PHARMACOGENETICS OF DRUG METABOLIZING ENZYMES AND CLINICAL IMPLICATIONS IN SELECTED KENYAN POPULATIONS

BY

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

To the memories of my dearly beloved late husband Dr. Justin Erimu Oluka, daughter Olivia Flora Among' and granddaughter Alexandra Ajowi who are so loud in their silence, ever so present in their absence.

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LIST OF ABBREVIATIONS AND ACRONYMS

ADR	Adverse drug reaction
AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
ARV	Anti-retro viral
AUC	Area under the curve
bp	Base pair
C	Cytosine
CYP	Cytochrome P450
Cl	Clearance
CCC	Comprehensive Care Centre
CD4	Subgroup of T lymphocytes carrying CD4 antigens
CL	Clearance
C_{max}	Maximum Plasma Concentration
CI	Confidence Interval
CV	Coefficient of variation
CYP	Cytochrome P_{450}
DNA	Deoxy-ribonucleic acid
DME	Drug metabolizing enzyme
EFZ	Efavirenz
EM	Extensive metabolizer
ERC	Ethics review committee
FBC	Full blood count
FGA	First generation antipyschotics
G	Guanine
HAART	Highly active antiretroviral therapy
HCC	Hepatocellular carcinorma
HIV	Human immune deficiency virus
HPLC	High performance liquid chromatography

IM	Intermediate Metabolizer
LLOQ	Lower limit of quantitation
Kb	Kilobase pair
KNH	Kenyatta National Hospital
LFTs	Liver function tests
NTC	Non template control
NRTIs	Nucleoside/Nucleotide reverse transcriptase inhibitors
NNRTIs	Non- Nucleoside/Nucleotide reverse transcriptase inhibitors
NVP	Nevirapine
PCR	Polymerase chain reaction
PD	Pharmacodynamics
PK	Pharmacokinetics
PI	Protease inhibitor
OR	Odds ratio
PM	Poor metabolizer
QC	Quality control
RD	Relative deviation
RNA	Ribonucleic acid
SD	Standard deviation
SDS	Sequence detection system
SNP	Single Nucleotide Polymorphism
RFLP	Restriction fragment length polymorphism
RT-PCR	Real time PCR
T	Thymidine
TB	Tuberculosis
ULOQ	Upper limit of quantitation
UV	Ultra violet
UM	Ultra-rapid metabolizer
WHO	World Health Organization

GLOSSARY

Active metabolite: A metabolite of a drug that produces a biological effect.

ADME: A common acronym used to describe the manner in which an agent is processed within an organism – *a*bsorption, *d*istribution, *m*etabolism and *e*xcretion.

Allele: In humans an allele is a member of a pair of different forms of a gene that occupies the same position (locus) on a chromosome.

Association: A statistical finding that the frequency of one or more genetic variants is significantly different in subjects with a phenotype than in those without the phenotype

Base pair: Two nitrogenous bases (adenine and thymidine or guanine and cytosine) held together by hydrogen bonds

Chromosome: The self-replicating genetic structure of cells containing the cellular DNA that bears in nucleotide sequence the linear array of genes

DNA sequence: A DNA sequence consists of a double strand of DNA molecules, which are made up of even smaller molecules known as nucleotides.

Enzyme: A protein molecule produced by living organisms that catalyses the chemical reaction of substances (including drugs).

Gene: The basic biological unit of heredity, determined by a specific sequence of purine and pyrimidine bases in DNA, located at a definite position (locus) on a particular chromosome and contributes to phenotype/function.

Genome: Sum total of the genetic material included in every cell of the human body, apart from the red blood cells.

Genotype: The genetic constitution of an individual, that is, the specific allelic makeup of an individual.

Haplotype: A set of genetic variants that are inherited together.

Heterozygote: A person who has two copies of an allele that are different.

Homozygote: A person who has two copies of an allele that are the same.

Locus: A specific position on the genome, for example, where a particular nucleotide is located.

Metabolism: Conversion of a compound *in vivo* to another chemical species

Mutation: A permanent transmissible change in the genetic material

Nucleotide: Small molecules that are the basic constituents of DNA.

Penetrance: The proportion of individuals carrying a particular genotype who also express a particular phenotype.

Polymorphism: The occurrence in the same population of multiple discrete allelic states of which at least one must have a frequency of not less than 1%

Pharmacogenetics: A term used to define inherited variability in response to drug treatment.

Pharmacogenomics: The study of the relationship between variants in a large collection of genes, up to the whole genome, and variable drug effects.

Pharmacodynamics: The study of the relationship between drug concentrations and drug effects.

Pharmacokinetics: The study of the relationship between drug dose and drug concentrations (often as a function of time) in plasma or tissue.

Phenotype: The observable physical or behavioral traits of an organism largely determined by the organism's genotype but also influenced by environmental factors.

Predictive value: Ratio of true-positive cases to combined true- and false-positive cases.

Prodrug: An agent that is administered in a significantly less active form, which, once administered, is metabolized in vivo into the active compound (active metabolite).

Pseudogene: Non-functional DNA sequences that is very similar to the sequences of known functional genes

Oxidative stress: Oxygen or free radical mediated damage in living organisms

Receptor: A protein molecule embedded in a membrane to which a signal molecule (ligand) such as a pharmaceutical drug may attach itself to and which usually initiates a cellular response (although some ligands merely block receptors without inducing any response).

Sensitivity: The proportion of true-positive cases that are correctly identified by a test.

Single-nucleotide polymorphism (SNP): The most common type of genetic variation in humans, which occurs when a single nucleotide [adenosine (A), guanine (G), cytosine (C) or thymidine (T)] in the genome sequence is changed.

Specificity: The proportion of true-negative cases that are correctly identified by a test.

Xenobiotics: Chemical substances that are foreign to the biological system such as drugs.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABSTRACT

Much of the inter-individual and interethnic variability in drug response is now attributable to genetic differences in drug metabolism. Drug metabolizing enzymes exhibit genetic polymorphism with certain allelic variants displaying striking variable ethnic distribution. The pharmacogenetics of drug metabolism has been extensively studied in Caucasian and Asian populations, yet much remains to be done in African populations. The main objective of this thesis was to investigate the genetic polymorphisms and clinical implications of genes encoding drug metabolizing enzymes namely, *CYP2B6, CYP2C19, CYP2D6,* N-acetyl transferase *(NAT2)* and Glutathione- S- transferases (*GSTs)* in selected Kenyan populations.

The first specific objective was to determine the distribution of pharmacogenetically relevant single nucleotide polymorphisms (SNPs) of *CYP2B6, CYP2C19, CYP2D6, NAT2, GSTM1* and *GSTT1* in selected Kenyan populations. A population of 350 Kenyans belonging to three ethno-linguistically distinct populations was recruited. The populations studied were the Western Nilotes (Luo) (100), the Eastern Nilotes (Maasai) (152) and the Bantu (Kikuyu) (102). Genotyping of allelic variants *CYP2B6**6(*516G>T); *CYP2C19**2(681G>A); *CYP2C19**3(638G>A); *CYP2D6**4(1846G>A); *CYP2D6**5(deletion); *CYP2D6**17(1023C>T); CYP*2D6**29(3183G>A); *NAT2**5(341T>C); N*AT2**6(590(G>A); *NAT2**7(857G>A); *NAT2**14 (191G>A); *GSTM1* and *GSTT1* was undertaken by polymerase chain reaction coupled with restriction fragment length polymorphism (PCR-RFLP).

Results showed that the distribution of *CYP2D6*, *GSTM1* and *GSTT1* allelic variants was highly variable between the three Kenyan ethnic populations. *CYP 2D6**4 was surprisingly higher in the Eastern Nilotes (9%) than in the Western Nilotes (3%) and the Bantu (2%) (P=0.002). *CYP2D6**17 was more prevalent in the Bantu (34%) than in the two Nilotic populations (18-23%) (P= 0.003). The frequency of *GSTM1**0/*0 (deletion) in the Eastern Nilotes (16%) was nearly half

that in the Western Nilotes and the Bantu (30%) (P=0.009) whereas that of *GSTT1*0/*0* (deletion) in the Eastern Nilotes (41%) was nearly double that found in the Bantu and Western Nilotes (22-26%) (P=0.005). The null allele CYP2C19*3 was undetected in the Bantu but was found at <1% in the Nilotes. There was homogeneity in the distribution of *CYP2C19**2 (10-18%), *CYP2B6**6 (34-37%) and the *NAT2* slow acetylator alleles (NAT2*5 (33-38%), NAT*6 (23-27%), NAT2*7(3-6%), NAT2*14 (9-14%) between the three Kenyan ethnic populations. The intermediate metabolizer (IM) was: for CYP2D6 (20-27%), CYP2C19 (20-27%), CYP2B6 516TT (12-16%) whereas NAT2 slow acetylation was 54-64%.

The second specific objective was to analyze clinically relevant genetic variants of *CYP2D6* and *CYP2C19* and evaluate their impact on psychotropic medication patterns in Kenyan psychiatric in-patients. A total of 193 psychiatric in-patients at Mathari Hospital were recruited and genotyped for *CYP2C19**2 and *CYP2C19**3; *CYP2D6*2*, *CYP2D6**4, *CYP2D6**5, *CYP2D6**17 and *CYP2D6**29 using PCR-RFLP methods. CYP2D6 and CYP2C19 metabolic phenotypes were predicted from observed genotypes according to published methods and related to the pattern and CYP metabolic profiles of psychotropic medications in the patient population. The distribution of SNPs of CYP2C19 and CYP2D6 in psychiatric in-patients was similar to that in the general population except for CYP2C19*3 which was higher in patients (8%) than in the general population (<1%). Predicted intermediate metabolizer (IM) of CYP2D6 and CYP2C19 was 24% and 26% respectively.

Predicted CYP2D6 IM phenotype was strongly associated with the use of high doses (15-20mg) of anticholinergic medication (68%) (P<0.001) indicative of serious extrapyramidal side effects; frequent hospital admission of 5-6 times (67%) per year (P<0.001) and exposure to antipsychotic polypharmacy of >5 drugs (P = 0.002). Psychotropic dosage regimens in the in-patients were higher

than the defined daily doses (DDD) for chlorpromazine (CPZ) (58%), haloperidol (94%) and amitriptyline (50%). Female gender (P=0.006) and non-exposure to substance abuse (P=0.031) were associated with lower doses of CPZ. There was a significant potential for drug-drug interactions with 96% of the prescriptions containing concurrent CYP2D6 substrates and inhibitors whereas 9% had both CYP2C19 substrates and an inducer. The main enzyme inducers were carbamazepine and phenobarbitone.

The third specific objective was to determine the prevalence of *CYP2B6* 516 G>T genotypes and assess their impact on nevirapine plasma levels and therapeutic outcomes in Kenyan HIV patients. A sample of 110 adult HIV positive patients on nevirapine based HAART at 6 months was recruited. They were genotyped for the *CYP2B6* 516G>T variant by real time polymerase chain reaction (RT-PCR). Nevirapine trough plasma levels were determined by a validated HPLC method. The frequency of *CYP2B6**6 (516T) variant allele was 44% which was higher than the 32% seen in Kenyan general populations. CYP2B6 516 GT was 50% whereas CYP2B6 516 TT was 19%.

Nevirapine plasma levels displayed great inter-individual variability ranging from 640 – 11800 ng/mL and was strongly correlated to *CYP2B6* genotypes with a significant gene dose effect. Patients bearing the *CYP2B6* 516 TT had 55% higher plasma levels than *CYP2B6* 516 GG (P< 0.001). Sixty six percent of HIV patients attained nevirapine levels above 4300 ng/mL which reportedly offers durable viral suppression. However 17% had levels of 3100– 4300 ng/mL defined as the mutant selection window whereas another 17% had levels below 3100 ng/mL associated with viral failure. Nevirapine plasma levels were not associated with toxicity and were not influenced by demographic characteristics except by the use of social drugs (16%) that resulted in 68% lower plasma levels (P = 0.006). Change in CD4 cell counts at 6 months of HAART was 170%, but was not influenced by nevirapine plasma levels or CYP2B6 genotypes.

This thesis research has provided novel genetic data on clinically important genes affecting drug therapy in Kenyan populations not studied before. Intra-ethnic, inter-ethnic and inter-population genetic variability was demonstrated by the variable didtribution of CYP2D6*4, CYP2D6*17, GSTM1*0 and GSTT1*0. These observations underscore the risk of generalizing population genetic data even within a country and the need for further studies covering other Kenyan ethnic populations. The clinical implications of CYP2D6*17(34%) is of concern since CYP2D6 metabolizes 25% of all drugs in clinical practice, notably for cancer, cardiovascular and central nervous system. The NAT2 slow acetylation (66%) could increase toxicity to isoniazid and co-trimoxazole, first-line agents for tuberculosis and pneumocystis pneumonia prophylaxis respectively. The deletion of GSTM1 (30%) and GSTT1 (40%) has been associated with increased incidence of hepatocellular carcinoma in the presence of hepatitis and aflatoxicosis, conditions that are common in Kenyan populations.

The clinical implications of pharmacogenetics have been demonstrated in this thesisis in psychiatric and HIV patients. These diseases afflict many Kenyans and are subject to inadequate treatment outcomes with attendant financial implications. Nevirapine pharmacokinetic studies incorporating CYP2B6 516 T (44%) could define plasma levels for optimal viral suppression in Kenyan HIV patients and stem antiretroviral resistance. Psychotropic dosage titrations incorporating CYP2D6 IM phenotype could provide a better algorithm for dosage optimization, improved therapy outcomes and reduced incidence of extrapyramidal effects instead of the observed rampant use of anti-cholinergic prophylaxis in psychiatric in-patients. Considerable genetic variability between Kenyan and Caucasian populations was evident, yet most drugs undergo clinical evaluation in largely Caucasian cohorts. There is need for local clinical studies incorporating the pharmacogenetics of CYP2D6 IM, *CYP2D6**17, *CYP2B6**6, *CYP219**2, *GSTM1**0, GSTT1*0 and *NAT2* slow acetylation to optimize dosage regimens for Kenyan populations.

PREFACE

Chapter one

An introductory chapter that has given an overview of genetic variability. It has explained some of the sources of genetic variability such as single nucleotide polymorphism (SNPs) and possible clinical consequences. It has also given the study justification and objectives.

Chapter two

This is the literature review that has given an exposition of drug metabolism and the genetic polymorphism of drug metabolizing enzymes. It has provided an overview of the cytochrome P450 enzyme system, NAT2 and GSTs and the impact of pharmacogenetics and pharmocgenomics on the therapy of common disease conditions such as HIV and mental disorders.

Chapter three

This is the experimental chapter that has described all the materials, methods and procedures used in generating genotypic data as well as the determination of nevirapine plasma levels.

Chapter four

This chapter has presented the population pharmacogenetic study. This is the study that described the distribution of pharmacogenetically relevant SNPs of genes such as *CYP2B6, CYP2C19, CYP2D6, NAT2, GSTM1* and *GSTT1* in three ethno-linguistically distinct populations of Kenya. The three populations studied were the Eastern Nilotes (Maasai), the Western Nilotes (Luo) and the Bantu (Kikuyu).

Chapter five

This chapter has described the analysis of the genetic variants of CYP2D6 and CYP2C19 in psychiatric in-patients and the evaluation of the impact of pharmacogenetics on the pattern of psychotropic medications in the patient population.

Chapter six

The chapter has described the frequency of CYP2B6 516 G>T genotypes in Kenyan HIV patients and how they impact on nevirapine plasma levels and therapeutic outcomes.

Chapter seven

This chapter has presented the general discussion of the major findings of this thesis work. The conclusions, recommendations and suggestions for further research are also included

PUBLICATIONS

The following papers and conference abstracts/ presentations arose from this thesis work:

Matimba A., **Oluka M.N.**, Ebeshi B.E., Sayi J. Bulaji O.O., Guantai A.N, and Masimirembwa C.M. Establishment of a Biobank and Pharmacogenetics Database of African Populations. *European Journal of Human Genetics, 2008, 2: April 1–4*

Oluka M.N., Matimba A. Guantai A N., Masimirembwa C.O., Pharmacogenetics of drug Metabolizing Enzymes in Kenya populations: Implications for Toxicity of Drugs and Environmental Toxicants – *Abstract in Drug Metabolism Reviews, 2007.*

Chapter One

INTRODUCTION

1.1 Genetic variability

The complete sequencing of the human genome has led to increased effort towards the identification of sequence variants throughout the genome that define inter-individual variation. This discipline focuses on the 0.1% of our genome that makes each individual genetically unique (Kruglyak and Nickerson 2001; Guttmacher *et al.*, 2010). Any two copies of the human genome are 99.9% identical, with variation being detected in approximately 0.1% of nucleotide sites, that is, one variant per 1,000 bases (Rozen *et al.*, 2009). The vast majority of these differences are single base pair changes in the DNA sequence such as deletions, insertions, or substitutions of individual bases. These polymorphisms are collectively referred to as single nucleotide polymorphisms or SNPs (Wolf *et al.*, 2000). It has been estimated that, in the human population, about 10 million sites vary, such that both alleles occur at a frequency of at least 1%. (Roses, 2000).

SNPs have long been recognized as ideal polymorphic markers for disease association studies and for fine-mapping efforts to identify disease-causing genes and their mutations (Collins *et al.*, 1997). Presently, this knowledge has greatly accelerated as a result of a technological revolution. This has allowed genome-wide association studies (GWASs) and gene expression studies in thousands of patients. These advances have greatly enhanced the understanding of how interactions between the entire genome and nongenomic factors influence health and disease as well as therapeutic response. The use of this knowledge offers great opportunities for the achievement of personalized medicine despite the many challenges.

Reports already show that the use of genomic information has graduated from SNPs to haplotypes and to the ultimate incorporation of a personal genomic

sequence into clinical patient assessment (Ashley *et al.*, 2010). A principal contributor to personalized medicine has been the use of pharmacogenomic biomarkers in tailoring drug treatment to individual patient characteristics (Meyer, 2004). This is highly dependent on the ability to classify patients into subgroups with predictable response to a specific treatment. Pharmcogenomic initiatives such as the international HapMap project (www.hapmap.org). The Pharmacogenomics Knowledge Base (www.pharmgkb.org) and the Pharmacogenomics Research Network (www.pgrn.org) are now available and provide rapid access to peer-reviewed, evidence-based recommendations for specific drug-gene pairs.

The international HapMap project was mandated to characterize haplotypes, specific single nucleotide polymorphisms (SNPs) and allele frequencies in various ethnic groups. The project is particularly useful for genetic variants that are relatively common in populations, such as those of drug metabolizing enzymes that contribute to considerable interindividual variability in drug response (Stojiljkovic *et al.*, 2011). This is particularly true for the SNPs that have been identified in genes encoding for the cytochrome P450 (CYP) enzyme system, which metabolizes the majority of drugs in clinical practice (Laing *et al.*, 2011). Some SNPs decrease the activity of these enzymes and lead to increased drug levels and the risk of toxicity, whereas others increase their activity resulting in sub-therapeutic drug concentrations. Tailoring drug dosage to unique SNP profiles may therefore represent a more rational approach to drug therapy.

1.2 Study Justification

The pharmacogenetics of drug metabolizing enzymes and the clinical consequences of their defective variant alleles have been better characterized in Caucasian and Asian populations, yet a wide gap in this knowledge still exists in African populations. A number of drugs that form first line chemotherapy for infectious diseases such as malaria, HIV and tuberculosis such as artemisisnin,

efavirenz, nevirapine, and ionized (WHO, 2012)are subject to polymorphic metabolism. Some of the key enzymes involved include CYP2B6, CYP2C19, N-acetylytransferase2 (NAT2) and Glutathione - S - transferases (GSTs). Furthermore, psychotropic drugs for mental disorders are mainly metabolized by CYP2D6 and CYP2C19 which are highly polymorphic. The extent of such genetic polymorphisms and possible impact on treatment outcomes in Kenyan populations is not known.

Resent advances in clinical pharmacogenetics have resulted in the inclusion of Pharmacogenomic biomarkers in drug product labeling requirements for some drugs (www.fda.gov/drug). For example, *CYP2D6* is listed as a biomarker with regard to the safety of commonly used drugs such as fluoxetine, terbinafine and tramodol. For the *CYP2C19*, examples include diazepam, nelfinavir and voriconazole whereas for *NAT1/NAT2*, labeling concerns have been raised for rifampicin, isoniazid and pyrazinamide. Presently, there is paucity of data on the pharmacogenetic variability of these biomarkers in Kenyan ethnic populations. This thesis work was therefore set up to illuminate Kenyan populations with regard to the distribution and clinical impact of pharmacogenetically relevant SNPs of genes responsible for the metabolism of drugs commonly used in Kenyan populations.

1.3 Objectives

1.3.1 Main Objective

The main objective of this thesis was to investigate the pharmacogenetics of drug metabolizing enzymes namely, CYP2D6, CYP2C19, CYP2B6, NAT2 and GSTs and to evaluate the therapeutic implications of their defective allelic variants on the therapy and treatment outcomes of common disease conditions in Kenyan populations

1.3.2 Specific objectives

1. To determine the genetic polymorphisms of *CYP2B6, CYP2C19, CYP2D6, NAT2,* GSTM1 and *GSTT1* in three ethno-linguistically distinct populations of Kenya.

2. To characterize clinically relevant genetic variants of *CYP2D6* and *CYP2C19* and to evaluate their relationship to the prescribing pattern of psychotropic medications and patient social habits in Kenyan psychiatric in-patients at Mathari Hospital

3. To determine the influence of *CYP2B6* genetic variants on nevirapine plasma levels and to assess effects on treatment outcomes in Kenyan HIV patients.

Chapter Two

LITERATURE REVIEW

2.1 Drug metabolism

Foreign chemicals found in the environment are continually absorbed by the human body through various routes of exposure. Such chemicals, which are collectively referred to as xenobiotics, include therapeutic drugs, industrial and agrochemical chemicals, dietary products and toxins from plants and animals. Unfortunately, the physical property that enables many xenobiotics to be absorbed through the skin and the gastrointestinal tract, namely their lipophilicity, is an impediment to their elimination from the body. Hence, the elimination of xenobiotics from the body requires conversion to water soluble derivatives. This process which is known as biotransformation is catalyzed by drug metabolizing enzymes. In the absence of biotransformation, lipophilic xenobiotics would be excreted from the body so slowly that they would eventually overwhelm and kill the organism (Parkinson, 1996). In fact, the process of biotransformation is the principal mechanism for the maintenance of homeostasis during exposure of an organism to small foreign molecules such as drugs or environmental pollutants. This is analogous to the immune system which comes into play during invasion by large foreign molecules including viruses and bacteria.

Biotransformation reactions occur mainly in the liver in two steps (Nebert *et al.*, 1999) and are generally classified as either phase I functionalization or phase II biosynthetic reactions. Phase I reactions involve the exposure or introduction of functional groups such as –OH, -NH2, -SH, -COOH on the compound by metabolic processes such as oxidation, reduction, and hydrolysis and confer a modest increase in hydrophilicity. Phase II reactions include glucuronidation, sulfation, acetylation, methylation or conjugation with glutathione or amino acids. The co-factors for these processes react with functional groups that are either

present on the compound or that had been introduced or exposed during phase I reactions. Hence phase II biotransformation may or may not be preceded by phase I reactions. Most phase II reactions result in a large increase in hydrophilicity and thereby greatly promote the excretion of xenobiotics.

Biotransformation may not only change the pharmacokinetic behavior of the xenobiotic but can also alter the biological effects. This is an important concept in pharmacology because some drugs require biotransformation to be converted to the active forms. On the other hand, biotransformation may convert xenobiotics to reactive electrophillic metabolites capable of forming protein and DNA adducts thus exerting toxic or tumourigenic effects (Nebert, 1997). In most instances however, biotransformation results in termination of biological activity and reduced toxicity. The process of biotransformation is catalyzed by enzymes referred to as drug metabolizing enzymes that are primarily located in the liver but are also present in the lungs, the enterocytes in the lining of the gastrointestinal tract and other organs (Kolars *et al.*, 1994). The main enzymes involved in phase I reactions are the cytochrome P450 monooxygenase system which are localized in the endoplasmic reticulum of hepatocytes whereas the phase II enzymes are mainly cytosolic. The primary biotransformation reactions and some of the enzymes responsible are shown in Table 2.1

Table 2.1: Metabolic reactions and enzymes involved in drug metabolism

PHASE I		Phase II	
Reactions	**Enzyme Family**	**Reactions**	**Enzyme Family**
Oxidation	Cytochrome P450	Glucuronide conjugation	UDP-glucuronosyltransferase
	Alcohol dehyrogenase	Glutathione conjugation	Glutathione - S- transferase
	Aldehyde dehyrogenase	Sulfate conjugation	Sulfotransferase
	Xanthine Oxidase	Acetylation	N-acetyltransferase
	Monoamine oxidase	Methylation	Methyltransferase
	Flavin - monooxygenase		
Reduction	DT-diaphorase		
	Cytochrome P450		
	Carbonyl reductases		
Hyrdrolysis	Carboxylestarases		
	Epoxide hydrolase		

Adapted from Parkinson, (1996)

2.1.1 The cytochrome P450 system

The cytochrome P450 (CYP) enzyme system constitutes a large superfamily of heme - thiolate proteins(NC-IUB, 1991). The signature name cytochrome P450 originates from a unique absorption spectrum at a wavelength of 450 nm. The spectrum is produced when carbon monoxide gets bound to the reduced heme as was first recognized by Martin Klingenberg (Klingenberg, 1958). The nature of the pigment as a hemeprotein was later characterized from rat liver (Omura and Sato, 1962, 1964). Subsequently, (Cooper *et al.*, 1965)provided the evidence that the CYP present in liver microsomes plays a central role in the metabolism of drugs and other xenobiotics. It is now well recognized that members of this ubiquitous enzyme superfamily play an important role in the metabolism and biosynthesis of a wide variety of both exogenous and endogenous compounds (Nebert and Russell, 2002).Mammalian CYP are found in all cell types except the

mature red blood cell and skeletal muscle cell. They are membrane bound and are mainly present in the endoplasmic reticulum with lesser amounts in the mitochondria, the nuclei and lysosomes.

The human genome contains 115 CYP genes, of which 57 are functional and the remaining are pseudogenes. These CYPs are grouped according to their sequence similarity into 18 families and 44 subfamilies (Nelson, 2009). Isoforms of families CYP1, CYP2 and CYP3 are collectively responsible for about 90% of most phase I biotransformation of drugs and other xenobiotics in human liver (Lewis, 2004; Ingelman-Sundberg *et al.*, 2007). In contrast, CYPs of families CYP4 to CYP51 are involved in endogenous metabolic pathways of steroids, fatty acids, prostaglandins and fat soluble vitamins (Guengerich *et al.*, 2005), (Table 2.2).

Table 2.2: Classification of Human CYP based on major substrate classes

	Substrates					
	Sterols	**Xenobiotics**	**Fatty Acids**	**Eicosanoids**	**Vitamins**	**Unknown**
CYP enzymes	1B1	1A1	2J2	4F2	2R1	2A7
	7A1	1A2	4A11	4F3	24A1	2S1
	7B1	2A6	4B1	4F8	26A1	2U1
	8B1	2A13	4F12	5A1	26B1	2W1
	11A1	2B6		8A1	26C1	3A43
	11B1	2C8			27B1	4A22
	11B2	2C8				4F11
	17A1	2C18				4F22
	19A1	2C19				4V2
	21A2	2D6				4X1
	27A1	2E1				20A1
	39A1	2F1				27C1
	46A1	3A4				
	51A1	3A5				
		3A7				

Adapted from Guengrich et al, (2005)

2.1.1.1 CYP evolution

The CYP represents an ancient superfamily of enzymes that seems to have evolved from a common ancestral gene which existed more than 3.5 billion years ago (Lewis *et al.*, 1989). Their origin lies in prokaryotes before the divergence of animals and plants and accumulation of molecular oxygen in the atmosphere. This has led to the suggestion that the CYP have played an important role in evolution (Nelson *et al.*, 1993). The diversification seemed to have arisen through extensive processes of gene duplications and possibly gene amplifications, conversions, genome duplications, gene loss and lateral transfers (Gonzalez and Nebert, 1990). A major gene duplication seemed to have occurred around 900 million years ago resulting in a split with one lineage of CYP enzymes continuing with endogenous activity whereas the other begun a new function as xenobiotic metabolizing enzymes.

The evolution of xenobiotic metabolizing enzymes has been attributed to the interaction between plants and animals, the so called animal-plant 'warfare' (Gonzalez and Nebert, 1990).As animals began consuming plants, plants responded by evolving newer genes to produce toxic metabolites for defense against animal and insect predators (Schuler, 1996). Consequently, to protect themselves from such new toxins, animals continuously adapted by evolving new xenobiotic metabolizing enzymes (Gonzalez and Nebert, 1990). Hence, the CYP have played a central role in the co-evolution of plants and animals and have largely determined the scope of compounds synthesized by plants and the type of toxins metabolized by animals.

2.1.1.2 CYP nomenclature

An update of the CYP sequences characterized in all organisms and the 'P450 nomenclature committee' is available from the David Nelson homepage (Nelson, 2009). A method of CYP classification has been established based on amino acid

sequence homology, phylogenetic criteria and gene organization (Nelson *et al.*, 1996). Hence, in the nomenclature, the root symbol CYP which denotes cytochrome P450 is followed by an Arabic numerical that designates the family, a capital letter that indicates the subfamily and an Arabic numerical designating the individual isoform. The CYP enzymes are thus classified by family, subfamily and isoform. Different families have less than 40% amino acid sequence similarity whereas each subfamily displays between 40 and 55% amino acid sequence similarity. Recommendations for the nomenclature of new human CYP alleles and haplotypes as well a listing of the different CYP alleles involved in xenobiotic metabolism have been established (Ingelman-Sundberg *et al.*, 2000; Sim and Ingelman-Sundberg, 2010)

2.1.2 CYP and drug metabolism

In humans, over90% of drug metabolism can be attributed to the activity of CYPs 1A2 (4%), 2A6 (2%), 2C9 (10%), 2C19 (2%),2E1 (2%), 2D6 (30%), and 3A4 (50%) (Rendic and Di Carlo, 1997).The relative abundance of the hepatic CYPs has been determined with CYP3A4 accounting for over 30% of CYP liver content relative to others such as CYP1A2 (13%), 2A6 (4%),2C (20%), 2D6 (2%), 2E1 (7%) (Shimada *et al.*, 1994). Variability in CYP content and activity can have profound influence on the *in vivo* response of humans to drugs (Nebert and Russell, 2002). Figure 2.1 depicts the relative contribution of phase I and phase II as well as that of individual CYPs to the metabolism of medicines in clinical use (Williams et al, 2004; Wienkers and Heath, 2005).

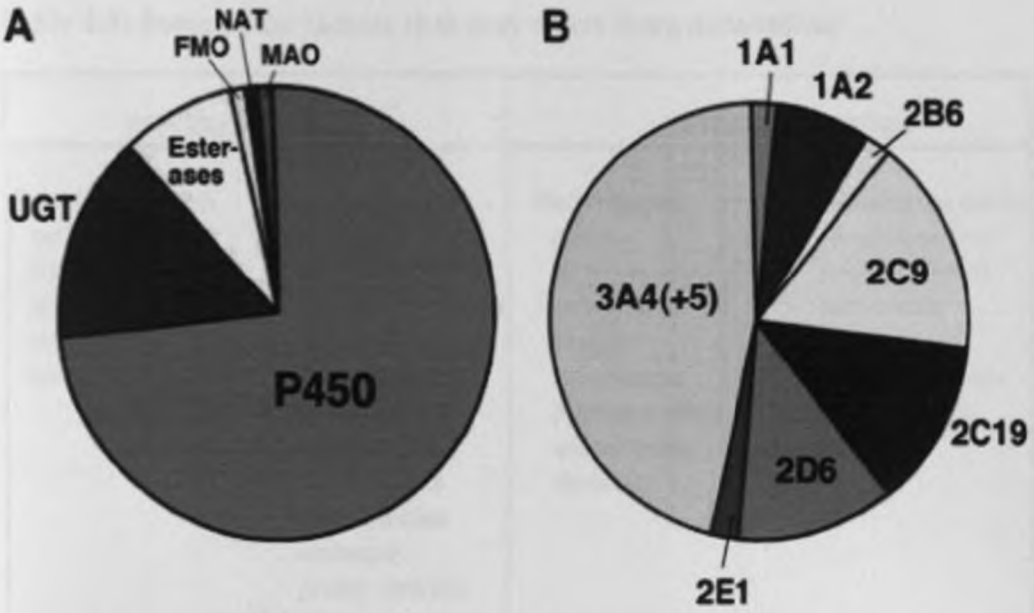


Figure 2.1: The contribution of enzyme systems to the metabolism of drugs

NB: A: Phase I and Phase II enzymes. B: Individual CYPs.
Adapted from Williams et al, (2004); Wienkers and Heath, (2005).

2.1.2.1 Factors affecting drug metabolism

Drug metabolizing enzymes are subject to variable expression and activity in individuals which may be attributed to various factors. Such influences include environmental, dietary, physiological, pathological, epigenetic and genetic factors (Table 2.3). Whereas most of these factors can change over time, the genetic make-up of an individual is virtually invariable. Hence, its determination will therefore provide life-long applicable information.

Table 2.3: Some of the factors that may affect drug metabolism

INTERNAL FACTORS		EXTERNAL FACTORS	
Physiological factors	**Pathological factors**	**Dietary factors**	**Environmental factors**
species variation	**Liver disease**	protein	petroleum products
Genetic variation	cirrhosis of the liver	fat	pyrolysis products
age	alcoholic liver disease	carbohydrates	heavy metals
sex	cholestatic jaundice	vitamins	pesticides
hormones	liver carcinoma	trace elements	industrial pollutants
	Endocrine disorders	pyrolysis products	pharmaceuticals
	diabetes mellitus	tobacco smoke	
	hypothyroidism	alcohol	
	hyperthyroidism		
	acromegaly		
	pituitary dwarfism		
	Infection		
	bacterial		
	viral		
	parasitic		
	inflammation		

Adapted from Parkinson, 1996

Drug metabolism may also be affected by agents that may cause induction or inhibition of drug metabolizing enzymes. Enzyme induction may lead to decreased efficacy whereas drugs with active metabolites can display increased drug effect and/or toxicity. Human CYP 1A1/2, 2A6, 2C9, 2C19, 2E1, and 3A4 are all known to be inducible with the exception of CYP2D6 (Ronis and Ingelman-Sundberg, 1999). On the other hand, enzyme inhibition occurs when two drugs sharing metabolism via the same isozyme compete for the same enzyme receptor site. Enzyme inhibition can cause a rapid and profound increase in blood levels of the parent drug leading to prolonged pharmacological activity or increased incidence of drug induced toxicity (Ferslew *et al.*, 1998). Enzyme induction or inhibition form a major basis for drug-drug interactions that are of particular importance with respect to adverse drug reactions. Table 2.4 shows important hepatic drug

metabolizing CYPs, and some examples of their substrates, inducers and inhibitors (Zanger *et al.*, 2008).

Table 2.4: Hepatic CYP and some selected substrates, inhibitors and inducers

CYP	Substrates	Inhibitors	Inducers
CYP1A2	Theophylline Acetominophen Propranolol	Cimetidine Ciprofloxacin Fluvoxamine	Omeprazole Cigarette smoke Broccoli
CYP2B6	Efavirenz Artemisinin Bupropion	Clopidogrel	Efavirenz Rifampicin Artemisinine
CYP2C8	Amodiaquine Paclitaxel	Trimethoprim Montelukast	Rifampicin
CYP2C19	(Es)omeprazole Diazepam	Clopidogrel Fluvoxamine	Carbamazepine Rifampicin
CYP2D6	Haloperidol Metoprolol Imipramine	Fluoxetine Quinidine	NONE
CYP3A4	Erythromycin Midazolam Verapamil	Ethinylestradiol Azole antimycotics Clarithromycin	Carbamazepine Efavirenz Nevirapine Rifampicin

Adapted from Zanger et al, 2008

2.1.3 Genetic Polymorphism of drug metabolizing enzymes

The metabolism of most drugs influences their pharmacological and toxicological effects. Drugs particularly affected are those with a narrow therapeutic window and those which are subject to considerable first pass metabolism (Thummel *et al.*, 1996). Table 2.5 above depicts some clinically relevant genetic polymorphism of CYP enzymes (www.cypallele.ki.se).

Table 2.5: Clinically relevant genetic polymorphisms of the human CYP

CYP allele	Key mutation	Protein effect effect	Functional effect
CYP1A2*1C	-3860G>A	Promoter	↓ inducibility
CYP1A2*1F	-163C>A	Intron 1	↑ inducibility
CYP2B6*4	18053A>G	K262R	↑ expression & activity
CYP2B6*6	15631G>T	Q172H	↓ expression & activity
CYP2B6*18	21011 T>C	I328T	↓ expression & activity
CYP2C8*2	11054A>T	I269F	↓activity
CYP2C8*3	2130G>A	R139K	↓activity
	30411A>G	K399R	↑activity
CYP2C9*2	3608C>T	R144C	↓activity
CYP2C9*3	42614A>C	I359L	↓↓ activity
CYP2C19*2	19154G>A	Splicing defect	Null allele
CYP2C19*3	17948G>A	W212X	Null allele
CYP2C19*17	-806C>T	Promoter	↑ expression & activity
CYP2D6*3	2549delA	Frameshift	Null allele
CYP2D6*4	1846G>A	Splicing defect	Null allele
CYP2D6*5	Recombination	Deletion	Null allele
CYP2D6*6	1707delT	Frameshift	Null allele
CYP2D6*10	100C>T	P34S	↓ expression & activity
CYP2D6*17	1023C>T	T107I	↓ expression & activity
CYP2D6*29	2850C>T	R296C	↓ expression & activity
CYP2D6*41	2988G>A	Splicing defect	↓ expression & activity
CYP2D6*Nxn	Recombination	Copy number variations	↑ expression & activity
CYP3A4*1B	-392A>G	Promoter	effect on transcription
CYP3A5*3	6986A>G	Splicing defect	↓ expression & activity

Compiled from Ingelman - Sundberg et al, (2007)

The term genetic polymorphism defines a monogenic trait resulting in at least two phenotypes that exist in a normal population. Neither of the traits should be rare and the least common phenotype should occur at a frequency greater than 1%

(Meyer, 1991). Such variation in DNA may result in strikingly exaggerated response or lack of effectiveness of drugs administered in the usual dosage.

Genetic polymorphism in drug metabolism gives rise to distinct populations namely, the extensive metabolizers (EM), the poor metabolizers (PM), and the ultra-rapid metabolizers (UM). The PM phenotype is monogenetically inherited as an autosomal recessive trait due to complete absence or marked decrease in the amount or activity of the enzyme. The UM phenotype on the other hand, is due to the presence of duplicated genes resulting in expression of increased amount of the enzyme (Ingelman-Sundberg, 1999b). A wide range of drug metabolizing enzymes are subject to genetic polymorphism (Kalow, 1993). These include those involved in phase I metabolic reactions such as dihydropyrimidine dehydrogenase (DPD) and the CYP super family of enzymes including CYPs 1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1 and CYP3A4.

2.1.3.1 Molecular mechanisms of genetic polymorphism

The molecular mechanisms underlying genetic polymorphisms have been elucidated. They may include deletion of a whole gene or insertions, deletions and substitution of a single base pair referred to as single nucleotide polymorphism (SNP) (Figure 2.2), (Ingelman – Sundberg et al, 1999b). By definition, single nucleotide polymorphisms (SNPs) are single base changes occurring at an allele frequency of at least 1% (Hoehe *et al.*, 2003). Anything with a frequency lower than this is referred to as a mutation. A SNP can either involve a transition (purine replaces a purine) or more rarely a transversion (purine replaces a pyrimidine).They can occur at regulatory (promoter), coding (exon) or in non-coding (intron) regions. They are referred to as synonymous if both alleles result in the expression of similar polypeptide sequences or non-synonymous if differing polypeptide sequences are produced. Other forms of DNA variability could be due

to starches of bases, repetitive tandem repeats, Alu segments and microsatellites (Meyer, 1994).

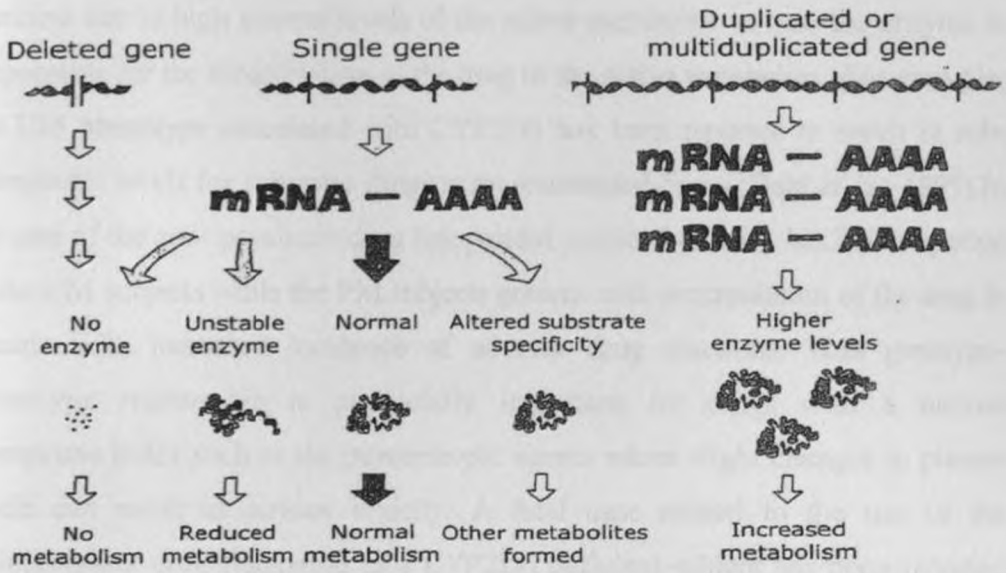


Figure 2.2: Overview of mechanisms that result in altered drug metabolism

Adapted from Ingelman – Sundberg et al, (1999b).

2.1.4 Consequences of genetic polymorphism

The consequences of genetic polymorphism seem to be extreme for subjects lacking the enzyme and are homozygous for the poor metabolizer phenotype (PM). This is also true for those with duplicated or amplified genes making them Ultra-rapid matabolizers (UM) (Meyer, 1994). Individuals who are homozygous for the PM phenotype may lack the enzyme or have markedly reduced activity leading to accumulation of substrate drugs. This may manifest as profound toxicity for medications that have a narrow therapeutic index and are inactivated by the enzyme as in the case of mercaptopurine, azathioprine and fluorouracil (Krynetski and Evans, 1998). There could also be loss of therapeutic efficacy in case the enzyme activates a pro-drug to an active metabolite.

On the other hand, the UM phenotype may not achieve therapeutic levels of the drug after standard doses due to rapid clearance which may manifest as lack of efficacy or therapeutic failure (Bertilsson *et al.*, 1993). Adverse effects may also manifest due to high plasma levels of the active metabolite in case the enzyme is responsible for the bioactivation of the drug to the active metabolite. For example, the UM phenotype associated with CYP2D6 has been reported to result in sub-therapeutic levels for substrate drugs at recommended doses (Dahl *et al.*, 1995).In the case of the anti- psychotic drug haloperidol, reduced efficacy has been reported in the UM subjects while the PM subjects present with accumulation of the drug in plasma with increased incidence of adverse drug reactions. This genotype-phenotype relationship is particularly important for drugs with a narrow therapeutic index such as the psychotropic agents where slight changes in plasma levels can result in serious toxicity. A fatal case related to the use of the antidepressant drug fluoxetine in a CYP2D6 deficient subject has been reported (Sallee *et al.*, 2000).

2.1.5 Genetic polymorphism and adverse drug reactions

Adverse drug reactions (ADRs) occur during drug development and with approved medicines during clinical practice (Edwards and Aronson, 2000). They are responsible for the termination of approximately 20% of investigational drugs in the pharmaceutical pipeline (Dimasi, 2001). Furthermore, about 1% of marketed drugs are withdrawn or restricted post-marketing due to safety-related issues. Some of the risk factors for ADRs include drug dose, environmental history and exposures, diet, concomitant medications, age, sex, ethnicity as well as genetic variants.

Table 2.6: Pharmacogenomic biomarkers predicting adverse drug reactions

Gene	Relevant Drugs	Specificity of Biomarker	% Patients with an ADR†
TPMT	Thiopurines	Very good	1 - 10
UGT1A*28	Irinotecan	Good	30 - 40
CYP2C9 and VKORC1	Warfarin	Good	5 - 40
CYP2D6	Tricyclic antidepressants	Relatively good	5 - 7
HLA-B*5701	Abacavir	Very good	5 - 8
HLA-B*1502	Cabamazepine	Very good	10
HLA-DRB1*07 and DQA1*02	Ximelagatran	Good	5 - 7

Adapted from Ingelman- Sundberg, (2008)

† Percentages are of affected caucasians except that for HLA-B*1502 which is for affected Asians.

The search for pharmacogenomic biomarkers of increased risk for drug toxicity has often focused on variation within genes encoding drug-metabolizing enzymes. Altered enzymatic activity can lead to elevated levels of the substrate drug, or alternatively, increased amounts of a reactive metabolite, either of which could have toxic effects. So far, only a limited number of pharmacogenomic biomarkers have been reported (Ingelman-Sundberg, 2008) (Table 2.6).

Among the potentially useful pharmacogenomic biomarkers for ADRs, the highest specificity has been exhibited by the HLA allelic variants. Thus, many more biomarkers remain to be identified. Unfortunately, much of the existing research in this area has been hampered by limitations in study design. Such limitations include poorly defined case and control groups, the use of retrospective and non-blinded study protocols and non-optimal selection of genetic variants. In addition, adverse drug reactions are subject to polygenic influences, treatment with multiple drugs and variation in the severity of clinically observed reactions that confound

genetic association. As a result, only a limited number of reports of a positive association between genetic characteristics and adverse drug reactions have been reproducible. There is a great need for large, randomized, double-blind, well controlled, prospective studies in the area of pharmacogenomics to clearly demonstrate the value of prospective genotyping in clinical practice.

2.2 Pharmacogenetics and Pharmacogenomics

2.2.1 Definition and Scope

Genetic determinants of drug disposition and effects remain stable for a person's lifetime and can have marked impact, accounting for 20-95% of the variability (Kalow *et al.*, 1998). The recognition of the genetic basis of this variability has since evolved into a science known as pharmacogenetics, which is defined as 'the study of variability in drug response due to genetic variations or heredity'. Pharmacogenetic variability can be divided into two types that which involves genes that determine drug absorption, distribution, metabolism and excretion, which is referred to as pharmacokinetic pharmacogenetics. On the other hand, is the variability that occurs at the level of genes encoding for drug target receptors and this is referred to as pharmacodynamic pharmacogenetics (Eichelbaum and Evert, 1996). The interplay of the variability in pharmacodynamic (PD) and pharmacokinetic (PK) properties of a drug on the one hand, and genetic variability on the other, can result in therapeutic failure or increased susceptibility to adverse effects in patients. Hence, the importance of the potential application of pharmacogenetics in the identification of a particular drug and dosage that is most likely to be safe and effective for each patient. This has become the new paradigm of modern drug therapy and is frequently described as 'personalized medicine'.

Pharmacogenomics on the other hand is a rapidly emerging field spawned by the human genome project, coupled with functional genomics and high-throughput screening methods. These methods have provided powerful new tools for the

elucidation of polygenic components of human health and disease. In this context, pharmacogenomics refers to the entire spectrum of genes that determine drug behavior and sensitivity, whereas pharmacogenetics is often used to define the narrower spectrum of inherited differences in drug disposition and effects. However, this distinction is often arbitrary and the two terms are commonly used interchangeably to define any kind of genetic information that helps to predict response to a particular drug. Hence, pharmacogenomics aims at elucidating the polygenetic basis of inter-individual differences in drug response, using genome wide approaches (McLeod and Evans, 2001). It capitalizes on information from the Human Genome Project and on advances in technology such as high-throughput sequencing, DNA and protein microarrays and bioinformatics. Ultimately, the integration of such sophisticated genomic tests with extensive phenotypic characterization of uniformly treated patients is a mandatory prerequisite in finding the definition of pharmacogenomics.

