GENOTYPIC FREQUENCIES OF CYP2B6, CYP3A5 AND SULT1A1 POLYMORPHISM AMONG HIV PATIENTS ON NEVIRAPINE-BASED ANTIRETROVIRAL THERAPY AT THE KORLE-BU TEACHING HOSPITAL

BY

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JULY, 2018
DECLARATION

I, Isaac Egyir Prah do hereby declare that, with the exception of quoted articles and references, this work was duly carried out by me and the results obtained herein are true reflection of the work done under the supervision of Dr Isaac Asiedu-Gyekye (Department of Pharmacology and Toxicology, School of Pharmacy) and Dr William Kudzi (Centre for Tropical Clinical Pharmacology and Therapeutics, School of Medicine and Dentistry), University of Ghana, Legon.

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DEDICATION

I dedicate this work to my parents, Mr and Mrs Prah; and my siblings, Alfred Attom Prah, Isaiah Prah and Emmanuel Annobil Prah. God richly bless them for their support.
ACKNOWLEDGEMENT

I would like to express my deep thanks to Dr William Kudzi of Centre for Tropical Clinical Pharmacology and Therapeutics, School of Medicine and Dentistry, University of Ghana, for his scientific support, guidance and invaluable supervision. I would like to express my thanks to him for kindly providing me with materials for this research work.

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To all staff of the Fevers Unit, Department of Medicine, Korle-Bu Teaching Hospital, I say thank you for your role in the sampling process. I also thank Rev Dr Charles Antwi-Boasiko and all staff of Physiology Department, School of Biomedical and Allied Health Sciences for their support during the laboratory analysis of the research samples.
ABSTRACT

Background: A major constraint in the management of People Living with Human Immunodeficiency Virus, with highly active antiretroviral therapy is the high incidence of adverse drug reaction among patients. Nevirapine, a non-nucleoside reverse transcriptase inhibitor is among the first-line antiretroviral therapy used in Ghana for the management of Human immunodeficiency virus-1 patients. Despite its usefulness, hypersensitivity reaction is a common complication that accounts for patients defaulting during therapy in Ghana. Nevirapine-induced hypersensitivity has been linked to various mutations in the gene encoding the drug metabolizing enzymes but these genetic associations are inconsistent among different populations.

General Aim: To determine the genotypic frequencies of CYP2B6, CYP3A5 and SULT1A1 among patients exposed to nevirapine-based antiretroviral therapy.

Method: The study was a case-control study that involved seventy Human Immunodeficiency Virus-1 infected patients accessing antiretroviral therapy at the Korle-Bu Teaching Hospital, Accra, Ghana. Based on Clinicians diagnosis, patients who have been on nevirapine combination antiretroviral therapy and have developed nevirapine hypersensitivity within six months after therapy initiation were identified and categorized as cases, and controls if otherwise. Clinical and demographic data of the participants were recorded and the genomic deoxyribonucleic acid was isolated from the whole blood samples. Samples were genotyped for specific single nucleotide polymorphisms in Cytochrome P450 (CYP2B6 983T>C and CYP3A5 14690G>A) and Sulfotransferase 1A1 (SULT1A1 638G>A) using Polymerase chain reaction-Restriction fragment...
length polymorphism. Logistic regression was used to find the association between the dependent and independent variables in the study.

**Outcome:** Twelve (12) out of the total 70 participants involved in the study, were identified as having nevirapine-induced hypersensitivity (cases) and all the remaining as a control. The mean age of study participants was recorded as 38 ± 9.47 years. Body mass index, CD4 count, sex and age of participants had no significant association with a nevirapine hypersensitivity reaction. The frequencies of *CYP2B6 983T>C* were 3% in the control group, and 4% in the case group. Also, *CYP3A5 14690G>A* frequencies were 50% in both case and control group. *SULT1A1 638G>A* frequencies were recorded as 21% and 22% in case and control group respectively.

Risk of nevirapine hypersensitivity development among study participants was estimated using odds ratio (OR) at a 95% confidence interval (CI). Individuals carrying at least one variant allele of *CYP2B6 983T>C* has 1.67 times more risk (OR=1.67, 95% CI=1.58-17.55, p =0.67) for developing nevirapine hypersensitivity whereas individuals carrying at least one variant allele of *SULT1A1 638G>A* has 1.35 times more risk (OR=1.35, 95% CI=0.38–4.84, p =0.64) and both were statistically not significant (p > 0.05). *CYP3A5 14690G>A* allele and genotypic frequency was constant in the study population.

**Conclusion:** There was no significant association of *CYP2B6 983T>C*, *CYP3A5 14690G>A*, *SULT1A1 638G>A* to nevirapine hypersensitivity development. The results may offer a preliminary basis for the more rational use of drugs that are substrates for CYP2B6, CYP3A5 and SULT in the Ghanaian population. This may be helpful to maximize optimal responses at the lowest doses, indirectly implying the reduction of unintended toxicities.
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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>ADR</td>
<td>Adverse drug reaction</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
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<tr>
<td>ARV</td>
<td>Antiretroviral</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral therapy</td>
</tr>
<tr>
<td>bp</td>
<td>Basepairs</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of differentiation 4</td>
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<tr>
<td>COOH</td>
<td>Carboxy</td>
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<td>CYP</td>
<td>Cytochrome P450</td>
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<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
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<tr>
<td>HAART</td>
<td>Highly Active Antiretroviral Therapy</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>INSTI</td>
<td>Integrase strand transfer inhibitors</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-nucleoside reverse transcriptase inhibitor</td>
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<tr>
<td>NRTI</td>
<td>Nucleoside reverse transcriptase inhibitors</td>
</tr>
<tr>
<td>OH</td>
<td>Hydroxy</td>
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<tr>
<td>PAPS</td>
<td>Phosphoadenosine – phosphosulfate</td>
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<tr>
<td>Acronym</td>
<td>Term</td>
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<td>-------------------------------------------</td>
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<tr>
<td>PI</td>
<td>Protease inhibitor</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SJS</td>
<td>Stevens-Johnson syndrome</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<tr>
<td>SUL</td>
<td>Sulphoxy</td>
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<tr>
<td>SULT</td>
<td>Sulfotransferase</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetic acid ethylenediamine tetra-acetic acid</td>
</tr>
<tr>
<td>TEN</td>
<td>Toxic epidermal necrolysis</td>
</tr>
<tr>
<td>UGT</td>
<td>Uridine glucuronosyltransferase</td>
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<td>WHO</td>
<td>World Health Organization</td>
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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Human Immunodeficiency Virus (HIV) infection is a burden in most resource-limited countries. It is one of the greatest health crises globally (Oreagba et al., 2014, Mbulaiteye et al., 2002). It is a global pandemic and all measures and efforts are geared to prevent and treat the infection. However, the challenges are multifaceted, from social and behavioural problems to the drug resistance of the virus (Nema and Singh, 2017).

The current estimate gives about 37 million people living with HIV and about 35 million people have died of AIDS by 2015 since the beginning of the epidemic (UNAIDS, 2017b). The epidemic is overwhelmingly centred on sub-Saharan Africa, where the adult prevalence is over 10 times greater than the prevalence in Europe (Rang et al., 2016). Sub-Saharan Africa remains the most severely affected region of the globe, accounting for at least 69% (about two-thirds) of the entire HIV infected population.

With more than thirty years of the HIV epidemic, there is still no cure or an effective vaccine. However, there have been major advances in treating HIV with the current available antiretroviral therapy (ART) (Kharsany and Karim, 2016). After the approval of the first antiretroviral (ARV), zidovudine by the United States-Food and Drug Administration, there has been a considerable progress in the treatment of HIV (Estes et al., 2007).
World Health Organization (WHO) guidelines for initiating ART recommends Highly active antiretroviral therapy (HAART) as a standard treatment for the management of HIV (WHO, 2016). The introduction of ART in the public health care system in Ghana dates back to June 2003 at two pilot sites in the Manya Krobo district in the Eastern region, Ghana (Ministry of Health and Ghana Health Service, 2016). Ghana aims to achieve the 90-90-90 fast-track treatment targets of 90% of people living with HIV knowing their HIV status, 90% of people who know their HIV-positive status are accessing treatment and 90% of HIV patients on treatment have suppressed viral loads by 2020 (Global Fund, 2017).

Antiretroviral therapy aims to provide maximum durable suppression of viral replication, which allows HIV patients to live long, restore and preserve immune function. It also aims to reduce HIV-related infectious and non-infectious morbidity and mortality (Manosuthi et al., 2006, Meintjes et al., 2017). Rapid scale-up of these ART has transformed what was inevitably a fatal disease to a chronic, manageable condition leading to notable declines in the worldwide rates of Acquired Immunodeficiency Syndrome (AIDS)-related deaths (Kharsany and Karim, 2016, Africa Constituencies Bureau, 2016). The scale-up of ART for HIV patients over the past 15 years is one of the most remarkable achievements in public health leading to a substantial decline in new HIV infection (Mikkelsen et al., 2017).

Despite the immense contribution by the introduction of ART, there are reports of accompanied adverse drug reactions (ADR) (Tozzi, 2010). HIV patients have a higher risk of developing hypersensitivity usually cutaneous reactions than the general population, which has a significant impact on patients’ current and future care options. The severity of cutaneous adverse reactions
varies greatly, and some may be difficult to manage. HIV-infected patients just at the beginning of ARV treatment can show a wide variety of adverse drug effects such as drug-induced hyperpigmentation, hair loss, toxic epidermal necrolysis (TEN) or Stevens-Johnson syndrome (SJS) and many more (Borras-Blasco et al., 2008). Among HIV-positive patients on HAART, adverse drug reaction (ADR) plays a major role in treatment modifications (substitution or switch) in Ghanaians (Tetteh et al., 2016). Specific ADRs may vary among patients, class of drugs and from one drug to another. The spectrum of adverse effects associated with ARVs may also vary between developed and developing countries (Subbaraman et al., 2007, Oreagba et al., 2014).

Genetic variations in drug-metabolizing enzymes, receptors, transporters, and other drug targets have been linked to inter-individual differences in the efficacy and toxicity of many medications (Tozzi, 2010, Pavlos and Phillips, 2012). Adverse events such as rash, hepatotoxicity, central nervous system side effects, hyperlipidaemia, renal toxicity, pancreatitis, peripheral neuropathy, lipodystrophy, and hyperbilirubinemia have been linked to genetic variations among patients on specific ART (Tozzi, 2010).

1.2 Problem statement

One of the constraints of HAART is the high prevalence of ADRs among patients receiving HAART. Adverse drug reactions are a very common complication of ART and a reason for patients defaulting during HIV therapy in the developing countries (O’Brien et al., 2003, Tetteh et al., 2016).
Nevirapine is a cornerstone drug for the treatment of HIV-1 infection, especially in resource-limited countries and for pregnant women to prevent mother to child HIV-1 transmission (Barco and Nóvoa, 2013, Jemmy and Alex, 2014). In Ghana, nevirapine is among the first line drugs prescribed in combination with tenofovir or zidovudine and lamivudine or emtricitabine for the management of HIV-1. However, it has been contraindicated in hypersensitivity rash and liver dysfunction (Ministry of Health and Ghana Health Service, 2008).

Nevirapine treated individuals develop allergenic reactions ranging from nevirapine-induced hepatotoxicity, rash to severe blistering skin reactions namely SJS as well as TEN (Figure 1.1). Rash and hepatotoxicity are the most frequent toxicities associated with nevirapine with about 6% to 7% of patients starting nevirapine therapy switching treatment due to the adverse effects (Sarfo et al., 2014). Approximately 10% of patients treated with nevirapine in clinical trials developed hepatotoxicity; however, 3.7% of these patients experienced symptomatic elevations of their liver enzymes, with nearly half (46%) of the patients with hepatotoxicity also developing a rash (Estes et al., 2007). This clinical manifestation usually occurs within the first 18 weeks after starting nevirapine therapy (Wit et al., 2008). In Malawi, 25% of HIV patients developed nevirapine-induced hypersensitivity following the administration of nevirapine to HIV-1 patients (Dickinson, 2014). A retrospective study in Ethiopia showed nevirapine hypersensitivity accounting for 26% and 8% as rash and hepatotoxicity respectively (Teklay et al., 2013).

