QATAR UNIVERSITY

COLLEGE OF PHARMACY

THE EFFECT OF GENETIC AND NON-GENETIC FACTORS ON WARFARIN DOSE VARIABILITY IN

QATARI POPULATION

BY

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ABSTRACT

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Title: The Effect of Genetic and Non-Genetic Factors on Warfarin Dose Variability in Qatari
Population

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Introduction: Genetic and non-genetic factors were shown to affect warfarin dosing; however,
their effect may vary from one population to the other. No previous studies were conducted on
the Qatari population to elucidate these factors.

Research question: What is the prevalence of *VKORC1*, *CYP2C9*, and *CYP4F2* genetic variants in
Qataris? and what is their contribution to warfarin dose variability?

Study design: An observational cross-sectional study

Methods: Hundred and fifty warfarin-treated Qatari patients on a stable dose and a therapeutic
INR for at least 3 consecutive clinic visits were recruited. Saliva samples were collected using
Oragene DNA self-collection kit, followed by DNA purification and genotyping via TaqMan Real-
Time-PCR assay.

Results: The minor allele frequency (MAF) of *VKORC1* (-1639G>A) was A 0.46, while the MAF's
for the *CYP2C9**2 and *3 and *CYP4F2**3 were T (0.12), C (0.04) and T (0.43), respectively. Carriers
of at least one loss of function *CYP2C9* allele (*2 or *3) required significantly lower warfarin doses

compared to non-carriers (24 mg/week vs. 34.1 mg/week, p<0.001). *VKORC1* (-1639G>A) and *CYP4F2**3 polymorphisms on the other hand were not associated with warfarin dose. Multivariate analysis on the derivation cohort showed that congestive heart failure (CHF) (P=0.002), and *CYP2C9**2 & *3 (P<0.001) were associated with lower warfarin dose while smoking (P=0.003) was associated with higher warfarin dose. These factors explained 24.1% of warfarin dose variability in Qatari patients. *CYP2C9**2 & *3 variants accounted for 11.8% of warfarin dose variability. In the validation cohort, correlation between predicted and actual warfarin doses was moderate (Spearman's rho correlation coefficient= 0.41, p=0.005).

Conclusion: This study showed that *CYP2C9**2 & *3 are the most significant predictors of warfarin dose along with CHF and smoking. Dose reduction should be considered in patients with CHF and those carrying at least one of the *CYP2C9**2 & *3 alleles. While dose increase should be considered in smokers.

DEDICATION

To my loving family

ACKNOWLEDGMENTS

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CHAPTER 1: Introduction

1.1 Pharmacogenetics and Pharmacogenomics

For many years, clinicians have relied on many environmental, behavioral and genetic aspects in the aim of personalizing medicine (1). Sir William Osler -Canadian physician- was the first to anticipate the concept of "personalized medicine" in the late 1800s (2). This concept went through many developments over the years. Among the current most prominent research areas in the field of personalized medicine is to study the effect of genetic biomarkers on drug safety and efficacy (2). In other words, the utilization of genetic information along with clinical/personal characteristics and family history to provide patients with more tailored therapies (3, 4). Pharmacogenomics; which is studying the relation between genomic information and drug response, and pharmacogenetics; which is studying the relationship between specific genes variability and drug response, are two terms used to describe this research area. While the definitions are not precisely the same, both terms are currently used interchangeably in the literature (2). Since the completion of Human Genome Project, pharmacogenomics (PGX) has been studied extensively and the number of identified pharmacogenetic associations have increased markedly over the years. Evidence for more than 2000 genes implicated in drug response has been annotated at the Pharmacogenomics Knowledge Base (1, 5). There are two main approaches used in PGX to identify genes that could be

clinically significant in certain treatments: candidate genes studies and genome wide association studies (GWAS).

Candidate genes is a directed attempt in which common variants in candidate genes are theorized to affect drug response (6).

GWAS is an approach in which the entire genome is screened for common variants. GWA studies can be followed by sequencing candidate gene, exomes, or entire genome to identify rare genetic variants in case of rare drug-induced adverse events (6).

Before deciding to use genetic information to guide in dosing any medication, The Evaluation of Genomic Application in Practice and Prevention (EGAPP) initiative has identified three important pieces of evidence to be considered: Analytic Validity, Clinical Validity, and Clinical Utility (7). The EGAPP defines these terms as follow:

Analytic Validity is the ability of a genetic test to measure the genotype of interest accurately and reliably, in the clinical laboratory and in specimens representative of the population in question (7).

Clinical Validity is the ability of a genetic test to predict the clinically defined disorder or phenotype of interest precisely and consistently (7).

Clinical Utility is the proof of enhanced measurable clinical outcomes, and its valuableness and added benefit to patient management decision making compared with existing management without genetic testing (7).

Two of the major areas where PGX had its early success are oncology and cardiovascular medicine.

1.2 Pharmacogenomics in Cardiovascular Diseases

Cardiovascular medications are among the most commonly used drugs (8). Despite many randomized controlled trials (RCT's) proving their efficacy, physicians are still faced with variations in response and serious adverse reactions when prescribing these medications. This highlights the fact that patients do not behave the same, in terms of drug response and that administering the same drug dose for everyone is probably not the ideal approach anymore (9). One of the reasons for altered drug response, which have been of great interest in recent years, is the genetic variation from one patient to another. Candidate genes studies and GWAS have demonstrated the associations of common genetic variants, involved in certain biological processes, with variations in response to specific drugs or class of medications such as; anticoagulants (warfarin), antiplatelet (clopidogrel and aspirin), antihypertensives (beta blockers and calcium channel blockers), statins, and antiarrhythmic agents (digoxin) (9).

Warfarin is an example of cardiovascular medications that have been extensively studied in PGX. GWAS and candidate gene studies have identified genetic variants involved in warfarin's pharmacokinetics and pharmacodynamics that are associated with the variability in warfarin stable dose requirements. The Clinical Pharmacogenetic Implementation Consortium (CPIC) in their 2017 updated guidelines, strongly

recommended the use of published PGX algorithms when calculating warfarin dose for patients who self-identify themselves as non-Africans (10).

1.3 Warfarin

Warfarin is the most widely used oral anticoagulant for the treatment and prevention of thromboembolic manifestations associated with atrial fibrillation, valve replacement, orthopedic surgery and venous thrombosis (11). It accounts for 0.5-1.5% of annually prescribed medications in the Western world (12).

Treatment with warfarin usually starts with an initial dose of 4-5 mg/day, followed by close INR monitoring and dose adjustments until patient reaches a therapeutic INR, then INR monitoring becomes less frequent and repeated dose adjustments are applied to maintain therapeutic range. Target INR range varies according to warfarin indication. Generally speaking, current clinical practice guidelines recommend a target INR range of 2-3 in patients with atrial fibrillation and venous thromboembolism, and 2.5-3.5 in patients with mechanical prosthetic heart valves (13). Once a stable INR is achieved, monitoring can be done every 4 weeks and up to 12 weeks. In some cases, loading doses of 10 mg can be used when initiating warfarin treatment; however, these loading doses can lead to extensive anticoagulation and put patients at risk of bleeding (13).

1.3.1 Historical Overview

Warfarin was first discovered by Karl Paul Link, a professor of agriculture chemistry at the University of Wisconsin (14). It all started when, in 1924, Frank Schofield concluded that a certain substance in damaged sweet clover is prolonging the clotting time in cattle and causing bleeding. Then, in 1931 Lee Roderick published a paper on "Sweet Clover Disease of Cattle" illustrating that a marked reduction in prothrombin was the cause of prolonged clotting time. In 1940, Link and his colleagues discovered that the substance causing the bleeding is a dicoumarol, after having it isolated in a crystalline form. Link pointed out the similarity in the chemical structure between the dicoumarol and vitamin K, and that vitamin K can reverse its hypothrombotic activity. In 1942, in his field trials on rodents control, Link found that the dicoumarol was not potent enough to be used in rodent control. He then asked his research assistants to reappraise 4-hydroxycoumarins analogs number 40 through 62, and they found that analog number 42 was more potent than the dicoumarol. In the year 1948, Link named this compound warfarin by combining the first letters of Wisconsin Alumni Research Foundation and introduced it as a rodenticide (14). Warfarin was then adopted for clinical use in 1955, after it showed success in treating 100 patients with myocardial infarction or deep venous thrombosis, including President Dwight D. Eisenhower who got treated for a heart attack that year (14).

1.3.2 Coagulation Process, Vitamin K Cycle, and Warfarin's Mechanism of Action

Coagulation Process: When a blood vessel is injured, blood is exposed to a transmembrane surface molecule called the tissue factor (TF). This exposure is the first

step in the coagulation cascade initiation. This tissue factor will bind to the small amount of active factor VII (VIIa), and this complex will cause more TF-VII complexes to be activated, thus converting factor IX into its active form (IXa). Factor IXa with its cofactor VIIIa activates factor X to factor Xa. Thrombin (IIa) is generated from prothrombin (II) by the binding of Xa to the cofactor Va. Thrombin production is the last step in the coagulation cascade that will lead to the formation of the fibrin clot (15). In the coagulation cascade, prothrombin (factor II), factors VII, IX, and X can only be activated in the presence of vitamin K, which plays an important role in the post-translational modification of specific glutamate (glu) residues on these factors in order to allow calcium binding in the liver, which is a prerequisite for the activation of these factors (15). Vitamin K, however, is not only responsible for the activation of the coagulation cascade, it also plays an important role in the activation of protein C and protein S which are natural anticoagulant factors (16, 17).

Vitamin K Cycle (Figure 1): Vitamin K is a group of naphthoquinone derivatives that can be synthesized by plants and eukaryotic bacteria (18). Humans can obtain vitamin K from food (mainly green leafy vegetables) or intestinal bacteria (19). Vitamin K is fat soluble, and it gets absorbed from the small intestines into the liver by an apolipoprotein E (APOE) receptor specific uptake (20). Its main role is to maintain redox homeostasis within the cells. In the liver, vitamin K is reduced to its hydroquinone form through two pathways: the first is performed by vitamin K epoxide reductase (VKOR) and uses vitamin K-epoxide and vitamin K as substrate; the second is through the microsomal enzyme NAD(P)H quinone dehydrogenase 1 (21). The microsomal epoxide hydrolase 1 (encoded by *EPHX1*),

which is a putative subunit of VKOR, harbors the vitamin K binding site (22). The reduced

vitamin K mediates the activation of the γ-carboxyglutamic acid (GGCX) enzyme (19). The

GGCX enzyme is responsible for the post-translational modification of several vitamin K

dependent coagulation factors. On these coagulation factors, the glutamic acid (Glu)

residues are changed into γ-carboxyglutamic acid (Gla), the Gla residue will then mediate

the binding of those factors to Ca^{++} in blood stream (19). This binding is essential for

platelets activation and other downstream coagulation factors. Calumenin (encoded by

CALU) can regulate the gamma glutamyl carboxylation by inhibiting the GGCX enzyme

(23). At the end of the γ-carboxylation process, reduced vitamin K is then oxidized to

vitamin K epoxide form and reduced to vitamin K through 2,3 vitamin K epoxide

reductase, to be engaged in the redox cycle again or removed from its cycle by CYP4F2

enzyme (vitamin k oxidase) (21, 24, 25).
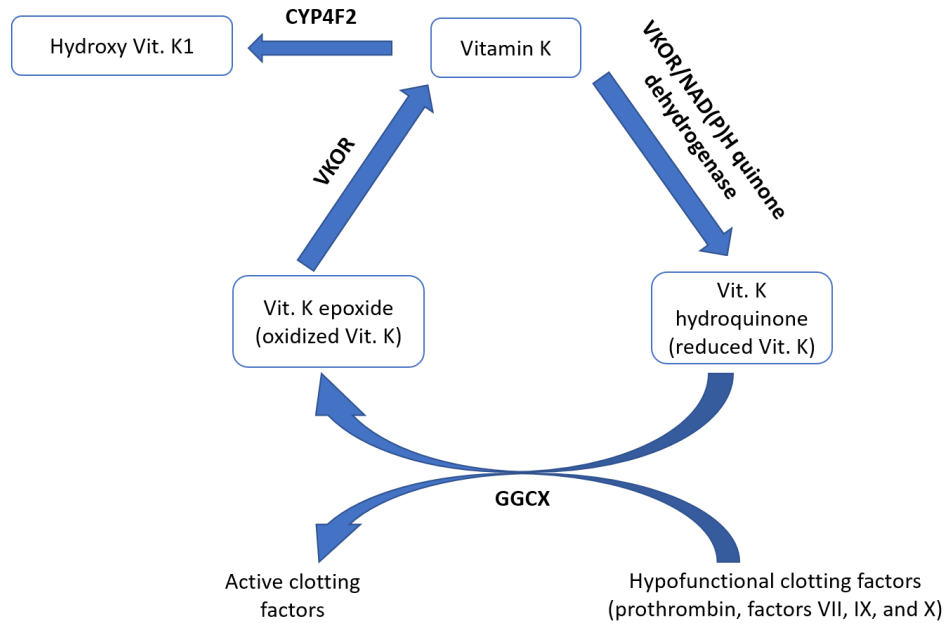
Figure 1 Vitamin K cycle.

Warfarin Mechanism of Action (Figure 2): Warfarin is a vitamin K antagonist. It inhibits the vitamin K cycle by binding to the oxidized VKOR and preventing the formation of sulfhydryles which are essential for vitamin K cycle activity (21, 24). This inhibits the production of reduced vitamin K, which is essential for the activation of: prothrombin, factors VII, IX, and X, protein C, and protein S. As a result, coagulation factors with impaired coagulant activity are produced in the liver. The effect of warfarin can be reversed by low doses of vitamin K1 (phytonadione) since it can bypass the VKOR (13).
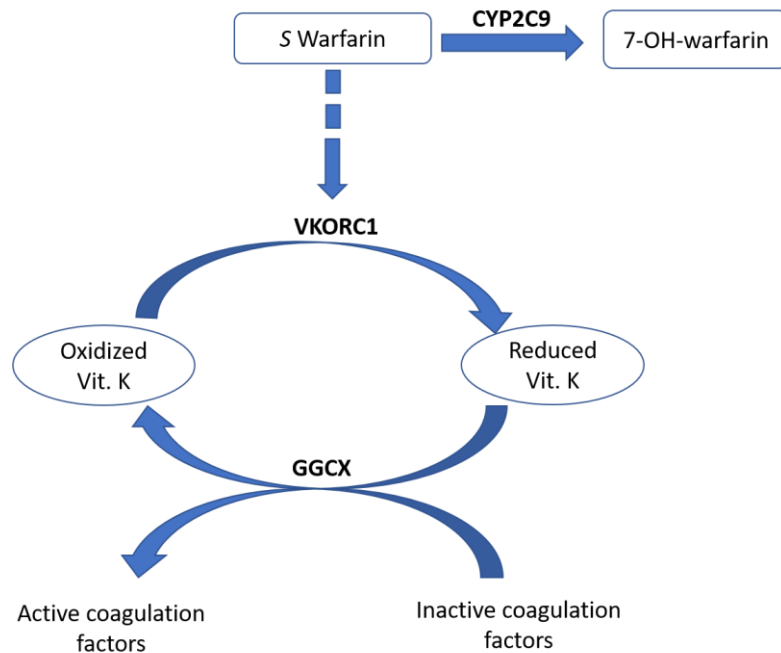
Figure 2 Warfarin mechanism of action.

