ANTI TUBERCULOSIS DRUG INDUCED HEPATOTOXICITY IN HIV POSITIVE AND NEGATIVE PATIENTS

BY

Getnet Yimer Ali, MD

A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfilment of the requirements for the Degree of Master of Science in Medical Pharmacology

June 2005

Acknowledgments

I would like to acknowledge all my supervisors for their valuable support, especially Dr. Abraham Aseffa for his input in the proposal development and thesis write up. Dr. Getachew Aderaye and Dr. Wondwossen Amogne are acknowledged for their input in the proposal development and scientific advice in the clinical aspects of the study. Prof. Eyasu Makonnen is greatly acknowledged for his unreserved support in every need I had from the start till the end of the study.

Dr. Eleni Aklilu has helped me in NAT2 genotyping and Prof. Lars Lindquist has covered all my expenses in my stay in Sweden. Dr. Howard Engers and Dr. Lawrence Yamuah helped me in progressively evaluating the study and had inputs in the final thesis write up.

I also want to thank W/o Kidist Bobosha for assisting me in the laboratory work, W/o Hiwot Tilahun and Ato Wondu Wagaye for entering and cleaning up my data. I would like to thank AHRI for funding most of my work in Ethiopia. I appreciate the support of Drug Administration and Control Authority (DACA) for allowing me to use their HPLC machine. Ato Bekele Abera has helped in teaching me and running samples on HPLC. I am also indebted to Dr. Klaus Golka Institute of Occupational Physiology, at the University of Dortmund, Germany, who sent me the protocol and the internal standard for caffeine analysis with HPLC.

CD4 count was done by the International Clinical Laboratories, Ethiopia against payment and their support in that respect is appreciated. I am grateful to the Department of Pharmacology, Medical Faculty, and School of Graduate Studies, AAU for allowing me to register for graduate studies and providing me with some funding. Staffs of St. Peter's TB Specialized hospital are not forgettable in their proper enrolment and follow up of patients.

In fact all the work would not have been done without the consent of the patients. They take a larger share of my appreciation.

Dedication

This work is dedicated to those patients enrolled in our cohort and those who died during the follow up period. Their lives could have been salvaged if they had been given antiretroviral drugs. May God keep their soul in heaven.

Table of content

List of Tables

List of Figures

List of Appendixes

List of Abbreviations

χ^2	Chi square
µl	Micro liter
1-MX	1-Methyl xanthine
A	Adenine
ADR	Adverse Drug Reaction
AFMU	5-acetylamino-6-formylamino-3-methyluracil
AHRI	Armauer Hansen Research Institute
AIDS	Acquired Immuno Deficiency Syndrome
ALERT	All African Leprosy and Tuberculosis Rehabilitation Research and Training center
ALT	Alanine amino transferase
Arg	Arginine
AST	Aspartate amino transferase
BMI	Body Mass Index
BSA	Bovine Serum albumin
C	Cytosine
CD	Cluster of Differentiation
CDC	Centers for Disease Control
CI	Confidence Interval
COA	Coenzyme A
CYP	Cytochrome P
DACA	Drug Administration and Control Authority
DIH	Drug Induced Hepatotoxicity
DNA	Deoxy Ribo Nucleic acid
dNTPs	Deoxy Nucleotide Tri Phosphates
DOTS	Directly Observed Therapy Short course
DSM	Diagnostic Statistical Manual
EDTA	Ethylene diamino Tetra Acetic acid
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
Gln	Glutamic Acid
Glu	Glutamine
Gly	Glycine
HBsAg	Hepatitis B Surface antigen
HCl	Hydro chloric acid
Hct	Hemathocrit
HCV	Hepatitis C Virus
HPLC	High Performance Liquid Chromatography
INH	Isonicotinic acid hydrazine
M	Molar

Max	Maximum
mg	Milli Gram
Min	Minimum
ml	Milli liter
mM	Milli Molar
mRNA	Messenger Ribo Nucleic acid
NaCl	Sodium Chloride
NAT	N-Acetyl transferase
ng	Nano gram
nmol	Nano Mole
^{o}C	Degree Celicius
PAS	Para-amino salsalic acid
PCR	Polymerase Chain Reaction
Pro	Proline
RFLP	Restriction Fragment Length Polymorphosim
RH	Rifampicin, Isoniazid (fixed dose)
RHZ	Rifampicin, Isoniazid, Pyrazinamide (fixed dose)
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
TBE	Tris Borate ethylene diamine tetra acetic acid
US	United States
VCT	Voluntary Counseling and Testing
WHO	World Health Organization
XO	Xanthine Oxidase

Abstract

Anti-tuberculosis drug induced hepatotoxicity (DIH) is a common problem in the management of tuberculosis. This study was intended to identify possible risk factors for development of DIH, including degree of immunosuppression. In this prospective 2-month cohort study, 103 HIV positive and 94 HIV negative newly diagnosed tuberculosis patients were followed after initiation of DOTS (direct observed treatment short course). CD4 count was measured for the HIV positive patients. All patients were also evaluated for different risk factors including HBsAg, Anti-HCV, alcohol intake, use of other drugs including traditional medicines, acetylation status and presence of chronic illness. Patients were monitored biochemically (by liver function tests) and clinically for development of DIH weekly in the first month and bi-weekly in the second month after start of therapy. Biochemical hepatotoxicity was seen in 17.3% of the patients. CD4 counts of these patients were 0-50 for 7 (35%), 51-100 for 8 (40%), 101-200 for 4 (20%), and > 200 for 1 (5%). Three patients were positive for HBsAg and none had anti-HCV. Five patients died of non-hepatic causes among the patients who developed DIH. Eight out of the 34 patients with biochemical hepatotoxicity (23.5%) developed clinical hepatotoxicity that necessitated discontinuation of their anti-TB drugs. Seven of the eight were HIV positive, seven were female, and 2 were positive for HBsAg. Biochemical hepatotoxicity was significantly associated with HIV co-infection (p=0.002), concomitant drug intake (p=0.008), decrease in CD4 count (p=0.001), high mortality (p=0.001), and having Wt/Wt allele for acetylation status (p=0.026). Clinical hepatotoxicity is also significantly associated with being female (p=0.027), HIV co-infection (p=0.043), concomitant drug intake (p=0.003), HBsAg (p=0.046), decrease in CD4 count (p=0.025), and high mortality (p=0.0001). No significant association was seen between hepatotoxicity with alcohol intake, age, body mass index, type of TB and anti HCV positivity. The findings would assist in selectively managing patients at risk. It is recommended to have a regular biochemical and clinical follow up for those patients who are at risk of developing DIH .These patients include HIV positive patients, with special emphasis to those with a lower CD4 count, and patients who take drugs other than their anti TB medication. We also recommend that further work should be done to explore the reason for the observed association between DIH and female sex, HBsAg positivity, and acetylation status.

Key words: Tuberculosis, HIV, Hepatotoxicity, Acetylation status, NAT2 gene

1. Introduction

Infectious diseases remain the largest cause of illness and death in the world, and tuberculosis is the world's leading cause of death among infectious diseases, with one person dying of it every 15 seconds across the globe (Bassey, *et al.*, 2005, Frieden, *et al.*, 2003). It is estimated that *Mycobacterium tuberculosis* has infected one-third of the world's population and 8-9 million new TB cases occur each year (Aaron, *et al.*, 2004, Bassey, et al., 2005, Brewer and Heymann, 2004, DISEASEWATCH, 2004, Gooze, 2003, Yepes, *et al.*, 2004). In 1993, WHO declared tuberculosis (TB) as a global health emergency.

Eighty percent of new cases of tuberculosis occur in 22 high burden countries, with sub-Saharan Africa having the highest incidence rate of tuberculosis (290 per100, 000 population) (Frieden, et al., 2003, Mwinga and Bernard Fourie, 2004). Ethiopia ranks 7th among the 22 high burden countries in the world, and is second only to Nigeria in Africa, in the number of tuberculosis (TB) patients. The annual risk of TB infection in Ethiopia is estimated at 2.2%. It is estimated that 177,428 Ethiopians (0.26% of the total population) have active TB of all kinds. According to hospital statistics data of the Ministry of Health (MOH) for 2003, tuberculosis is the leading cause of morbidity, the fourth cause of hospital admission, and the second cause of hospital death in Ethiopia (MOH, 2004).

1.1 Tuberculosis and HIV co-infection

The magnitude of the tuberculosis problem has increased globally since 1990 due to changing control practices, spread of human immunodeficiency virus (HIV) and population growth (Dye, *et al.*, 1999). In populations with high HIV prevalence, TB is the leading cause of morbidity and mortality. It has been estimated that about 11.5 million people were co-infected with HIV and *M. tuberculosis* by the end of 2000 and over 94% of these dually infected individuals reside in developing nations (Aaron, et al., 2004, Gooze, 2003, WHO, 2004). The population in these countries accounts for 95% of all tuberculosis cases and for about 98% of TB-related deaths. More than 85% of HIV deaths and 75% of TB deaths in developing countries occur in the economically productive age group, i.e., age 15 to 50 years (Pape, 2004). This situation is

compounded by the fact that on average, 30-40% of the TB cases in Africa are HIV positive and this figure reaching 70% in some countries (DISEASEWATCH, 2004, Gooze, 2003, Mwinga and Bernard Fourie, 2004).

TB can occur at any time during the course of HIV infection. In a study done in Haiti on patients with HIV and TB, $CD4^+$ cell count at TB diagnosis was above 350/µl in 23% and below 200/µl in 21% of the individuals (Pape, 2004). Indeed the immunosuppression induced by HIV modifies the clinical presentation and course of tuberculosis. On the other hand, TB influences the prognosis of HIV infection. The risk of death in HIV-infected patients with TB is twice that of HIV infected patients without TB with matched CD4 cell counts, with most deaths caused by progressive HIV infection rather than TB (Aaron, et al., 2004, Jones, *et al.*, 1994).

Although optimal treatment of those co-infected patients is essential to control the current epidemic of tuberculosis, prospective controlled trials documenting the efficacy and toxicity of regimens currently recommended for treatment of HIV infected patients with TB have not been reported. Retrospective studies of several different regimens have yielded variable results. Treatment failure rates ranged from 1-14% and adverse reactions resulted in modification of therapy in 4-18% of cases (Jones, et al., 1994). In some studies, adverse reactions to anti-TB medications were common in HIV-infected patients and serious adverse effects have been reported in 18-27% of patients (Pape, 2004).

2. Treatment of tuberculosis

2.1. DOTS

The goals of TB treatment are to ensure cure without relapse, prevent death, stop transmission, and prevent the emergence of drug resistance (Frieden, et al., 2003). Regularity and completeness is paramount in the treatment of tuberculosis, since default, interruption and incorrect dosing may cause failure or relapse and development of drug resistance (Deruaz and Zellweger, 2004).

The treatment of tuberculosis has witnessed many important changes over the years (Deruaz and Zellweger, 2004). WHO introduced Directly Observed Therapy, Short course, DOTS, as the global TB control strategy in 1993. It represents both an approach for treating active TB and a strategy for reducing the prevalence of TB in a population (Brewer and Heymann, 2004). The DOTS strategy produces cure rates of up to 95% even in the poorest countries and prevents new infections by curing infectious patients (WHO, 2005).

In Ethiopia DOTS coverage in Health facilities is 75%, excluding public health posts (MOH, 2004). It should also be noted that only 64% of Ethiopians have access to health service (MOH, 2004)

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 (Ligon-Borden, 2003). 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 the discovery of streptomycin, para-amino salicylic acid (PAS) in 1949 and isoniazid in 1952 became available; heralding the era of effective anti tuberculosis 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 (Jawahar, 2004).

The recommended anti-tuberculosis regimens are usually well tolerated. However, some patients may experience problems, usually due to the bulk of drugs, a single day's dose consisting of 6-7 tablets (Jawahar, 2004). Currently, the approach of using fixed dose drug combinations is being implemented.

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 bactericidal drugs isoniazid and rifampicin are necessary. 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 (Frieden, et al., 2003). The intensive phase is followed by a continuation phase in which 2 drugs (ethambutol and isoniazid or rifampicin and isoniazid) are administered for 4 or more months (Cheuk-ming, 2002, Yepes, et al., 2004). Second-line medications have been shown to be less effective and more toxic than the first-line agents or have not been studied extensively (Cheuk-ming, 2002, Yepes, et al., 2004).

2.2.1. Rifampicin

Rifampicin was introduced as a first-line anti-tubercular drug in the 1960s and became the most vital component of short-course chemotherapy in the 1970s. As it acts through the P-450 enzymatic pathway, its interaction with other drugs is an important consideration in planning maintenance therapy (Ali, 1996). The metabolism of rifampicin is mediated via the deacetylation route, resulting in a more water soluble compound that is excreted through the billary system (Ali, 1996).

2.2.2. Isoniazid

Isoniazid (isonicotinic acid hydrazine) has been used since 1952 as a frontline anti TB drug (Aaron, et al., 2004). It is also commonly used for prevention of TB infection (primary prophylaxis) and treatment of latent infection to prevent active TB (CDC, 1993, Richard, 2003). Isonicotinic acid hydrazine (INH) appears to be the only drug among the first line and major second line anti tubercular drugs in which plasma concentration and clearance may be related to hereditary differences in acetylator status (Ali, 1996).

2.3. Treatment of TB in HIV positive and negative patients

In HIV infected patients with TB, if a need arises to treat with both ART and anti TB, the priority is to treat TB because of public heath issues especially in smear-positive cases. Recommended treatment regimens are similar for HIV-infected and HIV negative tuberculosis patients. The most recent guidelines of the Centers for Disease Control (CDC) recommend that HIV-infected patients with drug-susceptible TB be treated with rifampicin, isoniazid, ethambutol and pyrazinamide which is similar to the regimen that is used for HIV-negative patients (Aaron, et al., 2004, Frieden, et al., 2003). The clinical, radiological and microbiological responses to short-course treatment are similar irrespective of HIV status, although death during anti-tuberculosis treatment is much more common in HIV-infected individuals (Frieden, et al., 2003).

3. Drug induced hepatotoxicity (DIH)

Liver damage caused by medication ingestion-also known as drug induced hepatotoxicity or DIH-has become an important public health problem contributing to more than 50% of acute liver failure cases, a fraction of whom require immediate transplantation because of the irreversible damage to the liver (Circle, 2003, Lee, 2003, Sturgill and Lambert, 1997).

Most drugs listed in the reference desk of the physicians and a number of over-the-counter preparations have been associated with different degrees of liver enzyme elevation. Resulting liver toxicity is the number one cause for Food and Drug Administration (FDA) non-approval or withdrawal of medications from the market (Circle, 2003). Reports of adverse drug reactions of any type have endangered fear and scepticism in the public about the actions of pharmaceutical industry and the FDA (Circle, 2003).

Despite rigorous preclinical studies and clinical trials prior to drug licensing, liver damage remains the most frequent type of adverse drug reactions (ADR) that can lead to the withdrawal of a drug from the market. Of drugs associated with hepatotoxicity in animal experiments, only one-third resulted in a rise in liver enzymes in humans (Circle, 2003). The reliable design of non-hepatotoxic molecules is not yet possible and the predictive value of animal and *in vitro* studies

to identify drug hepatotoxicity before use in humans remains disappointing (Bagheri, *et al.*, 2000).

Xenobiotic-induced liver injuries can be broadly classified as intrinsic and idiosyncratic. Intrinsic injuries are predictable in that threshold dose exists in all individuals typically leading to zonal liver cell necrosis accompanied by little or no signs of inflammation. These injuries are generally the result of phase I bioactivation with damage mediated by reactive drug metabolites. In contrast, the nature of idiosyncratic liver injuries suggests that most of them are mediated by an immune mechanism (Sturgill and Lambert, 1997). The reasons why the liver is prone to xenobiotic-induced injury are because of its central role in xenobiotic metabolism, its portal location within the circulation, and its anatomic and physiologic structure (Sturgill and Lambert, 1997).