The incidence of nevirapine hypersensitivity has been recorded in Ghana among HIV-1 patients who are given nevirapine as part of the combination ART regimen. A retrospective study in Ghana showed a cumulative frequency of 10.2% (n= 1,621) with 0.7% life-threatening
conditions associated with severe morbidity and occasional mortality of nevirapine-associated rash among HIV-1 patients at the Komfo Anokye Teaching Hospital, Kumasi (Sarfo et al., 2014).

A higher incidence of nevirapine hypersensitivity of 15.7% was recorded at the Korle-Bu Teaching Hospital, Accra, Ghana in a prospective cohort study (Kudzi et al., 2017). Reasons for variation in response to ART may include immunological, virological and pharmacological factors as well as behavioural differences among HIV-infected individuals. However, one potential factor that may contribute to these differences in ART response is genetic variability among the patients (Estes et al., 2007).

While the reasons for the adverse effects of nevirapine are still unclear, increasing evidence suggests that genetic factors have a role in the initiation of the toxic responses (Castroa et al., 2015, Pavlos et al., 2017).

Figure 1.1: Nevirapine associated adverse reaction among HIV-1 patients (a and b).

(a) Erosion of lips and mouth are characteristics of SJS and TEN  (b) SJS with discrete non-confluent small blisters, involving 10% of the body surface area

Source: Fagot et al. (2001).
1.3 Justification

Single nucleotide polymorphisms (SNPs) in the genes encoding the metabolizing enzymes Cytochrome P450 (CYP) and Sulfotransferase (SULT) among individuals have been shown to impact nevirapine pharmacokinetics in various populations (Marinho et al., 2013, Dickinson, 2014). Patients on HAART generally remain on the medication indefinitely. However, severe toxicity to ARTs may cause poor adherence to therapy and therefore treatment failure. Therefore, it is important to determine the genetic risk factors associated with the incidence of toxicity among the currently available first-line ART including nevirapine. Genotypic information about the metabolizing enzyme may indicate the need to change medication or adjust the dosage of a drug to minimize toxicity and maximize patient safety.

Nevirapine exposure could be influenced by polymorphisms in CYP2B6 and CYP3A4 affecting drug metabolism (Dickinson, 2014). However, little is known regarding genetic risk factors for nevirapine-induced hypersensitivity in sub-Saharan Africa (Carr et al., 2013). CYP2B6 516G>T and CYP2B6 983T>C have been reported in the literature to be significantly associated with an increased risk of nevirapine hypersensitivity in Malawian, Mozambique and Ugandans (Ciccacci et al., 2013, Carr et al., 2014b). Due to genetic variations among ethnic groups resulting in variability in response to ART, it is not recommendable to transpose results across different regions and populations (Mensah et al., 2017). It is, therefore, necessary to determine whether CYP2B6, CYP3A5 and SULT1A1 alleles are predisposing factors for nevirapine hypersensitivity in Ghanaian HIV-1 infected patients.
The findings will help to identify specific genetic markers for nevirapine hypersensitivity reaction that may be useful for tailored drug prescription which may be the best attempt to maximize optimal responses at the lowest doses, indirectly implying the reduction of unintended toxicities.

1.4 Aim

To determine the genotypic frequencies of CYP2B6, CYP3A5 and SULT1A1 among patients exposed to nevirapine-based antiretroviral therapy.

1.5 Specific objectives

1. To determine the genotypic frequencies of \textit{CYP2B6} 983T>C, \textit{CYP3A5} 14690G>A and \textit{SULT1A1} 638G>A among patients exposed to nevirapine-based antiretroviral therapy.

2. To determine the association between single nucleotide polymorphism (\textit{CYP2B6} 983T>C, \textit{CYP3A5} 14690G>A and \textit{SULT1A1} 638G>A) and nevirapine-induced hypersensitivity.
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Human Immunodeficiency Virus and its infection mechanism

HIV was identified in 1983 as it has evolved from a similar virus, Simian Immunodeficiency Virus that affects monkeys and apes. There are two forms of HIV. HIV-1 and HIV-2 (Fischer and Madden, 2011). However, HIV-1 comprises most HIV infections in Ghana (Cassels et al., 2014).

The early phase of HIV infection begins with recognition and binding of matured HIV-1 to cluster of differentiation 4 (CD4) cells such as macrophages, dendritic and T-helper cells (Figure 2.2). CD4 is a protein that normally functions in immune recognition. The binding occurs between the amino-terminal immunoglobulin domain of CD4 and the glycoprotein 120 of the virus (Fischer and Madden, 2011). HIV-1 and target cell membrane fusion are triggered by several chemokine receptors such as CXCR4 and CCR5 (Turner and Summers, 1999). Upon entering a host cell, it loses its outer envelope and its contents including the ribonucleic acid (RNA) and reverse transcriptase (RT) into the host cell. The RNA genome is used as a template and reverse-transcribed into viral deoxyribonucleic acid (DNA) by the viral RT in the cytosol (Abbas et al., 2000). The viral DNA is transported to the nucleus as part of a pre-integration complex (Miller et al., 1997).
The late phase begins with an expression of the integrated proviral genome, virus budding and maturation. Messenger RNA transcript is synthesized and transported out of the nucleus for translation (Turner and Summers, 1999). The viral proteins along with the genomic RNA are assembled into new virus particles by the action of the viral enzyme, protease (Marr, 1998). The new viral particles are released from the infected cell and go on to infect other cells in the host.

2.2 HIV Treatment

2.2.1 Antiretroviral Therapy

As of July 2017, 20.9 million HIV patients have been reported to receive ART, with a steady increase in the number of HIV infected women receiving ART for the prevention of Mother to child transmission (UNAIDS, 2017a).

There are more than 25 ARV drugs in six mechanistic pharmacologic classes that are approved by United States-Food and Drug Administration for treatment of HIV infection. These six classes include the Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), Non-nucleoside reverse transcriptase inhibitors (NNRTIs), Protease inhibitors (PIs), CCR5 antagonists, and Integrase strand transfer inhibitors (INSTIs) (Dalal et al., 2015, WHO, 2017). These classes of ART target different stages in the HIV replication cycle (Figure 2.2).
2.2.1.1 Nucleoside reverse transcriptase inhibitors

Non-nucleoside reverse transcriptase is a class of ARV drugs whose chemical structure constitutes a modified version of a natural nucleoside. They act as competitive inhibitors of RT to inhibit viral replication (Dalal et al., 2015).

The drugs in this class enter the host cells via endocytosis and they require intracellular phosphorylation in order to produce an active triphosphate form. The triphosphate derivatives then act as alternative substrates for the viral RT and compete with physiological nucleosides. This ultimately terminates viral DNA replication, as phosphodiester bridges can no longer be built to stabilize the DNA double-strand due to the absence of 3’ hydroxyl group essential for the addition of incoming new nucleotide. Blockade of the viral RT enzyme was the first attempt to inhibit the HIV life cycle, and in 1987 the first ARV, zidovudine was licensed for the treatment of HIV infection (Tozzi, 2010).

Examples of NRTI available include zidovudine, didanosine, stavudine, lamivudine, tenofovir, abacavir and emtricitabine. However, the recommended NRTIs used in Ghana (Table 2.1) are zidovudine, lamivudine, abacavir, tenofovir, and emtricitabine (Ministry of Health and Ghana Health Service, 2016).

2.2.1.2 Non-nucleoside reverse transcriptase inhibitors

Non-nucleotide reverse transcriptase inhibitors are a class of ARV drugs that act as non-competitive inhibitors of HIV-1 RT enzyme. These small molecules block retroviral reverse transcription through an allosteric mechanism of action (Figure 2.1) by binding to HIV-1 RT at a site distinct (allosteric site) from the active site of the RT enzyme (Kohlstaedt et al., 1992).
approved NNRTIs are strain specific and they are active against HIV-1 but not HIV-2 or other retroviruses. They have no activity against host cell DNA polymerases and they do not require intracellular phosphorylation for activity unlike NRTIs (Bruntun et al., 2008). NNRTIs includes nevirapine, efavirenz, delavirdine, etravirine and rilpivirine. However, nevirapine was the first NNRTI approved in 1996 by the United States-Food and Drug Administration for the treatment of HIV-1 infection, followed by delavirdine in 1997, efavirenz in 1998, etravirine in 2008, and rilpivirine in 2011 (Tozzi, 2010, Sluis-Cremer, 2018). NNRTIs recommended for use in Ghana include nevirapine and efavirenz (Ministry of Health and Ghana Health Service, 2016). Due to their unique antiviral potency, high specificity and low cytotoxicity, the NNRTIs have become an indispensable component of HAART regimen (Chen et al., 2011).

Figure 2.1: Mechanism of action of Non-nucleoside Reverse Transcriptase Inhibitors.

Source: Bruntun et al. (2008).
2.2.1.3 Protease inhibitor

HIV Protease inhibitors are peptide-like chemicals that competitively inhibit the action of the virus aspartyl protease which is responsible for the maturation of large polyproteins to structural and replicative enzymes (Pesoa et al., 2011). Human aspartyl protease contains only one polypeptide chain and is not significantly inhibited by HIV PIs (Bruntun et al., 2008). These drugs bind reversibly to the active site of the protease and prevent proteolytic cleavage of HIV proteins into essential structural and enzymatic components of the virus leading to the production of non-infectious viral particles (Justesen, 2006). Among all HIV-1 drugs, PIs have the highest intrinsic antiviral activity and are the only ARV drugs that have been successfully used in monotherapy (Rabi et al., 2013). Recommended PIs used in Ghana includes ritonavir, lopinavir, atazanavir and darunavir (Ministry of Health and Ghana Health Service, 2016).

2.2.1.4 CCR5 antagonists

A small molecule, chemokine receptor CCR5 antagonists are considered as allosteric inhibitors which bind to hydrophobic pockets within the transmembrane helices of CCR5, inhibiting HIV entry into its host cell (Lobritz et al., 2010). Targeting the chemokine receptor, the CCR5 receptor is a promising strategy for both prevention and therapy of HIV since CCR5 deficiency does not appear to carry any health disadvantage (Hartley et al., 2018). These findings highlight the importance of CCR5 in HIV transmission and suggest that pharmacologically replicating the phenotype of CCR5Δ32 homozygotes would be a promising strategy for HIV prevention. (Samson et al., 1996).
Maraviroc, the only FI, approved for clinical use, is a therapeutic chance for the treatment of the multi-experienced HIV patients (that is, those with resistance to traditional ARV drugs) (Aceti et al., 2015).

### 2.2.1.5 Integrase Strand Transfer Inhibitors

Integrase Strand Transfer Inhibitors are new ARV class which are metabolized by Uridine glucuronosyl transferase 1A1 (UGT1A1). Examples of INSTIs includes Raltegravir, Dolutegravir and Cabotegravir. Cabotegravir is a second-generation INSTI that has shown efficacy as a long-acting injectable Pre-Exposure Prophylaxis agent in macaque rectal and vaginal challenge models (Hartley et al., 2018). Dolutegravir and raltegravir are the INSTIs recommended for use in Ghana (Ministry of Health and Ghana Health Service, 2016).

### 2.2.2 Highly Active Antiretroviral Therapy

The Panel on ARV Guidelines for Adults and Adolescents emphasizes that monotherapy with any ARV drug should not be used due to increased risk of virologic failure and drug resistance (WHO, 2017). HAART is a standard ART which involves a potent combination of the different classes of ARV drugs that targets different stages of the viral life cycle (Figure 2.2) to effectively control HIV replication, infection and prevent resistance. HAART usually include two NRTIs with one NNRTI or PI (Colic et al., 2015).
Figure 2.2: HIV life cycle: Targets for antiretroviral agents.

Approved drugs categorized according to their mechanism of action. Only drugs potentially associated with pharmacogenetics markers of efficacy and/or toxicity are depicted.

Source: Pesoa et al. (2011)
Since these ARV combinations became available, their use has resulted in a significant reduction in morbidity and mortality among HIV patients (Estes et al., 2007). Several studies have demonstrated significant immunologic recovery and suppression of viral replication with HAART (Asensi et al., 2015, Black et al., 2014, Calmy et al., 2006). Not only does combined ART suppress viral replication to a level where infected individuals can live essentially free of symptoms but it has also been shown to strongly reduce onward transmission of the virus, providing a clear rationale for treatment of HIV-infected individuals as a means of preventing the spread of the epidemic (Hartley et al., 2018).