2.2.2 Clinical pharmacogenetics

Clinical pharmacogenetics is concerned with the contribution of genetic polymorphism to the efficacy and safety of therapeutic drugs (Table 2.7). It is well recognized that different patients respond in different ways to the same medication. These differences are often greater among members of a population

Table 2.7: Clinically relevant Pharmacogenetics of drug response

Enzyme	Substrate drug	Variable clinical effect	Possible mechanism	References
TPMT	Mercaptopurine Azathioprine	Bone marrow aplasia	Hypofunctional alleles	Evans et al, 2001
CYP2D6	Antidepressants Codein Antipsychotics	Increased side effects Reduced analgesia Decreased Efficacy	Hypofunctional alleles Hypofunctional alleles Gene duplication	Dahl and Bertilsson, 1993 Caraco, 1999
CYP2C19	Omeprazole	Better H. Pylori cure rate	Hypofunctional alleles	Furuta et al, 1998
UGT1A1	Irinotecan	Neutropenia	Reduced expression	Ramchandani et al, 2007
GSTM1 & GSTT1	Anti-cancer agents	Better breast cancer survival Poor outcome in AML	Gene deletion	Ambrosone et al, 2001 Davies et al, 2001
NAT2	Isoniazid Sulphonamides	Neurotoxicity Hypersensitivity	Slow acetylation	Spielberg, 1996 Alfirevic et al, 2003
MDR1	Digoxin Protease inhibitors	Increased bioavailability Immunological failure	Altered function	Sakaeda, et al 2001 Fellay et al, 2002
ADRB2	Inhaled β2-agonoists	Decreased brochodilation	Altered function	Drysdale et al, 2000
VKORC1 CYP2C9	Warfarin	Risk of bleeding	Variable expression Hypofunctional alleles	Aithal et al, 1999 Rost et al, 2004

than they are within the same person at different times (Vesell, 1989). The existence of large population differences with small intra-patient variability is consistent with inheritance as a determinant of drug response. Genetics factors are estimated to account for 20 to 95 percent of variability in drug disposition and

effects (Kalow *et al.*, 1998). There is a growing list of polymorphism in genes encoding drug-metabolizing enzymes, drug transporters, and drug targets that have been linked to clinically relevant effects (Evans and Johnson, 2001). More than 1.4 million SNPs were identified in the initial sequencing of the human genome (Sachidanandam *et al.*, 2001) with over 60,000 of them in the coding region of genes. Some of these SNPs have already been associated with substantial changes in the metabolism or effects of medications and are being used to predict clinical response (Yates *et al.*, 1997). For the most part, these represent the low lying fruit of pharmacogenetics as they are generally monogenic and highly penetrant with clearly recognizable drug induced phenotypic effects

However, monogenic drug response traits are rare and in general, drug response variability can be considered to be a complex trait, where many genes are implicated both at the pharmacokinetic and pharmacodynamic level. An excellent example of the contribution of multiple genes to variable drug response is the anticoagulant drug warfarin. Previous work had identified variable metabolism by CYP2C9 as a major contributor to variable responses to the drug (Aithal *et al.*, 1999). However, in 2004, coding-region mutations in VKORC1, encoding a subunit of the vitamin K epoxide reductase complex (the pharmacologic target for the drug), were found to cause a rare syndrome of warfarin resistance (Rost *et al.*, 2004). Subsequently, common variants in VKORC1have been found to account for a much greater fraction of variability in warfarin response (21%) compared to variants in CYP2C9(6%) (Rieder *et al.*, 2005).

2.3 Pharmacogenetics of HIV

By the end of 2010, it was estimated that 34 million people in the world were living with HIV (UNAIDS, 2010). While global HIV prevalence is 1.0%, sub-Saharan Africa is particularly affected, with an overall prevalence of 5.0% (Cohen, 2008). An estimated 7 million people in low and middle income countries were receiving antiretroviral therapy (ART) at the end of 2010, compared to 3 million in

2007 and 400,000 in 2003 (UNAIDS, 2010). The greatest increase in the number of people receiving ART was in sub-Saharan Africa. Six classes of antiretroviral (ARV) agents are available for combination of highly active antiretroviral treatment (HAART) regimens. These include the nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), the non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors (FIs), CCR5 antagonists and integrase inhibitors. Currently, preferred regimens use combinations of two NRTIs and either an NNRTI or a ritonavir-boosted PI. Both NNRTI- and PI-based regimens result in suppression of HIV RNA levels and CD4 T cell increases in a large majority of patients (Riddler *et al.*, 2008)

2.3.1 Variable response to HAART

Highly active antiretroviral therapy (HAART) has become the standard of care in HIV infection. The benefits are unquestionable as what was once a devastating illness has now been transformed into a manageable chronic disease (Mahungu *et al.*, 2009). Although HAART cannot eradicate HIV infection, it can suppress the level of viral replication, which helps to restore the immune system. Therapy is considered a success if it is able to suppress the viral load to a point below detection (Hammer *et al.*, 2008). There are often significant inter-individual differences in drug disposition despite standardized dosing. As a matter of fact, HIV-infected patients consist of individuals who differ genetically with regard to their response to both the virus and the antiretroviral (ARV) drugs. Furthermore, the response to HAART is a complex and variable phenomenon limited by the development of toxicity, the emergence of drug resistance and variable levels of drug adherence. Factors that could influence inter-individual variability include age, gender, body mass index, co-administered medications (including herbal preparations), food, co-morbidity and genetic differences. The genetic polymorphism of some genes relevant to antiretroviral drug disposition, toxicity and response is shown in Table 2.8.

Table 2.8: Pharmacogenetics of antiretroviral drug response

ARV class	Drugs	Genetic variants	Consequences
NRTI	Lamivudine	ABCC4 3724G>A, 4131 T>G	Higher triphosphate levels
	Zidovudine	ABCC4 3724G>A, 4131 T>G	Higher triphosphate levels
	Abacavir	HLA B*5701	Hypersensitivity reaction
	Tenofovir	ABCC2 CATC haplotype	Proximal renal tubulopathy
		TNFα - 238A	Accelerated lipoatrophy
		Mitochondrial DNA	Increased neuropathy
NNRTI	Efavirenz	CYP2B6 516G>T, CYP2B6 983T>C	Higher exposure
			Neurotoxocity
			Improved immune response
		HLA-DRB1*0101	Rash
	Nevirapine	CYP2B6 516G>T, CYP2B6 983T>C	Higher exposure
			Improved immune response
		HLA-DRB1*0101	Hypersensitivity reaction
		HLA-cw8	Hypersensitivity reaction
		ABCB1 (MDR1) 3435C>T	Reduced Hypersensitivity reaction
PI	Saquinavir	CYP3A5*3 A6986G (wild-type)	Faster oral clearance
	Indinavir	CYP3A5*3 A6986G (wild-type)	Faster oral clearance
		ORM1 (F1F1)	Increased clearance
		UGT1A1*28	Hyperbilirubinaemia
	Nelfinavir	CYP2C19*2 (681G>A)	Higher exposure
	Lopinavir	ORM1 (F1F1)	Increased clearance
	Atazanavir	UGT1A1*28	Hyperbilirubinaemia
	Ritonavir	APOA5 (non-*1/*1 haplotypes)	Hyperlipidaemia

Adapted from Mahungu et al, (2009)

The genetic basis of variability in response to HAART is supported by observations that patients from distinct ethnic groups had significantly different antiretroviral drug pharmacokinetics and clinical response (Kappelhoff *et al.*, 2005). The focus of pharmacogenomic studies has been the impact of SNPs on both metabolism and transport of antiretroviral drugs. However, their influence on each drug differs, depending on individual drug pharmacological properties. For example, the NRTIs are substrates for multi-drug resistance transporters, such as

MRP4 (Schuetz *et al.*, 1999)and BCRP/ABCG2. In contrast, the NNRTIs and PIs are primarily metabolized by the CYP enzyme pathways and are also substrates for the drug transporter P-glycoprotein (Störmer *et al.*, 2002).

Individualization of drug dosing guided by the measurement of drug levels by therapeutic drug monitoring (TDM) has been proposed to optimize therapeutic outcomes and prevent adverse effects (Back *et al.*, 2002). Nevertheless, the benefits of routine measurement of drug plasma levels in long-term antiretroviral therapy is still under debate (Aarnoutse *et al.*, 2003). The rising HIV-infected population consists of ethnically diverse individuals on complex and potentially toxic antiretroviral regimens on a long-term basis. These individuals would benefit greatly from predictive tests that identify the most durable regimens and pharmacogenetics could hold that promise. For this to be realized in African populations which are most affected by HIV, more studies need to be done to harness the unique genetic diversity inherent in this populations

2.4 Cytochrome P450 2B6

The cytochrome P450 2B6 (*CYP2B6)* gene has been mapped to chromosome 19 between 19q12 and 19q13.2, where it is located together with the pseudogene *CYP2B7P,* which spans a region of about 26 kb. It consists of nine exons encoding 491 amino acids (Yamano *et al.*, 1989). Studies in human liver microsomes suggest that the average CYP2B6 content in human liver is about 3–6% of the total microsomal CYP pool (Stresser and Kupfer, 1999). However, CYP2B6 is also found in various extrahepatic tissues, including the kidney, skin, brain, intestine, and lung (Gervot *et al.*, 1999). This enzyme was historically thought to play an insignificant role in human drug metabolism. However, increased interest has been stimulated by the discovery of polymorphic and ethnic differences in *CYP2B6* expression (Lang *et al.*, 2001). This has been coupled by the identification of additional substrates and evidence of coregulation with CYP3A4 (Faucette *et al.*, 2007). The expression of CYP2B6 protein and enzyme activity in human liver

reportedly varies more than 100-fold among individuals (Ekins *et al.*, 1998). This inter-individual variability has been associated with *CYP2B6* genetic polymorphism, which can influence expression and function of the enzyme (Lang *et al.*, 2001) as well as therapeutic outcomes of CYP2B6 metabolized drugs (Ingelman-Sundberg, 2004).

2.4.1 *CYP2B6* genetic variants

The genetic polymorphism of *CYP2B6* has been well characterized and to date at least 28 allelic variants and a series of sub-variants of *CYP2B6* (**1B* to **29*) have been described (Sim and Ingelman-Sundberg, 2010). The first systematic analysis of the genetic polymorphism in *CYP2B6* focused on the nine exons of the coding region (Lang *et al.*, 2001). This study led to the identification of nine single base mutations of which five were non-synonymous amino acid changes whereas four were silent mutations. Alone or in combination, these SNPs resulted in six different *CYP2B6* alleles designated as *CYP2B6**2 (64C>T), *CYP2B6**3 (777C>A), *CYP2B6**4 (785A>G), *CYP2B6**5 (1459C>T), *CYP2B6**6 (516G>T and 785A>G), and *CYP2B6**7 (516G>T, 785A>G and 1459C>T). The *CYP2B6**6, was found to occur in Caucasian (26%) (Ekins *et al.*, 1998), Asians (15 - 40%) (Hiratsuka *et al.*, 2004) and at over 50% in populations of African descent (Mehlotra *et al.*, 2006).

Analysis of human liver mRNA discovered that aberrant splicing of the *CYP2B6* gene resulted in the *CYP2B6**6 allele which lacks exon 4, 5 and 6 and thus displays a reduced expression of mRNA and protein (Hofmann *et al.*, 2008). Both heterozygous and homozygous carriers of *CYP2B6**6 allele displays remarkably lower catalytic activity and a significant decrease in protein expression, compared with wild-type*CYP2B6**1 allele carriers. Another polymorphic allele, *CYP2B6**18 is a non-synonymous allele that did not form a functional protein in transfected mammalian cells and may thus be a null allele (Klein *et al.*, 2005). It is absent in

Caucasian populations, but has been consistently found among black subjects at an allele frequency of 7- 9% (Mehlotra *et al.*, 2007; Wyen *et al.*, 2008).

2.4.2 CYP2B6 substrates and clinical relevance

CYP2B6 metabolizes about 8% of all pharmaceutical drugs to some extent. Clinically important drug substrates include the HIV drugs efavirenz and nevirapine (Turpeinen *et al.*, 2006), the antimalarial artemisinin (Svensson and Ashton, 1999), and the anesthetics propofol and ketamine (Svensson and Ashton, 1999). Other substrates include the antidepressant and smoking cessation agent bupropion (Faucette *et al.*, 2000), the synthetic opioid methadone, as well as the cytostatic cyclophosphamide. CYP2B6 is also involved in the metabolism of environmental chemicals (Hodgson and Rose, 2007) and procarcinogens such as aflatoxin B1 and 7, 12-dimethylbenz[*a*] anthracene (Code *et al.*, 1997). Additional substrates known to be metabolized in part by CYP2B6 include stimulants such as ecstasy (methylenedioxymethamphetamine (MDMA) (Kreth *et al.*, 2000) and nicotine (Yamazaki *et al.*, 1999). Like the rodent phenobarbital-inducible CYP2B genes, the human *CYP2B6* is strongly inducible by numerous drugs and chemicals, including rifampicin, barbiturates, cyclophosphamide, artemisinin, carbamazepine, efavirenz, nevirapine, and metamizole (Saussele *et al.*, 2007).

The clinical importance of CYP2B6 polymorphism has been demonstrated in HIV therapy. The 516G>T SNP has been reported to have a major impact on the pharmacokinetics and pharmacodynamics of efavirenz (Rotger *et al.*, 2005). For example, HIV-infected patients homozygous for *CYP2B6* 516G>T were reported to exhibit strongly elevated plasma levels of efavirenz, and in some studies, presented with increased risk for neurotoxicity (Telenti and Zanger, 2008). In clinical studies, subjects homozygous for combinations of the alleles *CYP2B6**6, *CYP2B6**16 and *CYP2B6**18 exhibited reduced metabolic capacity for CYP2B6 substrates, like efavirenz and nevirapine than expected from a linear gene–dose relationship (Rotger *et al.*, 2007). A study in Japanese HIV patients showed that

prospective, genotype-based dose adjustment successfully reduced the therapeutic dose of efavirenz and succeeded in improving CNS-related side effects (Gatanaga *et al.*, 2007). Studies have also reported that CYP2B6 516G>T was associated with nevirapine plasma concentrations in diverse populations (Mahungu *et al.*, 2009; Chou *et al.*, 2010). The *CYP2B6* genotypes also affected plasma levels of nevirapine in Ugandan HIV patients (Penzak *et al.*, 2007). It has also been reported that heterozygosity for the CYP2B6 983T>C SNP was associated with significantly higher nevirapine plasma levels in black patients (Wyen *et al.*, 2008).

2.5 Nevirapine

Nevirapine (NVP) belongs to a class of antiretroviral drugs known as the non-nucleoside reverse transcriptase inhibitors (NNRTIs) which act as noncompetitive inhibitors of the reverse transcriptase enzyme of HIV-1. Other licensed NNRTIs include efavirenz (EFV), delavirdine and etravirine (ETR). Most studies on pharmacogenetic determinants of NNRTIs disposition, efficacy and toxicity have focused on genes involved in the metabolism, transport and hypersensitivity reactions. Nevirapine is predominantly metabolized by CYP3A4 and CYP2B6 into its major metabolites, 2-hydroxynevirapine and 3-hyroxynevirapine, respectively, with a minor contribution from CYP3A5 (Erickson *et al.*, 1999) (Figure 2.4).

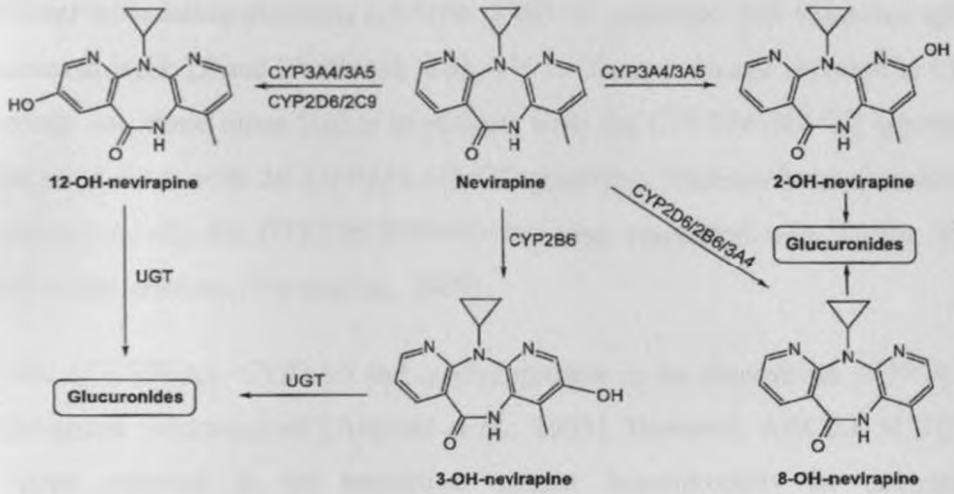


Figure 2.3: Metabolism of nevirapine by CYP2B6 and other CYPs

Adapted from Erickson et al., (1999)

2.5.1 Nevirapine disposition

Nevirapine is well absorbed with a bioavailability greater than □90% according to the innovator product information. The drug has a high volume of distribution (1.2 L/kg) in adults, and is approximately 60% bound to plasma proteins. The dose of nevirapine must be increased after 2 weeks of therapy from 200 mg once a day to 200 mg twice daily due to auto-induction of its metabolism. The drug crosses the placenta, resulting in a drug concentration ratio of 0.8 in cord blood compared with maternal blood (Mirochnick, 2000). Elimination is prolonged in women during labor and in newborn children (Mirochnick, 2000). The *in vitro* 50% inhibitory concentration (IC_{50}) of nevirapine is 100 nM against clinical isolates of HIV-1. The drug has been proven to be clinically effective, and its side-effect profile is well characterized (Milinkovic and Martinez, 2004).

Several studies have reported that the *CYP2B6* 516G>T polymorphism was associated with higher NVP plasma concentrations (Penzak *et al.*, 2007). A retrospective study on a pediatric cohort on NVP based regimens reported a

significant association between *CYP2B6* 516G>T genotype and immunological responses at week 12 and 24 (Saitoh *et al.*, 2007). The percentage increase in CD4 cell count was three times higher in patients with the *CYP2B6* 516 TT genotype compared to those with the *CYP2B6* 516 GG genotype. Findings have also shown that heterozygosity for *CYP2B6* 983T>C was also associated with higher NVP plasma concentrations (Wyen *et al.*, 2008).

The role of CYP3A4, CYP3A5 and p-glycoprotein in the disposition of NNRTIs has remained controversial (Almond *et al.*, 2005). However, ABCB1 3435C>T has been reported to be protective against hepatotoxicity in nevirapine hypersensitivity reactions (Ritchie *et al.*, 2006). NVP plasma levels have been shown to be predictive of treatment failure (de Vries-Sluijs *et al.*, 2003). The risk of virologic failure was reported to increase 5-fold when nevirapine trough concentrations were below 3.0 μg/Ml (González-de-Requena *et al.*, 2005). Studies have also found body weight, ethnicity, gender and underlying liver disease to be important in explaining nevirapine pharmacokinetics (Cooper and van Heeswijk, 2007).

2.5.2 Nevirapine toxicity

The use of NVP is limited by a potentially fatal, immune mediated hypersensitivity reaction. This reaction manifests as hepatotoxicity, fever and/or skin rash in approximately 5% of individuals (Dieterich *et al.*, 2004). However, clear relationship between plasma drug levels and NVP-associated hepatotoxicity or cutaneous disease has been reported (Kappelhoff *et al.*, 2005). Studies have reported that skin rash associated with NVP hypersensitivity reaction was more common with CD4 cell counts above 250 cells/mm in women and 400 cells/mm in men (Calmy *et al.*, 2007). Several studies have also reported an association between *HLA*-DRB1-0101 and NVP hepatotoxicity in predominantly Caucasian patients with CD4 cell percentages of above 25% (Martin *et al.*, 2005). In another study, the occurrence of isolated rash in Caucasian patients on EFV and NVP-

based regimens was associated with the presence of *HLA*-DRB1*01 but was not associated with CD4 cell percentages (Vitezica *et al.*, 2008). Further studies have also found *HLA*-cw8 to be a significant predictor of NVP hypersensitivity reaction (Gatanaga *et al.*, 2007). Despite the controversy surrounding the role of P-gp in NNRTI disposition, *ABCB1* variants continue to be implicated in the pharmacodynamics of NNRTIs. For example, *ABCB1* 3435C>T has been reported to be associated with a decreased risk of NNRTI-associated hepatotoxicity (Haas *et al.*, 2006). These findings need to be confirmed in other populations particularly those of African origin who are most affected by HIV if they are to be of any clinical utility.

2.6 Psychopharmacogenetics

Psychiatric drug treatment is characterized by large interindividual differences in both drug response and dosage requirement (Kahn *et al.*, 2008). The final clinical effect of a psychotropic drug depends on different factors that may influence the pharmacokinetics and pharmacodynamics of the drug. Pharmacogenetic studies have focused on molecular pathways hypothesized to be the mechanisms of action for psychotropic drugs. For example, dysfunction of the dopamine system has been known to underlie the pathophysiology of schizophrenia since 1960s. Dopamine has several receptor subtypes (D1 to D5), but only D2, D3, and D4 have been extensively studied in pharmacogenetics. First generation antipsychotics (FGAs), especially high potency drugs such as haloperidol, mainly bind to the D2 receptor (Kapur and Mamo, 2003). On the other hand, second generation antipsychotics (SGAs) have more diverse receptor binding profiles including the 5-HT_{2A} and 5-HT_{2C} receptors (Miyamoto *et al.*, 2005). Another area of interest is the pharmacokinetics of antipsychotic drugs, especially the influence of the cytochrome P450 family of enzymes that metabolize most antipsychotic drugs.

2.6.1 CYP and psychotropic drugs

Most antipsychotics and antidepressants are highly lipophilic compounds which are subject to extensive metabolism in the body by CYP enzymes (Bertz and Granneman, 1997). The most relevant CYP enzymes for the disposition of most antidepressant and antipsychotic drugs are CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (Table 2.9).

Table 2.9: Enzymes relevant to psychiatry and some psychotropic drug substrates

	CYP1A2	CYP2C19	CYP2D6	CYP3A4
Antidepressants	Amitriptyline Clomipramine Duloxetine Fluvoxamine Imipramine	Amitrptyline Citalopram Clomipramine Imipramine Sertraline	Amitriptyline Nortrityline Clomipramine Desipramine Fluoxetine Paroxetine Venlafaxine	
Antipsychotics	Clozapine Haloperidol Olanzepine		Chlopromazine Haloperidol Perphenazine Risperidone Thioridazine Zuclopenthixol	Haloperidol Bromperidol Rispesperidol Quetiapine Pimozide Ziprazidone

Adapted from Bertilsson et al, (1993).

Additionally, most of these compounds are converted into active metabolites that contribute to their pharmacological effects (Ghahramani *et al.*, 1997). A wide inter-individual variability has been reported in the biotransformation of psychotropic drugs leading to differences in steady-state plasma concentrations and possibly in therapeutic and toxic effects (Steimer *et al.*, 2005). This has implications for efficacy and toxicity particularly for drugs with a narrow therapeutic index.

For example, the tricyclic antidepressant drugs (TADs), such as amitriptyline, are metabolized by many CYP isoforms including CYP2D6, CYP3A4, and also CYP1A2, CYP2C19, CYP2C9. CYP2D6 is responsible for hydroxylation leading to inactivation (Brøsen *et al.*, 1991)whereas the latter isoforms catalyze *N*-demethylation of TADs leading to formation of active metabolites (Ghahramani *et al.*, 1997). The metabolism of the phenothiazine neuroleptics such as chlorpromazine and haloperidol is also under genetic control of hepatic CYP2D6 (Dahl and Bertilsson, 1993). The CYP2D6 isoenzyme mainly catalyze aromatic hydroxylation (Brockmoller *et al.*, 2002)and in some cases, sulfoxidation and *N*-demethylation (Daniel *et al.*, 2002). Thus, impaired activity of CYP2D6 may be a factor predisposing to over sedation and antipsychotic-induced extrapyramidal side effects due to accumulation of parent drugs (Scordo and Spina, 2002).

Variability in CYP activity may be caused by various factors. Besides endogenous factors such as age, gender and morbidity, exogenous factors such as co-medication, food components and smoking are important (Rostami-Hodjegan *et al.*, 2004). This is especially so in psychiatry where co-medication is the rule rather than the exception. Several drugs are known to interfere with certain CYP enzymes by either inhibition or induction. However, much of the inter-subject variability in enzyme activity is for a large part determined by genetic factors with most CYP enzymes being known to be polymorphic

Whereas the pharmacokinetic consequences of polymorphic metabolism has been well characterized for a number of antipsychotic drugs, the importance of this in relation to dosing and clinical response still needs to be elucidated (Spina *et al.*, 2000). The scenario is further complicated by the fact that the receptors through which the psychotropic drugs exert their psychopharmacological effects are also subject to genetic variation, consequently influencing drug response (Okey *et al.*, 2005; Arranz and Kerwin, 2003). Many antipsychotics and antidepressants drugs have a narrow therapeutic range, with concentration dependent adverse effects

occurring at concentrations only slightly higher than the therapeutic dose. Therefore patients would benefit from a 'tailor made' dosage. To accomplish this, classical therapeutic drug monitoring (TDM) can be complemented with pharmacogenetic tools. Studies have shown that CYP2D6 and CYP2C19 metabolic rates could guide dosage selection for psychotropic drugs (Kirchheiner *et al.*, 2004). This is a clear example of the potential application of clinical pharmacogenetics.

2.7 Cytochrome P450 2C19

The cytochrome P450 *2C19* (*CYP2C19)* gene is mapped to the long arm of chromosome 10 (10q24.1-q24.3), located in a densely packed region also containing genes encoding *CYP2C8, 2C9*, and *2C18* (Romkes *et al.*, 1991). It consists of nine exons and eight introns. The protein has a molecular weight of 55.9 kDa and encodes the CYP2C19 protein of 490 amino acids. The CYP2C19 enzyme is primarily present in hepatic tissue, but a significant amount is also found in the gut wall, particularly the duodenum.

The genetic polymorphism of *CYP2C19* is well established with at least 24 (**1B* to **25*) variants and a series of sub-variants of *CYP2C19*having been identified with *CYP2C19*1A* representing the wild type allele. The first *CYP2C19*variant allele discovered was *CYP2C19**2Acontaining 681G>A in exon 5 that causes a splicing defect (De Morais *et al.*, 1994b). *CYP2C19**3A and *3Bshare the 636G>A SNP, resulting in a premature stop codon in exon 4 (Fukushima-Uesaka *et al.*, 2005). The null alleles, *CYP2C19**2 and *CYP2C19**3result in complete loss of enzyme activity (De Morais *et al.*, 1994a). The majority of PMs of CYP2C19 are due to the presence these two variant alleles (Desta *et al.*, 2002). Other potentially defective variant alleles of *CYP2C19*have been discovered and designated as *CYP2C19**9through to *CYP2C19**19. Notably is the *CYP2C19**17allele containing two SNPs, 99C>T and 991A>G causing the I331V substitution that gives an ultrarapid phenotype (Sim *et al.*, 2006).

2.7.1 *CYP2C19* inter-ethnic variability

The distribution of common variant alleles of *CYP2C19* has been found to vary among different ethnic groups. The allelic frequency of *CYP2C19**2 is 17% in African Americans, 30% in Chinese, and 15% in Caucasians (Goldstein *et al.*, 1997). On the other hand, *CYP2C19**3has been shown to be more frequent in the Chinese (5%) and less frequent in African Americans (0.4%) and Caucasians (0.04%) (Allabi *et al.*, 2003). *CYP2C19**2is the dominant defective allele and accounts for 75–85% of PM phenotype in Asian and Caucasian populations (Lee*et al.*, 2007).. On the other hand, *CYP2C19**17has been reported in Swedish and Ethiopian populations at frequencies of 18% and in Chinese at 4% (Sim *et al.*, 2006). The PM phenotype is inherited as an autosomal recessive trait and the poor metabolizers (PMs) are unable to hydroxylate S-mephenytoin and other substrates of CYP2C19. The PM frequency ranges from 2-6% in Caucasians and black populations (Xie *et al.*, 1999a; Xie *et al.*, 1999b) as well as 13-23% in Asians (Bertilsson *et al.*, 1992). Three common types of *CYP2C19*genotypes responsible for the PM phenotype exist, including two homozygous genotypes, *CYP2C19**2/*2and *CYP2C19**3/*3, and one heterozygous genotype, *CYP2C19**2/*3. The homozygous CYP2C19*2/*2genotype is by far, the most frequent of the three defective PM genotypes (Desta *et al.*, 2002). For EMs, there are two genotypes that are heterozygous for the *CYP2C19* wild type, *CYP2C19**1/*2and *CYP2C19* *1/*3 and one genotype that is homozygous for the wild-type allele, *CYP2C19* *1/*1.

2.7.2 CYP2C19 substrates and clinical relevance

The CYP2C19 enzyme (S- mephenytion hydroxylase) is responsible for the metabolism of approximately 10% of clinically important drugs (Gardiner and Begg, 2006). These include proton pump inhibitors (PPIs) such as omeprazole, antidepressants such as amitriptyline and fluoxetine, benzodiazepines such as diazepam, anticonvulsants such as Phenobarbital and phenytoin, the antifungal

voriconazole and the antiretroviral nelfinavir. It is also involved in the bioactivation of the anti malarial proguanil to the active metabolite cycloguanil (Ward *et al.*, 1991). Omeprazol is mainly metabolized to 5 – hydroxyomeprazole in the liver (Ieiri *et al.*, 1996). In individuals with a PM phenotype of CYP2C19, the area under the curve (AUC) of omeprazole is markedly increased and the effect of omeprazole on acid secretion is enhanced (Furuta *et al.*, 1999). Asian populations with high proportions of the PM phenotype reportedly use lower dosages of diazepam compared to Caucasians (Bertilsson, 1995).

2.8 Cytochrome P450 2D6

The cytochrome P450 *2D6* (*CYP2D6)* gene is mapped to chromosome 22q13.1 and consists of nine exons with an open reading frame of 1,491 bp coding for 497 amino acids (Eichelbaum *et al.*, 1987; Kimura *et al.*, 1989; Heim and Meyer, 1990). *CYP2D6* belongs to a gene cluster of highly homologous inactive pseudogenes including *CYP2D7P* and *CYP2D8P* (Steen *et al.*, 1995). The CYP2D6 enzyme is primarily expressed in the liver where it accounts for less than 2% of all hepatic CYPs, but is also localized in brain neurons, where it may participate in local drug metabolism and in certain endogenous biotransformations.

2.8.1 *CYP2D6* genetic variants

CYP2D6 was the first CYP for which a classical pharmacogenetic polymorphism became known and is the most intensely studied of the CYP enzymes. To date, more than 105 different *CYP2D6* variants have been identified by the human cytochrome P450 allele nomenclature committee. Variant versions of the *CYP2D6* gene may result in a complete absence of enzyme activity, reduced activity, normal activity or even increased activity. Null alleles of *CYP2D6* do not encode a functional protein and there is no detectable residual enzymatic activity. They are responsible for the PM phenotype when present in homozygous or compound heterozygous constellations.

The mechanisms which lead to a total loss of function includes single base mutations or small insertions/deletions that interrupt the reading frame or interfere with correct splicing. This may lead to prematurely terminated protein/ stop codon as seen with variant alleles such as *CYP2D6*3, *4, *6, *8, *11, *15, *19, *20, *38, *40, *42* and **44* (Kagimoto *et al.*, 1990). There could also be non-functional full length coded alleles such as *CYP2D6*12, *14* and **18*) (Evert *et al.*, 1997) or the deletion of entire *CYP2D6* gene as a result of large sequence deletions as in the case of *CYP2D6*5, *13* and **16*) (Gaedigk *et al.*, 1991). On the other hand, extremely high CYP2D6 activity leading to the UM phenotype may result from gene duplication of functional allele **1* and **2* fused in a head to tail orientation, as a result of unequal crossover events and other mechanisms. This was noted by a molecular characterization of the *CYP2D6* locus in patients with extremely rapid metabolisms (Bertilsson *et al.*, 1993). The reduced activity allele, *CYP2D6*17* has four mutations (1023C>T, 1661G>C, 2850C>T, and 4180G>C) that result in decreased activity (Masimirembwa *et al.*, 1996). Two of which are non-synonymous mutations (1661G>C and 2850C>T) in common with the *CYP2D6*2* allele. This creates an alteration in the active site and hence an alteration in substrate specificity. The corresponding enzyme exhibits a five-fold higher apparent *K*m for codeine and the beta - adrenoceptor antagonist, bufuralol compared with the wild-type enzyme (Oscarson *et al.*, 1997). Individuals carrying this allele exhibit a diminished capacity (IM phenotype) for drug metabolism (Masimirembwa *et al.*, 1996).

2.8.2 *CYP2D6* inter-ethnic variability

The pattern of *CYP2D6* polymorphisms differs dramatically between individuals of different ethnic backgrounds. For example extremely high CYP2D6 activity (UM) occurs with allele frequencies of 1-2% in Caucasians (Griese *et al.*, 1998). However, much higher frequencies have been observed in Saudi Arabians (McLellan *et al.*, 1997) and Ethiopians (up to 29%) (Aklillu *et al.*, 1996). The

*CYP2D6*4* is by far the most frequent null allele in Caucasians. It occurs at a frequency of 20% to 25% and is responsible for 70% to 90% of all PMs in that population (Sachse *et al.*, 1997). The *CYP2D6*4* allele occurs with a frequency of about 1% in the Asian population (Wang *et al.*, 1993) and 6-7% in Africans and African- Americans (Leathart *et al.*, 1998). The deletion allele, *CYP2D6*5* seems to be present at similar frequencies in all these population groups. In Asian populations, the most common *CYP2D6* variant allele is *CYP2D6*10,* which has a Pro34Ser substitution in the proline-rich region near the NH_2-terminal and occurs with a frequency exceeding 50% (Wang *et al.*, 1993). In Caucasians, it occurs at a low frequency of about 2%. It however, accounts for 10-20% of individuals with the intermediate metabolizer (IM) phenotype (Griese *et al.*, 1998).

*CYP2D6*17* is virtually absent from Caucasian population but it occurs with a frequency of about 30% in populations of African descent (Masimirembwa *et al.*, 1996). It is the major variant in the black population and appears to explain why black Africans have a higher median metabolic ratio (Leathart *et al.*, 1998). The PM phenotype for CYP2D6 activity occurs at a frequency of 5 – 10% and 1 – 2% in Caucasian and Asian populations, respectively (Bradford, 2002). The PM phenotype is mainly attributed to the deficient *CYP2D6* gene variants *CYP2D6**3, *4,*5and *6withCaucasian allele frequencies of 0.3, 17, 3 and 0.6%, respectively (Bradford, 2002). Genotyping of these four *CYP2D6* variants identifies about 95 – 99% of Caucasian PMs. A substantial part of IM phenotype can be explained by the presence of the partially functional alleles *CYP2D6**9,*10, *17and *41 (Sistonen *et al.*, 2007; Bradford, 2002). In East Asian populations, the contribution of *CYP2D6*10* is greatly enhanced with frequencies as high as 39% (Sistonen *et al.*, 2007). For populations of African descent, the IM phenotype is mainly attributed to an allele with a lower metabolic rate identified as *CYP2D6**17 (Masimirembwa *et al.*, 1996; Bradford *et al.*, 1998). On the other hand, the frequency of UMs varies among ethnic groups, with studies reporting low prevalence in Caucasians of 0.8 - 1.0% (Bathum *et al.*, 1998) and a much higher

prevalence in populations from countries surrounding the Mediterranean Sea (8 - 10%) (Aynacioglu *et al.*, 1999).

2.8.3 CYP2D6 substrates and clinical relevance

CYP2D6 accounts for about 2%–5% of all hepatic CYP isozymes, but metabolizes approximately 25% of all clinically used medications. These include some cytotoxics, antiarrythmics, antiemetics, antidepressants, antipsychotics, and analgesics (Ingelman-Sundberg and Evans, 2001). Consequently, polymorphisms of the CYP2D6 gene have the potential to affect efficacy, drug–drug interactions, and adverse events in many diverse clinical settings. Clinical effects of CYP2D6 polymorphisms have been reported for a number of drug classes such as the antidepressants, neuroleptics, and 5-HT_3-receptor antagonists. For example, the use of tricyclic antidepressants (TADs), may lead to life threatening cardiac complications in individuals bearing CYP2D6 PM (Spina *et al.*, 1997). A higher incidence of adverse events has also been reported in PMs who receive neuroleptics (Brockmoller *et al.*, 2002). Parkinsonism and tardive dyskinesia have been shown to be more common in PMs of CYP2D6 (Jaanson *et al.*, 2002). The 5-HT_3-receptor antagonists all contain warnings for the risk of arrhythmias in their labeling. This risk may increase when these antiemetics are co-administered with other agents that have the ability to cause cardiac side effects, particularly in PM patients.

Lack of drug metabolizing capacity PMs may also impact on efficacy in cases in where s metabolic activation to an active metabolite. This is a concern for cancer patients being given pain relief with codeine. A lower analgesic effect of codeine has been reported in PMs, as conversion of the drug to morphine is CYP2D6-dependent (Sindrup *et al.*, 1990; Stamer *et al.*, 2003). This could also be an issue for the antiemetic dolasetron, as CYP2D6 is also required for its conversion to the active metabolite hydrodolasetron. Phenocopying can also occur when a CYP2D6 inhibitor is administered concurrently with an agent that is metabolized to its

active form by the CYP2D6 isozyme. For example, co-administration of the antiarrhythmic drug quinidine with codeine to an EM individual can result in the loss of the analgesic effect of codeine due to the inhibition of CYP2D6 by quinidine (Kroemer and Eichelbaum, 1995). Conversely, codeine given to UMs can result in excessive formation of morphine, leading to severe side effects such as abdominal pain (Dalen *et al.*, 1997).

2.9 Arylamine N-Acetyltransferase 2

Arylamine N- Acetyltransferase 2 *(NAT2)* together with *NAT1* and a pseudogene, *NATP* are located on the human chromosome 8p22 (Hickman *et al.*, 1994). It is derived from a single intronless protein coding exon with an open reading frame of 870 base pair that produces a 290 amino acid protein (Ebisawa and Deguch, 1991). *NAT1* and *NAT2* open reading frames are 87% identical and their proteins differ only in 55 amino acids (Grant, 2008)

2.9.1 *NAT2* genetic variants

Studies have identified various nucleotide substitutions in the human *NAT2* gene that result in reduction in substrate affinity, catalytic activity and protein stability with the NAT2*4 being considered as the wild type allele (Ferguson *et al.*, 1994). The slow acetylator phenotype is inherited as a recessive trait (Deguchi, 1992) and is associated with *NAT2* *5 (T341C), *NAT2**6 (G590A), *NAT2**7 (G857A), *NAT2**14 (G191A) and *NAT2**17 (A4234A). At the protein level, the slow acetylator phenotype is associated with a reduction of 10 – 20% in the quantity of NAT2 enzyme in the liver cytosol (Grant *et al.*, 1990) whereas the mRNA remains unchanged (Blum *et al.*, 1991).

Extensive polymorphism in *NAT2* gives rise to a wide interindividual variation in N-acetylation capacity. A clear bimodal distribution segregates the rapid acetylator phenotype, associated with a normal acetylation capacity, from the slow acetylator one, characterized by a reduced enzyme activity. These two main metabolic

phenotypes occur with varying prevalence in populations of different ethnic origin (Upton *et al.*, 2001). High frequencies of slow acetylators of 40 – 70% have been reported in Caucasian populations compared to only 10-30% found in populations of Asian origin. The *NAT2**14 gene variant is common to populations of African extraction but is virtually absent in Caucasian populations (Bell *et al.*, 1993).

2.9.2 NAT2 clinical relevance

The arylamine N-acetyltransferase 2 enzyme plays a crucial role in the metabolism of clinically useful drugs and exogenous chemicals present in the diet, cigarette smoke and the environment (Hein, 2002). Drug substrates include isoniazid which is a first line agent in the therapy of tuberculosis, sulphamethoxazole; a component of co-trimozaxole which is an important antibacterial agent used in the prophylactic management of pneumocystis jerovicii pneumonia (PCP) in HIV patients. Other substrates include sulphadoxine, a component of the anti-malarial sulphadoxine-pyrimethamine (SP) and dapsone for leprosy. The clinical consequences of the acetylation polymorphism can be severe if standard drug doses are applied, exposing patients to an increased risk of adverse drug reactions or a lack of therapeutic efficacy (Meisel, 2002). In addition, in the last decades, an increasing number of epidemiological studies have attempted to relate acetylation phenotype to a variety of complex human disorders, such as bladder cancer, atopic diseases, diabetes, Parkinson's disease and many others (Butcher *et al.*, 2002). However, these association studies have produced conflicting results among and within human populations and most findings have not been replicated. Hence, the need for further studies covering diverse populations including Kenyans.

2.10 Glutathione - S-transferase

The Glutathione –S- transferases (GSTs) superfamily belongs to the group of phase II enzymes that facilitate the detoxification of various carcinogens, therapeutic drugs, environmental toxins and products of oxidative stress (Strange

et al., 2001). Polymorphisms in the GSTs have the potential to alter an individual's susceptibility to carcinogens and toxins, and influence the toxicity and efficacy of drug treatment (Hayes and Strange, 2000). So far, eight distinct classes of the soluble cytoplasmic mammalian GST have been identified including alpha, kappa, mu, omega, pi, sigma, theta, and zeta (Strange et al, 2001). *GSTM1* is one of the genes encoding the mu class of enzymes located on chromosome 1p13.3 and contains 10 exons (Okcu *et al.*, 2004). They are believed to exert protective effects against numerous diseases ranging from cancer through to neurodegenerative disorders (Kearns *et al.*, 2003) In addition, they are involved in maintaining prostaglandin E_2 (PGE_2) regulated neurophysiologic functions (Beuckmann *et al.*, 2000). The theta class of GST enzymes is encoded by the *GSTT1* gene, which is mapped to chromosome 22q11.23 and contains six exons (Daniel, 1993).

The *GST* genes are highly polymorphic and frequently inducible. Among the numerous *GST* genes, *GSTM1* and *GSTT1* genes have been extensively examined in association with risk of cancer (Strange *et al.*, 2001) and clinical outcomes in cancer patients (Yang *et al.*, 2005). The most common variant of the *GSTM1* and *GSTT1* genes is the homozygous deletion (null genotype), which has been associated with the loss of enzyme activity and increased vulnerability to cytogenetic damage (Norppa, 2004). Reports show that the frequency of the *GSTM1* null genotype (*GSTM1**0) is 53% in Caucasians, 27% in African-American subjects, and 53% in Asians. On the other hand, the frequency of the *GSTT1* gene deletion (*GSTT1**0) is 20% in Caucasians, 47% in Asians and 40% in Africans (Garte *et al.*, 2001).

In the liver, GSTs may protect against hepatitis B virus related liver injury, which is partly manifested as extensive oxidative DNA damage (Hagen *et al.*, 1994). GSTs, in particular *GSTMl* also play an important role in detoxifying reactive metabolites of AFB1 (Liu *et al.*, 2003). Therefore, it has been proposed that the decreased production of *GSTM1* and *GSTT1* in the null genotypes is associated

with an increased hepatocellular carcinoma (HCC) risk in the presence of hepatitis B virus infection and/or aflatoxin exposure.

2.11 Population Genetics

Individuals within a population may differ from each other due to inherited genetic variation. The frequency of alleles in a population may be influenced by several factors such as natural selection, random genetic drift and interlocus sequence exchange. Natural selection is a process whereby genotype - environmental interactions occur at the phenotypic level. This may lead to differential survival and reproductive success of individuals and hence the modification of the gene pool of a population. Through natural selection, a single mutant may become the most dominant in the population if it is advantageous to the individuals carrying it. Random genetic drift are the changes in allele frequency that occur by chance and cause rapid changes in small populations but has little effect in large ones. On the other hand, sequence exchange can occur between different gene copies in a gene family that encodes essentially the same product and may result in one type of repeat increasing in population frequency (Strachan and Read, 1997). This process seemed to have been responsible for the origin and spread of many genes encoding the xenobiotic metabolizing enzymes (Gonzalez and Nebert, 1990). The generation of novel genes enabled populations to utilize new sources of plants and to better adapt to changes in the environment.

Genetic distance is a way of measuring the amount of evolutionary divergence in two separated populations of a species. It is determined by counting the number of allelic substitutions per locus that have cropped up in each population. Genetic distances are used to measure the global genetic differences between populations. Fig 2.5 depicts an example of a phylogenetic tree of human populations based on archeological, genetic and linguistic data (Cavalli- Sforza *et al.*, 1994).

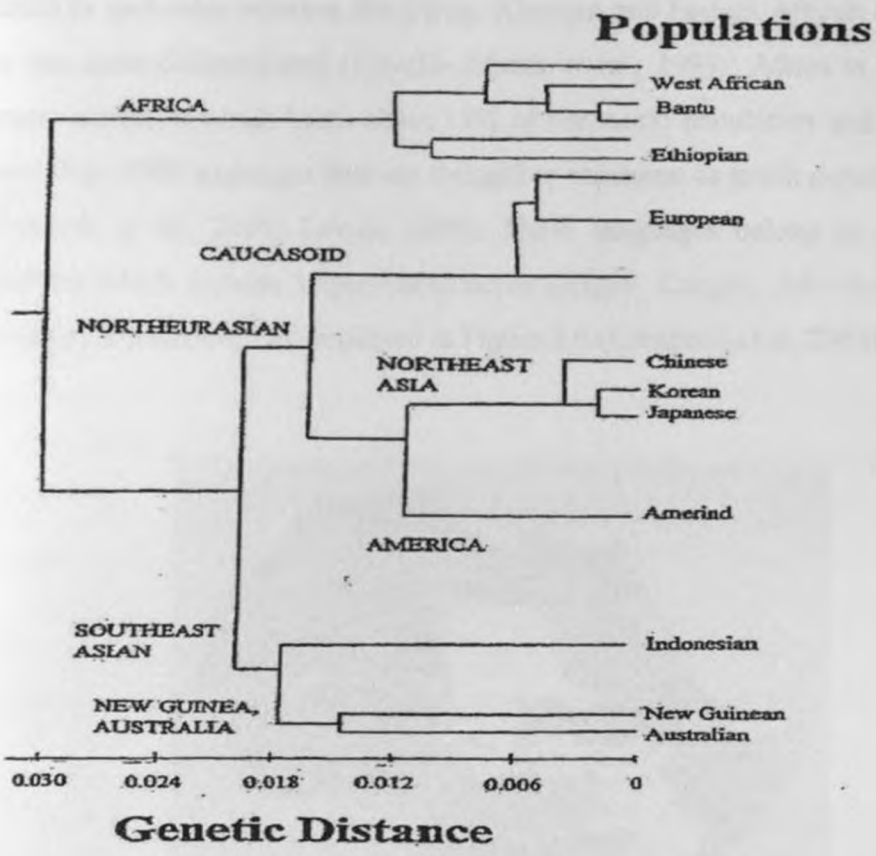


Figure 2.4: An example of a phylogenetic tree of human populations

Adapted from Cavalli- Sforza *et al.*, (1994).

2.11.1 Ethno - linguistic groups of Africa

It is postulated that the first human species (*Homo sapiens)* originated from Africa from where they spread to other parts of the world (McDougall *et al.*, 2005). Presently, the inhabitants of Africa include Aboriginal and Caucasoid groups in the north down to the southern border of the Sahara and Negroids in sub-Saharan Africa. The population of sub-Saharan Africa has been greatly affected by the Bantu expansion that originated near the confluence of the Niger and Benue rivers. Linguistic and genetic studies show that most sub-Saharan populations are closely

related to each other whereas the pigmy, Khoesan and Eastern African populations are the most differentiated (Cavalli- Sforza *et al.*, 1994). Africa is the second largest continent which hosts about 15% of the world population and is home to more than 2000 languages that are thought to represent as much genetic diversity (Tishkoff *et al.*, 2009; Lewis, 2009). These languages belong to four major families which include Niger-Kordofanian (Niger- Congo), Afro-Asiatic, Nilo-Saharan, and Khoesan as displayed in Figure 2.6 (Campbell et al, 2008).

Figure 2.5: The six main language families of Africa

Adapted from Campell et al (2008).

The Niger-Kordofanian (Niger-Congo) languages are spoken in western Africa, eastern Africa, central Africa, and southern Africa and include the Bantu languages. The Afro-Asiatic language family includes languages spoken in

northern, central, and eastern Africa such as Cushitic, Chadic, Semitic, and ancient Egyptian. The Nilo-Saharan language family is spoken in central and eastern Africa and includes the Sudanic and Nilotic languages. The Khoesan language family includes languages that contain click consonants and is spoken by hunter–gatherer populations in eastern (Hadza and Sandawe) and southern Africa [southern African Khoesan (SAK)]. It is the most contentious of the African language families because there is so much divergence among the Hadza, Sandawe, and SAK languages (Nurse, 1997;Sands, 1998; Tishkoff *et al.*, 2007).Linguistic barriers strengthen genetic isolation between groups speaking different languages, hence, people speaking the four major language families are thought to be genetically different. Reports show that Africans are genetically more diverse compared to Asians and Caucasians (Wooding *et al.*, 2002).