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 (Sturgill and Lambert, 1997).

The regional pattern of hepatocellular necrosis observed with some xenobiotic-induced liver injuries can be 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 cythochrome P450 enzyme activity, which frequently makes them most susceptible to xenobiotic-induced liver injury (Luster, *et al.*, 2001, Sturgill and Lambert, 1997).

The most frequent hepatotoxic drug reactions evoke moderate to severe injury to hepatocytes with a clinical picture that resembles viral hepatitis, characterized by a rapid onset of malaise and

jaundice in association with elevated aminotransferase levels. Each drug has its own pattern of injury, and if hepatocyte injury predominates, aminotransferase levels may be at least five times as high as normal elevations of alkaline phosphatase and bilurubin levels predominate in cholestataic syndromes (Lee, 2003).

3.1. Anti-tuberculosis DIH

Hepatotoxicity is one of the most important adverse drug reactions associated with anti-tuberculosis drugs that may limit their use and is also one of the most prevalent drug-induced liver injuries (Bassey, et al., 2005).

Among the first line anti-TB drugs, rifampicin, isoniazid and pyrazinamide are known to be potentially hepatotoxic (Barnes and Barrows, 1993, Cheuk-ming, 2002, Sharma, *et al.*, 2002, Ubaid, *et al.*, 2003). The efficacy and success rate of anti-tuberculosis treatment could be seriously compromised if any of these drugs were to be dropped from the therapeutic regimen due to hepatotoxic side effects (Ali, 1996).

Previous studies have shown transient elevations of serum hepatocellular enzymes (e.g. alanine aminotransferase and aspartate aminotransferase) (Ali, 1996) development in approximately 10% of patients who received a standard combination chemotherapy that includes isoniazid and rifampicin and that 1-2% of those patients would have to withdraw from the treatment because of severe hepatotoxicity that ultimately led to fulminant hepatitis. For example, the risk of hepatotoxicity based on data from four prospective Indian studies was 11.5% (with 95% CI: of 9.5-13.5) (Bagheri, et al., 2000, Dye, et al., 1999, Ungo, *et al.*, 1998), compared to only 4.3% (with 95% CI: of 3.4-5.3) in 14 published studies from the west (Sharma, et al., 2002).

Clarification of the etiological role of a particular agent in this group as the cause of hepatic injury has been complicated by the frequent use of several drugs in combination. Nevertheless, observations recorded from the period when PAS was employed alone or with streptomycin, and the more recent practice of treating 'tuberculin converters' with INH alone has permitted some deductions regarding the ability of each of these agents to produce hepatic injury. Streptomycin and dihydrostreptomycin appear to be free of hepatotoxic potential, but PAS can cause liver

damage. Ethambutol leads to very rare instances of cholistatic jaundice. Rifampicin also has been implicated in cases of hepatic injury. It appears to potentiate the ability of INH to produce hepatic injury and *vice versa*. By far the most important member of this group with regard to hepatotoxicity is INH (Kimmoun and Samuel, 2002, Macsween).

The mechanism of DIH by anti-tuberculosis agents involves direct cytotoxicity (by the drug or its metabolites). An immune-related component is believed to exist as well since both INH and rifampicin have documented immunologic effects, but the exact mechanism of this DIH remains unclear (Huang, *et al.*, 2003, Ungo, et al., 1998).

3.1.1. Isoniazid hepatotoxicity

Isoniazid hepatotoxicity is a common complication of anti-tuberculosis therapy that ranges in severity from asymptomatic elevation of serum transaminases to hepatic failure requiring liver transplantation (Huang, et al., 2003, Richard, 2003). Metabolic intermediates of isoniazid, instead of isoniazid *per se,* are incriminated as the cause of hepatotoxicity (Huang, et al., 2003). Even if the frequency of isoniazid hepatotoxicity depends on the threshold for making the diagnosis, it carries a 10-20% risk of biochemical hepatitis, which is commonly reversible despite continued therapy with isoniazid. However, 1-2 % of adult patients taking isoniazid develop severe hepatitis that may progress to liver failure and death if the drug is not stopped promptly (Nolan, *et al.*, 1999, Richard, 2003, Sarich, *et al.*, 1998).

The mild elevation of transaminases seen during the first 2 months of therapy may reflect direct toxicity from hydrazine metabolites which can covalently bind to cellular macromolecules, including DNA. The more severe hepatitis may be a consequence of the production of more reactive species by cythochrome P-450 enzyme system (Campos-Franco, *et al.*, 2004, Richard, 2003). In some patients an allergic mechanism has been proposed. Acetylation of hepatic macromolecules by acetyl hydrazine may lead to the release of antigenic macromolecules which induce the formation of antibodies directly against the liver, but still the biochemical mechanism of isoniazid hepatotoxicity remains incompletely defined (CDC, 1993, Richard, 2003, Ungo, et al., 1998).

Pathophysiologically, it is clear that isoniazid hepatotoxicity is not directly related to drug concentrations; hence, a direct toxic effect cannot be incriminated. The typical allergic manifestations of peripheral eosinopillia are also absent, and a successful rechallenge further rules out hypersensitivity mechanisms. The morphologic changes are probably indistinguishable from those associated with viral hepatitis. Ultrasound reveals an increase in liver size but there is no correlation between transaminase level and ultrasound findings (Ali, 1996).

3.1.2. Rifampicin hepatotoxicity

The margin of safety between the minimum effective dose and toxic levels of rifampicin is narrow, and hepatitis is associated with higher blood levels and longer period of exposure to the drug (Ali, 1996). Rifampicin causes DIH in 1-4% of patients when used alone and liver dysfunction associated with rifampicin is either hypersensitivity or cholistatic type (Ali, 1996).

Rifampicin is a potent inducer of enzyme Cytochrome P (CYP) 450, consequently increasing the metabolism of other drugs, such as isoniazid. Although its toxicity is low when administered alone, rifampicin hepatotoxicity has been observed in patients with underlying liver diseases (Kimmoun and Samuel, 2002).

3.1.3. Pyrazinamide hepatotoxicity

Pyrazinamide induces a cytolytic hepatitis by a mechanism of direct toxicity. Some cases of fulminant liver failure have been reported and hepatotoxicity occurs at relatively higher dosages of 40-50 mg/kg per day and usually after long periods of treatment (Ali, 1996, Kimmoun and Samuel, 2002).

3.1.4. Combination of INH and rifampicin hepatotoxicity

A meta-analysis of different studies has shown that regimens combining isoniazid and rifampicin generated a higher incidence of hepatotoxicity (2.5%) than did regimens without this combination (1.1%) (Ali, 1996, Roy, *et al.*, 2001).

3.2. Risk factors for anti TB DIH

While the occurrence of DIH is difficult to predict, it has been observed that certain patients are at higher risk of developing drug induced hepatitis during the course of anti-TB chemotherapy (Cheuk-ming, 2002).

Various studies have suggested that high alcohol intake, older age, acetylator status, pre-existing chronic liver disease, chronic viral infection due to hepatitis B and hepatitis C, HIV infection, advanced tuberculosis, Asian ethnicity, female sex, concomitant administration of enzyme-inducers (e.g. barbiturates and anaesthetic agents), inappropriate use of drugs and poor nutritional status increase the risk of anti-tuberculosis drug induced hepatitis (Barnes and Barrows, 1993, Bruchfeld, *et al.*, 2000, Campos-Franco, et al., 2004, Cheuk-ming, 2002, Huang, et al., 2003, Kimmoun and Samuel, 2002, Sharma, et al., 2002, Ungo, et al., 1998). On the other hand, Singh and associates evaluated the role of risk factors in predicting the development of hepatotoxic effects in patients receiving anti-TB drugs. They found that body-mass index was significantly lower in patients with DIH than in the controls but no significant difference was observed between the two groups with regard to other parameters such as age, sex, alcohol intake, presence of chronic liver disease, hepatitis B carrier state, or acetylator status (Ali, 1996).

The mechanisms for chronic alcohol intake to induce anti-TB hepatototoxicity are induction of CYP 450, that increases the production of toxic metabolites of isoniazid, and the decrease of hepatic glutathione reservoir that can be ascribed to high alcohol ingestion (Kimmoun and Samuel, 2002).

3.3. Anti-tuberculosis DIH and HIV infection

Patients with chronic viral illnesses, such as HIV or HCV infection appear to be more prone to develop hepatotoxicity due to anti-tuberculosis medications, possibly because of decrease in hepatocyte defense mechanisms, e.g. glutathione. In addition, patients with HIV infection are very likely to ingest a cocktail of drugs for treatment of opportunistic infections (Circle, 2003). A study has suggested that AIDS is significantly associated with developing hepatotoxicity. This

implies that an immunocompromised state may be an important factor in predicting hepatotoxicity (Ozick, *et al.*, 1995).

The reasons for marked increase in the incidence of adverse reactions in AIDS patients are currently unknown, but may reflect disease-induced alterations in bioactivation or detoxification of reactive metabolites, as well as immunodysregulation (Gross, *et al*., 1999).

In general there is a paucity of data available on the risk of anti-TB medications in patients infected with HIV with new or reactivated TB, leading to hospitalisation (Ozick, et al., 1995).

3.4. Anti-tuberculosis DIH and isoniazid acetylation status

3.4.1. Pharmacogenetics and drug metabolism

Once a drug is administered, it is absorbed and distributed to its site of action where it can interact with targets (such as receptors and enzymes), undergo metabolism, and is then excreted. Each of those processes could involve potentially clinically significant genetic variations (Weinshilboum, 2003). Metabolism usually converts drugs to metabolites that are more water soluble and thus more easily excreted. It can also convert pro-drugs into therapeutically active compounds, and it may even result in the formation of toxic metabolites (Weinshilboum, 2003).

The liver is the main organ in the metabolism of virtually every foreign substance. Most drugs and xenobiotics are lipophilic enabling them to cross the membranes of the intestinal cells. Drugs are rendered hydrophilic by biochemical processes in the hepatocyte yielding water-soluble products that are excreted in urine or bile (Lee, 2003). Genetic polymorphisms are thought to influence the chemical modification of drugs and its metabolites in the liver (Roy, et al., 2001).

The promise of pharmacogenetics, elucidating the role of inheritance in individual variations in drug response, lies in its potential to identify the right drug and dose for each patient. Even though individual differences in drug response can result from the effects of age, sex, disease, or drug interactions, genetic factors also influence both the efficacy of a drug and the likelihood of an adverse reaction (Anitha and Banerjee, 2003, Weinshilboum, 2003). The difference in the

effect of drugs in different individuals is most often due to variability in drug metabolizing genes. Polymorphisms in those genes are transmitted as autosomal recessive traits and cause impaired activation of numerous clinically important drugs or chemical carcinogens (Anitha and Banerjee, 2003).

Pathways of drug metabolism are generally classified as either phase I reactions (i.e., oxidation, reduction and hydrolysis) or phase II conjugation reactions (e.g. acetylation, glucorunidation, sulfation and methylation). Both types of reactions most often convert relatively lipid-soluble drugs into relatively more water-soluble metabolites (Osborne, *et al.*, 2003, Weinshilboum, 2003).

3.4.2. NAT2 gene

N-acetyl transferases (NAT) are phase II enzymes that metabolize drugs and xenobiotics. Molecular cloning studies subsequently demonstrated that there are two NAT genes in humans, NAT1 and NAT2. They differ both in their substrate specificity and in their prevalence 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 (Osborne, et al., 2003, Weinshilboum, 2003). 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 (Bakayev, *et al.*, 2004).

Polymorphism of human NAT2 has been known for more than 40 years. The inter-individual differences in the metabolism of isoniazid provided an important indication of this. N-acetylation of this drug precedes to a much lesser degree in a considerable percentage of people so that patients could be divided into two groups, the rapid and the slow acetylators. The elimination half-life for isoniazid is considerably shorter in the case of rapid acetylators. The biological activity of the majority of substances is reduced by acetylation. However, the reactivity of certain substances can also be enhanced. In human, NAT2 has been detected in the liver, the tissue of the

gastrointestinal tract , the bladder and the lungs (Gross, et al., 1999, Hein, *et al.*, 2000, Kukongviriyapan, *et al.*, 2003, Weinshilboum, 2003).

NAT1 and NAT2 are products of a single, intronless protein coding exons of 870-bp 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 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 (Anitha and Banerjee, 2003, Gross, et al., 1999, Hein, et al., 2000, Jetter, *et al.*, 2004, Kukongviriyapan, et al., 2003, Roy, et al., 2001).

Most or perhaps all populations of the world are polymorphic for 'rapid inactivation' versus 'slow inactivation'. The 'slow inactivator' individual is homozygous for a slow inactivator allele and the 'rapid inactivator' individual may be either homozygous or heterozygous for a rapid inactivator allele (Jetter, et al., 2004).

To date, 15 single nucleotide polymorphisms (SNPs) of NAT2 have been identified. They occur alone or in combination and lead to the 34 allele variations known at present and each allelic variant is a combination of one, two, three, or four nucleotide substitutions. Of the 15 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 unchanged (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) (Godfrey-Faussett and Ayles, 2003, Gross, et al., 1999, Jetter, et al., 2004, Kukongviriyapan, et al., 2003).

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 (Godfrey-Faussett and Ayles, 2003, Jetter, et al., 2004).

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 (Kukongviriyapan, et al., 2003).

3.4.2.1. Frequency of NAT2 in different ethnic groups

NAT2 gene polymorphism, like those in the genes for many other drug metabolizing enzymes, shows striking ethnic variation (Weinshilboum, 2003). The frequency of the specific mutations within the NAT2 locus depends on the racial and ethnic origin. In white individuals, the most common mutations are those at positions 481 T and 803 G, followed by the less frequent mutations at positions 590A and 857G, more than 50% of Caucasians show a decreased activity of the enzyme or lack of it (Gawronska-Szklarz, *et al.*, 2001).

By analyzing for the function impairing polymorphisms C_{282} T and $T_{341}C$, it is possible to adequately predict fast and slow acetylator phenotypes in a Caucasian population. In Black populations, $G_{191}A$ would also have to be genotyped as this is a common mutation in African but not in Caucasian or oriental populations (Hein, et al., 2000, Osborne, et al., 2003).

NAT2*4 is not the most common allele in many ethnic groups, including Caucasians and Africans. NAT2 alleles containing the $G_{191}A$, $T_{341}C$, $A_{434}C$, $G_{590}A$, and/or $G_{857}A$ mis-sense substitutions are responsible for the corresponding ethnic difference in the frequencies of slow acetylator alleles and phenotype (s) (Hein, et al., 2000).

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 (Gross, et al., 1999). Slow acetylation frequency in Asian populations ranges between 10-30%, while in most North American populations the frequency is between 40-70% (Hein, et al., 2000, Osborne, et al., 2003).

3.4.2.2. Relevance of NAT2 gene

The NAT2 acetylation polymorphism is very important in clinical pharmacology and toxicology because of its primary role in the activation and/or deactivation of a large and diverse number of aromatic amine and hydrazine drugs used in clinical medicine (Hein, et al., 2000). NAT2 is involved in the initial biotransformation metabolism of aromatic amines and hydrazines and catalyses the transfer of an acetyl group from acetyl COA to the nitrogen of the substrate (Godfrey-Faussett and Ayles, 2003).