In Ghana, dual or monotherapy is not recommended to be used in the management of HIV patients. Ghana uses a triple combination of ARV drug regimen based on the HAART (Ministry of Health and Ghana Health Service, 2016). The recommended first-line ART regimen used in Ghana for the management of HIV patients is shown in Table 2.1.
Table 2.1: Recommended first-line ART regimen used in Ghana for management of People living with HIV.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Contra-indications</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preferred Regimen</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tenofovir + Lamivudine (or Emtricitabine) + Efavirenz</td>
<td>Caution with Tenofovir in renal dysfunction</td>
<td>Monitor renal function including urinalysis</td>
</tr>
<tr>
<td></td>
<td><strong>Nevirapine</strong></td>
<td>Stop nevirapine if the client develops jaundice or severe rashes and call for advice or refer further management</td>
</tr>
<tr>
<td>Tenofovir + Lamivudine (or Emtricitabine) + Nevirapine</td>
<td>Caution with Tenofovir in renal dysfunction</td>
<td>Monitor renal function including urinalysis</td>
</tr>
<tr>
<td>Zidovudine + Lamivudine (or Emtricitabine) + Nevirapine</td>
<td>Zidovudine is contraindicated in severe anaemia</td>
<td>Tenofovir to be used where Hb is &lt; 8g/dl or drops &gt; 25 % drop from the baseline value in a client on zidovudine</td>
</tr>
<tr>
<td>Zidovudine + Lamivudine (or Emtricitabine) + Efavirenz</td>
<td>Zidovudine is contraindicated in severe anaemia</td>
<td>Tenofovir to be used where Hb is &lt; 8g/dl or drops &gt; 25 % drop from the baseline value in a client on zidovudine</td>
</tr>
</tbody>
</table>

Source: (Ministry of Health and Ghana Health Service, 2016)
2.2.3 Nevirapine

2.2.3.1 Pharmacokinetics and pharmacodynamics of nevirapine

Nevirapine like all NNRTs is a non-competitive inhibitor that inhibits the action HIV-1 RT enzyme (Black et al., 2014). It is metabolized by CYP2B6 into 3- and 8-hydroxynevirapine (OH-nevirapine), and by CYP3A4 into 2- and 12-OH-nevirapine. CYP3A5, CYP2C9 and CYP2D6 also play a role in these steps. Multiple dosing with 200–400 mg nevirapine per day results in auto-induction of these enzymes and leads to an increase in plasma clearance as well as a decrease in its half-life to 30 hours (Cooper and van Heeswijk, 2007). The therapeutic range of nevirapine plasma concentration at a steady state covers 11-30μM after administration of 400mg per day (Ingelheim, 2012). It is widely used as a first-line treatment for HIV infection in developing countries because of its low cost (Wit et al., 2008).

After oral administration, nevirapine is almost completely absorbed in the intestine with a mean bioavailability of more than 90%. It is distributed throughout the body with about 60% of the administered drug binding to plasma proteins. It is among an increasing number of drugs found to display sex differences in pharmacokinetics and adverse reactions upon biotransformation (Marinho et al., 2013). It also undergoes glucuronide conjugation (Figure 2.5) of the hydroxyl metabolites by the UGT enzyme (Whirl-Carrillo et al., 2012, Riska et al., 1999). In both humans and rats, nevirapine is metabolized into 2-, 3-, 8-, 12-OH-NVP and 4-carboxynevirapine (COOH-nevirapine) (Riska et al., 1999). The 12-OH-nevirapine may also be metabolized by Phase II metabolizing enzyme, SULT (Figure 2).
Figure 2. 3: Conversion of Nevirapine to 12-Hydroxy-nevirapine and subsequent activation to 12-sulphoxy-nevirapine.

Source: Caixas et al. (2012)

2.3 Drug-Induced Hypersensitivity Reaction

The immune system is a complex network of regulatory and effector cells and molecules whose primary function is discrimination of self from non-self to maintain the homeostasis of the body. (Descotes, 2004). However, the immune responses are not always beneficial. Thus, inadvertent immunological reactivity against “innocent antigens” such as drugs can lead to hypersensitivity reactions (Nijkamp and Parnham, 2005).

A drug-induced hypersensitivity reaction is an idiosyncratic and immune-mediated adverse effect of drugs characterized by a various combination of clinical features such as of skin rash, fever, multi-organ failure, hepatotoxicity and eosinophilia (Tozzi, 2010). These reactions depend on both environmental and genetic factors (Yun et al., 2012). It occurs 1-8 weeks after drug introduction and the hypersensitivity tend to resolve, following the withdrawal of the offending
agent and typically recurs on re-challenge, usually after a shorter time interval (Spengler et al., 2002, Ben m'rad et al., 2009). Skin reactions are the most common manifestation of Drug-induced hypersensitivity reaction. (Chaponda and Pirmohamed, 2010). SJS and TEN are the most severe and potentially life-threatening cutaneous complications of drug treatments. (Fritsch and Sidoroff, 2000).

### 2.3.1 Nevirapine-Induced Hypersensitivity

Nevirapine hypersensitivity tends to present within the first few weeks of therapy and cause a multisystem inflammatory syndrome, which can occasionally be fatal. However, nevirapine typically involves both rash and hepatotoxicity. The rash associated usually is presented as a diffuse, maculopapular rash, which can develop into SJS or TEN (Dieterich et al., 2004, Estes et al., 2007).

The relationship between drug exposure and the development of nevirapine hypersensitivity is unclear, but previous studies have reported higher plasma drug exposures in black African and Thai patients and higher rates of liver toxicity in patients receiving once-daily nevirapine than in those receiving twice-daily nevirapine, presumably due to higher maximum concentrations attained after dosing (Dickinson, 2014).

### 2.4 Genetic Polymorphism

When the genomic DNA sequences on the equivalent chromosome of any two individuals are compared, there is substantial variation in the sequence at many points throughout the genome.
Genetic polymorphism is the variation in DNA sequence among 1% or more of a population. These forms of genetic variations may include sequence repeats, recombination, SNPs, insertions and deletions. However, SNP which occurs as a result of a single base substitution of one nucleotide for another is the simplest and common form of genetic polymorphism (Fucharoen, 2007).

Previously, in some instances, genetic polymorphism was referred to by the particular methods used to detect it. For example, the first systematic studies of single base variants were pursued through the identification of restriction enzyme sites, where a single base pair change could result in the loss or gain of a restriction site. Digestion of a piece of DNA containing the relevant site with an appropriate restriction enzyme could then distinguish alleles or variants based on resulting fragment sizes via electrophoresis. This type of polymorphism was thus referred to as Restriction Fragment Length Polymorphism (RFLP) (Schork et al., 2000).

The study of variation at the DNA sequence level within the last 20 or so years has had an enormous impact on the belief that one could directly link specific variants with specific traits or diseases. Genome-wide association studies are increasingly being used to study drug response and susceptibility to adverse drug reactions, resulting in the identification of some novel pharmacogenetics associations (Daly, 2010).

Genetic polymorphisms are estimated to contribute about 20-50% to the variability in individual drug response and safety (Evans and McLeod, 2003). Genetic makeup affects inherent pharmacokinetics, giving rise to interpersonal differences in drug absorption, distribution, metabolism, and excretion. Antiretrovirals such as NNRTIs are particularly associated with
significant inter-individual variation in drug exposure, which is largely driven by genetic differences drug-metabolizing enzymes, transporters and nuclear receptors (Pavlos and Phillips, 2012).

2.4.1 Cytochrome P450 and Nevirapine-Induced Hypersensitivity

Cytochrome P450 plays a dominant role in the metabolism of drugs and other foreign chemicals. It, therefore, one of the primary means for Phase I xenobiotic detoxication. (Sevrioukova and Poulos, 2017). Approximately a dozen CYP isozymes of families CYP1, CYP2, and CYP3 are collectively responsible for most Phase I bio-transformations of drugs and other xenobiotics. Expression and function of these proteins are highly variable both inter- and intra-individually, and thus are a major contributor to unpredictable drug or metabolite plasma concentrations and to unforeseen drug responses (Telenti, 2004).

The expression of CYP3A5 in the human liver differs vastly between ethnic groups. CYP3A5*3 is the most frequently recognized truncated, nonfunctional allele and one of the most frequent polymorphisms among CYP genes being recognized. In Caucasians, the frequency of CYP3A5*3 has been shown to be ≥ 90% whilst the occurrence among black Africans differs between 11 and 78% (Marwa et al., 2014).

CYP3A5*6 / CYP3A5 14690G>A (transition in exon 2, leading to the skipping of exon 7) is associated with the lower CYP3A5 catalytic activity (Kumar et al., 2013). CYP3A5 polymorphisms may be of importance when considering African populations and metabolism of nevirapine, because CYP3A5 is more commonly expressed in Africans, particularly in comparison to Caucasians. (Dickinson, 2014).
Genetic polymorphism in CYP enzymes predicts the noted inter-individual variation in plasma concentrations of the nevirapine and efavirenz (Fellay et al., 2002). When two Bantu-speaking populations from Cameroon and South Africa were compared, they were found to be genetically similar with regard to CYP2A6 and CYP2B6. However, there were some statistically significant differences between the genotype frequencies seen in the two populations with respect to CYP3A5 (Swart et al., 2012). A study observed an association between \( \text{CYP3A5}^*3 \) and nevirapine exposure in 24 HIV-infected adults and children from Malawi (Brown et al., 2012).

CYP2B6 is one of the major CYP enzymes involved in the phase I biotransformation of many drugs. Clinically used drug substrates of CYP2B6 include cytostatic (cyclophosphamide), HIV drugs (efavirenz and nevirapine), antidepressants (bupropion), antimalarials (artemisinin), anaesthetics (propofol) and synthetic opioids (methadone). It is highly polymorphic and as a result, there is a considerable inter-individual variation in the expression and function of the gene (Barco and Nóvoa, 2013). CYP2B6 gene with several haplotypes (\( \text{CYP2B6}^*6 \), \( \text{CYP2B6}^*11 \), \( \text{CYP2B6}^*15 \) and \( \text{CYP2B6}^*18 \)) is associated with reduced catalytic activity and protein stability, resulting in an increased nevirapine plasma levels (Kranendonk et al., 2014).

Data shows a significant association between \( \text{CYP2B6}^*18 \) (\( \text{CYP2B6} \ 983T>C \)) polymorphism and nevirapine induced SJS / TEN (Table 2.2). \( \text{CYP2B6} \ 983T>C \) has a frequency of around 5-10% in African populations but is not observed in Caucasians, and this may, therefore, represent an ethnicity-specific predisposing factor (Carr et al., 2014a). In another study, the homozygous mutant for \( \text{CYP2B6} \ 983T>C \ (983CC) \) was not present in Malawian patients; however, the
presence of the heterozygote (983TC) in combination with CY2B6 516GG or 516GT resulted in decreased nevirapine clearance or bioavailability (Dickinson, 2014). Hepatic adverse events of nevirapine were not associated with CYP2B6 genotypes in whites but the risk for cutaneous adverse events was particularly high among Blacks with CYP2B6 516TT (Yuan et al., 2011).

For CYP2B6 516G>T, increasing T-allele count (Table 2.2) was significantly associated with progressively increased risk of cutaneous adverse events in Blacks and Whites, with a weak trend in Asians (Yuan et al., 2011).

Table 2.2: CYP450 alleles associated with increased risk of nevirapine-induced hypersensitivity reaction.