1.3.3 Warfarin Pharmacokinetics and Pharmacodynamics

Warfarin is administered as a racemic mixture of two optically active enantiomers, the *R*

and the *S* enantiomers, in almost equal proportions. After oral administration, warfarin is

readily absorbed in the gastrointestinal tract with high bioavailability and reaches

maximal blood concentration within 90 minutes of administration (13). Warfarin is almost

99% bound to albumin and plasma proteins, it accumulates in the liver to be then

metabolized through different pathways for each isomer. The *S* isomer is mainly

metabolized by the cytochrome P450 family 2 subfamily C polypeptide 9 (CYP2C9) and it

is 5 times more potent than the *R* isomer, but has faster clearance. The CYP2C9 enzyme

comprises around 20% of the total microsome P-450 enzymes in the liver. It has a key role in the metabolism of 15-20% of therapeutically important drugs including warfarin (26). This enzyme demonstrates great variability in its expression and catalytic activity which can result in either increased or decreased activity of certain drugs, leading to drug toxicity or treatment failure (27). Other P-450 isoenzymes that are involved in the metabolism of the *S*- and the *R*-warfarin include: 2C19, 2C8, 2C18, 1A2, and 3A4. Warfarin has a terminal half-life of 36 to 42 hours and is excreted through the urine (28).

1.3.4 Challenges with Warfarin Use

One of the major disadvantages of warfarin is its narrow therapeutic index which can lead to serious bleeding adverse events that can even cause death (29). In a study that estimated the frequency and rate of hospitalization due to adverse events in the elderly, warfarin was found to account for 33.3% of the hospitalizations (30). Another challenge that is faced with warfarin treatment is the inter- and intra-patient variability in the dose required to achieve the optimal anticoagulation response. Dose requirements can vary from 0.5 mg to 20 mg per day (31). To prevent the bleeding or thrombosis events that may be associated with the inappropriate warfarin dosing, an optimal coagulation assay, prothrombin time (PT), is used as a surrogate marker to indicate warfarin anticoagulant effect (11). International Normalized Ratio (INR) which is the standard unit used to measure PT is currently the cornerstone test used to assess warfarin response. An adequate response is reached when the INR is between 2-3 but other targets may be used based on the indication and patient's history. Thrombotic events are found to increase

with ratios less than 1.5 and hemorrhagic events are increased with ratios more than 4 (32, 33). Several factors can contribute to the inter-patient variability of warfarin dosing including: age, body surface area, ethnicity, and genotype (34, 35). While changes in vitamin k intake; having febrile or diarrheal disease; alcohol intake; diet; smoking; and others, contribute to intra-patient dose variability.

1.3.5 Factors Affecting Warfarin Dose Variation

1.3.5.1 Non-Genetic Factors

Concurrent Medications: Many medications can influence the effect of warfarin by either affecting its metabolizing enzymes activity (mainly CYP2C9, CYP3A4, and CYP1A2), competing with it on its binding site, or by inhibiting its absorption (11). Metronidazole, sulfamethoxazole-trimethoprim, azoles antifungals, statins (especially fluvastatin), amiodarone, and phenylbutazone are examples of medications that inhibit the clearance of *S* warfarin and intensify the effect of warfarin on prothrombin time (CYP2C9 enzyme inhibitors). While drugs like rifampicin, carbamazepine, and barbiturates increase the clearance of warfarin resulting in reduced effect (CYP2C9 enzyme inducers) (13).

Diet: The intake of large amounts of food rich in vitamin K (mainly green leafy vegetables) can counteract the anticoagulant effect of warfarin and expose the patient to the risk of thrombosis. Other foods such as cranberry, garlic, mango and grapefruit can potentiate the effect of warfarin and increase the risk of bleeding (28, 36). Having a diet with balanced amount of these types of food is crucial to avoid INR fluctuation.

1.3.5.2 Genetic Factors

VKORC1 is located on chromosome 16. It codes for the vitamin K epoxide reductase enzyme, the target protein for warfarin, and it is the biggest predictor of warfarin dose. Nine haplotypes (H1-H9) have been identified for this gene in Americans (37), out of these 9 haplotypes, 4 have shown to be significantly associated with warfarin dose variation. Haplotypes H1 and H2 were associated with lower warfarin dose requirements (warfarin sensitivity), while H7 and H9 were associated with higher warfarin requirements (warfarin resistance) (37). The VKORC1 has 4 star-haplotypes (*1, *2, *3, and *4), *1 being the reference haplotype. Variants in the *VKORC1* can explain up to 18-25% of warfarin dose variability in European Americans, 2-4.5% in African Americans, and 18.4% in Asians (37-39). The c.-1639G>A (rs9923231) is a single nucleotide polymorphism (SNP) located upstream of *VKORC1* in the promotor region, it belongs to *2 haplotype, and it is significantly associated with response to warfarin (26). Studies have shown that carriers of at least one A allele require much lower doses compared to those homozygous for the G allele. The -1639G>A variant has a MAF of 10.05% in African Americans (blacks), 37.8% in European Americans (whites), 91% in Asians (39). Other less common variants: Asp36Tyr (rs61742245), Val25leu (rs886043673), Arg58Gly (rs748906829), and Val45Ala (rs751708108) are missense mutations and are associated with much higher warfarin doses that can reach up to 70mg per week (40-42).

CYP2C9 is located on chromosome 10 (10q23.33), it codes for cytochrome P450 2C9 enzyme, the metabolizing enzyme for warfarin (43). It is a highly polymorphic gene, 60 star-haplotypes have been identified worldwide so far (more information on these

haplotypes are available here: http://www.cypalleles.ki.se/). The *CYP2C9*1 is the wildtype allele, *CYP2C9*2 and *CYP2C9*3 are two important SNPs that occurs in the coding region of *CYP2C9* and results in altered enzyme activity. *CYP2C9*2 (rs1799853) is a non-synonymous SNP where C nucleotide is replaced with a T at exon 3 at position 430, resulting in the replacement of amino acid arginine by cysteine at position 144 (p.R144C). While *CYP2C9*3 (rs1057910) has a SNP at position 1075 (A1075C) at exon 7 resulting in the replacement of leucine by isoleucine at amino acid position 359 (p.L395I) (44, 45). Both *2 and *3 variants are associated with reduced enzymatic activity, 12% and 5%, respectively. A meta-analysis on the effect of *CYP2C9* allelic variants on warfarin dose variation showed that compared to *1/*1 genotype, carriers of *1/*2, *1/*3, *2/*2, *2/*3, and *3/*3 required warfarin doses that were lower by: 19.6, 33.7, 36, 56.7, and 78.1%, respectively (46). The *2 variant has a MAF of 2.9% in African Americans, 8-15% in White Americans, and is almost absent in Asians (44). The *3 variant is less common in African Americans than the *2 (MAF: 0.5-2%), around 6.3% in White Americans, and 3% in Asians (44). Other less common variants in the *CYP2C9* are the *5, *6, *8, and *11, which are only observed in African Americans and are also associated with decreased enzymatic activity and lower dose requirement. These variant alleles are more common in AAs than the *2 and *3 alleles (47, 48).

CYP4F2 is located on chromosome 19p13 and it codes for CYP4F2 enzyme which is a vitamin K oxidase (49). Only two star-haplotypes were identified for this gene: *2 and *3. The *3 variant (c.1297G>A, g.15879621 C>T, rs2108622), is a SNP located in exon 2 and results in amino acid change at position 433 from valine to methionine (49). Caldwell et.

al. was the first to report that *CYP4F2*3* variant was associated with higher warfarin dose requirement in a cohort of European American patients (50). Replication studies in two other cohorts of patients showed that there is a 4-12% increase in warfarin dose per variant allele. A GWAS showed that *CYP4F2*3* variant accounted for 1.5% of warfarin dose variability (51). The MAF of *CYP4F2*3* variant is 17% in Asians, 8% in African Americans, and 30.3% in Europeans (52, 53). No significant association of this variant with warfarin dose was reported in African Americans (52).

Other less common genetic variants that are thought to contribute to warfarin dose variability include variants in the following genes: *EPHX1*, *GGCX1*, *APOE*, *CALU*, *MDR1*, *factor VII*, and *PZ (54)*.

1.4 Oral Anticoagulants and Warfarin Use in Qatar

In 2010, the first Direct Oral Anticoagulant (DOAC) – dabigatran -- was approved by the FDA for the prevention of stroke in patients with atrial fibrillation. Following dabigatran, other DOACs were approved including: rivaroxaban, dabigatran, apixaban, edoxaban, and betrixaban. Unlike warfarin, this new class has wide therapeutic index and less inter- and intra-patient variability. DOACs do not interact with food and have only few drug interactions compared to warfarin. Finally, DOACs are at least as efficacious as warfarin and are associated with lower risk of bleeding (55). On the downside, validated monitoring strategies available to detect therapy failure with DOACs are not very practical, they are contraindicated in patients with poor renal function, they do not have

antidote (except for dabigatran) and they have high acquisition cost (55). Warfarin on the other hand, is safe to use in patients with renal failure, has low cost, can be reversed by low doses of vitamin K and is monitored using INR which allows for dose adjustment, detecting therapy failure; non-adherence; and over anticoagulation (55). Therefore, warfarin remains to be the anticoagulant of choice for many clinicians and patients alike.

In Qatar, despite the introduction of DOACs, warfarin is still the mainstay oral anticoagulant. According to Elewa and colleagues, warfarin comprised 77% of oral anticoagulant use in Qatar in the year of 2015 (56).

1.5 Study Rationale

Warfarin is a drug with a narrow therapeutic index and a dose that can vary greatly from one patient to another. Genetic variations in the *VKORC1*, *CYP2C9*, and *CYP4F2* are known to affect patients' responses to warfarin, and together they could explain up to 35% of dose variability. Furthermore, warfarin is the most commonly used oral anticoagulant, comprising 77% of oral anticoagulant use in Qatar (56). Despite the importance of genetic variations in predicting initial warfarin doses, no previous warfarin PGX studies have been carried out on the Qatari population. The genetically admixed nature of the Qatari population and the existing high rate of consanguinity (35%) make it a good candidate for PGX studies (57). Therefore, we aimed in this thesis to fill in the gap about warfarin PGX in the Qatari population.

1.6 Study Objectives

The specific objectives of the present study were to:

1. estimate the frequencies of *VKORC1* (-1639G>A), *CYP4F2**3 and *CYP2C9**2 & *3 variants in Qataris.

2. estimate the mean difference in warfarin dose between carriers and non-carriers of these genetic variants.

3. determine the variability in warfarin dose explained by each genetic variant

4. develop and validate a dosing model/algorithm consisting of genetic and non-genetic predictors.

CHAPTER 2: Literature Review

2.1 Warfarin Pharmacogenetics

In August 2007, the Clinical Pharmacology Subcommittee of the Advisory Committee on Pharmaceutical Science of the US FDA, requested the manufacturers of warfarin to have a label on their products suggesting *CYP2C9* and *VKORC1* genotyping before initial dosing (58). At that time, there were no clinical trials or outcome research powered enough to prove the clinical utility of using genotype-guided dosing. Since then, many observational studies have been conducted worldwide to study the association of different genetic variants with warfarin dose variability in different populations. Dosing algorithms that included clinical and genetic information have been developed and validated. The International Warfarin Pharmacogenetic Consortium (IWPC) and Gage et. al. each developed a dosing algorithm that included clinical and genetic data derived from large cohorts of patients (59, 60). Randomized controlled trials then followed to study the clinical utility of those algorithms.

2.1.1 Evidence from Randomized Controlled Trials

The Clarification of Optimal Anticoagulation through Genetics Trial (COAG): This was a multicenter, double blind, randomized trial that tested the efficacy of genetic dosing of warfarin in comparison to clinical-guided dosing (61). In the genetic-guided arm, dose was calculated using the algorithm developed by Gage et. al., which included genotype data

for *CYP2C9**2 &*3 and *VKORC1*(-1639 G>A) along with clinical data. The percentage of time in therapeutic range (PTTR) from day 4 or 5 through day 28 of therapy was the primary outcome of the study. Results of the study showed no significant difference in the mean PTTR between both arms. However, subgroup analysis of race (black vs. non-black), showed that PTTR was significantly lower in blacks in the genetic-guided arms compared to the clinical dosing arm (61). This is probably because genotype-guided dosing algorithm did not account for other variants in the *CYP2C9* that are mostly common in blacks and are associated with lower warfarin dosage, including: *5, *6, *8, and *11.

European Pharmacogenetics of Anticoagulant Therapy Trial (EU-PACT): This was a multicenter, pragmatic, single blind, randomized, controlled trial, intended to determine whether genotype-guided dosing was superior to fixed dosing (62). The primary outcome measure was the PTTR during the first 12 weeks after warfarin initiation. To calculate the maintenance dose in genotype-guided arm, the researchers used the International Warfarin Pharmacogenetics Consortium algorithm with slight modification (60), the Avery et. al. algorithm for initial doses (63), and the Lenzini et. al. algorithm for dose revision (64). These dosing algorithms included genotype data for *CYP2C9**2 & *3 and *VKORC1*(-1639G>A) along with other non-genetic factors. The study was performed in a predominantly white population from Europe and it showed a significantly higher mean PTTR at 3 months in the genetic-guided group compared to the control group.

