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Genome-wide association and replication studies of anti-tubercular and anti-retroviral drugs-induced liver injury in Ethiopian TB/HIV patients

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Abbreviations and Acronyms

ABC	ATP-binding cassette
ADR	Adverse drug reaction
AGBL4	ATP/GTP binding protein-like 4
AIDS	Acquired immune deficiency syndrome
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ART	Anti-retroviral therapy
ARV	Antiretroviral
AST	Aspartate aminotransferase
ATD	Anti-tubercular drugs
ATP	Adenosine triphosphate
BMI	Body mass index
BSEP	Bile salt export pump
CGAS	Candidate gene association studies
CI	Confidence interval
CYP	Cytochrome P450
DILI	Drug-induced liver injury
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTG	Dolutegravir
EDTA	Ethylenediaminetetraacetic acid
EMB	Ethambutol
ER	Endoplasmic reticulum
ERN1	Endoplasmic reticulum to the nucleus signaling-1
FAM65B	Family with sequence similarity-65 member-B
FHAPCO	Federal HIV/AIDS Prevention and Control Office

FMoH	Federal Ministry of Health
FRET	Fluorescence resonance energy transfer
GRChg37	Genome reference consortium human genomes build 37
GST	Glutathione S-transferase
GWAS	Genome-wide association studies
HapMap	Haplotype mapping
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HWE	Hardy-Weinberg equilibrium
INH	Isoniazid
IRE1α	Inositol-requiring enzyme-1 alpha
LD	Linkage disequilibrium
LincRNA	Long intergenic non-coding RNA
MAF	Minor allele frequency
MHC	Major histocompatibility complex
MOMP	Mitochondrial outer membrane permeabilization
MPT	Mitochondrial permeability transition
MRP	Multidrug resistance-associated protein
MTB	Mycobacterium tuberculosis
NAT	N-Acetyltransferase
NRTI	Nucleoside/Nucleotide Reverse Transcriptase Inhibitor
OR	Odds ratio
PCA	Principal-component analysis
PCR	Polymerase chain reaction
PGP	Permeability glycoprotein
PLHIV	People living with HIV
PTPN2	Protein tyrosine phosphatase non-receptor type-2

PZA	Pyrazinamide
QQ	Quantile-quantile
RIF	Rifampicin
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rs	Reference SNP
RUCAM	Roussel Uclaf causality assessment method
SBE	Single-base extension
SEMA3A	Semaphorin 3A
SLCO	Solute carrier organic anion transporter
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
SSP	Sequence-specific primers
TB	Tuberculosis
TBE	Tris (hydroxymethyl) aminomethane-borate with EDTA
TBil	Total bilirubin
TE	Tris (hydroxymethyl) aminomethane-HCl with EDTA
TDF	Tenofovir
TNF	Tumor necrosis factor
UGT	Uridine diphosphoglucuronosyl-transferase
ULN	Upper limit of normal
UNAIDS	Joint United Nations program on HIV/AIDS
UPR	Unfolded protein response
WHO	World Health Organization
λ_{GC}	Genomic control inflation value

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Abstract

Background: Drug-induced liver injury (DILI) is a well-recognized adverse effect of anti-tubercular drugs (ATD) and antiretroviral therapy (ART) possibly associated with genetic variations. Liver injury induced by these drugs carries numerous risks that include morbidity and mortality due to liver failure, disease progression resulting from discontinuation of drug therapy, and drug resistance related to treatment interruption. Genome-wide association study (GWAS) is a genetic study used to identify single nucleotide polymorphisms (SNPs) that contribute to disease risk. Genetic risk variants identified by pharmacogenomic GWAS, and confirmed through replication studies can serve as biomarkers to predict treatment response, and advance clinical care through personalized medicine. The objective of this study was to identify and validate SNPs associated with ATD and ART-induced liver injury through GWAS and subsequent replication studies, and also to investigate the association of human leukocyte antigen (*HLA)-B* alleles with ATD and ART co-treatment induced liver injury in Ethiopian TB/HIV co-infected patients.

Methods: The present study had a case-control design using cases and controls obtained from a prospective cohort study conducted to determine the incidence and predictors for ATD and ART-induced liver injury in Ethiopia. Cases were those study participants who developed DILI due to ATD and/or ART in the follow up period. DNA samples from a total of 1,055 patients were used. The three treatment groups for the current study were TB patients treated with first-line ATD (75 cases, 571 controls), HIV patients without TB co-infection treated with Efavirenz-based ART (21 cases, 368 controls), and TB/HIV co-infected patients treated with both ATD and efavirenz-based ART concurrently (46 cases, 292 controls). Whole genome genotyping was done using Illumina Omni Express Exome BeadChip genotyping array on a total 89 cases and 488

controls. Replication study was carried out for the top SNPs with the lowest *P*-values in the GWAS using an independent cohort consisting of 45 cases and 425 controls. For the HLA study, genomic DNA from 46 cases, and sex and age matched controls from ATD and ART co-treatment group were typed for *HLA-B* alleles using low resolution Olerup SSP®*HLA-B* DNA typing kit. High resolution sub-typing was performed for *HLA-B* alleles that showed significant association with DILI. The association analysis for the GWAS and replication studies were done using Plink *v* 1.07, and we used SPSS *v* 22 for the *HLA-B* typing analysis.

Results: In the combined analysis of the GWAS and the replication study, the top SNP identified in the ATD treatment group was rs10946737 ($P = 4.4\times10^{-6}$, odds ratio (OR) = 3.4, 95% CI = 2.2-5.3) located in the intron region of family with sequence similarity-65 member-B (*FAM65B*). In addition, we identified cluster of SNPs with suggestive genome-wide significance in the intron of ATP/GTP binding protein like-4 (*AGBL4*). We identified a missense SNP rs199650082 (R919Q, $P = 1.4\times10^{-6}$, OR = 18.2, 95% CI = 7.1-46.9) in an endoplasmic reticulum to nucleus signaling-1 (*ERN1*) gene in the ART group. In the ATD and ART co-treatment group, we identified rs4842407 ($P = 5.3\times10^{-7}$, OR = 5.4, 95% CI = 2.8-10.3) a long intergenic non-coding RNA (*lincRNA*) transcript variant. In our HLA typing study, proportion of *HLA-B*57* allele carriers in the cases was significantly higher compared with the controls ($P = 0.002$). From *HLA-B*57* alleles detected, *HLA-B*57:02* and *HLA-B*57:03* accounted for 41.7 and 58.3%, respectively.

Conclusion: We identified genetic variants that may be associated with ATD and ART-induced liver injuries. Further studies with larger sample sizes are essential to confirm the findings.

Keywords: *AGBL4*, Anti-tubercular drugs, Anti-retroviral therapy, DILI, *ERN1*, Ethiopians, *FAM65B*, GWAS, Hepatotoxicity, *HLA-B*57*, *LincRNA*

1. Background

1.1. Tuberculosis and HIV infections

1.1.1. Tuberculosis infection

Tuberculosis (TB) is an airborne infectious disease caused by *Mycobacterium tuberculosis* complex (Forbes *et al.*, 2018). It is one of the top ten causes of death worldwide, and the leading cause of death from a single infectious agent ranking above human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) (WHO, 2019). In low and middle-income countries, TB continues to be a major cause of morbidity and mortality (Pai *et al.*, 2016).

According to the World Health Organization (WHO), in 2018, an estimated 10 million people developed TB globally, of whom more than 1.4 million died (WHO, 2019). There were TB cases in all age groups, but about 90% of cases were adults. The severity of TB epidemic in terms of the annual number of incident TB cases varied widely among countries (Figure 1). Most of the estimated number of incident cases occurred in South-East Asia and sub-Saharan Africa that accounted for more than 80% of TB cases (WHO, 2019). TB is the most prevalent opportunistic infection and the leading cause of death among HIV patients (Egelund *et al.*, 2017).

Ethiopia is listed among the top twenty high-TB burden countries globally (WHO, 2019). TB is slowly declining through effective diagnosis and treatment; though, the incidence rate is still high. In a nation-wide survey in 2011, adult population prevalence of bacteriological confirmed TB was 277 cases per 100,000 adult populations (Kebede *et al.*, 2014). The incidence in 2018 declined to an estimated 165 cases per 100,000 populations (WHO, 2019).

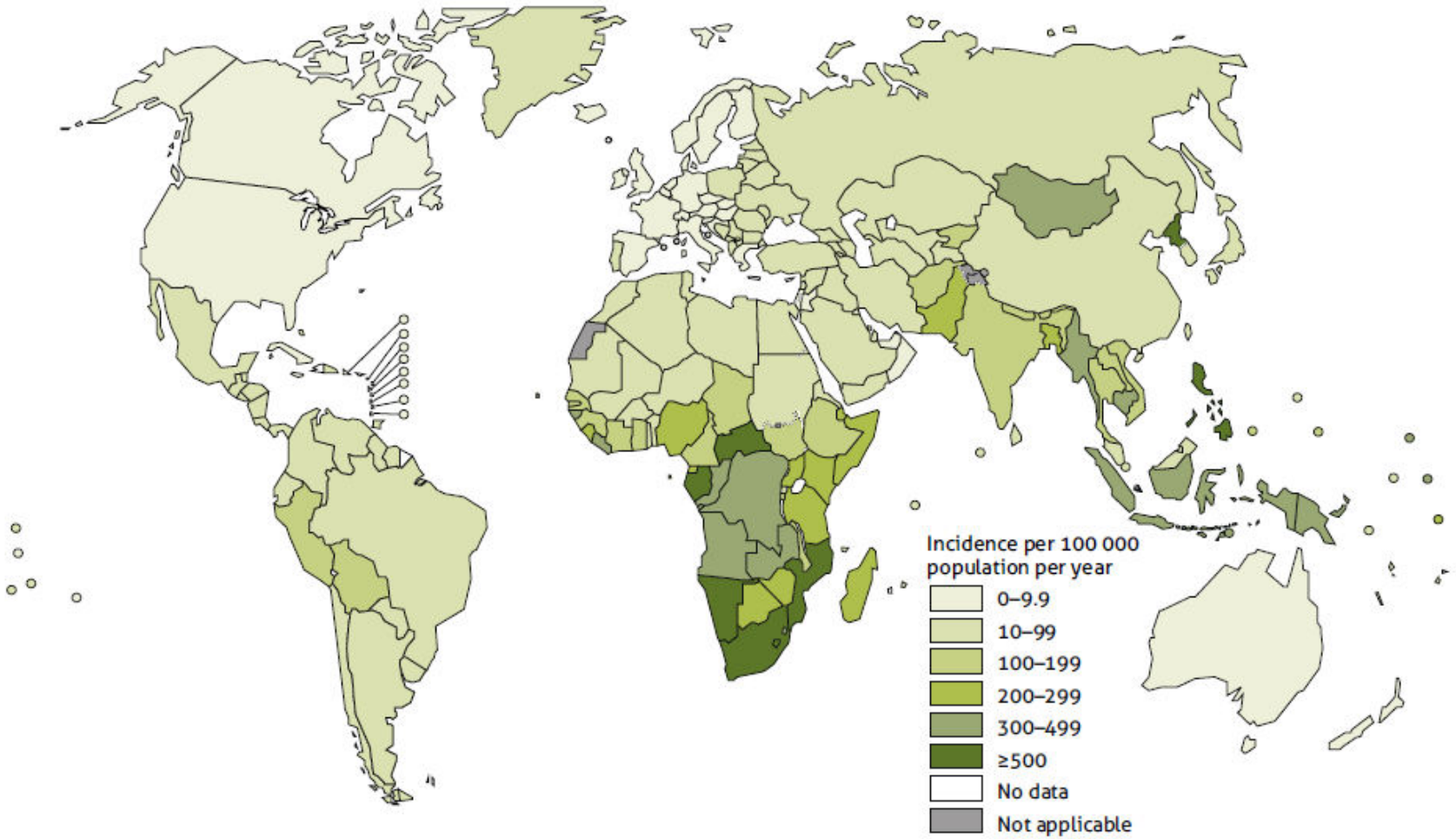

Figure 1. Estimated tuberculosis incidence rates in 2018 (WHO, 2019)

Mycobacterium tuberculosis (MTB) is a rod-shaped, non-spore-forming, aerobic and acid-fast bacterium (Pai *et al.*, 2016). The route of entry of the bacillus is via the respiratory tract. Although primarily a pulmonary pathogen, MTB can cause disease in almost any part of the body. Infection with the bacteria can range from containment in the host, in which the bacteria are isolated within granulomas (latent TB), to a contagious state in which the patient will show clinical symptoms that include persistent cough, fever, night sweat, and weight loss (active TB) (Zumla *et al.*, 2013).

For the diagnosis of active TB, the main techniques used are imaging, sputum smear microscopy, bacterial culture and molecular tests (Pai *et al.*, 2016). Chest X-ray (CXR), a rapid imaging technique with a high sensitivity, often used as a primary tool to detect pulmonary TB. The emergence of digital CXR technology offers superior quality imaging and computer-aided detection and interpretation of findings. The sputum smear microscopy continues to be the most widely used active TB test in low and middle-income countries (Kik *et al.*, 2014). A nucleic acid amplification molecular diagnostic test that can detect MTB complex and also identify rifampicin (RIF) resistance is called Xpert® MTB/RIF assay (Boehme *et al.*, 2011). This technology is now the first-line diagnostic test replacing smear microscopy as it permits rapid diagnosis of TB with high specificity and sensitivity (Boehme *et al.*, 2011). WHO has recommended Xpert® MTB/RIF as the preferred test in the diagnosis of suspected cases of active TB (Gilpin *et al.*, 2018, WHO, 2013).

Anti-tubercular drugs

The goals of TB treatment are to ensure cure without relapse, stop transmission, and prevent emergence of drug resistance (WHO, 2018). The standard treatment regimen of choice for drug-

susceptible TB in adults consists of a 2-month intensive phase of rifampicin, isoniazid, pyrazinamide and ethambutol followed by a 4-month continuation phase of rifampicin and isoniazid. These anti-tubercular drugs (ATD) are recommended on the basis of their capacity to achieve rapid culture conversion, shorten treatment duration needed to prevent relapse, better tolerability and low rate of drug resistance (Pai *et al.*, 2016). The most common adherence monitoring approach is directly observed therapy (DOT) in which treatment is directly supervised by a health professional (Brewer and Heymann, 2004). Treatment success rates of above 85% for cases of drug-susceptible TB are regularly reported (WHO, 2018). WHO 'End TB strategy' calls for a 90% reduction in TB deaths and an 80% reduction in TB incidence rate by 2030 compared to levels in 2015 (WHO, 2014).

The mechanisms of action of first line ATD

Isoniazid (INH): INH enters tubercle bacilli by passive diffusion, and will be biotransformed to its toxic form within the bacilli by catalase-peroxidase (KatG) (Argyrou *et al.*, 2007). KatG catalyzes the production of an isonicotinoyl radical that subsequently interacts with bacterial nicotinamide adenine dinucleotide (NAD^+) and $NADP^+$ to produce adducts. One of these adducts, nicotinoyl-NAD, inhibits the activities of enoyl-acyl carrier protein reductase (InhA) and β-ketoacyl-acyl carrier protein synthase (KasA). Suppression of these enzymes inhibits synthesis of mycolic acid, an essential component of mycobacterial cell wall, leading to bacterial cell death (Argyrou *et al.*, 2007). Another adduct, nicotinoyl-NADP, potently inhibits mycobacterial dihydrofolate reductase, thereby interfering nucleic acid synthesis (Argyrou *et al.*, 2006). Resistance to INH is associated with mutation or deletion of KatG with loss of ability to form adducts, or over-expression of the genes for InhA and KasA (Gumbo, 2018).

Adverse effects of INH include hepatotoxicity and peripheral neuritis (Gumbo, 2018). INH binds to pyridoxal 5′-phosphate to form INH-pyridoxal hydrazone, thereby depleting pyridoxal 5′-phosphate, interfering with pyridoxal phosphate-requiring reactions, and this is the likely mechanism of INH-induced peripheral neuropathy (Gumbo, 2018). The treatment or preventive approach is replenishment of pyridoxal 5′-phosphate with pyridoxine (vitamin-B6) supplement.

Rifampicin (RIF): RIF enters tubercle bacilli, and then binds to β subunit of deoxyribonucleic acid (DNA)-dependent RNA polymerase (encoded by *rpoB*) to form a stable drug-enzyme complex that suppresses RNA synthesis (Raviglione, 2018). Mutation in *rpoB* that leads to alteration of the target of the drug could results in drug resistance (Somoskovi *et al.*, 2001). In addition, mutations in efflux pump are also associated with RIF resistance (Li *et al.*, 2015). RIF is generally well tolerated during treatment (Raviglione, 2018). The common adverse effects are nausea, vomiting, fever and skin rash. Rarely, hepatitis and deaths due to liver failure have been observed in patients who received other hepatotoxic ATD with RIF (Abbara *et al.*, 2017, Saukkonen *et al.*, 2006).

Pyrazinamide (PZA): PZA is a synthetic pyrazine analogue of nicotinamide. Although the exact mechanism of action of PZA against MTB is unknown, several mechanisms have been proposed. It is widely accepted that PZA is a pro-drug requiring deamination to pyrazinoic acid, the active form, by the mycobacterial enzyme pyrazinamidase (encoded by *pncA*) (Anthony *et al.*, 2016). The acidification of the intracellular environment may inhibit vital enzymes and their functions thereby killing the bacteria (Anthony *et al.*, 2016, Zhang *et al.*, 2014). A specific target of PZA has also been proposed: inhibition of a ribosomal protein S1 (encoded by *RpsA*), a vital protein involved in protein translation, thus toxic proteins due to stress accumulate and kill the bacteria (Shi *et al.*, 2011). In addition, PZA target may include aspartate decarboxylase (encoded

by *panD*) which is involved in coenzyme-A biosynthesis in MTB (Zhang *et al.*, 2013a). Mutations in *pncA*, *RpsA* and *panD* genes that can lead to alteration of the target of the drug with reduced affinity for PZA could result in drug resistance (Zhang *et al.*, 2014). It has also been demonstrated that efflux rate of pyrazinoic acid predicts PZA resistance (Zimic *et al.*, 2012).

Liver injury is a serious adverse effect of PZA containing ATD regimen (Saukkonen *et al.*, 2006). Prior to administration of such regimen, patients should undergo hepatic function test, and the tests should be repeated at frequent intervals during the treatment period. If evidence of significant hepatic damage becomes apparent, therapy must be stopped. Another adverse effect of PZA is inhibition of excretion of urates contributing for hyperuricemia, which may precipitate acute episodes of gout. Other side effects include gastrointestinal disturbances, anorexia, fever, dysuria and malaise (Gumbo, 2018).

Ethambutol (EMB): EMB is effective against actively growing TB bacilli by interfering the formation of intact cell wall that comprises of mainly mycolic acid and arabinogalactan (Raviglione, 2018). Mycolic acid attaches to 5'-hydroxyl groups of D-arabinose residues of arabinogalactan, and forms mycolyl-arabinogalactan-peptidoglycan complex. EMB disrupts the transfer of arabinose for arabinogalactan biosynthesis by inhibiting the enzyme arabinosyl transferase (encoded by *embAB*). Interference of the arabinogalactan synthesis disrupts the assembly of mycobacterial cell wall (Gumbo, 2018). An important side effect of EMB is optic neuritis, resulting in decreased visual acuity and difficulty of color discrimination (Sarkar *et al.*, 2016), but recovery usually occurs when the drug is withdrawn (Raviglione, 2018).

When there is documented drug resistance for the first-line ATD, second-line drugs with prolonged course of multiple drugs should be initiated (Pai *et al.*, 2016). The second-line classes

of drugs include fluoroquinolones (levofloxacin, moxifloxacin and gatifloxacin); injectable agents (kanamycin, amikacin, streptomycin and capreomycin), other oral drugs (ethionamide, prothionamide, cycloserine, *p*-aminosalicylic acid, linezolid and clofazimine), and the recently approved add-on agents (bedaquiline and delamanid) (Raviglione, 2018). Because of lower degree of effectiveness and higher degree of intolerability (Sarkar *et al.*, 2016), the second-line drugs are generally used for the treatment of drug-resistant TB (Pai *et al.*, 2016).

1.1.2. HIV infection

Human immunodeficiency virus (HIV) is an RNA virus that belongs to the family of human retroviruses. Subtypes of the virus include HIV-1 and HIV-2. The most common cause of HIV/AIDS throughout the world is HIV-1 (Fauci *et al.*, 2018). The virus is transmitted primarily by sexual contact, blood and blood products, and from infected mothers to the child.

HIV/AIDS is a global public health problem. According to the Joint United Nations Program on HIV/AIDS (UNAIDS), at the end of 2018, an estimated 37.9 million people living with HIV (PLHIV) were reported globally; 1.7 million people became newly infected, and 770, 000 people died from AIDS-related illnesses (UNAIDS, 2019). TB remains the leading cause of death among PLHIV (WHO, 2018). The most affected region by HIV is the sub-Saharan Africa where an estimated 0.8 million people newly acquired HIV in 2018, and it is home for about 20.6 million PLHIV which is more than half of the global total (UNAIDS, 2019). Recent data offer signs of declining HIV incidence in many countries, although frequently at levels that still remain high (Fauci *et al.*, 2018).

HIV/AIDS is one of the key challenges for the overall development in Ethiopia, as it has led to a decrease in life expectancy and reduced workforce (FHAPCO, 2014). In 2018, there were an

estimated 613,000 PLHIV in Ethiopia (FMoH, 2018). Ethiopia is one of the twenty countries contributing to 80% of the global burden of HIV/AIDS (Feyissa *et al.*, 2019). A survey on Ethiopian population-based HIV impact assessment (EPHIA) conducted recently showed 3.0% prevalence of HIV among adults in urban Ethiopia, though the prevalence varies geographically (EPHIA, 2018).

The standard blood screening tests for the diagnosis of HIV infection are mainly based on the detection of antibodies to HIV (Fauci *et al.*, 2018). Commercial kits contain antigens from both HIV-1 and HIV-2 and thus are able to detect antibodies. A common platform is the Enzyme-linked immunosorbent assay (ELISA) technique with high sensitivity. In addition, a series of HIV rapid diagnostic tests (RDT) such as Determine®, Cappilus® and Unigold®, with a solid phase or particle coated with synthetic or recombinant HIV-1 and HIV-2 antigens, are used to detect antibodies usually in resource-limited settings with comparable sensitivity (Anzala *et al.*, 2008). CD4+ T-cell count is a laboratory test generally accepted as indicator of state of immunologic competence of the patient with HIV infection, and the measurement of copies of viral RNA has become an essential component for monitoring (Fauci *et al.*, 2018).

The clinical consequences of HIV infection ranges from an acute syndrome associated with primary infection to a prolonged asymptomatic state to advanced disease state (Fauci *et al.*, 2018). When the CD4+ T-cell count falls down, the resulting state of immunodeficiency will place the patient at high risk for opportunistic infections, and hence for clinically apparent disease. A diagnosis of AIDS is made with CD4+ T-cell count < 200 cells/μL (WHO clinical stage 3) and when developing HIV-associated diseases considered to be indicative of severe defect in the immune system (WHO, 2016).

Anti-retroviral therapy

Antiretroviral therapy (ART) is the core for the management of patients with HIV infection. Following the initiation of widespread use of ART in the world, there were marked reductions in morbidity and mortality, and improvement of quality of life of PLHIV (UNAIDS, 2018). Currently, ART is recommended for virtually all HIV-infected individuals, as soon as possible after HIV diagnosis, regardless of their CD4+ T-cell counts and WHO clinical stages (FMoH, 2017, Saag *et al.*, 2018, WHO, 2016). In 2018, an estimated 23.3 million PLHIV accessed ART globally (UNAIDS, 2019). In 2017, an estimated 436,000 PLHIV were taking ART in Ethiopia (FMoH, 2018). The scale of ART is increasing as part of achieving the UNAIDS '90–90–90' targets: ensuring that 90% of PLHIV know their HIV status; 90% of the PLHIV who know their HIV status are accessing treatment; and 90% of the PLHIV who are receiving treatment have suppressed viral load by the year 2020 (UNAIDS, 2014).

Understanding HIV replication cycle is important to understand rational therapy of the infection. HIV virus replication begins with high-affinity binding of viral glycoprotein gp120 on to CD4 receptors on host cell surface predominantly found on a subset of T-lymphocytes that are responsible in the immune system (Fauci *et al.*, 2018). Then, the gp120 protein undergoes conformational change that facilitates binding to one of the co-receptors - CCR5 or CXCR4. After this binding, fusion with the host cell membrane occurs via a newly exposed glycoprotein gp41 molecule penetrating the plasma membrane of the target cell. Following fusion, un-coating of the protein shell is initiated. Then, viral reverse transcriptase catalyzes reverse transcription of the RNA into double-stranded DNA (Figure 2).

The viral DNA then accesses the nuclear pore and transferred to the nucleus, where it integrates into the host cell genome through the action of another virally encoded enzyme, integrase. HIV proviral DNA integrates into the host genomic DNA preferentially in regions of active transcription (Fauci *et al.*, 2018). Following transcription, HIV messenger RNA (mRNA) is translated into proteins that undergo modification and cleavage. The viral particle is formed by the assembly of HIV proteins, enzymes and genomic RNA. During budding of the progeny virion through the lipid bilayer of the host cell membrane, viral encoded protease catalyzes post-translational cleavage to yield mature virion (Atta *et al.*, 2019).

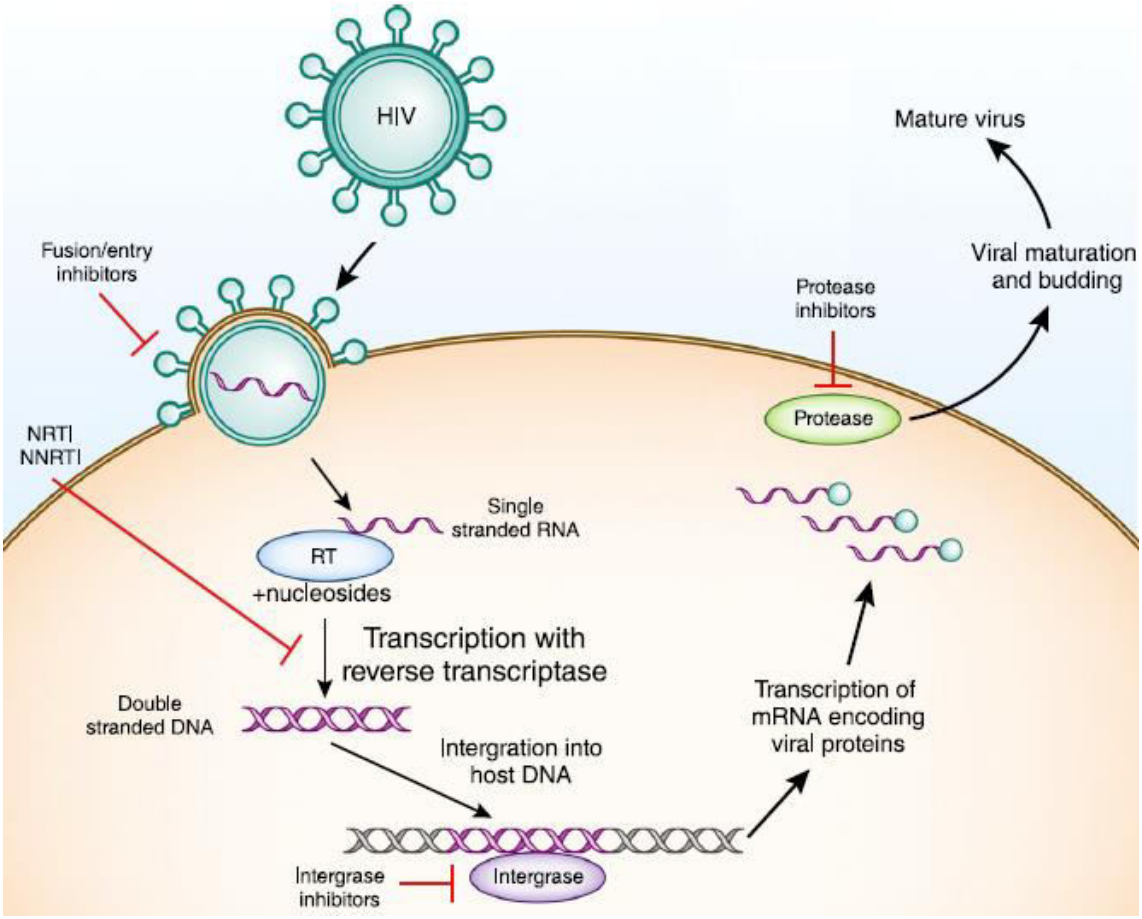

Figure 2. HIV replication cycle and sites of action of drugs. Adopted from (Atta *et al.*, 2019). HIV - Human immunodeficiency virus; NNRTI - Non-nucleoside reverse transcriptase inhibitor; NRTI - Nucleoside reverse transcriptase inhibitor; RT - Reverse transcriptase

The currently available antiretroviral (ARV) drugs for the treatment of HIV infection, as part of a combination regimen, fall into five categories (Atta *et al.*, 2019): Nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase inhibitors, protease inhibitors (PIs), and viral entry inhibitors. The sites of action of ARV drugs are shown in Figure 2.

The mechanisms of action of ARV drug classes

NRTIs: NRTIs include nucleoside analogues zidovudine (ZDV), lamivudine (3TC), stavudine (d4T), emtricitabine (FTC), abacavir (ABC), didanosine (ddI), and zalcitabine (ddC); and a nucleotide analogue tenofovir (TDF). Nearly all patients start ART with at least one agent from this class, and the drugs inhibit both HIV-1 and HIV-2 replication (Flexner, 2018).

The NRTIs block HIV replication cycle at the point of RNA-dependent DNA synthesis by competitive inhibition of HIV reverse transcriptase (Flexner, 2018). The drugs incorporation into the growing viral DNA chain causes premature chain termination. The selective toxicity of these drugs depends on their ability to inhibit HIV reverse transcriptase without inhibiting cellular DNA polymerase. However, some NRTIs inhibit mitochondrial DNA polymerase-γ, which encode mitochondrial enzymes (Flexner, 2018). For this reason, serious side effects vary among the NRTIs and include mitochondrial damage, peripheral neuropathy and pancreatitis. Lactic acidosis with or without hepatomegaly and hepatic steatosis is a rare but potentially fatal complication seen with ddI, d4T and ZDV; whereas, FTC, 3TC, and TDF have low affinity for DNA polymerase-γ and are largely devoid of mitochondrial toxicity (Fauci *et al.*, 2018). Because of the limited viral resistance and overall tolerability, nowadays TDF is the most widely used NRTI (Atta *et al.*, 2019).

NNRTIs: NNRTIs include efavirenz, nevirapine, delavirdine, etravirine and rilpivirine. NNRTIs bind to a hydrophobic pocket of the HIV reverse transcriptase in a site distant from the active site (Fauci *et al.*, 2018). The drugs induce conformational change in the structure of the reverse transcriptase enzyme that greatly reduces its activity, thus they act as non-competitive inhibitors. Because the binding site for NNRTIs is virus-strain specific, the drugs are active against HIV-1 infection (Fauci *et al.*, 2018). The NNRTIs have no activity against host cell DNA polymerases (de Béthune, 2010). They are well absorbed from the gastrointestinal tract; undergo hepatic metabolism. NNRTIs are susceptible to drug resistance caused by single amino acid change in their binding pocket, thus should be combined with other active drugs to avoid resistance (Kuritzkes, 2011).

Among drugs in NNRITs, efavirenz (EFV) has been used widely because of its convenience, effectiveness and tolerability (Flexner, 2018). The use of EFV in combination with other ARV drugs is associated with favorable long-term suppression of viremia and elevation of CD4+ lymphocyte counts (Flexner, 2018). EFV is a moderate inducer of hepatic enzymes especially Cytochrome P450 3A4 (CYP3A4). Adverse effects of EFV include CNS effects, skin rash and hepatotoxicity (de Béthune, 2010). CNS adverse effects (dizziness, impaired concentration, vivid dream, and insomnia) and skin rash are common in the first few weeks of starting treatment, and generally become more tolerable and resolve spontaneously within the first 4 weeks of therapy; rarely requiring drug discontinuation for these reasons. Increased hepatic transaminases and rare fatal hepatitis have been associated with the use of NNRTIs (de Béthune, 2010).

Integrase inhibitors: Integrase inhibitors include dolutegravir, raltegravir and elvitegravir. Integrase inhibitors act by blocking the catalytic activity of HIV integrase enzyme and thus prevent integration of the HIV provirus into the host cell genome in both HIV-1 and HIV-2 (Atta

et al., 2019). As they prevent formation of covalent bonds between host and viral DNA, they are also known as integrase strand transfer inhibitors (InSTIs). They are among the widely used ARV drugs currently because of their ARV activity and safety (Saag *et al.*, 2018).

Given its efficacy, tolerability and convenience, dolutegravir (DTG) is becoming increasingly popular as a part of combination ART starting agent in treatment-naïve HIV infected patients (Osterholzer and Goldman, 2014). A recent study in Cameroon on HIV-1 patients showed DTG-based regimen was non-inferior to EFV-based regimen with regard to viral suppression at 48th week (Kouanfack *et al.*, 2019). Although DTG has low potential to cause clinically significant drug interactions, liver microsomal enzyme inducers such as RIF and EFV can decrease its plasma concentration and hence require dose adjustment to counter this effect (Dooley *et al.*, 2013).

Protease inhibitors (PIs): PIs that include saquinavir, indinavir, ritonavir, nelfinavir, amprenavir, fosamprenavir, lopinavir, atazanavir, tipranavir, and darunavir are well absorbed after oral administration, and are active against both HIV-1 and HIV-2 (Flexner, 2018). During the later stages of HIV growth cycle, *Gag* and *Pol* gene products are translated into polyproteins that include essential structural and enzymatic components of the virus, and these become immature budding particles (Fauci *et al.*, 2018). The HIV protease is responsible for cleaving these precursor molecules to produce the final structural proteins of the mature virus. By preventing post-translational cleavage of the *Gag* and *Pol* polyproteins, PIs interrupt the processing of viral proteins into functional conformations, resulting in the production of immature and non-infectious viral particles (Flexner, 2018).

Most PIs are bio-transformed through hepatic metabolism mainly by CYP3A4, and hence they have the potential for drug interactions (Flexner, 2018). Some of these drugs inhibit CYP3A4, although the magnitude of inhibition varies greatly, with ritonavir being by far the most potent inhibitor. It is a common practice to combine PIs with a low dose of ritonavir as a pharmacokinetic enhancer which allows reduction in both dose and dosing frequency of the drug while increasing systemic concentrations (Cooper *et al.*, 2003). The common adverse effects of PIs are gastrointestinal side effects including nausea, vomiting, anorexia, abdominal pain, and diarrhea. Dose-dependent elevations in serum cholesterol and triglycerides have been reported (Fauci *et al.*, 2018).

Entry Inhibitors: Entry inhibitors act by inhibiting the binding of HIV to the host cell receptors thus interfering with the process of fusion (Fauci *et al.*, 2018). The two drugs available in this class are enfuvirtide and maraviroc. Enfuvirtide binds to gp41 subunit of the viral envelope glycoprotein, preventing conformational changes required for the fusion of the viral and cellular membranes (Tilton and Doms, 2010). The drug's cost and route of administration (parentral injection) limit its use. The prominent adverse effects of enfuvirtide are injection-site reactions: pain, erythema, and induration (Tilton and Doms, 2010).

Maraviroc is a chemokine receptor antagonist that binds to the host cell CCR5 co-receptor to block binding of the viral glycoprotein gp120 (Didigu and Doms, 2012). It is effective for use in patients infected with CCR5-tropic virus, but not active against viruses with CXCR4-tropism (Tilton and Doms, 2010). Maraviroc is generally well tolerated, but can cause dose-dependent orthostatic hypotension (Fauci *et al.*, 2018).

1.2. Structures and metabolic functions of liver

1.2.1. Structures of the liver

Liver is the largest gland in the body, accounting for about 2% of an adult body weight (Young *et al.*, 2013). It is a red-brown dome shaped organ covered by visceral peritoneum (Drake *et al.*, 2015). The liver lies mainly in the right upper quadrant of the abdomen, beneath the diaphragm and protected by the lower right ribs. It also extends across the midline towards the left upper quadrant of the abdomen. The liver is held on place through ligament attachments that suspend the organ to the diaphragm and the anterior abdominal wall (Abdel-Misih and Bloomston, 2010).

Liver is grossly divided into two parts: a large right lobe and a smaller left lobe (Drake *et al.*, 2015). The basic functional units of the liver are the liver lobules (Young *et al.*, 2013). The lobules are roughly hexagonal shape and consist of plates of hepatocytes radiating from the central vein (Figure 3A). The lobules are held together by fine connective tissues. A distinctive component of a lobule is the portal triad at the corners of each lobule. The portal triad consists of branches of the hepatic artery, hepatic portal vein, and bile duct. In between the hepatocyte plates, there are liver sinusoids through which blood from the hepatic portal vein (nutrient-rich blood) and the hepatic artery (oxygen-rich blood) enter into and supply the hepatocytes before draining into systemic circulation via hepatic venous system (Ozougwu, 2017). In between adjacent hepatocytes, grooves in the cell membranes provide room for bile canaliculi. These small ducts accumulate the bile produced by the hepatocytes, and then the biliary fluid exit via the hepatic bile ducts (Young *et al.*, 2013).

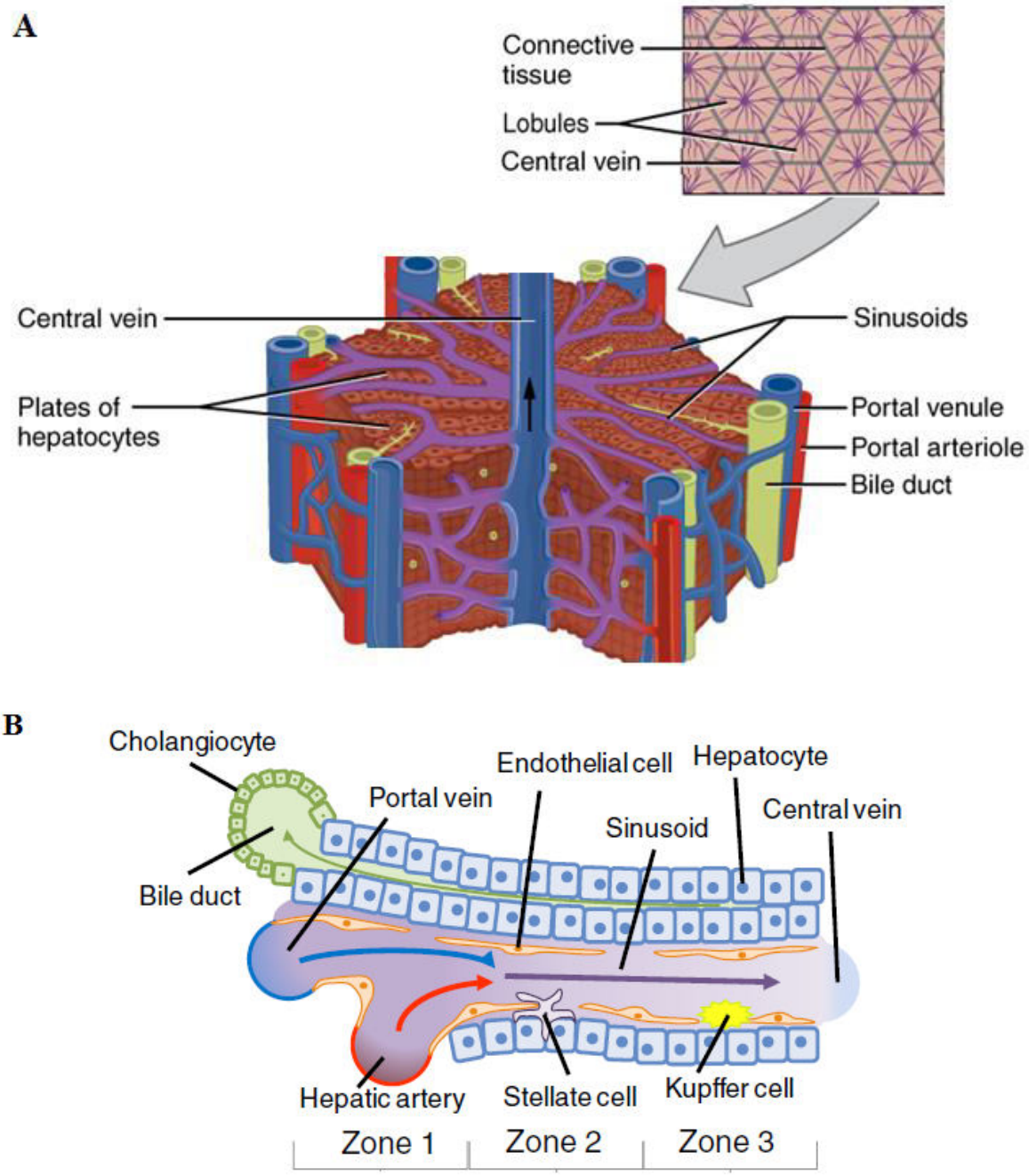

Figure 3. Liver lobule, main cell types of liver and zones of liver parenchyma. (A) Liver lobule, (B) Main cell types of liver and zones of liver parenchyma. Adopted from (Trefts *et al.*, 2017, Young *et al.*, 2013).

The liver is composed of several cell types (Trefts *et al.*, 2017) that include hepatocytes, cholangiocytes, sinusoidal endothelial cells, Kupffer cells, and stellate cells (Figure 3B). The hepatocytes make up the bulk of the lobule and perform many of the functions recognized to the liver. They are rich in organelles such as endoplasmic reticulum (ER) and golgi bodies that enable them to synthesize and secrete variety of proteins (Pineiro-Carrero and Pineiro, 2004). They also contain mitochondria which are large and many in numbers. Cholangiocytes are the second most abundant types of liver cells, and they line the lumen of the bile ducts. The walls of hepatic sinusoids are lined by fenestrated endothelial cells which serve as a barrier between the blood and the hepatocytes. The two other cell types are the Kupffer cells, which function as liver resident macrophages that engulf bacteria and worn out blood cells; and the stellate cells, which store fat and vitamin A (Pineiro-Carrero and Pineiro, 2004).

The structure and metabolic functions of the hepatocytes within the liver lobule differs depending on proximity to the portal triads (Trefts *et al.*, 2017), and thus liver parenchyma is divided into 3 metabolic zones (Figure 3B). The hepatocytes closest to the portal areas (zone-1) often have larger organelles. They receive the richest nutrient and oxygen supply, and mostly involved in the synthesis of glycogen and proteins. The hepatocytes in zone-3 (perivenular) are closest to the central veins, and the cells are involved in glycolytic energy production. In addition, the hepatocytes in this zone are rich in Cytochrome P450, a class of xenobiotic metabolizing enzymes, and thus the cells are more specialized in biotransformation reactions (Pineiro-Carrero and Pineiro, 2004). Zone-2 is an intermediate area between the two zones. Thus, hepatocytes more distant from the portal supply have a different enzymatic phenotype and respond differently to hypoxia and toxin exposure (Pineiro-Carrero and Pineiro, 2004).

1.2.2. Drug metabolism and transport in the liver

The liver has numerous functions which include secretion of bile, production of serum proteins (albumin and coagulation factors), metabolism of nutrients and regulation of blood glucose level, bilirubin metabolism, metabolic detoxification of xenobiotics, storage of minerals and vitamins, among other functions (Ozougwu, 2017). Toxicity caused by xenobiotics therefore can cause derangement in any of these functions (Akkara and Sabina, 2019).

A critical function of the liver is metabolism or detoxification of xenobiotics using enzyme-catalyzed processes (Ozougwu, 2017). These enzymes are collectively referred to as the hepatic microsomal enzymes (Gonzalez *et al.*, 2018). Cytochrome P450 (CYP) constitutes the major microsomal enzyme family capable of catalyzing the oxidative biotransformation of drugs and other lipophilic xenobiotics (Zanger and Schwab, 2013). Several of these enzymes exist in human beings, and include CYP3A4, CYP2B6, CYP2E1, CYP2D6, CYP2C9 and CYP2C19, among others (Zanger and Schwab, 2013).

Drug metabolism in the hepatocytes takes place in two stages: phase-I and phase-II reactions (Gonzalez *et al.*, 2018). Phase-I drug-metabolizing enzymes are largely located in the smooth ER, while phase-II enzymes are often cytosolic. Phase-I reactions create or unmask reactive functional groups via oxidation, reduction, or hydrolysis using the CYP enzymes. Phase-II reactions conjugate the metabolites generated in phase-I to make them more hydrophilic. Typical examples of phase-II drug-metabolizing enzymes include uridine diphosphoglucuronosyl-transferases (UGTs), glutathione-S-transferases (GSTs) and N-acetyltransferases (NATs) (Corsini and Bortolini, 2013).

UGT family of enzymes catalyzes conjugation of glucuronic acid to a wide range of xenobiotics and endogenous compounds such as bilirubin and bile acids (Rowland *et al.*, 2013). There are two major UGT families, UGT1 and UGT2 with many isoforms (Rowland *et al.*, 2013). Glutathione conjugation is catalyzed by GST family of enzymes which is important in protecting cells from reactive oxygen species (ROS) produced through cellular processes such as phase-I drug metabolism (Jancova *et al.*, 2010). GSTs are divided into five families that include GST mu (GSTM) and GST theta (GSTT) with genetic polymorphisms that express a null phenotype (Jancova *et al.*, 2010). NAT family of enzymes is responsible for the metabolism of drugs that contain aromatic amine or hydrazine group (Gonzalez *et al.*, 2018). The NAT family of enzymes include two isoforms, NAT1 and NAT2 (Jancova *et al.*, 2010). The NAT2 isoform is predominantly found in the liver and particularly important in the metabolism of drugs that include INH, hydralazine and sulfonamides (Gonzalez *et al.*, 2018).

In order for metabolism and elimination to occur within the hepatocytes, the compounds must first be taken into the cell. Large number of compounds enter the hepatocytes via membrane transporters (Corsini and Bortolini, 2013). Members of solute carrier (SLC) super-family play a major role in the transport of organic compounds to the hepatocytes through the sinusoidal membrane (Nigam, 2015). These transporters mediate uptake by either facilitated or active transport mechanisms with different substrate specificities. The major SLC-type uptake transporters include sodium taurocholate co-transporting polypeptide (NTCP/SLC10A1), organic anion transporters (OATs) and organic cation transporters (OCTs) classified in the SLC22A family, and organic anion transporting polypeptides (OATPs/SLCO) (Giacomini and Sugiyama, 2018) (Figure 4). The OATPs mediate uptake of a wide range of endogenous compounds such as bilirubin and bile acids, and many drugs including RIF (Roth *et al.*, 2012).

After compounds are taken up into the hepatocytes and metabolized, the modified products will be effluxed into either bile or blood for clearance from the system. The liver transporters involved in this process are mainly from ATP-binding cassette (ABC) super-family, which relies on ATP hydrolysis to actively pump the substrates across membranes (Nigam, 2015). These ABC transporters include multidrug resistance protein-1 (MDR1/ABCB1 or permeability glycoprotein (PGP)), bile salt export pump (BSEP/ABCB11), multidrug resistance-associated proteins (MRP/ABCC) family and breast cancer resistance protein (BCRP/ABCG2) (Corsini and Bortolini, 2013) (Figure 4). PGP and MRP have broad range of endogenous and drug substrates, and have overlapping substrate specificities (Nigam, 2015).

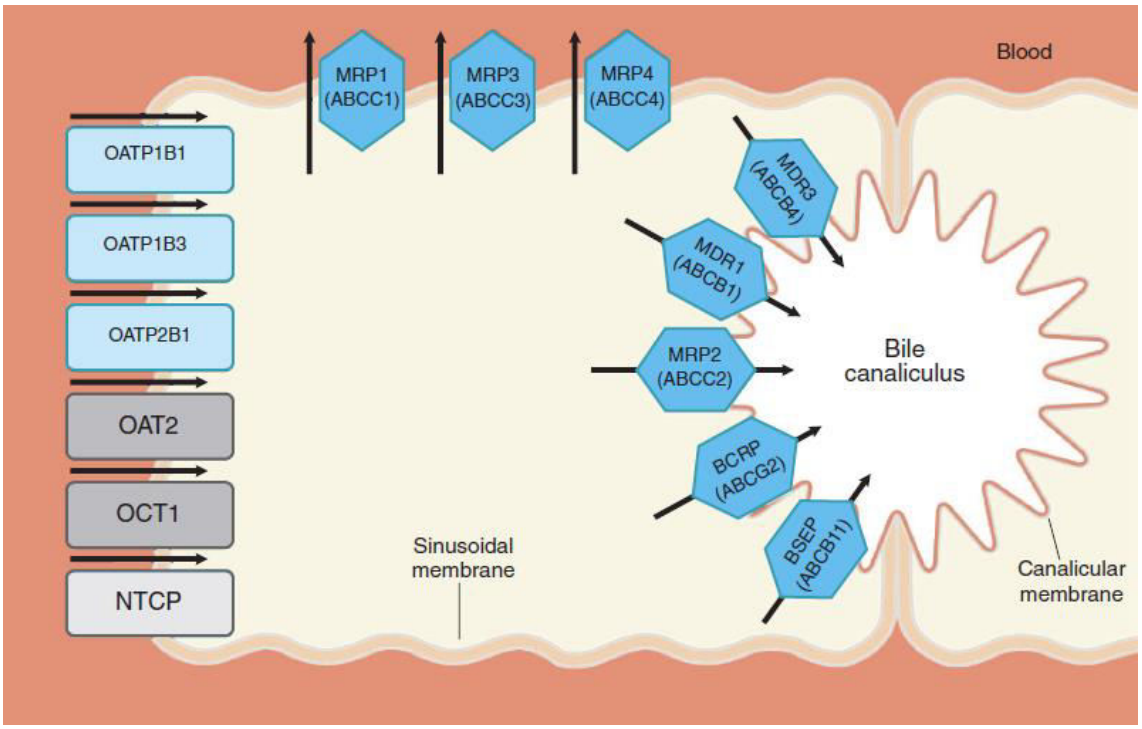

Figure 4. Uptake and efflux transporters in the hepatocytes (Giacomini and Sugiyama, 2018). Arrows show the primary direction of transport. ABC - ATP binding cassette; BCRP - Breast cancer resistance protein; BSEP - Bile salt export pump; MDR - Multidrug resistance protein; MRP - Multidrug resistance associated protein; NTCP - Sodium taurocholate co-transporting polypeptide; OAT - Organic anion transporter; OATP - Organic anion transporting polypeptides; OCT - Organic cation transporter

PGP is one of the most well-known drug efflux transporters located on the canalicular membrane and transports a wide range of substances into the bile (Finch and Pillans, 2014). BSEP is the main bile salt transporter along the canalicular membrane (Dietrich and Geier, 2014). MRP2 is responsible for secretion of wide range of conjugated xenobiotics and endogenous substances such as bilirubin. Efflux transporters located at the sinusoidal surface of the hepatocytes such as MRP3 and MRP4 play a role in the elimination of drug metabolites into the sinusoids for clearance via the renal system (Corsini and Bortolini, 2013). Inhibition of the various efflux transporter proteins by drugs or polymorphism in genes expressing the transporter proteins may cause cholestasis or hyperbilirubinemia (Giacomini and Sugiyama, 2018, Lang *et al.*, 2007).

