

**Population pharmacokinetic and
pharmacodynamic study of efavirenz in
HIV-1-infected children treated with first
line antiretroviral therapy in South Africa**

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M.Sc Pharmacology

Thesis submitted for the degree *Philosophiae Doctor*

in Pharmacology at the

North-West University (Potchefstroom Campus)

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August 2011

Potchefstroom

Acknowledgements

Dear Lord, thank you for giving me the grace, love, strength, good health, and the privilege to study and to have completed this undertaking.

To my dearest mother and father, Marlene and Polla, for your unconditional love and support throughout the years, for teaching me to never give up and to always persevere no matter what.

To my beloved son, Ruan, you are my pride and joy. Thank you for keeping me on my toes and knees at all times. My love for you is countless.

To my promoter, Dr Malie Rheeders, you have always believed in me; you have motivated and inspired me; you have been a wonderful mentor, colleague and friend. Your guidance was always with kindness, patience and remarkable skill and I thank you for that from the bottom of my heart.

To my co-promotor, Dr Hermien Gous, thank you for your always positive inspiration, support and special contributions.

To Prof Salome Kruger, thank you for all your assistance, guidance and expertise. I have learnt so much from you, I have the utmost respect and appreciation for what you have done for me.

To all my colleagues at the Department of Pharmacology, NWU, and friends (Rina, Liana, Sumei and Elsie), your genuine interest and assistance have always meant so much to me. Especially thank you to Prof Linda Brand, for your understanding and compassion.

To all the research staff of Harriet Shezi Children's Clinic, thank you for embracing me into your work place and to have made me feel welcome. Sister Matshediso,

Riki, Merleesa, Janet and Angela, you have all helped in so many ways, it was a real pleasure to have worked with you all.

To all the helpful laboratory advice and guidance from Ms Linda Malan, Ms Ellenor Rossouw, Dr Karin Conradie, Dr Du Toit Loots (Nutrition, NWU); Mr Peet Jansen van Rensburg (Biochemistry, NWU), Dr Collet Dandara (Human Genetics, UCT) with regards to the LC-/MS/MS apparatus; DNA handling, storing and isolation; and PCR techniques and Francois Viljoen (Pharmacology, NWU) for all his assistance with computer related problems.

To all the very special study participants and caregivers: for your time, effort, friendliness and most of all your trust. You have made such an impact on my philosophy of life. My prayers and thoughts are always with you. May God bless you all!

I would also like to acknowledge the North-West University, National Research Foundation and Medical Research Council for funding.

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Abstract

Highly active antiretroviral therapy (HAART) has improved the life expectancy of HIV-1-infected patients dramatically since it was launched in 1996, but there are still many challenges in the provision of HAART, especially to children in resource limited countries. Efavirenz (EFV), a non-nucleoside reverse transcriptase inhibitor (NNRTI) forms part of the recommended national first line antiretroviral treatment regimen for children older than 3 years and weighing more than 10 kg in South Africa. Limited pharmacokinetic information on EFV plasma concentrations in sub-Saharan HIV-1-infected children is available. EFV is primarily metabolised by hepatic CYP2B6 isoenzymes. The CYP2B6 gene is characterised by extensive inter-individual variability in hepatic expression and activity. The single nucleotide change, 516G>T, on the CYP2B6 gene has consistently been associated with elevated EFV plasma levels in different ethnic populations and these are more frequently observed in populations of African descent. The recommended therapeutic range of EFV plasma levels is 1-4 µg/ml and the C_{min} should be above 1 µg/ml.

In this prospective study (PK/PD.EFV.07) cohort, 60 black children, both genders, with no prior exposure to antiretroviral therapy and eligible for antiretroviral therapy (ART) were enrolled and followed up at 1, 3, 6, 12, 18 and 24 months post HAART initiation. They all attended the outpatient clinic at Harriet Shezi Children's Clinic, Chris Hani Baragwanath Hospital, Soweto, South Africa. The required ethics approval was obtained to conduct this study.

The objectives of this investigation were to: develop and validate a suitable LC-MS/MS method to accurately determine plasma EFV levels from this study population, determine the prevalence and effect of CYP2B6 516G>T polymorphism on EFV plasma levels, determine the population pharmacokinetic clearance (CL/F) value of EFV, identify covariates that influence the clearance of EFV in HIV-1-infected children, and investigate specific pharmacodynamic effects and therapeutic

outcomes of this EFV-based regimen within this paediatric population over the 24 months post-HAART initiation.

The main findings of the measured mid-dose EFV plasma concentrations showed that sub-therapeutic concentrations ($<1\text{ }\mu\text{g/ml}$) accounted for 18% (116/649), within therapeutic range ($1\text{--}4\text{ }\mu\text{g/ml}$) represented 52.5% (341/649), and concentrations above the therapeutic range ($>4\text{ }\mu\text{g/ml}$) represented 29.5% (192/649). A significant number of the samples (47.5%) were outside the accepted therapeutic range during this 24 month follow-up period. Possible reasons contributing to this include genetic variation in drug metabolism and non-adherence.

Genotype results on all 60 study participants were: 23% 516 T/T homozygotes, 42% 516 G/G homozygotes and 35% 516 G/T heterozygotes. The 516 T-allelic variant frequency was relatively high at 41%. This also supports and explains why such a large number (29.5%) of the mid-dose interval plasma samples were above ($>4\text{ }\mu\text{g/ml}$) the accepted therapeutic range.

Repeated measures ANOVA confirmed that CYP2B6 516 G/G, G/T and T/T genotypes were consistently predictive of the log EFV concentrations at all times ($P = 0.0001$). The total median (IQR) EFV plasma concentrations over the 24 months post-HAART when pooled, were 6.36 (3.47 – 7.28) for T/T, 2.55 (1.62 – 3.59) for G/T, and 1.41 (1.02 – 1.74) $\mu\text{g/ml}$ for G/G groups respectively ($P<0.00001$). Multiple comparisons by groups revealed that the EFV plasma concentrations between the T/T and G/G ($P=0.000002$) and between G/T and G/G ($P=0.009$) were statistically significant. However, the differences between the EFV plasma concentrations of the T/T and G/T groups were not significantly different ($P=0.074$). This supports previous results that the presence of the 516 T-allelic variant is responsible for the higher EFV plasma concentrations within individuals presenting with this single nucleotide mutation on the CYP2B6 gene.

This EFV-based treatment was well tolerated even at plasma concentrations above the therapeutic range ($>4\text{ }\mu\text{g/ml}$) and most side effects subsided spontaneously. 89% of the participants were virally suppressed at 24 months post-HAART. The efficacy of this EFV-based treatment did not affect the three genotype groups differently and they showed similar improvement in their immunological (CD4-cell

count and CD4%) markers and reduction in viral load over the 24 months post-HAART initiation. We found no association of the CYP2B6 516G>T polymorphism and side effects reported after 1 month of treatment within this study population.

The final population pharmacokinetic (PK) estimates for EFV clearance (CL/F) were, 2.46, 4.60, and 7.33 l/h for the T/T, G/T, and G/G respective genotype groups. The volume of distribution (V/F) estimate was 89.52 l. The importance of interoccasion variability (IOV) in a PK model for a longitudinal study was again highlighted by this investigation.

To our knowledge, this is the first study in black South African HIV-1-infected children with measured sequential EFV plasma concentrations which also investigated the influence of the CYP2B6 516G>T polymorphism on EFV plasma concentrations and the population clearance (CL/F) value of EFV in a longitudinal study over a period of 24 months post-HAART initiation.

Keywords: *Efavirenz, pharmacokinetics, pharmacodynamics, pharmacogenetics, clearance*

Opsomming

Hoogs aktiewe antiretrovirale terapie (HAART) het die lewensverwagting van HIV-1-infektiewe pasiënte dramaties verbeter sedert die bekendstelling daarvan in 1996. Daar is egter nog steeds verskeie hindernisse wat die voorsiening van HAART beïnvloed, veral aan kinders in lande met beperkte hulpbronne. Efavirens (EFV) is 'n nie-nukleosied trutranskriptase remmer wat deel uitmaak van die voorgestelde nasionale eerstelinie antiretrovirale behandeling vir kinders in Suid Afrika ouer as 3 jaar en wat meer as 10 kg weeg. Beperkte farmakokinetiese inligting van EFV-plasmavlakke in sub-Sahara HIV-1-geïnfekteerde kinders is beskikbaar. EFV word primêr deur die hepatiese sitochroom CYP2B6 iso-ensieme gemetaboliseer. Die CYP2B6-geen word gekarakteriseer deur aansienlike interindividuele variasie a.g.v. verskille in hepatiese uitdrukking en aktiwiteit. 'n Enkele nukleotied verandering, 516G>T, op die CYP2B6-geen word deurgaans geassosieer met verhoogde EFV-plasmavlakke in verskillende etniese populasies. Die mutasie kom veral baie meer voor onder swart populasies oorspronklik afkomstig uit Afrika. Die aanbevole terapeutiese grense vir EFV-plasmavlakke is 1-4 µg/ml en die C_{min} moet nie onder 1 µg/ml daal nie.

Hierdie prospektiewe kohort studie (PK/PD.EFV.07) het bestaan uit 60 swart kinders van beide geslagte, met geen vorige blootstelling aan antiretrovirale terapie (ART) nie en almal was geskik om met ART te begin. Na aanvang van ART is elkeen na 1, 3, 6, 12, 18 en 24 maande opgevolg. Al die studiedeelnemers was buite-pasiënte van die kliniek, Harriet Shezi Children's Clinic, Chris Hani Baragwanath Hospitaal in Soweto, Suid Afrika. Al die nodige etiese goedkeuring om die studie uit te voer is verkry.

Die doelstellings van die ondersoek was: ontwikkeling van 'n gevalideerde vloeistofchromatografie-massaspektrometriese metode wat die EFV-plasmavlakke van die studie akkuraat kon bepaal; bepaling van die frekwensie en effek van die

CYP2B6 516G>T polimorfisme; bepaling van die populasie farmakokinetiese opruiming (CL/F) van EFV, identifisering van kovariante wat die opruiming van EFV in die HIV-1-geïnfekteerde kinders beïnvloed, asook om die spesifieke farmakodinamiese effekte en terapeutiese uitkomst van die EFV-gebaseerde regime binne hierdie pediatriese populasie oor 24 maande na die aanvang van HAART te ondersoek.

Die hoofbevindings van die gemete EFV-plasmavlakke wat gedurende die middoseringsinterval geneem is, het aangetoon dat 18% (116/649) van die vlakke subterapeuties ($<1 \mu\text{g/ml}$) was, dat 52.5% (341/649) van die vlakke binne die terapeutiese grense ($1\text{--}4 \mu\text{g/ml}$) was en dat 29.5% (192/649) van die vlakke bo die terapeutiese grens ($>4 \mu\text{g/ml}$) was. 'n Beduidende hoeveelheid van die monsters (47.5%) was dus buite die aanvaarbare terapeutiese grens gedurende die 24-maande opvolgperiode. Moontlike redes wat daartoe kon aanleiding gee is genetiese variasie in die geneesmiddelmetabolisme en swak pasiënt-meewerkendheid.

Die genotiperingsresultate vir die 60 studiedeelnemers het die volgende aangetoon: 23% was 516T/T homosigoties, 42% was 516G/G homosigoties en 35% was 516G/T heterosigoties. Die CYP2B6 516T allel variantfrekwensie was hoog op 41%. Dit ondersteun en verklaar waarom so 'n groot hoeveelheid (29.5%) van die middoseringsinterval se EFV-plasmavlakke bo die aanvaarbare terapeutiese grens ($>4 \mu\text{g/ml}$) geval het.

Herhaalde metings deur ANOVA het ook bevestig dat die CYP2B6 516 G/G, G/T en T/T genotipes die log EFV-vlakke ten alle tye ($P = 0.0001$) voorspel het. Die totale mediaan (interkwartiel grens) EFV-plasmavlakke wat saamgevoeg is oor die 24 maande na ART was onderskeidelik 6.36 ($3.47 - 7.28$) vir T/T, 2.55 ($1.62 - 3.59$) vir G/T, en 1.41 ($1.02 - 1.74$) $\mu\text{g/ml}$ vir die G/G groepe ($P < 0.00001$). Veelvuldige vergelykings tussen die groepe het blootgelê dat die EFV-plasmavlakke tussen die T/T en G/G ($P = 0.000002$) en tussen die G/T en G/G ($P = 0.009$) genotipes wel statisties beduidend van mekaar verskil. Die verskil tussen die EFV-plasmavlakke van die T/T en G/T groepe was egter nie statisties beduidend nie ($P = 0.074$). Dit ondersteun vorige resultate dat die teenwoordigheid van die 516T allel variant

verantwoordelik is vir die hoër EFV-plasmavlakke by individue wat met die enkel nukleotiedmutasie op die CYP2B6-geen presenteer.

Die EFV-gebaseerde terapie is baie goed verdra, selfs by diegene met hoë plasmavlakke bo die terapeutiese grens ($>4 \mu\text{g/ml}$), en meeste van die nuwe-effekte wat wel gerapporteer is, het spontaan opgeklaar. Suksesvolle virale onderdrukking na 24 maande sedert die aanvang van ART is verkry by 89% van die studiedeelnemers. Die effektiwiteit van die EFV-gebaseerde behandeling het nie op statistiese verskille aangedui tussen die drie genotipes nie. Dieselfde verbetering in immunologiese (CD4 telling en CD4%) merkers en afname in virale lading is verkry gedurende die 24 maande sedert die aanvang van ART binne die drie groepe. Ons kon geen assosiasie vind tussen die CYP2B6 516G>T polimorfisme en nuwe-effekte wat na 1 maand van behandeling gerapporteer is nie.

Die finale populasie farmakokinetiese voorspellings vir EFV-opruiming (CL/F) was onderskeidelik 2.46, 4.60 en 7.33 l/h vir die T/T, G/T en G/G genotipes. Die volume van verspreiding (V/F) was 89.52 l. Die belangrikheid van interokkasie variasie (IOV) in 'n farmakokinetiese model wat 'n longitudinale studie moet kan voorspel en beskryf is weereens deur die ondersoek beklemtoon.

Hierdie studie is sover ons kennis strek die eerste studie in swart Suid-Afrikaanse HIV-1-geïnfekteerde kinders waar 'n reeks van opeenvolgende EFV-plasmavlakke gemeet is, die invloed van die CYP2B6 516G>T polimorfisme op EFV-plasmavlakke ondersoek is, en die populasie opruimingswaarde (CL/F) van EFV in 'n longitudinale studie oor 'n tydperk van 24 maande na die aanvang van HAART ondersoek is.

Sleutelwoorde: *Efavirens, farmakokinetika, farmakodinamika, farmakogenetika, opruiming*

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List of Abbreviations

AACTG	Adult Aids Clinical Trials Group
AAG	α 1 acid glycoprotein
AIC	Akaike's Information Criterion
AIDS	Acquired Immunodeficiency syndrome
ANOVA	Analysis of variance
ALT	Alanine aminotransaminase
ART	Antiretroviral treatment
ARV	Antiretroviral
AUC	Area under the curve

BMI	Body mass index
BIA	Bio-electrical impedance analysis
BSA	Body surface area

CCR5	Chemokine receptor 5
CD4-cell	CD4 T-lymphocyte cell
CI	Confidence interval
CDC	Centre for Disease Control
Chol	Cholesterol
C_{min}	Minimum concentration (trough level)
CNS	Central nervous system
CL	Clearance
CRF	Case report forms
CV	Coefficient of variance
CYP	Cytochrome

DNA	Deoxyribonucleic acid
DoH	Department of Health

EDTA	Ehtylenediaminetetraacetic acid
EFV	Efavirenz

F	Bioavailability
FBC	Full blood count
FLP	Fragment length polymorphism
FOCE	First-order conditional estimate
FOCE-I	First-order conditional estimate with interaction
GAM	Generalised additive modelling
GC-MS	Gas chromatography-mass spectrometry
G/G	Denotes the genotype; with base pairs: guanine/guanine
G/T	Denotes the genotype; with base pairs: guanine/thymine
HAART	Highly Active Antiretroviral Treatment
HIV	Human Immunodeficiency virus
HPV	Human Papilloma virus
HPLC	High performance liquid chromatography
HWE	Hardy Weinberg Equilibrium
IOV	Interoccasion variability
IRIS	Immune response inflammatory syndrome
IS	Internal standard
IQR	Inter quartile range
JCR	Journal Citation Report
K-W	Kruskal-Wallis
LAMB	Laboratory for Applied Molecular Biology
LC-MS/MS	Liquid chromatography tandem mass spectrometry
Log	Logarithm
LS	Least square
MDR	Multiple drug resistant
MeOH	Methanol
Mid	Midazolam
MNR	Medicine not returned
MRM	Multi reaction monitoring
mRNA	Messenger ribonucleic acid

NDoH	National Department of Health
NHLS	National Health Laboratory Services
NONMEM	Non-linear mixed effects modelling
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NtRTI	Nucleotide reverse transcriptase inhibitor
OFV	Objective function value
PACTG	Pediatric Aids Clinical Trials Group
PCR	Polymerase chain reaction
PI	Protease inhibitor
PMTCT	Prevention of mother to child transmission
PK	Pharmacokinetic(s)
PD	Pharmacodynamic(s)
PsN	Pearl speaks NONMEM
pcVPC	prediction-corrected visual predictive checks
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RSE	Residual standard error
SD	Standard deviation
SE	Standard error
SNP	Single nucleotide polymorphism
SOP	Standard operating procedure
SPE	Solid phase extraction
SS	Stock solution
TB	Tuberculosis
TDM	Therapeutic drug monitoring
T/G	Denotes the genotype; with base pairs: thymine/guanine
Trig	Triglyceride
T/T	Denotes the genotype; with base pairs: thymine/thymine
t_{1/2}	Half-life
UNAIDS	Joint United Nations Programme on HIV/AIDS
UV	Ultraviolet

VL	Viral load
VPC	Visual predictive checks
V	Volume of distribution
WHO	World Health Organization
WS	Working solution

SCIENTIFIC SYMBOLS AND UNITS

Ka	Absorption constant
cm	Centimetre
χ^2	Chi square
g	Gram
h	Hour/s
kg	Kilogram
l	Litre
μ	Micro
μg	Microgram
mg	Milligram
ml	Millilitre
min.	Minute
%	Percentage
Θ	Theta
σ	Sigma
ω	Omega

Chapter 1

Introduction

1.1 Problem Statement

In 2011 we commemorate two important milestones in the combat against the most challenging medical and social phenomenon of our time, the acquired immune deficiency syndrome (AIDS) pandemic. It is the 30th anniversary of the epidemic's commencement that was first reported and diagnosed in June 1981, and the 15th anniversary of the first launch of highly active antiretroviral therapy (HAART).

The 2006 UNAIDS, the global AIDS epidemic report stated, that “despite some notable achievements, the response to the AIDS epidemic to date has been nowhere near adequate. In just 25 years, HIV has spread relentlessly from a few widely scattered “hot spots” to virtually every country in the world, infecting 65 million people and killing 25 million”(UNAIDS, 2006).

However, the 2010 UNAIDS global AIDS epidemic report, was more optimistic, stating that: “The overall growth of the global AIDS epidemic appears to have stabilized.” A steady decline in new HIV infections and fewer AIDS-related deaths due to the increase of antiretroviral therapy (ART) are reported. The number of people living with HIV worldwide has increased due to the reduction in mortality. Sub-Saharan Africa still remains the worst HIV affected region in the world, although new HIV infections have either stabilized or are showing signs of decline. The estimated total number of people living with HIV in sub-Saharan Africa reached 22.5 million (20.9-24.2 million), which represents 68% of the global total. An estimated 1.8 million (1.6-2.0 million) people became infected in 2009. This is considerably lower than the estimated 2.2 million (1.9-2.4 million) people newly infected with HIV in 2001. South Africa's epidemic remains the largest in the world with an estimated 5.6 million (5.4-5.8 million) people living with HIV in 2009 (UNAIDS, 2010).

The South African National Department of Health completed an operational plan in November 2003 for the care and treatment of patients with HIV infection. This plan included the treatment and provision of ART to HIV-infected patients. The implementation of this HIV-roll out plan began shortly after the first sites, providing antiretroviral treatment, had been established early in 2004 (Mbewu & Simela, 2003; Ijumba *et al.*, 2004). Antiretroviral treatment guidelines were published by the National Department of Health in 2004, 2005 and 2010 (NDoH, 2004, 2005, and 2010). The South African public health care sector will have had 7 years of experience in the treatment of HIV/Aids on a national level in 2011.

Despite the fact that HAART has improved the life expectancy (prognosis and outcome) of HIV-1-infected children dramatically (de Martino *et al.*, 2000; Fraaij *et al.*, 2003; Palella *et al.*, 1998; Walker *et al.*, 2004; Fassinou *et al.*, 2004), there are still many challenges in the provision of HAART, especially to children. The following factors need to be taken into account when working with HIV-infected children within the South African context:

- i) Very large inter- and intra-patient variability is observed in the pharmacokinetics of antiretrovirals (ARVs), especially in the non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs)(Fraaij *et al.*, 2003; Back *et al.*, 2002). Unpredictable pharmacokinetics, caused by altered absorption, genetic variations in metabolism, and drug-drug interactions, frequently lead to either sub-therapeutic levels, and thus an increased risk of viral rebound or to toxic levels leading to related side-effects (de Martino *et al.*, 2000; Fraaij *et al.*, 2003; Palella *et al.*, 1998; Walker *et al.*, 2004).
- ii) The emergence of a high rate of virological failure and resistance to ARVs is a great concern, since it is a major contributor to the failure of long-term suppression of HIV replication (Fraaij *et al.*, 2003; Ledergerber *et al.*, 1999; Durant *et al.*, 1999; Aboulker *et al.*, 2004).
- iii) Adequate paediatric formulations have not yet been developed or adequately tested in children and a substantial number of dosage recommendations for children are still absent from the guidelines (Fraaij *et al.*, 2003; Ledergerber *et al.*, 1999).

- iv) Poor adherence to the prescribed therapy also contributes to treatment failure and plasma concentration variation (Ledergerber *et al.*, 1999; Durant *et al.*, 1999; Aboulker *et al.*, 2004).
- v) The optimal time to initiate HAART has evoked numerous debates and discussions (Walker *et al.*, 2004), and the new international guidelines published in July 2010 have stated to commence with HAART as soon as possible (Thompson *et al.*, 2010; WHO, 2010).

Lima *et al.*, (2009), concluded that the challenge still remains in the development of further strategies to sustain and maintain high levels of adherence to HAART, to sustain long-term viral suppression and to avoid or further prevent disease progression, death and drug toxicity.

1.2 Study Objectives

The clinical study (PK/PD.EFV.07), *Population pharmacokinetic and pharmacodynamic study of efavirenz in HIV-infected children treated with first line antiretroviral therapy in South Africa* (Principal Investigator: Mrs. M. Viljoen), was approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, Johannesburg (Ethics reference number: 070413) and the Ethics Committee of the North-West University, Potchefstroom (NWU-0015-07-A5) in June 2007. The recruitment commenced in June 2007 and the last 24-month follow-up was completed in March 2010.

The important novelty and contribution of this study to the current knowledge of efavirenz pharmacokinetics in South African black children, is that this study was conducted on HAART naïve children who were followed up on a longitudinal basis for 24 months post HAART initiation.

Various primary and secondary objectives were set out for this clinical trial; refer to the Approved Study Protocol (final amended version nr 8, March 2009) Addendum A1.

Only the primary and secondary objectives pertaining to this PhD thesis are reflected here.

1.2.1 Primary Objectives

- i) To develop, optimise and validate a LC-MS/MS method to determine plasma efavirenz levels accurately.
- ii) To collect a series of plasma efavirenz levels in this paediatric study population at 1,3,6,12,18 and 24 months post HAART, in order to investigate the levels of efavirenz in these children after chronic treatment.
- iii) To determine and investigate the prevalence of the CYP2B6 516G>T polymorphism and its effect on efavirenz plasma levels within this paediatric study population.
- iv) To determine the population pharmacokinetic clearance (CL/F) value, and covariates influencing the clearance of efavirenz in combination with 2 NRTIs in HIV-1 infected children (older than 3 years and weighing more than 10 kg), with a non-linear mixed effects modelling (NONMEM), using a routine clinic-based, sparse collection of blood samples.

1.2.2 Secondary Objectives

To investigate correlations between certain pharmacodynamic endpoints (CD4%, CD4-cell count; viral load; side effects and toxicity) and the efavirenz-based regimen within this paediatric population.

1.3 Brief Clinical Trial Layout

This clinical study (PK/PD.EFV.07) was a prospective, clinic-based, non-randomised open label study, in HIV-1-infected children who visited the Harriet Shezi Children's clinic on an outpatient basis. The attending clinicians, consulting with these patients, followed the antiretroviral treatment guidelines and first line regimen of the National Department of Health for the management of HIV-infected children (NDoH, 2005). No investigational drugs, dosages or combinations were used. The cohort consisted of 60 black children (3-14 years), both genders, with no prior exposure to ART. These children were eligible to commence with ART and were from resource-limited households. Each study participant was monitored and included in this study for a minimum period of 24 months (7 study related visits) and a maximum period of 30 months (including staging, screening and baseline monitoring). Figure 1.1 is a

schematic representation of this study layout and relevant study visits that were carried out from June 2007 up to March 2010.

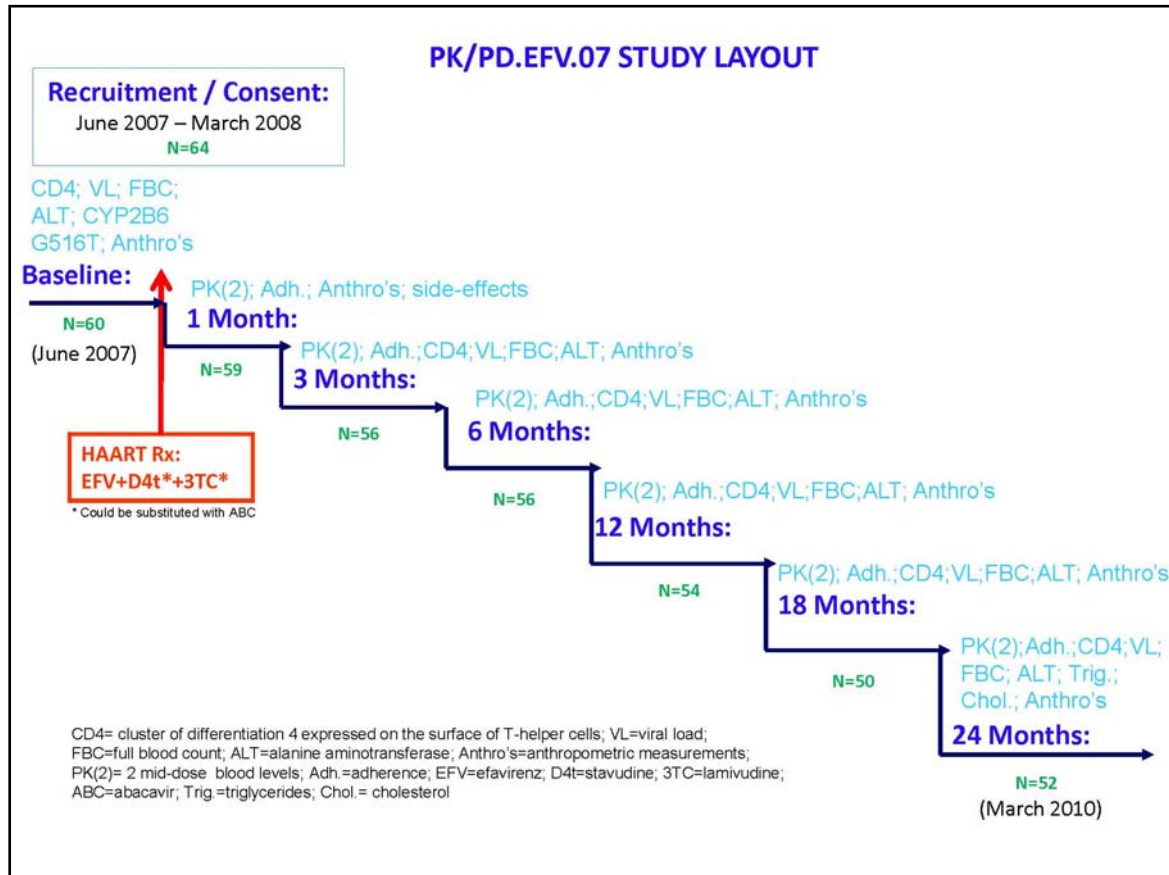


Figure 1.1: Schematic diagram of study layout of PK/PD.EFV.07.

1.4 Structure of this Thesis

This thesis was prepared and written to comply with the article format for thesis/dissertation submission, as approved by the North-West University (NWU); however, minor alterations were added. All chapters and manuscripts have their own reference list provided at the end of each chapter.

This article thesis format includes the following:

- Chapter 1:* Introduction
- Chapter 2:* Literature Review
- Chapter 3:* Materials and Methods
- Chapter 4:* Manuscript A (Published)
- Chapter 5:* Manuscript B (Submitted – March 2011)

Chapter 6: Manuscript C (Pro-forma article to be submitted)

Chapter 7: Additional results and discussions not covered in Manuscripts A-C

Chapter 8: Conclusions and Recommendations

Addendum A: Documentation pertaining to the clinical study (PK/PD.EFV.07), such as Approved Study Protocol, Consent Forms, Case Report Forms and Ethics approval.

Addendum B: Various standard operating procedures (SOP)

Addendum C: Guidelines and author instructions relevant to the accredited journals for which manuscripts were submitted or are going to be submitted.

Addendum D: Various descriptive statistics results and most relevant NONMEM analysis outputs.

1.5 Research Outputs

Accepted Publications relating to this clinical study PK/PD.EFV.07:

- Viljoen, M. Gous, H. Kruger, H.S. Riddick, A. Meyers, T.M. Rheeders, M. Efavirenz Plasma Concentrations at 1, 3 and 6 Months Post Antiretroviral Therapy Initiation in HIV-1-Infected South African Children. *Aids Research and Human Retroviruses*, 2010 26(6):613-619. (Chapter 4)
- Theron, A. Cromarty, D. Rheeders, M. Viljoen, M. Determination of salivary efavirenz by liquid chromatography coupled with tandem mass spectrometry. *Chromatography B*, 2010 878:2886-2890. *Not submitted as part of this thesis.*

Conference contributions (2008-2010) relating to this clinical study PK/PD.EFV.07:

- Viljoen, M. Loots, D.T. Rheeders, M. Gous, H. Routine drug level monitoring of first line ARV regimen in a South African paediatric HIV roll-out clinic. IXth World Conference on Clinical Pharmacology and Therapeutics, 27 July-1 Aug. 2008, Quebec, Canada. (Poster)
- Viljoen, M. Gous, H. Kruger, H.S. Riddick, A. & Rheeders, M. Efavirenz plasma concentrations at 1, 3 and 6 months post antiretroviral treatment in HIV-1 infected South African children. Ist International Workshop HIV Paediatrics, 17-18 July 2009, Cape Town, South Africa. (Poster)

- Kruger, H.S. Viljoen, M. Gous, Haupt, C. Improvement in nutritional status of HIV-infected children after starting highly active antiretroviral therapy. 1st International Workshop HIV Paediatrics, 17-18 July 2009, Cape Town, South Africa. (Poster)
- Theron, A. Viljoen, M. Rheeders, M. A pilot study to investigate the predictability of efavirenz plasma levels in HIV-1 infected South African children. 5th International Conference on Pharmaceutical and Pharmacological Sciences (ICPPS), 23-26 September 2009, North-West University, Potchefstroom Campus. (Podium)
- Kruger, HS. Viljoen, M. Haupt, C. Meyers, TM. Improved body composition of HIV-1 infected stunted children after one year of antiretroviral therapy. IUNS 5-8 October 2009, Bangkok, Thailand. (Poster)
- Theron, A. Viljoen, M. Cromarty, D. Rheeders, M. Jansen van Rensburg, PJ. Malan, L. Determination of efavirenz in human saliva by high performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). 2ND International Workshop on HIV Paediatrics, 16-17 July 2010, Vienna, Austria. (Poster)
- Viljoen, M. Dandara, C. Rheeders, M. Conradie, KR. Kruger, HS. Meyers, TM. Gous, H. Prevalence of CYP2B6-516G>T polymorphism in black South African HIV-infected children on efavirenz based antiretroviral therapy. 16TH World Congress of Basic and Clinical Pharmacology, 17-23 July 2010, Copenhagen, Denmark. (Poster)
- Rheeders, M. Viljoen, M. Theron, M. Meyers, TM. Saliva as a non-invasive alternative to plasma efavirenz. 16TH World Congress of Basic and Clinical Pharmacology, 17-23 July 2010, Copenhagen, Denmark. (Poster)

1.6 Contributions of Authors to the Manuscripts presented in this Thesis

The various contributions and responsibilities of the authors and researchers involved in this study and articles presented in this thesis are provided in Table 1.1.

Table 1.1: Respective contributions of the various collaborators

AUTHOR	AFFILIATION	ROLE
Mrs M Viljoen (PhD candidate)	Dept. of Pharmacology, School of Pharmacy, NWU.	Principal Investigator: PK/PD.EFV.07. Funding grants (NRF-Thuthuka: 2007-2010 & MRC: 2008-2010). Ethics approval and Study Protocol. Study visit recordings (baseline, 1-24 months). Case report forms and routine test results follow-up. Development, validation and analysis of EFV plasma (LC-MS/MS method). DNA isolation and PCR (CYP2B6 G516T). Statistical analysis in consultation with Statistical Consultation Services, NWU. Interpretation and writing up of manuscripts and all chapters presented herewith. First author: Manuscripts A-C.
Dr M Rheeders (Promoter)	Dept. of Pharmacology, School of Pharmacy, NWU.	Supervisor of M Viljoen. Co-author: Manuscripts A-C. Study Protocol. NONMEM fitting of data and modelling. Guidance with writing of thesis, results interpretation, problem analysis and solving.
Dr H Gous (Co-promoter)	Harriet Shezi Children's Clinic, Chris Hani Baragwanath Hospital, Dept. of Paediatrics, University of Witwatersrand.	Co-supervisor of M Viljoen. Co-author: Manuscripts A-C. Adherence assessments. Guidance with writing of thesis and clinical study logistics.

Prof HS Kruger	Centre of Excellence for Nutrition, Faculty of Health Sciences, NWU.	Co-author: Manuscripts A-B. Anthropometric measurements, Z-scores analysis and nutritional/social background recordings. Statistical guidance and interpretation.
Dr C Dandara	Division of Human Genetics, Faculty of Health Sciences, University of Cape Town.	Co-author: Manuscripts B-C. Guidance, expertise and interpretation of conventional PCR and relevant CYP2B6 polymorphisms.
Dr TM Meyers	Wits Institute for Sexual Reproductive Health HIV & Related Diseases, Faculty of Health Sciences, University of the Witwatersrand.	Co-author: Manuscripts A-C. Paediatrician and guidance with clinical aspects and Study Protocol. Interpretation of clinical care aspects.
Dr A Riddick	Dept. of Paediatrics, Tygerberg Hospital, Parow.	Co-author: Manuscript A. Physician, involved in the physical examination of study participants. Interpretation of clinical care aspects.
Prof MO Karlsson	Dept. of Pharmaceutical Biosciences Uppsala University, Sweden.	Co-author: Manuscript C. NONMEM modelling and interpretation.
With this signature I declare that I have approved the above contributions and my role in this study. I hereby provide consent that it may be published as part of the PhD thesis of Mrs M Viljoen.		

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Chapter 2

Literature Review

2.1 Introduction

This literature review explores the aspects of highly active antiretroviral therapy (HAART), antiretroviral drugs used in South Africa, their pharmacokinetics in general, and more specifically in children. The focus of this review will then change to highlight the pharmacokinetic and pharmacodynamic aspects of efavirenz, as this was the primary focus of this study.

The first awareness of AIDS (acquired immune disease syndrome) was evident in the summer of 1981 with the diagnosis of opportunistic infections such as pneumocystis pneumonia and Kaposi's sarcoma among homosexual men in the USA (CDC, 1981). Controversy and rivalry then developed between US and French virologists in claiming the party responsible for the identification of the human immunodeficiency virus (HIV) in 1983 as the etiological agent of AIDS (Gallo *et al.*, 2002; Montagnier, 2002). The Nobel committee awarded the Nobel Prize in Physiology or Medicine to the two French virologists, Francois Barre-Sinoussi and Luc Montagnier in 2008, which they shared with a German oncovirologist for his work on the human papilloma virus (HPV). Research contributions and scientific achievements on HIV/AIDS have been formidable over the last 25 years. This varied from the identification of HIV as the causative agent of AIDS, to the development of effective antiretrovirals (ARVs) and treatment strategies, increased knowledge of molecular virology, epidemiology and the pathogenesis of this virus to name just a few (Fauci, 2003).

2.2 Highly Active Antiretroviral Therapy (HAART)

Highly active antiretroviral therapy (HAART), a combination therapy of three antiretroviral agents from at least two different classes, was first eluded to by David

Ho, M.D, of the Aaron Diamond Aids Research Centre in New York with an editorial he published in The New England Journal of Medicine in 1995 (Ho, 1995). During the 11th International AIDS Conference in Vancouver in July 1996, various reports and study results were presented that introduced the decade of HAART (Grossman, 2006; Bartlett, 2006).

The introduction of HAART in 1996 substantially improved the life expectancy (prognosis and outcome) of HIV-1 infected patients (de Martino *et al.*, 2000; Fraaij *et al.*, 2003; Gulick *et al.*, 1997; Palella *et al.*, 1998; Walker *et al.*, 2004). HIV/AIDS is now classified as a treatable chronic disease where the median life expectancy for patients on HAART is 24 – 39 years (Gardner *et al.*, 2009). However, despite the significant clinical benefits of HAART, little evidence is available that HIV can be eradicated by antiretroviral drugs or by the immune system (Johnston, 2010). The virus has the ability to persist in stable latent reservoirs despite treatment. Ultra-sensitive assay methods are able to detect very low levels of the virus during treatment. Furthermore, studies have shown that the virus rebounds with little exception when HAART is stopped (Chun *et al.*, 1997; Davey *et al.*, 1999).

There is general disagreement on the type of cure (functional or sterilizing) that should be used for HIV, this should be approached by obtaining a better understanding and insight of the characteristics and mechanisms of these persistent viral reservoirs. The search for a cure for HIV is one of the most challenging and potentially rewarding research areas in AIDS research (Johnston, 2010).

The primary principles and goals of HAART are to:

- i) reduce and sustain viral replication below the threshold of detection of standard clinical assays (HIV-1 RNA < 50 copies / ml);
- ii) restore and preserve immunological function;
- iii) reduce HIV-related morbidity and prolong survival;
- iv) improve patient quality of life; and
- v) prevent HIV transmission (DHHS Panel on Antiretroviral Guidelines for Adults, 2009).

Other emerging goals of HAART that have been identified include:

- i) the reduction of the risk for immune response inflammatory syndrome (IRIS);
- ii) the reduction of morbidity and mortality due to non-AIDS-related disease;
- iii) the achievement of normal life expectancy;
- iv) the reduction of a lifetime risk for HIV transmission to others; and
- v) the reduction of drug toxicity and improvement of drug efficacy (DHHS Panel of Antiretroviral Guidelines for Adults and Adolescents, 2008).

2.2.1 Treatment Failure

With the evolution of HAART in the mid 1990s, it was clear that this strategy of combination treatment with ARVs was found to reduce and inhibit HIV replication profoundly. Furthermore, viral suppression can be sustained and maintained successfully if the correct HAART regimen was prescribed and adhered to (Flexner, 2006; Fraaij *et al.*, 2003; Walker *et al.*, 2004).

Both the CD4⁺T-lymphocyte (CD4)-cell counts and plasma HIV-1 viral load (VL) values have been shown to have prognostic value (Moore *et al.*, 2009). Evaluation of the therapeutic outcomes of HAART, generally involves the monitoring and assessments of the clinical disease progression, immunological tests (CD4-cell count and CD4% especially in children) and VL on a 3 monthly or 6 monthly (resource poorer countries) basis (DiPiro, 2009; NDoH, 2010). The CD4-cell count is an indication of the degree of immune suppression in adults, and the CD4% as a total of all lymphocytes is the best indicator of immunodeficiency in children. The CD4-cell count is regarded as a reliable marker of progressive immunodeficiency (NDoH, 2010). Assessment of viral load is an indicator of the number of HIV-1 RNA copies / ml in plasma and is a reflection of the degree of viral replication. Viral replication will lead to the development of resistance and ultimately treatment failure (Starr *et al.*, 1999).

HAART requires lifelong treatment, which is a daunting task for any healthcare practitioner and even more challenging for people living with HIV/AIDS. Optimisation

of HAART and the prevention of treatment failure are of paramount importance, it is a continuous dynamic process to ensure that the best and most effective HAART is prescribed and adhered to (Cressey *et al.*, 2007; Flexner, 2006).

Many factors have been reported to influence the efficacy of HAART, with pharmacokinetic variability of ARVs, adherence and viral resistance identified as the most important (Cressey *et al.*, 2007). There is a delicate and complex interplay between pharmacokinetic variability, adherence and resistance on virological suppression, which have been extensively investigated (Pham, 2009). The pharmacokinetic variability of ARVs, adherence and viral resistance will be discussed briefly to provide better insight into their roles and contributions to ART failure.

2.2.1.1 Pharmacokinetic variability

Pharmacokinetic variability, due to differences in absorption, distribution, metabolism, excretion, drug-drug interaction, drug-food interaction, age, sex, pregnancy and genetics, may lead to sub-therapeutic and or toxic plasma concentrations of ARVs (Adkins *et al.*, 1998, Cressey *et al.*, 2007, Flexner, 2006, Stocrin, 2004).

Sub-therapeutic plasma concentrations of ARVs are of great concern and will ultimately lead to viral resistance and consequently to treatment failure. Factors leading to sub-therapeutic plasma concentrations include the variability of drug pharmacokinetic parameters such as low bioavailability, high metabolic clearance due to genetic variations, drug interactions and food-drug interactions. Non-adherence is a key factor, since drug related toxicity can also lead to poor adherence and ultimately to sub-therapeutic levels (Cressey *et al.*, 2007, de Maat *et al.*, 2003).

A randomised controlled trial by Khoo and co-workers indicated that 38% of adult patients taking PIs and NNRTIs had persistent sub-therapeutic drug concentrations when measured. Adherence was identified as the main factor for this large inter-individual variability, but other factors such as ethnicity, sex, body weight, hepatitis status and host genetic variability may also have been responsible (Khoo *et al.*, 2006). An alarming statistic is that up to 50% of ARV-naïve patients discontinue their

first line regimen due to virological failure or toxicity in clinical practice (Kiser *et al.*, 2005).

Pharmacokinetic variability due to genetic mutations and specific polymorphisms that occur on the cytochrome (CYP) P450 enzyme systems are discussed in greater detail under Pharmacogenetics in this chapter, as this was one of the main objectives of the study.

2.2.1.2 Adherence

Poor adherence to HAART is recognized as one of the crucial challenges in achieving better clinical outcomes for patients (Cressey *et al.*, 2007). However, to maintain adequate levels of adherence to HAART is proving to be very difficult for the many people living with HIV. To obtain the optimum benefit from HAART, specific daily dosages at specific time intervals must be taken and these individuals face a lifetime of therapy. Lima and co-workers, reported that incomplete adherence to HAART was strongly associated with increased mortality. Patients being non-adherent on efavirenz-based NNRTI therapies were at a higher risk of mortality (Lima *et al.*, 2009). Non-adherence, as already stated, was reported to be one of the leading causes of sub-therapeutic ARV levels and subsequent treatment failure (Cressey *et al.*, 2007).

The “golden standard” for measuring adherence to HAART has not yet been established, despite various studies that have been conducted and the numerous tools available to monitor and enhance adherence (Nachega *et al.*, 2007).

The widely cited article by Paterson *et al.* (2000), reported that a protease inhibitor adherence of 95% or greater is required to obtain an optimised virological outcome for HIV-infected patients. This publication led to the adoption of the principle that $\geq 95\%$ adherence to ARV medication was required to achieve optimal treatment efficacy (Paterson *et al.*, 2000; Gulick, 2006). However, results from REACH concluded that less than 95% adherence to NNRTI-based regimens can still lead to successful viral suppression (Bangsberg, 2006). In another very large (n=2821) observational cohort study, conducted in the South-African private sector with HIV-infected adults, virologic outcomes improved in a linear dose-response manner when adherence to NNRTI-based regimens increased above 50%. These results suggest

that good clinical outcomes can be achieved in routine clinical practice even when adherence is not perfect on NNRTI-based regimens (Nachega *et al.*, 2007).

The ultimate primary goal with adherence outcomes remains to strive for the highest possible level of adherence. The emphasis that is placed on near-perfect adherence to ARV therapy is and will always remain crucial to improve HIV treatment outcomes (Bangsberg & Deeks, 2002; Bangsberg, 2006).

2.2.1.3 Resistance

When viral replication continues in the presence or selection pressure of drug exposure, antiretroviral resistance is likely to occur (Shafer, 2004). The NNRTI class and lamivudine single gene mutations, have demonstrated high-level resistance, which can emerge rapidly (Casado *et al.*, 2000; Delaugerre *et al.*, 2001; Walmsley *et al.*, 2001). High-level resistance to other NRTIs and to the PI class of drugs requires multiple mutations and therefore takes longer to develop.

Efavirenz rapidly selects resistant viruses when sub-optimal dosage regimens are used. The K103N point mutation was most frequently observed in patients failing efavirenz combination therapy. Other prevalent mutations observed as double mutants in combination with K103N, included V108I, P225H, K101E or K101 and L100I (Bachelier *et al.*, 2000).

Drug-resistant HIV is often resistant to several classes of ARVs, cross-resistance within the same class is frequent and this complicates further efforts to curb viral replication (Clavel *et al.*, 2004). The prevalence of NNRTI resistance was very low in 1998 (0.4%), but it has since increased and was found to be around 7% during the 2006-2007 interval (Wheeler *et al.*, 2007). The success of first-line regimens is under threat due to the transmission of the antiretroviral-resistant virus. The benefit of resistance testing prior to initial HAART initiation cannot be overstated (King, 2008).

The prevention of mother to child transmission (PMTCT) program recommended the use of a single dose of nevirapine for the mother and baby to prevent HIV infection. This method demonstrated an efficacy in the prevention of HIV transmissions to at least half of the infants who would likely have become infected with no intervention

(Guay *et al.*, 1999; Moodley *et al.*, 2003). However, a large proportion of women and infants develop NNRTI resistance rapidly following this treatment (Johnson *et al.*, 2005; Jones *et al.*, 2005; Lyons *et al.*, 2005). The PMTCT program in South Africa has been widely implemented since 2003, and although still inadequate in its coverage, more children are being exposed to nevirapine for PMTCT. However, since nevirapine resistance fades (Eshleman *et al.*, 2001), it is unlikely that these mutations will be present on standard population genotyping prior to commencing a first line regimen with efavirenz. It is prudent to have baseline genotyping of the virus, should resistance occur on therapy (DHHS 2008, 2009), but this is not standard practice in developing countries, including South Africa, as it is too costly.

It is thus extremely important that good adherence is advocated at every opportunity and with each patient encounter; therefore the absolute need for adherence must be reinforced at every follow-up visit. Inadequate therapeutic levels of ARV will predispose patients to the development of resistant strains (NDoH, 2010).

2.3 Antiretroviral Agents

The development of antiretroviral drugs for the HIV-1 infection, of which zidovudine was the first drug to be approved in 1987, has been hailed as one of the most important accomplishments in medical history. ART has transformed this fatal disease into a chronic condition that can be controlled by the appropriate use of various combinations of antiretroviral drugs (King *et al.*, 2009b).

Six different pharmacological classes of antiretroviral agents are being marketed worldwide (refer to Table 2.1). The USA approved 23 antiretroviral agents and 6 fixed-dose combinations by July 2010. Currently we only have the 3 original antiretroviral classes available in South Africa (NRTIs, NNRTIs and PIs). New investigational antiretroviral agents are continuously being investigated to improve efficacy and dosing, and to reduce viral resistance and adverse effects. Some of these new investigational agents include elvucitabine (NRTI), TMC-278 and IDX899 (NNRTIs), bracanavir (PI), vicriviroc and PRO 140 (CCR5 inhibitor), elvitegravir (integrase inhibitor), bevirimat (maturation inhibitor) and ibalizumab/TNX-355 (CD4 receptor inhibitor) (Safrin, 2009). However, in July 2010 Merck stopped all investigations of vicriviroc-cell due to disappointing study results (AVERT).

Table 2.1: FDA (Food and Drug Administration – USA) approved antiretroviral agents

Antiretroviral classes	Antiretroviral drugs – Year of registration by FDA
<i>Nucleoside Reverse Transcriptase Inhibitors (NRTIs)</i> <i>Nucleotide Reverse Transcriptase Inhibitors (NtRTIs)[#]</i>	Zidovudine (ZDV / AZT)* - 1987 Didanosine (ddI)* - 1991 Zalcitabine (ddC) - 1992 Stavudine (d4T)* - 1994 Lamivudine (3TC)* - 1995 Abacavir (ABC)* - 1998 Tenofovir ^{##} (TDF) - 2001 Emtricitabine* (FTC) - 2003
<i>Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs)</i>	Nevirapine* (NVP) - 1996 Delavirdine (DLV) - 1997 Efavirenz* (EFV) - 1998 Etravirine - 2008 Rilpivirine
<i>Protease Inhibitors (PIs)</i>	Saquinavir* (SQV) - 1995 Ritonavir* (RTV) - 1996 Indinavir* (IDV) - 1996 Nelfinavir* (NFV) - 1997 Amprenavir (APV) - 1999 Lopinavir/ritonavir (LPV/RTV)* - 2000 Atazanavir* (ATV) - 2003 Fosamprenavir* (FPV) - 2003 Tipranavir* (TPV) - 2005 Darunavir (DRV) - 2006
<i>Fusion Inhibitor</i>	Enfuvirtide (ENF) - 2003
<i>Chemokine Receptor 5 Receptor (CCR5) Antagonist</i>	Maraviroc - 2007
<i>Integrase Inhibitor</i>	Raltegravir – 2007

Adapted and updated from Chen and Aldrovandi, 2008, (<http://www.fda.gov/ForConsumers/ByAudience/ForPatientAdvocates/HIVandAidsActivities/ucml18915.htm> (accessed 29 Sept. 2010)) and Mims, 2011. * Registered and available in South Africa. (Chen *et al.*, 2008; Snyman, 2011)

2.3.1 Treatment Guidelines for HIV-infected South African Children

The latest treatment guidelines released in July 2010 are recommendations of the International AIDS Society – USA Panel - and apply to adults and mostly to an

international developed world setting (Thompson *et al.*, 2010). Guidelines for the developing world will have to be adjusted according to each country's individual needs and the availability of resources. The World Health Organization (WHO) recommendations were revised and also published in 2010. The most important impact of both of these recommendations was that earlier initiation of ART is recommended in both adults and children (Thompson *et al.*, 2010, WHO, 2010).

The South African National Department of Health – Guidelines for the management of HIV-infected children (2005), recommended the following first and second line regimens for HIV-infected children (refer to Table 2.2), (NDoH, 2005). These were the guidelines that were followed in the clinical study (PK/PD.EFV.07) from the initiation in June 2007 until completion of the last follow-up visit in March 2010.

Table 2.2: *First and second line regimens for ARV initiation in children*

	6 Months up to 3 years	> 3 years and > 10 kg
First Line	Stavudine* Lamivudine Lopinavir / Ritonavir (Kaletra®)	Stavudine* Lamivudine Efavirenz
Second Line	Zidovudine Didanosine Nevirapine	Zidovudine Didanosine Lopinavir / Ritonavir (Kaletra®)

* Abacavir may be used if adverse events occur with this NRTI's. National Department of Health – Guidelines for the management of HIV-infected children, 2005 – Section 10 Table 10 p. 82)

The dosages of efavirenz in combination with stavudine, lamivudine, abacavir and co-trimoxazole (trimethoprim + sulfamethoxazole) are reflected in the Approved Study Protocol (Addendum A1).

However, in 2010 changes were made to the guidelines for the management of HIV-infected children > 3 years and > 10 kg (Table 2.3). The most important amendment to the new guidelines was that all children less than 1 year, who test HIV positive, will be initiated on ART irrespective of their CD4 status. Another aim of the new ART guidelines for children was to decrease the use of stavudine and not to initiate children on stavudine any longer. Children already on stavudine, with regimens that are stable, should continue with it and they should be monitored for possible

lipodystrophy. Should lipodystrophy or other toxicities related to stavudine develop then a single drug substitution to abacavir is proposed (NDoH, 2010).

Table 2.3: First and second line regimens for ARV initiation in children > 3 years and > 10 kg

	< 3 years or < 10 kg	> 3 years and > 10 kg
First Line	Abacavir [#] Lamivudine Lopinavir / Ritonavir (Kaletra®)	Abacavir [#] Lamivudine Efavirenz
Second Line	To be referred	Zidovudine Didanosine Lopinavir / Ritonavir (Kaletra®)

[#] For all children already on stavudine with no side effects, stavudine may be continued. Abacavir should substitute stavudine once lipodystrophy is suspected. National Department of Health – Guidelines for the management of HIV-infected children, 2010 – Section 6 p. 30-32.

2.3.2 Mechanism of Action of Antiretroviral Drugs

2.3.2.1 Nucleoside reverse transcriptase inhibitors (NRTIs)

NRTIs are synthetic nucleoside analogues of natural occurring nucleosides (thymidine, adenosine, cytidine, guanosine and uridine). The NRTIs are prodrugs that require intracellular conversion via phosphorylation to the active triphosphate component in the host viral cell. These active forms compete with natural substrates by acting as false substrates for the reverse transcriptase enzymes. Once the false substrates are incorporated into the viral DNA, the NRTI prevents the formation of essential bonds and are thus able to terminate the elongation of the viral DNA. Most of these agents have activity against HIV-1 and HIV-2. The NtRTI, tenofovir, is similar to the NRTIs, except for a structural difference in the phosphorylation process where only two rather than three intracellular phosphorylations are required (Flexner, 2006; Safrin, 2009).

Figure 2.1 represents the HIV life cycle and reflects where the various antiretroviral agents act to inhibit the HIV replication.

2.3.2.2 Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

NNRTIs are potent direct inhibitors of the HIV-1 reverse transcriptase enzyme and do not require intracellular activation. They block the RNA and DNA dependent DNA polymerase activities in a non-competitive manner. NNRTIs have no *in vitro* activity against HIV-2 (Flexner, 2006, Safrin, 2009).

2.3.2.3 Protease inhibitors (PIs)

PIs competitively inhibit the action of HIV protease. This enzyme is responsible for the proteolytic cleavage of viral polyproteins, GaG and GaG-pol into smaller functional proteins. By blocking the post-translational cleavage of the GaG-pol polyprotein they prevent the processing of viral proteins into functional entities, resulting in the production of immature non-infectious virions. They are active against both HIV-1 and HIV-2 and they do not require intracellular activation as in the case of NRTIs (Flexner, 2006; Safrin, 2009).

2.3.2.4 Chemokine receptor 5 (CCR5) antagonists

For the HIV to enter a human cell, it has to attach itself to proteins on the cell's surface. This attachment to a host cell involves the binding of the viral envelope glycoprotein complex gp160 (consisting of gp120 and gp41) to a protein CD4 receptor. This binding process induces specific conformational changes that will allow access and exposure to the host cells. CCR5 is one of two co-receptors that are required for the HIV to enter into the CD4 cells. CCR5 inhibitors bind specifically and selectively to these receptors and are thus directly responsible for blocking the entry of the HIV into the host cells (Flexner, 2006; Safrin, 2009).

2.3.2.5 Fusion inhibitors

Fusion inhibitors such as enfuvirtide bind to the gp41 subunit preventing the conformational changes that is required for the fusion of the viral envelope with the host membrane. It has no activity against HIV-2 (Flexner, 2006; Safrin, 2009).

2.3.2.6 Integrase inhibitors

Integrase is an enzyme produced by HIV and it is responsible for the early stage replication of both HIV-1 and HIV-2. By blocking this integrase enzyme, strand transfer is blocked and the final step of the provirus integration is inhibited. The integrase inhibitor thus interferes with the final integration of the reverse-transcribed HIV DNA into the chromosomes of the host cells (Flexner, 2006; Safrin, 2009).

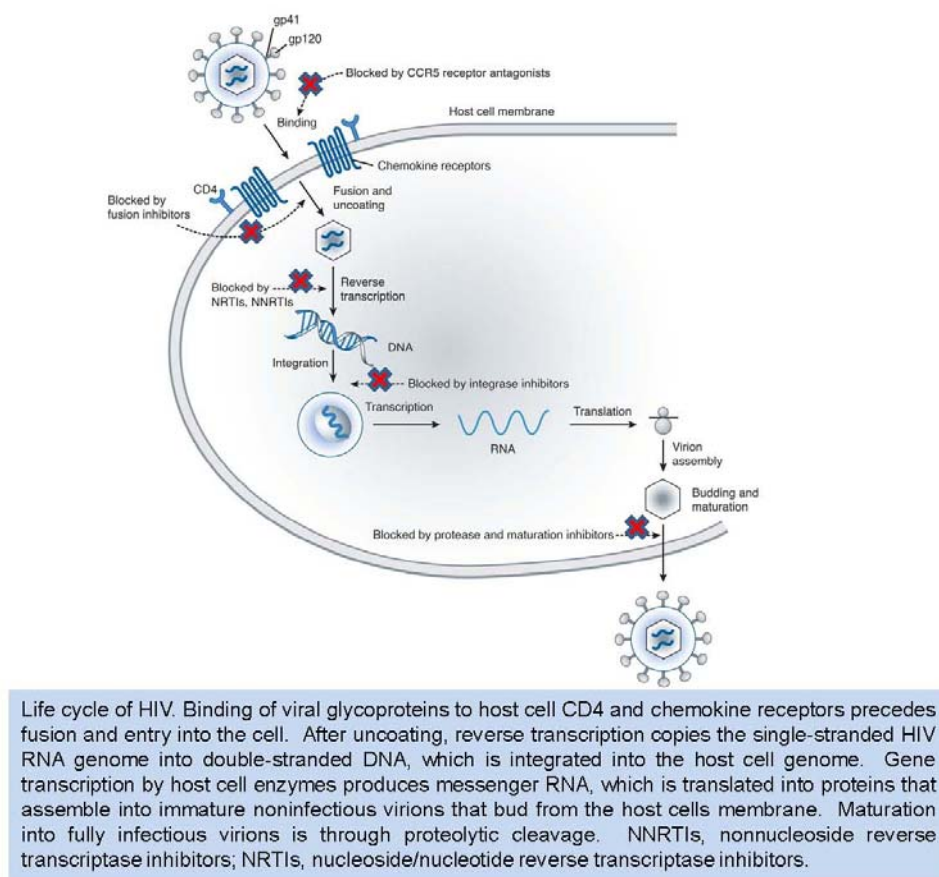


Figure 2.1: HIV life cycle and indication where the relevant ARVs act upon this life cycle of the HIV. (Taken from and used with permission from McGraw Hill, *Basic and Clinical Pharmacology*, Ed. Katzung, B.G.).

2.3.3 Pharmacokinetics and Therapeutic Drug Monitoring (TDM) of Antiretrovirals in HIV-infected Children

The early available guidelines for the treatment of children infected with HIV, recommended that the same principles of treatment for adults should also be applicable to children. However, the pharmacokinetic and pharmacodynamic

characteristics of several of these ARVs and regimens used in adults were not adequately defined in children (Fraaij *et al.*, 2004; Hoody *et al.*, 2003).

Significant inter-individual differences are observed in the pharmacokinetics (PK) of ARVs in children and in adults (Back *et al.*, 2002; Fraaij *et al.*, 2003; Fraaij *et al.*, 2004; Gerber *et al.*, 2003; Hoody *et al.*, 2003). Children are not just small adults and their pharmacokinetics and pharmacodynamics are distinctly different. Paediatric dosing is based on age, weight and or body surface, which are in contrast to adults who normally receive a standard fixed dose regardless of age or weight. Growth and development are dynamic, and these continuously changing parameters in children partly explain the differences in pharmacokinetics between adults and children.

Little data is available regarding the effects of HIV infection and antiretroviral drugs on body composition in children, particularly in resource restricted settings. Progressive subcutaneous fat wasting, fat accumulation, dyslipidaemia and insulin resistance in HIV-infected patients on antiretroviral therapy were attributed to the long-term toxicity of the PIs. Fat wasting however had been observed in patients who have never taken protease inhibitors in more recent studies, possibly implicating NRTIs. The effect of PIs is predominant and they may act synergistically with NRTIs. NRTIs appear to predispose individuals to slowly progressive fat loss, which is markedly accelerated when a PI and NRTI are combined. Stavudine leads to an earlier onset of clinical apparent fat wasting and a higher serum lactate concentration when compared to zidovudine (Mallal *et al.*, 2000). Studies have shown that ART dramatically improve growth in children. Height and weight are improved in children in whom a reduction of the viral load of at least 1.5 log or <500 copies/ml is achieved. In contrast to the increase of the body mass index (BMI) in adults on HAART, BMI did not increase in all children effectively treated with HAART. BMI increased more in children with an advanced stage of infection and a poor nutritional status as baseline (Verweel *et al.*, 2002; Chantry *et al.*, 2010).

The influence of changes in body composition (weight, height, growth, body fat, muscle mass or fat and muscle distribution) on the pharmacokinetic parameters of the antiretroviral drugs needs to be explored. These changes in body composition can be monitored by anthropometric measurements, which are affordable and non-invasive methods.

The pharmacokinetic differences between adults and children occur due to many factors, such as: physiological changes associated with growth and development, absorption and distribution of drugs, and renal and hepatic functions. The neonatal hepatic enzyme activity of the P450 enzyme system is approximately 20-70% of the adult values, it then increases to adult levels by 6-12 months, after which the activity exceeds adult levels at 1-4 years, and finally declines to normal adult levels at the end of puberty. This variability in the enzyme activity and maturation in the postnatal period, are thus responsible for significant alterations in the disposition of ARVs (de Wildt *et al.*, 1999; Fraaij *et al.*, 2004; Hoody *et al.*, 2003; Kennedy, 2008; King *et al.*, 2002).

The dosage selection of ARVs in a paediatric population cannot be based on age alone, due to the variability in weight among children of the same age, and this may lead to dosage errors. HIV-infected children tend to weigh less than non-infected children of the same age. Therefore the most accurate method for determining dosages of ARVs in children is based upon body surface area (BSA), which takes into account the patient's weight (kg) and height (cm) (King *et al.*, 2002).

HIV-infected children have much higher (10 to 100 fold) viral loads in their blood, which also necessitate a difference in management when compared to adults. Monitoring of plasma concentrations of the ARVs in children can be beneficial to the patient and reassure the clinician that the individual is receiving adequate exposure or to prevent toxicity (Aarnoutse *et al.*, 2003; King *et al.*, 2002).

The therapeutic ranges of antiretroviral drugs for children have not yet been formally defined or validated sufficiently in clinical trials. Concentration-response data are however available for most of the PIs and NNRTIs. It is further uncertain which pharmacokinetic parameter is the best to define the therapeutic and toxic exposures of drugs. Different pharmacokinetic parameters such as C_{min} (trough levels); C_{max} (peak levels) and area under the curve (AUC) are used for the assessment of TDM. The AUC is probably the most accurate, however, the analysis of the AUC is invasive, time consuming and complex. C_{min} is mostly monitored and accepted to determine efficacy, since it is the easiest to collect for both the patient and the investigator (Aarnoutse *et al.*, 2003; Fraaij *et al.*, 2004; Gerber *et al.*, 2003). A second method is the obtaining of randomly collected blood samples with a definite

recall of the time of the last dose, which is then correlated to population pharmacokinetic data for that specific drug. Inaccurate recall of the last dose by a few hours can produce inaccurate data especially in drugs with a short half-life such as the PIs (Gerber *et al.*, 2003).

TDM using plasma is considered not viable with the NRTI class, as the intracellular levels of the activated components need to be measured and require very specialized equipment and large quantities of blood, which is unsuitable for use in children (Flexner, 2006; Fraaij *et al.*, 2005; King *et al.*, 2002). TDM is more viable for the NNRTI and PI classes of antiretrovirals (Aarnoutse *et al.*, 2003; Back *et al.*, 2006; Flexner *et al.*, 2002; Fraaij *et al.*, 2004; Fraaij *et al.*, 2005; Marzolini *et al.*, 2001).

Measurements of most drugs in TDM utilise the total drug plasma concentration and not the unbound (free) fractions. Alternative drug measurements from biological matrixes, such as saliva and urine, are non-invasive with a lower or diminished risk of HIV-transmission to health workers, and could be a beneficial tool to investigate adherence (Kopecky *et al.*, 1997; Moeller *et al.*, 2008; van Heeswijk *et al.*, 2001).

Several pharmacokinetic studies have been performed over the years, especially in adults and very few in children. Various regimens of monotherapy and combination antiretroviral therapy have been investigated among children of different age groups. However, large scale clinical trials to establish the safety; efficacy and pharmacokinetic characteristics in children are still lacking for several reasons: different dosages and regimens used; too few participants in clinical trials (underpowered); different age groups (neonates-adolescents); varying duration of clinical trial periods (often only short term) and lack of controlled studies (Hoody *et al.*, 2003; King *et al.*, 2002; Saez-Llorens *et al.*, 2003).

The rationale for TDM in HIV infection had been the topic of discussion in numerous articles and was investigated in some clinical trials on different levels of intervention and application. It has been suggested that TDM could possibly: aid in preventing the development of viral resistance, prevent sub-optimal ARV plasma concentrations, be helpful in the management of drug interactions, be a tool to identify non-adherence, and could prevent avoidable toxicities and adverse reactions

(Aarnoutse *et al.*, 2003; Piscitelli *et al.*, 2001; Durant *et al.*, 2000; Acosta *et al.*, 2002; Back *et al.*, 2006).

2.4 Efavirenz

2.4.1 Background

Efavirenz, (S)-6-chloro-4-(cyclopropyl-ethynyl) 1,4-dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one ($C_{14}H_9ClF_3NO_2$), CAS-154598-52-4, has a molecular weight of 315.7, refer to Figure 2.2 (Flexner, 2006). It was first registered for use in 1998 by the DuPont Pharmaceuticals Company in the USA and marketed under the trade name Sustiva™ and later as Stocrin™ in countries such as South Africa. Efavirenz continues to play a very important role in HIV strategies and has contributed significantly to the evolution of HAART (Ruiz, 1999). It is recommended in various guidelines as part of first-line regimens in adults and children (Maggiolo, 2009).

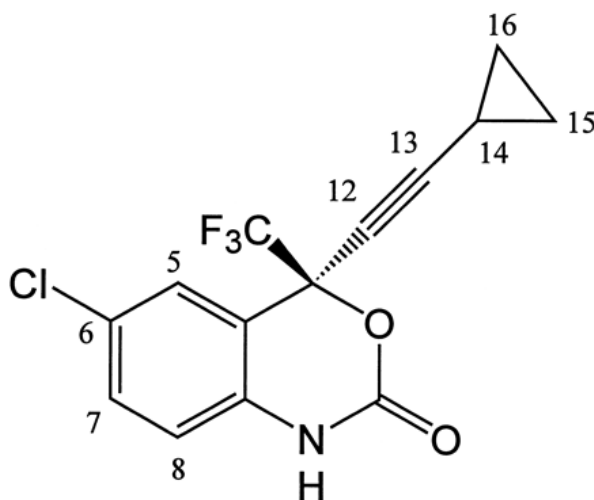


Figure 2.2: Chemical structure of efavirenz ($C_{14}H_9ClF_3NO_2$, $mm = 315.7$).

2.4.2 General Pharmacokinetics of Efavirenz

Efavirenz is well absorbed from the gastrointestinal tract and it reaches peak plasma levels within 5 hours. Increased doses can reduce absorption and high-fat content meals can increase the bioavailability (Adkins *et al.*, 1998; Stocrin, 2004). It is highly (> 99%) bound to plasma proteins, predominantly albumin (Aarnoutse *et al.*, 2003; Flexner, 2006; Stocrin, 2004). Certain clinical conditions and concurrent illnesses may influence or cause a fluctuation in the α_1 -acid glycoprotein (AAG) levels, it is

thus important to draw plasma samples under stable clinical conditions (Aarnoutse *et al.*, 2003).

Efavirenz is cleared slowly and it has an elimination half-life of 40 to 55 hours at steady state. This allows for dosing once a day, which contributes to the advantages of convenience and better patient compliance (Flexner, 2006; Stocrin, 2004). Efavirenz has been dubbed as “forgiving” for the occasional missed or delayed dose due to its long half-life, potency, convenience and tolerability (Gulick, 2006; King *et al.*, 2009b). However, the clearance can be faster and the half-life shorter in children. Half-lives of 14.7 hours and 12.93 hours have been reported in Burkino Faso (n=48) and German (n=11) children respectively (Hirt *et al.*, 2009; von Hentig *et al.*, 2006). The half-life reported in a study with HIV-1 infected adults (n=235) was 19 hours (95% CI, 13.2-24.2) (Csajka *et al.*, 2003) and the median estimated half-lives in HIV-infected adults (n=152) genotyped as G/G, G/T and T/T at position 516 of CYP2B6 were 23 (IQR, 18-35), 27 (IQR, 19-31) and, 48 (IQR, 39-77) hours respectively (Ribaud *et al.*, 2006). A more detailed discussion on the clearance of efavirenz in children is provided in § 2.4.3.

Less than 3% of the parent drug is excreted via the kidneys (Flexner, 2006), however 14 – 34% of efavirenz is excreted primarily in the urine, but also via biliary excretion as mostly the 8-hydroxyefavirenz glucuronide and less as the 7-hydroxyefavirenz metabolite (Mutlib *et al.*, 1999b; Ward *et al.*, 2003). Efavirenz is a substrate for CYP P450 enzymes and an inhibitor and inducer of CYP P450. Efavirenz is primarily metabolized by the hepatic CYP2B6, to a lesser extent by CYP3A4 and more recent findings reported on the involvement of CYP2A6 (di Lulio *et al.*, 2009; Kwara *et al.*, 2009). As a result of the involvement of the CYP P450 system, significant drug-drug interactions can and do occur (Flexner, 2006; Fraaij *et al.*, 2004; Fraaij *et al.*, 2005; King *et al.*, 2002). It was further reported that efavirenz enhances its own metabolism during repeated administration (Ngaimisi *et al.*, 2010; Stocrin, 2004; Ward *et al.*, 2003).

