The role of warfarin pharmacogenomics on the time it takes to reach stable therapeutic International Normalized Ratio (INR) and on warfarin dose required to maintain stable therapeutic INR in Black African and Mixed Ancestry South Africans: a focus on *CYP2C9* and *VKORC1*

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DECLARATION

I Edson Makambwa hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Preamble

This thesis, as part of the MMED, is organized in the form of two major chapters.

Chapter 1 is an introduction and review of the literature relevant to the topic. The chapter provides the opportunity to review the basics of pharmacogenomics and the basic principles of pharmacokinetics and pharmacodynamics. It also reviews the metabolism, indications and complications of warfarin, the potential role of genetic polymorphisms on dosing and the trial evidence of pharmacogenomic driven warfarin prescribing algorithms and their limitations. These identified gaps and limitations help inform the rationale for conducting the study.

Chapter 2 is presented as a journal ready manuscript for OMICS: Journal of Integrative Biology, thus the Abstract and Chapter 2 are presented in the format required by the Editorial team of OMICS: Journal of Integrative Biology. Chapter 2 is expected to be submitted for publication.

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Abbreviations

APOE – ApolipoproteinE

BA - Black Africans

CALU - CALUMEN

CYP1A1 - Cytochrome P450 Family 1 Subfamily A Member 1

CYP1A2- Cytochrome P450 Family 1 Subfamily A Member 2

CYP2B6- Cytochrome P450 Family 2 Subfamily B Member 6

CYP2C8 - Cytochrome P450 Family 2 Subfamily C Member 8

CYP2C9 – Cytochrome P450 Family 2 Subfamily C Member 9

CYP2C19 - Cytochrome P450 Family 2 Subfamily C Member 19

CYP2D6 - Cytochrome P450 Family 2 Subfamily D Member 6

CYP3A4 - Cytochrome P450 Family 3 Subfamily A Member 4

CYP4F2 - Cytochrome P450 Family 4 Subfamily F Member 2

EDTA – Ethylene Diamine Tetra Acetic acid

ESR - Estrogen receptor

G6PD - Glucose 6 phosphate dehydrogenase

GGCX – Gamma Glutamyl Carboxylase

HIV – Human Immunodeficiency Virus

HLA- Human leucocyte antigen

HWE – Hardy Weinberg Equilibrium

INR – International Normalized Ratio

MA - Mixed Ancestry

NAT- N-acetyltransferase

no. - Number

SNPs – Single Nucleotide Polymorphisms

TPMT - Thiopurine methyl-transferase

VKORC1 – Vitamin K epoxide Reductase Complex subunit 1

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Abstract

Warfarin, the most commonly prescribed anticoagulant, is principally metabolized by cytochrome P450 2C9 which functions by inhibiting the Vitamin K epoxide reductase. Genes *CYP2C9* and *VKORC1* code for these two proteins, respectively. *CYP2C9* and *VKORC1* exhibit genetic polymorphisms that have been shown to affect warfarin response and favorably facilitate warfarin dosing and improve clinical outcomes. However, none of these studies have involved populations from sub-Saharan Africa where the potential benefit of optimal dosing and reduced complications is greatest. Therefore, the thesis describes a study designed to investigate the role of genetic variations in *CYP2C9* and *VKORC1* on the time taken to reach a stable therapeutic international normalized ratio (INR) and warfarin dose required to maintain a therapeutic INR.

This was a cross-sectional study of patients on warfarin to determine the relationship between genetic polymorphism in *CYP2C9* and *VKORC1* amongst black and mixed ancestry South Africans and clinical surrogates of warfarin metabolism. Medical records were accessed to determine time to INR and warfarin doses. DNA was extracted from blood samples, and genotyping for polymorphism in *CYP2C9* (*2,*3,*8,*11) and *VKORC1* (*1173C>T, 1639G>A, 3730G>A*) was accomplished by PCR-RFLP, Sanger sequencing and iPlex Mass Sequencing.

Our results show that the genetic profile of *CYP2C9* and *VKORC1* differs between Black Africans (BA) and their Mixed Ancestry (MA) counterparts. *VKORC1-1639AA* genotype was observed at frequencies of 0.11 and 0.01 in the MA and BA, respectively. Time to stable INR was not influenced by *CYP2C9* and *VKORC1*. Furthermore, compared to known genetic polymorphisms in these genes from population out of Africa, both qualitative and quantitative differences were observed. Finally, we found that *VKORC1* genetic variation significantly affected the doses of warfarin in MA but had no effect in BA. These results suggest that further research in this area is warranted, and that it will be important to include populations from sub-Saharan Africa in future if the potential to develop personalized algorithms which integrate pharmacogenomics to assist with effective warfarin dosing and prevention of warfarin related complications is to be realized.

Keywords: Pharmacogenomics, variants, genotype, allele, Mixed Ancestry, Africa

CHAPTER 1: STRUCTURED LITERATURE REVIEW

Words 4852

1. Introduction

1.1 Pharmacogenomics

Pharmacogenomics deals with pharmacologic responses and their modification by hereditary factors (Kalow, 2005; Suarez-Kurtz and Botton, 2013). The primary focus of pharmacogenomics is to identify genetic variants that affect drug efficacy and safety by altering pharmacokinetics and pharmacodynamics (Relling and Evans, 2015; Mpye et al., 2017). These genetic variants, which are primarily in germline DNA are either inherited or result from spontaneous sequence changes. Pharmacogenomic effects can be investigated or reported at two broad levels: pharmacokinetics (how the drug is absorbed, distributed, metabolized and eliminated) and pharmacodynamics (the relationship between the concentration of a drug at its target and the pharmacological effects) (Relling and Evans, 2015). Pharmacokinetics and pharmacodynamics variation between individuals should be considered when assessing drugs for safety, dose and efficacy (Pirmohamed et al., 2013). This knowledge of inter-individual variability, clinical, environmental and lifestyle factors in response to standard therapy are of paramount importance in clinical practice as it leads to different treatment outcomes and adverse events (Kudzi et al., 2011). The ultimate aim is to improve efficacy and reduce significant adverse events. The human genome project and recent progress in genome characterization technology and analytical skills through bioinformatics have accelerated pharmacogenomic discoveries and translation into clinical practice (Relling and Evans, 2015).

Translation of pharmacogenomic knowledge to bedside use in developed world has assisted in the way countries respond to population specific diseases (Pang et al., 2009). In contrast, implementation has been slow in Africa due to various reasons such as lack of state-of-the-art facilities such as molecular biology and next generation sequencing techniques, lack of collaboration amongst established research facilities and scanty expert human resource. Most of the translated pharmacogenomic knowledge involve the genetic variants of genes coding for *CYP* enzymes (Dandara et al., 2014). Some of applications of the pharmacogenomic knowledge are shown in Table 1.1

Table 1.1 Application of pharmacogenomic knowledge (Adapted from Mpye et al. 2017 with changes)

Therapeutic area	**Drug**	**Biomarker**
Infectious diseases		
Anti-retroviral therapy	Abacavir	HLA-B
Anti-retroviral therapy	Efavirenz	*CYP2B6, CYP1A2, CYP3A4*
Anti-TB drug	Rifampicin	*CYP3A4*
Anti-TB drug	Isoniazid	NAT2
Anti-malarial drug	Chloroquine	G6PD, *CYP2D*6
Anti-malarial drug	Artemether	*CYP2C8, CYP3A4,CYP2B6*
Anti-malarial drug	Quinine	G6PD, *CYP2D6*
Cardiology		
Anti-failure therapy	Metoprolol	*CYP2D6*
Anti-failure therapy	Carvedilol	*CYP2D6*
Antiplatelet therapy	Clopidogrel	*CYP2C19*
Anticoagulant	Warfarin	*CYP2C9, VKORC1*
Psychiatry		
Antidepressant	Amitriptyline	*CYP2D6*
Antidepressant	Citalopram	*CYP2C19, CYP2D6*
Antipsychotic therapy	Clozapine	*CYP2D6*
Neurology		
Antiepileptic drug	Carbamazepine	HLA-B
Oncology		
Anticancer drug	Tamoxifen	ESR1, *CYP2D6*
Anticancer drug	Azathioprine	TMPT

Other important examples of the implementation of pharmacogenetics include the area of anticoagulation and immunosuppression. The variation in *VKORC1* and *CYP2C9* genes with respect to warfarin has been widely studied and genotype based algorithms for warfarin are now available for clinical use (Cavallari et al., 2013; Pirmohamed et al., 2013; Drozda et al., 2015). Furthermore, in order to reduce the risk of hematopoietic

toxicity, the dosage of thiopurine medications (mercaptopurine and azathioprine) can now be adjusted based on thiopurine methyl transferase (TPMT) genetic results (Relling and Evans, 2015).