The COAG and the EU-PACT were the first largest randomized controlled trials reported before 2017, studying the clinical utility of warfarin genotype-guided dosing. Although

both studies included genotype data for *CYP2C9* *2 & *3 and *VKORC1*(-1639G>A) and other clinical factors in their genotype-guided dosing algorithm, only the EU-PACT study found statistical significance in favor of the genotype-guided arm, this may have been due to several reasons. First, although the primary outcome in both studies was the percentage of time within therapeutic INR range (PTTR), it was after six weeks of treatment in the EU-PACT and only four weeks in the COAG study. Second, there was a difference in the comparator arm, clinical dosing (COAG) versus fixed dosing (EU-PACT), and the use of a loading dose only in the EU-PACT study. Another reason may have lied in the ethnic make-up of each study cohort. In the COAG study, 27% of the study cohort were black, while in the EU-PACT, study cohort consisted mainly of Europeans (98.5%). In the COAG study, algorithm used to calculate warfarin dose in the genetic-guided arm did not account for important variants affecting warfarin dose in black and a significant interaction was found between race and dosing strategy (p=0.003). Analysis of only the non-blacks in COAG showed small difference in mean %TTR between both arms, 48.8% in genotype guided arm vs. 46.1% in clinical arm. However, this difference was not statistically significant, p= 0.15. Such findings highlight the importance of considering the race or ethnicity of patients when using genotype-guided dosing.

Genetics-InFormatics Trial (GIFT): It is the most recent trial, published in October 2017. Its main objective was to determine whether genotype-guided dosing reduced adverse events (8). The pharmacogenetics dosing algorithm used included genotypes for *CYP2C9**2 and *3, *CYP4F2**3, and *VKORC1*-1639. The primary endpoint was composite of major bleeding, INR ≥ 4, venous thromboembolism, or death. This study was successful in

proving that genotype-guided dosing could reduce adverse events. In the genotype-guided arm, 10.8% of the participants had at least one composite endpoint, compared to 14.7% in the clinical arm, resulting in an absolute risk difference of 3.9% (95% CI, 0.7% to 7.2%; p = 0.02). Subgroup analysis showed no significant interaction in the following subgroups: high-risk subgroup, p = 0.88; black race, p = 0.74; *CYP2C9* genotype, p = 0.16; target INR of 1.8 vs 2.5, p = 0.70; or hip vs knee arthroplasty, p = 0.36. The genotype-guided group also showed benefit over the clinical group in the mean PTTR, 54.7% vs. 51.3%, (p=0.003). Subgroup analysis showed no significant interaction among black participants (p=0.48). Like the COAG trial, the GIFT trial included black patients; however, this group represented only 6% of the entire cohort while 91% were Whites. This may have been the reason why the GIFT trial did not find any significant effect of the black race on their results while the COAG did.

2.1.2 Evidence from Observational Studies in the MENA Region

From Morocco to Iran and down to Sudan, these are all countries of what is defined as the Middle East and North Africa (MENA) region. Most of these countries reside in a very strategic location which have kept them always at conflict. Various civilizations have migrated-in and -out of these countries, leaving behind genetically and racially admixed populations with Asian, Arab, African, and Caucasian ancestries. Additionally, some of these countries like Yemen and Qatar have high consanguinity rate (54% and 35%, respectively) (57, 65). While in other countries like Egypt and Oman, their strategic

location between Africa and Eurasia have left them with great intrapopulation diversity. All these reasons have made the MENA area of interest in genetic studies (54).

A good number of observational studies were conducted in different countries of the MENA, exploring the prevalence of genetic variants that are associated with warfarin dose. Moreover, many of these studies have investigated the effect of these genetic variants along with other clinical factors on warfarin dose variation.

To summarize the evidence on warfarin pharmacogenetics in MENA region and identify the gaps, we systematically reviewed studies that have estimated the impact of genetic and non-genetic factors on warfarin dose variability in populations of MENA. The detailed method of this systematic review was published elsewhere (54). Briefly, using the appropriate terms, we searched the following databases: PubMed, Scopus, Medline, PharmGKB (Pharmacogenomics Knowledge Base), and PHGKB (Public Health Genomics Knowledge Base). Our primary outcome was stable mean warfarin dose and the variability in the dose explained by genetic and non-genetic factors.

Seventeen studies in 8 different populations, that satisfied our inclusion criteria, were identified through the search. The studied populations were from Egypt, Iran, Lebanon, Turkey, Sudan, Oman, Kuwait, and two studies in occupied Palestine on Israeli population. Table 1 summarizes the characteristics of these studies.

In each of the included studies, targeted populations had to be on stable warfarin dose as part of the inclusion criteria. Although the definition slightly varied from one study to

another, in general, patients were considered stable if they had been on the same dose

for more than two clinic visits and their INR was in target range.

Table 1 *General Characteristics of Included Studies*

Study	Population	Sample size	Genetic factors explored	Non-genetic factors explored	Mean warfarin dose
Shahin et. al. 2011, 2013 (41, 66)	Egyptian	207	*CYP2C9*(*2, *3, *4, *5, *8), *VKORC1* (3673G>A, Asp36Tyr) *APOE, CYP4F2*(Val433Met), *CALU*	Sex, age, BSA, use of aspirin, indication for warfarin, concomitant disease & smoking status	36.8 ± 17.9 mg/wk
Bazan et. al. 2014 (67)	Egyptian	63	*CYP2C9*(*2, *3) *VKORC1*	Age & smoking status	7.3 ± 5.2 (1-30) mg/day
Ghozlan et. al. 2015 (68)	Egyptian with ACS	80	*CYP2C9* *VKORC1*	Age & height	4.8 ± 1.96 (2-10) mg/day
Issac et. al. 2014 & Ekladious et. al. 2013 (69, 70)	Egyptian	84 (50 for model, 34 for validation)	*CYP2C9*(1075A>C), *VKORC1*(1173C>T), *MR1*, (C3435T), *EPHX1*(H139R), *PZ*(A-13G)	Age & gender	
Namazi et. al. 2010 (71)	Iranian	100 total (100 CYP2C9, 99 CYP2C19, 81 VKORC1) 55 for the model	*CYP2C9* (*2, *3) *VKORC1* (-1639) *CYP2C19* (*2, *3)	Gender, age, BSA, weight & height	7.3 ± 5.2 (1-30) mg/day

Study	Population	Sample size	Genes	Other predictors	Dose
Loebstein et. al. 2005 (22, 72)	Israeli	100	*GGCX, CALU, VKORC1, EPHX1, CYP2C9* (*2, *3)	Age, weight, concurrent medication & total vit. K plasma concentration	5.7 ± 3.3 (1.1-20) mg/day
Alrashid et. al. 2016 (73)	Kuwaiti	108	*CYP2C9* *VKORC1*	Sex, BMI, and age were adjusted for in the model but not included as predictors	4.7 ± 2.7 mg/day
Esmerian et. al. 2011 (74)	Lebanese	43	*CYP2C9 (*2, *3)* *VKORC1* (-1639G>A, 1173 C>T)	N/A	31 ± 14 mg/week
Pathare et. al. 2012 (75)	Omani	212 (142 in the model derivation cohort, 70 for validation)	*CYP2C9* (*2, *3) *CYP4F2 *3* *VKORC1* (3673G>A, 5808T>G, 6009C>T, 6484C>T, 9041G>A)	Simvastatin, amiodarone, hypertension, diabetes, atrial fibrillation, deep vein thrombosis, mechanical valve, age, weight & gender	4.75 (3-5.5) mg/day
Shrif et. al. 2011 (76)	Sudanese	203 patients 180 healthy volunteers	*CYP2C9* (*2, *3, *5, *6, *8, *9, *11) *VKORC1* (20 tagSNPs)	Body weight, concurrent medication, target INR, BSA, height, age, indication for warfarin treatment & gender	5.58 ± 2.48 (1.5-22.5) mg/day

Study	Ethnicity	n	Genes	Covariates	Dose
Özer et. al. 2013 (77)	Turkish	107	*CYP2C9 (*1, *2) VKORC1*(-1639G>A, 1173C>T), *CYP4F2, EPHX1*	Age, height, weight, No. of cigarettes & daily consumed tea and green vegetables	5.16 ± 1.95 (1.43-10) mg/day
Ozgon et. al. 2008 (78)	Turkish	205	*CYP2C9 (*2, *3, *4, *5) VKORC1 (-1639G>A)*	Age & non-indication of VT	34.2 ± 16.78 (6.25-125) mg/wk
Yildirim et. al. 2014 (79)	Turkish	101	*CYP2C9 (*2, *3) VKORC1*(-1639G>A) *factor VII (-401G>T)*	Age, BMI & INR	4.07 ± 1.6(1.13-7.86) mg/day
Ozer et. al. 2010 (80)	Turkish	100	*CYP2C9(*2, *3), VKORC1(-1639G>A)*	Age & BSA	4.11 (1.16-9.33) mg/day

2.1.2.1 Prevalence of the Genetic Variants

The minor allele frequency (MAF) was used to evaluate the prevalence of different genetic variants in populations of MENA. Table 2 shows the MAF's of variants in the most commonly studied genes, *VKORC1*, *CYP2C9* and *CYP4F2*. While Table 3 represents the MAF's of the less commonly studied genetic variants.

Table 2 *Minor Allele Frequency of Most Common Genetic Variants*

Population	Gene	VKORC1	CYP2C9		CYP4F2
	Variant	-1639 G>A	*2	*3	*3
		(rs9923231)	(rs179853)	(rs1057910)	(rs2108622)
Egyptians (41, 66)		46.2%	11.7	9.2%	42%
Egyptians (70)		72.05%	N/A	10.7%	N/A
Egyptians (67)		51%	7%	9.6%	N/A
Egyptians (68)		30%	8%	4.3%	N/A
Iranian (71)		56%	27%	9%	N/A
Israeli (72)		N/A	12.5%	11%	N/A
Kuwaiti (73)		40%	14%	5%	N/A
Lebanese (74)		52%	15%	7%	N/A
Omani (75)		35%	6%	6%	30%
Sudanese (76)		37%	5%	0%	N/A
Turkish (78)		50%	13%	10%	N/A

Turkish (77)	49%	N/A	N/A	40%
Turkish (80)	40%	13%	15%	N/A
Turkish (79)	51%	17%	27%	N/A

Table 3 *Minor Allele Frequency of Less Common Genetic Variants*

Gene Variant	MAF	Population
EPHX1 (rs2292566)	16%	Turkish (77)
(rs1051740)	26.19%	Egyptians (69)
	25%	Israeli (22)
MDR1 C3435T	42.86%	Egyptians (69)
Protein Z A- 13G	0%	Egyptians (69)
APOE rs429358	6.7%	Egyptians (66)
rs7412	7.4%	
CALU rs339097	2.3%	Egyptians (66)
Factor VII (-401G>A)	35%	Turkish (79)
GGCX (rs699664)	29.5%	Israeli (72)

Variants of the *VKORC1* gene were the most prevalent among all populations. Carriers of the -1639G>A variant required lower warfarin dose compared to the wild type in all studies. Variants in the *CYP2C9* were less common than those of the *VKORC1* in all populations with the *2 variant being slightly more common than the *3 variant in most cases. The *3 variant was not detected in Sudanese and was highest in Turkish (27%) (76, 79), while *2 variant was highest among Iranian (27%) (71). Similarities in the MAF's of *2 and *3 variants were seen among Egyptian, Israeli, and Turkish populations (41, 72, 78). Both *2 and *3 variants were associated with decreased warfarin dose requirements in all populations.

2.1.2.2 Significant Predictors of Warfarin Dose

In all studies, the most significant predictors of warfarin dose and the most significant dosing models were identified through univariate and multivariate regression. Table 4 shows the significant predictors in the different populations and to what extent they could predict warfarin dose.

Table 4 *Most Significant Predictors and % Variability Explained*

Population	Significant Genetic Predictors	Significant Non-Genetic Predictors	Variability explained by the model
Egyptians **(41, 66)**	• V*KORC1* (-1639G>A) & (Asp36Tyr) • *CYP2C9*(*2, *3, *4, *5, *8) • *APOEε2*	• Age • Pulmonary embolism • Smoking status	36.5%
Egyptians **(68)**	• VKORC1(-1639G>A) • CYP2C9 *2 & *3	• Age • Height	30.6%
Egyptians **(67)**	• VKORC1 (-1639G>A) • CYP2C9*3	• Age • Smoking status	43.4%
Egyptians **(69, 70)**	• *VKORC1*(1173 C>T) • *MDR1* (p= 0.055)	• Age	20.9%
Iranian **(71)**	• *VKORC1* (-1639G>A)	• Age	41.3%

	• *CYP2C9* *2 & *3	• Sex	
		• Height	
Israeli **(22)**	• *VKORC1* (1542G>C)	• Age	63%
	• *CYP2C9* *2 & *3	• Weight	
Kuwaiti **(73)**	• *VKORC1* (-1639G>A)	• Age	N/A
	• *CYP2C9* *2 & *3	• Body Mass Index	
Lebanese **(74)**	• *VKORC1* (-1639G>A)	N/A	33.9%
	• *CYP2C9* *2 & *3		
Omani **(75)**	• *VKORC1* 3673(GA & AA)	• Atrial fibrillation	63%
	• *CYP2C9* (*2/*3, *3/*3)		
Sudanese **(76)**	• *VKORC1*(rs8050894, rs7199949, rs7294),	• Concurrent medication	36.75
	• *CYP2C9* (*2, *5, *6, *11)	• Indication for warfarin	
Turkish **(78)**	• *VKORC1* (3673 G>A)	• Age	43%
	• *CYP2C9* *2, *3, & *4	• Non-indication of VTE	
Turkish **(80)**	• *VKORC1*(-1639 G>A)	• Age	60.4%
	• *CYP2C9* *2 & *3	• Body surface area	
Turkish **(77)**	• *VKORC1*(-1639G>A)	• Age	39.3%
	• *CYP2C9* *2 &*3		
	• *CYP4F2* (rs2108622)		
Turkish **(79)**	• *VKORC1* (-1639G>A)		18.2%

The *VKORC1* variants were the most significant predictors of warfarin dose in all studies included in this systematic review, followed by those of *CYP2C9*. However, the frequency of these genetic variants and the extent of their effect on dose variability varied from one population to another. Similarities were seen between Sudanese and African Americans in that *CYP2C9**5, *6, and *11 were better predictors of warfarin dose than the *2 and *3 variants (81). Moreover, the variability explained by variants of *VKORC1* (rs9934438, rs9923231, rs8050894) were comparable to those of African Americans (81, 82).