Variability in the pharmacokinetics of efavirenz has been investigated and reported in numerous studies. Unpredictable toxicity and efficacy can be attributed to inter-individual variability. High levels of inter-individual variability (coefficient of variation, CV%), ranging from 40 to 143% have been reported for efavirenz plasma

concentrations in adults and children. The intra-individual variability of the efavirenz pharmacokinetics was far less with a CV% below 30% (Csajka *et al.*, 2003; Marzolini *et al.*, 2001; Pereira *et al.*, 2008; Stahle *et al.*, 2004). Various factors such as environmental (drug-drug interactions, drug-food interactions, recreational drug intake), physiological (gender, age, disease states and pregnancy), and genetic factors (polymorphisms and mutations) can contribute to this inter-patient variability (Adkins *et al.*, 1998; Cressey *et al.*, 2007; Ekhardt *et al.*, 2009; Stocrin, 2004; Telenti *et al.*, 2008). Gender was shown not to affect clearance (CL), volume of distribution (V) and bioavailability of efavirenz in adults (Burger *et al.*, 2006; Csajka *et al.*, 2003; Pfister *et al.*, 2003; Ngaimisi *et al.*, 2010). Controversial opinions exist on whether the multidrug-resistance transporter (MDR1) gene that encodes for P-glycoprotein (efflux transporter) is associated with altered efavirenz plasma concentrations (Winzer *et al.*, 2003; Tsuchiya *et al.*, 2004; Haas *et al.*, 2004; Ngaimisi *et al.*, 2010).

2.4.3 Therapeutic Drug Monitoring (TDM) and the Pharmacokinetics of Efavirenz in Children

Reviews focusing on the role of TDM to optimise ARV therapy were in consensus that most of the ARVs, specifically the NNRTIs and PIs were considered candidates for TDM, refer to § 2.3.3 (Aarnoutse *et al.*, 2003; Acosta *et al.*, 2002; Back *et al.*, 2002; Back *et al.*, 2006; Kappelhoff *et al.*, 2004; Csajka *et al.*, 2003). However, the TDM of ARV does not form part of routine clinical practice in most countries and it was not recommended as a requirement in the most recent guidelines released by the International Aids Society, USA panel (Thompson *et al.*, 2010). In the Netherlands, TDM programs are used more regularly with a strict protocol in which plasma levels of ARVs are routinely and frequently monitored in specific participating hospitals (Kappelhoff *et al.*, 2005a).

The TDM of efavirenz in adults has been investigated extensively (Khoo *et al.*, 2006; Lopez-Cortes *et al.*, 2005; Marzolini *et al.*, 2001; Pereira *et al.*, 2008; Stahle *et al.*, 2004). Most of these studies have used mid-dosing sampling times. Marzolini and co-workers (2001) proposed the first suitable target to be between 1-4 ug/ml, when sampling 8 to 20 hours post last dose and when updated guidelines support that the C_{min} is above 1 ug/ml (la Porte *et al.*, 2006).

Successful therapeutic drug monitoring relies on accurate, selective and sensitive analytical methods to quantify the specific drug(s) in question. Numerous analytical methods to determine efavirenz in plasma were developed and published since 1999. Analytical reviews have also focused on the various developed analytical methods to determine efavirenz in plasma (Saint-Marcoux *et al.*, 2007). The majority of developed methods incorporated liquid chromatography coupled with mass spectrometry (LC-MS/MS) due to its superior specificity and sensitivity (Colombo *et al.*, 2005; Egge-Jacobsen *et al.*, 2004; Fan *et al.*, 2002; Ghoshal *et al.*, 2003; Koal *et al.*, 2005; la Porte *et al.*, 2006; la Porte *et al.*, 2009; Mutlib *et al.*, 1999a; Mutlib *et al.*, 1999b; Rentsch, 2003; Rouzes *et al.*, 2004; Volosov *et al.*, 2002). Alternative analytical methods that involved high performance liquid chromatography (HPLC) with UV detection (Dailly *et al.*, 2004; Marzolini *et al.*, 2000; Mogatle *et al.*, 2009; Rezk *et al.*, 2006; Saras-Nacenta *et al.*, 2001) or gas chromatography-mass spectrometry (GC-MS) were also published (Lemmer *et al.*, 2005). A rare immunoassay detection method was also published (Roucairol *et al.*, 2007).

Several studies have shown that saliva drug levels can be used to estimate the unbound / free drug concentration in the plasma of several drugs, for example: theophylline, anticonvulsants (phenytoin, carbamazepine, and phenobarbital), digoxin, salicylic acid, acetaminophen and nevirapine (Kopecky *et al.*, 1997; van Heeswijk *et al.*, 2001). Indinavir saliva levels have been used to monitor patient compliance (Wintergerst *et al.*, 2000). A recent LC-MS/MS method published by Theron *et al.* (2010) from our department, established a novel and robust screening method that is sensitive and specific to detect low concentrations of efavirenz in the saliva of patients on ARV treatment. This is a non-invasive sampling method and small volumes (100 µl) of saliva are required in the process of using it as a possible screening tool for monitoring efavirenz compliance. However, it has not yet been established whether saliva drug concentrations of efavirenz would be an accurate estimate of the unbound drug concentration in the plasma. If saliva efavirenz levels can be correlated with the plasma efavirenz levels, then it would simplify the determination of whether the patient has adequate efavirenz plasma levels, due to easier sample collection and no specialized training needed for the staff (Theron *et al.*, 2010).

Studies on the pharmacokinetic properties of efavirenz in children are few and involve small subject numbers. In the performed studies and future studies to be performed, Pharmacokinetic variability will affect treatment outcomes and it will depend on various factors. Furthermore differences between study cohorts include different ethnic populations (genetic differences), sample sizes, selected doses/regimens/formulations and duration of follow-up (Wintergerst *et al.*, 2008).

Table 2.4 provides a summary of efavirenz pharmacokinetic parameters investigated in **children** since 1997, obtained with conventional pharmacokinetic studies compared to Table 2.9, which is a summary of clinical studies in which the efavirenz population pharmacokinetic parameters have been modelled with programs such as NONMEM in both **adults and children**.

It has been reported that the dosage of efavirenz liquid formulations in children needs to be increased (20% higher with liquid dose) due to the poorer bioavailability of the liquid formulation (Starr *et al.*, 2002; Wintergerst *et al.*, 2008; ter Heine *et al.*, 2008). Starr and co-workers (2002), reported that the AUC in children on liquid efavirenz is 198 $\mu\text{M}/\text{h}$ compared to 218 $\mu\text{M}/\text{h}$ in the children on efavirenz capsules (Starr *et al.*, 1999; Starr *et al.*, 2002).

The Paediatric AIDS Clinical Trials Group (PACTG) study 382 was one of the first studies to investigate the pharmacokinetics of efavirenz in children and the results were reported in the Sustiva and Stocrin patient information leaflets. PACTG 382 was an open-label, phase I/II, AUC controlled study investigating the pharmacokinetics, safety, tolerability and antiviral activity of efavirenz in combination with nelfinavir and a NRTI. It was conducted at 18 sites and enrolment commenced in October 1997 and the pharmacokinetic data of 50 children at weeks 2 and 6 after commencing on the drug was available (Starr *et al.*, 1999). Efavirenz daily dosages were allometrically scaled to body size according to the following formula:

Efavirenz (mg/day) = (weight (kg)/70)^{0.7} x 600. The doses were then rounded to the nearest 25 mg.

Other paediatric dosing schedules used in some of the other studies were in line with the recommended dosages as reflected by the manufacturer's product information leaflet (Stocrin, 2004; Sustiva, 2005) as shown in Table 2.5.

Table 2.4: Summary of efavirenz pharmacokinetic parameters derived from studies performed in children

Reference	mg/kg/day	AUC	Cl/F	C _{min} (mg/l)	Mid-dose level (mg/l)	t _{1/2} (h)	V (l)	Nationality	CYP2B6 516G>T frequency	Age years (range)	n
Sustiva PACTG 382	*15.4	*58 ± 45.4 mg*hr/ l	*0.34 ± 0.16 l/h/kg	*1.7 ± 1.5				9 White 33 Black 15 Hisp.		*8 (3-16)	48
Starr <i>et al.</i> , 2002 PACTG 382	*15.7 Liquid	*198 uM/l/h						3 White 11 Black 5 Hisp.		#5.3 (3.1-9.6)	19
Von Hentig <i>et al.</i> , 2006	#12.3 (10.2-15.6)	*63.6 mg*hr/l	*97 (73-120) ml/min 0.08 l/h/kg	*1.3 (0.89-1.7)		*12.93 (11.4-14.5)	*100.5 (58-142)	German		#8.7 (4.3-12)	11
Ren <i>et al.</i> , 2007	#13.9 (13.7-14.5)				*1.58 (0.67-2.2)			Black		#6.83 (5.4-9.1)	15
Saitoh <i>et al.</i> , 2007	(Weight/70) ^{0.7} x 600		T/T 3.0 l/h/m ² G/T 5.7 G/G 7.0					8 White 46 Black 16 Hisp. 1 Am.Ind.	T/T 17% G/T 39% G/G 44%	#6.2 (4.3-9.2)	71
Wintergerst <i>et al.</i> , 2008	13.3 (9.7-22.5)		*0.3 (0.07-1) l/h/kg		#2.8 (0.13-11.6)			24 White 7 Africans 2 Asian		#8.2 (2.1-16.7)	33
Puthanakit <i>et al.</i> , 2009	*12 (1.6)				*3.14 (3.3) T/T 11.6 G/T 2.6 G/G 1.6			Thai	T/T 11% G/T 41% G/G 48%	#12.3 (3.1-18.7)	63
Viljoen <i>et al.</i> , 2010	*13.9 (1M) (13.4-14.5) *13.7 (3M) (13.3-14.04); *13.4 (6M) (13.1-13.7)				#1.86 (1M) (0.21-9.11) #1.62 (3M) (0.55-10.7) #1.84 (6M) (0.14-10.46)			Blacks		*6.8 (3-14)	60
Fletcher <i>et al.</i> , 2008		#60 mg*hr/l (W2+W56)	#0.19 l/h/kg W2 #0.23 l/h/kg W56 *0.21 l/h/kg W2 *0.26 l/h/kg W56	#1.45 W2 #1.30 W56				Burkina Faso		*7.9 (3-16)	50 W2 34 W56

median *mean; Hisp.=Hispanic; Am.Ind. = American Indian; 1M=1 month post HAART; 3M=3 months post HAART ; 6M=6 months post HAART; W2=2 weeks post HAART; W56=56 weeks post HAART

Table 2.5: Paediatric dosing recommendations of efavirenz (Sustiva® and Stocrin®)

Body weight (kg)	Efavirenz dose (mg) / daily
13 to < 15	200
15 to < 20	250
20 to < 25	300
25 to < 32.5	350
32.5 to < 40	400
> 40	600

The efavirenz dosing as proposed by the South African National Department of Health – Guidelines for the management of HIV-infected children - published in 2005, differed slightly from the dosing as suggested by the manufacturer, refer to italic and bold printed values in Tables 2.5 and 2.6. The 2005 guidelines were implemented at the Harriet Shezi Children's Clinic for the complete duration of the clinical trial PK.PD/EFV.07 (refer to Table 2.6).

Table 2.6: Paediatric dosing of efavirenz according to the guidelines of the South African DoH (NDoH, 2005, 2010)

South African DoH guidelines (2005)		South African DoH guidelines (2009/10)	
Body weight (kg)	Efavirenz dose (mg) / daily	Body weight (kg)	Efavirenz dose (mg) / daily
10 - 14.9	200	10 - 13.9	200
15 - 19.9	250	14 - 19.9	250
20 - 24.9	300	20 - 24.9	300
25 - 32.9	350	25 - 29.9	350
33 - 40	400	30 - 39.9	400
> 40	600	> 40	600

The findings of various studies investigating efavirenz plasma concentrations in children suggested that the high prevalence of sub-therapeutic efavirenz plasma concentrations in children might be an indication that efavirenz could be under-dosed in a number of paediatric patients. Sub-therapeutic efavirenz concentrations may result in the rapid emergence of NNRTI resistant mutants and treatment failure (Ren *et al.*, 2007; ter Heine *et al.*, 2008; von Hentig *et al.*, 2006). The percentage of efavirenz plasma concentrations within the recommended target interval (AUC (60-120 mg/l) and mid-dose or C_{min} (1-4 µg/ml) was not very encouraging in several studies. The within target percentages reported were 50, 47, 36, 66, 66, 71 and 58% in several paediatric studies (Fletcher *et al.*, 2008; Ren *et al.*, 2007; von Hentig *et al.*, 2006; ter Heine *et al.*, 2008; Hirt *et al.*, 2009; Puthanakit *et al.*, 2009; Viljoen *et al.*, 2010).

CL is an important pharmacokinetic parameter that can also contribute to variability. Various published CL values of efavirenz are reflected in Tables 2.4 and 2.9. Mean efavirenz clearance (CL/F) values in children varied between 0.26 (Fletcher *et al.*, 2008), 0.30 (Wintergerst *et al.*, 2008), 0.19 (ter Heine *et al.*, 2008) and 0.21 l/h/kg (Hirt *et al.*, 2009). In adults, CL was slower and varied from 0.13 – 0.17 l/h/kg (8.82 to 11.7 l/h) (Pfister *et al.*, 2003; Csajka *et al.*, 2003; Kappelhoff *et al.*, 2005a; Kappelhoff *et al.*, 2005b; Ribaud *et al.*, 2006; Cabrera *et al.*, 2009). The influence of genetics (CYP2B6 516G>T) on the CL value of efavirenz is addressed under § 2.4.4.2. The influence of the different covariates on population pharmacokinetic parameters such as CL will be discussed in more depth in § 2.4.6.2.

As stated previously, TDM does not form part of routine clinical practice in most countries, but with the large inter-individual variation in children, the need to monitor efavirenz in the paediatric population is considered to have become more crucial (Back *et al.*, 2006; Fraaij *et al.*, 2004). The TDM of efavirenz was proposed as a useful tool for the optimisation of therapy in children and adults and to monitor adherence (Aarnoutse *et al.*, 2003; Back *et al.*, 2002; Back *et al.*, 2006; Fraaij *et al.*, 2003; Fraaij *et al.*, 2004; Marzolini *et al.*, 2001).

2.4.4 Pharmacogenetics

Pharmacogenetics is the study of the genetic basis of an individual for variation in drug response. With the advanced technology currently available in genomics, a reverse genetic genotype-to-phenotype approach is feasible, in which genetic polymorphisms can serve as the starting point to assess whether genomic variability translates into a phenotypic variation. Polymorphism can be defined as a variation in the DNA sequence that is present in an allele frequency of 1% or greater in a population. In the human phenotype two major types of sequence variation have been identified: single nucleotide polymorphisms (SNPs) and indels (insertion/deletions) (Relling *et al.*, 2006). SNP is a DNA sequence variation that occurs when a single nucleotide (adenine (A); thymine (T); cytosine (C) and guanine (G)) in a species specific genome sequence differs between members of that species. An allele can be defined as one of two or more alternate forms of a gene and is characterized by a single SNP or by a haplotype. Haplotype on the other hand, is collections of SNPs in one chromosome that is linked to each other or occurs together in individuals (Telenti *et al.*, 2008).

Genetic polymorphisms and variations on the level of CYP P450 enzymes, other drug-metabolising enzymes and transporters (efflux transporters) are the main pharmacogenetic sources of inter-individual variations in drug metabolism (Nguyen *et al.*, 2000).

The MDR1 C3435T polymorphism responsible for coding P-glycoprotein (efflux transporter) was associated with immune recovery. The MDR1, 3435T/T genotyped, patients showed a greater rise in CD4-cell count compared to the C/T and C/C genotypes at 6 months post ART initiation (Fellay *et al.*, 2002). However, different research groups reported conflicting results (Cressey *et al.*, 2007).

The literature review will now primarily continue to focus on the impact of CYP2B6 polymorphisms on efavirenz metabolism.

2.4.4.1 Cytochrome P450 enzymes and CYP2B6

The CYP P450 isoenzymes are the most intensely studied of all the xenobiotic metabolising enzymes as they are collectively responsible for the majority of phase I

biotransformations of drugs and other xenobiotics (Gonzalez *et al.*, 2006; Telenti *et al.*, 2008).

CYP P450 isoenzymes comprise of a large multi-gene family of hemethiolate proteins that are important in the metabolism of various xenobiotics and endobiotics (Ekins *et al.*, 1999; Lang *et al.*, 2001). The main focus for researchers in the field of drug metabolism was for a very long time focused on the human CYP3A, CYP2D, CYP2C and CYP1A subfamilies. The subgroup CYP3A in particular had been extensively investigated and is the most abundant of the P450 family of enzymes in the liver and intestine and among the most important of all of the human-drug metabolising enzymes (de Wildt *et al.*, 1999; Wilkinson, 1996). CYP2B6 was regarded to be of less importance in drug metabolism and only gained more attention later when it was shown to be significantly involved in the metabolism of a number of clinically important drugs, including bupropion, coumarin, cyclophosphamide, diazepam, **efavirenz**, halothane, ifosfamide, methadone, nevirapine, nicotine, promethazine, tamoxifen, temazepam, and valproic acid (Ekins *et al.*, 1999; Lang *et al.*, 2001; Ward *et al.*, 2003).

The CYP2B6 gene is located on chromosome 19 (between 19q12 and 19q13.2) and spans over a region of approximately 28 kb, it consists of 9 exons which encode for a protein with 491 amino acids, refer to Figure 2.3 (Hoffman *et al.*, 2001; Lang *et al.*, 2001; Yamano *et al.*, 1989).

CYP2B6 is mainly expressed in hepatic tissue, but extra-hepatic expressions at lower levels were found in the kidneys, intestine, lungs, uterine endometrium, broncho-alveolar macrophages and peripheral blood lymphocytes (Ekins *et al.*, 1999). The CYP2B6 gene is highly polymorphic and genotyping for functional SNPs was the focus of various studies. The first systematic investigation of genetic polymorphisms in the CYP2B6 gene was published in 2001. The most common SNP reported in this group of 215 **healthy Caucasian** was 785A>G, with an allele frequency of 32.6%. Other mutations reported in this group of healthy Caucasians were C64T, G516T, C777A and C1459T at frequencies of 5.3%, 28.6%, 0.5% and 14% respectively (Lang *et al.*, 2001). More than 50 alleles of this gene have since been identified and described. The variant CYP2B6 516G>T, Gln172His, c.516G>T, located in exon 4 is also a common polymorphism and a marker of several

haplotypes such as CYP2B6*6; *7; *9; *13; *19; *20; *26 and *29. (<http://www.cypalleles.ki.se/cyp2b6.htm>).

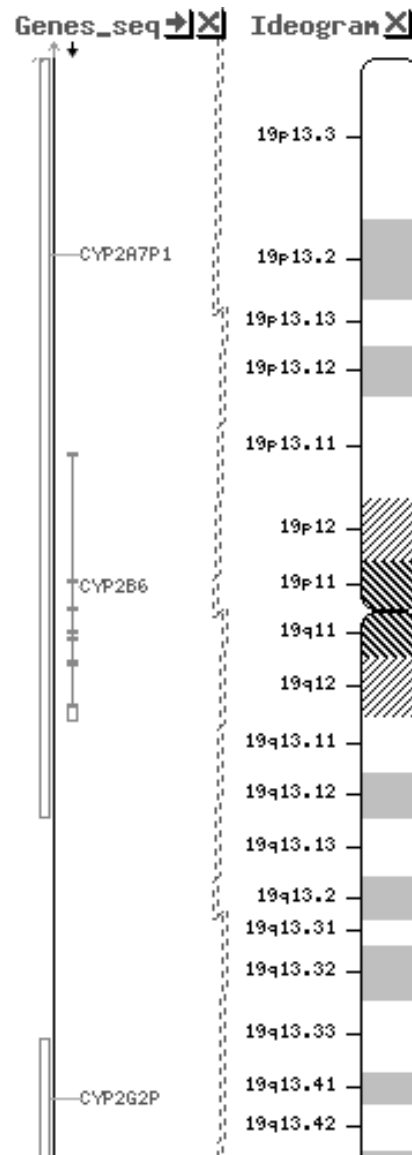


Figure 2.3: Ideogram CYP2B6 gene and cytogenetic coordinates 19q13.2 taken from <http://www.ncbi.nlm.nih.gov/mapview/maps>.

2.4.4.2 CYP2B6 G516T polymorphism and efavirenz pharmacokinetic variability

One of the first pharmacogenetic studies to report on the altered efavirenz metabolism associated with SNPs in the CYP2B6 gene was the Adult Aids Clinical Trials Group (AACTG) A5097s study. The CYP2B6 516TT genotype was associated with significantly greater efavirenz exposure due to lower clearance of the drug in African-Americans compared to Caucasians; the T/T genotype was 20% and 3% respectively. These inter-individual differences in the metabolism may in part be the

reason for central nervous system side effects associated with higher efavirenz plasma concentrations in African-Americans (Haas *et al.*, 2004). In another publication related to studies A5095 and A5097s, the median estimated efavirenz half-lives were 23, 27 and 48 hours in adult patients genotyped as G/G, G/T and T/T respectively, at codon 516 of the CYP2B6 gene (Ribaud *et al.*, 2006).

Numerous studies then followed, reporting that the CYP2B6 516G>T-allelic variant was consistently associated with reduced enzyme activity and elevated efavirenz plasma levels in different ethnic populations (Cohen *et al.*, 2009; Gatanaga *et al.*, 2009; Kwara *et al.*, 2009; Leger *et al.*, 2009; Puthanakit, *et al.*, 2009; Ramachandran *et al.*, 2009; Rodriguez-Antona *et al.*, 2000; Rotger *et al.*, 2005; Rotger *et al.*, 2007; Saitoh *et al.*, 2007; Tsuchiya *et al.*, 2004; Wyen *et al.*, 2008).

The overall reported allelic variant frequencies of CYP2B6 516G>T have generally been reported as high, regardless of race or ethnic background, although some of the earlier studies tend to show higher allelic variants in African-Americans compared to Caucasians and Asians (Haas *et al.*, 2004; Lang *et al.*, 2001; Tsuchiya *et al.*, 2004).

The T-allelic variant frequency at codon 516 in different ethnic populations (refer to Table 2.7) was 38% in African-Americans (Haas *et al.*, 2004), 21% in Caucasians (Haas *et al.*, 2004), 26% in the Swiss cohort (Rotger *et al.*, 2005), 26% in Whites, 28% in African-Americans, and 49% in Ghanaians (Klein *et al.*, 2005), 34% in Blacks (Wyen *et al.*, 2008), 29% in Caucasians (Wyen *et al.*, 2008), 45% in Ghanaians (Kwara *et al.*, 2009), 44% in South Indians (Ramachandran *et al.*, 2009) and most recently 32% (Cohen *et al.*, 2009) and 43% (Gounden *et al.*, 2010) in South African black adults.

An analysis of the CYP2B6 516G>T polymorphism in children were investigated and reported on by 3 research groups. Genotyping results from Saitoh and co-workers (2007), reported 44% G/G, 39% G/T and 17% T/T. The 516 T-allelic variant frequency was 44% in white non-Hispanic (n=8), 36% in black non-Hispanic (n=46) and 34% in Hispanic children (n=16), within this group of children participating in the PACTG 382 study. They found no difference among the 3 genotypes with regard to

gender, race/ethnicity and age. The overall 516 T-allele frequency in the whole cohort was 36% (Saitoh *et al.*, 2007).

Table 2.7: Reported frequencies of the T-allelic variant at codon 516 on the CYP2B6 gene in different ethnic groups

Reference	Black descent	Caucasian	Hispanic	Asian	Descent not specified
Haas <i>et al.</i> , 2004	38%	21%			
Rotger <i>et al.</i> , 2005					26%
Klein <i>et al.</i> , 2005 CYP2B6*6	28% A-A 49% G	26%		18% J 16% T 16% K	
Rodriguez-Novoa <i>et al.</i> , 2005			27% S		
Wyen <i>et al.</i> , 2008	34%	29%			
Saitoh <i>et al.</i> , 2007	36%	44%	34%		
Ramachandran <i>et al.</i> , 2009				44% SI	
Cohen <i>et al.</i> , 2009	32%				
Puthanakit <i>et al.</i> , 2009				31.75% TL	
Kwara <i>et al.</i> , 2009	45% G				
Gounden <i>et al.</i> , 2010	43%				

CYP2B6*6 (G516T and A785G) A-A: African-Americans; G: Ghanaians; J: Japanese; T: Tawaneese; K: Koreans; SI: South Indians; TL: Thai

In a Dutch cohort of 33 children, the genotyping results were 33%, 46% and 21% for the respective G/G, G/T and T/T genotypes (ter Heine *et al.*, 2008).

Puthanakit and co-workers (2009), reported that the genotyping of CYP2B6 516G>T in 63 Thai children is, 48% (G/G), 41% (G/T), 11% (T/T) and the 516 T-allelic variant frequency was 31.75%. In all of these studies it was shown that the CYP2B6 516T/T genotype was associated with elevated efavirenz plasma concentrations.

Studies reporting on measured efavirenz plasma concentrations at mid-dose interval in conjunction with the three genotypes (CYP2B6 516 G/G, G/T and T/T) are summarised in Table 2.8.

Table 2.8: Plasma efavirenz concentrations determined at mid-dose interval for CYP2B6 516 G/G, G/T, T/T genotype groups

	CYP2B6 516 G/G	CYP2B6 516 G/T	CYP2B6 516 T/T	Population description number of participants (n)	Reference
	Efavirenz plasma concentrations µg/ml				
Median	1.74	2.60	3.57	Spanish adults n=100 Cross sectional	Rodrigues- Novoa <i>et al.</i> , 2005
Median	1.779	2.299	6.248	Black and Caucasian adults n=174	Wyen <i>et al.</i> , 2008
Median	1.60	2.10	5.90	Black South African adults n=142	Cohen <i>et al.</i> , 2009
Median	1.282	1.560	8.320	Ghanaian adults n=65	Kwara <i>et al.</i> , 2009
Mean	1.604	2.635	11.582	Thai children n=63 Cross sectional	Puthanakit <i>et al.</i> , 2009
Median	2.260	3.858	7.136	Black South African adults n=80	Gounden <i>et al.</i> , 2010

Lang and co-workers (2001), reported that the G516T mutation was never found alone in their study population (healthy Caucasians), but always in combination with other mutations such as C64T, A785G and C1459T. Subsequent research papers were published reporting on CYP2B6 516G>T in combinations with other mutations on the CYP2B6 gene such as A785G and T983C (Haas *et al.*, 2009; Leger *et al.*, 2009; Nyakutira *et al.*, 2008; Tsuchiya *et al.*, 2004; Wyen *et al.*, 2008). In all of these reports, the various polymorphisms in linkage disequilibria with 516G>T were associated with increased plasma efavirenz concentrations.

The individual CL values of efavirenz in poor, intermediate and fast metabolisers (516 T/T; G/T, G/G genotypes respectively), were reported as 0.06, 0.10, 0.13 and

0.14, 0.26, 0.32 l/h/kg for Zimbabwean adults (n=74) and children (n=71) from mixed races respectively (Nyakutira *et al.*, 2008; Saitoh *et al.*, 2007). It is shown again that the CL of efavirenz in children is faster than in adults, even when the influence of CYP2B6 516G>T is taken into consideration.

Studies reporting on the association of efavirenz plasma levels and other CYP2B6 polymorphisms (not involving codon 516) and haplotypes were also published but are not further discussed here (Rotger *et al.*, 2007; Wang *et al.*, 2006; Wyen *et al.*, 2008).

2.4.5 Pharmacodynamics of Efavirenz

The efficacy and safety of efavirenz have been investigated and were well established in numerous clinical trials (Gulick *et al.*, 2004; Riddler *et al.*, 2008; Ruiz, 1999; Scherpbier *et al.*, 2007; Starr *et al.*, 1999; Staszewski *et al.*, 1999; van Leth *et al.*, 2004a; van Leth *et al.*, 2004b; van Leth *et al.*, 2004c). Efavirenz-based regimens are often preferred because of well established efficacy, excellent potency, convenience (daily dose), better tolerated and because they are less associated with long-term metabolic toxicity compared to PI-based regimens (Gulick *et al.*, 2004; King *et al.*, 2009a; Riddler *et al.*, 2008; Staszewski *et al.*, 1999). The only disadvantage of efavirenz-based regimens is the low genetic barrier to resistance. Failure of NNRTI-based regimens is often coupled with NNRTI resistance, and for this reason PI-based regimens may be preferred in patients who are judged more likely to be non-adherent. However, efavirenz-based regimens are considered to be the golden standard to which other antiretroviral regimens are compared (King *et al.*, 2009a; Riddler *et al.*, 2008).

2.4.5.1 Side effects and efficacy of efavirenz in combination therapy

PACTG 382 was one of the first studies to test the safety and efficacy of a then novel combination of efavirenz, nelfinavir and one or more NRTIs in children with ages ranging from 3.8 to 16.8 years. Fifty-seven (57) children were enrolled between October 1997 and February 1998 and they were monitored for 48 weeks. Prior to the enrolment, 55 of these children were already receiving one or more NRTI, but none were previously exposed to NNRTIs or PIs. They reported that the most

common side effects (moderate severity) associated with treatment were rash (30% of children), diarrhoea (18%), neutropenia (12%), and biochemistry abnormalities (12%). One (2%) life-threatening side effect of neutropenia was reported in a child who also had a concurrent varicella-infection. Central nervous system (CNS) side effects were uncommon, but dizziness or light-headedness was recorded (8/57, 14%) and it subsided once the efavirenz was administered at bedtime and not in the morning as per the original study protocol. Treatment was discontinued in 25% (14/57) of the children due to various reasons. Discontinuation of treatment was due to moderate rash (7%, 4/57), severe rash (2%, 1/57) and virological failure (11%, 6/57) as stated per their protocol. Other reasons included ineligibility to inclusion criteria (2%, 1/57), non-ability to take capsules (2%, 1/57) and non-compliance (2%, 1/57) (Starr *et al.*, 1999).

Efavirenz-based regimens are generally well tolerated by HIV-infected children with few side effects such as rash, while less fever and less frequent CNS symptoms were reported (Starr *et al.*, 1999; Starr *et al.*, 2002). Adverse events were visible in 28% (8/33) of children in a German cohort (Wintergerst *et al.*, 2008). Rash (16%, 10/63), CNS disturbances (22%, 14/63) and psychiatric problems (8%, 5/63) were evident in Thai children during the first few weeks post HAART (Puthanakit, *et al.*, 2009). In a South African paediatric population, none stopped efavirenz-based ART during the first 6 months post ART commencement due to reported side effects. A few side effects, such as nightmares (7%, 4/59), insomnia (2%, 1/59), rash and abdominal cramps were reported during the first month post-ART initiation. Low-grade transaminitis occurred in 11% (6/56) of the children 6 months post ART commencement (Viljoen *et al.*, 2010).

The well cited target range of efavirenz plasma concentrations between 1000–4000 µg/L (1-4 µg/ml) at mid-dosing interval was adopted (Marzolini *et al.*, 2001), together with the C_{min} not below 1 µg/ml (la Porte *et al.*, 2006), as guidelines for the monitoring and controlling of efavirenz plasma concentrations. Marzolini and co-workers (2001), reported that virological failure was observed in 50% of patients with efavirenz plasma levels of <1 µg/ml and the reversible CNS toxicity was approximately three times higher in patients with efavirenz levels of >4 µg/ml. Haas and co-workers (2004), further confirmed and established that the CYP2B6 G516T

variant was more common in African-Americans than in European-Americans and was associated with greater efavirenz plasma exposure and a plausible explanation for their susceptibility to efavirenz CNS side effects. Various other studies have also reported on the association of CNS side effects and higher efavirenz plasma concentrations in adults and children on efavirenz-based regimens (Cohen *et al.*, 2009; Gounden *et al.*, 2010; Gutiérrez *et al.*, 2005; Puthanakit *et al.*, 2009; Ramachandran *et al.*, 2009; Rotger *et al.*, 2005; Gatanaga *et al.*, 2007; Gatanaga *et al.*, 2009).

2.4.6 Population Pharmacokinetics of Efavirenz

2.4.6.1 Non-linear mixed effects model (NONMEM)

NONMEM is a program developed by the NONMEM project Group for estimating population pharmacokinetic parameters. It is written in FORTRAN and designed to fit general statistical (non-linear) regression-type models to data. NONMEM incorporates mixed effects (fixed and random) regression to estimate the various population means and variances of the relevant pharmacokinetics parameters and it can identify covariates that may influence these parameters (Beal *et al.*, 1998).

Sheiner and Beal from the University of California, San Francisco first introduced population pharmacokinetics in 1977. The advances in data analysis during the 1980s have opened up more comprehensive approaches, greater potential with more possibilities and applications (Whiting *et al.*, 1986; Barrett *et al.*, 2005). Simulation and modelling of clinical trial data has evolved significantly, it is being used as a predictive tool and proved very useful for optimum dose selection, hypothesis-generating purposes and optimisation of study designs. The Pharmaceutical industry also makes extensive use of simulation data derived from the pharmaceutical development phase and this contributes to the accelerated approval and labelling of drugs, especially in the field of antiretroviral agents (Holford *et al.*, 2000; Barrett *et al.*, 2005).

A crucial advantage of using NONMEM is that sparsely collected data can be used to generate population pharmacokinetics (Beal *et al.*, 1998). The future of population pharmacokinetics and pharmacodynamics for ARVs could evolve even more and

extend into the prediction of more effective drug candidate selection and the prediction of means to project specific therapeutic outcomes in individuals. The other benefit of modelling and simulation strategies is the support and enhancement of the development and quicker registration of newer ARVs (Barrett *et al.*, 2005).

2.4.6.2 Population pharmacokinetics of efavirenz in adults and children

The objectives of population pharmacokinetics are to characterize the main population pharmacokinetics parameters, CL and V of a specific drug and to identify the influence of various important covariates such as: gender, age, race group (genetic variation), weight, height and drug interactions that may be responsible for the variability in the drug exposure and response within the patient population. Pharmacodynamic explorations can be performed with population pharmacokinetics relevant to ARVs to investigate the relationship between the plasma ARV concentrations and treatment failure, time to virological failure, prediction of clinical failure and side effects (Barrett *et al.*, 2005).

Several population pharmacokinetic analyses of efavirenz in HIV-infected patients have been undertaken and published. The efavirenz population pharmacokinetic estimates and models where NONMEM was utilised are summarised in Table 2.9. The results of these studies mostly pertain to investigations in adults with some varying results that are discussed herewith. The influence of race and genotyping were the most profound in most of these studies.

Barret and co-workers (2002), described a two-compartment model for the population pharmacokinetics of efavirenz in a meta-analysis consisting of 16 Phase I studies in **healthy adult volunteers** (single and multiple doses) by using NONMEM. The CL of efavirenz after multiple dose administration was 10.2 l/h, but this was influenced by race and gender. CL was lower in Asians and Blacks than in Caucasians and the CL in the females was also slightly lower (9.08 l/h vs. 10.2 l/h) than in the males (Barrett *et al.*, 2002).

Pfister and co-workers (2003), reported that race influences CL, but not gender. The hepatic clearance of efavirenz in white **adults** (non-Hispanics) was 28% higher than in the African American and Hispanics (P=0.03). For the efavirenz population PK analysis, plasma measurements at 16, 24 and 48 weeks post-study treatment were

taken where permitted. Population PK measurements were spread out to ensure that not all samples were taken at the same time post dosing (Pfister *et al.*, 2003).

Csajka and co-workers (2003), concluded that no covariates (sex, height, age) or co-administration of antiretroviral drugs influenced the pharmacokinetics of efavirenz statistically in **adults**. A large inter-patient variability (CV, 54.6%) was reported to affect efavirenz's relative bioavailability but the intra-patient variability was relatively small (CV, 26%). The median number of plasma measurements per patient was 2 (range 1-12 over the 28 month period) and they further included full concentration-time profiles to be drawn between 0.3 and 20 hours post-last dose in 7 patients. No correlation was found between the population-based average (C_{av}) efavirenz concentrations and side effects and CD4-cell counts. A significant inverse correlation was found between the Log C_{av} and the log viral load (post-3 months of therapy). A significant association was also reported between the C_{av} and viral suppression (64% of patients ≤ 400 copies/mm³) for the entire duration of the study.

Kappelhoff and co-workers (2005), reported in their **adult** study that Asian race and baseline total bilirubin had a significant correlation with the bioavailability of efavirenz in the basic model. This however should be interpreted with caution, as the reduction in the minimal objective function value (OFV) and inter-individual variability in the final model was limited (Kappelhoff *et al.*, 2005a).

In the 2NN study by Kappelhoff and co-workers, which included HIV-1-infected **adult** patients from several sites in Europe, SA, Canada, USA, Argentina, Brazil, Australia and Thailand, the reported concomitant use of nevirapine increased the clearance of efavirenz by 43%. Efavirenz clearance from Thai patients was 53% higher compared to patients from SA and South America and 76% higher compared to the Western countries (Kappelhoff *et al.*, 2005b).

Ter Heine and co-workers (2007), investigated the population pharmacokinetic of efavirenz in **children**. They reported that the CYP2B6 genotype and drug formulation (oral liquid, tablets or capsules) significantly influenced the pharmacokinetics of efavirenz. The presence of the CYP2B6 516GG genotype in this population resulted in a 50-70% probability of them developing sub-therapeutic

trough levels, with only a 1-3% probability of being exposed to trough levels of >4 mg/l (ter Heine *et al.*, 2008).

Cabrera and co-workers (2009), developed a population pharmacokinetic model from TDM data in 131 HIV-infected **adult** patients. They investigated the effects of sex, age, total body weight, height, BMI, HIV treatment and in a smaller subgroup they also investigated the genetic polymorphisms of CYP2B6, CYP3A4 and MDR1. The presence of the 516 T/T and G/T polymorphisms resulted in lower efavirenz clearances of 50% and 75% respectively, compared to clearance values observed in the 516 G/G genotyped patients (Cabrera *et al.*, 2009).

Hirt and co-workers (2009), reported a mean CL/F value of 0.211 l/h/kg and a V/F value of 4.48 l/kg in West African **children** and that CL decreased significantly with age.

Two other studies, not reflected in Table 2.9, also analysed the population pharmacokinetics of efavirenz in **adults** but they utilised different software models than NONMEM. Villani and co-workers (1999), estimated the CL/F of efavirenz as 0.18 ± 0.072 l/h/kg when administered as mono-therapy and 0.16 ± 0.072 l/h/kg when administered in combination with nelfinavir in 22 adults with a 2-compartment model (Villani *et al.*, 1999). Ribaudó and co-workers (2006), assumed a 1-compartment, open, mixed-effect model with first-order absorption (NLME) and reported an estimated CL/F of 0.15 l/h/kg (10.7 l/h) and the median efavirenz half-lives as 23, 27 and 48 hours in 152 adult patients with the 516 G/G, G/T and T/T genotypes respectively, $P < 0.001$ (Ribaudó *et al.*, 2006).

Table 2.9: Summary of efavirenz population pharmacokinetic parameters in adults and children in different ethnic groups

Reference	Population	Daily Dose (mg)	CL/F (l/h)	CV %	V/F (l)	CV %	K _a (h ⁻¹)	CV %	t _{1/2} h	Model	CYP2B6 516G>T	Age (years)	n
Pfister <i>et al.</i> , 2003 AACTG 398	White (68%) Black (16%) Hispanic (11%) Asian (3%) Am.Indian (1%) Unknown (1%)	600	10.8 *	42	282* (7.2)	7.9	1.39* (10)	93		Well stirred 1-compart. First-order-abs.		40.2* (7.4)	139
Csajka <i>et al.</i> , 2003	White (82%) Black (13%) Hispanic (%) Asian (%)	600 800(2)	9.4* (8.7-10.3) (95% CI)		252* (200-326) (95% CI)		0.3*		19 (13.2-24.2) (95% CI)	1-compart. First-order abs.		38 [#] (18-75)	235
Kappelhoff <i>et al.</i> , 2005a	White (75%) Black (15%) Hispanic (7%) Latino (3%)	600 800 (5) 200-400 (7)	11.7* (4.4%) RSE		189* (14.6%) RSE					2-compart.. FOCE method		40 [#] (35.6 - 47.4, IQR)	172
Kappelhoff <i>et al.</i> , 2005b			8.82 (6.9%) RSE	60	418 (7.6%) RSE	54	0.287 (29.3) RSE			1-compart. First-order-abs. and elimination FOCE method		35.9* (8.9) 35.5 (8.8)	376 123
ter Heine <i>et al.</i> , 2008	Children	Liquid Capsules	13 ** (11.5%) RSE	52	424 (8.9%) RSE	40				1-compart. FOCE	TT=21% GT=46% GG=33%	6.5 [#] (0.9- 19)	33
Nyakutira <i>et al.</i> , 2008	Zimbabwean	600	GG - 9.4 (17%) RSE GT -7.2 (16%) RSE TT - 4 (19%) RSE		150 (98%) RSE		0.18 42% (RSE)			1-compart. First-order-abs. FOCE-I	TT=27% GT=44% GG=30%	39 (7)	58
Hirt <i>et al.</i> , 2009	West African children	14.4 mg/kg	0.21 l/h/kg (10%) RSE		4.48 l/kg 14% (RSE)		0.45 (23%) RSE		14.78	1-compart. FOCE		6.35 [#] (2.8-14.7)	48
Cabrera <i>et al.</i> , 2010	White 98% Black 2%	600 400 or 800	9.5 (4.06% SE)	37	311 (10.8% SE)	5	0.3 (fixed)			1-compart.	TT=18.8% GT=28.1% GG=53.1%	41.8* (9.1)	131

* mean values (± SD) # median (range or IQR, inter quartile range); ** allometrically scaled up to 70 kg; CV% coefficient of interindividual variation; FOCE: first-order conditional estimation method
FOCE-I: first-order conditional estimation method with interaction RSE: residual standard error SE: standard error CI: confidence intervals

2.5 References

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Chapter 3

Materials & Methods

3.1 Introduction

In this chapter, the following will be addressed:

- i) Practical aspects of the clinical trial (PK/PD.EFV.07).
- ii) Study design and population.
- iii) Analytical methods (liquid chromatography mass spectrometry (LC-MS/MS) and polymerase chain reaction (PCR) procedures.
- iv) Statistical methods.

Supporting documents such as Approved Study Protocol (final amended version No. 8, March 2009), Consent and Case Report forms (CRF) and Ethics Approval are included in Addendum A and relevant standard operating procedures (SOPs) are included in Addendum B.

3.2 Practical Aspects of the Clinical Trial (PK/PD.EFV.07)

3.2.1 Ethics Approval

The clinical trial (PK/PD.EFV.07): *Population pharmacokinetic and pharmacodynamic study of efavirenz in HIV-infected children treated with first line antiretroviral therapy in South Africa*, (Principal Investigator: M Viljoen), was approved by the Human Research Ethics Committee (Medical) University of the Witwatersrand, Johannesburg (Ethics reference number: 070413) and the Ethics Committee of the North-West University, Potchefstroom (NWU-0015-07-A5) in June 2007 (refer to Addendum A4). Informed consent was obtained from an

accompanying parent or legal guardian and/or assent from the child if he/she was older than 6 years. Parents, legal guardian(s) and study participants were provided with detailed information on the proposed study and what was expected from the study participant. Consent took place at the approved research site, Harriet Shezi Children's Clinic, Chris Hani Baragwanath Hospital, Soweto, South Africa, from 17 June 2007 to 2 March 2008. The consent process involved the principal investigator, study nurse and or nutritionist and a translator. A translator was always present during the procedures if the language medium was an obstacle and if the parent(s)/legal guardian(s) chose to have a translator present.

3.2.2 Funding

Funding was obtained from the NRF (Thuthuka – Researchers in training (RiT), 2007-2010), MRC (Self-initiative research grant 2008-2010), North-West University (2007-2010) and Drug Development and Research Focus area, School of Pharmacy, North-West University (2007-2011). These funding grants specifically pertained to the research aspects of this PhD study.

These grants also covered two related MSc projects in Pharmacology that were successfully completed for which this PhD candidate was the study supervisor:

- i) M du Plooy (2008): Blood levels of selective antiretroviral drugs over a period of time in Sprague-Dawley rats.
- ii) A Theron (2009): Plasma and saliva efavirenz levels in an HIV-infected paediatric population.

3.3 Study Design and Population

3.3.1 Study Design

This clinical trial (PK/PD.EFV.07) was a clinic-based, non-randomised open label study, performed on HIV-1-infected children who were attending the Harriet Shezi Children's clinic on an outpatient basis. The antiretroviral treatment guidelines and regimen of the National Department of Health (NDoH), South Africa, for the management of HIV-infected children were followed by the attending clinicians who

were consulting with these patients (NDoH, 2005). No investigational drugs; dosages or combinations were used. Each study participant was monitored and included in this study for a minimum period of 24 months (6 study related visits) and a maximum period of 30 months (including staging, baseline monitoring and screening).

Study visits were performed at baseline (0 months before commencement on highly active antiretroviral treatment - HAART), 1, 3, 6, 12, 18 and 24 months post-HAART commencement.

Baseline visit assessments:

Physical examination and blood samples for CD4-cell count, CD4%, viral load (VL), alanine aminotransferase (ALT), full blood count (FBC), *CYP2B6* 516G>T genotyping and population genotype sequencing for resistance mutations* were collected. The following anthropometric measurements: weight, height, mid-arm circumference*, waist circumference*, triceps and sub scapular* skin fold thicknesses and general personal background information. For children older than 9 years, assessment of Tanner stage* (physical maturity) was also recorded. Nutritional status, a 24-hour dietary recall* and general diet* questions were recorded by a qualified nutritionist.

Follow-up visit assessments (1, 3, 6, 12, 18 and 24 months post HAART):

According to the NDoH guidelines, routine 3-monthly visits for clinical assessments were scheduled for all patients attending this clinic, irrespective of their participation in this specific study. Repeat prescriptions for 3 months were issued for the specific antiretroviral (ARV) medication and the medicines were dispensed for 2 months and then 1 month, if no problems occurred, irrespective of their participation in this study.

The following information was collected and recorded at the specified follow-up visits:

- Two (2) blood samples (1.5 ml whole blood) were collected via an inserted cannula for the pharmacokinetic (PK) analysis. The exact time of the blood

* Genotype sequencing for resistance mutation investigations, certain anthropometric measurements, dietary recalls, saliva and urine sampling did not form part of the direct objectives of this PhD study but they were investigated by other study collaborators.

samples was meticulously recorded. The second PK sample was required to be taken 2 hours after the first PK sample to enable a better profile in the elimination phase.

- Blood samples for CD4-cell count, CD4%, VL, FBC and ALT were collected at (3, 6, 12, 18 and 24 months) and bloods for triglycerides (Trig.) and cholesterol (Chol.) were collected at 24 months only. Should virological failure occur (>1000 copies/ml, confirmed on a subsequent specimen within one month), then repeated resistance genotyping* was performed on blood specimens in between scheduled visits.
- Treatment adherence assessment was performed by the research pharmacist at the research site (diary card and counting/measuring of medicine).
- Dose and dosing schedule of the HAART regimen was checked and confirmed.
- Time of last 3 days doses of the HAART regimen (very specific for efavirenz) was recorded and/or checked on the diary cards provided.
- Dose and dosing schedules of all other possible and acceptable concomitant medication (e.g. co-trimoxazole and multivitamins) were recorded.
- The same anthropometric measurements and dietary assessments and recalls were recorded as stated for baseline visit*.
- Bio-electrical impedance analysis (BIA) to measure fat-free mass and fat mass was only measured at the 24 month visit*.
- Saliva and urine were collected only at 2 study visits (either 6 and 12 months or 12 and 18 months) from 20 study participants whom gave consent/assent for an additional MSc research study (refer to Approved Study Protocol, Addendum A1 for more details)*.

Figure 3.1 is a schematic diagram of the study design and layout.

* Genotype sequencing for resistance mutation investigations, certain anthropometric measurements, dietary recalls, saliva and urine sampling did not form part of the direct objectives of this PhD study but they were investigated by other study collaborators.

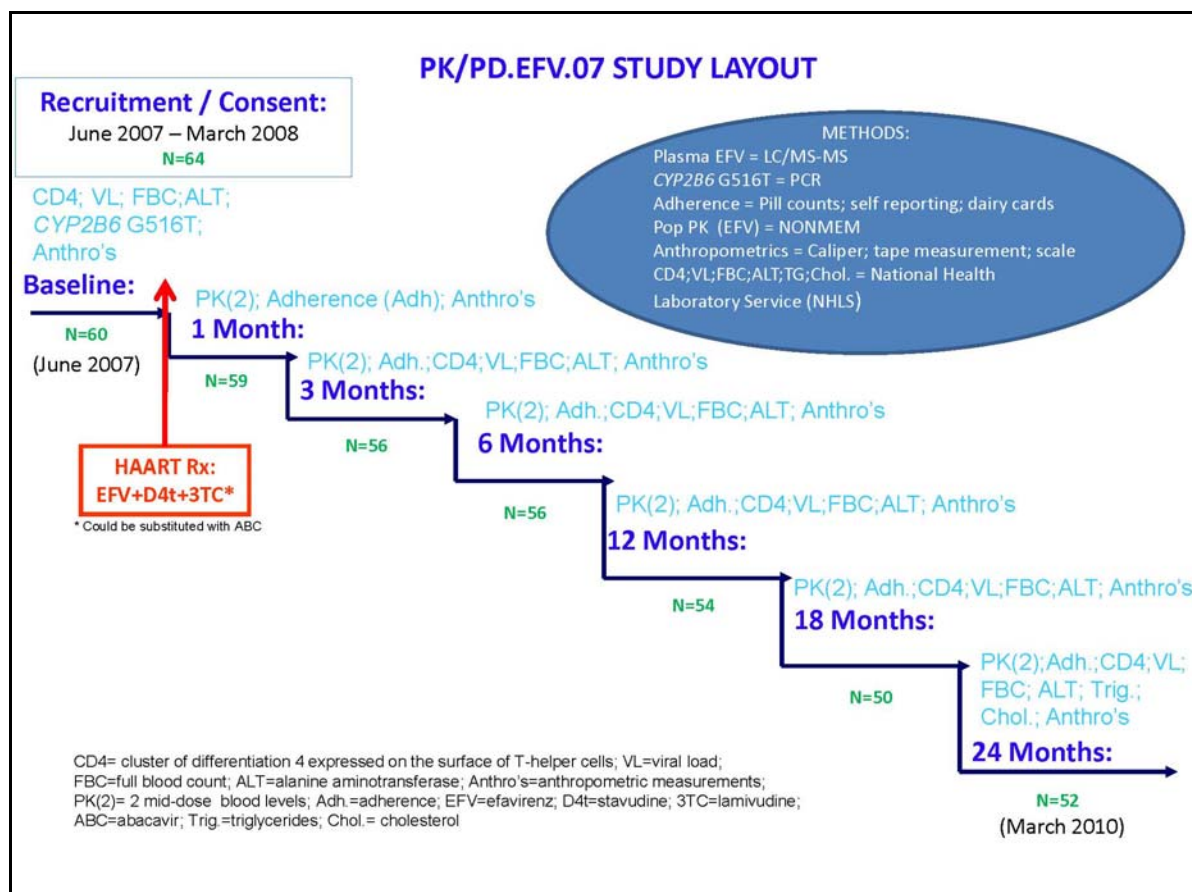


Figure 3.1: Schematic diagram of study layout and study visits.

3.3.2 Study Population

Sixty-four (64) black HIV-1-infected study participants were prospectively recruited and consent was obtained at the research site (Harriet Shezi Children's Clinic, Chris Hani Baragwanath Hospital, Soweto, South Africa) from June 2007 to March 2008. Only sixty (60) study participants (both genders) were finally enrolled onto the clinical trial (PK/PD.EFV.07). The cohort included children, 3-14 years, both genders, with no prior exposure to antiretroviral therapy (ART), eligible for ART and all were from resource limited households.

A summary of the approved inclusion and exclusion criteria follows (refer to Addendum A1 for Approved Study Protocol and for the sample size justification).

Inclusion Criteria:

- HIV-1 DNA PCR positive and, if possible, repeated at a second occasion.

- 3 to 16 years, both genders.
- Eligible to commence with first line HAART (efavirenz as part of their antiretroviral treatment).
- Concomitant use of multivitamins and co-trimoxazole.
- All other eligibility criteria as indicated by the NDoH – Guidelines for the management of HIV-infected children (NDoH, 2005).
- Signed informed consent from a legal guardian and, if applicable, signed informed assent.

Exclusion Criteria:

- Grade 3 - 4 defined toxicity according to the Pediatric AIDS Clinical Trial Group (PACTG) for laboratory tests and adverse effects.
- Use of midazolam or reserpine (internal standards in the LC-MS/MS method).
- Current use or recent exposure (within 4 weeks of the specific study visit in question) to any known cytochrome P450 inducers or inhibitors or any other drug at the discretion of the principal investigator.
- Any chronic medication taken on a regular daily basis besides the specified first line HAART regimen. Co-trimoxazole and amoxicillin for prophylaxis/treatment against opportunistic infections as specified by the DoH guidelines would be acceptable.
- Tuberculosis infection. Any study participant diagnosed with TB and started on anti-TB treatment while on the study to be withdrawn while on anti-tuberculosis regimen (at least 6 months). To re-enter post completion of anti-TB treatment after an additional 4 weeks wash-out period. This was added/amended and approved by the Ethics committee in October 2008.
- Known renal, hepatic or gastrointestinal tract disease resulting in mal absorption.
- Chronic diarrhoea.

3.3.3 Study Medication

The first line regimen of HAART and the dosages were according to the NDoH – Guidelines for the management of HIV-infected children (NDoH, 2005). The first line regimen consisted of efavirenz (non-nucleoside reverse transcriptase inhibitor, NNRTI) lamuvidine (nucleoside reverse transcriptase inhibitor, NRTI) and stavudine (nucleoside reverse transcriptase inhibitor, NRTI). Abacavir can be used if adverse effects were reported with the use of one of the NRTIs.

Table 3.1: Dosage guidelines for efavirenz according to the NDoH – Guidelines for the management of HIV-infected children (NDoH, 2005)

Body Weight (Kg)	Efavirenz capsules (mg) [#]
10 to 14.9	200
15 to 19.9	250
20 to 24.9	300
25 to 32.9	350
33 to 40	400
> 40	600

Stocrin™ Capsules (50 mg; 200mg; 600mg) were the only pharmaceutical products available at the state dispensary pharmacy until February 2009. Efavirenz generic products such as Aurobinda-Efavirenz™ (50 mg) capsules and Stocrin™ Tablets were phased in from March 2009 on an infrequent basis. All study participants were required to always report back to the Research Pharmacy at the Harriet Shezi Study site after collecting their ARVs from the state pharmacy for correct ARV checks. All efavirenz generic products were replaced with Stocrin™ Capsules (50 mg; 200mg; 600mg) where possible by the Research Pharmacist at the Harriet Shezi Study site.

3.3.4 Blood Sampling, Collection and Storage

On each follow-up visit, the two whole blood (1.5 ml) samples for the pharmacokinetic (PK) analyses were collected in ethylenediaminetetraacetic acid (EDTA) tubes. The PK blood samples were centrifuged at 3000 rpm for 10 minutes (min) within 1 hour of sampling. Plasma (top layer) was removed and stored in specific labelled Eppendorf tubes and stored at -80°C pending analysis with the validated LC-MS/MS method. The concentrated leucocytes (buffy coat), white thin second layer, was then removed and separately stored in nuclease free specific labelled Eppendorf tubes at -80°C pending DNA isolation. Refer to SOP-001 in Addendum B.

The routine blood analyses for CD4-cell count, CD4%, VL, FBC, ALT, Trig. and Chol. were sent to the National Health Laboratory Services (NHLS) at the Chris Hani Baragwanath Hospital, Soweto, Johannesburg, for analyses. The VL was determined with NucliSens EasyQ, Biomerieux, which is based on nucleic acid sequenced based amplification (NASBA). This VL assay can reliably detect HIV at a level of 357 IU/ml if 1 ml of plasma is tested; it has been optimised such that RNA copies/ml is comparable to IU/ml. The CD4-cell counts and CD4% were analysed by standard operation procedures using a light microscopic fluorescence technique.

3.4 Analytical Methods

3.4.1 LC-MS/MS Method for Efavirenz Determination in Plasma

3.4.1.1 Chemicals

Eluent solvents were high purity water, methanol (HPLC grade Burdick and Jackson Laboratory Co.); glacial acetic acid (SAARCHEM), and ammonium formate (Agilent Technologies). Efavirenz the reference standard was supplied by the World Health Organization: International Chemical Reference Substances (batch 104229). Midazolam (Dormicum® ampoule 5 mg/5 ml, batch Z7810 from Roche), internal standard (IS) was obtained from a local pharmacy with a prescription for research purposes only. Zinc sulphate was used as a precipitating agent. All chemicals were stored at the recommended and appropriate storage temperatures and conditions.

3.4.1.2 LC-MS/MS instrumentation

The chromatographic system consisted of an Agilent G1312A binary pump, a G1379B micro vacuum degasser and a temperature regulated auto sampler fitted with a six port injection valve with a 100 µl loop capillary. Efavirenz was separated from the internal standard (midazolam) on a Zobrax Eclipse XDB-C18 column (4.6 x 150 mm; 5 micron). The temperature of the column was maintained at 40°C. Efavirenz and midazolam (Mid) were detected with an Agilent 6410 triple quadrupole mass spectrometer in positive electrospray ionization mode. Manual product ion scanning was performed to select the best abundant fragment for each compound by adjusting the collision energy voltage and fragmentation voltage to provide the

highest sensitivity, as recorded in Table 3.2. The various multiple reaction monitoring (MRM) transitions that were measured for the analytes are reflected in Table 3.2. Nitrogen was used as a nebulizer gas and desolvation gas. The gas temperature (°C), gas flow (l/min), capillary voltage (V) and nebulizer pressure (psi) were set at 300, 12, 4000 and 40 respectively.

Table 3.2: Main mass spectrometry parameters for efavirenz and midazolam assay

Analyte	MRM	Collision Energy Voltage	Fragmentation Voltage
Efavirenz	316 → 244 [#]	10	120
	316 → 232 ^{##}	15	120
Midazolam (IS)	326 → 291 ^{##}	40	140
	326 → 223 [#]	40	140

MRM transitions used in the quantification of the efavirenz concentration. ## Qualifiers

The mobile phase was delivered at a flow rate of 0.5 ml/min and the gradient programme conditions are provided in Table 3.3. The total run time was 8 min. and the respective retention times (min.) were 6.7 and 7.2 for midazolam and efavirenz.

Table 3.3: Gradient elution programme

Time (min)	Flow (ml/min)	% Solvent A	% Solvent B
0	0.5	90	10
0.6	0.5	50	50
1.5	0.5	0	100
7.5	0.5	0	100
7.6	0.5	50	50
7.9	0.5	80	20
8	0.5	90	10

3.4.1.2.1 Mobile phases

Solvent A consisted of methanol (MeOH)-water (H₂O) (10:90 v/v). Solvent B consisted of a 5 mM ammonium formate buffer in 97% MeOH and 0.1% glacial

acetic acid. The pH of Solvent B should be between 5.6 - 5.9. Refer to SOP-003, Version 2 in Addendum B3.

3.4.1.2.2 Stock solutions, working solutions, plasma calibrants and quality control (QC) samples

Stock solutions (SS) of efavirenz were prepared by dissolving 5 mg EFV in 5 ml MeOH: H₂O (1:1) and stored at -20°C. Midazolam SS was obtained from Dormicum™ ampoules, 5 mg in 5 ml H₂O and stored at -20°C.

Working solution (WS) for efavirenz and midazolam were prepared by diluting the SS to a final concentration of 100 µg/ml in MeOH : H₂O (1:1) and H₂O, respectively.

Plasma donated from a healthy volunteer was used to prepare the plasma calibrators by serial dilution of the WS into plasma. Eight levels of plasma calibrators were used in the linear regression on a daily basis (refer to Table 3.4).

Table 3.4: *EFV plasma calibrators with corresponding theoretical concentrations*

Level (Plasma calibrant levels)	Concentration (µg/ml)
L 1	0.0937
L 2	0.1875
L 3	0.375
L 4	0.750
L 5	1.5
L 6	3
L 7	6
L 8	12

QC samples were prepared at L8, L6 and L2, and injected after every 15 samples in the LC-MS/MS system on a daily basis.

3.4.1.2.3 Sample preparation

The specific labelled clinical trial plasma samples were removed from the bio-freezer (-75 to -80°C) to thaw on the work bench. Plasma (100 µl) was transferred to clearly

individually marked Eppendorf tubes. The Eppendorf tubes were labelled according to the study participant number and the specific PK sampling time. The tubes were placed in a pre-heated oven for 30 min. at 56°C to de-activate the HIV-1 virus. Samples were removed and left on the work bench until room temperature was reached.

The following was added to the 100 µl plasma samples:

1. 40 µl IS (Mid – 1.325 µg/ml)
2. 160 µl MeOH (HPLC grade)
3. 40 µl Zinc sulphate (0.2 M)

The samples were vortexed for 10 seconds and centrifuged for 10 min. at 14 000 rpm. Visibly clear supernatant (180 µl) was transferred into a clearly marked insert/glass vial for injection into the LC-MS/MS system. The injection volume was 5 µl. Refer to SOP-002 and SOP-003 in Addendum B1 and B2.

3.4.1.2.4 Validation

This plasma LC-MS/MS method to quantify EFV was optimised and validated by modifying previously published methods by Koal and co-workers (2005) and Egge-Jacobsen and co-workers (2004). The general requirements of ISO/IEC/SANS 17025, 2005 were adhered to and the validation criteria of de Beer, 2006, were applied (ANON, 2005; De Beer, 2006). The validation results are reported in Chapter 7 (refer to § 7.2) and selective method validation results were published by Viljoen and co-workers (2010).

3.4.2 Polymerase Chain Reaction (PCR) for CYP2B6 516G>T Polymorphism

3.4.2.1 Deoxy nucleic acid (DNA) isolation

Genomic DNA was purified from 1.5 ml whole blood obtained at baseline and at subsequent PK study visits from each study participant. The whole blood was centrifuged and the concentrated leukocyte (white thin buffy coat) layer was stored in

specifically labelled nuclease free Eppendorf tubes at -80°C pending DNA isolation. DNA isolation was performed according to the instructions of the Flexigene DNA Kit (Qiagen GmbH, Hilden, Lot nr. 133221821) from the stored buffy coat (200 µl). The isolated DNA stock solutions (SS) were measured on a Nanodrop ND1000 to determine the DNA concentration from which working solutions (WS) were diluted with nuclease free water to 50 ng/µl. Each individual's, SS and WS, were specifically labelled and stored separately at -80°C pending further analysis.

3.4.2.2 *CYP2B6* 516G>T polymorphism

Genotyping of the target *CYP2B6* 516G>T gene polymorphism, located on chromosome 19, exon 4 was determined by PCR and restriction fragment length polymorphism (RFLP) analysis according to the method described by Lang and co-workers (2001). The PCR amplification produced a 526 base pair (bp) amplicon product with the forward primer 5'-GGTCTGCCCATCTATAAAC-3' and the reverse primer 5'-CTGATTCTTCACATGTCTGCG-3'. Tables 3.5-3.6 reflect the specific PCR equipment and conditions that were used.

Table 3.5: PCR equipment

Equipment	Source
Centrifuge (whole blood) – Beckman Coulter Allegra X-22	Beckman
Wealtec E-centrifuge	Wealtec Corporations
iCycler IQ version 3.0a Programmable thermal block – used as conventional PCR	Bio-Rad Laboratories
Nanodrop ND 1000 spectrophotometer	NanoDrop Technologies Inc.
Gel Doc™ EQ	Bio-Rad Laboratories
Electrophoretic tank and Power pack	Bio-Rad Laboratories

Table 3.6: PCR conditions

Forward primer	5'GGTCTGCCCATCTATAAAC 3'
Reverse primer	5' CTGATTCTTCACATGTCTGCG 3'
Reaction volume	25 µl
Cycling conditions	30 cycles of : 1: Denaturation at 95°C for 30s 2: Annealing at 59°C for 30s 3: Extension at 72°C for 60s Final extension 72°C for 5 min.
PCR Product	526 bp

The PCR reactions were performed in a total volume of 25 µl with 50 ng/µl of genomic DNA (refer to Addendum B4 for SOP-004). Tables 3.7-3.9 reflect the various quantities required for a single reaction volume, it can be multiplied with the number of samples intended to be analysed to calculate the quantities required for the respective master mix volumes.

Table 3.7: PCR reaction mix

	Volume (µl) X1	Master Mix Vol (µl) _____ samples
DNA (50 ng/l)	1.0	
Forward primer (10 µM)	1.25	
Reverse primer (10 µM)	1.25	
DreamTaq® Green Master Mix (Fermentas)	12.5	
Nuclease free H ₂ O	9.0	
Total	25	

The PCR product (526 bp) was digested with FastDigest® BseNI (Fermentas Life Science, Burlington, Canada) for 10 min. at 65°C and 5 min. at 80°C. The digested PCR products were electrophoresed on a 3% agarose gel with O'GeneRuler®, low

range, DNA ladder. Digestion of the homozygous wild type 516 G/G genotype yielded three fragments of 23, 236 and 267 bp, the homozygous 516 T/T genotype yielded two fragments of 23 and 503 bp and the 516 GT heterozygous yielded four fragments of 23, 236, 267 and 503 bp (refer to Figure 3.2). This analysis was performed at the Laboratory for Applied Molecular Biology (LAMB), Faculty of Health Science, School of Pharmacy, North-West University, Potchefstroom Campus.

Table 3.8: Restriction enzyme digestion (*FastDigest® BseNI*) mix

Component	Vol (μl) X1	Master Mix Vol (μl) ----- samples
PCR product (526 bp)	10	
10x FastDigest® Buffer	2.0	
FastDigest® BseNI Enzyme	1.0	
H ₂ O	17	
Total volume	30	

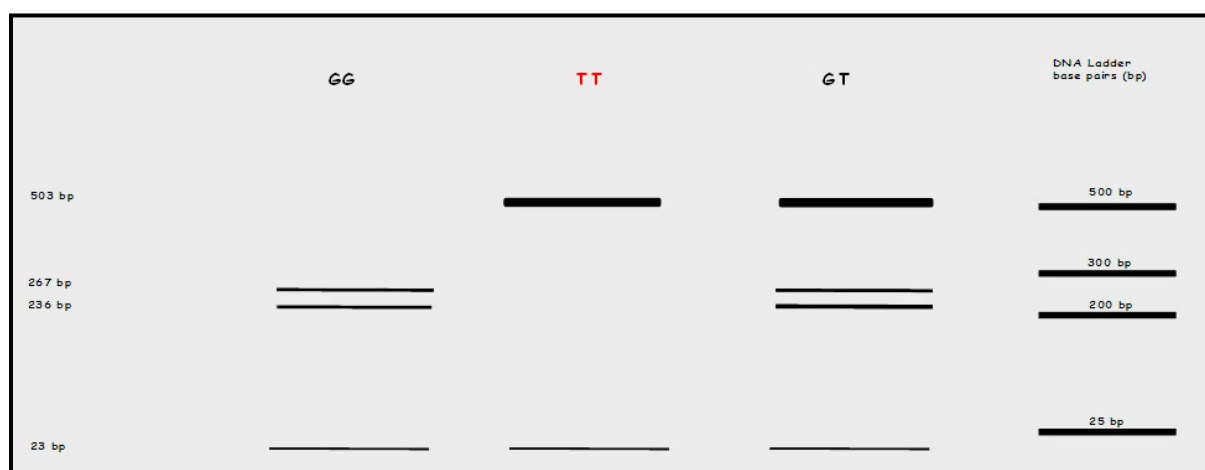


Figure 3.2: RFLP digestion pattern of CYP2B6 516 G/G, T/T and G/T, post *FastDigest® BseNI* enzyme digestion.

An alternative to the *FastDigest® BseNI* enzyme digestion was to use *BseNI* (BsrI). The PCR product (526 bp) was digested with *BseNI* (BsrI), Fermentas Life Science, Burlington, Canada, for 3 hours at 65°C in temperature controlled water bath or PCR

block (refer to Addendum B4 for SOP-004). The same fragments were yielded and were electrophoresed on a 2 % agarose gel with O'GeneRuler®, 50 bp, DNA ladder.

Table 3.9: Restriction enzyme digestion BseNI (Bsrl) mix

Component	Vol (μl) X1	Master Mix Vol (μl) ----- samples
PCR product (526 bp)	10	
10x Buffer B	2.0	
BseNI Enzyme	0.5	
H ₂ O	18	
Total volume	30.5	

3.5 Statistical Analysis

Descriptive statistics were used to describe results as mean ± standard (±SD) or median (minimum and maximum range) where relevant.

Repeated measure analysis of variance (ANOVA) with least square (LS) means and 95% confidence interval (CI) was used to investigate the change of variables over time (baseline to 24 months post-HAART) within this study population and when grouped according to the CYP2B6 516G>T genotype. Repeated measures ANOVA (LS means) only incorporated the complete data sets (all six occasions) over the 1-24 months. To exclude any **possible biased reporting, all PK1 and PK2** data were additionally repeated in a 2-way ANOVA analysis in The Mixed Procedure (model-based) with SAS (SAS Institute Inc. 2003, the SAS System for Windows Release 9.1 TS Level 1M3 Copyright© by SAS Institute Inc., Cary, NC, USA). These results were not repeated again in Chapter 7.

The Hardy-Weinberg exact test was performed on the study population for the genotype frequencies. Deviation of the genotype frequencies (observed and expected allele and genotype numbers) from the Hardy-Weinberg equilibrium (WHE) were assessed and compared with the Chi (χ^2) test.

The Kruskal-Wallis (K-W) test was used to investigate between-group comparisons of continuous variables and the χ^2 test for categorical variables. If the K-W test showed statistically significant differences between the groups, additional multiple comparisons by groups were performed to investigate exactly between which groups the differences occurred. Statistically significant differences were denoted when P-value < 0.05.

Correlations were determined by using the non-parametric Spearman Rank Order correlation when the EFV plasma data were not log transformed (Chapter 4, Manuscript A). A small, medium or strong correlation was defined as in Table 3.10.

Table 3.10: Relevance of *r*-values for small, medium or strong correlations

Correlation	Spearman r
Small	< 0.1
Medium	0.1 < r < 0.3
Strong	> 0.5

Correlation analysis of the log EFV plasma concentration data (parametric) was performed with Pearson's correlation (Chapter 5, Manuscript B).

