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Pharmacokinetic and Pharmacogenetic aspects of drug-drug interactions between antiretroviral and antituberculosis drugs in Ethiopian patients: Implication for optimization of TB-HIV co-treatment.

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ABSTRACT

TB and HIV are immuno-pathologically interacting epidemic infectious diseases affecting the lives of millions globally & sub-Saharan African region accounts the highest burden of both diseases. Although effective therapies are available for the management of each, TB-HIV co-treatment has faced challenges mainly due to drug-drug interactions & overlapping drug toxicities. To overcome these, efavirenz (EFV) based highly active antiretroviral therapy (HAART) is the preferred regimen while rifampicin (RIF) based anti-TB treatment regimen is a choice to treat TB-HIV co-infection in resource-limited settings. RIF is a known enzyme & drug transporter inducer and/or inhibitor. The dose of EFV to be used in the presence of RIF is, however, controversial. This thesis is primarily carried out to investigate the pharmacogenetic and pharmacokinetic aspect of drug-drug interaction between RIF & EFV aiming to optimize the dose of EFV to be used in TB-HIV co-infected Ethiopian patients.

This study was designed to be carried out in two sub-Saharan African countries (Ethiopia and Tanzania), owning to the heterogeneity of the region genetically and culturally. This thesis focuses on the Ethiopian population. The thesis was conducted by prospectively recruiting cohort of HIV infected individuals without TB (Arm 1; N = 285) in parallel to another cohort of HIV co-infected with active TB (Arm 2; N = 196). All study participants were adults with baseline CD_4 count less than 200 cells per mm^3 and were followed for a year. At baseline and follow up periods, clinical chemistry (liver and kidney function tests), hematological parameters (complete and differential blood cell counts) and HAART outcome monitoring (CD_4 counts and HIV RNA viral load) were done. In addition, genotyping for *CYP2B6*6, CYP3A5 (*3, *6, *7), UGT2B7*2, NAT2, ABCB1 (3435 C > T & 3842 A > G) & SLCO1B1 (*1b & *5)* were also done. Pharmacokinetic variables such as plasma/intracellular concentrations of EFV, 8-hydroxy-efavirenz (major metabolite) & metabolic ratio were determined at weeks 4 and/or 16, 16±1h post-dose. Besides, cholesterol, 4β-hydroxy-cholesterol (biomarker for *CYP3A* activity) & metabolic ratio at weeks 0, 4, 16 & 48 were also determined to investigate time-dependent effect of EFV on *CYP3A* enzyme. Socio-demographic factors (Age, sex, baseline body weight and BMI) were also recorded.

This thesis reports paradoxical increase in plasma/intracellular EFV concentrations by RIF co-therapy; coherent to this is improved immunological outcomes among individuals co-treated for TB and HIV with comparable virologic success to HAART than those without RIF co-treatment. The thesis also shows wide between-subject variability in the long-term auto-induction by EFV based on *CYP2B6* genotype. Between & within-subject variability in plasma EFV concentration and immunological outcome are shown to be influenced by RIF co-therapy, *CYP2B6* genotype and baseline body weight. Besides, the thesis demonstrates the influence of *CYP2B6* genotype on CYP3A auto-induction by EFV in a gene-dose dependent manner, *CYP2B6 (*6/*6 > *1/*6 > *1/*1).* Furthermore, the thesis reveals the importance of differences in ethnicity & environmental factors contributing to wide between-population variability in EFV auto-induction comparing Ethiopian & Tanzanian patients. In addition, associations of *CYP2B6, ABCB1 (3842A >G),* slow *NAT2* metabolizing genotypes & plasma concentration of EFV with increased incidences of drug-induced liver injury (DILI) and correlation of plasma and intracellular concentrations of EFV are reported in the thesis. The thesis also shows the long-term but not short-term effects of sex and *UGT2B7* genotype in predicting auto-induction as well as plasma concentration of EFV.

In conclusion, EFV dose-escalation from 600mg to 800mg is not required during TB-HIV co-treatment in Ethiopian patients. *CYP2B6*6* genotype is not only a strong predictor for EFV pharmacokinetics but also could predict EFV-based HAART outcomes, DILI & *CYP3A* auto-induction by EFV. In addition to pharmacogenetic variability, the importance of differences in ethnicity & environmental factors are highlighted to optimize HIV treatment across sub-Saharan Africa.

LIST OF PUBLICATIONS

I. **Habtewold A**, Amogne W, Makonnen E, Yimer G, Riedel KD, Ueda N, Worku A, Haefeli WE, Lindquist L, Aderaye G, Burhenne J, Aklillu E. Long-term effect of efavirenz autoinduction on plasma/peripheral blood mononuclear cell drug exposure and CD4 count is influenced by UGT2B7 and CYP2B6 genotypes among HIV patients. ***J Antimicrob Chemother. 2011 Oct;66(10):2350-61***

II. **Habtewold A**, Amogne W, Makonnen E, Yimer G, Nylén H, Riedel KD, Aderaye G, Bertilsson L, Burhenne J, Diczfalusy U, Aklillu E. Pharmacogenetic and pharmacokinetic aspects of CYP3A induction by efavirenz in HIV patients. ***Pharmacogenomics J. 2012 Oct 23. doi: 10.1038/tpj.2012.46.***

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IV. Yimer G, Ueda N, **Habtewold A**, Amogne W, Suda A, Riedel KD, Burhenne J, Aderaye G, Lindquist L, Makonnen E, Aklillu E. Pharmacogenetic & Pharmacokinetic Biomarker for Efavirenz Based ARV and Rifampicin Based Anti-TB Drug Induced Liver Injury in TB-HIV Infected Patients. ***PLoS One. 2011;6(12):e27810. doi: 10.1371/journal.pone.0027810. Epub 2011 Dec 6.***

V. **Abiy Habtewold,** Eyasu Makonnen, Wondwossen Amogne, Getnet Yimer, Klaus-Dieter Riedel, Getachew Aderaye, Leif Bertilsson, Jürgen Burhenne, Eleni Aklillu. Paradoxical effect of rifampicin on plasma/intracellular efavirenz pharmacokinetics and increased immunologic outcome: No need for efavirenz dose escalation with rifampicin co-therapy in Ethiopian HIV patients. ***[Submitted to CPT]***

*Shared first authorship with Eliford Ngaimisi.

The above papers are referred as Paper I - V in the text of this thesis.

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LIST OF ABBREVIATIONS

ABCB1	ATP-binding cassette transporter 1
AIDS	Acquired immunodeficiency syndrome
ALP	Alkaline phosphatase
ALT	Alanine aminotransaminase
AST	Aspartate aminotransferase
ARV	Antiretroviral drugs
Anti-TB	Anti-tubercular drugs
AZT	Zidovudine
4β-OHC	4β-hydroxy-cholesterol
CAR	Constitutively active receptor
CRF	Case report form
CYP450	Cytochrome P450
DDIs	Drug-drug interactions
DILI	Drug-induced liver injury
d4T	Stavudine
DOTs	Directly Observed Treatment Strategy
EFV	Efavirenz
EFV MR	Efavirenz metabolic ratio
FDA	Food and Drug Administration
FTC	Emtricitabine
FXR	Farnesoid X receptors
GCP	Good Clinical Practice
GST	Glutathione-S-transferase
GR	Glucocorticoid receptor
HAART	Highly active antiretroviral therapy
HIV	Human Immunodeficiency Virus
INH	Isoniazid
LC/MS/MS	Liquid chromatography–tandem mass spectrometry
MDR1	Multidrug resistance 1
NATs	N-acetyltransferases
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleoside reverse transcriptase inhibitors
NVP	Nevirapine
7-OH-EFV	7-hydroxy-efavirenz
8-OH-EFV	8-hydroxy-efavirenz
OATPs	Organic anionic transporter peptides
OCTPs	Organic cationic transporter peptides
PBMCs	Peripheral blood mononuclear cells
PPARƔ	Peroxisome proliferator activated receptors
P-gp	P-glycoprotein
PI	Proteases inhibitor
PXR	Pregnane X receptor
RIF	Rifampicin
RXR	Retinoid X receptor
SLCO	Solute carrier organic anion transporter
SNPs	Single-nucleotide polymorphisms
SOPs	Standard operating procedures
TB	Tuberculosis
TB-IRIS	TB immune reconstitution inflammatory syndrome
3TC	Lamivudine
TDF	Tenofovir disoproxil fumarate
TDM	Therapeutic drug monitoring
UGTs	UDP-glucuronosyl transferases
UNL	Upper normal limit
WHO	World Health Organization

1. INTRODUCTION

1.1. Common infectious diseases: an overview

More than 1400 species of infectious agents have been reported to cause diseases in humans. These include pathogens for some 347 diseases of sustained clinical importance, according to the recent review by Hay *et al* [1]. Although caused by vastly different pathogens, the world's three most serious infectious diseases are, tuberculosis (TB), malaria and human immunodeficiency virus (HIV) caused by mycobacteria, protozoa and retrovirus, respectively. Together, they cause ~5 million deaths per year and substantially affect the lives of a billion people world over [2]. Sub-Saharan African countries are hugely affected by these infectious diseases and the region harbors the highest burden of mortality and morbidity due to the diseases. Among these, TB and HIV are highly associated immuno-pathologically. Curable drug therapies are available for TB and malaria while HIV progression to AIDS could also be controlled by combination of antiretroviral drugs, although life-long administration of the drugs is required. There are immense challenges associated with the management and control of the diseases world over but worse in developing world due to expanding population, high disease burden and limited access to public health facilities.

1.1.1. HIV epidemiology & current treatment: global, regional & national scenario

Human immunodeficiency virus (HIV) is a chronic retroviral infection and a causative agent of acquired immunodeficiency syndrome (AIDS). The first case of HIV infection was identified in 1981 [3]. It has affected the lives of millions since and still continues to be one of the major leading cause of death [4-6] with cases virtually reported from every country. An estimated 34 million people were globally living with the virus at the end of 2010 [7]. Furthermore, an estimated 2.7 million new HIV infections and 1.8 million deaths from AIDS-related illnesses were reported during the same year. Sub-Saharan Africa remains the region most heavily affected by the virus, accounting for 68% of people living with HIV and 70% of new infections in 2010 [7].