<table>
<thead>
<tr>
<th>CYP450 Allele</th>
<th>Adverse reaction</th>
<th>Population</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2B6 516G&gt;T</td>
<td>SJS/TEN</td>
<td>Mozambique</td>
<td>Ciccacci et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>Cutaneous</td>
<td>Asians &amp; Thais</td>
<td>Yuan et al. (2011)</td>
</tr>
<tr>
<td></td>
<td>Adverse event</td>
<td>Blacks &amp; Whites</td>
<td></td>
</tr>
<tr>
<td>CYP2B6 c.983T&gt;C</td>
<td>Hypersensitivity</td>
<td>Malawian</td>
<td>Carr et al. (2014b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Uganda</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SJS/TEN</td>
<td>Mozambique</td>
<td>Ciccacci et al. (2013)</td>
</tr>
</tbody>
</table>

SJS; Steven Johnson Syndrome, TEN; Toxic epidermal necrolysis
2.4.2 Sulfotransferase and Nevirapine-Induced Hypersensitivity

Sulfotransferases are phase II metabolizing enzymes that catalyze the transfer of a sulfonate group from a donor molecule – usually 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to a great variety of hydroxyl and amine substrates in a process referred to as sulfonation. The PAPS is a universal donor of the sulphonyl moiety that enables the sulfo-conjugation of SULT substrates. Three PAPS synthase isoforms (PAPSS1, PAPSS2a and PAPSS2b), with different activities and tissue distributions, have been identified in humans (Xu et al., 2002). Based on the amino acid sequence and substrate specificity, three different classes of human cytosolic SULTs have been identified: Phenol SULTs, Hydroxysteroid SULTs and Estrogen SULTs (Wang et al., 2002).

Human SULT1A1 appears to be the main form of SULT involved in the biotransformation of xenobiotics due to its broad spectrum of substrates and high hepatic expression (Glatt and Meinl, 2004). It is highly polymorphic and that there are marked differences in the activities of SULT1A1 variants (SULT1A1*1, SULT1A1*2 and SULT1A1*3) (Nagar et al., 2006). Sex-divergent SULTs are mostly female predominant in mice (Alnouti and Klaassen, 2011). Female mice showed higher hepatic mRNA levels of SULT1A1 compared with male mice (Suzuki et al., 2012). Higher expression of SULT1D1 was reported in the canine female liver (Tsoi et al., 2001) and a female predominance in SULT2A1 or SULT2A2 has been reported in mice (Alnouti and Klaassen, 2011). The SULT1A1 638 G>A polymorphism is likely to play an important role in the susceptibility to some diseases. This polymorphism (G638A) in the SULT1A1 gene may cause
Arg213His amino acid change and consequently results in significantly reduced enzyme activity (Kumar et al., 2013).

Although the formation of glucuronides is a major route of elimination of nevirapine phase I metabolites, the involvement of sulfonation, cannot be excluded (Marinho et al., 2013). The bio-activation of the non-reactive metabolite, 12-OH-nevirapine by SULTs can generate 12-sulphoxynevirapine (SUL-nevirapine), a reactive metabolite that binds covalently to proteins and DNA (Caixas et al., 2012). Animal model studies have suggested that 12-OH-nevirapine (formed by oxidation of an exocyclic methyl group) can directly induce a skin rash, while the further metabolite quinone methide via sulfonation to generate a 12-SUL-nevirapine can induce both the skin rash and hepatotoxicity directly (Wongtrakul et al., 2016). The 12-SUL-nevirapine, which forms adducts with proteins (Figure 2.4) in the liver and skin, could explain the nevirapine-associated ADR such as hepatotoxicity and skin rash (Sharma et al., 2013).

The PAPSS1 expression is notably high in the skin where sulfonation of 12-OH-nevirapine has been associated with a nevirapine-induced skin rash (Venkatachalam, 2003, Sharma et al., 2013). Using 12-mesyloxy-nevirapine as a synthetic surrogate for 12-SUL-nevirapine, Antunes et al. (2010) showed that the reactive nevirapine metabolites covalently bind in vitro to several amino acids, human serum albumin as well as nucleosides and DNA.
Figure 2.4: Metabolic pathways of nevirapine, involving Phase I oxidation and Phase II sulfonation.

Source: Caixas et al. (2012)
CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Reagents and Equipment
The various reagents, equipment and preparation of buffers and solutions used in the study are described in Appendix II and III.

3.2 Study type
This was a case-control study which involved HIV patients exposed to nevirapine-based HARRT at the Korle-Bu Teaching Hospital. HIV-1 patients who have developed nevirapine-induced hypersensitivity within 6 months on nevirapine-based HAART initiation were recruited as cases, while those who did not as the controls. Cases and controls were recruited based on the clinicians’ diagnosis, as recorded in the HIV clinic’s database.

3.3 Study Sites
Whole blood samples (3 ml) were collected from HIV-1 infected patients at the Department of Medicine (Fevers Unit), at the Korle-Bu Teaching Hospital. The samples were analyzed at the Virology Laboratory, Department of Medical Microbiology and Molecular Biology Laboratory, Department of Physiology, School of Biomedical and Allied Health Sciences, University of Ghana.
Korle-Bu Teaching Hospital is a referral hospital with over 2000 beds for in-patients and it also has 21 clinical and diagnostic departments, as well as two “Centres of Excellence” (KBTH, 2016).

The HIV Clinic is run at the Fevers Unit for HIV patients by a team of clinicians, nurses, pharmacists, and counsellors, dieticians, social workers, and laboratory staff (Kudzi et al., 2017). Ethical clearance for the study was obtained from the Ethical and Protocol Review Committee of the University of Ghana College of Health Sciences with reference number CHS-Et/M.8-P 4.6/2016-2017.

3.4 Study population

The participants gave an informed consent (Appendix IV) after protocol approval by the Ethical and Protocol Review Committee, College of Health Sciences, University of Ghana. The recruitment involved a non-probability convenient sampling where only patients who willingly accepted to participate in the study were recruited. There are about 19,000 registered HIV patients attending the HIV clinic at the Fevers Unit of the Korle-Bu Teaching Hospital and out of which about 7,000 of them are on HAART. The HIV clinic is run three times each week, with about 50 patients attending each day (Kudzi et al., 2017).
3.5 Inclusion and exclusion criteria

3.5.1 Inclusion criteria

1. HIV-1 patients, who have been exposed to nevirapine-based HAART for at least 6 months.
2. Eighteen (18) years or above.

3.5.2 Exclusion criteria

1. Patients co-infected with hepatitis B virus.
2. Unavailability of patients for blood sample collection.
3. Patients previously on different ART before taking nevirapine-based HAART.

3.6 Sample size determination

The minimum number of study participants for this unmatched case-control study was calculated following an unmatched case-control formula (Kelsey et al., 1996) below:

\[ n_1 = \frac{(Z_{a/2} + Z_{1-\beta})^2 \times pq(r+1)}{r(p_1-p_2)^2} \]

And \( n_2 = r \times n_1 \)

Where \( n_1 = \) number of cases, \( n_2 = \) number of controls

\( Z_{a/2} = \) Standard normal deviate for the two-tailed test based on alpha level (relates to the confidence interval level)

\( Z_{1-\beta} = \) Standard normal deviate for the one-tailed test based on beta level (relates to the power level)
\[ r = \text{Ratio of controls to cases}, \quad p_1 = \text{Proportion of cases with exposure and} \quad q_1 = 1-p_1 \quad p_2 = \text{Proportion of controls with exposure and} \quad q_2 = 1-p_2 \]

With the following parameters: Using a ratio of 1:3 as cases (nevirapine hypersensitivity) to control (nevirapine tolerant), at 95% confidence interval, Power of 80%, the hypothetical proportion of cases and controls with the exposure is 70% and 30% respectively.

A minimum of 64 study participants was calculated as the minimum sample size for the study. However, a total of 70 participants were enrolled in the study.

### 3.7 Genomic DNA extraction

Genomic DNA was extracted from each whole blood sample using a Quick-gDNA Blood Miniprep (Inqaba Biotechnical, Pretoria, South Africa) according to the manufacturer’s instructions.

Genomic lysis buffer (400 ul) was added to 100µl of whole blood as a ratio of 4:1. The mixture was vortexed for 6 seconds and allowed to stand for 10 minutes at room temperature. The mixture was transferred into a zymo-spin \textsuperscript{TM} column in a collection tube and centrifuged at 10,000xg for 1 minute. The collection tube with the flow through was discarded.

The zymo-spin \textsuperscript{TM} column was transferred to a new collection tube. DNA Pre-wash buffer (200µl) was added to the spin column and centrifuged at 10,000xg for 1 minute. Genomic DNA wash buffer (500µl) was added to the spin column and centrifuged at 10,000xg for 1 minute. The collection tube with the flow through was discarded.
The zymo-spin™ column was transferred to a sterile Eppendorf tube. DNA elution buffer (50µl) was added to the spin column. The mixture was incubated for 5 minutes at room temperature and centrifuged at top speed (15,000xg) for 30 seconds to elute genomic DNA into the Eppendorf tube. The eluted genomic DNA (stored at -20 °C) was used to genotype the specific alleles of interest.

3.8 Genotyping

Genotyping of the specific alleles for \textit{CYP2B6} 983T\textgreater{}C, \textit{CYP3A5} 14690G\textgreater{}A and \textit{SULT1A1} 638G\textgreater{}A was performed by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) as previously described by Rohrich et al. (2016), Islam et al. (2014) and Wang et al. (2002) respectively with some modifications. The first step in a PCR-RFLP analysis was to amplify the fragment containing the gene variants of interest by PCR. For each PCR reaction, the gene of interest was amplified using specific primers for each of the genes under study. Primer sequences used for each gene in this study are shown in Appendix I. This was followed by digestion of the amplicon (PCR product) with an appropriate restriction enzyme. The presence or absence of a restriction enzyme recognition site results in the formation of restriction fragments of different sizes. Hence identification of an allele can be done by electrophoretic resolution of the restriction fragments in a gel matrix.
3.8.1 *CYP2B6 983T>C* Genotyping

Polymerase chain reaction for *CYP2B6 983T>C* was performed in a 20μl total reaction volume. The reaction mixture was made up of One Taq Quick-Load 2X Master Mix Standard Buffer (New England Biolabs), 10μM specific forward (F) and reverse (R) primers, nuclease-free water, genomic DNA. The details of each PCR mixture and cycling conditions are contained in Tables 3.1 and 3.2 respectively. For each reaction, a negative control of blank reaction tube containing all other reagents except genomic DNA was included.

Two per cent (2% w/v) agarose gel stained with ethidium bromide was used to separate DNA fragments after PCR. Five microliters (5μl) aliquot of the PCR product already containing a loading dye was loaded into each well in the agarose gel with 1X Tris-acetic acid Ethylenediamine tetra-acetic acid (TAE) buffer. The setup was electrophoresed at 110V for 40 minutes, visualized by ultraviolet transilluminator and photographed.

Aliquots of the PCR product was digested with appropriate restriction enzyme (*BsAmI*) (New England Biolabs) in a 15μl total mixture. The reaction mixture is found in Table 3.3. The reaction mixture in a sterile Eppendorf tube was incubated in a water bath for 2 hours at 55°C. Two per cent (2% w/v) agarose gel (stained with ethidium bromide) electrophoresis was run at 110V for the identification of the restriction fragments. A 100bp DNA ladder was run on each gel to allow for fragment size determination.

Expected fragments sizes (Figure 3.1) are 759bp for wildtype allele, 637bp and 122bp for homozygous mutant allele and 759bp, 637bp and 122bp for heterozygous.
Table 3.1: CYP2B6 983T>C PCR Reaction Mixture

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
<td>3</td>
</tr>
<tr>
<td>Forward primer</td>
<td>1</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1</td>
</tr>
<tr>
<td>Master Mix</td>
<td>11</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 3.2: CYP2B6 983T>C PCR Cycling Conditions

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>No. of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95</td>
<td>10min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>30sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>58</td>
<td>30sec</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>1min</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>10min</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 3. 3: Restriction digest reaction mixture for *CYP2B6 983T>C*

<table>
<thead>
<tr>
<th>Sample/Reagent</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Product</td>
<td>10</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>2</td>
</tr>
<tr>
<td>10X buffer</td>
<td>2</td>
</tr>
<tr>
<td><em>BsAmI</em></td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
</tr>
</tbody>
</table>

UF; undigested PCR product, wt; Wildtype (T), mt; Mutant (C), Heterozygous (T/C).