Univariate and multivariate analyses were performed to investigate the influences of various independent variables (gender; age; genotype; dose; VL; CD4; ALT; adherence; resistance/ lipodystrophy/ gynecomastia/ IRIS; Trig.; Chol.) on the log EFV plasma concentrations at 1, 3, 6, 12, 18 and 24 months post-HAART initiation.

Paired-wise multiple linear regression analysis was used for the selection of the independent variables in the multivariate model and each occasion (1, 3, 6, 12, 18 and 24 months) was treated separately.

Data and statistical analyses of the results reported in Manuscript A were performed using GraphPad® Prism version 5.00 for Windows, (GraphPad Software, San Diego, California, USA, www.graphpad.com).

Gender-specific height, weight and body mass index (BMI)-for-age z-scores were calculated using WHO Anthro (version 2.02), and WHO AnthroPlus software (version 1.0, www.who.int/childgrowth/software/en).

Data and statistical analyses reported in Manuscripts B, C and rest of Results and Discussion (Chapter 7) were performed using Statistica® (StatSoft, Inc., version 9.0, Tulsa, Oklahoma, USA, www.statsoft.com).

All statistical analyses were performed in consultation with the Statistical Consultation Services, North-West University, Potchefstroom Campus (Dr Suria Ellis and Prof. Faans (HS) Steyn).

3.5.1 Sample Size Justification (Power of the Study)

The justification of the sample size ($n=60$) of this study population, with regards to the pharmacokinetic parameters, was addressed in the Study Protocol, refer to Addendum A1.

Based on the sample size ($n=60$) and $e = 0.354$ (Addendum A1), representing the minimum deviation from the average C_{min} to be tolerated within the 95% confidence interval the power of the study was calculated as 0.77 by a power sample t-Test in Statistica®,

3.5.2 Non-linear Mixed Effects Modelling (NONMEM)

The non-linear mixed effects modelling program (NONMEM) was incorporated to generate population pharmacokinetic parameters of efavirenz from sparsely collected data. NONMEM (version 7 level 1.2) with Intel® Visual Fortran Compiler version 11.0 software was used to perform the analysis (Beal *et al.*, 1986-2006). The first-order conditional estimate method (FOCE) with interaction between inter-individual, intra-individual and residual error was used for all models tested and the **population approach** was applied. It is important to note that with the first-order method used in this analysis, the exponential error model is approximated by a proportional error model (Cabrera *et al.*, 2009). The prediction-corrected visual predictive checks (pcVPC) were generated with the Perl speaks NONMEM (PsN)

tool “vpc”, version 3.2.7 and the graphical presentations of the results were performed with Xpose, package, version 4.3.0 using R, version, 2.12.0.

The adequacy of the models was evaluated by the following processes:

- i) *Posteriori* estimation (POSTHOC option in NONMEM) between PK parameters and covariates (Yano *et al.*, 2001).
- ii) Diagnostic graphics were obtained with generalised additive modelling (GAM) using Xpose 4.3.0 with the R program 2.12.0. Xpose facilitates data set checkout, exploration and visualisation, model diagnostics, candidate covariate identification and model comparisons (Jonsson & Karlsson, 1999; Ihaka & Gentleman, 1996; Lindbom *et al.*, 2004).
- iii) Differences in OFV (objective function value) between models were used to evaluate whether or not the added covariate significantly improved the fit, (differences were regarded as significant when the OFV decreased by 3.84 ($P<0.05$) or 6.63 ($P<0.01$) points for one or two additional parameters respectively).
- iv) A reduction in inter-individual variability (IIV/ISV) was a good indication.
- iv) Improvement in the goodness-of-fit plots and graphic diagnostics (weighted residuals) were relevant (Ihaka & Gentleman, 1996).
- v) Akaike’s Information Criterion (AIC) was applied as an initial estimate to investigate which important covariates influenced the PK parameters.
- vi) A close relationship between predicted and observed concentrations was a good indication. The 95% confidence interval (CI) estimations using standard errors should not include zero values and that the percentage estimation error of fixed and random parameters should not be higher than 25 to 50% respectively.
- vii) Gender, age, weight, height, body surface area (BSA) and genotype (CYP2B6 G516T) were the covariates evaluated to establish their influence on the PK parameters (CL/F and V/F) of efavirenz within this paediatric study population.

AIC was incorporated initially to investigate which covariates influenced the CL/F and V/F values. All of these covariates were then also additionally tested on the basic model using the difference in the OFV to indicate significant improvement of the fit as described above (iii). In addition to the already stated covariates we also introduce the effect of interoccasion variability (IOV) to the final population PK model. IOV was added as a random effect measurement.

- viii) An additional validation process was introduced on the final selected model by introducing a non-parametric bootstrap ($n = 200$) method generated in Perl speaks NONMEM, PsN 2.2.3, to provide information on parameter uncertainty (Lindbom *et al.*, 2004). To best validate the final model, the parameters estimated from the bootstrap analysis must be close to the estimations obtained from the original population set data. pc-VPCs were used as informative diagnostic tools to allow inspection of model appropriateness across time as well as across covariate values

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Chapter 4

Manuscript A

In this chapter, a manuscript titled:

“Efavirenz Plasma Concentrations at 1, 3 and 6 Months Post-Antiretroviral Therapy Initiation in HIV Type-1 Infected South African Children “

is presented. It was ***approved and published*** as a full-length research report in *AIDS Research and Human Retroviruses*, 2010, 26(6):613-619. The impact factor of this journal according to Journal Citation Report (JCR Science Edition 2009) was 2.178. *Instructions to the Author* for this journal are presented in Addendum C1.

Title page

Efavirenz Plasma Concentrations at 1, 3 and 6 Months Post- Antiretroviral Therapy Initiation in HIV Type-1 Infected South African Children

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ABSTRACT

The aim of this study was to quantify the plasma efavirenz concentrations over 6 months in black HIV-1-infected South African children (3-14 years), from resource limited households, attending an outpatient clinic. The children were antiretroviral treatment (ART) naïve and received efavirenz in combination with 2 nucleoside reverse transcriptase inhibitors, according to South African national guidelines. Two blood samples were taken between 12 and 20 h after last efavirenz dose at 1 (n=58), 3 (n=54) and 6 (n=54) months post-ART initiation. A total of 328 efavirenz mid-dose plasma samples from 58 patients was determined with a validated liquid chromatography tandem mass spectrometry method. Viral suppression (<25 copies/ml) was achieved in 95% of the children after 6 months on ART. The median (range) plasma concentration at time points 1 and 2 were 2.06 (0.10-11.14) and 1.80 (0.14-10.70) µg/ml with respective mean (±SD) blood sampling times of 15.24 (2.03) and 16.91 (2.03) h post evening dose. Efavirenz plasma samples within the therapeutic range of 1-4 µg/ml accounted for 58%; 17% were <1 µg/ml and 25% were >4 µg/ml, over the 6 months. Efavirenz levels persistently >4 µg/ml were recorded for 13 (23%) children and 3 (5%) children had persistent efavirenz levels <1 µg/ml. Possible reasons for efavirenz plasma levels outside the accepted therapeutic range include genetic variation in drug metabolism, incorrect dosing, drug-drug interactions, and non-adherence. However, these need to be further explored and the importance of sequential plasma levels has been highlighted in this study.

INTRODUCTION

The UNAIDS report of 2008 estimates 5.7 million [4.9-6.6 million] people were living with HIV in South Africa in 2007, of whom 280 000 [230 000-320 000] were children (0-14 years).¹

Efavirenz, a nonnucleoside reverse transcriptase inhibitor (NNRTI) forms part of the recommended national first line antiretroviral treatment regimen for children older than 3 years and weighing more than 10 kg.² Efavirenz has a long half-life (40–55 h) and is therefore suitable for once daily dosing.³

The challenges and shortcomings of efavirenz as part of highly active antiretroviral therapy include very large inter and intra-patient variability in the pharmacokinetics of efavirenz in children and adults.⁴⁻⁵ Unpredictable pharmacokinetics caused by altered absorption, genetic variations in metabolism and drug-drug interactions frequently lead to either sub-therapeutic levels and thus an increased risk of viral rebound, or to toxicity associated with central nervous system side-effects.⁶⁻⁸ The virus has a low genetic barrier to the development of efavirenz resistance and this may result in treatment failure.⁹ Paediatric formulations have not yet been developed or adequately tested in children and a substantial number of dosage recommendations for children are still absent from the guidelines.^{6,10} Poor adherence to the prescribed therapy also contributes to treatment failure and plasma concentration variation.¹⁰⁻¹²

The need to monitor efavirenz levels, especially in adults, has been investigated and published fairly extensively.^{4,8,13-14} Some of these studies have investigated the plasma efavirenz levels and applied mid-dosing sampling times. Marzolini and co-workers recommended 1-4 µg/ml as a suitable target when sampling between 8 and 20 h post-dose.⁸ Updated guidelines published in 2006 support the C_{min} to be above 1 µg/ml.¹⁵

Several studies have reported on the altered efavirenz metabolism in HIV-infected patients associated with CYP2B6 polymorphisms. It has been shown that elevated efavirenz concentrations are more frequently observed in Africans due to variation of the CYP2B6 gene and more specifically due to polymorphic homogenous genotype, 516G>T, encoding for the CYP2B6 isoenzyme.^{7,17-18}

Limited information on efavirenz plasma concentrations in sub-Saharan HIV-1 infected patients (children and adults) is available.^{17,19} In this study we present data

on efavirenz plasma levels taken in South African HIV-infected children at 1, 3, and 6 months after the commencement of ART. To our knowledge, this is the only study in South African HIV-infected children that has measured serial efavirenz levels over a period of time, and only the second study to investigate efavirenz plasma levels in black South African children.

MATERIALS AND METHODS

Population and study design

This was a prospective study of children attending the outpatient clinic at Harriet Shezi Children's Clinic, Chris Hani Baragwanath Hospital, Soweto, South Africa. The cohort included children (3-14 years) both genders, with no prior exposure to antiretroviral therapy, eligible for ART, and all from resource limited households.

Exclusion criteria included concomitant or recent (within 4 weeks of study visit) use of any known P450 cytochrome inducers or inhibitors, tuberculosis infection, renal and or hepatic dysfunction and chronic diarrhea.

Ethics approval was obtained from the North-West University, Potchefstroom (NWU-0015-07-A5) and the University of the Witwatersrand, Johannesburg (reference nr. 070413). ART eligibility was in accordance with national guidelines for the management of HIV-infected children in South Africa. The first line ART regimen for those over 3 years of age and over 10 kg of weight was comprised of stavudine, lamivudine, and efavirenz. Efavirenz dosing was based on weight banding per the national guidelines; for children weighing: 10-14.9 kg, 15-19.9 kg, 20-24.9 kg, 25-32.9 kg, 33-40 kg, and > 40 kg, the dose was 200, 250, 300, 350, 400 and 600 mg daily, respectively.² All of the children were receiving co-trimoxazole as primary prophylaxis prior to ART and during ART.

At each scheduled study visit, children were assessed clinically, weight, height, dosages, and adherence were recorded. Laboratory investigations (haematology, chemistry, viral load, and CD4 cell count) were performed at baseline and every 6 months unless otherwise indicated. These tests were conducted at the National Health Laboratory Services (NHLS) at the Chris Hani Baragwanath Hospital. The viral load was detected with nucleic acid sequenced based amplification (NASBA) performed with Nuclisens EasyQ (Biomérieux). A successful antiviral response was defined as a viral load lower than the detectable 25 copies/ml at 6 months. A

virological “blip” was defined as a viral load measurement between 25 and 1000 copies/ml preceded and followed by repeated measurement below 25 copies/ml.

Assessment of adherence

Adherence was assessed verbally and checked on a pre-issued diary card at each study visit. Additional assessments of adherence were required whenever a renewal or repeat prescription was dispensed on a non study visit day.

Measured adherence (%) was calculated as $[(\text{Volume prescribed} - \text{Volume returned}) / \text{Volume prescribed}] \times 100$. Any discrepancies were addressed and additional adherence and counselling support provided. Adherence was defined as good when the measured adherence was calculated between 93-107%. Non-adherence was defined when the measured adherence was calculated as $< 93\%$ or $> 107\%$, this allowed for 2 doses to be missed or taken incorrectly during a 28 day prescription cycle.

Efavirenz blood sampling and analyses

Two consecutive mid-dose interval blood samples (between 12 and 20 h postdose) were taken at 1, 3, and 6 months using a canula. The exact times of the blood samples and the time of efavirenz administration for the three previous evenings prior to the study visit were recorded. Efavirenz plasma analyses were determined by an optimised and validated LC/MS/MS method adapted from previously published methods.²⁰⁻²¹ The calibration curve ($n=8$) was linear over the range of 0.094-12 $\mu\text{g/ml}$. The mean ($\pm\text{SD}$) accuracy obtained for the calibration standards were 100.37% (6.32%) for 171 plasma standards and the coefficient of variation (CV%) was less than 8%. The within (intra-) and between (inter-) day CV% were less than 6% and 10%, respectively for three quality control samples (low, middle, and high concentrations). The lower limit of quantification was set at 0.094 $\mu\text{g/ml}$. Retention times were 7.1 and 6.8 minutes for efavirenz and midazolam (internal standard), respectively. The following possible co-administered drugs- stavudine, lamivudine, sulfamethoxazole, trimethoprim, paracetamol, and amoxicillin- did not interfere with the chromatographic conditions to determine efavirenz accurately.

Statistical analyses

Descriptive statistics were used to describe demographic results as mean $\pm$ standard deviation ($\pm\text{SD}$), or median (minimum and maximum range) for the efavirenz plasma concentrations. Intra- and inter patient variability were expressed as coefficient of variation (CV%) and analysed by repeated measures ANOVA. The paired t-test was

used to investigate statistically significant differences ($p < 0.05$) between baseline and 6 month variables (refer to Table 1). The Friedman test (non-parametric) was applied to investigate differences between the median plasma concentrations at 1, 3, and 6 months respectively. Correlations were assessed by the non-parametric Spearman test. The Mann-Whitney U test (non-parametric) was used to determine differences in the median efavirenz plasma concentrations of the patients defined as adherent or nonadherent. Data and statistical analyses were performed using GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego, California, USA, www.graphpad.com). Gender-specific height-, weight-, and body mass index (BMI)-for-age z-scores were calculated using WHO Anthro (version 2.02), and WHO AnthroPlus (version 1.0, www.who.int/childgrowth/software/en) software. The statistical analyses were done in consultation with the Statistical Consultation Services, North-West University, Potchefstroom Campus.

RESULTS

Patient demographics and characteristics

Sixty HIV-1-infected children were enrolled at baseline. Patient characteristics at baseline and 6 month post-ART are summarized in Table 1. All of the children were black Africans and 52% were males. The mean ($\pm$ SD) age, and weight were 6.8 (2.9) years and 20.19 (6.70) kg, respectively, at baseline. Most children had relatively low weight, height, and BMI values for their age and gender at baseline, but their linear growth improved significantly ($p < 0.05$, Table 1) over 6 months.

The median (range) viral load decreased significantly ($p < 0.002$) after 6 months post-ART from 52,500 (640-3,000,000) to 25 (25-170,000) copies/ml, respectively. Viral load values lower than detectable (< 25 copies/ml) were measured for 95% (53/56) of the patients after 6 months. The remaining 5% (3/56) had viral load values of 780, 2800, and 170,000 copies/ml, respectively, at 6 months post-ART.

Efavirenz dose and plasma concentrations

The night time efavirenz doses were fairly evenly distributed between 200, 250, 300, and 350 mg (24%, 29%, 24.5%, 17.5%) with only 5% of the doses being either 400 or 600 mg during the 6 month period. The mean (95% confidence interval) efavirenz daily doses were 13.92 (13.38-14.45), 13.65 (13.26-14.04), and 13.41 (13.08-13.74) mg/kg/d at 1, 3, and 6 months, respectively.

A total of 328 efavirenz mid-dose plasma samples from 58 patients (n=58, 54, and 54 at 1, 3, and 6 months respectively) were utilized in the concentration analyses over the 6 month period (Table 2). Efavirenz plasma levels outside the calibration range (0.094-12 µg/ml) accounted for nine samples from three patients (Table 2); these results were excluded from all of the calculations, but are reported where relevant as persistently <1 µg/ml or >4 µg/ml.

A total of 164 efavirenz plasma samples were taken for the first time point at 1, 3, and 6 months respectively. The median (range) efavirenz plasma concentration over the six months was 2.06 (0.10-11.14) µg/ml at a mean (±SD) sampling time of 15.24 (± 2.03) h post-last dose. A total of 164 efavirenz plasma samples were also taken for the second time point at 1, 3, and 6 months respectively. The median (range) efavirenz plasma concentration was 1.80 (0.14-10.70) µg/ml at a mean (±SD) sampling time of 16.91 (2.03) h post-last dose. The median efavirenz plasma concentrations at time points 1 and 2, respectively, were similar over the 6 months and did not indicate significant differences when compared between 1, 3, and 6 months (Friedman test).

There was a high level of variability in the efavirenz plasma concentrations between the study individuals (inter), namely 137% and 143% at time points 1 and 2, respectively. Variability within the same individual (intra) was much lower at 25% and 18% at time points 1 and 2, respectively, over the 6-month period.

The measured efavirenz concentrations showed significant positive correlation ($p<0.05$) with age, dose and weight at 1, 3 and 6 months respectively (Spearman correlation coefficient $r>0.27-0.47$).

Efavirenz plasma samples at time points 1 and 2 within the target range (1-4 µg/ml), represented 58% (189/328) of all the samples taken over the 6-month period. Sub-therapeutic levels (<1 µg/ml) were reported for 17% (56/328) of samples and 25% (83/328) of samples were above 4 µg/ml taken during mid-dose interval.

The number of plasma concentrations <1 µg/ml accounted for 16% (18/114) at 1 month, 20% (22/108) at 3 months, and 15% (16/106) of the samples tested at 6 months. Persistent efavirenz concentrations <1 µg/ml were observed in 5% (3/56) of the children at 1, 3 and 6 months, with viral load values of 2800 and 170,000 copies/ml respectively in two of these children after 6 months. The viral load of the third child was <25 copies/ml, although the efavirenz levels were persistently below 1 µg/ml at 1, 3 and 6 months post-ART.

It was further observed that 23% (13/56) of the children had persistent efavirenz concentrations >4 $\mu\text{g/ml}$ at 1, 3, and 6 months. Although 25% (83/328) of the measured efavirenz plasma concentrations were above 4 $\mu\text{g/ml}$, none of the patients stopped taking the efavirenz based ART during the first 6 months due to side-effects or for any other reasons. Very few side-effects were reported in the study population in general. Mild side-effects such as dreaming (nightmares), rash, and abdominal cramps that were reported subsided spontaneously within the first few weeks of treatment. Nightmares were reported in 7% (4/59) of the children during the first four weeks on treatment and insomnia was reported in 2% (1/59). Among these children, four had efavirenz plasma levels between 1 and 4 $\mu\text{g/ml}$ and only one child who reported nightmares had elevated levels >4 $\mu\text{g/ml}$. This child was also one of the 13 patients that had persistently elevated efavirenz levels. Low grade transaminitis occurred in 11% (6/56) of the children of whom 7% (4/56) had efavirenz levels persistently above 4 $\mu\text{g/ml}$ after 6 months on treatment.

Figure 1 represents the combined efavirenz plasma concentration data vs. time at time point 1 (PK1) and time point 2 (PK2) over the 6-month period, indicating the plasma levels within the therapeutic range and outside the therapeutic range.

Adherence and efavirenz plasma concentrations

Good adherence was recorded for 59% (35/59), 54% (30/56), and 18% (10/56) of the children at 1, 3, and 6 months respectively. The number of adherence assessments (pill counts) was recorded for only 71% (42/59), 73% (41/56), and 59% (33/56) of the children resulting in a large number of patients (29%, 27%, and 41%) not returning their medicines and or not reporting for adherence assessments at 1, 3, and 6 months respectively.

The median efavirenz plasma concentration (post-1 month) in the adherent (2.43 $\mu\text{g/ml}$) vs. nonadherent (1.2 $\mu\text{g/ml}$) group was significantly ($p=0.02$) different, but no statistical significant difference was observed in the median efavirenz plasma concentration when grouped (adherent vs. nonadherent) at 3 and 6 months post-ART (Mann-Whitney U test).

Viral load values >25 copies/ml were recorded for 5% (3/56) of the patients. Persistent plasma efavirenz levels <1 $\mu\text{g/ml}$ were recorded for two of these patients at mid-dose intervals and the third had a borderline concentration of 1.01 $\mu\text{g/ml}$ at 16.67 h post-last dose. The adherence of all three of these patients was very poor:

two were nonadherent and the other never returned previous medicines for the specific adherence assessments.

DISCUSSION

To our knowledge, this is the first study to report on sequential measured efavirenz levels at mid-dosing interval in a relatively large (n=58) cohort of black South African HIV-infected children. It is well described that sub-therapeutic efavirenz concentrations (<1 µg/ml) could result in viral mutations conferring drug resistance and leading to virological failure.^{8,22-23} Conversely, central nervous system toxicity can occur at levels >4 µg/ml.⁸ In our study, 95% of the children were virally suppressed with viral loads below detectable levels (<25 copies/ml) after 6 months on ART. The remaining 5% of the children had efavirenz plasma concentrations <1 µg/ml or at borderline and were not virally suppressed. These results support the findings of Marzollini and co-workers that efavirenz levels <1 µg/ml during mid-dose sampling will increase the possibility of virological failure.⁸

Our findings of the median efavirenz plasma concentrations, 2.06 µg/ml and 1.80 µg/ml, taken at 15.24 and 16.91 h post-last dose are in agreement with other similar studies where plasma levels (median 2.19, 1.58, and 2.8 µg/ml and mean 3.14 µg/ml) were also sampled at mid-dose.^{8,19,24-25} Our mean efavirenz daily doses compared favorably to the average dose of PACTG 382 of 14.2 mg/kg/d and the median (range) dose of 13.3 (9.7-22.5) mg/kg/d of Wintergerst and co-workers.^{24,26}

Data from our study over a period of 6 months indicated that 58% of all the plasma samples were between 1 and 4 µg/ml, 17% were below 1 µg/ml, and 25% were above 4 µg/ml. It is important to emphasize that these efavirenz plasma levels were not taken at trough and are thus not actual C_{min} plasma levels. All of these samples (except for 1%) were taken between 12 and 20 h after the last dose intake and fall within the mid-dosing interval as implemented by various other studies.^{4,8,13,24-26} In a study conducted in Thailand, 71% of the samples were within 1-4 µg/ml, 13% were <1 µg/ml, and 10% were >4 µg/ml. The Thai children were, however, older (median of 12.3 years) and that could account for possible better compliance and similar dosages and thus the higher percentage of plasma levels within the 1-4 µg/ml target range.²⁵

Results from two independent studies in children suggested that efavirenz could be under dosed. Both these studies were however in small groups of children with

slightly different dosing.^{19,27} The study by Ren and co-workers in South African children reported that 40% had estimated trough levels (C_{min}) below 1 µg/ml and the median efavirenz plasma level was reported as 1.58 µg/ml, sampled at mid-dose interval.¹⁹ von Hentig and co-workers reported 64% of their children exhibited efavirenz plasma concentrations below 1 µg/ml.²⁷ However, in three other more recent studies, efavirenz plasma concentrations <1 µg/ml were reported for 8.8%, 13%, and 11% of the samples taken from 33 children respectively, in each of the studies.^{24-25,28}

The study performed in the Netherlands was also sequential (plasma samples taken after 1 day and after 2 and 6 weeks post-ART) and the age and weight of these children were similar to our study children.²⁸ It could be argued that the actual number of trough levels (C_{min}) <1 µg/ml from our study may be higher than the reported 17% as this was not taken at trough. Nevertheless, trough levels <1 µg/ml from our study are unlikely to be as high as in 40% or 64% due to the long half-life (40-55 h) of efavirenz. Possible explanations for the higher number of efavirenz trough concentrations (<1 µg/ml) in the studies by Ren and von Hentig could be that the efavirenz levels were measured on only one occasion (cross-sectional studies) compared to three occasions over six months in our study, and the number of children in our study was significantly larger compared to the 11 and 15 children, respectively, in the cited studies.^{19,27}

A significant larger percentage (25%) of our study efavirenz plasma levels were above 4 µg/ml when compared to the German (14.7%)²⁴ and Thai (10%)²⁵ studies but similar to the Netherlands study (23%) with sequential plasma sampling post-ART.²⁸ The correlation between higher levels and adverse effects was nonsignificant in the German cohort and in the Thai children, except for psychiatric issues that was prominent in five children. The same nonsignificant trend was observed in our study; although 25% of the measured efavirenz plasma concentrations were >4 µg/ml, none of the patients stopped taking the efavirenz-based ART during the first 6 months due to side-effects or any other reasons. A large number of children (23%) had persistent efavirenz concentrations >4 µg/ml at 1, 3, and 6 months with only one child reporting nightmares within the first few weeks of treatment. The prevalence of low-grade transaminitis was observed in 11% of the children, of whom four out of six had efavirenz levels persistently above 4 µg/ml. All of the children in this study were also taking co-trimoxazole and stavudine, thus the

low-grade transaminitis could not be solely attributable to elevated efavirenz plasma levels.

Although there was a decrease (59%, 54% and 18%) in adherence (as defined by our criteria), the number of plasma concentrations <1 $\mu\text{g/ml}$ did not change significantly (16%, 20% and 15%) over the 6-month period. The numbers of patients not returning their old medication and not reporting for adherence assessments were higher at 6 months (41%) compared to 29% and 27% at 1 and 3 months, respectively, and this could account for the decrease in adherence at 6 months post-ART.

In summary, several reasons could contribute to efavirenz plasma levels being outside the accepted therapeutic range of 1-4 $\mu\text{g/ml}$. These include genetic variation in drug metabolism, incorrect dosing, drug-drug interactions, and nonadherence. Genotypic investigations into possible genetic polymorphisms on the CYP2B6 gene in this study population would be useful and could further explain the high interindividual variation in the efavirenz plasma concentrations. A limitation of this study is that although all possible efforts were made to monitor adherence in this outpatient clinic scenario, we cannot conclude that adherence did not affect the efavirenz plasma levels in this study population. However, the reasons for children having efavirenz plasma concentrations outside the therapeutic range (1-4 $\mu\text{g/ml}$) need to be further explored. Therapeutic drug monitoring may be beneficial and could play a major role in elucidating these reasons as well as serving as a tool to evaluate adherence. The importance of sequential plasma measurements was highlighted in this study.

ACKNOWLEDGEMENTS

M. Viljoen received a Thuthuka research grant from the North-West University and the National Research Foundation (NRF). The authors gratefully acknowledge the contributions of the staff of the Harriet Shezi Children's Clinic especially the kind and very professional services of Sr Matshediso Moolwa. We also gratefully acknowledge the Department of Nutrition (North-West University, Potchefstroom Campus) for the use of the Agilent 1200 LC/MS/MS.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist

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Table 1: Baseline and 6 months post- ART characteristics

	Baseline (n=60)	6 Months post-ART (n=56)	P Value^a
AGE (years)			
Mean ($\pm$ SD)	6.8 (2.90)		
Range	3 - 14		
GENDER			
Male, n (%)	31 (52)	29 (52)	
WEIGHT (kg)			
Mean ($\pm$ SD)	20.19 (6.70)	21.59 (6.32)	<0.0001
Weight-for-Age-Z-Score ^{b,c}	-1.35 (0.95)	-1.21 (0.86)	0.0002
HEIGHT (cm)			
Mean ($\pm$ SD)	113.7 (17.94)	116.1 (16.50)	<0.0001
Height-for-Age-Z-Score ^c	-1.73 (1.06)	-1.65(0.96)	0.004
BSA^d (m ²)			
Mean ($\pm$ SD)	0.80 (0.19)	0.83 (0.18)	<0.0001
Body mass index-for Age-Z-Score ^c	-0.45 (1.04)	-0.14 (0.91)	0.003
Viral load (copies/ml)			
Median	52500	25	0.002
Range	640-3,000,000	25-170,000	

^a P Value < 0.05 denotes statistical significant difference.

^b Only calculated for children younger than 10 years as available on WHO Anthro software.

^c Z-scores calculated using WHO Anthro and WHO AnthroPlus software.

^d BSA, body surface area.

Table 2: Efavirenz plasma concentrations (µg/ml) after 1, 3, and 6 months on ART

	1 Month		3 Months		6 Months		Total over 6 months	
	PK1^a	PK2 ^a	PK1^a	PK2 ^a	PK1^a	PK2 ^a	PK1^a	PK2 ^a
Number of patients n	56^b	58 ^c	54^d	54 ^e	54^f	52 ^g	164	164
Mean (± SD) time after dose (h)	15.43 (3.15)	17.12 (3.08)	15.07 (1.15)	16.75 (1.22)	15.21 (1.03)	16.83 (0.96)	15.24 (2.03)	16.91 (2.03)
Median concentration (µg/ml)	2.19	1.86	1.79	1.62	2.12	1.84	2.06	1.80
Range (µg/ml)	0.25–9.95	0.21–9.11	0.54–11.14	0.55–10.70	0.10–10.69	0.14–10.46	0.10–11.14	0.14–10.70
1-4 µg/ml (%)	61	64	54	52	57	58	57	58
> 4 µg/ml (%)	23	21	28	26	28	27	26	24
<1 µg/ml (%)	16	16	18	22	15	15	17	18

^a PK1, Efavirenz concentration at time point 1. PK2, Efavirenz concentration at time point 2.

^b TB treatment (n=1); concentration > 12 µg/ml (n=1); Missing data (n=2).

^c TB treatment (n=1); concentration > 12 µg/ml (n=1).

^d TB treatment (n=4); concentration > 12 µg/ml (n=1); concentration < 0.094 µg/ml (n=1).

^e TB treatment (n=4); concentration > 12 µg/ml (n=1); concentration below < 0.094 µg/ml (n=1).

^f TB treatment (n=4); concentration > 12 µg/ml (n=1); concentration < 0.094 µg/ml (n=1).

^g TB treatment (n=4); concentration > 12 µg/ml (n=1); concentrations < 0.094 µg/ml (n=2); Missing data (n=1).

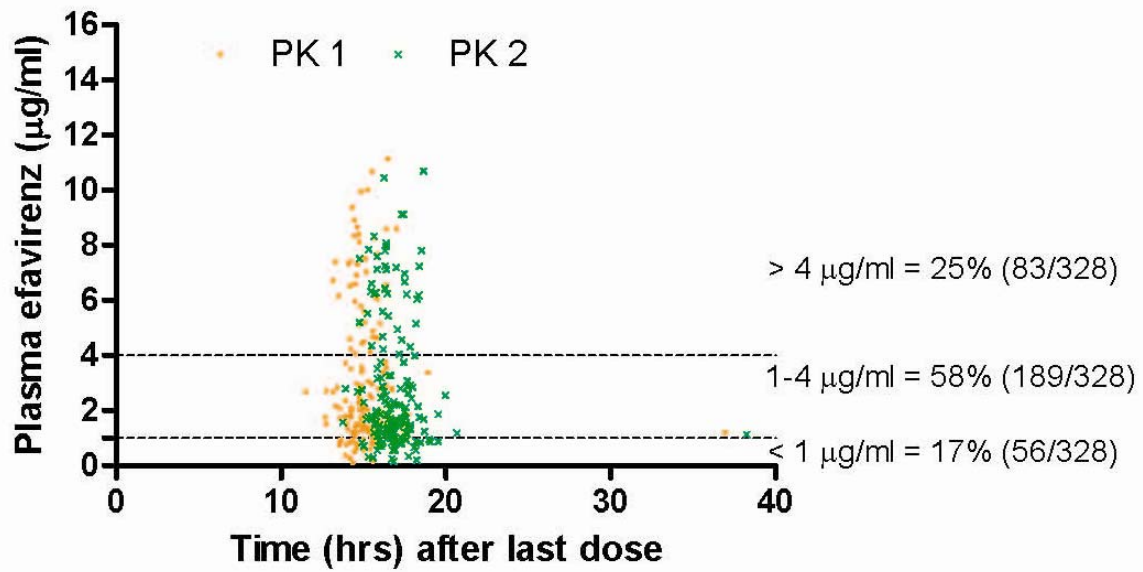


FIG. 1. Plasma efavirenz concentrations at 1, 3 and 6 months post ART.

n=164 plasma samples from 56 children at PK1 (time point 1)

n=164 plasma samples from 58 children at PK2 (time point 2)

Chapter 5

Manuscript B

In this chapter, a manuscript titled:

“Prevalence and impact of *CYP2B6* 516G>T polymorphism in black South African HIV-infected children on efavirenz-based antiretroviral therapy “

is presented. It was submitted to *Clinical Pharmacology and Therapeutics* as a full-length research paper. The impact factor of this journal according to Journal Citation Report (JCR Science Edition 2009) was 6.961. *Instructions to the Author* for this journal are presented in Addendum C2.

Title page

Prevalence and impact of *CYP2B6* 516G>T polymorphism in black South African HIV-infected children on efavirenz-based antiretroviral therapy

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ABSTRACT

We investigated the *CYP2B6* 516G>T polymorphism in association with the EFV plasma concentrations in a longitudinal study at 1,3,6,12,18 and 24 months post-HAART in 60 black South African HIV-infected children.

23% (14/60) were T/T homozygotes, 42% (25/60) were G/G homozygotes and 35% (21/60) were G/T heterozygotes with the 516T allele frequency at 41%. The total median (IQR) EFV plasma concentrations when pooled were, 6.36 (3.47 – 7.28) for T/T, 2.55 (1.62 – 3.59) for G/T and 1.41 (1.02 – 1.74) µg/mL for G/G groups respectively ($P<0.00001$). The *CYP2B6* 516G>T polymorphism was consistently predictive of the Log EFV concentrations at all times. EFV was well tolerated and most side effects subsided spontaneously, 89% of the participants were virally suppressed at 24 months post-HAART with no significant differences between the genotyped groups. We found no association of the *CYP2B6* 516G>T polymorphism with side effects reported after 1 month of treatment within this study population.

KEY WORDS

Efavirenz, *CYP2B6* 516G>T, pharmacogenetics, antiretroviral therapy

INTRODUCTION

The phase 1 cytochrome P450 2B6 (CYP2B6) isoenzyme is responsible for metabolizing several clinically important drugs including efavirenz (EFV) which is a key component of the backbone in highly active antiretroviral therapy (HAART) regimens. EFV, a nonnucleoside reverse transcriptase inhibitor (NNRTI) is primarily metabolized by hepatic CYP2B6 and to a lesser extent by CYP2A6, CYP1A2, CYP3A5 and CYP3A4.¹⁻² CYP2B6 is characterized by extensive interindividual variability in hepatic expression and activity.³ Factors that contribute to interindividual variation are induction and inhibition of the CYP isoenzymes by chemicals or drugs, dietary components, gender differences, age and genetic polymorphisms.³⁻⁸

The CYP2B6 gene is located on chromosome 19 and consists of 9 exons.⁹ It is highly polymorphic and genotyping for functional single nucleotide polymorphisms (SNPs) has been the focus in numerous studies. The single nucleotide polymorphism *CYP2B6* 516G>T is located on exon 4, and is a marker of several haplotypes *CYP2B6**6; *7; *9; *13; *19; *20; *26 and *29 (<http://www.cypalleles.ki.se/cyp2b6.htm>).

The single nucleotide change, 516G>T, in CYP2B6 has consistently been associated with elevated EFV plasma levels in different ethnic populations which resulted in altered EFV metabolism in HIV-infected patients.^{4,10-19}

The homozygous *CYP2B6* 516T/T genotype is associated with reduced CYP2B6 activity leading to higher EFV plasma concentrations and consequently more drug related side effects and toxicity.²⁰ It has been reported in several studies conducted in HIV-infected adults that elevated EFV plasma concentrations are more frequently observed in populations of African descent due to known and yet to be discovered mutations in the CYP2B6 gene.^{4,10-14,21-23} The recommended therapeutic range of EFV plasma levels is 1-4 µg/mL²⁴ and updated guidelines published in 2006 support the C_{min} to be above 1 µg/mL.²⁵

The *CYP2B6* 516G>T polymorphism in HIV-infected children was investigated in Thai children²⁶, a Dutch cohort²⁷ and in Pediatric AIDS Clinical Trials Group 382 (PACTG 382) which consisted mostly of black, Hispanic and white children.²⁸ In our study we investigated the influence of the *CYP2B6* 516G>T polymorphism in black

South African HIV-infected children on an EFV-based HAART regimen in a longitudinal study over a period of 24 months post-HAART initiation.

RESULTS

Subject demographics

Sixty HIV-1 infected children were enrolled at baseline. All of the children were black Africans and 52% (31/60) were males. The mean ($\pm$ SD) age and weight was 6.8 (2.9) years and 20.2 (6.7) kg respectively at baseline. Patient characteristics are summarized in Table 1. At baseline the distribution of gender (male vs. female) was equally distributed (χ^2 test, $P=0.99$) with no statistically significant difference between the ages (Kruskal-Wallis, $P=0.30$) within the 3 genotype groups (*CYP2B6* 516G/T, T/T and G/G), nor did the mean age of the males vs. females 6.77 (2.67) and 6.89 (3.12) years respectively, independent T-test, $P=0.87$) differ statistically.

Kruskal-Wallis analysis revealed no statistical significant differences between baseline Log VL, CD4%, CD4-cell counts, ALT, weight, and length within the 3 genotype groups. All concomitant medication 7 days prior to the study visit was recorded and no known inducers or inhibitors of CYP isoenzymes were orally co-administered within 4 weeks of any of the specified study visits.

CYP2B6 516G>T polymorphism and EFV plasma concentrations

Genotype results were available for all 60 participants (Table 1): 23% (14/60) were *CYP2B6* 516T/T homozygotes, 42% (25/60) were *CYP2B6* 516G/G homozygotes and 35% (21/60) were *CYP2B6* 516G/T heterozygotes and this was consistent with the Hardy-Weinberg equilibrium (χ^2 test $P=0.10$). The *CYP2B6* 516T allele variant frequency was 41%.

Figure 1 reflects the repeated measures of the Log EFV plasma concentrations (2nd PK's) for the 3 genotypes taken during mid-dose sampling at 1,3,6,12,18 and 24 months post-HAART initiation. Months post-HAART initiation (R1) nor the interaction (genotype*R1, $F(10,190) = 0.77$, $P=0.65$) influenced the Log EFV plasma significantly 1-24 months post-HAART. However, genotype independently influenced the Log EFV plasma concentrations ($P = 0.0001$). Therefore the total median (IQR) EFV plasma concentrations (average mean of each individual pooled over the 24 month period) were, 6.36 (3.47 – 7.28) for T/T, 2.55 (1.62 – 3.59) for G/T and 1.41 (1.02 – 1.74) $\mu\text{g/mL}$ for G/G groups respectively (Figure 2). The Kruskal-

Wallis test indicated that there was a significant difference ($H(2, N=60) = 25.74614$, $P < 0.00001$) in EFV plasma concentrations between the three genotype groups. Additional multiple comparisons by groups were calculated (Figure 2) and the EFV plasma concentrations between the T/T and G/G (6.36 vs 1.41 $\mu\text{g/mL}$, $P=0.000002$) and between G/T and G/G (2.55 vs 1.41 $\mu\text{g/mL}$, $P=0.009$) showed significant differences. However, the difference between the EFV plasma concentrations of the T/T and GT groups were not significant (6.36 vs. 2.55 $\mu\text{g/mL}$, $P=0.074$).

Gender (independent T-test, $P > 0.05$) and the blood sampling time between the 3 genotype groups (Kruskal-Wallis, $P > 0.05$) were not significantly different in terms of mean Log EFV concentrations at 1,3,6,12,18 and 24 months post-HAART.

Persistent EFV levels $> 4 \mu\text{g/mL}$ were measured for 10 patients at all 6 study visits of whom 8 (8/14, 57%) had the *CYP2B6* 516T/T genotype. Persistent extraordinarily high EFV concentrations ($> 14 \mu\text{g/mL}$, reaching levels as high as 20.4 $\mu\text{g/mL}$ at 17.3 hours post-last dose at 18 months) were measured in one other patient (female, 44 months at baseline) with the *CYP2B6* 516G/T genotype. Despite the high plasma concentration levels in this patient no side effects were ever reported over the 24 month follow-up period although ALT levels were mildly elevated at 6, 12 and 18 months (45, 46 and 31 U/L respectively and at baseline it was 19 U/L). Her medication was not returned at 4 of the 6 study visits. Only at the last 2 remaining visits was adherence monitored and this was acceptable based on pill counts performed.

Associations with EFV plasma concentrations

Genotype

In the univariate and multivariate analysis, genotype was the only consistent independent variable that was predictive of the Log EFV plasma concentrations at each of the respective study visits post-HAART initiation (all P -values < 0.006). Univariate and multivariate analyses of the 1 and 24 months post-HAART initiation are reflected in Tables 2 and 3.

Virological and Immunological Response

Viral load values lower than detectable (< 25 copies / mL) were recorded for 89% (47/53) of the children who completed the 24 month follow-up visit. The remaining 11% (6/53) had viral load levels ranging from 56 – 650 000 copies/mL at 24 months post-HAART initiation. Virological “blips” were recorded for two individuals.

No statistically significant associations (univariate and multivariate analysis) were found at 24 months post-HAART between the Log EFV plasma concentrations and the Log VL (Tables 2 and 3).

No statistically significant differences were observed between virological response (defined as plasma HIV RNA level < 25 copies/ml, χ^2 P > 0.05), Log VL, CD4% and CD4 counts (Kruskal-Wallis, P = > 0.05) when compared between the 3 genotype groups at any of the 6 study visits post-HAART initiation.

Side effects and reasons for changing EFV

Very few mild side effects were reported in the first month post-HAART commencement (7% nightmares; 2% insomnia). These subsided spontaneously and low grade transaminitis was reported in 11% of the patients at 6 months.³⁰ The side effects reported at 1 month post-HAART were not predictive of the Log EFV plasma concentrations nor were there any differences in reported adverse events between the 3 genotype groups (χ^2 , P = 0.62). For the remainder of the study the prevalence of reported side effects was very low. One (1/53) patient (male with CYP2B6-516G/G genotype) reported concentration problems in school at 13 months and EFV was switched on the insistence of the mother. The regimen of a second patient (male with CYP2B6 516G/G genotype) was changed due to the development of gynecomastia (associated with stavudine and possibly EFV) at 7 months. Grade 1 (DAIDS criteria, 32-63 UL)²⁹ transaminitis occurred in 9 % (5/53, 3 were T/T, 1 was G/T and 1 was G/G genotyped) of the patients at 24 months post-HAART initiation, only 1 patient was still taking co-trimoxazole. Three patients presented with persistent low grade transaminitis during the duration of the study. Two had the CYP2B6 516T/T genotype with persistent EFV concentrations > 4 µg/mL (1 to 24 months) while a third patient (CYP2B6 516G/T genotype) also presented with EFV concentrations > 4 µg/mL from 12 to 24 months of the study. The median ALT values at 24 months post-HAART were not significantly different between the 3 genotype groups (Kruskal-Wallis, P=0.51), no association (P > 0.05) was found between the ALT and the Log EFV concentrations at 24 months post-HAART.

Lipoatrophy and lipodystrophy were reported in 4% (2/55), 6% (3/51), 13 % (7/53) of the patients at 12, 18 and 24 months respectively and stavudine was substituted with abacavir in 5 patients at 12 and 18 months and for 1 patient at the actual 24 month follow-up. Other reasons for changing EFV during the 24 months follow-up included

virological failure with development of NNRTI resistance mutations (n=4) and side effects (concentrations problems and gynecomastia) reported at 7 and 13 months respectively post-HAART initiation by 2 participants.

Immune reconstitution inflammatory syndrome (IRIS) with related infections (primarily tuberculosis, 1 incident of oral thrush and molluscum contagiosum respectively) was reported for 8% (5/60) and 9% (5/54) of the patients at 1 and 3 months post-HAART initiation.

Adherence

Most diary cards were never returned or were incomplete. Adherence to EFV was assessed with pill counts. Adherence to EFV was significantly predictive of the Log EFV concentrations only at the first month post-HAART initiation ($P = 0.02$). Adherence to EFV did not show any significant differences between the 3 genotype groups at any of the months post-HAART initiation (χ^2 , $P > 0.05$).

DISCUSSION

To our knowledge, this is the first study to investigate the *CYP2B6* 516G>T polymorphism in association with six sequential EFV measurements over 24 months post-HAART initiation and the first in black HIV-1 infected South African children. The composition of our cohort was well distributed and well represented with regards to age, gender and clinical baseline values when compared between the 3 genotype groups.

CYP2B6 516T/T homozygotes results reported for HIV-infected patients from African descent varied in several reports from 13%, 20%, 19%, and 12.3%, and the 516T allele frequencies were 32%, 38%, 45% and 34% respectively.^{10-12,19} Our data are consistent with these previous reports with a large number (23%) of our participants genotyped as *CYP2B6* 516T/T homozygotes and the 516T allele frequency as 41%. It has been established that *CYP2B6* 516G>T polymorphism is associated with EFV metabolism variability and that the 516T allele is more common among Africans and is associated with elevated EFV exposure that can lead to toxicity.^{10-14,21}

Our data also confirmed that a significant difference in the EFV plasma concentrations occurred between the 3 genotypes at all of the 6 study visits (Figure 2). The pooled median EFV plasma concentrations were 6.36, 2.55 and 1.41 $\mu\text{g/mL}$ for the T/T, G/T and G/G genotypes respectively over the 24 month follow-up period

and this compared well with published median plasma levels of efavirenz from HIV-infected patients from African descent (Table 4). The *CYP2B6* 516G>T polymorphism was also consistently predictive of the Log EFV concentrations at all times.

Previous studies in adults reported that CNS side effects were associated with the *CYP2B6* 516T allele.^{11,17} Haas and co-workers reported that *CYP2B6* 516G>T could not be significantly associated with treatment failure in adults and findings by Saitoh and co-workers in children confirmed no associations between the *CYP2B6* 516G>T polymorphism and virological and immunological outcomes, toxicity or the development of viral resistance against EFV.^{21,28} Our results also confirmed no evidence of association of the *CYP2B6* 516G>T polymorphism with side effects reported after 1 month of treatment nor were any differences in the virological or immunologic response and ALT levels observed, between the 3 genotype groups at 24 months. Our results further confirmed that EFV is very well tolerated and that most side effects subsided spontaneously.

Viral suppression (VL < 25 copies / mL) was reported for 89% of our study participants at 24 months post-HAART with no significant differences between the 3 genotype groups. Ribaud and co-workers reported that the *CYP2B6* 516TT genotype may predict an increased risk of developing drug resistance among patients who discontinue EFV-containing regimens due to the long half-life of EFV.²² Few of our patients developed virological failure and resistance genotyping was performed in 4 subjects during the 24 month follow-up period (all at different stages). We were unable to make an association between genotyping and treatment failure. Factors other than genetics may influence and cause variability in the pharmacokinetics of EFV including different dosage forms, drug-drug interactions, food interactions and most importantly adherence. Our results however showed no difference between the adherences of the 3 genotype groups at any of the study visits, although adherence measured by pill counts was predictive of the Log EFV concentrations at 1 month post-HAART. This is not unanticipated as most individuals and caregivers are likely to be more conscientious during the commencement of life-long treatment. Although all effort was made to monitor adherence as best as possible in this outpatient scenario a large number of the study

participants did not return their medicines when required for the pill counts and this was experienced throughout the duration of the 24 month follow-up.

Possible limitations of our study included the following. Although *CYP2B6* G516T is associated with EFV concentration variations other single SNPs and the effects of haplotypes (combinations) have been reported to influence EFV concentrations but were not investigated in this study. However, Rodrigues-Novoa and co-workers provided evidence of the identification of a single mutation at c.516 G>T as the primary determinant for *CYP2B6* haplotypes *6 and *7.¹⁵ Complete sequencing of the *CYP2B6* gene in certain specific identified study participants will be undertaken in an additional future investigation. Assessment of reported side effects during the first month post-HAART and general side effects reported during the study was not evaluated with a specific structured questionnaire and this may have resulted in the under reporting of side effects. It is difficult to establish accurate reporting of side effects and to assess the different potential neuropsychological side effects of EFV in such young children as was the case in this cohort.

While genotyping is unlikely to form part of the standard treatment of care in HIV-infected patients its use as a research tool has demonstrated the benefit of genotyping in characterizing pharmacokinetic variability within populations. In conclusion, results from our study confirm that the *CYP2B6* 516G>T polymorphism influences the EFV plasma concentrations significantly but that *CYP2B6* 516G>T polymorphism could not be associated with toxicity within this study population. It is however important that clinicians be made aware of this phenomenon especially in resource limited countries where genotyping is too expensive to be carried out. Targeted TDM and vigilance for side effects in children who are initiated on EFV-based therapy may be helpful in managing children and in considering the appropriate dose of EFV in paediatric populations.

METHODS

Subjects and study design

In this prospective cohort study, 60 black children, both genders, with no prior exposure to antiretroviral therapy and eligible for ART (antiretroviral therapy) were included. All attended the outpatient clinic at Harriet Shezi Children's Clinic, Chris Hani Baragwanath Hospital, Soweto, South Africa. ART eligibility was in accordance

with national guidelines for the management of HIV-infected children in South Africa. Ethics approval was obtained from the North-West University, Potchefstroom and the University of the Witwatersrand, Johannesburg. If a participant became co-infected with tuberculosis they were withdrawn from the study and allowed to re-enter after completing the therapy for tuberculosis and following an additional 4 weeks wash-out period. A successful antiviral response was defined as a viral load (VL) lower than the detectable 25 copies / mL at 6 months post-HAART. A virological “blip” was defined as viral load measurement between 25 and 1 000 copies / mL preceded and followed by repeated measurement below 25 copies / mL.³⁰

EFV dosing and assay

EFV in combination with 2 nucleoside reverse transcriptase inhibitors (lamivudine, stavudine or abacavir) was prescribed and the doses were based on weight banding. EFV was self-administered at night and two blood samples (PK1 and PK2) were taken at mid-dose interval (>12 hours post-last dose and at least 2 hours apart) at 1,3,6,12,18 and 24 months post-HAART initiation. Blood samples were determined by an adapted, optimized and validated LC/MS/MS method.³⁰

CYP2B6-516G>T gene polymorphism

Genomic DNA was extracted from 1.5 ml whole blood using a FlexiGene DNA Kit (Qiagen, Hilden, Germany). Genotyping of the target *CYP2B6* 516G>T gene polymorphism was amplified by polymerase chain reaction (PCR) using the forward primer 5'-GGTCTGCCCATCTATAAAC-3' and the reverse primer 5'-CTGATTCTTCACATGTCTGCG-3'.³ The reactions were performed in a total volume of 25 µL with 50 ng of genomic DNA. The PCR conditions were: denaturation at 95°C for 3 min, amplification of 30 cycles at 95°C for 30 s, 59°C for 30 s, 72°C for 1 min and a final extension step at 72°C for 10 min. The PCR product (526 bp amplicon) was digested with FastDigest® BseNI (Fermentas Life Science, Burlington, Canada) for 10 min at 65°C and 5 min 80°C. Digestion of the homozygous wild type 516 G/G yielded three fragments of 23, 236 and 267 bp, the homozygous 516 T/T genotype yielded two fragments of 23 and 503 bp and the 516 G/T heterozygous yielded four fragments of 23, 236, 267 and 503 bp. The assay was performed at the Laboratory for Applied Molecular Biology (LAMB), Faculty of Health Science, School of Pharmacy, North-West University, Potchefstroom Campus.

Adherence assessment and side effects

Adherence assessments were done verbally, checked on pre-issued diary cards and measured adherence (pill counts) was required on each study visit or whenever a renewal or repeat prescription was dispensed. Adherence for EFV was defined as good when the measured adherence was calculated as between 93% to 107%. Poor adherence was defined when the measured adherence was calculated as <93% or >107%.³⁰ No structured questionnaire was used to report side effects, the physician's notes and general questions asked by the principal investigator were used.

Statistical Analysis

Descriptive statistics were used to describe results as mean $\pm$ standard deviation ($\pm$ SD), or median (IQR) for nonparametric data. Logarithmic transformation was applied to the EFV plasma concentrations (Log EFV) and the viral load (Log VL). Repeated measures ANOVA (analysis of variance, least square means with 95% confidence interval) was used to investigate the statistical significant influence of time (1, 3, 6, 12, 18 and 24 months post-HAART initiation) and the genotype (*CYP2B6* 516 T/T; G/T; G/G) on the Log EFV plasma concentrations. Only the second plasma sample (PK2's) of each occasion was used in the analysis as the PK2's were closer to the real time C_{min} (trough levels). It would not have been statistically acceptable to have used the mean value of PK1 and PK2 at each of the 6 occasions as these samples were not replicates. The observed and expected alleles and genotype numbers were compared using the Chi-square (χ^2) test for fit of the data to the Hardy-Weinberg equilibrium. The Kruskal-Wallis test was used to investigate between-group comparisons of continuous variables and the χ^2 test for categorical variables (gender, reported side effects post-1 month, adherence to EFV and virological failure). Two variables were used to describe the *CYP2B6* 516G>T polymorphism by coding G/T and T/T as = 1 and G/G = 0, in the univariate and multivariate analysis. Univariate analysis (adjusted R^2 and P-value) was applied to identify which independent variables (age at baseline; gender; genotype; time after last dose; weight; length; dose; Log VL; alanine aminotransferase (ALT); CD4%; reported side effects post-1 month and adherence to EFV) influenced the Log EFV plasma concentrations at 1, 3, 6, 12, 18 and 24 months post-HAART. P-value < 0.05 was considered to be statistical significant. Subsequent adjustments for genotype

and adherence to EFV in a paired-wise multiple linear regression model (adjusted R² and P-value) were then further applied at 1,3,6,12,18 and 24 months post-HAART, age at baseline, weight and height was added to the model for 24 months only. Statistical analyses were performed using Statistica® (StatSoft, Inc., version 9.0, Tulsa, Oklahoma, USA, www.statsoft.com). The statistical analyses were done in consultation with the Statistical Consultation Services, North-West University, Potchefstroom Campus.

ACKNOWLEDGEMENTS

Me M Viljoen received a Thuthuka research grant (North-West University and the National Research Foundation (NRF)) and a self-initiative grant from the Medical Research Council (MRC). The authors would like to thank the research staff of the Harriet Shezi Children's Clinic (Sr Matshediso Moilwa, Mrs Riki Badenhorst, Janet Grabe and Angela Oosthuizen) and Dr Karin Conradie (Centre of Excellence for Nutrition, North-West University) for her advice on PCR techniques. We are grateful to all study participants and their caregivers who have contributed to this study.

CONFLICT OF INTEREST

No competing financial interests exist.

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Table 1: Baseline, 3 and 24 months post HAART characteristics of study participants according to their genotype (*CYP2B6* 516 G/G, G/T or T/T).

	G/G	G/T	T/T	p-Values	All
No. of subjects	25	21	14		60
Genotype frequency %	42%	35%	23%	0.10	
Mean age (years) (± SD)	6.6 (2.8)	6.4 (3.1)	7.8 (2.5)	0.30	6.8 (2.9)
Male sex	13	11	7	0.99	31
Mean weight (kg) (± SD)	20.4 (7.6)	19.5 (6.6)	20.8 (5.3)	0.75	20.2 (6.7)
Log VL median (± SD)					
Baseline	4.86 (4.23-5.1)	4.68 (4.32-5.30)	4.45 (3.98-5.30)	0.85	4.72 (4.18-5.22)
3 Months	1.40 (1.40-1.40)	1.40 (1.40-1.40)	1.40 (1.40-1.40)	0.60	1.40 (1.40-1.40)
24 Months	1.40 (1.40-1.40)	1.40 (1.40-1.40)	1.40 (1.40-1.40)	0.30	1.40 (1.40-1.40)
CD4% median (IQR)					
Baseline	14.80 (9.29-18.50)	14.30 (9.00-21.20)	14.35 (6.96-17.10)	0.61	14.40 (8.44-18.80)
3 Months	22.20 (15.55-28.7)	25.95 (18.80-29.5)	21.50 (10.40-27.70)	0.71	24.20 (14.50-28.80)
24 Months	30.30 (25.10-36.20)	32.35 (25.80-36.8)	30.35 (25.60-37.40)	0.93	31.40 (25.10-36.80)
ALT level U/L median (IQR)					
Baseline	19 (15-24)	17 (14-24)	18 (12-29)	0.88	18.50 (14.50-25.50)
3 Months	18 (17-24)	23 (14-27)	24 (17-30)	0.41	20.50 (16-27)
24 Months	18 (15-20)	18 (13-22)	21 (15-29)	0.51	18.5 (14-22)

The P- values represent comparison between 3 genotypes. The Chi (χ^2) test was used for categorical values. The Kruskal-Wallis test was used for continuous data.

Table 2: Univariate analysis (b*-coefficient; P-value) of log EFV plasma concentrations at 1 and 24 months post-HAART.

Independent variables	Log1PK2		Log6PK2	
	b*	P	b*	P
Genotype	0.40	0.002	0.48	0.0004
Gender	-0.05	0.73	-0.05	0.72
Age (baseline)	0.18	0.17	0.32	0.02
Time post dose	-0.06	0.65	-0.10	0.50
Dose	0.16	0.23	0.26	0.07
Weight	0.12	0.36	0.30	0.03
Height	0.16	0.25	0.32	0.02
Side effects (1 month)	-0.01	0.94	NT	NT
CD4%	NT		0.28	0.06
Log VL	NT		-0.10	0.51
ALT	NT		0.27	0.06
Adherence	0.37	0.02	0.16	0.27

1PK2 (1 Month, 2nd PK); 6PK2 (24 Months, 2nd PK)

b*, beta-coefficient. NT, not tested.

Table 3: Multivariate regression model analysis (b*-coefficient, adjusted R²; P-value) of log EFV plasma concentrations at 1 and 24 months post-HAART

Independent variables	Log1PK2			Log6PK2		
	b*	R ²	P	b*	R ²	P
Genotype	0.39	0.25	0.005	0.51	0.30	0.0002
Adherence	0.35		0.01	0.24		0.07
Age (baseline)				0.65		0.06
Weight				0.13		0.75
Height				-0.50		0.37
Genotype				0.48	0.26	0.0005
Age (baseline)				0.43		0.18
Weight				0.16		0.70
Height				-0.30		0.59

1PK2 (1 Month, 2nd PK); 6PK2 (24 Months, 2nd PK)

b*, beta-coefficient. adjusted R², coefficient of determination

Table 4: Literature comparisons of median EFV plasma concentrations taken at mid-dose interval in HIV-infected patients according to their *CYP2B6* 516G>T polymorphism

Race	Median EFV (µg/mL) G/G	Median EFV (µg/mL) G/T	Median EFV (µg/mL) T/T	References
Spanish / Adults n=100	1.74	2.6	3.57	15
Africans / Adults n=74	1.8	2.3	6.3	19
Ghanaians / Adults n=74	1.3	1.6	8.3	12
Thai / Children n=63	1.6 (mean)	2.6 (mean)	11.6 (mean)	26
Black South African / Adults n=109	1.6	2.1	5.9	10
Black South African / Children n=60	1.41	2.55	6.36	Current study

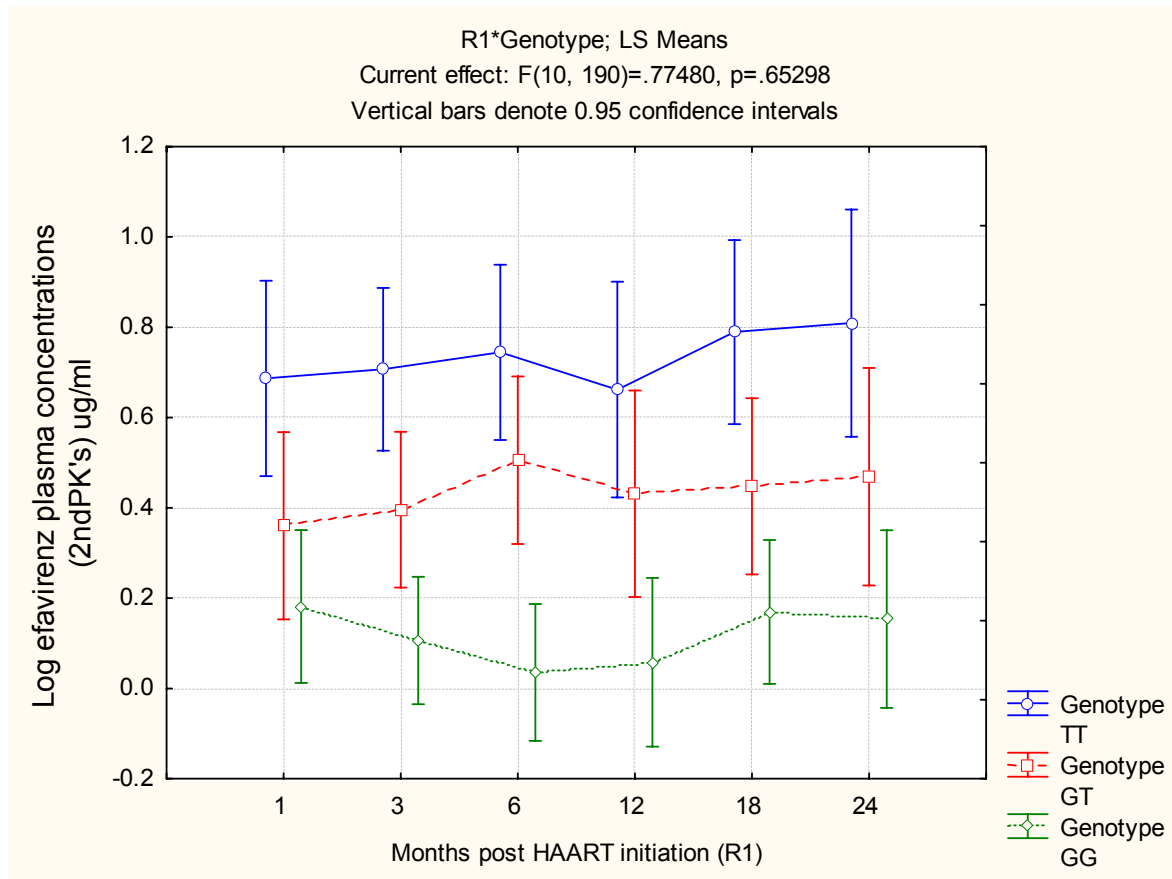


Figure 1: Repeated measures ANOVA of the log efavirenz plasma concentrations over time (1, 3, 6, 12, 18 and 24 months) post-HAART initiation.

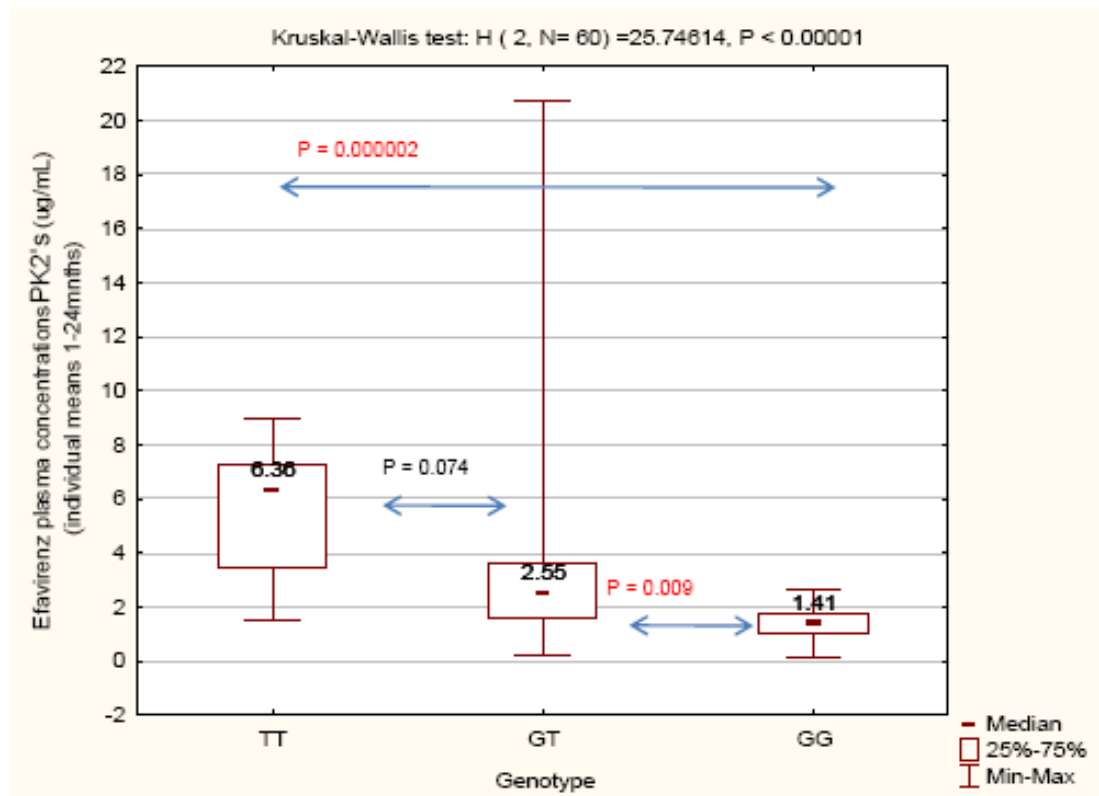


Figure 2: Pooled individual mean efavirenz plasma concentrations at 1, 3, 6, 12, 18 and 24 months post-HAART initiation.

Chapter 6

Manuscript C

In this chapter, a manuscript titled:

“Influence of *CYP2B6* 516G>T polymorphism and interoccasion variability (IOV) on the population pharmacokinetics of efavirenz in HIV-infected South African children”

is presented. It has been prepared for submission to *European Journal of Clinical Pharmacology* as a full-length research paper, and prepared according to the specific *Instructions to the Author* as presented in Addendum C3. The impact factor of this journal according to Journal Citation Report (JCR Science Edition 2009) was 2.743

Title page

Influence of *CYP2B6* 516G>T polymorphism and interoccasion variability (IOV) on the population pharmacokinetics of efavirenz in HIV-infected South African children

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ABSTRACT

Objective To investigate the influence of *CYP2B6* 516G>T polymorphism, as a covariate and interoccasion variability (IOV) on the oral clearance (CL/F) of efavirenz (EFV) in treatment-naïve black South-African children over a period of 24 months post-antiretroviral therapy (ART) initiation.

Methods HIV-infected black children (n=60), no prior exposure to ART, eligible to commence ART and attending an outpatient clinic were enrolled into this study. Blood samples were taken at mid-dose interval at 1,3,6,12,18 and 24 months post-ART initiation. EFV plasma samples were determined with an adapted and validated LC/MS/MS method. Genotyping of the *CYP2B6* G516T target gene was performed with polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP). NONMEM was used for the population pharmacokinetic modelling.

Results EFV concentrations below 1 µg/mL accounted for 18% (116/649), EFV concentrations >4 µg/mL accounted for 29.5% (192/649) and concentrations within the therapeutic range (1-4 µg/mL) represented 52.5% (341/649) of all the samples determined.

The covariates age, weight and *CYP2B6* 516 T/T, G/T and G/G genotypes were included in the final model with population estimates for CL/F determined as 2.46, 4.60 and 7.33 L/h for the T/T, G/T and G/G genotype groups, respectively.

Conclusions The inclusion of both age and weight to predict accurate EFV CL values for the respective genotype groups within this paediatric population was required whereas the addition of gender and body surface area did not improve the predictions. The importance of introducing IOV in a PK model for a longitudinal study with sparsely collected data was again highlighted by this investigation.

Keywords: Efavirenz, clearance, *CYP2B6* 516G>T, NONMEM

INTRODUCTION

Efavirenz (EFV) a non-nucleoside reverse transcriptase inhibitor is part of the backbone of highly active antiretroviral therapy (HAART), first line regimen for children (> 3 years > 10 kg) together with lamivudine and stavudine in South Africa since 2005 [1]. Stavudine has since been substituted with abacavir in the first line regimen for children who are initiated on HAART [1].

EFV is well absorbed after oral administration and peak plasma levels are reached within 5 hours. It is highly (> 99%) bound to plasma proteins, predominantly albumin [2-4]. EFV is a substrate for cytochrome P450 (CYP P450) enzymes and an inhibitor and inducer of CYP P450. EFV is primarily metabolised by the hepatic CYP2B6, to a lesser extent by CYP3A4 and more recent findings reported on the involvement of CYP2A6 [5-6]. As a result significant drug-drug interactions can and do occur [3,7-9]. It was reported that EFV enhances its own metabolism during repeated administration [2,10,11] and high-fat content meals can increase the bioavailability [2,12].

The clearance of EFV is slow and it has an elimination half-life of 40 to 55 hours at steady state and 52-76 hours after single dose administration in adults [2]. This allows for once daily dosing which contributes to the advantage of convenience and better patient compliance [2-3]. EFV has been dubbed as “forgiving” of the occasional missed or delayed dose and this is due to its long half-life, potency, convenience and tolerability [13-14]. However, the clearance can be faster and the half-life shorter in children. The half-life calculated for children (n=48) in Burkina Faso (CL = 0.211l/h/kg; V = 4.48 l/kg) was 14.7 hours [15] and for children (n=11) in Germany, 12.93 hours [16]. The median estimated half-lives in HIV-infected adults genotyped as G/G, G/T and T/T at position 516 of CYP2B6 were 23 (IQR, 18-35), 27 (IQR, 19-31) and 48 (IQR, 39-77) hours respectively [17].

Variability in the pharmacokinetics of EFV were investigated and reported in numerous studies. Unpredictable toxicity and efficacy can be attributable to inter-individual variability. High levels of inter-individual variability (coefficient of variation, CV%), ranging from 40 to 143% have been reported for EFV plasma concentrations in adults and children. Intra-individual variability of EFV pharmacokinetics (PK) was shown to be far less with CV% below 30% [18-22]. Various factors such as environmental (drug-drug interactions, drug-food interactions, recreational drug intake) physiological (gender, age, disease states and pregnancy), non-adherence

and genetic factors (polymorphisms and mutations) can contribute to this variability [2,12,23-25].

The *CYP2B6* 516G>T polymorphism is associated with slower EFV clearance, higher EFV plasma levels and increased CNS-related side effects [26-30].

Although various population pharmacokinetic studies of EFV in healthy adults [31], HIV-infected adults [17-18,26,28,32-34] and HIV-infected children [15,35] were published previously only a few of these studies investigated the influence of *CYP2B6* 516G>T polymorphism in HIV-infected adults [17,26,28] and in HIV-infected children [35].

