

From the Department of Clinical Science,
Intervention and Technology,
Division of Pediatrics
Karolinska Institutet, Stockholm, Sweden

**EFAVIRENZ THERAPY IN CHILDREN LIVING WITH HIV – THE ROLE OF
PHARMACOGENETICS FOR PLASMA LEVEL VARIATION AND
TREATMENT RESULTS**

Sandra Soeria-Atmadja



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EFAVIRENZ THERAPY IN CHILDREN LIVING WITH HIV - THE ROLE OF PHARMACOGENETICS FOR PLASMA LEVEL VARIATION AND TREATMENT RESULTS

Thesis for Doctoral Degree (Ph.D.)

By

Sandra Soeria-Atmadja

The thesis will be defended in public at 4U Solen, Alfred Nobels Allé 8, Entréplan, Campus Flemingsberg
October 18, 2024, 9.00 AM.

Principal Supervisor:

Associate Professor Lars Navér
Karolinska Institutet
Department of Clinical Science,
Intervention and Technology (CLINTEC)
Division of Pediatrics

Co-supervisor(s):

Professor Lars L Gustafsson
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Pharmacology

Associate Professor Jaran Eriksen
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Pharmacology

MD, PhD Johanna Rubin
Karolinska Institutet
Department of Clinical Science,
Intervention and Technology (CLINTEC)
Division of Pediatrics

MD, PhD Sarah Nanzigu
College of Health Sciences,
Makerere University, Kampala, Uganda
Department of Pharmacology and
Therapeutics

Opponent:

Professor Saye Khoo
Institute of Translational Medicine,
University of Liverpool, UK

Examination Board:

Professor Gareth Tudor-Williams
Imperial College, London, UK
Department of Infectious Disease
Faculty of Medicine

Professor Collen Masimirembwa
African Institute of Biomedical Science and Technology
(AiBST), Harare, Zimbabwe

Associate Professor Eva Wikström-Jonsson
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Pharmacology

To my father Hillman

Popular science summary of the thesis

Efavirenz (EFV) is a drug used in HIV treatment, but its effect can vary significantly from person to person due to genetic differences, particularly in the enzyme CYP2B6, which metabolizes the drug. In adults, this variability has important consequences for both the drug's effectiveness in controlling the HIV virus and its potential to cause side effects, particularly in the Central Nervous System (CNS). While studies in adults have led to adjusted dosing guidelines, optimizing EFV dosing in children remains a challenge, especially given the global rise of drug-resistant HIV.

This thesis aimed to investigate how genetic variations influence EFV levels in children living in Uganda and in Sweden and how EFV levels correlate with treatment success, side effects, and the development of drug-resistant HIV.

The research included two groups of children: from a multi-ethnic cohort in Sweden and a cohort of Ugandan children starting EFV for the first time. In Sweden, we examined previously collected data on EFV levels in 36 children under 18 years, linking these levels to genetic markers. In Uganda, we followed 99 children aged 3-12 over a 24-week period, measuring EFV and its metabolites including a newly discovered metabolite called EFAdeg. As far as we know, this is the first research to measure and describe EFV metabolites in children. We investigated how the concentrations of these metabolites depended on genetically controlled differences in CYP2B6 activity and if the metabolites were linked to CNS side effects. We also studied how well the virus was controlled depending on the blood levels of EFV.

The results revealed that in both groups, specific genetic variants, particularly in the CYP2B6 gene, were powerful predictors of EFV concentrations, accounting for up to 50% of the variability between individuals. Among the Ugandan children, 20% had HIV-drug resistance already before they started treatment, which put them at higher risk for poor virus control. Also, children with low EFV levels had worse viral control and were at higher risk of developing drug resistance, while those with very high levels experienced CNS-related side effects, such as dizziness and headache. Interestingly, while metabolites showed distinct patterns depending on genetic differences, they did not seem to contribute to CNS symptoms. The side effects were mostly mild and transient.

In conclusion, the study underscores the importance of exploring personalized medicine in HIV treatment of children. Tailoring EFV doses based on an individual's genetic makeup could enhance the drug's effectiveness while reducing harmful side effects.

Abstract

Background: Efavirenz (EFV) is used in HIV antiretroviral therapy (ART) and metabolized by CYP2B6, with great interindividual variability in plasma levels, due to pharmacogenetic variation. This impacts both viral efficacy and toxicity and has led to adjusted dosing for adults. Adult studies suggest that EFV hydroxy metabolites contribute to CNS toxicity, but pediatric data are lacking. Optimized EFV dosing in children remains challenging, especially as HIV drug resistance (HIVDR) is increasing.

Aims: To investigate the impact of pharmacogenetic variations on the plasma levels of EFV and its metabolites in children in Uganda and Sweden, and to explore how these concentrations correlate with viral outcomes, adverse effects, and HIVDR.

Methods: Study I was performed in a multi-ethnic cohort, Sweden. Retrospectively collected EFV plasma levels from 36 children <18 years with ongoing/previous EFV-therapy, were investigated for associations to single nucleotide polymorphisms (SNP) in EFV-metabolizing enzymes. Studies II-IV were prospective, observational and performed in 99 Ugandan ART-naïve children, aged 3-12 years. Plasma levels of EFV, phase I and phase II metabolites including a newly identified metabolite (EFAdeg) were quantified with LC-HRMS/MS (liquid chromatography high resolution with-tandem-mass-spectrometry) at 2, 6, 12 and 24 weeks after ART start and examined for association to SNPs, CNS toxicity and viral outcomes. Pretreatment HIVDR (PDR) was assessed.

Results: In study I, a multivariate mixed-effects restricted maximum likelihood regression model, (REML) identified genotypes CYP2B6*6 T/T, CYP2B6*11 G/G and CYP2A6*9 A/C, as independent predictors of EFV plasma concentrations, explaining 75% of interindividual variation. In study II-IV, CYP2B6 metabolizer phenotype (based on composite CYP2B6 c.516C>T/c.983T>C) and CYP2B6*11 G/G, predicted EFV plasma levels, with a REML model explaining 70% of EFV variation. No autoinduction was seen. Subtherapeutic EFV levels were linked to VL \geq 40 cop/ml and newly acquired HIVDR. The PDR prevalence was 20% and predicted poor virological outcomes. CNS symptoms were linked to supratherapeutic EFV levels, but not EFV metabolites. EFV metabolites, quantified for the first time in children, had a distinct distribution according to CYP2B6 metabolizer phenotype.

Conclusions: CYP2B6 and CYP2A6 genotypes were identified as key predictors of EFV plasma concentrations in Ugandan and Swedish children and explained a significant proportion of interindividual variability. Autoinduction was not seen. Subtherapeutic EFV levels were linked to poor viral outcomes, while supratherapeutic levels were linked to CNS toxicity. EFV metabolite profiles differed by CYP2B6 phenotype and had no correlation to CNS symptoms. These findings highlight the need for genotype-informed EFV dosing in children to optimize efficacy and minimize adverse effects.

List of scientific papers

- I. Soeria-Atmadja S, Österberg E, Gustafsson LL, Dahl ML, Eriksen J, Rubin J, Navér L. Genetic variants in CYP2B6 and CYP2A6 explain interindividual variation in efavirenz plasma concentrations of HIV-infected children with diverse ethnic origin. *PLoS One*. 2017;12(9).
- II. Sandra Soeria-Atmadja, Pauline Amuge, Sarah Nanzigu, Dickson Bbuye, Jaran Eriksen Johanna Rubin, Adeodata Kekitiinwa, Celestino Obua, Marja-Liisa Dahl, Madeleine Pettersson Bergstrand, Anton Pohanka, Lars L Gustafsson, Lars Navér. Sub- and supratherapeutic efavirenz plasma concentrations with risk for HIV-therapy failure are mainly genetically explained in Ugandan children: the prospective GENEFA cohort study. In press. *British Journal of Clinical Pharmacology*, September, 2024.
- III. Sandra Soeria-Atmadja, Madeleine Pettersson Bergstrand, Pauline Amuge, Sarah Nanzigu, Dickson Bbuye, Johanna Rubin, Adeodata Kekitiinwa, Celestino Obua, Marja-Liisa Dahl, Anton Pohanka, Lars L Gustafsson, Lars Navér, Jaran Eriksen. Plasma concentration profiles of efavirenz and its metabolites in Ugandan children during 6 months of follow-up show association between plasma concentrations and CNS toxicity, but no signs of autoinduction. Manuscript.
- IV. Soeria-Atmadja S, Amuge P, Nanzigu S, Bbuye D, Rubin J, Eriksen J, Kekitiinwa A, Obua C, Gustafsson LL, Navér L. Pretreatment HIV drug resistance predicts accumulation of new mutations in ART-naïve Ugandan children. *Acta Paediatr*. 2020;109(12):2706-2716.

Scientific papers not included in the thesis

Russo G, Paganotti GM, Soeria-Atmadja S, Haverkamp M, Ramogola-Masire D, Vullo V, Gustafsson LL. Pharmacogenetics of non-nucleoside reverse transcriptase inhibitors (NNRTIs) in resource-limited settings: Influence on antiretroviral therapy response and concomitant anti-tubercular, antimalarial and contraceptive treatments. *Infect Genet Evol.* 2016;37:192-207.

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Pettersson Bergstrand M, Soeria-Atmadja S, Barclay V, Tolic J, Navér L, Gustafsson LL, Pohanka A. Quantification of Efavirenz Hydroxymetabolites in Human Plasma Using LC-HRMS/MS. *Ther Drug Monit.* 2024;46(4):468-476.

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List of abbreviations

AIDS	Acquired Immunodeficiency Syndrome
ALB	Astrid Lindgrens Barnsjukhus (Astrid Lindgren Children's Hospital)
ANOVA	Analysis of variance
ART	Antiretroviral Treatment
AUC	Area Under the Curve
CLHIV	Children Living with HIV
CNS	Central Nervous System
CYP	Cytochromes P450
DRM	Drug resistance mutation
DTG	Dolutegravir
EFV	Efavirenz
8-OH-EFV	8-hydroxy efavirenz
7-OH-EFV	7-hydroxy efavirenz
EFAdeg	6-chloro-4-[3-cyclopropyl-1-hydroxy-1-(trifluoromethyl)-2-propyn-1-yl]-2(3H)-benzoxazolone
EID	Early Infant Diagnosis
EM	Extensive Metabolizer
FDC	Fixed Drug Combination
GENEFA	The importance of pharmacogenetic variation on Efavirenz levels and treatment effects in ART-naïve HIV-infected Ugandan children aged 3-12 years
HIV	Human Immunodeficiency Virus
HIVDR	HIV drug resistance
HPLC-UV	High-performance liquid chromatography
IM	Intermediate Metabolizer

INSTI	Integrase Inhibitor
JCRC	Joint Clinical Research Centre, Kampala
KI	Karolinska Institutet
LMIC	Low- and Middle-Income Countries
LC-HRMS/ MS	Liquid chromatography high-resolution tandem mass spectrometry
MakCHS	Makerere College of Health Sciences
MTCT	Mother-to-Child Transmission
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NVP	Nevirapine
PCR	Polymerase Chain Reaction
PEPFAR	President Emergency Plan for AIDS Relief
PDR	Pretreatment drug resistance
PK	Pharmacokinetic
PMTCT	Prevention of Mother-to-Child Transmission
PI	Protease inhibitor
REML	Multivariate Restricted Maximum Likelihood
SM	Slow Metabolizer
SNP	Single nucleotide polymorphisms
TB	Tuberculosis
TDM	Therapeutic Drug Monitoring
UGT	Uridine diphosphate (UDP)-glucuronosyltransferase
UNAIDS	Joint United Nations Programme on HIV/AIDS
VL	Viral Load
WHO	World Health Organization

ZDV

Zidovudine

Introduction

In 2009, when I joined the pediatric HIV team at Karolinska University Hospital as a resident, efavirenz (EFV) was one of the most widely prescribed antiretroviral drugs. At the time, we routinely monitored EFV plasma levels to avoid side effects from high concentrations and ensure viral control by preventing low levels. What immediately stood out was the significant variability in EFV concentrations among patients, despite them receiving similar doses.

By 2012, after returning from a pediatric HIV clinic in Ethiopia, I was invited to join a research project at Karolinska Institute, focusing on rational drug use in children. The project evolved into a study investigating the genetic basis of EFV plasma variability. With an established collaboration between Karolinska Institute (KI) and Makerere University (MakCHS), it was decided that the study would be conducted in Kampala, Uganda, in partnership with Baylor Uganda. We aimed to follow 100 children living with HIV over six months, collecting repeated plasma samples to analyze EFV levels, genetic polymorphisms, viral load, metabolites, and drug resistance. A retrospective study on children in Sweden was also incorporated to broaden the scope.

At that point, I had no idea what was ahead of me. What followed were two intense years of administrative preparation—negotiating budgets, drafting protocols, obtaining approvals from four institutional review boards, and setting up a database. This was coupled with numerous trips to Uganda and countless video meetings. The complexity of organizing a clinical study involving repeated blood samples from children, was both daunting and exciting. Especially since challenges in resource-limited settings like stigma and socio-economic factors may contribute to that participants are lost to follow-up. Fortunately, Baylor Uganda provided an excellent set-up, with a designated clinical study team that ensured that most participants remained engaged in clinical care and in the study. There was also the necessary infrastructure to handle the blood samples in a secure way for storage and transport. I visited the project every 3 months and had frequent digital meetings in between, with the coordinators from Baylor and MakCHS.

When recruitment finally began in 2015, progress was slower than expected. Originally, we planned to analyze EFV samples at MakCHS, but the methods we developed were not sensitive enough to measure metabolites. This setback required us to develop a new method at KI, which delayed the project but led to the unexpected discovery of a new substance.

Looking back, while this journey was long and filled with challenges, it was also one of the most rewarding experiences of my career. I learned more than I could have imagined, not just about the science but about collaboration, resilience, and the importance of flexibility in

research. In the end, we successfully collected and analyzed data from 99 children, allowing us to evaluate treatment outcomes, EFV concentrations, and genetic factors. Alongside the Swedish study, these results have contributed to our understanding of EFV treatment in children.

1 Literature review

1.1 Children living with HIV

HIV remains a global health challenge, with an estimated 39,9 million people living with HIV worldwide, whereof 1,4 million are children aged 0-14 years [1]. The majority is living in Eastern and Southern Africa, the region that has been most affected by the HIV pandemic [2] (Figure 1.)

Children (<15 years) estimated to be living with HIV | 2023

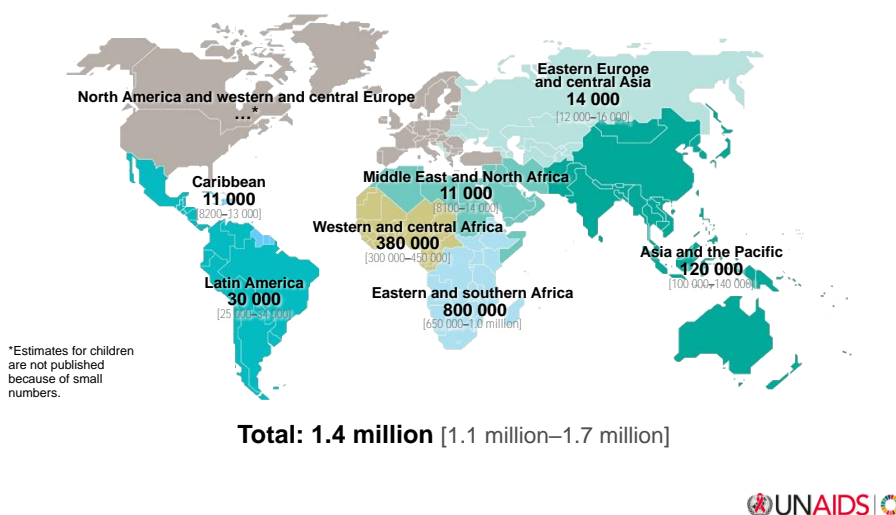


Figure 1. Source: Core Epidemiology Slides. Joint United Nations Programme on HIV/AIDS 2024 [3].

Most children living with HIV (CLHIV) have acquired the virus through vertical transmission—occurring either in utero, during birth, or through breastfeeding—from mothers who did not receive effective antiretroviral treatment. Without any intervention, the risk of mother-to-child transmission (MTCT) of HIV is estimated to be 15–45% globally, with higher transmission rates reported in resource-limited settings [4,5].

However, the expanded global availability of antiretroviral treatment (ART) has significantly reduced MTCT rates. By 2023, as many as 83% of pregnant women accessed ART [6]. Consequently, the number of new HIV cases among children aged 0-14 years dropped from 300,000 in 2010 to 150,000 in 2020 [2].

1.2 Prevention of mother to child transmission (PMTCT)

In 1994, a clinical trial found that administration of the nucleoside reverse transcriptase inhibitor (NRTI) zidovudine (ZDV) to the mother from gestational week 14, in labor and to the infant for 6 weeks postpartum reduced vertical transmission by 67% [7]. Trials performed in low and middle income countries (LMIC) proved that the risk vertical transmission could be significantly reduced by simplified regimens of ZDV or with single dose administration of the nonnucleoside reverse transcriptase inhibitor (NNRTI) nevirapine (NVP) to mother and infant [8,9]. NVP administered as a single dose reduced transmission rate [10] but also proved to be associated with acquisition of HIV viral drug resistance mutations [11].

