Optimization of HIV therapy in patients co-infected with tuberculosis

A pharmacogenetic and pharmacokinetic study of efavirenz in Rwandan adult patients undergoing HIV and tuberculosis co-treatment

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ABSTRACT

Tuberculosis (TB) is the most common opportunistic infection among people infected with human immunodeficiency virus (HIV). The co-management of HIV/TB co-infection is prone to multiple drug-drug interactions. In addition, the recommended HIV drug efavirenz (EFV) has a narrow therapeutic window which compromises its clinical safety and exhibits a highly variable pharmacokinetics (PK) between subjects due to genetic factors. A lack of genomic data on many African populations limits attempts aiming at optimizing therapies in general, HIV therapy in particular. This thesis specifically aimed to obtain genomic data in an African population and to investigate the pharmacokinetic and pharmacogenetic aspects of EFV exposure in the presence of TB therapy.

A clinical study was conducted in Rwandan HIV patients co-infected with TB. EFV plasma concentrations, CD4 cell counts and HIV-RNA copies were monitored. Genotyping for 13 single nucleotide polymorphisms (SNPs) with respect to five cytochrome P450 enzymes was conducted. A rapid and selective high performance liquid chromatography analytical method was developed for the quantification of EFV in plasma containing HIV and TB drugs.

Genetic variation was observed in 11 out of the 13 analyzed SNPs with minor allele frequencies for 12 SNPs. There was a significant difference between CYP1A2 -739T/G and T/T genotypes only in the presence of rifampicin-based TB treatment (RBT). In the presence and in the absence of RBT, CYP2B6 516T/T genotype was found to be associated with higher EFV plasma levels. CYP2A6 1093G>A, CYP2B6 516G>T and CYP2B6

983T>C SNPs were found to be independent predictors of EFV plasma levels accounting for 27%, 43%, and 29%, respectively, of the total variance in EFV plasma levels. There was a high positive predictive value for CYP2B6 516T/T and 983T/T genotypes in predicting supra-therapeutic EFV plasma levels. RBT was shown to significantly lower EFV plasma levels but did not affect HIV-treatment response. There were higher clearance (CL/F) values in patients with previous exposure to HIV therapy than in patients who were administered RBT prior to HIV therapy. Expectedly, carriers of CYP2B6 516G/G and T/T genotypes exhibited higher and lower CL/F, respectively, regardless of the previous treatment received by the patients.

In conclusion, CYP enzymes of the accessory metabolic pathways of EFV (CYP1A2 and CYP2A6) could explain variability in EFV exposure, in addition to CYP2B6 which proved to be the main pharmacogenetic determinant of EFV exposure in the patient population studied. As proven by the observed high positive predictive value, predictive genotyping in CYP2B6 SNPs may be useful in optimizing EFV-based HIV therapy. Not only should the patient genotype status with respect to CYP2B6 be taken into account, but also each individual patient treatment history, with caution to previous exposure to HAART. Even though it is clear from this thesis that specific CYP genotypes and co-medications do have a definite effect on EFV plasma levels causing its variation, this however does not seem to influence the efficacy of the EFV-based regimens in general.

Keywords: Clearance, CYP2B6, efavirenz, genotype, HIV, rifampicin, plasma level, SNP, tuberculosis.
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SAMMANFATTNING PÅ SVENSKA

Det övergripande målet för den här doktorsavhandlingen var att optimera HIV-behandlingen för patienter infekterade med både humant immunbristvirus (HIV) och tuberkulos (TB). Bland HIV-positiva personer är TB i särklass den vanligaste opportunistiska infektionen och denna grupp är också mer känslig mot tuberkelbakterien som orsakar TB. Kombinationsterapi, alltså behandling med mer än ett läkemedel, krävs för behandling av HIV/TB co-infektioner. Både antiretroviral behandling mot HIV och läkemedel mot TB spjälkas i kroppen genom samma metaboliska väg, nämligen genom cytokrom P450 (CYP)-systemet. Simultan behandling av HIV- och TB-infektion är därför benägen att leda till att de olika drogerna påverkar varandra i kroppen (läkemedelsinteraktion) vilket kan påverka läkemedlens effektivitet. Efavirenz (EFV) är det läkemedel som rekommenderas vid kombinationsterapi vid högintensiv HIV-behandling, HAART, hos patienter co-infekterade med TB. EFV har ett snävt terapeutiskt fönster som riskerar att påverka läkemedlets säkerhetsprofil hos patienten. Utöver detta uppvisar EFV en skiftande farmakokinetik, vilket gör att läkemedlet verkar olika mycket i patienter med olika genetisk uppsättning. Därtill saknas genomisk litteraturdata från vissa populationer i Afrika som också är den kontinent där båda dessa infektioner är vanligast förekommande och där den genetiska variabiliteten mellan individer är mycket stor.

Den här avhandlingen fokuserar bland annat på att kartlägga den genetiska uppsättningen hos personer i en population i Rwanda . De gener som särskilt studerats är involverade i spjälkningen av vanligt förekommande läkemedel som används vid behandling av olika typer av infektioner, inklusive antiretrovirala läkemedel. Dessutom undersöktes EFVs farmakokinetik och farmakogenetik då det användes tillsammans med TB-läkemedel. Effekterna av TB-behandling och långtidsterapi med HIV-läkemedel med avseende på farmakokinetiken hos EFV studerades också.

En klinisk studie utfördes i Rwanda där testpersonerna var co-infekterade med HIV och TB. EFV plasma-koncentrationer, antalet CD4-celler och antalet HIV-RNA kopior beräknades vid givna tidpunkter och fem CYP-enzymer genotypades. Samtidigt utvecklades en snabb, högpresterande vätskekromatografimetod för kvantifiering av EFV i plasma innehållande HIV- och TB-läkemedel.

Resultaten från genotypningen visade att den genetiska variationen mellan testpersonerna inom Rwanda-gruppen var mycket stor men resultaten skilde sig även från populationer från andra länder, så som Kamerun och Sydafrika. Den huvudsakliga genetiska faktorn som kunde kopplas direkt till skillnader i EFV-nivåerna i plasma var CYP2B6-genen som uttrycker enzymet cytochrome P450 2B6 involverat i spjälkningen av EFV-läkemedlet. Genetiska skillnader i CYP2B6-uttrycket mellan olika individer kunde förklara 72% av de totala variationerna i EFV-nivåerna. Ett positivt prediktivt värde upptäcktes för CYP2B6-varianterna och detta indikerade att det skulle kunna vara viktigt att använda genotypning som ett verktyg för att identifiera de patienter som finns i riskzonen för förhöjda EFV-nivåer. För höga nivåer av EFV i plasma kan leda till ökad neurotoxicitet och läkemedelsdosen bör i sådana fall justeras. Hos patienter som behandlas för kombinationen HIV/TB-infektion sker läkemedelsinteraktioner i kroppen mellan läkemedlen där särskilt TB-farmaka tenderar att kraftigt minska nivåerna av EFV i plasma och långtidsterapi med antivirala medel ökar elimineringen av EFV. Resultaten visar att TB-farmakas påverkan på EFV plasmanivåerna bör tas på allvar men justering av läkemedelsdosen bör övervägas endast då utfallet av behandlingen påverkas, särskilt i de fall då patienten genomgår HIV-terapi.

Sammanfattningsvis föreslår vi att den genetiska uppsättningen med avseende på CYP2B6 bör analyseras samtidigt som varje patients tidigare behandlingar bör beaktas, i synnerhet eventuell tidigare HIV-terapi.

Trots att våra resultat och tidigare publicerade studier visar att den genetiska bakgrunden ihop med behandling med en kombination av flera läkemedel påverkar EFV plasmanivåerna kan de inte kopplas direkt till en generellt minskad effektivitet av EFV-baserad terapi.

PAPERS DISCUSSED

This thesis is based on the following papers, referred to in the text by their Roman numerals assigned below:

I. **Bienvenu E**, Hoffmann KJ, Ashton M, Kayumba PC. A rapid and selective HPLC-UV method for the quantification of efavirenz in plasma from patients on concurrent HIV/AIDS and tuberculosis treatments. *Biomed. Chromatogr*. 2013; 27(11): 1554–1559.

II. **Bienvenu E**, Swart M, Dandara C, Ekman A, Äbelö A, Wonkam A, Ashton M. Frequencies of Single Nucleotide Polymorphisms in Cytochrome P450 Genes (CYP1A2, 2A6, 2B6, 3A4 and 3A5) in a Rwandan Population: Difference to Other African Populations. *Curr Pharmacogenomics Person Med.* 2013, 11(3): 237 – 246.

III. **Bienvenu E**, Swart M, Dandara C, Ashton M. The role of genetic polymorphisms in cytochrome P450 and effects of tuberculosis co-treatment on the predictive value of CYP2B6 SNPs and on efavirenz plasma levels in adult HIV patients. (*Submitted*)

IV. **Bienvenu E**, Ashton M, Äbelö A. Population pharmacokinetic modeling of efavirenz in Rwandan adult patients on concomitant HIV and tuberculosis treatments. (*In manuscript*)

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ABBREVIATIONS

3TC	=	Lamivudine
AIDS	=	Acquired immunodeficiency syndrome
ALAT	=	Alanine aminotransferase
ANOVA	=	Analysis of variance
ARV	=	Antiretroviral
ASAT	=	Aspartate aminotransferase
AUC	=	Area under the plasma drug concentration-time curve
AZT	=	Zidovudine
CD4	=	Helper T lymphocyte
CDC	=	Centers for Disease Control and Prevention
CDS	=	Clarity Chromatography Data Station
CI	=	Confidence interval
CL/F	=	Oral clearance
CNS	=	Central nervous system
C_{max}	=	Maximum plasma concentration
C_{min}	=	Minimum plasma concentration
CRF	=	Case report form
CV	=	Coefficient of variation
CYP	=	Cytochrome P450
DNA	=	Deoxyribonucleic acid
DOT	=	Directly observed therapy
E	=	Ethambutol
EDTA	=	Ethylene diamine tetra-acetic acid
EFV	=	Efavirenz
F	=	Bioavailability
FDA	=	Food and Drug Administration
FTC	=	Emtricitabine
FOCE	=	First-order conditional estimation
H	=	Isoniazid
HAART	=	Highly active antiretroviral therapy
HIV	=	Human immunodeficiency virus
HPLC	=	High performance liquid chromatography
HWE	=	Hardy-Weinberg equilibrium
ICH	=	International Conference on Harmonization
IQR	=	Interquartile range
IS	=	Internal standard
Ka	=	Absorption rate constant
LC	=	Liquid chromatography
LD	=	Linkage disequilibrium

MAF	=	Minor allele frequency
NNRTI	=	Non-nucleoside reverse transcriptase inhibitor
NRTI	=	Nucleoside reverse transcriptase inhibitor
NPV	=	Negative predictive value
NVP	=	Nevirapine
PCR	=	Polymerase chain reaction
PI	=	Protease inhibitor
PK	=	Pharmacokinetics
PPV	=	Positive predictive value
R	=	Rifampicin
RBT	=	Rifampicin-base tuberculosis therapy
RNA	=	Ribonucleic acid
RSD	=	Relative standard deviation
Rt	=	Retention time
RT	=	Reverse transcriptase
SD	=	Standard deviation
SNP	=	Single nucleotide polymorphism
TB	=	Tuberculosis
TDM	=	Therapeutic drug monitoring
TDF	=	Tenofovir
T_{max}	=	Time required to reach C_{max}
UGT	=	Uridine diphosphate glucuronosyltransferase
UV	=	Ultraviolet
V/F	=	Volume of distribution
VPC	=	Visual predictive check
WHO	=	World Health Organization
Z	=	Pyrazinamide

DEFINITIONS IN SHORT

Pharmacogenetics	The study of inter-individual genetic variation that gives rise to differing response (both safety and efficacy) to drugs [1].
Pharmacogenomics	The identification of the genes that influence drug response [1].
Pharmacokinetics	The study of how a drug is absorbed, distributed, metabolized, and excreted [2].

1 INTRODUCTION

1.1 Background

With the overall aim of contributing to the optimization of HIV therapy in the context of HIV and TB co-treatment, this thesis focuses mainly on the pharmacogenetics of EFV exposure in the presence of TB therapy and the effects of TB and long term HIV treatments on the pharmacokinetics (PK) of EFV.

Tuberculosis (TB) is the most common opportunistic infection among people infected with human immunodeficiency virus (HIV) [3]. HIV infected patients are more sensitive to develop TB than those who are HIV-negative [4]. The standard regimen for the treatment of TB is a combination of two to four drugs [5]. The recommended first line highly active antiretroviral therapy (HAART) for the management of HIV involves a combination of three drugs consisting of two nucleoside reverse transcriptase inhibitors (NRTIs) with a non-nucleoside reverse transcriptase inhibitor (NNRTI) [6].

The HIV drug of focus of this thesis is efavirenz (EFV). EFV is the NNRTI recommended as part of HAART either in patients infected with HIV alone or co-infected with TB, because of good tolerance with superior clinical outcomes compared to other NNRTIs [6-10]. Moreover, EFV itself has a PK profile that deserves a particular attention. EFV is highly protein bound (>99%), has a long steady-state half life (40-55 hours) and a narrow therapeutic window, which ranges between 1 and 4 μg/ml. Plasma levels of EFV below 1 μg/ml and above 4 μg/ml have been associated with increased risks of therapeutic failure and central nervous system (CNS) side effects and toxicity, respectively [11]. In addition, EFV has been shown to induce its own metabolism and hence accelerating its own elimination [12-14].

The relationship between a drug's pharmacokinetic and pharmacodynamic properties governs the time course of drug action. Thus, factors affecting the drug's PK could influence the expected treatment outcome [2]. High inter-individual variability in the PK of EFV following the administration of the fixed once daily adult dose of 600 mg has been reported [11, 15-19]. This is most likely caused by a combination of multiple factors including biologic

and genetic factors, as well as drug-drug interactions [20]. These are major concerns to address when attempts aiming at optimizing the therapy of HIV are made. Particularly, in the context of HIV and TB co-treatment drug-drug interactions represent an important challenge due to the fact that managing HIV and TB co-infections requires multidrug therapy.

Genetic polymorphism is the most important factor affecting the PK of antiretroviral (ARV) drugs, including EFV [20]. A lack of genomic data on many African populations limits attempts aiming at optimizing therapies in general, HIV therapy in particular. Currently, Africa is under-represented in genetic screening [21], whereas it is the most affected continent with respect to major infectious diseases [22-24] and presents with the most genetic diversity [25-27]. A pharmacogenomic study was performed in a Rwandan population with respect to genes coding for proteins involved in the metabolism of commonly used anti-infectious drugs, including antiretrovirals (**Paper II**).

EFV is mainly metabolized in humans by hydroxylation catalyzed by cytochrome P450 (CYP) 2B6, and to a lesser degree by CYP1A2, CYP2A6, CYP3A4 and CYP3A5 [31-38]. Thus, factors affecting the metabolism of EFV could influence EFV exposure and the treatment response. Therefore, this variability in EFV PK is thought to be caused by inter-individual differences in CYP activity and expression [18, 20-29, 32]. The effects of ten SNPs in CYP genes on EFV plasma levels and on treatment response in the absence and presence of TB therapy was investigated. Moreover, whether there are additional SNPs that could be used to enhance the prediction of EFV exposure (independent predictors) was analyzed (**Paper III**).

Therapeutic management of HIV/TB co-infection may result in complex PK drug-drug interactions taking place collectively between HAART components and TB drugs, impacting on blood levels of both HIV and TB drugs. Indeed, both ARV and TB drugs share metabolic pathways through the CYP system [33, 34], some being inhibitors or inducers of CYP enzymes [35]. As a consequence, multiple interactions take place during the co-management of HIV/TB co-infection, where EFV-based HAART is concomitantly administered with rifampicin-based TB treatment (RBT). The induction effect of rifampicin on EFV metabolizing enzymes (CYP2B6 and CYP3A4) has been demonstrated [36-39]. Particularly, CYP2B6 has been studied in different populations [39-41]. Moreover, EFV has been shown to induce CYP2B6 and CYP3A4, accelerating its own metabolism [12-14]. These issues were addressed in this thesis, by investigating the effects of

RBT on EFV exposure and on the clinical response (**Paper III**) and by estimating the clearance (CL/F) value taking into account not only CYP2B6 genetic polymorphism, but also enzyme induction arising from EFV-based HAART and from RBT (**Paper IV**).