The aim of this study was to investigate the influence of *CYP2B6* 516G>T polymorphism, as a covariate and interoccasion variability (IOV) on the oral clearance (CL/F) of EFV, in treatment-naïve black South-African children, after once daily (nocte) administration over a period of 24 months post-HAART initiation.

METHODS

Patients, dosing, study design and sampling

This cohort included 60 black HIV-infected children, both genders, with no prior exposure to antiretroviral therapy (ART). They all attended the outpatient clinic at Harriet Shezi Children's Clinic, Chris Hani Baragwanath Hospital, Soweto, South Africa. EFV was self-administered (based on weight banding - DOH 2005) at night and two blood samples (PK1 and PK2) were taken at mid-dose interval (12 hours post last dose and 2 hours apart) at 1,3,6,12,18 and 24 months post-ART initiation. Inclusion and exclusion criteria were previously described and no concomitant use of known inducers or inhibitors of the cytochrome P450 enzymes were reported during this study [22].

ART eligibility was in accordance with national guidelines for the management of HIV-infected children in South Africa [1]. Written informed consent and assent were obtained from all participants and legal guardians. Ethics approval was obtained from the North-West University, Potchefstroom and the University of the Witwatersrand, Johannesburg.

EFV assay and genotyping

Plasma samples were determined by an adapted, optimised and validated LC/MS/MS method as previously described [22].

Genotyping of the target *CYP2B6* G516T gene was performed with polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) method as previously described (Viljoen et al 2011 *in review*)

Population pharmacokinetic model strategy and analysis

The non-linear mixed effects modelling software program NONMEM (version 7 level 1.2) with Intel® Visual Fortran Compiler version 11.0 was used to perform the analysis [36]. The prediction-corrected visual predictive checks (pcVPC) were generated with the Perl speaks NONMEM (PsN) tool “vpc”, version 3.2.7 and the graphical presentations of the results were performed with Xpose, package, version 4.3.0 using R, version, 2.12.0 [37-40]. The first-order conditional estimate method (FOCE) was used for all models tested during the model development. Inter-individual variability was described by an exponential ($\theta_j = \theta' \exp \eta_{\theta_j}$) error model, where θ_j is the estimate for a PK parameter in the j^{th} individual as predicted by the model, θ' is the population mean of the PK parameter, and the η_{θ_j} represents the random variable with zero mean and variance ω^2 . Residual variability was described by a combination of additive and proportional ($C_{ij} = C'_{ij} (1 + \varepsilon_{\text{prop},ij} + \varepsilon_{\text{add},ij})$) error models. C_{ij} and C'_{ij} are the observed (C_{ij}) and the predicted (C'_{ij}) efavirenz concentrations in the j^{th} individual at time i , respectively and $\varepsilon_{\text{add},ij}$ and $\varepsilon_{\text{prop},ij}$ are the additive and proportional errors, with zero mean and variances σ_{add}^2 and σ_{prop}^2 .

Parameter estimation, model and covariate selection and validation

The fixed effect PK parameters estimated were, apparent clearance (CL/F) and volume of distribution (V/F). The absorption rate constant (k_a) was fixed at 0.287 h^{-1} [34], due to the sparse nature of the data in the absorption phase. This value was chosen as it resulted in the lowest objective function value (OFV) and most realistic CL and V values after subsequent testing of a number of published k_a -values ranging from 0.18 to 1.39 h^{-1} [15,18,26,28].

The adequacy of the models was evaluated by the following methods:

- 1) Bayesian maximum *a posteriori* estimation (POSTHOC option in NONMEM) between PK parameters and covariates [26,37].
- 2) Diagnostic graphics approach analysis via generalized additive modelling (GAM) was obtained in Xpose package, version 4.3.0 (<http://xpose.sourceforge.net/>), using R, version 2.12.0, to test the effect of each covariate and to identify outliers [37-40].

- 3) In NONMEM the difference in OFV between two models was used to evaluate if an added covariate significantly improves the ability to predict the observed plasma concentration. The difference in OFV between models is asymptotically chi-squared (χ^2) distributed. Differences were regarded as significant when the OFV decreased with 3.84 ($P < 0.05$) or 6.63 ($P < 0.01$) points for 1 additional parameter or 2 additional parameters respectively.
- 4) Interoccasion variability (IOV) was taken into account as proposed by Karlsson et al (1993), [41] for the final model selection.
- 5) A nonparametric bootstrap ($n=200$) method was generated in PsN, version 2.2.3 to provide information on parameter uncertainty.
- 6) pc-VPCs were used as informative diagnostic tools to allow inspection of model appropriateness across time as well as across covariate values [42].

RESULTS

Demographic results

All of the 60 HIV-infected children enrolled at baseline were black Africans and 52% (31/60) were males. The mean ($\pm$ SD) age, weight and length were 6.8 (2.9) years, 20.2 (6.7) kg and 113.7 (17.9) cm respectively. All other relevant subject demographics and characteristics at baseline, 3 and 24 months were previously published [22 and Viljoen et al *in review*].

EFV plasma concentrations

The total number of measured EFV plasma concentrations (PK1 and PK2) taken over the 24 months post HAART initiation during this longitudinal study was 649.

EFV concentrations below the targeted clinical level of 1 $\mu\text{g/mL}$ accounted for 18% (116/649). EFV concentrations >4 $\mu\text{g/mL}$ accounted for 29.5% (192/649). The EFV plasma concentrations within the therapeutic target range of 1-4 $\mu\text{g/mL}$ represented 52.5% (341/649) of all the samples.

One patient with persistent EFV levels at extraordinary high levels (>14 $\mu\text{g/mL}$) led to biased covariate identification and this patient's data was not included in the model building. All plasma levels below the limit of quantification (0.094 $\mu\text{g/mL}$) were included in the model analysis. However, plasma levels measured as 0 (in 4 patients) were excluded as the pill counts performed was indicative of non-compliance or in some cases due to admission of patient and or care giver that the medicine was not taken. Plasma levels >12 $\mu\text{g/mL}$ (upper limit of analytical linear

range) were diluted appropriately and re-analysed and results were included in the model building and population analysis.

CYP2B6 516G>T polymorphism

Genotyping results for all 60 participants were previously published (Viljoen et al 2011 *in review*) with 23%, 42% and 35% being T/T homozygotes, G/G homozygotes and G/T heterozygotes respectively and the CYP2B6 516T allele variant frequency was 41%.

Population pharmacokinetics

A one-compartment model was implemented with ADVAN 2 and TRANS 2 subroutines in NONMEM to describe the parameters CL/F and V/F.

The inclusion of interoccasion variability (IOV) in the model was pronounced and resulted in a large reduction of OFV and it was decided to evaluate the covariates with IOV included in the model. The population pharmacokinetic parameters estimates of EFV in the basic model (IOV included), final model and with bootstrapping are displayed in Table 1.

In the final model (OFV=170) covariates age, weight and CYP2B6 516 G/G, G/T and T/T genotype were included. The final population estimates for CL/F (RSE%) were 2.46 (10%), 4.60 (13%) and 7.33 (8.7%) L/h for the T/T, G/T and G/G genotype groups, respectively. The V/F (RSE%) was 89.5 L (11%). The omission of age and weight from the final model increased the OFV by 17 and 7 units respectively. When both age and weight were omitted (genotype included) the OFV was also increased by 7 units. The difference between the OFV (dOFV) of the basic model (OFV=216) compared to the model with genotype (OFV=177) included was 39 units. Genotype on CL/F therefore markedly improved the fit and CL/F was increased in the expected order. Addition of relations for gender and body surface area did not improve the OFV significantly.

Bootstrapping on the final model (weight, age, genotype and IOV) also confirmed that the model was stable. The bootstrapping estimates for the parameters were very similar to the population estimates reflected in the final model (Table 1).

Figures 1-3 represent the prediction and variance corrected VPCs for the three genotypes (Fig.1), age (Fig.2) and for weight (Fig.3).

DISCUSSION

The aim of the present study was to investigate the influence of *CYP2B6* 516G>T polymorphism and IOV on the CL/F of EFV within this paediatric HIV- infected population. This study contributes towards the knowledge of EFV population pharmacokinetics in HIV-infected children due to its longitudinal nature (24 month duration) with 6 follow-up visits post-ART initiation in EFV naïve children. It is also the first study to our knowledge where the influence of genotyping on EFV clearance in black HIV-infected South African children was studied.

The pharmacokinetics of efavirenz in HIV-infected patients were seemingly better described by a two-compartment model in the literature [33,43] but a large number of studies did assume one-compartment modelling [15,17-18,26,28,32,34-35]. Due to the design of the present study, sparse sampling with only two PK samples available in the elimination phase at each of the six study visits, the data was fitted by one-compartment open-kinetic model with first-order absorption and elimination (FOCE). The lack of data in the absorption phase made it necessary to fix the k_a value at 0.287 h^{-1} . Other considerations for fixing the k_a value were that a large number of the study population described by Kappelhoff and co-workers (2005b), were black adult South Africans [34]. Both Wade and co-workers and Cabrera and co-workers reported that the estimation of CL/F is not affected by the k_a value [26,44].

In this study the influences of several covariates (gender, age, weight, BSA and *CYP2B6* 516 G/G, G/T and T/T genotypes) and IOV were investigated by incorporating NONMEM. Statistically significant improvement in the OFV was observed when IOV, genotype, weight and age were added to the model.

Our study results concurred that no correlation was observed between gender and BSA and the CL/F or V/F of EFV. Gender was shown not to affect CL, V and bioavailability of efavirenz in adults [10,18,32,45], however in a Zimbabwean adult population (n=74) the women had slower CL values compared to the males [28].

The influence of age is contradictory in several studies. In results not shown in this article we have found a linear relationship with improved bioavailability relative to a typical 7-year old with age. Possible reasons could be that the hepatic enzyme activity at 7 years is similar to adult activity [46]. Saitoh and co-workers reported that the CL was significantly higher in children younger than 5 years [29]. In our study we did not distinguish between the different age groups, as discussed below.

However, when age without weight was added to the genotype model no improvement in OFV was observed, the OFV remained at 177. A combination of age and weight was necessary for an improvement in the model fit. Age was found to be related to the EFV disposition with similar relations for CL/F and V/F in our final model.

In a study with comparison age demographics as this present study (6.5 ± 2.5 years) ter Heine and co-workers (2008), reported that age did not influence CL in children ($n=33$, median 6.5 yrs (0.9-19)). Whereas, Hirt and co-workers (2009) reported that CL decreased with age (Burkina Faso children, $n=48$, median 6.35 years (IQR, 2.77-14.7) which was also reported by Wintergerst and co-workers (2008) in 33 children with median age 8.2 years (IQR, 2.1-16.7) [47]. Saitoh and co-workers (2007), reported that age and *CYP2B6* G516T were independently associated with CL in a multivariate analysis. The CL was higher in younger children < 5yrs with G/G than > 5yrs (9.7 L/h/m^2 vs 6.6 L/h/m^2). We did not distinguish between the different age categories in our study population. The age (mean (SD) and median (IQR)) variation of our study participants at baseline was 6.8 (2.9) and 7 (4.5-9.0) years.

As with age, the same phenomenon repeated with weight. When only weight was added to the genotype model, no improve in the fit was observed. When the combination of age and weight was added the OFV improved.

Weight did not influence EFV PK parameters significantly in adults [18,26,32]. In studies with children weight did influence the CL of EFV in a linear body weight model [15,35]. The weight variation of our paediatric population was possibly not distributed over a wide range to have an effect by itself (baseline - median 18.94 kg (IQR: 14.85-24.00); post-24 months – median 25.13 kg (IQR: 19.25-28.60). Although the dosage of children is normally calculated according to BSA it was shown in our study that BSA did not influence the CL of EFV in this study. However, our final population model showed that both age and weight should be included to predict accurate CL values for three genotype groups in this paediatric population.

The mean CL/F of EFV from our basic model (4.86 L/h, 0.24 L/h/kg) compared favourably with results (0.19 L/h/kg, 0.21 L/h/kg, 0.26 L/h/kg and 0.30 L/h/kg,) from the literature in other children [35,47-48].

Race/ethnicity and *CYP2B6* 516G>T polymorphism significantly influenced the CL of EFV in numerous studies performed in adults and children [17-18,26-28,32,34-35,45]. The ethnicity of our population was homogenous and could therefore not be

introduced as a covariate. When the respective CL/F parameters were calculated for the three genotype groups (516 T/T; G/T, G/G) an improvement in OFV was observed (dOFV=-39). The inter-individual variability on CL decrease from 63 to 41 % from the basic to the final model which compared favourable with most values (36.47%, 76%, 52.4%, 42%, 60%) from other studies that also employed NONMEM [26,28,35,32,34].

Studies reporting on the CL values of EFV in poor, intermediate and fast metabolisers (*CYP2B6* 516T/T, G/T, G/G genotypes) respectively were 0.06, 0.10, 0.13 L/h/kg in Zimbabwean adults (n=74) [28] and 3.0, 5.7 and 7 L/h/m² in children of mixed race (n=71) [29]. When comparing our results (0.12, 0.23 and 0.37 L/h/kg) with the results from Zimbabwean adults (0.06, 0.10, 0.13 L/h/kg) it is evident that the clearance in children is much faster. The only other study in children by Saitoh and co-workers (converted to L/h/kg with 20 kg) who also reported three CL values, 0.14, 0.26 and 0.32 L/h/kg, compared well with the results from our study. This also supports our results that the CL of EFV in children is faster compared to adults. The higher CL values also correspond with the reported shorter half-lives of efavirenz in children. The half-life calculated for children (n=48) in Burkina Faso (CL=0.211 L/h/kg; V=4.48 L/kg) was 14.7 hours [15] and for children (n=11) in Germany, 12.93 hours [16].

The VPCs (Figures 1-3) show that all the covariate effects are well captured in this final model although over prediction is observed with higher concentrations.

It is documented in the literature that CL/F is the parameter most accurately predicted with NONMEM in routinely collected data. Cabrera and co-workers (2009), stated that the wide range of V/F values reported in the literature could be due to sparsely collected data to predict the V/F parameters. We also observed with not including IOV that the V/F (187 L, results not shown) had unrealistic RSE% on the inter-individual variation (44%(1500)). However, when IOV was introduced into the model the scenario changed with a V/F value of 94.3 L and a reduction in inter-individual variability to 37%. This is more in line with published values of 100.5 L [16] and 89.6 L in children [15]. The importance of taking IOV into consideration was first described by Karlsson and Sheiner (1993), [41]. They argued that individual pharmacokinetics can change randomly over a period of time and it is important to take that into account. Our study was conducted over 24 months and compliance is

always a challenge and that can also be accounted for in the interoccasional variability.

The limitations of this study were:

- 1) No full PK profiles were available for any of the patients.
- 2) Assumption that this was a one-compartment pharmacokinetic model.
- 3) All PK samples were taken at very similar time points around the mid-dose interval during the 6 various follow-up visits, resulting in no PK's prior to 12hrs and very few between 20 and 24 hours post-last dose intake.

In conclusion, this is the first study to our knowledge where a large number of children (n=60) were followed up for 24 months with sequential EFV PK levels and we were able to distinguish three different CL values for the CYP2B6 516G/G, G/T and T/T genotype groups. The importance of introducing IOV in a PK model for a longitudinal study with sparsely collected data was again highlighted by this investigation.

ACKNOWLEDGEMENTS

Me M Viljoen received a Thuthuka research grant (North-West University and the National Research Foundation (NRF)) and a self-initiative grant from the Medical Research Council (MRC). The authors would like to thank the research staff of the Harriet Shezi Children's Clinic. We are grateful to all study participants and their caregivers who have contributed to this study.

CONFLICT OF INTEREST

No competing financial interests exist.

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Table 1: Efavirenz pharmacokinetic parameter estimates for basic (with IOV), final and bootstrap on the final model

Parameters	Basic Model (with IOV)	Final Model Mean (RSE%)	Bootstrap Mean (RSE%)
OFV	216	170	
CL/F(L/h)	4.86 (7.5)	2.46 (10) T/T	2.45 (13)
		4.60 (13) G/T	4.59 (14)
		7.33 (8.7) G/G	7.39 (7)
V/F (L/20kg)	94.30 (14)	89.52 (11)	92.89 (14)
Ka (h ⁻¹)	0.287 FIX	0.287 FIX	0.287 FIX
Add error	0.108 (8.3)	0.107 (7)	0.106 (11)
Prop error %	9.2(13)	9.3 (15)	8.9 (35)
ω CL/F %	63 (18)	41 (28)	40 (30)
ω V/F %	37 (63)	41 (51)	41 (53)
IOV _{CL} %		37(19)	37 (21)

IOV; interoccasion variability RSE; relative standard error ω inter-individual variability

Final model (Average child characteristics for this population: 20 kg, 7 years (84 months):

CL/F (L/h) = $\theta_x \cdot (WT/20)^{0.75} \cdot (AGE/7/12)^{-0.6}$ with $\theta_x = \theta_1$ (T/T) or θ_2 (G/T) or θ_3 (G/G)

V/F (L) = $\theta_4 \cdot (WT/20) \cdot (AGE/7/12)^{-0.6}$

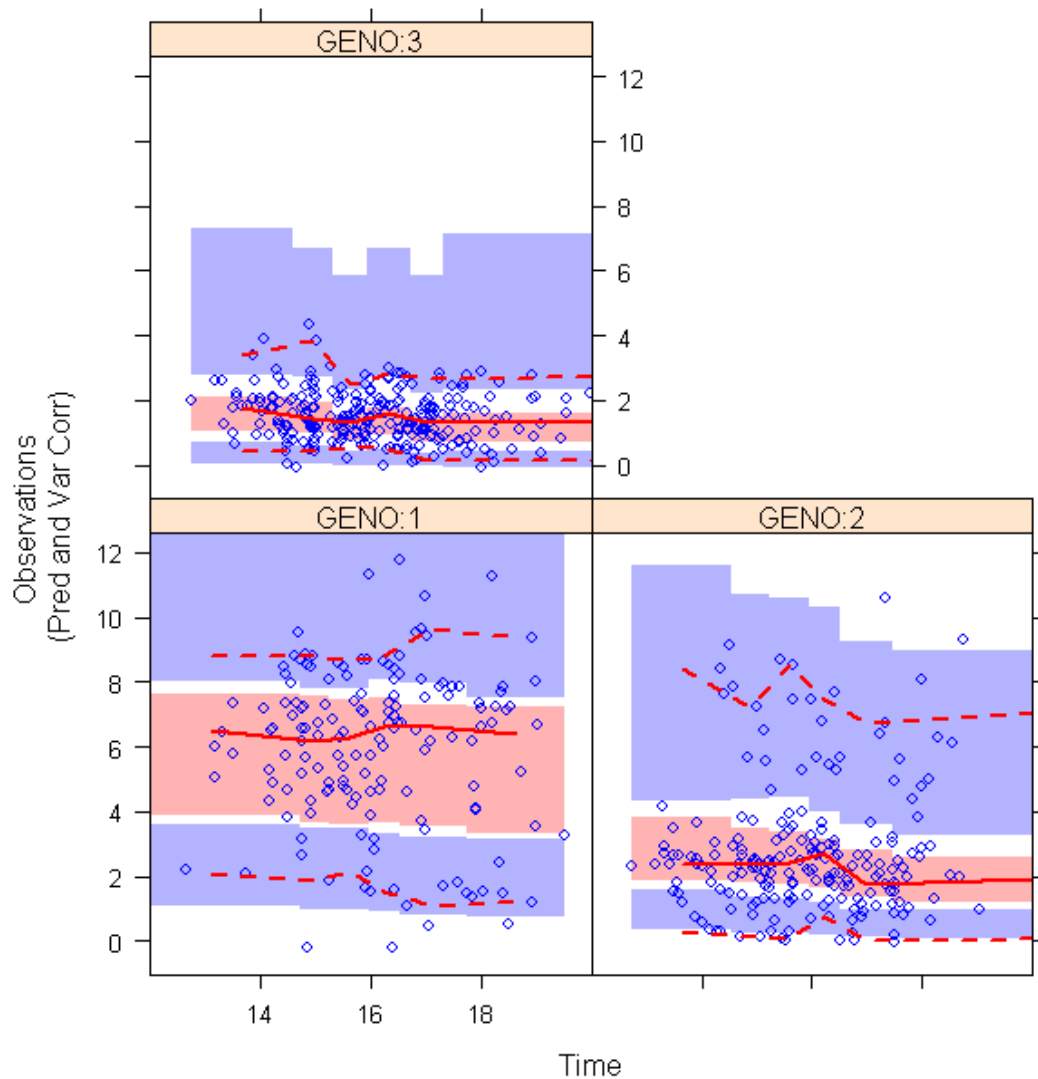


Fig. 1 Prediction-corrected visual predictive checks (pcVPC) for the EFV plasma concentration ($\mu\text{g/mL}$) over time post-last dose for final model

Geno1, T/T; Geno 2, G/T; Geno3, G/G. Solid red line is the median observed plasma concentration ($\mu\text{g/mL}$), semitransparent red field is the simulated 95% confidence interval for the predicted median. The observed 5% and 95% percentiles are presented by the dashed red lines and the 95% confidence intervals for the predicted percentiles are shown in the semitransparent blue fields.

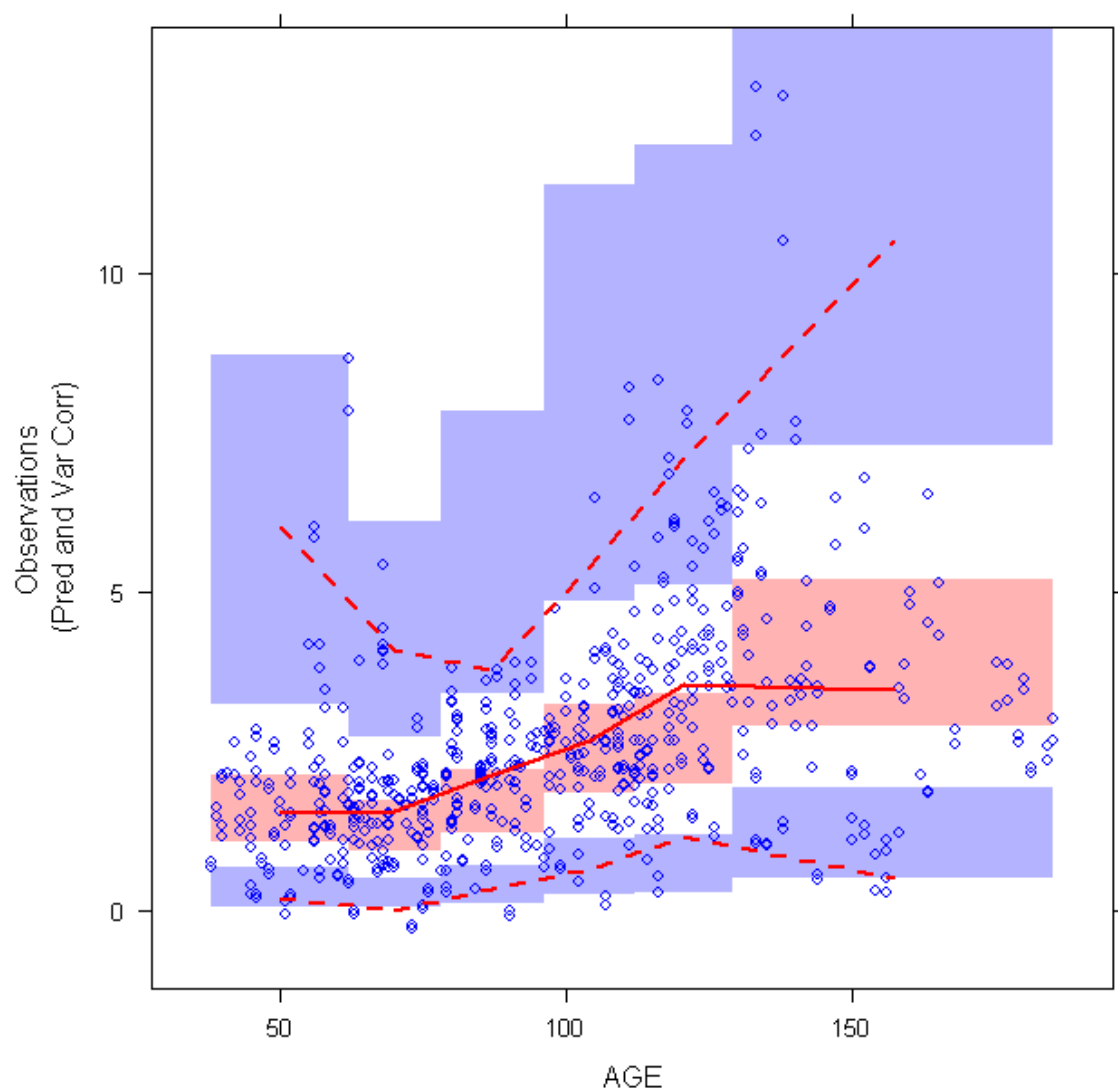


Fig. 2 Prediction-corrected visual predictive checks (pcVPC) for the EFV plasma concentration ($\mu\text{g/mL}$) vs. Age (months) for final model

Solid red line is the median observed plasma concentration ($\mu\text{g/mL}$), semitransparent red field is the simulated 95% confidence interval for the median. The observed 5% and 95% percentiles are presented by the dashed red lines and the 95% confidence intervals for the predicted percentiles are shown in the semitransparent blue fields

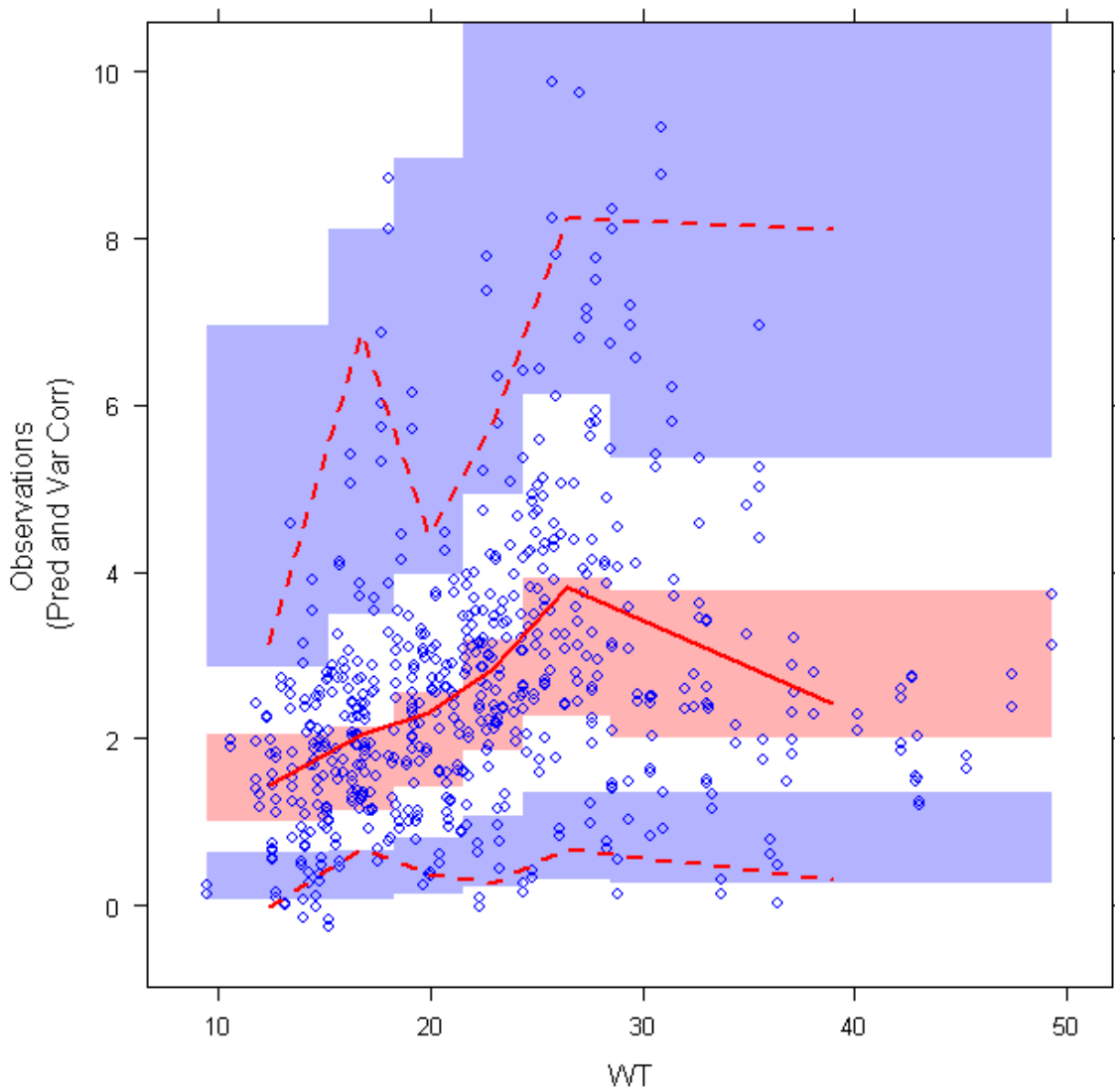


Fig. 3 Prediction-corrected visual predictive checks (pcVPC) for the EFV plasma concentration ($\mu\text{g/mL}$) vs. Weight (kg) for final model

Solid red line is the median observed plasma concentration ($\mu\text{g/mL}$), semitransparent red field is the simulated 95% confidence interval for the median. The observed 5% and 95% percentiles are presented by the dashed red lines and the 95% confidence intervals for the predicted percentiles are shown in the semitransparent blue fields

Chapter 7

Additional Results & Discussions

7.1 Introduction

This chapter reports on results from the clinical trial (PK/PD.EFV.07), *Population pharmacokinetic and pharmacodynamic study of efavirenz in HIV-1-infected children treated with first line antiretroviral therapy in South Africa*, as set out in the primary and secondary objectives of Chapter 1. The results presented here do not repeat the results, relevant discussions and conclusions as presented in the three manuscripts (Chapters 4-6) unless it was deemed necessary to project the results as a whole and to maintain a thread throughout this chapter. Some of the results (ANOVA and NONMEM) are explanatory and supportive to certain aspects presented in the manuscripts.

Results and discussions included herewith are:

- i) LC-MS/MS validation and optimisation of efavirenz (EFV) in plasma.
- ii) Subject demographics and characteristics at baseline.
- iii) EFV plasma concentrations at 1-24 months post-HAART initiation.
- iv) CYP2B6 516G>T single nucleotide polymorphism (SNP).
- v) Comparative subject demographics and characteristics at baseline for the CYP2B6 516 G/G, G/T and T/T genotype groups.
- vi) CYP2B6 516 G/G; G/T and T/T genotype and repeated measures of EFV plasma concentrations.
- vii) Pharmacodynamics: Therapeutic outcomes of the EFV-based regimen (absolute CD4-cell counts; CD4%; viral loads (VL), alanine aminotransferase

(ALT)) 3-24 months post-HAART of the whole group and dependent on the CYP2B6 516 G/G, G/T and T/T genotype groups.

- viii) Correlation and association analysis EFV plasma concentrations and various dependent variables.
- ix) Population pharmacokinetic (PK) analysis of EFV as performed with NONMEM.

7.2 LC-MS/MS Validation and Optimisation of Efavirenz in Plasma

The LC-MS/MS method development, validation and optimisation of a selective, accurate and repeatable method were one of the primary objectives of this investigation. Complete validation results are presented herewith; a brief summary of the method's validation was also published in Viljoen *et al.*, (2010).

7.2.1 Linearity and Calibration

The linear range of the EFV standards was between 0.094 – 12.000 µg/ml and the number of standards used in the calibration and regression analysis was 8 (L1 to L8).

Raw data (mass spectrometry response and measured concentrations of all EFV standards (L1-L8)) obtained over eight working days were incorporated to calculate mean values (Table 7.1) for each level in the regression statistical analysis. The calibration regression curve is presented in Figure 7.1.

Table 7.1: Regression statistical analysis data

N	Level	EFV Stds (µg/ml)	Mean EFV Abundance (m/z)	Mean measured EFV concentration (µg/ml)	Mean Accuracy %	Min. - Max. Accuracy %
	L0	0.000	0.00	0.000000		
13	L1	0.09375	1553.18	0.091689	97.80	83.67-110.18
13	L2	0.1875	3334.68	0.188058	100.30	93.16-108.26
13	L3	0.375	6975.38	0.376191	100.32	91.74-119.60
13	L4	0.75	14162.23	0.773567	103.14	94.35-110.28
13	L5	1.5	28229.95	1.456432	97.10	84.96-104.77
14	L6	3.000	60925.26	3.147074	104.90	96.05-113.96
13	L7	6.000	124230.2	6.112334	101.87	95.68-106.66
13	L8	12.000	272667.5	11.78496	98.21	93.57-105.23
Mean					100.46%	
SD					2.74%	
CV%					2.72%	

EFV, efavirenz. m/z, mass/charge ratio. Min., minimum. Max., maximum SD, standard deviation. CV%, coefficient of variation
Validation 22/10/2008-31/10/2008

The r^2 -value was 0.996 and the linear regression was, $Y = 22789.01X - 3696.62$.

A very good positive linearity was obtained and linearity was significant ($P < 0.000001$).

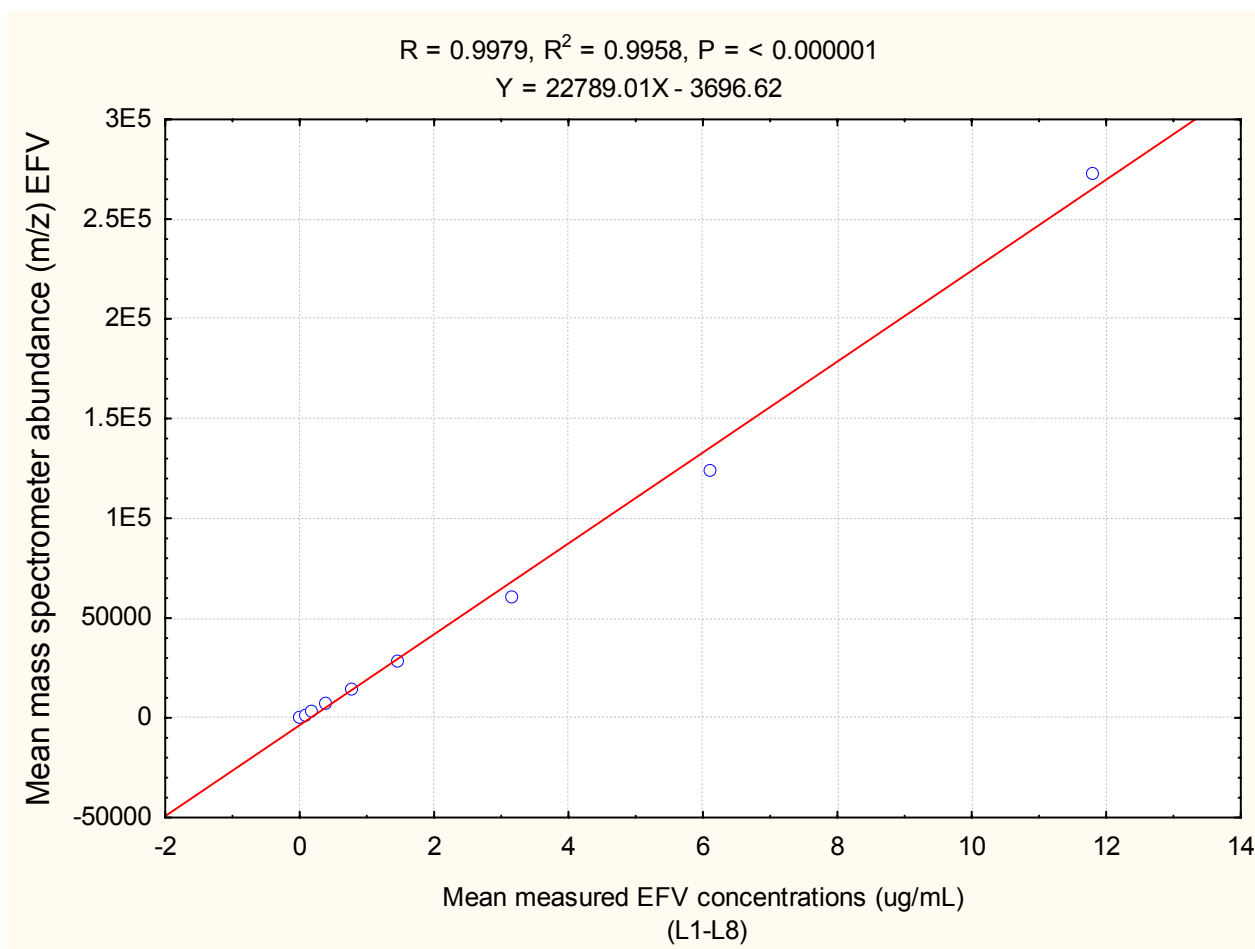


Figure 7.1: Regression curve of the mean efavirenz plasma standards (L1-L8).

7.2.2 Calibration Uncertainties (standard errors)

The following standard errors (SE) were calculated for the random calibration (Y/X), slope (m) and intercept (c):

Random calibration uncertainty (Y/X) = 6297.53

Uncertainty in slope (m) = 562.31

Uncertainty in intercept (c) = 2577.21

Since both standard error of slope (m) and standard error of intercept (c) were less than the standard error Y/X, it indicated that a good general selection of standards was applied. The working range that was applied (0.094 - 12,000 µg/ml) proved to be adequate as the standard error of slope (m) was less than the standard error of intercept (c).

7.2.3 Limit of Quantification (LOQ) and Limit of Detection (LOD)

The LOQ = 0.00001 µg/ml (SE = 0.024622) and the LOD = 0.000004 µg/ml (SE = 0.024622). It was, however, decided to use the **more stringent** set of guidelines and the LOQ was set at 0.094 µg/ml, corresponding to the lowest calibrant concentration in the linear range 0.094 – 12.000 µg/ml. Therefore all concentrations that were less < 0.094 µg/ml were incorporated in the NONMEM analysis, the concentrations values determined as 0 were not included in the NONMEM analysis. All concentrations that were > 12.000 µg/ml were diluted and re-analysed.

7.2.4 Precision: Repeatability and Reproducibility

Inter-day (between days) repeatability and reproducibility were determined by injecting low, medium and high spiked concentrations (quality control, QC) over 8 days (Table 7.2).

Table 7.2: Inter-day repeatability and reproducibility of standards

Day	0.1875 µg/ml (L2) (Relative Concentration)	3.000 µg/ml (L6) (Relative Concentration)	12.000 µg/ml (L8) (Relative Concentration)
Mean	0.1859	3.1584	12.3713
SD	0.0132	0.2137	1.1386
% RSD	7.10%	6.77%	9.20%

SD, standard deviation. RSD, relative standard deviation. Validation 22/10/2008-31/10/2008

Intra-day (same day) repeatability and reproducibility were determined by injecting low, medium and high (quality control, QC) spiked concentrations three times per day for 3 days (refer to Table 7.3).

Table 7.3: Intra-day repeatability and reproducibility of standards

Day	0.1875 µg/ml (L2) (Relative Concentration)	3.000 µg/ml (L6) (Relative Concentration)	12.000 µg/ml (L8) (Relative Concentration)
Day 1			
Mean	0.1866	2.9014	11.0892
SD	0.0068	0.0258	0.2485
% RSD	3.62%	0.89%	2.24%
Day 2			
Mean	0.1632	3.3197	12.6031
SD	0.0176	0.1277	0.3115
% RSD	10.78%	3.85%	2.47%
Day 3			
Mean	0.1834	2.9773	12.1415
SD	0.0077	0.1763	1.3537
% RSD	4.21%	5.92%	11.15%

SD, standard deviation. RSD, relative standard deviation. Validation 22/10/2008-31/10/2008

The % RSD of QC (0.1875, 3.000, 12.000 µg/ml) samples were acceptable as they were all less than 15% for both the inter-day and intra-day analyses performed. The method is thus repeatable as well as reproducible.

7.2.5 Stability of Efavirenz and Internal Standard (Midazolam)

Stability was determined by injecting three QC samples (0.1875, 3.000, 12.000 µg/ml) in triplicate directly after sample preparation and then repeated after 24, 48 and 72 hours, while being kept at 4°C in the auto sampler tray.

The respective accuracy values are reflected in Table 7.4.

Table 7.4: Accuracy (%) of EFV plasma samples stored at 4°C (auto sampler) for 24, 48 and 72 hours

	Time in auto sampler	L2 Accuracy %	L6 Accuracy %	L8 Accuracy %
Mean	0 hr	108.25	107.49	96.93
SD		2.05	7.80	2.91
RSD%		1.89%	7.26%	3.01%
Mean	24 hr	99.12	111.60	93.77
SD		1.12	3.53	0.61
RSD%		1.13%	3.17%	0.65%
Mean	48 hr	98.16	102.64	93.08
SD		18.23	2.05	1.78
RSD%		18.57%	2.00%	1.91%
Mean	72 hr	106.07	97.85	91.5
SD		2.4	4.39	1.04
RSD%		2.26%	4.48%	1.14%

SD, standard deviation. RSD, relative standard deviation. Validation 15/03/2010-18/03/2010

The % RSD was within the validation criteria of $\leq 15\%$ for all three levels after 24 hours. The % RSD for the lower concentration calibrant (L2, 0.1875 µg/ml) was outside this criterion at 48 hours. Samples were stable for at least 24 hours, therefore all analyses were performed on samples in the auto sampler tray within 24 hours.

7.2.6 Sensitivity and Specificity

The calibration sensitivity, slope (b) was 22789.01. As $b \neq 0$ the calibration sensitivity was acceptable.

The specificity was determined by injecting the following commonly used drugs: amoxicillin, paracetamol, sulphamethoxazole, trimetoprim, lamivudine and stavudine. Lamivudine and stavudine were co-analysed with EFV on the same LC-MS/MS

method but were not validated within this method. Their retention times are known as they overlap on the chromatograms of patient samples at 4.3-4.4 minutes not interfering with the EFV or internal standard (midazolam).

Typical total ion chromatograms (TIC) of EFV and midazolam (internal standard, IS) of a plasma calibrant (L3, 0.375 µg/ml), patient, blank, double blank and a plasma sample spiked with amoxicillin, paracetamol, sulphamethoxazole, trimetoprim are represented in Figures 7.2-7.6. The additional peaks shown at 4.3-4.4 minutes in the patient plasma sample (Figure 7.3) are the peaks of lamivudine and stavudine.

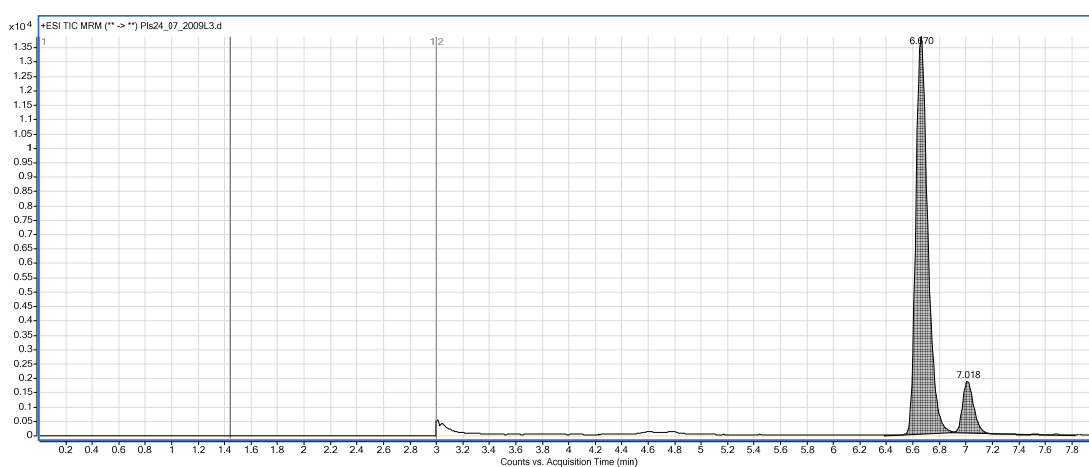


Figure 7.2: *Efavirenz plasma calibrant (L3, 0.375 µg/ml) at 7.018 min. and midazolam (IS) at 6.670 min.*

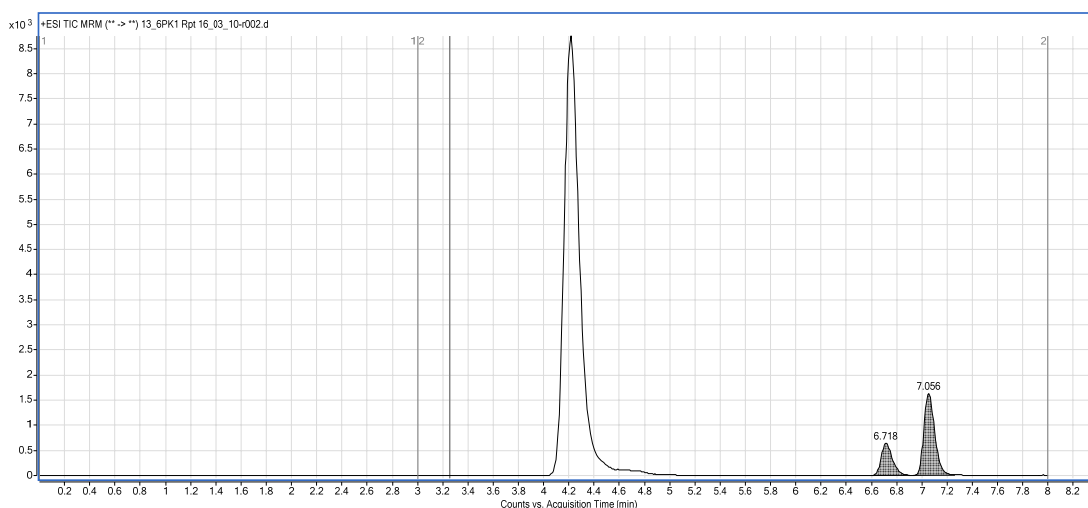


Figure 7.3: *Patient plasma sample (EFV = 5.30 µg/ml, at 7.056 min.) and IS (at 6.718 min.).*

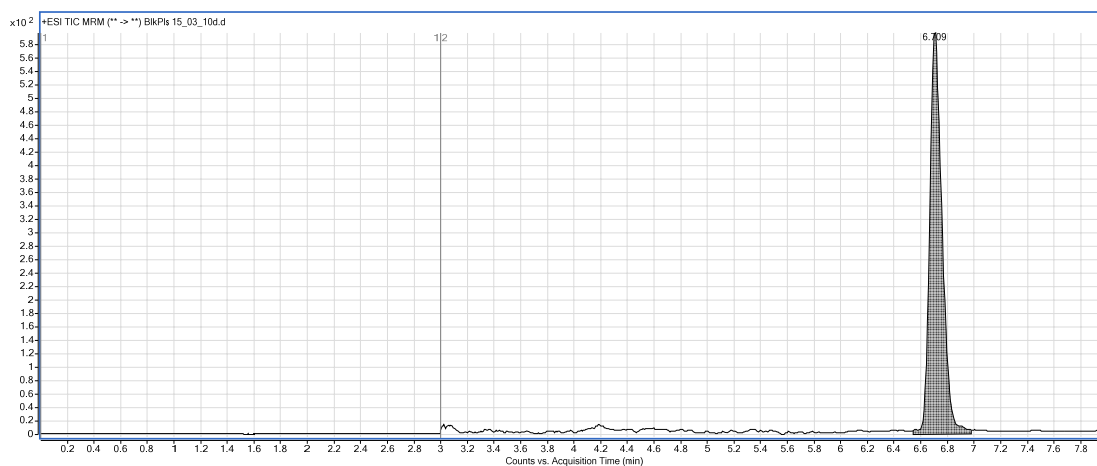


Figure 7.4: Blank plasma sample, IS (midazolam) at 6.709 min.

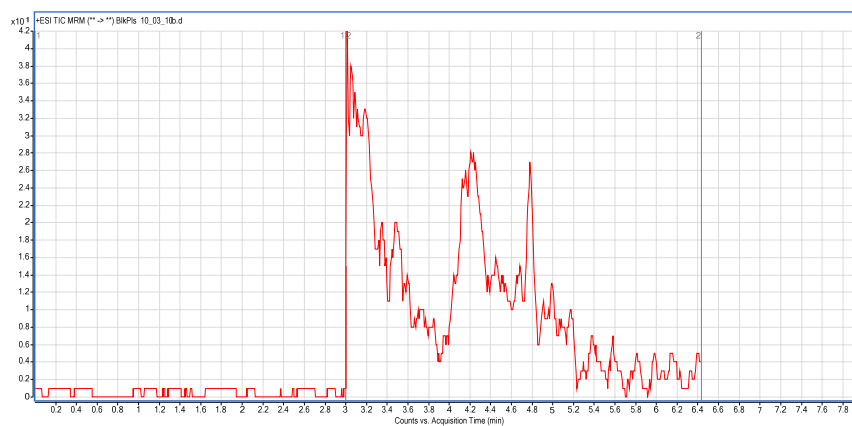


Figure 7.5: Double blank (no analyte or IS) plasma sample.

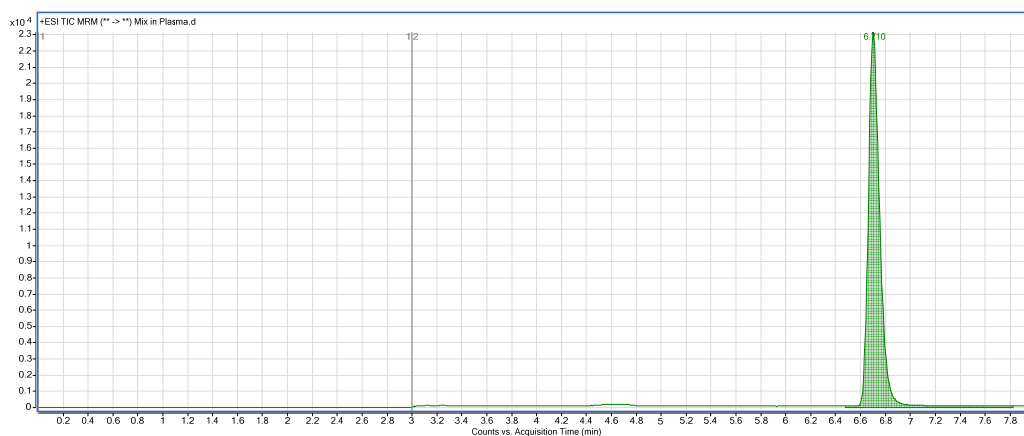


Figure 7.6: Plasma spiked with amoxicillin, paracetamol, sulphamethoxazole, trimethoprim and IS (midazolam) at 6.71 min.

No interference was found by any of the tested substances and this method was specific for efavirenz.

7.2.7 Accuracy

The mean (SD%) accuracy of the calibrants (L1-L8) calculated over 8 working days (n=13) was 100.46% (2.74%) and the coefficient of variation (CV%) was 2.72% over the complete range (Table 7.1).

The accuracy of the individual calibrants used in the daily calibration curves was between 83.67% and 119.60% for all of the calibrants. Table 7.1 reflects the minimum and maximum accuracy (%) of each calibrant (L1-L8) over 8 working days.

The accuracy did not deviate more than $\pm 20\%$. This method was validated as accurate for the range: 0.094 – 12.000 $\mu\text{g/ml}$.

7.2.8 Recovery

Two plasma standards (high and low concentrations) were prepared according to the sample preparation (precipitated). The standards were repeated three times, and injected into the LC-MS/MS system in triplicate. The average abundance (mass spectrometry response) of the plasma standards for EFV was evaluated against the theoretical concentrations of unprecipitated water standards for EFV. The recovery for two plasma standards (high and low concentrations) was $61.95\% \pm 5.86\%$. The recovery was consistent and thus acceptable.

7.3 Subject Demographics and Characteristics at Baseline

Sixty-four (64) black HIV-1 infected study participants were prospectively recruited from the Harriet Shezi Children's clinic, Chris Hani Baragwanath Hospital, Soweto, Johannesburg during June 2007 to March 2008. Only sixty (60) study participants (both genders) were enrolled in the clinical trial (PK/PD.EFV.07).

Reasons for not enrolling the other four recruited participants were: two (2) contracting TB, one (1) had received ART previously but did not disclose it at the time of consent and one (1) relocated to another city before the commencement of the first line regimen.

The mean age (SD) at baseline was 6.8 (2.9) years, the weight 20.29 (6.7) kg, height 113.7 (17.94) cm, body surface area 0.80 (0.19) and 52% were males (Table 7.5). The baseline clinical results for VL, CD4%, CD4-cell count and ALT are reflected in Table 7.5.

Table 7.5: Baseline demographic and clinical characteristics of study participants enrolled onto PK/PD.EFV.07

	Baseline (n=60)
AGE (years) Mean ($\pm$ SD)	6.8 (2.90)
GENDER Male, n (%)	31 (52)
WEIGHT (kg) Mean ($\pm$ SD)	20.19 (6.70)
Weight-for-Age-Z-Score ^{*#}	-1.35 (0.95)
HEIGHT (cm) Mean ($\pm$ SD)	113.7 (17.94)
Height-for-Age-Z-Score [#]	-1.73 (1.06)
BSA (m ²) Mean ($\pm$ SD)	0.80 (0.19)
Body mass index-for Age-Z-Score [#]	-0.45 (1.04)
VIRAL LOAD (VL) copies/ml	
Median (IQR range)	52 500 (14 500-172 500)
Log Median (IQR range)	4.72 (4.18-5.20)
CD4-CELL COUNT ($\times 10^6$ /L)	
Median (IQR range)	490 (211-657)
CD4%	
Median (IQR range)	14 (8.44-18.80)
Alanine aminotransferase (ALT) (U/L)	
Median (IQR range)	18.50 (14.5-25.50)

BSA, body surface area. IQR, inter quartile range. *Only calculated for children younger than 10 years as available on WHO Anthro software. # Z-scores calculated using WHO Anthro and WHO AnthroPlus software

All relevant descriptive statistics related to Table 7.5 are presented in Addendum D1.

7.4 Efavirenz Plasma Concentrations at 1-24 Months Post-HAART Initiation

The results for the first 6 months were published in Manuscript A: “Efavirenz plasma concentrations at 1, 3 and 6 months post-HAART initiation in HIV type 1-infected South African children”, and is presented in Chapter 4.

The EFV plasma concentrations of all the subjects over the 24-month period are briefly summarised in the following results and discussion section. The novelty and scientific contribution of this study was that ARV naïve patients were followed up for a period of 24 months with measured sequential EFV plasma concentrations at 1, 3, 6, 12, 18 and 24 months post-HAART initiation.

The median EFV plasma concentrations and the mean log transformed results of both the first (PK1) and the second blood samples (PK2) from 1-24 months are reported in Table 7.6. The respective mean times post-last evening dose, weight, height and median EFV doses are also shown in Table 7.6. All relevant descriptive statistics for all the PK1 and PK2, 1-24 months post-HAART, are presented in Addendum D1.

Table 7.7 and Figure 7.7 represent the percentage levels within and outside the therapeutic range over the 24-month post-HAART initiation. Gender comparison of the EFV plasma concentrations (1-24 months) is reflected in Table 7.8

Missing data points and plasma levels below and above the relevant analytical linear range (0.094-12 µg/ml) were differently presented in Manuscript A, compared to the results reflected in Table 7.6-7.7 and Figure 7.7. Very few missing data points (n=5) compared to the total number (n=649) of efavirenz plasma levels (PK1 and PK2) were recorded during the 24 month follow-up period and can thus be regarded as negligible. All concentrations that were less < 0.094 µg/ml (n=6 samples from 4 patients) were incorporated in the NONMEM analysis, the concentrations values determined as 0 µg/ml (n=10 samples from 4 patients) were not included in the NONMEM analysis.

Table 7.6: Median plasma and mean log transformed EFV concentrations ($\mu\text{g/ml}$) at each of the 6 follow-up study visits post-HAART initiation

	#1 Month		#3 Months		#6 Months		12 Months		18 Months		24 Months	
	1PK1	1PK2	2PK1	2PK2	3PK1	3PK2	4PK1	4PK2	5PK1	5PK2	6PK1	6PK2
Median EFV (ug/ml)	2.29 (1.40-3.77)	1.86 (1.29-3.76)	1.79 (1.12-4.35)	1.62 (1.03-4.32)	2.12 (1.22-4.77)	1.78 (1.12-4.22)	2.04 (1.11-4.47)	1.94 (0.90-4.16)	2.44 (1.64-5.61)	1.99 (1.33-5.17)	2.70 (1.56-5.94)	2.70 (1.42-6.03)
N	57	59	56	56	56	55	54	54	50	48	52	52
<i>Mean Log EFV (ug/ml)</i>	0.36 (0.40)	0.31 (0.40)	0.32 (0.45)	0.27 (0.48)	0.34 (0.44)	0.31 (0.45)	0.32 (0.51)	0.28 (0.53)	0.46 (0.38)	0.41 (0.40)	0.47 (0.46)	0.42 (0.46)
N^a	57	59	56	56	55	54	52	52	49	47	51	51
<i>Mean Time (hr)</i>	15.58 (3.15)	17.15 (3.06)	15.08 (1.14)	16.78 (1.21)	15.20 (1.02)	16.84 (0.94)	15.65 (3.08)	17.26 (3.44)	15.62 (3.44)	17.81 (3.54)	15.06 (1.12)	17.26 (1.08)
Median Dose (mg)	250 (200-300)		250 (250-300)		300 (250-325)		300 (250-350)		300 (250-350)		300 (250-350)	
<i>Mean Weight (kg)</i>	20.33 (6.85)		20.79 (6.58)		21.59 (6.62)		22.56 (7.11)		23.91 (7.26)		25.27 (7.71)	
<i>Mean Height (cm)</i>	114.05 (17.64)		114.65 (16.91)		116.12 (16.51)		119.18 (15.56)		122.95 (15.98)		125.78 (15.70)	

Median (IQR, 25% - 75%). Mean ($\pm$ SD). Concentrations > 12 ug/ml were diluted appropriately and reanalysed.

Concentrations = 0 were not included for the **log analysis** and therefore Na values will differ from N values as a log plasma concentration (Log 0) cannot be calculated. The actual missing efavirenz plasma values over the 24 month follow-up period accounted for n= 5 which is negligible compared to the total number (n=649) of samples.

Concentrations > 0 < 0.094 ug/ml were not included as they were outside the linear detection range.

Please note that some of the values for 1-6 months will differ from the published data (Manuscript A) as plasma concentrations greater than 12 ug/ml were diluted appropriately and reanalysed and included in Table 7.6 but were excluded from Table 2 (Manuscript A).

Table 7.7: Percentage EFV plasma concentrations within and outside the therapeutic range (1-4 µg/ml)

	1PK1	1PK2	2PK1	2PK2	3PK1	3PK2	4PK1	4PK2	5PK1	5PK2	6PK1	6PK2	Total PK1	Total PK2	TOTAL PK1+PK2
<1 µg/ml	16%	15%	19.5%	23%	16%	18%	24%	26%	10%	13%	13%	19%	54/325 16.5%	62/324 19%	116/649 18%
>4 µg/ml	24.5%	22%	27%	27%	29%	27%	33%	33%	34%	31%	37%	33%	99/325 30.5%	93/324 29%	192/649 29.5%
1-4 µg/ml	59.5%	63%	53.5%	50%	55%	55%	43%	41%	56%	56%	50%	48%	172/325 53%	169/324 52%	341/649 52.5%

All EFV plasma concentrations outside of the linear range (0.094-12 µg/ml) were included in these calculations.

% values of 1,3 and 6 months (1PK1; 1PK2; 2PK1; 2PK2; 3PK1; 3PK2) data will be slightly different from Manuscript A data (Table 2) where all data outside linear range were excluded.

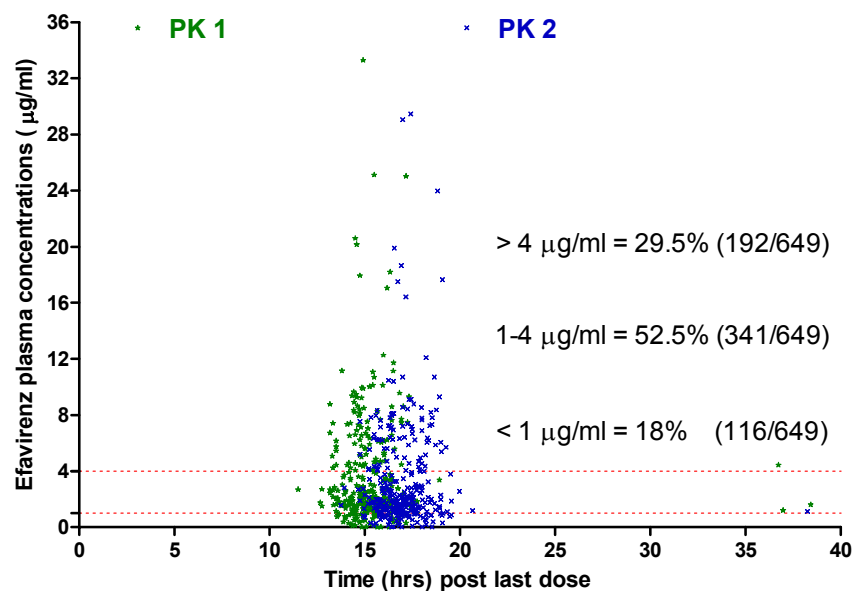
**Figure 7.7:** All efavirenz plasma concentrations (1st and 2nd PK's) taken at 1-24 months post-HAART vs. time post-last dose.

Table 7.8: Comparison of male vs. female mean log EFV plasma (PK2's) concentrations ($\mu\text{g/ml}$) at 1, 3, 6, 12, 18 and 24 months post-HAART

	Mean Log EFV plasma (PK2's) concentrations ($\mu\text{g/ml}$)											
	Log 1PK2	*P	Log 2PK2	*P	Log 3PK2	*P	Log 4PK2	*P-	Log 5PK2	*P-	Log 6PK2	*P-
Male	0.30	0.73	0.25	0.68	0.25	0.34	0.28	0.99	0.39	0.82	0.40	0.73
Female	0.33		0.30		0.37		0.28		0.42		0.44	

* P-value, Independent T-test (groups)

EFV plasma concentrations greater than 4 $\mu\text{g/ml}$ represented 29.5%, 1-4 $\mu\text{g/ml}$ represented 52.5% and less than 1 $\mu\text{g/ml}$ represented 18% of all the 649 samples taken during mid-dose interval at 1-24 months post-HAART initiation. These results were in line with the results for the first six months as reported in Manuscript A (25% >4 $\mu\text{g/ml}$; 58% between 1-4 $\mu\text{g/ml}$ and 17% <1 $\mu\text{g/ml}$). Although only 52.5% of the mid-dose interval plasma samples were within the therapeutic range the proportion of concentrations <1 $\mu\text{g/ml}$ (18 %) was in comparison with other studies also performed in children, 8.8% (Wintergerst *et al.*, 2008), 13% (Puthanakit *et al.*, 2009) and 11% (ter Heine *et al.*, 2008). Von Hentig *et al.*, (2006) and Ren *et al.*, (2007) reported 40% and 64% of their EFV plasma concentrations to be less than 1 $\mu\text{g/ml}$, but both these studies were performed with a very small number of children (n=15 and n=11 respectively) and only once-off cross sectional mid-dose concentrations were analysed. Bearing in mind that 23% of our study population were genotyped with the CYP2B6 516 T/T allele (slower metabolisers, refer to § 7.5.1), it is to be expected that a large (29.5%) number of the EFV plasma concentrations would be greater than 4 $\mu\text{g/ml}$ as was the case over the 24 months post-HAART initiation. Correlation and association analyses of EFV plasma concentrations and reported side effects from this study were addressed in Manuscripts A and B (Chapters 4 &5) and under § 7.7.1 to 7.7.2.

The gender results comparison showed a trend that the mean log EFV concentrations (PK2's) were slightly higher for the females compared to the males, however no statistically significant differences (independent T-test, $P>0.05$) were observed at any of the six follow-up study visits post-HAART initiation (Table 7.8) between the genders within this paediatric study population. Other studies also

reported that gender did not influence the EFV plasma concentrations in adults (Pfister *et al.*, 2003; Csajka *et al.*, 2003; Kwara *et al.*, 2009; Ngaimisi *et al.*, 2010). However, two studies did report that adult African women had significantly higher EFV plasma concentrations compared to their male counterparts and subsequently reported more side effects (Burger *et al.*, 2006; Nyakutira *et al.*, 2008).

7.5 CYP2B6 516G>T Single Nucleotide Polymorphism and Efavirenz Plasma Concentrations Over Time

7.5.1 CYP2B6 516 G/G, G/T and T/T Genotyping

Genotyping was performed on the isolated DNA samples of the 60 study participants to investigate whether some of the inter-individual variation in the EFV plasma levels (§7.4) could be explained by this single nucleotide polymorphism (SNP) on the CYP2B6 gene.

The genotyping results for the CYP2B6 516G>T polymorphism in the study participants are shown in Table 7.9 and discussed in Manuscript B (Chapter 5). The Chi-squared (χ^2) test was performed between observed and expected frequencies to fit data to the Hardy-Weinberg equilibrium (HWE). The proportions were found to be consistent with the HWE ($P=0.10$), a value <0.05 is an indication that data are not consistent with HWE.

Table 7.9: Genotype results and frequencies of CYP2B6 516G>T polymorphism

Genotypes	Observed Numbers	Expected Numbers	Observed Frequency
Homozygote (G/G) reference	25	21	41.66%
Heterozygote (G/T)	21	29	35%
Homozygote variant (T/T)	14	10	23.33%
Variant allele frequency (516T) = 0.41 (41%) χ^2 test P value = 0.10			

The high 516 T-allelic variant of 41% from our black paediatric South African population corresponds well with results from studies in other African-American and African descent populations which varied from 32% to 49% (Cohen *et al.*, 2009;

Haas *et al.*, 2004; Klein *et al.*, 2005; Kwara *et al.*, 2009; Wyen *et al.*, 2008). An in depth discussion and association analysis is presented in Manuscript B (Chapter 5).

The genotyping results were obtained as follows: the amplification of the target amplicon with conventional polymerase chain reaction (PCR) as described in Chapter 3 (§ 3.4.2.2) yielded a PCR product of 526 base pairs (bp), refer to Figure 7.8. This 526 bp product was then fast digested (FastDigest® BseNI) and fragment length polymorphism (FLP) analysis yielded:

- i) 3 fragments of 23, 236 and 267 bp for the homozygous wild type 516 G/G,
- ii) 4 fragments of 23, 236, 267 and 503 bp for the heterozygous 516 G/T and
- iii) 2 fragments of 23 and 503 bp for the homozygous 516 T/T (variant) refer to Figure 7.9.

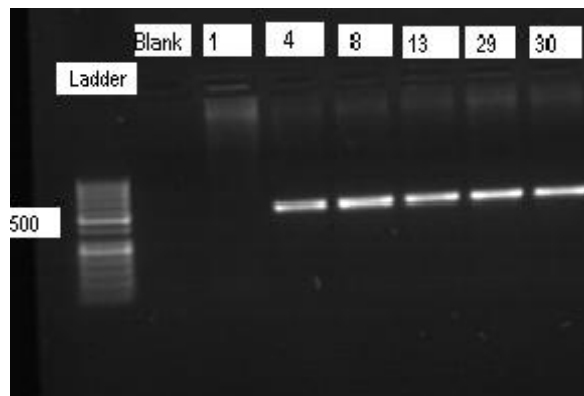


Figure 7.8: Gel electropherograms of the amplified 526 bp PCR product (CYP2B6 516G>T) of six DNA patient samples on 2% agarose gel.

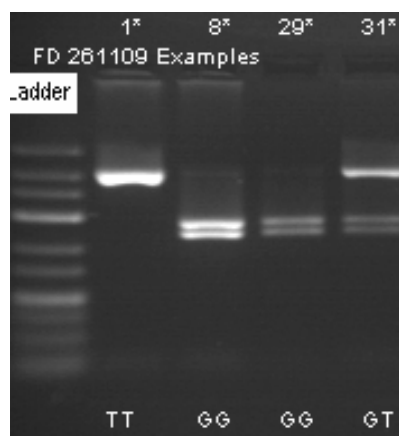


Figure 7.9: Gel electropherograms of the fast digested 526 bp and FLP analysis of the CYP2B6 516G>T genotypes (T/T, G/G and G/T) of four DNA patient samples on 3% agarose gel.

The 23 bp fragments are only very slightly visible at the bottom of each of the respective genotypes (T/T; G/G; G/G; G/T) in Figure 7.9, depending on the gel running time and time of UV exposure.

7.5.2 Comparative Subject Demographics & Characteristics at Baseline for the CYP2B6 516 G/G; G/T; T/T Genotypes

When considering that, within this study population, three distinct groups (fast, intermediate and slow metabolisers) based on the CYP2B6 516 G/G, G/T and T/T genotyping can be identified, it becomes important to compare the relevant baseline characteristics of the three respective genotype groups before any scientific conclusions can be drawn.

Table 7.10 presents the comparative baseline demographics and clinical characteristics of the three respective CYP2B6 516 G/G, G/T and T/T genotype groups.

All relevant descriptive statistics and corresponding box and whisker plots of baseline demographics and clinical characteristics of the three respective groups are presented in Addendum D1.

It is evident from Table 7.10 that the physical and clinical characteristics (VL; CD4-cell count; CD4%, ALT) among the respective CYP2B6 516 G/G, G/T and T/T genotype groups at baseline were not statistically different (Kruskal-Wallis, $P > 0.05$).

Table 7.10: Comparative baseline demographic and clinical characteristics of the CYP2B6 516 G/G, G/T and T/T genotype groups

CYP2B6 516G>T	516 G/G Genotype (n=25)	516 G/T Genotype (n=21)	516 T/T Genotype (n=14)	P
AGE (years)				
Mean (± SD)	6.6 (2.8)	6.4 (3.1)	7.8 (2.5)	0.30
GENDER				
Male, n (%)	13	11	7	0.99
WEIGHT (kg)				
Mean (± SD)	20.4 (7.6)	19.5 (6.6)	20.8 (5.3)	0.75
HEIGHT (cm)				
Mean (± SD)	113.9 (18.2)	112.1 (19.7)	115.9 (15.6)	0.68
VIRAL LOAD (copies/ml)				
Median (IQR range)	73 000 (17 000-130 000)	48 000 (21 000-200 000)	23 000 (9 500-130 000)	0.85
CD4-CELL COUNT (x 10⁶/l)				
Median (IQR range)	460 (241-648)	536 (245-775)	501 (176-580)	0.69
CD4 %				
Median (IQR range)	14.80 (9.29-18.50)	14.30 (9.00-21.20)	14.35 (6.96-17.10)	0.61
ALANINE AMINOTRANSFERASE (ALT) (U/l)				
Median (IQR range)	19 (15-24)	17 (14-24)	18 (12-29)	0.88

IQR, inter quartile range. P-value < 0.05 denotes statistical significant difference. The P values represent comparison between 3 genotypes. Chi (χ^2) test was used for categorical data.