WHO guidelines for PMTCT regimens in resource limited settings gradually evolved from single-dose NVP and short course zidovudine into more comprehensive regimens [12,13]. Since 2016, the WHO has recommended lifelong antiretroviral treatment for pregnant women living with HIV irrespective of CD4 count or clinical stage. For infants, a 4-6-week course of daily NVP or ZDV prophylaxis is recommended. The choice and duration of prophylaxis depend on whether the infant is breastfed or bottle-fed and the risk of transmission, such as late initiation of maternal ART, maternal viremia, or a late maternal HIV diagnosis [14,15].

With access to effective prevention of mother-to-child transmission (PMTCT) programs, including continuous antiretroviral treatment for mothers from pregnancy through breastfeeding, along with comprehensive antenatal and postnatal follow-up, transmission rates have been reduced to as low as <0.5% in high-resource settings [16] and <1% in resource-limited settings [17].

1.3 Antiretroviral treatment in children

1.3.1 Antiretroviral treatment- progress and challenges

Since 2015, WHO recommends that ART should be initiated in both adults and children diagnosed with HIV, regardless of age, clinical stage and CD4 count, which previously guided when to start ART [14]. Despite the overall improvements in ART access, children living with HIV, still face challenges in receiving efficient care and treatment, resulting in lower antiretroviral coverage compared to adults [17]. According to UNAIDS (Joint United Nations Program on HIV/AIDS), the global ART coverage between 2017 and 2023 increased from 57% to 77% for adults, but only from 47% to 57% for children [2]. Pediatric ART coverage also varies significantly across different regions. For instance, in 2023, only 35% of children living with HIV in West Africa had access to therapy, compared to 65% in Southern and Eastern Africa [2].

One significant barrier for timely initiation of ART in children is late diagnosis. Late diagnosis in perinatally exposed children may be caused by poor access to services providing early infant diagnosis (EID) or by caregiver-related challenges, such as long travel distances, financial constraints, or stigma associated with accessing testing and other difficulties to retain the mother-child pair in the PMTCT continuum [18-21].

Young children with perinatally acquired HIV, are at high risk of disease progression, irrespective of their CD4 T-lymphocytic status [22]. As many as 50% of children born with HIV die before two years of age [23] in the absence of treatment. The primary causes of illness and death among children under five years living with HIV in LMIC include severe bacterial infections, pneumonia (such as *Pneumocystis pneumonia*), tuberculosis (TB), severe acute malnutrition and diarrheal disease [24,25]. Wider access to ART has led to a significant reduction in global AIDS-related mortality among children aged 0-14, decreasing from 230,000 to 96,000 annual cases between 2017 and 2023 [2].

While pediatric ART has demonstrated excellent clinical efficacy in both high-income and resource-limited settings [26,27], young children on antiretroviral treatment may still experience poorer treatment outcomes compared to adolescents or adults. Mortality rates among infants and young children under five years of age on ART were significantly higher, with more frequent treatment interruptions and lower viral suppression rates observed in sites across 28 countries and regions receiving PEPFAR-supported treatment, during 2020-2022 (Figure 2) [28].

Figure 2. Annual percentage of reported deaths among persons living with and receiving antiretroviral treatment — U.S. President’s Emergency Plan for AIDS Relief, 28 supported countries and regions, 2021–2022

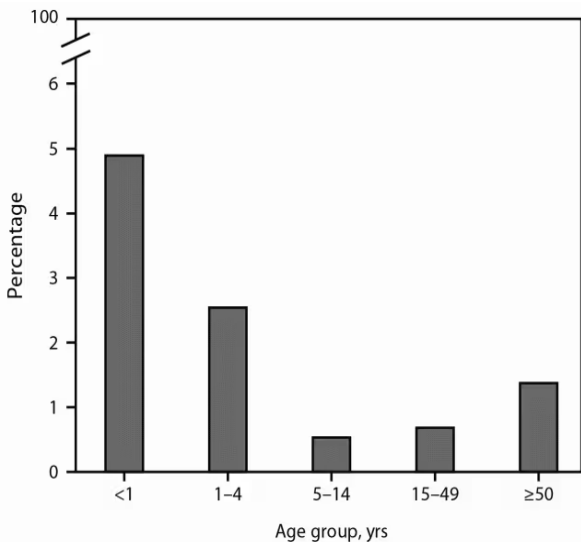


Figure 2. Abbreviations: ART = antiretroviral therapy; PEPFAR = U.S. President’s Emergency Plan for AIDS Relief. Adapted from “Mortality Among Children Aged <5 Years Living with HIV Who Are Receiving Antiretroviral Treatment — U.S. President’s Emergency Plan for AIDS Relief, 28 Supported Countries and Regions, October 2020–September 2022”. Agathis NT et al, MMWR Morb Mortal Wkly Rep 2023;72 [28].

1.3.2 Viral outcomes and treatment monitoring

Efficient antiretroviral treatment suppresses the viral burden, which results in improved health outcomes and reduces the risk of transmission and accumulation of HIV drug resistance mutations. Measurements of viral load is a way to monitor the effect of ART and is recommended by WHO to monitor adherence and to identify treatment failure that may require change in prescribed ART. A viral load (VL) < 50 copies/ml is defined as undetectable /virological suppression and is the ultimate viral outcome. Treatment failure should be suspected after repeated measurements of VL exceeding 1000 copies/ml, while VL ranging between 50-1000 copies is considered as low-level viremia that may require intensified support for adherence issues [15]. Although occasional elevations of VL up to < 200 copies/ml (“blips”) can be seen during efficient ART, persistent low-level viremia (200-500) were significantly associated with future treatment failure and acquisition of new HIV-drug resistance mutations in adults living with HIV [29].

1.3.3 Present and past WHO antiretroviral regimens for children

ART regimens recommended for children and adults living with HIV, typically include a backbone of two nucleoside reverse transcriptase inhibitors (NRTIs) combined with a third anchor drug from one of these antiretroviral drug classes: a non-nucleoside reverse transcriptase inhibitor (NNRTI), an integrase strand transfer inhibitor (INSTI) or a (pharmacokinetically boosted) protease inhibitor (PI).

Until 2018, WHO recommended an efavirenz (EFV)-based regimen as the first-line treatment for both adults and children living with HIV [30]. EFV is an NNRTI, that together with two NRTIs, was the preferred option for ART-naïve children from three years of age. EFV is not recommended in younger children as there is limited pharmacokinetic (PK) data from efavirenz treatment in this age group [15]. Efavirenz has been widely used as it is affordable, can be dosed once a day and is available in a fixed drug combination (FDC) for adults and adolescents weighing 40 kg or more. It has also been preferred to protease inhibitors when co-treating people living with HIV for tuberculosis as it is less affected by drug-drug interactions with rifampicin, the backbone of TB therapy in resource limited settings [15].

The most noted disadvantages of efavirenz treatment include its proneness to cause neuropsychiatric side-effects and a high susceptibility to the evolution of viral resistance [31].

From 2018, WHO guidelines were subsequently revised to recommend dolutegravir (DTG)-based regimens as first-line treatment for ART-naïve adults and children, where the integrase inhibitor DTG replaced EFV as the anchor drug. This recommendation was extended in 2019 to include women who are pregnant or of childbearing age [30]. DTG offers several advantages over EFV, including faster viral suppression, a high genetic barrier to HIV drug resistance, and a lower risk of drug-drug interactions [30,32,33].

In both ART-naïve adults and children above three years of age, regimens containing EFV are still considered as alternative options, provided that the national estimates of EFV HIV drug resistance is below 10 percent [30]. As of 2023, WHO observed that 79 out of 114 (69%) reporting countries had adopted DTG-regimens as first-line treatment for infants and children living with HIV [34], indicating that a significant number of children may still receive EFV-based therapies.

1.4 Efavirenz

1.4.1 Mechanism of action and metabolic pathway

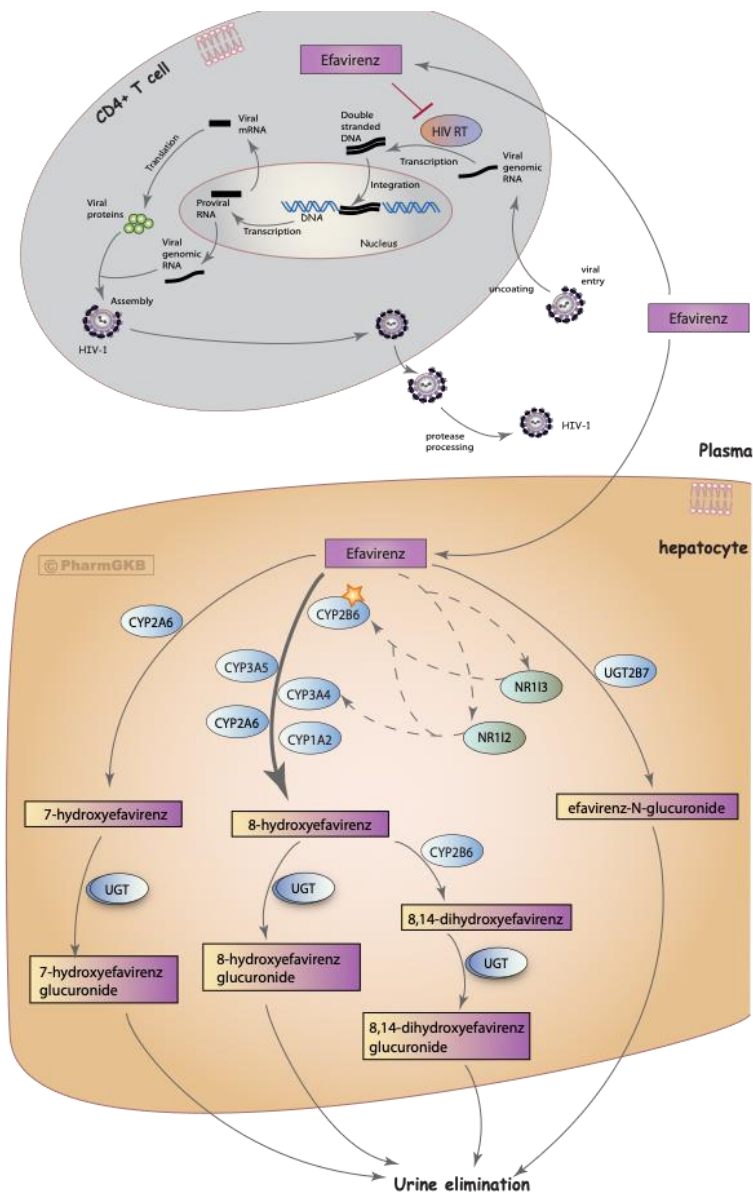
EFV is a non-nucleoside reverse transcriptase inhibitor (NNRTI) targeting HIV-1. by noncompetitive inhibition of HIV-1 reverse transcriptase. EFV does not inhibit HIV-2 reverse transcriptase [35]. The concentration of free EFV required to inhibit the replication of wild-

type laboratory-adapted strains and clinical isolates by 90-95% (EC90-95) in cell culture (lymphoblastoid cell lines, peripheral blood mononuclear cells (PBMCs), and macrophage/monocyte cultures) ranged from 1.7 to 25 nM [35]. The effect on viral suppression has also been confirmed in clinical settings [36-38].

Studies in adults show that the metabolism of efavirenz is complex. It is predominantly metabolized in the liver by the cytochrome P450 2B6 (CYP2B6). The clearance of EFV is mediated through hydroxylation to 8-hydroxyefavirenz (8-OH-EFV) by CYP2B6, with a minor contribution by CYP2A6, CYP3A4, CYP3A5, and CYP1A2 [39,40]. It is estimated that 80% of EFV is converted to 8-OH-EFV, while the remaining drug undergoes hydroxylation to 7-hydroxyefavirenz (7-OH-EFV)- mainly via CYP2A6, with potential involvement from CYP2B6 [39]. A small fraction of EFV is directly glucuronidated to EFV-N-glucuronide (EFV-N-gln) by uridine5-diphosphate-glucuronosyltransferase 2B7 (UGT2B7) [41,42].

Further metabolism of 8-OH- EFV by CYP2B6 results in the formation of 8,14-dihydroxy-EFV [40]. The three hydroxylated phase I metabolites of EFV—8-OH-EFV, 7-OH-EFV, and 8,14-dihydroxy-EFV—are subsequently conjugated by various UDP-glucuronosyltransferase (UGT) isoforms or undergo sulfation before being excreted via the kidneys [42-44].

Figure 3. Efavirenz metabolic pathway and its mechanism of action against HIV-1.



Abbreviations: CYP, cytochrome P450; NR1, transcription factors pregnane X receptor (PXR, NR1I2) and constitutive androstane receptor (CAR, NR1I3); RT, reverse transcriptase; UGT, UDP-glucuronosyltransferase. Adapted from "PharmGKB summary: Efavirenz pathway, pharmacokinetics", McDonagh E. M. et al, Pharmacogenetics and Genomics 2015 Vol. 25 Issue 7[45]. CC-BY-SA 4.0.

1.4.2 8-OH-EFV and 7-OH-EFV

The metabolites of EFV, are considered to lack antiviral activity [35,46], although *in vitro* studies have suggested that exposure to 7-OH-EFV and 8-OH-EFV may have toxic effects on neurons and astrocytes [47,48], why there has been an interest to investigate the exposure to these substances *in vivo*.

One study conducted in adults observed that the phase II metabolites 8-OH-EFV glucuronide and 8-OH-EFV sulfate were the predominant circulating metabolites, in plasma, with concentrations 64-fold and 7-fold higher, respectively, than those of the parent compound 8-OH-EFV. In accordance with previous studies [46], they also found that the phase II metabolites of 8-OH-EFV dominated in CSF and that their CSF concentration was higher than in plasma. The ratio of 8-OH-EFV/EFV in plasma was investigated as a phenotypic marker of CYP2B6 activity and was significantly associated with genetic polymorphisms affecting EFV plasma clearance [43].

1.4.3 EFAdeg, a newly discovered metabolite

We recently developed and validated a liquid chromatography high-resolution tandem mass spectrometry (LC-HRMS/MS) method to measure plasma efavirenz (EFV) and its phase I and II metabolites in children [49]. During analysis, a previously unknown metabolite, 6-chloro-4-[3-cyclopropyl-1-hydroxy-1-(trifluoromethyl)-2-propyn-1-yl]-2(3H)-benzoxazolone (EFAdeg), was discovered. This metabolite was detected in both patient samples and samples spiked with 8-OH-EFV. It was hypothesized that the formation of EFAdeg, likely resulted from a hydrolysis rearrangement with a ring-opening. Ring-opening has been previously described for EFV [50], while analogous rearrangements has been observed for the antiretroviral drug ritonavir [51]. Furthermore, we proposed that the rearrangement was an equilibrium reaction, in line with our observation that 8-OH-EFV was detected in blank plasma spiked with EFAdeg [49]. (Figure 4).

Figure 4. Suggested rearrangement of 8-hydroxyefavirenz and the newly discovered metabolite EFAdeg

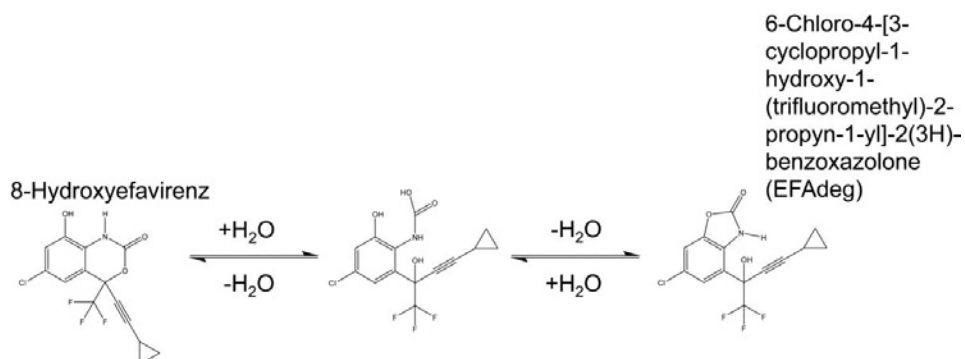


Figure 4. Source: Pettersson Bergstrand M et al. "Quantification of Efavirenz Hydroxymetabolites in Human Plasma Using LC-HRMS/MS". *Ther Drug Monit.* 2024;46(4)[49].

1.4.4 CYP2B6

CYP2B6 is responsible for metabolizing approximately 8–13% of clinically significant drugs and toxicologically relevant substances. Key drugs affected by CYP2B6 include efavirenz, nevirapine, methadone, bupropion, artemisinin, and ketamine, where the enzyme's activity significantly influences drug metabolism, response, and potential toxicity [52]. CYP2B6 is mainly expressed in the liver and to a lesser extent in the gastrointestinal tract, lungs, kidneys and brain. The expression is regulated by the constitutive androstane receptor (CAR, NR1I3) and pregnane X (PXR, NR1I2) receptor in the liver and is highly inducible by drugs like efavirenz and artemisinin [53]. As a result, with repeated dosing in healthy adults, efavirenz has been shown to induce its own metabolism (autoinduction) by upregulating the expression of CYP2B6 [54]. Effect of autoinduction with decreased EFV plasma exposure over time, has been observed in adults and is regarded as clinically significant for CYP2B6 extensive metabolizers [55-57]. Data in children is scarce and inconclusive [58,59].