One of the important aspects in therapy optimization is monitoring patients during treatment, especially when the drug has a narrow therapeutic window and an apparent correlation between high plasma levels and a high rate of side effects and toxicity. In the context of HIV management, monitoring patients' plasma levels (known as therapeutic drug monitoring or TDM) has been recommended for EFV [42] and genotyping has proven to be useful in the prediction of the pre-disposition to low or high EFV levels in clinical studies [43, 44]. However, the main challenges in implementing TDM are the availability of validated and simple quantification assays with high selectivity, small sample volume requirements, and rapid turnaround time for the measurements. In addition, the assay needs to be cost-effective in order to guarantee its applicability and sustainable use, especially in resource limited settings [35, 45]. In this perspective, a rapid and selective analytical method for the quantification of EFV in human plasma containing first line medications used for the management of both HIV/AIDS and TB was developed and validated as to contribute to the applicability of TDM in resource limited settings (**Paper I**). Furthermore, regarding monitoring patients through genotyping, there are still some concerns, about the clinical utility of routinely screening for genetic markers and the applicability to the broader population of data from stratified population [45, 46]. In **Paper III,** the validity of genotyping in each of CYP2B6 SNPs in predicting EFV plasma levels was evaluated.

This thesis is a presentation of studies that were conducted to shed more light on the optimization of HIV therapy. The overall aim of this thesis is described in Chapter 2, along with specific aims which were addressed through papers mentioned in above paragraphs. Multiple aims were addressed in some of the papers, whereas single aims were studied in others. Patients and methods are detailed in Chapter 3, while summary results and discussion are presented in Chapter 4. The main conclusions from this work are outlined in Chapter 5.

1.2 Human immunodeficiency virus

World Health Organization (WHO) recently reported that in 2012, an estimated 35.3 million people were living with HIV. HIV refers to human immunodeficiency virus. There are two types of HIV: HIV-1 and HIV-2. HIV-1 is responsible for the vast majority of HIV infections globally. These two viruses have been identified as the primary cause of acquired immunodeficiency syndrome (AIDS). In this thesis, HIV refers to both HIV-1 and HIV-2 unless otherwise indicated [47].

1.2.1 Pathogenesis of HIV infection

In the beginning, the newly HIV-infected individual exhibits no symptoms. Slight changes in the immune system follow and take place progressively; individuals can within two to four weeks after infection with HIV experience an acute retroviral syndrome or primary HIV infection. It is the body's natural response to the HIV infection taking up to 3 months for it to appear in some people. Although individuals may look healthy during primary infection, the virus is actively replicating. As a result, the immune system may get slowly damaged and individuals may develop symptoms of the late phase of HIV disease (AIDS) where individuals are susceptible to other opportunistic infections, such as infections with *Mycobacterium tuberculosis*, *Pneumocystis carinii*, toxoplasmosis and candidiasis, etc. Infected individuals are diagnosed as having AIDS status when their plasma HIV load is high and the CD4 cell counts falls below 200 cells/ mm^3(normal CD4 counts are between 500 and 1.600 cells/mm^3) [48, 49].

The primary and essential receptor for HIV is the CD4 lymphocyte cell which is involved in the induction of most immunologic functions. It has been suggested that the CD4 cell is the high-affinity receptor for the virus. The CD4 cell needs to use the chemokine receptor as essential co-receptor for HIV entry into other cell types such as monocytes and dendritic cells [48, 50]. After HIV binds to the CD4 cell, the virus is internalized and uncoated followed by the transcription of the genomic ribonucleic acid (RNA) to deoxyribonucleic acid (DNA) by the enzyme reverse transcriptase. The proviral DNA is integrated into the host chromosomal DNA (Figure 1). At this stage, the infection is in a latent phase until the infected cells are activated. Once the cell is activated, the proviral DNA transcribes viral genomic RNA and messenger RNA. Protein synthesis, processing, and virus

assembly occur with budding of the mature virion from the cell surface. When active replication of virus occurs, the host T-cell is usually killed. This results in progressive depletion of CD4 cells. At this stage, profound immune suppression occurs, resulting from removal of CD4 cells from the immune system rending it progressively weaker and unable to control subsequent infections [49].

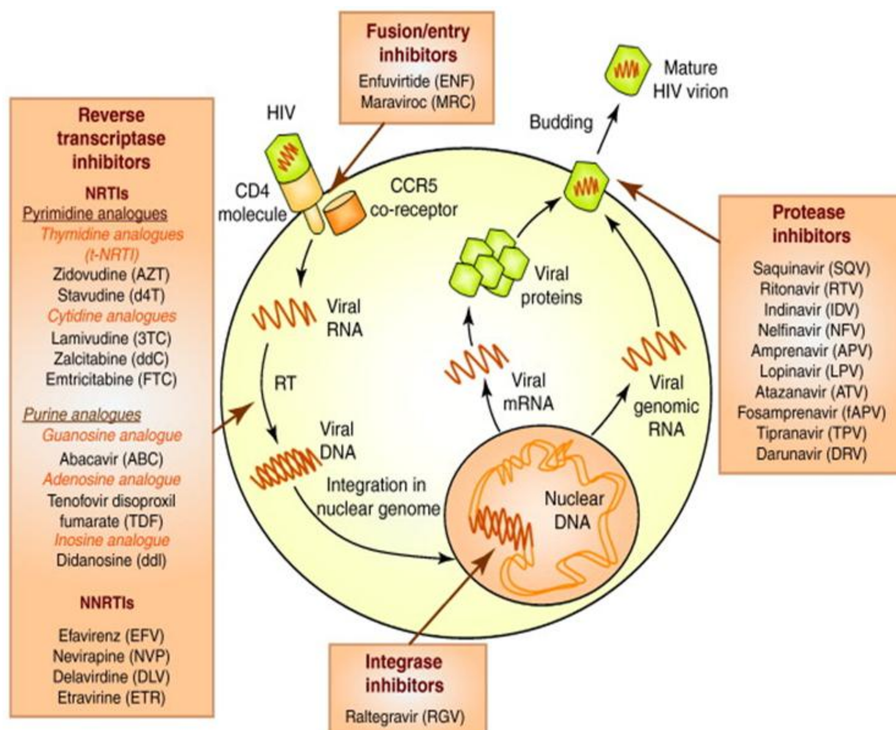

Figure 1. *HIV life cycle and the sites of action for available antiretroviral drugs. NRTIs: nucleoside reverse transcriptase inhibitors;*
NNRTIs: non-nucleoside reverse transcriptase inhibitors;
PIs: protease inhibitors; RT: reverse transcriptase;
CCR5: C-C chemokine receptor type 5. From Apostolova et al. (2011)[51].

1.2.2 Pharmacological approach to HIV treatment

1.2.2.1 Treatment goals

The primary goals for initiating antiretroviral therapy are to reduce HIV-associated morbidity and prolong the duration and quality of survival, restore and preserve immunologic function, maximally and durably suppress plasma HIV viral load, and prevent HIV transmission [42].

1.2.2.2 Current approach to treatment

The combined pharmacological approach to the treatment of HIV infection, known as highly active antiretroviral therapy (HAART) is a combination of antiretroviral drugs grouped in five classes according to the mechanism of which they interrupt the HIV life cycle [51] (Figure 1). The major classes are nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs). There are three new antiretroviral classes, namely integrase inhibitors, chemokine receptor type 5 (CCR5) inhibitors and fusion inhibitors [51-58] (Figure 1).

1.2.2.3 Recommend first-line antiretroviral therapy

Current WHO guidelines for the management of HIV infection recommend first-line treatment composed by two NRTIs combined with one NNRTI. The recommended HAART consists of an NNRTI (either nevirapine -NVP- or efavirenz -EFV-) plus two NRTIs, one of which being lamivudine (3TC) or emtricitabine (FTC) and the other zidovudine (AZT) or tenofovir (TDF). The less toxic and more convenient fixed-dose combination recommended for first-line HAART is once-daily regimen comprising TDF+3TC (or FTC)+EFV). This combination therapy is the preferred option to initiate HAART in adults, adolescents and children older than three years. For children younger than three years, a PI-based regimen is the preferred approach. If the above recommended combination is contraindicated or not available, one of the following options is recommended: AZT+3TC+EFV or AZT+3TC+NVP or TDF+3TC (or FTC)+NVP. Of note is that EFV is preferred to NVP because people receiving NVP are twice as likely as those receiving EFV to discontinue treatment because of adverse events. For pregnant and breastfeeding women, a once-daily fixed-dose combination of TDF+3TC (or FTC)+EFV is recommended as first-line HAART as well. Infants of mothers who are receiving HAART and are breastfeeding should receive six weeks of infant prophylaxis with daily NVP [47].

1.3 Tuberculosis

1.3.1 Pathogenesis of tuberculosis

TB is an infection caused by a bacterium called *Mycobacterium tuberculosis* that affects the lungs. It is transmitted after expectoration when an infected individual coughs, sneezes or talks, expelling the *Mycobacterium tuberculosis*, usually contained in droplet nuclei. *Mycobacterium tuberculosis* sometimes causes illness soon after the initial exposure (active TB), but an immune-competent system can prevent *Mycobacterium tuberculosis* from causing illness (latent TB). It may happen however, that *Mycobacterium tuberculosis* causes illness years later (reactivation TB). The droplets, when expelled, can remain airborne for minutes to hours. Once the infectious droplet nuclei are inhaled, *Mycobacterium tuberculosis* is taken up by alveolar macrophages, initiating a cascade of events that result in either a successful containment of the infection or progression to active disease [59]. The CD4 positive T lymphocytes cells are involved in these event cascades. After being ingested by alveolar macrophages, *Mycobacterium tuberculosis* replicates and spreads via the lymphatic system to the hilar lymph nodes. The infected macrophage releases interleukins 12 and 18, which stimulate CD4 T lymphocytes to release interferon γ. This interferon γ then stimulates the phagocytosis of *Mycobacterium tuberculosis* in the macrophage. Interferon γ also stimulates the macrophage to release tumor necrosis factor α, which is important in the formation of granuloma and control of the extent of infection. When the host immune response fails to contain the replication of *Mycobacterium tuberculosis* associated with initial infection, active disease occurs. In immune-competent individuals, granulomas are highly effective in containing, but not eliminating, the infection [60, 61].

1.3.2 Clinical management of tuberculosis

1.3.2.1 Aims of treatment

The aims of treatment of TB are to cure the patient and restore quality of life and productivity, to prevent death from active TB or its late effects, to prevent relapse of TB, to reduce transmission of TB to others, and to prevent the development and transmission of drug resistance [5].

1.3.2.2 Standard regimens for the treatment of TB

The TB drugs recommended in the WHO guidelines are ethambutol (E), isoniazid (H), rifampicin (R) and pyrazinamide (Z). Streptomycin is indicated when one of these drugs is contraindicated. New patients with pulmonary TB may receive a daily intensive phase treatment with RHZE for 2 months, followed by three times weekly continuation phase with RH, provided that each dose is directly observed ([$2(RHZE)_7/4(HR)_3$]; numbers before the letters indicate the months of duration of the treatment for the intensive treatment phase; subscripts indicate how often the treatment is taken each week). Three times weekly dosing throughout therapy ($2(RHZE)_3/4(HR)_3$) may be used when necessary as another alternative to above recommendation, provided that the patient is not living with HIV [5]. Otherwise the optimal dosing frequency for new patients with pulmonary TB is daily throughout the course of therapy wherever feasible [5].

1.4 HIV and tuberculosis co-infection

Infection with HIV is the most potent risk factor for TB, with an increased risk of more than 20-times compared to those not infected with HIV [59].

1.4.1 Epidemiology of HIV-related tuberculosis

The 2013 global TB report indicates that the prevalence of TB in the world was estimated to 8.6 million people in 2012 and the total number of those co-infected with HIV was 1.1 million (13%). Of these, 75% were in countries of the African Region. Regarding the number of deaths, of the 8.6 million cases of TB 1.3 million died and 320 000 (representing 25%) were co-infected with HIV. The same report further indicates that 26% of TB patients were co-infected with HIV in the year 2012 in Rwanda [3].

1.4.2 Interactions between HIV and tuberculosis

Without treatment, HIV and TB interact, accelerating the deterioration of immunological functions and shortening lifespan. It has been reported that TB infection exacerbates HIV infection. Epidemiological data showed that the incidence and mortality rates for new AIDS-defining opportunistic infections were higher if individuals with HIV were co-infected with TB [62, 63]. On the other hand, the progression from latent to active TB was reported to be increased in HIV/TB co-infected patients, compared to TB mono-infected patients. The depletion of CD4 cells by HIV infection is believed to be an important contributor to the increased risk of reactivation of latent TB [62, 64].

1.4.3 Co-management of HIV and tuberculosis

WHO guidelines recommend that HIV testing should be initiated for all patients with known or suspected active TB and that TB patients living with HIV receive the same duration of TB treatment as HIV-negative TB patients.

1.4.3.1 HIV/TB patients not yet receiving antiretroviral therapy

WHO recommended that TB treatment should be initiated first in all HIV/TB patients. HAART should follow as soon as possible, and within the first eight weeks regardless of CD4 cell counts [3, 47]. New HIV/TB patients should be treated with the TB regimens given in section 1.3.2.2, and with HAART consisting regimens described in section 1.2.2.3. EFV-based HAART regimens are preferred to NVP ones because of less interactions between EFV and TB drugs [5, 47].

1.4.3.2 Patients diagnosed with tuberculosis while on antiretroviral therapy

When TB is diagnosed in patients already receiving antiretroviral therapy, TB treatment should be started immediately. Before initiation of TB treatment, it must be considered whether HAART needs to be modified because of drug-drug interactions or to reduce possible overlapping toxicities.

1.5 Efavirenz

EFV is the recommended NNRTI, dosed once daily, for the treatment of HIV infection alone or of HIV/TB co-infection [47, 65]. EFV has a narrow therapeutic window ranging between 1 and 4μg/ml (3.2 and 12.6 μM, respectively) [11, 66, 67].

1.5.1 Pharmacokinetics of efavirenz

Pharmacokinetics is the study of how the body affects a drug, and is described by how a drug is absorbed, distributed, metabolized, and excreted [2].

1.5.1.1 Pharmacokinetic properties of efavirenz

Following EFV 600 mg once-daily administration, peak plasma concentrations (C_{max}) at steady-state were 12.9 ± 3.7 μM (mean ± SD), trough plasma concentrations (C_{min}) were 5.6 ± 3.2 μM, and areas under the plasma drug concentration-time curve (AUC) were 184 ± 73 μM*h. C_{max} was reached 3 - 5 hours after dose. Steady-state plasma concentrations were reached in 6 - 7 days. The oral bioavailability of EFV is 40-45% without food [65, 68]. Fat-containing meals increase absorption significantly [65, 68, 69]. EFV is highly bound (approximately 99.5-99.75%) to human plasma proteins, predominantly albumin. EFV is principally metabolized by the CYP system to essentially inactive hydroxylated metabolites followed by glucuronidation. Approximately 14-34% of the EFV dose is excreted renally primarily as metabolites and 16-61% of the dose is excreted in the feces as parent drug. EFV has a relatively long terminal half-life of 52-76 hours after single doses decreasing to 40-55 hours after multiple doses [65, 68]. EFV has been shown to induce CYP enzymes among which CYP2B6 [14, 68], resulting in the induction of also its own metabolism [12-14]. A population PK analysis performed to assess the PK profile of EFV in HIV patients reported an absorption rate constant (Ka) of 0.3 h^{-1}, a CL/F of 9.4 L/h and a volume of distribution (V/F) of 252 L [16].

1.5.1.2 Influence of biological factors on EFV pharmacokinetics

While genetic factors are considered as the most important determinant of the PK of EFV [20], there is no conclusive evidence to suggest that biological factors such as gender and body weight significantly influence EFV plasma levels. In some studies, higher EFV plasma levels were found in women

compared to in men [70], but in other studies no sex-related differences in EFV levels have been found [16, 71-73]. On the other hand, some authors found the body weight to be associated with clearance or EFV plasma levels [15, 73], while others did not find such association [70, 74, 75]. However, ethnicity has been consistently associated with EFV plasma levels such that Blacks or Asians tend to have higher EFV plasma levels than Whites [16, 70, 76, 72-75], which probably reflects host genetics. In this work, the relationship between demographic variables and EFV plasma levels was investigated in **Paper III**.

