapsing the proton motive force and affecting membrane transport as a possible mechanism of POA [42].

Pyrazinamide is preferentially active against semi-dormant, non-growing bacterial population, has good sterilizing activity and allows to shorten anti-TB therapy to a 6-month duration of treatment.

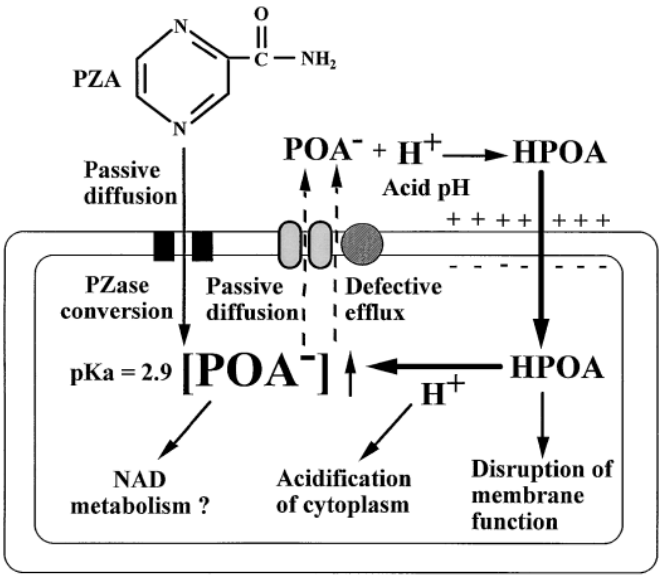

Figure 10. Mode of action of pyrazinamide. Reproduced from [42].

Pharmacokinetics

Pyrazinamide is well absorbed by the gastrointestinal tract and it is widely distributed throughout the body, including central nervous system, lungs and liver. Pyrazinamide is hydrolyzed into the liver to pyrazinoic acid, its major active metabolite. Subsequently, pyrazinoic acid is hydroxylated to 5-hydroxypyrazinoic acid, the main excretory compound. Pyrazinamide and its metabolites are excreted in the urine (70%), primarily via glomerular filtration. The plasma half-life is 9 to 10 hours in patients with normal renal function; however it can increase up to 26 hours in renal disease [39].

Untoward effects

Hepatotoxicity is the most severe adverse reaction of pyrazinamide. Jaundice, elevated hepatic enzymes, hepatitis, and a syndrome of fever, anorexia, malaise, hepatomegaly, and splenomegaly have been reported. In rare instances, hepatic necrosis and fatalities have occurred. This is a dose-related adverse reaction and is typically seen with large pyrazinamide dosages, such as 40-50 mg/kg/day for prolonged periods of treatment. Regimens employed currently (15 to 30 mg/kg/day) are much safer.

Moreover, pyrazinamide inhibits excretion of urate, resulting in hyperuricemia in nearly all patients. Other untoward effects that have been observed with pyrazinamide are arthralgias, anorexia, nausea and vomiting

Pharmacological interactions

Because pyrazinamide can increase serum uric acid levels and precipitate gouty attacks, the dosages of antigout agents, including allopurinol, colchicine, and probenecid, may need to be adjusted.

Dose

The dose of pyrazinamide for adults is 15 to 30 mg/kg/day orally, given as a single dose. The maximum quantity is 2 g per day, regardless of weight.

Ethambutol

Ethambutol is a water-soluble molecule, approved for the treatment of mycobacterial infections including TB and atypical mycobacterial infection by the FDA in 1967. Its structural formula is reported in **Figure 11**.

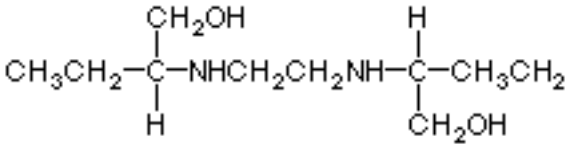

Figure 11. Ethambutol molecular structure.

Pharmacodynamics

The mechanism of action of ethambutol is unknown; however, it appears to block arabinosyl transferases involved in cell wall biosynthesis. Ethambutol is primarily bacteriostatic, although at higher doses it also exhibits bactericidal properties. Resistance to ethambutol develops very slowly in vitro.

In general, nearly all strains of *M. tuberculosis* and *M. kansasii* as well as a number of strains of *M. avium* complex are sensitive to ethambutol.

Pharmacokinetics

Ethambutol is administered orally. Approximately 75-80% of a dose is absorbed. Ethambutol is widely distributed, with high concentrations in the kidneys, lungs, and saliva. It penetrates inflamed meninges (10-50%) to reach therapeutic levels in the central nervous system. The drug crosses the placenta, resulting in plasma fetal concentrations that are 30% of the maternal ones and no adverse effects to the fetus have been reported. Ethambutol is partially metabolized in the liver. The parent drug and its metabolites are excreted primarily in the urine (65%), with the remaining 20-25%

excreted unchanged in the feces. The elimination half-life is 3.5 hours and can be up to 15 hours in renal disease.

Untoward effects

Optic neuritis, manifested by decreased visual acuity, loss of red-green color discrimination and constriction of visual fields is the most significant adverse reaction to ethambutol therapy. These visual changes are dose-related and usually reversible over a period of weeks to months following discontinuation of therapy. Optic neuritis is most frequently encountered with doses of 25 mg/kg/day and after 2 months of treatment.

Therapy with ethambutol results in an increased concentration of plasmatic urates in about 50% of patients, owing to decreased renal excretion of uric acid. The hepatotoxicity risk of ethambutol is not deemed relevant.

Dose

The usual adult dose of ethambutol is 15 mg/kg given once a day

2b. Hepatotoxicity risk development in the treatment of tuberculosis

The most serious adverse effect of antitubercular treatment is hepatotoxicity. It causes substantial morbidity and mortality either as a complication *per se* and by diminishing treatment effectiveness. In fact, by contributing to nonadherence, it eventually leads to treatment failure, relapse or the emergence of drug-resistance [43, 44].

The incidence of hepatotoxicity during standard multidrug TB treatment has been variably reported by different studies varying between 2.5% and 34.9% [45]. Nevertheless, when trying to characterize drug-induced hepatotoxicity during antitubercular therapy, some important issues should be considered.

First of all, the determination of exact incidence estimates is primarily impaired by the investigator's definition of hepatotoxicity as well as the population studied. Different definitions of hepatotoxicity have been endorsed by scientific societies and experts' boards during the past decades and even more heterogeneous criteria have been adopted by different authors in relation to the specific goals of their researches. Moreover, most studies on antitubercular drug-induced hepatotoxicity have been performed in Europe, Asia and the USA and the incidence varies between different world regions. Orientals are reported to have the highest rates, especially Indian patients. Hepatotoxicity in sub-Saharan Africa is mentioned in some papers, but incidence rates are not reported mainly because liver function tests are not routinely carried out in that context.

Secondly, active TB is treated with multiple drugs. Therefore there are limited data on toxicity rates of antitubercular drugs alone, expect for isoniazid (and few reports for rifampicin), which has been widely used as prophylactic monotherapy for latent TB infections. This undoubtedly complicates the attribution of the reaction to a specific medication, unless a clear temporal relationship between symptoms appearance/disappearance and drug initiation/withdrawal could be provided.

Thirdly, TB patients often presents with co-morbidities, and related co-treatments, that could further compromise hepatic function. In fact HIV, HBV, HCV, alcohol consumption and cirrhosis

are quite common conditions in this kind of patients. Moreover, the administration of certain therapies, for example the antiretroviral drugs for HIV, can ultimately aggravate the hepatic damage.

However, and bearing in mind the above considerations, current evidence on antitubercular drug-induced hepatotoxicity is summarized below.

Definition of hepatotoxicity

Many definition of antitubercular drug-induced hepatotoxicity have been used in the literature. International consensus criteria defined hepatotoxicity as an increase in serum alanine aminotransferase (ALT) level greater than two fold [46] or three fold [47] the upper limit of normal (ULN), or a more than two-fold increase in the ULN concentration of ALT alone or a serum ALT ratio/ALP ratio greater than 5, where ALP is the concentration of alkaline phosphatase, ALT ratio= ALT value/ULN of ALT and ALP ratio= ALP value/ULN of ALP [48]. The 2003 ATS document on the Treatment of TB defines drug-induced hepatotoxicity as a treatment-emergent increase in serum aspartate aminotransferase (AST) greater than three or five times the ULN, with or without symptoms of hepatitis, respectively [23]. This latter definition has been adopted by 2006 Official ATS Statement on the Hepatotoxicity of Antitubercular Therapy [49] as the transaminase threshold for drug suspension.

However, in an attempt to investigate the degree of severity of drug-induced hepatotoxicity, it is worth mentioning the WHO Toxicity Classification Standard [50], that stratifies hepatotoxicity according to the peak level of ALT, as reported in **Table 4**. The main advantages of using this classification are that, on the one hand, it is based only on laboratory (thus quantitative) data rather than on reporting subjective and often unrecognized or even absent symptoms and, on the other hand, it takes into account also mild elevation of transaminases, often the first step for more serious complications.

Table 4. Definition of hepatotoxicity according to the WHO Adverse Drug Reaction Terminology

WHO definition of hepatotoxicity	ALT concentrations
Grade 1 (mild)	<2.5 times ULN (ALT 51 – 125 UI/L)
Grade 2 (mild)	2.5-5 times ULN (ALT 126 – 250 UI/L)
Grade 3 (moderate)	5-10 times ULN (ALT 251 – 500 UI/L)
Grade 4 (severe)	>10 times ULN (ALT >500 UI/L)

ALT, alanine aminotransferase; ULN, upper limit of normal, i.e. 50 UI/L

Isoniazid-induced hepatotoxicity

Isoniazid-induced hepatotoxicity has been traditionally considered an idiosyncratic reaction, i.e. an adverse drug reaction that occurs unpredictably and that is unrelated to the dose administered. Nevertheless, newer evidences have shed light into the mechanism of isoniazid-induced hepatotoxicity and drug exposure appears to be pivotal in the development of hepatotoxicity. Among the different antitubercular drugs isoniazid has one of the highest risk potential [51]. The attributable rates of hepatotoxicity due to isoniazid could be derived from large data on patients treated with isoniazid alone in the context of latent TB infections. Overall, incidence rates according to the most strict criteria (three times ALT elevation with symptoms or five times ALT elevation with or without symptoms) have been reported between 0.1 and 0.56% [52, 53]. Even if, at a first glance, these percentages could seem small, clinical implications are worrisome. In fact, of the 17 cases of severe isoniazid-associated liver injuries reported to the CDC during the period 2004-2008 in patients treated with isoniazid monotherapy for latent TB infections, five patients underwent liver transplantation, including one child and five adults died, including a liver transplant recipient [54].

The mechanism of isoniazid induced hepatotoxicity is by far the most investigated amongst all the antitubercular drugs. Two metabolites of isoniazid are deemed responsible for toxicity, namely acetylhydrazine and hydrazine [55].

