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INCIDENCE, PREDICTORS AND BIOMARKERS FOR ANTIRETROVIRAL AND/OR ANTI- TUBERCULOSIS DRUGS INDUCED LIVER INJURY

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ABSTRACT

Anti-tuberculosis and/or antiretroviral drugs induced liver injury (DILI) is a major challenge when managing TB and/or HIV patients. The aims of this thesis were to identify incidence, risk factors, and management of DILI among 4 different treatment groups namely; HIV positive individuals with no TB co-infection and had a CD4 count of ≤ 200 cells/ μ l (taking ARV drugs alone = arm 1), TB-HIV co-infected patients with a CD4 count of ≤ 200 cells/ μ l (taking both anti-TB and ARV drugs = arm 2), TB-HIV co-infected patients with a CD4 count of > 200 cells/ μ l (taking anti-TB alone = arm 3), HIV negative TB patients (taking anti-TB alone = arm 4).

Newly diagnosed TB and/or HIV patients were prospectively followed for 56-weeks after initiation of anti-TB and/or ARV treatment. All patients were evaluated clinically and biochemically for development of DILI in each visit. Laboratory tests performed include; hepatitis B surface antigen and anti-hepatitis C virus antibody. Liver enzymes and function tests were measured before and during therapy. Associations of DILI with CYP2B6, CYP3A5, NAT2 and UGT2B7, ABCB1, SLCO1B1 genotypes as well as plasma efavirenz and 8-hydroxyefavirenz concentrations were evaluated.

In the pilot study which involved HIV positive and negative TB patients (n=197), who were taking anti-TB alone, the incidence of DILI was 17.3%. DILI was noted to have a statistically significant association with having a lower CD4 count and concomitant drug intake.

The main study, which involved the 4 different arms (n=953) showed that incidence of DILI was still high and significantly associated *with the specific arm the patient belonged to*. The highest incidence was observed in arm-2 (23.5%) >arm-3 (11.6%) >arm-1 (8.1%) >arm-4 (2.8%). DILI was significantly associated with lower baseline platelet, albumin, and CD4 count. Moreover, higher plasma viral load, EFV level, baseline ALT, AST, ALP, and CYP2B6*6 were also good predictors for development of DILI among arm 1 patients. Similarly, a statistically significant association between

DILI and female sex, higher plasma efavirenz level, efavirenz/8-hydroxyefavirenz ratio, baseline AST, ALT, lower haemoglobin, and serum albumin was observed among participants in arm 2. NAT2 slow-acetylator, CYP2B6*6/*6, and ABCB1 3435TT genotype were also seen to contribute for development of DILI in arm 2 patients. The median time for development of DILI was 1-2 weeks after initiation of treatment, depending on the arm, with the majority developing it in the first 8 weeks.

In conclusion anti-TB and/or ARV DILI is found to be a major problem among TB and/or HIV patients in Ethiopia. Hence, regular monitoring of liver enzymes during early therapy is recommended for better management. Particularly among those with an underlying risk factors; female, concurrent anti-TB and ART, advance HIV disease, elevated liver enzymes, lower haemoglobin, albumin and BMI at baseline, elevated plasma efavirenz level, having CYP2B6*6/*6 and ABCB13435TT genotype and slow acetylation status.

LIST OF PUBLICATIONS

- I. **Getnet Yimer**, Getachew Aderaye, Wondwossen Amogne, Eyasu Makonnen, Eleni Aklillu, Lars Lindquist, Lawrence Yamuah, Beniyam Feleke, and Abraham Aseffa. Anti-Tuberculosis Therapy-Induced Hepatotoxicity among Ethiopian HIV Positive and Negative Patients. PLoS ONE. 2008; 3(3): e1809.
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- IV. **Getnet Yimer**, Marcus Gry, Getachew Aderaye, Wondwossen Amogne, Abiy Habtewold, Eyasu Makonnen, Ina Schuppe-Koistinen, Lars Lindquist, Eleni Aklillu. TB-HIV co-infection and concomitant anti-TB and HAART increases the risk for drug induced liver injury: A prospective four arm cohort study. Manuscript

The papers will be referred in the text by their roman numerals (I-IV)

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LIST OF ABBREVIATIONS

χ^2	Chi square
μl	Micro liter
ADR	Adverse Drug Reaction
AIDS	Acquired Immuno Deficiency Syndrome
ALT	Alanine amino transferase
ARV	Antiretroviral
AST	Aspartate amino transferase
BMI	Body Mass Index
C	Cytosine
CD	Cluster of Differentiation
CDC	Centers for Disease Control
CI	Confidence Interval
CNS	Central nervous system
COA	Coenzyme A
CRF	Case report form
CYP	Cytochrome P
DILI	Drug Induced Liver Injury
DNA	Deoxy Ribo Nucleic acid
dNTPs	Deoxy Nucleotide Tri Phosphates
DOTS	Directly Observed Therapy Short course
EDTA	Ethylene diamino Tetra Acetic acid
EFV	Efavirenz
EH	Ethambutol, Isoniazid (fixed dose)
ERHZ	Ethambutol, Rifampicin, Isoniazid, Pyrazinamide (RHZ is fixed dose)
ESR	Erythrocyte Sedimentation Rate
FDA	Food and Drug Administration
FNA	Fine Niddle Aspirate
G	Guanine
GCP	Good clinical practice
GSTM	Glutathione <i>S</i> -transferase
GWAS	Genome-wide association study
HAART	Highly Active Antiretroviral Therapy
HBCs	High-TB burden countries
HBsAg	Hepatitis B Surface antigen
HCl	Hydro chloric acid
Hct	Hemathocrit
HCV	Hepatitis C Virus
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
INH	Isonicotinic acid hydrazine
LC/MS	Liquid chromatography–mass spectrometry
mRNA	Messenger Ribo Nucleic acid
MS	Mass spectrometry
MTB	Mycobacterium tuberculosis

NAT	N-Acetyl transferase
ng	Nano gram
NNRTIs	Nonnucleoside analog reverse transcriptase inhibitors
NRTIs	Reverse transcriptase inhibitors
NVP	Nevirapine
OH	Hydroxy
PAS	Para-amino salsalic acid
PCP	Pneumocystis jirovecii pneumonia
PCR	Polymerase Chain Reaction
PIs	Protease inhibitors
PLWHA	People living with HIV/AIDS
RFLP	Restriction Fragment Length Polymorphosim
RH	Rifampicin, Isoniazid (fixed dose)
RHZ	Rifampicin, Isoniazid, Pyrazinamide (fixed dose)
RIF	Resistance to rifampicin
rpm	Rotation per minute
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
SPSS	Statistical Package for Social Science
SRHZ	Streptomycin, Rifampicin, Isoniazid, Pyrazinamide (RHZ is fixed dose)
T	Thymine
TB	Tuberculosis
USA	United States of America
UNL	Upper normal limit
UNAIDS	Joint United Nations Programme on HIV/AIDS
VCT	Voluntary Counseling and Testing
WHO	World Health Organization

INTRODUCTION

1. BACKGROUND

WHO defines adverse drug reaction (ADR) as: a response to a drug that is noxious and unintended and occurs at doses normally used for the prophylaxis, diagnosis or therapy of disease, or for modification of physiological function [1]. Studies have shown that, development of adverse drug reactions is a major public health problem contributing for a sizable percentage of admissions, mortality, morbidity and increased healthcare costs [2,3]. Drug induced liver injury (DILI) is the most frequent single cause of adverse drug reactions that has been cited as a reason for withdrawal of an already approved drug from the market and for the non-approval of drugs by Food and Drug Administration (FDA) for the past 50 years [4,5].

1.1. DRUG INDUCED LIVER INJURY (DILI)

Drugs in general account for 20-50% of all instances of fulminant hepatic failure. Globally, data on the incidence of DILI caused by different drugs remain perplexing [6-8]. The incidence reported is also far beyond the number of actual cases detected during patient care [9]. Besides to its effect on health, DILI also causes a major economic impact as it is the most frequent cause of post-marketing withdrawal of new medications. The good thing about DILI, however, is most of the cases are benign, and improve after drug withdrawal. Hence, it is important to identify and withdraw the offending agent as quickly as possible to prevent its progression [10].

The major reasons for the liver to be prone for xenobiotic-induced injury are; its central role in xenobiotic metabolism, its portal location within the circulation, and its anatomic and physiologic structure [7]. Anatomically, the liver is divided into multiple lobules, each centered around a terminal hepatic (central) venule and surrounded peripherally by six portal triads. Afferent blood is supplied by the portal venule and hepatic arterioles of the portal triads, flow through the hepatic venous sinusoids, and empties into the terminal hepatic venule [7].

The regional pattern of hepatocellular necrosis observed with some xenobiotic-induced liver injuries can also be easily understood by dividing the liver into functional subunits referred to as acini. Each liver acinus is divided into three concentric zones of hepatocytes radiating from a portal triad and terminating at one or more adjacent terminal hepatic venules. Hepatocytes closest to the portal triad (zone one) receive blood most enriched with oxygen and other nutrients and are most resistant to injury. Hepatocytes more distal to the blood supply receive a lower concentration of essential nutrients, making them more susceptible to ischemic or nutritional damage. Most important for xenobiotic-induced hepatic damage, the centrilobar (zone three) hepatocytes are the primary sites of cytochrome P450 enzyme activity, which frequently makes them most susceptible to xenobiotic-induced liver injury [7,11].