Figure 3. 1: PCR–RFLP analysis for the detection of *CYP2B6 983T>C* alleles
3.8.2 *CYP3A5 14690G>A* Genotyping

Polymerase chain reaction for *CYP3A5 14690G>A* was performed in a 30μl total reaction volume. The reaction mixture was made up of One Taq Quick-Load 2X Master Mix Standard Buffer (New England Biolabs), 10μM specific forward and reverse primers, nuclease-free water, genomic DNA (Appendix I). The details of each PCR mixture and cycling conditions for *CYP3A5 14690G>A* amplifications are contained in Tables 3.4 and 3.5 respectively. For each reaction, a negative control of blank reaction tube containing all other reagents except genomic DNA was included.

Three per cent (3% w/v) agarose gel stained with ethidium bromide was used to separate DNA fragments after PCR. Five microliters (5μl) aliquot of the amplicon (PCR product) already containing a loading dye was loaded into each well in the agarose gel with 1X TAE buffer. The setup was electrophoresed at 110V for 40 minutes, visualized by ultraviolet transilluminator and photographed.

An aliquot of the PCR product was digested with appropriate restriction enzyme (*DdeI*) (New England Biolabs) in a 25μl total mixture. The reaction mixture is found in Table 3.6. The reaction mixture in a sterile Eppendorf tube was incubated in a water bath for 3 hours at 37°C. Four per cent (4% w/v) agarose gel (stained with ethidium bromide) electrophoresis was run for 40 minutes at 110V for the identification of the restriction fragments under ultraviolet transilluminator and was photographed. A 100bp DNA ladder was run on each gel to allow for fragment size determination. The restriction fragments (Figure 3.2) are 103bp, 74bp, 35bp and
25bp for wildtype allele (G/G), 128bp, 103bp, 74bp, 35bp and 25bp for heterozygous (G/A) and 128bp, 74bp and 35bp for homozygous mutant allele (A/A).

Table 3. 4: CYP3A5 14690G>A PCR Reaction Mixture

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
<td>5</td>
</tr>
<tr>
<td>10µM primer (forward)</td>
<td>1</td>
</tr>
<tr>
<td>10µM primer (reverse)</td>
<td>1</td>
</tr>
<tr>
<td>MasterMix</td>
<td>17</td>
</tr>
<tr>
<td>gDNA</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
</tr>
</tbody>
</table>
Table 3.5: *CYP3A5* 14690G>A PCR Cycling Conditions

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>No. of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>30 secs</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>58</td>
<td>30 secs</td>
<td>45</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>30 secs</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>5 min</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 3.6: Restriction digest reaction mixture for *CYP3A5* 14690G>A

<table>
<thead>
<tr>
<th>Sample/Reagent</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Product</td>
<td>25</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>2</td>
</tr>
<tr>
<td>10X buffer</td>
<td>2</td>
</tr>
<tr>
<td><em>DdeI</em></td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
</tr>
</tbody>
</table>
Figure 3. 2: PCR–RFLP analysis for the detection of \textit{CYP3A5} 14690G\textgreater{}A alleles

UF; undigested PCR product, wt; Wildtype (G), mt; Mutant (A), Heterozygous (G/A).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3_2.png}
\caption{PCR–RFLP analysis for the detection of \textit{CYP3A5} 14690G\textgreater{}A alleles}
\end{figure}

3.8.3: \textit{SULT1A1} 638G\textgreater{}A Genotyping

PCR reaction for \textit{SULT1A1} 638G\textgreater{}A was performed in a 24\textmu{}l total reaction volume. The reaction mixture was made up of One Taq Quick-Load 2X Master Mix Standard Buffer (New England Biolabs), 10\textmu{}M primers, nuclease-free water and genomic DNA. The details of each PCR mixture and cycling conditions for \textit{SULT1A1} 638G\textgreater{}A amplifications are shown in Tables 3.7 and 3.8 respectively. For each reaction, a negative control of blank reaction tube containing all other reagents except genomic DNA was included.
Three per cent (3% w/v) agarose gel stained with ethidium bromide was used to separate DNA fragments after PCR. Five microliters (5μl) aliquot of the PCR product already containing a loading dye was loaded into each well in the agarose gel with 1X TAE buffer. The setup was electrophoresed at 110V for 40 minutes using a mini-gel system, visualized by ultraviolet transillumination and photographed.

Aliquots of the PCR product was digested with appropriate restriction enzyme (HaeII) (New England Biolabs) in a 20μl total mixture. Reaction component is found in Table 3.9. The reaction mixture in a sterile Eppendorf tube was incubated in a water bath for 3 hours at 37°C. Three per cent (3% w/v) agarose gel (stained with ethidium bromide) electrophoresis was run at 110V for the identification of the restriction fragments. A 100bp DNA ladder was run on each gel to allow for fragment size determination.

Expected fragments sizes (Figure 3.4); the wildtype genotype (G/G) produces two bands (168bp and 165bp), and the homozygous mutant (A/A) yield one band (333bp) and heterozygous (G/A) shows 333bp, 168bp and 165bp.
Table 3. 7: *SULT1A1* 638G>A PCR Reaction Mixture

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
<td>3</td>
</tr>
<tr>
<td>Primer (forward)</td>
<td>2</td>
</tr>
<tr>
<td>Primer (reverse)</td>
<td>2</td>
</tr>
<tr>
<td>MasterMix</td>
<td>13</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>24</strong></td>
</tr>
</tbody>
</table>

Table 3. 8: *SULT1A1* 638G>A PCR Cycling Conditions

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>No. of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>94</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>30 secs</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>63</td>
<td>30 secs</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>30 secs</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>5 min</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 3. 9: Restriction digest reaction mixture for \textit{SULT1A1 638G>A}

<table>
<thead>
<tr>
<th>Sample/Reagent</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Product</td>
<td>10</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>6</td>
</tr>
<tr>
<td>10X buffer</td>
<td>3</td>
</tr>
<tr>
<td>\textit{BsAmI}</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
</tr>
</tbody>
</table>

Figure 3. 3: PCR–RFLP analysis for the detection of \textit{SULT1A1 638G>A} alleles

UF; undigested PCR product, wt; Wildtype (G), mt; Mutant (A), Heterozygous (G/A).
3.9 Statistical analysis

The data obtained were entered into Microsoft Excel 2016 table. This was then imported into IBM SPSS version 20 (Illinois, USA). All the statistical analyses were done using IBM SPSS statistics 20. The data were summarized as frequencies and proportions.

Logistic regression was used to explore the association between the observed allele and genotypic frequencies and the nevirapine hypersensitivity. Risk of nevirapine hypersensitivity development among study participants was estimated using odds ratio at a 95% confidence interval. All reported p-values were two-sided and were considered statistically significant at a level of p <0.05.
CHAPTER FOUR

4.0 RESULTS

4.1 Characteristics of the study participants

Out of the total 70 study participants, 12 (17.1%) developed nevirapine hypersensitivity (cases) and the remaining were nevirapine tolerant (control). Most of the study participants were females, accounting for 77.1% of the total study participants. Out of the cases identified, 91.7% were females. The mean age of the participants was 38 ± 9.47 years with the majority of the participants between ages 31-49 years. There was no significant association between all the participant characteristics recorded and nevirapine hypersensitivity.

The characteristics of the study participants in the study are shown in Tables 4.1 and 4.2.
Table 4.1: Demographic characteristics of study participants and their association with nevirapine hypersensitivity.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Participants, No. (%)</th>
<th>All</th>
<th>OR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NVP HS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NVP tolerant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age range (years)</td>
<td>Mean ± SD = (38 ± 9.47)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 and below</td>
<td></td>
<td>4</td>
<td>15 (25.9%)</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>19 (27.1%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ref.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31-49</td>
<td>6 (50.0%)</td>
<td>36</td>
<td>42 (60.0%)</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>(62.1%)</td>
<td></td>
<td>(0.15-2.54)</td>
<td></td>
</tr>
<tr>
<td>0.44</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 and above</td>
<td>2 (16.7%)</td>
<td>7</td>
<td>9 (12.9%)</td>
<td>1.07</td>
</tr>
<tr>
<td></td>
<td>(12.1%)</td>
<td></td>
<td>(0.16-7.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.67</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1 (8.3%)</td>
<td>15</td>
<td>16(22.9%)</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>(25.9%)</td>
<td></td>
<td>(0.31-2.19)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>11 (91.7%)</td>
<td>43</td>
<td>54 (77.1%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(74.1%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

No.; Number of responses obtained in each category, SD; Standard deviation, p; p-value, p ≤ 0.05 denotes significance, OR; odds ratio, CI; confidence interval. Ref.; Reference used for odds ratio calculation. NVP; nevirapine, HS; hypersensitivity.
Table 4.2: Clinical characteristics of study participants and their association with nevirapine hypersensitivity.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Participants, No. (%)</th>
<th>All</th>
<th>OR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NVP HS</td>
<td>NVP tolerant</td>
</tr>
<tr>
<td>CD4 cell count</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(cells/µl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 250</td>
<td>11 (15.9 %)</td>
<td>44 (63.8 %)</td>
<td>55 (79.7 %)</td>
<td>Ref.</td>
</tr>
<tr>
<td>250-350</td>
<td>1 (1.4 %)</td>
<td>9 (13.0 %)</td>
<td>10 (14.5 %)</td>
<td>0.44 (0.51-0.89)</td>
</tr>
<tr>
<td>&gt;350</td>
<td>0 (0.0 %)</td>
<td>4 (5.8 %)</td>
<td>4 (5.8 %)</td>
<td>Not Est.</td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Underweight</td>
<td>6 (50.0 %)</td>
<td>10 (17.2 %)</td>
<td>16 (22.9 %)</td>
<td>Ref.</td>
</tr>
<tr>
<td>Normal weight</td>
<td>6 (50.0 %)</td>
<td>34 (58.6 %)</td>
<td>40 (57.1 %)</td>
<td>0.29 (0.12-0.78)</td>
</tr>
<tr>
<td>Overweight</td>
<td>0 (0.0 %)</td>
<td>11 (19.0 %)</td>
<td>11 (15.7 %)</td>
<td>Not Est.</td>
</tr>
<tr>
<td>Obese</td>
<td>0 (0.0 %)</td>
<td>3 (5.2 %)</td>
<td>3 (4.3 %)</td>
<td>Not Est.</td>
</tr>
</tbody>
</table>

No.; Number of responses obtained in each category, BMI: Body mass index, p; p-value, p ≤ 0.05 denotes significance, OR; odds ratio, CI; confidence interval. Ref.; Reference used for odds ratio calculation, Not Est.; Not estimable. NVP; nevirapine, HS; hypersensitivity.
4.2: Identification of CYP2B6 983T>C genotypes by PCR-RFLP

The CYP2B6 983T>C allele and genotypes were identified by using 100bp DNA ladder separation on the PCR-RFLP electrophoregram compared with individual samples. For each sample, the digested product was run alongside the PCR product in the agarose gel electrophoresis. Below in Figure 4.1 is a sample of the PCR-RFLP electrophoregram of ethidium bromide stained 2% agarose gel that was used for the identification of the alleles in some participants. The CYP2B6 983T>C frequency among the study participants was 3%. The allele distribution of the CYP2B6 983T>C was similar in both cases and control as depicted in Table 4.3. Homozygous mutant, CC was not detected in the entire study participants. However, Individuals carrying the variant allele, C have about twofold high risk to develop nevirapine hypersensitivity compared to the wildtype, T as recorded in Table 4.4.

Figure 4. 1: CYP2B6 983T>C PCR-RFLP Gel electrophoregram.