NAT2 polymorphisms are major cause of drug toxicity, drug-drug interaction, and lack of therapeutic effect and causes inter-individual variations in the biotransformation of various drugs, pro-carcinogens and other xenobiotics with a primary aromatic or hydrazine structure. Examples of drugs that are metabolized by NAT2 enzyme are- isoniazid, sulfadimidine, sulfamethazine, hydralazine, dapsone, procainamide, sulfapyridine, nitrazepam, and caffeine (Bakayev, et al., 2004, Grant, *et al.*, 1990, Gross, et al., 1999, Jose, 2003, Lee, *et al.*, 2002, Sturgill and Lambert, 1997).

3.4.2.3. NAT2 gene and INH hepatotoxicity

Though several risk factors for the development of hepatotoxicity due to anti-tuberculosis drugs have been suggested, the involvement of genetic factors is not fully established (Roy, et al., 2001, Sharma, et al., 2002, Sturgill and Lambert, 1997).

Isoniazid undergoes extensive NAT2 catalyzed acetylation to acetylisoniazid which is then hydroxylated by cytochrome P450 enzymes to the hepatotoxic intermediate acetylhydrazine, a metabolite capable of forming covalent cellular adducts (Sturgill and Lambert, 1997).

In the 1980s, Weber and Associates and Gurumuthy *et al*. suggested that isoniazid is no more hepatotoxic in slow than in rapid acetylators. In their retrospective analysis, the Gurumurythy group showed that the incidence of jaundice among those two groups was 1.2% and 1.9% respectively, not a statistically significant difference. Therefore, it appears that the difference in

slow and rapid acetylation of isoniazid does not play a role in the development of isoniazid-induced hepatitis (Ali, 1996).

On the other hand, other studies have suggested that individuals who are slow acetylators are at greater risk suggesting that slow metabolism results in diversion of isoniazid metabolism to an alternate (e.g. CYP-450 mediated) pathway that may produce a toxic metabolite. This interpretation is supported by observation that drugs that induce CYP-450 levels (including rifampicin, which often is prescribed together with isoniazid) appear to increase the risk of isoniazid toxicity (Bruchfeld, et al., 2000, Richard, 2003).

It was also shown that not only development of hepatotoxicity but also more severe forms of hepatotoxicity are seen in slow than rapid acetylators (Kimmoun and Samuel, 2002). On the contrary, another study showed that individuals with rapid acetylator phenotype are more likely to develop hepatotoxicity (Richard, 2003). Generally the association of polymorphic NAT2 acetylator status and anti-tuberculosis DIH is still variable (Dye, et al., 1999).

3.4.2.4. NAT2 gene-associated diseases

NAT2 status has been associated with susceptibility to different cancers (Grant, et al., 1990, Gross, et al., 1999, Lee, et al., 2002). Slow acetylator phenotype has been associated with various cancers. This includes bladder cancer, particularly in individuals exposed to arylamines, oral/pharyngeal cancer, and malignant mesothelioma. Systemic lupus erythromytosis and Parkinson's disease have also been thought to be more frequent in slow acetylators. The fast acetylator phenotype has been linked to laryngeal cancer, adenoma, colorectal cancer and Alzheimer's disease with frequency of the NAT2*4 genotype in parallel with reduced frequency of the NAT2*6 genotype (Godfrey-Faussett and Ayles, 2003, Osborne, et al., 2003).

3.4.2.4. Determination of NAT2 gene

In the past several years several polymorphisms of drug metabolizing enzymes have been described and typing tests have been developed for *in vivo* evaluation of enzyme activities

(phenotyping) or for the detection of mutations that cause impaired metabolism (genotyping). These tests are currently being used to avoid adverse drug reactions in patients who have inherited impairment in drug metabolism (Jose, 2003).

Various methods have been used to determine NAT2 genotype status, including allele specific amplification and more commonly restriction fragment length polymorphism (RFLP) analysis (Osborne, et al., 2003). Concordance of NAT2 genotype and phenotype classification was found to be 97.8% so genotyping alone provides an efficient, accurate method of analysis for acetylator status (Gross, et al., 1999, Jetter, et al., 2004).

Depending on the probe drug and analytical method used, acetylation phenotypes are often not clearly separated or distinct but rather exhibit continued and overlapping variability due to numerous genetic and/or environmental factors, including diet, disease status, concurrent drug therapy, and the large number and diversity of NAT2 genotypes present in human populations (Grant, et al., 1990, Hein, et al., 2000, Kennedy, *et al*., 2004, Lee, et al., 2002).

Caffeine is most commonly used as a probe drug for NAT2 phenotype determinations, and excellent NAT2 genotype/phenotype correlations have been reported (Jetter, et al., 2004, Nyeki, *et al*., 2001). Calculation of urinary metabolite ratios of caffeine (1,3,7-trimethyl xanthine) is used for determining the bimodal NAT2, it also helps to assess the activity of cytochrome P450 isoenzyme 1A2 (CYP1A2) and xanthine oxidase (XO) (Jetter, et al., 2004, Nyeki, et al., 2001).

3.5. Management of anti-TB DIH

Drug-related adverse effects might be minor or major. In general, a patient who has minor adverse effects should be encouraged to continue the treatment with symptomatic measures such as use of antacids, anti-histamines, anti-emetics, or analgesics as indicated. If major adverse reactions occur, the regimen or the offending drug if identified, must be stopped. Further management depends on the nature of the adverse reactions and may have to be done in a hospital. Major adverse reactions to anti-tuberculosis drugs can cause significant morbidity and compromise treatment regimens for tuberculosis (Jawahar, 2004, Yee, *et al*., 2003).

Traditionally, therapy-related severe hepatotoxicity has been managed safely by the discontinuation and gradual reintroduction of therapy with potent-first line agents because of the indolent nature of tuberculosis. However, previous reports have documented a high early mortality among HIV-infected persons with tuberculosis, even with appropriate therapy. Because of this high mortality, discontinuing all anti-tuberculosis therapy when hepatotoxicity develops is not recommended. Although the role of the fluroquinolones in the treatment of drug-susceptible and drug-resistant tuberculosis has not been completely defined, a three-drug combination of ethambutol, ofloxacin, and an aminoglycoside may be a useful alternative to traditional hepatotoxicity management in HIV-infected individuals. In addition, a further microbiologic evaluation, especially for multi drug resistant tuberculosis is needed (Salomon, *et al.*, 1994, Ubaid, et al., 2003).

In the absence of ofloxacin and aminoglycosides DIH accompanied by clinical jaundice can be managed by withholding all drugs and instituting supportive treatment. Once liver function returns to normal, anti-tuberculosis drugs may be reintroduced. If jaundice recurs, rifampicin, isoniazid and pyrazinamide should be withdrawn and the patient be treated with streptomycin and ethambutol. If anti-tuberculosis drugs are considered essential during the course of jaundice, streptomycin and ethambutol should be used (Circle, 2003, Jawahar, 2004, Lee, 2003).

Existing data do not allow reliable prediction of the exact clinical course of asymptomatic patients with moderate degree of biochemical derangement. Opinions, therefore, differ as to at what cut-off level of liver dysfunction modification of treatment regimen should be initiated. For alanine transaminase levels, some recommend stopping the hepatotoxic drugs at three times or above that of normal, while others recommend at a rise of five times the normal level. The recommendations regarding the level of bilurubin are also not uniform (Cheuk-ming, 2002).

In some circumstances, patients who are severely affected may remain asymptomatic until potentially lethal liver damage has occurred. Thus, the risk of death cannot be avoided by monitoring the patient's symptoms but requires prospective monitoring of serum transaminases levels in patients at high risk (Barnes and Barrows, 1993, Richard, 2003).

4. Justifications of the study

- DIH in relation with fixed dose combination anti-TB drugs has not been reported.
- This type of prospective cohort study on identification of the risk factors for anti-TB DIH in Ethiopia has not been reported.
- Association of Acetylation status with anti-TB DIH and different health problems has not been reported.
- There is no National guideline for the diagnosis and management of anti-TB DIH.
- Association of polymorphic NAT2 and anti-TB DIH is still controversial:
    - Rapid acetylators at increased risk of anti-TB DIH (Richard *et.al.).*
    - Slow acetylators at increased risk of anti-TB DIH (Gurumurythy *et.al.).*
- HIV infection is reported to be a risk factor for development of DIH (Ungo *et.al.*).
- TB/HIV co-infection is high in Ethiopia.
- Clinicians encounter DIH frequently in practice.

5. Hypothesis

HIV co-infection and a slow acetylation status are risk factors for development of DIH in patients taking anti-TB drugs.

6. Study objectives

6.1. General objectives

The general objectives of this study are to assess and compare the prevalence, severity and prognosis of hepatitis induced by first- line anti- tuberculosis drugs in Ethiopian HIV positive and HIV negative patients and to identify associated risk factors.

6.2. Specific objectives

1. To determine the prevalence of DIH in tuberculosis patients with no HIV co-infection.
2. To determine the prevalence of DIH in HIV-associated tuberculosis.
3. To determine the association of immuno-suppression with the development of DIH.
4. To assess the role of acetylation status in the development of hepatotoxicity.
5. To assess the severity as well as outcome of anti-tuberculosis DIH in HIV infected patients.
6. To evaluate the need for special follow up and management for selected patients who might develop drug toxicity.

7. Methods

7.1. Study population

From August 2004 to March 2005, we prospectively evaluated 103 HIV positive and 94 HIV negative newly diagnosed male and female adult tuberculosis patients at St. Peter's TB Specialized Hospital in Addis Ababa, Ethiopia both from inpatient and outpatient departments.

Diagnosis of patients was based on sputum smear positivity, fine needle aspirate (FNA), clinical and radiological evidences (Appendix I). The criteria for diagnosis were based on the National guidelines for diagnosis of tuberculosis. Patients with smear positive, smear negative, extra pulmonary, and disseminated TB were included in the study. Patients were excluded if they had a history of prior tuberculosis, do not consent for HIV testing, are < 18 years of age and had clinical evidence of liver damage prior to starting medication. All patients were on DOTS for the full duration of the intensive phase.

7.2. Sampling

To answer the question that HIV infection is a risk factor for development of hepatotoxicity in patients taking anti-TB drugs, we set the level of significance and the power of the study at 5% and 80% respectively. A meta-analysis (2004, Ali, 1996, Roy, et al., 2001) had indicated 2.5% incidence rate of hepatotoxicity with combination treatment of anti-TB drugs. The relative risk in HIV positive patients for anti tuberculosis drug- induced hepatitis was found out to be 7.5 %; hence the incidence rate calculated is 18.75%. The ratio between the two groups is taken as 1:1. Therefore, the sample size calculated using the formula for comparing two groups is 67 in each study group. Assuming an attrition rate of 40%, the sample size in each study group is extended to 94 making the total sample 188 altogether.

7.3. Laboratory tests

All patients in the study had laboratory examinations for different tests that included HIV testing which were done by rapid test kits. Diagnosis of HIV was made based on the National HIV diagnosis algorithm. Blood was tested first by Determine® and if patient is negative the result interpreted as negative. If the result is positive, it will be reconfirmed by Cappilus®. If the results by the two tests were positive, the patient will be labelled as positive and if the results were controversial, Unigold® was used as a gold standard test for confirmation.

CD4 counts were done for HIV positive patients within the first week. CD4 counts were done by the International Clinical Laboratory, which is one of the few US accredited Clinical Laboratories in Africa.

Other laboratory tests done for all enrolled patients before initiation of anti TB drugs included; complete and differential blood counts, erythrocyte sedimentation rate (ESR), hematocrit (Hct), liver function tests (aspartate aminotransferase (AST), alanine aminotransferase (ALT), direct and total bilurubin and alkaline phosphatase), serological tests for hepatitis B surface antigen and anti-hepatitis C antibody. Liver function tests were performed before starting anti TB drugs and then at the 1^{st}, 2^{nd}, 4^{th}, 6^{th}, and 8^{th} weeks after starting DOTS.

7.4. Diagnosis of anti TB DIH

7.4.1. Biochemical DIH

Patients were diagnosed to have biochemical hepatotoxicity if they had no other apparent causes for the elevation of liver function tests and if they had at least one of the following parameters.

1. A rise of three times the upper limit of normal levels (32µ/l) of serum asparteate aminotransferase (AST).

2. A rise of three times the upper limit of normal levels (31µ/l) of serum alanine aminotransferase(ALT).
3. A rise in the level of serum total bilurubin >3mg/dl.

7.4.2. Clinical DIH

Clinical hepatotoxicity was diagnosed if a patient had biochemical hepatotoxicity plus jaundice.

7.5. Management of patients with DIH

If a patient developed only biochemical hepatotoxicity, he/she was followed closely both clinically and biochemically for the development of clinical hepatotoxicity. If a patient developed clinical hepatotoxicity, all the anti-TB drugs were discontinued. Patients were followed clinically and biochemically for the improvement of the jaundice and a decrement in the liver function tests. When the patient's jaundice improved, the anti-TB drugs were restarted in a stepwise manner where INH was first started at 50 mg dose and then increased to 100 mg after 3 days followed by 1 tablet RH plus 100 mg INH, then 2 tablets of RH plus 150 mg INH, then 1 tablet RHZ plus two tablets of RH and finally 3 tablets of RHZ. All tapering up was done with a 3 days gap. If a patient was taking a regimen that included ethambutol, the drug was started in full dose at the beginning of the reintroduction and all patients where put on EH during the continuation phase.

7.6. Determination of acetylation status

7.6.1. NAT2 phenotype determination

In vivo, NAT2 activity was measured with a caffeine-based assay as described by Blaszkewicz *et. al*. (Blaszkewicz, 2004) with some modification. Study participants ingest two tablets, each containing 100mg of caffeine following an overnight fast. They refrained from the consumption of coffee, tea, coca cola, pepsi cola, and chocolate 48 hours before taking caffeine tablets until the 2nd urine sample was collected. Two urine specimens at 4 and 5 hours were collected in

separate containers from each patient and kept immediately at $+4^0$C and the urine pH was adjusted to 2.5-3.5 by adding 2N HCl within 2 days of urine collection to prevent spontaneous deformylation (conversion of the caffeine metabolite 5-acetylamino-6-formylamino-3-methyluracil (AFMU)). Then, 5 ml urine sample was taken from each specimen and kept at -20^0C until analysis (2-8 months from the time of collection).

7.6.1.1. Urine analysis

Urinary AFMU and 1-methylxantine (1-MX) were determined by reverse-phase high performance liquid chromatography (HPLC). Six percent methanol in 0.01 M formic acid was used as mobile phase A and 100% HPLC grade methanol was used as mobile phase B. Both mobile phases were degassed before use.

7.6.1.1.2. Stock solution preparation

7.6.1.1.2.1. AFMU

Two milligram AFMU was weighed into a 10 ml volumetric flask (200 µg/ml = 0.88 µmol/ml). The flask was subsequently filled to its nominal volume with mobile phase A. The dissolution process was assisted by ultrasound treatment for 15 minutes. The volume was divided into 5 ml portions in plastic test tubes and stored at -20°C.

7.6.1.1.2.2. 1-MX

Two milligram 1-MX was weighed into a 10 ml volumetric flask (200 µg/ml = 1.2 µmol/ml). Eight ml 0.01 M formic acid was added while the flask was swirled occasionally. Twenty-five µL 25% ammonia was added with a pipette. The flask was subsequently filled to its nominal volume with 0.01 M formic acid. The dissolution process was assisted by treatment with ultrasound for 15 min. The volume was divided into 5ml portions in plastic test tubes and stored at -20°C. Stock solutions of AFMU and 1-MX were thawed immediately before use.

7.6.1.1.3. Sample preparation

Chloroform/isopropanol 97:3 v/v was used for the extraction of caffeine metabolites from urine. Before analysis, the samples were thawed and thoroughly mixed. One ml of the urine sample was diluted with 1.0 ml distilled water (step 1, Table 7.1) in a test tube. Using a pipette, 900 µl of the diluted sample and 100 µl of mobile phase A (blank sample) were placed in another test tube. Fifty µl AFMU stock solution, 900 µl of the diluted urine sample and 50 µl 1-MX stock solution (spiked sample) (step 2, Table 7.1) were added one after the other into a second test tube.