Presently, Africa is believed to be the ancestral homeland of modern humans (Jorde *et al.*, 1998). Mitochondrial DNA markers indicate that eastern Africa was the origin of both African and Eurasian expansion as well as an early exit route for *Homo-sapiens* from Africa (Tishkoff and Williams, 2002). Hence, studies on the levels and patterns of genetic diversity among ethnically different eastern African populations are deemed necessary. It is envisaged that such studies are likely to improve the understanding of evolutionary history and the genetic basis of phenotypic variation. Complex disease genes mapped in these populations by association between SNPs and disease phenotypes could have important implications for the understanding of genetic risk factors for many ethnic groups (Tishkoff and Williams, 2002).

2.11.1.1 Kenyan ethno – linguistic groups

Kenya is a country of great ethnic diversity being made up of over 42 ethnic groups speaking over 69 dialects (Lewis, 2009). Kenyan populations may be divided into three major ethno-linguistic groups, namely, the Bantu (67%), the

Nilotes (30%) and the Cushites (3%). Ethnologists believe the Kenyan Bantus originated from West Africa together with the other African Bantu groups during the great Bantu expansion. Their language belongs to the Niger-Kordofanian (Niger-Congo) family of African languages (Fig 2.6). In Kenya, the Kikuyu are the dominant Bantu tribe who inhabit mainly the central highlands and form about 20% of Kenyan population. The other major ethnic group in Kenya is the Nilotes who occupy the shores of Lake Victoria and the Great Rift Valley. Their language belongs to the Nilo-Saharan family of African languages (Fig 2.6).

The Nilotes are divided into three linguistic sub-groups which include the Eastern Nilotic group such as the Maasai (Plains Nilotes), the Southern Nilotic group such as the Kalenjin (Highland Nilotes) and the Western Nilotic group made up of the Luo (River- Lake Nilotes) (Greenberg, 1963; Creider, 1989). According to ethnologists, linguistics and oral history, the Nilotes originated from Chad and Sudan regions from where they spread to Uganda, Kenya and Tanzania (Ogot, 1967; Collins, 2006). The Luo are the fourth largest ethnic group in Kenya forming 13% of Kenyan population. They traditionally inhabit the shores of Lake Victoria (Herbich, 2002). On the other hand, the Maasai are a numerically minor ethnic group in Kenya (2.1%) but is culturally distinct and has been the focus of intense cultural tourism (ILO, 2000). According to oral history and archeological records, the Maasai originated from southern Sudan. They moved southwards displacing other ethnic groups as well as assimilating others such as the southern Cushites (Collins, 2006). They settled in a long tract of land stretching from northern Kenya to northern Tanzania.

2.11.2 Genetic characteristics of some Kenyan ethno - linguistic groups

Eastern Africa came to the attention of archeologists and anthropologists after the discovery of the ancient skeleton belonging to the *Australopithecus afarensis* dating 3-4 million years in Ethiopia (Johanson and Taib, 1976)and in Kenya (Brown *et al.*, 2001). Studies investigating the diversity of autosomal DNA and Y-

chromosomal DNA of Nilotic people of Eastern Africa have been reported. The three most populous southern Sudanese Nilotes (Dinka, Nuer and Shilluk) were reported to be characterized by the predominance of Y-haplogroups, A3b2 (53%), B (30%), and E1b1b (17.0%) (Hassan *et al.*, 2008). Another study analyzing human Y-chromosomal DNA in Nilotic populations including the Maasai (Kenya), Luo (Kenya) and Alur (Democratic Republic of Congo (DRC) reported that the predominant Y-DNA haplogroup in each of the three populations was different. For example, the Y-DNA haplogroup, E1b1b1-M35 was the most frequent among Maasai (50%), E1b1a-P1 was the most frequent among Luo (6/9 = 67%) whereas E2a-M41 was the most frequent among the Alur (67%) (Wood *et al.*, 2005). Furthermore, a study by Tishkoff *et al.*, (2007)tested a sample of 35 Datooga males, and reported that the E1b1b1-M35 was the most frequent Y-DNA haplogroup among them (54.3%).

In this present study, SNPs of genes encoding drug metabolizing enzymes were investigated in a sample of the general population of Kenyans drawn from two of the three main ethno-linguistic groups of Kenya, namely, the Bantu and the Nilotes. In addition, selected SNPs were characterized in Kenyan HIV and psychiatric patients and clinical implications assessed in various treatment covariates. Studies on the variation of genes encoding Drug metabolizing enzymes are deemed to have played an important role in the adaptation and survival of *homo sapiens*. Hence an investigation into their polymorphisms may reveal new insights into the genetic diversity of Kenyan populations and the interplay between genes and disease.

2.12 Methods for studying genetic polymorphism

Traditional methods of detecting genetic polymorphisms of drug metabolizing enzymes are based on observing differences between individuals or populations in terms of the gene encoding the enzyme (genotype) or function of the enzyme (phenotype). In *in-vivo* phenotyping studies, a probe drug is administered to

subjects and the metabolism of the drug is assessed by determination of parent drug to metabolite ratios (MR) in the urine, saliva or blood. Methods that enable the phenotyping of several CYPs using a cocktail of probe drugs have been described (Stewart *et al.*, 2011; De Bock *et al.*, 2012). Alternative methods include determination of CYP enzyme protein levels by immunological methods, CYP mRNA levels measured by various nucleic acid hybridization methods or by reverse transcription coupled with polymerase chain reaction (RT-PCR). In genotyping studies, mutations are traditionally detected by use of polymerase chain reaction (PCR) methods, restriction fragment length polymorphism (RFLP) analysis or single - strand conformation polymorphism (SSCP) analysis followed by direct sequencing. Genetic mutations characterized include deletion of a whole gene, point mutations within genes, deletion or insertions of fragments of DNA within genes and multiple copies of genes.

SNP genotyping technology has progressed to include DNA microarray technologies such as the Affymetrix DMET Plus (Dumaual *et al.*, 2007; Deeken, 2009). An important advantage of genotyping is the fact that a patient's genotype needs to be determined only once for any given gene, because, except for rare somatic mutations, it remains stable for a life time.

Chapter Three

EXPERIMENTAL

3.1 Genotyping methods

3.1.1 Introduction

Genotyping is a procedure for the analysis of variations or polymorphism in a gene encoding a specific protein. It requires a single tissue sample such as blood, buccal swabs or saliva, often in small quantities. High throughput methods that are highly sensitive, inexpensive and require less sample manipulation are increasingly available. For example genotyping chips and real-time polymerase chain reaction (PCR) are being used for simultaneous genotyping of several CYP alleles. In this study, genotyping methods employed for the detection of SNPs included polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) and real-time PCR. Some of the genes were also sequenced for validation of PCR-RFLP methods. The genes studied included *CYP2B6, CYP2C19, CYP2D6, NAT2*, *GSTM1* and *GSTT1* (Table 3.1)

Table 3.1: Allelic variants of drug metabolizing enzymes in this study

Gene	Allele	SNP	Effect on protein	Effect on enzyme activity
CYP2B6	*6	516G>T	Q172H	Reduced activity
CYP2C19	*2 *3	681G>A 638 G>A	Splicing W212X	Null allele Null allele
CYP2D6	*4 *5 *17 *29	1846G>A deletion 1023C>T 3183G>A	Splicing Deletion T107I V136M& V338M	Null allele No enzyme Reduced activity Reduced activity
NAT2	*5 *6 *7 *14	341T>C, 590G>A 857G>A 191G>A	I114T R197Q G286E R64Q	Reduced Activity (Slow acetylators)
GSTM1	*0	Deletion	deletion	No enzyme
GSTT1	*0	Deletion	deletion	No Enzyme

3.1.2 Methodology

3.1.2.1 Materials and reagents

Equipments included a Biosafety cabinet (Biofuge15) (Heraus Sepatech), PCR System 2700 GeneAMP® (Applied Biosystems), UV-VIS Spectrophotometer UV-160 (Shimadzu), GelPhoto System GFS1000 (Fran Techtum lab, Sweden), Video Copy Processor P90E (Mitsubishi), Spectroline TVD-1000A (Spectroline Corp, Westbury, USA), pH Meter Scholar 425 (Scholar), Microcentriguge MSE Mistraal 2000 (Labassco), 7500 Fast Real-Time PCR System (Applied Biosystems), Nanodrop 2000C, UV-VIS - Spectrophotometer (Thermo Scientific)and Vortex Vibromix (ThermoDenley, Electron Corporation, United Kingdom).

Common laboratory reagents for buffer preparation and agarose gel electrophoresis were bought from local agents but were originally from Sigma Aldrich, Germany and included ethylene diamine tetraacetic acid (EDTA), sodium chloride, potassium chloride, disodium hydrogen phosphate, ammonium chloride, potassium bicarbonate, glycerol, propanol, ethanol, trizma base, magnesium chloride glacial acetic acid, Agarose, bromophenol blue, Ficoll, xylene cyanolFF, ethidium bromide and synergel.

Molecular Biology reagents included QIAamp DNA blood minikit (Qiagen Inc, Germany), Eppendorf Perfect gDNA Blood minikit (Eppendorf, Germany) DNA ladder , dNTPs (New England Biolabs), oliginucleotide primers (Roche molecular Biochemicals), DNA polymerase such as PlatinumTaq high fidelity DNA polymerese, Jumpstart AccuTaq DNA polymerase, Jumpstart, RedTaq DNA polymerase (Sigma Chemical CO, St Louis, USA), restriction enzymes (*BSTU*I, *FOK*I, *Sma*I, *Kpn*I, *Bsr*I, *Bam*HI, *Asp*718, *Taq*I, *Alu*I, *Msp*I) were from SureCut Restriction, PCR buffers (Roche molecular Biochemicals), TaqMan® Drug Metabolism Genotyping Assay reagents (Applied Biosystems):

3.1.2.2 Blood sample collection and storage

Blood samples were collected from study subjects by venopuncture into EDTA vacuvette glass tubes and stored in a refrigerator at – 20^0C awaiting processing and analysis. The blood samples were processed for long-term storage on filter paper and an aliquot was used for DNA extraction. A total of 365 blood samples were collected. A special code was generated that linked the source population to each sample for ease of archiving and retrieval.

3.1.2.3 Blood on Filter Paper

An aliquot of blood was spotted on 20cm x 3.5cm strip of 2mm Whatmann® filter paper for long-term storage. Strips of filter paper measuring 20.0cm x3.5 cm were cut and a space measuring 1.5cm marked from one end for the blood sample code. Six spots separated by a distance of 2.5cm were marked on the filter paper and

spotted with 40µl of blood. The strips were left to dry for 6 hours at 25^0C in a well aerated secure place. The dry filter strips were stored at room temperature in transparent plastic filing sheets in box files labeled according to the source population (Figure 3.1).

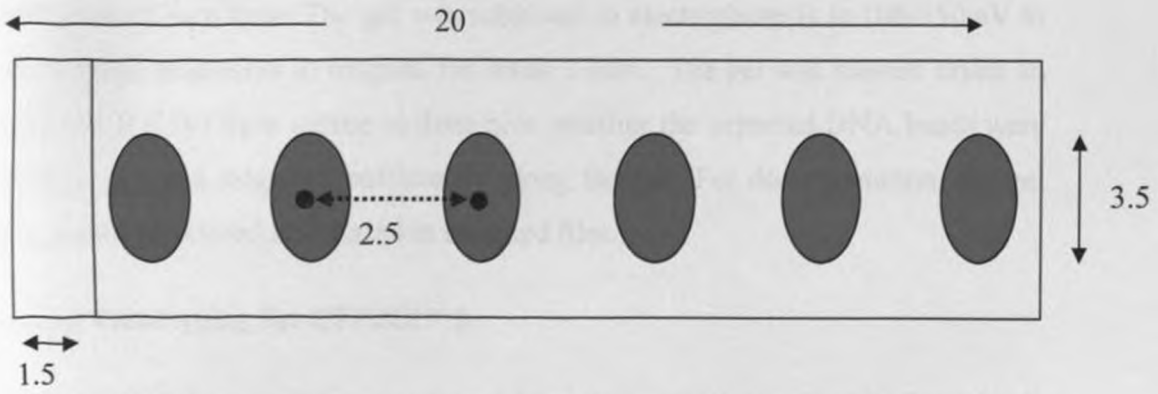


Figure 3.1: Blood impregnated on Filter Paper for long term storage

3.1.2.4 DNA Extraction

DNA was extracted from whole blood by the use of Eppendorf Perfect gDNA Blood Mini Kit (Eppendorf, Germany) for human and animal blood according to the manufacturer's protocol. The main components of the procedure included cell lysis by the use of proteinaseK. DNA purification by binding on spin columns, washing and final elution of the DNA with 200 uL of elution buffer. The extracted DNA was quantified by the use of UV- Vis Spectrophotometer.

3.1.2.5 Protocol for agarose gel electrophoresis

Agarose gel of 0.7% was used for the separation of DNA fragments >1 kb whereas 3.0% was used for the separation of DNA fragments up to 500bp. An appropriate amount of agarose powder was weighed for every 100 ml of gel solution prepared. The powder was mixed with TAE buffer in a volumetric glass flask and heated in the microwave to dissolve the agarose. The resultant gel was allowed to cool until tepid (about 55°C). Ethidium bromide was added to the cooled gel (10 mg/mL) and the gel poured onto a casting tray containing the required combs and allowed to cast at room temperature. Amplified DNA mixed with 2.0 µL of X10 loading dye was loaded on to the gel. DNA molecular weight marker was also loaded on both ends of each lane. The gel was subjected to electrophoresis at 100-150mV to allow DNA fragments to migrate for about 3.0cm. The gel was viewed under an ultra violet (UV) light source to determine whether the expected DNA bands were present and had migrated sufficiently along the gel. For documentation, the gel was scanned, printed and stored in assigned files.

3.1.2.6 Genotyping for *CYP2C19**2*

The genotyping procedure was adopted from the method described by De Morais *et al.*, (1994b) and optimized in our laboratory. A DNA fragment was amplified by PCR by the use of specific primers (Table 3.2) to generate a 169 bp product (Table

3.2).The PCR reaction mixture contained 1.0 µL of DNA incubated with 2.5 µL x10 PCR buffer (without $MgCl_2$), 6.4 µL dNTP mix (1.25 mM), 1.0 µL of 10 µM forward and reverse primers, 2.5µL $MgCl_2$ (25 mM), 0.7 µL Red Jumpstart DNA polymerase (1 U/µl) and 9.9 uL of water in a total reaction volume of 25 µL. Amplification was achieved on a PCR System 2700 GeneAMP® (Applied Biosystems). PCR conditions included initial denaturation step at 94^0C for 3 minutes followed by 35 cycles of three steps consisting denaturation at 94^0C for 10 seconds, annealing at 53^0C for 10 seconds and extension at 72^0C for 20 seconds respectively. A final extension step was carried out at 72^0C for 7 minutes. The amplified products were separated on a 3% agarose gel and viewed under UV light to confirm amplification. Details of primers and PCR conditions are shown in Table 3.2.

Table 3.2: Primers for CYPB6*6 and CYP2C19 *2 and CYP2C19*3 and RFLP

Allele	Primers	Annealing Temp	PCR pdt	RFLP	Fragment pattern
*CYP2B6*6* (516G>A)	F 5'-GGT CTG CCC ATC TAT AAA C-3' R 5'-CTG ATT CTT CAC ATG TCTG CG-3'	60^0C	526bp	*Bsr*I 60^0C	273+216+23bp(wt) 526 bp (mt)
*CYP2C19*2* (681G>A)	F 5'-AAT TAC AAC CAG GCT TGG C-3' R 5'-TAT CAC TTT CCA TAA AAG CAA G-3'	53^0C	169bp	*Sma*I 25^0CC	120 +49 bp (wt) 169 bp (mt)
*CYP2C19*3* (636G>A)	F 5'-TAT TAT TAT CTG TTA ACT AAT ATG A-3' R 5'-ACT TCA GGG CTT GGT CAA TA-3'	53^0C	329bp	*BamH*I 25^0C	233 + 96 bp (wt) 329 bp (mt)
329bp	*CYP2C19* wt- 5'-GCC TTA CCT GGA TT-3' *CYP2C19* mut- 5'- GCC TTA CCT GGA TC - 3'	50^0C	247bp		247 bp (wt) 247 (mt)

3.1.2.7 *CYP2C19* *2 genotype analysis by RFLP

RFLP analysis of amplified DNA products was achieved by digestion with the restriction enzyme *Sma*I at 25^{0}C according to the method ofDe Morais *et al.*, (1994a). The digestion mix consisted of 15 µL PCR product, 2.0 µL of X10 buffer A, 0.5 µL the restriction enzyme *Sma*I and 2.5 µL of water in a total reaction volume of 20 µL. The digestion reaction was carried out at 25^{0}C for 8 hours to ensure complete digestion and accurate interpretation of final banding patterns. The digested products were separated on 3% agarose gel and visualized under UV light. Details of expected fragment profiles are as shown in Table 3.2. A typical gel picture is shown in Figure 3.2 whereas an electropherogram showing nucleotide sequence at *CYP2C19**2 is shown in Appendix 7g.

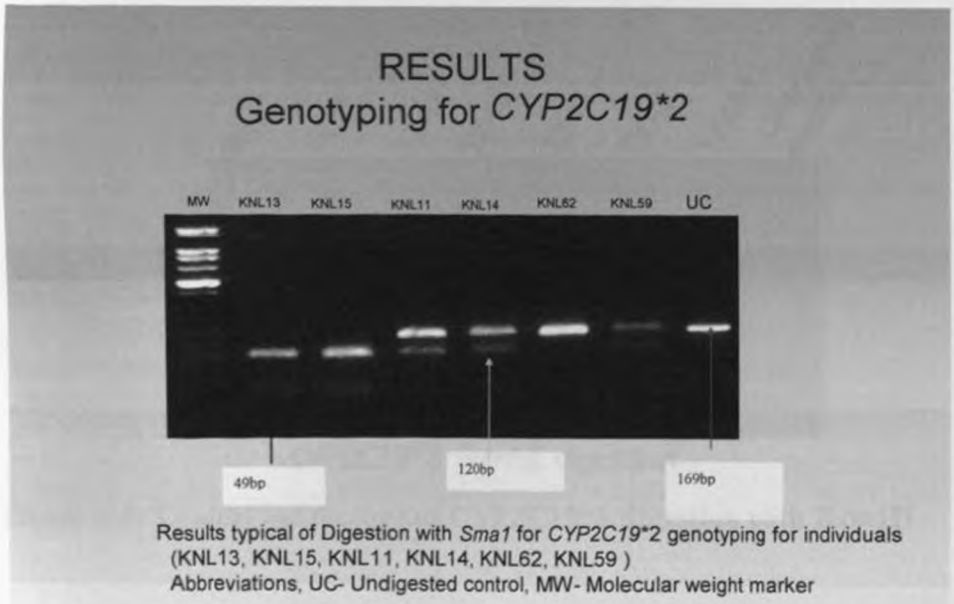


Figure 3.2: A typical gel picture of CYP2C19*2 digestion pattern with SmaI

3.1.2.8 Genotyping for *CYP2C19**3

Genotyping for this allele was performed by a two-step allelic - specific PCR method described De Morais *et al.*,(1994b). The first PCR reaction produced a 329 bp fragment covering exon 4 and the polymorphic site. Subsequently, an allele specific PCR reaction was carried out using 0.5uL of the product from the first PCR as the template. Allele specific amplification was achieved by the use of primers CYP2C19*wt* or *CYP2C19mut* annealing at 50^{0C} to produce a PCR product of 247 bp (Table 3.2). The products were separated on 2% agarose gel. Further analysis of some of the samples using the restriction enzyme *BamHI* RFLP method of De Morais *et al.*, (1994b) was undertaken to validate the allelic specific PCR

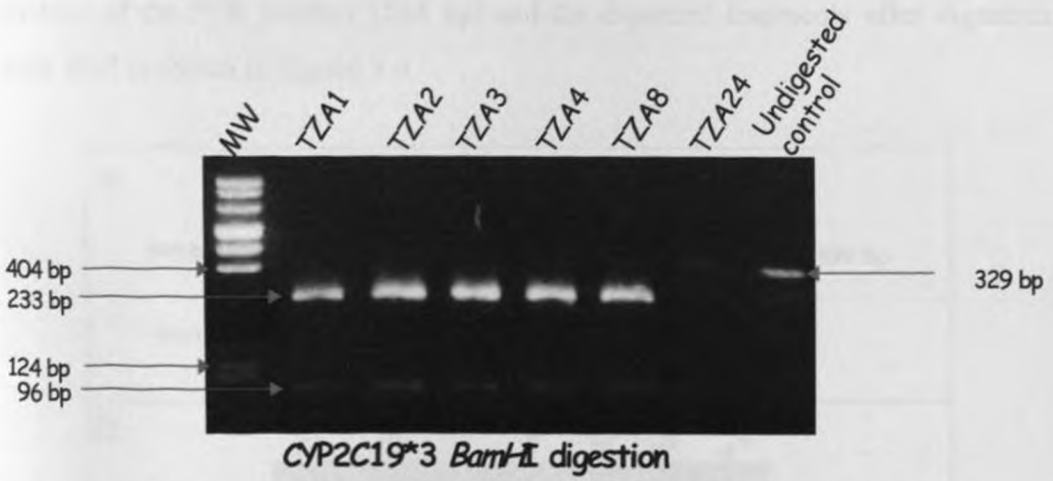


Figure 3.3: Typical gel picture of CYP2C19*3 digestion with BamHI

3.1.2.9 Genotyping for *CYP2B6**6 (516G>A) by PCR - RFLP

Amplification of DNA containing a fragment with *CYP2B6* *6 (516G>T was done according to the method described by Klein *et al.*, (2005). One microlitre of DNA

was incubated in a PCR mixture containing 1.5 µL X 10 thermopol buffer, 1.2 µL dNTPs (2.5mM), 0.6 µL of forward and reverse primers (10 µM), 0.6 µL of $MgCl_2$ (25 mM), 0.1 µL of NEB Taq polymerase (10U/ml) and 9.3 µL of water in a total reaction volume of 15 µL. Amplification was achieved on a PCR System 2700 GeneAMP® (Applied Biosystems). PCR cycling conditions consisted of 35 cycles of denaturation at 94^0C for 20s, annealing at 60^0Cfor 20s and extension at 72^0C for 60s. Details of primers, cycling conditions and PCR product are shown in Table 3.2.

Amplified products were subjected to RFLP analysis. A 10.0 µL sample of the PCR product was incubated with 0.1µL of the restriction enzyme *Bsr*I (10 000 U/ml), 1.5 µL X10 buffer and 3.4 µL of water in a total reaction volume of 15 µL at 60^0C for 8 hours. The digested products were loaded on a 3% agarose gel and visualized under UV light for genotype determination (Table 3.2). A typical gel picture of the PCR product (526 bp) and the expected fragments after digestion with *Bsr*I is shown in Figure 3.4.

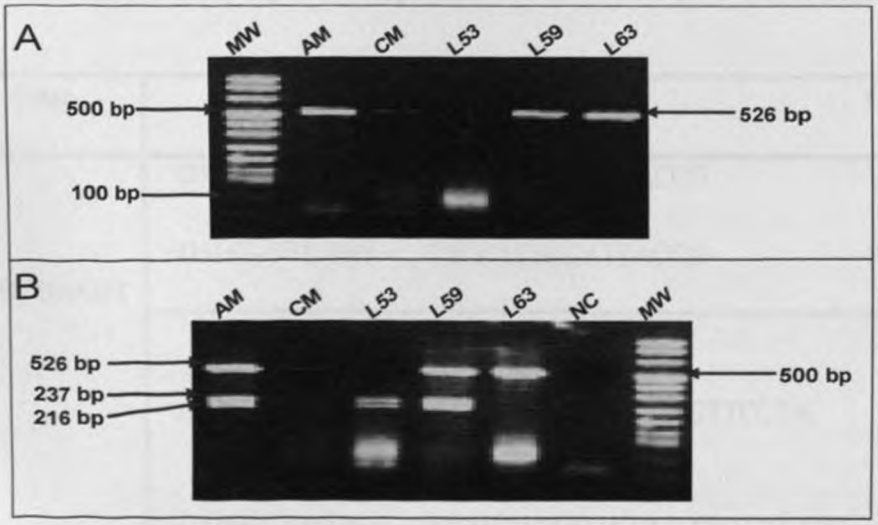


Figure 3.4: Typical picture of CYP2B6*6 amplification (A) and digestion with BsrI (B)

3.1.2.10 Genotyping for *CYP2B6* 516G>T by Real-Time PCR

CYP2B6 516G>T genotyping was also performed by a Real - Time PCR using the TaqMan® Drug Metabolism Genotyping Assay protocol (Applied Biosystems) as adapted from the one described by Mahungu T *et al.*, (2009). The first assay component was the Drug Metabolism Genotyping Assay Mix containing sequence specific primers and probes as shown in Table 3.3. The second one was the TaqMan Universal PCR Master Mix (No AmpErase UNG) containing AmpliTaq Gold DNA polymerase. Genomic DNA was quantified by the nanodrop system and diluted with an appropriate amount of DNase free water to produce a final DNA concentration of 20 ng/mL.

For a 96 well plate of 25 µL reaction volume, samples for each well contained DNA test samples diluted with DNase free water to make 11.25 µL (20ng DNA) and a PCR reaction Mix containing 12.50 µL X20 TaqMan Universal PCR master Mix (No AmpErase UNG) and 1.25 µL X20 Drug Metabolism Genotyping

Table 3.3: Primers and probe sequence for CYP2B6G>T Real-Time PCR assay

Gene	Allele	Probe sequence	Color
CYP2B6 516G>T	G516T-GTOT	TTCCAGTCCATTACCG	VIC
	G516T-GTOTM1	TTCCATTCCATTACCG	FAM
		Primer sequence	
	G516T-GTOTF	CTTGACCTGCTGCTTCTTCCTA	
	G516T-GTOTR	AGACGATGGAGCAGATGATGTTG	

VIC® - Green proprietary probe dye (Applied Biosystems)

FAM® - Blue non - proprietary probe dye

Assay Mix. Two wells of non-template controls (NTCs) containing only DNase free water and one well of a positive control was always included in every run. The plate was sealed with an optical adhesive cover and swirled to remove air bubbles and loaded onto Applied Biosystems 7500 Fast Real-Time PCR system. PCR cycling conditions included, activation at 95^0C for 10 min followed by 50cycles of denaturation at 92^0C for 15sec then annealing and extension at 60^0C for 90 sec. After the PCR amplification, Allelic discrimination plate read and analysis was performed using a sequence detection system (SDS) based on fluorescence values from each well according to the instrument manual.

The correlation between fluorescence signals and sequences in a sample was based on the intensity of fluorescence. A substantial increase in only the VIC dye fluorescence indicated homozygozity for allele 1, substantial increase in only the FAM dye fluorescence indicated homozygozity for allele 2 whereas a substantial increase in both VIC and FAM fluorescence indicated both allele 1 and allele 2 for heterozygozity. The alleles were then converted to genotypes.

3.1.2.11 Genotyping for *CYP2D6*

A polymerase chain reaction/restriction fragment length polymorphism-based genotyping strategy (PCR-RFLP) was employed according to the method described by (Gaedigk *et al.*, 1999). The first step involved performing a long-range PCR to specifically amplify the *CYP2D6* gene and discriminate it from the pseudogene *CYP2D7*. Subsequently, a series of nested PCR amplifications were performed in order to detect the variant alleles including *CYP2D6**2, *CYP2D6**4, *CYP2D6**5, *CYP2D6**17, and*CYP2D6**29. The resultant PCR products were subjected to restriction enzyme digestion and the resulting DNA fragments were analyzed by agarose gel electrophoresis. Assignment of genotypes was done by interpretation of the banding patterns observed.

3.1.2.11.1 Long-Range PCR for *CYP2D6* gene

DNA samples for amplification were selected together with negative and positive controls. A sample of 1.0 µL genomic DNA was incubated with a PCR reaction mix containing of 2.6 µL AccuTaq LA buffer, 6.0 µL dNTPs (2.5 mM), 1.0 µL of forward and reverse Primers(10 mM), 2.0 µL $MgCl_2$ (25mM), 0.7 µL DMSO (15%), 1.2 µL Jumpstart Redaccqutaq LA DNA polymerase (1U/µL), and 4.5 µL of water in a total reaction volume of 20.0 µL. Amplification was achieved on a PCR System 2700 GeneAMP® (Applied Biosystems). PCR reaction conditions consisted of initial denaturation step at 94^0C for 3 minutes followed by 34 consecutive cycles of denaturation at 94°C for 20 seconds, annealing at 58°C for 20 seconds, extension at 72°C for 10 minutes. Final extension was done at 72 °C for 15 minutes. Details of primers used and expected banding patterns are shown in Table 3.4. The PCR product was analyzed by agarose gel electrophoresis. A three microlitre of the long-range PCR product was loaded on 0.7% agarose gel together with 1kb DNA molecular weight marker. The fragments were viewed under UV transilluminator to confirm amplification of the *CYP2D6* gene. A typical gel picture of a long range PCR product is shown in Figure 3.5

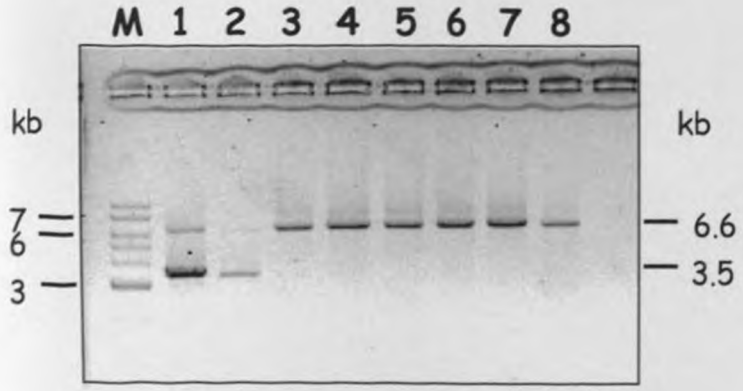


Figure 3.5: A representative gel of CYP2D6 long-range PCR

3.1.2.11.2 Nested PCR for *CYP2D6* Allelic variants

Genotyping for the various *CYP2D6* allelic variants including *CYP2D6**2, *CYP2D6**4, *CYP2D6**5, *CYP2D6**17 and *CYP2D6**29 was carried out by a method adopted from the algorithm developed by (Gaedigk *et al.*, 1999). Samples to be amplified were selected together with negative and positive controls. A sample of 0.8 µL long PCR product was incubated with a PCR reaction mixture containing 0.8 µL X10 PCR buffer (+$MgCl_2$), 0.5 µL dNTPs (2.5 mM), 0.15 µL of both forward and reverse primers (10uM), 0.06 µL Taq polymerase (1U/ml), and 5.54 µL of water in a total reaction volume of 8.0 µL. Amplification was achieved on a PCR System 2700 GeneAmp® (Applied Biosystem). Thermocycler program for nested PCR consisted of an initial denaturation at 94°C for 2 minutes followed by 34 consecutive cycles of denaturation at 94°C for 10 seconds, annealing at 58°C for 10 seconds, extension at 72°C for 20 seconds and final extension at 72°C for 6 minutes. Details of primers used for each specific allele and expected fragments are shown in Table 3.4. The resultant PCR products were separated on 3% agarose gel and viewed under UV light to confirm amplification after which they were subjected to RFLP analysis.

Table 3.4: Primers for CYP2D6 genotyping and RFLP fragment pattern

Gene/Allele	Primers	annealing Temp	PCR pdt (bp)	RFLP	Gel pattern
CYP2D6 Whole Gene	F 5'CCA GAA GGC TTT GCA GGC TTC AG-3' R 5'ACT GAG CCC TGG GAG GTA GGT AG-3'				6.6kb
CYP2D6 *5 (deletion)	F 5'CAC CAG GCA CCT GTA CTC CTC AG-3' (2D7 BP) R 5'CAG GCA TGA GCT AAG GCA CCC AGA C-3'	68^{0}C			3.5kb
CYP2D6 *2 (4180G>C)	F 5' AGG TGA GAG TGG CTG CCA CGG TGG-3' R 5' GAT GGG CTC ACG CTG CAC ATC-3'	62^{0}C	345bp	*Fsp*1 37^{0}C	345 wt 235 + 110 mt
CYP2D6 *4 (1846G>A)	F 5' AGA GGC GCTTCT CCG TGT CCA-3' R 5' CAG AGA CTC CTC GGT CTC TCG-3'	58^{0}C	394bp	*BstN*1 60^{0}C	194+161+37bp(wt) 355+37 bp (mt)
CYP2D6 *17 (1023C>T)	F 5' GTC GTG CTC AAT GGG CTG GCG GCC GTG CGC GAG GCG-3' R 5' GGC GAG GAC ACC GCC GAC CGC CCG CCT GTG CCC AGT A-3'	58^{0}C	254bp	*Fok*1 37^{0}C	180 + 74 bp (wt) 254 bp (mt)
CYP2D6 *29 (3183G>A)	F 5' TAT GGG CCA GCG TGG AGC GAG CAG AGG CGC TTC CGC-3' R 5' AGA TGC GGG TAA GGG GTC GCC TTC C-3'	58^{0}C	213bp	*BstU*1 60^{0}C	178 + 35 bp (wt) 213 bp (mt)

3.1.2.11.3 Genotyping for CYP2D6 alleles by RFLP

A sample of the nested PCR product for each of the *CYP2D6* alleles was subjected to RFLP analysis. An eight microlitre sample of the amplified product was incubated for 8 hours at temperatures specific for each of the restriction enzymes(Table 3.4) in a digestion master mix containing 0.9 µL X10 buffer, 0.25 µL restriction enzyme (10U/mL) and 6.86 µL of water in a total reaction volume of 16µL. The digested products were analyzed by agarose gel electrophoresis. The resultant banding patterns were viewed under a UV transilluminator, scanned and documented for genotype determination. Details of the restriction enzymes, incubation temperatures and fragment patterns are shown in Table 3.4. Typical gel pictures of digestion patterns for *CYP2D6**17 and *CYP2D6**4 are shown in Figure 3.6 and Figure 3.7 respectively.

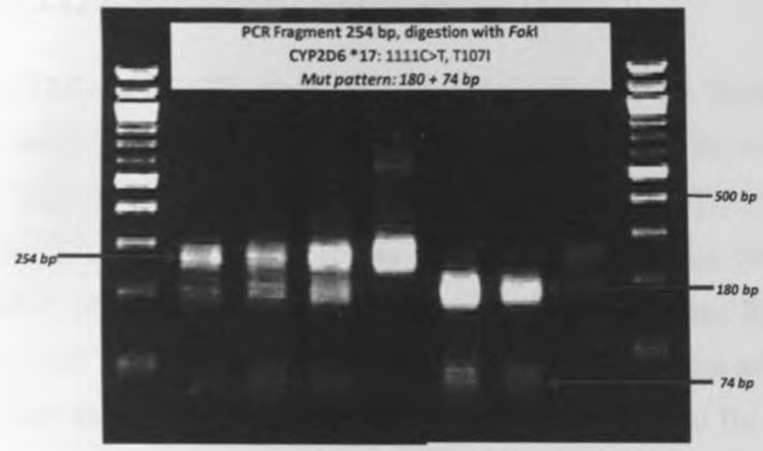


Figure 3.6: Typical gel picture of CYP2D6*17 after digestion with FokI

Electropherograms showing sequence patterns at *CYP2D6**17 and *CYP2D6**29 are also shown in Appendix 7a and Appendix 7b respectively.

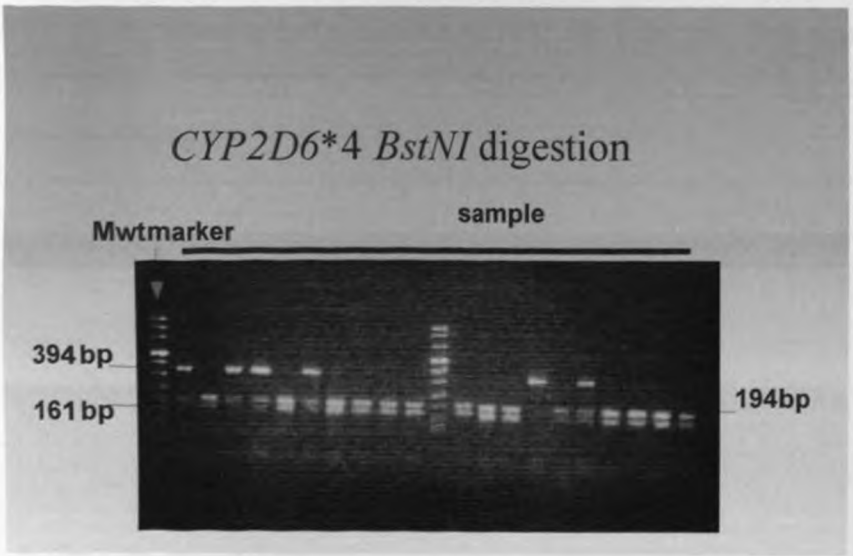


Figure 3.7: A typical gel picture of CYP2D6*4 digestion pattern with BstNI

3.1.2.12 Genotyping for *GSTMI* and *GSTTI*

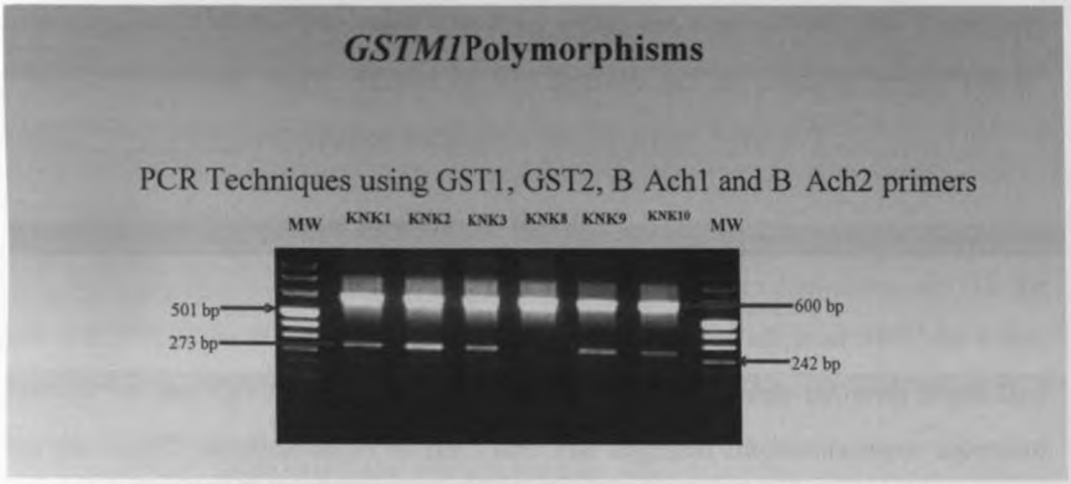
GSTMI and *GSTTI* were genotyped according to the method described by of (Dandara *et al.*, 2002). Amplification was achieved by the use of specific primers (Table 3.5). To determine the *GSTM1**0*0 and *GSTTI**0*0 polymorphism (deletion polymorphism) it was necessary to include the beta–actin primers (B-AcH1 and B-AcH2) which gives rise to a 600 base pair fragment as a positive control. The presence of the 600 bp fragment from beta-actin in the absence of either the 273 bp fragment for GSTM1 or the 480 bp for GSTT1 respectively signified the deletion allele. A sample of 1.0 uL of DNA was incubated in a PCR reaction mix containing 2.0 µL X10 PCR buffer (+$MgCl_2$), 2.0 µL dNTP mix (2.5 mM), 0.7 µL of both forward and reverse Primers (10 µM), 0.625 µL of both B-AcH1 (10 µM) and B-AcH2 (10 µM), 0.5 µL Taq DNA polymerase (2.5U /µL) and 13.3 µL water for a total reaction volume of 20.0 µL.

The amplification was achieved on a PCR System 2700 GeneAMP® (Applied Biosystems) with reaction conditions that included initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 10 seconds, annealing at 55°C for 10 seconds and extension at 72°C for 1 minute. This was followed by a final extension at 72°C for 7 min. Details of primers and fragment patterns are shown in Table 3.5

Table 3.5: Primers for *NAT2, GSTM1* and *GSTT1* PCR, RFLP and fragment patterns

Gene/allele	Primers	annealing Temp	PCR pdt	RFLP	Gel pattern
				Kpn I- NAT2*5	660 +433 bp (wt) 1093 bp (mt)
NAT2	N4 - TCTAGCATGAATCACTCTGC N5 - 5'-GGAACAAATTGGACTTGG	57°C	1093 bp	*Taq* I- NAT2*6	380+317+226+170 bp (wt) 396 bp (mt)
				Bam HI- NAT2*7	811+282 bp (wt) 1093 bp
				Msp I /*Alu* I- NAT2*14	759 + 189 wt 759 +280
GSTM1 *GSTM1*0/*0*	F 5'-CTG CCC TAC TTG ATT GAT GGG-3' R 5'-CTG GAT TGT AGC AGA TCA TGC-3'	53°C			273 bp (wt) + 600 bp no pdt + 600 bp
B-Ach 1 *B-Ach* 2	5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3' 5-'CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3'				600 bp
GSTT1 *GSTT1*0/*0*	F 5'-TTC CTT ACT GGT CCT CAC ATC TC-3' R 5'-TCA CCG GAT CAT GGC CAG CA-3'	56°C			480 bp (wt) no pdt + 600 bp

The PCR products were analyzed on 2% agarose gel and the resultant banding patterns viewed under UV light and documented for genotype determination. A typical gel picture of *GSTM1* polymorphism is depicted in Figure 3.8. It shows that samples for individuals KNK 1, 2, 3, 9 and 10 all carry the GSTM1*1 allele and that KNK 8 has a deletion (GSTM1*0/*0).. It is however not possible to determine which individuals would be heterozygous as the 273 bp fragment will still be present. Hence, this method was able to determine the homozygous deletion (null allele) but not able to discriminate between the heterozygous allele and the homozygous wild type.



Results typical of GST-M1 genotyping carried out on a 2 % agarose gel. Internal primers as control – beta Actin 1 and beta Actin 2 to obtain 600 bp band. The GST primers give a 273 band. GSTM1*1 is distinguished from GSTM1*0 by presence of 273 bp fragment

Figure 3.8: A typical gel picture showing GSTM1 deletion

3.1.2.13 Genotyping for *NAT2* allelic variants

Genotyping for *NAT2* allelic variants namely, *NAT2*5, NAT2*6, NAT2*7* and *NAT2*14* was done by a PCR-RFLP method adopted from that described by (Dandara *et al.*, 2003). Primers specific to the *NAT2* gene were used in the PCR reaction to generate a 1093 bp product which was subsequently subjected to RFLP with four restriction enzymes *Asp*718,*Taq*I, *Bam*HI and *Msp*I for the identification of *NAT2*5* (481 C>T), *NAT2*6* (590 G>A), *NAT2*7* (857G>A), *NAT2*14* (191G>A) respectively. A 1.0 µL DNA sample was incubated in a PCR reaction mix containing 2.5 µL x 10 PCR buffer (+$MgCl_2$), 2.0 µL dNTP Mix, 1.0 µL of both the forward and reverse primers, 0.7 µL Red jumpstart DNA polymerase and 16.8 µL of water in a total reaction volume 25 µL. Amplification was achieved on a PCR System 2700 GeneAMP® (Applied Biosystems). PCR conditions consisted of the initial denaturation at 94^0C for 3 minutes, followed by 34 cycles of denaturation at 94^0C for 20 seconds, annealing at 550C for 20 seconds and

extension at 72^0C for 1 minute. The final extension was at 72^0C for 7 minutes. The PCR products were viewed on 2% agarose gel to confirm amplification. Details of primers and reaction conditions are shown in Table 3.5

3.1.2.13.1 *NAT2* genotype analysis by RFLP

The PCR product was digested with four separate enzymes including *Asp*718 for the *NAT2**5 allele at 37°C for 2 hrs; *Taq*I for the *NAT2**6 allele at 56°C for 4 hrs; *Bam*HI for the *NAT2**7 allele at 37°C for 2 hrs; and a double cut with *Msp*I/*Alu*1 for the *NAT2**14 allele at 37°C for 2 hrs. The digested fragments were separated on 2% agarose gel for *NAT2**5, *NAT2**7, *NAT2**14 alleles and 3% agarose gel for *NAT2**6 allele and visualized under UV light to assign genotypes according to the digestion profile. The wild type allele, *NAT2**4 was assigned by exclusion of the four variant alleles (Hein et al, 2008). Details of fragment patterns seen on gel electrophoresis are shown in Table 3.5. Electropherograms showing nucleotide sequence for *NAT2** 5, *NAT2**6, *NAT2**7 and *NAT2**14 are shown in Appendix 7c- 7f.

3.2 HPLC method for the determination of nevirapine levels in plasma

3.2.1 Introduction

The biggest challenge in pharmacokinetic studies in patients undergoing HAART is the large number of additional drugs the patients are exposed to. For successful determination of nevirapine in plasma of patients on combination drug therapy, a selective analytical method is needed. Nevirapine has been assayed using high pressure liquid chromatography (HPLC) with ultraviolet detection (Kappelhoff *et al.*, 2003; Silverthorn and Parsons, 2006), or with tandem mass spectrometry (Chi *et al.*, 2003). Some of these methods are not adaptable in resource limited settings. In this study, a HPLC method for the determination of nevirapine plasma levels

with ultra violet detection and liquid-liquid sample extraction was adopted and validated. The chromatographic method was adopted from the one described by (Minzi and Ngaimisi, 2010). Method validation was done at the University of Nairobi - African Institute of Biomedical Science and Technology (UoN-AiBST) Biomedical analytical collaborative laboratory situated at the Department of Pharmacology and Pharmacognosy of the School of Pharmacy, in collaboration with the Government Chemist Laboratories.

3.2.2 Instrumentation

Mixing of reagents was done using a Vortex Vibromix from ThermoDenley, Electron Corporation, United Kingdom. Centrifugation was carried out using a microcentrifuge machine from Biofuse, Germany. Purification of water by reverse osmosis was carried out using Sartorius Water System machine Arium, United Kingdom. All materials were weighed using an analytic balance form Shimadzu Corporation, Tokyo, Japan.

The HPLC system consisted of a Shimadzu LC system from Shimadzu Corporation, Tokyo, Japan. It was made up of quaternary pumps (solvent delivery module LC-20AT prominence, Shimadzu) fitted with an online degasser (DGU-20A3/20AS prominence, Shimadzu). The eluent was monitored by a variable wavelength (200-800 nm) ultraviolet-visible spectrophotometric detector (SPD-20A/SPD-20AV prominence) fitted with an 18µL flow cell. The system was fitted with an on-line auto-sampler, SIL-20A/20AC and a column oven (CTO-10ASVP (15 – 60°C)). The stationary phase was reverse phase silica gel (C-18) of 5µm particle size in a stainless steel column (Phenomenex, Gemini, USA) 250 x 4.6 mm i.d. It was fitted with a guard column (Phenomenex, Gemini, USA). Control of chromatographic processes and peak integration was done using Labsolutions Release 1.22 SPI software.

3.2.3 Chemicals and reagents

Chemical structures of nevirapine and carbamazepine (IS) are shown in Figure 3.9. Pure reference standards of Nevirapine (NVP), zidovudine (AZT), stavudine (d4T), lamivudine (3TC), trimethoprim (TMP) and sulphamethoxazole (SM) were kind donations from the National Quality Control Laboratory (NQCL) which is the laboratory of the regulatory body, Pharmacy and Poisons Board of Kenya. Carbamazepine reference standard for use as internal standard (IS) was generously donated by Universal Corporation (K) Ltd. HPLC grade solvents and analytical grade chemicals were purchased from Sigma Aldrich (Darmstadt, Germany) and BDH Company (Poole, England) respectively. The solvents included methanol, acetonitrile and acetone. Drug free pooled plasma was obtained from the National Blood Transfusion Service (Kenya) whereas de-ionized water which was purified by reverse osmosis was obtained NQCL. Analytic grade sodium bicarbonate, sodium carbonate, potassium dihydrogen orthophosphate were obtained from Riedel-de Hach-Sigma Aldrich Chemie, Gmbh Germany.