Early studies initially suggested that acetylhydrazine was the causative metabolite inducing hepatotoxicity [56, 57]. More recently, the attention focused on hydrazine as the major metabolite responsible for isoniazid toxicity: its toxicity has been described as early as 1908 and it is known to

cause irreversible cellular damage. Moreover several hydrazine metabolites produced by oxidation such as nitrogen-centered radicals as well as hydrazones and nitrogen gas have been identified. However, several studies showed that subjects with the slow acetylator phenotype developed hepatotoxicity more often and also more severely as compared to fast acetylators [58, 59]. In fact, in slow acetylators more isoniazid is left for direct hydrolysis into hydrazine and also the accumulated acetylhydrazine can be converted into hydrazine. Huang et al. [58] demonstrated that the odd ratio of hepatotoxixcity of slow acetylators was of 3.7.

Once the isoniazid reactive intermediates are formed, the subsequent cellular mechanism of toxicity that can eventually involve the entire liver leading to patent hepatitis, is probably driven by at least some immune-mediated reactions. Some observations in fact support this conclusion. The fact that isoniazid can activate macrophages, that it can induce other immune responses – in particular autoimmunity similar to lupus with the presence of antinuclear antibodies, the fact that isoniazid-induced hepatotoxicity occurs with a delay of few weeks or more (typical of drug-induced autoimmunity) and the finding of a specific human leukocyte antigen genotype that is associated with an increased risk of isoniazid-induced hepatotoxicity, are just a few. Further studies on how these reactive metabolites induce hepatotoxicity in human are required [55].

In light of the pivotal role that NAT-2 enzyme plays in the metabolism of isoniazid and considering the relationship between the acetylation status and the risk of developing hepatotoxicity, a growing number of studies has recently demonstrated that genotyping the NAT-2 gene proved useful in reducing the risk of developing hepatotoxicity [60-67]. It is worth mentioning a meta-analysis based on a selection of 14 of these studies [68], showing that the odd ratio for NAT-2 slow acetylators compared with rapid acetylators was 4.70 irrespective of the concomitant antitubercular drug administered with isoniazid. A subgroup analysis of different treatment combinations identified the combined OR of 34.3, 4.09 and 2.36 for INH+RIF, INH+RIF+PZA+EMB and INH alone, respectively. Finally, a prospective, multicenter randomized control clinical trial involving 172 Japanese patients, compared a genotype-guided dosing regimen of isoniazid (with doses varying

from 2.5 mg/kg to 7.5 mg/kg according to the genetic profile of acetylation of different individuals) to the standard regimen (isoniazid dose of 5 mg/kg for all). In the intention-to treat analysis, isoniazid induced hepatotoxicity occurred in 78% of the slow acetylators in the standard treatment, while none of the slow acetylator in the pharmacogenetic treatment experienced either hepatotoxicity or early treatment failure [69].

Other gene polymorphism have been investigated in relation to isoniazid metabolism, namely the cytochrome P450 2E1 homozygous wild type and the glutathione S-transferase homozygous null genotype, with promising results.

Pyrazinamide-induced hepatotoxicity

When pyrazinamide was introduced in the 1950s, a high incidence of hepatotoxicity was reported and the drug was nearly abandoned. This appeared to be related to the high dosage of 40-70 mg/kg used at that time. Toxicity was no longer supposed to be a major problem when pyrazinamide was used at a daily dosage of 15–30 mg/kg.

In 2001, a 2-month prophylactic regimen with rifampicin and pyrazinamide for the treatment of latent TB infections gave higher hepatotoxicity rates (evaluated as an increase in ALT > 160) than a 6-month regimen with isoniazid alone (13% vs. 4%) [70]. Moreover, in a Canadian study involving 480 patients treated with first-line antitubercular drugs in the period 1990-1999, the incidence of all major adverse events was 1.48 per 100 person-months of exposure for pyrazinamide as compared to 0.49 for isoniazid and 0.3 for rifampicin [71].

Yet, the mechanism of pyrazinamide induced hepatotoxicity is at present unknown: it is unknown what enzymes are involved in pyrazinamide toxicity and whether toxicity is caused by pyrazinamide itself or its metabolites.

A recent pre-clinical study focused on the interactions that multiple antitubercular drugs have on the development of hepatotoxicity [72]. Surprisingly, researchers found that human hepatoma cells when pretreated with isoniazid or hydrazine had pyrazinamide IC_{50} (the drug concentration at which

cell survival is 50%) reduced by 30% and 38%, respectively. This means that the in vitro hepatotoxicity of pyrazinamide is increased by pre-treatment with isoniazid or its toxic metabolite hydrazine and that isoniazid exposure could behave as an enhancer for the subsequent development of pyrazinamide hepatotoxicity.

Rifampicin and Ethambutol-induced hepatotoxicity

The hepatotoxicity risk attributable specifically to rifampicin has been evaluated from the analysis of four published TB-related studies of patients treated for latent TB infections. Overall, these studies showed a non-significant elevation of transaminases and led to the conclusion that rifampicin is not associated to hepatotoxicity when administered alone, also because, at present, there is no evidence for the presence of a toxic metabolite [49].

Nevertheless, rifampicin activates hepatocytes pregnane X receptor, leading to induction of cytochromes, uridine diphosphate-glucuronosyl-transferases and P-glycoprotein, which are involved in the metabolism of other drugs. When combined to isoniazid, an increased risk of hepatotoxicity has been reported. This could happen because rifampicin induces isoniazid hydrolase, increasing hydrazine production, thus increasing isoniazid-induced hepatotoxicity [73].

As far as ethambutol is concerned, there has been only one report of ethambutol-related liver cholestatic jaundice, with unclear circumstances [74]. Generally, ethambutol is not considered to be hepatotoxic.

Other risk factors for hepatotoxicity

Many risk factors for antitubercular drug-induced hepatotoxicity have been reported. Among the most widely accepted risk factors are advanced age (above 60 years), female sex, low body mass index and denutrition, hepatitis B and C co-infection and alcoholism. The combined influence of some risk factors (in particular, advanced age, chronic liver disease, abuse of alcohol or other drugs or malnutrition) on the severity of antitubercular drug-induced hepatotoxicity has been

prospectively evaluated on 471 TB patients treated with first-line antitubercular drugs [75]. The risk factor group showed an OR of 3.5 for hepatotoxicity compared to the non-risk factor group and severe hepatotoxicity (transaminase >10 times the ULN) occurred in 6.9% of the risk factor group and in 0.4% (OR:17.7) of the group without risk factors.

Other well-recognized factors are HIV infection and combined TB/HIV treatment, for overlapping toxicities and drug-drug interactions. In particular nevirapine, among the non-nucleoside reverse transcriptase inhibitor (NNRTI), didanosine and stavudine among the nucleoside reverse transcriptase inhibitors (NRTI) and some protease inhibitors have demonstrated to negatively affect hepatic functionality [76].

As mentioned in previous sections, genetic polymorphisms in drug-metabolizing enzymes cause differences in treatment response and drug toxicity. The foremost example is represented by the N-acetyltransferase slow acetylator genotype along with the cytochrome P450 2E1 homozygous wild type and the glutathione S-transferase homozygous null genotype.

Management of hepatoxicity

Guidelines for the management of antitubercular drug-induced hepatotoxicity have been published by the American Thoracic Society (ATS), the British Thoracic Society (BTS) and the Task Force of the European Respiratory Society, the WHO and the Union Against Tuberculosis and Lung Disease [23, 77, 78].

A key role in early detection of hepatotoxicity, in deciding when to stop drug administration if hepatotoxicity occurs and in the subsequent follow-up phase until the end of therapy, is played by liver function monitoring by means of transaminase evaluation.

Although various guidelines as well as expert opinion documents have given varying advice on monitoring liver function strategies, the most comprehensive document, i.e. that published by the ATS in 2006, outlines a specific algorithm for monitoring liver function during antitubercular therapy and stipulates that all patients should have baseline liver function testing with subsequent

monitoring dependent on the presence of a number of risk factors that may increase the risk of developing hepatotoxicity. Briefly, a two times increase the ULN of ALT is considered a first warning signal for intensifying transaminase monitoring every week, while an increase of more than five times the ULN or more than three times the ULN in presence of symptoms are the criteria for drug suspension (**Figure 12**). Drug re-introduction should be performed in a stepwise manner, starting with rifampicin, then adding isoniazid and then pyrazinamide.

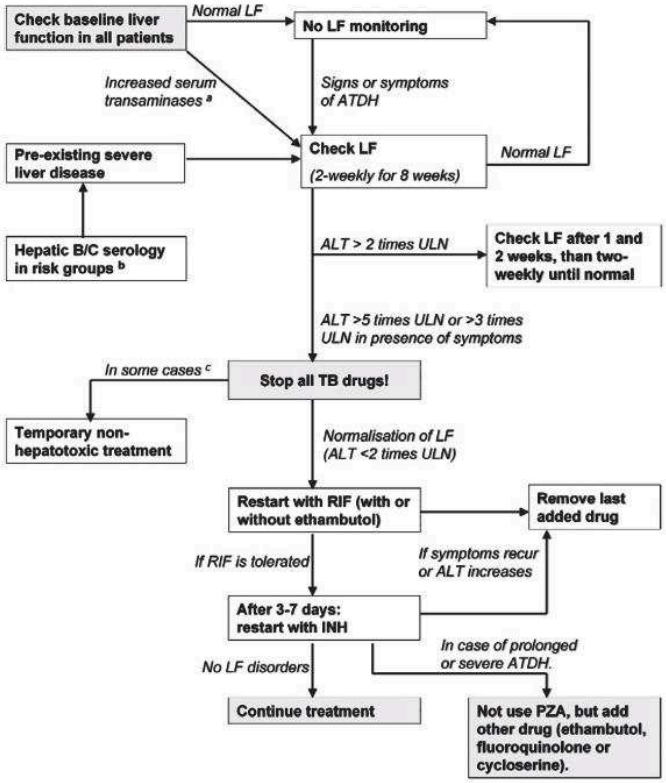

Figure 12. Flow chart for the management of antitubercular drug-induced hepatotoxicity. Reproduced from [35].

3. Drug Dosage Optimization

3a. Pharmacokinetic/pharmacodynamic relationship

The pharmacological characteristics of a drug are usually described according to pharmacodynamic and pharmacokinetic.

The pharmacodynamic (PD) approach focuses on the pharmacological effect that the drug produces on its target (i.e., a membrane receptor, an intracellular protein etc.) relative to its concentration at the site of action. From a graphical point of view, the *concentration vs. effect* graph resembles a sigmoidal curve, where at low concentrations drug effect parallels drug concentration, while at high concentrations drug effect reaches a plateau and no more effect could be obtained as target sites are saturated.

The pharmacokinetic (PK) approach focuses on the sequence of physiological processes the drug undergoes from the moment of its administration until its complete elimination from the organism. Traditionally, pharmacokinetic is made up of four distinct phases: absorption, distribution, metabolism and elimination (ADME). Even if these phases could be well identified and characterized by means of specific pharmacological parameters, they do not occur separately from each other but there is a wide overlap in their progression.

From a graphical point of view, the *concentration vs. time* graph is represented by the so-called area under the concentration-time curve (AUC). The AUC is one of the foremost pharmacokinetic determinants, as it represents the most appropriate parameter of drug exposure in the subject who has been administered the drug. The AUC is linked to drug dose and systemic drug clearance by means of the following formula:

$$AUC = Dose/Clearance$$

Put in another way, pharmacodynamic studies what the drug does to the organism, while pharmacokinetic studies what the organism does to the drug. These apparently opposite aspects can integrate with one another by means of the element they have in common: drug concentration.

