CYP2C9, VKORC1 AND CYP4F2 VARIANT FREQUENCIES IN PATIENTS ON EITHER LOW OR HIGH STABLE WARFARIN MAINTENANCE THERAPY IN THE GHANAIAN POPULATION

BY

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DECLARATION

I, Samuel Yao Ahorhorlu of the Department of Medical Biochemistry, University of Ghana Medical School, 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. Bartholomew Dzudzor at the Department of Medical Biochemistry of the University of Ghana Medical School, Dr. William Kudzi at the Center for Tropical Clinical Pharmacology and Therapeutics of the University of Ghana Medical School and Dr. Edeghonghon Olayemi at the Department of Haematology, University of Ghana Medical School.

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DEDICATION

I dedicate this work to Emefa my beloved wife for her understanding, care and support during the long hours of absence from home in pursuit of this research work. Thanks a lot "Sweetness". I also dedicate this work to my parents Mr. George K. Ahorhorlu and Mrs. Alice Kwadzo for their love and care.

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ABSTRACT

Warfarin is the most commonly prescribed oral anticoagulant drug for reducing thromboembolic events that often give rise to stroke, deep vein thrombosis, pulmonary embolism, or serious coronary malfunctions. Warfarin has a narrow therapeutic / toxic ratio and genetic factors have been associated with inter-individual variability in warfarin dose / response in different ethnic populations. The initiation of this drug has been associated with one of the highest adverse event rates for any single drug, particularly in the elderly. The aim of this study was to determine the frequency of CYP2C9, VKORC1 and CYP4F2 variant alleles in the Ghanaian population as bases to estimate the potential impact of these polymorphisms on warfarin maintenance dose. The study also sought to determine the clinical and demographic factors associated with warfarin maintenance dose in indigenous Ghanaian patients. One hundred and forty three adult Ghanaian patients on stable warfarin therapy at the Korle-Bu Teaching Hospital were genotyped for CYP2C9 (*2, *3), CYP4F2 rs2108622, and VKORC1_1639G > A polymorphisms using PCR-RFLP assay methods. The most common indications for warfarin use were valve replacement (n = 63, 44%), deep vein thrombosis (n = 52, 36.4%), pulmonary embolism (n = 18, 12.6%), and atrial fibrillation (n = 10, 7.0%). Warfarin dose was negatively correlated with patient age but not statistically significant (r = -0.024, 95% CI (-0.052-0.004), p = 0.090). Women were found to be taking a higher mean daily warfarin dose of 5.75mg (95% CI, 5.174-6.326) than males who took 5.46mg (95% CI, 4.907-6.022) although this was not statistically significant, p = 0.479). Warfarin dose was positively correlated with patient height but not statistically significant (r = 0.010, [-0.031-0.052], p = 0.630). BMI was found to have no influence on mean daily warfarin dose (OR: 0.571, p = 0.513) in this study population. Allelic frequencies for CYP2C9*3 were observed

at (23%) while genotype frequencies for CYP2C9*3 were observed at (10.87%). CYP2C9*2 alleles and genotypes were not detected in this study population. Allele frequencies for VKORC1_1639A were observed at (6%) however, the VKORC1_1639A genotype was not detected in this study. Allele frequencies for CYP4F2 rs2108622 (T) was observed at (41%) and its genotype (T/T) frequencies were observed at (6.84%). According to the combined effect of the CYP2C9, VKORC1 and CYP4F2 genotypes, patients having the wild-type (*1/*1) genotype of CYP2C9 in combination with the homozygous mutant (T/T) genotype of CYP4F2 and the wild-type (G/G) genotype of VKORC1 required the highest mean daily warfarin dose of 7.50mg/day (95% CI, 7.50-7.50, p = 0.096). It was also observed that patients with a combination of CYP2C9 wild-type (*1/*1), CYP4F2 wild-type (C/C) and VKORC1 wild-type (G/G) genotypes were treated with the lowest mean daily warfarin dose of 4.79mg/day (95% CI, 3.02-6.55, p = 0.096). Interestingly, carriers of the heterozygous genotype of CYP2C9*1/*3, and CYP4F2 (C/T), and VKORC1 wild-type (G/G) genotype were given 6.50mg (95% CI, 5.40-7.60, p = 0.027).

This study has established for the first time the combined effect of genotypes of CYP2C9, VKORC1 and CYP4F2 genes on mean daily warfarin dose in Ghanaian patients. VKORC1 and CYP4F2 variant alleles to our knowledge are being reported for the first time among the indigenous Ghanaian population.

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ABBREVIATIONS

ADRs	Adverse Drug Reactions
AF	Atrial Fibrillations
CYP2C9	Cytochrome P450 isoenzyme 2C9
CYP4F2	Cytochrome P450 isoenzyme 4F2
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMEs	Drug Metabolizing Enzymes
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediaminetetra acetic acid
EtBr	Ethidium Bromide
HGP	Human Genome Project
HWE	Hardy-Weinberg Equilibrium
INR	International Normalized Ratio
KBTH	Korle-Bu Teaching Hospital
PCR	Polymerase Chain Reaction
PGENI	Pharmacogenetics for Every Nation Initiative
RFLP	Restriction Fragment Length Polymorphism

SNPs	Single Nucleotide Polymorphisms
TAE	Tris acetate EDTA
Taq	Thermus aquaticus
USA	United States of America
VKORC1	Vitamin K Epoxide Reductase complex subunit 1

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Variations in the response of patients to medications have been noticed and documented since the 1950s (Alving *et al.,* 1956). This observation has been partly explained by factors such as age, body size, race, concurrent diseases, medications and genes of patients. Polymorphisms in genes coding for Drug Metabolizing Enzymes (DMEs) has also been shown to significantly influence patients' variability in drug response (Wadelius *et al.*, 2007).

Increasing numbers of relevant polymorphisms are being discovered. It is also known that the frequencies and distributions of both harmful and protective polymorphisms vary greatly between human populations (Nebert & Menon, 2001; Wilson *et al.*, 2001). Given all the above, it is valid to study traits that are predominantly expressed in specific populations (Nebert & Menon, 2001).

It has also been established that genetic variations within DMEs, drug transporters and drug targets do influence both the efficacy of drugs and the likelihood of Adverse Drug Reactions (ADRs) (Pirmohamed & Park, 2003). Knowledge of the allelic frequency distribution within a specified population can be useful in identifying potential risk groups for ADRs and appropriate dose adjustments could be made to achieve therapeutic efficacy.

Warfarin is the most commonly prescribed oral anticoagulant drug for reducing thromboembolic events that often give rise to stroke, deep vein thrombosis, pulmonary embolism, or serious coronary malfunctions (Daly & King, 2003). Although it has been used

for more than 50 years, initiation of therapy remains problematic because of inter-individual variability in the degree of anticoagulation achieved in response to standard warfarin dose. An appropriate warfarin dose in one patient may induce a haemorrhagic event in another. Warfarin has a narrow therapeutic window between too much and too little effect such that a small change in its dose can have quite a large effect on blood coagulation (Takahashi *et al.*, 2006b). For instance, overdosing and under-dosing can result in life-threatening events such as bleeding or thrombosis. It is estimated that 1% of patients die due to bleeding complications associated with warfarin and up to 15% of patients experience minor bleeding complications (Takahashi *et al.*, 2006b). Warfarin initiation has been associated with one of the highest adverse event rates for any single drug, particularly in the elderly (Takahashi *et al.*, 2006b). Physicians, therefore, find it very challenging to initiate therapy in patients where it is needed. It has been estimated that almost half of all atrial fibrillation (AF) patients who are eligible for and would benefit from warfarin therapy are not receiving the drug because of the associated risks and monitoring costs (Gedge *et al.*, 2000).

The efficacy of warfarin is dependent on maintaining a patient's anticoagulation within acceptable therapeutic range.

Dose adjustments are often necessary and are based on measuring the prothrombin time in blood and calculating the International Normalized Ratio (INR), which gives an indication of the time it takes for the patient's blood to clot. Genetic factors have been associated with inter-individual variability in warfarin dose in different ethnic populations (Kamali *et al.*, 2004).

1.2 Pharmacogenetics and Warfarin maintenance dose

Warfarin is an anticoagulant used in the prevention of thrombosis (the abnormal formation of blood clot in living vessels) and thromboembolism (the migration of blood clot in blood vessels to other parts of the body). It was introduced in 1948 as a pesticide against rats and mice but was found in the early 1950s to be effective and relatively safe for preventing thrombosis and embolism (Holbrook *et al.*, 2005).

Warfarin exists as a racemic mixture of two enantiomers (optical isomers) in equal amounts (Buckley & Dawson, 1992). These enantiomers are metabolized by different pathways and have different half-lives and potencies. The *S* (-) enantiomer of warfarin is 3-5 times more potent than the *R* (+) enantiomer (Buckley & Dawson, 1992).

The cytochrome P450 2C9 subfamily is responsible for the metabolism of *S*-warfarin. The cytochrome P450 isozymes 1A2, 3A4 and 2C are responsible for the metabolism of *R*-warfarin. *S*-warfarin is almost entirely oxidized to form *S*-7-hydroxywarfarin and some *S*-6-hydroxywarfarin (Buckley & Dawson, 1992). *R*-warfarin is oxidized and reduced to *R*-6-hydroxywarfarin and some *R*-7- hydroxywarfarin via the oxidative pathway and *R*, *S*-warfarin alcohol via the reductive pathway (Buckley & Dawson, 1992). Some warfarin metabolites are excreted in the bile, and there is significant enterohepatic circulation. Most warfarin metabolites are excreted in the urine.

A number of genes have been reported in a recent review by Wadelius and Pirmohamed to be involved in the biological pathway of warfarin (Wadelius & Pirmohamed, 2007a). The most

important of these genes are Cytochrome P450 isozyme 2C9 (CYP2C9), Vitamin K epoxide reductase (VKORC1) and Cytochrome P450 isozyme 4F2 (CYP4F2).

1.2.1 CYP2C9 and Warfarin maintenance dose

The primary enzyme involved in metabolism and subsequent inactivation of S-warfarin is the CYP2C9 which account for nearly 10% of the differences in people's responses to the drug (Wadelius *et al.*, 2007). CYP2C9 gene has two common variants, CYP2C9*2 (430C>T) and CYP2C9*3 (1075 A>C). These variants result in a protein with decreased function and nearly abolished function, respectively (Wadelius *et al.*, 2007).

Patients with variant CYP2C9*2 alleles have been shown to have reduced enzyme activity up to 12% compared to patients described as extensive metabolizers of warfarin with two wild-type CYP2C9*1 alleles. Patients with variant CYP2C9*3 alleles have been shown to have reduced activity up to 5% compared to extensive metabolizers (Crespi & Miller, 1997; Haining *et al.*, 1996). Patients with these variant alleles will require a lower dose of warfarin. Patients who carry these CYP2C9 gene variants are more likely to require more time to achieve steady state and a stable INR due to the longer half-life of the drug. Thus, dosing adjustments and INR determinations can be made when CYP2C9 variants are known to allow steady-state concentrations to be achieved more efficiently.

1.2.2 CYP4F2 and Warfarin maintenance dose

A newly discovered gene, CYP4F2 was also found to contribute 1% - 2% of the variability in warfarin dose and has an impact on stable warfarin dose (Takeuchi *et al*., 2009). Cytochrome P450 *4F2 (CYP4F2*) is a vitamin K1 (VK1) oxidase. Carriers of the V433M polymorphism (rs2108622: C>T nucleotide substitution) have lower hepatic concentrations of the enzyme, resulting in a reduced capacity to metabolise VK1. Elevated hepatic levels of VK1 are thought to render these individuals less sensitive to the anticoagulant effects of warfarin (McDonald *et al.*, 2009). Patients with homozygote mutant (T/T) alleles of CYP4F2 will require approximately 1 mg/day more of warfarin than those who carry the homozygote wild type allele (CC) (Caldwell *et al.*, 2007).

Results from the first genome-wide association study (GWAS) which searched the entire genome, reported that additional genes having a major influence on warfarin dose might not exist or be found in the near-term in Caucasians. Hence, clinical trials assessing patient benefit from individualized dose forecasting based on a patient's genetic makeup at VKORC1, CYP2C9 and possibly CYP4F2 could provide state-of-the-art clinical benchmarks for warfarin use during the foreseeable future (Takeuchi *et al.*, 2009).

1.2.3 VKORC1 and Warfarin maintenance dose

Vitamin K epoxide reductase (VKOR) is the site of action for warfarin and it is estimated that nearly 30% of warfarin dose variance is due to Single Nucleotide Polymorphisms (SNPs) in the warfarin drug target VKORC1 (D'Andrea *et al.*, 2005). The associated gene, VKORC1, has a common promoter variant (1639G>A) which reduces the expression of the gene, and

therefore lowers the amount of VKOR and leads to warfarin sensitivity. Variations in VKORC1 have been associated with both warfarin sensitivity and warfarin resistance. VKORC1 codes for an enzyme that recycles reduced vitamin K making it available for use by vitamin K-dependent coagulation factors and the mutant A allele decreases its action. In the VKORC1 _1639 SNP, the common G allele is replaced by the A allele. Because people with an A allele produce less VKORC1 than people with the G allele, lower warfarin doses are needed to inhibit VKORC1 and to produce an anticoagulant effect in carriers of the A allele (Rieder *et al.*, 2005).

The prevalence of these variants have been reported in literature to vary by race; 37% of Caucasians and 14% of Africans carry the A allele (Rieder *et al.*, 2005). The anticoagulant activity of warfarin is due to inhibition of the VKORC1 enzyme (Scott *et al.*, 2010). The common promoter mutation of G to A at position 1639 may explain much of the pharmacological variability in warfarin sensitivity. CYP2C9 and VKORC1 polymorphisms occur frequently in patients who are warfarin "sensitive" and require lower doses, whereas patients with VKORC1 missense mutations are warfarin "resistant" and require higher doses (Scott *et al.*, 2010).

The combination of the two CYP2C9 variants (*2 and *3) with the VKORC1 promoter mutation is estimated to account for 40% – 63% of the variability in therapeutic warfarin dose (Rieder *et al.*, 2005; Sconce *et al.*, 2005). Recent genome wide association studies have not only confirmed these observations but also identified a novel association between reduced hepatic CYP4F2, higher levels of hepatic vitamin K, and higher warfarin dose requirements (Singh *et al.*, 2011).

1.2.4 Clinical Implications of the Genetic Mutation

The three SNPs (CYP2C9*2, CYP2C9*3, and VKORC1 1639G>A) play key roles in determining (1) the dose of warfarin required to produce a therapeutic INR (typically 2.0 to 3.0); (2) the risk of bleeding or of producing supra-therapeutic INR (>4.0); and (3) the time required to achieve a stable therapeutic dose (Roth *et al.*, 2013).