The multi-gene panels or drug metabolising enzymes and transporters chips that analyse identified genes that are crucial in pharmacokinetics and pharmacodynamics of drugs do not cover African specific variants (Matimba et al., 2016). Despite this, a few examples of the potential for successful pharmacogenomics implementation in Africa do exist particularly in the sphere of anti-retroviral therapy. Human leukocyte antigen *(HLA) B*5701* allele screening for susceptibility to abacavir hypersensitivity was proven to be effective in preventing drug exposure with the potential to prevent severe hypersensitivity reactions. Similarly, the evaluation of efavirenz with regard to the role played by *CYP2B6* gene variation has resulted in the dose being lowered in patients who are Efavirenz sensitive (Matimba et al., 2016).

Pharmacogenomic studies with a focus on black and mixed ancestry Africans, by potentially optimizing dosing and reducing toxicity may be of particular benefit in sub-Saharan Africa given the limited facilities and capacity to monitor patients for toxicity and complications of drugs such as warfarin.

1.2 Pharmacodynamics and pharmacokinetics

1.2.1 General overview of pharmacodynamics and pharmacokinetics

The time course from absorption, distribution, metabolism and excretion of a drug describes its pharmacokinetics. Absorption, which is the migration of a drug from site of administration to the systemic circulation is assessed by bioavailability (fraction of drug reaching systemic circulation). The rate of drug movement is directly proportional to the concentration gradient across the membrane. A number of factors such as first pass metabolism (extraction of drug by liver before reaching systemic circulation), splanchnic circulation, gastric pH, drug-drug interaction determine the bioavailability. Acidic drugs are distributed bound to albumin, while on the other hand, basic drugs are distributed bound to alpha 1 glycoproteins. Only the unbound drug (free drug) has the physiologic effect. Metabolism terminates the action of the drug by creating water soluble metabolites suitable for excretion. The liver plays a crucial role in metabolism of drugs. There are two main types of reactions involved in metabolism of drugs; phase

1 oxidative reactions mediated by *CYP* isoenzymes and phase 2 conjugation reactions like glucuronidation. Water soluble metabolites are excreted through the biliary system, faeces, respiratory system and the kidneys. The kidneys play a major role in elimination of drugs through glomerular filtration and tubular secretion. The half-life of a drug is the time it takes for half of the drug concentration to be metabolised and eliminated. The steady state is reached when the amount of drug taken is in dynamic equilibrium with the drug eliminated. Usually it takes 4 to 5 times the half-life after regular dosing is started to reach steady state (Takimoto, 2001; Urso et al., 2002; Feucht and Patel, 2011; Currie, 2018).

The relationship between concentration of a drug at its site of action and the response elicited (therapeutic or adverse event) is referred to as pharmacodynamics. The drug response follows a sequence of steps initiated by the drug's binding with the receptor followed by signal transduction. Receptors can be ligand gated ion channels, G protein coupled receptors, ligand gated transmembrane proteins or even intranuclear receptors. The ability of a drug to bind to the receptor is its affinity, whereas its ability to elicit a response is its efficacy. The amount of a drug required to produce a certain amount of a response is called potency. Drugs that have got affinity for receptors and produce full effect are agonists whereas antagonists have affinity for a receptor but no efficacy. Establishment of dose- response relationship allows outcomes to be measured. The resultant effect is determined by sensitivity and specificity of the receptor, concentration of the drug at the receptor, density of receptors and regulatory factors that modify gene transcription and translation. (Takimoto, 2001; Feucht and Patel, 2011; Currie, 2018).

The pharmacokinetic and pharmacodynamic properties of a drug are influenced by genetic variation, clinical, environmental and lifestyle factors like age, weight, health status, alcohol consumption, renal failure, liver cirrhosis. Thus, these factors need to be considered if optimal drug response is to be accomplished (Pirmohamed et al., 2013; Relling and Evans, 2015).

1.2.2 Pharmacodynamics and pharmacokinetics of Warfarin

Warfarin is a coumarin anticoagulant that works by inhibiting vitamin K epoxide reductase (VKOR) encoded by vitamin K epoxide reductase complex subunit 1(*VKORC1*) gene (Li et al., 2004; Limdi and Veenstra, 2008). Warfarin's anticoagulant

effect is as a result of preventing regeneration of reduced Vitamin K from the epoxide form (Natarajan et al., 2013). Reduced vitamin K is an essential cofactor for gamma-glutamyl carboxylase (GGCX), an enzyme responsible for post translational gamma carboxylation of vitamin K dependent clotting factors II, VII, IX and X (Figure 1), thus it prevents maturation of vitamin K dependent clotting factors leading to reduced coagulation (Dahlback, 2005). Calumenin (CAL), an endoplasmic reticulum chaperone protein inhibits gamma-carboxylase (*GGCX*)(Wajih et al., 2004). Genetic variation in *VKORC1* or *GGCX* leads to inter individual variation in the effective warfarin dosing (Luxembourg et al., 2011).

Warfarin is a racemic mixture of R and S enantiomers with the S enantiomer being fivefold more potent (Hirsh et al., 2001; Ageno et al., 2012). S-warfarin is metabolized primarily by *CYP2C9* while R-warfarin is metabolized by *CYP3A4*, *CYP1A2*, *CYP1A1* (Redman, 2001) (Figure 1). CYP4F2 metabolize reduced vitamin K to hydroxyvitamin K which is inactive (Hirai et al., 2013). Genetic variations in *CYP2C9*, *CYP4F2*, *CYP3A4*, *CYP1A2* and *CYP1A1* lead to inter individual variation in the effective warfarin dosing (Kaminsky and Zhang, 1997; Thijssen et al., 2000; Hirai et al., 2013). In this study, we focused on two genes namely: *VKORC1* and *CYP2C9*.

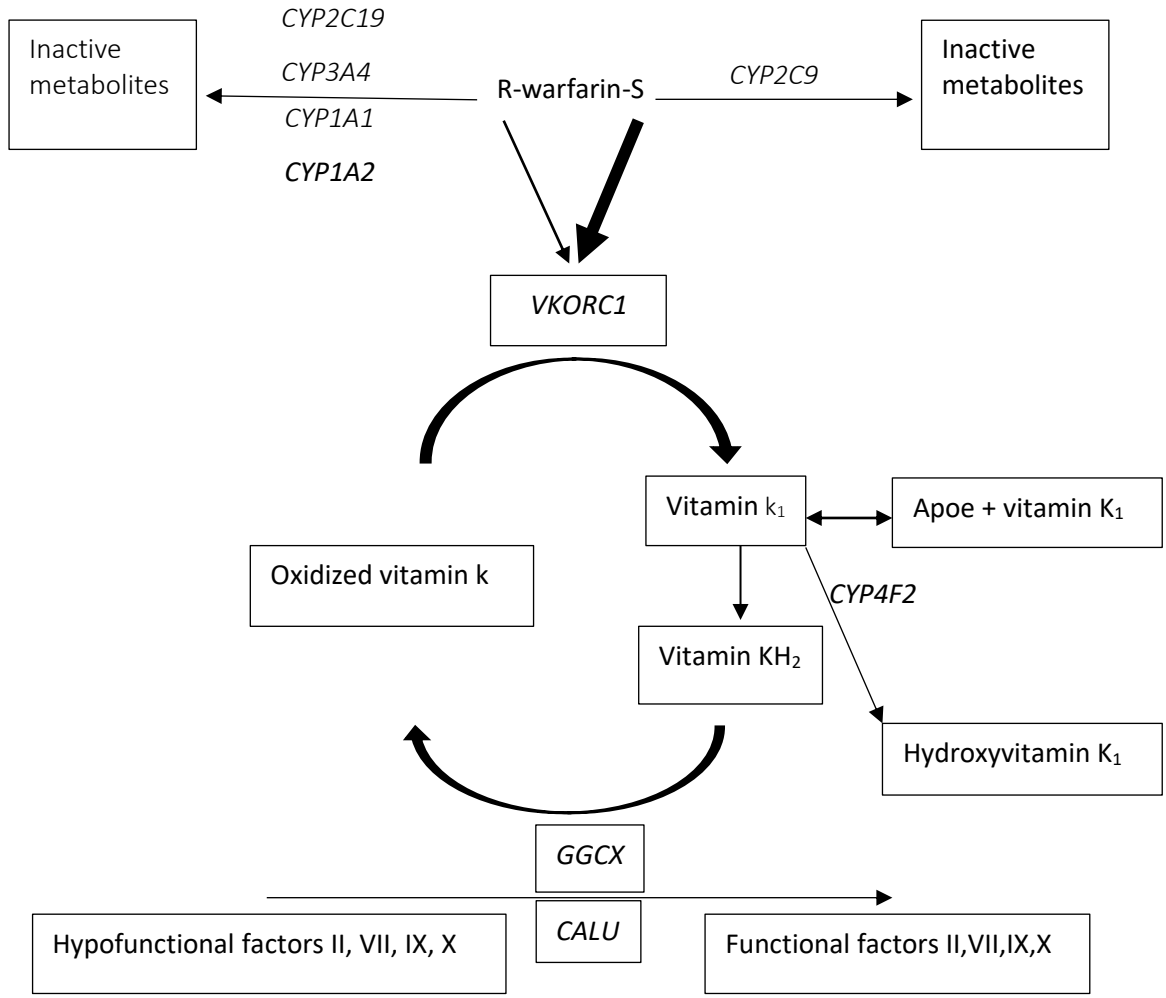

Figure 1 Warfarin interactive pathway. (Adapted from Suarez-Kurtz and Botton, 2013, with modifications)