1.3. Drug-induced liver injury

Liver is the principal organ responsible for maintenance of metabolic functions as well as for detoxification of exogenous compounds (Lee *et al.*, 2016). The liver is an organ prone to suffer toxic damage due to its anatomic location and its involvement in drug metabolism and detoxification of a wide variety of chemicals (Kolarić *et al.*, 2019). The liver is functionally located between the site of absorption of exogenous compounds and the systemic circulation. Thus, through its blood supply from the portal vein, the liver is directly exposed to ingested substances that are present in the portal blood. On the other hand, the liver is the most important site of drug metabolism due to the expression of metabolizing enzymes and the possibility of formation of drug-derived reactive species, make the liver an organ susceptible to drug-induced damage (Akkara and Sabina, 2019, Gonzalez *et al.*, 2018). In addition, the expression and activity of uptake transporters that facilitate accumulation of xenobiotics or endogenous toxic compounds in hepatocytes make this organ susceptible to injury by a wide variety of drugs and their metabolites (Kolarić *et al.*, 2019).

Drug-induced liver injury (DILI) is defined as liver injury caused by medications leading to liver dysfunction with a reasonable exclusion of other etiologies (Hoofnagle and Björnsson, 2019, Suk and Kim, 2012). DILI is an important cause of liver disease contributing to 10-15% of cases of acute liver failure (Hoofnagle and Björnsson, 2019) that often has significant morbidity and mortality (Colaci *et al.*, 2019, Montrief *et al.*, 2019). Worldwide, the estimated incidence rate of DILI is 14-24 per 100,000 populations per year (Bjornsson *et al.*, 2013, Shen *et al.*, 2019, Suk and Kim, 2012). However, experts believe that the actual incidence is higher due to under-reporting (Hassan and Fontana, 2019). DILI is the major adverse effect that leads to termination of clinical drug development programs, and it is a common reason for market withdrawal of medications (Stevens and Baker, 2009).

DILI can be classified as intrinsic or idiosyncratic injury (García-Cortés *et al.*, 2018). Intrinsic DILI is predictable, dose dependent and affect individuals to varying degree when exposed to sufficiently high dose (Lee *et al.*, 2016). In contrast, idiosyncratic DILI is unexpected on the basis of the known pharmacological actions of the drug and attributable to genetic differences among individuals (Aithal *et al.*, 2011). It is dose independent and varies in latency, presentation, and course of the reaction. Idiosyncratic DILI could be classified into immunologically and metabolically mediated reactions (Alempijevic *et al.*, 2017). The common classes of drugs with potential causes of idiosyncratic DILI are antimicrobials, non-steroidal anti-inflammatory drugs, and central nervous system acting drugs (Devarbhavi and Andrade, 2014).

The risk factors for DILI are complex involving both environmental and host genetic factors (Chalasani and Bjornsson, 2010, Kim and Naisbitt, 2016). DILI is likely to occur in the elderly, in females, and in patients with underlying chronic liver disease. Factors such as alcohol intake

and concurrent viral infections also contribute to the risk. Genetic factors are important in determining risk of idiosyncratic DILI, and are increasingly being identified (Urban *et al.*, 2014).

The standard approach for diagnosis of DILI relies on both clinical and biochemical information (Kullak-Ublick *et al.*, 2017). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and total bilirubin (TBil) are approved DILI biochemical markers in clinical practice (Chalasani *et al.*, 2014, Kullak-Ublick *et al.*, 2017). The recently used DILI diagnostic criteria were set by the International DILI Expert Working Group (Aithal *et al.*, 2011). The criteria are based on upper limit of normal (ULN) for the liver biochemical parameters. Clinical signs and symptoms of DILI include nausea, vomiting, anorexia, fatigue; right upper quadrant pain, itching, jaundice, ascites and encephalopathy (Devarbhavi, 2012).

Accurate diagnosis of DILI requires establishment of causal relationship with the suspected agent and exclusion of competing causes of liver injury (Hoofnagle and Björnsson, 2019). Roussel Uclaf Causality Assessment Method (RUCAM) is the most widely used approach to evaluate suspected cases of DILI (Chalasani *et al.*, 2014, Teschke *et al.*, 2017). RUCAM criteria include time to onset, course of response to treatment discontinuation, risk factors, concomitant medications, competing causes of liver injury, history of liver injury from the medication, and response to re-challenge. Each criterion is assigned to a score corresponding to probability of involvement of the medication being evaluated, and the causal relationship will be explained by the aggregate sum (Teschke *et al.*, 2017). The mainstay for the treatment of DILI is prompt removal of the offending agent, and clinical care ranging from general supportive therapy to intensive treatment and monitoring (Hassan and Fontana, 2019, Stine and Lewis, 2016). In rare instances, liver transplantation can be life saving (Biolato *et al.*, 2017).

1.3.1. Mechanisms of drug-induced liver injury

The underlying mechanisms of DILI are believed to be complex and not yet fully understood (Tailor *et al.*, 2015). The currently accepted mechanistic concept of DILI involves direct mitochondrial impairment, liver injury by direct cell stress or specific immune reactions with subsequent downstream events leading to mitochondrial permeability transition (MPT) resulting in apoptotic or necrotic cell death (Russmann *et al.*, 2009). The three step mechanistic model for DILI is shown in Figure 5.

First, the parent drug or its reactive metabolite(s) can exert initial injury through a wide range of mechanisms including depletion of glutathione, intracellular accumulation of substrates, binding to macromolecules, ER stress, or mitochondrial dysfunction (Pessayre *et al.*, 2010, Vuda and Kamath, 2016). The major mechanisms of mitochondrial dysfunction are over production of ROS, impairing mitochondrial respiratory chain, mitochondrial DNA damage or interfering its replication, inhibition of β-oxidation of fatty acids, or directly causing MPT pores in the mitochondrial inner membrane (Pessayre *et al.*, 2012, Pessayre *et al.*, 2010, Vuda and Kamath, 2016). Hepatotoxic drugs have specific initial mechanism of injury; however, a single drug may concomitantly act through several initial mechanisms and for many drugs some of the involved mechanisms currently remain unknown (Russmann *et al.*, 2009).

Second, if the initial mechanism does not directly impair mitochondrial function, mitochondrial dysfunction occurs in two principal ways: either via an intrinsic pathway initiated by severe intracellular stress or via an extrinsic death receptor-mediated pathway triggered by specific immune reaction (Iorga *et al.*, 2017, Malhi and Gores, 2008)**.**

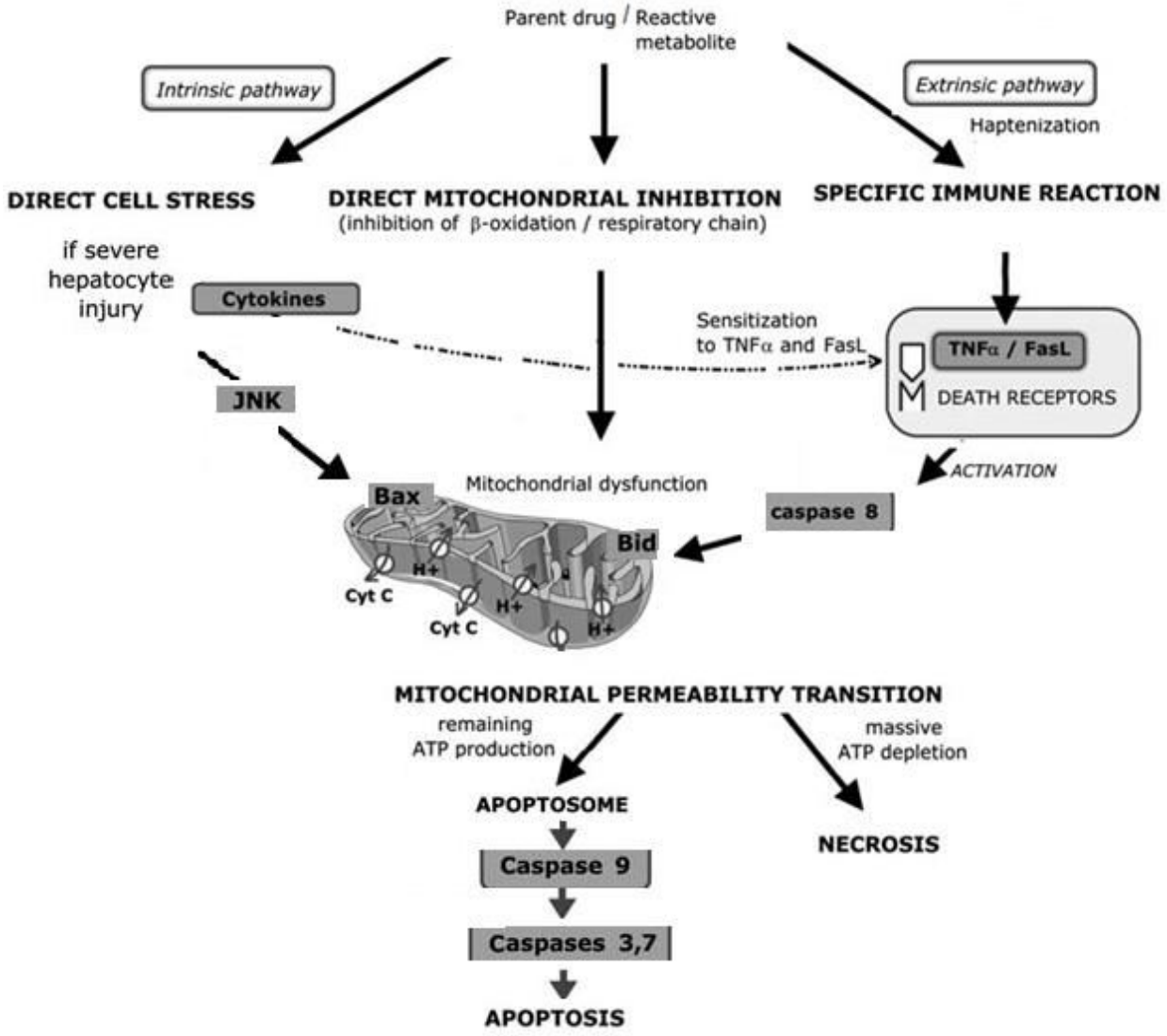

Figure 5. Mechanistic model of DILI. Adopted from (Russmann *et al.*, 2009).

Bid and Bax - Proapoptotic proteins; Cyt *C* - Cytochrome *C*; DILI - Drug induced liver injury; FasL - Fas ligand; JNK - c-Jun N-terminal kinase; TNFα - Tumor necrosis factor alpha

In the intrinsic pathway, severe intracellular stress activates ER stress, lysosomal permeabilization, or c-jun N-terminal kinase (JNK) activity (Russmann *et al.*, 2009). Subsequent activation of proapoptotic proteins Bax/Bak form pores on mitochondria inducing mitochondrial outer membrane permeabilization (MOMP). The extrinsic pathway is activated when the initial event is a specific immune reaction where human leukocyte antigen (HLA)-dependent antigen presentation will activate the release of tumor necrosis factor alpha (TNFα) and Fas ligand (FasL) from Kupffer cells and cytotoxic T-cells (Russmann *et al.*, 2009). Then, TNFα and FasL bind to intracellular death receptors on the hepatocytes leading to formation of death-inducing signaling complex (DISC) with initiator caspase-8 activation, subsequent proapoptotic protein Bid cleavage and activation inducing MOMP (Iorga *et al.*, 2017, Malhi and Gores, 2008).

Third, MPT allows massive influx of protons which stops mitochondrial ATP synthesis (Vuda and Kamath, 2016). The MOMP leads to release of Cytochrome *C* and other pro-apoptotic mitochondrial proteins into the cytosol (Iorga *et al.*, 2017). Impaired mitochondrial function and energy production leads to apoptotic or necrotic cell death (Shehu *et al.*, 2017). In the case of apoptosis, Cytochrome *C* combines with apoptotic protease-activating factor (Apaf-1) and caspase-9, forming an apoptosome. This activates caspase-9 which in turn activates effector caspases (caspase-3 and -7) resulting in programmed cell death characterized by cytoplasmic and nuclear condensation and fragmentation without loss of membrane integrity (Iorga *et al.*, 2017). If the initial injury is so severe that MPT quickly occurs in all mitochondria, or if rapid and severe mitochondrial ATP depletion occurs, it will result in necrosis characterized by cell swelling and lysis (Shehu *et al.*, 2017). Necrotic cell lysis induces inflammatory responses including release of cytokines that may amplify the initial injury (Russmann *et al.*, 2009).

1.3.2. ATD-induced liver injury

Anti-tubercular drugs (ATD) are the most common drugs underlying idiosyncratic DILI in the developing world (Huang, 2014, Ramappa and Aithal, 2013). ATD-induced liver injury is a major adverse effect of ATD treatment (Bao *et al.*, 2018). The reported incidence rate of ATD-induced liver injury among patients treated with the first-line combination ATD treatment varied from 2-28% (Richardson *et al.*, 2019, Tostmann *et al.*, 2008). ATD-induced liver injury can be fatal, with reported mortality rates of 5-23% due to acute liver failure if the drugs are not promptly stopped (Devarbhavi *et al.*, 2013, Kumar *et al.*, 2010).

In a study done in south-west Ethiopia to determine mortality attributable to adverse drug reactions (ADRs) and to identify the drugs implicated in ADR-related mortality in patients presenting to a hospital, the primary suspected cause of death was DILI, and first-line ATD and ARV drugs were the commonly implicated drugs (Angamo *et al.*, 2018). In studies done on Ethiopian adult TB patients, ATD-induced liver injury was observed in 4-17% of the patients (Abera *et al.*, 2016, Hassen Ali *et al.*, 2013, Yimer *et al.*, 2008).

Among the first-line ATD, INH, RIF and PZA are known to cause DILI that ranges from mild to severe forms (Huang, 2014). The mechanism of INH-induced liver injury could be due to accumulation of toxic intermediates from its metabolism (Tostmann *et al.*, 2008). In the hepatocytes, INH is first acetylated into acetylisoniazid via NAT2 and then hydrolyzed into monoacetylhydrazine (MAH) (Figure 6). MAH could be oxidized by CYP2E1 into hepatotoxic reactive metabolites, which could bind covalently with macromolecules resulting in liver injury (Gumbo, 2018). INH also undergoes hydrolysis and forms hepatotoxic hydrazine, which is then

metabolized to MAH by NAT2 (Huang, 2014). NAT2 is also responsible for further acetylation of MAH to non-toxic diacetylhydrazine (Huang, 2014).

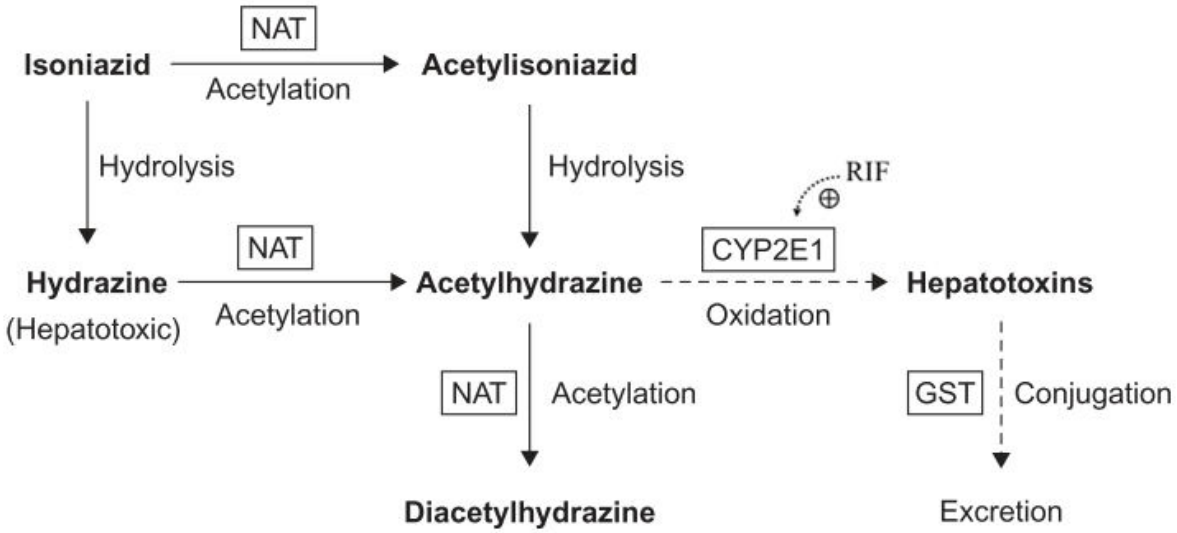

Figure 6. The metabolism of isoniazid in the liver. Adopted from (Huang, 2007).
CYP2E1 - Cytochrome P450 2E1; GST - Glutathione S-transferase; NAT - N-acetyltransferase; RIF - Rifampicin

Glutathione is an important antioxidant that protects against oxidative damage by providing a thiol (-SH) group for conjugation with the reactive metabolites (Ramappa and Aithal, 2013). Depletion of glutathione from cellular storage or deficiency of GST activity because of polymorphism may promote formation of protein adducts in the mitochondria increasing the risk of INH-induced liver injury (Yew *et al.*, 2018). In susceptible individuals, INH itself could be directly bioactivated to a reactive metabolite that could be converted to immunogens by covalently binding to endogenous proteins to initiate immune response and liver injury (Metushi *et al.*, 2016, Metushi *et al.*, 2014). RIF is potent inducer of hepatic enzymes, and induces INH metabolism resulting in increased hydrazine production and CYP2E1 activity which may explain increased risk of liver toxicity of the combined use of RIF and INH (Tostmann *et al.*, 2008).

ATD could also cause toxicity to hepatocytes due to accumulation of drugs, toxic metabolites or endogenous compounds by altering activity of transporter proteins (Corsini and Bortolini, 2013). For example, RIF interferes with BSEP (Guo *et al.*, 2015), and down-regulates expression of MRP2 (Chen *et al.*, 2015b). As BSEP is important for bile acid transport, and MRP2 is responsible for the transport of conjugated bilirubin through canalicular membrane, the inhibitory action of RIF may disrupt normal transport of these endogenous molecules leading to cholestasis and liver injury (Chen *et al.*, 2015b, Qu *et al.*, 2018). The abnormal accumulation of bile acids in the hepatocytes not only induces changes in gene expression but also induces damage on cellular membranes, mitochondrial dysfunction and cellular death due to its detergent nature (Dietrich and Geier, 2014, Padda *et al.*, 2011, Yang *et al.*, 2013).

PZA is a widely used ATD; however, hepatotoxicity is a life-threatening adverse effect reported in some patients (Yew *et al.*, 2018). In a case-control study, the odds ratio of hepatotoxicity for continuation-phase regimen incorporating PZA relative to the standard regimens was shown to be about three fold (Chang *et al.*, 2008). Although the exact mechanism by which PZA induces hepatotoxicity is not clear yet, animal model-based studies showed that the drug increased ROS generation; and changes in the activities of major antioxidant enzymes such as superoxide dismutase (SOD) and antioxidant molecules such as glutathione were found significantly associated with PZA-induced liver injury (Taziki *et al.*, 2018, Zhang *et al.*, 2013b). These findings could indicate a state of increased oxidative stress in the pathogenesis of PZA-induced liver injury. Researchers also identified that the metabolite 5-hydroxypyrazinoic acid is responsible for PZA-induced hepatotoxicity (Shih *et al.*, 2013). Thus, drug metabolizing enzymes, drug transporters, antioxidant response and immune mediated reactions may play roles in ATD-induced liver injury.

Although factors such as age, gender, malnutrition, alcohol consumption and hepatitis B and C viral co-infections contribute towards the development of ATD-induced liver injury, genetic variations are also known to have significant contribution (Bao *et al.*, 2018). Genetic polymorphisms in genes for drug metabolizing enzymes: *NAT2* (Huang, 2014, Khan *et al.*, 2019, Richardson *et al.*, 2019, Wattanapokayakit *et al.*, 2016, Yang *et al.*, 2019, Zhang *et al.*, 2018), *CYP2E1* (Cai *et al.*, 2012, Deng *et al.*, 2012, Lee *et al.*, 2010, Yang *et al.*, 2019), *GSTM1* (Feng *et al.*, 2014, Lucena *et al.*, 2008), *UGT1A1* (Chang *et al.*, 2012, Tao *et al.*, 2018) and *UGT2B7* (Sun *et al.*, 2019); transporter protein genes: *ABCB11* (Chen *et al.*, 2016), *SLCO1B1* (Chen *et al.*, 2015a)); immune response related genes such as HLA (Kim and Naisbitt, 2016, Perwitasari *et al.*, 2018, Sharma *et al.*, 2002), and antioxidant response genes such as *SOD2* and glutathione peroxidase (Bao *et al.*, 2018, Huang *et al.*, 2007, Kim *et al.*, 2015, Lucena *et al.*, 2010, Nanashima *et al.*, 2012, Yew *et al.*, 2018) have been suggested to play roles in predisposing to ATD-induced liver injury.

1.3.3. ART-induced liver injury

The use of combined anti-retroviral therapy (ART) for HIV infection has resulted in marked suppression of viral replication and reduced morbidity and mortality (UNAIDS, 2018). First-line ART regimens preferred for treatment-naïve adult HIV patients consists of a combination of two NRTIs, and EFV or DTG (Apostolova *et al.*, 2017, FMoH, 2017, WHO, 2016). However, it is not uncommon for patients to discontinue or change treatment due to serious ADRs (Lubomirov *et al.*, 2011). One of the most common ADRs leading to HIV treatment interruption is liver toxicity (Jones and Nunez, 2012, Singh *et al.*, 2019b), with up to 30% of patients experience on EFV-based ART (Pugh *et al.*, 2009). ART-induced liver injury can lead to adverse patient outcomes either from acute liver failure or AIDS following discontinuation of treatment

(Segamwenge and Bernard, 2018). In studies done on Ethiopian HIV patients, EFV-based ART-induced liver injury was observed in 8-16% of the patients (Yimer *et al.*, 2012, Yimer *et al.*, 2014). Concomitant use of ARV and ATD increases the risk of developing DILI (Puri *et al.*, 2017). In studies done on TB/HIV co-infected Ethiopian adult patients on EFV-based ART and first line ATD co-treatment, DILI was observed in 18-24% of the patients (Yimer *et al.*, 2014, Yimer *et al.*, 2011).

Molecular mechanisms responsible for EFV-based ART-induced liver toxicity remain unknown, but reports have highlighted features of mitochondrial dysfunction (Ezhilarasan *et al.*, 2017, Pessayre *et al.*, 2012). EFV may inhibit mitochondrial complex-I resulting in a decrease in ATP production, an increase in ROS generation, metabolic alterations, induction of oxidative stress, triggering MOMP and apoptosis (Apostolova *et al.*, 2017, Pessayre *et al.*, 2012). Apostolova *et al.* reported that EFV induces endoplasmic reticulum (ER) stress in hepatocytes (Apostolova *et al.*, 2013). EFV also inhibits bile acid transport resulting in cholestasis and liver injury (Ezhilarasan *et al.*, 2017). While structural differences contribute to varying degree of mitochondrial dysfunction, NRTIs generally inhibit mitochondrial DNA polymerase-γ, leading to impaired mitochondrial respiration, ATP depletion, and subsequent downstream effects that results in liver toxicity (Ezhilarasan *et al.*, 2017, Vuda and Kamath, 2016).

The bases of individual susceptibility to ART-induced liver injury are multi-factorial, and genetic variation is believed to play an important role (Lubomirov *et al.*, 2011, Mahungu *et al.*, 2009). Genetic polymorphisms in genes for drug metabolizing enzymes such as *CYP2B6* (Yimer *et al.*, 2012) and in genes for drug transporter proteins such as *ABCB1* (Ritchie *et al.*, 2006, Yimer *et al.*, 2011) have been suggested to play roles in predisposing to EFV-based ART-induced liver injury.

1.3.4. HLA and ATD/ART-induced liver injury

Human leukocyte antigens (HLAs) are complex of genes located on chromosome-6 and encode Major Histocompatibility Complex (MHC) proteins that play integral role in the immune system (Mosaad, 2015). HLA genes are classified into MHC class I, II, and III. MHC class I include the classic genes HLA-A, HLA-B, and HLA-C; class II genes contain HLA-DP, HLA-DQ and HLA-DR, and class III consists of genes for complement components (Sukasem *et al.*, 2014).

MHC class I and class II loci encode distinct highly polymorphic cell surface molecules whose function is to capture and display various antigenic peptides to T-lymphocytes, and play a key role in the induction and regulation of immune responses (Mosaad, 2015). The HLA molecules present peptides to T-cell receptors (TCRs) on CD8+ cytotoxic T-cells or CD4+ T-helper cells leading to immune mechanisms that destroy target cells (Kaliyaperumal *et al.*, 2018, Neefjes *et al.*, 2011). The peptide-binding region of HLA molecules is highly polymorphic, and allelic differences among the genes are often due to non-synonymous substitutions encoding amino acid differences within this region (Neefjes *et al.*, 2011).

Genetic variations in HLA genes are implicated with susceptibility to immune mediated ADRs to a wide range of drugs, making them candidate genes relevant to pharmacogenetic studies (Barbarino *et al.*, 2015, Driessche and Fourches, 2017). For example, *HLA-B*57:01* allele carriers were found to be at a higher risk of developing abacavir-induced hypersensitivity reaction, and genetic screening for this allele and subsequent treatment modifications have been shown to reduce incidence of life-threatening drug toxicity, and led to a recommendation of routine screening before treatment initiation (Mallal *et al.*, 2008).

HLA-mediated immunological reactions are also implicated in DILI (Daly, 2010a). HLA alleles reported to have association with the risk of idiosyncratic DILI include *HLA-DQB1*02:01* and *HLA-DQB1*05* with ATD-induced liver injury (Chen *et al.*, 2015c, Sharma *et al.*, 2002), *HLA-B*58:01* and *HLA-DRB1*01:02* with nevirapine-containing ART-induced liver injury (Phillips *et al.*, 2013). The HLA alleles were also reported to have association with the risk of developing idiosyncratic DILI to some other drugs including *HLA-B*57:01* with flucloxacillin (Phillips and Mallal, 2013) and *HLA-B*44:03* with ticlopidine (Hirata *et al.*, 2008)-induced liver injuries, among others.

Several models have been proposed to explain drug-induced T-cell activation in idiosyncratic DILI. The main models are Hapten hypothesis, pharmacological interaction (pi) concept, and altered repertoire model (Grove and Aithal, 2015, Mak and Uetrecht, 2017, Mosedale and Watkins, 2017). Hapten hypothesis states that an individual's susceptibility to DILI is determined by the covalent binding of a drug or its metabolites to a cellular protein and the interaction of the resultant complex with the peptide-binding groove of a specific HLA molecule (Mak and Uetrecht, 2017). Alternatively, the pi concept proposes that a drug or its metabolite can directly bind to a specific HLA molecule or T-cell receptor to trigger T-cell activation, leading to immune-mediated reaction (Grove and Aithal, 2015). Recently, altered repertoire model has been proposed (Mosedale and Watkins, 2017), drugs may non-covalently occupy sites within the peptide binding groove of a specific HLA molecule and change the shape and chemistry of the peptide-binding cleft (alter specificities). This results in presentation of self-peptide that are different to those normally bind, which in turn leads to an adaptive immune response (Grove and Aithal, 2015, Mosedale and Watkins, 2017).

Various methods are used for determining whether an individual carries a particular HLA allele. These strategies include serological testing and polymerase chain reaction (PCR)-based molecular methods such as sequence-specific primer (SSP)-PCR and direct DNA sequencing methods (Erlich *et al.*, 2001). Serological testing for HLA antigens, which uses antibodies of known specificity, has long been used. However, unavailability of certain antibodies and problems of cross-reactivity have led to the use of DNA based methods (Erlich *et al.*, 2001). SSP-PCR method employs pairs of PCR primers complementary to a polymorphic position that distinguishes specific alleles (Martin *et al.*, 2005). If an individual possesses the allele of interest, the PCR will lead to a product whose presence and size is typically identified by agarose gel electrophoresis. The HLA typing can be performed by choosing many pairs of primers in independent reactions to cover all allele groups. The SSP-PCR method is highly sensitive and specific, and thus commonly used method that offers effective screening for HLA alleles (Martin *et al.*, 2005, Martin *et al.*, 2012).

1.4. Candidate gene association studies

The human genome is a complete set of nucleic acid sequences encoded as DNA within 23 pairs of chromosomes: 22-pairs of autosomal and 1-pair of sex chromosomes. DNA is a long sequence of nucleotides consists of two strands that form double helix carrying genetic information. Each nucleotide is composed of a deoxyribose sugar, a phosphate group and a nitrogenous base adenine (A), thiamine (T), cytosine (C) or guanine (G). The nucleotides are joined to one another in a chain by covalent bonds between the sugar of one nucleotide and the phosphate of the next, resulting in an alternating sugar-phosphate backbone. The nitrogenous bases of the two strands are bound together, according to base pairing rules (A with T, and C with G), with hydrogen bonds to make the double-stranded DNA.

An estimated 3.2 billion base pairs exist in the human genome (Kwok and Chen, 2003). Although the vast proportions of DNA sequences are the same among individuals, the remaining portions of the sequences make us all unique (Speliotes, 2015). Single nucleotide polymorphism (SNP) is one of the most abundant forms of genetic variations that refer to a single nucleotide difference in the DNA sequence (Speliotes, 2015). Basically, a SNP exists in two variants (alleles) with a frequency of at least 1% in the human population (Schork *et al.*, 2000). Since SNPs are the most abundant form of genetic variations and relatively easier to type, they are often used as markers in genetic association studies (Bush and Moore, 2012).

Approaches to discover population-based association between genetic variants such as SNPs and common diseases or complex traits fall into two categories: candidate-gene and genome-wide approaches (Zondervan and Cardon, 2007). Until recently, genetic risk variants for diseases were primarily investigated through candidate gene association studies (CGAS) (Patnala *et al.*, 2013). In such studies, DNA samples from cases and controls will be genotyped for SNPs in a particular gene for which prior knowledge suggested a role in the pathogenesis of the disease of interest or has functional relevance (Hirschhorn *et al.*, 2002). For example, various CGAS have identified susceptible genetic variants for idiosyncratic DILI linked to pharmacokinetics, oxidative stress and immune system (Daly and Day, 2012, Russmann *et al.*, 2010, Urban *et al.*, 2014).

Although CGAS have been able to identify genetic risk factors that are known to contribute to susceptibility for common diseases and pharmacogenetic traits, such studies have a number of limitations (Daly and Day, 2001, Daly and Day, 2009, Russmann *et al.*, 2010). The major limitation is that the selection of candidate genes is based on the current understanding of disease pathogenesis, and genetic variants that are related to unknown mechanisms, which might have relevance to disease susceptibility, will not be detected (Patnala *et al.*, 2013, Russmann *et al.*,

2010). Another limitation is that, genetic polymorphisms chosen for investigations were often selected in a rather arbitrary way thus lack comprehensiveness (Daly and Day, 2009). Many CGAS also relied on the analysis of limited number of SNPs and did not consider regions that regulate gene expression (Russmann *et al.*, 2010). In addition, inconsistent results were reported in a number of CGAS (Chatterjee *et al.*, 2010, Singh *et al.*, 2019a, Tang *et al.*, 2013, Yadav *et al.*, 2019). In this sense, the other population based association study approach may need to be considered to understand genetic variants associated with common diseases or complex diseases.

1.5. Genome-wide association studies

Genome-wide association studies (GWAS) are genetic association studies in which dense array of genetic markers usually SNPs that capture substantial proportion of common variations in the genome sequence are typed in a set of DNA samples that are informative for a trait of interest (McCarthy *et al.*, 2008). The goal of GWAS is to identify DNA sequence variants that affect risk to a disease or response to drug treatment through detection of association between allele or genotype frequencies and the trait status (Awany *et al.*, 2019, Dehghan, 2018). GWAS techniques have evolved over the last decade into a powerful tool for investigating the genetic risk factors for human diseases (Bush and Moore, 2012). It is increasingly being applied in the area of pharmacogenomics (Daly, 2010b).

In contrast to CGAS, GWAS investigate the possible association of genetic variations in the entire human genome (Daly, 2012, Karlsen *et al.*, 2010). Therefore, GWAS represents comprehensive and unbiased scan of the genome even in the absence of evidence regarding the function or location of the causal genes (Daly, 2012, Grant and Hakonarson, 2007, Hirschhorn and Daly, 2005). In contrast to candidate gene approach, which is limited to the current

knowledge of pathogenesis of diseases, GWAS approach allow the identification of novel susceptibility variants that may provide better biological understanding of the phenotypes (Motsinger-Reif *et al.*, 2013). GWAS enable to elucidate the involvement of multiple genes or previously unrecognized biological pathways in disease development (Manolio, 2013). These studies are particularly suitable for simultaneous identification of several risk variants in a single study, and thus they are relevant for complex traits where multiple risk variants could contribute to the disease (Russmann *et al.*, 2010).

In 2005, the first GWAS was conducted on age-related macular degeneration which identified an association between complement factor-H and the risk of developing the disease (Klein *et al.*, 2005). Subsequently, GWAS of multiple diseases carried out by the Wellcome Trust Case-Control Consortium began the era of large-scale GWAS (WTCCC, 2007). GWAS is then increasingly being popular approach for identifying genetic variants influencing common diseases and complex traits (Amos, 2007, Marees *et al.*, 2018). According to the recent summary of GWAS catalog, there are hundreds of unique SNP-trait associations reported for many human diseases (Buniello *et al.*, 2019).

The availability of comprehensive data on human genetic variation from Human Genome project (Austin, 2004) and International Haplotype Mapping (HapMap) Project (International-HapMap-Project, 2003) together with high-throughput genotyping technologies that allow large numbers of SNPs to be genotyped simultaneously with very low error rates (Amos, 2007, Perkel, 2008), and the decline of cost per-SNP genotyping (Perkel, 2008) have made GWAS technically possible. GWAS are becoming increasingly feasible to conduct large-scale studies, presenting the opportunity for the discovery of genetic variations (Awany *et al.*, 2019).

The HapMap Project genotyped millions of SNPs on samples representing European, African, and Asian populations and characterize the patterns of linkage disequilibrium (LD) across the genome (International-HapMap-Consortium, 2005). SNPs in the genome have groups of neighbors that are nearly in correlation with each other (Gabriel *et al.*, 2002). Once the patterns of LD are known for a given region of the genome, a minimal set of correctly chosen variants (tag SNPs) can thereby serve as proxy for many others and provide adequate information about most of the common variations within the genomic region (Hirschhorn and Daly, 2005).

GWAS make use of microarray chip technology that can process up to a million of SNPs (Lambert *et al.*, 2013). The two main supplier companies of the chips are Illumina (San Diego, California (CA), USA) and Affymetrix (Santa Clara, CA, USA) (Bush and Moore, 2012, Distefano and Taverna, 2011). A key assumption in GWAS is the 'common-disease common-variant (CDCV) hypothesis' (Wang *et al.*, 2005). It states that common disorders are likely influenced by genetic variations that are common in the population (minor allele frequency (MAF) > 5%), and GWAS approach is well powered to detect common variants with modest effect (Wang *et al.*, 2005).

The most commonly used GWAS design to date has been the case-control study design in which allele frequencies in individuals with the disease of interest are compared with those in a disease-free group (Pearson and Manolio, 2008). A typical GWAS has the following steps: (i) careful selection of cases and controls from the population, (ii) DNA isolation, SNP genotyping, and quality control measures, (iii) appropriate statistical tests to identify differences in allele and genotype frequencies between cases and controls for the SNPs that passed the quality filter, (iv) careful interpretation of the results, and then replication of the discovery GWAS findings with an independent set of cases and controls (Pearson and Manolio, 2008).

In GWAS systematic stepwise standard quality control filtering processes are performed on the samples as well as on the raw genotype data (Anderson *et al.*, 2010, Ellingson and Fardo, 2016, Marees *et al.*, 2018, Turner *et al.*, 2011, Zeng *et al.*, 2015). These processes include:-

- Checking for sample identity: checking reported sex of individuals against predicted sex by the genetic data is one way to discover sample mix-up (Anderson *et al.*, 2010).
- Missing call rate: Both the fraction of missing calls per SNP over samples, and the fraction per sample over SNPs could show incompleteness of the data. Low genotype calling may be indicative of a poor-quality sample (Turner *et al.*, 2011).
- Sample relatedness: If study subjects are closely related then their genotype will be correlated, and thus statistical tests of association will be violated (Purcell *et al.*, 2007, Turner *et al.*, 2011). The degree of relatedness between every pair of individuals could be identified using identity-by-state (IBS) (Anderson *et al.*, 2010).
- Population stratification (PS): PS is allele frequency difference between cases and controls related to difference in genetic ancestry (Price *et al.*, 2006). It is a major confounder for GWAS in heterogeneous population (Turner *et al.*, 2011). If undetected and not accounted during analysis, PS could lead to spurious association (Price *et al.*, 2006). Therefore, accounting for PS can reduce confounding effect of genetic heterogeneity, and increase power for finding associations. One of the steps towards reducing PS is careful selecting cases and control samples from the same population (Zeng *et al.*, 2015). In addition, methods such as genomic control (GC) and principal component analysis (PCA) to detect and address the problem of PS could be used (Dehghan, 2018, Price *et al.*, 2006, Zeng *et al.*, 2015).
- Minor allele frequency (MAF): SNPs with low MAF (< 1%) decrease statistical power and inflate false positive rate, and thus usually filtered out in GWAS (Ziegler, 2009).

- Hardy-Weinberg equilibrium (HWE): HWE describes that genotype and allele frequencies are expected to remain constant in randomly mating population (Marees *et al.*, 2018, Zeng *et al.*, 2015). When there is a systematic genotyping error, this distribution will be distorted (Marees *et al.*, 2018). Thus, SNPs that deviate from expected HWE usually discarded.

Since GWAS involve testing of multiple markers, large test statistics (small *P*-values) are expected just by chance along with the genuine disease associated alleles (Hirschhorn and Daly, 2005, Karlsen *et al.*, 2010); therefore, appropriate statistical considerations are essential. The most widely applied approach is correcting *P*-values by Bonferroni correction, i.e., the threshold for genome-wide significance computed by dividing the level of significance ($\alpha = 0.05$) with the number of marker SNPs tested (Bland and Altman, 1995, Karlsen *et al.*, 2010, Teo, 2008).

The commonly used strategy for identifying genetic risk variants involve multi-stage testing for associations whereby the initial GWAS findings will be confirmed in a subsequent replication study in order to reduce false positive results (Chanock *et al.*, 2007, Kwak *et al.*, 2009, McCarthy *et al.*, 2008). The replication study helps determine which of the findings arising from the initial GWAS reflect true reproducible associations (McCarthy *et al.*, 2008). Replication of positive findings from genetic association studies in an independent population has served as the gold standard for establishing validity of observed genotype-phenotype associations (Chanock *et al.*, 2007, Jiang and Yu, 2016). This is because repeated observations add convincing statistical evidence, and can provide more accurate estimate of the effect measures of the genetic risk variants being studied (Bush and Moore, 2012, Konig, 2011). The recommended strategy for analyzing such multistage data is to separately and jointly analyze the data from the discovery GWAS and the replication study (Skol *et al.*, 2009), and such strategy increase power to detect genetic associations (Kwak *et al.*, 2009, Skol *et al.*, 2009).

Replication study is carried out for the top genetic markers identified in the initial GWAS using a different genotyping method such as invader or Taqman assay (Kim and Misra, 2007, Koch, 2004). This will minimize the chance of false positive associations that arise as a result of technical genotyping artifacts (Hirschhorn and Daly, 2005). Replication of an association in the same population either in a split samples or repeat independent samples give evidence in favor of the variant being associated with the phenotype (Kraft *et al.*, 2009). Credibility increases when multiple investigators find the same association in independent samples (McCarthy *et al.*, 2008).

Invader assay is one of the commonly used SNP genotyping techniques in replication studies (Koch, 2004). This assay uses invader and allele-specific (primary) probes for each SNP, fluorescence resonance energy transfer (FRET) cassette probes mix and Cleavase (Olivier, 2005). As illustrated in Figure 7, the invader probe is complementary to target sequence 3' of the SNP site and ends with a non-matching base overlapping the SNP nucleotide. The allele-specific probes contain complementary base of the SNP allele and extend to the sequence 5' of the SNP site with flap sequence which is not complementary to target sequence (FAM-flap and VIC-flap).

When the invader and complementary allele-specific probe anneal to the target DNA, they form a three-dimensional invader structure over the SNP site that can be recognized by Cleavase, a structure-specific 5'-flap endonuclease (Koch, 2004). The enzyme cleaves the probe 3' of the base complementary to the polymorphic site. The 5'-flap is released by cleavage and hybridizes to one of the two FRET cassettes in a secondary reaction, thereby creating another Cleavase substrate. Cleavage of the secondary substrate separates the fluorophore (F) from the internal quencher (Q) molecule, and generates measurable fluorescent signal. In contrast, if the allele specific probe does not match the SNP allele present in the target DNA, then no overlapping invader structure is formed, and the probe will not be cleaved (Koch, 2004, Olivier, 2005).

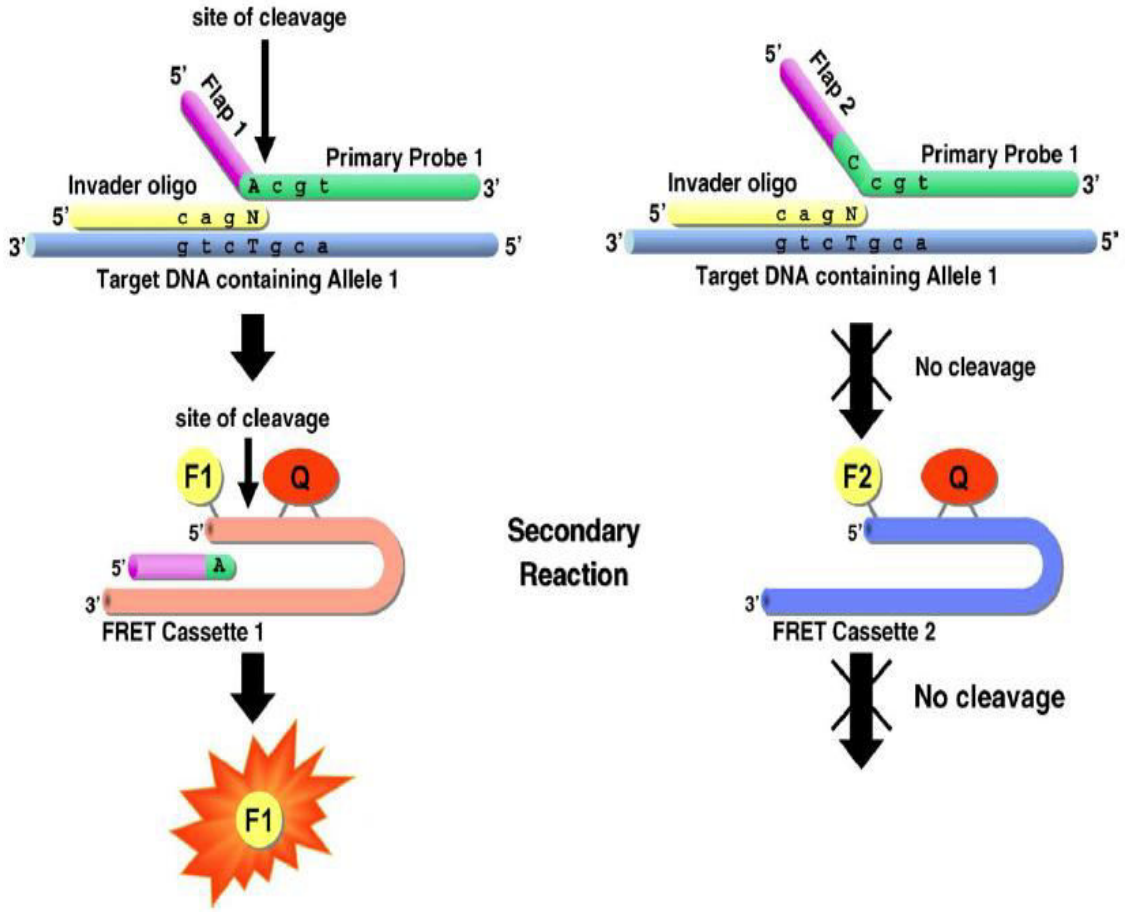

Figure 7. Schematic representation of Invader assay. Adopted from (Olivier, 2005).

F - Fluorophore; FRET - Fluorescence resonance energy transfer; N - Non matching base; Q - Quencher

1.5.1. GWAS for drug-induced liver injury

One of the successful applications of GWAS has been in the area of pharmacogenomics (Bush and Moore, 2012), the goal of which is identifying DNA sequence variations that are associated with drug response (Stankov *et al.*, 2013). GWAS could allow discovery of previously unknown genetic risk variants that influence pathogenesis of serious ADRs (Motsinger-Reif *et al.*, 2013).

To date, a few GWAS (Daly *et al.*, 2009, Lucena *et al.*, 2011, Nicoletti *et al.*, 2017, Parham *et al.*, 2016, Singer *et al.*, 2010, Suvichapanich *et al.*, 2019, Urban *et al.*, 2012) have investigated genetic factors that could explain variations in the risk of developing idiosyncratic DILI. However, the identified genetic variants had substantially higher effect sizes compared to GWAS involving the majority of other complex diseases (Aithal and Grove, 2015). The associated markers of these GWAS for DILI are summarized in Table 1.

Daly *et al.* reported the earliest GWAS for flucloxacillin-induced liver injury (Daly *et al.*, 2009). A strong association was identified with a reference SNP (rs) 2395029, which is in LD with *HLA-B*57:01* and it was replicated in an independent cohort. Subsequent GWAS for lumiracoxib (Singer *et al.*, 2010), Amoxicillin-clavulanate (Lucena *et al.*, 2011) and lapatinib (Parham *et al.*, 2016)-induced liver injuries identified strong associations with SNPs in LD with *HLA-DRB1*15:01, HLA-DQB1*06:02* and *HLA-DRB1*07:01*, respectively, indicating the important role of HLA variants in DILI. These findings were consistent with earlier CGAS reported (Hautekeete *et al.*, 1999, Schaid *et al.*, 2014), and also replicated using high resolution HLA typing (Lucena *et al.*, 2011, Singer *et al.*, 2010). The GWAS finding for lumiracoxib implicates the potential to improve safety profile of the drug by identifying individuals at high risk, and may be considered as a possible means of re-introducing the drug to market (Daly, 2012).

Table 1. GWAS identified genetic variants associated with idiosyncratic DILI

Drug	Cases/Controls		SNP	Gene/allele	*P*	OR	Reference
	GWAS	Replication					
Flucloxacillin	51/282		rs2395029	*HLA-B*57:01*	$8.7x10^{-33}$	45.0	(Daly *et al.*, 2009)
			rs10937275	*ST6GAL1*	$1.4x10^{-8}$	4.1	
		23/64	rs2395029	*HLA-B*57:01*	$9.0x10^{-19}$	80.6	
Lumiracoxib	41/176		rs9270986	*HLA-DRB1*	$2.8x10^{-10}$	5.3	(Singer *et al.*, 2010)
		137/577	HLA typing	*HLA-DRB1*15:01*	$6.8x10^{-25}$	7.5	
Amoxicillin-Clavulanate	201/532		rs9274407	*HLA-DQB1*06:02*	$4.8x10^{-14}$	3.1	(Lucena *et al.*, 2011)
		177/219	HLA typing	*HLA-DQB1*06:02*	$4.6x10^{-10}$	4.2	
Lapatinib	34/810		NR	*HLA-DRB1*07:01*	$7.8x10^{-11}$	NR	(Parham *et al.*, 2016)
			rs7828135	*TPD52*	$4.5x10^{-8}$	NR	
Multiple drugs	783/3,001		rs35709459	*PCDH11X*	$2.0x10^{-7}$	NR	(Urban *et al.*, 2012)
Diclofenac	30 cases		rs17036170	*PPARG*	$1.0x10^{-8}$	11.3	
Multiple drugs	862/10,588		rs114577328	*HLA-A*33:01*	$2.4x10^{-8}$	2.7	(Nicoletti *et al.*, 2017)
			rs72631567	Intergenic SNP	9.7×10^{-9}	2.0	
Terbinafine	14 cases		rs114577328	*HLA-A*33:01*	$6.7x10^{-10}$	40.5	
Anti-tuberculosis drugs	79/239		rs1495741	Intergenic SNP	$6.9x10^{-11}$	6.0	(Suvichapanich *et al.*, 2019)
			rs2756263	*PRND*	$6.9x10^{-6}$	4.5	

DILI - Drug-induced liver injury; GWAS - Genome-wide association studies; HLA - Human leukocyte antigen; NR - Not reported; OR - Odds ratio; *P* - The smallest *P*-values in the genome-wide and subsequent replication studies; *PCDH11X* - Protocadherin-11-X-linked; *PPARG* - Peroxisome proliferator-activated receptor gamma; *PRND* - Prion-like 243 protein Doppel; SNP - Single nucleotide polymorphism; *ST6GAL1* - ST6 β-galactosamide α-2,6-sialyltranferase-1; rs - Reference SNP; *TPD52* - Tumor protein D52; *TRPM2* - Transient receptor potential cation channel subfamily-M member-2

GWAS to identify genetic risk variants associated with DILI from various drugs was conducted to elucidate common mechanistic pathways underlying DILI (Urban *et al.*, 2012). Although the study did not find a significant association, it suggested that risk alleles may be drug specific. Among drug specific tests, analysis revealed a SNP significantly associated with diclofenac-induced liver injury (Urban *et al.*, 2012). By expanded the number of cases, the researchers conducted the largest GWAS of DILI genetics to date (Nicoletti *et al.*, 2017). The most significantly associated SNPs were rs114577328 in *HLA-A*33:01* and rs72631567 (an intergenic SNP) on chromosome-2. Breakdown by drugs showed strong association of the HLA allele signal with terbinafine-induced liver injury, providing further evidence for the role of adaptive immune system in DILI. The most recently conducted GWAS in DILI identified rs1495741 in an intergenic region closer to *NAT2* gene which was significantly associated with ATD-induced liver injury (Suvichapanich *et al.*, 2019).

2. Statement of the problem

DILI is one of the leading causes of acute liver failure (Colaci *et al.*, 2019, Lee, 2013). It is a well-recognized adverse effect of ATD and ART (Schutz *et al.*, 2012). In Ethiopia, ATD and ART-induced liver injuries are among those important problems associated with drug treatment for TB/HIV infections (Hirpa *et al.*, 2013, Yimer *et al.*, 2014). Liver injury due to ATD and/or ART carries numerous risks that include morbidity and mortality due to liver failure, disease progression as a result of discontinuation of optimal therapy, and drug resistance related to treatment interruption (Hirpa *et al.*, 2013, Lubomirov *et al.*, 2011, Tostmann *et al.*, 2008). Currently, there are no diagnostic tests available to predict risk of developing idiosyncratic DILI before initiating drug treatment (Bagheri *et al.*, 2000).

Although CGAS contribute to the discovery of genetic risk variants associated with ATD and ART-induced liver injury (Daly and Day, 2012, Mahungu *et al.*, 2009), the discovered variants account only for a small fraction of the risk variants (David and Howard, 2009). In addition, most of the studies focused on drug metabolizing enzymes and transporter proteins. Furthermore, some of the reported findings of the CGAS were inconsistent (Chatterjee *et al.*, 2010, Tang *et al.*, 2013). The polygenic nature of DILI (Andrade *et al.*, 2009) also limited the ability of CGAS approach to explain much of the inter-individual variations. Thus, CGAS approach is inadequate to fully explain genetic basis of ATD and ART-induced liver injury.

To our knowledge, genetic variants that contribute to ATD and ART-induced liver injury in Ethiopian patients has not been studied through GWAS, and also the impact of *HLA* gene variants on ATD and ART-induced liver injury have not yet been explored.

3. Significance of the study

GWAS represents a comprehensive and unbiased scan of the genome that allows identification of novel susceptibility variants for diseases (Scherer and Christensen, 2016). This approach is suitable for simultaneous identification of multiple risk variants in a single study (Russmann *et al.*, 2010). Genetic risk variants identified by GWAS and confirmed through replication studies can have impacts on clinical medicine through prediction of outcomes, or through elucidation of underlying biology (Manolio, 2013). Much of the immediate focus is on genetic testing that utilizes common variants as biomarkers to predict disease occurrence or progression, to monitor treatment response, or to avoid serious ADRs, and thus advance clinical care through personalized medicine (Barrett, 2019). The identification of additional common genetic variants helps describe new biological pathways and therapeutic targets, which can in turn provide clues for the development of novel preventive and treatment approaches. It is now possible and indeed the norm to conduct GWAS to find associations between disease phenotypes and genetic variants that may predispose to the diseases (Marees *et al.*, 2018).