Indeed, by combining the variation of drug concentration in time with the variation of the effect relative to drug concentration, the result is the variation of the effect in time. This is an extremely important parameter, directly related to clinical efficacy (**Figure 13**).

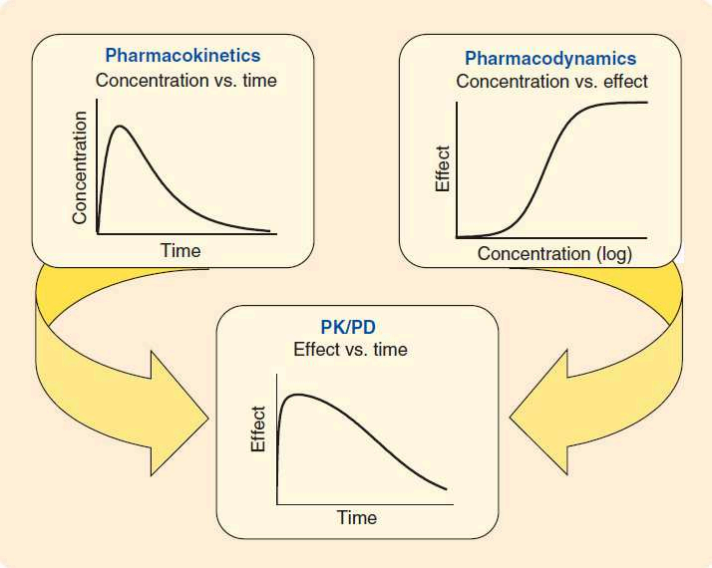

Figure 13. Integration of pharmacokinetic and pharmacodynamic into pharmacokinetic/pharmacodynamic (PK/PD) relationship. Reproduced from [79].

PK/PD parameters for efficacy and safety issues

Antimicrobial chemotherapy has largely used PK/PD models for the last twenty years, mainly with the intent of identifying the most appropriate drug dose and posologic regimen against different strains of bacteria in different patient populations in which drug pharmacokinetic is significantly altered. This approach proved undoubtedly useful in terms of optimizing efficacy. In particular, several different strategies have been developed, such as minimum inhibitory concentrations (MIC)-based PK/PD indices, time-kill analysis and advanced modelling and simulation techniques such as population pharmacokinetics and Monte Carlo Simulation [79].

Traditionally, the MIC is the most commonly used indicator of antimicrobial efficacy. It is defined as the lowest concentration that inhibits visual growth of the organism after 16-20 h of incubation with an inoculum size normally standardized to 10^5 to 5×10^5 CFU per mL. The MIC is a standard

PD parameter routinely determined in microbiology laboratories. Consequently, the MIC serves as the PD input for the most widely used PK/PD approaches for antimicrobials.

MIC-based PK/PD indices are:

- C_{max}/MIC: antimicrobial peak concentration to MIC ratio
- %t>MIC: time of antibiotic concentration above the MIC
- AUC/MIC: area under the concentration vs. time to MIC ratio

On the basis of these indices, antimicrobials could be classified in two categories: concentration-dependent drugs (such as aminoglicosides, quinolones, daptomycin - whose antimicrobial activity is best explained by the parameters of C_{max}/MIC and AUC/MIC) and time-dependent drugs (such as beta-lactams, glycopeptides, azoles, etc. - whose antimicrobial activity is best explained by %t>MIC).

Generally speaking, different PD parameters could be selected and related to one or more PK determinants. Therefore, PK/PD relationship could be applied also for safety purposes. In this case the pharmacodynamic parameter could be expressed as the dicotomic variable of presence or absence of toxicity, while the AUC usually remains the pharmacokinetic parameter to be used. For example, this approach has been adopted by Drusano et al [80] in investigating the safety profile of daptomycin (a new antigram-positive antimicrobial) in patients with *Staphylococcus aureus* bacteremia, and allowed these authors identifying the drug concentration threshold that minimized the likelihood of a potential life-threatening adverse event (in that case creatine phosphokinase elevation and skeletal myopathy) while ensuring clinical efficacy at the same time.

PK/PD parameters in antitubercular chemotherapy.

Few studies have investigated the bactericidal activity of isoniazid by means of a PK/PD approach. The first pre-clinical research, conducted both in vitro and in a murine aerosol infection model of TB, showed that $AUC_{0\text{-}24h}$/MIC correlated best with the bactericidal efficacy, followed by C_{max}/MIC [81]. Similar results confirmed that the $AUC_{0\text{-}24h}$/MIC was the PK/PD index most

explanatory of isoniazid killing activity, with an EC_{50} (i.e. the $AUC_{0\text{-}24h}$/MIC ratio mediating 50% of the maximal antimicrobial effect on bacillary density with isoniazid therapy - or E_{max}) of 61.55. Moreover the authors could predict by simulation the probability of achieving at a population level the desired PK/PD target when administering the usual isoniazid dose of 300 mg/die, and demonstrated a suboptimal early bactericidal activity attainment [82].

More recently, Jeena et al. [83] studied the probability of attaining an $AUC_{0\text{-}24h}$/MIC ratio of 287.2 in children 10 years old infected from TB. This value, derived from [82], was associated with 80% of maximal kill (80% effective concentration [EC_{80}]) and therefore considered as an optimal PK/PD parameter. Surprisingly, the authors showed that the standard isoniazid doses recommended in children of 10 to 15 mg/kg/day were largely inadequate in achieving the pre-specified target of efficacy. Only under the very limited circumstances of children who were slow acetylators and had disease limited to pneumonia that target could be achieved.

Now, it is worth noting that for isoniazid, but this holds true also for the other antitubercular drugs [84], the PK/PD approach has been used only for efficacy purposes and that the last two studies cited above used Monte Carlo Simulation techniques in order to test the likelihood that different isoniazid doses had in achieving a pre-specified target of efficacy at a larger (population) scale. No applications of the PK/PD concepts or Monte Carlo Simulation techniques have ever been used with the intent of investigating the safety profile of isoniazid.

3b. The role of therapeutic drug monitoring

Therapeutic Drug Monitoring (TDM) is defined as the practice of measuring plasmatic drug concentration with the intent to providing informed dosing to ensure adequate exposure [85]. TDM is recognized as one of the key activities within the discipline of Clinical Pharmacology. It represents, along with pharmacogenetic, one of the most feasible and precise tool for predicting drug target attainment for drug therapy personalization.

However, to be properly managed, TDM requires several considerations and the adoption of some methodological issues.

As a rule of thumb, TDM is beneficial only for a few drugs or drug classes. Indeed, only a relatively small number of molecules fulfills the pharmacological characteristics for TDM feasibility and, on the contrary, for many drugs easier and more rapid methods for efficacy evaluation exist (e.g. blood pressure measurement or cholesterol determination for antihypertensive and statin therapy efficacy evaluation, respectively).

The first and foremost requirement that a drug or drug class should have in order to be eligible for TDM is the presence of a good concentration-effect relationship, the effect being either efficacy or toxicity. In fact, only in this case, one might ignore drug dose as a parameter for guiding drug exposure, while relying entirely on plasmatic drug concentrations to derive the correct dose. Put in other words, TDM helps clinicians finding the correct dose regardless of the type of dose-concentration relationship.

Secondly, the drug should have a narrow "therapeutic window", i.e. a small difference between the toxic and sub-therapeutic concentrations. This implies that when the drug is given at standard doses, there is a high risk of under or over-exposure (**Figure 14**).

On these basis, drug classes that traditionally undergo TDM are represented by immunosuppressors, antiepileptics, antineoplastics, some cardiovascular drugs and antibiotics. In particular for antibiotics, recent years have seen a resurgence of TDM, and at least two factors have contributed to

it. On the one hand, a better comprehension of the pathophysiological mechanism of pharmacokinetic variability, such as altered renal clearance, altered volume of distribution, drug-drug interactions, obesity and old age have played a major role. On the other hand, the increasing antimicrobial resistance in Gram-negative and Gram-positive bacteria, and in *M. tuberculosis* as well, coupled with a dearth of new antimicrobial molecules in the market, have forced clinicians optimizing the administration of currently available antibiotics.

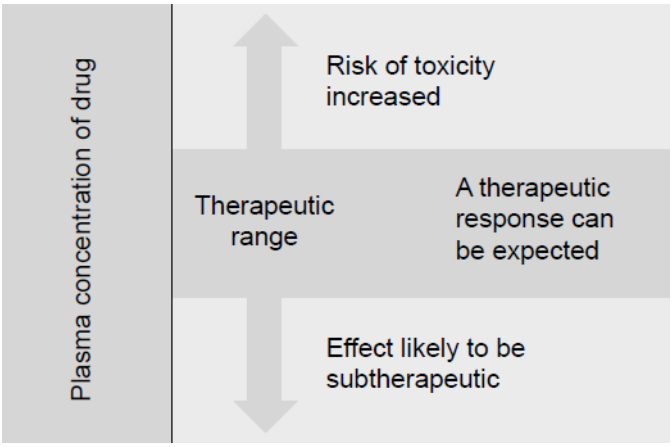

Figure 14. Concept of the therapeutic range. Reproduced from [86].

TDM in clinical practice

The pharmacokinetic parameter that best represents drug exposure after a single or multiple dose is the area under the concentration-time curve, or AUC. Usually it is expressed as a 24-hours AUC (or $AUC_{0\text{-}24h}$) and it is calculated from the analysis of a multiple and consecutive set of blood samples, starting from time zero (t=0, that corresponds to the pre-dose concentration, i.e. the concentration immediately before drug administration) and then drawn at different post-dose times. Obviously, the more samples are drawn, the more the AUC estimation is precise. Nevertheless, in the clinic AUC is rarely calculated as it is time-consuming both for healthcare workers and for patients. Therefore, for those drugs in which a positive correlation between AUC and the pre-dose concentration (or trough concentration or C_{min}) has been demonstrated, this parameter is currently

utilized. Moreover, for those drugs with a concentration-dependent activity, it is advisable to also collect a 2-hours post-dose concentration (or peak concentration or C_{max}). A TDM session is then always made up of a C_{min}, and, in some circumstances, also of a C_{max}.

Once TDM results come out from the lab, they should be carefully interpreted. This step is critical and requires both advanced technological skills and a clinical background to contextualize values at the patient level. There is a multitude of pharmacokinetic software that aid in drug concentration evaluation, either for research purposes and for the routine clinical intent. Depending on the specific aims, the so-called population approach forces input data in a set of predetermined pharmacokinetic models for drug absorption, distribution and covariates and, by means of nonlinear regression analysis, it estimates how well these data fit those of the model. The statistical packages now available (NONMEM, Winnonlin, Pmetrics, ADAPT, just to name a few) are very powerful and sophisticated, but require a steep-learning curve and are somewhat time-consuming, thus quite unpractical to use in the everyday clinical routine. Conversely, one of the most used approaches to optimize dosing regimen is the MAP Bayesian technique that combines two sets of information to estimate the individual patient's pharmacokinetic parameters. Results from previous population pharmacokinetic studies are used as prior information. Then, patient concentrations are combined with prior information to estimate the patient specific pharmacokinetic parameters. From the estimated PK parameters, one can dose the patient to achieve a specific therapeutic target.