1.3 Genetic polymorphisms of *CYP2C9* and their effects on warfarin dosing

CYP2C9 is a highly polymorphic gene with more than 50 allelic variants; it is mapped to the long arm of chromosome 10 (Ansell et al., 2008; Sim and Ingelman-Sundberg, 2010). CYP2C9 being the major enzyme responsible for metabolism of S-warfarin (Van Booven et al., 2010) accounts for at least 10% of variation in warfarin dose (Verhoef et al., 2014). Variant alleles result in different enzymatic activities (Yin and Miyata, 2007). *CYP2C9* allelic variants vary according to racial and ethnic differences (Allabi et al., 2003; Suarez-Kurtz and Botton, 2013). *CYP 2C9*2* allele frequency ranges from 11- 20% in Caucasians yet its only 1% in African Americans (Limdi and Veenstra, 2008). *CYP2C9*2* has not been detected in South African population (Dandara et al., 2011; Mitchell et al., 2011). *CYP2C9*3* variant allele has a frequency

of 7- 8.5% in Caucasians (Yasar et al., 1999; Limdi et al., 2007) with catalytic activity reduced by 80% (Crespi and Miller, 1997; Takanashi et al., 2000). Functional effects of *CYP2C9* polymorphisms are summarized in Table 1.2. *CYP2C9*8* and *CYP2C9*11* alleles were detected in Blacks (Allabi et al., 2003; Limdi et al., 2007) and Black South African women (Mitchell et al., 2011). The *CYP2C9*8* variant allele is more frequent in Africans at 5.6% compared to 0.03% in Europeans with reduced activity towards warfarin in both races (Daly et al., 2017). The *CYP2C9*11* variant allele has reduced activity towards warfarin with a frequency of 2.1% and 0.2% in Africans and Europeans respectively (Daly et al., 2017).

T**able 1.2** *CYP2C9* polymorphisms with functional effects. Adapted from (Yin and Miyata, 2007) with changes.

Alleles	Nucleotide change in cDNA	Enzymatic activity
*CYP2C9*2*	*430C>T*	Decrease. 30-50% lower turnover of warfarin
*CYP2C9*3*	*1075A>C*	Decrease. 90% lower turnover of -warfarin
*CYP2C9*8*	*449G>A*	2-fold increase
*CYP2C9*11*	*1003C>T*	Decrease. A modest decrease

1.4 Genetic polymorphism of VKORC1 and their effects on warfarin dosing

VKORC1 gene is located on chromosomes 16p11.2 and spans about 52 base pairs encompassing 3 exons and 2 introns (Li et al., 2004; D'Andrea et al., 2005). *VKORC1 c.1173, c.3730* and *c.1639* are the most common single nucleotide polymorphisms (Yang et al., 2010). Promoter region of single nucleotide polymorphism (SNP) *VKORC1 1639 g-G>A* accounts for 20-35% inter individual difference (Takahashi et al., 2006; Dean, 2012). *VKORC1 1173 g-C>T* genotypes differ significantly among Asians, Caucasians and African Americans, with a frequency of 11%, 58% and 91% of *VKORC1 c.1173T* allele respectively (Kovac et al., 2010; Moon et al., 2011). Table 1.3 shows allelic frequencies of *VKORC1* SNPs among different African populations. Homozygous *VKORC1 1639AA, 1173TT* are associated with lower doses of warfarin (Jia et al., 2017). The polymorphism in *VKORC1 c.-1639G>A* results in changes on the binding site for VKORC1 promoter region with the resultant effect of lower *VKORC1*

mRNA levels. This causes the person carrying this variant to be warfarin sensitive as a result of reduced levels of *VKORC1* in the liver (Linder et al., 2009).

Table 1.3: Allele frequency of *VKORC1* polymorphisms in selected African populations (Adapted from Zinhle Cindi's MSc (Med) Human genetics, University of Cape Town- November 2017)

Population	**c.-1639A**	**c.1173T**	**c.3730A**	**Reference**
Mozambican	0.035	N/A	0.389	Suarez-Kurtz et al., 2010
South African	N/A	0.04	0.40	Dandara et al., 2011
Angolan	0.027	N/A	0.404	Suarez-Kurtz et al., 2010

1.5 Warfarin use as anticoagulant

Warfarin is the most commonly prescribed anticoagulant worldwide since its introduction in 1950s (Hirsh et al., 2001; Cho et al., 2016). It is inexpensive, and effective (despite its narrow therapeutic window) and therefore, remains the anticoagulant of choice in sub-Saharan Africa (Stambler and Ngunga, 2015). Annually, more than 30 million prescriptions of warfarin are issued in USA (Kirley et al., 2012) whereas in South Africa, just over 400 thousand prescriptions are dispensed (Blaauw, 2012). Warfarin is taken orally and has a long half-life and narrow therapeutic range thus requiring frequent monitoring by measuring prothrombin time that is expressed as the international normalized ratio (INR) (Higashi et al., 2002; Oden et al., 2006). As a monitoring index, INR enables comparison and exchange of information in research as well as between laboratories (Kamali and Wynne, 2010; Wells et al., 2014). To minimize the adverse events such as bleeding and clot formation, the INR has to be held in the range 2-3.5 depending on the indication (Hirsh et al., 2001; Sonuga et al., 2016).

1.6 Indications for warfarin therapy

Warfarin therapy is used for prevention of systemic thrombo-embolization in patients at high risk such as atrial fibrillation, prevention of thrombotic complications such as valve thrombosis in those with mechanical valves and as treatment for established

venous thromboembolism and its complications such as deep vein thrombosis and stroke (Daly and King, 2003; Ageno et al., 2012; Lee and Klein, 2013).

In the developed world, atrial fibrillation is the most common indication. The prevalence of atrial fibrillation is higher than that in Africa because of the increasing aging population (Fuster et al., 2011). It is estimated that by 2050, atrial fibrillation will be higher in Africa than the rest of the world (Rahman et al., 2014). In sub-Saharan Africa, there is high burden of valvular atrial fibrillation as a result of the high incidence of rheumatic heart disease (Bloomfield et al., 2013). It has been observed that ischemic strokes secondary to atrial fibrillation are not only increasing in prevalence but are also more disabling (Camm et al., 2010). Stroke remains a leading cause of both morbidity and mortality in Africa (Moran et al., 2013) with an estimated annual incidence of approximately 244 per 100 000 persons in South Africa (Maredza and Chola, 2016).

1.7 Warfarin dosing

There are many models for initiation of warfarin therapy. Traditionally, individualisation of warfarin dosing has been empiric but because of severe adverse events associated with warfarin therapy, different prediction models have been devised (Doi, 2007).

For stable patients who can be managed as outpatients, initiation dose of 5-10mg is advised. However, a dose of 5mg is advised in the elderly and patients with heart failure, liver disease or high risk of bleeding such as severe hypertension and concomitant use of antiplatelet drugs. The INR is then measured after 48-72 hrs and the warfarin dose is adjusted accordingly using prediction tables. Initially the INR is measured daily and once it is stable, the testing frequency can be done at 4-6 weekly intervals. If the dose adjustment is required again, the high intense monitoring is repeated until stable therapeutic levels are achieved (Ageno et al., 2012; Holbrook et al., 2012). Rapid anticoagulation is advised in patients with acute veno-thromboembolism (VTE). Warfarin therapy is initiated on day 2 of low molecular weight heparin or unfractionated heparin cover. Once therapeutic INR is achieved, low molecular weight heparin and unfractionated heparin are discontinued (Ageno et al., 2012; Holbrook et al., 2012). Once they have been commenced on warfarin, patients are usually followed up at anticoagulation clinics (in countries with such facilities) or in medical outpatients. The emergence of point of care INR testing devices has seen the

introduction of patient self-management as an option, thus reducing the need for frequent visits to anticoagulation clinic or outpatient departments which in Africa may sometimes be quite some distance from where patients live (Connock et al., 2007).