A 2% agarose gel electrophoregram showing RFLP results for CYP2B6 983T>C using BsAmI restriction enzyme. L–100bp ladder, Lane: 1-5, 8-15 = wt/wt = (TT), Lane: 6 and 7 = wt/mt = (TC).
Table 4.3: Allele and Genotypic frequencies of CYP2B6 983T>C

<table>
<thead>
<tr>
<th></th>
<th>Allele</th>
<th>n</th>
<th>Frequency (%)</th>
<th>Genotype</th>
<th>N</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>T</td>
<td>136</td>
<td>0.97 (97)</td>
<td>TT</td>
<td>66</td>
<td>0.94 (94)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>4</td>
<td>0.03 (3)</td>
<td>TC</td>
<td>4</td>
<td>0.06 (6%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>0</td>
<td>0.00 (0)</td>
</tr>
<tr>
<td>Cases</td>
<td>T</td>
<td>23</td>
<td>0.96 (96)</td>
<td>TT</td>
<td>11</td>
<td>0.92 (92)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1</td>
<td>0.04 (4)</td>
<td>TC</td>
<td>1</td>
<td>0.08 (8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>0</td>
<td>0.00 (0)</td>
</tr>
<tr>
<td>Control</td>
<td>T</td>
<td>113</td>
<td>0.97 (97)</td>
<td>TT</td>
<td>55</td>
<td>0.95 (95)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3</td>
<td>0.03 (3)</td>
<td>TC</td>
<td>3</td>
<td>0.05 (5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>0</td>
<td>0.00 (0)</td>
</tr>
</tbody>
</table>

n: the number of alleles, N: the number of genotypes, T: Wildtype allele, C: Variant allele. TT: Homozygous wildtype, TC: Heterozygous, CC: Homozygous mutant.
Table 4.4: Association of CYP2B6 983T>C genotypes with nevirapine hypersensitivity among Ghanaians

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Participants, No. (%)</th>
<th>OR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NVP HS</td>
<td>NVP tolerant</td>
<td>All</td>
</tr>
<tr>
<td>TT</td>
<td>11 (91.67)</td>
<td>55 (94.82)</td>
<td>66 (94.29)</td>
</tr>
<tr>
<td>TC</td>
<td>1 (8.33)</td>
<td>3 (5.17)</td>
<td>4 (5.71)</td>
</tr>
</tbody>
</table>

No.; Number of responses obtained in each category, p; p-value, p < 0.05 considered to be significant. CI; Confidence interval. OR; Odds ratio. Ref.; Reference used for odds ratio calculation. TT; Homozygous wildtype, TC; Heterozygous. NVP; nevirapine, HS; hypersensitivity.

4.3 Identification of CYP3A5 14690G>A genotypes by PCR-RFLP

The CYP3A5 14690G>A allele and genotypic frequencies were also identified by using the DNA ladder separation on the PCR-RFLP electrophoregram compared with individual subjects. A sample of the PCR-RFLP electrophoregram used to identify the alleles in some of the participants is depicted in Figure 4.2. The variant allele, A, was detected in all the 58 samples analyzed in the study. Allele and genotypic frequencies observed was 0.5 for both cases and control. All the participants were heterozygous, GA for the CYP3A5 14690G>A as shown in Table 4.5. The allele frequency observed in this study was compared to a similar study in Ghanaians and other populations (Table 4.6).
Figure 4. 2: CYP3A5 14690G>A PCR-RFLP Gel electrophoregram.

A 4% agarose gel electrophoregram showing RFLP results for CYP3A5 14690G>A. L–100bp DNA ladder. All Samples / Lanes: 1-3 are heterozygous (GA).
Table 4. 5: Allele and Genotypic frequencies of *CYP3A5 14690G>A*

<table>
<thead>
<tr>
<th></th>
<th>Allele</th>
<th>n</th>
<th>Frequency (%)</th>
<th>Genotype</th>
<th>N</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All</strong></td>
<td>G</td>
<td>58</td>
<td>0.50 (50)</td>
<td>GG</td>
<td>0</td>
<td>0.00 (0)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>58</td>
<td>0.50 (50)</td>
<td>GA</td>
<td>58</td>
<td>1.00 (100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AA</td>
<td>0</td>
<td>0.00 (0)</td>
</tr>
<tr>
<td><strong>Cases</strong></td>
<td>G</td>
<td>10</td>
<td>0.50 (50)</td>
<td>GG</td>
<td>0</td>
<td>0.00 (0)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>10</td>
<td>0.50 (50)</td>
<td>GA</td>
<td>10</td>
<td>1.00 (100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AA</td>
<td>0</td>
<td>0.00 (0)</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>G</td>
<td>48</td>
<td>0.50 (50)</td>
<td>GG</td>
<td>0</td>
<td>0.00 (0)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>48</td>
<td>0.50 (50)</td>
<td>GA</td>
<td>48</td>
<td>1.00 (100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AA</td>
<td>0</td>
<td>0.00 (0)</td>
</tr>
</tbody>
</table>

n; the number of alleles, N; the number of genotypes, GG; Homozygous wildtype, GA; Heterozygous, AA; Homozygous mutant.
Table 4. 6: Frequency distribution for \textit{CYP3A5 14690G\textgreater{}A} genotypes among Ghanaians and other previously studied populations.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Population</th>
<th>Total</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>G</td>
</tr>
<tr>
<td>This study</td>
<td>Ghanaian</td>
<td>58</td>
<td>0.5</td>
</tr>
<tr>
<td>Kudzi et al. (2010)</td>
<td>Ghanaian</td>
<td>194</td>
<td>0.86</td>
</tr>
<tr>
<td>Lavandera et al. (2011)</td>
<td>Argentinian</td>
<td>141</td>
<td>1.00</td>
</tr>
<tr>
<td>Swart et al. (2012)</td>
<td>Cameroonian</td>
<td>71</td>
<td>0.82</td>
</tr>
<tr>
<td>Swart et al. (2012)</td>
<td>South African</td>
<td>162</td>
<td>0.83</td>
</tr>
<tr>
<td>Fukuen et al. (2002)</td>
<td>Japanese</td>
<td>200</td>
<td>1.00</td>
</tr>
</tbody>
</table>

4.4: Identification of \textit{SULT1A1 638G\textgreater{}A} genotypes by PCR-RFLP.

Figure 4.3 shows an RFLP electrophoregram of 3\% agarose gel stained with ethidium bromide for the identification \textit{SULT1A1 638G\textgreater{}A} allele and genotype. Out of the 70 samples genotyped, 43 were identified as having homozygous wildtype, GG; 24 as heterozygous, GA and the remaining as a homozygous mutant, AA. The genotypic and allele distribution within the cases and control are as shown in Table 4.7 below. The distribution of the \textit{SULT1A1 638G\textgreater{}A} alleles also had no significant difference between cases and controls although the cases had no
homozygous mutant detected compared to the control. *SULT1A1 638G>A* variants were not significantly associated with nevirapine hypersensitivity. However, individuals carrying the variant allele, A have about 1.4 high risks to develop the nevirapine hypersensitivity compared to the wildtype, G (Table 4.8). The *SULT1A1 638G>A* allele frequency (21%) in this study was compared to observed frequencies in other study populations (Table 4.9).

Figure 4.3: *SULT1A1 638G>A* PCR-RFLP Gel electrophoregram.

A 3% agarose gel electrophoregram showing RFLP results for *SULT1A1 638G>A* using *HaeII* restriction enzyme. L–100bp ladder, Sample / Lane: 2, 3, 4, 5, 7, 9-12 and 15 = wt/wt (GG). Sample / Lane 1 and 8 = mt/mt (AA). Sample / Lane 6, 13 and 14 = wt/mt = (GA).
<table>
<thead>
<tr>
<th>Allele</th>
<th>n</th>
<th>Frequency (%)</th>
<th>Genotype</th>
<th>N</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>G</td>
<td>110</td>
<td>GG</td>
<td>43</td>
<td>0.62 (62)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>30</td>
<td>GA</td>
<td>24</td>
<td>0.34 (34)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AA</td>
<td>3</td>
<td>0.04 (4)</td>
</tr>
<tr>
<td>Cases</td>
<td>G</td>
<td>19</td>
<td>GG</td>
<td>7</td>
<td>0.58 (58)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>5</td>
<td>GA</td>
<td>5</td>
<td>0.42 (42)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AA</td>
<td>0</td>
<td>0.00 (0)</td>
</tr>
<tr>
<td>Control</td>
<td>G</td>
<td>91</td>
<td>GG</td>
<td>36</td>
<td>0.62 (62)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>25</td>
<td>GA</td>
<td>19</td>
<td>0.33 (33)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>AA</td>
<td>3</td>
<td>0.05 (5)</td>
</tr>
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</table>

Table 4.8: Association of SULT1A1 638G>A genotypes and nevirapine hypersensitivity among Ghanaians

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Participants, No. (%)</th>
<th>OR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NVP HS</td>
<td>NVP tolerant</td>
<td>All</td>
</tr>
<tr>
<td>GG</td>
<td>8 (67)</td>
<td>36 (62)</td>
<td>43 (62)</td>
</tr>
<tr>
<td>GA</td>
<td>4 (33)</td>
<td>19 (33)</td>
<td>24 (34)</td>
</tr>
<tr>
<td>AA</td>
<td>0 (0.00)</td>
<td>3 (5)</td>
<td>3 (4)</td>
</tr>
</tbody>
</table>

No.; number of responses obtained from each category, p; p-value, p < 0.05 considered to be significant. CI; Confidence interval. OR; Odds ratio. Ref.; Reference used for odds ratio calculation. GG; Homozygous wildtype, GA; Heterozygous, AA; Homozygous mutant. NVP; nevirapine, HS; hypersensitivity.
Table 4. 9: Frequency distribution for *SULT1A1 638G>A* genotype among Ghanaians and other previously studied populations.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Population</th>
<th>Total</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>G</td>
</tr>
<tr>
<td>This study</td>
<td>Ghana</td>
<td>70</td>
<td>0.79</td>
</tr>
<tr>
<td>Sun et al. (2005)</td>
<td>Sweden</td>
<td>775</td>
<td>0.61</td>
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<tr>
<td>Liang et al. (2004)</td>
<td>China</td>
<td>1614</td>
<td>0.89</td>
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<tr>
<td>Shen et al. (2012)</td>
<td>Taiwan</td>
<td>300</td>
<td>0.91</td>
</tr>
<tr>
<td>Gjerde et al. (2007)</td>
<td>Norway</td>
<td>151</td>
<td>0.66</td>
</tr>
<tr>
<td>Wang et al. (2002)</td>
<td>America</td>
<td>948</td>
<td>0.65</td>
</tr>
<tr>
<td>Arslan et al. (2009)</td>
<td>Turkey</td>
<td>377</td>
<td>0.76</td>
</tr>
<tr>
<td>Tamaki et al. (2011)</td>
<td>Japan</td>
<td>395</td>
<td>0.81</td>
</tr>
<tr>
<td>Pachouri et al. (2006)</td>
<td>India</td>
<td>225</td>
<td>0.59</td>
</tr>
</tbody>
</table>
CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

5.1 Discussion

This case-control study was conducted to determine the genotypic frequencies of selected nevirapine metabolizing enzymes alleles and their link to the development of nevirapine-induced hypersensitivity among Ghanaian patients exposed to nevirapine-based antiretroviral therapy at the Korle-Bu Teaching Hospital. Nevirapine-induced hypersensitivity is linked to a number of genes but varies within races and ethnic groups. The identification of genetic factors, particularly SNPs within metabolizing enzymes involved in the pharmacokinetics of ARV drugs is of paramount interest. Hence the need to elucidate the differences in genotypic frequencies of SNPs and their significant association to influencing variation in therapy response among patients on nevirapine-based antiretroviral therapy.

Eleven (11) out of the 12 participants who developed nevirapine hypersensitivity were females (Table 4.1). This supports the findings that there is a high risk for women to develop nevirapine hypersensitivity reaction (Ben m'rad et al., 2009, Prosperi et al., 2012). Sex-related differences in antiretroviral response and toxicity may be due to differences in gene expression which leads to peculiar pharmacokinetics influencing drug response. Age and BMI of participants had no association with nevirapine hypersensitivity in the study.
There was no significant association of CD4 cell count of participants with nevirapine hypersensitivity, suggesting that CD4 count has no influence on the development of nevirapine hypersensitivity (Table 4.2) similar to reports in other studies (Vitezica et al., 2008, Yuan et al., 2011). However, other studies were contradictory to the finding reported in this study (Martin et al., 2005, Wit et al., 2008, Issah, 2018). The contradiction was not totally unexpected since most of the patients in this study initiated nevirapine combined ART at a CD4 count below 250 cells/µl.