At the same time patient's clinical information (drug dose, route of administration, diagnosis, date of therapy initiation, renal and hepatic function evaluations, type and dose of co-administered medications), along with microbiological information (bacterial susceptibility in terms of MIC and site of infection) if dealing with TDM of antibiotics, should be taken into account.

Finally, a clinical pharmacological advice (CPA) reporting drug concentrations coupled with a clinical pharmacological interpretation of these results to the specific patient, should be provided to requesting physician in a reasonable time frame to let him make all the necessary posologic adjustments [87].

3c. The role of Monte Carlo Simulation

Simulation is a general technique that uses mathematics to mimic, or simulate, the operations of a real-world process. It affects our life every day through our interactions with the automobile, airline and entertainment industries, just to name a few [88].

Simulations are based on models that are built on data and look back in time. The purpose of any simulation is to determine the expected outputs of a model in the context of a hypothetical set of inputs and look forward in time.

The models in question can be *deterministic*, in which all the inputs are fixed, or *stochastic*, in which some or all the model parameters have some degree of random variability associated with them. Stochastic elements in a model tend to reflect real-world phenomena, whereas models without stochastic properties tend to reflect ignorance of the system and result in simulations for which there is no uncertainty in the outcome [88].

What is Monte Carlo simulation

Monte Carlo simulation (MCS) was firstly developed and used systematically during the Manhattan Project, the American World War II effort to develop nuclear weapons. John von Neumann and Stanislaw Ulam suggested it to investigate properties of neutron travel through radiation shielding, and named the method after the Monte Carlo Casino in Monaco. They, along with others, used simulation for many other nuclear weapon problems and established most of the fundamental methods of Monte Carlo simulation [89].

Monte Carlo simulation is now a much-used scientific tool for problems that are analytically intractable and for which experimentation is too time-consuming, costly, or impractical. Researchers explore complex systems, examine quantities that are hidden in experiments, and easily repeat or modify experiments [89].

Mathematically, Monte Carlo simulation is a method for *iteratively* evaluating deterministic and stochastic models using sets of random numbers as inputs. This method is often used when the model is complex, nonlinear, or involves more than just a couple uncertain parameters. A simulation can typically involve over 10 000 evaluations of the model, a task which in the past was only practical using super computers. The Monte Carlo method is just one of many methods for analyzing uncertainty propagation, where the goal is to determine how random variation, lack of knowledge, or error affects the sensitivity, performance, or reliability of the system that is being modeled. Monte Carlo simulation is categorized as a sampling method because the inputs are randomly generated from *probability distributions* to simulate the process of sampling from an actual population.

An accurate choice of the type of probability distribution for the inputs is essential for the Monte Carlo technique to give outputs as close as possible to the "real" population. Usually such distributions are those that matches at best data that we already have, or best represents our current state of knowledge [90].

Applications of Monte Carlo simulation in clinical pharmacology

Monte Carlo simulation is essentially the use of a computer software via simulation platforms to "expand" the sample size of a study to provide predictions of the likely result of different therapeutic approaches. MCS allows researchers and clinicians to ask the many 'what if?' questions about different dosing regimens and targets in virtual clinical trials without the capital and human cost of conducting the many possible clinical trials in patient populations [91].

Antimicrobial chemotherapy is probably the pharmacological context in which Monte Carlo simulation has been applied with the greatest success. Indeed, in recent years, hundreds of papers have performed MCS for efficacy purposes with almost any antibiotic, and only some references are here reported [92-98]. Several factors have contributed to it. The first is the identification of affordable PK/PD parameters that well describe the antibacterial killing activity. Except for

antimicrobials, few PK/PD indexes have been reported, and even less are currently used at the patient bedside. The second is the easy availability of the MIC that constitutes the PD parameter of reference. The third is represented by the increasing rates of antimicrobial resistance that advocates for testing of different therapeutic regimens by altering drug dose, frequency of administration, duration of intravenous infusion etc.

In the context of antibiotic dosing, the principal requirements to perform MCS are: (1) a well-evaluated and robust PK model with defined distribution and covariance of PK parameters; (2) a covariate model that provides information about how the PK parameters change with respect to observable physiological signs, symptoms and patient demographics (e.g. how drug clearance changes with renal function); and (3) a PD model with a defined interrelationship between PK and PD. Therefore, to perform MCS to guide antibiotic dosing, the minimum data requirement are the inputs, as described in **Figure 15**.

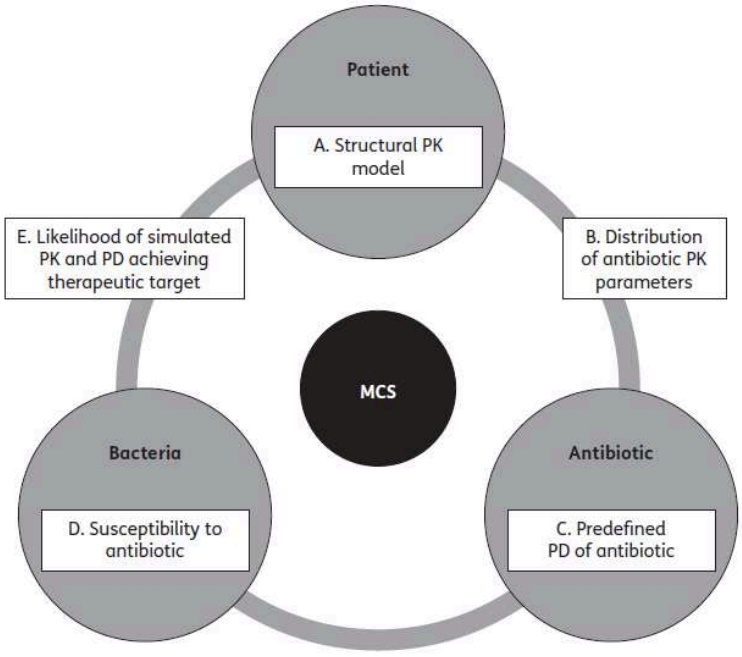

Figure 15. Interrelationship between factors necessary for MCS. Reproduced from [91].

As depicted in **Figure 15**, using the structural PK model and covariate model A (developed in a population PK analysis of patient data), the MCS generates a set of PK parameter values, usually clearance and volume of distribution, by random sampling of values within the predefined PK parameter distribution in B for each simulated patient. From these PK parameters a full antibiotic concentration–time profile, usually AUC, is generated for each simulated patient, which can then be evaluated against susceptibility data, D, in light of antibiotic PD in C. The likelihood of achieving the predefined therapeutic target E, or clinical outcome, is calculated for the entire simulated population.

The probability that the simulated patient can attain the predefined PK/PD target (such as a 40%T>MIC or a specific AUC/MIC value) for a given range of MICs is called *probability of target attainment* (PTA) and it is usually expressed as a percentage of the total predicted attainment. From the analysis of the different PTAs generated by MCS at each MIC, scientists can derive the most appropriate drug regimen that ensure efficacy with respect to the selected pre-specified target.

By introducing different PD parameters with already known PK distributions, MCS could be a useful tool in order to investigate the probability of attaining not even an efficacy threshold, but a toxicity level over which an adverse drug event is most likely to occur. So far, this approach has been attempted by few, as it is very difficult to obtain mathematical functions that describe, for instance, the relationship between drug concentration and the occurrence of a particular toxic effect. This because rate of ADR occurrence is generally low; ADR could be misdiagnosed or caused by co-administered drugs; drug concentration is rarely measured when the ADR presents; ADR could not be dose-related. The only relevant examples of such an investigations have been conducted by Drusano et al. The first paper pertains Monte Carlo analysis on the probability of developing nephrotoxicity at various dosing level of gentamycin [99]. The second is the already mentioned paper on the risk of creatine-phosphokinase elevation when using daptomycin at different doses

[80]. The paramount results of this latter research led to the identification of the trough concentration level of daptomycin not to exceed to minimize the safety risk.

AIMS

The aims of this research are:

1. to investigate the isoniazid dose-concentration relationship in a cohort of TB patients treated with first-line antitubercular drugs
2. to investigate the isoniazid concentration-toxicity relationship in the aforementioned cohort
3. to identify the isoniazid exposure threshold that best distinguishes between patients who will or will not develop hepatotoxicity
4. to investigate the time to hepatotoxicity development since start of therapy
5. to investigate, by means of Monte Carlo simulation, the probability of developing hepatotoxicity at different isoniazid doses both in slow and rapid acetylators

MATERIALS AND METHODS

Study design

This was a retrospective study conducted over eight years of clinical activity (from january 2005 to december 2012) among adult patients affected by TB whose diagnosis, treatment and clinical follow-up were carried out at the Clinic of Infectious Disease of the Azienda Ospedaliero-Universitaria of Udine and who underwent TDM of isoniazid for dosage optimization at the Institute of Clinical Pharmacology of the same hospital, a tertiary-care institution in the North-East of Italy. The study was approved by the regional Ethics Committee, with central notification at the Italian Medicines Agency (AIFA).

Patients suggestive for TB were initially hospitalized until microbiological confirmation allowed for definitive diagnosis and patients became non-contagious, with negative sputum at the microbiological direct examination. Then, usually in the first two months from presentation, patients were discharged from hospital with their first-line antitubercular therapy, and clinical follow-up was undertaken at an ambulatory level directly linked to the Clinic of Infectious Disease and managed by the same health-care givers of that ward.

First-line antitubercular therapy included the standard four-drug combination of isoniazid, at 5 mg/kg daily, rifampicin at 10 mg/kg daily, pyrazinamide at 15-20 mg/kg daily and ethambutol at 15 mg/kg daily for two months; then pyrazinamide and ethambutol were discontinued, and therapy went on only with isoniazid and rifampicin.

Each patient diagnosed with TB underwent a structured and time-scheduled program of ambulatory visits in which laboratory, microbiological and pharmacological examinations were performed until the end of therapy. Patient's complete blood count, renal function (serum creatinine and BUN), hepatic function (ALT, AST, bilirubin, alkaline phosphatase), C-reactive protein were assessed at baseline, i.e. one or two days before the start of therapy, and then at day 7, 14, 30, 60, 90, 120, 150

and 180 of therapy. Sputum examination, even if negative, was repeated monthly and once more after the end of therapy.

As far as the pharmacological monitoring is concerned, approximately one week after the start of therapy nine blood samples for isoniazid exposure determination were drawn on the isoniazid morning administration. One sample was collected shortly before dose administration for C_{min} evaluation and eight samples were collected at 30 minutes and then at 1, 2, 3, 5, 7, 9, 12 hours after dose administration. On the basis of laboratory results, a non-compartmental pharmacokinetic analysis with Winnonlin version 1.1 was carried out. Isoniazid systemic clearance, half-life and $AUC_{0-\infty}$ were then calculated. Patients whose half-life was greater than 130 minutes were considered slow acetylators, the others were globally referred to rapid acetylators. The observed $AUC_{0-\infty}$ was considered equivalent to AUC_{0-24h} as it was calculated over a 24-hours dose interval. Finally a clinical pharmacological advice was provided to requesting physician including isoniazid pharmacokinetic parameters (AUC_{0-24h} and half-life), patient's acetylation status and suggestions for dose adjustments in order to avoid excessive exposure. In this regard, the AUC_{0-24h} threshold considered for dose reduction was 47.95 mgh/L. This value was derived from [100] and corresponded to the mean + 1 SD of slow acetylators AUC_{0-24h}.