Genotype based algorithms have emerged as an alternative way of initiation of warfarin. The assessment of environmental, demographic, clinical and genetic factors may predict the dose of warfarin. Trials incorporating genetic variation in *VKORC1* and *CYP2C9* have shown genotype based algorithms to be superior to traditional warfarin dosing (Cavallari et al., 2010; Pirmohamed et al., 2013; Drozda et al., 2015). Comparison of the two main warfarin dosing algorithms is shown in Table 1.4. However, it is important to note that none these trials recruited patients from Africa and hence applicability of the results to black and mixed ancestry Africans is limited.

Table 1.4 Comparison of two main warfarin dosing algorithms

Variable	**Units/Allowed values**	**IWPC**	**WD**
Age	Years	✓	✓
Height	Centimeters	✓	✓
Weight	Kilograms	✓	✓
VKORC1c.-1639G>A	A/A, A/G, G/G, unknown	✓	✓
CYP2C9 genotype	*1/*1,*1/*2,*1/*3,*2/*2,*2/*3,*3/*3	✓	✓
Race	Asian/Black/Caucasian/Unknown	✓	✓
Ethnicity	Hispanic/Non-Hispanic		✓
Taking enzyme inducer	Y/N		✓
Taking amiodarone	Y/N		✓
Statin	Y/N		✓
Azole	Y/N		✓
Bactrim	Y/N		✓
Liver disease	Y/N		✓
Smokes	Y/N		✓

IWPC- International Warfarin Pharmacogenetic Consortium
WD- www.warfarindosing.org
Y – Yes ; N- No

1.8 Adverse drug events and challenges associated with warfarin therapy

Bleeding is the most important adverse event associated with warfarin and is directly related to the degree of anticoagulation assessed with INR (Ageno et al., 2012; Li et al., 2015a). The associated risk of bleeding is high if the INR is greater than 4, with a 25% chance of bleeding compared to those in the therapeutic range (Ageno et al., 2012; Pirmohamed et al., 2013). Close to 26% of elderly population in USA have had warfarin discontinued because of bleeding (Hylek et al., 2007). It is because of these significant adverse events that tight monitoring through INR is required making warfarin therapy more expensive (Anderson et al., 2002; Stambler and Ngunga, 2015). Prediction models for bleeding risk have been proposed but none of these achieved sufficient required accuracy to be recommended for clinical use (Ageno et al., 2012). One such prediction model, is the HAS-BLED score; it was developed to assess the risk of major bleeding at one year in patients taking warfarin for atrial fibrillation. The prediction model identified hypertension, abnormal liver and renal function, previous stroke, previous bleeding, age >65, labile INR, drugs and alcohol as risk factors for bleeding (Pisters et al., 2010).

Polymorphisms in *VKORC1* and *CYP2C9* are associated with high bleeding risk especially early on after initiation of warfarin (Schwarz et al., 2008). Other risk factors associated with increased likelihood of bleeding while on warfarin include advanced age, weight, gender, chronic co-morbid conditions like liver disease, chronic kidney disease and hypertension, co-medications with medications like amiodarone and non-steroidal anti-inflammatory drugs, and lifestyle practices like smoking and alcohol consumption (Kamali and Wynne, 2010; Dean, 2012).

The first week of warfarin therapy is associated with acute thrombotic events like limb gangrene and skin necrosis. The pathogenesis of these complications remains unknown but skin necrosis results from extensive thrombosis of capillaries and venules in the subcutaneous tissue whereas limb gangrene is as result of thrombosis in the venous circulation of limbs (Ageno et al., 2012).

Warfarin related nephropathy occurs in patients with either normal kidneys or those with underlying chronic kidney disease. The dominant mechanism for the kidney impairment is likely obstruction of kidney tubules by red blood cell casts (Brodsky et al., 2011; An et al., 2013). Currently there are no guidelines on how to initiate warfarin

in patients with kidney impairment or titrate doses in those who develop warfarin related nephropathy. At the present moment, patients with kidney impairment are managed the same as those with normal kidney function (Genovesi et al., 2008; An et al., 2013). Ideally, patients with chronic kidney disease or warfarin related nephropathy should be assessed for warfarin dose requirements since they require lower initiating or maintenance doses (Limdi et al., 2010).

Warfarin resistance is one of the challenges associated with warfarin use. This occurs when patients require very high doses of more than 105mg a week to attain a therapeutic INR. Causes can either be hereditary or acquired (Vaes and Chyka, 2000; Osinbowale et al., 2009). Some of the acquired factors include non-compliance with warfarin, increased consumption of vitamin k, co-medication with enzyme inducers of warfarin metabolism leading to rapid clearance as well as decreased absorption of warfarin (Cain et al., 1997; Vaes and Chyka, 2000; Daly and Aithal, 2003). Hereditary causes of warfarin resistance are not well understood (Osinbowale et al., 2009). There is some evidence that an individual based approach to dosing which takes into account genetic factors allows clinicians to identify patients who are likely to bleed thus allows for a much safer approach to dosing (Ageno et al., 2012).

1.9 Addressing challenges associated with warfarin use

Educating the patient on the need for anticoagulation, warfarin side effects like bleeding and action to take in the event of early signs of bleeding, importance of regular INR testing and interactions of warfarin with other drugs, herbs and diet is key to successful warfarin therapy (Tideman et al., 2015). Contraindications like decompensated liver disease, uncontrolled severe hypertension and coagulation defects must be assessed before initiation of warfarin therapy. (Blann et al., 2003; Long et al., 2010; Tideman et al., 2015). Discontinuation of antiplatelet therapy may be considered if risk of bleeding is deemed high (Ageno et al., 2012). To reverse the effects of bleeding, warfarin must be temporarily stopped, followed by administering vitamin K, fresh frozen plasma (FFP) or prothrombin complex concentrates (PCCs) (Tran et al., 2013).

To address the issue of frequent patient visit to INR clinic, patient self-management has been introduced and has proved to be less expensive (Connock et al., 2007). This was made possible by introduction of hand-held INR testing devices that are so simple to

use to the extent that patients can now check INR at home. (Pirmohamed et al., 2013). This has resulted in considerable enthusiasm and time in therapeutic range (TTR).

Erratic INR and bleeding especially in the first month of warfarin initiation can be significantly lowered by adopting genotyping at initiation to allow rapid adoption of the optimum dose. This results in more rapid achievement of stable therapeutic INR and more time therapeutic range (TTR) (Pirmohamed et al., 2013). The challenge now is to produce point of care devices that will allow genotyping to be done in under 2 hours.

Another strategy to improve the safety and utility of anticoagulation is the emergence of drugs called new oral anticoagulants (NOACs). These drugs target factors of the clotting cascade (Barnes et al., 2015). These novel drugs, dabigatran, rivaroxaban, edoxaban and apixaban have a better safety profile and are non-inferior to warfarin in terms of efficacy (Barnes et al., 2015).

1.10 Factors affecting warfarin dose response

One of the challenges associated with warfarin dosing is large inter-individual variation in maintenance dose. Sixty percent of the warfarin dose variability can be explained by both genetic and clinical factors (Yin and Miyata, 2007). Much of the variability can be attributed to genetic polymorphism mainly involving *VKORC1* gene (Geisen et al., 2005) that accounts for 30% (D'Andrea et al., 2005; Wadelius et al., 2005) and *CYP2C9* gene (Moridani et al., 2006). While, *VKORC1* and *CYP2C9* combined account for 40% of the variation attributed to genetic factors, non-genetic factors contribute 15% of the variance observed (Takeuchi et al., 2009). Other candidate genes like *gamma* glutamyl carboxylase (*GGCX)*, *CYP4F2*, *epoxide hydroxylase 1(EPHX1)*, *calumenin* (*CALU*) and *CYP2C19* might explain inter-individual variation (Wadelius et al., 2007). Concurrent medications, co-morbid conditions and demographic factors such as age, gender, weight and height influence maintenance dose in a predictable manner (Skov et al., 2012). Warfarin dose decrease with age and increase with increasing height, weight and body surface area (Sconce et al., 2005). Hence a combination of genetic and non-genetic factors can explain this inter-individual variability (Lindh et al., 2005; Wadelius et al., 2009). Table 1.5 shows percentage contributions of different factors affecting warfarin dose response. Dosing algorithms combining both genetic and clinical factors have been developed (Klein et al., 2009; Lubitz et al., 2010), however,

these algorithms can only explain a third to half of the variation (Lubitz et al., 2010) but perform better than algorithms based on clinical factors only (Klein et al., 2009). Little is known about the impact of behavioral factors like diet, exercise, smoking and alcohol (Skov et al., 2012).