The *VKORC1* (-1639G>A) variant was the most significant predictor of warfarin dose in all populations. This variant alone explained 45% of the dose variability in Omani (75). The *VKORC1* Asp36Tyr was significantly associated with higher warfarin doses in Egyptians and when added to Shahin et al. dosing model it further explained the variability by only 2% (41). Only 3 studies in three different populations of MENA (Egyptian, Omani, and Turkish) have investigated the association between warfarin dose and *CYP4F2**3. A significant association between *CYP4F2* variants and warfarin dose was among Turkish by Özer et. al (77), and carriers of the variant allele required higher warfarin doses than those with the wild-type. However, no significant association between this variant and warfarin dose was detected in Egyptians or Omani despite its high frequency (66, 75). In the Egyptian cohort studied by Shahin. et. al., the *APOE ε2* gene variant explained 2.5% of the dose variability, and carriers of this variant required lower doses compared to wild type. No association was found between *MDR1* C3435T, *EPHX1* H139R, *GGCX* and *protein Z* A-13G gene variations and warfarin dose in populations of MENA. However, when combined, these genetic variants were able to explain 3% of the dose variability in

Egyptians (69). Shahin et al. was able to confirm the association of *CALU* rs339097 gene polymorphism with increased doses of warfarin, however, when added to the regression model it failed to show any significance (66). The authors reported that failing to show significance may have been due to lack of power, since only nine variant carriers were found in their cohort. In African Americans, it was shown, that for every *CALU* rs339097 variant allele warfarin dose increases by almost 11% (83). Despite its high frequency in Turkish (35%), *factor VII* -401G>T variant was able to predict only 2.2% of warfarin dose variability (79). Lower warfarin dose requirements were seen among carriers of the minor allele as opposed to carriers of the major allele (79).

In the Omani cohort, Pathare et al. compared the performance of their algorithm with that of the IWPC in predicting warfarin dose (75). Pathare et al. algorithm explained 63% of the dose variability, while the IWPC algorithm explained only 33.6% of the variability (75). Furthermore, warfarin dose predicted by the IWPC algorithm was higher than the patients' actual dose by 13% (75). Although the IWPC algorithm has been developed and validated using clinical and genetics information from different racial groups including Asians, Blacks, and Whites across four different countries, and even though Omanis come from Asian, African and Caucasian ancestries, the IWPC algorithm failed to show good performance in the Omani population (75).

2.2 Research Question

Our review of literature has shown that despite the shared ancestries between some of the populations of MENA, great discrepancies are still found in the frequencies of the genetic variants and their effect on warfarin dose. Moreover, variations were seen in the performances of the different dosing algorithms. Such variations could be owed to the variability in ethnic/racial definitions, clinical variations, social/lifestyle perspectives, population migration and historical perceptions. These observations highlight the importance of having dosing algorithms that are more population tailored. Because evidence about warfarin PGX in the Qatari population is lacking, we aimed in this thesis to answer the following questions:

1. What is the prevalence of *VKORC1*, *CYP2C9*, and *CYP4F2* genetic variants in Qatari population?

2. What is the extent of *VKORC1*, *CYP2C9*, and *CYP4F2* contribution into warfarin dose variability?

CHAPTER 3: Methods

3.1 Research Design and Ethics

Study Design: Our study was a cross sectional observational study involving a cohort of Qatari patients taking varying doses of warfarin for various indications.

Ethics: Since our study involved human subjects, we obtained ethical approvals from the Institutional Review Board (IRB) of Hamad Medical Corporation (HMC) for each participating site (Appendix 1 & 2), and from Qatar University (QU) IRB (Appendix 3). The approval of QU Institutional Bio-safety Committee was also obtained (Appendix 4), since our study involved working with potentially biohazardous substances.

3.2 Study Setting and Timeline

Patients' recruitment was conducted at 3 different sites, which are all parts of the Hamad Medical Corporation, the biggest medical institution in Qatar. Recruitment started in September 2016, and completed in March 2017. While DNA samples were processed and analyzed at the laboratories of Qatar University, between November 2016 and June 2017.

First site was the pharmacist-managed anticoagulation clinic at Al-Wakra Hospital. Al-Wakra Hospital was established in 2012. It is a general hospital that delivers comprehensive, high quality healthcare to people of all ages. It provides emergency care, general medicine, surgery and highly specialized treatments. It has a capacity of 325 beds,

comprising 248 beds for general and acute patients and 77 beds reserved for critical care, high dependency and burn patients. The anticoagulation clinic at Al-Wakra Hospital was established in 2013. The clinic is managed by a full-time pharmacist and a full-time nurse and its activities are supervised by the Head of the Cardiology Unit at the hospital. Second site was the anticoagulation clinics at the Heart Hospital. The Heart Hospital is a specialized hospital for cardiology and cardiothoracic patients. It comprises 20-bed coronary care unit, 12-bed cardiothoracic intensive care unit (ICU), a 24-bed surgical high-dependency unit (HDU) and a 60-bed ward. It has 3 anticoagulation clinics that are either physician-managed or pharmacist-managed clinics. Two clinics are managed by 2 full-time doctors and 2 full-time nurses, and 1 clinic is managed by 2 full-time pharmacists. Third site was the anticoagulation clinics at the Hamad General Hospital. This hospital is a 603-bed facility. It provides a wide range of medical care and clinical services including trauma, emergency medicine, pediatrics, critical care, specialized and sub-specialized surgery, specialized medicine, laboratory medicine, diagnostic imaging and adult rehabilitation services. The anticoagulation clinics at Hamad General Hospital are either physician-managed or pharmacist-managed. It has two anticoagulation clinics managed by 2 full-time pharmacists and 2 full-time nurses, and one clinic managed by a full-time doctor and a full-time nurse. At all sites, INR testing was performed using point-of-care (POC) device.

3.3 Study Population and Sampling

This study included warfarin-treated patients of the Qatari nationality only (identified as being Qatari passport holders). Due to the limited number of Qatari patients on warfarin, we used a sample of convenience in this study. This means that any patient coming to the clinic for a follow-up visit was screened. If patient was found to meet the inclusion/exclusion criteria, then patient was approached for recruitment.

Patients were considered eligible if they had been on warfarin for at least six weeks, had been on a stable warfarin dose for at least three consecutive clinic visits with their INR in therapeutic range, agree to participate in the study and sign a written informed consent form. A stable warfarin dose was defined as a dose that did not vary by more than 10% between clinic visits (66).

We excluded any patients: who had liver cirrhosis, had advanced malignancies, were hospitalized within the previous four weeks or had a diarrheal or febrile disease within the previous 2 weeks.

3.4 Patient Recruitment

We screened through 470 records to identify possible eligible Qatari patients. Forty records were excluded for the following reasons: patients were deceased; not on warfarin; or not Qatari. Out of the remaining 430 patients, 169 were approached, and 150 patients gave consent with an 82% response rate. Only 149 patients were included in the analysis due to poor quality in one of the samples where no DNA was detected (Figure 3).
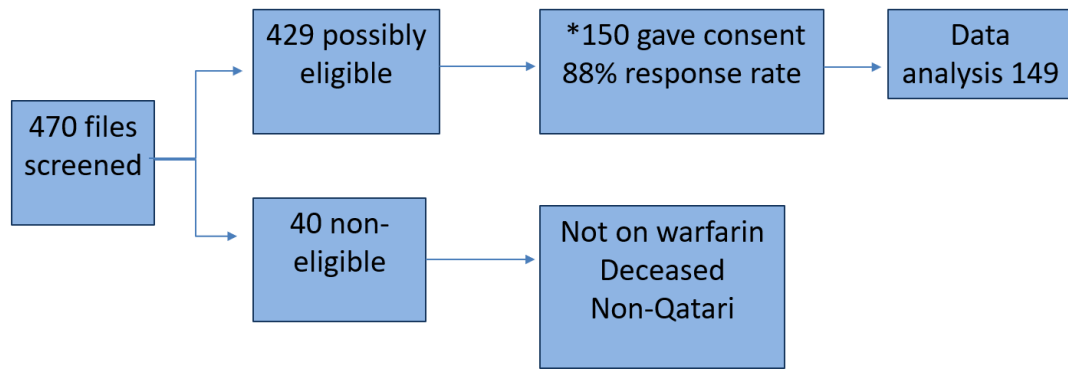
Figure 3 Patient recruitment flow diagram.

[*]A total 169 patients were approached

3.5 Data Collection and Outcome Measures

After signing an informed consent form, patients were asked to provide the following demographic information; height, weight, gender, the origin of parents, smoking status, alcohol consumption and vitamin k intake.

Clinical data including: recent bleeding and/or thromboembolic incidences, indication for warfarin, target INR, treatment duration, concurrent medications and past medical history were collected from hospital electronic medical records.

The primary outcome of our study was stable weekly warfarin dose defined as: mean weekly dose at which INR readings were in the therapeutic range for 3 consecutive clinic visits, with at least 5 days between readings and no more than 10% variation in the dose between clinic visits. When screening patients for eligibility, we allowed for a margin of

error of ± 0.2 in INR readings, since we were relying on the point-of-care device for measuring INR.

3.6 Genetic Samples Collection

Prior to study initiation, we optimized our genetic samples collection techniques by assessing the quantity and quality of saliva DNA versus blood DNA. Assessment results showed that saliva has a higher DNA yield (Appendix 6). Based on these results and the convenience to the patients, saliva was chosen as the first choice for DNA collection.

After signing a written informed consent, patients were asked to provide saliva sample. They were asked to use Oragene•DNA (OG-500) self-collection kit (DNA genotek, USA) and fill it with 2 mL of saliva. Before collection of samples, we insured that patients did not eat or drink in the past 30 minutes. During the collection, patients were asked to rub their tongue to the inner side of their cheek to allow the production of more epithelial cells. After collection, the tube was shaken for 5 seconds and kept at room temperature (RT) for long-term storage. Figure 4 illustrates the entire process of saliva collection.
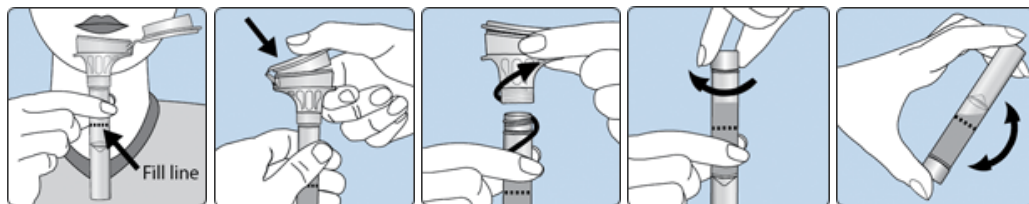
Figure 4 Saliva collection using Oragene DNA (OG-500) self-collection kit.

For patients who found it hard or inconvenience to provide saliva, blood samples were taken instead. Around 5 mL of blood were drawn by a trained nurse/phlebotomist using BD Vacutainer® K3 EDTA 12.15 mg (15% Sol, 0.081 mL) glass collection tubes (Reference number 366450). Tubes were inverted 8 times after collection, then stored at -20°C for further analysis.

3.7 DNA Extraction and Quantification

3.7.1 DNA from Blood

Genomic DNA was extracted from fresh frozen whole blood samples using the PureLink® Genomic DNA mini kits, Invitrogen™, following the manufacturer protocol (84).

Preparing the Lysate: Frozen whole blood was first thawed in the lab at room temperature. Then, 20 µl of proteinase A and 20 µl of RNase A (both supplied with the kit) were added to 200 µl of whole blood into a sterile 1.5 mL centrifuge tube, vortexed for 5 seconds then incubated for 2 minutes at room temperature. Next, 200 µl of PureLink® Genomic Lysis/Binding Buffer were added, vortexed to ensure homogeneity and incubated in a heating block for 10 minutes at 55 °C to ensure protein digestion. After incubation was completed, 200 µl of 96-100% ethanol was added to the lysate and vortexed for 5 seconds.

Binding DNA: Prepared lysate was added to the PureLink® spin column and centrifuged at 10,000 x g for 1 minute at RT. Collection tube was then discarded and the column was placed in a clean collection tube (supplied with the kit).

Washing DNA: 500 µl of washing buffer 1 (supplied with kit) were added to the column followed by centrifugation at 10,000 x g at RT for 1 minute. Collection tube was then discarded and a new one was placed. Next, 500 µl of washing buffer 2 (supplied with the kit) were added to the column, followed by centrifugation at room temperature for 3 minutes at maximum speed, collection tube was discarded at the end.

Eluting DNA: After placing the spin column in a clean sterile 1.5 mL centrifuge tube, 50-100 µl of PureLink® elution buffer (supplied with the kit) was added, followed by incubation for 1 minute at RT and then centrifuge for 1 minute at maximum speed at room temperature. The elution contained purified DNA and was kept at -20°C.

3.7.2 DNA from Saliva

Extraction of DNA from saliva samples was performed using the prepIT®•L2P manual protocol for the purification of DNA from 0.5 sample (85). Before starting the extraction procedure, all samples (in their original tubes) were incubated at 50°C in air incubator overnight. After incubation was completed, in a 1.5mL sterile centrifuge tube, 20 µl of prepIT•L2P (catalog #: PT-L2P) reagent was added to a 500 µl of the saliva, vortexed for few seconds, incubated on ice for 10 minutes and centrifuged for 5 minutes at 15,000 x g at room temperature. After transferring the clear supernatant into a clean micro-centrifuge tube, 600 µl of 95-100% ethanol was added, mixed gently by inversion 10 times, left to stand at RT for 10 minutes and followed by centrifugation at RT for 2 minutes at 15,000 x g. The supernatant was then completely removed, and the DNA pellet was

washed with 250 µl 70% ethanol, left to stand for 1 minute at RT, followed by complete

removal of ethanol. Last step was the addition of 100 µl of the elution buffer (TE Buffer:

10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to dissolve the pellet and vortexed for 5 seconds.

Samples were incubated overnight at room temperature to ensure the complete

rehydration of DNA.

After each extraction step, DNA was quantified, and samples were stored in aliquots at -

20°C.

3.7.3 DNA Quantification

To assess the quality of the purified DNA and quantify it, we used the Nanodrop 2000c

Spectrophotometer (Thermo Fisher Scientific). Usually a volume of 1-2 µl is used in

Nanodrop measurements. However, since the presence of DNA in the samples may

reduce the surface tension, we used 2 µl in our measurements to ensure reproducibility.

All samples were measured twice and TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was

used as a blank. For further details or troubleshooting, we referred to the user manual

(86).

3.8 Single Nucleotide Polymorphism Detection and Genotyping

For SNP detection and genotyping, we used the Real-Time Polymerase Chain Reaction

(RT-PCR) 7500 Fast System with TaqMan Drug Metabolizing Enzyme (DME) genotyping

assay, Applied Biosystems™, Life Technologies. The RT-PCR TaqMan software provided

us with more than 95% call rate. The reaction mix was used according to the volumes

listed in Table 5. The context sequence used for each probe are listed in Table 6, while

PCR conditions used are shown in Table 7.