1.4.5 Variability in efavirenz plasma concentration

Plasma levels of EFV correlate to treatment outcomes and a therapeutic range of 1000-4000 ng/ml, sampled at mid-dose interval has been suggested [60] in adults. Levels below 1000 ng/ml were reported to cause viral failure in ART naïve adults whereas supra-therapeutic levels were associated with an increased risk of CNS adverse events [60].

Early studies from healthy adults indicate that EFV is well absorbed, reaching peak plasma levels approximately 5 hours after oral administration [35]. Absorption is further enhanced

when co-administered with food. A single 600 mg dose taken with high-fat food increased the area under the curve (AUC) by 28% and the maximal plasma concentration (C_{max}) by 79% compared to administration on an empty stomach [35]. EFV is highly albumin-bound in plasma (>99%) and initial data suggested a plasma terminal half-life ranging from 35 to 55 hours. However, clinical studies have reported much longer plasma half-life of EFV, with one study reporting a median half-life of 148 hours among five participants who were followed after discontinuing EFV and switching their ART-regimen [35,61,62].

The plasma levels of EFV display high grade of intra and inter-individual variation in both adults and children [63-65] and there has been concerns regarding the appropriate EFV dosing schedules and treatment outcomes [59,66]. Pediatric studies have highlighted issues related to both underdosing [66] and [67] overdosing, while in adults, the primary concern has been the risk of overdosing.

Intra-individual variations may be caused by inconsistent adherence, drug-drug interactions, altered absorption or by CYP2B6 autoinduction [68]. Regarding potential causes behind interindividual difference between individuals many factors have been investigated whereas inter-individual variation is largely attributed to genetic polymorphic expression of enzymes involved in EFV metabolism and disposition. The effect of gender on plasma efavirenz levels is unclear [69,70].

1.4.6 EFV plasma concentrations and pharmacogenetics

Studies in both adults and children living with HIV have shown that this interindividual variation is largely due to genetic polymorphisms in the enzymes responsible for EFV metabolism and disposition [71]. The distribution of pharmacogenetic polymorphisms vary across ethnic groups which may explain observed differences in PK parameters between different populations [72]. Most studies have investigated CYP2B6, the major efavirenz metabolizing enzyme. It is highly polymorphic and has several single-nucleotide polymorphisms (SNPs) associated with loss of variable degree of function in the expressed enzyme [52,71].

1.4.6.1 Genetic polymorphisms in CYP2B6

The best characterized allelic variant of CYP2B6 is the SNP 516G>T (rs3745274) which changes G to T and significantly predicts increased efavirenz plasma exposure within homozygous genotype (T/T) compared to heterozygous (G/T) and wildtype (G/G) genotypes, in both children and adults [73-77]. Bienzak et al showed that clearance in African children reduced with 34 % in the presence of 1 variant allele and with 72 % in homozygous individuals which is in accordance with previous studies [70,74]. The proportion of slow metabolizers varies among different populations and is relatively high among black Africans [65,78,79] with an estimated minor allele frequency (SNP 516G>T) of 30-36% [69,72] compared to 15 % in Europeans [72].

Another SNP in the CYP2B6 gene, 983T>C (rs28399499) is found almost exclusively in Africans with a frequency of 7% and is also associated with increased plasma efavirenz levels in both children and adults [72,74]. Other SNPs of CYP2B6 that seem to reduce efavirenz clearance are 785A>G (rs2279343) [69] and 15582C>T (rs4803419) [78,79].

Conversely, a possible gain- of- function SNP, (CYP2B6 18492T>C), has been described in efavirenz treated Thai adults, co-infected with HIV and tuberculosis. Patients who carried a heterozygous or homozygous mutant of the polymorphism and lacked minor allele of SNP CYP2B6 516G>T (rs3745274) had markedly lower plasma efavirenz concentrations [80].

The combined effect on plasma efavirenz of composite genotypes (haplotypes) have been studied as well. The presence of a single variant allele in 983T>C can significantly modify the effect of SNP 516G>T (in wild type/GG 516>T individuals) on clearance with increased efavirenz exposures as a result [74]. Similarly Reay et al investigated the haplotypes of 516G>T, 785A>G and 983T>C in South African children and found that the haplotype T-G-T significantly increased EFV plasma levels compared to G-A-T [75,81] at repeated measurements during a 24 months follow-up.

1.4.6.2 Genetic polymorphisms in other enzymes involved in EFV metabolism

Evidence on pharmacogenetic association between other enzymes and efavirenz plasma levels is scarce. A few studies have investigated CYP2A6 and found that the minor allele CYP2A6*9 g.-48T >G (rs28399433) may predict higher efavirenz plasma levels in children [63] and in adults [82,83], especially in individuals with reduced clearance due to carrier status of CYP2B6 516G>T [84].

No association between CYP3A5 polymorphisms and with EFV plasma levels have been found [77,85] while there are conflicting results regarding the impact on efavirenz levels by the genetic variant c.4036A>G (rs3842A>G) for the transporter ABCB1 [69,85]. Carrier status of genotype UGT2B7*1a allele was reported to contribute to raised EFV levels in CYP2B6 516G>T slow metabolizers [82].

1.4.7 EFV and HIV drug resistance

Efavirenz and other NNRTIs have a low genetic barrier to viral resistance. A single point mutation in the gene for the reverse transcriptase can be associated with clinically significant phenotypic resistance [35]. There is also a high degree of cross-resistance between the different NNRTIs [86]. The most common NNRTI mutations are K103N/S, L100I, K101E/P, V106A/M, Y181C/I/V, Y188C/H/L, G190A/S/E, and M230L and each of them confer resistance to EFV [87,88].

Viral NNRTI resistance can arise due to direct exposure to NNRTI treatment or can be transferred from one individual to another. PCR-amplification and population sequencing

techniques can detect genotypic resistance if present in <15-20% of a viral population [86,89]. If the frequency of resistance mutations is low and present as minority variants within the HIV population of an infected individual, standard assays will fail to detect them [89].

Presence of minority NNRTI resistance variants has been associated with a dose-dependent increased risk of virologic failure in patients treated with NNRTI-based first line ART [90]. Minority NNRTI-resistance variants have also been detected in infants due to direct or indirect exposure to nevirapine in PMTCT-programs [91]. Thus, children who acquire HIV despite PMTCT exposure, have an increased risk of pretreatment HIV drug resistance (PDR). PDR has high clinical relevance as it is associated with poor response to first-line therapy and further accumulation of drug resistance mutations [92,93]. In a study of ART-naïve Ugandan children, the group with PDR were 15 times more likely to experience viral failure and 3 times more likely to acquire new HIV drug mutations (HIVDRM), within a two-year period from treatment initiation [94].

In 2021, WHO published HIV drug resistance (HIVDR) prevalence data from 14 national surveys including information from adults and children below 18 months old. The prevalence of NNRTI PDR among adults has increased significantly; In Africa, the NNRTI PDR prevalence ranges from 8% in Cameroon up to 15% in Uganda with a higher prevalence (18-35%) among individuals with prior ART exposure compared to ART-naïve patients [95].

Data in children is emerging and indicate a high prevalence of pre-treatment HIVDR as well [96-99]. A systematic review [100] from 2017 estimated the pooled PDR prevalence from 19 African studies to 43% and 13 % in children with and without PMTCT exposure and also found that PDR among children has increased. The majority of the studies included performed focused on children below 3 years of age and further studies on pre-treatment drug resistance in children of all ages need to be undertaken, especially with consideration given to the adolescent group as few studies address children above 12 years. Pretreatment resistance studies in infants and young children need to be ongoing as PMTCT programs change over time [95].

1.4.8 Efavirenz and adverse effects

Efavirenz treatment in adults is associated with EFV concentration dependent CNS symptoms such as dizziness, abnormal dreams, headache, light-headedness and impaired concentration [60]. These side effects typically occur during the first weeks of treatment and thereafter decline in severity [101,102] even if symptoms persisting up to one year or longer after EFV initiation have been described [103,104].

In a systematic review of 42 trials comparing EFV based vs non EFV based regimens, nearly one third of the patients experienced some kind of CNS related adverse event, often

transient, but with an increased risk of treatment discontinuation [102]. Suicidality and other severe adverse events have been described as well. Ataxia and encephalopathy were reported in 20 South African women with EFV plasma levels ranging from more than twice the upper level of therapeutic range up to above the upper limit of assay detection [105].

Fewer studies address side-effects and tolerability in children, but reported symptoms tend to be transient and include neuropsychiatric symptoms such as dizziness, headache, nightmares, sleeping disorder and nausea and rash [106-108]. Rash is more frequently reported in children compared to adults and was the most common side effect (25%) in a study in Indian children [107] while CNS-symptoms appear in 14-36% of cases [106].

More severe but rare adverse events have also been observed in children. Four African children that experienced either general seizures, absence seizures or cerebellar dysfunction had plasma efavirenz levels ranging from 20000-60000 ng/ml, 5-15 times the upper limit of the suggested reference range [109].

Attempts to explain the underlying mechanism behind efavirenz linked neurotoxicity has been made both in *vitro* and in adults living with HIV. It has been suggested that 8-OH efavirenz could play a role in the development of CNS related adverse events [110,111].

One study found that EFV, 7-OH-EFV, and 8-OH-EFV each caused neuronal damage in a dose-dependent manner in vitro and that 8-OH-EFV showed toxic effects at much lower concentrations than EFV or 7-OH-EFV. The same study showed that all three compounds were found in CSF sampled from EFV exposed individuals living with HIV within the same range, that damaged neurons in culture [47,111].

Mood changes were linked to 8-OH plasma in a case control study [112] whereas another study found no increased risk of EFV treatment discontinuation due to CNS adverse events in relation to plasma levels of EFV, 8-OH-EFV or other metabolites [43]. Thus, the relation between EFV, its metabolites and the mechanisms for CNS toxicity *in vivo* remains inconclusive.

To our knowledge there is no pediatric study where 8-OH efavirenz and other EFV-metabolites have been either quantified or investigated for association with neurotoxicity.

1.4.9 Pharmacogenetic- guided EFV dosing

The standard adult dose of EFV was originally set at 600 mg once daily. However, a randomized study showed that a 400 mg dose provided similar efficacy with slightly improved tolerability [113]. As a result, the WHO recommends the 400 mg dose as an alternative first-line treatment for HIV in adults. Additionally, there are dosing schedules developed for adults, where doses are adjusted for metabolizer phenotype predicted by

different CYP2B6 [71] genotypes. Genotype informed dosing have also been suggested to improve plasma exposure in children.

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2 Research aims

2.1 Overall aim:

To investigate the impact of pharmacogenetic variations on the plasma concentrations of efavirenz (EFV) and its metabolites in children Uganda and Sweden, and to explore how these concentrations correlate with viral outcomes, adverse effects, and HIV drug resistance.

2.2 Specific aims

2.2.1 Study I

- To evaluate how the plasma concentration of efavirenz vary in a multiethnic cohort of children living with HIV in Sweden.
- To evaluate if pharmacogenetic variation is more important than weight, age and gender for the variation of plasma concentration of efavirenz (EFV) in children living with HIV in Sweden.
- To identify the genetic variants most important for variation in plasma concentration of EFV.
- To examine the importance of age at start of treatment and time from treatment start for variation in plasma concentration of EFV.

2.2.2 Study II-IV

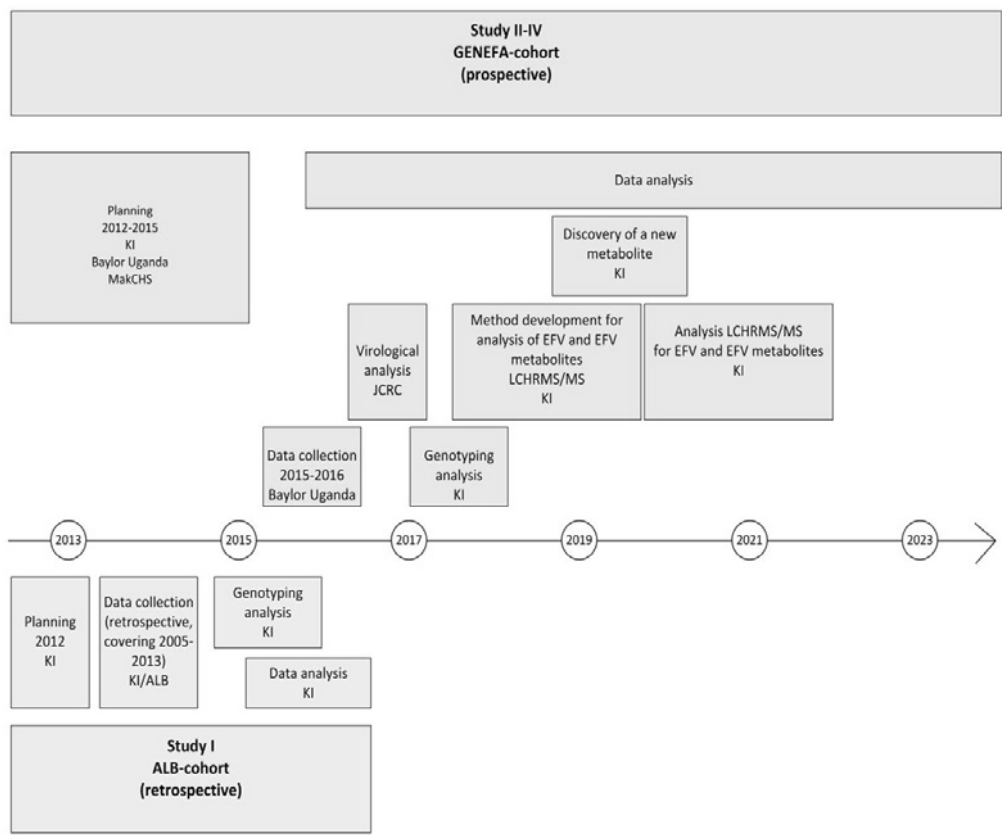
- To investigate the importance of CYP2B6 genotypes for variations in EFV concentrations in ART naive Ugandan children (**study II**).
- To examine signs EFV autoinduction.
- To evaluate if EFV plasma levels and the CYP2B6 genotypes are correlated to treatment outcomes (HIV- RNA levels, viral resistance) after 24 weeks of EFV-based ARV treatment (**study II**).
- To perform a quantitative analysis of a selection of phase I and II EFV metaboliteplasma profiles in children, and to relate them to potential adverse effects and CYP2B6 genotypes (**study III**).
- To assess the prevalence of pretreatment drug resistance (PDR) and its association with virologic outcomes after 24 weeks of ART, within a cohort of Ugandan children (**study IV**).

3 Materials and methods

3.1 Overview

This thesis is built upon four studies. Study I was a retrospective analysis conducted at the pediatric HIV outpatient clinic of Astrid Lindgren's Children's Hospital (ALB), Karolinska University Hospital, Stockholm, Sweden, covering the period from June 2005 to October 2013. Studies II to IV were part of the GENEFA project ("The importance of pharmacoGENetic variation on EFAvirenz levels and treatment outcomes in ART-naïve HIV-infected Ugandan children aged 3-12 years"), a collaborative initiative involving Baylor College of Medicine Children's Foundation-Uganda (Baylor Uganda), Makerere University College of Health Sciences (MakCHS), Uganda, and Karolinska Institutet (KI), Sweden. These were prospective studies conducted at the pediatric outpatient clinic of Baylor Uganda in Kampala, with data collected between 2015 and 2016.

Fig 5. The timeline for studies I-IV.



JCRC: Joint Clinical Research Centre, Kampala, LCHRMS/MS: liquid chromatography high-resolution with tandem mass spectrometry.