Carriers of CYP2C9*2 and CYP2C9*3 require, on average, a 19% and 33% reduction of warfarin respectively, per allele in warfarin dose verses those who carry the CYP2C9*1 allele (Higashi *et al.*, 2002; Schwarz *et al.*, 2008). Carriers of the VKORC1 A allele require, on average, a 28% reduction of warfarin per allele in their warfarin dose compared to those who carry none (Higashi *et al.*, 2002; Schwarz *et al.*, 2008). The effect of CYP2C9 and VKORC1 variants on warfarin dosage were reported by (Scibona *et al.*, 2012) (Table 1.1).

As expected, using standard dosing algorithms in patients with these variants leads to adverse clinical and laboratory outcomes because of their genetically mediated sensitivity to the drug. In particular, standard dosing algorithms lead, on average, to a 2- to 3-fold increased risk of serious or life threatening bleeding or an out-of-range INR (>4.0) in carriers of the *2 or *3 alleles of CYP2C9 (Higashi *et al.*, 2002). Similarly, carriers of the VKORC1 A allele are also at a 2- to 3-fold higher risk of an INR >4.0 during initiation of warfarin therapy when standard dosing algorithms are used (Schwarz *et al.*, 2008).

Finally, as a result of the sensitivity of these patients to warfarin and the additional dose adjustments required, the time required to achieve a "stable" INR between 2.0 and 3.0 is significantly delayed in carriers of all three SNPs (CYP2C9*2, CYP2C9*3, and VKORC1 1639G>A) (Higashi *et al.*, 2002; Schwarz *et al.*, 2008). Overall, using a combination of

genetic and clinical factors to predict the warfarin maintenance dose may be more accurate than using clinical factors alone (Klein *et al.*, 2009).

Table 1.1: **CYP2C9 and VKORC1 Polymorphism and Sensitivity to Warfarin**

CYP2C9	VKORC1_1639G>A	Sensitivity to Warfarin
CYP2C9*1	G/G	Low
CYP2C9*2	G/A	Intermediate
CYP2C9*3	A/A	High

Source: (Scibona *et al.*, 2012).

Genetic polymorphisms of CYP2C9 and VKORC1 variants have been reported in different ethnic populations. CYP2C9*2 (430C>T) variant allele is found in 8% -13% of Caucasian, 2% - 6% of Asian and less than 1% of African- Americans populations. CYP2C9*3 (1075A>C) variant is 6% - 10% in Caucasians, less than 1% in Asians and 1% - 4% in African-American population (Takahashi *et al.*, 2006b). VKORC1 (1639G>A) is 42% in Caucasians, 89% in Asians and 8% in African Americans populations (Takahashi *et al.*, 2006b). While the Asian population generally has the low-dose variation of VKORC1, African- Americans have the high-dose version. European Americans fell in the middle between the Asians and African Americans (Rettie & Tai, 2006; Rieder *et al.*, 2005).

Some of these studies mentioned earlier investigated the influence of pharmacogenetics on warfarin treatment response focusing only on variants within individual CYP2C9, VKORC1 or CYP4F2 genes. Other studies however assessed the combined effect of these variants in these genes together with various environmental factors (Higgins, 2005; Lee *et al.*, 2006;

Takahashi *et al.*, 2006a; Wadelius *et al.*, 2005). Based on the outcome of some of these investigations, several warfarin dosing procedures incorporating polymorphisms of VKORC1, CYP2C9 and in some cases, CYP4F2 as covariates, were explored in a study by Jonas and McLeod (Jonas & McLeod, 2009). The predictive power of these warfarin dosing procedures were observed to have varied markedly across different populations. The study compared the combined frequency of variant VKORC1, CYP2C9 and CYP4F2 alleles among African-Americans, Asian, Caucasian, Hispanic and Ashkenazi Jewish populations (Scott *et al.*, 2010). This approach was further extended to populations that are consistently under-represented in pharmacogenetics databases, namely Amerindians (Native Americans), sub-Saharan Africans (Mozambicans) and an admixed Brazilian cohort, with African, European and Amerindian ancestral roots (Vargens *et al.*, 2011). The study revealed that Mozambicans and African-Americans differed significantly in the frequency of CYP2C9 and VKORC1 polymorphisms. This observation was explained in part by the authors as a European admixture in African-Americans (Suarez-Kurtz, 2005) and may be reflecting the genetic diversity of sub-Saharan African populations (Tishkoff *et al.*, 2009). It was established that in the context of warfarin dosing algorithms, VKORC1, CYP2C9*2 and CYP2C9*3 are rare or absent in Mozambicans (Tishkoff *et al.*, 2009).

Considering the major contribution of these SNPs to the performance of most warfarin algorithms, it might be anticipated that such algorithms will perform poorly in Mozambicans, as they do in other cohorts of African descent (Cavallari *et al.*, 2010; Gage & Lesko, 2008; Klein *et al.*, 2009). There is very limited pharmacogenetics data on warfarin dose requirement in many other indigenous African populations. There is currently no pharmacogenetics data

on warfarin metabolism to assist in the management of warfarin patients in the Ghanaian population. Therefore it will be of clinical relevance to explore the impact of VKORC1, CYP2C9, and CYP4F2 polymorphisms on warfarin dosage to ascertain patient sensitivity or resistance.

1.3 Problem Statement

Warfarin, the most commonly prescribed anticoagulant, exhibits large interpatient variability in dose requirements (Kaminsky & Zhang, 1997). Patient-specific factors such as age, body weight, race, concurrent diseases, and medications, explain some of the variability in warfarin dose but genetic factors influencing warfarin response explain a significantly higher proportion of the variability in dose (Wadelius & Pirmohamed, 2007b).

Candidate-gene association studies (Sconce *et al.*, 2005) have identified 2 genes: CYP2C9, which codes for the enzyme cytochrome P450 2C9 that metabolizes S-warfarin (Kaminsky & Zhang, 1997), and VKORC1, which codes for warfarin's target, vitamin K epoxide reductase (Li *et al.*, 2004), as responsible for the main proportion of the genetic effect. The influence of CYP2C9 and VKORC1 has also been confirmed by genome-wide association studies among Caucasians (Takeuchi *et al.*, 2009).

Personal communication with Physicians at Korle-Bu Teaching Hospital revealed that there are some Ghanaian patients on high warfarin maintenance dose (>5mg per day) and others on low warfarin maintenance dose (≤5mg per day). Warfarin has a narrow therapeutic window and patient response varies widely. Giving a high dose of warfarin to a patient who requires a low dose, may induce bleeding events whilst administering a low dose of warfarin to a patient

who requires a high dose may lead to thrombosis both of which may be life-threatening. Specific alleles have been reported to be distributed differently in different race/ethnic populations (Kittles & Weiss, 2003). However, pharmacogenetic data on these clinically relevant genes which could help in managing warfarin patients are scarce in indigenous African populations and are unavailable in Ghana.

1.4 Justification

It is expected that genetic information, especially for CYP2C9 and VKORC1 genes in addition to patient-specific factors such as age, body size, race, concurrent diseases, and medications could potentially improve management of warfarin dose/response in Ghanaian patients on either high or low warfarin maintenance dose.

1.5 Aim

To determine the frequency of CYP2C9, VKORC1 and CYP4F2 variant alleles in the Ghanaian population as basis to estimate the potential impact of these polymorphisms in patients on either low or high warfarin maintenance dose.

1.6 Specific Objectives

1. To determine the clinical and demographic factors associated with both high and low stable warfarin doses in indigenous Ghanaian patients.
2. To genotype CYP2C9, VKORC1 and CYP4F2 variants in patients on high warfarin maintenance dose (>5mg per day), and those on low warfarin maintenance dose (≤ 5mg per day).

3. To ascertain the genetic effect on dose variation for patients who are warfarin ‘sensitive’ and those who are warfarin ‘resistant’.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Genetic Effect on Drug Efficacy

Research over the past 50 years have shown that variation between individuals that is influenced by genes and other factors is relevant to the efficacy of all drugs (Meyer, 2004). It is now known that metabolic enzymes are affected not only by Single Nucleotide Polymorphisms, SNPs (of which the human genome contains more than 10 million), but also by other genomic variation, such as gene duplications and deletions, mutations in regulatory genes, and probably by recently-described large-scale copy number variations (Iafrate *et al.*, 2004; Sebat, 2004).

Such Pharmacogenomics studies might provide a molecular basis for population differences in DMEs for example Cytochrome P450 (Xie *et al.*, 2001), sulfotransferases (Falany, 1997) and methyltransferases (Weinshilboum, 2003), transporters such as ABC1 (Ameyaw *et al.*, 2001), receptors such as adrenergic receptors (Tate & Goldstein, 2004) and other factors that are involved in differential drug responses and disease susceptibility. Many of the population-group differences that are documented are likely to have important medical and public-health implications (Taylor, 2004). It is evident that drugs for which the metabolism is carried out by a polymorphic enzyme and where pronounced differences exist because of genetic variations, data generated in one population cannot be extrapolated into other populations. The responses to drugs vary between individuals and differences have also been shown to exist between different populations (Vesell, 1989). These differences in response to drugs can be partially attributed to physiological and environmental factors such as age, renal and liver function,

drug interactions, and the presence of disease. Investigating the genetic variation within genes encoding DMEs, drug transporters and drug targets by studying key polymorphisms help in understanding the genotype-phenotype correlation and the allelic frequency distribution within different populations. Pharmacogenetics information of this nature is increasingly becoming useful for improving drug therapy and explaining individual and inter-ethnic differences due to drug response (Grasmäder *et al.*, 2004). It is also being used to predict and explain ADRs which are estimated to account for about 106,000 deaths in the USA each year, which was more than auto accidents, suicides, and homicides combined (Landow, 1998). ADRs are one of the top six causes of death in the USA (Marwick, 1997).

2.2 Pharmacogenetics and Pharmacogenomics

The field of pharmacogenetics, deals with inherited variations in drug responses and refers to genetic differences in metabolic pathways which can affect individuals responses to drugs, both in terms of therapeutic as well as adverse effects (Klotz, 2007). Pharmacogenomics reflects the evolution of pharmacogenetics into the study of the entire spectrum of genes that determine drug efficacy and safety. The ultimate aim of pharmacogenetics is to lead to personalized treatment of individual patients based on their genetic profile; “the right drug at the right dose to the right patient at the right time” (Evans & Relling, 1999). However, the identification of individual genetic differences (polymorphisms) within populations can also be useful in improving the quality of healthcare in that specified population. It is becoming increasingly important to derive data from different populations to build a database which can then be used in epidemiological investigations to better understand the genetic risk factors

which affect many diseases and to be in a better position to predict these risk factors in the future (Nebert, 1999).

The proteins CYP2C9, CYP4F2, VKOR, and calumenin are encoded by polymorphic genes that provide significant contributions to warfarin disposition (*CYP2C9*), vitamin K disposition (*CYP4F2*), and clotting factor activation (*VKOR* and *calumenin*). CYP = cytochrome P450; *VKOR* = vitamin K epoxide reductase (Fig. 2.2).

With the progress of pharmacotherapy in the world, it is now known that inter-ethnic differences in clinical outcome are often greater than inter-individual differences, and it is well-accepted that clinical outcome of pharmacotherapy depends on genetic factors (Evans & McLeod, 2003; Kalow, 2002). Since the completion of the Human Genome Project (HGP), pharmacogenetics has been expanding rapidly over the past 10 years with great potential for improving drug efficacy and the ultimate goal of individualized therapy. It is also due to improved genotyping technologies over this period. The HGP is an international scientific research project initiated by the USA with a primary goal of determining the sequence of the chemical base pairs which make up DNA, and of identifying and mapping the approximately 20,000-25,000 genes of the human genome from both a physical and functional standpoint (Arledge *et al.*, 2001).

2.3 Warfarin's Mechanism of Action

Warfarin, a coumarin derivative, inhibits clotting by limiting hepatic production of the biologically active vitamin K-dependent clotting factors which are activated factors II, VII, IX, and X (Hirsh *et al.*, 2001). Precursors of these factors undergo a carboxylation reaction to be converted to their activated forms. Warfarin is a vitamin K antagonist which interferes with

this reaction. Reduction in the amount and activity of these factors produces the anticoagulant response. However, warfarin also interferes with production of the body's natural anticoagulants, protein C and protein S, and can therefore sometimes exert a procoagulant response (Hirsh *et al.*, 2001) (Fig. 2.1).

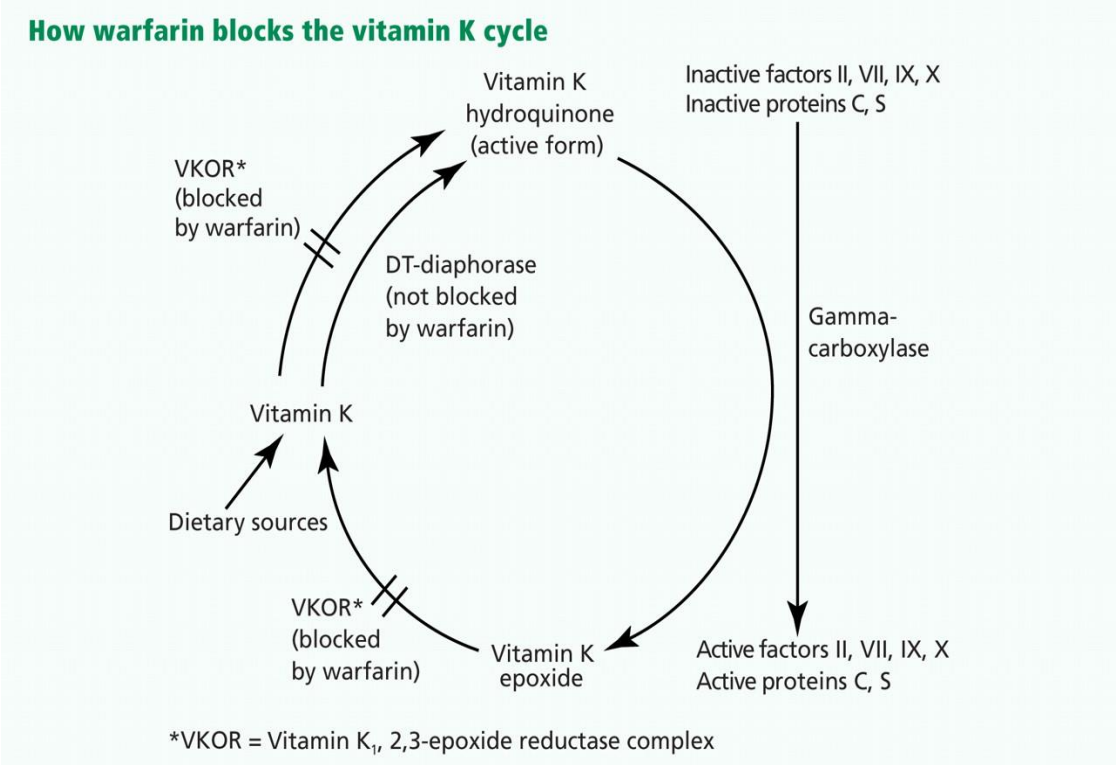

Fig. 2.1: How warfarin blocks the vitamin K cycle.

