THE EFFECTS OF CYP2B6 POLYMORPHISMS ON CD4 CELL COUNT IN HIV PATIENTS ON NEVIRAPINE BASED REGIMENS AT KENYATTA NATIONAL HOSPITAL

By

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A thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Science in molecular pharmacology of the university of Nairobi

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NOVEMBER 2015

DECLARATION

I, James Angima Angima, do hereby declare that this thesis is my original work and that this work has not been presented for the award of any other degree or to any other university.

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DEDICATION

This thesis is dedicated to my dear and lovely wife Mellen Bosibori Onyoni, my daughter Faith Kerubo Angima and my son Nehemiah Maragia Angima.

"I can do all things through Christ which strengtheneth me."

Philippians 4:13

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the University of Nairobi, College of Health Sciences, School of Pharmacy and Department of Pharmacology and Pharmacognosy, for letting me fulfill my dream of being a student here. My special thanks first and foremost go to my supervisors Dr. Faith A. Okalebo, Dr. Margaret N. Oluka and Dr. George O. Osanjo for having sacrificed their precious time and resources to guide me till I successfully completed this thesis.

I am also deeply grateful to all my lecturers who took me through the course work and saw to it that I complete my course successfully. Their help, professionalism, guidance and academic support are much appreciated.

My special thanks also go to Mr. Daniel Juma of the School of Pharmacy, Department of Pharmacology and Pharmacognosy and Mr. Meshack Wadegu of the Center of Public Health and Research, Walter Reed project for their sacrifice in guiding me through DNA extraction and genotyping. Also special thanks to Martin Maina of Africa Biosystems, for technical support throughout the genotyping assays. May God bless them.

Finally I must express my profound gratitude to my Mum, my lovely wife and children for their prayers together with my friends, colleagues and classmates. Their unfailing moral support and continuous encouragement through my years of study and research and writing this thesis are much appreciated. This accomplishment would not have been possible without them.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABCC	ATP Binding Cassette sub-family C
ADR	Adverse Drug Reaction
AIDS	Acquired Immunodeficiency Syndrome
ALDH	Aldehyde dehydrogenase
BMI	Body Mass Index
CCC	Comprehensive Care Centre
CD4	Cluster of differentiation antigen 4
CNS	Central Nervous System
CYP450	Cytochrome P450
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
FDC	Fixed Dose Combination
HAART	Highly Active Anti-retroviral Therapy
HIV	Human Immunodeficiency Virus
KNH	Kenyatta National Hospital
MGB	Minor Groove Binder
MHC	Major Histocompatibility Complex
NFQ	Nonfluorescent quencher
NNRTIs	Non Nucleoside Reverse Transcriptase Inhibitors
NVP	Nevirapine
PMTCT	Prevention of Mother to Child Transmission
SNP	Single Nucleotide Polymorphisms

OPERATIONAL DEFINITIONS

Allele: one of two or more alternative forms of a gene that arise by mutation and are found at the same place on a chromosome

Gene: Is the functional and physical unit of heredity passed from parent to offspring.

Genotype: the entire set of genes in a cell or an organism.

Haplotype: Is a group of genes which is inherited together by an offspring from a single parent.

Isoenzyme: Any of a group of enzymes that catalyze the same reaction but have different amino acid sequence.

Isoform: Is different forms of a protein that may be produced from different genes or from the same gene by alternative splicing.

Mutation: Is a permanent change of the nucleotide sequence of the genome of an organism.

Pharmacogenetics: The convergence of pharmacology and genetics which deals with genetically determined responses to drugs.

Phenotype: The observable physical or biochemical characteristics of an organism as determined by both genetic makeup and environmental influences.

Polymorphism: Natural variation in a gene, DNA sequence or chromosomes that have no adverse reactions on the individual and occur with fairly high frequency in the general population.

Single nucleotide polymorphism: A variation at a single position in a DNA sequence among individuals.

ABSTRACT

Background:

The CD4 cell count has become a valuable indicator of immunological function in the management of HIV infection. Regular measurements of CD4 levels have been utilized to assess immunological response to antiretroviral drugs in HIV/AIDS patients. Fluctuations in CD4 cell counts can be influenced by genetic characteristics of HIV/AIDS patients. CYP2B6 gene is a drug metabolizing enzyme that may influence CD4 cell counts due to its effect on the disposition of antiretroviral drugs.

Objectives:

The main objective of this study was to determine the prevalence of polymorphisms of CYP2B6 516G>T and CYP2B6 983T>C and the influence of these polymorphisms on the CD4 cell levels on patients on nevirapine based regimens at Kenyatta National Hospital.

Methodology:

DNA extraction and genotyping were performed on 204 archived blood samples of HIV positive patients who had been on nevirapine based antiretroviral regimens. The DNA extraction was performed using PureLink® Genomic DNA extraction protocol (Life Technologies, Carlsbad, CA). Genotyping was performed using TaqMan® Drug Metabolism genotyping assay protocol (Life Technologies, Carlsbad, CA). Multi-linear regression was performed using STATA version 11 to establish association between CYP2B6 516G>T and 983T>C single nucleotide

polymorphisms and CD4 cell counts using patient social-demographic data adapted from a study conducted by Makori et al 2014.

Results:

One hundred and ninety four (194) blood samples were genotyped for the expression of CYP2B6 516G>T SNP. The number of samples that expressed CYP2B6 516GG wild-type genotype was 89 (45.9%). Seventy three (37.6%) samples expressed the heterozygous GT genotype while 32 (16.5%) expressed the homozygous TT genotype. The allele frequencies for CYP2B6 516G>T SNP in the study population were; 64.7% for G and 35.3% for the T allele (χ^2= 6.04; P<0.014). Two hundred and four (204) blood samples were genotyped for the expression of CYP2B6 983T>C SNP. One hundred and eighty three (89.7%) samples expressed CYP2B6 983TT genotype, while 19 (9.3%) and 2 (1%) expressed the heterozygous TC and homozygous CC genotypes respectively. Allele frequencies for the CYP2B6 983T>C SNP were 94.4% for T allele and 5.6% for C allele (χ^2= 3.165; P<0.075). On bivariable analysis, there was no association between CYP2B6 516G>T and 983T>C genotypes and baseline, current and the rate of change of CD4 cell counts.

Discussion

This study has shown that the prevalence of CYP2B6 516G>T and 983T>C genotypes in the study population was similar to those reported in other African populations. There was no association between CYP2B6 516G>T and 983T>C genotypes and CD4 cell counts which was at variance with previous studies.

Conclusion:

This study found no association between the expression of CYP2B6 516G>T and 983T>C genotypes and CD4 cell counts. Nevirapine plasma concentrations need to be determined to infer any association between these genotypes and CD4 cell counts.

CHAPTER ONE: INTRODUCTION

1.1 Background

The emergence of Human Immunodeficiency Virus (HIV) in early eighties has caused a global AIDS epidemic. HIV causes progressive deterioration of the immune system manifesting as Acquired Immunodeficiency Syndrome (AIDS). The weakened immune system is susceptible to opportunistic infections and malignant tumors (1). The hallmark of HIV is the depletion of CD4 lymphocytes leading to cellular immunodeficiency and multiplication of the virus to millions of viral copies (2).

Treatment intervention in the management of HIV involves use of antiretroviral drugs which suppress the virus while enhancing immune recovery by boosting CD4 cell counts. This treatment intervention involves use of three antiretroviral drugs (2 nucleoside reverse transcriptase inhibitors and one non nucleoside reverse transcriptase inhibitor or a protease inhibitor). This combination is termed as Highly Active Antiretroviral Therapy (HAART). The most important markers and monitoring tools used to evaluate the outcome of HAART are the CD4 cell count and viral load levels. Indicators of plasma virus load such as quantitative culture, p24 antigen, and HIV RNA generally increase as the CD4 cell count falls and decrease with the initiation of HAART, and thus they can be used as prognostic guides (3). In the Kenyan setting measurements of CD4 cell counts at treatment initiation and at regular intervals of 6 months during follow up is the most common indicator of disease progression (4).

It is projected that the CD4 levels for a normal uninfected adult/adolescent in good health ranges from 500 cells/mm^3 to 1200 cells/mm^3. Persons who are HIV positive and with CD4 cell counts below 200 cells/mm^3 are considered to have attained AIDS stage 3 infection. Fluctuations in the CD4 cell counts are used in clinical staging of AIDS and also as an indicator on when to initiate antiretroviral therapy in persons infected with HIV. Previously, CD4 cell count of below 250 cells/mm^3 was used as an indicator to initiate HAART. However in the current guidelines, HAART initiation is commenced when CD4 cell counts fall below 500 cell/mm^3 (4). As CD4 cell counts increase in the course of therapy, the patient's response to therapy is deemed to be good while a decline is deemed to be immunological failure.

Long term pattern of CD4 cell count is determined by an interaction between baseline CD4 cell count, duration of therapy and patients social demographic characteristics (5). The CD4 cell counts are normally measured for all patients before initiating antiretroviral therapy. This forms the basis for the need to initiate antiretroviral therapy or prophylaxis of opportunistic infections. Most opportunistic infections occur when the CD4 cell counts fall below 200 cells/mm^3, but some can occur even at higher CD4 cell counts (6).

Nevirapine is a non-nucleoside reverse transcriptase inhibitor, recommended as a first line antiretroviral drug in HIV/AIDS management. It is used in combination with two nucleoside reverse transcriptase inhibitors. It is also used as monotherapy in the prevention of mother to child transmission (PMTCT) of HIV in babies for six months or till two weeks after cessation of breastfeeding. It is metabolized in the liver by cytochrome P450 enzymes and less that 3% is excreted unchanged in urine. It is majorly metabolized by CYP2B6 and CYP3A4 enzymes.

1.2 Study justification

Clinically, the CD4 cell count is used to monitor the response to antiretroviral therapy. Some patients show a good response while other patients show a poor response. The various reasons that have been advanced for low CD4 cell counts include infections with resistant viral strains, poor diet, poor adherence to antiretroviral drugs and genetic characteristics of the individuals.

Studies have reported that medical and social-demographic characteristics influence fluctuations of CD4 cell counts in HIV positive patients on nevirapine based regimens (7). However the influence of genetic factors on CD4 cell count has not been adequately studied. CYP2B6 516G>T and 983T>C single nucleotide polymorphisms (SNP) affect the disposition of nevirapine which may influence CD4 cell counts.

CYP2B6 516G>T and 983T>C single nucleotide polymorphisms (SNPs) have higher prevalence in Africans populations than in the Caucasian and Asian populations (3) (9). An association has been suggested between the CYP2B6 983T>C SNP and increased CD4 cell counts (8). In Kenyan populations, only one study has been conducted to determine the influence of CYP2B6 516G>T and 983T>C genotypes on CD4 cell counts and possible clinical outcomes. That study was conducted on a small sample size (8). More studies are therefore required to evaluate the influence of these polymorphisms on the CD4 cell counts.

1.3 Research question

What is the impact of CYP2B6 516G>T and 983T>C single nucleotide polymorphisms on the baseline CD4 cell count, the rate of change in CD4 cell count and the current CD4 cell counts attained in HIV patients on nevirapine based ART regimens?

1.4 Objectives

1.4.1 Main objective

The main objective of this study was to investigate the effect of CYP2B6 516G>T and 983T>C SNPs on CD4 cell counts in Kenyan HIV patients on nevirapine based ART regimens at the Comprehensive Care Centre (CCC) of Kenyatta National Hospital (KNH).

1.4.2 Specific objectives

The specific objectives of this study were to determine:

1. The prevalence of CYP2B6 516G>T and 983T>C SNPs in Kenyan HIV patients on nevirapine based ART regimens.

2. Characterize the CD4 response of HIV patients on nevirapine based ART regimens.

3. Describe clinical and demographic characteristics of the HIV patients on nevirapine based ART regimens.

CHAPTER TWO: LITERATURE REVIEW

2.1 HIV infection

HIV enters the body and binds to dendritic cells which carry the virus to CD4+ T cells in the lymhoid tissue. Binding to the dendritic cells is through DC-SIGN, a glycoprotein expressed on dendritic cells (10). HIV has affinity for CD4+ T cells and monocytes. Viral entry into CD4+ T cells is through binding to viral glycoproteins. Viral glycoproteins are transmembrane protein gp41 and gp120 whereby gp120 is non-covalently linked to gp41 (11). Viral binding to the CD4+ T cells induces a conformational change within gp120 which exposes co-receptor binding sites in gp120. The co-receptor binds chemokine receptor CXCR4 or CCR5 depending on the type of HIV particle. The M-tropic HIV particles recognize CCR5 and infects macrophages and primary T-cells. T-tropic particles recognize CXCR4 and induce syncitia. The interaction between gp120 and host's chemokine receptors enables gp41 to bind to host's heparan sulfate which triggers fusion of the host's and viral membranes followed by the entry of the capsid into the cytoplasm (12).