Table 1. Methods summary for the Swedish ALB-cohort and the Ugandan GENEFA-cohort

ALB-cohort (study I)			GENEFA-cohort (study II-IV)
Study Design		Retrospective cohort study.	Prospective observational cohort study
		EFV-plasma concentrations from TDM monitoring from children on standard EFV therapy.	EFV-plasma concentrations in ART naïve children, sampled before initiation of standard EFV therapy, 2,6,12,24 weeks thereafter.
Study period		2005–2013	2015–2016
Study site		Pediatric HIV outpatient clinic, ALB, Karolinska University hospital	Baylor Uganda, Kampala, Uganda
Intended sample size		–	100 participants based on power calculation
Eligible for inclusion		Children on current or previous EFV-based therapy < 18 years of age	ART-naïve children aged 3–12 years followed in Baylor starting EFV - based therapy
Exclusion criteria		–No available plasma concentration of EFV –Taking EFV as syrup or in the morning	Concomitant treatment with St Johns worth, carbamazepine, rifampicin
Number of children	screened	43	120
	included	36	99
Origin of participants	African	30 (83%)	99
	Asian	4 (11%)	
	Latin America	2 (6%)	
Median (IQR) Age (years)		9	6
Gender	Female	16 (44%)	59 (60%)
	Male	20 (56%)	40 (40%)
Adverse drug reactions	Collection	From electronic medical record	–Clinician's assessment –Participant questionnaire administered at each study visits from week 0
	Definition	Any symptom/event described in electronic medical record, by clinician as suspected ADR	Any symptom/event reported as suspected ADR, by clinician and/or in questionnaire, week 2–24
Adherence		From electronic medical record: viral load, history of adherence	Pill count

		ALB- cohort (study I)	GENEFA- cohort (study II- IV)
Plasma levels of EFV and EFV metabolites	Sampling	- Routine therapeutic drug monitoring: 2- 3 weeks after treatment start and then at least yearly monitoring. - Only EFV	- Before treatment start and at 2,6,12 and 24 weeks thereafter - EFV and metabolites
	Method of quantification	HPLC, Karolinska University Hospital	LC- HRMS/ MS. Karolinska University Hospital
Variant alleles tested			
CYP2B6	CYP2B6 g.- 82T>C	-	✓
	CYP2B6 g.1582C>T	-	✓
	CYP2B6 g.18492 C>T	✓	✓
	CYP2B6 c.516G>T (CYP2B6*6)	✓	✓
	CYP2B6 c.136A>G (CYP2B6* 11)	✓	✓
	CYP2B6 c.983T>C (CYP2B6* 18)	✓	✓
CYP2A6	CYP2A6 g.- 48T>G (CYP2A6*9)	✓	✓
CYP3A4	CYP3A4_22 G>A	✓	-
CYP3A5	CYP3A5 g.6986A>G (CYP3A5*3)	✓	✓
	CYP3A5 g.14690 G>A (CYP3A5*6)	✓	✓
	CYP3A5 g.727131_27132insT (CYP3A5*7)	✓	✓
ABCB1	ABCB1c.3435 A>G	✓	✓
	ABCB1c.4036 C>T	✓	✓
	Laboratory analysis	Karolinska University Hospital	Karolinska University Hospital
Viral outcomes			
Viral Load	undetectable	<50 cop/ml <20 cop/ml*	<40 cop cop/ml
	viremia/viral failure	>50 cop/ml >20 cop/ml*	≥40 cop cop/ml
	Laboratory analysis	Karolinska University Hospital	Joint Clinical Research Centre, Kampala
HIV drug resistance	System used to define		Stanford HIVdb
	Laboratory analysis		Joint Clinical Research Centre, Kampala

*From 2007

3.2 Material and methods, study I (the ALB-cohort)

3.2.1 Study subjects and design

This was a retrospective study, covering the period of 2005-2013. Enrollment took place in the HIV pediatric outpatient clinic Astrid Lindgrens Barnsjukhus, Karolinska University Hospital, Stockholm. Children with current/ previous EFV-based ART and who had initiated therapy before 18 years of age were eligible if they had at least one EFV plasma concentration, sampled in therapeutic drug monitoring (TDM). In clinical routine, EFV plasma concentration were analyzed 2-3 weeks after EFV initiation. Initial dosing was weight based and followed PENTA-guidelines. Dose adjustments were made in case of suspected adverse drug reactions or to target the therapeutic interval of 1000-4000 ng/ml.

Clinical and laboratory data, including sex, age, ethnicity, weight, adverse effects, EFV dose and plasma concentration, CD4-cell count, and viral load, were obtained from medical records and the Swedish HIV quality register (InfCare HIV) [114]. Viral suppression was defined as VL < 50 cop/ml .

Out of the 43 identified children, 36 were included in the study, while 7 were excluded due to missing data or different medication routines. Genotyping for single nucleotide polymorphisms in genes coding for CYP2B6 and other enzymes of relevance for EFV metabolism and disposition, was performed.

3.2.2 Data and statistical analysis

3.2.2.1 Outcome variable ($\log(e)$ EFV plasma concentration/(dose/weight))

To account for variations that could impact EFV plasma concentration during treatment and across individuals, such as changes in weight and dosage based on EFV levels, the primary outcome variable was EFV plasma concentration divided by (dose/weight). This variable was log-transformed to normalize its distribution before being included as the outcome ($\log(e)$ EFV plasma concentration/(dose/weight)) in a multivariate regression model to explore factors influencing EFV plasma concentrations.

3.2.2.2 Statistical Analysis

We used descriptive statistics, linear regression, Student t-test, Fisher's exact test, and mixed-effects REML regression models to analyze the relationship between EFV plasma concentrations and variables such as weight, dose, sex, age, and gene polymorphisms. Explaining variables that were identified as significant in univariate model, were further examined using multivariate mixed models. EFV plasma concentration/(dose/weight) was log-transformed to normalize the distribution. Data analysis was conducted using JMP 12.1.0 (SAS Institute Inc.Cary, USA) and Stata version 13.1 (Statacorp, Texas, USA), with a significance threshold of $p < 0.05$. No power calculation was performed. Bryk/Raudenbush

R-squared level[115] was used to calculate R² to estimate how much of inter-and intraindividual variation in EFV plasma could be explained by the REML-model.

3.2.3 Laboratory Analyses

3.2.3.1 Quantification of EFV concentration in plasma ALB-cohort

Plasma EFV concentrations were analyzed between June 2005 and October 2013 during clinical follow-ups as a part of routine TDM. Blood samples were collected 14 to 20 hours post-dose and were analyzed at the Department of Clinical Pharmacology, Karolinska University Hospital, by high-performance liquid chromatography and UV detection (HPLC-UV). After centrifugation, a 500 µL aliquot of plasma was stored at -20°C until analysis. After protein precipitation, a 6 µL supernatant sample was injected into an HPLC-UV system (Agilent 1100 HPLC-UV system, equipped with a Luna reversed-phase column (2.5 µm, 50 × 2 mm, Phenomenex, Torrance, CA). Separation was achieved by isocratic elution, and EFV was detected at 210 nm, with quantification at 11 minutes post-injection. The method was calibrated using in-house prepared controls and spiked human plasma and had a quantification range of 158 ng/ml to 31 600 ng/ml. The precision expressed as the coefficient of variation (CV) was 5.7% at 2500 ng/ml. The method performance was monitored by analysis of internal quality control samples and participation in external antiretroviral proficiency testing scheme.

3.2.3.2 Genotyping

Genomic DNA was isolated from 1.5 mL whole blood collected in EDTA using χ (QIAGEN GmbH, Hilden, Germany) and stored at -20°C until analysis. The analysis was carried out in at the Department of Clinical Pharmacology, Karolinska University Hospital as described in 3.2.62.

3.2.3.3 T-cell Populations and Viral Load

VL were routinely analyzed using flow cytometry and Cobas Amplicor (Roche Molecular Systems Inc., Branchburg, New Jersey, USA, respectively, in Karolinska University Hospital). The detection limit for VL was 50 copies/mL before June 2007 and 20 copies/mL thereafter.

3.2.4 Ethical considerations

Written informed consent was obtained from participants and/or their legal guardians after the families had received both oral and written information about the study. The study was approved by the Regional Ethical Review Board in Stockholm, Sweden (No: 2012/1696-31/1). A sample for genotyping was collected alongside the routine blood draws performed during regular clinical visits, to minimize any additional burden on the participants. All other data were extracted from electronic medical records.

3.3 Material and methods, study II-IV (the GENEFA-cohort)

3.3.1 Study subjects and overall design

GENEFA was an observational prospective 6-months study, with data collection performed 2015-2016. Enrollment and follow-up took place in Baylor Uganda, which is located within Mulago National Referral Hospital in Kampala and provides outpatient HIV services to children and their families.

ART-naïve children living with HIV, aged 3-12 years and weighing at least 10 kg, were enrolled. The intended sample size was 100 and was determined using ANOVA, with a significance level of 0.05 and 80% power to detect a mean difference of at least 3 $\mu\text{mol/L}$ (947 ng/mL) in EFV concentrations across CYP2B6 c.516G>T genotypes (GG, GT, TT).

Treatment followed Uganda's national HIV guidelines, with EFV in combination with two NRTIs (abacavir and lamivudine). Mid-dose plasma samples of EFV and its metabolites were collected and quantified at 2, 6, 12, and 24 weeks post-ART initiation. Genotyping was conducted for single nucleotide polymorphisms in genes for CYP2B6, CYP2A6, CYP3A5 and ABCB1, (Table 1). Clinical data was collected at each visit. VL and HIV drug resistance were tested both before and after 24 weeks of ART. Plasma EFV levels were correlated to CYP2B6 metabolizer phenotype, other SNPs and to weight, age, sex, dose, treatment adherence, reported adverse events and treatment results measured as viral failure and acquisition of new HIV drug resistance mutations towards NRTI/ NNRTIs at week 24.

At each visit, adverse drug events and adherence to ART were monitored. Adherence was estimated on a scale from 0 to 100%, based on pill count. A designated clinical study team comprising of a physician, nurse, counselor, laboratory technician and a community officer followed all participants and recorded data into clinical report forms, that were entered into REDCap [116] after quality check. Participants who failed to attend a visit, were contacted by phone and/or a home visit, to ensure follow-up.

3.3.2 Common definitions for data and outcomes, study II-IV

3.3.2.1 CYP2B6 metabolizer phenotype

Participants were categorized according to their composite genotype of the two SNPs CYP2B6 c.516G>T and CYP2B6 c.983T>C, and thereafter assigned a predicted metabolizer phenotype as extensive metabolizer (EM), 516GG|983TT, intermediate metabolizer (IM), 516GG|983TC or 516GT|983TT, slow metabolizer (SM) 516GT|983TC or 516TT|983TT as described [74,117]. No ultraslow metabolizer (USM), 516GG|983CC was identified in our population

3.3.2.2 EFV therapeutic interval

EFV plasma concentrations ranging between 1000, and 4000 ng/ml were considered therapeutic, while concentrations above and below were defined as supra- and sub-therapeutic concentrations respectively.

3.3.2.3 HIV drug resistance and viral suppression

Drug resistance mutations (DRMs) were identified by the 2015 IAS-USA mutations list [118] and was classified according to Stanford HIV Drug Resistance Database (HIVdB), scoring predicted viral resistance levels as *susceptible*, *potentially low*, *low*, *intermediate* or *high* [119].

Pretreatment HIVDR was defined as the presence of baseline DRMs known to confer *any* level of impaired susceptibility to any NRTI/ NNRTI before treatment initiation.

HIVDR at week 24: Only samples with VL \geq 500 copies/mL could be sequenced. The total occurrence of HIVDR at 24 weeks included children who acquired new DRMs and all children with pretreatment HIVDR. Children with PDR but no viremia at week 24, were included as they were considered to still harbor their pretreatment DRMs. Children with VL < 500 copies/mL and no PDR were classified as not having HIVDR in week 24 data analysis.

Acquired HIVDR was defined as acquisition of \geq one new HIVDR mutation after 24 weeks of treatment.

Viral suppression was defined as VL < 40 cop/ml and viral failure as VL \geq 40 cop/ml after 24 weeks of treatment.

3.3.3 Methods study II

All data analysis was based on unconjugated EFV plasma concentration. EFV measurements below the lower limit of quantifications were assigned a concentration value of “0” in statistical computations.

The distribution of plasma EFV across CYP2B6 metabolizer phenotype and visits, were with median (IQR). Comparisons of EFV-concentrations were made in between visits to investigate changes over time, as a sign of autoinduction.

To investigate factors influencing plasma EFV levels a multivariate Restricted Maximum Likelihood (REML)[120] regression was used, where age (years), sex, dose (mg/kg), time from treatment initiation (days), assessed individual mean adherence (%), gene polymorphisms and metabolizer phenotype was correlated to the outcome variable $\log(e)(\text{EFV ng /ml})$. In twelve samples (after week 0), EFV levels were detectable but below the lower limit of quantification (LLOQ), 100 ng/ml. In order not to disregard the effect of

potential polymorphisms leading to increased EFV clearance, the values below LLOQ were assigned an EFV concentration of 99 ng/ml before log(e) transformation.

The impact of sub-therapeutic EFV levels, pretreatment drug resistance and CYP2B6 metabolizer phenotype on viremia and acquired HIV drug resistance (HIVDR) after 24 weeks of standard therapy was assessed.

3.3.4 Methods study III

3.3.4.1 EFV metabolite concentrations, metabolite/EFV ratios and CYP2B6 metabolizer phenotype

To display the relative quantity of phase I and phase II metabolites compared to EFV in plasma, metabolite /EFV ratios were calculated. The metabolites analyzed are shown in Figure 9. and further described in section 3.3.5.1

The metabolite ratio of 8-OH-EFV/EFV has been proposed as a marker for CYP2B6 activity. However, in this study, the ratio of (8-OH-EFV + EFAdeg)/EFV was selected instead. This choice was based on two key assumptions: first, that 8-OH-EFV and EFAdeg exist in constant equilibrium, meaning both metabolites should be included when evaluating CYP2B6 activity; and second, that the (8-OH-EFV + EFAdeg)/EFV ratio was more accurate, compared to ratios involving total metabolites (8-OH-EFV-tot and EFAdeg-tot), which also captures phase II metabolism.

The association between factors as CYP2B6 metabolizer phenotype, gender, age, mean adherence, time on treatment and EFV dose/weight with log(8-OH-EFV + EFAdeg/EFV) was investigated using REML.

CYP2B6 autoinduction

CYP2B6 autoinduction was investigated in the REML using log(e)(8-OH-EFV+ EFAdeg/EFV) as the outcome measure. An interaction term (metabolizer phenotype × time on treatment) was included to assess differences in metabolite/EFV ratios over time in between different metabolizer phenotypes.

3.3.4.2 Adverse drug reactions

Adverse events were monitored using a questionnaire adapted from Gounden et al [121], administered by the physician at baseline and all ensuing visits to the caretakers or directly to children aged eight and above. It assessed symptoms experienced in the two weeks prior to the visit, including CNS symptoms, gastrointestinal symptoms and rash. Participants indicated how often symptoms affected their daily activities (“never,” “sometimes,” or “most times”). The clinician assessed and graded the severity of adverse reactions based on WHO standards. CNS-symptoms was investigated for association with EFV therapeutic intervals, the distribution of EFV metabolite concentrations and with CYP2B6 metabolizer phenotype.

3.3.5 Statistical methods study II-IV

We used the software Stata version 14.2 and 17.0, StataCorp LLC, Texas. P-values <0.05 were considered significant and were calculated two-sided.

Table 2. Statistical methods in Study II-IV

Statistical Analysis	Data and Outcomes Analyzed
Multivariate mixed-effects restricted maximum likelihood regression model (REML)	Used random intercepts for individuals, random slopes for treatment time, and other variables as fixed effects (Study II and III).
Bryk/Raudenbush R-squared level	Estimated inter- and intra-individual variation in EFV plasma levels explained by the REML model (Study II).
Wald test with Holm correction	Post-regression estimation to assess the impact of time on log(e)EFV, depending on metabolizer phenotype (Study III).
Kruskal-Wallis (KW) test	Analyzed differences across metabolizer phenotypes for adherence, EFV and metabolite plasma concentrations (Study III).
Conover-Iman test (Holm-adjusted)	Followed KW test for pairwise comparisons, correcting for multiple testing (p-value < 0.025 considered significant) (Study II-III).
Wilcoxon Rank Sum test	Investigated EFV concentration distribution by gender and compared EFV metabolite distributions between participants with/without CNS symptoms. (Study II-III).
χ^2 test and Fisher's exact test	Compared categorical variables (Study II- IV).
Wilcoxon Signed Rank Test	Tested within-subject changes in EFV levels over time (Study II).
Logistic regression	Assessed the association between baseline characteristics and 24-week outcomes virologic suppression and acquired HIVDR (Study IV).
T-tests	Compared means between groups. (Study IV)
Mann-Whitney U test	Compared medians between groups. (Study IV)

3.3.5.1 Quantification of EFV and metabolites concentration in plasma

Samples for EFV and EFV metabolites were collected 14-20 hours after medicine intake. They were stored at -80°C. The samples were transported to Sweden and were analyzed using a liquid chromatography high-resolution tandem mass spectrometry (LC-HRMS/MS) method developed by the Department of Clinical Pharmacology at the Karolinska University Hospital [49].

After protein precipitation, one aliquot was used to measure unconjugated EFV and phase I metabolites (7-OH-EFV and 8-OH-EFV), while the second aliquot was hydrolyzed (mediated by β -glucuronidase/arylsulfatase) to measure total concentrations of phase I and II metabolites: EFV-tot (the total concentration of EFV + EFV-N-glucuronide), 8-OH-EFV-tot

(the total concentration of 8-OH-EFV+ 8-OH-EFV-glucuronide/sulfate) and 7-OH-EFV-tot (the total concentration of 7-OH-EFV + 7-OH- glucuronide/sulfate) (Fig. 10).

Additionally, the newly identified compound EFAdeg (6-Chloro-4-[3-cyclopropyl-1-hydroxy-1-(trifluoromethyl)-2-propyn-1-yl]-2(3H -benzoxazolone) was quantified, EFAdeg. The plasma concentrations were measured for unconjugated EFAdeg (EFAdeg), and for the total concentration of EFAdeg + EFAdeg-conjugates (EFAdeg-tot), as described for EFV and 7-OH-EFV and 8-OH-EFV. The phase II metabolites of 8-OH-EV, 7-OH-EFV and EFAdeg were not individually quantified, as the method could not differentiate between the contribution from glucuronidated and sulfated substances.

Chromatographic separation was achieved using a Dionex Ultimate 3000RS UHPLC system with an RP C18 column. Detection was carried out using a Q-Exactive system in both full MS and PRM mode. The measurement range was 100-50,000 ng/mL for EFV and related compounds, 125-25,000 ng/mL for 7-OH-EFV, and 200-10,000 ng/mL for EFAdeg.