1.5.2 Clinical safety of efavirenz

EFV-related neurotoxicity is the most important among EFV adverse reactions [68, 81, 82]. Most common disorders in the CNS following EFV use are confusion, dizziness, insomnia, somnolence, impaired concentration and abnormal dreaming, anxiety and depression [68, 81, 82]. Other common EFV adverse reactions are metabolic and nutrition disorders (increases in fasting triglycerides, total and lipoprotein cholesterol), hepatobiliary disorders (elevation of liver enzymes), skin and subcutaneous rashes [68].

1.5.3 Pharmacogenetics of efavirenz

Pharmacogenetics refers to the study of inter-individual genetic variation that gives rise to differing response (both safety and efficacy) to drugs [1]. Indeed, host genetic polymorphisms in drug metabolizing enzymes influence drug clearance and response [83].

1.5.3.1 Role of CYP enzymes in the metabolism of efavirenz

The suggested metabolic pathways of EFV are shown in figure 2. The drug is mainly metabolised by the CYP system through hydroxylation into two primary metabolites, namely 8-hydroxy-EFV (8-OH-EFV) and 7-hydroxy-EFV (7-OH-EFV) [31, 84]. The 8-OH-EFV metabolite is the main one [29-31, 84] accounting for 77% of the overall EFV metabolism [30]. It is principally formed by CYP2B6 [30, 31, 84], with small contribution of CYP1A2, CYP3A5, and CYP3A4 [31]. *In vitro*, CYP2A6 seems to contribute to the formation of 8-OH-EFV [30]. 7-OH-EFV is the second most important metabolite [29-31, 84] accounting for 23% and mainly formed by CYP2A6 [15] and to some extent by CYP2B6 [29]. In addition to the primary hydroxylation, about 17% of 8-OH-EFV formed was found *in vitro* to

subsequently oxidize mainly by CYP2B6 [31] and to a lesser extent by CYP2C9 [31, 41] to the secondary metabolite 8,14-dihydroxy-EFV.

In addition, EFV also undergoes direct conjugation by uridine diphosphate glucuronyltransferase (UGT) to form N-glucuronide [30, 15, 48]. Both 7-OH-EFV and 8-OH-EFV subsequently undergo urinary and biliary excretion after glucuronidation [28, 15]. Alternate pathways such as 7–hydroxylation by CYP2A6 and glucuronidation by UGT were reported to play an important role in individuals with reduced CYP2B6 activity [29].

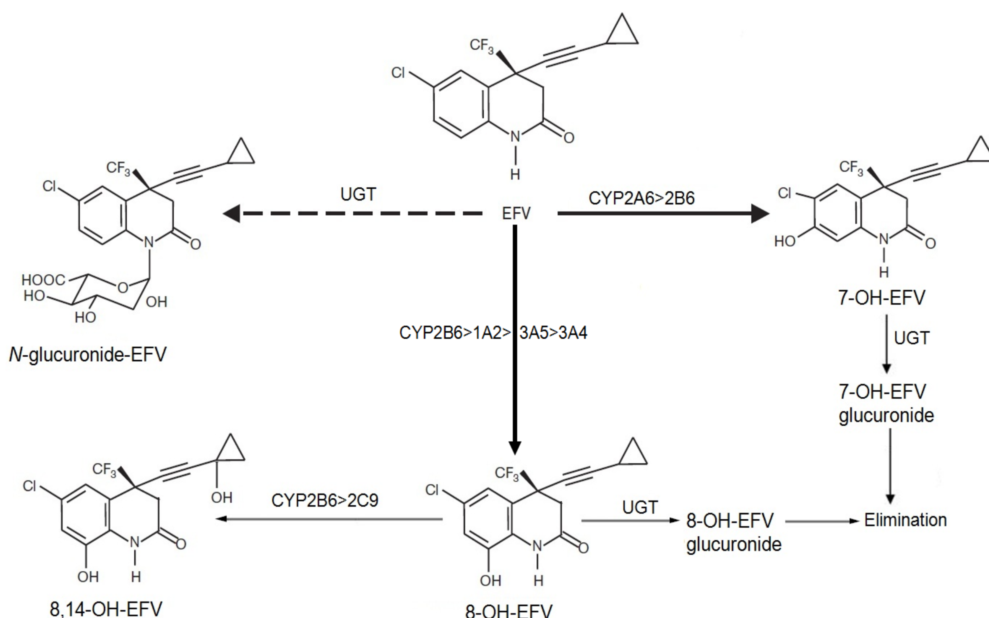

Figure 2. *Suggested metabolic pathways for efavirenz and its catalytic hepatic enzymes.*

1.5.3.2 Influence of genetic variants on efavirenz pharmacokinetics

Inter-individual variability in EFV plasma levels following the administration of the fixed adult dose of 600 mg of EFV was found to be up to 120% [11], whereas the reported coefficient of variation in oral clearance of EFV ranged from 40 to 55% [15-17]. Inter-individual variability in the PK of EFV was thought to be caused by a combination of multiple factors including biologic, exogenous and genetic factors; the latter being considered as the most important factor [20].

The gene that encodes for CYP2B6, the main metabolizing enzyme of EFV, is highly polymorphic. CYP2B6 is thus subject to large inter-individual variability in expression and activity [18, 32, 84, 85, 76]. CYP2B6 516G>T SNP is the most important; CYP2B6 516T/T genotype has been consistently associated with reduced enzyme activity and higher EFV exposure in different patient populations when compared with G/T or G/G genotypes [43, 70, 76, 86-89]. The frequency of the 516T/T variant was higher in African-Americans (20%) and European-Americans (3%) [90]. CYP2B6 983T>C SNP shares similar characteristics with CYP2B6 516G>T [41, 74, 76, 91], appears to be exclusively found in populations of African descent and was also found to be associated with lower enzyme activity and higher EFV levels [18, 32, 85]. CYP2B6 785A>G SNP was also reported to be associated with reduced enzyme activity and elevated EFV levels [41, 46]. Other CYP2B6 polymorphisms that have been identified have minimal impact on EFV metabolism [92]. Recently, CYP2A6 genetic polymorphisms [15, 29, 74], CYP3A4*1B and CYP3A4_rs4646437 [15] have also been found to influence EFV plasma concentrations or clearance. In this work, the genomics of an African population in five genes coding for proteins involved in the metabolism of commonly used anti-infective drugs, including antiretrovirals was investigated in **Paper II** and the role of ten SNPs of these genes on EFV plasma levels was studied in **Paper III**.

1.5.3.3 Pharmacogenetics of efavirenz-related neurotoxicity

EFV plasma levels above 4 μg/ml have been associated with increased risks of CNS toxicity [11, 41, 77-80]. 516G>T SNP of CYP2B6 is associated with increased plasma EFV exposure as described in 1.5.3.2. This is suggestive of association between CYP2B6 516T/T genotype and CNS toxicity. This has been demonstrated in different studies where individuals harboring CYP2B6 516T/T genotype were found to present with a higher incidence of CNS toxicity during the first week of therapy [76, 88, 89]. Since CYP2B6 785A>G and 983T>C SNPs are associated with increased EFV levels [9, 16, 49, [41, 46], individuals with these SNPs could develop EFV-related CNS toxicity [41, 46, 76, 88, 89].

1.6 Pharmacokinetic drug-drug interactions

Pharmacokinetic drug-drug interactions are consequences of the effect of one drug that alters the absorption, distribution, metabolism or excretion of another drug. This could lead to altered levels of exposure to the latter and possibly altered drug response [33, 36, 37]. Interactions at the level of hepatic excretion were reported to be the most important when TB and ARV drugs are co-administered [33].

1.6.1 Interactions between tuberculosis and HIV drugs

Drug-drug interactions between ARV and TB drugs are one of the major challenges that face the therapeutic management of HIV/TB co-infection [35, 93]. Many ARV and TB drugs share the same metabolic pathways through the CYP system. Many of them induce or inhibit the CYP system, and may affect the clearance of co-administered drugs [33, 34]. For example, EFV has been shown to induce hepatic CYP2B6 and CYP3A4 [14, 94, 95], hence to accelerate not only its own metabolism [33, 12-14], but also that of co-administered TB drugs that share these metabolic pathways [33, 34]. On the other hand, some recommended TB drugs were reported to affect ARV metabolizing enzymes [36, 37, 41, 35, 96]. Rifampicin was reported to be a potent inducer of CYP enzymes, such as CYP2B6, CYP1A2 and CYP3A4 enzymes [19, 36, 37, 41] and UGT-1A1 enzyme [33]. Isoniazid and pyrazinamide were reported to exhibit the inhibitory effects *in vitro* on EFV hydroxylation in CYP1A2, CYP2A6, CYP2B6 and CYP3A4 enzymes [97, 98]. In addition isoniazid has been shown to induce CYP3A enzyme [37]. Thus, the therapeutic management of HIV/TB co-infection may result in complex PK drug-drug interactions taking place collectively between HAART components and TB drugs, impacting on blood levels of both HIV and TB drugs. The clinical relevance of their consequences has been reported controversially and still needs validation [99-102].

1.6.2 Influence of tuberculosis therapy on efavirenz pharmacokinetics

Rifampicin induces the function and activity of CYP2B6, the main metabolizing enzyme for EFV [19, 41]. In primary human hepatocytes, the increase in CYP2B6 activity due to rifampicin varies widely from 2.5- to 13-fold [103-105]. *In vivo*, co-administration of EFV with RBT was reported to lead to accelerated EFV clearance with reduction in EFV exposure differing from one cohort to another [19, 83, 99-102, 106]. Reduction in EFV exposure was either modest [19, 83] or significant [99-102, 106] during co-treatment of HIV and TB. The variability in EFV plasma levels was found to be greater in the presence of RBT than without RBT, which is probably a manifestation of inherent differences in the inducibility of CYP2B6 variants in patients [99, 108]. Reports on whether EFV doses need to be modified to be compatible with RBT are still conflicting (see section 1.6.3). Nevertheless, TDM is thought to be a useful tool to monitor EFV levels and should prompt assessment of drug adherence after determining the drugs and doses to use [42]. In this work, the influence of RBT on EFV plasma levels in the population studied was investigated in **Paper III**.

Paradoxically, elevated EFV plasma levels in the presence of RBT were recently reported by Gengiah *et al.* (2012) [109] and Luetkemeyer *et al.* (2013) [110], both pointing out that the underlying mechanism for this observation remains unclear.

1.7 Dose adjustment for efavirenz during co-treatment

Two reasons may dictate dose adjustment for EFV during co-management of HIV and TB. First, EFV is associated with significant inter-individual variability in plasma levels [11, 16, 66] as described in section 1.5.3.2. Secondly, concurrent HIV and TB therapy is necessary for the management of both infections, but at the same time it is challenged by managing the consequences of interactions between EFV and rifampicin that may decrease EFV exposure [11, 16, 66]. The concern is that the fixed daily dose of EFV 600 mg for adults may consequently result in supra- or sub-therapeutic EFV levels [18, 32, 85, 76], leading to CNS toxicity and treatment failure followed by development of drug resistance, respectively [11, 16, 66, 67]. Therefore, the goal of adjusting the dose for EFV when co-administered with RBT is to avoid EFV plasma levels outside the therapeutic window, thus preventing related consequences. Tailoring EFV dose should be done at individual level and take into account the individual genotype. For example, carriers of CYP2B6 516 T/T genotype (slow metabolizers) are at risk of high EFV levels even in the presence of RBT [75, 111]. Thus, increase in EFV dose during RBT may not be necessary in slow metabolizers.

Several reports on increase in EFV clearance followed by reduction in its plasma exposure after concurrent administration of RBT and EFV has led some experts to recommend an increased EFV dose to 800 mg/day when co-administered with RBT [6, 35, 112]. Comparative studies were conducted and no clinical advantages of the increased dose have been found [100, 113]. A recent clinical study in South Africans comparing 600 mg to 800 mg of EFV during TB treatment concluded that dose escalation of EFV 600 mg to 800 mg is not required during concomitant TB therapy in South Africans, based on the fact that the proportion of virologically suppressed individuals at 48 weeks was similar in both groups [113]. These findings were consistent with those of another previous randomized study on EFV 600 mg vs 800 mg daily conducted in Thai patients where no difference in virological outcome between the two groups was observed [100]. In addition, another study that recruited predominantly native Africans reported that the increased dose of 800 mg was associated with a high frequency of CNS and hepatic toxicities associated with high EFV plasma levels [114]. In conclusion, there is no sufficient evidence to support an increase in EFV dose to 800 mg/day in HIV/TB co-infected patients; this was also concluded in a review of available literature conducted in 2008 by FDA [115].

1.8 Patient monitoring

1.8.1 Predictive genotyping

1.8.1.1 Definitions

The term "*allele*" indicates a different form of a gene at a particular locus on a chromosome. *Genotype* refers to the pair of alleles present at a single locus of an individual (genetic make-up). Thus, *genotyping* consists of determining the genetic constitution (the genotype) of an individual by examining the deoxyribonucleic acid (DNA) sequence. The genotype defines a given characteristic, condition or disease. Hence, genotyping helps in finding the origin and the pre-disposition of a disease, a characteristic or a condition. DNA sequence variations occurs when a single nucleotide in the genome sequence is altered and this refers to *single nucleotide polymorphisms* (SNP). Variations in the DNA sequences (SNPs) influence how humans develop diseases, respond to drugs, etc. Thus, knowledge of this variation in SNPs helps in understanding and treating human diseases [116].

A *predictive* genetic test is performed with the purpose to investigate whether an individual presents a pre-disposition to a disease or to developing drug toxicity [117].

1.8.1.2 Predictive genotyping for patients treated with efavirenz

In section 1.5.3.3, the association between CYP2B6 SNPs and increased EFV plasma levels and high incidence of CNS toxicity was discussed. Mallal *et al.* 2007 [46] indicated that genotyping could be a useful patient management tool for identifying individuals at risk of genetically influenced drug toxicity. Drugs of concern are those exhibiting a narrow therapeutic window and those metabolized by the polymorphic enzymes [46]. EFV itself exhibits a narrow therapeutic window that may put patients at risk of CNS toxicity [11] and is metabolized by a polymorphic enzyme (CYP2B6) potential to alter plasma levels [43, 70, 76, 86-89]. Accordingly, genotyping for CYP2B6 variants in patients receiving EFV has been suggested as a clinical tool that may help identifying patients at greatest risk of developing CNS toxicity and who may need adjusted EFV dose [43, 44]. FDA has approved some pharmacogenomics tests and published the guidance for genomic data submissions, which indicates a supportive approach to personalized drug therapy [118]. The evaluation of the usefulness of genotyping has been recommended and prognostic values were suggested as important indicators

to assess [41, 46]. The nature of prognostic values usually evaluated is described in section 3.3.4 and were determined in **Paper III**.

1.8.2 Therapeutic drug monitoring for efavirenz

Therapeutic drug monitoring (TDM) refers to measurement of drug plasma levels in the clinic with the aim of adjusting the dose or intervening with respect to adherence counselling or switching therapy [121]. As described in sections1.5.3.3 and 1.7, EFV exhibits high inter-individual variability in plasma levels that may pre-dispose patients to either CNS toxicity or treatment failure. Since there is no sufficient evidence to support an increase in dose of EFV to 800 mg/day in HIV/TB co-infected patients, TDM was suggested as a useful tool in order to closely monitor EFV plasma levels and adjust the dose when proven necessary [11, 42].

TDM remains a tool for therapy optimization for selected indications. However, implementing TDM requires the availability of validated and simple quantification assays with high selectivity, small sample volume requirements, and rapid turnaround time for the measurements. In addition, the assay needs to be cost-effective in order to guarantee its sustainable use in resource limited settings [45, 74]. In **Paper I**, a selective and rapid analytical method suitable for the quantification of EFV in less resourced settings was developed.

2 AIMS OF THE THESIS

This thesis aimed at contributing to the optimization of HIV therapy in the context of HIV and TB co-treatment by investigating the pharmacokinetic and pharmacogenetic aspects of EFV plasma levels in the absence and presence of rifampicin-based TB therapy (RBT).