7.5.3 CYP2B6 516 G/G; G/T; T/T Genotype & Repeated Measures of Efavirenz Plasma Concentrations

The prevalence and impact of CYP2B6 516G>T polymorphism in black South African HIV-infected children on efavirenz-based antiretroviral therapy (Manuscript B) are presented in Chapter 5. The focus in Manuscript B was on the log EFV plasma concentrations of the second PK's (1-24 months) only as they were the

nearest to the true trough level ($C_{\min}$). The results of the log PK2's (Manuscript B) are repeated here to provide continuity to these results and discussions.

All relevant descriptive statistics on all the EFV and log EFV concentrations of both the PK1's and PK2's sampled during mid-dose intervals at 1, 3, 6, 12, 18 and 24 months post-HAART initiation for the three genotyped groups are presented in Addendum D1.

The graphical repeated measures (PK1's and PK2's) of all the EFV and log EFV concentrations are presented in Figure 7.10 (A1-2:PK1's; B1-2:PK2's). In order to pool all the EFV concentration data together it was necessary to investigate what the influence of the interaction (months post-HAART R1 * genotype) was on the plasma EFV concentrations within these three genotyped groups. The graphical presentations (Fig.7.10) and the P-values (Table 7.11) illustrate the influence of genotype, months post-HAART initiation (R1) and the interaction between R1*genotype on the EFV plasma and log EFV plasma concentrations. It is evident from Table 7.11 that genotype independently influenced the EFV plasma and log EFV plasma concentrations significantly ($P=0.001$ (PK1); $P=0.00001$ (log PK1); $P=0.001$ (PK2); $P=0.0001$ (log PK2)). Table 7.11 shows that neither months post-HAART initiation (R1) nor the interaction (R1*genotype) influenced the EFV plasma or the log EFV plasma concentrations ($P > 0.05$) of the PK1's or PK2's taken at 1-24 months. Therefore the individual EFV plasma concentrations (PK1's and PK2's) were pooled to obtain the mean individual EFV plasma concentrations and are presented in Figure 7.11 (A&B). The Kruskal-Wallis test indicated that there were statistically significant differences ($P < 0.00001$) in the EFV plasma concentrations for both the PK1's and PK2's between the three genotypes (1-24 months post-HAART). Additional multiple comparisons by groups were calculated and the respective P-values between the three genotype groups are shown on Figure 7.11. The EFV plasma concentrations for both the PK1's and PK2's were significantly different between the T/T and G/G groups (6.96 vs. 1.63 $\mu\text{g/ml}$, $P = 0.000002$ for PK1's; 6.36 vs. 1.41 $\mu\text{g/ml}$, $P = 0.00002$ for PK2's). The EFV plasma concentrations for both the PK1's and PK2's were also significantly different between the G/T and G/G groups (2.82 vs. 1.63 $\mu\text{g/ml}$, $P = 0.01$ for PK1's; 2.55 vs. 1.41 $\mu\text{g/ml}$, $P = 0.009$ for PK2's). However, no statistically significant differences were evident between the

EFV plasma concentrations for both the PK1's and PK2's of the T/T and G/T groups (6.96 vs. 2.82 µg/ml, $P = 0.059$ for PK1's; 6.36 vs. 2.55 µg/ml, $P = 0.074$ for PK2's), this could possibly be contributed to the outlier values ($n=14$, re-analysed after dilution as the values were >12 µg/ml) that were not excluded from the G/T analysis. This is an indication that the presence of the 516 T-allele at CYP2B6 is responsible for higher EFV plasma concentrations (corresponding to intermediate and slow metabolisers) when compared to the wild type (516G/G) genotype which is the faster metabolisers. Table 2.8 (refer to § 2.4.4.2) provides a summary of comparative EFV plasma concentrations determined at mid-dose interval in various patients from different study populations that have been identified as CYP2B6 516 G/G, G/T and T/T genotypes. It is clear from this summary that our results compare well to results obtained from African descent populations and that the differences in EFV plasma concentrations between G/G and T/T genotypes are greatly significant among all populations that were investigated. Manuscript B (Chapter 5) refers to the differences in EFV plasma exposures between the three genotypes (PK2's) and comparisons were made to other relevant published data.

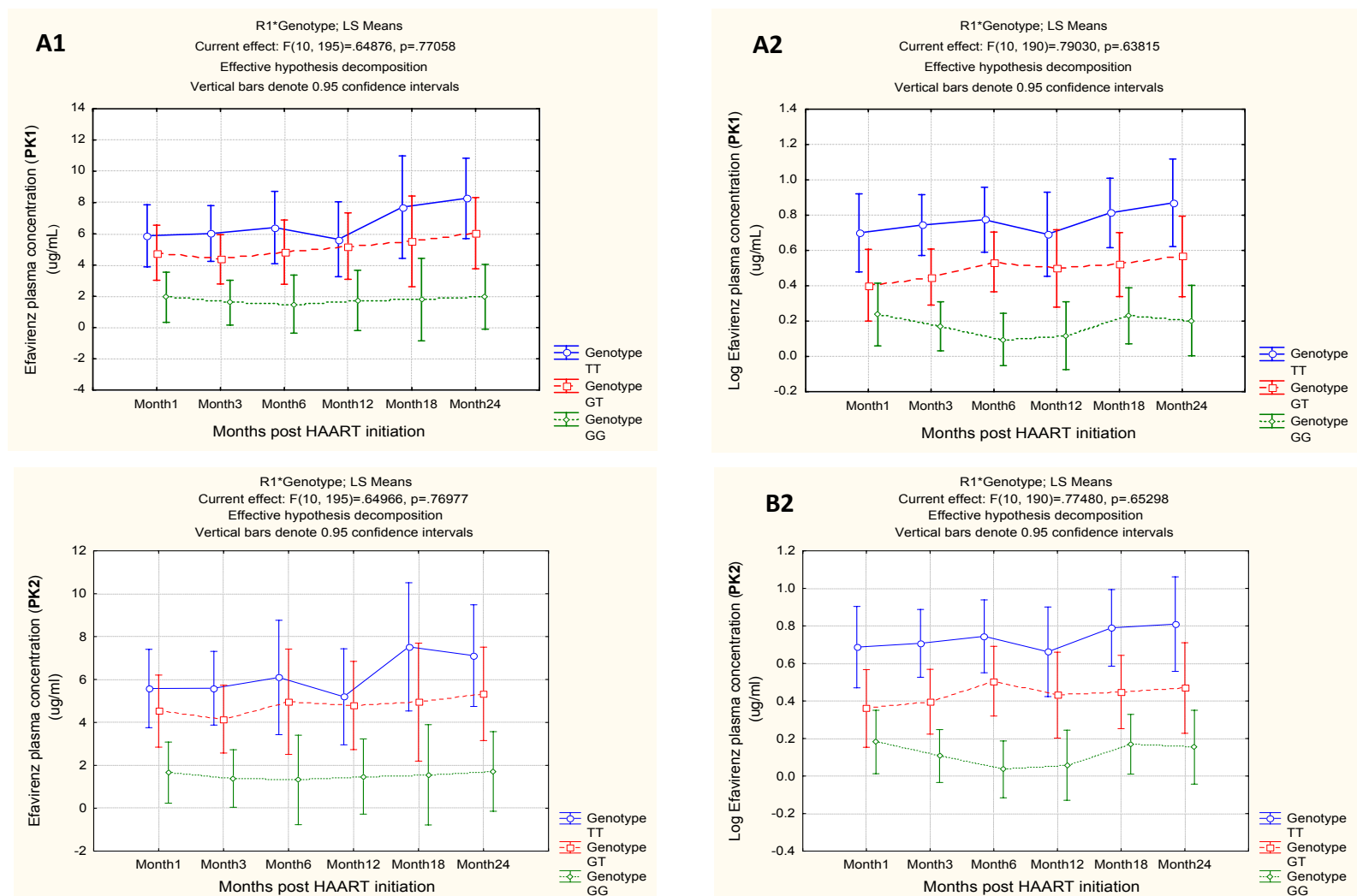
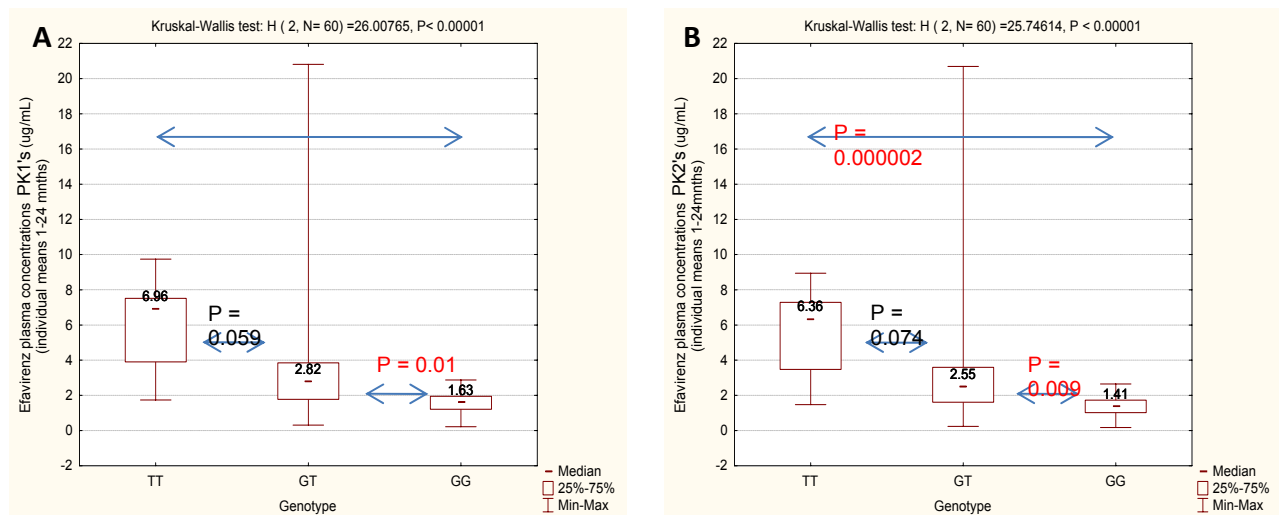


Figure 7.10: Repeated measures ANOVA of efavirenz plasma (A1:PK1 & B1:PK2) and the log EFV plasma (A2:PK1 & B2: PK2) concentrations 1-24 months post-HAART initiation.

Table 7.11: The influence of genotype, time post-HAART (R1) and the interaction (R1* Genotype) on the efavirenz plasma and log efavirenz plasma concentrations with repeated measures ANOVA

EFV concentrations	PK1's	Log PK1's	PK2's	Log PK2's
Genotype P-value	0.001	0.00001	0.001	0.0001
R1 (Time) P-value	0.06	0.20	0.17	0.39
Interaction: R1*Genotype P-value	0.77	0.64	0.77	0.65



Additional multiple comparison tests by groups as the Kruskal-Wallis test $P < 0.00001$. **No outlier data points (G/T) were excluded** from these calculations

Figure 7.11: Individual mean efavirenz plasma concentrations µg/ml (1-24 months combined) for PK1's and PK2's.

Figure 7.12 (A&B) reflects the repeated measures ANOVA, of the time post-last evening dose (in hours) of EFV for both the first blood sample (PK1) and the second blood sample (PK2), 1-24 months post-HAART for the three genotype groups. The specific design of the study and the scheduled visits took place at specified times. In the majority of the cases the study participants reported to the clinic on time from 08:00 to 09:00 and were then taken for the various blood PK samples. The **mean**

time post-last dose for the PK1's only varied slightly from 15.06 to 15.65 hours and for the PK2's, 16.78 to 17.81 hours, over the 24 months post-HAART initiation. The **median** time post-last dose for the PK1's also varied minimally from 14.96 to 15.05 hours and for the PK2's, 16.58 to 17.33 hours, over the 24 months post-HAART initiation. Figure 7.13 (A,B &C) shows the repeated measures ANOVA of weight (kg), height (cm) and the EFV night time dose (mg), 1-24 months post-HAART initiation for the three genotype groups. It is evident from these graphs and Table 7.12 that the months post-HAART initiation (R1) independently increased the weight, height and EFV night time dose significantly ($P < 0.000001$). This was, however, expected as the children were growing and thriving post-HAART initiation and they should naturally grow and gain weight over 24 months and subsequently increase in the EFV dose. Although the LS means of the weight, height and consequently the EFV night time dose of the T/T genotype group were consistently higher than those of the G/T and G/G genotype groups, no statistically significant differences ($p > 0.05$) between the three genotype groups were evident for any of these variables over the 24 months post-HAART initiation. The mean weight and height values of the T/T genotype group at baseline (Table 7.10) was slightly higher compared to the G/T and G/G groups to begin with. The interaction (genotype* R1) did not affect any of these three variables (weight, height and EFV night time dose) significantly ($P = 0.29$, $P = 0.41$ and $P = 0.29$ respectively).

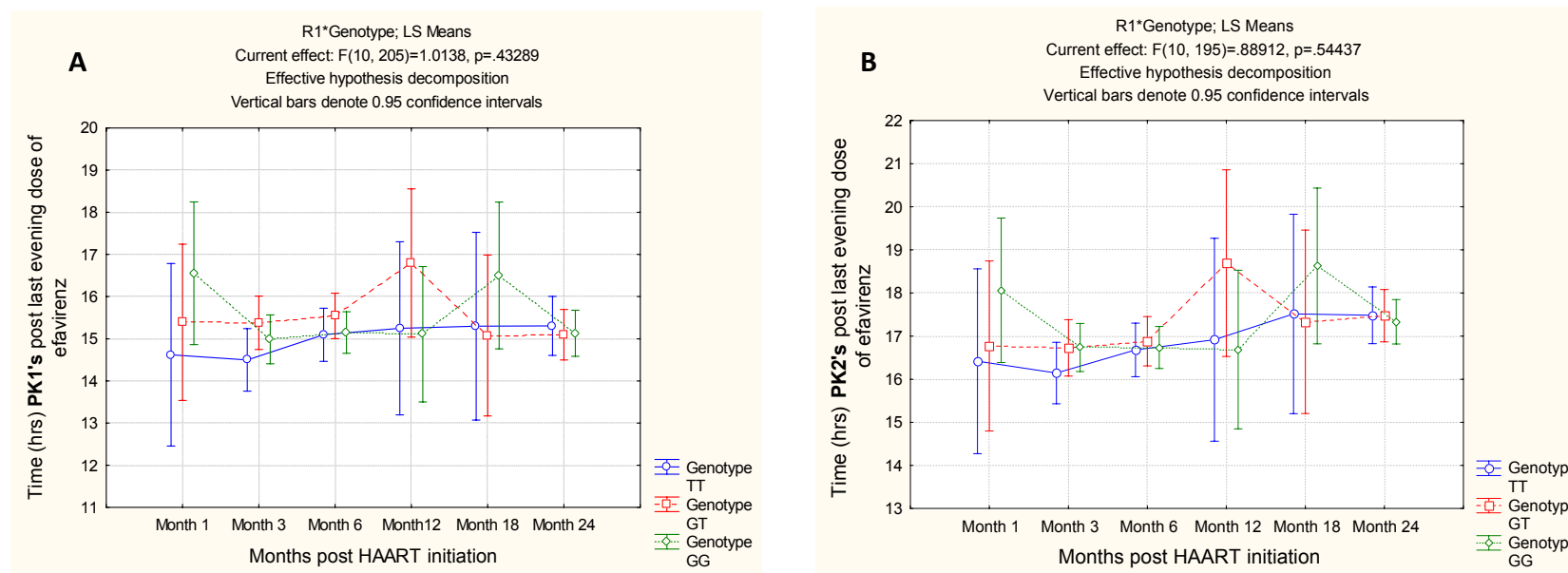


Figure 7.12: Repeated measures ANOVA of time post-last efavirenz dose for PK1's & PK2's (1-24 months post-HAART initiation).

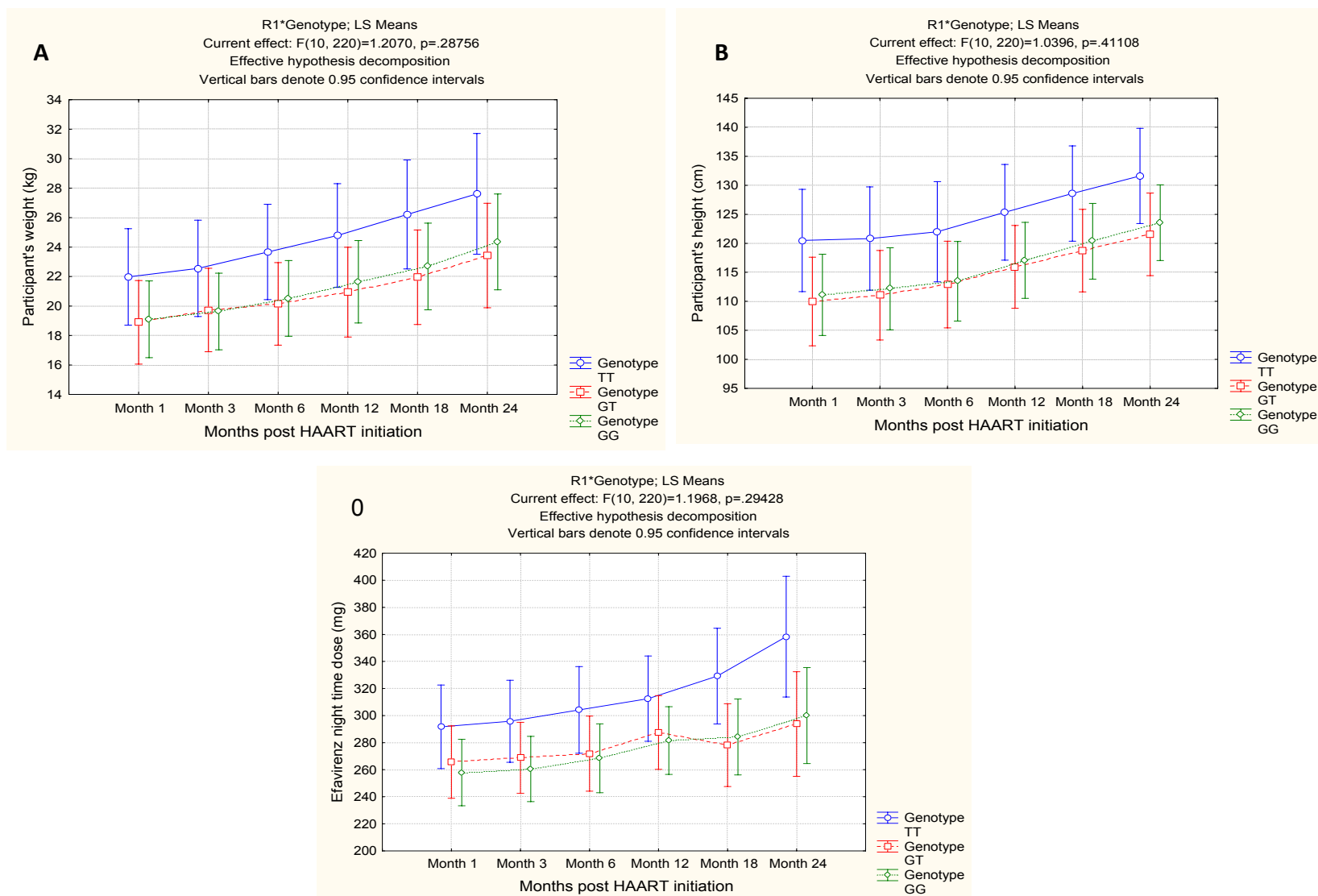


Figure 7.13: Repeated measures ANOVA of weight, height and EFV night time dose 1-24 months post-HAART initiation.

Table 7.12: *The influence of genotype, months post-HAART initiation (R1) and the interaction on the weight, height and EFV nocte dose, 1-24 months post-HAART initiation (repeated measures ANOVA)*

	Weight (kg)			Height (cm)			EFV nocte dose (mg)		
CYP2B6 516 Genotype	T/T	G/T	G/G	T/T	G/T	G/G	T/T	G/T	G/G
LS Means	24.471	20.852	21.325	124.81	115.03	116.29	315.28	277.60	275.44
Genotype P-values	P > 0.05			P > 0.05			P > 0.05		
R1 (Months) P-values	P < 0.000001			P < 0.000001			P < 0.000001		
Interaction (R1*Genotype) P-values	P = 0.29			P = 0.41			P = 0.29		

LS, least square

7.6 Pharmacodynamic Outcomes of Efavirenz-Based Regimen, 3-24 months post-HAART Initiation

It is important to emphasise that these pharmacodynamic outcomes are the result of the HAART combination which includes EFV, lamivudine and stavudine.

7.6.1 Immunological and Virological Marker Results: 3-24 months post-HAART Initiation (independent of genotype groups)

The prognostic value of immunological markers (CD4-cell counts and CD4%) and virological markers (viral load) are important in clinical practice to evaluate and monitor treatment response or progression of the HIV infection (Grabar *et al.*, 2000; Anastos *et al.*, 2004; Moore *et al.*, 2009). However, in children, the percentage of CD4-cells (CD4%) expressed as a total of all lymphocytes is considered the best indicator of immunodeficiency provided that the lymphocyte count is not very low (NDoH, 2010).

Table 7.13 shows the mean (SD) and median (IQR) results of absolute CD4-cell count; CD4%; viral load (VL) and alanine aminotransferase (ALT) values recorded at 3, 6, 12, 18 and 24 months post-HAART initiation. Cholesterol and triglyceride tests

were only performed at 24 months post-HAART initiation and did not form part of the routine 6-monthly testing by the Department of Health guidelines for the management and care of HIV in children (NDoH, 2005). All descriptive statistics relevant to these clinical markers are presented in Addendum D1.

A comparative dependent (paired) T-test was performed to investigate the change of absolute CD4-cell count, CD4%, VL, log VL and ALT from baseline to 24 months post-HAART initiation within this study population (independent of genotype distinction). The Box and Whisker plots in Figure 7.14 (A & B) show that the immunological markers (absolute CD4-cell count and CD4%) drastically increased ($P < 0.005$) from baseline to 24 months post-HAART initiation within this study population. The VL and log VL values decreased significantly ($P < 0.005$), Figure 7.14 (C & D), from baseline compared to 24 months. Figure 7.15 depicts the comparative results of ALT at baseline and 24 months, indicating that the ALT values did not significantly ($P = 0.36$) change over the 24-month period post-HAART initiation.

This marked improvement in the clinical (immunological and virological) markers is supportive of many other clinical studies that have shown that EFV-based regimens are effective in improving the immunological status and viral suppression in HIV infected adults and children (Starr *et al.*, 1999; Starr *et al.*, 2002; Staszewski *et al.*, 1999; Riddler *et al.*, 2008; Ruiz, 1999; Gulick *et al.*, 2004; Marzolini *et al.*, 2001). Comparable results of VL values in other studies were 90% <50 copies/ml after 48 weeks (Staszewski *et al.*, 1999), 63% <50 copies/ml after 48 weeks (Starr *et al.*, 1999), 89% <200 copies/ml after 32 weeks (Gulick *et al.*, 2004), 67% <50 copies/ml after 96 weeks (Scherpbier *et al.*, 2007) and 89% <50 copies/ml after 96 weeks (Riddler *et al.*, 2008) of EFV-based treatment. In our study at 24 months, 89% (47/53) of the children had VL values lower than the detectable 25 copies/ml (Viljoen *et al.*, 2011 under review, Manuscript B). Virological suppression, treatment failure and side effects reported from this study were addressed in the manuscripts reflected in Chapters 4 and 5. However, Table 7.14 provides additional information on eleven ($n=11$) study participants that were not virally suppressed (VL <25 copies/ml) at 6 months and onwards post-HAART initiation.

Table 7.13: Clinical marker results at 3, 6, 12, 18 and 24 months post- HAART initiation (independent of CYP2B6 516 genotype)

	N*	Mean	SD	Median	IQR (25%)	IQR (75%)
3 Months post-HAART						
CD4-cell count	52	663.0	376.8	602.00	404.50	913.0
CD4%	52	22.1	9.3	24.15	14.50	28.8
Viral load (VL)	55			25.00	25.00	25.0
ALT	54	23.2	10.5	20.50	16.00	27.0
6 Months post-HAART						
CD4-cell count	53	678.7	365.5	624.00	370.00	865.0
CD4%	53	24.7	8.6	24.80	19.10	29.7
Viral load (VL)	56			25.00	25.00	25.0
ALT	56	23.9	12.4	21.00	16.50	26.0
12 Months post-HAART						
CD4-cell count	54	788.6	400.4	723.50	535.00	1029.0
CD4%	54	27.0	7.8	28.10	22.10	32.3
Viral load (VL)	55			25.00	25.00	25.0
ALT	55	21.9	11.7	19.00	14.00	27.0
18 Months post-HAART						
CD4-cell count	51	805.4	342.1	744.00	613.00	921.0
CD4%	51	29.6	7.8	29.40	25.10	34.2
Viral load (VL)	51			25.00	25.00	25.0
ALT	49	20.7	10.6	17.00	16.00	23.0
24 Months post-HAART						
CD4-cell count	51	823.6	299.4	809.00	590.00	986.0
CD4%	51	30.5	7.5	31.40	25.10	36.8
Viral load (VL)	53			25.00	25.00	25.0
ALT	50	19.7	8.2	18.50	14.00	22.0
Cholesterol	52	3.9	0.8	3.85	3.30	4.5
Triglycerides	52	1.1	0.4	1.00	0.80	1.4

* Reasons for inconsistent N- values were insufficient blood samples or clotted blood samples and could therefore not be tested. SD, standard deviation. IQR, inter quartile range. ALT, alanine aminotransferase

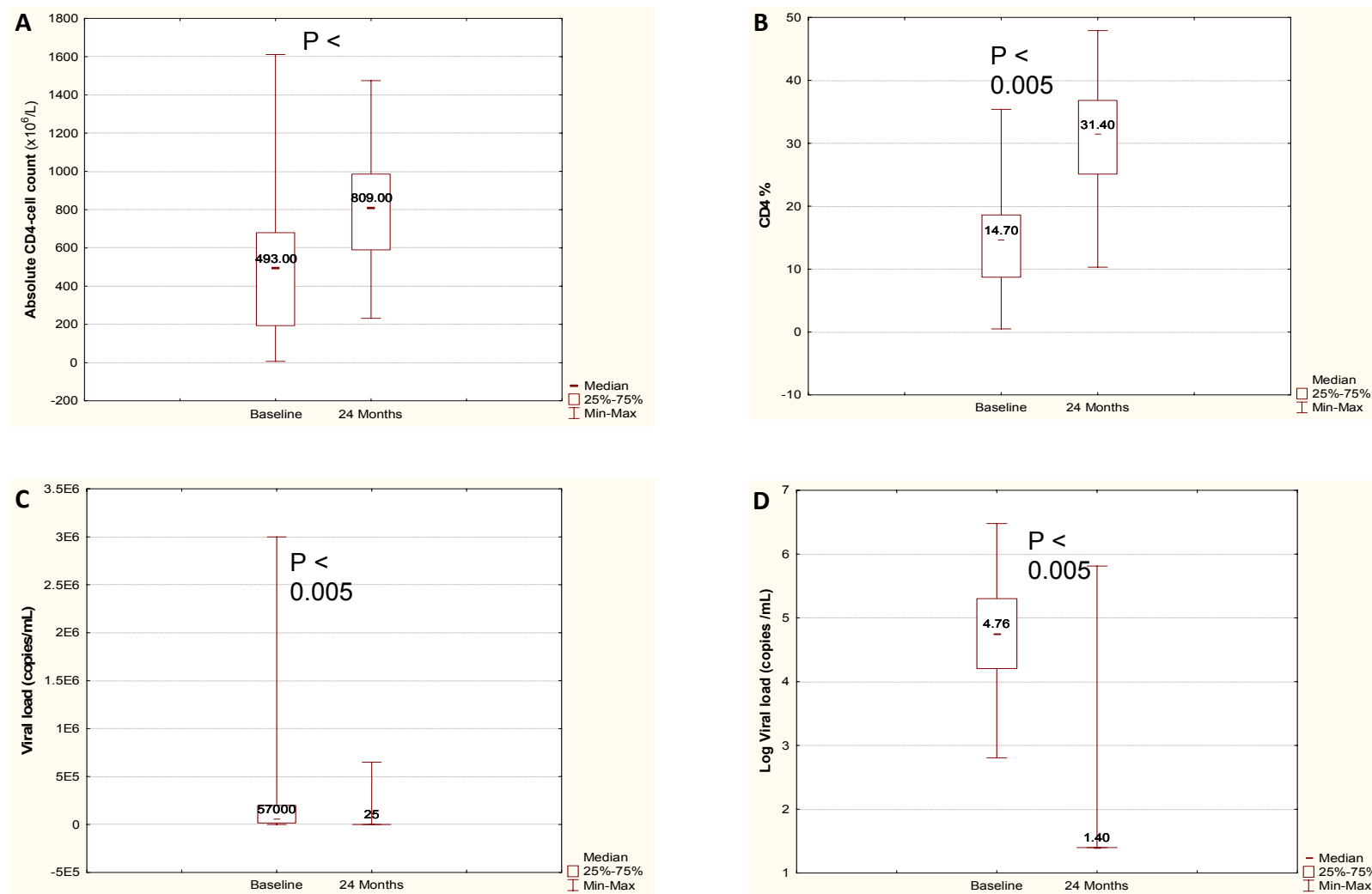


Figure 7.14: Dependent (paired) T-test of clinical markers compared at baseline and at 24 months post-HAART initiation for CD4-count (A), CD4%(B), viral load (C) and log viral load (D).

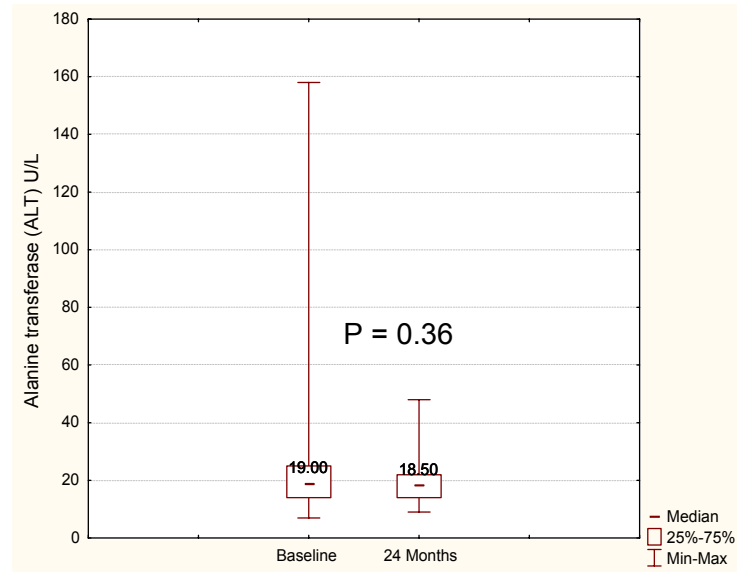


Figure 7.15: Dependent (paired) T-test of ALT at baseline compared to 24 months post-HAART initiation.

Re-testing of VL for five participants (26-32 months post-HAART and after the study monitoring ended) were performed to exclude possible viral “blips” and it was confirmed that the VL values of two participants were indeed only “blips” (refer to Table 7.14).

Table 7.14: Characteristics of patients with VL >25 copies/ml during the 24 month follow-up period

Subject	Months post-HAART	VL (copies/ml)	EFV (PK2) (µg/ml)	Genotype	Adherence to EFV ^a	Resistance test ^b	VL (26-32 months post-HAART)
9TK	24	270	2.80	GT	Good		49
12LS	6 12	780 56	1.01 0.45	GG	Poor Poor	ND	
16HN	18 24	84 000 2 000	2.60 0	GT	MNR Poor	Yes	
19SS	12	990	0	TT	Poor	Yes	
24TM ^c	6 12	170 000 2 100	0 0.46	GG	MNR	Yes	
35KN ^c	6 12	2 800 23 00	<0.094 0	GT	MNR	^d	
48SS ^c	6	3 100	1.73	GG	MNR	Yes	
54PM	24	1 300	7.97	TT	Poor	No	<25
59TP	24	61	4.31	GT	Good	No	116
63TM	24	650 000	0.70	GG	Poor	No	<25
64NM	24	56	3.96	GT	Poor	No	312

^a Refer to manuscript (Chapter 4 and 5) for more information on definition of adherence to EFV.

^b Resistance testing did not fall within the scope of the objectives of this PhD. Resistant tests were performed by CLS/NHLS.

^c No viral load (VL) < 25 copies/ml were ever reached during their participation within this study

^d Lost to follow up prior to resistance testing. ND – not done as regimen was changed due to concentration problems, 13 months post-HAART initiation and prior to resistant test. MNR – medicines not returned for pill counts.

7.6.2 Repeated Measures of Immunological and Virological Markers of the CYP2B6 G/G, G/T and T/T Genotypes

Repeated measures ANOVA with least square (LS) mean values were used to graphically present results of absolute CD4-cell count, CD4% and ALT values recorded at 3, 6, 12, 18 and 24 months post-HAART initiation for the three genotype groups. Repeated measures for the VL results could not be presented since no analysis of variance existed from 3 months onwards as both the median and IQR (25% and 75%) were at 25 copies/ml.

Table 7.15 reflects the LS mean values of absolute CD4-cell counts, CD4% and ALT and the respective P-values of the influence of genotype, months post-HAART initiation (R1) and the interaction (R1*Genotype) on these variables. It is evident from Table 7.15 and Figure 7.19 (A-C) that neither genotype nor the interaction (R1*Genotype) influenced ($P > 0.05$) the absolute CD4-cell counts, CD4% and ALT values as they were similar over time within the three genotype groups. However, months post-HAART initiation (R1) independently influenced the absolute CD4-cell count, CD4% and ALT values of the genotype groups across the board significantly ($P < 0.0001$), refer to Table 7.15. It is also evident from Figures 7.16 A&B that the CD4-cell counts and CD4% values increased over time for all three genotype groups. This was supported by the dependent T-test (Figure 7.14) results that CD4-cell count and CD4% increased significantly within the whole group from baseline to 24 months post-HAART.

Table 7.15: *The influence of genotype, months post-HAART initiation (R1) and the interaction (R1*Genotype) on the absolute CD4-cell counts, CD4% and ALT over 24 months post-HAART initiation (repeated measures ANOVA)*

CYP2B6 516 Genotype	Absolute CD4-cell counts			CD4%			ALT		
	T/T	G/T	G/G	T/T	G/T	G/G	T/T	G/T	G/G
LS Means	690.51	758.64	758.85	26.77	26.37	26.66	27.33	22.4	18.4
Genotype P-values	P = 0.80			P = 0.99			P = 0.07		
R1 (Months) P-values	P < 0.0001			P < 0.0001			P < 0.00001		
Interaction (R1*Genotype) P-values	P = 0.60			P = 0.78			P = 0.36		

LS, least square ALT, alanine aminotransferase

It was established at baseline (Kruskal-Wallis tests in Table 7.10) that the VL, log VL, CD4-cell counts, CD4% and ALT values did not show any significant statistical differences between the CYP2B6 516 G/G, G/T and T/T genotype groups. Additional Kruskal-Wallis tests were performed at 3, 6, 12, 18 and 24 months for each of these respective variables and also showed no statistically significant differences between the genotype groups for the VL, CD4-cell counts and CD4% values (all P values > 0.05) at any of the months post-HAART initiation. For the ALT values at 12 months the genotype did influence the ALT values ($P = 0.04$). However, when tested with multiple comparisons between the three genotype groups no statistically significant differences ($P > 0.05$) were observed between the three respective groups (Figure 7.20B). The various median (IQR) values of each of these variables at 3, 12 and 24 months post-HAART initiation are presented in Figures 7.17-7.20.

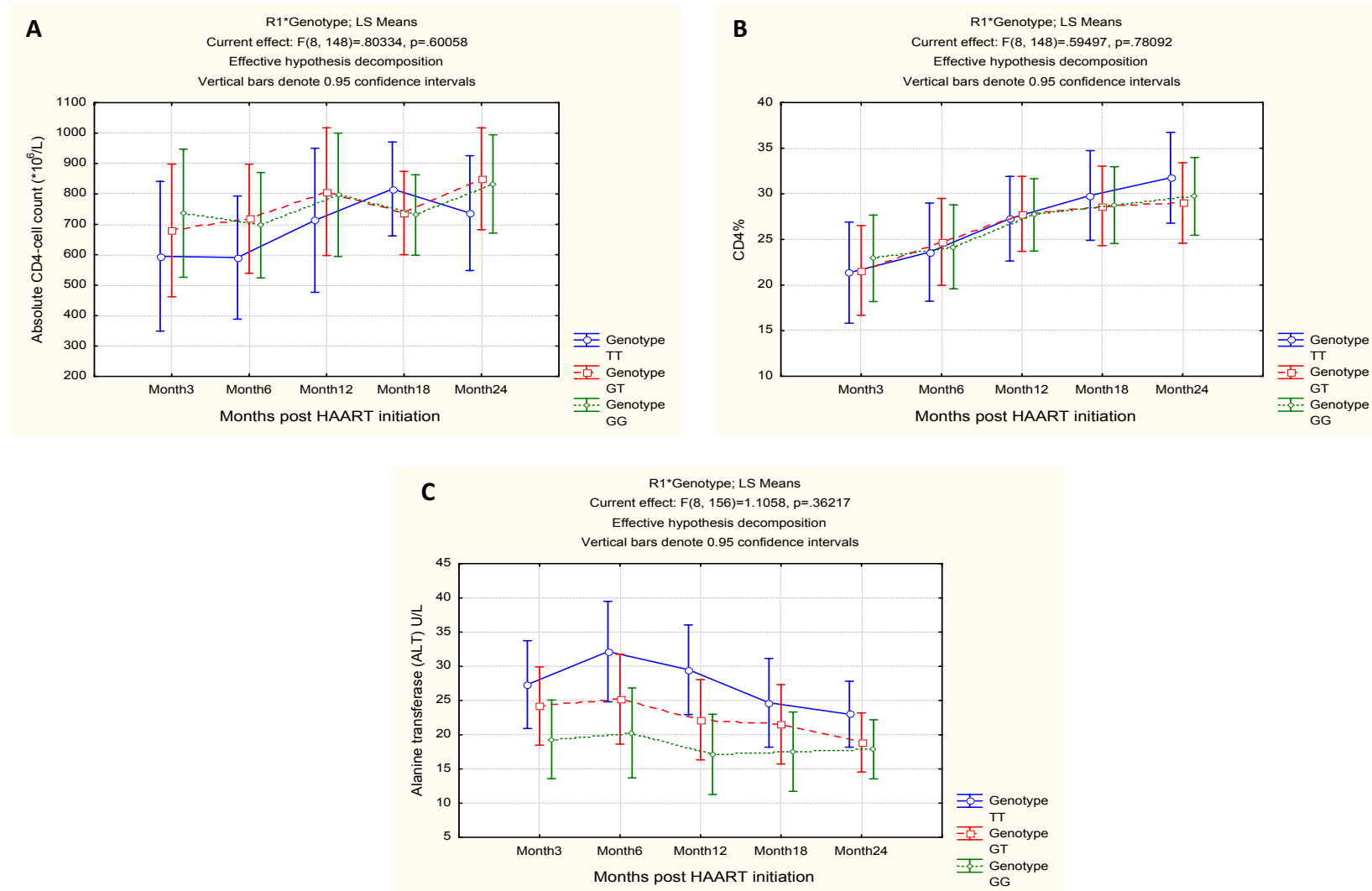


Figure 7.16: Repeated measures ANOVA of absolute CD4-cell counts; CD4% and ALT during 3-24 months post-HAART initiation for CYP2B6 516 G/G, G/T and T/T genotypes

No beneficial clinical outcome benefit nor treatment disadvantage was established between the CYP2B6 516 G/G, G/T and T/T genotype groups as the efficacy of this EFV-based treatment did not affect the three genotype groups differently and they showed similar improvement in the immunological (CD4-cell count and CD4%) markers and reduction in the viral load values among the three genotype groups over the 1-24 months post-HAART initiation. Haas and co-workers (2005) and Saitoh and co-workers (2007) also reported that no significant differences were found between the CYP2B6 516 G/G, G/T and T/T genotypes and their respective immunological and virological responses. The efficacy of this EFV-based regimen supports previous reports that the CD4-cell count, CD4% and VL were significantly improved from baseline at different months post-HAART initiation (Gulick *et al.*, 2004; Riddler *et al.*, 2008; Ruiz, 1999; Scherpbier *et al.*, 2007; Starr *et al.*, 1999; Staszewski *et al.*, 1999).

This EFV-based regimen was well tolerated by the majority of this study's participants over the 1-24 months post-HAART initiation. Correlation and association analyses of EFV plasma concentrations and reported side effects from this study were addressed in Manuscripts A and B (Chapters 4 & 5) and under § 7.7.1 to 7.7.2. Various studies could not establish an association with EFV plasma concentration and CYP2B6 516G>T polymorphism and toxicity (Ramachandran *et al.*, 2009; Rotger *et al.*, 2005; Saitoh *et al.*, 2007). Several studies did, however, report association of CYP2B6 516 T/T with CNS toxicity (Haas *et al.*, 2005) whereas some studies reported an increase in the prevalence of side effects, especially CNS related which correlated with EFV plasma levels greater than 4 µg/ml (Cohen *et al.*, 2009; Marzolini *et al.*, 2001; Puthanakit *et al.*, 2009; Gounden *et al.*, 2010). Results from our study could not corroborate that an association existed between high EFV plasma levels and reported side effects, specifically 1 month post-HAART.

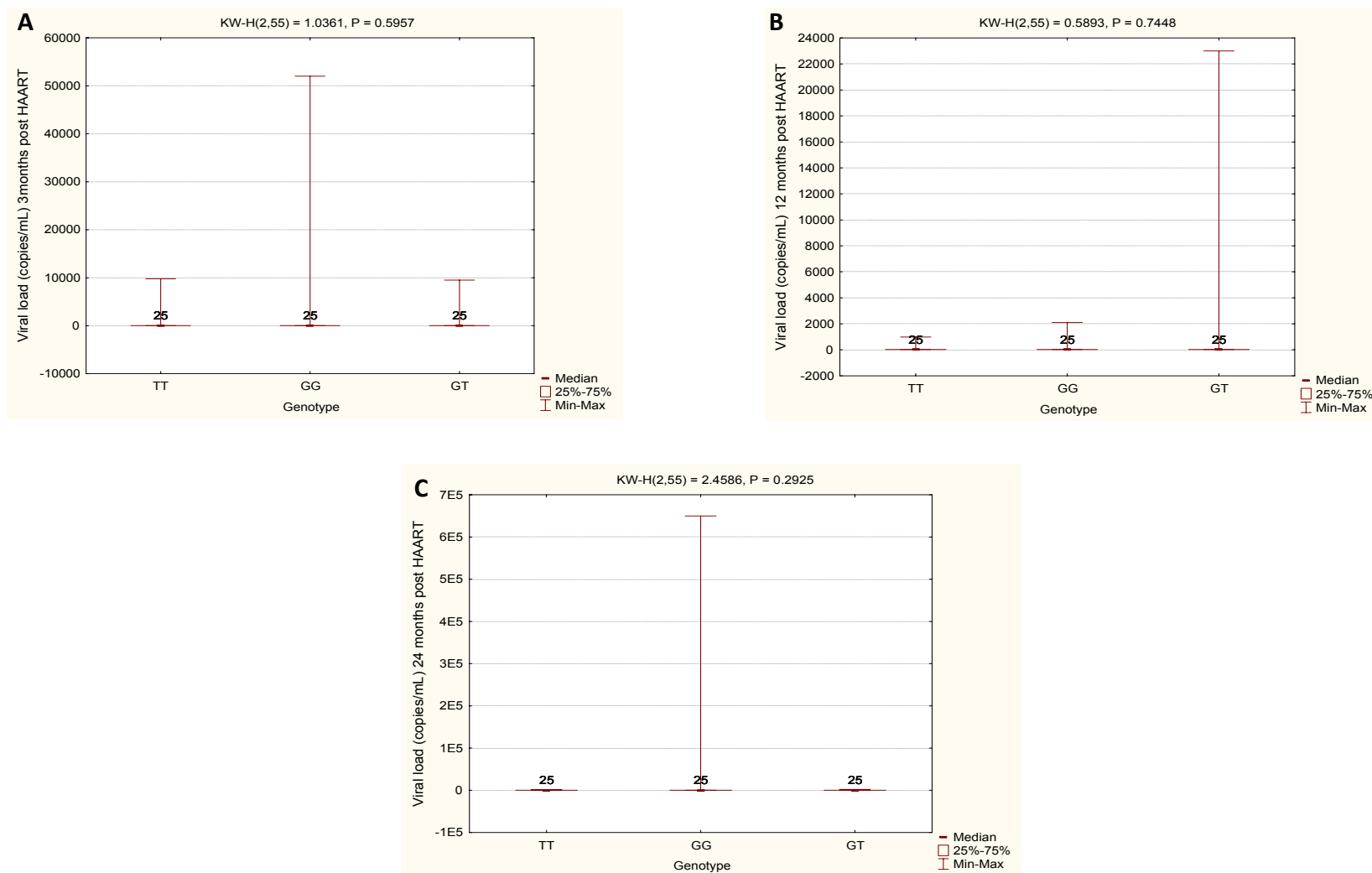


Figure 7.17: Box and Whisker plots of median viral load (VL) values of the three genotype groups (G/G; G/T; T/T) at 3, 12 and 24 months post-HAART initiation.

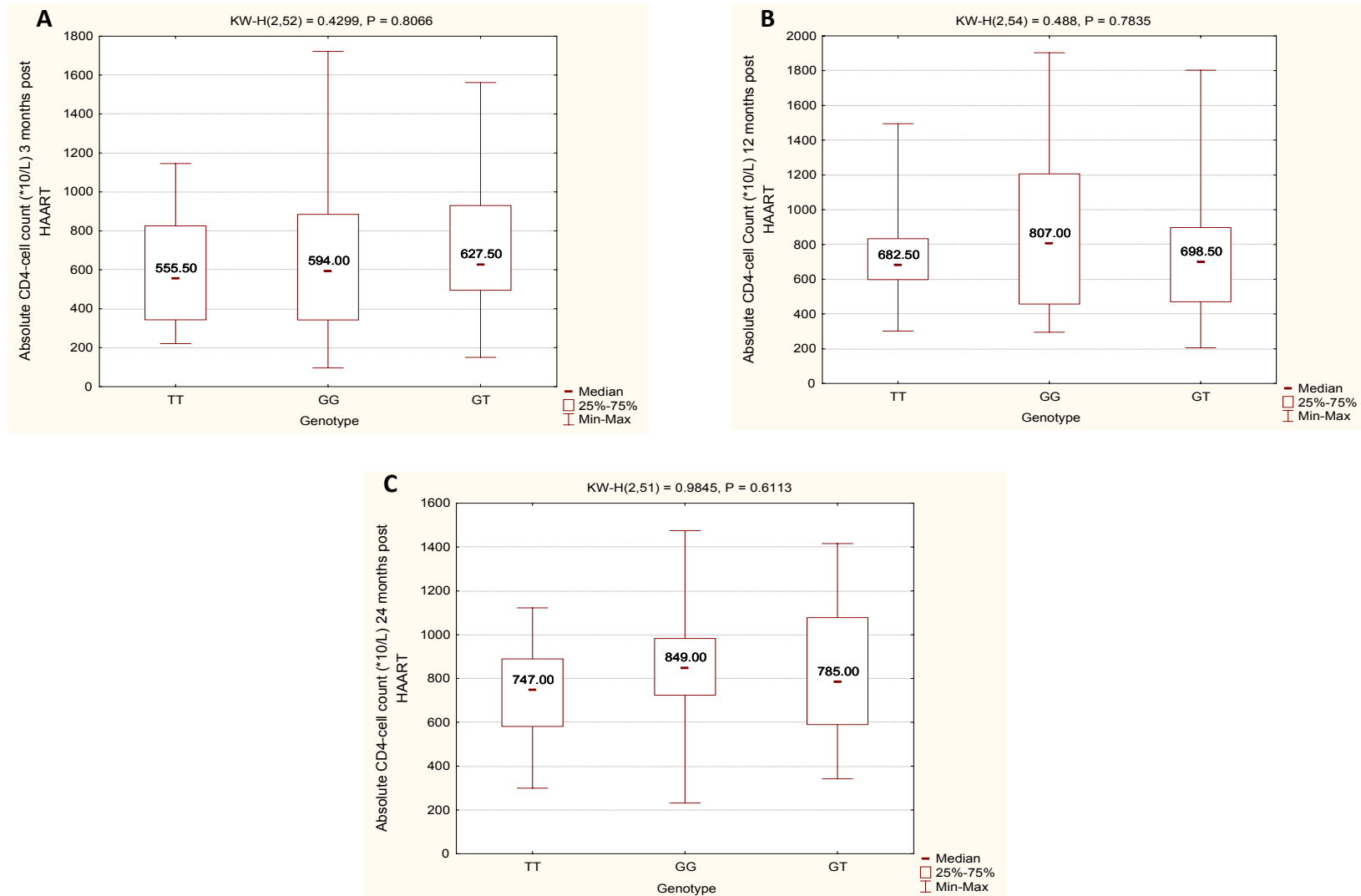


Figure 7.18: Box and Whisker plots of median CD4-cell count values of the three genotype groups (G/G; G/T; T/T) at 3, 12 and 24 months post-HAART initiation.

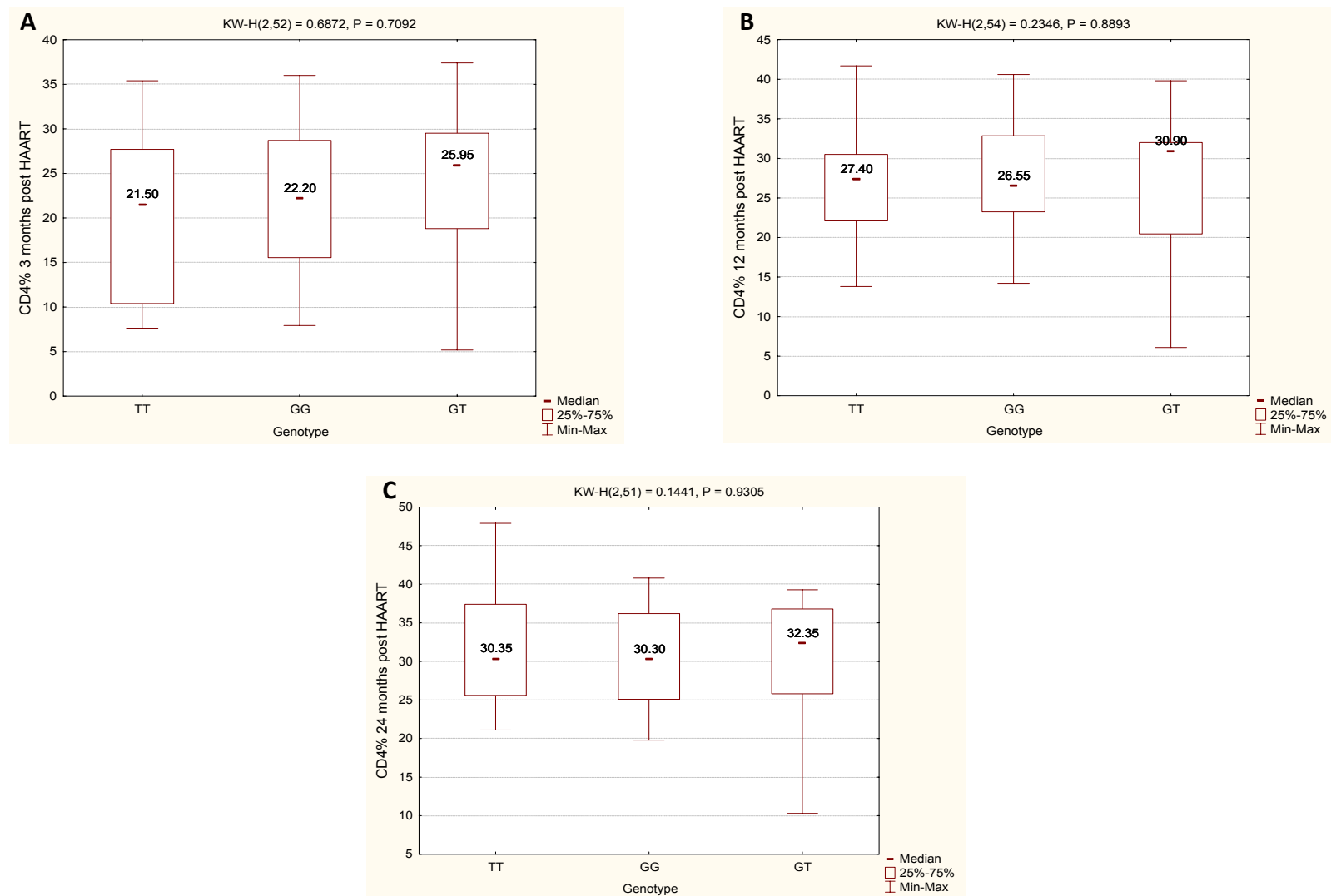


Figure 7.19: Box and Whisker plots of median CD4% values of the three genotype groups (G/G; G/T; T/T) at 3, 12 and 24 months post-HAART initiation.

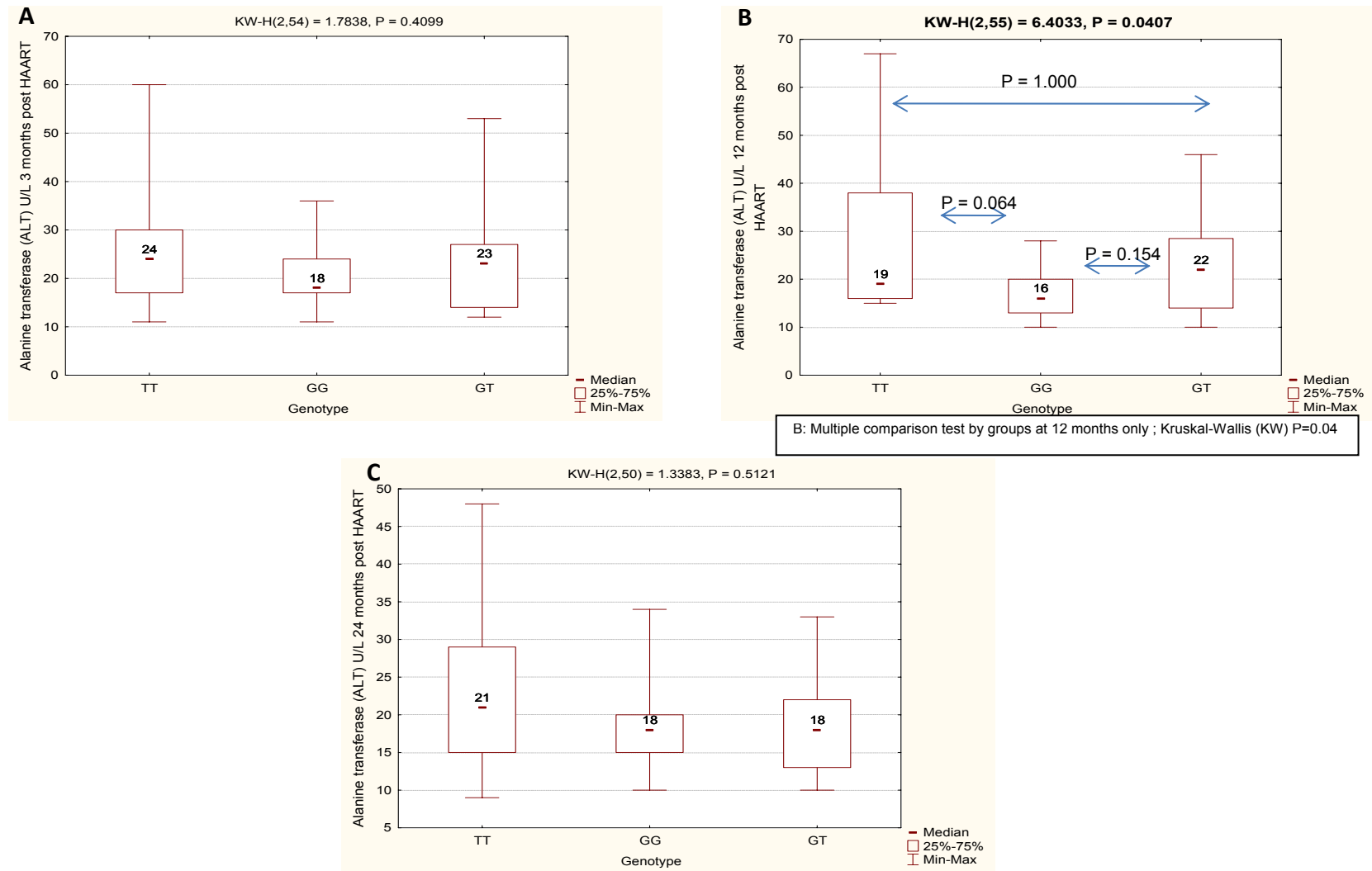


Figure 7.20: Box and Whisker plots of median ALT values of the three genotype groups (G/G; G/T; T/T) at 3, 12 and 24 months post-HAART initiation

7.7 Correlation and Association Analysis between Plasma Efavirenz Concentrations and Dependent Variables

The relationship between plasma EFV concentrations and certain dependent variables was investigated by correlation, univariate and multivariate methods.

Although two plasma levels (PK1 and PK2) were taken at each study visit at mid-dose intervals, the decision was made to use only the second plasma concentration (log PK2) values for each of the respective study visits (1, 3, 6, 12, 18 and 24 months) in the correlation and associations analysis. **The second plasma level (PK2) were closest to the actual C_{min} (trough) level** and would thus be more relevant in the correlation and association analysis compared to the first level (PK1) taken 1-2 hours earlier. The respective **PK1 and PK2** from each study visit **were not replicates** from each other and it would thus be **statistically inappropriate to have used the mean value** of PK1 and PK2.

7.7.1 Correlation Analysis

Spearman's correlation analysis (non-parametric) were performed on the EFV plasma concentrations and related variables, refer to Manuscript A (Chapter 4). Correlation analysis of the log EFV plasma concentration data (parametric) were performed with Pearson's correlation. Table 7.16 reflects the respective r-values for each of the variables correlated with the log EFV plasma concentrations. Marked (asterisk*) correlations that were significant ($p < 0.05$) are shown in Table 7.16.

Genotype consistently correlated positively with the log EFV concentrations at each of the respective study visits, indicating that the T/T and G/T (denoted as = 1) genotyped individuals had higher log EFV plasma concentrations than the G/G (denoted as = 0) genotype individuals. This was previously confirmed with the repeated measures results discussed in this chapter (refer to § 7.5.3). Other positive correlations were with age, weight and height at 24 months only, log VL at 3, 6 and 12 months and ALT at 6 and 18 months.

Table 7.16: Pearson's correlation values

Log EFV conc. vs. variables	Log EFV (Mnth1)	Log EFV (Mnth3)	Log EFV (Mnth6)	Log EFV (Mnth12)	Log EFV (Mnth18)	Log EFV (Mnth24)
Genotype	0.40*	0.47*	0.51*	0.40*	0.56*	0.48*
Age (baseline)	0.18	0.08	0.25	0.17	0.02	0.32*
Gender	-0.05	-0.06	-0.13	0.00	-0.03	-0.05
Dose	0.16	0.01	0.23	0.11	0.05	0.26
Weight	0.12	-0.05	0.20	0.08	-0.02	0.30*
Height	0.16	0.04	0.21	0.11	-0.03	0.32*
Log VL	ND	-0.60*	-0.33*	-0.40*	-0.00	-0.09
ALT	ND	-0.00	0.35*	0.24	0.49*	0.27
Adherence	0.36*	0.21	0.17	-0.20	-0.05	0.16
CD4%	ND	0.16	-0.02	0.12	0.07	0.28

EFV, efavirenz

conc., concentration

ND, not tested at 1 month post-HAART

7.7.2 Association Analysis

Univariate and multivariate regression analyses were performed to investigate the contributions of various independent variables on the log transformed EFV plasma concentrations at 1, 3, 6, 12, 18 and 24 months post-HAART initiation. The respective adjusted R^2 and P-values at each of the month's post-HAART are shown in Tables 7.17-7.18.

Only associations of independent variables with P-values < 0.05 (bold and italic in Table 7.17) were used and built into the subsequent multivariate regression model (Table 7.18). Adjustments for genotype; log VL; ALT and adherence to EFV were applied to the multiple regression model with the respective adjusted R^2 and P-values at 1, 3, 6, 12, 18 and 24 months post-HAART shown in Table 7.18. The influence of weight and height was introduced at 24 months post-HAART as the Pearson's correlation values were only significant at 24 months for these variables.

The respective mean ($\pm$ SD) contribution of the influence of genotype on the log EFV plasma concentrations in the multivariate analysis model was 42.83% (4.71%) and varied from 38% to 49% over the six study visits (1-24 months post-HAART) when the model was adjusted for genotype; log VL; ALT and adherence to EFV (results not shown in Table 7.18). The coefficients of determination (adjusted R^2) for this multivariate model were 0.25, 0.55, 0.27, 0.31, 0.43 and 0.24 (Table 7.18) for the respective month's post-HAART. It is clear that genotype was consistently associated with a large influence on the log EFV concentrations at each of the respective study visits within this model adjusted for genotype; log VL; ALT and adherence to EFV. Genotype was also the most important covariate in the NONMEM analysis (refer to § 7.8.1.2)

Table 7.17: Univariate regression analysis (*b**-coefficient; *P*-value) of the second EFV PK level

Independent variables	Log EFV (Mnth1) <i>b</i> * <i>P</i>		Log EFV (Mnth3) <i>b</i> * <i>P</i>		Log EFV (Mnth6) <i>b</i> * <i>P</i>		Log EFV (Mnth12) <i>b</i> * <i>P</i>		Log EFV (Mnth18) <i>b</i> * <i>P</i>		Log EFV (Mnth24) <i>b</i> * <i>P</i>	
Genotype	0.40	0.002	0.47	0.0002	0.51	< 0.0001	0.40	0.004	0.55	0.0001	0.48	0.0004
Gender	-0.05	0.73	-0.05	0.68	-0.13	0.34	0.001	0.99	-0.03	0.82	-0.05	0.72
Age (baseline)	0.18	0.17	0.08	0.56	0.25	0.07	0.17	0.24	0.02	0.89	0.32	0.02
Time post- dose	-0.06	0.65	-0.10	0.44	-0.17	0.23	0.16	0.26	-0.09	0.55	-0.10	0.50
Dose	0.16	0.23	0.01	0.92	0.23	0.10	0.11	0.45	0.05	0.72	0.26	0.07
Weight	0.12	0.36	-0.05	0.73	0.20	0.15	0.08	0.58	-0.02	0.91	0.30	0.03
Height	0.16	0.25	0.04	0.79	0.21	0.12	0.11	0.44	-0.03	0.82	0.32	0.02
Side effects (1 month)	-0.01	0.94										
CD4%	ND		0.16	0.26	-0.02	0.91	0.12	0.39	0.07	0.62	0.28	0.06
Log VL	ND		-0.60	< 0.0001	-0.33	0.01	-0.40	0.003	-0.004	0.98	-0.10	0.51
ALT	ND		-0.0006	0.99	0.35	0.01	0.24	0.10	0.49	0.001	0.27	0.06
Adherence	0.37	0.02	0.21	0.18	0.17	0.35	NI		-0.05	0.80	0.16	0.27
Cholesterol											0.12	0.42
Triglyceride											0.003	0.98

*b** - beta coefficient

ND – not tested at 1 month post-HAART

NI – not included in univariate analysis, missing adherence data due to not returning medicines for medicine checks

Table 7.18: Multivariate regression model analysis (b*-coefficient; P-value) of the second EFV PK level

Independent variables	Log EFV (Mnth1)		Log EFV (Mnth3)		Log EFV (Mnth6)		Log EFV (Mnth12)		Log EFV (Mnth18)		Log EFV (Mnth24)	
	b*	P	b*	P	b*	P	b*	P	b*	P	b*	P
Genotype	0.39	0.005	0.46	0.0001	0.39	0.02	0.39	0.003	0.49	0.001	0.46	0.0008
Log VL		ND	-0.61	<0.0001	-0.20	0.21	-0.42	0.0007	0.02	0.87	-0.02	0.89
ALT		ND	0.07	0.50	0.21	0.20	0.10	0.43	0.44	0.004	0.21	0.11
Adherence	0.35	0.01	0.05	0.62	0.12	0.43		NI	-0.17	0.24	0.17	0.22
Genotype											0.42	0.002
Log VL											-0.06	0.67
ALT											0.21	0.10
Adherence											0.12	0.38
Weight											0.02	0.97
Height											0.23	0.59

b* - beta coefficient

ND – not tested at 1 month post-HAART

model, missing adherence data due to not returning medicines for medicine checks

NI – not included in multivariate analysis

7.8 Population Pharmacokinetics (PK) Analysis of Efavirenz Performed with NONMEM

The influence of interoccasion variability (IOV) and covariates CYP2B6 516G>T polymorphism, age and weight on the population pharmacokinetics of efavirenz in HIV-1-infected South African children is presented in a pro-forma article (Manuscript C, Chapter 6) intended for submission and peer review.

The results and discussions presented herewith are ***explanatory*** to the steps and methods used in building up to the final PK model presented in Manuscript C.

7.8.1 Additional NONMEM Analysis

The population PK model strategy and analysis, parameter estimations (CL/F and V/F), error models and covariate selections were reported and discussed in Manuscript C. A one-compartment model with first-order conditional estimate (FOCE) method was implemented. The ADVAN 2 and TRANS 2 subroutines in NONMEM (non-linear mixed effects modelling), version VII level 1.2, with Intel® Visual Fortran Compiler version 11.0 were used to perform these analyses. The prediction-corrected visual predictive checks (pcVPC) were generated with the Perl speaks NONMEM (PsN) tool “vpc” version 3.2.7 and the graphic presentations of the results were performed with Xpose package version 4.3.0 using R, version 2.12.0.

7.8.1.1 Identification of outliers

Population pharmacokinetic analysis allows for inter- and intra-individual variation, however, some individuals “skew” the data unrealistically and may contribute to inaccuracies. The stepwise generalised additive model (GAM) by Xpose allows the user to identify these extreme outliers. Figure 7.21 is an example of a GAM output analysis to identify outliers. An individual (subject 51) was identified and excluded from the original data set used in NONMEM. This individual was the same individual that persistently had EFV plasma levels well above 4 µg/ml as was reported in Manuscript A and B (Chapters 4 & 5). An additional three outliers (subjects 40, 42 and 56) were also identified in the final model (Figure 7.22) with GAM. However, when these three subjects were excluded from the final model analysis, the parameter estimations did not change and it was decided not to exclude these three

subjects. Thus only subject number 51 was excluded from the final NONMEM data set.

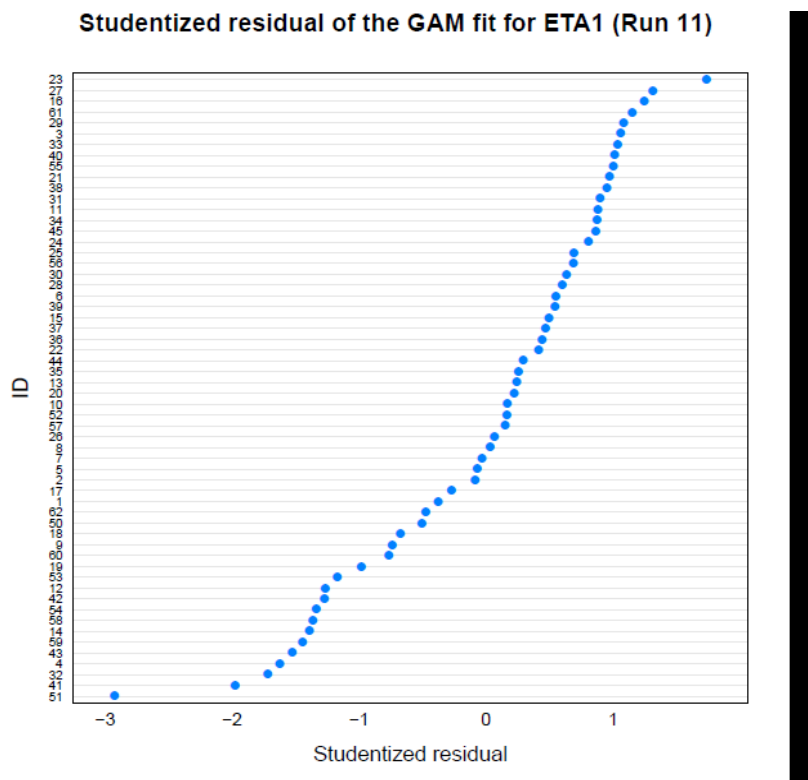


Figure 7.21: Studentised residual of the generalised additive model (GAM) to identify subject outlier (nr. 51).

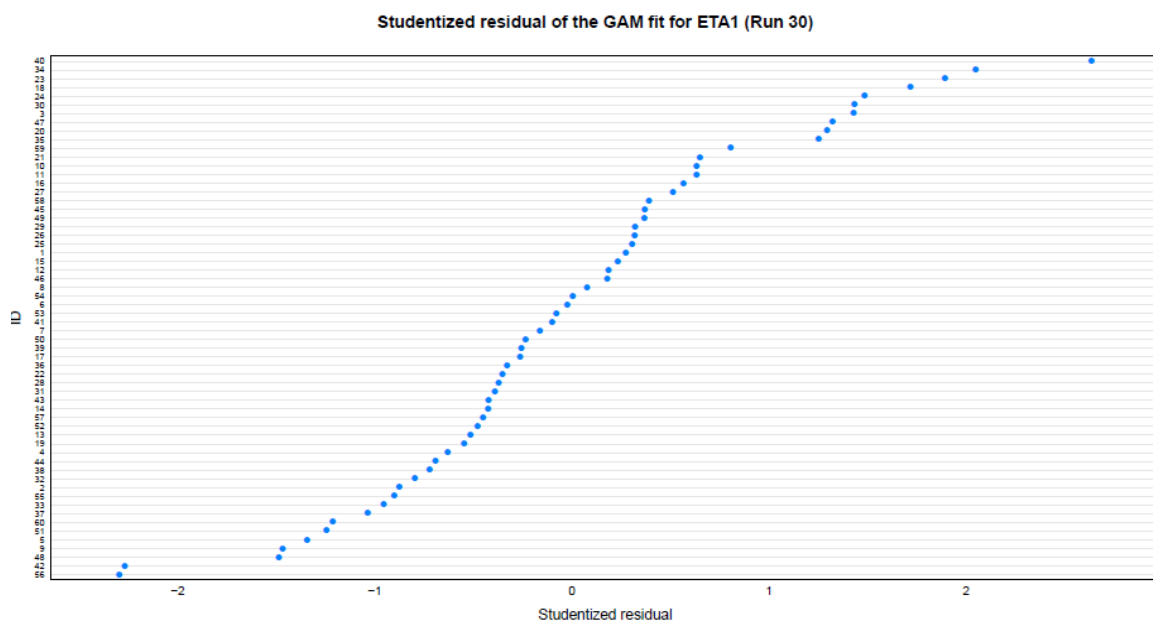


Figure 7.22: Studentised residual of the generalised additive model (GAM) to identify subject outliers (nr. 40, 42 and 56).