3.3.5.2 Genotyping

We reviewed the literature to identify polymorphisms in EFV drug-metabolizing enzyme genes and ABCB1 (Table 1). The analysis was performed at the Department of Clinical Pharmacology, Karolinska University Hospital. Genomic DNA was extracted from 1.5 mL of whole blood collected in EDTA tubes using the QIAamp DNA MiniKit (QIAGEN GmbH, Hilden, Germany). Samples were stored initially at -80°C in Kampala and stored in -20 °C after transport to Sweden. Genotyping was performed using validated TaqMan assays (Thermo Fisher Scientific) following the manufacturer's protocols, with analysis conducted on a StepOnePlus Real-Time PCR system (Life Technologies).

3.3.5.3 Viral Load and HIV drug resistance mutations

Genotypic resistance was assayed by sequencing of the reverse transcriptase region using 3730xl Applied Bio-systems platform (Life Technologies). Sequences were edited in REcall (beta V3.01), and a web-based HIV drug resistance sequence analysis software (BC Centre for Excellence in HIV/AIDS) and then entered in HIVdB to obtain drug resistance profiles and HIV subtypes. Viral load was detected and quantified with the Abbottm2000sp/rt platform (Abbott Laboratories) using Abbott RealTime HIV-1 assay. The lower limit of detection was 40 copies/ml. The analyses were performed in Joint Clinical Research Centre, (JCRC), Kampala ,Uganda.

3.3.6 Ethical considerations

Written informed consent was obtained from participants and/or their legal guardians after the families had received both oral and written information about the study. The study was approved by the Ethical Institutional Review Boards of School of Biomedical Sciences and Higher Degrees, Makerere University College of Health Sciences (SBS-HDREC 174), Uganda,

National Council for Science and Technology UNCST (HS1659), Baylor College of Medicine Children's Foundation IRB Texas (H35946) and the Regional Ethical Review Board in Stockholm, Sweden228 (2016/1026-31). Treatment and services offered followed the standard clinical routines of Baylor Uganda.

4 Results

4.1 Study populations

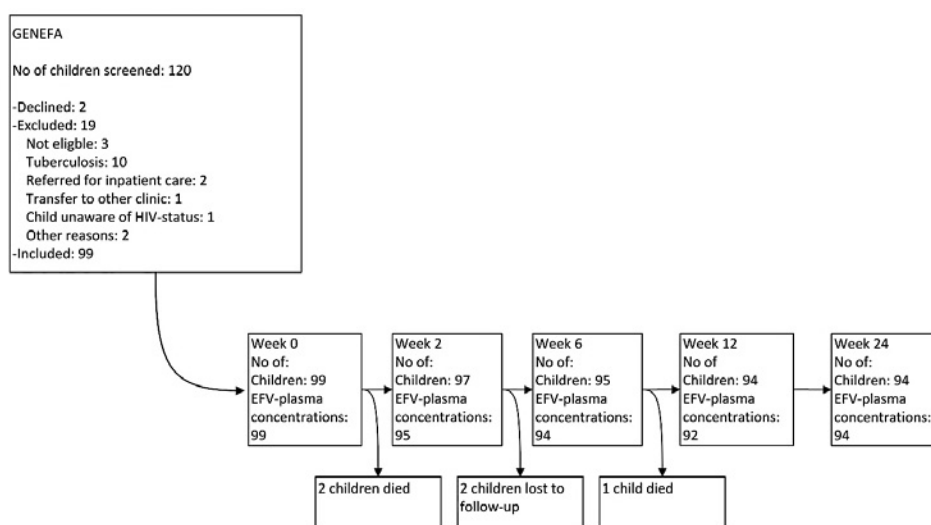
4.1.1 The ALB-cohort (study I)

Thirty-six children were included, 56% boys and 44 % girls. The cohort included individuals from diverse ethnic backgrounds, with 83% of African origin, 11% of Asian origin and 6% of Latin American origin. The median age at treatment start was 9 years (range 2-17 years) and the median time of EFV based therapy was 48 months (range 1-121 months).

For 12 children, dose adjustments directed by therapeutic drug monitoring had been made. Thirteen participants discontinued their EFV-based therapy within the study period due to various reasons including suspected adverse effects (n = 5), viral resistance to EFV (n = 6) and other reasons (n = 2). There were no deaths.

4.1.2 The GENEFA-cohort (study II-IV)

Figure 5. GENEFA study flow chart



Out of 120 screened, 99 ART-naïve Ugandan children aged 3-12 years were enrolled and initiated EFV based antiretroviral therapy, 2015-2016 in Baylor Uganda (Figure 5). The median age at enrolment was 6 years with 60% girls and 40% boys. Out of the 99 participants included, only one child reported a history of PMTCT. All other participants reported either no (85%) or unknown history (14%) of PMTCT. The adherence reported by pill count was high with a visit median (range) varying 96% -97% (52–100%) across the

study. There were no significant differences in adherence based on sex, age, or metabolizer phenotype.

Table 3. Baseline characteristics of the GENEFA-cohort

TABLE 1 Baseline characteristics of a cohort of 99 ART-naïve Ugandan children aged 3-12 y, followed for 24 wk after initiating efavirenz-based ART at Baylor Uganda during 2015-2016

Variable		Percentage
Sex (Females)	59	60
Age (years)	6.2 (4.2-8.3)	
WHO Clinical stage 3/4	18	18
CD4 ⁺ absolute count (cells/μL)	545 (249-880)	
CD4 ⁺ (%)	17 (8-24)	
Immunodeficiency [†]		
- None	38	39
- Mild	16	16
- Advanced	32	32
- Severe	13	13
Primary caregiver		
- Mother/Father	59	60
- Other	40	40
Education, primary caregiver		
- None	10	10
- Primary	50	51
- Secondary or higher	39	39
Maternal PMTCT [‡]		
Yes	1	1
No	84	85
Unknown	14	14
Infant PMTCT		
Yes	0	0
No	83	84
Unknown	16	16
Viral load (copies/mL) (LoB ₁₀)	108,164 (29,666-423 365) 5.04 (4.42-5.63)	
[§] PDR (n = 90)	NNRTI and/or NRTI ^{††}	18 20
	NNRTI drug resistance	16 18
	NRTI drug resistance	4 4
	EVF/NVP drug resistance ^{‡‡}	12 13
HIV subtype (n = 90)	A	57 63
	D	25 28
	C	6 7
	B	1 1
	CRF02_AG [¶]	1 1

[†]According to WHO classification of immunodeficiency, based on CD4 percentage (% CD4⁺) and CD4⁺ absolute count of cells/μL (CD4⁺): None: % CD4⁺ >25% in children <5 y; CD4⁺ ≥500 in children ≥5 y; Mild: % CD4⁺ = 20%-35% in children <5 y; CD4⁺ = 350-499 in children ≥5 y; Advanced: % CD4 = 15%-19% in children <5 y; CD4⁺ = 200-349 in children ≥5 y; and Severe: % CD4 < 15% in children < 5 y; CD4⁺ < 200 or % CD4⁺ <15% in children ≥ 5 years.

[‡]PMTCT: prevention of mother-to-child transmission.

[§]PDR pretreatment drug resistance. CRF02_AG: Subtype A/G recombinant form.

^{††}NNRTI: non-nucleoside reverse transcriptase inhibitors, NRTI: nucleoside reverse transcriptase inhibitors.

^{‡‡}EVF/NVP: efavirenz and/or nevirapine. N = 99 unless otherwise stated.

Table 3. Adapted from Soeria-Atmadja et al. "Pretreatment HIV drug resistance predicts accumulation of new mutations in ART-naïve Ugandan children". Acta Paediatr 109(12): 2706-2716 [122].

4.2 EFV plasma concentration - variation and therapeutic interval

In the ALB cohort, 182 measurements of EFV plasma concentrations, were included in the analysis. In average, the participants contributed with 5 measurements each (range 1-11). In the GENEFA-cohort, 97 participants contributed with a total of 375 EFV-measurements from week 2 and onwards.

As expected, there was considerable intra- and interindividual variation in EFV plasma concentrations in both cohorts. In the ALB cohort for instance, EFV levels fluctuated up to 21-fold between measurements, while in the GENEFA cohort, the typical within-individual variation was 2-fold (median value), though in one participant, it reached as high as 113-fold.

EFV plasma levels outside the recommended therapeutic interval were common in both cohorts. In the GENEFA cohort, 60 to 66% of individuals had plasma EFV levels within the recommended therapeutic range across the sampling periods. Meanwhile, 12 to 17% of children had levels below the therapeutic range, and 21 to 24% had levels above it. Among participants in the ALB-cohort, the individuals had EFV plasma levels at first visit, that were below, within and above the target range in 8%, 70% and 22% respectively (unpublished).

Mean EFV concentrations among participants varied in the ALB-cohort, although they had similar mean EFV dose/weight across the study, Figure 6.

Figure 6. Interindividual variability in mean EFV plasma concentrations and mean dose/weight.

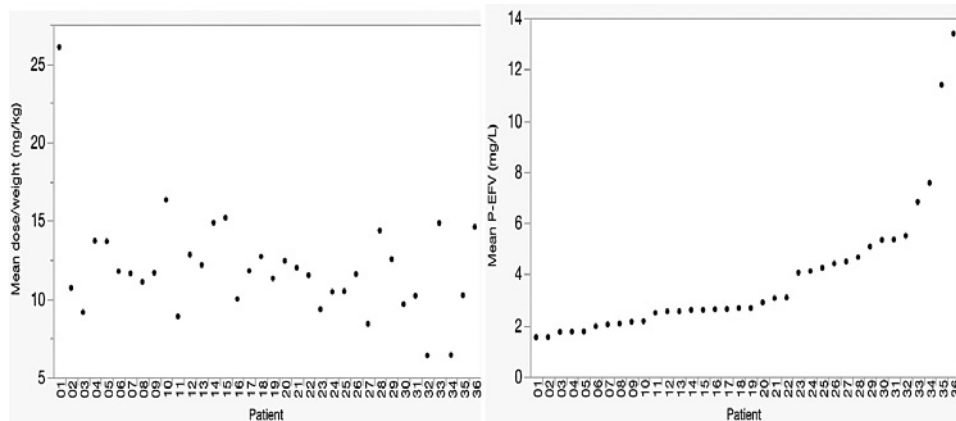


Figure 6. Variation in the mean dose/weight (left) and EFV mean plasma concentration (right) in all included patients (n = 36). Soeria-Atmadja S, et al. “Genetic variants in CYP2B6 and CYP2A6 explain interindividual variation in efavirenz plasma concentrations of HIV-infected children with diverse ethnic origin”. PLoS One. 2017;12(9) [63].

In both cohorts, the (weight-adjusted) efavirenz (EFV) dose, did not appear to account for the variation in plasma EFV concentrations. In the ALB cohort, plasma EFV concentrations from the initial sampling—before any dose adjustments—showed no significant correlation with dose per weight in a linear regression model ($p = 0.63$). Similarly, in the GENEFA cohort, EFV dose per weight was not identified as a predictor of plasma EFV concentration in a multivariate REML regression analysis.

4.3 Pharmacogenetic influence on EFV plasma concentrations

EFV plasma concentration was significantly associated with several polymorphisms in CYP2B6 in both cohorts (Table 4). Additionally, in the ALB cohort, EFV plasma levels showed a significant correlation with a polymorphism in CYP2A6. However, no significant associations were found between EFV plasma concentrations and polymorphisms in CYP3A4, CYP3A5, or ABCB1 in either cohort.

4.3.1 Distribution of genotypes, minor allele frequency and metabolizer phenotype

Table 4 summarizes the distribution of the genotypes and minor allele frequency for all SNPs found to have a significant effect on EFV plasma concentration in any of the two cohorts. It also displays the distribution for the probable metabolizer phenotypes, predicted by the composite CYP2B6 c.516G>T and CYP2B6 c.983T>C genotype.

The metabolizer phenotype data for the ALB-cohort is previously unpublished and indicated that 36.3%, 42.3% and 21,4% of participants were extensive, intermediate and slow metabolizers respectively. This distribution varied significantly among participants of different geographical origin ($p=0.027$). All 4 children of Asian origin were extensive metabolizers, while the 2 children of Latin American origin were both intermediate metabolizers. Among participants of African origin, 27%, 50%, and 23% were extensive, intermediate and slow metabolizers respectively.

Table 4. Distribution of genotypes, minor allele frequency and metabolizer phenotype

	Genotype	ALB-cohort					GENEFA-cohort	
		African	Asian	Latin American	N	Frequency %	N	Frequency %
CYP2A6*9 (rs28399433)	AA	26	2	1	29	81	87	90
	AC	4	1	1	6	17	9	9
	CC	0	1	0	1	3	1	1
Minor allele	C	4	3	1	8	11	11	6
CYP2B6*6 (rs3745274)	GG	10	4	1	15	42	38	39
	GT	15	0	1	16	44	48	50
	TT	5	0	0	5	14	11	11
Minor allele	T	25	0	1	26	36	60	36
CYP2B6*11 (rs35303484)	AA	28	4	2	34	94	96	99
	AG	1	0	0	1	3	1	1
	GG	1	0	0	1	3	0	0
Minor allele	G	3	0	0	3	4	1	0.5
CYP2B6*18 (rs28399499)	CC	0	0	0	0	0	0	0
	CT	4	0	1	5	14	14	14
	TT	26	4	1	31	86	83	86
Minor allele	C	4	0	1	5	7	14	7
CYP2B6 g.15582C>T (rs4803419)	CC	-	-	-	-	-	86	88
	CT	-	-	-	-	-	9	9
	TT	-	-	-	-	-	2	2
Minor allele	T	-	-	-	-	-	13	7
Metabolizer phenotype	Composite							
Extensive		8	4	0	12	33	28	29
Intermediate		15	0	2	17	47	54	56
Slow		7	0	0	7	20	15	15

Table 4. Distribution of SNPs that were found to have a significant impact on plasma EFV concentrations in any of the two cohorts. In the GENEFA cohort, the CYP2B6 c.516G>T and CYP2B6 c.983T>C genotypes were analyzed together as composite genotypes and grouped by metabolizer phenotypes, meaning their combined effect was assessed.

4.3.2 CYP2B6 metabolizer phenotype and EFV plasma concentrations

In the GENEFA cohort, median EFV plasma concentrations per visit varied significantly ($p=0.0001$) according to participants' metabolizer phenotypes, as predicted by the composite genotype of two SNPs: CYP2B6 c.516G>T and CYP2B6 c.983T>C. (Table 5, Figure 7). Slow metabolizers consistently exhibited markedly higher median EFV levels compared to the other metabolizer phenotypes throughout the study ($p<0.0001$, week 2-24), while the difference between intermediate and extensive metabolizers became significant from week 6 and onwards. Notably, four intermediate metabolizers repeatedly experienced very high EFV levels.

Table 5. Distribution of metabolizer phenotype over EFV plasma concentration intervals and median EFV concentrations per visit

Metabolizer classification based on composite genotypes	Number (%) of children at different concentration intervals (defined by individual median EFV plasma concentrations)				Median EFV concentration (ng/ml) (IQR) per visit			
	<1000 ng/ml(%)	1000–4000 ng/ml(%)	>4000 ng/ml(%)	N (100 %)	Week 2*	Week 6*	Week 12*	Week 24*
All	9 (9.3)	66 (68.0)	22 (23.7)	97	2204 (1358–3954)	2003 (1285–3225)	2473 (1536–3761)	2618 (1524–3875)
EM	5 (17.9)	23 (82.1)	0	28	1577 (1181–2185)	1525 (1073–1853)	1909 (1486–2340)	1537 (1058–2196)
516GGI983TT	5 (17.9)	23 (82.1)	0	28				
IM	4 (7.4)	43 (79.6)	7 (13)	54	2120 (1358–3142)	2237 (1197–2977)	2456 (1425–3410)	2786 (1767–3294)
516GGI983TC	1 (10)	7 (70)	2 (20)	10				
516GTI983TT	3 (6.8)	37 (84.1)	4 (9.1)	44				
SM	0	0	15 (100)	15	10052 (7078–12821)	11621 (7155–14961)	9864 (5121–16260)	12725 (8192–18706)
516GTI983TC			4	4				
516TTI983TT			11	11				

Distribution over different EFV concentration intervals varied significantly between metabolizer phenotypes ($p=0.000$). *Kruskal Wallis equality of population rank test showed statistically significant differences in EFV plasma levels among categories of slow (SM), intermediate (IM) and extensive metabolizers (EM) across the study period: visit 2 ($\chi^2=36.541$, $p=0.0001$); visit 6 ($\chi^2=32.525$, $p=0.0001$); visit 12 ($\chi^2=34.759$, $p=0.0001$) and visit 24 ($\chi^2=44.127$, $p=0.0001$).