Table 5 *Reagents and Volumes Used When Preparing the Reaction Mix for PCR*

Reaction Component	Volumes per well (96-Well Plate)
TaqMan Master Mix 2X	12.5 µl
20X Assay Working Stock	1.25 µl
Nuclease Free Water	9.25 µl
DNA	2 µl
Total volume per well	25 µl

Table 6 *Context Sequences of the Probes Used in Genotyping*

SNP ID*	Gene	Context Sequence [**VIC/FAM**]
rs1799853	*CYP2C9*	GATGGGGAAGAGGAGCATTGAGGAC**[C/T]**GTGTTCAAGAGGAAGCCCGCTGCCT
rs1057910	*CYP2C9*	TGTGGTGCACGAGGTCCAGAGATAC**[C/A]**TTGACCTTCTCCCCACCAGCCTGCC
rs9934438	*VKORC1*	CCCCGACCTCCCATCCTAGTCCAAG**[A/G]**GTCGATGATCTCCTGGCACCGGGCA
rs2108622	*CYP4F2*	CCCCGCACCTCAGGGTCCGGCCACA**[C/T]**AGCTGGGTTGTGATGGGTTCCGAAA

Table 7 *Temperature, Duration, and Number of Cycles Used in each Step of the PCR Reaction*

Step	Temperature	Duration	Cycles
AmpliTaq GoldR, UP, **Enzyme Activation**	95°C	10 minutes	HOLD
Denaturation	95°C	15 seconds	50
Annealing/Extention	60°C	90 seconds	50

3.9 Statistical Analysis

Descriptive statistics were used to analyze baseline demographics. Depending on their normal distribution, numerical data were presented as mean with standard deviation or median and interquartile range. Categorical variables were presented as frequencies and percentages. Chi-square- Goodness of Fit was used to make sure that all allele frequencies fit the Hardy- Weinberg equilibrium.

We ran normality tests and plots for the continuous variables, including Kolmogorov-Smirnov and Shapiro-Wilk. We also used box plot and stem-and-leaf to identify any outliers. When data were normally distributed, we used independent sample t-test or ANOVA to estimate the difference in mean warfarin dose between the different genotype groups. For data that were not normally distributed, we used Mann-Whitney *U* or Kruskal-Wallis tests.

Sample Size Calculation: Since regression usually requires higher sample sizes than other statistical tests, we did our sample size calculation only for the regression objective. For an anticipated effect size (f^2) of 0.2, statistical power of 0.8, 15 predictors, and an alpha level of 0.05, it was estimated that a sample size of 107 would be required. To account for patients' withdrawal and model validation a total of 150 patients was considered adequate. Sample size calculations were performed using Soper, D.S. (2017). A-priori Sample Size Calculator for Multiple Regression, available from http://www.danielsoper.com/statcalc.

Stratification and Randomization: To assure the homogeneity of both the derivation and validation cohorts, samples were first stratified by dose using the interquartile range. Samples in the first quartile were considered as low, samples in the fourth quartile were considered high, and anything between the second and third were considered as moderate. After that, samples were randomized to have 70% in the derivation cohort and 30% in validation cohort, using computer randomization.

Simple linear regression (SLR) modeling was used to estimate the effect of each genetic and non-genetic factors studied on warfarin dose variability. Furthermore, we relied on the results of SLR to decide which factors to include in model development, any factor with a p-value 0.2 and below was included.

Multiple linear regression modeling with both backward elimination selection and stepwise selection was used to develop the dosing model. We tried all three models: recessive; dominant; and additive, and chose the one with the highest R-square value. To

evaluate the validity of our model, before using it in dose prediction, we assessed four assumptions. First, we assessed the Durbin-Watson value (2 being the perfect value indicating no correlation between errors). Second, we looked for any outlier, an observation was considered an outlier if it had a studentized residual (T_{ei}) ≥ 3. Third, we investigated for any influential observation using the Cook's distance statistic (D_1), an observation was said to be influential if it satisfied the following: $D_1 > 4/n$. Lastly, we inspected the standardized residual plot to ensure randomity of errors.

To validate the dosing-model we calculated Spearman's correlation coefficient and mean absolute error.

All Statistical tests were carried using the IBM Statistical Package for Social Sciences, SPSS v. 24.0 (IBM Corp., Armonk, NY, USA).

CHAPTER 4: Results

4.1 Patient Recruitment and Study Population Characteristics

We screened through 470 records to identify possible eligible patients. Forty of them were excluded for the following reasons: patients were deceased; not on warfarin; or not Qatari. Out of the remaining 430 patients, 169 were approached, and 150 patients gave consent with an 82% response rate. Only 149 patients were included in the analysis due to poor quality in one of the samples where no DNA was detected.

Based on the interquartile range (IQ) patients were stratified into three groups: low dose (≤ 21 mg/week), intermediate dose (>21 and < 43.75 mg/week), and high dose (≥ 43.75 mg/week). After that, patients were randomized to have 70% in the derivation cohort and 30% in the validation cohort.

Table 8 shows the demographic characteristics of our studied population. More than half of our studied population were females (59.7%), with mean of age of 62, and mostly overweight or obese. Median warfarin dose was 29.1 (21-45.5) mg/week in the derivation cohort and 32.2 (21.7-43.7) mg/week in the validation cohort. Warfarin weekly dose in our entire cohort ranged from 9.5 to 91 mg/week (9.58-fold inter-patient dose variability). The most common indication for warfarin was atrial fibrillation (65.1%), followed by valve replacement (16.1%) and venous thromboembolism (12.8%). More than 60% of patients in both cohorts were on statins, and around 18-20% were using either amiodarone or digoxin.

Table 8 *Demographic and Clinical Characteristics*

Variable	Derivation cohort (N=104)	Validation cohort (N=45)	P Value	Total (N=149)
Age (years) mean ± SD	62.47 ± 12.8	62.95 ± 13.6	0.63	62.61 ± 13.02
Gender no. (%)			0.014	
Male	38 (36.5)	22 (48.9)		60 (40.3)
Female	66 (63.5)	23 (51.1)		89 (59.7)
Weight (Kg) mean ± SD	83.75 ± 18.45	81.56 ± 14.7	0.48	83.09 ± 17.39
Height(cm) mean ± SD	161.06 ± 10.7	161.93 ± 9.7	0.64	161.32 ± 10.39
BMI (kg.m^{-2}) mean ± SD	32.41 ± 7.3	31.25 ± 5.9	0.35	32.06 ± 6.94
Smoker no. (%)mean ± SD	8 (7.7)	3 (6.7)	0.6	11 (7.4)
Weekly Warfarin Dose (mg/week)			0.73	
Median (IQR)	29.1 (21 – 45.5)	32.2 (21.7 – 43.7)		31.5 (21.08 – 43.7)
Target INR Range no. (%)			0.09	
2-3	79 (76)	35 (77.8)		114 (76.5)
2.5-3.5	13 (12.5)	7 (15.6)		20 (13.4)
Other	12 (11.5)	3 (6.6)		15 (10.1)

Indication for warfarin no. (%)			<0.001	
Atrial fibrillation	64 (61.5)	33 (73.3)		97 (65.1)
Valve replacement	17 (16.3)	7 (15.6)		24 (16.1)
Venous thromboembolism	15 (14.4)	4 (8.9)		19 (12.8)
Other*	8 (7.7)	1 (2.2)		9 (6.1)
Concomitant disease no. (%)				
Diabetes	61 (58.7)	33 (46.1)	0.01	85 (57)
Hypertension	66 (63.5)	33 (73.3)	0.02	99 (66.4)
CHF	12 (11.5)	4 (8.9)	0.29	16 (10.7)
Cancer	1 (1)	2 (4.4)	0.09	2 (2)
Dyslipidemia	27 (26)	14 (31)	0.43	41 (27.5)
Concurrent medications no. (%)				
Statins	71 (68.3)	31 (69.8)	0.54	102 (68.5)
Antiplatelets	31 (29.8)	13 (28.9)	0.53	44 (29.5)
Antiarrythmics**	19 (18.3)	9 (20)	0.24	28 (18.8)
Thyroidal Hormones	14 (13.5)	8 (17.8)	0.23	22 (14.8)
Genotype Frequencies no. (%)				

VKORC1(-1639G>A)			0.35
GG	30 (28.8)	11 (24.4)	41 (27.5)
AG	56 (53.8)	24 (53.3)	80 (53.7)
AA	18 (17.3)	10 (22.2)	28 (18.8)
*CYP2C9 *2 & *3*			0.64
*1*1	74 (71.2)	31 (68.9)	105 (70.5)
*1*2/*2*2	20 (21.1)	11 (24.4)	33 (22.1)
*1*3/*3*3	7 (7.7)	3 (6.7)	11 (7.4)
*CYP4F2*3 (C>T)*			0.022
CC	32 (30.8)	18 (40)	50 (33.6)
CT	50 (48.1)	21 (46.7)	71 (47.7)
TT	22 (22.2)	6 (13.3)	28 (18.8)

*Thrombophilia, LV thrombus, or cardiomyopathy
**Amiodarone or digoxin

4.2 Prevalence of *VKORC1*, *CYP2C9* and *CYP4F2* variants

To estimate the prevalence of our studied genetic variants we calculated their minor allele frequency (MAF), which are all shown in Table 9 along with MAF's in other populations of MENA. The genotype frequencies are shown in Table8, for both the validation and the derivation cohort. No deviations from Hardy-Weinburg equilibrium were seen for any of the genotype frequencies (Table 10). The MAF of *VKORC1*(-1639G>A), *CYP2C9 *2*, *CYP2C9*3*, and *CYP4F2*3* were 46%, 12%, 4%, and 43%, respectively.

Carriers of the *VKORC1*(-1639G>A) represented 72.5% of our population and 66.5% were carriers of the *CYP4F2*3* minor allele. For both variants, 18.8% of the total cohort were homozygous for the minor allele. While about 30% of our population were identified as carriers for at least one of the *CYP2C9* minor alleles, only two patients were homozygous for *2 and only one was homozygous for *3.

Table 9 *Minor Allele Frequencies of Studied Variants in Qatari Compared to Other Populations of MENA*

	VKORC1 -1639G>A	*CYP2C9*2*	*CYP2C9*3*	*CYP4F2*3*
Qatari (our study) N=149	0.46	0.12	0.04	0.43
Qatari (87) N= 100	--	0.12	0.02	0.38

UAE	0.50	--	--	--
N=213				
Saudi (88)	0.42	0.13	0.02	--
N=131				
Jordan (89)	--	0.13	0.06	--
N= 263				
Egypt (66)	0.46	0.11	0.09	0.42
N= 207				
Iran (71)	0.56	0.27	0.09	--
N= 100				
Israeli (72)	--	0.12	0.11	--
N= 100				
Kuwait (73)	0.40	0.14	0.04	--
N= 108				
Lebanon (74)	0.52	0.15	0.07	--
N=43				
Oman (75)	0.35	0.06	0.06	0.3
N=212				
Sudan (76)	0.37	0.05	0	--
N= 203				
Turkey (77) (78)	0.49	0.13	0.1	0.4
N= 107, 205				

Table 10 *Chi-Square Goodness-of-fit for Hardy-Weinburg Equilibrium*

SNP	VKORC1(1639G>A)	CYP2C9*2(C>T)	CYP2C9*3(A>C)	CYP4F2*3(C>T)
Chi-Square value	1.00	0.002	2.58	0.10
P-value	0.31	0.96	0.11	0.75

4.3 Association of Genetic and Non-Genetic Factors with Warfarin Dose

Weekly warfarin dose in the derivation cohort was skewed so log transformation was performed before carrying on with the analysis.

Univariate analysis showed that the minor loss of function allele (*CYP2C9* *2 & *3) was significantly associated with warfarin dose and that carriers of this allele required lower mean weekly doses (MWD) compared to the wild type (Carriers 23.98 mg/week, WT 34.11 mg/week, p< 0.001) (Figure 5). One-Way ANOVA and post-hoc analysis showed that mean dose requirements were significantly lower in the heterozygous genotype (*1/*2 or *1/*3) compared to the wild type (24.26 mg/week vs. 34.11, p= 0.002). Patients homozygous for *2 or *3 required much lower doses than all other genotype groups (19.52mg/week), however, this difference was not statistically significant (p= 0.097). The lowest dose in our cohort belonged to the patient with *3*3 genotype (9.5 mg/week). Table 11 shows dose requirements by each of the *CYP2C9* genotype groups.

There were no significant differences in dose requirement across the different genotype groups of *VKORC1*(-1639G>A) or *CYP4F2**3 (Figure 6 & Figure 7).
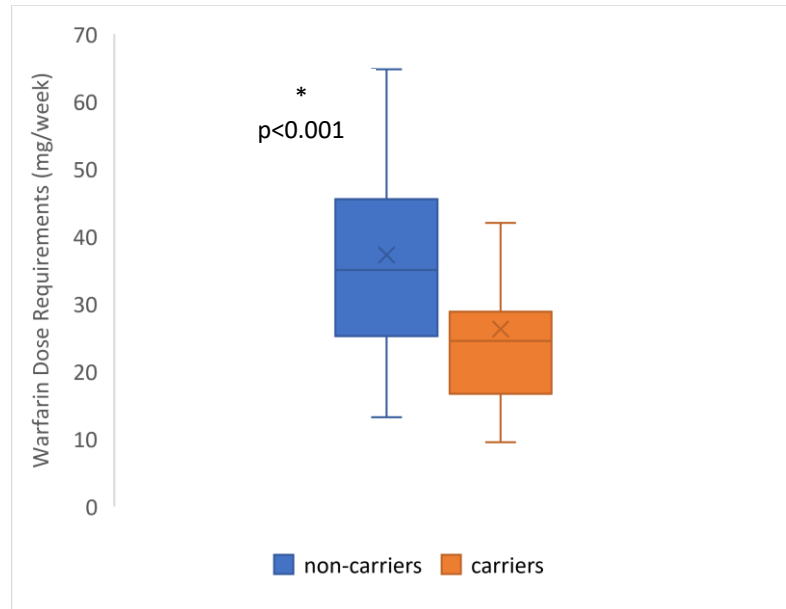
Figure 5 Effect of *CYP2C9* variants on warfarin dose in Qatari (n=104).

Box-and-Whisker plots showing the distribution of weekly warfarin dose in carriers and non-carriers of CYP2C9 variant alleles. Boxes represent median and interquartile range. Lines above and below the boxes represents maximum and minimum values.