Specific aims were to:

- develop and validate a rapid and selective analytical method for the quantification of EFV in human plasma containing first line medications used for the management of both HIV/AIDS and TB, and the major metabolites of EFV and rifampicin. (**Paper I**)

- obtain pharmacogenomic data in a Rwandan population in genes coding for proteins involved in the metabolism of commonly used anti-infectious drugs, including antiretrovirals. (**Paper II**)

- investigate the effects of 10 SNPs in five drug-metabolizing enzymes on EFV plasma levels and treatment response in the absence and presence of RBT. (**Paper III**)

- investigate the predictive factors of EFV plasma levels and the prognostic values of CYP2B6 genotyping in predicting supra-therapeutic EFV levels in the absence and presence of RBT. (**Paper III**)

- evaluate the effects of RBT on EFV plasma levels and on the clinical response. (**Paper III**)

- estimate the clearance of EFV in patients under both HIV and TB treatments by accounting for CYP2B6 genetic polymorphism and for DDIs caused by enzyme induction arising from both treatments. (**Paper IV**)

3 METHODS

3.1 HPLC-UV analytical method development

3.1.1 HPLC instruments

The high performance liquid chromatography (HPLC) instrument was an Agilent 1260 Infinity LC system consisting of a thermostated Hip-ALS autosampler, a binary pump, a degasser, and a thermostated column compartment. Analytes were monitored using an ultra-violet (UV) detector (SPD-10A, Shimadzu, Japan). Clarity Chromatography Data Station (CDS) (www.dataapex.com) was the software package used for system control and acquisition of EFV data during bioanalysis.

3.1.2 Development of the chromatographic system

Since EFV is a weak acid [122], reversed phase liquid chromatography was chosen using a C_{18} column. Ritonavir was selected as internal standard (IS) because the compound exhibits good UV absorbance and is well separated from EFV.

The aim was to develop and validate a rapid and selective method in the presence of HIV/AIDS and TB medications, but also using at the same time an extraction procedure and a mobile phase that are straightforward. The extraction procedure employed was based on protein precipitation with acetonitrile containing the IS followed by sample centrifugation. The mixture acetonitrile:water (80:20 % v/v) at a flow-rate of 0.3 ml/min was selected as mobile phase for initial experiments while working with stock solutions. The injection volume was 10 µL. The post run time was set to 1 minute to allow for equilibration prior to injection of the next sample. EFV and ritonavir (IS) were monitored with the UV detector set at 247 nm.

During method development, the proportions (% v/v) of the ingredients in the mobile phase were considered as adequate based on producing good peak resolution between the IS and peaks in blank plasma, and reproducibility of

the analyte response. Without trying the addition of new chromatographic modifiers, the proportions of the initial mobile phase (acetonitrile:water) were continuously modified, together with adjusting the pH (varying concentrations of formic acid) and the flow rate, until these criteria were met. The analysis time was progressively adjusted during the experiment and after obtaining a satisfactory mobile phase, chromatographic conditions were re-adjusted to shorten the analysis time as much as possible.

3.1.3 Method validation

Validation of the method was carried out in terms of linearity, lower limit of quantification, selectivity, accuracy, precision, recovery and stability (at room temperature, during freeze–thaw cycles and heat application), in accordance with FDA bioanalytical method validation guidelines [123].

3.1.4 Application of the method to plasma samples

Final chromatographic conditions were tested by analysing seven plasma samples collected from a representative patient co-infected with HIV/AIDS and TB, enrolled in the clinical study described in sections below. The patient was treated with EFV-based HAART (TDF+3TC+EFV) (EFV dosed 600 mg) concomitantly with TB combination therapy (RHZE). Blood samples were collected between 0 and 8 hours after oral administration of EFV-based HIV treatment (EFV dosed 600 mg) to a patient on TB drugs since six weeks. Plasma samples were heated for 60 min at 56°C to inactivate HIV virus.

3.2 Clinical study

3.2.1 Design outline

An open-label clinical study was conducted among adult HIV/AIDS patients co-infected with TB, receiving standard treatments, according to national treatment guidelines. Naive HIV patients initiating a standard TB treatment (HIV treatment deferred) were recruited. In addition, previous exposure to HIV treatment was allowed as in the previous design by ter Heine *et al.* (2008) [124]. The study involved scheduled weekly visits to the clinics for blood sampling and patient follow up. Participating patients were monitored before, during and after the intensive initiation phase of TB therapy on a weekly basis. Genotyping was performed for all participants. EFV plasma concentrations, viral load and CD4 cell counts were measured throughout the study. This study was conducted in Rwanda at four sites throughout the country. The recruitment of patients started on August 15th, 2009 and the follow up occasions were concluded in November 2010.

3.2.2 Study treatments

3.2.2.1 Nature of medications

Medications were administered in accordance with the Rwandan guidelines for the management of HIV/AIDS [125]. HAART combination was composed of one NNRTI and 2 NRTIs. The NNRTIs used by participating patients were EFV (76 patients) and NVP (4 patients), while the combination of 2 NRTIs was 3TC plus either AZT or abacavir or stavudine or TDF. TB treatment consisted of the following rifampicin-based fixed dose-combination regimen: $2(RHZE)_7/4(RH)_3$ (for the meaning of letters, numbers before the letters and subscripts, refer to section 1.3.2.2). Streptomycin which is normally indicated when one of these drugs is contraindicated [5] was given to only 8 patients of this cohort. Both treatments were administered in a pre-existing directly observed therapy (DOT) program.

3.2.2.2 Adherence to medications

Adherence to the treatments was assessed at each weekly visit by means of patient self-report. The patient received a diary and was explained how to use it, writing down the date and time of every dose intake. The diary was brought to the clinic at every visit, where the Investigator asked the patient adherence related questions to assess the accuracy of the diary records.

3.2.3 Inclusion and exclusion criteria

In order to be eligible for inclusion, a patient (male or female) had to fulfil all of the following criteria: adults (21-65 years of age), HIV antibody positive with newly diagnosed HIV (naive) or on an HIV regimen, diagnosed for TB, anti-tubercular naive, literacy in the native language (Kinyarwanda), English or French, and provision of signed informed consent. The presence of any of the following was used as exclusion criteria from entry into the study: any clinically significant laboratory value or any known concurrent disease likely to limit life expectancy to less than 6 months, concomitant medication taken less than 14 days prior to the study that is known to cause interactions with the study medication, participation in another clinical drug trial within 3 months prior to or during the study, and factors suggesting inability to comply with the protocol. Withdrawal criteria were voluntary decision of the patient to discontinue the participation in the study, decision of the Investigator to exclude the patient based on the patient health status or due to severe protocol violation. The reason for and date of subject removal from the study were documented in the Case Report Form (CRF).

3.2.4 Participating patients

One hundred forty seven adult HIV/AIDS patients co-infected with TB were screened. One hundred and five patients who met inclusion criteria were recruited. Twenty five patients were withdrawn from the study during the follow up period in accordance with the study protocol. Eighty patients who completed the study were genotyped. Of these, four patients had been treated with NVP-containing therapy and were excluded from EFV studies; the remaining 76 patients had received EFV-based therapy. Among them, were naive patients who initiated RBT, with HAART to be initiated after two to eight weeks if CD4 cell counts were below 500 cells/µL, in accordance with the local treatment guidelines (Group A; n=41). A second category was composed of patients who were diagnosed for TB after several months of HIV treatment (Group B; n=35) and had immediately initiated RBT with HAART continuing. Schematic representations of the flow of the recruitment process of the patients and the study design are depicted in figure 2 and figure 3, respectively.

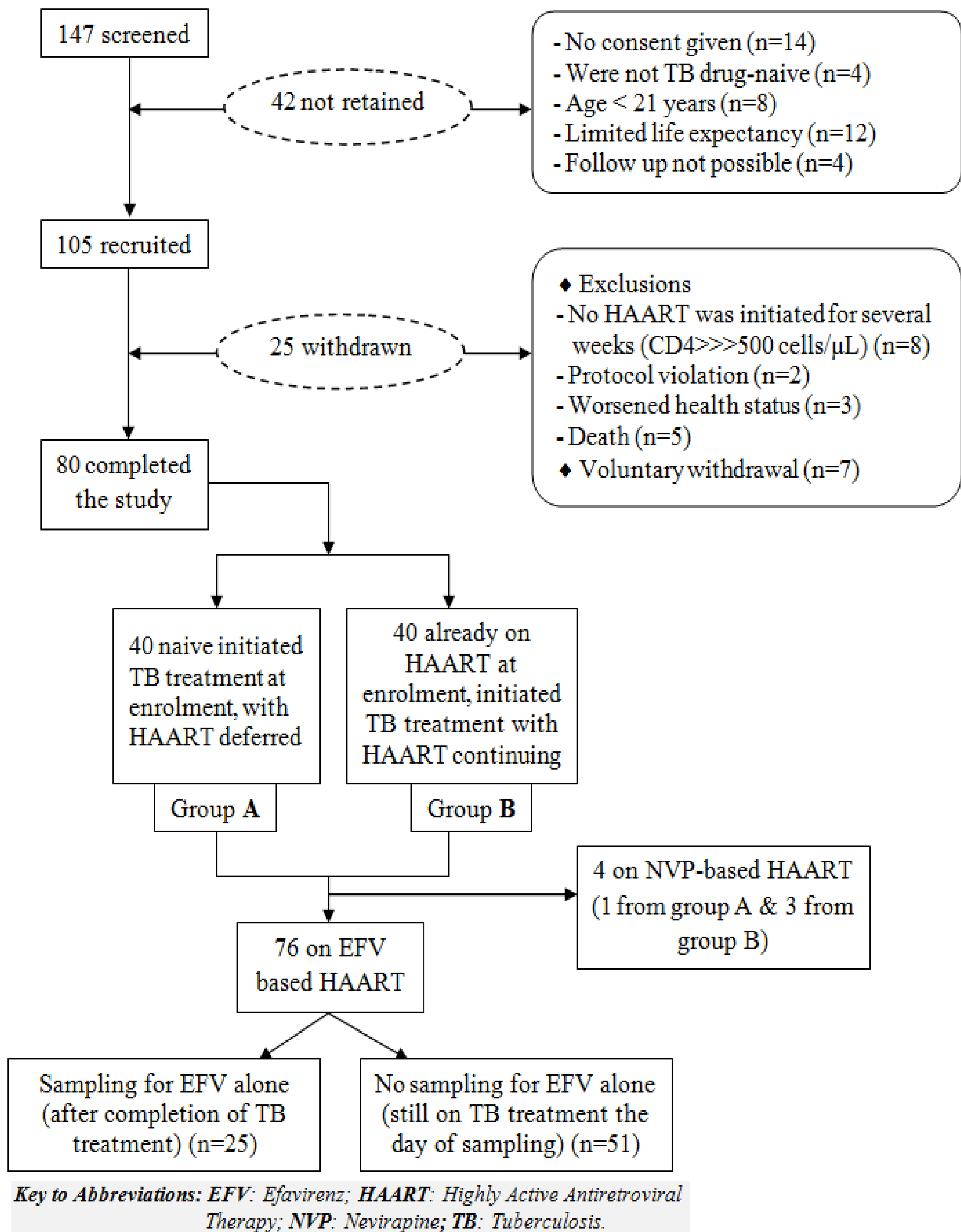

Figure 2*. A schematic representation of the flow of the recruitment process of the patients.*

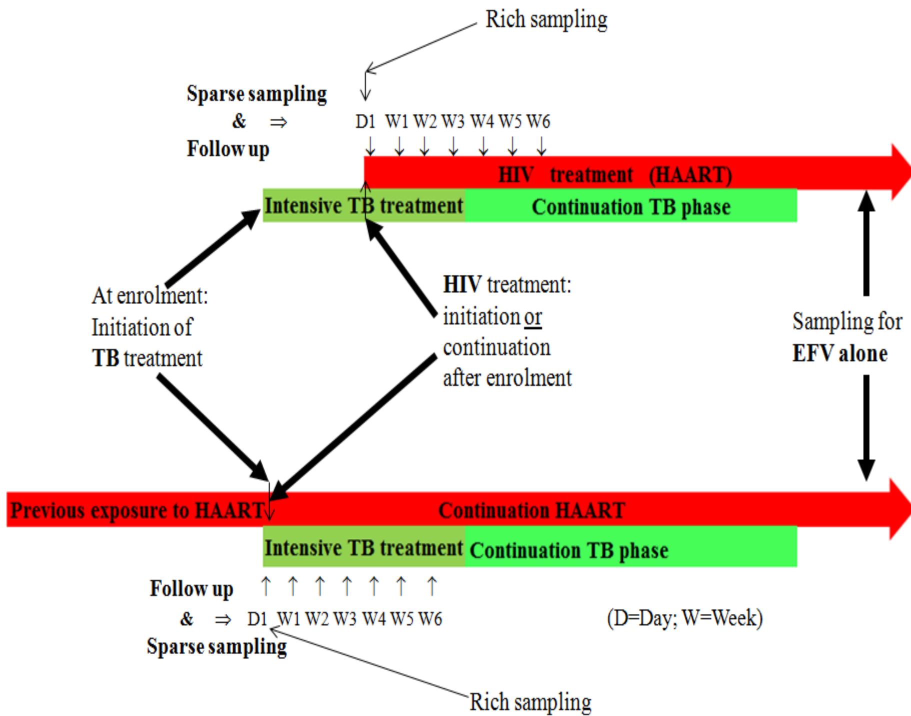

Figure 3*. Schematic illustration of the study design, including occasions for treatment initiation and sampling.*

3.2.5 Collection of blood samples

Patients had taken an EFV dose (600 mg) in the evening as per the current treatment guidelines. Samples were collected in the morning at arrival at the clinic, at the convenient time of the patient, approximately 13 hours after the previous evening EFV dose. Mid-dose sampling (sampling performed between doses, usually between 8 - 20 hours) was used because it is usually used in clinical studies of EFV disposition for patient convenience, given that EFV dose is invariably taken at bedtime. In addition, mid-dose levels of EFV have been reported to be highly associated with EFV area under the curve values when measured at steady state [74, 111].

3.2.5.1 Baseline visit

The day of initiation of TB treatment was the day of enrolment in the study. At enrolment after the obtainment of the patient´s written informed consent,

demographic variables (patient gender, age and body weight) were recorded, and blood samples collected for baseline clinical chemistry tests (alanine transaminase -ALAT-, aspartate transaminase -ASAT-, total bilirubin), and baseline CD4 cell counts and viral loads. An additional blood sample was collected at enrolment for genotyping.

3.2.5.2 Sparse sampling

After initiation of both HIV and TB treatments, patients were monitored on a weekly basis to collect blood samples for determination of EFV and treatment response data (CD4 cell counts and HIV-RNA copies). EFV was monitored over six weeks following the initiation of HAART. An additional sample was collected after at least two weeks of completion of TB treatment (n=25) (Figures 2 and 3).

3.2.5.3 Rich sampling

At initiation of the treatment after enrolment, rich sampling was performed at pre-dose and after 1, 2, 3, 4, 6 and 8 hours of initiation of HAART (Figures 2 and 3).

3.2.5.4 Blood sample handling

All blood samples were collected into ethylene diamine tetra-acetic acid (EDTA)-containing tubes (4 ml), stored first at −30°C in clinics for one week, and then transferred at −80°C until analysis. Samples for determination of EFV concentrations were centrifuged (10 minutes at 10500 g) before storage. On the day of analysis, plasma samples were heated for 60 min at 56°C to inactivate HIV virus, using the technique described elsewhere [126, 127].

3.2.5.5 Defining the data used

Blood samples collected in patients taking concomitantly HIV treatment and RBT were used for determination of EFV in the presence of TB drugs. EFV plasma concentrations in the absence of TB drugs (data for EFV alone) were quantified in blood samples collected after at least two weeks of completion of TB therapy. Data were considered as complete for each patient when one of the variables to use was not missing (plasma concentration or genotype, or HIV-RNA copies or CD4 cell counts, depending on the envisaged investigation). In each analysis, only patients with complete data were considered.

3.2.6 Laboratory analyses

3.2.6.1 Bioanalysis for the quantification of efavirenz

EFV was quantified in the Unit for Pharmacokinetics and Drug Metabolism, Department of Pharmacology of University of Gothenburg, using a developed and validated high-performance liquid chromatography method with ultraviolet detection as described in **Paper I**.