1.1.1. DEFINING DRUG INDUCED LIVER INJURY

One of the major challenges that researchers face when conducting studies on DILI is the absence of standard definition. One of the most commonly used definitions for DILI is what is taken from Council for International Organizations of Medical Sciences (CIOMS) scale which defines DILI by combining basic principles of 'chronological criteria' (establishing a temporal relationship between the drug treatment and reaction) and 'clinical criteria' (the exclusion of alternative causes for the particular pattern of liver injury) to determine the probability of the reaction being related to the drug [12]. The other criteria which is widely used to define DILI is the Hy's rule, it defines DILI as a serum ALT level greater than or equal to 3 times the ULN plus a serum bilirubin level greater than or equal to 2 times the ULN and this has also been advocated by the US Food and Drug Administration for use in the assessment of the hepatotoxicity of newly developed drugs [13].

The other major challenge that researchers encounter when trying to define DILI is, the practice of using several delineations for UNL of liver enzymes such as ALT with no recognized global reference [14], which is highly dependent on the population being studied and the reagent and instrument used to measure the enzymes. Consequently, the

definition of DILI remains to be a challenge until a reliable and accessible biomarkers are developed.

Consequently, whenever DILI is suspected, it is essential to gather additional clinical and laboratory information necessary to rule out other differential diagnosis. It is also always important to observe the timing in relation to drug intake with the course of injury, and to seek other potential causes for the liver injury. Some of the common differential diagnosis which could give similar picture as that of DILI include: acute viral hepatitis A, B, or C; concomitant use of a hepatotoxic drug or exposure to other hepatotoxins including traditional drugs; autoimmune or alcoholic hepatitis; biliary tract disorders; and circulatory problems of hypotension or right heart congestive failure that may cause ischemic or hypoxic hepatopathy. As part of the investigation, it is therefore important to assess patients for previously existing liver disease, such as chronic hepatitis C or nonalcoholic steatohepatitis. It should also be recognized that DILI may occur in individuals with pre-existing liver disease as a superimposed problem [10,14-35].

1.1.2. PATHOPHYSIOLOGY AND MECHANISM OF DRUG INDUCED LIVER INJURY

The pathophysiology of DILI covers a broad spectrum of abnormalities, from acute hepatic necrosis, chronic hepatitis, and vascular injury to bile ductular injury and neoplasms. These injuries resemble almost all known liver diseases and there are no pathognomonic findings, even upon liver biopsy, that make diagnosis of DILI certain. In general, the type of pathology depends on the duration of injury and the histological location of damage. DILI could also be categorized as acute or chronic, and either as hepatitis, cholestatic, or a mixed pattern of injury [10,14-35]. The hepatitis pattern is characterized by hepatocyte necrosis and is associated with a poor prognosis. There are however, three types of cholestatic DILI: bland cholestasis is the result of abnormal biliary secretion, and is not accompanied by significant hepatocellular damage while cholestatic hepatitis (mixed type) refers to cholestasis with concomitant hepatic

parenchymal damage; and the third form of cholestasis is defined by the presence of bile duct injury or cholangitis [10,14-35].

Drugs induce liver injuries by different mechanisms. The mechanisms identified so far can be broadly categorised as intrinsic (drugs that directly affect the liver) and idiosyncratic (drugs that mediate an immune response).

1.1.2.1. Intrinsic (Direct) Drug Induced Liver Injury

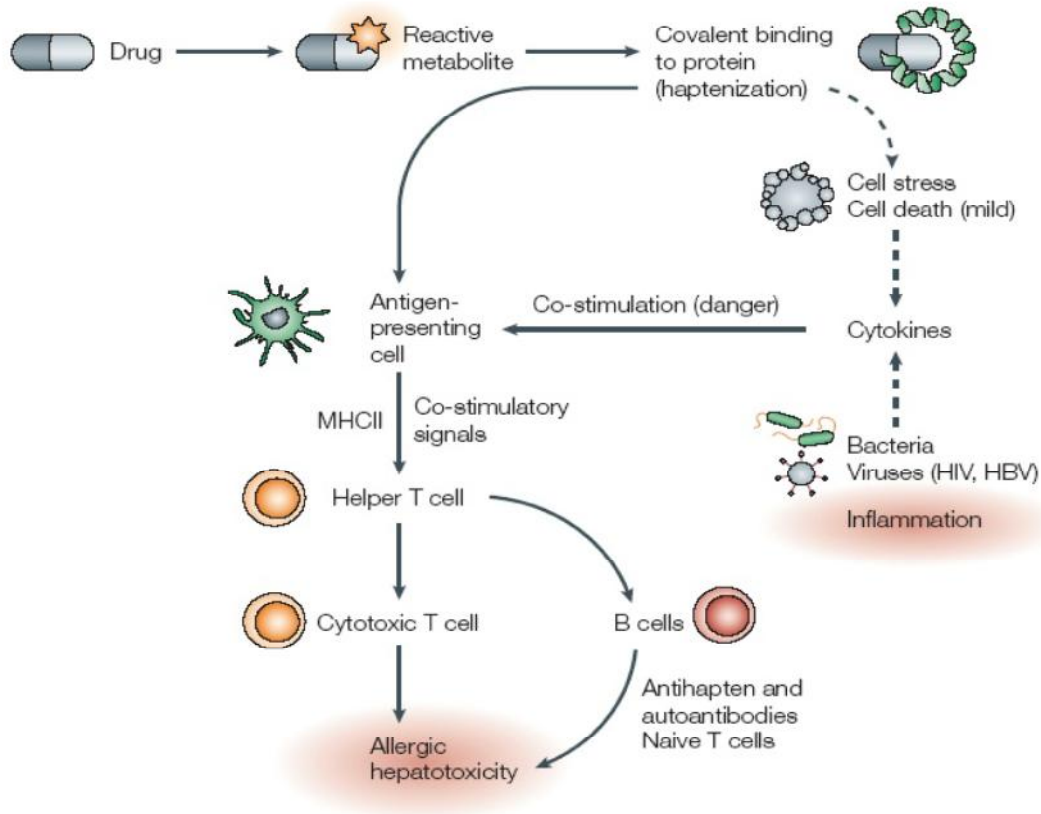
Intrinsic injuries are predictable and reproducible in that threshold dose exists in all individuals typically leading to zonal liver cell necrosis accompanied by little or no signs of inflammation. The injury can be due to the drug itself or to a metabolite and are generally the result of phase I bioactivation with damage mediated by reactive drug metabolites. Fortunately, drug candidates that induce significant direct hepatotoxicity at therapeutic doses are more likely to be detected during preclinical toxicity screening and thus rarely reach the pharmaceutical market [7,10,15-17,22-24,36].

1.1.2.2. Idiosyncratic (Immune-Mediated) Drug Induced Liver Injury

The nature of idiosyncratic liver injuries suggests that most of them are mediated by an immune mechanism. Idiosyncratic drug reactions can still be subdivided into those who have hypersensitivity or immunoallergic (drugs causing hypersensitivity reactions) and those that are metabolic-idiosyncratic (reaction occurs through an indirect metabolite of the offending drug) [7,10,15-17,22-24,36].

Moreover, in most instances of DILI, it appears that hepatocyte damage triggers the activation of other cells, which in turn can initiate an inflammatory reaction and/or an adaptive immune response. These secondary events may overwhelm the capacity of the liver for adaptive repair and regeneration, thereby, contributing to the pathogenesis of liver injury [7,10,15-17,22-24,36].

Figure 1: Mechanisms of liver injury [36].



Not all drugs fall precisely into one of these categories when causing DILI. An overlapping mechanisms may also occur with some drugs [37]. The Pathophysiologic mechanisms of DILI could also be different for different drugs and it includes the following: disruption of the hepatocyte involving covalent binding of the drug to intracellular proteins causing a decrease in ATP levels, leading to actin disruption. Disruption of the transport proteins at the canalicular membrane interrupting the bile flow leading to loss of villous processes and interruption of transport pumps thereby preventing the excretion of bilirubin, causing cholestasis. This leads to cytolytic T-cell activation which involves covalent binding of a drug to the P-450 enzyme acting as an immunogen, activating T cells and cytokines and stimulating a multifaceted immune

response. Other mechanisms involved include activation of the apoptotic pathways by the tumor necrosis factor- α , inhibition of mitochondrial function resulting in decreased ATP production and bile duct injury by toxic metabolites excreted in bile [37].

1.1.3. DRUGS ASSOCIATED WITH LIVER INJURY

Of the estimated 10,000 documented human drugs, more than 1,000 have been associated with DILI, although causality has not always been established clearly [38]. In the last few years, the US FDA has withdrawn 2 drugs from the market for causing severe liver injury: bromfenac and troglitazone. Other drugs that have significant limitations of use because of their hepatotoxic effects include felbamate, an antiepileptic used for complex partial seizures; zileuton, indicated for asthma; tolcapone, used for Parkinson disease; trovafloxacin, an antibiotic; benoxaprofen, an NSAID; and tienilic acid, a diuretic [37]. Moreover, three of the first-line anti-TB drugs i.e. isoniazid, rifampicin and pyrazinamide and all classes of antiretroviral drugs are also known to cause DILI [39-46].