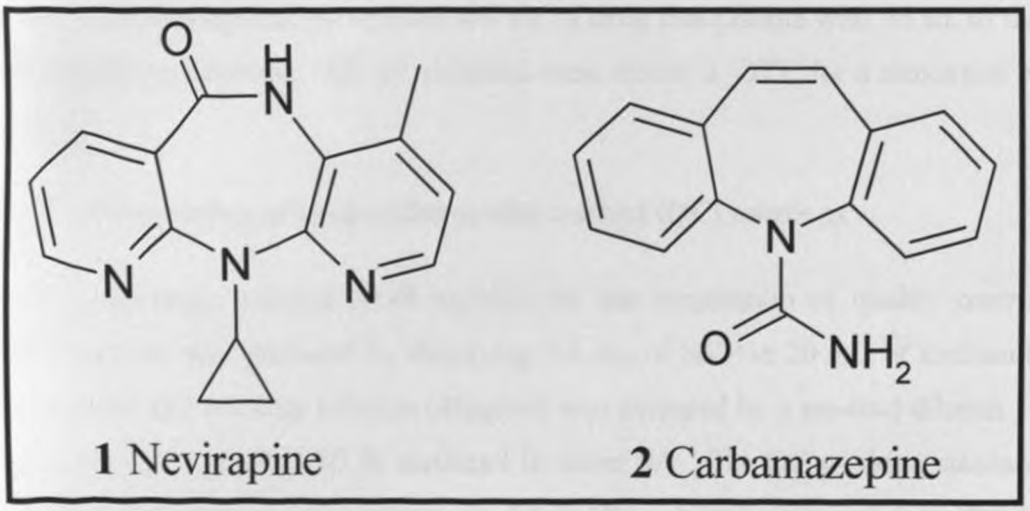


Figure 3.9: Chemical structures of Nevirapine (1) and Carbamazepine (2)

3.2.4 Preparation of solutions

3.2.4.1 Preparation of buffer solutions

Carbonate buffer pH 9.8 was prepared by dissolving 8.4g of sodium hydrogen carbonate and 10.6g of sodium carbonate in 500 mL of water. The pH was adjusted to 9.8 using sodium hydroxide (30% w/v) solution. Phosphate buffer pH 7.5 (0.2M) was prepared by dissolving 27.22 g of potassium dihydrogen orthophosphate in 930 mL of water. The pH of the solution was adjusted to 7.5 with potassium hydroxide solution (30% w/v).

3.2.4.2 Preparation of nevirapine standard solutions

Nevirapine (NVP) stock solution (0.86 mg/mL) was prepared by dissolving 17.2 mg of nevirapine in 10 mL of methanol. The solution was sonicated for 5 minutes and diluted to 20 mL using de-ionized water. A total of 12 nevirapine working solutions were prepared with concentrations ranging from 1.7 to 86 µg/mL by appropriate dilutions of the stock solution. Nevirapine calibration standard solutions were prepared by spiking 450 µL of drug free plasma with 50 µL of the NVP working solutions. All the solutions were stored at -20°C for a maximum of 7 days.

3.2.4.3 Preparation of nevirapine quality control (QC) solutions

Nevirapine stock solution (0.48 mg/mL) for the preparation of quality control (QC) samples was prepared by dissolving 9.6 mg of NVP in 20 mL of methanol. Nevirapine QC working solution (48µg/ml) was prepared by a ten-fold dilution of the stock solution using 50 % methanol in water v/v. The QC working standard solution was used to spike plasma to obtain QC solutions designated as: QC-H (4.8µg/mL); QC-M (2.4) µg/mL and QC-L (1.2 µg/mL) respectively.

3.2.4.4 Preparation of carbamazepine (CBZ) solutions

Carbamazepine (CBZ) was used as the internal standard (IS). Carbamazepine stock solution (1.16 mg/mL) was prepared by dissolving 23.2 mg of CBZ in 20 mL of methanol. Two CBZ working solutions were prepared by separately diluting 0.1 and 0.3 mL of the CBZ stock solution to 1 mL. The resultant concentration of the CBZ working solutions was 11.6 and 34.4μg/mL respectively.

3.2.5 Sample pre-treatment

Prior to analysis, all frozen plasma samples were thawed to room temperature. Sample clean - up was achieved by liquid-liquid extraction under basic conditions. An aliquot of 100 μL of spiked plasma (QC samples or calibration standards) and plasma from study subjects was transferred into a 2 mL microcentrifuge tube. Twenty five microliters of IS (CBZ) solution was added and mixed gently. For plasma samples whose NVP concentration was above 5.4 μg/mL, the concentration of the IS solution used was 34.4μg/mL. For plasma samples whose NVP concentration was less than 5.0 μg/mL, the concentration of IS solution used was 11.6 μg/mL.

Twenty microliters of carbonate buffer pH 9.8 was added to the mixture and vortexed for 20 seconds. The drugs were then extracted from the plasma by the addition of 500 μL of ethyl acetate followed by vortexing for 5 minutes. The mixture was centrifuged at 10000 g for 15 minutes to separate the organic layer. Subsequently, 350 μL of the upper organic layer was carefully transferred into a glass test tube and evaporated to dryness at room temperature with a gentle stream of nitrogen gas. The dried residue was reconstituted with 120 μL of methanol and transferred to vial inserts and placed in the autosampler tray for injection into the LC column.

3.2.6 Chromatographic conditions

Separation of the analytes was achieved on a Phenomenex C18 (250 X 4.6 mm id, 5μm particle size) reversed phase analytical column (Gemini, USA) maintained at ambient temperatures. The injection volume was 90 μL and detection was done at 282 nm. The mobile phase consisted of acetonitrile and phosphate buffer at pH 7.5 in the ratio of 6:4. The flow rate of the mobile phase under isocratic conditions was 0.8 mL/min.

3.2.7 Validation procedures

The LC method was validated according to the FDA guidelines for validation of bioanalytical assays (FDA, 2001).

3.2.7.1 Calibration Curve

Two types of calibration curves were constructed. The first curve was used for the analysis of samples with high NVP concentrations ranging from 1.72 to 10.32μg/ml. The second curve was used for the analysis of samples with low NVP concentrations of less than3.44 μg/mL. The calibration solutions were prepared by spiking 450 μL of drug free plasma with 50 μL NVP working solution. The spiked plasma samples were extracted by liquid-liquid extraction as previously described followed by chromatographic analysis. Two QC samples (low and high) were always included in each analysis. The resulting peak area ratios of the analyte and were plotted versus the nominal NVP concentrations.

3.2.7.2 Linearity of the calibration curve

Three calibration curves were obtained from analysis of calibration plasma samples on three separate days. Linearity was determined by linear regression analysis without weighting. The regression equation (slope, intercept, coefficient of correlation) was documented. Nevirapine concentration in unknown samples was calculated from the best-fit equation ($y = mx + c$), where y is the peak area

ratio. From the parameters of the regression line, the concentrations of nevirapine calibration solutions were recalculated. The calibration curve was accepted if not more than 75 % of relative deviations (RD) of the calculated concentrations from the nominal values did not exceed ± 15 %.

3.2.7.3 Lower limit of quantitation (LLOQ)

The LLOQ was determined from the calibration curve for the quantitation of nevirapine concentrations less than 3.44µg/mL. The lower limit of quantitation was the highest concentration at which the relative deviation for the recalculated calibration solution was consistently greater than 20 %.

3.2.7.4 Precision and accuracy

The precision of the analytical method was determined by analyzing QC samples with nevirapine concentrations at the lower limit of quantification (LLOQ) and in low, medium and high concentration ranges of the calibration curves. For each concentration, 6 runs were carried out on the same day to determine intra-day precision. Inter-day precision was determined from 6 replicate determinations of NVP concentrations carried out on 6 different days. Precision was expressed as the standard deviation (SD) and coefficient of variation (CV) of the calculated concentrations from the nominal values. The precision of the method was accepted to be adequate if the maximum coefficient of variation (CV) was not greater than 15%. At the lower limit of quantitation (LLOQ), a maximum CV of 20 % was accepted.

The accuracy of the analytical method was evaluated to determine the closeness of the values obtained by the method to the nominal concentrations of nevirapine in QC samples. The QC samples were analyzed against the calibration curve, and the obtained concentrations were compared with the nominal values. The accuracy was reported as the percent relative deviation (RD) from the nominal value.

Accuracy was evaluated for the values of QC samples obtained within the same day (inter-day accuracy) and on different days (inter-day accuracy). The accuracy of the method was deemed acceptable when the maximum percentage relative deviation from the nominal value was ± 15 %. At least two thirds of the QC samples at each concentration were expected to fulfill this acceptance criterion. At the LLOQ, a maximum relative deviation of ± 20 % was accepted.

3.2.7.5 Extraction recovery

Recovery represents the extraction efficiency of a method. To determine the extraction recovery of the method, a quality control (QC) spiked plasma sample containing nevirapine with a NVP concentration of 9.6µg/mL was prepared. A solution of NVP in water with the same concentration as the QC standard was prepared using the procedure used for the preparation of QC standards. The spiked plasma QC samples were extracted using the sample pre-treatment procedure. The plasma and water based samples were subjected to chromatographic analysis. Peak areas from extracted drugs and directly injected NVP standard solutions were compared in order to determine the extraction recovery using Equation 3.1. Six replicate determinations were carried out.

Equation 3.1: Calculation of Extraction Recovery

$$\text{Percentage recovery} = \frac{\text{Mean Peak Area of the Extracted spiked QC plasma standard}}{\text{Mean Peak area of the Unextracted QC standards.}} * 1.37 * 100$$

3.2.7.6 Selectivity

Selectivity of the analytical method was evaluated with regard to interference by drugs that are commonly used by HIV patients and other endogenous substances.

Stock solutions of drugs commonly used by HIV/AIDS patients on HAART were prepared by separately dissolving 23.2 mg of each of drugs in 20 mL of methanol. Working solutions of the drugs were prepared by separately diluting 0.3 ml of the respective stock solutions to 1.0 mL to obtain a final concentration of 34.4µg/mL. One hundred microliters of the working solutions was separately used to spike 500 µL of blank plasma. Twenty microliters of carbamazepine were added to aliquots of 100µL of the spiked plasma which was then subjected to liquid-liquid extraction and chromatography as previously described.

Six QC samples whose NVP concentration were at LLOQ and ULOQ were also extracted and assayed as above. Selectivity was evaluated by calculating the percentage relation of the area of the interfering peak in comparison to the mean peak area found at LLOQ at the retention time of nevirapine. If there was a peak present at the retention time of nevirapine, then the area should not have been greater than 20 % of the mean peak area for nevirapine at LLOQ.

In addition, six blank plasma samples were extracted and analyzed. At least five of the six different blank plasma samples should have fulfilled the above criterion. The chromatographs were acceptable if there was good separation of interfering drug peaks from peaks associated with the internal standard and nevirapine. The percentage interference was calculated using Equation 3.2.

Equation 3.2: Calculation of interference at the retention time of Nevirapine

% Interference = area of the interfering peak*100 / mean area of NVP at LLOQ

3.2.7.7 Carryover

Six plasma QC samples of nevirapine with concentrations at the upper limit of quantitation (ULOQ) (17.2 µg/ml) were extracted and assayed as described

previously. Immediately after running each sample, a blank sample of methanol was run. Percentages carryover was calculated using Equation 3.3.

Equation 3.3: Calculation of sample Carryover

Carry over (%) = Peak at retention time of NVP / Mean peak area at LLOQ (n=6)

Any carryover from a sample at ULOQ should have presented a response that is not greater than 20 % of the mean peak area found for NVP at LLOQ and a response that is not greater than 2 % of the mean peak area found for the internal standard. If the response was more than 20 % for NVP at LLOQ response, carryover should not have been greater than 1 % of ULOQ, but at most half the peak area of the LLOQ sample.

3.2.7.8 Influence of hemolysis

The influence of hemolysis was determined by calculating the percentage relation of the mean peak area ratio of the quality control samples with hemolysis in comparison to the mean peak area ratio of the quality control samples without hemolysis.

Hemolysed plasma was prepared from freshly collected blood. A 100 mL sample of freshly collected blood was centrifuged and the upper layer of plasma transferred into an epperndorf tube. A total of 6 samples of plasma with 1% hemolysis were prepared by suspending 10 µL of packed red blood cells into 990 µL of plasma and hemolysis was induced by freezing the sample at -20°C followed by thawing.

Spiked plasma QC samples without hemolysis (QC-M) and with 1% hemolysis (QC-M1) with a NVP concentration of 2.4μg/mL were prepared. One hundred microliter aliquots of each of these QC samples were extracted and assayed as previously described. A total of six replicate determinations were made. For each QC-M1sample (1% hemolysis), the mean peak area ratio (to 5 decimal places) and the relative deviation from that of the QC-M sample without hemolysis was calculated. The peak areas of samples were compared using the unpaired Student t-test.

3.2.7.9 Bench top and Freeze-Thaw Stability of Nevirapine Plasma Samples

Two types of stability were evaluated: freeze-thaw stability and bench top stability. Spiked QC NVP plasma samples were stored frozen at minus twenty degrees centigrade and thereafter subjected to three freeze–thaw cycles. At the end of the cycle, the samples were analyzed and the peak areas compared to those of the freshly analyzed plasma samples. Six replicate determinations were done. The ratio of the peaks areas of the freshly analyzed and frozen samples were documented as percentage values and compared using the unpaired Student t-test. Similarly, bench top stability was determined from the peak area ratio of QC samples that had been left standing on a bench at ambient temperatures for 24 hours.

3.2.8 Analysis of samples from study subjects

The validated method was adopted for the determination of nevirapine levels in the plasma of HIV patients who had been stabilized on a nevirapine based HAART for not less than six months.. Patient samples were processed as described above. Every analytical run of patient samples also included a blank sample (processed plasma sample without analyte and IS); a zero sample (processed plasma with IS), calibration standards at a minimum of 6 concentration levels and the 3 levels of

QC samples (low, medium and high) in duplicate. The concentration of nevirapine in patient samples expressed in "µg/mL" were calculated using Equation 3.4

Equation 3.4: Calculation of concentration for nevirapine from the calibration curve

$$X = \left(\frac{Area1}{Area2} - a\right)/b$$

X = Unknown concentration, Area1= Peak area of nevirapine, Area2=Peak area of CBZ (IS), a = intercept, b=slope

3.2.9 Results

3.2.9.1 Chromatography and detection

A number of modifications were made to the method described by (Minzi and Ngaimisi, 2010). Liquid-liquid extraction of the plasma samples was done using ethyl acetate instead of di-isopropyl ether. This is because ethyl acetate was more readily available and less costly. In addition, there was less loss of solvent due to evaporation during the sample treatment process.

Separation of the analytes was achieved on a Phenomenex C18 (250 X 4.6 mm id, 5µm particle size) reversed phase analytical column of similar specifications as the Microsorb®, C18 column (Gemini, USA) used in the method described in literature. Furthermore, the elution of nevirapine in relation to the IS, concurrent drugs and endogenous substances was optimized with a higher proportion of, acetonitrile in phosphate buffer at 6:4 instead of 5:16. Hence, the retention times of nevirapine and carbamazepine were 3.687 and 5.217 minutes respectively. Representative chromatograms of plasma spiked with QC samples of nevirapine with IS is shown in Figure 3.10.

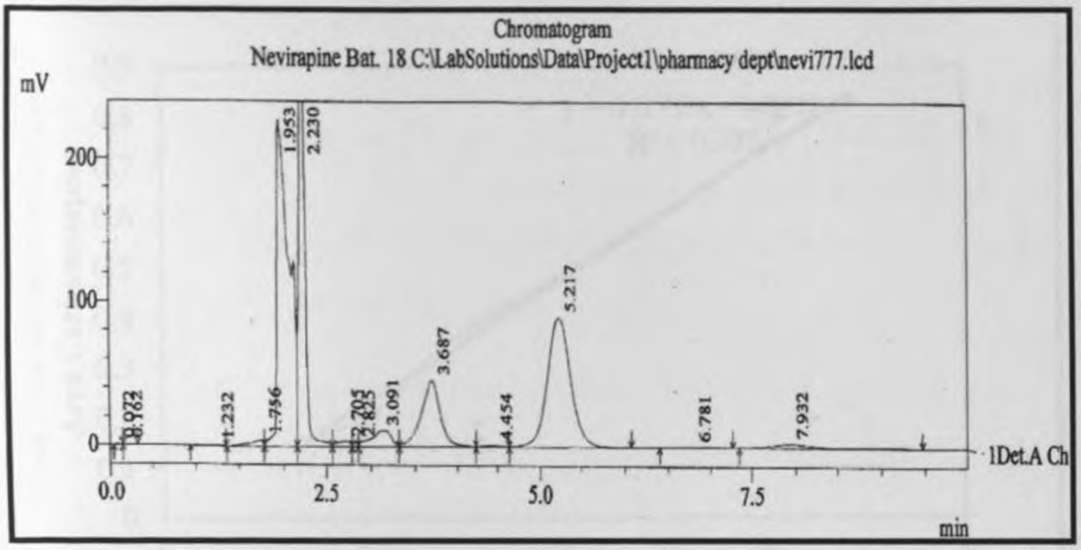


Figure 3.10 Representative chromatogram of QC sample with NVP and CBZ:

3.2.9.2 Linearity of calibration curve

Linearity was determined by linear regression analysis of the plot of the nominal nevirapine concentration against ratio of the peak areas of the NVP and CBZ peaks. Two calibration graphs were used: one for determination of concentrations of NVP above 3.44µg/ml and a second for the determination of concentrations below 3.44µg/ml. The two representative calibration curves are presented in Figures 3.11 and 3.12

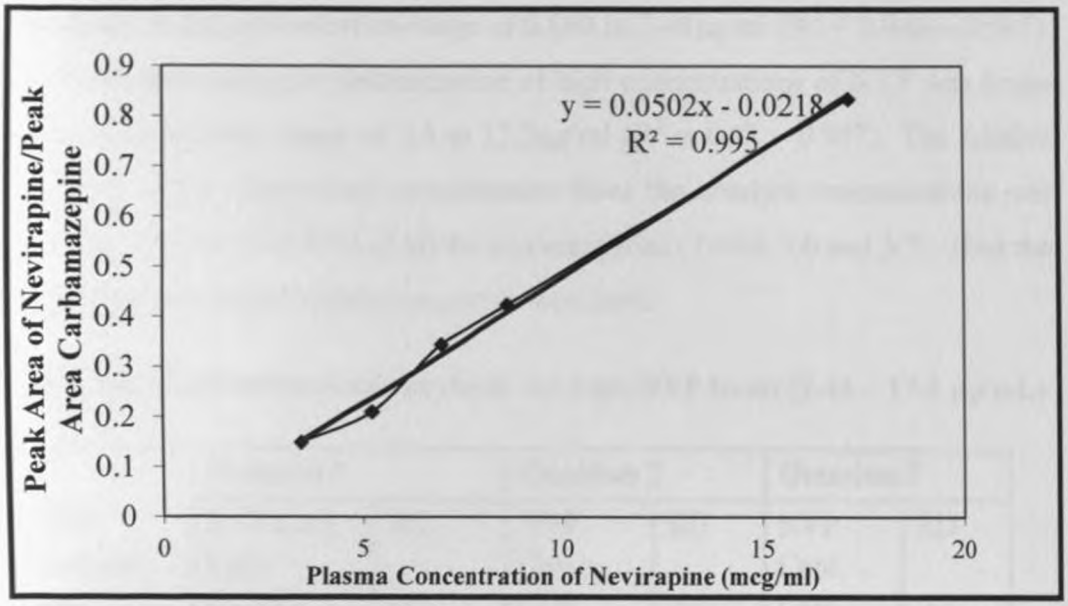


Figure 3.11: A calibration graph for NVP concentrations above 3.44µg/ml

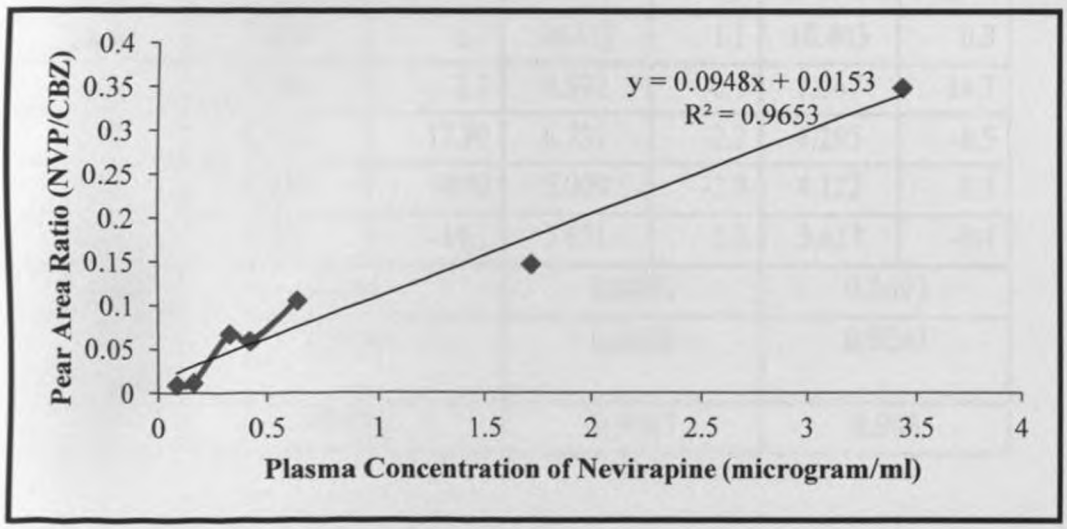


Figure 3.12: A calibration graph for NVP concentrations below 3.44µg/ml

The calibration concentrations were recalculated from the response for each calibration curve. Details of data generated for the calibration curves are shown in Table 3.6. The calibration curve for determination of low concentrations of NVP

was linear in the concentration range of 0.860 to 3.44µg/ml (R^2 = 0.940 – 0.965). The calibration curve for determination of high concentrations of NVP was linear in the concentration range of 2.4 to 17.2µg/ml (R^2 = 0.93 – 0.997). The relative deviation of the recalculated concentration from the nominal concentrations was less than 15% for over 80% of all the concentrations (Tables 3.6 and 3.7). Thus the acceptance criteria for calibration curves were met.

Table 3.6: Calibration concentrations for high NVP levels (3.44 – 17.2 µg/mL)

	Occasion 1		**Occasion 2**		**Occasion 3**	
NVP Nominal conc. (µg/mL)	NVP Conc. Calc. (µg/mL)	RD (%)	NVP Conc. Calc. (µg/mL	RD (%)	NVP Conc. Calc. (µg/mL)	RD (%)
17.2	17.066	-0.78	17.136	-0.37	16.958	-1.41
10.32	9.624	-6.7	10.437	1.1	10.403	0.8
8.6	8.794	2.3	8.592	-0.1	7.367	-14.3
6.88	8.112	17.90	6.731	-2.2	6.295	-8.5
5.16	4.928	-4.50	5.009	-2.9	4.122	0.1
3.44	2.955	-14.1	3.631	5.5	3.427	-0.4
X-coeff	0.064		0.0805		0.0693	
Y-intercept	0.1644		0.0588		0.0263	
R^2	0.9271		0.9963		0.993	

Table 3.7: Calibration concentrations for low NVP levels (0 to 3.44 µg/mL)

NVP nominal Concentration (µg/mL)	NVP/CBZ	RD%	NVP/CBZ	RD%	NVP/CBZ	RD%
3.44	3.308	-3.8	3.762	9.4	3.530	2.6
2.58			2.422	-6.1	2.571	-0.4
1.72	1.993	15.9	1.271	-26.1	1.401	-18.6
0.86	0.736	-14.4	0.834	-3.0	1.011	17.5
0.645	0.956	48.2	0.985	52.7	0.962	49.1
X coefficient	0.1889		0.186		0.2321	
Y intercept	0.0968		0.2277		0.2007	
R^2	0.969		0.9546		0.9401	

3.2.9.3 Lower limit of quantitation (LLOQ)

The lower limit of quantification (LLOQ) was the lowest concentration of nevirapine in a sample which could be quantified reliably with an acceptable accuracy and precision. The calibration curve for the determination of low NVP concentrations was used to determine LLOQ. From the results presented in Table 3.7, the LLOQ for nevirapine was 0.860µg/mL. At this value the relative deviation of the calculated concentration from the nominal concentration had a maximal value of 17.5%. At concentrations below this value, the relative deviation from the nominal value was consistently above the acceptable value of ± 20% (Table 3.7).

3.2.9.4 Precision and accuracy

Precision was derived by assessment of the coefficient of variation of replicate runs of QC. The intra and inter-day precision and accuracy were within acceptable

limits for the calibration curve for the determination of higher NVP concentrations (Tables 3.8 and 3.9 respectively).

Table 3.8: Intra-day accuracy and precision for NVP levels from the calibration curve (3.44 to 17.2 µg/ml)

RUN	QC-H (7.68 µg/ml)		QC-M (5.66 µg/ml)		QC-L (4.8 µg/ml)	
	Calculated Conc.	RD%	Calculated Conc.	RD%	Calculated Conc.	RD%
1	7.170	-0.9	5.0	-12.5	4.733	-1.4
2	8.036	4.6	5.0	-11.8	4.681	-2.5
3	8.224	7.1	5.7	0.08	4.719	-1.7
4	7.707	0.4	5.9	3.6	4.717	-1.7
5	7.368	-4.1	4.9	-13.6	4.733	-1.4
6	-	-	5.5	-2.2	-	-
Mean	7.68	1.42	5.66	-2.2	4.8	-1.7
SD	0.753		0.421		0.628	
CV	7.963		6.64		6.084	

Table 3.9: Inter-day accuracy and precision of NVP plasma levels from the calibration curve (3.44 to 17.2 µg/ml)

	QC-H (7.68 µg/ml)		QC-M (5.66 µg/ml)		QC-L (4.8 µg/ml)	
Run	Calculated Conc.	RD%	Calculated Conc.	RD%	Calculated Conc.	RD%
1	7.880	2.6	5.203	-8.1	4.180	-12.9
2	7.572	-1.4	5.395	-4.7	4.390	-8.5
3	7.170	-6.6	5.980	5.6	4.681	-2.5
Mean	7.541	-1.814	5.526	-2.373	4.417	-7.981
SD	0.356		0.405		0.252	
CV (%)	4.716		7.324		5.704	

Similarly, intra and inter-day precision and accuracy were within acceptable limits for the calibration curve for the determination of lower NVP concentrations (Tables 3.10. and 3.11) respectively.

Table 3.10: Intra-day accuracy and precision of NVP plasma levels from the calibration curve (0 to 3.44 µg/ml)

RUN	QC-H (2.4 µg/ml)		QC-M (1.2 µg/ml)		QC-LLOQ (0.860 µg/ml)	
	Calculated Conc.	RD%	Calculated Conc.	RD%	Calculated Conc.	RD%
1	2.63	9.78	1.26	5.02	0.81	-5.27
2	2.72	13.32	1.01	-15.88	1.00	15.78
3	2.19	-8.55	1.17	-2.56	0.80	-7.39
4	2.65	10.30	1.25	3.81	0.89	3.59
5	2.46	2.69	1.52	26.94	0.97	13.31
Mean	2.53	5.51	1.24	3.47	0.89	4.00
SD	0.21		0.19		0.09	
%CV	8.31		15.00		10.10	

Table 3.11: Inter-day accuracy and precision of NVP plasma levels from the calibration curve (0 to 3.44µg/ml)

RUN	QC-H (2.4 µg/ml)		QC-M (1.2 µg/ml)		QC-LLOQ (0.860 µg/ml)	
	Calculated Conc.	RD%	Calculated Conc.	RD%	Calculated Conc.	RD%
1	2.431	1.3	1.357	13.1	0.736	-14.4
2	2.262	-5.8	1.429	19.1	0.834	-3.0
3	2.360	-1.7	1.107	-7.8	1.011	17.5
Mean	2.35	-2.0	1.30	8.1	0.86	0.0
SD	0.09		0.17		0.14	
%CV	3.62		13.03		16.15	

3.2.9.5 Extraction recovery

The total recovery for nevirapine for the high QC concentration (7.68µg/ml) was 96%.

3.2.9.6 Selectivity and specificity

The chromatogram of blank pooled plasma is presented in Figure 3.13. Pooled plasma showed no interference from endogenous substances since there were no peaks at the retention times associated with NVP and CBZ.

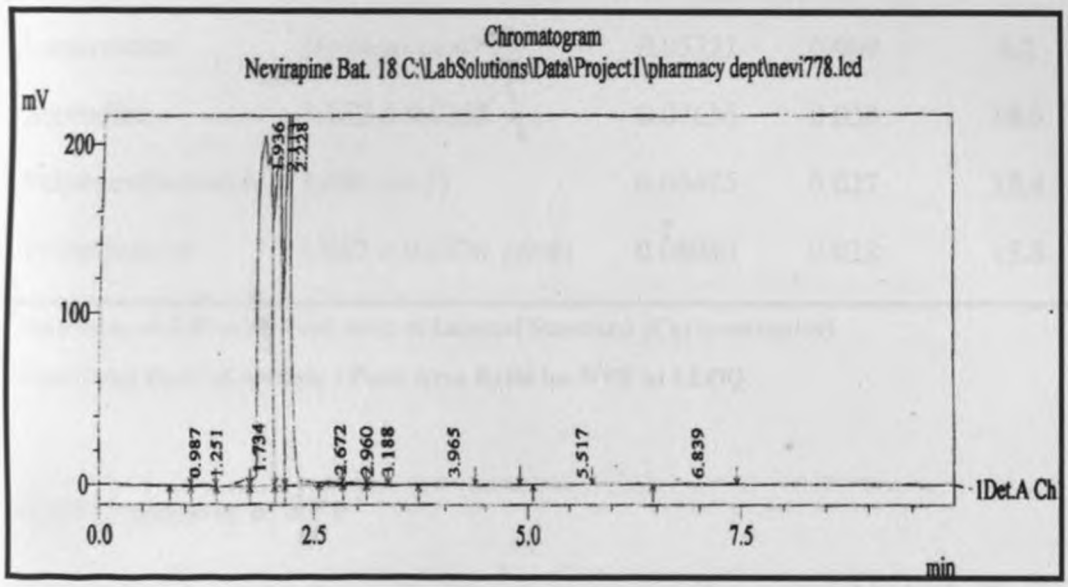


Figure 3.13: Chromatogram of pooled plasma

Data for the determination of interference by concurrent medications are shown in Table 3.12. Retention times for co-administered drugs were different from that of nevirapine or were not detected with the described analytical method. Lamuvidine did not show any peaks that could be attributed to these two drugs. Sulphamethoxazole, trimethoprim and AZT gave prominent peaks that had retention times of about 3.10 minutes. These peaks were clearly separated from

that of NVP and CBZ. The interference at the retention time of NVP was within acceptable levels.

Table 3.12: Degree of Interference by drugs commonly used by patients on HAART

Analyte	Retention Time of Analyte	Peak Area Ratio[a] N=3	SD	Interference[b] (%)
(LLOQ 0.86μg/mL)	**3.692 ± 0.0402 (n=20)**	**0.17759**	**0.014**	-
AZT	3.133 ± 0.0274 (n=4)	0.06645	0.026	**14.7**
Lamuvidine	No clear peak	0.05721	0.009	**5.2**
Stavudine	3.162 ± 0.0355	0.07636	0.033	**18.5**
Sulphamethoxazole	3.086 (n=1)	0.06476	0.027	**15.4**
Trimethoprim	3.067 ± 0.0476, (n=4)	0.08040	0.028	**15.8**

[a] **Peak Area at 3.69 min / Peak area of Internal Standard (Carbamazepine)**

[b] **Peak Area Ratio of Analyte / Peak Area Ratio for NVP at LLOQ**

3.2.9.7 Carryover of NVP

The data generated for the evaluation of carryover is shown in Table 3.13. From the results, there was no carryover of the IS since there was no peak at its retention time. However, there was carryover of NVP and the degree of carryover is presented in Table 3.13. The peak area of the carryover peak was 37.3% which was less than 50% of the peak area of NVP at LLOQ and about 1% the peak area at ULOQ. Therefore the acceptance criteria for carryover were met.

Table 3.13: Determination of Carryover effects

Replicate samples	Carryover Peak Area	LLOQ Peak Area	ULOQ Peak Area
1	37682	121944	5018732
2	14578	63655	3155215
3	30830	90565	3134523
4	61854	103065	3210982
5	61635	166530	2859502
6	37682		5132147
Mean Peak Area	40710.16667	109151.8	3751850.17
% Carryover		37.2968349	1.0850691

3.2.9.8 Influence of hemolysis

Results for the determination of the influence of hemolysis are shown in Table 3.14. There was no detectable influence of 1% hemolysis on the determination of nevirapine. The deviation in peak area ratio between non hemolysed plasma and those with 1% hemolysis was low at 4%.

Table 3.14: Determination of the influence of hemolysed plasma samples on nevirapine

	Peak Area Ratio	
Run	**Plasma without hemolysis**	**Plasma with1% hemolysis**
1	0.327527	0.344564
2	0.361288	0.346075
3	0.26372	0.387251
4	0.429168	0.383246
5	0.377651	0.368753
6	0.323956	0.36278
Mean	**0.347218**	**0.361192**
SD	**0.056108**	**0.018445**
diff		0.013973
RD		4.024332
t-test		**0.548657**

There was no statistically significant difference in the peak area ratios QC High sample obtained from plasma that was free of hemolysis and plasma that had hemolysed red blood cells.

3.2.9.9 Freeze Thaw and Bench Top Stability

Table 3.15 shows the influence of storage at minus twenty degrees centigrade on the stability of nevirapine in plasma samples. Results show that there was no significant difference between the concentration of nevirapine in freshly prepared samples and those that had been subjected to freeze – thaw cycle.

Table 3.15: The influence of storage conditions on the stability of nevirapine samples

Run	Bench Top Stability Samples	Freeze –Thaw Stability Samples	Freshly analyzed samples
1	17.082	13.332	15.144
2	15.314	17.168	16.584
3	16.221	15.957	14.992
4	11.251	17.874	19.478
5	16.246	16.727	17.281
Mean	15.223	16.211	16.696
%age of freshly Analyzed sample	91.2%	97.2%	100
SD	2.306436	1.753723	1.831
T-test	0.296	0.680	-

There was no statistically significant difference in the calculated concentrations of NVP between freshly analyzed samples and samples stored under various conditions. However, the concentration of NVP reduced by about 9.8% after 3 freeze-thaw cycles. It also reduced by about 2.8% after exposure to ambient conditions for 24 hours.

3.2.10 Discussion

The chromatographic system exhibited good separation for NVP and CBZ (IS) after injection of a standard solution and extracted spiked plasma samples. There was no interference from endogenous substances. Inspection of the chromatograms showed that the retention times of NVP and IS in spiked plasma samples and those obtained from patient samples corresponded well to those obtained from a standard solution. The calibration curves were linear in the concentration range of 0.645 to 17.32µg/ml. Intra and inter-day variations were within acceptable limits. The limit of quantitation for nevirapine was 0.86 µg/mL which was higher than that in the method described byKappelhoff *et al.* (2003). The latter study reported a LLOQ of 0.249 µg/ml. This could be due to differences in sample pre-treatment methods. It is possible that liquid-liquid extraction was less effective compared to protein precipitation as a method for sample pre-treatment.

Selectivity of the analytical method evaluated the interference by drugs that were commonly used by HIV patients in this study. The method was found to exhibit good specificity for the analyte and IS without any interference from endogenous substances. The degree of interference from the concurrent drugs tested was in the range of 14 – 19% which was less than 20% of the Peak Area Ratio of NVP at LLOQ (0.86µg/ml).The carryover effect was within acceptable limits. There was no carryover effect for the IS since there was no detectable peak at its retention time. On the other hand, the carryover effect recorded for nevirapine was within acceptable limits. This is because the carryover peak area was 37.3% which was less than 50% of the peak area of NVP at LLOQ and about 1% at ULOQ.

This study found that the concentration of NVP reduced by about 9.8% after 3 freeze-thaw cycles. This finding is comparable to studies in literature that reported a 7.9 % reduction in NVP concentration after 3 freeze-thaw cycles(Kappelhoff *et al.*, 2003). Unlike the findings of the later study, there was a 2.8% reduction in the levels of NVP after exposure to ambient conditions for 24 hours. The European

study (Kappelhoff *et al.*, 2003), reported a 10.6% decline in levels of NVP. This discrepancy could be due to differences in the ambient temperatures at the time of the local and European studies.

Chapter Four

CHARACTERIZATION OF THE GENETIC VARIANTS OF *CYP2B6, CYP2C19, CYP2D6, NAT2, AND GSTS* IN SELECTED POPULATIONS OF KENYA

4.1 Introduction

Available data suggest wide differences in allele specificity and frequencies between populations of varied ethnic origin. For example, a study investigating the frequency of reduced activity alleles of Catechol O-methyltransferase (COMT) in Caucasians, Asians and Kenyans (McLeod *et al.*, 1999) revealed that the frequency of these alleles was much lower in Kenyans (32%) compared to Caucasians (54%), and South-west Asians (49%). Maxwell *et al.*, (2005) evaluated the frequency of three clinically important human beta-2 adrenergic receptor (beta2AR) polymorphisms in 8 distinct ethnic populations (Chinese, Filipino, Southwest Asian, Saudi, Ghanaian, Kenyan, Sudanese, and European from Scotland). The results of that study showed that most haplotypes had a geographically different distribution pattern.

There is lack of data on the genetic polymorphism of CYP enzyme superfamily and other clinically important drug metabolizing enzymes in Kenyan populations, yet, most drugs used in clinical practice require CYP enzymes for phase I metabolism. In addition, most drugs used in developing countries such as Kenya are developed and clinically evaluated in Western countries. Local populations are subsequently exposed to these drugs without any regard to the inherent genetic variability between ethnically different populations. Furthermore, the population of a country such as Kenya is made up of three major ethno-linguistic groups which can be broken down to more than 42 different dialects envisaged to represent as much genetic diversity. This study was set up to investigate the

variability in genes encoding clinically relevant drug metabolizing enzymes in three ethno-linguistic populations in Kenya.

4.1.1 Drug metabolizing enzymes in this study

The enzymes included in this study were selected on the basis of their relevance to the metabolism of commonly used drugs in Kenya. The selected enzymes included CYP2B6, CYP2C19, CYP2D6, N-acetyltransferase2 (NAT2) and GSTs which metabolizes medicines listed in the WHO essential medicines list (EML) and Kenyan Standard Treatment Guidelines (STGs) for the therapy of common infectious diseases and chronic illnesses in Kenya (Table 4.1).

Table 4.1: Drug metabolizing enzymes selected for this study.

Enzyme	Drug substrate/Agent	Disease / condition
CYP2B6	Efavirenz	HIV & HIV - TB
	Nevirapine	HIV & PMCT
	Artemisinin	Malaria
	Cyclophosphamide	Cancer
CYP2C19	Omeprazole	Peptic ulcer disease
	Nelfinavir	HIV
	Fluconazole	Systemic candiasis
	Voriconazole	Systemic Aspergillosis
CYP2D6	Chlorpromazine	Schizophrenia
	Haloperidol	Schizophrenia
	Amitriptyline	Depression
	Metoprolol	Hypertension
	Carvedilol	Hypertension
NAT2	Isoniazid	TB
	Sulphamethoxazole *	HIV- PCP
GST	Aflatoxin B1	Aflatoxicosis

HIV-TB - HIV-TB co- infection
PMCT - Prevention of mother to child transmission
PCP - Pneumocystis jiroveci pneumonia
*Sulphamethoxazole plus trimethoprim (Cotrimoxazole)

4.2 Objectives

4.2.1 Main objective

The main objective of this study was to determine the distribution of genetic variants of *CYP2B6, CYP2C19, CYP2D6, NAT2* and *GSTs* in selected Kenyan general population

4.2.2 Specific Objectives

The specific objectives of this study were:

1. To determine the population frequencies of clinically relevant genetic variants of *CYP2B6, CYP2C19, CYP2D6, NAT2*, *GSTM1* and *GSTT1* in three ethno-linguistically distinct populations of Kenya.

2. To evaluate the inter-ethnic genetic variation of *CYP2B6*, *CYP2C19, CYP2D6, NAT2*, *GSTM1* and *GSTT1*in the three ethno-linguistically distinct populations of Kenya.

4.3 Methodology

4.3.1 Ethical consideration

The study was conducted according to the Declaration of Helsinki which governs research in human beings. Ethical approval was obtained from the joint institutional review board (IRB) of the University of Nairobi and the Kenyatta National Hospital (KNH/UoN-ERC) before initiation of the study (Appendix 1). Each study participant was furnished with comprehensive verbal and written information about the study. This included details about study aims, procedures and potential benefits and risks. Upon verbal consent, each participant signed a voluntary consent form (Appendix 2) before enrollment into the study. To ensure confidentiality of study participant filled questionnaires were identified by a code and held in safe custody.

4.3.2 Study design

A cross-sectional study was conducted to determine the frequency of clinically relevant genetic variants of drug metabolizing enzymes in three ethnic populations of Kenya. Study subjects were recruited in the year 2004 from the Bantu and two Nilotic ethnic groups of Kenya.

4.3.3 Study Population

The regional distribution of study population in Kenya is shown in Figure 4.1. The study

Figure 4.1: Kenyan map showing distribution of study population

Subjects belonged to two ethno-linguistically distinct groups in Kenya, namely, the Bantu and the Nilotic ethnic groups. The Bantus were made up of members of the Kikuyu tribe whereas the Nilotes were made up of the Luo (Western Nilotes) and the Maasai (Eastern Nilotes). The Kikuyu traditionally occupy the central highlands in the Central Province of Kenya whereas the Western Nilotes (Luos) normally reside in the lake basin of Nyanza Province. The Eastern Nilotes (Maasai) are found in the plains of southern Rift Valley and parts of northern Tanzania (Figure 4.1).

4.3.4 Sample Size

The sample size was calculated according to the Hardy–Weinberg principle for population pharmacogenetics. The principle gives a formula for establishing the pharmacogenetic status of a known trait in a population. This is based on the estimated prevalence of the variable of interest, the desired level of confidence and the acceptable margin of error. The formula is given as follows:

$\mathbf{n = t^2 x p (1-p)/m^2}$ Where:

n = The required sample size. t = Confidence level at 95 % (standard value of 1.96)
p = Estimated prevalence of the pharmacogenetic trait in the population, (0.01)
m = Margin of error at 5% (standard value of 0.05).

Hence, for genetic polymorphism studies, the recommended sample size was given as:
$n = (1.96)^2 \times 0.01(1-0.01)/0.05^2 = 152$ or approximately, 160

Since each person has two alleles of a gene, the minimum number of individuals required to confidently detect a genetic polymorphism with a prevalence of 1% in the population was 80 individuals.

4.3.5 Inclusion and exclusion criteria

Study subjects were required to be above 18 years of age and not related to a subject already recruited into this study. They were also required to confess fidelity of ethnicity up to the first generation grandparents. They were to be free from an acute or chronic illness, willing to sign a voluntary consent form and provide a blood sample for genetic studies. Subjects of mixed parentage and those not willing to provide blood sample for genetic analysis were excluded.

4.3.6 Sampling and recruitment procedure

A notice of intention to recruit study subjects was placed on notice boards around the College of Health Sciences (CHS) and Kenyatta National Hospital (KNH). Potential study participants were interviewed using a questionnaire that particularly required information on ethnicity up to the grandparents and absence of acute and chronic illness. Participants meeting study criteria were recruited sequentially by a convenient sampling strategy till the required sample size was attained. The Bantus and the Western Nilotes were recruited from a population of medical students and staff of the College of Health Sciences (CHS), University of Nairobi. The Eastern were recruited from a population living around Kajiado district headquarters and Kitengela Township. On recruitment, a 5.0 ml blood sample was collected by venopuncture into an EDTA vacutainer and stored at minus twenty degrees centigrade (-20^0C) until analyzed

4.3.7 Genotype determination

Genotyping for clinically relevant allelic variants of *CYP2B6, CYP2C19, CYP2D6, NAT2* and *GSTs* was carried out by polymerase chain reaction coupled with restriction fragment length polymorphism (PCR-RFLP). The specific allelic variants analyzed included *CYP2B6* 516 G>T, *CYP2C19**2 and *CYP2C19**3, CYP2D6 (*1, *2, *4, *5, *17, *29), NAT2 (*4, *5, *6, *14), and GSTs (*GSTM1*

and *GSTT1*). Genotyping protocols are described in detail in Chapter Two, sections 2.1.3.7 to 2.1.3.12.

4.4 Data analysis

Allele and genotype frequencies were calculated from observed genotypes using a population genetic analysis program POPGENE version 1.3 software, Deviation from Hardy–Weinberg equilibrium (HWE) was tested by the chi-square goodness of fit test. *GSTM1 or GSTT1*genotypes were coded as positive (wild-type homozygotes and deletion heterozygous) or as negative (homozygous deletion), thereby making direct calculation of Hardy - Weinberg equilibrium impossible. Allele and genotype frequencies were calculated for individual Kenyan populations then averaged across the three populations to derive the population mean and 95% confidence interval. Predicted drug metabolism phenotypes were derived from observed genotypes. For the CYP enzymes, this was done use of the semi- quantitative gene dose (SGD) algorithm described by Steimer *et al.*, (2004) as shown in Table 4.2.

Table 4.2: Semi-quantitative gene dose algorithm for deriving predicted drug metabolism phenotypes from observed genotypes.

Allele	Example	Activity	Gene dose	Genotype	SGD rating	Predicted phenotype
Wild type (wt)	*CYP2D6**1, *CYP2D6**2 *CYP2C19**1, CYP2B6*1	Full	1	wt/wt	2	EM
Mutant (mt-p)	*CYP2D6**17, *CYP2D6**29 *CYP2B6**6	Partial	0.5	wt/mt-P	1.5	EM
				mt-p/mt-p	1	IM
Mutant (mt)	*CYP2D6**4, *CYP2C19**2 *CYP2C19**3, *CYP2B6**18	None	0	mt/mt	0	PM
				wt/mt	1	IM
				mt/mt-P	0.5	IM
Deletion	*CYP2D6**5	None	0	del/del	0	PM
				wt/del	1	IM
				mt/del	0	PM
				mt-P/del	0.5	IM
NAT2						
NAT2 *wt	*NAT2**4	Full	1	*4/*4	2	Rapid Acetylation
NAT2 *mt	*NAT2**5,*6,*7,*14	Partial	0.5	*4/*mt-p	1.5	Rapid Acetylation
				mt-p/mt-p	1	Slow Acetylation
GST						
GST X1	*GSTM1, GSTT1*	Full	1	*GSTX1* /*1	2	Positive conjugation
Deletion	*GSTM1* *0, *GSTT1* *0	None	0	*GSTX1* /*0	0	Non-conjugation

SGD rating - Semi -quantitative gene dose rating: **EM** = 1.5 -2, **IM** = 0.5 - 1, **PM** = 0.
mt-P- mutant allele with partial activity. **mt**- mutant allele with no activity. **wt**- wild type allele with full activity
EM - extensive metabolizer, **IM** - intermediate metabolizer, **PM** - poor metabolizer.

In the SGD rating scale, nonfunctional alleles such as *CYP2D6**4. *CYP2D6**5, *CYP2C19**2and *CYP2C19**3were assigned a gene dose value of 0, partially functional alleles (*CYP2D6**17, *CYP2D6**29, *CYP2B6**6), a gene dose value of 0.5, fully functional alleles (*CYP2D6**1, *CYP2D6**2, *CYP2B6**1, *CYP2C19**1), a gene dose of value of 1. The SGD of an individual is the sum of the gene dose values of both alleles in the genotype. In this respect, the poor metabolizer phenotype (PM) (with 2 nonfunctional alleles), was assigned an SGD 0, the

intermediate metabolizer phenotype (IM) (with either 1 nonfunctional and 1 partially functional alleles; 1 nonfunctional and 1 fully functional or 2 partially functional alleles) had an SGD of 0.5 - 1 whereas the extensive metabolizer phenotype (EM) (with either 1 partially functional and 1 fully functional or 2 fully functional alleles) was assigned as SGD of 1.5 – 2, (Table 4.2).

For *NAT2*, observed genotypes were designated as *NAT2**5(341T>C), *NAT2**6 (590G>A), *NAT2**7 (857G>A) and *NAT2**14 (191G>A) according to the banding pattern. The wild type allele *NAT2**4 was assigned in the absence of any of the four mutations above. All genotypes containing at least one wild type allele (*NAT2**4) were categorized as rapid acetylators. Genotypes containing combinations of *NAT2**5, *NAT2**6, *NAT2**7 and *NAT2**14 were categorized as slow acetylators (Table 4.2). For *GSTs*, the presence of *GSTM1* and *GSTT1* represented positive conjugation. The deleted genotypes were designated *GSTM1**0 and *GSTT1**0 and represented non conjugation phenotypes (Table 4.2).