Source: (Osinbowale *et al.*, 2009)

2.4 Pharmacokinetics and Pharmacodynamics of Warfarin

Warfarin is a racemic mixture of a right-handed and a left-handed stereoisomer, designated R and S respectively. This racemic mixture has a half-life of approximately 36 to 42 hours

(Jaffer & Bragg, 2003). The S-isomer is five times more potent as a vitamin K antagonist than the R-isomer (Breckenridge *et al.*, 1974).

Absorption of warfarin is rapid and complete. It is highly protein bound (> 98%), primarily to albumin. Only the free drug is pharmacologically active (O'Reilly, 1969). If the serum albumin level is low such as in the nephrotic syndrome, the free fraction of warfarin is increased, but so is its plasma clearance (Ganeval *et al.*, 1986). Therefore, such conditions are not likely to lead to significant changes in the INR.

The hepatic metabolism of the two isomers differs, with clinically significant implications for drug interactions. The S-isomer is primarily metabolized by cytochrome P450 2C9 (and to a lesser degree by P450 3A4) and is eliminated in the bile (Hirsh *et al.*, 2001). The R-isomer, in contrast, is primarily metabolized by cytochrome P450 1A2 and P450 3A4 and is excreted in the urine as inactive metabolites. Since the S-isomer is much more potent than the R-isomer, medications that inhibit or induce the P450 2C9 pathway lead to the most significant drug interactions. Most drug interactions that affect the R-isomer are not significant (Hirsh *et al.*, 2001).

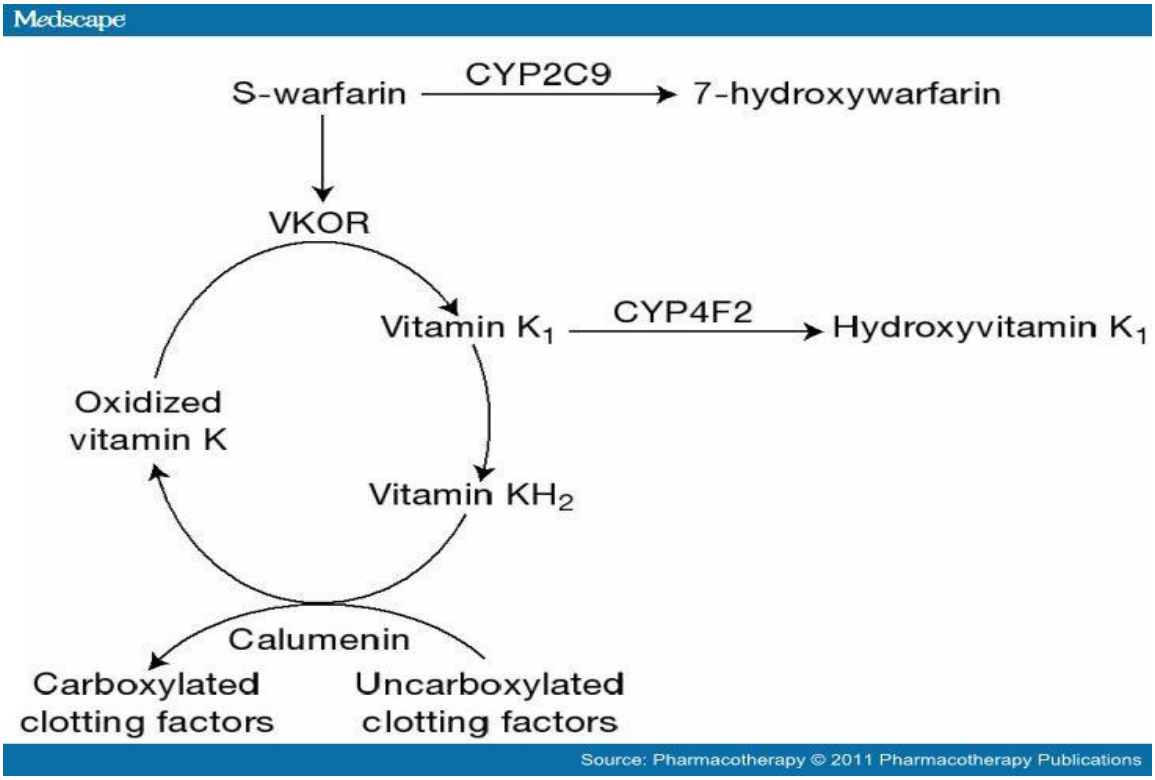

Fig. 2.2: Genes involved in the pharmacokinetics and pharmacodynamics of warfarin.
Source: Pharmacotherapy ©2011, Pharmacotherapy publications

2.5 Warfarin Multiple Interactions with other Drugs, Diet, and Disease States

There are many causes of high or low INR values. The most common, that is likely to lead to significant changes in the INR and increase the propensity for bleeding or clotting are drug interactions (Jaffer & Bragg, 2003). Drug interactions with warfarin can be defined as either pharmacokinetic or pharmacodynamic. Pharmacokinetic interactions involve alterations in the absorption, protein binding, and hepatic metabolism of warfarin. Conversely, pharmacodynamic interactions affect the tendency for bleeding or clotting through either antiplatelet effects or increases or decreases in vitamin K catabolism (Jaffer & Bragg, 2003). A list of common drugs that interacts with warfarin can be found in appendix II. Excessive vitamin K consumption can promote increased production of the vitamin K clotting factors,

decreasing the anticoagulant response to warfarin (Booth *et al.*, 1997). Alternatively, decreased vitamin K consumption can increase the anticoagulant response to warfarin. The foods that contain the highest amount of vitamin K per serving are the green leafy vegetables such as spinach, broccoli, and turnip greens (Booth *et al.*, 1997).

2.5.1 Concomitant diseases

Certain diseases can influence anticoagulation control (Demirkan *et al.*, 2000). Congestive heart failure can cause hepatic congestion of blood flow and inhibit warfarin metabolism. This can be troublesome in patients with frequent exacerbations of heart failure. Hypothyroidism decreases the catabolism of the vitamin K clotting factors. Therefore, hypothyroidism of new onset or due to inadequate replacement therapy could be suspected if there is a general trend toward decreased INR values with a need for increased warfarin doses (Jaffer & Bragg, 2003). Hyperthyroidism, on the other hand, increases the catabolism of the vitamin K clotting factors and could be suspected if there is a general trend toward increased INR values with a need for decreased warfarin doses (Demirkan *et al.*, 2000). Hepatic failure may significantly elevate the INR due to decreased production of clotting factors.

2.6 INR Monitoring and Warfarin dose determination

2.6.1 Adherence, missed doses

High or Low INR could mean that the patient is not taking the warfarin medication as required. The actual dose of warfarin taken can be confirmed by ruling out the possibility that the patient took a higher or lower than the prescribed dose. The possibility of a missed warfarin dose could also affect the INR results. In general, a missed dose of warfarin may be

reflected in the INR within 2 to 5 days after the dose is missed (Jaffer & Bragg, 2003). This could be important even though the INR value of the patient may be in the therapeutic range. For example, a patient with a therapeutic INR value who reports missing a dose of warfarin 2 days ago would very likely have had a higher than the therapeutic INR if he or she had not missed the dose (Jaffer & Bragg, 2003). This is particularly essential when interpreting INR results of a patient recently started on warfarin.

The patient's response to warfarin as reflected by the INR determines his or her warfarin dose required to attain therapeutic levels. The frequency of INR testing is dictated by dose response and current clinical information. High INR predisposes patients to increased risk of bleeding while low INR means a patient is at risk of thrombosis.

2.7 The Cytochrome P450 2C9 Gene

The human CYP enzymes are involved in the metabolism of a wide range of substances including pharmaceutical agents usually leading to their inactivation and excretion (Nelson *et al.*, 1996). They can also activate prodrugs or transform exogenous compounds into reactive intermediates that can act as therapeutic agents or as carcinogens. CYP enzymes are highly polymorphic and responsible for the metabolism of nearly 20% of clinically used drugs (Goldstein, 2001). Understanding these polymorphic variations within different populations is becoming increasingly important because of the drug interactions that result from enzyme inhibitions or inductions.

The official name of this gene is "cytochrome P450, family 2, subfamily C, polypeptide 9" and its official symbol is CYP2C9. This gene encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze

many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids (Guengerich, 2008). This protein localizes to the endoplasmic reticulum and its expression is induced by rifampin (Vormfelde *et al.*, 2009). The enzyme is known to metabolize many xenobiotics, including phenytoin, tolbutamide, ibuprofen and S-warfarin (Miners & Birkett, 1998). Studies identifying individuals who are poor metabolizers of phenytoin and tolbutamide suggest that this gene is polymorphic. The gene is located within a cluster of cytochrome P450 genes on chromosome 10q24 (Gray *et al.*, 1995).

The CYP2C9 gene helps the body to break down warfarin. There are several variants in the CYP2C9 gene that can cause the CYP2C9 enzyme to be less active. When the CYP2C9 enzyme is less active, warfarin stays in the body for a longer period of time. This means that lower warfarin doses are needed in people with certain (reduced function) CYP2C9 genetic variants. Variation in the CYP2C9 gene is responsible for up to 18% of variability in warfarin response (Johnson *et al.*, 2011).

The nomenclature for the CYP2C9 SNPs is unique: the normal, or wild-type, variant is referred to as *1 ("star 1"), the two polymorphic versions are *2 ("star 2") and *3 ("star 3"), and each person can carry any two versions of the SNP. For example, a person with two normal copies would be *1/*1, a person with only one polymorphism could be *1/*2, and a person with both polymorphisms could be *2/*3. The prevalence of each variant varies by race; 10% and 6% of Caucasians carry the *2 and *3 variants, respectively, but both variants are rare (< 2%) in those of African or Asian descent (Au & Rettie, 2008).

The CYP2C9 gene constitutes 50% of the CYP2C enzyme subfamily and is the most abundant member of this subfamily (Gerbal-Chaloin *et al.*, 2001). The CYP2C9 gene is located on chromosome 10q24.2 and spans approximately 55kb with 9 exons and encodes a protein of 490 amino acids (De Morais *et al.*, 1994). The CYP2C9 gene is predominantly expressed within the liver and also in smooth muscle and endothelial cells. It metabolizes approximately 10% of the clinically important drugs such as warfarin, tolbutamide, glipizide, losartan and phenytoin (Miners & Birkett, 1998). It also plays an important role in the metabolism of endogenous substances such as arachidonic acid (Miners & Birkett, 1998). The CYP2C9 gene exhibits inter-individual variability in its expression and catalytic activity due to genetic variations (Lindh *et al.*, 2005). This can result in either drug toxicity or therapeutic failure in some patients.

CYP2C9*1 metabolizes warfarin normally, CYP2C9*2 reduces warfarin metabolism by 30%, and CYP2C9*3 reduces warfarin metabolism by 90% (Shah & Voora, 2013). Because warfarin given to patients with *2 or *3 variants will be metabolized less efficiently, the drug will remain in circulation longer, so lower warfarin doses will be needed to achieve anticoagulation. The CYP2C9*2 and CYP2C9*3 variants alleles are the most common and are associated with decreased metabolism of different substrates (Xie *et al.*, 2002). The CYP2C9*2 variant allele, a C to T polymorphism at position 430 within exon 3, results in an arginine to cysteine substitution and impaired enzyme activity (Sullivan-Klose *et al.*, 1996). The CYP2C9*3 variant allele is an A to C polymorphism within exon7 at position 1075 which encodes for an isoleucine to leucine amino acid change also leading to impaired enzyme activity (Sullivan-Klose *et al.*, 1996).

Earlier studies have shown CYP2C9 polymorphisms leading to amino acid changes in the composition of this enzyme affecting both activity and specificity of the enzyme and therefore leading to inter-individual variability in the elimination of CYP2C9 substrates in different ethnic groups (Xie *et al.*, 2002; Yasar *et al.*, 1999). This genetic variability can lead to drug toxicity such as warfarin induced bleeding complications and to inadequate drug efficacy or therapeutic failure in some patients (Goldstein, 2001; Schwarz, 2003). CYP2C9*2 and CYP2C9*3 allele variants show decrease clearance for warfarin and phenytoin compared with the wild type allele (CYP2C9*1). Identifying individuals with this allele could help to predict the dose requirements of these drugs.

Standard 5mg/day dosing of warfarin in patients with CYP2C9 and/or VKORC1 variants can lead to excessive warfarin exposure, resulting in an exaggerated anticoagulant response and a risk of serious or life-threatening bleeding complications (Takahashi *et al.*, 2006b; Voora *et al.*, 2005). Patients with CYP2C9 variants are more likely to require more time to achieve steady state and a stable INR due to the longer half-life of the drug. Thus, dosing adjustments and INR determinations can be made when CYP2C9 variants are known to allow steady-state concentrations to be achieved more efficiently.

A recently evaluated warfarin genetics (WARG) cohort in approximately 1500 Swedish patients, the largest study to date, has shown likely benefit from genetic forecasting of dose (Wadelius *et al.*, 2009). The report also confirmed that SNPs in VKORC1 and CYP2C9 predict approximately 40% of dose variance while non-genetic factors (age, sex, body mass

index) jointly account for another nearly 15%. The robust and now widely replicated associations of warfarin dose with VKORC1 and CYP2C9 have provided one of the most successful applications of pharmacogenetics to date (Rettie & Tai, 2006) and offer promise for genetic prediction of required dose in a clinical setting (Wadelius *et al.*, 2009).

A previous study showed that a combination of genetic and non-genetic factors cause Caucasians to exhibit 20-fold inter-individual variation in warfarin dose needed to achieve the usual therapeutic level of anticoagulation as measured by the prothrombin INR (Takahashi & Echizen, 2003). Thus, in the absence of genotypic, clinical and other relevant information for predicting each patient's required warfarin dose, initial prescribed doses may be too low or too high. Warfarin's risks of serious side effects, narrow therapeutic range, and wide inter-individual variation in dosage, have focused attention on the need to better predict dose in the initial stage(s) of treatment (Bozina, 2010).