Even if a drug is identified to cause DILI, a number of other factors will also be considered, and is not always the case that the drug will be withdrawn from the market because of the already predicted adverse effects.

1.1.4. RISK FACTORS FOR DRUG INDUCED LIVER INJURY

Even if a number of clinical studies have been conducted to identify risk factors for DILI, it is not yet identified as to why only some people develop mild or severe DILI in response to a hepatotoxic drug, while others show no DILI or seem to be adapting to the possible side effects. FDA claims that, to identify risk factors and to extend the understanding, it is currently working with industry, academia, and other experts on the biochemical and genetic bases of DILI. Some of the risk factors proposed from different clinical studies [29,30,32] involve a complex relationship between the chemical properties of the drug, as well as environmental and genetic factors. Risk factors identified so far include: age (elderly persons are at increased risk of hepatic

injury because of decreased clearance, drug-drug interactions, reduced hepatic blood flow, variation in drug binding, poor diet, more susceptibility to infections, multiple hospitalizations, and lower hepatic volume), sex (although the reasons are unknown, hepatic drug reactions are more common in women as compared to men), alcohol ingestion (alcohol induces liver injury and cirrhotic changes that alter drug metabolism and in addition it causes depletion of glutathione stores that make the person more susceptible to toxicity by drugs), liver disease (though not uniformly, patients with chronic liver disease are at increased risk of hepatic injury), and concurrent infections: patients with AIDS, patients with hepatitis B or C virus infection and malnutrition [37,47-54].

2. TUBERCULOSIS

It is estimated that *Mycobacterium tuberculosis* has infected more than 2 billion people with 8-9 million new TB cases occurring each year with a yearly mortality ranging from 1.3-2 million [55]. Global incidence figures on TB shows that the peak was around 2003 with a slow decline to reach 8.8 million in 2010 [55].

In 2010, from the total cases notified, 82% were from the 22 high-TB burden countries (HBCs) with China and India taking the lion share, i.e., 40% from the total cases [55].

Ethiopia ranks 9th among the high burden countries with an incidence rate of 220 per 100,000 populations [55].

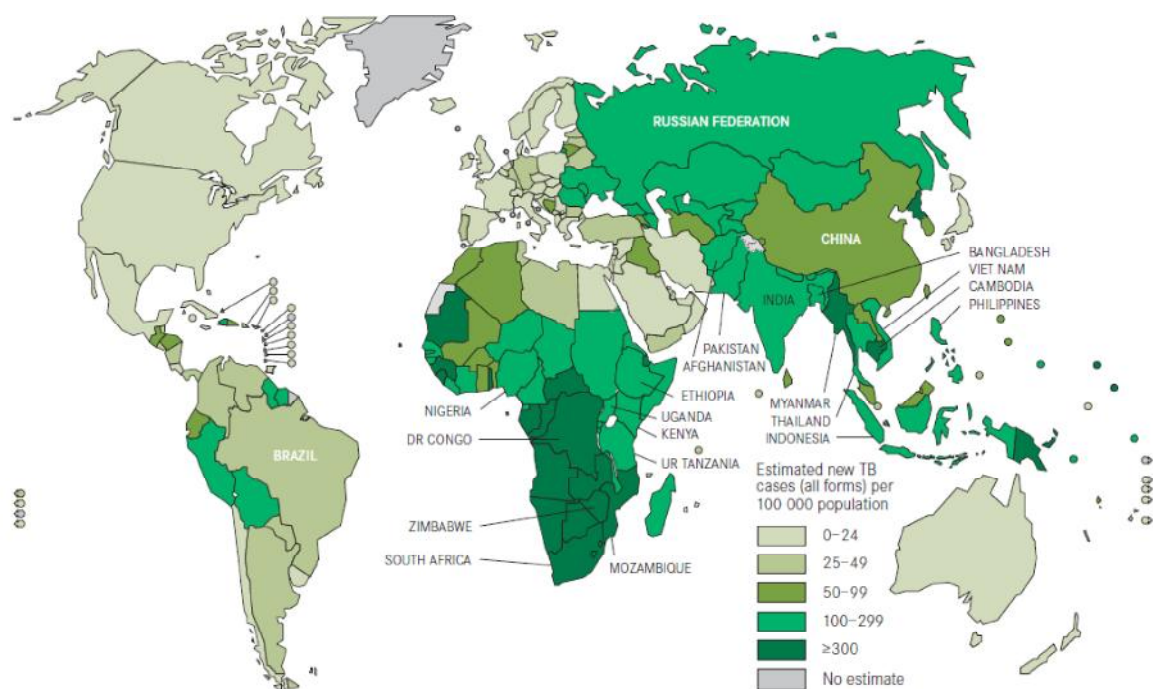


Figure 2. Estimated TB incidence rates, 2010 (WHO)

2.1. TREATMENT OF TUBERCULOSIS

WHO recognizes the goals of TB treatment to be; ensuring cure without relapse, preventing death, stopping transmission, and preventing the emergence of drug resistance [56]. Regularity and completeness is paramount in the treatment of TB, since default, interruption and incorrect dosing may cause failure or relapse and development of drug resistance [57].

The treatment of TB in general has witnessed a number of important changes over the years [57,58]. Directly Observed Therapy, short-course (DOTS) was introduced by WHO in 1993 as the global TB control strategy and it represents both an approach for treating active TB and a strategy for reducing the prevalence of TB in the population [59]. The DOTS strategy produces cure rates of up to 95% even in the poorest countries and prevents new infections by curing infectious patients [60].

In Ethiopia, the geographical coverage of DOTS has reached 95%, however, due to limited health infrastructure in the country, only 64 % of the population has access to the services [61,62].

2.2. ANTI-TUBERCULOSIS DRUGS

For a disease that has been in existence since antiquity and has left its imprint on the history of mankind, specific treatment aimed at the causative agent became available for the first time only in 1944 with the discovery of streptomycin by Selman Waksman [63]. Before that time, empirical measures such as blood letting, horse riding, sea voyages, graded exercise, absolute bed rest, injections of extracts of gold or other heavy metals, artificial pneumothorax, thoracoplasty, and various other exotic remedies were practised, usually in the setting of a sanatorium, and without much success. Soon after streptomycin, para-amino salicylic acid in 1949 and isoniazid in 1952 became available; heralding the era of effective anti-TB treatment. This treatment required prolonged duration of up to 18-24 months. The discovery of rifampicin in the late 1960s and the re-discovery of the anti-mycobacterial activity of pyrazinamide soon after, were major breakthroughs in the treatment of TB that made it feasible to shorten the duration of treatment considerably [64].

Almost all recommended treatment regimens have two phases, based on extensive evidence from controlled clinical trials. There is an initial intensive phase where at least two mycobactericidal drugs, isoniazid and rifampicin, are used. Pyrazinamide is given in the intensive phase allowing the duration of treatment to be reduced from 9 to 6 months, with the addition of ethambutol benefiting the regimen when initial drug resistance may be present or the burden of organisms is high [56]. The intensive phase is followed by a continuation phase in which 2 drugs; rifampicin and isoniazid are administered for 4 or more months [47,65].

3. HIV

The estimated number of people living with HIV in 2010 was 33.3 million (31.6-35.2 million) of whom the total number of deaths due to AIDS in the same year was 1.8 million (1.6-1.9 million). The majority of the deaths (76%) occurred in sub-Saharan Africa where more than two thirds (68%) of adults and nearly 90% of children who were infected with HIV reside [66,67].

Ethiopia is among the countries most heavily affected by the HIV epidemic. The current cumulative number of PLWHA in Ethiopia is about 1.5 million, with 95,000 under 15 years of age [67,68].

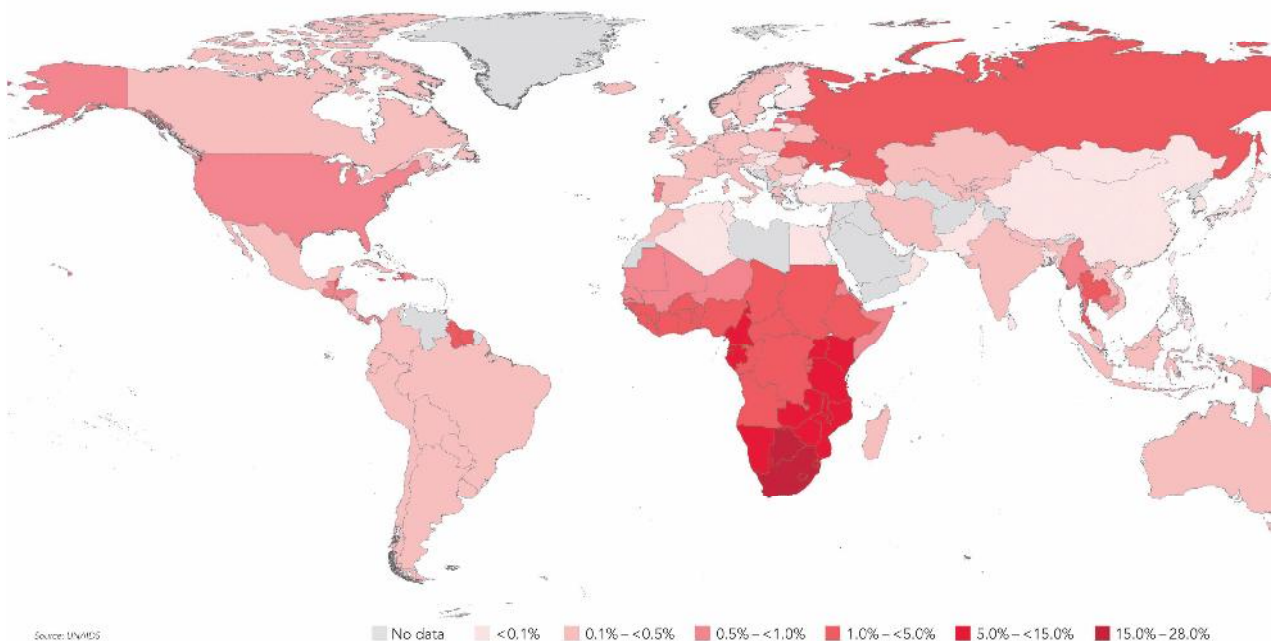


Figure 3. Estimated HIV prevalence rates, 2009 (UNAIDS)