Ammonium sulphate (140 mg) was then placed in an empty test tube for the blank and the spiked samples. Then 200 µl of the samples from step 2 and 3 ml chloroform/isopropanol (97:3; v/v) were added with a Pasteur pipette into the ammonium sulphate. Each tube was tightly sealed immediately after addition of the extraction solution (step 3, Table 7.1). The test tubes were then vortexed briefly and centrifuged for 5 minutes at 3,000 rpm.

Table 7. 1. Processing scheme

Step	Designation	**Spiking volumes or weights**	
1	Urine sample	1 ml	
	Water	1 ml	
2	Sample from step 1	900 µl	900 µl
	Mobile phase A	100 µl	-
	AFMU stock solution	-	50 µl
	1-MX stock solution	-	50 µl
3	Sample from step 2	200 µl	200 µl
	Ammonium sulphate	140 mg	140 mg
	Chloroform/isopropanol	3 ml	3 ml
		Blank sample	**Spiked sample**

Two ml of the lower (organic phase) was transferred with a Pasteur pipette into a fresh tube. The organic phase was then evaporated at 45°C for 7 hours in a water bath. The residue was taken up in 1 ml of mobile phase A and then centrifuged for 5 minutes at 5,000 rpm. Five hundred µl of the processed sample were then pipetted into an HPLC autosampler vial for the subsequent HPLC separation.

7.6.1.1.7. Chromatographic system and condition

A Shimadzu HPLC machine in the laboratory of Ethiopian Drug Administration and Control Authority (DACA) was used for analysis. The chromatographic system consisted of a stainless steel HPLC column with 250 mm length, 4.6 mm inner diameter and Superspher 100 RP-18; 4 µm package. The oven temperature was set at 30°C, elution was gradient and the machine was adjusted to take 100% from mobile phase A from 0 to 18 minutes, 100% from mobile phase B from 18 to 19 minutes and 100% from mobile phase A from 19.0 to 24 minutes flow rate was 1.0 ml/min and injection volume was 50 µl.

The AFMU and 1-MX concentrations were determined using the following formula

$$C_{\text{AFMU (blank sample)}}\ \text{nmol/ml} = \frac{C_{\text{AFMU (spiked sample)}}\ \text{nmol/ml} \times \text{Area}_{\text{AFMU (blank sample)}} \times 2}{\text{Area}_{\text{AFMU (spiked sample)}} - \text{Area}_{\text{AFMU (blank sample)}}}$$

$$C_{\text{1-MX (blank sample)}}\ \text{nmol/ml} = \frac{C_{\text{1-MX (spiked sample)}}\ \text{nmol/ml} \times \text{Area}_{\text{1-MX (blank sample)}} \times 2}{\text{Area}_{\text{1-MX (spiked sample)}} - \text{Area}_{\text{1-MX (blank sample)}}}$$

Acetylation status was finally determined from the molar ratio of AFMU to 1-MX in the urine samples. To obtain the quotient, the molar AFMU concentration was divided by the molar 1-MX concentration. If the quotient (AFMU/1-MX) was less than 1, the test person is assigned to the slow acetylators and if it is greater than or equal to 1, the person is a rapid acetylator.

7.6.2. NAT2 genotype determination

When a patient came for the last visit, 8-10 ml of venous blood was taken into tubes containing ethylenediamine-tetra-acetic acid (EDTA) as anti-coagulant. The blood sample was put at $+4^0$C and transported for DNA isolation within 48 hours. DNA was isolated according to Boodram *et. al*. (Boodram, 2004) with some modification.

7.6.2.1. DNA extraction procedure

7.6.2.2. Buffers

The following buffers were used for DNA extractions: Buffer A (Red Blood Cell lysis buffer) is composed of 0.32 M sucrose, 10 mM Tris HCl, 5 mM $MgCl_2$, 0.75% Triton-X-100 and buffer B (Proteinase K buffer) is composed of 20 mM Tris-HCl, 4 mM Na_2EDTA, 100 mM NaCl, were first prepared and the pH of buffer A was adjusted to 7.6 and that of buffer B was adjusted to 7.4. Both buffers were autoclaved. However, Buffer A was autoclaved prior to addition of Triton-X-100.

7.6.2.3. Extraction procedure

Ten ml of buffer A was added to 10 ml of blood in a 50 ml plastic test tube and then 20 ml of cold, sterile, distilled, deionised water was added. The tube was then inverted 6-8 times and left to incubate on ice for 2-3 minutes. The mixture was then centrifuged at 4,500 rpm for 10 minutes. The supernatant was then discarded and the residue was vortexed for 30 seconds at medium speed in 2 ml of buffer A and 6 ml of water. It was centrifuged again at 4,500 rpm for 10 minutes until the pellet became white to cream colour.

Five ml of buffer B and 500 µl of 10% SDS were added to the pellet, and it was re-suspended by vortexing vigorously for 30-60 seconds. Then 50 µl of refrigerated Proteinase K solution (20mg/ml) was added. It was then left to incubate at 55^oC for 2 hours in a water bath and then

cooled for 2-3 minutes on ice. Four ml of 5.3 M NaCl solution was then added and vortexed gently for about 15 seconds.

It was then centrifuged at 4,500 rpm for 20 minutes at 4^{o}C and supernatant was poured off into a fresh tube very gently not to dislodge the pellet. Then, approximately equal volume of cold isopropanol (stored at -20^{o}C) was added into it and inverted 5-6 times to precipitate DNA.

DNA was removed with a Pasteur pipette and transferred to a tube and then 1 ml of 70% ethanol was added and vortexed gently to wash the DNA and then the ethanol was discarded. Finally DNA was re-suspended in 500 µl of Tris HCl, pH 8.5 and left overnight at room temperature to re-dissolve and then stored at -20^{o}C till analysis (4-8 months).

7.6.2.4. Genotyping procedure

A combination of PCR-RFLP and allele specific PCR were applied for genotyping NAT2 gene polymorphism.

7.6.2.4.1. PCR RFLP

7.6.2.4.1.1. First PCR

Master mix was first prepared by mixing 5 µl of 10X PCR buffer, 1 µl of 10mM dNTPs, 4 µl of 2 mM $MgCl_2$, 5 µl of 0.1 µg of primer 5'-GGCTATAAGAACTCTAGGAAC-3',
7 µl of 0.1 µg of 5'-AAGGGTTTATTTGTTCCTTATTCTAAAT-3', 0.25 µl of 5 unit/µl Taq DNA polymerase and 22.8 µl of sterile distilled water and then 45 µl of the mix was taken and added into a labelled PCR tube after that 5 µl DNA sample (approximately100 ng DNA/µl) was pipetted into each PCR tube. The PCR tubes were then closed tightly and centrifuged briefly (1,000 rpm) to remove any bubbles. Then the tubes were placed in the PCR device (Gene Amp ® PCR system 9700), which was programmed as a 5-min pre-treatment at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, and a 5-min extension step at 72°C. The amplicon was then checked for success of the reaction by gel electrophoresis.

7.6.2.4.1.2. Electrophoresis 1

The PCR product was checked by running on a carried out by means of electrophoresis on 1.2% agarose gel electrophoresis with 10X TBE buffer. Eight µl of the PCR product and 4 µl gel-loading buffer (including glycerol) were mixed and applied into the gel wells. The loading buffer ensures that the samples stay in the application wells. The electrophoresis was performed at 124 Volts for approximately 1 hour. The DNA was visualised under UV illumination and picture of the gel was taken using Polaroid film.

7.6.2.4.1.3. Restriction cleavage of the first PCR product

After successful amplification, a total of three aliquots of the PCR products of the first amplification per sample were further processed separately and subjected to cleavage by restriction enzymes.

Master mix for each enzyme was first prepared for all the three enzymes (see Table 7.2); then 8 µl of the appropriate restriction enzyme master mix were then added to each separate 12 µl aliquot of the product from the first PCR, and the samples were incubated in 37°C water bath over night (see Table 7.2). Mutations at different restriction fragment sites were then identified by analysing the different restriction DNA fragment band pattern (see Table 7.3) after the second agarose gel electrophoresis.

7.6.2.4.1.4. Electrophoresis 2

The products of restriction cleavage were subjected to gel electrophoresis. Two percent agarose gels with 10X TBE buffer. Two litres of 1 X TBE buffer was used as a running buffer. A 15 µl enzyme digestion product and 4 µl of loading buffer were added into the wells and then run for approximately 4 hours. A photo of the electrophoresis result was then taken and results of the enzyme digestion were interpreted according to the result of the enzyme digestion with or without single nucleotide polymorphism to see for the presence or absence of point mutations.

Samples were then labelled accordingly into wild type heterozygous mutant and homozygous mutant for all the 3 enzymes.

Table 7. 2. Restriction cleavage for amplification products of the first PCR

		Volumes for one sample		
		***Taq* I**	***Msp* I**	***BamH* I**
Product 1st PCR		12 µl	12 µl	12 µl
Master mix	Restriction enzyme	0.80 µl (10 U)	0.50 µl (5 U)	0.80 µl (10 U)
	10-fold restriction enzyme buffer	2 µl	2 µl	2 µl
	BSA	0.20 µl	Without BSA	0.20 µl
	H_2O sterile	5 µl	5.50 µl	5 µl
Incubation time		Overnight	Overnight	Overnight
Incubation temp.		37°C	37°C	37°C
Agarose gel		2%	2%	2%

Table 7. 3. Enzyme digestion pattern of the first PCR product

Restriction Enzyme	Nucleotide change	Amino acid change	PCR I product	Wild type fragment pattern	Mutated Fragment pattern
Taq-I	590G>A (NAT2*6)	Arg197Gln	866	332+226+170+137	396+332+137
Bam H-I	857G>A (NAT2*7)	Gly286Glu	866	826+39	866
Msp-I	191G>A (NAT2*14)	Arg64Gln	866	706+93+66	799+66
Msp-I	434A>C (NAT2*17)	Gln145Pro	866	706+93+66	462+244+93+66

7.6.2.4.1.5. Allele-specific PCR

PCR master mix was first prepared for detection of the wild type and the mutant type NAT2 gene in separate tubes. The tube for the wild type contained a mix of 2.5 µl of 10X PCR buffer for Ampli-Taq Gold, 0.5 µl of 10mM dNTPs, 1.2 µl of 1.2 mM $MgCl_2$, 0.625 µl of 10 µM of the reverse primer 5'-GAAGATGTTGGAGACGTCTGCAGG-3', and 0.625 µl of 10 µM of the forward primer 5'-TTCTCCTGCAGGTGACCAT-3', 0.125 µl of 5 unit/µl Ampli-Taq Gold DNA polymerase and 18.43 µl of sterile distilled water, and the tube for the mutant type contains the same as for the wild type except a change in the forward primer which was 5'-TTCTCCTGCAGGTGACCAC-3'. Twenty-four µl of the mix was then taken and added in to labelled PCR tubes for both the wild type and mutant samples. After that 0.5 µl of the first PCR product was added into each PCR tube. The PCR tubes were then closed tightly and centrifuged briefly. Then the tubes were placed in the PCR device (Gene Amp ® PCR system 9700), which was programmed as a 10-min pre-treatment at 95°C, followed by 23 cycles of 20 sec at 95°C, 20 sec at 56°C, and 1 min at 72°C, and a 7-min extension step at 72°C.

The presence or absence of a mutation at 341T>C (NAT*5) was then checked by the presence or absence of the allele specific amplifications after gel electrophoresis.

7.6.2.4.1.6. Electrophoresis 3

A check for the PCR product was carried out by means of electrophoresis on 1.2% agarose gel with 10X TBE buffer. Fifteen µl of the PCR product, and 4µl gel-loading buffer was loaded on the gel.

7.6.2.4.1.7. Haplotyping

Haplotype frequencies were estimated from raw genotypic data by maximum-likelihood method based on the expected maximization algorithm, using the Alriquin program (Version 2000).

7.7. Materials

Reagents for determination of liver chemistry and kits for HIV testing (Determine®, Cappilus®, Unigold®) were bought from pharmaceutical importers in Ethiopia. AFMU and 1-MX were obtained from Dr. Klaus Golka at the Institute of Occupational Physiology, University of Dortmund, Germany. Caffeine tablets 200 mg were bought from a Pharmacy in Stockholm, Sweden. The HPLC column was bought from Set Point Company, South Africa. The other chemicals used for phenotyping were HPLC grade and purchased from Sigma- Aldrich Chemical Company. Primers for PCR I were synthesized by Med Probe Company, Norway and all the other necessary reagents and chemicals including primers for PCR II for genotyping were provided by Prof. Leif Bertilsson at Department of Clinical Pharmacology, Karolinska Institute, Sweden.

7.8. Ethical consideration

The study protocol was approved by Ethics Review Committees at different levels. The hospital ethical review committee at St. Peter's TB Specialised Hospital, AHRI/ALERT Ethical Review Committee, Faculty of Medicine Research and Publication committee at Addis Ababa University, and National Ethical Review Committee provided approval of the protocol. Written informed consent was obtained from all participants prior to the commencement of the study (Appendixes

II & III). Opportunistic infections were treated free of charge and patients were refunded for their transportation to and from the hospital. Antiretroviral drugs were not provided for the patients due to logistic reasons. However, patients are linked to the VCT Center at St. Peter's TB Specialised Hospital.

7.9. Statistical analysis

Data were double entered by two different data entry clerks on different computers by Armauer Hansen Research Institute data management office. Verification was performed in order to clean the data with crosschecking. SPSS version 11.0 was used for analysis of different risk factors. Chi-square and Fishers exact tests were used to test for the level of significance. Variables with $p<0.05$ were considered potential predictors of DIH.

8. Results

8.1. Patients

A total of 103 HIV positive and 94 HIV negative newly diagnosed tuberculosis patients were enrolled in the cohort to be evaluated for the development of hepatotoxicity. One hundred and five (52.8%) were male and 92 (47.2%) female; the mean age of the patients was 31 yr (range, 18 to 67 yr). The Body Mass Index (BMI) of the patients was < 18.5 for 108 (58.7%), 18.5-24.9 for 71 (38.6%), and 25-29.9 for 5 (2.7%) patients. Fourteen patients (7.1%) had a past history of jaundice, 5 (2.5%) had past history of blood transfusion, another 5 (2.5%) had history of chronic illness, 35 (17.8%) had history of traditional medicine intake for the TB treatment and 74 (37.6%) had history of alcohol intake before starting anti-TB treatment even if none fulfilled the DSM IV criteria for being alcoholic (Table 8.1).

Among the 197 patients one hundred and forty six (74.9%) had pulmonary tuberculosis of whom 99 (71.7%) were smear positive and 39 (28.3%) smear negative, 32 (16.4%) had extra pulmonary tuberculosis and 17 (8.7%) had disseminated tuberculosis.

One hundred and forty two patients (72.1%) were treated with SRHZ, 10 (5.1%) took ERHZ and 44 (22.3%) took RHZ during the intensive phase of treatment. Fifty-five (27.9%) patients were taking drugs other than anti-TB drugs in the intensive phase, 14 (7.2%) patients were positive for HBsAg and 4 (2.2 %) were positive for anti-HCV.

The CD4 count profile of the HIV positive patients was 0-50/mm^3 for 15 (17.2%), 51-100 for 32 (36.8%), 101-200 for 22 (25.3%), and > 200 for 18 (20.7%) of the patients (Table 6.2).