As TB and HIV infections remain major problems in sub-Saharan Africa and treatment is scaled up, identification of genetic biomarkers that predict safety of ATD and ART is important to match patients with alternative, effective and safe medications. Identifying additional and strong genetic risk variants that contribute for the development of ATD and ART-induced liver injury help screen patients susceptible to DILI, individualize and optimize the treatments, minimize impact of liver injury associated with the use of these drugs and likely results in an improved outcome. Thus, the present study may provide nation-wide genomic information on the risk of developing ATD and ART-induced liver injury, and contribute to the advancement of clinical care through individualized medicine.

4. Objectives

4.1. General objective

- To identify and validate genetic risk variants associated with ATD and ART-induced liver injury through GWAS and replication studies, and also to investigate the association of *HLA-B* alleles with ATD and ART co-treatment induced liver injury in Ethiopian TB/HIV patients.

4.2. Specific objectives

- To identify genetic risk variants for ATD-induced liver injury, using GWAS
- To identify genetic risk variants for ART-induced liver injury, using GWAS
- To identify genetic risk variants for ATD and ART co-treatment induced liver injury, using GWAS
- To validate the genetic risk variants for ATD-induced liver injury, using replication study
- To validate the genetic risk variants for ART-induced liver injury, using replication study
- To validate the genetic risk variants for ATD and ART co-treatment induced liver injury, using replication study
- To identify *HLA-B* alleles associated with ATD and ART co-treatment induced liver injury

5. Materials and Methods

5.1. Study design, participants and drug treatment

The present study is a case-control study. The participants were obtained from a prospective cohort study conducted to determine the incidence and predictors of ATD and ART-induced liver injury in Ethiopia from June 2007 to June 2011. The patients were recruited from Kazanchis, Arada and Beletshachew Health Centers, and Tikur-Anbessa Specialized Hospital, Addis Ababa, Ethiopia. The eligibility criteria were TB and/or HIV-confirmed men and non-pregnant women of age ≥18 years. The study participants were grouped based on the disease types and drug treatments received as follow:

Group-I: TB infected patients treated with first-line ATD only. This group also included TB/HIV co-infected patients with baseline CD4 counts > 200 cells/μL (treated with ATD but not eligible for ART according to the National Treatment Guideline valid during the prospective cohort study period). DILI cases in this group also included TB/HIV co-infected patients on ATD that developed DILI before starting ART.

Group-II: HIV-positive patients with CD4 counts ≤ 200 cells/μL and without TB co-infection, treated with ART only.

Group-III: TB and HIV co-infected patients with CD4 counts ≤ 200 cells/μL and treated with both ATD and ART at the same time.

The drug treatments provided were according to the Ethiopian National TB Treatment Guideline (Yimer *et al.*, 2014). Briefly, all TB patients received daily fixed dose ATD treatment consisting of RIF (150 mg), INH (75 mg), PZA (400 mg) and EMB (275 mg) for the first two months

followed by RIF (150 mg) and INH (75 mg) for the next four months. The treatment dosage was based on the weight of the patients: 20-29 kg (1½ tablet), 30-37 kg (2 tablets), 38-54 kg (3 tablets) and ≥ 55 kg (4 tablets). HIV positive patients received ART consisting of efavirenz (EFV, 600 mg once daily) and lamivudine (3TC, 150 mg twice daily) along with stavudine (d4T, 30 mg twice daily) or zidovudine (ZDV, 300 mg twice daily) or tenofovir (TDF, 300 mg once daily). The patients were not on other known hepatotoxic drugs concurrently, except for co-trimoxazole 960 mg/day, which was given for HIV positive patients with lower CD4 count according to the Ethiopian National Treatment Guideline for TB/HIV infections. Liver function tests were assessed at baseline and at the 1st, 2nd, 4th, 8th, 12th and 24th weeks after initiation of treatment. All the laboratory tests were performed in the International Clinical Laboratories (ICL) which is one of the Joint Commission International (USA) accredited Clinical Laboratories in Africa (https://www.jointcommissioninternational.org/).

5.2. Sample storage and analysis site

DNA samples for the current study were stored at -20°C in deep freezer at the Division of Clinical Pharmacology, Department of Laboratory Medicine, Karolinska Institutet, Karolinska University Hospital - Huddinge, Stockholm, Sweden. The samples were obtained from peripheral blood using QIAamp DNA Maxi Kit. The DNA samples were shipped and genotyping conducted at RIKEN Center for Integrative Medical Sciences, Yokohama - Japan.

5.3. Case definitions

For DILI case definition and categorization, the criteria set by the International DILI Expert Working Group were used (Aithal *et al.*, 2011). The Group developed DILI definition criteria as follow: Alanine transaminase (ALT) cut-off point of 5 times the upper limit of normal (ULN), 3

times the ULN with total bilirubin (TBil) values exceeding 2 times the ULN; or more than 2 times the ULN of alkaline phosphatase (ALP). The ULN for liver biochemical parameters used in this study were ALT (33 U/L for male; 29 U/L for female), AST (41 U/L), ALP (128 U/L) and TBil (1 mg/dL) (Yimer *et al.*, 2014). Based on ALT to ALP ratio relative to their respective ULN, DILI categorized as hepatocellular ($R \geq 5$), cholestatic ($R \leq 2$), or mixed ($2 < R < 5$) types.

DILI severity grading was determined as explained by the Expert Working Group (Aithal *et al.*, 2011) and Yimer *et al* (Yimer *et al.*, 2014) with slight modification as follow:- Mild: elevated ALT/ALP values reaching criteria for DILI but TBil < 2×ULN; Moderate: elevated ALT/ALP values reaching criteria for DILI and TBil ≥ 2×ULN, or symptomatic hepatitis; Severe: elevated ALT/ALP values reaching criteria for DILI, TBil ≥ 2×ULN, and one of the followings: ALT ≥ 10×ULN, ascites and/or encephalopathy with disease duration < 26 weeks, other organ failure, liver transplantation or death due to DILI. Causality assessment for DILI was performed using RUCAM (Chalasani *et al.*, 2014). Patients on the same drug treatment but who did not fulfill the DILI case definitions and presented no clinical symptoms for DILI during the follow-up period were considered as controls.

5.4. Inclusion and exclusion criteria

Inclusion criteria

- complete clinical data, and adequate DNA concentration for genome genotyping

Exclusion criteria

- higher baseline liver enzyme biochemistry or known liver disease prior to treatment
- positive for hepatitis B virus surface antigen or anti-hepatitis C virus antibody
- poor quality DNA: missing call rate > 1%; gender mismatch; ancestral deviation

5.5. Sample size determination

To determine the effective (minimum) sample size required to undertake the current study, we used QUANTO software, a program that computes sample size and power for genetic association studies (Gauderman, 2002). Testing a single SNP marker requires 15 cases and 60 controls while testing half a million SNP markers requires 45 cases and 180 controls per treatment group under the assumption of 10% DILI prevalence, risk allele frequency of 0.2, 1:4 case to control ratio at GWAS significance level ($\alpha = 1.0\times10^{-7}$), 80% power, an odds ratio (OR) of five and 5% error rate. Studying a phenotype in a case-control study of 1:4 is one way to achieve higher statistical power (Hong and Park, 2012). Analysis using a larger number of markers requires a larger sample size; whereas, a lower sample size is required for common SNPs with strong effect sizes (Hong and Park, 2012).

DNA samples obtained from the prospective cohort study available for the current study were as follow: TB patients treated with first-line ATD only (75 cases, 571 controls), HIV patients without TB co-infection treated with EFV-based ART only (21 cases, 368 controls), and TB/HIV co-infected patients treated with both first-line ATD and EFV-based ART concurrently (46 cases, 292 controls).

5.6. DNA quantification and whole genome genotyping

Illumina uses the molecular probe PicoGreen assay using spectrofluorometer to quantify double-stranded DNA samples, and genome-wide SNP genotyping done on GWAS samples using Illumina Omni Express Exome BeadChip genotyping array that captures 951,117 SNPs (Illumina Inc., San Diego, CA, USA) according to Infinium® HD assay protocol (Illumina®, 2010) with the provided supplies (**Annex-I**). The whole genome genotyping protocol employs single-base

extension (SBE) to achieve allele discrimination (Lambert *et al.*, 2013, Steemers and Gunderson, 2007). SBE is a method for SNP genotyping that involves annealing a primer adjacent to the SNP site followed by its extension using dideoxynucleotide terminators (ddNTPs) to block further incorporation of nucleotides (Kim and Misra, 2007). The Infinium® HD assay utilizes several enzymatic and chemical steps (**Annex-II**) yielding fluorescently-labeled products, which upon scanning and image processing, provide genotype calls. The assay requires 400 ηg of DNA as input.

The workflow of Infinium® HD assay:-

- DNA samples were amplified (to increase the amount of DNA uniformly).
- The amplified product was fragmented by a controlled end-point enzymatic process.
- The fragmented DNA was precipitated, dried, and re-suspended in hybridization buffer.
- The re-suspended genomic DNA product was hybridized to a BeadChip (anneal to locus-specific primers) with the use of a capillary flow-through chamber.
- After the un-hybridized DNA was washed away, the probes were extended using SBE.
- The SBE product was stained with dual-color florescent staining labeled anti-dinitrophenol antibody or streptavidin.
- The BeadChips were scanned through iScan system.
- Using a laser to excite the fluorescently modified signal of the SBE product, high-resolution images of the light emitted from the fluorophores were recorded by the scanner.
- The digital images were converted to genotype information using GenomeStudio software.

5.7. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was performed on the replication study samples by a standard method (Ohnishi *et al.*, 2001).

- To amplify DNA fragments, primers (primer3 program designed) were used (**Annex-III**).
- Master mix was prepared in such a way that each well of the PCR tube had a reaction volume of 18 µl containing 3.2 µl of 10X buffer with $MgCl_2$, 1.2 µl of 25 mM deoxynucleotide triphosphates mix (dNTP), 0.05 µl of each of 100 ρmol/µL primers, 0.4 µl of 2.5 U/µL Ex Taq HS DNA polymerase, and distil water to make the final volume.
- Then 2 µl of 5 ηg/µL template DNA was added to the reaction wells.
- PCR was performed in thermal cycler with the following PCR conditions: Hot start at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 45 sec and extension at 72°C for 3 min; and a final extension at 72°C for 5 min completed the reaction. PCR product was kept at 4°C.

5.8. Agarose gel electrophoresis

- Agarose gel was made by melting 1.5 gm agarose powder in 100 ml 1X TBE (Tris (hydroxymethyl) aminomethane-borate with EDTA) buffer in a microwave oven.
- The gel was casted with addition of 5 µl Ethidium bromide, and using plastic combs.
- Once set, the gel was submerged in TBE buffer in an electrophoresis tank.
- Samples pre-mixed with loading dye were kept into gel wells.
- DNA size marker was loaded in one well per row.
- The gel box was covered, and electrophoresis was run for 10 min at 200 mV.
- The amplified PCR product was visualized under UV illuminator.

5.9. Invader assay

To verify findings of the GWAS analysis, the top SNPs that showed the smallest *P*-values (top SNPs) evaluated in an independent set of samples using PCR-based invader assay (Ohnishi *et al.*, 2001) with ABI prism 7900HT (Applied Biosystems, Foster City, CA, USA). The Invader assay uses FRET cassette probes mix, invader probe, allele-specific probes and Cleavase (Olivier, 2005). The probes designed with melting temperature of 53°C for allele-specific probes and 66°C for invader probe using RIKEN probes designer tool (http://www.riken.go.jp/lab-www/help2/members/kagawa/tmnumber.html). Invader assay steps:-

- Invader reaction master mix was prepared in such a way that each reaction well had 3 µl containing 0.125 µl invader buffer, FRET probes mix and Cleavase enzyme; 0.25 µl ROX and 0.25 µl invader probes mix; and distil water to make the final volume.
- The reaction mixture was dispensed to each of 384-well plate.
- PCR product (2 µl) was diluted 1:10 and incorporated into each reaction well using Beckman Coulter Biomek liquid handler, and then spin-down.
- The Invader reaction was done in ABI prism 7900HT at 95°C for 5 min then 63°C for 5 min, 10 min, 20 min and 30 min of reaction time in a thermal cycler.

5.10. *HLA-B* typing

Genomic DNA from 46 cases and age and sex matched controls from ATD and ART co-treatment group were used for the *HLA-B* genotyping study. We performed allele-specific PCR using sequence-specific primers (SSP) of Olerup SSP® *HLA-B* typing kit according to the protocol and recommendations of the manufacturer (Olerup SSP AB, Franzengatan 5, SE-112 51 Stockholm, Sweden).

HLA-B typing steps:-

- Primer tray & Master Mix of low-resolution Olerup SSP® *HLA-B* typing kit were thawed, and the Master Mix was vortexed.
- 162 µl Master Mix and 270 µl distil water were added into a 1.5 ml eppendorf tube.
- 8 µl Master Mix – distil water mix and 2 µl distil water were added into negative control.
- 92 µl of 5 ηg/µl DNA sample was added to remaining Master Mix – distil water mix.
- 10 µl of reaction mixture was dispensed into each well, except the negative control.
- The plate was covered and pulse centrifuge, then spin down.
- PCR was performed in thermal cycler with the following PCR conditions: 94°C for 2 min, followed by 10 cycles of denaturation at 94°C for 10 sec, annealing and extension at 65°C for 60 sec; followed by 20 cycles of denaturation at 94°C for 10 sec, annealing and extension at 61°C for 50 sec, and extension at 72°C for 30 sec.
- The amplified PCR product was loaded onto 2% agarose gel.
- DNA size marker was loaded in one well per row, and the gel box was covered to run electrophoresis in 0.5X TBE buffer for 15 min at 10 mV.
- The presence or absence of specific PCR products visualized under UV illuminator.
- Positive lanes were marked in Helmberg-Score software to determine allele types.
- Low-resolution allele typing results were recorded with 2-digit code.
- High resolution (4-digits) sub-typing was conducted for *HLA-B* alleles that showed significant association with DILI on the low-resolution data (*HLA-B*57* allele carriers) using Olerup SSP® *HLA-B*57:01* typing kit (Olerup SSP AB, Franzengatan 5, Stockholm, Sweden) according to the manufacturer's protocol.

5.11. Quality assurance

For data cleaning, systematic stepwise standard quality filtering was performed on the samples as well as on the raw genotyping data using Plink *v* 1.07 (Purcell *et al.*, 2007). From an initial full set, those SNPs not mapped on autosomal chromosomes were filtered out. In addition, SNPs with a call rate less than 99%, MAF < 0.01, or deviated from expected HWE ($P < 1.0\times10^{-6}$ in the controls) were removed from further analysis (Anderson *et al.*, 2010, Turner *et al.*, 2011). Individuals were checked for gender concordance between recorded clinical data and genotype determined sex. For every sample, the overall genotype missingness was determined to exclude samples with > 1% missing call rate. Outlier samples from principal component analysis (PCA) for population stratification were excluded from further analysis in the GWAS.

5.12. Statistical analysis

For data analysis, continuous variables were presented as mean and standard deviation, and categorical variables as numbers and percentages. After the quality filter, the test for associations for the GWAS was undertaken using Plink *v* 1.07, a computationally efficient software to handle large data set (Purcell *et al.*, 2007). For each SNP, the association between the risk allele and ATD and/or ART-induced liver injury was evaluated using logistic regression analysis adjusted for sex, HIV status, body mass index (BMI), CD4 count and HIV viral load as covariates. Odds-ratio (OR) and 95% confidence intervals (95% CI) were computed taking the non-risk allele as a reference. We also examined the association using Fisher's exact test in the dominant, recessive and allele frequency genetic inheritance models (Bush and Moore, 2012, Lewis, 2002).

The *P*-values for the combined (joint) analysis of the GWAS and the replication studies were also computed (de Bakker *et al.*, 2008, Skol *et al.*, 2009). We performed sub-group GWAS analysis based on the pattern of liver injury for the ATD treatment group. From the initial

GWAS result, we also assessed whether there is any indication of contribution (association of SNPs) from genes related to pharmacokinetics, autoimmune diseases, oxidative stress and *HLA* region (Ahmadi *et al.*, 2005, Hindorff *et al.*, 2009) with ATD and/or ART-induced liver injury. The threshold for genome-wide significance computed by dividing the level of significance (α = 0.05) with the number of marker SNPs tested (Teo, 2008). A marker SNP, which is significant after Bonferroni correction ($P < 7.5\times10^{-8}$) declared genome-wide significant. SNPs with *P*-values below 1.0×10^{-5} were considered suggestive of genome-wide significance (Dahlin *et al.*, 2015). For the overall estimation of statistical power, we used QUANTO program (Gauderman, 2002) at genome-wide significance level for the study.

Quantile-quantile (QQ) plots comparing the expected and observed *P*-values were constructed to evaluate deviations from the expected chi-square test statistic distribution. Manhattan plots for the distribution of log-transformed *P*-values of the GWAS across the chromosomes were generated using R-statistical environment version 3.1.0 (http://www.r-project.org) to visualize the results, and genomic control inflation factors (λ_{GC}) were computed to detect population stratification (Devlin and Roeder, 1999). The effect of population structure was also assessed through principal component analysis (PCA) (Price *et al.*, 2006, Ringnér, 2008) of the GWAS data of our study population with the HapMap reference populations (Utah residents of Northern and Western European ancestry; Yoruba trios from Ibadan, Nigeria; Han Chinese individuals from Beijing, China; Japanese individuals from Tokyo, Japan) using Eigenstrat program in Eigensoft, and outlier samples excluded from further analysis (Price *et al.*, 2006).

The statistical analysis for the *HLA-B* association study was performed using SPSS *v* 22 (IBM Corp., Armonk, NY, USA). To reduce bias in estimating the OR, Haldane's modification was employed (Low *et al.*, 2013), i.e., when a zero-count cell was encountered, 0.5 was added to all

cells in the 2x2 table. The level of significance $P < 0.05$ was considered statistically significant. The corrected *P*-value (*P*c) was adjusted by using Bonferroni's correction for multiple comparisons to account for the number of *HLA-B* alleles observed in the study.

5.13. Ethical consideration

The Institutional Review Board (IRB) of College of Health Sciences, Addis Ababa University (Protocol No. 011/15/Pharma); and the National Research Ethics Review Committee (NRERC) of the Federal Democratic Republic of Ethiopia Ministry of Science and Technology (MoST) (Ref. No. 3.10/081/2015) approved the current study (**Annex-IV**).

5.14. Operational definitions

Bonferroni correction: It is an adjustment made to *P*-values when multiple hypothesis statistical testing is performed simultaneously on a single data set. To perform this correction, divide significance threshold *P*-value ($\alpha = 0.05$) by the number of tests (SNP loci). A marker SNP significant after Bonferroni correction ($P < 7.5\times10^{-8}$) declared genome-wide significant.

DILI case: DILI cases had at least one of the following criteria: - (1) ALT ≥ 5xULN, (2) ALP ≥ 2xULN, or (3) ALT ≥ 3xULN and TBil ≥ 2xULN; where ULNs were ALT (33 U/L for male; 29 U/L for female), AST (41 U/L), ALP (128 U/L) and TBil (1 mg/dL).

DILI type: Based on the ratio of ALT to ALP relative to their respective ULN, DILI categorized as hepatocellular type ($R \geq 5$), cholestatic type ($R \leq 2$), or mixed type ($2 < R < 5$).

DILI grade: Mild: - Elevated ALT or ALP values reaching criteria for DILI but TBil < 2×ULN. Moderate: - Elevated ALT or ALP values reaching criteria for DILI and TBil ≥ 2×ULN, or symptomatic hepatitis. Severe: - Elevated ALT or ALP values reaching criteria for DILI, TBil ≥

2×ULN, and one of the followings: ALT ≥ 10×ULN, ascites or encephalopathy, disease duration < 26 weeks, other organ failure, liver transplantation or death due to DILI.

Genomic control inflation value (λ_{GC}): λ_{GC} is the ratio of the median of the observed Chi-square (χ^2) association statistic for a set of genome-wide tests to the expected median of that statistic under the null hypothesis. $\lambda_{GC} < 1.05$ considered to have no population stratification.

Hardy-Weinberg Equilibrium (HWE): In a large random mating population, if p and q are frequencies of allele A and B of a given locus, the expected genotype frequencies are p^2, 2pq and q^2 for AA, AB and BB, respectively. Chi-square test is used to investigate whether the HWE holds in a given population. When there is a systematic genotyping error, HWE will be violated. Thus, SNPs that deviate from expected HWE ($P < 1.0\text{x}10^{-6}$) were removed from further analysis.

Minor Allele Frequency (MAF): It is the frequency of the less common allele of a polymorphic locus. When MAF < 1%, power is lacking for detecting association; thus, such SNPs were excluded from the GWAS analysis.

Missingness: Missingness could be at individual level or at SNP level, and shows incompleteness of the data. Individual level missingness (low genotype call) is the number of SNPs missing for a specific individual. High level of individual missingness can be indicator of poor DNA quality. SNP level missingness is the number of individuals for whom information on a specific SNP is missing. SNPs with a high level of missingness can potentially lead to bias. SNPs with an overall missing call rate > 1% were excluded from further analysis.

Principal Component Analysis (PCA): PCA is a dimension-reduction tool that can be used to reduce a large set of variables to a small set that still contains most of the information in the large set. It is used to infer population structure in genetic data. The goal of PCA is to extract the

important information from the data and express this information as a set of new orthogonal variables called principal components (obtained as linear combinations of the original variables). PCA represents the pattern of similarity of the observations by displaying them as points on a plot. Outliers were excluded using standard settings in Eigenstrat (more than 6 standard deviations from the mean along the first ten principal components).

Roussel Uclaf Causality Assessment Method (RUCAM): RUCAM is a DILI diagnostic algorithm that uses a scoring system based on time to onset, clinical course of the reaction, risk factors, role of concomitant therapy, non-drug related causes, previous information on hepatotoxicity of the drug regimen and validation of the reaction by positive re-challenge (**Annex-V**). It yields a summed score grouped into likelihood levels as 'excluded' (score < 1), 'unlikely' (score 1–2), 'possible' (score 3–5), 'probable' (score 6–8), and 'definite or highly probable' (score > 8).

Sex discrepancy: It is the difference between sex of individuals recorded in the clinical dataset and their sex determined from genetic data. Sex discrepancy likely point miscoded sex or sample mix-ups. Plink software uses X-chromosome homozygosity estimate (XHE) i.e., male: XHE > 0.80, female: XHE < 0.20, to determine sex then reports individuals for whom the sex recorded in the data set does not match the predicted sex based on genetic data. It is a fairly sensitive test to detect sample swaps.

6. Results

6.1. Study participants and markers that passed quality filters

In the present study, we used data from a total of 1,055 study participants. All the samples were checked for gender concordance between recorded clinical data and genotype determined sex, and there was no DNA sample excluded for gender mismatch. All the genomic DNA samples passed the threshold for genotype call rate. All except four DNA samples met the minimal concentration required for whole genome genotyping.

As shown in Figure 8, the participants grouped into three categories based on the disease types and the drug treatments received. For the statistical analysis, we used 89 cases and 488 controls in the GWAS (48, 14 and 27 cases; and 354, 293 and 159 controls for ATD, ART, and ATD and ART co-treatment groups, respectively), and 45 cases and 425 controls in the replication study (27, 7 and 11 cases; and 217, 75 and 133 controls for ATD, ART, and ATD and ART co-treatment groups, respectively). The numbers of SNPs that passed the quality filter were 660, 206 for the ATD group, 662, 282 for the ART group and 662, 807 for the ATD-ART co-treatment group (Table 2). The Principal component analysis (PCA) plot (Figure 9) indicated that our study population is genetically distinct from the HapMap reference populations, even from the West African cluster.

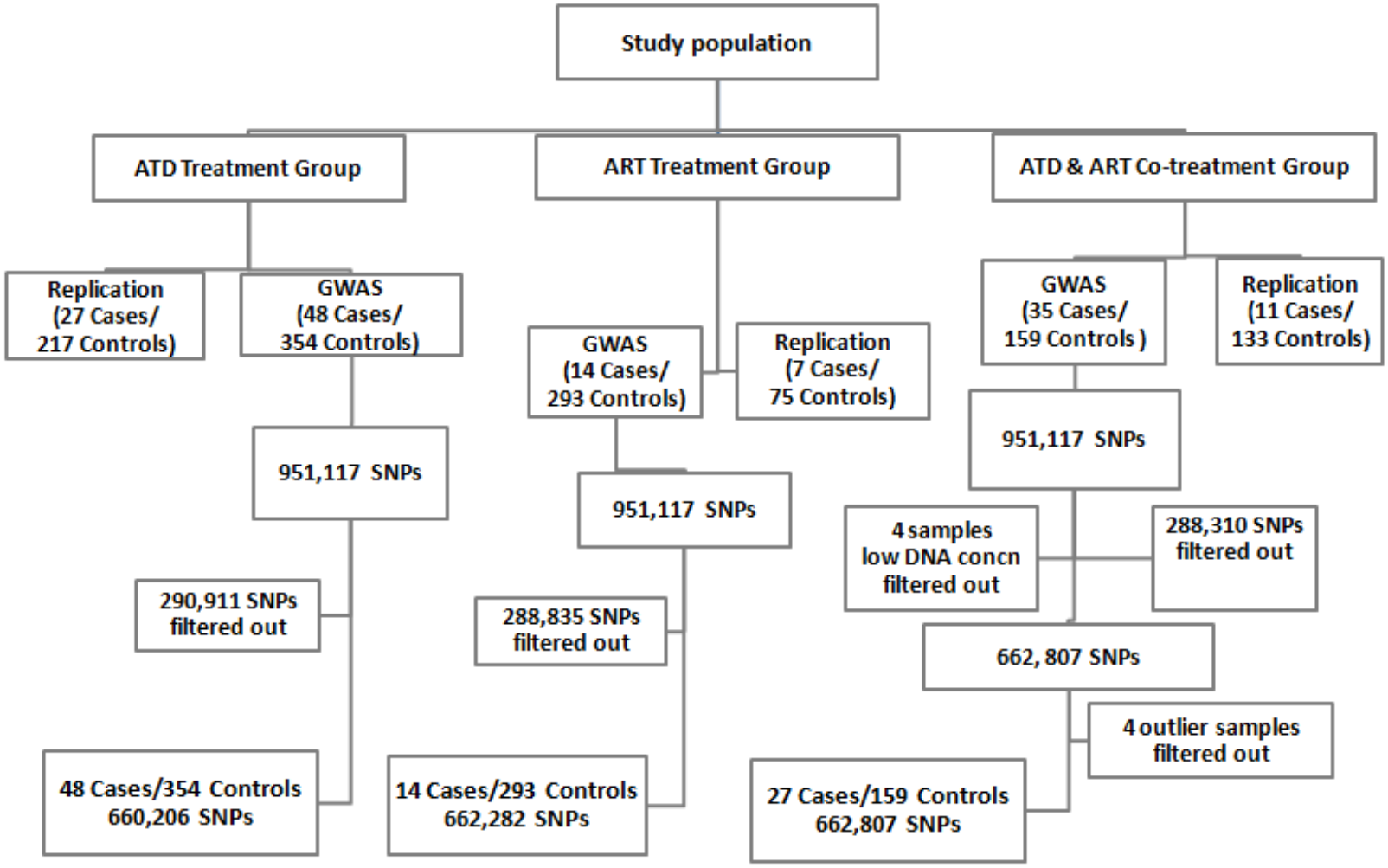

Figure 8. Treatment groups, number of study participants, and quality filter flow chart

ART - Anti-retroviral therapy; ATD - Anti-tubercular drugs; GWAS - Genome wide association study; SNP - Single nucleotide polymorphism

Table 2. Number of SNPs that passed the quality filtering

Quality filters	Treatment groups		
	ATD	**ART**	**ATD & ART co-treatment**
Full set of SNPs genotyped	951, 117	951 117	951, 117
SNPs on autosomal chromosomes	925, 436	925, 436	925, 436
Non-polymorphism	175, 058	177, 715	183, 193
Polymorphic SNPs on autosomal chromosomes	750, 378	747, 721	742, 243
SNPs genotyped successfully (call rate $\geq$ 99%)	695, 426	695, 079	688, 380
Genotype call rate < 0.01	54, 952	52, 642	53, 863
Minor allele frequency (MAF) < 0.01	35, 192	32, 788	25, 568
Hardy-Weinberg equilibrium, $P < 1.0 \times 10^{-6}$	28	9	5
Total number of SNPs excluded	290, 911	288, 835	288, 310
Total number of SNPs passed quality filtering	**660, 206**	**662, 282**	**662, 807**

ATD - Anti-tubercular drugs; ART - Anti-retroviral therapy; SNPs - Single nucleotide polymorphisms

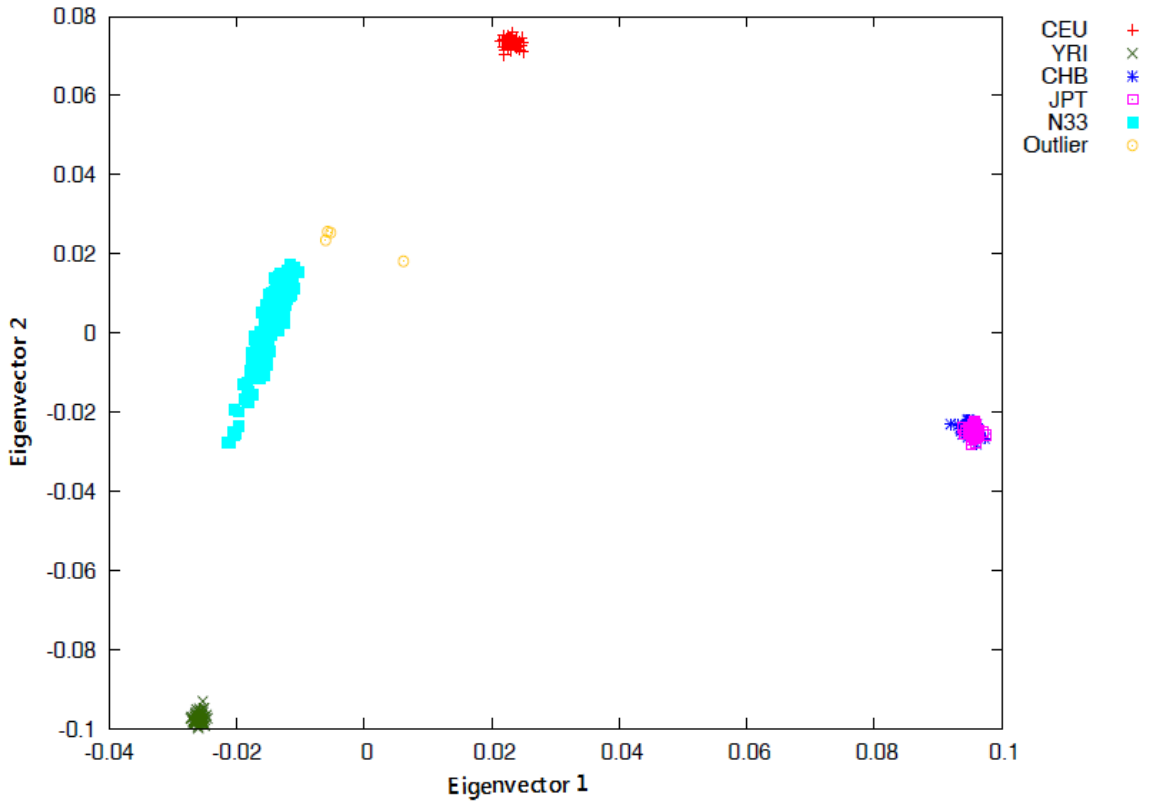

Figure 9. PCA plot of our study population (N33) with the HapMap reference populations

CEU - Utah residents of Northern and Western European ancestry; YRI - Yoruba trios from Ibadan, Nigeria; CHB - Han Chinese individuals from Beijing, China; JPT - Japanese individuals from Tokyo, Japan; PCA - Principal component analysis

6.2. Baseline and follow up clinical characteristics of the study participants

6.2.1. Baseline clinical characteristics of the study participants

The baseline clinical characteristics of the study participants in the GWAS and the replication studies are described in Tables 3 and 4, respectively. In the ATD treatment group, there were statistically significant differences ($P < 0.05$) in sex, HIV status, CD4 count and viral load between cases and controls in the GWAS cohort. There was also a statistically significant difference in BMI and HIV status in the replication study. In the same treatment group; however, there was no statistically significant difference in sex, CD4 count and viral load between cases and controls in the replication study that may be attributed to the smaller sample size of the replication cohort. In the ATD and ART co-treatment group, there was a statistically significant difference in the viral load between cases and controls in the GWAS cohort. For the rest of the baseline clinical characteristics, there were no statistically significant differences between cases and controls in both the GWAS and the replication studies.

Table 3. Baseline clinical variables of the study participants in the GWAS

Variables	ATD group			ART group			ATD & ART co-treatment group		
	Cases	**Controls**	***P***	**Cases**	**Controls**	***P***	**Cases**	**Controls**	***P***
No. of patients	48	354[*]	-	14	293[*]	-	27	159	-
Sex (M, F)	19, 29	203, 151	0.02	5, 9	115, 178	0.79	15, 12	73, 86	0.35
Age (yr), M (SD)	35.6 (10.4)	35.7 (11.5)	0.93	35.3 (12.6)	37.1 (10.6)	0.31	37.9 (10.7)	35.7 (9.8)	0.30
BMI (kg/m^2), M (SD)	19.0 (3.2)	19.3 (3.0)	0.55	20.6 (5.0)	19.3 (2.8)	0.11	18.9 (2.4)	19.0 (2.6)	0.92
Hemoglobin (g/dL), M (SD)	11.8 (3.2)	12.3 (3.5)	0.32	12.2 (1.9)	12.0 (2.4)	0.71	11.1 (1.8)	11.3 (2.5)	0.58
WBC count ($x10^3$ per μL), M (SD)	6.0 (2.5)	6.3 (2.9)	0.41	5.1 (2.6)	5.5 (2.8)	0.63	6.0 (2.9)	6.2 (2.5)	0.85
Neutrophils (%), M (SD)	66.5 (13.4)	66.2 (13.2)	0.92	63.1 (10.0)	60.3 (13.8)	0.45	71.0 (12.6)	67.0 (12.3)	0.23
Platelets ($x10^4$ per μL), M (SD)	31.6 (15.5)	29.6 (12.6)	0.11	21.8 (8.3)	23.3 (12.1)	0.28	32.5 (14.8)	32.1 (13.3)	0.87
ALT (U/L), M (SD)	22.8 (5.8)	21.8 (6.1)	0.40	23.0 (8.4)	21.7 (6.4)	0.55	22.8 (5.3)	21.4 (6.1)	0.40
AST (U/L), M (SD)	31.2 (5.7)	29.7 (7.2)	0.32	28.6 (5.1)	29.2 (6.8)	0.81	28.1 (7.1)	29.9 (6.5)	0.39
ALP (U/L), M (SD)	97.6 (20.9)	99.5 (19.5)	0.67	99.8 (23.5)	95.1 (20.8)	0.35	82.9 (17.6)	87.7 (19.5)	0.29
TBil (mg/dL), M (SD)	0.8 (0.5)	0.7 (0.5)	0.54	0.5 (0.3)	0.6 (0.5)	0.27	0.7 (0.5)	0.7 (0.6)	0.54
Albumin (g/dL), M (SD)	3.3 (0.7)	3.5 (0.9)	0.25	3.7 (0.7)	3.8 (2.2)	0.81	4.1 (3.8)	3.5 (0.7)	0.44
Urea (mg/dL), M (SD)	27.4 (7.0)	29.1 (15.2)	0.29	30.9 (16.9)	27.5 (11.2)	0.31	27.2 (9.2)	29.6 (16.8)	0.46
Creatinine (μmol/L), M (SD)	0.9 (0.2)	1.0 (0.7)	0.71	0.9 (0.3)	0.9 (0.5)	0.98	0.9 (0.2)	1.1 (0.9)	0.41
HIV status, N (%)	44 (91.7)	225 (63.6)	< 0.01	14 (100)	293 (100)	-	27 (100)	159 (100)	-
CD4 count (cells/μL), M (SD)	96.6 (78.5)	129.3 (120.8)	0.03[a]	96.2 (64.2)	99.2 (53.6)	0.84	74.7 (55.1)	90.6 (49.9)	0.13
HIV viral load, log M (SD)	5.3 (0.9)	4.9 (0.9)	0.03[a]	5.0 (0.9)	4.9 (1.0)	0.64	5.3 (0.6)	4.9 (0.9)	0.04

ATD - Anti-tubercular drugs; ART - Anti-retroviral therapy; M(SD) - Mean (standard deviation); BMI - Body mass index; *P* - *P* values; ALT - Alanine aminotransferase; AST - Aspartate aminotransferase; ALP - Alkaline phosphatase; TBil - Total bilirubin; N - Number; [*]Include controls from ATD and ART co-treatment group; [a] Data from HIV positive patients only.

Table 4. Baseline clinical variables of the study participants in the replication study

Variables	ATD group			ART group			ATD & ART co-treatment group		
	Cases	**Controls**	***P***	**Cases**	**Controls**	***P***	**Cases**	**Controls**	***P***
No. of patients	27	217	-	7	75	-	11	133	-
Sex (M, F)	12, 15	85, 132	0.60	1, 6	8, 67	0.77	5, 6	69, 64	0.68
Age (yr), M (SD)	32.0 (7.4)	33.4 (10.3)	0.48	36.1 (8.8)	33.0 (7.1)	0.28	37.6 (9.5)	37.3 (10.0)	0.92
BMI (kg/m^2), M (SD)	17.5 (3.0)	18.9 (3.0)	0.02	19.4 (2.8)	19.6 (2.6)	0.85	19.8 (3.9)	18.7 (3.2)	0.29
Hemoglobin (g/dL), M (SD)	11.3 (2.3)	11.9 (2.1)	0.22	11.9 (1.8)	12.6 (1.7)	0.35	11.6 (2.1)	11.6 (2.2)	0.30
WBC count (x10^3 per μL), M (SD)	6.8 (2.8)	6.5 (2.4)	0.52	4.5 (2.4)	4.7 (1.5)	0.84	5.1 (2.6)	6.3 (2.3)	0.21
Neutrophils (%), M (SD)	69.1 (10.0)	66.0 (12.5)	0.23	54.7 (14.5)	54.8 (14.5)	0.99	63.2 (11.0)	67.0 (11.8)	0.16
Platelets (x10^4 per μL), M (SD)	31.3 (12.8)	33.3 (14.4)	0.49	30.4 (14.1)	26.2 (12.6)	0.40	27.4 (10.3)	30.7 (13.2)	0.41
ALT (U/L), M (SD)	23.5 (7.0)	22.3 (6.5)	0.27	17.0 (1.4)	22.4 (6.6)	0.26	20.8 (7.5)	19.1 (7.0)	0.55
AST (U/L), M (SD)	30.1 (5.8)	28.1 (7.0)	0.31	30.2 (2.8)	29.4 (7.3)	0.24	30.0 (6.8)	28.8 (7.1)	0.63
ALP (U/L), M (SD)	88.8 (28.9)	92.2 (21.2)	0.33	99.9 (20.1)	96.1 (17.6)	0.33	95.6 (23.6)	96.8 (23.8)	0.43
TBil (mg/dL), M (SD)	0.5 (0.3)	0.6 (0.4)	0.60	0.4 (0.4)	0.5 (0.3)	0.59	0.4 (0.3)	0.6 (0.4)	0.30
Albumin (g/dL), M (SD)	3.2 (0.9)	3.4 (0.8)	0.21	3.9 (0.8)	4.1 (0.8)	0.28	3.3 (0.6)	3.5 (0.8)	0.51
Urea (mg/dL), M (SD)	26.8 (8.5)	25.2 (10.5)	0.30	23.0 (6.5)	24.2 (8.0)	0.70	25.2 (4.8)	26.3 (12.0)	0.75
Creatinine (μmol/L), M (SD)	0.8 (0.2)	0.9 (0.3)	0.68	0.7 (0.3)	0.8 (0.2)	0.73	0.9 (0.2)	0.9 (0.4)	0.91
HIV status, N (%)	25 (92.6)	158 (72.8)	0.03	7 (100)	75 (100)	-	11 (100)	133 (100)	-
CD4 count (cells/μL), M (SD)	116.8 (98.3)	138.2 (121.0)	0.33[a]	93.4 (68.1)	109.4 (59.2)	0.51	109.2 (61.6)	89.8 (52.8)	0.23
HIV viral load, log M (SD)	5.0 (0.8)	4.9 (0.9)	0.54[a]	4.9 (0.9)	4.7 (1.2)	0.54	5.0 (0.9)	4.9 (0.9)	0.71

ATD - Anti-tubercular drugs; ART - Anti-retroviral therapy; M(SD) - Mean (standard deviation); BMI - Body mass index; *P* - *P* values; ALT - Alanine aminotransferase; AST - Aspartate aminotransferase; ALP - Alkaline phosphatase; TBil - Total bilirubin; N - Number; [a] Data from HIV positive patients only.

6.2.2. Follow up clinical characteristics of the study participants

In the follow up period after treatment was initiated, there were statistically significant differences ($P < 0.05$) in the mean of the peak values of liver biochemical tests between cases and controls in the GWAS and the replication studies of all the treatment groups (Tables 5 and 6). With regard to the different EFV-based ART regimens, there were no statistically significant differences in the number of cases and controls taking a specific regimen. More than one-third of cases in the ATD treatment group, and about two-third of the cases in the ART alone and with ATD co-treatment groups had cholestatic type of DILI. About 10% of the cases had severe grade of liver injury. The DILI causality assessment was definite for more than 60% of the cases in the treatment groups.

Table 5. Liver biochemical tests, ART regimens, and characteristic of DILI in the GWAS

Variables	ATD group			ART group			ATD & ART co-treatment group		
	Cases	**Controls**	***P***	**Cases**	**Controls**	***P***	**Cases**	**Controls**	***P***
No. of patients	48	354	-	14	293	-	27	159	-
Liver biochemical tests M (SD)*									
ALT (U/L)	296.6 (162.3)	44.6 (18.0)	< 0.01	235.6 (179.1)	45.5 (16.1)	< 0.01	105.6 (19.4)	50.2 (19.0)	< 0.01
AST (U/L)	303.8 (129.3)	51.7 (19.8)	< 0.01	251.3 (149.5)	51.2 (18.4)	< 0.01	172.1 (55.9)	60.8 (23.1)	< 0.01
ALP (U/L)	312.8 (162.9)	124.2 (33.2)	< 0.01	362.7 (266.6)	174.0 (36.1)	< 0.01	397.7 (206.7)	145.7 (41.3)	< 0.01
TBil (mg/dL)	1.91 (1.82)	0.85 (0.27)	< 0.01	2.23 (1.20)	0.73 (0.26)	< 0.01	1.85 (1.44)	0.86 (0.31)	< 0.01
ART regimens, N (%)									
EFV/3TC/D4T	NA			7 (50.0)	148 (50.5)	0.97	11 (40.7)	65 (40.8)	0.99
EFV/3TC/ZDV	NA			4 (28.6)	91 (31.1)	0.84	7 (25.9)	47 (29.6)	0.70
EFV/3TC/TDF	NA			3 (21.4)	54 (18.4)	0.78	9 (33.3)	47 (29.6)	0.69
DILI pattern, N (%)									
Cholestatic	19 (39.6)			10 (71.4)			21 (77.8)		
Hepatocellular	10 (20.8)			2 (14.3)			-		
Mixed	19 (39.6)			2 (14.3)			6 (22.2)		
Severity grade, N (%)									
Mild-to-moderate	43 (89.6)			13 (92.6)			24 (88.9)		
Severe	5 (10.4)			1 (7.4)			3 (11.1)		
RUCAM score, N (%)									
Definite (score > 8)	32 (66.7)			9 (64.3)			17 (63.0)		
Probable (score 6–8)	10 (20.8)			3 (21.4)			7 (25.9)		
Possible (score 3–5)	6 (12.5)			2 (14.3)			3 (11.1)		

*Mean of peak values; ATD-Anti-tubercular drugs; ART-Anti-retroviral therapy; DILI-Drug induced liver injury; M(SD)-Mean (standard deviation); *P* - *P* values; ALT-Alanine aminotransferase; AST-Aspartate aminotransferase; ALP-Alkaline phosphatase; TBil-Total bilirubin; N-Number; D4T-Stavudine; 3TC-Lamivudine; EFV-Efavirenz; ZDV-Zidovudine; TDF-Tenofovir. NA-Not applicable; RUCAM-Roussel Uclaf Causality Assessment Method

Table 6. Liver biochemical tests, ART regimens, and characteristic of DILI in the replication study

Variables	ATD group			ART group			ATD & ART co-treatment group		
	Cases	**Controls**	***P***	**Cases**	**Controls**	***P***	**Cases**	**Controls**	***P***
No. of patients	27	217	-	7	75	-	11	133	-
Liver biochemical tests M (SD)*									
ALT (U/L)	268.6 (109.0)	40.7 (18.4)	< 0.01	160.1 (115.5)	43.2 (17.3)	< 0.01	171.2 (25.1)	47.6 (21.7)	< 0.01
AST (U/L)	326.4 (124.7)	48.5 (17.5)	< 0.01	184.6 (101.7)	44.4 (17.5)	< 0.01	186.3 (13.6)	58.3 (24.0)	< 0.01
ALP (U/L)	379.0 (198.5)	119.5 (35.2)	< 0.01	373.9 (252.0)	143.7 (79.5)	< 0.01	322.8 (210.9)	132.9 (64.0)	< 0.01
TBil (mg/dL)	2.40 (3.10)	0.78 (0.30)	< 0.01	1.16 (0.64)	0.82 (0.31)	< 0.01	2.05 (1.80)	0.72 (0.32)	< 0.01
ART regimens, N (%)									
EFV/3TC/D4T	NA			3 (42.9)	33 (44.0)	0.96	2 (18.2)	29 (21.8)	0.78
EFV/3TC/ZDV	NA			4 (57.1)	42 (56.0)	0.95	3 (27.3)	32 (24.1)	0.81
EFV/3TC/TDF	NA			-	-	-	6 (54.5)	72 (54.1)	0.98
DILI pattern, N (%)									
Cholestatic	15 (55.6)			5 (71.4)			6 (54.5)		
Hepatocellular	5 (18.5)			1 (14.3)			2 (18.2)		
Mixed	7 (25.9)			1 (14.3)			3 (27.3)		
Severity grade, N (%)									
Mild-to-moderate	24 (88.9)			6 (85.7)			10 (90.9)		
Severe	3 (10.1)			1 (14.3)			1 (9.1)		
RUCAM score, N (%)									
Definite (score > 8)	19 (70.4)			4 (57.1)			7 (63.6)		
Probable (score 6–8)	5 (18.5)			2 (28.6)			3 (27.3)		
Possible (score 3–5)	3 (11.1)			1 (14.3)			1 (9.1)		

*Mean of peak values; ATD-Anti-tubercular drugs; ART-Anti-retroviral therapy; DILI-Drug induced liver injury; M(SD)-Mean (standard deviation); *P* - *P* values; ALT-Alanine aminotransferase; AST-Aspartate aminotransferase; ALP-Alkaline phosphatase; TBil-Total bilirubin; N-Number; D4T-Stavudine; 3TC-Lamivudine; EFV-Efavirenz; ZDV-Zidovudine; TDF-Tenofovir. NA-Not applicable; RUCAM-Roussel Uclaf Causality Assessment Method

6.3. Association of SNPs with DILI in the treatment groups

6.3.1. Association of SNPs with ATD-induced liver injury

Quantile-quantile (QQ) plot for the observed versus expected *P*-values, and Manhattan plot of the GWAS analysis for the ATD-induced liver injury treatment group are shown in Figure 10. The genomic control inflation value (λ_{GC}) was one indicating no systemic test statistic inflation, and population stratification was reasonably controlled.

As shown in Figure 10B and Table 7, the top SNP in the GWAS of ATD treatment group after adjustment for covariates was rs10946739 ($P = 4.1x10^{-6}$, OR = 3.4, 95% CI = 2.0-5.6) located in the intron region of family with sequence similarity-65 member-B (*FAM65B*). The top SNP in the replication study was rs319952 ($P = 1.0x10^{-2}$, OR = 2.3, 95% CI = 1.2-4.4) located in the intron of ATP/GTP binding protein-like-4 (*AGBL4*) in chromosome-1 (Table 8). In the combined analysis (Table 9), the top SNP was rs10946737 ($P = 4.4x10^{-6}$, OR = 3.4, 95% CI = 2.2-5.3) located in the intron region of *FAM65B*. Out of the top six SNPs in the combined analysis, four (rs320035, rs393994, rs319952, and rs320003) were clustered in a region located in the intron of *AGBL4*. Details of genotype distributions of the top SNPs in the cases and controls of the treatment groups are shown in **Annex-VI**.

For sub-group GWAS analysis based on the pattern of liver injury (Table 10), the top SNPs for cholestatic, hepatocellular and mixed patterns of DILI were rs10182566 ($P = 4.1x10^{-6}$, OR = 6.0, 95% CI = 2.8-12.8) in 3'-untranslated region of chromosome-2 open reading frame-71 (*C2orf71*), rs1990046 ($P = 3.7x10^{-6}$, OR = 28.4, 95% CI = 6.9-117.3) in the intron region of semaphorin-3A (*SEMA3A*) in chromosome-7, and rs12603186 ($P = 8.1x10^{-6}$, OR = 7.2, 95% CI = 3.0-17.2) in shisa family member-6 (*SHISA6*) in chromosome-17, respectively.