Plasma concentrations of isoniazid were analyzed by means of a validated HPLC method, as previously described [101]. Precision and accuracy were assessed by performing replicate analysis of quality controls samples against calibration standards. Intra- and inter-assay coefficients of variation were always < 10%. The low limit of detection was 0.20 mg/L.

Patients' selection criteria

A total of 392 patients were identified as eligible for inclusion in the study. This represented the initial pool of patients for whom a pharmacokinetic study of isoniazid exposure was requested at the Institute of Clinical Pharmacology in the study period. Then, after electronic requests' examination

and medical records' evaluations, a stepwise procedure of patients' exclusions was conducted, according to the following exclusion criteria:

- availability of clinical and laboratory data for all the patients
- diagnosis consistent only with TB and with isoniazid-susceptible strains
- appropriateness of blood sampling for pharmacokinetic assessment
- appropriateness of transaminase monitoring evaluation (patients were excluded if without baseline transaminases assessments and/or transaminases monitoring lasting less than 30 days)
- presence of concomitant liver disease and/or potentially hepatotoxic drugs in addition to antitubercular drugs
- isoniazid dose reduction after the finding of an elevated $AUC_{0\text{-}24h}$ irrespective of transaminase concentration (as an interpretation of exposure-response relationship could not be correctly derived).

Diagram of patients' selection is depicted in **Figure 16**.

Data collection

For each patient included in the study the following data were retrieved: demographic characteristics, clinical characteristics of TB and of any co-morbidity, type, posologic regimen and route of administration of antitubercular drugs and of any co-administered medications, duration of treatment, isoniazid plasma concentrations determined at the day of TDM along with the pharmacokinetic parameters of $AUC_{0\text{-}24h}$, half-life and drug clearance. Hepatic function parameters (ALT, AST, bilirubin and alkaline phosphatase) were retrieved both at baseline and during therapy, according to the pre-defined monitoring schedule.

All clinical information were stored in a MS Access electronic database.

Definition of hepatotoxicity

Hepatotoxicity was defined as an increase of ALT over the ULN (i.e. 51 UI/L) occurred any time during antitubercular treatment. Hepatotoxicity was then classified according to the WHO definition of hepatotoxicity [50]:

- grade 1 (mild): ALT between 51 and 125 UI/L
- grade 2 (mild): ALT between 126 and 250 UI/L
- grade 3 (moderate): ALT between 251 and 500 UI/L
- grade 4 (severe): ALT greater than 500 UI/L

Clinical management of hepatotoxicity was in accordance to the international guidelines (drug suspension when in presence of an ALT increase of more than five times the ULN or more than three times the ULN in presence of symptoms), though at physician discretion in the cases of fewer ATL elevation. Drug re-challenge or definitive drug withdrawal was also decided by the clinician on a case by case approach.

Pharmacokinetic/pharmacodynamic analysis by means of Monte Carlo simulation

A 10.000-subject Monte Carlo simulation was performed (with Systat version 13, Systat Software, Inc., USA) for five different dosing regimens (2.5 mg/kg every 24 hours, 5.0 mg/kg every 24 hours, 7.5 mg/kg every 24 hours, 200 mg every 24 hours and 300 mg every 24 hours) both for slow and rapid acetylators, with the intent to assess the probability of an $AUC_{0\text{-}24h}$ greater than the identified cut-off for hepatotoxicity.

During each simulation a set of clearances and body weights were randomly generated, according to each mean and standard deviation of the population parameters. Two distribution of clearances, one for slow acetylators and the other for rapid acetylators, were derived from patient pharmacokinetic analysis. Likewise, distributions of body weights of the age class 18-65 years old, were calculated from the NHANES data [102]. Clearances were varied according to a log-normal distribution,

whereas body weights were assumed to follow a normal distribution. These two parameters, along with the selected drug dose, directly determined the $AUC_{0\text{-}24h}$ of isoniazid for each patient.

Statistical analysis

The Kolmogorov-Smirnov test was used to assess whether data were normally or non-normally distributed. Accordingly, the mean ± SD or median and IQR were used in the descriptive statistics. Likewise, the strength of a trend between two variables was expressed by means of the Pearson correlation coefficient or the Spearman rank correlation coefficient. Categorical variables were compared by the χ^2 test with Yate's correction or Fisher exact test as necessary, and continuous variables were compared using the Student t-test or Mann-Whitney test. A p-value < 0.05 was required for statistical significance.

Logistic regression analysis was performed using isoniazid $AUC_{0\text{-}24h}$ as independent predictor of hepatotoxicity. Receiver Operator Characteristic curve (ROC curve) was performed in order to identify the $AUC_{0\text{-}24h}$ cut-off that best discriminates between patients with and without hepatotoxicity.

Statistical analysis was performed with Systat version 13 (Systat Software, Inc., USA).

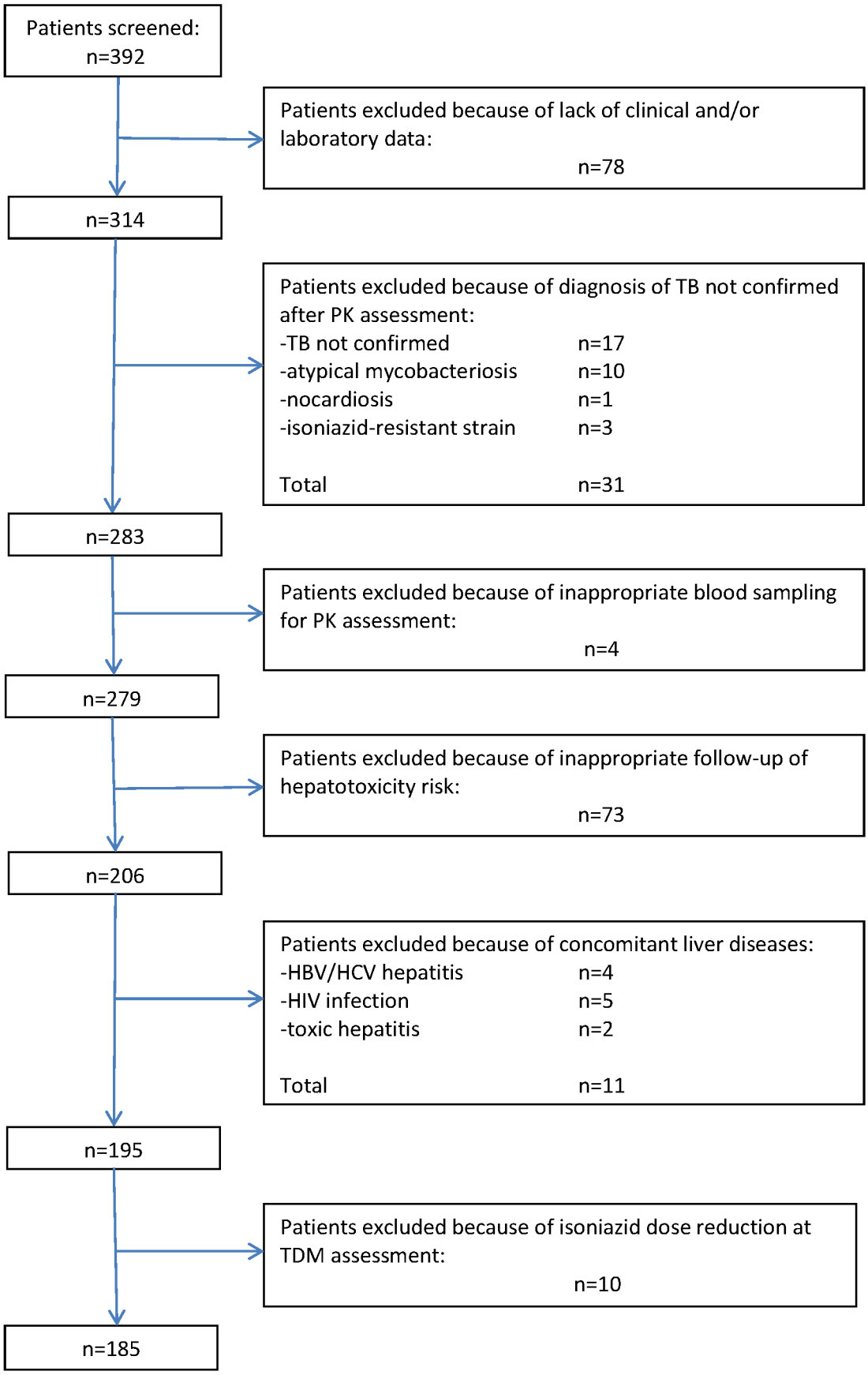

Figure 16. Patients' selection for inclusion in the final study population.

RESULTS

A total of 185 patients have been included for final evaluations. Patients' demographic and clinical characteristics are reported in **Table 5**. The group was quite homogeneous in terms of origin, as it was composed mainly by European and African patients, these ethnicities accounting for 90.8% of the total population. The most represented sites of TB infection were the lungs and the bone in 81% of patients. In 42.2% of patients, other ailments co-existed and thus other co-medications were administered; however, none of them underwent known clinical drug-drug interactions with first-line antitubercular drugs. The rest of the patients did not assume other co-medications. Hepatic function evaluated at baseline was normal in all the patients.

Isoniazid dose was of 300 mg daily, per oral route of administration, in 92% of patients. Fourteen patients started with 200 mg daily, one patient with 150 mg daily. These doses, that corresponded to a median of 4.92 mg/kg daily, thus very closed to the recommended 5 mg/kg daily, gave a median isoniazid $AUC_{0\text{-}24h}$ of 36.51 mgh/L, though with a wide range of variability (between 7.7 and 142.18 mgh/L).

Phenotypic characterization of acetylation status identified 145 slow acetylators (78.4% of patients) and 40 rapid acetylators (21.6% of patients). Mean (±SD) isoniazid half-life, $AUC_{0\text{-}24h}$ and systemic clearance values were 3.56±1.16 h, 42.22±18.07 mgh/L and 8.62±5.43 L/h for slow acetylators, and 1.87±0.2 h, 19.48±8.05 mgh/L and 17.73±7.34 L/h for rapid acetylators, respectively.

Isoniazid concentrations at each sampling time for slow and rapid acetylators are reported in **Figure 17**. In both groups, isoniazid serum concentration declined monoexponentially. A large isoniazid interindividual variability was observed at all the different post-dose concentrations, not only according to the acetylator status, but also inside each group. The isoniazid C_{max} values (range 0.37 to 13.89 mg/L for slow and between 0.33 and 14.71 mg/L for rapid acetylators) were lower than the desired range (<3 mg/L) in 18.6% (27/145) of slow and 20% (8/40) of rapid acetylators, and higher

than the proposed desired range (>5 mg/L) in 66% (96/145) of slow and in 50% (20/40) of rapid acetylators.