For inter-ethnic comparisons, genotype and allele frequencies as well as proportions of predicted phenotypes were compared between the three Kenyan ethnic populations. For inter- population comparisons, the data from the three ethnic populations of Kenya were compared with those reported for other African, Caucasian and Asian populations. All comparisons between ethnic groups and populations were performed by the Chi-square or Fisher's exact test using SPSS, version 17.0 (SPSS Inc., Chicago, IL, USA). A P value of less than 0.05 was considered as statistically significant.

4.5 Results

4.5.1 Demographic characteristics

Demographic characteristics of study population are shown in Table 4.3. The study population consisted of 354 unrelated Kenyans recruited from the general population of whom 65% were males who were mainly non-smokers (84%) with

the majority not taking alcohol (94%). In terms of ethnicity, the study population consisted of 102 Bantus (29%), 100 Western Nilotes (28%) and 152 Eastern Nilotes (43%).

Table 4.3: Demographic characteristics of the three Kenyan populations

Characteristics	n	%				
Gender						
Male	229	64.5				
Female	125	35.3				
Age group (Yrs)						
<20	32	9.0				
21 - 24	211	59.6				
25 - 29	62	17.5				
>30	49	13.8				
Alcohol						
Yes	21	5.9				
No	333	94.1				
Smoking						
Yes	58	16.4				
No	296	83.6				
Ethnicity			**Tribe**	**Region**	**Linguistic family**	**Historic origin**
Bantu	102	28.8	Kikuyu	Central	Niger - Kordofanian	West Africa
Eastern Nilotes	152	42.9	Maasai	Nyanza	Nilo - Saharan	Sudan
Western Nilotes	100	28.2	Luo	Rift-valley	Nilo - Saharan	Sudan
TOTAL	354					

n - Number of subjects

4.5.2 *CYP2D6* Genotype and allele frequencies

CYP2D6 genotypes and allele frequencies in the three Kenyan populations are shown in Table 4.4. Comparison of observed to predicted *CYP2D6* genotypes showed conformity to Hardy – Weinberg expectations. Inter-ethnic analysis of CYP2D6 genotypes revealed significant variability between the three Kenyan populations (Table 4.4). *CYP2D6**1/*17, *CYP2D6**2/*17, and *CYP2D6**17/*17 had significantly varied distribution between the three Kenyan populations.. The

CYP2D6 PM genotypes of *CYP2D6**4/*4, *4/*5 and *5/*5 were found at very low frequencies of less than 2% in the three Kenyan populations. Predicted drug metabolism phenotypes derived from observed genotypes are shown in Table 4.4. The proportion of intermediate metabolism (IM) was high in the three populations ranging between 20 – 36%. The poor metabolism (PM) phenotype occurred at very low proportions of 1.0 – 5.0%.

CYP2D6 allele frequencies were also compared between the three Kenyan populations as shown in Table 4.4. There was significant variability in the distribution of *CYP2D6**1, *CYP2D6**4 and *CYP2D6**17 between the three ethnic populations of Kenya. The null allele *CYP2D6*4*, was significantly higher in the Eastern Nilotes at 9% compared to the Western Nilotes (2.5%) and the Bantus (1.7%) (P = 0.002). The diminished or reduced activity allele *CYP2D6**17, was significantly more prevalent in the Bantus (34%) compared to the Eastern Nilotes (18%) and the Western Nilotes (23%) (P = 0.003).

Table 4.4: Inter-ethnic comparison of *CYP2D6* genotypes, predicted phenotypes and allele frequencies in Kenyan populations.

CYP2D6	Prevalence (%)						
Genotypes	**Bantu**	**Eastern Nilotes**	**Western Nilotes**	χ^2	**P**	**Mean**	**95% CI**
EM							
*1 /*1	11.4	21.3	16.3	3.03	0.23	16.1	(12.1-21.2)
*1/*2	11.4	6.3	15.0	3.19	0.203	10.9	(7.6-15.4)
***1/*17**	**11.4**	**13.8**	**25.0**	**6.31**	**0.043**	**16.5**	**(12.4-21.7)**
*1/*29	4.5	7.5	1.3	3.69	0.158	4.4	(2.5-7.8)
*2/*2	11.4	13.8	15.0	0.5	0.779	13.3	(9.6-18.1)
***2/*17**	**11.4**	**3.8**	**2.5**	**6.89**	**0.032**	**6.0**	**(3.7-9.7)**
*2/*29	1.1	0	1.3	0.97	0.617	0.8	(0.2-2.9)
IM							
*1/*4	1.1	6.3	1.3	5.06	0.08	2.8	(1.4-5.7)
*1/*5	1.1	2.5	1.3	0.59	0.745	1.6	(0.6-4.1)
*2/*4	1.1	2.5	2.5	0.53	0.766	2	(0.9-4.6)
*2/*5	1.1	1.3	1.3	0.01	0.997	1.2	(0.4-3.5)
*4/*17	1.1	2.5	0	2.1	0.35	1.2	(0.4-3.5)
*4/*29	0	0	0			0	
*5/*17	2.3	2.5	1.3	0.36	0.835	2	(0.9-4.6)
*5/*29	0	0	0			0	
***17/*17**	**17.0**	**6.3**	**7.5**	**6.32**	**0.042**	**10.5**	**(7.3-14.9)**
*17/*29	6.8	1.3	1.3	5.64	0.06	3.2	(1.6-6.2)
*29/*29	4.5	3.8	5	4.23	0.12	4.4	(2.5-7.8)
PM							
*4/*4	0	2.5	0	4.23	0.12	0.8	(0.2-2.9)
*4/*5	0	1.3	1.3	1.11	0.574	0.8	(0.2-2.9)
*5/*5	1.1	1.3	1.3	0.01	0.997	1.2	(0.4-3.5)
n	88	80	80				
Phenotypes							
EM	62.5	66.3	76.3	2.08	0.353	68.1	(62.1-73.6)
IM	36.4	29.0	21.3	4.65	0.098	29.0	(23.7-35)
PM	1.1	5.0	2.5	2.33	0.313	2.80	(1.4-5.7)
n	88	80	80				
Alleles							
***1**	**26.1**	**39.4**	**38.1**	**8.08**	**0.018**	**34.3**	**(30.2-38.7)**
*2	24.4	20.6	26.3	1.46	0.482	23.8	(20.3-27.7)
***4**	**1.7**	**8.8**	**2.5**	**12.01**	**0.002**	**4.2**	**(2.8-6.4)**
*5	3.4	5.0	3.8	0.60	0.742	4.0	(2.6-6.2)
***17**	**33.5**	**18.1**	**22.5**	**11.38**	**0.003**	**25.0**	**(21.4-29.0)**
*29	10.8	8.1	6.9	1.72	0.424	8.7	(6.5-11.5)
N	176	160	160				

n - number of subjects. N - Total number of alleles

EM - extensive metabolism, **IM** - intermediate metabilism, **PM** - poor metabolism.

4.5.3 *CYP2C19* genotype and allele frequencies

Observed and predicted genotype frequencies of *CYP2C19* conformed to Hardy – Weinberg proportions. Inter- ethnic analysis showed that there were no significant differences in the distribution of *CYP2C19*681G>A and *CYP2C19* 636G>A genotypes in the three Kenyan populations (Table 4.5). The IM genotype *CYP2C19**1/*2 occurred at a high frequency of 20 – 27% whereas *CYP2C19* *1/*3 was found a very low frequency of less than 1%. The PM genotypes *CYP2C19**2/*3 and *CYP2C19**3/*3 were not detected in any of the three populations. Further inter- ethnic comparisons revealed that the proportion of CYP2C19 IM (20 - 27.0%) and PM (0.8 - 5%) were similar in the three Kenyan populations (Table 4.5).

In terms of allele frequencies, the null allele *CYP2C19**2 was distributed in a range of 10 – 18% in the three Kenyan populations whereas *CYP2C19**3 was undetected in the Bantus while occurring at low frequencies of less than 1.0% in the Nilotic populations ($P < 0.01$) (Table 4.5).

Table 4.5: Inter-ethnic comparison of CYP2C19 genotypes, predicted phenotypes and allele frequencies in Kenyan populations

CYP 2 C19	Prevalence (%)						
Genotypes	**Bantu**	**Eastern Nilotes**	**Western Nilotes**	χ^2	**P**	**Mean**	**95% CI**
EM							
***1/*1**	70.7	78.0	68.5	3.32	0.19	73.33	(68.2-77.9)
IM							
***1/*2**	27.2	19.7	25.0	1.78	0.41	23.5	(19.2-28.5)
***1/*3**	0	0.8	1.1	0.92	0.63	0.63	(0.17-2.3)
PM							
***2/*2**	2.2	0.8	5.4	4.84	0.09	2.50	(1.3-4.9)
***2/*3**	0	0	0				
***3/*3**	0	0	0				
n	92	131	92				
Phenotypes							
EM	71.0	78.0	69.0	3.32	0.190	73.3	(68.2-77.9)
IM	27.0	20.6	26.0	1.54	0.462	24.1	(19.7-29.2)
PM	2.2	0.8	5.4	4.84	0.089	2.50	(1.3-4.9)
n	90	130	87				
Alleles							
***1**	84.2	88.9	81.5	5.04	0.0805	85.40	(82.4-87.9)
***2**	15.8	10.7	17.9	5.1	0.0781	14.30	(11.8-17.2)
***3**	**0.0**	**0.4**	**0.5**	**223.35**	**<0.01**	**0.32**	**(0.09-1.2)**
N	184	262	184				

n - number of subjects. N - Total number of alleles

EM- extensive metabolizers, IM- intermediate metabolizers, PM- poor metabolizers

4.5.4 *CYP2B6 516* G>T genotype and allele frequencies

Genotype frequencies of *CYP2B6* 516G>T in the three ethnic populations of Kenya are shown in Table 4.6. Differences between observed and expected *CYP2B6* 516 G>T genotype frequencies were within the Hardy – Weinberg expectations. Inter- ethnic analysis showed that there were no significant differences in the distribution of *CYP2B6* genotypes, predicted phenotypes and alleles between the three populations. The EM genotypes *CYP2B6**1*1 and *CYP2B6**1/*6 occurred at frequencies of 40 – 50% whereas the IM genotype *CYP2B6**6/*6 was found at 12 – 16% across the three populations. The allele

frequency of *CYP2B6**6 was 35 – 37% across the three populations (P = 0.81) (Table 4.6).

Table 4.6: Inter-ethnic comparison of CYP2B6 genotypes, predicted phenotypes and allele frequencies in Kenyan populations.

CYP2B6	Prevalence (%)						
Genotypes	Bantu	Eastern Nilotes	Western Nilotes	χ^2	P	mean	95% CI
EM							
*1/*1	46.4	49.2	40.4	1.64	0.44	45.8	(40.3-51.4)
*1/*6	38.1	38.3	44.9	1.18	0.55	40.2	(34.8-45.8)
IM							
*6/*6	15.5	12.5	14.6	0.42	0.81	14.0	(10.5-18.3)
N	84	128	89				
Phenotypes							
IM	15.5	12.5	14.6	0.42	0.811	14.0	(10.5-18.3)
N	84	128	89				
Alleles							
*6	34.5	35.2	37.1	0.28	0.871	35.5	(31.8-39.5)
N	168	256	178				

EM- extensive metabolizers, IM- intermediate metabolizers, PM- poor metabolizers

4.5.5 *NAT2* genotypes and allele frequencies

Inter- ethnic analysis of *NAT2* genotype frequencies in the three ethnic populations of Kenya is shown in Table 4.7. Differences between observed and expected *NAT2* genotypes were in conformity with the Hardy – Weinberg proportions. There were no significant differences in the distribution of *NAT2* genotypes between the three Kenyan populations. *NAT2**4/*5 was the most abundant rapid acetylator genotype with a frequency of 18 - 20% whereas the slow acetylator genotypes of *NAT2**5/*5 (13 – 18%) and *NAT2**5/*6 (11 – 19%) were the most prevalent

(Table 4.7). Further inter – ethnic comparison of *NAT2* allele frequencies in the three Kenyan populations is shown in Table 4.7. There was no significant

Table 4.7: Inter-ethnic comparison of NAT2, GSTM1 and GSTT1frequencies in three ethnic populations of Kenya

	Prevalence (%)						
Genotypes	Bantu	Eastern Nilotes	Western Nilotes	χ^2	P	Mean	95% CI
NAT2							
RA							
*4/*4	8.2	4.4	8.7	2.09	0.35	6.70	(4.5-9.98)
*4/*5	18.4	20.4	19.6	0.2	0.93	19.60	(15.6-24.2)
*4/*6	5.1	7.3	10.9	2.28	0.32	7.60	(5.2-11.0)
*4/*7	2.0	0.7	1.1	0.83	0.66	1.20	(0.48-3.1)
*4/*14	2.0	1.5	5.4	3.51	0.17	2.80	(1.5-5.2)
SA							
*5/*5	15.3	17.5	13.0	0.85	0.66	15.60	(12.1-19.9)
*5/*6	16.3	19.0	11.9	2.0	0.37	16.20	(12.6-20.6)
*5/*7	2.0	1.5	1.1	0.29	0.86	1.50	(0.7-3.5)
*5/*14	9.2	7.3	6.5	0.52	0.77	7.60	(5.2-11.0)
*6/*6	9.2	10.9	8.7	0.37	0.83	9.80	(7.0-13.5)
*6/*7	2.0	0.7	1.1	0.83	0.66	1.20	(0.48-3.1)
*6/*14	4.1	4.4	4.3	0.01	0.99	4.30	(2.6-7.1)
*7/*7	1.0	1.5	1.1	0.11	0.95	1.20	(0.48-3.1)
*7/*14	4.1	1.5	1.1	2.55	0.28	2.10	(1.0-4.4)
*14/*14	1.0	1.5	5.4	4.84	0.09	2.40	(1.2-4.8)
N	98	137	92				
Alleles							
NAT2							
*4	21.9	19.3	27.2	3.92	0.1411	22.3	(19.3-25.7)
*5	38.3	41.6	32.6	3.78	0.1508	38.1	(34.4-41.9)
*6	22.9	26.6	22.8	1.21	0.5458	24.5	(21.3-27.9)
*7	6.1	3.7	3.3	2.45	0.2943	4.3	(3.0-6.1)
*14	10.7	8.8	14.1	3.29	0.1933	10.9	(8.7-13.5)
N	196	274	184				
***GSTM1* *0**	**29.2**	**15.6**	**30.9**	**9.44**	**0.0089**	**23.6**	**(19.4-28.5)**
N	89	147	94				
***GSTT1* *0**	**26.4**	**40.6**	**21.9**	**10.63**	**0.0049**	**31.3**	**(26.5-36.5)**
N	87	143	96				

EM- extensive metabolizers, IM- intermediate metabolizers, PM- poor metabolizers
RA-rapid acetylators. SA- slow acetylators

difference in the frequencies of *NAT2* alleles between the three Kenyan populations. *NAT2*5* (30 – 42%) and *NAT2*6* (20 – 27%) were the most common slow acetylator alleles (Table 4.7). The proportion of slow acetylation phenotype ranged between 54 – 66% in the three populations.

4.5.6 *GSTM1* and *GSTT1* frequencies

Inter-ethnic distribution of *GSTM1* and *GSTT1* alleles in the three Kenyan populations is shown in Table 4.7. There was a significant difference in the distribution of *GSTM1*0* (deletion) and *GSTT1*0* (deletion) in the three Kenyan populations. The frequency of *GSTM1*0* in the Eastern Nilotes was 16% which was nearly half that found in the Western Nilotes and the Bantu (29-31%) (P = 0.009). On the other hand, the frequency of *GSTT1*0* was also significantly higher in the Eastern Nilotes (41%) compared to that seen in the Bantu and the Western Nilotes (22 - 26%) (P = 0.005).

4.6 Discussion

4.6.1 Population pharmacogenetics

This study determined the genetic polymorphism of *CYP2B6, CYP2C19, CYP2D6, NAT2, GSTM1* and *GSTT1* in ethno-linguistically distinct populations of Kenya. The study population consisted of one Bantu and two Nilotic ethnic populations in Kenya. This is the first study to investigate the inter-ethnic genetic diversity of these genes in the Bantu and Nilotic populations of Kenya. Many clinically relevant polymorphisms have been demonstrated to vary between different populations (Engen *et al.*, 2006). Results from this study have revealed a rich inter – ethnic and inter – population diversity of genes encoding drug metabolizing enzymes.

4.6.2 *CYP2D6* genetic diversity

CYP2D6 was found to exhibit the greatest inter- ethnic genetic diversity between the Bantu and Nilotic populations of Kenya in this study. This was with respect to the variable distribution of *CYP2D6**4 and *CYP2D6**17 between the Bantu and Nilotic ethnic populations. Results seem to set the Eastern Nilotes apart by the sharp difference in the frequency of *CYP2D6**4 (9%) which was more than threefold that seen in the Bantu (1.7%) and the Western Nilotes (2.5%). The IM genotype *CYP2D6**17/*17 was also found to be more than two-fold higher in the Bantu (17%) compared to Nilotes (6 – 8%). The predicted phenotypes were also distributed in an ethnically specific manner with the proportion of IMs being higher in the Bantu (36%) compared to the Nilotes (21 – 29%) even though the differences did not reach statistical significance.

Comparison of the distribution of CYP2D6 alleles in Kenyan population with other Africans, Caucasians and Asian populations is shown in Table 4.8. *CYP2D6**4 is a splice - site mutant allele that is reported to be the most frequent null allele in Caucasians, occurring at a frequency of 17.2% (Sistonen *et al.*, 2007). The surprising presence of this European specific allele in the Kenyan Eastern Nilotes (Maasai) at 9% was at variance with genotyping reports from other East African populations (1 – 3%), (Wennerholm *et al.*, 2001; Dandara *et al.*, 2001) but was similar to that of African Americans (7%) (Gaedigk *et al.*, 2005) and the Ghanaians (7%) (Griese *et al.*, 1999; Yen-Revollo *et al.*, 2009). This disparity could be in part due to gene flow between the Maasai (Eastern Nilotes) and Caucasoid / Cushitc populations in the wake of their migration to East Africa (Ehret, 1998; Tishkoff, 2007).

The *CYP2D6**17 (T107I, R296C) is a variant allele that exhibits substrate dependent diminished affinity and activity of up to 50% (Oscarson *et al.*, 1997). In this study, *CYP2D6**17 occurred at a higher frequencies in the Kenyan Bantu (34%) than in the two Nilotic populations (18 – 23%). This observation was

Table 4.8: Inter-population comparison of CYP2D6 allele frequencies in Kenyans with other Africans, Caucasians and Asian populations

CYP2D6	Prevalence (%)								
	Kenyans			Other Africans					
Alleles	Bantu	EN	WN	Ghana	Zimbabwe	Venda	Tanzania	χ^2	P
*4	1.7	**8.8**	2.5	7.0	2.0	3.3	1.4	14.36	**0.006**
*17	**33.5**	18.1	22.5	27.7	34.0	24.0	20.3	12.23	**0.016**
*29	10.8	8.1	6.9	ND	ND	ND	19.8	17.43	**<0.001**
N	176	160	160	386	228	152	212		
	Kenyans			Caucasians					
	Bantu	EN	WN	German	Turkish	American			
*4	1.7	**8.8**	2.5	18.9	11.3	19.7		**66.2**	**<0.001**
*17	**33.5**	18.1	22.5	0.1	0.1	0.43		**367.7**	**<0.001**
*29	10.8	8.1	6.9	ND	ND	0.14		**31.31**	**<0.001**
N	176	160	160	672	404	374			
	Kenyans			Asians					
	Bantu	EN	WN	Chinese	Korean				
*4	1.7	**8.8**	2.5	0.0	0.25			**33.59**	**0.002**
*17	**33.5**	18.1	22.5	0.0	0.0			**259**	**<0.001**
*29	10.8	8.1	6.9	0.0	0.0				**<0.001**
N	176	160	160	100	400				

N - Total number of alleles. EN - Eastern Nilotes. WN - Western Nilotes ND - Not determined

Ref: Dandara et al, 2001, Gaedigk et al, 2008, Yen-Revollo et al, 2009, Zhou et al, 2009.

similar to that reported for other Bantu populations in Africa such as in Ghana (28%) and Zimbabwe (34%) (Masimirembwa *et al.*, 1995; Griese *et al.*, 1998). Another diminished activity allele, *CYP2D6**29 was also found in Kenyans at 7 - 11% which was in contrast to that reported for Tanzanians (20%) (Wennerholm *et al.*, 2001). Hence, the *CYP2D6* locus seems to exhibit remarkable inter – ethnic and inter-population genetic diversity between Kenyan populations, other

Africans, Caucasian and Asian populations (Bertilsson *et al.*, 1992, (Zhou *et al.*, 2009). This variability is underscored by the distribution of three important CYP2D6 alleles. These are CYP2D6*4 which is Caucasian specific, CYP2D6*17 which is African specific and CYP2D6*10 that is Asian specific (Masimirembwa *et al.*, 1993).

The variable distribution of *CYP2D6* alleles between populations is of great importance. This is because the CYP2D6 enzyme metabolizes 25% of all drugs in clinical use, most of which target the cardiovascular and central nervous system (Gardiner and Begg, 2006). The proportion of the IM was higher in the Bantu (36%) than in the Nilotes (21 -29%) whereas the PM was higher Eastern Nilotes (5%) even though the differences did not reach statistical significance. The low proportion of predicted PM found in this study was attributed to the low frequency of CYP2D6*4 and CYP2D6*5 and was similar to reports from phenotyping studies in similar populations. However, the proportion of predicted intermediate metabolizer (IM) was found to be high (21 – 36%) particularly in the Bantu was attributed to high prevalence of*CYP2D6*17 and *CYP2D6*29. These observations were similar to those reported in other studies covering the Bantu of East and Southern Africa (Dandara *et al.*, 2001). The clinical implications of such distribution in different ethnic populations of Kenya remain to be determined in well designed clinical studies. A study on the distribution of *CYP2D6* and *CYP2C19* genotypes in psychiatric patients and impact on psychotropic drug medications is reported elsewhere in this thesis.

4.6.3 *GSTM1* and *GSTT1* inter-ethnic diversity

Inter-population comparison of *GSTM1*0* and *GSTT1*0* (deletion) frequencies found in Kenyans with those reported for other Africans, Caucasians and Asian populations is shown in Table 4.9. Differences in *GSTT1* and *GSTM1* frequencies in populations worldwide have been described (Nelson *et al.*, 1995; Garte *et al.*, 2001). This study is the first to genotype *GSTM1* and *GSTT1* in Kenyan

populations. The low frequency of *GSTM1**0 (16%) and the high frequency of *GSTT1**0 (41%) in the Eastern Nilotes seem to take an ethnic specific dimension. On the other hand, the Bantu and the Western Nilotes displayed similarity in *GSTM1**0 (29 – 31%) and *GSTT1**0 (21 – 26%) allele frequencies. The observed disparity of the Eastern Nilotes on the one hand and the apparent convergence of the Bantu and the Western Nilotes on the other is consistent with reports from studies correlating African genetic and linguistic variation (Tishkoff *et al.*, 2009). This is believed to be a reflection of the high levels of admixture among the Western Nilotes (Luo) and geographically nearby Bantu populations.

Studies have shown that *GST* genotype frequencies are distributed population wise according to the various ethnic and geographical patterns (Garte *et al.*, 2001; Hatagima A, 2004). In this study, the frequency of *GSTM1**0 in Kenyan Bantu (29.2% and the Western Nilotes (31%) was in agreement with that reported for other African populations such as Ivory Coast (36%) and Tanzania (Dandara *et al.*, 2002) but different from that reported for Egyptians (55%) (Hamdy *et al.*, 2003) reflecting the diversity of African populations. The frequencies of *GSTT1**0 in Kenyan Bantu (26.4%) and Western Nilotes (22%) were also in concert with reports from Tanzania (25%) (Dandara *et al.*, 2002). Hence the observed low frequency of *GSTM1**0 in the Eastern Nilotes (16%) and high prevalence of *GSTT1**0 (41%) in this study is so far at variance with studies in other African populations probably as a result of differences in population evolutionary history. In Africa, the variation in the genetic structure of many otherwise genetically "neutral" systems, such as mitochondrial DNA has been demonstrated (Salas *et al.*, 2002).

Table 4.9: The frequency of GSTM1*0 and GSTT1*0 in Kenyans, other Africans, Caucasians and Asian populations

GST deletion	Prevalence (%)							
	Kenyans			other Africans				
	Bantu	EN	WN	Ivory Coast	Tanzania	African Americans	χ^2	P
GSTM1 *0	29.2	15.6	30.9	36.0	33.0	28.0	71.69	<0.001
N	89	147	94	133.0	106.0	271		
GSTT1 *0	26.4	40.6	21.9	33.0	25.0	17.0	36.05	<0.001
N	87	143	96	133	106	271		
	Kenyans			Caucasians				
	Bantu	EN	WN	British	Turkish	American		
GSTM1 *0	29.2	15.6	30.9	50.8	51.9	52.0	73.27	<0.001
N	89	147	94	178	133	392		
GSTT1 *0	26.4	40.6	21.9	16.9	17.3	16.0	30.34	<0.001
N	87	143	96	178	133	392		
	Kenyans			Asians				
	Bantu	EN	WN	Japanese	Chinese			
GSTM1 *0	29.2	15.6	30.9	55.7	51.0		77.36	<0.001
N	89	147	94	88	477			
GSTT1 *0	26.4	40.6	21.9	44.3	46.0		75.7	<0.001
N	87	143	96	88	477			

N - total number of alleles. EN - Eastern Nilotes. WN - Western Nilotes

Ref: Dandara et al 2002, Santotivo et al, 2010, Hamdy et al, 2003, Wefare et al, 1999, Ada et al,2004, Mishra et al, 2004.

The implications of such inter-ethnic diversity in the distribution of *GST* null genotypes in Kenyan populations will require further studies to elucidate. However, the presence of *GSTM1**0 and *GSTT1**0 (deletion or null genotype), has been associated with loss of enzyme activity and increased vulnerability to cytogenetic damage (Norppa, 2004). In the liver, GST has been proposed to protect against hepatitis B virus-related injury, which is partly manifested as extensive oxidative. From the results of this study, It can be postulated that the high frequencies of *GSTM1**0 of 29 – 31% found in the Western Nilotes and the Bantu could be associated with increased risk of HCC in these populations compared to the Eastern Nilotes (16%). This warrants further investigation by well

designed epidemiological studies. This within the backdrop of frequent aflatoxin poisoning outbreaks and the rising incidence of various cancers in Kenyan populations

4.6.4 *CYP2B6* 516G>T inter-population variability

Results from this study have demonstrated that the distribution of the C*YP2B6**6allele (34 – 37%) and *CYP2B6**6/*6 (TT) (12 -16%) was similar across the three Kenyan populations. Inter- population comparisons of *CYP2B6* 516 G>T allele frequencies between the Kenyans and those reported for other Africans, Caucasians and Asians is shown in Table 4.10. Variability was however, noted between some African populations such Ghanaian (46%) and Zimbabwean (49%) (Nyakutira *et al.*, 2008). This variability between African populations could be attributed to the diversity in population history as due to selective pressure of exposure to environmental chemicals known to cause induction of *CYP2B6* expression (Sueyoshi and Negishi, 2001). Studies have reported that the *CY2B6**6 variant is more prevalent in people of African origin compared to Caucasians and Asians (Barrett *et al.*, 2002; Haas *et al.*, 2009; Gounden *et al.*, 2010). The same trend was seen in this study, where the frequency in Kenyans (36%) was 10 percent higher than those reported for Caucasian populations such as the Germans (26%) (Kirchheiner *et al.*, 2003). On the other hand, *CYP2B6**6 frequency in Asian populations such as the Japanese (18%) and Korean (16%) are reportedly nearly half that found in Kenyans (Hiratsuka *et al.*, 2002; Klein *et al.*, 2005).

In Kenya, CYP2B6 major substrates form first line chemotherapeutic agents according to the WHO EML and Kenyan treatment guidelines. Among these are first line antiretroviral drugs, efavirenz (EFV) (Ward *et al.*, 2003) and nevirapine (NVP) (Erickson *et al.*, 1999) which are agents used for treatment and control of HIV infection. It also metabolizes the antimalarial drug artemisinin (Svensson and Ashton, 1999) and its derivatives artesunate (Li *et al.*, 2003) and β-arteether (Grace *et al.*, 1998). Other substrates include the anticancer drug

cyclophosphamide (Xie *et al.*, 2003).The clinical impact of *CYP2B6* 516 G>T genotypes on nevirapine treatment outcomes in Kenyan HIV patients has been addressed elsewhere in this thesis.

Table 4.10: Inter-population comparison of CYP2C19 and CYP2B6 allele frequencies in Kenyans and other Africans, Caucasians and Asian populations

Prevalence (%)								
CYP2C19		Kenyans		other Africans				
	Bantu	EN	WN	Zimbabwe	Venda	Tanzania	χ^2	P
*2	15.8	10.7	17.9	13.1	21.7	17.9	6.74	0.080
*3	0	0.4	0.5	0.0	0.0	0.0	1.69	0.639
N	184	262	184	168	152	212		
		Kenyans		Caucasians				
	Bantu	EN	WN	Italian	Turkish	Caucasian		
*2	15.8	10.7	17.9	11.1	12.0	12.7	2.47	0.481
*3	0	0.4	0.5	0.0	0.4	0.9	2.84	0.418
N	184	262	184	360	404	273		
		Kenyans		Asians				
	Bantu	EN	WN	Japanese	Chinese	Korean		
*2	15.8	10.7	17.9	26.1	27.1	28.0	34.4	0.0016
*3	0	0.4	0.5	12.8	8.2	9.8	68.28	<0.001
N	184	262	184	250	100	377		
CYP2B6		Kenyans		other Africans				
	Bantu	EN	WN	Ghana	Zimbabwe	Ivorian	χ^2	P
*6	34.5	35.2	37.1	46.0	49.0	38.0	15.96	0.003
N	168	256	178	88	142	82		
	Bantu	EN	WN		Caucasian			
*6	34.5	35.2	37.1		25.6		11.56	<0.001
N	168	256	178		430			
					Asians			
	Bantu	EN	WN	Japanese	Taiwanese	Korean		
*6	34.5	35.2	37.1	18.0	16.2	15.9	26.2	<0.001
N	168	256	178	50	68	88		

N- total number of alleles. EN - Eastern Nilotes. WN - Western Nilotes.

Ref: Dandara et al, 2001, Scordo et al, 2004, Aynacioglu et al, 1999, Zhou et al, 2009 Mehlotra et al, 2006, Nyakutira et al, 2008, Haas et al, 2005, Klein et al, 2005,

4.6.5 *CYP2C19* inter- population diversity

This study observed a similar distribution of the null allele *CYP2C19**2 (16 – 18%) and *CYP2C19**1/*2 (19 – 27%) in the three Kenyan populations. Inter-population comparison of *CYP2C19* allele frequencies between the Kenyans and those reported for other Africans, Caucasians and Asian populations is shown in Table 4.10. These results were also similar to those reported for other African and Caucasian populations (Aynacioglu *et al.*, 1999; Dandara *et al.*, 2001;Scordo *et al.*, 2004). The null Allele *CYP2C19**3 was detected in Kenyan Nilotes at very low frequencies (<1%) while being undetected in the Kenyan Bantu in similarity to other Bantu populations across the African continent (Masimirembwa *et al.*, 1995; Dandara *et al.*, 2001; Dandara *et al.*, 2011) *CYP2C19**3 is predominantly an Asiatic allele and its presence in the Kenyan Nilotes could be attributed to gene flow between the Nilotes and the Cushitic populations (AfroAsiatic) possibly during the period of Nilotic migration Eastern Africa (Tishkoff *et al.*, 2007). The distribution of common variant alleles of *CYP2C19* has been reported to vary among different ethnic groups (Strom *et al.*, 2012). This was evident in this study where there was stark differences in the distribution of *CYP2C19**3 (<1%) in Kenyans and that reported for Asian populations such as the Japanese (12.8%) (Fukushima-Uesaka *et al.*, 2005) and Korean (8%) (Lee *et al.*, 2007).

In this study, *CYP2C19* observed genotypes predicted a PM phenotype of 2 – 5% in the three ethnic populations of Kenya. This observation was similar to that reported from phenotyping studies in Caucasian populations (1 -6%) and other African populations (1 -7.5%) (Xie *et al.*, 1999a; Xie *et al.*, 1999b). This study also predicted an intermediate metabolizer phenotype of 24% which could have implications for commonly used substrate drugs in Kenyan populations. CYP2C19 is responsible for the metabolism of drugs such as omeprazole, diazepam, amitriptyline, voriconazole, nelfinavir, and the prophylactic anti-malarial proguanil. These drugs are important in the therapy of diseases such as malaria,

HIV and fungal infections that are a burden in tropical countries such as Kenya. The distribution of *CYP2C19* SNPs in psychiatric patients and consequences on the use of psychotropic medications has been investigated elsewhere in this thesis

4.6.6 *N*-acetyl transferase 2 inter-ethnic diversity

Inter- population analysis of the distribution of *NAT2* alleles between the Kenyans and those reported for other African, Caucasian and Asian populations is shown in Table 4.11. The pattern of distribution of *NAT2**5 (33 - 42%), *NAT2**6 (22 – 27%), *NAT2**7(3 - 7%) and NAT2*14 (9 - 14%) in the three Kenyan populations was in similarity with other African populations (Dandara *et al.*, 2003). The results from this study were in conformity with observations in other studies that *NAT2**5 is more prevalent in Caucasians and Africans (40-60%), whereas *NAT2**7 was more common in Asians (10 -12%). (Cascorbi *et al.*, 1995; Okumura *et al.*, 1997; Lee *et al.*, 1998). NAT2*14 is said to be African specific and was detected in Kenyan populations (9- 14%) in similarity to other African populations (Deloménie *et al.*, 1996; Patin *et al.*, 2006; Sabbagh *et al.*, 2011).

This study report NAT2 slow acetylation of 62% in the three Kenyan populations. NAT2 drug substrates include isoniazid which is a first line agent in the therapy of tuberculosis and sulphamethoxazole, a component of co-trimozaxole which is an important antibacterial agent presently used in the prophylactic management of pneumocystis jiroveci pneumonia (PCP) in HIV patients. The question whether slow acetylation status is a risk factor for hepatotoxicity has been controversial (Huang *et al.*, 2002). Given that the incidence of drug resistant tuberculosis (TB) is rising in Kenya, studies are required to elucidate the role of NAT2 acetylation status in anti-TB drug resistance, treatment failure and adverse reactions.

Table 4.11: Inter – population comparison of NAT2 slows acetylator alleles in Kenyans, other Africans, Caucasians and Asian populations

NAT2	Prevalence (%)							
Alleles	**Kenyans**			**other Africans**				
	Bantu	**EN**	**WN**	**Gabon**	**Zimbabwe**	**Tanzania**	χ^2	**P**
*5	38.3	41.6	32.6	40.0	30.7	33.8	9.38	0.095
*6	22,9	26.6	22,8	22.0	20.9	20.5	2.58	0.765
*7	6.1	3.7	3.3	2.0	5.8	3.5	3.98	0.553
*14	10.7	8,8	14.1	9.0	13.8	11.5	5.22	0.400
N	196	274	184	102	326	234		
	Kenyans			**Caucasians**				
	Bantu	**EN**	**WN**	**German**	**Swedish**	**American**		
***5**	**38.3**	**41.6**	**32.6**	**46.5**	**50.7**	**43.0**	**14.11**	**0.015**
*6	22,9	26.6	22,8	27.8	27.8	31.0	6.35	0.274
***7**	**6.1**	**3.7**	**3.3**	**1.3**	**2.1**	**2.0**	**15.39**	**0.009**
***14**	**10.7**	**8,8**	**14.1**	**0.0**	**0.0**	**0.0**	**194.0**	**<0.001**
N	196	274	184	844	72	421		
	Kenyans			**Asians**				
	Bantu	**EN**	**WN**	**Japanese**	**Chinese**	**Korean**		
***5**	**38.3**	**41.6**	**32.6**	**2.4**	**5.0**	**2.0**	**202.7**	**<0.001**
*6	22,9	26.6	22,8	19.3	30.0	18.0	14.49	**0.276**
***7**	**6.1**	**3.7**	**3.3**	**9.7**	**12 .0**	**11.0**	**32.99**	**<0.001**
***14**	**10.7**	**8,8**	**14.1**	**0.0**	**0.0**	**1.0**	**64.5**	**<0.001**
N	196	274	184	145	254	85		

N - total number of alleles. EN - Eastern Nilotes, WN - Western Nilotes.
Ref: Delomenie et al, 1996, Dandara et al, 2003, Bell et al, 1993, Smith et al, 1997, Lee et al, 1998

Beyond its medical relevance, the possible role of *NAT2* in evolutionary genetics has generated considerable interest (Mortensen *et al.*, 2011). This is particularly so for the high prevalence of slow acetylators in humans populations worldwide (> 50%) and the possible role *NAT2* has played in human adaptation. Studies have reported that acetylation status in human populations has been driven by dietary and lifestyle changes in the last 10,000 years. High prevalence of slow acetylation has been said to occur in sedentary agriculturalists compared to pastoralists and hunter-gatherer populations (Luca *et al.*, 2008). However, this trend was not

apparent in this study whereby the prevalence of slow acetylation in the Kenyan Bantus and the Western Nilotes (agriculturalists) on the one hand and the Eastern Nilotes (pastoralists) on the other was not evident. Further studies

4.7 Summary of major findings and conclusion

The diversity of genes affecting drug metabolism namely, *CYP2D6, CYP2B6, CYP2C19, NAT2* and *GSTs* in the Bantu and Nilotic populations of Kenya is reported for the first time in this study. The main findings of this study are:

CYP2D6 genetic variants were distributed in an ethnic specific manner in Kenyan populations. *CYP2D6**4 was preferentially distributed in the Eastern Nilotes (9%) which was three fold higher than in the Bantu and Western Nilotes and was indicative of Caucasoid gene flow. *CYP2D6**17 was more common in Kenyan Bantus at 34% compared to the Nilotic (18 – 23%) and was in agreement with other Bantu populations in Africa. The proportion of *CYP2D6* predicted IM phenotype was high in Kenyans (21 - 36%) compared to the low predicted PM (1-5%). *CYP2C19**3 was rare in Kenyan population being detected at low frequencies of less than one percent in the Nilotes. *CYP2C19**2 occurred at 10 - 18% and was equally distributed in the Kenyan ethnic populations. The proportion of *CYP2C19* predicted IM phenotype was high (20 -27%) whereas the PM was low (1 – 5%)

CYP2B6 *6 occurred at high frequencies (34-37%) and was equally distributed in Kenyan ethnic populations. *NAT2*alleles (*4,*5,*6, *7, *14) were equally distributed in Kenyan ethnic populations with a proportion of slow acetylators of 54 – 66%. *GSTM1**0 and *GSTT*0 (d*eletion) were distributed in an ethnic specific manner in Kenyan populations. The Eastern Nilotes had low frequenciesof*GSTM1**0 *(16%)* that was nearly half that of the other two Kenyan ethnic groups whereas the frequency of *GSTT1**0 of 41% in that population was nearly double that of the other two Kenyan ethnic groups. . The deletion

frequencies found in the Eastern Nilotes were significantly different from those reported for other African populations.

In conclusion, this study has reported on novel frequency data as regards the genetic polymorphisms of *CYP2B6, CYP2C19, CYP2D6*, *NAT2*, *GSTM1* and *GSTT1* in three ethnic ethno-linguistic populations of Kenya. In this respect, this study has extended and added new data to existing knowledge obtained from previous pharmacogenetic studies in other African populations. This study has confirmed that genetic variability exists among different ethnic groups in a country. Hence, caution should be exercised when making generalized treatment recommendations for population subgroups. This is particularly important for African populations who are known to exhibit great genetic diversity. However in the absence of individual genomic sequencing, genotyping of a larger variety of ethnic groups as has been initiated in this study should be carried out, so that treatment guidelines can be customized at least at the national level.

Chapter Five

AN ANALYSIS OF *CYP2D6* AND *CYP2C19* GENETIC VARIANTS AND THE PATTERN OF PSYCHOTROPIC MEDICATIONS IN PSYCHIATRIC IN-PATIENTS AT MATHARI HOSPITAL

5.1 Introduction

Psychiatric disorders such as depression and schizophrenia contribute significantly to worldwide morbidity and mortality (Ustün *et al.*, 2004; Alonso, 2012). In depression and schizophrenia, effective drug therapy is available, but 30 to 50% of all patients do not respond sufficiently to the initial treatment regime (Fava *et al.*, 2012). Moreover, psychotropic drug efficacy may not occur until 3 – 6 weeks after initiation of drug treatment (Conley *et al.*, 2007; Stauffer *et al.*, 2012). Hence, the time period before a clinician can determine whether a specific treatment is ineffective and consider alternative pharmacotherapy can be lengthy. During this period, treated patients may experience continuous psychiatric symptoms, employment loss and social dysfunction. In fact, a significant proportion of patients with psychosis and affective disorders even commit suicide (Kaplan *et al.*, 1994). On the other hand, there is also a subset of patients who develop drug-induced adverse events that may range from the troublesome to the life threatening (Zhang and Malhotra, 2011).

Earlier efforts to identify predictors of psychotropic drug response focused on clinical or biological variables with limited success (Szymanski *et al.*, 1995; Baeza *et al.*, 2009). Presently, molecular genetic approaches such as pharmacogenomics, attempt to provide novel methods of dissecting the heterogeneity of psychotropic drug response. Both the pharmacokinetic and the pharmacodynamic action of drugs that determine therapeutic and adverse effects are subject to extensive genetic variation. Genes encoding for CYP enzymes are subject to functional polymorphisms that alter their metabolic rate, resulting in enzyme variants either

with no activity, diminished activity or ultrarapid activity (Coutts and Urichuk, 1999). CYP2D6 and CYP2C19 enzymes are involved in the biotransformation and elimination of many psychotropic drugs (Bertilsson, 2007; Porcelli *et al.*, 2011), (Table 5.1).

Table 5.1: Some psychotropic drug substrates of CYP2D6 and CYP2C19

	CYP2C19	**CYP2D6**
Antidepressants	Amitrptyline Citalopram Clomipramine Imipramine Sertraline	Amitriptyline Nortrityline Clomipramine Desipramine Fluoxetine Paroxetine Venlafaxine
Antipsychotics		Chlopromazine Haloperidol Perphenazine Risperidone Thioridazine Zuclopenthixol

Adapted from Bertilsson et al, (1993).

Individuals presenting with CYP2D6 poor metabolizer (PM) variants are reportedly more likely to develop extrapyramidal side effects (EPS), tardive dyskinesia (TD) and weight gain (Kobylecki *et al.*, 2009). Studies report that the metabolic status of CYP2D6 and CYP2C19 may influence the therapeutic doses of antidepressants and antipsychotics (Kirchheiner *et al.*, 2004). This could form a basis for the potential clinical application of pharmacogenetic information, especially when combined with relevant environmental and clinical data. Consequently, *CYP2D6*and *CYP2C19* genotyping has been used to predict phenotype and other clinical covariates such as increased risk of adverse reactions, length of hospitalization and switching of drugs (Patsopoulos *et al.*, 2005; Kropp

et al., 2006). However, there is paucity of data on the pharmacogenetics of psychotropic drug therapy in psychiatric patients of African origin. This is despite reports that some African ethnic groups such as the Bantu have a high proportion of CYP2D6 intermediate metabolizer (IM) phenotype (Masimirembwa *et al.*, 1996; Dandara *et al.*, 2001). Hence, the goal of this study was to investigate the relationship between *CYP2C6* and *CYP2C19* genetic variants and the pattern of psychotropic medications in Kenyan psychiatric patients.

5.2 Objectives

5.2.1 Main Objective

The main objective of this study was to analyze the genetic variants of *CYP2D6*and *CYP2C19*and to evaluate their relationship on the pattern of prescribing of psychotropic medications and patient behavior in Kenyan psychiatric in-patients.

5.2.2 Specific Objectives

The specific objectives of this study were:

1. To determine the distribution of clinically relevant SNPs of *CYP2D6* and *CYP2C19* in psychiatric in-patients at Mathari Hospital.

2. To investigate the pattern of prescribing of psychotropic medications and establish the P450 enzymes involved in their metabolism in psychiatric in-patients at Mathari Hospital

3. To evaluate the impact of*CYP2D6* and *CYP2C19*genetic variants and predicted phenotypes on the prescribing pattern of psychotropic medications in psychiatric in – patients at Mathari Hospital.
4. To characterize the behavioral habits of Kenyan psychiatric in-patients at Mathari Hospital

5.3 Methodology

5.3.1 Ethical considerations

The study was conducted according to the Declaration of Helsinki regarding research in human beings. Ethical approval was granted by the joint institutional review board (IRB) of the Kenyatta National Hospital and the University of Nairobi (KNH/UoN-ERC) (Appendix 1). Two psychiatric nurses working at Mathari Hospital assisted in explaining details of the study to potential study participants subjects and in the administration of recruitment procedures. Details of the study and intention to recruit study participants was explained to potential participants in the wards in a language the patients understood. Those who were willing to take part in the study were further provided with details of study procedures, benefits and risks. Upon verbal consent, each participant was presented with a written informed consent form (Appendix 2) for signature. Approval was also sought from one other person selected by the patient to act as an advisor who was in most cases a spouse, a parent or a guardian.

5.3.2 Study Area

Mathari Hospital is a national teaching and referral psychiatric hospital situated in Nairobi province, within Nairobi North District, Starehe constituency. It is located along the Thika super highway, opposite the Muthaiga police station, 5 km to the north of the city centre. The hospital has a total bed capacity of 700 patients, 300 of which are in the maximum security unit (MSU) which caters for forensic patients. There are of twelve wards on the civil side and four in the MSU. It also runs 8 outpatient clinics for psychiatric and general out-patient services and attends to an average of 550 patients daily. The institution works closely with the University of Nairobi, the Kenya Medical Training College (KMTC) and other public and private institutions. It is the only institution that offers specialized postgraduate training in mental health in East and Central Africa. Students from

Uganda, Rwanda, Tanzania and Burundi have benefited from training facilities offered in this hospital.

5.3.3 Study population

The study population consisted of adult male and female psychiatric in - patients who were admitted to the civil wards of the Mathari Hospital. The study was conducted in the year 2009.

5.3.4 Study design

A cross – sectional study was carried out to determine the prevalence of *CYP2C19* and *CYP2D6* genotypes in psychiatric in – patients in Mathari Mental Hospital. A retrospective medical record review was undertaken to extract data pertaining to the clinical and medication history of the patients.

5.3.5 Sample size determination

The sample size was calculated according to the Hardy–Weinberg principle for population pharmacogenetics. The principle gives a formula for establishing the pharmacogenetic status of a known trait in a population based on the estimated prevalence of the variable of interest, the desired level of confidence and the acceptable margin of error. The formula is as follows:

$n= t^2 x p (1-p)/m^2$ Where: n = the required sample size.

t = Confidence level at 95 % (standard value of 1.96).
p = Estimated prevalence of the pharmacogenetic trait in the population, (0.01).
m = Margin of error at 5% (standard value of 0.05). For genetic polymorphism studies, the recommended sample size is given as: $n = (1.96)^2 x 0.01(1-0.01)/0.05^2 = 152$ or approximately, 160.

Since each person has two alleles of a gene, the minimum number of individuals required to confidently detect a single nucleotide polymorphism (SNP) with a

prevalence of 1% in the population is 80 individuals. Hence for this study, a minimum of 80 study participants were required but where feasible, more participants were recruited.

5.3.6 Sampling method and recruitment

A total of 200 psychiatric in – patients were recruited from the civil male and female wards in the year 2009 by a convenient sampling strategy. The recruitment exercise was conducted by two psychiatric nurses who were conversant with the ward procedures. Patients whose mental state enabled them to participate individually in daily ward procedures such as queuing for meals, bathing, and changing clothes were deemed fit to participate in the study. Those meeting study criteria and had a signed consent were recruited by filling the first part of a comprehensive data collection instrument that captured details of the in-patient number, biodata and demographic characteristics. On recruitment, a 5.0 ml blood sample was obtained from each subject for genotyping studies.

5.3.7 Inclusion and exclusion criteria

The study participants were male or female adult psychiatric in - patients admitted to the civil wards of Mathari Mental Hospital. They were to be in a mental state that enabled them to communicate individually and to voluntarily participate in study procedures by signing the consent form. Only patients who had stabilized on maintenance therapy were included. The patients were required to have the support of a spouse, parent or a guardian. Patients who were too sick to participate individually in study procedures were excluded. Also excluded were those who were newly admitted and undergoing therapy for acute illness as well as those admitted to the maximum security unit.