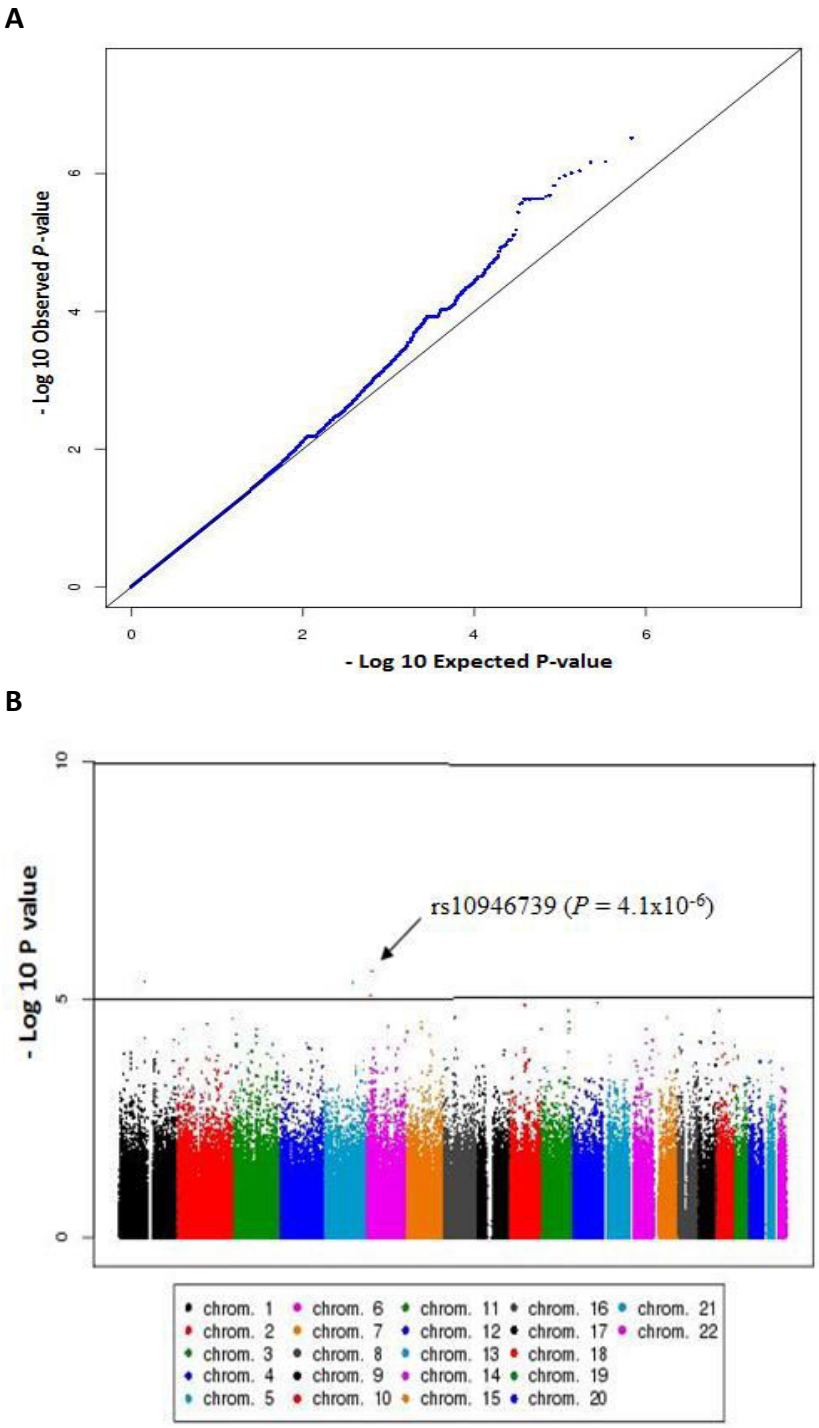

Figure 10. QQ and Manhattan plots for the GWAS in the ATD treatment group. **(A).** QQ plot for the observed versus expected *P*-values ($\lambda_{GC} = 1.00007$), **(B)**. $-\text{Log}_{10}P$ values of logistic regression across chromosomes.

ATD - Anti-tubercular drugs; GWAS - Genome wide association study; QQ - Quantile-quantile

Table 7. Top SNPs in the GWAS of anti-tubercular drugs-induced liver injury

SNP	Chr (loci)	Allele 1/2 (RA)	11vs	22vs	1vs2	*P*_min	*P*_adj	OR (95% CI)	MAF	Nearest gene
rs10946739	6 (24993127)	A/G (A)	$1.1x10^{-3}$	$2.5x10^{-4}$	$9.6x10^{-6}$	$9.6x10^{-6}$	$4.1x10^{-6}$	3.4 (2.0-5.6)	0.19	*FAM65B*
rs7708937	5 (123169574)	T/C (T)	$4.0x10^{-2}$	$1.1x10^{-5}$	$4.8x10^{-6}$	$4.8x10^{-6}$	$4.4x10^{-6}$	4.0 (2.4-6.7)	0.11	*KRT18P16*
rs591141	1 (108694894)	A/G (G)	$2.2x10^{-5}$	$1.0x10^{0}$	$4.5x10^{-5}$	$2.2x10^{-5}$	$4.9x10^{-6}$	4.2 (2.9-6.5)	0.05	*SLC25A24*
rs10946737	6 (24967240)	A/G (A)	$1.2x10^{-1}$	$2.4x10^{-5}$	$2.0x10^{-5}$	$2.0x10^{-5}$	$9.7x10^{-6}$	4.3 (2.5-7.4)	0.10	*FAM65B*
rs2089910	11 (1874404)	T/C (T)	$1.2x10^{-2}$	$2.2x10^{-5}$	$5.3x10^{-6}$	$5.3x10^{-6}$	$4.2x10^{-5}$	2.7 (1.8-4.2)	0.30	*LSP1*
rs4903067	14 (73286300)	T/C (C)	$9.9x10^{-6}$	$2.9x10^{-1}$	$6.1x10^{-5}$	$9.9x10^{-6}$	$8.1x10^{-5}$	3.0 (1.7-5.1)	0.17	*DPF3*
rs320035	1 (49089197)	A/G (G)	$2.5x10^{-3}$	$4.9x10^{-5}$	$3.5x10^{-6}$	$3.5x10^{-6}$	$1.3x10^{-4}$	2.4 (1.5-3.8)	0.48	*AGBL4*
rs393994	1 (49108745)	T/C (C)	$2.5x10^{-3}$	$9.6x10^{-5}$	$6.1x10^{-6}$	$6.1x10^{-6}$	$1.7x10^{-4}$	2.4 (1.5-3.7)	0.48	*AGBL4*
rs2069912	2 (128178191)	T/C (T)	$2.9x10^{-5}$	$9.3x10^{-2}$	$1.2x10^{-5}$	$1.2x10^{-5}$	$1.7x10^{-4}$	5.3 (2.2-12.5)	0.22	*PROC*
rs320003	1 (49126778)	A/G (A)	$2.1x10^{-4}$	$2.6x10^{-3}$	$1.7x10^{-5}$	$1.7x10^{-5}$	$2.3x10^{-4}$	2.3 (1.5-3.7)	0.48	*AGBL4*
rs319952	1 (49113622)	A/G (G)	$2.5x10^{-3}$	$2.1x10^{-4}$	$1.1x10^{-5}$	$1.1x10^{-5}$	$2.8x10^{-4}$	2.3 (1.5-3.6)	0.48	*AGBL4*
rs239319	22 (34120608)	A/G (G)	$1.1x10^{-1}$	$3.7x10^{-5}$	$1.3x10^{-4}$	$1.3x10^{-6}$	$2.8x10^{-4}$	2.3 (1.5-3.6)	0.39	*LARGE*
rs3959930	11 (15835975)	T/C (T)	$7.8x10^{-1}$	$7.2x10^{-7}$	$5.4x10^{-4}$	$7.2x10^{-7}$	$1.1x10^{-3}$	2.1 (1.4-3.3)	0.27	*SOX6*
rs3892834	12 (16486288)	A/G (A)	$2.0x10^{-5}$	$4.0x10^{-1}$	$6.5x10^{-5}$	$2.0x10^{-5}$	$1.1x10^{-3}$	3.1 (1.6-6.3)	0.26	*MGST1*
rs4733759	8 (128804840)	T/C (T)	$1.0x10^{-5}$	$1.6x10^{-1}$	$6.1x10^{-4}$	$1.0x10^{-5}$	$1.6x10^{-3}$	2.1 (1.3-3.2)	0.36	*PVT1*
rs2835109	21 (37107446)	A/G (A)	$2.0x10^{-5}$	$3.7x10^{-1}$	$9.8x10^{-6}$	$9.8x10^{-6}$	$6.1x10^{-3}$	16.1 (2.2-116)	0.14	*RPS20P1*

SNP - Single nucleotide polymorphism; Chr (loci) - Chromosome, and chromosomal loci based on GRChg37; RA - Risk allele; 11vs - Dominant or recessive-inheritance model of Fisher's exact test depending on inheritance mode of allele 1; 22vs - Dominant or recessive-inheritance model of Fisher's exact test depending on inheritance mode of allele 2; 1vs2 - Allelic model of Fisher's exact test; *P*_min - Minimum *P*-value among the genetic models of Fisher's exact test; *P*_adj - Logistic *P*-value after adjustment for covariates; OR - Odds ratio; 95% CI - 95% Confidence interval; MAF - Minor allele frequency.

Table 8. Top SNPs in the replication study of anti-tubercular drugs-induced liver injury

SNP	Chr (loci)	Allele 1/2	11vs	22vs	1vs2	*P*_min	*P*_adj	OR (95% CI)	MAF	Nearest gene
rs319952	1 (49113622)	A/G	$1.5x10^{-1}$	$1.3x10^{-2}$	$1.2x10^{-2}$	$1.2x10^{-2}$	$1.0x10^{-2}$	2.3 (1.2-4.4)	0.50	*AGBL4*
rs320003	1 (49126778)	A/G	$2.0x10^{-2}$	$3.0x10^{-1}$	$1.9x10^{-2}$	$1.9x10^{-2}$	$1.2x10^{-2}$	2.3 (1.2-4.5)	0.50	*AGBL4*
rs320035	1 (49089197)	A/G	$2.4x10^{-1}$	$4.2x10^{-3}$	$5.8x10^{-3}$	$4.2x10^{-3}$	$1.2x10^{-2}$	2.2 (1.9-3.9)	0.50	*AGBL4*
rs393994	1 (49108745)	T/C	$2.4x10^{-1}$	$7.9x10^{-3}$	$1.4x10^{-2}$	$7.9x10^{-3}$	$1.4x10^{-2}$	2.1 (1.2-4.0)	0.50	*AGBL4*
rs10946737	6 (24967240)	A/G	$1.0x10^{0}$	$3.8x10^{-2}$	$5.0x10^{-2}$	$3.8x10^{-2}$	$8.6x10^{-2}$	2.2 (0.9-5.4)	0.10	*FAM65B*
rs7708937	5 (123169574)	T/C	$1.0x10^{0}$	$1.5x10^{-1}$	$1.8x10^{-1}$	$1.5x10^{-1}$	$1.3x10^{-1}$	2.6 (0.8-8.7)	0.12	*KRT18P16*
rs2835109	21 (37107446)	A/G	$1.0x10^{0}$	$1.0x10^{0}$	$9.0x10^{-1}$	$9.0x10^{-1}$	$1.3x10^{-1}$	1.1 (0.4-3.7)	0.10	*RPS20P1*
rs10946739	6 (24993127)	A/G	$5.5x10^{-1}$	$1.1x10^{-1}$	$1.1x10^{-1}$	$1.1x10^{-1}$	$1.8x10^{-1}$	1.7 (0.8-3.6)	0.18	*FAM65B*
rs2069912	2 (128178191)	T/C	$1.0x10^{0}$	$4.8x10^{-1}$	$4.0x10^{-1}$	$4.0x10^{-1}$	$2.5x10^{-1}$	1.6 (0.7-3.5)	0.21	*PROC*
rs591141	1 (108694894)	A/G	$8.1x10^{-1}$	$1.0x10^{0}$	$1.8x10^{-1}$	$1.8x10^{-1}$	$1.8x10^{-1}$	1.5 (0.5-8.6)	0.05	*SLC25A24*
rs2089910	11 (1874404)	T/C	$7.0x10^{-1}$	$5.2x10^{-1}$	$4.1x10^{-1}$	$4.1x10^{-1}$	$2.7x10^{-1}$	1.5 (0.7-3.1)	0.31	*LSP1*
rs4903067	14 (73286300)	T/C	$6.3x10^{-1}$	$1.0x10^{0}$	$4.0x10^{-1}$	$4.0x10^{-1}$	$3.2x10^{-1}$	1.6 (0.6-4.0)	0.15	*DPF3*
rs4733759	8 (128804840)	T/C	$7.0x10^{-1}$	$1.0x10^{0}$	$7.4x10^{-1}$	$7.0x10^{-1}$	$6.9x10^{-1}$	1.2 (0.6-2.3)	0.32	*PVT1*
rs3892834	12 (16486288)	A/G	$1.0x10^{0}$	$4.8x10^{-1}$	$7.5x10^{-1}$	$4.8x10^{-1}$	$8.1x10^{-1}$	1.1 (0.6-2.0)	0.28	*MGST1*
rs3959930	11 (15835975)	T/C	$6.5x10^{-1}$	$8.4x10^{-1}$	$7.4x10^{-1}$	$6.5x10^{-1}$	$9.0x10^{-1}$	1.1 (0.5-2.0)	0.09	*SOX6*
rs239319	22 (34120608)	A/G	$8.2x10^{-1}$	$7.9x10^{-1}$	$1.0x10^{0}$	$7.9x10^{-1}$	$9.5x10^{-1}$	1.1 (0.6-1.8)	0.43	*LARGE*

SNP - Single nucleotide polymorphism; Chr (loci) - Chromosome, and chromosomal loci based on GRChg37; RA - Risk allele; 11vs - Dominant or recessive-inheritance model of Fisher's exact test depending on inheritance mode of allele 1; 22vs - Dominant or recessive-inheritance model of Fisher's exact test depending on inheritance mode of allele 2; 1vs2 - Allelic model of Fisher's exact test; *P*_min - Minimum *P*-value among the genetic models of Fisher's exact test; *P*_adj - Logistic *P*-value after adjustment for covariates; OR - Odds ratio; 95% CI - 95% Confidence interval; MAF - Minor allele frequency.

Table 9. Top SNPs in the combined analysis of anti-tubercular drugs-induced liver injury

SNP	Chr (loci)	Alleles (RA)	Study	Cases/ Controls	MAF	*P*_min	*P*_adj	OR (95% CI)	Nearest gene
rs10946737	6 (24967240)	A/G (A)	GWAS	48/354	0.10	$2.0x10^{-5}$	$9.7x10^{-6}$	4.3 (2.5-7.4)	*FAM65B*
			Rep	27/216	0.10	$3.8x10^{-2}$	$8.6x10^{-2}$	2.2 (0.9-5.4)	
			Comb	75/570	0.10	$6.3x10^{-7}$	$4.4x10^{-6}$	3.4 (2.2-5.3)	
rs320035	1 (49089197)	A/G (G)	GWAS	48/354	0.48	$3.5x10^{-6}$	$1.3x10^{-4}$	2.4 (1.5-3.8)	*AGBL4*
			Rep	27/216	0.50	$4.2x10^{-3}$	$1.2x10^{-2}$	2.2 (1.9-3.9)	
			Comb	75/570	0.49	$8.2x10^{-7}$	$5.1x10^{-6}$	2.3 (1.6-3.3)	
rs10946739	6 (24993127)	A/G (A)	GWAS	48/354	0.19	$9.6x10^{-6}$	$4.1x10^{-6}$	3.4 (2.0-5.6)	*FAM65B*
			Rep	25/209	0.18	$1.1x10^{-1}$	$1.8x10^{-1}$	1.7 (0.8-3.6)	
			Comb	73/563	0.19	$4.7x10^{-6}$	$5.1x10^{-6}$	2.7 (1.8-4.1)	
rs393994	1 (49108745)	T/C (C)	GWAS	48/354	0.48	$6.1x10^{-6}$	$1.7x10^{-4}$	2.4 (1.5-3.7)	*AGBL4*
			Rep	27/216	0.50	$7.9x10^{-3}$	$1.4x10^{-2}$	2.1 (1.2-4.0)	
			Comb	75/570	0.49	$1.9x10^{-6}$	$7.6x10^{-6}$	2.3 (1.6-3.3)	
rs319952	1 (49113622)	A/G (G)	GWAS	48/354	0.48	$1.1x10^{-5}$	$2.8x10^{-4}$	2.3 (1.5-3.6)	*AGBL4*
			Rep	26/216	0.50	$1.2x10^{-2}$	$1.0x10^{-2}$	2.3 (1.2-4.4)	
			Comb	74/570	0.49	$2.5x10^{-6}$	$8.5x10^{-6}$	2.3 (1.6-3.3)	
rs320003	1 (49126778)	A/G (A)	GWAS	48/354	0.48	$1.7x10^{-5}$	$2.3x10^{-4}$	2.3 (1.5-3.7)	*AGBL4*
			Rep	23/208	0.50	$1.9x10^{-2}$	$1.2x10^{-2}$	2.3 (1.2-4.5)	
			Comb	71/562	0.49	$4.6x10^{-6}$	$8.3x10^{-6}$	2.3 (1.6-3.4)	

SNP - Single nucleotide polymorphism; Chr (loci) - Chromosome, and chromosomal loci based on GRChg37; RA - Risk allele; GWAS - Genome wide association study; Rep - Replication study; Comb - Combined analysis using inverse variance method; MAF - Minor allele frequency; *P*_min - Minimum *P*-value among allelic, dominant and recessive models of Fisher's exact test, and *P*-value of inverse variance combined analysis; OR - Odds ratio; 95%CI - 95% Confidence Interval; *P*_adj - Logistic *P*-value after adjustment for covariates; *AGBL4* - ATP/GTP binding protein-like-4; *FAM65B* - Family with sequence similarity-65 member-B

Table 10. Top SNPs in the GWAS of the pattern of anti-tubercular drugs-induced liver injury

DILI Pattern	SNP	Chr (loci)	Alleles	Cases/Controls	*P*_min	*P*_adj	OR (95% CI)	MAF	Nearest gene
Cholestatic	rs10182566	2 (29287708)	T/C	19/354	$1.2x10^{-6}$	$4.1x10^{-6}$	6.0 (2.8-12.8)	0.24	*C2orf71*
	rs12969241	18 (12842480)	T/G	19/354	$1.9x10^{-4}$	$6.8x10^{-6}$	18.2 (5.1-64.4)	0.03	*PTPN2*
	rs9507038	13 (23737972)	A/C	19/354	$4.1x10^{-6}$	$1.5x10^{-5}$	6.2 (2.7-14.0)	0.34	*SGCG*
	rs10504112	8 (52101444)	T/C	19/354	$1.0x10^{-5}$	$4.0x10^{-5}$	4.6 (2.2-9.6)	0.18	*PXDNL*
	rs3176320	6 (36646788)	T/C	19/354	$4.5x10^{-6}$	$3.7x10^{-4}$	9.1 (2.7-30.7)	0.42	*CDKN1A*
Hepatocellular	rs1990046	7 (83747642)	A/G	10/354	$1.0x10^{-6}$	$3.7x10^{-6}$	28.4 (6.9-117.3)	0.05	*SEMA3A*
	rs7183361	15 (57343199)	A/C	10/354	$9.8x10^{-6}$	$1.4x10^{-5}$	11.6 (3.8-35.1)	0.06	*TCF12*
	rs10865177	2 (41829089)	A/G	10/354	$3.9x10^{-6}$	$2.0x10^{-5}$	8.1 (3.1-21.2)	0.06	*LDHAP3*
	rs7615167	3 (107514134)	A/G	10/354	$6.9x10^{-6}$	$3.7x10^{-5}$	8.8 (3.1-24.8)	0.08	*BBX*
	rs1926559	10 (114052917)	A/G	10/354	$7.7x10^{-6}$	$4.6x10^{-5}$	7.2 (2.8-18.5)	0.11	*TECTB*
Mixed	rs12603186	17 (11218707)	A/G	19/354	$4.8x10^{-6}$	$8.1x10^{-6}$	7.2 (3.0-17.2)	0.11	*SHISA6*
	rs2089910	11 (1874404)	T/C	19/354	$3.9x10^{-7}$	$9.5x10^{-6}$	5.8 (2.7-12.6)	0.29	*LSP1*
	rs1432988	5 (164771289)	A/G	19/353	$2.7x10^{-6}$	$1.3x10^{-5}$	5.5 (2.6-11.8)	0.32	*LOC100507193*
	rs8039957	15 (74664851)	A/G	19/354	$2.8x10^{-6}$	$1.3x10^{-5}$	5.6 (2.6-12.2)	0.16	*CYP11A1*
	rs1983476	7 (102065322)	T/C	19/354	$7.7x10^{-6}$	$1.7x10^{-5}$	7.3 (2.9-18.1)	0.05	*LOC100630923*

DILI - Drug induced liver injury; SNP - Single nucleotide polymorphism; Chr (loci) - Chromosome, and chromosomal loci based on GRChg37; RA - Risk allele; *P*_min - Minimum *P*-value among the genetic models (dominant, recessive or allelic) of Fisher's exact test; *P*_adj - Logistic *P*-value after adjustment for covariates; OR - Odds ratio; 95% CI - 95% Confidence interval; MAF - Minor allele frequency

6.3.2. Association of SNPs with ART-induced liver injury

The QQ plot for the observed versus expected *P*-values, and the Manhattan plot for the GWAS analysis for the ART-induced liver injury treatment group are shown in Figure 11. The λ_{GC} was very closer to one indicating no systemic test statistic inflation.

As shown in Figure 11B and Table 11, the top SNP in the GWAS of ART treatment group after adjustment for covariates was a non-synonymous SNP rs199650082 ($P = 2.3x10^{-6}$, OR = 21.4, 95% CI = 7.5-60.6) in endoplasmic reticulum to nucleus signaling-1 (*ERN1*) gene on chromosome-17. The top SNPs for the replication study are shown in Table 12. In the combined analysis (Table 13), the top SNP was also rs199650082 ($P = 1.4x10^{-6}$, OR = 18.2, 95% CI = 7.1-46.9) in *ERN1* gene.

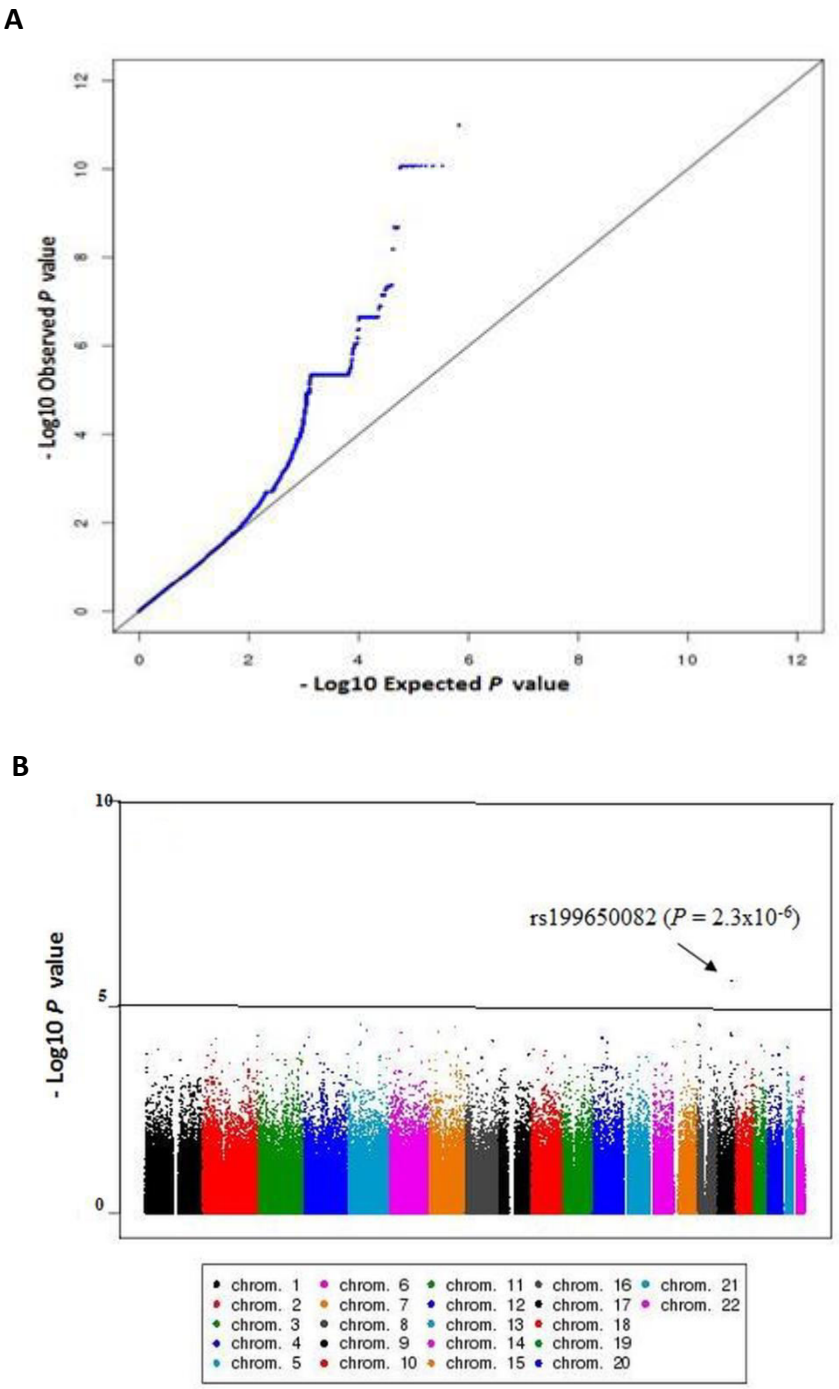

Figure 11. QQ and Manhattan plots for the GWAS in the ART group. **(A).** QQ plot for the observed versus expected *P*-values ($\lambda_{GC} = 0.98100$), (**B**). $-\text{Log}_{10}P$ values of logistic regression across chromosomes.

ART - Anti-retroviral drugs; GWAS - Genome wide association study; QQ - Quantile-quantile

Table 11. Top SNPs in the GWAS of anti-retroviral therapy-induced liver injury

SNP	Chr (loci)	Allele 1/2 (RA)	11vs	22vs	1vs2	*P*_min	*P*_adj	OR (95% CI)	MAF	Nearest gene
rs199650082	17 (62121526)	T/C (T)	$4.6x10^{-2}$	$2.6x10^{-5}$	$4.3x10^{-6}$	$4.3x10^{-6}$	$2.3x10^{-6}$	21.4 (7.5-60.6)	0.03	*ERN1*
rs32498	5 (55548797)	A/G (A)	$7.1x10^{-6}$	$2.1x10^{-2}$	$8.8x10^{-6}$	$7.1x10^{-6}$	$2.6x10^{-5}$	11.5 (4.6-28.9)	0.38	*ANKRD55*
rs7196606	16 (15941025)	T/C (C)	$5.6x10^{-4}$	$1.9x10^{-3}$	$2.9x10^{-5}$	$2.9x10^{-5}$	$2.8x10^{-5}$	9.6 (3.3-27.8)	0.07	*MYH11*
rs7804397	7 (116857547)	A/C (A)	$1.8x10^{-2}$	$4.4x10^{-5}$	$7.3x10^{-6}$	$7.3x10^{-6}$	$3.0x10^{-5}$	7.1 (3.1-16.2)	0.09	*ST7*
rs7206999	17 (61733668)	A/G (A)	$1.1x10^{-2}$	$3.1x10^{-4}$	$1.5x10^{-5}$	$1.5x10^{-5}$	$4.7x10^{-5}$	7.4 (2.8-19.4)	0.05	*MAP3K3*
rs16947045	17 (61770954)	T/C (T)	$1.1x10^{-2}$	$3.1x10^{-4}$	$1.5x10^{-5}$	$1.5x10^{-5}$	$4.7x10^{-5}$	7.4 (2.9-18.7)	0.05	*MAP3K3*
rs196911	17 (62120843)	T/C (C)	$2.4x10^{-4}$	$8.9x10^{-2}$	$6.9x10^{-5}$	$6.9x10^{-5}$	$6.1x10^{-5}$	8.1 (2.9-22.5)	0.04	*ERN1*
rs2305599	8 (121210250)	A/G (A)	$7.5x10^{-4}$	$9.0x10^{-4}$	$3.6x10^{-5}$	$3.6x10^{-5}$	$6.6x10^{-5}$	6.1 (2.5-14.8)	0.19	*COL14A1*
rs152343	5 (55560577)	T/C (T)	$1.6x10^{-6}$	$4.0x10^{-2}$	$4.6x10^{-6}$	$1.6x10^{-6}$	$7.3x10^{-5}$	10.5 (3.6-30.5)	0.43	*PSMC1P4*
rs12632280	3 (87722254)	T/C (C)	$5.0x10^{-2}$	$1.7x10^{-5}$	$6.5x10^{-5}$	$1.7x10^{-5}$	$2.0x10^{-4}$	4.9 (2.1-11.4)	0.26	*PSMC1P6*
rs7615453	3 (174909952)	T/C (C)	$2.4x10^{-5}$	$5.1x10^{-1}$	$6.4x10^{-4}$	$2.4x10^{-5}$	$5.6x10^{-4}$	4.6 (1.9-10.8)	0.22	*NAALADL2*
rs7487755	12 (7631190)	A/C (A)	$9.0x10^{-6}$	$9.8x10^{-2}$	$2.5x10^{-4}$	$9.0x10^{-6}$	$3.0x10^{-4}$	5.0 (2.0-10.0)	0.27	*CD163*
rs17064971	5 (165015242)	A/G (G)	$5.3x10^{-1}$	$3.9x10^{-6}$	$3.1x10^{-4}$	$3.9x10^{-6}$	$8.9x10^{-4}$	5.9 (2.1-16.9)	0.48	*LOC574080*
rs971048	4 (13955163)	A/G (A)	$1.4x10^{-5}$	$5.8x10^{-1}$	$1.1x10^{-3}$	$1.4x10^{-5}$	$1.2x10^{-3}$	3.3 (1.7-10.0)	0.37	*LOC391636*
rs937303	15 (33940768)	T/C (C)	$7.0x10^{-1}$	$8.5x10^{-6}$	$2.1x10^{-4}$	$8.5x10^{-6}$	$1.8x10^{-3}$	11.6 (2.5-54.3)	0.39	*RYR3*

SNP - Single nucleotide polymorphism; Chr (loci) - Chromosome, and chromosomal loci based on GRChg37; RA - Risk allele; 11vs - Dominant or recessive-inheritance model of Fisher's exact test depending on inheritance mode of allele 1; 22vs - Dominant or recessive-inheritance model of Fisher's exact test depending on inheritance mode of allele 2; 1vs2 - Allelic model of Fisher's exact test; *P*_min - Minimum *P*-value among the genetic models of Fisher's exact test; *P*_adj - Logistic *P*-value after adjustment for covariates; OR - Odds ratio; 95% CI - 95% Confidence interval; MAF - Minor allele frequency.

Table 12. Top SNPs in the replication study of anti-retroviral therapy-induced liver injury

SNP	Chr (loci)	Allele 1/2	11vs	22vs	1vs2	*P*_min	*P*_adj	OR (95% CI)	MAF	Nearest gene
rs12632280	3 (87722254)	T/C	$2.0x10^{-1}$	$1.0x10^{0}$	$3.0x10^{-1}$	$2.0x10^{-1}$	$7.7x10^{-2}$	20.0 (0.7-56.1)	0.26	*PSMC1P6*
rs199650082	17 (62121526)	T/C	$1.0x10^{0}$	$2.6x10^{-1}$	$2.6x10^{-1}$	$2.6x10^{-1}$	$2.3x10^{-1}$	6.8 (0.3-15.8)	0.02	*ERN1*
rs17064971	5 (165015242)	A/G	$4.0x10^{-1}$	$6.7x10^{-1}$	$4.0x10^{-1}$	$4.0x10^{-1}$	$2.6x10^{-1}$	2.0 (0.6-6.7)	0.49	*LOC574080*
rs2305599	8 (121210250)	A/G	$1.0x10^{0}$	$2.0x10^{-1}$	$2.8x10^{-1}$	$2.0x10^{-1}$	$3.0x10^{-1}$	2.0 (0.5-7.0)	0.18	*COL14A1*
rs7615453	3 (174909952)	T/C	$1.0x10^{0}$	$2.3x10^{-2}$	$3.5x10^{-1}$	$2.3x10^{-2}$	$3.5x10^{-1}$	1.9 (0.5-7.3)	0.25	*NAALADL2*
rs152343	5 (55560577)	T/C	$1.0x10^{0}$	$4.1x10^{-1}$	$5.8x10^{-1}$	$4.1x10^{-1}$	$4.7x10^{-1}$	1.5 (0.5-4.7)	0.43	*PSMC1P4*
rs196911	17 (62120843)	T/C	$3.4x10^{-1}$	$1.0x10^{0}$	$3.4x10^{-1}$	$3.4x10^{-1}$	$3.5x10^{-1}$	3.7 (0.2-59.1)	0.03	*ERN1*
rs937303	15 (33940768)	T/C	$6.5x10^{-1}$	$3.8x10^{-1}$	$4.3x10^{-1}$	$3.8x10^{-1}$	$5.4x10^{-1}$	2.9 (0.7-12.3)	0.48	*RYR3*
rs971048	4 (13955163)	A/G	$5.8x10^{-1}$	$4.1x10^{-1}$	$1.0x10^{0}$	$4.1x10^{-1}$	$9.8x10^{-1}$	1.1 (0.3-3.3)	0.40	*LOC391636*
rs32498	5 (55548797)	A/G	$5.8x10^{-1}$	$7.0x10^{-1}$	$1.0x10^{0}$	$5.8x10^{-1}$	$9.8x10^{-1}$	1.1 (0.3-3.4)	0.38	*ANKRD55*
rs7487755	12 (7631190)	A/C	$5.2x10^{-1}$	$6.7x10^{-1}$	$1.0x10^{0}$	$5.2x10^{-1}$	$9.9x10^{-1}$	1.1 (0.3-9.4)	0.37	*CD163*
rs7804397	7 (116857547)	A/C	$1.0x10^{0}$	$5.9x10^{-1}$	$6.1x10^{-1}$	$5.9x10^{-1}$	$1.0x10^{0}$	NA	0.08	*ST7*
rs7196606	16 (15941025)	T/C	$1.0x10^{0}$	$1.0x10^{0}$	$1.0x10^{0}$	$1.0x10^{0}$	$1.0x10^{0}$	NA	0.08	*MYH11*
rs16947045	17 (61770954)	T/C	$1.0x10^{0}$	$1.0x10^{0}$	$1.0x10^{0}$	$1.0x10^{0}$	$1.0x10^{0}$	NA	0.05	*MAP3K3*
rs7206999	17 (61733668)	A/G	$1.0x10^{0}$	$1.0x10^{0}$	$1.0x10^{0}$	$1.0x10^{0}$	$1.0x10^{0}$	NA	0.05	*MAP3K3*

Chr (loci) - Chromosome, and chromosomal loci based on GRChg37; 11vs - Dominant or recessive-inheritance model of Fisher's exact test depending on inheritance mode of allele 1; 22vs - Dominant or recessive-inheritance model of Fisher's exact test depending on inheritance mode of allele 2; 1vs2 - Allelic model of Fisher's exact test; *P*_min - Minimum *P*-value among allelic, dominant and recessive models of Fisher's exact test; OR - Odds ratio; 95%CI - 95% Confidence interval; *P*_adj - Logistic *P*-value after adjustment for covariates; MAF - Minor allele frequency; NA - Not applicable.

Table 13. Top SNPs in the combined analysis of anti-retroviral therapy-induced liver injury

SNP	Chr (loci)	Alleles (RA)	Study	Cases/ Controls	MAF	*P*_min	*P*_adj	OR (95% CI)	Nearest gene
rs199650082	17 (62121526)	T/C (T)	GWAS	14/293	0.03	$4.3x10^{-6}$	$2.3x10^{-6}$	21.4 (7.5-60.6)	*ERN1*
			Rep	7/66	0.02	$2.6x10^{-1}$	$2.3x10^{-1}$	6.8 (0.3-15.8)	
			Comb	21/359	0.03	$1.8x10^{-6}$	$1.4x10^{-6}$	18.2 (7.1-46.9)	
rs7804397	7 (116857547)	A/C (A)	GWAS	14/293	0.09	$7.3x10^{-6}$	$3.0x10^{-5}$	7.1 (3.1-16.2)	*ST7*
			Rep	7/66	0.08	$5.9x10^{-1}$	$1.0x10^{0}$	NA	
			Comb	21/359	0.09	$1.1x10^{-5}$	$3.0x10^{-5}$	7.1 (3.4-14.9)	
rs196911	17 (62120843)	T/C (C)	GWAS	14/293	0.04	$7.3x10^{-6}$	$6.1x10^{-5}$	8.1 (2.9-22.5)	*ERN1*
			Rep	7/66	0.03	$3.4x10^{-1}$	$3.5x10^{-1}$	3.7 (0.2-59.1)	
			Comb	21/359	0.04	$4.4x10^{-5}$	$3.8x10^{-5}$	8.3 (3.0-22.6)	
rs16947045	17 (61770954)	T/C (T)	GWAS	14/293	0.05	$1.5x10^{-5}$	$4.7x10^{-5}$	7.4 (2.9-18.7)	*MAP3K3*
			Rep	7/66	0.05	$1.0x10^{0}$	$1.0x10^{0}$	NA	
			Comb	21/359	0.05	$3.4x10^{-5}$	$4.7x10^{-5}$	7.4 (3.1-17.5)	

SNP - Single nucleotide polymorphism; Chr (loci) - Chromosome, and chromosomal loci based on GRChg37; RA - Risk allele; GWAS - Genome wide association study; Rep - Replication study; Comb - Combined analysis; MAF - Minor allele frequency; *P*_min - Minimum *P*-value among allelic, dominant and recessive models of Fisher's exact test, and *P*-value of inverse variance combined analysis; OR - Odds ratio; 95%CI - 95% Confidence Interval; *P*_adj - Logistic *P*-value after adjustment for covariates; NA - Not applicable; *ERN1* - Endoplasmic reticulum to nucleus signaling-1; *MAP3K3* - Mitogen-activated protein kinase-3; *ST7* - Suppression of tumorigenicity-7

6.2.3. Association of SNPs with ATD and ART co-treatment induced liver injury

The QQ plot for the observed versus expected *P*-values, and the Manhattan plot of the GWAS analysis for the ATD and ART co-treatment induced liver injury treatment group are shown in Figure 12. The λ_{GC} was close to one indicating no systemic test statistic inflation, and population stratification was reasonably controlled.

As shown in Figure 12B and Table 14, the top SNP in the GWAS of ATD and ART co-treatment group was rs4842407 ($P = 7.2x10^{-6}$, OR = 5.9, 95% CI = 2.7-12.8) a long intergenic non-coding RNA (*lincRNA*) transcript variant located between simian leukemia viral oncogene homolog-A pseudogene (LOC642550) and synaptotagmin-1 (*SYT1*) genes on chromosome-12. The top SNPs for the replication study are shown in Table 15. In the combined analysis (Table 16), the top SNP was also rs4842407 ($P = 5.3x10^{-7}$, OR = 5.4, 95% CI = 2.8-10.3).

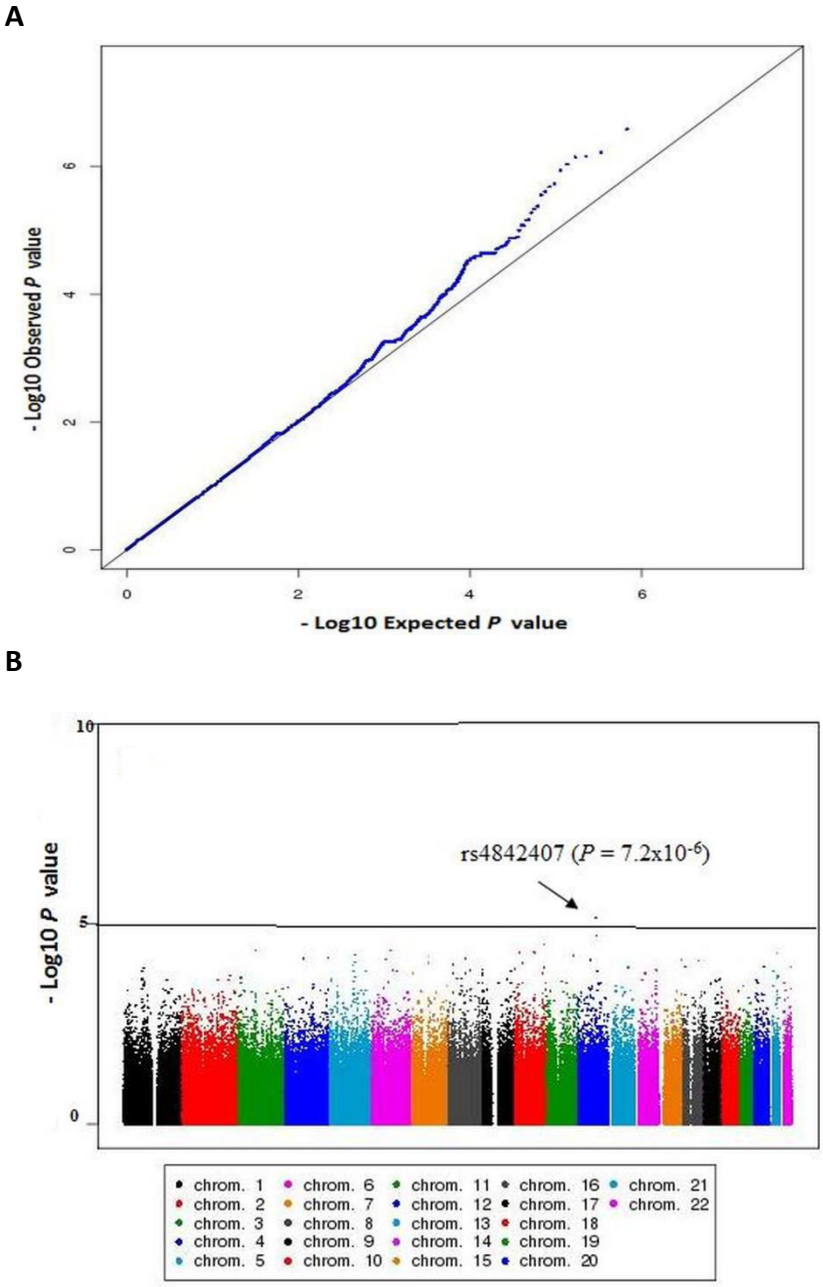

Figure 12. QQ and Manhattan plots for the GWAS in the ATD and ART- co-treatment group. **(A).** QQ plot for the observed versus expected *P*-values (λ_{GC} = 1.01457), **(B)**. $-\text{Log}_{10}P$ values of logistic regression across chromosomes.

ART - Antiretroviral; ATD - Anti-tubercular drugs; GWAS - Genome wide association study; QQ - Quantile-quantile

Table 14. Top SNPs in the GWAS of ATD and ART co-treatment induced liver injury

SNP	Chr (loci)	Allele 1/2 (RA)	11vs	22vs	1vs2	*P*_min	*P*_adj	OR (95% CI)	MAF	Nearest gene
rs4842407	12 (79201073)	T/C (C)	$1.1x10^{-5}$	$3.4x10^{-5}$	$2.3x10^{-7}$	$2.3x10^{-7}$	$7.2x10^{-6}$	5.9 (2.7-12.8)	0.44	*LOC642550*
rs10862812	12 (84464616)	T/C (T)	$4.2x10^{-4}$	$1.6x10^{-3}$	$4.5x10^{-5}$	$4.5x10^{-5}$	$2.0x10^{-5}$	4.7 (2.6-8.5)	0.27	*LOC100128335*
rs868567	10 (129266910)	T/G (G)	$6.3x10^{-4}$	$2.3x10^{-4}$	$1.4x10^{-5}$	$1.4x10^{-5}$	$3.3x10^{-5}$	4.8 (2.3-10.1)	0.29	*DOCK1*
rs16870561	6 (84230450)	T/C (C)	$4.5x10^{-5}$	$1.0x10^{0}$	$7.8x10^{-5}$	$4.5x10^{-5}$	$4.5x10^{-5}$	10.6 (3.4-32.8)	0.04	*PRSS35*
rs11012476	10 (21292923)	A/G (A)	$1.0x10^{-0}$	$5.7x10^{-5}$	$8.7x10^{-5}$	$5.7x10^{-5}$	$5.1x10^{-5}$	13.7 (4.3-43.5)	0.03	*NEBL*
rs2835071	21 (37032177)	T/C (C)	$1.2x10^{-4}$	$1.8x10^{-3}$	$1.7x10^{-5}$	$1.7x10^{-5}$	$5.4x10^{-5}$	4.4 (2.2-9.1)	0.30	*EZH2P1*
rs251891	5 (115050362)	A/C (A)	$2.0x10^{-2}$	$5.6x10^{-4}$	$1.2x10^{-4}$	$1.2x10^{-4}$	$6.0x10^{-5}$	5.8 (2.7-12.2)	0.10	*TMED7*
rs10809892	9 (1315843)	A/C (A)	$2.4x10^{-5}$	$3.2x10^{-2}$	$8.0x10^{-6}$	$8.0x10^{-6}$	$1.4x10^{-4}$	6.2 (2.7-14.0)	0.40	*RPS27AP14*
rs714046	8 (97631573)	A/C (A)	$1.6x10^{-1}$	$2.2x10^{-5}$	$9.2x10^{-5}$	$2.2x10^{-5}$	$1.4x10^{-4}$	4.1 (2.0-8.3)	0.21	*SDC2*
rs7044362	9 (1316816)	A/G (G)	$8.5x10^{-2}$	$5.4x10^{-6}$	$5.1x10^{-6}$	$5.1x10^{-6}$	$1.9x10^{-4}$	7.1 (2.5-19.9)	0.35	*RPS27AP14*
rs2270476	2 (211525764)	A/G (A)	$5.5x10^{-1}$	$7.4x10^{-6}$	$5.3x10^{-5}$	$7.4x10^{-6}$	$2.0x10^{-4}$	4.1 (1.9-8.3)	0.13	*CPS1*
rs2597553	4 (121786273)	A/G (A)	$3.9x10^{-5}$	$3.7x10^{-1}$	$6.9x10^{-5}$	$3.9x10^{-5}$	$3.9x10^{-4}$	5.0 (2.5-10.0)	0.29	*PRDM5*
rs7149043	14 (95116292)	A/G (A)	$4.6x10^{-1}$	$1.3x10^{-5}$	$5.8x10^{-4}$	$1.3x10^{-5}$	$4.1x10^{-4}$	3.3 (1.7-5.0)	0.29	*SERPINA13*
rs10809893	9 (1315952)	T/C (T)	$2.0x10^{-5}$	$2.9x10^{-2}$	$3.6x10^{-6}$	$3.6x10^{-6}$	$8.0x10^{-4}$	10.0 (2.5-98.0)	0.30	*RPS27AP14*

ART - Antiretroviral therapy; ATD - Anti-tubercular drugs; SNP - Single nucleotide polymorphism; Chr (loci) - Chromosome, and chromosomal loci based on GRChg37; RA - Risk allele; 11vs - Dominant or recessive-inheritance model of Fisher's exact test depending on inheritance mode of allele 1; 22vs - Dominant or recessive-inheritance model of Fisher's exact test depending on inheritance mode of allele 2; 1vs2 - Allelic model of Fisher's exact test; *P*_min - Minimum *P*-value among the genetic models of Fisher's exact test; *P*_adj - Logistic *P*-value after adjustment for covariates; OR - Odds ratio; 95% CI - 95% Confidence interval; MAF - Minor allele frequency.

Table 15. Top SNPs in the replication study of ATD and ART co-treatment induced liver injury

SNP	Chr (loci)	Alleles (1/2)	11vs	22vs	1vs2	*P*_min	*P*_adj	OR (95% CI)	MAF	Nearest gene
rs251891	5 (115050362)	T/C	$2.0x10^{-1}$	$1.6x10^{-2}$	$8.9x10^{-3}$	$8.9x10^{-3}$	$1.3x10^{-2}$	4.5 (1.4-13.9)	0.13	*TMED7*
rs11012476	10 (21292923)	A/G	$1.0x10^{0}$	$2.4x10^{-2}$	$2.5x10^{-2}$	$2.4x10^{-2}$	$1.9x10^{-2}$	16.9 (1.6-178)	0.02	*NEBL*
rs4842407	12 (79201073)	T/C	$4.9x10^{-2}$	$8.4x10^{-2}$	$1.6x10^{-2}$	$1.6x10^{-2}$	$2.2x10^{-2}$	4.2 (1.2-14.4)	0.44	*LOC642550*
rs2835071	21 (37032177)	T/C	$1.4x10^{-1}$	$3.6x10^{-1}$	$1.3x10^{-1}$	$1.3x10^{-1}$	$4.0x10^{-2}$	4.0 (1.1-14.9)	0.25	*EZH2P1*
rs2597553	4 (121786273)	A/G	$2.8x10^{-2}$	$5.9x10^{-1}$	$2.4x10^{-2}$	$2.4x10^{-2}$	$5.2x10^{-2}$	8.7 (0.9-77.9)	0.32	*PRDM5*
rs868567	10 (129266910)	T/G	$1.4x10^{-1}$	$1.0x10^{0}$	$1.0x10^{0}$	$1.4x10^{-1}$	$1.1x10^{-1}$	3.6 (0.8-17.1)	0.32	*DOCK1*
rs10862812	12 (84464616)	T/C	$1.0x10^{0}$	$4.6x10^{-1}$	$3.7x10^{-1}$	$3.7x10^{-1}$	$2.6x10^{-1}$	2.5 (0.5-11.9)	0.25	*LOC100128335*
rs10809893	9 (1315952)	T/C	$7.3x10^{-1}$	$2.2x10^{-1}$	$4.1x10^{-1}$	$2.2x10^{-1}$	$2.7x10^{-1}$	1.8 (0.6-5.2)	0.33	*RPS27AP14*
rs10809892	9 (1315843)	A/C	$1.0x10^{0}$	$6.1x10^{-1}$	$6.0x10^{-1}$	$6.0x10^{-1}$	$4.7x10^{-1}$	1.5 (0.5-4.4)	0.42	*RPS27AP14*
rs7149043	14 (95116292)	A/G	$5.6x10^{-1}$	$1.0x10^{0}$	$7.9x10^{-1}$	$5.6x10^{-1}$	$7.1x10^{-1}$	1.2 (0.4-3.8)	0.34	*SERPINA13*
rs2270476	2 (211525764)	A/G	$1.0x10^{0}$	$1.0x10^{0}$	$1.0x10^{0}$	$1.0x10^{0}$	$8.0x10^{-1}$	1.2 (0.3-4.5)	0.20	*CPS1*
rs7044362	9 (1316816)	A/G	$2.9x10^{-1}$	$4.8x10^{-1}$	$1.0x10^{0}$	$2.9x10^{-1}$	$9.8x10^{-1}$	1.1 (0.3-3.0)	0.38	*RPS27AP14*
rs714046	8 (97631573)	A/C	$1.0x10^{0}$	$4.9x10^{-2}$	$4.6x10^{-2}$	$4.6x10^{-2}$	$1.0x10^{0}$	NA	0.02	*SDC2*
rs16870561	6 (84230450)	T/C	$1.0x10^{0}$	NA	$1.0x10^{0}$	$1.0x10^{0}$	$1.0x10^{0}$	NA	0.06	*PRSS35*

ART - Antiretroviral therapy; ATD - Anti-tubercular drugs; Chr (loci) - Chromosome, and chromosomal loci based on GRChg37; 11vs - Dominant or recessive-inheritance model of Fisher's exact test depending on inheritance mode of allele 1; 22vs - Dominant or recessive-inheritance model of Fisher's exact test depending on inheritance mode of allele 2; 1vs2 - Allelic model of Fisher's exact test; *P*_min - Minimum *P*-value among allelic, dominant and recessive models of Fisher's exact test; OR - Odds ratio; 95%CI - 95% Confidence interval; *P*_adj - Logistic *P*-value after adjustment for covariates; MAF - Minor allele frequency; NA - Not applicable.

