Pharmacogenomics of Warfarin: Comprehensive Evaluation of Important Warfarin Genomic Response Factors

By

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List of Publication included in this thesis:

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1. **Ndadza A**, Cindi Z, Makambwa E, Chimusa E, Wonkam A, Kengne AP, Ntsekhe M and Dandara C. **Warfarin Dose and CYP2C Gene Cluster: An African Ancestral-Specific Variant Is a Strong Predictor of Dose in Black South African Patients.** OMICS. 2019;23(1):36-44. https://doi.org/10.1089/omi.2018.0174

2. **Ndadza A,** Thomford NE, Mukanganyama S, Wonkam A, Ntsekhe M and Dandara C**. The Genetics of Warfarin Dose-Response Variability in Africans: An Expert Perspective on Past, Present, and Future.** OMICS. 2019;23(3):152-66. https://doi.org/10.1089/omi.2019.0018

3. **Ndadza A*,** Muyambo S*, Mntla P, Wonkam A, Chimusa E, Kengne AP, et al. **Profiling of warfarin pharmacokinetics-associated genetic variants: Black Africans portray unique genetic markers important for an African specific warfarin pharmacogenetics-dosing algorithm.** J Thromb Haemost. 2021;19(12):2957-73**.** https://doi.org/10.1111/jth.15494
   *Joint first co-authors

4. Muyambo S*, **Ndadza A***, Soko ND, Kruger B, Kadzirange G, Chimusa E, et al. **Warfarin Pharmacogenomics for Precision Medicine in Real-Life Clinical Practice in Southern Africa: Harnessing 73 Variants in 29 Pharmacogenes**. OMICS. 2022;26(1):35-50. https://doi.org/10.1089/omi.2021.0199
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Abstract

Introduction:

Warfarin is the most widely prescribed anticoagulant for the prevention and treatment of thromboembolic diseases. However, warfarin use is complicated by its narrow therapeutic range and inter-individual variability in the starting dose required to achieve a stable international normalised ratio (INR). Warfarin is initiated clinically at 5mg/day then subsequent doses are adjusted accordingly to achieve a stable targeted INR. However, inter-individual variability in response to the warfarin starting dose has been observed and this is reported to be attributed to by various genetic and non-genetic factors. Non-genetics factors implicated in the warfarin dose variability include age, gender, body weight, comorbidities and concomitant drugs. Genetic factors affecting warfarin dose variability include variation in genes encoding the warfarin metabolising enzymes and targeted proteins. Genetic variants in *CYP2C9* and *VKORC1* have been extensively studied on how they affect warfarin dose variability, culminating in several dosing algorithms incorporating genetic (i.e., *CYP2C9*2, CYP2C9*3* and *VKORC1 g.-1639G>A*) and non-genetic factors (i.e., age, body surface area, amiodarone, race, targeted INR, smoking and thromboembolism). However, these studies have often excluded African populations, therefore missing variants that might be important in the prediction of warfarin doses among Africans. Data on variants that specifically affect warfarin dose variability among Africans is lacking, with no dosing algorithms tailored specifically for Africans developed to date. Thus, the main aim of the study is to conduct a comprehensive evaluation of important genetic and non-genetic factors affecting warfarin response, and further make recommendations on variables important for the development of appropriate algorithms for warfarin dosing among black Africans and the Mixed Ancestry population group in Southern Africa.

Method:

A total of 302 black Africans and 277 Mixed Ancestry adults undergoing warfarin treatment were recruited at INR clinics in the Western Cape Province, South Africa and Harare, Zimbabwe. Their DNA samples were extracted and utilised for downstream

analyses. A total of 73 candidate variants involved in either pharmacokinetics or pharmacodynamics of warfarin, were genetically characterised using a combination of allelic discrimination, Sanger sequencing, restriction fragment length polymorphism and iPLEX PGx74 Mass Array platform. Various statistical packages in STATA, R, haploview and plink were employed to determine frequency distribution, linkage disequilibrium and haplotype mapping of the studied genetic variants. Furthermore, genetic and non-genetic variables were correlated with warfarin maintenance dose and their cumulative effect on warfarin dose variability measured through a multivariate step-wise regression analysis in both the black African and Mixed Ancestry cohorts. Whole exome sequencing was done using the ion torrent Sequence ion S5 system in selected black African individuals presenting with extreme phenotypes (i.e., very low dose or very high dose) but who did not harbour variants known to significantly affect warfarin dose requirements. A workflow which applied various bioinformatics tools was employed for the analyses of the resultant raw BAM files, subsequently, population structure and frequency distribution patterns were described among our cohort and individuals in the 1000 Genomes project. Specific variants identified through WES were prioritised according to clinical significance and further genotyped in an enhanced sample size of 252 black Africans, to confirm their effect on warfarin dose requirements.

Results:

The common comorbidities among participants were hypertension (43-46%), heart failure (39-45%), diabetes mellitus (18%), arrhythmia (25%) and HIV infection (15%). Accordingly, the most common co-prescribed drugs were antihypertensives, antiarrhythmic drugs, antidiabetics and antiretroviral therapy. Our results further revealed quantitative and qualitative differences in the allele frequency distribution and linkage disequilibrium mapping of the studied genetic markers between black Africans and the Mixed Ancestry group. For instance, *CYP2C9*3, VKORC1*2, VKORC1*4* and *CYP4F2*2* occurred at higher frequencies in the Mixed Ancestry group compared to black Africans, whilst variants *CYP2C9*8* and *VKORC1 g.6171T* were only reported among black Africans. Furthermore, the WES data showed inter-population variation in allele frequency distribution patterns of 49 variants extracted from 10 actionable

pharmacogenes (i.e., *CYP2B6, CYP2C9, CYP2C19, NUDT15, CYP3A5*, *CYP4F2, NAT2, SLCO1B1, UGT1A1* and *VKORC1*) when African populations (i.e., our black African cohort in Southern Africa, Kenyans (Luhya), Nigerians (Yoruba) and Gambians) were compared with Hispanic Americans, Europeans, East and South Asians. For instance, over 90% of the variants that were identified among black Africans (our study) across all 10 actionable genes were also present in all other African population groups from the 1000 genomes project, whilst approximately 40% of variants which included *CYP2B6* rs28399499, rs34749331, *CYP2C9* rs2256871, *CYP2C19* rs17884712, *CYP3A5* rs41303343, rs546268184, rs531042654, rs200312875, rs8175345 and *NAT2* rs12720065 were either rare or absent in either Hispanic Americans, Europeans, East Asians or South Asians.

SNPs *CYP2C rs12777823G>A, CYP2C8 c.805A>T (*2), CYP2C9 c.449G>A (*8), CYP2C9 c.1008C>T (*11), CYP3A5 c.624G>A (*6)* and *MTHFR c.677C>T* were significantly associated ($p<0.05$) with warfarin dose requirements among black Africans. Furthermore, *PROC c.423G>T* (rs5936) and *EPHX1 g.26978G>C* (rs2260863) prioritised from the WES data showed a significant effect ($p=0.04$) on warfarin dose requirements in a recessive and codominant genetic model, respectively. Thus, SNPs *CYP2C rs12777823G>A, CYP2C8 c.805A>T (*2), CYP2C9 c.449G>A (*8), CYP2C9 c.1008C>T (*11), CYP3A5 c.624G>A (*6), MTHFR c.677C>T, PROC c.423G>T* and *EPHX1 g.26978G>C* together with age, deep venous thrombosis and mechanical valve replacement explained 40% of the warfarin dose variability among black Africans. In contrast, *VKORC1* SNPs *g.-1639G>A (rs9923231), c.1173C>T (rs9934438)* and g.*9041G>A (rs7294)* were the main genetic factors significantly affecting warfarin dose requirements among the Mixed Ancestry group. Thus, a *VKORC1* haplotype comprised *of VKORC1 g.-1639G>A, c.1173C>T* and g.*9041G>A* coupled with age and BMI explained 22% of warfarin dose variability among the Mixed Ancestry.

Discussion and conclusion:

Pharmacogenetic profiles present with high inter-ethnic variability which further influences the variability in drug response. We report of genetic variants that have not

previously been shown to be important in warfarin pharmacogenetics. Thus, genetic markers *CYP2C9*2, CYP2C9*3* and *VKORC1 g.-1639G>A* which are regarded as the most informative pharmacogenetic profiles for warfarin dose requirements appear rare and seem to have little or no influence on the warfarin dose variability that is observed among black Africans of Bantu origin. However, variants occurring uniquely among Africans which include *CYP2C9*5, CYP2C9*6, CYP2C9*8* and *CYP2C9*11* seem to be the important drivers in explaining the warfarin dose variability among Africans. Furthermore, the warfarin dose variability among Africans seems to be affected by genetic variation in other genes not traditionally studied for warfarin pharmacogenetics and these genes include *CYP2C8, CYP3A5, MTFHR, EPHX1* and *PROC*.

The contribution of markers in various genes among black Africans further buttresses the importance of including a wide spectrum of genes in pharmacogenetic analyses instead of focusing only on genes described to be principally involved in the drug's pathway. Furthermore, to ensure that pharmacogenetics is effectively applied in Africans, dosing guidelines which are inclusive of African-specific pharmacogenetic profiles are required and should be prioritised to further ensure health equity. Thus, future research should focus on carrying out collaborative studies that will capture a large-scale of genomic data that is inclusive of various African population groups. This will ensure confirmation of the current warfarin pharmacogenetic data whilst also decoding additional informative genetic profiles. Collaborative studies should also focus on ensuring that warfarin pharmacogenetic-based dosing algorithm tailored specifically for Africans are developed and validated through conducting trials. For pharmacogenetics of warfarin to be fully applied, it is time that African-specific genetic variants reported in this work be considered for implementation to further improve warfarin-dosing outcomes in African populations.

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Abbreviations

ABCB1:	ATP binding cassette subfamily B member 1
ADE:	Adverse drug event
ADME:	Absorption, distribution, metabolism and excretion
ADRs:	Adverse drug reactions
AF:	Atrial fibrillation
AIDS:	Acquired immunodeficiency syndrome
APOE:	Apolipoprotein E
ARVs:	Antiretrovirals
BA:	Black Africans
BMI:	Body mass index
BSA:	Body surface area
CALU:	Calumenin
CAR:	Constitutive androstane receptor
CDC:	Centres of disease control and prevention
CHD:	Coronary heart diseases
CHF:	Congestive heart failure
CNS:	Central nervous system
COMT:	Catechol-O-methyltransferase
COPD:	Chronic obstructive pulmonary disease
CPIC:	Clinical Pharmacogenetics Implementation Consortium
CVD:	Cardiovascular diseases

CYP:	Cytochrome P450
CYP1A2:	Cytochrome P4501A2
CYP2C9:	Cytochrome P4502C9
CYP3A4:	Cytochrome P4503A4
CYP4F2:	Cytochrome P4504F2
dbSNP:	Single Nucleotide Polymorphism Database
DNA:	Deoxyribonucleic acid
DP:	Read depth
DOACs:	Direct oral anticoagulants
DRD2:	Dopamine receptor D2
DTG:	Dolutegravir
DVT:	Deep vein thrombosis
EFV:	Efavirenz
EPHX1:	Epoxide hydrolase 1
Exo I:	Exonuclease I
F2:	Coagulation factor 2
F5:	Coagulation factor 5
FDA:	Food and Drug Administration
GATK:	Genome Analysis Toolkit
GCHC:	Gugulethu Community Health Centre
gDNA:	Genomic DNA
GGCX:	Gamma-glutamyl carboxylase

GLP1R:	Glucagon-like peptide 1 receptor
GQ:	Genotype quality
GSH:	Groote Schuur Hospital
GWAS:	Genome wide association study
HIV:	Human immunodeficiency virus
HWE:	Hardy-Weinberg equilibrium
IDT:	Integrated DNA Technologies
IHD:	Ischemic heart disease
IM:	Intermediate metabolisers
INDELS:	Insertions and deletions
INR:	International normalised ratio
IWPC:	International Warfarin Pharmacogenetics Consortium
KBP:	Kilobase pairs
KH2:	Vitamin K hydroquinone
LD:	Linkage disequilibrium
LWK:	Luhya in Webuye Kenya
MA:	Mixed Ancestry
MHVR:	Mechanical heart valve replacement
MICE:	Multivariate imputation by chained equations
MPC:	Mitchell's Plain Community Health Centre
MRCZ:	Medical Research Council of Zimbabwe
mRNA:	Messenger RNA

MTHFR:	Methylenetetrahydrofolate reductase
MVD:	Mixed valve disease
NCBI:	National Center for Biotechnology Information
NGS:	Next-generation sequencing
NR1I2:	Nuclear receptor subfamily 1 group I member 2
NR1I3:	Nuclear receptor subfamily 1 group I member 3
OPRM1:	Opioid receptor Mu1
PCA:	Principal component analysis
PCR:	Polymerase chain reaction
PE:	Pulmonary embolism
PGH:	Parirenyatwa Group of Hospitals
P-gp:	P-glycoprotein
PGx:	Pharmacogenomics/pharmacogenetics
PM:	Poor metabolisers
PNPLA5:	Patatin like phospholipase domain containing 5
PXR:	Pregnane X receptor
RFLP:	Restriction fragment length polymorphism
RI:	Responsible innovation
SA:	South Africa
SAMRC:	South African Medical Research Council
SD:	Standard deviation
SLCO1B1:	Solute carrier organic anion transporter family member 1B1

SNP:	Single-nucleotide polymorphism
SNVs:	Single nucleotide variants
SSA:	Sub-Saharan Africa
SULT4A1:	Sulfotransferase family 4A member 1
TB:	Tuberculosis
TIA:	Transient ischemic attack
TMAP:	Torrent Mapping Alignment Program
UCT-HREC:	University of Cape Town Human Research Ethics Committee
UGT1A1:	UDP glucuronosyltransferase 1 family polypeptide A1
VHD:	Valvular heart diseases
VKORC1:	Vitamin K epoxide reductase complex 1
VTE:	Venous thromboembolism
WES:	Whole exome sequencing
WGS:	Whole genome sequencing
YRI:	Yoruba in Ibadan Nigeria

Chapter 1: Introduction and Literature review

Synopsis:

Chapter 1 comprises of 4 sections which will include the following:

1. Background of the study which gives an overview of statistics on the use of warfarin, epidemiology of warfarin indications, risk factors of warfarin response, outline of the warfarin pathway and the genetics or pharmacogenetics of warfarin response.

2. A published review focusing on outlining the pharmacogenetics of warfarin among African populations. The citation of the review is given below:
   "Ndadza A, Thomford NE, Mukanganyama S, Wonkam A, Ntsekhe M and Dandara C. The Genetics of Warfarin Dose-Response Variability in Africans: An Expert Perspective on Past, Present, and Future. https://doi.org/10.1089/omi.2019.0018."

3. The study rationale.

4. The study aims and objectives.

1.1. General Background to the project

Warfarin, a coumarin derivative, is an anticoagulant that has been used for over 70 years [1] and still remains highly prescribed globally and an anticoagulant of choice in resource limited settings such as Africa [2]. In 2004, over 30 million prescriptions of warfarin were reportedly distributed in the United States alone [3], and as of 2022 the warfarin prescription is estimated to be over 11 million, ranking at position 58 among the top 200 most prescribed medications in the United States [4]. The decline in warfarin dose prescription in developed economies, is attributed to the introduction of direct oral anticoagulants (DOACs), classified as direct thrombin inhibitors (i.e., dabigatran) and oral direct factor Xa inhibitors (i.e., apixaban, rivaroxaban, edoxaban and betrixaban). DOACs act by inhibiting specific proteins within the clotting cascade, hence they are considered to be safer with better safety profiles [5], however in low resourced settings such as in Africa, warfarin is persistently still the highly used anticoagulant [2]. This is documented in studies that have reported high usage of warfarin in their respective hospitals in various parts of Africa [6-8]. For instance, a study in 2020 which evaluated anticoagulation services in five Ugandan and South African hospitals, reported that DOACs (i.e., rivaroxaban) was only available in one of the five hospitals, whilst warfarin was the primary anticoagulant being offered across the five hospitals evaluated [6]. The consistent high usage of warfarin in developing countries such as those in Africa, is attributed to warfarin's low cost and familiarity of use by physicians as compared to newer anticoagulants which are expensive and whose use is limited to patients with private health insurance [9].

Warfarin is essential in the treatment and prevention of thromboembolism in individuals with mechanical heart valves, atrial fibrillation (AF), pulmonary embolism (PE) and deep vein thrombosis (DVT) [10-12]. Although the prevalence of warfarin treated conditions is not well defined in Africans, they form part of cardiovascular diseases that contribute to increased global disease burden and cause of numerous deaths in developing economies [13-15]. Furthermore, these conditions often lead to complications such as stroke and ischemic heart disease which account for about 25% mortality rate worldwide [16]. Warfarin dosing is however challenging owing to its narrow therapeutic

range and the inter-individual variability in dose requirements, which makes it difficult to titrate the optimal starting dose of 5mg/day to reach a stable recommended international normalised ratio (INR) of 2-3 [17]. Poor anticoagulation control (defined as having a time in therapeutic INR range (TTR) of <65%) has been observed among African patients due to the lack of applicable dose-prediction models available in this region [6]. The difficult in identifying the appropriate warfarin starting dose results in under or over anticoagulation related adverse drug reactions (ADRs), such as persistent thrombotic events or excessive bleeding, respectively, which often lead to hospital admissions and death in certain cases [1, 18]. Hence, patients on warfarin treatment are regularly monitored and doses regularly adjusted in an effort to reach a stable targeted INR with minimum failure [19].

Warfarin optimal dosing is further complicated by various non-genetics and genetics factors, which is evidenced by the inter-individual variability observed in the way patients respond to standard warfarin starting dose. Non-genetics factors include demographic characteristics (e.g., age, gender and body weight) [20-22], clinical variables (e.g., comorbidities and concomitant drugs) [21-23] and social behaviour (e.g., diet, alcohol consumption and smoking status) [22, 23]. Warfarin dose sensitivity is reported to be heightened among older patients, thereby resulting in the need for generally decreased warfarin dose requirements [24, 25]. It is important to note that vitamin K is a pre-requisite for the activation of clotting factors, thus, an inconsistent intake of vitamin K rich diet such as green vegetables and vegetable oils results in warfarin dose requirements variability. Individuals with decreased vitamin K intake are sensitive to warfarin [26], whereas those with higher vitamin K intake are generally resistant to warfarin [27]. Other factors such as smoking, alcohol consumption and the use of concomitant drugs affect warfarin dose by either inhibiting or activating warfarin metabolism. Drugs that interact with warfarin include amiodarone and rifampicin which act through inhibition and induction of warfarin metabolism, respectively, consequently increasing or decreasing the INR levels [28-30]. It has also been reported that the use of warfarin concurrently with the medicinal herb, St John's wort decreases the anticoagulant effect of warfarin by prematurely inducing warfarin metabolism and

consequently elevating its clearance [31]. Hence clinical warfarin dosing prediction models which incorporate, variables such as age, height, weight (or body surface area), race, smoking and interacting drugs have been proposed [20].

Genetic factors affecting warfarin dose variability include variation in genes encoding enzymes involved in either the warfarin pharmacokinetics (PK) or pharmacodynamics (PD) pathways. The warfarin PK pathway includes enzymes responsible for the transport, metabolism and clearance of warfarin, whilst the PD pathway includes enzymes that are directly or indirectly targeted by warfarin. Warfarin exerts its anticoagulant effect by disrupting the vitamin K cycle, through inhibiting the functioning of the vitamin K epoxide reductase complex 1 (VKORC1) in recycling vitamin K epoxide to reduced vitamin K [32]. The biotransformation or hydrolysis of VKORC1 is through the microsomal epoxide hydrolase (EPHX1) [33]. Vitamin K is a co-factor for the activation of procoagulant factors II, VII, IX, and X, as well as antithrombotic protein C, protein S and protein Z through the post-translational gamma-carboxylation of their glutamic acid residues by gamma-glutamyl carboxylase (GGCX) [34]. The action of GGCX is regulated through inhibition by a molecular chaperone calumenin which is encoded by CALU [35]. The uptake of vitamin K to the liver, where it becomes functionally transformed to reduced vitamin K and participate in the vitamin K cycle, is through apolipoprotein (APOE) bound primarily to chylomicrons [36]. Vitamin K is eliminated in circulation through oxidation from vitamin K1 to hydroxyvitamin K1 by CYP4F2 [37].

Warfarin exists and is administered as a racemic mixture of S- and R-enantiomers, of which the former is 3-5 folds potent than the latter [38]. Upon administration, warfarin is immediately absorbed from the gastrointestinal (GI) tract with 100% bioavailability [39]. It reaches the liver bound primarily to albumin, whereby S-warfarin is principally metabolised by CYP2C9 to its inactive metabolite 7-hydroxywarfarin and to a lesser extent 6-hydroxywarfarin [40, 41]. In contrast, R-warfarin is mainly metabolised by CYP1A1, 1A2, CYP3A5 and 3A4 to its inactive metabolites that include 6-, 8-hydroxywarfarin and 10-hydroxywarfarin [40, 41]. Other enzymes involved in warfarin metabolism include CYP2C19, CYP2C8 and CYP2C18, whose contribution is limited

[40]. Warfarin excretion is through the kidney and bile, with the latter mediated by an ATP-dependent trans-membrane efflux transport, P-glycoprotein (P-gp), which is encoded by the ATP-binding transporter B1 (*ABCB1*) gene [42]. Figure 1.1 illustrates the warfarin interactive pathway encompassing warfarin metabolism and mode of action.

Owing to their principal roles in warfarin disposition, *CYP2C9* and *VKORC1* have been the most widely studied genes for their pharmacokinetic and pharmacodynamic effects on warfarin response variability, respectively. *CYP2C9* is located in the chromosomal region 10q24 clustering together with other members of the CYP2C family, in the following order; *CYP2C18, CYP2C19, CYP2C9* and *CYP2C8* [20]. Generally, in CYP nomenclature, the *1 allele (e.g., *CYP2C9*1)* is regarded as the wild type allele producing protein with expected functional activity and all other alleles are compared to it. Thus, in terms of warfarin dosing and metabolism, it is expected that individuals homozygous for the *1 allele would perform the expected function normally. There are several *CYP2C9* alleles [43] that have been reported which include *CYP2C9*2* (*430C>T, Arg144Cys* in exon 3) and *CYP2C9*3 (1075A>C, Ile359Leu* in exon 7) [20, 44, 45]. Alleles *CYP2C9*2* and *CYP2C9*3* are associated with reduced enzyme activity of ~30% and 80%, respectively [46]. Individuals carrying these variants in hetero- or homozygous state require reduced warfarin doses to reach similar effects [47-49]. Given standard doses, individuals, possessing *CYP2C9*2* and *CYP2C9*3* are likely to present with plasma warfarin levels which lead to bleeding episodes [50, 51]. It is important to note that additional CYP variants such as *CYP2C9*5 (1080C>G), CYP2C9*6 (818delA), CYP2C9*8 (449G>A)* and *CYP2C9*11 (1003C>T)* have also been reported and act similar to *CYP2C9*2* and *CYP2C9*3* [52-57].

VKORC1 gene located in the chromosomal position 16p11.2, is roughly 5 kilobase pairs (kbp) long, comprising of 3 exons and 2 introns [58, 59]. SNP *VKORC1 g.-1639G>A* located in the *VKORC1* promoter region has been reported to result in reduced expression levels of VKORC1 [60], subsequently requiring reduced warfarin dose requirements by over 30% [48, 49]. Although *VKORC1 g.-1639G>A* is the most widely studied *VKORC1* marker, Rieder et al [61], previously mapped and reported on 10

noncoding *VKORC1* SNPs including *VKORC1 g.-1639G>A*. Reports show that *VKORC1 g.-1639G>A* together with four other SNPs (i.e., *VKORC1 381C>T, 6484T>C, 6853G>C,* and *7566T>C*) are in strong linkage disequilibrium ($r^2 \geq 0.9$). Furthermore, the studied SNPs were used to infer common haplotypes of which the haplotype group A comprised of SNPs significantly associated with a low dose whilst group B was significantly associated with a high warfarin dose. However, *VKORC1* haplotypes do not explain more variability than any of the single *VKORC1* SNPs which have been significantly associated with warfarin dose [61]. Hence, studies usually consider SNP *VKORC1 g.-1639G>A* or alternatively *VKORC1 c.1173C>T* for warfarin pharmacogenetics [59]. As a result, *VKORC1 g.-1639G>A* alongside *CYP2C9*2* and *CYP2C9*3* have been incorporated with various non-genetic variables (e.g., age, body surface area, amiodarone, race, targeted INR, smoking and thromboembolism) to develop pharmacogenetic-guided warfarin dosing algorithms for the prediction of suitable warfarin initial dose [62-65].

The current available pharmacogenetic-guided warfarin dosing algorithms are reportedly highly effective among population groups such as Europeans explaining over 50% of warfarin dose variability, whilst among Africans they explain as low as 26% warfarin dose variability [65-67]. The inter-ethnic variability in the effectiveness of the various pharmacogenetic dosing algorithm is a result of data used to develop these algorithms being extracted mainly from Europeans while missing African ancestry genetic profiles that could be informative on warfarin response [66]. There are nearly 30 genes involved in the warfarin disposition pathway, however, current dosing algorithms concentrate on genetic variation in *CYP2C9* and *VKORC1* genes [68], failing to capture genetic markers in other warfarin-related genes that could be informative in predicting warfarin dose. Thus, to close the genetic characterisation gap we set out to review literature and see the extent of exclusion of certain genes and populations, then develop a rationale of what we intended to do. A review scoping the past, present and future status of pharmacogenetics of warfarin in Africans was undertaken.

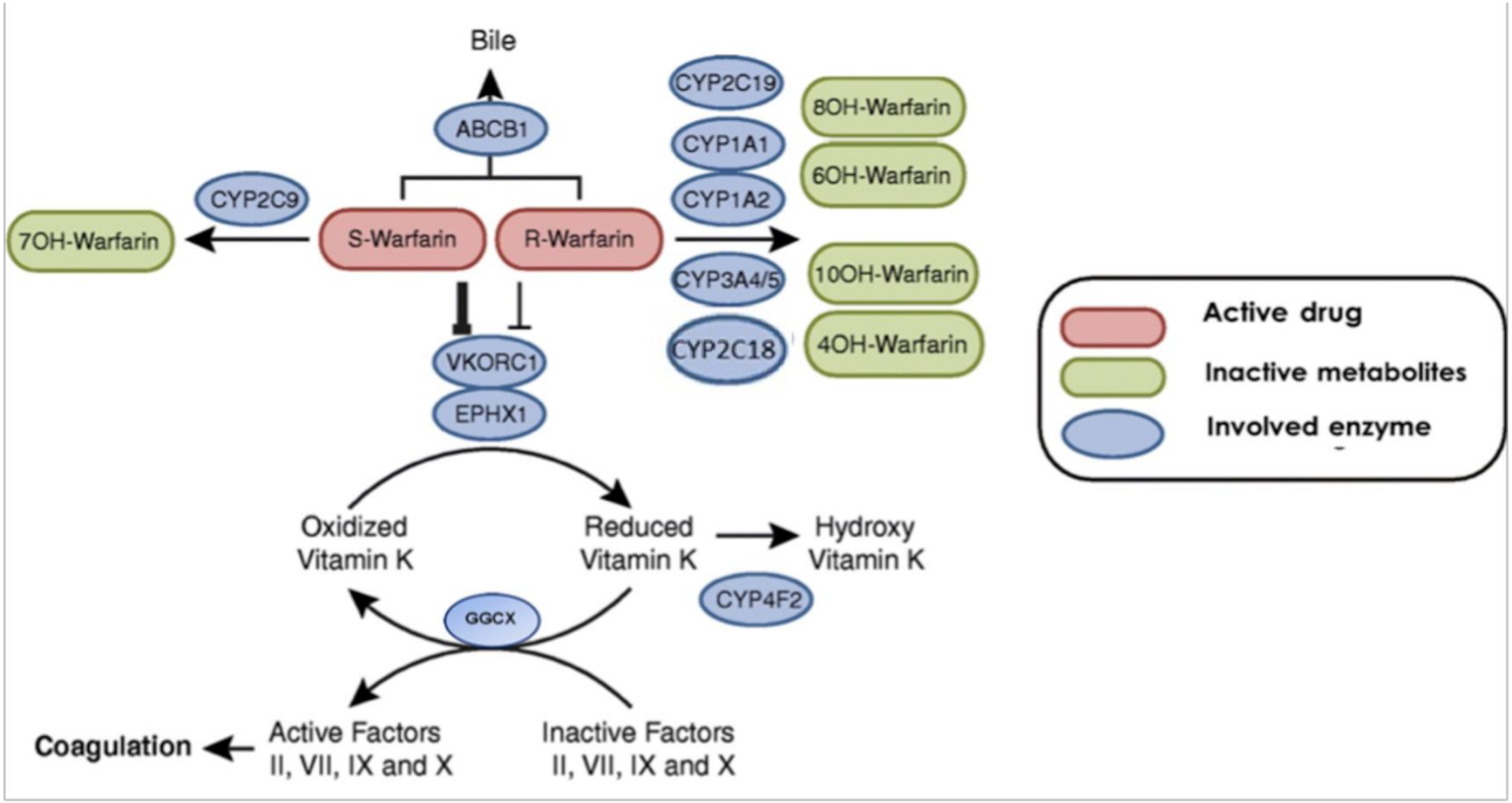

Figure 1.1: The warfarin interactive pathway, illustrating the warfarin metabolism, mode of action and enzymes involved. (Adapted with modifications from: Ingelman-Sundberg et al [69])

1.2. Literature review

1.2.1. The Genetics of Warfarin Dose-Response Variability in Africans: An Expert Perspective on Past, Present, and Future

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CD: idea conceptualisation, supervised all components as principal investigator and reviewed the manuscript drafts.

All authors contributed to the final version of the article. The authors read and approved the final manuscript.

The Genetics of Warfarin Dose–Response Variability in Africans: An Expert Perspective on Past, Present, and Future

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Abstract

Coumarins such as warfarin are prescribed for prevention and treatment of thromboembolic disorders. Warfarin remains the most widely prescribed and an anticoagulant of choice in Africa. Warfarin use is, however, limited by inter-individual variability in pharmacokinetics and a narrow therapeutic index. The difference in patients' pharmacodynamic responses to warfarin has been attributed to genetic variation in warfarin metabolism and molecular targets (e.g., *CYP2C9* and *VKORC1*) and host–environment interactions. This expert review offers a synthesis of human genetics studies in Africans with respect to pharmacogenetics-informed warfarin dosing. We identify areas that need future research attention or could benefit from harnessing existing pharmacogenetics knowledge toward rational and optimal therapeutics with warfarin in African patients. A literature search was conducted until January 2019. A total of 343 articles were retrieved from nine African countries: Botswana, Ethiopia, Egypt, Ghana, Kenya, South Africa, Sudan, Tanzania, and Mozambique. We found 19 studies on genetics of warfarin treatment specifically among Africans. Genes examined included *CYP2C9, VKORC1, CYP4F2, APOE, CALU, GGCX*, and *EPHX1*. *CYP2C9*2* and **3* alleles were highly frequent among Egyptians, while rare in other African

populations. *CYP2C9*5, *8, *9*, and **11*, and *VKORC1 Asp36Tyr* genetic variants explained warfarin variability in Africans better, compared to *CYP2C9*2* and **3*. In Africa, there is limited pharmacogenetics data on warfarin. Therefore, future research and funding commitments should be prioritised to ensure safe and effective use of warfarin in Africa. Lessons learned in Africa from the science of pharmacogenetics would inform rational therapeutics in haematology, cardiology, and surgical specialties worldwide.

Keywords: global pharmacogenetics, anticoagulation, warfarin, Africa, *CYP2C9, VKORC1*

1.2.1.1. Introduction

Coumarins such as warfarin, phenprocoumon, and acenocoumarol are prescribed for the prevention and treatment of thromboembolic disorders [70, 71]. Furthermore, direct oral anticoagulants (DOACs) with better safety profiles, which include rivaroxaban, apixaban, dabigatran, and edoxaban, have recently been introduced in the market [72, 73]. In Africa, the most common indications for anticoagulation include atrial fibrillation, venous thromboembolism, and their associated complications such as stroke [14, 74, 75]. Although the prevalence of these conditions is not well defined in Africans, they form part of cardiovascular diseases that contribute to increased global disease burden and cause of death in developing economies [13-15]. Thus, warfarin remains the most widely prescribed and an anticoagulant of choice in Africa due to extensive knowledge of its use by physicians and low cost [15], while other anticoagulants such as phenprocoumon, acenocoumarol, and DOACs are expensive and their use is limited to patients on private health insurance and those in resource-rich countries [76, 77].

Warfarin use is beset by inter-individual variability and a narrow therapeutic index, which makes it difficult to titrate the optimal starting doses to reach the international normalised ratio (INR) [17, 44]. Prolonged titration to identify the appropriate dose to reach INR is associated with adverse drug effects and the need for hospitalisation [17, 78]. Therefore, warfarin requires rapid and continuous monitoring of the INR, before reaching stable INR of 2 to 3 [20]. The differences in patients' responses to warfarin

have been attributed to both environmental and genetic factors [79]. Environmental factors include the following: race, age, gender, body weight, comorbidities, comedications, and lifestyle variables such as diet, alcohol consumption, and smoking [20, 80, 81]. For instance, warfarin dose requirements are reported to decrease with increasing age [25, 81]. With respect to diet, an inconsistent intake of vitamin K-rich foods such as green vegetables and vegetable oils affects warfarin dose requirements [82]. Thus, individuals with decreased vitamin K intake are said to be sensitive to warfarin, whereas those with higher vitamin K intake may be resistant to warfarin [26]. Warfarin activity is also affected by medications such as amiodarone and rifampin through inhibition and induction of metabolism, respectively, consequently increasing or decreasing the INR levels [28-30]. Genetic factors include variants in genes involved in warfarin metabolism and genes coding for warfarin-targeted proteins [48, 58].

Warfarin is administered as a racemic mixture of S- and R-enantiomers. The S-form is the most potent [40, 45]. Warfarin exerts its anticoagulant effects by inhibiting the action of vitamin K epoxide reductase complex 1 (VKORC1), an enzyme involved in the interconversion of vitamin K-2,3-epoxide (vitamin K epoxide) to reduced vitamin K hydroquinone (KH2) [32]. The S-warfarin form is principally metabolised by CYP2C9, while R-warfarin is mainly metabolised by CYP1A2 and CYP3A4, with other enzymes such as CYP2C18, CYP2C19, and CYP2C8 playing minor roles [40, 41]. Single-nucleotide polymorphisms (SNPs) in *CYP2C9, VKORC1*, and *CYP4F2* have largely been shown to affect warfarin dose variability [48, 50, 58, 83-85]. Furthermore, variants in *CYP2C9* and *VKORC1* have been consistently shown to account for up to 40% of the total variability in warfarin dose requirements among Caucasians and Asians [45, 48, 49, 86]. Hence, data on effects of genetic variants have been incorporated together with environmental factors in developing pharmacogenetics-based warfarin dosing algorithms.

These algorithms enable better prediction of warfarin starting dose that allows quick achievement of the required INR. The algorithms mostly include the following SNPs: *VKORC1 g.-1639G>A, CYP2C9 c.430C>T (*2),* and *CYP2C9 c.1075A>C (*3),* due to their inactivating effects on carrier enzymes [63, 65, 87]. Although warfarin

pharmacogenetics algorithms have been applied in Caucasians and Asians, they have not been considered in native Africans [66, 67, 88]. Furthermore, data used to develop these algorithms are extracted mostly from Europeans and Asians [66, 89]. Therefore, it is of importance to determine variants that influence warfarin variability in African populations with an effort to reduce the mismanagement of warfarin use and its associated side effects. This expert review evaluates the human genetics studies in African patients with respect to warfarin dose–response variations. The ultimate goal is identifying areas that still need attention or could benefit in making informed recommendations on the management of warfarin in Africans.

1.2.1.2. Literature Search

A literature search was conducted from the earliest data available until January 2019 using the following databases: PubMed, Google Scholar, Academic Search Premier, and MEDLINE. The keywords used individually or in combination in the search included the following: ‘‘Warfarin AND Africa,’’ ‘‘Pharmacogenetics AND warfarin AND Africa,’’ ‘‘Warfarin algorithm AND Africa,’’ and ‘‘Warfarin AND Blacks.’’ A total of 343 articles were retrieved from the search and studies were filtered down using the study selection criteria illustrated in Figure 1.2. After identification and screening, 34 studies were eligible for review.

1.2.1.2.1. Selection criteria

The inclusion criteria were as follows: (1) studies focusing on warfarin therapy in African populations, (2) articles focused on either genetics of warfarin alone or with both genetics and non-genetic factors, (3) research articles, and (4) articles published in English. Articles excluded comprised review articles, studies on warfarin in non-African populations, studies focused on the use of other anticoagulants such as other coumarin derivatives and DOACs, and studies reporting on warfarin indications only.

1.2.1.2.2. Data extraction

For each study, the following data were extracted: the investigators, title of the study, the year of publication, study country, patients' data (e.g., demographic data), clinical data (indications and comorbidities), and studied genes.

1.2.1.3. Results

A total of 34 studies conducted in native Africans undergoing warfarin treatment were identified for the purpose of the expert review. The studies were conducted in individuals from the following nine African countries: Botswana, Ethiopia, Egypt, Ghana, Kenya, South Africa, Sudan, Tanzania, and Mozambique. The most common indication for warfarin in African patients was mechanical heart valve replacement (19–89%), followed by vascular heart disease (17–75%), atrial fibrillation (6–65%), and venous thromboembolism (4–65%) (Table 1.1). More than 30% of the studies were conducted in Egypt (n=11). Genes explored included *CYP2C9, VKORC1, CYP4F2, APOE, CALU, GGCX*, and *EPHX1* (Table 1.1).

The warfarin indications were accompanied by comorbidities such as HIV/AIDS and tuberculosis co-infections [75, 90]. The medications used for the treatment of comorbidities pose risks of drug–drug interactions with warfarin [91]. For example, some studies have shown that antiretroviral therapy potentiates an extended anticoagulation and reduces INR when co-administered with warfarin [75, 92]. The risks of warfarin use have led to recommendations for warfarin anticoagulation monitoring clinics as services to improve anticoagulation control [93, 94]. Anticoagulation control refers to when patients would spend 60% of the time within the required warfarin therapeutic range [95-97]. However, it is worth noting that pharmacogenetics should be accounted for when managing warfarin treatment as it has been reported to explain over 40% of warfarin variability [48, 86, 98].

CYP2C9 is the main enzyme involved in the metabolism of warfarin, reducing S-warfarin to 6- and 7-hydroxywarfarin metabolites [41]. Hence, *CYP2C9* is the most studied pharmacokinetic gene with regard to warfarin response [45]. Although there are several *CYP2C9* variants that have been reported [43], *CYP2C9*2* and *CYP2C9*3* are

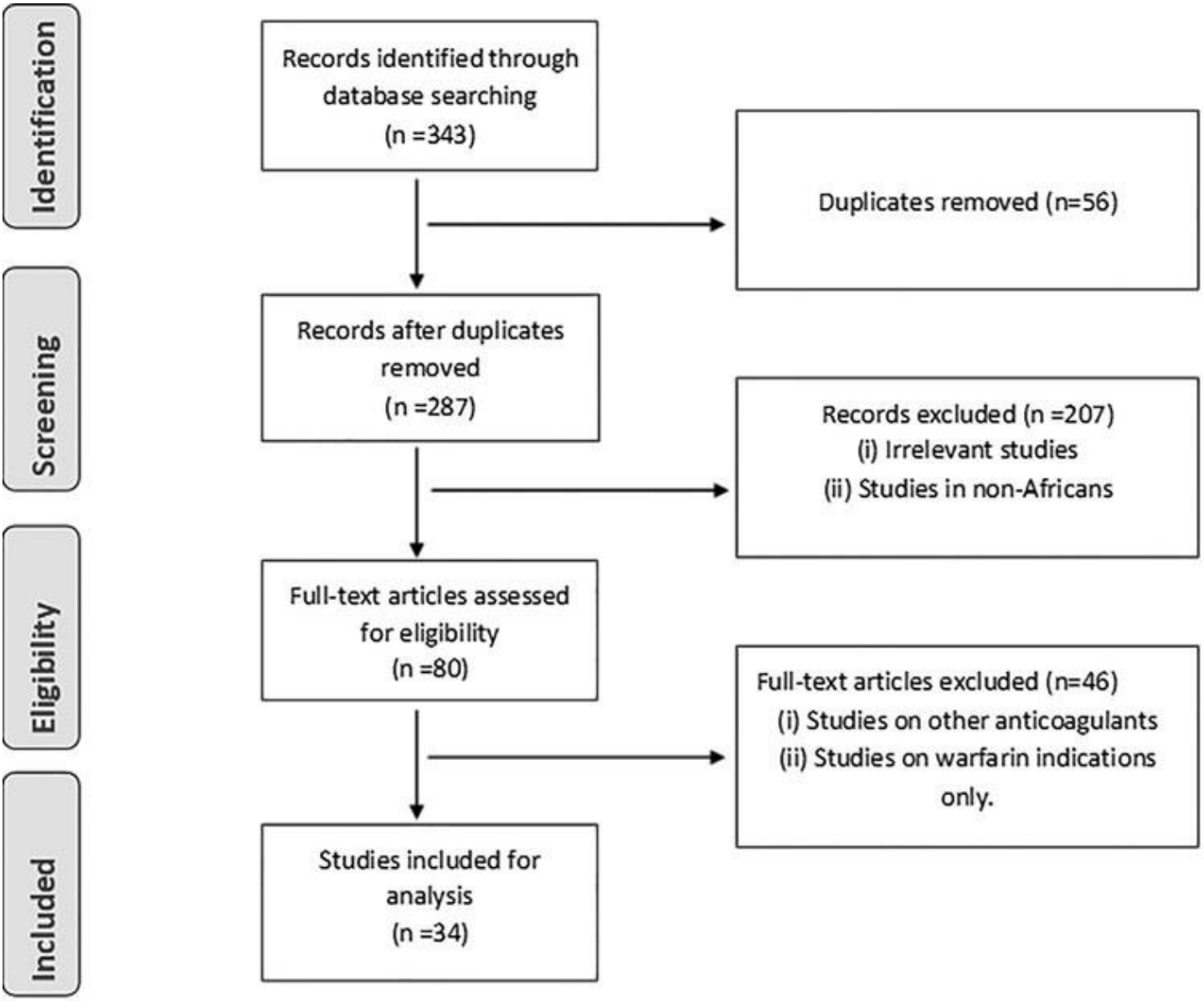

Figure 1.2: Flowchart of the literature search employed in this review

the most studied with regard to warfarin response, especially in Caucasians and Asians [48, 50, 84, 86, 98-100]. *CYP2C9*2* and *CYP2C9*3* alleles reduce enzyme activity, thereby decreasing warfarin metabolism by ~30% and 80%, respectively [45, 50]. However, studies conducted across different African population groups (including African Americans) have reported absence or rarity of both *CYP2C9*2* and *CYP2C9*3* [101-109] (Table 1.2). Hence, a lack of influence of these variants on warfarin variability is notable in populations with an African ancestry [92, 107, 110, 111].

Understandably, African populations such as Egyptians and Tunisians have reported the presence of *CYP2C9*2* and *CYP2C9*3* (Table 1.2), and this can be explained by their pronounced admixture with Asian and Caucasian populations [112-115]. Thus, *CYP2C9*2* and *CYP2C9*3* have been significantly associated with low warfarin dose requirements in adults of Egyptian descent, explaining over 5.2–15.6% of warfarin variability (Table 1.3) [104, 113-115]. Due to the rare nature of *CYP2C9*2* and *CYP2C9*3* in Africans, focus has now shifted to alternative *CYP2C9* variants. Other *CYP2C9* variants that are being explored in Africans include the following: *CYP2C9*5, *6, *8,* and **11* [47, 52]. *CYP2C9*5, *6, *8*, and **11* highly contribute to warfarin response in Africans as their occurrence has been associated with a reduced warfarin response in both African Americans and native Africans [57, 109, 111, 115, 116]. The combined analysis of these variants with either *CYP2C9*2* or **3* has been shown to improve warfarin dose predictions by explaining ~5% and 6% to 7% in Sudanese and African Americans, respectively [57, 109, 116] (Table 1.3). The inclusion of these variants improve warfarin dosing model from 30% to 36% in African Americans [57]. Furthermore, the Clinical Pharmacogenetics Implementation Consortium guideline recommends the warfarin pharmacogenetics-based dosing to be utilised in African Americans only when information on the *CYP2C9*5, *6, *8*, and **11* genotypes is available [45].

VKORC1 is the principal pharmacodynamic gene of interest in warfarin response. Genetic variation in *VKORC1* has been described to be the major contributor to warfarin variability, explaining ~15–34% of the variability [48, 49, 86]. *VKORC1 g.-1639G>A* is the most studied *VKORC1* SNP and occurs in the *VKORC1* promoter region. *VKORC1*

Table 1.1: Clinical, demographic and genetic outlook in Africans undergoing warfarin therapy

Study	Country (Region)	Mean age ±SD in years	Major warfarin indications (%)	Comorbidities	Comedication	Genes characterised	Mean warfarin maintenance dose ±SD mg/week
Bazan et al [103]	Egypt (Cairo)	45.9 ±12.5	AF (35), MHRV (30) and VTE (27)	CHD, dyslipidemia, and hypertension.		*VKORC1* and *CYP2C9*	51 ±5.2
El-Din et al [113]	Egypt Cairo)	41 ±14	MHVR (89) and AF (5)			*VKORC1* and *CYP2C9*	55 ±12
Shahin et al [115]	Egypt (Cairo)	47.4 ±14.7	MHVR (52), DVT (16) and cerebrovascular accident (11)	Hypertension, hyperlipidemia, and diabetes mellitus	Aspirin and glimepiride	*VKORC1, CYP2C9, CYP4F2, APOE* and *CALU*	36.8 ±17.9
Ghozlan et al [114]	Egypt (Cairo)	54 ±8	Acute coronary syndrome			*VKORC1* and *CYP2C9*	33.6 ±13.3
El-Din et al [117]	Egypt (Cairo)	6.59 ±2.97	Cardiac valve replacement (63)			*VKORC1* and CYP2C9	34.1 ±9.3
Ekladious et al [118]	Egypt (Cairo)		VHD (75), DVT (14) and AF (6)	Hypertension, diabetes mellitus, arrhythmia, and CHF		*VKORC1* and *CYP2C9*	38.5 (30.4–49.0)[a]
Hamadeh et al [119]	Egypt (Cairo)	47.4 ±14.7	AF and MHVR			*GGCX, STX1B* and *FPGS*	
Issac et al [120]	Egypt (Cairo)	40.9 ±13.3	VHD (75), DVT (14) and AF (6)	Hypertension diabetes mellitus, arrhythmia, and CHF		*MDR1*, *EPHX1* and *PZ*	42 (31.5-49)[a]
Njovane et al [121]	South Africa (Cape Town)		AF (47), MVD (42), MHVR (39) and VTE/PE (24)	Diabetes mellitus, hypertension, arthritis, COPD, and peptic ulcers	Quinolones, beta-lactams, metronidazole, paracetamol, and anti-ulcer drugs	None	
Schapkaitz & Sithole [92]	South Africa (Johannesburg)	56.1 ±16.3	AF (36), VHD (27) and VTE (23)	HIV and TB	antiepileptics, rifampicin and antiretrovirals	*VKORC1* and *CYP2C9*	Blacks: 37.8 ±20.3 Whites: 26.6
Mbokota et al [94]	South Africa (Johannesburg)	53 ±15	VTE (57), AF (21) and VHD (18)			None	
Laas & Naidoo [7]	South Africa (KwaZulu-Natal)		MHVR (47), AF (42), DVT (9) and PE (4)	Diabetes mellitus, hypertension, dyslipidemia, COPD and IHD		None	
Ebrahim et al [96]	South Africa (Cape Town)	MPC: 57 (48-66) [a] GSH: 53 (41-62)[a]	VHD (46) and AF (25)			None	
Schapkaitz et al [91]	South Africa (Johannesburg)	55 ±16	VTE (31), AF 30) and MHVR (25)	Myocardial infarction and diabetes mellitus	Analgesics, anti-inflammatory drugs and antibiotics	None	
Ndadza et al [107]	South Africa (Cape Town)	55 ±15	MHVR (43), AF (24), DVT (18) and PE (9)	Hypertension, diabetes mellitus, heart failure and HIV	Statins & efavirenz	*CYP2C cluster, CYP2C9* and *VKORC1*	BA: 41 ±14.7 MA: 33 ±13.8
Jacobs et al [122]	South Africa (Cape Town)		AF (38), MHVR (19) and DVT (17)	CVD and HIV	Simvastatin, aspirin, clopidogrel, amiodarone & efavirenz	None	

Study	Country (Region)	Mean age ±SD in years	Major warfarin indications (%)	Comorbidities	Comedication	Genes characterised	Mean warfarin maintenance dose ±SD mg/week
Sonuga et al [123]	South Africa (Cape Town)		AF (65), VHD (17), MHVR (13) and DVT (13)	Hypertension, diabetes mellitus, IHD and others	Simvastatin, aspirin, amiodarone, and others.	None	
Kudzi et al [110]	Ghana (Accra)		MHVR (45), DVT (36), PE (11) and AF (7)			*VKORC1, CYP2C9* and *CYP4F2*	
Shrif et al [109]	Sudan (Khartoum)	39 ±13.9	MHVR (76), AF (14) and DVT (4)	Hypertension, diabetes mellitus, gout, epilepsy, hypothyroidism, and hypertension	Aspirin, amidorone, propranolol, oral antidiabetic drugs and antiepileptic drugs	*VKORC1, CYP2C9* & *PLO3S*	39 ±17.4
Ahmed et al [95]	Sudan (Khartoum)		MHVR (61)	AF, heart failure, mitral stenosis, diabetes mellitus and asthma	Diuretics, β-blockers, and digoxin	None	
Manji et al [97]	Kenya (Eldoret)		VTE (65), valvular dysfunction (19) and MHVR (8)	HIV and TB	ARVs	None	
Kanyi et al [75]	Kenya	40 ±9.91	VTE	HIV	ARVs	None	
Mariita et al [124]	Kenya (Nairobi)		VTE (48), MHVR (34) and heart disease (18)			None	
Fenta et al [125]	Ethiopia (Addis Ababa)		VHD (48), AF (46) and MHVR (30)	Congestive heart failure, hypertension, asthma, and hyper/hypothyroidism		None	
Mwita et al [126]	Botswana (Gaborone)		MHVR (45), DVT (28), AF (17) and intracardiac thrombus (9)	HIV, hypertension, stroke, heart failure, tuberculosis, diabetes mellitus and renal failure		None	35 (35-52.5)[a]

[a]Median (interquartile range).

AF, atrial fibrillation; ARVs, antiretrovirals; BA, black Africans; CHD, coronary heart diseases; CHF, congestive heart failure; COPD, chronic obstructive pulmonary disease; CVD, cardiovascular diseases; DVT, deep vein thrombosis; GSH, Groote Schuur Hospital; IHD, ischemic heart disease; MA, Mixed Ancestry; MHVR, mechanical heart valve replacement; MPC, Mitchell's Plain Community Health Centre; MVD, mixed valve disease; PE, pulmonary embolism; SD, standard deviation; TB, tuberculosis; VHD, valvular heart diseases; VTE, venous thromboembolism

Table 1.2: Minor Allelic distribution of variants in African populations in comparison with other world populations

Gene	Variant name and rs number	Africans					Other populations			References
		Egypt (%)	Ghana (%)	South Africa (%)	Sudan (%)	Others Africans (%)	African Americans (%)	Europeans (%)	Asians (%)	
CYP2C9	*CYP2C9*2 rs1799853*	7-12	0	2-4	5	0[a, c],4[b],9[d], 1[e,f]	9.9	12	0.1	[101, 102, 104, 105, 107-110, 112, 115, 127-129]
	*CYP2C9 *3 rs1057910*	5-12	0	0.5-5	0	1[a], 2[b], 0[c, d], 8[f]	3.7	7	3.3	[101, 102, 106-109, 112, 113, 117, 127-129]
	*CYP2C9*5 rs28371686*	1	0	0.01	1	2[a], 1.8[c]	0.14	0	0	[102, 106, 109, 111, 115]
	*CYP2C9*6 rs9332131*	-	-	0.5	2	0[a], 2.7[c]	0	0	0	[102, 109, 111, 129]
	*CYP2C9*8 rs7900194*	0.8	-	8-13	1	15[a], 8.6[c], 2[e]	0.1	0.2	0-0.5	[102, 107, 109, 111, 115, 128, 129]
	*CYP2C9*9 rs2256871*	-	-	12-18	-	16[c], 6[e]	0.1	0.8	0	[102, 111, 128]
	*CYP2C9*11 rs2837168*	-	2	4.5	5	2[a], 2.7[c], 3[e]	15	16	34	[102, 106, 107, 109, 128, 129]
VKORC1	*g.-1639A rs9923231*	46-51	6	12-31	37	3.5[a],31[b]	13	39	88	[107, 109, 110, 129, 130]
	1173T rs9934438	52-72	-	5	36	31[b]	13	39	88	[105, 109, 113, 117, 130]
	V66M rs72547529	-	-	0.4-2.7	-	-	0.1	0	0	[111]
	L120L rs7200749	-	-	20-26	-	-	1.3	0	0	[111]
	g.9041A rs7294	-	-	40-44	-	38-42[b]	40	36	11	[105, 111, 130]
	Asp36Tyr rs61742245	2.5	0	0	6	15[b]	0	0.1	0	[111, 130, 131]

	2255T rs2359612	-	-	24	42	31-32[b]	5	61	12	[105, 109, 130]
	497G rs2884737	-	-	-	32	26[b]	17.5	26	0.1	[109, 130]
	1452C rs8050894	-	-	-	40	31-34[b]	44	40	88	[109, 130]
CYP4F2	*V433M rs2108622*	42	41	-	-	8.7[a]	8.2	29	21	[110, 115, 129]
APOE	*ε2 rs429358*	7	-	-	-	-	10	1	8.6	[115]
	ε4 rs7412	7	-	-	-	-	5	6	10	[115]
CALU	*rs339097*	2.3	-	-	-	-	1	0	1	[115]

The African American, European, and Asian allele frequencies were extracted from [170].
[a]Mozambique, [b]Ethiopia, [c]Benin, [d]Morocco, [e]Gambia, and [f]Tunisia.
-, no data.

Table 1.3: Warfarin dose-response variation explained by genetic and environmental factors

Study	Population	Significant Genetic Predictors (r^2)	Significant nongenetic predictors (r^2)	Overall r^2/variability explained by the model
Ghozlan et al [114]	Egyptians	*CYP2C9*2* and **3* (5.2%) *VKORC1 g.-1639G>A* (12.5%)	Age (9.4%) Height (3.3%)	30.6%
El-Din et al [113]	Egyptians	*CYP2C9*2* and **3* (15.6%) *VKORC1 C1173T* (31.7%)	Age, sex, and weight (14%)	61.3%
Ekladious et al [118]	Egyptians	*VKORC1 C1173T* (7.3 %)	Age (13.2 %)	20.5 %
Issac et al [120]	Egyptians	*VKORC1 C1173T* (13%) *MDR1 C3435T* (3%)	Age (5%)	21%
Shahin et al [115]	Egyptians	*VKORC1 3673G>A* (10%) *CYP2C9*2, *3, *5* and **8* (5%) *APOE E2* (2.5%)	Age (8%), Pulmonary embolism (3%) Smoking status (1.8%)	31%
Bazan et al [104]	Egyptians	*VKORC1 1693G>A* (19.5%) *CYP2C9 *2* and **3* (9.3%)	smoking status (8.1%) Age (12%) Atrial fibrillation (11%)	34.5%
Shrif et al [109]	Sudanese	*VKORC1 G1452C, A3730G* (20%) *PLO3S p. Pro406Ala (7%)* *CYP2C9 *2, *5, *6,* and **11* (5%)	Body weight (1%) Concurrent medication (3%) Indication for warfarin (2.5%)	36.7%
Mitchell et al [111]	South Africans	*CYP2C9*8* (5.9%) *CYP2C9*2/*3* (0%) *VKORC1 L120L* (6.9%) *VKORC1 G3730A* (5.3%)	β-blockers (9.7%)	45.2%
Ndadza et al [107]	South Africans	*CYP2C rs12777823G>A*[a] *CY2C9*3* and *VKORC1 g.−1639G>A (11%)* [b]	Age and BMI (9%) [b]	12%[a] 20%[b]
Cavallari et al [57]	African American	*VKORC1 g.−1639G>A* (7.3%), *CYP2C9*2, *3, *5, *6, *8,* and **11* (7.7)	Age (10%), BSA (9%), and stroke/TIA (2%)	36%
Alzubiedi and Saleh [87]	African American	*VKORC1 g.-1639G>A, CYP2C9, CYP2C rs12777823* and *CYP4F2 rs2108622*	Weight, age, amiodarone, and Congestive heart failure	38%

[a]Black Africans and [b]Mixed Ancestry.
BMI, body mass index; BSA, body surface area; TIA, transient ischemic attack.

g.-1639A is associated with reduced level of expression of the VKORC1, subsequently reducing warfarin dose requirements by over 30% [45, 59, 98]. The occurrence of *VKORC1 g.-1639A* variant varies among different African population groups, occurring at a high allele frequency of 46–51%, 37%, and 31% in Egyptians, Sudanese, and South Africans of Mixed Ancestry, respectively, compared to black South Africans (12%), Ghanaians (6%), and Mozambicans (3.5%) [104, 107, 109, 110, 114, 115, 129] (Table 1.2). The influence of *VKORC1 g.-1639G>A* on warfarin response is reportedly low among Africans compared to non-African populations, as a 10–13% and 4–7% warfarin variability has been reported in Egyptians and African Americans, respectively, compared to the 15–34% reported in Caucasians and Asians [57, 114, 115, 132].

VKORC1 g.-1639G>A occurs at high linkage disequilibrium (LD) with *C1173T (rs9934438), G1542C (rs8050894), C2255T (rs2359612),* and *T4931C (rs7196161)* (collectively defined as *VKORC1*2*) in Caucasians and Asians (61, 133); however, in Africans, the LD is only strong between *VKORC1 g.-1639G>A* and *C1173T (rs9934438)* (r^2 = 0.95) [109, 132, 134, 135]. Hence, in other African studies, *VKORC1 C1173T* is the most targeted SNP, as it is also associated with low warfarin dose requirements [104, 109, 113-115, 118]. There are other *VKORC1* polymorphisms that have been associated with warfarin resistance and these include the following: *VKORC1 g.9041G>A* located in the 3′ untranslated region, *VKORC1 Asp36Tyr* and *Val66Met* both located in the *VKORC1* coding regions. *VKORC1 g.9041A* occurs at a high frequency in Africans (43–52%) and Europeans (35–37%) compared to Asians (10–17%) [61, 136]. However, the influence of *VKORC1 g.9041G>A* on warfarin response is inconsistent among Africans, as it has been significantly associated with a high warfarin dose in Sudanese and black South Africans [109, 111], whereas a nonsignificant difference in maintenance dose between the *VKORC1 g.9041G>A* genotypes has been reported in African Americans [57, 137].

VKORC1 Asp36Tyr has been reported in Ethiopians, Kenyans, Sudanese, and Egyptians at a frequency of 15%, 6%, 6%, and 2.5%, respectively [130, 131]. However, it is reported to be absent or rare in black South Africans and West African descendants such as Ghanaians and African Americans [111, 131]. The presence of *VKORC1*

Asp36Tyr has been reported to increase warfarin variability by 5.5% in Egyptians [131]. *VKORC1 Val66Met* has been described in Brazilians of African descent, who are warfarin resistant; furthermore, a Tanzanian male who was taking 21 mg/day of warfarin was reported to be heterozygous for *VKORC1 Val66Met* [138, 139]. *EPHX1, CALU, GGCX, APOE*, and *CYP4F2* are other pharmacodynamic genes that have been described. These genes have not been extensively studied in native Africans, as they have been mainly explored in Egyptians [115, 119, 120]. *EPHX1 rs1057140, CALU rs2290228, GGCX rs699664, GGCX rs12714145 (C>T)*, and *CYP4F2 p.V433M* have been reported to have a lack of influence on warfarin dose in Egyptians and African Americans [47, 115, 137, 140, 141].

APOE-E2 has been associated with a low warfarin dose requirement, explaining 2.5% of warfarin variability in Africans [115, 142], while *CALU rs339097* has been associated with an increased warfarin dose requirement and accounted for 5.7% of warfarin variability in African Americans and Egyptians [115, 143]. A microsatellite (CAA) tandem repeats in intron 6 of *GGCX (rs10654848)* has been described to play a role in warfarin response. The *GGCX (CAA) rs10654848* was associated with an increased warfarin dose in African Americans, possessing at least 16 CAA repeats and further explained 2% of the warfarin variability [144], while in Egyptians, only one individual carried the 16 CAA repeats with a higher warfarin dose of 70 mg/week [119]. Furthermore, the increased warfarin dose tended to be higher with the increase in number of CAA repeats (<15 vs. ≥15 CAA repeats); however, the difference was not statistically significant (p=0.16) [119].