Table 11 *Mean Weekly Warfarin Dose Requirements by CYP2C9 Genotype*

CYP2C9 Genotype	Mean Weekly Dose ± SD (mg/week)	P-Value Compared to WT
*1*1 **(N= 105)**	34.4 ± 1.5	
*1*2 **(N= 31)**	25.7 ± 1.5	P= 0.007
*1*3 **(N= 10)**	22.9 ± 1.3	P=0.029
*2*2 **(N= 2)**[*]	28 ± 2.3	
*3*3 **(N= 1)**[*]	9.5	

One-Way-ANOVA was used to compare long-transformed mean weekly dose between genotype groups of the

entire cohort (n=149), p-values refer to any significant difference between the genotype group and wildtype

[*]**Because of the limited number of cases in these groups, statistical comparison in mean dose requirements was**

not possible
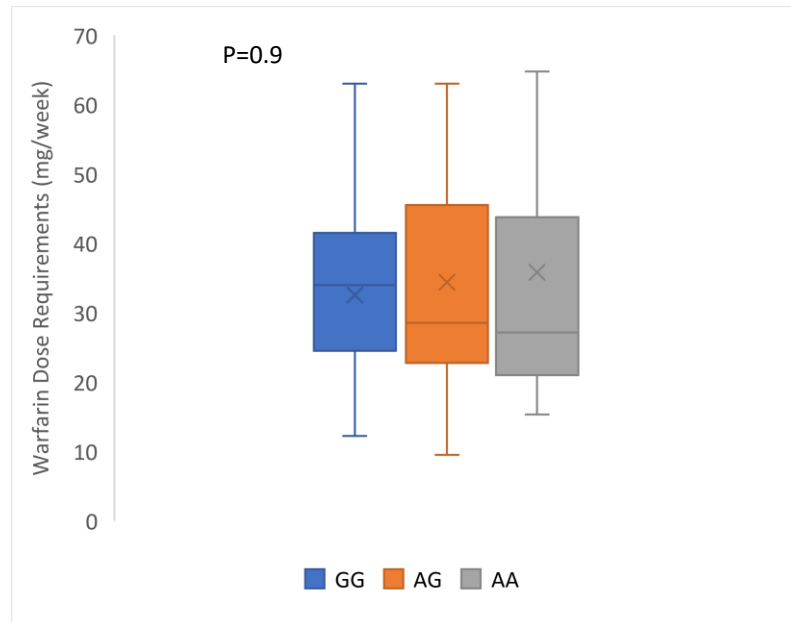
Figure 6 Effect of *VKORC1*(-1639G>A) genotypes on warfarin dose in Qatari (n=104).
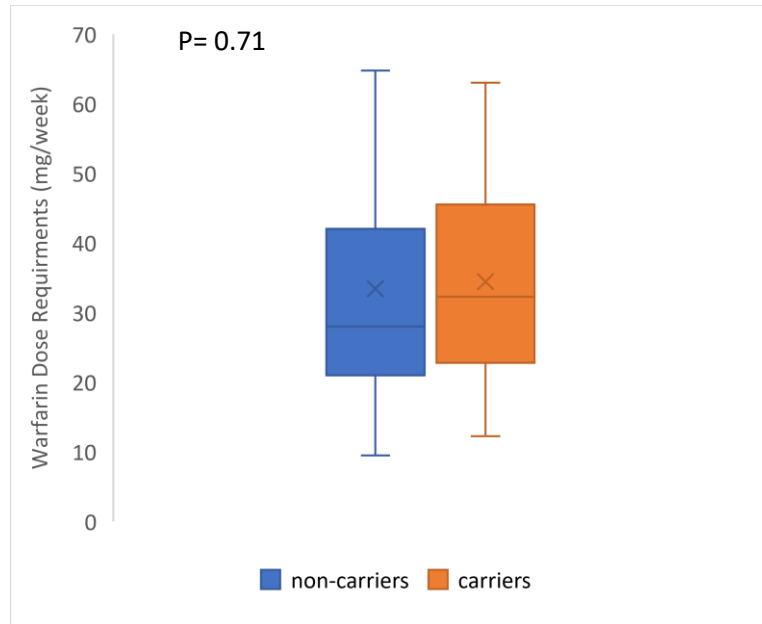
Figure 7 Effect of *CYP4F2**3 on warfarin dose in Qatari (n=104).

*Box-and-Whisker plots showing the distribution of weekly warfarin dose between CYP4F2*3 carriers and non-carriers. Boxes represent median and interquartile range. Lines above and below the boxes represents maximum and minimum values.*

To estimate possible reasons for the insignificant effect of VKORC1 on warfarin dose, we considered the combined effect of variants of the *CYP2C9* and *VKORC1*. Table 12 shows the distribution of warfarin dose between different combined genotype groups. We

found that more than 50% of *VKORC1*-1639G>A had the *CYP2C9**1*1 genotype. The only

significant difference in mean weekly warfarin dose was seen between the group of

carriers of at least one variant allele for each gene and those only carrying the minor allele

for the *VKORC1*-1639G>A (24.04mg/week vs. 34.47mg/week, p=0.012). Furthermore, we

considered the difference in mean age between carriers and non-carriers of G1639A

minor allele. We found that carriers of the minor allele were younger compared to non-

carriers (61.41±12.17 vs. 65.06±14.13 years, respectively). However, this difference in

mean age did not reach statistical significance (p=0.19).

Table 12 *Frequency of Different Combined Genotype Groups of CYP2C9 And VKORC1 and the Distribution of Weekly Warfarin Dose between these Groups*

Genotype group	Frequency (%), N=104	Mean weekly dose (SD) mg/week
WT/WT	20 (20.1)	33.25 (1.53)
CYP2C9WT/VKORC1 carriers	53 (51)	34.47 (1.53)
Carrier/carrier	21 (20.2)	24.04 (1.63)
CYP2C9carrier/VKORC1WT	9 (9.6)	23.09 (1.47)

To identify possible predictors of warfarin dose we ran simple linear regression between log-transformed warfarin dose and each of the following variables: age, height, weight, BMI, gender, atrial fibrillation, diabetes, hypertension, congestive heart failure (CHF), cancer, smoking, statins, antiarrhythmics, antiepileptics, antibiotics, antiplatelet, thyroid hormones, and variants of the *VKORC1*, *CYP2C9* and *CYP4F2.* Results for the simple linear regression are shown in table 13. We found that age, height, hypertension, CHF, smoking, statin, and *CYP2C9* are all significant predictors of warfarin dose with p-value less than 0.05. The *CYP2C9* had the lowest p-value (<0.001) and the highest adjusted-R^2, it explained 11.8% of the dose variability. This was followed by smoking, age, and CHF, which explained 7.6, 6.5, and 5.6% of the variability in dose, respectively. While statins, height, and hypertension explained only 3-4.4% of the variability.

Table 13 *Univariate Analysis of Factors Affecting Warfarin Dose Variability (derivation cohort, n=104)*

Factor Explored	Constant	Coefficient	Adjusted R^2	P-Value
Age	1.754	-0.271	0.065	0.005
Gender	1.561	-0.108	0.002	0.270
Height	0.868	0.205	0.032	0.037
Weight	1.418	0.076	-0.004	0.440

BMI	1.543	-0.062	-0.006	0.530
AF	1.523	-0.145	0.011	0.143
Diabetes	1.514	-0.112	0.003	0.250
Hypertension	1.545	-0.215	0.037	0.028
CHF	1.506	-0.256	0.056	0.009
Smoking	1.471	0.291	0.076	0.030
Antibiotics	1.489	-0.033	-0.009	0.700
Statins	1.557	-0.230	0.044	0.019
Antiarrythmics	1.486	0.022	-0.009	0.820
Antiplatelet	1.488	-0.001	-0.010	0.990
Thyroid Hormones	1.482	0.070	-0.005	0.470
Antiepileptics	1.486	0.037	-0.008	0.71
VKORC1	1.474	0.042	-0.008	0.67
CYP2C9	1.533	-0.355	0.118	<0.001
CYP4F2	1.476	0.037	-0.008	0.7

4.4 Derivation and Validation of the Dosing Model/Algorithm

After running the simple linear regression analysis for all factors, we only included factors with a p-value of 0.2 or less in the model development. These included: age, height, hypertension, smoking, statins, CHF, and *CYP2C9*. Although *VKORC1* and *CYP4F2* had a p-

value more than 0.2 in SLR, we still included them in the multiple linear regression (MLR) because of their known important effect on warfarin dose in previous studies. Using the dominant model for *CYP2C9, VKORC1,* and *CYP4F2* variants, multiple linear regression, in both backward elimination and stepwise selection, showed that having CHF, being a smoker, and carrying at least 1 of the *CYP2C9* minor alleles were the only significant predictors of warfarin dose, and together they explained 24.1% of the dose variability (Table 14).

Table 14 *Multiple Linear Regression-Association between Weekly Warfarin Dose and Genetic and Non-Genetic Factors*

Predictor	Coefficient	Standard error	p-value
Intercept	1.535	0.022	<0.001
CHF	-0.268	0.053	0.002
Smoking	0.258	0.065	0.003
CYP2C9	-0.337	0.038	<0.001
Model adj-R^2 = 0.241			<0.001

Based on the coefficient of regression estimates, the proposed warfarin dosing model for

Qataris is:

Warfarin dose (mg/week) = 10 ^ [1.535 − 0.268 (CHF)[a] + 0.258 (smoking)[b] − 0.337

(*CYP2C9*)[c]

    [a] presence of CHF (1), absence of CHF (0)

    [b] smoker (1), non-smoker (0)

    [c] carrying at least 1 of the *2 or *3 minor alleles (1), non-carriers (0)

To evaluate the validity of our model we calculated the Durbin-Watson value and it was

1.795, which is close to the perfect value of "2", indicating that errors were random and

not correlated. We inspected for any outliers or influential observations and we have not

found any values with a $T_{ei} \geq 3$ or $D_1 > 4/n$. Our standardized residuals plot (Figure 8) was

relatively random also indicating the absence of correlation between errors.
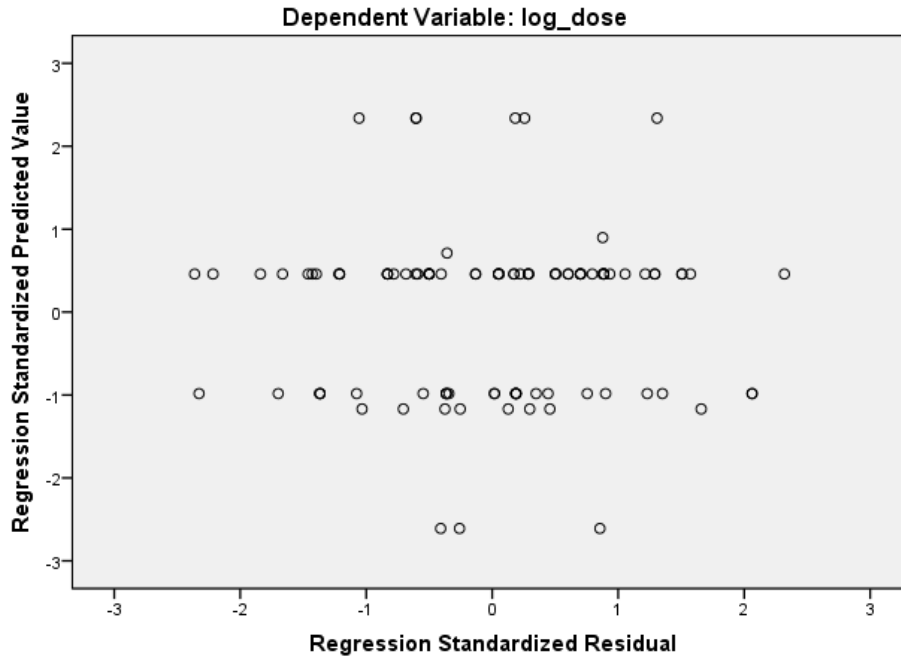
Figure 8 Standardized residuals plot.

To validate the regression model produced from the derivation cohort, we calculated the predicted warfarin doses using the dosing model and then compared it to the actual doses in the validation cohort. Figure 9 shows the comparison between actual doses from the validation cohort and predicted doses calculated using the regression model. No significant differences were found between the validation and the derivation cohort in terms of all demographic, clinical, and genetic characteristics except for: gender, indication for warfarin, having diabetes or hypertension, and genotype frequencies of *CYP4F2**3. We found significant correlation between the predicted and observed doses,

Spearman's rho correlation coefficient was 0.412 (p=0.005). However, our dosing model failed to show a good prediction of warfarin dose in the validation cohort, the mean absolute error (MAE) was somewhat high (MAE = -6.82 ± 15.11). Only 31% of patients had their predicted dose within 20% of their actual stable therapeutic dose, while 46.6% of the predicted doses were underestimated by more than 20% of the actual stable therapeutic dose.
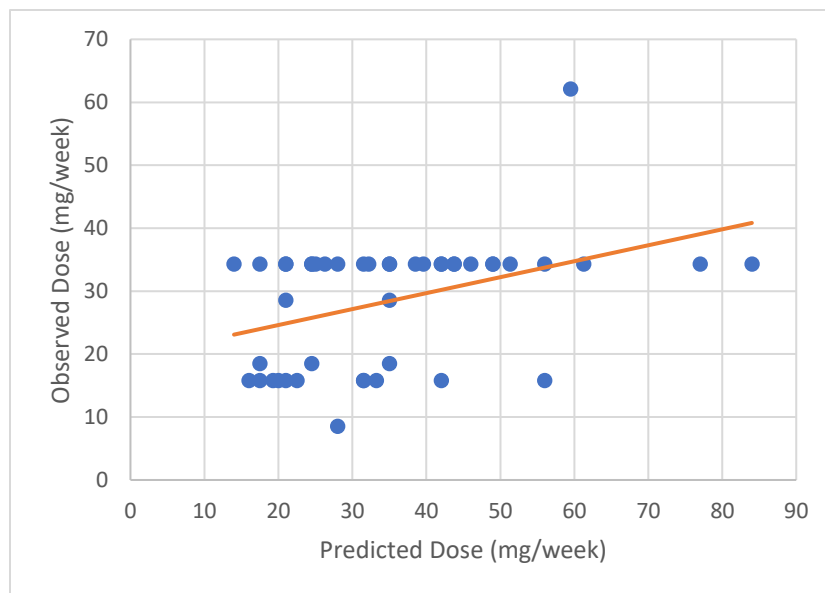
Figure 9 Observed versus predicted weekly warfarin doses in the validation cohort (n=45).

Solid line indicates perfect prediction.