Table 16. Top SNPs in the combined analysis of ATD and ART co-treatment group

SNP	Chr (loci)	Alleles (RA)	Study	Cases/ Controls	MAF	*P*_min	*P*_adj	OR (95% CI)	Nearest gene
rs4842407	12 (79201073)	T/C (C)	GWAS	27/159	0.44	$2.3x10^{-7}$	$7.2x10^{-6}$	5.9 (2.7-12.8)	*LOC642550*
			Rep	8/108	0.44	$1.6x10^{-2}$	$2.2x10^{-2}$	4.2 (1.2-14.4)	
			Comb	35/267	0.44	$3.0x10^{-7}$	$5.3x10^{-7}$	5.4 (2.8-10.3)	
rs11012476	10 (21292923)	A/G (A)	GWAS	27/159	0.03	$5.7x10^{-5}$	$5.1x10^{-5}$	13.7 (4.3-43.5)	*NEBL*
			Rep	8/107	0.02	$2.4x10^{-2}$	$1.9x10^{-2}$	16.9 (1.6-178)	
			Comb	35/266	0.03	$9.6x10^{-7}$	$2.8x10^{-6}$	14.3 (5.3-39.9)	
rs251891	5 (115050362)	A/C (A)	GWAS	27/159	0.10	$1.2x10^{-4}$	$6.0x10^{-5}$	5.8 (2.7-12.2)	*TMED7*
			Rep	8/104	0.13	$8.9x10^{-3}$	$1.3x10^{-2}$	4.5 (1.4-13.9)	
			Comb	35/263	0.11	$3.5x10^{-6}$	$2.5x10^{-6}$	5.2 (2.9-9.5)	
rs2835071	21 (37032177)	T/C (C)	GWAS	27/159	0.30	$1.7x10^{-5}$	$5.4x10^{-5}$	4.4 (2.2-9.1)	*EZH2P1*
			Rep	8/104	0.25	$1.3x10^{-1}$	$4.0x10^{-2}$	4.0 (1.1-14.9)	
			Comb	35/263	0.28	$7.4x10^{-6}$	$6.0x10^{-6}$	4.3 (2.3-8.1)	

ART - Antiretroviral therapy; ATD - Anti-tubercular drugs; SNP - Single nucleotide polymorphism; Chr (loci) - Chromosome, and chromosomal loci based on GRChg37; *LOC642550* - v-ral simian leukemia viral oncogene homolog A pseudogene; RA - Risk allele; GWAS - Genome wide association study; Rep - Replication study; Comb - Combined analysis; MAF - Minor allele frequency; *P*_min - Minimum *P*-value among allelic, dominant and recessive models of Fisher's exact test, and *P*-value of inverse variance combined analysis; OR - Odds ratio; 95% CI - 95% Confidence Interval; *P*_adj - Logistic *P*-value after adjustment for covariates; NA - Not applicable; *LOC642550* - simian leukemia viral oncogene homolog-A pseudogene; *NEBL* - nebulette; *TMED7* - transmembrane p24 trafficking protein-7

6.3.4. Association of SNPs in autoimmune disease, oxidative stress, pharmacokinetic genes, and HLA region with ATD and/or ART-induced liver injury

The top SNPs in genes related to autoimmune diseases, oxidative stress, pharmacokinetic, and HLA region in the GWAS of DILI in the treatment groups are shown in Table 17. The SNPs with the lowest *P*-values in the ATD treatment group include rs12969241 ($P = 1.1x10^{-5}$, OR = 9.1, 95% CI = 3.3-24.9) located in the intron region of protein tyrosine phosphatase non-receptor type-2 (*PTPN2*) and rs2842997 ($P = 7.1x10^{-3}$, OR = 5.5, 95% CI = 1.6-19.0) in the vicinity of SOD2 related to autoimmune diseases and oxidative stress, respectively. For genes related to pharmacokinetics, SNPs with the lower *P*-values in the ATD treatment group include rs12543818 near *NAT2*, rs4148328 in *UGT1A8* and rs885622 in dihydropyrimidine dehydrogenase (*DPYP*) gene. The SNPs rs11642957 ($P = 1.1x10^{-4}$, OR = 4.9, 95% CI = 2.3-10.7) in ATP-binding cassette subfamily-C member-1 (*ABCC1*, membrane bound drug transporter), and rs9276370 ($P = 1.1x10^{-3}$, OR = 5.1, 95% CI = 2.0-12.7) in the vicinity of *HLA-DQA2* were among those with the lower *P*-values in the ART alone and with ATD co-treatment groups, respectively.

Table 17. Top SNPs for GWAS of ATD and/or ART induced liver injury in genes related to autoimmune disease, oxidative stress, pharmacokinetic, and *HLA* region.

Group	SNP	Chrloc	Alleles	*P*_min	*P*_adj	OR (95% CI)	MAF	Nearest gene
ATD	rs12969241	18 (12842480)	T/G	$1.3x10^{-4}$	$1.1x10^{-5}$	9.1 (3.3-24.9)	0.03	*PTPN2*
	rs7958375	12 (111640017)	A/G	$8.8x10^{-5}$	$1.2x10^{-5}$	11.3 (3.8-33.5)	0.02	*CUX2*
	rs2103025	3 (188070570)	A/G	$1.7x10^{-4}$	$4.9x10^{-4}$	2.9 (1.6-5.2)	0.32	*LPP*
	rs4479187	15 (38711486)	T/C	$7.2x10^{-4}$	$1.3x10^{-3}$	2.1 (1.3-3.3)	0.37	*FAM98B*
	rs2842997	6 (160063852)	A/G	$5.1x10^{-3}$	$7.1x10^{-3}$	5.5 (1.6-19.0)	0.35	*SOD2*
	rs2758331	6 (160105070)	T/G	$8.8x10^{-3}$	$7.7x10^{-3}$	5.3 (1.6-17.9)	0.34	*SOD2*
	rs885622	1 (97691005)	T/C	$2.2x10^{-3}$	$2.3x10^{-3}$	2.7 (1.5-5.0)	0.20	*DPYD*
	rs4148328	2 (234677659)	A/G	$4.3x10^{-3}$	$4.6x10^{-3}$	1.9 (1.2-3.0)	0.26	*UGT1A8*
	rs12543818	8 (18271912)	A/C	$1.9x10^{-3}$	$1.1x10^{-2}$	3.4 (1.7-6.9)	0.37	*NAT2*
	rs7670819	4 (70481880)	T/C	$2.9x10^{-3}$	$4.5x10^{-1}$	1.2 (0.8-1.8)	0.45	*UGT2A1*
ART	rs11642957	16 (16124008)	T/C	$9.5x10^{-5}$	$1.1x10^{-4}$	4.9 (2.3-10.7)	0.18	*ABCC1*
	rs9380345	6 (33080359)	T/C	$4.6x10^{-3}$	$1.0x10^{-3}$	8.6 (2.4-31.2)	0.03	*HLA-DPB2*
	rs2857204	6 (32744347)	A/C	$5.3x10^{-3}$	$8.8x10^{-3}$	3.3 (1.1-10.0)	0.42	*HLA-DQB2*
	rs13199787	6 (32705276)	T/C	$6.6x10^{-3}$	$2.3x10^{-2}$	1.9 (0.6-6.4)	0.43	*HLA-DQA2*
	rs2860975	10 (96766934)	A/C	$2.0x10^{-3}$	$2.5x10^{-2}$	7.8 (1.7-35.7)	0.27	*CYP2C9*
	rs1569343	4 (70341463)	A/C	$4.6x10^{-3}$	$2.6x10^{-2}$	4.9 (1.6-15.2)	0.46	*UGT2B4*
	rs12024396	1 (97880883)	A/G	$1.1x10^{-3}$	$2.8x10^{-2}$	6.7 (2.0-21.8)	0.46	*DPYD*
ATD & ART	rs9276370	6 (32707295)	A/C	$2.8x10^{-4}$	$1.1x10^{-3}$	5.1 (2.0-12.7)	0.36	*HLA-DQA2*
	rs7773149	6 (32706042)	A/G	$3.5x10^{-4}$	$1.2x10^{-3}$	3.8 (1.7-8.3)	0.36	*HLA-DQA2*
	rs9341244	2 (38303095)	A/G	$3.8x10^{-3}$	$2.1x10^{-3}$	8.8 (2.2-35.3)	0.02	*CYP1B1*
	rs7592624	2 (234602906)	A/G	$3.3x10^{-3}$	$5.2x10^{-3}$	4.5 (1.7-11.6)	0.38	*UGT1A8*
	rs11203943	8 (18070927)	T/C	$3.9x10^{-3}$	$6.6x10^{-3}$	4.7 (1.7-12.9)	0.06	*NAT1*
	rs7670819	4 (70481880)	T/C	$2.9x10^{-3}$	$6.0x10^{-2}$	16.8 (9.2-30.9)	0.45	*UGT2A1*

ATD - Anti-tubercular drugs; ART- antiretroviral therapy; Chr (loci) - Chromosome, and chromosomal loci based on GRChg37; *HLA* - Human leukocyte antigen; *P*_min - Minimum *P*-value among the genetic models (allelic, dominant, recessive and allelic) of Fisher's exact test; *P*_adj - Logistic *P*-value after adjustment for covariates; OR - Odds ratio; 95% CI - 95% Confidence interval; MAF - Minor allele frequency.

6.4. Association of HLA-B alleles with ATD and ART co-treatment induced liver injury

The clinical characteristics of participants in the *HLA-B* typing study are described in Table 18. In multivariate logistic analysis, baseline CD4 count was significantly associated with ATD and ART co-treatment induced liver injury ($P < 0.05$). There were no statistically significant differences between cases and controls in the baseline liver biochemical tests. There were also no statistically significant differences in the number of cases and controls with regard to the different ART regimens. About two-third of the DILI cases developed cholestatic type of liver toxicity, and more than 10% had developed severe DILI. The causality assessment was very likely for more than 60% of the cases.

The proportion of *HLA-B* allele carriers' in the cases and controls of ATD and ART co-treatment group is presented in Table 19. A total of 18 *HLA-B* alleles were detected. In a multivariate analysis, the proportion of *HLA-B*57* and *HLA-B*14* allele carriers were significantly higher in the cases than the controls. The association of *HLA-B*57* remained significant after correction for multiple testing. An illustrative agarose gel electrophoresis for *HLA-B*57* allele carrier DNA sample is shown in Figure 13.

The proportions of *HLA-B*57* allele carriers were significantly higher in the cholestatic and mixed patterns of DILI, and in the mild-to-moderate DILI cases than the controls (Table 20). In addition, the proportion of *HLA-B*14* allele carriers was significantly higher in the severe DILI cases than the controls. However, the proportion of *HLA-B*41* allele carriers was significantly lower in the mild-to-moderate DILI cases compared with the controls.

Table 18. Clinical variables of the study participants in the *HLA-B* typing study

Variables	Cases	Controls	Univariate analysis		Multivariate analysis	
	(n = 46)	(n = 46)	*P*	OR (95% CI)	*P*	OR (95% CI)
Sex (M, F)	22, 24	22, 24	-	-		
Age (yrs), M (SD)	35.4 (9.3)	35.3 (8.7)	0.95	0.99 (0.95-1.05)		
BMI (kg/m^2), M (SD)	18.4 (2.4)	19.1 (3.5)	0.25	1.09 (0.94-1.26)		
CD4 count (cells/μL), M (SD)	69.3 (48.7)	94.2 (51.4)	0.02	1.01 (1.01-1.02)	0.03	1.01 (1.00-1.02)
Viral load (copies/mL), log M (SD)	5.3 (1.0)	5.0 (1.0)	0.20	0.72 (0.44-1.19)		
Karnofsky score	82.8 (13.6)	88.7 (13.4)	0.04	1.03 (1.00-1.07)	0.06	1.03 (0.99-1.07)
Liver biochemical tests, M (SD)						
Baselline ALT (U/L)	25.5 (9.8)	27.8 (8.3)	0.22	1.03 (0.98-1.08)		
Baseline AST (U/L)	32.7 (10.3)	31.9 (9.6)	0.70	0.99 (0.95-1.03)		
Baselline ALP (U/L)	99.5 (19.3)	104.3 (19.3)	0.23	1.01 (0.99-1.04)		
Baseline TBil (mg/dL)	0.7 (0.5)	0.7 (0.4)	0.71	0.84 (0.34-2.10)		
ART regimen, N (%)						
EFV/3TC/TDF	15 (32.6)	12 (26.1)	0.49	1.37 (0.56-3.38)		
EFV/3TC/ZDV	16 (34.8)	16 (34.8)	1.00	1.00 (0.42-2.36)		
EFV/3TC/D4T	15 (32.6)	18 (39.1)	0.52	0.75 (0.32-1.78)		
Pattern of liver injury, N (%)						
Cholestatic	29 (63.0)					
Hepatocellular	2 (4.4)					
Mixed	15 (32.6)					
Severity grade, N (%)						
Mild-to-moderate	39 (84.8)					
Severe	7 (15.2)					
RUCAM score, N (%)						
Definite (score > 8)	30 (65.2)					
Probable (score 6–8)	12 (26.1)					
Possible (score 3–5)	4 (8.7)					

3TC - Lamivudine; ALP - Alkaline phosphatase; ALT - Alanine aminotransferase; ART- antiretroviral therapy; AST - Aspartate aminotransferase; ATD - Anti-tubercular drugs; BMI - Body mass index; CI - Confidence Interval; D4T - Stavudine; EFV - Efavirenz; *HLA* - Human leukocyte antigen; M (SD) - mean (standard deviation); N - Number; OR - Odds ratio; RUCAM - Roussel Uclaf Causality Assessment Method; TBil - Total bilirubin; TDF - Tenofovir; ZDV - Zidovudine

Table 19. Proportion of *HLA-B* allele carriers in the cases (N = 46) and controls (N = 46)

***HLA-B* alleles**	**Cases**	**Controls**		**Univariate**		**Multivariate**
	N (%)	**N (%)**	***P***	**OR (95% CI)**	***P***	**OR (95% CI)**
*B*57*	17 (37.0%)	1 (2.2%)	0.002[a]	26.38 (3.33-209.07)	0.002	30.08 (3.44-263.11)
*B*14*	10 (21.7%)	3 (6.5%)	0.05	3.98 (1.02-15.58)	0.01	7.51 (1.50-37.68)
*B*41*	3 (6.5%)	10 (21.7%)	0.05	0.25 (0.06-0.98)	0.11	0.26 (0.05-1.37)
*B*15*	8 (17.4%)	16 (34.8%)	0.06	0.40 (0.15-1.05)	0.53	0.68 (0.21-2.23)
*B*08*	0	4 (8.7%)	0.12	1.48 (0.01-1.94)[b]		
*B*18*	0	3 (6.5%)	0.24	0.13 (0.01-2.66)[b]		
*B*44*	2 (4.3%)	5 (10.9%)	0.25	0.37 (0.07-2.03)		
*B*13*	5 (10.9%)	8 (17.4%)	0.37	0.58 (0.17-1.93)		
*B*58*	4 (8.7%)	2 (4.3%)	0.41	2.10 (0.36-12.05)		
*B*51*	6 (13.0%)	4 (8.7%)	0.50	1.58 (0.41-6.00)		
*B*53*	4 (8.7%)	3 (6.5%)	0.70	1.37 (0.29-6.47)		
*B*07*	10 (21.7%)	9 (19.6%)	0.80	1.14 (0.42-3.14)		
*B*49*	10 (21.7%)	10 (21.7%)	1.00	1.00 (0.37-2.69)		
*B*35*	1 (2.2%)	0	1.00	3.07 (0.12-77.25)[b]		
*B*37, *39, *50, *73*	0	2 (4.3%)	0.50	0.19 (0.01-4.10)[b]		

[a] Corrected *P*-values (*P*c) = 0.036, *P*-values adjusted by using Bonferroni's correction for multiple comparisons to account for the observed 18 *HLA-B* alleles; [b]Haldane's modification; ART - Antiretroviral therapy; ATD - Anti-tubercular drugs; CI - Confidence Interval; *HLA* - Human leukocyte antigen; OR - Odds ratio; *P* - *P* values calculated by Fisher's exact test comparing the positive alleles in cases with those of controls; variables with $P < 0.1$ in the univariate logistic analysis included in the multivariate analysis.

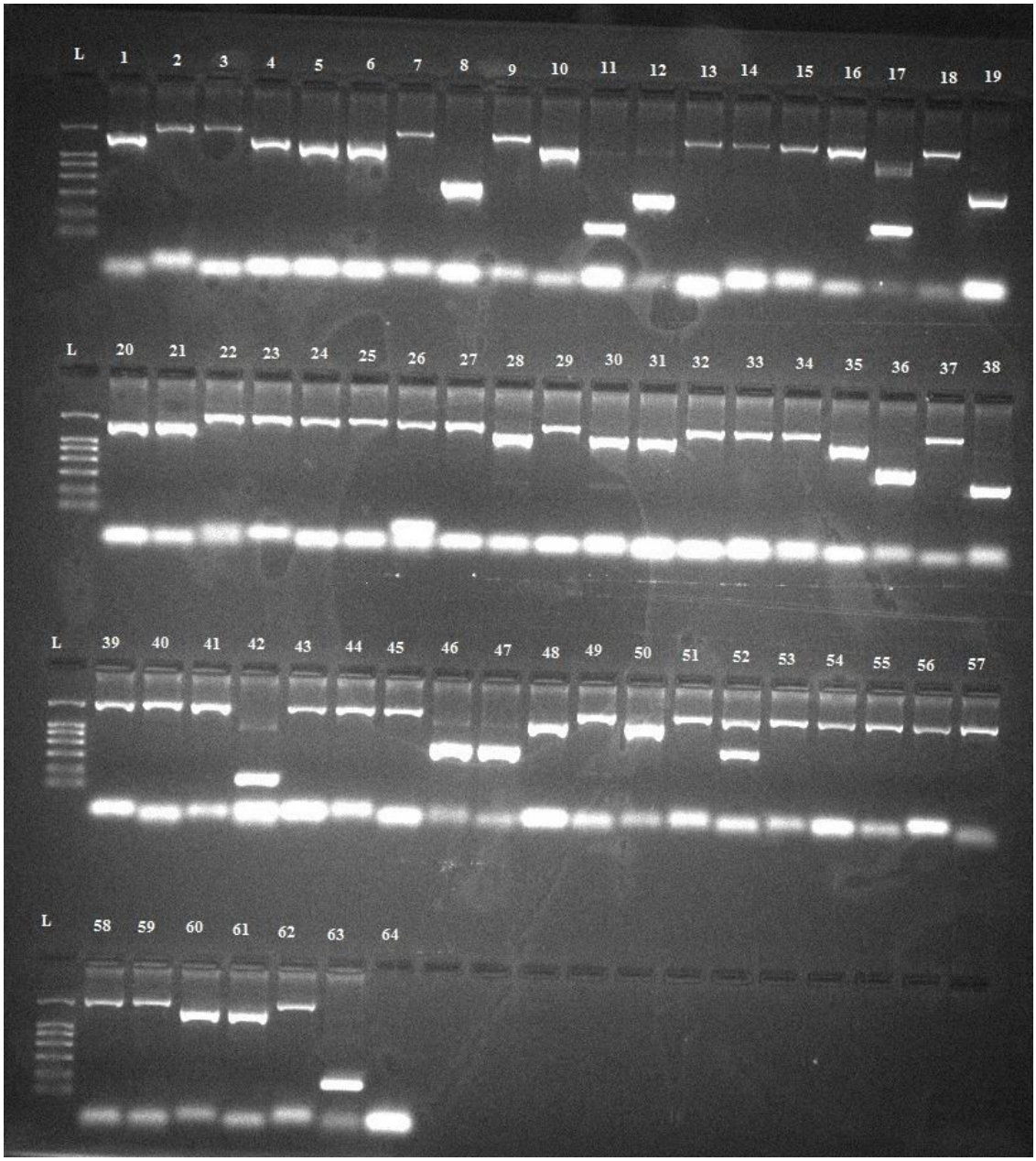

Figure 13. Representative agarose gel electorphoresis of *HLA-B*57* allele carrier DNA sample. L - DNA ladder; Amplified PCR product in lane number 8, 11, 12, 17, 19, 36, 38, 42, 46, 47, 52 and 63. Internal control band of 1,070 base pairs (bp) for most wells, or a band of 800 bp for some wells was observed except in the negative control (Lane 64).

Table 20. Association of *HLA-B* alleles with the pattern and severity of DILI

Characteristics	*HLA-B*57* carriers			*HLA-B*14* carriers			*HLA-B*41* carriers		
	N (%)	***P***	**OR (95% CI)**	**N (%)**	***P***	**OR (95% CI)**	**N (%)**	***P***	**OR (95% CI)**
DILI Pattern									
Controls (n = 46)	1 (2.2)	-	-	3 (6.5)	-	-	10 (21.7)	-	-
Cholestatic (n = 29)	13 (44.8)	< 0.01	36.6 (4.4-302.3)	5 (17.2)	0.17	3.0 (0.7-13.6)	2 (6.9)	0.11	0.3 (0.1-1.3)
Hepatocellular (n = 2)	0	1.00	6.1 (0.2-190.1)*	1 (50.0)	0.16	14.3 (0.7-29.4)	0	1.00	0.7 (0.1-15.6)*
Mixed (n = 15)	4 (26.7)	0.02	16.4 (1.7-161.3)	4 (26.7)	0.05	5.2 (1.0-26.8)	1 (6.7)	0.22	0.3 (0.1-2.2)
DILI Severity Grade									
Controls (n = 46)	1 (2.2)	-	-	3 (6.5)	-	-	10 (21.7)	-	-
Mild-to-moderate (n = 39)	16 (41.0)	< 0.01	31.3 (3.9-251.0)	7 (17.9)	0.12	3.1 (0.8-13.1)	2 (5.1)	0.04	0.2 (0.1-1.0)
Severe (n = 7)	1 (14.3)	0.17	2.7 (0.6-11.7)	3 (42.9)	0.01	10.8 (1.6-71.9)	1 (14.3)	0.65	0.6 (0.1-5.6)

*Haldane's modification; ART - Antiretroviral therapy; ATD - Anti-tubercular drugs; CI - Confidence Interval; DILI - Drug induced liver injury; *HLA* - Human leukocyte antigen; N - Number; OR - Odds ratio; *P* - *P* values

The result of high resolution typing for *HLA-B*57* allele carrier study participants is presented in Table 21. Of *HLA-B*57* alleles identified, *HLA-B*57:02* and *HLA-B*57:03* alleles accounted for 41.7 and 58.3%, respectively. The *HLA-B*57:03* and *HLA-B*57:02* allele frequencies in the cases (15.2 and 9.8%) were also higher than the controls (0 and 1.1%), respectively. The overall allele frequency of *HLA-B*57* was higher in the cases compared to the controls (25.0% vs. 1.1%).

Table 21: High resolution genotyping for *HLA-B*57* allele carriers' stratified by DILI types

Participant	Age	Sex	Status	DILI type	Allele 1	Allele 2
1	25	F	Case	Cholestatic	*57:03*	*57:03*
2	34	F	Case	Cholestatic	*57:03*	*57:03*
3	45	M	Case	Cholestatic	*57:03*	*57:03*
4	25	M	Case	Cholestatic	*57:02*	*57:02*
5	28	F	Case	Cholestatic	*57:03*	*58:01*
6	37	F	Case	Cholestatic	*57:02*	*51:08*
7	28	F	Case	Cholestatic	*57:03*	*49:01*
8	55	M	Case	Cholestatic	*57:02*	*39:12*
9	45	F	Case	Cholestatic	*57:02*	*58:01*
10	30	F	Case	Cholestatic	*57:03*	*53:01*
11	60	M	Case	Cholestatic	*57:03*	*13:02*
12	30	F	Case	Cholestatic	*57:03*	*53:01*
13	30	F	Case	Cholestatic	*57:02*	*44:02*
14	30	F	Case	Mixed	*57:03*	*57:03*
15	31	M	Case	Mixed	*57:02*	*57:02*
16	49	M	Case	Mixed	*57:03*	*58:01*
17	38	F	Case	Mixed	*57:02*	*49:01*
18	32	F	Control	None	*57:02*	*41:02*

ART - Antiretroviral therapy; ATD - Anti-tubercular drugs; DILI - Drug induced liver injury; *HLA* - Human leukocyte antigen

7. Discussion

As an effort to provide safer treatment options, genetic determinants of drug response are currently on the global health research agenda. In this broader and global context, in a larger prospective cohort study, Yimer and co-researchers investigated the genetic variants for ATD and ART-induced liver injury in TB/HIV infected patients in Ethiopia using candidate gene approach (Yimer *et al.*, 2012, Yimer *et al.*, 2011). In the present study, we investigated additional genetic markers for ATD and ART-induced liver toxicity using GWAS and replication approach in a total of 1,055 study participants from the prospective cohort study. We also investigated the association of *HLA-B* alleles with the risk of ATD and ART co-treatment induced liver injury. Identifying additional genetic risk variants could help develop genetic tests to prevent DILI, and to match the patients with alternative, effective and safe medications as well as for better monitoring. To our knowledge, this is the first study to conduct GWAS for ATD and ART-induced liver injury, and to report the association of *HLA-B*57* alleles with ATD and ART co-treatment induced liver injury among Ethiopians.

The incidence of ATD and ART-induced liver injury displays wide variability among populations. While receiving the same type of treatment, TB/HIV patients from Ethiopia presented a higher incidence of DILI compared with patients from other nations for example Tanzanians for ATD (4% vs. 1%), ART (9% vs. 6%) and ATD and ART co-treatment (18% vs. 10%) (Mugusi *et al.*, 2012, Tostmann *et al.*, 2010, Yimer *et al.*, 2008, Yimer *et al.*, 2014, Yimer *et al.*, 2011). Ethiopians also display a distinct pharmacogenetic profile and CYP enzyme activities compared with other populations. Higher *CYP3A* (Gebeyehu *et al.*, 2011), *CYP2A6* (Aklillu *et al.*, 2014), *CYP2B6* (Ngaimisi *et al.*, 2013) and *CYP2D6* (Aklillu *et al.*, 1996) enzyme activities, and unique distribution of the variant alleles in Ethiopians were reported. Indeed, the

PCA plot of the GWAS genotyping data in the present study also indicated distinct cluster for Ethiopians compared with the HapMap data for Caucasian, Chinese, Japanese, and Nigerian. This finding further substantiates the need for more population-specific pharmacogenetic studies in Ethiopians to identify genetic markers for drug-induced adverse effects such as liver injury.

In the current study, the top SNP (rs10946737) in the GWAS and the combined analysis of ATD-induced liver injury was found in the intron of *FAM65B* gene. The product of this gene play roles in myoblast differentiation, and it is up-regulated during early stage of the process (Yoon *et al.*, 2007). Inhibition of expression of this gene in myoblasts causes marked decrease in myogenin expression with consequent lack of myoblast differentiation and muscle regeneration; and its over-expression induces formation of cellular protrusions (Balasubramanian *et al.*, 2014). *FAM65B* protein expression and phosphorylation also finely tune T-lymphocyte motility (Froehlich *et al.*, 2016, Megrelis *et al.*, 2018). Although the *FAM65B* gene is known to play a role in liver-associated inflammatory diseases (Stoyanov *et al.*, 2015), only a few studies have investigated the relationship between genetic polymorphisms in *FAM65B* and human diseases (Diaz-Horta *et al.*, 2014, Pan *et al.*, 2019).

After we reported our GWAS finding on the possible association of rs10946737 in the *FAM65B* gene with ATD-induced liver injury (**Paper-I**), a recent case-control study was carried out in Han Chinese TB patients on ATD treatment to validate our findings (Pan *et al.*, 2019). The researchers reported that patients with polymorphisms at rs10946737 in the *FAM65B* gene were at increased risk of moderate-to-severe liver injury ($P = 0.032$, OR = 2.15, 95% CI = 1.07-4.32). This finding is consistent with the results of our GWAS in ATD treatment group. The researchers also carried out expression quantitative trait loci (eQTL) analysis of rs10946737 with the expression of nearby genes in liver samples and showed that the 'A' allele was associated

significantly with an elevated expression of a specific protein (Pan *et al.*, 2019). Future studies in larger and varied populations may help validate this relationship and elucidate the functional importance of polymorphisms in *FAM65B* for the development of ATD-induced liver injury.

In our study, another possible association with ATD-induced liver injury was identified by a cluster of four SNPs in the intron of *AGBL4* with *P*-values suggestive of genome-wide significance. This gene encodes an enzyme that catalyzes deglutamylation of polyglutamate side chains generated by post-translational modification of target proteins such as tubulins in microtubules (Rodriguez *et al.*, 2013). The Han Chinese validation study did not reproduce the relationship on the SNPs of *AGBL4* (Pan *et al.*, 2019).

Some genetic variations are specific to a population with particular ancestry, and allele frequencies for the genetic variants may vary across populations (McCarthy *et al.*, 2008) which in turn make difference in their risk for diseases (Pearson and Manolio, 2008) and present challenges for replication findings (Kraft *et al.*, 2009). Thus, the lack of replication with the SNPs in the *AGBL4* gene in the Han Chinese study may be due to the allele frequency difference between the two populations. For example, the MAF of the SNP rs320035 in Han Chinese is 21%; whereas, it is 48% in our study cohort. Similar differences in the MAFs were observed in the other suggestive genome-wide significant SNPs in the *AGBL4* gene. Further analysis is required to explain the role of *AGBL4* gene and its contribution to individual differences for susceptibility to ATD-induced liver injury.

It is increasingly evident that genetic variants can determine an individual's susceptibility to develop a particular pattern of liver injury (Padda *et al.*, 2011). Therefore, we performed sub-group GWAS analysis based on the pattern of ATD-induced liver injury. The SNP (rs1990046)

with the lowest *P*-value after adjustment for covariates was identified in the hepatocellular type of ATD-induced liver injury. This SNP is located in the intron region of *SEMA3A*, a member of the semaphorin family. This gene encodes a protein which is vital for normal neuronal pattern development (Boczek *et al.*, 2014), and also plays a role in the pathogenesis of allergic conditions (Vadasz *et al.*, 2014). Further studies are required to elucidate the roles of *SEMA3A* gene in hepatocellular pattern of ATD-induced liver injury.

In the present study, we identified SNPs that are suggestive of importance to predict ART-induced liver injury. The top SNP (rs199650082) in the combined analysis of ART treatment group was a missense SNP (2756G>A [R919Q]) in *ERN1* gene. This gene encodes inositol-requiring enzyme-1 alpha (IRE1α), a trans-membrane protein. This protein contains two functional catalytic domains (protein kinase and endoribonuclease), important in altering gene expression in response to ER stress signals (Chen *et al.*, 2014a, Itzhak *et al.*, 2014).

ER stress is an important intracellular stress pathway in hepatocytes that can result in cell death (Dara *et al.*, 2011). It occurs when ER homeostasis is disturbed with accumulation of unfolded/misfolded proteins due to stress signals such as ROS, drugs or toxins (Shehu *et al.*, 2017). ER stress has recently been shown to have an important role in the pathogenesis of EFV-based ART-induced liver injury (Apostolova *et al.*, 2017, Chen *et al.*, 2014b, Foufelle and Fromenty, 2016). The IRE1α protein functions as a key sensor of unfolded proteins in the ER and triggers a series of adaptive cellular responses to the ER stress termed as unfolded protein response (UPR) that help restore the proper functions of ER (Maiers and Malhi, 2019).

When an ER stress occurs, the cell initiates the UPR (Abdullah and Ravanan, 2018, Liu and Green, 2019). It starts with activation of IRE1α which possesses a kinase activity leading to its

autophosphorylation and activation of the ribonuclease activity. This leads to splicing of X-box binding protein-1 (XBP1) mRNA, which is translated into an active transcription factor that up-regulates expression of target genes to promote protein folding, degradation of misfolded proteins, restoration of ER homeostasis and promotion of cell survival (Liu and Green, 2019, Maiers and Malhi, 2019). IRE1α also plays a crucial role in liver regeneration following damage caused by various toxic agents (Rashid *et al.*, 2017, Sano and Reed, 2013). In our study, the ARV drugs might have attributed to the ER stress, and polymorphism in *ERN1* gene that encodes the IRE1α protein (which plays a major role in UPR pathway) might have contributed to individual differences in response to the ER stress, predisposing to ART-induced liver injury.

Another top SNP (rs16947045), a coding sequence variant, identified in the combined analysis of ART treatment group was located in mitogen-activated protein kinase-3 (*MAP3K3*). This gene directly regulates stress-activated protein kinase pathway that participate in the regulation of cellular responses to various stress signals (Ellinger-Ziegelbauer *et al.*, 1997). Although further analysis is required to clarify functional importance of *ERN1* and *MAP3K3* genes, altered proteins encoded by these genes or variations in protein expression may contribute to individual differences for susceptibility to ART-induced liver injury.

In the present study of ATD and ART co-treatment group, the top SNP (rs4842407) had a consistently strong signal in the GWAS, the replication study and the combined analyses, both before and after adjustment for covariates. This SNP is a long intergenic non-coding RNA (*lincRNA*) transcript variant located between LOC642550 and Synaptotagmin-1 genes on chromosome-12 (**Paper-II**). *LincRNAs* are non-protein coding RNA transcripts with longer than 200 nucleotides that exhibit various biological functions and have gained attention recently (Ransohoff *et al.*, 2018, Shi *et al.*, 2013). They may broadly serve to fine-tune the expression of

neighboring genes with notable tissue specificity (Ransohoff *et al.*, 2018). *LincRNAs* also play roles in alternative splicing, protein stability, and modulating signal transduction pathways (Noh *et al.*, 2018, Sun *et al.*, 2018).

Previous GWAS revealed some SNPs within *lincRNAs* to be disease associated (Hirano *et al.*, 2015, Radtke *et al.*, 2009). A *lincRNA* was also reported to be involved in the progression of liver fibrosis (Yu *et al.*, 2016). In fact, there was also a recent report of a *lincRNA* variant significantly associated with ATD-induced liver injury (Li *et al.*, 2017). The suggestive genome-wide significant association of a *lincRNA* transcript variant (rs4842407) in our study may indicate its relevance in determining predisposition to DILI in TB/HIV co-infected patients receiving ATD and ART co-treatment. Alternatively, this SNP may be in LD with other SNPs that are implicated in the development of DILI. If this finding is replicated in larger sample sizes, the SNP may serve as potential genetic biomarker for susceptibility to ATD and ART co-treatment induced liver injury.

CGAS conducted on genetic risk factors contributing to ATD and/or ART-induced liver injury identified genetic variants in genes involved in drug metabolism (*NAT2, CYP2B6*) (Mugusi *et al.*, 2012, Richardson *et al.*, 2019, Yimer *et al.*, 2011), drug transporter (*ABCB1, SLCO1B1*) (Chen *et al.*, 2015a, Yimer *et al.*, 2011), HLA region (Huang, 2014, Phillips *et al.*, 2013, Sharma *et al.*, 2002), in genes related to oxidative stress (*SOD2*) (Chen *et al.*, 2015b, Huang *et al.*, 2007, Yew *et al.*, 2018) and autoimmune diseases (Urban *et al.*, 2012). In the current GWAS, we did not find SNPs that passed genome-wide significance in pharmacokinetic, immune system or oxidative stress-related genes, which may be related to the limited sample sizes used for the study. But we found some SNPs with lower *P*-values: rs12969241 located in *PTPN2*, rs11642957 in *ABCC1*, and rs9276370 in the vicinity of *HLA-DQA2* in ATD, ART, and

ATD/ART co-treatment groups, respectively, and may be important in the development of DILI. The SNP rs12969241 in the *PTPN2* gene was also among the top SNPs in the GWAS of cholestatic pattern of ATD-induced liver injury (Table 17). The protein encoded by *PTPN2* gene is an intracellular tyrosine specific phosphatase expressed in epithelial cells (Moron *et al.*, 2013). This protein was shown to play an important role in epithelial barrier function during inflammation by acting as negative regulator of pro-inflammatory cytokine interferon-γ (Penrose *et al.*, 2013). This finding may support the role of an immune related mechanism in ATD-induced liver injury.

The SNP rs2842997 in the vicinity of *SOD2*, and rs12543818 near *NAT2* for genes related to oxidative stress and pharmacokinetics, respectively, were also among the SNPs with lower *P*-values in the ATD treatment group. The product of *SOD2* gene detoxifies highly reactive superoxide radicals generated by mitochondrial respiration (Candas and Li, 2014). This finding is in line with a previous CGAS (Huang *et al.*, 2007), which reported common polymorphisms in *SOD2* as predictor of ATD-induced liver injury. CGAS reports showed that among genes relevant to drug metabolism, the best replicated association is between *NAT2* slow acetylation status and ATD-induced liver injury (Daly, 2010a, Khan *et al.*, 2019). From the findings of the current study, we think that DILI susceptibility due to ATD and/or ART may be related to the combined effect of the newly identified variants, pharmacokinetic, oxidative stress, and immune-related gene variants.

In our HLA typing study, we investigated the association of *HLA-B* alleles with the risk of developing liver toxicity in the ATD and ART co-treatment group (**Paper-III**). The matched case-control design used in this study minimizes effects of potential confounders and may increase power to identify associations. We found that the proportion of *HLA-B*57* allele carriers

who developed liver injury was significantly higher compared with the controls. This may indicate that *HLA-B*57* allele carriers might be at a higher risk of developing liver injury. Thus, the *HLA-B*57* allele may play an important role in the pathogenesis of immune-mediated liver toxicity, and could be a potential predictor for ATD/ART co-treatment induced liver injury.

Our HLA study also indicated positive associations of *HLA-B*57* allele with mild-to-moderate liver injury, and *HLA-B*14* allele with severe liver injury. On the other hand, *HLA-B*41* allele was negatively associated with mild-to-moderate liver injury. These findings show that the association of *HLA-B* alleles may depend on the severity of liver injury. The *HLA-B*57* allele may be important for the initiation of immune response to cause DILI and the *HLA-B*14* allele for progression to severe degree of liver injury. On the other hand, the *HLA-B*41* allele seems to play a role in prevention of development of mild-to-moderate liver injury. These findings require further investigation in a larger number of case samples for each severity grade of liver injury.

The frequency and subtypes of *HLA-B*57* alleles display inter-ethnic variability globally, ranging from 0-22.5% (http://www.allelefrequencies.net/). *HLA-B*57* allele frequency was shown in up to 5% in Asians and Caucasians. *HLA-B*57:03* and *HLA-B*57:02* alleles commonly occur in black population reaching up to 3 and 7% allele frequencies, respectively. Allele frequency of *HLA-B*57* in our study population was 13% which is relatively high. *HLA-B*57:01* allele was not detected, and it may be rare or absent in Ethiopians similar to other black Africans where the allele frequency is less than 1%. The *HLA-B*57:03* and *HLA-B*57:02* allele frequencies in our study population were 7.6 and 5.4%, respectively, although the frequencies in the general population of Ethiopians is yet unknown.

There are some limitations in the current study. First, as DILI is relatively rare event, identifying a large number of affected individuals was a constraint. This resulted in a relatively small number of case samples for sub-group analysis. We estimated power of the study using QUANTO (Gauderman, 2002), and determined what magnitude of effect, in terms of OR, would likely have been missed in our analysis. The overall power estimated was 80, 90 and 97% to detect effects with OR 3.3, 3.6 and 4.0, respectively, considering 10% prevalence of DILI, 15% risk allele frequency and $\alpha = 7.5x10^{-8}$ genome-wide significance level. The study was well powered to detect common variants with fairly large effect sizes.

Second, populations of African ancestry display greater genetic diversity and lower LD among chromosomal loci compared with non-African populations (Campbell and Tishkoff, 2008, Teo *et al.*, 2010). Although the low levels of LD in African ancestry is considered as a powerful tool for fine mapping of causal variants (Peprah *et al.*, 2015), it is disadvantageous when screening the genome for associations using the current SNP-genotyping approach for GWAS which essentially relies on the principle of LD mapping (Hirschhorn and Daly, 2005). However, our exploratory study for the discovery of new DILI biomarkers represents an important first step in applying GWAS to identify the variants in African populations.

The third limitation is that as drug combinations are the current treatment protocols for TB/HIV infections, we cannot say that the risk variants identified correspond only to a single drug. As treatment of TB/HIV infection consists of combination therapy, it is not possible to study individual ATD or ARV drugs for ethical reasons. Thus, identification of genetic risk factors for ATD and ART-induced liver injury is important for future clinical applications, particularly in the current situation in Africa where ATD and ART being extensively used. Identifying individuals at risk for DILI before drug treatment would improve drug safety.

8. Conclusions

The results of the GWAS provide evidence that additional genetic variants may also contribute to the risk of developing ATD and ART-induced liver injury other than what has been discovered through CGAS. The identified genetic variants in the current study with suggestive genome-wide significance include:-

- rs10946737 in the intron of *FAM65B* gene, and a cluster of SNPs (rs320035, rs393994, rs319952, and rs320003) in the intron of *AGBL4* with ATD-induced liver injury
- rs199650082, a missense SNP in the *ERN1* with ART-induced liver injury
- rs4842407, *lincRNA* transcript variant with ATD/ART co-treatment induced liver injury

Using HLA-B typing study, we also identified *HLA-B*57* allele as a genetic risk variant for the development of ATD & ART co-treatment induced liver injury.

9. Recommendations

Replication of research findings is a common approach for validation of scientific discoveries. Replication studies are essential to confirm associations discovered via GWAS, provide convincing statistical evidence for association and help rule out associations due to biases. As validation can improve the credibility of an association study, we recommend subsequent replication studies on larger scale to verify the genetic risk variants identified in our study. Upon successful replication of the findings, fine mapping to identify the casual variants could further be considered.

GWAS explores common SNPs that account only for a portion of the genetic variations using the current SNP-Chip array platforms. Therefore, studies such as next-generation sequencing (NGS) that explores DNA sequence variations in the entire genome including rare variants (Sazonovs and Barrett, 2018) should also be given attention. Alternatively, it is important to explore variants through exome sequencing as a likely source for the discovery of rare variants.

Identifying non-genetic biomarkers to predict individuals at risk for DILI may have a potential for prevention of DILI. Large-scale 'omics' technologies (transcriptomics, proteomics, metabolomics) besides genomics may offer the potential to reveal biomarkers for idiosyncratic DILI (Fontana, 2014, Mikus *et al.*, 2017). The integration of data from different research platforms (genomics and other omics) may provide an opportunity to identify biomarkers for the prediction of ATD and ART-induced liver injury.

10. References

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11. Annexes

Annex I: Equipment, materials and chemicals

Equipment and materials

- PicoGreen spectrofluorometer
- Beckman Coulter Biomek liquid handlers
- ABI prism 7900HT Sequence Detection System (SDS)
- Hybridization oven, and Hybex micro-sample incubator
- Illumina iScan reader and Genome Studio software
- Infinium HD BeadChips
- Thermal cyclers
- Barcode printer
- Fluotrac flat bottom plate, DNA plates, cap mats, and adhesive film seal
- Vacuum desiccator, Wash dish and staining rack
- Electrophoresis tank and UV trans-illuminator
- Multichannel micropipettes
- Polymerase chain reaction (PCR) tubes
- 96-well 0.8 ml microtiter plate and 384-well plates
- Measuring cylinders, micro-plate shaker, and absorbent pad
- Multi-sample BeadChip alignment fixture, chamber gasket and insert
- Flow through chamber (with black frames, spacer, glass back plate, and clamp)
- Water bath with flow-through rack, tube rack, cleaning brush, dismantling flat spatula
- BeadChip carriers, heat sealer, and slide storage box

Chemicals

- PicoGreen reagent
- Ethylene-diamine-tetraacetic acid (EDTA)
- TE (10mN Tris-HCl [tris (hydroxymethyl) aminomethane-HCl] pH 8.0 in 1mM EDTA)
- TBE buffer (Tris-borate, EDTA pH 8), NaOH (0.1N)
- Isopropanol, 100% ethanol, 95% formamide with 1 mM EDTA, deionized distilled water
- Agarose gel powder, Ethidium bromide, marker DNA, loading dye
- Infinium kit (Illumina® reagents: multi-sample amplification mix 1, mix 2 and master mix (MA1, MA2, MSM), fragmentation solution (FMS), precipitation solution (PM1); re-suspension, hybridization, and wash solution (RA1); humidifying buffer (PB2), two-color extension master mix (TEM), anti-stain two-color extension master mix (ATM), hybridization preparation buffer (PB1), superior two-color master mix (STM) and XStain BeadChip solution (XC1-4).
- Hot start (HS) Taq DNA polymerase, primers
- Deoxynucleotide triphosphates (dNTP) mix [dATP, dCTP, dGTP, and dTTP]
- Invader set (buffer, fluorescence resonance energy transfer (FRET) probes mix, Cleavase, allele probes (FAM and VIC), and invader probe)
- Olerup SSP® HLA typing kit [containing dried pre-optimized sequence-specific primers (SSP) for PCR amplification of HLA alleles, PCR Master Mix and adhesive seals]

Annex II: DNA quantification and whole genome genotyping protocol

DNA quantification

PicoGreen assay with spectrofluorometer was used to quantify double-stranded DNA samples according to Illumina Infinium® HD assay protocol.

PicoGreen DNA quantification steps:-

- PicoGreen was thawed to room temperature, and microtiter plate (Standard DNA) and two fluotrac plates (Standard QNT and Sample QNT) were hand-labeled.
- Serial of dilution in the Standard plate were made from 233.3 µL stock lambda DNA (75 ηg/µL) at A1 using 1X TE. i.e., after adding 1X TE 66.7 µl to B1 and 100 µl to C1- H1, 133.3 µL of Lambda DNA was transferred from A1 into B1. Then 100 µl was transferred from B1 into C1. The 100 µL transfer was repeated for D1 to G1. H1 served as the blank.
- Dilute PicoGreen (1:200 in 1X TE for 96-wells) was prepared.
- 195 µL diluted PicoGreen, and 2 µL of stock lambda DNA dilutions (Standard DNA plate) were transferred into each well of columns 1 and 2 of Standard QNT fluotrac plate, and the plate was covered and centrifuged at 280×g for 1 min.
- 195 µL dilute PicoGreen, and 2 µL of stock DNA sample were transferred into 96-wells of Sample QNT fluotrac plate, and the plate was covered and centrifuged (280×g for 1 min).
- The Standard QNT plate was placed into the spectrofluorometer (SoftMax program), and a standard curve graph was viewed from the known concentrations.
- The Sample QNT plate was loaded and read. An output data file (*.pda) was saved, and exported as text file to open in Microsoft Excel.
- Ninety-nine point six percent of the genomic DNA samples met the minimal concentration required for whole genome genotyping (400 ηg); four samples were excluded.

Whole genome genotyping

Genome-wide SNP genotyping was done using Illumina Omni Express Exome BeadChip genotyping array (Illumina Inc., San Diego, CA, USA), according to the manufacturer's protocol. The protocol employs single-base extension (SBE) to achieve allele discrimination. Locus-specific probes selectively hybridize to the SNP of interest, stopping one base before the interrogated SNP. Specificity is subsequently conferred by enzymatic SBE, which incorporates terminating biotinylated ATP and TTP and terminating dinitrophenolated CTP and GTP nucleotides. Dual-color florescent staining is then used to detect the incorporated, modified nucleotide(s) with cyanine-3 labeled streptavidin and cyanine-5 labeled anti-dinitrophenol antibodies binding to the A and T, and C and G nucleotides, respectively. Genotype calls are derived from allele-specific fluorescence signals that are read-out using a microarray scanner. The red and green color signals specify each allele, where homozygotes are indicated by red/red or green/green signals, and heterozygotes are indicated by yellow (red/green) signals (Lambert *et al.*, 2013).

The Assay workflow is divided into 3 sections: (i) Sample preparation & DNA amplification, (ii) DNA fragmentation & hybridization, and (iii) Single-base extension (SBE), staining & scanning.

Sample preparation and DNA amplification

- Whole genome (WG)-DNA barcode was applied to a new microtiter plate
- From stock DNA samples, 20 µL of 50 ηg/µL was prepared by re-suspended in 1X TE
- Multi-sample amplification mix 1, mix 2 and master mix (MA1, MA2, MSM) were thawed
- MSA1 barcode was applied to a new 0.8 mL microtiter plate ('reaction plate' = 'MSA1 plate')
- 20 µL MA1, and 4 µL DNA sample were transferred into each well of the MSA1 plate

- 4 µL 0.1N NaOH was dispensed into each well of the MSA1 plate, sealed, vortexed (1600 rpm - 1 min), and incubated for 10 min at room temperature.
- 68 µL MA2 & 75 µl MSM were dispensed into each well MSA1, sealed, vortexed as above
- The MSA1 plate was placed in Illumina hybridization oven (37^{o}C) and incubated (24 hr)

DNA fragmentation, precipitation, Re-suspension and hybridization

Fragmentation, precipitation and re-suspension

- Fragmentation solution (FMS) was thawed, and pulse centrifuged at room temperature.
- The reaction plate was removed from the oven, centrifuged, and the cap mat removed.
- 50 µL FMS was added to each well, mixed, sealed and vortexed (1600 rpm for 1 min)
- The sealed plate was placed at 37°C in the Hybex micro-sample incubator for 1 hr.
- Precipitation solution (PM1) was allowed to equilibrate, and pulse centrifuged.
- The plate was removed from the incubator, and 100 µL PM1 was added to each well, sealed, vortexed (1600 rpm for 1 min) and incubated (37^{o}C for 5 min).
- 300 µL 2-propanol (100%) was added to each well, sealed, mixed thoroughly, spin down and incubated (4°C for 30 min), then the plate was centrifuged (3,000×g at 4°C for 20 min).
- The supernatant was decanted by quickly inverting the plate, dumping the liquid into a sink then firmly tapping onto an absorbent pad several times. The plate was then left uncovered and inverted on a tube rack for 1 hr at room temperature to dry the pellets.
- Re-suspension, hybridization and wash solution (RA1) was thawed, pulse centrifuged.
- 46 µL RA1 was added to each well of the MSA1 plate and sealed.
- The plate was vortexed (1800 rpm for 1 min) and left at room temperature.

Hybridization

- 400 µL humidifying buffer (PB2) was added into Hyb reservoirs, and covered with a lid.
- The MSA1 plate was placed on Hybex micro-sample incubator (95°C for 20 min) then removed and placed on bench top for 30 min, pulse-centrifuged, and the foil seal removed.
- BeadChip was removed from the package, and placed in Hyb Chamber insert.
- For 8x1 HD BeadChip, 26 µL of each DNA sample was dispensed onto BeadChip section.
- The Chamber insert was placed inside the Hyb Chamber, then kept into Illumina hybridization oven and incubated (48°C for 20 hrs, rocker speed set to five).

SBE, staining and scanning

Washing BeadChip

- The Hyb Chamber was removed from the Illumina Hybridization oven and cooled.
- Two wash dishes were filled with 200 mL of hybridization preparation buffer (PB1).
- Wire handle was attached to a wash rack and submerged the rack in the first wash dish.
- BeadChip was removed from Hyb Chamber insert, and IntelliHyb Seal from BeadChip.
- The BeadChip was put immediately into wash rack, completely submerged in PB1.
- The wash rack was moved up and down for 1 min with gentle agitation; and removed and placed immediately into the other PB1 wash dish.

Assembling flow-through chamber

- BeadChip frame was placed into BeadChip alignment fixture filled with 150 mL PB1, and the BeadChip was placed into the frame fully immersed in PB1.
- Clear spacer was placed on top of BeadChip using the grooves in the alignment fixture.
- Clean glass back plate was placed on top of the spacer covering the BeadChip to create a reservoir against the BeadChip surface.

- Two metal clamps were attached onto each flow-through chamber, and the assembled flow-through chambers were removed from the alignment fixture.
- Water circulator reservoir was filled with water to the appropriate level, and set to a temperature that brought Chamber rack to 44°C.
- Re-suspension, hybridization, and wash solution (RA1), 95% formamide with 1 mM EDTA, XStain BeadChip solutions (XC1-4), two-color extension master mix (TEM), superior two-color master mix (STM), and anti-stain two-color extension master mix (ATM) were thawed.

Extension and staining BeadChip

- Flow-through chamber was placed into the Chamber rack with the reservoir facing up.
- Into the reservoir of each flow-through chamber: 150 µL RA1 was added and incubated for 30 sec, repeated 5X; 450 µL XC1 was added and incubated for 10 min; 450 µL XC2 was added and incubated for 10 min; 200 µL TEM was added and incubated for 15 min; 450 µL 95% formamide with 1 mM EDTA was added and incubated for 1 min; incubated for 5 min.
- 450 µL XC3 was added, and incubated for 1 min; repeated once.
- Into the reservoir of the flow-through chamber, 250 µL STM was added and incubated for 10 min; 450 µL XC3 was added and incubated for 1 min, repeated once, and then incubated for 5 min; 250 µL ATM was added and incubated for 10 min; 450 µL XC3 was added and incubated for 1 min, the above steps repeated one more time
- 250 µL STM was added and incubated for 10 min; 450 µL XC3 was added and incubated for 1 min, repeated once, and then waited 5 min
- The flow-through chamber was removed from the chamber rack and placed horizontally onto an absorbent pad on a lab bench.