Isoniazid dose-concentration relationship both for slow and rapid acetylators is depicted in **Figure 18**. The mean (±SD) total daily exposure to isoniazid ($AUC_{0\text{-}24h}$) was more than two-fold higher in slow than in rapid acetylators (42.22±18.07 *vs.* 19.48±8.05 mgh/L, p<0.001).

No correlation between isoniazid $AUC_{0\text{-}24h}$ and daily dose was observed in any of the two acetylator groups, with an r^2 of 0.0087 for slow and 0.115 for rapid acetylators.

Among the 185 patients treated with first-line antitubercular drugs, 22.16% of patients, corresponding to 41 subjects, developed hepatotoxicity, and were classified according to the WHO classification of hepatotoxicity as reported in **Table 6**. Overall, when considering the relationship between ALT concentrations and $AUC_{0\text{-}24h}$ in the total population, a mild correlation between the two variables emerged (Spearman's rho coefficient = 0.34; p≤0.001). Interestingly, mean (±SD) isoniazid $AUC_{0\text{-}24h}$ of patients who developed hepatotoxicity was significantly higher than that observed in patients who did not develop hepatotoxicity (58.33±18.59 *vs.* 31.28±13.96 mgh/L, p<0.001), as shown in **Figure 19**.

From the ROC analysis, the accuracy of $AUC_{0\text{-}24h}$ in discriminating between patients with and without hepatotoxicity was 0.90 (95% CI: 0.85 – 0.95). The optimal cutoff that minimized the false positives and negatives was 55.0 mgh/L and it correctly classified 87% of subjects (**Figure 20).**

The logistic regression model was utilized to estimate the probability of hepatotoxicity at different isoniazid $AUC_{0\text{-}24h}$. The estimated probability of hepatotoxicity was 50% in the presence of an $AUC_{0\text{-}24h}$ of 53.7 mgh/L and 90% in the presence of an $AUC_{0\text{-}24h}$ of 70.0 mgh/L (**Figure 21**).

The Kaplan-Meier toxicity estimate at 1 month is 21.08%. Among the patients who developed hepatotoxicity, 93% of patients experienced it within the first month (**Figure 22)**.

Finally, a Monte Carlo simulation analysis with the intent of estimating the likelihood of developing hepatotoxicity when using different isoniazid posologic regimens in the two populations of slow and rapid acetylators was carried out (**Table 7**). Isoniazid clearance values were those derived from

the 145 slow acetylators and 40 rapid acetylators as reported above, while mean (±SD) total body weight included in simulations was 79.96±20.73kg. The pharmacodynamic target of toxicity was that identified by the ROC analysis, i.e. an $AUC_{0\text{-}24h}$ of 55.0 mgh/L. On the basis of simulation results, it is predicted, for instance, that a slow acetylator receiving a 2.5- or 5-mg/kg once-daily isoniazid dose will have, respectively 8.89% or 45.7% probability of ALT elevation. Conversely, the probabilities for a rapid acetylator, being administered the same isoniazid doses, will be 0.03% and 3.46%, respectively.

Table 5. Patients' characteristics

Demographics	
Total	185
Sex (male/female)	108/77
Age (years), mean±SD	46.88±21.04
Body weight (kg), mean ±SD	61.84±12.61
BMI, mean ±SD	21.43±4.11
Ethnicity, *n (%)*	
EUR, AMR	139 (75.1)
AFR	29 (15.7)
EMR, SEAR, WPR	17 (9.2)
Diseases's characteristics	
TB localization, *n (%)*	
Lungs	131 (70.8)
Bone	19 (10.2)
Lymph nodes	12 (6.5)
Disseminated	6 (3.2)
Abdominal	4 (2.2)
Laryngeal	2 (1.1)
Meningeal	2 (1.1)
Urinary	2 (1.1)
Other sites	7 (3.8)
Underlying diseases, *n (%)*	
Patients with co-morbidities	78 (42.2)
Co-morbidities, median (IQR)	1.5 (1.0-3.0)
Assessment of hepatic function	
Baseline ALT (UI), median (IQR)	18.0 (13.0-28.0)
Baseline AST (UI), median (IQR)	21.0 (17.0-28.3)
Pharmacological treatments' characteristics	
Isoniazid therapy	
Duration of treatment (days), median (IQR)	204.5 (186.5-278.25)
Day of TDM assessment, median (IQR)	7.0 (6.0-9.0)
Dose/kg/day, median (IQR)	4.92 (4.29-5.29)
$AUC_{0\text{-}24h}$ (mgh/L), median (IQR)	36.51 (21.69-49.39)
Clearance (L/h/kg), median (IQR)	0.13 (0.09-0.21)
Co-medications, *n (%)*	
Patients with rifampicin+ pyrazinamide+ethambutol	185 (100)
Patients with drugs other than antituberculars	77 (41.6)
Co-medications, median (IQR)	3.0 (2.0-6.0)

BMI, body mass index; EUR, Europe; AMR, America; AFR, Africa; EMR, East-Mediterranean Region; SEAR, South-east Asiatic Region; WPR, West-pacific Region; TDM, therapeutic drug monitoring.

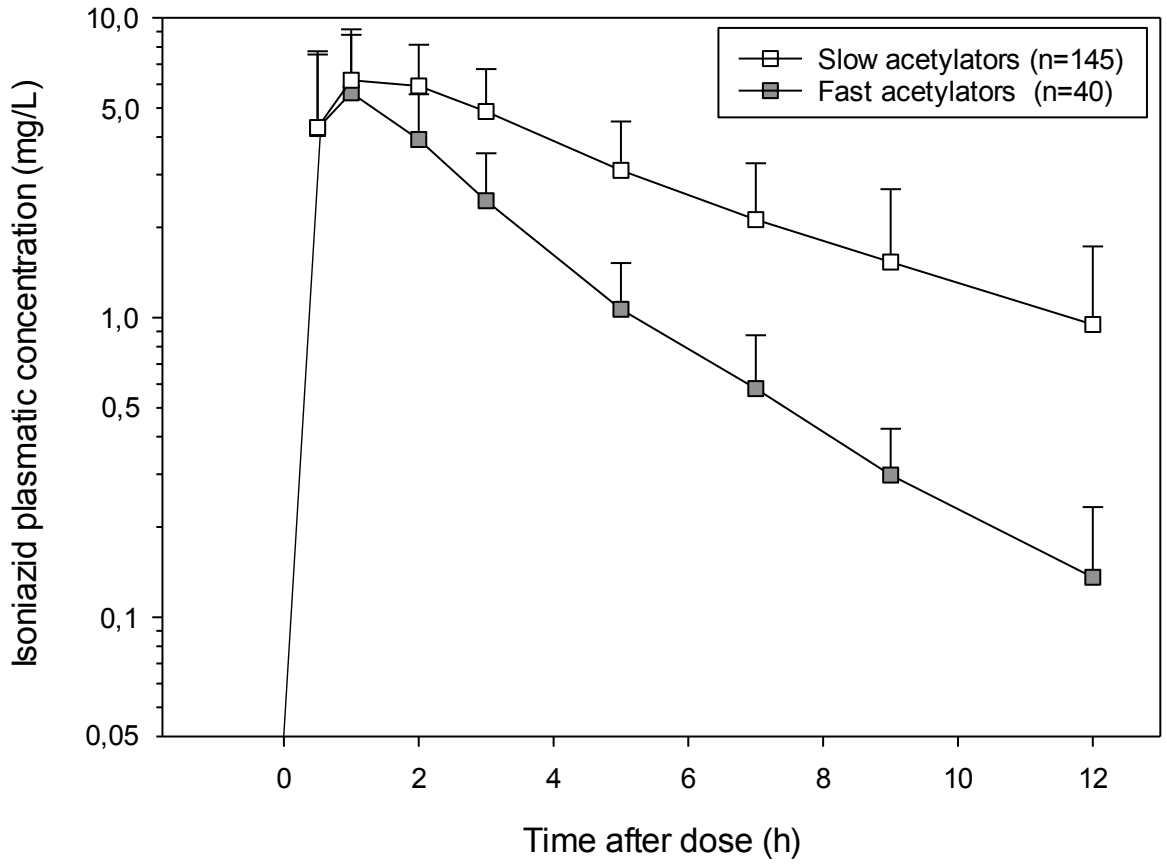

Figure 17. Mean (±SD) isoniazid serum concentration-time curves after single dose in slow and rapid acetylators.

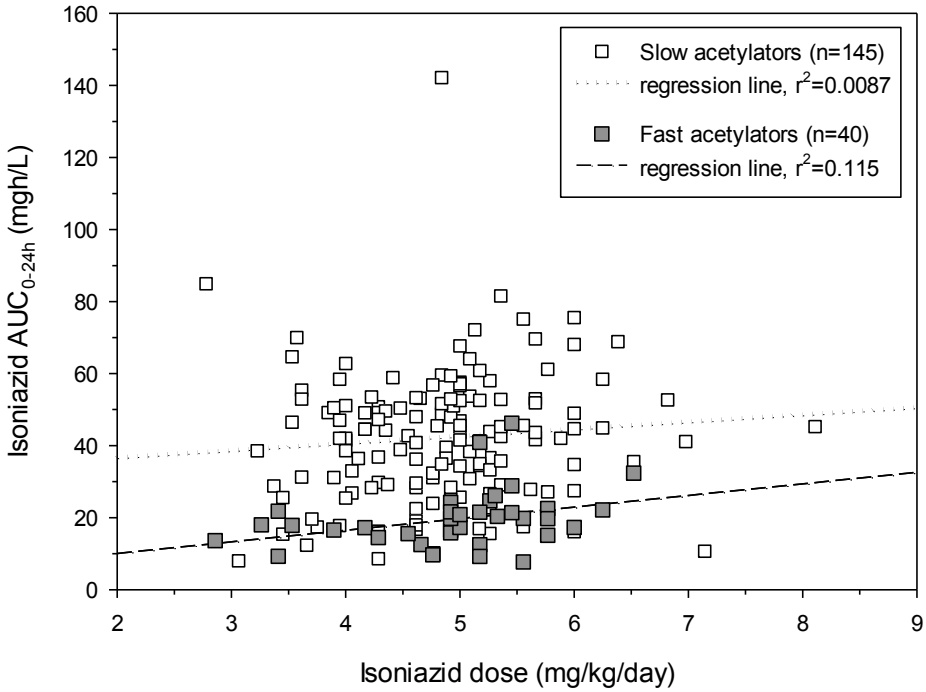

Figure 18. Isoniazid dose-concentration relationship both for rapid and slow acetylators. Each square indicates the plasmatic isoniazid exposure, expressed in terms of $AUC_{0\text{-}24h}$, that corresponds to an administered specific isoniazid dose. Dotted and dashed lines represent regression line between isoniazid $AUC_{0\text{-}24h}$ and dose for slow and rapid acetylators, respectively.

Table 6. Classification of patients who experienced (n=41 patients) or not experienced (n=144) hepatotoxicity among the study population (n=185 patients), according to the WHO definition of hepatotoxicity.