7.8.1.2 Covariate model building results

The importance of modelling is to investigate the influence of various covariates on the PK parameters (CL/F and V/F) that are being investigated. The Akaike's Information Criterion (AIC) is an initial estimate to investigate which important covariates influenced the PK parameters. In Table 7.19 the AIC is provided for CL (ETA1) and V (ETA2) that were performed on the basic model (Run1).

Table 7.19: Akaike's Information Criterion (AIC) to investigate which covariates influence the PK (CL and V) parameters of efavirenz

Start:		
ETA1 (CL) ~ 1; AIC= 132.8131 (<i>no covariates added to CL estimates</i>)		
Trial:	ETA1 ~ SEX + 1 + 1 + 1 + 1;	AIC= 134.707
Trial:	ETA1 ~ 1 + GENO + 1 + 1 + 1;	AIC= 108.2329
Trial:	ETA1 ~ 1 + 1 + WT + 1 + 1;	AIC= 133.933
Trial:	ETA1 ~ 1 + 1 + 1 + AGE + 1;	AIC= 134.8131
Trial:	ETA1 ~ 1 + 1 + 1 + 1 + HT;	AIC= 134.4861
Step : ETA1 ~ GENO ; AIC= 108.2329		
Trial:	ETA1 ~ SEX + GENO + 1 + 1 + 1;	AIC= 110.093
Trial:	ETA1 ~ 1 + GENO + WT + 1 + 1;	AIC= 109.0576
Trial:	ETA1 ~ 1 + GENO + 1 + AGE + 1;	AIC= 110.0041
Trial:	ETA1 ~ 1 + GENO + 1 + 1 + HT;	AIC= 109.5718
Start:		
ETA2 (V)~ 1; AIC= -115.8718 (<i>no covariates added to V estimates</i>)		
Trial:	ETA2 ~ SEX + 1 + 1 + 1 + 1;	AIC= -113.8958
Trial:	ETA2 ~ 1 + GENO + 1 + 1 + 1;	AIC= -115.0077
Trial:	ETA2 ~ 1 + 1 + WT + 1 + 1;	AIC= -122.458
Trial:	ETA2 ~ 1 + 1 + 1 + AGE + 1;	AIC= -119.3974
Trial:	ETA2 ~ 1 + 1 + 1 + 1 + HT;	AIC= -122.0513
Step : ETA2 ~ WT ; AIC= -122.458		
Trial:	ETA2 ~ SEX + 1 + WT + 1 + 1;	AIC= -120.6114
Trial:	ETA2 ~ 1 + GENO + WT + 1 + 1;	AIC= -121.6657
Trial:	ETA2 ~ 1 + 1 + ns(WT, df = 2) + 1 + 1;	AIC= -121.7787
Trial:	ETA2 ~ 1 + 1 + WT + AGE + 1;	AIC= -121.1551
Trial:	ETA2 ~ 1 + 1 + WT + 1 + HT;	AIC= -120.4636

CL, clearance. V, volume of distribution. GENO, CYP2B6 516 G/G, G/T and T/T genotypes. WT, weight. HT, height

From these results (Table 7.19) it was evident that genotype (CYP2B6 516 G/G, G/T and T/T) was an important covariate that affected the CL/F of EFV within this study population. The same trend was observed with the correlation and univariate analysis (refer to § 7.7.1 and 7.7.2). However, all of these covariates were still additionally tested in various NONMEM models (refer Table 7.20 and Table 7.21).

7.8.1.3 Model validation and results of various NONMEM runs (Basic to Final Model)

The adequacies of the various models were evaluated and discussed in Manuscript C (Chapter 6) extensively and will not be repeated.

The extent of shrinkage, associated with poor individual parameter estimates, was calculated for all parameters using the equation:

$$\text{Shrinkage} = 1 - \frac{SD_{\eta_{CL}}}{\omega_{CL}}$$

Where $SD_{\eta_{CL}}$ was the standard deviation of the individual estimate of η for CL and ω_{CL} was the standard deviation of the estimated population variance. If the two values were similar, the influence of shrinkage was low and desirable (Savic & Karlsson., 2009). The shrinkage percentage (%) is obtained from Xpose and the different shrinkage % is shown in Table 7.22. Values higher than 30% are an indication of a poor estimation.

The modelling was performed in two stages. A basic model (Run 1) was constructed and the influences of the different covariates were tested in an added step-wise manner (Table 7.20). The OFV value decreased dramatically when IOV (Run 41) was added to the model. The model with IOV included was then used to construct the final model (Run 36). The different covariates were then omitted from the final model to evaluate the importance of each covariate's contribution to the final model (Table 7.21).

Table 7.20: Summary of the analysis of covariates influencing the pharmacokinetic parameters of efavirenz in the basic model

Covariates	OFV	DOFV	P-value	CV _{CL/F} (%)	CV _{V/F} (%)	σ (%)
*Basic model (Run 1)	700			72	44	59
Does WEIGHT influence CL/F?	744	+ 44	>0.05	76	<1	63
Does WEIGHT influence V/F?	704	+4	>0.05	73	88	60
Does AGE influence CL/F?	795	+95	>0.05	85	<1	63
Does AGE influence V/F?	705	+5	>0.05	73	75	60
Does GENDER influence CL/F? (Male=1)	700	0		72	43	58
Does GENDER influence V/F? (Male=1)	700	0		72	43	58
Does BSA influence CL/F?	768	+68	>0.05	77	76	65
Does BSA influence V/F?	704	+4	>0.05	74	73	59
Does GENOTYPE (3) influence CL/F? (Run 6)	660	-75	<0.01	48	55	59
Does GENOTYPE (2) influence CL/F? (Run 7)	673	-27	<0.01	56	68	59
Does IOV influence CL/F? (Run 41))	216	-484	<0.01	63	41	FIX

* Basic model results: CL= 5.01 L/h, V=183 L, Ka=0.287 h⁻¹, F=1 and OFV=700

OFV; objective function value DOFV; difference in objective functions between models σ ; residual error

BSA; body surface area genotype (3); CYP2B6 516 G/G; T/T; G/T

genotype (2); CYP2B6 516 G/G and (T/T+G/T) IOV; interoccasion variability

The following visual checks were applied: the changes in DV (dependent variable) vs. PRED (population prediction), DV vs. IPRED (individual prediction), IWRES (individual weighted residual) vs. IPRED and WRES (weighted residual) vs. TIME were used to investigate the visual improvement in the fitting of the various models. Basic goodness-of-fit plots are presented in Figures 7.23 for some of the runs (Run 1, 41 and 36) that built up to the final model (Run 36).

In § 7.5.3 it was reported that no statistically significant difference existed between the EFV plasma concentrations of the T/T and G/T groups. For that reason, the

genotypes T/T and G/T were grouped together and denoted a value of 1 and the genotype G/G was denoted as 0 in Run 7. In Run 6 the CYP2B6 516 T/T, G/T and G/G genotypes were modelled separately. The reduction in OFV was 75 units when.

3 groups (Run 6) were modelled in comparison with 27 units when genotypes T/T and G/T were grouped (Run 7) together.

A summary of the most significant NONMEM run outputs (Runs 1, 41 and 36) generated in Xpose are presented in Addendum D2.

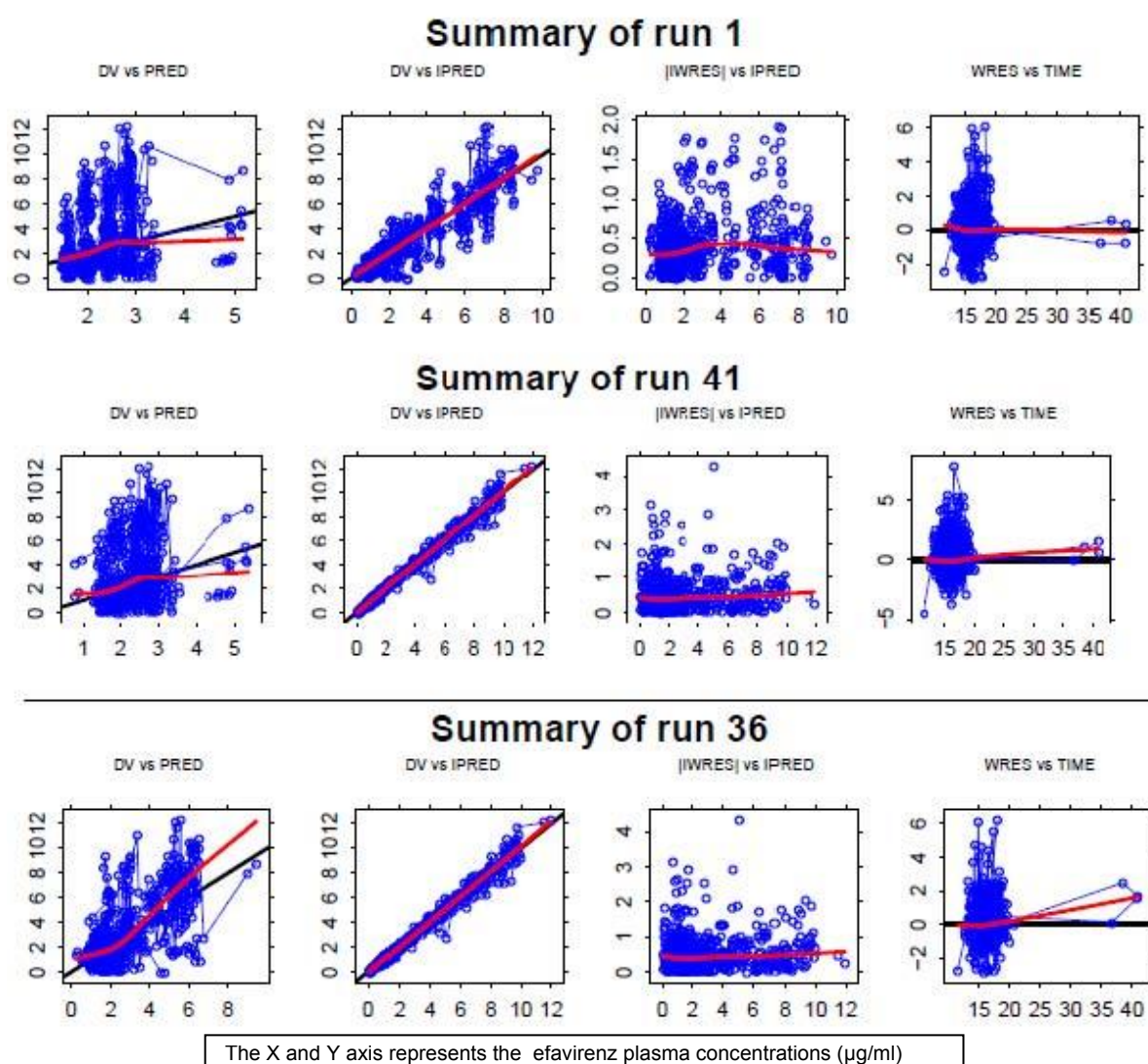


Figure 7.23: Goodness-of-fit plots of various relevant models tested.

Table 7.21: Summary of the analysis of patient covariates influencing the pharmacokinetic parameters of efavirenz in the final model

Covariates	OFV	DOFV	P-value	CV _{CL/F} (%)	CV _{V/F} (%)	CV _{IOV} (%)
*Final model (Run 36)	170			41	41	37
Does AGE influence CL/F and V/F in final model? (Run 37)	187	17	<0.01	44	41	37
Does WEIGHT influence CL/F and V/F in final model? (Run 39)	177	7	<0.01	43	43	37
Does GENOTYPE influence final model? (Run 40)	177	7	<0.01	43	43	37
Does IOV influence final model? (Run 41)	216	39	<0.01	63	37	37

OFV; objective function value DOFV; difference in objective functions between models CV: coefficient of variance
 Genotype; CYP2B6 516 G/G; T/T; G/T IOV; interoccasion variability σ ; residual error was fixed

Although weight and age, added on their own did not improve the fit (Tables 7.20 and 7.21), an improvement in fit was observed when weight and age was combined in the final model. These results indicate the importance of both age and weight to predict accurate CL values of EFV in this paediatric population.

The efavirenz PK parameter estimates for the IOV model (Run 41) and the final model (Run 36) are presented in Table 7.22 including the bootstrap and shrinkage evaluations that were performed to validate the models.

Table 7.22: *Efavirenz pharmacokinetic parameter estimates for the basic, IOV and final model including the bootstrap evaluation*

	IOV Model (Run 41)	Final Model (Run 36)	Bootstrap evaluation on the final model
OFV	216	170	
Parameters	Mean (RSE%)	Mean (RSE%)	Mean (RSE%)
CL/F(l/h)	4.86 (7.5)	2.46 (10) T/T	2.45 (13) T/T
		4.60 (13) G/T	4.59 (14) G/T
		7.33 (8.7) G/G	7.39 (7) G/G
V/F (l)	94.3 (14)	89.52 (11)	92.89 (14)
Ka (h ⁻¹)	0.287 FIX	0.287 FIX	0.287 FIX
Add error	0.108 (8.3)	0.107 (7)	0.106 (11)
Prop error %	9.2 (13)	9.3 (15)	8.9 (35)
Exponent θ_8		-0.6 (23)	-0.57 (26)
ω CL/F %	63 (18)	41 (28)	40 (30)
ω V/F %	37 (63)	41 (51)	41 (53)
IOV _{CL} %	37 (20)	37 (19)	37 (21)
σ %	FIX	FIX	FIX
Shrinkage (%)	CL=58.6; V=47.2	CL_{TT}=10.9; CL_{GT}=42.7; CL_{GG}=26.4; V=29.7	

IOV model with interoccasion variability .

Final model with IOV, age, weight and CYP2B6 516 G/G, G/T, T/T.

RSE; relative standard error Exponent θ_8 ; Theta 8 σ ; residual variability ω ; inter-variability

Bootstrapping on the final model (Run 36) indicated that the model was stable and provided an additional internal validation step. The means and coefficient of variation of the parameter estimates from the bootstrap method were nearly identical to the final model.

The final equations for CL/F and V/F of efavirenz from this study population were as follows:

$$\text{CL/F (l/h)} = \theta_x \cdot (\text{WT}/20)^{0.75} \cdot (\text{AGE}/7/12)^{\theta_8},$$

$$\text{V/F (l)} = \theta_4 \cdot (\text{WT}/20) \cdot (\text{Age}/7/12)^{\theta_8},$$

with $\theta_x = \theta_1(\text{T/T}); \theta_2(\text{G/T}); \theta_3(\text{G/G})$, $\theta_8 = -0.6$; weight (WT) is the body weight (kg) and age in months.

The efavirenz CL/F values for the CYP2B6 516 T/T, G/T and G/G genotypes respectively were 2.46, 4.60 and 7.33 l/h and the V/F was 89.52 l, for this specific study population with an average weight of 20 kg and age of 7 years. It was evident that three distinct CL values of EFV could be identified, within this black paediatric South African study population, based on the CYP2B6 516 T/T, G/T and G/G genotypes. Further in depth discussions and comparisons of these results are presented in Manuscript C (Chapter 6).

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Chapter 8

Overall Conclusions & Recommendations

In this clinical study (PK/PD.EFV.07), 60 children who attended the outpatient clinic at Harriet Shezi Children's Clinic, Chris Hani Baragwanath Hospital, Soweto, South Africa, were prospectively enrolled. This cohort included children (3-14 years at baseline) of both genders, with no prior exposure to antiretroviral therapy, eligible for ART, and all from resource limited households.

The most important contribution and impact of this investigation to the scientific knowledge of EFV, as part of HAART in HIV-1-infected children was the longitudinal nature of this study. It was performed in black ART naïve South African children who were followed up for a period of 24 months with sequentially measured EFV plasma concentrations at 1, 3, 6, 12, 18 and 24 months post-HAART initiation.

8.1 Study Objectives Achieved

All of the set primary and secondary study objectives (Chapter 1, § 1.2.1 and 1.2.2) were successfully achieved, and included:

- i) the development, optimisation and validation of a LC-MS/MS method to determine plasma EFV levels accurately,
- ii) the collection and analysis of plasma EFV samples from this paediatric study population at 1, 3, 6, 12, 18 and 24 months post-HAART,
- iii) the determination and prevalence of the CYP2B6 516G>T polymorphism and its effect on EFV plasma levels within this paediatric study population,
- iv) the determination of the population pharmacokinetic clearance (CL/F) value and covariates influencing the clearance of EFV in HIV-1-infected children

with NONMEM using routine clinic-based, sparsely collected blood samples, and

- v) the investigation of specific pharmacodynamic effects and therapeutic outcomes (CD4%, CD4 count; viral load; side effects and toxicity) of this EFV-based regimen within this paediatric population over the 24 months post-HAART initiation.

8.2 Overall Discussion and Conclusions

Very limited pharmacokinetic information on EFV plasma concentrations in sub-Saharan HIV-1-infected children is available. To our knowledge, this is the first study in black South African HIV-infected **children** that has measured **serial EFV plasma** concentrations, investigated the influence of the **CYP2B6 516G>T** polymorphism on EFV plasma concentrations and determined the **population clearance** (CL/F) value of EFV in a longitudinal study over a period of 24 months post-HAART initiation. Ren and co-workers (2007) reported EFV plasma concentrations in a cross sectional study in a small number (n=14) of South African children. Studies reporting on the effect of CYP2B6 516G>T polymorphism on EFV concentrations in South African adults were published by Cohen and co-workers (2009) and Gounden and co-workers (2010).

By successfully developing and validating a LC-MS/MS method, EFV were determined in plasma accurately, selectively and with good repeatability within a relative short time period. In this study, data on EFV plasma concentrations taken from South African HIV-infected children at 1, 3, 6, 12, 18 and 24 months after the commencement of HAART are presented. A total number of 649 EFV plasma concentrations were determined, taken at mid-dose interval, during 1-24 months post-HAART initiation. Sub-therapeutic concentrations (<1 µg/ml) accounted for 18% (116/649) of the samples analysed. The concentrations within the therapeutic range (1-4 µg/ml) represented 52.5% (341/649), and above the therapeutic range (>4 µg/ml) 29.5% (192/649) of the samples. Evidently a significant number of the samples (47.5 %) were outside the accepted therapeutic range (1-4 µg/ml) and this is a great cause for concern. Sub-therapeutic levels pose the risk for the

development of viral resistance against EFV and levels >4 $\mu\text{g/ml}$ may result in toxicity.

Various investigators have expressed concerns that underdosing in children occurs with the current guidelines based on once-off cross sectional studies (von Hentig *et al.*, 2006; Ren *et al.*, 2007) with a small number of children (ter Heine *et al.*, 2008). They suggested an increase in the EFV dose in children to be further investigated in larger studies to define optimal dosing strategies in children, and to take into consideration the influence of CYP2B6 genotyping and differences in formulation bioavailability for children.

Nyakutira and co-workers (2008) suggested a priori dose reduction in Zimbabwean adults from 600 mg to 400 mg daily for genetically defined poor metabolisers as a frequency of 49% of the CYP2B6*6 allele was observed within their study population. Ramachandran and co-workers (2009) suggested that further clinical studies are required to investigate lower EFV doses in the south Indian population as they also present with high frequencies of the CYP2B6 516 T-allele. It was shown by Gatanaga and co-workers (2007) that genotype-based EFV dose reductions in Japanese CYP2B6 *6/6* and CYP2B6 *6/*26 carriers were feasible and that it can reduce EFV-associated side effects.

In our study we did not change the recommended first line EFV dosage as set by the National Department of Health (NDoH), however possible reasons for efavirenz plasma levels outside the accepted therapeutic range were investigated. This included genetic variation in drug metabolism (CYP2B6 516G>T polymorphism), incorrect dosing, drug-drug interactions and non-adherence (non-compliance). Recording of any co-administered drugs during the duration of this study (drug-drug interactions) and dosage checks were performed after the medication was issued at every study visit and for the in between pharmacy visits.

The influence and prevalence of the CYP2B6 516G>T polymorphism on EFV plasma concentrations within this relatively large (n=60) cohort of black South African HIV-infected paediatric population were investigated. Genotype results were available for all 60 study participants: 23% (14/60) were CYP2B6 516 T/T homozygotes, 42% (25/60) were CYP2B6 516 G/G homozygotes and 35% (21/60) were CYP2B6

516G/T heterozygotes. The relatively high CYP2B6 516 T-allelic variant (41%) frequency from our black paediatric South African population corresponds well with other published results. Studies performed in African-American and African descent populations, showed that this variant varied from 32% to as high as 49% (Cohen *et al.*, 2009; Haas *et al.*, 2004; Klein *et al.*, 2005; Kwara *et al.*, 2009; Wyen *et al.*, 2008; Nyakutira *et al.*, 2008). The contribution of the presence of the 516 T-allelic variant (41% frequency within our population) explains the high number (29.5%) of mid-dose interval plasma samples that were >4 $\mu\text{g/ml}$ within individuals presenting with this single nucleotide polymorphism (SNP) mutation on the CYP2B6 gene.

Repeated measures of ANOVA showed that the CYP2B6 516 G/G, G/T and T/T genotypes was consistently predictive of the log EFV concentrations at all times ($P = 0.0001$). Neither time (months post-HAART initiation (R1)), nor the interaction (genotype*R1), $F(10,190) = 0.77$, $P=0.65$) influenced the log EFV plasma concentrations at 1-24 months post-HAART. Therefore all the EFV plasma concentrations over the 24 months post-HAART were pooled, the total median (IQR) EFV plasma concentrations were, 6.36 (3.47 – 7.28) for T/T, 2.55 (1.62 – 3.59) for G/T and 1.41 (1.02 – 1.74) $\mu\text{g/ml}$ for G/G groups respectively (Kruskal-Wallis, $P<0.00001$). Additional multiple comparisons by groups revealed that the EFV plasma concentrations between the T/T and G/G (6.36 vs 1.41 $\mu\text{g/ml}$, $P=0.000002$) and between G/T and G/G (2.55 vs 1.41 $\mu\text{g/ml}$, $P=0.009$) were statistically significantly different. However, the difference between the EFV plasma concentrations of the T/T and G/T groups were not statistically significantly different (6.36 vs. 2.55 $\mu\text{g/ml}$, $P=0.074$), possibly due to the outlier values ($n=14$) in the G/T group, mostly from one patient with persistently high levels ($n=10$) and from 2 other G/T genotyped individuals ($n=4$). Even though we observed such an alarming number (29.5%) of the plasma samples >4 $\mu\text{g/ml}$, which can be attributed to the high frequency (41%) of the 516 T-allelic variant, this EFV-based regimen was very well tolerated by the majority of this study's participants over the 24 months post-HAART initiation. Various studies could not establish an association with EFV plasma concentration and CYP2B6 516G>T polymorphism and toxicity (Ramachandran *et al.*, 2009; Rotger *et al.*, 2005; Saitoh *et al.*, 2007). Several studies did, however, report an association of CYP2B6 516 T/T with CNS toxicity (Haas *et al.*, 2005; Gatanaga *et al.*, 2007) whereas some studies reported an increase in the prevalence

of side effects, especially CNS related, which correlated with EFV plasma levels greater than 4 µg/ml (Cohen *et al.*, 2009; Marzolini *et al.*, 2001; Puthanakit *et al.*, 2009; Gounden *et al.*, 2010). Results from our observational study could thus not corroborate that an association existed between high EFV plasma levels and reported side effects (1 month post-HAART). An association between the CYP2B6 516G>T polymorphism and reported side effects within the T/T genotype group could not be established, although the pooled median (IQR) EFV plasma levels were 6.36 (3.47 – 7.28) µg/ml during the 24 months post-HAART observation. Most of the reported side effects were mild and subsided spontaneously. Possible reasons for the low frequency of side effects reported during the 24 month follow-up period, could be the development of tolerance despite persistently high plasma EFV levels, as was also suggested by Haas and co-workers (2004) in a study where adults were followed up for 6 months. The difficulty in quantifying and assessing neuropsychological side effects in such young children has also been raised by Saitoh and co-workers (2007).

Successful VL suppression was observed in 89% of our study participants at 24 months post-HAART. The efficacy of this EFV-based treatment did not affect the three genotype groups differently and they showed similar improvement in their immunological (CD4-cell count and CD4%) markers and reduction in viral load and viral suppression over the 24 months post-HAART initiation. The fact that the clinical outcomes were the same in all three of the groups, are encouraging and an indication that this HAART regimen is successful and effective. Haas and co-workers (2005) reported no association of CYP2B6 G516T polymorphism with treatment failure (VL, toxicity and all-cause treatment failure) in a large cohort of adults after 144 weeks of follow-up. Saitoh and co-workers (2007) also reported no immunologic and virological outcome differences among children with the CYP2B6 G516T polymorphism.

In the population pharmacokinetic investigation of EFV, the influence of several covariates (gender, age, weight, BSA and CYP2B6 516 G/G, G/T and T/T genotypes) and interoccasion variability (IOV) were investigated with NONMEM. The inclusion of age, weight, CYP2B6 516 G/G, G/T and T/T genotypes and IOV in the final regression model provided the following final population estimates for Cl/F

(RSE%): 2.46 (10%), 4.60 (13%) and 7.33 (8.7%) l/h for the T/T, G/T and G/G genotype groups respectively. The V/F (RSE%) was 89.52 l (11%). This is the first study to our knowledge where a large number of children (n=60) were followed up for 24 months with sequential EFV PK levels and we were able to identify three distinct CL/F values for EFV when the CYP2B6 516 G/G, G/T and T/T genotype groups were taken into consideration. The importance of introducing IOV in a PK model for a longitudinal study with sparsely collected data was highlighted again by this investigation.

In summary, we have identified three CYP2B6 516 T/T, G/T and G/G genotype groups with three distinct CL/F values for EFV within this study population, we have also confirmed that the presence of the 516 T-allelic variant was responsible for the higher EFV plasma exposures. However, the clinical success outcome (CD4%, CD4-count and VL reduction) of this EFV-based first line regimen showed no significant differences between the three genotyped groups nor did it influence the side effects that were reported. Thus, no beneficial clinical outcome or treatment disadvantage could have been established between the three genotype groups. It however remains important that clinicians must be made aware of this pharmacogenetic phenomenon, especially in resource-limited countries where genotyping is too expensive to be carried out. It could be used as an additional investigative tool in problematic and challenging cases where extremely high EFV plasma levels have been found or where excessive side effects or toxicities have been reported.

The question however still remains, should TDM or CYP2B6 genotyping be used to optimise efavirenz therapy or not (Rotger *et al.*, 2008). The answer to this question will depend on the country in question and its financial and human resource capabilities. In most African and third world countries, the HIV treatment and management burden is just too large to introduce routine TDM and or genotyping. It is already considered a luxury and not part of the current WHO guidelines to perform routine VL testing compared to the routinely monitored guidelines of the National Health Institute (WHO, 2010; NHI 2011). As shown by Nyakutira *et al.* (2008) and Gatanaga *et al.* (2007), a reduction in EFV dose, based on genotyping maybe beneficial to the patient's clinical outcome but it also holds a financial benefit with a

reduction in cost. It could be argued that baseline genotype testing could early identify individuals with the relevant polymorphism to make early dose adjustments and therefore add cost saving benefits on the long-term treatment of such individuals. Gatanaga and co-workers (2009) referred to a commercially available CYP2B6 516 genotype detection system in Japan and other commercially available kits (IILA-B*5701) that are already available to screen for abacavir hypersensitivity. It would be ideal, even for third world countries, to have such commercial kits available that are able to test or screen for a large number of possible polymorphisms. These kits would not require intensive staff training and expensive PCR equipment. Rotger and co-workers (2008) concluded quite amicably that the reconciliation of pharmacogenetics and TDM in a formal prospective clinical study will be an important step toward evaluating the cost benefit of genotype-based tailored EFV treatment.

8.3 Shortcomings

A limitation of this study was that although all possible efforts were made to monitor adherence in this outpatient clinic scenario, the researchers cannot conclude that adherence did not affect the efavirenz plasma levels in this study population.

The evaluation of reported side effects during the first month post-HAART initiation, and general side effects reported during the study was not assessed with a specific structured questionnaire. This may have resulted in the under reporting of side effects. It is extremely difficult to establish accurate reporting of side effects and to assess the different potential neuropsychological side effects of EFV in such young children as was the case in this cohort and echoed by Saitoh and co-workers (2007).

Study limitations with regard to the population pharmacokinetic aspects investigated included:

- i) No full PK profiles were available for any of the patients.
- ii) The assumption that this was a one-compartment pharmacokinetic model.
- iii) The two PK (at least 2 hours apart) samples at each study visit was taken at very similar time points around the mid-dose interval post-last evening dose

over the 24 month period. This allowed for little variation over the time range post-last dose, resulting in no PKs prior to 12 hrs (in the absorption phase) and very few between 20 and 24 hrs post-last dose intake.

However, due to the nature of this clinic-based study it was not practical to take earlier blood samples (prior to 8:00 daily) or to keep children out of school and constrained to the clinic environment for an entire day to take blood samples over a wider range of time post-last dose, especially during the absorption phase (1-4 hours post-last dose). Mid-dose interval (8-20 hours) EFV levels have been used with success, in numerous research studies over the years as discussed in the literature, and the advantage of investigating population pharmacokinetics with NONMEM is that sparsely collected data can be used (Cabrera *et al.*, 2009; Cohen *et al.*, 2009; Csajka *et al.*, 2003; Kappelhoff *et al.*, 2005; Marzolini *et al.*, 2001; Nyakutira *et al.*, 2008; Pereira *et al.*, 2008; Puthanakit *et al.*, 2009; Ramachandran *et al.*, 2009; Ren *et al.*, 2007; Stahle *et al.*, 2004; Viljoen *et al.*, 2010; Wintergerst *et al.*, 2008).

8.4 Recommendations

Reasons for children having efavirenz plasma concentrations outside the therapeutic range (1-4 µg/ml) need to be further explored. TDM may be beneficial and could play a role in elucidating these reasons as well as serving as a tool to evaluate adherence more accurately. From a clinical perspective, it could be recommended that future targeted TDM and vigilance for side effects in larger cohort studies of children who are initiated on EFV-based therapy should be performed. This will shed light and be helpful in managing children and will contribute to appropriate dose strategies of EFV, especially in children where the CYP2B6 G516T polymorphism has been identified. Correctly planned clinical studies of this nature will then also answer the question whether it is financially viable to amend EFV doses as a priori in such genotyped-based studies or not. The contribution and influence of other single nucleotide polymorphisms and haplotype organisations of the CYP2B6 gene or other genes (CYP1A2, CYP2A6, CYP3A4) associated with an influence on the EFV metabolism and plasma concentrations could also be further investigated within the South African population.

Possible future expansion on the current clinical study could include further investigations on complete sequencing of the CYP2B6 gene in certain identified study participants that showed persistent deviations from the therapeutic range of EFV.

I would like to conclude with the words as published in Hope for HIV in Africa: Observations by an Eldoret Journeyman (Hannan, 2006): "It is impossible to define the emotions when one stands in a ward of 50 people dying of HIV. So I am privileged to be here. I have also seen that when "there is no other way, there is hope." We have changed care in Africa in the management of HIV. Whether we will win, time will tell. At least we have lit candles in the darkness of African HIV care and management".

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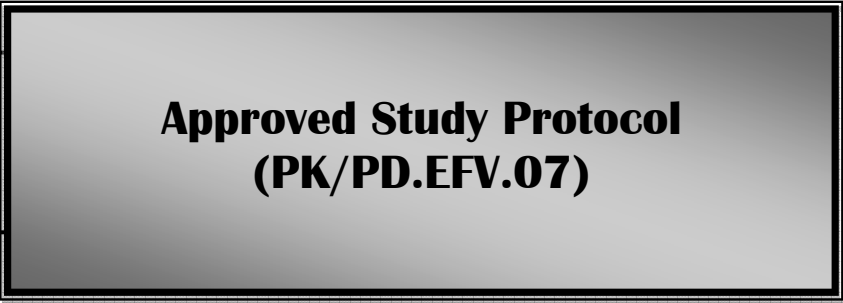
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Addendum A1



Approved Study Protocol (PK/PD.EFV.07)



PROTOCOL

Population Pharmacokinetic and Pharmacodynamic study of Efavirenz in HIV-1-infected children treated with First Line Antiretroviral Therapy in South Africa

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 Date: 21/05/07 HREC (WMA) approved: 04/12/07 (Study participant numbers), 04/06/08 (Urine/Saliva), 20/10/08 (Re-enter post TB Rx), 27/02/09 (BIA/Consent)
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2. HAART fails to control HIV-1 replication and thus complete viral suppression is not always achieved^{10,11}. The Panita 7 study indicated that incomplete viral suppression in 70% (14 of 20) of the infants was associated with genotypic resistance mutations in 30% (6 out of 20) of the infants¹².
3. The emergence of a high rate of virological failure and resistance to antiretroviral drugs is of great concern, it is a major contributor to the failure of long-term suppression of HIV replication^{10,11,12,19}.
4. The optimal time to initiate HAART remains unclear^{2,12}.
5. The optimal dosage and/or dosing regimens in children need to be established as paediatric formulations have not yet been developed or adequately tested in children and a substantial number of dosage recommendations for children are still absent in guidelines^{14,15}.
6. Poor adherence to the prescribed therapy also contributes to one of the leading causes for treatment failure^{10,19,20}. Adherence to medication schedules is mostly a function of the parent's or guardian's commitment and in the case of the protease inhibitors the palatability of liquid formulations¹.
7. Drawing blood, from especially a child, is painful and a traumatic experience¹⁰, but plasma efavirenz levels can be useful in determining treatment adherence¹⁴ and optimize dosage of therapy since various studies has either shown a correlation between low plasma efavirenz levels and virological failure or a correlation between higher plasma concentrations and virological response²⁵.

2 ANTIRETROVIRAL AGENTS

Numerous antiretroviral agents are now available and registered in South Africa (refer to Table 1). The antiretroviral drugs must always be prescribed in combination of three or more agents. Highly active antiretroviral therapy (HAART) usually consists of 2 NRTI's and 1 or 2 PI's or 1 NNRTI and has become the standard of treatment of HIV infections.

Table 1: Antiretrovirals currently available in South Africa

NRTI's	NNRTI's	PI's
Zidovudine	Efavirenz	Lopinavir
Stavudine	Nevirapine	Ritonavir
Lamivudine		Nelfinavir
Didanosine		Indinavir
Zalcitabine ^a		Saquinavir
Abacavir		Amprenavir
Tenofovir ^b		Fosamprenavir
		Atazanavir

^a May be discontinued ^b Registration restricted to adults

2.1 Nucleoside Reverse Transcriptase Inhibitors (NRTI's)

NRTI's are nucleoside analogues that act as false substrates for reverse transcriptase and are thus able to terminate the elongation of the viral DNA. The NRTI's are prodrugs that require intracellular conversion via phosphorylation to the active triphosphate component in the host viral cell. Therapeutic drug monitoring (TDM) using plasma is thus not viable with this group of drugs as the intracellular levels of the activated components need to be measured and requires very specialised laboratories and large amounts of blood which is unsuitable for use in children^{14,20,22}.

2.2 Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI's)

NNRTI's are potent inhibitors of the HIV reverse transcriptase enzyme and do not require intracellular activation. TDM is thus more viable for this class of antiretrovirals^{14,15,22,23}. Measurements of most drugs in therapeutic drug monitoring (TDM) comprise of the total drug concentration and not the unbound fraction that is free to exert an effect or to be distributed. This also applies to the analysis of efavirenz which is highly (> 95%) bound to plasma proteins, predominantly albumin^{22,23}. Certain clinical conditions and intercurrent illnesses may influence or cause a fluctuation in the α -acid glycoprotein (AAG) levels. It is thus important to take note of this and to draw blood samples in stable clinical conditions²⁴.

1 INTRODUCTION

The year 2006 was very significant; we commemorated 2 very important milestones in the fight against the greatest medical and social challenge of our time, the AIDS epidemic. It was the 25th anniversary of the epidemic's start which was first reported in June 1981. It was also the 10th anniversary since the first launch of highly active antiretroviral treatment (HAART).

The 2006 UNAIDS global report stated 'despite some notable achievements, the response to the AIDS epidemic to date has been nowhere near adequate. In just 25 years, HIV has spread relentlessly from a few widely scattered 'hot spots' to virtually every country in the world, infecting 65 million people and killing 25 million.'¹

This UNAIDS global report of May 2006 estimated 38.6 million [33.4-46 million] people world-wide were living with HIV at the end of 2005.

2.3 million Children under the age of 14 years of which an estimate of 2 million children were in Sub-Saharan Africa¹.

Sub-Saharan Africa remains the worst affected region in the world, with almost 64% (24.5 million) of all people living with HIV. The AIDS epidemic in South Africa is one of the worst in the world and shows no evidence of decline. The report estimate 5.5 million [4.9-6.1 million] people were living with HIV in South Africa during 2005. The estimate for South African children living with HIV was 240 000 [93 000-500 000] compared to the 200 000 estimate at the end of 2003. Almost one in three pregnant women attending public antenatal clinics were living with HIV in 2004 and trends show a gradual increase in HIV prevalence. Around 190 000 people were receiving HAART therapy by the end of 2005 this represents less than 20% of those in need¹.

The Department of Health completed an operational plan for the care and treatment of people with HIV infection which included the treatment and provision of antiretroviral drugs (ARV's) for HIV infected patients in SA in November 2003. The plan was to provide one service point in every health district by the end of the first year of implementation, and within five years, one service point in every local municipality to provide more accessibility to South Africans and permanent residents who require comprehensive care and treatment for HIV/AIDS². Implementation began shortly after that with many of the first sites beginning to provide antiretroviral treatment from early 2004.

National antiretroviral treatment guidelines were published by the National Department of Health in 2004 and 2005^{3,5}. Recommended first line treatment regimens for children are lamivudine stavudine as the nucleoside reverse transcriptase inhibitor (NRTI) backbone together with lopinavir/ritonavir a protease inhibitor (PI) for children under 3 years of age or efavirenz, a nonnucleoside reverse transcriptase inhibitor (NNRTI) for those from age 3 years.

Despite the fact that highly active antiretroviral therapy (HAART) has improved the life expectancy (prognosis and outcome) of HIV-1-infected children dramatically^{2,5,15} there are still many challenges in the provision of HAART to children. The following factors need to be taken into account when considering the short comings of HAART, especially in children:

1. Very large inter and inpatient variability are observed in the pharmacokinetics (PK) of ARV's in children, especially in the PIs and NNRTI's^{4,15}. Unfavorable pharmacokinetics caused by altered absorption, genetic variations in metabolism and drug-drug interactions frequently lead to antiretroviral drug concentrations below the inhibitory concentration for 50% of the viral quasispecies (IC50) and loss of viral suppression^{10-14,20}.

A uniform standard for the classification of children according to age have not been established. The following classifications are commonly accepted:

- neonate – younger than 1 month,
- infant – 1 to 24 months,
- child – 2 to 12 years,
- adolescent – 13 to 17 years.

A neonate may further be characterised as premature if the birth occurred before 38 weeks of gestation, or as term, if birth was after 38 weeks.^{11,12}

The dosage selection of ARV's in the paediatric population can not be based alone on age due to the variation in weight among children of the same age as it can lead to dosage errors. HIV-infected children tend to weigh less than non-infected children of the same age. Therefore the most accurate method for determining dosages of ARV's in children is based upon body surface area (BSA), which takes into account the patient's weight (kg) and height (cm).²⁰

Antiretroviral exposure in adults may differ from that of children. HIV-infected children have much higher (10 to 100 fold) levels of HIV in their blood, which also necessitate a difference in management compared to adults. Monitoring of plasma concentrations of the ARV's in children can be beneficial to the patient and reassure the clinician that the individual is receiving adequate exposure or to prevent toxicity.^{20,21}

The therapeutic ranges (target concentrations) of antiretroviral drugs for children have not yet been formally defined or validated sufficiently in clinical trials. Concentration-response data are however available for most of the P's and NNRTI's. It is further uncertain which pharmacokinetic parameter is the best to define the therapeutic and toxic exposures of drugs. Different pharmacokinetic parameters such as C_{min} (trough levels), C_{max} (peak levels) and area under the curve (AUC) are used for the assessment of therapeutic drug monitoring (TDM). The AUC is probably the most accurate however the analysis of the AUC is invasive, time consuming and complex. C_{min} (C_{trough}) is mostly monitored and accepted to determine efficacy as it is the easiest to collect for both the patient and investigator.^{18,19,21} A second method is to obtain randomly taken blood samples with a definite recall of the time of the last dose which is then correlated to population pharmacokinetic data for that specific drug. Inaccurate recall of the last dose by a few hours can produce incorrect data especially in drugs with a short half-life such as the P's.¹⁸

Saliva and urine are both non-invasive and painless methods of specimen collection, with a lower or diminished risk of HIV-transmission to the health worker.^{18,19,21}

Several studies have shown that saliva drug levels can be used to estimate the unbound/free drug concentration in the plasma of several drugs for example: theophylline,²² anticonvulsants (phenytoin, carbamazepine, phenobarbital), digoxin, salicylic acid, acetylsalicylic acid,²³ nevirapine.²⁴ Indirectly, saliva levels have been used to monitor patient compliance.²⁵ However, it has not yet been established if saliva drug concentrations of efavirenz would be an accurate estimate of the unbound drug concentration in the plasma.

Efavirenz undergoes primary oxidative hydroxylation to 8-hydroxyefavirenz (major metabolite and excreted in urine) and 7-hydroxyefavirenz (minor metabolite and excreted in the bile) and secondary metabolism to 8,14-dihydroxyefavirenz. The enzyme that is mainly responsible for this metabolism is cytochrome P450 (CYP2B6). CYP2B6 is the principal catalyst metabolising 17% of the 8-hydroxyefavirenz metabolite further to the 8,14-dihydroxyefavirenz.²⁶ If the quantity of this metabolite can be determined in the urine and correlated with plasma efavirenz levels, it would be of value in determining the plasma concentration and provide possible information about the CYP2B6 system.

Virological failure has been observed in significantly more patients with low efavirenz plasma concentrations (< 1 µg/ml) and reversible central nervous system side effects are associated with patients with plasma concentrations above 4 µg/ml in adults.²⁷ Haas and co-workers found that the CYP2B6 allelic variant is more common in African-Americans than in European-Americans and this was associated with greater efavirenz plasma exposure and a plausible explanation for their susceptibility to efavirenz central nervous system side effects.²⁸

A randomised controlled trial by Koo and co-workers indicated that 38% of adult patients taking P's and NNRTI's had persistently sub-therapeutic drug concentrations when measured. Adherence is undoubtedly responsible for this large inter-individual variability but other factors such as ethnicity, sex, body weight, hepatitis status and host genetic variability may also be responsible.²⁹

Cytochrome CYP3A and CYP2B6 are responsible for the hepatic metabolism of these drugs. As a result significant drug-drug interactions can and do occur.^{30,31,32,33}

Efavirenz is well absorbed from the gastrointestinal tract and it reaches peak plasma levels within 5 hours. Increased doses can reduce absorption and high-fat meals can increase the bioavailability. The clearance of efavirenz is slow and it has an elimination half-life of 40-55 hours at steady state. Less than 3 % of the parent drug is excreted renally,³⁴ however 14 - 34% of efavirenz is excreted in the urine as metabolites and the major metabolite is the 8-hydroxyefavirenz glucuronide.³⁵

2.3 Protease inhibitors (PI's)

Pi's competitively inhibit the action of the viral aspartyl protease, preventing the post-translational cleavage of the gag and pol polyproteins into smaller functional proteins. Blocking the action of the protease enzymes result in the production of immature noninfectious virions.³⁶ TDM of the P's has been proposed as a useful tool for the optimisation of therapy in children and adults.^{37,38}

All protease inhibitors are substrates for the CYP enzymes, predominantly CYP3A4, some are inducers and/or inhibitors of CYP3A4. Other enzymes such as CYP3A5 may also play a role.³⁹ The inherent variability of the quantity of CYP enzymes among humans, coupled with age-related changes in metabolic capability in children produces even higher interpatient variability in the pharmacokinetics of these drugs in children compared to adults.⁴⁰

Most of the protease inhibitors are also substrates for the P-glycoprotein drug transporter (P-gp), an efflux pump encoded by the MDR1 gene. The expression of this protein can limit the entry of the drug into the central nervous system, semen and gastrointestinal tract.^{22,23,34}

It is evident that many drug substrates of P-gp are also substrates of particularly CYP3A4.²³ It is thus likely that CYP3A and P-gp both contribute to poor bioavailability of protease inhibitors, but the relative contribution of each protein remains unknown.⁴¹

Lopinavir and ritonavir are highly (98-99%) protein bound. Lopinavir binds to both to α-acid glycoprotein (AAG) and to albumin, however it has a higher affinity for AAG.^{42,43} Ritonavir also binds to both with comparable affinities.⁴⁴

In vitro experiments performed with human hepatic microsomes indicate that lopinavir primarily undergoes oxidative metabolism. Lopinavir is almost exclusively metabolised by the CYP3A isozyme. Ritonavir is a potent CYP3A inhibitor and when combined with lopinavir, it increases the plasma levels of lopinavir.⁴⁵ Low dose ritonavir in combination with lopinavir (1:4) is used as a pharmacokinetic enhancer and allows for a reduction in the dose and dosing frequency of lopinavir. Furthermore lower doses of ritonavir are much better tolerated and the bioavailability of lopinavir is improved.⁴⁶

Ritonavir is metabolised, primarily by CYP3A and to a lesser extent by CYP2D6. Ritonavir has also shown to induce the metabolic enzymes resulting in the induction of its own metabolism and most likely the induction of lopinavir metabolism, this appear to stabilise after approximately 2 weeks of treatment.^{47,48}

3 PHARMACOKINETICS OF ANTIRETROVIRALS IN HIV-1 INFECTED CHILDREN

The current available guidelines for the treatment of children infected with HIV, recommend that the same principles of treatment for adults should also be applicable to children. However, the pharmacokinetic and pharmacodynamic characteristics of several of these ARV's and regimens used in adults have not been defined adequately in children.⁴⁹

Significant inter-individual differences are observed in the pharmacokinetics (PK) of ARV's in children and in adults.^{16,17,49,50} Children are not just small adults and their pharmacokinetics and pharmacodynamics are distinctly different. Paediatric dosing is based on age, weight and/or body surface which are in contrast to adults who normally receive a standard fixed dose regardless of age or weight. Growth and development are dynamic and continuously changing parameters in children partly explain the differences in pharmacokinetics between adults and children.

The pharmacokinetic differences that occur are due to many factors such as; physiologic changes associated with growth and development, absorption and distribution of drugs; renal and hepatic functions.^{16,17,50} Hepatic enzyme activity and maturation are not comparable to that of the adult in the postnatal period and are thus responsible for significant alterations in the disposition of this class of antiretrovirals.^{16,17,50} The neonatal activity of the P450 enzyme system is approximately 20-70% of the adult values, it then increases to adult levels by 6-12 months after which the activity exceeds adult levels at 1-4 years and finally declines to normal adult levels at the end of puberty.^{15,16}

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The subgroup CYP3A4 in particular has been extensively investigated and is the most abundant of the P450 family of enzymes in the liver and intestine and among the most important of all of the human drug-metabolising enzymes^{14,17,20,23}.

Efavirenz is principally metabolised via oxidative metabolism by CYP2B6 and to a lesser extent by CYP3A4. Efavirenz is a moderate inducer of CYP3A4 and undergoes limited auto-induction^{22,28,35}. It is however not required to alter the dose during the first few weeks of therapy due to its long half-life²².

The hepatic enzyme CYP2B6 is one of the least characterised and investigated P450 enzyme. Haas and co-workers observed a polymorphism in the gene variant G516T that encodes for the hepatic enzyme that is primarily responsible for the metabolism of efavirenz. This CYP2B6 allelic variant was associated with significantly greater efavirenz exposure due to lower clearance of the drug in African-Americans compared to European-Americans. These inter-individual differences in the metabolism may in part be the reason for greater central nervous system symptoms associated with higher efavirenz plasma concentrations in this study population³⁶.

The MDR1 gene that encodes for P-glycoprotein (efflux transporter) was found not to play a role in the efavirenz metabolism by Haas and co-workers³⁶.

5 RESISTANCE

When viral replication continues in the presence of selection pressure of drug exposure, antiretroviral resistance is likely to occur³⁷. For drugs of the NNRTI class and lamivudine, single gene mutations have been demonstrated to confer high level resistance which can emerge rapidly^{38,39}. High level resistance to other NRTIs and to the PI class of drugs requires multiple mutations and therefore takes longer to develop.

The prevention of mother to child transmission (PMCT) program currently recommends the use of single dose nevirapine for mother and baby to prevent HIV infection. This method has demonstrated efficacy in preventing HIV transmissions to at least half of infants who would likely become infected with no intervention^{40,41}.

However, a large proportion of women and infants develop NNRTI resistance rapidly following this treatment. These mutations fade over⁴². The PMCT program in South Africa has been widely rolled out since 2003, and although still inadequate in its coverage, more children are being exposed to nevirapine for PMCT. However, since nevirapine resistance fades, it is unlikely that these mutations will be present on standard population genotyping prior to commencing first line regimen with efavirenz (an NNRTI) to which resistance mutations to nevirapine cross react⁴³. Efavirenz is recommended as part of the antiretroviral regimen for children over the age of 3 years⁴⁴. Although we expect to have wild-type virus at baseline in this population it is prudent to have baseline genotyping of virus should resistance occur on therapy.

Whether archived resistance mutations reemerge following exposure to ARV treatment and how rapidly this occurs remains to be established.

If, as suggested above, there is wide inter- and intra-patient variability in drug metabolism, some children who start ARV's may have inadequate drug levels predisposing them to development of resistance with concomitant virological failure. If salivary efavirenz levels can be correlated with the plasma efavirenz levels, then it would simplify determining if the patient has adequate efavirenz plasma levels, due to the fact of easier sample collection and no specialized training needed for the staff⁴⁵.

Standard methods for detection of resistance mutations rely on the presence of a viral load above 1000 copies/ml and will be done at baseline and should virological failure occur. However, since ongoing viral replication is apparent even when viral load is below 50 copies per ml it is conceivable that resistance mutations may develop early. Standard population based sequencing methods such as the Celera Viroseq assay and the Bayer TruGene assay is unable to detect viral DNA mutations at low viral loads.

6 THE EFFECT OF HAART THERAPY ON BODY COMPOSITION OF HIV-INFECTED CHILDREN

The effect of antiretroviral therapy on body composition has been studied in adults in various studies. The progressive, selective wasting of subcutaneous fat from the face and limbs of HIV-infected patients treated with HAART therapy first emerged in 1997⁴⁶. Very little data is known about the effects of HIV infection and antiretroviral drugs on body composition in children particularly in resource restricted settings. HIV-infected children are at high risk of malnutrition and have unique nutritional

3.1 Pharmacokinetic studies with antiretroviral therapy in children

Several pharmacokinetic studies have been performed over the years, especially in adults but also in children. Various regimens of monotherapy and combination antiretroviral therapy have been investigated among children of different age groups^{14,17,20,23}.

Studies have shown that exposure to PIs may gradually decrease over time^{47,48}. Very little information on the change in the pharmacokinetic parameters after prolonged PI therapy in children is available.

King *et al.*, 2002, published a review article on antiretroviral pharmacokinetics in paediatric population. Table 2 refers to the pharmacokinetic parameter values of certain antiretroviral drugs in paediatric patients.

Table 2: Pharmacokinetic parameter values of certain antiretroviral drugs in paediatric patients (taken from King *et al.*, 2002.)

Drug	Dosage	AUC (mg hL) ⁻¹	CL/F (L/h/kg)	C _{min} (mg/L)	C _{max} (mg/L)
Nucleoside reverse transcriptase inhibitors (NRTIs)					
Stavudine	2 mg/kg/day	1.63			1.1
Lamivudine	4 mg/kg bid	7.14		0.07	2.21
Abacavir	8 mg/kg q12h	9.8 ± 4.6	1.13		3.71 ± 1.4
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)					
Efavirenz	15.4 mg/kg/dose	58 ± 45.4	0.34 ± 0.16	1.7 ± 1.5	
Protease Inhibitors					
Ritonavir	400 mg/m ² /dose bid	97	4.3 ^a		11.4
Lopinavir	230/67.5 mg/m ² bid	72.6 ± 31.1 ^c	3.4 ± 2.2 ^c		8.2 ± 2.9 ^c

AUC = area under the concentration-time curve; bid = twice daily; CL/F = apparent (oral) systemic clearance; C_{max} = peak concentration; C_{min} = minimum (trough) concentration; qd = once daily; qh = every x hours; tid = three times daily; Lop = Lopinavir and Rtl = Ritonavir.

^a Lopinavir and Rtl are at steady state except for Nevirapine values which are derived from a single-dose pharmacokinetic study.

^b Lopinavir

^c For lopinavir

Large scale clinical trials to establish the safety, efficacy and pharmacokinetic characteristics in children are still lacking for several reasons: different dosages and regimens used too few participants in clinical trials (underpowered), different age groups (neonates-adolescents), varying duration of clinical trial periods (often only short term) and lack of controlled studies.

4 PHARMACOGENETICS

Pharmacogenetics is the study of the genetic basis for variation in drug response. With the advanced technology currently available in genomics, a reverse genetic genotype-to-phenotype approach is feasible where genetic polymorphisms can serve as the starting point to assess whether genomic variability translates into a phenotypic variation⁴⁹.

Polymorphism can be defined as a variation in the DNA sequence that is present in an allele frequency of 1% or greater in a population. In the human phenotype two major types of sequence variation have been identified: SNP (single nucleotide polymorphisms) and indels (insertion/deletions).

Genetic polymorphisms and variations in the levels of the cytochrome P450 (CYP) enzymes; other drug-metabolising enzymes and transporters (efflux transporters) are the main pharmacogenetic sources of intra- and inter-individual variations in drug metabolism⁵⁰.

The CYPs are the most intensely studied of all the xenobiotic metabolising enzymes as they are responsible for the greatest majority of metabolism of the therapeutic drugs⁵¹.

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8.2 Secondary Objectives

- To investigate the relationship between the pharmacodynamics (CD4 count, viral load, side-effects and toxicity) and the treatment regimen in this specific paediatric population. Patient and treatment covariate effects will be identified by incorporating the NONMEM program.
- To investigate genetic polymorphisms and variations in the expression of CYP2B6 and CYP3A4 in whole blood, to determine the inter-individual and intra-individual variations that may occur. Phenotype investigations to establish the inter-individual variation on CYP2B6 within this study population. To investigate changes in the liver metabolism via the cytochrome P450 (CYP2B6 and CYP3A4) enzyme system prior to efavirenz treatment and during the 24 month study period by taking whole blood dried spot samples.
- To establish the effect of long term antiretroviral therapy on body composition (weight, height, growth, body fat, muscle mass or fat and muscle distribution).
- To establish if the changes in body composition with regard to lipidolipid affect the pharmacokinetic characteristics of efavirenz in combination with 2 NRTI's by excluding the contribution of normal aging and growth.
- To examine the relationship between pharmacokinetic parameters and the development of NNRTI resistance mutations as well as time to virological failure of the first line regimen.
- To describe resistance profiles emerging in children on NNRTI-based treatment with HIV subtype C.
- To determine if a correlation exists between unbound efavirenz plasma levels and efavirenz levels in the saliva.
- To determine if a correlation exists between urine efavirenz metabolite and efavirenz plasma levels.

9 STUDY DESIGN

9.1 General

This proposed study is a clinic-based, non-randomised open label study, performed in HIV-1 infected children who are attending the Harriet Shezi Children's clinic on an out patient basis. The antiretroviral treatment guidelines and regimen of the National Department of Health (DoH) for the management of HIV-infected children are followed by the attending clinicians who are consulting with these patients. No investigational drugs, dosages or combinations will be used. Efavirenz blood concentrations will be determined on the routinely scheduled visits to the Harriet Shezi Children's clinic. Each study participant will be monitored and included in this study for a minimum period of 24 months (6 PK study related visits) and a maximum period of 30 months (including staging and baseline monitoring and screening). All of the participants will be children who have not been on antiretroviral therapy previously (excluding possible exposure during pregnancy and or single dose therapy to prevent mother to child transmission).

Routine 3 monthly visits for clinical assessments are scheduled for all patients attending the clinic, irrespective of their participation in this specific study. Repeat prescriptions for 3 months are issued for the specific ARV medication and the medicines are dispensed for 2 months and then 1 month, if no problems occur, irrespective of their participation in this study. Blood is drawn for routine laboratory tests at staging, baseline and then 6 monthly (refer to table 5) for all patients attending the clinic, irrespective of their participation in this study.

Two pharmacokinetic blood samples (1.5 ml whole blood respectively) will be collected to determine the projected trough level at steady state (collected > 10 hours post dose, previous evening) for efavirenz (StocromTM) and again after 1-2 hours at 1, 3, 6, 12, 18, 24 months to coincide with the scheduled clinical assessment and routine blood tests according to the standard treatment and management of HIV-infected children.

On 2 study visits (either 6 and 12 months or 12 and 18 months after starting on ARV's), urine and saliva will be collected in addition to the pharmacokinetic blood samples, to establish if a correlation between the urine and plasma efavirenz levels, and the saliva and plasma efavirenz levels exists. This will only be performed as a pilot study in 20 of the already recruited study participants that have given their consent and assent where applicable.

Sparsely collected pharmacokinetic data will be incorporated into the nonlinear mixed effects modelling (NONMEM) software to generate population pharmacokinetic parameters. A single plasma concentration does not offer adequate estimates of pharmacokinetic parameters. Inclusion of data from a second time point improve estimates and provides the most uniformly accurate and precise predictions on the parameters and intra-individual variability^{2,3,20}.

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needs due to the physiological demands of growth and development. Malnutrition in children results in growth retardation, increased susceptibility to infection and decreased functional capacity¹.

Anthropometry is an affordable and non-invasive method of assessment of body composition and can be used to monitor changes in body composition and nutritional risk of children with HIV infection. Measurements in children should include serial measurements of height (or length) and weight, with calculation of body mass index (BMI) and plotted on the Centers for Disease Control (CDC) growth charts (children > 5y old)²³ or WHO growth charts (0-5y old)²⁴. Mid arm circumference, subscapular and triceps skin folds and mid arm muscle area in children > 1y reflect fat and lean body mass stores²⁵. Lean body mass changes measured by anthropometry agree reasonably well with lean body mass changes measured by dual X-ray absorptiometry (DXA) in patients with HIV infection²⁶. Changes in waist circumference (WC) can be measured to detect visceral fat accumulation over time²⁷. WC percentiles have been published for children 5-17y²⁸ and can be used to monitor normal and excessive increase in WC over time.

The Paediatric AIDS Clinical Trials Group (PACTG) study 1010 reported at the XVI International AIDS Conference (IAC) in Toronto that HIV treatment dramatically improved growth and body composition in HIV-positive children after 48 weeks of starting treatment or switching to a protease inhibitor based regimen for the first time²⁹. Varvel and co-workers also reported a positive influence on the growth of HIV-1-infected children treated with HAART³⁰. Height and weight are improved in children in whom a reduction of the viral load or at least 1.5 log₁₀ or to <500 copies/ml is achieved. In contrast to the increase of the body mass index (BMI) in adults on HAART, BMI did not increase in all children effectively treated with HAART. BMI increased more in children with an advanced stage of infection and a poor nutritional status at baseline³¹.

Progressive subcutaneous fat wasting, fat accumulation, dyslipidaemia and insulin resistance in HIV-infected patients on antiretroviral therapy has been attributed to the long-term toxicity of HIV protease inhibitors³². Fat wasting however had been observed in patients who have never taken protease inhibitors in more recent studies, possibly implicating the effect of nucleoside analogue reverse transcriptase inhibitor (NRTI) therapy. Protease inhibitors have a predominant influence and may act synergistically with NRTIs. NRTIs appear to predispose individuals to slowly progressive fat loss, which is markedly accelerated when a PI and NRTIs are combined. Stavudine leads to an earlier onset of clinically apparent fat wasting and higher serum lactate concentration compared with zidovudine³³.

The influences of changes in body composition (weight, height, growth, body fat, muscle mass or fat and muscle distribution) on the pharmacokinetic parameters of the antiretroviral drugs need to be explored. These changes in body composition can be monitored using anthropometric measurements.

7 NONLINEAR MIXED-EFFECTS MODELS (NONMEM)

NONMEM is a program developed by the NONMEM project Group for estimating population pharmacokinetic parameters. It is written in FORTRAN, designed to fit general statistical (nonlinear) regression-type models to data³⁴.

The advantage is that sparsely collected pharmacokinetic data can be used to generate population kinetic analysis²³.

8 STUDY OBJECTIVES

To establish a large data base for population pharmacokinetic parameters of efavirenz in children (>3 years and weight > 10 kg) who are being treated with the first line regimen (2NRTI's + efavirenz) as set out in the National Guidelines for the Management of HIV-infected children in South Africa³⁵. This will be executed using routine clinic-based, sparse sampling methods and population pharmacokinetic (PK) analysis.

8.1 Primary Objectives

- To investigate the population pharmacokinetic parameters (projected C_{max}) and clearance (Cl) of efavirenz in combination with 2 NRTI's in HIV-1 infected children (older than 3 years and weighing more than 10 kg) by incorporating the data into NONMEM.

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Table 3: ARV drug choices for children (taken from National Department of Health – Guidelines for the management of HIV-infected children¹, 2005 – Section 10 Table 10 p. 82)

First Line	> 3 Years old and > 10 kg
	Stavudine (d4T) ² Lamivudine (3TC) ² Efavirenz (EFV)

¹Children may receive 2 antiretroviral drugs without the third² – provided no reported resistance at some point in the future as part of the first line regimen but the exact date of this implementation is not yet known.

The dosages of efavirenz, in combination with stavudine, lamivudine, abacavir and co-trimoxazole (trimethoprim + sulfamethoxazole) are reflected in Table 4.

The following anthropometric measurements will be done at 0, 1, 3, 6, 12, 18, 24 months: weight on an electronic Defecto scale, height with a stadiometer, or recumbent length on a Seca paediatric length mat (children < 2y), mid arm circumference, waist circumference (children > 5y) with a Lurkin steel tape, triceps and subscapular skin fold thickness with a John Bull skin fold calliper. For children older than 9 years, assessment of Tanner stage will also be done to record their physical maturation. No other additional tests, monitoring or invasive procedures will be performed on any of the study participants.

Whole blood (1-1.5 ml) which will allow for a repeat if there is failure will be taken at baseline for population based sequencing using an NIH VOA, certified in-house methodology with primers optimised for HIV subtype C. If the patient fails virologically with confirmed viral load >1000 copies/ml, further genotyping will be performed on whole blood.

9.2 Sample size justification

Very little data on efavirenz levels in children have been published to be used as a guide for sample size estimations.

The determining of the minimum justified sample size was based on the following calculation²⁸:

$$n \geq \left(\frac{2\sigma}{e} \right)^2$$

n = minimum sample size

σ = standard deviation

e = the minimum deviation from the average (x), which can be tolerated with a probability of 0.95. A 20% deviation from the average C_{min} value was chosen.

The Sustiva™ and Stocrin™ package inserts indicate the C_{min} = 5.6 ± 4.1 μM (1.77 ± 1.29 μg/ml) efavirenz in 48 paediatric patients after once daily administration adjusted to body size based on weight.

Based on the available limited published data on the pharmacokinetics of efavirenz the statistical calculation of a justified sample size was calculated as 64 (53.1 + 10.6)²⁸ participants.

Since e = 0.2 x 1.77 = 0.354

$n \geq (2 \times 1.29 / 0.354)^2 = 53.1$ and 10.6 added (20%²⁸ to account for non-completers and withdrawals).

²⁸ Study participant numbers approved by Wits-HREC on 4 December 2007.

Only 20 of the current recruited participants of the main study will take part in this proposed pilot study involving additional saliva and urine sampling. Two saliva samples (one hour apart) will be collected from each participant in the pilot study when the pharmacokinetic blood from the main study is drawn. No additional blood will be drawn from these subjects. Urine will be collected from these 20 subjects during a 4 hour period on the same study visit.

The 2 blood and 2 saliva samples will enable the investigator to investigate if the following exist:

- Correlation between plasma and saliva at different time points in one dosing interval.
- Intra-individual variation in the same individual over time (6 months interval) in the total and free efavirenz concentration.
- The correlation coefficient between total and free efavirenz concentrations.
- A regression equation between the efavirenz saliva and blood samples.

9.3 Study treatment regimen

The study regimen is in line with to the first line regimen of the DoH for the management of HIV-infected children, 2005, which are reflected in Table 3. The study regimen is the combination of efavirenz and 2 NRTIs in children older than 3 years and weighing more than 10 kg.

9.4 Selection of participants

Children older than 3 and younger than 16 years, both genders, attending the study site at Harriet Shezi Children's Clinic, Chris Hani Baragwanath Hospital, Soweto, South Africa, as out patients and from whom consent/assent was obtained.

For the saliva and urine collection, 20 participants older than 3 years and younger than 16 years, both genders, attending the study site at Harriet Shezi Children's Clinic, Chris Hani Baragwanath Hospital, Soweto, South Africa, as out patients and from whom consent/assent was obtained and who's already participating in the main approved study.

9.5 Criteria

9.5.1 Inclusion Criteria

- HIV-1 DNA PCR positive at 0 (baseline) and if possible at a second occasion, if the PCR testing is not available then HIV ELISA and again at 12 and 18 months if the child was diagnosed with HIV before 6 months of age.
- 3 years to 16 years.
- The concomitant use of multivitamins and co-trimoxazole would be acceptable.
- All other eligibility criteria as indicated by the National DoH - Guidelines for the management of HIV-infected children.
- Signed informed consent from a legal guardian and, if indicated, signed informed assent or waiver of assent.

9.5.2 Exclusion Criteria

- Grade 3-4 defined toxicity according to the Paediatric AIDS Clinical Trial Group (PACTG) for laboratory tests and adverse events.
- Any of the following laboratory findings at entry: absolute neutrophil count <1000 cells/mm³; platelet count <100 (000 cells/mm³); AST >3 times upper limit of age adjusted normal values; ALT >3 times upper limit of age adjusted normal values; serum creatinine >1.2mg/dL.
- Use of the internal standard (reserpine) as it is used in the LC-MS method.
- Current use or recently exposed (within 4 weeks of the specific study visit in question) to any known P450 cytochrome inducers or inhibitors such as rifampicin, phenobarbital, carbamazepine, isoniazid (INH), erythromycin, cyclosporine, ketoconazole, metronidazole, ciprofloxacin, corticosteroids and cimetidine or any other drug at the discretion of the principle investigator.
- Any chronic medication that is taken on a regular daily basis besides the specified first line HAART regimen. Co-trimoxazole for prophylaxis against opportunistic infections as specified in the DOH guidelines would be acceptable.
- Tuberculosis infection. Any study participant diagnosed with TB and started on TB treatment while on the study will be withdrawn while on the anti-tuberculosis regimen (at least 6 months). After completion of TB treatment and after a further 4 weeks, the participant will re-enter the study. This is to ensure the exclusion of enzyme inducing effects of Rifampicin (and INH) on the pharmacokinetic blood level determinations of efavirenz for the remainder of the study period.
- Known renal, hepatic or gastrointestinal tract disease (malabsorption).
- Chronic diarrhoea.

9.6 Informed consent

Written informed consent will be obtained from the parent or legal guardian of eligible patients and assent will be sought from the child if he/she is old enough. The investigator or designated assistant will fully explain the nature, conditions and consequences of the study to the responsible parent or legal guardian, and the child if he/she is able to understand the nature of the study. A competent translator, familiar with the study will assist in the informed consent process when necessary. Subject information and consent documents will be available in Zulu, Afrikaans and English. A witness independent of the study will countersign the consent documents of illiterate parents or legal guardians. Each parent or legal guardian will be given a copy of the signed consent document. No patient will be enrolled prior to or without the written informed consent of parent or legal guardian.