3.2.6.2 Genotyping

Patients were genotyped using a PCR-based technology with respect to 13 SNPs; CYP1A2 (-739T>G, -163C>A, -729C>T and 2159G>A); CYP2A6 (479T>A, 1436G>T, -48T>G and 1093G>A); CYP2B6 (516G>T, 785A>G and 983T>C); CYP3A4 (-392A>G) and CYP3A5 (6986A>G). The background, genotyping methodology and findings including the frequency of alleles, genotypes and haplotypes are described in **Paper II**.

3.2.6.3 CD4 cell counts and plasma HIV-RNA quantification

CD4 cell counts were determined using FACS Count System (Becton Dickinson, San Jose, CA, USA) at the Butare University Teaching Hospital Laboratory in Rwanda. Plasma HIV-RNA was quantified using the COBAS Amplicor, version 2.0 (Roche Diagnostics, Branchburg, NJ) at the Department of Infectious Diseases of University of Gothenburg. The lower detection limit for HIV-RNA level was 50 copies/mL.

3.2.7 Ethical considerations

The clinical study was conducted in accordance with the principles laid down in Helsinki Declaration of 2008, and the ICH (International Conference on Harmonization) guidance for Good Clinical Practice. Prior to inclusion in the study, patients were given full and adequate verbal and written information regarding the nature of the study and explained that participation in the study is voluntary. All information regarding participating patients (e.g. the patient's identity) was kept confidential during data analysis and reporting. Ethics approval was sought from and given by the National Ethics Committee of the Ministry of Health in Rwanda on December 9th, 2008 for 12 months, and was renewed on December 12th, 2009 for another 12 months.

3.2.8 Quality assurance

The source data was the Case Report Form (CRF). Results on lab-report printouts were immediately transferred into the CRF to avoid missing some data or later confusions of data. Hospital records were consulted whenever needed.

Adherence to the protocol and the accuracy of records in the CRFs were monitored on a regular basis, and facilities at study sites checked regularly. Patient visits to the study clinics were scheduled within each week in a way allowing the study coordinator to be present at each site the days of patient visits in order to allow providing the study personnel with support and advice.

3.3 Data analysis

3.3.1 Statistical analyses

The statistical tests were selected according to whether the data were normally distributed or not, whether the analysis involves two or more than two sample groups, and whether they were independent or not. The data to be investigated (EFV plasma levels, HIV-RNA copies, CD4 cell counts) were first tested for normality using the Shapiro-Wilk test. A p-value <0.05 was considered to be statistically significant. Predictive Analytics SoftWare (PASW) Statistics 18 (Chicago, IL) was used to perform statistical analyses.

3.3.1.1 Assessment of relationships between variables

The Spearman's correlation by rank was used to measure statistical dependence between dependent and independent variables studied. Investigating whether independent variables were associated with the dependant variable (EFV levels in our case) was performed using non-parametric tests, Kruskal-Wallis and Mann-Whitney for groups of three and two, respectively, if the data for the dependant variable were non-normally distributed and unrelated; and using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test if the data for the dependant variable were normally distributed. EFV data were log-transformed when necessary. For prediction analyses aiming at investigating whether various independent variables could predict the dependant variable, regression analysis was performed. Other specific non-parametric tests were used where appropriate (Pearson chi-square (χ^2) and Fisher's exact test).

In this thesis:

- Pearson χ^2 test was used **to perform Hardy–Weinberg equilibrium (HWE) analysis** by comparing expected and observed genotype frequencies for SNPs during genotyping (**Paper II**).
- Fisher's exact test was performed **to investigate the differences between populations** with regards to allele and genotype distributions (**Paper II**).
- The Spearman's correlation by rank was used to assess **correlations between EFV plasma levels and patient baseline characteristics** (**Paper III**).
- **The association of genotype groups with EFV levels** was performed using Kruskal-Wallis test for groups of three, followed by Mann-Whitney test for pairwise comparisons. Mann-Whitney test was also used where only two genotype groups were present (**Paper III**).

- One-way ANOVA followed by Tukey's multiple comparison test was used to **compare $\log_{10}$ EFV plasma levels over the six weeks of follow up** (**Paper III**).
- Linear regression analysis was used to **determine the predicting effects** of patients' baseline characteristics and genotypic variants and their contribution to overall variability in EFV plasma levels (**Paper III**).

In performing the regression analysis, genotypes were dichotomized according to the dominant genetic model (wild-type = 0; heterozygote/homozygote variants = 1) [129], as shown under Table 4. Univariate analyses of the effects of patients' baseline characteristics and CYP enzyme genotypes on $\log_{10}$ EFV levels (dependant variable) were assessed by linear regression. The percentage change in EFV plasma levels, with the 95% confidence interval (CI), was calculated as 100 x regression coefficient. Multivariate regression analysis, using a stepwise backward elimination method, was conducted by including variables that achieved statistical significance in the univariate analyses. To avoid confounding effects on the relation between selected genotypes and the end-points, covariates that had a p-value of less than 0.20 in univariate analyses were included in the multivariate analyses. Plasma levels of EFV were log-transformed in order to achieve data normality for the linear regression analysis and equal variance.

3.3.1.2 Comparison of related samples

Two related sample groups were compared using Wilcoxon signed-ranks test if the data were non-normally distributed, and paired sample t-test if the data were normally distributed. In order to assess the **effect of RBT on EFV plasma levels and clinical response** (**Paper III**), within-patient comparisons were conducted using:

- Wilcoxon signed-ranks test to compare
    - EFV levels with and without RBT, and
    - HIV-RNA copies at baseline and after six weeks of HAART;
- Paired sample t-test to compare CD4 cell counts at baseline and after six weeks of HAART.

3.3.2 Linkage disequilibrium analysis

When genotyping, the extent to which association mapping can be used in a population needs to be determined. Linkage disequilibrium (LD) measures the degree to which SNP alleles at two or more sites in the same region of the genome are associated. In a population, two linked SNP alleles occur at a frequency higher or lower than expected on the basis of the gene frequencies of the individual genes. If SNP alleles at the two sites are not randomly associated, then there will be a deviation (D') in the expected frequencies. The parameter D' is the coefficient of LD. D' varies between 0 and 1 and allows assessing the extent of linkage disequilibrium relative to the maximum possible value it can take. If $D'=0$, then the two sites are in linkage equilibrium, meaning that the genotype present at one site is independent of the genotype at a second site. If $D'\neq 0$, the two sites are in LD indicating that genotypes at the two sites are not independent of another (dependency). LD=1 implies that the markers (genotypes) provide exactly the same information. When LD=1, the two sites are said to be in complete or tight LD [128, 129]. LD analysis was performed using SHEsis software platform in order to evaluate the degree of association between SNPs which were selected for genotyping the population studied (**Paper II**).

3.3.3 Hardy-Weinberg equilibrium analysis

When carrying out genotyping, the quality of the assay needs to be validated. Hardy-Weinberg equilibrium is a common criterion with which to assess the quality of a genotyping assay [130].The Hardy–Weinberg principle states that both allele and genotype frequencies in a population remain constant which mean that they are in equilibrium, referred to as Hardy-Weinberg equilibrium (HWE). Under HWE, alleles segregate randomly in the population, allowing expected genotype frequencies to be calculated from allele frequencies. A comparison of the expected and observed genotype frequencies provides a test of HWE; deviation from HWE is generally tested for by using the Pearson chi-square (χ^2). Thus, the degree of deviation from HWE indicates the quality of genotyping. A p-value below a pre-determined threshold indicates a deviation from HWE [129]. The deviation from HWE was tested for all the SNPs when genotyping the population studied, using Pearson χ^2 test (**Paper II**).

3.3.4 Estimation of prognostic values

In clinical practice, prognostic values are usually evaluated before introducing a diagnostic technology. Methods for evaluating a disease or a characteristic are compared against a gold standard, which is the best or the preferred method of diagnosing a particular disease or a characteristic [119]. Usually evaluated prognostic values are the clinical sensitivity, specificity, negative (NPV) and positive (PPV) predictive values.

The PPV is the proportion of patients with positive test result who actually have the disease or the characteristic, whereas the NPV is the proportion of patients with a negative test result who do not have the disease or the characteristic [119, 120]. The PPV indicates the chance of having the disease or the characteristic among those that test positive, while the NPV indicate the chance of not having the disease or the characteristic among those that test negative [119, 120]. The clinical sensitivity (proportion of patients that are known to have the disease/characteristic and are tested positive for it) and the clinical specificity (proportion of patients that are known not to have the disease/characteristic and are tested negative for it). Using the methods adapted from Sackett *et al*., (1991) [119], these prognostic values were determined in **Paper III** to assess the validity of CYP2B6 genotyping in predicting EFV plasma levels above the upper limit of the therapeutic range (4 μg/ml), which was the characteristic to be evaluated.

3.3.5 Pharmacokinetic modelling

The population PK analysis was performed in NONMEM computer program, Version 7.1.2 and goodness-of-fit plots were constructed with Xpose4 library (version 4.3.2, 2010) run in R program (version 2.13.0, 2011). A one-compartment model with first-order absorption and elimination was used to describe the data, using the NONMEM subroutine ADVAN5/TRANS1 and first order conditional estimation (FOCE) method. Inter-individual variability (ω^2) was estimated for all the structural parameters of the model using an exponential error model, assuming that the individual value for a structural parameter was log-normally distributed. Residual variability (σ^2) was estimated as an additative residual error on log-transformed data corresponding to an approximately exponential residual model.

Population PK parameters including CL/F, V/F and Ka with a lag time (alag) were first estimated in the basic PK model. CL/F values were estimated at baseline and at steady state to take into account the potential for the previous treatment to induce EFV metabolism. CL/F at baseline corresponded to the CL/F of the first day of initiation of HAART for group A patients (naive) after some weeks of RBT, and the CL/F at the day of enrolment in the study after several months of EFV-based HAART in group B patients. CL/F at steady state corresponded to the CL/F estimated for the two patient groups over six weeks with patients being under concomitant HIV and TB treatments. Different CL/F for each of the three CYP2B6 516G>T genotypes were estimated in the two groups in order to quantify the impact of genetic polymorphism in that gene on the metabolism of EFV. The CL/F at baseline and at steady state was modelled according to the following equations:
At baseline (time = 0): $CL/Fi = \theta * \exp(\eta i)$
At steady-state (time>0): $CL/Fi = \theta * (1 + Fract) * \exp(\eta i)$
Where CL/F_i is the apparent clearance of the ith individual; θ is the typical value of clearance in the population; η_i is the inter-individual variability in clearance; and Fract represents the fractional increase in baseline clearance.

The final model selection was based on standard goodness-of-fit plots, agreement in the observed and individual and population predicted data, lack of trend or pattern in scatter plots of weighted residuals versus predicted concentrations or conditional weighted residuals versus time, the precision of the parameter estimates expressed as relative standard error (%RSE), based upon the known PK of EFV, agreement between the observed data and the 95% confidence interval (CI) of the data and comparison of NONMEM objective function values (OFV; it is expressed for convenience as minus twice the log of the likelihood, and is a single number that provides an overall summary of how closely the model predictions match the data; the lower the OFV, the best the fit). A reduction in OFV of at least 3.84 was considered significant ($p = 0.05$). Model validation was performed using bootstrap with 200 re-sampled datasets, and a simulation-based visual predictive check (VPC) to evaluate whether model predictions adequately described the observed data [131] (**Paper IV**).

4 RESULTS AND DISCUSSION

4.1 Rapid and selective HPLC method for efavirenz quantification

Chromatographic conditions

Chromatographic separation was performed using a C_{18} (100 × 4.6 mm, 2.6 μm particle size) analytical column equipped with a short guard column (Kinetex®, Phenomenex®, Torrance CA, USA). A reversed phase isocratic elution of the analytes was achieved using a mobile phase consisting of 25% solvent A (Milli-Q water; pH adjusted at 3.2 using 0.1% formic acid) and 75% solvent B (acetonitrile), pumped at a flow rate of 0.3 ml/min at ambient temperature. The analysis time was 6 min. The lower limit of quantification was set to 0.06 μg/mL with deviation from the nominal concentrations being <20%, in accordance with FDA bioanalytical method validation guidelines [123].

Validation parameters

- Linearity: the response was linear with a correlation coefficient of 0.9997, a slope of 0.189 and an intercept of 0.003. The relative standard deviation for the slope was 5.474%.
- Accuracy and precision: the accuracy ranged between 98 and 115% (intraday) and between 99 and 117% (interday). The precision ranged from 1.670 to 4.087% (intraday) and from 3.447 to 13.347% (interday).
- Recovery and stability: the recovery ranged from 98 to 132%, and the stability between 99 and 123%.
- Selectivity: the selectivity was demonstrated by the analysis of drugs used for the management of HIV/AIDS and TB, and the major metabolites of EFV and rifampicin. Obtained retention times (Rt) are given in table 1.

Table 1. *Retention times of drugs commonly used in the Treatment of HIV/AIDS and TB, and metabolites of EFV and rifampicin. Adapted from Paper I.*

	Drug	Retention time* (min)
Antiretrovirals	Abacavir	3.3
	Lamivudine	3.2
	Nevirapine	3.6
	Stavudine	3.1
	Tenofovir	3.1
	Zidovudine	3.4
TB drugs	Ethambutol[c]	-
	Izoniazid	3.1
	Pyrazinamide	3.2
	Rifampicin	3.4
	Streptomycin[c]	-
Adjuvant drugs	Pyridoxin	3.0
	Sulfamethoxazole[c]	-
	Trimethoprim	3.0
Metabolites	8-hydroxy-Efavirenz	3.3
	Deacetyl-Rifampicin	3.5

Retention times of drugs listed in this table were compared to those of EFV (5.6 minutes) and ritonavir (IS) (4.6 minutes).
[c]*Not detected by UV*

Ethambutol, streptomycin and sulfamethoxazole were not detected by the current method due to their lack of absorption in UV. All other drugs were well separated from the analytes (EFV; Rt = 5.6 min) and IS (Rt = 4.6 min). This is also illustrated by a representative chromatogram from a plasma sample collected 3 hours after oral administration of EFV-based treatment from a patient on concomitant treatment of HIV and TB (Figure 4). The data demonstrated no interference from co-medications with the peak of EFV and IS, respectively.

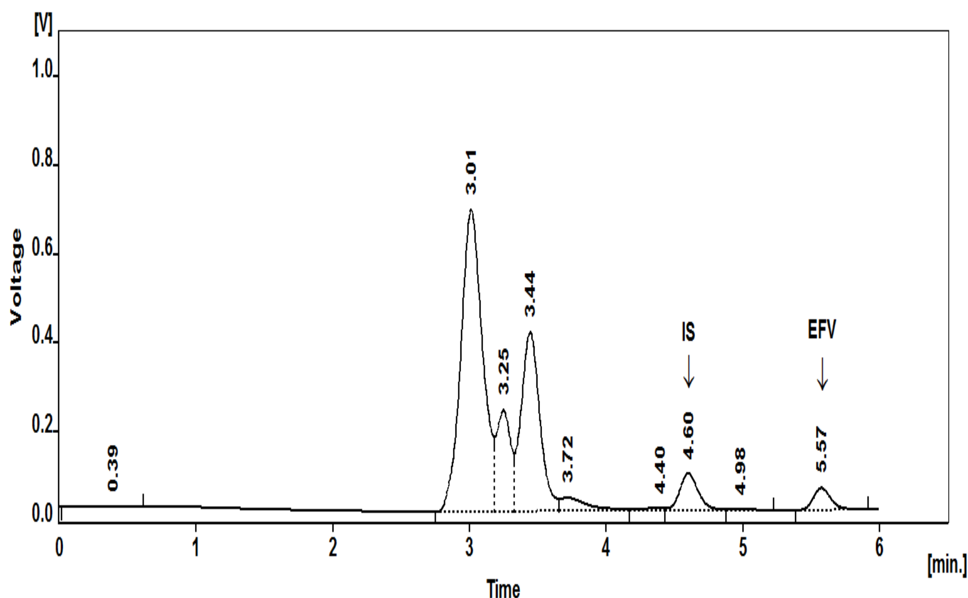

Figure 4*. A representative chromatogram from a plasma sample collected 3 hours after oral administration of efavirenz-based treatment (EFV dosed 600 mg) from a patient on concomitant treatment of HIV and TB. From Paper I.*

Application of the method

The method was applied by analyzing plasma samples containing HIV and TB drugs as described in the section 3.1.4. A representative chromatogram from a plasma sample collected 3 hours after oral administration of EFV-based treatment is shown in figure 4.