Although data on warfarin pharmacogenetics are still lacking in Africans, the few studies conducted have shown that inclusion of additional variants other than *CYP2C9*2*, **3* and *VKORC1 g.-1639A* improve prediction of warfarin variability in Africans (Table 1.3). Therefore, further identification of novel variants through application of molecular techniques that detect multiple variants simultaneously such as Mass Arrays and whole exome analysis from next generation sequencing (NGS) is warranted. Technological advancement in the pharmacogenetics of warfarin in African Americans has allowed identification of novel and rare variants important for warfarin response. These include

the *rs12777823G>A* polymorphism that was identified in the CYP2C cluster upstream of *CYP2C18*. The *rs12777823G>A* was identified by Perera et al [145] in a genome-wide association study (GWAS) and was found to reduce warfarin dose requirement by 10–25%, exclusively in African Americans. The effect of the *rs12777823G>A* polymorphism was further confirmed by our group, wherein this SNP reduced warfarin dose requirement in black South Africans, thereby explaining 12% of warfarin variability [107]. Another novel variant (*rs7856096)* was identified in a study that applied whole exome sequencing. The *rs7856096* is located upstream of exon 2 of the folylpolyglutamate synthase gene and it improves the variance explained by the International Warfarin Pharmacogenetics Consortium (IWPC) dose equation of 30% with an addition of 3.3% in African Americans [146].

1.2.1.4. Outlook

The field of pharmacogenomics entails the utilisation of an individual's genetic composition to personalise therapeutic regimens and improve treatment outcomes. The application of pharmacogenomics is important in drugs such as warfarin that are difficult to use due to their narrow therapeutic range and inter-individual variability. The Food and Drug Administration (FDA) has recommended pharmacogenetics testing in warfarin dosing [147]. Since the FDA recommendation, there has been an increased development in the pharmacogenetics of warfarin. Studies have identified environmental and genetics variants that explain over 50–60% of warfarin variability [45, 148]. Several groups, including Gage et al [63] and the IWPC [65], have developed pharmacogenetics-based algorithms for use in warfarin dosing. Native Africans are not represented in the development of warfarin pharmacogenetics algorithms because most data available are obtained from Caucasians and Asians. Extrapolating data from other population groups to Africans is not appropriate as Africans are genetically diverse and allelic frequencies vary between different population groups [149, 150]. Furthermore, there is a heightened admixture in the African continent [151, 152].

The large genetic diversity in native Africans is highlighted in this expert review as different allelic frequencies varied among African populations. For example, *CYP2C9*2* and *CYP2C9*3* are frequent among Egyptians, while rare in black South Africans and

Ghanaians (Table 1.2). Therefore, the contribution of each genetic variation on warfarin response differs from one population to the next, with specific variants playing significant roles in particular populations. The variability in the influence of specific variants on warfarin response among Africans is also driven by the non-homogeneous nature of other population groups such as Egyptians and Sudanese. Egyptian and Sudanese gene pools are contributed by sub-Saharan Africans, Europeans, and Asians. Hence, genetic polymorphisms affecting warfarin response in Egyptians are skewed toward both Europeans and Africans. This is highlighted by the increased contribution *of CYP2C9 c.430C>T (*2), CYP2C9 c.1075A>C (*3)*, *and VKORC1 g.-1639G>A* in warfarin variability in Egyptians. Yet, these polymorphisms play an insignificant role in sub-Saharan African populations. Thus, data on warfarin pharmacogenetics in one African population cannot be generalised or be used to infer on other African populations that have not been studied previously.

Africa is comprised of low- and middle-income economies, suggesting that there are few or limited resources and infrastructure to conduct expensive genomic research. Hence, warfarin pharmacogenetics studies are sparse and limited to <15% of the African countries. Furthermore, studies undertaken in Africans are mostly of candidate genes of variants described in either Europeans or Asians. Candidate gene studies limit detection of novel or rare SNPs that are only functional in Africans, such as the *rs12777823G>A* identified through a GWAS and was described to explain 5% of warfarin variability exclusively in African Americans. Candidate gene studies undertaken in Africans mostly focus on *CYP2C9, VKORC1,* and *CYP4F2*, and to a lesser extent, *APOE, CALU*, and *GGCX*. However, there are over 30 genes that are involved in warfarin disposition, including *CYP1A2, CYP3A4, CYP2C18, CYP2C19*, and *CYP2C8* [68]. Variants in some of the abovementioned genes have been described in Africans independent of warfarin response. For instance, *CYP2C8*2* has been widely explored in Africans and it occurs at an allele frequency ranging from 9% to 24% [153-156]. Furthermore, at least one copy of the mutant allele exhibits decreased CYP2C8 enzyme activity in the metabolism of anticancer (paclitaxel) and antimalarial (amodiaquine) drugs [153].

Consideration of variants in genes that have not been previously studied could increase the warfarin variability explained in Africans. The importance of inclusion of variants less studied or with no contribution in Europeans and Asians was highlighted in the review [157], as inclusion of variants such as *CYP2C9*5, *8, *9,* and **11, VKORC1 Asp36Tyr*, and *VKORC1 L120L* increased the warfarin variability explained by commonly studied variants such as *CYP2C9*2, CYP2C9*3*, and *VKORC1 g.-1639A* (Table 1.3). In addition to consideration of African-specific genetic variants, it is also important to consider inclusion of demographic, environmental, and social variables when developing pharmacogenetics dosing algorithms. This is highlighted on this expert review, as the inclusion of age, body weight, and concurrent medications improved warfarin variability explained by genetic factors (Table 1.3). However, there are no data on the effect of nutrition and usage of herbal medications on warfarin response in Africans. Consideration of nutrition is of paramount importance as vitamin K-rich diet has an effect on warfarin activity. Furthermore, in some African populations, particularly in the rural areas, there is high consumption of traditional and indigenous vegetables such as the African green leafy vegetables (African spinach), which are the main source of the vitamin K and could ultimately affect warfarin response.

Although studies have considered the effect of other conventional medications on warfarin response, it is worth noting that in most African countries, in addition to prescribed medications that are administered for diseases, patients also co-administer herbal medications or supplements with their prescribed drugs. The course of herbs and prescribed medication also potentiates herb–drug interaction and adverse effects. Similar incidences of herb–drug interactions with consequential adverse effects have been reported in other populations. An example is the induction of CYP3A4, CYP1A2, and CYP2C9 activity by St. John's wort (a herbal medication), thereby resulting in increased warfarin clearance and reduced effects [31]. The challenge in African populations is that despite the widely accepted view that patients are co-administering herbal products with their medications, no conscious effort has been made to study the effects of these herbs on warfarin indications and bleeding events.

Having considered the limitations that are hindering development of warfarin pharmacogenetics in Africans, several recommendations can be made to improve on the accumulation of locally useful data that can assist in warfarin management. Collaboration among researchers from across Africa is essential, as this will enhance sample sizes and African countries studied. Furthermore, extension of collaboration with researchers from developed countries can leverage financial support for carrying out more extensive GWAS or NGS-anchored studies. This is exemplified by the International Warfarin Pharmacogenetics Consortium [65], which developed a warfarin pharmacogenetics-based algorithm through collaboration of 21 research groups from 9 countries and 4 continents, thereby attaining genetic data from a total of 5700 patients. Enhanced sample size and financial support will further enable studies such as GWAS to be undertaken. This would also allow application of technologically advanced molecular techniques such as MassArray platforms and whole exome sequencing by NGS. Technologically advanced molecular approaches enable the detection of novel and rare variants that have not been reported with regard to warfarin response and are specific for Africans. The increase in pharmacogenetics data on warfarin use in Africans will potentially allow for the development of pharmacogenetics algorithm best suited for Africans and possible future clinical trials.

1.2.1.5. Concluding Remarks

Warfarin is an essential drug in the management of thromboembolic disorders and will be more important clinically if optimal dosing algorithms are also available. Implementation of pharmacogenetics testing has the potential of allowing safe and quality anticoagulation. In Africa, there are limited pharmacogenetics data on warfarin; therefore, research in this regard should be prioritised to ensure safe and quality anticoagulation. However, the genetic diversity and the heightened admixture in Africans should be taken into consideration to generate data that will be beneficial to all. Collaboration and utilisation of techniques that create and harness Big Data simultaneously are desirable and would accelerate discoveries in African pharmacogenetics studies. Finally, new practices are emerging that impact on how we do science so as to cultivate innovations that are meaningful locally, and thus

sustainable and equitable. In particular, there are attempts to understand the broader sociotechnical context as well as human values that cocreate scientific outcomes and innovation products. One example is the emerging field of responsible innovation (RI) that can help identify the human values that shape and steer collaboration among scientific groups, for collaboration is not a simple or a value- neutral practice.

Greater competency of scientists in understanding the human values embedded in scientific decisions and choices would contribute to RI and help remedy the ‘‘two cultures divide’’ among laboratory sciences and critical social sciences [158, 159]. Such broader skills, if included in scientific training, would also help decipher and make transparent the embedded opaque values that drive scientific collaborations. Thus, the practices and principles of RI are often considered ‘‘socio-technical integration research’’ and enable collaborations that are enriched by interdisciplinary or multidisciplinary perspectives. Ultimately, this would allow for robust and enduring responses to the broader social and equity dimensions of science in the 21st century as we generate, analyse, and contextualise Big Data to meaningfully improve the health of African patients and populations.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

1.3. Study Rationale

Warfarin remains the anticoagulant of choice in reducing the burden of thromboembolic disorders and their associated complications, especially in low income economies such as Southern Africa. This is owed to its efficiency in reducing the resultant stroke at low cost as compared to other alternative anticoagulants in the market. However, its efficiency is compromised by complications associated to its use which include bleeding complications and thrombotic events due to overdosing and underdosing, respectively. It is now accepted that patients' response to drugs is a product of both the individual's genetic makeup and environmental factors. Therefore, the use of clinicodemographic information, genetic variants in genes involved in the warfarin metabolism and excretion pathway to predict warfarin dose has been adopted in reducing warfarin-related complications through the development of warfarin pharmacogenetics dosing algorithms. However, these dosing algorithms have proved to be impractical among individuals of African ancestry because data that has been used to develop these algorithms is extracted from other population groups such as Europeans. Thus, making it difficult to be applied among Africans as the genomic profiles of Africans differ from that of Europeans with the former being highly diverse as highlighted in the review in section 1.2. Additionally, African data is excluded in the available pharmacogenetics dosing algorithms because there are very few studies that have investigated the pharmacogenetic markers affecting warfarin dose among Africans making data very limited in this regard. Thus, to bridge that gap and improve the efficiency of pharmacogenetic data to predict warfarin dose among Africans, studies that focus on mining for African-specific pharmacogenetic data affecting warfarin response are warranted so that designing of dosing algorithms better suited for Africans can be made possible. The importance of this study is to contribute to the limited available data by extensively characterising Africans of Bantu origin and Mixed Ancestry from Southern Africa that are on warfarin treatment, in order to identify and recommend important genetic variants and non-genetic variables affecting warfarin response among Africans. Thereby, assisting with the development of pharmacogenetics-based warfarin dosing algorithms that can better predict warfarin starting doses suited for Africans with reduced complications.

1.4. Aims and objectives

1.4.1. Aim of the study:

The aim of the study is to investigate and identify important pharmacogenomic profiles that could be potential predictors for warfarin dose among African populations.

1.4.2. Study objectives:

1. To recruit cross-sectionally, patients on warfarin treatment, obtain blood samples for downstream genetic analyses and determine patient's demographic and clinical parameters through access of medical records.
2. To characterise genetic variants in genes that affect the pharmacokinetics (PK) and pharmacodynamics (PD) of warfarin, subsequently report and compare their frequencies with other world populations.
3. To correlate observed allelic variants, clinical and demographic variables with effects on warfarin maintenance dose.
4. To identify additional known and novel pharmacogene variants through the application of whole exome analysis.
5. To report on a set of variants and non-genetic variables that are important in the prediction of the best starting dose for warfarin among Africans.

Chapter 2: Overview of methodology used in the project

Synopsis:

This chapter will give an overview of all the methods used in the project and show how each is connected to the other. Specific and detailed methods will be highlighted in each manuscript/publication. This chapter is comprised of the following sections:

1. Study design
2. Study population and ethical consideration
3. Data collection and data capture
4. Genetic characterisation and data analysis

The numbers of participants (sample size) in this section may differ and will always be more or equal to numbers in each of the manuscripts because some participants are left out in subsequent characterisation and analyses due to depletion of biological samples. Below is the flowchart of the methods applied in the study.

2.1. Study design

This was a cross-sectional study that recruited adults (18 years and older) that were undergoing warfarin treatment at anticoagulation clinics in Groote Schuur Hospital (GSH), Gugulethu Community Health Centre (GCHC) in the Western Cape Province, South Africa, and Parirenyatwa Group of Hospitals (PGH) in Harare, Zimbabwe.

2.2. Study population and ethical consideration

A total of 579 (302 black Africans and 277 Mixed Ancestry) adults undergoing warfarin treatment at anticoagulation clinics in GSH, GCHC in the Western Cape Province, South Africa and PGH in Harare, Zimbabwe were recruited into the study. Before enrolling into the study, the participants provided informed consent and anonymity was fully observed by replacing patient names with laboratory generated numbers. The study was granted ethical approval by the University of Cape Town Human Research Ethics Committee (UCT-HREC REF No. 581/2015), Faculty of Medicine, University of Zimbabwe Ethics Committee (JREC/160/13) and the Medical Research Council of Zimbabwe (MRCZ/A/1815) before recruitment commenced.

2.3. Data collection and Data capture

Whole blood samples (5 ml) were drawn from participants that provided informed consents and were enrolled in the study. The blood samples were stored at -20^{o}C freezers before DNA extraction for genomic analyses. Study participants completed questionnaires to provide demographic information (e.g., age, gender, ethnic group, height and weight) and social lifestyles (e.g., dietary intake, alcohol and smoking history). Ancestry for these patients was determined both through self-identity and family histories preceding three generations. Clinical data which include; primary indication for warfarin treatment, medical history, use of concomitant medications and current warfarin maintenance dose were retrieved from the hospitals' patient records/files. The patients' demographic, social lifestyles and clinical information were captured in the study database with the corresponding patient's laboratory generated number.

2.4. Genetic characterisation and Data analysis

Deoxyribonucleic acid (DNA) was extracted from whole blood samples using the salting-out DNA purification method (modified from Gustafson et al [160]) and Qiagen Blood Mini Kit according to manufacturer's instructions (Qiagen, Hilden, Germany). The quantity, purity and quality of the purified DNA were subsequently examined using a NanoDrop® spectrophotometry and agarose gel electrophoresis, respectively. Genetic characterisation was done at the pharmacogenetics and drug metabolism research lab in Human Genetics, UCT; Inqaba biotec in Pretoria and DNA Sequencing Unit at Stellenbosch University in the Western Cape Province. Targeted genetic characterisation was performed using a combination of iPLEX® PGx 74 Panel MassARRAY® System (Agena Bioscience Inc., San Diego, CA), Polymerase Chain Reaction (PCR), Restriction Fragment Length Polymorphism (RFLP), Taqman genotyping assay and Sanger Sequencing. Additionally, whole exome sequencing was done using the ion torrent Sequence ion S5 system in selected black African individuals presenting with extreme phenotypes (i.e., very low dose or very high dose) but who did not harbour variants known to significantly affect warfarin dose requirements. Specific variants identified through whole exome sequencing (WES) were prioritised according to clinical significance and further genotyped in an enhanced sample size of 252 black Africans, to confirm their effect on warfarin dose variability. Detailed protocols and targeted genetic markers are highlighted in chapter 3 in the respective publications. Various statistical tests were employed in statistical packages such as STATA, R and graph-pad prism for descriptive and comparative analyses. Various bioinformatics tools such as Torrent Mapping Alignment Program (TMAP), Genome Analysis Toolkit (GATK) and ANNOVAR were employed to analyse the WES data. Specific data analyses are detailed in Chapter 3 in the respective publications or manuscripts. Figure 2.1 depicts the flowchart of the broad methodology employed in the study.

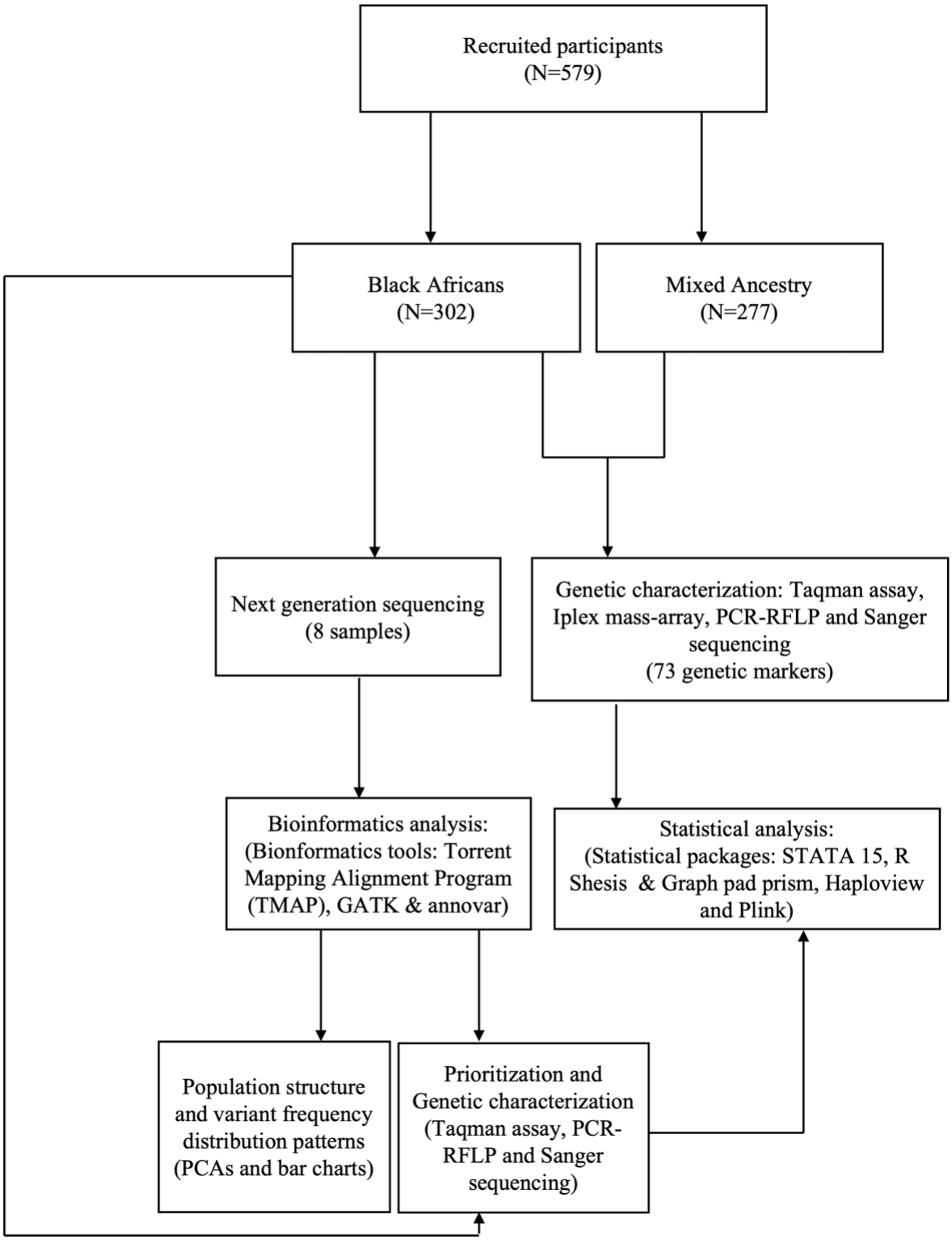

Figure 2.1: Flowchart of the methodology employed in the study

Chapter 3: Results

Synopsis:

This chapter will present the findings as each of the objectives was investigated. There are 5 objectives and 5 manuscripts responding to the respective objectives. The section will start with a synopsis of the overall results, and then include the manuscripts. Each objective will form a section of the results as follows:

3.1. Recruitment of patients on warfarin treatment and report on their demographic and clinical profiles.

3.2. Characterisation of genetic variants in genes that affect the PK and PD of warfarin and report on their frequency distribution among our studied cohorts in comparison with other world populations.

3.3. Correlation of observed allelic variants, clinical and demographic variables with effects on warfarin maintenance dose.

3.4. Identification of additional known and novel pharmacogene variants through the application of whole exome analysis.

3.5. Reporting on a set of variants and non-genetic variables that are important in the prediction of the best starting dose for warfarin among Africans.

3.1. Recruitment of patients on warfarin treatment and report on their demographic and clinical profiles

Synopsis: This section focuses on the first objective of the study which seeks to present on the recruitment, demographic, and clinical characteristics of the study participants. The detailed clinicodemographic profiles of the study participants are outlined in publications https://doi.org/10.1089/omi.2021.0199, https://doi.org/10.1089/omi.2018.0174 and https://doi.org/10.1111/jth.15494 which will be presented on section 3.2 and 3.3 of the thesis. Thus, this section will only include an overview of the recruitment and clinicodemographic profiles.

Overview:

A total of 148 and 277 black Africans and Mixed Ancestry, respectively, were recruited and enrolled into the study in 2016 from INR clinics at Groote Schuur Hospital (GSH), Gugulethu Community Health Centre (GCHC) in the Western Cape Province, South Africa. Additionally, 154 black African participants were recruited from Parirenyatwa Group of Hospitals (PGH) in Harare, Zimbabwe. Ancestry for the patients was determined both through self-identity and family histories preceding three generations. The participants provided full consent to be enrolled in the study after ethical approval was granted by the University of Cape Town Human Research Ethics Committee (UCT-HREC REF No. 581/2015), University of Zimbabwe Ethics Committee (JREC/160/13) and the Medical Research Council of Zimbabwe (MRCZ/A/1815). Demographic variables and clinical parameters were collected through a questionnaire and retrieval of patients' medical/hospital records, respectively. Whole blood (5 mL) was collected in EDTA-coated tubes and used for extraction of deoxyribonucleic acid (DNA). The most common warfarin indications were; atrial fibrillation (26-14%), deep venous thrombosis (18-31%), mechanical valve replacement (21-45%) and pulmonary embolism (6-10%). Patients presented with comorbidities such as hypertension (43-46%), heart failure (39-45%), diabetes mellitus (6-18%), arrhythmia (25%), and HIV infection (3-15%), resulting in the prescription of co-medications such as statins, furosemide, enalapril and antiretroviral therapy to be used concurrently with warfarin.

3.2. Characterisation of genetic variants in genes that affect the pharmacokinetics (PK) and pharmacodynamics (PD) of warfarin, subsequently report and compare their frequencies with other world populations

Synopsis: This section presents on the allele frequency distribution of genetic variants characterised in the study participants in comparison to other global populations. The section further highlights on how the occurrences of the various pharmacogenetic variants influence the prescription of warfarin and other comedications in various population groups. Publication https://doi.org/10.1089/omi.2021.0199 has been included here to respond to the set objective.

3.2.1. **Warfarin pharmacogenomics for precision medicine in real life clinical practice in Southern Africa: harnessing 73 variants in 29 pharmacogenes**

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Candidate's Contribution: conceptualised the idea, generated and analysed data, incorporated changes from co-authors and finalised manuscript draft.

Co-Authors Contribution:

SM: Conceptualised the idea, generated and analysed data.

NDS: analysed data and drafted the manuscript.

BK: Generated data.

EC: Reviewed manuscript**.**

GK, CM, CFBN, MN and CD: conceptualised the ideas, supervised all components and reviewed the manuscript drafts.

All authors contributed to the final version of the article. The authors read and approved the final manuscript.

Warfarin Pharmacogenomics for Precision Medicine in Real-Life Clinical Practice in Southern Africa: Harnessing 73 Variants in 29 Pharmacogenes

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Abstract

Pharmacogenomics is universally relevant for worldwide modern therapeutics and yet needs further development in resource-limited countries. While there is an abundance of genetic association studies in controlled medical settings, there is a paucity of studies with a naturalistic design in real-life clinical practice in patients with comorbidities and under multiple drug treatment regimens. African patients are often burdened with communicable and non-communicable comorbidities, yet the application of pharmacogenomics in African clinical settings remains limited. Using warfarin as a

model, this study aims at minimising gaps in precision/personalised medicine research in African clinical practice. We present, therefore, pharmacogenomic profiles of a cohort of 503 black Africans (n=252) and Mixed Ancestry (n=251) patients from Southern Africa, on warfarin and co-prescribed drugs in a naturalised noncontrolled environment. Seventy-three (n=73) single nucleotide polymorphisms (SNPs) in 29 pharmacogenes were characterised using a combination of allelic discrimination, Sanger sequencing, restriction fragment length polymorphism, and Sequenom Mass Array. The common comorbidities were hypertension (43–46%), heart failure (39–45%), diabetes mellitus (18%), arrhythmia (25%), and HIV infection (15%). Accordingly, the most common co-prescribed drugs were antihypertensives, antiarrhythmic drugs, antidiabetics, and antiretroviral therapy. We observed marked variation in major pharmacogenes both at interethnic levels and within African subpopulations. The Mixed Ancestry group presented a profile of genetic variants reflecting their European, Asian, and African admixture. Precision medicine requires that African populations begin to capture their own pharmacogenetic SNPs as they cannot always infer with absolute certainty from Asian and European populations. In the current historical moment of the COVID-19 pandemic, we also underscore that the spectrum of drugs interacting with warfarin will likely increase, given the systemic and cardiovascular effects of COVID-19, and the anticipated influx of COVID-19 medicines in the near future. This observational clinical pharmacogenomics study of warfarin, together with past precision medicine research, collectively, lends strong support for incorporation of pharmacogenetic profiling in clinical settings in African patients for effective and safe administration of therapeutics.

Keywords: warfarin, Africa, pharmacogenomics, personalised medicine, gene-drug interaction, genetic variation, drug transporters

3.2.1.1. Introduction

Pharmacogenomics is universally relevant for worldwide modern therapeutics and yet needs further development in resource-limited countries and in real-life clinical practice contexts. Moreover, for pharmacogenomics to be implemented in routine medical practice, genetic variation type and frequencies ought to be established not only for a

given prescribed drug but also for all other commonly co-administered drugs. To this end, Sub-Saharan Africa (SSA) presents with a high burden of communicable diseases such as HIV/AIDS, TB, and malaria, with a rising incidence of noncommunicable cardiovascular disease (CVD) comorbidities [15]. Managing the colliding pandemic of infectious diseases and a growing burden of CVDs faces the potential of numerous drug-drug interactions [13, 161]. Of great significance to SSA are thromboembolic disorders, which are one of the leading causes of CVDs [13]. Despite the introduction of direct oral anticoagulants (DOACs), the vitamin K antagonist warfarin remains the mainstay of thromboembolic management in many African countries because of its efficacy, low cost, and familiarity of its use by physicians [14, 15, 97, 123, 162]. Analysis of the clinicodemographic profiles of African patients on warfarin treatment confirms the need to manage comorbidities requiring concomitant medications, as this poses huge risks of drug-drug interactions, which may lead to adverse drug events (ADEs) [75, 107].

Genotyping and haplotyping data provide extensive evidence of genomic profiles varying significantly between different ethnic groups, with groups in Africa exhibiting 60% heterogeneity compared to Asians and European groups [150]. Very few African populations have been studied for the genetics of warfarin response, yet warfarin use now encourages the use of pharmacogenetics algorithms [163]. It is our contention that such algorithms generated with little or no data from African populations may not be effective in Africans because of quantitative and qualitative differences in important pharmacogenomic variants that affect warfarin response [164]. In the era of precision medicine, pharmacogenomics has an important role, thus the growing need to genetically characterise a wide range of different African ethnic groups. Due to African genetic diversity, one African ethnic group cannot be used as representative for all African populations. Understanding African population genetics and issues of admixture will allow pharmacogenetics-guided safe and efficacious warfarin dosing in African populations. This study therefore aimed to decode the pharmacogenomic profiles of Africans in Southern Africa, concentrating on genes/pathways associated with warfarin and comedication disposition, by reporting on 73 single nucleotide polymorphisms

(SNPs) in 29 genes. The study seeks to contribute to knowledge on the prevalence of genetic variation in Africans and its implications for rational drug use in a region with colliding epidemics of communicable and noncommunicable diseases.

3.2.1.2. Materials and Methods

3.2.1.2.1. Study participants

The study included an analysis of 503 participants on warfarin treatment recruited from Groote Schuur Hospital, Gugulethu Community Health Centre (GCHC) in the Western Cape, South Africa, and Parirenyatwa Group of Hospitals (PGH) in Harare, Zimbabwe. The South African group comprised of 98 black Africans and 251 Mixed Ancestry, while all the 154 Zimbabweans were of black African ancestry.

3.2.1.2.2. Ethical considerations

Ethical approval was obtained from the University of Cape Town Human Research Ethics Committee (UCT-HREC Ref. No. 581/2015), the University of Zimbabwe College of Health Sciences, Joint Research Ethics Committee (JREC; Ref. 160/13), and Medical Research Council of Zimbabwe (MRCZ; Ref. MRCZ/B/1815). All participants provided written informed consent before recruitment.

3.2.1.2.3. Genotyping

Genomic DNA was extracted from 5 mL of whole blood drawn from each participant, using a modified salting out DNA purification method (modified from Gustafson et al [160]) and Qiagen Blood Mini Kit according to manufacturer's instructions (Qiagen, Hilden, Germany). Genotyping of 73 variants in 29 genes (Supplementary Table S1) was done using a combination of allelic discrimination, Sanger sequencing, restriction fragment length polymorphism, and Sequenom Mass Array (iPLEX PGx74 platform) (full methods are described in Supplementary Data). Genotype and allele frequencies of *CYP1A1, CYP2C9 (rs2256871),* microsomal epoxide hydrolase 1 (*EPHX1*), nuclear receptor subfamily 1 group I member 2 (*NR1I2)*, and nuclear receptor subfamily 1 group I member 3 (*NR1I3*) were obtained from work previously published in our group [165-167]. However, Mixed Ancestry was not characterised in these studies.

3.2.1.2.4.Data analysis

Genotype and allele frequencies for the studied SNPs among both the black Africans and Mixed Ancestry were determined using an online software platform, Shesis [168]. Chi-squared analysis was performed on R (version 4.0.3 [October 10, 2020]) to compare the allele frequency distribution among the studied populations and previously reported data among world populations, which included West Africans, East Africans, African Americans, East Asians, and Europeans. Data from the 1000 genome project phase 3 and NCBI ALFA published on Ensembl [169] and NCBI single nucleotide polymorphism Database (dbSNP) [170] were utilised to obtain the comparative data. All statistical tests were performed taking a 5% significance level.

3.2.1.3. Results

We present results on the pharmacogenomic profiling of participants from Southern Africa (i.e., black Africans and Mixed Ancestry), focusing on 73 SNPs in 29 genes (Table 3.1) involved in the pharmacology of drugs prescribed together with warfarin to treat comorbidities in African populations. The findings are primarily important in understanding the determinants of response to warfarin treatment as well as responses to the comorbidities, which could improve treatment outcomes. In addition, the results contribute to knowledge on the prevalence of genetic variation in Africans and further inform on rational drug use in a region with colliding epidemics of infectious and noncommunicable diseases, taking into account pharmacogenomics. The reasons for warfarin treatment for this cohort have been previously reported [107, 171]. Among black Africans, the most common warfarin indication (Table 3.2) was deep vein thrombosis (31%) and the most common comorbidities were hypertension (46%), heart failure (45%), and arrhythmia (25%), respectively. In contrast, mechanical valve replacement (45%) was the most common reason for warfarin treatment among the Mixed Ancestry, with hypertension (43%), heart failure (39%), and diabetes mellitus (18%) as common comorbidities, respectively. Figure 3.1 highlights the burden of additional concomitant drug usage in patients already on warfarin treatment among black Africans and the Mixed Ancestry. At least 21% (53/251) of Mixed Ancestry patients on warfarin also had statin co-prescription. However, among the black Africans,

45% took herbal supplements, followed by the diuretic furosemide, statins, and the ACE inhibitor enalapril. Both furosemide and enalapril are used to treat high blood pressure and heart failure. Antihypertensives constituted 8 out of the top 12 (75%) co-prescribed drugs among black Africans on warfarin. Of the 38 HIV-positive black African participants on warfarin, 25 (10%) were also on antiretroviral therapy consisting of efavirenz (EFV), nevirapine, and lamivudine.

The genotype and allele frequency distribution of the 73 SNPs involved in the pharmacology of drugs co-prescribed with warfarin are presented in Tables 3.1 and 3.3, respectively. Allele combinations (i.e., genotypes) in Table 3.1 are displayed as RR (homozygous reference allele), RO (heterozygous reference and alternate/variant alleles), and OO (homozygous alternate/variant allele). We report here qualitative and quantitative differences in the distribution of variant allele frequencies among black Africans and the Mixed Ancestry. For example, the Mixed Ancestry group presents with an allele frequency of 18% for opioid receptor Mu1 (*OPRM1) rs1799971* compared to 0% reported among black Africans. Although over 90% of the variants reported showed similar allele frequencies when comparing black Africans from Southern, West, and East Africa, *CYP2C9 rs2256871* exhibited a high frequency of 58% among black Africans from Southern Africa compared to black Africans from East Africa (15%) and West Africa (9%). In contrast, the frequency for *CYP2D6 rs16947* was significantly lower among black Africans in Southern Africa (12%) when compared to their West African (65%) and East African counterparts (56%).

There are differences in the distribution of pharmacogene variants when comparing global populations, with over 60% of the variants having varying allele frequencies when comparing black Africans to Europeans and Asians. This is supported by the varying minor allele frequency profiles for *CYP2C8 rs11572103* among Africans (20%), Asians (0%), and Europeans (0%), and by the skewed distribution of solute carrier organic anion transporter family member 1B1 (*SLCO1B1) rs4149056* minor allele as seen in black Africans in Southern Africa (0%), Asians (13%), and Europeans (16%). Furthermore, variants involved in warfarin disposition, which include *CYP2C9*2 (c.430T, rs1799853), CYP2C9*3 (c.1075C, rs1057910), VKORC1*2 (g.-1639A,*

rs9923231), Vitamin K epoxide reductase complex subunit 1 (*VKORC1) g.6484T (rs9934438), VKORC1*4 (g.6009T, rs17708472),* and *CYP4F2*2 (c.1297C>T, rs2108622)* presented with significantly high frequencies (p≤0.05) among Europeans, Asians, and the Mixed Ancestry group compared to black Africans from Southern Africa (Table 3.3). Variants *CYP2C9*8 (c.449A, rs7900194)* and *VKORC1 (g.6171T, rs13336384)* seem exclusively reported among populations of African ancestry. In addition, we report on actionable drug-gene interaction according to recommendations by the Clinical Pharmacogenetics Implementation Consortium [172] (Figure 3.1). Mixed Ancestry participants had high actionable variants for warfarin compared to the black African participants (47 vs. 16), while black Africans had high actionable variants for tacrolimus and codeine among 94 and 59 participants, respectively, compared to 56 and 37 participants among Mixed Ancestry participants.

3.2.1.4. Discussion

The place of pharmacogenomics in precision medicine is not contested, thus making it imperative that pharmacogenomics knowledge permeates clinical practice for the health and well-being of patients as we respond to sustainable development goals. Historically, clinical observational pharmacogenomics studies have been missing in the precision medicine literature and this article addresses this knowledge gap in a context of warfarin. Warfarin anticoagulant is a good example to illustrate how the integration of pharmacogenomics is central to the success of precision medicine. At least 14.6 million warfarin scripts [173] were prescribed in the United States alone in 2019. The 29 genes evaluated and analysed in this study metabolise drugs that are used in the treatment of a spectrum of disorders. For example, some of the genes affected by the variants metabolise drugs used in psychiatry (e.g., CYP2D6 and atomoxetine), hematology (coagulation factor 2/coagulation factor 5 [F2/F5] and avatrombopag), cardiology (CYP2D6 and carvedilol), rheumatology (e.g., CYP2C19 and Carisoprodol), gastroenterology (CYP2C9 and dexlansoprazole), cardiology (Prasugrel and CYP2C9, 2C19, 2B6, and 3A5), and infectious diseases (EFV and CYP2B6).

Although warfarin is administered globally, it is administered in different settings and under different backgrounds of disease burden. In European countries, warfarin is

administered in a background of cardiovascular comorbidities, while in developing or poor countries, warfarin is administered in a background of infectious disease pandemics colliding with an increasing burden of CVDs. Thus, across the world, patients on warfarin treatment present with a diverse spectrum of comorbidities and therefore response patterns. Such comorbidities also require treatment with additional drugs that potentially interact with warfarin, thereby affecting treatment outcomes. For example, in this study among Africans in Southern Africa, it was observed that at least 80% of respondents had at least one other concurrent illness that required treatment with an additional drug, which compares well to the 70% reported in another South African study [123] and 100% reported respondents in a study among Kenyans [174], and is substantially higher than the 35–42% reported in a Swedish study [175]. Besides the number of concurrent illnesses, it is important to note that, the type of illness also differs across population groups, thereby pointing to the different outcomes of warfarin treatment due to different interacting drugs in different geographical areas. An additional important factor is the underlying genomic variation that affects drug response, the pharmacogenomics footprint.

Comorbidities can potentially enhance pathology of conditions indicated for warfarin, as observed with atrial fibrillation (AF), where both valvular heart disease and hypertension have been identified as the most common risk factors [14]. Long-standing hypertension is associated with AF, while AF itself is a major clinical indication for initiation of warfarin therapy [176]. Hypertension was a predominant concurrent illness in our study cohort and is known to be a major illness among Africans, most of the time asymptomatically in males [177]. Similarly, hypertension was a predominant comorbidity in similar studies in Sweden [175], South Africa [123, 176], and Brazil [178]. As much as there seems to be some similarity with respect to predominant comorbidities among patients on warfarin, outcomes in such cases are often comparatively different due to underlying pharmacogene variant profiles across global populations [171]. For example, the observed differences in the frequencies of *CYP2B6 c.516T*, with nearly 40% among

Table 3.1: Genotype Distribution of the 73 Single Nucleotide Polymorphisms in 29 Genes Among Black Africans and Mixed Ancestry

Gene	dbSNP number	Black Africans			Mixed Ancestry		
		RR (n)	RO (n)	OO (n)	RR (n)	RO (n)	OO (n)
ABCB1	rs1045642	0.82 (106)	0.17 (22)	0.01 (1)	0.35 (40)	0.50 (56)	0.15 (17)
APOE	rs429358	0.62 (77)	0.34 (45)	0.04 (6)	0.67 (75)	0.32 (36)	0.01 (1)
APOE	rs7412	0.65 (88)	0.28 (34)	0.08 (6)	0.84 (94)	0.14 (16)	0.02 (2)
CALU	rs1043550	0.86 (132)	0.13 (21)	0.01 (1)	0.68 (72)	0.28 (30)	0.04 (4)
CALU	rs339097	0.62 (94)	0.35 (54)	0.03 (5)	0.82 (87)	0.15 (16)	0.03 (3)
COMT	rs4680	0.41 (48)	0.49 (65)	0.10 (15)	0.44 (49)	0.41 (46)	0.15 (16)
CYP1A1[a]	rs1048943	1.00 (244)	0 (0)	0 (0)	-	-	-
CYP1A2	rs2069514	0.58 (68)	0.32(46)	0.10 (9)	0.73(80)	0.25 (27)	0.03 (3)
CYP1A2	rs762551	0.20 (30)	0.48 (61)	0.32 (32)	0.07 (8)	0.58 (64)	0.35 (38)
CYP2B6	rs28399499	0.81 (103)	0.18 (23)	0.01 (2)	0.94 (105)	0.06 (7)	0 (0)
CYP2B6	rs3745274	0.45 (52)	0.42 (53)	0.13 (23)	0.50 (56)	0.37 (41)	0.13 (15)
CYP2C cluster	rs12777823	0.49 (115)	0.43 (110)	0.08 (20)	0.59 (148)	0.33 (83)	0.08(21)
CYP2C cluster	rs12772169	0.30 (62)	0.57 (115)	0.14 (31)	0.33 (49)	0.57 (85)	0.10 (16)
CYP2C8	rs11572105	0.94 (126)	0.05 (7)	0.01 (1)	0.97 (30)	0.03 (1)	0 (0)
CYP2C8	rs11572103	0.65 (120)	0.31 (58)	0.04 (10)	0.87 (122)	0.13 (19)	0 (0)
CYP2C8	rs1058930	0.99 (190)	0.01 (3)	0	0.98 (146)	0.02 (3)	0 (0)

CYP2C8	rs188934928	1.00 (194)	0.00 (0)	0	1.00 (149)	0.00 (0)	0 (0)
CYP2C8	rs11572101	0.68 (125)	0.27 (51)	0.05 (7)	0.58 (82)	0.36 (51)	0.06 (9)
CYP2C8	rs11572100	0.84 (149)	0.15 (31)	0.01 (2)	0.89 (126)	0.11 (16)	0 (0)
CYP2C8	rs1926705	0.79 (148)	0.20 (34)	0.01 (1)	0.52 (73)	0.39 (55)	0.09 (13)
CYP2C9	rs1799853	0.98 (176)	0.02 (4)	0 (0)	0.92 (220)	0.08 (19)	0 (0)
CYP2C9	rs1057910	1.00 (190)	0 (0)	0 (0)	0.90 (197)	0.10 (23)	0 (0)
CYP2C9	rs28371686	0.98 (113)	0.02 (4)	0 (0)	1.00 (105)	0.00 (0)	0 (0)
CYP2C9	rs9332131	0.995 (116)	0.05 (1)	0 (0)	1.00 (105)	0.00 (0)	0 (0)
CYP2C9	rs7900194	0.81 (146)	0.17 (30)	0.02 (4)	0.96 (228)	0.04 (10)	0 (0)
CYP2C9[b]	rs2256871	0.17[c]	0.49[c]	0.34[c]	-	-	-
CYP2C9	rs28371685	0.98 (175)	0.02 (5)	0 (0)	0.98 (213)	0.02 (5)	0 (0)
CYP2C9	rs9332239	1.00 (117)	0 (0)	0 (0)	0.99 (104)	0.01 (1)	0 (0)
CYP2C19	rs12248560	0.01 (2)	0.27 (34)	0.72 (85)	0.03 (3)	0.21 (22)	0.76 (82)
CYP2C19	rs4244285	0.71 (83)	0.26 (33)	0.03 (5)	0.63 (69)	0.30 (33)	0.06 (7)
CYP2C19	rs4986893	1.00 (121)	0 (0)	0 (0)	0.96 (105)	0.04 (4)	0 (0)
CYP2C19	rs28399504	1.00 (121)	0 (0)	0 (0)	0.99 (108)	0.01 (1)	0 (0)
CYP2D6	rs1065852	0.88 (85)	0.10 (8)	0.02 (1)	0.80 (63)	0.20 (16)	0 (0)
CYP2D6	rs28371706	0.68 (61)	0.27 (28)	0.05 (5)	0.95 (75)	0.03 (2)	0.02 (2)
CYP2D6	rs59421388	0.71 (65)	0.28 (28)	0.01 (1)	0.99 (78)	0.01 (1)	0 (0)
CYP2D6	rs35742686	1.00 (94)	0 (0)	0 (0)	0.97 (22)	0.03 (2)	0 (0)

CYP2D6	rs3892097	0.96 (91)	0.04 (3)	0 (0)	0.79 (62)	0.20 (16)	0.01 (1)
CYP2D6	rs28371725	0.94 (87)	0.06 (7)	0 (0)	0.91 (72)	0.08 (6)	0.01 (1)
CYP2D6	rs5030655	1.00 (94)	0 (0)	0 (0)	1.00 (78)	0 (0)	0 (0)
CYP2D6	rs5030656	1.00 (94)	0 (0)	0 (0)	0.99 (78)	0.01 (1)	0 (0)
CYP2D6	rs16947	0.78 (68)	0.21 (24)	0.01 (2)	0.63 (50)	0.28 (22)	0.09 (7)
CYP2D6	rs72549357	0.94 (91)	0.02 (1)	0.04 (2)	0.97 (77)	0 (0)	0.03 (2)
CYP3A4	rs35599367	1.00 (182)	0 (0)	0 (0)	0.94 (104)	0.06 (7)	0 (0)
CYP3A5	rs776746	0.73 (90)	0.26 (33)	0.01 (2)	0.19 (21)	0.46 (50)	0.35 (38)
CYP3A5	rs10264272	0.58 (75)	0.38 (47)	0.04 (3)	0.91 (99)	0.08 (9)	0.01 (1)
CYP3A5	rs41303343	0.73 (94)	0.26 (29)	0.01 (2)	0.92 (100)	0.08 (9)	0 (0)
CYP4F2	rs2108622	0.86 (230)	0.10 (24)	0.04 (9)	0.48 (127)	0.35 (92)	0.17 (43)
DRD	rs1800497	0.37 (46)	0.49 (60)	0.14 (23)	0.43 (48)	0.50 (56)	0.08 (9)
EPHX1[d]	rs1051740	0.65 (74)	0.33 (38)	0.02 (4)	-	-	-
EPHX1[d]	rs2234922	0.51 (96)	0.42 (77)	0.07 (14)	-	-	-
F2	rs1799963	1.00 (182)	0 (0)	0 (0)	0.97 (106)	0.03 (3)	0 (0)
F5	rs6025	1.00 (182)	0 (0)	0 (0)	0.96 (108)	0.04 (4)	0 (0)
GGCX	rs12714145	0.27 (41)	0.48 (74)	0.25 (39)	0.37 (39)	0.47 (50)	0.14 (15)
GLP1R	rs1042044	0.12 (16)	0.51 (59)	0.37 (50)	0.14 (16)	0.52 (56)	0.34 (36)
GLP1R	rs2300615	0.81 (106)	0.17 (21)	0.02 (2)	0.55 (61)	0.39 (42)	0.06 (7)
GLP1R	rs6923761	0.98 (127)	0.00 (0)	0.02 (2)	0.83 (94)	0.15 (17)	0.02 (2)

MTHFR	rs1801131	0.73 (102)	0.26 (26)	0.01 (1)	0.46 (52)	0.43 (49)	0.11 (12)
MTHFR	rs1801133	0.86 (110)	0.14 (28)	0.01 (1)	0.72 (81)	0.25 (28)	0.03 (3)
NR112[e]	rs3732356	0.61 (95)	0.32 (50)	0.08 (12)	-	-	-
NR112[e]	rs2472677	0.37 (57)	0.48 (75)	0.15 (23)	-	-	-
NR112[e]	rs6785049	0.85 (128)	0.14 (21)	0.007 (1)	-	-	-
NR113[e]	rs2307424	0.88 (138)	0.12 (19)	0.00 (0)	-	-	-
NR113[e]	rs3003596	0.33 (51)	0.46 (72)	0.22 (34)	-	-	-
NR113[e]	rs2502815	0.56 (88)	0.34 (53)	0.10 (15)	-	-	-
OPRM1	rs1799971	1.00 (129)	0.00 (0)	0.00 (0)	0.66 (75)	0.310 (35)	0.03 (3)
PNPLA5	rs5764010	0.94 (120)	0.05 (8)	0.01 (1)	0.78 (88)	0.20 (23)	0.02 (2)
SLCO1B1	rs4149056	0.995 (180)	0.005 (2)	0 (0)	0.86 (96)	0.13 (15)	0.01 (1)
SULT4A1	rs763120	0.94 (119)	0.06 (10)	0.00 (0)	0.78 (87)	0.20 (22)	0.03 (3)
VKORC1	rs9923231	0.85 (208)	0.14 (32)	0.01 (3)	0.49 (123)[c]	0.41 (102)[c]	0.10 (26)[c]
VKORC1	rs9934438	0.75 (210)	0.20 (51)	0.05 (3)	0.54 (141)	0.36 (95)	0.10 (26)
VKORC1	rs7294	0.34 (87)	0.45 (121)	0.21 (55)	0.28 (73)	0.49 (127)	0.23 (59)
VKORC1	rs17708472	1.00 (154)	0 (0)	0 (0)	-	-	-
VKORC1	rs13336384	0.90 (138)	0.10 (16)	0 (0)	-	-	-

[a]Adapted from Dandara et al [165].

[b]Adapted from Soko (PhD thesis, personal communication).

[c]Genotype frequencies inferred from allele frequencies. NB: the following 15 polymorphisms, CYP1A2*2, CYP2C8*14, CYP2C9*2, *3, *12, CYP2C19*3, *4, CYP2D6*3, *6, *9, *15, CYP3A4*22, VKORC1*4, F2 rs1799963, F5 rs6025, were monomorphic.

[d]Adapted from Masimirembwa et al [166].

[e]Adapted from Swart et al [167].
ABCB1, ATP binding cassette subfamily B member 1; APOE, apolipoprotein E; CALU, Calumenin; COMT, catechol-O-methyltransferase; CYP, cytochrome P450; dbSNP, Single Nucleotide Polymorphism Database; DRD2, dopamine receptor D2; EPHX1, microsomal epoxide hydrolase 1; F2, coagulation factor 2; F5, coagulation factor 5; GGCX, gamma glutamyl carboxylase; GLP1R, glucagon like peptide 1 receptor; MTHFR, methylenetetrahydrofolate reductase; NR1/2, nuclear receptor subfamily 1 group 1 member 2; NR1/3, nuclear receptor subfamily 1 group 1 member 3; OPRM1, opiod receptor Mu1; PNPLA5, patatin like phospholipase domain containing 5; SLCO1B1, solute carrier organic anion transporter family member 1B1; SULT4A1, sulfotransferase family 4A member 1; VKORC1, Vitamin K epoxide Reductase Complex subunit 1.

Africans and less than 20% in other geographical populations, would differentially affect the outcomes of patients on warfarin and antiretroviral (ARV) drug EFV, with more Africans being affected, while the *SLCO1B1* rs4149056 of 0% among Africans, but up to 16% among Europeans, would affect warfarin outcomes among patients on statins, and the effects would be felt more among Europeans than Africans.

Comorbidities lead to requirements for concurrent medications, which increases risks of drug-to-drug interactions. Data in this study show that at least 31% of the drugs co-prescribed with warfarin are contraindicated for warfarin therapy [179]. Our findings support observations in the United States of at least 70–80% of patients on warfarin being co-administered with potentially interacting drugs [180] and about 13% of these being contraindicated for warfarin therapy. Risk of bleeding increases with concurrent illness owing to drug-to-drug interactions. It is even more complicated with herbal medicines that are commonly used throughout African populations, as evidenced by the 50% respondents in this study using herbal supplements alongside warfarin therapy [181, 182]. The major complication with herbal therapy is the lack of information on their effects when used together with other drugs, thus requiring a new impetus to understand herb-drug interactions [183]. However, there is little known about warfarin-herb interactions, and in African populations, this paucity of knowledge is further mystified by the limited knowledge on the actual herbal supplements taken, as well as limited research on common African herbal supplements [184, 185]. Herbal medicines together with food interactions are cited numerously as

Table 3.2: Distribution of Parameters Observed Among Participants on Warfarin

Parameters	Black Africans (N=252), N (frequencies)	Mixed Ancestry (N=251), N (frequencies)
Warfarin indication**		
Atrial fibrillation	34 (0.14)	65 (0.26)
Deep venous thrombosis	77 (0.31)	44 (0.18)
Mechanical valve replacement	53 (0.21)	114 (0.45)
Pulmonary embolism	14 (0.06)	25 (0.10)
Cardiomyopathy	8 (0.03)	0
Congestive cardiac failure	26 (0.10)	0
Rheumatic heart disease	17 (0.07)	0
Stroke	6 (0.02)	1 (0.004)
others	17 (0.07)	2 (0.008)
Comorbidity*		
Hypertension	11 (0.46)	107 (0.43)
Diabetes mellitus	16 (0.06)	45 (0.18)
Heart failure	113 (0.45)	97 (0.39)
HIV positive	38 (0.15)	7 (0.03)
Arrhythmia	64 (0.25)	0
Dyslipidemia	6 (0.02)	0
Concomitant drugs**		
ARVs	25 (0.10)	3 (0.01)
Aspirin	3 (0.01)	2 (0.01)
Allopurinol	0	3 (0.01)
Amiodarone	2 (0.01)	2 (0.01)
Amlodipine	7 (0.03)	8 (0.03)
Digoxin	13 (0.05)	2 (0.01)
Enalapril	30 (0.12)	0
Furosemide	61 (0.24)	4 (0.02)
Lorsatan	16 (0.06)	1 (0.004)
Statins	22 (0.09)	53 (0.21)
Spironolactone	28 (0.11)	1 (0.004)
Herbal medicine	72 (0.29)	0
Other drugs (rifampicin, nifedipine, atenolol, propranolol,)	106 (0.42)	57 (0.23)

Adapted with modifications from [107;171]. *Denotes global p-value <0.01.
**Denotes global p-value <0.0001.
ARVs, antiretroviral drugs (i.e., efavirenz, nevirapine and lamivudine).

one of the main causes of unstable International Normalised Ratio (INR) [183, 186]. Importantly, the lack of knowledge on herbal medicines should not always be viewed as a negative on herbal medicines, but should be viewed as a gap in indigenous knowledge translation [181]. Thus, a strong call for research on herbal medicines is made and this has a huge potential to unlock a whole health value chain.

Differences in pharmacogene variants are evident among African populations, and these run along ethnic or geographical lines. African populations show marked heterogeneity [161, 187, 188]; thus, the need for pharmacogenetic profiling and testing among African populations are large and should be considered for effective treatment and management of disease, especially in cases of concurrent illnesses [161]. For instance, the efflux transporter variant ATP binding cassette subfamily B member 1 (*ABCB1) rs1045642 (c.3435C>T, p.Ile1145Ile)* is associated with reduced tramadol, digoxin [189], and nevirapine [190] response. This variant has a frequency of 40% and 57% in South Africans of Mixed Ancestry and among East/Central Africans, respectively, while it only occurs in about 10% among other African populations. Thus, the use of tramadol and digoxin in the South African population, for example, is bound to result in different outcomes among the Mixed Ancestry and the black African groups, with pronounced effects in the Mixed Ancestry population group. This difference in the profile of pharmacogene variants is likely to differentially affect drug interactions that involve warfarin. Thus, our current study on the pharmacogenetic profiles of Africans on warfarin treatment, with special emphasis on the major concurrent medications prescribed to patients, is a step in setting a stage for precision medicine in Africa. Evaluating the 73 SNPs in the 22 genes shows that several groups of drugs (e.g., statins, antiretrovirals, antidiabetics, and antihypertensives), which are potentially prescribed together with warfarin, need to be taken into account to avoid drug-drug interactions and improve the health and well-being of patients.

3.2.1.4.1. *Statin pharmacogenetics*

Dyslipidemia was observed at a frequency of 21% among patients on warfarin in this study, which agrees with an average of 25% reported for population-based prevalence

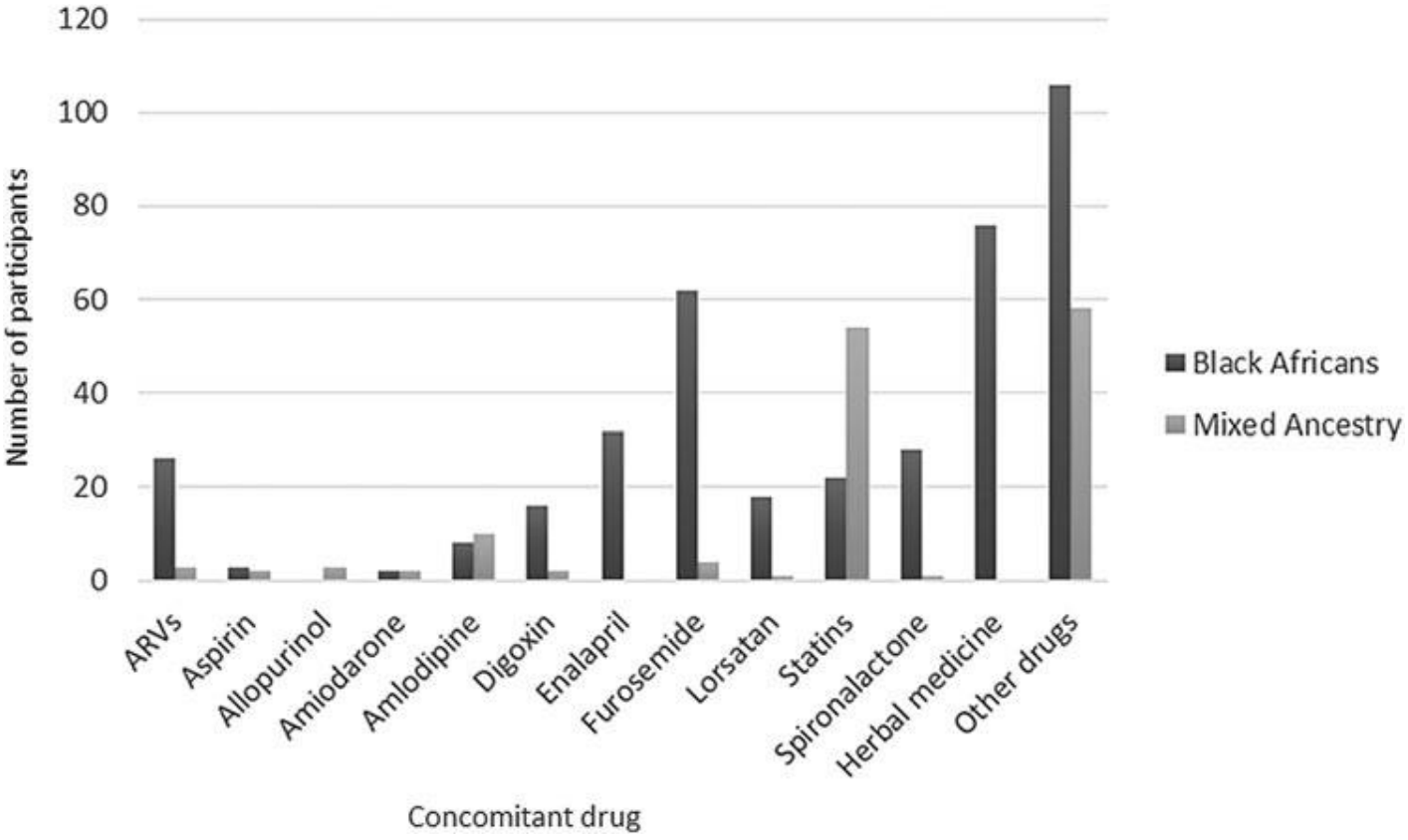

Figure 3.1: Burden of various concomitant drug use among Black Africans and Mixed Ancestry.

for dyslipidemia in African populations [191], implying at least a quarter of Africans on the continent have elevated cholesterol. Statins are the drugs of choice in the treatment of dyslipidemia and are prescribed in at least 5% of patients on warfarin in this study. Influx transporter *SLCO1B1* encoding organic anion transporter protein (OATPIBI) is an important pharmacogene in statin pharmacokinetics. This transporter is partially responsible for the hepatic uptake of statins. The liver is the site for both therapeutic effect and elimination of the hydrophilic statins pravastatin and active (acidic) simvastatin and rosuvastatin. The variant *SLCO1B1 rs4149056 c.521T>C, p.Val174Ala* has been implicated in statin-induced myopathy [192]. The *SLCO1B1 c.521C* allele has been shown to increase the odds of statin-induced myopathy in patients on statins through reduced transport or influx of the statin into the liver [193]. Consequently, the CPIC [194] issued precaution on patients with *SLCO1B1 c.521C* and considering statin therapy.

There is marked interethnic variability in the presence of *SLCO1B1 c.521C* in global populations. Indeed, in African populations, *c.521C* allele is significantly lower (p>0.0003) [195] when compared to European and Asian populations. Similarly, in our study, *SLCO1B1 c.521C* variant occurred at a frequency of 1% in black African populations compared to 13% in the Mixed Ancestry who have significantly higher European admixture [196]. This discrepancy strengthens the call for pharmacogenetic profiling among African populations as some subsets of African populations may require different doses of the same drug for effective and safe therapeutic outcome. Statin therapy is also affected by pharmacodynamic effects and the ability of effective response by the patient. Apolipoprotein E (*APOE)* has been associated with varying response to statin therapy. The protein APOE has three isoforms E2, E3, and E4. Of the three isoforms, E2 is associated with better response to statin therapy [197], while E4, defined by the presence of *APOE4 rs429358C* and *rs7412C* polymorphisms, shows the lowest response to statin therapy [198]. In patients on warfarin therapy in our study, *rs429358C* was observed at frequencies of 0.22 and 0.17 in black Africans and the Mixed Ancestry group respectively (Table 3.3). These frequencies are much higher than what is observed in both Asian (0.06) and European (0.04) populations. This has bearing on the efficacy of statins in Africans with dyslipidemia with a tendency toward poor outcomes among black Africans compared to their Mixed Ancestry counterparts. *APOE4* may therefore be an important pharmacogene variant in treatment of dyslipidemia in African populations on warfarin therapy.