Table 1.5: Factors affecting warfarin dose response and their contribution.

Variable	Estimated contribution	Reference
VKORC1	37%	Bodin et al. 2005
CYP2C9	22%	D'Andrea et al. 2005
Age	17%	Sconce et al. 2005
Gender	8.1%	Yin and Miyata 2007
Weight	7.8%	Yin and Miyata 2007

Despite our current knowledge on pharmacogenomics and clinical factors, 40% of warfarin dose variability cannot be explained (Yin and Miyata, 2007), additional genetic factors might be responsible for the observed inter individual variability.

1.11 Existing warfarin genotype-based algorithms and their extrapolation to African population

The two main dosing algorithms used in clinical trials were derived from mostly European population (Drozda et al., 2015). Genotyping was limited to *VKORC1c.-1639G>A, CYP2C9*2* and *CYP2C9*3* polymorphisms. Recent clinical trials incorporating allelic variants *CYP2C9*2* and *CYP2C9*3* have shown genotype-based algorithms to be superior to clinical algorithms in Caucasians (Pirmohamed et al., 2013), however genotype-based warfarin dosing performed worse when African Americans were included (Kimmel et al., 2013). Trials incorporating *CYP2C9*5*, *CYP2C9*6, CYP2C9*8* and *CYP2C9*11* in African American performed better and improved the predicted warfarin dose required to reach INR (Cavallari et al., 2010; Drozda et al., 2015).

Africans differ in their extent of recent admixture with non-Africans and specifically their European counterparts (Suarez-Kurtz and Botton, 2013). The European ancestry proportion in African American ranges 4-35% (Suarez-Kurtz and Botton, 2013), whilst

its 0-86% in Cape Mixed Ancestry of Cape Town South Africa (Tishkoff et al., 2009), 44-73% in Brown Brazilians and 29-54% in Black Brazilians (Pena et al., 2011). The black African population in sub-Saharan Africa has high level of admixture of distinct ancestral African clusters making it very diverse (Tishkoff et al., 2009). The data from self-identified African Americans cannot be extrapolated to populations from Southern Africa who already differ in some of these polymorphisms from West Africans (Suarez-Kurtz and Botton, 2013).

2. Motivation and rationale for the study focusing on blacks and mixed ancestry in South Africa.

Warfarin is the most commonly prescribed anticoagulant in sub-Saharan Africa and the developing world despite the existence of newer and presumably better anticoagulants (Ogilvie et al., 2010; Stambler and Ngunga, 2015). However, warfarin has a narrow therapeutic index resulting in fluctuation of INR (Poller, 2004) leading to a significant incidence of adverse drug reactions (ADRs) like bleeding or thrombotic events (Merli and Tzanis, 2009; Ageno et al., 2012). Bleeding is the most common complication of warfarin anticoagulation (Li et al., 2015a). Adverse drug reactions rank as one the leading cause of mortality and in the USA it has been estimated to cost in excess of $100 billion annually (Sultana et al., 2013). In South Africa, 14% of hospital admissions are thought to be due to adverse drug reactions (Mehta et al., 2008), double what has been reported internationally and the costs are not known. The contribution of warfarin related complications to disease is unknown but given the large number of people who use warfarin and its narrow therapeutic window, it is likely that the proportional contribution to this burden of adverse drug reactions is high.

Genotype based warfarin dosing has been shown to be superior to clinical based algorithms in both Caucasians (Pirmohamed et al., 2013) and African Americans (Cavallari et al., 2010; Drozda et al., 2015) resulting in reduced adverse drug reactions. None of the available genotype-based warfarin dosing algorithms can be used in sub-Saharan Africa where the Black African and Africans of mixed ancestry were not included in any of the above efficacy studies and in whom the distribution of polymorphism of *CYP* and *VKOR* genes is known to be different from the populations

included in the studies. Furthermore, given the relative lack of capacity to monitor INRs adequately and the resources for close follow up of patients on warfarin, a personalized pharmacogenomic approach to warfarin dosing based may offer major advantages compared to the current nurse led clinic-based monitoring strategies which are currently the standard of care in much of the sub-continent.

It is thus imperative that genetic variants that are relevant to local African population are sought and found so that they may benefit from effective and safe drug administration algorithms. We therefore set out to find out the role of known polymorphisms in *CYP2C9* and *VKORC1* on the time it takes to reach stable therapeutic INR, mean weekly dose of warfarin and adverse events in Blacks and Africans of Mixed Ancestry in South Africa.

3. AIMS and OBJECTIVES

3.1 Aim

To determine the role of genetic variation in *CYP2C9* and *VKORC1* on the time it takes to reach stable therapeutic INR as well as on dose required to maintain INR in the therapeutic range.

3.2 Objectives

This was approached through a number of objectives as follows:

1. To determine time, it took patients to achieve required INR and the current warfarin maintenance dose
2. To determine allele frequency for *VKORC1* and *CYP2C9* variants
3. To determine the common variant genotypes
4. To determine the association between genetic variants and adverse drug reactions

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CHAPTER 2: JOURNAL READY MANUSCRIPT

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The role of warfarin pharmacogenomics on the time it takes to reach stable therapeutic target International Normalized Ratio (INR) and on dose of warfarin required to maintain stable therapeutic INR in Black African and Mixed Ancestry South Africans: a focus on *CYP2CP* and *VKORC1*

Short running title: Pharmacogenomics of warfarin in South Africans.

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Abstract

Warfarin, the most commonly prescribed anticoagulant, is principally metabolized by cytochrome P450 2C9 which functions by inhibiting the Vitamin K epoxide reductase. Genes *CYP2C9* and *VKORC1* code for these two proteins, respectively. *CYP2C9* and *VKORC1* exhibit genetic polymorphisms that have been shown to affect warfarin response and favorably facilitate warfarin dosing and improve clinical outcomes. However, none of these studies have involved populations from sub-Saharan Africa where the potential benefit of optimal dosing and reduced complications is greatest. Therefore, the thesis describes a study designed to investigate the role of genetic variations in *CYP2C9* and *VKORC1* on the time taken to reach a stable therapeutic INR and warfarin dose required to maintain a therapeutic INR.

In a cross-sectional study of patients on warfarin, medical records were used to collect data on time to INR and warfarin doses. DNA was extracted from blood samples, and genotyping for polymorphism in *CYP2C9* (*2,*3,*8,*11) and *VKORC1* (*1173C>T, 1639G>A, 3730G>A*) was accomplished by PCR-RFLP, Sanger sequencing and iPlex Mass Sequencing.

Our results show that the genetic profile of *CYP2C9* and *VKORC1* genetic variation differs between Black Africans (BA) and their Mixed Ancestry (MA) counterparts. *VKORC1-1639AA* genotype was observed at frequencies of 0.11 and 0.01 in the MA and BA, respectively. Furthermore, compared to known genetic polymorphisms in these genes from population out of Africa, both qualitative and quantitative differences were observed. Finally, we found that *VKORC1* genetic variation significantly affected the doses of warfarin in MA but had no effect in BA. These results suggest that further research in this area is warranted, and that it will be important to include populations from sub-Saharan Africa in future if the potential to develop personalized algorithms which integrate pharmacogenomics to assist with effective warfarin dosing and prevention of warfarin related complications is to be realized.

Keywords: Pharmacogenomics, variants, genotype, allele, Mixed Ancestry, African

INTRODUCTION

Warfarin, a coumarin derivative, is the most commonly prescribed anticoagulant for the prevention and treatment of thromboembolic diseases (Hirsh et al., 2001; Cho et al., 2016). It works by inhibiting Vitamin K epoxide reductase multi protein complex (*VKORC1*) (Wallin and Hutson, 2004; Limdi and Veenstra, 2008) which recycles Vitamin K 2,3 Epoxide to Vitamin K hydroquinone, a cofactor that is essential for post translational gamma carboxylation of clotting factors (Dahlback, 2005). It has a narrow therapeutic index and wide inter-individual variability and therefore requires close monitoring by measuring the prothrombin time expressed as the International Normalized Ratio (INR) (Wadelius et al., 2009; Ageno et al., 2012). Bleeding is the most common complication of warfarin anticoagulation; other important adverse events common in patients using warfarin are related to inadequate anticoagulation and include venous thromboembolisms and systemic emboli (Ageno et al., 2012; Li et al., 2015b).