3.1. ANTIRETROVIRAL DRUGS

In recent years, Highly Active Antiretroviral Therapy (HAART) has dramatically reduced HIV related morbidity making AIDS a chronic rather than mortal illness. Standard antiretroviral therapy (ART) consists of the use of at least three antiretroviral

(ARV) drugs to maximally suppress the HIV virus and stop the progression of disease. By the end of 2010, nearly 14 million people were estimated to be in need of ARV with only 6.7 million people accessing it. While current options have permitted rapid scale-up, the cost in terms of side-effects has also been considerable [55,67].

Currently, 6 classes of antiretroviral drugs have received FDA approval: nucleoside/nucleotide analog reverse transcriptase inhibitors (NRTIs), nonnucleoside analog reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors, Entry Inhibitors - CCR5 co-receptor antagonist, and HIV integrase strand transfer inhibitors [69]. Among the different groups of antiretroviral drugs, the NRTIs, NNRTIs, and PIs are currently the groups of drugs recommended by WHO for use in developing countries like Ethiopia (Table 1) [66].

Table 1: WHO recommendation on which antiretroviral combination to start, 2010

Target Population	2010 ART guideline	2006 ART guideline
HIV+ ARV-naïve adults and adolescents	No change, but in settings where d4T regimens are used as the principal option for starting ART a progressive plan to move towards AZT-based or TDF-based first – line regimens should be developed, based on an assessment of cost and feasibility	AZT or TDF + 3TC (or FTC) + EFV or NVP
HIV+ pregnant women	AZT preferred but TDF acceptable EFV included as a NNRTI option (but do not initiate EFV during first trimester) Benefits of NVP outweigh risks where CD4 count is 250-350cells/μl In HIV+ women with prior exposure to MTCT regimens, see ART recommendation on section 13.2 of the WHO guideline	AZT + 3TC + NVP
HIV/TB co-infection	No Change ART should be initiated as soon as possible in all HIV/TB co-infected patients with active TB (within 8 weeks after the start of TB treatment)	AZT or TDF + 3TC (or FTC) + EFV
HIV/HBV co-infection	NNRTI regimens that contain both TDF + 3TC (or FTC) are required	TDF + 3TC (or FTC) + EFV

4. TUBERCULOSIS AND HIV CO-INFECTION

The magnitude of the problem associated with TB has increased globally since 1980s due to changing control practices, population growth and spread of human immunodeficiency virus (HIV) [70,71]. Although, the distribution and usage of anti-TB and ARV drugs in the world has improved in recent years, TB and HIV remain to be the foremost causes of death among the population living in low income countries [55].

Among people living with HIV, TB is the most frequent life-threatening opportunistic infection and a leading cause of death [55,61,66]. Globally, just over one in ten of the 8-9 million people who develop TB each year are HIV-positive, equivalent to 1.1 million new TB cases among people living with HIV in 2010 [55,61,66,72]. Over 82% of these new TB cases that were living with HIV in the same year lived in Africa [73-75].

On the other hand, TB influences the prognosis of HIV infection by stimulating HIV replication directly by *M. tuberculosis* and its cellular components and/or indirectly by releasing cytokines. The risk of death in HIV-infected patients with TB is substantially higher than that of HIV infected patients without TB with matched CD4 cell counts. Though it depends on the CD4 count, death rate for HIV-infected TB patients during TB treatment is particularly high, ranging from 20% to 50% for those with a CD4 count of < 200 cells/ μ l [76-78]. Most deaths caused by TB among HIV positive individuals include: multidrug resistant TB, disseminated TB, progressive HIV infection, pneumonia, cerebral toxoplasmosis, and *Pneumocystis jirovecii* pneumonia (PCP) [74,79].

4.1. CHALLENGES IN TB/HIV TREATMENT

Studies have confirmed that TB and HIV related mortality can be reduced through early initiation of ART and use of appropriate anti-TB and ARV regimens [77,78]. However, concomitant treatment of TB and HIV is complicated due to a number of reasons, of which, increased frequency and intensity of adverse drug reactions, high pill burden,

drug interactions affecting safety and efficacy of drugs, complexity of dose adjustment, mal absorption reducing drug bioavailability, and knowing the exact timing to initiate HAART in TB patients especially for those with a higher CD4 count i.e. CD4 > 350 cells/ μ l are the foremost [55,66,77,78,80].

4.2. ANTI-TB AND ARV DRUGS INDUCED LIVER INJURY

4.2.1. ANTI-TB DRUGS INDUCED LIVER INJURY

Mortality associated with Anti-TB DILI is not uncommon which makes it one of the most important ADR to consider during TB treatment [81,82]. The extent of the injury resulting from it ranges from mild to severe with an incidence ranging from 1-47% depending on the anti-TB regimen and definition of DILI used in the study [47,49,53,83].

The mechanisms of DILI by anti-TB drugs involve direct cytotoxicity (by the drug or its metabolites) or an immune-mediated, which is believed to exist since both INH and rifampicin has been documented for their immunologic effects. The exact mechanism, however, remains unclear [48,50]. Different studies has also revealed that the production and elimination of the toxic metabolites from the anti-TB drugs, predominantly isoniazid, depends on the activities of several enzymes, such as N-acetyl transferase 2 (*NAT2*), cytochrome P450 oxidase (*CYP2E1*) and glutathione *S*-transferase (*GSTM1*) [54,84].

Although toxicity is low when administered alone, DILI caused by rifampicin has been observed in patients with underlying liver diseases [52]. Pyrazinamide is found to induce a cytolytic hepatitis by direct toxicity, most notably after long periods of treatment. Some cases of fulminant liver failure has been reported mainly at relatively higher dosages, 40-50 mg/kg per day [52,82].

4.2.2. ANTIRETROVIRAL DRUGS INDUCED LIVER INJURY

HAART is associated with DILI which has been identified in 6-30% of treated patients [44-46,85]. A study conducted in an HIV clinical in Johns Hopkins Hospital indicated a finding, where 15.6% of patients who were prescribed NVP and 8.0% of those prescribed EFV had severe DILI [86].

When taking HAART, DILI may lead to treatment interruption, which again will cause a greater health impact both for the patient and for the community at large as it might lead to development of resistance secondary to reduced adherence [85-88]. The risk of DILI seems to also be higher with NNRTIs when compared to other classes of ARV drugs [85,87,88].

The mechanism for DILI caused by nevirapine seems to be the result of hypersensitivity reaction which also has genetic predisposition. However, hypersensitivity reactions to efavirenz are found to be less frequent. There is also no clear explanation on the mechanism of production of toxic hepatitis in patients receiving NNRTI who has not exhibited symptoms of a hypersensitivity reaction [78,85,88]. Mitochondrial toxicity, lipodystrophy syndrome, and steatohepatitis are some of the already identified mechanisms for NRTIs [89].

5. PHARMACOGENOMICS AND DRUG INDUCED LIVER INJURY

Pathways of drug metabolism are generally classified as either phase I reactions i.e., oxidation, reduction and hydrolysis or phase II conjugation reactions i.e. acetylation, glucuronidation, sulfation and methylation. Either type of reactions often convert relatively lipid-soluble drugs into a more water-soluble metabolite [90,91].

Studies has indicated that the activity of drug-metabolizing enzymes shows significant inter-individual and interethnic variation. Whether or not a genetic polymorphism of a drug-metabolizing enzyme has clinical relevance depends on its functional role in the metabolism of a drug [92,93]. Most drug-metabolising enzymes are polymorphic, due to

the presence of polymorphisms, leading to abolished, reduced, altered or increased enzyme activity. The inter-individual genetic variation could be due to complete gene deletions, single nucleotide polymorphisms that occur isolated or combined, or gene duplications [94,95].

An increased prevalence and severity of ADRs, including DILI, could be expected among subjects carrying enzyme inactivating mutations, when receiving drugs that are substrates of the defect enzyme. In her analysis of the past, present, and future of DILI, Ann K Daly, has indicated that very little information was available on genetic susceptibility to DILI in the past, while both GWAS and candidate gene studies have confirmed an important role of genetic variations with DILI in the present time. In the future, she also assumed that both candidate gene studies and GWAS will continue to provide insights into genetic factors affecting susceptibility to DILI [96].