3.2.1.4.2. *Antiretroviral pharmacogenetics*

The global burden of HIV disproportionately affects Southern Africa. In 2019, 1.4 million and 7.7 million people were living with HIV and AIDS in Zimbabwe and South Africa alone, respectively [199]. EFV has been the backbone of first- and second-line regimens in the fight against HIV and AIDS for the past decade. Concurrent administration of antiretroviral therapy in African patients on warfarin is inevitable. Indeed, in this study, the most prescribed ARV drug was EFV. EFV is metabolised predominantly by CYP2B6 and to a lesser extent by CYP1A2 and CYP3A5. *CYP2B6*6*, defined by *c.516G>T* and *c.785A>G* allele, is the most clinically elucidated

polymorphism of *CYP2B6* and is associated with decreased expression and function of CYP2B6 enzyme [200]. Individuals expressing this variant consistently demonstrate significantly reduced levels of the 8-hydroxylation of EFV and increased circulating plasma levels of the parent EFV compound, which is associated with increased neurotoxicity and central nervous system (CNS) side effects [201, 202]. Increased neuropsychiatric events have been reported among Zimbabwean [201] and South African patients [203, 204] harbouring the *CYP2B6*6 c.516T* allele when compared to their counterparts expressing the *CYP2B6*6 c.516C* allele. Frequency of the *CYP2B6*6 c.516T* allele is higher in African populations than both Asian and European populations [161, 202], as is confirmed in our study cohort where frequency of *CYP2B6*6 c.516T* ranged from 0.28 to 0.49 in African populations (Table 3.3), being lowest in South African Mixed Ancestry populations, while frequency was as low as 0.17 in Asians and 0.03 in Europeans, respectively. Thus, *CYP2B6*6 c.516T* pharmacogenetic profiling may be necessary in warfarin patients co-prescribed EFV and any other CYP2B6 metabolised drugs to reduce potential adverse drug reactions and enhance safe treatment.

CYP1A2 variation is more frequent in African populations with one study done in Ethiopia showing individuals with total absence of CYP1A2 activity [205]. *CYP1A2*1C* was higher in our study cohort among the black Africans when compared to the Mixed Ancestry (Table 3.3) and was similarly higher than in global populations such as the Europeans and African Americans. Both CYP1A2 and CYP2B6 expression are regulated by the transcription factors pregnane X receptor (PXR) and constitutive androstane receptor (CAR). The genes *NR1I2* and *NR1I3* encode PXR and CAR, respectively. Both PXR and CAR have been implicated in variation in EFV plasma levels in South African patients [167]. *NR1I3 rs2307424* was associated with increased levels of EFV and hence increased risk of CNS adverse drug response [167]. This polymorphism occurs at a significantly lower frequency in African patients [167] than in European and Asian patients, likewise this trend was observed among the black African warfarin patients (Table 3.3). Polymorphisms *NR1I2 rs2472677, rs6785049*, *NR1I3 rs3003596* and *rs2502815* were all associated with reduced EFV

Table 3.3: Comparison of Pharmacogene Variant Distribution Among Major Global Populations

			This study		Other African populations				
Gene	**dbSNP No.**	**Variant Allele**	**Black Africans**	**Mixed Ancestry**	**West Africans (YRI)**	**East Africans (LWK)**	**African Americans**	**East Asians**	**Europeans**
ABCB1	rs1045642	T	0.09*,†,‡,§	0.40‡,§,**,††	0.13	0.14	0.23	0.40	0.52
APOE	rs429358	C	0.22†,‡,**	0.17‡,**	0.24	0.38	0.05	0.09	0.16
APOE	rs7412	T	0.22*,†,‡,§,**,††	0.09§	0.11	0.05	0.10	0.10	0.06
CALU	rs1043550	G	0.07*,‡,§	0.18*,‡,§	0.09	0.11	0.17	0.07	0.42
CALU	rs339097	G	0.21*,‡,§	0.10*,‡,§	0.19	0.18	0.14	0.01	0.00
COMT	rs4680	A	0.35	0.35	0.31	0.29	0.31	0.24	0.50
CYP1A1[a]	rs1048943	G	0.00*,†	-	0.00	0.00	0.02	0.25	0.04
CYP1A2	rs2069514	A	0.26*	0.15*,**,††	0.32	0.32	0.21	0.28	0.02
CYP1A2	rs762551	A	0.55	0.64	0.54	0.48	0.60	0.67	0.68
CYP2B6	rs28399499	C	0.10*,‡,§	0.03§,††	0.12	0.06	0.07	0.00	0.00
CYP2B6	rs3745274	T	0.35	0.32	0.40	0.36	0.35	0.22	0.24
CYP2C cluster	rs12777823	A	0.30*	0.25*	0.29	0.27	0.25	0.31	0.15
CYP2C cluster	rs12772169	T	0.42*	0.39*	0.44	0.41	0.38	0.37	0.22
CYP2C8	rs11572105	T	0.03	0.02	0.04	0.05	0.04	0.00	0.02

CYP2C8	rs11572103	A	0.21*,†,‡,§	0.07*,†,§,††	0.20	0.14	0.07	0.00	0.00
CYP2C8	rs1058930	C	0.005*	0.01*	0.005	0.00	0.02	0.001	0.06
CYP2C8	rs188934928	G	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CYP2C8	rs11572101	G	0.19†	0.24	0.19	0.17	0.19	0.38	0.19
CYP2C8	rs11572100	C	0.09 †	0.06†	0.11	0.14	0.09	0.00	0.04
CYP2C8	rs1926705	T	0.89†	0.71†	0.88	0.87	0.80	0.45	0.69
CYP2C9	rs1799853	T	0.01*,‡	0.04*	0.00	0.00	0.07	0.001	0.12
CYP2C9	rs1057910	C	0.00*,§	0.05§	0.00	0.00	0.02	0.03	0.07
CYP2C9	rs28371686	G	0.01	0.00	0.03	0.01	0.006	0.00	0.00
CYP2C9	rs9332131	delA	0.003	0.00	0.02	0.00	0.005	0.00	0.00
CYP2C9	rs7900194	A/T	0.11*,†,‡,§	0.02§	0.05	0.07	0.02	0.00	0.002
CYP2C9[b]	rs2256871	G	0.58*,†,‡,**,††	-	0.09	0.15	0.07	0.00	0.001
CYP2C9	rs28371685	T	0.01	0.01	0.05	0.02	0.02	0.00	0.002
CYP2C9	rs9332239	T	0.00	0.005	0.00	0.00	0.001	0.00	0.003
CYP2C19	rs12248560	T	0.14†	0.13†	0.25	0.18	0.22	0.02	0.22
CYP2C19	rs4244285	A	0.17†	0.22	0.17	0.21	0.18	0.31	0.15
CYP2C19	rs4986893	A	0.00†	0.00†	0.00	0.01	0.00	0.06	0.00
CYP2C19	rs28399504	G	0.00	0.00	0.00	0.00	0.001	0.001	0.001
CYP2D6	rs1065852	T	0.07*,†,‡	0.10*,†	0.11	0.04	0.19	0.57	0.20

CYP2D6	rs72549357	T	0.05	0.03	-	-	-	-	-
CYP2D6	rs28371706	T	0.19*,‡,§	0.04‡,§,**,††	0.26	0.19	0.14	0.00	0.00
CYP2D6	rs59421388	A	0.15*,‡,§	0.006‡,§,**,††	0.11	0.17	0.07	0.00	0.00
CYP2D6	rs35742686	delA	0.00	0.00	0.00	0.00	0.008	0.00	0.02
CYP2D6	rs3892097	A	0.02*,‡,§	0.11†,§,**	0.06	0.03	0.15	0.00	0.19
CYP2D6	rs28371725	A	0.03	0.05††	0.009	0.03	0.09	0.04	0.09
CYP2D6	rs5030655	delA	0.00	0.00	0.00	0.00	0.01	0.00	0.02
CYP2D6	rs5030656	CT	0.00	0.006	0.00	0.00	0.01	0.00	0.03
CYP2D6	rs16947	T	0.12*,‡,**,††	0.23‡,**,††	0.56	0.65	0.46	0.14	0.34
CYP3A4	rs35599367	T	0.00*	0.03	0.00	0.00	0.00	0.00	0.05
CYP3A5	rs776746	G	0.15*,†,‡,§	0.58*,‡,§,**,††	0.17	0.12	0.31	0.71	0.95
CYP3A5	rs10264272	A	0.24*,†,‡,§	0.05*,†,§,**,††	0.17	0.24	0.12	0.00	0.003
CYP3A5	rs41303343	T	0.14*,†,‡,§	0.04§,**,††	0.12	0.12	0.04	0.00	0.00
CYP4F2	rs2108622	T	0.10*,‡,§	0.34‡,§,**,††	0.06	0.11	0.12	0.21	0.29
DRD	rs1800497	A	0.39*	0.33	0.41	0.37	0.34	0.41	0.19
EPHX1[c]	rs1051740	C	0.19†	-	0.10	0.20	0.19	0.48	0.30
EPHX1[c]	rs2234922	G	0.28†	-	0.42	0.33	0.32	0.12	0.16
F2	rs1799963	A	0.00	0.01	0.00	0.00	0.00	0.00	0.008
F5	rs6025	A	0.00	0.02	0.00	0.00	0.005	0.00	0.02
GGCX	rs12714145	T	0.49	0.40	0.52	0.46	0.39	0.38	0.42

GLP1R	rs1042044	C	0.63	0.60	0.61	0.67	0.57	0.55	0.56
GLP1R	rs2300615	G	0.11[†,§]	0.26[‡,§,**,††]	0.06	0.09	0.09	0.44	0.18
GLP1R	rs6923761	A	0.03*	0.09*	0.00	0.005	0.09	0.01	0.33
MTHFR	rs1801131	C	0.15*[,§]	0.32[§,††]	0.12	0.19	0.17	0.22	0.31
MTHFR	rs1801133	T	0.08*[,†]	0.15*[,†]	0.11	0.07	0.13	0.30	0.36
NR112[d]	rs3732356	G	0.23*	-	0.31	0.30	0.26	0.12	0.06
NR112[d]	rs2472677	T	0.35*[,†]	-	0.36	0.40	0.40	0.62	0.66
NR112[d]	rs6785049	A	0.04*[,†,‡]	-	0	0.05	0.12	0.40	0.62
NR113[d]	rs2307424	T	0.05*[,†,‡]	-	0.10	0.09	0.16	0.52	0.35
NR113[d]	rs3003596	C	0.42	-	0.61	0.52	0.49	0.57	0.44
NR113[d]	rs2502815	T	0.23[†,**,††]	-	0.44	0.38	0.31	0.44	0.25
OPRM1	rs1799971	G	0.00*[,†,‡,§]	0.18[†,‡,§]	0.00	0.005	0.05	0.39	0.16
PNPLA5	rs5764010	T	0.03*[,‡,§]	0.12[‡,§,**,††]	0.04	0.006	0.04	0.19	0.10
SLCO1B1	rs4149056	C	0.005*[,†,‡,§]	0.08[§,††]	0.009	0.02	0.04	0.12	0.16
SULT4A1	rs763120	G	0.03*[,‡,§]	0.13[§,**,††]	0.04	0.01	0.05	0.29	0.10
VKORC1	rs9923231	A	0.09*[,‡,§]	0.31[†,‡,§,**,††]	0.03	0.04	0.12	0.88	0.39
VKORC1	rs9934438	T	0.12*[,‡,§]	0.28[†,‡,§,**,††]	0.03	0.04	0.11	0.88	0.39
VKORC1	rs7294	A	0.44[†]	0.47[†]	0.51	0.43	0.46	0.11	0.37
VKORC1	rs17708472	T	0.02*	-	0.02	0.06	0.07	0.00	0.23
VKORC1	rs13336384	T	0.05*[,†]	-	0.04	0.09	0.05	0.00	0.00

*p-Value ≤0.05 between studied population and Europeans.
†p-Value ≤0.05 between studied population and Asians.
‡p-Value ≤0.05 between studied population and African Americans.
§p-Value ≤0.05 between the studied populations (i.e., Black Africans and Mixed Ancestry).
**p-Value ≤0.05 between studied population and East African.
††p-Value ≤0.05 between studied population and West Africans.
[a]Adapted from Dandara et al [165].
[b]Adapted from Soko (PhD thesis, personal communication).
[c]Adapted from Masimirembwa et al [166].
[d]Adapted from Swart et al [167].
LWK, Luhya in Webuye Kenya; YRI, Yoruba in Ibadan Nigeria.

plasma levels in South African HIV/AIDS patients. The presence of these polymorphisms is postulated to increase expression of CYP2B6 and CYP1A2, hence increasing metabolism of EFV and lowering its concentration in the blood. *NR1I2 rs2472677C>T* occurs at a significantly higher frequency in African populations than in both Asian populations, implying the failure of viral suppression maybe higher in African patients with HIV on warfarin treatment co-prescribed EFV, than their European and Asian counterparts.

In 2019, the WHO recommended the use of an integrase inhibitor Dolutegravir (DTG) as a first- and second-line regimen to gradually replace EFV therapeutic use in HIV patients. DTG has good tolerability and predictable pharmacokinetics in adults [206] when compared to EFV. However, concerns surround plasma level-linked neuropsychiatric events [207] and weight gain [208] in patients switching from DTG to EFV. DTG is primarily metabolised by UDP glucuronosyl-transferase 1 family, polypeptide A1 (UGT1A1), and partly by CYP3A. Variations in the *UGT1A1* gene, in particular *UGT1A1*28* and **6*,

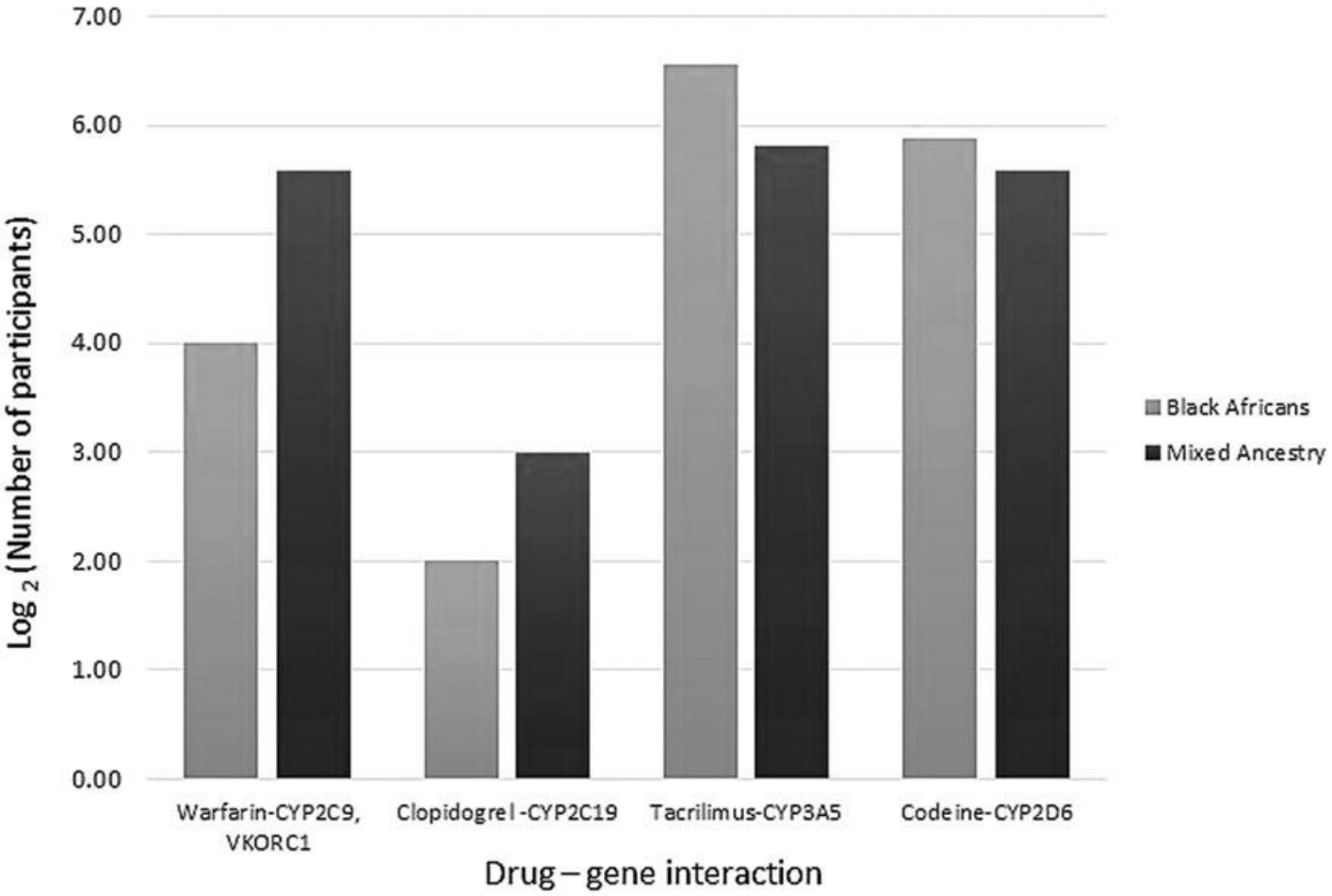

Figure 3.2: Clinically actionable drug-genotype interactions among Black Africans and Mixed Ancestry. The definition of actionable variants as adapted (with modifications) from Goh et al. (2017) is defined as follows: (1) warfarin, *CYP2C9*2, *3, *8, *11* heterozygote or homozygote with VKORC1 GA or AA genotype and CYP2C9*1 with VKORC1 AA genotype; (2) clopidogrel, CYP2C19*2 and *3; (3) tacrolimus, CYP3A5*1 heterozygote or homozygous; and (4) codeine, CYP2D6 poor and intermediate metabolisers. VKORC1, Vitamin K epoxide Reductase Complex subunit 1.

have been associated with increased levels of DTG [207]. Transporter genes *SLC22A2* [208] and *ABCG2* [209] have also been implicated in increased plasma levels of DTG. Frequency of both *UGT1A1*28* and **6* in African populations is low; these polymorphisms are likely to play a minor role in the pharmacogenomics of DTG therapy in African HIV patients. Similarly, *ABCG2 rs2231142 (c.421C>A, pGln141Lys),* which is associated with increased plasma levels of DTG, also occurs in significantly ($p<0.001$) lower levels in African populations (1%) than in both European (10%) and Asian (19%)

populations [195]. It may be necessary to investigate the African-specific pharmacogenetic variants that may play a role in inter-individual variability in response and toxicity to DTG than rely on polymorphisms identified in Asian or European populations.

3.2.1.4.3. *Antihypertensive pharmacogenetics*

The pharmacogenetics of antihypertensives has a huge bearing on patients on warfarin therapy in both Zimbabwe and South Africa. Hypertension was the predominant co-morbidity in this study, with at least 40% of the respondents co-prescribed the diuretic furosemide, while the antihypertensives enalapril, losartan, carvedilol, atenolol, hydrochlorothiazide spironolactone, and nifedipine were among the most common co-prescribed drugs in this study population (Table 3.2). Antihypertensives are themselves a broad class of drugs with differing pharmacokinetics and pharmacodynamics. Little is known of the pharmacokinetics and pharmacodynamics of furosemide, which is the most prescribed anti-hypertensive in our study cohort; however, it is believed the drug is metabolised predominantly in the kidney [210] by glucuronidation by UGT1A9, UGT2B7, and UGT1A6. However, variants in genes encoding *CYP2C9, CYP2D6*, and *CYP3A5* have all been implicated in the pharmacogenetics of antihypertensive drugs (211). Transporter genes *SLC4A1* and *SLCO1B1* have also been associated with the pharmacogenetics of antihypertensives [211]. Indeed, *SLCO1B1 *15/*15* carriers showed a 6.94-fold increased risk (95% confidence interval=1.30–37.07, p=0.020) of enalapril-induced cough as an ADE to hypertension therapy. As *SLCO1B1*15/*15* occurs in close to 0% in Southern African populations, it is unlikely this polymorphism can be used for enalapril toxicity, instead, it is possible that other uniquely African variants [195] may play a role in enalapril pharmacogenetics.

The enzyme CYP2C9 is an important enzyme in warfarin pharmacogenetics. Poor metabolism of warfarin leads to prolonged bleeding in patients. The CPIC in 2017 [45] provided a guideline for warfarin dosing using a pharmacogenomics algorithm. Due to interethnic variability in warfarin pharmacogenetics, patients are grouped into non-African ancestry and African ancestry. Genotypes with impact on warfarin dosing for non-Africans are *CYP2C9 *2/*3* and *3/*3*. Numerous studies show that *CYP2C9*2*

(R144C) and **3 (I359L),* which are predominantly European polymorphisms, increase risk of prolonged bleeding in warfarin patients. Similarly, *CYP2C9*5, *6, *8*, and **11,* predominantly African variants, are incorporated into the African warfarin pharmacogenetics algorithm [45, 171] as they too affect warfarin therapy. Indeed, the effects of *CYP2C9*2* and **3* variants are seen in the angiotensin II receptor blocker, losartan. Losartan is metabolised to its active form E-3174 by CYP2C9. Individuals expressing the *CYP2C9*3* variant showed reduced metabolism of oral losartan [212], while *CYP2C9*2* showed a slightly better clearance of plasma levels of oral losartan compared to the *CYP2C9*3* variant [213]. Losartan was co-prescribed in 10% of participants in this study (Table 3.2), implying reduced CYP2C9 would affect not only losartan metabolism but also warfarin metabolism. A uniquely African variant *CYP2C9*9* variant, first reported by Matimba et al [214], occurs at a frequency of 58% in black Africans (Zimbabweans) and is postulated to reduce CYP2C9 activity, and hence increase toxicity to drugs metabolised by CYP2C9. This implies that at least 10% of Zimbabwean patients could have adverse drug reactions to both losartan and warfarin, thus decreasing the safe and efficacious use of these two drugs in this population. As *CYP2C9* is an important gene in warfarin treatment, pharmacogenetic testing of *CYP2C9* focusing on uniquely African variants should be of paramount importance in African populations where comorbidities, especially hypertension, are high.

CYP3A4 and CYP3A5 are also important genes/enzymes in the drug-drug interaction of warfarin. CYP3A4 activity is more dominant in European populations, while CYP3A5 is more active in individuals of African ancestry [215]. CYP3A5 and CYP3A4 have overlapping specificities and together are responsible for the metabolism of 50–60% of all clinical drugs, including erythromycin, nevirapine, lopinavir, tamoxifen, EFV, and the statins (lovastatin, simvastatin, and atorvastatin), drugs that are co-prescribed in warfarin patients, as observed in this study. The CYP3A isoforms are also responsible for the metabolism of antihypertensives amlodipine and nifedipine, which were also reportedly co-prescribed in patients on warfarin in this study. *CYP3A4*1B* and **22* are the most characterised SNPs of *CYP3A4*. *CYP3A4*22* is associated with reduced messenger RNA (mRNA) and enzyme expression that resulted in elevated statin

plasma levels [216]. *CYP3A4*22* is rare in global populations; its highest occurrence is 5% in European populations. In our study, *CYP3A4*22* was only observed among Mixed Ancestry (3%), probably owing to European admixture, and was virtually absent in the black population groups. *CYP3A4*22* is unlikely to play a major role in the pharmacogenetics of antihypertensives, or any other CYP3A cluster drug in Southern African populations.

*CYP3A5*3 (rs776746)* is the most documented non-functional variant of *CYP3A5*. *CYP3A5*3* is defined by the presence of the *c.6986A* allele. The A allele creates an alternatively spliced form that alters the reading frame, and introduces a premature stop codon resulting in a non-functional truncated protein [217]. Thus, carriers of the *CYP3A5*3/*3* genotype do not express CYP3A5. *CYP3A5*3* occurs at a frequency of 95% in Europeans, 71% in Asians, 58% in South Africans of Mixed Ancestry, and 15% black Africans. The total absence of the *1 wild-type allele in European individuals implies there is reduced activity of CYP3A5 in drug disposition; hence, CYP3A5 plays little role in drug pharmacogenetics in European and Asian individuals. However, variations in *CYP3A5* have an effect on inter-individual differences among populations of African ancestry; thus, CYP3A5 inhibitors may have a more profound effect on drug metabolism in black Africans than those of Mixed Ancestry. As CYP3A5 metabolises over 50% of clinical drugs, including the antihypertensives (amlodipine and nifedipine), this enzyme may play a significant role in the pharmacogenetics of drugs co-prescribed with warfarin in African patients.

CYP2D6 metabolises up to 25% of drugs commonly used in clinical practice. It is the enzyme primarily responsible for metabolising the β-blocker carvedilol [218]. Carvedilol, which is used to treat hypertension and heart disease, was co-prescribed to 10% of Zimbabwean participants on warfarin treatment. CYP2D6 is highly polymorphic, giving rise to four main phenotypes; poor metabolisers (PM) who lack a single functional allele, intermediate metabolisers (IM) who have one null allele, extensive metabolisers who are the normal phenotype, and ultra-metabolisers who possess more than one copy of the CYP2D6 gene (i.e., gene duplications). PM have been shown to have two to three times higher R-carvedilol plasma levels, which lead to higher risk of drug-related

dizziness (219). PM status occurs at frequencies of 5–10% in Europeans [220] and is rare in both Asian and African individuals. *CYP2D6*17 (rs28371706, c.1023C>T)* occurs in at least 30% of African individuals [220, 221] and is associated with reduced enzyme activity. Individuals carrying the *CYP2D6*17* allele are classified as IM.

CYP2C19 plays a minor role in the metabolism of the beta-blocker propranolol, which was co-prescribed to 2% of the Zimbabwean respondents. Two important SNPs observed in this study, *CYP2C19*17 (c.-806C>T, rs12248650),* occurring 13–17% in the three African populations, are rare among Asians (4%) and highest in Europeans (26%). *CYP2C19*17* is associated with increased activity of the enzyme [222] and therefore increased clearance of CYP2C19 substrates. However, *CYP2C19*17* carriers may have reduced response to propranolol, especially in the presence of reduced activity of CYP2D6, the principal metabolising enzyme. The second variant, *CYP2C19*2 (c.618G>A, rs4244285),* is the most common loss of function allele known to *CYP2C19* (223). *CYP2C19*2* creates an aberrant splice variant in exon 5 that alters the mRNA reading frame producing a truncated non-functional protein [224]. *CYP2C19*2* occurs at a frequency of 17% in black Africans, 22% in South Africans of Mixed Ancestry, and 31% Asians and 15% in Europeans. Evidently, the effect of reduced CYP2C19 is expected to be more pronounced in South Africans of Mixed Ancestry than among their black African counterparts.

3.2.1.4.4. *COVID-19 therapy or interventions*

In July of 2021, the WHO [225] reported that there were 191,773,590 confirmed COVID-19 cases globally; the WHO Africa Region accounted for 4,688,762 of these cases. Within the African region, South Africa bore half of the COVID-19 burden with 2,327,472 confirmed cases, while Zimbabwe had 91,120 cases. Since December 2019, COVID-19 has grown into a global pandemic that has affected every nation on earth. It can, therefore, be expected that patients on warfarin are likely to be treated for COVID-19. Although during our recruitment, COVID-19 was not yet a global threat to the population under study, concurrent exposure to COVID-19 by warfarin patients in Africa cannot be ignored; hence, the pharmacogenomics of COVID-19 therapy is worth considering. Pharmacogenomics of COVID-19 is critical among warfarin patients in Africa as it may

point clinicians to first-line choices and initial dosages, given that the list of approved drugs keeps growing and recommendations are still evolving. This will allow reduced risk of drug efficacy in patients who cannot afford ineffective therapy in the face of a life-threatening infection. Furthermore, as patients with severe symptoms tend to also have comorbidities [226], drug toxicity needs to be reduced. Using a combination of online sources, Takahashi et al [227] identified several drug-gene pairs that may play a role in the pharmacogenomics of COVID-19 therapy. These included hydroxychloroquine/chloroquine (*CYP2C8, CYP2D6, SLCO1A2*, and *SLCO1B1*), azithromycin (*ABCB1*), ribavirin (*SLC29A1, SLC28A2,* and *SLC28A3*) and lopinavir/ritonavir (*SLCO1B1, ABCC2*, and *CYP3A*).

The macrolide ivermectin is currently under trial in both Zimbabwe and South Africa for possible application in COVID-19 therapy. Being lipophilic, ivermectin is widely distributed in the body, but it is metabolised in liver microsomes into ten different metabolites by CYP3A4 [228]. Pharmacogenomics of anti-COVID-19 drugs is likely to be African specific as studies have already shown that pharmacogene variants are ethnically biased [161], a variant that may play a significant role in one population may not play a major role in another. Furthermore, our study confirms the heterogeneity of African populations as seen by the variation among the South African Mixed populations when compared to their black counterparts. Therefore, it is imperative for African populations to profile pharmacogenetic variants within their populations to achieve safe and efficacious administration of COVID-19 treatment in patients co-prescribed with warfarin. COVID-19 is a systemic disease whose long-term effects are far reaching and go beyond the lungs. Long-term effects of COVID-19 include damage to the lungs, heart, and brain [229]. The potential of a myriad of therapeutic agents on an already burdened African warfarin patient cannot be ignored. As such, COVID-19 introduces a new drug-drug dilemma for warfarin patients in Africa. Pharmacogenomic biomarkers are vital [230] for inclusion in drug development, on-going clinical trials, and drug repurposing as they offer a conceptual and practical steering wheel for therapeutic management of COVID-19. As African populations display significant inter-individual and population differences in drug treatment outcomes, and pharmacogenomic markers

in Africans differ from other global populations, it is vital to include African pharmaco-genomic biomarkers in emerging clinical trials for COVID-19 medicines and their therapeutic implementation strategies.

3.2.1.4.5. *Antimalarial and antidiabetic therapy*

CYP2C8, which is implicated in the metabolism of hydroxychloroquine and chloroquine, is also involved in the metabolism of antidiabetics (meglitinides and thiazolidinedione), the antimalarials (chloroquine and aminoquinoline), the anticonvulsant carbamazepine, and the anti-inflammatory pain killers diclofenac and ibuprofen [231]. Diabetes was a concurrent illness in 5% of the Zimbabwean patients, while none of the patients was taking antimalarials during recruitment. However, as malaria is endemic in Zimbabwe, it can be expected that during the course of warfarin therapy, some patients will receive concurrent treatment of malaria. A total of 4/154 (3%) Zimbabwean patients were also co-prescribed CYP2C8 substrate, carbamazepine. CYP2C8 comprises 7% of the total hepatic CYP content of the liver [232]. It shares 74% sequence homology with CYP2C9 and is therefore regulated by similar transcription factors like PXR. Therefore, pharmacogenomic variants that affect PXR function will ultimately affect CYP2C8 transcription and hence overall function in patients. CYP2C8 variants show ethnic bias with *CYP2C8*2* occurring at a frequency of 21% in black African patients co-prescribed warfarin. However, *2 is rare in individuals of European descent and occurs in 7% of the mixed Ancestry South Africans. *CYP2C8*2 (rs11572103)* is associated with lowered intrinsic clearance of the enzyme's metabolites and may therefore be an important variant in African populations, especially those with less European admixture. Similarly, *CYP2C8*3*, another *CYP2C8* variant with lowered activity, is rare in African populations, but occurs in 20% of European populations [231]. *CYP2C8*4 (rs1058930),* another variant associated with low enzyme activity, is rare in African populations, but occurs at a frequency of 1% in European individuals. Both *CYP2C8*3* and *CYP2C8*4* therefore may play a minor role in African populations co-prescribed CYP2C8 substrates when compared to their European counterparts. However, CYP2C8 has an active site like CYP3A4; as a result, CYP3A4 and CYP2C8 have overlapping substrates. The effects of

CYP2C8 are therefore unlikely to be detrimental in African populations co-prescribed warfarin as most concurrent drugs will be metabolised by CYP3A4.

3.2.1.5. Conclusions

African patients on warfarin tend to have a high incidence of comorbidities owing to the high burden of both communicable and noncommunicable diseases on the African continent. We present the pharmacogenetic profiles of 503 individuals from Southern African on warfarin therapy. Most of these patients were also on concurrent treatment for hypertension, heart failure, dyslipidemia, diabetes, and/or HIV infection. Thus, pharmacogenetic profiles presented in this study were for drug-metabolising enzymes, receptors, and transporters involved in the pharmacology of drugs co-prescribed with warfarin in our study cohort. Black African patients present with similar pharmacogenetic profiles. However, their profiles are distinct from both Asian and European populations, especially in pharmacogenetic SNPs predominantly useful among Asian and European populations. Similarly, South Africans of Mixed Ancestry have pharmacogenetic profiles somewhat different from the black Africans, showing the effect of admixture in African subpopulations. Hence, to ensure safe and efficacious warfarin therapy, pharmacogenetic profiling of African subpopulations should be enhanced. Precision medicine requires African populations begin to capture their own pharmacogenetic SNPs as they cannot infer with absolute certainty from Asian and European populations. African heterogeneity also requires subpopulation investigation as seen by the differences between African blacks when compared to South Africans of Mixed Ancestry.

Authors' Contributions

S.M. and A.N. conceptualised the idea, generated and analysed data and drafted the article. B.K. generated data. E.C. analysed data and reviewed the article. G.K., C.M.M. and C.F.B.N. conceptualised the idea, supervised the work, and reviewed the article. M.N. and C.D. conceptualised the ideas, supervised all components, and reviewed the article drafts. All authors made a significant intellectual contribution to the final revised version.

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Author Disclosure Statement

The authors declare they have no financial conflicts of interest.

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3.3. Correlation of observed allelic variants, clinical and demographic variables with effects on warfarin maintenance dose.

Synopsis: This section focuses on associating the studied genetic markers, clinical and demographic factors with warfarin response measured by the warfarin weekly maintenance dose. Two published papers (https://doi.org/10.1089/omi.2018.0174 and https://doi.org/10.1111/jth.15494) and an unpublished manuscript are included in this section responding to this objective.

3.3.1. Warfarin Dose and CYP2C Gene Cluster: An African Ancestral-Specific Variant Is a Strong Predictor of Dose in Black South African Patients

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Co-Authors Contribution:

ZC: Recruitment, collected demographic, clinical and biological data.

EM: Recruitment of the study participants.

APK and EC: Data analysis and reviewed the manuscript draft.

AW and MN: co-supervised all components, reviewed the manuscript.

CD: conceptualised the ideas, supervised all components and reviewed the manuscript draft.

All authors contributed to the final version of the article. The authors read and approved the final manuscript.

Warfarin Dose and CYP2C Gene Cluster: An African Ancestral-Specific Variant Is a Strong Predictor of Dose in Black South African Patients

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Abstract

Warfarin is a widely prescribed anticoagulant with a narrow therapeutic index. The *rs12777823G>A* single-nucleotide polymorphism (SNP) in the CYP2C gene cluster has been shown to influence optimal warfarin doses in African Americans. We report here effects of *rs12777823G>A* SNP on warfarin dose requirements in two South African population groups, black Africans (BA) and Mixed Ancestry (MA). A total of 425 participants on warfarin treatment were enrolled in the study. The age group of the studied population ranged between 18 and 90 years, with 69% females enrolled. Genetic characterisation of the *rs12777823G>A* was done using the TaqMan SNP genotyping assay. To further compare effects of *rs12777823G>A* to those of other SNPs, *VKORC1 g.–1639G>A* and 4 SNPs in *CYP2C9* gene (i.e., *CYP2C9 c.430C>T, c.1075A>C, c.449G>A, and c.1003C>T*) were analysed. The *rs12777823A* variant allele frequencies were 0.28 and 0.25 in the BA and MA, respectively. The *rs12777823A/A* genotype was associated with significantly (p=0.002) reduced mean warfarin dosage (27±5.3 mg/week) compared with the G/G genotype (45±16.1 mg/week) among BA, but not among the MA. The *rs12777823G>A* is located in a nongenomic region, suggesting that this SNP might be in linkage disequilibrium with another, likely causal SNP that is

present in BA only. Given ongoing worldwide efforts to identify clinically relevant human genetic variation impacting on optimal warfarin dose selection, the African ancestry-specific genetic variant in the CYP2C cluster and others warrant further research and consideration in development of future warfarin dosing algorithms for precision medicine guidelines.

Keywords: warfarin, South Africa, Black African, Mixed Ancestry, CYP2C cluster, polymorphism

3.3.1.1. Introduction

Warfarin is the most widely used anticoagulant worldwide and the anticoagulant of choice in sub-Saharan Africa, due to its efficiency in reducing stroke rates and its relatively low cost [15, 233]. However, warfarin use is complicated by a narrow therapeutic index, difficulty with dose titration, and adverse effects that often result in hospital admissions [17, 78]. Huge differences have been reported on the dose requirements to reach the international normalised ratio (INR) [17, 44]. Therefore, warfarin requires constant monitoring of the INR, and significant efforts in reaching and maintaining a stable therapeutic ratio of 2–3 [20]. The inter-individual variability observed in warfarin starting and maintenance doses is attributed to environmental and genetic factors [79]. Environmental factors include demographic parameters (e.g., race, age, gender, and body weight), clinical parameters (such as comorbidities and comedications), and social or lifestyle variables (e.g., diet, alcohol consumption, and smoking) [20, 80, 81]. Variants in genes involved in warfarin metabolism and warfarin target proteins play a significant role in warfarin dose requirements and response variabilities [48, 58].

Warfarin exists and is administered as a racemic mixture of S- and R-enantiomers, of which the S-form is the most potent [45]. It exerts its anticoagulant effects by inhibiting the actions of the vitamin K epoxide reductase complex subunit 1 (VKORC1), an enzyme involved in catalysing the interconversion of vitamin K 2,3 epoxide (vitamin K epoxide) to reduced vitamin K hydroquinone (KH2) [32]. The S-warfarin form is

principally metabolised by CYP2C9, while R-warfarin is mainly metabolised by CYP1A2 and CYP3A4, with other enzymes such as CYP2C18, CYP2C19, and CYP2C8 playing minor roles [40, 41]. Single-nucleotide polymorphisms (SNPs) in *CYP2C9, VKORC1*, and *CYP4F2*, which are located in chromosome 10, 16, and 19, respectively, have largely been shown to affect dose variability [48, 50, 58, 83, 85]. Studies across world populations have consistently shown that genetic variants in *CYP2C9* and *VKORC1* account for up to 40% of the total variability in warfarin dose requirements among Caucasian and Asian populations, but are poor predictors of variability in African populations. *VKORC1 g.–1639G>A* SNP and *CYP2C9* allelic variants (*CYP2C9*2, CYP2C9*3*) are now part of several warfarin dosing algorithms [45, 59, 63, 67, 87, 88, 234]. However, because of the quantitative and qualitative differences in the distribution of genetic variants that affect warfarin response in different populations, these algorithms are limited to specific ethnic groups such as Europeans and Asians, and they perform poorly or play no role in the prediction of warfarin dose requirements among native Africans [66, 89].

Recently, a number of studies and reports have described additional important variants such as *CYP2C9*5, *6, *8*, and **11* to play a more prominent role in affecting warfarin doses among African Americans [45, 57, 116]. For example, a genome-wide association study (GWAS) identified an *rs12777823G>A* SNP in the CYP2C cluster to be strongly associated with reduced warfarin dose among African Americans [145]. The effects of *CYP2C9*5, *6, *8*, and **11* have been confirmed in several African studies [109, 111, 115]. However, despite recent emerging data from populations of African ancestry, there are no data on the effects of *rs12777823G>A* SNP in indigenous African populations with respect to warfarin response. This is important because the available information suggests that this SNP is African ancestral specific [47, 56, 145]. Considering, the huge genetic diversity and admixture in Africans, it would be important to determine the influence of this variant in warfarin response among indigenous African populations. Therefore, we set out to examine the effects of *rs12777823G>A* SNP on warfarin dose requirements in two South African population groups: black Africans (BA) and individuals of Mixed Ancestry (MA).

3.3.1.2. Methods and Materials

3.3.1.2.1.Patient cohort

Four hundred twenty-five (n=425) participants on warfarin treatment were recruited from INR clinics at the Groote Schuur Hospital (GSH) and Gugulethu Community Health Centre (GCHC) in the Western Cape Province, South Africa, in 2016. Ancestry for these patients was determined both through self-identity and family histories preceding three generations. The participants comprised 148 BA and 277 MA individuals. The participants provided full consent to be enrolled in the study after ethical approval was granted by the University of Cape Town Human Research Ethics Committee (UCT-HREC REF No. 581/2015). Demographic variables (e.g., age, gender) and clinical parameters (e.g., warfarin indications, comedications, and comorbidities) were collected through a questionnaire and retrieval of patients' medical/hospital records, respectively. Information collected was recorded in the study database. Whole blood (5 mL) was collected in EDTA-coated tubes and used for extraction of deoxyribonucleic acid (DNA). For anonymity, patients' names were replaced by a laboratory-generated number.

3.3.1.2.2.Genetic characterisation for rs12777823G>A, CYP2C9, and VKORC1 g.–1639G>A SNPs

Genetic analysis was conducted in the Pharmacogenomics and Drug Metabolism Research Laboratory, Division of Human Genetics, University of Cape Town. DNA was extracted from whole blood using the salting-out DNA purification method (modified from Gustafson et al [160]). The *rs12777823G>A* genotypes were determined using a commercially available TaqMan SNP genotyping assay (catalog No. 4351379) obtained from Thermo Fisher Scientific (Applied Biosystems, Foster City, CA). The assay was carried out on a CFX Real Time Quantitative Polymerase Chain Reaction (q-PCR) detection system in 96-well plates containing a 10 µL reaction mix that comprised 10 ng DNA, 5 µL of TaqPath ProAmp Master Mix, and 0.5 µL of 20X TaqMan SNP genotyping assay. The q-PCR cyclic conditions were as follows: pre-read at 60°C for 30 sec, followed by the initial denaturation at 95°C for 5 min, then 40 cycles of denaturation at 95°C for 5 min, and annealing at 60°C for 30 sec. The reaction was incubated at 60°C for 30 sec to complete the reaction. The resulting data were analysed using the CFX

Manager™ software (BioRad, CA). The TaqMan SNP genotyping assay results were validated using PCR, coupled with Sanger sequencing. For sequencing, the dye was obtained from Applied Biosystems. Oligonucleotides were designed using the Integrated DNA Technologies (IDT) PrimerQuest tool (https://eu.idtdna.com/ PrimerQuest/Home/Index) and were blasted on National Center for Biotechnology Information (NCBI) Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_ LOC=BlastDescAd).

PCR was carried out in a 25 µL reaction containing 0.4 µM of forward primer: 5′ GCCTGAAGGGACTAGAGTCTTA 3′ and reverse primer: 5′ GTTGAAGATGTCCTCTGCTTACA 3′ each, 0.4 mM of dNTPs, 0.02U Go-Taq polymerase, 1X Go-Taq Flexi buffer, 10 ng DNA, and 3 mM MgCl2, made up to 25 µL with 12.37 µL of nuclease free H_2O. PCR cyclic conditions involved an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 63°C for 30sec, and extension at 72°C for 30 sec, followed by a final incubation at 72°C for 5 min, to allow extension. PCR products were cleaned up and sequenced using Big-Dye Terminator sequencing according to the manufacturer's protocol (Applied Biosystems). The sequencing reaction included a 10 µL reaction mix that comprised 1.5 µL of PCR product, 5X of Big-Dye Terminator mix, 1X of Big-Dye Terminator buffer, and 8.5 µL of nuclease-free H_2O, carried out in an Applied Biosystems SimpliAmp Thermal Cycler with the following conditions: an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 96°C for 30sec, annealing at 50°C for 15sec, and extension at 60°C for 4 min (Applied Biosystems). The sequencing products were analysed on the ABI 3730XL DNA Analyzer (Applied Biosystems) and evaluated using the DNAStar-SeqMan Pro Sequence Assembly software (DNAStar , Madison®, WI). To further compare the effects of *rs12777823G>A* with those of other SNPs, four SNPs in *CYP2C9* gene, namely, *CYP2C9 c.430C>T (*2), c.1075A>C (*3), c.449G>A (*8), c.1003C>T (*11)* and a *VKORC1* SNP (*VKORC1 g.–1639G>A*) were also analysed. *CYP2C9* SNPs were genetically characterised using the iPLEX PGx74 Mass Genotyping Array (Inqaba biotec, South Africa). *VKORC1 g.–1639G>A* genotypes were determined using the PCR, coupled with Sanger sequencing

as described above, with the following primers: *VKORC1–1639G>A*-forward: 5′ GAG CCA GCA GGA GAG GGA AAT AT 3′, *VKORC1–1639G>A*-reverse: 5′ GTT TGG ACT ACA GGT GCC TGC C 3′.

3.3.1.2.3.Statistical analysis

Statistical analysis was performed using STATA for windows (version 12). Representation of the patients' characteristics and clinical data was done by calculating the frequency [presented as n (%)] and mean ±standard deviation for the categorical and continuous variables, respectively. Continuous data were tested for normality using the Shapiro–Wilks test, and further comparison done using parametric (ANOVA) and nonparametric (Wilcoxon rank-sum/Mann–Whitney and Kruskal–Wallis rank test) testing for normally and non-normally distributed data, respectively. The mean warfarin weekly maintenance doses were compared between the racial groups by the Wilcoxon rank-sum/Mann–Whitney U test. The mean warfarin weekly maintenance doses were further compared by the Wilcoxon rank-sum/Mann–Whitney or Kruskal–Wallis rank test between characteristic groupings (e.g., age categories, gender, and HIV status) and genotypes for *rs12777823G>A, CYP2C9* SNPs, and *VKORC1 g.– 1639G>A* in each racial group. Categorical data were compared using the chi-squared or Fisher's exact test. Linear regression was undertaken for association of the warfarin weekly maintenance doses with patients' characteristics and genetic variants. The *rs12777823G>A, CYP2C9* SNPs, and *VKORC1 g.–1639G>A* were tested for Hardy–Weinberg equilibrium. All statistical tests were performed taking a 5% significance level.

3.3.1.3. Results

3.3.1.3.1. Demographic and genotype characteristics

Although 425 individuals were enrolled in the study, detailed pharmacogenetic analyses were completed on only 340 (i.e., 89 BA and 251 MA individuals), due to poor DNA in 58 samples and lack of warfarin dose information in 27 individuals. The demographic characteristics of the study participants are presented in Table 3.4. The mean age of the study population was 55±15 years, with predominantly more females (69%). The main indications for warfarin treatment among BA were mechanical valve replacement (48%),

deep vein thrombosis (20%), atrial fibrillation (17%), and pulmonary embolism (8%) and these were similar among the MA. Efavirenz (14%) and statins (17%) were the most concurrently prescribed in both BA and MA participants, respectively. Among the BA, 25% were HIV positive compared with 3% in the MA group ($p<0.0001$). In contrast, there were a high number of individuals who were tobacco smokers in the MA group (62%) compared with only 20% in the BA ($p<0.0001$). The allele frequencies for *rs12777823G>A, CYP2C9* variants, and *VKORC1 g.–1639G>A* are shown in Table 3.5. The allelic variants that differed significantly between the BA and MA group included *CYP2C9*3, *8,* and *VKORC1 g.–1639A*, with *CYP2C9*3* allele being virtually absent in the BA group. None of the genotypes deviated from Hardy–Weinberg equilibrium ($p>0.05$).

3.3.1.3.2. Correlation of warfarin maintenance dose with demographic characteristics, CYP2C cluster SNPs, and VKORC1 g.–1639G>A

Warfarin maintenance dose ranged from 17.5 to 85 mg/week in the BA population group, significantly differing to what was observed among the MA (7.5 to 77.5 mg/week). Comparison of the mean maintenance doses between the BA (41±14.7 mg/week) and MA (33±13.8 mg/week) showed significant differences ($p<0.0001$). Generally, warfarin maintenance dose was reduced with increasing age, but was significant only among the MA, with a high warfarin dose of 39±16.2 mg/week reported for under 40 years age group compared with the 29±12.5 mg/week reported for the over 70 years age group ($p=0.01$). Other factors such as comorbidities, smoking, and alcohol consumption did not seem to significantly affect warfarin maintenance dose in both groups. In BA, the maintenance dose varied significantly with *rs12777823G>A* genotypes, with individuals possessing the G/G, G/A, and, A/A requiring mean maintenance doses of 45±16.1, 39±11.5, and 27± 5.3 mg/week, respectively ($p=0.002$) (Figure 3.3a). However, there were no significant differences in the mean warfarin maintenance doses among the Mixed Ancestry when comparing different *rs12777823G>A* genotypes (Figure 3.3b). The CYP2C *rs12777823G>A* SNP significantly reduced warfarin dosage by 18 mg/week on average in BA possessing the

Table 3.4: Clinical and Demographic Characteristics of South African Patients on Warfarin Treatment

Characteristics	Black Africans (89)	Mixed Ancestry (251)	p-value
Age: mean±SD(range)	39± 4 (18-80)	57±15.1(19-90)	<0.0001
Weight: mean±SD(range)	76±17.7 (44- 134)	75±17.4 (41- 152)	0.46
Height: mean ±SD (range)	1.64±0.08 (1.48-1.84)	1.65±0.09(1.42-1.95)	0.72
BMI: mean ±SD (range)	30±6.9 (17.7-47)	27±5.7 (15.8-52.6)	0.03
Female Gender, *n* (freq)	70 (0.79)	163 (0.65)	0.017
Warfarin maintenance dose(mg/week): mean ±SD (range)	41±14.7 (17.5-85)	33±13.8 (7.5-77.5)	<0.0001
International normalised ratio categories, *n* (freq)			
<4	33 (0.37)	101 (0.40)	
>4<10	48 (0.54)	130 (0.52)	0.86
>10	8 (0.09)	20 (0.08)	
Warfarin indications, *n* (freq)			
Atrial fibrillation	15 (0.17)	65(0.26)	
Deep vein thrombosis	18 (0.20)	44 (0.18)	
Mechanical valve replacement	43 (0.48)	104 (0.41)	
Pulmonary embolism	7 (0.08)	25 (0.10)	0.97
Mechanical replacement (AF first)	6 (0.07)	10 (0.04)	
Hip replacement	-	1 (0.004)	
Deep vein & stroke	-	1 (0.004)	
Pulmonary embolism & Deep vein	-	1 (0.004)	
Concomitant Drugs, *n* (freq)			
None	62 (0.69)	174 (0.69)	
Statin	7 (0.08)	53 (0.21)	
Efavirenz	12 (0.14)	2 (0.008)	<0.001
Others/low frequent used drugs	8 (0.09)	22 (0.09)	
Comorbidities, *n* (freq)			
Hypertension	32 (0.36)	107 (0.43)	0.27
Diabetes Mellitus	8 (0.09)	45 (0.18)	0.05
Heart failure	31 (0.35)	97 (0.39)	0.52
HIV positive	22 (0.25)	7 (0.03)	<0.0001
History of bleeding on warfarin, *n* (%)	23 (0.26)	50 (0.20)	0.24
Current smokers, *n* (freq)	18 (0.20)	156 (0.62)	<0.0001
Previous smokers n (freq)	16 (0.18)	156 (0.62)	<0.0001
Current alcohol consumption, *n* (freq)	28 (0.31)	120 (0.48)	0.008
Previous alcohol consumption *n* (freq)	30 (0.34)	122 (0.49)	0.015

BMI, body mass index; freq, frequency; SD, standard deviation.

Table 3.5: Genotype and allele frequency distribution of CYP2C cluster and VKORC1 single-nucleotide polymorphisms among Black Africans and Mixed Ancestry

	Black Africans (N=89)		Mixed Ancestry (N=251)		
SNP ID	**Genotype n (freq)**	**Allele freq**	**Genotype n (freq)**	**Allele freq**	**p-value**
CYP2C rs12777823G>A					
GG	47 (0.53)		148 (0.59)		
GA	33 (0.38)		83 (0.33)		
AA	8 (0.09)		21 (0.08)		
A allele		0.28		0.25	0.37
*CYP2C9 c.430C>T (*2)*					
CC	85 (0.96)		220 (0.92)		
CT	4 (0.04)		19 (0.08)		
T allele		0.02		0.04	0.24
*CYP2C9 c.1075A>C (*3)*					
AA	89 (1)		197 (0.90)		
AC	0		23 (0.10)		
C allele		0		0.05	0.02
*CYP2C9 c.449G>A (*8)*					
GG	73 (0.82)		228 (0.96)		
GA	15 (0.17)		10 (0.04)		
AA	1 (0.01)		0		
A allele		0.09		0.02	<0.0001
*CYP2C9 c.1003C>T (*11)*					
CC	88 (0.89)		213 (0.98)		
CT	1 (0.01)		5 (0.02)		
T allele		0.006		0.01	0.49
VKORC1 g.–1639G>A					
GG	71 (0.80)		123 (0.49)		
GA	15 (0.17)		102 (0.41)		
AA	3 (0.03)		26 (0.10)		
A allele		0.12		0.31	<0.0001

Freq, frequency; SNP, single-nucleotide polymorphism

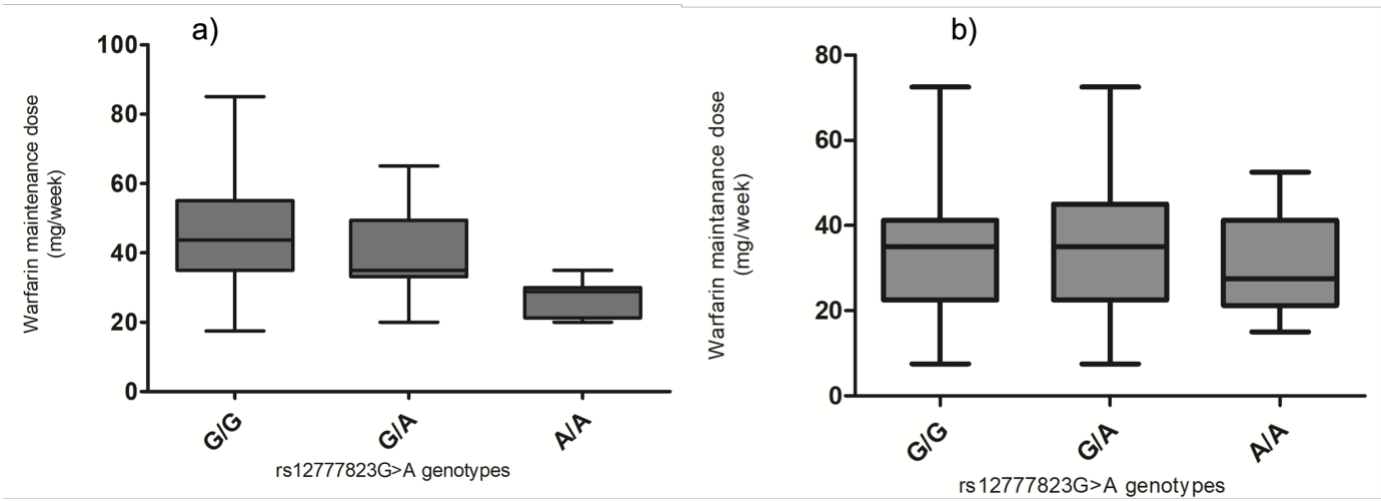

Figure 3.3: Effects of rs12777823G>A SNP on warfarin maintenance dose among a) black South Africans (p=0.002) and b) mixed Ancestry (p=0.78)

A/A genotype compared with those carrying the G/G genotype (p=0.002). Effects of the *rs12777823G>A* SNP on maintenance dose distribution were higher among BA (Cohen's d=1) (Table 3.6).

Furthermore, BA patients possessing the *rs12777823A/A* genotype, normal *CYP2C9* (i.e., *CYP2C9*1*), and normal *VKORC1* genotype (i.e., g.–1639G/G) required a maintenance dose of ≤30mg/week, compared with those possessing the *rs12777823G/G* genotypes (Table 3.7), further confirming the effects of this SNP. The warfarin maintenance dose did not vary significantly with respect to *CYP2C9* and *VKORC1* genotypes among the BA (Table 3.6). However, among the MA group, the maintenance dose varied significantly due to *CYP2C9 c.430C>T (*2)* and *VKORC1 g.–1639G>A* genotypes (p=0.02 and 0.0001, respectively). A stepwise regression model of warfarin weekly maintenance dose against environmental and genetic variables is represented in Table 3.8. None of the environmental factors affected warfarin maintenance dose variability in the BA group. However, in the MA group, age and body mass index (BMI) affected warfarin maintenance dose variability and remained significant in the final model that included genetic variables (Table 3.9). The *CYP2C rs12777823G>A* SNP was the only genetic variable that significantly affected warfarin dose requirement explaining 12% of variability (p=0.001) in the BA group. However, the

effects of the *CYP2C rs12777823G>A* SNP were reduced in the presence of *CYP2C9 c.449G>A (*8)* in the model.

Linkage disequilibrium (LD) results suggest that *CYP2C rs12777823G>A* and *CYP2C9 c.449G>A (*8)* are in partial linkage (D′=0.67, R^2=0.13) in the BA group but not in the MA group (D′=0.43, R^2=0.01). Among the MA, *VKORC1g.–1639G>A* was the main genetic variable that affected warfarin dose variability (R^2=11%, p<0.001). Furthermore, the *VKORC1 g.–1639G>A* SNP influenced the effect of *CYP2C9 c.1075A>C (*3)* on warfarin variability in MA group. The final model for the BA group explained 12% variability and only included the *CYP2C rs12777823G>A* SNP (p=0.001). In the MA group, the final model explained 20% variability and it comprised of age, BMI, *VKORC1g.– 1639G>A*, and *CYP2C9 c.1075A>C (*3)* (Table 3.9).

3.3.1.4. Discussion

Warfarin is used extensively for prevention and reduction of thromboembolic disorders [70]. Its efficiency is, however, accompanied by huge inter-individual variability in the starting and maintenance doses, often leading to adverse drug effects. Hence, there has been a growing interest in identifying environmental and genetic factors associated with warfarin response. In the developed countries, advanced technologies have allowed identification of genetic polymorphisms associated with warfarin response, with a breakthrough in the development of pharmacogenetic algorithms. Research on the pharmacogenetics of warfarin has not been inclusive of native African populations, as studies have mostly been done in Caucasian and Asian populations [67, 88, 235]. Hence, variants reported to affect warfarin response are not completely informative in Africans. In recent years, there has been progress in identifying genetic variants in genes affecting warfarin response in African Americans [57, 116, 137]. Such developments have opened the way to the realisation that there could be variants that are only found in populations with African ancestry and some of these could affect warfarin response and thus require to be identified and incorporated in African-targeted warfarin dosing pharmacogenetic algorithms [47, 56]. African-specific genetic variation identified include *rs12777823G>A* located in the CYP2C cluster upstream of *CYP2C18* gene [145].

Table 3.6: Comparison of warfarin mean weekly maintenance dose between rs12777823G>A, CYP2C9 and VKORC1 genotypes

	Black Africans			Mixed Ancestry		
Genotype	**Maintenance dose (mg\week), Mean ±SD (range)**	**p-value**	**Cohen's d (95% CI)**	**Maintenance dose (mg\week), Mean ±SD (range)**	**p-value**	**Cohen's d (95% CI)**
CYP2C rs12777823G>A						
GG	45±16.1 (17.5-85)			33±13.8 (7.5-72.5)		
GA	39±11.5 (20-65)	0.002	1.2 (0.4 to 1.9)	34±14.6 (7.5-72.5)	0.78	0.2 (-0.3 to 0.6)
AA	27±5.3 (20-52.5)			31±12.1 (15-52.5)		
CYP2C9 c.430C>T (*2)						
CC	40±13 (17.5-85)	0.32	0.4 (-0.6 to 1.4)	34±14 (7.5-72.5)	0.02	0.6 (0.06 to 1)
CT	34±9 (27.5-47.5)			27±13 (10-62.5)		
*CYP2C9 c.1075 A>C (*3)*						
AA	40±12 (17.5-85)	-	-	34±14 (7.5-72.5)	0.32	0.2 (-0.2-0.7)
AC				31±15 (10-62.5)		
*CYP2C9 c.449G>A (*8)*						
GG	41±12 (17.5-85)			35±17 (17.5-72.5)		
GA	34±11 (20-57.5)	0.07	0.5 (-0.03 to 1.1)	34±14 (7.5-72.5)	0.88	-0.07 (-0.7 to 0.6)
AA	25			-		
*CYP2C9 c.1003C>T (*11)*						
CC	40±12 (17.5-85)	0.12	-	34±14 (10-72.5)	0.48	0.4 (-0.5 to 1.3)
CT	20			28±12 (7.5-37.5)		
VKORC1 g.–1639G>A						
GG	41±14.6 (17.5-85)			37±12.8 (10-72.5)		
GA	39±14.2 (17.5-70)	0.90	-0.3 (-1.5 to 0.9)	31±13.5 (7.5-72.5)	0.0001	1.3 (0.8 to 1.7)
AA	45±24.1 (27.5-72.5)			21±11.6 (10-62.5)		

CI=confidence interval

The role for *rs12777823G>A* SNP in warfarin response was first reported by Perera et al [145] in a GWAS among African Americans. Their findings showed that this SNP affected warfarin dose requirement by 10–25%, exclusively in African Americans. Furthermore, its effects occurred independently of *CYP2C9*2* and *CYP2C9*3*, a result that we confirm in this present report. In fact, *rs12777823G>A* SNP was only associated with a reduced warfarin maintenance dose among BA and had no effects in the MA group, despite *rs12777823A* allele frequencies not differing significantly between the two groups. The lack of association of the *rs12777823G>A* SNP with warfarin maintenance dose among the MA cohort can be explained by the non-homogenous nature of this population group, as Caucasians, BA, and Malaysians contribute to the gene pool [236]. Therefore, this SNP could be in LD with another causal SNP that is present in BA. Although Perera et al [145] did not find any linkage between the CYP2C

Table 3.7: Comparison of the effect of rs12777823G>A Single-Nucleotide Polymorphism Genotypes on Warfarin Maintenance Dose for Selected Participants Without Genetic Variation in CYP2C9 and VKORC1

Laboratory ID	rs12777823G>A	*CYP2C9*	*VKORC1 g.–1639G>A*	Maintenance dose (mg\week)
6348	AA	*1/*1	GG	20
6332	AA	*1/*1	GG	30
6320	AA	*1/*1	GG	25
6293	AA	-	GG	27.5
6208	AA	*1/*1	GG	30
6008	GG	*1/*1	GG	45
6042	GG	1/*1	GG	55
6070	GG	1/*1	GG	40
6259	GG	1/*1	GG	67.5
6321	GG	1/*1	GG	70
6326	GG	1/*1	GG	52.5

rs12777823G>A SNP and any known *CYP2C9* variants, our results suggest that *CYP2C rs12777823G>A* could be in linkage with *CYP2C9 c.449G>A (*8)*, which has been described to affect warfarin variability exclusively in Africans. However, this warrants confirmation of these findings on a larger sample size. Furthermore, if the hypothesis is that *rs12777823G>A* is in LD with a yet to be identified causal variant, whole-exome sequence analysis with a closer look in the CYP2C cluster among BA could decode the causative variant. In the meantime, this confirmation of the presence of unique African-specific genetic variants calls for the development of warfarin pharmacogenetic algorithms that take into account the African genome architecture.