Ethiopia is geographically located in the eastern horn of Africa. It is second most populous African country, with an estimated inhabitant for over 80 million people in 2008 [8]. Like most other sub-Saharan African countries, Ethiopia has experienced a severe HIV epidemic since the mid-1980s [9, 10] and HIV-1 infection was first documented in 1984 in Ethiopia [11]. Subsequently, the first clinically overt case of AIDS was diagnosed in 1986 [12]. The epidemic has expanded rapidly and reached a plateau around the mid-1990s and Ethiopia remained one

of the hardest hit countries by the epidemic with an estimated adult HIV prevalence rate of 1.5% in 2011 [13].

The introduction of highly active antiretroviral therapy (HAART) has substantially resulted in reductions of HIV-related morbidity and mortality globally [14-16] WHO 2010 [7]. Current WHO guidelines recommend use of a standard first-line regimen consisting of either two nucleos(t)ide reverse transcriptase inhibitors (NRTIs) plus a non-nucleoside reverse transcriptase inhibitors (NNRTIs), efavirenz or nevirapine, or a triple NRTI regimen, and a second-line regimen consisting of a boosted Proteases inhibitor (PI) with at least one NRTI [7]. The currently available antiretroviral drugs (ARV) are effective in suppressing viral replication and reduce the plasma viral load to the level below the detection limit, significantly restore the immune function and reducing HIV-associated morbidity and mortality [17-19].

1.1.2. TB and HIV co-infection: epidemiology and co-treatment challenges

Tuberculosis (TB) and HIV are interacting epidemic diseases. TB is the commonest opportunistic infection and leading cause of death among HIV infected individuals [20]. Likewise HIV complicates TB infection and is associated with a more rapid clinical decline. Infection with HIV increases the risk of reactivating latent TB infection and HIV-infected individuals who acquire new TB infections have high rates of disease progression [21, 22]. TB-HIV co-infected individuals are at high risk of death [23, 24].

The lifetime risk of developing active TB in HIV uninfected individuals is approximately 10%; but the annual risk among HIV-infected patients is ~10%, while the lifetime risk approaches 50% among them, as reviewed by Habib [25]. According to the recent WHO report [26], in 2010, there were 8.8 (range, 8.5–9.2) million incident cases of TB, 1.1 (range, 0.9–1.2) million deaths from TB among HIV uninfected individuals and an additional 0.35 (range, 0.32–0.39) million deaths from HIV-associated TB globally. The highest rates of TB-HIV co-infection are in the African Region, where 44% of TB patients with an HIV test result were HIV infected (range among high TB-HIV burden countries, 8%–82%).

Ethiopia is among the first 22 TB burden countries and also among the 41 high TB-HIV co-infection burden countries [26]. As reviewed by Seyume and Legesse [27], during the year 2011, a total of 159,017 TB cases and about 38,000 HIV-associated new TB cases were notified in Ethiopia. Among 65,140 TB patients who were screened for HIV infection, 5,442 were found to be co-infected with HIV. The rate of TB and HIV co-infection is high in Ethiopia ranges from 25% to 57% in different regions of the country [28, 29].

In co-infected patients, the priority is to treat TB with anti-tubercular (anti-TB) drugs before the initiation of highly active antiretroviral therapy (HAART) in anti-retroviral-naïve patients [30]. The anti-TB drugs used to treat drug-sensitive TB are universally the same, independent of HIV status although the thrice a week TB treatment regimen is recently disfavored by WHO among TB-HIV co-infected individuals due to higher rate of relapsing TB [26]. HAART reduces TB rates by up to 90% at an individual level, by 60% at a population level and reduces TB recurrence rates by 50% [31, 32], indicating the importance of initiating HAART for all people living with HIV and active TB disease, irrespective of CD_4 cell count [26].

However, TB-HIV co-treatment poses several management challenges [33]. The optimum time to initiate HAART in TB-HIV co-infected individuals remains unclear [34]. Among the three milestone studies, SAPIT (Starting Antiretroviral therapy at three Points In Tuberculosis therapy) of South Africa [35] and CAMELIA (Cambodian Early versus Late Introduction of Antiretroviral Drugs) of Cambodia [36] showed reduced incidence of all-cause mortality, hence improved survival when HAART was introduced simultaneous to anti-TB treatment in cohorts of TB-HIV co-infected patients. Even AIDS Clinical Trial Group (ACTG) study called STRIDE (Immediate Versus Deferred Start of Anti-HIV Therapy in HIV-Infected Adults Being Treated for Tuberculosis), association of earlier HAART with a lower rate of new AIDS-defining illnesses and death was reported among individuals with CD_4 counts less than 50 cells per mm^3 [37]. The above studies solidify the benefits of HAART introduction during anti-TB treatment. On the other hand, concurrent treatments of TB-HIV co-infection create several management challenges, including pharmacokinetic drug interactions, overlapping drug toxicities, TB immune reconstitution inflammatory syndrome (TB-IRIS) and high pill burden, which potentially affects adherence [38]. To minimize these risks, optimization of TB-HIV co-treatment is of paramount importance either through selection of appropriate HAART regimens or optimized dosage.

1.2. Antitubercular & anitretroviral drugs: challenges of concomitant use

WHO recommends that the first-line HAART regimen contain two nucleoside reverse transcriptase inhibitors (NRTIs) plus one non-nucleoside reverse transcriptase inhibitor (NNRTI). The preferred NRTI backbone is zidovudine (AZT) or tenofovir disoproxil fumarate (TDF), combined with either lamivudine (3TC) or emtricitabine (FTC) [7]. By and large, NRTIs remain the backbone of HAART during TB-HIV co-treatment due to their safety, efficacy and favorable drug-drug interaction profiles, reviewed by Aaron [30]. However, potentially deleterious outcomes of simultaneous co-administrations of the anti-TB drugs with

NNRTIs are challenging due to pharmacokinetic drug – drug interactions (DDIs) and overlapping drug toxicities [33, 39].

1.2.1. Pharmacokinetic interactions: emphasis on rifampicin & efavirenz

Drug interaction between current ARV and anti-TB drugs is a problem. The majorities of the clinically significant drug interactions are pharmacokinetic-based [40], mainly through cytochrome P450 (CYP450) enzyme induction or inhibition in the liver [30]. The isoform CYP3A4 is particularly important as it is the main enzyme responsible for the metabolism of PIs and to a lesser extent, NNRTIs [41, 42]. Amongst the potent inducers of CYP3A4 are the rifamycin family [43]. Rifampicin (RIF) is a cornerstone anti-TB drug. It is a potent inducer of many genes controlling drug metabolism and transport, including CYP3A4 and the drug efflux pump p-glycoprotein [44]. Induction of CYP3A4 may reduce plasma concentrations of concomitantly administered ARV drugs, potentially leading to inferior HAART outcomes [45, 46]. Besides, increased activity of the drug transporter (P-glycoprotein) may affect the absorption, distribution and elimination of PI [47, 48].

NRTIs are mostly metabolized by glucuronidation and are free of significant interactions with rifamycins, as reviewed by Schutz C [49]. Clinically significant interactions may occur between RIF and NNRTIs. Pharmacokinetic data showed that RIF reduced nevirapine (NVP) to sub-therapeutic concentration [46, 50]. Therefore, the data to date suggest that if RIF and an NNRTI are to be used then efavirenz (EFV) is preferred [7], although it is unclear whether this has any clinical impact. Besides, there is no agreement on the appropriate dose of EFV to be co-administered with RIF.

Pharmacokinetic studies have found a 20–30% reduction in EFV plasma levels when co-administered with RIF [51] and that increasing the dose of EFV from 600 mg to 800mg is effective and safe [51, 52]. Conversely cohort studies employed the standard 600mg dose of EFV with RIF but clinical efficacies were not compromised [53-55]. A large cohort study conducted in South Africa showed no difference in rates of virological suppression when comparing patients on EFV-containing HAART alone and those on EFV-containing HAART and RIF-based TB treatment concurrently. EFV was used at a dose of 600 mg irrespective of body-weight in this cohort [56].

In contrast, an observational cohort, looking at body-weight stratified pharmacokinetic and clinical outcomes, suggested that for patients weighing 60kg, a dose of 800mg of EFV should be prescribed [50], although this was not observed in African patients [45]. A major problem is

large between-subject variability in EFV concentrations [57] and this is compounded by the fact that EFV levels and associated toxicity are increased in individuals with polymorphisms in CYP2B6, present in over 20% of the black population compared with 3% of whites [58, 59]. This may explain some of the variability and high rates of clinical toxicity in some studies [60]. Drug-gene interactions studies were also recommended [61]. Based on available data, standard doses of EFV can be given to patients weighing 60kg, but in patients weighing 60kg an increase to 800mg can be considered [33, 47].

1.2.2. Overlapping toxicities: emphasis on drug induced liver injury (DILI)

HIV infection *per se* results in an increased rate of serious adverse events in patients on TB treatment [62]. In addition, shared toxicities with anti-TB and ARV drugs are common: rash, fever, peripheral neuropathy and drug-induced liver injury (DILI) being highly common adverse effects [30, 49]. Amongst which, DILI is the most commonly shared toxicity by anti-TB drugs and NNRTIs and PIs. Close monitoring of liver functions are required. Severe DILI was found in 7.7 per 100 person-years in an African cohort; although in TB-HIV co-infected patients, treatment of TB increased its risk by 8.5-fold [63].

Among the first-line anti-TB drugs pyrazinamide, isoniazid and RIF have all been associated with DILI [64]. There are concerns about increased incidence of DILI when NNRTIs are concomitantly used with the anti-TB drugs. Increased propensity of DILI was seen when both EFV [65, 66] and NVP [66] were administered concomitantly with TB treatment. Boosted PIs with RIF-based TB treatment may also result in DILI [67-69]. Development of deranged liver functions may significantly complicate treatment of co-infected patients [70].

1.3. Pharmacogenetics

Individuals differ in response to administration of equal dose of a drug. Various factors have been described to contribute between-subject variability in drug response; amongst those factors, the contribution genetic variability has received much focus. Pharmacogenetic is a scientific discipline that deals with the identification and description of genetic diversity to ascribe differences in drug response through candidate-gene approach. The related discipline often confused with pharmacogenetics is pharmacogenomics which uses whole human genome to explain between-subject variability in drug response through genome-wide association studies (GWAS).

Mutational changes on certain candidate-genes affect pharmacokinetics processes, mainly drug metabolism and transmembrane drug transport processes. Identification of single-nucleotide

polymorphisms (SNPs) on genes affecting these processes significantly impact inter-individual and inter-population variability on drug exposure parameters, which, in turn, may influence drug responses at both individual and population level, respectively [71].