Initiation of nevirapine-based HAART is contraindicated in females with CD4 above 250 cell/µl and males with a CD4 count above 400 cells/µl (Popovic et al., 2010). However, the CD4 count may not necessarily be the sole predisposing factors inducing nevirapine hypersensitivity, hence the variation in a report by different studies in different populations. For example, a study showed that high CD4 counts before initiation of nevirapine and HLA-DRB1*0101 were linked to a high risk of developing nevirapine hypersensitivity. However, neither HLA-DRB1*0101 nor the CD4 count alone was linked to nevirapine-induced hypersensitivity (Martin et al., 2005).

The genotypic frequencies observed in this study for the CYP2B6 983T>C as recorded in Table 4.3 and Table 4.4) showed no significant difference between cases and controls. However, the genotypic frequency in this study confirms that CYP2B6 983T>C is prevalent in Africans (Wang et al., 2006). The frequency (3%) of CYP2B6 983T>C in this study is in agreement to those reported by Klein et al. (2005) who observed CYP2B6 983T>C alleles in African-Americans (2.9%) and also Mehlotra et al. (2007) reported 1.6% in Guinea population.
More importantly, the allele frequencies of 97% T and 3% C reported in this study closely replicate studies involving Ghanaians that recorded 96% T and 4% C (Sarfo et al., 2013); 96% T and 4% C (Kwara et al., 2009) and 96.9% T and 3.1% C (Klein et al., 2005). However, Mehlotra et al. (2007) reported a higher frequency of 7.6% for the variant allele, C in a Ghanaian population which may be due to a large number of study participants used in that study. Contrary to this study, no allele variant (983C) was identified in any of the samples analyzed from Papua New Guinea (Mehlotra et al., 2007). CYP2B6 983T>C occurs with an allele frequency of 4–9% in the African population (Thorn et al., 2010).

Caucasian-Americans and Asian-Americans had no allele variant detected but was detected in African-Americans (7.5%) and Hispanic-Americans (1.1%). This is an indication that there is a genetic variation between Africans and the Caucasians with respect to CYP2B6 metabolizing enzymes, hence variations in response to drugs metabolized by CYP2B6 among different populations (Lamba et al., 2003). The homozygous mutant for CYP2B6 983T>C (983CC) was not detected in this study which is similar to what was reported in another study in Malawi population (Dickinson, 2014) and Ghanaians (Sarfo et al., 2014). However, Kwara et al. (2009) reported a very low genotypic frequency of 0.001 (1 out of 701 study participants) for CC in Ghanaian participants. This may imply that, although the variant allele is present in Africans, the homozygous mutant which describes poor metabolizer of the CYP2B6 substrate may be rare in the Ghanaian and the African populations.

There is high variation in CYP2B6 messenger RNA expression, ranging from 20 to 250-fold, which may be attributed to differential transcriptional regulation and inherited genetic variation.
(Zanger et al., 2007) CYP2B6 gene has several haplotypes (CYP2B6*6, CYP2B6*11, CYP2B6*15 and CYP2B6*18) which is associated with reduced catalytic activity and protein stability, resulting in an increased nevirapine plasma levels (Kranendonk et al., 2014). CYP2B6 983T>C shows about 15-30% decreased expression as compared to the wild-type (Wang et al., 2006). CYP2B6 983T>C (CYP2B6*18) is associated with high plasma drug concentrations in patients treated with nevirapine (Mehlotra et al., 2007). However, this study did not determine the plasma concentration of nevirapine in the study participants and its association to CYP2B6 983T>C polymorphism. Although sex has also been shown to influence CYP2B6 expression, there is a conflicting report in the literature, which may, in turn, be because of confounding factors such as race and environment (Zanger et al., 2007).

There was no statistically significant association of CYP2B6 983T>C to the development of nevirapine hypersensitivity. However, this was contrary to other studies where CYP2B6 983T>C was significantly associated with nevirapine hypersensitivity in Malawians and Ugandans (Carr et al., 2014b) and Mozambicans (Ciccacci et al., 2013). CYP2B6 functional polymorphism 983T>C can be linked with 785A>G as a CYP2B6*16 allele, hence resulting in a haplotype effect (Mehlotra et al., 2007). However, this study did not target CYP2B6*16 in the study population which is common in Central, Western, and Southern Africa (Wang et al., 2006). This suggests ethnic as well as intra-regional differences among African populations with respect to CYP2B6 983T>C (either alone or as a haplotype).
A significant association of the CYP2B6 983T>C to nevirapine hypersensitivity could be a combined effect of two CYP2B6 SNPs. For example, the presence of the heterozygote (983TC) in combination with CY2B6 516GG or 516GT resulted in decreased nevirapine clearance or bioavailability (Dickinson, 2014). However, 516G>T and 983T>C together were associated with up to five-fold higher mean plasma efavirenz plasma concentrations, suggesting an additive effect of these polymorphisms (Wang et al., 2006). Nuclear receptor genes, NR112 and NR113 regulate the transcription of CYP2B6 (Thorn et al., 2010). Variations in these nuclear receptors genes that regulate CYP2B6 may be possible genetic factors associated with nevirapine hypersensitivity among participants in this study. However, these regulatory genes were not considered in this study. A study by Ciccacci et al. (2013) identified that the variant allele, C was significantly associated with a higher risk to develop SJS/TEN with a higher frequency of heterozygotes in cases than in controls. However, the frequencies of the heterozygous in this study were comparable between the cases and controls, hence resulting in an insignificant association.
CYP3A5 although is not a major metabolizing enzyme of nevirapine, it is known to impact on the pharmacokinetics of nevirapine among HIV patients. Both CYP3A5*3 and CYP3A5 14690G>A (CYP3A5*6) polymorphism results in absent CYP3A5 enzymatic activity (Pinto and Eileen Dolan, 2011). In this study, the majority of the genotypes observed for CYP3A5 14690G>A among the participants indicated an absence of activity. It is anticipated that CYP3A5 14690G>A would be associated with a poor nevirapine metabolism phenotype and might cause elevated plasma nevirapine concentrations among study participants. There was a significant genotypic difference of CYP3A5 between two Bantu-speaking populations from Cameroon and South Africa (Swart et al., 2012). Genotypic frequencies of CYP3A5 14690G>A has been reported in different countries and races (Table 4.6). The analysis of CYP3A5 14690G>A allele showed that all the subjects studied were carrying this allele. However, this was contrary to other previous studies among the Ghanaian population, where about 27% of the studied population were carrying the variant allele (Kudzi et al., 2010). The contradiction may also be due to the small sample size used, as a similar study among Ghanaians involved a large number of participants. Although the allele variant was not statistically significant to nevirapine hypersensitivity, it is of concern to pharmacogenomics studies due to its significant variation between Ghanaians and the Caucasians (Table 4.6). The report by this study also contradicts findings by Lavandera et al. (2011) and Fukuen et al. (2002) who indicated that no variant allele was detected among Argentinian and Japanese studied population respectively. A similar finding was reported among the Bangladeshi population with all the subjects indicating only the wildtype with no variant allele (Islam et al., 2014). Solas et al. (2007) showed in a piece of work
carried out in a French population, that none of the participant's was carrying the \textit{CYP3A5*6/*6} genotype which agrees with the result found in this study.

The result obtained in this study and other studies suggest that \textit{CYP3A5 14690G>A} is a common allele variant in the Ghanaian population, but it is absent or rare allelic variant in a Caucasian population (Table 4.6). It is also reported that the highest prevalence of \textit{CYP3A5*1} allele is found to be common in African-Americans. Therefore, non-Caucasians may be more likely to experience higher clearance of some drugs metabolized by \textit{CYP3A5} and less likely to experience drug toxicities (Fukuen et al., 2002).

Human SULT1A1 is an important enzyme in the metabolism of endogenous and exogenous carcinogens. \textit{SULT1A1 638G>A} polymorphism results in a reduced enzyme activity and thermostability. \textit{SULT1A1 638G>A} genotypes were investigated to determine whether they have an effect on the individual susceptibility to nevirapine hypersensitivity. SULTs catalyze 12-OH-nevirapine, a non-reactive metabolite to a reactive metabolite, 12-SUL-nevirapine which is an important pathway in the phase II metabolism of nevirapine. The 12-SUL-nevirapine can bind covalently to protein, forming a protein adduct. This could explain the nevirapine hypersensitivity characterized by skin rash and hepatotoxicity in some patients (Caixas et al., 2012, Marinho et al., 2013). Sharma et al. (2013) demonstrated that 12-OH-nevirapine sulphate causes induction of skin rash in both humans and Brown Norway rats. However, this study did not consider nor measured plasma concentration of the substrate (12-OH-nevirapine) of SULT and its reactive metabolite (12-SUL-nevirapine).
Literature is limited with respect to genotypic frequencies of SULT1A1 638G>A and its association with the development of nevirapine hypersensitivity. This study identified all the genotypes of SULT1A1 638G>A in the study population (Table 4.8). It recorded genotypic frequencies of 79% G and 21% A. The observed SULT1A1 638G>A frequency (21%) in this study was high than those reported in China (11%) by Liang et al. (2004) and Taiwan (9%) by Shen et al. (2012), which indicate a variation in response of drugs metabolized by SULT1A1. The homozygous mutant (AA) was absent in the cases and had no significant association with nevirapine hypersensitivity. SULT1A1 638G>A polymorphism is expected to lead to a decreased 12-SUL-nevirapine plasma concentration. A study observed that SULT1A1 inhibitor, dehydroepiandrosterone, did not prevent the skin rash, although it affected the plasma concentration of nevirapine and 12-OH-nevirapine (Sharma et al., 2013). This may depict that another phase II enzymes may play a role in the generation of nevirapine-derived reactive electrophiles, which may be responsible for nevirapine-rash (Antunesa et al., 2011). Patients with combined polymorphisms of glutathione S-transferase, GSTT1 and GSTM1 null genotypes have an increased risk of developing nevirapine hypersensitivity (Singh et al., 2017). This is an indication that SULT may not be the only phase II metabolizing enzyme to be associated with the development of nevirapine hypersensitivity. The 12-OH-nevirapine which is a substrate for SULT can also be metabolized to 4-COOH-nevirapine, escaping the SULT metabolizing pathway (Caixas et al., 2012) which may not result in nevirapine hypersensitivity.
5.2 Conclusion

This study to the best of my knowledge has established for the first time, the genotypic frequencies of SULT1A1 638G>A among the Ghanaian population. There were no statistically significant differences of CYP2B6 983T>C, CYP3A5 14690G>A and SULT1A1 638G>A alleles between nevirapine hypersensitive and nevirapine tolerant patients.

Individual with one or more one variant allele of CYP2B6 983T>C has a 1.67-time risk (OR=1.67, 95% Cl=1.58-17.55, p=0.67) for having nevirapine hypersensitivity whereas participants with one or more variant allele of SULT1A1 638G>A has 1.35 times more risk (OR=1.35, 95% Cl=0.38–4.84, p=0.64) to develop nevirapine hypersensitivity. The distribution of genotypic frequencies of CYP2B6 983T>C, CYP3A5 14690G>A and SULT1A1 638G>A was not significantly different among the HIV patients on nevirapine-based antiretroviral therapy. These SNPs showed no significant association with the development of nevirapine-induced hypersensitivity.

The results may offer a preliminary basis for the more rational use of drugs that are substrates for CYP2B6, CYP3A5 and SULT in the Ghanaian population. Also, the findings in this study will serve as a guide to help identify specific genetic markers for nevirapine hypersensitivity reaction that may be useful for tailored drug prescription. This may be helpful to maximize optimal responses at the lowest doses, indirectly implying the reduction of unintended toxicities.
RECOMMENDATION

It is recommended that a large number of study participants should be used so that if any, a correlation of the genotypic frequencies can reflect the larger Ghanaian population.

Whole haplotype or exon genotyping for CYP2B6, CYP3A5, SULT1A1, HLA and other Phase II metabolizing enzymes preferably by sequencing to help identify unique alleles that are specific to the Ghanaian general population. The study must also involve major hospitals across the country for the possible and significant association of other genetic alleles.
REFERENCES


INGELHEIM, B. 2012. Virmaune Prescription Information, Ridgefield, CT, USA Boehringer Ingelheim Pharmaceuticals, Inc.