The reported HPLC-UV method quantitated EFV in relatively short time (6 minutes) and used a mobile phase and plasma extraction procedure that were straightforward. The extraction procedure produced clear supernatants from human plasma with no interference from endogenous compounds and both IS and analyte completely resolved to baseline. HPLC using UV is, due to lower cost and greater robustness, still widely used for relatively less complex bioanalytical assays [132]. Several analytical methods have previously been reported for the determination of EFV in human plasma using HPLC-UV [133-135]. However, some HPLC-UV methods use procedures resulting in long analysis time [132, 134, 136, 137], hence limiting the scopes of their application.

Furthermore, the selectivity of this method was tested in the presence of a wide range of medications used for the management of HIV/AIDS and TB, specifically all first line antiretrovirals and TB drugs, as well as other medications used as adjuvant to HIV/AIDS and TB therapies, and the major metabolite of EFV (8-hydroxyefavirenz) and rifampicin (deacetyl-rifampicin). The chromatography yielded a well-resolved EFV peak without interference from co-medications with the detection process of the analyte. Indeed, published methods have studied the interference of only some TB drugs, without testing interference from other medications used as adjuvant. No data demonstrating a lack of interference of EFV and rifampicin metabolites with the parent drugs when using HPLC-UV are available, while metabolites may interfere when analyzing parent drugs [138]. The reported method was characterized by a high selectivity allowing its applicability in the quantification of EFV from HIV/TB patients.

In comparison to several other published methods for the quantification of EFV, the reported HPLC method presents the advantage of being simultaneously rapid, selective in the presence of a wide range of HIV and TB medications, and straightforward by using a mobile phase and extraction procedure that are simple. Therefore, this HPLC method is suitable not only for PK studies, but also for TDM in patients with HIV/AIDS alone or co-infected with TB at sites with less resourced settings.

4.2 Pharmacogenomics in a Rwandan population

Allele and genotype frequencies showed genetic variation in a Rwandan population

Genetic variation was observed in 11 of the 13 SNPs with two being monomorphic (CYP1A2 -729C>T and CYP2A6 479T>A). These two monomorphic SNPs presented with a single allele each (C for CYP1A2 -729C>T and T for CYP2A6 479T>A); consequently only one genotype group for these SNPs could be identified (Table 2). The minor allele frequencies (MAF) were identified for 10 SNPs, such as CYP1A2 -739G, CYP1A2 2159A, CYP2A6 1436T, CYP2A6 1093A, CYP2A6 -48G, CYP2B6 516T, CYP2B6 785G, CYP2B6 983C, CYP3A4 -392A and CYP3A5 6986G (Table 2). Positions -739 and -163 in CYP1A2 gene and positions 516 and 785 in CYP2B6 gene were in complete LD (coefficient of LD = 1.000). All the SNPs conformed to HWE. Haplotypes were also inferred; results are shown in table 3.

Differences to other African populations

A comparison of allele and genotype frequency between the Rwandan population studied and other African populations revealed significant differences in the distribution of allelic variants when the Rwandan subjects were compared to Cameroonian and South African populations with respect to CYP2A6 1093G>A SNP (P=0.0033 and 0.019, respectively) and CYP3A4 -392A>G SNP (P=0.0001 and 0.0084, respectively). There were differences in the distribution of the CYP1A2 -163C>A SNP between the Rwandan subjects and the South African population (P=0.0001), but not with the Cameroonian population (P=0.265). The distribution of CYP3A5 6986A>G SNP differed between the Rwandan subjects and the Cameroonian population (P=0.017) but not with the South African population (P=0.097). The CYP2B6 516G>T polymorphism did not show significant differences (P>0.05) between the Rwandans, Cameroonians and South Africans in the distribution of the 516T variants. This is consistent with earlier results from Svärd *et al.* [21] and Kwara *et al.* [74, 139] who reported the high prevalence of the same allelic variants in other African populations.

***Table 2**. Allele and genotype frequencies in Rwandan subjects for selected SNPs. From Paper II.*

Gene	Reference SNP	SNP	Allele	Frequency (N)	95% CI	Genotype	Frequency (N)	95% CI
CYP1A2	rs2069526	T-739G	T	0.893 (125)	0.831-0.934	T/T	0.800 (56)	0.691-0.877
			G	0.107 (15)	0.066-0.169	T/G	0.186 (13)	0.112-0.293
						G/G	0.014 (1)	0.003-0.076
	rs12720461	C-729T	C	1.000 (140)	0.974-1.000	C/C	1.000 (70)	0.949-1.000
			T	0.000 (0)	0.000-0.026	C/T	0.000 (0)	0.000-0.051
						T/T	0.000 (0)	0.000-0.051
	rs762551	C-163A	C	0.559 (76)	0.475-0.640	C/C	0.324 (22)	0.224-0.442
			A	0.441 (60)	0.360-0.525	C/A	0.471 (32)	0.356-0.588
						A/A	0.025 (14)	0.127-0.317
	rs2472304	G2159A	G	0.887 (126)	0.825-0.929	G/G	0.803 (57)	0.695-0.878
			A	0.113 (16)	0.071-0.175	G/A	0.169 (12)	0.010-0.273
						A/A	0.028 (2)	0.009-0.097
CYP2A6	rs5031017	G1436T	G	0.816 (111)	0.743-0.872	G/G	0.691 (47)	0.573-0.788
			T	0.184 (25)	0.128-0.257	G/T	0.250 (17)	0.163-0.365
						T/T	0.059 (4)	0.024-0.142
	rs28399454	G1093A	G	0.979 (137)	0.939-0.992	G/G	0.957 (67)	0.881-0.984
			A	0.021 (3)	0.008-0.061	G/A	0.043 (3)	0.016-0.119
						A/A	0.000 (0)	0.000-0.051
	rs1801272	T479A	T	1.000 (136)	0.973-1.000	T/T	1.000 (68)	0.948-1.000
			A	0.000 (0)	0.000-0.027	T/A	0.000 (0)	0.000-0.052
						A/A	0.000 (0)	0.000-0.052
	rs28399433	T-48G	T	0.899 (124)	0.837-0.938	T/T	0.811 (56)	0.703-0.886
			G	0.101 (14)	0.062-0.163	T/G	0.173 (12)	0.103-0.280
						G/G	0.014 (1)	0.003-0.077
CYP2B6	rs3745274	G516T	G	0.669 (91)	0.586-0.743	G/G	0.439 (29)	0.315-0.540
			T	0.331 (45)	0.257-0.414	G/T	0.439 (29)	0.315-0.540
						T/T	0.121 (8)	0.061-0.214
	rs2279343	A785G	A	0.667 (92)	0.584-0.740	A/A	0.449 (31)	0.337-0.567
			G	0.333 (46)	0.260-0.416	A/G	0.435 (30)	0.324-0.553
						G/G	0.116 (8)	0.061-0.213
	rs28399499	T983C	T	0.919 (125)	0.861-0.954	T/T	0.838 (57)	0.733-0.907
			C	0.081 (11)	0.046-0.139	T/C	0.162 (11)	0.093-0.267
						C/C	0.000 (0)	0.000-0.052
CYP3A4	rs2740574	A-392G	G	0.529 (74)	0.446-0.609	G/G	0.272 (19)	0.181-0.386
			A	0.471 (66)	0.391-0.554	G/A	0.514 (36)	0.399-0.628
						A/A	0.214 (15)	0.135-0.324
CYP3A5	rs776746	A6986G	A	0.703 (97)	0.622-0.773	A/A	0.478 (33)	0.364-0.594
			G	0.297 (41)	0.227-0.378	A/G	0.449 (31)	0.337-0.567
						G/G	0.073 (5)	0.032-0.159

***Table 3**. Haplotype frequencies in Rwandan subjects for selected SNPs. From Paper II.*

SNPs	**Haplotype**	**Frequency (N)**
CYP1A2 rs762551-rs12720461-rs2069526-rs2472304	A-C-G-G	0.099 (13)
	A-C-T-A	0.114 (15)
	A-C-T-G	0.220 (29)
	C-C-T-G	0.568 (75)
CYP2A6 rs28399433-rs1801272-rs28399454-rs5031017	G-T-G-G	0.092 (12)
	T-T-G-G	0.703 (89)
	T-T-G-T	0.174 (22)
	T-T-A-G	0.020 (3)
CYP2B6 rs3745274-rs2279343-rs28399499	G-A-C	0.082 (11)
	G-A-T	0.605 (81)
	T-G-T	0.313 (42)

The existence of single genotype groups for each of the two monomorphic SNPs implies same characteristics in the phenotype for all subjects carrying these genotypes in the cohort studied. The MAF observed showed alleles that appear less frequently than the others in this population. The SNPs in both CYP1A2 and CYP2B6 genes being in complete LD indicated a total dependency between these SNPs, as previously reported by Svärd *et al*. [21] and Swart *et al*. [140]. The observed genetic variation in the Rwandan subjects was also reported for other populations [140, 141]. Genetic variation has been shown to influence both efficacy and safety of drugs, which is attributed to polymorphisms in the CYP gene family that confer rapid versus slow metabolizers of drugs [142]. The clinical relevance of such genetic variations observed in the population studied needs to be explored further.

In summary, the presented genomic information on a Rwandan population, especially in terms of genetic variation, indicates a need for further genomic and genotype-phenotype investigations to explore the clinical influence of polymorphisms in the CYP system and its relevance in the population studied. Differences observed between the Rwandans and other African populations are congruent with genetic diversity earlier reported for Africans. This suggests genomic screening for all African populations in order to be able to predict responses to drugs based on specific rather than general genetic data.

4.3 Correlation between population characteristics and efavirenz plasma levels

Highlights for population characteristics

The gender ratio (male:female) was 1.05 (39:37) and the mean (range) age was 38.0 (21-57) years. Other baseline characteristics of the participating patients were described in papers III and IV.

Highlights for EFV plasma levels

The coefficients of variation within and between subject plasma levels were 28% and 88%, respectively. 32% of the patients had plasma EFV levels outside the expected therapeutic range (1-4 µg/ml) with 14% of these having plasma levels above 4 µg/ml and 18% having plasma levels below 1µg/ml, whereas 68% presented with plasma EFV levels within the expected therapeutic range.

No correlation between population characteristics and EFV levels

There were no statistically significant associations when baseline characteristics (patient gender, age, body weight, ALAT, ASAT, total bilirubin and baseline CD4 cell counts and viral loads) were compared to EFV plasma levels, whether when with or without concomitant RBT.

4.4 Effects of genetic polymorphisms on efavirenz plasma levels

CYP2B6 516T/T genotype associated with high EFV levels

With respect to CYP2B6 516G>T, EFV plasma levels significantly differed by genotype in the absence (P=0.006) and presence (P=0.0005) of RBT. Pairwise comparisons revealed that CYP2B6 T/T genotype was associated with high EFV plasma levels compared to G/G (P=0.009) and G/T (P=0.004) genotypes when EFV-based therapy was given alone (Figure 7A), and when it was given concurrently with TB treatment (P=0.00002 and 0.005, respectively; Figure 7B). When comparing follow up occasions during concomitant HIV and TB treatment, a significant difference in EFV plasma levels was observed between CYP2B6 516T/T genotype and the other two genotypes (G/T and G/G) (P=0.0001) over the six weeks of follow-up (Figure 8C). Further, the analysis showed a statistically significant difference between CYP2B6 516G/T and G/G genotypes when patients were treated with EFV-based therapy combined with RBT (P=0.037; Figure 7B), but not when it was given alone (P=0.073; Figure 7A).

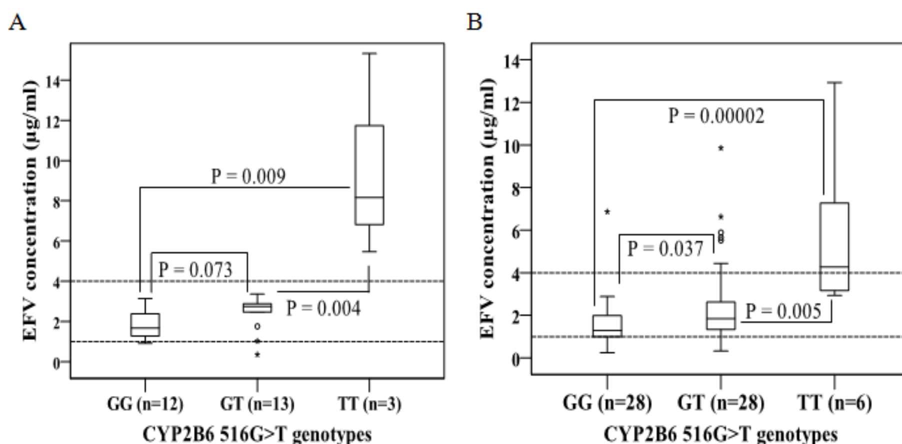

Figure 7. *Effects of CYP2B6 516G>T genotypes on EFV plasma levels when patients were treated with EFV-based therapy alone (A) and when it was combined with TB treatment (B). Boxes represent interquartile ranges; horizontal lines within boxes, median values. o and * indicate outlier and extreme values, respectively. Dotted lines represent the therapeutic window for EFV (1 – 4 µg/ml). From Paper III.*

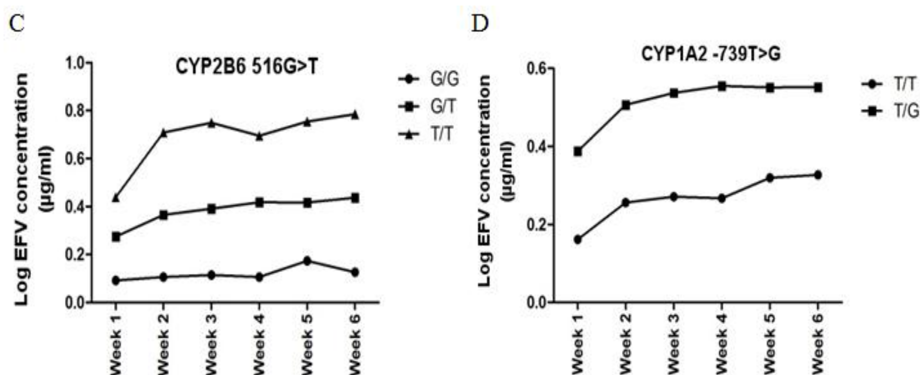

Figure 8. *Comparisons over the six weeks of follow up between CYP2B6 516G>T genotypes (C) and between CYP1A2 -739T>G genotypes. Over six weeks, CYP2B6 516T/T genotype was significantly different in EFV levels compared to G/T and G/G genotypes (C) (P=0.0001), and the difference between CYP1A2 -739T/G and T/T genotypes (D) was also significant (P=0.0001). From Paper III.*

Similar observations have been made in other cohorts, where high EFV levels in carriers of CYP 2B6 516T/T were observed, with examples among Thai [143, 144], Caucasian [145] and South African patients [77]. Since, EFV levels above the therapeutic range were found to cause CNS toxicity in HIV patients [11, 77-80], carriers of the CYP 2B6 516T/T genotype in the population studied treated with EFV-based HAART are at high risk of developing the same side effects. The same applies for carriers of CYP2B6 785G/G genotype due to the fact that 516G>T and 785A>G SNPs in CYP2B6 gene were in complete LD as discussed in section 4.2.

CYP2B6 983T/T genotype associated with higher EFV levels

There was no statistically significant difference between CYP2B6 983T>C genotypes (P > 0.05) when compared for the association with EFV plasma levels; over the six weeks however, the CYP2B6 983T/T genotype was associated with significantly higher EFV levels (P=0.0035) compared to T/C genotype.