CHAPTER 5: Discussion

5.1 Discussing Main Study Findings

Qatar is a peninsula in the Persian/Arabian Gulf. It resides at the eastern edge of the Arabian Peninsula which makes it at the center of migration patterns that took part in the region over the era of human history (90). Early Arab tribes have migrated through Qatar as trade prospered with ancient Mediterranean civilizations. The region was colonized, before the 20th century, by Portuguese, Ottomans, and British. It also witnessed an inflow of Persian traders and African slaves brought in by Oman trade routes. All these events have created a genomic admixed Qatari population consisting mainly of Arabic (mainly Bedouins), Persian (Iran, Pakistan and Afghanistan), and African (Sub-Saharan Africa) ancestry (90). A high rate of consanguinity still exists among Qataris (35% in 2010), with first cousins' marriages being widely accepted and predominant (57). Such high levels of consanguinity may have had a profound influence on the genetic make-up of this population (91).

Warfarin is a commonly used oral anticoagulant in Qatar and worldwide. Genome wide association studies have shown that variants in the *VKORC1*, *CYP2C9*, and *CYP4F2* can contribute to the dose variability from one patient to another (51). Since no previous warfarin pharmacogenetics studies were conducted in Qatar, the main goal of the present study was to estimate the prevalence and the association of *VKORC1*-1639G>A, *CYP2C9* *2 & *3, and *CYP4F2**3 with warfarin dose variability in Qataris.

5.1.1 Prevalence of the Studied Genetic Variants

In our study we found that the MAF of *CYP2C9**2 was 12%, which was the same as what was previously found by Sivadas and others from whole exome datasets in Qataris (87). The MAF of *CYP2C9**3 and *CYP4F2**3 were 4% and 43% in our study, 2% and 38% in the previous study (87). This study is the first to report the MAF of *VKORC1*-1639G>A in Qataris and it was 46%, resembling Saudi (42.4%), Egyptian (46.2%), Turkish (49%), and Kuwaiti populations (40%) (66, 73, 77, 88). The *CYP2C9**2 MAF was comparable to Saudi, Israeli, Jordanian, Egyptian, Kuwaiti, and Turkish populations which were all between 11-14% (66, 72, 73, 77, 78, 88, 89). Furthermore, *CYP2C9**3 frequency was the same as Kuwaiti and close to Saudi (2%), but lower than in other populations of MENA which were between 6-11%, except for Sudanese where this variant was not detected (73, 76, 88). Even though a good number of Qataris descent from at least one Iranian parent, we did not find good similarities between our population and Iranians. The *CYP2C9**2 and *3 frequencies are twice as high in Iranian compared to Qatari (71).

Looking outside the MENA region, the *VKORC1* and *CYP2C9**2 MAF's were closer to Europeans (36.1% & 12.5%) than to African Americans (10.6% & 1.9%) or Asians (87% & 0%) (39, 44). The *CYP2C9**3 frequency on the other hand, resembled Asians (3%) as compared to Europeans (6.3%) or African Americans (1.9%) (44).

5.1.2 Effect of the Studied Genetic Variants on Warfarin Dose

CYP2C9: We found a significant association between dose requirements and *CYP2C9**2 & *3. Patients carrying any loss-of-function CYP2C9 alleles (*1*2, *1*3, *2*3, or *3*3) had significantly lower doses than those with the wild type genotype (*1*1) (23.98±1.53 mg/wk vs. 34.11±1.58 mg/wk, p= 0.002). These findings greatly resemble those found in Egyptians by Ghozlan et al. (carriers 24.5 mg/week vs. WT 38.5mg/week, p< 0.001) and Shahin et. al. (26.6 mg/week *1*2 & *1*3 vs. 40.3 mg/week WT, p< 0.01) (66, 68). They were also comparable to what was reported by Ozer et al in Turkish (*1*3/*2*3: 23.24 mg/week, *1*1: 33.18 mg/week, p < 0.05); however, Turkish patients with *1*2 genotype showed no significant difference in their dose requirements compared to other genotype groups (80). While Bazan et al found a significant difference only in carriers of the *3 variant (19.67 mg/week vs. 57.47 mg/week, p< 0.001) and no difference was reported by Ekladious et al in Egyptians (67, 70). Our results also matched those found in Kuwaiti (carriers: 23.1 mg/week, WT: 33.26 mg/week, p=003) and Iranian (carriers 24.38mg/week, WT: 33.26 mg/week, p<0.05) (71, 73). Variants of the *CYP2C9* were the biggest predictors of warfarin dose in our population explaining 11.8% of the variability. Close findings were seen in Iranian (12.9%), Egyptians (11.3%), and Lebanese (12%). However, these findings only belonged to *2 variant in Iranian and only *3 variant in the other populations (67, 71, 74). In Turkish, both variants were responsible for 8.1% of the dose variability, 16.5% in Omani, and 20% in Israeli (72, 75).

VKORC1: The *VKORC1* is the target enzyme for warfarin and it was shown to be the biggest predictor of warfarin dose worldwide. Carriers of the G-1639A variant usually require significantly lower doses compared to non-carriers. In 2008, Limdi et. al. reported the *VKORC1*-1639 to be a predictor of warfarin dose in Asians, Blacks, and Whites. However, the contribution of this variant to dose variability varied from one race to another reflecting its varying frequencies in different race groups. It could explain 11-32% of dose variability in Caucasians, 30% in Asians, and only 4-11% in African Americans (38). In populations of MENA, this variant resulted in a significant decrease in dose requirements every time it was studied (54). Nevertheless, the size of its effect and contribution to dose variability varied between populations. In Turkish, the *VKORC1*-1639 accounted for 14.7-43% of dose variability (77, 79, 92), 12.5-19.5% in Egyptians (67, 68), 20-22% in Lebanese, Israeli, Iranian, and Sudanese (22, 71, 74, 76). Despite its high frequency, the *VKORC1*-1639G>A did not show any significant association with warfarin dose in Qatari population. Mean weekly dose requirements were almost the same in all genotype groups (GG 29.8 mg/week, AG 31.04 mg/week, AA 31.36 mg/week, p=0.9), and when added to the multivariate analysis it did not show significance. The reason we could not find any association between this variant and warfarin dose may have been due to the existence of less common missense mutations that are associated with warfarin resistance including: Asp36Tyr, Tyr139Cys, Val45Ala, Val25leu, Arg58Gly (42). The presence of two variants with opposing effect could have resulted in an insignificant overall effect on dose. However, the effect of these variants has not been widely studied except for the *VKORC1* Asp36Tyr. Shahin et al, in their study on the prevalence of this variant, have reported its

existence mainly in all Northern African and Middle Eastern countries and its absence in

West Africans or African Americans and Peruvians (41). Its highest frequency was seen in

Sudanese and Kenyans (6%), while it was less in Ashkenazi Jew (4%) (93), Egyptians (2.5%)

and Saudi (3%) (41). In the study reported by Shahin et al, 10 Egyptian patients carried

this variant and their mean(SD) weekly dose was significantly higher compared to the

non-carriers, carriers: 57.1(29.4) vs. non-carriers: 35.8(16.6) mg/week, p=0.03 (41). When

added to their dosing model, it improved the adjusted-R^2 from 31% to 36.5%. These

findings highlighted the importance of Asp36Tyr variant in predicting warfarin resistance.

In a large cohort of Ashkenazi Jews, the presence of one Asp36Tyr variant allele resulted

in an increase in weekly warfarin dose to a median of 43.7 mg (IQR: 40.5 to 47.2 mg/week)

(93).

The lack of association between *VKORC1*-1639G>A and warfarin dose in this study could

be also attributed to the presence of other factors that are associated with higher

warfarin doses in the group of G1639A carriers. These include having the *CYP2C9**1*1

genotype, having younger age, and having a larger BMI (94, 95). There was no significant

difference in the frequencies of *CYP2C9* variants among *VKORC1*-1639G>A WT and

carriers in the derivation cohort (see Appendix 6). Furthermore, estimating the combined

effect of *CYP2C9* and *VKORC1* variants showed no significant difference in dose

requirements between *CYP2C9**1*1-*VKORC1*GG group and *CYP2C9**1*1-*VKORC1*AG/AA

group. The only significant difference found was between

*CYP2C9*carriers/*VKORC1*carriers and *CYP2C9**1*1-*VKORC1*AG/AA (p=0.012), with dose

being significantly lower in the former group. Such findings approve that the effect on

dose was mainly due to variants of the *CYP2C9*. Mean age was slightly lower in the carriers of G1639A allele compared to non-carriers (61.41±12.17 years vs. 65.06±14.13 years). However, this difference was not statistically significant, p=0.19. No significant difference was found in mean BMI between carriers and non-carriers (see Appendix 6).

CYP4F2: CYP4F2 is the metabolizing enzyme for vitamin K. Previous studies have shown that the Val433Met variant in the gene coding for this enzyme is associated with higher warfarin dose requirements, and when added to *VKORC1* and *CYP2C9* it could further explain the variability in warfarin dose (50, 96). This variant is highly prevalent in Europeans and Middle Eastern countries (30-40%), and less common in Asians (17%) and African Americans (8%) (52, 53, 66, 75, 77). Asians carrying the variant *3 allele require higher doses of warfarin compared to the wildtype, (carriers: 26.25mg/week vs WT: 21mg/week, p=0.033). Moreover, this variant accounted for 3% of the dose variability in Asians (53). It was shown to be a significant predictor of warfarin dose in African Americans and European Americans. Furthermore, European American patients homozygote for *3 allele had a statistically significant increase in dose by 13.2% (52). The *CYP4F2**3 was not widely studied in Middle Eastern countries. In Omani, patients homozygous for this variant required significantly higher warfarin daily doses compared to non-carriers, (*3*3: 5.8±4.3mg/day vs. *1*1: 4.6±1.9, p<0.05) (97). However, when combined with *VKORC1* and *CYP2C9* in multiple analysis, the *CYP4F2**3 was no longer significant. In Turkish, Özer et al reported significant association between warfarin dose and *CYP4F2**3. Carriers of at least one *3 allele required significantly higher mean daily

doses compared to non-carriers, *1*3: 5.58±2.24 mg/day, *3*3: 5.2±1.1 mg/day, *1*1: 4.53±1.73 mg/day, p=0.032 (77). When incorporated into the multivariate analysis, *CYP4F2*3 still explained 2.8% of dose variability in Turkish. In our study, we did not find any significant association between weekly warfarin dose and *CYP4F2*3, carriers: 29.5±1.6mg/week, WT: 30.9±1.6mg/week, p=0.71. Our findings are consistent with Egyptians, where no significant difference was found between carriers and non-carriers of *CYP4F2*3, p=0.314 (66). Both results are also in agreement with the most recent findings of Shendre et al in African Americans, in which they reported no statistically significant association between warfarin dose and *CYP4F2*3 (52). However, such finding was attributed to the low prevalence (n=2) of this variant in their study cohort.

5.1.3 Effect of Studied Clinical Factors on Warfarin Dose

Apart from genetic factors, univariate analysis revealed the following clinical factors to be independent significant predictors of warfarin dose: age, height, hypertension, CHF, smoking status, and being prescribed statins. All were negatively correlated with mean weekly warfarin dose, except for height and smoking status. While multiple regression analysis showed that only CHF and smoking status along with *CYP2C9* are predictive of warfarin dose and together they explained 24.1% of the variability in dose.

Age: As the human body ages its requirements to warfarin dose decreases. Such observation was once attributed to the negative correlation between age and liver size (r

= -0.41; P =0.01) (98). Wynne reported in 1995 that liver size was around 28% lower in patients over the age of 65 years compared to ones under 40 years (98). Wynne found negative correlation between age and dose (r = -0.53; P = < 0.001) and positive correlation between dose and liver volume (r = 0.49; P = 0.002), and that liver volume and age accounted for 43% of dose variability. Another study concluded that lower dose requirements in elderly are attributed to decreased hepatic clearance, 1% decrease per year (99). Increased sensitivity to warfarin in the elderly could be also attributed to reduced vitamin k intake or reduced absorption, which can decrease the capacity of the liver to synthesize clotting factors; or it could simply be a result of polypharmacy that can lead to drug-drug interaction, potentiating the effect of warfarin (100). In the present study we found that mean weekly dose decreases as age increases and that age could explain 6.5% of dose variability, such findings are consistent with the literature (66-71, 73, 101). In Egyptians age resulted in 8.11-12% decrease in dose (66, 67). In a retrospective study by Ghada Khoury including 96 patients from the anticoagulation clinic at Celebration Health Florida Hospital, age was found to be negatively correlated with warfarin dose. A statistically significant lower mean total weekly dose was seen in patients in the 80–89 age group as compared to patients in 20–29, 30–39, 40–49, and 50–59 age groups (P<0.05). Mean total weekly dose of patients in group 20–29, 30–39, and 40–49 was around 51 mg as compared to almost half of that dose (24.82 mg), in group 80–89 (P<0.05) (102). In a study that included mainly White Americans dose requirements of more than 5mg/day was associated with younger age <55 years (19). In the algorithm developed by Gage et al, age was reported as a significant predictor of warfarin dose

(p<0.001), and as age increased warfarin dose decreased by 7% per decade (-9% to -6%) (59). It was also reported as a significant predictor of warfarin dose in the IWPC algorithm (p<0.001), in which it explained 6.7% of the dose variability (60).

In the final regression model of the present study age did not show significant association with warfarin dose. Although, in many studies it was an essential contributor to dose variability (59, 60). Our finding could be limited by the small sample size used. It could also be owed to the association between age and CHF. Heart failure is one of the common age-related disease, studies have shown that increasing age is associated with increased risk for HF (103, 104). In the current study we found significant association between age and CHF, patient with CHF had significantly higher mean age (see Appendix 6). When age was added to final regression model it still did not show any significant association with warfarin dose (p=0.26), although it did slightly enhance dose prediction by 0.2%. On the other hand, adding age to the final regression model with the exclusion of CHF resulted in marked decrease in the adjusted-R^2 value from 24.1% to 19% and age had a p-value that tended to be significant (p=0.06) (see Appendix 6). Such findings indicate that the effect of age on the dose variability was better explained by CHF. Future studies with larger sample size could better explain our findings.