Washing and Coating BeadChip

- XC4 reagent was re-suspended with 330 mL 100% ethanol, and vigorously shaken
- Two wash dishes were set up: 300 mL of hybridization preparation buffer (PB1) and 300 mL of XC4 were poured to wash dishes labeled 'PB1' and 'XC4', respectively.
- Flow-through chamber was disassembled to remove the clamp and the BeadChip.
- The BeadChip was kept on a staining rack, then the rack was moved up and down repeatedly, and then the BeadChip was soaked for additional 5 min.
- Staining rack was removed from PB1 dish, and placed into XC4 containing dish, and then the rack moved up and down repeatedly, and soaked the chip for additional 5 min.
- Clean tube rack was prepared by placing kimwipes under tube rack.
- The BeadChip was removed from the staining rack, and placed on the tube rack.
- The tube rack was placed in a vacuum desiccator, and the BeadChip was dried for 1 hr.
- The BeadChip was scanned on the iScan system within 72 hrs.

Imaging the BeadChip on iScan

- When iScan Reader was turned on, the iScan control Software application started.
- The BeadChip was placed into the iScan Reader tray, and scanned. As BeadChip section scanned its status was indicated by green color (scan and registration successful).
- After images scanned and registered, intensities were extracted for every bead type and saved in intensity data (*.idat) files on the iScan Reader hard drive and network under the array ID (barcode) in the Output Path folder.
- Genotyping data from intensity data files created by the Illumina iScan system were extracted from Illumina Genome Studio software package.

Annex III: Primers and probes

	Oligo sequence (5' to 3')	Oligo name
1	ATGGCAAGTCCTTCAAGGTG	rs10946737_F1
2	ATGACATGCTCCTTCCCAAG	rs10946737_R1
3	CTTCAAATCAACCACTTTCTTTCAATATCCTGGAGGCAGTGCT	rs10946737_INV1
4	CGCGCCGAGGAGACACGGACAGAATGTC	rs10946737_A_VIC1
5	ATGACGTGGCAGACGGACACGGACAGAATGT	rs10946737_G_FAM1
6	GGCCATACCCAAGCTGATAA	rs320035_F1
7	TTGCTTGTTGCCATTGAGAG	rs320035_R1
8	CTGGTCCTTTTCTTATCAGAATTCAGCTGTTTCCACCACAGTAAT	rs320035_INV1
9	CGCGCCGAGGATAATGGACAAATTGGGCATC	rs320035_A_VIC1
10	ATGACGTGGCAGACGTAATGGACAAATTGGGCAT	rs320035_G_FAM1
11	GGGGGCACCTTTTTGTTATT	rs393994_F1
12	CTGGCAAGGCTCTCACCTAC	rs393994_R1
13	CAGCTAACATGTGTAGCACTTATCTCAATCTAGATGGTGTCCTA	rs393994_INV1
14	CGCGCCGAGGTGGCATTTTACCTGTATGATTTC	rs393994_T_VIC1
15	ATGACGTGGCAGACCGGCATTTTACCTGTATGATTT	rs393994_C_FAM1
16	TGGATGGTGTTGTTGGTAGC	rs319952_F1
17	GTGCTGCAGTGTGTGTTGTG	rs319952_R1
18	AAGGATCTGGGACCTAGGAGTCTTGAGGGATGAAA	rs319952_INV1
19	CGCGCCGAGGTATTGTAACTTCAGGTATTACAGAG	rs319952_T_VIC1
20	ATGACGTGGCAGACCATTGTAACTTCAGGTATTACAG	rs319952_C_FAM1
21	TAGTTCCCATCCCTGAGTGG	rs320003_F1
22	TGCCTGCATTGTGGATCTAA	rs320003_R1
23	GAGACCAGCCCTAGACCTTACACAGCTCT	rs320003_INV1
24	CGCGCCGAGGATGGTATAAAGAGGGAAAAACAAATAT	rs320003_A_VIC1
25	ATGACGTGGCAGACGTGGTATAAAGAGGGAAAAACA	rs320003_G_FAM1
26	CGTTTCCTAGGGCAGTCTCA	rs7708937_F1
27	AATCTCCAGGCTTCCTTTCC	rs7708937_R1
28	TGGAAGATTTGTGGGGAGGATCCAAACCATCTTCCTT	rs7708937_INV1
29	CGCGCCGAGGATTTACATCAGGTCTTTGGTC	rs7708937_A_VIC1
30	ATGACGTGGCAGACGTTTACATCAGGTCTTTGGT	rs7708937_G_FAM1
31	TTGGCCCCTGTTTATCTGTC	rs591141_F1
32	TGTGCCTGTACTCAGGATGC	rs591141_R1
33	TCCACCAGTCTTGAGGCACAGTGAACCACA	rs591141_INV1
34	CGCGCCGAGGTTTCAGTCTCTTCAGTGAGTG	rs591141_T_VIC1
35	ATGACGTGGCAGACCTTCAGTCTCTTCAGTGAG	rs591141_C_FAM1
36	AAGACTTCCTGCCACCTGTCT	rs2089910_F1
37	AGAAAGCACTGAAAGCCACAG	rs2089910_R1
38	TTACCCCAGCAACTCTTCCCGCTCCTCT	rs2089910_INV1

	Oligo sequence (5' to 3')	Oligo name
39	CGCGCCGAGGAGCACCCGGGTCAC	rs2089910_A_VIC1
40	ATGACGTGGCAGACGGCACCCGGGTC	rs2089910_G_FAM1
41	CCAGGCTGTTGGTTTCATTT	rs2069912_F1
42	TCCTCCCCCAACCAAGTAT	rs2069912_R1
43	AGCTCAGGCATACCCTCCTCTAGGATGCCTTTA	rs2069912_INV1
44	CGCGCCGAGGTCCCCCATCCCTTCTTG	rs2069912_T_VIC1
45	ATGACGTGGCAGACCCCCCCCATCCCTTC	rs2069912_C_FAM1
46	AGACCACGGGTCTTTCTTGA	rs239319_F1
47	CAGCTTGTCCCAGACACTCA	rs239319_R1
48	CCACAGGCTATGGTACGGGAGACCCATA	rs239319_INV1
49	CGCGCCGAGGTCCTCCCAGAAAATGGTG	rs239319_T_VIC1
50	ATGACGTGGCAGACCCCTCCCAGAAAATGGT	rs239319_C_FAM1
51	CCCAATTCAGCCCTTTTGTA	rs3959930_F1
52	AAAATCCCACCAAACATAGCC	rs3959930_R1
53	GCTACATCTTCAAGTAGATTTTTCTGGAAGCTCACATGGGTTAAATATTTCCA	rs3959930_INV1
54	CGCGCCGAGGTAAGACTTAAATATTTGAGAATAACTTTCTATT	rs3959930_T_VIC1
55	ATGACGTGGCAGACCAAGACTTAAATATTTGAGAATAACTTTC	rs3959930_C_FAM1
56	ATGAGAGCTGGAGACCCAGA	rs4733759_F1
57	TGAGCTGCAGTTGGCATCTA	rs4733759_R1
58	CTGGGCATCAACTGTCAGGACATGGACAGAGAA	rs4733759_INV1
59	CGCGCCGAGGTGTCTGCCATTAGAAGCTG	rs4733759_T_VIC1
60	ATGACGTGGCAGACCGTCTGCCATTAGAAGC	rs4733759_C_FAM1
61	TGTTACACACTGGGGTTGGA	rs2835109_F1
62	GCGTGGCCTCTGAGTTACAT	rs2835109_R1
63	TGGGGACAGGGATGGGATCTGGCATATGA	rs2835109_INV1
64	CGCGCCGAGGTAAGAGGAAACAGCCAACC	rs2835109_T_VIC1
65	ATGACGTGGCAGACCAAGAGGAAACAGCCAAC	rs2835109_C_FAM1
66	AAAGAAATGGGGGAAAATGG	rs4903067_F1
67	AGGCTGGTCTTGAACTCCTG	rs4903067_R1
68	TCACCAGGCCCTGTTTTAAG	rs3892834_F1
69	ATTGGGAAACCTGGGAAGAG	rs3892834_R1
70	CCAAGGCAATCCTAAGCAA	rs10946739_F1
71	CACAATTAGAAATGCTGAACAA	rs10946739_R1
72	GTAACTAAAACAGCATGGTACTGGTACAAATCTGGATTTACATGTAAAACCTC ATGATTTTAAAACAAA	rs10946739_INV1
73	CGCGCCGAGGTGACAGCAATTCAAACTTAAATATCC	rs10946739_VIC1
74	ATGACGTGGCAGACCGACAGCAATTCAAACTTAAATATC	rs10946739_FAM1
75	GGCATCAAGCTCTAATTGTGG	rs199650082_F1
76	AGGCGACTTTACTCCTCCTGA	rs199650082_R1
77	GGAGGGACCCCAGCGTCTCCA	rs199650082_INV1
78	CGCGCCGAGGTGCACCTCTGCAGG	rs199650082_T_VIC1

	Oligo sequence (5' to 3')	Oligo name
79	ATGACGTGGCAGACCGCACCTCTGCAGG	rs199650082_C_FAM1
80	TTGCACATGGAGAATCTGGA	rs7804397_F1
81	TGGGATTAGAGCAGAGCCAGT	rs7804397_R1
82	CTTCCCAGAAGAGATTTTAGTGTATCCTATAAAGGCACTAAAAGGCTTATTTTTGTTTTTGTTTGTTTTTGTA	rs7804397_INV1
83	CGCGCCGAGGTGGGGGTGTTTTGTTCTC	rs7804397_T_VIC1
84	ATGACGTGGCAGACGGGGGGTGTTTTGTTCT	rs7804397_G_FAM1
85	CTCGTGACCTACAGGGAACTG	rs196911_F1
86	GAGCAGCCTCTTCATTTCTGA	rs196911_R1
87	GGAGTTCAGAGGGTGTCGTCCTGCAGTT	rs196911_INV1
88	CGCGCCGAGGATGAGATTTCTCATTGATCACAG	rs196911_A_VIC1
89	ATGACGTGGCAGACGTGAGATTTCTCATTGATCACA	rs196911_G_FAM1
90	TGTGTGGTGAGTGTAGGTCCA	rs16947045_F1
91	GGAGAAGCAGCAACTTCACTG	rs16947045_R1
92	GTGGTGGAGATGCTGACAGAGAAACCACCA	rs16947045_INV1
93	CGCGCCGAGGTTGGGCAGAGTATGAAGC	rs16947045_T_VIC1
94	ATGACGTGGCAGACCTGGGCAGAGTATGAAG	rs16947045_C_FAM1
95	GGTTTATGCTTTCCTTCATCCA	rs32498_F1
96	TCATACACAGGAGCCCATTG	rs32498_R1
97	ATTTTCAACATCAGGCTATTAATAATATTTGATGGGAATAAAGGTTGTTATGGTGATCTCTGAATTCC	rs32498_INV1
98	CGCGCCGAGGTTTTTGTCAGTGATAAAGTCTACAC	rs32498_T_VIC1
99	ATGACGTGGCAGACCTTTTGTCAGTGATAAAGTCTAC	rs32498_C_FAM1
100	GTCCACTTTACTTGTCATAGTGCTTAG	rs7196606_F1
101	TTTGCACATCAAGCGTACTT	rs7196606_R1
102	ACACAGTTCAGTAGCATTAAGTATATTGATATTCTTGTGCAATAATGAAGAGTTTGTGCAGT	rs7196606_INV1
103	CGCGCCGAGGATGGTAGCCAATTACATCC	rs7196606_A_VIC1
104	ATGACGTGGCAGACGTGGTAGCCAATTACATCC	rs7196606_G_FAM1
105	TTGCAGAACATTCTCCCATTC	rs7206999_F1
106	AGAAGGTCCCTGAGGGTACAA	rs7206999_R1
107	GAGTGAGCCACCACGCCTGGTCAGATTTTATACAA	rs7206999_INV1
108	CGCGCCGAGGTGTATGGTAAACACAAAATCAAGC	rs7206999_T_VIC1
109	ATGACGTGGCAGACCGTATGGTAAACACAAAATCAAG	rs7206999_C_FAM1
110	CTCCACTCTGTTGGTCTCAGG	rs2305599_F1
111	ATTATTCCTGGTGCGTTTCCT	rs2305599_R1
112	CTGAGTTTGGGAAAACAGCCATCAAAGAATCCTAAGTGCTAGTTTAATTAAAACATTATGATAACT	rs2305599_INV1
113	CGCGCCGAGGACTTACAAAGTGGCAAAATGAG	rs2305599_A_VIC1
114	ATGACGTGGCAGACGCTTACAAAGTGGCAAAATGA	rs2305599_G_FAM1
115	TTCAATTTCACCAGGCTTCAC	rs152343_F1
116	GAGAAGTCACAAGCACCAAGC	rs152343_R1
117	TCCAATCTCCTGATACCCTGA	rs12632280_F1

	Oligo sequence (5' to 3')	Oligo name
118	ACTTGTTCCGTTTCACAGCAG	rs12632280_R1
119	CTTGATTTTTCTTTTTTGGTTTTTGTGAAACAAGATGATAATAAATTCTTTTTTT CACCTAAAATATTCTCTATATATTTGCTCCAT	rs12632280_INV1
120	CGCGCCGAGGATATGTATACAGACAAATCTATAATTAAGTTAA	rs12632280_A_VIC1
121	ATGACGTGGCAGACGTATGTATACAGACAAATCTATAATTAAG	rs12632280_G_FAM1
122	GCCTGAAAGTGGCAGTTAGC	rs7615453_F1
123	ACGACAAACGGATTTATGAGTG	rs7615453_R1
124	TTATTATTCCTAAAATAGGTAAAACAAATATCAAACACTTTGGGCTTTACTGGAGTGTTC CTGGAAATA	rs7615453_INV1
125	CGCGCCGAGGTGTTGCAAGATGTACTTCATCT	rs7615453_T_VIC1
126	ATGACGTGGCAGACCGTTGCAAGATGTACTTCAT	rs7615453_C_FAM1
127	GGGGAAGACTACATCTAGAAATGA	rs7487755_F1
128	GATTGCTTTCTCCAAAGGCTA	rs7487755_R1
129	TCTATTAGATGTTACACATACACATACATACAATTTACATTCACATATATTAAC CCTTTTGTGCTCTGTRCAGTTTT	rs7487755_INV1
130	CGCGCCGAGGATAAGAACTCTATGTATCTATGTGT	rs7487755_A_VIC1
131	ATGACGTGGCAGACCTAAGAACTCTATGTATCTATGTG	rs7487755_C_FAM1
132	TGATTTGAGAGGCAGTGTAGGA	rs17064971_F1
133	ATACCAAGGAGCCGACTATGG	rs17064971_R1
134	TTTCCCTGACTCAATGTTAACTCAGAGTGGATTTATGTCTGGATTTACTTTTCT TTTCA	rs17064971_INV1
135	CGCGCCGAGGTACCTTCAGAAAACAGGACTTT	rs17064971_T_VIC1
136	ATGACGTGGCAGACCACCTTCAGAAAACAGGAC	rs17064971_C_FAM1
137	GATCCATGTTCTCAAGCAAGG	rs971048_F1
138	AAGCTGACCTGAGAGCAACAC	rs971048_R1
139	AAAACAACCTCAAAATATAGGTATCATGATCCTCAGGATACAGGTTAGAAAA AGAGAGATTA	rs971048_INV1
140	CGCGCCGAGGTTTTAACTTGCTGCAAGTCACA	rs971048_T_VIC1
141	ATGACGTGGCAGACCTTTAACTTGCTGCAAGTCA	rs971048_C_FAM1
142	AATCAGGAGATGCTGTGGATG	rs937303_F1
143	CTGGCAGTGTCAAGATTCAAG	rs937303_R1
144	GGAAGTCTAGTGCTTTTTTCTGGAAAGTCTTGAAAGAGGTTGCTA	rs937303_INV1
145	CGCGCCGAGGTAAGAAAAAGTTCATCTCATGGC	rs937303_T_VIC1
146	ATGACGTGGCAGACCAAGAAAAAGTTCATCTCATGG	rs937303_C_FAM1
147	CTACTGGAGCGGGACATCAG	rs4842407_F1
148	TTGGGAGCTAGAAGGTGAGAC	rs4842407_R1
149	TATCTCTGGGCATAAGATCAGACTGGAAATGGATGAAGTAAGGA	rs4842407_INV1
150	CGCGCCGAGGTAGTGACCCTAAAAAATTTAATGCTAA	rs4842407_T_VIC1
151	ATGACGTGGCAGACCAGTGACCCTAAAAAATTTAATGC	rs4842407_C_FAM1
152	CTGTTGTTACCAAATATCCACTGC	rs11012476_F1
153	CGGTTGTACACATCCTGCTG	rs11012476_R1
154	AAAAAATTTCAGGTTACAATGCATTCTGTTGTTTTGAACTACAGGAATCAAAC CAGAATTTTTAGCCT	rs11012476_INV1
155	CGCGCCGAGGATGATACACATTTGATACATTCATG	rs11012476_A_VIC1

	Oligo sequence (5' to 3')	Oligo name
156	ATGACGTGGCAGACGTGATACACATTTGATACATTCATG	rs11012476_G_FAM1
157	AGGCTGGTCTCAACGAACTCT	rs2835071_F1
158	TAGCATGGGTGAAGATGTCTG	rs2835071_R1
159	ATCAGAGACATCGTTGATACTTCTGGGCAATTTGCCAATTAAYAGTAGAAA CAA	rs2835071_INV1
160	CGCGCCGAGGTCATAATTAACACTTATCATCCAAACT	rs2835071_T_VIC1
161	ATGACGTGGCAGACCCATAATTAACACTTATCATCCAAA	rs2835071_C_FAM1
162	GGAAAGCTAGATTTGATTGCTC	rs251891_F1
163	GCTATTTGCTGACACCATGC	rs251891_R1
164	AAGTAGCCTGCTCTACACTGCATGTTTAAAATGAATTTCTTAGGAGCAAGT	rs251891_INV1
165	CGCGCCGAGGATTTGGCCTCCAAACAAGC	rs251891_A_VIC1
166	ATGACGTGGCAGACCTTTGGCCTCCAAACAAG	rs251891_C_FAM1
167	ACAAATCGTGGAAGACACCAG	rs10862812_F1
168	TGAGGTCAGAGAGAGGACAGC	rs10862812_R1
169	CTCAAAACAGCCCTATATGAACTGGACATTCTGTAGAATATTACATTAGTCCA ATATTGATTTTATCAT	rs10862812_INV1
170	CGCGCCGAGGATGATATCGCAATAAGATCTGATG	rs10862812_A_VIC1
171	ATGACGTGGCAGACGTGATATCGCAATAAGATCTGA	rs10862812_G_FAM1
172	TGAGTCTCCTGAGTCGTCAGC	rs868567_F1
173	GGGCTTTCCTAGTGCTCAGTC	rs868567_R1
174	GAGAACCACTCTCCTTATTCTGTGCAGGGGTT	rs868567_INV1
175	CGCGCCGAGGAGGGACATCCCTTGCT	rs868567_A_VIC1
176	ATGACGTGGCAGACCGGGACATCCCTTG	rs868567_C_FAM1
177	GCCAAATATCCACGTCAAGAG	rs16870561_F1
178	GCTGTGAGAATGTGAACGTGA	rs16870561_R1
179	TAGCCAGACTTTTAAGAAAGCYTCATCAGAGTTGAATTGTATTTAAGTATAA TTTTATTTCTGGCTATGTAGATGT	rs16870561_INV1
180	CGCGCCGAGGACACTGGTTTATAAAGCTATTGTTC	rs16870561_A_VIC1
181	ATGACGTGGCAGACGCACTGGTTTATAAAGCTATTG	rs16870561_G_FAM1
182	TCCTGTGAGGATTTGACTTGC	rs10809892_F1
183	GAAGGTTGTTGGAAGATGCAG	rs10809892_R1
184	GCCTGGGGTGTTTATCAGAGCACCTCCTTT	rs10809892_INV1
185	CGCGCCGAGGATTAATGGGCCTTGACCTA	rs10809892_A_VIC1
186	ATGACGTGGCAGACCTTAATGGGCCTTGACC	rs10809892_C_FAM1
187	ATGGAGGAGATCTGATGATGC	rs714046_F1
188	TGCTAACAAATCCTGGGTGAC	rs714046_R1
189	AGACAAGGTATAATGTTAAAAGCTAAAGGAGTGAAGGAAAAGGAGTGAGG TTA	rs714046_INV1
190	CGCGCCGAGGTATTGAGGGAATGGATAATGAC	rs714046_T_VIC1
191	ATGACGTGGCAGACGATTGAGGGAATGGATAATGA	rs714046_G_FAM1
192	ATGGCAATTCTTGAACCTGTG	rs7044362_F1
193	ATATGGAAGACATTGGCGAAG	rs7044362_R1
194	TCGTTTTCACTCTGCCTCATATGCCTCTTATGCTCTTTCTATAGTA	rs7044362_INV1

	Oligo sequence (5' to 3')	**Oligo name**
195	CGCGCCGAGGTGCCCATGTCCATCCTT	rs7044362_T_VIC1
196	ATGACGTGGCAGACCGCCCATGTCCATC	rs7044362_C_FAM1
197	CACTAATCCTCATGCCTGTCC	rs2270476_F1
198	AGCTGACACATGATGGTGCT	rs2270476_R1
199	CCACTGAAGAAAATATATACTAAGCAAAGGTGGTCAATGTGGTAGGAATTT AATCAACA	rs2270476_INV1
200	CGCGCCGAGGTCCACAGGCTAACCCTT	rs2270476_T_VIC1
201	ATGACGTGGCAGACCCCACAGGCTAACC	rs2270476_C_FAM1
202	ACTTGGAGTGTCCCAAATTCC	rs2597553_F1
203	GAAGCACCAACTCAGAAGAGC	rs2597553_R1
204	AAGAAAACCAGGGCTTCTTCCCTTGATGTSATTTTCTTACACCATA	rs2597553_INV1
205	CGCGCCGAGGTAAGTGCCTGAACCAAAGG	rs2597553_T_VIC1
206	ATGACGTGGCAGACCAAGTGCCTGAACCAAAG	rs2597553_C_FAM1
207	GCTCCGAGAGAGAAAGTCACC	rs7149043_F1
208	AAATTCCTGAAACGAGGAAGC	rs7149043_R1
209	CTGTGTGCGGCAGGATCTCCTGTTCCT	rs7149043_INV1
210	CGCGCCGAGGAATGTTGCCAGTGCTGC	rs7149043_A_VIC1
211	ATGACGTGGCAGACGATGTTGCCAGTGCTG	rs7149043_G_FAM1
212	AGACAGTTAGGGTGGGAGCAA	rs10809893_F1
213	CAGAAGGTTGTTGGAAGATGC	rs10809893_R1
214	AGTTAGGGTGGGAGCAAATCACCTTAATCCAATAAAAATTAGGCTGTTTTGT	rs10809893_INV1
215	CGCGCCGAGGAGCTCTAGTCCTGTGAG	rs10809893_A_VIC1
216	ATGACGTGGCAGACGGCTCTAGTCCTGTGA	rs10809893_G_FAM1

Annex IV: Ethical approval letters

ADDIS ABABA UNIVERSITY COLLEGE OF HEALTH SCIENCES (IRB)
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Institutional Review Board

ANNEX 3
Form AAUMF 03-008

IRB's Decision

Meeting No: 006/2015 Date: July 29, 2015
Protocol number: 011/15/Pharma Assigned No.....

Protocol Title: Genome wide association and replication studies of anti-TB and ARV drug induced liver injury on Ethiopian TB and /or HIV patients		
Principal Investigators:	Zelalem Petros	
Institute:	SoM-CHS-AAU	
Elements Reviewed (AAUMF 01-008)	☑ Attached	☐ Not attached
Review of Revised Application ☐ Yes ☐ No	Date of Previous review:	
Decision of the meeting:	☑ **Approved** ☐ Approved with Recommendation ☐ Resubmission ☐ Disapproved	

I. Elements approved-
1. Protocol Version No. ...2.....
2. Protocol Version Date.....
3. Informed consent Version No. ...2.....
4. Informed Consent Version Date .....

II. Obligations of the PI-
1. Should comply with the standard international & national scientific and ethical guidelines
2. All amendments and changes made in protocol and consent form needs IRB approval
3. The PI should report SAE within 10 days of the event
4. End of the study, including manuscripts and thesis works should be reported to the IRB

III. **TO NERC** ■

Institution Review Board (IRB) Approval: Period from 30/07/2015 to 29/07/2016

Follow up report expected in
3 Months ___ 6 months ___ 9 months ___√___ one year ____

Chairperson, IRB
Dr. Yimtubezinash Woldeamanuel
Signature _____
Date: 30/07/15

Associate Director for Research and Technology
Signature _____
Date 31/8/2015

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የሳይንስና ቴክኖሎጂ ሚኒስቴር
The Federal Democratic Republic of Ethiopia
Ministry of Science and Technology

ቁጥር Ref. No. ፫፤10/081/2015
ቀን Date Dec 21, 2015

To: Addis Ababa University Collage of Health Science, Ethics Committee

Addis Ababa

Re: Genome-wide association and replication studies of anti-tuberculosis and anti-retroviral drugs induced liver injury on Ethiopian TB and/or HIV patients

Dear Sir/Madam//Mr./Mrs./Dr,

The National Research Ethics Review Committee (NRERC) has reviewed the aforementioned project protocol in an expedited manner. We are writing to advise you that NRERC has granted

Full Approval

To the above named project, for a period of **one year (December 21, 2015- December 20, 2016).** All your most recently submitted documents have been approved for use in this study. The study should comply with the standard international and national scientific and ethical guidelines. Any change to the approved protocol or consent material must be reviewed and approved through the amendment process prior to its implementation. In addition, any adverse or unanticipated events should be reported within 24-48 hours to the NRERC. Please ensure that you submit biannual progress report once in six months and annual renewal application 30 days prior to the expiry date.

We, therefore, request you as PI and your esteemed organization to ensure the commencement and conduct of the study accordingly and wish for the successful completion of the project.

With regards,

Yohannes Sitotaw
Secretary of NRERC

CC._ Mr. Zelalem Petros (PI)

_ NRERC chairperson

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You may Contact

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ፋክስ Fax +251-011-4-66 02 41

Annex V: RUCAM Score

Hepatocellular [H] Type			Cholestatic [C] or Mixed Type		
1. Time to onset	**Initial treatment**	**Re-challenge**	**Initial treatment**	**Re-challenge**	**Score**
Drug start	5 – 90 d < 5 d or > 90 d	1 – 15 d > 15 d	5 – 90 d < 5 d or > 90 d	1 – 90 d > 90 d	+2 □ +1 □
Drug stop	≤ 15 d	≤ 15 d	≤ 15 d	≤ 15 d	+1 □
2. Course (after stopping)	**Change in ALT [peak value & ULN]**		**Change in ALP/TBil**		
Highly suggestive	Decrease ≥ 50% within 8 d		Not applicable		+3 □
Suggestive	Decrease ≥ 50% within 30 d		Decrease ≥ 50% within 180 d		+2 □
Compatible	Not applicable		Decrease < 50% within 180 d		+1 □
Inconclusive	No information or decrease ≥ 50% after 30 d		Persistence or increase/no information		+0 □
Against the role of the drug	Decrease < 50% after 30 d, or recurrent incr.		Not applicable		-2 □
If drug continued: Inconclusive	All situations		All situations		+0 □
3. Risk Factors Alcohol:- presence (+1) or absence (0)					+1 □
Age: - ≥ 50 yrs (+1) or < 50 yrs (0)					+1 □
4. Concomitant drug(s) **-** None or no information or concomitant drug with incompatible time to onset					+0 □
- Concomitant drug with suggestive or compatible time to onset					-1 □
- Concomitant drug known to be hepatoxic with a suggestive time to onset					-2 □
- Concomitant drug with clear evidence for its role (positive re-challenge)					-3 □
5. Exclusion of other causes of liver injury			- All causes in G-I & II ruled out		+2 □
G-I (6 categories of causes):			- 6 causes of G-I ruled out		+1 □
Acute viral hepatitis [HAV, HBV or HCV]; biliary obstruction; alcoholism			- 5 or 4 causes of G-I ruled out		+0 □
(AST/ALT ≥ 2); hypotension, shock/ischemia (within 2 wks)			- < 4 causes of G-I ruled out		-2 □
G-II (2 categories of causes):			- Non drug cause highly probable		-3 □
Complications of autoimmune hepatitis, sepsis, chronic HBV/HCV, cholangitis					
Clinical features of serologic/virologic test indicating acute CMV, EBV or HSV					
6. Previous information on hepatotoxicity of the treatment - Reaction labeled in the product characteristics					+2 □
- Reaction published but unlabeled					+1 □
- Reaction unknown					+0 □
7. Response to re-admin Positive	Doubling of ALT for [H] ALP/ TBil for [C] with drug alone				+3 □
Compatible	Doubling of ALT for [H], ALP/ TBil for [C] with combined with another drug				+1 □
Not done	Other situations				+0 □
Negative	Increase of ALT for [H], ALP/ TBil for [C] but less than ULN with drug alone				-2 □
Total (Add checked scores)					

ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; [C]: Cholestatic; d: days; [H]: Hepatocellular; TBil: Total bilirubin.
Modified from: Danna G and Benichou C. J Clin Epidemiol 1993; 46: 1323-30.

Annex VI: Genotype distributions of the top SNPs among the treatment groups

A. Genotype distributions of the top SNPs in the cases and controls of anti-tubercular drugs (ATD) treatment group

						GWAS						Replication study							
			Alleles			Cases			Controls			Cases			Controls				
SNP	Chr	Chrloc	1	2	RA	11	12	22	11	12	22	11	12	22	11	12	22	Nearest gene	Relative loci (bp)
rs10946739	6	24993127	A	G	1	7	21	20	9	97	248	1	11	13	6	57	146	FAM65B	0
rs7708937	5	123169574	T	C	1	3	19	26	4	54	296	0	3	23	3	50	161	KRT18P16	196413
rs591141	1	108694894	A	G	2	33	15	0	326	28	0	25	2	0	194	22	0	SLC25A24	0
rs10946737	6	24967240	A	G	1	1	21	26	0	59	295	0	10	17	1	38	177	FAM65B	0
rs2089910	11	1874404	T	C	1	9	31	8	25	144	185	1	10	13	20	94	98	LSP1	0
rs4903067	14	73286300	T	C	2	19	27	2	257	90	7	20	5	0	154	48	8	DPF3	0
rs320035	1	49089197	A	G	2	6	16	26	119	149	86	4	9	14	58	108	50	AGBL4	0
rs393994	1	49108745	T	C	2	6	17	25	120	150	84	4	10	13	56	112	48	AGBL4	0
rs2069912	2	128178191	T	C	1	42	6	0	203	128	23	19	7	1	129	78	8	PROC	0
rs320003	1	49126778	A	G	1	24	18	6	83	153	118	11	9	3	47	108	53	AGBL4	0
rs319952	1	49113622	A	G	2	6	18	24	119	152	83	3	11	12	55	114	47	AGBL4	0
rs239319	22	34120608	A	G	2	13	15	20	141	169	44	8	12	4	73	81	44	LARGE	0
rs3959930	11	15835975	T	C	1	3	35	10	32	113	209	2	10	14	12	82	121	SOX6	152020
rs3892834	12	16486288	A	G	1	40	6	2	182	141	31	14	10	3	114	83	17	MGST1	-13788
rs4733759	8	128804840	T	C	1	17	16	15	34	170	150	1	12	11	19	93	91	PVT1	-1939
rs2835109	21	37107446	A	G	1	47	1	0	256	87	11	21	5	0	170	41	1	RPS20P1	-9982
rs7074842	10	65633037	T	G	2	27	17	4	291	61	2	21	3	0	172	39	0	RPL7AP50	29108
rs9507038	13	23737972	A	C	1	14	14	20	23	179	152	5	11	10	26	92	96	SGCG	-17088
rs2835119	21	37118059	A	G	1	47	1	0	260	84	10	23	3	0	176	38	1	RPS20P1	-20595
rs1619379	6	29785235	T	C	1	24	12	12	71	201	82	5	16	6	59	105	51	MICG	-4766
rs6477968	9	115708164	T	G	2	2	28	18	108	174	72	8	16	2	54	117	36	MUP	13250
rs13096001	3	170128750	A	C	2	4	27	17	122	178	54	11	9	5	66	92	48	CLDN11	-7903
rs7183361	15	57343199	A	C	1	2	14	32	1	36	317	0	4	23	1	28	185	TCF12	0
rs1611717	6	29829577	A	G	2	7	17	24	78	202	74	5	17	4	47	110	53	3.8-1.4	4115
rs624931	11	117922486	A	G	2	23	19	6	255	93	6	17	10	0	148	60	6	LOC100526771	0
rs6999996	8	51533503	A	G	1	4	18	26	1	77	276	0	5	22	3	41	170	SNTG1	0
rs9298341	8	51523824	T	C	1	4	18	26	1	77	276	0	6	21	3	43	170	SNTG1	0
rs2166318	11	105483661	T	C	2	4	33	11	133	149	72	8	13	5	68	102	43	GRIA4	0

SNP - Single nucleotide polymorphism; Chr (Chrloc) - Chromosome, and Chromosomal loci based on GRChg37; RA - Risk allele; bp - base pairs

B. Genotype distributions of the top SNPs in the cases and controls of antiretroviral therapy (ART) group

						GWAS						Replication study							
			Alleles			Cases			Controls			Cases			Controls				
SNP	**Chr**	**Chrloc**	**1**	**2**	**RA**	**11**	**12**	**22**	**11**	**12**	**22**	**11**	**12**	**22**	**11**	**12**	**22**	**Nearest gene**	**Relative loci (bp)**
rs199650082	17	62121526	T	C	1	1	5	8	0	11	282	0	1	6	0	2	64	ERN1	0
rs32498	5	55548797	A	G	1	9	4	1	32	145	116	0	5	2	10	30	26	ANKRD55	-19611
rs7196606	16	15941025	T	C	2	7	5	2	261	32	0	7	0	0	59	7	0	MYH11	0
rs7804397	7	116857547	A	C	1	2	7	5	3	38	252	0	0	7	0	12	54	ST7	0
rs7206999	17	61733668	A	G	1	2	4	8	2	17	274	0	0	7	0	7	59	MAP3K3	0
rs16947045	17	61770954	T	C	1	2	4	8	2	17	274	0	0	7	0	7	59	MAP3K3	0
rs196911	17	62120843	T	C	2	8	5	1	275	17	1	6	1	0	63	3	0	ERN1	0
rs2305599	8	121210250	A	G	1	4	7	3	7	90	196	0	4	3	3	16	47	COL14A1	0
rs152343	5	55560577	T	C	1	11	2	1	49	142	102	1	5	1	13	29	23	PSMC1P4	10326
rs12632280	3	87722254	T	C	2	4	3	7	169	107	17	5	1	0	33	30	3	PSMC1P6	-41439
rs7615453	3	174909952	T	C	2	1	12	1	187	90	14	4	1	2	35	30	1	NAALADL2	0
rs7487755	12	7631190	A	C	1	7	3	4	15	120	158	1	2	3	7	35	24	CD163	0
rs17064971	5	165015242	A	G	2	2	1	11	73	166	54	3	3	1	18	29	19	LOC574080	194106
rs971048	4	13955163	A	G	1	9	1	4	35	141	117	0	6	1	11	31	24	LOC391636	-23627
rs937303	15	33940768	T	C	2	1	0	13	40	157	96	1	3	3	13	39	14	RYR3	0
rs173236	16	18086557	A	C	2	0	10	4	164	103	26	4	2	1	40	19	7	RPL7P47	59355
rs4902750	14	70186440	T	C	1	2	11	1	13	90	190	0	2	5	3	26	37	KIAA0247	4579
rs7796747	7	127017001	T	C	2	7	2	5	206	82	5	4	3	0	45	17	4	ZNF800	0
rs11690142	2	225022657	A	G	1	9	2	3	37	133	123	1	3	3	10	32	24	LOC100420435	-12777
rs3115263	2	34968286	A	C	1	11	3	0	73	145	75	2	5	0	17	35	14	LOC100130842	476157
rs1153462	3	3157051	A	G	1	3	11	0	20	113	160	1	2	4	4	24	38	IL5RA	-4993
rs17029711	2	60784033	T	G	1	6	3	5	11	104	178	0	3	4	6	22	38	BCL11A	-3400
exm160316	1	234603322	A	T	1	4	1	9	2	65	226	0	2	5	1	21	44	TARBP1	0
exm2268014	17	61055390	A	G	2	5	6	3	228	62	3	7	0	0	47	17	2	LOC729667	-26841
rs12599288	16	11822066	A	G	2	6	6	2	248	43	2	6	1	0	57	9	0	TXNDC11	0
rs2098985	2	60817254	A	G	1	1	8	5	0	49	244	0	1	6	1	14	51	BCL11A	-36621
rs7794242	7	42015020	T	C	1	2	6	6	0	52	241	0	3	4	0	15	51	GLI3	0

SNP - Single nucleotide polymorphism; Chr (Chrloc) - Chromosome, and Chromosomal loci based on GRChg37; RA - Risk allele; bp - base pairs

C. Genotype distribution of the top SNPs in the cases and controls of ATD and ART co-treatment group

						GWAS						Replication study							
			Alleles			Cases			Controls			Cases			Controls				
SNP	**Chr**	**Chrloc**	**1**	**2**	**RA**	**11**	**12**	**22**	**11**	**12**	**22**	**11**	**12**	**22**	**11**	**12**	**22**	**Nearest gene**	**Relative loci (bp)**
rs4842407	12	79201073	T	C	2	0	13	14	60	77	22	0	4	4	41	44	23	LOC642550	-13179
rs10862812	12	84464616	T	C	1	8	12	7	8	57	94	0	2	6	6	43	59	LOC100128335	377793
rs868567	10	129266910	T	G	2	5	14	8	88	64	7	6	2	0	47	50	11	DOCK1	16130
rs16870561	6	84230450	T	C	2	18	9	0	152	7	0	7	1	0	96	12	0	PRSS35	0
rs11012476	10	21292923	A	G	1	0	8	19	0	5	154	0	2	6	0	2	105	NEBL	0
rs2835071	21	37032177	T	C	2	4	16	7	87	64	8	2	5	1	61	42	5	EZH2P1	59624
rs251891	5	115050362	A	C	1	2	10	15	0	22	137	1	4	3	2	19	83	TMED7	-88486
rs10809892	9	1315843	A	C	1	21	5	1	53	72	34	2	4	2	35	57	16	RPS27AP14	-150976
rs714046	8	97631573	A	C	1	3	17	7	7	41	111	0	0	8	4	36	64	SDC2	7536
rs7044362	9	1316816	A	G	2	1	3	23	27	71	61	2	2	4	13	52	39	RPS27AP14	-151949
rs2270476	2	211525764	A	G	1	1	15	11	4	22	133	0	3	5	4	35	68	CPS1	0
rs2597553	4	121786273	A	G	1	23	4	0	67	81	11	7	1	0	47	44	13	PRDM5	0
rs7149043	14	95116292	A	G	1	3	21	3	12	58	89	1	4	3	10	53	45	SERPINA13	2961
rs10809893	9	1315952	T	C	1	24	3	0	73	63	23	3	3	2	50	47	11	RPS27AP14	-151085
rs12886622	14	95116465	T	C	1	4	20	3	13	59	87	1	4	3	10	49	48	SERPINA13	3134
rs10140228	14	95141693	T	C	2	11	9	7	99	58	2	4	4	0	62	39	3	LOC100288028	0
rs8134246	21	17926550	A	G	2	1	18	8	69	69	21	2	4	2	43	42	20	LINC00478	0
rs17148947	7	8491534	T	G	2	18	7	2	149	10	0	8	0	0	90	16	2	NXPH1	0
rs4965087	15	98081701	T	C	2	0	3	24	14	70	75	1	1	6	9	39	54	LOC91948	204145
rs10493958	1	102069696	T	C	1	1	18	8	5	38	116	0	4	3	2	25	76	RPSAP19	182126
rs1895191	5	159995502	A	G	1	8	11	8	9	51	99	0	4	4	6	41	61	ATP10B	0
rs4921305	5	160003685	T	C	1	8	11	8	9	51	99	0	4	4	5	41	58	ATP10B	0
exm2266643	8	75217116	T	C	2	3	16	8	80	65	14	4	4	0	44	47	14	JPH1	0
rs8097559	18	59682216	A	G	1	27	0	0	103	51	5	8	0	0	69	32	2	PIGN	29241
rs11254509	10	17340342	A	C	2	0	6	21	27	69	63	2	4	2	18	51	39	ST8SIA6	22334
rs10739255	9	111023899	T	C	2	0	9	18	30	85	44	1	5	2	29	46	33	LOC100128657	-163768
rs7900449	10	33913412	A	C	1	3	13	11	1	35	123	0	2	6	2	34	70	LOC100505583	135229
rs9309764	3	77325741	T	C	1	0	14	13	0	23	136	0	2	6	0	26	82	ROBO2	0
rs3010815	9	113434178	A	G	2	10	8	9	61	79	19	2	5	1	43	48	17	MUSK	0

SNP - Single nucleotide polymorphism; Chr (Chrloc) - Chromosome, and Chromosomal loci based on GRChg37; RA - Risk allele; bp - base pairs

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Genome-wide association and replication study of anti-tuberculosis drugs-induced liver toxicity

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Abstract

Background: Drug-induced liver injury (DILI) is a well-recognized adverse event of anti tuberculosis drugs (ATD) possibly associated with genetic variations. The objective of this study was to perform genome-wide association study (GWAS) to identify genetic variants associated with the risk for ATD induced liver toxicity in Ethiopian patients.

Result: Treatment-naïve newly diagnosed tuberculosis patients ($n = 646$) were enrolled prospectively and treated with rifampicin based short course anti-tuberculosis therapy. Whole genome genotyping was done using Illumina Omni Express Exome Bead Chip genotyping array with 951,117 single nucleotide polymorphisms (SNPs) on 48 DILI cases and 354 ATD tolerants. Replication study was carried out for 50 SNPs with the lowest *P*-values (top SNPs) using an independent cohort consisting of 27 DILI cases and 217 ATD tolerants. In the combined analysis, the top SNP identified was rs10946737 ($P = 4.4 \times 10^{-6}$, OR = 3.4, 95 % confidence interval = 2.2–5.3) in the intron of *FAM65B* in chromosome 6. In addition, we identified a cluster of SNPs with suggestive genome-wide significance in the intron of ATP/GTP binding protein-like 4 (*AGBL4*).

Conclusion: We identified genetic variants that are potentially associated with ATD induced liver toxicity. Further studies with larger sample sizes are essential to confirm the findings.

Keywords: Anti-tuberculosis, Drug induced liver injury, Ethiopian, *FAM65B*, C6ORF32, GWAS, *AGBL4*, Hepatotoxicity, Africa, Tuberculosis

Background

Liver toxicity associated with drug treatment, known as drug-induced liver injury (DILI) is implicated in most cases of acute liver failure [1]. It can limit patient access to drugs that might otherwise be beneficial [2]. DILI is a major adverse event that leads to termination of clinical drug development programs and regulatory measures on approved drugs [3]. The largest population-based study reported on the incidence of DILI was from Iceland with a crude incidence rate of 19.1 cases per 100,000 inhabitants per year [4]. Although the causes of DILI can be various, studies have shown that genetic variations in genes involved in drug disposition, cellular stress, and immune response may contribute to DILI susceptibility [5, 6].

Anti-tuberculosis drugs (ATD) are among the most reported anti-microbial drugs incriminated to be potential causes of DILI [7]. ATD induced liver injury (ATDILI) is one of the most prevalent hepatotoxicities reported in many countries [8]. A previous study in Ethiopian tuberculosis (TB) patients showed 17.3 % incidence of ATDILI [9]. Incidence of treatment induced liver toxicity varies between populations. Higher incidence of concomitant

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ATD and antiretroviral (ARV) drugs induced liver toxicity in Ethiopian (30 %) compared to Tanzanian (10 %) TB and human immunodeficiency virus (HIV) coinfected patients has been reported [10, 11] Among the first line ATD, isoniazid, rifampicin, and pyrazinamide are known to cause DILI [8]. Genetic variations contribute to inter-individual ATDIL susceptibility [12]. Polymorphisms in drug metabolizing genes such as N-acetyltransferase 2 (*NAT2*), cytochrome P450 family 2 subfamily E polypeptide 1 (*CYP2E1*), glutathione S-transferase mu 1 (*GSTM1*), uridinediphosphate-glucuronosyltransferase1 family polypeptide A1 (*UGT1A1*) [8, 13], human leukocyte antigen (*HLA*) region [5, 8] and superoxide dismutase-2 mitochondrial (*SOD2*) gene [8, 14, 15] have been suggested to play roles in ATDILI.

Sub-Saharan Africa is disproportionally affected by high burden of TB and HIV. According to the latest WHO report, Ethiopia is listed among the top ten high-TB burden countries globally and one of the high multi-drug resistant TB (MDR-TB) burden countries [16]. DILI is one of the important adverse events of anti-TB drugs, particularly during the intensive phase of TB therapy [17]. Treatment has to be discontinued in those patients who developed severe ATD induced liver toxicity, and treatment interruption may increase the risk for emergence of multidrug-resistant TB. Increased risk of developing MDR-TB in Ethiopian TB patients who encountered adverse events during the first course of TB treatment is reported recently [18]. Thus identification of genetic markers that predispose patients for ATD induced liver toxicity using GWAS in high TB burden sub-Saharan African countries, such as Ethiopia is imperative.

Using candidate gene approach, we previously identified genetic variation in *NAT2*, *CYP2B6*, and *ABCB1* genes as risk factors for ATD and antiretroviral (ARV) drugs co-treatment induced liver toxicity in TB-HIV co-infected patients [10, 11, 19]. Although candidate gene studies contribute to the discovery of genetic risk variants associated with ATDILI, the discovered genetic factors may account only for a proportion of the genetic variations, and some of the studies led to inconsistent results [20–22]. Therefore, we aimed to identify additional genetic variants through genome-wide association study (GWAS) for ATDILI in Ethiopian TB patients.

Results

A total of 646 TB patients participated in this study and 75 (11.6 %) of them met the criteria for DILI diagnosis while on ATD treatment. Whole genome genotyping was done using genomic DNA from 48 DILI cases and 354 ATD tolerants. Replication study for 50 SNPs with lowest *P*-values (top SNPs) was done using genomic DNA from an independent cohort consisting of 27 DILI cases and 217ATD tolerants. The difference between the GWAS and the replication cohorts was based on time of first presentation. The first groups of patients were used for the GWAS, and the subsequent group of patients used for the replication study. The study area, TB diagnostic methods, case definitions, inclusion and exclusion criteria, and ATD treatment regimens used were all the same. The demographics and clinical characteristics of the study participants are presented in Table 1. There were statistically significant differences ($P < 0.05$) in HIV status and liver function test values between cases and treatment tolerants in both the GWAS and replication cohorts. There were statistically significant differences in sex, CD4 count and viral load between cases and treatment tolerants in the GWAS cohort but not in the replication study, which may be attributed to the smaller sample size of the replication cohort. More than one-third of the cases in our study had cholestatic pattern of DILI, and the rest had hepatocellular or mixed pattern.

The Quantile-quantile (QQ) plot for the observed versus expected *P*-values, and Manhattan plot for the regression analysis are shown in Figs. 1 and 2, respectively. The top SNP in the GWAS after adjustment for sex, HIV status, CD4 count and HIV viral load was rs10946739 ($P = 4.1 \times 10^{-6}$, odds ratio (OR) = 3.4, 95 % CI = 2.0–5.6) located in the intron region of family with sequence similarity 65 member B (*FAM65B*), which is also named as chromosome 6 open reading frame 32 (*C6ORF32*) (Additional file 1: Table S1). The top SNP in the replication study after adjustment for covariates was rs319952 ($P = 1.0 \times 10^{-2}$, OR = 2.3, 95 % CI = 1.2–4.4) located in the intron of ATP/GTP binding protein-like 4 (*AGBL4*) in chromosome 1 (Additional file 1: Table S2). In the combined analysis, the top SNP after adjustment for covariates was rs10946737 ($P = 4.4 \times 10^{-6}$, OR = 3.4, 95 % CI = 2.2–5.3) located in the intron region of *FAM65B* (Table 2). In addition, four of the top SNPs (rs320035, rs393994, rs319952 and rs320003) were clustered in the intron of *AGBL4*.

For the sub-group analysis based on the pattern of liver injury, the top SNPs for cholestatic, hepatocellular and mixed patterns of DILI were rs10182566 ($P = 4.1 \times 10^{-6}$, OR = 6.0, 95 % CI = 2.8–12.8) in 3′-untranslated region of chromosome 2 open reading frame 71 (*C2orf71*), rs1990046 ($P = 3.7 \times 10^{-6}$, OR = 28.4, 95 % CI = 6.9–117.3) in the intron of semaphorin3A (*SEMA3A*) in chromosome 7, and rs12603186 ($P = 8.1 \times 10^{-6}$, OR = 7.2, 95 % CI = 3.0–17.2) in shisa family member 6 (*SHISA6*) in chromosome 17, respectively (Additional file 1: Table S3).