In 1980s, effect of HLA class II genotype and N-acetyltransferase 2 (*NAT2*), an enzyme important in isoniazid metabolism were identified as increasing in susceptibility to DILI, relating to co-amoxiclav and isoniazid induced respectively [96]. There are also a number of other genetic polymorphisms reported to have association with DILI. Some of these genes involved in drug biotransformation and associated with DILI include: *CYP1A2*, *CYP2C9*, *CYP2C19*, *CYP2D6*, *CYP2E1*, *CYP3A4*, *CYP3A5*, *NAT2*, *GSTM1*, *GSTT1*, *UGT1A1*, *UGT1A3*, *UGT1A9*, and *UGT2B7*. While drug transporter (*ABCB11* and *ABCC2*), HLA genes (*HLA-B*, *HLA-DRB1*, and *HLA-B*), Superoxide dismutase (*SOD2*), and Cytokine genes (*IL-4*, *IL-6*, *IL-10*) were also part of the investigated genes for a possible association with DILI [92,96].

5.1. CYTOCHROME P450

CYP450 is the generic name given to a large family of highly versatile enzymes that are mainly involved in phase I metabolism which metabolise more than 50% of the currently available drugs including anti-TB, ARV and a countless number of other chemicals of toxicological importance [95].

Multiple forms of CYP enzymes exist which play important roles in the oxidation of structurally diverse drugs. Despite the large number of human *CYP450s*, the bulk of drug metabolism is catalysed by a relatively small number of *CYP450* enzymes found in families 1, 2 and 3. The predominant drug-metabolising *CYP450s* include *CYP1A2*, *CYP2C9*, *CYP2C19*, *CYP2D6*, *CYP2E1*, *CYP3A4* and *CYP2B6* (<http://medicine.iupui.edu/flockhart>; <http://www.imm.ki.se/CYPalleles/>) [97].

5.1.1. *CYP 2B6*

CYP2B6 is one of the most polymorphic *CYP450* genes in humans with over a 100 different SNPs known and with 29 complex haplotypes and distinct ethnic frequencies. SNPs in *CYP2B6* may thus influence the efficacy of drugs as it is the primary metabolizer of a number of drug substrates, such as, efavirenz, nevirapine, bupropion, ketamine, ifosfamide, cyclophosphamide, pethidine and propofol [98]. The most well investigated functional variant allele is *CYP2B6**6 consisting of two linked SNPs (516G>T; 785A>G) resulting in significantly reduced enzyme activity. Several studies have reported association of *CYP2B6**6 variant allele with significantly higher plasma nevirapine and efavirenz levels which is associated with development of different ADRs including CNS toxicities [99,100]. Females had higher amounts of *CYP2B6* mRNA (3.9-fold), protein (1.7-fold) and activity (1.6-fold) than males do. Furthermore, 7.1% of females and 20% of males were poor *CYP2B6* metabolizers. Striking differences among different ethnic groups were also observed: *CYP2B6* activity was 3.6 and 5.0 fold higher in Hispanic females than in Caucasian or African-American females [98,101,102]. Drug, including efavirenz, concentration and pharmacogenomic data are however scarce in an African population.

5.2. N-ACETYL TRANSFERASES (*NAT 2*)

Molecular cloning studies demonstrated that there are two NAT genes in humans, *NAT1* and *NAT2*. They differ both in their substrate specificity and in their distribution in various tissues. Although both enzymes are expressed as polymorphic forms and both are of great toxicological and clinical relevance, *NAT2* has been more widely researched

[90,91]. *NAT2* are phase II enzymes that metabolize drugs and xenobiotics. Genetic polymorphism of *NAT2* is responsible for some of the observed inter-individual variation in the metabolism of several therapeutic drugs, carcinogens and other xenobiotics [103].

N-acetylation of INH takes place to a much lesser degree in a considerable percentage of people compared to others. Based on this, individuals are categorized into two groups; the rapid and the slow acetylators. The elimination half-life for INH is considerably shorter in the case of rapid acetylators leading to reduction in the biological activities of the majority of substances. However, the effect of certain substances can be enhanced by N-acetylation as well. In human, *NAT2* has been detected in the: liver, the tissue of the gastrointestinal tract, the bladder and the lungs [90,103-105]. The application of the knowledge on acetylator status could ultimately help to individualize selection and drug dosing to predict toxicity and improve clinical outcome of therapy [106].

Both *NAT1* and *NAT2* are products of a single, intronless protein coding exons of 870bp open reading frame encoding 290 amino acids. The genes for both *NAT1* and *NAT2* have been assigned to chromosome 8p 21.3-23.1. A third *NAT* gene (*NAT3*) has also been identified in the mouse. Among its three iso-enzymes, *NAT-2* is expressed predominantly in the liver. *NAT1* and *NAT2* share 87% nucleotide homology in the coding region, yielding 55 amino acid differences. Whereas *NAT1* derives its entire transcript from a single exon, *NAT2* mRNA is derived from both the protein coding exon and a second non-coding exon of a loop located about 8kb upstream of the translation start site [103-105,107-109].

To date, more than 26 single nucleotide polymorphisms (SNPs) of *NAT2* have been identified. They transpire in single or in combination eventually leading to more than 34 allele variations. Each allelic variant is a combination of one, two, three, or four nucleotide substitutions. Of the 26 SNPs, four (¹⁹¹*NAT2*, ³⁴¹*NAT2*, ⁵⁹⁰*NAT2*, ⁸⁵⁷*NAT2*) have an amino acid exchange that leads to a reduction in the enzyme activity. The

enzyme activity remains at the same level (high) in the case of three SNPs (²⁸²*NAT2*, ⁴⁸¹*NAT2*, ⁸⁰³*NAT2*) and the phenotypic effect is still unknown for eight of the SNPs (¹¹¹*NAT2*, ¹⁹⁰*NAT2*, ³⁶⁴*NAT2*, ⁴³⁴*NAT2*, ⁴¹¹*NAT2*, ⁴⁹⁹*NAT2*, ⁷⁵⁹*NAT2*, ⁸⁴⁵*NAT2*) [103,104,108,110].

Homozygotes for the *NAT2**4 wild type allele are fast acetylators; heterozygotes for *NAT2**5, *6, or *7 allele have reduced activity, and mutant type homozygotes are slow acetylators [108,110].

Like in other genes involved in drug metabolism, *NAT2* gene polymorphism also shows striking ethnic variation [90]. The reported incidence of fast acetylators is 80-90% in Eskimos, Japanese, and Chinese, 17% in Egyptians and 10% in Moroccans. In central Europe, approximately 40% of the populations are fast acetylators. Of the allelic variations *NAT2**5B occurs with a frequency of 45% and *NAT2**6A is found in 30% of this population. *NAT2**5A, *NAT2**5C and *NAT2**7B have an incidence of only 1%, 3%, and 1% respectively [104]. Slow acetylation frequency in Asian populations ranges between 10-30%, while in most North American populations the frequency is between 40-70% [91,105,111].

The cascade of events which takes place during INH metabolism is: INH will first be acetylated to acetylhydrazine which then undertake further metabolism by *CYP450* to a toxic metabolite, monoacetylhydrazine, (causing isoniazid-induced DILI) or by *NAT2* to the less toxic diacetylhydrazine. It was, therefore, hypothesized that fast acetylators with normal levels of *NAT2* will form diacetylhydrazine efficiently, then levels of both acetylhydrazine and the toxic P450 metabolites will be low in these individuals. As various studies have also confirmed, it is expected that the incidence of INH induced liver injury is lower among fast acetylators [112,113].

6. THIS THESIS

6.1. RATIONALE OF THE THESIS

Even if effective therapies are available to treat both TB and HIV, it is not without a problem. Some of the challenges encountered when treating these diseases, either separately or together, include: high pill burden, adherence issues, drug-drug interactions, paradoxical immune reconstitution reactions and overlapping drug toxicities [55,66].

Drug toxicities has been implicated as major causes of discontinuation of therapy for both TB and HIV [46,82,114,115]. DILI is one of those toxicities which lead to discontinuation of treatment, poor adherence, and consequently towards the development of resistance, and mortality if not handled properly. Among the 1st line anti-TB drugs: isoniazid, rifampicin and pyrazinamide are known to cause DILI, while all classes of ARV drugs are also incriminated as a potential cause [39-44].

DILI secondary to anti-TB and ARV drugs is in general an area that has been less researched and the available few published data has limited emphasis on the incidence, risk factors, management and prognosis. Moreover, the available few studies has limitation on representing an African population, particularly when compared to other continents [39-44,116]. Thus, it is evident that more studies are needed to explore this in Africa.

This thesis therefore, investigates; the incidence, risk factors, timing, prognosis, and management of patients with DILI when taking anti-TB and/or ARV drugs. The thesis also determined pharmacogenetic profiles of participants for selected relevant drug metabolising and transporter genes. In addition, the thesis has evaluated the impact of polymorphisms of these genes and the association of efavirenz pharmacokinetics with development of DILI.