This is proposed with the full realisation that African genomes are diverse; it may not be surprising that different African population groups could require specific variants to be incorporated into their algorithms to improve warfarin responses. Findings from this study and previous studies conducted in African Americans highlight the importance of this SNP in prediction of warfarin doses in Africans. Our results show that this SNP alone accounts for ~12% of warfarin variability, which is comparable with the warfarin variability of ~4–18% that has been previously reported to be explained by *CYP2C9*2* and *CYP2C9*3* in Caucasians [48, 98, 237]. Previous studies have shown that inclusion

Table 3.8: Stepwise regression model of weekly warfarin maintenance dose associated with multiple variables in the Black Africans and Mixed Ancestry

	Black Africans			Mixed Ancestry		
Variable	**RMSE**	**p-value**	**R^2**	**RMSE**	**p-value**	**R^2**
Demographic factors (age+gender+BMI)	14.51	0.32	0.05	13.47	0.0003	0.11
Age+ gender	13.35	0.25	0.03	13.58	0.0001	0.07
Gender+BMI	14.45	0.21	0.04	13.92	0.03	0.05
BMI+age	14.52	0.30	0.03	13.56	0.0004	0.09
Genetic factors						
*CYP2C rs12777823G>A+ CYP2C9 c.430 C>T (*2) + CYP2C9 c.1075 A>C (*3) + CYP2C9 c.449G>A (*8)* + CYP2C9 c.1003C>T (*11) + *VKORC1 g.–1639G>A*	12.33	0.03	0.17	13.19	<0.0001	0.16
*CYP2C rs12777823G>A + CYP2C9 c.430 C>T (*2)*	12.22	0.01	0.11	13.96	0.07	0.02
CYP2C rs12777823G>A+ CYP*2C9 c.449G>A (*8)*	12.27	0.02	0.11	14.10	0.84	0.002
CYP2C rs12777823G>A+ CYP2C9 c.1003C>T (*11)	12.22	0.009	0.13	14.20	0.68	0.004
CYP2C rs12777823G>A+ VKORC1 g.–1639G>A	13.31	0.006	0.12	12.99	<0.0001	0.13
Demographic and genetic factors						
Age + Gender+ BMI +*CYP2C rs12777823G>A+ CYP2C9 c.430 C>T (*2) + CYP2C9 c.1075 A>C (*3) + CYP2C9 c.449G>A (*8)+ CYP2C9 c.1003C>T (*11)+ VKORC1 g.–1639G>A*	13.44	0.10	0.27	12.61	0.0001	0.25
Demographic and genetic factors						
(VKORC1 g.–1639G>A excluded) Age+ Gender+ BMI+ *CYP2C rs12777823G>A+ CYP2C9 c.430 C>T (*2) + CYP2C9 c.1075 A>C (*3) + CYP2C9 c.449G>A (*8) + CYP2C9 c.1003C>T (*11)*	13.67	0.14	0.23	13.48	0.03	0.13
Demographic and genetic factors						
(VKORC1 g.–1639G>A and CYP2C9*2/*3 excluded) Age+ Gender+ BMI+ *CYP2C rs12777823G>A+ CYP2C9 c.449G>A (*8) + CYP2C9 c.1003C>T (*11)*	13.70	0.13	0.21	13.49	0.02	0.11
Demographic factors and rs12777823G>A						
Age+ Gender+ BMI + *CYP2C rs12777823G>A*	14.25	0.02	0.19	13.25	0.002	0.11
rs12777823G>A only	13.23	0.001	0.12	13.90	0.78	0.0003

Table 3.9: Final Regression Model of the Association Between the Warfarin Maintenance Dose with Environment and Genetic Variables in Both Black Africans and Mixed Ancestry

Population group	Variable	Standardized Coefficient	Standard Error	p-value	R^2
Black Africans	*rs12777823G>A*	-7.27	2.18	0.001	
	Total Model			0.001	0.12
Mixed Ancestry	Age	-0.23	0.08	0.005	
	BM1	0.48	0.20	0.021	
	VKORC1 g.–1639G>A	-7.01	1.75	<0.001	
	*CYP2C9 c.1075 A>C (*3)*	-10.2	3.77	0.008	
	Total Model			<0.0001	0.20

of the *rs12777823A* variant concurrently with other alleles such as *CYP2C9*5, *6, *8,* and **11*, which are specific to individuals of African ancestry, improves dosing estimation in Africans compared with the dosing prediction made using available pharmacogenetic algorithms such as Clarification of Oral Anticoagulation through Genetics algorithm [47, 56]. Alzubiedi and Saleh [87] reported that a linear regression model that included the *rs12777823G>A* explained 38% of warfarin variability in African Americans, which was higher compared with the 26% variability explained by the International Warfarin Pharmacogenetics Consortium (IWPC) [65] dosing algorithm, excluding the *rs12777823G>A* SNP.

The Clinical Pharmacogenetics Implementation Consortium (CPIC) guideline has made recommendations on the inclusion of *rs12777823G>A* together with *CYP2C9*5, *6, *8,* and **11* when predicting warfarin doses in African Americans [45]. However, we feel that, in addition to the *rs12777823G>A* SNP, there could be more African-specific variants that affect warfarin and response to many other drugs. The improvement of warfarin prediction in Africans when considering African-specific variants is motivation that identification of novel variants affecting warfarin response in Africans should be a priority. The significant association observed between race and warfarin response could be a proxy for the genetic variants that are yet to be discovered.

3.3.1.5. Conclusion

In a South African cohort that comprised 425 warfarin-treated patients, we set out to evaluate the contribution of genetic variation in CYP2C cluster on inter-individual variability of warfarin response. We conclude that the CYP2C *rs12777823G>A* polymorphism is a strong predictor in warfarin response among South African black patients but seems to be not important in the MA population group. Therefore, consideration and inclusion of the CYP2C *rs12777823G>A* polymorphism have a high potential of improving warfarin dosing in South African blacks. However, further analyses on the polymorphism are warranted for better understanding of its functionality on warfarin disposition, or for the identification of causal SNPs that it may be linked with.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

3.3.2. Profiling of warfarin pharmacokinetics-associated genetic variants: Black Africans portray unique genetic markers important for an African specific warfarin pharmacogenetics-dosing algorithm.

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Co-Authors Contribution:

SM: conducted experimental activities, data analysis and reviewed manuscript draft.

PM: reviewed the manuscript draft.

APK and EC: data analysis and reviewed the manuscript draft.

AW and MN: co-supervised all components and reviewed the manuscript draft.

CD: conceptualised the ideas, supervised all components and reviewed the manuscript drafts.

All authors contributed to the final version of the article. The authors read and approved the final manuscript.

Profiling of warfarin pharmacokinetics-associated genetic variants: Black Africans portray unique genetic markers important for an African specific warfarin pharmacogenetics-dosing algorithm

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Abstract

Warfarin dose variability observed in patients is attributed to variation in genes involved in the warfarin metabolic pathway. Genetic variation in *CYP2C9* and *VKORC1* has been the traditional focus in previous studies that evaluated warfarin dose variability, with little focus on other genes. Thus, we set out to evaluate 27 single nucleotide polymorphisms (SNPs) in the *CYP2C9*, *VKORC1*, CYP2C cluster loci, and 6 additional genes (*ABCB1, CYP2C19, CYP2C8, CYP1A2, CYP3A4*, and *CYP3A5)* involved in pharmacokinetics of warfarin. 503 participants were recruited among black Africans and Mixed Ancestry population groups, from South Africa and Zimbabwe, and a blood sample taken for DNA. Clinical parameters were obtained from patient medical records, and these were correlated with genetic variation. Among black Africans, the SNPs *CYP2C rs12777823G>A, CYP2C9 c.449G>A (*8), CYP2C9 c.1003C>T (*11)* and *CYP2C8 c.805A>T (*2)* were significantly associated with warfarin maintenance dose. Conversely, *CYP2C9 c.430C>T (*2), CYP2C8 c.792C>G (*4)* and *VKORC1 g.-1639G>A* were significantly associated with maintenance dose among the Mixed Ancestry. The presence of *CYP2C8*2* and *CYP3A5*6* alleles was associated with increased mean warfarin maintenance dose, whereas *CYP2C9*8* allele was associated with reduced warfarin maintenance dose. African populations present with a diversity of variants that are important in predicting pharmacogenetics-based warfarin dosing in addition to those reported in *CYP2C9* and *VKORC1*. It is therefore important, to include African populations in pharmacogenomics studies to be able to identify all possible biomarkers that are potential predictors for drug response.

Keywords: pharmacogenetics, pharmacokinetics, South Africa, Southern Africa, warfarin, Zimbabwe

Essentials

• Warfarin is a widely prescribed anticoagulant, that is highly affected by genetic and non-genetic factors.

• Genetic variants affecting warfarin dose were investigated among Southern Africans recruited in Cape Town, South Africa and Harare, Zimbabwe.
• Single nucleotide polymorphisms which included *CYP2C8 c.805A>T (*2), CYP2C9 c.449G>A (*8)* and *CYP2C rs12777823G>A* affected warfarin dose variability among black Africans.
• Age, BMI *and VKORC1 g.-1639G>A* explained 22% of warfarin dose variability among Mixed Ancestry.

3.3.2.1. Background

Warfarin is a widely prescribed anticoagulant for prevention and treatment of thromboembolic disorders and associated complications such as stroke [70, 71]. In Africa, warfarin remains the most prescribed anticoagulant despite the availability of alternative anticoagulants, due to its relatively low cost and extensive knowledge of use by physicians [15]. Warfarin use is however complicated by its narrow therapeutic range, and difficulties in establishing standard doses that allow reaching stable international normalised ratio (INR) of 2–3.5, in a relative short space of time [17, 44]. Consequently, patients often require different starting doses to reach the same INR range, leading to a proportion of patients presenting with warfarin associated adverse drug reactions (ADRs) and increased risk for hospitalisation or death after administration of standard starting doses [17, 78]. Differences in warfarin starting dose requirements among patients are due to both genetic and environmental/non-genetic factors [79]. Non-genetics factors include demographic variables (e.g., age and gender), lifestyle (e.g., diet, tobacco smoking and alcohol consumption) and clinical factors (e.g., comorbidities, co-medications, and physiological aspects) [20, 80]. Whilst genetic factors associated with the warfarin dose variability include variants in genes encoding enzymes involved in the warfarin disposition pathway [48, 58].

Warfarin exerts its effects by targeting the vitamin K epoxide reductase complex 1 (VKORC1) through blocking its recycling of vitamin K, consequently inhibiting the activation of clotting factors [32], thus, *VKORC1* gene has become an important gene to evaluate. CYP2C9 principally metabolises the most potent form of warfarin, S-warfarin, whilst CYP3A4/5 and CYP1A2 metabolise the R-warfarin form. Other enzymes involved

in the metabolism of warfarin include CYP2C8, CYP2C18 and CYP2C19 [40, 41]. Polymorphisms in *CYP2C9* and *VKORC1* have been widely studied with respect to warfarin dosing variability. *CYP2C9*2 (rs1799853), CYP2C9*3 (rs1057910)* and *VKORC1 g.-1639G>A (rs9923231)* are the most investigated variants and have been widely associated with reduced warfarin dose requirements [48, 50, 84, 85]. Consequently, *CYP2C9*2, CYP2C9*3* and *VKORC1 g.–1639G>A* variants coupled with some non-genetic factors have been incorporated in warfarin pharmacogenetics-based dosing algorithms to assist in predicting the best warfarin starting doses [63, 87]. However, these variants show qualitative and quantitative differences in their frequencies across world populations, with generally higher frequencies among European and Asian populations, when compared to African populations [48, 84, 102, 105].

Recent studies have started to expand on the repertoire of genetic variants important in warfarin dosing, reporting on *CYP2C9*5, CYP2C9*8, CYP2C9*11* and *CYP2C rs12777823G>A* [57, 107, 109, 111, 115, 145]. The additional variants have started unravelling the causes of variability in warfarin doses observed in African populations, even after taking into account the most commonly studied *CYP2C9* and *VKORC1* polymorphisms. Although, several algorithms for estimating warfarin starting doses have been proposed, the effectiveness of the available pharmacogenetics algorithms in African populations remains in doubt due to very little data from Africans [66, 67, 88]. Furthermore, most studies focus on the two principal genes, *CYP2C9* and *VKORC1*, excluding other genes involved in warfarin disposition. Thus, we present here the determination in Southern Africa populations (i.e., black Africans, and the Mixed Ancestry) the combined effects of 27 single nucleotide polymorphisms (SNPs) in 8 genes (i.e., *ABCB1, CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP3A4, CYP3A5* and *VKORC1*) plus additional loci in the CYP2C cluster, which play a role in the pharmacokinetics of warfarin. We included participants from black African and Mixed Ancestry population groups in the study as they are the dominating population groups in Southern Africa and have been poorly characterised with respect to warfarin pharmacogenes. Furthermore, previous reports have shown that individuals of the Cape Mixed Ancestry (i.e., included here), are an admixed population group with a gene pool

comprising of European, African, South Asian and Indonesian ancestry, whilst black Africans are an indigenous African population group [150, 236, 238]. Thus, distinct genomic profiles of black Africans and the Mixed Ancestry will allow us to compare and determine whether the influence of the studied SNPs on warfarin variability varies between the two population groups. Additionally, we also set out to present variables that could be included in an African-specific pharmacogenetics-based warfarin dosing algorithm.

3.3.2.2. Methods and Materials

3.3.2.2.1. Patient cohort

Sample size was determined using a method by Naing [239] described as $n=(z/\Delta)^2 p(1-p)$. The sample size for a SNP with a lowest frequency of 2% was determined as follows; the proportion (p) of the sampled patients that will take abnormally long to reach INR was assumed to be 10%, then the required sample size to achieve a 95% confidence interval of width 0.10 (i.e., $\Delta=0.05$) was calculated as $n=(1.96/0.05)^2$ 0.10 (1–0.10)=138. The minimum participants required for the study was 138 each for both black Africans and Mixed Ancestry. Thus, the study included 503 participants recruited between 2016 and 2017 from INR clinics at Groote Schuur Hospital (GSH), Gugulethu Community Health Centre (GCHC) in the Western Cape Province, South Africa, and Parirenyatwa Group of Hospitals (PGH) in Harare, Zimbabwe. Two hundred and fifty-two (252) participants were of black African (BA) descent and 251 participants were of Mixed Ancestry (MA) descent. The participants provided consent to be enrolled in the study after ethical approval was granted by the University of Cape Town Human Research Ethics Committee (UCT-HREC REF No. 581/2015), Faculty of Medicine, University of Zimbabwe Ethics Committee (JREC/160/13) and the Medical Research Council of Zimbabwe (MRCZ/A/1815). Recruitment of the patients including accessing of demographic variables (e.g., age, gender), clinical parameters (e.g., warfarin indications, comedications, and comorbidities) and biological samples was described before [107]. For anonymity, participant names were replaced by a laboratory generated number.

3.3.2.2.2. Genetic characterisation for pharmacogenetic variants

DNA was extracted from whole blood using the salting-out DNA purification method (modified from Gustafson et al [160]). Fifty (50) µl of 50ng of purified DNA was sent to Inqaba biotec (Pretoria, South Africa) for genotyping using the iPLEX PGx74 Mass Genotyping Array platform (Agena Bioscience, Inc. San Diego, CA). The iPLEX PGx74 panel targets 69 SNPs/INDELs and 5 CNVs in 20 genes of pharmacogenetics importance (Agena Bioscience, Inc. San Diego, CA). However, from the 20 genes targeted, the present paper reports on only 6 genes of pharmacokinetics relevance on warfarin, namely, *ABCB1, CYP1A2, CYP2C9, CYP2C19, CYP3A4* and *CYP3A5*. In addition to the genes targeted by the iPLEX PGx74 panel, SNPs in the CYP2C cluster non-genomic loci, *CYP2C8* and *VKORC1* have also been included in the study. Genetic characterisation of *CYP2C rs12777823G>A* and *VKORC1 g.-1639G>A* has been described before [107]. The *CYP2C rs12772169C>T* SNP and seven *CYP2C8* SNPs were genotyped using PCR-RFLP and Sanger sequencing, respectively.

Oligonucleotides for the *CYP2C8* and *CYP2C rs12772169C>T* SNPs were designed using the Integrated DNA Technologies (IDT) PrimerQuest tool (https://eu.idtdna.com/PrimerQuest/Home/ Index) and were blasted on National Center for Biotechnology Information (NCBI) Primer-BLAST (www.ncbi.nlm.nih.gov/tools/ primer-blast/index.cgi?LINK_LOC=BlastDescAd). A restriction enzyme was identified using the NEBcutter tool (http://nc2.neb.com/ NEBcutter2/) for the CYP2C rs12772169C>T RFLP. For *CYP2C8* SNPs, PCR was carried out in a 25 µl reaction containing 0.4 µM forward primer: 5′-CCA TCG TTC TCA GCA TAC TAT CAC-3′ and reverse primer: 5′-CTA TGC ATT CTA GCC ATT GGA CAAT-3′ each, 0.4 mM of dNTPs, 0.02 U/µl Go-Taq polymerase, 1X Go-Taq Flexi buffer, 10 ng DNA, and 3 mM MgCl2, made up to 25 µl with 12.37 µl of nuclease free H_2O. PCR cyclic conditions involved an initial denaturation at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 65°C for 30s, and extension at 72°C for 30s, followed by a final incubation at 72°C for 5 min, to allow extension. PCR products were cleaned up in an enzymatic reaction of Exonuclease I (Exo I) and Shrimp Alkaline Phosphatase, according to the manufacturer's protocol (New England Biolabs, Ipswich, MA), and then

sequenced using Big-Dye Terminator sequencing according to the manufacturer's protocol (Applied Biosystems, Foster City, CA).

The sequencing protocol and results analysis were done as described by Ndadza et al [107] For the *CYP2C rs12772169C>T* SNP, PCR was carried out as described above using the following primer set; forward primer: 5′-AAG ACA GTT CTC TCT ACA GGA GT-3′ and reverse primer: 5′-GTT GCA GTG TTA AAA CTA GCT GGA-3′, with an annealing temperature of 56.9°C. The PCR products were then digested using the AatII (GACGTC) restriction enzyme, in a 15 µl reaction containing 3 µl of the PCR product, 0.13 U/µl of the restriction enzyme, and 1X of cutsmart buffer made up to 15 µl with 9.9 µl of nuclease free H_2O. The reaction was then incubated for 2 hrs at 37°C and then the enzyme was inactivated at 80°C for 20 min. The RFLP results were then validated with Sanger sequencing as described before [107].

3.3.2.2.3. Statistical data analysis

Twenty-seven (n=27) SNPs from 8 genes (i.e., *ABCB1, CYP1A2, CYP2C8,CYP2C9, CYP2C19, CYP3A4, CYP3A5* and *VKORC1*) and 2 non-genomic loci in the CYP2C cluster (Table S2) were successfully characterised and assessed on their contribution to warfarin maintenance dose. The analysis included previously published data on the following 6 SNPs; *CYP2C rs12777823G>A, CYP2C9 c.430C>T (*2), CYP2C9 c.1075A>C (*3), CYP2C9 c.449G>A (*8), CYP2C9 c.1003C>T (*11) and VKORC1 g.-1639G>A*, allowing re-analysis with increased sample size. Statistical analysis was performed using STATA for windows version 15 (StataCorp, College Station, Texas, USA) and various statistical packages in R (version 4.0.3 [2020–10–10]) as follows; patients' characteristics and clinical data were determined by calculating the frequency (presented as n [freq]) and mean ±standard deviation for the categorical and continuous variables, respectively. The distribution of weekly warfarin maintenance dose was assessed for normality using the Shapiro wilks test. Further comparison of the maintenance dose distribution according to SNPs genotypes, demographic and clinical characteristics groupings were done using the appropriate tests depending on the distribution of the data. To assess the association or the allele occurrence of SNPs located in the CYP2C cluster region to each other and to identify important haplotypes,

linkage disequilibrium (LD) was calculated and visualised for 19 SNPs in the CYP2C cluster in both the studied cohorts using the HaploView software (https://www.broad institute.org/haploview).

Continuous variables such as age and BMI were grouped according to categories to allow comparison of the maintenance dose distribution. Age was categorised into three groups; <50 years, 50–70 years and >70 years as described by Shendre et al [240]. BMI was grouped into four categories; underweight (<18.5), normal (18.5–24.9), overweight (25–29.9) and obese (≥30), as described by the centres of disease control and prevention (CDC) (https://www.cdc.gov/obesity/adult/defining.html). Box plots were constructed using GraphPad prism (version 6) to compare the maintenance dose distribution according to age categories. The effect size was determined through the Cohen's d, comparing the mean maintenance dose between two groups for each variable, for variables with more than 2 groups the upper and lower groups mean maintenance dose were then compared (e.g., homozygous wild-type and homozygous mutant genotypes were compared for each SNPs). Stepwise regression analysis was done to control for confounding factors and to determine the interaction and cumulative influence of the various studied genetic and non-genetic variables. The variables that were eligible to enter the stepwise regression analysis were those with a $p \leq 0.20$ for their effect in univariable analysis. To control for missing data and reduce bias, imputation was done on variables that were eligible for the stepwise regression analysis and have missing data using the multivariate imputation by chained equations (MICE) package in R. Supplementary Table S3 outlines the missing data according to each variable. All statistical tests were performed taking a 5% significance level.

3.3.2.3. Results

3.3.2.3.1. Correlation of demographic and clinical characteristics with warfarin maintenance dose

The demographic and clinical characteristics of these cohorts have been previously reported [107]. However, data for black Africans was re-analysed to accommodate an increased sample size (Supplementary Table S4). Table 3.10 presents the correlation

between various demographic and clinical characteristics with warfarin maintenance dose. Similar to observations from other studies [25, 81], increasing age was significantly inversely correlated with reduced warfarin weekly maintenance dose ($p<0.05$) among both the black African and the Mixed Ancestry patients with an effect size of 0.64 (95% CI: 0.22 to 1.06) and 0.74 (95% CI: 0.38 to 1.09), respectively (Figure 3.4). For example, patients <50 years of age required mean warfarin maintenances doses of nearly 40 mg/week compared to the much lower 30 mg/week for patients >70 years of age ($p<0.05$; Table 3.10). Deep venous thrombosis (DVT) was associated with higher mean warfarin maintenance dose requirements in the Mixed Ancestry group ($p=0.004$), whilst mechanical valve replacement was significantly associated with higher warfarin maintenance dose among black Africans ($p<0.05$). Hypertension and heart failure were significantly correlated ($p=0.04$ and 0.03, respectively) with a reduced warfarin dose requirement among black Africans only. Other variables such as BMI, gender, concomitant drug use, diabetes mellitus, HIV, tobacco smoking, and alcohol consumption did not significantly affect the required mean weekly warfarin maintenance doses in both black Africans and Mixed Ancestry.

3.3.2.3.2. Distribution of pharmacogenes variants among the black Africans and Mixed Ancestry group

We present an analysis on 27 SNPs with pharmacokinetics relevance on the disposition of warfarin (except *VKORC1 g.-1639G>A*, included for its central role in warfarin mechanism of action) on their association with warfarin maintenance dose (Table 3.11). Data for 6 SNPs has previously been presented by Ndadza et al [107]. For the 27 SNPs characterised, there was quantitative and qualitative differences in the distribution and effects of variant SNPs, when comparing black Africans to the Mixed Ancestry group. Two SNPs, *CYP2C9 c.1076T>C (*4), and CYP2C8 c.712G>C (*14)* were monomorphic in both the black African and Mixed Ancestry groups, thus, were therefore excluded from further analysis. *CYP2C9 c.1075A>C (*3)* and *CYP3A4 g.15389C>T (*22)* were monomorphic among black Africans, and *CYP2C9 c.1080C>G (*5)* was monomorphic among the Mixed Ancestry group. Supplementary Table S5 shows the variant allele frequencies for the 27 SNPs in the two population groups. The variants that significantly

varied according to the distribution between the black African and Mixed Ancestry group included *ABCB1 c.3435T, CYP2C8*2, CYP2C9*8, CYP3A5*3, CYP3A5*6* and *VKORC1 g.-1639A.*

3.3.2.3.3. Linkage disequilibrium mapping of the CYP2C cluster including CYP2C8, 9 and 19

The profile of linkage disequilibrium (LD) mapping in the CYP2C cluster region varied between the black African and Mixed Ancestry population groups (Supplementary Figure S1). The strongest LD was observed among black Africans between SNPs *CYP2C19 c.681G>A (*2)* (rs4244285) and *CYP2C8 rs11572101T>C* (D'=0.94, r^2=0.80), whilst, *CYP2C rs12777823G>A* had a moderate LD with *CYP2C rs12772169C>T* and *CYP2C8 rs11572101T>C* in the black Africans (D'=0.91, r^2=0.48; D'=0.79, r^2=0.32, respectively) and in the Mixed Ancestry (D'=0.77, r^2=0.30; D'=0.56, r^2=0.31,respectively). However, a moderate LD was observed between SNPs *CYP2C19 g.-806C>T (*17) (rs12248560)* and *CYP2C8 c.805A>T (*2) (rs11572103)* only *among* black Africans (D'=0.72, r^2=0.45), whilst SNPs *CYP2C rs12772169C>T* and *CYP2C8 rs1926705G>A* had a moderate LD only among the Mixed Ancestry ([D'=0.61, r^2=0.10]). Detailed LD results with complete LD parameters (i.e. LOD score, D' and r^2) for the studied CYP2C cluster SNPs among both black Africans and Mixed Ancestry is outlined in Supplementary Table S6.

3.3.2.3.4. Correlation of SNP genotypes with warfarin weekly maintenance dose

The SNP genotypes were evaluated for their association with mean warfarin weekly maintenance doses required to keep INR in the normal range for the two population groups, black Africans and the Mixed Ancestry. The comparison of mean weekly warfarin maintenance dose for genotypes were done using additive, dominant and recessive genetic models. Among black Africans, the maintenance dose distribution was significantly correlated ($p \leq 0.05$) with the following SNPs in an additive model: *CYP2C8 c.805A>T (*2), CYP2C9 c.449G>A (*8), CYP2C9 c.1003C>T (*11)* and *CYP2C rs12777823G>A* with the following effect size −1.06 (95% CI: −1.68 to −0.43),

Table 3.10: Effects of demographic and clinical characteristics on warfarin maintenance dose among Black Africans and Mixed Ancestry

	Black Africans (N=252)			Mixed Ancestry (N=251)		
Variable	**Maintenance dose (mg/week), mean ±SD (range)**	***p* value**	**Cohen's d (95%CI)**	**Maintenance dose (mg/week), mean ±SD (range)**	***p* value**	**Cohen's d (95%CI)**
Age						
<50 years	39 ± 13 (17.5-72.5)	0.03	0.64	38 ± 15 (10 -72.5)	<0.0001	0.74
50-70 years	39 ± 13 (12.5-75)		(0.22 to 1.06)	33 ± 13 (7.5-72.5)		(0.38 to 1.09)
>70 years	31 ± 12 (17.5-70)			28 ± 12 (7.5-52.5)		
Gender						
Female	39 ± 13 (17.5-75)	0.49	0.15	32±14 (7.5-72.5)	0.25	-0.17
Male	37 ± 13 (12.5-70)		(-0.14 to 0.45)	35±15 (10-72.5)		(-0.43 to 0.09)
BMI						
<18.5	43 ± 18 (17.5-70)	0.73	0.38	31 ± 13 (17.5-52.5)	0.05	-0.20
18.5-24.9	37 ± 14 (17.5-75)		(-0.26 to 1.02)	30 ± 13 (7.5-72.5)		(-0.91 to 0.51)
25-29.9	39 ± 13 (12.5-70)			36 ± 16 (12.5-72.5)		
>30	38 ± 12 (12.5-70)			34 ± 14 (10 -72.5)		
Warfarin indication						
Atrial fibrillation						
No	39 ± 13 (17.5-75)	0.16	0.29	33 ± 14 (7.5-72.5)	0.81	0.002
Yes	35 ± 13 (12.5-70)		(-0.06 to 0.66)	33 ± 15 (10-72.5)		(-0.28 to 0.28)
Deep venous thrombosis						
No	37 ± 13 (12.5-75)	0.11	-0.20	32 ± 14 (7.5-72.5)	0.004	-0.41
Yes	40 ± 12 (17.5-70)		(-0.47 to 0.07)	38 ± 12 (12.5-65)		(-0.74 to -0.09)
Mechanical valve replacement						
No	37 ± 12 (12.5-70)	0.05	-0.38	34 ± 15 (7.5-72.5)	0.17	0.17
Yes	42 ± 15 (20-75)		(-0.68 to -0.08)	32 ± 14 (10-72.5)		(-0.08 to 0.42)
Pulmonary embolism						
No	38 ± 13 (12.5-75)	0.61	0.18	34 ± 14 (10-72.5)	0.33	0.19
Yes	36 ± 12 (17.5-57.5)		(-0.36 to 0.72)	31 ± 17 (7.5-62.5)		(-0.21 to 0.61)
Comorbidities						
Hypertension						
No	40 ± 13 (12.5-72.5)	0.04	0.27	34 ± 13 (10-72.5)	0.41	0.07
Yes	36 ± 13 (12.5-75)		(0.02 to 0.52)	33 ± 15 (7.5-72.5)		(-0.17 to 0.32)
Diabetes mellitus						
No	39 ± 13 (12.5-75)	0.32	0.32	33 ± 13 (7.5-72.5)	0.38	-0.24
Yes	34 ± 14 (12.5-55)		(-0.18 to 0.83)	36 ± 17 (10-72.5)		(-0.56 to 0.08)
Heart failure						
No	40 ± 12 (17.5-72.5)	0.03	0.25	34 ± 14 (10-72.5)	0.16	0.17
Yes	37 ± 14 (12.5-75)		(-0.004 to 0.49)	32 ± 14 (7.5-72.5)		(-0.08 to 0.42)
Co-treatments						
Statins						
No	39 ± 13 (12.5-72.5)	0.06	0.35	33 ± 13 (7.5-72.5)	0.73	-0.15
Yes	34 ± 15 (12.5-75)		(-0.09 to 0.79)	35 ± 17 (10-72.5)		(-0.45 to 0.15)

Efavirenz						
No	38 ± 13 (12.5±75)	0.77	-0.15	33 ± 14 (7.5-72.5)	0.20	-0.61
Yes	40 ± 15 (17.5-70)		(-0.66 to 0.36)	42 ± 6 (35-47.5)		(-1.75 to 0.53)
Herbal supplements						
No	38± 14 (12.5-70)	0.43	0.13	-	-	-
Yes	36± 12 (17.5-70)		(-0.19 to 0.45)			
Alcohol consumption						
No	38 ± 12 (12.5-75)	0.23	-0.33	32 ± 14 (7.5-72.5)	0.18	-0.17
Yes	42 ± 16 (17.5-72.5)		(-0.70 to 0.04)	35 ± 14 (7.5-72.5)		(-0.41 to 0.08)
Tobacco Smoking						
No	38±13 (12.5-75)	0.35	-0.23	33±14 (7.5 -72.5)	0.98	<0
Yes	41±13 (20-70)		(-0.66 to 0.19)	33±14 (7.5-72.5)		(-0.25 to 0.25)

Abbreviation: CI, confidence interval.

1.09 (95% CI: −0.05 to 2.24), 0.94 (95% CI: 0.05 to 1.84) and 0.98 (95% CI: 0.46 to 1.50), respectively. *CYP2C rs12772169C>T* and *CYP3A5 c.624G>A (*6)*, were borderline correlated (*p*=0.05 and 0.04, respectively) with the warfarin maintenance dose with the effect size of 0.54 (95% CI: 0.09 to 0.98) and −0.52 (95% CI: −1.68 to 0.64), respectively, among black Africans.

The variants *CYP2C9 c.449A* (i.e., GG=40±13 mg/week vs. AA+GA=31±10 mg/week), *CYP2C rs12777823A* (i.e., GG=41±14 mg/week vs. AA+GA=35±12 mg/week), *CYP2C rs12772169T* allele (i.e., CC=40±13 mg/week vs. TT+CT=36±13 mg/week) were associated with decreased mean weekly warfarin maintenance doses. In contrast, *CYP2C8 c.805T (*2)* (i.e., AA=36±12 mg/week vs. TT+AT=40±14 mg/week), and *CYP3A5 c.624A (*6)* (i.e., GG=35±13 mg/week vs. AA+GA=40±12 mg/week) variants were associated with significantly higher mean weekly warfarin maintenance dose (*p<0*.05) requirements. Among the Mixed Ancestry population group, the SNPs that exhibited statistically significant correlation with warfarin maintenance dose in an additive model, included, *CYP2C9 c.430C>T (*2)*, *CYP2C8 c.792C>G (*4)* and *VKORC1 g.-1639G>A* (*p*=0.02, 0.03 and <0.0001, respectively). *CYP2C9 c.430C>T (*2)* had a borderline association with warfarin maintenance dose with the effect size of 0.55 (95% CI:0.06 to 1.03), whilst *CYP2C8 c.792C>G (*4)* and *VKORC1 g.-1639G>A*

had a significant association with the warfarin maintenance dose with the effect size of 1.47 (95% CI:−2.63 to −0.31) and 1.25 (95% CI:0.79 to 1.69), respectively. There were no important haplotypes identified that had an influence on weekly warfarin maintenance dose requirement, as the SNPs with strong linkage (i.e., *CYP2C19 c.681G>A (*2)* (rs4244285) and *CYP2C8 rs11572101T>C)* did not have any individual statistical significance correlation with mean weekly warfarin maintenance dose among both black Africans and Mixed Ancestry.

The interaction and cumulative influence of the various studied genetic and non-genetic variables on the warfarin maintenance dose was analysed using a stepwise regression model. Table 3.12 outlines the stepwise regression analysis for the studied population groups, starting with a full model comprising non-genetic variables and SNPs with a $p \leq 0.20$ for their effect in univariable analysis. Variables were then eliminated in the subsequent models, in a stepwise fashion, starting with variables with the highest *p*-value until variables with $p \leq 0.05$ remained in the final model. In a univariate analysis, deep venous thrombosis did not have a statistically significant effect on warfarin dose variability ($p=0.14$, $R^2=0.004$) among black Africans, however the effect of deep venous thrombosis was increased when factors such as mechanical valve replacement, *CYP2C8 c.805A>T (*2)* and *CYP3A5 c.624G>A (*6)* were entered in the stepwise regression analysis. In contrast, the effect of age and mechanical valve replacement were independent of other variables as they both had a statistical significance influence on warfarin dose variability in a univariate analysis among black Africans. Thus, age, deep venous thrombosis and mechanical valve replacement were non-genetic factors that were significantly associated ($p \leq 0.05$) with the mean maintenance dose among black Africans, together with five SNPs, *CYP2C rs12777823G>A*, *CYP2C8 c.805A>T*

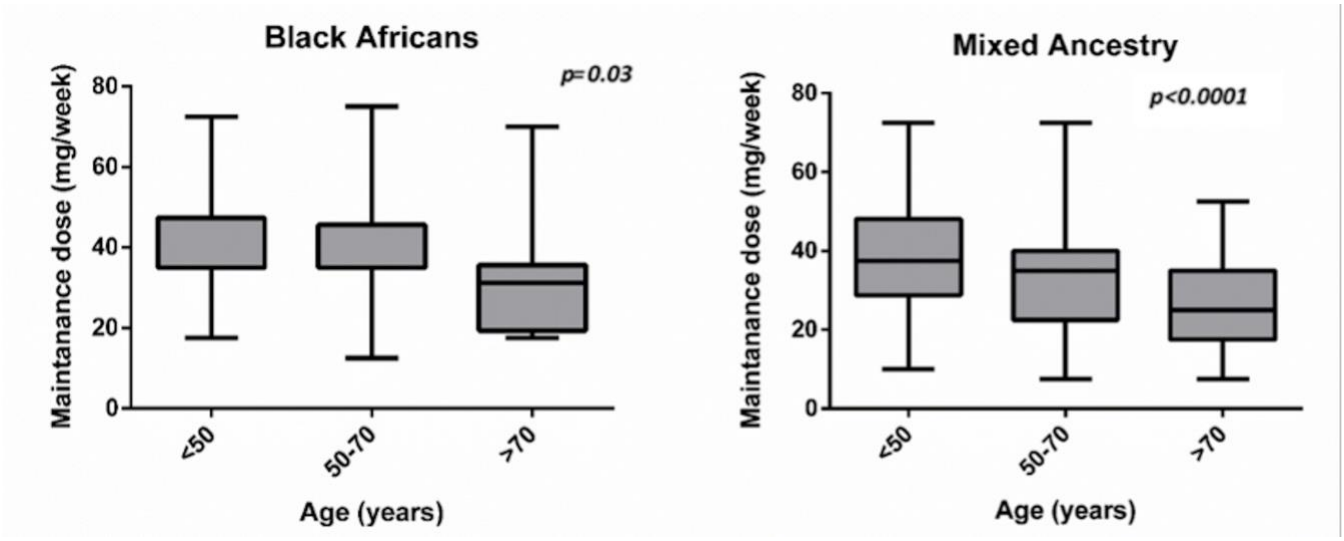

Figure 3.4: Comparison of the warfarin weekly maintenance dose distribution according to age groupings among Black Africans (recruited from Cape Town, South Africa and Harare Zimbabwe) and the Mixed Ancestry (recruited from Cape Town)

*(*2)*, *CYP2C9 c.449G>A (*8)*, *CYP2C9 c.1003C>T (*11)*, and *CYP3A5 c.624G>A (*6)* which exhibited a consistent significant association ($p \leq 0.05$).

The maintenance dose variability explained by non-genetic (i.e., age, deep venous thrombosis, mechanical valve replacement) and genetic factors (i.e. *CYP2C8 c.805A>T (*2)*, *CYP2C9 c.449G>A (*8)*, *CYP2C9 c.1003C>T (*11)*, *CYP2C rs12777823G>A* and *CYP3A5 c.624G>A (*6))*, among black Africans were 9.2% and 18.8%, respectively, thus explaining 28% of the warfarin variability ($p<0.0001$). In a univariate analysis for the Mixed Ancestry, non-genetic variables such as deep venous thrombosis, age and BMI had a statistical significance influence on warfarin dose variability, however when entered in a stepwise regression analysis, deep venous thrombosis lost its significance effect. Thus, a stepwise regression analysis for the Mixed Ancestry group resulted in age and BMI being the non-genetics factors remaining in the final model complimented by *VKORC1 g.-1639G>A* as a single genetic factor that was significantly ($p \leq 0.05$) associated with the weekly maintenance dose in the final model, taken together explaining 22% of warfarin variability ($p<0.0001$). Non-genetic factors (i.e. age and BMI)

and genetic factors (i.e. *VKORC1 g.-1639G>A*) contributed 10% and 12%, respectively, to the cumulative (22%) maintenance dose variability among the Mixed Ancestry group.

In a model combining the two populations groups and adjusted for ethnicity; age, deep venous thrombosis, *CYP2C8 c.805A>T (*2)*, *CYP2C9 c.449G>A (*8)*, *CYP2C9 c.1003C>T (*11)*, *CYP3A5 c.624G>A (*6)*, and *VKORC1 g.-1639G>A* stayed in the final model explaining 23% of the warfarin maintenance dose variability ($p<0.0001$) (Table S7). When interaction terms were included in the stepwise regression model of the combined ethnicities, ethnicity*age and ethnicity*BMI were the only interaction terms that were significant in the final model but they did not the improve the variability ($R^2=0.23$) explained by the model. Thus, factors that are of possible importance regardless of ethnic groups were age, deep venous thrombosis, *CYP2C8 c.805A>T (*2)*, *CYP2C9 c.449G>A (*8)*, *CYP2C9 c.1003C>T (*11)*, *CYP3A5 c.624G>A (*6)*, and *VKORC1 g.-1639G>A*. Table 3.13 outlines factors that we are proposing to be considered for inclusion in warfarin-dosing pharmacogenetic algorithms among African populations. We present here parameters/variables that should be considered in warfarin pharmacogenetics-dosing algorithm among black Africans and the Mixed Ancestry population groups.

3.3.2.4. Discussion

Warfarin remains a very important therapeutic drug widely used across the world and particularly in poorly resourced healthcare settings including many African countries. However, the variability in the dose required to reach and maintain warfarin international normalised ratio, differs greatly among patients. Whereas several studies have decoded the factors associated with this variability, there is little or no information on genetic variation and its contribution to the variability observed in African populations [163, 164]. This study is a contribution in decoding the pharmacogenomics of Africans using populations from Southern Africa. The study investigated genetic variation in the genes that affect the pharmacokinetics of warfarin with respect to their contribution to the variability in warfarin dosing. The study particularly focused on SNPs in *ABCB1, CYP1A2, CYP2C8, CYP2C19, CYP3A4* and *CYP3A5*, in addition to the traditionally characterised *VKORC1* and *CYP2C9* genes.

Evaluating current pharmacogenetics-guided warfarin algorithms and the presented data on African populations here, it is interesting to note that variants such as *CYP2C9*2* and *CYP2C9*3* which are an important inclusion in most warfarin-dosing algorithms appear to play no significant role among black Africans, because they are rare among Africans [107, 110, 111]. Some of the common variables included in warfarin dosing algorithms include genetic factors *CYP2C9 c.430C>T (*2), CYP2C9 c.1075A>C (*3)* & *VKORC1 g.–1639G>A* and non-genetics factors age, body surface area, amiodarone, race, targeted INR, smoking, thromboembolism, height, weight, enzyme inducer status, stroke indication, diabetes, and Fluvastatin use, in different combinations (Table 3.13) [63-65]. We present variables that are important in African populations and these include deep venous thrombosis, BMI, variants in *CYP2C8*, *CYP2C9* and *CYP3A5*, which have not been included in most of the available warfarin dosing algorithms.

Age is the only non-genetic variable that seems to be important across population or geographical populations as reflected in the various warfarin dosing algorithms (Table 3.13). What is clear is that African populations present with a wide range of important genetic variants markers that are important for warfarin-dosing, further buttressing the genomic diversity of African populations and the short linkage disequilibrium (LD). The genomic diversity among Africans is further confirmed by the varying LD profiles of the CYP2C cluster SNPs observed between the two population groups included in the present study. Our results further show the important role of gene-environmental interactions on warfarin maintenance dose variability in various population groups, as non-genetic factors such as deep venous thrombosis and mechanical valve replacement contributed to warfarin dose variability among black Africans only and not in Mixed Ancestry.

The common denominator of existing warfarin-dosing algorithms is their exclusion or lack of consideration of variants that are of pharmacogenomics importance among Africans. We report here on additional variants in *CYP2C9* (*CYP2C9*8* & *CYP2C9*11*), and in genes that were peripheral in warfarin disposition, *CYP2C8* and *CYP3A5* on their

Table 3.11: Effects of genetic variation in warfarin-associated pharmacogenes on warfarin maintenance dose among Black Africans and Mixed Ancestry

	Black Africans (N=252)			Mixed Ancestry (N=251)		
SNP genotype	**Maintenance dose (mg/week), mean ± SD (range)**	***p* value**	**Cohen's d (95%CI)**	**Maintenance dose (mg/week), mean ± SD (range)**	***p* value**	**Cohen's d (95%CI)**
ABCB1 c.3435C>T, rs1045642						
CC	37 ± 13 (12.5-70)	0.29	0.25	32 ± 14 (10-65)	0.77	0.09
CT	34 ± 9 (17.5-47.5)		(-0.22 to 0.72)	33 ± 14 (10-72.5)		(-0.52 to 0.69)
TT	52.5 (52.5-52.5)			31 ± 14 (12.5-52.5)		
CYP1A2 g.-3860G>A (*1C)*, *rs2069514						
GG	36 ± 12 (12.5-70)	0.67	0.20	33 ± 15 (10-72.5)	0.87	-0.28
GA	39 ± 14 (12.5-70)		(-0.49 to 0.89)	33 ± 15 (12.5-65)		(-1.69 to 1.13)
AA	34 ± 15 (17.5-57.5)			36 ± 4 (32.5-40)		
CYP1A2 g.-163C>A (*1F), rs762551						
CC	34 ± 13 (12.5-70)	0.28*	-0.16	26 ± 14 (17.5-52.5)	0.38	-0.40
CA	39 ± 12 (17.5-70)		(-0.59 to 0.27)	35 ± 16 (10-72.5)		(-1.21 to 0.41)
AA	38 ± 14 (12.5-70)			31 ± 11 (15-52.5)		
CYP2C8 rs1926705G>A						
GG	41 ± 13 (27.5-52.5)	0.83	0.26	35 ± 17 (17.5-70)	0.76	0.03
GA	37 ± 12 (17.5-70)		(-0.88 to 1.41)	32 ± 14 (12.5-72.5)		(-0.66 to 0.74)
AA	37 ± 13 (12.5-72.5)			34 ± 16 (7.5-72.5)		
CYP2C8 rs11572100A>G						
AA	37 ± 13 (12.5-72.5)	0.85	0.47	33 ± 15 (7.5-72.5)	0.96	-0.78
AG	38 ± 11 (17.5-70)		(-0.93 to 1.86)	35 ± 17 (15-70)		(-0.63 to 0.48)
GG	31 ± 19 (17.5-45)			-		
CYP2C8 rs11572101T>C						
TT	37 ± 13 (12.5-72.5)	0.98	-0.13	32 ± 14 (7.5-72.5)	0.63	-0.11
TC	37 ± 13 (12.5-70)		(-0.94 to 0.69)	36 ± 17 (10-72.5)		(0.84 to 0.63)
CC	39 ± 17 (20-67.5)			34 ± 11 (15-52.5)		
CYP2C8 c.792C>G (*4), rs1058930						

CC	37 ± 13 (12.5-72.5)	0.76	0.19	33 ± 14 (7.5-72.5)	0.03	1.47
CG	35 (35-35)		(-1.21 to 1.58)	54 ± 14 (45-70)		(-2.63 to -0.31)
CYP2C8 c.805A>T (*2)*, *rs11572103						
AA	36 ± 12 (12.5-70)	0.04*	-1.06	33 ± 14 (7.5-72.5)	0.19	-0.38
AT	38 ± 12 (17.5-70)		(-1.68 to -0.43)	38 ± 16 (17.5-72.5)		(-0.90 to 0.15)
TT	49 ± 19 (20-72.5)			-		
CYP2C8 rs11572105C>A						
CC	37 ± 13 (17.5-70)	0.41	-0.33	33 ± 13 (10 -62.5)	0.16	-
CA	41 ± 12 (35-70)		(-1.04 to 0.39)	15 (15-15)		
AA	45 (45-45)					
CYP2C9 c.430C>T (*2), rs1799853						
CC	38 ± 13 (12.5-70)	0.52	0.29	34 ± 14 (7.5-72.5)	0.02	0.55
CT	34 ± 9 (27.5-47.5)		(-0.71 to 1.28)	27 ± 13 (10-62.5)		(0.06 to 1.03)
CYP2C9 c.1075 A>C (*3), rs1057910						
AA	38 ± 13 (12.5-70)	-	-	34 ± 14 (7.5-72.5)	0.32	0.21
AC	-			31 ± 15 (10-62.5)		(-0.22 to 0.65)
CYP2C9 c.1080C>G (*5), rs28371686						
CC	38 ± 13 (12.5-70)	0.43	-0.33	34 ± 14 (7.5-72.5)	-	-
CG	42 ± 9 (35-52.5)		(-1.32 to 0.67)	-		
CYP2C9 c.449G>A (*8), rs7900194						
GG	40 ± 13 (12.5-70)	0.001*	1.09	35 ± 17 (17.5-72.5)	0.88	-0.07
GA	32 ± 10 (17.5-57.5)		(-0.05 to 2.24)	34 ± 14 (7.5-72.5)		(-0.74 to 0.59)
AA	26 ± 9 (17.5-25)			-		
CYP2C9 c.1003C>T (*11), rs28371685						
CC	39 ± 13 (12.5-70)	0.03	0.94	34 ± 14 (10-72.5)	0.48	0.41
CT	27 ± 8 (17.5-35)		(0.05 to 1.84)	28 ± 12 (7.5-37.5)		(-0.48 to 1.29)
CYP2C19 c.681G>A (*2), rs4244285						

GG	37 ± 13 (12.5-70)	0.52	0.30	33 ± 15 (10-70)	0.78	0.27
GA	38 ± 12 (17.5-70)		(-0.70 to 1.31)	34 ± 15 (15-72.5)		(-0.51 to 1.05)
AA	33 ± 5 (25-35)			29 ± 12 (17.5-47.5)		
CYP2C19 c.636G>A (*3), rs4986893						
GG	37 ± 13 (12.5-70)	-	-	33 ± 14 (10-47.5)	0.76	0.19
GA	-			30 ± 19 (10-47.5)		(-0.82 to 1.19)
CYP2C19 c.1A>G (*4), rs28399504						
GG	37 ± 12 (12.5-70)	-	-	33 ± 14 (10-72.5)	0.63	-
GA				37.5 (37.5-37.5)		
CYP2C19 g.-806C>T (*17), rs12248560						
CC	36 ± 12 (12.5-70)	0.64	0.08	33 ± 14 (10-70)	0.69	0.49
CT	39 ± 14 (17.5-700		(-1.32 to 1.49)	34 ± 16 (12.5-72.5)		(-0.67 to 1.64)
TT	35 (35-35)			26 ± 13 (15-40)		
CYP2C rs12777823G>A						
GG	41 ± 14 (12.5-75)	0.0001*	0.98	33 ± 13 (7.5-72.5)	0.78	0.16
GA	37 ± 12 (17.5-70)		(0.46 to 1.50)	34 ± 15 (7.5-72.5)		(-0.29 to 0.62)
AA	28 ± 8 (12.5-45)			31 ± 12 (15-52.5)		
CYP2C rs12772169C>T						
CC	40 ± 13 (17.5-72.5)	0.05*	0.54	33 ± 15 (10-70)	0.38	0.36
CT	37 ± 13 (12.5-70)		(0.09 to 0.98)	34 ± 14 (10-72.5)		(-0.24 to 0.95)
TT	33 ± 12 (12.5-52.5)			28 ± 13 (7.5-47.5)		
CYP3A4 g.15389C>T (*22), rs35599367						
CC	37 ± 13 (12.5-70)	-	-	33 ± 15 (10-72.5)	0.91	0.004
CT	-			33 ± 12 (17.5 -52.5)		(-0.82 to 0.83)
CYP3A5 g.6986A>G (*3), rs776746						
AA	37 ± 13 (12.5-70)	0.44	-0.79	29 ± 14 (10-62.5)	0.64	-0.06
AG	40 ± 14 (12.5-70)		(-2.18 to 0.62)	34 ± 13 (10-72.5)		(-0.63 to 0.52)
GG	48 ± 18 (35-60)			34 ± 16 (12.5-70)		
CYP3A5 c.624G>A (*6),						

rs10264272						
GG	35 ± 12 (12.5-70)	0.04*	-0.52	33 ± 15 (10-72.5)	0.93	0.008
GA	40 ± 12 (17.5-70)		(-1.68 to 0.64)	31 ± 13 (17.5-52.5)		(-0.82 to 0.83)
AA	41 ± 5 (35 -45)			27.5 (27.5- 27.5)		
CYP3A5 g.27131_27132insT (*7)*, *rs41303343						
AA	38 ± 13 (12.5-70)	0.63	0.22	32 ± 15 (10-72.5)	0.25	-0.36
AT	34 ± 12 (12.5-52.5)		(-1.18 to 1.62)	37 ± 12 (17.5-50)		(-1.08 to 0.37)
TT	35 (35-35)			-		
VKORC1 g.-1639G>A, rs9923231						
GG	38 ± 13 (12.5-75)	0.49	0.52	37 ± 13 (10-72.5)	0.0001*	1.25
GA	38 ± 11 (17.5-70)		(-0.87 to 1.91)	31 ± 14 (7.5-72.5)		(0.79 to 1.69)
AA	31 ± 5 (27.5-35)			21 ± 12 (10-62.5)		

Abbreviation: CI, confidence interval.
*p value ≤.05 for the dominant genetic model.

significant association with mean weekly warfarin maintenance doses among black Africans. The effect of *CYP2C9 c.449G>A (*8)* and *CYP2C9 c.1003C>T (*11)* on warfarin maintenance dose that we report here, is an extension of our earlier report [107] which showed a trend towards significant association between these SNPs and mean weekly warfarin maintenance dose. Thus, here we report with a larger sample size for the black African group (n=252), firmly confirming our earlier report of the influence of *CYP2C9 c.449G>A (*8)* and *CYP2C9 c.1003C>T (*11)* on warfarin maintenance dose and this finding is consistent with other studies that characterised populations with considerable African ancestry such as African Americans [47].

We further confirm in this study, our earlier report [107] where we showed *CYP2C rs12777823G>A* to be strongly associated with prediction for warfarin maintenance dose among black Africans. Although the effect of the *CYP2C*

rs12777823G>A and *CYP2C9* variants on warfarin maintenance dose has been described before, the effect of *CYP2C8* and *CYP3A5* variants among Africans have only being examined on other drugs other than warfarin [153, 241-243]. Furthermore, *CYP2C8* phenotype-genotype correlation is not well characterised in humans as data available has mainly focused on reporting the allelic distribution of the *CYP2C8* variants [155, 156, 244] and in vitro studies investigating the CYP2C8 enzyme activity [241, 245, 246]. The variants *CYP2C8*2* and *CYP3A5*6*, which we are proposing for consideration for inclusion in the warfarin pharmacogenetics-based dosing algorithm for African populations, affect metabolism of the isomer, R-warfarin [247]. The results presented here suggest that there is also an important role for R-warfarin on pharmacodynamics, confirming findings by Maddison et al [248].

This study shows that both *CYP2C8*2* and *CYP3A5*6*, increase warfarin dose requirements. However, this is in contrast to the expected outcome as these variants are associated with reduced enzyme activity and gene expression. For instance, The *CYP2C8 c.805T* variant (coding for *CYP2C8*2* allele) results in functionally impaired CYP2C8.2 variant enzyme, exhibiting reduced intrinsic clearance of paclitaxel and amodiaquine [241, 245]. *CYP3A5*6* is reported to reduce expression of CYP3A5 due to a splicing defect caused by the presence of a *G14690A* transition in exon 7, resulting in the splicing deletion of exon 7, thereby, affecting the availability of CYP3A5 enzyme [217, 249]. Thus, it would be expected that with respect to warfarin pharmacokinetics, both these variants should be associated with reduced warfarin-dose requirements. However, the conflicting findings we report here with other previous studies could be alluded to the fact that *CYP2C8* phenotype-genotype correlation is not well characterised in humans and available data has mainly been from in vitro studies investigating the CYP2C8 enzyme activity, as previously mentioned [241, 245, 246]. Furthermore, considering the low clearance rate of R-warfarin compared to that of S-warfarin (mean half-life of 58 h vs. 33 h) [250], the binding affinity that the metabolising enzymes have with the R-warfarin might be different with that of other substrates.

Table 3.12: Stepwise regression model of the effect of multiple variables on warfarin weekly maintenance dose among Black Africans and Mixed Ancestry

Black Africans				**Mixed Ancestry**			
Variable	**Coefficient**	**95% CI**	***p* value**	**Variable**	**Coefficient**	**95% CI**	***p* value**
Model including variables with p≤0.2 in univariate analysis: R^2=0.30, p <0.0001				**Model including variables with p≤0.2 in univariate analysis: R^2=0.25, p<0.0001**			
Age	-0.14	-0.25 to -0.03	0.009	Age	-0.22	-0.33 to -0.11	<0.0001
Atrial fibrillation	1.43	-3.32 to 6.18	0.56	Gender	2.75	-0.72 to 6.23	0.12
Deep venous thrombosis	4.00	-0.39 to 8.39	0.07	BMI	0.46	0.13 to 0.78	0.006
Mechanical valve replacement	6.62	2.14 to 11.1	0.004	Deep venous thrombosis	0.78	-3.97 to 5.54	0.74
Hypertension	0.12	-3.17 to 3.42	0.94	Mechanical valve replacement	-0.44	4.16 to 3.27	0.81
Diabetes mellitus	-1.72	-8.00 to 4.57	0.59	Diabetes mellitus	2.93	-1.54 to 7.40	0.19
Heart failure	-2.13	-5.71 to 1.44	0.24	Heart failure	-2.30	-5.78 to 1.18	0.19
Statins	-1.83	-7.48 to 3.82	0.52	HIV	0.51	-8.77 to 9.78	0.91
Alcohol consumption	-1.49	-6.41 to 3.43	0.55	Alcohol consumption	1.74	-1.60 to 5.08	0.31
*CYP2C8 c.805A>T (*2), rs11572103*	13.3	6.36 to 20.3	0.0002 (0.001) *	*CYP2C8 c.805A>T (*2), rs11572103*	0.50	-3.99 to 4.99	0.83
*CYP2C9 c.449G>A (*8), rs7900194*	-5.65	-9.99 to -1.31	0.01 (0.06) *	*CYP2C8 c.792C>G (*4), rs1058930*	4.31	-1.34 to 9.95	0.13
*CYP2C9 c.1003C>T (*11), rs28371685*	-7.82	-14.3 to -1.30	0.02 (0.12) *	*CYP2C9 c.430C>T (*2), rs1799853*	-3.63	-9.28 to 2.02	0.23
CYP2C rs12777823G>A	-6.54	-13.8 to 0.71	0.07	*VKORC1 g.-1639G>A, rs9923231*	-15.1	-20.7 to -9.41	<0.0001 (0.0004)*
CYP2C rs12772169C>T	1.21	-5.16 to 7.58	0.71	-	-	-	-
*CYP3A5 c.624G>A (*6), rs10264272*	8.88	5.44 to 12.3	<0.0001 (0.0006) *	-	-	-	-
Model including variables with p≤0.05: R^2=0.28, p<0.0001				**Model including variables with p≤0.05: R^2=0.22, p<0.0001**			
Age	-0.14	-0.24 to -0.05	0.004	Age	-0.24	-0.35 to -0.14	<0.0001
Deep venous thrombosis	4.91	1.52 to 8.28	0.005	BMI	0.49	0.23 to 0.77	0.0004

Mechanical valve replacement	6.36	2.67 to 10.0	0.0008	*VKORC1 g.-1639G>A, rs9923231*	-16.1	-21.5 to -10.7	<0.0001
*CYP2C9 c.449G>A (*8), rs7900194*	-5.62	-9.82 to -1.42	0.009 (0.05) *	-	-	-	-
*CYP2C9 c.1003C>T (*11), rs28371685*	-7.97	-14.3 to -1.63	0.01 (0.05) *	-	-	-	-
CYP2C rs12777823G>A	-6.49	-12.4 to -0.60	0.03 (0.15)*	-	-	-	-
*CYP3A5 c.624G>A (*6), rs10264272*	8.27	5.15 to 11.4	<0.0001 (0.0005)*	-	-	-	-

Abbreviation: CI, confidence interval.
*Adjusted p value for the Bonferroni correction.