Figure 7. Distribution of EFV plasma levels over CYP2B6 metabolizer phenotype

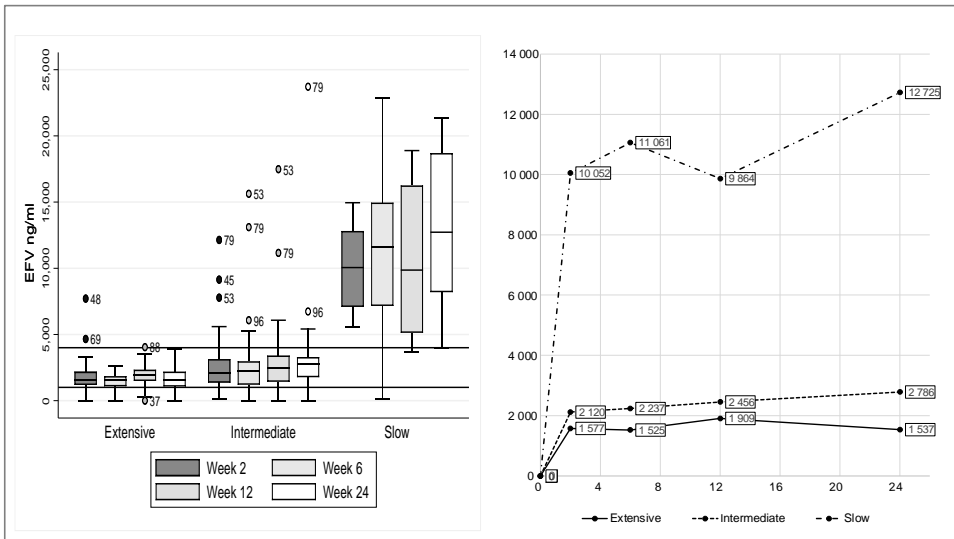


Figure 7: Extensive (EM), intermediate (IM) and slow metabolizer (SM) type based on composite genotype of CYP2B6 516G>T/983T>C, with 28, 54 and 15 children in each group. In total, 97 individuals contributed to 375 EFV plasma samples with 95, 94, 92 and 94 samples at week 2, 6, 12 and 24, respectively.

Left: EFV-concentrations (ng/ml) week 2-24. Line inside box denote median, while lower and upper box boundaries represent 25th and 75th percentiles, respectively. Data points more than 1,5 box-lengths away from 25th or 75th percentiles are represented by dots, marked with study-ID. Horizontal lines display therapeutic EFV plasma concentration interval of 1000-4000 ng/ml.

Right: Median EFV concentration per visit and metabolizer type, by weeks from therapy start. EFV concentrations (ng/ml) on y-axis and weeks on x-axis.

In the GENEFA cohort, a multivariate mixed-effects model (REML) regression analysis, adjusted for gender, time on treatment, age, mean adherence, dose per weight and other polymorphisms, confirmed that the CYP2B6 metabolizer phenotype was a significant predictor of EFV plasma concentrations and that both IM and SM phenotype were significantly associated with higher log(e) EFV plasma concentration compared to EM ($p=0.03$ and 0.00 , respectively).

In the ALB-cohort, median EFV plasma levels were 1910 ng/mL in extensive metabolizers, 2710 ng/mL in intermediate metabolizers, and 9310 ng/mL in slow metabolizers ($p < 0.0001$). Pairwise comparisons (with Holm-adjusted Conover-Iman test) demonstrated

that these differences were statistically significant between slow and intermediate metabolizers ($p < 0.0001$) and between slow and extensive metabolizers ($p = 0.0003$). (Unpublished.)

4.3.3 Other polymorphisms in CYP2B6 and CYP2A6 influencing EFV plasma concentrations

In the ALB cohort, all genotypes were analyzed in individual REML regression models adjusted for age, sex, and time on treatment. A significant positive association with log-transformed EFV concentration/(dose/weight) emerged for the following genotypes: CYP2A6*9 A/C (CYP2A6 g.-48T>G), CYP2B*6 g.18492 C/T, CYP2B6*6 T/T (CYP2B6 c.516G>T), and CYP2B6*11 G/G (CYP2B6 c.136A>G). When these genotypes were examined together in an adjusted multivariate REML analysis, the significant association persisted for **CYP2A6*9 A/C** (CYP2A6 g.-48T>G), **CYP2B6*6 T/T** (CYP2B6 c.516G>T), and **CYP2B6*11 G/G** (CYP2B6 c.136A>G), indicating that carriers of these genotypes had higher EFV plasma concentrations compared to individuals with the corresponding “wildtype” variant. CYP2B6*18 T>C (CYP2B6 c.983T>C) was not included in the multivariate regression model, as it was not identified as a SNP associated with EFV plasma concentration in individual REML.

In the GENEFA cohort, in addition to metabolizer phenotype (predicted by the combined effect of CYP2B6 c.983T>C and c.516G>T), two more variants in CYP2B6 displayed a significant correlation to log(e) EFV plasma concentrations in the adjusted multivariate REML. **CYP2B6 g.15582C>T/T** genotype predicted lower log(e) EFV, while **CYP2B6*11 A/G** (CYP2B6 c.136A>G) predicted higher log(e) EFV plasma concentration.

Interestingly, **CYP2B6*11** (CYP2B6 c.136A>G) was found in three outliers. Two had been classified as extensive metabolizers in the ALB cohort, but had median EFV concentrations of 5560 and 6340 ng/ml. One was an intermediate metabolizer in GENEFA with a median EFV-concentration of 12500 ng/ml.

4.3.4 EFV-variability explained by pharmacogenetics

Bryk/Raudenbush R-squared [115] metrics assessed to what extent the variability in EFV plasma levels were explained by the genetic polymorphisms and other factors in the REML-models. Level 1 represents intraindividual variation, while level 2 reflects interindividual variation. In the GENEFA cohort, the multivariate REML model explained 1.04% of the variation within individuals (level 1) and 70.1% of the variation between individuals (level 2). In the univariate REML models, these values were 0.03% and 49.9% for metabolizer phenotype, 0% and 3.22% for CYP2B6 c.136A>G, and -0.06% and 12.8% for CYP2B6 g.15582C>T.

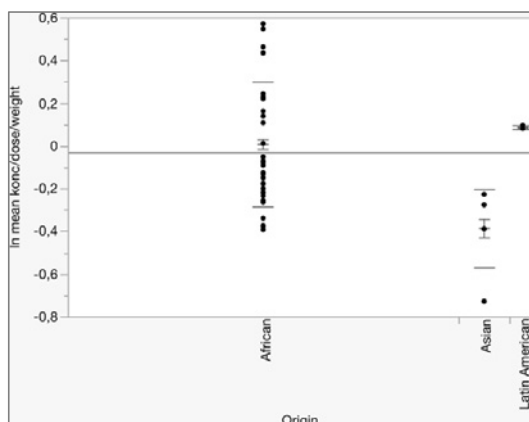
Similarly, in the ALB cohort, the multivariate REML model accounted for 6% of the variation within individuals and 75% of the variation between individuals in EFV plasma

concentrations. The univariate models showed 0.2% (level 1) and 21% (level 2) for CYP2B6*6, -0.05% and 11% for CYP2B6*11, and -0.5% and 14% for CYP2A6*9.

4.4 Other factors influencing EFV plasma concentration

The multivariate REML models identified time on treatment as a positive predictor of EFV plasma concentration in both cohorts. Age was significantly associated with higher EFV plasma concentrations, among participants from the ALB cohort. In the GENEFA cohort, the individual mean adherence showed a significant positive correlation with EFV plasma concentration. No significant gender difference in EFV-plasma concentrations was found in either cohort. Unpublished results indicate that the distribution of the initial EFV plasma concentration (measured before dose adjustments) differed among the three ethnic groups and was significantly higher in children of African heritage compared to children of Asian origin ($p=0.0023$).

Figure 8. The distribution of $\log(e)$ mean plasma EFV concentration according to the ethnicity of participant



Number of patients 36 (African 30, Asian 4, Latin American 2), number of samples 182. Mean, standard deviation and 95% confidence interval are incorporated. Adapted from Soeria-Atmadja, S., et al. (2020). "Pretreatment HIV drug resistance predicts accumulation of new mutations in ART-naïve Ugandan children." *Acta Paediatr* 109(12): 2706-2716

4.5 EFV metabolites (study III)

The phase I and II metabolites of EFV (including the newly found substance EFAdeg), were only investigated in the GENEFA cohort and are shown in Figure 9.

Figure 9. Efavirenz and metabolites detected and quantified in GENEFA

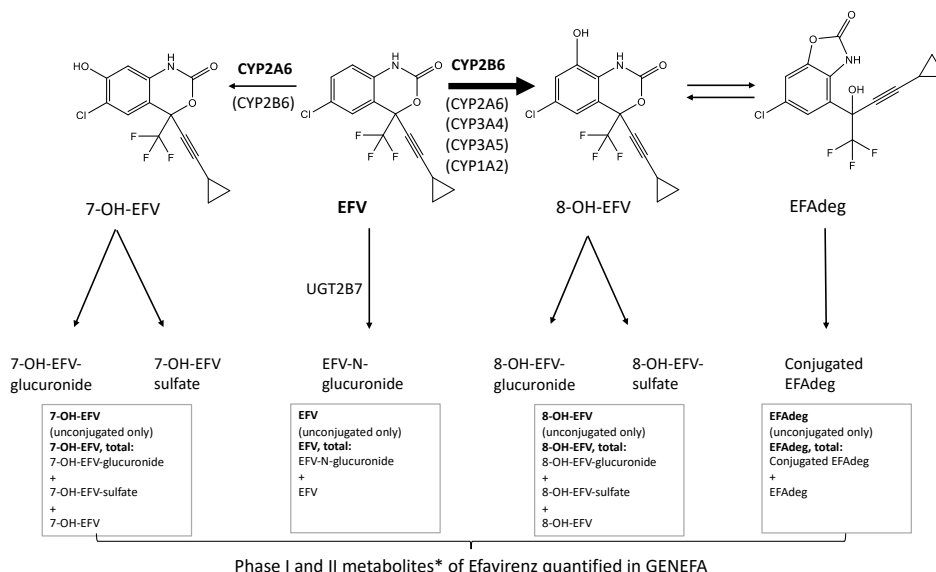
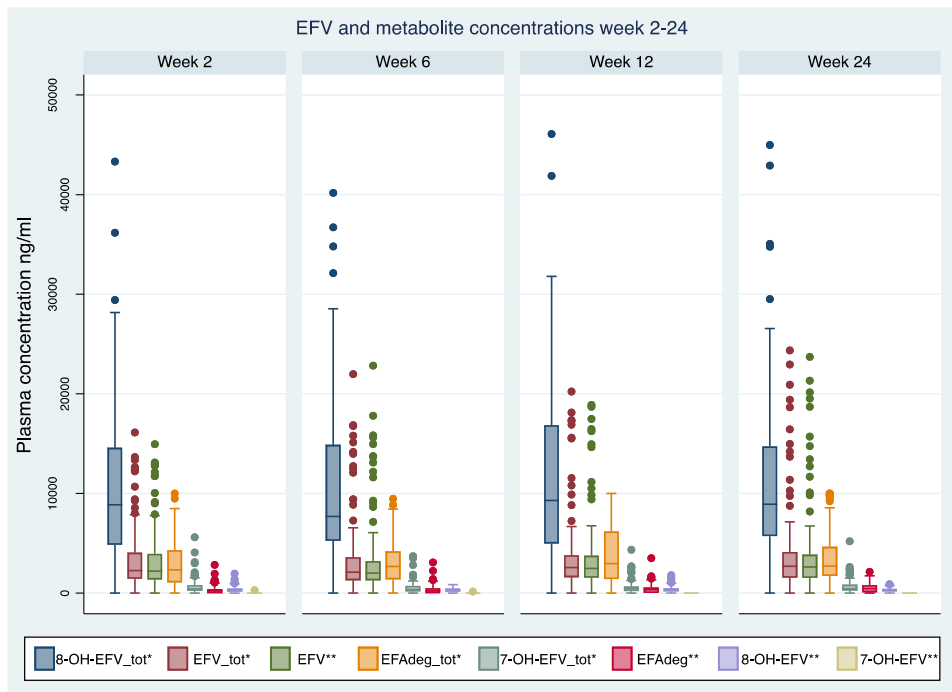


Figure 9. Plasma EFV and metabolites were detected and quantified with a method using liquid chromatography, high-resolution tandem mass spectrometry (LC–HRMS/MS). Total concentrations include both phase I (8-OH-EFV, 7-OH-EFV) and phase II metabolites as the phase II metabolites 8-OH-EFV glucuronide, 8-OH-EFV sulfate, 7-OH-EFV glucuronide, 7-OH-EFV sulfate and EFV-N-glucuronide were detected but could not be individually quantified. During development of the LC–HRMS/MS method, a new substance was found, hypothesized to be an 8-OH-EFV degradation product ("EFAdeg"). *Desta Z et al. [71].

For the entire cohort, 8-OH-EFV tot (including both unconjugated and conjugated forms) was the most abundant analyte, followed by EFAdeg-tot (conjugated and unconjugated), as the second most common. Throughout the study, 8-OH-EFV and EFAdeg made up a minor part of the 8-OH-EFV-total and EFAdeg-total quantified meaning that phase II metabolites 8-OH-EFV sulfate/ 8-OH-EFV glucuronide dominated in plasma. 7-OH-EFV could only be quantified in two samples, both from slow metabolizers. The EFV-N-glucuronide contribution to circulating EFV remained limited across the study visits with a median EFV-tot/EFV unconjugated ratio of 1:1.

Figure 10. The distribution of the mid dose plasma concentrations for EFV and its metabolites by weeks 2, 6, 12 and 24.



*Total concentration of unconjugated and conjugated substance. **Concentration for unconjugated substance only. Number of successful measurements per analyte and visit for week 2,6, 12, 24: 8-OH-EFV-tot 88, 92, 87, 92; EFV-tot 95, 91, 92, 94; EFV 95, 94, 92, 94; EFAdeg-tot 87, 91, 88, 92; 7-OH-EFV-tot 95, 93, 92, 94; EFAdeg 87, 91, 88, 92; 8-OH-EFV 89, 92, 87, 92; 7-OH-EFV 95, 93, 92, 94. Line inside box denotes the median, while lower and upper box boundaries represent the 25th and 75th percentiles, respectively. Data points more than 1,5 box-lengths away from 25th or 75th percentiles are represented by dots.

4.5.1 Efavirenz metabolite profiles and CYP2B6 metabolizer phenotypes

Distinct differences in metabolite profiles according to metabolizer phenotypes could be discerned. Among the extensive and intermediate metabolizers, 8-OH-EFV-tot dominated, while EFV-tot was predominant in slow metabolizer. The distribution of EFV and EFV metabolites plasma concentrations and their corresponding metabolite/EFV ratios were investigated by metabolizer phenotypes and is displayed in Figure 11.

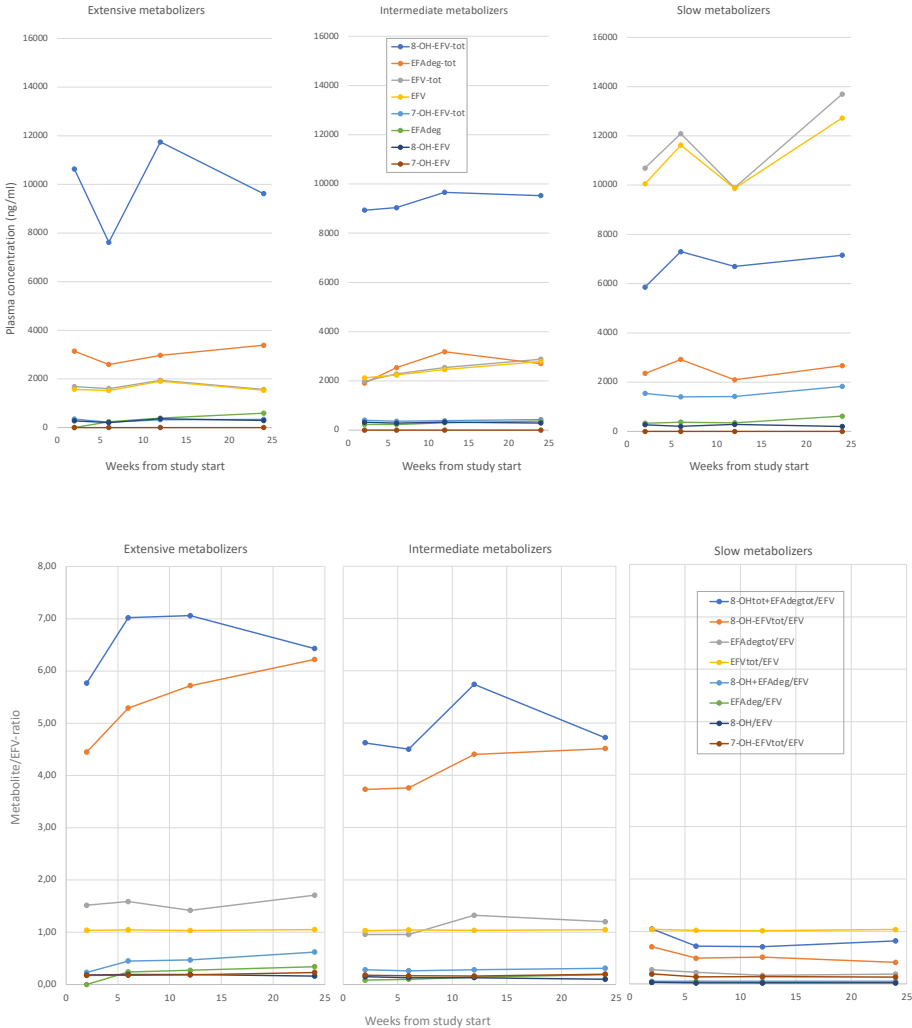
In this study, the ratio of (8-OH-EFV + EFAdeg)/EFV was selected as a marker for CYP2B6 activity. This metabolite/EFV ratio varied significantly between the three metabolizer phenotypes, where slow metabolizers had significantly lower median values for 8-OH-EFV-

tot, EFAdeg-tot, 8-OH-EFV, and EFAdeg, ($p < 0.0001$ to $p = 0.0097$), compared to the others. These differences were confirmed through multivariate REML analysis, using the log-transformed metabolite/EFV ratio of (8-OH-EFV + EFAdeg). In this analysis, metabolizer phenotype was a key predictor, with significantly higher log-transformed metabolite/EFV ratios in intermediate metabolizers (IM, coefficient=1.7) and extensive metabolizers (EM, coefficient=2.3) compared to slow metabolizers (SM, coefficient=-2) ($p < 0.0001$). A significant difference was also discerned between EM and IM.