The sum of the number of patients may not be 197 for all the demographic and clinical variables and this is because some (<5%) patients had some missed variables. Lose to follow up was very much minimal since all patients were on DOTS and since patients were made aware of the use of having the follow up.

8.2. Association between biochemical hepatotoxicity and demographic factors

Biochemical hepatotoxicity was seen in 14 (13.3%) males and 20 (21.7%) females, the ages were < 35 in 22 (16.8%) patients and ≥35 in 11 (17.2%). The mean time for development of Biochemical DIH is 2.8 weeks (Range 1-8 weeks). Generally there was no statistically significant association between biochemical hepatotoxicity and the demographic characteristics (Table 8.3) studied.

Table 8. 1. Frequency distribution of demographic variables of newly diagnosed TB patients at St. Peter TB Specialized Hospital, Addis Ababa, August 2004-March 2005

Variables	Status	Number of patients	Total number patients	Percentage
Age (years)	<35	131	195	67.2
	≥35	64		32.8
Sex	Male	105	197	53.3
	Female	92		46.7
Body mass index	<18.5	108	184	58.7
	18.5-24.9	71		38.6
	25-29.9	5		2.7
History of Jaundice	Yes	14	194	7.2
	No	180		92.8
History of blood transfusion	Yes	5	196	2.6
	No	191		97.4
History of chronic illness	Yes	5	196	2.6
	No	191		97.4
Traditional medicine intake	Yes	35	192	18.2
	No	157		81.8
Alcohol intake	Yes	74	194	38.1
	No	120		61.9

Table 8. 2. Frequency distribution of clinical variables of newly diagnosed TB patients at St. Peter TB Specialized Hospital, Addis Ababa, August 2004-March 2005

Variables	Status	Number of patients	Total number of patients	Percentage
HIV status	Positive	103	197	52.3
	Negative	94		47.7
Type of TB	Pulmonary	146	195	74.9
	Extra pulmonary	32		16.4
	Disseminated	17		8.7
Anti-TB drugs	SRHZ	142	196	72.4
	ERHZ	10		5.1
	RHZ	44		22.5
Concomitant drug intake	Yes	55	193	28.5
	No	138		71.5
HBsAg	Positive	14	195	7.2
	Negative	181		92.8
Anti-HCV Ab	Positive	4	181	2.2
	Negative	177		97.8
CD4 count/mm^3	0-50	15	87	17.2
	51-100	32		36.8
	101-200	22		25.3
	>200	18		20.7

Table 8. 3. Demographic risk factors for development of biochemical hepatotoxicity in the newly diagnosed TB patients at St. Peter TB Specialized Hospital, Addis Ababa august 2004-March 2005

Variable	Status	Biochemical DIH		p-value
		Yes	No	
Age (years)	<35	22 (16.8%)	109 (83.2%)	0.467
	≥35	11 (17.2%)	53 (82.8%)	
Sex	Male	14 (13.3%)	91 (86.7%)	0.119
	Female	20 (21.7%)	72 (78.3%)	
Body mass index	<18.5	22 (20.4%)	86 (79.6%)	0.607
	18.5-24.9	9 (12.7%)	62 (87.3%)	
	25-29.9	1 (20.0%)	4 (80%)	
History of Jaundice	Yes	2 (14.3%)	12 (85.7%)	0.741
	No	32 (17.8%)	148 (82.5%)	
History of blood transfusion	Yes	0 (0%)	5 (100%)	0.589
	No	34 (17.3%)	157 (82.7%)	
History of chronic illness	Yes	0 (0%)	5 (100%)	0.589
	No	34 (17.3%)	157 (82.7%)	
Traditional medicine intake	Yes	7 (20.0%)	28 (80.0%)	0.695
	No	27 (17.7%)	130 (82.8%)	
Alcohol intake	Yes	13 (17.6%)	61 (82.4%)	0.871
	No	20 (16.6%)	100 (83.3%)	

Table 8. 4. Clinical risk factors for development of biochemical hepatotoxicity in the newly diagnosed TB patients at St. Peter TB specialized Hospital, Addis Ababa, August 2004-March 2005

Variable	Status	Biochemical DIH		p-value
		Yes	No	
HIV status	Positive	26 (25.2%)	77 (74.8%)	0.002
	Negative	8 (8.5%)	86 (91.5%)	
Type of TB	Pulmonary	25 (17.1%)	121 (82.9%)	0.325
	Extra pulmonary	4 (12.5%)	28 (87.5%)	
	Disseminated	5 (29.4%)	12 (70.6%)	
Anti-TB drugs	SRHZ	27 (19.0%)	115 (81.0%)	0.175
	ERHZ	3 (30.0%)	7 (70.0%)	
	RHZ	4 (9.1%)	40 (90.9%)	
Concomitant drug intake	Yes	16 (29.1%)	39 (70.9%)	0.008
	No	18 (13.0%)	120 (87.0%)	
HBsAg	Positive	3 (21.4%)	11 (78.6%)	0.683
	Negative	31 (17.1%)	150 (82.9%)	
Anti-HCV Ab	Positive	0 (0%)	4 (100%)	1.000
	Negative	33 (18.6%)	144 (81.8%)	
CD4 count/mm^3	0-50	8 (53.3%)	7 (46.7%)	0.001
	51-100	8 (25.0%)	24 (75.0%)	
	101-200	4 (18.2%)	18 (81.8%)	
	>200	1 (5.6%)	17 (94.4%)	
Patient death	Died	5 (71.4%)	2 (28.6%)	0.002
	Not Died	29 (15.3%)	161 (84.7%)	
Acetylation status phenotypic	Slow	5 (16.7%)	25 (83.3%)	0.240
	Rapid	1 (4.0%)	24 (96.0%)	
Acetylation status Genotypic	Wt/Wt	5 (35.7%)	9 (64.3%)	0.026
	Wt/Mu	4 (7.7%)	48 (92.3%)	
	Mu/Mu	8 (14.0%)	49 (86.0%)	

8.3. Association between biochemical hepatotoxicity and clinical variables

Twenty five (17.1%) of those patients who had pulmonary TB, 4 (12.5%) of extra pulmonary and 5 (29.4%) of those with disseminated TB had biochemical hepatotoxicity. Twenty seven (19.0%) of the patients who took SRHZ, 3 (30.0%) who took ERHZ and 4 (9.1%) who took RHZ had developed biochemical hepatotoxicity. A statistically significant association (p=0.008) was seen between development of biochemical hepatotoxicity and concomitant drug intake where 16 (29.1%) of the patients who took other drugs on top of anti TB drugs (Tables 8.4 & 8.5) developed biochemical hepatotoxicity. Drugs taken by those patients who developed hepatotoxicity were: amoxicillin (6 patients), antiacid syrup (4 patients), phenobarbitone (2 patients), phenobarbitone plus antiacid syrup (1 patient), ketoconazole (1 patient), and sulfamethoxazole/trimethoprim (2 patients).

Twenty six (25.2%) of the HIV positive but only 8 (8.5%) of the HIV negative patients developed biochemical hepatotoxicity and this was shown to be statistically significant (p=0.002) (Table 8.4).

Three (21.4%) of the HBsAg positive and 31 (17.1%) of the HBsAg negative study participants developed biochemical hepatotoxicity whereas none of the 4 anti-HCV positive patients had hepatotoxicity and no patient was found to be positive for both HBsAg and anti-HCV.

Out of the patients whose CD4 count was 0-50/mm^3 8 (53.3%), 51-100/mm^3 8 (25.0%), 101-200/mm^3 4 (18.2%) and >200/mm^3 1(5.6%) developed biochemical hepatotoxicity. In this study there was a statistically significant association (p=0.001) between biochemical hepatotoxicity and a decrease in CD4 count (Tables 8.4 & 8.5).

Five (16.7%) of those phenotypically slow acetylators had biochemical hepatotoxicity whereas only one (4%) of the phenotypically rapid acetylators had such toxicity. Five (71.4%) of the 7 patients who died during the follow up had biochemical hepatotoxicity. There was a statistically significant association between development of biochemical hepatotoxicity and patient death.

Table 8. 5. The Odds Ratio of an association between biochemical hepatotoxicity with different clinical variables in the newly diagnosed TB patients at St. Peter TB Specialized Hospital, Addis Ababa, August 2004-March 2005

Variable	Status	OR (95% CI)
HIV status	Positive	3.6 (1.5-8.5)
	Negative	1.0
Concomitant drug intake	Yes	2.7 (1.2-5.8)
	No	1.0
CD4 count/mm3	0-50	20.5 (2.1-195.6)
	51-100	5.9 (0.6-52.2)
	101-200	4.2 (0.4-41.7)
	>200	1.0
Patient death	Died	13.9 (2.6-74.9)
	Not Died	1.0

8.4. Association between clinical hepatotoxicity and demographic factors

A statistically significant association (p=0.027) was observed between clinical hepatotoxicity and only sex of patient where 7 (7.6%) of the females and 1 (1.0%) of the males had clinical hepatotoxicity (Table 8.6).

8.5. Association between clinical hepatotoxicity and clinical variables

HIV infection was shown to have a statistically significant association (p=0.043) with clinical hepatotoxicity where 7 (6.8%) of the HIV positive and only 1 (1.1%) of the HIV negative patients had clinical hepatotoxicity (Table 8.7).

There is a statistically significant association between concomitant drug intake and development of clinical hepatotoxicity. Six (10.9%) of those patients who took additional drugs on top anti TB drugs and 2 (1.4%) who did not take additional drugs concomitantly had clinical hepatotoxicity.

Two (14.3%) of those HBsAg positive patients and 6 (3.3%) of the HBsAg negative patients had clinical hepatotoxicity which is statistically significant (p=0.046). A decrease in the CD4 count was shown to have a statistically significant association (p=0.025) with development of clinical hepatotoxicity where 3 (20%) of those with a CD4 count 0-50 /mm^3, 1 (3.1%) with CD4 count 51-100 /mm^3, 1 (4.5%) with CD4 count 101-200 /mm^3 and none with CD4 count >200 /mm^3 patients had clinical hepatotoxicity (Table 8.7).

Clinical hepatotoxicity had also a statistically significant association (p=0.0001) with death of patients, 3 (42.9%) of those who died and 5 (2.6%) of those alive during the follow up period had clinical hepatotoxicity (Table 8.7). The other clinical variables studied did not show a significant association with clinical hepatotoxicity.

Restarting of treatment was possible in all the patients who necessitated discontinuation of anti-TB due to DIH based on our management except one patient who re-develop jaundice again but by then she already has completed the intensive phase.

Table 8. 6. Demographic risk factors for development of clinical hepatotoxicity in the newly diagnosed TB patients at St. Peter's TB Specialized Hospital, Addis Ababa, August 2004-March 2005

Variable	Status	Clinical DIH		p-value
		Yes	No	
Age (years)	<35	4 (3.1%)	127 (96.9%)	0.467
	≥35	4 (6.3%)	60 (93.8%)	
Sex	Male	1 (1.0%)	104 (99.0%)	0.027
	Female	7 (7.6%)	85 (92.4%)	
Body mass index	<18.5	5 (4.6%)	103 (95.4%)	0.796
	18.5-24.9	2 (2.8%)	69 (97.2%)	
	25-29.9	0 (0%)	5 (100%)	
History of Jaundice	Yes	0 (0%)	14 (100%)	0.420
	No	8 (4.4%%)	172 (95.6%)	
History of blood transfusion	Yes	0 (0%)	5 (100%)	0.810
	No	8 (4.2%)	183 (95.8%)	
History of chronic illness	Yes	0 (0%)	5 (100%)	0.856
	No	8 (4.3%)	180 (95.7%)	
Traditional medicine intake	Yes	3 (8.6%)	32 (91.4%)	0.149
	No	5 (3.2%)	152 (96.8%)	
Alcohol intake	Yes	1 (1.4%)	73 (98.6%)	0.122
	No	7 (5.8%)	113 (94.2%)	

Table 8. 7. Clinical risk factors for development of clinical hepatotoxicity in the newly diagnosed TB patients at St. Peter's TB Specialized Hospital, Addis Ababa, August 2004-March 2005

Variable	Status	Clinical DIH		p-value
		Yes	No	
HIV status	Positive	7 (6.8%)	96 (93.2%)	0.043
	Negative	1 (1.1%)	93 (98.9%)	
Type of TB	Pulmonary	7 (4.8%)	139 (74.3%)	0.431
	Extra pulmonary	0 (0%)	32 (100%)	
	Disseminated	1 (5.9%)	16 (94.1%)	
Anti-TB drugs	SRHZ	8 (5.6%)	134 (94.4%)	0.205
	ERHZ	0 (0%)	10 (100%)	
	RHZ	0 (0%)	44 (100%)	
Concomitant drug intake	Yes	6 (10.9%)	49 (89.1%)	0.003
	No	2 (1.4%)	136 (98.6%)	
HBsAg	Positive	2 (14.3%)	12 (85.7%)	0.046
	Negetive	6 (3.3%)	175 (96.7%)	
Anti-HCV Ab	Positive	0 (0%)	4 (100%)	0.833
	Negative	8 (4.5%)	169 (95.5%)	
CD4 count/mm^3	0-50	3 (20.0%)	12 (80.0%)	0.025
	51-100	1 (3.1%)	31 (96.9%)	
	101-200	1 (4.5%)	21 (95.5%)	
	>200	0 (0%)	18 (100%)	
Patient death	Died	3 (42.9%)	4 (57.1%)	0.0001
	Not Died	5 (2.6%)	185 (97.4%)	

Table 8. 8. Observed NAT2 haplotypes and their frequency in 128 newly diagnosed TB patients in Ethiopia and their expected frequency analysed using Alriquin software

SNP NAT-2	Nucleotide change	SNP frequency (%) observed	SNP frequency (%) predicted from alriquin	χ^2 test
NAT-2*4	No change	32.0	32.0	p>0.99, (df=3)
NAT-2*5	341T>C	44.5	44.5	
NAT-2*6	590G>A	18.0	17.9	
NAT-2*7	857G>A	5.5	5.4	
NAT-2*14	191G>A	0.0	0.0	
NAT-2*17	434A>C	0.0	0.0	

8.6. NAT2 SNPs haplotyping and genotyping

We tested the SNPs in NAT2 gene at positions 191, 341, 434, 590, and 857 in 128 of the patients. NAT2 *5 (341T>C) was the most frequently seen SNP (44.5%) followed by the wild type NAT2*4 (32%) and NAT2*6 (590G>A) which was 18%. The least frequent SNP was NAT2*7 which was seen only in 5.5% and no patient had NAT2*14(191G>A), which is thought to be a Black specific SNP and NAT2*7 (434A>C) SNPs. The observed frequencies of the SNPs were found to be similar with the expected frequencies according to Alriquin software programme (p>0.995) (Table 8.8).

The genotype data also showed that *4/*5 is the most frequent genotype with a frequency of 35 (27.3%) patients followed by *5/*5 26 (20.3%) patients, and *5/*6 20 (15.6%) of the patients. The observed genotype frequency was in accordance with the expected genotype frequency according Hardy-Weinberg rule (p>0.77) (Table 8.9).

8.7. Hepatotoxicity and NAT2 genotype

The frequency of rapid acetylators taking the homozygous and heterozygous wild type genes as rapid acetylators was 68 (53.1 %) and that of slow acetylators was 60 (46.9%). A statistically significant association between NAT2 genotype and DIH was observed where patients who have wild type homozygous gene are at a higher risk compared to that of patients with heterozygous mutant and homozygous mutant genotypes ($p<0.03$) (Tables 8.4 & 8.10).