After the HIV virus has infected host cells, it undergoes reverse transcription and integration followed by transcription and translation then viral assembly, budding and maturation (2). Reverse transcription and integration enables the virus to convert the viral RNA into DNA using HIV reverse transcriptase. This DNA is then transported to the cell nucleus, where it is inserted into the human DNA by the enzyme integrase. In the transcription and translation stage, HIV provirus is activated and converts HIV genes into messenger RNA then the messenger RNA is transported outside the nucleus to form the blueprint for producing new HIV proteins and enzymes. The strands of messenger RNA contain complete copies of HIV genetic material.

These assemble with newly made HIV proteins and enzymes to form new viral copies. Budding of the HIV releases the new viral particles from the cell. The new viral particles after budding are ready to infect other cells and the process of replication starts all over again (2).

2.1.1 Immunological response to HIV infection

HIV infection is characterized by several effects on the host's immune system. The number and function of B cells decline and CD4+ T-cells decline due to the toxicity of HIV antigens and distortion of cytokine regulation (13) (14). Non asymptomatic HIV patients show more T_H1-type cytokine like interleukin-2 (IL-2) and interferon alpha (IFN-α). Elevated response by CD4+ T-cells and cytotoxic CD8+ T-cells against HIV is observed in these patients. The Virus may counter these reactions by preventing an effective immune response and overwhelming the immune system by varying antigenic sites (15). The virus may also reduce the major histocompatibility complex (MHC) on the surface of cells and by reducing the number of CD8+ T-cells (16).

2.1.2 Antiviral strategies to HIV infection

HIV infection has no cure; therefore most antiviral strategies involve inhibition of normal viral functions. Antiretroviral drugs inhibit viral replication and form the cornerstone of HIV therapy. These drugs act by targeting enzymes involved in different stages of virus replication (2). Three types of antiretroviral drugs are currently employed in the fight against HIV. These are nucleoside reverse transcriptase inhibitors, non nucleoside reverse transcriptase inhibitors and protease inhibitors. Other minor antiretroviral drugs are integrase inhibitors and fusion inhibitors.

Nucleoside reverse transcriptase inhibitors act by binding to the active site of the enzyme reverse transcriptase and getting added to the DNA chain which halts normal 5' to 3' synthesis. Non nucleoside reverse transcriptase inhibitors bind to the reverse transcriptase enzyme at a site distal from the active site, inducing detrimental conformational change within the enzyme. Protease inhibitors inhibit the enzyme protease. These drugs are used in combination called Highly Active Antiretroviral Therapy (HAART) which is composed of three types of antiretroviral drugs. The most common combination is two nucleoside reverse transcriptase inhibitors and a non nucleoside reverse transcriptase inhibitor or a protease inhibitor (17). The first line antiretroviral therapy in adolescents and adults is illustrated in Table 2.1

Table 2.1: First Line ART in Adolescents and Adults. Adapted from NASCOP (2014)

Patient category	Preferred regimen	Alternative regimen
Adolescents (≥15 years) and Adults	TDF + 3TC + EFV	TDF + 3TC + NVP AZT + 3TC + EFV AZT + 3TC + NVP
HIV-infected sexual partner in sero-discordant relationship	TDF + 3TC +EFV	TDF + 3TC + NVP AZT + 3TC + EFV AZT + 3TC + NVP
For pregnant women and breastfeeding mothers	TDF + 3TC +EFV	TDF + 3TC + NVP AZT + 3TC + EFV AZT + 3TC + NVP
First line ART regimen to start in all women with previous exposure to NVP through PMTCT		
<than 24 months since previous NVP exposure	TDF + 3TC + ATV/r	TDF + 3TC + LPV/r AZT + 3TC + ATV/r AZT + 3TC + LPV/r
>More than 24 months since previous NVP exposure	TDF + 3TC +EFV	TDF + 3TC + NVP AZT + 3TC + EFV AZT + 3TC + NVP

3TC = lamivudine;AZT=Zidovudine; TDF= Tenofovir; EFV= Efavirenz;LPV/r= Lopinavir/ritonavir; ATV/r= Atanavir/ritonavir

2.1.3 Importance of CD4 cell count in HIV disease

CD4 cell count is the result of the clinical investigation which measures the patient's CD4 levels, as an indicator of the progress of Human Immunodeficiency Virus infection. The estimation of CD4+ T- cell counts is used to decide the initiation of antiretroviral therapy, monitor antiretroviral therapy and to start treatment of opportunistic infections in HIV positive patients (18). The threshold CD4 cell count to initiate antiretroviral therapy is updated regularly as current research may dictate. Previously a decline of CD4 cells count below 250 cells/mm^3 was used as an indicator to initiate antiretroviral therapy (19). Current research has shown that initiating antiretroviral therapy at high CD4 cell counts leads to overall better immunological response than at low CD4 cell counts (20).

2.1.4 Fluctuations of CD4 cell counts

The CD4 cell counts may fluctuate in the course of treatment for the HIV positive patients. Longitudinal analysis of the trajectories of CD4 cell counts have shown that clinical progression of HIV infection results in a decline in CD4 cell counts over time (21). CD4 cell counts tend to steadily increase in the first few years of therapy. This increase may reach a point in time when further increase is not possible and the patient attains maximum CD4 cell count, which may be followed by a decline. Social-demographic and genetic factors may also influence fluctuations in CD4 cell counts.

2.2. Nevirapine

Nevirapine is a potent non nucleoside reverse transcriptase inhibitor effective against HIV – 1. It is one of the most extensively prescribed antiretroviral drugs currently used to treat HIV-AIDS in Kenya (17). It is used in combination with nucleoside analogues in HAART. It has a molecular weight of 266.2979 g/mol and its chemical formula is $C_{15}H_{14}N_4O$. The structure of nevirapine is illustrated in Figure 2.1

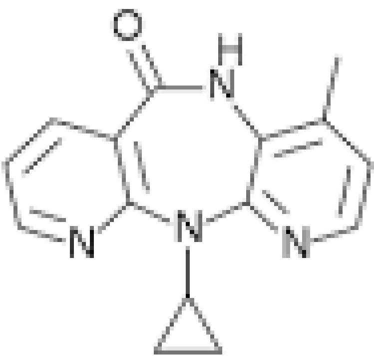


Figure 2.1: Structure of nevirapine

2.2.1. Mechanism of action

Nevirapine acts by binding to the tyrosine residues at position 181 and 188 of the HIV-1 reverse transcriptase enzyme (22). The position at which NVP binds to the HIV-1 reverse transcriptase is distant from the active site. The moment nevirapine binds to the tyrosine residues at position 181 and 188; a conformational change is induced in the HIV-1 reverse transcriptase which blocks the RNA-dependent and DNA-dependent DNA polymerase activities by causing a disruption of the enzyme's catalytic site. Any single mutation at codon 181 of the HIV-1 reverse transcriptase will induce resistance to nevirapine. Other mutations at codons 103, 100, 106, 108, 188 and 190 will also induce resistance. There is cross resistance to all non nucleoside reverse transcriptase inhibitors (2).

2.2.2. Pharmacokinetics of nevirapine

Nevirapine has very good oral bioavailability of more than 90% and freely partitions to all tissues including the brain (2). The presence or absence of food does not affect its oral bioavailability. It is estimated that following a single dose administration of nevirapine 50mg tablet, absolute bioavailability of 93 ± 9% (mean ± SD) can be achieved. The in vitro inhibitory concentration (IC_{50}) of nevirapine ranges from 10 to 100nM. Less than 60% of nevirapine that reaches systemic circulation is bound to plasma proteins. Nevirapine crosses the placenta and has been found in breast milk, due to this property, this drug has found a major application in preventing mother to child transmission (23).

2.2.3 Metabolism of nevirapine

Nevirapine is metabolized in the liver by oxidative metabolism by Cytochrome P450 class of enzymes. Specifically it is metabolized by CYP3A4 and CYP2B6 subclass of the larger super family Cytochrome P450 enzymes. Nevirapine is eliminated by glucoronide (ether) conjugation and urinary excretion of the ether-glucoronidated-oxidized metabolites (24). Four hydroxyl metabolites are produced after nevirapine metabolism at position 2, 3, 8, and 12. The metabolite 12-hydroxynevirapine is further metabolized by oxidation to 4-carboxynevirapine by the enzyme ALDH. These hydroxyl metabolites undergo glucoronidation by glucoronosyl transferase and finally are excreted in urine (25).

2.2.4 Pharmacogenetics of nevirapine metabolism

Nevirapine is metabolized to five hydroxyl metabolites at positions 2, 3, 8, and 12 and to 4-carboxynevirapine (26). It has been suggested that CYP3A4 metabolizes nevirapine to 2-hydroxynevirapine, while CYP2B6 metabolizes it to 3- hydroxynevirapine. CYP2D6 is involved in the formation of other metabolites. The CYP450 enzymes involved in nevirapine metabolism are illustrated in figure 2.2.

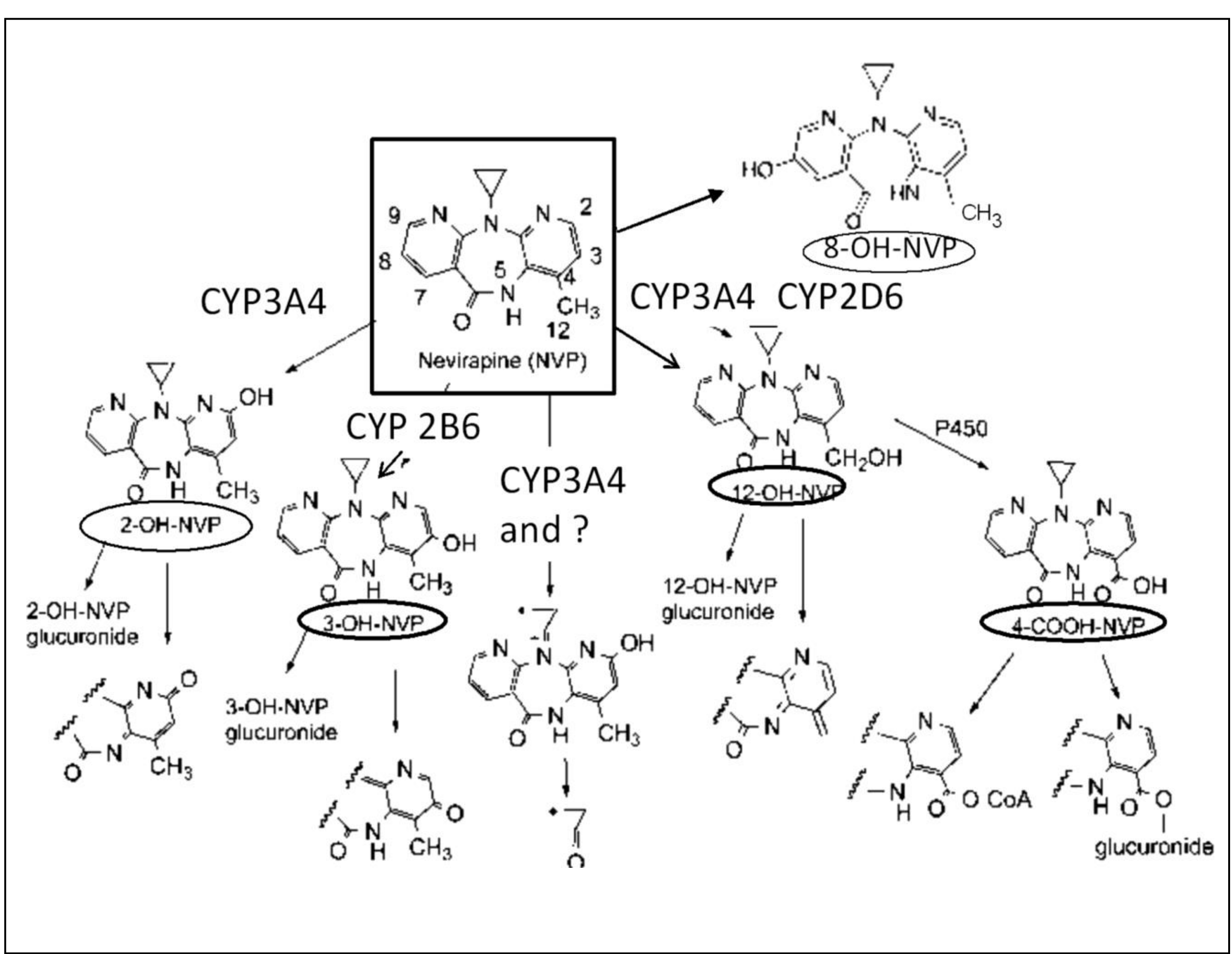


Figure 2.2: Enymes involved in nevirapine metabolism and its end products. Adapted from Chen (2008)

Nevirapine is also metabolized by CYP3A5, CYP2D6, and CYP2C9 enzymes but to a lesser degree than CYP3A5 and CYP2B6. CYP2D6 metabolizes nevirapine to 8-hydroxynevirapine and 12-hydroxynevirapine. CYP3A5 metabolizes nevirapine to 2-hydroxynevirapine and 12-hydroxynevirapine while CYP2C9 metabolizes nevirapine to 12-hydroxynevirapine.