5.3.8 Data Collection

A data collection instrument was designed to be filled in two parts. The first part was filled during recruitment and blood sample collection. It was used to record

in-patient file number as well as to collect patient demographic data including age, sex, ethnicity and ethnicity of parents, place of residence and contact address. The second part of the form was filled during the review of patient medical records.

5.3.9 Review of Medical Records

The in-patient file number previously recorded on recruitment was used to retrieve medical records pertaining to the clinical management of the patient in the last 12 months. The files were reviewed and relevant information recorded in the second part of the data collection instrument. The information collected included the admission and discharge dates, diagnosis of mental illness, concurrent illness, psychotropic medications (drug name, strength, dose, and duration), concurrent medications, adverse drug reactions and management and number of admissions during the last 12 months.

5.3.10 Determination of CYP2C19 and CYP2D6 genotypes

Genotyping was done according to protocols described in details in Chapter Three, section 3.2.1.3.7 for *CYP2C19* and section 3.1.3.10 for *CYP2D6*. Observed genotypes of *CYP2D6* and *CYP2C19* were recorded from PCR-RFLP banding patterns.

5.3.11 Case definitions

5.3.11.1 Predicted drug metabolism phenotypes

Predicted drug metabolism phenotypes were derived from observed genotypes of *CYP2C19* and *CYP2D6* according to a semi - quantitative gene dose (SGD) algorithm described by Steimer *et al.* (2004). In this method, observed genotypes were rated according to the number of functional alleles (Table 5.2). A nonfunctional allele (such as *CYP2D6**4, *CYP2D6**5, *CYP2C19**2 and *CYP2C19**3) was assigned a gene dose value of 0, a partially functional allele (such as *CYP2D6**17 and *CYP2D6**29), a gene dose value of 0.5, a fully

functional allele (*CYP2D6**1, *CYP2D6**2 or *CYP2C19**1), a gene dose value of 1. The SGD of an individual is the sum of the gene dose value of both alleles in the observed genotype. In this respect, the poor metabolizer phenotype (PM) with two non-functional alleles has a gene dose of 0, the intermediate metabolizer phenotype (IM) which could have either a non-functional and a partially functional, a non-functional and a functional, or two partially functional alleles has gene dose of 0.5 – 1. The extensive metabolizer phenotype (EM) with either a partially functional and a functional allele or two functional alleles has a gene dose of 1.5 – 2 (Table 5.2.)

Table 5.2: Predicted drug metabolism phenotypes by the Semi-quantitative gene dose

Allele	Example	Activity	Gene dose	Genotype	SGD rating	Predicted phenotype
wt	*CYP2D6**1, *CYP2D6**2 *CYP2C19**1,	Full	1	wt/wt	2	EM
mt-p	*CYP2D6**17, CYP2D6*29	Partial	0.5	wt/mt-P	1.5	EM
				mt-p/mt-p	1	IM
mt	*CYP2D6**4, *CYP2C19**2 *CYP2C19**3,	None	0	mt/mt	0	PM
				wt/mt	1	IM
				mt/mt-P	0.5	IM
deletion	*CYP2D6**5	None	0	del/del	0	PM
				wt/del	1	IM
				mt/del	0	PM
				mt-P/del	0.5	IM

SGD rating - Semi -quantitative gene dose rating: **EM** = 1.5 -2, **IM** = 0.5 - 1, **PM** = 0.
mt-P- mutant allele with partial activity. **mt**- mutant allele with no activity. **wt**- wild type allele with full activity
EM - extensive metabolizer, **IM** - intermediate metabolizer, **PM** - poor metabolizer.

5.3.11.2 Classification of psychotropic drugs

Psychotropic drugs in this study were classified into therapeutic classes according to the anatomical, therapeutic and chemical (ATC) classification guidelines (WHOCC, 2012). ATC is a classification system in which drugs are divided into different groups depending on the target organ of action and their chemical, pharmacological, and therapeutic properties.

5.3.11.3 Psychotropic dosage regimen

Dosage regimens prescribed in this study were defined in terms of the prescribed daily dose (PDD) which was the total dose of a particular drug per day for oral medications or monthly for injections. The PDD was expressed as multiples of the defined daily dose (DDD) as shown in Table 5.3. This allowed for the calculation of cumulative drug consumption for each patient. The DDD is a theoretical unit of measurement which is defined as the assumed average maintenance dose per day for a drug used for its main indication in adults(WHOCC, 2012). A PDD/DDD ratio of 1 indicated that the dose prescribed was equal to the DDD of that drug, a ratio greater than 1 indicated a dosage higher than the DDD of that drug; whereas a ratio less than 1 meant that the PDD was lower than the DDD of that drug. The DDD used in this study were obtained from the WHO Collaborating Centre for Drug Statistics Methodology website as shown in Table 5.3. For each new therapeutic agent introduced in the market, the WHOCC determines the appropriate DDD and a regularly updated list of all medications with the corresponding DDDs is accessible at the website. In cases where the DDD was not available for a particular drug, as was the case of benzhexol, the Kenyan Clinical Guidelines, 2010 edition was used (Table 5.3).

Table 5.3: Defined Daily Dose (DDD) for psychotropic drugs in this study

	ATC	DDD (mg)			
Psychotropic drugs	**ATC code**	**Oral**	**Parenteral**	**Depot**	**Rectal**
Chlorpromazine	NO5AA01	300	100	–	300
Haloperidol	NO5AD01	8	8	3.3	–
Fluphenazine	NO5AB02	10	–	1	–
Trifluoperazepine	NO5AB06	20	8		20
Zuclopenthixol	NO5AF05	30	–	15	–
Flupenthixol	–	–	–	–	–
Thioridazine	NO5AC02	300	–		–
Benzhexol	–	–	–	–	–
Carbamazepine	NO3AF02	1000	–	–	1000
Amitriptyline	NO6AA09	75	75	–	–
Diazepam	NO5BA01	10	–	–	10
Phenobarbitone	–	–	–	–	–

– Not available

5.3.11.4 Mental diseases

The mental diseases encountered in this study were defined and coded according to the WHO International Classification of Diseases and Health related Problems, Tenth Revision, (ICD 10, 2010).

5.3.11.5 Psychotropic drugs and CYP metabolism

The prescribed psychotropic drugs were categorized according to their metabolism as substrates, inhibitors or inducers of CYP2D6, CYP2C19, CYP3A4 and CYP1A2 (Flockhart, 2007). These CYPs are the main enzymes involved in the metabolism of psychotropic drugs. In this study, CYP metabolism profiles were

derived from the Cytochrome P450 Drug Interactions Table, by the division of Clinical Pharmacology, Indiana University, School of Medicine, version 5.0, 2009.

5.4 Data Analysis

A database was constructed in Excel 2007 containing data fields on patient codes, demographics, mental diseases, medications, dosage regimens, genotypes, phenotypes and metabolic profiles. Observed and predicted genotypes were compared by the chi-square goodness of fit test. Allele frequencies were calculated from observed genotypes according to the Hardy-Weinberg equation, $p2 + 2pq + q2 = 1$.

CYP2D6 and *CYP2C19* genotype and allele frequencies were compared between psychiatric in-patients and Kenyan Bantu controls by the use of the chi-square or Fishers Exact test. Categorical data including demographic characteristics, medication patterns, genotypes, allele frequencies as well as predicted phenotypes were grouped and analyzed using chi square or Fisher's exact test. These data were presented in tables and charts using frequencies and percentages. Both bivariate and multivariate analyses were used to determine the medication patterns withCYP2D6 and CYP2C19 phenotypic characteristics (extensive, intermediate and poor metabolism). In bivariate analyses, odds ratios (OR) and 95% confidence intervals (CI) for the association between enzyme (CYP2D6 and CYP2C19) phenotypic characteristics among the patients and their characteristics were calculated using ordered logistic regression.

Bivariate analyses were used to identify the key covariates that influenced patient dependent variables. In multivariate analyses, a manual backward elimination approach was utilized to reach the most parsimonious model including factors that were independently associated with enzyme phenotypic characteristics. The significance level was set at $p \leq 0.05$. All statistical analyses were performed using STATA v 9.2 (StataCorp LP, Texas USA).

5.5 Results

5.5.1 Baseline characteristics of the psychiatric in-patients

Of the 200 psychiatric in-patients recruited into the study, complete data was available for 193. Seven patients with incomplete data were excluded from the analysis. The demographic characteristics of the 193 psychiatric in-patients are shown in Table 5.4.

Table 5.4: Baseline characteristics of the psychiatric in-patients at Mathari Hospital

Characteristic	Proportion of patients				
	n	%	χ^2	df	P
Age in Years					
> 20	14	7.3			
21 - 30	**92**	**47.7**	104.301	4	**0.0001**
31 - 40	48	24.9			
41 - 50	30	15.5			
<51	9	4.7			
Sex					
Female	54	28.0			
Male	**139**	**72.0**	**38.348**	**1**	**0.0001**
Ethinicity					
Bantus	**165**	**85.5**	**236.311**	**2**	**0.0001**
Nilotes	15	7.8			
Cushites	13	6.7			
Type of illness					
Bipolar Disorder	19	10			
Depressive illness	9	4.7			
Drug induced psychosis	26	13.7			
Epileptic psychosis	6	3.2			
Psychotic disorder	27	14.2			
Schizoaffective disorder	8	4.2			
Schizophrenia	97	50.3	264.063	7	0.0001
Senility	1	0.5			
Hospital adminisions					
1 to 2	**97**	**50.3**	**111.011**	**3**	**0.0001**
3 to 4	78	40.4			
5 to 6	18	9.3			
Substance abuse					
Yes	80	43.2	3.378	1	0.066
No	113	58.5			

The mean age of the 193 in-patients was 31.3 years (range 14 – 88 years) with the majority being aged 21 to 30 years (48%). The majority of the participants were males (72.0%) whereas 28.0% were females. The Bantus were the majority (85.5%) while the Nilotes (7.8%) and the Cushites (6.7%) had almost an equal proportion. Mental diseases which were encountered in the psychiatric in-patients are shown in Table 5.4. Schizophrenia was the most common mental disease (50.3%). Various types of psychotic disorders were also common (31.0%), particularly drug induced psychosis (14%). Other diseases included bipolar disorder (10%), depressive illness (5%) and one case of senility of old age.

The Majority of the psychiatric in-patients (50.3%) had been admitted 1 to 2 times in the course of the 12 months preceding the study as shown in Table 5.4. The proportion of psychiatric in- patients who used substances of abuse was 43% (Table 5.4). The substances of abuse encountered in this study included alcohol, marijuana, cannabis, khat, heroin, solvents and cigarettes.

5.5.2 Medications used by the psychiatric in- patients

Table 5.5 shows the six different classes of the psychotropic drugs administered to the psychiatric in-patients during this study. These different medications were prescribed 629 times to the patients.

Table 5.5: Therapeutic classes of medications used in the psychiatric in-patients at Mathari Hospital

Therapeutic class	Drugs	Proportion of prescriptions	
Antipsychotics		n	%
	Chlorpromazine	168	26.7
	Haloperidol	81	13
	Fluphenazine	55	8.7
	Trifluoperazepine	17	2.7
	Zuclopenthixol	13	2.1
	Flupenthixol	3	0.5
	Thioridazine	1	0.2
		338	53.9
Anticholinergics	Benzhexol	168	26.7
Anticonvulsant & mood stabilizer	Carbamazepine	76	12.1
Antidepressants	Amitriptyline	32	5.1
Anxiolytics	Diazepam	11	1.7
Anticonvulsants	Phenobarbitone	4	0.6
	TOTAL	**629**	

The most prescribed drugs belonged to antipsychotic class (54%) as shown in Table 5.5. The antipsychotic drugs used included chlorpromazine (27%), haloperidol (13%), fluphenazine (9%), trifluoperazepine (3%), zuclopenthixol (2%), flupenthixol (0.5%) and thioridazine (0.2%), (Figure 5.1). The other drugs prescribed for this patient population included the anti-cholinergic agent benzhexol (trihexyphenidyl) (27%), the anti-convulsant and mood stabilizer drug, carbamazepine (12%), the antidepressant drug, amitriptyline (5%), the anxiolytic drug diazepam (2%) and the anticonvulsant drug, phenobarbitone(1%).The most frequently used drug combination was chlorpromazine given orally together with

benzhexol. The Non psychotropic drugs used in this patient population were antimalarials (amiodiaquine), metronidazole, vitamins, analgesics and amoxicillin. However the frequency of use of these agents was less than 0.5 %.

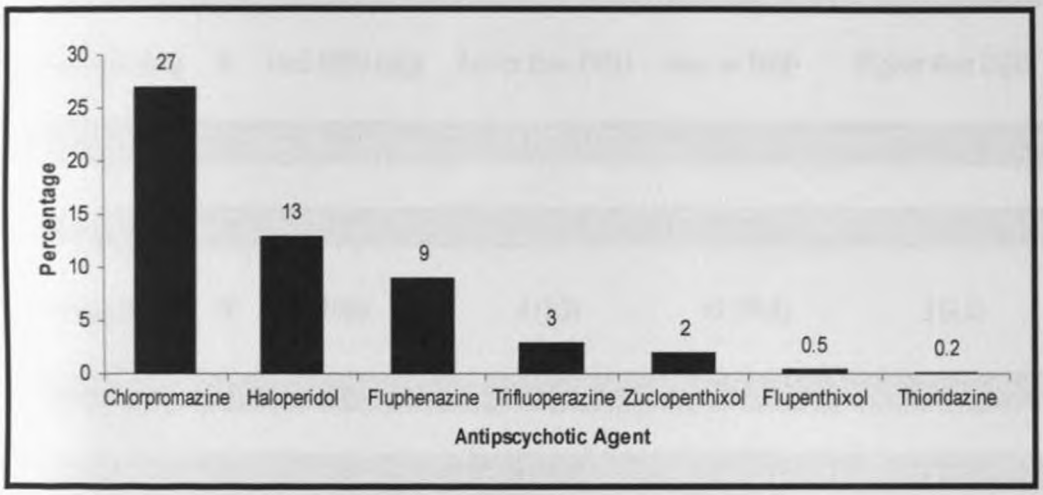


Figure 5.1: Frequency of prescription for various antipsychotic agents used in psychiatric in-patients at Mathari Hospital.

5.5.3 Psychotropic dosage regimens

Comparison of the prescribed daily dose (PDD) of oral psychotropic drugs with the corresponding defined daily dose (DDD) is shown in Table 5.6. For chlorpromazine and amitriptyline, over 50% of the patients received doses higher than the DDD whereas in the case of haloperidol, over 90% of the doses were higher than the DDD. On the other hand, over 90% of the patients on carbamazepine received doses that were similar to the DDD. As for benzhexol, over 50% of patients received doses that were lower than those recommended by the Kenya Clinical Guidelines for the treatment of psychotropic drug induced extrapyramidal effects (EPS).

Table 5.6: Comparison of psychotropic dosage regimens with DDD in psychiatric in-patients

			Proportion of oral prescriptions - n (%)		
Psychotropic drug	N	Oral DDD (mg)	Lower than DDD	Same as DDD	Higher than DDD
Chlorpromazine	156	300	53 (34.0)	12 (7.7)	91(58.3)
Benzhexol	168	15*	87 (51.8)	68 (40.5)	13 (7.7)
Carbamazepine	76	1000	4 (5.3)	69 (90.8)	3 (4.0)
Haloperidol	33	8	0(0)	2 (6.1)	31 (94.0)
Amitriptyline	32	75	8 (25.0)	8 (25.0)	16 (50.0)
Trifluoperazine	17	20	5 (29.4)	7 (41.2)	5 (29.4)

* - From Kenya Clinical Guidelines, 2010.
Oral DDD (mg) - www.whocc.no/atcddd
N - number of oral prescriptions

5.5.4 Number of drugs prescribed

The number of drugs prescribed for this psychiatric in-patient population is shown in Figure 5.2. The majority of psychiatric in- patients were taking 3 to 4 (68%) different drugs, 22% were taking 1 to 2 different drugs whereas 10% were taking more than 5 different drugs. ($\chi 2 = 107.389$; $P < 0.001$).

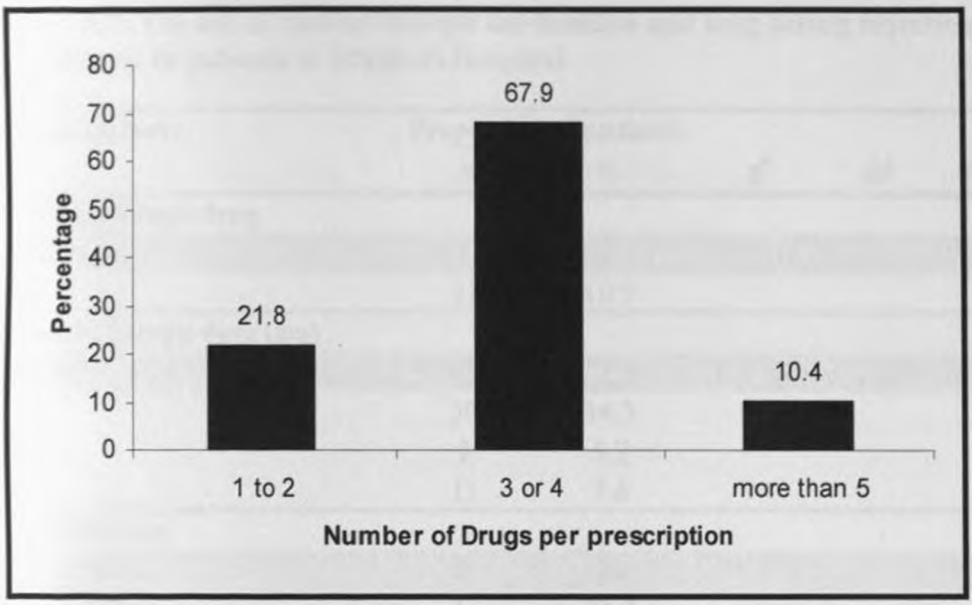


Figure 5.2: The numbers of drugs prescribed for psychiatric in-patients at Mathari Hospital

5.5.5 Use of anti - cholinergic medication

The use of anticholinergic medication and the dosage regimen are shown in Table 5.7. The majority of the psychiatric in-patients were on the anticholinergic agent benzhexol (89%) ($\chi 2 = 118.14$; $P < 0.001$). The dosage ranged from 5mg to 20mg per day with the majority taking 5mg (53%). Doses ranging from 10 – 15 mg were administered to 39.5% of the in-patients. A high dose of 20mg was being administered to 8% of the in - patients ($\chi 2 = 107.389$; $P < 0.001$).

Table 5.7: The use of anticholinergic medication and long acting injections by psychiatric in-patients at Mathari Hospital

Characteristic	Proportion of patients				
	n	%	χ^2	df	P
Anticholinergic drug					
Yes	172	89.1	118.14	1	0.0001
No	21	10.9			
Anticholinergic dose (mg)					
5	91	52.9	107.349	3	0.0001
10	59	34.3			
15	9	5.2			
20	13	7.6			
LA Injection					
Fluphenazine	54	28	70.211	3	0.0001
Haloperidol	47	24.4			
Zuclopenthixol	11	5.7			
Flupenthixol	2	1			
No LA injection	79	40.9			

5.5.6 Use of long acting injections

The use of long acting (LA) injections and dosage regimen is shown in Table 5.7. A total of 114 (59%) of the psychiatric in-patients were on long acting injections of different types. The most commonly used long acting injection was fluphenazine injection (28%), ($\chi 2 = 70.211$; $P<0.001$).

5.5.7 Psychotropic drugs and CYP metabolic profiles

The psychotropic drugs prescribed and relationship to metabolism by CYP2D6, CYP2C19, CYP1A2 and CYP3A4 is shown in Table 5.8. Most of the prescriptions contained substrates (97%) and inhibitors (94%) of CYP2D6 whereas 19% had CYP2C19 substrates and 38% had CYP2C19 inducers; 60% had CYP1A2 substrates whereas 95% and 39% had CYP3A4 substrates and inducers respectively.

Table 5.8: Psychotropic drugs used in psychiatric in-patients and CYP metabolism profiles

Therapeutic class	Prescription		CYP Metabolic profile			
	n	%	CYP2D6	CYP2C19	CYP1A2	CYP3A4
Antipsychotic						
Chlorpromazine	168	27.0	substrate &inhibitor		substrate	substrate
Haloperidol	81	13.0	substrate &inhibitor		substrate	substrate
Fluphenazine	55	9.0	substrate &inhibitor		substrate	
Trifluoperazepine	17	3.0			substrate	
Zuclopenthixol	13	2.0	substrate			
Flupenthixol	3	0.9	substrate		substrate	substrate
Thioridazine	1	0.3	substrate		substrate	
Anticholinergic						
Benzhexol	168	27.0				
Mood stabilizers						
Carbamazepine	76	12.0		inducer		substrate&inducer
Antidepressant						
Amitriptyline	32	5.1	substrate &inhibitor	substrate		substrate
Anxiolytic						
Diazepam	11	1.7		substrate	substrate	substrate
Anticonvulsant						
Phenobarbitone	4	0.6		substrate &inducer	inducer	inducer
TOTAL	**629**					

compiled from Flockhart, 2007

5.5.8 *CYP2D6* genotype and allele frequencies in psychiatric in-patients

Observed and expected genotypes of *CYP2D6* and *CYP2C19* were in conformity with Hardy – Weinberg proportions. Genotype frequencies for *CYP2D6* in the in-patient population are shown in Table 5.9. The most common genotype was *CYP2D6**1*17 (26%) while the least common genotypes (1% each) were *CYP2D6**1*5, *2*4, *2*29, *4*17, *5*5, *5*29, and *29*29 ($P < 0.001$). Observed genotypes were used to predict metabolic phenotypes. The majority of

the these genotypes predicted CYP2D6 extensive metabolism (EM) (74%) (P <0.001) (Table5.9). CYP2D6 genotype frequencies were also compared between psychiatric in-patients and Kenyan Bantu controls shown in Table 5.9

Table 5.9: CYP2D6 genotype frequencies in psychiatric in-patients in comparison to Kenyan Bantu controls

CYP2D6	Kenyan Bantu		Psychiatric in-patients			
Genotype	n	%	n	%	χ^2	P
EM						
*1 /*1	10	11.4	30	15.5	0.86	0.353
*1/*2	10	11.4	27	13.5	0.24	0.625
***1/*17**	**10**	**11.4**	**50**	**26**	**7.59**	**0.006**
*1/*29	4	4.5	13	6.7	0.51	0.476
***2/*2**	**10**	**11.4**	**8**	**4.1**	**5.23**	**0.022**
*2/*17	10	11.4	13	6.7	1.72	0.19
*2/*29	1	1.1	2	1.0	0.01	0.940
IM						
*1/*4	1	1.1	3	1.6	0.08	0.784
*1/*5	1	1.1	2	3.1	0.97	0.326
*2/*4	1	1.1	2	1	0.01	0.940
*2/*5	1	1.1	1	0.5	0.33	0.568
*4/*17	1	1.1	2	1.0	0.01	0.940
*4/*29	0	0	0	0		
*5/*17	2	2.3	3	1.6	0.18	0.673
*5/*29	0	0	2	1	0.92	0.339
*17/*17	15	17.0	27	14	0.44	0.506
***17/*29**	**6**	**6.8**	**3**	**1.6**	**5.38**	**0.020**
*29/*29	4	4.5	2	1.0	3.41	0.060
PM						
*4/*4	0	0	0	0		
*4/*5	0	0	1	0.5	0.46	0.500
*5/*5	1	1.1	2	1.0	0.01	0.940
N	88		193			
Phenotypes						
EM	55	62.5	143	73.6	3.52	0.060
IM	32	36.4	47	24.4	2.86	0.091
PM	1	1.1	3	1.6	0.08	0.784
N	88		193			

N - number of subjects

EM - extensive metabolizers. IM - intermediate metabolizers. PM - poor metabolizers

There were significant differences in the distribution of *CYPD6**1/*17 (P = 0.006), *CYP2D6**2/*2 (P = 0.022) and *CYP2D6**17/*29 (P = 0.020). However there was no difference in the proportions of CYP2D6 EM, IM and PM between the two populations. *CYP2D6* allele frequencies in psychiatric in-patients are shown in Table 5.10. The most abundant allele was *CYP2D6**1 (40%) followed by the defective active allele *CYP2D6**17 (32.4%). The null allele *CYP2D6**4 (2%) and the deletion *CYP2D6**5 (3%) occurred at very low frequencies. Allele frequencies were also compared between psychiatric in-patients and Kenyan Bantu controls as shown in Table 5.10. There were significant difference in the distribution of *CYP2D6**1 (P = 0.012) and *CYP2D6**2 (P = 0.012).

Table 5.10: CYP2D6 and CYP2C19 allele frequencies in psychiatric in-patients in comparison to Kenyan Bantu controls

Allele	Kenyan Bantu		Psychiatric in-patients			
CYP2D6	n	%	n/N	%	χ^2	P
***1**	**46**	**26.1**	**152**	**39.4**	**9.27**	**0.002**
***2**	**43**	**24.4**	**60**	**15.5**	**6.37**	**0.012**
***4**	3	1.7	8	2.1	0.09	0.77
***5**	6	3.4	15	3.9	0.12	0.729
***17**	59	33.5	125	32.4	0.07	0.790
***29**	19	10.8	32	8.4	0.92	0.338
N	176		386			
CYP2C19						
***1**	155	84.2	310	80.3	1.28	0.258
***2**	29	15.8	68	17.6	0.3	0.582
***3**	**0**	**0**	**8**	**2.1**	**3.86**	**0.049**
N	184		386			

n - number of alleles.

N - Total number of alleles

5.5.9 CYP2C19 genotype and allele frequencies in in-patients

Observed and expected genotypes of *CYP2C19* were in conformity with Hardy – Weinberg proportions. *CYP2C19* genotype frequencies are shown in Table 5.11. The most common genotype was *CYP2C19**1*1 (67%) while the least common genotypes was *CYP2C19**1*3 (1.6%) ($P < 0.001$). Observed genotypes were used to predict metabolic phenotypes. The majority (67%) of these genotypes predicted CYP2C19 extensive metabolism ($P < 0.001$) (Table 5.11). Comparison of CYP2C19 phenotype frequencies between psychiatric in-patients and Kenyan Bantu controls is also shown in Table 5.11. There were no significant differences between the two populations.

Table 5.11: CYP2C19 genotype frequencies in psychiatric in-patients in comparison to Kenyan Bantu controls

CYP2C19	Kenyan Bantu		Psychiatric Patients			
Genotypes	n	%	n	%	χ^2	P
EM						
*1/*1	65	70.7	130	67.4	0.31	0.577
IM						
*1/*2	25	27.2	47	24.4	0.26	0.609
*1/*3	0	0	3	1.6	1.44	0.23
PM						
*2/*2	2	2.2	8	4.1	0.71	0.399
*2/*3	0	0	5	2.6	2.42	0.120
*3/*3	0	0	0	0		
Phenotypes						
EM	65	71.0	130	67.4	0.31	0.577
IM	25	27.0	50	26.0	0.05	0.821
PM	2	2.2	13	6.7	2.59	0.107
N	92		193			

N - number of subjects

EM - extensive metabolizers. IM - intermediate metabolizers. PM - poor metabolizers

*CYP2C19*allele frequencies in psychiatric patients are shown in Table 5.10. The wild type allele CYP2C19*1 (80.3%) was most abundant allele whereas the null allele *CYP2C19*3 (2.1%) was the least. *CYP2C19* allele frequencies were also compared between psychiatric patients and Kenyan Bantu controls as shown in Table 5.10. The only significant difference was found in the distribution of *CYP2C19*3 which was not detected in the Kenyan Bantu controls but occurred at a low frequency of 2% in the psychiatric in-patients (P = 0.049).

5.5.10 Influence of *CYP2D6* and *CYPP2C19* on psychotropic dosage regimens

Dosage regimens in psychiatry are often done empirically, hence, exploratory analysis was undertaken to determine phenotypic factors that influenced dose selection. The influence of CYP2D6 and CYP2C19 predicted phenotypes on dose selection was evaluated by ordered logistic regression. Chlorpromazine, benzhexol and carbamazepine were selected as the marker drugs since they were the most widely prescribed agents in this patient population. In the case of chlorpromazine, there was no correlation between the predicted phenotypes and the dose prescribed (Table 5.12). However, substance abuse, (OR 0.492; 95% CI 0.252 – 0.9359, P = 0.037) and gender (OR 0.35; 95% CI 0.165 – 0.741; P = 0.006) were the most significant predictors of chlorpromazine dose. There was an association between the dose of chlorpromazine and substance abuse as well as gender whereby patients without a history of substance abuse as well as females tended to receive lower doses of chlorpromazine (Table 5.12).

Table 5.12: Determinants of chlorpromazine dose in psychiatric in-patients at Mathari Hospital

Variable	Crude Odds Ratio	95 % CI	P value	Adjusted OR	95 % CI	P value
Substance Abuse	**0.492**	**0.252 - 0.959**	**0.037**	**0.458**	**0.225 - 0.932**	**0.031**
Ethno-linguistic group	0.9	0.509 -1.592	0.718			
Ethnic group	1.021	0.913- 1.142	0.72			
Diagnosis	0.906	0.759 -1.080	0.27			
CYP2D19 phenotype	0.709	0.690 -1.724	0.709			
CYP2D6 phenotype	0.744	0.304 -1.823	0.518			
Gender	**0.35**	**0.165 - 0.741**	**0.006**	**0.336**	**0. 156 - 0.726**	**0.006**
Age	1.029	0.993 - 1.065	0.113			
Number of drugs prescribed	0.606	0.660 - 1.275	0.606			
Use of Benzhexol	1.391	0.732 - 2.645	0.313			
Diazepam	2.15E-17	n/a	1			
Amitryptyline	1.177	0.394 - 3.517	0.77			
Carbamazepine	0.318	0.019 - 5.428	0.429			
Trifluoperazine	6	0.221 - 162.53	0.287			
Injection use	0.97	0.692 - 1.361	0.862			
CYP2C19 Substrate	0.56	0.233 - 1.352	0.198			
CYP2D6 Substrate	2.02E-08	n/a	n/a			

Determinants of Benzhexol dose are shown in Table 5.13. Bivariate analysis was undertaken to explore the factors that determined the dose of benzhexol in this patient population. A statistically significant association between the dose of benzhexol and CYP2D6 IM phenotype, the use of CYP2C19 substrates and the number of drugs prescribed was observed (Table 5.13). These associations remained significant even after controlling for confounding except for the use of CYP2C19 substrates.

Table 5.13: Determinants of benzhexol dose in psychiatric in-patients at Mathari Hospital

Variable	Crude OR	95 % CI	P value	Adjusted OR	95 % CI	P value
Substance Abuse	0.653	0.343 - 1.242	0.193			
Ethno-linguistic group	0.882	0.520 - 1.497	0.642			
Ethnic group	0.993	0.894 - 1.103	0.897			
Diagnosis	0.882	0.739 - 1.053	0.166			
CYP2D6 phenotype	**1.999**	**1.189 - 3.360**	**0.009**	**1.77**	**1.111- 2.819**	**0.016**
CYP2C19 phenotype	1.949	0.801 - 4.740	0.141	2.217	0.871 - 5.640	0.095
Gender	0.643	0.312 - 1.323	0.231			
Age	1.01	0.982 - 1.038	0.501			
Number of Drugs	**0.675**	**0.479 - 0.951**	**0.025**	**0.687**	**0.473 - 0.997**	**0.048**
Diazepam	1.00E+00	0.020 - 50.397	1			
Amitriptyline	1.129	0.408 - 3.125	0.816			
Carbamazepine	2.716	0.247 - 29.882	0.414			
Trifluoperazine	1.044	0.240 - 4.553	0.954			
Haloperidol	0.000001	no convergence				
Injection use	0.847	0.558 - 1.285	0.436			
CYP2C19 Substrate	**0.334**	**0.131 - 0.855**	**0.022**	**0.473**	**0.171 - 1.304**	**0.148**
CYP2C19 Inhibitor	n/a					
CYP2C19 Inducer	0.707	0.361 - 1.385	0.312			
CYP2D6 Substrate	4.23E+00	0.380 - 47.10	0.241			
CYP2D6 Inhibitor	collinearity					
CYP1A2 Substrate	0.881	0.461 - 1.683	0.701			

Determinants of carbamazepine dose are shown in Table 5.14. There was a significant association between the dose of carbamazepine and CYP2C19 phenotypes (OR 0.171; (95% CI 0.036 – 0.810; P = 0.026). The association was such that lower doses of carbamazepine were prescribed for patients who were CYP2C19 poor metabolizers.

Table 5.14: Determinants of carbamazepine dose in psychiatric in-patients at Mathari Hospital

Variable	Crude Odds Ratio	95 % CI	P value
Substance Abuse	1.109	0.147 - 8.395	0.92
Ethno-linguistic group	0.414	0.059 - 2.889	0.374
Ethnic group	0.888	0.632 - 1.2484	0.494
Diagnosis	1.518	0.854 - 2.696	0.155
CYP2C19 phenotype	**0.171**	**0.036 - 0.810**	**0.026**
CYP2D6 phenotype	0.412	0.041 - 4.114	0.45
Gender	3.754	0.265 - 53.263	0.328
Age	0.99	0.888 - 1.104	0.862
Number of drugs prescribed	0.671	0.246 - 1.830	0.436
Chlorpromazine	5.56E-01	0.133 - 2.336	0.423
Amitriptyline	1	0.833 -11.998	1
Benzhexol	2.108	0.272-16.336	0.475
Haloperidol	collinearity		
Injection use	1.317	0.750 - 2.311	0.338
CYP2C19 Inhibitor	n/a		
CYP2D6 Substrate	1.82E-01	0.016 - 203.8	0.804
CYP1A2 Inducer	collinearity		
CYP1A2 Substrate	0.944	0.125 - 7.097	0.955
CYP3A4 inducer	1.874	0.476 - 73.832	0.738
CYP3A4 inhibitors	no observations		
CYP3A4 inhibitors	collinearity		

5.5.11 Influence of *CYP2D6* and *CYP2C19* on medication patterns

The influence of predicted phenotypes of CYP2D6 and CYP2C19on psychotropic medication patterns is shown in Table 5.15 and Table 5.16. The use of more than 5 drugs per prescription was strongly associated with CYP2D6 IM ($P = 0.002$). Patients being admitted in hospital 1-4 times were more likely to be extensive metabolizers of CYP2D6 whereas those who had been admitted more than 5 times were more likely to be intermediate metabolizers of CYP2D6 ($P < 0.001$). The majority of patients in this study were on treatment with the anti-cholinergic drug benzhexol. Those taking a minimum dose of 5mg, were more likely to be EM (87%) of CYP2D6 ($P = 0.001$) whereas the use of 15 – 20 mg was strongly

associated with CYP2D6 IM ($P < 0.01$). The use of LA depot antipsychotic injections was also significantly associated with CYP2D6 EM ($P = 0.005$).

Table 5.15: Impact of *CYP2D6* and *CYP2C19* on medication patterns in psychiatric in – patients

Characteristic	CYP2D6				CYP2C19			
	N	EM	IM	P	N	EM	IM	P
Number of drugs								
1 to 2	42	34	8	0.334	42	26	13	0.397
3 to 4	131	100	28	0.19	131	86	34	0.865
> 5	20	9	11	**0.002**	20	17	3	0.173
Hospital admissions								
1 to 2	97	87	9	**<0.001**	97	70	24	0.453
3 to 4	78	49	26	**0.012**	78	45	23	0.17
5 to 6	18	6	12	**<0.001**	18	14	3	0.405
Anticholinergic drug								
Yes	172	125	44	0.024	172	113	46	0.403
No	21	18	3	**0.024**	21	16	4	0.403
Anticholinergic dose(mg)								
5	91	79	12	**<0.001**	91	66	21	0.346
10	59	41	17	0.484	59	31	23	**0.033**
15	9	2	5	**0.014**	9	6	0	**0.051**
20	13	3	10	**<0.001**	13	10	2	0.276
LA injection								
Yes	114	76	36	**0.005**	114	77	29	0.837
No	79	67	11	**0.005**	79	52	21	0.837

Table 5.16: Summary of factors influencing psychotropic medication patterns in psychiatric in-patients

Clinical covariates	Variable	Bivariate OR (95% CI)	P value	Multivariate OR (95% CI)	P value
Hospital admission					
1 to 2	**CYP2D6 EM**	**1.41 (1.07 - 2.00)**	**0.055**	**2.57 (1.09 - 6.03)**	**0.031**
5 to 6	**CYP2D6 IM**	**0.27 (0.13- 0.59)**	**0.001**	**0.14 (0.05 - 0.39)**	**0.0001**
Substance abuse	**CYP2D6 IM**	**3.47 (4.99 - 12.29)**	**0.054**	NS	NS
Number of drugs					
>5	**CYP2D6 IM**	**2.88 (1.16 - 7.18)**	**0.022**	NS	NS
LA injection					
Haloperidol	**CYP2D6 IM**	**0.21 (0.05 - 0.97)**	**0.046**	NS	NS
Flupenthixol	**CYP2D6 IM**	**0.14 (0.03 - 0.63)**	**0.01**	**0.14 (0.03 - 0.63)**	**0.01**
Chlorpromazine dose	CYP2D6 IM	0.74 (0.30 - 1.82)	0.52	NS	NS
	CYP2C19	0.71 (0.69 - 1.72)	0.71	NS	NS
	Substance abuse	**0.49 (0.25-0.96)**	**0.037**	**0.46 (0.23 - 0.09)**	**0.031**
	Gender	**0.35 (0.17 -0.74)**	**0.006**	**0.34 (0.16 - 0.73)**	**0.031**
Benzhexol dose	**CYP2D6 IM**	**1.20 (1.19 - 3.36)**	**0.009**	**1.77 (1.11 - 2,82)**	**0.016**
	Number of drugs	**0.68 (0.48 - 0.95)**	**0.025**	**0.69 (0.47 - 0.99)**	**0.048**
	CYP2C19 substrates	**0.33 (0.13 - 0.86)**	**0.022**	NS	NS
Carbamazepine dose	**CYP2C19**	**0.17 (0.04 - 0.81)**	**0.026**	NS	NS

OR - odds ratio, NS- not significant, EM- extensive, IM - intermediate, PM - poor metabolizers

5.6 Discussion

5.6.1 *CYP2D6* genetic variability in psychiatric patients

This study characterized clinically relevant SNPs of *CYP2D6* (*4,*5,*1*29) in Kenyan psychiatric in-patients. The CYP2D6 loci in this patient population was characterized by the high prevalence of the defective (reduced activity) alleles *CYP2D6**17 (32%) and *CYP2D6**29 (8%). This translated into a proportion of CYP2D6 slow metabolizers (CYP2D6 IM phenotype) of 24%. The majority of

psychiatric patients in this study were schizophrenics (54.4%). There is reported evidence of the involvement of CYP2D6in the endogenous metabolism of neuroactive amines and possibly in the pathogenesis of mental disorders such as schizophrenia (LLerena *et al.*, 1993; Stingl *et al.*, 2012). Studies report the involvement of CYP2D6 in the biotransformation of several neurotransmitters which may be functionally impaired in psychiatric disorders including schizophrenia (Niwa *et al.*, 2004; Peñas-LLedó *et al.*, 2009). Such neurotransmitters include dopamine, serotonin and neuroactive steroids (Hiroi *et al.*, 1998; Yu *et al.*, 2003).

Hence, there is a hypothesis that CYP2D6 is not only associated with the interindividual variability in response to antipsychotic drugs, but also with the vulnerability to psychiatric disorders such as schizophrenia. However, the possible implications of the high prevalence of the defective allele, *CYP2D6**17 in African populations and its possible role in the etiology and prognosis of schizophrenia warrants further investigations

The results of this study also revealed a low prevalence of CYP2D6 PMs (1.6%) in Kenyan psychiatric patients which is expected in the African setting. This observation was similar to reports from studies in other African psychiatric patients (2.3%) (Dandara *et al.*, 2001) and is attributed to the low prevalence of the null allele, *CYP2D6**4 in African populations. Similarly, a study in Spanish schizophrenics has also reported a low frequency of PM (2.3%) in psychiatric patients compared to healthy controls (8.5%) (Llerena *et al.*, 2007). That same study also reported that the frequencies of the null allele *CYP2D6**4 and the deletion allele CYP2D6*5 were lower in schizophrenics. However, in this study, there was no significant differences between the frequencies of *CYP2D6**4 (2.1%) and *CYP2D6**5 (4%) in psychiatric patients and those observed in Kenyan Bantu controls of *CYP2D6**4 (1.7%) and *CYP2D6**5 (3.4%).

5.6.2 CYP genotype based psychotropic dose adjustment

Chlorpromazine (27%) and haloperidol (13%) were the most commonly used antipsychotic drugs in this study. Most of the prescriptions had doses that were higher than the DDD in the case of chlorpromazine (58%) and haloperidol (94%). Chlorpromazine and haloperidol are benchmark antipsychotic drugs which are frequently used as standards in antipsychotic drug trials (Joy *et al.*, 2006; Adams *et al.*, 2007). Hence, the importance of defining factors that influence dosage regimens in different populations. The disparity between the published DDD and the dosages in this study is probably a reflection of the differences in the dosage regimens standardized for Caucasian populations and those being practically used in Kenyans.

In this study, both bivariate and multivariate analysis revealed that chlorpromazine dosage regimens were not correlated to CYP2D6 metabolic status (OR 0.744; 95% CI 0.304 – 1.823; P = 0.518) and CYP2C19 metabolic status (OR 0.709; 95% CI 0.690 – 1.724; P = 0.709) genotypes. Reports suggest that genetic determination of metabolic status is expected to bring clinical benefits to patients with regard to dosage adjustments aimed at reducing adverse drug reactions (Arranz and de-Leon, 2007). Methods for dosage adjustments based on CYP2D6 and CYP2C19 genotypes have been developed and published (Kirchheiner and Seeringer, 2007; Kirchheiner and Rodriguez-Antona, 2009). Depending on the impact of the CYP2D6-enzyme activity on the metabolism of a specific drug, dosage recommendations propose a 30 -70% dose reduction for PM and 135 -180% dose elevation for UM patients (Maier and Zobel, 2008). However such studies may have limited application in Asian and African populations where the CYP2D6 PM and UM status are rare. The prevalence of CYP2D6 PMs and IMs in this study was 1.6% and 23% respectively whereas that for CYP2C19 was 7% and 26% respectively. Hence, genotype dosage adjustments need to be optimized for

different populations taking into account the respective population pharmacogenetics as well as other relevant environmental covariates.

In this study, female gender and not using substances of abuse were significantly associated with the use of lower doses of chlorpromazine (OR 0.35, CI 0.156 – 0.741, P = 0.006). The use of lower antipsychotic dosage in women in this study was in agreement with other studies in drug naïve as well as chronically ill psychiatric patients (Melkersson *et al.*, 2001; Groleger and Novak-Grubič, 2010). The prevalence of substance abuse in this study was 43% with 14% of the patients being diagnosed with substance induced psychosis which was similar to reports of other studies (Duke *et al.*, 1994). Other studies have shown that smokers require higher doses of narcoleptics, possibly due to CYP enzyme induction and increased renal clearance by tobacco (Jarvik and Schneider, 1992; Ziedonis *et al.*, 1994).

Exposure to psychoactive substances (PAS) such as alcohol, cannabis, cocaine and tobacco is associated with negative consequences such as psychotic relapses, depressive episodes, homelessness, unemployment as well as legal and health problems (Mueser *et al.*, 1998). Such negative manifestations often culminate in raised antipsychotic drug dosage in an attempt to achieve better symptom control. Hence, this could explain observations in this study that lower doses of chlorpromazine were being used in patients who were not exposed to PAS. Environmental factors such as cigarette smoking, alcohol and diet can induce or inhibit metabolic pathways, which may affect plasma levels of antipsychotic drugs and metabolites. Therefore, the knowledge of diet, alcohol and smoking habits could be used as an indicator for the adjustment of therapeutic doses (Nebert and Dieter, 2000; Katoh *et al.*, 2010).

5.6.3 Extrapyramidal side effects

There was a high rate (89%) of prescribing the anticholinergic drug benzhexol (trihexyphenidyl) of at a low dose of 5mg. Centrally acting anticholinergic drugs

are used for the amelioration of extrapyramidal side effects (EPS) induced by antipsychotic medications (Gjerden *et al.*, 2009). Hence, the prescribing rate of benzhexol in this study was assumed to be a surrogate for the prevalence of EPS in this psychiatric patient population. Other studies have reported that the use of anticholinergic medication is highly correlated with clinical indices of extrapyramidal symptoms (Hong and Bishop, 2010; Peluso *et al.*, 2012). The Kenyan Clinical Guidelines recommend the use of 7.5 - 15mg benzhexol for the treatment of EPS. Hence, it is postulated that the high rate of low dose anticholinergic therapy at 5mg was a routine prophylaxis against EPS. The use of anticholinergic drugs for prophylaxis against EPS in long-term antipsychotic therapy has been reported in other studies (Ghio *et al.*, 2011).

This study reports that the use of high doses of benzhexol of 15 -20mg per day 22/172 (13%) which has been assumed to be a surrogate for severe episodes of EPS was significantly associated with CYP2D6 slow metabolism (CYP2D6 IM) (P =0.009) as well as the number of drugs prescribed. Other Studies have reported increased side effects due to antipsychotic drug therapy in CYP2D6 intermediate metabolizers (39%) compared to 22% CYP2D6 extensive metabolizers (Kobylecki *et al.*, 2009).It is also noteworthy that all the patients in this study were being treated exclusively with first generation, typical antipsychotic agents (FGA) such as chlorpromazine and haloperidol which are subject to a high incidence of EPS compared to the second generation, atypical antipsychotics (Ghio *et al.*, 2011; Peluso *et al.*, 2012).

5.6.4 Antipsychotic polypharmacy

The majority of patients in this study were on 3- 4 drugs (68%) whereas 20/193 (10.4%) were taking more than 5 drugs. In this study, antipsychotic polypharmacy of more than 5 drugs per prescription was associated impaired CYP2D6 metabolism. Patients having more than 5 drugs were 88% more likely to be CYP2D6 IMs compared to those taking 1-2 drugs (P = 0.022). In fact, of the 20

patients taking more than 5 drugs per prescription, 11/20 (55%) were intermediate metabolizers of the CYP2D6 enzyme (P = 0.002). Patients in this study were prescribed first generation typical antipsychotics (FGA) in combination therapy with other drugs including another antipsychotic, anticholinergic, antidepressants, mood stabilizers, anxiolytics or anticonvulsants. Studies report that most patients with schizophrenia and other psychotic illness have symptoms that either do not or only partly respond to monotherapy with antipsychotic medications (Loga-Zec and Loga, 2011; Bernardo *et al.*, 2012). The sustained use of combinations of antipsychotic medications also called "antipsychotic polypharmacy" is a common treatment strategy (Tiihonen *et al.*, 2012). Antipsychotic polypharmacy with two typical antipsychotic drugs has been reported with frequencies ranging from 10–69% (Procyshyn *et al.*, 2001; Englisch and Zink, 2012).

5.6.5 Frequency of hospitalization

This study reports that frequent hospitalization of up to 5- 6 times was associated with CYP2D6 IM status. Other studies have reported frequent hospitalization to be a predictive marker of non-adherence to antipsychotic therapy, severity or excercebation of mental illness, poor prognosis and use of depot injections (Law *et al.*, 2008). This study also observed that patients with 5-6 admissions were more likely to be CYP2D6 IMs compared to those with 3 - 4 admissions (P = 0.051). In fact, of the 18 patients who had more than 5 admissions, 12 (67%) were IMs of CYP2D6. Studies have reported that increased length of hospitalization was correlated to the CYP2D6 PM phenotype (Kropp *et al.*, 2006). The PM phenotype (predicted from genotype) has also been reported to be associated with more frequent switching of drugs and changes in dosage regimen, indicating an unsatisfactory response to treatment (Mulder *et al.*, 2006). Hence, *CYP2D6* genotyping has the potential to identify subgroups of patients likely to show unsatisfactory response and adverse effects or no effect at all on treatment with certain psychotropic drugs.

5.6.6 Use of depot Antipsychotics

Depot antipsychotics may help patients adhere to treatment because depot agents are administered by injection every two to four weeks, eliminating the need for daily dosing (Kane, 2011). In this study comprising 193 in-patients, 114 (59.1%) were on typical antipsychotic depot injections with the majority being given fluphenazine decanoate (28%). The other depot injections included haloperidol, zuclopenthixol and flupenthixol. Treatment guidelines for schizophrenia reportedly recommend that clinicians strongly consider depot medication for patients who may be non adherent to antipsychotic treatment regimens (Valenstein *et al.*, 2006; Peng *et al.*, 2011). In this study, the majority of patients on LA injections were EM of CYP2D6 (67%), P = 0.005). The use of these antipsychotic depot injections has been shown to reduce the risk of relapse and hospitalization and help improve patient long-term functional outcomes (Lieberman *et al.*, 2005; Law *et al.*, 2008).