1.3.1. Pharmacogenetics of drug metabolizing enzymes

Approximately all systemically absorbed drugs undergo biotransformations mediated by mainly hepatic and rarely extrahepatic enzymes through phases I and II. Phase I biotransformation involves mainly oxidation reactions mediated by hepatic and extrahepatic enzyme systems called Cytochrome P450 (CYP450) and Phase II metabolism involves conjugation reactions mainly glucouridation by UGT enzyme system.

1.3.1.1. Cytochrome P450 (CYP450) enzymes

The most important eliminating pathways for lipophilic drugs are CYP450 dependent oxidation to polar metabolites. Mo *et al* [72] reviewed that the human hepatic CYP system consist of over 30 related isoenzymes with different, sometimes overlapping substrate specificities. The enzymes belonging to the families CYP1, CYP2 and CYP3 catalyse biotransformations of xenobiotics including 90% of clinically used drugs [73, 74]. Mutant alleles carrying certain nucleotide substitutions, deletions or insertions result in abnormal activity and variability in the contents of the CYP [74]. This, in turn, leads to between-subject variability of CYP metabolic rates [75]. Evidences exist that difference in CYP enzymes metabolic capacities leading to inter-individual variability in therapeutic responses of drugs [76-78]. Most CYPs are subject to enzyme induction and inhibition, leading to DDIs. Clinically significant DDIs between anti-TB and ARV drugs have been explained through inductions at CYP2B6 and CYP3A enzyme systems [79, 80].

1.3.1.1.1. CYP2B6

The human CYP2 cluster is located on chromosome 19q 13.2. This cluster contains the loci for six functional genes including CYP2A6 and CYP2B6 subfamilies [81]. CYP2B6 is mainly expressed in liver, accounting for 6% of the total microsomal CYPs as reviewed by Mo *et al* [72]. Although, this enzyme was historically thought to play insignificant role in drug metabolism, the discovery of genetic polymorphisms and inter-ethnic differences in CYP2B6 expression [82, 83], identification of additional substrate drugs [72] as well as evidences of its co-regulation with CYP3A [84-86] stimulated renewed interest. Currently, CYP2B6 enzyme is known to metabolize ~8 - 10% of drugs including chemotherapeutics, opioids and ARV drugs such as NVP and EFV [87].

Certain SNPs at CYP2B6 gene have been associated with high between-subject variations on substrate drugs exposure. Four exonic SNPs, 516G > T, 714G > A, 785A > G and 983T > C were identified [88]. The relative importance of each of the SNP varies among different races and ethnicity. Worldwide, inter-population diversity in CYP2B6 516G>T allele frequency ranges between 25 to 69% [89]. CYP2B6 516G>T SNP is abundant among black Africans [90]. The inter-population variability in the allele frequency of mutated CYP2B6 516G>T SNP was reported to vary between 20 to 50% among Africans, suggesting ethnic differences in the expression of the enzyme within the same race [91].

The identification of CYP2B6 enzyme as the major metabolic pathways for the first-line ARV drugs, such as NVP and EFV [92], has also stimulated renewed interest. Differences in NVP and EFV exposures were reported, owing to the existence of between-subject variability due to differences in the expression of CYP2B6 at individual level [45, 93]. Individuals with non-functional (mutated) CYP2B6 gene showed higher drug exposures, possibly leading to higher incidences of toxicities [72]. Evidences for CYP2B6 inducibility by RIF [85], its auto-inducibility by EFV and NVP [94] and inhibition by certain drugs, have led to investigations to identify clinically relevant drug - drug interactions. Moreover, the intracellular co-regulation similarities of CYP2B6 and CYP3A enzymes have also been the basis for DDI studies [84-86].

CYP2B6 is induced by a large number of compounds with diverse structures, as reviewed by Tompkins [95]. Constitutively active receptor (CAR) is a nuclear receptor containing functional domains, including a DNA-binding domain and a ligand-binding domain. CAR was shown to have much smaller ligand-binding domain than PXR. In addition, unique structural conformations were identified that may explain CAR's ligand independent activities [96]. The induction of CYP2B family is activation. Along with CYP2B induction, up-regulation of other genes including CYP2C, CYP3A, glucuronosyltransferases and transporters occurs with CAR activation [97].

1.3.1.1.2. CYP3A

The CYP3A iso-enzymes constitute the largest portion of CYP450 protein in the liver and small intestine [98-100] and are located adjacent to each other on chromosome 7q21. The CYP3A family is composed of four enzymes: CYP3A4 (the major isoform), CYP3A5, CYP3A43 and CYP3A7. Among these, CYP3A4 and CYP3A5 account for the majority of the catalytic activity of the enzyme subfamilies [91, 101]. They are responsible for the metabolism of 50–60% of all currently available drugs as well as endogenous substances such as cholesterol and steroidal hormones. 4β-hydroxy-cholesterol (4β-OHC) is metabolic product of

cholesterol via CYP3A. Its plasma level was shown to be an endogenous biomarker for CYP3A enzyme activity [102, 103]. However, in cases where plasma cholesterol levels are expected to vary, 4β-OHC to Cholesterol (4β-OHC/Chol) ratio was suggested to be used as marker for the enzyme [104].

Multiple nuclear receptor binding sites control both the basal and inducible expression of CYP3A and ensure sensitivity to a number of structurally diverse substrates [105]. Expressions of CYP3A enzymes involve the nuclear hormone receptors pregnane X receptor (PXR) and retinoid X receptor (RXR) [106]. Substantial inter-individual differences in CYP3A enzyme expression contribute to variation in oral bioavailability and systemic clearance of CYP3A substrates. Not only the expression but also the induction of CYP3A shows large inter-individual variations [95]. Among many, rifampicin (RIF) and efavirenz (EFV) are known to induce [107] while most PIs inhibit CYP3A activity [108, 109].

The contribution of CYP3A5 to drug metabolism was shown to vary from 6 to 99% of the total CYP3A activity in different populations [101, 110]. CYP3A5 is reported in detectable amounts in only 10 to 30% of adult white people and Asians, whereas 60% of African Americans express the protein [110], wide intra-continental variation in the expression of CYP3A5 enzyme has been reported within African continent [111].These variations may be due to the modulation of CYP3A expression through a wide array of environmental factors, drug-drug interactions and genetic variations. CYP3A5 is polymorphic and displays inter-individual and interracial differences in expression and CYP3A-dependent drug clearance and responses hence may be an important genetic contributor [112]. The main reason for variability of CYP3A5 activity is SNPs at 27289C<A, 6986 A<G, G14690A and 27131-32insT yielding prematurely truncated and thus non-functional enzymes namely CYP3A5*2, CYP3A5*3, CYP3A5*6 and CYP3A*7 variants, respectively [110]. Individuals carrying at least one CYP3A5*1 allele can express high levels of CYP3A5 enzyme.

1.3.1.2. Conjugating enzymes: emphasis on UGTs and NATs

Phase II enzymes conjugates phase I metabolites or parent drugs for renal and biliary excretions. Phase II enzymes include glutathione-S-transferase (GST), Thiopurine-S-methyltransferase (TPMT), UDP-glucuronosyl transferases (UGTs), N-acetyltransferases (NATs) and others. UGTs are the major conjugation drug biotransformation pathway while inter-individual and inter-population variability due to polymorphisms on NATs has been identified early.

The UGTs are distributed in many tissues including liver, small intestine, kidney, colon and brain [113]. UGTs mediate metabolisms of various endogenous substrates such as bile and steroid hormones as well as chemically diverse xenobiotics, as reviewed by Lin and Wang [114]. A great deal of drugs from almost all therapeutic classes including ARV drugs such as zidovudine (NRTI) and efavirenz (NNRTI) undergo glucuronidation [115]. Similar to some CYP450 enzymes, the expressions of UGTs is inducible and the mechanism of their cellular regulation was suggested to be similar as that of CYPs through activations of PXR and CAR nuclear receptors, reviewed by Zhou *et al* [116]. In addition, Zhou *et al* described other nuclear receptors which regulate transcriptions of UGTs such as farnesoid X receptors (FXR), peroxisome proliferator activated receptors (PPARɣ) and glucocorticoid receptor (GR). Many classical CYP450 inducers including RIF and phenobarbital were shown to induce UGTs *in vitro* and in animal studies [117, 118]. A number of UGT isoforms have been identified in human, encoded either by UGT1A or UGT2B gene subfamilies. UGT2B7 enzyme system is the major UGT isoform, highly polymorphic and is involved in the metabolism of ARV drugs like zidovudine and efavirenz [89, 115, 119].

N-acetyltransferases (NATs) are involved in the metabolism of drugs and environmental toxins. They catalyze the acetyltransfer from acetyl-coenzyme A. Sequence variations in the human NAT1 and NAT2 result in the production of NAT proteins with variable enzyme activity or stability, leading to slow or rapid acetylation [120, 121]. Genetic polymorphisms in NAT1 and NAT2 have been associated with drug-induced toxicities and disease, according to the reviews [122-124]. NAT1 and NAT2 occur in the liver, with NAT2 being 2 to 10 times the level of NAT1, depending on the NAT2 status of slow or rapid acetylator, respectively [125]. There are two distinct phenotypes for NAT2: ‘fast’ and ‘slow’ acetylators, measured in vivo using substrates such as isoniazid, dapsone and caffeine. The slow isoform of NAT2 is present in up to 90% of some Arab populations, in 40–60% of Caucasians and Indians and in 5–25% of East Asians [126-128]. Genetic polymorphism of the NAT2 is strongly implicated in differential susceptibility to adverse drug reactions [122, 129].

The NAT2 acetylation polymorphism was discovered over fifty years ago when individual variability in isoniazid (INH) neurotoxicity was attributed to genetic variability in N-acetylation [130]. Among the first-line therapeutic drugs for TB, INH is the drug principally associated with drug-induced hepatotoxicity [131]. In the liver, INH is first metabolized into acetylisoniazid via NAT2 [132] followed by hydrolysis to acetylhydrazine, subsequently acetylhydrazine is oxidised into hepatotoxic intermediates [133, 134].

1.3.2. Pharmacogenetics of drug transporter proteins

A drug molecule passes through several biological membranes during pharmacokinetic processes. The extent of transmembrane movement of drugs is affected by the physicochemical properties of a drug. In addition, membrane transporters have a significant role in facilitating or preventing drug movement. Drug transporters may be either influx (uptake into cell) or efflux (out of cell) transporters, which are typically located either at the basolateral or apical membrane in polarized cells, reviewed by Kalliokoski *et al* [135]. The level of expressions and functionalities of membrane transporters are critical modulators for pharmacokinetic processes [136].