6.2. OBJECTIVES

6.2.1. General objectives

The general objectives of the thesis were to assess and compare the incidence, identify the risk factors, severity, and prognosis of anti-TB and/or ARV drugs induced liver injury (DILI) and to evaluate the effects of genetic polymorphisms and serum efavirenz level on development of DILI among Ethiopians.

6.2.2. Specific objectives

1. To determine the incidence and identify risk factors of DILI in HIV positive non-TB co-infected patients receiving efavirenz based HAART alone.
2. To determine the incidence and identify risk factors of DILI in TB-HIV co-infected patients with CD4 count ≤ 200 cells/ μ l receiving rifampicin based anti-TB and efavirenz based HAART.
3. To determine the incidence and identify risk factors of DILI in TB-HIV co-infected patients with CD4 count > 200 cells/ μ l taking anti-TB alone.
4. To determine the incidence and identify risk factors of DILI among HIV negative TB patients receiving anti-TB alone.
5. To assess the distribution and evaluate the effect of *NAT2*, *CYP2B6*, *CYP3A5*, *ABCB1*, *UGT2B7* and *SLCO1B1* polymorphisms on development of DILI among TB-HIV co-infected patients.
6. To assess the distribution and evaluate the effect of *CYP2B6*, *CYP3A5*, *ABCB1* 3435C/T and *UGT2B7**2 polymorphisms on development of DILI among HIV positive non-TB co-infected patients.
7. To assess the association between plasma efavirenz and 8-hydroxyefavirenz levels and development of DILI among patients taking efavirenz based HAART.

7. MATERIALS AND METHODS

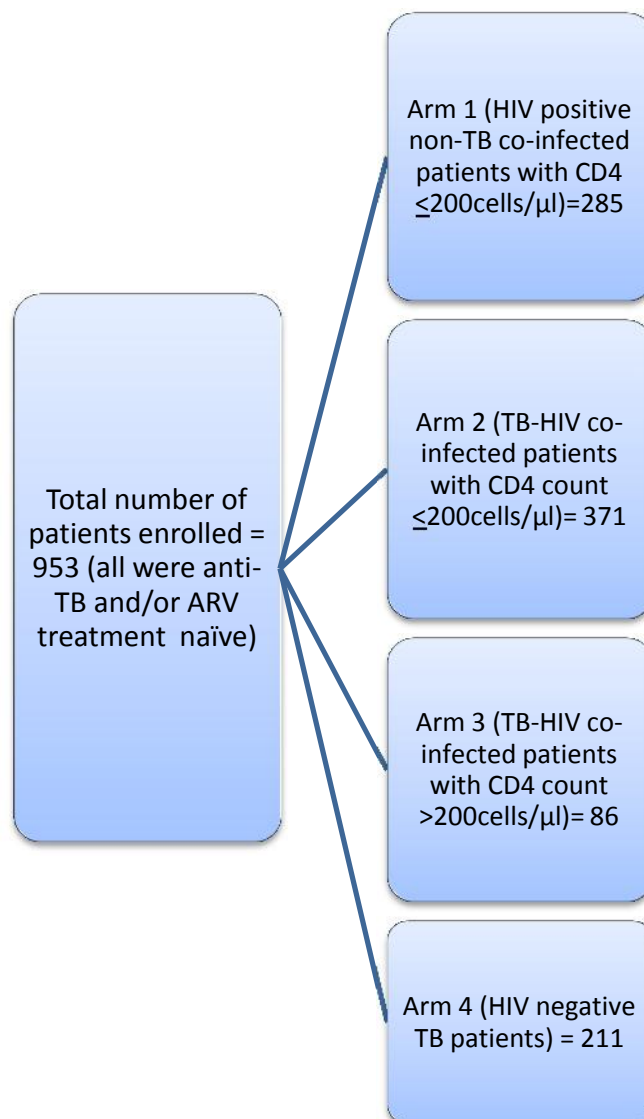
7.1. STUDY DESIGN AND POPULATION

This was an open label prospective cohort study conducted on TB and/or HIV positive individuals from Ethiopia. The pilot study was conducted from August 2004 to March 2005, during which we prospectively enrolled and assessed participants for development of DILI on a total of 103 HIV positive and 94 HIV negative newly diagnosed male and female adult TB patients from St. Peter's TB Specialized Hospital in Addis Ababa, Ethiopia.

The main study, with similar study design, was then conducted during June, 2007 to June, 2011. It was a prospective cohort study which enrolled ARV and anti-TB treatment naïve TB and/or HIV positive patients from four study sites: Kazanchis, Arada, and Beletshachew Health Centers, and *Black Lion* specialized referral Hospital in Addis Ababa, Ethiopia. A total of 953 patients, grouped into 4 study arms depending on their disease condition and type of treatment received, were enrolled. Follow up period and type of investigations done was also different for the different arms. Arm 1 consisted of HIV positive individuals with CD4 count ≤ 200 cells/ μ l who had no TB co-infection and receiving efavirenz based HAART alone, arm 2 consisted of TB-HIV co-infected patients with CD4 count ≤ 200 cells/ μ l receiving rifampicin based anti-TB drugs together with efavirenz based HAART, arm 3 consisted of TB-HIV co-infected patients with CD4 count > 200 cells/ μ l taking anti-TB drugs alone, arm 4 consisted of HIV negative TB patients receiving anti-TB drugs alone.

The eligibility criteria for both the pilot and main study were: both male and female, age > 18 years, non-pregnant women, receiving no other known hepatotoxic drugs concurrently (except co-trimoxazole, 960 mg per day, which was given for all HIV positive patients whose CD4 count was ≤ 200 cells/ μ l before enrolment and during the follow up period according to the treatment guideline. None of the participants received isoniazid prophylaxis and treatment of TB at least two years before enrolment into the study.

Fig 4. Distribution of patients enrolled into the main study by ARM



7.1.1. Diagnosis of TB

TB was diagnosed based on sputum smear positivity, fine needle aspirate, and clinical and radiological evidences. The criteria for diagnosis were based on the national guideline for diagnosis of TB. Patients with smear positive, smear negative, extra pulmonary, and disseminated TB were enrolled.

7.1.2. Diagnosis of HIV and determination of CD4 count

All patients in the study had laboratory investigations for different parameters that included HIV testing which was done by rapid test kits. Diagnosis was made following the national HIV diagnosis algorithm. Blood was tested first by Determine® and if the result became negative it is then interpreted as negative. If the result read positive, it needed to be reconfirmed by Cappilus®. If the results by the two tests read positive, the patient was considered positive but if the two tests read controversial results, Unigold® was then used as a tiebreaker. CD4 count and HIV RNA measurements were done for HIV positive patients, all within 1 week from the date of enrolment.

7.1.3. Other clinical Laboratory tests

These tests were done for all participants before initiation of anti-TB and /or ARV drugs and tests include; complete and differential blood counts, erythrocyte sedimentation rate (ESR), hematocrit (Hct), liver enzymes and function tests (aspartate aminotransferase (AST), alanine aminotransferase (ALT), direct and total bilirubin and alkaline phosphatase), serological tests for hepatitis B surface antigen and anti-hepatitis C antibody. Liver enzymes and function tests were also assessed at the 1st, 2nd, 4th, 8th, 12th, 24th, 48th, and 56th weeks after initiation of treatment for arms 1 and 2 and at 1st, 2nd, 4th, 8th, and 12th weeks after initiation of anti-TB for arms 3 and 4. All Laboratory tests were done by a company called International Clinical Laboratory, Addis Ababa, Ethiopia which is accredited by Joint Commission International (USA).

7.2. TREATMENT REGIMENS

7.2.1. Anti-TB regimen

All TB patients were initiated on anti-TB drugs provided by Ministry of health, Ethiopia and they were put on DOTS for the duration of TB treatment.

Table 2. Treatment regimen and duration of treatment recommended by MOH, Ethiopia

Duration of treatment	Drugs	Weight in kilograms			
		20-29	30-37	38-54	>55
Intensive phase (2 months)	Ethambutol (275mg) Rifampicin (150mg) Isoniazid (75mg) Pyrazinamide (400mg)	1½ tablets	2 tablets	3 tablets	4 tablets
Continuation phase (4 months)	Rifampicin (150mg) Isoniazid (75mg)	1½ tablets	2 tablets	3 tablets	4 tablets

7.2.2. Antiretroviral regimen

Patients enrolled in the study received a standard ARV regimen recommended by Ministry of health, Ethiopia. The regimens used include:

- Stavudine/Lamivudine/Efavirenz (D4T/3TC/EFV)
- Zidovudine/Lamivudine/Efavirenz (AZT/3TC/EFV)
- Tenofovir/Lamivudine/Efavirenz (TDF/3TC/EFV)

All regimens had the same non-nucleoside reverse transcriptase backbone (NNRTI) i.e. efavirenz (EFZ).

7.3. DNA EXTRACTION AND GENOTYPING

When a patient came for the second visit, i.e. 1 week after initiation of anti-TB or ARV drugs, 4-5 ml of venous blood was taken into tubes containing ethylenediamine-tetra-acetic acid (EDTA) as anti-coagulant. The blood sample was then put at -80⁰C and transported to KI for DNA isolation and genotyping.

7.3.1. DNA extraction

DNA was isolated from peripheral blood leukocytes using QIAamp DNA Maxi Kit (QIAGEN GmbH, Hilden, Germany).