WIT, F. W., KESSELRING, A. M., GRAS, L., RICHTER, C., VAN DER ENDE, M. E.,
BRINKMAN, K., LANGE, J. M., DE WOLF, F. & REISS, P. 2008. Discontinuation of
nevirapine because of hypersensitivity reactions in patients with prior treatment
experience, compared with treatment-naive patients: the ATHENA cohort study. *Clinical
Infectious Diseases*, 46, 933-940.

WONGTRAKUL, J., PAEMANEE, A., WINTACHAI, P., THEPPARIT, C., ROYTRAKUL, S.,
Nevirapine induces apoptosis in liver (HepG2) cells. *Asian Pacific Journal of Tropical
Medicine*, 9, 547-553.

Human 3'-phosphoadenosine 5'-phosphosulfate synthetase 2 (PAPSS2)
pharmacogenetics: gene resequencing, genetic polymorphisms and functional

YUAN, J., GUO, S., HALL, D., CAMMETT, A. M., JAYADEV, S., DISTEL, M., STORFER,
S., HUANG, Z., MOOTSIKAPUN, P., RUXRUNGTHAM, K., PODZAMCZER, D. &
HAAS, D. W. 2011. Toxicogenomics of nevirapine-associated cutaneous and hepatic
adverse events among populations of African, Asian, and European descent. *AIDS*, 25,
1271-1280.

YUN, J., ADAM, J., YERLY, D. & PICHLER, W. J. 2012. Human leukocyte antigens (HLA)
associated drug hypersensitivity: consequences of drug binding to HLA. *Allergy*, 67,
1338-1346.
APPENDIX I

Primer sequences, amplicon sizes and restriction enzymes used

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<th>Primers</th>
<th>Sequence 5’-3’</th>
<th>Amplicon size</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2B6 983T&gt;CF</td>
<td>AGGAAT CCACCCACCTCAAC</td>
<td></td>
<td>BsmAI</td>
</tr>
<tr>
<td>CYP2B6 983T&gt;CR</td>
<td>GATAAGGCAGGTGAAGCAATCA</td>
<td>759bp</td>
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*F = Forward primer, R = Reverse primer*

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence 5’-3’</th>
<th>Amplicon size</th>
<th>Restriction enzyme</th>
</tr>
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<tr>
<td>CYP3A5 14690G&gt;AF</td>
<td>GTGGGTTTCTTGCTGCATGT</td>
<td>237bp</td>
<td>DdeI</td>
</tr>
<tr>
<td>CYP3A5 14690G&gt;AR</td>
<td>GCCCACATACTTAT TGAGAG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*F = Forward primer, R = Reverse primer*

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence 5’-3’</th>
<th>Amplicon size</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>SULT1A1 638G&gt;AF</td>
<td>GTTGGCTCTGCAG GGT TTCTAGGA</td>
<td>333bp</td>
<td>Haell</td>
</tr>
<tr>
<td>SULT1A1 638G&gt;AR</td>
<td>CCCAAACCCCTGCTGGCCAGCACC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*F = Forward primer, R = Reverse primer*
APPENDIX II

Reagents and Equipment

Reagents:

1. Ethidium bromide
2. PCR tubes (0.2 ml)
3. Eppendorf tubes (2.5 ml)
4. One Taq Quick-Load 2X Master Mix with Standard Buffer
5. Nuclease-free water
6. 50X TAE Buffer
7. Quick-Load Purple 100 bp DNA ladder
8. Distilled Water
9. Double distilled water
10. NaOH
11. HCl
12. Absolute and 70 % ethanol
Equipment:

1. Micro Pipettes (0.5-20μl, 5-50μl, 20-200μl, 100-1000μl),

2. Microwave Oven

3. Thermocycler

4. Electrophoretic tray and tank

5. Autoclave

6. Gel Imaging Systems

7. pH Meter

8. Glass stirrer

9. Spatula

10. Vortex

11. Analytical balance
APPENDIX III

Preparation of reagents

Preparation 1X TAE Buffer

Ten (10) millilitres of stock 50X TAE buffer was measured into a 500ml volumetric flask. The volume was adjusted to 500ml with double distilled water. The flask was covered and inverted several times for thorough mixing. The buffer was stored in a clean glass bottle at room temperature before use.

Two per cent (2%) agarose gel preparation

One gram (1g) of agarose powder was weighed using analytical electronic balance into a heat-resistant conical flask and 50ml of 1X TAE buffer was added to it. The mixture was then allowed to melt in a microwave oven for about 60 seconds. The solution was allowed to cool just above room temperature. Three microliters (3µl) ethidium bromide was added and mixed well by swirling the mixture gently. The solution was poured into the casting tray with inserted combs for the creation of wells in the gel.

For 3% and 4% agarose gel, 1.5g and 2.0g of agarose powder were weighed respectively and dissolved into 50ml of 1X TAE buffer.

Preparation of 100µM Primer Stock Solution

From the equation;

\[ \text{Concentration (C)} = \frac{\text{Amount of Substance (n)}}{\text{Volume (V)}} \]

\[ V = \frac{n}{C} \]
**CYP2B6**

\( n \ (CYP2B6 \ 983T>CF) = 44.116 \text{nmoles} \)

Substituting it into the equation above

\[ V = \frac{44.16 \times 10^{-9} \text{ moles}}{100 \times 10^{-6} \text{ mol/dm}^3} \]

\[ V = 0.4416 \times 10^{-3} \text{ dm}^3 = 441.6 \mu\text{L}. \]

Therefore, to reconstitute 100µM \( CYP2B6 \ 983T>CF \) stock primer solution, 441.6µL of sterile double distilled water (pH 7.6) was added to the lyophilized primer supplied (44.16 nmol) and vortex for 10 seconds. The reconstituted primer stock solutions were stored at -20°C until used.

\( n \ (CYP2B6 \ 983T>CR) = 50.34 \times 10^{-9} \text{ moles} \)

To prepare 100µM primer stock solution, volume of double distilled water required, \( V = \frac{n}{C} = 0.05034 \mu\text{M} / 100\mu\text{M} = 503.4\mu\text{L} \). Thus, 503.4µL of double distilled water (pH 7.6) was added to the lyophilized primer content to make the 100µM stock primer solution.

**CYP3A5**

\( n \ (CYP3A5 \ 14690G>AF) = 63.26 \times 10^{-9} \text{ moles} \)

To prepare 100µM primer stock solution, volume of double distilled water required, \( V = \frac{n}{C} = 0.06326 \mu\text{M} / 100\mu\text{M} = 632.6\mu\text{L} \). Thus, 632.6µL of ddH\(_2\)O (pH 7.6) was added to the lyophilized primer content to make the 100µM stock primer solution.

\( n \ (CYP3A5 \ 14690G>AR) = 49.68 \times 10^{-9} \text{ moles} \)
To prepare 100µM primer stock solution, volume of double distilled water required, V = n/C = 0.04968µM / 100µM = 496.8µL. Thus, 496.8µL of ddH₂O (pH 7.6) was added to the lyophilized primer content to make the 100µM stock primer solution.

**SULT1A1**

n (SULT1A1 638G>AF) = 52.66 x 10⁻⁹ moles

To prepare 100µM primer stock solution, volume of ddH₂O required, V = n/C = 0.05266µM / 100µM = 526.6µL. Thus, 526.6µL of double distilled water (pH 7.6) was added to the lyophilized primer content to make the 100µM stock primer solution.

n (SULT1A1 638G>AR) = 48.85 x 10⁻⁹ moles

To prepare 100µM primer stock solution, volume of double distilled water required, V = n/C = 0.0488.5µM / 100µM = 488.5µL. Thus, 488.5µL of ddH₂O (pH 7.6) was added to the lyophilized primer content to make the 100µM stock primer solution.

**Working primer Solutions**

For each primer used in this study, a 10.0µM primer working solution of 100µL was prepared from the 100.0µM primer stock solution.

From the equation;
\[ C_1 \times V_1 = C_2 \times V_2, \text{ where } C_1 = \text{ primer stock concentration (100\mu M)}, \ V_1 = \text{ volume of primer stock concentration to be pipetted to make the working solution}, \ C_2 = \text{ primer working concentration (10\mu M)} \text{ and } V_2 = \text{ total volume of primer working solution to be prepared (100\mu L)}. \]

\[ V_1 = C_2 \times V_2 / C_1 \]

\[ = 10\mu L \times 100\mu L / 100\mu M \]

\[ = 10\mu L \]

Therefore, to prepare 10ul primer solution (forward and reverse), 10\mu M of the primer stock solution (100\mu M) was pipetted into a sterile Eppendorf tube and the volume was adjusted with sterile nuclease-free water to 100\mu L. The Eppendorf tube was vortexed for a few seconds and also stored at -20\degree C until used.
APPENDIX IV

Consent form

Genotypic frequencies of CYP2B6 983T>C, CYP3A5 14690G>A and SULT1A1 638G>A polymorphism among HIV patients on nevirapine-based antiretroviral therapy at the Korle-Bu Teaching Hospital.

Nevirapine is an important member of a drug regimen being used in Ghana for treating HIV-1 infection, which has greatly improved HIV-1 patient survival. Nevirapine use has been associated with hypersensitivity reaction characterized by rash and hepatotoxicity in some patients which may be due to genetic factors. The results of this study may be useful to health care providers, policymakers, and other stakeholders in Ghana for the management of HIV-1 patients. This will improve the efficacy of the drug, safety of the patients through reduced adverse effect and also reduce financial loss in the drug sector.

This study will take a small amount of blood (5ml) from you by inserting a needle into your forearm. The risk involved in this blood collection procedure is negligible and it will cause only minimal pain and bruise. This sample will be collected once and will be used for analyses of genotypic frequencies and its association with nevirapine-induced hypersensitivity.

It will be appreciated if you will agree to take part in this study. Your participation in the study is voluntary, and not participating will not at without any disadvantage concerning your medical care at this Hospital. All information gathered will be treated with strict confidentiality.
If you have further questions on the study you can contact;

Isaac Egyir Prah (0249204839), Department of Pharmacology and Toxicology, School of Pharmacy, University of Ghana.

Consent

I ................................................................................................................................................................
of .......................................................................................................................................................... give my
consent for my sample to be used for the research project stated above which has been explained
to me.

By..........................................................................................................................................................

Patient’s signature............................................Date..........................................................

Doctor’s signature............................................Date..........................................................
Data Collection Form

This form should be filled out by the attending Clinician for each Patient enrolled in the Nevirapine-based antiretroviral therapy study.

Date: ........../ .......... / ........... ] Patient ID: .................

Section I: Demographics of participant

1. The current age of the participant.
   [      ] years

2. Sex of the participant.
   [  ] Male
   [  ] Female

3. The weight of the participant before the initiation of therapy
   [   ] kg

4. The height of the participant before the initiation of therapy
   [   ] m

5. Ethnic group of the participant:

.................................................................

6. Date of Initial HIV diagnosis of participant
   [   ][  ]/[   ][  ]/[   ][  ][  ][  ]

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Section II: Evaluation of the participant

7. Mark one answer for each question in the table below.

<table>
<thead>
<tr>
<th>Statement</th>
<th>Baseline</th>
<th>Week 2</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Did the participants have nevirapine-induced hypersensitivity on this period?</td>
<td>[ ] Yes</td>
<td>[ ] Yes</td>
<td>[ ] Yes</td>
</tr>
<tr>
<td></td>
<td>[ ] No</td>
<td>[ ] No</td>
<td>[ ] No</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Statement</th>
<th>Week 8</th>
<th>Week 12</th>
<th>Week 16</th>
<th>Week 24</th>
</tr>
</thead>
<tbody>
<tr>
<td>b) Did the participant have nevirapine-induced hypersensitivity on this period?</td>
<td>[ ] Yes</td>
<td>[ ] Yes</td>
<td>[ ] Yes</td>
<td>[ ] Yes</td>
</tr>
<tr>
<td></td>
<td>[ ] No</td>
<td>[ ] No</td>
<td>[ ] No</td>
<td>[ ] No</td>
</tr>
</tbody>
</table>

Section III: Laboratory results of the participant

8. HIV status:

   Type 1 [ ]   Type 2 [ ]

9. Hepatitis B virus status:

   Positive [ ]   Negative [ ]

<table>
<thead>
<tr>
<th>Test</th>
<th>Baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 count</td>
<td></td>
</tr>
</tbody>
</table>