2.2.5 Auto induction of CYP2B6 and CYP3A4 by nevirapine

Nevirapine is a moderate inducer of CYP2B6 and CYP3A4 and hence nevirapine induces its own metabolism. This auto induction of metabolism decreases its half life from 45 hours during initiation to 25 to 30 hours after 2 weeks. Nevirapine is initiated at a dose of 200mg once daily in the first two weeks when the patient is starting treatment and then 200mg twice daily if no hypersensitivity reactions have been observed (17).

2.3 CYP2B6

CYP2B6 is a member of the CYP450 superfamily that is very important in the metabolism of many drugs. CYP2B6 makes up approximately 2-10% of total hepatic cytochrome P (CYP) content. It is not only found in the hepatic cells but it is also found in the brain where it is believed to play an important role in the metabolism of central nervous system drugs and neurological side effects of drugs. It's a major metabolizer of non nucleoside reverse transcriptase antiretroviral drugs.

2.3.1 Genetic polymorphisms of CYP2B6

CYP2B6 is located within the CYP2B6 gene cluster on chromosome 19 (27), and this gene contributes 2 -10% of total CYP content (28). CYP2B6 exhibits genetic polymorphisms, thus far

29 allelic variants have been characterized (CYP2B6*1 to CYP2B6*29), and more than 100 single nucleotide polymorphisms have been described for CYP2B6 gene (29).

2.3.2 The prevalence of CYP2B6516GT and 983TC genotypes in various populations

The prevalence of CYP2B6 516G>T SNP varies among different ethnic groups. The prevalence of this SNP is 15 to 40% in Asians, 25% in Caucasians, and more than 50% in African Americans and black Africans (30). In a study that was conducted in an Iranian ethnic group the prevalence of genotypes for CYP2B6 516G>T SNPs were 28.1% for GT, 16% for GG and 4.8% for TT genotype(31). The prevalence for CYP2B6 516TT genotype was not different from Caucasians, Japanese and Chinese populations, but it was lower than in West African populations (17%) and Papua New Guinea (43%). Masebe et al have reported the prevalence of genotypes for CYP2B6 516G>T SNPs in South African HIV-1 patients to be 9.5% for the GG, 78.4 for GT and 12.1% for the TT genotype (3).

CYP2B6 983T>C SNP seems to be prevalent in African and African – American populations but not in Caucasians or Asians (9). Mehlotra et al 2007 have reported the allele frequency for the C allele to be 7.6% in Ghanians, 1.6% in Guinea, 5.5% in Ivory Coast, 3.8% in Sierra Leone and 6.2% among the Senegalese. In North America the prevalence for the allele was 7.5% for African-Americans and 1.1% for Hispanics. The allele was not expressed among the Caucasians and Asians (9).

2.3.3 The influence of CYP2B6 516G>T and 983T>C SNPs on nevirapine plasma levels

The study of polymorphisms of drug metabolizing enzymes has mainly been carried out in resource rich developed countries. Sub-Saharan Africa being resource limited has depended on research done in other countries to guide its prescribing patterns. Studies in Asian populations have been done to investigate the influence of CYP2B6516G>T SNP on the disposition of nevirapine (32). A study conducted on Cambodian HIV positive participants has revealed that genetic variability of CYP2B6516G>T SNP influenced the apparent clearance of nevirapine (32).

In one multivariable analysis (33), it was shown that CYP2B6 516G> T SNP and non Caucasian ethnicity remained significant predictors of nevirapine trough concentrations. In the same study it was shown that CYP2B6 516GT homozygosity had the greatest effect in predicting nevirapine trough concentrations. In a study conducted on a rural HIV positive Burundian population, it was shown that CYP2B6 516G>T and 983T>C genotypes influenced nevirapine plasma concentrations (34).In that study it was shown that nevirapine trough concentrations were above the target concentration in 84% of participants due to polymorphisms in these single nucleotide polymorphisms.

The influence of polymorphisms of CYP2B6 516G>T SNP on nevirapine plasma levels has been studied in ethnic Kenyan populations. An unpublished study done on the Luo, the Maasai, and the Kikuyu have shown significant influence of CYP2B6516G>T on plasma levels of nevirapine. The study showed that nevirapine plasma levels displayed greater inter-individual variability and were strongly associated to CYP2B6 516G>T single nucleotide polymorphisms (35).

CHAPTER THREE: METHODOLOGY

3.1 Study design

This study was nested under a larger study that was conducted in 2014 to determine patterns of ALT changes, and risk factors for ALT elevation among HIV positive patients on nevirapine based antiretroviral regimens at KNH Comprehensive Care Center (36). In that study blood samples from the study participants were to be genotyped for the expression of CYP2B6 516G>T and CYP2B6 983T>C single nucleotide polymorphisms but this was not performed. Therefore the purpose for this current study was to genotype the archived blood samples of the study participants for the expression of CYP2B6 516G>T and CYP2B6 983T>C single nucleotide polymorphisms and relate this polymorphisms with CD4 cell counts. The baseline characteristics, retrospective data and blood samples collection was done by Makori et al in 2014 (36). Genotyping of the blood samples was performed in 2015.

3.2 Study site

The study site was previously described by Makori et al 2014 (36). The DNA extraction from blood samples was done at the African Institute of Biomedical Science and Technology (AiBST) of the University of Nairobi, School of Pharmacy, Department of Pharmacology and Pharmacognosy. The African Institute of Biomedical Science and Technology was founded in 2002 by Professor Collen Masimirembwa and at the time its activities were mainly organization of international courses on the science and technologies of drug discovery and development. Its branch at the University of Nairobi focuses on pharmacogenetics of drug metabolism. DNA genotyping was conducted from the laboratories of The Centre for Public Health and Research, Walter reed project Nairobi. This laboratory specializes in research on viral infections.

3.3 Study population

The study population was archived blood samples obtained from the cohort study conducted by Makori et al 2014 (36).

3.4 Inclusion and exclusion criteria

The inclusion and exclusion criteria were previously described by Makori et al 2014 (36). The blood samples were from patients who were above 18 years but below 55 years and were on any nevirapine based ART regimen for at least 6 months. Patients were of either sex, and were not having major illness such as severe hepatotoxicity at the time of the study. Blood samples from patients who were less than 18 years and above 55 years were excluded. Samples from patients on nevirapine based ART regimen for less than 6 months were also excluded.

3.5 Sample size

The expected outcome of interest is the baseline CD4 levels, the rate of change of CD4 cell counts and current CD4 cell counts attained. Consequently the Twisk (2003) formula for the estimation of sample size of a continuous outcome variable was used (37).

$$N = \frac{(Z\,(1-\alpha/2) + Z\,(1-\beta))\ 2\alpha 2\ (r+1)\ [1+(T-1)\rho]}{V2rT}$$

Where,

N is the sample size,

$Z\,(1-\alpha/2)$ is the $(1-\alpha/2)$ percentile point of the standard normal distribution,

$Z\,(1-\beta)$ is the $(1-\beta)$ percentile point of the standard normal distribution,

Σ is the standard deviations of the outcome variable,

r is the ratio of the number of subjects in the compared groups,

T is the number of follow-up measurements

ρ is the correlation coefficient of the repeated measurements and

v is the difference in mean value of the outcome variable between the groups.

Therefore the sample size needed to make a 0.2 difference in a continuous outcome variable statistically significant on a 5% level with a power of 80% with different within-subject correlation coefficients (ρ) of 0 and 4 repeated measurements is 130. To accommodate for missing files and missing data entries, the calculated sample size was inflated by 20%. Therefore a minimum sample size of 156 participants was targeted.

3.6 Sampling and participant recruitment

Patient sampling and participant recruitment was done in 2014 as previously described by Makori et al 2014 (36).

3.7 Data Collection

Blood sample collection and processing, data collection and retrospective review of patient files was done in 2014 as previously described by Makori et al 2014 (36).

3.7.1 Adherence measurement

Adherence was measured by an oral interview conducted in 2014 by Makori et al (36) using an adherence tool to antiretroviral therapy. The study Participants were questioned with regard to: frequency in which they took medication 2 hours before or after the stipulated time, number of days the participants missed a dose in the previous week and how long ago the participants

missed a dose. These variables were used to compute a summary index of adherence on a scale of 1 to 14. A measure of 1 to 12 indicated poor adherence while a measure of 13 and above indicated good adherence.

3.8 Genotyping

Genotyping was conducted in 2015 at the African Institute of Biomedical Science and Technology (AiBST) and Centre for Public Health and Research, Walter Reed Project of the Kenya Medical Research Institute (KEMRI).

3.8.1 Materials and reagents

PureLink® Genomic DNA Kits for purification of genomic DNA were used for DNA extraction. The kits were composed of Proteinase K (20 mg/mL in storage buffer), RNAse A (20 mg/mL in 50 mMTris-HCL, pH 8.0, 10 mM EDTA) and buffers. The buffers included PureLink® Genomic Lysis/ Binding buffer, PureLink® Genomic Wash buffer 1, PureLink® genomic Wash buffer 2 and PureLink® Genomic Elution buffer (10 mMTris-HCL, pH 9.0, 0.1 mM EDTA). Other materials that were used were a heat block, sterile microcentrifuge tubes (supplied with the kit), pipettes, spin columns, collection tubes (supplied with the kit), vortexing machine (Thermal Electron Corporation, Denley VibroMix) and a centrifuge (Biofuge Pico, Heraeus Instruments).

The reagents for genotyping consisted of two tubes of 20X TaqMan Drug Metabolism Genotyping Assay with assay ID of C_7817765_60 and C_60732328_20, 2X TaqMan Genotyping Master Mix and distilled water for PCR (dH_2O). The TaqMan Drug Metabolism Genotyping assay consisted of 20X mix of unlabeled PCR primers and TaqMan MGB (minor groove binder) probes. The TaqMan MGB probes consisted of target-specific oligonucleotides with a reporter dye linked to the 5' -end of each probe (VIC dye for allele 1 probe and FAM dye for allele 2 probe). The probes also contained a non-fluorescent quencher (NFQ) at the 3'-end of the probe. The primers in the genotyping assay were for amplifying the sequence of interest and the two probes were for allele detection and allowed genotyping two possible variant alleles at the polymorphic site in a DNA target sequence. The minor groove binder was for increasing the melting temperature without increasing probe length. The 2X TaqMan Genotyping Master Mix contained AmpliTaq Gold DNA Polymerase to catalyze the reaction, dNTPs with dUTP as building blocks for new DNA strands and to minimize PCR cross contamination respectively, passive Reference to normalize for differences in fluorescence levels and optimized buffer components (GeneAmp 10X PCR Buffer). The GeneAmp 10X PCR Buffer contained 500mM potassium chloride, 100mM Tris-HCL (pH 8.3 at room temperature), 15 mM magnesium chloride and 0.01% (w/v) gelatin. The equipment that were not supplied with the reagents included the 7500 Fast Real Time PCR machine (Applied Biosystems, Foster City, CA)

3.8.2 Protein and RNA digestion

The DNA was extracted from frozen whole blood using PureLink® Genomic DNA Kit as per the manufacturer's protocol (Life Technologies Carlsbad CA). All the proteins were lysed by the addition of the enzyme Proteinase K. This was followed by hydrolysis of RNA by the addition

of 20µlof RNAse (20 mg/mL in 50 mMTris-HCL, pH 8.0, 10 mM EDTA). The contents were mixed well by vortexing and incubated at room temperature for 2 minutes. Two hundred microlitres of PureLink® Genomic Lysis/ Binding buffer was added and vortexed to obtain a homogenous solution, and incubated the lysate at 55°C for ten minutes to promote protein digestion. The genomic DNA separated from cellular proteins into solution. To the lysate we added 200µL 96 – 100% ethanol to concentrate and desalt the genomic DNA in aqueous solution for downstream applications. The lysate was mixed well by vortexing for 5 seconds to obtain a homogenous solution.