Table 8. 9. NAT2 SNPs and genotyping combinations, in 128 newly diagnosed TB patients from Ethiopia according to Hardy-Weinberg

341T>C	590G>A	857G>A	191G>A	434A>C	Genotype	Observed frequency	Expected frequency	χ^2 test
C/C	G/G	G/G	G/G	A/A	*5/*5	26	25.3	p>0.77 (df=7)
T/C	G/A	G/G	G/G	A/A	*5/*6	20	20.5	
T/C	G/G	G/A	G/G	A/A	*5/*7	7	6.3	
T/C	G/G	G/G	G/G	A/A	*4/*5	35	37.3	
T/T	A/A	G/G	G/G	A/A	*6/*6	7	4.2	
T/T	G/A	G/G	G/G	A/A	*4/*6	12	14.7	
T/T	G/G	G/A	G/G	A/A	*4/*7	7	4.5	
T/T	G/G	G/G	G/G	A/A	*4/*4	14	13.2	

Table 8. 10. NAT2 genotype as a risk factor for development of clinical and biochemical hepatotoxicity in the newly diagnosed TB patients at St. Peter's TB Specialized Hospital, Addis Ababa, August2004-March2005

Genotype	Clinical DIH		Biochemical DIH	
	Yes	**No**	**Yes**	**No**
*4/*4	1 (7.1%)	13 (92.9%)	5 (35.7%)	9 (64.3%)
*4/*5	0 (0%)	33 (100%)	1 (3.0%)	32 (97.0%)
*4/*6	0 (0%)	12 (100%)	1 (8.3%)	11 (91.7%)
*4/*7	0 (0%)	7 (100%)	2 (28.6%)	5 (71.4%)
*5/*5	1 (4.2%)	23 (95.8%)	4 (16.7%)	20 (83.3%)
*5/*6	0 (0%)	20 (100%)	2 (10.0%)	18 (90.0%)
*5/*7	0 (0%)	7 (100%)	1 (14.3%)	6 (85.7%)
*6/*6	0 (0%)	6 (0%)	1 (16.7%)	5 (83.3%)

- p-values for association between different genotypes and clinical hepatotoxicity is p=0.655 and that of biochemical hepatotoxicity is p=0.143.

8.8. Concordance between phenotypic and genotypic NAT2 determinations

The concordance rate of our genotypic and phenotypic determination of acetylation status is 50.9% and the concordance between the different alleles and the AFMU/1MX ratio was not statistically significant when analyzed by a non-parametric test (Kruskal Walis) p=0.19 (Figs.8.1, 8.2 and Table 8.11).

Table 8. 11. NAT2 genotype and phenotype concordance in newly diagnosed TB patients in Ethiopia

Genotype	Mean AFMU/1-MX	Phenotype SD	95%CI for Phenotyping	
*5/*5	2,113000	3,200892	-0,1768	4,40278
*5/*6	2,498000	3,646652	-2,0299	7,02592
*5/*7	0,880000	0,925131	-0,5921	2,35209
*4/*7	0,050000	0,042426	-0,3312	0,43119
*4/*6	5,678571	8,283776	-1,9826	13,33979
*4/*5	5,314500	6,724684	2,1673	8,46175
*4/*4 •	0,267500	0,186793	-0,0297	0,56473
*6/*6	2,425000	3,019346	-24,7027	29,55275

- p-value=0.19

Figure 8. 1. A bar graph showing AFMU/1-MX metabolic ratio frequency in 54 newly diagnosed TB patients in Ethiopia

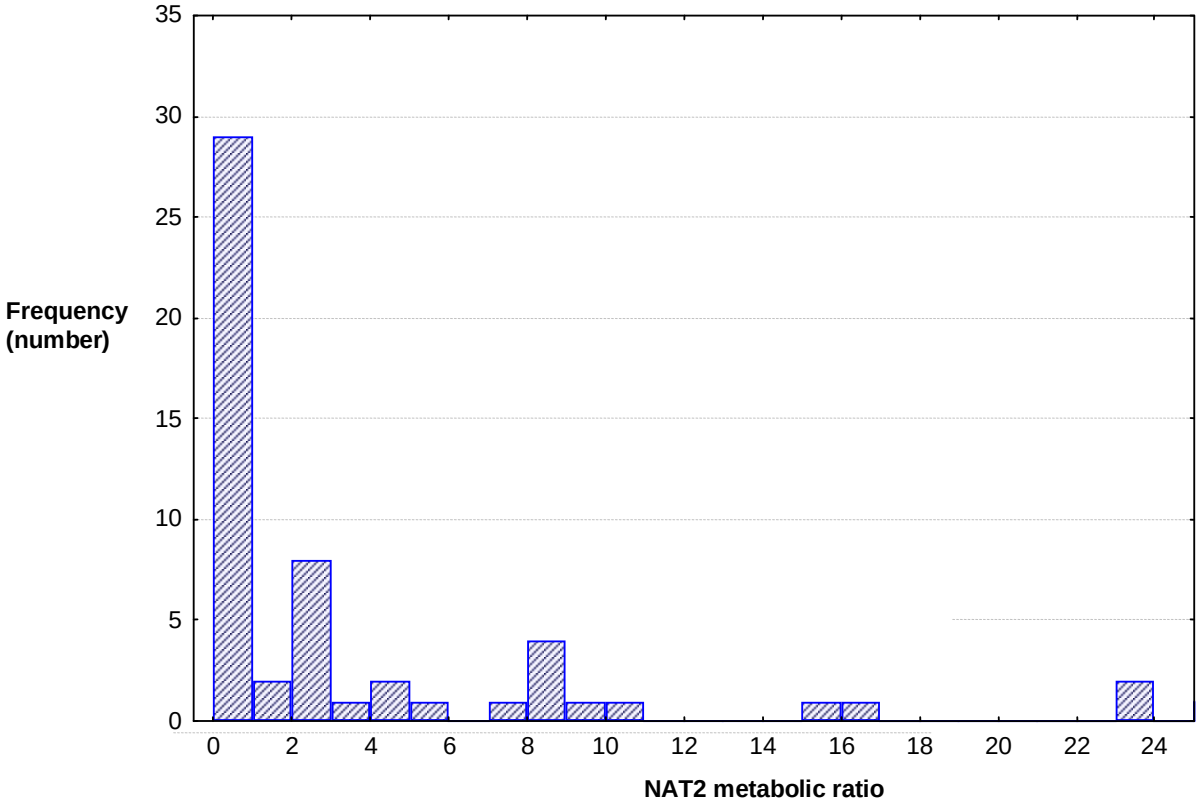
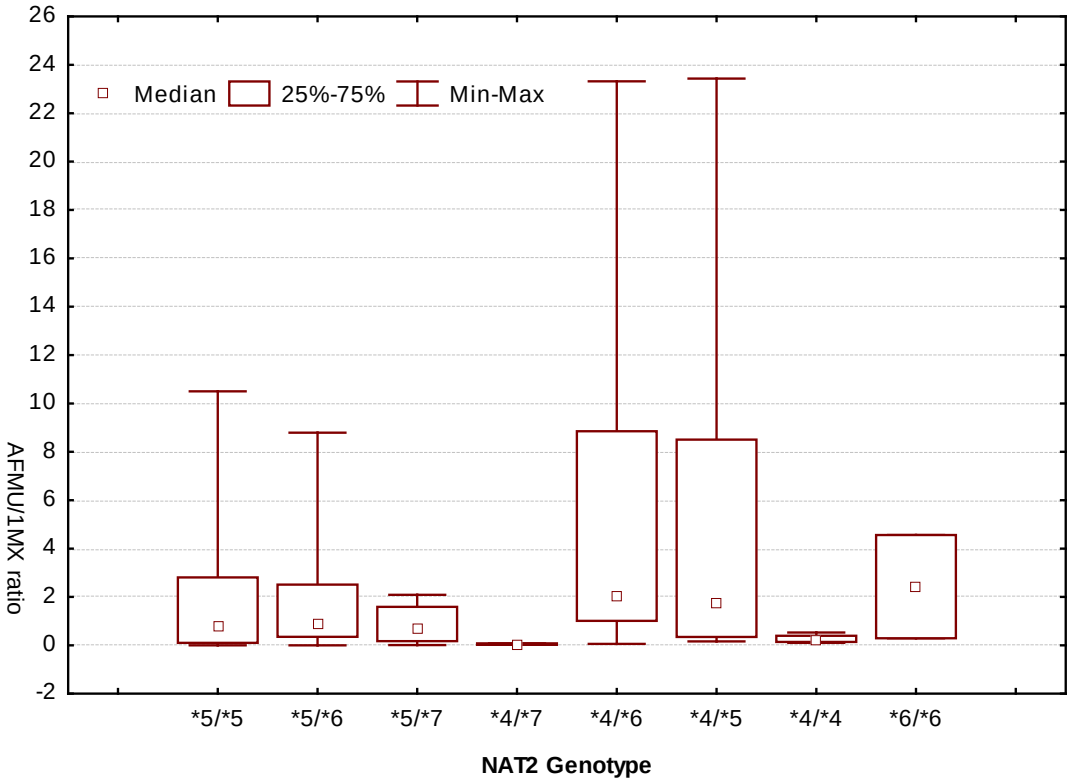


Figure 8. 2. A box plot showing NAT2 genotype and phenotype concordance in newly diagnosed TB patients from Ethiopia



9. Discussion

The prevalence of biochemical hepatotoxicity among newly diagnosed TB patients who started anti-TB drugs according to DOTS regimen was 17.3%. The mean time for development of biochemical DIH in both HIV positive and negative patients was 2.8 weeks and in HIV positive patients the mean time was 2.7 and the range (2-4 weeks). Prevalence of clinical hepatotoxicity was 4.1% in our study, and this finding was similar to a previous study done elsewhere (Ungo, et al., 1998). There are different proposed risk factors for the development of hepatotoxicity as indicated by previous studies. These factors include: female sex, older age, poor nutritional status, high alcohol intake, pre-exciting liver disease, inappropriate drug use, HIV and HCV infections and acetylator status.

More of the females study participants were observed to have biochemical hepatotoxicity in the present study, and a statistically significant association was seen between female sex and clinical hepatotoxicity. These results indicate that females are not only predisposed to hepatotoxicity but also are more prone to severe hepatotoxicity as observed by other investigators (Sharma, et al., 2002). On the other hand, advanced age was not observed to have a significant association with both biochemical and clinical hepatotoxicity. This may be because a larger proportion of our study population were of age below 35 years of age. Also most of the HIV infected patients were below 35, which may predispose this population to DIH and thus increase the number of patients in this age group who developed DIH. Previous larger studies that showed age as a risk factor for DIH were performed before HIV was prevalent and this idea is supported by another study (Ungo, et al., 1998).

Malnutrition as a risk for DIH was suggested by some studies, but in our study we did not see any significant association between malnutrition measured by BMI. This may be because a large number of our study population had lower BMI which makes it difficult to compare with both biochemical and clinical hepatotoxicity. This finding is supported by another study (Sharma, et al., 2002). No significant association was observed between factors such as history of jaundice, blood transfusion, chronic illness and traditional medicine intake and biochemical hepatotoxicity. No previous study has tried to associate these factors with DIH either.

Being alcoholic was observed to be an independent risk factor for DIH in different studies (Sharma, et al., 2002) but in our study all patients denied drinking alcohol heavily.

In addition to HIV predisposing to TB, studies show that it also predisposes to the development of DIH secondary to the use of anti-TB agents (Ungo, et al., 1998). This was confirmed by our study that a statistically significant number of HIV positive patients were seen to be predisposed not only to development of biochemical hepatotoxicity but also to severe clinical hepatotoxicity which necessitated discontinuation of anti TB drugs. This may partly be explained by the different drugs used by these patients for treatment of AIDS related opportunistic infections. We have also seen that both clinical and biochemical hepatotoxicity had a statistically significant association with decrease in the immune status of the patients as measured by their CD4 count. This phenomenon has not been shown in any other study previously. This may indicate that an immunologic mechanism may play a role for the development of anti TB DIH since the mechanism of hepatotoxicity is not elucidated.

We did not see any significant association between different anti-TB regimens, and type of TB with development of clinical and biochemical hepatotoxicity.

We found that patients who took drugs other than anti-TB drugs are found to be at risk for development of both biochemical and clinical hepatotoxicity. Other studies have failed to show this relation and it was only an association between hepatotoxicity with INH alone or in combination with RIF that was studied in detail. Drugs that were concomitantly taken with the anti TB drugs by the patients were: penicillins- which may induce hepatotoxicity rarely, sulfamethoxazole/trimethoprim, which may induce hepatotoxicity occasionally, ketoconazole, which is an inhibitor of the liver enzymes especially CYP 3A4 and thereby diverting the metabolism of the hepatotoxic drugs to another metabolic pathway that may result in a more hepatotoxic metabolite, phenobarbitone which may increase hepatotoxicity induced by other drugs (Thiim and Friedman, 2003).

Anti-HCV positivity did not show any association with hepatotoxicity unlike in other studies (Ungo, et al., 1998). This may be attributed to the small number of Anti-HCV positive patients enrolled in the study. On the other hand HBsAg positivity was shown to have a statistically significant association with clinical hepatotoxicity but not with biochemical hepatotoxicity. This may suggest that an underlying liver disease may aggravate drug-induced hepatotoxicity. The reason why biochemical hepatotoxicity was not observed may be because of the small number of HBsAg positive patients employed in the present study.

Death of patients was seen to have a statistically significant association with development of biochemical and clinical hepatotoxicity in our study. This could show that those patients who are seriously sick might be at a risk of developing hepatotoxicity which was also observed in other studies (Sharma, et al., 2002).

Having a homozygous wild type NAT2 gene in our study patients was shown to predispose patients to biochemical DIH significantly. Previously some studies suggested that being a slow acetylator is a risk factor (Ali, 1996). Others studies contradicted these findings. Rapid acetylators were shown to be at risk of developing hepatotoxicity from anti TB drugs. But it is still a major point of debate (Ali, 1996). The percentage of slow and rapid acetylators in our study population is similar to those reported from other Africans, European and North American populations (Gross, et al., 1999). To our knowledge, there is no reported study to date in Ethiopians on genotypic and phenotypic determination of NAT2 gene. Another finding from our study is none of the study participants demonstrate the black-specific SNP, NAT2*14 and this finding was reported in a study that tested different gene on Ethiopian samples (Passarino, *et al.*, 1998).

Concordance between phenotypic and genotypic determination of acetylation status in our study population is very much lower than the concordance reported earlier (Gross, et al., 1999). This may be because of the drug interaction between caffeine and anti-TB drugs at the level of metabolism. This indicates that caffeine is probably not the best probe for determination of acetylation status in patients taking anti-TB drugs. No previous study, however, has shown the effect of the anti-TB drugs on caffeine metabolism. We have considered this before and tried to

use INH as a probe but there was no loose formulation of INH for our current research participants. We also anticipated a technical difficulty that since it is a cohort study and not a cross-sectional study there might be patient compliance problem as blood has to be drawn every hour for 6 hours to determine acetylation status by using INH as a probe and our study itself necessitated blood drawing every week to see development of DIH. Over all this study shows the different risk factors associated with development of anti-TB DIH and also the distribution of NAT2 gene and its association with anti-TB DIH in Ethiopians. Findings from this study will help clinicians in the management of TB by identifying patients who are at risk of anti-TB DIH.

10. Conclusions

- Anti-tuberculosis drug-induced hepatotoxicity is a problem in the management of tuberculosis.
- HIV infection and decreased immunity status (CD4 count particularly below 50/µl) were risk factors for development of anti-tuberculosis drug-induced hepatotoxicity.
- Patients taking other additional drugs while on anti-tuberculosis drug are more likely to develop anti- tuberculosis drugs induced hepatotoxicity.
- Having a homozygous wild type NAT2 gene was a risk factor for biochemical DIH among our study participants.
- Female patients were at a higher risk of developing severe forms of DIH.
- HBsAg carrier TB patients were at a higher risk to develop a severe form of DIH.
- Patients who developed clinical DIH could safely be managed by stepwise reintroduction of the same anti TB drugs.
- The percentage of slow and rapid acetylators in our study population was similar to percentages of other African, European and North American populations.
- Ethiopians appear to have different NAT2 alleles as compared to other Black populations.