According to the present data, CYP2B6 983T allelic variant had a higher frequency (0.919; Table 2) in the Rwandan subjects. This was consistent with reports stating that CYP2B6 983T>C SNP is found not only in non-African

populations [147, 148], but also in African populations or in populations of African descent [74, 146]. Since CYP2B6 983T>C SNP shares similar characteristics with CYP2B6 516G>T polymorphisms [74, 91] and since CYP2B6 516G>T polymorphisms has been associated with high EFV levels in the cohort studied as discussed above, it is possible that CYP2B6 983T>C SNP exhibits in some individuals the same trends in the phenotype as CYP2B6 516G>T. In other studies, CYP2B6 983T>C SNP has shown to be associated with elevated EFV plasma levels [46, 120]

Difference between CYP1A2 -739T/G and T/T genotypes only in the presence of RBT

There was a significant difference between CYP1A2 -739T/G and T/T genotypes with respect to EFV plasma levels when patients were treated with EFV-based therapy combined with RBT (P=0.027; Figure 9F), but not when EFV-based therapy was given alone (P=0.145; Figure 9E). The difference in EFV levels between CYP1A2 -739T/G and T/T genotypes was also significant over the six weeks of follow up (P=0.0001; Figure 8D).

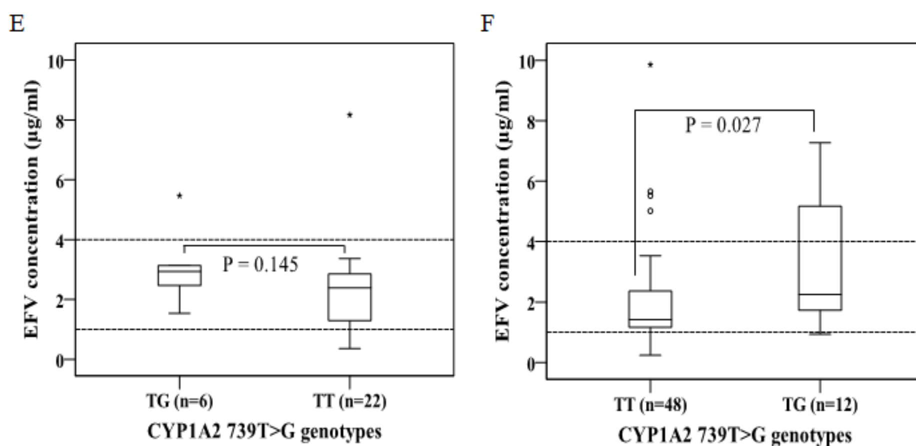

Figure 9. *Effects of CYP1A2 739T>G genotypes on EFV plasma levels when patients were treated with EFV-based therapy alone (E) and when it was combined with TB treatment (F). Boxes represent interquartile ranges; horizontal lines within boxes, median values. o and * indicate outlier and extreme values, respectively. Dotted lines represent the therapeutic window for EFV (1–4 µg/ml). From Paper III.*

Given the scarceness of data concerning relationships between EFV exposure and SNPs in CYP enzymes other than CYP2B6, the present findings on CYP1A2 could not be compared with the data from other cohorts. CYP1A2

and CYP2A6 are part of the accessory pathways for EFV metabolism [30, 31, 84]. CYP1A2 participates in 8-hydroxylation of EFV together with other CYP enzymes with the rank order of CYP2B6 > CYP1A2 > CYP3A5 > CYP3A4 (Figure 2) [29]. CYP2A6 gets involved in the second metabolic accessory pathway for 7-hydroxylation of EFV with the rank order of CYP2A6 > CYP2B6 (Figure 2) [30, 31, 84]. di Iulio *et al.* (2009) [29] reported a contribution of CYP2A6 alleles among individuals characterized as CYP2B6 slow metabolizers. Therefore, the present results indicating that the difference in association with EFV plasma levels between CYP1A2 -739T/G and T/T genotypes becomes significant during concomitant treatment with RBT, hypothetically suggest that 8-hydroxylation of EFV may be redirected to CYP1A2 accessory pathway in slow CYP2B6 metabolizers. This shall be expected for CYP1A2 -163C>A given that -739T>G and -163C>A in CYP1A2 gene were in complete LD as described in section 4.2 above. It would be interesting to study the importance of these observations in larger groups.

SNPs with no association with EFV levels

No statistically significant associations were found between EFV plasma levels and the genotypes of the following SNPs, CYP1A2 (-163C>A, and 2159G>A); CYP2A6 (1436G>T and -48T>G); CYP3A4 (-392A>G) and CYP3A5 (6986A>G) whether when with or without concomitant RBT.

No association between genotypic variants and HIV treatment response

There were no statistically significant associations between HIV-RNA copies and CD4 cell counts, and CYP1A2, CYP2A6, CYP2B6, CYP3A4 and CYP3A5 genotypes and haplotypes.

4.5 Predictors of efavirenz plasma levels

CYP2A6 1093G>A, CYP2B6 516G>T and CYP2B6 983T>C are independent predictors of EFV plasma levels

Univariate linear regression analysis was conducted in order to determine the effects of patients' baseline characteristics (gender, age, body weight, ALAT, ASAT, baseline CD4 cell counts, baseline viral loads, total bilirubin), and the genotypes with respect to the 10 SNPs, CYP1A2 (-739T>G, -163C>A, and 2159G>A), CYP2A6 (1436G>T, 1093G>A and -48T>G), CYP2B6 (516G>T, 983T>C), CYP3A4 (-392A>G) and CYP3A5 (6986A>G) as independent variables on $\log_{10}$ EFV plasma levels (dependant variable) collected from patients on concurrent HIV and TB treatment. Total bilirubin and the genotypes of CYP1A2 -739T>G, CYP2A6 1436G>T, CYP2A6 1093G>A, CYP2B6 516G>T and CYP3A4 -392A>G SNPs statistically significantly predicted EFV levels (Table 4). Thus, they were included in the multivariate analysis. The covariates that had a p-value of less than 0.20 (CYP1A2 -163C>A and CYP2B6 983T>C) were also included to avoid confounding effects on the relation between selected genotypes and the end-points. Multivariate analysis was then performed to identify independent predictors of EFV plasma levels and to evaluate their contribution to overall variability in EFV plasma levels. Three independent variables with $P<0.05$ remained in the final model, including CYP2A6 1093G>A (P=0.023), CYP2B6 516G>T (P=0.0004) and CYP2B6 983T>C (P=0.016; Table 4). The coefficient of determination (R^2) for the regression was 0.302 (P=0.0002), indicating that the model explained 30% of the variability in EFV plasma levels. The standardized regression coefficients indicated that CYP2A6 1093G>A, CYP2B6 516G>T and CYP2B6 983T>C accounted for 27%, 43%, and 29% of the total variance in EFV plasma levels, respectively.

The performed regression analysis identified CYP2A6 1093G>A and CYP2B6 983T>C as additional independent predictors of EFV levels beyond that provided by CYP2B6 516G>T polymorphism. Regarding the latter, its role in explaining variability in EFV levels has been previously reported extensively [11, 20, 43, 70, 76, 86-89], and the present data has also demonstrated its association with EFV levels in a Rwandan population, when with and without RBT as described in section 4.4 (Figure 7). As discussed in the same section, CYP2B6 983T>C SNP sharing similar characteristics with CYP2B6 516G>T polymorphisms [74, 91] could present with the same phenotypic trends as CYP2B6 516G>T. As described in section 4.4, CYP2A6 is part of the accessory pathways for EFV metabolism [30, 31, 84].

Its contribution to EFV metabolism among individuals characterized as CYP2B6 slow metabolizers has been also reported [29]. In addition, an association between slow metabolizer CYP2A6 alleles and increased EFV concentration was mentioned by Kwara *et al*. (2009) [74]. The current data suggest that CYP2A6 1093G>A and CYP2B6 983T>C SNPs have the potential to contribute to identifying patients who could be treated with adjusted dose of EFV through predictive genetic testing. Thus, in addition to CYP2B6 516G>T, genotypic data for CYP2A6 1093G>A and CYP2B6 983T>C SNPs should be taken into consideration when estimating the appropriate dose of EFV for HIV patients.

Table 4. *Regression analysis of association between EFV plasma levels and influential factors. From Paper III.*

Independent variable		% Log_{10} EFV (95% CI)	p-value	R^2
UNIVARIATE				
Gender		- 5.4 (- 20.6 to 13.5)	0.679	0.003
Age (years)		- 2.6 (- 1.2 to 1.0)	0.843	0.001
Body weight (kg)		8.8 (- 0.6 to 1.3)	0.495	0.008
Baseline CD4* (cells/µL)		10.7 (- 0.03 to 0.1)	0.429	0.011
Baseline log_{10} HIV-RNA* (copies/mL)		15.1 (- 2.6 to 8.7)	0.289	0.023
Alanine aminotransferase (U/ml)		- 2.7 (- 0.4 to 0.3)	0.836	0.001
Aspartate aminotransferase (U/ml)		- 4.1 (-0.3 to 0.2)	0.749	0.002
Total bilirubin (mg/dl)		25.8 (0.3 to 15.6)	0.043	0.067
CYP1A2	-739T>G	30.5 (4.6 to 44.7)	0.017	0.093
	-163C>A	17.5 (-6.3 to 31.9)	0.186	0.031
	2159G>A	16.8 (-7.9 to 36.5)	0.203	0.028
CYP2A6	1436G>T	27.6 (1.8 to 36.8)	0.031	0.076
	1093G>A	-28.8 (-100.1 to -7.5)	0.023	0.083
	-48T>G	3.3 (-19.5 to 25.1)	0.805	0.001
CYP2B6	516G>T	37.1 (8.6 to 40.6)	0.003	0.138
	983T>C	22.1 (-3.2 to 42.8)	0.090	0.049
CYP3A4	-392A>G	-28.2 (-41.9 to -2.7)	0.026	0.079
CYP3A5	6986A>G	-8.9 (-23.3 to 11.4)	0.494	0.008
MULTIVARIATE			0.0002	0.302
CYP2A6	1093G>A	-26.5 (-90.3 to -7.0)	0.023	
CYP2B6	516G>T	43.3 (13.5 to 44.4)	0.0004	
	983T>C	28.7 (4.9 to 46.1)	0.016	

**Refer to baseline before any HIV treatment, after enrolment.*
Genotypes were dichotomized and organized as follows: TT vs TG/GG for CYP1A2 -739T>G; CC vs CA/AA for CYP1A2 -163C>A; GG vs GA/AA for CYP1A2 2159G>A; GG vs GT/TT for CYP2A6 1436G>T; GG vs GA/AA for CYP2A6 1093G>A; TT vs TG/GG for CYP2A6 -48T>G; GG vs GT/TT for CYP2B6 516G>T; TT vs TC/CC for CYP2B6 983T>C; AA vs AG/GG for CYP3A4 -392A>G and for CYP3A5 6986A>G.

4.6 Prognostic values of CYP2B6 genotyping

High PPV for CYP2B6 516T/T and 983T/T genotypes

Sensitivity, specificity, PPV and NPV were evaluated to determine whether CYP2B6 SNPs could be used in predicting EFV plasma levels above the upper limit of the therapeutic range (Table 5). Overall, the predictive values for CYP2B6 SNPs were shown to change in the presence of RBT. Of note in this analysis was the higher PPV percentage (100%) for CYP2B6 516T/T and 983T/T genotypes in the absence of RBT, which decreased in the presence of RBT by 3.3-fold for CYP2B6 516T/T genotype and by 1.4-fold for CYP2B6 983T/T genotype. The latter genotype also had high specificity in the absence (100%) and in the presence (93%) of RBT.

Table 5. *Sensitivity, specificity and predictive values for CYP2B6 SNPs♣ on EFV plasma levels. From Paper III.*

Genotype	n	Sensitivity (%)	Specificity (%)	PPV* (%)	NPV* (%)
		In the absence of TB treatment			
CYP2B6 516G>T					
G/G	12	0.00	80.00	0.00	48.00
G/T	13	0.00	81.25	0.00	52.00
T/T	3	10.71	-	100.00	0.00
CYP2B6 983T>C					
T/T	23	42.86	100.00	100.00	83.33
T/C	4	0.00	57.14	0.00	16.67
		In the presence of TB treatment			
CYP2B6 516G>T					
G/G	28	3.85	75.00	10.00	51.92
G/T	28	16.67	84.62	60.00	42.31
T/T	6	5.77	30.00	30.00	5.77
CYP2B6 983T>C					
T/T	50	50.00	93.48	70.00	86.00
T/C	10	6.52	50.00	30.00	14.00

*Negative (NPV) and Positive (PPV) Predictive Values**

CYP2B6 516G>T and 983T>C SNPs were shown to be independent predictors of EFV levels (section 4.5); beyond being associated with EFV levels (section 4.4). Both 516G>T and 983T>C share similar characteristics, suggesting similar phenotype [74, 91]. In addition, the results in section 4.2 demonstrated that CYP2B6 516G>T and 785A>G were in complete LD. Given this demonstrated relationship between EFV plasma levels and CYP2B6 SNPs in the population studied, the observed higher PPV for CYP2B6 516T/T and 983T/T genotypes in predicting supra-therapeutic EFV plasma levels is suggestive of predictive genetic genotyping for CYP2B6 SNPs to identify patients who could be at risk of CNS toxicity and need adjusted EFV dose.

4.7 Effects of tuberculosis therapy on efavirenz plasma levels

EFV plasma levels were significantly lowered in the presence of RBT

The effect of RBT on EFV plasma levels was assessed to determine whether variability in EFV plasma levels in the cohort studied is due to genetic polymorphism and RBT, and to assess its impact on the clinical response. The effect of RBT on EFV plasma levels was evaluated by comparing EFV levels measured from samples collected during concomitant HIV and TB treatments with those collected from the same patients after completion of RBT. Overall, compared to when EFV was alone, EFV plasma levels were lowered during concomitant HIV and RBT for 80% of patients. The medians (IQR) EFV plasma levels were 2.7 (1.5-3.1) µg/ml and 1.8 (1.4-2.3) µg/ml in the absence and in the presence of RBT respectively, corresponding to a statistically significant decrease (P=0.004) of EFV plasma levels by 1.5-fold, following concomitant use of RBT (Figure 10G).

The observed decrease of EFV plasma levels could partly be attributed to the reported induction effect of rifampicin on metabolizing enzymes of EFV as discussed in section 1.6.2, but also to overlapping EFV auto-induction. However, based on the data from his study cohort when HIV and TB were treated concomitantly, Ngaimisi *et al.* (2011) [12] demonstrated that EFV auto-induction does not exhibit significant additive or synergistic effects over and above ongoing RBT. This implies that a decrease of EFV levels observed in both groups of the cohort studied during HIV and TB co-treatment could be attributed mainly to RBT effects and to a non significant extent to EFV auto-induction. This is however to be confirmed in other prospective study cohorts. In line with the objective to ascertain whether both genetic polymorphism and RBT contribute to the variability in EFV plasma levels, the present data suggest that deciding on EFV dosages for HIV/TB co-infected patients should take into accounts both CYP2B6 polymorphisms and RBT effects.

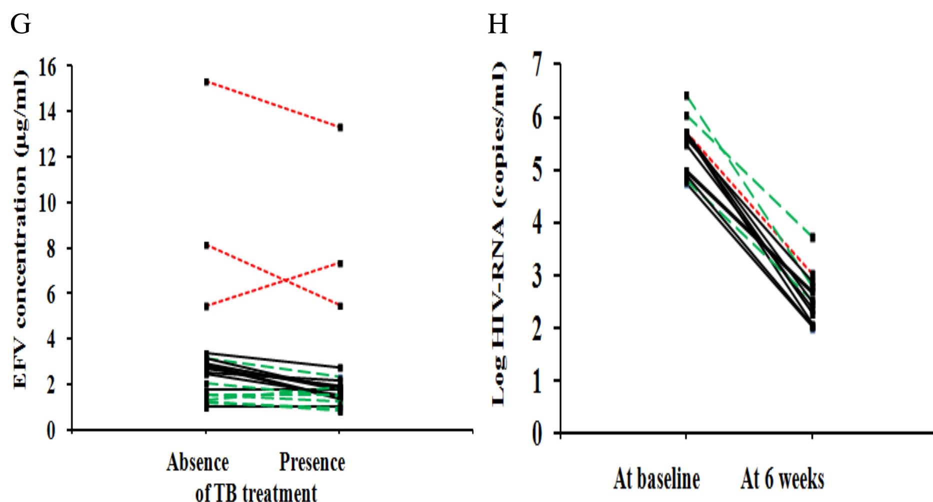

Figure 10. *Changes in EFV plasma concentrations (µg/ml) (G) and HIV-RNA (copies/mL) (H) after six weeks of initiation of antiretroviral therapy in the presence of TB treatment. In the presence of TB treatment, EFV plasma levels were statistically significantly lowered (P=0.004) and HIV-RNA copies were reduced significantly (P=0.002). Dotted, dashed and solid lines show carriers of CYP2B6 516T/T, G/G and G/T genotypes, respectively. From Paper III.*

Significant reduction in HIV-RNA copies in the presence of RBT

Whether the statistically significant decrease in EFV levels in the presence of RBT had an impact on the treatment response was further explored. The effect of RBT on clinical response was evaluated by comparing HIV-RNA copies measured from group A patients (naive) at baseline and after six weeks of initiation of HAART in the presence of RBT. Only naive patients were included (Group A) to avoid bias that may be caused by patients enrolled in the study while they were previously on HAART (Group B). Wilcoxon signed-ranks test implied a statistically significant (P=0.002) reduction in HIV-RNA copies (median; IQR) from baseline (446407 copies/mL; 83514 - 538809) to six weeks of initiation of HAART (328 copies/mL; 141 - 7403) in the presence of RBT (Figure 10H), indicating no effect of the latter on the clinical response. CD4 cell counts were increased in some patients and decreased in others and the difference was not statistically significant.