5.6.7 Potential for drug – drug interactions

Results from this study showed that nearly all the patients who were CYP2D6 and CYP2C19 PMs and IMs were taking the anticholinergic drug against extrapyramidal side effects (EPS). Further, CYP2D6 IM was significantly associated with higher doses of benzhexol. This observation is suggestive of impaired hydroxylation of the antipsychotics in patients bearing CYP2D6 PM and IM status leading to accumulation of active drugs, higher incidence of EPS and higher doses of the anticholinergic drug. Other CYP isoforms such as CYP1A2, 2C19 and 3A4 are involved in *N*-demethylation and ring sulfoxidation pathways (Wójcikowski *et al.*, 2010). Studies report that the genetic polymorphism of CYP2D6 and CYP2C19 leads to interindividual differences in the degree of psychotropic metabolism and in plasma drug concentrations (Zanger *et al.*, 2004).

Potential for drug - drug interactions between tricyclic antidepressants (TAD) and neuroleptics exists in this study. Results showed that amitriptyline was prescribed to a total 32 patients where it was in combination with one or two neuroleptics such as chlorpromazine and fluphenazine or haloperidol. Hence, the neuroleptics could cause inhibition of hydroxylation reactions of CYP2D6 and possibly lead to accumulation of amitriptyline. However, it is also noteworthy that these patients were also on therapy with the mood stabilizer carbamazepine, which is a potent inducer of CYP2C19. CYP2C19 is the major CYP responsible for the N-demethylation of amitriptyline leading to the formation of the potent active metabolite nortripyline. Hence, it is important to note that in a study such as this which was carried out in a naturalistic, uncontrolled setting, polypharmacy and other pharmacotherapy practices come into play to modulate response to medications.

5.7 Summary of major findings and conclusion

This is the first study to characterize SNPs of *CYP2D6* and *CYP2C19* in Kenyan psychiatric inpatients and to evaluate their influence on psychotropic medication patterns.

This study has established that psychiatric inpatients were mainly male schizophrenics aged 20 – 40 years, who had been hospitalized at least twice within the year with the majority receiving up to 4 drugs of antipsychotic combination therapy. *CYP2D6*and CYP2C19 genotypes in psychiatric in-patients predicted a low prevalence of poor metabolism phenotype but a high rate of slow metabolism phenotype (IM) of 24% and 26% respectively which was similar to that seen in Kenyan Bantus controls

CYP2D6 intermediate metabolism (IM) was strongly associated with covariates of psychotropic medication patterns with regard to polypharmacy of more than 5

drugs (55%) , more frequent hospitalization of 5- 6 times a year (67%) and the use of high doses (15 - 20 mg) of the anticholinergic drug benzhexol (68%).

Antipsychotic dosage regimens were at variance with DDD for chlorpromazine (58%). Haloperidol (94%) and was found to be influenced by gender and exposure to psychoactive substances of abuse. There was a high rate of anti-cholinergic use of 89% with 53% using a low dose (5 mg) as prophylactic, 40% (10 15 mg) for treatment of EPS and 8% (20 mg) as an indication of serious toxicity (8%). Benzhexol dosage was influenced by CYP2D6 IM, number of drugs prescribed and the use of CYP2C19 substrates.

The impact of genetic factors on psychotropic drug response in this patient population was also under the modifying influence of environmental factors such as diet, smoking, alcohol and other PAS. This, together with the high rate of antipsychotic polypharmacy of 3 - 5 drugs (78%) posed a high risk of drug – drug interactions in this patient population.

Prospective studies are required to elucidate the clinical implications and cost effectiveness of CYP2D6 and CYP2C19 intermediate metabolizer status on psychotropic treatment outcomes in psychiatric patients of African origin. Of further interest is a follow up on the high rate of antipsychotic polypharmacy in terms of cost implications and potential for drug – drug interaction and toxicity in well designed prospective studies.

Chapter Six

DETERMINATION OF *CYP2B6* GENETIC VARIANTS AND IMPACT ON NEVIRAPINE PLASMA EXPOSURE IN HIV PATIENTS AT THE KENYATTA NATIONAL HOSPITAL

6.1 Introduction

The WHO/UNAIDS estimates that over 33 million people are living with the human immunodeficiency virus type 1 (HIV-1) infection globally. While global HIV prevalence is only 1.0%, sub-Saharan Africa is particularly affected, with an overall prevalence of 5.0% (UNAIDS/WHO, 2011). In Kenya, an estimated 1.4 million adults are living with HIV-1 infection, with a reported prevalence of 7.4% among 15-64 year olds and a female to male ratio of 1.6 (NASCOP, 2007; Oluoch *et al.*, 2011). First-line ART in Kenya typically contains a backbone of two nucleoside reverse transcriptase inhibitors (NRTIs) plus one non-nucleoside reverse transcriptase inhibitor (NNRTI) which is either nevirapine or efavirenz or a protease inhibitor (PI). NNRTI-based regimens have been a preferred choice for many patients compared to PI-based regimens because of lower pill burden. This allows for better compliance to antiretroviral therapy (Trotta *et al.*, 2003) and the better lipid profile associated with NNRTI usage (Young *et al.*, 2005).

The use of nevirapine is limited by two main factors. These include an immune-mediated hypersensitivity reaction that manifests as hepatotoxicity, fever and/or skin rash (Lopez-Delgado *et al.*, 2012; Dong *et al.*, 2012) and a fragile genetic barrier to the development of drug resistance (Ngo-Giang-Huong *et al.*, 2012). Highly drug resistant viral strains rapidly emerge in suboptimal regimens. The etiology of sub-optimal responses is multi-factorial and may include variability in plasma drug exposure attributable to poor adherence, drug – drug interactions or variability in genes responsible for antiretroviral drug disposition (Darwich *et al.*, 2008; Khienprasit *et al.*, 2011).

Nevirapine is metabolized primarily by the cytochrome P450 3A4 and 2B6 enzymes into its major metabolites, 2-hydroxynevirapine and 3-hydroxynevirapine, respectively. There is also a minor contribution from CYP3A5 (Erickson *et al.*, 1999). Several studies have reported that the *CYP2B6* 516G>T variant allele is predictive of nevirapine plasma concentrations (Saitoh *et al.*, 2007; Heil *et al.*, 2012). Other studies have also found body weight, ethnicity, gender and underlying liver disease to be important in explaining nevirapine pharmacokinetics (Cooper and van Heeswijk, 2007).

The relationship between plasma exposure to nevirapine and virological response has been examined in various studies. Studies exploring the association of exposure to nevirapine with virologic response have yielded a target trough concentration of 3000 ng/mL (de Vries-Sluijs *et al.*, 2003). In another study, a nevirapine trough concentration greater than 4300 ng/mL was found to be predictive of maintenance of viral suppression for more than 3 years (González-de-Requena *et al.*, 2005). However, these values have not been confirmed among Kenyan HIV patients and the influence of CYP2B6 genetic variants has not been evaluated. The aim of this study was to determine the influence of *CYP2B6* genetic polymorphisms and other host characteristics on nevirapine plasma exposure and to evaluate their therapeutic impact in Kenyan HIV patients.

6.2 Objectives

6.2.1 Main objective

The main objective of this study was to determine the influence of CYP2B6 genetic variants on nevirapine plasma levels and to assess the impact on therapeutic outcomes in Kenyan HIV patients.

6.2.2 Specific objectives

The specific objectives of this study included:

1. To determine the prevalence of *CYP2B6*516 G>T variant in Kenyan HIV patients on nevirapine based antiretroviral therapy.

2. To determine the steady state nevirapine plasma concentrations in Kenyan HIV patients on nevirapine based antiretroviral therapy. .

3. To correlate CYP2B6 516 G>T genotypes and other host characteristics to nevirapine steady state plasma concentrations at 6 months of HAART

4. To evaluate the relationship between nevirapine plasma exposure at 6 months and therapeutic outcomes of HAART in Kenyan HIV patients.

6.3 Methodology

6.3.1 Ethical considerations

The study was conducted in accordance with the basic principles defined in Guidance for Good Clinical Practice and the principles enunciated in the Declaration of Helsinki(WMA, 2008). Ethical approval was obtained from the joint institutional review board (IRB) of the University of Nairobi and the Kenyatta National Hospital (KNH/UoN-ERC) before initiation of the study (Appendix 1). Each potential participant was furnished with comprehensive verbal and written information about the study. This included details about study aims, procedures and potential benefits and risks. Upon verbal consent, each subject signed a voluntary consent form (Appendix 2) before enrollment into the study. To ensure confidentiality of study subjects, no personal identification information including names of study subjects were collected. Filled questionnaires were identified by a code and held in safe custody.

6.3.2 Study design

A cross-sectional study was conducted to determine the distribution of *CYP2B6* genetic variants and steady state nevirapine plasma concentrations in Kenyan HIV patients. The subjects were required to have been on nevirapine based antiretroviral therapy for 6 months. They were recruited at the Comprehensive Care Centre (CCC) of Kenyatta National Hospital in the month of July 2009.

6.3.3 Study Population

Study subjects consisted of adult black Africans of both gender, who were HIV sero-positive and attending the comprehensive care clinic (CCC) at the Kenyatta National Hospital on a regular basis.

6.3.4 Sample size

The sample size was calculated according to the Hardy–Weinberg principle for population pharmacogenetics. The principle gives a formula for establishing the pharmacogenetic status of a known trait in a population based on the estimated prevalence of the variable of interest, the desired level of confidence and the acceptable margin of error. The formula is given as follows: $\mathbf{n = t^2 x p (1-p)/m^2}$ Whereby n is the required sample size; t is the Confidence level at 95 %(standard value of 1.96); p is the estimated prevalence of the pharmacogenetic trait in the population, (0.01) and m is the margin of error at 5% (standard value of 0.05).Hence, for genetic polymorphism studies, the recommended sample size was given as:

$n = (1.96)^2 \times 0.01(1-0.01)/0.05^2 = 152$ or approximately, 160. Since each person has two alleles of a gene, the minimum number of individuals required to confidently detect a genetic polymorphism with a prevalence of 1% in the population was 80 individuals.

6.3.5 Inclusion/ exclusion criteria

Study subjects were required to be Kenyans of black African origin who were above 18 years of age and HIV seropositive. They were willing to declare their ethnicity and that of their parents and grandparents. They were regular attendees of the CCC clinic as confirmed by the CCC records. They were also required to have been on nevirapine based antiretroviral drugs for6 months and have been collecting antiretroviral (ARV) drugs as scheduled as per the Pharmacy records. They were to be willing to participate in study procedures by signing a voluntary consent form and to provide a blood sample for genetic and nevirapine plasma level determination. Those who had been on antiretroviral therapy (ART) for less than 6 months as well as those subjects with interracial parentage were excluded. Also excluded were subjects who were not willing to sign a voluntary consent form and to provide a blood sample.

6.3.6 Sampling and recruitment

Recruitment of study subjects was based at the CCC Pharmacy. A list of HIV positive patients on nevirapine based HAART was prepared with details of scheduled monthly appointment visits to the CCC. Potential study subjects were those who were visiting the CCC pharmacy for their monthly drug refill of ARV drugs. The subjects were approached with intention of recruitment after going through the pharmacy drug dispensing procedures. Details of study procedures were explained and subjects were given an opportunity to make an independent decision to participate in the study. Those who met study criteria were sequentially enrolled after signing the consent form in duplicate and were issued with a study number. A questionnaire was administered to every subject to collect demographic characteristics, current HAART regimen, duration of ART, change of ART, concurrent medications, adverse drug reactions, concurrent illness and use of contraceptives, herbal preparations, alcohol and smoking. Eligible subjects were

recruited consecutively on the scheduled visit until the desired sample size was attained.

6.3.7 Blood sampling procedure:

Upon recruitment, study subjects were sent to the CCC laboratory for blood sample collection. A single 5.0 mL blood sample was collected from each subject by veno-puncture at 8 – 14 hours after the last dose of nevirapine. A 2.0 mL aliquot of the blood was collected into EDTA vacutainer tubes (Plymouth, UK) and stored at minus 20^0C awaiting DNA extraction for genotyping procedures. Plasma was prepared from the remaining 3.0 mL aliquot of the blood by centrifugation at 10, 000g for 10 minutes. Thereafter; plasma was decanted and stored frozen at minus 20 °C until analyzed for nevirapine plasma concentrations.

6.3.8 Review of patient medical records

Clinical data pertaining to the study subjects were further collected by review of patient medical records in the central registry of Kenyatta National Hospital. This was necessary to confirm the data collected through patient interviews and questionnaires. It was necessary to collect baseline clinical and laboratory parameters at the initiation of ART and at the time of recruitment into the study. Laboratory parameters collected included CD4 cell counts, Alanine aminotransferase (ALT), and complete blood count.

6.3.9 Case definitions

6.3.9.1 Alanine aminotransferase levels

Alanine aminotransferase (ALT) is a liver enzyme that is released during liver injury and is used as a proxy for hepatotoxicity. In Kenya, ALT levels are measured in patients with HIV infection as a baseline laboratory test before initiation of ART. It is also measured periodically in the course of ART to monitor drug induced hepatotoxicity particularly in nevirapine based HAART. In this

respect, ALT is measured at 1-2 months, 3 months, 6 months, 12 months and thereafter every 6 months in the course of ART. In this study, serum ALT levels were graded according to the criteria established by the AIDS Clinical Trial Group (ACTG, 1996). Grade 0: 1.25x upper limit of normal (ULN); grade 1: 1.25–2.5x ULN; grade 2: 2.6–5.0x ULN, grade 3: 5.1–10x ULN; grade 4: >10x ULN. Baseline ALT was defined as a pre-treatment measurement taken before initiation of ART whereas final ALT was the most current measurement at 6 months of ART.

6.3.9.2 CD4 cell counts

CD4 cell count serves as the most important biological (laboratory) indicator of the degree of immunosuppressant in Patients with HIV infection. It is also the most important prognostic indicator for patients starting ART. In Kenya, CD4 cell counts are measured at baseline and thereafter every 6 months to monitor efficacy of ART. In this study, baseline CD4 cell count was that measurement taken before the initiation of ART whereas final CD4 cell count was the most current measurement after 6 months of ART.

6.3.9.3 Hemoglobin levels

Anemia is defined as a decrease in the circulating red blood cell mass; the usual criteria is a hemoglobin (Hb) of less than 12 gm/dl (Haematocrit; HCT <36%) in women and less than 14gm/dl (HCT<41%) in men. Anemia can be graded in terms of severity as mild (8-10 g/dl); moderate (6.5-7.9g/dl); severe (< 6.5g/ dl). In this study, baseline Hb level was that measurement taken before the initiation of ART whereas final Hb level was the most current measurement after 6 months of antiretroviral drug therapy.

6.3.10 Determination of CYP2B6 516 G>T genotypes

Genotyping of the CYP2B6 516G>T SNP was done by real-time quantitative polymerase chain reaction (RT-PCR)-based allelic discrimination assay adopted from the method ofMahungu *et al.* (2009).Details of the genotyping protocol are described in Chapter Three section3.1.3.9.

6.3.11 Determination of nevirapine plasma levels.

Nevirapine plasma concentrations were determined by a validated reversed phase high performance liquid chromatographic (HPLC) method adapted from the method of Ngaimisi *et al.* (2010). Details of the analytical procedure and method validation are provided in Chapter Three, section 3.2

6.4 Data analysis

All variables were subjected to descriptive data analysis. Allele frequencies were estimated from the total number of copies of individual alleles divided by the number of all alleles in the study population. Deviation of genotypes from Hardy Weinberg equilibrium expectation was estimated by the use of the Pearson's chi-square goodness of fit test. CYP2B6 phenotypes were inferred from observed genotypes by the use of a semi-quantitative gene dose rating algorithm of Steimer *et al.* (2004). A one-way analysis of variance (ANOVA) was used to determine whether there were significant differences in nevirapine plasma concentrations among the three genotype groups. P_{ANOVA} was used to refer to the statistical significance of the differences among all three groups after simultaneous comparison. For continuous variables the mean, median and interquartile ranges were determined. The Chi square test was used to compare categorical variables. Relationships in all variables with $P < 0.05$ in univariate analysis were included in a multivariable linear regression analysis. Statistical calculations were performed with SPSS, software version 17.0 (SPSS Inc., Chicago, IL, USA).

6.5 Results

6.5.1 Baseline characteristics of study population

Patient demographic characteristics are summarized in Table 6.1.

Table 6.1: Baseline demographic characteristics of study population

Characteristic	Median (Range)	Proportion of patients		P
		n	%	
Age in Years				
Range	44 (66 - 22)			
< 40		56	51	
>= 40		54	49	0.495
Weight (Kg)				
Range	62.7 (108.9 - 46.2)			
< 60		40	36.4	
>= 60		70	63.6	0.001
Gender				
Female		83	75.5	0.0001
Male		27	24.5	
Ethnicity				
Bantu		87	79.1	0.0001
Nilotes		23	20.9	
Marital Status				
Married		72	65.5	0.0001
Single		38	34.5	
Social Habits				
Yes		18	16.4	0.001
No		92	83.6	
Total		110		

A total population of 110 HIV Patients were recruited into the study of which 76% were females Bantus (79%) and 66% were married. Median age and weight were 40 years and 65 kg respectively. Smoking was found in 3%, alcohol (9%) and herbal medications (6%). Concurrent medications taken by 25.5% of the patients

during the study the period are shown in Figure 6.1. Dapsone (21.4%) and fluconazole (14.3%) were most commonly used concurrent drugs.

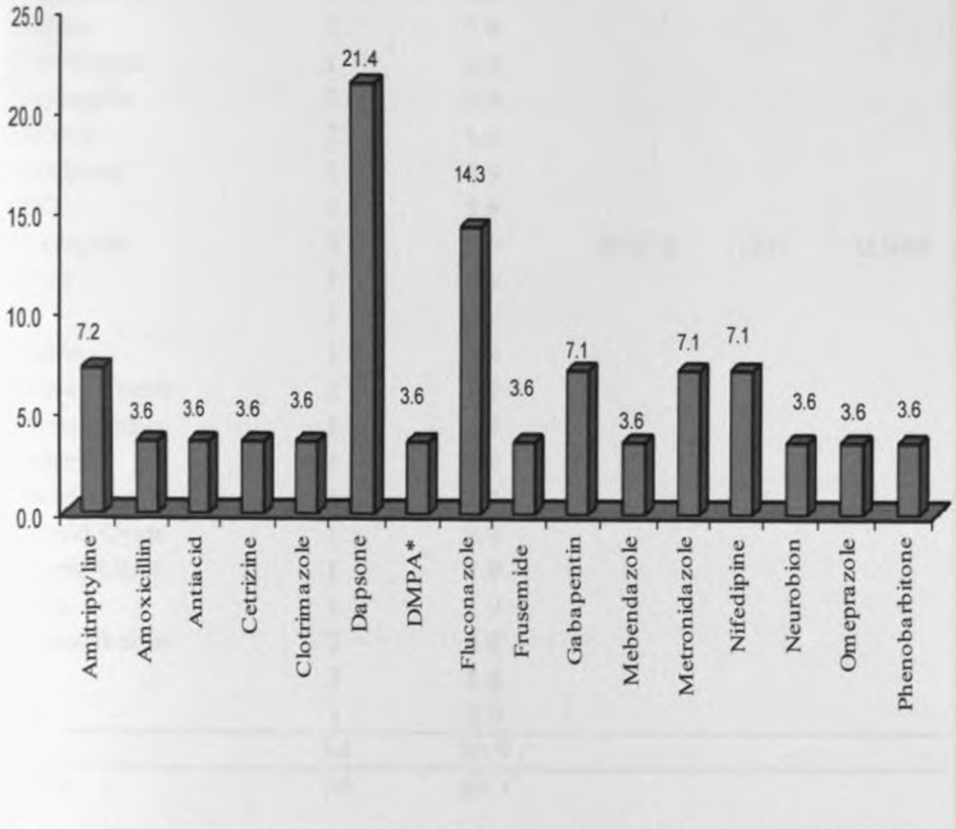


Figure 6.1: Concurrent medications used by HIV patients in addition to HAART

Co-morbidity (Table 6.2) occurred in 31% of the patients. Tuberculosis and stomach pains were the most common at 8% each.

Table 6.2: Concurrent diseases found in the study population

Concurrent Diseases	Proportion of patients				
	n	%	χ^2	df	P
Allergy	2	5.9			
Ammenorhea	1	2.9			
Asthma	2	5.9			
Convulsions	1	2.9			
Menengitis	2	5.9			
Diabetis	2	5.9			
Diarrhoea	2	5.9			
DVT	2	5.9			
Dyspepsia	2	5.9	6.118	21	0.999
Fever	1	2.9			
Flu	1	2.9			
Hernia	1	2.9			
Herpes Zoster	2	5.9			
Hypertension	1	2.9			
Malaria	1	2.9			
Oedema	1	2.9			
Parotid Cysts	1	2.9			
Peprtic Ulcer	1	2.9			
Rhitis	1	2.9			
Stomach ache	3	8.8			
TB	3	8.8			
UTI	1	2.9			
N	34	30.9			
None	76	69.1			

Baseline clinical traits of patient population are shown in Table 6.3. Baseline CD4 cell counts ranged from 19 to 374 cells per mL whereas baseline ALT levels ranged from 7 to 90 U/L. Baseline hemoglobin (Hb) levels ranged from 12.4 to 19.8g/dl.

Table 6.3: Baseline clinical traits of study population

Characteristic	Median (range)	Proportion of patients n	%	P
Baseline CD4 (cells/mL)	162 (19 - 374)			
< 200		59	53.6	0.446
≥ 200		51	46.4	
Baseline ALT (U/L)	21.0 (7 -90)			
<45		68	61.8	**0.0001**
≥ 45		42	38.2	
Baseline Hb (g/dl)	12.8 (12.4 - 19.8)			
< 12		53	48.2	0.144
≥12		57	51.8	

6.5.2 *CYP2B6* genotypes and allele frequencies

CYP2B6 genotype and allele frequencies are shown in Table 6.4. In this patient population, three genotypes were detected with a frequency of 50% for *CYP2B6* *1*6, followed by 30.1% for *1/*1 and 19.1% for *6/*6 ($\chi 2 = 16.055$; P =0.003). Comparison of observed versus predicted genotype frequencies showed conformance with HW proportions. The majority of the patients were CYP2B6 extensive metabolizers 89/110 (81%) whereas 21/110 (19%) were intermediate metabolizers ($\chi 2 = 16.036$; P = 0.0001). In terms of allele frequencies, *CYP2B6**1 occurred at a frequency of 56% whereas *CYP2B6**6 was at a frequency of 44%.

Table 6.4: CYP2B6 genotypes and allele frequencies in study population

Characteristic	Proportion of patients			
	n	%	χ^2	P
CYP2B6 516 G>T				
Genotypes				
*1/*1	34	30.9	31.3	
*1/*6	55	50.0	49.3	0.003
*6/*6	21	19.1	19.5	
N	110			
Phenotypes				
EM	89	80.9	80.6	
IM	21	19.1	19.5	0.0001
N	110			
Alleles				
*1	123	55.9		
*6	97	44.1		
N	220			

EM - extensive metabolizer, IM- intermediate metabolizer.

6.5.3 Anti-retroviral regimens used by study population

Antiretroviral drug regimens in this patient population are shown in Table 6.5. The standard nevirapine-containing regimen included nevirapine 200 mg daily for 14 days, then 200 mg twice daily. The NRTI backbone was made up of tenofovir (TDF), stavudine (D4T) or zidovudine (AZT) plus lamivudine (3TC). The ARVs drugs were all at standard doses according to Kenyan national guidelines (NASCOP, 2011). The most common combination of HAART was the one that contained nevirapine together with tenofovir and lamivudine (TDF/3TC/NVP (51%) as shown in Table 6.5. The duration of ART for all the patients in this study was 6 months.

Table 6.5: Antiretroviral regimens and nevirapine plasma trough concentrations found in the HIV patients

Characteristic					
Nevirapine plasma C_{trough} (ng/mL) Mean 5287.7 Median 5035.0 Range 11160 (11800 - 640)					
	Proportion of patients				
	n	%	χ^2	df	P
Nevirapine C_{trough} categories					
< 3100	19	17.3			
3100 - 4300	19	17.3	51.073	2	0.001
> 4300	72	65.5			
ARV regimen					
AZT.3TC,NVP	20	18.2			
D4T,3TC,NVP	34	30.9	17.964	2	0.001
TDF,3TC,NVP	56	50.9			

AZT-zidovudine, 3TC - lamivudine, D4T - stavudine, TDF- tenofovir, NVP - nevirapine

6.5.4 Nevirapine plasma levels and categorization

Nevirapine plasma concentrations are shown in Table 6.5. There was considerable inter-individual variability in nevirapine trough concentrations (C_{trough}) ranging from a minimum value of 640 ng/mL to a maximum value of 11800 ng/mL (mean 5287.73, median 5035) (Table 6.5). Categorization of nevirapine C_{trough} concentrations is shown in Figure 6.2. The three nevirapine C_{trough} cutoff levels were, levels above 4300ng/mL, levels ranging from 3100-4300 ng/mL and levels below 3100 ng/mL. The majority of the patients 72/110 (65.5%) had nevrapine plasma levels above 4300 ng/mL ($\chi 2$ = 34.087; P = 0.001). Of the remaining patients, 19 (17.3%) had a nevirapine concentration of less than 3100 ng/mL

whereas another 19 subjects (17.3%) had a nevirapine concentration in the range of 3100 – 4300 ng/mL as shown in Figure 6.2.

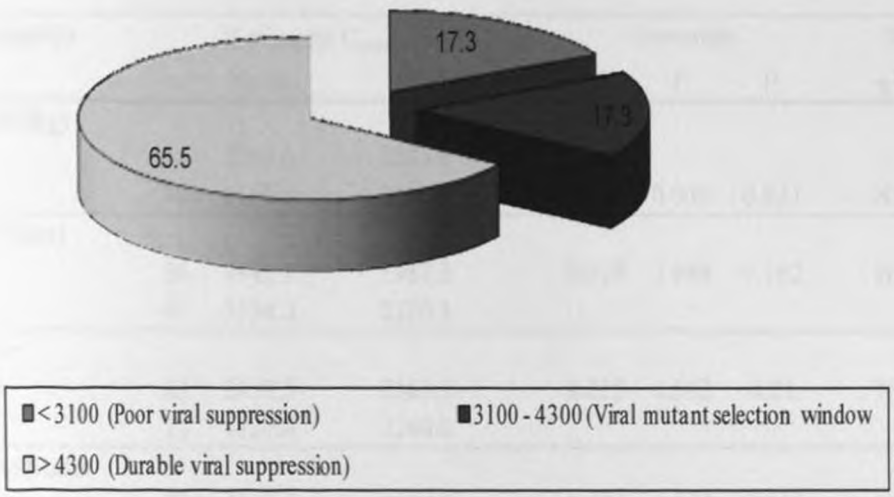


Figure 6.2: Categorization of nevirapine plasma concentrations found in the HIV patients

6.5.5 Factors influencing Nevirapine plasma concentrations in HIV patients

6.5.5.1 Demographic factors

Table 6.6 shows the relationship between demographic characteristics and nevirapine plasma concentrations. All demographic factors including age, weight, gender, marital status, ethnicity and social habits were entered into a univariate regression model to search for potential relationships with nevirapine plasma concentrations. Subsequently, factors that were significantly associated with nevirapine exposure were added stepwise in a multivariate analysis. Only social

habits was significantly associated with nevirapine plasma levels on univariate analysis (P= 0.006) but was found not to be significant on multivariate analysis. However, there was a trend to elevated nevirapine plasma levels in females, but the difference did not reach statistical significance (P = 0.21).

Table 6.6: The relationship between demographic factors and NVP plasma levels in HIV patients

Demographics		Nevirapine C_{trough} (ng/mL)		Univariate			Multivariate		
	n	Mean	SD	R^2	F	P	R^2	F	P
Weight (Kg)									
< 60	35	5263.1	2283.6						
≥ 60	70	5166.9	2125.5	0.009	0.046	0.831	NS		
Age (Years)									
< 40	56	4942.0	1987.3	0.019	1.986	0.162	NS		
≥ 40	49	5534.3	2320.1						
Gender									
Female	83	5437.5	2163.2	0.015	1.592	0.21	NS		
Male	27	4827.4	2240.8						
Marital Status									
Married	72	5316.9	2202.9	0.001	0.005	0.946	NS		
Single	34	5285.9	2247.4						
Ethinicity									
Bantu	87	5189.3	2249	0.008	0.841	0.331	NS		
Nilotes	23	5660.0	1940.1						
Social habits									
Yes	13	3752.3	1673.9						
No	97	5493.5	2173.4	0.067	7.706	**0.006**	NS		

n = proportion of patients; SD = Standard Deviation; NS = Not significance; R = Regression coefficient; F = Fisher's exact value; P = Level of significance

6.5.5.2 *CYP2B6* Genotypes

Table 6.7 summarizes the association between *CYP2B6* 516G>T genotypes and predicted phenotypes with nevirapine plasma concentrations. There was a

significant association between *CYP2B6* 516G>T genotypes and nevirapine trough concentrations (P = 0.01). There was also a significant association between *CYP2B6* 516G>T predicted phenotypes and nevirapine trough concentrations (P> 0.01).

Table 6.7: The impact of CYP2B6516 G>T genotypes and predicted phenotypes on nevirapine plasma levels in HIV patients

CYP2B6	Nevirapine C_{trough} (ng/mL)			Univariate			Multivariate		
	n	Mean	SD	R^2	F	P	R^2	F	P
Genotypes									
*1*1	34	4075.3	1368						
*1*6	55	5249.6	2038.9	0.267	19.5	**0.0001**	0.6	7.2	**0.001**
*6*6	21	7350.5	2192.9						
Phenotypes									
EM	34	4075.3	1368						
IM	76	5830.1	2273.7	0.139	17.4	**0.0001**	0.14	8.1	**0.005**

n = Proportion of patients; SD = Standard Deviation; NS = Not significance; R = Regression coefficient; F = Fisher's exact value; P = Level of significance,

Figure 6.3 also shows the influence of *CYP2B6* 516 G>T genotypes on nevirapine plasma concentrations.

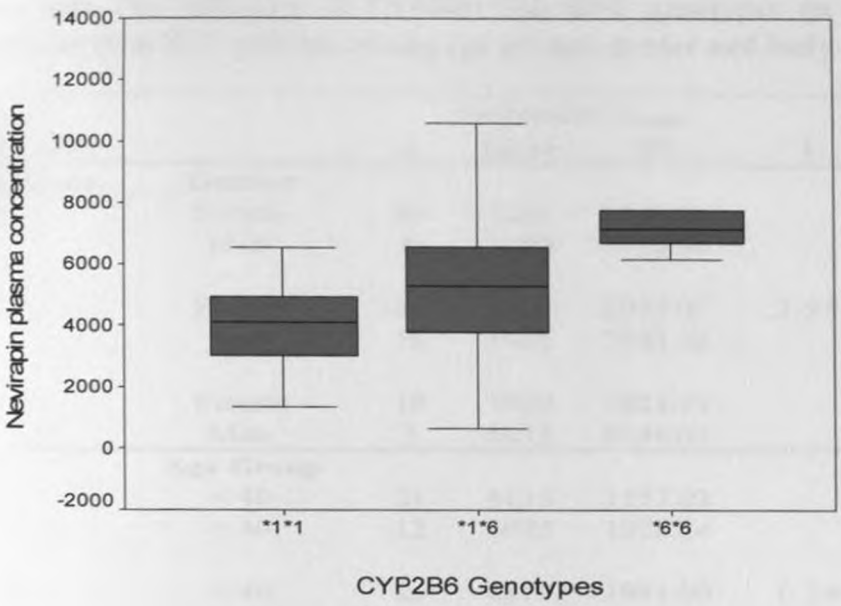


Figure 6.3: The impact of *CYP2B6* 516G>T genotypes on nevirapine plasma levels in HIV patients.

The influence of *CYP2B6* genotypes on nevirapine plasma concentrations was also tested among various demographic characteristics such as age, gender and body weight as shown in Table 6.8. However, there was no association between nevirapine plasma concentrations and genotypes among the genders (P = 0.083), age groups (P = 0.752) and body weight (P = 0.890).

Table 6.8: The influence of CYP2B6 516 G>T genotypes on nevirapine plasma levels in HIV patients among age groups, gender and body weight

		Nevirapine C_{trough}				
CYP2B6		n	Mean	SD	F	P
Genotype	**Gender**					
*1*1	Female	26	4225	1353.23		
	Male	8	3589	1388.64		
*1*6	Female	39	5188	1959.07	2.547	0.083
	Male	16	5401	2282.28		
*6*6	Female	18	7730	1828.29		
	Male	3	5073	3256.03		
	Age Group					
*1*1	< 40	21	4135	1557.03		
	> 40	12	4075	1028.84		
*1*6	< 40	26	4877	1991.09	0.285	0.752
	> 40	27	5407	1958.66		
*6*6	< 40	9	7013	1466.79		
	> 40	10	7629	2927.29		
	Body Weight					
*1*1	< 60	6	3620	1597.2		
	> 60	26	4070	1291.65		
*1*6	< 60	22	5023	2246.87	0.117	0.890
	> 60	32	5264	1767.21		
*6*6	< 60	7	7426	1177.89		
	> 60	12	7286	2823.88		

n = Proortion of patients, SD = Standard Deviation; F = Fisher's exact value; P = Level of s

6.5.5.3 Antiretroviral regimens and other clinical parameters

Table 6.9 shows the relationship between ART regimens, use of CYP inhibitors, skin rash and co-morbidity in the patient population and nevirapine plasma concentrations. These Factors were entered into a univariate regression model to search for potential associations with nevirapine plasma concentrations. Subsequently, factors that were significantly associated with nevirapine exposure

were added stepwise in a multivariate analysis. No significant association with nevirapine plasma concentrations was detected (P>0.05).

Table 6.9: Variation of nevirapine plasma levels with ART regimens and other clinical parameters in HIV patients

Medical Traits		Nevirapine C_{trough} (ng/mL)		Univariate			Multivariate		
	n	Mean	SD	R^2	F	P	R^2	F	P
ARV Regimen									
AZT,3TC,NVP	20	5904.5	2285.1						
D4T,3TC,NVP	34	5453.8	2407.2	0.027	1.51	0.226	NS		
TDF,3TC,NVP	56	4966.6	1986.7						
Skin Rash									
Yes	22	5701.4	1986.1						
No	88	5184.3	2234.2	0.009	0.983	0.324	NS		
CYP Inhibitors									
Yes	9	5171.1	1463						
No	101	5298.1	2246.3	0.001	0.028	0.868	NS		
Concurrent Disease									
Yes	34	5574.4	2079.4						
No	76	5159.5	2236.4	0.008	0.884	0.36	NS		

n = Proportion of patients; SD = Standard Deviation; NS = Not significance; R = Regression coefficient; F = Fisher's exact value; P = Level of significance

Other clinical traits such as CD4 cell counts, ALT and Hb levels of the patient population were also correlated with nevirapine plasma concentrations (Table 6.10). These factors were entered into a univariate regression model to search for potential association with nevirapine plasma concentrations. Subsequently, factors that were significantly associated with nevirapine exposure were added stepwise in a multivariate analysis. There was no significant association between either the

baseline or final parameters of these clinical traits and nevirapine concentrations ($P > 0.05$).

Table 6.10: The Association of CD4cell counts, ALT and HB levels with nevirapine plasma concentrations in HIV patients

Clinical Traits	Nevirapine C_{trough} (ng/mL)			Univariate			Multivariate		
	n	Mean	SD	R^2	F	P	R^2	F	P
Baseline CD4 (cells/mL)									
< 200	59	4609.2	2292.9						
≥ 200	51	5869.1	2289.8	0.061	1.946	0.128	NS		
Final CD4									
< 200	6	5625	1660						
200 - 500	68	5114.7	2243.9	0.005	0.226	0.798	NS		
≥ 500	36	5373.1	2324.1						
Baseline ALT									
> 20	37	5279.7	2046.1						
21 - 50	33	5332.1	2480.5	0.006	0.251	0.779	NS		
> 50	40	5783.1	2254.4						
Final ALT									
> 20	13	4643.1	1752.9						
21 - 50	40	5343.8	2371	0.045	1.358	0.261	NS		
51 - 100	30	4576.3	2295.7						
> 101	27	5892.4	2149.9						
Baseline Hb > 12									
Yes	57	5569.7	2085.6						
No	53	5031.5	2203	0.015	1.403	0.239	NS		
Final Hb > 12									
Yes	18	6059.4	1866.5						
No	92	4993.9	2291.2	0.036	3.344	0.071	NS		

n = Proportion of patients; SD = Standard Deviation; NS = Not significance; R = Regression coefficient; F = Fisher's exact value; P = Level of significance

6.5.6 The influence of nevirapine on selected treatment outcomes

The treatment outcomes were defined as changes in clinical traits such as CD4 cell counts, ALT and Hb levels from baseline values measured at initiation of ART to the final values measured at 6 months of ART. Table 6.11 shows the magnitude of change in clinical traits from baseline to the final values at 6 months of ART. Adverse drug reactions associated with ART were also taken as treatment outcomes. The two major adverse drug reactions recorded in the patient population were lipodystrophy (42%) and skin rash (34%).

Table 6.11: Changes in clinical traits in HIV patients as a measure of treatment outcomes

Trait	Median (range)	Proportion of patients n (%)			
CD4 (cell/mL)		<200	200 - 500	>500	P
Baseline	162.0 (19 - 374)	59 (53.6)	51 (46.4)	0	0.446
Final	445.0 (75 - 980)	6 (6.6)	68 (61.8)	36 (32.7)	**0.006**
Change	282.5 (298 - 823)	53 (90.0)	17 (33.3)	36 (32.7)	
ALT (U/L)		<45	45 - 90	>90	
Baseline	21.0 (7 - 90)	68 (61.8)	42 (38.2)	0	**0.001**
Final	35.0 (8 - 146)	53 (48.2)	30 (27.3)	27 (24.5)	**0.001**
Change	18.75 (74 - 108)	15 (22.0)	12 (28.6)	27 (24.5)	
HB (g/L)		<12	>12		
Baseline	12.8 (12.4 - 19.8)	53 (48.2)	57 (35.5)		0.144
Final	13.3 (5.4 - 18.9)	92 (83.6)	18 (16.4)		**0.001**
Change	0.7 (15.4 - 15.7)	39 (73.6)	39 (68.4)		

6.5.6.1 Change in CD4 counts and nevirapine trough concentration

The profile of CD4 cell counts from initiation of ART (Baseline) to 6 months of ART (Final) is shown in Table 6.11. Final CD4 cell counts ranged between 75 – 980 cells/mL and only a few patients (7%) still had CD4 cell counts below 200 cells/mL compared to (54%) at baseline. There was a significant increase in CD4 cell counts in the course of 6 months of ART whereby 33% of the patients had CD4 cell counts above 500 cells per mL at 6 months of ART compared to none at baseline ($P = 0.006$). However, there was no association between the change in CD4 cell counts and nevirapine trough concentrations. ($P = 0.621$). Other factors such as age ($P = 0.229$), gender ($P = 0.885$), body weight ($P = 0.779$) and *CYP2B6* genotypes ($P = 0.312$) did not seem to have an influence on the relationship between change in CD4 cell counts and nevirapine trough concentrations.

A total population of 110

6.5.6.2 Change in ALT levels and nevirapine trough concentration

The change in ALT levels in the course of 6 months of ART is shown in Table 6.11. Final ALT levels ranged from 8 to 146 U/L. There was a significant increase in ALT levels at 6 months of ART with patients having ALT levels above 45 U/L being 52% compared to 38% at baseline ($P = 0.001$). In fact, 25% of the patients had ALT levels above 90 U/L at 6 months compared to none at baseline ($P< 0.01$).There was a mean increase of 85% in ALT levels from baseline to 6 months of ART. However, there was no association between the change in ALT levels in the course of ART and nevirapine concentration ($P > 0.05$). Further analysis revealed that an increase in ALT levels above 45U/L was associated with elevated nevirapine plasma levels in patients with body weight above 60 kg ($P = 0.003$), (Figure 6.4). Other factors such as age ($P = 0.29$), gender ($P =0.659$) and *CYP2B6*516 G>T genotypes ($P = 0.735$) showed no influence on the relationship

between the change in ALT levels and nevirapine trough concentrations in the course of ART at 6 months.

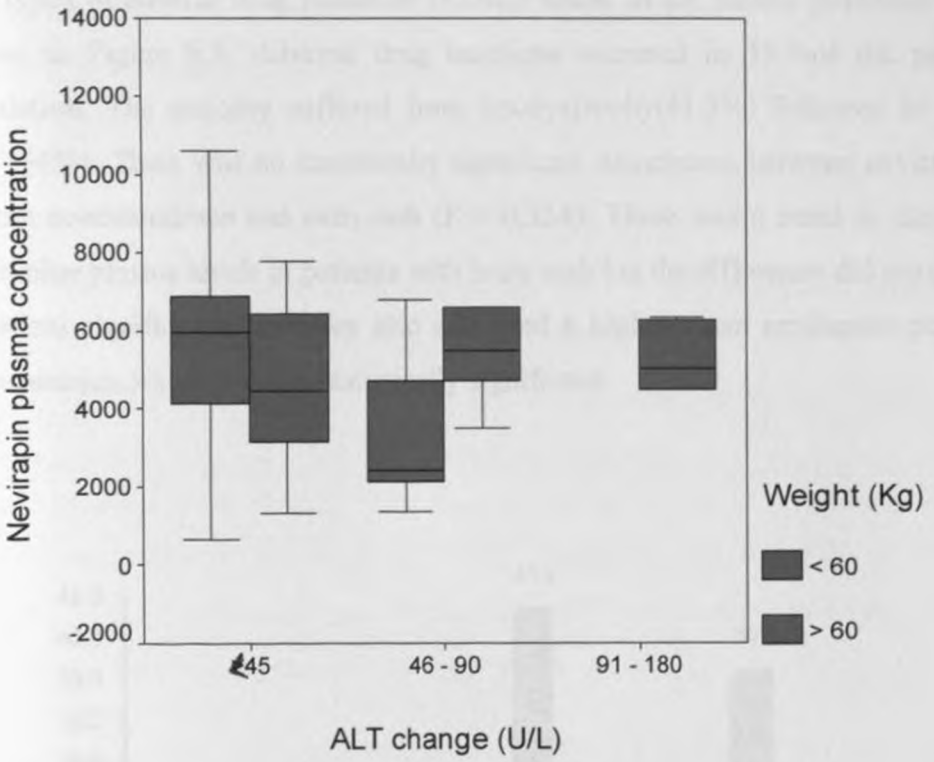


Figure 6.4: The association between nevirapine plasma levels and change in ALT levels in HIV patients of different body weights

6.5.6.3 Change in Hb levels and Nevirapine trough concentration

The change in Hb levels in the course of 6 months of ART is shown in Table 6.11. The final Hb ranged from 5.4 to 18.9 g/L with the majority 84% having Hb levels less than 12g/dl($P < 0.05$). The number of patients with Hb levels below 12g/dl increased by 74% (Table 6.11). The mean change in Hb levels from baseline to 6

months of ART was 6%. However, there was no significant relationship between change in Hb levels and nevirapine trough concentrations (P> 0.05).

6.5.6.4 Adverse drug reactions

The types of adverse drug reactions (ADRs) found in the patient population are shown in Figure 6.5. Adverse drug reactions occurred in 59.%of the patient population. The majority suffered from lipodystrophy(41.5%) followed by skin rash (34%). There was no statistically significant association between nevirapine plasma concentrations and skin rash (P = 0.324). There was a trend to elevated nevirapine plasma levels in patients with body rash but the difference did not reach statistical significance. Females also exhibited a higher mean nevirapine plasma concentration which was not statistically significant

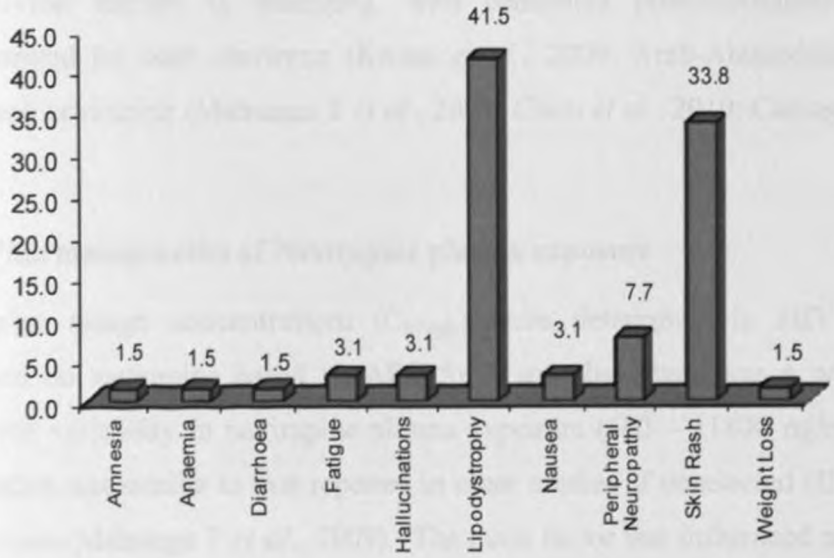


Figure 6.5: The types of adverse drug reactions in the HIV patients

6.6 Discussion

This study characterized *CYP2B6* 516G>T genotypes and determined their influence on nevirapine plasma trough concentrations and assessed the subsequent impact on therapeutic outcomes in Kenyan HIV patients. This is the first study to investigate the relationship between *CYP2B6* 516G>T and nevirapine concentration in Kenyan HIV patients. Nevirapine is metabolized by CYP2B6, a highly polymorphic enzyme with various SNPs and associated haplotypes (Zanger *et al.*, 2007; Watanabe *et al.*, 2010). This study reports the frequency of the *CYP2B6**6 (516G>T) variant allele in HIV patients of 44% which was much higher than that seen in healthy Kenyan populations of 36% as reported elsewhere in this thesis. However, these results were similar to those observed in other studies genotyping *CYP2B6* 516G>T in HIV patients of African descent in Tanzania (Ngaimisi *et al.*, 2010), Zimbabwe (Nyakutira *et al.*, 2008) and South Africa (Masebe *et al.*, 2012). The significance of CYP2B6 pharmacogenetics antiretroviral therapy is emerging, with consistent pharmacokinetic effects demonstrated for both efavirenz (Kwara *et al.*, 2009; Arab-Alameddine *et al.*, 2009) and nevirapine (Mahungu T *et al.*, 2009; Chou *et al.*, 2010; Calcagno *et al.*, 2012).

6.6.1 Pharmacogenetics of Nevirapine plasma exposure

Nevirapine trough concentrations (C_{trough}) were determined in HIV patients stabilized on nevirapine based HAART for 6 months. There was a wide inter-individual variability in nevirapine plasma exposure (640 – 11800 ng/mL). This observation was similar to that reported in other studies of unselected HIV patient populations (Mahungu T *et al.*, 2009). The main factor that influenced nevirapine plasma exposure in this study was *CYP2B6* 516 G>T SNP. There was a significant gene dose - effect between nevirapine C_{trough} and CYP2B6 516 G>T genotypes. Patients bearing the *CYP2B6* 516 TT had 55% higher plasma levels than *CYP2B6* 516 GG. Similar results have been reported in other African HIV

patients (Penzak *et al.*, 2007; Nyakutira *et al.*, 2008). A study in an ethnically diverse cohort also reported that subjects who were homozygous to the variant (*CYP2B6* 516 TT) had a 108% higher nevirapine plasma exposure than those bearing the wild type (*CYP2B6* 516 GG) (Mahungu T *et al.*, 2009). A significant gene-dose effect associated with *CYP2B6516* G>T genotypes was also reported by Schipani *et al.* (2011). In that study, the presence of *CYP2B6* 516 TT genotypes led to a decrease in nevirapine clearance by 37% compared to the CYP2B6 516 GT (15%) and *CYP2B6* 516 GG genotypes. Hence, these results have consistently demonstrated the influence of *CYP2B6* 516G>T polymorphism on nevirapine clearance in different populations. It is noteworthy that the *CYP2B6* 516 G>T SNP is one of the most commonly occurring *CYP2B6* variants, leading to a Gln172His change in amino acid. Although it is not directly in the protein active site, residue 172 along with additional nearby SNP residues (in linkage disequilibrium), probably affects ligand binding (Gay *et al.*, 2010) altering the size and flexibility of the active site.