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Table 4: ARV drugs and paediatric dosing chart for use in resource constrained settings (National DoH - Guidelines for the management of HIV-infected children, 2009 - Appendix 3A p. 124-126)

Weight	Effavirenz 3TC daily (loading dose)	Savathine D4T Twice daily	Lamivudine 3TC Twice daily	Abacavir ABC Twice daily	Trimethoprim Sulfa-methoxazole Once daily
5-9 kg	250 mg	100 mg	150 mg	20 mg	100 mg
10-14 kg	250 mg	100 mg	150 mg	20 mg	100 mg
15-19 kg	250 mg	100 mg	150 mg	20 mg	100 mg
20-24 kg	250 mg	100 mg	150 mg	20 mg	100 mg
25-29 kg	250 mg	100 mg	150 mg	20 mg	100 mg
30-34 kg	250 mg	100 mg	150 mg	20 mg	100 mg
35-39 kg	250 mg	100 mg	150 mg	20 mg	100 mg
40-44 kg	250 mg	100 mg	150 mg	20 mg	100 mg
45-49 kg	250 mg	100 mg	150 mg	20 mg	100 mg
50-54 kg	250 mg	100 mg	150 mg	20 mg	100 mg
55-59 kg	250 mg	100 mg	150 mg	20 mg	100 mg
60-64 kg	250 mg	100 mg	150 mg	20 mg	100 mg
65-69 kg	250 mg	100 mg	150 mg	20 mg	100 mg
70-74 kg	250 mg	100 mg	150 mg	20 mg	100 mg
75-79 kg	250 mg	100 mg	150 mg	20 mg	100 mg
80-84 kg	250 mg	100 mg	150 mg	20 mg	100 mg
85-89 kg	250 mg	100 mg	150 mg	20 mg	100 mg
90-94 kg	250 mg	100 mg	150 mg	20 mg	100 mg
95-99 kg	250 mg	100 mg	150 mg	20 mg	100 mg
100-104 kg	250 mg	100 mg	150 mg	20 mg	100 mg
105-109 kg	250 mg	100 mg	150 mg	20 mg	100 mg
110-114 kg	250 mg	100 mg	150 mg	20 mg	100 mg
115-119 kg	250 mg	100 mg	150 mg	20 mg	100 mg
120-124 kg	250 mg	100 mg	150 mg	20 mg	100 mg
125-129 kg	250 mg	100 mg	150 mg	20 mg	100 mg
130-134 kg	250 mg	100 mg	150 mg	20 mg	100 mg
135-139 kg	250 mg	100 mg	150 mg	20 mg	100 mg
140-144 kg	250 mg	100 mg	150 mg	20 mg	100 mg
145-149 kg	250 mg	100 mg	150 mg	20 mg	100 mg
150-154 kg	250 mg	100 mg	150 mg	20 mg	100 mg
155-159 kg	250 mg	100 mg	150 mg	20 mg	100 mg
160-164 kg	250 mg	100 mg	150 mg	20 mg	100 mg
165-169 kg	250 mg	100 mg	150 mg	20 mg	100 mg
170-174 kg	250 mg	100 mg	150 mg	20 mg	100 mg
175-179 kg	250 mg	100 mg	150 mg	20 mg	100 mg
180-184 kg	250 mg	100 mg	150 mg	20 mg	100 mg
185-189 kg	250 mg	100 mg	150 mg	20 mg	100 mg
190-194 kg	250 mg	100 mg	150 mg	20 mg	100 mg
195-199 kg	250 mg	100 mg	150 mg	20 mg	100 mg
200-204 kg	250 mg	100 mg	150 mg	20 mg	100 mg
205-209 kg	250 mg	100 mg	150 mg	20 mg	100 mg
210-214 kg	250 mg	100 mg	150 mg	20 mg	100 mg
215-219 kg	250 mg	100 mg	150 mg	20 mg	100 mg
220-224 kg	250 mg	100 mg	150 mg	20 mg	100 mg
225-229 kg	250 mg	100 mg	150 mg	20 mg	100 mg
230-234 kg	250 mg	100 mg	150 mg	20 mg	100 mg
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240-244 kg	250 mg	100 mg	150 mg	20 mg	100 mg
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260-264 kg	250 mg	100 mg	150 mg	20 mg	100 mg
265-269 kg	250 mg	100 mg	150 mg	20 mg	100 mg
270-274 kg	250 mg	100 mg	150 mg	20 mg	100 mg
275-279 kg	250 mg	100 mg	150 mg	20 mg	100 mg
280-284 kg	250 mg	100 mg	150 mg	20 mg	100 mg
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370-374 kg	250 mg	100 mg	150 mg	20 mg	100 mg
375-379 kg	250 mg	100 mg	150 mg	20 mg	100 mg
380-384 kg	250 mg	100 mg	150 mg	20 mg	100 mg
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390-394 kg	250 mg	100 mg	150 mg	20 mg	100 mg
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405-409 kg	250 mg	100 mg	150 mg	20 mg	100 mg
410-414 kg	250 mg	100 mg	150 mg	20 mg	100 mg
415-419 kg	250 mg	100 mg	150 mg	20 mg	100 mg
420-424 kg	250 mg	100 mg	150 mg	20 mg	100 mg
425-429 kg	250 mg	100 mg	150 mg	20 mg	100 mg
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455-459 kg	250 mg	100 mg	150 mg	20 mg	100 mg
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475-479 kg	250 mg	100 mg	150 mg	20 mg	100 mg
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485-489 kg	250 mg	100 mg	150 mg	20 mg	100 mg
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495-499 kg	250 mg	100 mg	150 mg	20 mg	100 mg
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670-674 kg	250 mg	100 mg	150 mg	20 mg	100 mg
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680-684 kg	250 mg	100 mg	150 mg	20 mg	100 mg
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720-724 kg	250 mg	100 mg	150 mg	20 mg	100 mg
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745-749 kg	250 mg	100 mg	150 mg	20 mg	100 mg
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755-759 kg	250 mg	100 mg	150 mg	20 mg	100 mg
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770-774 kg	250 mg	100 mg	150 mg	20 mg	100 mg
775-779 kg	250 mg	100 mg	150 mg	20 mg	100 mg
780-784 kg	250 mg	100 mg	150 mg	20 mg	100 mg
785-789 kg	250 mg	100 mg	150 mg	20 mg	100 mg
790-794 kg	250 mg	100 mg	150 mg	20 mg	100 mg
795-799 kg	250 mg	100 mg	150 mg	20 mg	100 mg
800-804 kg	250 mg	100 mg	150 mg	20 mg	100 mg
805-809 kg	250 mg	100 mg	150 mg	20 mg	100 mg
810-814 kg	250 mg	100 mg	150 mg	20 mg	100 mg
815-819 kg	250 mg	100 mg	150 mg	20 mg	100 mg
820-824 kg	250 mg	100 mg	150 mg	20 mg	100 mg
825-829 kg	250 mg	100 mg	150 mg	20 mg	100 mg
830-834 kg	250 mg	100 mg	150 mg	20 mg	100 mg
835-839 kg	250 mg	100 mg	150 mg	20 mg	100 mg
840-844 kg	250 mg	100 mg	150 mg	20 mg	100 mg
845-849 kg	250 mg	100 mg	150 mg	20 mg	100 mg
850-854 kg	250 mg	100 mg	150 mg	20 mg	100 mg
855-859 kg	250 mg	100 mg	150 mg	20 mg	100 mg
860-864 kg	250 mg	100 mg	150 mg	20 mg	100 mg
865-869 kg	250 mg	100 mg	150 mg	20 mg	100 mg
870-874 kg	250 mg	100 mg	150 mg	20 mg	100 mg
875-879 kg	250 mg	100 mg	150 mg	20 mg	100 mg
880-884 kg	250 mg	100 mg	150 mg	20 mg	100 mg
885-889 kg	250 mg	100 mg	150 mg	20 mg	100 mg
890-894 kg	250 mg	100 mg	150 mg	20 mg	100 mg
895-899 kg	250 mg	100 mg	150 mg	20 mg	100 mg
900-904 kg	250 mg	100 mg	150 mg	20 mg	100 mg
905-909 kg	250 mg	100 mg	150 mg	20 mg	100 mg
910-914 kg	250 mg	100 mg	150 mg	20 mg	100 mg
915-919 kg	250 mg	100 mg	150 mg	20 mg	100 mg
920-924 kg	250 mg	100 mg	150 mg	20 mg	100 mg
925-929 kg	250 mg	100 mg	150 mg	20 mg	100 mg
930-934 kg	250 mg	100 mg	150 mg	20 mg	100 mg
935-939 kg	250 mg	100 mg	150 mg	20 mg	100 mg
940-944 kg	250 mg	100 mg	150 mg	20 mg	100 mg
945-949 kg	250 mg	100 mg	150 mg	20 mg	100 mg
950-954 kg	250 mg	100 mg	150 mg	20 mg	100 mg
955-959 kg	250 mg	100 mg	150 mg	20 mg	100 mg
960-964 kg	250 mg	100 mg	150 mg	20 mg	100 mg
965-969 kg	250 mg	100 mg	150 mg	20 mg	100 mg
970-974 kg	250 mg	100 mg	150 mg	20 mg	100 mg
975-979 kg	250 mg	100 mg	150 mg	20 mg	100 mg
980-984 kg	250 mg	100 mg	150 mg	20 mg	100 mg
985-989 kg	250 mg	100 mg	150 mg	20 mg	100 mg
990-994 kg	250 mg	100 mg	150 mg	20 mg	100 mg
995-999 kg	250 mg	100 mg	150 mg	20 mg	100 mg
1000-1004 kg	250 mg	100 mg	150 mg	20 mg	100 mg
1005-1009 kg	250 mg	100 mg	150 mg	20 mg	100 mg
1010-1014 kg	250 mg	100 mg	150 mg	20 mg	

Possible risks imposed on participants are minimal. Drawing of blood is done as part of routine medical care, it may result in faintness, redness, swelling, pain, bruising or bleeding where the cannula punctures the skin. The insertion of a cannula will cause less discomfort and trauma especially as blood samples will be taken at 2 separate times during the specified PK visits. Emla™ or Topi™ cream will be applied onto the skin prior to the cannula insertion to minimise the pain and discomfort that may be experienced by the participant.

Collecting saliva has a small risk of the participant swallowing the cotton roll, but the participant will be well supervised to minimize this risk. Collection of urine can be unhygienic, but participants will be well informed about good hygiene during sanitation.

The identity of all the participants will be confidential.

9.7 Study procedures

At baseline (0 months) – prior to commencement of ARV therapy:

The following data will be recorded by the investigator or designated assistant from eligible participant's once written informed consent has been obtained (Refer to attached Appendix I for Schedules of Study Evaluations).

- Date of birth
- Sex
- Height
- Weight
- Mid arm circumference
- Waist circumference
- Sub scapular and triceps skin folds
- Tanner stage or physical maturation
- Viral load, full blood count and ALT (part of routine standard of care treatment and management of HIV-1 infected children, Table 5).
- Whole blood (2 drops) will be sampled at the same time as for the above tests and blotted on specific filter papers (Guthrie cards or FTA cards) for the required DNA, PCR-analysis to investigate the occurrence of polymorphism on CYP2B6 within this study population.
- Whole blood (2 drops) will be sampled at the same time as for the above tests and blotted on specific filter papers (Guthrie cards or FTA cards) for the required DNA and or m-RNA PCR-analysis to investigate the baseline expression of CYP2B6 and CYP3A4 in blood prior to the commencement of the specified ARV treatment within this study population.
- Whole blood (1-1.5 ml) which will allow for a repeat if there is failure will be taken at baseline for population based sequencing using an NIH VQA certified in-house methodology with primers optimised for HIV subtype C. If the patient fails virologically with confirmed viral load >1000 copies/ml, further genotyping will be performed on whole blood.
- Dose and dosing schedules of all other possible and acceptable concomitant medication (e.g. co-trimoxazole and multivitamins) will be recorded.
- A 24-hour dietary recall and diet questions will be posed to the patient, parent or guardian, to establish a typical daily diet of the patient. Alcohol, caffeine, grapefruit juice, garlic and pepper should be avoided.

Each patient and his/her parent/guardian will be counselled about adherence and their treatment.

The first pharmacokinetic (PK) assessment day will be scheduled and confirmed with the patients/parents/guardians. They are specifically instructed to record the time of the HAART dosing for the previous 3 days prior to their clinic visit (this should be done at about 8h00 and 20h00 respectively) on their supplied diary cards. Diary cards (2 monthly + 1 monthly) will be issued to assist with adherence/compliance and scheduled clinic visits will be reflected on the cards.

PK assessment visits (1, 3, 6, 12, 18, 24 months) post commencement of ARV therapy:

- Participants are requested to report to the clinic at 8h00 on the day of the clinic visit.

- A cannula will be inserted and 1.5 ml whole blood will be collected for the predicted trough levels of efavirenz. This will be performed by an experienced study nurse. The exact time of this blood sample must be recorded. Emla™ or Topi™ cream will be applied onto the skin prior to insertion of the cannula.

- The second blood sample (1.5 ml whole blood) will be collected concomitantly with the required routine blood sample to be drawn from each patient (via the already inserted cannula) that is used for routine testing (see routine laboratory evaluations, Table 5). This will be performed by an experienced study nurse or the attending clinician. The exact time of this blood sample will be recorded. This 2nd PK sample is not required to be taken at exactly 1 or 2 hours after the initial projected trough sample – the exact recording of the time of this sample is however very crucial. The study-related visits at 1 and 3 months are the only study related visits that does not form part of the normal routine laboratory monitoring visits as set out in the DoH guidelines and management of HIV-1 infected children. Patients are always seen 3 monthly for clinical visits but will only have blood draws 6 monthly hereafter unless there is a clinical indication to repeat bloods in between.

- Saliva will be collected on 2 study visits (either 6 and 12 months OR 12 and 18 months), by using Salivettes on 20 study participants who have given consent / assent. No fluids or bubblegum may be chewed during this period. A cotton roll will be given to the participant, every time blood is drawn, and he/she will be asked to chew the cotton roll for 1 minute.

- Urine will be collected in a specially labelled container on the same scheduled study visit for a period of 4 hours from the same 20 study participants who have given consent / assent to the saliva and urine sampling while the participants are at the clinic.

- The following data will be recorded on the day of PK assessment (Refer to attached Appendix I for Schedules of Study Evaluations):

- Height
- Weight
- Mid arm circumference
- Waist circumference
- Subscapular and triceps skin folds
- bio-electrical impedance analysis (BIA) to measure fat-free mass and fat mass (only at 24 month visit)
- Tanner stage
- Viral load, CD4+ cell count, full blood count and ALT – 6 monthly (part of routine standard of care treatment and management of HIV-1 infected children, Table 5). Should virological failure occur (>1000 copies/ml confirmed on a subsequent specimen within one month), repeat genotyping will be done on blood specimens in between scheduled visits.
- Whole blood (2 drops) will be sampled at the same time as for the above tests and blotted on specific filter papers (Guthrie cards or FTA cards) for the required DNA and or m-RNA PCR-analysis to investigate the expression of CYP2B6 and CYP3A4 in blood after exposure to the specified ARV treatment within this study population.
- Treatment adherence assessment (diary card and counting/measuring of medicine)
- Dose and dosing schedule of the HAART regimen
- Time of last 3 days doses of the HAART regimen (specific for efavirenz), this will have to be specifically specified and recorded on their provided diary cards.
- Dose and dosing schedules of all other possible and acceptable concomitant medication (e.g. co-trimoxazole and multivitamins)
- General dietary questions and a 24-hour dietary recall will be posed to the patient, parent or guardian, to establish a typical daily diet of the patient. Alcohol, caffeine, grapefruit juice, garlic and pepper should be avoided.

Each patient and his/her parent/guardian will be counselled about adherence and their treatment.

Laboratories) are available for these analyses. Relatively small quantities of PCR product (DNA, c-DNA or RNA) can be quantified and the applications of the real time PCR in this study include:

- a) Quantitative m-RNA expression studies.
- b) DNA copy number measurements in genomic or viral DNA's.
- c) Allelic discrimination assays or SNP genotyping.

9.11 Routine laboratory evaluations

Routine 3 monthly visits for clinical assessment and dispensing of the specific ARV medicines are scheduled for all patients attending the clinic, irrespective of their participation in this proposed study. Blood samples are drawn from the patients at the staging phase, baseline and then on a 6 monthly basis as part of the routine monitoring tests, irrespective of their participation in a clinical trial. Routine tests performed are reflected in Table 5 and the analyses are done according to standard operating procedures in the laboratories of the NHLS (National Health Laboratory Services).

Table 5: Paediatric HAART regimen 1 and routine monitoring during treatment

First-line regimen > 3 years < 10kg	Test	Frequency
Stavudine (d4T)*	CD4	Staging, 6 monthly
Lamivudine (3TC)*	VL	Baseline, 6 monthly
Efavirenz	ALT and FBC	Baseline, 6 monthly

9.12 Resistance

Population genotype sequencing for resistance mutations will be performed on 1.5ml of blood at baseline and subsequent to a confirmed rise in viral load >1000copies/ml after ARV therapy commences.

9.13 Pharmacokinetic and statistic analysis

For the population pharmacokinetic analysis, a non-linear mixed effects model (NONMEM) describing the pharmacokinetics of efavirenz in the study population will be generated, using data from the samples collected during the scheduled clinic visits. The model will be used to identify significant covariate effects and to predict the individual trough C_{min} concentrations and clearance (Cl).

Population methods and regression analyses will be used to identify significant covariate factors and to adjust for these factors in regression models.

Linear regression will be performed using the SAS system for windows 9.1 for the proposed pilot study.

9.14 Data storage

A case record form (CRF) will be completed for each participant to collect specific details, study-related blood test results (apart from efavirenz concentrations), drug dosing details and the exact times of pharmacokinetic sampling. The raw data relating to the efavirenz concentrations will be kept in a study specific document. All study related documents will be stored for at least 15 years following study completion. The duplicate plasma samples will be kept at -80°C until one year after study completion.

9.15 Compensation for participation

The participants or parents / guardians / caregivers will not be paid for their child's participation in this study. Each participant's parent or caregiver will be compensated to cover travel costs and inconvenience for each study-related clinic-based visit, an amount of R50 will be provided and signed for (staging and or baseline, 1, 3, 6, 12, 18, 24 months after ARV therapy had been started). An additional amount of R50 will be paid to the 20 participants who have agreed to take part in the additional urine and saliva sampling as this may prolong the study visit at the clinic and they would need to buy or make arrangements for lunch for those specific visits.

The dates for the next PK sampling visit (second (3 months), third (6 months), fourth (12 months), fifth (18 month) or sixth (24 months)) will be confirmed with the patients/parents/guardians on their routine visits to the clinic. Patients, parents or guardians will be informed to note the time of ingestion of the HAART medication for the previous 3 days (evening and morning doses) prior to their clinic visit on the supplied diary card. Diary cards (2 monthly + 1 monthly) will be issued to assist with adherence/compliance and scheduled clinic visits will be reflected on the cards.

Follow-up data:

If and when a participant completes this particular study, the participant will continue on the antiretroviral therapy according to the National Department of Health regimens, all within the discretion of the attending clinician. If a participant withdraws from this study for what ever reason the patient will still continue on antiretroviral therapy and be able to attend the clinic according to the National DoH guidelines.

The same routine monitoring tests will be done at the same time interval as set out in the National DoH - Guidelines for the management of HIV-infected children. No additional blood (1.5 ml) will be drawn for the detection of the blood concentrations of the antiretroviral after the study have been completed nor would any additional dried blood spot sampling be performed.

The clinic visits are booked on a 3 monthly basis for as long as the patient attends the clinic and the routine laboratory testing are performed as outlined in Table 5.

9.8 Data blinding and recording

All data will be recorded in the study specific case record form of each participant.

The pharmacology laboratory will be blinded to any details relating to the study participants.

The attending clinician will have access to the plasma PK data within 14 days of the sample receipt by the laboratory.

9.9 Efavirenz concentration determination

Venous blood (2 x 1.5 ml) will be collected in EDTA labelled vacutainers.

The 1.5 ml blood samples must be kept on ice if not being centrifuged immediately. Centrifugation must be performed for 10 minutes at 3000 rpm within 1 hour of collection. From each plasma sample, 2 x 0.4 ml aliquots of plasma will be transferred into duplicate labelled polypropylene tubes (Ependorph tubes suitable for storage at -80°C). Within 12 hours of sampling, plasma samples must be stored at -20°C for up to 1 week before longer term storage at -80°C until analysis. The label on each polypropylene storage tube must include the subject number, sampling occasion, the sampling time and date.

The saturated cotton wool is placed in the suspended insert of the Salivette, which fits into the polypropyl tube. The Salivette will first be stored at 4°C, and after the blood, it will be centrifuged at 3000 rpm for 5 minutes within 2 hours. After centrifugation the Salivette will be stored at below -20°C pending analysis. The label on each Salivette will contain the subject number, sampling occasion, the sampling time and date.

Urine samples will be collected over a period of 4 hours. All urine will be pooled together and stored at -20°C until further analysis.

A validated LC-MS method have already been developed to determine efavirenz, lopinavir, ritonavir, lamivudine, stavudine and abacavir plasma concentrations in the Analytical Technology Laboratory (ATL), School of Pharmacy, North-West University, Potchefstroom. The analysis of lamivudine, stavudine and abacavir is not for pharmacokinetic purposes but can be used to measure compliance or adherence.

9.10 Pharmacogenetic analysis

Genetic polymorphisms will be investigated by using real-time polymerase chain reaction (PCR-RT). An iCycler Thermal Cycler with optical system software, iCycler IQ version 3.0a (Bio-Rad

10 INSURANCE

The North-West University and the University of the Witwatersrand subscribe to insurance policies which include liability for research conducted under the auspices of the University. The study participants are receiving antiretroviral treatment regimens identical to generally accepted treatment guidelines as part of their medical management. Their participation in the study does not change this.

11 ETHICAL CONSIDERATIONS

Ethical approval of the protocol will be sought from the Human Research Ethics Committee of the University of the Witwatersrand. All protocol amendments will be submitted to the ethics committees for approval, prior to implementation. The Ethics Committee of the North-West University will be notified after the approval of the Human Research Ethics Committee of the University of the Witwatersrand and had been obtained. No subject will be enrolled in the study before they (if old enough) and their legal guardian or parent has been fully informed about the study verbally and by means of a subject information document, with the aid of a translator if necessary, and the parent or legal guardian's signed consent has been obtained on the study specific consent form. Additional written assent will be sought from children 7 years and older.

The study is of an observational nature, as the treatment regimen (first line) is in line with current guidelines and the risk imposed on participants is minimal. The additional analysis of the drug levels is in the best interests of the individual participant as it will contribute to additional information to the attending clinician. Furthermore, the study is of benefit to the care of this patient population as it may further guide dosing policies of efavirenz in children, especially in the Southern African region.

The study will be conducted in accordance with the Declaration of Helsinki 2000 (Refer to Appendix II) and according to National Guidelines of Good Clinical Practice, Department of Health.

A report of the study findings will be submitted to the relevant ethic committees.

12 REFERENCES

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13 APPENDIX I: SCHEDULE OF EVALUATIONS – PK/PD.EFV.07

Evaluations	Blood Volume (ml)	All Subjects (after ARV therapy)					
		Pre-entry and Entry (Baseline)	1 Month ¹	3 Months ¹	6 Months	12 Months	24 Months
History		X	X	X	X	X	X
Physical Exam ²		X	X	X	X	X	X
Adherence ³ & concomitant meds ⁴		X	X	X	X	X	X
Weight		X	X	X	X	X	X
Mid arm circumference		X	X	X	X	X	X
Sub scapular and triceps skin folds		X	X	X	X	X	X
Tanner stage of physical maturation ⁵		X	X	X	X	X	X
# Blood tests	2 x 1.5	X	X	X	X	X	X
# Hepatology ⁶	1	X	X	X	X	X	X
# Liver function tests ⁷	1	X	X	X	X	X	X
# CD4 ⁸	2	X	X	X	X	X	X
# Viral Load ⁹	2	X	X	X	X	X	X
Pharmacogenetics ¹⁰	0.5	X	X	X	X	X	X
Resistance mutations ¹¹	1.5	X	X	X	X	X	X
Dietary questions and adherence ¹²		X	X	X	X	X	X
Urine + Saliva samples ¹³		X	X	X	X	X	X
Total Blood Volume (ml)		8	3.5	11	11	11	11

1. This phase may include staging and or baseline at enrollment depending on when the participant/guardian was seen for the first time. Record previous antiretroviral ARV treatment (if applicable); present CDC classification for HIV disease; CD4+%, and absolute cell count and highest VL; all previous HIV-related and AIDS-defining diagnoses; significant chronic or past illnesses (other than HIV), including hepatitis A and C, diabetes, hypertension, asthma and malignancies; and all present symptoms and diagnoses. Record smoking status (if applicable).
2. **Subsequent visits:** Record smoking status; CDC classification for HIV disease; symptoms present at the visit; and new and resolved diagnoses since the preceding study visit.
3. Complete physical exam, including assessment of current signs & symptoms and vital signs as are done with all standard examinations.
4. **Pre-entry visit:** Record all ARV meds the subject has ever taken (if applicable). **Entry visit:** Record all concomitant medications. **Subsequent visits:** Record continued, discontinued and new ARVs; and targeted concomitant medications since the preceding study visit.
5. For children older than 5 years.
6. Complete blood count, cell differential, platelet count.
7. ALT, liver function test.
8. **Staging + Subsequent visits:** CD4+ cell percentages and absolute counts may be performed. Not to be done at baseline.
9. **Pre-entry visit:** Viral load (baseline). **Subsequent visit:** 3 months then 6 monthly. Should virological failure occur (>1000 copies/mL confirmed on a subsequent specimen within one month), repeat genotyping will be done on blood specimens at between scheduled visits.

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10. DBS will be used in these investigations. **Preentry visit:** Genotype polymorphism variant of CYP2B6 will be investigated by using real-time polymerase chain reaction (PCR-RT). **Preentry and Subsequent visit:** The expression of CYP2B6 and CYP3A4 in whole blood - using PCR-RT. **11.** These are the only study related visits that does not form part of the normal public laboratory monitoring visits as they are not necessary for the management of HIV. Patients will be asked to provide 3-4 ml of blood for each clinical visit but will only have blood drawn 6 monthly thereafter unless there is a clinical indication to repeat bloods in between.

12. Only 20 participants will provide urine and saliva samples after 6 and 12 months OR after 12 and 18 months of treatment. After consent and assent had been obtained.

All these evaluations form part of standard routine test for caring, monitoring and managing HIV infected children as described in the Guidelines of the Department of Health (DoH). The tests are done according to standard operating procedures in the laboratories of the NHLS (National Health Laboratory Services).

14 APPENDIX II: THE DECLARATION OF HELSINKI 2000

WORLD MEDICAL ASSOCIATION DECLARATION OF HELSINKI Ethical Principles for Medical Research Involving Human Subjects

Adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, and amended by the 29th WMA General Assembly, Tokyo, Japan, October 1975
35th WMA General Assembly, Venice, Italy, October 1983
41st WMA General Assembly, Hong Kong, September 1989
48th WMA General Assembly, Somerset, West, Republic of South Africa, October 1996
and the 52nd WMA General Assembly, Edinburgh, Scotland, October 2000
Note of Clarification on Paragraph 29 added by the WMA General Assembly, Washington 2002

A. INTRODUCTION

1. The World Medical Association has developed the Declaration of Helsinki as a statement of ethical principles to provide guidance to physicians and other participants in medical research involving human subjects. Medical research involving human subjects includes research on identifiable human material or identifiable data.

2. It is the duty of the physician to promote and safeguard the health of the people. The physician's knowledge and conscience are dedicated to the fulfillment of this duty.

3. The Declaration of Geneva of the World Medical Association binds the physician with the words, "The health of my patient will be my first consideration," and the International Code of Medical Ethics declares that "A physician shall act only in the patient's interest when providing medical care which might have the effect of weakening the physical and mental condition of the patient."

4. Medical progress is based on research, which ultimately must rest in part on experimentation involving human subjects.

5. In medical research on human subjects, considerations related to the well-being of the human subject should take precedence over the interests of science and society.

6. The primary purpose of medical research involving human subjects is to improve prophylactic, diagnostic and therapeutic procedures and the understanding of the aetiology and pathogenesis of disease. Even the best-proven prophylactic, diagnostic, and therapeutic methods must continuously be challenged through research for their effectiveness, efficiency, accessibility and quality.

7. In current medical practice and in medical research, most prophylactic, diagnostic and therapeutic procedures involve risks and burdens.

8. Medical research is subject to ethical standards that promote respect for all human beings and protect their health and rights. Some research populations are vulnerable and need

special protection. The particular needs of the economically and medically disadvantaged must be recognized. Special attention is also required for those who cannot give or refuse consent for themselves, for those who may be subject to giving consent under duress, for those who will not benefit personally from the research and for those for whom the research is combined with care.

9. Research investigators should be aware of the ethical, legal and regulatory requirements for research on human subjects in their own countries as well as applicable international requirements. No national ethical, legal or regulatory requirement should be allowed to reduce or eliminate any of the protections for human subjects set forth in this Declaration.

B. BASIC PRINCIPLES FOR ALL MEDICAL RESEARCH

10. It is the duty of the physician in medical research to protect the life, health, privacy, and dignity of the human subject.

11. Medical research involving human subjects must conform to generally accepted scientific principles, be based on a thorough knowledge of the scientific literature, other relevant sources of information, and on adequate laboratory and, where appropriate, animal experimentation.

12. Appropriate caution must be exercised in the conduct of research, which may affect the environment, and the welfare of animals used for research must be respected.

13. The design and performance of each experimental procedure involving human subjects should be clearly formulated in an experimental protocol. This protocol should be submitted for consideration, comment, guidance, and where appropriate, approval to a specially appointed ethical review committee, which must be independent of the investigator, the sponsor or any other kind of undue influence. This independent committee should be in conformity with the laws and regulations of the country in which the research experiment is performed. The committee has the right to monitor ongoing trials. The researcher has the obligation to provide monitoring information to the committee, especially any serious adverse events. The researcher should also submit to the committee, for review, information regarding funding, sponsors, institutional affiliations, other potential conflicts of interest and incentives for subjects.

14. The research protocol should always contain a statement of the ethical considerations involved and should indicate that there is compliance with the principles enunciated in this Declaration.

15. Medical research involving human subjects should be conducted only by scientifically qualified persons and under the supervision of a clinically competent medical person. The responsibility for the human subject must always rest with a medically qualified person and never rest on the subject of the research, even though the subject has given consent.

16. Every medical research project involving human subjects should be preceded by careful assessment of predictable risks and burdens in comparison with foreseeable benefits to the subject or to others. This does not preclude the participation of healthy volunteers in medical research. The design of all studies should be publicly available.

17. Physicians should abstain from engaging in research projects involving human subjects unless they are confident that the risks involved have been adequately assessed and can be satisfactorily managed. Physicians should cease any investigation if the risks are found to outweigh the potential benefits or if there is conclusive proof of positive and beneficial results.

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18. Medical research involving human subjects should only be conducted if the importance of the objective outweighs the inherent risks and burdens to the subject. This is especially important when the human subjects are healthy volunteers.
19. Medical research is only justified if there is a reasonable likelihood that the populations in which the research is carried out stand to benefit from the results of the research.
20. The subjects must be volunteers and informed participants in the research project.
21. The right of research subjects to safeguard their integrity must always be respected. Every precaution should be taken to respect the privacy of the subject, the confidentiality of the patient's information and to minimize the impact of the study on the subjects' physical and mental integrity and on the personality of the subject.
22. In any research on human beings, each potential subject must be adequately informed of the aims, methods, sources of funding, any possible conflicts of interest, institutional affiliations of the researcher, the anticipated benefits and potential risks of the study and the discomfort it may entail. The subject should be informed of the right to abstain from participation in the study or to withdraw consent to participate at any time without reprisal. After ensuring that the subject has understood the information, the physician should then obtain the subject's freely given informed consent, preferably in writing, if the consent cannot be obtained in writing, the non-written consent must be formally documented and witnessed.
23. When obtaining informed consent for the research project the physician should be particularly cautious if the subject is in a dependent relationship with the physician or may consent under duress. In that case the informed consent should be obtained by a well-informed physician who is not engaged in the investigation and who is completely independent of this relationship.
24. For a research subject who is legally incompetent, physically or mentally incapable of giving consent or is a legally incompetent minor, the investigator must obtain informed consent from the legally authorized representative in accordance with applicable law. These groups should not be included in research unless the research is necessary to promote the health of the population represented and this research cannot instead be performed on legally competent persons.
25. When a subject deemed legally incompetent, such as a minor child, is able to give assent to decisions about participation in research, the investigator must obtain that assent in addition to the consent of the legally authorized representative.
26. Research on individuals from whom it is not possible to obtain consent, including proxy or advance consent, should be done only if the physical/mental condition that prevents obtaining informed consent is a necessary characteristic of the research population. The specific reasons for involving research subjects with a condition that renders them unable to give informed consent should be stated in the experimental protocol for consideration and approval of the review committee. The protocol should state that consent to remain in the research should be obtained as soon as possible from the individual or a legally authorized surrogate.
27. Both authors and publishers have ethical obligations. In publication of the results of research, the investigators are obliged to preserve the accuracy of the results. Negative as well as positive results should be published or otherwise publicly available. Sources of funding, institutional affiliations and any possible conflicts of interest should be declared in the publication. Reports of experimentation not in accordance with the principles laid down in this Declaration should not be accepted for publication.

C. ADDITIONAL PRINCIPLES FOR MEDICAL RESEARCH COMBINED WITH MEDICAL CARE

28. The physician may combine medical research with medical care, only to the extent that the research is justified by its potential prophylactic, diagnostic or therapeutic value. When medical research is combined with medical care, additional standards apply to protect the patients who are research subjects.
 29. The benefits, risks, burdens and effectiveness of a new method should be tested against those of the best current prophylactic, diagnostic, and therapeutic methods. This does not exclude the use of placebo, or no treatment, in studies where no proven prophylactic, diagnostic or therapeutic method exists.
 30. At the conclusion of the study, every patient entered into the study should be assured of access to the best-proven prophylactic, diagnostic and therapeutic methods identified by the study.
 31. The physician should fully inform the patient which aspects of the care are related to the research. The refusal of a patient to participate in a study must never interfere with the patient-physician relationship.
- in the treatment of a patient, where proven prophylactic, diagnostic and therapeutic methods do not exist or have been ineffective, the physician, with informed consent from the patient, must be free to use unproven or new prophylactic, diagnostic and therapeutic measures, if in the physician's judgment it offers hope of saving life, re-establishing health or alleviating suffering. Where possible, these measures should be made the object of research, designed to evaluate their safety and efficacy. In all cases, new information should be recorded and, where appropriate, published. The other relevant guidelines of this Declaration should be followed.

Addendum A2



Informed Consent Forms (PK/PD.EFV.07)

INFORMATION LEAFLET AND INFORMED CONSENT FOR PARENT/LEGAL GUARDIAN AND PARTICIPANT/ASSENT

POPULATION PHARMACOKINETIC AND PHARMACODYNAMIC STUDY OF EFAVIRENZ IN HIV-1 INFECTED CHILDREN TREATED WITH FIRST LINE ANTIRETROVIRAL THERAPY IN SOUTH AFRICA

PK/PD.EFV.07

Department of Pharmacology, School of Pharmacy, North-West University in collaboration with Harriet Shezi Children's Clinic, Chris Hani Baragwanath Hospital, Soweto, Johannesburg

Principal investigator: Me Michelle Viljoen Contact No: 018 2992232 or 074 101 3728

The co-investigators: Dr Tammy Meyers Contact No: 011 938 7784 or 083327 0066
Dr Hermien Gous Contact No: 011 938 8189 or 083 283 8360
Dr Lee Kleynhans Contact No: 011 9339629 or 082 780 9997
Prof Salome Kruger Contact No: 018 2992482 or 072 737 2235
Dr Ramoteme Mamabolo Contact No: 018 2992466 or 083 368 9084
Dr Megan Palmer Contact No: 011 9339629 or 083 327 3790

DATE OF FIRST INFORMED CONSENT DISCUSSION:

		20
day	month	Year

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Michelle Viljoen (PI)
Informed consent/assent.

Participant Initials: _____
Participant No: _____
Date approved by Wits HREC: 21/05/2007

INFORMATION LEAFLET AND INFORMED CONSENT FOR PARENT/LEGAL GUARDIAN:

INTRODUCTION:

Good day, you are kindly invited to consider your child taking part in this research study:

Population pharmacokinetic and pharmacodynamic study of efavirenz in HIV-1 infected children treated with the first line antiretroviral therapy in South Africa

The study doctor's name is **Dr Tammy Meyers** and all other attending doctors at the Harriet Shezi Children's Clinic involved in the standard care and management of the children attending this clinic.

The study / attending doctor's institution is:

Harriet Shezi Children's Clinic, Chris Hani Baragwanath Hospital, Soweto, Johannesburg

You can contact the following 24-hour telephone numbers:

Cell: 076 730 1769 for any study related emergency or

Cell: 074 101 3728 for any research information.

Before agreeing to allow your child to take part, it is important that you read and understand the purpose of the study, the study procedures, benefits, risks, discomforts, and precautions.

Your child's participation in this study is entirely voluntary and you can decline to participate, or stop at any time, without stating any reason.

It is your right to withdraw your child from the study at any time. Withdrawal of your child from the study will not affect his/her access or your access to medical treatment in any way.

This information leaflet is to help you to decide if you would like your child to take part. You should fully understand what is involved before you agree that he/she take part in the study. You should not agree that your child take part unless you are satisfied about all the procedures involved. If you have any questions, do not hesitate to ask the study doctor or principal investigator.

For the study to be useful, it is important that all the information you give the attending doctor / principal investigator / co-investigators / study

nurse is truthful and that you follow the study instructions carefully.

Your child will receive antiretroviral (ARV) medications for the human immunodeficiency virus (HIV), irrespective of your child's participation in this study or not. The ARV medications your child will be receiving will not change if you decide not to let your child participate in this study. The attending doctor(s) could recommend as part of the standard care and management guidelines a change in the ARV medication at any time and this will be in accordance with the Department of Health's guidelines irrespective of your child's participation in this study.

If you decide that your child may take part in this study, you will be asked to sign this document to confirm that you understand the study and agree to your child's taking part. You will be given a copy to keep.

PURPOSE OF THE STUDY:

One of the ARV medicines that your child will receive for treatment of the virus is efavirenz (Stocrin™).

The quantity of efavirenz will be measured in the blood of your children while taking all of the ARV medicines prescribed by the doctor according to the Department of Health's guidelines.

The testing of the levels of efavirenz will help in building up a data base to enable doctors and researchers to predict or simulate specific properties of the efavirenz in children of the same age, weight and on the same ARV therapy.

Other aims of the study include:

1. To investigate the effect of this treatment on your child's growth (length, weight, lean body mass); CD4 cell count, viral load and full blood count over time.
2. To establish whether possible change in your child's body composition will affect the blood levels of the efavirenz medication.

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3. To investigate changes in the liver metabolism (how the liver is able to break down the medication) over time. This is important to determine if there is variation between all the participants in this study or not.

4. To investigate the virus mutation and if the virus is or becoming resistant to the ARV therapy.

STUDY PARTICIPANTS:

Children (boys and girls) who weigh at least 10 kg, are between 3-16 years, are medically stable and who do not have chronic diarrhoea, or problems with their liver or intestines will be able to take part. Children who are taking certain medicines will not be able to take part.

Children attending the Harriet Shezi Children's Clinic will be invited to take part.

PROCEDURES:

Once you agree that your child may take part in this study, you and where possible your child will be asked questions and your child will be examined to see if he/she qualify to take part in this study.

During the 24-30 months of this study your child will participate in **7 study related visits** (including the first visit or enrolment before medication is started).

During the study related visits the following procedures will be followed:

1. The study nurse or co-investigators will record your child's age, sex, weight, length, certain measurements around the arm, waist and a skin fold on the shoulder, and details of her/his medicines and the results of his/her last HIV-tests in the study folder at 0; 1; 3; 6; 12; 18; 24 months.

2. Before receiving any antiretroviral medication, blood samples need to be drawn. Three blood samples (together amounting to less than 1 teaspoon or 5 ml) will be taken to test the liver, the red cells in the blood and to count the virus (viral load). These samples will be sent to the routine contracted laboratory that is responsible for all the required tests as stipulated in the Department of Health guidelines as they are routine, irrespective of your child's participation in this study. At the same time of collecting the above, 4 blood drops (less than ¼ teaspoon) will be taken and blotted

on a filter paper to dry for specific DNA testing (read and sign Supplementary Informed consent on DNA sampling) and 1-1.5 ml (less than ¼ teaspoon) will be taken to test for virus mutations. The total amount of blood drawn at this initial visit will be less than 2 teaspoons (8 ml) and it is all done at the same time.

3. The dose of the efavirenz and other ARV medicines will be monitored and prescribed by an attending doctor according to the guidelines of the Department of Health at each visit.

4. After ARV treatment had started 2 x 1.5 ml blood samples (approximately ¼ of a teaspoon each) at 1; 3; 6; 12; 18 and 24 months will be taken to determine the efavirenz blood concentration. A cannula (flexible plastic needle like the end of a drip) will be inserted into your child's vein for drawing the blood samples. A cream will be applied before the blood is drawn to reduce any possible pain or discomfort during the blood drawing process. 4 Blood drops (less than ¼ teaspoon) will be blotted on a filter paper to dry for DNA testing (read and sign Supplementary Informed consent on DNA sampling).

5. At 3; 6; 12; 18; 24 months after ARV treatment had started four blood samples (together amounting to less than 2 teaspoons, 6 ml) will be taken to test the liver, the red cells in the blood, the CD4 count and to count the virus (viral load). All of these blood samples will have to be taken even if your child is not taking part in this study as this is part of the standard treatment and management of HIV. These blood samples will be taken at the same time as the blood samples mentioned in point 4 except for the 1 month interval that it not relevant. These samples will be sent to the routine contracted laboratory that is responsible for all the required tests as stipulated in the Department of Health guidelines.

6. The total amount of blood drawn at 1 month after treatment would be approximately ¾ of a teaspoon (3.5 ml). The total amount of blood drawn at 3; 6; 12; 18 and 24 months after treatment would be less than 3 teaspoons (11 ml) respectively.

7. At each visit the study nurse / investigators will ask you some questions about your diet and medicines you have taken in the last few days, and when you took them

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8. The date and time for the next study visit will be arranged and confirmed. All of these study related visits will be at the same time as the normal 3 or 6 monthly visits to receive the required medication and to be seen by an attending doctor.

The study visits will all take place at Harriet Shezi Children's Clinic.

You, the child or the child's minder, will be asked to write down the exact time on the provided diary card, that the child takes his/her efavirenz dose on the 3 evenings before each of the study visits. All of the ARV medication must be brought to the clinic on each study related visit.

PRIVACY:

Your child will be identified by his/her initials and the hospital number. The information obtained about your child for the purposes of the study will be private. Only the study staff will see the information. If they wish to inspect the study records, officials of the Human Research Ethics Committee of the University of Witwatersrand and the Department of Health might also see the information. Any publications arising from the study will not be linked to your child's identity in any way.

POTENTIAL RISKS:

Your child will be exposed too little risk if he/she takes part. The drawing of blood is normally done as part of routine medical care and presents a slight risk of discomfort. It may result in faintness, redness, swelling, pain, bruising or bleeding where the needle punctured the skin. There is also a slight possibility of infection. However, experienced staff will perform the procedures under sterile conditions. A total of 18 ml of blood (less than 3 teaspoons) will be collected for determining the efavirenz concentration over the course of the entire study. A total of 28 drops of blood (less than 3 teaspoons) will be blotted on filter paper to dry over the course of the entire study. The other bloods (approximately 6 teaspoons) that are drawn form part of the standard routine medical care that would have to be done in any case even if the child does not take part in this study.

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POTENTIAL BENEFITS:

The study will help doctors and investigators to give better treatment over a longer period to children with HIV.

As the attending doctor will be given the results of the measurements in your child's blood, he/she will be able see if the amount of efavirenz in your child's blood is within the recommended range to be effective against the virus and to reduce the possibility of side effects.

WITHDRAWAL:

Your decision to withdraw your child at any time will not affect access to the standard treatment and care of HIV-1 infected patients.

The study doctor or investigators will provide you with any additional information that becomes available during the study, which may affect your willingness for your child to continue on the study.

The attending doctor has the right to withdraw your child from the study if it is in your child's best interest. The attending doctor has the right to change your child's antiretroviral medication at any time if it is in your child's best interest and this may mean that your child will no longer be able to continue in this study.

If you or your child did not give accurate information for the study, or did not follow the guidelines of the study or the rules in the study clinic, your child may be withdrawn from the study.

Pregnancy (only for girls): If your daughter is pregnant or breastfeeding or planning to become pregnant during the study period, she may not then take part in this study. Suitable contraceptive measures during the study and while on efavirenz treatment **should always** be taken. Should she become pregnant during this study, she would have to be withdrawn from this study immediately as her efavirenz medication will have to be changed. If your daughter is 14 years or older then she must read and sign the Supplementary Informed Consent for Woman of Childbearing Potential. All aspects of healthcare related to this pregnancy and infant will be your/her responsibility.

Participant Initials: _____
Participant No: _____
Date approved by Wits HREC: 21/05/2007

MONEY MATTERS:

You / your child will not be paid for any of the study related visits or procedures.

Your child will not be paid to participate in this study but R50 will be given to cover transport expenses for each of the **7 study related visits** to the Harriet Shezi Clinic (i.e. outpatient participants).

INSURANCE:

The ARV treatments in this study are the same as those that are widely used and recommended by the Department of Health. The medicines are taken as part of your child's standard medical care. If your child suffers illness or disability that results from study-specific procedures, the investigators will ensure that your child receives medical care until the related injury or illness resolves. You should notify the study / attending doctor immediately of any complications, side effects and/or injuries during the study.

North-West University in collaboration with Harriet Shezi Children's Clinic will provide compensation for reasonable medical expenses incurred as a result of study-related injury or illness, determined according to the guidelines laid down by the Association of the British Pharmaceutical Industry (ABPI Guidelines), and Guidelines for Good Practice in the Conduct of Clinical Trials in Human Participants in South Africa. You have to notify the study doctors or investigators immediately of any complications, side effects and/or injuries during the study and the nature of the expenses to be covered. If a research related injury occurs, you have not waived any of the legal rights which you otherwise would have as a participant in this study by signing this form.

NEW FINDINGS:

Any additional information that becomes available during the study, which may affect your willingness to allow your child to continue in this study, will be provided to you.

ETHICAL APPROVAL:

The study protocol has been submitted to the Human Research Ethics Committee (Medical) University of Witwatersrand. These committees are established to protect the rights of people taking part in research studies. The protocol has been granted approval by the committees.

IMPORTANT CONTACT DETAILS:

If at any time between your visits, you feel that any of your child's symptoms are causing problems, or you have any questions during the study, please do not hesitate to contact the study or attending doctor or the principal investigator listed on the front page of this document.

If you want any information regarding your child's rights as a research participant, or have complaints regarding this research study, you may contact Prof. Cleaton-Jones, the **Chairperson of the Research Ethics Committee** at the University of Witwatersrand (**011 717 2301**).

If you have consulted your doctor or the ethics committee and if they have not provided you with answers to your satisfaction, you should write to the Medicines Control Council (MCC) South Africa at: The Registrar, Medicine Control Council SA, Department of Health, Private Bag X828, Pretoria, 0001.

PERSONAL DOCTOR / SPECIALIST NOTIFICATION OPTION

Please indicate below, whether you want us to notify your personal doctor or your specialist of your child's participation in this study:

- **YES**, I want you to inform my personal doctor / specialist of my participation in this study.
- **NO**, I do not want you to inform my personal doctor / specialist of my participation in this study.
- **I do not have** a personal doctor / specialist.

INFORMATION LEAFLET FOR PARTICIPANT ASSENT

Good day, you are kindly invited to take part in a research study. Before you agree to take part it is important that you understand what will happen to you during the study, what will be asked of you, and what good things and bad things might be caused by the study. If you have any questions, feel free to ask your study or attending doctor.

WHY THE STUDY IS BEING DONE

The quantity of efavirenz will be measured in your blood while you are taking all of your prescribed ARV medicines.

The testing of the levels of efavirenz will help in building up a data base to enable doctors and researchers to predict specific properties of the efavirenz in children of the same age, weight and on the same ARV therapy.

It is also important for doctors to monitor your growth while you are taking the ARV medicines. This information will help them in making decisions in the future that can also possibly help you and other children on the same medication.

WHAT DOES THE STUDY INVOLVE?

Once you agree to take part you will be asked questions and you will be examined to see if you qualify to take part in this study.

During the 24-30 months of this study you will participate in **7 study related** visits. You will visit the clinic on the same routine schedule, every 3 or 6 months to see a doctor and to collect your medication as would any other patient of this clinic.

The following procedures will be followed:

1. The study nurse or investigators will record your personal and treatment details in the study folder. Certain measurements around the arm, waist and skin fold on the shoulder; weight and length will be taken and recorded.
2. Before receiving any antiretroviral medication, blood samples need to be drawn. Three blood samples (together amounting to less than 1 teaspoons or 5 ml) will be taken to test the liver, the red cells in the blood and to count the virus (viral load). Additional 4 drops (less than ¼ teaspoon) of blood will be taken and blotted on a filter paper to dry for DNA testing (read and sign

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Supplementary Informed consent on DNA sampling) and 1-1.5ml to test for virus mutations.

3. The doctor will check the dose of efavirenz and the other ARV medication that you will have to take and will adjust it if it is necessary according to your weight and length.

4. After you had started with your ARV treatment blood will have to be taken from you after 1; 3; 6; 12; 18 and 24 months of treatment. A cannula (flexible plastic needle like the end of a drip) will be inserted into your vein for drawing the blood samples. A cream will be applied before the cannula is inserted to numb your skin and to reduce possible pain. 2 x 1.5 ml blood samples (approximately ¼ of a teaspoon each); will be taken to determine the efavirenz blood concentration. 4 Blood drops (less than ¼ of teaspoon) will be blotted on a filter paper to dry for testing. Other bloods (less than 2 teaspoons) will also be taken at 3; 6; 12; 18; 24 months for routine tests (liver function; red cells; CD4 count and virus count), this would be done even if you do not take part in this study.

5. At each visit the study nurse / investigators will ask you some questions about your diet and medicines you have taken in the last few days, and when you took them.

6. The date and time for the next study visit will be arranged and confirmed.

The study visits will all take place at Harriet Shezi Children's Clinic

You, your parent or guardian will be asked to write down the exact time that you take your efavirenz dose on the 3 evening before the study visits on the provided diary cards.

WHO WILL KNOW ABOUT YOU?

The information about you will not be given to anybody except the study staff, and officials of the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, or the Health Department if they wish to inspect the study records. Your name will not be used in any publications.

Participant Initials: _____
Participant No: _____
Date approved by Wits HREC: 21/05/2007

WHAT ARE THE RISKS?

There is a risk of feeling faint, pain, bruising or bleeding when the needle is put into your vein for taking blood. There is also a slight possibility of infection. Blood is often taken as part of medical care. Your protection is that experienced doctors or nurses will take the blood and a cream will be applied to your skin before drawing the blood which should help to numb your skin against the feel of the needle prick. A total of approximately 10 teaspoons of blood will be collected over the entire duration of the study for the various routine and study tests to be performed.

HOW YOU MIGHT BENEFIT

The study will help doctors to give better treatment to children using efavirenz and to monitor their and your growth process.

Your doctor will be given the results of the efavirenz measurements in your blood. So, he/she will be able to see if the amount of efavirenz in your blood is enough, but not too much.

WITHDRAWAL

It is your right to stop taking part at any time. If you stop taking part, you will still receive normal treatment for your illnesses now and in the future.

Your parent or guardian has the right to stop you from taking part. Your doctor also has the right to

stop you from taking part if it is best for you to stop.

If the information you give for the study is untruthful, or if you disobey the rules of the study or the study clinic, you might be stopped from taking part.

Pregnancy (only for girls): If you are pregnant; breastfeeding or planning to become pregnant during the study period, you may then not take part in this study. Suitable contraceptive measures during the study and while on efavirenz treatment **should always** be taken. You will have to be withdrawn from this study immediately if you fall pregnant as the efavirenz medication will have to be changed. If you are 14 years or older then you must read and sign the Supplementary Informed Consent for Woman of Childbearing Potential. All aspects of healthcare related to pregnancy and infant will be your own responsibility

INFORMED CONSENT (parents/legal guardians) and ASSENT (participants):

..... (Study doctor / Investigators) has provided me with a copy of the Participant Information Leaflet and Consent regarding the clinical study, **protocol number: PK/PD.EFV.07**, and has fully explained to me the nature, risks, benefits and purpose of the study.

- The study doctor/investigators have given me the opportunity to ask any questions concerning the study procedures, the potential benefits and risks.
- It has been explained to me that I will be free to withdraw my child from the study at any time, without any disadvantage to future care.
- I have understood everything that has been explained to me and I consent for my child to take part in this clinical study.
- I am aware that the results of the study, including personal details regarding sex, age, date of birth, initials plus hospital number and diagnosis will be anonymously processed into a study report.

PARENT/LEGAL GUARDIAN:

Printed Name	Signature / Mark or Thumbprint	Date and Time

PARTICIPANT ASSENT: (7 years old and above)

Printed Name	Signature / Mark or Thumbprint	Date and Time

STUDY DOCTOR:

Printed Name	Signature	Date and Time

TRANSLATOR / OTHER PERSON EXPLAINING INFORMED CONSENT:

Printed Name	Signature	Date and Time

WITNESS (If applicable):

Printed Name	Signature	Date and Time

VERBAL PARTICIPANT INFORMED CONSENT:*(Applicable when participants cannot read or write or are incapable of giving consent)*

- I, the undersigned, ... (Study doctor / Investigator) have read and have explained fully to the participant, named, and/or his/her relative/friend/legal representative, ..., the participant information leaflet.
- The account I have given has explained both the possible risks and benefits of the study as well as the alternative treatments available for his/her illness. The participant and/or his/her relative/friend/legal representative understand these.
- The participant and/or his/her relative/friend/legal representative indicated that he/she understands that the participant will be free to withdraw from the study at any time for any reason and without jeopardising his/her subsequent treatment.
- I have also informed the participant and/or his/her relative/friend/legal representative of the existence of relevant compensation arrangements in case of an injury attributable to the medicine(s) used in the clinical study, to which he/she agrees.

I hereby certify that, the participant and/or his/her relative/friend/legal representative, acting on his/her behalf, has agreed to participate in this study.

PARTICIPANT:

Printed Name	Mark or Thumbprint (if applicable)	Date and Time

STUDY DOCTOR / INVESTIGATOR:

Printed Name	Signature	Date and Time

TRANSLATOR / OTHER PERSON EXPLAINING INFORMED CONSENT:..... (DESIGNATION)

Printed Name	Signature	Date and Time

PARENT/LEGALGUARDIAN/LEGALREPRESENTATIVE/FRIEND:..... (RELATIONSHIP)

Printed Name	Signature / Mark or Thumbprint	Date and Time

WITNESS:

Printed Name	Signature	Date and Time

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Participant Initials: _____
 Participant No: _____
 Date approved by Wits HREC: 21/05/2007

SUPPLEMENTARY INFORMED CONSENT FOR WOMEN OF CHILDBEARING POTENTIAL (14 YEARS AND OLDER)

- I understand that as I am a woman of childbearing potential and in order to participate in this study, I should not be pregnant or breastfeeding.
- If I am sexually active, I must be using an effective method of contraception.
- I must have a negative pregnancy test before I can enter this study. A pregnancy test will be performed before and at various intervals during the study.
- I understand that **Efavirenz (Stocrin™)** is approved by the South African Medicines Control Council (MCC). This medicine is contra-indicated during pregnancy and lactation and the use of this medicine may involve risks to my unborn child, should I become pregnant while receiving this medicine.
- If I should become pregnant, I will be immediately withdrawn from the study and be referred for medical care for my pregnancy. All aspects of health care related to my pregnancy and infant will be my own responsibility.
- The study doctor and/or a member of the study staff have reviewed information on pregnancy prevention for women of childbearing potential with me.
- I understand that I am receiving medicine and should not become pregnant while I am participating in this study or on this medication.
- I understand that I should immediately call or inform..... *(Study doctor / Investigator)* at **The Harriet Shezi Children's Clinic** if:
I am or think I may be pregnant
I have missed my menstrual cycle or it is late, or I have a change in my usual menstrual cycle (for example, heavier bleeding during my menstrual cycle or bleeding between cycles)
- I should also call if I have changed or plan to change my birth control method, or if I need to take any medication not given to me by the study doctor.
- This supplementary informed consent has been explained to me to my satisfaction and in my home language.
- I have been given the chance to ask questions and they have been answered to my satisfaction.
- I hereby voluntarily consent to participate in this study.
- I understand that I will receive a copy of this supplementary consent form.

PARTICIPANT:

Printed Name	Signature / Mark or Thumbprint	Date and Time

STUDY DOCTOR:

Printed Name	Signature	Date and Time

TRANSLATOR / OTHER PERSON EXPLAINING INFORMED CONSENT: (DESIGNATION)

Printed Name	Signature	Date and Time

WITNESS (if applicable):

Printed Name	Signature	Date and Time

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 Michelle Viljoen (PI)
 Informed consent/assent.

Participant Initials: _____
 Participant No: _____
 Date approved by Wits HREC: 21/05/2007

**INFORMATION LEAFLET AND INFORMED CONSENT FOR DNA SAMPLING
PARENT/LEGAL GUARDIAN AND PARTICIPANT/ASSENT**

**POPULATION PHARMACOKINETIC AND PHARMACODYNAMIC STUDY OF EFAVIRENZ IN HIV-1
INFECTED CHILDREN TREATED WITH FIRST LINE ANTIRETROVIRAL THERAPY IN SOUTH
AFRICA**

PK/PD.EFV.07

Department of Pharmacology, School of Pharmacy, North-West University in collaboration with Harriet Shezi Children's Clinic, Chris Hani Baragwanath Hospital, Soweto, Johannesburg

Principal investigator: Me Michelle Viljoen Contact No: 018 299 2232 or 0741013728

The co-investigators: Dr Tammy Meyers Contact No: 011 938 7784 or 0833270066
Dr Lee Kleynhans Contact No: 011 9339629 or 0827809997
Dr Hermien Gous Contact No: 011 938 8189 or 0832838360
Prof Salome Kruger Contact No: 018 299 2482 or 072 737 2235
Dr Ramoteme Mamabolo Contact No: 018 299 2466 or 0833689084

DATE OF FIRST INFORMED CONSENT DISCUSSION (**DNA**):

		20
day	month	Year

INTRODUCTION:

Good day, you are kindly invited to consider your child taking part in this research study and to carefully read the following information sheet.

You have already been informed about the research study:

Population pharmacokinetic and pharmacodynamic study of efavirenz in HIV-1 infected children treated with the first line antiretroviral therapy in South Africa

The study doctor's name is:

Dr Tammy Meyers and all other attending doctors at the Harriet Shezi Children's Clinic who are involved in the standard care and management of the children attending the clinic....

The study / attending doctor's institution is:

Harriet Shezi Children's Clinic, Chris Hani Baragwanath Hospital, Soweto, Johannesburg

You can contact the following 24-hour telephone numbers

Cell: 076 730 1769 for any study related emergency or

Cell: 074 101 3728 for any research information.

In addition to all of the other study information already provided we need to specifically inform you of the DNA sampling; storing and testing that will be done with the dried blood drops to be collected from your child during this study as described and explained in the other informed consent document.

Before agreeing to allow the sampling of DNA from your child, it is important that you read and understand the following information, study procedures, benefits and risks.

Your child's participation in this study is entirely voluntary and you can decline his/her participation, or stop at any time, without stating any reason.

This information leaflet is to help you to decide if you would like your child to take part. You should fully understand what is involved before you agree that he/she take part in the study. You should not agree that your child take part unless you are satisfied about all the procedures involved. If you have any questions, do not hesitate to ask the study doctor or principal investigator.

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Me Michelle Viljoen (PI)
Informed consent / assent DNA sampling

Participant Initials: _____
Participant No: _____
Date approved by Wits HREC: 21/05/2007

If you decide that your child may take part in this study, you will be asked to sign this document to confirm that you understand the study and agree to your child's participation. You will be given a copy to keep.

DNA SAMPLING; HANDLING AND STORAGE:

As explained in the general Informed Consent document, blood will be drawn at specific time intervals during this study.

Four (4) blood drops will be blotted on special cards before any antiretroviral (ARV) medication will be started and 1 – 1.5 ml of blood will be sampled to check for virus mutations (0 month or enrolment).

The other 4 blood drops at 1; 3; 6; 12; 18 and 24 months will also be blotted on special cards after the ARV medication had been started.

All of the dried blood spots collected on the special cards will show the following details.

- Your child's hospital number and initials
- Date and time of blood spots taken
- Study protocol number

These cards will be stored in a safe dedicated locked filing cabinet with limited access, under the direct supervision of Me Michelle Viljoen at the Department of Pharmacology, North-West University, Potchefstroom Campus.

The testing done on the sampled dried blood spots for specific DNA identification of the liver enzymes at 0; 1; 3; 6; 12; 18; 24 months will be done at LAMB (Laboratory for Applied Molecular Biology); Department of Pharmacology, North-West University, Potchefstroom Campus.

The cards will be stored for the duration of this study and for additional 2 years should certain tests or results be required to be checked or re-done.

The resistance or mutation testing on the virus will be performed by CLS (Contracted Laboratory Services), Johannesburg. The used cards as well as unused cards will have to be returned back to Department of Pharmacology, North-West University, Potchefstroom Campus for storage after resistance testing had been performed.

Protocol: PK/PD.EFV.07 Version: 2 08/05/2007
Me Michelle Viljoen (PI)
Informed consent / assent DNA sampling

RISKS AND CONFIDENTIALITY:

There are few risks related to storing your child's DNA samples. The greatest being your child's privacy. We will do everything we can to protect your child's privacy. Your child's name will not be shown on the cards with the sampled dried blood spots. Your child's hospital number and initials will be used to label the cards. No other tests or investigators (refer to above investigators) will be allowed to access your child's DNA samples. Any additional information that becomes available during the study, which may affect your willingness to allow your child to continue in this study, will be provided to you.

North-West University in collaboration with Harriet Shezi Children's Clinic will provide compensation for reasonable medical expenses incurred as a result of study-related injury or illness, determined according to the guidelines laid down by the Association of the British Pharmaceutical Industry (ABPI Guidelines), and Guidelines for Good Practice in the Conduct of Clinical Trials in Human Participants in South Africa.

You have to notify the study doctors or investigators immediately of any complications, side effects and/or injuries during the study and the nature of the expenses to be covered.

If a research related injury occurs, you have not waived any of the legal rights which you otherwise would have as a participant in this study by signing this form.

POTENTIAL BENEFITS:

There are no direct benefits to you or your child. The information that are gathered from this study will help in learning more about this disease and its complications in order to help all people who have HIV.

WITHDRAWAL:

It is your right to withdraw your child from the study at any time. Withdrawal of your child from the study will not affect his/her access or your access to medical treatment in any way.

All sampled DNA will be destroyed after the withdrawal of your child from this study. Every effort will be made to respect the wishes of the parent; legal guardian and participant in this regard.

Participant Initials: _____
Participant No: _____
Date approved by Wits HREC: 21/05/2007

ETHICAL APPROVAL:

The study protocol has been submitted to the Human Research Ethics Committee (Medical) University of Witwatersrand. These committees are established to protect the rights of people taking part in research studies. The protocol has been granted approval by the committees.

IMPORTANT CONTACT DETAILS:

If at any time you have questions about allowing your child to take part or about storage or use of the DNA samples, please contact the principal investigator listed on the front page of this document.

If you want any information regarding your child's rights as a research participant, or have complaints regarding this research study, you may contact Prof. Cleaton-Jones, the **Chairperson of the Research Ethics Committee** at the University of Witwatersrand (**011 717 2301**).

If you have consulted your doctor or the ethics committee and if they have not provided you with answers to your satisfaction, you should write to the Medicines Control Council (MCC) South Africa at: The Registrar, Medicine Control Council SA, Department of Health, Private Bag X828, Pretoria, 0001.

INFORMATION LEAFLET FOR PARTICIPANT/ASSENT: EFAVIRENZ

Good day, you are kindly invited to take part in this research study and to carefully read the following information sheet. You have already been informed about the research study:

Population pharmacokinetic and pharmacodynamic study of efavirenz in HIV-1 infected children treated with the first line antiretroviral therapy in South Africa

In addition to all of the other study information already provided we need to specifically inform you of the DNA sampling; storing and testing that will be done with the dried blood drops that will be collected from you during this study as described and explained in the other informed consent document.

Before agreeing to allow the sampling of your DNA, it is important that you read and understand the following information, study procedures, benefits and risks.

Your participation in this study is entirely voluntary and you can decline to participate, or stop at any time, without stating any reason.

This information leaflet is to help you to decide if you would like to take part. You should fully understand what is involved before you agree to take part in the study. If you have any questions, do not hesitate to ask the study doctor or principal investigator.

If you decide to take part in this study, you will be asked to sign this document to confirm that you understand the study and agree to take part. You will be given a copy to keep.

DNA SAMPLING; HANDLING AND STORAGE:

As explained in the general Informed Consent document, blood will be drawn at specific time intervals during this study.

Four (4) blood drops will be blotted on special cards before any antiretroviral (ARV) medication will be started and 1 – 1.5 ml of blood will be

sampled to check for virus mutations (0 month or enrolment).

The other 4 blood drops at 1; 3; 6; 12; 18 and 24 months will also be blotted on the special cards after you had started your ARV treatment.

All of the dried blood spots collected on the special cards will show the following details.

- Your hospital number and initials
- Date and time of blood spots taken
- Study protocol number

These cards will be stored in a safe dedicated locked filing cabinet with limited access, under the direct supervision of Me Michelle Viljoen at the Department of Pharmacology, North-West University, Potchefstroom Campus.

The cards will be stored for the duration of this study and for additional 2 years should certain tests or results be required to be checked or re-done.

The testing done on the sampled dried blood spots for specific DNA identification of the liver enzymes at 0; 1; 3; 6; 12; 18; 24 months will be done at LAMB (Laboratory for Applied Molecular Biology); Department of Pharmacology, North-West University, Potchefstroom Campus.

The resistance or mutation testing on the virus will be performed by CLS (Contracted Laboratory Services), Johannesburg. The used cards as well as unused cards will have to be returned back to Department of Pharmacology, North-West University, Potchefstroom Campus for storage after resistance testing had been performed

WHAT ABOUT THE RISKS?

The information about you will not be given to anybody except the study staff, and officials of the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, or the Health Department if they wish to inspect the study records. Your name will not be used in any publications. We will do everything we can to protect your privacy. Your name will not be shown on the cards with the sampled dried blood spots. Your hospital number and initials will be used to label the cards. No other tests or

Protocol: PK/PD.EFV.07 Version: 2 08/05/2007
Me Michelle Viljoen (PI)
Informed consent / assent DNA sampling

Participant Initials: _____
Participant No: _____
Date approved by Wits HREC: 21/05/2007

investigators (refer to above investigators) will be allowed to access your DNA samples.

HOW YOU MIGHT BENEFIT

There are no direct benefits to you. The information that are gathered from this study will help in learning more about this disease and its complications in order to help all people who have HIV.

WITHDRAWAL

It is your right to stop taking part at any time. If you stop taking part, you will still receive normal treatment for your illnesses now and in the future. All your sampled DNA will be destroyed after your withdrawal from this study. Every effort will be made to respect your wishes in this regard.

IMPORTANT CONTACT DETAILS:

If at any time you have questions about the storage or use of the DNA samples, please contact the principal investigator listed on the front page of this document.

If you want any information regarding your rights as a research participant, or have complaints regarding this research study, you may contact Prof. Cleaton-Jones, the **Chairperson of the Research Ethics Committee** at the University of Witwatersrand (**011 717 2301**).

If you have consulted your doctor or the ethics committee and if they have not provided you with answers to your satisfaction, you should write to the Medicines Control Council (MCC) South Africa at: The Registrar, Medicine Control Council SA, Department of Health, Private Bag X828, Pretoria, 0001.

INFORMED CONSENT (parents/legal guardians) and ASSENT (participants):

..... (STUDY DOCTOR/INVESTIGATORS) has provided me with a copy of the Participant Information Leaflet and Consent regarding the clinical study, **protocol number: PK/PD.EFV.2007**, and has fully explained to me the nature, risks, benefits and purpose of the study.

- The study doctor/investigators have given me the opportunity to ask any questions concerning the study procedures, the potential benefits and risks.
- It has been explained to me that I will be free to withdraw my child from the study at any time, without any disadvantage to future care.
- I have understood everything that has been explained to me and I consent for my child to provide his/her DNA samples to be taken for testing as specified.