Rettie et al [251] reported that the R-warfarin metabolites were produced from low binding affinity reactions between R-warfarin metabolising enzymes and the R-warfarin substrate, thus the low R-warfarin clearance could be due to a low binding affinity between the R-warfarin and the metabolising enzymes produced by the wild-type alleles. Therefore, the CYP2C8.2 variant enzyme produced by the *CYP2C8*2* allele could be having an increased binding affinity with R-warfarin, thereby increasing warfarin clearance resulting in increased warfarin dose requirement. This phenomenon is similar to that reported by Kaminsky and Zhang [40] where variant CYP1A1-Val462 had a high R-warfarin binding affinity and increased metabolic rate compared to the wild type CYP1A1-Ile462. In addition, our results also show that the effect of *CYP2C8*2* is independent of other SNPs in the CYP2C cluster that have been shown before to affect warfarin dose variability, as the LD results displayed a weak linkage between *CYP2C8*2* with *CYP2C9* SNPs and *CYP2C rs12777823G>A.* With regards to *CYP3A5*6*, previous studies have reported that the CYP3A5 expression and metabolic activity is not modulated by *CYP3A5* SNPs alone but by multiple factors which include endogenous molecules (e.g., circulating hormones and drug-drug interactions) and exogenous molecules (e.g., diet and environmental conditions) [252, 253]. Thus, the influence of *CYP3A5*6* on warfarin maintenance dose could be driven

Table 3.13: Proposed variables for possible inclusion in an African-specific warfarin pharmacogenetics-based dosing algorithm compared to other algorithms reported in different world populations

References	Genetics variables	Demographic and clinical variables
This study	*VKORC1 g-1639 G>A, CYP2C rs12777823G>A, CYP2C8 c.805A>T (*2), CYP2C9 c.449G>A (*8), CYP2C9 c.1003C>T (*11), CYP3A5 c.624GA (*6)*	Age (in years), Gender, BMI, Deep venous thrombosis, mechanical valve replacement
Gage et al [63]	*VKORC1 3673G>A, CYP2C9*3*, *CYP2C9*2*	BSA (per 0.25 m^2), Age (per decade), target INR (per 0.5 increase), Amiodarone, Current smoker, African American race, Venous thromboembolism
International Warfarin Pharmacogenetics Consortium [65]	*VKORC1 g-1639 G>A, CYP2C9 *2, CYP2C9 *3*	Age in decades, height (in cm), weight (in kg) Race, Enzyme inducer status, Amiodarone status
Lenzini et al [64]	*VKORC1 g.-1639G>A, CYP2C9*2, CYP2C9*2*	Natural logarithm (INR), $dose_{-3}$ (per mg), age (per year), BSA (per 0.25 m^2), target INR, African ancestry, stroke indication, $dose_{-4}$ (per mg), $Dose_{-2}$ (per mg), diabetes, amiodarone use, fluvastatin use
www.warfarindosing.org (modified from Gage et al [63]	*VKORC1 g.-1639G>A, CYP2C9*2, CYP2C9*3, CYP2C9*5, CYP2C9*6, CYP4F2 V433M, GGCX rs11676382*	Age, sex, race, ethnicity, weight (kg or lbs.), height (feet and inches or cm), smokes, liver diseases, indication, baseline INR, target INR, amiodarone/cordarone® dose (mg/day), statin/HMG CoA reductase inhibitor, any azole and Sulfamethoxazole/Septra/Bactrim/Cotrim/ Sulfatrim

by additional factors not considered in the study. Considering that R-warfarin is less potent than S-warfarin, the increased warfarin dose associated with variants of genes encoding enzymes metabolising R-warfarin could also suggest that to increase the R-warfarin potency an increased dose may be required. This is supported by Maddison et al [248] where they reported that R-warfarin reached a pharmacodynamic response when they administered a higher warfarin dose of 80 mg of R-warfarin compared to the 12.5 mg for S-warfarin. More pharmacokinetics-pharmacodynamics studies on the influence of variants involved in R-warfarin metabolism are warranted to further confirm

our findings and to better elucidate the functional role they play on R-warfarin metabolism.

We further report on the lack of significant association between SNPs *ABCB1 c.3435C>T, CYP1A2 g.-3860G>A (*1C), CYP2C19 c.681G>A (*2), CYP3A4 g.15389C>T (*22)* and *CYP3A5 g.6986A>G (*3)* with warfarin maintenance dose. However, *CYP1A2*1C*, has been reported to be associated with significantly high warfarin dose requirements among Chinese patients [254]. The role of *ABCB1 c.3435C>T* SNP on warfarin dose requirements has been contradictory, with various studies reporting a lack of association with warfarin dose [120, 255, 256], while others such as Wadelius et al [257] and Tavares et al [258] have reported its association with low warfarin dose requirements among Swedish and Brazilian patients, respectively. The lack of association between warfarin dose requirements and *CYP3A5 g.6986A>G (*3)* observed in this study, further confirms observations by Wadelius et al [257] In addition, to SNPs studied here, it is worth noting that there are other SNPs not reported here but have been studied before in other Sub-Saharan African populations to contribute to warfarin dose variability and should be considered for the African-specific warfarin pharmacogenetics-based dosing algorithm. This include, SNPs *VKORC1 Asp36Tyr* and *VKORC1 Val66Met* which have been associated with warfarin resistance, the former was reported among Ethiopians, Kenyans, Sudanese, and Egyptians at a frequency of 15%, 6%, 6%, and 2.5%, respectively, whilst the latter was reported among Tanzanians and Brazilians of African descent [130, 131, 138, 139].

Although our study investigated multiple SNPs in various genes of warfarin pharmacokinetic relevance, this candidate gene approach introduces bias and limits the identification of other novel variants not investigated in the study that could be playing a role in warfarin maintenance dose distribution. Hence, the variables that we identified to be of importance among black Africans and Mixed Ancestry explained only 28% and 22% of warfarin dose variability, respectively. Therefore, a high percentage of the warfarin dose variability remain unexplained, and this could be achieved by decoding more variants important for warfarin dose variability through employing high-throughput

techniques such as next generation sequencing (NGS). Our study included individuals recruited at specific areas (i.e., Western Cape Province and Harare) in South Africa and Zimbabwe, thus, the results presented here does not give a full representation of various factors affecting warfarin dose variability in the different populations in Southern Africa or Africa as a whole. However, the results presented here gives a perspective of some of the important genetic and non-genetic predictors of warfarin dose that can be applied in various African populations, especially populations that migrated from the southern West Africa, known as populations of "Bantu origin". Missing data on some of the SNPs targeted was a limitation in our study, which however the genetic variant information was imputed to allow us to draw conclusions on the important genetic variants for warfarin dose among the population studied.

Another the limitation is our study design being cross-sectional and not longitudinal, thus it does not allow us to identify the role of genetics in terms of patients reaching the time to therapeutic range. Considering all the limitations that we have outlined, we postulate that 19%, 3%, 52%, 38% and 40% individuals in the black African population carry the *CYP2C9*8, CYP2C9*11, CYP2C rs12777283A*, *CYP2C8*2* and *CYP3A5*6* alleles, respectively, either in the homozygous or heterozygous genotypes. Thus, possibly affecting their warfarin maintenance dose. Furthermore, the difference in warfarin maintenance dose requirements between the homozygous wild-type and homozygous mutant genotypes in these SNPs of interest was over 30% except for *CYP3A5 c.624G>A* which was 17%. Thus, outlining the increased variability in warfarin dose distribution according to alleles in the different SNPs. However, to further confirm the measured clinical significance of these SNPs reported here, additional studies which follow a longitudinal design are required.

3.3.2.5. Conclusion

Our study highlights the importance of widening the populations that are investigated for pharmacogenetics tests and the inclusion of a wide array of genes contributing to pathways that involve warfarin disposition. The current approach of focusing on selected genes (e.g., *VKORC1* and *CYP2C9*) is difficult to apply to different populations.

Additionally, our study advocates for inclusion of diverse populations in pharmacogenomics research in order to identify population-specific pharmacogenes variants. We identified 2 SNPs that are potential warfarin dose predictors which have not been studied before with regards to warfarin response in an African setting. We propose an African-specific warfarin pharmacogenetics-based dosing algorithm should include, *VKORC1 g.-1639G>A, CYP2C rs12777823G>A, CYP2C8 c.805A>T (*2), CYP2C9 c.449G>A (*8), CYP2C9 c.1003C>T (*11)* and *CYP3A5 c.624GA (*6)* in addition to the various demographic and clinical variables. In addition to these warfarin-pharmacokinetics associated genetic variants reported here, it is important to characterise the whole spectrum of warfarin-pharmacodynamics associated genes for their contribution to warfarin response.

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CONFLICT OF INTEREST

None of the authors reported any conflict of interest.

AUTHOR CONTRIBUTION

AN and SM conceptualised the idea, generated the data, analysed the data and drafted the manuscript; PM reviewed the manuscript draft; APK assisted with data analysis and reviewed the manuscript draft, EC assisted with data analysis and reviewed the

manuscript; AW and MN co-supervised all components and reviewed the manuscript draft; CD conceptualised the ideas, supervised all components as principal investigator and reviewed the manuscript drafts. All authors contributed to the final version of the article. The authors read and approved the final manuscript.

3.3.3. Pharmacodynamic determinants of warfarin dose variability in 17 genes among Africans: significant roles for VKORC1, MTHFR and CYP2D6

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Nature of Manuscript: Original research article

Candidate's Contribution: conceptualised the idea, conducted experimental activities, data analysis, drafted the manuscript and incorporated changes from co-authors and reviewers

Co-Authors Contribution:

SM: conducted experimental activities, and reviewed manuscript draft.

MN: co-supervised all components and reviewed the manuscript draft and is the clinical expert on the project.

CD: conceptualised the ideas, supervised all components and reviewed the manuscript drafts.

All authors contributed to the final version of the article. The authors read and approved the final manuscript.

Pharmacodynamic determinants of warfarin dose variability in 17 genes among Africans: significant roles for VKORC1, MTHFR and CYP2D6

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Abstract

Warfarin therapy is challenging to manage clinically, due to its narrow therapeutic range and difficulty in predicting the accurate starting dose for each patient, which leads to adverse treatment outcomes. Pharmacogenetics-guided warfarin prescriptions seem to be improving patient safety profiles. Most warfarin pharmacogenetics studies have focused on *VKORC1* and *CYP2C9* and less on genes coding for enzymes involved in warfarin minor pathways. In addition, data on African populations is generally lacking. We set out to evaluate 34 SNPs in 17 genes (i.e., *APOE, F2, F5, GGCX, CYP4F2, CALU*, *VKORC1*, *COMT, CYP2B6, CYP2D6, DRD2, GLP1R, MTHFR, OPRM1, PNPLA5, SLCO1B1* and *SULT4A1*) on their effects on warfarin maintenance dose among black Africans and Mixed Ancestry population groups in Southern Africa.

We report on a significant association of *MTHFR c.677T* variant *(*rs1801133*)* with lower warfarin dose requirement among black Africans, as indicated by the significant difference (p=0.04) in the maintenance doses of the *MTHFR c.677C/C* genotype (38±12 mg/week) and that of *MTHFR c.677T/T* genotype (17.5 mg/week). *CYP2D6 g.100C>T (*10) (*rs1065852*), VKORC1 c.1173C>T (*rs9934438*)* and *VKORC1 g.9041G>A (rs7294)* showed significant (p<0.05) effect on warfarin mean maintenance dose among the Mixed Ancestry population group. The haplotypes in *GLP1R* (rs6923761, rs2300615 and rs1042044) *G-G-C* and *MTHFR* (rs1801131 and rs1801133) T-A were significantly (p=0.02) associated with increased and reduced warfarin maintenance dose variability among black Africans, respectively. Conversely, haplotypes in *CALU* (rs339097 and rs1043550) *A-G*, in *VKORC1 (*rs7294, rs9934438 *and* rs9923231*) G-T-A,* and *A-C-G* were significantly associated with warfarin dose variability (p=0.02; p=5.678e-08 and p=1.461e-05, respectively) in the Mixed Ancestry population group. *MTHFR c.677C>T (*rs1801133*)* seems to contribute to about 6% of the observed warfarin maintenance dose variability among black Africans. It is important to now integrate the pharmacokinetic and pharmacodynamic genetic markers in one coherent model that can be used to improve warfarin starting dose decisions.

Keywords: warfarin, pharmacodynamics, pharmacogenetics, Southern Africa, VKORC1, MTHFR, Mixed Ancestry & Black Africans

3.3.3.1 Introduction

Although warfarin is challenging in predicting the starting dose required to achieve the international normalised ratio, and its narrow therapeutic index, it remains one of the most widely used drug globally [44]. Warfarin is the most highly prescribed anticoagulant for the treatment of thromboembolic disorders and associated conditions, particularly in low-income economies such as Africa, where it is considered to be cost effective as compared to other alternatives such as the direct oral anticoagulants (DOACs) [2]. The application of pharmacogenetics has become attractive in guiding prescription of warfarin therapy. However, pharmacogenetics of warfarin has mostly focused on genes affecting the pharmacokinetics of warfarin, particularly *CYP2C9* and *VKORC1* [63]. Although *CYP2C9* and *VKORC1* are primary enzymes interacting with

warfarin, there are several other genes/enzymes that play a role in the disposition of warfarin and could serve as important additional pharmacogenetic markers in warfarin starting-dose decision making. These enzymes include, CYP1A2, CYP2C8, CYP2C18, CYP2C19, CYP3A4, CYP3A5, and ABCB1 which are also involved in the warfarin metabolism and transport [40], and other enzymes such as CYP4F2, GGCX, CALU and APOE, involved either in the recycling inter-conversion or transport of the cofactor vitamin K, a cycle targeted by warfarin [34-37]. Additionally, coagulation factors II (prothrombin), VII, IX, and X as well as antithrombotic protein C and protein S are affected by the action of warfarin as their activation is dependent on the interconversion of vitamin K [34].

To date, most warfarin pharmacogenetics studies barely evaluate variants in genes other than *VKORC1* and *CYP2C9*, thus hindering the advancement of warfarin pharmacogenetics and decoding genetic variants that could add to the explanation of the observed vast inter-individual variability in warfarin response that remains unexplained (163, 164). For the full utility of pharmacogenetics to be exploited in warfarin prescription, genetic profiles of the full range of genes encoding enzymes involved in the warfarin interactive pathways need to be comprehensively evaluated, that is, genes encoding enzymes involved both the pharmacokinetics and pharmacodynamics of warfarin [259]. Although, advancement in research on the pharmacogenetics of warfarin has been evident in European and Asian populations, where they have moved beyond just investigating markers only in the warfarin principal genes, there is still paucity of research and data in African populations. The huge genetic diversity in African populations therefore means that pharmacogenetics data from other populations is not completely useful in an African setting [260, 261].

The few Africans studies have shown some specific genetic markers (i.e., *CYP2C9 c.449G>A (*8), CYP2C9 c.1003C>T (*11) and CYP2C rs12777823G>A)* that seem to play important roles in warfarin dose variability and could be useful in the development of African specific warfarin-dosing algorithms [107, 111, 115, 171, 261]. Indeed, our earlier work has reported on African specific warfarin pharmacogenetic predictive markers (i.e., *CYP2C9 c.449G>A (*8), CYP2C9 c.1003C>T (*11) and CYP2C*

rs12777823G>A), as part of over 20 warfarin pharmacokinetic-related genetic markers we evaluated. In the study [171], we reported for the first time an association of warfarin dose variability with SNPs *CYP2C8 c.805A>T (*2)* and *CYP3A5 c.624G>A (*6),* culminating in a recommendation for the inclusion of *CYP2C8 c.805A>T (*2)* (rs11572103) and *CYP3A5 c.624G>A (*6)* (rs10264272) in pharmacogenetic dosing algorithms tailored for African populations [171]. The present study is aimed at profiling and establishing the effect of genetic variants in genes affecting the pharmacodynamics of warfarin among black Africans and Mixed Ancestry populations in Southern Africa. We further report on genes that are not directly involved in the warfarin pathway, but interact with the primary genes, to impact on warfarin dose variability. This study is done to add to the comprehensive characterisation of variants that could serve as predictive markers in an African-specific warfarin pharmacogenetic dosing algorithm.

3.3.3.2. Methods and Materials

3.3.3.2.1. Patient characteristics

This is a secondary genetic characterisation; thus, sample size was limited by the samples which had been comprehensively characterised for the warfarin pharmacokinetics-associated genes [171] and comprised of 503 participants (252 black Africans and 251 Mixed Ancestry). The participants were recruited from Groote Schuur Hospital (GSH), Gugulethu Community Health Centre (GCHC) in the Western Cape Province, South Africa, and Parirenyatwa Group of Hospitals (PGH) in Harare, Zimbabwe. Ethical clearances were granted by the University of Cape Town Human Research Ethics Committee (UCT-HREC REF No. 581/2015), Faculty of Medicine, University of Zimbabwe Ethics Committee (JREC/160/13) and the Medical Research Council of Zimbabwe (MRCZ/A/1815). The process of collection of biological samples, demographic and clinical information has been reported before [107].

DNA was extracted from 5ml of whole blood using the salting-out method as modified from Gustafson et al [160] and Qiagen Blood Mini Kit according to manufacturer's instructions (Qiagen, Hilden, Germany). Genetic characterisation was done using a

combination of restriction fragment length polymorphism (RFLP), Sanger sequencing and the iPLEX PGx74 Mass Genotyping Array panel. The iPLEX PGx74 Mass Genotyping Array panel targets 69 SNPs/INDELs and 5 CNVs in 20 pharmacogenes. Data on 6 additional genes, namely, *ABCB1, CYP1A2, CYP2C9, CYP2C19, CYP3A4* and *CYP3A5*, has already been reported before [171]. Thus, this study reports on the remaining 14 genes *APOE, F2, F5*, *VKORC1, COMT, CYP2B6, CYP2D6, DRD2, GLP1R, MTHFR, OPRM1, PNPLA5, SLCO1B1* and *SULT4A1*. Variants in genes not included in the iPLEX PGx74 panel (i.e., *GGCX, CYP4F2* and *CALU*) were genotyped using RFLP and Sanger sequencing and detailed methodology has been reported before [259].

3.3.3.2.2. Data analysis

Statistical analysis was conducted using STATA for Windows version 15 (StataCorp, College Station, Texas, USA), various statistical packages in R (version 4.0.3 [2020–10–10]) and plink (http://pngu.mgh.harvard.edu/purcell/plink/) (version 1.07). Warfarin maintenance dose was tested for normality using the Shapiro wilks test, subsequently, comparison of the maintenance dose distribution according to SNPs genotypes was done using the appropriate tests depending on the distribution of the data. Effect size was determined through the Cohen's d, comparing the mean maintenance dose between two groups for each variable, for variables with more than 2 groups the upper and lower groups mean maintenance dose were then compared (e.g., homozygous wild-type and homozygous mutant genotypes were compared for each SNPs).

SNPs that were in the same gene or chromosome were tested for linkage disequilibrium (Supplementary Table S8) using the HaploView software (https://www.broad institute.org/haploview). Subsequently, haplotypes were inferred, their frequency calculated and their association with warfarin maintenance dose determined through a conditional regression test on plink (http://pngu.mgh.harvard.edu/purcell/plink/) (version 1.07). Haplotypes were inferred in genes with either two or more SNPs, which included *APOE* (rs429358 and rs7412), *CALU* (rs339097 and rs1043550), *CYP2B6* (rs3745274 and rs28399499), *GGCX* (rs10179904 and rs2592551), *GLP1R* (rs692376, rs2300615

and rs1042044), *MTFHR* (rs1801131 and rs1801133) and *VKORC1* (rs7294, rs9934438 and rs9923231). Haplotypes were not inferred for *CYP2D6* due to its complicated nature. SNPs and haplotypes with a p≤0.20 for their effect in univariate analysis were entered into a stepwise regression analysis to control for covariates and to determine their cumulative influence on warfarin maintenance dose variability. Variables previously associated with warfarin maintenance dose variability [171] in the studied cohort were also included in the multivariable regression model. To control for missing data and reduce bias, imputation was done on genetic variants that were eligible for the stepwise regression analysis and have missing data using the multivariate imputation by chained equations (MICE) package in R.

3.3.3.3. Results

3.3.3.3.1. Distribution of the studied pharmacogene variants among Black Africans and the Mixed Ancestry group

We present here an analysis of 34 SNPs in 17 genes on their effect on warfarin maintenance dose among black Africans and Mixed Ancestry population groups in Southern Africa. Six genes (i.e*., APOE, F2, GGCX, CYP4F2, CALU* and *VKORC1*) reported here are directly affected by warfarin, whilst the other eleven genes (i.e., *COMT, CYP2B6, CYP2D6, DRD2, GLP1R, MTHFR, F5, OPRM1, PNPLA5, SLCO1B1* and *SULT4A1*) are indirectly involved either in the disposition of warfarin through either pharmacokinetics and pharmacodynamics or other pathways that regulate thrombosis. The participants' clinical, demographic characteristics, genotype and minor allele distribution of the studied SNPs have been reported before [107, 171, 259]. *CYP2D6 g.137-138insT (*15) rs72549357* was monomorphic in both the black Africans and the Mixed Ancestry, whilst *CYP2D6 g.2549delA (*3) (rs35742686), CYP2D6 2613_2615TAG (*9) (rs5030656), CYP2D6 1707delT (*6) (rs5030655), F2 20210G>A (rs1799963), F5 c.1601G>A (rs6025) and OPRM1 c.118A>G (rs179997)* were monomorphic in the black Africans.

3.3.3.3.2. Correlation of SNP genotypes and haplotypes with warfarin weekly maintenance dose

The effect of individual SNPs on the warfarin maintenance dose was evaluated through comparison of the mean warfarin maintenance dose distribution according to SNP genotypes in both black Africans and the Mixed Ancestry (Table 3.14). The effect of *VKORC1 g.-1639G>A* has been reported before [107, 171], and was included here for LD and haplotype analysis with other *VKORC1* SNPs. Among black Africans*, MTHFR c.677C>T (rs1801133)* SNP displayed a significant effect (p=0.04) on the mean warfarin maintenance dose, with an effect size of 0.46 (-0.02 to 0.96). Individuals carrying the *MTHFR c.677C*/C genotype presented with significantly (p=0.04) higher warfarin maintenance dose (38±12 mg/week) compared to the heterozygotes (32±13 mg/week) and the *MTHFR c.677T/T* (17.5 mg/week) (Figure 3.5).

*CYP2D6 g.100C>T (*10) (rs1065852), VKORC1 c.1173C>T (rs9934438)* and *VKORC1 g.9041G>A (rs7294)* were significantly associated (p≤0.05) with warfarin dose requirement in the Mixed Ancestry group with an effect size of 0.59 (0.02 to 1.17), 0.95 (0.55 to 1.35) and -0.89 (-1.28 to -0.50), respectively. Individuals presenting with the homozygous variant genotype for *VKORC1 c.1173 T/T (*25±12 mg/week*)* required a low warfarin maintenance dose compared to heterozygotes (*VKORC1 c.1173* C/T=30±13 mg/week) and homozygous wild-type genotype (*VKORC1 c.1173* C/C=37±13 mg/week) (Figure 3.5). Individuals presenting with the heterozygous *CYP2D6 g.100* C/T required a significantly lower warfarin dose (26±13 mg/week) as compared to the homozygous wild-type genotypes *CYP2D6 g.100* C/C=34±15 mg/week (p=0.04). In contrast, individuals possessing the *VKORC1 g.9041A/A* genotypes presented with a significantly (p=0.0001) higher warfarin dose *(*39±13 mg/week) as compared to individuals carrying the G/G (26±14 mg/week) and G/A (35±13 mg/week) genotypes (Figure 3.5).

Haplotypes were inferred in genes with either two or more SNPs, the haplotype frequencies distribution for the studied SNPs in the respective genes are represented in Table 3.15. *APOE, CALU* and *GGCX* haplotypes were comparable among black Africans and the Mixed Ancestry groups, whilst haplotypes *GLP1R G-T-C* and *VKORC1-G-C-G* occurred at a higher frequency of 0.52 and 0.49 in black Africans as

compared to the 0.24 and 0.23 in the Mixed Ancestry, respectively, whilst haplotypes *GLP1R G-G-C*, *MTFHR C-C* and *VKORC1 G-T-A* occurred at a higher frequencies of 0.25, 0.32 and 0.26, respectively, in Mixed Ancestry compared to 0.10, 0.10 and 0.06 in black Africans, respectively. Haplotypes in the studied genes were further tested for their association with warfarin dose variability through a conditional regression model (Table 3.15). Haplotypes *GLP1R G-G-C* and *MTFHR A-T* were significantly (p=0.02) associated with the warfarin maintenance dose variability among black Africans. Conversely, haplotypes *CALU A-G*, *VKORC1 G-T-A* and *VKORC1 A-C-G* had a significant association with warfarin dose variability (p=0.02, $p=5.678x10^{-8}$ and $p=1.461x10^{-5}$, respectively) in the Mixed Ancestry. However, only the overall *VKORC1* haplotype (inclusive of all haplotypes) had a significant association ($p=9.51x10^{-7}$) with warfarin dose explaining 14% of the variability in the Mixed Ancestry.

The SNPs and/or haplotypes were tested for their cumulative effect on warfarin maintenance dose variability together with previously reported variables [171] through a stepwise-multivariable regression model. Among black Africans, none of the haplotypes contributed to the multivariable regression model, whilst *MTHFR c.677C>T* (rs1801133) became an additional genetic marker that contributed to the final regression model. Thus, improving a previously reported model [171] that included *CYP2C8 c.805A>T (*2), CYP2C9 c.449G>A (*8), CYP2C9 c.1003C>T (*11)*, *CYP2C rs12777823G>A* and *CYP3A5 c.624G>A (*6)* allowing the improvement in the contribution of the model in explaining 34% from a previous 28% of warfarin dose variability. Among the Mixed Ancestry group, *VKORC1 g.-1639G>A* (rs9923231), *VKORC1 c.1173C>T* (rs9934438) and *VKORC1 g.9041G>A* (rs7294) when analysed, the model relied heavily on the contribution of *VKORC1 g.-1639G>A*. Indeed, there is strong linkage between *VKORC1* SNPs*, with VKORC1 c.1173C>T* (rs9934438) and *VKORC1 g.-1639G>A* (rs992323) (D′=0.83, r^2=0.66) in strong LD which is expressed as well with a moderate to high linkage with *VKORC1 g.9041G>A* (rs7294) (D′=0.88, r^2=0.28; D′=0.94, r^2=0.33, respectively) (Figure S2). Therefore, a multivariate regression model that included the *VKORC1* haplotypes and previously reported variables, age and BMI, explained 22% (p=0.0008) of warfarin dose variability among the Mixed Ancestry [171].

Table 3.14: Effects of genetic variation in warfarin-associated pharmacogenes on warfarin maintenance dose among Black Africans and Mixed Ancestry

SNP genotype	Black Africans (N=252)			Mixed Ancestry (N=251)		
	Maintenance dose (mg/week), mean ±SD (range)	**P value**	**Cohen's d (95%CI)**	**Maintenance dose (mg/week), mean ±SD (range)**	**P value**	**Cohen's d (95%CI)**
APOE c.388T>C, rs429358						
TT	37±12 (12.5-70)	0.53	-0.36	31±15 (10-72.5)	0.26	-0.29
TC	35±11(17.5-70)		(-1.19 to 0.47)	35±13 (15-65)		(-0.71 to 0.13)
CC	42±19 (12.5-70)			27.5 (27.5-27.5)		
APOE c.526C>T, rs7412						
CC	35±12 (12.5-70)	0.25	-0.44	32±15 (10-72.5)	0.61	-0.16
CT	39±12 (17.5-70)		(-1.27 to 0.39)	34±12 (15-52.5)		(-0.71 to 0.39)
TT	41±12 (27.5-57.5)			38±4 (35-40)		
COMT c.322G>A, rs4680						
GG	39±13 (17.5-70)	0.44	0.22	34±16 (10-72.5)	0.63	0.29
GA	35±12 (12.5-70)		(-0.37 to 0.80)	33±13 (10-65)		(-0.34 to 0.94)
AA	36±10 (17.5-60)			29 ±11 (15-50)		
CALU g.29809A>G, rs1043550						
AA	36±13 (12.5-70)	0.86	-0.05	34±14 (10-72.5)	0.20	0.38
AG	37±11(17.5-52.5)		(-0.51 to 0.42)	29±12 (10-57.5)		(-0.04 to 0.80)
GG	35 (35-35)			26±11 (12.5-40)		
CALU g.24879A>G, rs339097						
AA	39 ±12 (17.5-70)	0.34	0.31	31±13 (10-72.5)	0.18	-0.56
AG	34 ±13 (12.5-70)		(-0.09 to 0.69)	39 ±15(17.5-70)		(-1.09 to -0.03
GG	38 ±6 (35-45)			32±14 (17.5-50)		
CYP2B6 c.516G>T, rs3745274						
GG	37±12 (12.5-70)	0.95	-0.005	31±15 (10-70)	0.33	-0.36
GT	36±14 (12.7-70)		(-0.51 to 0.49)	32±14 (10-72.5)		(-0.94 to 0.22)
TT	37±16 (17.5-60)			37±13 (15-52.5)		
CYP2B6 c.983T>C, rs28399499						
TT	37±11 (12.5-70)	0.34	0.08	32±14 (10-72.5)	0.81	-0.007
TC	36±14 (17.5-70)		(-0.37 to 0.53)	33±11 (17.5-42.5)		(-0.83 to 0.82)
CC	24 (12.5-35)			-		
CYP2D6 g.2850C>T (*2), rs16947						

CC CT TT	35±12 (12.5-70) 37±12 (17.5-70) 44±12 (35-53)	0.59	-0.13 (-0.59 to 0.34)	33±16 (10-72.5) 30±13 (10-52.5) 39±8 (22.5-50)	0.27	-0.42 (-1.21 to 0.38)
CYP2D6 g.2549delA (*3), rs35742686						
AA A/T	36±12(12.5-70) -	-	-	33±15 (10-72.5) 17.5±4 (15-20)	0.12	1.05 (-0.37 to 2.46)
CYP2D6 g.137-138insT (*15), rs72549357/ rs774671100						
AA	36±12(12.5-70)	-	-	33±15 (10-72.5)	-	-
CYP2D6 g.1846G>A (*4), rs3892097						
GG GA	36±12(12.5-70) 34±16 (17.5-50)	0.83	0.15 (-0.99 to 1.30)	33±15 (10-72.5) 3±13 (15-52.5)	0.67	0.15 (-0.43 to 0.73)
CYP2D6 g.1023C>T (*17), rs28371706						
CC CT TT	36±13(12.5-70) 36±8 (17.5-52.5) 39±13 (17.5-52.5)	0.74	-0.20 (-1.11 to 0.71)	32±15 (10-72.5) 36±9 (30-42.5) 31±19 (17.5-45)	0.87	0.08 (-1.32 to 1.49)
CYP2D6 g. 1659G>A (*29), rs61736512						
GG GA AA	35±11 (12.5-60) 37±13 (17.5-70) 52.5 (52.5-52.5)	0.32	-0.17 (-0.61 to 0.28)	32±15 (10-72.5) 42.5 (42.5-42.5) -	0.44	-
CYP2D6 g.100C>T (*10), rs1065852						
CC CT	36±12(12.5-70) 33±8 (17.5-45)	0.45	0.29 (-0.39 to 0.98)	34±15 (10-72.5) 26±13 (10-52.5)	**0.04**	0.59 (0.02 to 1.17)
CYP2D62988G>A (*41), rs28371725						
GG GA AA	36±11 (17.5-70) 36±17(12.5-60 -	0.97	-0.04 (-0.81 to 0.73)	32±15 (10-72.5) 37±19 (25-70 40 (40-40)	0.73	-0.29 (-1.20 to 0.62)
CYP2D6 2613_2615TAG (*9), rs5030656						
AA TAG/-	36±12 (12.5-70) -	-	-	33±15 (10-72.5) 17.5 (17.5-17.5)	0.29	-
CYP2D6 1707delT (*6), rs5030655						
TT T/-	36±12 (12.5-70) -	-	-	33±15 (10-72.5) 30 (30-30)	0.96	-
CYP4F2 c.1297C>T rs2108622						

GG	42±14(17.5- 75)	0.49	0.08	34±14 (7.5-72.5)	0.74	0.02
GA	38±11 (20- 65)		(-0.62 to 0.78)	32±14 (7.5-72.5)		(-0.34 to 0.37)
AA	41±15 (25-70)			34±13 (10- 72.5)		
DRD2 c.2137G>A (Taq1A), rs1800497						
GG	38±12 (17.5-70)	0.54	0.24	31±15 (15-72.5)	0.62	-0.22
GA	36±12 (12.5-70)		(-0.27 to 0.74)	33±14 (10-65)		(-0.98 to 0.53)
AA	35±13 (12.5-70)			35±14 (17.5-62.5)		
F2 c.*97G>A, rs1799963						
GG	37±12 (12.5-70)	-	-	32±14 (10-72.5)	0.21	-0.61
GA	-			41±5 (35-45)		(-1.77 to 0.54)
F5 c.1601G>A, rs6025						
GG	37±12 (12.5-70)	-	-	32±14 (10-72.5)	0.71	-0.19
GA	-			35 (35-35)		(-1.59 to 1.21)
GGCX c.1218C>T, rs2592551						
CC	41±15 (17.5-75)	0.39	0.17	32±14 (7.5-72.5)	0.27	-0.29
CT	37±13 (22.5-67.5)		(-0.63 to 0.96)	34±14 (7.5-72.5)		(-0.76 to 0.19)
TT	39±6 (32.4-47.5)			36±15 (12.5-62.5)		
GGCX c.1242C>T, rs10179904						
CC	40±14 (17.5-75)	0.05	1.24	34±15 (7.5-72.5)	0.41	0.21
CT	43±14 (20-70)		(0.05 to 2.41)	31±13 (10-72.5)		(-0.10 to 0.53)
CT	23±8 (17.5-32.5)			34±15 (17.5-52.5)		
GLP1R c.780A>C, rs1042044						
AA	38±15 (17.5-70)	0.58	0.10	31±13 (15-52.5)	0.50	-0.30
AC	35±11 (12.5-70)		(-0.46 to 0.67)	32± 14(10-70)		(-0.92 to 0.32)
CC	37±12 (17.5-70)			35±16 (10-72.5)		
GLP1R c.510-1135T>G, rs2300615						
TT	35±12 (12.5-70)	0.16	-0.57	32±13 (15-72.5)	0.76	0.15
TG	42±13 (25-70)		(-1.04 to -0.09)	33±14 (10-70)		(-0.63 to 0.94)
GG	43±21 (27.5-57.5)			30±21 (10-65)		
GLP1R c.502G>A, rs6923761						
GG	37±12 (12.5-70)	0.34	-	32±14 (10-70)	0.70	-0.02
GA	30±7 (25-35)			32±12 (17.5-52.5)		(-0.58 to 0.55)
AA	-			48±35 (22.5-72.5)		
MTHFR c.1298A>C rs1801131						
AA	37±12 (12.5-70)	0.83	0.005	35±15 (10-72.5)	0.23	0.21
AC	37±13 (12.5-70)		(-0.43 to 0.44)	30±13 (12.5-52.5)		(-0.47 to 0.89)
CC	-			32± 18(10-70)		

MTHFR c.677C>T, rs1801133						
CC	38±12 (12.5-70)	0.04	0.46	33±15 (10-72.5)	0.27	0.11
CT	32±13(12.5-60)		(-0.02 to 0.96)	31±13 (10-52-5)		(-0.35 to 0.56)
TT	17.5 (17.5-17.5)			46±2 (45-57.5)		
OPRM1 c.118A>G, rs1799971						
AA	37±12(12.5-70)	-	-	33±14 (10-70)	0.24	0.16
AG	-			31±15 (10-72.5)		(-0.27 to 0.59)
GG	-			15 (15-15)		
PNPLA5 g.C>T, rs5764010						
CC	37±12 (12.5-70)	0.44	0.15	32±14 (10-72.5)	0.38	-0.009
CT	35±13 (17.5-52.5)		(-0.53 to 0.83)	32±15 (10-70)		(-0.50 to 0.48)
TT	25 (25-25)			45±11 (37.5-52.5)		
SLCO1B1 g.37041T>C (*5) rs4149056						
TT	37±12 (12.5-70)	0.34	-	33±14 (10-72.5)	0.46	-0.09
TC	28 (27.5-27.5)			34±14 (17.5-52.5)		(-0.65 to 0.48)
CC	-			17.5 (17.5-17.5)		
SULT4A1 c.*1113A>G, rs763120						
AA	37 ±12 (12.5-70)	0.58	0.16	33±14 (10-72.5)	0.18	0.19
AG	35±12 (17.5-52.5)		(-0.46 to 0.78)	30±16 (10-70)		(-0.31 to 0.69)
GG	-			44±8 (37.5-52.5)		
VKORC1 c.1173C>T, rs9934438						
CC	39±14(12.5-75)	0.59	0.05	37±13 (10-72.5)	0.0001	0.95
CT	38±12(17.5-75)		(-0.31 to 0.41)	30±13 (7.5-70)		(0.55 to 1.35)
TT	50 (50-50)			25±12 (10-62.5)		
VKORC1 g.9041G>A, rs7294						
GG	39 (17.5-70)	0.52	0.12	26±14 (7.5-70)	0.0001	-0.89
GA	37 (17.5-70)		(-0.26 to 0.49)	35±13 (10-72.5)		(-1.28 to -0.50)
AA	37 (12.5-75)			39±13 (17.5-72.5)		

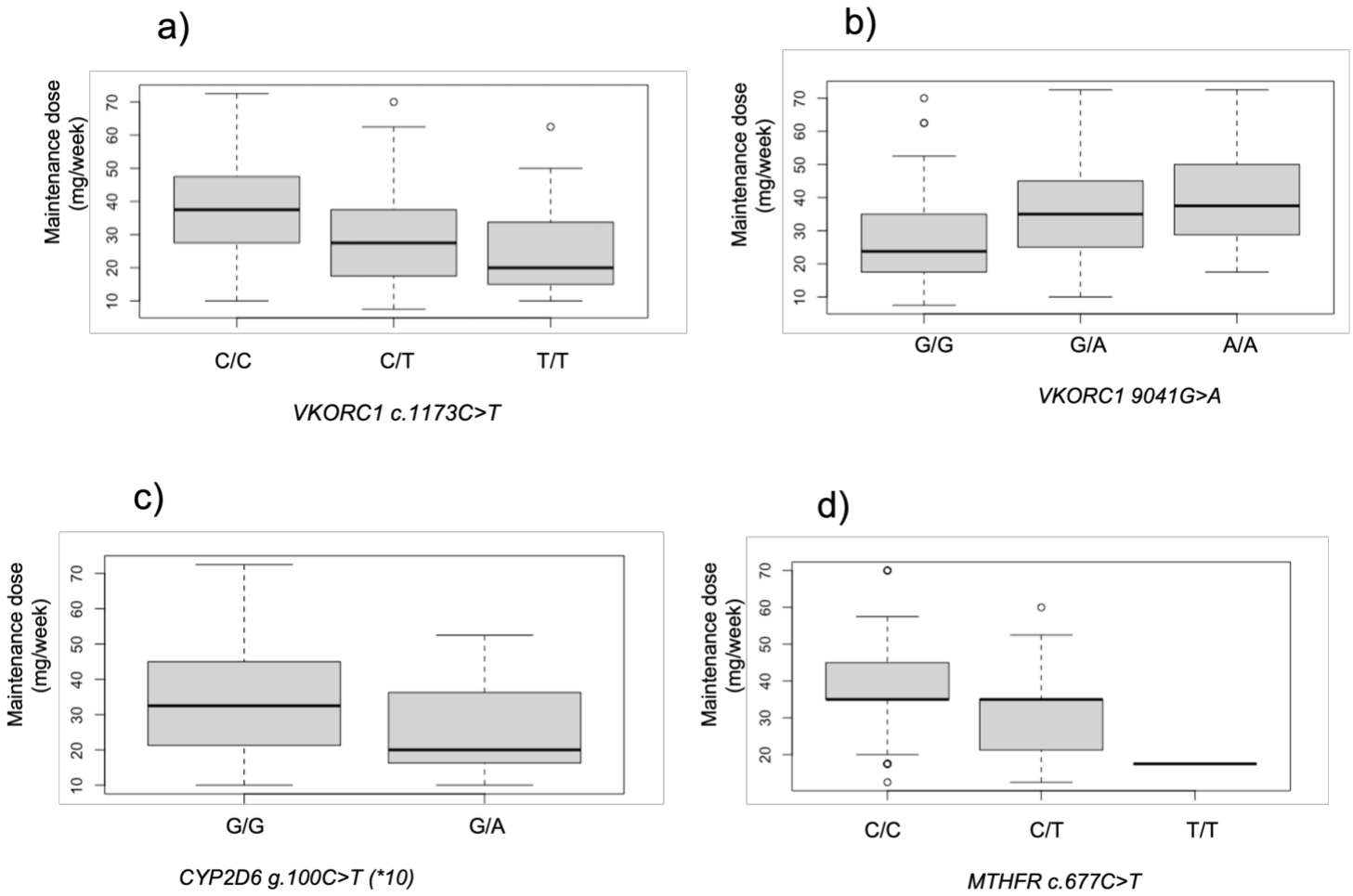

Figure 3.5: Distribution of the warfarin weekly maintenance dose according to genotypes in SNPs: **a)** *VKORC1 c.1173C>T, rs9934438* (p=0.0001) **b**) *VKORC1 G>A, rs7294* (p=0.0001), **c**) *CYP2D6 g.100C>T (*10), rs1065852* (p=0.04) in Mixed Ancestry and **d**) *MTHFR c.677C>T,rs1801133 (p=0.04)* in Black Africans

Table 3.15: Regression model testing for the association of the haplotype groupings on warfarin maintenance dose among Black Africans and Mixed Ancestry

Genes (SNPs IDs)	Haplotype	Black Africans				Mixed Ancestry			
		Freq	Beta	R^2	P value	Freq	Beta	R^2	P value
APOE (rs429358|rs7412)	T-T (ε2)	0.18	3.09	0.02	0.11	0.10	2.41	0.006	0.46
	C-C (ε4)	0.22	0.06	0.000006	0.97	0.18	3.29	0.01	0.25
	T-C (ε3)	0.60	-2.07	0.01	0.18	0.72	-3.33	0.02	0.15
CALU (rs339097|rs1043550)	A-G	0.11	0.38	0.0002	0.89	0.17	-5.85	0.05	0.02
	G-A	0.21	-2.70	0.01	0.22	0.11	3.08	0.01	0.24
	A-A	0.68	1.72	0.008	0.35	0.71	1.58	0.006	0.43
	G-G	-	-	-	-	0.01	5.27	0.006	0.43
CYP2B6 (rs3745274|rs28399499)	G-C	0.11	-2.59	0.009	0.29	0.03	0.09	2.17×10^{-6}	0.99
	T-T	0.39	-0.05	7.75×10^{-6}	0.99	0.33	2.23	0.01	0.26
	G-T	0.50	0.94	0.004	0.51	0.64	-2.09	0.01	0.27
GGCX (rs10179904|rs2592551)	C-T	0.24	-2.29	0.01	0.34	0.26	2.15	0.01	0.15
	T-C	0.12	-3.02	0.01	0.33	0.14	-2.15	0.006	0.27
	C-C	0.64	3.05	0.03	0.14	0.60	-0.71	0.001	0.59
GLP1R (rs6923761|rs2300615|rs1042044)	G-T-A	0.37	-0.08	1.68×10^{-5}	0.96	0.42	-2.39	0.01	0.27
	G-G-C	0.10	5.78	0.04	0.02	0.25	-0.13	3.19×10^{-5}	0.96
	A-T-C	0.01	-3.25	0.004	0.46	0.09	3.17	0.009	0.34
	G-T-C	0.52	-1.7	0.009	0.27	0.24	1.35	0.003	0.57
MTFHR (rs1801131|rs1801133)	T-A	0.08	-6.99	0.05	0.02	0.15	0.83	0.0009	0.77
	G-G	0.10	0.20	0.00005	0.94	0.32	-2.94	0.02	0.18
	T-G	0.82	3.57	0.02	0.09	0.53	2.09	0.01	0.29
VKORC1 (rs7294|rs9934438|rs9923231)	G-T-A	0.06	-0.23	0.00003	0.93	0.26	-7.19	0.11	5.68×10^{-8}
	G-C-A	0.005	-	-	-	0.03	-4.02	0.006	0.23

A-T-G	0.008	-	-	-	0.01	2.54	0.0008	0.65
G-T-G	0.02	0.82	0.0001	0.85	0.02	-6.38	0.009	0.14
A-C-G	0.40	-1.57	0.007	0.19	0.44	5.58	0.07	$1.46x10^{-5}$
G-C-G	0.49	1.14	0.004	0.35	0.23	2.46	0.009	0.12

3.3.3.4. Discussion

There are notable advances on the research and translation of pharmacogenetics of warfarin throughout the years, with Africans coming to the scene [171, 261]. Huge genetic diversity is reported among Africans, thus, mining for genetic markers in African populations to explain warfarin response is of global importance [164]. The trend of mainly targeting candidate genes, in the case of warfarin, *VKORC1* and *CYP2C9* is now shifting to whole genome approaches using genome wide association studies (GWAS) or whole exome/genome sequencing (WES/WGS). Our study [171] demonstrated the importance of investigating a wider array of variants and widening the populations studied, in order for equitable implementation of pharmacogenetics to be realised. Extending on our previous work, this study evaluated additional genes, some secondarily associated with warfarin disposition.

The current study adds on already presented data for 26 other genetic markers among Africans [171] by reporting on six important warfarin pharmacodynamic relevant genes, namely *VKORC1, CYP4F2, GGCX, F2, CALU* and *APOE*. However, only *VKORC1* SNPs had a significant association with warfarin maintenance dose requirement exclusively in the Mixed Ancestry group. The *VKORC1* SNPs (*VKORC1 g.-1639G>A, VKORC1 c.1173C>T* and *VKORC1 g.9041G>A)* reported here, have been extensively investigated in the context of warfarin among various population groups [57, 61, 109, 111, 115, 136]. The effect of *VKORC1 g.-1639G>A* and *VKORC1 c.1173C>T o*n warfarin dose requirement, has been consistent in the various reported studies and effects have been reported across global populations [48, 86, 132, 109, 115, 171]. The two *VKORC1* SNPs *(VKORC1 g.-1639G>A* and *VKORC1 c.1173C>T)* are in strong LD [61], which is supported by our results (Supplementary Figure S2).

The association of *VKORC1 g.9041G>A* with increased warfarin maintenance dose requirement reported here, among the Mixed Ancestry cohort, is consistent with previous reports in Europeans and Sudanese [61, 109]. However, among black Africans reports have been inconsistent, as *VKORC1 g.9041A* variant was associated with increased warfarin dose requirement in a study that included black South Africans [111], but no significant association was observed in studies that included African Americans [57, 137]. The lack of association between *VKORC1 g.9041G>A* and warfarin dose requirement observed among African Americans [57, 137] is consistent with our current findings in the black African cohort. Our study further confirmed reports by Limdi et al [132] which showed that the inclusion of other *VKORC1* SNPs or haplotype does not improve a regression model that includes *VKORC1 g.-1639G>A.* This was demonstrated when *VKORC1 g.-1639G>A, VKORC1 c.1173C>T* and *VKORC1 g.9041G>A* were included in a regression model and *VKORC1 g.-1639G>*A was the only genetic marker that remained significant. Furthermore, when the *VKORC1* haplotype was entered in a regression model it had the same effect and explained the same variability as *VKORC1 g.-1639G>A* [171].

In addition to *VKORC1* markers, genetic variants in *CYP4F2, APOE, GGCX* and *CALU* are hypothesised to have an effect in the prediction of warfarin dose requirement as these genes seem to play important roles in the warfarin pharmacodynamic pathway. With regards to *CYP4F2*, Caldwell et al [83] reported on SNP *CYP4F2* c.1297C>T (*V433M,* rs2108622) and that variant rs2108622T is associated with increased warfarin dose requirements. Furthermore, individuals with homozygous T/T genotype require a median warfarin maintenance dose of 1 mg/day higher than those harboring the wild type C/C genotype. This has driven the *CYP4F2* to be one (together with *CYP2C9* and *VKORC1*) of the highly recommended marker for the prediction of warfarin starting doses [45, 261]. However, in our study *CYP4F2* rs2108622 did not have any significant association with warfarin maintenance dose in both black Africans and Mixed Ancestry cohorts, which is in agreement with reports in other African population where *CYP4F2* rs2108622 has not been shown to affect warfarin dose requirements [47, 115, 262, 263].

APOE plays a role in the uptake of chylomicron bound vitamin K to the liver, where the latter acts as a cofactor for the activation of specific coagulation factors [36].

Three major *APOE* isoforms (i.e., ε2, ε3 and ε4) have been described and are derived from the combination of two SNPs, *APOE c.388T>C (rs429358)* and *APOE c.526C>T (rs7412)* [264]. The three *APOE* isoforms result in varying APOE binding affinity to the low-density lipoprotein receptor, thus influencing the rate of chylomicron clearance. *APO-ε2* presents with low binding affinity which leads to reduced clearance rate while ε3 and ε4 present with higher binding affinity which supports fast clearance [264]. Based on the differing binding affinities and clearance rates for the *APOE* isoforms possess, there is no doubt that it is likely to manifest as differences in the uptake of vitamin K, which eventually affects the warfarin dose requirements.

Although, the effects that *APOE* SNPs have on warfarin dose requirement has been inconsistent, our present findings are in agreement with reports from previous studies among Italians, Caucasian Americans, African Americans, Iranian and Jordanians, of minimal or no significant role for *APOE* SNPs on warfarin dose requirements [57, 142, 265-267]. However, our findings contrast with reports among African Americans, that reported ε4 carriers to require a significantly higher warfarin dose than non-carriers owing to the increased binding affinity [142]. In contrast, Li et al [256] among Chinese patients reported that *APOE rs7412*, CC wild-type carriers (2.82 ±1.14 mg/d) required significant higher warfarin dose than CT and TT genotype carriers (2.49 ±0.64 and 2.13 ±0.34 mg/d, $p<0.05$, respectively), whilst de Oliveira Almeida et al [268] reported that *ε4* carriers (41.5 ±18.5) required 21% lower warfarin dose as compared to non-*ε4* carriers (52.5 ±37.5 mg) ($p=0.038$) among Brazilian patients.

Gamma-glutamyl carboxylase encoded by *GGCX* participates in the vitamin K cycle through carboxylation of glutamate residues of vitamin K-dependent coagulation proteins needed for their activation [34]. Thus, SNPs in *GGCX* have been investigated for their effect on warfarin dosing in different population groups [119, 269-271]. For the first time in a native African cohort, we evaluated the effect of *GGCX c.1218C>T* (rs2592551) and *GGCX c.1242C>T* (rs10179904) on warfarin maintenance dose. We report the presence of genetic variants but no significant association with warfarin maintenance dose. Our findings on the two *GGCX* SNPs are consistent with reports among Europeans, Japanese and Africans Americans [137, 269, 270]. However, our findings contradict what Kamali et al [272] reported on

GGCX rs2592551 where the rs2592551T variant was significantly associated with increased warfarin dose requirements among Chinese atrial fibrillation patients. The actions of *GGCX* are reported to be regulated through inhibition by a molecular chaperone calumenin which is encoded by *CALU* [35]. Thus, it has been suggested that SNPs that regulate the action of calumenin could be associated with increased warfarin dose requirement owing to the inhibitory effect calumenin has on GGCX [35].

The two *CALU* SNPs (i.e., CALU *g.24879A>G (*rs339097*)* and *g.29809A>G* (rs1043550)) investigated in our cohort did not show any significant associations with warfarin maintenance dose among both the black Africans and the Mixed Ancestry cohorts. Interestingly, in our cohort, when *CALU* rs339097-rs1043550 haplotypes were evaluated on their effects on warfarin maintenance dose variability, haplotype A-G was statistically significantly (p=0.02) associated with negative effect (beta coefficient=-5.85) on warfarin dose, explaining 5% of the variability among the Mixed Ancestry cohort. Although the functional effects of *CALU g.24879A>G* and *g.29809A>G* have not been well defined, previous studies have suggested that the G allele for the intronic *CALU g.24879A>G* result in higher expression of *CALU* thereby resulting in higher warfarin dose requirement as compared to the individuals carrying the A allele [115, 141], whilst the G allele of the 3'-UTR *CALU g.29809A>G* results in a lower mRNA stability as compared to the A allele resulting in low expression of *CALU* which may results in reduced warfarin dose requirement [273]. Therefore, the combined effects of *CALU g.24879A* and *g.29809G* could possibly lead to a reduced warfarin dose requirement, corroborating the low warfarin dose variability associated with the *CALU* A-G haplotype observed here in the Mixed Ancestry cohort.

The vitamin K cycle is essential for the activation of procoagulant factors II (prothrombin), VII, IX, and X as well as antithrombotic protein C and protein S. Thus, the action of warfarin ultimately alters the normal functioning of these proteins which could also suggest that variation in genes encoding the vitamin K-dependent proteins might have an influence on warfarin dose variability. In the present study we evaluated polymorphisms in vitamin-K dependent coagulant factor II (*F2 c.*97G>A,* rs1799963) and coagulant factor V Leiden (*F5 c.1601G>A*, rs6025) on their effect on warfarin dose requirement. SNPs *F2* rs17999630 and *F5* rs6025 have been reported

before to highly increase the risk of thrombotic events by three to fivefold [274]. However, the assumption that the influence of *F2 c.*97G>A* and *F5 c.1601G>A* have on thrombotic manifestation will subsequently affect warfarin dose variability remains to be determined, as a previous study [275] could not find any association between these SNPs and warfarin dose requirements, which is consistent with our findings.

Interestingly, we report on significant effect *of MTHFR c.677C>T* (rs1801133) SNP on warfarin maintenance dose requirement among black Africans, which improved a previous multivariate regression model we reported before [171] by 6%. The *MTHFR* gene encodes the methylenetetrahydrofolate reductase, an enzyme responsible for the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a cofactor for homocysteine re-methylation to methionine [276]. Thus, deficiency in the MTHFR enzyme results in elevated levels of homocysteine consequently leading to venous thrombosis [277]. There are contradictory reports on the role of *MTHFR c.677C>T (*rs1801133) SNP on warfarin dosing, although it is known that the variant allele leads to almost 50% reduction in the activity of MTHFR enzyme, which has been suspected to lead to thrombotic events due to hyperhomocysteinemia [274, 276, 278]. Our results paint a different picture when the expected functional effects of the mutant variant are taken into account, where one would expect carriers to require higher dose of warfarin compared to non-carriers, but we report reduced warfarin maintenance dose requirements. It could be that our study's end point is maintenance dose, and this variant could still have a different effect on dose required to reach therapeutic range, or our results could suggest that *MTHFR* may be influenced by other factors. For instance, the effect of reduced MTHFR activity on homocysteine levels is reported to be dependent on folate intake [279], thus, homocysteine levels are about 25% higher in *MTHFR c.677T* carriers only when plasma folate concentration is low [279]. Furthermore, León-Cachón et al [280] reported that *MTHFR c.677T* allele augments the clearance of atorvastatin but the mechanism in which this variant influence drug response is yet to be determined in both atorvastatin and warfarin.

Previously, we reported [259] on the importance of evaluating the genetic markers that interact with other drugs that are concurrently prescribed with warfarin resulting in drug-drug interactions. In addition to polymorphisms in warfarin-pharmacodynamic related genes, we also investigated effects of genetic markers in other

pharmacogenes that do not directly affect warfarin disposition, which included *CYP2B6, CYP2D6, SLCO1B1* and *SULT4A1*. A statistically significant association with warfarin maintenance dose was observed for *CYP2D6 g.100C>T (*10) (rs1065852) SNP,* whose variant was significantly associated with reduced warfarin dose requirement in the Mixed Ancestry group. The SNP *CYP2D6* rs1065852 is known to be common among populations of Asian Ancestry and affects the metabolism of several drugs including tamoxifen [281, 282]. The presence of the *CYP2D6*10* allele among the Mixed Ancestry group is further confirmation of the historical descendance of the Mixed Ancestry population of ancestry from Africans, Europeans and Asians. To our knowledge this is the first study to find an association between *CYP2D6 g.100C>T (*10)* and warfarin dose requirements. Thus, the mechanism in which *CYP2D6 g.100C>T (*10)* affect warfarin dose requirement needs to be further investigated.

3.3.3.5. Conclusion

VKORC1 is a major genetic predictor for warfarin dose requirement among Mixed Ancestry, which aligns with reports from Europeans which have described *VKORC1* markers to contribute approximately 15–34% of warfarin dose variability. Although, our study was only limited to three *VKORC1* SNPs, the lack of association between the studied *VKORC1* SNPs and warfarin dose requirement among black Africans calls for additional *VKORC1* variants to be evaluated as it is the principal gene targeted by warfarin. Furthermore, expanding on a repertoire of genes beyond those directly linked with warfarin is imperative in decoding more African specific genetic variants that are important in explaining warfarin dose variability. This is in reference to the *MTHFR c.677C>T* which affects warfarin dose requirements but is not directly linked to the warfarin interactive pathway. Thus, we propose the *MTHFR c.677C>T* to be considered for an African specific warfarin dosing algorithm in addition to the *VKORC1* markers, *CYP2C8 c.805A>T (*2), CYP2C9 c.449G>A (*8), CYP2C9 c.1003C>T (*11), CYP2C rs12777823G>A* and *CYP3A5 c.624G>A (*6)* that we reported before.

3.4. Identification of additional known and novel pharmacogene variants through the application of whole exome sequencing (WES) analysis

Synopsis: This section presents data on an objective that seeks to identify additional known and possible novel/unknown pharmacogene variants in the black African study population. The search for additional known and novel variants was done through whole exome sequence analysis of data generated from individuals presenting with extreme warfarin maintenance dose requirements (i.e., either very low or high warfarin dose) but who do not harbor any known warfarin genetic predictors. The main purpose for this objective was to screen for important warfarin genetic predictors that could have been missed through the candidate variant approach presented on the previous sections. An unpublished manuscript has been included to respond to this section's objective.

3.4.1. Application of Whole Exome Sequencing (WES): in Search of Informative African-Specific Pharmacogenetic Profiles for Warfarin

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Nature of Manuscript: Original research article

Candidate's Contribution: conceptualised the idea, conducted experimental activities, data analysis, drafted the manuscript and incorporated changes from co-authors and reviewers.

Co-Authors Contribution:

KE: Conceptualised the idea and data analysis.

SM: Collected biological data.

AW and MN: Co-supervised all components and reviewed the manuscript draft.

CD: Conceptualised the ideas, supervised all components and reviewed the manuscript drafts.

All authors contributed to the final version of the article. The authors read and approved the final manuscript.

Application of Whole Exome Sequencing (WES) in Search of Informative African-Specific Pharmacogenetic Variants for Warfarin

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Abstract

Pharmacogenetics has become a field of interest worldwide in unravelling the observed inter-individual variability in response to therapeutic treatments, which often result in increased risks of adverse drug reactions. Although, the field of pharmacogenetics is swiftly advancing culminating in the development of pharmacogenetic guidelines for various drugs, it is anticipated that additional variants which could improve the available guidelines are yet to be decoded, especially in genetically diverse populations such as Africa. The application of NGS has been proposed to be a better approach in comprehensively analysing informative variants, thus promoting advancement of pharmacogenetics, and

accelerating its integration clinically. The study aims to carry out an in-depth analysis of various genetic regions by employing whole exome sequencing on selected black African participants presenting with extreme warfarin-related phenotypes and further compare their genome architecture with that of other world population groups. A total of 8 black African individuals (males and females) presenting with extreme phenotype (i.e., either very low or very high warfarin dose) and not carrying the *CYP2C9 or VKORC1* variants previously associated with warfarin dose were selected and analysed though whole exome sequencing (WES). WES was carried out using the ion torrent Sequence ion S5 system, subsequently a workflow which applied various bioinformatics tools was employed for the analyses of the resultant raw BAM files. Principal component analysis (PCA) and allele frequency distribution patterns were conducted among our cohort and individuals in the 1000 Genomes project to assess the population structure and pharmacogenetic variation, respectively. Genetic characterisation and association with warfarin dose requirements were carried out in a cohort of 252 black Africans for variants prioritised. Over 90% of variants identified among black Africans in Southern Africa across 10 extracted actionable genes were present in all African population groups from the 1000 Genome, whilst approximately 40% of variants were completely absent in either Hispanic Americans, Europeans, East Asian or South Asians. *PROC c.423G>T* (rs5936) and *EPHX1 g.26978G>C* (rs2260863) were significantly associated (p=0.04) with warfarin dose requirements, displaying a recessive and codominant effect, respectively. Thus, contributing a 6% warfarin dose variability on a previously reported multivariate regression model. Africans present with unique pharmacogenetic markers which are informative in the development of African specific dosing guidelines. Thus, the application of NGS data offers an opportunity for a wide variety of informative pharmacogenetic profiles to be captured, further accelerating the integration of pharmacogenetic clinically.

Keywords: Warfarin, whole exome sequencing, pharmacogenetics, Africans, next generation sequencing

3.4.1.1. Introduction

Pharmacogenetics has become a field of interest worldwide in unravelling the observed inter-individual variability on the response to therapeutic treatments, which

often result in increased risks of adverse drug reactions [283, 284]. Various pharmacogenetics studies have revealed that the inter-individual variability in drug response is driven by variants in genes that encode enzymes affecting the drug's pharmacokinetics which is comprised of drug absorption, distribution, metabolism, and excretion (ADME) [285, 286], also proteins targeted by the drug which are those driving the pharmacodynamic effects [286, 287]. The effects of the various genetic markers on drug response are further exacerbated by the various environmental, demographic, pathophysiology, drug-drug and herb-drug interactions [188, 288]. This information has driven the development of pharmacogenetic guidelines according to the various drug's actionable genetic variants, an initiative by the Clinical Pharmacogenetics Implementation Consortium (CPIC) [172, 289].

Although, the field of pharmacogenetics has taken off on a positive trajectory and steadily advancing, it is anticipated that additional variants which could improve the available data and guidelines are yet to be decoded, as well as make it easier for pharmacogenetics to be integrated clinically [69, 290, 291]. Particularly in low- and middle-income settings and diverse populations such as African where studies that have applied pharmacogenetics are very limited and the available guidelines are not as informative, as they have been developed using data from other population groups such as Europeans [292, 293]. Furthermore, most of the pharmacogenetic studies undertaken in African populations, especially in the context of warfarin, apply the candidate gene/variants approach utilising conventional quantitative PCR, primer extension methods and Sanger sequencing [292, 294, 295]. Thus, hindering the in-depth analysis of various genetic regions that could be highly informative in the application of pharmacogenetics. With the advancement in technology, application of next generation sequencing (NGS) platforms has been proposed as a better approach in comprehensively analysing informative variants in the application and advancement of pharmacogenetics [296-298].