1.3.2.1. Efflux transporters: ABCB1

Efflux transporters are involved in energy-dependent efflux pump of xenobiotics from inside to outside via the plasma membrane. The most widely known efflux transporter proteins, p-glycoproteins (P-gps) are transmembrane proteins present in intestinal epithelial cells, biliary canalicular cells, blood-brain barrier, lymphocytes and on the luminal surface of proximal tubule kidney cells, hence virtually affecting all pharmacokinetic processes [137]. Evidences show that a large group of drugs, including ARV drugs like most PIs were shown as substrates to this efflux transporter protein [138].

Multidrug resistance 1 gene (MDR1), also called ATP-binding cassette transporter (ABCB1), codes for P-gps. Several SNPs have been described on ABCB1 gene, the SNP 3435C >T, located in exon, has been shown to be associated with lower intestinal P-gp expression and activity in vivo [139-141]. Individuals homozygous for the mutation at position 3435 (TT) had significantly lower P-gp expression in the small intestine compared to those with functional variants and showed the highest plasma concentrations of some drugs after oral administration [139-141]. Pronounced ethnic difference in the alleles distribution of ABCB1 gene was also described [142-144]. The cellular expression of ABCB1 gene could either be induced or inhibited.

1.3.2.2. Influx transporters: SLCO1B1

Contrary to efflux system, influx transporters are ATP-independent pumps, importing xenobiotics from outside to inside across cellular transmembrane [135]. They are either organic cationic transporter peptides (OCTPs) or anionic transporter peptides (OATPs) [145].OATPs are expressed in a wide variety of tissues including liver, kidney, brain and small intestine [146]. A number of human OATP families have been identified, of those the roles of

OATP1B1, OATP1A2, OATP1B3 and OATP2B1 were characterized in drug pharmacokinetics [146].

OATPs are encoded by genes of the solute carrier organic anion transporter (SLCO) family. The genes encoding human OATP1 family members are located in chromosome 12. Articles have reviewed the pharmacogenomics of OATPs and the role of OATP1B1 in drug elimination [135, 136, 147]. According to the reviews, numerous SNPs had been identified in SLCO genes [148,149]. These polymorphisms may lead to significant consequences on drug pharmacokinetics.

OATP1B1 is mainly expressed on the sinusoidal membrane of human hepatocytes, also possibly in the small intestinal enterocytes [150, 151]. Substrates of OATP1B1 include a large number of structurally diverse drugs including statins, rifampicin and many others. Rifampicin was shown to be both inhibitor and inducer of OATP1B1 *in vitro* [152, 153]. In addition, some PIs were identified as inhibitors for OATP1B1 [154-156]. According to the review by Niemi [147], a large number of SNPs and other sequence variations have been described in the gene region of SLCO1B1 [149]. Of the variants identified, 521T>C SNP has been associated with a markedly reduced transport activity *in vitro* using several OATP1B1 substrates, including rifampicin. The 521T>C variant is common in non-African populations, with the variant allele frequency ranging between 10 and 20%. Another common variant associated with altered transport activity in vitro is 388A>G SNP [147, 157].

1.3.3. Pharmacogenetics of efavirenz

Efavirenz (EFV) displays nearly complete absorption from the gut and the Cmax reaches up to 5h after oral administration. It is highly bound (over 99%) to plasma proteins, mainly to albumin. It is also widely distributed in different body fluids. Elimination of EFV is through both oxidation and conjugation by CYP450 and UGT enzyme systems, respectively, to its inactive metabolites and excreted mainly in urine.

Efavirenz (EFV) undergoes complex metabolic processes. It is metabolized mainly to 8-hydroxy-efavirenz (8-OH-EFV), primarily by CYP2B6 to a lesser extent by CYP3A4/5 [158, 159]. These enzymes are genetically polymorphic. Polymorphisms at 516G>T (Q172H) and 785 A>G (K262R) have been associated with pronounced decreases in CYP2B6 expression and activity as well as low rate of EFV hydroxylation [160]. CYP3A5 polymorphisms *per se* did not seem to affect EFV exposures significantly as its role in 8-hydroxylation of EFV is minimal. However, among individuals with non-functional genotypes for CYP2B6, the role of

CYP3A5 polymorphism may be important. Besides, EFV is also metabolized to 7-hydroxy-efavirenz (7-OH-EFV) by CYP2A6 [158] and evidences showed that EFV also undergoes direct glucuronidation by UGT2B7 to efavirenz-N-glucuridine [115]. UGT2B7 was also shown to be polymorphic, although evidences lack the influence of the polymorphism in relation to EFV exposure among individuals with distinct CYP2B6 genotypes [161]. The major metabolite of EFV, 8-OH-EFV, also undergoes secondary hydroxylation to 8,14-dihydroxy-efavirenz again by CYP2B6 and glucuronidation to 8-hydroxy-glucuridine by UGT2B7 enzyme [162-164].

Attempts to investigate transmembrane transport of EFV and pharmacogenetics of drug transporter proteins on EFV are a few. No conclusive report exists whether EFV is substrate to MDR1 transporters as previous reports are conflicting [165-168]. Moreover, reports are a few whether EFV is a substrate or inducer/inhibitor of influx transporter proteins, mainly OATPs, except that Janneh *et al* [167] suggested that intracellular accumulation of EFV may be due to interaction with SLCO3A1 while Weiss *et al* [169] showed that EFV up-regulated expressions of SLCO3A1and SLCO2B1 *in vitro*.

2. THIS THESIS

2.1. Rationale of the thesis

The current WHO guideline for treatment of HIV recommends the use of efavirenz-based HAART as the preferred first-line regimen in the presence and absence of TB co-infection, complying with this are most sub-Saharan African countries including Ethiopia. The fact that appreciable proportion of HIV infected individuals also develops TB in Ethiopia, by and large; co-treatment of TB with rifampicin-based anti-TB drugs and HIV with EFV-based HAART is inevitable. Owing to enzyme induction by RIF, plasma exposure of EFV is described to reduce, as a consequence, may lead to HAART failure.

There is no agreement on the appropriate dose of EFV to administer with RIF. This thesis primarily investigated whether EFV dose escalation is necessary or not when co-treating HIV in TB and HIV co-infected Ethiopian patients receiving RIF-based anti-TB treatment. Earlier studies conducted on Caucasians showed 20–30% reduction in EFV levels when administered with RIF, recommending dose increment of EFV from 600 mg to 800mg. Conversely, recent cohort studies in non-Caucasian populations showed that when standard dose of EFV (600mg) was given with RIF, clinical efficacy of HAART was not compromised. Current recommendations suggest that when EFV is to be used concomitantly with RIF, the standard dose of EFV (600mg) to be used or to be adjusted upward to 800mg based on the body-weight. Even then, there is no agreement in the body-weight cut-offs.

The existence of extensive heterogeneity in relation to genetic diversity, environmental exposures and dietary habits coupled with pharmacogenetically under explored facts among populations in sub-Saharan African countries also stimulated this thesis. The fact that Ethiopia is the 2nd highly populous country in Africa, and one of the worst affected countries by HIV and TB burden, the distinct genetic make-up and dietary habits of the population in Ethiopia as opposed to other sub-Saharan African countries had been another impetus for the design of this study to be conducted parallel in two sub-Saharan African countries, namely Ethiopia and Tanzania.

Given the genetic heterogeneity of sub-Saharan African population, wide inter-population differences in the distribution of allele frequencies of EFV metabolizing enzymes and transporter proteins are expected. This thesis also describes the pharmacogenetics of EFV and investigates the effects of long-term administration of EFV on its pharmacokinetics and linking this to treatment outcomes in Ethiopian HIV patients. The thesis also attempts to characterize

the influences of pharmacogenetic factors, demographic variables, biochemical lab parameters and hepatitis B and C co-infections on pharmacokinetics and pharmacodynamic of EFV in Ethiopian TB and HIV co-infected patients. This thesis also investigated the link between pharmacogenetic based pharmacokinetic differences resulting drug-induced liver injury (DILI) among TB and HIV co-treated Ethiopian patients. In addition, the thesis characterized the factors affecting EFV pharmacokinetics and pharmacodynamic differences in HIV patients based on ethnic differences by comparing these from two sub-Saharan African countries, namely Ethiopia and Tanzania.

2.2. Objectives

2.2.1. General objective

To investigate the pharmacokinetic and pharmacogenetic aspects of drug – drug interactions aiming to optimize efavirenz dose to be co-administered with rifampicin in Ethiopian patients.

2.2.2. Specific objectives

1. To investigate impacts of long-term auto-induction by efavirenz and pharmacogenetic factors on plasma/intracellular drug exposure and immunologic outcome over time.
2. To assess pharmacogenetic and pharmacokinetic influences of efavirenz on CYP3A induction over time using 4β-hydroxy-cholesterol metabolic ratio as biomarker.
3. To characterize impacts of ethnicity and pharmacogenetic factors on pharmacokinetics of efavirenz and immunologic outcomes in two sub-Saharan African countries.
4. To assess effects of efavirenz pharmacokinetic and pharmacogenetic factors on drug-induced liver injury (DILI).
5. To investigate influence of rifampicin co-therapy on plasma/intracellular efavirenz pharmacokinetics and immunologic and virologic outcomes of HAART.

3. METHODS

3.1. Study design

The study was designed to be conducted in two different African countries, namely Ethiopia and Tanzania and similar protocol has been employed to conduct the trial. This thesis focused mainly on Ethiopia as one of the two participating countries. The study was an open-label, parallel design consisting of cohorts of two arms followed prospectively for a year. HIV infected adults without active TB at the time of enrolment, naïve for HAART with absolute CD_4 count less than 200 cells per mm^3, hereafter called Arm 1 **(Papers I, II & III)**, while HIV co-infected with active TB, naïve for both HAART and anti-TB regimens, hereafter called Arm 2 (**Papers IV & V**).

3.2. Study sites and population

This study was primarily conducted by the Departments of Pharmacology and Internal Medicine, School of Medicine, Addis Ababa University (Ethiopia). Study subject enrolments and follow ups were conducted at HIV and TB clinics, which ran separately at the start of this study. The study sites were one tertiary level referral teaching hospital (*Tikur Anbessa General Specialized Hospital*) and six other health centers (*Arada, Beletshachew, Bole, Kazan'chiz, Lideta and Meshua'lekia*) in Addis Ababa. The capital city of Ethiopia is Addis Ababa, the inhabitant of most nations and nationalities of Ethiopia as well as people of varied socio-economic status. This ensured the heterogeneity of the study population as a representative of Ethiopia.