Discussion

In this study, we carried out GWAS and replication analysis on a total of 646 patients treated with ATD to identify novel genetic variants associated with DILI. Previously we investigated pharmacogenetic markers for

Table 1 Demographics and clinical variables of the study participants

Variables	GWAS			Replication study		
	DILI Cases	Treatment tolerants	*P*	DILI cases	Treatment tolerants	*P*
No. of patients	48	354	-	27	217	-
Sex (M, F)	19, 29	203, 151	0.02	12, 15	85, 132	0.60
Age (yr), M (SD)	35.6 (10.4)	35.7 (11.5)	0.93	32.0 (7.4)	33.4 (10.3)	0.48
BMI (kg/m^2), M (SD)	19.0 (3.2)	19.3 (3.0)	0.55	17.5 (3.0)	18.9 (3.0)	0.02
HIV positive, N (%)	44 (91.7)	225 (63.6)	<0.01	25 (92.6)	158 (72.8)	0.03
CD4 count, M (SD)	96.6 (78.5)	129.3 (120.8)	0.03	116.8 (98.3)	138.2 (121.0)	0.33
Viral load, log M (SD)	5.3 (0.9)	4.9 (0.9)	0.03	5.0 (0.8)	4.9 (0.9)	0.54
ALT (U/L), M (SD)	69.7 (37.2)	30.4 (14.4)	<0.01	67.2 (42.1)	30.7 (14.1)	<0.01
AST (U/L), M (SD)	101.2 (52.7)	40.5 (16.2)	<0.01	103.6 (71.9)	38.7 (13.6)	<0.01
ALP (U/L), M (SD)	187.7 (72.2)	121.1 (51.7)	<0.01	225.8 (139.9)	114.0 (63.1)	<0.01
T Bil (mg/dL), M (SD)	1.2 (1.0)	0.6 (0.4)	<0.01	1.1 (0.7)	0.5 (0.3)	<0.01
DILI pattern, N (%)						
Cholestatic	19 (39.6)			15 (55.6)		
Hepatocellular	10 (20.8)			5 (18.5)		
Mixed	19 (39.6)			7 (25.9)		

ALP Alkaline phosphatase, *ALT* Alanine aminotransferase, *AST* aspartate aminotransferase, *BMI* Body mass index, *DILI* Drug induced liver injury, *GWAS* Genome wide association study, *HIV* Human immunodeficiency virus, *M (SD)* Mean (standard deviation), *N* Number, *P - P* values, *T Bil* Total bilirubin

concomitant ARV and ATD co-treatment induced DILI in TB-HIV co-infected patients ($n = 353$) using candidate gene approach [10]. As a continuation, we conducted a large prospective cohort study in 1060 patients, where we evaluated the patterns of ATD and/or ARV drugs induced liver toxicities [23]. In the present study, we investigated for possible genetic markers for ATD induced liver toxicity using genome wide association approach in 646 selected study participants from the recent large prospective cohort study by considering DILI cases developed during anti-TB treatment only. Identifying the risk variants could help developing clinical tests to prevent DILI,

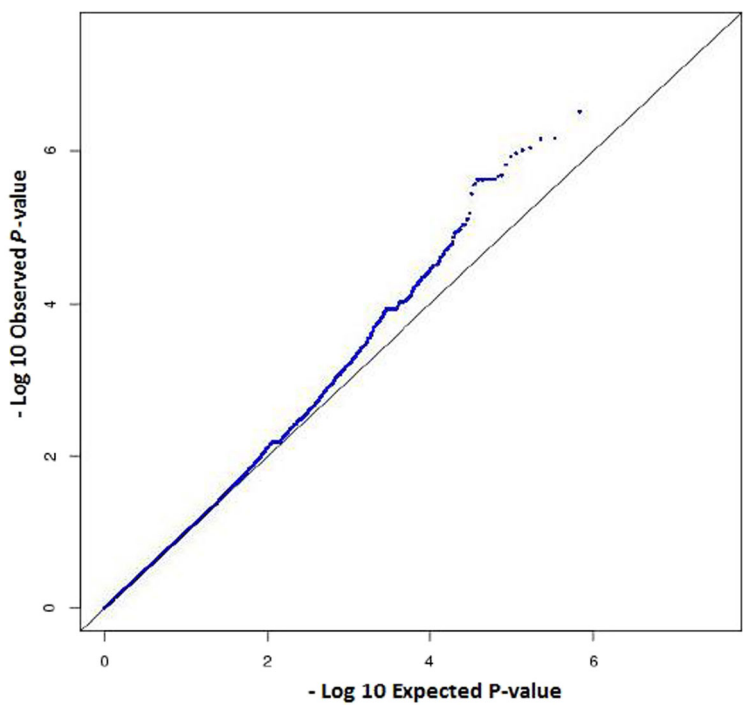

Fig. 1 Quantile-quantile (QQ) plot for the observed versus expected *P*-values in trend test ($\lambda_{GC} = 1.00007$)

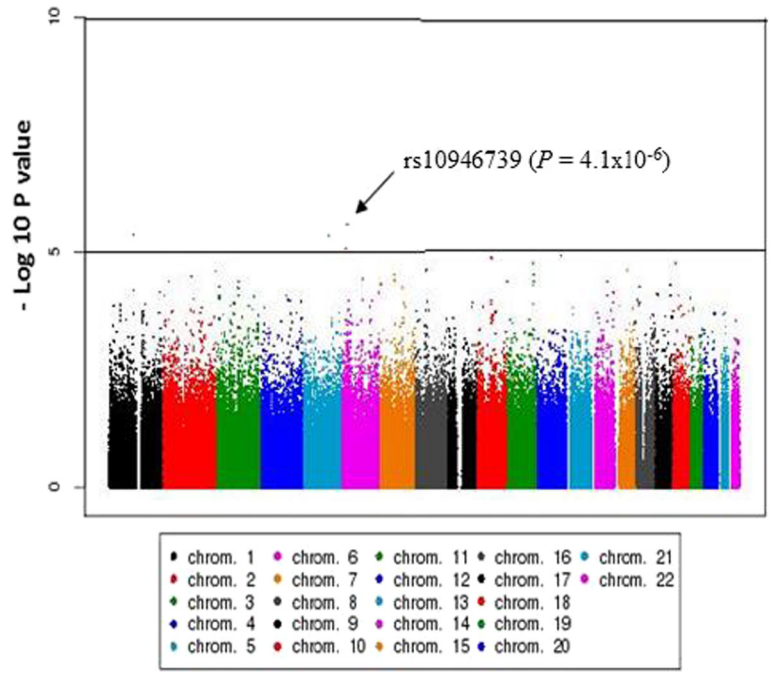

Fig. 2 $-\text{Log}_{10}P$ values of logistic regression across chromosomes in the GWAS

Table 2 Top SNPs in the combined analysis of the GWAS and the replication study

SNP	Chr (loci)	Alleles (RA)	Study	Cases/controls	MAF	*P*_min	*P*_adj	OR (95 % CI)	Nearest gene
rs10946737	6 (24967240)	A/G (A)	GWAS	48/354	0.10	2.0×10^{-5}	9.7×10^{-6}	4.3 (2.5–7.4)	*FAM65B*
			Rep	27/216	0.10	3.8×10^{-2}	8.6×10^{-2}	2.2 (0.9–5.4)	
			Comb	75/570	0.10	6.3×10^{-7}	4.4×10^{-6}	3.4 (2.2–5.3)	
rs320035	1 (49089197)	A/G (G)	GWAS	48/354	0.48	3.5×10^{-6}	1.3×10^{-4}	2.4 (1.5–3.8)	*AGBL4*
			Rep	27/216	0.50	4.2×10^{-3}	1.2×10^{-2}	2.2 (1.9–3.9)	
			Comb	75/570	0.49	8.2×10^{-7}	5.1×10^{-6}	2.3 (1.6–3.3)	
rs10946739	6 (24993127)	A/G (A)	GWAS	48/354	0.19	9.6×10^{-6}	4.1×10^{-6}	3.4 (2.0–5.6)	*FAM65B*
			Rep	25/209	0.18	1.1×10^{-1}	1.8×10^{-1}	1.7 (0.8–3.6)	
			Comb	73/563	0.19	4.7×10^{-6}	5.1×10^{-6}	2.7 (1.8–4.1)	
rs393994	1 (49108745)	T/C (C)	GWAS	48/354	0.48	6.1×10^{-6}	1.7×10^{-4}	2.4 (1.5–3.7)	*AGBL4*
			Rep	27/216	0.50	7.9×10^{-3}	1.4×10^{-2}	2.1 (1.2–4.0)	
			Comb	75/570	0.49	1.9×10^{-6}	7.6×10^{-6}	2.3 (1.6–3.3)	
rs320003	1 (49126778)	A/G (A)	GWAS	48/354	0.48	1.7×10^{-5}	2.3×10^{-4}	2.3 (1.5–3.7)	*AGBL4*
			Rep	23/208	0.50	1.9×10^{-2}	1.2×10^{-2}	2.3 (1.2–4.5)	
			Comb	71/562	0.49	4.6×10^{-6}	8.3×10^{-6}	2.3 (1.6–3.4)	
rs319952	1 (49113622)	A/G (G)	GWAS	48/354	0.48	1.1×10^{-5}	2.8×10^{-4}	2.3 (1.5–3.6)	*AGBL4*
			Rep	26/216	0.50	1.2×10^{-2}	1.0×10^{-2}	2.3 (1.2–4.4)	
			Comb	74/570	0.49	2.5×10^{-6}	8.5×10^{-6}	2.3 (1.6–3.3)	
rs7958375	1 (2 111640017)	A/G (A)	GWAS	48/354	0.02	8.8×10^{-5}	1.2×10^{-5}	11.3 (3.8–33.5)	*CUX2*
			Rep	27/216	0.02	$1.0\times10^{+0}$	7.4×10^{-1}	1.5 (0.2–13.1)	
			Comb	75/570	0.02	1.7×10^{-4}	4.6×10^{-5}	7.6 (2.9–20.0)	

Chr (loci) Chromosome, and chromosomal loci based on NCBI built 37, *CI* Confidence Interval, *Comb* Combined analysis using inverse variance method, *GWAS* Genome wide association study, *MAF* Minor allele frequency, *OR* Odds ratio, *P_adj* Logistic *P*-value after adjustment for sex, HIV status, CD4 count and HIV viral load; *P_min* Minimum *P*-value among allelic, dominant and recessive models of Fisher's exact test, and *P*-value of inverse variance combined analysis; *RA* Risk allele, *Rep* Replication study, *SNP* Single nucleotide polymorphism

and to match the patients with alternative, effective and safe medications. To our knowledge, this is the first GWAS for ATDILI in an African population.

The top SNP in the GWAS analysis after adjustment for covariates was in the intron of *FAM65B*. This gene encodes a cytoplasmic protein that plays a role in myoblast differentiation, and it is transiently up-regulated during early stage of the process [24]. Alternative splicing of this gene results in multiple transcript variants. Inhibition of expression of this gene in myoblasts causes marked decrease in myogenin expression with consequent lack of myoblast fusion; and its over-expression induces formations of cellular protrusions [25]. It is suggested that *FAM65B* may possibly play a role in myoblast migration, and mutations could affect muscle development and human muscle diseases; however, its exact role is still largely unknown [25]. According to the human Protein Atlas data, *FAM65B* is also a mitochondrial protein expressed in the liver hepatocytes, gall bladder and bile duct [26]. Recent studies indicate that *FAM65B* plays a role in cancer and liver inflammation [27]. Further analysis is necessary to explain functional importance of *FAM65B* gene in ATDILI.

Strong association with ATDILI was identified by a cluster of four SNPs with *P*-values suggestive of genome-wide association significance in the intron of *AGBL4* (*CCP6*). This gene encodes an enzyme that catalyzes deglutamylation of polyglutamate side chains generated by post-translational modification of target proteins like tubulins in microtubules [28]. Further analysis is required to explain the role of *AGBL4* gene and its contribution to individual differences for susceptibility to ATDILI. The identified genetic risk variants in our study if replicated in larger sample sizes and in other populations, they may serve as genetic biomarkers for ATDILI.

It is increasingly evident that genetic variants can determine an individual's susceptibility to develop a particular pattern of liver injury [29]. Therefore, we performed sub-group GWAS analysis based on the pattern of DILI. The SNP (rs1990046) with the smallest *P*-value after adjustment for covariates ($P = 3.7 \times 10^{-6}$) was identified in the hepatocellular type of DILI. This SNP is located in the intron region of *SEMA3A*, a member of the semaphorin family. This gene encodes a protein with an immunoglobulin-like domain and sema domain, which is vital for normal neuronal pattern development [30], and also plays a role in the pathogenesis of allergic conditions such as allergic rhinitis [31]. However, further studies are required to elucidate the role of *SEMA3A* gene in hepatocellular pattern of ATDILI.

In our previous candidate gene study [10], genetic variants in genes involved in drug metabolism of ATD like *NAT2* were associated with DILI. Variants in other drug metabolizing genes [8, 13], *HLA* region [5, 8], and in genes related to oxidative stress [32] and autoimmune diseases [2] were also reported to have association with susceptibility to ATDILI. In our GWAS, we did not find genetic variants that passed genome-wide significance in these genes, which may be related to the limited sample sizes used for the study. But we found possible association SNPs rs12969241 ($P = 1.1 \times 10^{-5}$) located in the intron region of protein tyrosine phosphatase non-receptor type 2 (*PTPN2*), rs2842997 ($P = 5.1 \times 10^{-3}$) in the vicinity of *SOD2*, and rs12543818 ($P = 1.9 \times 10^{-3}$) near *NAT2* for genes related to autoimmune diseases, oxidative stress and pharmacokinetics, respectively (Additional file 1: Table S4). The SNP rs12969241 in the *PTPN2* gene was also among the top in the GWAS of cholestatic pattern of DILI ($P = 6.8 \times 10^{-6}$) (Additional file 1: Table S3). The protein encoded by the *PTPN2* gene is an intracellular tyrosine-specific phosphatase, which is expressed in epithelial cells, fibroblasts or endothelial cells [33]. This protein was shown to play an important role in the protection of epithelial barrier function during inflammation by acting as negative regulator of pro-inflammatory cytokine interferon-γ [34]. This finding may indicate the implication of an immune related mechanism in ATDILI. The product of *SOD2* gene, which was identified for genes related to oxidative stress, detoxifies highly reactive superoxide radicals generated by mitochondrial respiration [35]. This finding is in line with a previous study [15], which reported common polymorphisms in *SOD2* as predictor of ATDILI. We speculate that ATDILI may be related to the combined effect of the new variants identified, pharmacokinetic, oxidative stress, and immune-related gene variants.

Sub-Saharan African population is the most genetically heterogeneous population globally, characterized by extensive population substructure, and less linkage disequilibrium (LD) among loci compared to non-African populations.[36]. Although GWAS in populations of African ancestry is challenging due to less degree of LD; the high level of genetic diversity and weak LD with neighboring SNPs in Africans ancestry is considered as a powerful tool for fine mapping causal variants that underlie common diseases or complex traits found globally [37]. The advantage of conducting GWAS in African ancestry populations in the context of addressing existing and emerging global health conditions is reported recently [37]. The present study exploring ATDILI risk variants through GWAS in Ethiopia, the second most densely populated country in Africa, will not only provide national genomic information for personalized medicine but also may contribute to the advancement of pharmacogenomics in Africa.

There were some limitations for this study. First, as the DILI cases are rare and were difficult to collect (four years were required to identify 75 ATDILI cases), this resulted in small number of case samples particularly for

sub-group analysis based on the pattern of DILI. Second, populations of African ancestry, as in case of our study population, have greater genetic diversity and lower levels of linkage disequilibrium (LD) among chromosomal loci [38]. The low levels of LD are disadvantageous when screening the genome for disease associations using the current SNP-genotyping approaches that essentially rely on the principle of LD mapping. Therefore, additional studies with higher density SNP array or next generation sequencing may be required to discover susceptibility variants in such population. Ethiopian population display distinct pharmacogenetic variations compared to other black African population [39–42], and thus results from this study may not be directly extrapolated to other sub-Saharan African population. However, our exploratory study using homogenous well-characterized clinical samples for the discovery and replication of new DILI biomarkers, represents an important first step in applying GWAS to identify genetic variants for ATDILI. The third limitation is that the current protocol of TB treatment consists of combinations of drugs, thus we cannot affirm that the risk variants identified corresponds only to a single drug or multiple drugs in the treatment regimen.

Conclusion

Using genome-wide wide associations study, we identified potential genetic variants associated with ATDILI. The results provide evidence that in addition to genetic variants identified by candidate gene studies, other variants also influence the risk of developing DILI by ATD. Further replication studies are essential to confirm the findings.

Methods

Study participants and treatment

The participants for the present GWAS were selected from a recent prospective cohort study where patterns of antiretroviral therapy (ART) and/or anti-TB treatment induced liver toxicity was investigated [23]. In brief newly diagnosed treatment naïve patients enrolled into one of the following study arms were considered for the present study:

1) TB infected patients (with out HIV co-infection) treated with rifampicin based ATD only.
2) TB-HIV co-infected patients with baseline CD4 count >200 cells/mm^3 (not eligible for ART, following the national and WHO treatment guideline valid during the study period) and treated with rifampicin based ATD only.
3) TB-HIV co-infected patients with baseline CD4 count <200 cells/mm^3 and (eligible for ART co-treatment) and rifampicin based ATD was initiated first followed by efavirenz based ART (delayed up to 8 weeks after starting ATD). Patients who developed DILI while on ATD treatment only (before starting ARV therapy) were included in the current GWAS, but patients who developed DILI after initiating ARV co-treatments were excluded from this study.

Patients were recruited from three health institutions: Kazanchis and Beletshachew health centers and Black Lion specialized referral and teaching university hospital in Addis Ababa, Ethiopia [23]. Diagnosis of TB was based on sputum smear, fine needle aspirate, clinical and radiological evidences. The eligibility criteria were TB confirmed men and non-pregnant women, age ≥18 years and receiving no other known hepatotoxic drugs concurrently. Patients who had abnormal liver enzyme biochemistry at baseline, positive serological test for either hepatitis B virus surface antigen or anti-hepatitis C virus antibody or known liver injury prior to starting treatment were excluded.. Written informed consent was obtained from all the study participants prior to study enrolment. The study protocol was approved by the Institutional Review Board of College of Health Sciences, Addis Ababa University, Ethiopia; Ethical Review Board of Karolinska Institutet, Sweden; and Ethical Review Committee of RIKEN, Japan.

Drug treatment was initiated according to World Health Organization (WHO) and Ethiopian National TB Treatment Guidelines as described previously [23]. All patients received short-course ATD treatment consisting of rifampicin (150 mg), isoniazid (75 mg), pyrazinamide (400 mg) and ethambutol (275 mg) for the first two months in fixed dose combinations given daily under direct observed therapy during the intensive phase, followed by rifampicin (150 mg) and isoniazid (75 mg) for the next four months in fixed dose combinations given daily. The treatment dosage was based on the weight of the patient: 20–29 kg (1½ tablets), 30–37 kg (2 tablets), 38–54 kg (3 tablets) and ≥55 kg (4 tablets). Liver function tests were carried out at baseline and on the 1st, 2nd, 4th, 8th, 12th and 24th weeks after initiation of treatment.

Case definitions

For DILI case definitions, the criteria set by the International DILI expert working group were used [43]. The upper limit of normal (ULN) for liver biochemical parameters used for the study population were alanine aminotransferase (ALT 33 U/L, male; 29 U/L, female), aspartate aminotransferase (AST, 41 U/L), alkaline phosphatase (ALP, 128 U/L), and 1.0 mg/dL for total bilirubin [23]. All cases recruited met at least one of the following criteria: – (1) ALT ≥5xULN, (2) ALP ≥2xULN, or (3) ALT ≥3xULN along with total bilirubin ≥2xULN. All cases had a minimum score of three ('possible') in Roussel Uclaf Causality Assessment Method (RUCAM) scoring system for DILI. The pattern of liver injury was defined using

R-values where, R = (ALT/ULN)/(ALP/ULN). Cases were categorized as having hepatocellular ($R \geq 5$), cholestatic ($R \leq 2$), or mixed ($2 < R < 5$) pattern of DILI [43]. Treatment tolerants for the study were defined as individuals who were also on short course ATD treatment but did not fulfill the case definitions for DILI, and had not presented clinical signs and symptoms consistent with DILI in the follow up period [23].

Whole genome genotyping and quality control

Genomic DNA was isolated from whole blood samples using QIAamp DNA Maxi Kit (QIAGEN GmbH, Hilden, Germany). Genotyping was conducted in RIKEN Center for Integrative Medical Sciences, Yokohama, Japan. Whole genome genotyping was done using Illumina Omni Express Exome Bead Chip genotyping array (Illumina Inc., San Diego, CA, USA) according to the manufacturer's protocol. This array captures 951,117 single nucleotide polymorphisms (SNPs). To further validate the results of the GWAS analysis, replication study was then carried out for 50 SNPs with the lowest *P*-values (top SNPs) using an independent cohort. Genotyping for the replication study was done using multiplex polymerase chain reaction (PCR) based Invader assay [44] with ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

For data cleaning, systematic stepwise quality filtering of raw genotyping data was done using PLINK [45]. From an initial full set, those SNPs not mapped on autosomal chromosomes were filtered out. In addition, SNPs with a call rate less than 99 %, minor allele frequency less than 0.01, or deviated from expected Hardy-Weinberg equilibrium ($P < 1.0 \times 10^{-6}$) were removed. A total of 660, 206 SNPs that passed the quality filter were used for further analysis. Individuals were checked for gender concordance between recorded clinical data and genotype determined sex. Samples with genotyping call rate greater than 99 % were included in the analysis. Quantile-quantile plot comparing the expected and observed *P*-values was performed in R-statistical environment, and genomic control inflation factor (λ_{GC}) was computed to detect population stratification [46].

Statistical analysis

After the quality filter, the tests of associations were done using PLINK v1.07 [45]. For each SNP, Fisher's exact test using the three genetic inheritance models (dominant, recessive, allele frequency) were carried out to compare allele and genotype frequencies between DILI cases and treatment tolerants. SNPs were rank-ordered according to the minimum *P*-value in the genetic models. The threshold for genome-wide significance for associated SNPs was determined using Bonferroni correction ($P < 7.6 \times 10^{-8}$). SNPs with *P*-values below 10^{-5} were considered suggestive of genome-wide significance. Logistic regression analysis adjusted for sex, HIV status, CD4 count and HIV viral load as covariates was performed. These variables were associated with DILI as described previously [9, 10]. Combined analysis of GWAS and replication study was conducted using inverse-variance method [47]. Manhattan plot was generated using Haploview software to visualize the results [48]. We also performed sub-group GWAS analysis based on the pattern of liver injury.

Additional file

Additional file 1: Table S1. Top fifteen SNPs in the GWAS of ATDILI. **Table S2.** Top fifteen SNPs in the replication study of ATDILI. **Table S3.** Top SNPs in the GWAS of the pattern of ATDILI. **Table S4.** Top SNPs for GWAS of ATDILI in genes related to autoimmune diseases, oxidative stress, pharmacokinetic, and HLA region. (PDF 57 kb)

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Authors' contributions

Conceived and designed the experiments: EA EM MK TM GYAH MTML ZP. Performed the experiments: ZP YZ EA GY AH. Analyzed the data: AT MTML ZP EA. Contributed reagents/materials/analysis tools: EA MK MTML TM AT EM GY AH WA GA ISK. Wrote the paper: ZP MTML EA EM MK TM. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Paper II

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Genome-Wide Association and Replication Study of Hepatotoxicity Induced by Antiretrovirals Alone or with Concomitant Anti-Tuberculosis Drugs

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Abstract

Drug-induced hepatotoxicity (DIH) is a common adverse event that is associated with both antiretroviral (ARV) and anti-tuberculosis drugs (ATD). Moreover, the genetic variations predisposing ARV- and ARV-ATD-induced liver toxicity in African populations are not well investigated, despite the two diseases being the major global health problems in sub-Saharan Africa. We performed a genome-wide association study (GWAS) and replication study to identify the genetic variants linked to the risk of developing DIH due to ARV drugs alone, and ARV-ATD co-treatment in Ethiopian HIV-positive patients. Treatment-naïve newly diagnosed HIV patients ($n=719$) with or without tuberculosis (TB) co-infection were enrolled prospectively and received efavirenz-based ARV therapy with or without rifampicin-based short course ATD, respectively. Whole-genome genotyping was performed by using the Illumina Omni Express Exome Bead Chip genotyping array with 951,117 single nucleotide polymorphisms (SNPs) on a total of 41 cases of DIH, and 452 people without DIH (treatment tolerants). The replication study was carried out for 100 SNPs with the lowest *p*-values (top SNPs) by using an independent cohort consisting of 18 DIH cases and 208 treatment tolerants. We identified a missense SNP rs199650082 (2756G→A, R919Q, $p=1.4\times10^{-6}$, odds ratio [OR] = 18.2, 95% confidence interval [CI] = 7.1–46.9) in an endoplasmic reticulum to the nucleus signaling-1 (*ERN1*) gene on chromosome 17 to be associated with DIH in the ARV-only cohort. In the ARV-ATD co-treatment groups, rs4842407, a long intergenic noncoding RNAs (lincRNAs) transcript variant on chromosome 12, was associated with DIH ($p=5.3\times10^{-7}$, OR = 5.4, 95% CI = 2.8–10.3). These genetic variants that are putatively associated with DIH due to ARV drugs alone and ARV-ATD co-treatment establish a foundation for future personalized medicine in people with HIV and TB and call for larger studies in independent populations.

Keywords: antiretroviral drugs, anti-tuberculosis drugs, drug-induced hepatotoxicity, genome-wide association study, GWAS, OMICS, and Africa

Introduction

Antiretroviral therapy (ART) has dramatically lowered HIV-/AIDS-related mortality globally, but treatment-associated liver toxicity remains a challenge (Fink and Bloch, 2013; Ugiagbe et al., 2012; Yimer et al., 2011, 2012, 2014). To end the AIDS epidemic, several African countries, including Ethiopia, are currently making important progress in scaling up HIV testing and ART in response to the UNAIDS (2014) 90–90–90 target: 90% of people with HIV diagnosed, 90% of diagnosed

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people on treatment, and 90% of treated people having fully suppressed viral replication by 2020. On the other hand, antiretroviral (ARV) treatment-associated hepatotoxicity is of increasing concern in the management of patients with HIV/AIDS (Jones and Nunez, 2012; Reisler et al., 2003; Turkova et al., 2009; Ugiagbe et al., 2012; Walker, 2007).

Tuberculosis (TB) is the most common opportunistic infection in people living with HIV/AIDS, and concomitant treatment of the two diseases is challenging because of drug interactions and overlapping toxicities. The risk of developing drug-induced hepatotoxicity (DIH) after treatment initiation is much higher for TB-HIV co-infected patients than for those with HIV or TB mono-infection (Araujo-Mariz et al., 2016; Mugusi et al., 2012; Yimer et al., 2011, 2014). ARV- and anti-tuberculosis drug (ATD)-induced hepatotoxicity have become major clinical challenges because of high morbidity, mortality, and frequent hospitalization as a leading cause of acute liver failure and complications requiring liver transplantation (Elsharkawy et al., 2013; Fink and Bloch, 2013; Nunez, 2010; Turkova et al., 2009).

The type and incidence of DIH varies between populations and geographical locations (Lamar and Nunez, 2011; Mugusi et al., 2012; Yimer et al., 2011). The mechanisms and risk factors in DIH are complex, involving both host genetics and environmental factors (Daly, 2010; Nunez, 2010). Previous candidate gene association studies have reported the potential role of genetic variation in Cytochrome P450 (*CYP*) *2B6* and ATP-binding cassette subfamily-B member-1 (*ABCB1*) for ARV-DIH with efavirenz- or nevirapine-based regimens (Mugusi et al., 2012; Ritchie et al., 2006; Yimer et al., 2011, 2012). Studies also reported an association between polymorphisms in drug-metabolizing genes (N-acetyltransferase-2 [*NAT2*], *CYP2E1*, glutathione S-transferase-1 [*GSTM1*], uridine diphosphateglucuronosyltransferase-1A1 [*UGT1A1*]) and mitochondrial superoxide dismutase (*SOD2*) with ATD-induced hepatotoxicity (Huang, 2014; Kim et al., 2015; Yimer et al., 2011).

Although candidate gene studies contribute to the discovery of genetic risk variants that are associated with ARV- and ATD-induced hepatotoxicity, the identified variants may account only for a proportion of the genetic variations. Genome-wide association studies (GWAS) have identified variant alleles that are associated with an increased risk of developing DIH after treatment intentions with various classes of drugs (Aithal and Grove, 2015; Daly et al., 2009; Urban et al., 2014). Recently, using GWAS in Ethiopian TB patients, we have reported genetic variations in ATP-/GTP-binding protein-like-4 (*AGBL4*) and family with sequence similarity 65 member-B (*FAM65B*) as potential risk factors for developing ATD-induced liver toxicity (Petros et al., 2016).

The genetic variations predisposing ARV- and ARV-ATD-induced liver toxicity in black African populations are not well investigated, despite the two diseases being the major public health problems in sub-Saharan Africa. As the scale of ART is increasing in sub-Saharan African countries, it is imperative to identify the genetic markers that are associated with an increased risk of ARV-induced liver toxicity for subsequent treatment interventions. This study reports on GWAS and replication studies of genetic variants in relation to DIH risk with ARV drugs alone, and ARV-ATD co-treatment, in Ethiopian HIV-positive people with or without TB co-infection.

Materials and Methods

Study participants

The study participants for the present GWAS were included from a recent observational, prospective cohort study where the incidence and patterns of ARV drugs and/or ATD-induced liver toxicities were investigated (Yimer et al., 2014). In brief, newly diagnosed treatment naïve patients enrolled into the following study arms were considered for the present study:

1. HIV-positive patients with CD4 count ≤200 cells/mm^3 without TB co-infection receiving efavirenz-based ARV therapy only.
2. TB and HIV co-infected patients with CD4 count ≤200 cells/mm^3 receiving both rifampicin-based short-course anti-TB drugs and efavirenz-based ARV therapy at the same time (Fig. 1). Following the World Health Organization (WHO) and national TB-HIV treatment guidelines valid during the study period, all TB-HIV patients initiated rifampicin-based ATD regimen and initiation of ARV was delayed for a maximum of 8 weeks after starting ATD treatment. Patients who developed DIH while on anti-TB drugs alone (before starting ARV therapy) were not included in the present study.

The study participants were recruited from three health institutions: the Kazanchis and the Beletshachew Health Centers and the Black Lion specialized referral and teaching university hospital in Addis Ababa, Ethiopia. The inclusion criteria were HIV-positive men and non-pregnant women of age ≥18 years. Patients were excluded if they had a history of prior treatment for TB/HIV, known preexisting liver disease, or abnormal liver biochemistry before starting treatment; if they were positive for either hepatitis B virus surface antigen or anti-hepatitis C virus antibody, they were also excluded.

The study protocol and consent procedure were approved by the Institutional Review Board of College of Health Sciences, Addis Ababa University; the National Research Ethics Review Committee of Ethiopia, Ethical Review

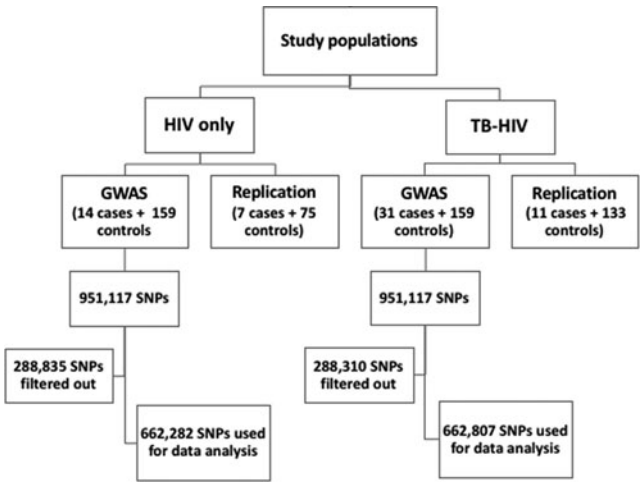

FIG. 1. Study design, genotyping, and quality control flow chart. GWAS, genome-wide association study; HIV, human immunodeficiency virus; SNP, single nucleotide polymorphism; TB, tuberculosis.

Board of Karolinska Institutet, Sweden, and Ethical Review Committee of RIKEN, Japan. Written informed consent was obtained from all the study participants before their inclusion in the study.

Drug treatment and patient follow-up

All patients received treatment according to the national and the WHO treatment guidelines for HIV and TB as previously described (Yimer et al., 2014). In brief, all HIV patients received ARV drugs containing efavirenz and lamivudine with stavudine, zidovudine, or tenofovir. TB-HIV coinfected patients also received short-course ATD consisting of rifampicin, isoniazid, pyrazinamide, and ethambutol for the first 2 months followed by rifampicin and isoniazid for the next 4 months. The patients were not on other known hepatotoxic drugs concurrently (except co-trimoxazole, which was given for TB and HIV co-infected patients according to the National Treatment Guideline). Liver function tests were carried out at baseline and on the 1st, 2nd, 4th, 8th, 12th, and 24th weeks after initiation of treatment.

Case definitions, severity grade, and pattern of hepatotoxicity

The criteria set by the International DIH Expert Working Group were used for DIH case definitions and pattern of hepatotoxicity (Aithal et al., 2011). The upper limit of normal (ULN) for liver biochemical parameters used for the study population were alanine aminotransferase (ALT 33 U/L, male; 29 U/L, female), aspartate aminotransferase (AST, 41 U/L), alkaline phosphatase (ALP, 128 U/L), and 1.0 mg/dL for total bilirubin (T Bil) (Yimer et al., 2014). All cases met at least one of the following criteria: (1) ALT $\geq 5 \times$ ULN, (2) ALP $\geq 2 \times$ ULN, or (3) ALT $\geq 3 \times$ ULN along with T Bil $\geq 2 \times$ ULN. Patients on ARV drugs alone or ARV-ATD co-treatment but who did not fulfill the case definitions and presented no clinical symptoms for DIH during the follow-up period were considered as treatment tolerant controls for the ARV drugs alone and ARV-ATD co-treatment groups, respectively.

The pattern of hepatotoxicity was defined by using an R-value, where R = (ALT/ULN)/(ALP/ULN). Cases were categorized as a hepatocellular ($R \geq 5$), cholestatic ($R \leq 2$), or mixed ($2 < R < 5$) pattern of DIH (Aithal et al., 2011). Clinical severity grading was determined by employing the highest measured values for each of the biochemical parameters during the course of DIH. Patients with grades 1 and 2 severities were grouped into a ''mild-to-moderate'' group, and those with grades 3 and 4 were classified into a ''severe'' group. Causality assessment for DIH was performed by using the Roussel Uclaf Causality Assessment Method (RUCAM) (Danan and Benichou, 1993).

Genotyping and quality control

Blood samples were collected, and DNA was extracted by using the QIAamp DNA Maxi Kit (QIAGEN GmbH, Hilden, Germany). Genotyping was conducted in RIKEN Center for Integrative Medical Sciences (Yokohama, Japan). For GWAS analysis, genotyping was performed on a total of 41 cases and 452 controls with Illumina Omni Express Exome Bead Chip genotyping array (Illumina, Inc., San Diego, CA, USA) according to the manufacturer's protocol. This array captures 951,117 single nucleotide polymorphisms (SNPs). Replication studies were then carried out for the top 100 SNPs (the lowest *p*-value) by using an independent cohort of 18 cases and 208 controls. Genotyping for the replication studies was done by using a multiplex polymerase chain reaction (PCR)–based Invader assay (Ohnishi et al., 2001) with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

For data cleaning, as shown in Figure 1, systematic stepwise quality filtering of raw genotyping data was done by using PLINK v1.07 (Purcell et al., 2007). From an initial full set, those SNPs that were not mapped on autosomal chromosomes were filtered out. In addition, SNPs with a call rate <99%, minor allele frequency (MAF) <0.01, or deviated from expected Hardy-Weinberg equilibrium ($p < 1.0 \times 10^{-6}$) were removed from further analysis. A total of 662,282 and 662,807 SNPs passed the quality filter for the ARV drugs alone and ARV-ATD co-treatment groups, respectively. Individuals were checked for gender concordance between recorded clinical data and genotype-determined sex. Samples with a genotyping call rate greater than 99% were included in the analysis. The effect of population structure was assessed through principal component analysis (PCA) implemented in Eigenstrat (Price et al., 2006). Quantile-quantile (QQ) plots comparing the expected and observed *p*-values were performed in an R-statistical environment. To detect population stratification, genomic control inflation factor (λ_{GC}) was also calculated (Devlin and Roeder, 1999).

Statistical analysis

After the quality filter, the tests of associations were done by using PLINK (Purcell et al., 2007). For each SNP, Fisher's exact test using the three genetic inheritance models (dominant, recessive, allele frequency) were carried out to compare allele and genotype frequencies between cases and controls. SNPs were rank ordered according to the minimum *p*-value in the genetic models. A threshold for genome-wide significance was set as $p < 7.5 \times 10^{-8}$ after Bonferroni correction. SNPs with *p*-values below 10^{-5} were considered suggestive of genome-wide significance (Dahlin et al., 2015). Logistic regression analysis adjusted for clinical variables such as sex, body mass index, baseline CD4 count, and HIV viral load as covariates was also performed. These variables were associated with DIH as previously described (Yimer et al., 2008, 2011). A combined analysis of GWAS and replication studies was conducted by using the inverse-variance method (de Bakker et al., 2008). Manhattan plots were generated to visualize the results by using Haploview software (Barrett et al., 2005).

Results

For the GWAS analysis, there were 14 cases and 293 controls in the ARV drug-alone treatment group, and 27 cases and 159 controls in the ARV-ATD co-treatment group that passed the quality filter. The demographics and clinical characteristics of the study participants for ARV-alone and ARV-ATD co-treatment groups are described in Tables 1 and 2, respectively. There were statistically significant differences ($p < 0.05$) in liver function test values between cases and controls in the GWAS and the replication cohorts of both treatment groups. There were no statistically significant differences in the number of DIH cases and treatment tolerants

Table 1. Demographics and Clinical Variables of the Study Participants in Antiretroviral Drug-Alone Treatment Group

	GWAS			*Replication*		
Variables	*Cases*	*Controls*	p	*Cases*	*Controls*	p
No. of patients	14	293		7	75	
Sex (M, F)	5, 9	115, 178	0.79	1, 6	8, 67	0.77
Age (year), mean (SD)	35.3 (12.6)	37.1 (10.6)	0.31	36.1 (8.8)	33.0 (7.1)	0.28
BMI (kg/m^2), mean (SD)	20.6 (5.0)	19.3 (2.8)	0.11	19.4 (2.8)	19.6 (2.6)	0.85
CD4 count, mean (SD)	96.2 (64.2)	99.2 (53.6)	0.84	93.4 (68.1)	109.4 (59.2)	0.51
Viral load, log mean (SD)	5.0 (0.9)	4.9 (1.0)	0.64	4.9 (0.9)	4.7 (1.2)	0.54
ALT (U/L), mean (SD)	43.6 (15.4)	30.1 (13.2)	<0.01	51.9 (48.3)	31.8 (16.0)	<0.01
AST (U/L), mean (SD)	48.4 (13.0)	36.1 (16.7)	<0.01	66.4 (23.5)	34.2 (14.3)	<0.01
ALP (U/L), mean (SD)	199.8 (75.1)	120.3 (63.1)	<0.01	203.5 (89.3)	118.0 (36.2)	<0.01
T Bil (mg/dL), mean (SD)	0.7 (0.9)	0.5 (0.3)	0.70	0.6 (0.3)	0.6 (1.1)	0.97
ARV drugs, *N* (%)						
D4T/3TC/EFV	7 (50.0)	148 (50.5)	0.97	3 (42.9)	33 (44.0)	0.96
ZDV/3TC/EFV	4 (28.6)	91 (31.1)	0.84	4 (57.1)	42 (56.0)	0.96
TDF/3TC/EFV	3 (21.4)	54 (18.4)	0.78			
DIH type, *N* (%)						
Cholestatic	10 (71.4)			5 (71.4)		
Hepatocellular	2 (14.3)			1 (14.3)		
Mixed	2 (14.3)			1 (14.3)		
Severity grade, *N* (%)						
Mild to moderate	13 (92.6)			6 (85.7)		
Severe	1 (7.4)			1 (14.3)		
RUCAM scale, *N* (%)						
Definite (score >8)	9 (64.3)			4 (57.1)		
Probable (score 6–8)	3 (21.4)			2 (28.6)		
Possible (score 3–5)	2 (14.3)			1 (14.3)		

3TC, lamivudine; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ARV, antiretroviral; AST, aspartate aminotransferase; BMI, body mass index; D4T, stavudine; DIH, drug induced hepatotoxicity; EFV, efavirenz; GWAS, genome-wide association study; RUCAM, Roussel Uclaf Causality Assessment Method; SD, standard deviation; TDF, tenofovir; T Bil, total bilirubin; ZDV, zidovudine.

Table 2. Demographics and Clinical Variables of the Study Participants in Antiretroviral and Anti-Tuberculosis Drugs Co-Treatment Group

	GWAS			*Replication*		
Variables	*Cases*	*Controls*	p	*Cases*	*Controls*	p
No. of patients	27	159		11	133	
Sex (M, F)	15, 12	73, 86	0.35	5, 6	69, 64	0.68
Age (year), mean (SD)	37.9 (10.7)	35.7 (9.8)	0.30	37.6 (9.5)	37.3 (10.0)	0.92
BMI (kg/m^2), mean (SD)	18.9 (2.4)	19.0 (2.6)	0.92	19.8 (3.9)	18.7 (3.2)	0.29
CD4 count, mean (SD)	74.7 (55.1)	90.6 (49.9)	0.13	109.2 (61.6)	89.8 (52.8)	0.23
Viral load, log mean (SD)	5.3 (0.6)	4.9 (0.9)	0.04	5.0 (0.9)	4.9 (0.9)	0.71
ALT (U/L), mean (SD)	49.3 (19.8)	30.5 (13.1)	<0.01	63.3 (28.2)	31.1 (14.7)	<0.01
AST (U/L), mean (SD)	69.8 (40.2)	36.2 (15.3)	<0.01	91.5 (54.8)	37.6 (16.4)	<0.01
ALP (U/L), mean (SD)	220.7 (94.4)	121.3 (68.8)	<0.01	206.5 (95.8)	118.0 (64.6)	<0.01
T Bil (mg/dL), mean (SD)	0.9 (0.5)	0.6 (0.3)	<0.01	0.8 (0.5)	0.5 (0.4)	0.06
ARV drugs, *N* (%)						
D4T/3TC/EFV	11 (40.7)	65 (40.8)	0.99	2 (18.2)	29 (21.8)	0.78
ZDV/3TC/EFV	7 (25.9)	47 (29.6)	0.70	3 (27.3)	32 (24.1)	0.81
TDF/3TC/EFV	9 (33.3)	47 (29.6)	0.69	6 (54.5)	72 (54.1)	0.98
DIH type, *N* (%)						
Cholestatic	21 (77.8)			6 (54.5)		
Hepatocellular	–			3 (27.3)		
Mixed	6 (22.2)			2 (18.2)		
Severity grade, *N* (%)						
Mild to moderate	24 (88.9)			10 (90.9)		
Severe	3 (11.1)			1 (9.1)		
RUCAM scale, *N* (%)						
Definite (score >8)	17 (63.0)			7 (63.6)		
Probable (score 6–8)	7 (25.9)			3 (27.3)		
Possible (score 3–5)	3 (11.1)			1 (9.1)		

with regard to the different ARV regimens. In both treatment groups, more than half of the cases had a cholestatic pattern of DIH. Ten percent of the cases developed a severe grade of hepatotoxicity in either a hepatocellular or a mixed pattern. Four participants in each treatment group had developed jaundice and necessitated discontinuation of treatment. There were no cases of liver transplantation. All of the cases had a minimum score of three (''possible'') in the RUCAM scoring system for DIH.

The QQ plots for the observed versus expected *p*-values are shown in Figure 2. The genomic inflation values were closer to one (λ_{GC} 0.98 and 1.00), indicating no systemic test statistic inflation, and population stratification was reasonably controlled. The PCA plot presented in Figure 3 indicated that our study population is distinct from the HapMap reference samples, even from the West African cluster. The Manhattan plots for the GWAS analyses are shown in

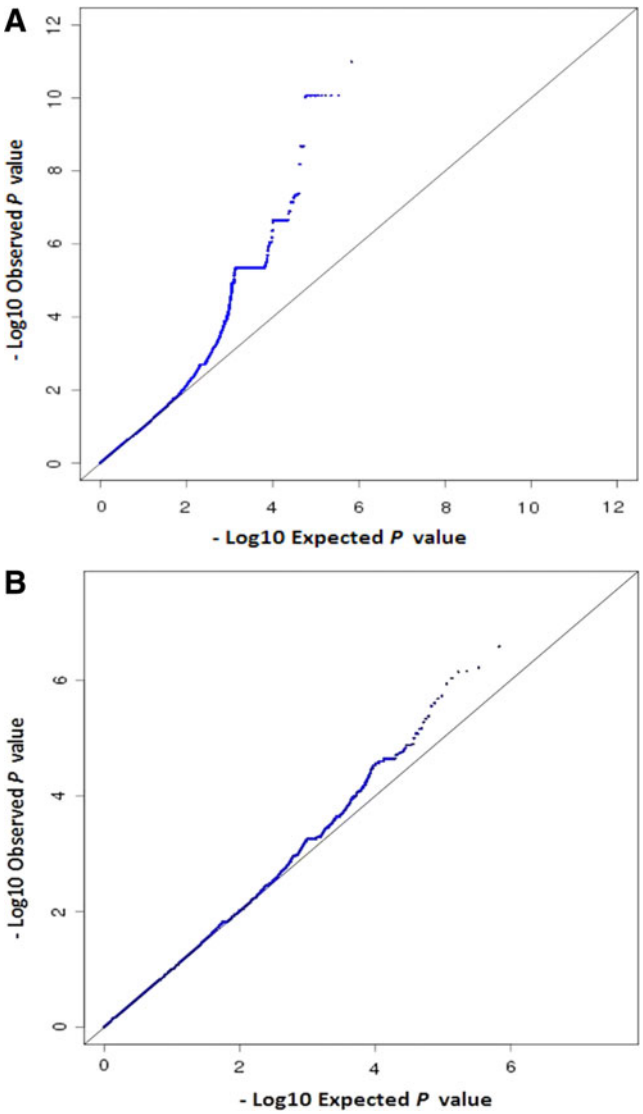

FIG. 2. QQ plots for the observed versus expected *p*-values. **(A)** ARV drug-alone treatment group ($\lambda_{GC} = 0.98$). **(B)** ARV-ATD drug co-treatment group ($\lambda_{GC} = 1.00$). ARV, antiretroviral; ARV-ATD, antiretroviral and anti-tuberculosis; QQ, quantile-quantile.

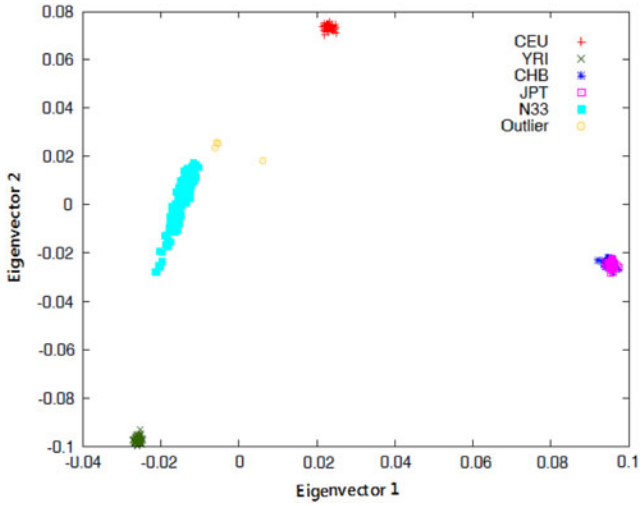

FIG. 3. PCA plot of the study population. Ethiopians coded as N33 with the HapMap reference population. CEU, Utah residents of Northern and Western European ancestry; CHB, Han Chinese individuals from Beijing, China; JPT, Japanese individuals from Tokyo, Japan; PCA, principal component analysis; YRI, Yoruba trios from Ibadan, Nigeria.

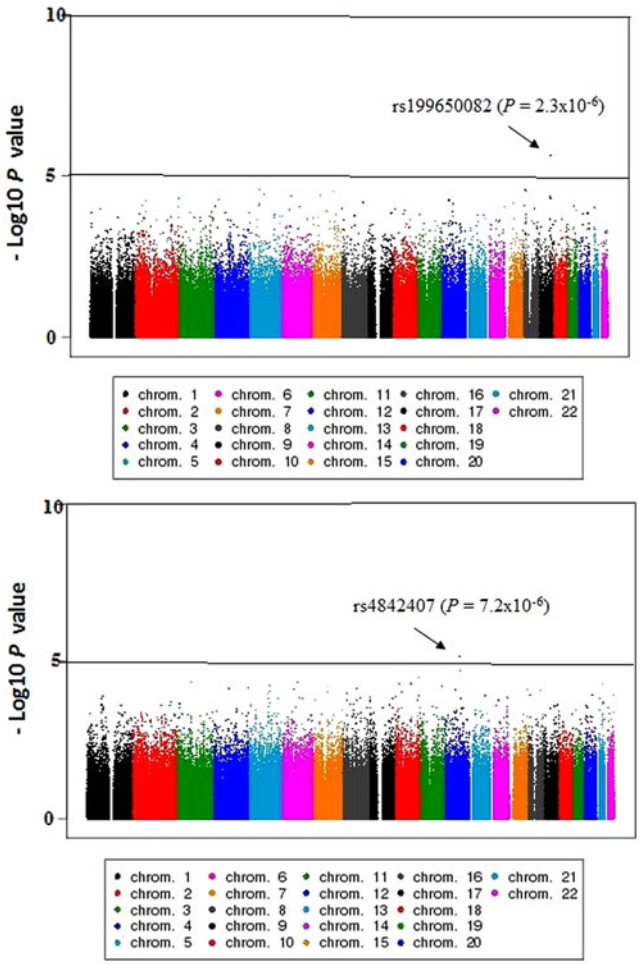

FIG. 4. $-\text{Log}_{10}$ *p* values of logistic regression across chromosomes. **(A)** ARV drug-alone treatment group. **(B)** ARV-ATD drugs co-treatment group.

Table 3. Top Single Nucleotide Polymorphisms Associated with Drug-Induced Hepatotoxicity in the Combined Analysis for Antiretroviral Drug-Alone and Antiretroviral and Anti-Tuberculosis Drugs Co-Treatment Groups

Group	*SNP*	*Chr (loci)*	*Alleles (RA)*	*Study*	*Cases/ controls*	*MAF*	*p_min*	*p_adj*	*OR (95% CI)*	*Gene/loci*
ARV	rs199650082	17 (62121526)	T/C (T)	GWAS	14/293	0.03	4.3×10^{-6}	2.3×10^{-6}	21.4 (7.5 – 60.6)	*ERN1*
				Rep	7/66	0.02	2.6×10^{-1}	2.3×10^{-1}	6.8 (0.3–15.8)	
				Comb	21/359	0.03	1.8×10^{-6}	1.4×10^{-6}	18.2 (7.1–46.9)	
	rs7804397	7 (116857547)	A/C (A)	GWAS	14/293	0.09	7.3×10^{-6}	3.0×10^{-5}	7.1 (3.1–16.2)	*ST7*
				Rep	7/66	0.08	5.9×10^{-1}	1.0×10^{0}	NA	
				Comb	21/359	0.09	1.1×10^{-5}	3.0×10^{-5}	7.1 (3.4–14.9)	
	rs16947045	17 (61770954)	T/C (T)	GWAS	14/293	0.05	1.5×10^{-5}	4.7×10^{-5}	7.4 (2.9–18.7)	*MAP3K3*
				Rep	7/66	0.05	1.0×10^{0}	1.0×10^{0}	NA	
				Comb	21/359	0.05	3.4×10^{-5}	4.7×10^{-5}	7.4 (3.1–17.5)	
ARV-ATD	rs4842407	12 (79201073)	T/C (C)	GWAS	27/159	0.44	2.3×10^{-7}	7.2×10^{-6}	5.9 (2.7–12.8)	*LOC642550*
				Rep	8/108	0.44	1.6×10^{-2}	2.2×10^{-2}	4.2 (1.2–14.4)	
				Comb	35/267	0.44	3.0×10^{-7}	5.3×10^{-7}	5.4 (2.8–10.3)	
	rs11012476	10 (21292923)	A/G (A)	GWAS	27/159	0.03	5.7×10^{-5}	5.1×10^{-5}	13.7 (4.3–43.5)	*NEBL*
				Rep	8/107	0.02	2.4×10^{-2}	1.9×10^{-2}	16.9 (1.6–178)	
				Comb	35/266	0.03	9.6×10^{-7}	2.8×10^{-6}	14.3 (5.3–39.9)	
	rs251891	5 (115050362)	A/C (A)	GWAS	27/159	0.10	1.2×10^{-4}	6.0×10^{-5}	5.8 (2.7–12.2)	*TMED7*
				Rep	8/104	0.13	8.9×10^{-3}	1.3×10^{-2}	4.5 (1.4–13.9)	
				Comb	35/263	0.11	3.5×10^{-6}	2.5×10^{-6}	5.2 (2.9–9.5)	

ARV-ATD, antiretroviral and anti-tuberculosis drugs co-treatment; Chr (loci), chromosome, and chromosomal loci based on NCBI built 37; CI, confidence interval; Comb, combined analysis; *ERN1*, endoplasmic reticulum to nucleus signaling-1; MAF, minor allele frequency; NA, not applicable; OR, odds ratio; *p*_adj, logistic *p*-value after adjustment for covariates; *p*_min, minimum *p*-value among allelic, dominant, and recessive models of Fisher's exact test, and *p*-value of inverse variance combined analysis; RA, risk allele; Rep, replication study; SNP, single nucleotide polymorphism.

Figure 4. The SNPs with the lowest *p*-value (top SNPs) in the GWAS after adjustment for sex, body mass index, CD4 count, and HIV viral load were nonsynonymous SNP (rs199650082, $p=2.3\times10^{-6}$, odds ratio [OR] = 21.4, 95% confidence interval [CI] = 7.5–60.6) in an endoplasmic reticulum to nucleus signaling-1 (*ERN1*) gene on chromosome 17, and rs4842407 ($p=7.2\times10^{-6}$, OR = 5.9, 95% CI = 2.7–12.8) within the intron of non-coding RNA on chromosome 12 for ARV drug-alone and ARV-ATD co-treatment groups, respectively (Supplementary Table S1).