WHO definition of hepatotoxicity	Number of patients	Isoniazid $AUC_{0\text{-}24h}$ (mgh/L)	ALT (UI/L)
No hepatotoxicity	144	29.40 (18.81-42.08)	32.0 (21.0-41.0)
Grade I (mild)	22	56.65 (47.54-65.27)	74.9 (68.0-104.0)
Grade II (mild)	16	57.59 (44.99-69.26)	176.0 (134.8-213.8)
Grade III (moderate)	2	50.80 (45.58-84.95)	259.0 (250.0-399.0)
Grade IV (severe)	1	55.38	1064.0

Values are expressed as median and IQR

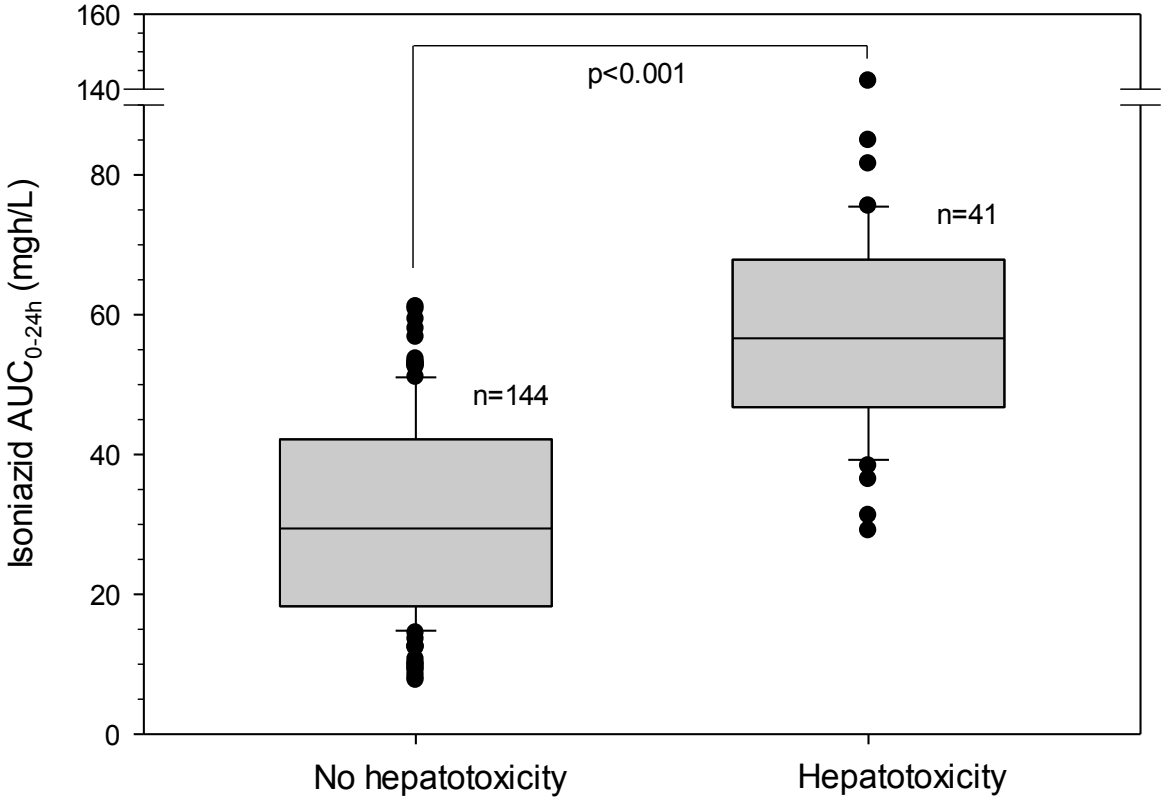

Figure 19. Box (median and 25th to 75th percentile) and whisker (5th to 95th percentile) plots of isoniazid $AUC_{0\text{-}24h}$ observed in patients who experienced and didn't experience hepatotoxicity. Filled circles are outliers.

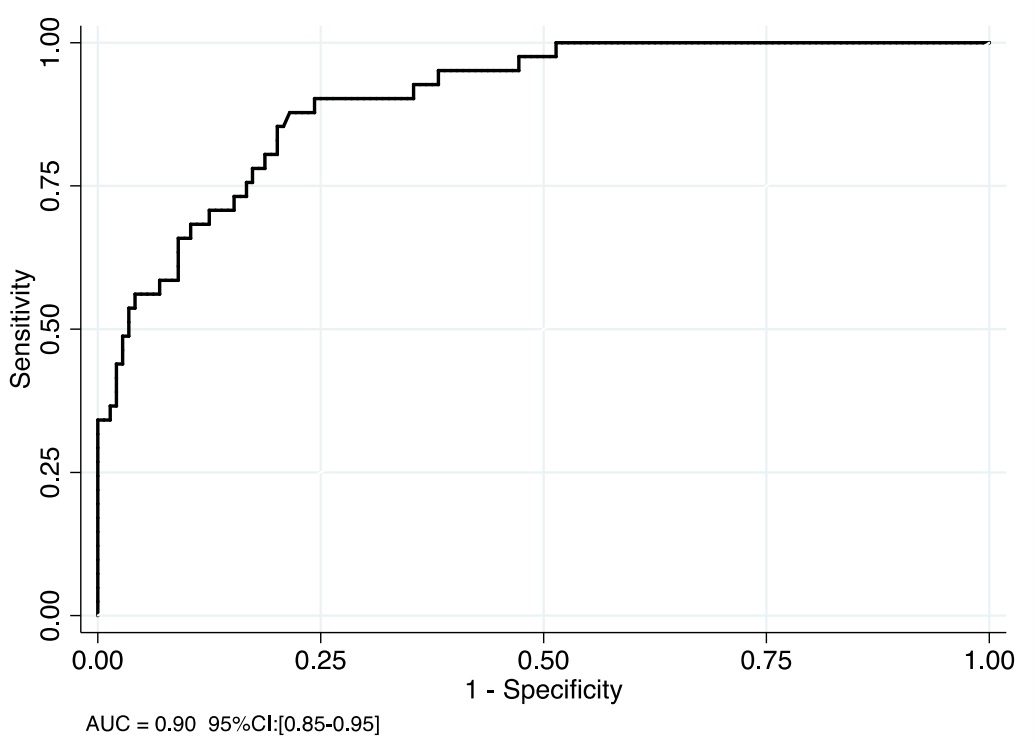

Figure 20. Receiver Operating Characteristic (ROC) curve that illustrates the performance of isoniazid $AUC_{0\text{-}24h}$ in the binary discrimination between the likelihood of developing or not developing hepatotoxicity.

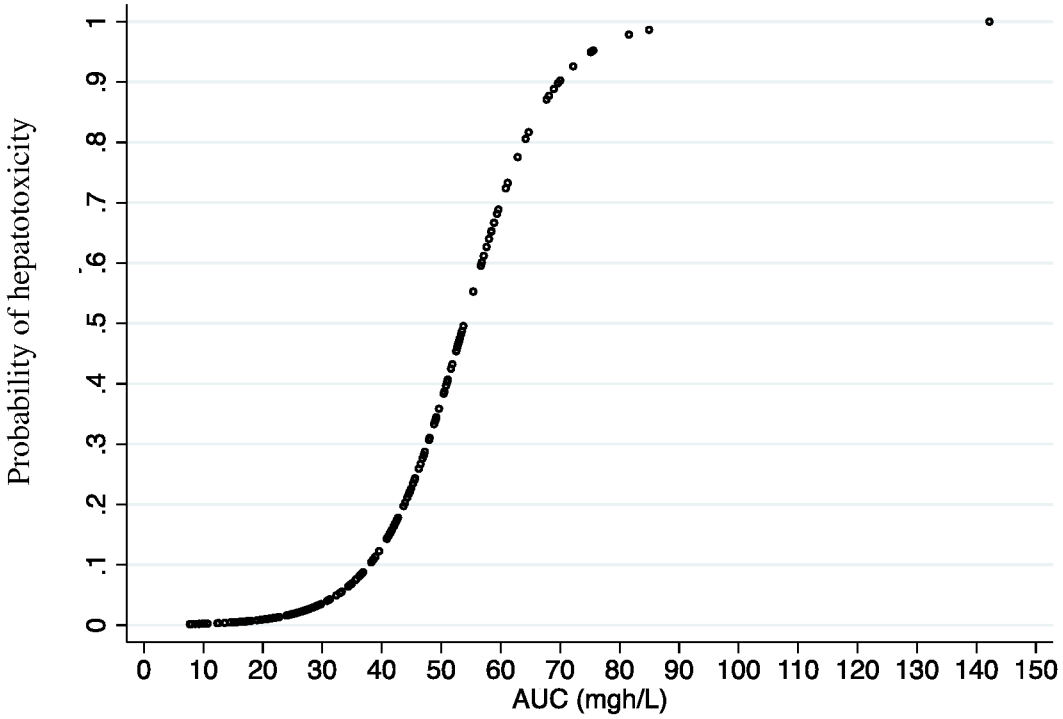

Figure 21. Isoniazid $AUC_{0\text{-}24h}$ and logistic regression model for hepatotoxicity. The symbols refer to the $AUC_{0\text{-}24h}$ observed over time in each patient with or without hepatotoxicity and are imposed on the line of logistic regression.

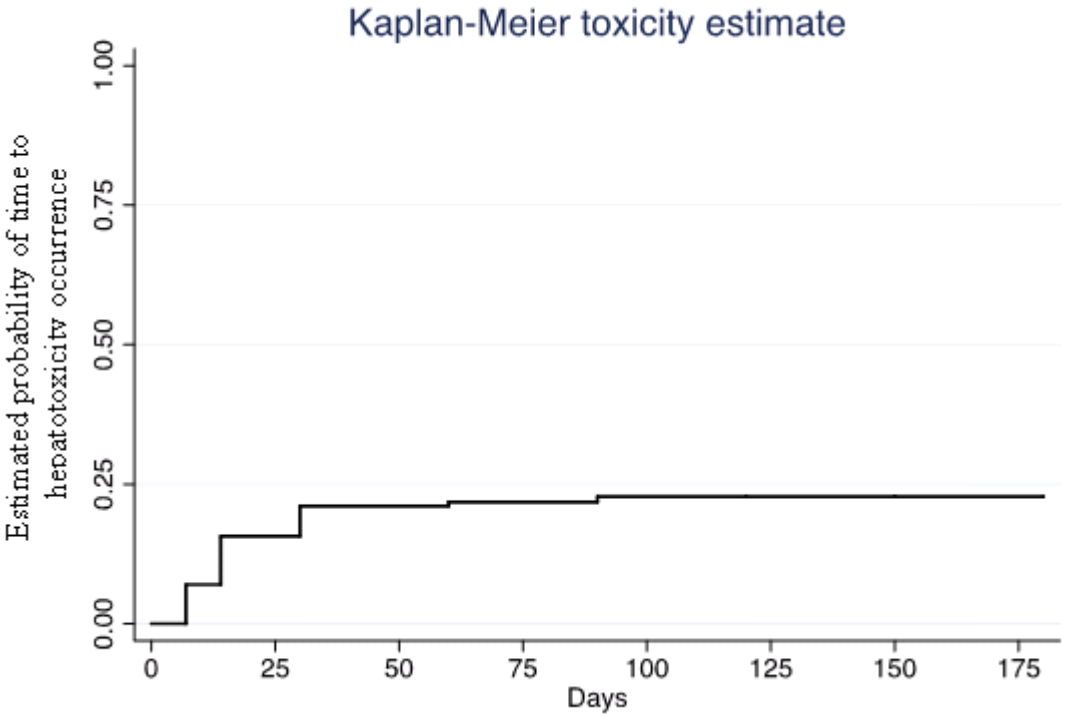

Figure 22. Kaplan-Meier plot showing time to hepatotoxicity occurrence.