Warfarin is a racemic mixture of R and S enantiomers (Herman et al., 2005; Ageno et al., 2012). The more potent of the two is the S-enantiomer, it is metabolized primarily by CYP2C9 (Redman, 2001; Ageno et al., 2012) and is encoded by a highly polymorphic gene. *CYP2C9* has more than 50 allelic variants (Lee et al., 2002; Sim and Ingelman-Sundberg, 2010) varying along racial and ethnic lines (Allabi et al., 2004; Suarez-Kurtz and Botton, 2013) and accounts for at least 10% of dose variation encountered in patients on warfarin (Verhoef et al., 2014).

The promoter region of single nucleotide polymorphism (SNP) *VKORC1 1639g-G>A* accounts for 20-35% inter-individual difference observed in warfarin dose response (Takahashi et al., 2006; Dean, 2012). *VKORC1 c.1173*, *VKORC1 c.3730* and *VKORC1 c.-1639* are the most common single nucleotide polymorphism in warfarin dosing response (Yang et al., 2010).

Recent clinical trials incorporating allelic variants *CYP2C9 *2* and *CYP2C9*3* have shown genotype-based algorithms to be superior to clinical algorithms in Caucasians with a more predictable time to stable INR and a reduction in warfarin related toxicity (Pirmohamed et al., 2013). However, genotype-based warfarin dosing performed poorly when African Americans were included (Kimmel et al., 2013) and none of the studies included black African and mixed ancestry populations from Africa, thereby excluding these populations from the potential benefit of safer pharmacogenetic based

dosing strategies. Given the relative lack of capacity to monitor INRs adequately and the inability to follow up patients on warfarin closely across much of sub- Saharan Africa, clinical research on pharmacogenomic approaches to warfarin dosing may offer major advantages compared to the current nurse led clinic-based monitoring strategies, which are currently the standard of care in much of the sub-continent. In order to begin to address this important gap in knowledge on the impact of warfarin pharmacogenomics on the safety and efficacy of treatment with warfarin, we performed a study to determine the role of known *CYP2C9* and *VKORC1* genotype variants on the time it takes to reach target stable therapeutic INR, mean weekly dose and adverse events in Black African and African Mixed Ancestry patients in South Africa.

METHODS

Study design and participants

This was a cross-sectional analysis of genetic data with subsequent correlation of the results with information derived from the clinical records. The participants were selected from a cohort of patients who attended the INR clinic at Groote Schuur hospital in Cape Town from 2007 to 2016.These patients all had stable doses of warfarin and had recently been genotyped for *CYP2C9* and *VKORC1* for a concurrent and related pharmacogenomics of warfarin study (Ndadza et al., 2019). We included consenting patients above the age of 18 years, who were Black African or African of Mixed Ancestry, with genotype results available, and complete medical records from the time of initiation of warfarin to the time they reached stable therapeutic INR. The latter was defined as three consecutive INRs, tested greater than one week apart and found to be 2.5 to 3.5 if indication was a mechanical valve and 2-3 for any other indication). The warfarin dose was considered stable if there was <10% variability in the warfarin dose while maintaining a therapeutic INR for three consecutive months (De Caterina et al., 2013). For the study an adverse event was defined an INR> 4, the value which has been shown to correspond to an increased risk of bleeding (Hylek and Singer, 1994; Higashi et al., 2002), thromboembolic event like stroke while on warfarin and bleeding while on warfarin.

Data collection

A trained nurse collected demographic and clinical information. Five milliliters of blood were drawn into an EDTA tube for genotyping using Tagman SNP assay (*CYP2C9)* and polymerase chain reaction (PCR) coupled to restriction fragment length polymorphism (RFLP) for *VKORC1*. Variables collected included- age, sex, weight, height, smoking and alcohol history, diet, co-morbidities, concomitant drugs and bleeding. INR results were retrieved from the National Health Laboratory Service (NHLS), a robust and reliable electronic record system. Study was approved by Human Research Ethics Committee, University of Cape Town.

Data analysis

The data was analyzed using the R statistical software (version 3.0.3[2014-03-06], The R Foundation for statistical computing, Vienna, Austria) and the packages 'genetics' and 'SNPassoc'. General characteristics of the study were summarized as count and percentage for dichotomous traits, mean, and standard deviation (SD) or median and 25^{th}-75^{th} percentiles for quantitative traits. Traits were log transformed to approximate normality, where necessary, prior to analysis. Single Nucleotide Polymorphisms (SNPs) were tested for departure from Hardy-Weinberg Equilibrium (HWE) expectation via a chi square goodness of fit test. Linkage disequilibrium was estimated using a D' statistic. Linear regression models were used for analysis of quantitative traits and logistic regression models for dichotomous traits assuming various genetic models for the SNPs. Using linear and regression models enabled us to adjust all analyses for known confounders. Adjustment for multiple testing was conducted via Bonferroni Kaplan Meir survival curves were also done to compare the different categorical variables. A *p* value of less than 0.05 was considered statistically significant.

RESULTS

Four hundred and forty-six potential participants were screened for enrollment into the study. Of the eligible patients, 48 were excluded because of incomplete genotype information and 82 patients were excluded because they did not meet the criteria for a stable warfarin dose. Detailed analysis was done on only 316 individuals; 228 (72.1%) were mixed ancestry (MA) and 88 (27.9%) were Black African (BA).

Overall, females were 68% of the study population (Table 2.1). There was a statistically significant difference (p=0.001) in the median ages of the BA (47 years) compared to MA (58 years). HIV infection affected 8.1% of the study participants, however, it was significantly (p<0.0001) more prevalent in BA at 22.7% compared to 2.6% in MA. Among the BA participants, 20% were smokers compared to 62% in MA (p<0.0001). The most prescribed co-medications were efavirenz (14%) and statins (17%) in both BA and MA, respectively. The main indication for warfarin therapy was mechanical valve replacement with hypertension as the most common co-morbid condition.

The allele frequency for *CYP2C9* and *VKORC1* variants were shown in Table 2.2. Among the MA participants the most common variant *VKORC1* genotype was *VKORC1 c.3730GA* (0.47), followed by *VKORC1c.-1639GA* (0.41) and the least common was *VKORC1c.-1639AA* (0.11) while among the BA participants, the frequencies for *VKORC1c.3730GA*, *VKORC1c.-1639GA* and *VKORC1c.-1639AA* were 0.35, 0.17 and 0.01 respectively. The allelic variants *VKORC1 c.-1639A* and *VKORC1*c.1173T differed significantly (p<0.0001) between the two ethnic groups. The *CYP2C9 c.449A* (**8*) allelic variant was significantly more frequent in BA (0.07) compared to MA (0.02) (p<0.0001). In contrast, in MA participants the allelic variant *CYP2C9 c.1075C* (**3*) was more frequent (0.04) than it was in their BA counterparts (0.01) (p=0.02). The observed frequencies for *VKORC1* genotypes did not fit into Hardy-Weinberg Equilibrium except for *VKORC1 1173* in BA participants. In contrast none of the *CYP2C9* genotypes deviated from Hardy-Weinberg Equilibrium (p>0.05).

All the patients included in the study met the definition for stable therapeutic INR. The mean weekly warfarin dose was significantly lower in MA (33 ± 12.9mg/week) compared to BA (41± 14.1mg/week) (p<0001). The mean weekly warfarin dose by genotype are shown in Table 2.3. Among the MA group, the mean maintenance dose varied significantly (p<0.0001) with the *VKORC1* genotypes. In contrast, the BA group did not vary significantly with *VKORC1* genotypes. A significant (p<0.0001) weekly dose reduction of 16mg was noted in MA group possessing the *VKORC1 c.-1639A/A* genotype compared to those carrying *VKORC1 c.-1639G/G*. MA participants carrying the *CYP2C9 c.430C>T* SNP had a significantly (p=0.02) lower mean weekly warfarin dose compared to BA participants. There was a tendency (p=0.07) towards a lower mean weekly warfarin dose in BA participants carrying the *CYP2C9 c.449G>A SNP*.

Among the BA participants carrying *CYP2C9 c.449A/A*, the mean weekly warfarin dose was 16mg significantly (p=0.002) less compared to those possessing *CYP2C9 c.449G/G*. Furthermore, carriers of *CYP2C9 c.430C/T* in BA group required 14mg less mean weekly warfarin compared to *CYP2C9 c.430C/C* carriers (p=0.002).