3.8.3 Binding DNA

Six hundred and fifty microlitresof the lysate was transferred to sterile PureLink® Spin Columns in collection tubes. The genomic DNA was bound to the columns. The ethanol in the lysate also enhanced the binding of DNA to the column. The contents were centrifuged at 10,000 x g for 1 minute at room temperature. The impurities passed into the collection tubes while genomic DNA remained bound to the spin columns. The collection tubes were discarded and the spin columns placed into clean PureLink® Collection Tubes.

3.8.4 Washing DNA

Five hundred microlitres of wash buffer 1 prepared with ethanol was added to the spin columns and centrifuged the columns at room temperature at 10,000 x g for 1 minute. The collection tubes were discarded and the spin columns placed into clean PureLink® Collection tubes. The washing of genomic DNA allowed for removal of impurities, proteins and polysaccharides while

the DNA remained bound to the column. Wash buffer 1 contained chaotropic salt whose purpose was to remove proteins and colored contaminants. Five hundred microlitres of wash buffer 2 prepared with ethanol was added to the spin columns and centrifuged the columns at maximum speed for 3 minutes at room temperature and discarded the collection tubes. The purpose for wash buffer 2 was to remove the salts leaving pure genomic DNA bound to the spin columns.

3.8.5 Eluting DNA

The spin columns were placed in sterile 1.5-mL microcentrifuge tubes and added 100µL PureLink® Genomic Elution Buffer (10 mMTris-HCL, pH 9.0, 0.1 mM EDTA) to the spin columns. The contents were incubated at room temperature for 1 minute and centrifuged the columns at maximum speed for 1 minute at room temperature. The 1.5-mL microcentrifuge tube contained purified genomic DNA. The spin columns were removed and discarded. The extracted DNA was quantified using NanoDrop microvolume quantitation of nucleic acids technique (NanoDrop 2000c spectrophotometer, Thermal Fisher Scientific Inc) and stored in a freezer at -20°C for further processing.

3.9 Real time PCR amplification

The PCR amplification consisted of three stages; preparation of the reaction mix, preparation of the reaction plate and performing the PCR.

3.9.1 Preparation of the reaction mixture

The reaction mix was composed of 20X TaqMan Drug Metabolism Genotyping Assay, TaqMan Genotyping Master Mix and nuclease-free water. The reaction mix was prepared by wet DNA method and the total volume of each component needed for each assay was calculated as shown in Table 3.1

Table 3.1: Preparation of the reaction mix for wet DNA method.

Component	**96- well plate (25 µL reaction)**
2X Taqman Master Mix (No AmpeRase)	12.50 µL
20X TaqMan Drug Metabolism Genotyping Assay working stock	1.25 µL
Nuclease free water	-
Total volume per well	**13.75 µL**

The 2X TaqMan Genotyping Master Mix was swirled gently to mix the contents. The 20X TaqMan Drug Metabolism Genotyping Assay was vortexed and centrifuged so as to mix properly. The required volumes of 2X TaqMan Genotyping Mix, 20X TaqMan Drug Metabolism Genotyping Assay and nuclease-free water were pipette into sterile tubes and capped then vortexed briefly to mix the components. The air bubbles were eliminated from the solution by centrifuging and spinning down the contents.

3.9.2 Preparation of the reaction plate

The reaction plate was prepared with wet DNA method. Each DNA samples were diluted in nuclease free water to deliver 1-15 ng of DNA per well. The reaction mix was pipetted into each well of the reaction plate using volumes listed in table 1. The reaction plate was covered with MicroAmp Optical Adhesive Film. The plate was gently swirled to spin down the contents and to eliminate air bubbles from the solutions. The MicroAmp Optical Adhesive Film was removed and into each well of the plate, pipetted 11.25 μL of DNA samples diluted in nuclease free water, and included a well for a no template control to check for contamination. The reaction plate was again covered with MicroAmp Optical Adhesive Film and sealed with the plate with a MicroAmp Adhesive Film Applicator. The contents were swirled gently to eliminate air bubbles from the solutions.

3.9.3 Allelic discrimination pre-read test

The reaction plate was introduced into the reaction chamber of the Fast Real Time PCR (Applied Biosystems, Foster City, CA) and the sequence detection software activated. Allelic discrimination pre-read test was performed in a total reaction mixture of 25µl.(4.25µl genomic DNA, 12.5µl TaqMan® Universal PCR Master Mix (NoAmperase UNG), 1.25µl TaqMan® Drug Metabolism Genotyping Assay Mix and 7µl DNAse free water). This was carried out at 60°C for 1 minute.

3.9.4 Fast Real Time PCR amplification

The DNA amplification was done using 7500 Fast Sequence Detection System (Applied Biosystems, Foster City, CA).Instrumentation parameters consisted of initial hold cycle for 10 minutes at 95ºC, followed by 50 cycles of denaturation at 92ºC for 15 seconds and then annealing and extension at 60ºC for 1 minute and 30 seconds. A negative control was included in the reactions to detect contamination.

3.9.5 Allelic discrimination post-read test

The Sequence Detection Software plotted R_n values using fluorescence measurements made during the plate read, based on the fluorescence signals from each well and then determined which alleles were in each sample. The general process consisted of creating and setting up a post read-PCR plate read document, performing a post-PCR plate read on a real-time PCR instrument, analyzing the experiment, making automatic or manual allele calls and verifying allele types. Each assay contained two allele specific probes and a primer that detected the specific SNP of interest. Increase in red dye (VIC) fluorescence indicated the presence of allele 1. Increase in blue dye (FAM) fluorescence indicated the presence of allele 2. The increase in green fluorescence indicated the presence of both allele 1 and allele 2. Sequence Detection Software was used to determine which genotypes were present using color codes of the alleles. The red dye (VIC) indicated the presence of homozygous wild-type allele, the blue dye (FAM) indicated the presence of homozygous variant allele while green indicated the presence of heterozygous allele. This was conducted at 60ºC for 1 minute.

3.10 Case definitions

Composite CYP2B6 516G>T and 983T>C genotypes were used to assign metabolic phenotypes in the study population (38). Participants that did not express a variant allele at either position 516 or 983 were classified as extensive metabolizers. Participants who expressed a single variant allele at either position 516 or 983 but not both were classified as intermediate metabolizers. Participants with 2 variant alleles at position 516 or 983 were classified as slow metabolizers.

3.11 Variables and outcomes

There were three outcome variables of interest. The main outcome variable of interest was the rate of change in the CD4 cell count. The other outcome variables of interest were the baseline CD4 cell count and current CD4 cell counts. The main co-variate of interest were polymorphisms of CYP2B6 516G>T and 983T>C genotypes. The other co-variates of interest were patient demographic characteristics and disease parameters such as renal and liver function.

3.12 Data analysis

Data analysis was done in three stages, descriptive, exploratory and regression data analysis.

3.12.1 Descriptive and exploratory data analysis

All variables were subjected to descriptive data analysis. The Shapiro-Wilk test was used to determine whether the continuous variables were normally distributed. Variables that were not normally distributed were expressed as the median and inter- quartile range. Variables that were normally distributed were expressed as mean and standard deviation of the mean.

Exploratory data analysis was conducted to identify key correlations and associations between variables. To determine the response profile, empirical growth plots of CD4 cell counts against time were generated for each individual. The optimal response profile was selected by conducting log transformation of the CD4 cell count and quadratic transformation of the time variable. The Hardy-Weinberg equilibrium was calculated using excel spreadsheet obtained online (39).

3.12.2 Regression analysis

For the two outcome variables, baseline CD4 count and current CD4 count attained, multi-linear regression was done. Model building was done in all regression analysis using a forward stepwise approach. Variables that had a P value of 0.002 on bivariable analysis were considered for model building.

3.12.3 Multi-level regression analysis for longitudinal changes in CD4 cell count

The data was subjected to linear mixed effects modeling. In model building we began by fitting both the unconditional means model and unconditional growth models. Next, the individual independent variables were added singly to obtain the unadjusted effects of the predictors. The models were then modified to determine if the predictors affected the rate of change, initial status or both. After identifying the key predictor variables, forward stepwise model building was conducted to identify the most important predictor variables. The Deviance and Hausaman tests were conducted to determine if the random effects should be dropped from the model. In the

final parsimonious model the question predictors were added. Regression analysis was done using STATA version 11 software and all variables with P values of 0.05 or less were considered to be statistically significant.

3.13 Ethical considerations

Permission to conduct the study was granted by the KNH/UoN Research and Ethics committee (Ref: KNH-ERC/R/48 and KNH-ERC/MOD/145). The letters granting ethical approval are appended in appendix A and B respectively.

CHAPTER FOUR: RESULTS

The results of this study are presented in three different parts: the medical social-demographic characteristics of the study participants; the prevalence and allelic frequency of CYP2B6 516G>T and 983T>C genotypes of the study participants; and factors that influenced the CD4 cell counts.

4.2 PART A: Social-demographic characteristics of the study participants

4.2.1 Baseline characteristics of the study participants

The baseline characteristics of the study participants were adapted from those previously reported by Makori et al (2014) (36). A total of 241 participants took part in the study of which 185 (76.8%) were females, and 56 (23.2%) were males. The median age of the study participants was 39 years [inter-quartile range (IQR) 35, 44]. The number of study participants with a baseline body mass index below 18.5kg/m^2 was 13(5.4%). The median body weight at baseline was 62 kg ranging from 56 to 70kg. The number of married participants was 154 (64.3%). The Bantu ethno-linguistic group had the most number of participants at 184 (76.7%), followed by the Nilotes with 43 participants (18%). The Cushites and the other participants who did not report their ethnicity constituted the remaining 14 (5.3%) participants. The median ALT levels at initiation of HAART was 22 [17-32]. The median creatine levels study recruitment was 80 [66-92] while for estimated glomerular filtration rate was 101.7 [80.7-120.2]. The median baseline CD4 cell count was 206 [127-270]. A summary of the baseline characteristics of the study participants is presented in the Table 4.1.

Table 4.1: Baseline characteristics of the study participants

Variables	n (%) or median (IQR)
Duration of therapy	4.8 [3.3-6.6]
Sex	
Male	56 (23.2)
Female	185 (76.8)
Age at diagnosis in years	39 [35-44]
Weight at diagnosis (Kg)	62 [56-70]
Height (cm)	162 [158-168]
BMI at HAART initiation	
≤18.5	147 (61)
≥18.5	94 (39)
Ethnolinguistic groups	
Bantu	184 (76.7)
Nilotes	43 (18)
Cushites and others	14 (5.3)
Regimens at initiation of HAART	
TDF,3TC,NVP	71 (29.5)
AZT,3TC,NVP	78 (32.4)
D4T,3TC,NVP	85 (35.3)
D4T,3TC,EFV	1 (0.4)
AZT, 3TC,EFV	3 (1.2)
ALT at initiation of HAART	22 [17-32]
Normal (≤40UI/L)	209 (86.7)
Elevated (≥40UI/L)	26 (10.8)
Missing	6 (2.5)
Creatinine levels	80 [66-92]
Normal (≤120 up/dl)	211 (87.6)
Elevated (≥120 up/dl)	10 (4.2)
Missing	20 (8.3)
Estimated GRF (ml/min/1.73m^2)	101.7 [80.7-120.2]
eGFR ≥50	214 (88.9)
eGFR ≤50	5 (2.1)
missing values	22 (9.1)
CD4 cell counts x 10^9/L	206 [127-270]
≤250	158 (65.6)
≥250	68 (28.2)
Missing values	15 (6.2)
Concurrent illness	
None	182 (75.5)
Hypertension	36 (14.9)
Diabetes	3 (1.2)
PUD	4 (1.7)
Asthma	3 (1.2)
Chronic pain	5 (2.1)
Other conditions	8 (3.6)

Adapted from Makori 2014

Abbreviations: ART, antiretroviral therapy; BMI, body mass index, calculated as the weight in kilograms divided by the square of height in meters; IQR, inter-quartile range; PUD, peptic ulcer disease; n= proportion per category.