2.8 The Vitamin K Epoxide Reductase complex subunit 1 Gene (VKORC1)

The human body uses vitamin K in the blood clotting process. If vitamin K recycling is less efficient, then a person's blood will not clot easily. The VKORC1 gene codes for an enzyme that helps with the recycling of vitamin K. Genetic variation in VKORC1 can reduce the ability of this enzyme to recycle vitamin K. If the ability of the blood to clot is reduced, then a lower dose of warfarin may be needed. Higher doses of warfarin could be dangerous, because too much warfarin could slow clotting to the point where bleeding cannot stop (Johnson *et al.*, 2011).

The "A" variant (rs9923231) of the VKORC1 gene causes less efficient vitamin K recycling. This variant is responsible for 10% to 30% of the variability in warfarin response (Johnson *et al.*, 2011).

2.9 The Cytochrome P450 4F2 Gene (CYP4F2)

The CYP4F2 gene also plays a role in helping the body use vitamin K in the blood clotting process. CYP4F2 helps to reduce blood clotting by decreasing the amount of vitamin K.

People with the 'T' variant in the CYP4F2 gene could have increased vitamin K levels and may therefore need a higher warfarin dose to prevent clotting (Borgiani *et al.*, 2009). The differences in CYP4F2 genotype account for approximately 1-7% of the variability in warfarin response between individuals (Borgiani *et al.*, 2009; Pautas *et al.*, 2010).

2.10 Pharmacogenomics in Africa

CYP2C8, CYP2C9 and CYP2C19 polymorphisms were characterized in a healthy Ghanaian population. Allele frequency distributions for CYP2C8, CYP2C9 and CYP2C19 among the Ghanaian population were comparable to data previously reported in other populations of African origin but differ from that observed in Caucasian and Asian populations (Kudzi *et al.*, 2009). Variant allele frequencies for CYP2C9 and CYP2C19 were reported for the first time among indigenous Ghanaian population (Kudzi *et al.*, 2009).

Allelic frequencies were obtained for CYP2C8*2 (17%), CYP2C8*3 (0%), CYP2C8*4 (0%), CYP2C9*2 (0%), CYP2C9*3 (0%), CYP2C9*4 (0%), CYP2C9*5 (0%), CYP2C9*11 (2%), CYP2C19*2 (6%) and CYP2C19*3 (0%) (Kudzi *et al.*, 2009).

These results provide additional information on polymorphisms of this CYP2C subfamily of enzymes in an indigenous African population which is scarce in published literature (Kudzi *et al.*, 2009).

Information regarding genetic influences of warfarin dosage variability in the South African black population is very little. Novel CYP2C9 and VKORC1 gene variants associated with warfarin dosage variability in the South African black population were determined for patients on warfarin therapy (Mitchell *et al.*, 2011). Findings from that research indicated that 26 novel SNPs and seven previously described CYP2C9 variants and three previously described but not novel VKORC1 SNPs were observed (Mitchell *et al.*, 2011). Only 11 of the CYP2C9 variants and two of the VKORC1 variants were observed at high enough allele frequencies to assess their impact on warfarin dosage (Mitchell *et al.*, 2011). The researchers demonstrated that CYP2C9*8 and two novel CYP2C9 SNPs (g.16179 and g.46028) were associated with a decrease in warfarin dosage, β-blockers were independently associated with a decrease in warfarin dosage and two known VKORC1 variants (rs7200749 and rs7294) were associated with an increase in warfarin dosage. The CYP2C9 and VKORC1 variants and a small subset of environmental factors used in the study explained approximately 45% of warfarin dosage variability in the South African black population (Mitchell *et al.*, 2011).

2.11 Amplicon sizes of Genes under study and the Expected Restriction digest fragment Sizes

The CYP2C9*2 variant allele, a ‘C to T’ polymorphism at position 430 within exon 3, results in an arginine to cysteine substitution and impaired enzyme activity (Sullivan-Klose *et al.*,

1996). In CYP2C9*2 genotyping, the C430 > T substitution in exon 3 'abolishes' the *Ava*II restriction site (GGACC). Treatment of the 375 bp PCR product generated by the primer sets with *Ava*II leaves the fragment undigested at 375 bp when run on a 2% agarose gel. The wild-type (CYP2C9*1) and the mutant (CYP2C9*3) alleles were digested by *Ava*II restriction enzyme to give two smaller fragments of 296 bp and 79 bp (Seng *et al.*, 2003).

The CYP2C9*3 variant allele is an 'A to C' polymorphism within exon 7 at position 1075 which encodes an isoleucine to leucine amino acid change also leading to impaired enzyme activity (Sullivan-Klose *et al.*, 1996). In CYP2C9*3 genotyping, the A1075 >C substitution in exon 7 creates a *Kpn*I restriction site (GGTACC). Samples containing CYP2C9*3 produced 85 bp and 20 bp *Kpn*I digestion products. However, samples containing CYP2C9*1 and CYP2C9*2 produce a single undigested fragment of 105 bp. These possible outcomes were reported in a study by (Scibona *et al.*, 2012) (Table 2.1).

VKORC1 wild-type has two *Nci*I sequence recognition sites. An *Nci*I restriction enzyme treatment of the 636 bp PCR product generated smaller fragments of 472 bp, 114 bp and 50 bp for the wild-type allele (G/G). A 'G to A' polymorphism at position 1639 abolishes one of the *Nci*I restriction sites leading to only two smaller fragments of 522 bp and 114 bp being produced in the mutant allele (A/A). The heterozygous genotype with one wild-type and one variant allele (G/A) leads to four DNA fragments being resolved on the agarose gel (522 bp, 472 bp, 114 bp and 50 bp) (Aomori *et al.*, 2009).

CYP4F2 wild-type has a single *Pvu*II sequence recognition site. A *Pvu*II restriction enzyme digestion of the 439 bp PCR product generated two smaller fragments of 379 bp and 60 bp for the wild-type allele (C/C). A 'C to T' Polymorphism abolishes this recognition site and treatment with *Pvu*II restriction enzyme leaves the mutant allele (T/T) undigested. The heterozygous genotype with one wild-type and one variant allele (C/T) results in three DNA fragments being resolved on the agarose gel (439 bp, 379 bp and 60 bp).

Table 2.1: **CYP2C9 genotyping with restriction enzymes**

Genotypes											
*1/*1		*1/*2		*1/*3		*2/*2		*2/*3		*3/*3	
*Ava*II	*Kpn*I	*Ava*II	*Kpn*I	*Ava*II	*Kpn*I	*Ava*II	*Kpn*I	*Ava*II	*Kpn*I	*Ava*II	*Kpn*I
	105	375	105		105	375	105	375	105		
296		296		296	85			296	85	296	85
79		79		79	20			79	20	79	20

Restriction fragment sizes in base pairs (bp) generated during CYP2C9 genotyping using *Ava*II and *Kpn*I restriction enzymes. **Source:** (Scibona *et al.*, 2012).

2.12 Hardy-Weinberg Equilibrium (HWE) Determinations

In 1908, two scientists, Godfrey H. Hardy, an English mathematician, and Wilhelm Weinberg, a German physician, independently worked out a mathematical relationship that related genotypes to allele frequencies (Dorak, 2005).

Their mathematical concept, called the Hardy-Weinberg (HWE) principle, is a crucial concept in population genetics. It predicts how gene frequencies will be inherited from generation to

generation given a specific set of assumptions (McClean, 1997). The Hardy-Weinberg principle states that in a large randomly breeding population, allelic frequencies will remain the same from generation to generation assuming that there is no mutation, gene migration, selection or genetic drift (Thompson *et al.*, 1991). This principle is important because it gives biologists a standard from which to measure changes in allele frequency in a population. It is employed in genetic association study, to guard against genotyping errors and population stratification. HWE is an ideal state that never occurs in nature because there is always a disturbing influence present in nature. Examples of disturbing influences include: non-random mating, mutations, selection, limited population size, random genetic drift, and gene flow. The observed and the expected allele frequencies in a studied population are said to be in HWE if $p > 0.05$. A population of alleles must meet all the following rules in order to be considered "in equilibrium": (i) no gene mutations, hence no change in alleles; (ii) no migration of individuals either in or out of the population; (iii) random mating must occur, individuals mate by chance; (iv) no genetic drift must occur, a chance change in allele frequency may occur; (v) no natural selection, a change in allele frequency due to environment may occur. A deviation from the HWE results when $p < 0.05$ and this indicates evolution of species. Deviations of genotype frequencies from HWE can affect the validity of tests of association using allele-based contrasts (Schaid & Jacobsen, 1999). Testing of HWE is therefore used as a quality control step in the statistical analysis of genetic data.

2.12.1 Hardy-Weinberg Equation

There are two equations necessary to solve a HWE question: (a) $p + q = 1$, and (b) $p^2 + 2pq + q^2 = 1$, where p is the frequency of the dominant allele, q is the frequency of the recessive

allele, p^2 is the frequency of individuals with the homozygous dominant genotype, 2qp is the frequency of individuals with the heterozygous genotype and q^2 is the frequency of individuals with the homozygous recessive genotype.

For instance: a population containing the genotypes A/A, aa, and Aa, the frequency of A/A will always be p^2, aa, will be q^2, and Aa will be 2pq at equilibrium, where p is the frequency of A and q is the frequency of a.

CHAPTER THREE

3.0 METHODS

3.1 Study Design

This cross sectional study involved adult patients on either high or low stable warfarin maintenance dose. The patients were classified into two groups after their demographic data and clinical histories were recorded. Blood samples were taken from all participating patients for INR measurement and genotyping.

Ethical clearance for the study was obtained from the Protocol and Ethical Review Committee of the University of Ghana Medical School with reference number MS-Et/M.6-P.4.5/2011-2012.

3.2 Study site and study population

This study was conducted at the Korle-Bu Teaching Hospital (KBTH). KBTH is a referral hospital with over 1800 beds for in-patients and has several specialist clinics, wards, pharmacies, laboratories and a reference laboratory.

Patients were recruited from the Cardiothoracic Center and the Anticoagulation clinic of Haematology department. A stable warfarin patient was defined as one whose warfarin dose requirement had remained constant for at least the 3 previous clinic visits over a minimum period of 3 months, and with an International Normalized Ratio (INR) of the prothrombin time within the target range of 2.0 to 3.0 or 2.5 to 3.5 for heart valve replacement patients. A total of 143 individuals were recruited for this study. Eighty five (85) of these individuals were patients on low daily warfarin maintenance dose and 58 of these individuals were

patients on high daily warfarin maintenance dose. In this study, high daily warfarin dose was defined as > 5 mg/daily and low daily warfarin dose was defined as ≤ 5mg/daily based on personal interaction with some senior medical officers who administer the drug in Korle-Bu Teaching Hospital to reflect common practice already available in the hospital.

3.3 Inclusion Criteria

Patients of both genders were included in the study. Patients who required warfarin for at least 3 months and were between 18 to 77 years with any of the indications listed below: atrial fibrillation/flutter (AF), deep vein thrombosis (DVT), pulmonary embolism (PE), and mitral valve replacement (MVR) were enrolled for the study.

3.4 Exclusion Criteria

Patients with the following medical conditions were excluded from the study: history of GI bleeding or peptic ulcer disease, significant liver disease (active hepatitis or chronic HBV/HCV infection), uncontrolled hypertension, chronic diarrhoea or malabsorption syndrome, viral or bacterial infection prior to enrolment, active or previous infective endocarditis, hospital stay > 30 days as a result of septicaemia, mediastinitis or pneumonia, cardiac cachexia, and morbid obesity.

3.5 Procedures

Demographic data and clinical history of all patients were taken. Patients who satisfied the inclusion criteria were enrolled into the study after they have given their consent to be part of the study. Relevant study data was extracted from their folders onto a study data sheet (Appendix III). Patient height and weight were recorded to determine the Body Mass Index

(BMI). A whole blood sample (5mL) was collected from all patients. Aliquots of this sample (3.2 ml) were contained in EDTA specimen tubes for DNA extraction and genotyping of CYP2C9, VKORC1 and CYP4F2 variants. The remaining volume (1.8 mls) of the blood sample was collected into citrate tubes and used for INR measurement.

3.5.1 INR Measurement

The HumaClot Duo ^{plus} device was used to measure the INR for all the samples. The device is a 2 channel photo-optical instrument which offers clotting, chromogenic and immuneturbidimetric testing capabilities. The result (in seconds) for a prothrombin time (PT) performed on a normal individual may vary according to the type of analytical system employed. This may be due to the variations between different batches of manufacturer's tissue factor used in the reagent to perform the test. The INR was devised to standardize the results. Each International Sensitivity Index (ISI) value assigned by each manufacturer. For any tissue factor the manufacturer indicates how a particular batch of tissue factor compares to an international reference tissue factor. The ISI is usually between 1.0 and 2.0. The INR is therefore the ratio of a patient's prothrombin time (PT_{test}) to a control sample (PT_{normal}) raised to the power of the ISI value for the analytical system used and is calculated by the following formula:

$$\mathrm{INR} = \left(\frac{\mathrm{PT_{test}}}{\mathrm{PT_{normal}}}\right)^{\mathrm{ISI}}$$

3.5.2 Genomic DNA Extraction

Genomic DNA was isolated from whole blood samples using a QIAamp DNA blood Maxi Kit (QIAGEN, USA) following the manufacturer's protocol. Briefly, 500 µL of Qiagen protease was added to 3ml of whole blood and made up to 5ml with 1x PBS in a 50ml tube and mixed well. Lysis buffer AL (6ml) was added to the sample and vortexed for 1 minute. The sample was then incubated at 70°C for 10 minutes, after which 5ml of ethanol (96-100%) was added and the content vortexed. The solution was then transferred into the QIAamp Maxi column placed into a 50ml centrifuge tube and centrifuged at 3000 rpm for 3 min. The QIAamp maxi column was removed, the filtrate discarded and the column was placed back into the 50ml centrifuge tube. Buffer AW1 (5ml) was added to the QIAamp maxi column and the contents centrifuged at 5000 rpm for 1 min after which Buffer AW2 (5ml) was added to the column and the contents centrifuged at 5000rpm for 15 min. The QIAamp maxi column was then placed in a clean 50ml centrifuge tube and the collection tube containing the filtrate was discarded. Buffer AE (600µl) equilibrated to room temperature (25^0C) was then added to the QIAamp maxi column, incubated at room temperature for 5 min and then centrifuged at 5000rpm for 2 min. To get a maximum concentration of DNA, the eluate (600µl) containing the DNA was reloaded onto the membrane of the QIAamp maxi column, incubated at room temperature for 5 min and finally centrifuged at 5000rpm for 5 min. Less than 600µl was eluted from the column.