11. Recommendations

- HIV infected patients, especially those with lower CD4 counts and particularly those with < 50 /µl need to have a regular monitoring of their liver function test during treatment of tuberculosis.
- A follow-up study on those patients who are managed by discontinuation of their anti-TB drugs needs to be done to evaluate possible development of drug resistance, treatment failure and recurrence of tuberculosis.
- There is a need for safe second line or alternative first line drugs for managing tuberculosis.
- The underling biochemical, cellular and immunological mechanisms for anti tuberculosis DIH need to be investigated.
- It would also be good to study the development of DIH when patients are taking both anti TB and antiretroviral drugs at the same time.
- Further studies should be pursued to elucidate the background for the association of severe anti-TB DIH with female sex.
- Prescribing concomitant drugs for patients taking anti-TB drugs has to be done with caution.

12. Limitations of the study

- Since this study was conducted in St. Peter TB Specialized Hospital which is a referral center for TB patients the findings of the study may not be representative of all TB patients so similar study needs to be done in other health institutions.
- The sample size of this study was calculated considering HIV positive and negative TB patients, so in order to make a stronger recommendation on other associations similar study has to be done considering other risk factors while calculating sample size.

13. References

Aaron, L., D. Saadoun, I. Calatroni, O. Launay, N. Memain, V. Vincent, G. Marchal, B. Dupont, O. Bouchaud, D. Valeyre, and O. Lortholary. 2004. Tuberculosis in HIV-infected patients: a comprehensive review. Clin Microbiol Infect **10:**388-98.

Ali, J. 1996. Hepatotoxic effects of tuberculosis therapy. A practical approach to a tricky management problem. Postgrad Med **99:**217-20, 30-1, 35-6.

Anitha, A., and M. Banerjee. 2003. Arylamine N-acetyltransferase 2 polymorphism in the ethnic populations of South India. Int J Mol Med **11:**125-31.

Bagheri, H., F. Michel, M. Lapeyre-Mestre, E. Lagier, J. P. Cambus, P. Valdiguie, and J. L. Montastruc. 2000. Detection and incidence of drug-induced liver injuries in hospital: a prospective analysis from laboratory signals. Br J Clin Pharmacol **50:**479-84.

Bakayev, V. V., F. Mohammadi, M. Bahadori, M. Sheikholslami, A. Javeri, M. R. Masjedi, and A. A. Velayati. 2004. Arylamine N-acetyltransferase 2 slow acetylator polymorphisms in unrelated Iranian individuals. Eur J Clin Pharmacol **60:**467-71.

Barnes, P. F., and S. A. Barrows. 1993. Tuberculosis in the 1990s. Ann Intern Med **119:**400-10.

Bassey, E. B., M. A. Momoh, S. O. Imadiyi, E. B. Udofia, F. S. Miri, K. C. Anukam, J. Epoke, M. O. Benka-Coker, and H. S. Aluyi. 2005. The trend of pulmonary tuberculosis in patients seen at DOTS clinics in the Federal Capital Territory, Abuja, Nigeria. Public Health **119:**405-8.

Blaszkewicz, M. 2004. N-Acetyltransferase 2 (phenotyping: caffeine test).

Boodram, L. 2004. Extraction of genomic DNA from whole blood. Protocol online from internate.

Brewer, T. F., and S. J. Heymann. 2004. To control and beyond: moving towards eliminating the global tuberculosis threat. J Epidemiol Community Health **58:**822-5.

Bruchfeld, J., G. Aderaye, I. B. Palme, B. Bjorvatn, G. Kallenius, and L. Lindquist. 2000. Sputum concentration improves diagnosis of tuberculosis in a setting with a high prevalence of HIV. Trans R Soc Trop Med Hyg **94:**677-80.

Campos-Franco, J., A. Gonzalez-Quintela, and M. R. Alende-Sixto. 2004. Isoniazid-induced hyperacute liver failure in a young patient receiving carbamazepine. Eur J Intern Med **15:**396-7.

CDC. 1993. Severe Isoniazid-Associated Hepatitis. MORBIDITY AND MORTALITY WEEKLY REPORT **42**.

Cheuk-ming, T. 2002. MONITORING FOR HEPATOTOXICITY DURING ANTITUBERCULOSIS TREATMENT.

Circle, M. W. 2003. Drug-associated Liver Disease during HAART: Impact of HCV Coinfection. HCV Advocate.

Deruaz, J., and J. P. Zellweger. 2004. Directly observed therapy for tuberculosis in a low prevalence region: first experience at the Tuberculosis Dispensary in Lausanne. Swiss Med Wkly **134:**552-8.

DISEASEWATCH, F. 2004. Tuberculosis. NATURE REVIEWS/MICROBIOLOGY **2:**930-2.

Dye, C., S. Scheele, P. Dolin, V. Pathania, and M. C. Raviglione. 1999. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. Jama **282:**677-86.

Frieden, T. R., T. R. Sterling, S. S. Munsiff, C. J. Watt, and C. Dye. 2003. Tuberculosis. Lancet **362:**887-99.

Gawronska-Szklarz, B., A. Pawlik, G. Czaja-Bulsa, W. Gornik, T. Luszawska-Kutrzeba, and J. Wrzesniewska. 2001. Genotype of N-acetyltransferase 2 (NAT2) polymorphism in children with immunoglobulin E-mediated food allergy. Clin Pharmacol Ther **69:**372-8.

Godfrey-Faussett, P., and H. Ayles. 2003. Can we control tuberculosis in high HIV prevalence settings? Tuberculosis (Edinb) **83:**68-76.

Gooze, L. 2003. Tuberculosis and HIV. Current American Thoracic Society/CDC Treatment Recommendation.

Grant, D. M., K. Morike, M. Eichelbaum, and U. A. Meyer. 1990. Acetylation pharmacogenetics. The slow acetylator phenotype is caused by decreased or absent arylamine N-acetyltransferase in human liver. J Clin Invest **85:**968-72.

Gross, M., T. Kruisselbrink, K. Anderson, N. Lang, P. McGovern, R. Delongchamp, and F. Kadlubar. 1999. Distribution and concordance of N-acetyltransferase genotype and phenotype in an American population. Cancer Epidemiol Biomarkers Prev **8:**683-92.

Hein, D. W., M. A. Doll, A. J. Fretland, M. A. Leff, S. J. Webb, G. H. Xiao, U. S. Devanaboyina, N. A. Nangju, and Y. Feng. 2000. Molecular genetics and epidemiology of the NAT1 and NAT2 acetylation polymorphisms. Cancer Epidemiol Biomarkers Prev **9:**29-42.

Huang, Y. S., H. D. Chern, W. J. Su, J. C. Wu, S. C. Chang, C. H. Chiang, F. Y. Chang, and S. D. Lee. 2003. Cytochrome P450 2E1 genotype and the susceptibility to antituberculosis drug-induced hepatitis. Hepatology **37:**924-30.

Jawahar, M. S. 2004. Current trends in chemotherapy of tuberculosis. Indian J Med Res **120:**398-417.

Jetter, A., M. Kinzig-Schippers, M. Illauer, R. Hermann, K. Erb, J. Borlak, H. Wolf, G. Smith, I. Cascorbi, F. Sorgel, and U. Fuhr. 2004. Phenotyping of N-acetyltransferase type 2 by caffeine from uncontrolled dietary exposure. Eur J Clin Pharmacol **60:**17-21.

Jones, B. E., M. Otaya, D. Antoniskis, S. Sian, F. Wang, A. Mercado, P. T. Davidson, and P. F. Barnes. 1994. A prospective evaluation of antituberculosis therapy in patients with human immunodeficiency virus infection. Am J Respir Crit Care Med **150:**1499-502.

Jose, A. 2003. NAT2 Genotyping: Equilibrium Between Accuracy and Feasibility in Routine Analyses. The Journal of Applied Research in clinical and Experimental Therapeutics **3**.

Kennedy, M. J., S. M. Abdel-Rahman, A. D. Kashuba, and J. S. Leeder. 2004. Comparison of various urine collection intervals for caffeine and dextromethorphan phenotyping in children. J Clin Pharmacol **44:**708-14.

Kimmoun, E., and D. Samuel. 2002. Antituberculous drugs in patients with chronic liver disease. J Gastroenterol Hepatol **17 Suppl 3:**S408-S12.

Kukongviriyapan, V., A. Prawan, W. Tassaneyakul, J. Aiemsa-Ard, and B. Warasiha. 2003. Arylamine N-acetyltransferase-2 genotypes in the Thai population. Br J Clin Pharmacol **55:**278-81.

Lee, S. Y., K. A. Lee, C. S. Ki, O. J. Kwon, H. J. Kim, M. P. Chung, G. Y. Suh, and J. W. Kim. 2002. Complete sequencing of a genetic polymorphism in NAT2 in the Korean population. Clin Chem **48:**775-7.

Lee, W. M. 2003. Drug-induced hepatotoxicity. N Engl J Med **349:**474-85.

Ligon-Borden, B. L. 2003. Selman A. Waksman, PhD (1888-1973): Pioneer in Development of Antibiotics and Nobel Laureate. Seminars in Pediatric Infectious Diseases **14:**60-3.

Luster, M. I., P. P. Simeonova, R. M. Gallucci, A. Bruccoleri, M. E. Blazka, and B. Yucesoy. 2001. Role of inflammation in chemical-induced hepatotoxicity. Toxicol Lett **120:**317-21.

Macsween, N. Hepatic injury due to drugs and toxins. Pathology of the Liver **4 th edition:**677-702.

MOH. 2004. TB and HIV/ADIS. Center for National Health Development in Ethiopia.

Mwinga, A., and P. Bernard Fourie. 2004. Prospects for new tuberculosis treatment in Africa. Trop Med Int Health **9:**827-32.

Nolan, C. M., S. V. Goldberg, and S. E. Buskin. 1999. Hepatotoxicity associated with isoniazid preventive therapy: a 7-year survey from a public health tuberculosis clinic. Jama **281:**1014-8.

Nyeki, A., J. Biollaz, U. W. Kesselring, and L. A. Decosterd. 2001. Extractionless method for the simultaneous high-performance liquid chromatographic determination of urinary caffeine metabolites for N-acetyltransferase 2, cytochrome P450 1A2 and xanthine oxidase activity assessment. J Chromatogr B Biomed Sci Appl **755:**73-84.

Osborne, A., C. Bell, F. Grant, F. Dick, A. Seaton, and N. Haites. 2003. A rapid method of screening for N-acetyltransferase (NAT2) phenotype by use of the WAVE DNA fragment analysis system. Biochem Genet **41:**405-11.

Ozick, L. A., L. Jacob, G. M. Comer, T. P. Lee, J. Ben-Zvi, S. S. Donelson, and C. P. Felton. 1995. Hepatotoxicity from isoniazid and rifampin in inner-city AIDS patients. Am J Gastroenterol **90:**1978-80.

Pape, J. W. 2004. Tuberculosis and HIV in the Caribbean: approaches to diagnosis, treatment, and prophylaxis. Top HIV Med **12:**144-9.

Passarino, G., O. Semino, L. Quintana-Murci, L. Excoffier, M. Hammer, and A. S. Santachiara-Benerecetti. 1998. Different genetic components in the Ethiopian population, identified by mtDNA and Y-chromosome polymorphisms. Am J Hum Genet. **62:**420-34.

Richard, A. 2003. Isoniazid Hepatotoxicity. eMedicine.

Roy, B., A. Chowdhury, S. Kundu, A. Santra, B. Dey, M. Chakraborty, and P. P. Majumder. 2001. Increased risk of antituberculosis drug-induced hepatotoxicity in individuals with glutathione S-transferase M1 'null' mutation. J Gastroenterol Hepatol **16:**1033-7.

Salomon, N., D. C. Perlman, and S. Goldstein. 1994. Special problems in treating tuberculosis. Ann Intern Med **120:**440-1; author reply 1.

Sarich, T. C., S. P. Adams, and J. M. Wright. 1998. The role of l-thyroxine and hepatic reductase activity in isoniazid-induced hepatotoxicity in rabbits. Pharmacol Res **38:**199-207.

Sharma, S. K., A. Balamurugan, P. K. Saha, R. M. Pandey, and N. K. Mehra. 2002. Evaluation of clinical and immunogenetic risk factors for the development of hepatotoxicity during antituberculosis treatment. Am J Respir Crit Care Med **166:**916-9.

Sturgill, M. G., and G. H. Lambert. 1997. Xenobiotic-induced hepatotoxicity: mechanisms of liver injury and methods of monitoring hepatic function. Clin Chem **43:**1512-26.

Thiim, M., and L. S. Friedman. 2003. Hepatotoxicity of antibiotics and antifungals. Clin Liver Dis **7:**381-99, vi-vii.

Ubaid, R. S., K. M. Anantrao, J. B. Jaju, and M. Mateenuddin. 2003. Effect of Ocimum sanctum (OS) leaf extract on hepatotoxicity induced by antitubercular drugs in rats. Indian J Physiol Pharmacol **47:**465-70.

Ungo, J. R., D. Jones, D. Ashkin, E. S. Hollender, D. Bernstein, A. P. Albanese, and A. E. Pitchenik. 1998. Antituberculosis drug-induced hepatotoxicity. The role of hepatitis C virus and the human immunodeficiency virus. Am J Respir Crit Care Med **157:**1871-6.

Weinshilboum, R. 2003. Inheritance and drug response. N Engl J Med **348:**529-37.

WHO. 2004. TB/HIV. TB/HIV A CLINICAL MANUAL **second edition**.

WHO, R. 2005. Global Tuberculosis Control Surveillance, Planning, Financing.

Yee, D., C. Valiquette, M. Pelletier, I. Parisien, I. Rocher, and D. Menzies. 2003. Incidence of serious side effects from first-line antituberculosis drugs among patients treated for active tuberculosis. Am J Respir Crit Care Med **167:**1472-7.

Yepes, J. F., J. Sullivan, and A. Pinto. 2004. Tuberculosis: medical management update. Oral Surg Oral Med Oral Pathol Oral Radiol Endod **98:**267-73.