These data suggesting that viral loads decreased significantly in the presence of RBT are congruent with reports compiled by Avihingsanon *et al*. (2009) [7] on lack of association between virological failure and decrease of EFV levels following concomitant use of RBT. The fact that significant reduction of HIV-RNA copies was not affected during co-medication with RBT suggests that taking into account TB treatment effects during dose adjustment could be made rather at individual than at population level. Specifically, the impact of RBT on EFV plasma levels could be assessed through TDM in patients suspected being at risk of sub-therapeutic levels and dictate dose adjustment only when the clinical response is affected.

4.8 Efavirenz clearance by CYP2B6 516G>T and treatment nature

Long term exposure to HAART increases the CL/F of EFV

Oral clearance values of EFV were estimated using population PK modelling approach. The population CL/F values of the final model for EFV at baseline and at steady state stratified by CYP2B6 516G>T genotypes and by treatment nature are given in table 6. The fractional increases of CL/F were 0.51 and 0.55 in patients who had initiated TB treatment prior to HAART and in group of those who had previous exposure to HAART for a long period (from weeks to years), respectively.

Table 6. *Population clearance of the final model for efavirenz by genotype and by nature of previous treatment. From Paper IV.*

Parameter		Estimate (%RSE*)		%IIV (%RSE*)
		♣TB_{PREV}	♣$HAART_{PREV}$	
$CL/F_{G/G}$ (L/h)	(a)	10.2 (14)	14.5 (10)	54 (37)
	(b)	15.4	22.5	
$CL/F_{G/T}$ (L/h)	(a)	7.6 (15)	9.3 (16)	53 (33)
	(b)	11.5	14.4	
$CL/F_{T/T}$ (L/h)	(a)	3.4 (15)	1.6 (4)	32 (59)
	(b)	5.1	2.5	

CL/F: oral clearance stratified by CYP2B6 516 G>T genotypes, at baseline (a) and at steady state (b). IIV: Inter-individual variability. RSE: relative standard error. HAART: Highly Active Antiretroviral Therapy. TB_{PREV}: Group of patients who had TB treatment prior to HAART. $HAART_{PREV}$: Group of patients who had previous exposure to HAART before being diagnosed for TB and initiation of its treatment.

**Estimated from bootstrap (n=200 samples).*

♣The fractional increases (%RSE) of CL/F were 0.51 (15.3) and 0.55 (9.6) in TB_{PREV} group and in $HAART_{PREV}$ group, respectively.

As can be seen, the CL/F at baseline and steady state was always higher in patients with previous exposure to HAART ($HAART_{PREV}$) compared to those who were naive, and who initiated TB treatment prior to HAART (TB_{PREV}). Exception to this was for carriers of CYP2B6 516T/T genotype; probably because of the limited sample size (only one T/T patient in the second group). As one could expect, carriers of CYP2B6 516G/G genotype (extensive

metabolizers) in all patient groups exhibited higher CL/F compared to carriers of other genotypes, whereas lower CL/F were seen with carriers of CYP2B6 516T/T genotype (poor metabolizers). The present results are in agreement with the trends of measured EFV plasma concentrations as plotted in figure 12, where patients with previous exposure to HAART showed lower concentrations compared to those who had prior TB treatment.

The basic goodness of fit plots for the final PK model for EFV is shown in figure 13. Upper right and left plots show that observed concentrations match predicted concentrations, indicating that the suggested model described the PK of EFV adequately. Lower right and left plots show that residuals are evenly distributed around the reference line over time (constant variance). The final model was evaluated, and the results of the VPC are displayed in figure 11, as the prediction of the median concentration (solid line), and the 95% CIs (grey area). The VPC results confirmed the adequacy of the model predictions in describing the observed data.

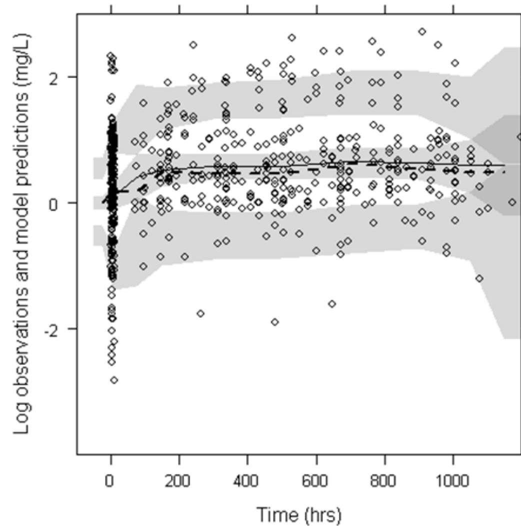

Figure 11. *Visual predictive check (95% prediction interval) of the pharmacokinetic model. Observed data is depicted as circles. The solid and dashed lines are the median of the predicted and observed data, respectively. The grey shaded areas are the 95 confidence interval of the simulated median, 2.5th and 97.5th percentile, respectively. From Paper IV.*

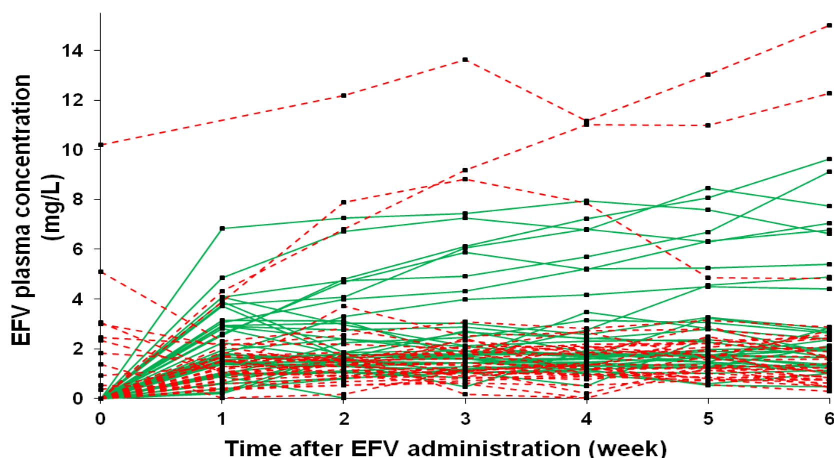

Figure 12. *Observed EFV plasma concentrations over a 6-week follow up in 71 patients on concomitant HIV and TB treatment. Generally, the figure shows lower concentrations in patients who have been on HAART since several months before initiation of TB treatment (represented by dashed red lines) compared to naive patients who initiated HAART after some weeks of TB treatment (represented by solid green lines). From Paper IV.*

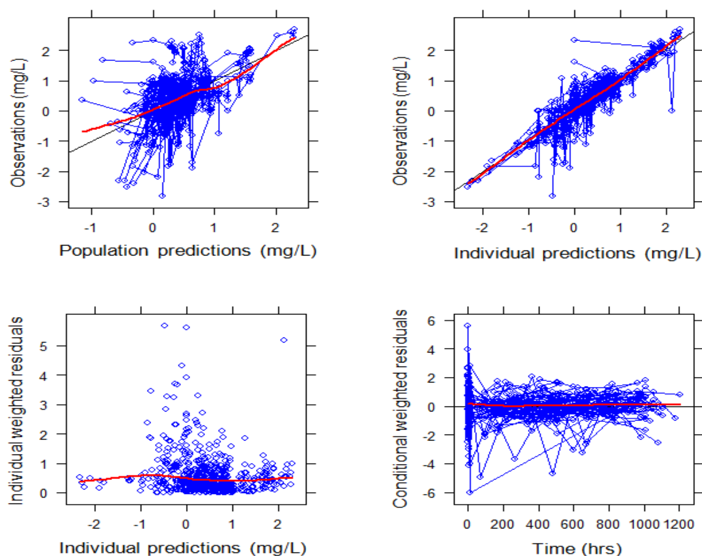

Figure 13. *Goodness of fit plots for the final pharmacokinetic model for efavirenz. The fine solid lines are the line of identity or the zero reference lines. The heavy solid red lines represent the tendency curve. From Paper IV.*

In patients pre-treated for TB (Group A), it appears that the CL/F of EFV was under the influence of enzyme induction which was elicited by the previous TB treatment before the initiation of EFV-based HAART. While in patients with previous exposure to EFV-based HAART (Group B), the estimated CL/F of EFV was auto-induced by EFV itself. On one hand, rifampicin, one of the TB drugs has been shown to induce EFV metabolizing enzymes such as CYP2B6 [19, 75, 149-151] and CYP3A4 [38, 39]. Enzymes metabolizing EFV were reported to be induced to a maximum degree during the early stage of RBT (first eight weeks after RBT administration), indicating no prolonged enzyme induction to affect EFV kinetics over time [12]. This suggests that rifampicin enzyme induction was still ongoing the day of initiation of EFV-based HAART in group A patients who had less than eight weeks of TB treatment, which was the case for 85% of the patients of this group. This indicates that enzyme induction had not yet reached its maximum, explaining the lower CL/F of EFV at baseline for patients of this group compared to group B patients. On the other hand when treating HIV alone, EFV was reported to accelerate its own metabolism by inducing CYP2B6 and CYP3A4 up to a prolonged period of 3 months to reach almost its maximum [13, 14]. This indicates that in group B, EFV auto-induction was still ongoing for patients who have been treated with HAART for a period of less than 3 months, whereas it has reached its maximum for those who have been treated with HAART for a period of more than 3 months. Since patients of this group may have been under HIV treatment for several months, EFV metabolizing enzymes were highly induced up to the maximum in patients of this groups, which could explain the higher efavirenz CL/F values observed at baseline for group B compared to group A patients.

Furthermore, when treating both HIV and TB, Ngaimisi *et al.* (2011) [12] reported an early induction of EFV metabolizing enzymes by RBT and a non-significant additive or synergistic effects for EFV auto-induction over and above ongoing RBT. The present data however, showing an increase of 51% and 55% of the CL/F in group A and B patients, respectively, argue for a significant contribution of EFV auto-induction to the overall induction. This indicates that the increase of the CL/F observed was dependant on the inducing effects of both RBT and EFV.

An influence of CYP2B6 genetic polymorphism on the CL/F of EFV was observed, according to which carriers of G/G genotype had higher CL/F compared to carriers of G/T and T/T genotypes. This is comparable to the significantly higher EFV trough concentrations and exposure in T/T than in G/T and G/G genotype patients reported by Ramachandran *et al.* (2009) [75]

in Indians, and the longest half-life for T/T genotype individuals reported by To *et al.* (2009) [152] in Chinese; all these implying a lower and higher CL/F of EFV for T/T and G/G genotype patients, respectively. Similar trends were also reported in African populations, such as South Africans [77], Ghanaians [74] and Zimbabwean patients [87]. Our results imply that genetic polymorphism in CYP2B6 516G>T should be taken into account when adjusting EFV dose and patients under EFV-based treatment monitored closely since carriers of T/T and G/G genotypes may be at risk of supra-therapeutic levels (responsible for CNS side effects) and sub-therapeutic levels of EFV (responsible for treatment failure), respectively. Overall, estimated CL/F values for EFV were in agreement with those previously reported [16, 17, 87].

Paper IV aimed to estimate the CL/F of EFV by accounting simultaneously for CYP2B6 genetic polymorphisms and for DDIs caused by enzyme induction. Reported CL/F values of EFV differed according to the treatment nature (whether the patients were under TB treatment before initiation of EFV-based HAART or whether they had previous exposure to HAART before being diagnosed for TB and initiation of its treatment). CL/F values were also different according to the CYP2B6 516G>T genotype carried by the patients (G/G, G/T and T/T for extensive, intermediate and poor metabolizers, respectively). The present results indicated higher CL/F values in patients with previous exposure to HAART and in carriers of CYP2B6 516G/G genotype in the two patient groups, and similarly lower CL/F values were observed in carriers of CYP2B6 516T/T genotype in the two patient groups. Overall, this indicates that previous exposure to HAART increased the CL/F, regardless of CYP2B6 516G>T polymorphisms, and CYP2B6 516G>T polymorphisms determined the CL/F regardless of the previous treatment received by the patients. This suggests that the CL/F of EFV in the population studied was variable due to whether the patients were previously mono-treated for TB or HIV and to CYP2B6 genetic polymorphisms, implying that both CYP2B6 genetic polymorphisms and the current treatment nature should be taken into account when adjusting EFV dose, with caution in patients who have been treated with HAART since several months.

5 GENERAL CONCLUSION

Taken together, the data from this thesis demonstrated that CYP2B6 polymorphism is the main pharmacogenetic determinant of EFV exposure in the patient population studied, being associated with higher EFV plasma levels and predicting 72% of the variability in EFV plasma levels. In addition to CYP2B6, CYP enzymes of the accessory metabolic pathways of EFV (CYP1A2 and CYP2A6) were shown to explain variability in EFV plasma levels. During the management of HIV/TB co-infection, both HIV and TB therapies are involved in drug-drug interactions, with TB therapy significantly decreasing EFV plasma levels and long term HAART increasing the clearance of EFV. Even though it is clear from this thesis that specific CYP genotypes and TB co-treatment do have a definite effect on EFV plasma levels causing its variation, this however does not seem to influence the efficacy of the EFV-based regimens in general.

The specific findings were:

- ❖ In comparison to several other published methods for the quantification of EFV, the developed HPLC method presents the advantage of being simultaneously rapid, highly selective, and straightforward, making it suitable not only for PK studies, but also for therapeutic drug monitoring in patients with HIV/AIDS alone or co-infected with TB at sites with less resourced settings.

- ❖ Investigated allele and genotype frequencies showed genetic variation in almost all SNPs studied, indicating a need for further genomic and genotype-phenotype investigations to explore the clinical influence of polymorphisms in the cytochrome P450 and its relevance in the population studied.

- ❖ A difference between CYP1A2 -739T/G and T/T genotypes was observed only in the presence of RBT, suggesting that this SNP may be of interest in slow CYP2B6 metabolizers.

- ❖ CYP2B6 516T/T genotype was found to be associated with higher EFV plasma levels, which could pre-dispose carriers of this genotype to CNS toxicity.

- ❖ CYP2A6 1093G>A and CYP2B6 983T>C were identified as additional independent predictors of EFV levels beyond that provided by CYP2B6 516G>T polymorphism, indicating that genotypic data for these SNPs should be taken into consideration when estimating the appropriate dose of EFV for the management of HIV/AIDS.

- ❖ A high PPV for CYP2B6 SNPs was observed in predicting supra-therapeutic EFV plasma levels, indicating the validity of predictive genotyping in CYP2B6 SNPs to identify patients who could be at risk of EFV-related CNS toxicity and need adjusted EFV dose.

- ❖ Rifampicin-based TB therapy was shown to significantly lower EFV plasma levels but not HIV-treatment response, suggesting that taking into account TB treatment effects during dose adjustment for EFV could be made rather at individual level. Specifically, the impact of Rifampicin-based TB therapy on EFV plasma levels could be assessed in patients suspected being at risk of sub-therapeutic levels and dictate dose adjustment only when the clinical response is affected.

- ❖ Long term exposure to HAART was found to increase the clearance of EFV, indicating that not only should the patient genotype status with respect to CYP2B6 be taken into account, but also each individual patient treatment history, with caution to previous exposure to HAART.

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