3.3. Recruitment of study participates

As per the then national guideline, serologically diagnosed and confirmed HIV infected individuals with CD_4 counts less than 200 cells per mm^3 were eligible for HAART initiation. At HIV clinics, those HAART eligible HIV infected patients without active TB (Arm 1; N=285) were recruited **(Papers I – V)**. Similar recruitment procedure was employed by TB clinics to recruit TB and HIV co-infected individuals (Arm 2; N=196) **(Papers IV and V)**. TB diagnosis in Arm 2 patients was done either by microbiological, radiographical, clinical or combination of any of these. The inclusion criteria to participate in the study in both Arms were, age greater than or equal to 18 years, able to give written consent, who were not on any medication known to interact with the study drugs and did not have any significant hematological, liver and/or renal impairments at baseline **(Papers I – V)**.

3.4. Treatments, clinical and laboratory monitoring

Treatment of TB in TB-HIV co-infected individuals (Arm 2) was employed by a combination of body-weight adjusted dose of rifampicin (RIF) plus INH plus ethambutol plus pyrazinamide for 8 weeks (intensive phase) which was followed by 16 weeks of daily dose of RIF plus INH (continuation phase). TB treatment was done all through DOTs **(Papers IV and V)**. While all recruited patients (both Arms 1 and 2) received combination of the first-line highly active antiretroviral therapy (HAART) consisting of 600mg daily dose of efavirenz (EFV) with zidovudine plus lamuvidine (AZT+3TC) or stavudine plus lamuvidine (d4T+3TC) or tenofovir plus lamuvidine (TDF+3TC) **(Papers I – V)**. The EFV-based HAART was initiated on the 4th week of starting RIF-based anti-TB therapy in TB and HIV co-infected patients (Arm 2).

At baseline and scheduled follow-up periods, all recruited patients had clinical examinations consisting of physical diagnoses and clinical laboratory measurements such as liver function tests (ALT, AST, ALP, total and direct bilirubin), renal function test (serum creatinine), hematological parameters (complete and differential blood counts, hemoglobin, hematocrit and albumin), the presence or absence of viral hepatitis co-infections (such as Hepatitis B surface antigen and Hepatitis C viruses), immunological parameter (CD_4 count) and HIV-RNA viral load **(Papers I – V)**.

3.5. HIV treatment outcome and DILI monitoring

HIV treatment response was mainly evaluated by change in absolute CD_4 count **(Papers I, II, III & V)** and undetectable (< 50 copies per mL) HIV-RNA viral load **(Paper V)** at weeks 12, 24 and 48 from the baseline value. Drug induced liver injury (DILI) monitoring was done at weeks 1, 2, 4, 8, 12, 24, 48 and 52 weeks after starting anti-TB therapy in Arm 2 while at weeks 1, 2, 4, 8, 12, 24, and 48 weeks after starting HAART in Arm 1. Liver biochemical parameters more than two times the upper normal limit (UNL) value were considered as DILI. Those greater than or equal to 5 times the UNL or equal to threefold elevation in ALT and simultaneous elevation of total bilirubin concentration greater than or equal to 2 times UNL were considered as severe DILI **(Paper IV)**.

3.6. Pharmacokinetic and pharmacogenetic specimen collection, isolation, storage and transport

Specimens intended for pharmacokinetic and pharmacogenetic studies, treatment outcome monitoring and routine laboratory measurements were collected from brachial veins. To

determine plasma and intracellular concentrations of efavirenz (EFV) and 8-hydroxy-efavirenz (8-OH-EFV), duplicate blood samples (~8mL each) were collected 16±1h post-dose of efavirenz into CPT vacutainers (Becton Dickinson), at least once or at most twice on either week 4 or 16 or both, after start of HAART **(Papers I – V)**. Standard operating procedures (SOPs) for isolations of the specimens from whole blood were described by Burhenne *et al* [166] and subsequently in **Papers I and II**. The specimens were stored in -80^{0}C until transport in dry-ice pack to the Divisions of Clinical Pharmacology and Clinical Chemistry, Karolinska Institutet, Stockholm (Sweden) for genotyping analysis and determination of cholesterol and 4β-OHC, respectively **(Papers I - V)**. Specimens for quantifications of efavirenz (EFV) and 8-hydroxyefavirenz (8-OH-EFV) were similarly stored and transported to the Division of Clinical Pharmacology and Pharmacoepidemiology, University of Heidelberg (Germany) for analysis **(Papers I – V)**.

3.7. Quantifications of pharmacokinetics indexes

3.7.1. Efavirenz and 8-hydroxy-efavirenz by LC/MS/MS

Plasma and intracellular EFV and 8-OH-EFV concentrations were determined by liquid chromatography–tandem mass spectrometry (LC/MS/MS). In brief, protein precipitation with ice-cold acetonitrile containing the deuterated and ^{13}C-labelled internal standards was used for sample preparation and extraction. Extracts underwent chromatography on a Phenomenex® Synergi Fusion RP column with an eluent consisting of acidified 5mM ammonium acetate buffer, acetonitrile and methanol. Efavirenz and 8-hydroxyefavirenz were quantified using $^{13}C_6$-efavirenz and $^2H_4$-8-hydroxyefavirenz as internal standards and electrospray tandem mass spectrometry in the selected reaction monitoring mode. The lower limits of quantification in plasma were 10.0 ng/mL for efavirenz and 0.4 ng/mL for 8-hydroxyefavirenz. The efavirenz (8-hydroxyefavirenz) calibration range was 10–10000 ng/mL (0.4–400 ng/mL). Linear regression with 1/X weighting resulted in correlation coefficients of $r^2>0.99$. The accuracy and precision (intrabatch and interbatch) of the assay fulfilled all the recommendations of the FDA guidelines. Quality control results for accuracy and precision were in the range accepted by the FDA guidelines.

3.7.2. Cholesterol and 4β-hydroxy-cholesterol

Cholesterol concentrations were measured by a commercial enzymatic method (Cholesterol CHOD-PAPP, Roche Diagnostics GmbH, Mannheim, Germany) run on a Roche/Hitatchi Modular instrument. The between-day variation was 1.3% (at 5 mmol l-1). The determination

of 4β-hydroxy-cholesterol (4β-OHC) was performed by isotope-dilution gas chromatography–mass spectrometry using deuterium-labeled 4β-OHC as an internal standard as described previously and modified by Diczfalusy *et al* [170]. The relative between-day variation (CV) was 4.9% (at 26.5 ng ml-1) **(Paper II).**

3.8. Genotyping

Genomic DNA was isolated from peripheral blood leukocytes using QIAamp DNA Maxi Kit (QIAGEN GmbH. Hilden. Germany). Genotyping was carried out at the Division of Clinical Pharmacology, Department of Laboratory Medicine, Karolinska University Hospital-Huddinge, Karolinska Institutet, Stockholm (Sweden). The SOPs for genotyping analysis were described in **Papers I – V**.

3.9. Data collection, management and analysis

3.9.1. Data collection and management

Standard case report form (CRF) database was designed in MS Access. Both hard and soft copies of the database were employed to capture the study participants' socio-demographic, pharmacogenetic parameters, baseline and follow-up laboratory values (pharmacokinetic parameters, biochemical, hematological, immunological and virological values). Besides, detailed baseline and scheduled follow-up clinical parameters including past medical history, physical diagnosis, karnofisky score, concomitant disease state (if any), concomitant use of any medication and assessment of self-reporting adherence.

The data were collected by Good Clinical Practice (GCP) certified physicians, clinical nurses and lab technicians at real-time on the hardcopy of the CRF for each study participant then the same was entered by two independent data encoders. The double-entry was cross-checked by an independent data-manager periodically. Data cleanings were done before and after extraction of the data in MS excel spreadsheet format in preparation of transcription into relevant statistical software.

3.9.2. Statistical analyses

Normal distribution of the data was diagnosed by either Shapiro–Wilk or Kolmogorov–Smirnov test or visual diagnostic checks before statistical analyses **(Papers I - V)**. When abnormal distribution was diagnosed, the data were transformed to log to base 10. Independent t-test or Mann-Whitney U test and chi-square tests were used to compare independent groups

of continuous and categorical variables, respectively **(Papers I – V)**. Kruskal–Wallis analysis of variance (ANOVA) was used for multiple comparisons **(Papers I & II)**. Wilcoxon matched paired test was used to evaluate within-subject comparisons **(Papers I, II III & V)**. Repeated measure and Freidman two-way ANOVA were used to investigate the influence of certain factors on change over time **(Papers I, II, III & V)**.

Univariate regression analysis was used to identify potential predictors and multiple linear regression analysis was employed to build the final models **(Papers III, IV & V)**. Logistic regression was used to identify factors predicting virologic response, defined as non-detectable HIV RNA viral load < 50 copies/mL, **(Paper V)**. Nonlinear mixed effect model and stepwise covariate model building were implemented **(Paper III)**. Pearson and Spearman correlation analyses were also used **(Papers I & II)**.

Statistical analyses were performed using either IBM SPSS versions 15, 19 and 20 **(Papers I – V)** for Windows (IL, USA), Statistica version 10 (StatSoft Inc., Tulsa, OK, USA), NonMem Version 7.2 and PsN 3.5.3.or combinations of the above softwares **(Paper III)**. GraphPad Prism 5 was used for graphical presentation of the data **(Papers I – V)**. P values ≤ 0.05 were considered as significant.

3.10. Ethical considerations

The study had obtained ethical approvals by Institutional Review Boards of the Faculty of Medicine, Addis Ababa University (Ethiopia) and Karolinska Institutet (Sweden). Ethical approval was also obtained from Addis Ababa City Administration Health Bureau and National Health Ethics Committee at Ministry of Science and Technology. Drug Administration and Control Authority of Ethiopia granted final approvals to conduct the clinical study. Written informed consent was obtained from all study participants upon recruitment. The study was conducted as per guidelines laid in the International Conference on Harmonization of Good Clinical Practice (ICH-GCP).