7.3.2. TaqMan Methods

Allelic discrimination reactions were performed using TaqMan® (Applied Biosystems, CA, USA) genotyping assays: (C__7586657_20 for *ABCB1* 3435C>T, C__11711730_20 for *CYP2B6* 516G>T [*CYP2B6**6], C__30720663_20 for *UGT2B7* -372G>A [*UGT2B7**2b,*2c,*2d,*2f], C__26201809_30 for *CYP3A5* 6986A>G [*CYP3A5**3], C__30203950_10 for *CYP3A5* 14690G>A [*CYP3A5**6], C__32287188_10 for *CYP3A5* g.27131_27132insT [*CYP3A5**7], C__1901697_20 for *SLCO1B1* 388A>G (*1b) and C__30633906_10 for *SLCO1B1* 521T>C (*5) on ABI 7500 FAST (Applied Biosystems, Foster City, CA). The final volume for each reaction was 10µl, consisting of 2x TaqMan Universal PCR Master Mix® (Applied Biosystems), 20 X drug metabolising genotype assay mix and 10 ng genomic DNA. The PCR profile consisted of an initial step at 50⁰C for 2 min and 50 cycles with 95⁰C for 10 minutes and 92⁰C for 15 sec.

7.3.3. *NAT2* gene sequencing

The coding regions of *NAT2* gene was amplified by PCR using a forward primer (5'-GTCACACGAGGAAATCAAATGC-3') and a reverse primer (5'-GTTTTCTAGCATGAATCACTCTGC-3') as described previously [117]. The PCR products were purified using ExoSAP-IT[®] (USB Corporation, Cleveland, OH) PCR Purification kit. Sequencing was done in forward and reverse directions using PCR primers described above and an internal reverse primer (5'-GGATGAAAGTATTTGATGTTTAGG-3'). Sequencing reaction was done using the ABI PRISM[™] BigDye[®] terminator cycle sequencing ready reaction kit v3.1 (Applied Biosystems, Foster City, CA), and analyzed on an ABI Prism 377 DNA sequencer. *NAT2* sequence chromatograms were visually inspected and analyzed using software program FinchTV version.1.4.0 (<http://www.geospiza.com>) and aligned with the *NAT2* reference sequence (<http://www.ncbi.nlm.nih.gov>; GenBank reference: NM 000015.2) for SNPs identification.

7.4. QUANTIFICATION OF PLASMA EFAVIRENZ AND 8-HYDROXYEFAVIRENZ CONCENTRATION

On the 4th week after initiation of efavirenz based HAART, 8 ml of blood samples were collected 16 hrs post efavirenz dosing in a vacutainer CPT (Becton Dickinson, Heidelberg, Germany), centrifuged (1700 g for 20 min), and 2 mL plasma aliquot was stored at -80°C for determination of efavirenz and its metabolite concentrations. Plasma samples were sent in dry ice to the Department of Clinical Pharmacology and Pharmacoepidemiology, University of Heidelberg, Germany. Plasma efavirenz and 8-hydroxyefavirenz concentration were determined by LC/MS/MS. The lower limits of quantification in plasma were 10.0 ng/mL for efavirenz and 0.4 ng/mL for 8-hydroxyefavirenz. The efavirenz and 8-hydroxyefavirenz calibration ranges were 10-10000 ng/mL (0.4-400 ng/mL). Linear regression with 1/x weighing resulted in correlation coefficients of $r^2 > 0.99$. Accuracy and precision (within-batch and batch-to-batch) of the assay fulfilled all recommendations by FDA.

7.5. DATA MANAGEMENT AND ANALYSIS

7.5.1. Data management

Data collection was done by clinicians and nurses who were trained on good clinical practice (GCP) which was then verified by a clinical monitor. Pre-piloted structured case report form (CRF) was used for data collection. It was then double entered and crosschecked for consistence on an accesses database.

7.5.2. Statistical analysis

Data analysis at different times was done using versions 11.0, 15.0 and 19.0 (SPSS, Chicago, IL, USA) for complete data analysis and Statistica version 10 (StatSoft Inc., Tulsa, OK, USA) for graphical data presentation. Chi-square, Fisher's exact tests and multivariate analysis were used to test for the level of significance for **paper 1**, while independent t-test, Univariate and multivariate Cox proportional hazards mode and Kaplan-Meier were used for **papers 2, 3 and 4**. In addition, Efron approximation for ties in the time point of DILI onset and the variable selection method (varSelRF) was used on the data set to rank the importance of the variable for **paper 4**. Variables with $p < 0.05$ were considered to determine the level of significance.

7.5.3. Ethical consideration

The study protocol was approved by the Regional Ethical Review Board in Stockholm at Karolinska Institutet, Sweden; Institutional Review Board at the Faculty of Medicine, Addis Ababa University as well as the National Ethics Review Committee at Science and technology Ministry in Ethiopia.

8. RESULTS

8.1. EFFECT OF HIV INFECTION, CD4 COUNT AND CONCOMITANT DRUG INTAKE ON DEVELOPMENT OF DILI (PAPER I)

The cohort in this group is HIV positive and negative TB patients who were taking rifampicin based anti-TB alone (HAART wasn't freely available in Ethiopia when conducting this particular study). This was the first study which reported on the incidence of anti-TB DILI among Ethiopians. In this study, we noted that the incidence of DILI was higher among Ethiopians when compared to other population, though a similar finding was also observed in other countries [43,82,118]. Sub-clinical hepatotoxicity was observed in 17.3% of the patients and 8 out of the 197 (4.1%) developed severe clinical hepatotoxicity.

8.1.1. Contribution of HIV infection and lower CD4 count to the development of DILI

The study reported a statistically significant association between development of sub-clinical DILI with HIV co-infection ($p = 0.002$), and decrease in CD4 count ($p = 0.001$). Out of the 34 patients with subclinical DILI, twenty six (76.5%) were HIV positive. The odds of developing subclinical DILI was 20.5 times higher among those with a CD4 count of $< 50/\mu\text{l}$ when compared with those who had a baseline CD4 count of $> 200/\mu\text{l}$. There were only limited studies done on this area when we conducted this study which was later complimented by a number of other studies.

8.1.2. Effect of concomitant drug intake on development of DILI

In this study we observed that patients who took other drugs concomitantly with the anti-TB were at a higher risk for DILI. Concomitant drugs taken by the participants include: amoxicillin, antacids, omeprazole and promethazine to mention some. Similar findings have been reported earlier and the explanation given for that, which we also agree with, is the additive or synergistic hepatotoxic effect and the potential drug-drug interactions [39,43,78,116,119].

8.2. EFFECT OF GENETIC POLYMORPHISMS AND PLASMA EFAVIRENZ LEVEL ON DEVELOPMENT OF DILI (PAPER II)

This study comprises of HIV positive individuals with a CD4 count $\leq 200/\mu\text{l}$ who had no TB co-infection and received efavirenz based ARV drugs alone. The data from a total of 261 patients were analysed and we reported the incidence of DILI to be 15.7% (27.9 per 100 person-years). Though similar findings were reported previously, this is a relatively higher figure when compared to most of the studies which were conducted on a similar cohort [44,46,89,116,120].

8.2.1. Effect of genetic polymorphism on development of DILI

From this cohort, though we performed the analysis on possible association of different genotypes with DILI i.e. *CYP2B6*, *ABCB1 3435C>T*, *CYP 3A5*, and *UGT2B7-372G>A*, we found a statistically significant association only with *CYP2B6*6* ($p=0.01$).

8.2.2. Efavirenz pharmacokinetics and other risk factors for DILI

Comparison of plasma EFV, 8-hydroxyefavirenz and EFV/8-hydroxyefavirenz ratio between DILI cases and controls in our study showed that, a higher steady-state plasma EFV concentration significantly increases the risk for DILI (95% CI=1.31–14.55; $P: 0.017$). Other risk factors include; higher baseline AST (95% confidence interval (CI)=1.01–1.05; $P: 0.01$), ALT (95% CI=1.01–1.06; $P: 0.035$), and ALP (95% CI= 1.00–1.01; $P: 0.016$). In addition, lower baseline albumin (95% CI=56.21–16.14; $P: 0.03$), and platelet levels (95% CI=0.29–0.96; $P: 0.037$), were also noted to predict forthcoming DILI.

8.3. THE PHARMACOGENETIC & KINETIC PREDICTORS FOR DILI IN TB-HIV CO-INFECTED PATIENTS (PAPER III)

This study, which was conducted on TB-HIV co-infected patients with CD4 count ≤ 200 cells/ μl re-confirmed that the incidence of DILI in Ethiopia was still high, 30.0% or

14.5 per 1000 person-week. Incidence of severe DILI was 18.4% or 7.49 per 1000 person-week.

8.3.1. Effect of genetic polymorphism on development of DILI

Like the cohort in paper II, we have performed genotyping, this time on *NAT2*, *CYP2B6*, *CYP3A5*, *ABCB1*, *UGT2B7* and *SLCO1B1* genes. The result after performing relevant statistical analysis showed that, it is only *NAT2* slow-acetylator genotype ($p=0.039$) and *ABCB1* 3435TT genotype ($p=0.001$) which significantly increase the risk for DILI.