Table 7. Probability of elevated alanine-aminotransferase (ALT) level, stratified by dose, as predicted by Monte Carlo simulation.

Daily dose	Probability of ALT elevation for slow acetylators, %	Probability of ALT elevation for rapid acetylators, %
2.5 mg/kg	8.89	0.03
5.0 mg/kg	45.7	3.46
7.5 mg/kg	72.5	18.6
200 mg/die	7.34	0
300 mg/die	26.01	0.28

DISCUSSION

This study aimed at investigating the relationship between isoniazid exposure and the development of hepatotoxicity in adults patients affected by tuberculosis.

In our study population, phenotypic identification by means of the isoniazid half-life cut-off of 130 minutes well identified the two subgroups of slow and rapid acetylators [37]. Slow acetylators accounted for 78.4% of patients: considering the heterogeneity of the patient case-mix (75.1% of Caucasians patients and 15.7% of African patients), this is in line with previously reported data, describing 40 to 70% of slow aceylators in European and North American subjects [103] and up to 86.8% in south-African individuals [104].

The absence of any significant relationship between isoniazid dose and concentration has also been previously reported [65]. Nevertheless, even if slow acetylators showed a two-fold higher mean $AUC_{0\text{-}24h}$ compared to rapid acetylators, thus suggesting a major role of the genetic component in affecting plasmatic exposure, taking acetylator status into account was not helpful in disentangle any association, the regression coefficient between isoniazid dose and $AUC_{0\text{-}24h}$ being very poor within both sub-populations. The clinical consequences of these observations have resulted in the implementation of therapeutic drug monitoring in guiding treatment with isoniazid, as well as with the other antitubercular drugs, with the intent of ensuring adequate concentrations and prevent the development of resistance [105-109].

In our patients, mean isoniazid clearances in rapid and slow acetylators were 17.73 and 8.62 L/h, respectively. Compared to our results, three studies reported similar values. Wilkins et al [104] by pooling together and analyzing by means of a nonlinear mixed-effects model 235 concentration-time measurements from two clinical studies carried out among south-African TB patients, obtained mean apparent clearances of 21.6 L/h and 9.70 L/h for rapid an slow acetylators respectively, and $AUC_{0\text{-}\infty}$ values also similar to ours.

Kinzig-Schippers et al. [110] assessed isoniazid exposure in 18 healthy Caucasian volunteers of 30 years of mean age, with respect to their relative NAT2 genotype, after administration of isoniazid at 100 and 300 mg orally and 200 mg intravenously and interpreted observations by means of a two-compartmental population pharmacokinetic model. Slow acetylators were predicted to have an apparent clearance of 10.0 L/h, intermediate acetylators an apparent clearance of 19.2 L/h and fast acetylators were predicted to have an average apparent clearance of 28.4 L/h.

Donald et al., [65] by analyzing isoniazid serum concentrations from four studies during which a spectrum of isoniazid doses (from 0.2 to 12 mg/kg) was given to groups of adult patients and in whom the NAT2 genotype was also ascertained, tough not reporting isoniazid clearance values, produced $AUC_{0-\infty}$ estimates similar to ours, with the greatest variation associated to the 5 and 6 mg/kg doses, as previously mentioned.

In contrast, Peloquin et al. [111] in a phase I study conducted in 24 north-American healthy males, reported apparent clearances of approximately 15.0 L/h in slow acetylators and 50.0 L/h in rapid acetylators. Also a small steady-state study conducted in Kenya among 29 African patients, half of whom were HIV-infected patients, reported AUC_{0-12h} values substantially lower than ours (4.05 and 12.02 mgh/L in rapid and slow acetylators, respectively), suggesting an enhanced drug clearance in this population [112].

The main reasons for these discrepancies could rely both on differences in study populations (healthy volunteers, HIV-infected subjects, young vs. elderly patients) and on methodological issues in estimating the pharmacokinetic parameters. With respect to this latter point, it should be emphasized that, from a pharmacokinetic point of view, AUC_{0-24h} is the most correct drug exposure parameter that should be taken into account for clearance estimates of any drug. Nevertheless, due to the short isoniazid half-life of elimination, isoniazid trough values at steady state fall below undetectable level within the 24 hours post-dose period of administration both in rapid and slow acetylators; thus the collection of samples up to the 12^{th} hour and the subsequent calculation of an $AUC_{0-\infty}$ correctly estimates the AUC_{0-24h} in each patient. Conversely, while using AUC_{0-12h} as

estimates of $AUC_{0\text{-}24h}$ could be a correct practice for rapid acetylators, it might underestimate drug exposure in the slow phenotype.

Occurrence of hepatotoxicity represented the pharmacodynamic part of this investigation. There is neither a unique definition of hepatotoxicity nor a shared scheme for ALT monitoring among different centers. The ATS recommendation for drug discontinuation (a 3-times increase of ALT concentrations above the ULN with symptoms or a 5-times increase of ALT concentrations above the ULN with or without symptoms) detects hepatic damage only when it has already occurred [49]. Conversely, the WHO classification by including all the spectrum of hepatic damage in terms of ALT increase, offers the advantage of taking into account also less severe transaminase increases (i.e. < 2.5 times he ULN) that could represent the initial evolution of a patent hepatic injury. Using this approach, 22.16% of our patients were classified as having experienced hepatotoxicity, even if only three patients had moderate or severe liver injury. When evaluating isoniazid exposure, patients who experience hepatotoxicity had significantly higher isoniazid mean $AUC_{0\text{-}24h}$ compared to those who did not develop hepatotoxicity (51.33 vs. 31.28 mgh/L). The ROC analysis confirmed that isoniazid $AUC_{0\text{-}24h}$ was a very good biomarker in distinguishing between subjects who developed or not hepatotoxicity, and identified an $AUC_{0\text{-}24h}$ of 55 mgh/L as the most accurate cut-off for this discrimination.

The need for some biochemical indicators besides ALT that might prove useful in the early detection of antitubercular drug-induced hepatotoxicity with the intent to quickly arrest the harmful process, has been recently advocated [113]. Moreover, the kinetic of serum ALT concentration both in predicting the occurrence of drug-induced liver injury and in monitoring its development has also been questioned, especially when considering the current ATS recommendation of measuring ALT at baseline and at 2 weeks only in patients with putative-predictive factors of hepatotoxicity (chronic hepatitis B and C, alcoholic hepatitis, HIV co-infection, pregnant women).

Although a mild increase in ALT concentrations shortly after the introduction of antitubercular drugs has often been documented and it has been commonly referred to as hepatic adaptation, it is difficult to say, by using only ALT monitoring, whether this initial ALT increase will spontaneously resolve or will aggravate toward a true hepatic injury. In this regard, by assessing isoniazid plasmatic exposure along with ALT determination in the first weeks from starting therapy, clinicians could avoid the development of severe hepatotoxicity by reducing drug dose in those subjects having higher isoniazid $AUC_{0\text{-}24h}$ in the presence of an initial elevation of transaminases.

Isoniazid-induced hepatotoxicity is classified as an idiosyncratic ADR thus unpredictable in nature and not related to the pharmacological properties of the drug [114]. Nevertheless, the mechanism underlying isoniazid toxicity by the accumulation of toxic metabolites with slow acetylators having a significantly higher exposure to these molecules with consequent higher rate of toxic effect, has been largely demonstrated [58, 59, 115]. The finding that a mild correlation between isoniazid $AUC_{0\text{-}24h}$ and ALT concentrations has emerged and that patients who developed hepatotoxicity had significantly higher $AUC_{0\text{-}24h}$ than those who did not experienced it, supports the existence of a concentration-toxicity relationship. Compensatory mechanism of detoxification and variable hepatic functions among different patients could explain the remaining part of this variability.

It is worth mentioning a recent prospective study conducted in India in 110 tuberculosis patients who were administered the standard four-drug antitubercular regimen, had plasmatic $AUC_{0\text{-}4h}$ of all drugs assessed at 1, 7 and 14 days of treatment and were followed for the development of drug-induced hepatotoxicity [116]. Surprisingly, the authors found that only plasma rifampicin concentrations independently predicted subsequent development of hepatotoxicity, these being the only significantly higher concentrations in the 15 cases compared to controls. Generally speaking, rifampicin is considered by far less hepatotoxic than isoniazid and pyrazinamide and it contributes to isoniazid toxicity by inducing isoniazid hydrolase thus increasing hydrazine production [73]. Moreover, the assessment of isoniazid exposure by means of an $AUC_{0\text{-}4h}$ is an important flaw of

that investigation, as it seriously underestimated all drug exposures, especially those of isoniazid in which differences in the acetylation status primarily impact on the elimination phase.

Some authors have proposed the idea of adjusting isoniazid dosage on the acetylator status, administering a lower dosage in slow acetylators to reduce the risk of hepatotoxicity and a higher dosage in fast acetylators to increase the early bactericidal activity and thereby lower the probability of treatment failure [35]. In particular, instead of the standard 5 mg/kg isoniazid dose, 2.5 mg/kg, 5 mg/kg and 7.5 mg/kg daily should be administered to subjects with none, one or two rapid NAT2 alleles, respectively, to achieve similar isoniazid exposure [110]. This seems logical, and our results from Monte Carlo simulation support this approach in terms of risk of developing hepatotoxicity with respect to the different isoniazid doses investigated. Indeed, administering 2,5 mg/kg instead of 5 mg/kg to slow acetylators reduced the risk of ALT increase of more than 5-fold. However, attention should be paid in using 7.5 mg/kg in fast acetylators, as a non-negligible risk appeared even in this group.

This study has some limitations. The retrospective nature of this investigation and the concurrent administration of other potential hepatotoxic drugs are probably the most relevant. Although isoniazid $AUC_{0\text{-}24h}$ was significantly different between patients with and without hepatotoxicity and resulted a valuable pharmacokinetic parameter in discriminating between the two groups, a synergistic role of pyrazinamide and rifampicin in causing hepatotoxicity could not be definitely ruled out. Additionally, an overestimation of the risk of developing serious hepatic damage could be generated, as not all of the patients with a mild ALT increase even if in presence of an elevated isoniazid plasmatic exposure would develop serious hepatotoxicity.

In conclusion, this study, by showing an association between higher plasma isoniazid concentrations and the risk of ALT increase, helps in furthering our understanding of the development of isoniazid-induced hepatotoxicity. Plasma isoniazid $AUC_{0\text{-}24h}$ is a reliable tool in estimating those patients more likely to develop ALT increases, and could be more widely implemented into clinical practice. To achieve this goal, both confirmatory results from prospective

clinical studies and the adoption of less demanding strategies for estimating isoniazid plasmatic exposure, such as limited sampling strategies, are clearly warranted.

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