4.2.2 Antiretroviral regimens and patient adherence

The antiretroviral regimens of the participants were previously described by Makori et al 2014(36). Two hundred and thirty four (97%) of the participants were initiated on nevirapine based regimens. Seven (3%) participants had been started on efavirenz based regimens but had switched to nevirapine based regimen at the time of recruitment into the study. The regimens of the study participants are outlined in the Table 4.2.

Table 4.2: Regimens of study participants at ART initiation and change of regimen on follow up (Adapted from Makori et al 2014).

Regimen	Participants at treatment initiation n (%)	Participants who switched to regimen n (%)	Participants on Current regimen n (%)
D4T+3TC+NVP	85 (35.3)	0	0
TDF+3TC+NVP	71 (29.5)	80 (87)	151 (62.7)
AZT+3TC+NVP	78 (32.4)	11 (11.9)	89 (36.9)
AZT+3TC+EFV	3 (1.2)	0	0
TDF+3TC+EFV	3 (1.2)	0	0
ABC+3TC+EFV	1 (0.4)	0	0
ABC+3TC+NVP	0	1 (1.1)	1 (0.4)
Total	**241**	**92**	**241**

TDF: Tenofovir; 3TC: Lamivudine; NVP: Nevirapine; AZT: Zidovudine, D4T: Stavudine; ABC: Abacavir; EFV: Efavirenz

Of the 241 participants who participated in the study, 92 switched regimens in the course of their therapy. Stavudine based side effects were the cause of most regimen switches. This caused the participants to develop lipodystrophy and peripheral neuropathy. One hundred and ninety four

(194%) of the study participants had good adherence and 47 (19%) had poor adherence. The key findings with regard to adherence are summarized in Table 4.3

Table 4.3: Adherence parameters of the study participants

Adherence measure	Frequency n (%)
Adherence scale	
Scale <12 (poor)	47 (19.5)
Scale 12 > (good)	194 (80.5)
N	**241**
Adherence score	
5	1 (0.4)
6	1 (0.4)
7	1 (0.4)
8	13 (5.4)
9	8 (3.3)
10	7 (2.9)
11	16 (6.6)
12	20 (8.3)
13	116 (48.1)
14	58 (24.1)
N	**241**

4.3 PART B: Prevalence and Allelic frequencies of CYP2B6 genotypes

4.3.1 CYP2B6 516G>T and 983T>C genotypes and allele frequencies

The prevalence of the CYP2B6 516GG wild-type genotype was 89 (45.9%) while that of the heterozygous GT genotype was 73 (37.6%), and the TT variant genotype was 32 (16.5%). For the CYP2B6 983T>C SNP, the prevalence of the heterozygous TC genotype was 19 (9.3%).

The wild-type TT genotype had a prevalence of 183 (89.7%), while the CC variant genotype had the lowest prevalence of2 (1%).

Table 4.4: CYP2B6 516G>T and 983T>C genotypes and allele frequencies in the study population

Characteristic	Proportion of patients			
	n	%	$\chi 2$	P
CYP2B6 516G>T				
Genotypes				
GG	89	45.9		
GT	73	37.6		
TT	32	16.5		
N	194	100		
Alleles				
G	251	64.7	6.04	**0.014**
T	137	35.3		
N	388			
CYP2B6 983T>C				
Genotypes				
TT	183	89.7		
TC	19	9.3		
CC	2	1		
N	204	100		
Alleles				
T	385	94.4	3.165	**0.075**
C	23	5.6		
N	408			

The genotype and allele frequencies in the study population are presented in Table 4.4. The G allele for CYP2B6 516G>T SNP occurred at a frequency of 64.7% while the frequency for T allele was 35.3% (χ^2= 6.04; P<0.014). Comparison between observed versus predicted genotype frequencies for this SNP did not conform to Hardy-Weinberg equilibrium proportions. The T allele for CYP2B6 983T>C SNP occurred at a frequency of 94.4% while the frequency for C allele was 5.6% (χ^2 = 3.165; P = 0.075). Comparison between observed and predicted genotype frequencies for this single nucleotide polymorphism conformed to Hardy-Weinberg proportions.

4.3.2 CYP2B6 516G>T and 983T>C genotypes and allele frequencies in the ethno-linguistic groups

The distribution of CYP2B6 516G>T and 983T>C genotypes were estimated in three major Kenyan ethno-linguistic groups namely: the Bantus, Nilotes and Cushites. There was no statistically significant difference in the distribution of these genotypes across the ethno-linguistic groups (P= 0.631 and 0.898 for CYP2B6 516G>T and 983T>C respectively). There were 73 (82%) Bantus and 15 (16.9%) Nilotes that expressed the CYP2B6 516GG genotype. This genotype was not expressed in Cushites and only 1(1.1%) participant from other ethnic groups expressed this genotype. For the heterozygous GT genotype, there were 57 (78.1%), 12 (16.4%), 1 (1.4%) and 3 (4.1%) participants among the Bantus, Nilotes, Cushites and other ethno-linguistic groups who expressed this genotype respectively. The homozygous variant TT genotype was only expressed among the Bantus 26 (81.2%) and Nilotes 6 (18.8%). For the CYP2B6 983T>T wild type genotype, the prevalence among the Bantus was 147 (80.3%), Nilotes 30 (16.4%), Cushites 1 (0.5%) and others were 5 (2.8%). The heterozygous TC genotype was only expressed among the Bantus 14 (73.7%) and Nilotes 5 (26.3%). Only Bantus 2 (100%) expressed the homozygous CC variant genotype.

Table 4.5: Prevalence of CYP2B6 516G>T and 983T>C genotypes in the study population

	Bantu	Nilotes	Cushites	Others	N	P value
CYP2B6 516G>T						
CYP2B6 516GG	73 (82%)	15 (16.9%)	0 (0%)	1 (1.1%)	89	0.631
CYP2B6 516GT	57 (78.1%)	12 (16.4%)	1 (1.4%)	3 (4.1%)	73	
CYP2B6 516TT	26 (81.2%)	6 (18.8%)	0 (0%)	0 (0%)	32	
N	156	33	1	4	194	
CYP2B6 983T>C						
CYP2B6 983TT	147 (80.3%)	30 (16.4%)	1 (0.5%)	5 (2.8%)	183	
CYP2B6 983TC	14 (73.7%)	5 (26.3%)	0 (0%)	0 (0%)	19	0.898
CYP2B6 983CC	2 (100%)	0 (0%)	0 (0%)	0 (0%)	2	
N	163	35	1	5	204	

The prevalence of CYP2B6 516G>T and 983T>C genotypes are illustrated in Table 4.5. The prevalence of the CYP2B6 516 G allele in Bantus was 203 (65.1%), in Nilotes it was 42 (63.6%) while in Cushites and other ethno-linguistic groups it was 1 (50%) and 5 (62.5%)` respectively. The prevalence for T allele was 109 (34.9%), 24 (36.4%), 1 (50%) and 3 (37.5%) among the Bantus, Nilotes, Cushites and other ethno-linguistic groups respectively. Only the prevalence in Bantus did not conform to Hardy-Weinberg equilbrium. The prevalence of CYP2B6 983 T allele in Bantus was 308 (94.5%), 65 (92.9%) among the Nilotes, 2 (100%) among the Cushites and 10 (100%) in other ethno-linguistic groups. For the C allele, the prevalence in Bantus was 18 (5.5%) and 5 (7.1%) in the Nilotes. The Cushites and other ethno-linguistic groups did not express this allele. The comparison of observed versus predicted allele

frequencies did not conform to Hardy-Weinberg proportions for the Bantus only. The allele frequencies of CYP2B6 516G>T and 983T>C genotypes are presented in table 4.6.

Table 4.6: Allele frequencies of CYP2B6 516G>T and 983T>C genotypes in the study population.

	Bantu	Nilotes	Cushites	Others	N
CYP2B6 516G>T SNP					
G	203 (65.1%)	42 (63.6%)	1 (50%)	5 (62.5%)	251
T	109 (34.9%)	24 (36.4%)	1 (50%)	3 (37%)	137
N	312	66	2	8	388
χ^2	6.0098	1.5153	-	1.4400	
P	**0.014**	0.2183	0.3173	0.2301	
CYP2B6 983T>C SNP					
T	308 (94.5%)	65 (92.9%)	2 (100%)	10 (100%)	385
C	18 (5.5%)	5 (7.1%)	0 (0%)	0 (0%)	23
N	326	70	2	10	408
χ^2	5.0932	0.2071	-	-	
P	**0,024**	0.649	0.3173	0.2301	

4.3.3 Prevalence of CYP2B6 516G>T and 983T>C genotypes by sex

There was no statistically significant difference in the distribution of CYP2B6 516G>T (P<0.808) and 983T>CSNPs (P<0.531) by sex. For the CYP2B6 516G>T SNP, 69 (77.5%) female participants expressed the homozygous wild-type GG genotype as compared to 20 (22.5%) males. Fifty six (76.8%) female participants and seventeen (23.2%) male participants expressed the heterozygous GT genotype while 23 (71.9%) and 9 (28.1%) females and males respectively expressed the TT genotype. For the CYP2B6 983T>C SNP, 139 (76%) females expressed the homozygous wild-type TT genotype as compared to 44 (24%) males. The number of female participants with the heterozygous TC genotype was 16 (84.2%) and for males were 3 (13.8%). Only 2 (100%) females expressed the homozygous mutant genotype. This genotype was not expressed by males. The key findings are presented in Table 4.7.

Table 4.7: Prevalence of CYP2B6 516G>T and 983T>C genotypes by sex in the study population

Genotypes	Females	Males	N	P value
		CYP2B6 516G>T SNP		
CYP2B6 516GG	69 (77.5%)	20 (22.5%)	89	
CYP2B6 516GT	56 (76.8%)	17 (23.2%)	73	0.808
CY2B6 516TT	23 (71.9%)	9 (28.1%)	32	
N	148	46	194	
		CYP2B6 983T>C SNP		
CYP2B6 983TT	139 (76%)	44 (24%)	183	
CYP2B6 983TC	16 (84.2%)	3 (15.8%)	19	0.531
CYP2B6 983CC	2 (100%)	0 (0%)	2	
N	157	47	204	

4.3.4 Predicted phenotypes on the basis of genotypic profile

Composite CYP2B6 516G>T and 983T>C genotypes were used to assign metabolic phenotypes in the study population (38). Participants that did not express a variant allele at either position 516 or 983 were classified as extensive metabolizers. Participants who expressed a single variant allele at either position 516 or 983 but not both were classified as intermediate metabolizers. Participants with 2 variant alleles at position 516 or 983 were classified as slow metabolizers. The STATA command *generatecombiphenotype = phenotypeone + phenotypetwo*categorized 189 study participants as extensive, intermediate or poor metabolizers. On this basis, 34 (18%) participants were predicted to be slow metabolizers. Eighty three (44%) participants were predicted to be intermediate metabolizers and 71 (38%) were predicted to be extensive metabolizers. The prevalence of the phenotype frequencies are presented in Table 4.8

Table 4.8: Predicted phenotype frequencies in the study population

Phenotype	Bantus	Nilotes	Cushites	Others	Totals	P value
			CYP2B6 516G>T			
Extensive metabolizer	73 (83%)	14 (16%)	0 (0%)	1 (1%)	88	
Intermediate metabolizer	57 (78.1%)	12 (16.4%)	1 (1.4%)	3 (4.1%)	73	0.626
Poor metabolizer	26 (81.3%)	6 (18.7%)	0 (0%)	0 (0%)	32	
			CYP2B6 983T>C			
Extensive metabolizer	147 (80.3%)	30 (16.4%)	1 (0.5%)	5 (2.7%)	183	
Intermediate metabolizer	14 (8.7%)	5 (14.3%)	0 (0%)	0 (0%)	19	0.902
Poor metabolizer	2 (1.2%)	0 (0%)	0 (0%)	0 (0%)	2	

4.4 PART C: Factors that influenced CD4 cell count

4.4.1 Description of baseline CD4 cell counts of the study population

At the initiation of HAART, 158 (65.6%) participants had CD4 cell counts below 250cells/mm^3. Sixty eight (28.2%) participants had CD4 cell counts above 250cells/mm^3. Baseline CD4 cell counts for fifteen (6.2%) participants were not reported. The median range for baseline CD4 cell counts was 206 [127 - 270]. The Shapiro Wilk test for normality showed that the baseline CD4 cell count at treatment initiation was not normally distributed ($P < 0.01$). The median and inter-quartile levels for baseline and current CD4 levels are presented in Table 4.9.