The top SNPs for the replication studies are shown in Supplementary Table S2. The top SNPs in the combined analysis after adjustment for covariates were rs199650082 ($p=1.4\times10^{-6}$, OR = 18.2, 95% CI = 7.1–46.9), and rs4842407 ($p=5.3\times10^{-7}$, OR = 5.4, 95% CI = 2.8–10.3) for ARV drug-alone and ARV-ATD co-treatment groups, respectively (Table 3). Top SNPs for DILI in pharmacokinetic genes and human leukocyte antigen (HLA) region for ARV drug-alone and ARV-ATD co-treatment groups are indicated in Supplementary Table S3.

Discussion

Genetic determinants of HIV treatment response are currently firmly on the global health research agenda as efforts to improve access to treatment scale up (Soko et al., 2016). In this broader and global context, using a candidate gene approach, we have investigated pharmacogenetic markers for ARV alone ($n=285$) or with ATD co-treatment-induced liver toxicity ($n=353$) (Yimer et al., 2011, 2012). As a continuation, we evaluated the patterns of ARV and/or ATD drug-induced liver toxicities in a large prospective cohort study ($n=1060$) (Yimer et al., 2014). In the present study, we carried out GWAS and a replication study in a total of 719 patients treated with either ARV drugs alone or ATD to identify genetic variants associated with ARV-induced liver toxicity. Our preliminary finding indicates the potential role of a missense SNP rs199650082 (2756G → A, R919Q) in an *ERN1* gene on chromosome 17, and rs4842407 located in the long intergenic noncoding RNA (lincRNA) on chromosome 12 for ARV-alone- and ARV-ATD-induced liver toxicity, respectively. To our knowledge, this is the first GWAS for ART-associated DIH.

The participants for the current study were selected from a recent prospective cohort study (Yimer et al., 2014) by considering DIH cases developed during ART treatment only (in HIV only patients) or TB-HIV patients who developed DIH after initiation of ART therapy. In the latter cohort, anti-TB therapy was initiated 4–8 weeks before ART initiation. The median time for DIH onset among Ethiopian HIV patients who initiated ART alone was 4 weeks; whereas for TB-HIV patients receiving anti-TB therapy alone, the median DIH onset was 2 weeks after treatment initiation (Yimer et al., 2014). TB-HIV patients who developed DIH during prior anti-TB therapy (before starting ART) were excluded from this study.

All the TB-HIV patients who developed liver toxicity (cases) in the present study tolerated the prior anti-TB therapy and developed DIH after initiating ART. Therefore, it is plausible to assume that the DIH event is more likely due to ART but not due to the concomitantly administered anti-TB drugs, although synergistic effects between ARV and anti-TB drugs to elicit the event cannot be ruled out. If this assumption holds true, findings from the present study may reflect possible genetic markers for ARV-induced liver toxicity.

In the present study, we identified SNPs that are suggestive of importance to predict DIH. The top SNP (rs199650082) in the combined analysis of ARV drugs treatment group is a missense SNP (R919Q [2756G → A]) in the *ERN1* gene. The protein encoded by this gene is the endoplasmic reticulum (ER) to nucleus signaling 1 protein, a human homologue of

the yeast serine/threonine-protein kinase/endoribonuclease inositol-requiring enzyme 1 (*IRE1*) gene product (Tirasophon et al., 1998). This protein possesses intrinsic kinase and endoribonuclease activities, and it is important in altering gene expression as a response to ER-based stress signals (Chen et al., 2014a; Itzhak et al., 2014). Emerging evidence indicates that stress in ER makes a substantial contribution to the pathogenesis of DIH (Chen et al., 2014b). Perhaps the ARV drugs have contributed to stress in ER.

Another top SNP (rs16947045) identified in the combined analysis of the ARV drug treatment group was located in mitogen-activated protein kinase-3 (*MAP3K3/MEKK3*). This gene directly regulates the stress-activated protein kinase pathway that participates in the regulation of cellular responses to various extracellular signals (Ellinger-Ziegelbauer et al., 1997). Although further analysis is required to clarify the functional importance of *ERN1* and *MAP3K3* genes, variation in the expression of proteins that are encoded by these genes might contribute to individual differences for ARV-DIH susceptibility.

In the ARV-ATD co-treatment group, the top SNP (rs4842407) had a consistently strong signal in the GWAS, the replication study, and the combined analyses, both before and after adjustment for covariates. The SNP rs4842407 is a lincRNAs transcript variant located between LOC642550 and SYT1 on chromosome 12. Long noncoding RNAs (lncRNAs) are transcribed RNA molecules (>200 nucleotides in length) that structurally resemble mRNAs but do not encode proteins. Thousands of lincRNAs are now known; however, many of their functions are still unknown.

Recently, lincRNAs are emerging as important regulators in a wide range of biological processes, including proliferation, apoptosis, and differentiation (Cai et al., 2016; Shi et al., 2013). Liver-expressed lincRNA promoters show greater enrichment for proximal binding of liver transcription factors than protein-coding gene promoters, which may reflect the higher conservation of liver lincRNA promoters (Melia et al., 2016). In fact, recently, hepatic lncRNAs are reported to be involved in the progression of liver fibrosis (He et al., 2014; Yu et al., 2016; Zheng et al., 2015). Previous GWAS studies revealed some SNPs within the lincRNAs as disease associated (Hirano et al., 2015; Radtke et al., 2009).

The significant association of rs4842407 with ARV-ATD-induced hepatotoxicity in the present study may indicate its relevance in determining predisposition to DIH in TB-HIV coinfected patients receiving dual treatment for the two diseases. Alternatively, this SNP might be in linkage disequilibrium (LD) with other SNPs that are implicated in the development of DIH. If the results are replicated in a larger sample size, rs4842407 may serve as a marker for DIH susceptibility during ARV-ATD co-treatment.

Candidate gene studies conducted on genetic risk factors contributing to ARV drugs and/or ATD-induced hepatotoxicity identified genetic variants in genes involved in drug metabolism (*CYP2B6, NAT2*), drug transporter proteins (*ABCB1*), and *HLA* region (Daly, 2016; Mugusi et al., 2012; Yimer et al., 2011, 2012). In addition, variants in *AGBL4* and *FAM65B* were identified as potential risk factors for ATD-induced liver toxicity through GWAS (Petros et al., 2016). In the current study, we did not find genetic variants that passed genome-wide significance level in both the pharmacokinetic-related genes and *HLA* region. But we found a possible association between SNPs rs11642957 ($p = 9.5 \times 10^{-5}$) in ATP-binding cassette subfamily-C member-1 (*ABCC1*, membrane-bound drug transporter) and rs9276370 ($p = 2.8 \times 10^{-4}$) in the vicinity of *HLA-DQA2* in the ARV drug-alone and ARV-ATD co-treatment groups, respectively (Supplementary Table S3). Due to these drugs, DIH may be related to the combined effect of the newly identified variants, the pharmacokinetic and immune-related gene variants.

The incidence of ARV- and ATD-induced liver toxicity displays wide variability among populations. While receiving the same type of treatment regimen, HIV (16% vs. 6%) and TB-HIV co-infected (30% vs. 10%) patients from Ethiopia presented a higher incidence of DIH compared with patients from Tanzania (Mugusi et al., 2012; Yimer et al., 2011 2012, 2014). Ethiopians display a distinct pharmacogenetic profile and CYP enzyme activities compared with other populations, including Caucasian, Asians, and other Black African populations. Higher *CYP3A* (Gebeyehu et al., 2011), *CYP2A6* (Aklillu et al., 2014), *CYP2B6* (Ngaimisi et al., 2013), *SLCO1B1* (Aklillu et al., 2016), and *CYP2D6* (Aklillu et al., 1996) enzyme activities and unique distribution of the respective variant alleles in Ethiopians compared to other populations were reported previously.

Indeed, the PCA plot of the whole-genome genotyping data in the present study indicated distinct clusters for Ethiopians compared with HapMap data for Caucasians, Chinese, Japanese, and Nigerians (Fig. 3). This further corroborates the need for more population-specific pharmacogenetic studies in Africa to identify genetic markers for drug-induced adverse events such as liver toxicity. Black Africans are characterized by greater levels of genetic diversity, more within-group genetic heterogeneity, and low LD between loci compared with non-African populations (Campbell and Tishkoff, 2008; Teo et al., 2010). HIV and TB infection remain major problems in sub-Saharan Africa, and treatment is scaled up. Hence, the identification of genetic biomarkers that predict the safety and efficacy of ARV and ATD treatment in different black African populations is crucial.

There are several limitations of this study. First, as DIH is a rare event, identifying a large number of cases for GWAS can be a constraint for achieving large study samples. For the present study, notably 4 years of research and work were required to collect the 59 cases, which resulted in a relatively limited number of case samples for sub-group analyses. Second, populations of African ancestry display greater genetic diversity and lower levels of LD among chromosomal loci (Campbell and Tishkoff, 2008; Teo et al., 2010). The low levels of LD are disadvantageous when screening the genome for associations by using the current SNP-genotyping approaches that essentially rely on the principle of LD mapping.

Although GWAS in populations of African ancestry is challenging due to a less degree of LD, the high level of genetic diversity and weak LD with neighboring SNPs in Africans ancestry is considered a powerful tool for fine mapping causal disease or phenotype-associated variants globally (Campbell and Tishkoff, 2008; Peprah et al., 2015). Therefore, additional studies with a larger sample size using higher-density SNP array or next-generation sequencing may be required to discover susceptibility variants. Our study may represent an important first step in applying GWAS to identify genetic variants of ARV drug-alone- or ARV-ATD co-treatment-induced hepatotoxicity in black African populations.

The third limitation is that as drug combinations are the current treatment protocols for TB/HIV infections, we cannot affirm that the risk variants identified correspond only to a single drug or multiple drugs. As treatment of TB/HIV consists of combination therapy, it is not possible to study individual ARV or ATD drugs in TB-/HIV-infected patients for ethical reasons. Thus, the identification of genetic risk factors for ARV-ATD co-treatment-induced liver toxicity is both important and relevant for future clinical applications, particularly in the current era when ARV drugs are being rolled for extensive use in Africa that bears a large burden of HIV and TB.

Identifying individuals at risk for DIH before drug treatment would improve drug safety. Although genetic associations with ATD- and ARV drug-induced hepatotoxicity susceptibility have been reported, none have yet been strong enough to be useful for managing DIH in the clinical practice. Identifying non-genetic factors, such as measurement of biomarkers in body fluids to predict individuals at risk for DIH before drug treatment, could have a potential for prevention. Large-scale ''-omics'' technologies are powerful tools for molecular profiling of complex disorders such as idiosyncratic DIH. The application of the new technologies, such as transcriptomics (study alterations in gene expression as a result of toxic compounds), proteomics (characterizes patterns of altered protein expression), and metabonomics (characterizes patterns of altered metabolites in blood or urine) besides pharmacogenomics, offer the potential to reveal biomarkers, identify individuals at risk, and clarify the pathogenesis of idiosyncratic hepatotoxicity (Fontana, 2014; Thulin, 2014) (No. 3458).

Proteomics investigations revealed elevated levels of cadherin 5 (CDH5) and fatty acid-binding protein (FABP) as protein biomarkers for DIH (Mikus et al., 2016). However, the detection of idiosyncratic hepatotoxicants with the currently available *in vitro* methods remains challenging, as idiosyncratic drug reactions are unpredictable and mostly immune mediated (Van Summeren et al., 2012). The integration of data from different research platforms (proteomics, transcriptomics, metabolomics, genomics) by using a systems biology approach may provide an opportunity to identify biomarkers for the prevention of idiosyncratic DIH in clinical practice.

Conclusions

Using genome-wide genotyping, we identified potential genetic variants associated with DIH due to ARV drugs alone and ARV-ATD co-treatment in Ethiopian HIV-infected patients with or without TB co-infection. The results provide evidence that in addition to genetic variants previously identified by candidate gene association studies, other variants also influence the risk of developing DIH by ARV or ARV-ATD drugs. These genetic variants that are putatively associated with DIH due to ARV drugs alone and ARV-ATD co-treatment establish a foundation for future personalized medicine in people with HIV and TB in Africa and call for larger discovery and replication studies in independent populations.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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Abbreviations Used

ABCB1 = ATP-binding cassette subfamily-B member-1
AGBL4 = ATP-/GTP-binding protein-like-4
ALP = alkaline phosphatase
ALT = alanine aminotransferase
ART = antiretroviral therapy
ARV = antiretroviral
AST = aspartate aminotransferase
ATD = anti-tuberculosis drugs
CI = confidence interval
CYP = cytochrome P450
DIH = drug-induced hepatotoxicity
ER = endoplasmic reticulum
ERN1 = endoplasmic reticulum to nucleus signaling-1
FAM65B = family with sequence similarity 65 member-B
GWAS = genome-wide association studies
HIV/AIDS = human immunodeficiency virus/acquired immune deficiency syndrome
HLA = human leukocyte antigen
LD = linkage disequilibrium
lincRNAs = long intergenic noncoding RNAs
lncRNAs = long noncoding RNAs
MAP3K3 = mitogen-activated protein kinase-3
NAT2 = N-acetyltransferase-2
OR = odds ratio
PCA = principal component analysis
PLINK = PuTTY Link
QQ = quantile-quantile
RUCAM = Roussel Uclaf Causality Assessment Method
SNP = single nucleotide polymorphism
TB = tuberculosis
T Bil = total bilirubin
ULN = upper limit of normal
WHO = World Health Organization

Paper III

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ORIGINAL RESEARCH
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*HLA-B*57* Allele Is Associated with Concomitant Anti-tuberculosis and Antiretroviral Drugs Induced Liver Toxicity in Ethiopians

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Drug-induced liver injury (DILI) is a known adverse effect of both anti-tuberculosis (anti-TB) and antiretroviral (ARV) drugs. Recent studies highlight the implications of genetic predispositions to DILI. We performed a case-control study to identify Human Leukocyte Antigen-B (HLA-B) variant alleles associated with anti-TB and ARV co-treatment induced liver toxicity in Ethiopian TB and HIV co-infected patients. A total of 495 newly diagnosed TB and HIV co-infected patients were enrolled and received rifampicin based anti-TB and efavirenz based ARV therapy. Change in liver enzyme level from baseline was monitored 1st, 2nd, 4th, 8th, 12th, and 24th weeks after treatment initiation to identify patients who developed DILI (cases) and those who did not (treatment tolerants). Genomic DNA from 46 cases and 46 sex and age matched treatment tolerants were genotyped for HLA-B variant alleles using Olerup SSP®HLA-B DNA Typing Kits. The proportion of *HLA-B*57* allele carriers in DILI cases (37.0%), particularly in those who developed cholestatic type of DILI (44.8%) was significantly higher compared with those who tolerated the treatment (2.2%). The *HLA-B*57* allele frequency was significantly higher in cases (25%) than treatment tolerants (1.1%). In a multivariate logistic analysis, the proportion of patients carrying *HLA-B*57* ($P = 0.002$) and *HLA-B*14* ($P = 0.014$) alleles were significantly higher in DILI cases compared with treatment tolerants. *HLA-B*57* was significantly associated with cholestatic ($P = 0.001$) and mixed ($P = 0.017$) types of liver toxicity, and mild-to-moderate severity ($P = 0.001$). Of all *HLA-B*57* alleles detected, *HLA-B*57:03* accounted 58.3% and *HLA-B*57:02* accounted 41.7%. *HLA-B*57:01* was not detected. The variant allele frequencies of *HLA-B*57:03* (15.2 vs. 0%) and *HLA-B*57:02* (9.8 vs. 1.1%) were significantly higher in the DILI cases than treatment tolerants ($P < 0.03$). We conclude that *HLA-B*57* alleles (*B*57:03* and *B*57:02*) confer susceptibility to the development of anti-TB and ARV drugs co-treatment induced liver toxicity, which is mainly of cholestatic type. The possible association of *HLA-B*14* with anti-TB and ARV drugs co-treatment induced liver toxicity requires further investigations.

Keywords: antiretroviral drugs, anti-tuberculosis, drug induced hepatotoxicity, DILI, Ethiopian, HIV, HLA, *HLA-B*57*

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INTRODUCTION

Tuberculosis (TB) is the most common opportunistic infection associated with human immunodeficiency virus (HIV) infection, and co-treatment of the two diseases is recommended (Harries et al., 2009). However, simultaneous treatment of TB and HIV infections is challenging due to drug interactions and overlapping toxicities (Cohen and Meintjes, 2010). Antiretroviral (ARV) and anti-tuberculosis (anti-TB) Drugs-induced liver injury (DILI) is a common adverse event, which can be fatal if therapy is not interrupted or changed on time (Devarbhavi et al., 2013; Naidoo et al., 2015; Shamanna et al., 2016). TB-HIV co-infected patients on anti-TB and ARV co-therapy are at a higher risk of developing DILI than TB or HIV only infected patients receiving monotherapy (Yimer et al., 2011, 2014; Mugusi et al., 2012). A recent study in TB/HIV patients on anti-TB and antiretroviral therapy (ART) with high levels of immune activation demonstrated impaired isoniazid clearance, implicating the need for exploring immune response and the risk of DILI (Vinnard et al., 2016). Up to 32% of HIV patients on ART discontinue their treatment or switch therapy mainly due to DILI (Bica et al., 2001), and genetic predisposition contributes partly (Lubomirov et al., 2011). Treatment interruption may increase the risk for developing of multidrug-resistant TB (MDR-TB) and HIV/AIDS (Hirpa et al., 2013). Thus, identifying genetic markers for drug-induced liver toxicity is valuable to identify high-risk patients and to introduce appropriate measures.

Both HIV and TB remain a major problem and co-infection is common in most Sub-Saharan African (SSA) countries including Ethiopia, the second most densely populated country in Africa with an estimated population size of 100 million. Ethiopia is listed among the top 20 high-TB burden countries globally, and one of the high MDR-TB burden countries (Biadglegne et al., 2014; World-Health-Organization, 2016). The rate of new HIV infection in Ethiopia is declining with the estimated number of people living with HIV being 769, 600 in 2014 (World-Health-Organization[1]). The scale of ART is increasing in the country as part achieving the UNAID/WHO "90-90-90" target: to diagnose 90% of all HIV positive people, provide ART for 90% of those diagnosed and achieve viral suppression for 90% of those treated, by 2020 (UNAIDS, 2014). ART and anti-TB drug-induced liver toxicity is a common problem in Ethiopia causing treatment discontinuation and hence MDR-TB (Hirpa et al., 2013; Yimer et al., 2014).

Genetic variations in HLA gene is implicated with susceptibility to T-cell mediated adverse events to a wide range of pharmaceuticals making it a candidate gene relevant to pharmacogenetic studies (Barbarino et al., 2015). HLA alleles that are reported to be association with increased risk of idiosyncratic DILI include: HLA-*DQB1*02:01* and *DQB1*05* to anti-TB drugs (Sharma et al., 2002; Chen et al., 2015), and *HLA-B*58:01* and *DRB1*01:02* to nevirapine-containing ARV regimens (Phillips et al., 2013). *HLA-B*57:01* and *A*33:03* variant alleles were also reported as genetic markers for idiosyncratic liver injury induced by flucloxacillin (Daly et al., 2009) and ticlopidine (Hirata et al., 2008) respectively. Susceptibility to amoxicillin-clavulanate-induced liver injury is influenced by multiple HLA class I and II alleles (Lucena et al., 2011). A large genome-wide association study found a strong association of amoxicillin-clavulanate induced liver injury with *HLA-A*02:01*, HLA-DQB1*06:02, and *DRB1*15:01* variant alleles (Lucena et al., 2011). Genetic screening for *HLA-B*57:01* and subsequent treatment modifications have been shown to reduce incidence of life-threatening hypersensitivity to abacavir in HIV/AIDS patients carrying the allele (Hughes et al., 2008; Mallal et al., 2008) and *HLA-B*15:02* to carbamazepine in Southeast Asian carriers (Amstutz et al., 2014).

Most previous reports investigating genetic risk factors for anti-TB and ARV drugs-induced liver toxicity focused on drug metabolizing enzymes and transporter proteins (Lee et al., 2010; Yimer et al., 2011, 2012). Previously we reported the association of high efavirenz plasma concentration and *CYP2B6*6* genotype with DILI in TB-HIV patients (Yimer et al., 2011, 2012; Mugusi et al., 2012). However, only a few studies have explored the association of HLA genes with anti-TB or ARV drugs-induced liver toxicity. Therefore, in this study, we aimed to investigate the possible associations between HLA-B alleles, and anti-TB and ARV drugs co-treatment induced liver injury in TB and HIV co-infected patients in Ethiopia.

METHODS

Study Design and Participants

Using a case-control comparative study design, we analyzed data from newly diagnosed TB and HIV co-infected patients, who were enrolled and followed up prospectively to identify the incidence, the pattern, and severity of anti-TB and ARV drugs-induced liver toxicity in Ethiopian patients (Yimer et al., 2014). In brief, 495 TB and HIV co-infected patients with CD4 count $\leq$200 cells/mm^3 were recruited from three health institutions: Kazanchis and Beletshachew health centers, and Tikur Anbessa Specialized Hospital in Addis Ababa, Ethiopia, from June 2007 to June 2012. The inclusion criteria were TB and HIV co-infected men and non-pregnant women with age 18 years old and above. Patients were excluded if they had a history of prior treatment for TB/HIV or known pre-existing liver disease.

The study protocol was approved by the Institutional Review Board of College of Health Sciences, Addis Ababa University, the National Research Ethics Review Committee of Ethiopia, and Ethical Review Board of Karolinska Institutet, Sweden. Written informed consent was obtained from all the study participants in accordance with the Declaration of Helsinki.

Drug Treatment

All the study participants received first line ARV drugs containing efavirenz and lamivudine with tenofovir, zidovudine, or stavudine. A short-course anti-TB regimen consisting of rifampicin, isoniazid, pyrazinamide, and ethambutol for the first 2 months followed by rifampicin and isoniazid for the next 4 months was given. The patients did not receive other known hepatotoxic drugs concurrently, except co-trimoxazole

[1]World Health Organization. Ethiopia. HIV/AIDS. Available online at: http://www.afro.who.int/en/ethiopia/country-programmes/topics/4480-hivaids.html (Accessed January 29, 2017).

prophylaxis that was given for TB and HIV co-infected patients according to the National Treatment Guideline. Change in liver enzymes levels from baseline was monitored on the 1st, 2nd, 4th, 8th, 12th, and 24th weeks after initiation of treatment.

Case Definitions, Severity Grade, and Pattern of Liver Toxicity

The criteria set by the International DILI Expert Working Group were used for DILI case definitions and pattern of liver injury determination (Aithal et al., 2011). The upper limit of normal (ULN) for liver enzymes used for the study population were alanine aminotransferase (ALT 33 U/L for male; 29 U/L for female), aspartate aminotransferase (AST, 41 U/L), alkaline phosphatase (ALP, 128 U/L), and 1.0 mg/dL for total bilirubin (Yimer et al., 2014). All cases recruited met at least one of the following criteria: (1) ALT $\geq$ 5xULN, (2) ALP $\geq$ 2xULN, or (3) ALT $\geq$ 3xULN along with total bilirubin (T Bil) $\geq$ 2xULN. Treatment tolerants (controls) were individuals who were on anti-TB and ARV drugs co-treatment but did not fulfill the case definitions for DILI and had not presented with clinical signs and symptoms consistent with DILI in the follow-up period.

The pattern of liver toxicity was defined using *R*-value, where $R = (\text{ALT/ULN})/(\text{ALP/ULN})$. Cases were categorized as hepatocellular ($R \geq 5$), cholestatic ($R \leq 2$), or mixed ($2 < R < 5$) pattern of DILI. Clinical severity grading was determined by employing the highest measured values for each of the biochemical parameters (Yimer et al., 2014). Patients with grades one and two severities were grouped together into a "mild-to-moderate" group and those with grades three and four into a "severe" group. Causality assessment for DILI was performed using Roussel Uclaf Causality Assessment Method (RUCAM; Danan and Benichou, 1993).

Among the 495 TB and HIV co-infected patients involved in the initial cohort, 120 experienced DILI in the follow-up period (Yimer et al., 2014). Of these, 80 cases and 275 treatment tolerants had adequate DNA available for further analysis. After excluding patients that had abnormal liver biochemistry prior to starting treatment, or patients who had serological test positive for either hepatitis B virus surface antigen or anti-hepatitis C virus antibody, 46 cases and 46 treatment tolerants that have complete clinical data and matched with respect to gender and age in a 1:1 ratio were used for the current study.

HLA-B Genotyping

Genomic DNA was isolated from peripheral blood using QIAamp DNA Maxi Kit (QIAGEN GmbH. Hilden. Germany). We first screened for HLA-B variant alleles using a low-resolution (two digits) genotyping. HLA-B genotyping was performed using low-resolution Olerup SSP®HLA-B Typing Kit (Olerup SSP AB, Franzengatan 5, SE-112 51 Stockholm, Sweden). Allele-specific polymerase chain reaction (PCR), using sequence-specific primers was done according to the protocol and recommendations of the manufacturer. The amplified PCR products were analyzed using 2% agarose gel, and the HLA-B allele types were determined using HELMBERG-SCORE software. Low-resolution typing results were recorded with the 2-digit code to ensure a uniform level of HLA resolution for the alleles.

As a next step, high resolution (four digits) typing were done for HLA-B variant alleles that showed significant association with DILI based on the low-resolution genotyping data. HLA-B*57 exhibited a significant association with DILI, and high-resolution subtyping was performed for all *HLA-B*57* allele carriers using Olerup SSP® *HLA-B*57:01* Typing Kit (Olerup SSP AB, Franzengatan5, SE-112 51 Stockholm, Sweden) according to the protocol and recommendations of the manufacturer.

Statistical Analysis

Continuous variables were presented by a mean and standard deviation, and categorical variables as numbers and percentages. Univariate logistic regression analysis was used to identify potential independent risk factors for anti-TB and ARV drugs co-treatment induced liver toxicity. Variables with $P < 0.1$ in the univariate analysis were included in a multivariate logistic analysis. The strength of the associations was estimated by calculating the odds ratio (OR) and 95% confidence interval (CI). Fisher's exact test was used for HLA-B alleles with <5 expected cell count in a 2×2 table. To reduce bias in estimating the OR, Haldane's modification was employed (Haldane, 1956) i.e., whenever a zero-count cell was encountered, 0.5 was added to all cells in the 2×2 table. $P < 0.05$ were considered statistically significant. The corrected *P*-values (*Pc*) were adjusted by using Bonferroni's correction for multiple comparisons (18 tests) to account for the number of HLA-B alleles observed in the study participants. The statistical analysis was performed using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA).

RESULTS

A total of 92 TB and HIV co-infected patients on anti-TB and ARV drugs co-treatment were involved in this study; 46 treatment induced liver toxicity cases and 46 sex and age matched treatment tolerants. The demographics and clinical characteristics of the study participants are described in **Table 1**.

In the univariate analysis, there were statistically significant differences in the CD4 count and Karnofsky score between DILI cases and treatment tolerants ($P < 0.05$). In a multivariate logistic analysis, baseline CD4 count remained as significant predictors of anti-TB and ARV drugs co-treatment induced liver injury. There were no statistically significant differences in the baseline liver enzyme levels and type of ARV regimens used between the DILI cases and treatment tolerants. More than half of the DILI cases developed the cholestatic type of liver toxicity, and 85% of the cases had mild-to-moderate severity of liver toxicity. All of the cases had a minimum score of three ("possible") in RUCAM scoring system for DILI.

HLA-B genotype result from the low resolution typing for each study participant is presented in Supplementary Table 1. Comparison of *HLA-B* allele carriers' proportions between patients who developed DILI vs. treatment tolerants is presented in **Table 2**. A total of 18 *HLA-B* variant alleles were detected (**Table 2**). In the univariate analysis, the proportion of *HLA-B*57*

TABLE 1 | Demographics and clinical characteristics of the study participants.

Characteristics	Cases	Treatment tolerants	Univariate analysis		Multivariate analysis	
	(*N* = 46)	(*N* = 46)	*P*	OR (95% CI)	*P*	OR (95% CI)
Sex (M/F)	22/24	22/24	1.00	1.00 (0.44–2.27)		
Age (yrs), mean (*SD*)	35.4 (9.3)	35.3 (8.7)	0.95	0.99 (0.95–1.05)		
BMI (kg/m^2), mean (*SD*)	18.4 (2.4)	19.1 (3.5)	0.25	1.09 (0.94–1.26)		
CD4 count (per mm^3), mean (*SD*)	69.3 (48.7)	94.2 (51.4)	**0.02**	1.01 (1.01–1.02)	**0.03**	1.01 (1.00-1.02)
Viral load (copies/ml), log mean (*SD*)	5.3 (1.0)	5.0 (1.0)	0.20	0.72 (0.44–1.19)		
Karnofsky score	82.8 (13.6)	88.7 (13.4)	**0.04**	1.03 (1.00–1.07)	0.06	1.03 (0.99–1.07)
LFT VALUES, MEAN (*SD*)						
Baseline ALT (U/L)	25.5 (9.8)	27.8 (8.3)	0.22	1.03 (0.98–1.08)		
Baseline AST (U/L)	32.7 (10.3)	31.9 (9.6)	0.70	0.99 (0.95–1.03)		
Baseline ALP (U/L)	99.5 (19.3)	104.3 (19.3)	0.23	1.01 (0.99–1.04)		
Baseline TBil (mg/dL)	0.7 (0.5)	0.7 (0.4)	0.71	0.84 (0.34–2.10)		
ARV REGIMEN, *N* (%)						
TDF/3TC/EFV	15 (32.6)	12 (26.1)	0.49	1.37 (0.56–3.38)		
ZDV/3TC/EFV	16 (34.8)	16 (34.8)	1.00	1.00 (0.42–2.36)		
D4T/3TC/EFV	15 (32.6)	18 (39.1)	0.52	0.75 (0.32–1.78)		
PATTERN OF LIVER INJURY, *N* (%)						
Cholestatic	29 (63.0)					
Hepatocellular	2 (4.4)					
Mixed	15 (32.6)					
SEVERITY GRADE, *N* (%)						
Mild-to-moderate	39 (84.8)					
Severe	7 (15.2)					
RUCAM SCALE, *N* (%)						
Definite (score > 8)	30 (65.2)					
Probable (score 6–8)	12 (26.1)					
Possible (score 3–5)	4 (8.7)					

3TC, Lamivudine; ALP, Alkaline phosphatase; ALT, Alanine aminotransferase; ARV, Antiretroviral; AST, Aspartate aminotransferase; BMI, Body mass index; CI, Confidence Interval; D4T, Stavudine; EFV, Efavirenz; HIV, Human immunodeficiency virus; LFT, Liver function test; OR, Odds ratio; RUCAM, Roussel Uclaf Causality Assessment Method; SD, Standard deviation; T Bil, Total bilirubin; TB, Tuberculosis; TDF, Tenofovir; ZDV, Zidovudine. P-value < 0.05 is highlighted in bold.

and *HLA-B*14* alleles carriers were significantly higher in DILI cases than treatment tolerants. Association of being a carrier of *HLA-B*57* with increased risk for DILI remained significant after correction for multiple testing. On the other hand, *HLA-B*41* was negatively associated with DILI. The multivariate logistic analysis retained *HLA-B*57* and *HLA-B*14* as significant predictors of concomitant anti-TB and ARV drugs induced liver injury. For *HLA-B*57*, the association maintained after correcting for multiple comparisons ($Pc = 0.036$).

Associations between *HLA-B*57*, *HLA-B*14*, and *HLA-B*41* with the pattern and severity of liver toxicity was evaluated by comparing the proportion of allele carriers between DILI cases and treatment tolerants (**Table 3**). Compared with treatment tolerants, the proportion of *HLA-B*57* allele carriers were significantly higher in cholestatic and mixed types of DILI. In addition, the proportion of *HLA-B*14* allele carriers were higher in the mixed type of DILI cases than the treatment tolerants. There were no statistically significant differences in the hepatocellular type of DILI. Compared with treatment tolerants, the proportion of patients carrying *HLA-B*57* allele was significantly higher in the mild-to-moderate DILI group, and those carrying *HLA-B*14* in the severe DILI group were over-represented. There was a statistically significant difference in the proportion of *HLA-B*41* allele carriers in the mild-to-moderate DILI group compared with treatment tolerants.

HLA-B variant alleles that showed significant association with DILI from the low-resolution genotyping were further subjected to high resolution (four digit) testing to identify the sub-variant allele. The result from high resolution genotyping was interpreted in conjunction with the results from the prior low-resolution typing. *HLA-B*57* was the only variant allele that exhibited a significant association with DILI after correcting for multiple testing. Thus, high-resolution typing was done for all subjects who were genotyped as carriers of HLA-B*57 in the low-resolution typing (see **Table 4**).

Of all *HLA-B*57* alleles identified, *HLA-B*57:03* accounted 58.3% and *HLA-B*57:02* accounted 41.7%. *HLA-B*57:01* was not detected. Of the *HLA-B*57* allele carrier cases, 10 (58.8%) had *HLA-B*57:03* (four homozygous) and the rest seven (41.2%) had *HLA-B*57:02* (two homozygous). There was only one *HLA-B*57* allele carrier (heterozygous for *HLA-B*57:02)* out of the 46 treatment-tolerants. The proportion of *HLA-B*57:03* and

TABLE 2 | Comparison of proportion of *HLA-B* allele carriers between patients who developed ARV and anti-tuberculosis drugs induced liver injury (cases) and patients who did not (treatment tolerants).

HLA-B alleles	DILI cases (*N* = 46)	Treatment tolerants (*N* = 46)	Univariate analysis			Multivariate analysis	
	N (%)	*N* (%)	*P*	*Pc*	OR (95% CI)	*P*	OR (95% CI)
*B*57*	17 (37.0%)	1 (2.2%)	**0.002**	**0.036**	26.38 (3.33–209.07)	**0.002**	30.08 (3.44–263.11)
*B*14*	10 (21.7%)	3 (6.5%)	**0.047**	0.845	3.98 (1.02–15.58)	**0.014**	7.51 (1.50–37.68)
*B*41*	3 (6.5%)	10 (21.7%)	**0.047**	0.845	0.25 (0.06–0.98)	0.112	0.26 (0.05–1.37)
*B*15*	8 (17.4%)	16 (34.8%)	0.06	1.000	0.40 (0.15–1.05)	0.526	0.68 (0.21–2.23)
*B*08*	0	4 (8.7%)	0.12		1.48 (0.01–1.94)*		
*B*18*	0	3 (6.5%)	0.24		0.13 (0.01–2.66)*		
*B*44*	2 (4.3%)	5 (10.9%)	0.25		0.37 (0.07–2.03)		
*B*13*	5 (10.9%)	8 (17.4%)	0.37		0.58 (0.17–1.93)		
*B*58*	4 (8.7%)	2 (4.3%)	0.41		2.10 (0.36–12.05)		
*B*51*	6 (13.0%)	4 (8.7%)	0.50		1.58 (0.41–6.00)		
*B*53*	4 (8.7%)	3 (6.5%)	0.70		1.37 (0.29–6.47)		
*B*07*	10 (21.7%)	9 (19.6%)	0.80		1.14 (0.42–3.14)		
*B*49*	10 (21.7%)	10 (21.7%)	1.00		1.00 (0.37–2.69)		
*B*35*	1 (2.2%)	0	1.00		3.07 (0.12–77.25)*		
*B*37, *39, *50, *73*	0	2 (4.3%)	0.50		0.19 (0.01–4.10)*		

*Haldane's modification.

ARV, Antiretroviral; CI, Confidence Interval; HLA, Human Leukocyte Antigen; OR, Odds ratio; TB, Tuberculosis.

P, P-values were calculated by Fisher's exact test comparing the positive alleles in cases with those of treatment tolerants.

Pc, Corrected P-values were adjusted by using Bonferroni's correction for multiple comparisons to account for the observed 18 HLA-B alleles. P-value < 0.05 is highlighted in bold.

TABLE 3 | Association of HLA-B alleles with the pattern and severity of drug-induced liver injury.

Characteristics	*HLA-B*57* carriers			*HLA-B*14* carriers			*HLA-B*41* carriers		
	N (%)	*P*	OR (95% CI)	*N* (%)	*P*	OR (95% CI)	*N* (%)	*P*	OR (95% CI)
DILI PATTERN									
Treatment tolerants (*n* = 46)	1 (2.2)	–	–	3 (6.5)	–	–	10 (21.7)	–	–
Cholestatic (*n* = 29)	13 (44.8)	**0.001**	36.6 (4.4–302.3)	5 (17.2)	0.17	3.0 (0.7–13.6)	2 (6.9)	0.11	0.3 (0.1–1.3)
Hepatocellular (*n* = 2)	0	1.00	6.1 (0.2–190.1)*	1 (50.0)	0.16	14.3(0.7–29.4)	0	1.00	0.7 (0.1–15.6)*
Mixed (*n* = 15)	4 (26.7)	**0.017**	16.4 (1.7–161.3)	4 (26.7)	**0.048**	5.2 (1.0–26.8)	1 (6.7)	0.22	0.3 (0.1–2.2)
SEVERITY GRADE									
Mild-to-moderate (*n* = 39)	16 (41.0)	**0.001**	31.3 (3.9–251.0)	7 (17.9)	0.12	3.1(0.8–13.1)	2 (5.1)	**0.04**	0.2 (0.1–1.0)
Severe (*n* = 7)	1 (14.3)	0.173	2.7 (0.6–11.7)	3 (42.9)	**0.01**	10.8 (1.6–71.9)	1 (14.3)	0.65	0.6 (0.1–5.6)

*Haldane's modification.

CI, Confidence Interval; HLA, Human Leukocyte Antigen; OR, Odds ratio. P-value < 0.05 is highlighted in bold.

*B*57:02* allele carriers were significantly higher in the DILI cases than treatment tolerants [$P = 0.01$, OR = 26.8 (1.5–47.2) and $P = 0.03$, OR = 8.1 (1.0–68.6)], respectively.

Comparisons of *HLA-B*57*, **14*, and **41* (from the low-resolution genotyping) as well as HLA-*B*57:02* and *B*57:03* (from the high-resolution genotyping) allele frequencies between DILI cases and treatment tolerants is presented in **Table 5**. The overall allele frequency of *HLA-B*57* was higher (13.0%) than that of the other *HLA-B* alleles (7.6% for *B*14* and 7.1% for *B*41*). The allele frequency of *HLA-B*57* was higher in DILI cases (25.0%) compared to treatment tolerants (1.1%). The *HLA-B*57:03* and *B*57:02* allele frequencies in DILI cases (15.2 and 9.8%) were higher than the treatment tolerants (0 and 1.1%), respectively.

DISCUSSION

In the present study, we investigated the association of *HLA-B* variant alleles with risk for concomitant anti-TB and ARV drugs induced liver toxicity. The proportion of *HLA-B*57* allele carriers in Ethiopian patients who developed anti-TB and ARV drugs induced liver toxicity (37.0%), particularly in those who developed cholestatic type of liver toxicity (44.8%) was significantly higher compared with those who tolerated the treatment (2.2%). The proportion of *HLA-B*14* allele carriers who developed DILI (21.7%) was also significantly higher compared with the treatment tolerants (6.5%). These indicate that *HLA-B*57* and *B*14* allele carriers might be at a higher risk of developing anti-TB and ARV drugs co-treatment induced liver

toxicity. Accordingly, these variant alleles might play important roles in the pathogenesis of immune-mediated liver toxicity during anti-TB and ARV drugs co-treatment. The *HLA-B*57* and *B*14* molecules may function as endogenous antigen presenting molecules for the drugs/metabolites to HLA-restricted cytotoxic T-cell activation (Pichler, 2002). To our knowledge, this is the first report to investigate the association of *HLA-B*57*, *B*14*, and *B*41* alleles with anti-TB and ARV drugs co-treatment induced liver toxicity.

The *HLA-B*57* variant allele, which was observed in significantly higher proportion among DILI cases than treatment tolerants, had a high specificity (97.8%) and positive predictive value (94.4%). Hence, *HLA-B*57* is likely to be an important predictor for anti-TB and ARV drugs co-treatment induced liver injury. However, there could be additional yet unidentified genetic markers and non-genetic risk factors involved in the pathogenesis of DILI. The matched case-control design used in this study minimizes effects of potential confounders and may increase power to identify genetic associations. Although this limits us from exploring associations of the matching variables such as sex, body mass index, Karnofsky score, CD4 count, and HIV viral load which were independently and significantly associated with the risk of developing DILI (Yimer et al., 2011, 2014). Association of CD4 cell counts and Karnofsky score as risk factors for DILI were also found in this study, although the others were not significant.

Studies suggest that a particular *HLA-B* allele may exert a protective effect against certain adverse drug reactions as evidenced by lower allele carrier frequencies in cases compared with treatment tolerants. *HLA-DQA1*01:02* was identified as a protective variant for anti-TB drugs induced hepatotoxicity (Sharma et al., 2002). *HLA-B*40:01* and *HLA-B*07:02* were also identified as protective variant alleles for carbamazepine-induced severe cutaneous adverse reactions (Alfirevic et al., 2006; Hung et al., 2006). In our study, statistically significant lower allele carrier rate of *HLA-B*41* was noted in the DILI cases compared with the treatment tolerants (6.5 vs. 21.7%), but this effect did not reach statistical significance after correcting for multiple comparisons. Further, analysis is required to clarify the role of *HLA-B*41* in the prevention of development of anti-TB and ARV drugs co-treatment induced liver injury.

Our result indicated a positive association of *HLA-B*57* allele with mild-to-moderate liver injury and the *HLA-B*14* allele with severe liver injury. On the other hand, *HLA-B*41* allele was negatively associated with mild-to-moderate liver injury. Accordingly, the association of *HLA-B* alleles with anti-TB and ARV drugs co-treatment induced liver injury may seem to depend on the severity of liver injury. The *HLA-B*57* allele may be critical for the initiation of the immune response to cause DILI and the *HLA-B*14* allele for the progression to severe degree of liver injury. On the other hand, the *HLA-B*41* allele seems to play a role in the prevention of development of mild-to-moderate liver injury due to anti-TB and ARV drugs co-treatment. These findings warrant further investigation in a larger DILI case samples for each severity grade of liver injury.

DILI can be hepatocellular (predominant rise in ALT), cholestatic (predominant rise in ALP), or mixed type liver injury (Hussaini and Farrington, 2014). Recently, we conducted

TABLE 4 | High resolution genotyping data for all *HLA-B*57* allele carriers stratified by DILI type.

Patient No.	Age	Sex	Status	DILI type	Allele 1	Allele 2
1	25	F	Case	Cholestatic	**B*57:03**	**B*57:03**
2	34	F	Case	Cholestatic	**B*57:03**	**B*57:03**
3	45	M	Case	Cholestatic	**B*57:03**	**B*57:03**
4	25	M	Case	Cholestatic	**B*57:02**	**B*57:02**
5	28	F	Case	Cholestatic	**B*57:03**	B*58:01
6	37	F	Case	Cholestatic	**B*57:02**	B*51:08
7	28	F	Case	Cholestatic	**B*57:03**	B*49:01
8	55	M	Case	Cholestatic	**B*57:02**	B*39:12
9	45	F	Case	Cholestatic	**B*57:02**	B*58:01
10	30	F	Case	Cholestatic	**B*57:03**	B*53:01
11	60	M	Case	Cholestatic	**B*57:03**	B*13:02
12	30	F	Case	Cholestatic	**B*57:03**	B*53:01
13	30	F	Case	Cholestatic	**B*57:02**	B*44:02
14	30	F	Case	Mixed	**B*57:03**	**B*57:03**
15	31	M	Case	Mixed	**B*57:02**	**B*57:02**
16	49	M	Case	Mixed	**B*57:03**	B*58:01
17	38	F	Case	Mixed	**B*57:02**	B*49:01
18	32	F	Control	Treatment tolerant	**B*57:02**	B*41:02

*DILI, Drug induced liver injury. B*57:02 and B*57:03 alleles are highlighted in bold.*

TABLE 5 | Comparison of *HLA-B* allele frequencies distribution between patients who developed ARV and anti-tuberculosis drugs induced liver toxicity (cases) and patients who did not (treatment tolerants).

HLA-B allele	DILI cases (*N* = 46)		Treatment tolerants (*N* = 46)		*P*-value	Odds ratio	
	Observed frequency	95% CI	Observed frequency	95% CI		OR	95% CI
*HLA-B*57*	0.250	0.162–0.338	0.011	0.004–0.032	<0.001	30.33	4.00–230.3
*HLA-B*14*	0.130	0.062–0.199	0.033	0.004–0.069	0.06	3.30	1.02–10.65
*HLA-B*41*	0.033	0.004–9.069	0.109	0.045–0.172	0.08	0.28	0.07–1.04
*HLA-B*57:03*	0.152	0.079–0.226	0	0			
*HLA-B*57:02*	0.098	0.037–0.159	0.011	0.001–0.032			

OR, Odds Ratio; CI, Confidence interval; HLA, Human leukocyte antigen.

a prospective observational study to evaluate the incidence, type, severity, and predisposing risk factors for DILI in a large well-defined TB and/or HIV patient cohort receiving either anti-TB drugs alone, ARV drugs alone or concomitant anti-TB and ARV therapy (Yimer et al., 2014). We found rates of hepatocellular DILI being highest among patients treated with anti-TB drugs alone than patients treated with ARV drugs alone or co-treated with anti-TB drugs. On the other hand, the rates of cholestatic DILI was highest among patients treated with efavirenz based-ARV drugs alone than patients treated with anti-TB drugs alone or with ARV drugs (Yimer et al., 2014). DILI due to anti-TB drugs in TB mono-infected patients is known to be more of hepatocellular type. In the present study, most of the TB-HIV co-infected patients treated with concomitant anti-TB and efavirenz based-ARV drugs developed cholestatic DILI cases. Apparently, there is a significant contribution from efavirenz based-ARV drugs toward developing cholestatic type of DILI. Indeed, the significant association of *HLA-B*57* variant allele with cholestatic type DILI identified in the present study might reflect for ARV-drugs induced hepatotoxicity. However, the role of HLA variant alleles for predisposition to anti-TB DILI cannot be ruled out (Sharma et al., 2002; Chen et al., 2015). Further, studies are necessary to investigate the association of HLA allele carrier status with anti-TB drugs alone as well as ARV drugs alone-induced liver injury.

Major histocompatibility complex (MHC) class I and class II-mediated immunological reactions are implicated in DILI, particularly in the cholestatic type that involves damage to the biliary system (Andrade et al., 2004; Daly, 2010). In line with this, carrier status of *HLA-B*57* was significantly higher in patients who presented with the cholestatic type of DILI (44.8%) and mixed type (26.7%) compared with those who tolerated the treatment (2.2%). None of the patients who developed hepatocellular DILI were carriers of *HLA-B*57* variant allele.

*HLA-B*57* allele is associated with long-term non-progressive chronic HIV-1 infection by restricting cytotoxic T-lymphocyte response (Goulder et al., 2000). HLA-*B*57:01* and *B*57:03* are the most prevalent *HLA-B*57* subtypes in Caucasian and African populations, respectively (Pelak et al., 2010; Apps et al., 2013). The *HLA-B*57:01* and *B*57:03* alleles are protective against HIV disease progression, and appear to present identical Gag epitopes (Payne et al., 2014). *HLA-B*57:01*, *B*57:02*, and *B*57:03* share more than 90% sequence homology and as such have peptide-binding repertoires which substantially overlap (Illing et al., 2012; Ogese et al., 2017). Although in ART naïve patients *HLA-B*57* (*B*57:01* in Europe and US, *B*57:03* in black Africans) confers protective effect against HIV-1 disease progression to AIDS (Costello et al., 1999; Migueles et al., 2000; López-Larrea et al., 2005; Frater et al., 2007), it may exert contradictory effect on treatment outcome when the disease course is altered by ARV therapy (Dold et al., 2015). Previous studies reported the association of *HLA-B*57* with increased all causes of mortality (Dold et al., 2015) and reduced virological responses during ARV therapy (Kuniholm et al., 2011). *HLA-B*57* allele is also known to be associated with immune-mediated drug-induced hypersensitivity reactions. Carriers of *HLA-B*57:01* allele are at higher risk of developing abacavir-induced hypersensitivity reactions (Hetherington et al., 2002), whereas *HLA-B*57:03* is associated with spondylarthropathies (López-Larrea et al., 2005). Indeed, genetic screening for *HLA-B*57:01* variant allele has been shown to reduce drug toxicity and subsequently led to a labeled recommendation of routine screening before treatment initiation (Hughes et al., 2008; Mallal et al., 2008).

The frequency and subtypes of *HLA-B*57* variant alleles display wide inter-ethnic variability globally ranging from 0 to 22.5% (http://www.allelefrequencies.net/). The overall frequency of *HLA-B*57* in our study population from Ethiopia is 13% which is relatively high. *HLA-B*57:01* occurs in Asians and Caucasians (up to 5%). The *HLA-B*57:03* and *B*57:02* variant alleles commonly occur in black population reaching up to 3 and 7% allele frequencies, respectively. Interestingly *HLA-B*57:01* was not detected, and it may be rare or absent in Ethiopians similar to other black Africans where the allele frequency is <1%. The overall *HLA-B*57:03* and *B*57:02* allele frequencies in our TB/HIV co-infected study population was 7.6 and 5.4%, respectively, although the frequencies in healthy Ethiopians is yet unknown. Interestingly, *HLA-B*57:03* and *B*57:02* allele frequencies in DILI cases (15.2 and 9.8%) were significantly higher than the allele frequencies in the treatment-tolerants (0 and 1.1%), respectively.

There were some limitations in this study. First, as DILI is a rare event, it was not easy to get large number of cases (four years were required to collect the DILI cases in this study), which subsequently resulted in a small number of samples for sub-group analysis. The second limitation is that as drug combinations are the current treatment protocols for TB and HIV co-infections, and hence we cannot link the risk variant allele to a specific drug or class of drug(s). Since first line anti-TB and HIV treatment regimen consists of combination therapy, it is not possible to study individual drug-induced liver toxicity in TB and HIV co-infected patients for ethical reasons. However, our study represents an important first step in applying HLA-B typing to identify genetic variants for anti-TB and ARV drugs co-treatment induced liver injury.

Identification of genetic risk factors for anti-TB, and ARV drugs co-treatment induced liver injury is essential for patient safety. The HLA risk alleles predisposing to immune-mediated anti-TB and ARV drugs induced liver toxicity in black African population are not well investigated. A common problem encountered in HLA genotyping is inability to determine the variant alleles accurately using a simple genotyping procedure. This is mainly due to the extensive genetic diversity in HLA gene locus. Accurate allele-level HLA typing using the current methods requires high workload, cost and time. Because of extreme genetic variation of the HLA locus, pharmacogenetic testing for routine clinical practice is increasingly challenging in resource limited settings. However, the recent development of second-generation sequencing methods provides the possibility of sequencing a single DNA strand in isolation. Establishing a straightforward and affordable genotyping method for accurate HLA typing to identify patients at risk of developing drug-induced adverse events may lay the ground for the future application of pharmacogenetic testing in clinical practice for globalized personalized medicine.

In conclusion, HLA-B variant alleles may play important roles in determining the risk and severity of concomitant anti-TB and ARV drugs induced liver toxicity. *HLA-B*57* variant alleles (*HLA-B*57:03* and *HLA-B*57:02*) are risk factors to develop anti-TB and ARV drugs co-treatment induced liver injury, mainly of cholestatic type and mild DILI cases. The possible risk association of *HLA-B*14* allele with severe DILI and the protective association of *HLA-B*41* require further investigations. Additional studies are necessary to understand the roles of the identified *HLA-B* variant alleles in the pathogenesis of anti-TB and ARV drugs co-treatment induced liver toxicity.

AUTHOR CONTRIBUTIONS

EA, JK, and EM conceived and designed the study; EA, GY, AH, EM collected the data; EA, JK, and ZP performed the experiment and analyzed the data; EA and ZP wrote this paper. All authors revised/edited the manuscript and approved for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphar.2017.00090/full#supplementary-material

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