For the entire study cohort, the median time taken to reach a stable INR was 28.5 days. The only demographic variable which influenced both the time taken to reach stable therapeutic INR (p=0.030) and mean weekly dose (p=0.0001) was age. The mean weekly warfarin dose requirement was inversely associated with age in both MA and BA patients (p<0.0001 and p=0.042, respectively). In contrast, time to stable INR increased with increasing age in both MA and BA patients. Ethnicity had no effect on time taken to reach target stable INR (0.492). The time to stable INR was not influenced by *VKORC1* and *CYP2C9* genotypes (Figure 1). However, there was a tendency towards effects of *CYP2C9*8* (p=0.06) and *VKORC1 c.1169* (p=0.056) in Mixed Ancestry on the time to therapeutic INR.

Ninety two percent (92%) of the study population experienced an INR>4 making this the most common study defined adverse event (68%) amongst those who had an AE. Demographic and clinical variables were not associated with adverse events. *VKORC1 c.-1639A/A* genotype (p=0.047), *VKORC1 c.3730G/A* (p=0.046) and *VKORC1 c.3730G/G* (p=0.015) were all associated with developing an INR>4 in MA patients. The only other variables significantly associated with an adverse event were age (p=0.002) and heart failure (p=0001).

DISCUSSION

In this study, we have shown that time to stable therapeutic INR, was not influenced by *CYP2C9* and *VKORC1* genotypes. This is in accordance with previous studies, which showed no association between *CYP2C9* and *VKORC1* genotypes in patients of diverse ethnicity (Kealey et al., 2007; Schelleman et al., 2007; Limdi et al., 2008a). Interestingly, several studies done in patients of European descent showed that indeed these same variants prolong time to stable INR (Taube et al., 2000; Higashi et al., 2002; Lindh et al., 2005; Schalekamp et al., 2006; Kealey et al., 2007; Schelleman et al., 2007). The outcome could have been influenced by a number of factors. First, different protocols for warfarin initiation and dose adjustment were used resulting in different

trends and rates at which time to stable INR was reached. An example is that of a patient post mechanical valve replacement, who is discharged after therapeutic INR is reached and a patient with atrial fibrillation managed as an outpatient at local clinic with erratic follow up. Second, frequent interruption of warfarin therapy after occurrence of adverse events and non-compliance affected time taken to reach stable INR. Third, it could that there are other variants yet to be discovered in the South African population.

We have also shown that the inter-ethnic variability in frequencies of variant genotypes and alleles. *VKORC1 c.1639G>A* and *VKORC1 c.1173C>T* genotypes were significantly more frequent in Mixed Ancestry patients compared to Black Africans. Both *VCORC1 c.1639A* and *VKORC1 c.1173T* variant alleles were more prevalent in Mixed Ancestry patients compared to Blacks. The observed difference could be explained by: (a), the small sample size of Black patients and (b), the fact that Africans differ in the extend of recent admixture with non-Africans specifically Europeans (Suarez-Kurtz and Botton, 2013) with the proportion of European ancestry ranging from 0 to 0.86 in Cape Mixed Ancestry of Cape Town (Tishkoff et al., 2009). In Black Africans, the frequency of *VKORC1 c.-1639A* variant allele was 0.10. This is in agreement with a study done on African Americans by Geisen et al. which showed a frequency of 0.14 (Geisen et al., 2005). Interestingly, Dandara et al observed a frequency of 0.28 with respect to *VKORC1 c.1173T* variant allele in Mixed Ancestry in South Africa (Dandara et al., 2011). This is comparable to our own finding of 0.27. Variant allele frequencies in *CYP2C9 c.1075C* and *CYP2C9 c.449A* were 0.01 and 0.07 respectively in Blacks. The low prevalence in *CYP2C9*2* and high prevalence in *CYP2C9*8* relative to other races was also observed by Cavallari and Allabi in a study of African American (Allabi et al., 2004; Cavallari et al., 2010). In the same study by Cavallari, *CYP2C9 c.449G>A* was higher in Blacks compared to other races and genetic algorithms incorporating both *CYP2C9*8* and *CYP2C9* 11* performed better (Cavallari et al., 2010; Drozda et al., 2015).

Our study has shown that ethnicity plays a role in the determination of mean weekly dose. Our findings suggest that patients of Mixed Ancestry required lower doses of warfarin compared to Blacks in all the three *VKORC*1 SNPs. A previous study which was done on African Americans and Caucasians by Schelleman (2007) noted that carriers of *VKORC1 c.1639* A/A required lower daily or weekly doses than *VKORC1*

c.1639G/G or *VKORC1 c.1639G G/A* carriers. This observation was replicated in our study in patients of Mixed Ancestry. We also found that *CYP2C9 c.430C>T* and not *CYP2C9 c.1075A>C* influence mean warfarin dose in patients of Mixed Ancestry only. This finding contrasts other studies done in patients of European origin which showed that *CYP2C9 c.1075A>C* was associated with a lower dose than *CYP2C9 c.430C>T* (Yamazaki et al., 1998; Kirchheiner and Brockmoller, 2005).

The association with the risk of over-anticoagulation by *CYP2C9* and VKORC1 was inconsistent across races in a study involving African Americans and Americans of European origin and in addition the likelihood of over-anticoagulation was higher in Europeans with *VKORC1 c.1173C>T* variant (Limdi et al., 2008b). In our study, genetic prediction for INR>4 was only possible with *VKORC1 c.1639A/A* (p=0.05) and VKORC1 c.3730G/G (p=0.02) in Mixed Ancestry patients. However, our data collection was incomplete in terms of adverse events. INR>4 was not correlated to time from the initiation of warfarin. Bleeding was significant in our study at 22% of the patients who had an adverse event, unfortunately we did not correlate bleeding to INR>4 and to time from initiation of warfarin. Furthermore, bleeding was not categorized into either minor or major bleeding events as stated by the criteria used in the Second Copenhagen Atrial Fibrillation, Aspirin, and Anti- coagulation study (Gullov et al., 1999).

This study had limitations. First, warfarin dosing was not protocol driven. Close to half of our patients (48.4%) were on warfarin for mechanical valves meaning they were closely monitored in hospital whereas the other half were started as outpatients and the dosing regimen differed from one practitioner to the other. Second, sample size was small especially in Black Africans after a batch of more than 100 samples from Black patients was discarded for failure to adhere to protocol. Third, we did not check warfarin levels for both confirming compliance and correlating warfarin levels to adverse events. Fourth, very basic information was collected on adverse events which was not adequate to correlate bleeding and INR>4 to time from warfarin initiation.

These findings are important, as they are the first to investigate the impact for pharmacogenomics on measure of warfarin metabolism in African populations in sub-Saharan Africa. Warfarin remains the most commonly used anticoagulation in Africa despite its adverse event profile and the emergence of new oral anticoagulants with a

better safety profile (Ogilvie et al., 2010; Stambler and Ngunga, 2015). A need therefore arises to improve warfarin dosing regimens and reduce toxicity. Genetic based dosing algorithms have already been developed outside of African which have been shown to be superior to clinical algorithms (Pirmohamed et al., 2013). However, the two main dosing algorithms used in clinical trials were derived from mostly European populations (Drozda et al., 2015). Africans are a diverse and heterogenous population with high level of admixture of distinct ancestral African clusters (Tishkoff et al., 2009). It's not surprising that the dosing algorithms developed for the European population performed poorly in the black African populations. African specific genetic variants need to be identified and incorporated into dosing algorithms. Our study was able to examine how the already known single nucleotide polymorphisms of *CYP2C9* and *VKORC1* affect time to stable therapeutic INR and mean weekly dose in Black and Mixed Ancestry Africans in South Africa which is an important step.

In conclusion, the main findings were: (1) There was no association between variant genotypes and time taken to reach stable INR; (2) Variant genotypes and alleles are more frequent in Mixed Ancestry patients; (3) Mixed Ancestry patients required significantly lower mean weekly dose of warfarin; and (4) *VKORC1* g-1639 and *VKORC1* c.3730 were associated with over anticoagulation as defined by an INR>4 in Mixed Ancestry patients. These preliminary findings suggest that there is a need to conduct more research and explore the possibility of developing personalized algorithms to assist with achieving the required INR and prevent warfarin related complications.

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Table 2.1 General characteristics of South African patients on warfarin

Variable	N(frequency/range*)
Sex	
Male	101(0.32)
Female	215(0.68)
#Ethnicity	
Black African	88(0.28)
Mixed Ancestry	228(0.72)
Median age	
Black African	47(18-80)
Mixed Ancestry	58(20-87)
Indication for warfarin therapy	
Mechanical valve replacement	153(0.48)
Atrial fibrillation	79(0.25)
Deep vein thrombosis	54(0.17)
Pulmonary embolism	30(0.10)
Co-morbidity	
Diabetes	50(0.16)
Hypertension	130(0.41)
Heart failure	115(0.36)
HIV	26(0.08)
Adverse effects	292(0.92)
Bleeding	65(0.22)
INR>4	198(0.68)
Thromboembolism (on warfarin therapy)	29(0.10)
Co-medication	
Statin	60(0.19)
Efavirenz	14(0.04)

*Refers to interquartile range

#Ethnicity was reported by patient

Percentages may not add up to 100 because of rounding.