8.3.2. Efavirenz pharmacokinetics and other risk factors for DILI

Comparison of plasma EFV, 8-hydroxyefavirenz and EFV/8-hydroxyefavirenz ratio between DILI cases and controls in this cohort showed that, a higher steady-state plasma EFV concentration and efavirenz/8-OH efavirenz metabolic ratio significantly increase the risk for DILI with p values of 0.009 and 0.036 respectively.

Other risk factors include; female sex ($p= 0.001$), higher baseline AST ($p=0.022$) and ALT ($p=0.014$). Moreover, lower haemoglobin ($p=0.008$), and serum albumin ($p=0.007$) were also noted to predict impending DILI.

8.4. FOUR ARM COMPARATIVE STUDY ON THE INCIDENCE AND RISK FACTORS FOR DILI (PAPER IV)

This study constituted of four parallel arm cohorts namely: HIV positive patients without TB co-infection receiving efavirenz based ARV alone with CD4 count $\leq 200/\mu\text{l}$ (arm-1), TB-HIV co-infected patients with CD4 count $\leq 200/\mu\text{l}$ receiving concomitant anti-TB and ARV drugs (arm-2): TB-HIV co-infected patients with CD4 count of $> 200/\mu\text{l}$ receiving anti-TB drugs alone (arm-3), HIV negative TB patients receiving anti-TB drugs alone (arm 4).

The study showed that incidence of DILI was significantly associated with type of treatment ($p<0.0001$). The highest incidence was for arm-2 (23.5%) >arm-3 (11.6%) >arm-1 (8.1%) >arm-4 (2.8%). Concomitant intake of anti-TB and ARV drugs increased the risk of DILI 9 times compared to anti-TB alone (least risk).

8.4.1. Clinical and biochemical risk factors for development of DILI

The median time for development of DILI was 2 weeks, post initiation of treatment for arm 1 and 4, while it was 1 week for arm 2 and 3. Lower BMI was significantly associated with development of DILI. Moreover, taking HAART concomitantly, having a lower baseline CD4 count and higher viral load were significantly associated with increased risk for development of DILI. There was also a statistically significant association between DILI and a decrease in baseline serum albumin, independent of the type of treatment that patients were taking. When stratified by arms, among arm-3 patients alone, effect of Hepatitis B on development of DILI was significant ($p=0.01$) and Hepatitis C showed a tendency ($p=0.16$).

9. DISCUSSION

Incidence of DILI and role of HIV infection and CD4 count was initially determined during the pilot study **(I)**. The thesis further examined the effect of pharmacogenetic and kinetic differences as a potential risks for development of DILI, while taking EFV based ARV drugs alone **(II)**, and when taking EFV based ARV drugs and rifampicin based anti-TB drugs concomitantly **(III)**. Furthermore, incidence, predictors, timing and prognosis of DILI were assessed among different arms of patients at a larger cohort **(IV)**. Different sociodemographic, clinical and biochemical variables were also assessed for a potential risk for DILI **(I-IV)**. Though slightly different from time to time, we have used internationally accepted definitions for DILI.

Findings from the pilot study were helpful in providing the first information on the incidence of DILI (which is higher than expected) and impact of HIV infection, CD4 count, and concomitant drug intake among Ethiopians. Our succeeding studies have also re-confirmed that DILI was indeed a common problem among patients who took anti-TB and/or ARV drugs in Ethiopia **(II-IV)**. Even though we noticed similar findings from elsewhere, studies from Tanzania revealed a lower incidence of DILI, indicating that there indeed exists a population difference which might be attributed to genetic, environmental or other confounding factors [74,78,121,122].

Papers II and III, in addition to assessment of the incidence, sociodemographic, clinical and biomedical associations with DILI, have provided information on the association of DILI with different pharmacogenetic and kinetic variables. Thus, in addition to giving complementary scientific knowledge on the distribution of different genotypes among Ethiopians, the studies have also indicated a statistically significant association between different polymorphisms and development of DILI. After assessment of *CYP2B6**6, *CYP3A5**3, *CYP3A5**6, *CYP3A5**7, *ABCB1* 3435C4T and *UGT2B7*- 372G4A for those non TB co-infected HIV positive individuals with CD4 count $\leq 200/\mu\text{l}$ **(paper II)**, we reported that it was only patients with *CYP2B6**6/*6 genotype who had a 2.7 times higher risk to develop DILI when compared with homozygous wild-type (*1/*1). Other

genetic polymorphisms assessed in the study didn't show significant association with development of DILI. Similarly, we have determined genotyping of *NAT2*, *CYP2B6*, *CYP3A5*, *ABCB1*, *UGT2B7* and *SLCO1B1* genes in patients who were taking anti-TB and ARV drugs concomitantly (III). The result from this study showed that, having *NAT2* slow-acetylator genotype and *ABCB1* 3435TT genotype in addition to having *CYP2B6**6/*6 were significantly associated with DILI. Studies have shown similar findings on the association of DILI with slow acetylator status (mainly because of the building-up of monoacetylhydrazine (MAH)) [17,112,123,124]. Our study was, however, the first to demonstrate the association of anti-TB and ARV DILI with *ABCB1* 3435TT and *CYP2B6**6/*6. This could mainly be due to increased efavirenz plasma concentration among these groups of patients complementing that fact that efavirenz causes a direct hepatotoxicity (III, IV).

Assessment on the impact of efavirenz pharmacokinetics in our studies indicated that increased plasma efavirenz concentration and efavirenz/8-OH-efavirenz metabolic ratio were significantly associated with DILI (II, III). These findings were not affected by concomitant intake of anti-TB drugs (III). Other studies have also shown evidence to support the hypothesis that efavirenz reduces cellular proliferation and triggers apoptosis *in vitro*. Moreover, clinically relevant concentration of EFV has been shown to be mitotoxic in human hepatic cells in a concentration dependent manner, related to direct efavirenz induced hepatotoxicity [125]. A more recent study on this has also reported that increased efavirenz level exceeding a certain threshold of mitochondrial dysfunction was associated with an autophagic overload or stress and may constitute a new mechanism implicated in the pathogenesis of efavirenz induced liver injury [87].

In agreement with other studies, our studies have also revealed that female sex (III), elevated baseline alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase (II,III,IV), and viral load (II,IV), and lower haemoglobin, platelet (II,IV), serum albumin (II,III, IV), and CD4 count (I, II, III, IV) increase the risk for DILI significantly. Though there is no clear evidence, the association of female sex with DILI was reported to be linked with hormonal differences, while the effect of elevated baseline AST, ALT, and ALP indicate that pre-treatment liver enzymes could be good

predictors for DILI and this has been re-confirmed by other studies too [21,39,118]. Furthermore, both lower haemoglobin and platelet count were noted to have a strong association with DILI, which wasn't reported earlier from other similar studies.

In addition, population prevalence of hepatitis B surface antigen and hepatitis C antibody positivity in TB and/or HIV cohorts have been reported in our work **(I,II,III,IV)**. The prevalence was similar to what was reported from the general population in Ethiopia. However, the association of DILI with hepatitis B and C was more of a trend, as the number of positive patients in our studies were too small [86,116,121,126].

In general, this thesis presented major findings on the incidence, predictors, severity and prognosis of anti-TB and/or ARV DILI and evaluated its association with different genetic polymorphisms which are involved in the metabolism of those drugs. In addition, serum efavirenz and its metabolite levels and the association with DILI have been determined. Findings from this thesis will provide clinicians, researchers, guideline developers and policy makers with fundamental information about DILI in general and anti-TB and ARV DILI in particular.

10. CONCLUSIONS AND RECOMMENDATIONS

10.1. CONCLUSIONS

- Incidence of anti-TB and/or ARV DILI is relatively higher among Ethiopians.
- Females and those patients who took concomitant drugs are at a higher risk to develop DILI.
- HIV disease progression, as measured by CD4 count and viral load, significantly increases the risk for DILI.
- Elevated baseline ALT, AST, ALP and lower baseline haemoglobin and albumin are good predictors for development of anti-TB and/or ARV DILI.
- Elevated plasma efavirenz level and efavirenz/8-OH efavirenz ratio increase the risk of DILI. This could potentially indicate that the most likely mechanism for EFV induced liver injury is a direct liver injury than immunologic.
- *CYP2B6**6/*6 and *ABCB1*3435TT genotype could be potential biomarkers for EFV induced liver injury.
- Majority of Ethiopians are slow acetylators and they are at a higher risk of developing anti-TB DILI.
- Concomitant administration of anti-TB and ARV drugs increase the risk for DILI.
- DILI is a major challenge in the management of TB and/or HIV, especially during the first 2-8 weeks, with the majority of cases developing it in the first 2 weeks after initiation of treatment.

10.2. RECOMMENDATIONS

1. Individuals at risk of developing DILI need close follow up and monitoring especially during the first 8 weeks after initiation of anti-TB and/or ARV treatment.
2. The existing diagnostic markers for DILI aren't very sensitive and specific; there is a strong need to look for a more reliable biomarker, which might include genome-wide association study (GWAS) and discovery of new peptides.
3. Availing a point of care diagnostic assay which could easily pick an already identified potential risk factors like *CYP2B6**6/*6 would significantly improve the early detection and management of DILI.
4. There is always a need to further explore the exact mechanism of anti-TB and/or ARV DILI so that a better management and prevention strategies could be sought.

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