Participants who at the study initiation were less than 40 years and whose baseline BMI was above 18.5 had higher baseline CD4 cell counts than their counterparts. Females generally had higher baseline CD4 cell counts than men. The study participants who had an estimated glomerular filtration rate of ≥ 50ml/min/1.73m^2 had higher baseline CD4 cell counts compared to those with ≤ 50ml/min/1.73m^2. Study participants with normal liver function (≤40UI/L) had lower baseline and current CD4 cell counts.

Table 4.9: **Baseline and current CD4 levels attained in the study population**

patient characteristics	n	baseline CD4 levels median [iqr]	p value	n	current CD4 levels attained median [iqr]	p value
Sex						
Male	50	198[115-261]	0.267	55	575[406-761]	0.161
Female	175	210[128-281]		185	627[447-818]	
Age						
≤40	117	216[153-299]	**0.002**	125	605[438-794]	0.664
≥40	108	189[107-249]		115	627[436-833]	
Regimens at initiation						
AZT,3TC,NVP	78	144[128-246]		3	345[144-606]	
D4T,3TC,NVP	85	191[126-205]		6	537[416-737]	
ABC,3TC,NVP	1	238[238-238]	0.518	1	872[872-872]	**0.041**
D4T,3TC,EFV	1	199[100-279]		69	761[599-888]	
AZT,3TC,EFV	3	238[142-261]		3	420[287-805]	
TDF,3TC,EFV	3	219[219-219]		3	561[489-959]	
Current regimen						
TDF,3TC,NVP	138	208[123-286]		147	657[481-836]	
AZT,3TC,NVP	78	206[142-249]	0.762	82	513[397-743]	**0.024**
D4T,3TC,NVP	8	177[122-242]		10	490[448-644]	
ABC,3TC,NVP	1	238[238-238]		1	872[872-872]	
CYP2B6 516G>T SNP						
CYP2B6 516GG	84	208[143-282]		89	594[436-815]	
CYP2B6 516GT	68	206[119-264]	0.445	73	644[482-833]	0.450
CYP2B6 516TT	30	193[128-235]		32	624[393-735]	
CYP2B6 983T>C SNP						
CYP2B6 983TT	173	200[128-263]		183	616[447-812]	
CYP2B6 983TC	17	235[192-286]	**0.089**	19	657[493-874]	0.721
CYP2B6 983CC	2	335[281-388]		2	675[515-835]	
eGFR (ml/nin/1.73m^2)						
Normal (≥50)	213	210 [127-273]	0.147	214	611 [433-815]	0.281
Poor (≤50)	5	144 [57-180]		5	609 [308-627]	
Liver function						
Normal (≤40UI/L)	210	206[124-270]	0.391	220	608[434-813]	0.925
Elevated (≥40UI/L)	14	218[145-306]		14	631[515-727]	
Concurrent illness						
Hypertension	34	200[123-246]		36	680[489-826]	
Diabetes	3	147[135-199]		3	420[326-710]	
PUD	4	205[146-251]	0.692	4	635[491-799]	0.569
Asthma	3	153[115-237]		3	797[375-812]	
Chronic pain	5	235[232-395]		3	649[572-657]	
cancer	2	190[147-233]		5	474[436-511]	

Participants who at the study recruitment were on D4T, 3TC and EFV had the lowest baseline CD4 cell count. Only one participant was on ABC, 3TC and NVP, this participant had the highest baseline CD4 cell counts. Participants who expressed the homozygous CYP2B6 516TT variant genotype had the lowest baseline CD4 cell counts compared to those who expressed the homozygous wild-type and heterozygous genotypes of this genotype. Participants who expressed the CYP2B6 983TT homozygous wild-type genotype had lowest baseline CD4 cell counts compared to those who expressed the homozygous mutant and heterozygous genotypes. The participants who expressed homozygous wild-type 516GG and 983CC variant genotypes had the highest baseline CD4 cell counts compared to those who expressed other genotypes.

4.4.2 Factors affecting baseline CD4 cell counts levels

On bivariable analysis, factors that affected the baseline CD4 levels were age, baseline BMI, and alcohol use as shown in Table 4.10. Other factors that were associated with baseline CD4 cell counts were severity in liver dysfunction and severity in adverse drug reactions. All other factors had no significant effect on baseline count. On adjustment for confounding only age of the study participants and baseline BMI were associated with the baseline CD4 cell counts. The study participants who were 40 years and above had CD4 cell counts declining by -0.01 units (P<0.022). The CYP2B6 516G>T and 983T>C genotypes did not have any significant effect on the baseline CD4 cell count.

Table 4.10: Factors that affected the baseline CD4 cell counts in the study population

Variable	**Crude Beta coefficient (95% CI)**	**P value**	**Adjusted Beta coefficient (95% CI)**	**P value**
Age	-0.013 (-0.023, -0.003)	**0.013**	-0.01 (-0.019, -0.001)	**0.022**
Initial BMI	0.031 (0.015, 0.047)	**0.000**	0.026 (0.128, 0.04)	**0.000**
Alcohol use	0.146 (0.016, 0.276)	**0.028**	-	-
Severity of liver dysfunction	-0.089 (-0.193, 0.014)	**0.091**	-	-
Severity in ADRs	0.162 (-0.024, 0.347)	**0.088**	-	-

4.4.3 Changes in the CD4 cell counts during antiretroviral therapy

A scatterplot was generated to examine how CD4 counts changed with time. At baseline there was a lot of variance in the CD4 counts, with outliers. With time the variance seemed to reduce. The CD4 levels showed non-linear change reaching towards an upper asymptote. This is illustrated in the scatter plot of the first 50 participants in the Figure 4.1. The CD4 response plateaued and then started to fall. The CD4 response plateaued between the 4th and 6th years of treatment and thereafter declined slightly. The response profile was approximately quadratic.

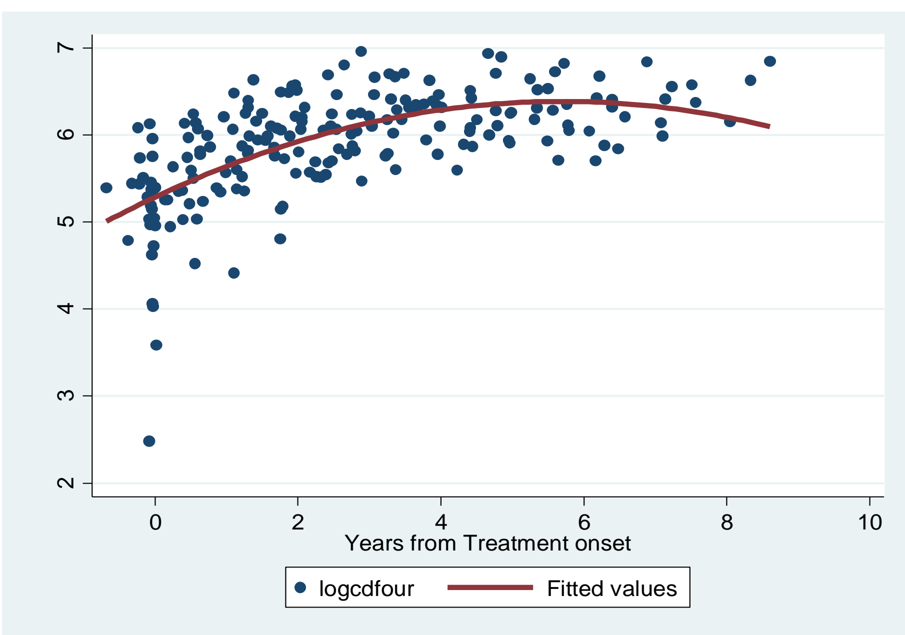
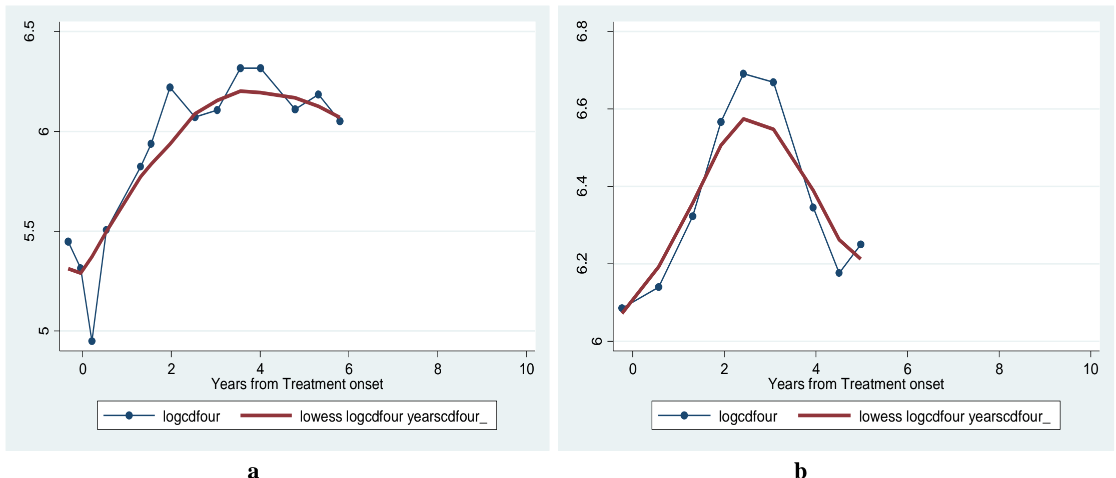


Figure 4.1: Scatter plot of CD4 levels of the first 50 participants with a fitted quadratic predictor

Study participants especially those who had been on treatment for less than two years gave a simple linear response profile. Participants who had been on treatment for more than three years gave a curve-linear response profile with CD4 cell count plateauing at log CD4 values of above 6.

Fifty six participants had a discontinuous response profile with initial rapid increase in CD4 cell count; and this was followed by a phase of decline in rate of increase. For seventy one participants, a plateau phase was not achieved even after 6 years of therapy and the CD4 cell count continued to increase. Two selected participants showed a clear decline in CD4 cell count (Figure 4.2a and b). In one patient the decline was very severe and almost reached the baseline value.



a **b**

Figure 4.2: The CD4 cell count of two selected participants

4.4.4 Factors that affected the rate of change in the CD4 cell count

Hierarchical regression modeling was conducted to identify variables that affected the rate of change in the CD4 cell count. The results are presented in Table 4.11. The duration of therapy and eGFR at various time points, were the main factors that significantly affected the rate of change of CD4 cell counts. When adjustment for confounding for estimated glomerular filtration rate at various time points was done, duration of therapy, age of the study participants, baseline BMI and changes in BMI at various time points were the main factors that affected the rate of change of CD4 cell counts. The effect of CYP2B6 983TC genotype also became significant ($p<0.04$).