6.6.2 Potential for drug –drug interactions

A proportion of patients in this study (12 %) engaged in smoking, alcohol intake and use of concomitant herbal preparations. These variables were grouped together to form one variable termed social habits. On univariate analysis, the data showed that engaging in these social habits was associated with a 70% lower mean nevirapine plasma concentrations (P = 0.006), but this effect was not maintained on multivariate analysis. These observations could be attributed to CYP2B6 induction by these environmental covariates. Infact, some authors have observed a significantly higher level of CYP2B6 expression in the brains of smokers and alcoholics (Miksys *et al.*, 2003). Other studies have also reported the induction of CYP2B6 by cigarette smoke extract through activation of the human constitutive androstane receptor (CAR) (Washio *et al.*, 2010).While the rate of concomitant use of herbal products was low in this study, other studies in Kenya and Uganda

have reported that up to 70% of HIV patients use herbal products together with antiretroviral therapy (ART) (Nagata *et al.*, 2011; Lubinga *et al.*, 2012). Various herbal preparations have been reported to cause metabolic enzyme induction (Parkinson *et al.*, 2004). This calls for collaboration and communication between clinicians and herbalists. Also needed are Pharmacological, toxicological, and ART-herb interaction studies targeting the possible interaction between HAART and indigenous African herbal products.

In this study, all the patients were on concurrent therapy with cotrimoxazole (trimethoprim and sulphamethoxazole) as prophylaxis against PCP as well as multivitamins to boost immunity. Data from this study showed that 26% of the patients were taking additional medications including dapsone (22%) and fluconazole (14%). Some of these co-medications are reportedly CYP inhibitors such as fluconazole, metronidazole, amitriptyline and omeprazole. However, their presence did not seem to have any significant association with nevirapine plasma concentrations in this patient population. Drug- drug interactions is a common phenomenon in ART both by design and practice, due to combination therapy. Hence, patients are often taking concomitant drugs such as herbal or nutritional supplements together with drugs for the treatment of opportunistic infections (Katende-Kyenda *et al.*, 2012; Desai *et al.*, 2012).Since hepatic CYP2B6 is highly inducible by a broad array of clinical drugs including nevirapine itself, the likelihood of induction related drug- drug interactions is an ever present concern.

6.6.3 Nevirapine plasma exposure and predicted viral suppression

From the results of this study, 66% of the patients achieved nevirapine C_{trough} above 4,300 ng/mL which has been associated with durable viral suppression (González-de-Requena *et al.*, 2005). Studies have reported a relationship between the risk of virological failure and nevirapine plasma concentrations below 3000 ng/mL (de Vries-Sluijs *et al.*, 2003; Duong *et al.*, 2005). In this study, 17.3% of HIV patients had nevirapine C_{trough} below 3,100 ng/mL which could result in poor

viral suppression. A similar proportion (17.3%) attained levels between 3,100 and 4,300 ng/mL which reportedly lies in the mutant selection window. These cutoff points are based on observations that nevirapine plasma exposure is a contributory factor to the durability of viral suppression over time (González-de-Requena *et al.*, 2005; Aizire *et al.*, 2012). In addition some guidelines for therapeutic drug monitoring of antiretroviral agents recommend 3,000 ng/mL as the cut-off value for nevirapine trough concentrations (Kappelhoff *et al.*, 2004). A study on Chinese HIV patients have set the therapeutic nevirapine C_{trough} cutoff level at 3900 ng/mL (Wang *et al.*, 2011). It is also important to recall that nevirapine reportedly has a low "genetic barrier" to the development of drug resistance. SNPs at critical sites of the *pol* gene reportedly result in a 100-fold loss of sensitivity to nevirapine(Lalonde *et al.*, 2007; Ravich *et al.*, 2010). Thus, from the results of this study, 35% of the patients who had nevirapine C_{trough} below 4,300ng/mL could be at risk of viral failure with attendant development of nevirapine resistant strains. The Chinese study reported a significant correlation between nevirapine C_{trough} and viral load after six months of ART (Wang *et al.*, 2011). However, this present study did not have virologic data of the study participant. Studies are urgently required to determine similar cutoffs for Kenyan HIV patients.

6.6.4 Nevirapine associated hepatotoxicity

Results from this study showed that the majority of the patients (62%) had no manifestation of hepatotoxicity at initiation of ART (ALT < 45 IU/L) whereas 15% had grade 1 hepatotoxicity (baseline ALT = 45 – 90 IU/L). At six months of ART, there was an 85% increase in ALT levels with 16% having grade 2 hepatotoxicity (Final ALT = 91 – 180 IU/L). However, there were no cases of grade 3 or 4 hepatotoxicity. In the Kenyan setting, ALT levels are routinely determined in every patient before initiation (baseline) of ART and on follow up visits at month 1, 3, 6, 12 and thereafter at every six months. Screening for hepatotoxicity during ART is primarily based on measurements of ALT levels and

clinical guidelines recommend discontinuation of nevirapine at grade 3 or 4 of hepatotoxicity (Bartlett and Gallant, 2007). The low levels of hapatotoxicity in this patient population was similar to that reported in other studies in Kenya (Peters *et al.*, 2012), Uganda (Kalyesubula *et al.*, 2011), the Niger (Meyssonnier *et al.*, 2008), and in Thailand (Phanuphak *et al.*, 2007). Some studies have reported that sex-dependent CD4 cell count was predictive of hepatotoxicity (Stern *et al.*, 2003), but this was not apparent in this study. However, this study found a strong association between nevirapine C_{trough} and change in ALT levels when stratified by body weight ($P = 0.003$). Patients with body weight above 60 kg had a greater increase in ALT levels from grade 1 to grade 2. This observation was contrary to reports from the South African study (Chu *et al.*, 2010) and requires further investigations.

6.6.5 Nevirapine associated Skin rash

The prevalence of skin rash in this study was 34%, affecting mainly women. Skin rash is reportedly the most common adverse reaction associated with nevirapine (Pollard *et al.*, 1998; Phillips and Mallal, 2008). The rate attributable to nevirapine is 16% and about 7% of the patients experience grade 3 - 4 skin rash. This study found no association between nevirapine C_{trough} and skin rash. A study in Thailand reported that risk factors for nevirapine associated skin rash included a history of drug allergy, lower body weight and higher CD4 cell count (Kiertiburanakul *et al.*, 2008). This present study did not find any influence of gender, body weight as well CYP2B6 genotypes on the occurrence of skin rash.

Some studies have suggested that that ALT elevations and rash are related to nevirapine plasma C_{trough} (Gonzalez-de-Requena *et al.*, 2002; de-Maat *et al.*, 2003), however, conclusive evidence is still lacking (Knobel *et al.*, 2004). For example, the substudy of 2NN investigating the relationship between adverse events and nevirapine concentrations found no association (Kappelhoff *et al.*, 2005). Furthermore, no clear cut-off value of nevirapine plasma concentration

associated with increased risk for adverse events has been identified (Kappelhoff *et al.*, 2005).

6.6.6 Nevirapine plasma levels and clinical outcomes

In this study, there was no association between nevirapine plasma trough concentration and change in CD4 cell counts (P = 0.621). The relationship was also not influenced by gender, age, body weight and *CYP2B6* genotypes. However, there was a significant change in baseline CD4 cell counts of 170% which was not related to nevirapine plasma levels but is a clear evidence of the efficacy of ART in totality. A retrospective study on a pediatric cohort on nevirapine-based regimens reported a significant association between *CYP2B6* 516G>T genotype and immunological responses at both week 12 and week 24 (Saitoh *et al.*, 2007). Higher plasma levels of nevirapine have been reported to be associated with improved virological response and reduced selection of resistant mutations (de Vries-Sluijs *et al.*, 2003) and there are conflicting reports on the association between nevirapine plasma levels and the development of adverse events (Kappelhoff *et al.*, 2005). Previous studies have found ethnicity, gender, weight and underlying hepatic disease to be predictive of nevirapine plasma concentrations (Stöhr *et al.*, 2008). This study was conducted in a naturalistic setting and did not have access virological data since the parameter is not routinely monitored according to WHO and national guidelines in Kenya

6.6.7 Demographic characteristics and nevirapine plasma exposure

In this study, none of the demographic factors including age and body weight were associated with nevirapine plasma levels. However, there was a trend towards a higher nevirapine C_{trough} in women but the effect did not reach statistical significance. Other factors reported to influence nevirapine plasma levels include gender; body mass index, age, ethnicity, social habits and use of concurrent medications (Kappelhoff *et al.*, 2005; de-Maat *et al.*, 2005). This study did not also

find any influence of age on nevirapine plasma exposure in relation to *CYP2B6* 516G>T genotypes. However, a study on HIV patients made up of both Caucasian and Black HIV patients treated with nevirapine based regimens reported that age was significantly associated with nevirapine concentrations (Wyen *et al.*, 2008). In that particular study, neither gender, ethnicity, BMI, alcohol consumption nor smoking was related to nevirapine concentrations. However, a study by Schipani *et al.* (2011) found that body weight influenced nevirapine clearance whereby there was an increase of 5% in clearance with body weight increase of 10 kg. Hence, further studies with adequate sample size are recommended.

6.7 Summary of major findings and conclusion

This is the first study investigating the influence of *CYP2B6* 516 G>T on nevirapine exposure in Kenyan HIV patients. The study has established that:

CYP2B6 516 G>T was highly prevalent in Kenyan HIV patients at 44% compared to that in healthy population 35% population. Nevirapine plasma exposure in Kenyan HIV patients was subject to a wide inter-individual variability with a range of 640 – 11800 ng/mL. There was a strong influence of *CYP2B6* 516G>T genotypes with a notable gene dose effect of 1.8 for CYP2B6 516 GG versus CYP2B6 516 TT. Exposure to smoking, alcohol or herbal products resulted in 68% lower nevirapine plasma levels. . Nevirapine displayed a relatively good safety which was underscored by the absence of grade 3 or 4 hepatotoxicity and a low incidence of grade 2 hepatotoxicity of 16% at 6 months of ART in this patient population .The effectiveness of nevirapine based HAART was demonstrated by the great change in baseline CD4 cell counts of 170% at 6 months of ART. A high proportion of the HIV patients (66%) attained nevirapine plasma levels above 4 3100 ng/mL associated with durable viral suppression. However, of concern and interest are the 34% who are within the mutant selection window (17%) and poor viral suppression range (17%) due to high the risk of emergence of ARV drug resistance and virological failure.

In conclusion, this study has quantified the effects of *CYP2B6* 516G > T polymorphism on nevirapine exposure in an unselected HIV patient population in Kenya. This adds to the current evidence implicating *CYP2B6* polymorphisms in the variability of nevirapine exposure. The observed wide interindividual variability as well as the evident influence of environmental factors require further investigations. Attention should also focus on the role of *CYP2B6* polymorphism in nevirapine associated ARV drug resistance especially in those patients who fail to attain durable viral suppression in the course of HAART. Finally, only a well-powered prospective trial will allow us to address the clinical utility of prospective genotyping by assessing the impact of these variants on clinical outcomes.

Chapter Seven

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

7.1 General Discussion

This thesis has described studies designed to investigate the distribution and influence of genetic polymorphisms of genes encoding drug metabolizing enzymes in Kenyan populations. The genes of concern were *CYP2B6, CY2C19, CYP2D6, NAT2, GSTM1* and *GSTT1* which had been selected due to their clinical relevance to the therapy of common disease conditions in Kenyan populations. It has also characterized and assessed the clinical impact of gene polymorphisms of *CYP2D6* and *CYP2C19* in psychiatric patients. The influence of *CYP2B6* genotypes together with patient characteristics on nevirapine plasma levels in HIV patients was also investigated.

Results from the population study revealed significant variability in allele frequencies not only between the three Kenyan populations but also at the ethno-linguistic divide. For example, *CYP2D6**4* was preferentially distributed in the Eastern Nilotes while occurring at low frequencies in the Western Nilotes and the Bantus. Similarly, *CYP2C19**3* while being rare in African populations (Dandara *et al.*, 2001) was detected solely in the Nilotes albeit at low frequencies but was undetected in the Bantus. These results indicate that genotype data from one group or subgroups both at the national or ethnic level may not be overly generalized and applied to genetically distinct groups.

In contrast, however, results from this study also detected a considerable degree of genetic homogeneity with regard to some genes. *CYP2B6 516* G>T and *NAT2* alleles *(NAT2*5, NAT2*6, NAT2*&, NAT*2*14)* were found to exhibit homogeneity in their distribution between the three Kenyan populations. Therefore, although it is indeed true that great variation across the human genome

occurs in African populations, this is not observed when the focus is on a small number of causative SNPs. This suggests that genotyping studies within a country may be applied to other regions of the country, provided that subgroups known to be genetically isolated are not included.

Kenyan populations may be divided into three major ethno-linguistic groups, namely, the Bantu, the Nilotes and the Cushites which are made up of more than 42 dialects believed to represent as much genetic diversity. This study only compared the Bantu and the Nilotes, hence, genetic variability between other regions or tribes within Kenya may be present and may not be accounted for in this study. It is widely acknowledged that using genotype data from the national population is a poor surrogate for individual genotyping, even where genetic homogeneity exists. Results from this study confirms that using one country to estimate the allele frequency of a larger region is insufficient, particularly for African populations, which have been shown to exhibit greater genetic diversity (Tishkoff and Williams, 2002). This view maybe attested for by the data for the distribution of *GSTM1**0 (deletion) and *GSTT1**0 (deletion) in this study which not only exhibited wide variability between Kenyan population but also between other African, Caucasian and Asian populations. Hence, results from this study seem to further suggest that if individual genotype data are not available, it would be unwise to extrapolate an individual's genotype based on genetic data from beyond the national population.

This study compared genotyping data for Kenyan populations with those reported for Caucasian and Asian populations. This is because, drug efficacy and dosing studies are frequently performed in populations that are primarily Caucasian. Results from this study suggest that Kenyans and other African populations may in some instances, have different responses to drug treatment regimens that have been optimized in Caucasian cohorts. This is particularly so with regard to the distribution of certain genes such as *CYP2D6.* Genotyping data from this study

and others, consistently reveal that the defective alleles *CYP2D6**17 and *CYP2D6**19 are preferentially distributed in African populations (Matimba *et al.*, 2008) while being virtually absent in Caucasian and Asian populations. Results from this study has revealed a high prevalent of *CYP2D6**17 (34%) and *CYP2D6**29 (10.8%) in Kenyan Bantu population as well as in psychiatric patients. CYP2D6 metabolizes more than 25% of drugs in clinical practice and is presently listed as a pharmacogenomic biomarker in the labeling requirements for important commonly used drugs such as tamoxifen (FDA, 2012; Pharmgkb, 2012).

The ultimate goal of pharmacogenetic research is the clinical application of genetic information for optimizing treatment for each patient. In this regard, this project conducted clinical studies to evaluate the therapeutic implications of selected genetic polymorphisms observed in the Kenyan general population study. An evaluation of the influence of *CYP2B6* 516G>T genotypes on nevirapine plasma levels was undertaken in HIV patients after 6 months of HAART. One of the major findings of concern in this HIV study was the wide inter-individual variability of nevirapine plasma exposure of 54%. Results showed that 66% of the HIV patients attained nevirapine trough concentrations above 4,300 ng/mL that has been associated with durable viral suppression (González-de-Requena *et al.*, 2005). However, of concern are the 34% of the patients who did not achieve this cut off levels. Of the patients who did not achieve this cut off level, 17% achieved plasma levels of 3100 – 4300 ng/mL which reportedly represents the viral mutant selection window while a similar percentage had levels below 3100 ng/mL which has been associated with virologic failure (González-de-Requena *et al.*, 2005).

No target nevirapine trough concentration has been established for treatment success in Kenyan HIV patients. This is in terms of durable virologic suppression, adequate immunologic response as well as emergence of ARV drug resistance. Available treatment guidelines recommend various target thresholds for nevirapine trough concentrations, but these data are predominantly from studies in Caucasian

and African American patients, which may not be applicable to Kenyan HIV patients. Additionally, the current recommendations for nevirapine C_{trough} thresholds are not completely in agreement (de Vries-Sluijs *et al.*, 2003; Duong *et al.*, 2005). A Chinese study has reported that nevirapine threshold levels of 3000 ng /mL was associated with an unfavorable sensitivity (40%) leading to a high incidence of virologic failure in that population.

That same study recommended raising nevirapine C_{trough} threshold by 30% to 3900 ng/mL for Chinese HIV patients with improved sensitivity of 60%. This variability in nevirapine threshold levels in HIV patients of diverse origin could be explained by differences in racial/ ethnicity as well as environmental factors that influence nevirapine pharmacokinetics. This study observed a significant influence of *CYP2B6* 516 G>T genotypes on nevirapine plasma exposure in Kenyan HIV patients. Unfortunately, this study did not have virologic data for the patient study population. Hence, a prospective study is urgently required in Kenyan HIV patients to examine the role of *CYP2B6* genotypes on nevirapine plasma exposure in relation to virologic and immunologic outcomes as well as emergence of ARV drug resistance.

In the psychiatric study, the medication pattern of psychotropic drugs was characterized by a high rate of prescription of the anticholinergic drug benzhexol (89%). Of these, up to 50% were on a low dose of 5mg for apparent 'initial prophylaxes' against extrapyramidal side effects (EPS). It is important to note that the patient files had no entries of occurrence of EPS. Hence, the high rate of prescription of low dose benzhexol was taken as a surrogate for EPS in this study. Studies report that EPS are very common in antipsychotic therapy (75%) (Dewan and Koss, 1989) and that the practice of initial prophylaxis remains controversial (Keepers *et al.*, 1983) and against WHO consensus guidance (WHO, 1989). A search of the literature did not reveal any guidelines on the practice in Kenya and hence warrants further investigation and action. It is s also noteworthy that the

psychiatric patients in this hospital were on sole treatment with typical first generation antipsychotics that are highly prone to causing EPS. Hence none of the patients had the benefit of using the new generation atypical second generation antipsychotic drugs that cause fewer side effects.

Further analysis revealed that serious EPS which was represented by the prescription of high dose benzhexol drug (15 - 20 mg) in 11% of the patients was significantly associated with CYP2D6 IM status. Similar observations have made in other studies (Kobylecki *et al.*, 2009). Genetic determination of metabolic status is expected to bring clinical benefits to patients with regard to dosage adjustments aimed at reducing adverse drug reactions (Arranz and de-Leon, 2007). Methods for dosage adjustments based on *CYP2D6* and *CYP2C19* genotypes have been developed and published (Kirchheiner and Seeringer, 2007; Kirchheiner and Rodriguez-Antona, 2009). However such studies may have limited application in African populations where the CYP2D6 PM and UM status are rare. On the other hand, the prevalence of CYP2D6 PMs and IMs in this study was 1.6% and 23% respectively whereas that for CYP2C19 was 7% and 26% respectively. Hence, genotype dosage adjustments need to be optimized for different populations taking into account the respective population pharmacogenetics as well as other relevant environmental covariates. For Kenyans and other African populations, it is the impact of the CYP2D6 IM status that would be of interest. This calls for the integration of pharmacogenetics in drug discovery, development and clinical trials in different populations especially for Africans.

The clinical implications of the results of the studies in this thesis could be postulated for Kenyan populations. For example, *CYP2D6*and *GSTM1* and *GSTT1*displayed greatest inter-ethnic genetic variability. CYP2D6 30% impaired function in Kenyan populations could have negative consequences for breast cancer treatment outcomes with tamoxifen, a drug of choice for adjuvant treatment of breast cancer in Kenya (Goetz *et al*, 2008). Potential for drug-drug interactions

also exist with commonly used CYP2D6 inhibitors such as quinine, fluoxetine and amodiaquine which could convert CYP2D6 IM to poor metabolizers in a phenomenon known as phenocopying. On the other hand, results from this study also report *GSTM1* deletion of 24%, which together with the NAT2 slow acetylation of 62% could have implications for the incidence of isoniazid and sulphonamide induced hepatotoxicity in Kenyan populations in similarity to that reported in other studies (Yimer et al, 2008; Kim et al, 2010).

The deletion alleles of *GSTM1**0 (24%) and *GSTT1**0 (31%) were found in this study. This could prove deleterious in the presence of chronic exposure to aflatoxin from commonly consumed food products such as maize and milk (Kangethe et al, 2007). Members of *GST* family including *GST-mu (GSTM1)* and *GST-theta (GSTT1)* are important in the susceptibility to aflatoxin associated hepatocellular carcinoma (HCC) (White et al, 2008; Yu et al, 2011). A Kenyan study has previously pointed out the possible association of HBV and other risk factors such as aflatoxin and non A/non B hepatitis viruses with the causation of liver disease in Kenya (Mwangi and Gatei, 1993). The prevalence of HBV in Kenyan populations is about 10%, hence, the interplay between *GSTM1* and *GSTT1* deletion, HBV and aflatoxin in the etiology of liver cancer in Kenyan populations warrants further investigations. Further studies are also needed to clarify the clinical significance of CYP2C19 impaired metabolism in the antimalarial efficacy of proguanil-atovaquone antimalarial prophylaxis in Kenya as well as the impact of the high prevalence of CYP2B6*6 variant on the dosage regimens for efavirenz and nevirapine .

7.2 Conclusion

In conclusion, this thesis has achieved its main objective of investigating the genetic variability of genes encoding drug metabolizing enzymes in Kenyan general population and the influence of defective variant alleles on therapeutic outcomes in two common disease conditions. The project successfully set up PCR-

RFLP and HPLC methods for genotype determination and blood drug level measurements with adequate accuracy and precision. The general population study has revealed that clinically relevant SNPs of CYP2B6, CYP2C19, CYP2D6, NAT2 and GSTs, particularly CYP2D6*4, CYP2C19*3 GSTM1 and GSTT1 deletion alleles are variably distributed between the three ethno-linguistically distinct populations of Kenya, other Africans, Caucasian and Asian populations. The psychiatric study has shown that CYP2D6 IM status significantly influenced covariates of psychotropic medication pattern such as polypharmacy of more than five drugs, frequent hospitalization and use of high doses of benzhexol against EPS. The HIV study has established that CYP2B6 516 G>T was highly prevalent and strongly influenced Nevirapine plasma levels

The data obtained from these studies is applicable in the optimization of therapeutic outcomes in HIV and psychiatry. Further benefits could accrue from the introduction of pharmacogenomic testing. However, the challenge is the development and eventual marketing of a pharmacogenomic test that is cost-effective and has a high predictive value in African populations. Presently, however, using national genetic data as a substitute for individual genotyping seem to be a practicable option, due to financial and technological limitations. Thus, for the foreseeable future, knowledge of allele frequencies within each country is deemed essential for individuals to receive genetically tailored drug therapy. Unfortunately, there is currently minimal genotype data for most African countries, which will continue to exclude most African individuals from genetically based healthcare recommendations. The results of this study have therefore contributed to bridging this gap.

The limitations of this project hinge on the limited number of SNPs tested for each gene mainly due to financial limitations. There was also a weakness in the use of convenient sampling of study populations which could introduce bias. Availability

of sequencing could also have enabled the project to explore hitherto unknown SNPs.

7.3 Recommendations

Results from this thesis research suggest the following recommendations:

Further population pharmacogenetic studies covering diverse Kenyan populations particularly those who are known to be unique such as the Luhyia who are highly heterogeneous in dialect, the isolated tribes such as the Ogiek, the Mijikenda and the cushites.

Genotyping of a wider array of genes involved in drug metabolism and adverse drug reactions including the *HLA* and membrane transporters in Kenyan populations.

Randomized control trials or well-designed prospective clinical studies testing sound hypotheses to evaluate the clinical impact of pharmacogenetically tailored therapy on treatment outcomes.

Formulation of pharmacogenetic tests of high predictive value in African populations and cost-effectiveness studies to evaluate their cost-benefit effects in clinical settings in resource constraint healthcare delivery systems.

Genome wide association studies (GWAS) to relate the upsurge of certain diseases such as cancer, exposure to noxious environmental pollutants and defective xenobiotic metabolism due to genetic polymorphisms in relevant genes.

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APPENDICES

APPENDIX 1: Ethical approval

KENYATTA NATIONAL HOSPITAL
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Ref: KNH-ERC/ MOD/402

21st October 2009

Dr. Margaret Oluka
School of Pharmacy
University of Nairobi

Dear Dr. Oluka

Re: Approval of modifications on the study titled "Genetic Polymorphism of drug metabolizing Enzymes and clinical implications in Kenyan population" (P72/7/2003)

Your communication of September 24, 2009.

The KNH/UON-ERC has reviewed and approved the following modifications:

1. Two new consent forms.
2. Additional subjects populations which includes:
   - HIV/AIDs patients on Nevirapine based ARV drug therapy at K.N.H.
   - Patients on Thiopurine drugs including 6-mercaptopurine and Azathioprine at Kenyatta National Hospital and other hospitals and clinics around Nairobi.
3. Revised proposal incorporating the two additional specific objectives which include:
   - The influence of CYP2B6 on response to Nevirapine in HIV/AIDS patients.
   - The influence of the enzyme Thiopurine-S- methyl transferase and other risk factors on the safety and tolerability of Thiopurine drugs.

Yours sincerely

DR. L. MUCHIRI
AG SECRETARY, KNH/UON-ERC

c.c. Prof. K.M. Bhatt, Chairperson, KNH-ERC
The Deputy Director CS, KNH
The Dean, School of Pharmacy, UON

APPENDIX 2: Informed consent form for general population

VOLUNTEER INFORMTION AND CONSENT FORM

CONSENT FOR ENROLMENT IN THE STUDY

(To be administered in English or Kiswahili translations

Preamble

You are being asked to volunteer freely in this study .Before you decide to join, we would like to provide you with information about the study. This document is a consent form, it has information about the study and will be discussed with you by the investigators. Please study it carefully and be free to seek any clarification especially concerning terminologies or procedures that may not be clear to you. If you agree to join this study, you will be asked to sign this consent form and a copy will be given to you for safe keeping.

Purpose of the Study

The main purpose of this study is to investigate the occurrence and influence of genetic polymorphism of drug metabolizing enzymes in the African Kenyan population. We shall focus mainly on cytochrome P450 (CYP) super family of enzymes and some phase II drug metabolizing enzymes. The study will particularly focus on the isoenzymes, CYP2D6, CYP2B6, CYP2C19, NAT2 and Glutathione S transferase(GSTs) which are involved in the metabolism and disposition of therapeutic drugs including neuroleptic and anti-depressant, antiretrovirals , Proton pump inhibitors, antitubecular and Anti neoplastic agents. These enzymes have been shown to have many polymorphic variants which are associated with increased, reduced or absent enzyme activity. The occurrence and distribution of the polymorphic variants in a population depends on ethnic origin. Hence this study will investigate the presence and frequency of occurrence of polymorphic variants of these enzymes in the African Kenyan population

Study Procedures

Genotyping studies

With your consent, 10.0ml of blood will be withdrawn from your arm by venopuncture and collected into a clean sterile bottle. Strict observance of aseptic conditions will be ensured. This blood sample will be used to determine your enzyme polymorphic status.

Screening and eligibility If you show interest in participating in this study, you will undergo a medical assessment to establish the state of your health including: Physical Examination, past medical history as well as well as past and present medication use. Tobacco and alcohol use.

Your sample. Only blood samples will be collected from your which will be used only for genotyping for polymorphic drug metabolizing enzymes and their allelic variants.

Risks and Discomforts Participating in this study will be associated with minimum risk and discomfort. Possible risks and discomfort during blood collection includes: Pain , Bleeding , Swelling

Benefits

You may not benefit directly from this research study. The findings of this study may or may not benefit you or your community.Some possible benefits are: Increased knowledge about cytochrome P450 enzyme system and other polymorphic enzymes. Increased knowledge about genetic polymorphism of drug metabolizing enzymes. Adjustment of drug dosages for indigenous population. Reduced adverse effects. Improved patient compliance. Improved clinical out comes

Confidentiality

The study staff will take utmost care to keep your participation in this study confidential. Your samples will be identified only by a coded number. Information from this study will be used in reports, published papers or presented in public but your name will never be used. Your name will only be known to the principal investigators for purposes of follow-up.

Voluntary participation/ withdrawal from study

The decision to take part in this research study is your choice. You may choose not to take part or to stop participating at any time.

Questions

You are free to ask questions at any time about the study and regarding your right as a research volunteer. You will not be giving up any of your legal rights by signing this consent form.

Further Information

For further information about this study you may contact Ms Margaret N. Oluka, who is a principal investigator as well as the coordinator of the study at the Department of pharmacology and pharmacognosy at the Faculty of Pharmacy, University of Nairobi, P.O. Box 19498, Nairobi.Tel;2725099/2726771,0722-604216.

For questions related to your rights as a volunteer in this research study; You may contact Prof. A. N. Guantai, Secretary to the Kenyatta National Hospital Ethics and Research Committee (KNH - ERC), Faculty of Pharmacy, P. O. Box 19676, Tel; 2711132/ 2726770).

STATEMENT OF CONSENT

I have read this consent form. I have had the chance to discuss this research study with the investigator. I have had my questions answered in a language I understand. The risks and benefits have been explained to me. I understand that my participation in this study is voluntary and that I may choose to withdraw at any time. I freely agree to participate in this research study.

By signing this consent form, I have not given up any of the legal rights that I have as a participant in a research study.

- I agree to participate in this research study

  YES/N

  O
- I agree to have my blood collected and analysed for enzyme genotypes

  YES/N

  O

Participant's signature _____ Date _____

Participants printed name _____

I, the undersigned have fully explained the relevant details of this research study to the participant named above and believe that the participant has understood and has knowingly given his consent.

Printed Name _____ Date _____

Signature _____

Role in study _____

APPENDIX 3: Psychiatric patient recruit questionaire

CYP P450 STUDY IN KENYA

PSYCHIATRIC PATIENTS VOLUNTEER ENROLMENT FORM

1. ENROLLMENT NUMBER: _____
2. AGE _____ SEX _____
3. ETHNICITY (e.g.: Kamba, Kikuyu, Luo, etc.)

   _____
4. ETHNICITY OF PARENTS:

   Father: _____ Mother: _____
5. RESIDENCE:

   i. Residence in Nairobi

   Road: _____

   Estate: _____

   House No.: _____

   ii. Permanent Residence (if different from above)

   Road: _____

   Estate: _____

   House No.: _____
6. ORIGIN / BIRTHPLACE

   Province: _____ District: _____

   Location: _____ Sub-Location: _____

   Village: _____
7. TYPE OF MENTAL ILLNESS (e.g.: Depression, Schizophrenia)

   1. _____

   2. _____

8. OTHER MEDICAL CONDITIONS

   1. _____

   2. _____

9. MEDICATIONS

   Medications in the last six months (Name, Disease, Duration)

1. _____
2. _____

10. HOSPITAL ADMISSIONS (Last 2 years) YES / NO

If yes, give details: (when, duration, medication)

_____

_____

11. OUT-PATIENT CONSULTATIONS (Last 2 years) YES / NO

If yes, give details: (when, medication, etc.)

_____

12. ADVERSE DRUG REACTIONS YES / NO

If yes, give details: (drug, symptoms, management)

_____

_____

13. PROGNOSIS / CLINICAL OUTCOME

_____

_____

APPENDIX 4: HIV patient assesment and interview during blood sampling

CRITERIA CHECK

SECTION ONE: STUDY ELIGIBILITY CHECK LIST

Inclusion Criteria: (if any of the criteria is marked "NO" the participant is not eligible for enrollment)

		YES	NO
1.	Participant has been attending Comprehensive Care Center, Kenyatta		
2.	Participant has been on a Nevirapine based regimen for a t least 6months .		
3.	Participant is aged above 18 years but below 55 years		
4.	Participant has signed the informed consent form		

Exclusion Criteria: (if any of the criteria is marked "YES" the participant is not eligible for enrollment)

		YES	NO
1.	Participant pregnant		

2. Participant is not on any Nevirapine containing regimen

3. Participant has not signed the informed consent form

Section B: Patient Interview

B.I Case Adherence Index questionnaire (Mannheimer et al., 2006; Wakibi et al., 2011)

Please ask each question and circle the corresponding number next to the answer, then add up the numbers circled to calculate Index score.

B.1.1 How often do you feel that you have difficulty taking your HIV medications on time? By 'on time' we mean no more than two hours before or two hours after the time your doctor told you to take it.

4 Never, 3 Rarely, 2 Most of the time, 1 All of the time

B.1.2 On average, how many days per week would you say that you missed at least one dose of your HIV medications?

1 Everyday2 4–6 days/week3 2–3 days/week, 4 Once a week , 5 Less than once a week, 6 Never

B.1.3 When was the last time you missed at least one dose of you HIV medications?

1 Within the past week, 2 1–2 weeks ago, 3 3–4 weeks ago, 4 Between 1 and 3 months ago, 5 More than 3 months ago

6 Never

INDEX SCORE: _____ >10 = good adherence ≤10 = poor adherence

B.2 PATTERNS OF NON PRESCRIPTION DRUG/HERBAL PRODUCTS USE

1.In the last 7 days have you ever used any drugs or herbal medicines that were obtained elsewhere?

YES _____ NO _____

3. If yes the above, what products have you used?

PRODUCT	DOSE, DURATON OF USE	WHERE SOURCED	REASONS FOR USE

B.3 SELF REPORTED ADVERSE DRUG REACTIONS

B.3.1 Have you ever reacted badly to the medicines that you are given in this clinic?

TYPE OF REACTION	YES	NO
Skin Problems (rash, itching, open		

wounds)		
Stomach problems (stomach ache, diarrhea, vomiting, heartburn, ulcers)		
Liver problems (Yellow Eyes)		
Any Other		

B.3.1 If yes to any of the above, were you able to carry out your normal duties such as reporting to work and carry out normal household chores?

1. Fully active and was able to continue with normal duties with no restrictions

2. Could not carry out normal duties fully but could do light work in the office and at home

3. Could do no work at all

If answer is 1, then skip the next two questions (B.3.2 to B.3.3)

B.3.2 If you could not do much work, where you able to take care of yourself (bathing, feeding)?

Yes _____ No _____

B.3.3 During day time, How much time did you spend in bed or resting in a chair when you reacted to the drug?

1. Continued with usual activities

2. Most of my waking hours /

3 Was admitted in hospital

B.3.4 Were you admitted to hospital as a result of the reaction to the drug?

Yes _____ No _____

B.3.4 When did the reaction occur?

1. Within the first month of starting treatment

2. Within first 6 months of starting treatment

3. Other _____

4. Cannot remember

B.3.5 Did you inform the doctors in CCC about the reaction to the drug?

YES _____ NO _____

GUIDE FOR RATING THE SEVERITY OF THE ADVERSE DRUG REACTION (FOR USE BY THE PI AND CO PIs ONLY)

	B.3.1(Ability to carry out normal duties)	**B.3.2** (Ability to take care of oneself)	B.3.3 (Proportion of waking hours spend in bed/sitting)	B.3.4 Hospitalization was required
0	1 Fully active	Yes	1	No
1	2.Could carry out light work	Yes	2	No
2	3 Could not do my normal duties	Yes	Up and about more than 50% of waking hours	No
3	3 Could not do my normal duties	No	Capable of only limited self care, confined to bed or chair more than 50% of waking hours	No
4	3 Could not do my normal duties	No	Completely disabled. Cannot carry on any self care. Totally confined to bed or chair	Yes

APPENDIX 5: Data collection form for extraction of information from patient files

SECTION B: SOCIO-DEMOGRAPHIC CHARACTERISTICS OF THE PARTICIPANT

Date of Birth _____

GENDER ☐ MALE ☐ FEMALE

MARITAL STATUS ☐ MARRIED ☐ SINGLE ☐ DIVORCED

HIGHEST EDUCATION LEVEL ☐ DEGREE ☐ DIPLOMA HIG☐OL PRIMA☐

OCCUPATION ☐ EMPLOYED ☐ UNEMPLOYED ☐ SELF EMPLOYED

ETHNICITY

_____

SMOKER ☐ YES ☐ NO

ALCOHOL USE ☐ NEVER ☐ OCCASIONALLY ☐ REGULARLY

SECTION C: MEDICAL HISTORY

DATE DIAGNOSED WITH HIV/AIDS

_____

DATE COMMENCED HAART

_____

CONCURRENT MEDICAL CONDITIONS AT THE TIME OF DATA COLLECTION

1.
2.
3.
4.
5.

ALLERGIES _____

SECTION D: SELECTED LABORATORY VALUES AT BASELINE

PARAMETER	DATE	VALUE	NORMAL VALUE
ALT			
AST			
BILURIBIN			
CREATININE			
CD4 COUNTS			
VIRAL LOAD			

SECTION E: MEDICATION HISTORY

(Only list medications that patient has been taking at least 2 weeks before and after the date of sample collection)

DRUG NAME	DOSE	FREQUENCY	
ANTIRETROVIRALS			
1.			
2.			
3.			
4			
OTHERS			
5			
6			
7			

8			
9			
10			
11			
12			

SECTION F: LABORATORY VALUES DURING TREATMENT

DATE						
PARAMETER	**1st**	**2nd**	**3rd**	**4rd**	**5th**	**6th**
ALT						
AST						
BILURIBIN						
CREATININE						
CD4 COUNTS						
VIRAL LOAD						
DATE						
PARAMETER	**7th**	**8th**	**9th**	**10th**	**11th**	**12th**
ALT						
AST						
BILURIBIN						
CREATININE						
CD4 COUNTS						
VIRAL LOAD						

SECTION G: ADVERSE DRUG REACTIONS AND ADHERENCE

(Examine the clinical notes for following)

DATE

Did Patient complain of rash ☐ **YES** ☐ **NO**

If Yes to the above, what was the severity? ☐ **MILD** ☐ **SEVERE**

Is the patient noted to be non-adherent? ☐ **YES** ☐ **NO**

APPENDIX 6: HIV volunteer information and consent form

PREAMBLE

Nevirapine is a (NNRTI) antiretroviral agent used as a component of HAART (highly active antiretroviral therapy). It is used in combination with other anti-retroviral drugs.

Studies show that blood levels of nevirapine can vary greatly between individuals. Some patients have very high levels and others have very low levels. These differences be caused by fact that some people destroy the drug very fast and other very slowly. One of the enzymes that destroy Nevirapine in the body is called CYP2B6. There are different types of this enzyme that makes it work faster or much slower.

We therefore wish to carry out a study to check blood levels of Nevirapine and the types of CYP2B6 in patients treated with the drug.

We are requesting you to volunteer freely in this study. Before you decide to join, we would like to provide you with information about the study. This document is a consent form; it has information about the study and will be discussed with you by the investigators. Please, study it carefully and feel free to seek any clarification especially concerning terminologies or procedures that may not be clear to you. If you agree to join this study, you will be asked to sign this consent form and a copy will be given to you for safekeeping.

PURPOSE OF THE STUDY

The main objective of this study is to determine the blood levels of drug nevirapine and to determine whether these levels are affected by genotype of an individual. In addition, the plasma levels may be influenced by adherence, body mass and other factors. The second objective is to determine whether patients with low or high blood levels respond better or worse to medication.

STUDY PROCEDURES

With your consent, 5.0 ml of blood will be withdrawn from your arm using a clean needle and syringe and transferred into a clean EDTA tube. This blood sample will be used to determine the concentrations of nevirapine in your blood. With your permission, you make donate a second blood sample at least 2 weeks later. You will be asked a few questions about your ethnicity, if you are using any other drugs or herbal products, whether you drink or smoke, how regularly you take the drugs and whether you have ever experienced any bad reactions to the drugs that you are taking and what time you took the medication. In addition your weight and height will be measured. The researchers will also check your medical records to see how well you have been responding drugs. The information that will be obtained from the files will be the levels of white blood cells (CD4 counts), viral load, history of skin reactions and the function of your liver (Alaninine Transaminases, Aspartate Transaminase and biluribin levels).

INCLUSION/EXCLUSION CRITERIA

In order to participate in the study, you should meet the following criteria:

1. You must be have been attending Comphrensive Care Care, KNH for at least one year.
2. You must be gave been taking Nevirapine for at least 5 weeks.
3. You must be aged above 18 years and less than 55 years.
4. You must have agreed to take part in the study.

Exclusion criteria

You cannot take part in this study if:

1. If you are a child less than 18 years of age.
2. If you are a pregnant woman.
3. If you decline to participate

Your sample

Only blood samples will be collected from you, which will be used solely to determine the plasma concentration of the drug nevirapine and whether you have genes that enable you to metabolise nevirapine very fast or slowly.

Risks and Discomforts

Participating in this study may be associated with minimum risk and discomfort during blood collection which may include the following reactions at the injection site:

1. Pain
2. Slight bleeding
3. Slight swelling

Benefits

This study may be of benefit to you in that you will be could be used to adjust your treatment to avoid treatment failure or toxicity. The findings of this study may be of benefit to other HIV patients in Kenya. The information gained from the study may be used to:

1. Adjustment of drug dosages.

2. Reduce adverse reactions and treatment failures.
3. Reduce the development of resistance to anti-retroviral drugs.
4. Improved clinical outcomes in HIV treatment.
5. Improved rational use of drugs.

Confidentiality

The study staff will take utmost care to keep your participation in this study confidential. Your samples will be identified only by a coded number. Information from this study will be used in reports, published papers or presented in public but your name will never be used. Your name will only be known to the principal investigators for purpose of follow-up.

Voluntary participation/withdrawal from study

The decision to take part in this research study is your choice. You may choose not to take part or to stop participating at any time.

Questions

You are free to ask any questions at any time about the study and regarding your right as a research volunteer. You will not be giving up any of your legal rights by signing this consent form.

Further Information

For further information about this study you may contact:

Dr Margaret Oluka , Department of pharmacology and Pharmacognosy,
School of Pharmacy, University of Nairobi,
P O Box 19676, Nairobi.. Tel: 722604216, +254 02 2725099, 0727499537.
For questions related to your rights as a volunteer in this research study; you may contact
Prof A. N. Guantai, Chairman of theUniversityof Nairobi/Kenyatta National Hospital Research and Ethics Committee (UoN/KNH – ERC), School of Pharmacy, P. O. Box 19676, Nairobi. Tel: +254 020 2726300 Ext 44102.

STATEMENT OF CONSENT

I have read, or have had this consent form read to me. I have had the chance of discussing this research study with the investigators. I have had my questions answered in a language I understand. The risks and benefits have been explained to me. I understand that my participation in this study is voluntary and that I may choose to withdraw at any time. I freely agree to participate in this research study.

By signing this consent form, I have not given up any of the legal rights that I have as a participant in a research study.

I have read, or have had it read to me **YES / NO**

I agree to participate in this research study **YES / NO**

I agree to have my blood collected and analyzed for plasma concentrations of Nevirapine for the gene CYP2B6. **YES / NO**

Participant's signature: _____ Date: _____

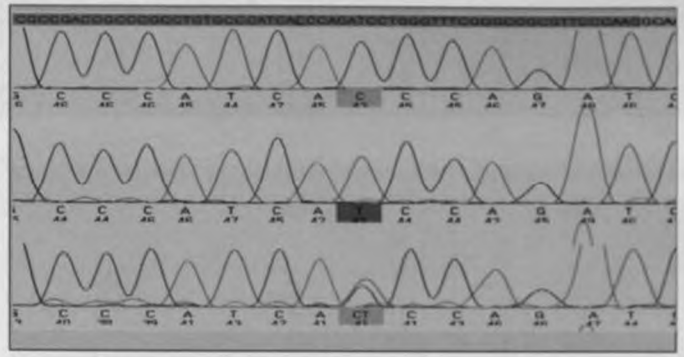
I, the undersigned have fully explained the relevant details of this research study to the participant named above and believed that the participant has understood and has knowingly given his consent.

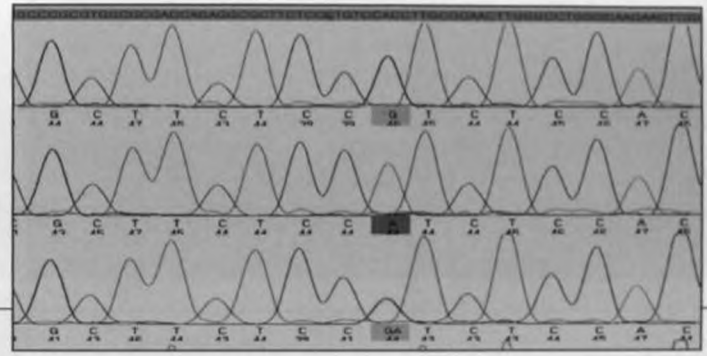
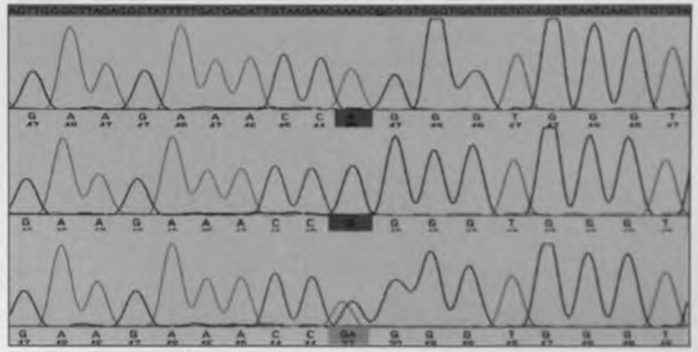
Printed Name: _____ Date: _____

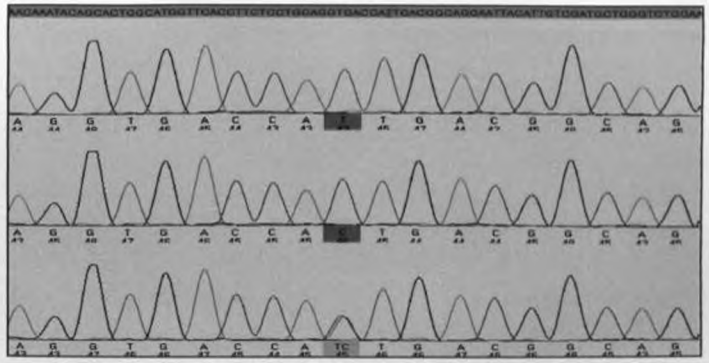
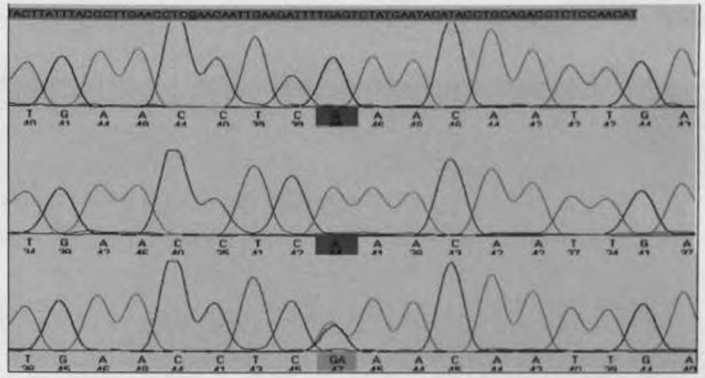
Signature: _____

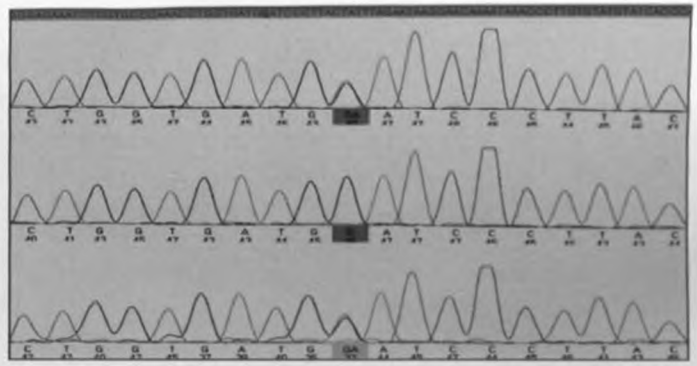
Role in this study: _____

APPENDIX 7: Electropherograms showing neucletide sequences at some of the SNPs in this study

CYP2D6*17 **(1023C>T)**	**Appendix 7a: Nucleotide sequence at *CYP2D6*17***	
		

CYP2D6*29 **(3183 G>A)**	**Appendix 7b: Nucleotide sequence at *CYP2D6*29***	
NAT2*14 **(191 G>A)**	**Appendix 7c: Nucleotide sequence at *NAT2*14***	

NAT2*5 **(341 T>C)**	**Appendix 7d: Nucleotide sequence at *NAT2*5**	
***NAT2*6** **(590G>A)**	**Appendix 7e: Nucleotide sequence at *NAT2*6**	

NAT2*7 **(857 G>A)**	**Appendix 7f: Nucleotide sequence at *NAT2*7**	
CYP2C19*2 **(681G>A)**	**Appendix 7g: Nucleotide sequence atCYP2C19*2**