NGS allows for an in-depth analysis of a wide range of genetic regions either through whole genome sequencing (WGS) which allows analysis of the entire genome, whole exome sequencing (WES) which targets the genome's coding regions or targeted sequencing through multigene panels [299-302]. NGS technologies in pharmacogenetic studies have mainly been applied through targeted sequencing of

ADME and drug target genes utilising the custom capture-based pharmacogenetic panels/arrays [303-306]. However, African populations are rarely represented in these NGS-based pharmacogenetics studies, which suggest that there is a vast majority of an African genome that has not been decoded in the pharmacogenetics context [293, 294, 307]. Thus, delaying the integration of pharmacogenetics clinically and the development of African specific pharmacogenetic-based dosing algorithms [292].

Recently, we applied the iPlex PGx74 Massarray panel in our black Africans and Mixed Ancestry warfarin cohorts and reported the targeted genetic markers frequency distribution and effects on warfarin dose variability [171, 259]. However, there were individuals that presented with extreme phenotypes (i.e., very high or very low warfarin dose) but who did not harbour any variants that explained warfarin dose variability in black Africans. Thus, in an effort to screen for additional informative African-specific pharmacogenetic markers not captured through the candidate variant approach and describe the African genome in the context of pharmacogenetics. The study aims to carry out an in-depth analysis of various genetic regions by employing whole exome sequencing on selected black African participants presenting with extreme warfarin-related phenotypes and further compare their genome architecture with that of other world population groups.

3.4.1.2. Methods

3.4.1.2.1. Study participants and whole exome sequencing

Ethical approval for the study was granted by the University of Cape Town Human Research Ethics Committee (UCT-HREC REF No. 581/2015). Sample and data collection for the study participants has been described before [107]. A database with study participants' demographic, clinical and treatment outcome information was evaluated. Subsequently, 8 black African individuals (males and females) were selected for whole exome sequencing (WES) based on the following inclusion criteria; presented with extreme phenotype (i.e., either very low or very high warfarin dose), do not carry the *CYP2C9*2, CYP2C9*3*, *VKORC1 g.-1639A* or any variants that we previously [107, 171] associated with warfarin maintenance dose. Genomic DNA (gDNA) (20ul of 50ng/ul) of selected individuals was sent to the Central Analytical Facilities DNA Sequencing unit at Stellenbosch University in the Western

Cape Province, South Africa, for WES with the ion torrent Sequence ion S5 system. Quality control for the gDNA was done by evaluating the Genome Quality Score (GQS) determined through electrophoresis using the PerkinElmer LabChip and evaluation of the gDNA concentration using both the nanodrop spectrophotometer and qubit fluorometer. Libraries were then constructed for all samples that passed quality control; the resultant libraries were then loaded onto the Sequence Ion S5 540 chip for WES. Quality check for the post sequencing reads was done and processed as raw BAM files using the Torrent Suite software.

3.4.1.2.2. Whole-exome sequencing data analysis

3.4.1.2.2.1. Quality check and alignment

Computational analyses were performed using facilities provided by the University of Cape Town's ICTS High Performance Computing team (hpc.uct.ac.za). A workflow which applied various bioinformatics tools was employed for the analyses of the raw BAM files (Figure 3.6). For quality control and preprocessing of the raw reads, the raw BAM files were sorted and transformed to fastq files using samtools software (version 1.9). Quality control of the fastq files was performed using the FASTX toolkit (version 0.0.6) (http://hannonlab.cshl.edu/fastx_toolkit/commandline.html). First, the FASTQC (version 0.11.9) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and MULTIQC (version 1.9) (cite https://academic.oup.com/bioinformatics/article/32/19/3047/2196507) packages were used to check the initial reads quality to inform the FASTX filtering parameters. FASTX was then used to clip the IonTorrent adapters and trim the reads by removing the five base pairs (bp) in the 5' end, retaining only reads with a minimum length of 36 bp and maximum length of 350 bp, as well as to filter the reads by retaining only reads for which at least 90% of the bases have minimum quality score of 12.

The Torrent Mapping Alignment Program (TMAP: version 3.4.0) software (https://github.com/iontorrent/TMAP) was then used to align the reads to the human reference genome in build 38 (hg38) coordinates using the recommended default settings of the mapall command. The resulting BAM files were sorted according to chromosome and base pair position (coordinate) using the samtools software (version 1.9) (https://github.com/samtools/samtools), and then FASTQC and

MULTIQC were used again to assess the reads quality. Duplicate reads were marked using the GATK (version 4.2.5.0) (https://github.com/broadinstitute/gatk) MarkDuplicates command, and base quality scores were recalibrated using the GATK BQSRPipelineSpark command. After base quality score recalibration, the reads qualities were checked again using FASTQC and MULTIQC. The QC metrics of the raw FASTQ, after filtering the FASTQ reads and after alignment and recalibration of base quality scores are outlined in Supplementary Table S9.1 to S9.3 and Figure S3.1 to S9.3.

3.4.1.2.2.2. Variant calling, filtering, phasing, and annotation

Variant calling was performed using DeepVariant version 1.3.0 (https://github.com/google/deepvariant) for single-sample variant calling and GLNexus version 1.4.3 (https://github.com/dnanexus-rnd/GLnexus) for joint variant calling (https://academic.oup.com/bioinformatics/article/36/24/5582/6064144). Variant filtering was performed based on four tunable parameters implemented within GLNexus following extensive benchmarking. Additional filtering involved excluding variants with read depth (DP) <10 and genotype quality (GQ) <20. The variants were then left-normalised using the bcftools norm command and subsequently phased and annotated for the respective downstream analyses. Each chromosome was phased against the 1000 Genomes reference haplotype panel in build 38 coordinates using the EAGLE software (version 2.4.2) (cite https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5096458/), whilst variant annotation was performed using the ANNOVAR software (cite: https://academic.oup.com/nar/article/38/16/e164/1749458).

3.4.1.2.3. Population structure and *allele frequency distribution analyses*

Principal component analysis (PCA) was performed among our cohort and individuals from Zimbabwe, as well as among individuals in the 1000 Genomes project to assess the clustering of our cohort relative to global populations. Firstly, genotype data of the Zimbabwe individuals was obtained from another project in our group and phased with the EAGLE software and then merged with our cohort to retain only variants that were present in both cohorts. The merged data set was then merged with the 1000 Genomes phased data set, again retaining only variants that were present in the two data sets. This was achieved using the bcftools merge

command. Thereafter, additional variant filtration was performed using PLINK2 (cite https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4342193/) to exclude variants with minor allele frequency <1%, variants with genotype call rate <95%, variants that failed the Hardy-Weinberg equilibrium (HWE) test at a p-value threshold of $1x10^{-4}$, and variants with linkage disequilibrium >0.2.

The PLINK2 --pca command was then used to compute five (5) principal components (PCs) and a custom R program was used for plotting the first two PCs while coloring by the individual ancestry. To compare the variant frequency distribution patterns between our Southern Africa cohort (black South African and Zimbabwean) with other global populations, variants in nine actionable pharmacogenes as described by US-FDA (https://www.fda.gov/medical-devices/precision-medicine/table-pharmacogenetic-associations#updates) and CPIC (https://cpicpgx.org/), were extracted from the merged data using coordinates retrieved from the ENSEMBL database and then allele frequencies computed using the PLINK2 --freq command. Histograms of the allele frequencies across the populations were then generated for visualisation.

3.4.1.2.4. Variant prioritisation, validation, and replication of identified variants

Variants were prioritised according to clinical significance and variant pathogenicity predictions which were based on the SIFT, PolyPhen2, mutation assessor, mutation taster, MetaLR, CADD, and LR algorithmic scores. All variants with clinical significance classification of drug response were extracted into a separate file and all “Pathogenic” or “Likely Pathogenic” variants were also extracted into a separate file. Additionally, unknown coding variants predicted by several pathogenicity prediction algorithms to have a high pathogenic consequence and deleterious were extracted in a separate file, so as intronic/splicing unknown variants predicted by regSNP-intron to be either damaging or possibly damaging. Subsequently, Gene enrichment and network pathways were then assessed in genes of prioritised variants using online web-based tools Enrichr (https://maayanlab.cloud/Enrichr/) and string pathway (https://string-db.org/).

Based on the enrichment analysis of the prioritised genes, variants were selected for validation in a previously described cohort of 252 black Africans [171, 259]. The selected variants were genetically characterised using either PCR-RFLP, Taqman SNP genotyping assay or Sanger sequencing accordingly. Oligonucleotides for the characterisation of the selected variants were designed using the IDT primer quest tool (https://eu.idtdna.com/ PrimerQuest/Home/Index) and blasted using the NCBI blast tool (www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_ LOC=BlastDescAd), subsequently, restriction enzymes for the respective variants were designed using the online tool NEBcutter (http://nc2.neb.com/NEBcutter2/). The detailed characterisation methods, oligonucleotides, restriction enzymes and Taqman assay IDs for the respective variants are outlined in Supplementary Table S10.

Statistical analysis for the characterised SNPs and resultant haplotypes was subsequently conducted using various statistical packages in R (version 4.0.3 [2020–10–10]) and plink (http://pngu.mgh.harvard.edu/purcell/plink/). The variants were tested for their effect on warfarin dose requirements through comparison of the warfarin weekly maintenance dose distribution according to genotypes. Additionally, haplotypes were inferred for SNPs in the same chromosome or gene, and subsequently associated with warfarin maintenance dose variability through a conditional regression analysis in plink. *VKORC1* and CYP2C cluster haplotypes also included SNPs described in section 3.3 of the thesis (i.e., *VKORC1* rs9923231, rs9934438 and rs7294) and (CYP2C rs12777823, rs1799853, rs7900194, rs28371685, rs1057910 and rs11572103). Subsequently, SNPs and haplotypes with a univariate effect p value of ≤0.20 were entered into a multivariate regression analysis to determine their cumulative effect with statistically significant variables that have been previously reported.

3.4.1.3. Results

3.4.1.3.1. Identifications of sequenced variants, population structure and variant distribution

From a total of 8 participants (three with low warfarin dose and five with higher warfarin dose) whose genome underwent whole exome sequencing, a total of 220312 single nucleotide variants (SNVs) (including insertion and deletions (indels))

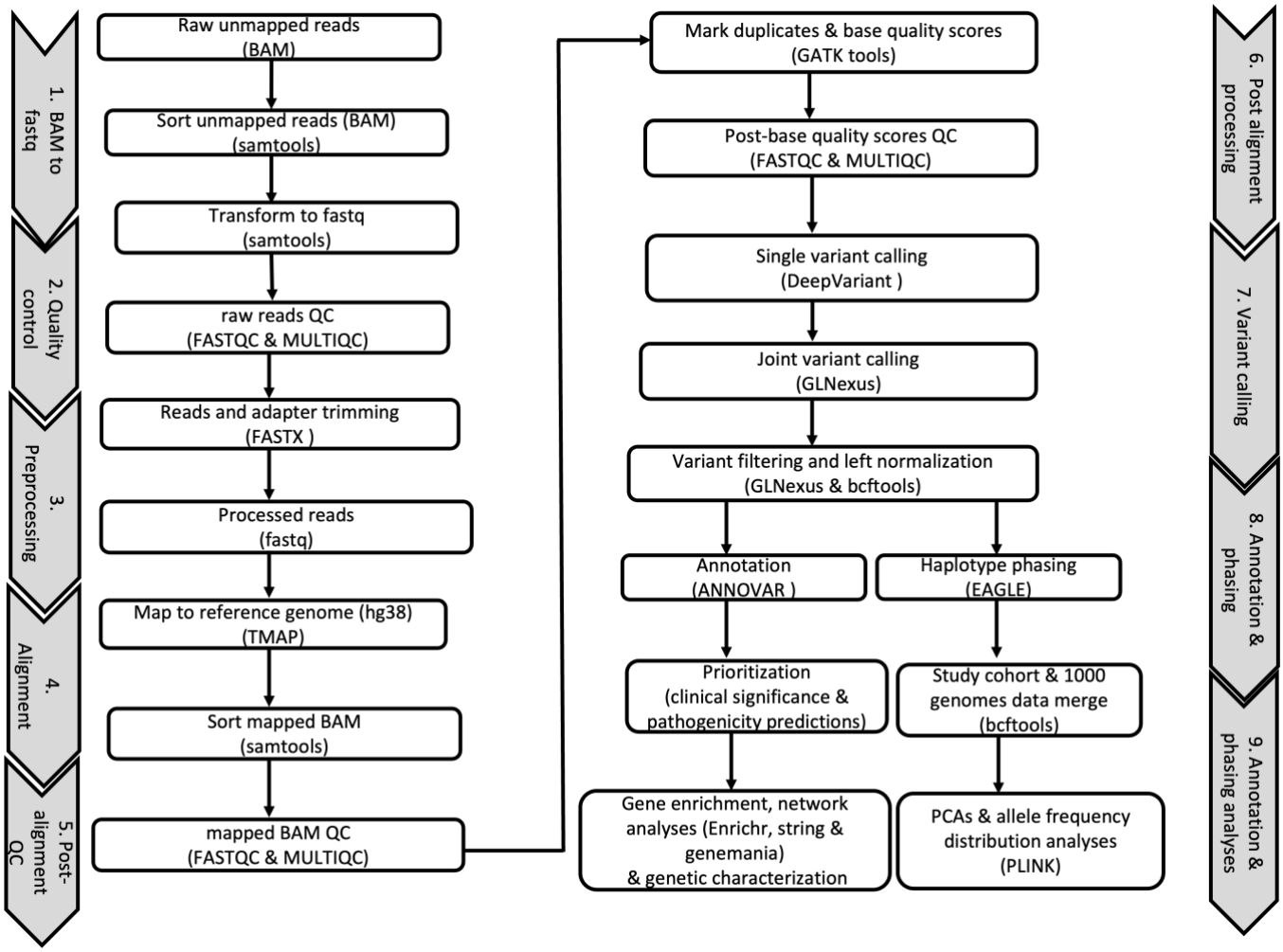

Figure 3.6: Bioinformatics workflow employed for whole exome sequencing data analysis

were successfully called and filtered according to read depth (i.e., DP ≥8), and genotype quality (i.e., GQ ≥20). Among the 220312 variants identified, 9261 were identified in various groups of pharmacogenes and their distribution were as follows: phase 1 pharmacogenes=739, phase 2 pharmacogenes=1124, transporters=6326 and other pharmacogenes=1072 variants (Supplementary Figure S10a). The distribution of the known and unknown variants across the various pharmacogenes is illustrated in Supplementary Figure S10b. All variants identified (pharmacogenes and non-pharmacogenes variants) underwent phasing and those similar between our black South African cohort and Zimbabwean cohort from another study in our group (included here to enhance the sample size for population genetics analyses) were merged with the 1000 Genomes project and principal component analysis (PCA) was done for population structure evaluation.

The PCA revealed relatedness among various African populations with black South African clustered together with other African populations from the 1000 genomes project (Figure 3.7). This was further confirmed by the varying allele frequency distribution patterns of the extracted 49 variants in 10 actionable pharmacogenes (i.e., *CYP2B6, CYP2C9, CYP2C19, NUDT15, CYP3A5, CYP4F2, NAT2, SLCO1B1, UGT1A1* and *VKORC1*) when African populations (i.e., our black African cohorts in Southern Africa, Kenyans (Luhya), Nigerians (Yoruba) and Gambians) were compared with Hispanic Americans, Europeans, East and South Asians (Figure 3.8A and B). For instance, over 90% of the variants that were identified among black Africans (our study) across all 10 actionable genes were also present in all other African population groups from the 1000 genomes project, whilst approximately 40% of variants which included *CYP2B6* rs28399499, rs34749331*, CYP2C9* rs2256871*, CYP2C19* rs17884712, *CYP3A5* rs41303343, rs546268184*,* rs531042654, rs200312875, rs8175345 and *NAT2* rs12720065 were either rare or absent in either Hispanic Americans, Europeans, East Asians or South Asians.

3.4.1.3.2. Prioritisation, gene enrichment and network analyses

Following filtration of variants according to clinical significance, a total of 43 genes were extracted from a list of 71 variants predicted to have a drug response significance, whilst 191 genes were extracted from a list of 207 variants predicted to be pathogenic. Subsequently, the extracted genes both from the drug response and pathogenic list were entered into a gene enrichment and gene-gene network analyses. The Enrichr analysis for the drug response gene list revealed significant association of *VKORC1, CYP2C9, CYP4F2* and *CDHR3* genes with anticoagulant and warfarin by the PhenGenI Association 2021 (OR= 57.16; p= 0.000001) and dbGaP (OR= 973.32; p= 3.342×10^{-10};) respectively, whilst the GWAS catalog (OR= 285.00; p=5.913×10^{-7}) significantly associated only *VKORC1, CYP2C9* and *CYP4F2* with the warfarin maintenance dose. Additionally, *ABCB1* and *NQO1* were significantly associated with warfarin resistance using String pathway's disease-gene associations catalog (p= 0.00034), together with *VKORC1* and *CYP4F2*. Genes that had either a co-expression, co-localization, genetic or physical interactions with *CYP2C9* and *VKORC1* included*, EPHX1, APOB, PROC* and *CYP4F2* (Figure 3.9).

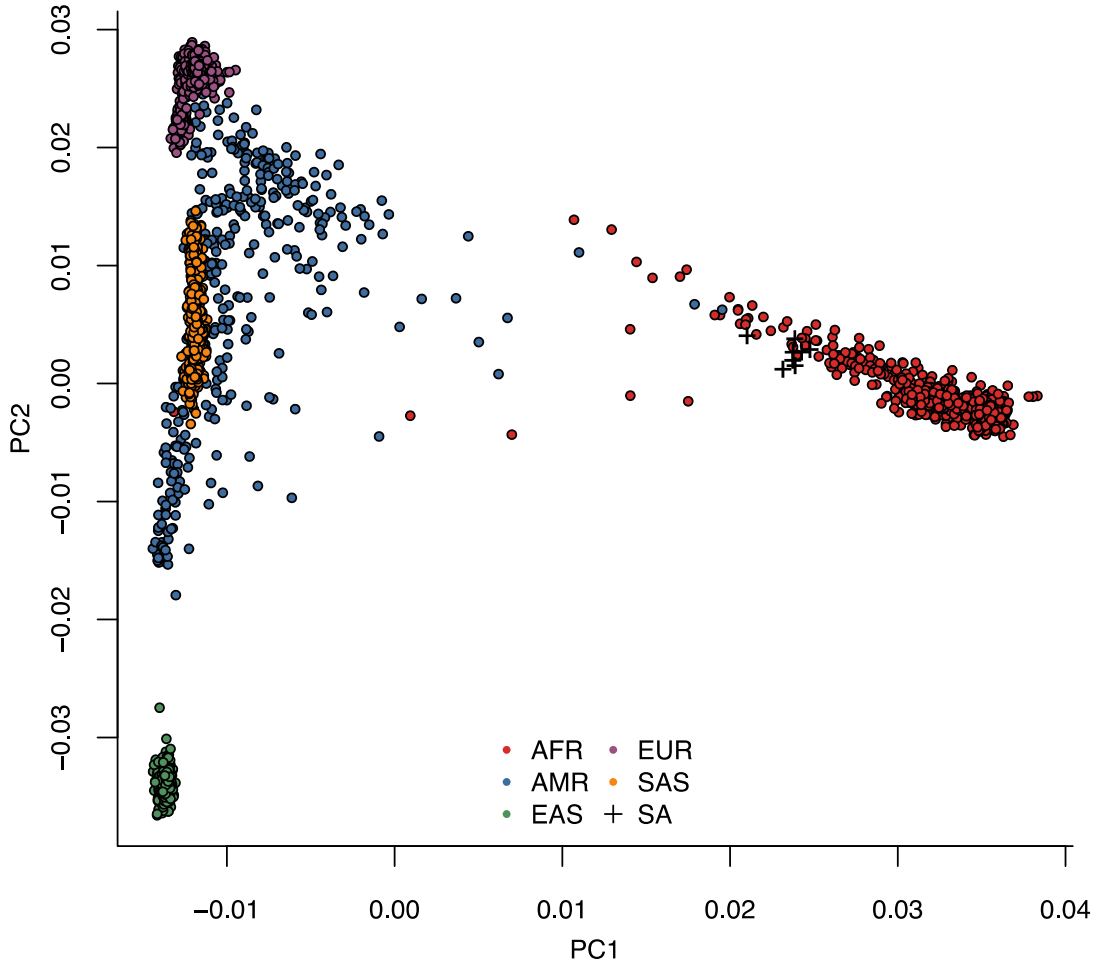

Figure 3.7: PCA of black Africans (current study) in comparison with other global populations from the 1000 Genomes project represented as follows; AFR=Africans, AMR=Americans, EUR=Europeans, EAS=East Asians, and SAS=South Asians. The South African cohort clustered with other population groups as represented by the plus (+).

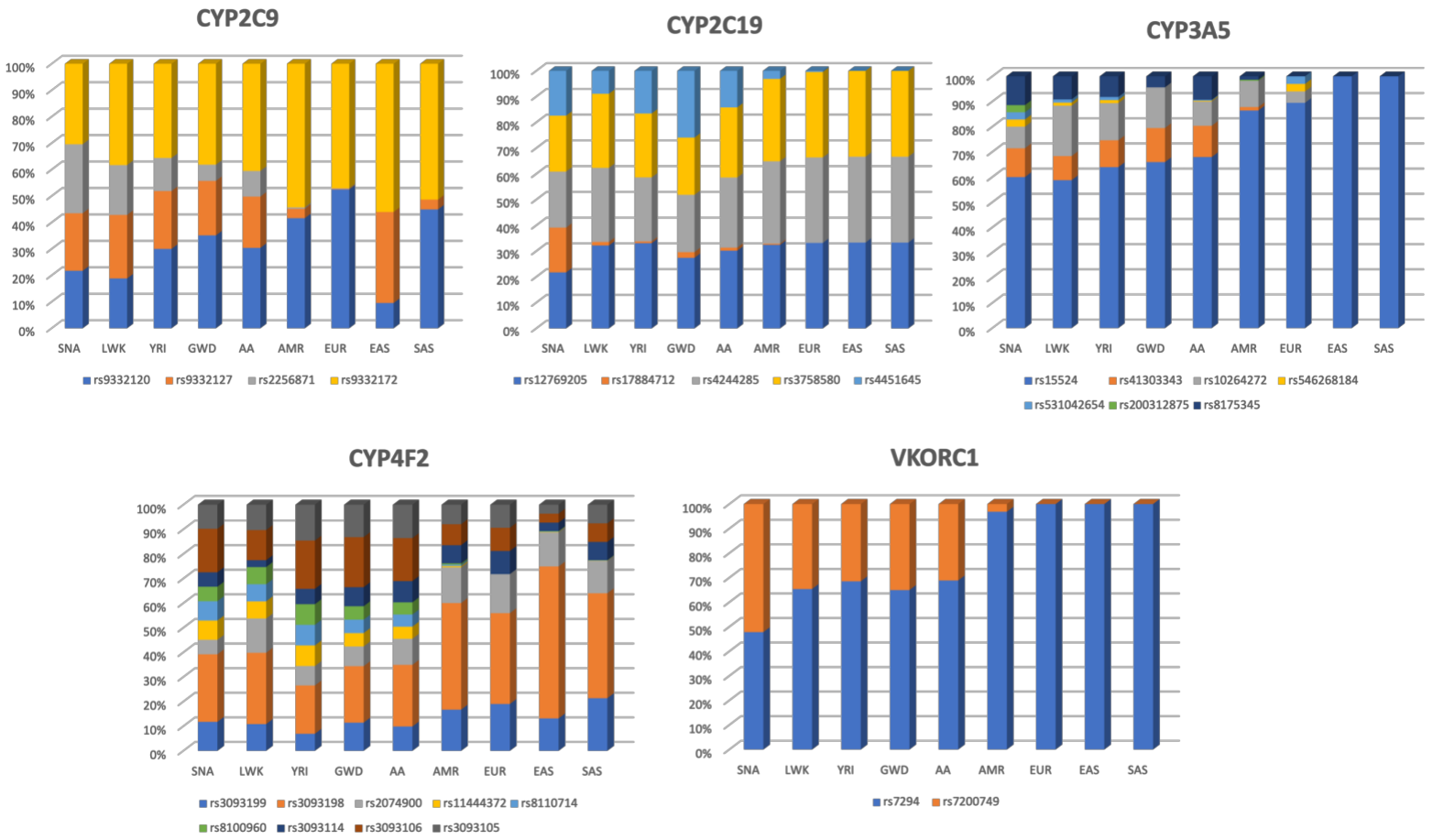

Figure 3.8A: Allele frequency distribution patterns of identified variants in actionable pharmacogenes *CYP2C9, CYP2C19, CYP3A5, CYP4F2,* and *VKORC1* among Southern Africans (South Africans and Zimbabweans) and other global populations from the 1000 Genomes project dataset. Represented as follows; SNA=Southern Africans, LWK=Luhya in Kenya, YRI=Yoruba in Nigeria, GWD=Gambian, AA=African Americans, AMR= Hispanic Americans, EUR=Europeans, EAS=East Asians and SAS=South Asians.

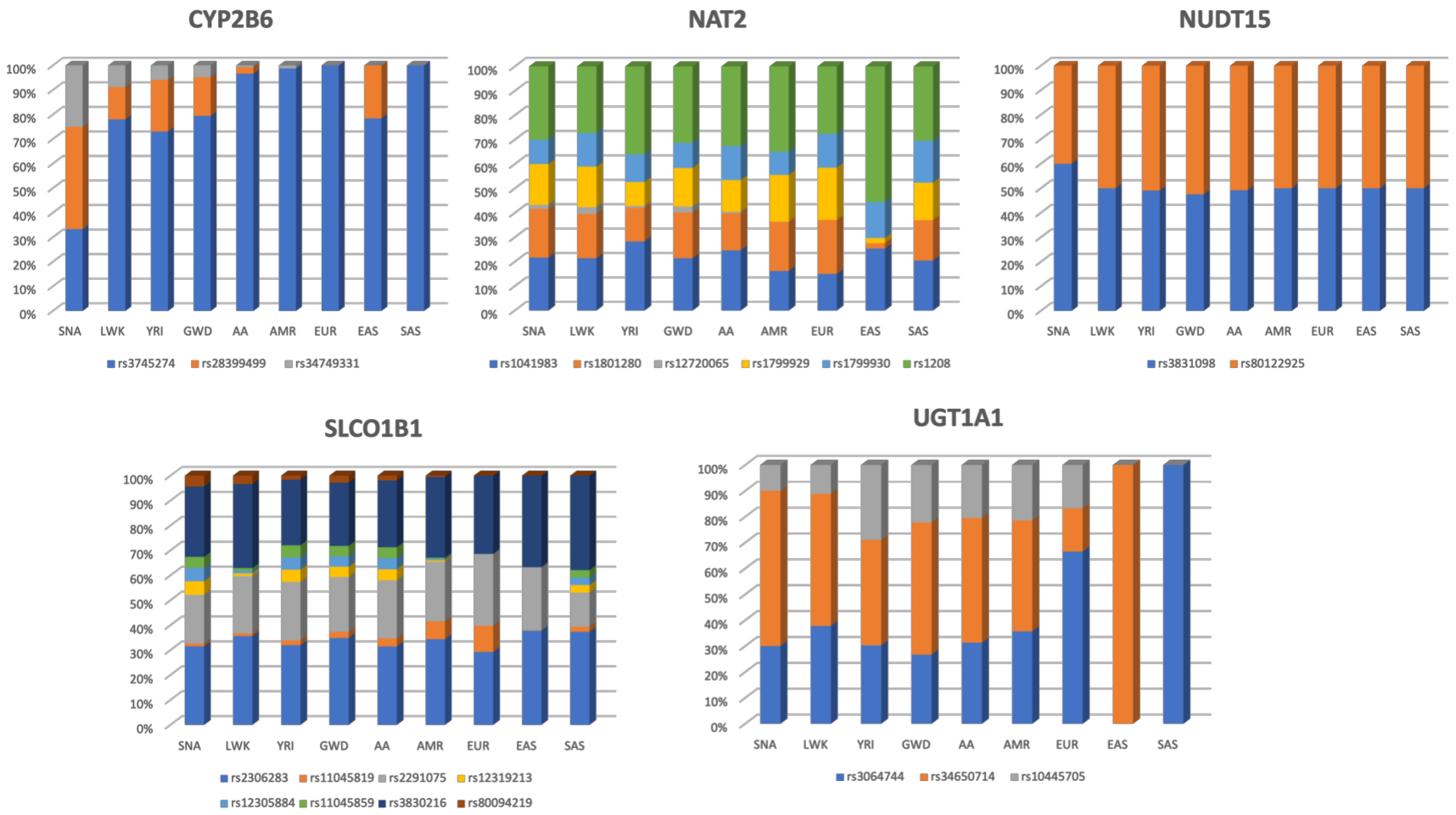

Figure 3.8B: Allele frequency distribution patterns of identified variants in actionable pharmacogenes *CYP2B6, NAT2, NUDT15, SLCO1B1, UGT1A1* and *VKORC1* among Southern Africans (South Africans and Zimbabweans) and other global populations from the 1000 Genomes project dataset. Represented as follows; SNA=Southern Africans, LWK=Luhya in Kenya, YRI=Yoruba in Nigeria, GWD=Gambian, AA=African Americans, AMR= Hispanic Americans, EUR=Europeans, EAS=East Asians and SAS=South Asians.

In contrast, none of the 191 genes extracted from the list of variants predicted to be pathogenic were directly associated with warfarin or biological process related to the warfarin disposition pathway, furthermore, they had no interaction with either *CYP2C9* or *VKORC1*. Unknown variants predicted with a high pathogenic consequence by the mutation assessor and to be deleterious by at least 10 of the 17 variant pathogenicity predictors in genes *CYP2C8, F7, F9, GGCX, LRP1, PROZ, HMOX1* and *STAB2* were also prioritised for validation, however, they all failed validation as they could not be identified through Sanger sequencing in the 8 samples analysed by WES. Thus, they were not carried forward for further analysis.

3.4.1.3.3. Association of prioritised SNPs with warfarin maintenance dose

Based on the gene enrichment and network analyses, 18 SNPs from the drug response significance list *ABCB1, APOB, CYP2C9, CDH3, EPHX1, GSTP1, NQO1, PROC* and *VKORC1* genes were prioritised, genotyped, and tested for their effect on warfarin dose requirements. The *CYP4F2* rs2108622 (predicted to have a drug response here) and *VKORC1* rs7294 have already been tested for their effects on warfarin dose requirements in this cohort. As presented on section 3.3.3, *CYP4F2* rs2108622 *and VKORC1* rs7294 do not have a significant effect on warfarin maintenance dose requirement among black Africans studied here. Variants in *VKORC1 c.358C>T* (rs7200749), *NQO1 c.343C>T* (rs1800566), *CYP2C9 c.752AG* (rs2256871), *CYP2C9 c.482-65G>C* (rs9332127), *ABCB1 c.*89A>T* (rs17064), *EPHX1 g.26936C>T* (rs373523374) and *PCSK6 c.1694C>T* (rs1058291) showed trends towards requirements for reduced warfarin maintenance doses, which did not reach statistical significance (p>0.05) (Table 3.16). In contrast, *PROC c.423G>T (*rs5936*)* showed trends towards requirement for increased warfarin maintenance dose among homozygous *PROC c.423T/T* (40±14 mg/week) genotype carriers compared to heterozygous *c.423G/T* (35±12 mg/week) carriers and homozygous *c.423G/G* (34±13 mg/week) genotype carriers (p=0.05). Thus, in a recessive genetic model, *PROC c.423T/T (*40±14 mg/week) was significantly associated (p=0.02) with higher mean

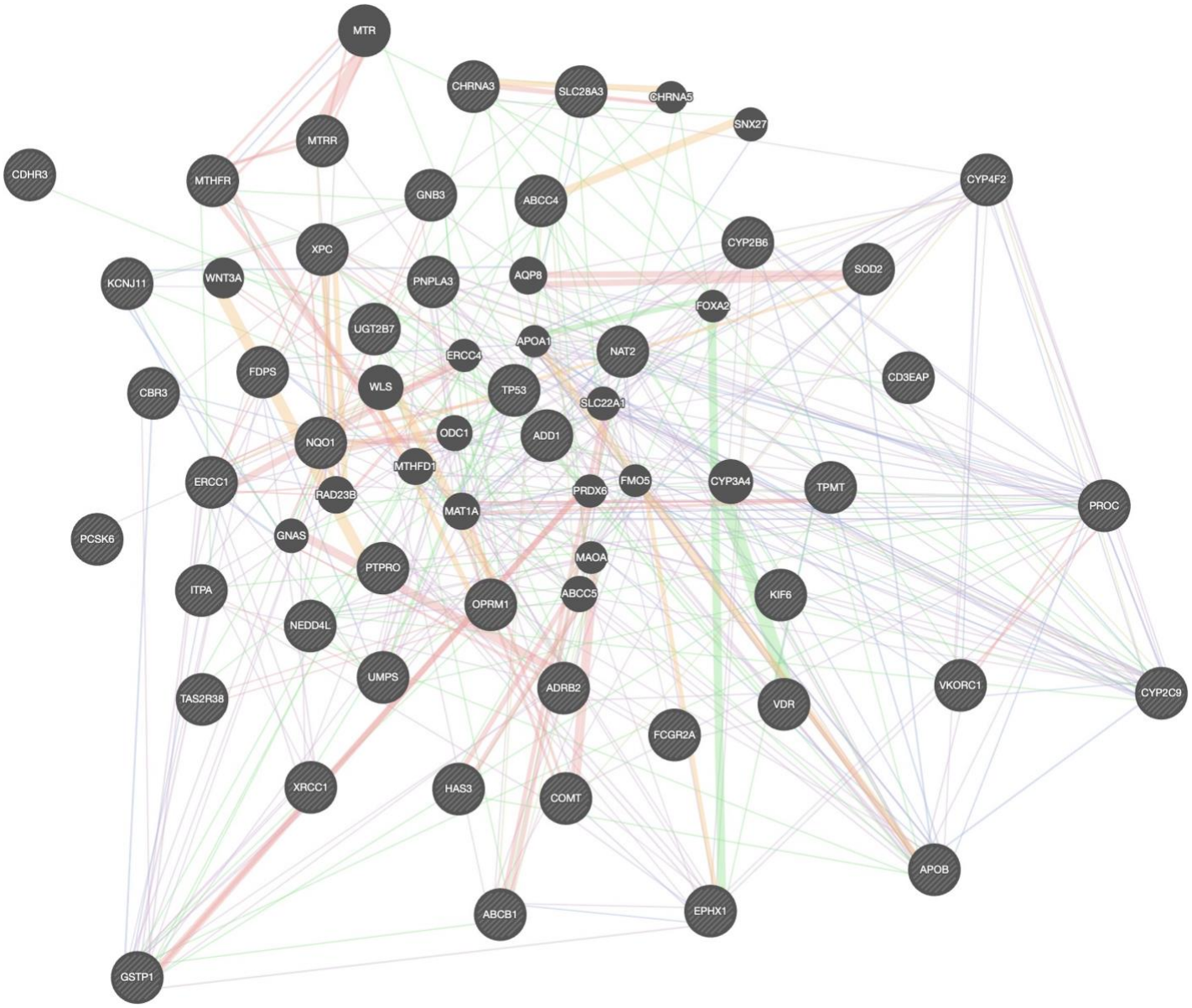

Figure 3.9: Illustration of the network analysis of the gene extracted from the drug-response variants list and their interaction with *CYP2C9* and *VKORC1*.

warfarin maintenance as compared to *PROC c. 423*GG/GT (35±12 mg/week) (Figure 3.10). The effect of *EPHX1 g.26978G>C* (rs2260863) on warfarin maintenance dose requirement displayed a codominant trend with individuals possessing the heterozygous G/C genotype requiring significantly (p=0.04) lower warfarin maintenance doses (34±12 mg/week) compared to the G/G (40±15 mg/week) and C/C (39±12 mg/week) genotype carriers.

From the haplotypes inferred, haplotype *PROC* C-G-T from the three SNPs, rs2854696, rs5936 and rs5937 had a borderline significant association (p=0.05) with reduced warfarin dose variability, however the overall *PROC* haplotype model did not reach statistically significant effect (Table 3.17). In contrast, the haplotypes from the *CYP2C* loci (comprising rs12777823, rs1799853, rs7900194, rs9332127, rs2256871, rs28371685, rs1057910 and rs11572103) had haplotypes, G-C-G-G-A-C-A-T and G-C-G-G-A-C-A-A significantly associated with increased warfarin dose variability (p=0.02, p=0.01, respectively), whilst A-C-A-G-A-C-A-A was significantly associated with reduced warfarin dose variability (p=0.0002). Furthermore, the overall CYP2C cluster model explained 14% of warfarin dose variability (p=0.002), and when entered into a multivariable regression model inclusive of age, deep venous thrombosis, mechanical valve replacement, *CYP3A5 c.624G>A (*6)* and *MTHFR c.677C>T* it explained the same variability as the model *(i.e.,* R^2=34%) described in section 3.3.3. However, the inclusion of *PROC c.423G>T* and *EPHX1 g.26978G>C* improved a model inclusive of age, deep venous thrombosis, mechanical valve replacement, *CYP2C8 c.805A>T (*2), CYP2C9 c.449G>A (*8), CYP2C9 c.1003C>T (*11)*, *CYP2C* rs12777823G>A, *CYP3A5 c.624G>A (*6)* and *MTHFR c.677C>T* from 34% to 40% variability (p=0.0003).

3.4.1.4. Discussion

Pharmacogenetics is a fundamental tool in ensuring therapeutics prescription result in little to no adverse drug associated complications. However, for pharmacogenetics to be fully integrated clinically, genetic profiles affecting various drugs need to be comprehensively evaluated at a population level, specifically in low- and middle-income countries such as in Africa, as these countries are lagging compared to Europeans and

Asians [292, 293]. The application of NGS in pharmacogenetics has presented an opportunity for the field of pharmacogenetics to be fully accelerated, thus enabling comprehensive evaluation of informative pharmacogenetics profiles. Newer genomic technologies including NGS, have been fully embraced in highly resourced settings, it is not the case in low and middle economies as most pharmacogenetic studies still apply the candidate variant approach focusing on genetic markers reported in population groups such as European. Our study successfully carried out an in-depth search of informative pharmacogenetic profiles in various genetic regions through the application of whole exome sequencing. Furthermore, we were able to demonstrate the importance of carrying out population specific NGS-anchored pharmacogenetic studies as making inference using data from other population groups means we miss important African specific genetic markers.

The inter-population difference in the frequency distribution of pharmacogenetic markers was notable in the present study when we compared the distribution of identified variants across 10 known actionable pharmacogenes between our studied cohort and populations from the 1000 Genome. From the variants extracted in known actionable pharmacogenes, variants rs28399499, rs34749331, rs2256871, rs17884712, rs41303343, rs546268184, rs531042654, rs200312875, rs8175345 and rs12720065 occurred specifically in the various African populations and not in Europeans, Hispanic Americans, East and South Asians. Furthermore, these variants were identified in genes (i.e., *CYP2C9, CYP2B6, CYP2C19, CYP3A5* and *NAT2*) important for the pharmacogenomic response of drugs such as warfarin, efavirenz, clopidogrel, citalopram, omeprazole, tacrolimus, and isoniazid. Thus, there are studies that have documented the effect of some of these African specific variants on the respective drug response. For instance, *CYP2B6* rs28399499 has been consistently associated with increased efavirenz plasma concentrations in various African population groups [308-310], whilst *CYP2C19* rs17884712 is associated with a decreased metabolism of omeprazole among Black South Africans [311] and *CYP3A5 rs*41303343 has been associated with decreased metabolism of tacrolimus among African Americans [312, 313]. The occurrence of these variants exclusively in African individuals support the

Table 3.16: Univariate analysis of prioritised genetic markers of SNPs on their effects on warfarin maintenance dose requirements among black Africans

SNP genotype	Maintenance dose (mg/week), mean ±SD (range)	P value	Cohen's d (95%CI)
*ABCB1 c.*89A>T*, rs17064			
T/T	40±14 (12.5-75)	0.61	0.32 (-0.31 to 0.94)
T/A	37±13 (17.5-70)		
A/A	35±9 (17.5-52.5)		
APOB c.1853C>T, rs679899			
CC	39±14 (12.5-75)	0.32	0.59 (-0.41 to 1.58)
CT	38±14 (17.5-72.5)		
TT	31±5 (25-35)		
APOB c.13013G>A, rs1042034			
GG	37±4 (35-42.5)	0.36	-0.10 (-1.10 to 0.89)
GA	35±15 (12.5-70)		
AA	38±13 (12.5-72.5)		
CDH3 c.2334T>C, rs10270308			
TT	37±14 (12.5-70)	0.40	-0.19 (0.64 to 0.26)
TC	36±13 (17.5-72.5)		
CC	40±14 (12.5-70)		
CYP2C9 c.752AG, rs2256871			
A/A	38±14 (12.5-75)	0.45	3.70 (3.28 to 4.12)
A/G	37±11 (17.5-70)		
G/G	27±16 (17.5-45)		
CYP2C9 c.482-65G>C, rs9332127			
G/G	38±14 (12.5-75)	0.56	3.94 (3.57 to 4.31)
G/C	38±13(17.5-70)		
C/C	30±7(25-35)		
EPHX1 c.A416G, rs2234922			
A/A	37±12 (12.5-75)	0.50	0.05 (-0.51 to 0.59)
A/G	41±16 (12.5-72.5)		

G/G	37±16 (17.5-70)		
EPHX1 c.T337C, rs1051740			
T/T	37±13 (12.5-70)	0.26	-0.45 (-1.45 to 0.56)
T/C	34±13 (12.5-70)		
C/C	43±11 (35-57.5)		
EPHX1 c.357G>A, rs1131873			
G/G	37±13 (12.5-70)	0.23	-0.13 (-0.59 to 0.33)
G/A	38±14 (12.5-70)		
A/A	17.5 (17.5-17.5)		
EPHX1 g.26936C>T, rs373523374			
C/C	37±13 (12.5-70)	0.12	0.76 (-0.24 to 1.76)
C/T	28±9 (17.5-35)		
EPHX1 g.26948G>A, rs112043151			
G/G	37±13 (12.5-70)	0.93	-0.12 (-1.01 to 0.77)
G/A	39±23 (17.5-70)		
EPHX1 g.26978G>C, rs2260863			
G/G	40±15 (17.5-70)	0.04	0.09 (-0.35 to 0.55)
G/C	34±12 (12.5-70)		
C/C	39±12 (17.5-70)		
GSTP1 c.A313G, rs1695			
A/A	39±13 (17.5-75)	0.74	0.02 (-0.39 to 0.44)
A/G	38±14 (12.5-70)		
G/G	39±13 (17.5-72.5)		
NQO1 c.C343T, rs1800566			
C/C	39±14 (12.5-72.5)	0.56	0.47 (-0.68 to 1.61)
C/T	38±14 (12.5-75)		
T/T	33±4 (27.5-35)		
PROC c.G423T, rs5936			
G/G	34±13 (17.5-70)	0.05	-0.41 (-0.89 to 0.06)
G/T	35±12 (12.5-70)		

T/T	40±14 (12.5-72.5)		
PROC c.T768C, rs5937			
T/T	36±12 (12.5-70)	0.44	-0.17 (-0.75 to 0.41)
T/C	38±13 (12.5-70)		
C/C	38±13 (17.5-70)		
PROC g.5398T>C, rs2854696			
C/C	37±13 (12.5-70)	0.48	0.28 (-0.72 to 1.27)
C/T	37±11 (17.5-70)		
T/T	40±5 (35-45)		
VKORC1 c.358C>T, rs7200749			
CC	38±12 (17.5- 70)	0.20	0.70 (-0.69 to 2.10)
CT	36±15 (12.5- 72.5)		
TT	30±7 (25- 35)		

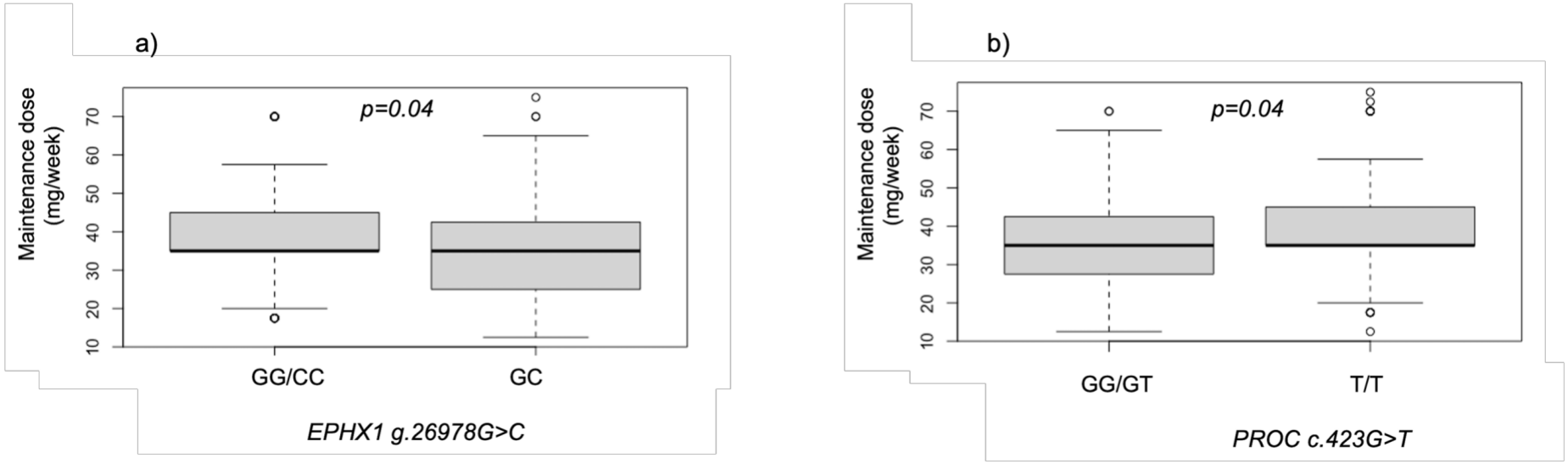

Figure 3.10: Distribution of the warfarin weekly maintenance dose according to genotypes for a) EPHX1 g.26978G>C, rs2260863 codominant model (p=0.04 b) *PROC c.423G>T, rs5936 recessive model* (p=0.04) in black Africans.

Table 3.17: Regression model testing for the association of the haplotype groupings on warfarin maintenance dose among black Africans

Genes (SNPs ID)	Haplotype	β coefficient	R^2	P value
APOB	G-A	-1.39	0.0005	0.76
(rs1042034|rs679899)	A-A	-1.59	0.002	0.52
	G-G	-1.97	0.004	0.42
	A-G	1.96	0.007	0.26
CYP2C cluster/CYP2C9	G-C-G-G-A-C-A-T	4.66	0.03	0.03
(rs12777823|rs1799853|rs7900194|rs9332127	A-C-G-G-A-C-A-A	-1.58	0.0006	0.75
|rs2256871|rs28371685|rs1057910| rs11572103)	G-C-G-G-G-C-A-A	-1.16	0.001	0.63
	A-C-G-C-A-C-A-A	-2.17	0.005	0.36
	G-C-G-G-A-C-A-A	4.32	0.04	0.01
	A-C-G-C-A-C-A-T	5.69	0.007	0.29
	A-C-A-G-A-C-A-A	-9.25	0.08	0.0002
	G-C-A-G-A-C-A-A	1.64	0.0005	0.78
	A-C-G-G-A-T-A-A	-10.3	0.02	0.11
EPHX1	T-A-C-G-G-G	-0.09	$3.71x10^{-6}$	0.98
(rs1051740|rs1131873|rs373523374|rs112043151	T-G-C-G-G-G	-0.03	$6.82x10^{-7}$	0.99
|rs2260863|rs2234922)	C-G-C-A-C-G	-2.66	0.001	0.63
	T-G-C-G-C-G	4.32	0.01	0.13
	T-A-C-G-G-A	-7.72	0.02	0.08
	T-G-C-G-G-A	1.24	0.003	0.48
	C-G-C-G-C-A	2.34	0.007	0.25
	T-G-C-G-C-A	-1.99	0.009	0.21
PROC	C-T-C	1.97	0.009	0.21
(rs2854696|rs5936|rs5937)	T-G-T	-0.06	$4.16x10^{-7}$	0.99
	C-G-T	-2.92	0.02	0.05
	T-T-T	0.95	0.0009	0.69
	C-T-T	0.97	0.002	0.54
VKORC1	G-G-T-A	-1.29	0.0009	0.64
(rs7294|rs7200749|rs9934438|rs9923231)	A-A-C-A	6.12	0.004	0.31
	G-G-T-G	-0.51	$3.45x10^{-5}$	0.93
	A-A-C-G	-2.79	0.01	0.12
	A-G-C-G	0.10	$1.85x10^{-5}$	0.95
	G-G-C-G	1.37	0.005	0.27

notion that Africans possess additional unique genetic architecture different from the highly studied populations (i.e. Europeans and Asians) and the importance of increasing pharmacogenetic studies that include Africans.

From the WES data, we prioritised and tested for the effect of *CYP2C9* and *VKORC1* variants on warfarin dose requirements, together with variants predicted to have a clinical drug response significance and are identified in genes enriched or interacting with warfarin associated genes (i.e., *ABCB1, APOB, CDH3, EPHX1, GSTP1, NQO1* and PROC). Variants *CYP2C9*2, *3* and African specific *CYP2C9*8, *5* and **11* which we have reported before in the presence cohort, are the center of attention with regards to warfarin pharmacogenetics. However, in the presence WES data we detected four *CYP2C9* variants (rs9332120, rs9332127, rs2256871 and rs9332172) that we had not reported on before and prioritised variants rs2256871 and rs9332127 to test for their effect on warfarin dose requirements in a black African cohort. Although effect size was high (>3) and showed a trend of reduced warfarin dose requirement, their effect was however not statistically significant (P>0.05). Furthermore, the *CYP2C* haplotypes (G-C-G-G-A-C-A-T, G-C-G-G-A-C-A-A and A-C-A-G-A-C-A-A) which were significantly associated with warfarin dose requirements was comprised of the wild type A and G allele of the rs2256871 and rs9332127, respectively.

Interestingly, in a study by Schelleman et al [137], variant rs2256871 was reported to account for more than 10% of variability in log-transformed warfarin maintenance dose requirements in African Americans, however this was not statistically significant, and they ruled it out to be due to chance. Additionally, Suriapranata et al [314] reported variant rs2256871 to not influence patient-specific warfarin dose among Indonesians. Thus, the functional effect of rs2256871 is yet to be determined as phenotypic studies in African individuals have shown no effect on the metabolism of the antiepileptic drug phenytoin [214]. The effect of *CYP2C9* rs9332127 on warfarin dose requirement has never been tested among Africans but it is highly reported among Chinese patients with contradictory results. Other studies have reported that *CYP2C9* rs9332127 significantly influences warfarin dose requirements whilst other studies have reported its lack of effect on warfarin dose requirements [315-318].

VKORC1 g.9041G>A (rs7294) and *c.358C>T* (rs7200749) were the only *VKORC1* variants identified from the WES data, however the effect of *VKORC1 g.9041G>A* (rs7294) on warfarin dose requirements has been tested before in the black African cohort from our study (described in section 3.3.3) and it was found to have no significant association with warfarin dose requirements. Thus, *VKORC1 c.358C>T* (rs7200749) was the only *VKORC1* SNP that we had not captured in the previous candidate variant analysis we undertook. Although in the present analysis *VKORC1 c.358C>T* (rs7200749) showed a trend of reduced warfarin maintenance dose requirements, the effect was not statistically significant in our black African cohort. Thus, supporting findings by Limdi et al [134] which showed no significant association between *VKORC1 c.358C>T* (rs7200749) and warfarin dose requirements among African Americans. However contradicting findings by Mitchell et al [111] which reported that *VKORC1 c.358C>T* (rs7200749) was significantly associated with increased warfarin dose requirement in black South Africans.

Although *ABCB1, NQO1* and *CDH3* were enriched to be significantly associated with warfarin, SNPs (i.e., *ABCB1 c.*89A>T*, (rs17064), *CDH3 c.2334T>C* (rs10270308) and *NQO1 c.C343T*, rs1800566) identified in these genes through WES did not have any significant association with warfarin dose requirement among the black African cohort in our study. *ABCB1* codes for the multidrug efflux pump P-glycoprotein (P-gp), responsible for the transport of various xenobiotics through the kidney and liver, including warfarin which it mediates its excretion out of the liver through the bile [42]. *ABCB1 c.3435C>T* (rs1045642) has been the mostly studied *ABCB1* marker with regards to warfarin dose requirements yielding inconsistent results [120, 255, 257, 258], we have also previously reported its effect on warfarin dose requirement in our black African and Mixed Ancestry cohort and did not find any significant association in both cohorts [171]. Thus, to our knowledge this is the first study to report on the effect of *ABCB1 c.*89A>T*, (rs17064) in a warfarin context, its lack of effect on warfarin dose requirement may explain why it is not considered in warfarin pharmacogenetic studies.

CDH3 c.2334T>C (rs10270308) was first tested for its association on warfarin dose variability by Alghamdi et al [319] among cardiovascular patients from Amman-Jordan,

and their findings were similar to ours as they could not establish any significant association between this SNP and warfarin sensitivity or responsiveness during the maintenance phase of therapy. CDH3 is a member of transmembrane proteins which are involved in the homologous cell adhesion processes that are important for epithelial polarity, cell-cell interaction, and tissue differentiation [320-322], however the mechanism in which *CDHR3* influences warfarin activity has not been described before in previous literature, thus further studies are required to explain the basis in which this gene is significantly associated with warfarin dose in the enrichment analysis. In contrast, the role of *NQO1* in the warfarin pathway has been clearly described, as it encodes NAD(P)H dehydrogenase quinone 1, an enzyme responsible for the reduction of vitamin K to vitamin K hydroquinone during the recycling interconversion of vitamin K, a cycle targeted by warfarin [323]. Thus, individuals carrying the *NQO1 c.343*T/T or C/T genotypes have been reported to have no or reduced NQO1 activity, respectively [324]. However, the effect of *NQO1 c.343C>T* (rs1800566) on warfarin dose requirement has been contradictory with other studies reporting that it results in increased warfarin dose requirement [140, 325], whilst Chung et al [326] reported a reduced warfarin dose requirement and other studies found no association between *NQO1 c.343C>T* (rs1800566) and warfarin dose requirement [256, 327, 328]. Similarly, we could not establish any significant association between *NQO1 c.343C>T* (rs1800566) and warfarin dose requirement, confirming a meta-analysis by Asiimwe et al [163] which reported that *NQO1* rs1800566 has no effect on warfarin dose requirement among black African patients.

The *EPHX1* gene encodes microsomal epoxide hydrolase, a phase 1 biotransformation enzyme important for the hydrolysis of various epoxide intermediates including vitamin K epoxide [33], a derivate of the gamma carboxylation of glutamate residues in coagulation factor II, VII, IX, X, protein C, S and Z [323]. From the WES data we prioritised and further characterised six *EPHX1* SNPs in the black African cohort, however, we could not find any significant association between five of these SNPs and warfarin dose requirements. In contrast, *EPHX1 g.26978G>C*, rs2260863 showed a co-dominant significant effect on warfarin dose requirement, with individuals possessing a

G/C (34±12 mg/week) genotype requiring low warfarin dose as compared to G/G (40±15 mg/week) and C/C (39±12mg/week), further contributing to 3% of warfarin dose requirement in a multivariate regression model. However, the effect of *EPHX1 g.26978G>C* on warfarin dose requirements has been contradictory, Pautas et al [330] reported that *EPHX1 g.26978G>C* has no effect on warfarin dose among Caucasians, whilst Liu et al [254] and Lin et al [329] reported that Chinese patients carrying the *EPHX1 g.26978G/C* genotype require a high warfarin dose as compared to those carrying the C/C genotype. To our knowledge, this is the first African study to report on the effect of *EPHX1 g.26978G*>C on warfarin dose and this contradictory effect of this SNP among various ethnic group could suggest that its effect is population specific being influenced by another population-specific markers. The lack of effect of other *EPHX1* SNPs we reported here such as *EPHX1 c.A416G* (rs2234922) and *EPHX1 c.T337C* (rs1051740) is supported by other studies which have reported similar findings in various population groups [120, 137, 330, 331].

Our analysis also included *APOB c.1853C>T* (rs679899) and *APOB c.13013G>A (rs1042034)*, although there was no association between both these SNPs and warfarin dose requirements, *APOB c.1853C>T* (rs679899) showed a trend of reduced warfarin dose requirement with individuals harboring the C/C and C/T genotypes requiring higher warfarin dose as compared to those with T/T genotypes. Thus, this finding explains Yee et al [332] reports which revealed *APOB c.1853C>T* was significantly associated with warfarin related bleeding complications with individuals carrying the C allele experiencing more bleeding that those with T/T genotypes. Yee et al [332] further confirmed the lack of association between *APOB c.13013G>A* and warfarin related associated bleeding complications, consistent with the lack of effect of this SNP with warfarin dose requirement we reported here. Protein C encoded by *PROC* is a vitamin K dependent anticoagulant protein whose activation is disrupted by the action of warfarin. Wadelius et al [68] reported that *PROC* SNPs (i.e., rs1799809, rs2069901, rs2069910 and rs2069919) are significantly associated with warfarin through their influence on prothrombin time which is a determinant of a required warfarin dose [333]. In our study we identified and reported on the effect 3 *PROC* SNPs on warfarin dose

requirements, however, only *PROC c.G423T*, rs5936 showed a trend towards significant in an additive model but was significantly associated with warfarin dose requirements in a recessive model, explaining 3% of warfarin dose variability in a multivariate regression model. The findings reported here confirm a study by Lee et al [334] that *PROC c.G423T* significantly affects warfarin dose requirements among Han Chinese, with individuals carrying the T/T genotypes requiring a higher warfarin dose (23.1 mg/week) as compared to individuals harboring the G/G (18.8mg/week) and G/T (19.3 mg/week) genotypes. To our knowledge this is the first study to confirm the effect of *PROC c.423G>T* on warfarin dose requirements in an African setting, thus inclusion of this SNP could potentially serve as a genetic predictor in an African-specific dosing algorithm.

3.4.1.5. Limitations

Although WES allowed us to scan and carry out an in-depth analysis of various genetic regions in our black African warfarin patients presenting with extreme phenotypes, our study design had some limitations. The WES sequencing was undertaken in only 8 individuals, which means we could not carry out any genome wide association analysis in all the variants identified, thus we prioritised specific variants based on clinical significance and genotyped them in an enhanced sample size for association with warfarin maintenance dose. However, this approach means we are still utilising a candidate variant approach therefore missing important variants that have not been described before to have any clinical significance and could explain the warfarin inter-individual variants among black Africans. Another thing to note is that there is no established pipeline that has been developed for the prioritisation of pharmacogenetics data generated through NGS. Thus, most of the pharmacogenetics studies that utilised NGS platforms usually prioritise variants in either known pharmacogenes or only known actionable pharmacogenes, introducing the bias of not analysing variants in other genes that could be indirectly affecting drug response through a pathway not enriched for that specific drug. For NGS data to be fully useful in the application of pharmacogenetics, a genome wide association analysis is a better approach as it allows for a more in-depth association of genetic variants identified. However, genome wide analysis need a more

enhanced sample sizes to account for multiple testing, which also mean the cost of sequencing will be quite high. Hence, such studies are lacking in low- and middle-income economies where resources are limited. Another limitation with high throughput data such as NGS generated data, it results in identification of false positives variants. In the present study we prioritised some novel or unknown variants, but we failed to detect these variants through Sanger sequencing, and we could not move forward with association for these variants. For NGS platforms to be fully embraced in the field of pharmacogenetics such limitations need to be considered.

3.4.1.6. Conclusion

The application of NGS data is a solution in making sure a wide variety of informative pharmacogenetic profiles are captured, thus accelerating the integration of pharmacogenetic clinically. In the present study we were able to apply WES data to scan through multiple genetic regions, identify African specific markers in various actionable genes and identify additional pharmacogenetic markers that could serve as important predictors for an African specific dosing algorithm.

3.5. Reporting on a set of variants and non-genetic variables that are important in the prediction of the best starting dose for warfarin among Africans.

Synopsis: This section presents on a set of genetic and non-genetic variables that are important for the prediction of warfarin dose in our study. Furthermore, recommend for their inclusion in an African specific dosing algorithm. The variables explaining warfarin dose variability were described in the publications (https://doi.org/10.1089/omi.2018.0174 and https://doi.org/10.1111/jth.15494) and the two unpublished manuscript presented in previous sections as they were entered into a multivariate regression analysis each time variables were added into the analysis. Thus, in this section we will only include an overview of the various variables significantly explaining warfarin dose variability in our study.

Overview:

Genetic and non-genetics variables significantly affecting warfarin dose requirements were tested for their cumulative effect on warfarin dose variability among our cohorts through a stepwise multivariable regression model as described above in the respective manuscripts. The final model for black Africans was comprised of age, deep venous thrombosis, mechanical valve replacement, *CYP2C8 c.805A>T (*2), CYP2C9 c.449G>A (*8), CYP2C9 c.1003C>T (*11),* CYP2C rs12777823G>A, *CYP3A5 c.624G>A (*6)*, *MTHFR c.677C>T*, *PROC c. G423T* (recessive model) and *EPHX1 g.26978G>C* (codominant model), explaining 40% of the warfarin dose variability (p=0.0003). In contrast the final model for Mixed Ancestry was comprised of *VKORC1* haplotypes, age and BMI, explaining 22% of the warfarin dose variability (p=0.0008). Thus, the variables we are recommending to be considered for an African specific dosing algorithm are outlined in Table 3.18 in comparison with other dosing algorithms reported before in other world populations.