Table 4.11: Factors that the rate of change of CD4 cell counts in the study population

Log CD4	**Crude Beta coefficient (95% CI)**	**P value**	**Adjusted Beta coefficient (95% CI)**	**P value**
Duration of therapy	0.314 (0.263 – 0.366)	**0.000**	0.269 (0.213, 0.325)	**0.000**
Duration of therapy Squared	-0.000 (-0.000 - -0.000)	**0.003**	-0.034(-0.043, -0.024)	**0.000**
Demographic characteristics				
Age	0.002 (-0.000, 0.004)	0.100	-0.100(-0.019, -0.001)	**0.022**
Baseline BMI	-0.003 (-0.006, 0.001)	0.116	0.026 (0.013, 0.04)	**0.000**
Change BMI	-	-	-0.031 (-0.054, -0.007)	**0.011**
			-	**-**
ARV regimens				
AZT/3TC/NVP	0.002 (-0.013, 0.016)	0.835	-	-
D4T/3TC/NVP	-0.002 (-0.045, 0.040)	0.914	-	-
			-	
CYP2B6 516G>T and 983T>C genotypes				
CYP2B6 983TC	-0.018 (-0.059, 0.023)	0.382	-0.057 (-0.11, -0.003)	**0.040**
CYP2B6 516GT	-0.007 (-0.027, 0.013)	0.490	-	-
Kidney function				
eGFR at various time timepoints	-0.000 (-0.001, -0.000)	**0.004**	**-**	**-**
Measures of Adherence				
Adherence score	0.000 (-0.008, 0.008)	0.954	**-**	**-**
Delay in taking drugs 2 hours before/after the stipulated time	-0.026 (-0.052, 0.000)	**0.054**	**-**	**-**
How long ago the patient missed a dose	0.004 (-0.007, 0.014)	0.462	**-**	**-**
Co-morbidities			**-**	**-**
Concurrent illness	0.0001 (-0.007, 0.008)	0.897		
allergies	-0.002 (-0.012, 0.008)	0.686		

On bivariable analysis, factors that were found to affect the rate of change of log CD4 cell count were: duration of therapy, estimated glomerular filtration rate and delay in taking antiretroviral drugs two hours before or after the stipulated time. The composite adherence score, co-morbidities, CYP2B6 516G>T and 983T>C genotypes and antiretroviral regimens did not have a significant effect on the rate of change of CD4 cell counts. During forward model building, these factors did not have a statistically significant effect on rate of change of CD4 cell count. In

the parsimonious model, the key determinants of the log CD4 cell count were BMI, CYP2B6 983TC genotype, age and duration of therapy.

4.4.5 The effect of CYP2B6 516G>T and 983T>C genotypes on the rate of change of CD4 cell count

On bivariable analysis, the expression of CYP2B6 516GT and 983TC genotypes had a negative association with the rate of change of CD4 cell counts [crude β =-0.007 (-0.027, 0.013), $P<0.490$] and [Crude β=-0.018 (-0.059, 0.023), $P<0.382$] respectively. However this association was not statistically significant. When adjustment for confounding for estimated glomerular filtration rate was performed, only CYP2B6 983TC genotype was associated with the rate of change of CD4 cell counts [Adjusted β=-0.057 (-0.11, -0.03), $P<0.04$].

4.5 Factors that affected current CD4 cell count of the study population

The square root of current CD4 cell count attained was obtained and regressed against the covariates as presented in Table 4.12. The Shapiro-Wilk test for normality showed that the current CD4 cell count was not normally distributed. The current CD4 count ranged from 92 to 2735, with a median CD4 count of 615 and an inter-quartile range of 437 – 812.5.

Table 4.12: Factors that the current CD4 cell counts in the study population

Square root of current CD4	Beta coefficient (95% CI)	P value	Adjusted beta coefficient (95% CI)	P value
Duration of therapy		**0.000**	1.079 (0.719, 1.439)	**0.000**
Baseline CD4 count			0.179 (0.11, 0.025)	**0.000**
Demographic characteristics				
Sex	-1.393 (-2.947, 0.16)	**0.079**	-1.594 (-3.02, -0.168)	**0.029**
Initial BMI	0.178 (0.015, 0.341)	**0.032**	-	**-**
Change in BMI	0.266 (0.042, 0.489)	**0.020**	-	**-**
Current BMI			0.239 (0.075, 0.404)	**0.004**
CYP2B6 516G>T and 983T>C genotypes				
CYP2B6 516GT	0.76 (-1.364, 2.88)	0.481	-	-
CYP2B6 516TT	0.906 (-3.53, 5.339)	0.688		
CYP2B6 983TC	0.558 (-1.14, 2.275)	0.522	-	-
CYP2B6 983CC	-1.012 (-3.16, 1.119)	0.349		
Regimen at initiation				
D4T/3TC/NVP	5.051 (-1.97, 12.06)	0.156	-2.138 (-3.622, -0.654)	**0.005**
ABC/3TC/NVP	11.13 (4.94, 17.328)	**0.001**	-	**-**
D4T/3TC/EFV	8.737 (2.426, 15.05)	**0.007**	-	**-**
AZT/3TC/EFV	11.25 (2.832, 19.68)	**0.009**	-	**-**
TDF/3TC/EFV	2.531 (-3.66, 8.727)	0.419	-	**-**
Current regimen				
AZT/3TC/NVP	-1.502 (-3.084, 0.08)	**0.063**		
D4T/3TC/NVP	-2.418 (-4.47, -0.37)	**0.021**		
ABC/3TC/NVP	4.078 (3.2, 4.957)	**0.000**		
			-	-
Concurrent illness				
Cancer	-3.171 (-4.69, -1.65)	**0.000**		
URTI	-11.38 (-12.3, -10.5)	**0.000**		
Anemia	-1.228 (-2.11, -0.34)	**0.007**		
Others	-5.021 (-10.21, 0.17)	**0.058**		

On bivariable analysis, there was a positive correlation between duration of therapy, baseline CD4 cell counts and current CD4 cell counts attained in the study population.

All the regimens at initiation of ART except TDF/3TC/EFV and D4T/3TC/NVP were associated with current CD4 cell counts attained. The current regimens were positively associated with the current CD4 cell counts attained by the study participants. The presence of concurrent illness

was negatively associated with current CD4 cell counts attained. Concurrent illness that had the most important association were; cancer [Crude β=-3.171 (-4.687, -1.656)], URTIs [Crude β=-11.386 (-12.271, -10.501)] and anemia [Crude β=-1.229 (-2.114, -0.343)]. The polymorphisms of CYP2B6 516G>T and 983T>C genotypes did not have any association with the current CD4 cell count attained.

On adjusting for confounding, duration of therapy [Adjusted β=1.079 (0.719, 1.439)], baseline CD4 cell counts [Adjusted β=0.179 (0.11, 0.025)] and current BMI [Adjusted β= 0.239 (0.075, 0.409)] were positively associated with current CD4 cell counts attained by the study population. Sex [Adjusted β=1.394 (-3.02, -0.168)] and D4T/3TC/NVP regimen [Adjusted β=- 2.138 (-3.622, -0.654)] were negatively associated with current CD4 cell counts attained.

CHAPTER FIVE: DISCUSSION

This study found no association between CYP2B6 983TC genotype and the rate of change of CD4 cell counts on bivariable analysis [Crude β=-0.018 (-0.059, 0.023), $P<0.382$]. The expression of CYP2B6 516G>T and 983T>C genotypes were also not associated with baseline, and current CD4 cell counts achieved.

5.1 Prevalence of CYP2B6 516G>T and 983T>C single nucleotide polymorphisms

The prevalence of CYP2B6 516G>T and CYP2B6 983T>C genotypes and allele frequencies followed a similar pattern to that reported in other African populations (40). This study detected the presence of rare homozygous mutant CYP2B6 983CC genotype in two individuals (1%). Other studies that have been done in African populations have not detected this variant genotype. A study conducted by Oluka et al in a Mombasa cohort did not detect this genotype (41). A study done in Malawi by Dickison et al also did not report the expression of this variant genotype (42). The allele frequencies of the Bantu ethnic group did not conform to Hardy-Weinberg equilibrium. This might indicate genotyping errors, batch effects or population stratification.

5.2 Factors that affected baseline CD4 cell count

In this study the males had lower CD4 levels at baseline which predicted their poor CD4 response during the rest of the course of the study. This is in concurrence with studies that have been done to compare CD4 response in men and women. In a random-intercept linear mixed model conducted by Mathieu et al 2013, after adjusting for baseline CD4 count, women had on

average better CD4 response than men after two and half years on HAART (43). Women might have better CD4 response than men because they seek medical intervention earlier than men. Women are also likely to visit ART clinic regularly than men. A multivariate analysis was conducted by Ndawinz et al 2013 to study which gender was likely to start ART late (44). They showed that being a male younger than 45 years and living in a rural setting was more likely to predispose to starting ART late as compared to women of the same age group and living in the same setting (44).

There was an interesting positive association between alcohol use and baseline CD4 cell count. Study participants who regularly took alcohol had lower baseline CD4 cell counts than those who never or occasionally took alcohol. This is in harmony with recent studies which have indicated that alcohol use may enhance HIV progression. Marianna et al 2010 have shown that frequent alcohol use (two or more drinks daily) were more likely to present a CD4 cell count decline to less than 250 cells/μl independent of other patient demographic characteristics (45). Alcohol may exacerbate HIV progression by suppressing the immune system by causing defects in both cell-mediated and humoral immunity (46).

5.3 Factors that affected the rate of change of CD4 cell counts.

There seemed to be an association between CYP2B6 983TC genotype and the rate of change of CD4 cell counts [Adjusted $\beta=-0.057$ (-0.11, -0.003), $P<0.04$] on adjusting for confounding for estimated glomerular filtration rate. However this might be a spurious association and should be approached cautiously because nevirapine plasma levels were not determined to determine whether they influenced the rate of change of CD4 cell counts. Current evidence has not found a

direct association between CYP2B6 983TC genotype and CD4 cell counts, because less than 3% of nevirapine is excreted unchanged in urine while more than 80% is excreted as glucorinidated inactive metabolites in urine and 10% in feces (47).

The baseline CD4 cell counts were shown to consistently affect the rate of change of CD4 cell counts during the duration of therapy. Lower baseline CD4 cell counts did not predict better changes in the rate of change of CD4 cell counts. This was in variance with findings from a study done by Mathieu et al 2013, that reported faster increase in CD4 cell counts in patients with lower baseline CD4 cell counts (43). In this study, participants who had higher baseline CD4 cell counts at study recruitment had higher rate of increase of CD4 cell counts. This finding suggests that better CD4 response can be obtained if HIV/AIDS patients are initiated on HAART when baseline CD4 cell counts are higher.

A significant association between age and the rate of change of CD4 cell count was noted on adjustment for confounding [Adjusted β=-0.100 (-0.019, -0.001)]. As the age of the participants increased, the rate of change of CD4 cell counts declined. This was in deviance with a study conducted by Wright et al on the correlation between age and CD4 cell counts. They found out that an increase in age do not result in decreasing mean changes in CD4 cell counts for HIV patients(48). This might be due to the observation that the level of immune recovery achieved during the first years of treatment may be sustained through the rest of the years on HAART (48).

One study has shown that duration of therapy is strongly correlated with the CD4 cell response in all age groups (49). In that study younger age was associated both with higher absolute CD4 cell gain and shorter time to current response independent of baseline CD4 response. This is corroborated with a multilevel analysis study done by Montarroyos et al 2014 to determine factors related to changes in CD4 over time in patients living with HIV/AIDS (7).This might be attributed to age related changes in immune function and reduced metabolism as the patients get older.

5.4 Factors that affected current CD4 cell count

The general effect of duration of therapy on the current CD4 cell counts was positive. The participants CD4 cell counts continued to increase till a time point when further increase was impossible. When this situation occurred, the participants were deemed to have attained current CD4 cell counts. It was observed that some participants reached a plateau phase before a decline was noted while others had sharp decline and did not have a plateau phase.

Males were independently associated with lower current CD4 cell counts compared to women. Studies that have compared gender response to antiretroviral regimes have reported better response in women as compared to men (44).The reasons advanced for this observation might be poor adherence to antiretroviral regimens by men and poor ART clinic attendance. Therefore it can be assumed that males were at a greater risk of developing immunological failure compared to women.

Participants with low baseline CD4 cell counts attained lower current CD4 cell counts compared to those who had higher baseline CD4 cell counts. This was in concurrence with studies conducted by Asfaw et al 2015 that indicated a directly proportional relationship between baseline CD4 cell counts and current CD4 cell counts attained (20).This association is of clinical importance because it was a significant predictor of subsequent CD4 cell recovery. Since participants with higher baseline CD4 cell counts had better immunological response and higher current CD4 cell counts, then it can concluded that initiating HAART at higher baseline CD4 cell counts is better than at low baseline levels.