The pattern was similar, for both for 8-OH-EFV and EFAdeg when investigated as log-transformed metabolite/EFV ratio, in (separate) REML analyses, where significant differences were observed for EM vs SM, IM vs SM and between EM and IM. However, when repeating the analysis for 8-OH-EFV-tot and EFAdeg-tot (both representing phase I *and* phase II metabolism), significant differences were only observed between EM/IM and SM.

The median plasma concentration of total 7-OH-EFV was consistently higher in SM compared to IM and EM ($p < 0.0001$), a result confirmed by multivariate REML analysis of log-transformed 7-OH-EFV levels ($p < 0.0001$). No significant differences were found in the EFV-tot/EFV ratio across the three metabolizer phenotypes.

Figure 11. Plasma levels for EFV, metabolites and metabolite/EFV-ratio by CYP2B6 metabolizer phenotype



Top figure: Plasma concentrations, median per visit and metabolizer type, by weeks from therapy start. 8-OH-EFVtot (8-OH-EFV + 8-OH-EFV-sulfate+ 8-OH-glucuronide), 7-OH-EFVtot (7-OH-EFV + 7-OH-EFV-sulfate+ 7-OH-glucuronide), EFV-tot (EFV + EFV-N-glucuronide), EFAdeg-tot (EFAdeg + EFAdeg-conjugate). Bottom figure: Metabolite: EFV ratio, median per visit and metabolizer type, by weeks from therapy start. EFAdeg was hypothesized to be an 8-OH-EFV degradation product in equilibrium with 8-OH-EFV, why metabolite: EFV ratio was calculated also for the combined concentrations of 8-OH-EFV and EFAdeg and the combined concentrations of 8-OH-EFV-tot + EFAdeg-tot.

4.6 Autoinduction

Changes in EFV plasma concentrations over time was used to estimate autoinduction. In the ALB cohort, multivariate REML analysis revealed a significant increase in EFV concentrations with time $\log(e)(\text{EFVconc}/\text{mg}/\text{kg})$. Similar observations were made in the GENEFA cohort, both when comparing potential changes in distribution across visits within individuals and in a multivariate REML analysis of predictors for $\log(e)(\text{EFV})$ plasma concentration (study II).

Autoinduction was further explored (GENEFA), in the multivariate REML analysis where $\log(e)((8\text{-OH-EFV} + \text{EFAdeg})/\text{EFV})$ was the outcome variable. An interaction term (days on treatment \times metabolizer phenotype) was added to assess whether there was an effect of time on the outcome, depending on metabolizer phenotype. The slopes for "days on treatment" were 0.0026 for extensive (EM), 0.00060 for intermediate (IM), and -0.0013 for slow metabolizers (SM), with only the EM slope significantly different from zero ($p=0.027$). Pairwise comparisons suggested a potential difference between EM and SM slopes ($p=0.037$), but this became non-significant after adjusting for multiple testing ($p=0.107$). Overall, the model found no evidence of decreasing EFV plasma concentrations in the full cohort or within metabolizer subgroups, suggesting a lack of autoinduction (study IV).

4.7 Virological outcomes-ALB-cohort (Study I)

The final viral load sampled during the study period was used to assess viral suppression. Viral suppression was achieved in 28 participants, while eight participants had viral loads exceeding 50 copies/ml. There was no significant difference in treatment effect as indicated by viral load status (above or below 50 copies/mL), in relation to the mean EFV plasma concentration (Fisher's exact test, $p = 1.0$). Pretreatment or acquired HIV-drug resistance was not investigated.

4.8 Virological outcomes-GENEFA-cohort (study II and IV)

4.8.1 Pretreatment HIV-drug resistance (study IV)

Before treatment initiation, genotypic HIV drug resistance data were available for 90 out of 99 participants with 20% showing resistance to NNRTIs or NRTIs.

Figure 12. Drug resistance profiles and NRTI and NNRTI drug resistance mutations

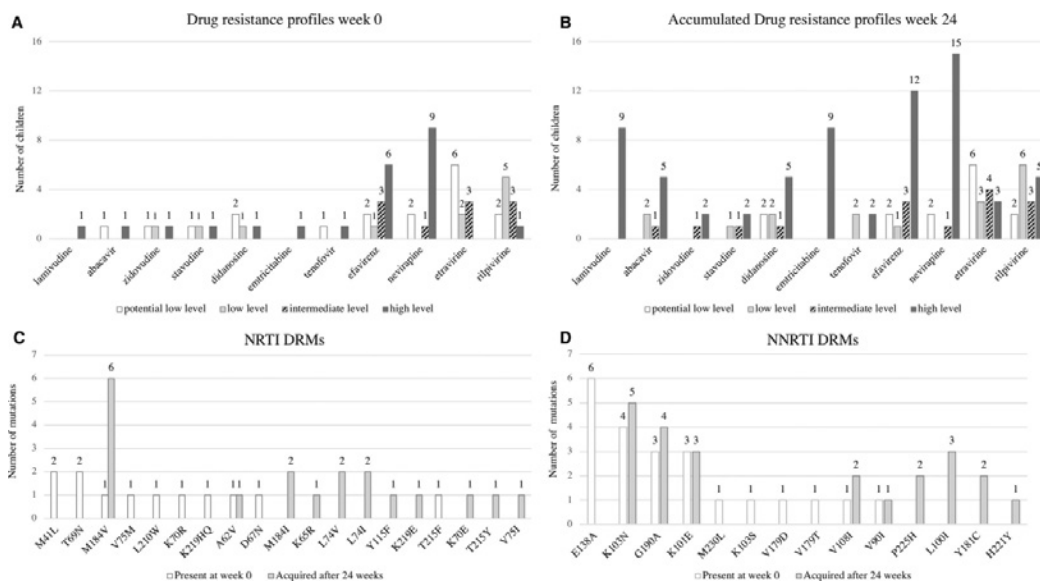


Figure 12. Distribution of individual drug resistance profiles and NNRTI and NRTI drug resistance mutations in ART-naïve Ugandan children at baseline and after 24 wk of efavirenz-based therapy. A: Drug resistance profiles among eighteen children with pretreatment drug resistance. Ninety out of 99 children had a successful HIVDR assay. The number of children with virus susceptible to the individual drugs is not shown. B: Accumulated drug resistance profiles at week 24 (ie pretreatment and acquired drug resistance). At week 24, 92 children were sampled for VL. Out of them, successful HIVDR assay was performed in 12/13 children with (VL) ≥ 500 copies/ml. Newly acquired DRMs were detected in 5 children without pretreatment drug resistance at baseline. Children with baseline pretreatment drug resistance (n = 18) were considered to still harbor drug resistant virus, irrespective of VL level at week 24. In summary, 23 children were considered to have HIVDR at week 24. Children with VL < 500 copies/mL and no pretreatment drug resistance were classified as having virus susceptible to all drugs. The number of children with virus susceptible to the individual drugs is not shown. C-D. NRTI and NNRTI DRMs detected in HIVDR assay at week 0 and at week 24. DRMs, drug resistance mutations; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor. Adapted from Soeria-Atmadja, S., et al. (2020). "Pretreatment HIV drug resistance predicts

accumulation of new mutations in ART-naive Ugandan children." *Acta Paediatr.* 109(12): 2706-2716.

Specifically, 18% had NNRTI resistance, 4% had NRTI resistance, and 2% had dual-class resistance. Overall, 14% of children had reduced viral susceptibility to at least one drug in their current regimen. Additionally, 13% had reduced susceptibility to both EFV and NVP, with 10% showing intermediate and/or high resistance. A total of 33 DRMs were identified in 18 children with pre-treatment drug resistance (PDR), where NNRTI mutations accounted for 64%.

Viral suppression and acquired HIV-drug resistance at week 24 (study IV)

After 24 weeks of therapy viral load results were available for 93 children. Seventy-two children (77%) had undetectable viremia ($VL < 40$ copies/ml), while 80 (86%) reached a $VL < 400$ copies/ml. Eighty-five children had VL-data and HIV drug resistance data from both week 0 and week 24. Out of 67 children without pretreatment HIV drug resistance, 82% (55/67) achieved $VL < 40$ copies/mL, in contrast to only 56% (10/18) of those with HIV drug resistance at baseline ($P = .02$).

Baseline characteristics and potential risk factors for viremia ($VL \geq 40$ copies/mL) at 24 weeks were evaluated. The odds of having $VL \geq 40$ copies/mL increased 9.6 times for every log₁₀ increase in baseline VL ($P = .003$) and 3.6 times for each additional baseline DRM ($P = .023$). At the time of analysis, results for EFV-plasma concentrations were not yet available and were therefore not included as a risk factor.

After 24 weeks on EFV-therapy, HIV drug resistance was successfully analyzed in 12 of 13 children with a viral load ≥ 500 copies/ml. Eleven of these children acquired new drug resistance mutations including five who had wild-type virus at baseline. By week 24, in total 25% (23/92) of the cohort had HIVDR.

Thirteen percent of children had high-level resistance to EFV, compared to 6.7% at baseline. Six children (6.5%) had intermediate or high resistance to all prescribed antiretrovirals, and three had intermediate or high resistance to zidovudine and/or tenofovir, second-line NRTI options.

Key predictors of acquired HIVDR included high viral load at baseline, poor adherence, and the presence of baseline DRMs. The odds of acquiring at least one new drug resistance mutation by week 24 increased 3.2-fold with each additional DRM present at baseline. The most common NNRTI and NRTI mutations were K103N and M184V.

4.8.2 Therapeutic EFV interval linked to virological outcomes week 24 (study II)

In study II, virological outcomes were investigated in relation the suggested EFV therapeutic interval of 1000-4000 ng/ml. Viremia was more common (in those with a median EFV

concentration ($P = 0.013$) or week 24 EFV concentration ($P = 0.025$) below 1000 ng/mL, compared to those with EFV concentrations within or above the therapeutic interval. Children with median EFV levels below 1000 ng/mL were also more likely to develop new drug resistance mutations ($P = 0.035$). Interestingly, among participants who had EFV levels within or above the therapeutic range, both persisting viremia and the development of new HIV drug resistance mutations were significantly associated with pre-treatment HIVDR. Table 6 and Figure 13.

Figure 13. EFV plasma concentrations, virological outcomes and pretreatment HIVDR

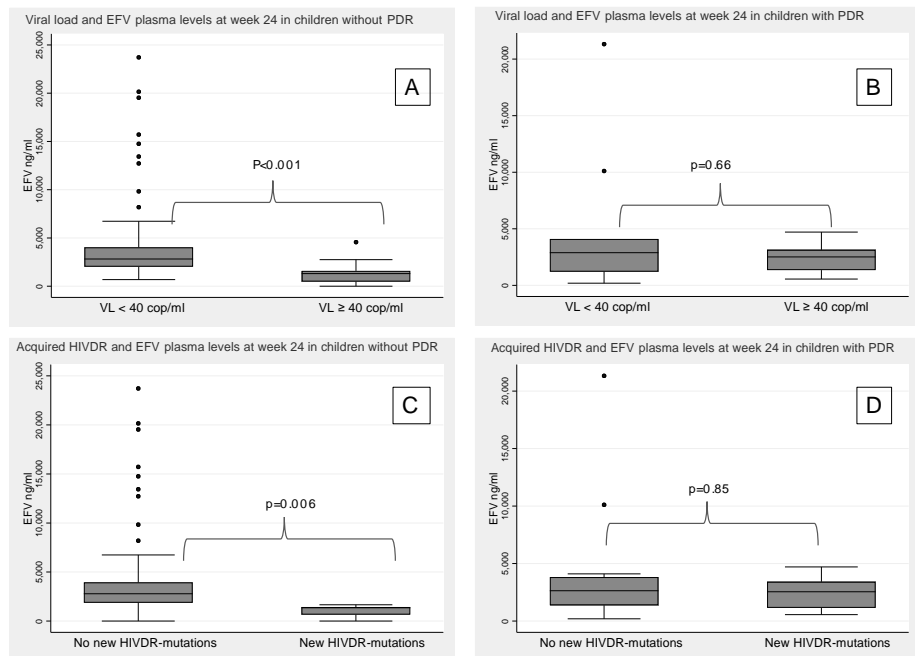


Figure 13. (unpublished). A and C: Among sixty-seven participants without pretreatment HIV drug resistance (PDR), participants with persistent viremia and acquisition of new HIVDR-mutations at week 24, had significantly lower EFV-plasma concentrations, compared to those with viral suppression and no new HIVDR-mutations B and D: Eighteen participants experienced PDR. Among them, there was no significant difference in EFV-plasma concentrations according to virological outcomes. Horizontal reference lines: therapeutic interval 1000-4000 ng/ml.

There was no significant difference in occurrence of PDR, viral suppression, or the emergence of new drug resistance mutations by week 24, when comparing extensive and intermediate metabolizer phenotypes with slow metabolizers.

Table 6. Association between EFV-concentrations, pretreatment HIV drug resistance and viral outcomes at week 24

a/ Viral outcomes and individual median/week 24 EFV –concentration below and within/above therapeutic interval							
		Viremia (VL>40 copies/ml)	No viremia (VL<40 copies/ml)	p- value	New DRM	No new DRM	p-value
Individual EFV median concentration							
n=8	Subtherapeutic (<1000 ng/ml)	5 (62.5 %)	3 (37.5%)	n=7	3 (42.9%)	4 (57.4%)	
n=8 5	Therapeutic– Suprathereapeutic (≥1000 ng/ml)	16 (18.8%)	69 (81.2%)	n=8 5	8 (9.4%)	77 (90.6)	
				0.013	0.035		
EFV concentration at week 24							
n=12	Subtherapeutic (<1000 ng/ml)	6 (50%)	6 (50%)	n=12	3 (25%)	9 (75%)	
n=81	Therapeutic– Suprathereapeutic (≥1000 ng/ml)	15 (18.5%)	66 (81.5%)	n=8 0	8 (10%)	72 (90%)	
N=9 3				0.025 N=9 2	0.153		
b/ Viral outcomes and pretreatment HIV drug resistance according to individual median EFV concentration below or within/above therapeutic intervals							
		Viremia (VL≥ 40 copies/ml)	No viremia (VL<40 copies/ml)	p- value	New DRM	No new drug mutations	p-value
Individual EFV median concentration ≥1000 ng/ml							
n=16	Pretreatment DRM	7 (43.8%)	9 (56.2%)	n=16	11 (68.8%)	5 (31.2%)	
n=61	No pretreatment DRM	8 (13.1%)	53 (86.9%)	n=61	3 (4.9%)	58 (95.1%)	
N=7 7				0.006 N=7 7	0.008		
Individual EFV median concentration <1000 ng/ml							
n=3	Pretreatment DRM	1 (33.3%)	2 (66.7%)	n=2	1 (50%)	1 (50%)	
n=8	No pretreatment DRM	5 (62.5%)	3 (37.5%)	n=5	2 (40%)	3 (60%)	
N=11				1.00 N=7	1.00		

4.9 Adverse events and suprathereapeutic concentrations

In the ALB-cohort fifty-three percent of the participants reported adverse events, primarily CNS-related symptoms, and gastrointestinal complaints (65). Most reported adverse events (23/31) were transient and occurred during the first weeks of EFV treatment. While 14 %

discontinued the therapy due to EFV related symptoms, there were no observations of severe adverse events related to EFV, such as hospital admissions, intensive care, or deaths. Individuals with supratherapeutic mean EFV concentrations were more likely to experience adverse events ($p=0,041$).

Table 7. Adverse events reported in the ALB-cohort

Adverse events reported		
	Count	Percentage
All participants in study	36	100%
Participants reporting adverse events	19	53%
Therapy ended due to adverse effects	5	14%
Total adverse events in study	31	100%
Gastrointestinal	Nausea, malaise, stomach pain	7
	Other gastrointestinal complaints	2
	Total	9 29%
CNS symptoms	Lightheadedness, vertigo, unsteadiness	3
	Affected cognition, loss of concentration	3
	Headache	3
	Disrupted sleep patterns, nightmares	3
	Weariness, fatigue, tiredness	2
	Total	14 45%
Other symptoms	Skin rash (nettle-rash/eczema)	3 10%
	Gynecomastia	1 3%
	Hyperlipidemia	4 13%

Also, in the GENEFA-cohort, CNS-symptoms and gastrointestinal symptoms were frequently reported. Symptoms were in general perceived as mild or moderate and no severe clinical adverse event or discontinuation of therapy was reported. A total of 35 participants reported experiencing at least one CNS symptom during the follow-up period. Among them, 34% (12/35) had consistently supratherapeutic EFV plasma levels, compared to 15% (9/59) of those whose EFV levels remained within or below the therapeutic range ($P = 0.032$). CNS symptoms were also more frequent in slow metabolizers (60%) compared to extensive and intermediate metabolizers (33%) ($P = 0.046$). When comparing the median plasma concentrations of EFV phase I and phase II metabolites at each visit, no significant differences were found between participants who reported and those who did not report CNS symptoms throughout the study.