Table 3.18: Proposed variables for possible inclusion in an African-specific warfarin pharmacogenetics-based dosing algorithm compared to other algorithms reported in other populations

References	Genetics variables	Demographic and clinical variables
This study	*VKORC1 g-1639 G>A (or haplotype), CYP2C rs12777823G>A, CYP2C8 c.805A>T (*2), CYP2C9 c.449G>A (*8), CYP2C9 c.1003C>T (*11), CYP3A5 c.624GA (*6), MTHFR c.677C>T*, *PROC c. 423G>T* and *EPHX1 g.26978G>C*	Age (in years), Gender, BMI, Deep venous thrombosis, mechanical valve replacement
Gage et al [63]	*VKORC1 3673G>A, CYP2C9*3*, *CYP2C9*2*	BSA (per 0.25 m^2), Age (per decade), target INR (per 0.5 increase), Amiodarone, Current smoker, African American race, Venous thromboembolism
International Warfarin Pharmacogenetics Consortium [65]	*VKORC1 g-1639 G>A, CYP2C9 *2, CYP2C9 *3*	Age in decades, height (in cm), weight (in kg) Race, Enzyme inducer status, Amiodarone status
Lenzini et al [64]	*VKORC1 g.-1639G>A, CYP2C9*2, CYP2C9*2*	Natural logarithm (INR), $dose_{-3}$ (per mg), age (per year), BSA (per 0.25 m^2), target INR, African ancestry, stroke indication, $dose_{-4}$ (per mg), $Dose_{-2}$ (per mg), diabetes, amiodarone use, fluvastatin use
www.warfarindosing.org (modified from Gage et al [63]	*VKORC1 g.-1639G>A, CYP2C9*2, CYP2C9*3, CYP2C9*5, CYP2C9*6, CYP4F2 V433M, GGCX rs11676382*	Age, sex, race, ethnicity, weight (kg or lbs.), height (feet and inches or cm), smokes, liver diseases, indication, baseline INR, target INR, amiodarone/cordarone® dose (mg/day), statin/HMG CoA reductase inhibitor, any azole and Sulfamethoxazole/Septra/Bactrim/Cotrim/ Sulfatrim

Chapter 4: Overall Discussion and Conclusion

4.1. Summary Discussion

This study was undertaken in order to decode the pharmacogenomics of warfarin and generate data including pharmacogenes variants that could be utilised in the development of a pharmacogenetic or pharmacogenomic test to inform warfarin dosing decisions. Warfarin therapy presents with a narrow therapeutic range which makes its prescription cumbersome due to difficulties in dose prediction to enable the quickest achievement of the international normalised ratio (INR). Delays in reaching the INR is associated with adverse complications, a proportion of which is accompanied by hospitalisations or mortality [44]. Although, shortcomings associated with warfarin have been described, and that now alternative anticoagulants are available, warfarin still remains the most commonly prescribed anticoagulant used in low- and middle-income countries such as those existing in Africa, due to its low cost, and familiarity of use among healthcare practitioners [2]. Pharmacogenomics/pharmacogenetics (PGx) provides a solution through understanding and then using human genetic variation to improve warfarin treatment outcomes.

PGx has been swiftly advancing, which has resulted in the curation and development of 26 dosing guidelines for over 140 drugs by the clinical pharmacogenetics implementation consortium (CPIC) [172]. Furthermore, the pharmacogenetic guidelines for specific drugs such as warfarin have been endorsed by the United States-Food and Drug Administration (US-FDA) through the inclusion of the recommended PGx parameters on the specific drug labels [179]. Although the advantages of PGx are obvious, implementation of pharmacogenomics to support clinical decision dose making has taken too long due to many factors, the major ones being the poor characterisation of African populations, financial, regulatory, and ethical issues [292, 335]. Considering the persistent high usage of warfarin therapy among Africans, coupled with limited data on the pharmacogenomic profiles important for the development of informative dosing algorithms tailored specifically for Africans. For the first time in an African setting, we

carried out a comprehensive evaluation of various genomic regions in search of informative pharmacogenomics profiles for the prescription of warfarin dosing in Africans.

Studying the various pharmacogenetic variation patterns in black Africans and the Mixed Ancestry cohort allows us to compare the profile of important pharmacogenes for different populations globally. Our results highlight inter-ethnic differences in the frequency distribution of the various pharmacogenetic markers. Thus, confirming previous studies explaining the modern human origins which have deemed Africans genetically and phenotypically diverse with heightened level of admixture and lower levels of linkage disequilibrium (LD) compared to non-Africans [187, 336]. The inter-ethnic variation on the allele frequency distribution of various pharmacogenetic markers further drives drug response, resulting in inter-ethnic variability in response to various drugs. For instance, in the context of warfarin, Africans generally present with high warfarin dose requirements compared to their Europeans counterparts [157]. Hence, we reported on a black African cohort that seems to require a much higher warfarin dose as compared to the Mixed Ancestry cohort, reflecting the European ancestry contribution to the latter's admixture. Our observations must be read considering the diversity of the genomes of African populations and therefore with no intention to sound as if this data represents what would be observed across Africa. The studied cohort is only but a small component in a huge continent blessed with genetic diversity.

It is thus, expected that the variability observed among patients with respect to warfarin treatment, also taps from the underlying genetic diversity in warfarin pharmacogenes. Inter-ethnic variation to drug response is driven by the varying allele frequency distribution of informative pharmacogenetic markers in various population groups. In the context of warfarin pharmacogenetics, this is reflected by genetic markers in the principal metabolising gene *CYP2C9* and the targeted gene *VKORC1*. For instance, *CYP2C9*2, CYP2C9*3* and *VKORC1 g.-1639G>A* are regarded as the most informative pharmacogenetic profiles for warfarin dose requirements explaining roughly 30-40% of warfarin variability and regularly included in the various warfarin dosing algorithms

developed to date [47, 49, 63]. However, among Africans, *CYP2C9*2* and *CYP2C9*3* are virtually absent and only reported in individuals with admixture such as the South African Mixed Ancestry cohort (this study), Egyptians [115], and Sudanese [109]. Thus, *CYP2C9*2* and *CYP2C9*3* have little to no influence on the warfarin dose variability observed among black Africans, which we confirmed in the present study. Whilst the *CYP2C9* variants occurring uniquely among Africans which include *CYP2C9*5, CYP2C9*6, CYP2C9*8* and *CYP2C9*11* are the alternatives in explaining the warfarin dose variability in Africans, further corroborating our findings [56, 57, 111]. *VKORC1 g.-1639G>A* is reportedly present in different population groups, although at varying frequencies, ultimately influencing warfarin dose requirements. For instance, *VKORC1 g.-1639G>A* reportedly explains approximately 18-25% of warfarin dose variability among Asians and Europeans [63, 132, 337], whilst among Africans its effect has only been observed in the admixed populations which include the Mixed Ancestry cohort (this study), Egyptians, and African Americans explaining 12%, 10–13% and 4–7% of warfarin dose variability, respectively [57, 114, 115, 132].

The lack of influence of *VKORC1 g.-1639G>A* among black Africans has driven researchers to identify SNPs such as *VKORC1 g.9041G>A (*rs7294*), Asp36Tyr (*rs61742245*) and Val66Met* (rs72547529) to be alternative *VKORC1* predictors for warfarin dose among Africans [111, 131, 138, 139]. However, in our present study, we could not establish any significant *VKORC1* SNP that could be driving warfarin dose variability in the black African cohort. Thus, suggesting that genetic markers in other alternative genes could be dominating the *VKORC1* gene on the effect they have on warfarin dose variability among the black African cohort. This is reflected by the warfarin dose variability among the black African cohort being significantly explained by various genetic markers in genes regarded as playing minor roles in warfarin disposition. In addition to the *CYP2C9* variants (*CYP2C9*8* and *CYP2C9*11*), the warfarin dose variability of the black African cohort included in our study, was significantly explained by a combination of markers in the CYP2C cluster, *CYP2C8, CYP3A5*, *MTFHR, EPHX1* and *PROC* genes. Thus, implying that the warfarin dose variability observed among black Africans is due to a collective effort of markers in various genomic positions, as

compared to the available warfarin dosing algorithms which are mainly contributed for by markers in the principal genes *CYP2C9, VKORC1* and to a lesser extent *CYP4F2*. The contribution of markers in various genes among black Africans further buttresses the importance of including a wide spectrum of genes in pharmacogenetic analyses instead of focusing only on genes described to be principally involved in the drug's pathway.

Interestingly, the proportion of warfarin dose variability (31%) explained by a collective of 8 genetic markers (i.e., CYP2C rs12777823G>A*, CYP2C9*8, CYP2C9*11, CYP2C8*2, CYP3A5*3, MTHFR c.677C>T, EPHX1 g.26978G>C* and *PROC c. G423T*) among our black African cohort is comparable to the 30-40% among Europeans and Asians that is explained by only *CYP2C9*2, CYP2C9*3* and *VKORC1 g.-1639G>A*. This could be attributed to the highest level of genetic variation and lower levels of LD that exist among Africans, suggesting that multiple genetic variation could be acting on the disposition of warfarin dose among Africans in comparison to few genetic variation observed among Europeans and Asians. Furthermore, the highest levels of LD among non-Africans means although a repertoire of genetic variants could be affecting warfarin dose requirements, if there are in high LD only one marker is useful in explaining the warfarin dose variability. For instance, haplotypes inferred from multiple *VKORC1* markers affecting warfarin dose requirements, reportedly do not explain more variability that any of the single *VKORC1* marker affecting warfarin dose requirements [61]. Similarly, the warfarin dose variability (12%) explained by the *VKORC1 g.-1639G>A* among the Mixed Ancestry cohort was equivalent to that explained by a haplotype comprised of *VKORC1 g.-1639G>A, c.1173C>T* and g.*9041G>A*. Furthermore, the warfarin dose variability observed among the Mixed Ancestry was not explained by characterised genetic markers associated with warfarin dose requirement among Africans. Thus, the genetic predictors for warfarin dose among the Mixed Ancestry resemble more of their European ancestry.

Considering the inter-ethnic variability in the occurrences of various pharmacogenetic profiles and in warfarin response. It is evident that the available warfarin

pharmacogenetic based dosing algorithms are inadequate in the prediction of warfarin dose among Africans, as the profiles explaining warfarin dose variability among Africans are unique to them and do not include any of the genetic markers important in the prediction of warfarin dose among Europeans and Asians. Thus, it is imperative to prioritise the curation and development of warfarin dosing guidelines that include informative pharmacogenetic profiles tailored specifically for Africans. The present study captures some important predictive genetic markers that are important in explaining the warfarin dose variability among Africans and we recommend including them in a pharmacogenetic dosing algorithm that could potentially improve the quality of coagulation among Africans and account for the warfarin dose variability that exists. Although we were able to capture an array of variants in multiple genomic regions and carry out association analysis of over 80 genetic markers in more than 30 genes affecting the warfarin PK and PD. Due to some limitations in our study, we believe we might have not captured or carry out association analysis in some other variants that could account for the warfarin dose variability that remains unexplained. For instance, the small sample size characterised through whole exome sequencing could only be carried out on eight samples. Furthermore, our study design was cross-sectional, thus we were unable to measure the clinical significance of the genetic variants identified through assessing their role in patients reaching the time to therapeutic range, as this requires a longitudinal study design approach. Furthermore, due to the cross-sectional study design we were unable to collect relevant information to enable the assessment of activity of the warfarin enantiomers and the effect of diet, particularly vitamin K-rich diet, on warfarin activity.

4.2. Conclusions and Future perspectives

Warfarin is anticipated to remain as the anticoagulant of choice for a very long time in low- and middle-income settings such as Africa. Thus, pharmacogenetics remains as the most applicable solution in ensuring that warfarin therapy is clinically prescribed effectively. To ensure that pharmacogenetics is effectively applied in Africans, dosing guidelines which are inclusive of African-specific pharmacogenetic profiles are required

and should be prioritised. Furthermore, this will ensure health equity, improve practice of care, anticoagulation control and further accelerate the translation of pharmacogenetics clinically. Thus, the future with respect to warfarin pharmacogenomics should focus on collaborative studies that cut across the African continent in order to capture the large-scale genomic diversity so that whatever algorithms are able to cover a wider variety of African population groups. This will ensure confirmation of the current warfarin pharmacogenetic data (including our current findings) whilst also decoding additional informative genetic profiles. The collaborative studies should also focus on ensuring that warfarin pharmacogenetic-based dosing algorithm tailored specifically for Africans are developed and validated through conducting of clinical trials. For pharmacogenetics of warfarin to be fully applied, research and implementation policies need to also be considered. In addition to the application of pharmacogenetics in the context of warfarin, it is important that physicians start to embrace the use of newer drugs, such as DOACs that appear to have better safety profiles, although currently these are prohibitively expensive.

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Appendices

Appendix 1: Supplementary materials

Publication: Muyambo S, Ndadza A, Soko ND, Kruger B, Kadzirange G, Chimusa E, Masimirembwa CM, Ntsekhe M, Nhachi CFB and Dandara C. Warfarin pharmacogenomics for precision medicine in real life clinical practice in Southern Africa: harnessing 73 variants in 29 pharmacogenes. OMICS: 26,(1): 35-50. https://doi.org/10.1089/omi.2021.0199

Supplementary Data: Detailed Methods

1. Detailed Methods

i. DNA extraction

Genomic DNA was extracted from 5ml of whole blood drawn from each participant, using a modified salting out DNA purification method (modified from Gustafson et al [1]) and Qiagen Blood Mini Kit (Qiagen, Hilden, Germany).

ii. TaqMan Genotyping assay

Allelic discrimination (Applied Biosystems, California, USA) was employed to genotype for *CYP2C9* rs12777823G>A (c_31983399_10), *CALU* rs339097 A>G (c_617432_20) and *GGCX* rs12714145 C>T (c_31839079) on a CFX Real Time Quantitative PCR (qPCR) detection system (BioRad Laboratories, California, USA).

iii. Sanger Sequencing

Ten SNPs in *CYP2C8* (rs11572105, rs11572103, rs1058930, rs188934928, rs11572101, rs11572100, rs1926705), and *VKORC1* (rs9934438, rs17708472, rs13336384)) were genotyped using PCR coupled with Sanger Sequencing. Primers were designed using IDT Primer Quest Tool (Integrated DNA Technology, Iowa, USA) and are provided as

supplementary material. The PCR reaction was a 25µL reaction consisting of 100 ng DNA template, 1U GoTaq Polymerase (Promega Cooperation, Madison, USA), 0.4µM each of primers (Integrated DNA Technologies, Illinois, USA), x1 Go Taq Flexi Colourless Buffer (Promega Cooperation, Madison, USA), 0.4mM dNTPs), (Kappa Biosystems, Cape Town, South Africa) and 1.5 mM magnesium chloride (ThermoScientific, Waltham, USA) except *VKORC1* which required 3 mM magnesium chloride. The reaction mixes were made up to 25 µL with nuclease free water (ThermoScientific, Waltham, USA). The PCR reaction was performed on a BioRad T100 Thermal Cycler (Biorad Laboratories, California, USA) and proceeded the PCR conditions are provided as supplementary material. Amplicons were viewed on a 1.5% agarose gels stained with gel red (Biotium, California, USA), cleaned and thereafter sequenced using Big-Dye Terminator V3.1 cycle sequencing kit (Life Technologies, California, USA) on an Applied Biosystems SimpliAmp Thermal Cycler (Applied Biosystems, California, USA). Post sequencing ethanol precipitation was done coupled with salting out using sodium acetate. Capillary electrophoresis of sequencing products was done on the ABI 3730 xl DNA Analyzer (Applied Biosystems, California, USA). The sequencing data was analysed using the DNAStar SeqMan Pro Sequence Assembly software (DNAStar, Madison, USA). All SNPs that were characterised by a non-sequencing method, were confirmed using Sanger Sequencing.

iv. Restriction Fragment Length Polymorphism (RFLP)

RFLP was used to genotype *VKORC1* g.-1639G>A; *VKORC1* g.9041 G>A; *CYP4F2* c.1297C>T and *CALU* g.29809A>G according to Cen et al [2], Natajaran et al [3], van Schaik et al [4], Swart et al [5], Gonzalez-Conejero et al [6] and Argalacsova et al [7], respectively. Restriction enzymes *Msp1, Aci1, PvuII* and *Fnu4H1* restriction enzymes were used for *VKORC1 g.-1639G>A, VKORC1 g.9041G>A, CYP4F2 c.1297C>T* and *CALU g.29809A>G*, respectively.

v. Sequeman Mass Array

The Sequenom Mass Array Iplex PGx platform at Inqaba Biotec, South Africa was used to genotype the following 36 SNPs: *CYP1A2* (rs2069514, rs762551), *CYP2C9* (rs1799853, rs1057910, rs28371686, rs93321321, rs7900194, rs28371685, rs9332239), *CYP2C19* (rs12248560, rs4244285, rs4986893, rs28399504), *CYP3A4* (rs35599367), *CYP3A5* (rs776746, rs10264272, rs41303343), *VKORC1*(rs9923231), *APOE* (rs429358, rs7412), *ABCB1* (rs1045642), *F2* (rs1799963), *F5* (rs6025) *CYP2B6* (rs28399499, rs3745274), *CYP2D6* (rs1065852, rs28371706, rs59421388, rs35742686, rs3892097, rs28371725, rs5030655, rs35030656, rs16974, rs72549357), and *SLCO1B1* (rs4149056) using Matrix-Assisted Laser Desorption/Ionization Time-of-flight mass spectrometry (MALDI-TOF MS). Results were visualised on the MassArray analyser 4 system (Sequenom, California, USA) where molecular weight of oligonucleotides was used to infer genotypes.

Table S1: Pharmacogenes variants included in the study

Gene	dbSNP number	Allele (Nucleotide Change)	Variant Type (Effect)	Chromosome	Genomic Region	Functional Consequence
ABCB1	rs1045642	(c.3435C>T)	Synonymous (I1145I)	7p21	Exon 26	Decreased
APOE	rs429358	(c.388T>C)	Missense (C130R)	19q13	Exon 4	
APOE	rs7412	(c.526C>T)	Missense (R176C)	19q13	Exon 4	
CALU	rs1043550	(g.29809A>G)		7q32	3'UTR	
CALU	rs339097	(g.24879A>G)		7q32	Intron 4	
COMT	rs4680	(c.472G>A)	Missense (V158M)	22q11	Exon 4	Decreased
CYP1A1	rs1048943	*2C (c.2454A>G)	Missense (I462V)	15q22	Exon 7	Normal
CYP1A2	rs2069514	*1C (g.3860G>A)	Enhancer	15q24		Increased
CYP1A2	rs762551	*1F (g.-163C>A)		15q24	Intron 1	Decreased
CYP2B6	rs28399499	*18 (c.983T>C)	Missense (I328T)	19q13	Exon 7	Decreased
CYP2B6	rs3745274	*6 (c.516G>T)	Missense (Q172H)	19q13	Exon 4	Decreased
CYP2C cluster	rs12777823	(G>A)		10q24	Intergenic	Decreased
CYP2C cluster	rs12772169	(C>T)		10q24	Intergenic	Decreased
CYP2C8	rs11572105	(g.16290G>T)		10q24	Intron 5	-
CYP2C8	rs11572103	*2 (c.805A>T)	Missense (I269F)	10q24	Exon 5	Decreased
CYP2C8	rs1058930	*4 (c.792G>C)	Missense (I264M)	10q24	Exon 5	Decreased
CYP2C8	rs188934928	*14 (c.712C>G)	Missense (A238P)	10q24	Exon 5	Decreased
CYP2C8	rs11572101	(g.15893A>G)		10q24	Intron 5	-
CYP2C8	rs11572100	(g.15853T>C)		10q24	Intron 5	-
CYP2C8	rs1926705	(g.15837C>T)		10q24	Intron 5	-
CYP2C9	rs1799853	*2 (c.430C>T)	Missense (R144C)	10q24	Exon 3	Decreased
CYP2C9	rs1057910	*3 (c.1075A>C)	Missense (I359L)	10q24	Exon 7	Decreased
CYP2C9	rs28371686	*5 (c.1080C>G)	Missense (D360E)	10q24	Exon 7	Decreased
CYP2C9	rs9332131	*6 (c.818 delA)	Frame shift	10q24	Exon 5	Inactive
CYP2C9	rs7900194	*8 (c.449G>A)	Missense (R150H)	10q24	Exon 3	Decreased
CYP2C9	rs2256871	*9 (c.752A>G)	Missense (H251R)	10q24	Exon 5	Decreased
CYP2C9	rs28371685	*11 (c.1008C>T)	Missense (R335W)	10q24	Exon 7	Decreased
CYP2C9	rs9332239	*12 (c.1465C>T)	Missense (P489S)	10q24	Exon 9	Decreased
CYP2C19	rs12248560	*17 (g.-806C>T)	Regulatory	10q24	Promoter	Increased
CYP2C19	rs4244285	*2 (c.681G>A)	Splicing defect	10q24	Exon 5	Inactive
CYP2C19	rs4986893	*3 (c.636G>A)	Stop-gain (W212X)	10q24	Exon 4	Inactive

CYP2C19	rs28399504	*4 (c.1A>G)	Initiator codon	10q24	Exon 1	Inactive
CYP2D6	rs1065852	*10 (g.100C>T)	Missense (P34S)	22q13	Exon 1	Decreased
CYP2D6	rs28371706	*17 (g.1023C>T)	Missense (T107I)	22q13	Exon 2	Decreased
CYP2D6	rs59421388	*29 (g.3183G>A)	Missense (V338M)	22q13	Exon 7	Decreased
CYP2D6	rs35742686	*3 (g.2549delA)	259 Frameshift	22q13	Exon 5	Inactive
CYP2D6	rs3892097	*4 (g.1846G>A)	Splicing defect	22q13	Intron 3	Inactive
CYP2D6	rs28371725	*41 (g.2988G>A)	Splicing defect	22q13	Intron 6	Decreased
CYP2D6	rs5030655	*6 (g.1707delT)	118 Frameshift	22q13	Exon 3	Inactive
CYP2D6	rs5030656	*9 (g.2613_2615delAGA)	Inframe deletion (K281del)	22q13	Exon 5	Decreased
CYP2D6	rs16947	*2 (g.2850C>T)	Missense (R296C)	22q13	Exon 6	Normal
CYP2D6	rs72549357	*15 (g.137_138insT)	Frameshift	22q13	Exon 1	Inactive
CYP3A4	rs35599367	*22 (g.15389C>T)		7q21	Intron 6	Decreased
CYP3A5	rs776746	*3 (g.6986A>G)	Splicing defect	7q21	Intron 3	Inactive
CYP3A5	rs10264272	*6 (c.624G>A)	Splicing defect	7q21	Intron 6	Inactive
CYP3A5	rs41303343	*7 (g.27131_27132insT)	Frame shift	7q21	Intron 11	
CYP4F2	rs2108622	*2 (c.1297C>T)	Missense (V433M)	19p13	Exon 2	Decreased
DRD2	rs1800497	(c.2137G>A)	Missense (E724K)	11q23	Exon 8	Decreased
EPHX1	rs1051740	(c.337T>C)	Missense (Y113H)	1q42	Exon 3	Decreased
EPHX1	rs2234922	(c.416A>G)	Missense (H139R)	1q42	Exon 4	Increased
F2	rs1799963	(g.20210G>A)		11p11	3'UTR	
F5	rs6025	(c.1601G>A)	Missense (R397Q)	1q24	Exon 10	
GGCX	rs12714145	(g.6317C>T)		2p12	Intron 2	
GLP1R	rs1042044	(c.780A>C)	Missense (L260F)	6p21	Exon 7	
GLP1R	rs2300615	T>G		6p21	Intron	
GLP1R	rs6923761	(c.502A>G)	Missense (G173R)	6p21	Exon 4	Decreased
MTHFR	rs1801131	(c.1298A>C)	Missense (E429A)	1p36	Exon 8	
MTHFR	rs1801133	(c.677C>T)	Missense (A222V)	1p36	Exon 5	
NR1/2	rs3732356	(g.34783T>G)		3q11	Intron 3	-
NR1/2	rs2472677	(g. 24087C>T)		3q11	Intron 1	Increased[#]
NR1/2	rs6785049	(g. 39403G>A)		3q11	Intron 5	Increased[#]
NR1/3	rs2307424	(c.540C>T)	Synonymous (P180P)	1q23	Exon 5	
NR1/3	rs3003596	(g.8784T>C)		1q23	Intron 3	

NR1/3	rs2502815	(g.9774C>T)		1q23	Intron 3	
OPRM1	rs1799971	(c.118A>G)	Missense (N40D)	6q25	Exon 1	
PNPLA5	rs5764010	(g. C>T)		22q13	Intron	
SLCO1B1	rs4149056	*5 (c.521T>C)	Missense (V174A)	12p12	Exon 5	Decreased
SULT4A1	rs763120	(c.1113A>G)		22q13	Intron 2	
VKORC1	rs9923231	*2 (g.-1639G>A)		16p11	Promoter	Decreased
VKORC1	rs9934438	(g.6484C>T)		16p11	Intron 1	Decreased
VKORC1	rs7294	*3 (g.9041G>A		16p11	3'UTR	Increased
VKORC1	rs17708472	*4 (g.6009C>T)		16p11	Intron 1	Decreased
VKORC1	rs13336384	(g.6171C>T)		16p11	Intron 1	

[#]Increases expression of CYP3A4; CYP, cytochrome P450; ABCB1, ATP Binding Cassette Subfamily B Member 1; APOE, Apolipoprotein E; CALU, Calumenin; COMT, Catechol-O-methyltransferase; DRD2, Dopamine Receptor D2; EPHX1, Microsomal epoxide hydrolase 1; F2, Coagulation Factor 2; F5, Coagulation Factor 5; GGCX, Gamma glutamyl carboxylase; GLP1R, Glucagon like peptide 1 receptor; MTHFR, Methylenetetrahydrofolate reductase; NR1/2, Nuclear Receptor subfamily 1 group 1 member 2; NR1/3, Nuclear Receptor subfamily 1 group 1 member 3; OPRM1, Opiod Receptor Mu1; PNPLA5, Patatin Like Phospholipase Domain containg 5; SLCO1B1, Solute carrier organic anion transporter family member 1B1; SULT4A1, Suphotransferase Family 4A Member 1; VKORC1, Vitamin K epoxide Reducatse.

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Table S2: List of single nucleotide polymorphisms characterised among Black Africans and Mixed Ancestry

Gene	SNP ID	Nucleotide change (allele)
ABCB1	rs1045642	*ABCB1 c.3435C>T*
CYP1A2	rs2069514	*CYP1A2 g.-3860G>A (*1C)*
CYP1A2	rs762551	*CYP1A2g.-163C>A (*1F)*
CYP2C8	rs1926705	*G>A*
CYP2C8	rs11572100	*A>G*
CYP2C8	rs11572101	*T>C*
CYP2C8	rs188934928	*CYP2C8 c.712G>C (*14)*
CYP2C8	rs1058930	*CYP2C8 c.792C>G (*4)*
CYP2C8	rs11572103	*CYP2C8 c.805A>T (*2)*
CYP2C8	rs11572105	*C>A*
CYP2C9	rs1799853	*CYP2C9 c.430C>T (*2)*
CYP2C9	rs1057910	*CYP2C9 c.1075A>C (*3)*
CYP2C9	rs56165452	*CYP2C9 c. 1076T>C(*4)*
CYP2C9	rs28371686	*CYP2C9 c.1080C>G (*5)*
CYP2C9	rs7900194	*CYP2C9 c.449G>A (*8)*
CYP2C9	rs28371685	*CYP2C9 c.1003C>T (*11)*
CYP2C19	rs12248560	*CYP2C19 g.-806C>T (*17)*
CYP2C19	rs4244285	*CYP2C19 c.681G>A (*2)*
CYP2C19	rs4986893	*CYP2C19 c.636G>A (*3)*
CYP2C19	rs28399504	*CYP2C19 c.1A>G (*4)*
CYP2C cluster	rs12777823	*G>A*

CYP2C cluster	rs12772169	*C>T*
CYP3A4	rs35599367	*CYP3A4 g.15389C>T (*22)*
CYP3A5	rs776746	*CYP3A5 g.6986A>G (*3)*
CYP3A5	rs10264272	*CYP3A5 c.624G>A (*6)*
CYP3A5	rs41303343	*CYP3A5 g.27131_27132insT (*7)*
VKORC1	rs9923231	*VKORC1 g.-1639G>A*

Table S3: Missing data according to variables among Black Africans and Mixed Ancestry

Variable	**(Black Africans, N=252)**	**(Mixed Ancestry, N=251)**
BMI	27	40
*CYP1A2*1F A(rs762551)*	7	2
*CYP1A2*1C A(rs2069514)*	7	2
CYP2C8 rs1926705G	7	6
CYP2C8 rs11572100G	7	6
CYP2C8 rs11572101C	7	6
*CYP2C8*14 c.712C*	7	6
*CYP2C8*4c.792G*	7	6
*CYP2C8*2 c.805T*	7	6
CYP2C8 rs11572105A	7	6
*CYP2C9 (*2) c.430T (rs1799853)*	12	22
*CYP2C9 (*3) c.1075C (rs1057910)*	17	22
*CYP2C9 (*5) c.1080G (rs28371686)*	17	22
*CYP2C9 (*8) c.449A (rs7900194)*	17	22
*CYP2C9 (*11) c.1003T (rs28371685)*	17	22
*CYP2C19*2 681A (rs4244285)*	9	3
*CYP2C19*3 636A (rs4986893)*	9	3
*CYP2C19*4 c.1G (rs28399504)*	9	3
*CYP2C19 g.-806C>T (*17) (rs12248560)*	9	3
CYP2C rs12777823A	14	7
*CYP3A4*22 T(rs35599367)*	6	2
*CYP3A5*3 6986G (rs776746)*	5	4
*CYP3A5*6 14690A (rs10264272)*	5	4
*CYP3A5*7 27131/27132T (rs41303343)*	5	4

Table S4: Clinical and demographic characteristics of Black African patients on warfarin treatment

Characteristics	Black Africans (N=252)
Age: mean±SD (range)	49±15 (18-87)
Weight: mean±SD (range)	73±16.9 (41.9-134)
Height: mean±SD (range)	1.63±0.08 (1.47-1.9)
BMI: mean±SD (range)	28±5.9 (16.5-47.1)
Female gender, n (freq)	195 (0.77)
Warfarin maintenance dose (mg/week): mean ±SD (range)	38±13 (12.5-75)
Warfarin indications, n (freq)	
Atrial fibrillation	34 (0.14)
Deep venous thrombosis	77 (0.31)
Mechanical valve replacement	46 (0.18)
Pulmonary embolism	14 (0.05)
Mechanical replacement (AF first)	7 (0.03)
Cardiomyopathy	8 (0.03)
Congestive cardiac failure	26 (0.10)
Rheumatic heart disease	17 (0.07)
Stroke	6 (0.02)
others	17 (0.07)
Comorbidities, n (freq)	
Hypertension	11 (0.46)
Diabetes mellitus	16 (0.06)
Heart failure	113 (0.45)
HIV positive	38 (0.15)
Arrhythmia	64 (0.25)
Concomitant drugs, n (freq)	
Statins	22 (0.09)
Efavirenz	16 (0.06)
Aspirin	3 (0.01)
Frusemide	61 (0.24)
Other drugs	10 (0.04)
Current smokers, n (freq)	23 (0.09)
Previous smokers, n (freq)	21 (0.08)
Current alcohol consumption, n (freq)	32 (0.13)
Previous alcohol consumption, n (freq)	69 (0.27)

Table S5: Allele Frequency Distribution of the studied single-nucleotide polymorphisms among Black Africans and Mixed Ancestry

Gene	SNP ID	Black Africans (freq)	Mixed Ancestry (freq)	P value
ABCB1	*ABCB1 c.3435T, rs1045642*	0.10	0.40	<0.0001
CYP1A2	*CYP1A2*1F A(rs762551)*	0.56	0.64	0.47
	*CYP1A2*1C A(rs2069514)*	0.26	0.15	0.09
CYP2C8	*rs1926705G*	0.11	0.28	0.006
	rs11572100G	0.10	0.06	0.32
	rs11572101C	0.17	0.25	0.22
	*CYP2C8*14 c.712C*	0	0	-
	*CYP2C8*4c.792G*	0.01	0.01	1
	*CYP2C8*2 c.805T*	0.22	0.07	0.005
	rs11572105A	0.03	0.02	0.65
CYP2C9	*CYP2C9 (*2) c.430T (rs1799853)*	0.01	0.04	0.18
	*CYP2C9 (*3) c.1075C (rs1057910)*	0	0.05	0.03
	*CYP2C9 (*5) c.1080G (rs28371686)*	0.01	0	0.32
	*CYP2C9 (*8) c.449A (rs7900194)*	0.10	0.02	0.02
	*CYP2C9 (*11) c.1003T (rs28371685)*	0.02	0.01	0.56
CYP2C19	*CYP2C19*2 681A (rs4244285)*	0.17	0.21	0.52
	*CYP2C19*3 636A (rs4986893)*	0	0.02	0.16
	*CYP2C19*4 c.1G (rs28399504)*	0	0.005	0.82
	*CYP2C19 g.-806C>T (*17) (rs12248560)*	0.16	0.14	0.72
CYP2C cluster	*CYP2C rs12777823A*	0.30	0.25	0.50
	CYP2C rs12772169T	0.42	0.40	0.83
CYP3A4	*CYP3A4*22 T(rs35599367)*	0	0.03	0.08
CYP3A5	*CYP3A5*3 6986G (rs776746)*	0.14	0.58	<0.0001
	*CYP3A5*6 14690A (rs10264272)*	0.21	0.04	0.0006
	*CYP3A5*7 27131/27132T (rs41303343)*	0.13	0.04	0.03
VKORC1	*VKORC1 g.-1639A, rs9923231*	0.08	0.31	0.0002

Table S6: Linkage disequilibrium mapping of the CYP2C cluster SNPs among Black Africans and Mixed Ancestry

Black Africans					Mixed Ancestry				
Marker 1	**Marker 2**	**D'**	**LOD**	**r^2**	**Marker 1**	**Marker 2**	**D'**	**LOD**	**r^2**
rs12772169	rs12777823	0.91	19.1	0.48	rs12772169	rs12777823	0.77	9.57	0.30
rs12772169	rs12248560	1.0	4.34	0.16	rs12772169	s1799853	1.0	1.42	0.03
rs12772169	rs4244285	0.94	7.2	0.24	rs12772169	rs7900194	1.0	0.79	0.03
rs12772169	rs1799853	1.0	0.44	0.008	rs12772169	rs28371685	1.0	0.4	0.006
rs12772169	rs7900194	0.79	2.69	0.09	rs12772169	rs1057910	1.0	1.22	0.07
rs12772169	rs28371685	1.0	0.35	0.03	rs12772169	rs11572103	1.0	0.66	0.05
rs12772169	rs56165452	1.0	1.18	0.02	rs12772169	rs1058930	1.0	0.59	0.02
rs12772169	rs11572103	0.54	1.29	0.05	rs12772169	rs11572101	0.62	4.7	0.19
rs12772169	rs1058930	1.0	0.13	0.009	rs12772169	rs11572100	0.21	0.03	0.002
rs12772169	rs11572101	0.75	5.25	0.16	rs12772169	rs1926705	0.61	2.14	0.10
rs12772169	rs11572100	0.87	3.08	0.07	rs12777823	rs1799853	1.0	0.98	0.01
rs12772169	rs1926705	0.48	0.57	0.02	rs12777823	rs7900194	0.44	0.41	0.01
rs12777823	rs12248560	1.0	2.65	0.08	rs12777823	rs28371685	1.0	0.66	0.02
rs12777823	rs4244285	1.0	17.3	0.49	rs12777823	rs1057910	0.67	0.55	0.009
rs12777823	rs1799853	1.0	0.47	0.005	rs12777823	rs11572103	1.0	0.41	0.02
rs12777823	rs7900194	0.82	6.49	0.19	rs12777823	rs1058930	0.47	0.26	0.008
rs12777823	rs28371685	1.0	1.16	0.04	rs12777823	rs11572101	0.56	9.12	0.31
rs12777823	rs56165452	1.0	1.53	0.03	rs12777823	rs11572100	1.0	0.66	0.02
rs12777823	rs11572103	0.76	2.26	0.06	rs12777823	rs1926705	0.32	0.38	0.01
rs12777823	rs1058930	1.0	0.42	0.02	rs1799853	rs7900194	1.0	0.11	0.001
rs12777823	rs11572101	0.79	10.7	0.32	rs1799853	rs28371685	1.0	0.04	0.0
rs12777823	rs11572100	0.78	1.35	0.03	rs1799853	rs1057910	1.0	0.35	0.003
rs12777823	rs1926705	1.0	2.79	0.05	rs1799853	rs11572103	0.23	0.0	0.0
rs12248560	rs4244285	1.0	1.93	0.04	rs1799853	rs1058930	1.0	0.05	0.0
rs12248560	rs1799853	1.0	0.0	0.0	rs1799853	rs11572101	1.0	0.4	0.02
rs12248560	rs7900194	0.66	0.14	0.008	rs1799853	rs11572100	0.09	0.0	0.0
rs12248560	rs28371685	0.47	0.01	0.001	rs1799853	rs1926705	1.0	0.41	0.02
rs12248560	rs56165452	0.42	0.01	0.001	rs7900194	rs28371685	1.0	0.02	0.0
rs12248560	rs11572103	0.72	11.4	0.45	rs7900194	rs1057910	1.0	0.16	0.001
rs12248560	rs1058930	1.0	0.16	0.002	rs7900194	rs11572103	1.0	0.14	0.001
rs12248560	rs11572101	0.75	1.07	0.03	rs7900194	rs1058930	1.0	0.03	0.0

rs12248560	rs11572100	0.02	0.01	0.0	rs7900194	rs11572101	0.21	0.02	0.0
rs12248560	rs1926705	1.0	0.99	0.02	rs7900194	rs11572100	0.09	0.07	0.003
rs4244285	s1799853	1.0	0.0	0.0	rs7900194	rs1926705	0.09	0.01	0.001
rs4244285	rs7900194	1.0	1.5	0.02	rs28371685	rs1057910	1.0	0.05	0.0
rs4244285	rs28371685	1.0	0.33	0.004	rs28371685	rs11572103	1.0	0.03	0.0
rs4244285	rs56165452	0.63	0.62	0.03	rs28371685	rs1058930	1.0	0.01	0.0
rs4244285	rs11572103	1.0	1.28	0.05	rs28371685	rs11572101	1.0	0.13	0.002
rs4244285	rs1058930	1.0	0.92	0.04	rs28371685	rs11572100	1.0	0.03	0.0
rs4244285	rs11572101	0.94	25.3	0.80	rs28371685	rs1926705	1.0	0.15	0.002
rs4244285	rs11572100	1.0	1.09	0.03	rs1057910	rs11572103	0.56	0.02	0.001
rs4244285	rs1926705	0.84	0.26	0.02	rs1057910	rs1058930	1.0	0.06	0.001
rs1799853	rs7900194	1.0	0.15	0.001	rs1057910	rs11572101	1.0	0.58	0.02
rs1799853	rs28371685	1.0	0.02	0.0	rs1057910	rs11572100	0.43	0.01	0.001
rs1799853	rs56165452	1.0	0.01	0.0	rs1057910	rs1926705	1.0	0.76	0.02
rs1799853	rs11572103	0.48	0.25	0.01	rs11572103	rs1058930	1.0	0.08	0.001
rs1799853	rs1058930	1.0	0.01	0.0	rs11572103	rs11572101	1.0	0.5	0.02
rs1799853	rs11572101	1.0	0.27	0.003	rs11572103	rs11572100	1.0	0.39	0.004
rs1799853	rs11572100	0.15	0.05	0.002	rs11572103	rs1926705	1.0	0.57	0.03
rs1799853	rs1926705	0.58	0.62	0.03	rs1058930	rs11572101	1.0	0.91	0.04
rs7900194	rs28371685	0.21	0.0	0.0	rs1058930	rs11572100	1.0	0.08	0.001
rs7900194	rs56165452	1.0	0.2	0.002	rs1058930	rs1926705	1.0	0.43	0.005
rs7900194	rs11572103	1.0	1.52	0.03	rs11572101	rs11572100	0.47	0.18	0.005
rs7900194	rs1058930	1.0	0.1	0.001	rs11572101	rs1926705	1.0	4.74	0.14
rs7900194	rs11572101	1.0	1.72	0.03	rs11572100	rs1926705	0.69	0.66	0.01
rs7900194	rs11572100	1.0	0.95	0.02	-	-	-	-	-
rs7900194	rs1926705	1.0	1.24	0.01	-	-	-	-	-
rs28371685	rs56165452	1.0	0.03	0.0	-	-	-	-	-
rs28371685	rs11572103	1.0	0.14	0.004	-	-	-	-	-
rs28371685	rs1058930	1.0	0.02	0.0	-	-	-	-	-
rs28371685	rs11572101	1.0	0.44	0.004	-	-	-	-	-
rs28371685	rs11572100	0.23	0.18	0.008	-	-	-	-	-
rs28371685	rs1926705	1.0	0.24	0.002	-	-	-	-	-
rs56165452	rs11572103	1.0	0.27	0.003	-	-	-	-	-

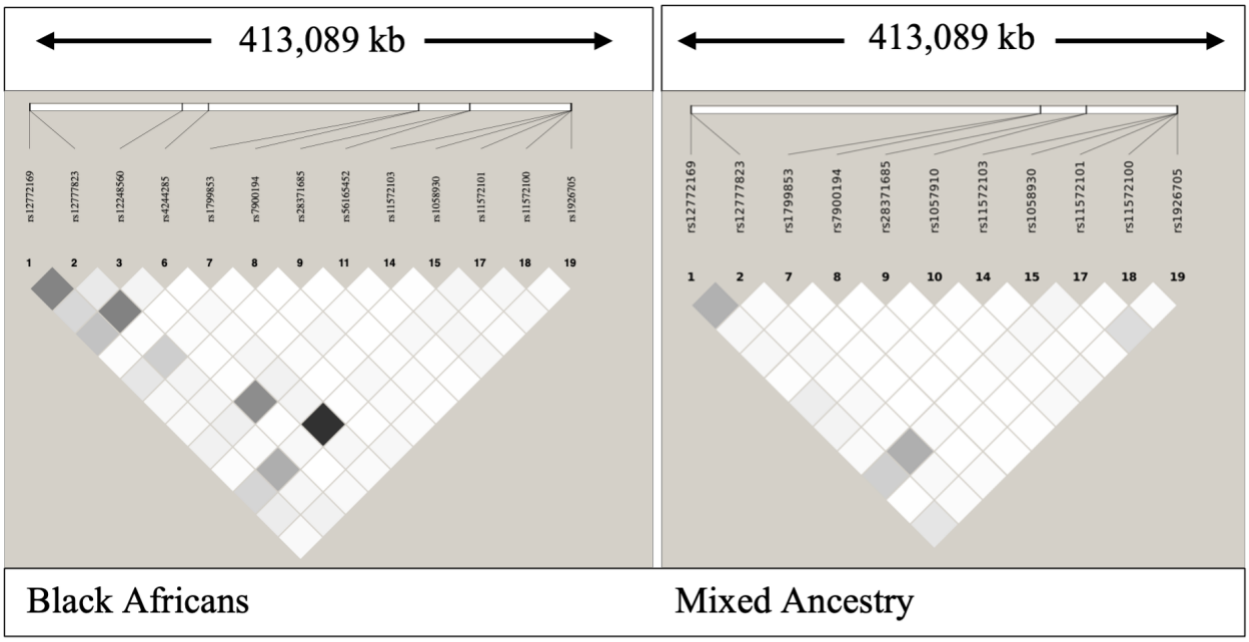
rs56165452	rs1058930	1.0	0.01	0.0	-	-	-	-	-
rs56165452	rs11572101	0.61	0.57	0.03	-	-	-	-	-
rs56165452	rs11572100	1.0	0.21	0.002	-	-	-	-	-
rs56165452	rs1926705	1.0	0.19	0.002	-	-	-	-	-
rs11572103	rs1058930	1.0	0.19	0.002	-	-	-	-	-
rs11572103	rs11572101	0.69	0.85	0.03	-	-	-	-	-
rs11572103	rs11572100	1.0	1.05	0.03	-	-	-	-	-
rs11572103	rs1926705	1.0	1.41	0.03	-	-	-	-	-
rs1058930	rs11572101	1.0	0.89	0.03	-	-	-	-	-
rs1058930	rs11572100	0.36	0.17	0.008	-	-	-	-	-
rs1058930	rs1926705	0.37	0.18	0.008	-	-	-	-	-
rs11572101	rs11572100	1.0	1.38	0.03	-	-	-	-	-
rs11572101	rs1926705	1.0	0.68	0.03	-	-	-	-	-
rs11572100	rs1926705	1.0	0.68	0.01	-	-	-	-	-

Table S7: Stepwise regression model of the effect of multiple variables on warfarin weekly maintenance dose for the combined ethnicities

Variable	Coefficient	95% CI	P value
Model including variables with p≤0.2 in univariate: R^2= 0.26, P<0.0001			
Race	0.06	-3.12 to 3.23	0.97
Age	-0.16	-0.25 to -0.09	<0.0001
BMI	0.15	-0.05 to 0.35	0.14
Atrial fibrillation	-0.11	-3.15 to 2.93	0.94
Deep venous thrombosis	3.19	0.18 to 6.22	0.04
Pulmonary embolism	-2.27	-6.52 to 1.97	0.29
Hypertension	-0.31	-2.68 to 2.05	0.79
Heart failure	-1.28	-3.71 to 1.14	0.29
Efavirenz	-0.51	-3.71 to 1.14	0.87
Tobacco smoking	0.77	-2.01 to 3.55	0.59
*CYP2C8 c.805A>T (*2), rs11572103*	8.05	2.75 to 13.3	0.003
*CYP2C8 c.792C>G (*4), rs1058930*	4.32	-0.82 to 9.46	0.09
*CYP2C9 c.430C>T (*2), rs1799853*	-3.92	-8.77 to 0.93	0.11
*CYP2C9 c.1075 A>C (*3), rs1057910*	4.76	-8.94 to -0.59	0.03
*CYP2C9 c.449G>A (*8), rs7900194*	-7.01	-10.9 to -3.06	0.0005
*CYP2C9 c.1003C>T (*11), rs28371685*	-10.0	-15.5 to-4.60	0.0003
CYP2C rs12777823G>A	-2.51	7.61 to 2.58	0.33
CYP2C rs12772169C>T	-3.01	-7.59 to 1.56	0.19
*CYP3A5 c.624G>A (*6), rs10264272*	6.56	3.66 to 9.46	<0.0001
VKORC1 g.-1639G>A, rs9923231	-15.0	-20.2 to -9.79	<0.0001
Model including variables with p≤0.05: R^2=0.23, p<0 0.0001			
Age	-0.18	-0.25 to-0.11	<0.0001
Deep venous thrombosis	4.12	1.53 to 6.73	0.002
*CYP2C8 c.805A>T (*2), rs11572103*	9.47	4.62 to 14.3	0.0001
*CYP2C9 c.449G>A (*8), rs7900194*	-5.96	-9.70 to-2.22	0.002
*CYP2C9 c.1003C>T (*11), rs28371685*	-10.5	-15.8 to -5.16	0.0001
*CYP3A5 c.624G>A (*6), rs10264272*	6.71	4.06 to 9.35	<0.0001

VKORC1 g.-1639G>A, rs9923231	-14.2	-19.2 to -9.26	<0.0001
Final model variables and interaction terms: R^2=0.24, p<0 0.0001			
Age	-0.91	-0.22 to 0.05	0.19
Deep venous thrombosis	3.67	-4.03 to 11.4	0.35
*CYP2C8 c.805A>T (*2), rs11572103*	9.11	4.21 to 14.0	<0.001
*CYP2C9 c.449G>A (*8), rs7900194*	-6.10	-9.98 to -2.22	0.002
*CYP2C9 c.1003C>T (*11), rs28371685*	-10.2	-15.6 to -4.85	<0.001
*CYP3A5 c.624G>A (*6), rs10264272*	6.39	3.59 to 9.19	<0.001
VKORC1 g.-1639G>A, rs9923231	-15.1	-20.3 to -9.92	<0.001
Ethnicity*age	-0.65	-0.14 to 0.01	0.09
Ethnicity*BMI	0.12	0.001 to 0.24	0.05
Ethnicity*deep venous thrombosis	0.34	-4.92 to 5.60	0.89
Ethnicity*mechanical valve replacement	0.56	-0.98 to 2.10	0.47
Model including variables with p≤0.05: R^2=0.23, p<0 0.0001			
Deep venous thrombosis	3.48	0.84 to 6.13	0.01
*CYP2C8 c.805A>T (*2), rs11572103*	8.69	3.83 to 13.6	<0.001
*CYP2C9 c.449G>A (*8), rs7900194*	-6.69	-10.5 to -2.91	0.001
*CYP2C9 c.1003C>T (*11), rs28371685*	-10.3	-15.6 to -4.92	<0.001
*CYP3A5 c.624G>A (*6), rs10264272*	5.77	3.08 to 8.45	<0.001
VKORC1 g.-1639G>A, rs9923231	-14.7	-19.8 to -9.64	<0.001
Ethnicity*age	-0.11	-0.15 to -0.07	<0.001
Ethnicity*BMI	0.16	0.07 to 0.26	0.001

CI=confidence interval



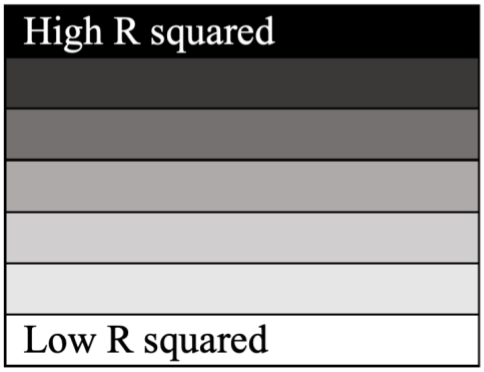


Figure S1: Linkage disequilibrium (LD) mapping of the CYP2C cluster SNPs spanning ~400 kb region among both Black Africans and Mixed Ancestry. The LD was visualised through the r squared colour scheme as determined by the HaploView software.

Table S8: Linkage disequilibrium mapping of *APOe, CALU, CYP2B6, CYP2D6, GGCX, GLP1R, MTHFR* and *VKORC1* SNPs among Black Africans and Mixed Ancestry

			Black Africans			Mixed Ancestry		
Gene	**Marker 1**	**Marker 2**	**D'**	**LOD**	**r^2**	**D'**	**LOD**	**r^2**
APOE	rs429358	rs7412	1.0	2.7	0.06	1.0	0.51	0.02
CALU	rs339097	rs1043550	1.0	0.57	0.03	0.46	0.3	0.007
CYP2B6	rs3745274	rs28399499	1.0	2.66	0.08	1.0	0.15	0.02
CYP2D6	rs28371725	rs16947	0.13	0.0	0.0	1.0	0.24	0.02
	rs28371725	rs5030656	-	-	-	1.0	0.02	0.0
	rs28371725	rs35742686	-	-	-	1.0	0.04	0.001
	rs28371725	rs3892097	1.0	0.05	0.001	0.053	0.04	0.001
	rs28371725	rs5030655	-	-	-	1.0	0.02	0.0
	rs28371725	rs61736512	1.0	0.55	0.008	1.0	0.02	0.0
	rs28371725	rs28371706	1.0	0.39	0.01	1.0	0.13	0.002
	rs28371725	rs1065852	0.05	0.04	0.002	1.0	0.33	0.006
	rs16947	rs5030656	-	-	-	1.0	0.33	0.022
	rs16947	rs35742686	-	-	-	1.0	0.24	0.004
	rs16947	rs3892097	1.0	0.21	0.003	1.0	1.13	0.03
	rs16947	rs5030655	-	-	-	1.0	0.12	0.002
	rs16947	rs61736512	0.39	0.06	0.005	1.0	0.12	0.002
	rs16947	rs28371706	0.81	0.26	0.03	1.0	0.42	0.013
	rs16947	rs1065852	1.0	0.66	0.009	1.0	0.66	0.04
	rs5030656	rs35742686	-	-	-	1.0	0.01	0.0
	rs5030656	rs3892097	-	-	-	1.0	0.04	0.001
	rs5030656	rs5030655	-	-	-	1.0	0.0	0.0
	rs5030656	rs61736512	-	-	-	1.0	0.0	0.0
	rs5030656	rs28371706	-	-	-	1.0	0.02	0.0
	rs5030656	rs1065852	-	-	-	1.0	0.05	0.001
	rs35742686	rs3892097	-	-	-	1.0	0.09	0.001
	rs35742686	rs5030655	-	-	-	1.0	0.01	0.0
	rs35742686	rs61736512	-	-	-	1.0	0.01	0.0
	rs35742686	rs28371706	-	-	-	1.0	0.04	0.001
	rs35742686	rs1065852	-	-	-	0.38	0.2	0.02
	rs3892097	rs5030655	-	-	-	1.0	0.04	0.001
	rs3892097	rs61736512	1.0	0.23	0.003	1.0	0.04	0.001
	rs3892097	rs28371706	1.0	0.29	0.004	1.0	0.26	0.004
	rs3892097	rs1065852	0.265	0.29	0.023	1.0	0.38	0.012
	rs5030655	rs61736512	-	-	-	1.0	0.0	0.0
	rs5030655	rs28371706	-	-	-	1.0	0.02	0.0
	rs5030655	rs1065852	-	-	-	1.0	0.05	0.001
	rs61736512	rs28371706	1.0	1.08	0.05	1.0	0.02	0.0
	rs61736512	rs1065852	0.02	0.0	0.0	1.0	0.05	0.001
	rs28371706	rs1065852	1.0	0.29	0.01	1.0	0.28	0.005
GGCX	rs10179904	rs2592551	1.0	0.98	0.05	1.0	3.16	0.06
GLP1R	rs6923761	rs2300615	1.0	0.09	0.001	1.0	1.2	0.03
	rs6923761	rs1042044	1.0	0.4	0.005	1.0	2.41	0.07
	rs2300615	rs1042044	1.0	1.98	0.06	1.0	7.42	0.24
MTHFR	rs1801131	rs1801133	0.18	0.01	0.0	1.0	2.3	0.08
VKORC1	rs7294	rs9934438	0.72	1.75	0.04	0.88	21.5	0.28
	rs7294	rs9923231	0.46	0.84	0.01	0.94	24.6	0.33
	rs9934438	rs9923231	0.76	17.2	0.50	0.83	53.2	0.66

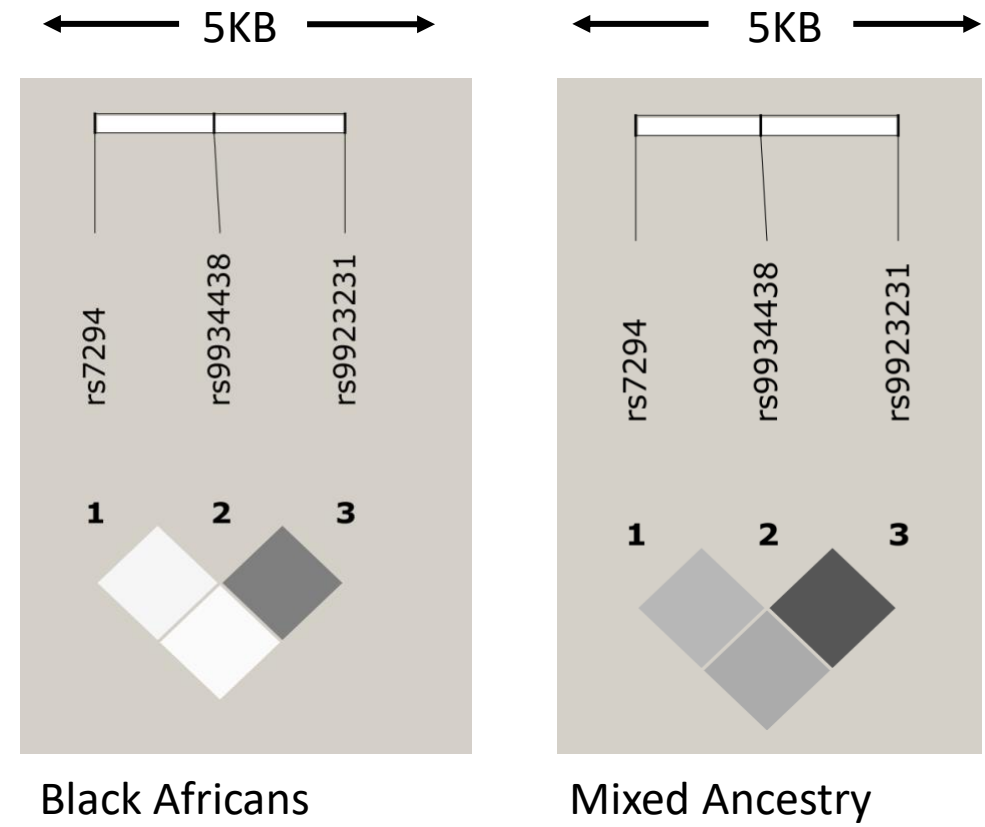


Figure S2: Linkage disequilibrium (LD) mapping of the VKORC1 (g.*9041G>A (rs7294), c.1173C>T (rs9934438) and g.-1639G>A (rs992323))* SNPs spanning ~5 kb region among both Black Africans and Mixed Ancestry. The LD was visualised through the r squared colour scheme determined by the HaploView software

Unpublished manuscript: Ndadza A, Esoh K, Muyambo S, Soko ND, Mnta P, Wonkam A, Ntsekhe M and Dandara C. Application of Whole Exome Sequencing (WES): in Search of Informative African-Specific Pharmacogenetic Profiles for Warfarin.