Table 2.2 Observed genotype frequency in BA and MA patients

SNP	Genotype	BA(*n*=88)		MA(*n*=228)		*p value*
		n	**frequency**	**n**	**frequency**	
VKORC1 c.-1639G>A	AA	1	0.01	24	0.11	
	GA	15	0.17	94	0.41	
	GG	72	0.82	110	0.48	
	A allele	17	0.1	142	0.31	0.0001
VKORC1 c.1173C>T	TT	1	0.01	22	0.10	
	CT	16	0.18	81	0.35	
	CC	71	0.81	125	0.55	
	T allele	18	0.1	125	0.27	0.0001
VKORC1 c.3730G>A	AA	21	0.24	52	0.23	
	GA	31	0.35	106	0.47	
	GG	36	0.41	67	0.29	
	N/A	0	0	3	0.01	
	A allele	73	0.41	210	0.46	0.31
*CYP2C9(*2) c.430C>T*	CT	4	0.05	18	0.08	
	CC	73	0.82	188	0.82	
	N/A	11	0.13	22	0.10	
	T allele	4	0.02	18	0.04	0.24
*CYP2C9(*3) c.1075A>C*	CC	1	0.01	0	0.00	
	AC	0	0.00	17	0.08	
	AA	71	0.81	168	0.74	
	N/A	16	0.18	43	0.18	
	C allele	2	0.01	17	0.04	0.02
*CYP2C9(*8) c.449G>A*	AA	1	0.01	0	0	
	GA	11	0.13	10	0.04	
	GG	65	0.74	196	0.86	
	N/A	11	0.13	22	0.10	
	A allele	13	0.07	10	0.02	0.0001
*CYP2C9(*11) c.1003C>T*	CT	1	0.01	4	0.02	
	CC	71	0.81	181	0.79	
	N/A	16	0.18	43	0.19	
	T allele	1	0.006	4	0.01	0.49

BA- Black African

MA- Mixed Ancestry

N/A- patients with no genotype data

n-number

SNP- single nucleotide polymorphism

Table 2.3 Mean weekly warfarin dose by genotype in BA and MA South Africans

SNP	Genotype	Ethnicity			
		Black African		Mixed Ancestry	
		Mean dose(mg/wk) ±SD (range)	**P value**	**Mean dose(mg/wk) ±SD (range)**	***P value***
VKORC1 c.-1639G>A	AA	45 ± 24(27-72)	0.99	21 ± 11.6(15-25)	<0.0001
	GA	40 ± 14(29-50)		31 ± 13.5(20-38)	
	GG	41 ± 13(32-50)		37 ± 12.8(28-48)	
VKORC1 c.1173C>T	TT	50 ± 0	0.69	25 ± 12(15-35)	<0.0001
	CT	40 ± 13(30-45)		30 ± 13(17-37)	
	CC	41 ± 13(32-52)		37 ± 13(27-47)	
VKORC1 c.3730G>A	AA	42 ± 17(25-42)	0.73	39 ± 13(29-50)	0.001
	GA	39 ± 14(27-40)		35 ± 13(25-45)	
	GG	40 ± 10(35-50)		27 ± 14(15-35)	
*CYP2C9(*2) c.430C>T*	CT	34 ± 9(28-41)	0.32	27 ± 13(17-35)	0.02
	CC	40 ± 13(33-50)		34 ± 14(22-42)	
*CYP2C9(*3) c.1075A>C*	CC	-	-	-	0.32
	AC	-		31 ± 15(17-37)	
	AA	40 ± 12(30-47)		34 ± 14(22-42)	
*CYP2C9(*8) c.449G>A*	AA	25 ± 0	0.07	-	0.87
	GA	34 ± 11(25-40)		34 ± 14(22-44)	
	GG	41 ± 12(35-50)		35 ± 17(25-35)	
*CYP2C9(*11) c.1003C>T*	CT	20 ± 0	0.12	28 ± 12(25-35)	0.48
	CC	40 ± 12(32-47)		34 ± 14(22-42)	

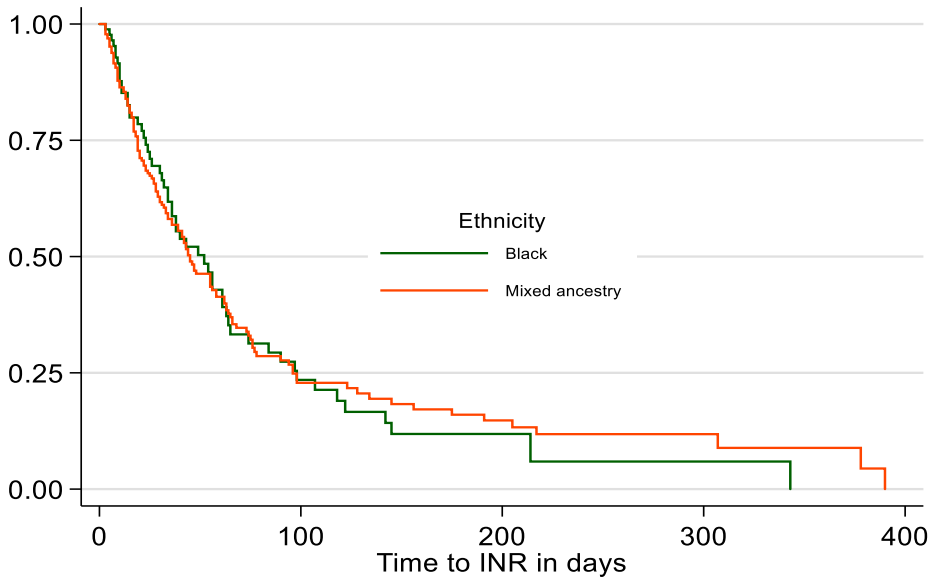
wk- week

BA- Black African

MA- Mixed Ancestry

SD- Standard deviation from the mean

	Overall	Black	Mixed Ancestry	*p*-value
Median time to INR (days)	28.5	30.5	28	0.202



Number at risk					
Black	88	12	2	1	0
Mixed ancestry	228	23	11	4	0

Figure 2 Survival analysis showing association between ethnicity and Time to INR

INR- International Normalized Ratio

Race was reported by patient

Appendices

Appendix 1. Data Capture sheet

PROJECT TITLE: Role of pharmacogenomics on time it takes to reach stable INR

Participant name: _____ Participant number:

Date of birth (yyyy/mm/dd)	**Gender**		**Height**	**Weight**
	Male	Female		
Ethnicity (tick where applicable)				

Zulu	Swazi	Mixed ancestry		
Sotho	Tswana	Venda	Swazi	
Tsonga	Xhosa	Ndebele	Other (specify)	

Do you smoke Yes No	If you have stopped, when was that?		Alcohol Yes No	If you have stopped, when was that?

Level of Qualification	None	Primary	High School	Matric	Tertiary
Employment State type of employment	Studying	Employed	Unemployed	Retired	

Conditions indicated for:	Atrial fibrillation	Ventricular clot	Mechanical valves	Stroke	Deep vein thrombosis	Pulmonary embolism
Initiation date for treatment (yyyy/mm/dd)						
Number of warfarin dose adjustment before reaching INR (mg/d)	2^{nd} dose	3rd dose	4th dose	More		
Date when INR was achieved (3 readings in range one week apart)	Date	Dose of warfarin (mg/d)				
Adverse events on warfarin(bleeding, INR>4)	Yes	No	Date & description :			
Concomitant drugs use (Name and duration)						
Other comorbidities e.g. blood pressure, diabetes etc.	Yes	No	Comments:			

Appendix 2. Ethics approval letter

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6626
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Website: www.health.uct.ac.za/fhs/research/humanethics/forms

26 July 2016

HREC REF: 410/2016

Prof M Ntsekhe
Cardiology
E17, NGSH

Dear Prof Ntsekhe

PROJECT TITLE: THE ROLE OF PHARMACOGENOMICS ON THE TIME IT TAKES TO REACH STABLE THERAPEUTIC TARGET INR IN BLACKS AND AFRICANS OF MIXED ANCESTRY IN SOUTH AFRICA (MMed-candidate-Dr E Makambwa) Sub-study linked to 581/2015

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30th July 2017.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval before the research may occur.

The HREC acknowledge that the student Dr Edson Makambwa will also be involved in this study.

Yours sincerely

signature removed to avoid exposure online

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

HREC 410/2016