Figure 14. Adverse drug reactions in Ugandan children on EFV-therapy.

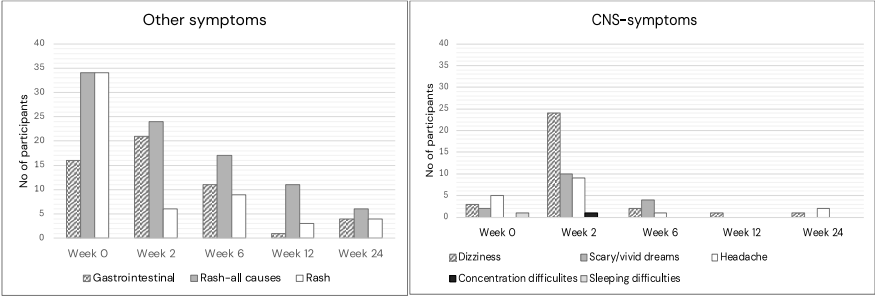


Figure 14. Before starting ART at week 0 and during the subsequent four visits, participants reported adverse drug reactions (ADRs) using a questionnaire. The number of participants at each time point was 99, 97, 95, and 94 for weeks 0, 2, 6, 12, and 24, respectively. From week 2 to week 24, a study clinician evaluated and graded potential ADRs. For central nervous system (CNS) and gastrointestinal symptoms, the clinician’s assessment of ADRs aligned with the participants’ reports. However, many of the rashes reported by participants were not considered adverse reactions by the clinician. The graph illustrates both the total number of rashes reported by participants (“Rash - all causes”) and those assessed as potentially efavirenz (EFV)-related by the clinician (“Rash - EFV”). Gastrointestinal symptoms included nausea, abdominal pain, and vomiting.

4.10 Summary of findings study I-IV

4.10.1 ALB cohort (study I):

- There was considerable interindividual variability in EFV plasma concentrations but no signs of autoinduction.
- A mixed-effects REML regression model identified CYP2B6*6 T/T, CYP2B6*11 G/G, CYP2A6*9 A/C genotypes, age at treatment and time from treatment initiation as independent factors with a significant positive correlation to log(e) EFV plasma concentration/(dose/weight) that together explained 75% of the interindividual variation in EFV plasma concentrations.
- Adverse effects were reported in 50% of participants, but mostly mild and transient and were more frequent among those with supratherapeutic EFV-concentrations.

4.10.2 GENEFA cohort (study II-IV)

- A mixed-effects REML regression model identified several predictors of EFV plasma exposure among Ugandan children, including therapy duration, adherence, the CYP2B6 g.15582C>T/T genotype (CYP2B6*11 A/G), and CYP2B6 metabolizer phenotype (based on composite genotype for CYP2B6 c.516G>T and CYP2B6 c.983T>C). The model accounted for 70% of the interindividual variation in EFV plasma concentrations, with CYP2B6 metabolizer phenotype alone contributing to nearly 50% of this variability.
- Over a third of EFV plasma measurements fell outside the recommended range, with subtherapeutic levels leading to increased risk of viral failure and acquisition of HIV drug resistance. However, therapeutic-supratherapeutic EFV levels only seemed protective in terms of virological outcomes, when pretreatment drug resistance was absent.
- Pretreatment drug resistance was present in 20% of ART-naïve Ugandan children, yet 77% achieved virological suppression after 24 weeks of EFV-based therapy. Pretreatment drug resistance predicted higher rates of viremia and acquisition of new HIV drug resistance mutations.
- This study-the first to measure EFV hydroxy metabolites in children, identified distinct metabolite patterns dependent on CYP2B6 metabolizer phenotype for phase I and phase II metabolites including a newly identified metabolite EFAdeg, proposed to be a degradation product of 8-OH-EFV.
- Time on treatment was included in multivariate REML models predicting log(e)EFV plasma concentrations and log(e)metabolite/EFV ratio for 8-OH-EFV+EFAdeg. There was no sign of decreasing EFV concentrations over time, neither signs of increasing metabolite/EFV ratio over time. This was interpreted as absence of autoinduction.

- CNS-related adverse effects were linked to supratherapeutic levels and slow metabolizer phenotype but were generally mild and transient. The study found no link between specific EFV metabolite levels in plasma and CNS toxicity.

5 Discussion

This thesis explored key aspects of EFV therapy in two pediatric cohorts from different settings. The GENEFA cohort (studies II-IV) in urban Kampala, Uganda (HIV prevalence 5.1% [2]), followed a prospective design, while Study I (ALB cohort) in Stockholm, Sweden (HIV prevalence 0.08%, Sweden, a high resource setting with a HIV-prevalence of 0.08% [123].

In both cohorts, multivariate mixed model regression (REML), accounting for genetic polymorphisms, explained 70-75% of the variability in EFV plasma levels. Among Ugandan children, the CYP2B6 metabolizer phenotype explained nearly 50% of this variability, with SNPs c.516C>T and c.983C>T being key predictors, consistent with previous findings in African populations [74,75]. In the Swedish cohort, 21% of the variability was explained by CYP2B6 c.516C>T, but c.983T>C was not significant, possibly due to the smaller sample size.

The effect of SNP 516G>T may change significantly by the presence of 983T>C [74,78,79]. One study estimated that the presence of a single variant allele in 983T>C caused a 43% drop in EFV-clearance within individuals who had the “wildtype” SNP 516G>T (genotype G/G). It is therefore argued that genotyped-based EFV dosing schedules should account for these CYP2B6 SNPs [71,74], which is supported by our findings.

Other SNPs also influenced EFV levels. Three outliers with CYP2B6*11 (CYP2B6 c.136A>G) showed high EFV concentrations, despite being categorized as extensive and intermediate metabolizers. CYP2A6*9 was the only SNP outside of CYP2B6 that was identified as a significant predictor and explained 14% of variation in the ALB cohort. This SNP, along with other loss-of-function alleles in CYP2A6, may be clinically important for CYP2B6 slow metabolizers who rely on this alternative pathway for EFV metabolism. [124]. In this study, it was found in six intermediate and one slow metabolizer.

To the best of our knowledge, this was the first study to quantify EFV phase I and II metabolites in children (the GENEFA cohort only). Measuring metabolites in children provides insights into the development of metabolic pathways and the pharmacokinetic and pharmacodynamic properties of drugs with toxic metabolites [125]. The main metabolites 8-OH-EFV and 7-OH-EFV have both been implicated as neurotoxic agents *in vitro* and *in vivo* studies in adult populations [47,48,126]. We also described EFAdeg, a recently identified substance and a proposed degradation product of 8-OH-EFV.

There was a distinct patterns of metabolite distribution based on metabolizer phenotype, when investigating the metabolite/EFV ratios. In extensive and intermediate metabolizers, the conjugates of 8-OH-EFV and EFAdeg dominated in plasma, while the parent drug EFV was in abundance in slow metabolizers, in line with previous data in adults [43]. It was observed in adults that the glucuronides are more abundant than sulfated metabolites in plasma. However, our study couldn't determine the relative contribution of glucuronidated

versus sulfated metabolites in children. The stable EFV-tot/EFV ratio across the metabolizer phenotypes and visits confirmed that EFV-N-glucuronidation is a minor pathway [43].

In adults, autoinduction of EFV metabolism may lead to reduced plasma levels in extensive metabolizers but has less impact on those with slower metabolism [57,127,128]. A similar pattern was observed in an Ethiopian pediatric study, where children with CYP2B6 516 GG (EM) and GT (IM) genotypes showed a non-significant increase in EFV clearance between weeks 1 and 8 [128]. In a small Dutch pediatric cohort, increased EFV clearance was seen within the first two weeks for CYP2B6 516 GG subjects. We found no significant signs of autoinduction in either cohort.

In our material, there was no significant difference in plasma concentrations of any of the metabolites, between participants with/without CNS-symptoms. Instead, these symptoms were more common among those with supratherapeutic EFV levels and with slow metabolizer phenotype. Interestingly, it has been observed that exposure to 8-OH-EFV in plasma and CSF do not correlate [129] and that 8-OH-EFV levels in CSF is independent from CYP26516G>T polymorphism [130]. Thus perhaps the EFV-hydroxy metabolites levels in plasma do not predict their potential toxic effect on the CNS.

CNS symptoms like headache, dizziness, and sleep disturbances were commonly reported in both cohorts, generally mild and transient. In the GENEFA study, 35 individuals reported CNS symptoms, slightly higher than the 30% seen in a systematic review of adults and children on EFV therapy [102]. There were no severe CNS-manifestations observed in either cohort, possibly due to few participants (ALB-cohort) and a short follow-up time (GENEFA). In adults, EFV-related cases of encephalopathy and ataxia have been observed among CYP2B6 slow metabolizers with high EFV levels in plasma, with symptoms presenting after (median) two years on treatment [131]. Other factors contributing to CNS symptoms, such as preexisting neurocognitive issues or traumatic experiences, were not examined in our cohorts.

Few studies have explored the link between EFV plasma concentrations and HIV drug resistance in children. A previous study in ART-naïve children found no correlation, but it may have been underpowered. [73]. In the GENEFA cohort, subtherapeutic EFV levels were associated with a higher risk of poor viral control, with nearly two-thirds experiencing viral failure and 40% acquiring new drug resistance mutations. Among those with EFV levels above 1000 ng/mL, less than 20% had viremia, and only 9% developed new resistance mutations. However, pre-existing HIVDR reduced the protective effect of high EFV levels.

Only one child reported prior antiretroviral exposure through PMTCT, yet 20% of participants had pretreatment drug resistance, possibly due to vertical transmission. A 2021 WHO report estimated that 17% of Ugandan women starting ART had resistance to EFV or NVP, and 30% of newly diagnosed infants showed resistance [132], while the prevalence

was approximately 30% among newly diagnosed HIV-positive infants with no or unknown exposure to PMTCT.

By 24 weeks, 77% of the GENEFA cohort were virally suppressed, slightly lower than another cohort of Zambian/Ugandan children, which had 80% viral suppression after 36 weeks on EFV therapy [130]. Viremia at week 24 was strongly associated with pretreatment drug resistance (PDR), aligning with a recent metareview predicting viral failure in children with PDR [133]. Viremia at week 24 was significantly associated with PDR, which is in line with a recent metareview including pediatric and adult cohorts from sub-Saharan Africa, where PDR among children was estimated to 14 percent and predicted viral failure [134]. Accumulation of new HIVDR mutations were significantly linked to PDR. This may be a concern in future ART, particularly for the NRTIs that are a part of DTG-regimens.

5.1 Strengths and limitations

5.1.1 Study I

A key strength of the study was its long duration of eight years, allowing for numerous EFV plasma concentration measurements in a small cohort. However, the retrospective design had limitations, such as the lack of systematic collection of adverse reactions. Clinicians may have scrutinized adverse events more in children with very high EFV levels, leading to biased reporting. Additionally, children with extreme EFV concentrations might be overrepresented due to more frequent plasma sampling, and dose adjustments were made based on these concentrations. A restricted maximum likelihood mixed model was used to account for variability in sample frequency and dose changes.

5.1.2 Study II-IV

The study had several strengths, including its prospective design with frequent EFV concentration measurements, a relatively large cohort, and a low rate of loss to follow-up. The follow-up was thorough and systematic, led by a designated team, and included comprehensive analysis of EFV plasma concentrations, metabolites, pharmacogenetics, and clinical outcomes. Despite the ADR questionnaire not being validated for children, adverse drug reactions were consistently reported.

However, the study had limitations. EFV intake was unobserved, and adherence measured by pill count may be unreliable. Factors like socioeconomic status, malnutrition, co-medication, and HIV status may have influenced EFV metabolism, potentially leading to inaccurate assessments of SNP effects. Viral suppression was based on a single viral load (VL) measurement after 24 weeks, which could misclassify temporary elevations as viral failure. The short 24-week follow-up was also a limitation for assessing virological outcomes.

Pretreatment drug resistance (PDR) may have been underestimated, as the assay couldn't detect minority variants, possibly misclassifying baseline drug resistance as newly acquired. Additionally, poor viral suppression may have been underestimated by excluding participants who died or were lost to follow-up. Lastly, the genotyping focused on known SNPs, potentially overlooking rare or unknown variants.

.

6 Conclusions

This thesis highlight the significant impact of pharmacogenetic variations on EFV plasma concentrations and treatment outcomes in children living with HIV. Across a Swedish multiethnic cohort and a Ugandan cohort, we observed substantial interindividual variability in EFV plasma levels. In the ALB- cohort, 75 % of this variability was explained in a multivariate mixed model, based on gender, age, time on treatment and genetic polymorphisms in CYP2B6 and CYP2A6. A similar model including mean adherence, gender, age, time on treatment, dose/kg, genetic polymorphisms for CYP2B6 and metabolizer phenotype explained 70% of interindividual variation in the GENEFA cohort.

The three metabolizer phenotypes (extensive, intermediate and slow) were based on the composite genotype of CYB2B6 c.516C>T/c.983T>C. At each visit, significant differences in EFV plasma levels were found between the metabolizer phenotypes.

EFV phase I and phase II metabolites were identified and quantified for the first time in children. We observed distinct patterns of metabolite distribution based on metabolizer phenotype, when investigating the metabolite/EFV ratios; in extensive and intermediate metabolizers, the conjugates of 8-OH-EFV dominated in plasma, while the parent drug EFV was in abundance in slow metabolizers.

Additionally, we report a recently identified compound, EFAdeg, which is hypothesized to be in constant equilibrium with 8-OH-EFV. Its clinical relevance is yet unclear, but it may inadvertently influence the measurements of 8-OH, if using a method that is not specific enough and should be considered when quantifying EFV-metabolites. The ratio 8-OH-EFV+EFAdeg/EFV was used as an index for CYP2B6 metabolic activity and was significantly associated with the three metabolizer phenotypes. Overall, we found no signs of autoinduction in either cohort.

Current standard dosing based on weight/age led to EFV-concentrations outside the suggested therapeutic interval in over a third of the participants. Extensive metabolizers were more likely to have subtherapeutic EFV plasma exposure which in turn, conferred a higher risk for viral failure and accumulation of new HIV drug resistance mutations. In contrast, all slow metabolizers had EFV concentrations above the therapeutic range, which was linked to experiencing CNS-related adverse drug reactions at least once during the treatment period. In both cohorts, the adverse reactions reported were frequent, but mild and transient. None of the metabolites could be linked to CNS-toxicity.

Pretreatment drug resistance conferring reduced susceptibility towards NRTIs and NNRTIs were found in one fifth of the ART-naïve Ugandan children and this significantly increased the odds of viral failure and the acquisition of new HIV drug mutations. In participants who

had EFV plasma concentrations within/above the therapeutic range, the risk for poor virological outcomes increased in the presence of PDR.

In summary, the findings suggest that individualized EFV dosing, guided by pharmacogenetic profiling could help more children to achieve optimal EFV concentrations and that this approach may benefit children from diverse ethnic backgrounds.

7 Clinical implications and future perspectives

The findings in this thesis support that individualized EFV dosing, guided by pharmacogenetic profiling should be considered, to optimize EFV plasma exposure in children. Further, the studies indicate that the CYP2B6 SNPs c.516C>T and c.983C>T should be included when defining CYP2B6 metabolizer phenotypes. For adults and children weighing more than 40 kg, phenotype-based dosing guidelines have been suggested by CPIC (Clinical Pharmacogenetics Implementation Consortium) [71].

Most children living with HIV are found in Africa, where the genetic diversity is extensive [135], and the distribution of different SNPs vary across the continent [136]. For instance, the minor allele frequency for CYP2B6 SNPs c.516C>T and c.983C>T, ranges from 22-47% and 2-19% in different African populations [137]. The genetic diversity seen is not only limited to CYP2B6 but extends to other enzymes responsible for the metabolism of drugs used in various diseases [135]. This underlines the potential benefits of individualized dosing of EFV and other drugs in African settings, if the infrastructure and methods for pharmacogenetic testing would be available. Considering that the distribution of genetic variants for drug metabolizing enzymes differ according to ethnic background, pharmacogenetic testing may have to be adapted to specific populations

Our studies were not designed to suggest specific dosing. Yet, the data could be further explored in a more advanced population pharmacokinetic model, where modeling of 7-OH-EFV, 8-OH-EFV and EFAdeg alongside EFV could be included to tailor appropriate doses with simulations. However, significant proportion of children with PDR, experienced viral failure at 24 weeks despite having EFV-plasma concentrations above the recommended threshold of 1000 ng/ml. This suggests that even if therapeutic EFV levels can be achieved with a CYP2B6-phenotype-based dosing regimen, high rates of HIVDR may still challenge successful viral suppression in pediatric populations.

The GENEFA study can serve as a model for designing future studies focused on personalized medicine, particularly when evaluating treatments for children with drugs that have a narrow therapeutic range or significant interindividual variability. Furthermore, simplified approaches, such as assessing metabolizer phenotypes based on a selected number of SNPs, could provide a practical alternative to full-scale genetic testing, allowing for more accessible patient categorization in settings where comprehensive genetic testing may not be feasible.

8 Acknowledgements

The first idea for this work was sparked more than ten years ago, and it wouldn't have become a reality without the support and understanding, from my supervisors, colleagues, friends, and family.

I had no idea what to expect when I initially joined this project, but I am so grateful for getting this opportunity to learn and to work with so many accomplished and dedicated people, that together made sure that we could see these studies through.

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