3.5.3 Genotyping

Genotyping of CYP2C9 alleles was performed by Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) as previously described (Burian *et al.*, 2002)

with some modifications. PCR-RFLP also known as Cleaved Amplified Polymorphic Sequence (CAPS) is a popular technique for genetic analysis. It is applied for the detection of intraspecies as well as interspecies variation. The first step in a PCR-RFLP analysis is the amplification of a fragment containing the variation of interest. This is followed by treatment of the amplified fragment with an appropriate restriction enzyme. Since the presence or absence of the restriction enzyme recognition site results in the formation of restriction fragments of different sizes, allele identification can be done by electrophoretic resolution of the fragments in a gel matrix.

3.5.4 PCR for Genes under study

In each reaction, genomic DNA was amplified using gene specific primers for each of the genes under study. Sequences of primers used for each gene are shown in Appendix I. Each PCR reaction was performed in a 25µl mixture made up of 10X PCR buffer containing Mg^{2+}, 10mM dNTP mix, 20µM specific forward and reverse primers for each gene, 1U of Taq polymerase and 10µl of genomic DNA. Details of each PCR mix / thermal cycling conditions for CYP4F2, VKORC1 and CYP2C9 amplifications are contained in Tables 3.1, 3.1.1, 3.2, 3.2.1, 3.3, 3.3.1, 3.4, and 3.4.1 respectively. A blank reaction tube containing all other reagents except DNA was included as negative control in each run. Preparation of reagents is contained in Appendix VI.

3.5.5 Gel Electrophoresis of PCR Products

Two percent (2% w/v) agarose gels were used to separate DNA fragments after PCR. Five microliters (5µl) aliquots of the PCR products already containing a loading dye were loaded

onto the gel and electrophoresed to confirm PCR amplification before proceeding with RFLP analysis. A 100 bp DNA molecular weight marker was run on each gel to allow for fragment size determination.

3.5.6 PCR protocol for CYP4F2 rs2108622

The CYP4F2 gene was amplified using the following reaction compositions and thermal cycling conditions. The primer sequences used are contained in Appendix I.

Table 3.1: Reaction composition / mix for CYP4F2

Reagents	1xµl	11xµl
DNA Sample	10	-
10x PCR Buffer +15mM Mg^{2+}	5.0	55
10µM dNTPs		
dTTP	0.5	5.5
dATP	0.5	5.5
dCTP	0.5	5.5
dGTP	0.5	5.5
Primer 1 (F)	0.25	2.75
Primer 2 (R)	0.25	2.75
Taq Polymerase	0.125	1.375
Nuclease free water	7.375	81.125
Total Reaction Volume	25µ	

Table 3.1.1: Thermal Cycling conditions for CYP4F2

Initial Denaturation	95^0C for 5 mins	1 Cycle
Denaturation	94^0C for 30 secs	35 Cycles
Annealing	50^0C for 30secs	
Extension	72^0C for 1 min	
Final Extension	72^0C for 7 mins	1 Cycle

3.5.7 PCR protocol for VKORC1 _1639G > A

The VKORC1 gene was amplified using the following reaction compositions and thermal cycling conditions. The primer sequences used are contained in Appendix I.

Table 3.2: Reaction composition / mix for VKORC1

Reagents	1xµl	11xµl
DNA Sample	10	-
10x PCR Buffer +15mM Mg^{2+}	5.0	55
10µM dNTPs		
dTTP	0.5	5.5
dATP	0.5	5.5
dCTP	0.5	5.5
dGTP	0.5	5.5
Primer 1 (F)	0.25	2.75
Primer 2 (R)	0.25	2.75
Taq Polymerase	0.125	1.375
Nuclease free water	7.375	81.125
Total Reaction Volume	25µl	

Table 3.2.1: Thermal Cycling conditions for VKORC1

Initial Denaturation	95^0C for 5 mins	1 Cycle
Denaturation	95^0C for 60 secs	35 Cycles
Annealing	51^0C for 30 secs	
Extension	72^0C for 2 mins	
Final Extension	72^0C for 10 mins	1 Cycle

3.5.8 PCR protocol for CYP2C9*2

The CYP2C9*2 gene variant was amplified using the following reaction compositions and thermal cycling conditions. The primer sequences used are contained in Appendix I.

Table 3.3: Reaction composition / mix for CYP2C9*2

Reagents	1xµl	11xµl
DNA Sample	10	-
10x PCR Buffer +15mM Mg^{2+}	5.0	55
10µM dNTPs		
dTTP	0.5	5.5
dATP	0.5	5.5
dCTP	0.5	5.5
dGTP	0.5	5.5
Primer 1 (F)	0.25	2.75
Primer 2 (R)	0.25	2.75
Taq Polymerase	0.125	1.375
Nuclease free water	7.375	81.125
Total Reaction Volume	25µl	

Table 3.3.1: Thermal Cycling conditions for CYP2C9*2

Initial Denaturation	95^0C for 10 mins	1 Cycle
Denaturation	95^0C for 5 secs	35 Cycles
Annealing	53^0C for 10 secs	
Extension	72^0C for 15 secs	
Final Extension	72^0C for 5 mins	1 Cycle

3.5.9 PCR protocol for CYP2C9*3

The CYP2C9*3 gene variant was amplified using the following reaction compositions and thermal cycling conditions. The primer sequences used are contained in Appendix I.

Table 3.4: Reaction composition / mix for CYP2C9*3

Reagents	1xµl	11xµl
DNA Sample	10	-
10x PCR Buffer +15mM Mg^{2+}	5.0	55
10µM dNTPs		
dTTP	0.5	5.5
dATP	0.5	5.5
dCTP	0.5	5.5
dGTP	0.5	5.5
Primer 1 (F)	0.25	2.75
Primer 2 (R)	0.25	2.75
Taq Polymerase	0.125	1.375
Nuclease free water	7.375	81.125
Total Reaction Volume	25µl	

Table 3.4.1: Thermal Cycling conditions for CYP2C9*3

Initial Denaturation	94^0C for 5 mins	1 Cycle
Denaturation	94^0C for 45 secs	35 Cycles
Annealing	53^0C for 45 secs	
Extension	72^0C for 1 min	
Final Extension	72^0C for 5 mins	1 Cycle

3.5.10 Restriction Fragment Length Polymorphism (RFLP) For Genes under Study

Aliquots of each PCR product (10µl) were digested with appropriate restriction enzymes obtained from *Biolabs* (*Ava*II for CYP2C9*2, *Kpn*I for CYP2C9*3, *Nci*I for VKORC1 and *Pvu*II for CYP4F2) at 37^0C for 2hrs each except *Kpn*I digestion of CYP2C9*3 which was carried out for 1hr to avoid star activity. Each reaction was performed in 20µl mixture containing 7µl of nuclease free water, 2µl of 10X NEBuffer, and 1µl restriction enzyme. The DNA fragments were electrophoresed on 2% agarose gel cast with Ethidium bromide (EtBr). Bands were detected (see Fig. 4.1, 4.2, 4.3, and 4.4), using Gel Logic 200 Imaging System from KODAK company, USA.

3.6 Statistical Analysis

Data was initially entered into Microsoft Excel™ 2010 table and then imported into Stata™ version 10 (StataCorp, College Station, Texas, United States). All statistical analyses were done using Stata™ version 10. Logistic regression was used to explore the association if any between categorical predictor / independent variables and categorical outcome variables. The data was summarized as frequencies and proportions. Chi square tests were performed to test for association between categorical variables. Observed genotype frequencies were compared with those expected under Hardy-Weimberg equilibrium using the χ^2 test. Warfarin dosages were summarized as means with accompanying standard deviations, and compared between patients of different genotypes using t-tests and analysis of variance (ANOVA). All reported p-values were two-sided and considered statistically significant at a level of $p < 0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 Demographic and Clinical Data

Influence of various patient-specific factors on daily warfarin maintenance dose (DD) was determined. A population of 143 subjects (55.9% females and 44.1% males) was recruited for the study. The most common indications for warfarin use were valve replacement (n = 63, 44%), deep vein thrombosis (n = 52, 36.4%), pulmonary embolism (n = 18, 12.6%), and atrial fibrillation (n = 10, 7.0%) (Table 4.1). Mean daily warfarin dose was negatively correlated with patient age but not statistically significant (r = -0.024, 95% CI (-0.052-0.004), p = 0.090) (Table 4.2). Warfarin dose was positively correlated with patient height but not statistically significant (r = 0.010, 95% CI (-0.031-0.052), p = 0.630) (Table 4.2). Body Mass Index (BMI) has no influence on mean daily warfarin dose; (OR = 0.571, 95% CI (0.107-3.051), p = 0.513) (Table 4.2). Females were found to be taking a higher mean daily warfarin dose 5.75mg (95% CI, 5.174-6.326) than males 5.46mg (95% CI, 4.907-6.022), p = 0.479) although this was not statistically significant as (Table 4.3).

Table 4.1: Comparison of High and Low Warfarin dose Patient Populations

Characteristics		Dosage		Total (143)	*p*
		High (>5mg) N=58, %	Low (≤5mg) N=85, %		
Median Age, IQR,		46.5, (33-58)	48 (36.59)	(100)	0.170, 0.650
Gender	*Female*	32 (55.2)	48 (56.5)	80 (55.9)	0.88076
	Male	26 (44.8)	37 (43.5)	63 (44.1)	
Diagnosis	*AF*	2 (3.4)	8 (9.4)	10 (7.0)	0.17068
	DVT	23 (39.7)	29 (34.1)	52 (36.4)	0.4965
	PE	8 (13.8)	10 (11.8)	18 (12.6)	0.71884
	MVR	25 (43.1)	38(44.7)	63 (44)	0.8493
BMI status	*Obese*	23 (39.65)	27 (31.76)	50 (34.96)	0.20408
	Overweight	11 (18.97)	26 (30.58)	37 (25.87)	0.69654
	Underweight	2 (3.45)	5 (5.88)	7 (4.90)	0.64552
	Normal	22 (37.93)	27 (31.76)	49 (34.27)	0.5485

N values indicate number of responses obtained in each category, BMI, body mass index, $p \leq 0.05$ denotes significance, IQR, interquartile range, DVT, deep vein thrombosis, PE, pulmonary embolism, MVR, mitral valve replacement, AF, atrial fibrillation.

Table 4.2: Relationship between some patient-specific factors and daily warfarin dose

Factor	Statistics	95% CI	*p*
Age	-0.024*	-0.052-0.004	0.090
Height	0.010*	-0.031-0.052	0.95
BMI	0.571^{+}	0.107-3.051	0.513

*…… linear regression coefficient

$^{+}$ ……Odds ratio

$P \leq 0.05$ denotes significance

Table 4.3: Gender versus Mean daily warfarin dose (mg)

	Mean dose	95% CI	*p*
Male	5.46	4.907-6.022	0.479
Female	5.75	5.174-6.326	

4.2 PCR-RFLP Gel Electrophoreses Results

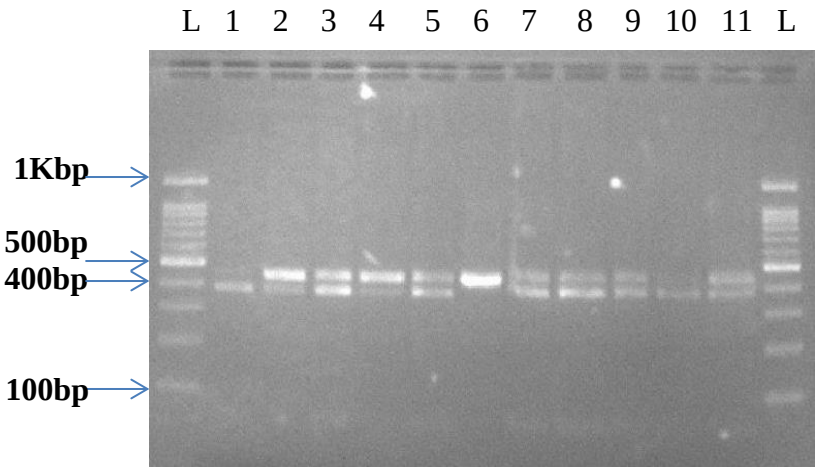

Fig.4.1: CYP4F2 PCR - RFLP Gel Electrophoregram
A 2% agarose gel electrophoregram showing RFLP results for CYP4F2 using *Pvu*II restriction enzyme. Lane L – 100 bp DNA ladder; lanes 1 and 10, samples homozygous to the wild-type allele (**C/C**). Lanes 3, 5, 7, 8, 9 and 11, heterozygous samples (**C/T**). Lanes 2 and 4, samples homozygous to the mutant allele (**T/T**). Lane 6, the uncut PCR product.

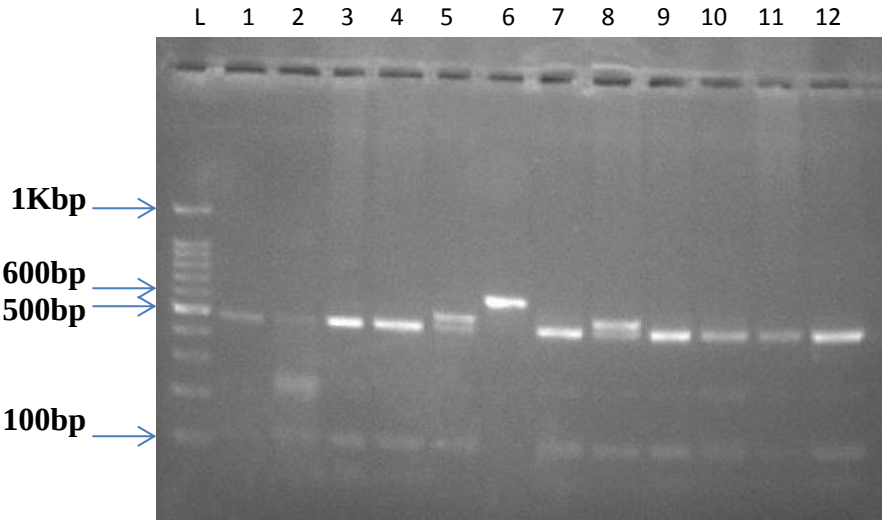

Fig. 4.2: VKORC1 PCR - RFLP Gel Electrophoregram
A 2% agarose gel electrophoregram showing RFLP results for VKORC1 using *Nci*I restriction enzyme. L- 100 bp DNA ladder. Lanes 5 and 8, the heterozygous samples (**G/A**). Lane 6, the uncut PCR product. Lanes 1, 2, 3,7,9,10,11 and 12, samples homozygous to the wild-type allele (**G/G**). No homozygous mutant (**A/A**) was detected in this study.