Appendix I. Questionnaire

Questionnaire for new untreated TB cases to assess anti tuberculosis drug induced hepatotoxicity

Format 1

I. Identification of the patient:

Name: _____

Address: Wereda _____Kebele _____House No_____ Phone No_____

Card No_____ Date of examination _____ Site of Treatment _____

II. Clinical variables

6. Age (years)_____
7. Sex 1. Male ☐ 2. Female ☐
8. If female are you pregnant? 1.yes ☐ 2.No ☐
9. Ethnicity 1. Amhara ☐ 2. Oromo ☐ 3. Tigre ☐ 4. Gurage ☐
   5. Other(specify)_____
10. Weight (Kg) _____
11. Height (cm) _____
12. Body Mass Index (BMI) (to be filled by data manager) _____
13. Type of TB 1. Pulmonary only ☐ 2. EPTB ☐ 3. Disseminated ☐
    8.1. If Pulmonary only 1. Smear +ve ☐ 2. Smear –ve ☐
    8.2. If EPTB specify the organ/s. (Tbc pleurisy is taken as EPTB) _____
    8.2.1. How is the diagnosis made? _____
    8.3. If disseminated TB, specify the organs involved. (Pleuroparenchymal Tbc is taken as disseminated TB.) _____
9. Anti-TB drugs being taken in the intensive phase 1.SRHZ ☐ 2. ERHZ ☐
   3. RHZ ☐
10. History of jaundice 1. Yes ☐ 2. No ☐ 3. Do not remember ☐
    10.1. If yes when _____
11. History of blood transfusion 1.Yes ☐ 2. No ☐ 3. Do not remember ☐
    11.1. If yes when _____

12. History of chronic illness: 1.Yes ☐ 2. No ☐ 3. Do not remember ☐

12.1. If yes fill the table below:

Type of Illness	Time of diagnosis

13. Do you use traditional medicine? 1. Yes ☐ 2. No ☐

13.1. If yes fill the table below

Name of herb (scientific name not necessary)	How long ago you started taking the herb (in weeks)	How frequent (in weeks)	Last time you took (d/m/y)

14. Do you drink Alcohol? 1. Yes ☐ 2. No ☐

14.1. If yes fill the table below

Type of Alcohol	Amount (Per week)	For how long (In months)	Last time you took (d/m/y)
Tella			
Tej			
Araki			
Beer/Draft			
If other specify			

15. Any drugs being taken by the patient currently 1. Yes ☐ 2.No ☐

15.1. If yes fill the table below

Name of Drug	How long ago you started taking the drug (in days)	Dose (per day)	Last time you took (d/m/y)

III. Physical examination

16. Blood Pressure (in mm Hg) Systolic_____ Diastolic_____

17. Pulse rate (BPM) _____

18. Temperature (^{o}c) _____

19. RR (per minute) _____

20. HEENT: Abnormal findings 1. Yes ☐ 2. No ☐

If yes specify _____

_____

21. Lymphatic and glandular system 1. Yes ☐ 2. No ☐

If yes specify _____

_____

22. Respiratory system 1. Yes ☐ 2. No ☐

If yes specify _____

_____

23. Cardiovascular system 1. Yes ☐ 2. No ☐

If yes specify _____

_____

24. Abdomen 1. Yes ☐ 2. No ☐

If yes specify _____

_____

25. Genitourinary 1. Yes ☐ 2. No ☐

If yes specify _____

_____

26. Nervous system 1. Yes ☐ 2. No ☐

If yes specify _____

_____

27. Skin 1. Yes ☐ 2. No ☐

If yes specify _____

_____

28. Musculoskeletal 1. Yes ☐ 2. No ☐

If yes specify _____

IV. Laboratory findings

29. Hemoglobin (Hgb) _____

30. White Blood Cell Total (mm^3) _____

31. White Blood Cell differential count

WBC	**Percent**
Neutrophil	
Lymphocyte	
Eosinophil	
Basophile	
Monocyte	

32. Erythrocyte Sedimentation Rate (ESR) (mm/hr) _____

33. Sputum culture for AFB 1.positive ☐ 2. Negative ☐ 3. Not available ☐

34. HBsAg 1.positive ☐ 2. Negative ☐ 3. Not available ☐

35. Anti-HCV Ab 1.positive ☐ 2. Negative ☐ 3. Not available ☐

36. Abnormal chest radiographic features 1. Present ☐ 2. Absent ☐

If present specify _____

_____

37. Abnormal ultrasonographic finding of the liver 1. Present ☐ 2. Absent ☐

If present specify _____

_____

38. Acetylation status: (to be filled by the Principal investigator)

i. Phenotypically 1. Rapid ☐ 2. Slow ☐ 3. Intermediate ☐

ii. Genotypically 1. Rapid ☐ 2. Slow ☐

39. Table on physical examination and history

Clinical variables	Values Before Treatment	Values at Week					Remark
		1st	2nd	4th	6th	8th	
Jaundice (Yes/No)							
Nausea (Yes/No)							
Anorexia (Yes/No)							
Vomiting (Yes/No)							
Right upper quadrant pain (Yes/No)							
Skin rash (Yes/No)							
Arthralgia (Yes/No)							
Abdominal discomfort (Yes/No)							
Visual problems (Yes/No)							
Other side effects of anti TB specify_____ (Yes/No)							

40. Table for Lab. result

Lab Tests	Normal Values Range	Values Before Treatment	Values at Week					Remark
			1st	2nd	4th	6th	8th	
AST								
ALT								
Bilirubin Total								
Bilirubin Direct								
Alkaline Phosphate								

41. Does the patient develop anti TB drug induced Hepatotoxicity? 1. ☐ 2. ☐

41.1. If yes fill the management information sheet

Form filled by: _____ Signature: _____ Date: _____

Questionnaire for new untreated TB cases to assess anti tuberculosis Drug induced hepatotoxicity

Format 2

Date: ___/ ___/ ____

1. Patients Code No _____
2. HIV status: 1. Positive ☐ 2. Negative ☐

   If positive answer Q3 & 4
3. WHO stage of HIV/AIDS (see protocol) 1. Stage **I** ☐ 2. Stage **II** ☐

   3. Stage **III** ☐ 4. Stage **IV** ☐
4. CD4 count /mm^3 _____

Name of Physician _____ Signature: _____ Date: _____

Name of Supervisor _____ Signature: _____ Date: _____

Questionnaire for new untreated TB cases to assess anti tuberculosis Drug induced hepatotoxicity

Format 3

IV. Management interventions and measurements of out-come in patients with Drug Induced Hepatotoxicity (DIH).

1. Number of days treated before diagnosis of DIH is made? _____
2. Is the anti tuberculosis drug regimen totally discontinued? 1. Yes ☐ 2. No ☐
3. Drug 1st started after total discontinuation:
   1. INH ☐ 2. Rifampicin ☐ 3. Pyrazinamide ☐
   4. Ethambutol ☐ 5. Streptomycin ☐
4. Drug started next to the first restarted drug:
   1. INH ☐ 2. Rifampicin ☐ 3. Pyrazinamide ☐
   4. Ethambutol ☐ 5. Streptomycin ☐
5. How many days after the diagnosis of DIH is the anti tuberculosis drug treatment fully re-instituted? _____
6. Final out come of the DIH 1. Recovered ☐ 2. Death ☐ 3. Do not know ☐

   6.1. If you don't know the final out come. What is the reason for not knowing?

   _____

   6.2. If the final out come is death, indicate the cause of death.

   1. Hepatic failure ☐ 2. Non-hepatic ☐

   6.2.1. If non hepatic (specify) _____

Further remarks on course

_____

_____

_____

_____

_____

Form filled by: _____ Signature: _____ Date: _____

Appendix II. English consent form

I have been informed that I have tuberculosis and I am going to take anti tuberculosis drugs for the prescribed number of months. I have been requested to participate in a research project that aims at determination of risk factors for development of liver damage while taking Anti TB drugs. This requires that I be tested for HIV after pre- and- post test counseling. It also requires that about 5 ml (one teaspoonful) of blood will be taken from me for different investigations at $1^{st,}$ $2^{nd,}$ $4^{th,}$ 8^{th}, 12^{th} week of anti TB treatment. In addition, I may be requested to take two tabs of caffeine and get my urine tested after 5 hours to determine how my body breaks down the drugs.

I have been informed that all information I will be giving to the interviewer will be kept confidential and that the nature of the questionnaire is private. I understand that the risks I am exposed to will be the risk from blood drawing. I will have to come for repeated visits and extra investigations. The investigations will help to recognize and manage any damage to my liver early. I also know that I have the right to withhold information, refuse cooperation or to drop out of the study at any time I want without any need to explain the reason to anyone, and that my actions will have no effect on the overall management of my disease. I know that I have the right to ask and get clarifications at any time. I also know that I will not get anti retroviral drugs paid for by the project. But I will be linked to a VCT facility or the like where I can get support for HIV if I happen to be seropositive.

I have asked for clarification and I have received satisfactory responses in a language I understood. I have been given sufficient time to respond. I finally confirm my agreement by putting my signature below.

Signature of patient _____ Date _____

Signature of physician _____ Date _____

Signature of witness _____ Date _____

Appendix III. Amharic consent form

የስምምነት ቅፅ

እኔ በኮድ ቁጥር ---------- የተመዘገብኩት የቲቢ በሽተኛ የሆነኩና የታዘዘለኝን የቲቢ መድኃኒት ለሚታዘዝልኝ ጊዜ ያህል እንደምወስድ አውቄ በምወስድበት ጊዜ በቲቢ መድሀኒቶች ሳቢያ ጉበት ላይ ስለሚደርስ ጉዳት በሚደረግ ጥናት ላይ እንድሳተፍ ብግያሳ እና በጥናቱ ወቅት ለጥናቱ የሚያስፈልጉ ነገሮችን ማለትም የኤች አይ ቪ ምርመራ ከቅድመ እና ድህረ ምርመራ ምክር አገልግሎት ጋር፣የአንድ የሻይ ማንኪያ ያህል ደም በ1ኛ፣2ኛ፣4ኛ፣ 8ኛ እና 12 ሳምንት (ህክምናው ከተጀምረ ጀምሮ) እንደሚወሰድልኝ፣ ለጥናቱ አስፈላጊ ሆኖ ከተገኘ እና ከተጠየቅኩኝ ሁለት ያህል በውስጣቸው ቡና ውስጥ ያለውን ንጥረነገር የያዙ እንክብሎችና (ከሁለት ስኒ የተፈላ ቡና ጋር ተመጣጣኝ ነው) እንደምወስድና ከአምስት ሰዓታት በኃላ የሽንት ምርመራ እንደሚደረግልኝ፣ በቃለምልልስ ወቅት የምሰጠው እና ከሚደረግልኝ የላብራቶሪ ምርመራ የሚገኘው ውጤት በሚስጥር እንደሚያዝ እያወኩና በምርምሩ የተነሳ የሚደርሱብኝን ጉዳቶች ማለትም በመርፌ መወጋት፣ በጤና ተቋሙ መመላለስ እና ምንአልባትም ከሚጠበቀው በላይ መመርመር እንዳለ አውቄ ከምርምሩ የሚገኙ ጥቅሞችንም ለምሳሌ ያለምንም ክፍያ የኤች አይ ቪ ምርመራ፣ ጉበቴ ላይ መጠኑ የከፋ ጉዳት ከመድረሱ በፊት የሚከ ታተለኝ የጤና ባለሙያ እንዲያውቅና ለእኔም ተገቢውን ህክምና በተገቢው ጊዜ እንዲያደረግልኝ እንደሚረዳ እያወኩ ምርምሩ በሙሉ ፈቃደኝነት ላይ የተመሰረተ፣ መንገር ያልፈለኩትን ነገር እንድነግር የማልገደድበትና ከጥናቱ በፈለኩት ጊዜ ማቋረጥእንደምችል እና ይህን ማድረጌ በህክምና አሰጣጡ ላይ ምንም አይነት እዋጽእንደማይኖረው እየተገነዘብኩ በጥናቱ ውስጥ እንደታቀፍኩም ማንኛውም ተገቢ ጥያቄ የመጠየቄና ለጥያቄም ተገቢውን ምላሽ እና ማብራሪያ በሚገባኝ ቋንቋ እንደማገኝ አውቄ እንዲሁም የኤች አይ ቪ ቫይረስ በደሜ ውስጥ ከተገኘ የእድሜ ማራዘሚያ መድሀኒት ከጥናቱ ጋር በተያያዘ እንደማላገኝ እና ነገር ግን በጥናቱ አስተባባሪ አማካኝነት የቅርብ ክትትልእና የምክር አገልግሎት ከሚሰጡ ድርጅቶች ጋር እንደምገናኝ አውቄ እና ፈቅጀ በጥናቱ ለመሳተፍ በቂ ጊዜ ተሰጥቶኝ እና አስቤበት መስማማቴን በፊርማዬ አረጋግጣለሁ::

ቀን _____

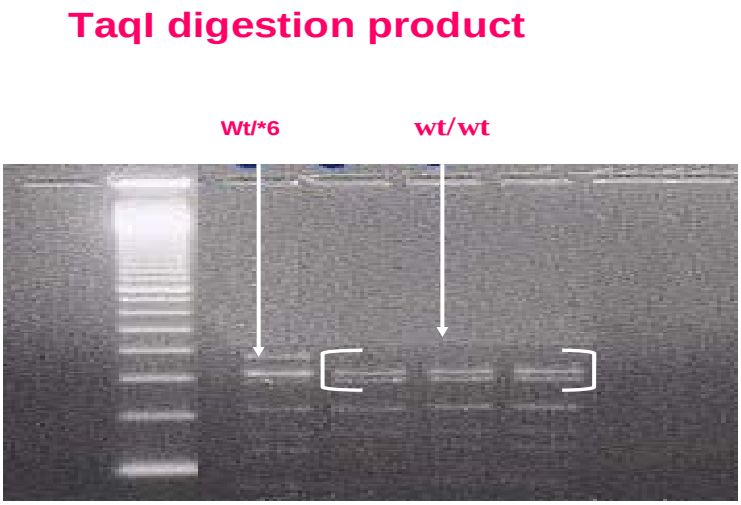
የህመምተኛ ፊርማ _____

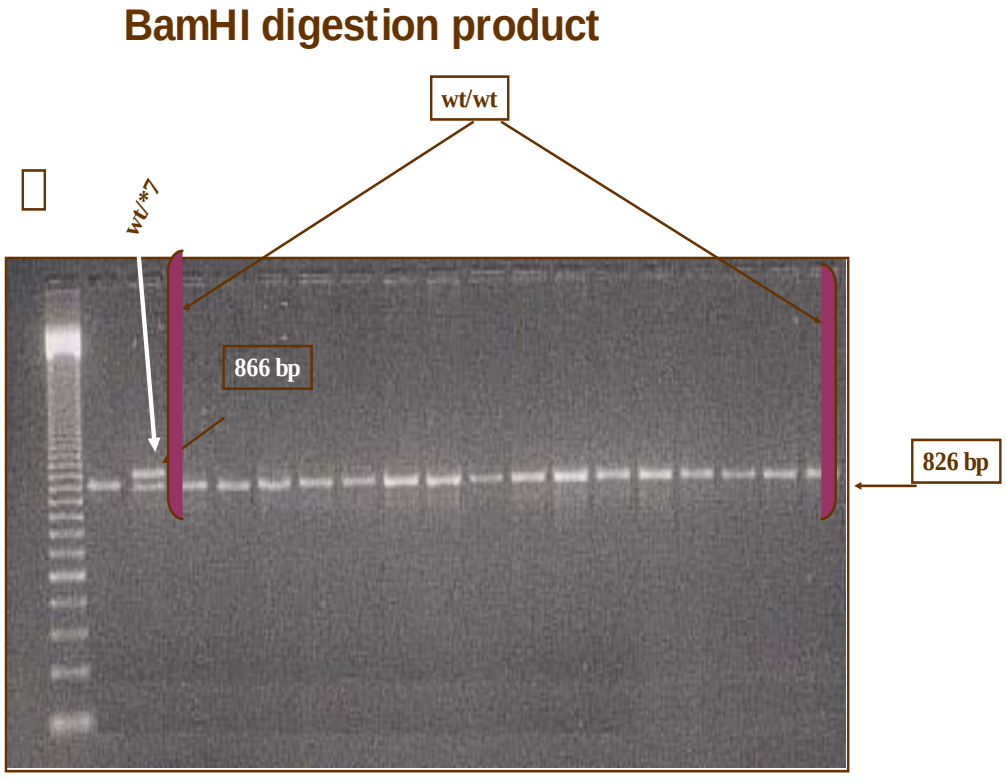
የጤና ባለሙያ ፊርማ _____

የታዛቢ ፊርማ _____

ጥናቱን በተመለከተ ጥያቄ ወይም አስተያየት ካሎት በስልክ ቁጥር 53 49 45 ዶ/ር የማነ ተሽላይ ብለው ማነጋገር ይችላሉ።

Appendix IV. Gel pictures of NAT2 gene





MspI digestion product and allele specific PCR respectively