Stavudine based regimens had a negative association with the current CD4 cell counts attained [Adjusted β= 2.138 (-3.622, -0.654)]. Study participants who were on stavudine, lamivudine and nevirapine had lower current CD4 cell counts compared to those from other regimens. This observation might be attributed to the emergence of adverse drug reaction caused by stavudine. Another reason for this interaction might be due to the development of resistance to antiretroviral drugs by the study participants as a result of mutations among patients on antiretroviral therapy. Harrigan et al 2005 have documented mutations against stavudine and stavudine based regimens which rendered the patients resistant to these drugs with consequent decline in CD4 cell counts (50).

Study strength and limitations

The major strength of this study is that, blood samples used were obtained from participants who were seen at a major referral hospital having patients from diverse backgrounds and multiple ethnic groups. Secondly sample size was larger than that of a similar Kenyan study that was conducted in Mombasa.

The major limitation of this study was complex regression analysis which required higher software versions which were not easily available. Another limitation of this study was the poor representation of the Cushitic ethno-linguistic group; therefore the effect of the polymorphisms of drug metabolizing enzymes could not be adequately studied in this group.

CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS

Conclusion

There was no association between the expression of CYP2B6 516G>T and 983T>C genotypes and CD4 cell counts. Nevirapine plasma concentrations need to be determined to infer any association between these genotypes and CD4 cell counts.

Recommendations

It is recommended that nevirapine plasma levels be taken into account when determining whether CYP2B6 516G>T and 983T>C single nucleotide polymorphisms influence CD4 cell counts in patients on nevirapine based antiretroviral regimens. It is also recommended that robust statistical tools be employed when doing regression analysis for this kind of study so eliminate making spurious results.

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APPENDICES

APPENDIX A: KNH/UoN ETHICAL APPROVAL –THESIS RENEWAL LETTER

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Ref: KNH-ERC/ R/48

29th March, 2015

Dr. James Angima Angima
School of Pharmacy
MSC Molecular Pharmacology
University of Nairobi

Dear Dr. James

Re: Approval of annual study renewal –Evaluation of the Effects of CYP2B6 polymorphisms on CD4 levels in HIV/AIDS patients on nevirapine based regimes at Kenyatta National Hospital (P10/01/2014)

Your communication of 17th March, 2015 refers.

This is to acknowledge receipt of the study progress report and hereby grant you annual extension of approval for ethical research protocol P10/01/2014.

The study renewal dates are 29th March, 2015 to 28th March, 2016.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH/UoN ERC before implementation.
c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH/UoN- ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH/UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH/UoN-Ethics & Research Committee for each batch of shipment.

g) Submission of an *executive summary* report within 90 days upon completion of the study
This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

Protect to discover

For more details consult the KNH/UoN -ERC website **www.uonbi.ac.ke/activities/KNHUoN**

Kindly forward the informed consent documents for endorsement with updated stamp.

Yours sincerely

PROF. M.L. CHINDIA
SECRETARY, KNH/UON-ERC

c.c. The Principal, College of Health Sciences, UoN
The Deputy Director CS, KNH
The Principal, College of Health Sciences, UoN

APPENDIX B: KNH/UoN ETHICAL APPROVAL - THESIS MODIFICATION LETTER

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Ref: KNH-ERC/ MOD/145

16th April, 2015

Dr. James Angima Angima
U52/69554/2013
School of Pharmacy
University of Nairobi

Dear Dr James

Re: Approval of modification: Evaluation of CYP2B6 Polymorphisms on CD4 Levels in HIV/AIDS Patients on Nevirapine Based Regimens at Kenyatta National Hospital (P10/01/2014)

Refer to your communication of 27th March, 2015.

The KNH/UoN-ERC has reviewed and approved modification of the following:

1. Genotype and estimate of the levels of nevirapine in the blood samples collected.
2. A change of the Investigator from Dr. Makori to Dr. James Angima
3. Change of title "Evaluation of the effects of CYP2B6*6 polymorphisms on plasma levels of nevirapine and the CD4 response in HIV/AIDS patients at Kenyatta National Hospital"

Yours sincerely,

PROF. M. L. CHINDIA
SECRETARY, KNH/UON-ERC

c.c. The Principal, College of Health Sciences, UoN
The Deputy Director CS, KNH
The Chair, KNH/UoN-ERC

KENYATTA NATIONAL HOSPITAL
APPROVED
16 APR 2015
KNH/UoN-ERC
P.O. Box 20723-00202 NRB.

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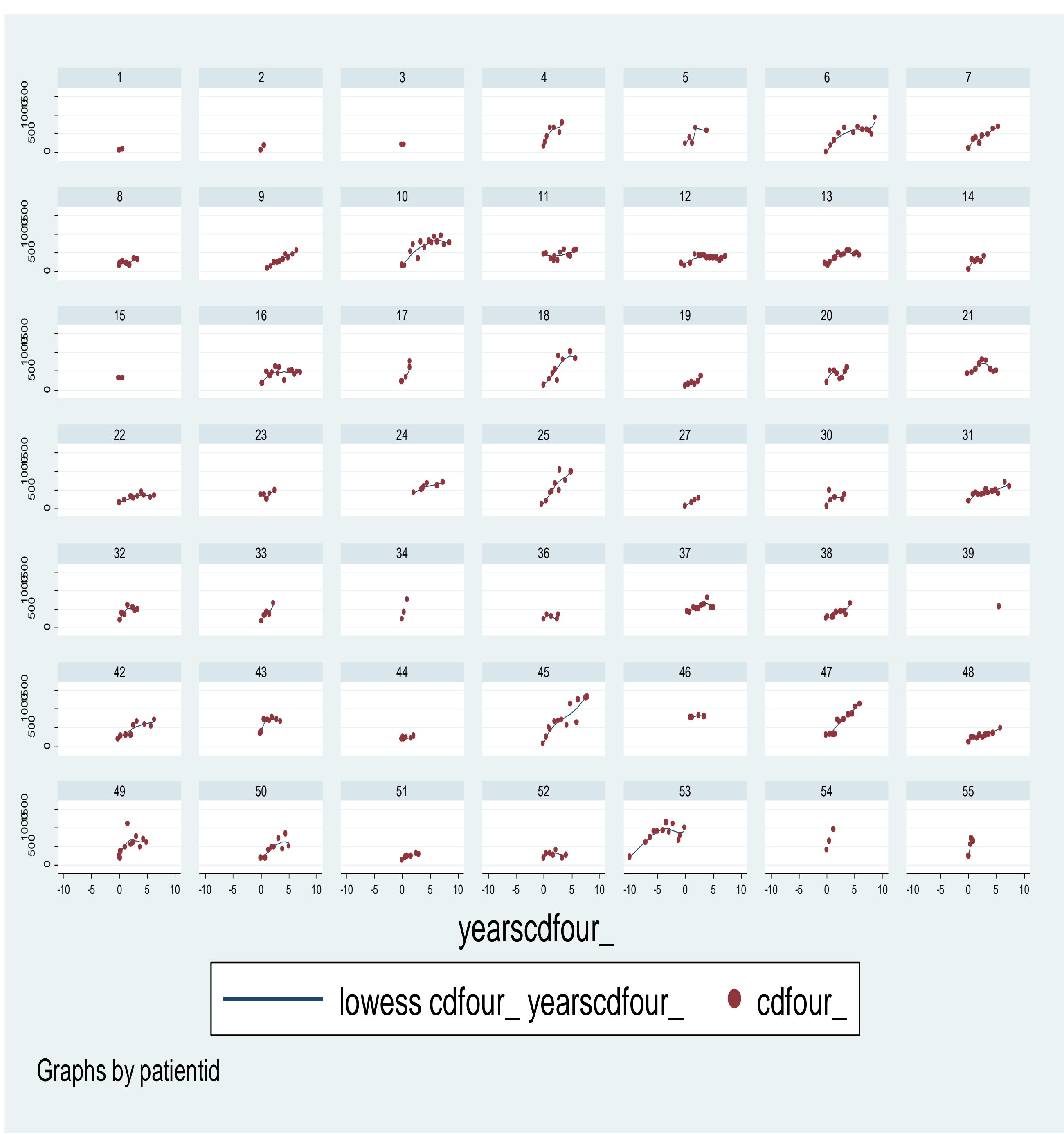
APPENDIX C: SAMPLING FORM

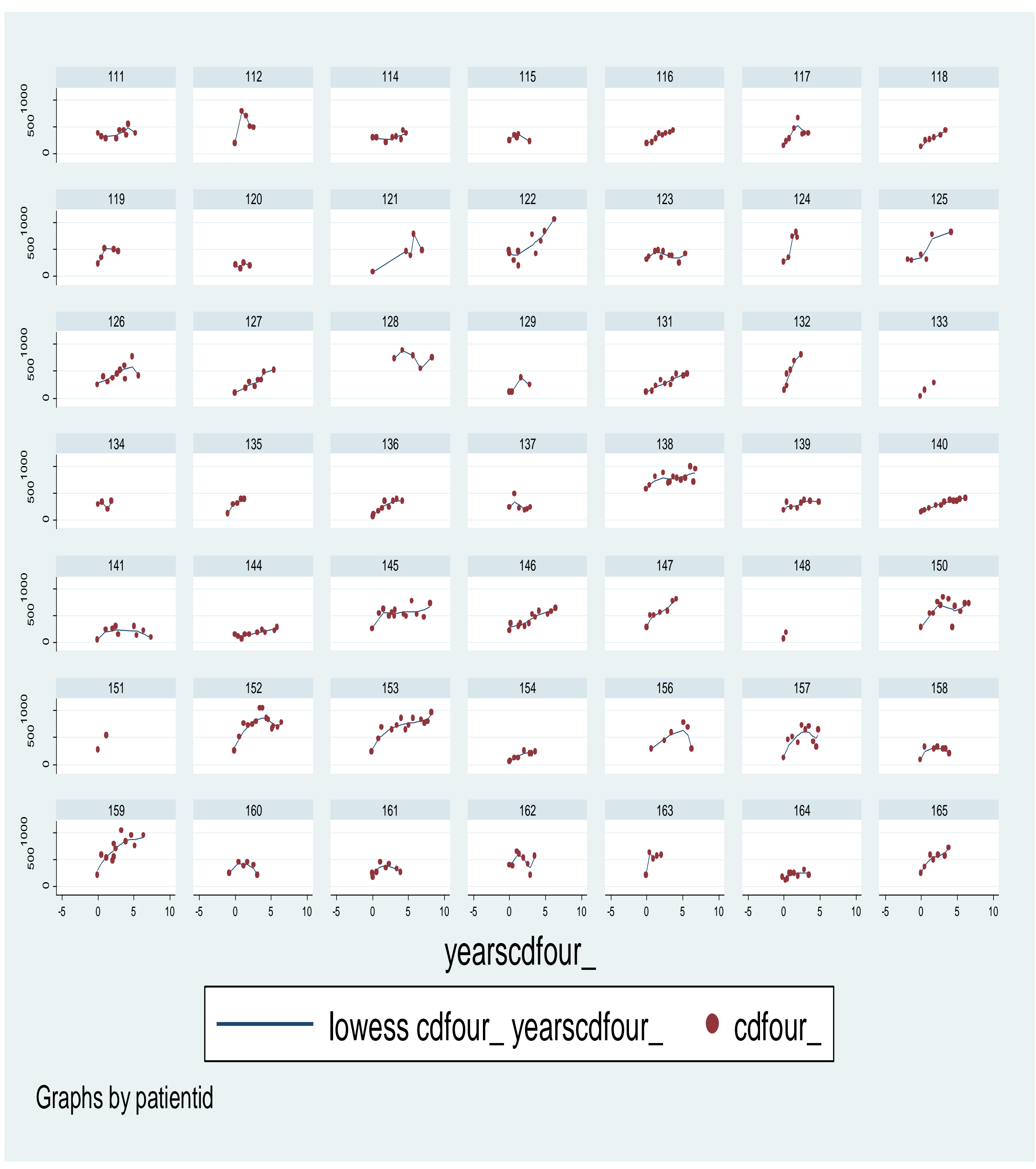
Study number	CCC Clinic Number	KNH File Number

APPENDIX D: EMPIRICAL PLOTS FOR EACH INDIVIDUAL



The empirical growth plots of the entire cohort

the empirical growth plots continued......



the empirical growth plots continued......