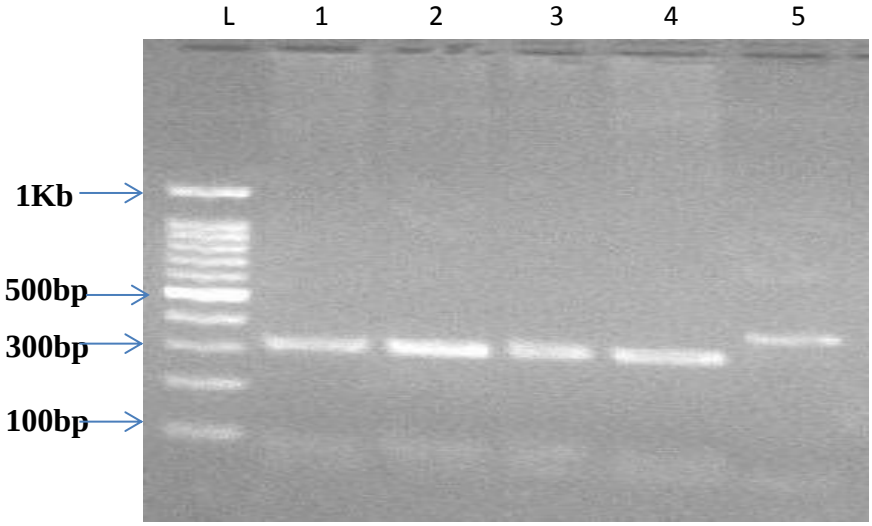

Fig. 4.3: CYP2C9*2 PCR - RFLP Gel Electrophoregram
A 2% agarose gel electrophoregram showing RFLP results for CYP2C9*2 using *Ava*II restriction enzyme. Lane L- 100 bp DNA ladder. Lanes 1, 2, 3 and 4, samples with either the wild-type (CYP2C9*1) or the mutant (CYP2C9*3) alleles. Lane 5, the uncut PCR product. There was no sample with the mutant variant CYP2C9*2 allele.

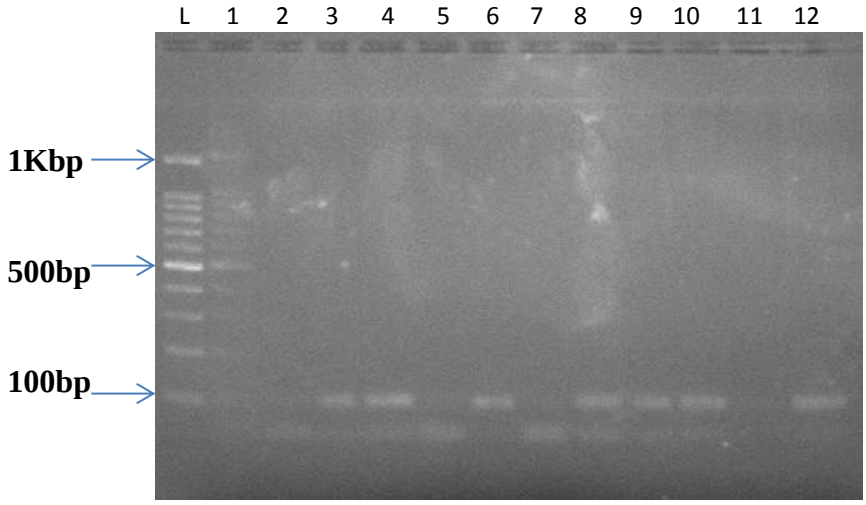

Fig. 4.4: CYP2C9*3 PCR - RFLP Gel Electrophoregram
A 2% agarose gel electrophoregram showing RFLP results for CYP2C9*3 using *Kpn*I restriction enzyme. Lane L-100 bp DNA ladder. Lanes 2, 5 and 7, samples with the variant CYP2C9*3 allele. Lane 6, the uncut PCR product. Lanes 3, 4, 8, 9, 10 and 12, samples with the wild-type (CYP2C9*1) allele.

4.3 Allele and Genotype Frequencies for CYP2C9

4.3.1 CYP2C9*3

The Hardy-Weinberg Equilibrium (HWE) was calculated using the formula in sections 2.12 and 2.12.1. The observed allele frequencies of CYP2C9*3 for the studied population and the low warfarin dose category both deviated significantly from the HWE ($p < 0.05$). The observed allele frequencies for high warfarin dose category were however in HWE ($p > 0.05$).

Table 4.4: Allele and Genotype frequencies of CYP2C9*3

SNP	n	Allele	Freq	N	Genotype	Observed (Expected†) frequencies (%)	
CYP2C9*3	142	*1	0.77	60	*1/*1	65.22	(59.56)
	42	*3	0.23	22	*1/*3	23.91	(35.23)
				10	*3/*3	10.87	(5.21)
CYP2C9*3 (Low Dose)	83	*1	0.80	37	*1/*1	71.15	(63.69)
	21	*3	0.20	9	*1/*3	17.31	(32.23)
				6	*3/*3	11.54	(4.08)
CYP2C9*3 (High Dose)	59	*1	0.74	23	*1/*1	57.50	(54.39)
	21	*3	0.26	13	*1/*3	32.50	(38.72)
				4	*3/*3	10.0	(6.89)

N = number of genotypes, n = number of alleles, SNP = single nucleotide polymorphism. † predicted Hardy-Weinberg frequencies.

4.3.2 CYP2C9*2

The CYP2C9*2 allele frequencies as well as genotype frequencies and their further categorization into low dose and high dose are summarized in Table 4.5

In the current study, all individuals (100%) had the common (C) allele and none carried the minor (T) allele for CYP2C9*2. The same observation was made in subjects on either low or high daily warfarin dose.

Table 4.5: Allele and Genotype frequencies of CYP2C9*2

SNP	n	Allele	Freq	N	Genotype	Freq
CYP2C9*2	174	C	1.00	87	C/C	1.00
	0	T	0.00	0	C/T	0.00
				0	T/T	0.00
CYP2C9*2	92	C	1.00	46	C/C	1.00
(Low Dose)	0	T	0.00	0	C/T	0.00
				0	T/T	0.00
CYP2C9*2	82	C	1.00	41	C/C	1.00
(High Dose)	0	T	0.00	0	C/T	0.00
				0	T/T	0.00

N = number of genotypes, n = number of alleles, SNP = single nucleotide polymorphism, C = Common allele, T = Minor Allele.

4.4 Allele and Genotype Frequencies for VKORC1

The VKORC1 allele frequencies as well as genotype frequencies and their further categorization into low dose and high dose groups within the Ghanaian population are summarized in Table 4.6

The observed allele frequencies of VKORC1 for the studied population were in HWE ($p > 0.05$), likewise the observed allele frequencies of both the low dose and high dose patient categories.

Table 4.6 Allele and Genotype frequencies of VKORC1

SNP	n	Allele	Freq	N	Genotype	Observed (Expected†) Frequencies (%)	
VKORC1-1639G>A	182	G	0.94	85	G/G	87.63	(88.01)
	12	A	0.06	0	G/A	12.37	(11.61)
				12	A/A	0.00	(0.38)
VKORC1-1639G>A (Low Dose)	105	G	0.91	47	G/G	81.03	(81.93)
	11	A	0.09	0	G/A	18.97	(17.17)
				11	A/A	0.00	(0.90)
VKORC1-1639G>A (High Dose)	77	G	0.99	38	G/G	97.44	(97.45)
	1	A	0.01	0	G/A	2.56	(2.53)
				1	A/A	0.00	(0.02)

N = number of genotypes, n = number of alleles, SNP = single nucleotide polymorphism. † predicted Hardy-Weinberg frequencies.

4.4 Allele and Genotype Frequencies for CYP4F2

The CYP4F2 (V433M: rs2108622: C>T) allele frequencies as well as genotype frequencies and their further categorization into low dose and high dose groups within the Ghanaian population are summarized in Table 4.7

The observed allele frequencies of CYP4F2 significantly deviated from HWE ($p < 0.05$), likewise the allele frequencies observed for both the low dose and the high dose groups.

Table 4.7: Allele and Genotype frequencies of CYP4F2

SNP	n	Allele	Freq	N	Genotype	Observed (Expected†) Frequencies (%)	
CYP4F2	137	C	0.59	28	C/C	23.93	(34.28)
	97	T	0.41	81	C/T	69.23	(48.54)
				8	T/T	6.84	(17.18)
CYP4F2	80	C	0.63	18	C/C	28.13	(39.06)
(Low Dose)	48	T	0.37	44	C/T	68.75	(46.88)
				2	T/T	3.13	(14.06)
CYP4F2	57	C	0.54	10	C/C	18.87	(28.92)
(High Dose)	49	T	0.46	37	C/T	69.81	(49.72)
				6	T/T	11.32	(21.37)

N = number of genotypes, n = number of alleles, SNP = single nucleotide polymorphism. † predicted Hardy-Weinberg frequencies.

4.5 Prevalence of CYP2C9, VKORC1 and CYP4F2 genotypes and Mean Daily Warfarin Dosage

According to the CYP2C9 genotypes, the highest daily warfarin dosages were administered to carriers of the heterozygous (*1/*3) genotype, while carriers of the wild-type CYP2C9*1/*1 genotype were treated with lower daily warfarin dosages of (6.82 ± 0.56 mg/day and 5.84 ±0.25 mg/day) respectively. Carriers of the homozygous mutant (*3/*3) were given intermediate daily warfarin dosages of (6.50 ±0.67 mg/day).

The carriers of the homozygous mutant (T/T) genotype for CYP4F2 were treated with higher daily warfarin dosages (6.88 ±0.41 mg/day) compared to the heterozygous (C/T) genotype (6.13 ±0.22 mg/day). Patients with the homozygous wild-type (C/C) were given intermediate daily warfarin dosages of (6.16 ±0.55 mg/day).

The carriers of the homozygous wild-type (G/G) genotype for VKORC1, were given higher daily warfarin dosages (6.14 ±0.22 mg/day), than carriers of the heterozygous (G/A) genotype

(4.97 ±0.55 mg/day) (Table 4.8). There were no carriers of the homozygous mutant genotype (A/A) in this study.

Table 4.8: The prevalence of CYP2C9, VKORC1 and CYP4F2 Genotypes and Mean Daily Warfarin Dosage

Genotype	Prevalence n (%)	Daily Warfarin Dosage mean(SD), mg	*p*
CYP2C9*2	0 (0.00)%	-	-
CYP2C9*3			
*1/*1	60(65.2%)	5.84(0.25)	0.169
*1/*3	22(23.9%)	6.82(0.56)	
*3/*3	10(10.9%)	6.50(0.67)	
CYP4F2			
C/C	28(23.9%)	6.16(0.55)	0.651
C/T	81(69.2%)	6.13(0.22)	
T/T	8(6.8%)	6.88(0.41)	
VKORC1			
G/G	85(87.6%)	6.14(0.22)	
G/A	12(12.4%)	4.97(0.55)	0.065
A/A	-	-	

CYP2C9*3: *1/*1 = homozygous wild-type genotype, *1/*3 = heterozygous genotype, *3/*3 = homozygous mutant genotype, CYP4F2: C/C = wild-type genotype, C/T = heterozygous genotype, T/T = homozygous mutant genotype, VKORC1: G/A = heterozygous genotype, G/G = homozygous wild-type, and A/A = homozygous mutant genotype.

4.6 The Mean Daily Warfarin Dosage According to Combined CY2C9, VKORC1 and CYP4F2 Gene Variants

According to the combined CYP2C9, VKORC1 and CYP4F2 genotypes, patients having the wild-type (*1/*1) genotype of CYP2C9 in combination with the homozygous mutant (T/T) genotype of CYP4F2 and the wild-type (G/G) genotype of VKORC1 required the highest mean daily warfarin dosage of 7.50mg/day (95% CI 7.50-7.50), p = 0.096). It was also

observed that patients with a combination of CYP2C9 wild-type (*1/*1), CYP4F2 wild-type (C/C) and VKORC1 wild-type (G/G) genotypes were treated with the lowest mean daily warfarin dosage of 4.79mg/day (95% CI 3.02-6.55), p = 0.096). The findings also revealed that carriers of CYP2C9 wild-type (*1/*1), who are heterozygous (C/T) for CYP4F2 and also carry VKORC1 wild-type (G/G) genotypes required an intermediate mean daily warfarin dosage of 5.78mg/day (95% CI 5.10-6.45), p = 0.096). Furthermore, carriers of the homozygous mutant (*3/*3) for CYP2C9, heterozygous (C/T) for CYP4F2 and the wild-type (G/G) genotype for VKORC1 were administered 6.67mg/day (95% CI 5.00-8.33). Also, carriers of the heterozygous (*1/*3) genotype for CYP2C9, heterozygous (C/T) genotype for CYP4F2 and the wild-type (G/G) genotype for VKORC1 were given 6.50mg/day (95% CI 5.40-7.60), p = 0.027).

Table 4.9: The Mean Daily Warfarin Dosage According to Combined CYP2C9, VKORC1 and CYP4F2 Gene Variants

CYP2C9*3	CYP4F2	VKORC1	freq(%)	mean(95%CI)	dose class	*p**
*1/*1	C/C	G/A	1(1.45)	5(-)	Low dose	
***1/*1**	**C/C**	**G/G**	7**(**10.14**)**	**4.79(3.02-6.55)**	Low dose	
*1/*1	C/T	G/A	1(1.45)	5(-)	Low dose	**0.096**
***1/*1**	**C/T**	**G/G**	27**(**39.13**)**	**5.78(5.10-6.45)**	High dose	
*1/*1	T/T	G/A	1(1.45)	5(-)	Low dose	
***1/*1**	**T/T**	**G/G**	5(7.25)	**7.50(7.50-7.50)**	High dose	
*1/*3	C/C	G/G	1(1.72)	15(-)	High dose	
*1/*3	C/T	G/A	1(1.45)	5(-)	Low dose	**0.027**
***1/*3**	**C/T**	**G/G**	15(21.74**)**	**6.50(5.40-7.60)**	High dose	
*1/*3	T/T	G/G	0	-	-	
***3/*3**	**C/T**	**G/G**	9(13.04**)**	**6.67(5.00-8.33)**	High dose	**-**
*3/*3	T/T	G/A	0	-	-	
			68(100%)			

CYP2C9*3: *1/*1 = homozygous wild-type, *1/*3 = heterozygous genotype, *3/*3 = homozygous mutant genotype, CYP4F2: C/C = wild-type genotype, C/T = heterozygous genotype, T/T = homozygous mutant, VKORC1: G/A = heterozygous genotype, and G/G = homozygous wild-type.