4. RESULTS

4.1. Impacts of efavirenz long-term auto-induction and pharmacogenetic factors on plasma/intracellular drug exposure and immunologic outcome. (Paper I)

4.1.1. Effect of pharmacogenetic variation and sex on between and within subject variability of plasma and intracellular efavirenz pharmacokinetics

The allele frequencies of CYP2B6*6, CYP3A5*3, CYP3A5*6, ABCB1 3435C_T and UGT2B7*2 (2327G_A, rs7662029) were 29.7%, 66.9%, 10.6%, 20.9% and 48.1%, respectively. There was no significant difference between the observed and expected genotype frequencies according to the Hardy–Weinberg equilibrium.

Wilcoxon matched paired tests were used to compare within-subject variability (weeks 4 and 16) for the median plasma and intracellular concentrations of EFV, 8-OH-EFV and EFV MR. No between-occasion variability was observed in both plasma and intracellular concentrations of EFV. However, significant differences in plasma 8-OH-EFV concentrations, with the corresponding decreases in EFV MR, were shown between weeks 4 and 16.

Kruskal–Wallis ANOVA was employed to see the effects CYP2B6, CYP3A5, UGT2B7 and ABCB1 genotypes on plasma and intracellular concentrations of EFV, 8-OH-EFV and EFV MR of between-subject variability at each occasion (either at week 4 or 16). CYP2B6 genotype showed significant influence in both plasma and intracellular concentrations of EFV, 8-OH-EFV and EFV MR at week 4. In addition, CYP2B6 genotype maintained significant influence on plasma concentrations of EFV at week 16. Controlling for the effect of CYP2B6 genotype, significant change over time (within-subject variability) in plasma concentrations of increased 8-OH-EFV and decreased EFV MR among *1/*1 and *1/*6 genotypes but not among *6/*6. Similarly, intracellular concentrations of EFV and EFV MR showed significant difference over time, where in intracellular EFV MR decreased in *1/*1 and *1/*6 genotypes but increased in *6/*6 genotype.

UGT2B7 genotype significantly affected plasma concentrations of 8-OH-EFV and EFV MR in long-term (week 16) but not during short-term (week 4). However, it did not influence plasma and intracellular concentrations of EFV during short-term but only with a trend during long term (P=0.07). UGT2B7 genotype significantly affected plasma concentration of 8-OH-EFV and EFV MR and a significant trend in EFV concentration over time. This within-subject

variability was pronounced among *2/*2 but not *1/*1 genotypes. However, CYP3A5 and ABCB-1 3435C >T genotypes did not significantly affect both between and within-subject variabilities in plasma and intracellular pharmacokinetics of EFV.

Pearson and Spearman correlation tests indicated a significant positive correlation between the plasma and intracellular concentrations of EFV and the EFV MR at both weeks 4 and 16. Besides, a significant correlation between plasma and intracellular concentrations of 8-OH-EFV at week 4 (P=0.02), but not at week 16 (P=0.44) was shown.

The extent of change in plasma concentrations of 8-OH-EFV over time was significantly affected by sex (repeated measures ANOVA, P=0.015), where females at week 16 displayed higher concentrations than the respective value at week 4. Considering male and females separately, there was a significant increase in the 8-OH-EFV level (P=0.0001) and a decrease in the EFV MR (P=0.001) in females at week 16 compared to week 4. However, no significant change was observed in males (P=0.20 and P=0.74, respectively). In addition, sex had no significant effect on the intracellular EFV concentration at week 4, but displayed a tendency to influence it at week 16 (P=0.08).

4.1.2. Effect of plasma and intracellular efavirenz pharmacokinetics on CD_4 count over time

Regression analysis revealed that plasma concentration of EFV at week 4 predicted CD_4 count at week 24 (P=0.01, F=5.43) and a trend at week 12 (P=0.09) but not at week 48 (P=0.75). In addition, the plasma concentration of EFV at week 4 significantly affected within-subject variability in the CD_4 count (change over time). EFV plasma concentration at week 4 influenced the change in CD_4 count from baseline until week 12 (P=0.038). Inclusion of intracellular and plasma EFV concentrations at weeks 4 and 16 in a stepwise forward regression model, the intracellular concentration at week 16 was found to be a significant predictor of CD_4 count at week 24 (F=6.36, P=0.01).

4.1.3. Effect of pharmacogenetic variations on the change in CD_4 counts over time

Friedman two-way ANOVA indicated significant increases in CD_4 count (P=0.0001) from baseline over time during the 48 weeks treatment with efavirenz-based HAART in all patients. Significant within-subject variability was shown: week 0 versus week 12 (P=0.0001); week 12 versus week 24 (P=0.08); and week 24 versus week 48 (P=0.028).

Regardless of sex or genotype differences, there was a sharp increase in the CD_4 counts from baseline until week 12. However, the pattern of change in the CD_4 count was different between the different CYP2B6 and UGT2B7 genotype groups after week 24. Among the CYP2B6*1/*1 and UGT2B7*1/*1 genotype groups there was no significant change after week 24, whereas the mean CD_4 count continued to increase until week 48 among CYP2B6*6/*6 and UGT2B7*2/*2 genotype groups.

Sex, CYP2B6, CYP3A5, UGT2B7, ABCB1 genotypes were included in a forward stepwise regression model, but none of them predicted CD_4 count at week 24, but CYP2B6 (P=0.02) and UGT2B7 became significant predictors of CD_4 count at week 48.

4.2. Pharmacogenetic and pharmacokinetic influences of efavirenz on CYP3A induction (Paper II)

4.2.1. Change in 4β-hydroxy-cholesterol metabolic ratio over time

The median plasma 4β-OHC concentration was increased by 1.4-, 1.8- and 3.5-fold at weeks 4, 16 and 48, respectively. Compared with the baseline value, the median plasma 4β-OHC/Chol ratios increased by 2.6-, 2.9- and 1.7-fold at weeks 4, 16 and 48, respectively (Friedman two-way ANOVA test, P=0.0001). Dunn's multiple comparison test of pairwise contrast from baseline indicated a significant increase in the median 4β-OHC/Chol ratios (P=0.001) at each study time point. Despite the constant increase in 4β-OHC concentrations, the median plasma 4β-OHC/Chol ratio at week 48 was lower than week 16 merely due to the relatively larger increase in the denominator cholesterol concentration.

4.2.2. Effect of sex and pharmacogenetic factors on between and within subject variability of 4β-hydroxy-cholesterol metabolic ratio

Kruskal–Wallis one-way ANOVA was used to investigate effects of genotype on between-subject variability of plasma 4β-OHC/Chol ratios at each time point. None of the investigated genotypes including CYP3A5 had a significant effect except the CYP2B6 genotype whose influence became apparent and significant at weeks 16 and 48 but had no effect at baseline or at week 4. The within subject contrast test between adjacent concentrations indicated a significant effect of the CYP2B6 genotype on the change in 4β-OHC/Chol ratio (P=0.029, F=3.71). Friedman two-way ANOVA indicated that there were significant differences in the extent of change in 4β-OHC/Chol ratio over time between the different CYP2B6 genotypes; being highest in CYP2B6*6/*6 > *1/*6 > *1/*1 genotypes (P=0.04). The level increased

significantly in carriers of the CYP2B6*6 allele at weeks 16 and 48. No effect of sex and CYP3A5, ABCB1 and UGT2B7 genotypes was shown on the short- or long-term induction of CYP3A.

4.2.3. Correlation between plasma efavirenz & 4β-OHC/Chol ratio concentrations

There was a significant positive correlation between 4β-OHC/Chol ratio and plasma EFV concentration both at week 4 (P=0.02, r^2=0.08) and at week 16 (P=0.001; r^2=0.17. The higher the plasma EFV concentration, the higher the respective 4β-OHC/Chol ratio.

4.3. Impacts of ethnicity and pharmacogenetic factors on pharmacokinetics of efavirenz and immunologic outcomes (Paper III)

4.3.1. Comparisons of allele frequencies and efavirenz concentrations between Ethiopia and Tanzania populations

Genotype frequencies were determined in Ethiopian (n=262) and Tanzanian patients (n=184). Haplotype analyses indicated no linkage between CYP3A5 SNPs. The frequencies of all variant alleles determined in this study such as CYP2B6, CYP3A5, UGT2B7, ABCB1 and SLCO1B1 genes were significantly different between the two populations.

Independent *t-test* indicated higher mean log plasma EFV concentration in Tanzanians than Ethiopians at both weeks 4 (P<0.0001) and 16 (P=0.002). Within country analyses using paired samples t-test revealed significant decrease in the mean plasma EFV concentration over time in Tanzanians (p=0.006), but not in Ethiopians (P=0.84). Univariate analysis indicated the following as significant predictors of plasma efavirenz concentrations (percent explained variability, P value); CYP2B6*6 genotype (10.7%, P < 0.0001) patient country (4%, P < 0.0001), co-infection with Hepatitis B at recruitment (1.3%, P=0.001), CYP3A5 genotype (0.8%, P=0.02), ABCB1 3435C >T (0.7%, P=0.02), ABCB1 3842A>G (0.98%, P=0.01), SLCO1B1D130N (0.7%, P=0.02), SLCO1B1 A174V (0.6%, P=0.031).

4.3.2. Influences of ethnicity, pharmacogenetic and other factors on plasma and intracellular concentrations of efavirenz

Time on therapy (week at which EFV plasma levels assessed) was not a significant predictor (p=0.3557). However, time on EFV therapy showed significant interaction with country (p=0.02). Therefore, further assessments of factors affecting EFV plasma level at week 4 and week 16 were done separately.

Univariate linear regression analysis identified the following variables as predictors of EFV plasma concentration at week 4; CYP2B6*6 genotype (9.8%, p<0.0001), country (4.8%, p<0.0001), co-infection with Hepatitis B at recruitment (0.6%, p=0.061), CYP3A5 genotype 11 (1.5%, p=0.019), ABCB1 3435C>T (0.8%, p=0.082), ABCB1 3842 A>G (2.4%, p=0.003), SLCO1B1 D130N (0.8%, p=0.084). Multivariate regression analysis for EFV plasma concentration at week 4: CYP2B6 genotypes (p<0.0001), country (p=0.035) and ABCB1 3842 A>G genotypes (p=0.002) were associated with significant increase in model explained inter-individual variability of week 4 plasma concentration of EFV. The overall model explained for 16% of inter-individual variability in week 4 plasma concentrations of EFV (p<0.0001). When models were built for each country separately, CYP2B6*6 contributed 8.3% and 11% while ABCB1 3842 A>G contributed 0.6% and 8.3% of the inter-individual variability in week 4 plasma EFV concentration in Ethiopian and Tanzanian patients, respectively.