Table S9.1: Summary statistics of the WES QC metrics of the raw FASTQ files

Sample Name	% Dups	% GC	M Seqs
IonCode_0357_rawlib_processed	90.0%	52%	62.5
IonCode_0358_rawlib_processed	89.9%	52%	62.8
IonCode_0359_rawlib_processed	91.4%	53%	71.9
IonCode_0360_rawlib_processed	89.3%	52%	83.5
IonCode_0361_rawlib_processed	88.5%	53%	86.7
IonCode_0362_rawlib_processed	87.7%	53%	75.7
IonCode_0363_rawlib_processed	90.0%	53%	74.2
IonCode_0364_rawlib_processed	89.0%	53%	65.3

Table S9.2: Summary statistics of the WES QC metrics after filtering the FASTQ reads

Sample Name	% Dups	% GC	M Seqs
IonCode_0357_rawlib_processed.filtered	87.8%	52%	53.7
IonCode_0358_rawlib_processed.filtered	87.8%	52%	53.7
IonCode_0359_rawlib_processed.filtered	89.0%	52%	62.6
IonCode_0360_rawlib_processed.filtered	87.5%	52%	71.8
IonCode_0361_rawlib_processed.filtered	86.9%	53%	74.5
IonCode_0362_rawlib_processed.filtered	86.2%	53%	64.3
IonCode_0363_rawlib_processed.filtered	87.8%	53%	64.2
IonCode_0364_rawlib_processed.filtered	87.0%	53%	56.2

Table S9.3: Summary statistics of the WES QC metrics after alignment and recalibration of base quality scores

Sample Name	% Dups	% GC	M Seqs
IonCode_0357_rawlib_processed.bqsr	61.5%	52%	53.7
IonCode_0358_rawlib_processed.bqsr	61.4%	52%	53.7
IonCode_0359_rawlib_processed.bqsr	62.5%	52%	62.6
IonCode_0360_rawlib_processed.bqsr	60.9%	52%	71.8
IonCode_0361_rawlib_processed.bqsr	59.2%	53%	74.5
IonCode_0362_rawlib_processed.bqsr	60.7%	53%	64.3
IonCode_0363_rawlib_processed.bqsr	62.1%	53%	64.2
IonCode_0364_rawlib_processed.bqsr	61.5%	53%	56.2

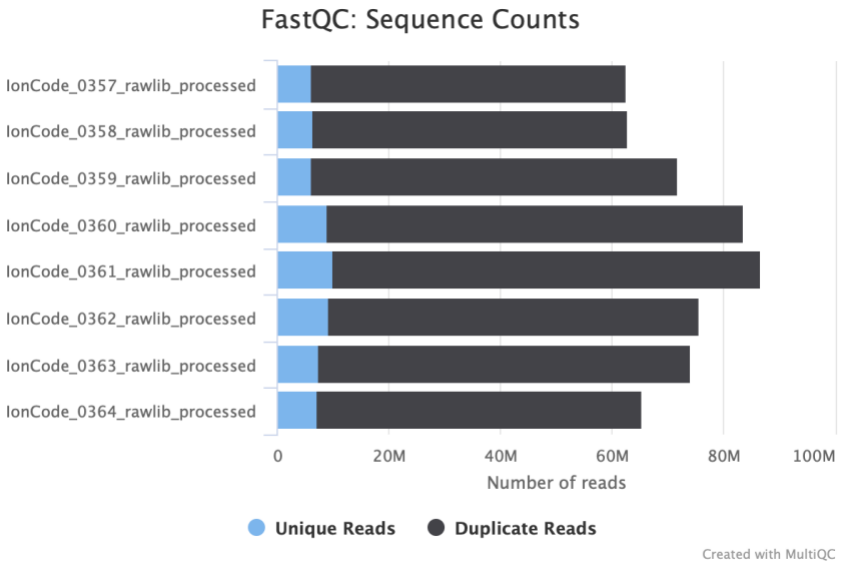


Figure S3.1: FastQC sequence counts of the raw FASTQ files

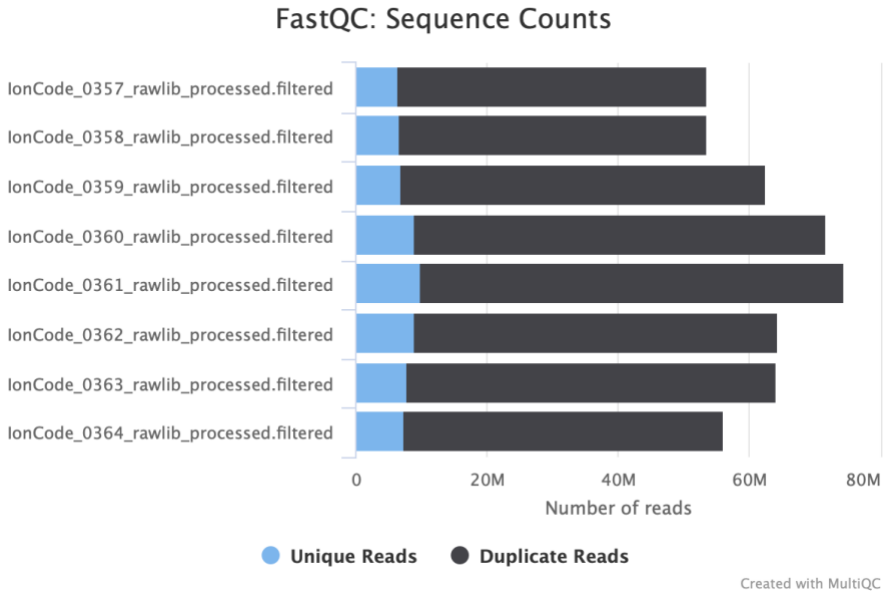


Figure S3.2: FastQC sequence counts after filtering the FASTQ reads

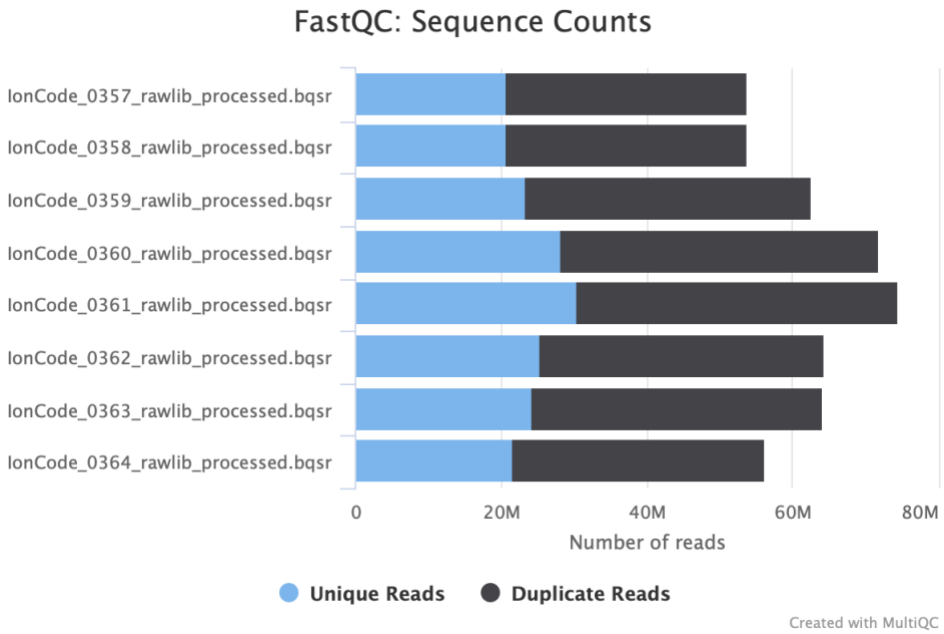


Figure S3.3: FastQC sequence counts after alignment and recalibration of base quality scores

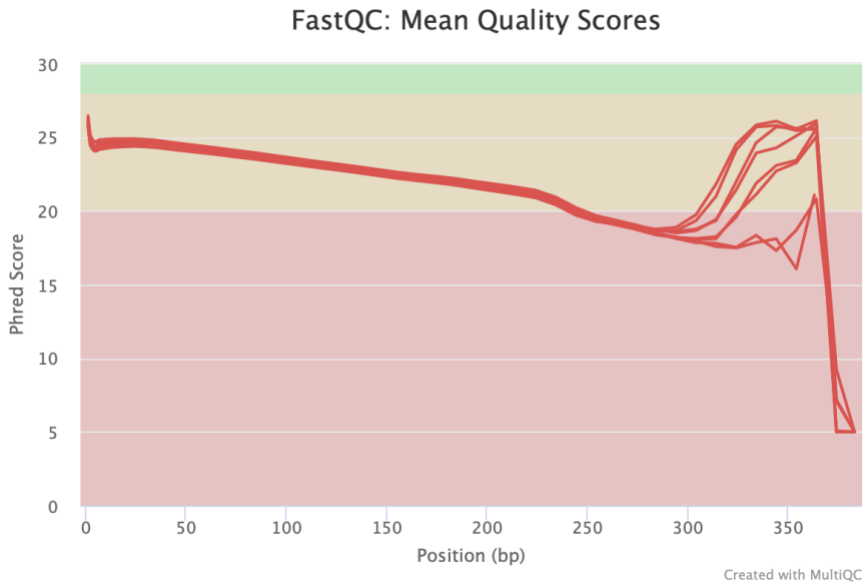


Figure S4.1: FastQC mean quality value across each base position in the read of the raw FASTQ files

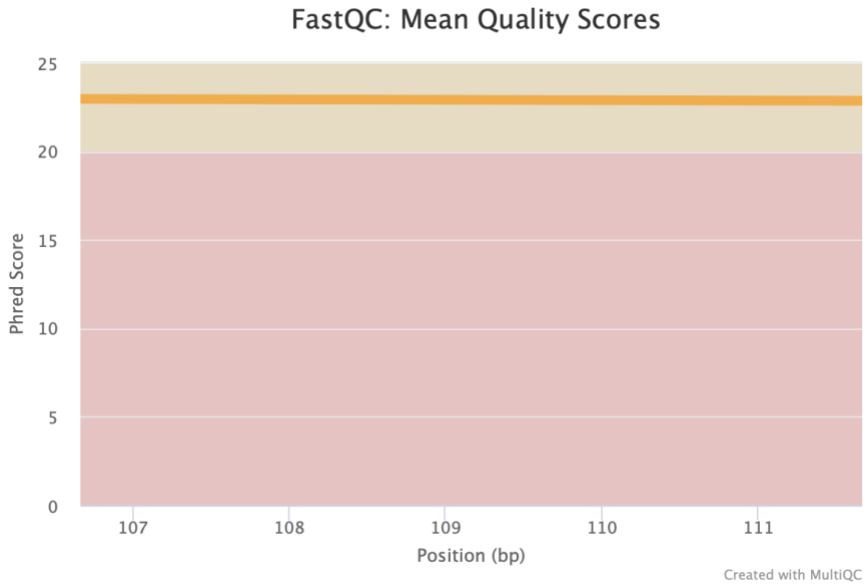


Figure S4.2: FastQC mean quality value across each base position in the read after filtering the FASTQ reads

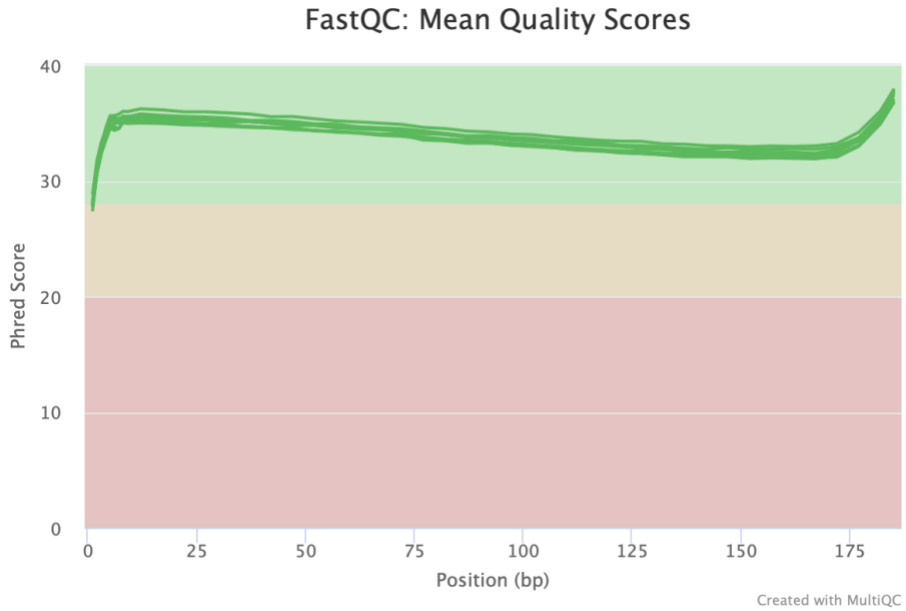


Figure S4.3: FastQC mean quality value across each base position in the read after alignment and recalibration of base quality scores

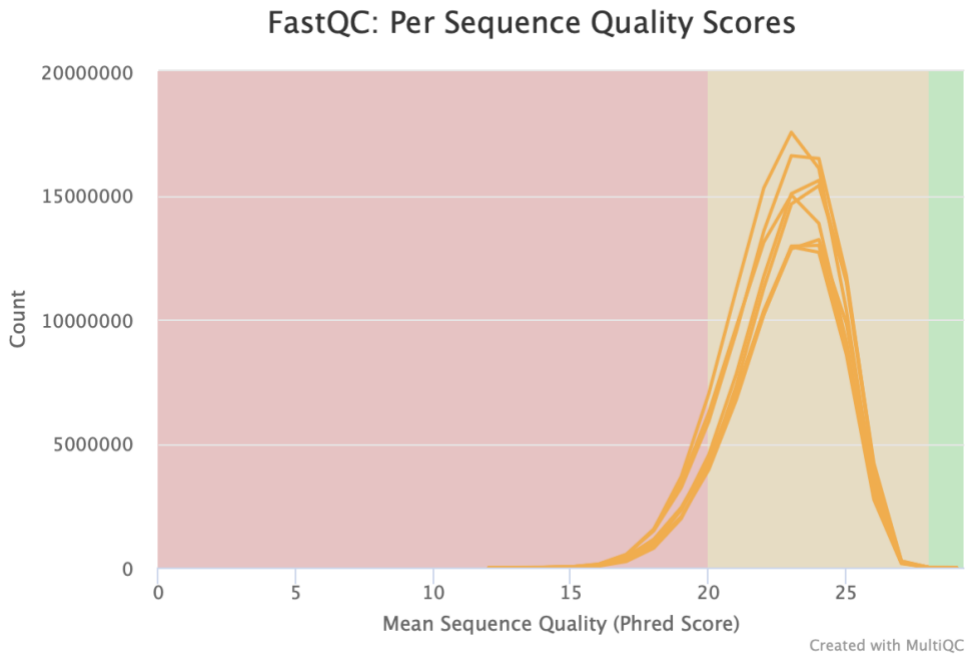


Figure S5.1: FastQC: the number of reads with average quality scores of the raw FASTQ files

Figure S5.2: FastQC number of reads with average quality scores after filtering the FASTQ reads

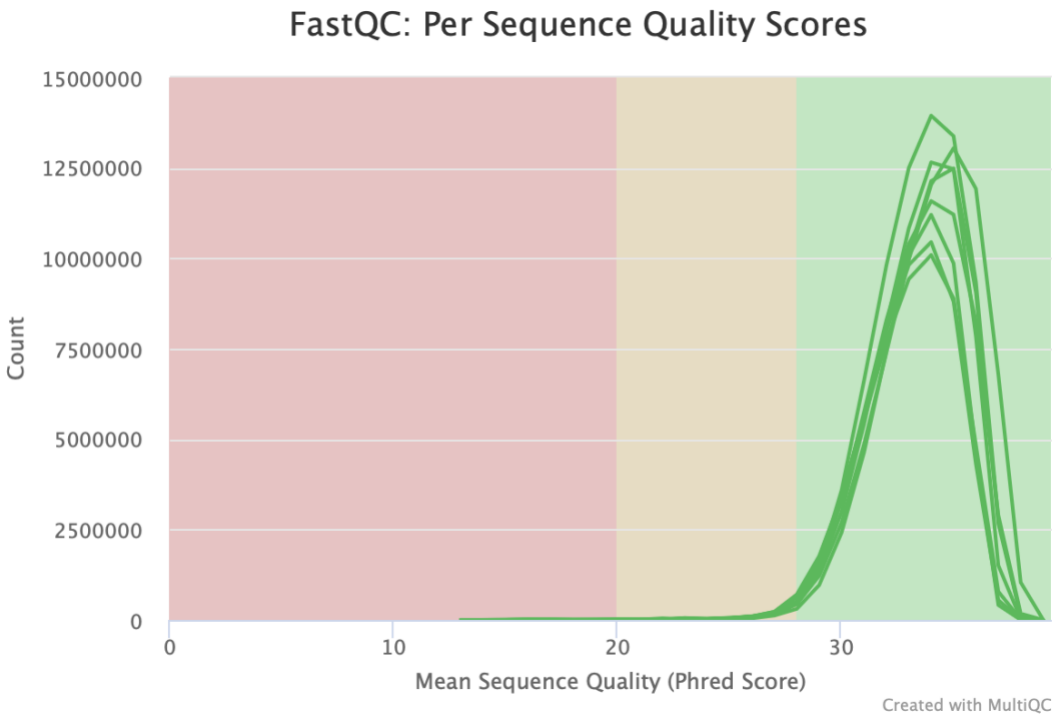


Figure S5.3: FastQC number of reads with average quality scores after alignment and recalibration of base quality scores

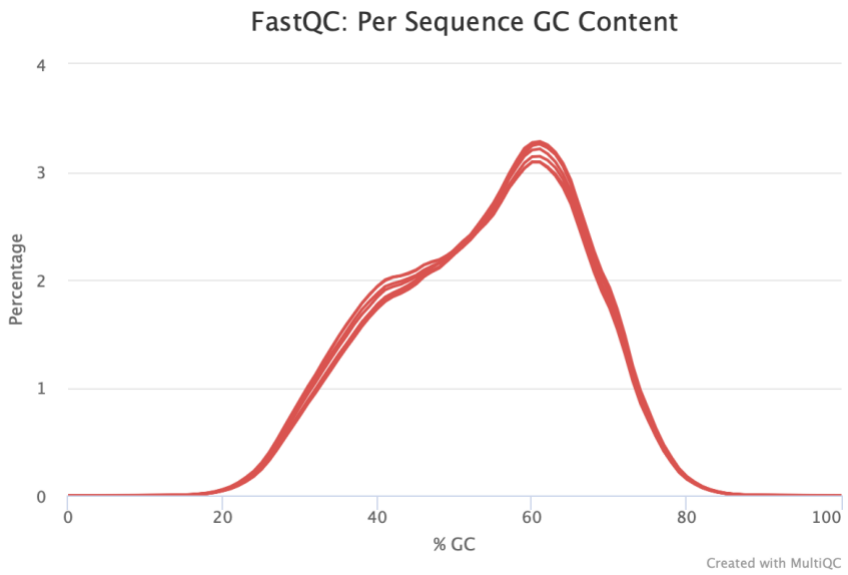


Figure S6.1: FastQC average GC content of reads of the raw FASTQ files

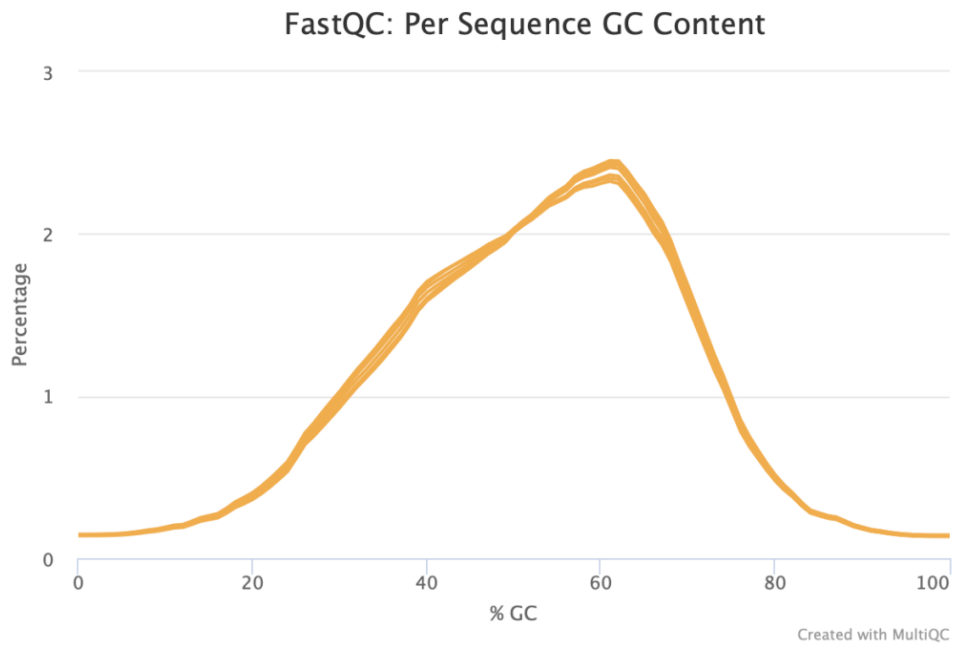


Figure S6.2: FastQC average GC content of reads after filtering the FASTQ reads

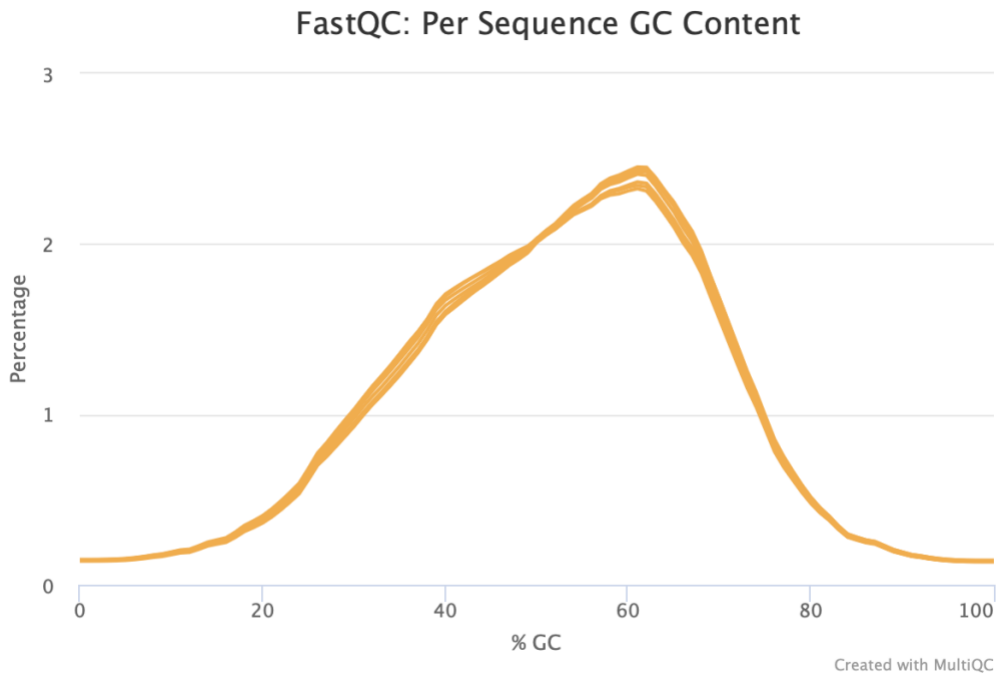


Figure S6.3: FastQC average GC content of reads after alignment and recalibration of base quality scores

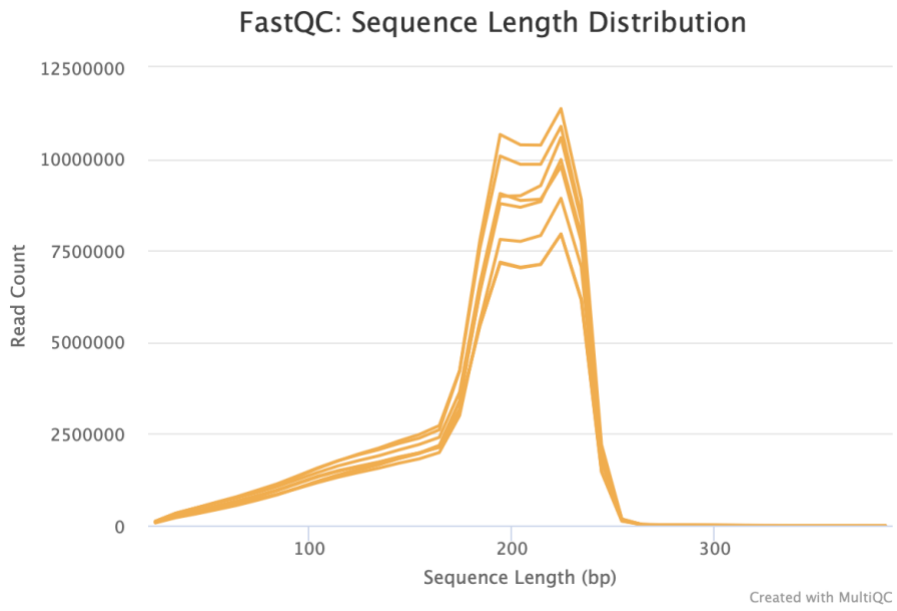


Figure S7.1: FastQC: The distribution of fragment sizes (read lengths) of the raw FASTQ files

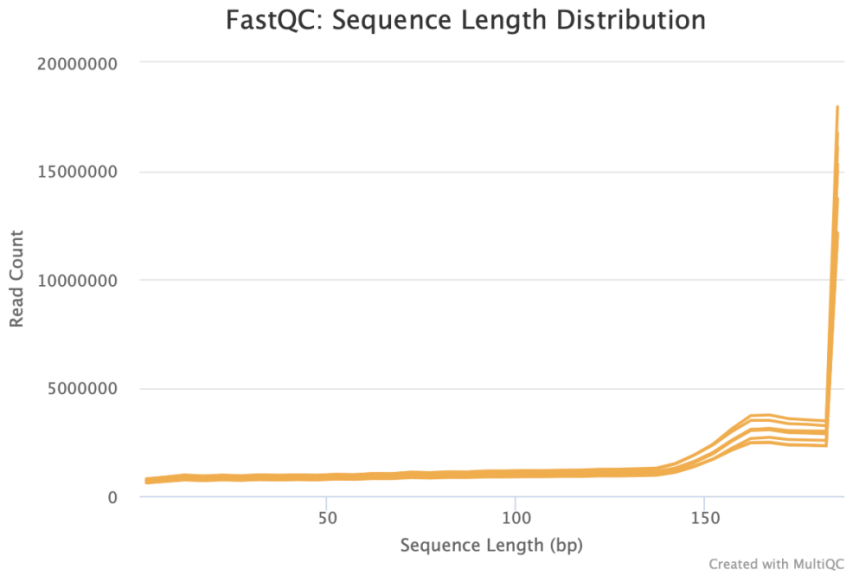


Figure S7.2: FastQC: The distribution of fragment sizes (read lengths) after filtering the FASTQ reads

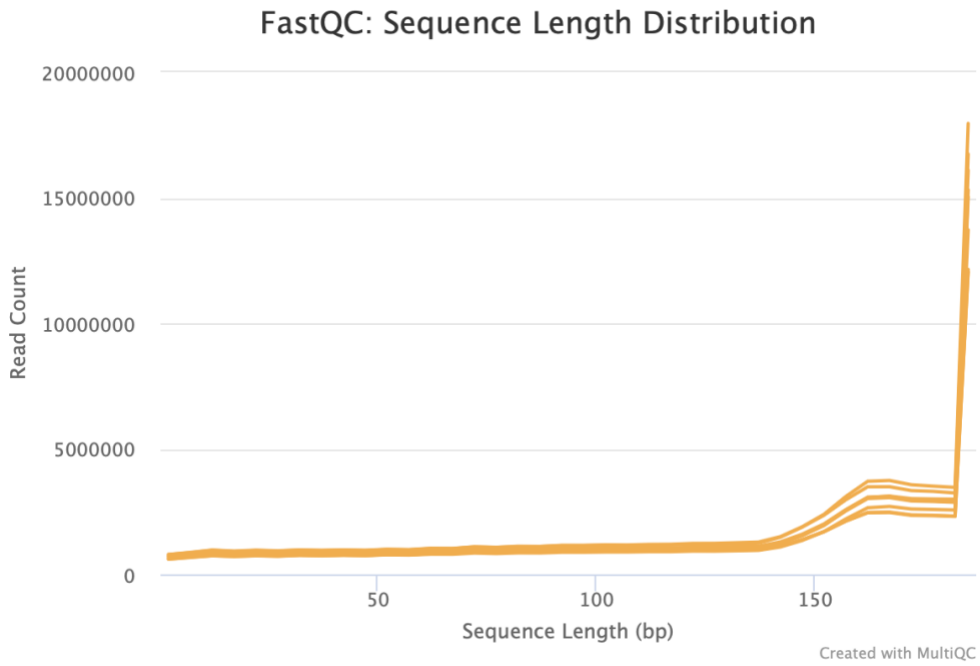


Figure S7.3: FastQC: The distribution of fragment sizes (read lengths) after alignment and recalibration of base quality scores

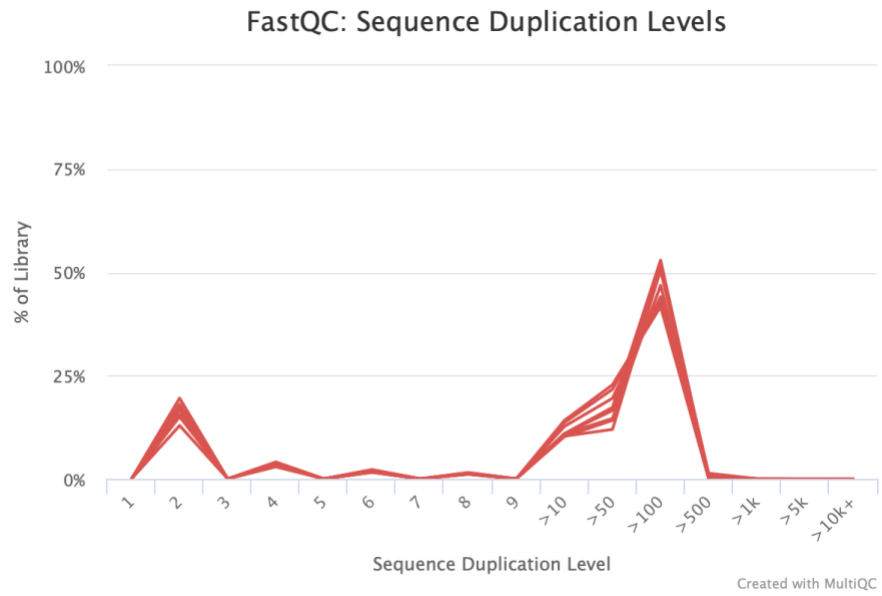


Figure S8.1: The relative level of duplication found for every sequence of the raw FASTQ files

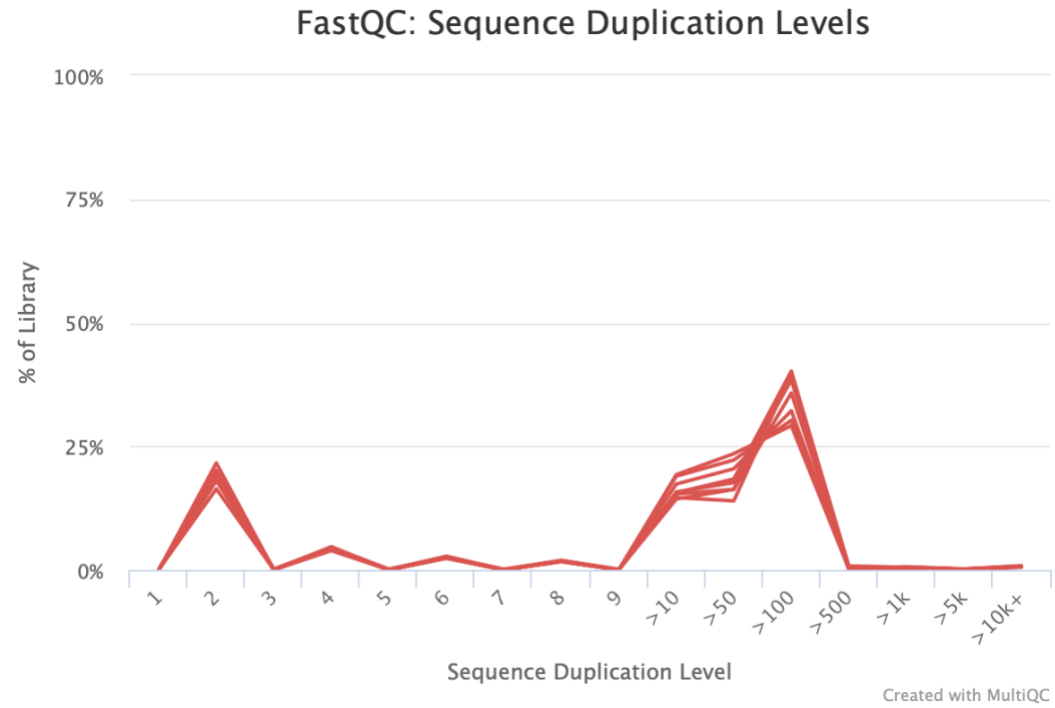


Figure S8.2: The relative level of duplication found for every sequence after filtering the FASTQ reads

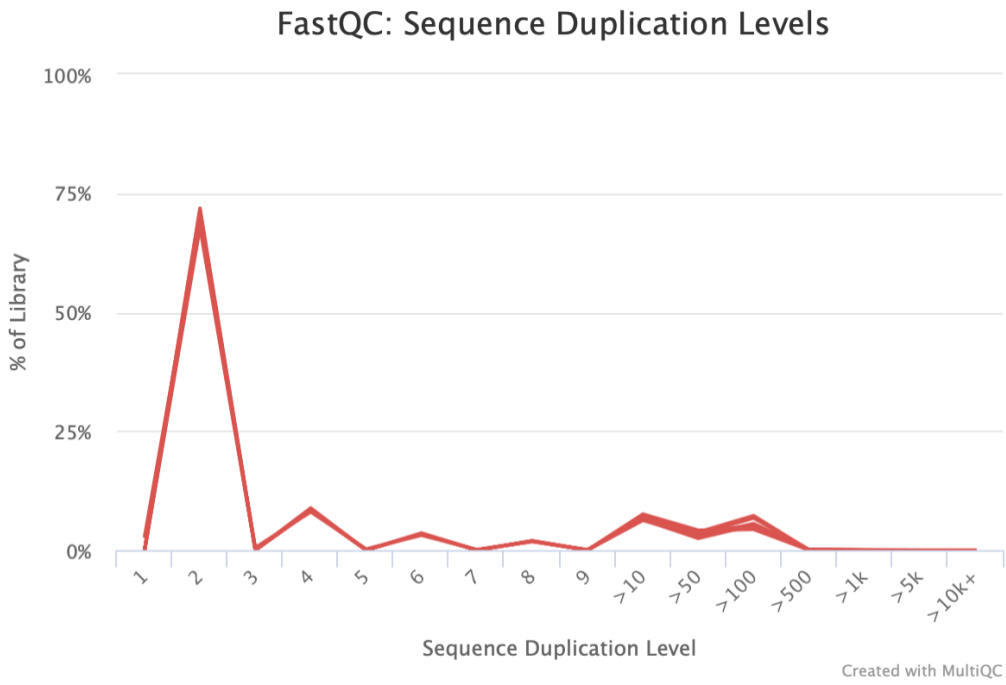


Figure S8.3: The relative level of duplication found for every sequence after alignment and recalibration of base quality scores

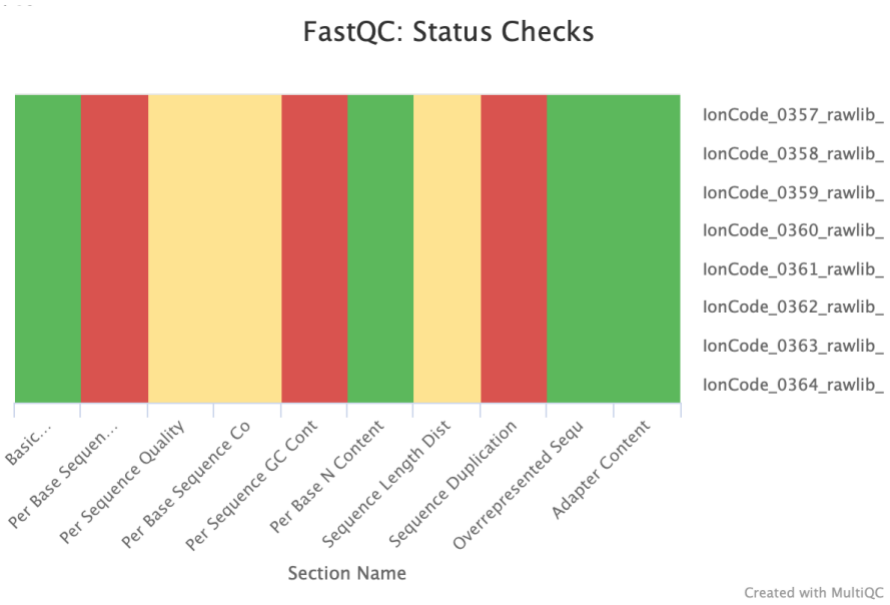


Figure S9.1: Status for each FastQC section showing whether results seem entirely normal (green), slightly abnormal (orange) or very unusual (red) of the raw FASTQ files

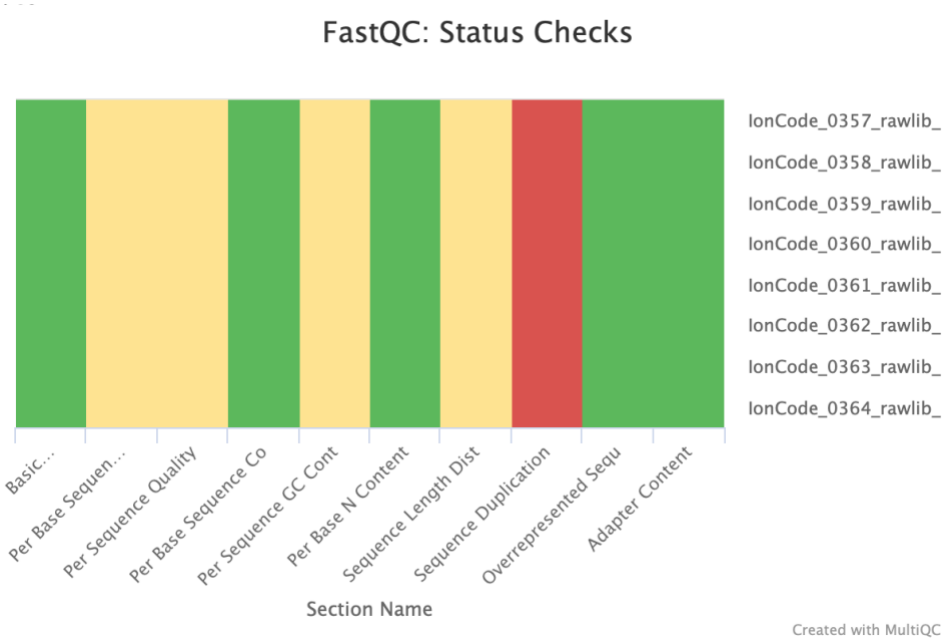


Figure S9.2: Status for each FastQC section showing whether results seem entirely normal (green), slightly abnormal (orange) or very unusual (red) after filtering the FASTQ reads

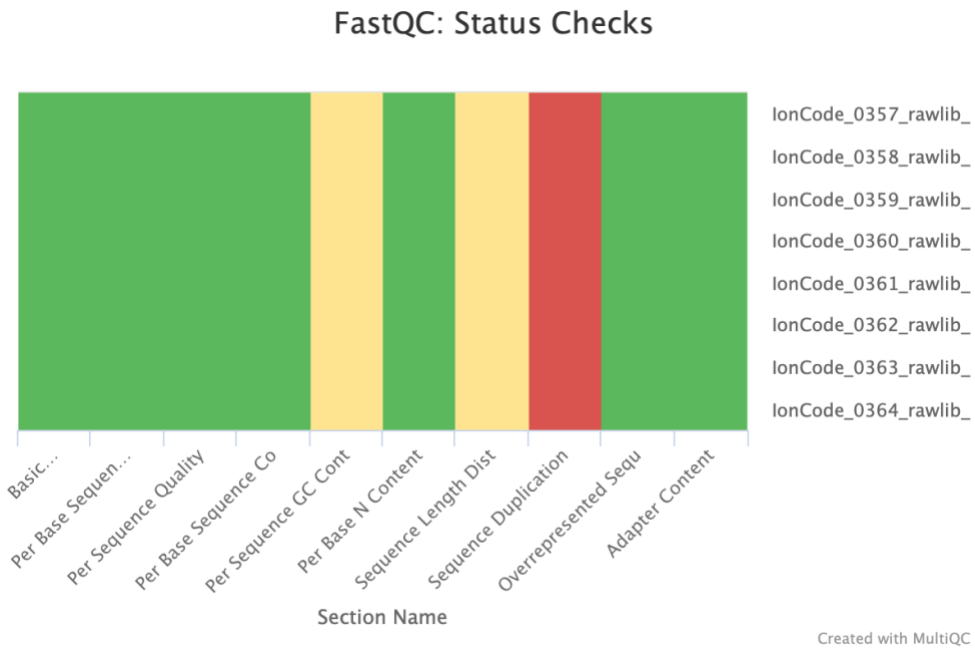


Figure S9.3: Status for each FastQC section showing whether results seem entirely normal (green), slightly abnormal (orange) or very unusual (red) after alignment and recalibration of base quality scores

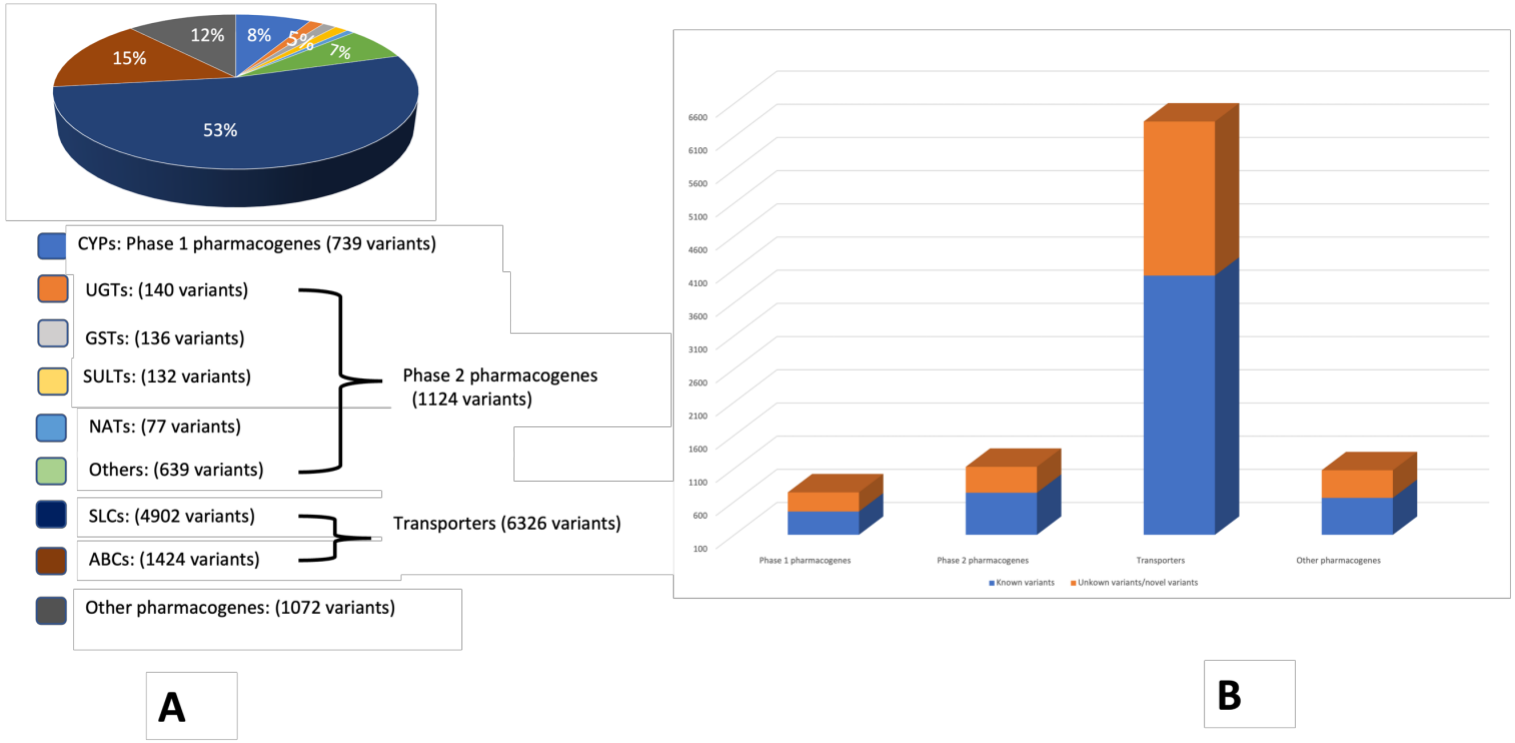


Figure S10: a) Illustration of the distribution of the 9 261 variants identified across the various pharmacogenes, 53% variants identified in SLCs transporters genes, followed by 15% identified among ABCs transporter genes, 12% among other pharmacogenes, 8% among CYPs (phase 1 pharmacogenes), 7% among other phase pharmacogenes and lastly 5% identified across the phase 2 pharmacogenes namely the UGTs, GSTs, SULTs and NATs. b) Distribution of the known and unknown variants identified across the various pharmacogenes as follows; phase 1 pharmacogenes: 451 known and 288 unknown variants, phase 2 pharmacogenes: 734 known and 390 unknown variants, transporter genes: 4005 known and 2321 unknown variants and other pharmacogenes: 656 known and 416 unknown variants.

Table S10: List of SNPs prioritised and method of characterisation

Gene	SNP rsID/genomic position	Method of characterisation	Primer sequence	Annealing Temp	Restriction enzyme/Taqman ID	Digestion conditions
ABCB1	rs17064	RFLP	Forward: 5'-GTCTCTGAAGACTCTGAACTTGAC-3' Reverse: 5'-CACCATCCAGAATGCAGACTTA-3'	61.1°C	ApoI: RAATTY	Incubation: 50°C Heat inactivation:80°C
ApoB	rs1042034	Taqman	-	-	C___7615376_20	-
	rs679899	Taqman	-	-	C___1026583_10	-
CDH3	rs10270308	Taqman	-	-	C___2618666_10	-
CYP2C9	rs2256871	Sanger sequencing	Forward: 5'-TTAGAATTGATCCTCTGGTCAG-3' Reverse: 5'-ACAAGCAGTCACATAACTAAGC-3'	55.7°C	-	-
	rs9332127	Sanger sequencing	Forward: 5'-ACCAGCTAGGTTGTAATGGTC-3' Reverse: 5'-TAATGGAGCAGATCACATTGC-3'	55.7°C	-	-
EPHX1	rs1051740	Sanger sequencing	Forward: 5'-AGCTGCTTCCACTATGGCTTCAAC-3' Reverse: 5'-ATGTATGTGTTCCTGCCTAGCTC-3'	61.1°C	-	-
	rs1131873	Sanger sequencing	Forward: 5'-AGCTGCTTCCACTATGGCTTCAAC-3' Reverse: 5'-ATGTATGTGTTCCTGCCTAGCTC-3'	61.1°C	-	-
	rs373523374	Sanger sequencing	Forward: 5'-AGCTGCTTCCACTATGGCTTCAAC-3' Reverse: 5'-ATGTATGTGTTCCTGCCTAGCTC-3'	61.1°C	-	
	rs112043151	Sanger sequencing	Forward: 5'-AGCTGCTTCCACTATGGCTTCAAC-3' Reverse: 5'-ATGTATGTGTTCCTGCCTAGCTC-3'	61.1°C	-	-
	rs2260863	Sanger sequencing	Forward: 5'-AGCTGCTTCCACTATGGCTTCAAC-3' Reverse: 5'-ATGTATGTGTTCCTGCCTAGCTC-3'	61.1°C	-	-
	rs2234922	RFLP	Forward: 5'-TGTGAGTGTGAAACCAGTGTCAG-3' Reverse: 5'-TACCCTTCTTGGAGGATGCCTCTG-3'	61.1°C	BceAI: ACGGC	Incubation: 37°C Heat inactivation:65°C
GSTP1	rs1695	RFLP	Forward: 5'-ATCCTTCCACGCACATCCTCTTC-3' Reverse: 5'-TACTTGGCTGGTTGATGTCCCAG-3'	61.1°C	HpyCH4IV: ACGT	Incubation: 37°C Heat inactivation:65°C
NQ01	rs1800566	RFLP	Forward: 5'-AATCCTGCCTGGAAGTTTAGGTC-3' Reverse: 5'-TATGGAGAGGCAGAGAAATGCAC-3'	61.1°C	TfiI: GAWTC	Incubation: 65°C Heat inactivation: none
PROC	rs5936	Taqman	-	-	C___1841978_20	-
	rs5937	RFLP	Forward:5'-TCCTGCTGGACTCAAAGAAGAAGC-3' Reverse:5'-CTTCTGTGGAGCTCAAGAAGCC-3'	61.1°C	BtsCI: GGATG	Incubation: 50°C Heat inactivation: 80°C
	rs2854696	Taqman	-	-	C__16071201_10	-
VKORC1	rs7200749	Taqman	-	-	C__29057362_10	-

CYP2C8	95064999	Sanger sequencing	Forward: 5'-ATCTTGGCCTTACCTGGATCCATG-3' Reverse: 5'-TGTGAATAACCACATGCGAAATGAC-3'	58.8°C	-	-
F9	139561919	Sanger sequencing	Forward:5'-ATGACATTGCCCTTCTGGAACTG-3' Reverse:5'-AGTTGACATACCGGGATACCTTG-3'	58.8°C	-	-
F7	113118926	Sanger sequencing	Forward: 5'-ATCACGGAGTACATGTTCTGTG-3' Reverse:5'-AGTCTCTTTGAATCTTGGAGTCTC-3'	58.8°C	-	-
HMOX1	35386980	Sanger sequencing	Forward:5'-TCTACTTCCCAGAAGAGCTGCAC-3' Reverse:5'-TGGATGTTGAGCAGGAACGCAG-3'	58.8°C	-	-
	35386978	Sanger sequencing	Forward:5'-TCTACTTCCCAGAAGAGCTGCAC-3' Reverse:5'-TGGATGTTGAGCAGGAACGCAG-3'	58.8°C	-	-
LRP1	57193674	Sanger sequencing	Forward:5'-TGCAAGAAGACTTTCCGGCAGTG-3' Reverse:5'-ATCGTCAGGCCAGAGTTTTCTCTG-3'	58.8°C	-	-
GGCX	85552409	Sanger sequencing	Forward:5'TCAGCATGTTATTGCCAGTCCAG-3' Reverse:5'-AGGATCATGCAGACATGCTGAAGC-3'	58.8°C	-	-
STAB2	103638121	Sanger sequencing	Forward:5'-AGCAAATTCTTAGAGCAGGGCTTAG-3' Reverse:5'-TACCTGTGACACATAGTGGCTTGTC-3'	58.8°C	-	-
PROZ	113159340	Sanger sequencing	Forward:5'-TTCTACCACCAGCCACTTCACTG-3' Reverse:5'-ACTTATACATTGCAGCAGGCTCTG-3'	58.8°C	-	-

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Appendix 2: Published papers excluded, with significant contribution of the candidate

Editorial

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Pharmacogenomics

The importance of including African populations in pharmacogenetics studies of warfarin response

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> "Simply expressed, African population groups differ from each other more than Africans differ with either European or Asian populations, because the latter two, are subsets of the African population."

Tweetable abstract: Warfarin will remain in use for a long time to come, thus inclusion of African populations in pharmacogenomics is essential to be able to identify all possible biomarkers that are potential predictors for warfarin drug response.

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Keywords: warfarin • pharmacogenomics • Africa

Warfarin is a widely used blood thinning agent whose clinical indications include recurrent thrombosis in patients with venous thromboembolism. Its low cost ensures warfarin will continue to be prescribed, particularly in poorly resourced healthcare settings such as those found in many African countries. In sub-Saharan Africa, warfarin costs anything from US$0.05 to US$0.20 for a 5 mg tablet in comparison with direct oral anticoagulants (DOACs), whose cost–effectiveness is still not known [1]. While DOACs may appear as a solution to the challenges faced with using warfarin, DOACs remain unaffordable in resource limited developing countries in Africa [1]. Early studies have also shown that DOACs have additional limitations which include their short-half lives, which makes adherence a huge problem, most importantly drug–drug interactions with drugs that are used for treating infectious diseases such as tuberculosis (TB) [2]. Africa already carries a high burden of infectious diseases [3,4], which is now coupled with an increasing burden of non communicable diseases. Based on this, it is anticipated that an increasing need for anticoagulation will ensure warfarin remains an important drug on the African continent for the foreseeable future.

Warfarin is the most evaluated drug in pharmacogenetic-guided dosing studies. However, gaps remain regarding the influence of the genetic polymorphisms in the commonly studied genes *CYP2C9, VKORC1* and *CYP4F2* and other genes thought to play 'minor' roles on specific pharmacodynamic parameters like the warfarin sensitivity index and international normalized ratio (INR). Large ethnic and population-level differences are observed in the allele frequencies of *CYP2C9, VKORC1* and *CYP4F2* variants [4,5]. However, these alleles show qualitative and quantitative differences in their frequencies across world populations when comparing among European, Asian and African populations. These differences emphasize the need for population-specific algorithms for warfarin dose optimization.

It is important to note that nearly all genetic variants with functional significance seen through their influence on disease risk, have human-specific origins [6]. Humans as they are currently dispersed across the world, owe their being to the 'out-of-Africa origin', thus understanding human evolution and the genetic architectures of diseases, can help explain how and why modern humans become ill and how they respond to medication while on treatment. Importantly, populations today are found in geographical areas markedly different from those of their ancestors.

Future Medicine

This evolutionary 'mismatch' results in genetic variants that were adaptive in the past now being associated with an increased risk of disease or poor therapeutic drug response due to changed gene–environment interactions. The above is best illustrated by the human apolipoprotein L1 protein that is encoded by *APOL1* gene. Genetic variants in *APOL1* confer resistance to infection by *Trypanosoma brucei* parasites that cause African sleeping sickness, a positive selection signature; however, the same *APOL1* variants are associated with an increased risk for kidney disease in African Americans [7]. Thus, inclusivity of worldwide populations in warfarin pharmacogenomics research can improve equity.

The frequencies of several SNPs of important ADME genes that impact on warfarin disposition, including *CYP2C9* and *VKORC1*, are markedly different in African populations compared with Europeans and Asians [4,8]. In a study by Ndadza *et al.* [4] it was observed that among black Africans, the SNPs *CYP2C rs12777823G >A, CYP2C9 c.449G >A (*8), CYP2C9 c.1003C > T (*11)* and *CYP2C8 c.805A > T (*2)* were significantly associated with warfarin maintenance dose, while among the Mixed Ancestry, *CYP2C9 c.430C > T (*2), CYP2C8 c.792C >G (*4)* and *VKORC1 g.-1639G >A* were the important pharmacogenes variants associated with variability in warfarin maintenance dose. In addition, variants *CYP2C8*2* and *CYP3A5*6*, not commonly evaluated in European and Asian populations, were reportedly associated with increased mean warfarin maintenance dose and *CYP2C9*8* with reduced warfarin maintenance dose [4]. However, among Europeans variants that contribute significantly to warfarin dose variability are *VKORC1 g.-1639G >A* and *CYP2C9* variants (i.e., *CYP2C9*2 and CYP2C9*3*), explaining approximately 30 and 11% of warfarin dose variability, respectively, while among populations of African ancestry these variants explain less than 10% [9–11]. Among Northern Indians *CYP2C9*3* polymorphism alone accounts for 29% of variation in dose response and can be seen as large compared to reports on African studies [12]. Thus, highlighting the high differences in genetic markers that influence warfarin dose requirements among various population groups.

Several algorithms proposed for estimation of warfarin starting doses include both pharmacogenetics and clinical iterations [4,13]. Evaluating emerging data on pharmacogenetics-guided warfarin algorithms, it is clear that the pharmacogenetic data (i.e., *CYP2C9*2 and CYP2C9*3*) included in available dosing algorithms play insignificant roles in warfarin dosing variability among Africans. Thus, advocating for inclusion of African specific pharmacogenetic markers in warfarin dosing algorithms is necessary. Ndadza *et al.* [4] reported on other variants that seem to play a more significant roles on warfarin variability in black Africans and these include genetic variants *CYP2C rs12777823G >A, CYP2C8 c.805A > T (*2), CYP2C9 c.449G >A (*8), CYP2C9 c.1003C > T (*11)* and *CYP3A5 c.624GA (*6)*. Thus, among black Africans, a warfarin-dosing algorithm that should include genetic variants *VKORC1 g.-1639 G >A, CYP2C rs12777823G >A, CYP2C8 c.805A > T (*2), CYP2C9 c.449G >A (*8), CYP2C9 c.1003C > T (*11)* and *CYP3A5 c.624GA (*6)* in conjunction with demographic and clinical variables age, gender, BMI, presence of deep venous thrombosis and mechanical valve replacement, is proposed. More efforts should be put in decoding the contribution of other less studied genes that are involved in vitamin K recycling, such as NPC1L1, a key transporter of vitamin K (VK) intestinal absorption, which may modulate the anticoagulant effect of warfarin [14].

Most warfarin dosing algorithms have been developed in Asians and Europeans and may not be applicable to underserved populations [13]. Figure 1 compares the frequencies among African, Asian and European populations, of seven pharmacogenes variants in genes reported to play a role in warfarin disposition. There are quantitative and qualitative differences in the distribution and frequencies of variant alleles among the African, Asian and European populations, respectively, this is shown by unpublished work in our group which highlights the following respective allele frequency distributions; *ABCB1* rs1045642T (0.09, 0.40 and 0.52), *CALU* rs1043550G (0.07, 0.07 and 0.42), *CALU* rs339097G (0.21, 0.01 and 0.00), *CYP2C* cluster rs12777823A (0.30, 0.31 and 0.15), *CYP2C8* rs11572105T (0.21, 0.00 and 0.00), *CYP2C9* rs1799853T (0.01, 0.001 and 0.12) and *CYP2D6* rs59421388A (0.15, 0.00 and 0.00) (Dandara, personal communication). Whereas, all the seven pharmacogene variants are present in black African populations, three variants, *CALU* rs339097G, *CYP2C8* rs11572105T and *CYP2D6* rs59421388A, are either rare or absent in both Asian and European populations [15]. For these selected genetic variants, Africans exhibit the greatest genetic diversity. This is true for most of the genome variation as it has been widely accepted that black African populations are the most genetically diverse [16,17]. Thus, given that most therapeutic drugs are developed supported by clinical trials conducted mostly in European and Asian populations, the contribution of black African-specific genetic variants is mostly missed in the drug discovery and development phases and their effects or contribution only realized when adverse drug reactions are observed after these drugs are released for use among African populations, post-approval. Simply expressed, African population groups differ from each other

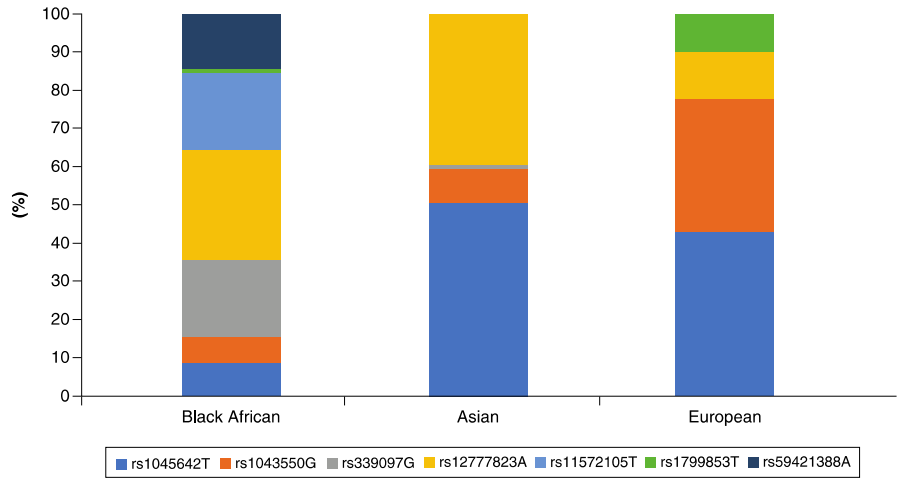

Figure 1. Comparison of frequencies of pharmacogenes variants among black Africans, Asians and Europeans.

more than Africans differ with either European or Asian populations, because the latter two, are subsets of the African population. Thus, the rich genomic diversity of African populations and the possibility of missing African-specific genetic variants is a strong motivation for inclusion of African populations in pharmacogenomics studies in general [18,19] and those investigating genetic contribution to variability observed in warfarin responses [17].

Conclusion

African populations present with a diversity of variants that are important in predicting pharmacogenetics-based warfarin dosing in addition to those reported in *CYP2C9* and *VKORC1*. In an effort to identify all possible biomarkers that are potential predictors of drug [4], African populations should be included in pharmacogenomics studies. Warfarin seems will be in use for a long time to come; thus, pharmacogenomics studies that include populations from poor or developing countries will assist in health equity.

Financial & competing interests disclosure

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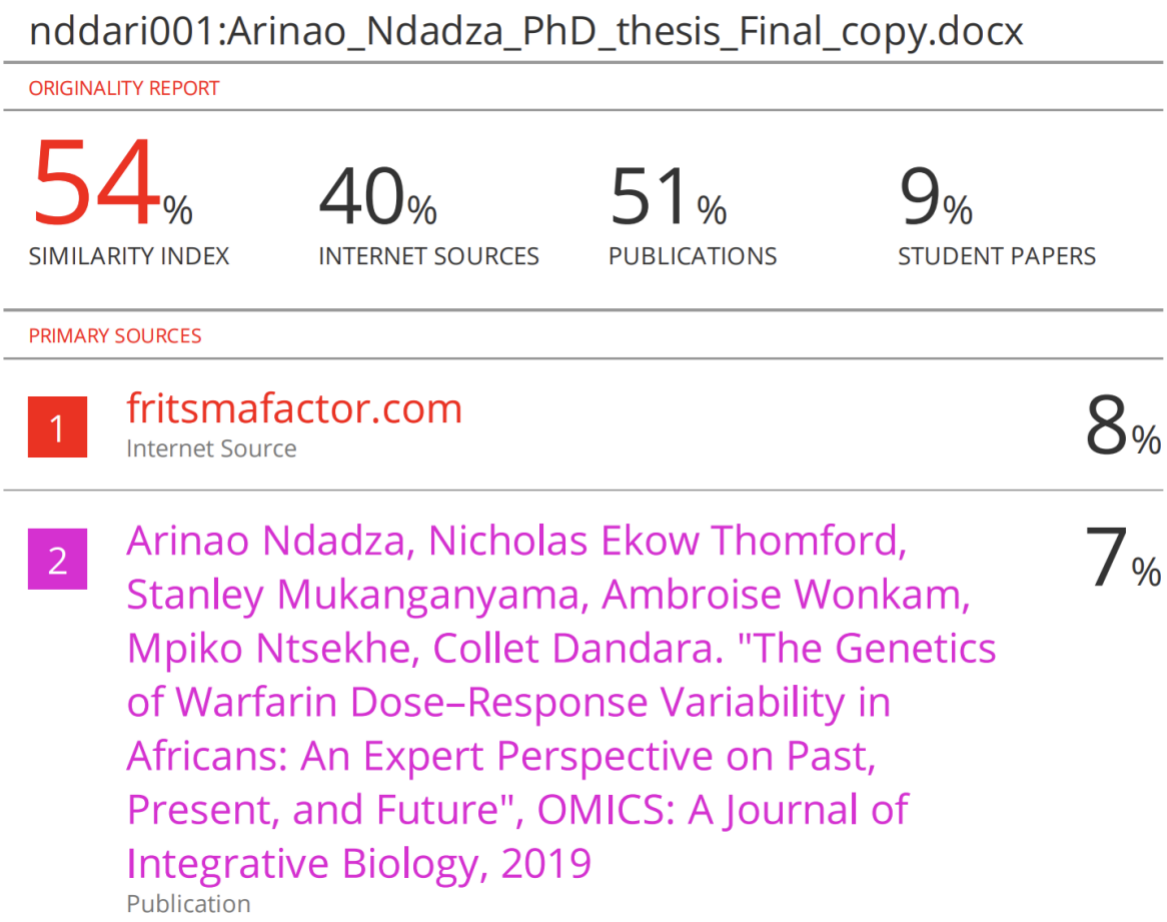

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