*Analysis of variance (ANOVA).

Table 4.10: Frequency distribution for CYP2C9 alleles in Ghanaian and other previously studied populations.

Ethnicity	N	*1	*2	*3	References
African					
Ghanaian	184	0.77	0.0	0.23	This study
Ghanaian	195	0.98	0.0	0.0	(Kudzi *et al.*, 2009)
Beninese	107	0.955	0.0	0.0	(Allabi *et al.*, 2003)
Ethiopian	150	0.977	0.043	0.023	(Scordo *et al.*, 2001)
African-American	107	0.914	0.033	0.023	(Dreisbach *et al.*, 2005)
Caucasians					
Belgian	121	0.822	0.10	0.074	(Allabi *et al.*, 2003)
Canadian	325	0.78	0.15	0.07	(Gaedigk *et al.*, 2001)
Swedish	430	0.82	0.107	0.074	(Yasar *et al.*, 1999)
Russian	290	0.82	0.11	0.07	(Gaikovitch *et al.*, 2003)
Asians					
Chinese	102	0.95	0.0	0.05	(Gaedigk *et al.*, 2001)
Japanese	218	0.98	0.0	0.02	(Nasu *et al.*, 1997)
Korean	574	0.99	0.0	0.01	(Yoon *et al.*, 2001)

N = number of Alleles

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSIONS

5.1 DISCUSSION

This study was designed to determine the frequencies of CYP2C9*1, *2, *3 allele variants, VKORC1_1639G>A and CYP4F2 _1347C>T gene polymorphisms in Ghanaian patients on either low or high warfarin maintenance therapy to ascertain the genetic basis of dose variation. The effects of patient specific factors such as age, sex, height, and body mass index on daily warfarin dosage were also assessed.

The most prevalent indications for warfarin use were valve replacement (n = 63, 44%), deep vein thrombosis (n = 52, 36.4%), pulmonary embolism (n = 18, 12.6%), and atrial fibrillation (n = 10, 7.0%) (Table 4.1) and these differed from 10.8% (valve replacement), 24.5% (deep vein thrombosis), 12.2% (pulmonary embolism), and 49.6% (atrial fibrillation) reported by Whitley (Whitley *et al.*, 2007) in an African-American population. Warfarin dose was negatively correlated with patient age but statistically not significant (r = -0.024) 95% CI (-0.052-0.004), p = 0.090). This observation compares with what was reported for an African-American population where it was observed that total weekly warfarin dosage was 2.4 mg less for each additional decade of patient age (Whitley *et al.*, 2007). In this study, women were found to be taking a higher mean daily warfarin dose of 5.75mg (95% CI (5.174-6.326) compared to men who were administered 5.460mg (95% CI (4.907-6.022), p = 0.479), although this was not statistically significant. This observation was however different from what was reported in an African-American population where women were found to require 2.55mg lower total weekly warfarin dosage compared to men, though not statistically significant (Whitley *et al.*, 2007). Previous studies also reported that women require an

average of 4.5mg less of warfarin per week (Ansell *et al.*, 2004; Garcia *et al.*, 2005). Warfarin dose was positively correlated with patient height but statistically not significant (r = 0.010, (95% CI (-0.031-0.052), p = 0.630) and this differed from what was reported in Lithuanian patients where higher daily warfarin dosages were prescribed for heavier and taller patients (Tatarunas *et al.*, 2011). In this study, BMI has no influence on mean daily warfarin dose (OR = 0.571, p = 0.513) and this was consistent with findings from previous studies which also reported no relationship between BMI or body weight and warfarin dose (Blann *et al.*, 1999; Gurwitz *et al.*, 1992; Oates *et al.*, 1998). This observation was however in contrast with what was reported in an African-American population where a weak correlation was found between BMI and Total Weekly warfarin Dose (TWD) (r = 0.08) though not statistically significant (Whitley *et al.*, 2007). Other studies have also indicated that height has greater predictive value of warfarin dose than does body weight or BMI (Sconce *et al* 2005). However, Singla and Morrill reported that BMI influences TWD as equal as gender (r2 = 5.3, p = 0.001) (Singla & Morrill, 2005).

Warfarin, the most commonly prescribed anti-clotting drug is a drug of choice for testing the hypothesis that pharmacogenetics can predict and reduce the incidence of adverse drug reactions. Warfarin has a narrow therapeutic/toxic ratio and is affected by common genetic polymorphisms. Use of clinical and patient-specific factors such as age, body size, race, concurrent diseases, and medications) explain some of the variability in warfarin dose but genetic factors influencing warfarin response explain a significantly higher proportion of the variability in dose (Wadelius & Pirmohamed, 2007b). The combination of the two CYP2C9 variants (*2 and *3) with the VKORC1 promoter mutation is estimated to account for 40% –

63% of the variability in therapeutic warfarin dose (Rieder *et al.*, 2005; Sconce *et al.*, 2005). Recent genome wide association studies have not only confirmed these observations but also identified a novel association between reduced hepatic CYP4F2, higher levels of hepatic vitamin K, and higher warfarin dose requirements (Singh *et al.*, 2011).

In the present study, allele frequencies for CYP2C9*3 were observed at (23%) and this observation differed from an earlier report in a Ghanaian population by (Kudzi *et al.*, 2009) where no CYP2C9*3 variant alleles were detected. The allele frequency observed for CYP2C9*3 in this study was high compared to frequencies obtained within the African-American and Ethiopian populations which were both 2.3% (Dreisbach *et al.*, 2005; Scordo *et al.*, 2001). This variant was prevalent at 1% in Korean, 2% in Japanese and 5% in Chinese populations (Gaedigk *et al.*, 2001; Yoon *et al.*, 2001) (Table 4.10).

Genotype frequencies for CYP2C9*3 were observed at (10.87%). This was higher than that reported in the Egyptian (0.40%) population (Hamdy *et al.*, 2002). This finding was also different from those reported in Moroccan (2%) and Libyan (3.3%) populations (Nakai *et al.*, 2005).

The CYP2C9*2 genotype and variant alleles were not detected in this study and this was consistent with an earlier report in a Ghanaian population (Kudzi *et al.*, 2009). This finding agrees with those reported in Moroccan and Libyan populations (Nakai *et al.*, 2005) as well as in a Beninese population (Allabi *et al.*, 2003) (Table 4.10). This observation was however different from that reported in the Egyptian (2.43%) population (Hamdy *et al.*, 2002) as well as in an African-American population and Ethiopian populations. The CYP2C9*2 variant

allele was reported at 3.3% in an African American population (Dreisbach *et al.*, 2005) and 4.3% in an Ethiopian population (Scordo *et al.*, 2001). The CYP2C9*2 variant allele has been reported at 10-15% among the Caucasian populations (Allabi *et al.*, 2003) but was absent from Asian populations (Table 4.10).

Allele frequencies for VKORC1_1639A were observed at (6%). This observation is similar to that reported for African-Americans (10.8%) (Scott *et al.*, 2009) but lower than that reported for Asians (66.7%), Caucasians (40.6%), Hispanics (43.6%) (Scott *et al.*, 2010) and Ashkenazi Jewish (46.7%) populations (Scott *et al.*, 2008).

The VKORC1_1639A genotype (A/A) was not detected in this study and this is consistent with a previous study which reported that this genotype is very rare (1%) in Africans (Huang *et al.*, 2009). This observation however differed from that reported in other ethnic populations; African-American (2.0%), Asians (55.9%), Caucasians (17.9%), Hispanics (17.8%) and Ashkenazi Jewish (22.7%) populations (Scott *et al.*, 2008; Scott *et al.*, 2009).

Allele frequencies for CYP4F2 rs2108622 (T) was observed at (41%). This observation is higher than that reported in African-Americans (11.7%), Asians (30.5%), Caucasians (34.2%), Hispanics (23.3%) and Ashkenazi Jewish (32.8%) populations (Scott *et al.*, 2010).

Genotype frequencies for CYP4F2 rs2108622 (T/T) was observed at (6.84%). This observation was lower than that reported for Caucasians (11%), Ashkenazi Jewish (9%) and Asians (9%) populations (Scott *et al.*, 2010) but was higher than that reported in African-Americans (1.3%), and Hispanics (5.3%) populations (Scott *et al.*, 2010).

From the CYP2C9 genotypes, the highest mean daily warfarin dosages in this study were administered to carriers of the heterozygous (*1/*3) genotype, while carriers of the wild-type CYP2C9*1/*1 genotype were treated with lower daily warfarin dosages (6.82 ± 0.56 mg/day) and 5.84 ± 0.25 mg/day respectively (p = 0.169). Carriers of the homozygous mutant (*3/*3) genotype were given intermediate daily warfarin dosages of 6.50 ± 0.67 mg/day. These findings were in contrast with those reported in the Lithuanian population where the highest daily warfarin dosages were given to carriers of CYP2C9 wild-type (*1/*1) genotype, while carriers of the CYP2C9 heterozygous (*1/*3) were treated with lower daily warfarin dosages 5.84 ± 2.84 mg/day versus 4.28 ± 1.92 mg/day (Tatarunas *et al.*, 2011).

The carriers of the homozygous minor (T/T) genotype for CYP4F2, were treated with higher daily warfarin dosages (6.88 ± 0.41 mg/day) than carriers of the heterozygous (C/T) genotype 6.13 ± 0.22 mg/day (p = 0.651). Patients with the homozygous wild-type (C/C) genotype were given intermediate daily warfarin dosages of 6.16 ± 0.55 mg/day. This observation was slightly different from that reported in Asian adult patients where carriers of the heterozygous (C/T) and carriers of the homozygous minor (T/T) genotypes of CYP4F2 required a 25% higher warfarin dosage than carriers of the wild-type (C/C) genotype (Singh *et al.*, 2011).

In this study, the carriers of the homozygous wild-type (G/G) genotype for VKORC1, were given higher daily warfarin dosages (6.14 ± 0.22 mg/day), than carriers of the heterozygous (G/A) genotype (4.97 ± 0.55 mg/day) (p = 0.065). There were no carriers of the homozygous mutant (A/A) genotype in this population. These findings were similar to those reported for the Lithuanian population where higher daily warfarin dosages were administered to carriers of the VKORC1 wild-type (G/G) genotype as compared to carriers of the heterozygous (G/A)

(6.20 ± 2.78 mg/day and 5.60 ± 2.77 mg/day (p = 0.04) respectively. Carriers of the homozygous mutant (A/A) genotype were treated with significantly lower daily warfarin dosages (3.75 ± 1.40 mg/day (*p* = 0.04) than those carrying the wild-type (G/G) genotype (Tatarunas *et al.*, 2011).

Using the combined effect of the CYP2C9, VKORC1 and CYP4F2 genotypes, patients having the wild-type (*1/*1) genotype of CYP2C9 in combination with the homozygous mutant (T/T) genotype of CYP4F2 and the wild-type (GG) genotype of VKORC1 required the highest mean daily warfarin dosage of 7.50 mg/day to achieve the required therapeutic effect. This observation is consistent with previous reports which indicated that carriers of CYP2C9*1/*1 and VKORC1 (G/G) genotypes exhibit low sensitivity to warfarin (Scibona *et al.*, 2012) while carriers of the homozygous mutant (T/T) genotype of CYP4F2 require higher daily warfarin dosages (Singh *et al.*, 2011). This suggests that the standard administration of 5 mg of warfarin to initiate treatment in such patients could lead to hyper-coagulation which may result in thrombotic complications.

It was also observed that patients with a combination of CYP2C9 wild-type (*1/*1), CYP4F2 wild-type (C/C) and VKORC1 wild-type (GG) genotypes were treated with the lowest mean daily warfarin dosage of 4.79mg/day. This also confirms what was reported in other studies that carriers of the wild-type (C/C) genotype of CYP4F2 require 25% lower warfarin dosages than those with either the heterozygous (C/T) or the homozygous mutant (T/T) genotypes (Singh *et al.*, 2011). This suggests that the standard administration of 5 mg of warfarin to initiate treatment in such patients could lead to hypo-coagulation which may cause bleeding complications. The findings further revealed that carriers of CYP2C9 wild-type (*1/*1), who

are heterozygous (C/T) for CYP4F2 and also carry VKORC1 wild-type (G/G) genotypes required an intermediate mean daily warfarin dosage of 5.78mg/day.

5.2 LIMITATIONS

The sample size of 143 may not be large enough to extrapolate findings from this study to cover warfarin dose / response for the entire Ghanaian population. This limitation was as a result of lack of relevant resources and the limited time frame within which this thesis has to be submitted for examination.

5.3 CONCLUSIONS

The current study has led to the determination of allelic variants of CYP2C9, VKORC1 and CYP4F2 in a Ghanaian population. With the exception of CYP2C9 which has already been reported in a Ghanaian population by a previous study, VKORC1 and CYP4F2 variant alleles to our knowledge are being reported for the first time among the indigenous Ghanaian population.

This study has also established for the first time, the combined effect of genotypes of CYP2C9, VKORC1 and CYP4F2 genes on mean daily warfarin dosage where carriers of the wild-type genotypes of CYP2C9 (*1/*1) and VKORC1 (G/G) together with the homozygous mutant (T/T) genotype for CYP4F2 required the highest mean daily warfarin dosages of 7.50 mg/day at 95% CI (7.50-7.50), $p = 0.096$, compared to those with the wild-type genotypes of all three genes who required 4.79 mg/day at 95% CI (3.02-6.55), $p = 0.096$. Patients who had the heterozygous (C/T) genotype for CYP4F2 in addition to the wild-type genotypes of

CYP2C9 and VKORC1 were given an intermediate daily warfarin dosage of 5.78 mg/day at 95% CI (5.10-6.45), $p = 0.096$.

Also, carriers of the heterozygous genotypes of CYP2C9 (*1/*3), and CYP4F2 (C/T) and the wild-type (G/G) genotype of VKORC1 were given 6.50 mg/day at 95% CI (5.40-7.60), p = 0.027. Subjects with a combination of CYP2C9*3/*3, (C/T) for CYP4F2, and (G/G) for VKORC1 genotypes were given 6.67 mg/day at 95% CI (5.00-8.33).