PARENT/LEGAL GUARDIAN:

Printed Name	Signature / Mark or Thumbprint	Date and Time

PARTICIPANT ASSENT: (7 years old and above)

Printed Name	Signature / Mark or Thumbprint	Date and Time

STUDY DOCTOR:

Printed Name	Signature	Date and Time

TRANSLATOR / OTHER PERSON EXPLAINING INFORMED CONSENT:

Printed Name	Signature	Date and Time

WITNESS (If applicable):

Printed Name	Signature	Date and Time

Addendum A3



Case Report Forms (PK/PD.EFV.07)

CRF: PK/PD.EFV.07**Efavirenz (EFV)****Patient Enrolment****STUDY PARTICIPANT DETAILS:**

Study number			
Hospital & folder number			
Date of Birth			
Gender	Male	Female	
Language: Zulu Xhosa Sesotho Tswana Venda Pedi			
Date of consent:	(DD/MM/YYYY)		

CONTACT INFORMATION:

Parent's / Guardian's name	
Telephone number	
Address	
Other parent/guardian/ caregiver	
Telephone number	

Completed by: _____ Signature: _____ Date: _____ (DD/MM/YYYY)

ELIGIBILITY – Dr to complete

Include if:	✓	Exclude if:	✓
Age > 3-16 years and > 10 kg		Kidney, liver or GIT disease	
Medically stable		Chronic diarrhoea	
CONSENT of parent/ guardian		Expect PK drug-drug interactions	
Efavirenz based HAART (First line regimen)		Medication such as rifampicin; phenobarbitone; carbamazepine; isoniazide (INH); erythromycin; cyclosporine; ketoconazole; metronidazole; ciprofloxacin; corticosteroids; cimetidine.	
Cotrimoxazole prophylaxis if applicable		TB infection	

DIAGNOSIS & HAART MEDICATION – Dr to complete

Date RVD diagnosed	(DD/MM/YYYY)
Date HAART started	(DD/MM/YYYY)
Current staging	
Latest CD 4 count	(number) (%)
Latest viral load	
Latest ALT	
Latest FBC	

Treatment adherence assessment

Diary Card completion	Medication count

Pharmacokinetics

	Exact time	Time interval (hr)
EFV DOSE given/taken last night =		
1 st Blood sample (clinic arrival) taken		
2 nd Blood sample (1-2 hours after clinic arrival)		

NEXT STUDY VISIT SCHEDULE

2 nd PK sampling date (after 3 months)	(DD/MM/YYYY)
time to arrive at clinic	
3 rd PK sampling date (after 6 months)	(DD/MM/YYYY)
Time to arrive at clinic	
4 th PK sampling date (after 12 months)	(DD/MM/YYYY)
Time to arrive at clinic	
5 th PK sampling date (after 18 months)	(DD/MM/YYYY)
Time to arrive at clinic	
6 th PK sampling date (after 24 months)	(DD/MM/YYYY)
Time to arrive at clinic	

Completed by: _____ Signature: _____ Date: _____ (DD/MM/YYYY)

Anthropometric measurements

Weight				
Height				
Middle arm circumference				
Waist circumference				
Skin fold (sub scapular)				
Skin fold (triceps)				
BIA: Resistance / Reactance				
Has the child any signs of dehydration, oedema?				
Tanner stage (breast/genital dev.)				
Tanner stage (hair distribution)				
Girls: Started menstruation Y N	If yes, age at menarche: _____			
Diet checklist:				
Garlic	Daily	2-3/week	1/week	seldom never
Grapefruit	Daily	2-3/week	1/week	seldom never
Coffee	Daily	2-3/week	1/week	seldom never
Pepper	Daily	2-3/week	1/week	seldom never
Alcohol	Daily	2-3/week	1/week	seldom never
St John's Wort	Daily	2-3/week	1/week	seldom never

Completed by: _____ Signature: _____ Date: _____ (DD/MM/YYYY)

CRF:	PK/PD.EFV.07	Efavirenz (EFV)

Occasion: 1; 3; 6; 12; 18; 24 Months

Study number		
Hospital & folder number		
Date of Birth	(DD/MM/YYYY)	
Gender	Male	Female

Medication and doses:

HAART- First line regimen		
Medication:	Dose:	
Clinical condition / Signs of toxicity or ARV side effects		
Medication other than HAART (including supplements, herbal remedies and complementary medication)		
Medication:	Dose:	

Completed by: _____ Signature: _____ Date: _____ (DD/MM/YYYY)

Routine tests done as per DOH guidelines

Regimen	Test	Frequency * Except for 3 month visit	Date of test	Result
Efavirenz (+ 2 NRTI's)	CD4	*6-monthly		(%)
	Viral load	*6-monthly		
	ALT	*6-monthly		
	FBC: WBC	*6-monthly		
	FBC: Hb	*6-monthly		
	FBC: Plt	*6-monthly		
	Cholesterol	24-months		
	Triglycerides	24-months		

Completed by: _____ Signature: _____ Date: _____ (DD/MM/YYYY)

Dept. Pharmacology, North-West University in collaboration with Harriet Shezi Children's Clinic
(Chris Hani Baragwanath Hospital)

HAART medication & doses	Medication:	Dosage:

Dr. Completed: _____ Signature: _____ Date: _____ (DD/MM/YYYY)

MEDICATION OTHER THAN HAART		
Medication:	Dosage:	
Received TB treatment before	Yes	No
If "Yes", state the medication used for TB treatment		
Medication:	Dosage:	
Date of treatment completed	(DD/MM/YYYY)	

Pharmacist : _____ Signature: _____ Date: _____ (DD/MM/YYYY)

NEXT STUDY VISIT SCHEDULE

1 st PK sampling date (after 1 month)	(DD/MM/YYYY)
time to arrive at clinic	
2 nd PK sampling date (after 3 months)	(DD/MM/YYYY)
Time to arrive at clinic	

Pharmacist : _____ Signature: _____ Date: _____ (DD/MM/YYYY)

MEASUREMENTS & DIETARY ASSESMENT

Weight				
Height				
Middle arm circumference				
Waist circumference				
Skin fold (sub scapular)				
Skin fold (triceps)				
Tanner stage (breast/genital dev.)				
Tanner stage (hair distribution)				
Girls: Started menstruation Y N	If yes, age at menarche:			
<u>Diet checklist:</u>				
Garlic	Daily	2-3/week	1/week	seldom never
Grapefruit	Daily	2-3/week	1/week	seldom never
Coffee	Daily	2-3/week	1/week	seldom never
Pepper	Daily	2-3/week	1/week	seldom never
Alcohol	Daily	2-3/week	1/week	seldom never
St John's Wort	Daily	2-3/week	1/week	seldom never

Nutritionist to complete: _____ Signature: _____ Date: _____ (DD/MM/YYYY)

Addendum A4

**Ethics Approval
(PK/PD.EFV.07)**



Wits Clinical Research

8 Blackwood Avenue, Parktown, 2193, South Africa
Tel. +27-11-274-9200, Fax: +27-11-274-9281
Postnet Suite 189, Private Bag x2600, Houghton, 2041

FAXED & COURIE

Mrs M Viljoen,

Harriet Shezi Children's Clinic
Chris Hani Baragwanath Hospital
Old Potch Road
Soweto
2013

Fax: 018 299 2225

Dear Mrs Viljoen,

PROTOCOL NO: PK/PD.EFV.07

**PROTOCOL TITLE: Population Pharmacokinetic and Pharmacodynamic Study of
Efavirenz in HIV-1 Infected Children Treated with First Line Antiretroviral Therapy in
South Africa**

PRC REFERENCE NUMBER: 070413

Please be advised that your trial application was:

APPROVED

The Expert Reviewers were: Dr M Mokhachane

Also reviewed by: Mrs R Wills: Acting Chairperson Protocol Review Committee
Dr S Khan: Gauteng Department of Health
Dr W Manning - Chris Hani Baragwanath

Yours sincerely


Acting **MS RENÉ WILLS**
Chairperson: Protocol Review Committee
21 May 2007

cc.

National Research Foundation Ms M Viljoen

Tel 018 299 2232 Cell 074 101 3728 Fax 018 299 2225



NORTH-WEST UNIVERSITY
YUNIBESITHI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT

Ethics Committee

Tel +27 18 +27 18 299 1366

Fax +27 18 +27 18 297 5308

Email Ethics@nwu.ac.za

Dear Ms M Viljoen (project leader)

25 June 2007

ETHICS APPROVAL OF PROJECT

The North-West University Ethics Committee (NWU-EC) hereby approves your project as indicated below. This implies that the NWU-EC grants its permission that, provided the special conditions specified below are met and pending any other authorisation that may be necessary, the project may be initiated, using the ethics number below.

Project title: Population Pharmacokinetic and Pharmacodynamic study of Efavirenz in HIV-1 infected Children Treated with First Line Antiretroviral Therapy in South Africa															
Ethics number:	N	W	U	-	0	0	0	1	5	-	0	7	-	A	5
	Institution				Project Number					Year			Status		
Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation															
Approval date: 25 June 2007										Expiry date: 25 June 2012					

Special conditions of the approval (if any):

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- The project leader (principle investigator) must report in the prescribed format to the NWU-EC:
 - annually (or as otherwise requested) on the progress of the project,
 - without any delay in case of any adverse event (or any matter that interrupts sound ethical principles) during the course of the project.
- The approval applies strictly to the protocol as stipulated in the application form. Would any changes to the protocol be deemed necessary during the course of the project, the project leader must apply for approval of these changes at the NWU-EC. Would there be deviation from the project protocol without the necessary approval of such changes, the ethics approval is immediately and automatically forfeited.
- The date of approval indicates the first date that the project may be started. Would the project have to continue after the expiry date, a new application must be made to the NWU-EC and new approval received before or on the expiry date.
- In the interest of ethical responsibility the NWU-EC retains the right to:
 - request access to any information or data at any time during the course or after completion of the project;
 - withdraw or postpone approval if:
 - any unethical principles or practices of the project are revealed or suspected,
 - it becomes apparent that any relevant information was withheld from the NWU-EC or that information has been false or misrepresented,
 - the required annual report and reporting of adverse events was not done timely and accurately,
 - new institutional rules, national legislation or international conventions deem it necessary.

The Ethics Committee would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the Ethics Committee for any further enquiries or requests for assistance.

Yours sincerely

Prof M Löwes
(chair NWU Ethics Committee)

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Addendum B1

**SOP-001
(PK/PD.EFV.07)**

DEPARTMENT OF PHARMACOLOGY - NWU			
STANDARD OPERATING PROCEDURE			
Subject:	VENOUS BLOOD COLLECTION FOR EFAVIRENZ PLASMA LEVEL DETERMINATIONS AND GENOTYPING – PK/PD.EFV.07		
Document no.:	SOP-001	Amendment no.:	
Effective date:	September 2007	Revision date:	
Compiled by:	M.Viljoen	Signature:	
Approved by:	Dr M Rheeders	Signature:	

1. PURPOSE

To ensure that venous blood is collected correctly and safely during the PK/PD.EFV.07 clinical trial to determine efavirenz plasma levels and genotyping of the study participants.

2. SCOPE

This procedure outlines the collection; handling, centrifuging and storage of venous blood from study participants of PK/PD.EFV.07.

3. RESPONSIBILITIES

Study doctor, study nurse or pharmacist involved in study PK/PD.EFV.07.

4. DEFINITIONS AND ABBREVIATIONS

PK- Pharmacokinetic

EDTA-

1PK1 – 1st PK visit, first sample

1PK2 - 1st PK visit, second sample

2PK1 – 2nd PK visit, first sample

2PK2 – 2nd PK visit, second sample

5. PROCEDURE

5.1 Collection of venous blood

Emla™ cream is to be placed onto the skin (preferably the arm fold) when the study participant reports to the clinic. A canulae is inserted into the vein approximately 20 minutes post Emla™ application by the study doctor or research nurse.

PK blood is to be collected twice on each study visit after 1;3;6;12;18;24 months of anti-retroviral therapy commenced.

DEPARTMENT OF PHARMACOLOGY - NWU			
STANDARD OPERATING PROCEDURE			
Subject:	VENOUS BLOOD COLLECTION FOR EFAVIRENZ PLASMA LEVEL DETERMINATIONS AND GENOTYPING – PK/PD.EFV.07		
Document no.:	SOP-001	Amendment no.:	
Effective date:	September 2007	Revision date:	
Compiled by:	M.Viljoen	Signature:	
Approved by:	Dr M Rheeders	Signature:	

The first (*1PK1*) blood sample is taken when the canulae is inserted. 3 ml of whole blood (*1PK1*) for the efavirenz determination is collected into an EDTA vacutainer marked with a label reflecting the participant number; sampling occasion; sampling time and date.

The exact time of this *1PK1* sample must be recorded on the CRF and 3 printed labels provided for the 1 EDTA vacutainer and 2 polypropylene tubes (Epindorph tubes).

Sterile normal saline 0.9% for intravenous use is placed into a smallbore extension set and directly attached to the inserted canulae after the *1PK1* blood sample is taken.

The second (*1PK2*) blood sample is taken approximately 1-2 hours after *1PK1*. The saline is evacuated from the smallbore extension set before 3 ml of whole blood is to be sampled. This blood is collected into an EDTA vacutainer marked with a label reflecting the participant number; sampling occasion; sampling time and date.

The exact time of this *1PK2* sample must be recorded on the CRF and 3 printed labels provided for the 1 EDTA vacutainer and 2 polypropylene tubes (Epindorph tubes).

5.2 Dried Blood Spot (DBS) prior to centrifuging of whole blood

4 x 125 µl is taken from the first PK (*1PK1*; *2PK1*; *3PK1*) sample and spotted separately on Whatman FTA cards. The FTA card must be marked with participant number; GT number; sampling occasion (baseline; *1PK1*; *2PK1*; *3PK1*) and date. The FTA card is allowed to dry at room temperature for 60 minutes before it is placed into a plastic bag with silica gel granules for long term storage. All of the DBS must be securely stored in a locked cabinet.



Page 2 of 4

DEPARTMENT OF PHARMACOLOGY - NWU			
STANDARD OPERATING PROCEDURE			
Subject:	VENOUS BLOOD COLLECTION FOR EFAVIRENZ PLASMA LEVEL DETERMINATIONS AND GENOTYPING – PK/PD.EFV.07		
Document no.:	SOP-001	Amendment no.:	
Effective date:	September 2007	Revision date:	
Compiled by:	M.Viljoen	Signature:	
Approved by:	Dr M Rheeders	Signature:	



5.3 Centrifuge of whole blood; storage of plasma and buffy coat

The collected whole blood in the marked EDTA vacutainer is kept on ice if not being centrifuged immediately. The temperature is set at 4°C and centrifugation must be performed for 10 minutes at 3000 rpm within 1 hour of collection. From each of the centrifuged whole blood samples, 2 x 0.4-0.5 ml aliquots of plasma will be transferred into duplicate labelled Epindorph tubes suitable for storage at -80°C. Store the sampled plasma at -20°C for up to 1 week or for long term storage at -80°C until analysis. The label on each Epindorph tube must include the participant number, sampling occasion (1PK1; 1PK2; 2PK1; 2PK2; 3PK1; 3PK2 etc), the sampling time and date.



The remaining buffy coat (BC) in the labelled EDTA vacutainers must be separated from the red blood cells with a plastic pipette. The quantity of BC should be more than 100 µl and must be stored in a marked Epindorph tube labelled with participant number, sampling occasion, sampling

DEPARTMENT OF PHARMACOLOGY - NWU			
STANDARD OPERATING PROCEDURE			
Subject:	VENOUS BLOOD COLLECTION FOR EFAVIRENZ PLASMA LEVEL DETERMINATIONS AND GENOTYPING – PK/PD.EFV.07		
Document no.:	SOP-001	Amendment no.:	
Effective date:	September 2007	Revision date:	
Compiled by:	M.Viljoen	Signature:	
Approved by:	Dr M Rheeders	Signature:	

time and date. Store at -20°C for up to 1 week or for longer term storage at -80°C until DNA or RNA isolation is carried out.



5.4 Discard of whole blood

All remaining whole blood in EDTA tubes must be safely discarded into provided bio-hazard container after plasma and buffy coat have been removed/separated.

Addendum B2

**SOP-002
(PK/PD.EFV.07)**

DEPARTMENT OF PHARMACOLOGY - NWU			
STANDARD OPERATING PROCEDURE			
Subject:	EFAVIRENZ PLASMA PREPARATION PRIOR TO LC/MS – PK/PD.EFV.07		
Document no.:	SOP-002	Amendment no.:	Version 2
Effective date:	March 2010	Revision date:	9 March 2010
Compiled by:	M.Viljoen	Signature:	
Approved by:	Dr M Rheeders	Signature:	

1. PURPOSE

To prepare and clean up the plasma prior to injection into LC/MS to determine efavirenz plasma levels of the study participants.

2. SCOPE

This procedure outlines the precipitation; handling and centrifuging of plasma samples.

3. RESPONSIBILITIES

LC/MS analyst in study PK/PD.EFV.07.

4. DEFINITIONS AND ABBREVIATIONS

1PK1 – 1st PK visit, first sample

1PK2 - 1st PK visit, second sample

5. PROCEDURE

Remove the specific labelled samples from freezer storage (-75 to -80°C) to thaw on work bench. Transfer 100 µl of the plasma to a clearly individually marked eppendorf tubes. The eppendorf tubes must be labelled according to the study participant number and the specific PK sampling time. Place the transferred 100 µl samples in the pre-heated oven (Labcon Incubator) for 30 minutes at 56°C to de-activate the HIV-1 virus. Remove samples from oven and allow to reach room temperature.

Add the following to the 100 µl plasma samples:

1. 40 µl I.S. (Midasolam – 1.325 µg/ml. Diluted from working solution of Diazepam Ampulles)
2. 160 µl MeOH (LC/MS grade)
3. 40 µl ZnSO₄ (0.2 M)

DEPARTMENT OF PHARMACOLOGY - NWU			
STANDARD OPERATING PROCEDURE			
Subject:	EFAVIRENZ PLASMA PREPARATION PRIOR TO LC/MS – PK/PD.EFV.07		
Document no.:	SOP-002	Amendment no.:	Version 2
Effective date:	March 2010	Revision date:	9 March 2010
Compiled by:	M.Viljoen	Signature:	
Approved by:	Dr M Rheeders	Signature:	

Vortex for 10 sec (important step, not to be skipped). Centrifuge for 10 min at 14 000 rpm (4.4 x 10 – Nutrition Department). Transfer 180ul of supernatant (must be visibly clear) into clearly marked inserts/glass vials ready for LC/MS injection.

Calculations: 0.2 M ZnSO₄

ZnSO₄·7H₂O = 287.54 mm

1 M/5 = 287.54 g in 1000 ml

Thus 0.2 M = 57.51 g in 1000 ml

Or 2.88 g in 50 ml of Water (LC/MS grade)

Addendum B3

**SOP-003
(PK/PD.EFV.07)**

DEPARTMENT OF PHARMACOLOGY - NWU			
STANDARD OPERATING PROCEDURE			
Subject:	EFAVIRENZ PLASMA ANALYSIS: LC/MS – PK/PD.EFV.07		
Document no.:	SOP-003	Amendment no.:	Version 2
Effective date:	March 2010	Revision date:	9 March 2010
Compiled by:	M.Viljoen	Signature:	
Approved by:	Dr M Rheeders	Signature:	

1. PURPOSE

To set up the method for efavirenz plasma analysis on the LC/MS.

2. SCOPE

This procedure outlines the preparation of mobile phase; injection volume; flow rate; retention times and quantitative analysis (regression).

3. RESPONSIBILITIES

LC/MS analyst in study PK/PD.EFV.07.

4. DEFINITIONS AND ABBREVIATIONS

Efv – Efavirenz

LC/MS – Liquid chromatography / Mass spectrometer

5. PROCEDURE

Place all samples to be analysed (post preparation according to SOP-002) in the sample tray at 4°C. Open Acquisition on the Mass Hunter Software. Search and choose the correct Method Name (D:\MassHunter\methods\ARV_Mid08_MRMFinal.m):

Flow rate (LC) = 0.5 ml/min;

Retention times (minutes) : Lam = 3.2-3.5; Mid = 6.7-6.8 ; EFV= 7.1-7.2

Injection volume = 5 ul

Record all settings as required on additional log book of the specific LC/MS apparatus.

Allow for at least 20 minutes for the mobile phase to reach equilibrium after pump has been switched on before samples are injected.

DEPARTMENT OF PHARMACOLOGY - NWU			
STANDARD OPERATING PROCEDURE			
Subject:	EFAVIRENZ PLASMA ANALYSIS: LC/MS – PK/PD.EFV.07		
Document no.:	SOP-003	Amendment no.:	Version 2
Effective date:	March 2010	Revision date:	9 March 2010
Compiled by:	M.Viljoen	Signature:	
Approved by:	Dr M Rheeders	Signature:	

Set up a worklist and use the correct file names to correspond with plasma samples (1_1PK1; 30_2PK2); calibrators (PIs/date/L1 to L8); QC's (QC2/date; QC6/date; QC8/date) and blank (Blk/date). Data file: d:\Masshunter\Data\ARV\MidasolamIstd\PKSamples (Date??)

Preparation of Mobile Phase A & B:

Mobile Phase A:

10% Methanol / 90% Water (LC/MS Grade)

Mobile Phase B:

LC-MS Grade Reagents	1000 ml	500 ml	250 ml
MeOH	968 ml	484	242
H ₂ O	30 ml	15	7.5
CH ₃ COOH 0.1%	1 ml	0.5	0.25
NH ₄ Formate 5M	1 ml	0.5	0.25

Final NH₄Formate = 5mM

Record pH (5.6-5.9) =

Record Date:

Quantitative Analysis (via PC) – Regression/Calibration curve/Batch Analysis

1. File ; New Batch – Type in Batch File Name according to analysis Date
2. File; Add Samples – Choose Calibs (L1-L8)
3. Method; New Method from acquired data – Opens up Quantitative Analysis screen. Select L5 Calibrant to do the method/regression setup.

Go through all the steps under Method Setup Tasks

- a) MRM Compound setup: Delete reference to Compound 1 (optional).

DEPARTMENT OF PHARMACOLOGY - NWU			
STANDARD OPERATING PROCEDURE			
Subject:	EFAVIRENZ PLASMA ANALYSIS: LC/MS – PK/PD.EFV.07		
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Compiled by:	M.Viljoen	Signature:	
Approved by:	Dr M Rheeders	Signature:	

EFV - Prefix = L # Levels=8; Enter highest concentration = 12; Dilution 1:2; Create levels
 Lamivudine - Prefix = L # Levels=8; Enter highest concentration = 12; Dilution 1:2; Create levels

Midasolam – No levels to be created. Enter concentration 1.325 only

- b) Check Retention Time Setup
 - c) ISTD Setup – Select Mid as Istd for EFV and Lamivudine. Enter Midasolam Istd concentration = 1.325
 - d) Concentration setup – Change units to ug/ml for all 3 components
 - e) Check Qualifier setup (Uncertainty = 20)
 - f) Check Calibration Curve Setup – Default settings
4. Validate – No errors, then continue
 5. Save As – Choose correct folder to save method file (quantmethod.xml) – Use date of analysis as file name.
 6. Exit – Would you like to apply this method to batch – YES.
 7. Return to Batch Analysis for calibration curve / regression or to add samples to be added to batch for further analysis.

Addendum B4

**SOP-004
(PK/PD.EFV.07)**

Exp no.:	SOP-004	Experimenter:	M.Viljoen
Date:	2009-09-09	Study Leader:	Dr M Rheeders
Ethics#:	NWU-00015-07-A5	Lab:	LAMB (building G16, room G14)
Title:	CYP2B6 (G516T) - (PK/PD.EFV.07)		

1. Check List

I calculated below (with understanding) how much will be needed of each and every chemical, etc..	☒	I checked that sufficient quantities of all chemicals, consumables, etc. needed are available.	☒
I ensured that all apparatus needed are available and reservations made where applicable.	☒	I understand all assays & protocols and operation of instruments to be used in the experiment.	☒
I understand all potential hazards and have all safety measures in place for the experiment.	☒	I reported all chemicals, consumables, etc. that are nearly finished and that needs to be re-ordered.	☒
My study leader checked and approved the experimental protocol.	☐	My study leader checked and approved the results (after experiment).	☐
Stamp 1: Protocol Approval		Stamp 2: Results & Filing Approval	

2. Background & Objectives

Explanation: Why do you need to perform this experiment and what do you wish to investigate? Refer to results of previous experiments, any questions that needs to be answered, any standardisation needed, any repeats of experiments, etc. The person who reads this, should understand why the experiment is done, even if he/she is not familiar with the project, and also what you are trying to investigate by performing this experiment.

To investigate the presence or not of the mutation (G516T) on the CYP2B6 human gene in the study population of the approved clinical trial PK/PD/EFV.07

3. Experimental Layout

Explanation: Describe here how you have planed the experiment (controls, test groups, test drugs, etc.) You may describe or give a flow chart. Do not give details of assays, chemicals, quantities, etc. here.

DNA obtained from buffy coat and dried blood spots (FTA-Whatman cards) of study participants as per Qiagen FlexiGene DNA Kit.

4. Procedures, Assays & Protocols

Explanation: Describe here which techniques you wish to employ. Describe in detail the procedures assays and protocols almost like a recipe describing the apparatus, drugs, chemicals & solutions, animal objects/biological tissue/cultures etc. and each step.

The standard PCR amplified product is run on a 2% agarose gel for the 526 bp fragment and again after the digestion with BseNI (BsrI) restriction enzyme for the possible fragments of 23; 236; 267 and 503 bp on a 3% agarose gel.

Normal Digest: BseNI (BsrI) - Fermentas. PCR product in a tightly closed vial was heated in a temperature block for 3 hours at 65°C according to the manufacturer's instructions.

FastDigest BseNI - Fermentas. PCR product in a tightly closed vial was heated in a temperature block for 10 min at 65°C and 5 min at 80°C according to the manufacturer's instructions.

4.1. Procedure/Assay/Protocol 1

Conventional PCR for genotyping of CYP2B6 exon 4 polymorphism G516T (Gln172His).

PCR reaction x1 (25ul):

Dream Taq 12.5 ul

FW2 1.25 ul

RW2 1.25 ul

H2O 9.0 ul

DNA (50ng/ul) 1.0 ul

FW2 primer (Lang, Klein et al 2001): 5'GGTCTGCCCATCTATAAAC 3'

RW2 primer (Lang, Klein et al 2001): 5' CTGATTCTTCACATGTCTGCG 3'

PCR Amplification conditions:

1. denaturation at 95°C for 3 min,

2. 30 Cycles of: denaturation at 95°C for 30s, annealing at 59°C for 30s and extension at 72°C for 1 min.

3. A final extension step at 72°C for 10 minutes completed the reaction.

The 526bp PCR product (4 ul) can be loaded on a 2% Agarose gel with a DNA ladder (O'GeneRuler 50 bp, Fermentas Life Science, Burlington, Canada), 3.5 ul.

-
-

4.2. Procedure/Assay/Protocol 2

BseNI (BsrI) Enzyme Digestion (FAST)

The amplified PCR product (526 bp amplicon) is digested with FAST BsrI restriction enzyme (Fermentas) at the following conditions:

10 min 65°C incubation followed by 5 min at 80°C to inactivate the enzyme. The digested PCR products (10 ul) are electrophoresed on 3% agarose gel and loading O'GeneRuler (Low range) DNA ladder (4 ul).

The homozygous wild-type or normal genotype (516G/G) resulted in THREE fragments that were 23, 236 and 267 base pairs.

The homozygous mutant genotype (516T/T) resulted in TWO digested fragments of 23 and 503 base pairs.

The heterozygous genotype (516G/T) resulted in FOUR fragments 23, 236, 267 and 503 base pairs.

The normal digest reaction (30.5 ul):

PCR product	10ul
10 x Buffer B	2ul
Bsrl Enzyme	0.5 ul
H2O	18ul

The FastDigest reaction (30ul):

PCR product	10ul
Fast Digest Buffer	2ul
Bsrl Enzyme	1ul
H2O	17ul

•

4.3. Procedure/Assay/Protocol 3

NOT APPLICABLE

•

•

5. Calculations

Explanation: Type detailed calculation here (e.g. exactly how much you will need of every substance mentioned in an assay, how to prepare cocktails and solutions, where substances are kept, etc.). Always allow about 10% extra. You should have all calculations done before the experiment starts, since there may be insufficient time to struggle with this during the experiment. You should also use the calculation to ensure that you have enough of each ingredient before the experiment starts.

NOT APPLICABLE

5.1. Calculation 1

NOT APPLICABLE

5.2. Calculation 2

5.3. Calculation 3

Addendum C1

Instructions to the Author: AIDS Research & Human Retroviruses

<http://www.liebertpub.com/products/manuscript.aspx?pid=2>

AIDS Research and Human Retroviruses is a peer-reviewed journal in the English language. The Journal will publish Full and Short Communications, Letters to the Editor, Perspectives, Sequence Notes, Mini-reviews, and Conference or Workshop Summaries as deemed appropriate.

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Animal Models

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Other Human Retroviruses

Mitsuaki Yoshida, Ph.D., Dept. of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa Chiba, Japan.

Sequence Notes

Brian Foley, Ph.D., HIV Sequence Database, Los Alamos National Laboratory, Los Alamos, NM.

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SEQUENCE DATA

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Addendum C2



Instructions to the Author: Clinical Pharmacology & Therapeutics

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Clinical Pharmacology & Therapeutics

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Title Page

Abstract

Introduction

Results

Discussion

Methods (must contain IRB or IACUC approval: see **Informed Consent and Ethics** below)

Acknowledgements

Conflict of Interest/Disclosure

References

Figures Legends

Figures

Tables

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Clinical Pharmacology & Therapeutics

CLINICAL TRIALS: GUIDE TO AUTHORS

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ACKNOWLEDGMENTS

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Addendum D1



Descriptive Statistics

Table D1.1: Descriptive statistics (baseline and 1-24 months post-HAART)

	N	Mean	CI -95%	CI +95%	Geo. Mean	Median	Minimum	Maximum	IQR 25%	IQR 75%	SD	CV
AgeBaseline	60	6.3	6.1	7.6	6.22	7.00	3.0000	14	4.50	9.0	2.9	42.1017
CDcountBase	60	504.3	408.2	600.3	338.24	490.00	7.0000	1611	210.50	657.0	371.7	73.7158
CD%Base	60	14.5	12.5	16.5	11.33	14.40	0.4800	35	8.44	18.8	7.8	53.8997
VLBase	60	188009			49392.7	52500	640.0000	3000000	150000.0	1650000.0	424827.4	225.96
ALTBase	60	22.0	17.1	27.0	19.08	18.50	7.0000	158	14.50	25.5	19.3	87.4250
WeightBase	60	20.2	18.5	21.9	19.17	18.94	9.2000	41	14.85	24.0	6.7	33.2151
LengthBase	60	113.7	109.1	118.4	112.35	113.93	82.1000	162	100.55	125.6	17.9	15.7790
1PK2 (1Mnth)	59	3.1	2.3	3.9	2.06	1.86	0.2100	18	1.29	3.8	3.0	98.9488
Log1PK2	59	0.3	0.2	0.4		0.27	-0.6778	1	0.11	0.6	0.4	128.0037
Time2	59	17.2	16.4	17.9	16.98	16.58	13.7500	38	16.03	17.8	3.1	17.8333
1PK1	57	3.40	2.5	4.3	2.30	2.29	0.2500	18.2	1.39	3.77	3.27	96.3167
Log1PK1	57	0.36	0.3	0.47		0.36	-0.6000	1.26	0.14	0.58	0.40	111.5615
Time1	57	15.5	14.6	16.3	15.3	14.9	11.5000	36.97	14.33	15.83	3.1	20.2091
Dose1	59	274.6	255.9	293.2	266.75	250.00	200.0000	600	200.00	300.0	71.5	26.0557
Weight1	59	20.3	18.5	22.1	19.29	19.50	9.4500	42	14.75	23.9	6.8	33.6811
Length1	59	114.0	109.5	118.6	112.73	114.85	83.0000	164	100.00	124.2	17.6	15.4654
2PK2 (3Mnth)	56	3.0	2.2	3.8	1.87	1.62	0.0100	16	1.03	4.3	3.0	102.2981
Log2PK2	56	0.3	0.1	0.4		0.21	-2.0000	1	0.01	0.6	0.5	177.3665
Time2	56	16.3	16.5	17.1	16.74	16.71	14.6600	21	15.83	17.5	1.2	7.2271
Dose2	56	276.3	258.0	295.5	269.37	250.00	200.0000	600	250.00	300.0	70.0	25.3054
2PK1	56	3.19	2.3	4.04	2.08	1.79	0.02000	17.06	1.12	4.35	3.17	99.2616
Log2PK1	56	0.32	0.1	0.40		0.25	-1.69897	1.23	0.05	0.64	0.45	141.4983
Time1	56	15.03	14.78	15.39	15.04	14.99	12.75000	18.92	14.46	15.83	1.14	7.5327
Weight2	56	20.3	19.0	22.5	19.86	20.05	11.7500	43	15.50	24.2	6.6	31.6713
Length2	56	114.6	110.1	119.2	113.44	115.60	83.0000	165	101.90	125.7	16.9	14.7490
CD4count2	52	663.0	558.1	768.0	551.63	602.00	96.0000	1722	404.50	913.0	376.8	56.8357
CD4%2	52	22.1	19.6	24.7	19.74	24.15	5.1800	37	14.50	28.8	9.3	41.9619
VL2	55	1413.4	-533.3	3360.1	44.84	25.00	25.0000	52000	25.00	25.0	7201.0	509.4643
ALT2	54	23.2	20.3	26.1	21.28	20.50	11.0000	60	16.00	27.0	10.5	45.4226
3PK2 (6Mnth)	55	3.3	2.1	4.5	1.78	1.78	0.0010	29	1.12	4.2	4.3	130.6299
Log3PK2	55	0.3	0.1	0.4		0.25	-3.0000	1	0.05	0.6	0.6	252.8034
Time3	55	16.3	16.6	17.1	16.82	16.78	14.9200	19	16.20	17.6	0.9	5.5512
Dose3	56	283.9	264.8	303.1	276.16	300.00	200.0000	600	250.00	325.0	71.4	25.1538

Table D1.1: Descriptive statistics (baseline and 1-24 months post-HAART) cont.

	N	Mean	CI -95%	CI +95%	Geo. Mean	Median	Minimum	Maximum	IQR 25%	IQR 75%	SD	CV
3PK1	56	3.42	2.37	4.47		2.12	0.0000	25.12	1.22	4.77	3.92	114.6886
Log3PK1	55	0.34	0.22	0.46		0.33	-1.0000	1.40	0.09	0.69	0.44	128.3537
3Time1	56	15.19	14.92	15.47	15.16	14.96	13.3000	17.75	14.45	15.78	1.02	6.6938
Weight3	56	21.6	19.8	23.4	20.69	21.12	12.3000	45	16.25	25.1	6.6	30.6553
Length3	56	116.1	111.7	120.5	114.98	117.07	88.0500	166	103.07	126.6	16.5	14.2223
CD4count3	53	678.7	578.0	779.5	588.81	624.00	120.0000	2089	370.00	865.0	365.5	53.8512
CD4%3	53	24.7	22.3	27.1	22.88	24.80	5.7100	43	19.10	29.7	8.6	34.9730
VL3	56	3184.9	-2895.2	9265.0	38.77	25.00	25.0000	170000	25.00	25.00	22703.7	712.8522
ALT3	56	23.9	20.6	27.2	21.58	21.00	9.0000	68	16.50	26.0	12.4	51.8404
4PK2 (12Mnth)	54	3.2	2.1	4.2	1.44	1.94	0.0010	24	0.90	4.2	3.7	118.0466
Log4PK2	54	0.2	-0.1	0.4		0.29	-3.0000	1	-0.05	0.6	0.8	512.3434
Time4	54	17.3	16.3	18.2	17.06	16.66	14.7500	41	16.08	17.5	3.4	19.9450
Dose4	55	296.4	277.8	314.9	289.47	300.00	200.0000	600	250.00	350.0	68.6	23.1541
4PK1	54	3.44	2.35	4.53		2.04	0.0000	25.02	1.11	4.47	3.98	115.9328
Log4PK1	52	0.33	0.18	0.47		0.31	-1.52288	1.40	0.09	0.65	0.51	157.1115
4Time1	54	15.65	14.81	16.49	15.46995	15.05	13.5000	36.73	14.50	16.00	3.08	19.6821
Weight4	55	22.6	20.6	24.5	21.57	21.55	12.4500	43	16.72	25.6	7.1	31.5360
Length4	55	119.2	115.0	123.4	118.19	115.25	91.8500	161	105.60	130.6	15.6	13.0593
CD4count4	54	788.6	679.3	897.8	694.27	723.50	206.0000	1903	535.00	1029.0	400.4	50.7718
CD4%4	54	27.0	24.8	29.1	25.53	28.10	6.1000	42	22.10	32.3	7.8	28.8619
VL4	55	502.4	-337.0	1341.7	35.11	25.00	25.0000	23000	25.00	25.0	3104.9	618.0614
ALT4	55	21.9	18.7	25.0	19.57	19.00	10.0000	67	14.00	27.0	11.7	53.3320
5PK2 (18Mnth)	48	4.0	2.5	5.5	2.17	1.99	0.0010	29	1.33	5.2	5.1	127.7095
Log5PK2	48	0.3	0.2	0.5		0.30	-3.0000	1	0.12	0.7	0.6	188.4727
Time5	48	17.3	16.8	18.8	17.61	17.31	15.2500	41	16.70	18.0	3.5	19.8940
Dose5	51	301.0	279.8	322.1	291.53	300.00	100.0000	600	250.00	350.0	75.2	24.9717
5PK1	50	4.30	2.76	5.83		2.44	0.0000	33.30	1.64	5.61	5.41	125.7552
Log5PK1	49	0.46	0.35	0.57		0.39	-0.46852	1.52	0.23	0.75	0.38	84.2184
5Time1	50	15.62	14.64	16.60	15.40	15.05	13.17000	38.42	14.50	15.93	3.44	22.0298
Weight5	51	23.9	21.9	26.0	22.92	22.93	14.0000	47	18.25	27.4	7.3	30.3465
Length5	51	122.9	118.5	127.4	121.93	123.45	96.1500	166	109.20	134.8	16.0	12.9955
CD4count5	51	805.4	709.1	901.6	745.74	744.00	271.0000	2105	613.00	921.0	342.1	42.4788
CD4%5	51	29.6	27.4	31.8	28.42	28.40	8.6600	48	25.10	34.2	7.8	26.4820

Table D1.1: Descriptive statistics (baseline and 1-24 months post-HAART) cont.

	N	Mean	CI -95%	CI +95%	Geo. Mean	Median	Minimum	Maximum	IQR 25%	IQR 75%	SD	CV
VL5	51	3083.2	-1229.3	7395.7	34.70	25.00	25.0000	84000	25.00	25.0	15333.1	497.3058
ALT5	49	20.7	17.7	23.8	18.97	17.00	8.0000	64	16.00	23.0	10.6	51.2327
6PK2 (24Mnth)	52	4.1	2.9	5.2	2.25	2.70	0.0010	20	1.42	6.0	4.2	102.0771
Log6PK2	52	0.4	0.2	0.5		0.43	-3.0000	1	0.15	0.8	0.7	185.8768
Time6	52	17.3	17.0	17.6	17.23	17.33	15.4300	19	16.38	18.0	1.1	6.2258
Dose6	53	317.9	294.0	341.8	304.05	300.00	50.0000	600	250.00	350.0	86.7	27.2564
6PK1	52	4.58	3.30	5.85		2.70	0.00000	20.60	1.56	5.94	4.57	99.9670
Log6PK1	51	0.47	0.34	0.60		0.44	-0.92082	1.31	0.20	0.79	0.46	97.7660
6Time1	52	15.06	14.75	15.37	15.02	15.00	13.17000	17.17	14.04	16.00	1.12	7.4544
Weight6	53	25.3	23.1	27.4	24.22	25.13	14.3500	49	19.25	28.6	7.7	30.5129
Length6	53	125.3	121.5	130.1	124.83	125.95	98.6000	168	112.90	136.6	15.7	12.4781
CD4count6	51	823.6	739.4	907.9	765.76	809.00	232.0000	1475	590.00	986.0	299.4	36.3513
CD4%6	51	30.5	28.4	32.6	29.45	31.40	10.3000	48	25.10	36.8	7.5	24.5501
VL6	53	12355.9	-12250.6	36962.3	38.28	25.00	25.0000	650000	25.00	25.0	89272.1	722.5066
ALT6	50	19.7	17.4	22.1	18.29	18.50	9.0000	48	14.00	22.0	8.2	41.7165
Chol6	52	3.9	3.7	4.1	3.80	3.85	2.3000	6	3.30	4.5	0.8	20.0018
TG6	52	1.1	1.0	1.2	1.05	1.00	0.5000	3	0.80	1.4	0.4	35.7910

CI, confidence interval Geo., geometric IQR, inter quartile range SD, standard deviation SE, standard error CV, coefficient variance

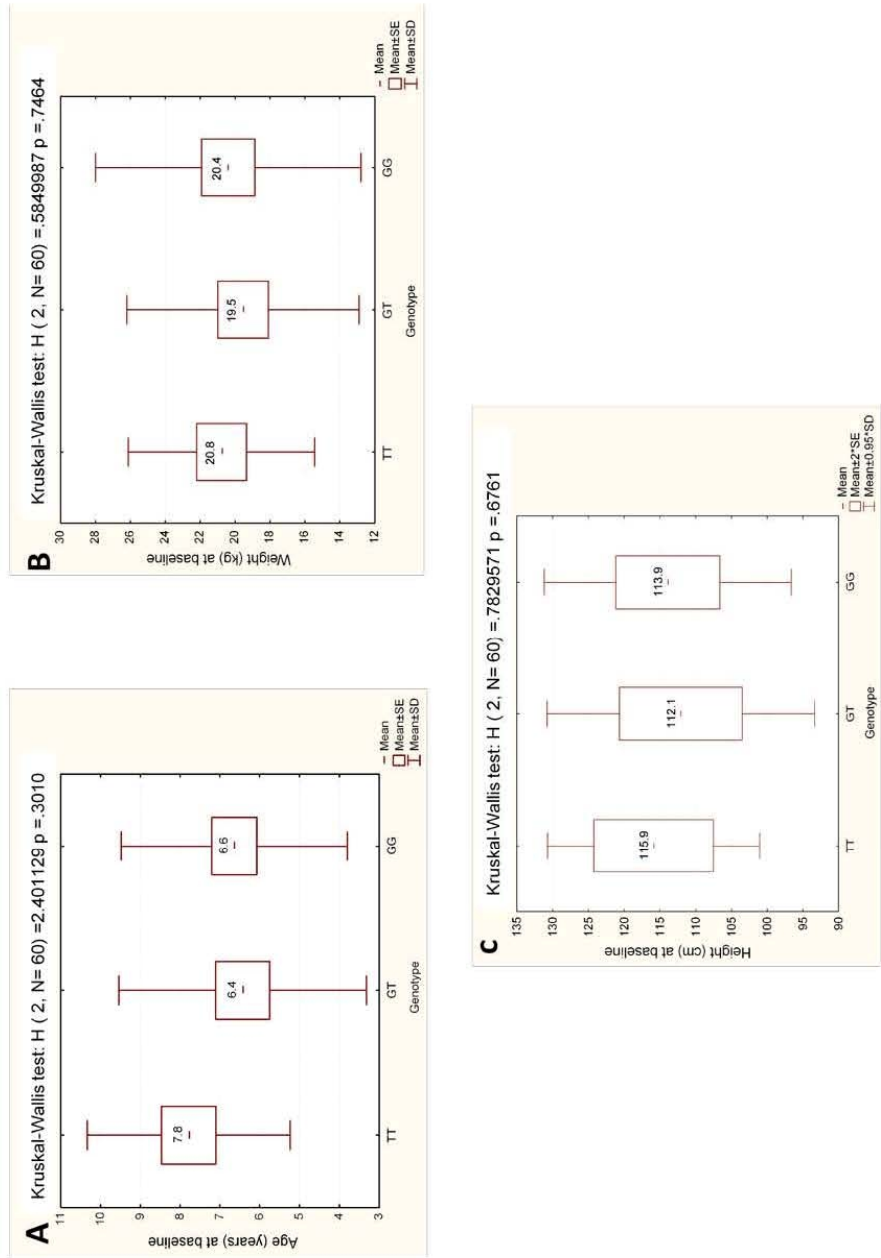


Figure D1.1: Box and whisker plots of baseline demographics (age, weight and height) of the respective CYP2B6 516 G/G, G/T and T/T genotype groups

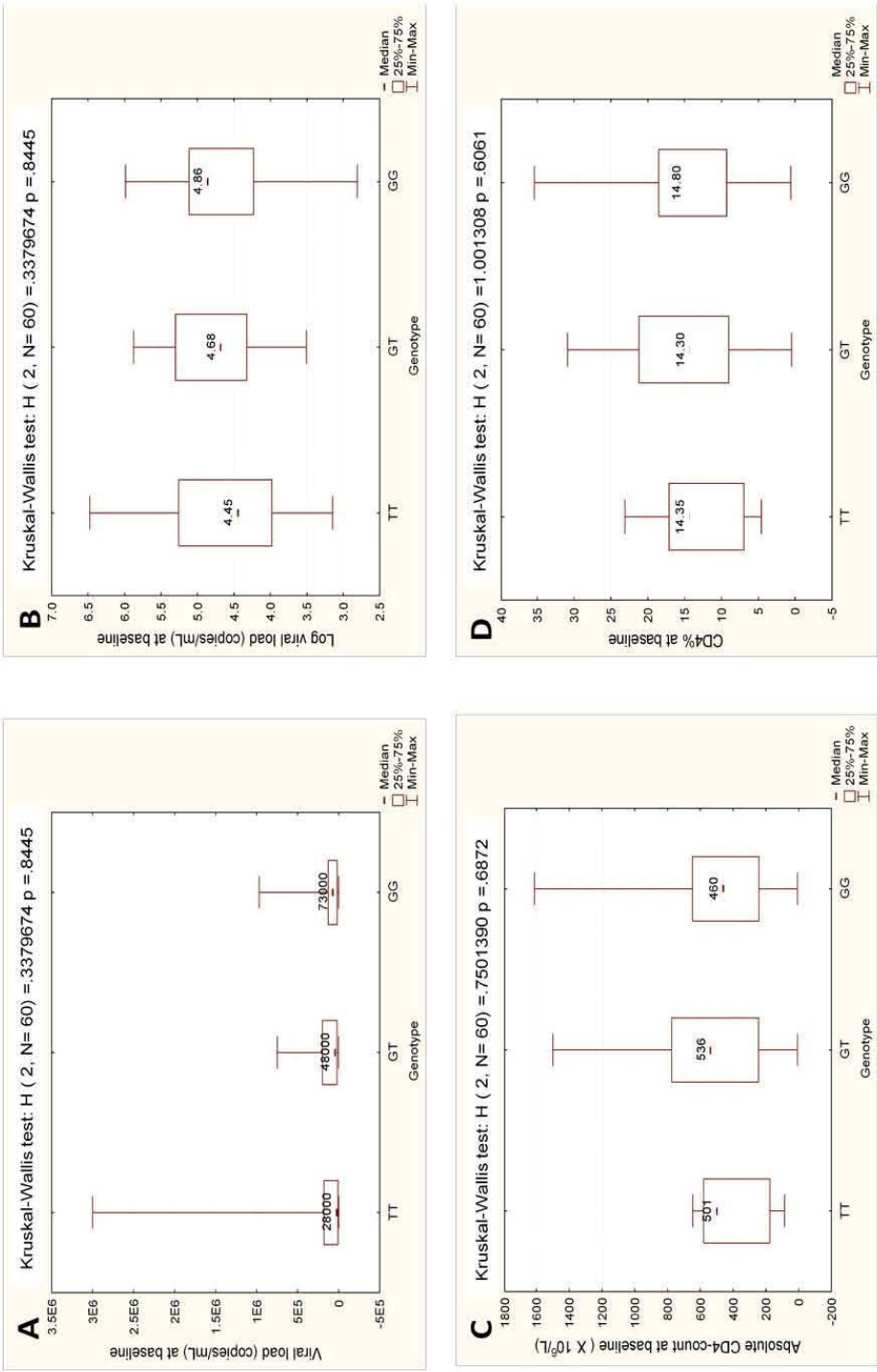


Figure D1.2: Box and whisker plots of clinical characteristics (VL, log VL, CD4-count, CD4%) at baseline of the respective CYP2B6 516 G/G, G/T and T/T genotype groups

Table D1.2: Descriptive statistics of the efavirenz concentrations (1-24 months) of the CYP2B6 516 G/G genotype

EFV conc. µg/ml	N	Mean	Geo. Mean	Median	Minimum	Maximum	IQR 25%	IQR 75%	SD	CV	SE
1PK1	24	1.73	1.48	1.47	0.25	4.50	1.20	1.85	0.98	56.99	0.20
Log1PK1	24	0.17		0.17	-0.60	0.65	0.08	0.27	0.26	151.82	0.05
1PK2	25	1.53	1.35	1.42	0.21	3.26	1.16	1.72	0.72	46.95	0.14
Log1PK2	25	0.13		0.15	-0.68	0.51	0.06	0.24	0.24	186.98	0.05
2PK1	23	1.44	1.16	1.40	0.02	3.38	0.97	1.77	0.70	48.74	0.15
Log2PK1	23	0.06		0.15	-1.70	0.53	-0.01	0.25	0.42	662.34	0.09
2PK2	23	1.28	1.01	1.19	0.01	2.85	0.83	1.60	0.62	48.34	0.13
Log2PK2	23	0.002		0.08	-2.00	0.46	-0.08	0.20	0.47	21945.52	0.10
3PK1	23	1.39		1.35	0.00	3.41	0.75	1.95	0.82	59.35	0.17
Log3PK1	22	0.09		0.14	-0.80	0.53	0.00	0.29	0.29	338.51	0.06
3PK2	23	1.23		1.21	0.00	3.26	0.73	1.60	0.76	61.25	0.16
Log3PK2	22	0.03		0.09	-0.85	0.51	-0.08	0.20	0.29	870.09	0.06
4PK1	20	1.58	1.18	1.28	0.13	7.50	0.95	1.91	1.51	95.62	0.34
Log4PK1	20	0.07		0.11	-0.89	0.88	-0.03	0.28	0.35	477.45	0.08
4PK2	20	1.38	1.04	1.24	0.15	5.70	0.65	1.79	1.18	85.61	0.26
Log4PK2	20	0.02		0.09	-0.82	0.76	-0.20	0.25	0.34	1902.41	0.08
5PK1	21	1.72	1.62	1.78	0.68	2.61	1.31	2.17	0.56	32.91	0.12
Log5PK1	21	0.21		0.25	-0.17	0.42	0.12	0.34	0.16	77.33	0.04
5PK2	21	1.53	1.44	1.60	0.60	2.42	1.11	1.91	0.52	33.69	0.11
Log5PK2	21	0.16		0.20	-0.22	0.38	0.05	0.28	0.16	102.87	0.04
6PK1	21	1.91	1.59	1.62	0.30	4.43	1.13	2.52	1.06	55.44	0.23
Log6PK1	21	0.20		0.21	-0.52	0.65	0.05	0.40	0.30	146.02	0.06
6PK2	21	1.71	1.44	1.51	0.31	3.22	0.99	2.41	0.89	52.10	0.19
Log6PK2	21	0.16		0.18	-0.51	0.51	-0.004	0.38	0.28	177.14	0.06

EFV, efavirenz Geo., geometric IQR, inter quartile range SD, standard deviation CV, coefficient variance SE, standard error

Table D1.3: Descriptive statistics of the efavirenz concentrations (1-24 months) of the CYP2B6 516 G/T genotype

EFV conc. µg/ml	N	Mean	Geo. Mean	Median	Minimum	Maximum	IQR 25%	IQR 75%	SD	CV	SE
1PK1	19	4.09	2.44	2.71	0.26	18.20	2.03	4.44	4.35	106.16	0.99
Log1PK1	19	0.39		0.43	-0.59	1.26	0.31	0.65	0.50	128.66	0.11
1PK2	20	3.61	2.10	2.43	0.25	17.64	1.27	3.87	4.08	113.26	0.91
Log1PK2	20	0.32		0.39	-0.60	1.25	0.08	0.59	0.50	154.28	0.11
2PK1	19	3.56	2.28	2.14	0.62	17.06	1.10	3.72	4.19	117.68	0.96
Log2PK1	19	0.36		0.33	-0.21	1.23	0.04	0.57	0.39	109.71	0.09
2PK2	19	3.39	2.07	1.95	0.55	16.42	0.85	3.74	4.06	119.85	0.93
Log2PK2	19	0.32		0.29	-0.26	1.22	-0.07	0.57	0.42	133.25	0.10
3PK1	19	3.84	2.17	2.35	0.10	25.12	1.27	3.39	5.54	144.29	1.27
Log3PK1	19	0.34		0.37	-1.00	1.40	0.10	0.53	0.49	146.61	0.11
3PK2	18	4.04	2.15	2.25	0.08	29.04	1.20	2.91	6.54	162.12	1.54
Log3PK2	18	0.33		0.35	-1.10	1.46	0.08	0.46	0.51	153.32	0.12
4PK1	20	4.09		2.35	0.00	25.02	1.59	4.44	5.48	133.88	1.23
Log4PK1	19	0.35		0.42	-1.52	1.40	0.21	0.65	0.63	181.83	0.15
4PK2	20	3.88		2.09	0.00	23.96	1.38	4.13	5.26	135.81	1.18
Log4PK2	19	0.30		0.33	-1.70	1.38	0.15	0.62	0.67	219.01	0.15
5PK1	17	5.10		3.03	0.00	33.30	1.82	4.75	7.64	149.85	1.85
Log5PK1	16	0.51		0.51	-0.47	1.52	0.28	0.71	0.44	85.52	0.11
5PK2	15	4.69		2.57	0.00	29.46	1.18	4.68	7.21	153.65	1.86
Log5PK2	14	0.45		0.41	-0.57	1.47	0.30	0.67	0.47	104.60	0.13
6PK1	19	5.18		3.47	0.00	20.60	1.73	5.45	5.93	114.45	1.36
Log6PK1	18	0.51		0.55	-0.92	1.31	0.35	0.74	0.52	102.49	0.12
6PK2	19	4.76		2.80	0.00	19.90	1.47	4.35	5.60	117.78	1.28
Log6PK2	18	0.46		0.51	-1.10	1.30	0.28	0.64	0.54	118.73	0.13

EFV, efavirenz Geo., geometric IQR, inter quartile range SD, standard deviation CV, coefficient variance SE, standard error

Table D1.4: Descriptive statistics of the efavirenz concentrations (1-24 months) of the CYP2B6 516 T/T genotype

EFV conc. µg/ml	N	Mean	Geo. Mean	Median	Minimum	Maximum	IQR 25%	IQR 75%	SD	CV	SE
1PK1	14	5.32	4.49	5.38	1.59	9.95	2.69	7.68	2.91	54.66	0.78
Log1PK1	14	0.65	0.58	0.73	0.20	0.99	0.43	0.89	0.28	42.52	0.07
1PK2	14	5.04	4.28	5.34	1.44	8.07	2.43	7.60	2.55	50.56	0.68
Log1PK2	14	0.63	0.55	0.73	0.16	0.91	0.39	0.88	0.28	43.87	0.07
2PK1	14	5.55	4.80	6.10	0.83	10.02	4.11	6.72	2.38	42.87	0.64
Log2PK1	14	0.68		0.79	-0.08	1.00	0.61	0.83	0.29	42.24	0.077
2PK2	14	5.21	4.52	5.62	0.92	9.11	4.30	6.33	2.26	43.30	0.60
Log2PK2	14	0.66		0.75	-0.04	0.96	0.63	0.80	0.28	42.79	0.08
3PK1	14	6.20	5.72	6.04	2.14	10.69	4.88	8.11	2.38	38.32	0.63
Log3PK1	14	0.76	0.73	0.78	0.33	1.03	0.69	0.91	0.19	25.28	0.05
3PK2	14	5.79	5.22	6.32	1.75	10.46	4.22	7.23	2.39	41.33	0.64
Log3PK2	14	0.72	0.67	0.80	0.24	1.02	0.63	0.86	0.22	30.82	0.06
4PK1	14	5.16		4.61	0.00	10.13	3.91	7.71	2.89	56.12	0.77
Log4PK1	13	0.68		0.67	-0.03	1.01	0.64	0.89	0.28	40.52	0.08
4PK2	14	4.72		4.59	0.00	8.09	3.78	7.02	2.50	52.92	0.67
Log4PK2	13	0.65		0.70	-0.06	0.91	0.61	0.85	0.27	41.71	0.08
5PK1	12	7.69	6.57	6.62	1.75	17.94	5.90	9.45	4.30	55.99	1.24
Log5PK1	12	0.82	0.76	0.82	0.24	1.25	0.77	0.98	0.27	33.10	0.08
5PK2	12	7.40	6.15	6.36	1.62	17.50	5.17	9.40	4.40	59.43	1.27
Log5PK2	12	0.79	0.71	0.80	0.21	1.24	0.71	0.97	0.30	37.73	0.09
6PK1	12	8.28	7.50	9.12	1.54	11.73	6.68	10.01	2.87	34.68	0.83
Log6PK1	12	0.88	0.81	0.96	0.19	1.07	0.82	1.00	0.24	27.50	0.07
6PK2	12	7.13	6.52	7.67	1.47	10.40	6.03	8.55	2.40	33.58	0.70
Log6PK2	12	0.81	0.76	0.88	0.17	1.02	0.78	0.93	0.23	27.83	0.07

EFV, efavirenz Geo., geometric IQR, inter quartile range SD, standard deviation CV, coefficient variance SE, standard error

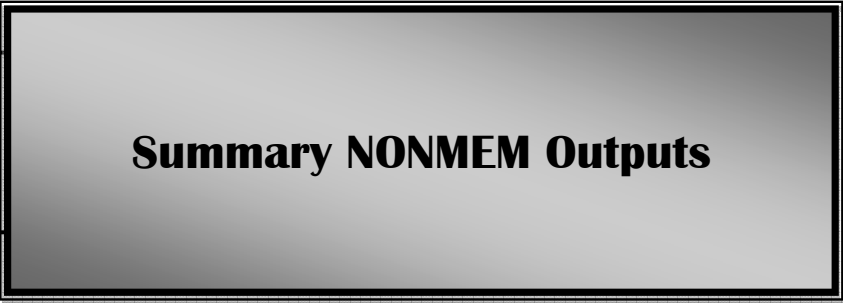
Table D1.5: Descriptive statistics of the clinical (pharmacodynamic) markers 3-24 months post-HAART initiation

	N	Mean	CI -95%	CI +95%	Geo. Mean	Median	Min.	Max.	IQR 25%	IQR 75%	SD	CV	SE	Skew ness	Kurto sis
CD4count2	52	663.0	558.1	768.0	551.63	602.00	96.00	1722	404.50	913.0	376.8	56.84	52.26	0.78	0.44
CD4%2	52	22.1	19.6	24.7	19.74	24.15	5.18	37	14.50	28.8	9.3	41.96	1.29	-0.250	-1.07
VL2	55	1413.4	-533.3	3360.1	44.84	25.00	25.00	52000	25.00	25.0	7201.0	509.46	970.98	6.71	47.30
ALT2	54	23.2	20.3	26.1	21.28	20.50	11.00	60	16.00	27.0	10.5	45.42	1.43	1.49	2.47
CD4count3	53	678.7	578.0	779.5	588.81	624.00	120.00	2089	370.00	865.0	365.5	53.85	50.20	1.29	2.96
CD4%3	53	24.7	22.3	27.1	22.88	24.80	5.71	43	19.10	29.7	8.6	34.97	1.19	-0.17	-0.40
VL3	56	3184.9	-2895.2	9285.0	38.77	25.00	25.00	170000	25.00	25.0	22703.7	712.85	3033.91	7.48	55.93
ALT3	56	23.9	20.6	27.2	21.58	21.00	9.00	68	16.50	26.0	12.4	51.84	1.66	1.93	4.12
CD4count4	54	788.6	679.3	897.8	694.27	723.50	206.00	1903	535.00	1029.0	400.4	50.77	54.48	0.90	0.42
CD4%4	54	27.0	24.8	29.1	25.53	28.10	6.10	42	22.10	32.3	7.8	28.86	1.06	-0.54	0.02
VL4	55	502.4	-337.0	1341.7	35.11	25.00	25.00	23000	25.00	25.0	3104.9	618.06	418.67	7.31	53.87
ALT4	55	21.9	18.7	25.0	19.57	19.00	10.00	67	14.00	27.0	11.7	53.33	1.57	1.75	3.50
CD4count5	51	805.4	709.1	901.6	745.74	744.00	271.00	2105	613.00	921.0	342.1	42.48	47.90	1.76	4.87
CD4%5	51	29.6	27.4	31.8	28.42	29.40	8.66	48	25.10	34.2	7.8	26.48	1.10	-0.001	0.26
VL5	51	3083.2	-1229.3	7395.7	34.70	25.00	25.00	84000	25.00	25.0	15333.1	497.31	2147.06	4.94	23.51
ALT5	49	20.7	17.7	23.8	18.97	17.00	8.00	64	16.00	23.0	10.6	51.23	1.52	2.73	9.09
CD4count6	51	823.6	739.4	907.9	765.76	809.00	232.00	1475	590.00	986.0	299.4	36.35	41.93	0.32	-0.27
CD4%6	51	30.5	28.4	32.6	29.45	31.40	10.30	48	25.10	36.8	7.5	24.55	1.05	-0.30	-0.07
VL6	53	12355.9	-12250.6	36962.3	38.28	25.00	25.00	650000	25.00	25.0	89272.1	722.51	12262.47	7.28	52.99
ALT6	50	19.7	17.4	22.1	18.29	18.50	9.00	48	14.00	22.0	8.2	41.72	1.16	1.39	2.49
Chol6	52	3.9	3.7	4.1	3.80	3.85	2.30	6	3.30	4.5	0.8	20.00	0.11	0.09	-0.69
TG6	52	1.1	1.0	1.2	1.05	1.00	0.50	3	0.80	1.4	0.4	35.79	0.06	0.94	1.38

CI, confidence interval Geo., geometric Min., minimum Max., maximum IQR, inter quartile range SD, standard deviation CV, coefficient variance SE, standard error

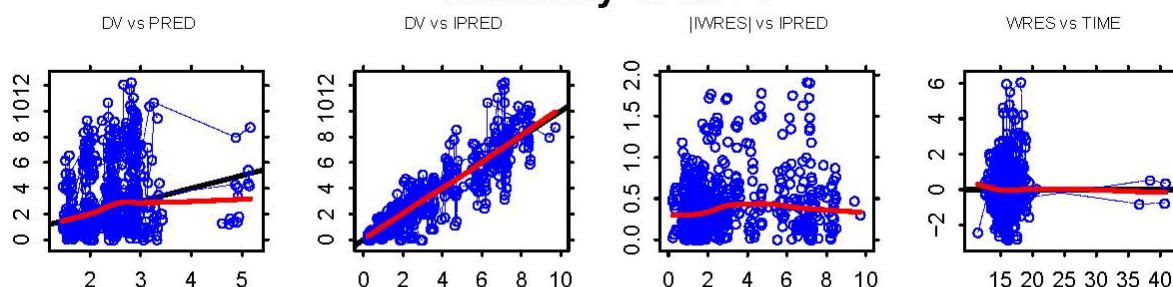
VL, viral load ALT, alanine aminotransferase Chol, cholesterol TG, triglyceride

Addendum D2



Summary NONMEM Outputs

Summary of run 1



MINIMIZATION SUCCESSFUL

NO. OF FUNCTION EVALUATIONS USED: 103

NO. OF SIG. DIGITS IN FINAL EST.: 4.4

ETABAR IS THE ARITHMETIC MEAN OF THE ETA-ESTIMATE ERROR AND THE P-VALUE IS GIVEN FOR THE NULL HYPOTHESIS

ETABAR: -7.0621E-03 -2.2038E-02

SE: 9.0984E-02 1.1512E-02

P VAL.: 9.3813E-01 5.5567E-02

ETAsrink(%): 1.2789E+00 7.9668E+01

EPSshrink(%): 4.7755E+00

Objective: 702.442455490047

Par	Val	RSE	Par	Val	RSE
TH1	5.00737	8.5e-02	SI1:1	0.59	0.15
TH2	187.573	1.4e-01			
TH3	0.287	3.5e+10			
OM1:1	0.72	0.15			
OM2:2	0.44	1.5			

\$PROB EFavirenz ADVAN 2

;; RUN 1 ADVAN2 TRANS2 met makliker error model

;; LDV = log DV LAMT = log AMT

\$INPUT ID SEX AGE TIME DV LDV WT LAMT AMT II SS EVI

\$DATA efavirenz34.txt

\$SUBROUTINES ADVAN 2 TRANS 2

\$PK

;K=WT**0.425

;KK=HT**0.725

;BSA=(K*KK)*0.007184

TVCL=THETA (1)

CL = TVCL * EXP (ETA(1))

TVV = THETA (2)

V= TVV*EXP (ETA(2))

KA = THETA (3)

;KA = KA * EXP (ETA (3))

S2=V

S2=SQRT(F)

;W=F

Y=F+W*EPS(1)

IPRED=F

IRES= DV-IPRED

IWRES= IRES/W

\$THETA

(0,5,20)

(0,184,300)

0.287 FIXED

;\$OMEGA DIAGONAL (2)

0.520

0.185

;\$SIGMA

1

;\$EST SIGDIGITS=4 PRINT=10 MSFO=msfb8 METH=1 INT

;\$COV

;\$TABLE ID TIME IPRED IWRES NOPRINT ONEHEADER F

;\$TABLE ID CL V ETA1 ETA2 NOPRINT ONEHEADER FILE

;\$TABLE ID WT AGE HT NOPRINT ONEHEADER FILE= cot

;\$TABLE ID SEX GENO NOPRINT ONEHEADER FILE= cat

;\$SCAT DV VS PRED

;\$SCAT CL VS WT

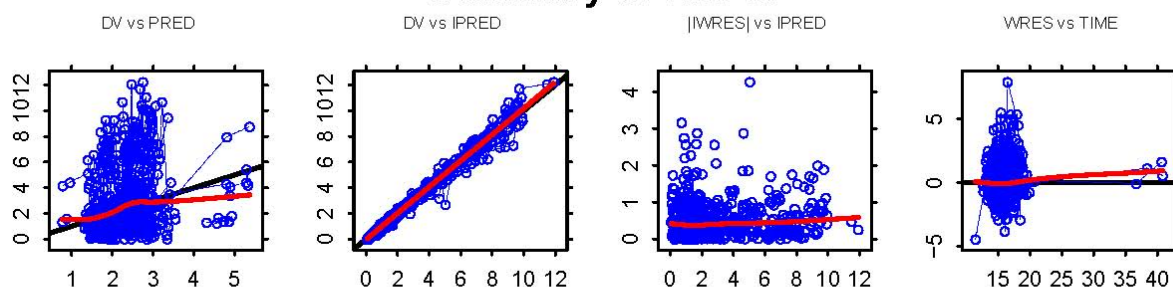
;\$SCAT IWRES VS IPRED

;\$SCAT IPRED VS DV

;\$SCAT IWRES VS IPRED

Figure D2.1: Summary of NONMEM output Run 1 (Basic Model)

Summary of run 41



Objective: 216.080143251337

Par	Val	RSE
TH1	4.85989	7.5e-02
TH2	94.3013	1.4e-01
TH3	0.287	3.5e+10
TH4	0.107665	8.3e-02
TH5	0.0917579	1.3e-01
OM1:1	0.63	0.18
OM2:2	0.37	0.63
OM3:3	0.37	0.19
OM4:4	0.37	
OM5:5	0.37	
OM6:6	0.37	
OM7:7	0.37	
OM8:8	0.37	
SI1:1	1	0

\$PROB EFVIRENZ INTEROCCASION + GENOTYPE

;; RUN 41 efavirenz2011.txt IOV

;; LDV=log DV LAMT=log AMT

\$INPUT ID SEX AGE TIME DV LDV WT LAMT AMT II SS EVI

\$DATA efavirenz2011.txt

\$SUBROUTINES ADVAN 2 TRANS 2

\$PK

IF(OCC.EQ.1) THEN

BOV=ETA(8)

ENDIF

IF(OCC.EQ.2) THEN

BOV=ETA(3)

ENDIF

IF(OCC.EQ.3) THEN

BOV=ETA(4)

ENDIF

IF(OCC.EQ.4) THEN

BOV=ETA(5)

ENDIF

IF(OCC.EQ.5) THEN

BOV=ETA(6)

ENDIF

IF(OCC.EQ.6) THEN

BOV=ETA(7)

ENDIF

TVCL=THETA(1)

CL = TVCL * EXP(ETA(1)+BOV)

TVV = THETA (2)

V= TVV*EXP (ETA(2))

KA = THETA (3)

;KA = KA * EXP (ETA (3))

S2=V

\$ERROR

W=SQRT((F*THETA(4)+1E-6)**2+THETA(5)**2)

Y=F+W*EPS(1)

IPRED=F

IRES= DV-IPRED

IWRES= IRES/W

;THETA (0,5,2,200)

(0.87,1)

0.287 FIX

(0,0.10800000000000001,0.3)

(0,0.0897,0.2)

;SOMEGA DIAGONAL(2)

0.195

0.166

;SOMEGA BLOCK(1)

0.1

;0.111385 ; BOV

;SOMEGA BLOCK(1) SAME

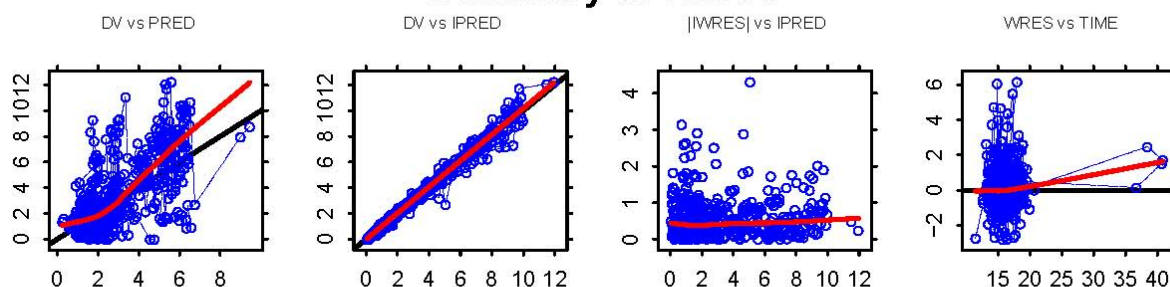
;SOMEGA BLOCK(1) SAME

;SOMEGA BLOCK(1) SAME

;SOMEGA BLOCK(1) SAME

Figure D2.2: Summary of NONMEM output Run 41 (IOV Model)

Summary of run 36



```

MINIMIZATION SUCCESSFUL
NO. OF FUNCTION EVALUATIONS USED: 194
NO. OF SIG. DIGITS IN FINAL EST.: 4.5
ETABAR IS THE ARITHMETIC MEAN OF THE ETA-ESTIMATES
AND THE P-VALUE IS GIVEN FOR THE NULL HYPOTHESIS $PK
ETABAR: -2.5332E-02 -1.3313E-01 2.9102E-02 3.350F (GENO.EQ.1)TVCL=THETA(1)
SE: 4.7141E-02 3.0175E-02 3.4575E-02 3.3007E-IF (GENO.EQ.2)TVCL=THETA(2)
P VAL.: 5.9101E-01 1.0250E-05 3.9995E-01 9.1914 IF (GENO.EQ.3)TVCL=THETA(3)
IF(OCC.EQ.1) THEN
BOV=ETA(8)
ENDIF
IF(OCC.EQ.2) THEN
BOV=ETA(3)
ENDIF
IF(OCC.EQ.3) THEN
BOV=ETA(4)
ENDIF
IF(OCC.EQ.4) THEN
BOV=ETA(5)
ENDIF
IF(OCC.EQ.5) THEN
BOV=ETA(6)
ENDIF
IF(OCC.EQ.6) THEN
BOV=ETA(7)
ENDIF
CL = TVCL * EXP(ETA(1)+BOV)*(WT/20)**0.75*(AGE/7/12 **THETA(8)
TVV = THETA (4)
V= TVV*EXP (ETA(2))*(WT/20)*(AGE/7/12)**THETA(8)
KA = THETA (5)
;KA = KA * EXP (ETA (3))
S2 =V
$ERROR
W=SQRT((F*THETA(6)+1E-6)**2+THETA(7)**2)
Y=F+W*EPS(1)

; RUN 30 efavirenz2011.txt Finale model
; LDV=log DV LAMT= log AMT
$INPUT ID SEX AGE TIME DV LDV WT LAMT AMT II SS E'
$DATA efavirenz2011.txt
$SUBROUTINES ADVAN 2 TRANS 2
$PK
ETAShrink(%): 1.0931E+01 4.2732E+01 2.6364E+01 2.9
EPSshrink(%): 2.8152E+01

Objective: 170.837311187409

Par Val RSE
TH1 2.4579 1.0e-01
TH2 4.59632 1.3e-01
TH3 7.3275 8.7e-02
TH4 89.5179 1.1e-01
TH5 0.287 3.5e+10
TH6 0.107036 7.0e-02
TH7 0.0934513 1.5e-01
TH8 -0.598035 2.3e-01
OM1:1 0.41 0.28
OM2:2 0.41 0.51
OM3:3 0.37 0.19
OM4:4 0.37
OM5:5 0.37
OM6:6 0.37
OM7:7 0.37
OM8:8 0.37
SI1:1 1 0
$PROB EFAVIRENZ INTEROCCASION + GENOTYPE

```

Figure D2.3: Summary of NONMEM output Run 36 (Final Model)