Similarly, Univariate linear regression identified the following variables as predictors (r^2, p value) of EFV plasma concentration at week 16; CYP2B6*6 genotype (17.9%, p<0.0001), country (2.5%, p<0.0001), co-infection with Hepatitis B at recruitment (2.6%, p=0.003). Only CYP2B6*6 genotypes (p<0.0001) and baseline hepatitis B co-infection (p=0.002) were associated with significant increase in model explained inter-individual variability of week 16 EFV plasma concentration. Country had a trend to influence variability (p=0.08). The model explained for 20% of inter-individual variability in week 16 plasma EFV concentrations (p<0.0001). On separate model building for each country, CYP2B6*6 contributed 13.3% and 20.6% of inter-individual variability in EFV plasma concentration at week 16 in Ethiopian and Tanzanian patients, respectively.

Factors influencing EFV intracellular concentrations individually at week 4 were; EFV plasma concentrations (29.2%, p<0.0001), country (11.9%, p<0.0001), CYP2B6*6 genotypes (4%, p=0.02), body mass index (2.2%, p=0.009), baseline ALT levels (1.9%, p=0.014), SLCO1B1*5 (1.8%, p=0.039), SLCO1B1*1b (1.5%, p=0.056) and CYP3A5 (3.6%, p=0.004). In multivariate model, only EFV plasma concentrations at week 4 (p<0.0001) and country (p= 0.041) remained significant predictor of intracellular EFV concentrations at week 4.

Factors influencing EFV intracellular concentrations individually at week 16 were; EFV plasma concentration at week 16 (25%, p<0.0001), country (10.2%, p<0.0001), CYP2B6*6 (6.9%, p<0.0001), SLCO1B1 D130N (*1b) (2.1%, p=0.034), SLCO1B1 A174V (*5) (1.5%, p=0.056) baseline Hepatitis B co-infection (2.1% p=0.014). However, only EFV plasma concentrations at week 16 and country were significant predictors of the intracellular EFV

concentrations. The model explained for 32.4% of inter-individual variability in week 16 intracellular EFV concentration (p<0.0001).

4.3.3. Effect of ethnicity and CYP2B6 genotype on immunologic outcomes

At weeks 24 (p=0.004) and 48 (p=0.002) of EFV therapy, the mean CD_4 cell count was significantly higher in Tanzanians than Ethiopians. Within subject tests of repeated measure ANOVA showed a significant increase in CD_4 over time (p<0.0001). Bonferroni *post hoc* test indicated that major increases were between week 0 and 12 (p<0.0001) and between week 24 and 48 (p=0.008). No significant difference was shown between week 12 and 24 (p=0.57).

Between subject effect tests of repeated measure ANOVA showed a significant effect for country (p=0.004) but not CYP2B6 (p=0.29). Splitting the data by country, between subject effect test for CYP2B6 genotype was not significant for Tanzanians (p=0.52). However, a trend of having higher CD4 gain for being carrier of CYP2B6*6 allele in gene-dose dependent manner for Ethiopians (p=0.11). Having the same CYP2B6 genotype, Tanzanians displayed higher CD4 gain than Ethiopians except in CYP2B6*6 genotype groups. Multivariate linear regression model building indicated intracellular EFV concentrations at week 4 (p=0.013) was a significant predictor of CD4 gain by week 12, while patient country (p=0.059) had a trend.

4.4. Effects of efavirenz pharmacokinetic & pharmacogenetic factors on drug-induced liver injury (DILI) (Paper IV)

4.4.1. Effect of efavirenz concentration on DILI

Significant associations were shown between DILI and increased plasma EFV concentration/EFV MR (p=0.036).

4.4.2. Associations of CYP2B6, ABCB1 and NAT2 genotypes with DILI

Cox-regression analysis showed that there was a statistically significant association between DILI with CYP2B6*6, ABCB1 3435TT and NAT2 slow-acetylator genotypes (p=0.04, 0.02 and 0.039 respectively). There was significant difference in the proportion of subjects with ABCB1 3435TT genotype between cases (12.2%) and controls (3.8%) wherein the proportion was higher in those who developed DILI. A nearly significant effect of UGT2B7*2/*2 genotype with DILI was also noted (P=0.08).

4.5. Influence of rifampicin co-therapy on plasma/intracellular efavirenz pharmacokinetics & immunologic and virologic outcomes of HAART. (Paper V)

4.5.1. Effect of rifampicin co-treatment on plasma/intracellular concentrations of efavirenz

Independent *t-test* revealed that early in co-therapy (week 4), plasma concentration of EFV was higher in patients receiving EFV concomitantly with RIF co-treatment (Arm 2) than patients receiving EFV only (Arm 1) (p=0.04). Similar difference was noted, though a trend (p=0.1) during later stage of co-treatment. A nearly significant higher EFV intracellular concentration was observed in RIF co-treatment group than without RIF at week 4 (p=0.08) but no difference was seen at week 16. Stratifying patients based on baseline body weight, no difference was seen in plasma concentrations of EFV between the two arms at both occasions (weeks 4 and 16) among patients with body weight less than 50Kg. On the contrary, higher plasma EFV concentrations were shown in RIF co-treatment group than without RIF at both occasions (weeks 4 and 16) among patients with body weight greater than or equal to 50Kg at baseline.

Stratifying patients by CYP2B6, higher EFV plasma concentrations were noted in RIF co-treatment group than without RIF at week 4 (p=0.04) and a similar trend (p=0.1) was seen at week 16 among CYP2B6*6 carriers. However, no difference was shown between the groups at both occasions among CYP2B6*1/*1 genotype. Regardless of CYP2B6 genotype stratification, there was no difference in EFV intracellular concentration between RIF co-treatment group and the group without RIF at both occasions.

4.5.2. Predictors of plasma and intracellular concentrations of efavirenz

Factors affecting log plasma efavirenz concentrations at week 4 and week 16 separately were analyzed using regression analysis. Treatment group, demographic, type of HAART, baseline biochemical parameters, and CYP2B6 genotype were used as predictors and plasma EFV concentrations as dependent variable. Factors that influence log plasma concentrations of EFV with p<0.3 in the Univariate were included in multivariate linear regression model to build the final model. Backward elimination method was used to obtain predictors in the final multivariate linear regression model. Treatment group (Arm), sex and CYP2B6 genotype remained significant predictors of plasma efavirenz concentration at both week 4 and 16 but not body baseline body weight categorization.

4.5.3. Immunologic and virologic outcome comparisons between HIV and TB-HIV co-treatment groups

Repeated measure ANOVA indicated a tendency of having higher median CD_4 over time in Arm-2 (TB-HIV co-infected) patients compared (p=0.06). However repeated measure ANOVA within each treatment group did not show significant effect of baseline body weight groups on change in CD_4 cell count over time. Immunologic response to EFV-based HAART was higher among individuals co-treated with RIF containing anti-TB regimen than without. Having the same CYP2B6 genotypes, patients' co-treated with RIF displayed higher CD_4 gain than those treated with EFV-based HAART only.

Univariate regression analyses were done on CD_4 count at week 24 as outcome variable. Factors in final models predicting efavirenz plasma concentration were, Arm (p=0.0001) and HBsAg (p=0.02) at week 4 and Arm (p=0.0001), HBsAg (p=0.02), and CYP2B6 genotypes (P=0.02) at week 16 predicted CD_4 count at week 24. Among efavirenz intracellular concentration predictors, Arm (p=0.05), Sex (p=0.001), Baseline BMI (p=0.02) and CYP3A5 genotypes (p=0.07) at week 4 affected CD_4 count at week 24.

Proportion of patients with detectable HIV RNA (> 50 copies/mL) at week 24 was significantly lower among HIV patients receiving EFV based HAART alone (9%) than TB-HIV co-infected patients receiving concomitant RIF co-therapy (23%), regardless of baseline body weight category. No significant differences in the proportion of HIV virologic responders were observed at 48 weeks between the two treatment groups and stratified by body weight category. To evaluate relationships between EFV concentration, presence or absence of RIF co-therapy (Arm) and baseline body weight category, we performed Univariate and multivariate binary logistic regression analysis. No significant effect of plasma EFV concentration or CYP2B6 genotype on virologic response was observed at both study time points. Baseline body weight, presence of TB co-infection and RIF co-treatment had significant effect on virologic response at week 24 but not at week 48. The effect of baseline body weight on virologic response was observed, irrespective of presence or absence of RIF co-treatment.

5. DISCUSSION

This thesis reports several major findings. One of the main findings of this thesis is paradoxical effect of rifampicin (RIF) co-therapy on plasma and intracellular concentrations of efavirenz (EFV), consistent to this, improved immunologic response to HAART is reported among RIF co-treated cohort than without. These effects were pronounced among CYP2B6 slow metabolizers or CYP2B6*6 carriers **(Paper V)**. In addition, there was no difference in the proportions of virologic successes between the two groups, irrespective of pretreatment body-weight **(Paper V)**. The thesis also reports substantial between subject variability in the effect of long-term efavirenz (EFV) auto-induction, systemic plasma and intracellular exposures of EFV as well as treatment response over time, partly due to time-dependent effects of sex, CYP2B6 and UGT2B7 genotypes **(Paper I)**. In addition, this thesis reports that induction of CYP3A by EFV is concentration and time-dependent and influenced by CYP2B6 genotypes in a gene-dose dependent manner **(Paper II)**. The thesis also demonstrates importance of ethnicity and environmental factors, besides pharmacogenetic variations in CYP2B6 and ABCB1 genotypes to substantially contribute differences in EFV plasma/intracellular systemic exposure as well as immunologic recovery between Ethiopian and Tanzanian HIV patients receiving similar HAART **(Paper III)**. Moreover, this thesis reveals associations of elevated EFV plasma concentration and pharmacogenetic biomarkers such as ABCB1 3435TT, CYP2B6*6/*6 and NAT2 slow-acetylator genotypes with the development DILI in Ethiopian TB-HIV co-infected patients **(Paper IV)**.

Pharmacokinetic studies reported reduced plasma exposures of EFV due to co-administration of RIF, owing to the ubiquitous enzyme inducing nature of RIF [42]. To the effect, escalation of EFV dose from 600mg to 800mg was recommended when EFV was co-administered with RIF [171]. However, recent studies from diverse population rebut the dose escalation recommendation [57, 93, 161, 172-174]. In agreement with the later, the thesis does not recommend escalation of EFV dose in TB-HIV co-infected Ethiopian patients through this thesis with pharmacokinetic supported TB-HIV treatment outcomes **(Paper V)**.