5.4 RECOMMENDATIONS

It is recommended that this study be carried out with a larger sample size so that the correlations established can reflect that of the larger Ghanaian population. It is possible that there may be other mutations in the genes genotyped in this study which may have some effect on warfarin dose / response in the Ghanaian population. It is therefore recommended that further investigation be carried out to ascertain this.

These results provide additional information on polymorphisms of CYP2C9, CYP4F2, and VKORC1 subfamily of enzymes in an indigenous African population which is scarce in published literature and can be added to Pharmacogenetics for Every Nation Initiative (PGENI) database. PGENI has been set-up with the main objective of integrating pharmacogenetics into public health care for all global populations and aims to determine the baseline frequencies of known DNA variants present in 154 genes which are involved with the action of 206 drugs.

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APPENDIX I

PRIMER SEQUENCES, AMPLICON SIZES AND RESTRICTION ENZYMES USED

CYP2C9 specific primer sequences				
Primers	**Sequence 5'-3'**	**Detection of allelic variant**	**Amplicon size**	**Restriction Enzyme**
F_a	CACTGGCTGAAAGAGCTAACAGAG	CYP2C9*2	375 bp	*Ava* II
R_a	GTGATATGGAGTAGGGTCACCCAC			
F_b	TGCACGAGGTCCAGAGGTAC	CYP2C9*3	105 bp	*Kpn* I
R_b	ACAAACTTACCTTGGGAATGAGA			

CYP4F2 specific primer sequences				
Primers	**Sequence 5'-3'**	**Detection of allelic variant**	**Amplicon size**	**Restriction Enzyme**
F_a	CGGAACTTGGACCATCTACA	*CYP4F2*rs2108622	439bp	*Pvu*II
R_a	CCTACTCTCCCACAGGCATTA			

VKORC1 specific primer sequences				
Primers	**Sequence 5'-3'**	**Detection of allelic variant**	**Amplicon size**	**Restriction Enzyme**
F_a	ATCCCTCTGGGAAGTCAAGC	*VKORC1*_1639G>A	636bp	*Nci* I
R_a	CACCTTCAACCTCTCCATCC			

APPENDIX II

COMMON POTENTIAL DRUG-DRUG INTERACTIONS WITH WARFARIN

The following drugs may increase INR (i.e. a lower warfarin dose may be necessary):

Amiodarone

Certain Antibiotics (TMP-SMZ/Bactrim, Metronidazole)

Antifungals (fluconazole, miconazole, voriconazole)

Antiretrovirals (delaviradine,efavirenz)

Cimetidine

Corticosteroids

Fibrates

Griseofulvin

Isoniazid

Leflunomide (Arava)

Mifepristone

Orlistat (Alli, Xenical)

PPIs (e.g. omeprazole)

Statins (e.g. simvastatin)

Tamoxifen

Thyroid replacement

Tramadol (Ultram)

The following drugs may decrease INR (i.e. a higher warfarin dose may be required):

Antacids

Certain Antibiotics (dicloxacillin, nafcillin)

Anticonvulsants (eg phenytoin)

Barbiturates

Bile acid resins

Cyclosporine

Rifampin

Con-commitant use of the following drugs may increase bleeding risk on warfarin:

Anti-depressants (e.g. SSRIs)

Antiinflammatories (note: celecoxib may still cause bleeding but may be an option instead of other NSAIDs)

Antiplatelet agents (e.g. aspirin/salicylates, clopidogrel (Plavix), prasugrel (Effient), aspirin/extended-release dipyridamole (Aggrenox)

Other anticoagulants (e.g. heparin, LMWH, fondaparinux).

APPENDIX III

CONSENT FORM

CYP2C9, VKORC1 AND CYP4F2 VARIANT FREQUENCIES IN PATIENTS ON EITHER LOW OR HIGH STABLE WARFARIN MAINTENANCE THERAPY IN GHANAIAN POPULATION.

Warfarin is an anti-clotting drug for reducing thromboembolic events such as stroke, deep vein thrombosis, pulmonary embolism and other serious coronary malfunctions. Warfarin has been used for more than 50 years, with varied degree of anticoagulation activity. An appropriate warfarin dose in one patient can induce a bleeding event in another. As a result of this variation, some patients are put on a low warfarin dose while others are on a high warfarin dose. Variation in warfarin dose in patients has been associated with genetic variations of patients among Caucasians and other ethnic populations. Little information exists on indigenous Africans populations and no data is available on Ghanaian populations.

This study will take a small amount of blood (5ml) from you as a patient on warfarin by inserting a needle in your forearm. The risk involved in this procedure is negligible and it will cause only minimal pain and bruising. The sample will be used to determine the genetic variations of CYP2C9, VKORC1, and CYP4F2 genes and the International Normalized Ratio (INR), which gives an indication of the time it takes for your blood to clot. No other tests will be conducted on your blood sample. Results of this study may help Clinicians treat Patients on warfarin in Ghana better. All information gathered will be treated in strict confidentiality.

It will be appreciated if you agree to take part in this study. You may choose not to take part in the study however; this will not affect your medical care in this Clinic.

If you have any problems or further questions, please contact: Dr. William Kudzi of the University of Ghana Medical School. Mobile: 0246703400

Consent:

I……..of …….....

……give my consent to the research procedures above, the nature, purpose and possible consequences of which have been described to me

By……

Patient's signature…….Date……..

Doctor's signature……

APPENDIX IV

DATA COLLECTION SHEET

This form should be filled by a Clinician on duty or the MPhil Student for each patient enrolled in the warfarin study.

A. DEMOGRAPHY OF PATIENT

Participant ID: [ ] [ ] [ ] **Date:** [ ] [ ] / [ ] [ ] / [ ] [ ] [ ] [ ]

1) Please indicate the current age of patient (Yrs)……

2) Please indicate the sex of the patient ….. ……..Male ☐

Female ☐

3) Please describe the patient's ethnic group:

Akan ☐ Ga/Dangbe ☐ Ewe ☐

Mole ☐ Dagbani ☐ Guan ☐

Hausa ☐ Gruma ☐ Grussi ☐

Other

(specify)……

CLINICAL DATA OF PATIENT

4) Please record the Main Diagnosis of the patient ……

……

(a) Please indicate whether the patient takes any other medications aside warfarin?

Yes…..……☐

No……..☐

(b) If yes, please name the drug (s) and indicate their duration of use ……

……

5) Please indicate how long the patient has been on warfarin medication. ……

6) Please record the patient's daily warfarin dosage ……

7) (a) Please indicate if the patient has ever experienced any adverse effects after taken his/her warfarin medication? Yes……..……☐

No ……☐

(b) If yes, what adverse effects did he/she experienced?……..

8) (a) Please indicate if the patient eats a lot of Vegetables (such as kontonmire, garden eggs, cabbage, lettuce, etc)? Yes….……☐

No……..……☐

(b) If yes, how often does the patient eat these vegetables? Daily ☐ Weekly ☐ Monthly ☐

(a) Please indicate if the patient takes fruit juice (such as orange juice etc)?

Yes……☐

No……☐

(b) If yes, how often does the patient drink these fruit juices? Daily ☐ Weekly ☐ Monthly ☐

9) Folder Study

Height (Cm)		BMI:		
Weight (Kg)				
		INR	INR	

APPENDIX V

REAGENTS AND MATERIALS USED

Reagents:

1. Agarose
2. PCR tubes (0.2ml)
3. Eppendorf tubes (2.5ml)
4. Microcentrifuge tube (0.5ml)
5. Ethidium bromide
6. dNTPs (deoxynucleoside triphosphates)
7. Taq Polymerase
8. 10X PCR Buffer with 15mM $MgCl_2$
9. TAE Buffer (50X)
10. Loading Dye
11. DNA ladder (100bp)
12. Nuclease free water
13. Distilled Water
14. 500g Tris base {Tris (hydroxymethyl)-aminomethan. Aminomethylidintrimethanol $C_4H_{11}NO_3$
15. Glacial acetic acid
16. 0.5M EDTA pH 8.0
17. ddH_2O
18. NaOH
19. EDTA powder

Equipments:

1. Micro Pipettes (0.5-10µl, 10-100µl, 100-200µl, 200-1000µl)
2. Microwave Oven
3. Electrophoretic tray & tanks
4. Autoclave
5. Analytical Balance
6. KODAK Gel Logic 200 Imaging Systems
7. pH Meter
8. Glass stirrer
9. Spatula

APPENDIX VI

PREPARATION OF REAGENTS

Primer stock solutions

CYP2C9*2

Amount of substance, n (CYP2C9-Fa) = 153.4 nMoles

Converting to µMoles gives $153.4 \times \frac{1}{1000} = 0.1534$ µMoles

To prepare a 100µM stock primer solution, volume of water required V (L) = n / C

= 0.1534 / 100 = 0.001534L or 1534µl. Thus, 1534 µl of nuclease free water was added to the lyophilized primer content to make a 100 µM stock primer solution.

Amount of substance, n (CYP2C9-Ra) = 151.9 nMoles

Converting to µMoles gives $151.9 \times \frac{1}{1000} = 0.1519$ µMoles

To prepare a 100µM stock primer solution, volume of water required V (L) = n / C

=0.1519 / 100 = 0.001519L or 1519µl. Thus, 1519 µl of nuclease free water was added to the lyophilized primer content to make a 100 µM stock primer solution.

CYP2C9*3

Amount of substance, n (CYP2C9-Fb) = 163.1 nMoles

Converting to µMoles gives $163.1 \times \frac{1}{1000} = 0.1631$ µMoles

To prepare a 100µM stock primer solution, volume of water required V (L) = n / C

= 0.1631 / 100 = 0.001631L or 1631µl. Thus, 1631 µl of nuclease free water was added to the lyophilized primer content to make a 100 µM stock primer solution.

Amount of substance, n (CYP2C9-Rb) = 164.1 nMoles

Converting to µMoles gives $164.1 \times \frac{1}{1000} = 0.1641$ µMoles

To prepare a 100µM stock primer solution, volume of water required V (L) = n / C

= 0.1641 / 100 = 0.001641L or 1641µl. Thus, 1641 µl of nuclease free water was added to the lyophilized primer content to make a 100 µM stock primer solution.

CYP4F2

Amount of substance, n (CYP4F2-Fa) = 168.1 nMoles

Converting to µMoles gives $168.1 \times \frac{1}{1000} = 0.1681$ µMoles

To prepare a 100µM stock primer solution, volume of water required V (L) = n / C

= 0.1681 / 100 = 0.001681L or 1681µl. Thus, 1681 µl of nuclease free water was added to the lyophilized primer content to make a 100 µM stock primer solution.

Amount of substance, n (CYP4F2-Ra) = 196.9 nMoles

Converting to µMoles gives $196.9 \times \frac{1}{1000} = 0.1969$ µMoles

To prepare a 100µM stock primer solution, volume of water required V (L) = n / C

= 0.1969 / 100 = 0.001969L or 1969 µl. Thus, 1969 µl of nuclease free water was added to the lyophilized primer content to make a 100 µM stock primer solution.

VKORC1

Amount of substance, n (VKORC1-Fa) = 151.5 nMoles

Converting to µMoles gives $151.5 \times \frac{1}{1000} = 0.1515$ µMoles

To prepare a 100µM stock primer solution, volume of water required V (L) = n / C

= 0.1515 /100 = 0.001515L or 1515 µl. Thus, 1515 µl of nuclease free water was added to the lyophilized primer content to make a 100 µM stock primer solution.

Amount of substance, n (VKORC1-Ra) = 193.3 nMoles

Converting to µMoles gives $193.3 \times \frac{1}{1000} = 0.1933$ µMoles

To prepare a 100µM stock primer solution, volume of water required V (L) = n / C

= 0.1933 / 100 = 0.001933L or 1933 µl. Thus, 1933 µl of nuclease free water was added to the lyophilized primer content to make a 100 µM stock primer solution.

NB: All the stock solutions prepared were stored at -20^0C for future use.

Working Primer solutions

To prepare 20µM working primer solution from the 100µM stock primer solution in a final volume of 200µl for each of the genes listed above, the dilutions formula below was used.

Stock concentration (C_1) x Volume of stock concentration required to prepare working concentration (V_1) = Working concentration (C_2) x Volume of working concentration needed (V_2). Thus, $C_1V_1 = C_2V_2$

C_1 = 100 µM, V_1 =? C_2 = 20 µM, V_2 = 200µl; $V_1 = C_2V_2 / C_1$

V_1 = 20 µM x 200 µl / 100 µM = 40 µl

Therefore, 160 µl of nuclease free water was added to an empty 2ml eppendorf tube and 40 µl of primer stock was added to make a 20 µM primer working solution.

Preparation of dNTP Set

To prepare 10mM dNTP set from 100mM dNTP stock solution in a final volume of 200µl, the dilutions formula $C_1V_1 = C_2V_2$ was used. C_1 = 100mM, C_2 = 10mM, V_2 = 200µl

$V_1 = C_2V_2 / C_1$ Hence V_1 = 10mM x 200 µl / 100mM = 20µl.

Therefore 20 µl of each dNTP (dATP, dCTP, dGTP, dTTP) was added to separate empty eppendorf tubes and 180µl of nuclease free water was added to make a 10mM dNTP set in a final volume of 200µl.

Preparation of 0.5M Ethylenediaminetetra acetic acid (EDTA)

The analytical balance was used to weigh 29.23g of the EDTA powder (FW = 292.25) into a clean glass bottle and 200 ml of distilled water was added to dissolve content. The pH of this solution was adjusted to 8.0 using concentrated NaOH solution.

Preparation of 50X Tris-acetate-EDTA (TAE) Buffer Stock

The analytical balance was used to weigh 242g of Tris base into a clean glass bottle and 800 ml of ddH_2O was added and dissolved by stirring. Then 57.1 ml of glacial acetic acid and 100

ml of 0.5M EDTA was added and mixed. This was followed by the addition of 800 ml ddH_2O to a final volume of 1 L. Finally, the stock buffer was sterilized by autoclaving.

Preparation of 1X TAE Buffer from 50X TAE Buffer Stock

Twenty milliliters of the stock buffer was measured in a 100ml measuring cylinder and poured into a 1 L volumetric flask and 980 ml of double distilled water was used to make it up to the 1 L mark. The flask was then stoppered and inverted several times to mix after which the buffer is stored away in a clean glass bottle at room temperature for future use.

Two Percent (2%) Agarose Gel Preparation and Casting

The analytical balance was used to weigh 2g of agarose powder into a heat resistant bottle and 100 ml of 1X TAE buffer was added and mixed. The solution was then heated in a microwave oven to melt the agarose and then allowed to cool down to just above room temperature. Two microliters of ethidium bromide was added and mixed and the resultant solution was poured into a gel casting tray with combs inserted to create wells.