Proportion of patients with detectable HIV RNA viral load (>50 copies/mL) was significantly higher in EFV group by week 24 irrespective of baseline body weight category **(Paper V)**. However, there was no difference in proportions of virologic responders between groups containing EFV-based HAART only and RIF co-treatment with EFV-based HAART by week 48, indicating that concurrent TB-HIV co-treatment takes longer time to reach virologic success compared to treating HIV only infection **(Paper V)**. Besides, analysis indicated higher

proportion of patients with detectable HIV RNA viral load in those weighting > 50 kg at baseline as compared to those weighing ≤ 50kg in both EFV-based HAART only and RIF co-treatment with EFV-based HAART groups. This might indicate that effect of pretreatment body weight on viral response is not specific to presence of rifampicin co-treatment **(Paper V)**.

By and large, Sub-Saharan countries obtain ARV drugs through donations from various international initiatives. The above pharmacokinetic and pharmacodynamic findings in the thesis highlight the use of standard dose of EFV during TB-HIV co-treatments, irrespective of pretreatment body-weight to minimize dose-related EFV toxicities, in addition to the cost reductions that could have been incurred due to EFV dose-escalation from 600mg to 800mg.

The thesis also reveals the long-term auto-inductions by EFV, as evident by the significant increase in the main metabolite, 8-hydroxyefavirenz (8-OH-EFV), in plasma (32%) and in PBMCs (53%) with a corresponding decrease in EFV metabolic ratio (20% and 5%, respectively) over time. The extent of change was significantly affected by sex, CYP2B6 and UGT2B7 genotypes **(Paper I)**. EFV is primarily metabolized to 8-OH-EFV mainly by CYP2B6, also by others such as CYP1A2, CYP3A5/4 and CYP2C9 [158, 159]. Auto-induction of EFV may involve some if not all of these enzymes as most of them are inducible [175-177]. The long-term auto-induction effect of EFV on CYP3A was reported in this thesis, using an endogenous biomarker for CYP3A activity, 4β-OHC/Chol ratio **(Paper II)**. Besides, the auto-induction on CYP3A by EFV was shown to be influenced by CYP2B6 genotype in gene-dose dependent manner, being highest in slow metabolizers with CYP2B6*6/*6 genotype followed by *1/*6 and *1/*1. This was further substantiated by a positive correlation, though weak, between plasma EFV concentration and 4β-OHC/Chol ratio both at two different times **(Paper II)**. The implication of long-term auto-inductions by EFV may warrant potential DDIs between EFV and substrates to CYP3A and CYP2B6 enzymes.

The plasma and intracellular concentrations of EFV did not change significantly over time, despite changes in 8-OH-EFV and EFV MR over time, as above **(Paper I)**. This is in contrast to a report on Tanzanian HIV patients, where there was an overall significant reduction in the EFV concentration over time [176]. The two studies were designed and conducted in parallel to investigate the effect of EFV auto-induction on its systemic exposure over time in two pharmacogenetically different African populations. This thesis indicates not only the presence of inter-individual but also interethnic variation in the extent of EFV auto-induction. EFV pharmacokinetic variability is not solely explained by the 8-hydroxylation pathway or the CYP2B6 genotype, because large between-subject variability in the plasma EFV exposure

remains unexplained, even after accounting for known CYP2B6 genetic variations. This was the basis for evaluation and identification of predictors of plasma and intracellular concentrations of EFV by combining the similarly designed and parallelly conducted data from Ethiopian and Tanzanian HIV patients **(Paper III)**.

The thesis reports that geographic differences (patient country), CYP2B6*6 and ABCB1 3842 A < G genotypes as significant predictors of EFV plasma and intracellular concentration **(Paper III)**. The allele frequency distributions of both CYP2B6*6 and ABCB1 3842A<G alleles was significantly higher in Tanzanians than Ethiopians. Likewise EFV plasma and intracellular concentrations as well as immunological gain were significantly different between the two populations; being higher in Tanzanians than Ethiopians **(Paper III)**. Besides, the extent of EFV auto-induction overtime was pronounced in Tanzanians whereas no significant change was observed in Ethiopians **(Papers I & III)**. This thesis demonstrates not only the existence of between population differences in EFV pharmacokinetics and pharmacogenetics but also variations in the extent of EFV auto-induction between populations **(Paper III)**. Furthermore, to optimize EFV dosage, the thesis emphasizes the importance of ethnicity and environment factors in addition CYP2B6 and ABCB1 genotype, which may in turn influence the immunological outcomes **(Paper III)**. This is in line with the results by Dandara *et al* [111] who also found inter-ethnic and population differences in the distribution of various allele frequencies for other CYPs among Africans. This thesis reaffirms the need for extensive population specific pharmacogenetic research for optimized antiretroviral therapy across Africa.

The thesis also reports the association of elevated plasma concentration of EFV with DILI **(Paper IV)**. The association of higher EFV plasma concentration with DILI was regardless of concomitant RIF based anti-TB therapy, whereas no association of the metabolite (8-hydroxyefvairenz) was observed. Furthermore, ABCB1 3435TT, CYP2B6*6/*6 and NAT2 slow-acetylator genotypes were identified as pharmacogenetic biomarkers for the development DILI in Ethiopian TB-HIV co-infected patients **(Paper IV)**. Direct liver toxicity by higher EFV plasma concentration could be a possible mechanism for EFV-based HAART induced liver injury in HIV patients. In support of this argument were associations of DILI with CYP2B6*6 and UGT2B7*2, the variant alleles associated with increased EFV plasma concentration **(Paper IV)**. The associations of these genetic biomarkers with DILI emphasize the need to use pharmacogenetic tools to predict and identify HIV treatment toxicities *a priori*.

In addition to the above major findings, this thesis also reports several outcomes. One of the results reported in the thesis is the following allele frequencies in Ethiopian population: CYP2B6*6, CYP3A5*3, CYP3A5*6, UGT2B7*2, ABCB1 3435C >T, ABCB1 3842A<G, SLCO1B1-D130N (*1b), SLCO1B1-A174V (*5) were 29.7%, 66.9%, 10.6%, 48.1%, 20.9%, 14.5%, 61.1% and 79.1% respectively **(Papers I & III)**. Besides reporting these allele frequencies in Ethiopian population as new information, the comparisons of the allele frequencies between the two sub-Saharan African countries, the thesis also shows significant differences between Ethiopians and Tanzanians, reaffirming the need for extensive population specific pharmacogenetic studies to optimized antiretroviral therapy across Africa **(Paper III)**.

The thesis also shows positive correlations of plasma and intracellular concentrations of EFV **(Paper I)**. Most ARV drugs including EFV are known to exert their effect intracellularly [178, 179]. Drug concentrations at the site of action are regarded as the true indicative for treatment successes. The result of the correlation between plasma and intracellular concentrations of EFV suggested that plasma exposure could be surrogate marker for intracellular activity **(Paper I)**. This is in agreement with previous reports [180, 181]. This thesis also reports higher incidence of drug induced liver injury (DILI) due to concomitant anti-TB and EFV-based HAART among TB-HIV co-infected Ethiopian patients **(Paper IV)**.

This thesis also demonstrates the influence of pharmacogenetic variations on immunologic outcome among HIV infected Ethiopians **(Paper I)**. By and large, good immune recovery, as indicted by a sharp increase in the CD_4 count during early (3 months) on HAART. This was regardless of sex or genotype **(Paper I)**. However, the pattern of CD_4 gain was observed to be variable between the different CYP2B6 and UGT2B7 genotype groups 6 and 12 months after HAART. There was no significant change in the CD_4 count between 6 and 12 months among subjects with CYP2B6*1/*1 and UGT2B7*1/*1, while the CD_4 count continued to increase among carriers of CYP2B6*6/*6 and UGT2B7*2/*2 **(Paper I)**. The thesis indicates that the effect of genetic variation in CYP2B6 and UGT2B7 on the change in the CD_4 count becomes apparent in the long run as therapy continues **(Paper I)**. Genotype variability may be considered as additional factor to explain differences in immunologic response for HAART. The thesis pioneers to stratify immunologic outcomes of EFV-based HAART based on genotypes.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

- Rifampicin (RIF) co-therapy caused paradoxical effect of plasma and intracellular concentrations of efavirenz (EFV), subsequent to that it caused better immunologic response and comparable virologic success to HAART than HAART without RIF.
- Substantial between-subject variability was shown in the effect of long-term efavirenz (EFV) auto-induction, systemic plasma and intracellular exposures of EFV and HAART response over time, partly due to time-dependent effects of sex, *CYP2B6* and *UGT2B7* genotypes.
- Induction of *CYP3A* by EFV was influenced by *CYP2B6* genotypes in concentration and time-dependent in a gene-dose dependent manner.
- Importance of ethnicity and environmental factors substantially contributed to the differences in EFV plasma/intracellular systemic exposure as well as immunologic recovery.
- Associations of elevated EFV plasma concentration and *ABCB1 3435TT, CYP2B6*6/*6* and slow *NAT2* metabolizing genotypes with the development of DILI in Ethiopian TB-HIV co-infected patients were observed.
- Allele frequencies of *CYP2B6*6, CYP3A5*3, CYP3A5*6, UGT2B7*2, ABCB1 3435C >T, ABCB1 3842A>G, SLCO1B1 D130N (*1b), SLCO1B1 A174V (*5)* in Ethiopian population were reported.
- Positive correlation between plasma and intracellular concentrations of EFV was established.
- Impact of pharmacogenetic variations on immunologic outcome among HIV infected Ethiopians was demonstrated.
- RIF co-therapy, Baseline Body Weight and *CYP2B6* genotypes predicted plasma and intracellular concentrations of EFV during short-term and long-term EFV administration. Among these factors, RIF co-therapy and *CYP2B6* genotypes were associated with CD_4 recovery at 6 months EFV-based HAART.

6.2. Recommendations

- Escalation of efavirenz (EFV) dose from 600mg to 800mg is not needed in co-treating TB/HIV co-infected Ethiopian.
- Potential drug-drug interactions may be warranted on substrates of *CYP3A* and *CYP2B6* enzymes due to long-term induction effect of EFV.

- Extensive population specific pharmacogenetic studies for optimized antiretroviral therapy across Africa should be carried out.
- Therapeutic drug monitoring (TDM) in association with pharmacogenetic tools may be employed to routinely monitor HAART toxicities and responses.
- Plasma concentrations of EFV may be surrogate markers for intracellular EFV exposure.

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