Smoking: Warfarin is administered as a racemic mixture of two pharmacologically active enantiomers, *R* and *S.* The more potent S enantiomer is mainly metabolized by the CYP2C9 enzyme and to a smaller extent by the CYP3A4. The R enantiomer is mainly

metabolized by CYP1A2 and CYP3A4 (11). Polycyclic aromatic hydrocarbons (PAH), main component of tobacco smoke, are potent inducers of CYP1A1 and 1A2 (105). Thus, pharmacokinetics interactions occur with smoking and drugs that are substrates for these enzymes, including warfarin. In the present study, we found a significant association between smoking status and warfarin dose (p=0.03), being a smoker resulted in 7.6% increase in warfarin dose. Moreover, in the final dosing model, smoking remained to be one of the significant predictors of mean weekly warfarin dose. Our findings are consistent with other studies as well. In Egyptian population, 8-9.4% of the increase in dose was attributed to smoking in 2 studies (67, 68), while only 1.88% was reported by Shahin (66). No significant association between tobacco smoking and warfarin dose was reported in other populations of MENA. A meta-analysis concerning the interaction between smoking and warfarin dose, was published in 2011 and included one experimental pharmacokinetic study and 12 cross-sectional studies (106). It concluded the association of smoking with 12.13% (95% CI, 6.999-17.265; P=0.001) increase in required warfarin doses and an addition of 2.26 mg (95% CI, 2.529-7.042; P 5 .355) per week compared with nonsmoking (106). In the algorithm developed by Gage et al, using data of a derivation cohort that consisted mainly of Caucasians (83%), smoking was a significant predictor of warfarin dose. Furthermore, smoking increased dose requirements by 10% (3-16%), p<0.001 (59).

CHF: Having CHF was the concomitant disease to show the most significant association with warfarin dose in our population. Having CHF resulted in 5.6% dose reduction,

p=0.009. Such finding is consistent with African Americans, where significant negative correlation was reported, beta coefficient= -0.51, p<0.05 (107). Visser reported that after adjusting for confounding variables, heart failure (HF) was significantly associated with increased risk of over-anticoagulation with coumarin treatment (108). Moreover, patients with HF required much lower doses of coumarins compared to those without, regardless of the higher INR levels seen in patient with HF. The enhanced response to coumarin in patients with HF is thought to be a result of impaired liver function resulting from the congestion. It is hypothesized that causes of enhanced response to coumarin is rather pharmacodynamic (impaired synthesis of clotting factors) than pharmacokinetic (decreased coumarin hepatic clearance) (109, 110).

Statins: Sixty-eight percent of our derivation cohort (n=71) were taking lipid lowering agents, mainly atorvastatin (64.8%) and rosuvastatin (28%). Being on statins was shown to be significantly associated with decreased warfarin dose requirements and it could explain 4.4% of dose variability in univariate analysis. Statins are a large group of medications and their interaction with warfarin and increased risk of bleeding is debated. In 2010, it was reported that initiation of statins that inhibit the CYP3A4 enzyme, including atorvastatin, was associated with increased odds ratios for gastrointestinal bleeding [1.39 (95% CI, 1.07-1.81) for the first prescription; 1.05 (95% CI, 0.73-1.52) for the second prescription] (111). While other statins like pravastatin, which are mainly excreted unchanged, posed no increased risk. Although atorvastatin is classified as a drug that has no interaction with warfarin, such conclusion was based only on one study that did not

show any significant increase in INR readings in 12 warfarin treated patients after 15 days of initiating atorvastatin (112). Moreover, one study concluded that the co-administration of any of the statins agents is associated with decreased risk for bleeding in patients with atrial fibrillation (113). The effect of rosuvastatin on warfarin pharmacokinetics and pharmacodynamics was only studied in two small randomized controlled trials (114). The two trials concluded that rosuvastatin can enhance the anticoagulant effect of warfarin; however, the mechanism of their interactions remains unclear.

Hypertension: Univariate analysis showed that having hypertension is associated with lower warfarin dosages (R^2 = 3.7%, p=0.028). There was no reporting of the significant association of hypertension with warfarin dosage in populations of MENA. It is well known that having hypertension increases the risk of bleeding during anticoagulation treatment. A prediction model for risk of bleeding (HEMORR2HAGES), that developed with the use of data registry on patients with AF, included uncontrolled hypertension as one of the risk factors (115). BLACK BOX warning on warfarin includes hypertension as a risk factor for bleeding (28). In patients with moderate to severe hypertension, decision makers must weigh the risks of using warfarin against the benefits before deciding on its administration (28). Out of the different antihypertensive drug classes, beta-blockers are listed as drugs that may increase the INR response when co-administered with warfarin. While diuretics are listed to have both an increasing and decreasing effect (28). In our study, we did not include detailed data about the severity of hypertension condition or medications used to treat it, nor did we investigate for any bleeding events. Hence, we cannot draw any

solid conclusions or justifications for our finding. We speculate that the lower doses could be attributed to the decision of the healthcare providers to initiate patients with hypertension on lower doses of warfarin to avoid the risk of bleeding.

5.1.4 Combined Effect of Genetic and Non-Genetic Factors on Warfarin Dose

Our final dosing model included: CHF, smoking, and *CYP2C9* and explained 24.1% of dose variability. Significant correlation was found between predicted and actual mean weekly doses (r=0.412, p=0.005). The final dosing model showed good prediction for 31% of the validation cohort, where patients' predicted doses were within 20% of their actual doses. The relatively low R^2 value of the dosing model could be attributed to the small sample size. It could also be attributed to not including other variants of the *CYP2C9* or *VKORC1* in our analysis, as well as other clinical variables (vitamin K intake, adherence to treatment, patient education, race or ethnicity).

5.2 Limitations of the Study

Small Sample Size: Although we attempted to power the study by using adequate sample size, our calculations were only limited to the model development and not the model validation. Moreover, in our sample size calculation, we did not account for any of the minor allele frequencies of the studied genetic variants.

Study Population: The Qatari population is highly admixed but at the same time it is structured. It consists mainly of Arabic (mainly Bedouins), Persian (Iran, Pakistan and Afghanistan), and African (Sub-Saharan Africa) ancestry (90). In this study, Qatari patients were identified based on what is indicated on their passports. The genetic sub-structure of the Qatari population was not accounted for in the present study, which may have had a major influence on our study results. Future studies must incorporate this important variable in the analysis.

Confounding Variables: Dietary vitamin K has been previously shown to be a major contributing factor to anticoagulation stability (116). However, due to lack of documentation in some patients and inconsistency of documentation in others, we could not include vitamin K intake in our analysis. We also did not account for adherence to treatment or patient anticoagulant education. Additionally, some of the recruited patients were followed by physicians, while others were followed by pharmacists. It was previously shown by Elewa et. al. that pharmacist-managed anticoagulation has a better INR control than doctor-managed anticoagulation in Qatar (117). Not accounting for this in our analysis may have affected our results.

Patients Included: In this study, we included patients on a stable warfarin dose, regardless of their target INR range. However, other studies usually limit their study population to only those with a target INR of 2-3. Not doing so in our study may have

affected our multivariate analysis. However, we did not find any changes in results when we excluded any patients with a target INR other than 2-3 (data not shown).

Study Design and Outcomes Measured: Our study was cross-sectional observational which have served our exploratory purpose. However, a longitudinal study, that starts with treatment initiation and continues with a follow up period, could have given better overview of the effect of the genetic variants not only on warfarin dose but also on other clinical outcomes. These outcomes include: percent time in therapeutic INR range, time to therapeutic INR, thromboembolic and bleeding events.

Genetic Variants Studied: Our focus in this study was on *VKORC1*-1639G>A, *CYP2C9**2 & *3, and *CYP4F2**3 and their association with warfarin dose. However, other less common variants may have had an impact on warfarin dose requirement in Qatari. Not including these variants in our analysis may have affected the study results.

Quality of Genotyping: Due to lack of feasibility, we could not confirm our genotyping results accuracy by repeating the genotyping in a different laboratory, or by using another genotyping technique in our laboratory.

5.3 Strengths of the Study

Our study is the first study in Qatar to explore the association of common genetic variants with warfarin dose requirements. The only study reported about Qatari population was concerning the frequencies of these variants but not their effect on dose variability (87). Evidence regarding warfarin pharmacogenetics in MENA region is still lacking. The current study adds to the body of literature in this region and sets the way for further studies to be conducted on the Qatari population. The *CYP4F2**3 was only reported in Egyptians, Omani, and Turkish, which makes our study only the fourth to explore this variant in the region.

Warfarin is highly prescribed in Qatar, and it is part of the treatment for cardiovascular diseases, which is a national health priority in Qatar. This makes our study in-line with the national health vision for the country.

Patient recruitment can be very challenging, having a good team of collaborators working by our side have greatly facilitated the recruitment process. Furthermore, using saliva samples as means for genomic DNA collection have increased patients' enrollment rate.

5.4 Conclusions

This study showed significant association between warfarin dose requirements and the *CYP2C9**2 & *3 variants in Qatari population. Dose reduction should be considered in patients carrying any of the *CYP2C9* variants alleles. There was no significant association

found between warfarin dose and *VKORC1* (-1639G>A) or *CYP4F2**3 in Qatari, despite their high frequency.

This study also showed that a dosing algorithm consisting of smoking, CHF, and *CYP2C9**2 & *3 could predict warfarin dose for the Qatari population to some extent. Underestimation was seen in the greater part of doses predicted by the dosing algorithm. This could be attributed to not including genetic variants or other clinical variables, associated with warfarin resistance, in the algorithm.

Despite the shared ancestry between our population and some of the MENA populations, great variations were found between our findings and theirs. This further highlights the importance of studying each population alone in a step towards personalizing warfarin treatment.

5.5 Future Directions

Further studies are needed with larger sample size and a longitudinal follow up, to assess the effect of genetic variants on clinical outcomes in warfarin patients. A study that accounts for adherence, vitamin K intake and patient anticoagulant education level is preferable. In addition, inclusion of less common variants in *CYP2C9*, such as *4; *5; and *8, should be considered. To our knowledge, our study is the first to show lack of significant association between *VKORC1*-1639 and warfarin dose. More studies are needed to confirm and explain such finding. Studies that investigate other variants in this gene, including the less common ones that are associated with increased dose

requirements (Asp36Try, Tyr139Cys, Val45Ala, Val25leu, Arg58Gly) are warranted. A GWAS would help identifying any rare genetic variants that could be associated with warfarin dose requirements in Qataris. Future studies could explore the effect of epigenetics and metabolomics on warfarin dose variability.

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APPENDICES

Appendix 1 Hamad Medical Corporation Institutional Review Board Approval

Hamad Medical Corporation

Institutional Review Board

Approval Notice:

Protocol Title: The Effect Of Genetic Variants On Appropriate Warfarin Dosing To Achieve Optimal Therapeutic Outcome
Study Number: 15230
Date of Approval: 22 March 2016
HMC Principal Investigator: Hazem Elewa
Review Type: Expedited
Decision: Approved for Renewal
Approved HMC Enrollment: 150

The IRB has reviewed the submitted documents of the above titled research and approval to continue the study has been granted. List of the approved documents is attached.

IRB oversight expires 12 months from the date of approval indicated above.
It is the responsibility of the Investigator to ensure timely renewal of study oversight. Progress reports for continuing review must be approved prior to expiration date; therefore submissions must be received by the IRB 60 to 90 days prior to the expiration date.

As the Principal Investigator of this research project, you are ultimately responsible for:
- Protecting the rights, safety and welfare of research subjects
- Following the IRB-approved protocol (application and any materials submitted with it).
- Following the requirements of HMC policies, especially with regard to obtaining prior approval of changes to the research, reporting events or new information and final reports.
- The conduct of the study team with regards to all of the above.

Requested Resolutions: None

Any resolutions submitted must include a letter indicating that the submission is a follow up request by the IRB; this will ensure that resolutions are processed appropriately and in a timely manner.

If you have any questions or need additional information, Please contact IRB at the above mentioned email address or telephone number.

Sincerely,

Prof. David Barlow
Chairman Institutional Review Board
Hamad Medical Corporation

Cc: MRC Project File

Hamad Medical Corporation

Institutional Review Board

Protocol Title: The Effect Of Genetic Variants On Appropriate Warfarin Dosing To Achieve Optimal Therapeutic Outcome
Study Number: 15230
Date of Approval: 22 March 2016
HMC Principal Investigator: Hazem Elewa
Review Type: Expedited
Decision: Approved for Renewal
Approved HMC Enrollment: 150

List of Approved Documents:

1) 15230_DataCollectionTool_vNov2015_1page
2) 15230_ICF_v17Jan16_8pages
3) 15230_InitialApplication_v28Nov2015_6pages
4) 15230_Protocol_v28Nov2015_12pages
5) 15230_SchemeOfDelegation_ 2pages

مـؤسسـة حمـد الطبيـة
Hamad Medical Corporation
HEALTH · EDUCATION · RESEARCH   صحة · تعليم · بحوث

مركز البحوث الطبية
Medical Research Center

Ref no.: MRC0355/2016
Date: 24 March 2016

Dr. Hazem Elewa
Clinical Pharmacist
Al Wakra Hospital

Dear Dr. Hazem,

Research Protocol 15230/15:" The effect of genetic variants on appropriate warfarin dosing to achieve optimal therapeutic outcomes"

The above titled Research Proposal submitted to the Medical Research Center has been approved to be conducted in HMC provided that the continuing approval from the Institutional Review Board (IRB) is renewed as per the committee terms. The Research Center has acknowledged the IRB approval letter dated 22 March, 2016- 21 March, 2017.

This research study should be conducted in full accordance with all the applicable sections of the Rules and Regulations for Research at HMC and you should notify the Medical Research Center immediately of any proposed changes in study conduct that may affect the resource utilization at HMC. It is the Principal Investigator's responsibility to obtain review and continued approval if there is any modification to the approved protocol.

A study progress report should be submitted bi-annually and a final report upon study's completion.

We wish you all success and await the results in due course.

Yours sincerely,

Ms. Angela Ball,
Asst. Executive Director of Research and
Business Development
Medical Research Center

Cc:

1. Osama ELbadry, Al Wakra Hospital, HMC
2. Mohamed Kasem, Heart Hospital, HMC

Appendix 3 Qatar University Institutional Review Board ethical approval

Qatar University Institutional Review Board
QU-IRB

September 29, 2016

Dr. Hazem Elewa
College of Pharmacy
Qatar University
Tel.: 4403-5616
Email: hazem.elewa@qu.edu.qa

Dear Dr. Hazem Elewa,

Sub.: **Research Ethics Review Exemption**
Ref.: **Project titled, "The Effect of Genetic Variants on Appropriate Warfarin Dosing To Achieve Optimal Therapeutic Outcomes"**

We would like to inform you that your application along with the supporting documents provided for the above proposal, is reviewed and having met all the requirements, has been exempted from the full ethics review for the interview part. The questionnaire when developed must be approved by QU-IRB prior to the implementation.

Please note that any changes/modification or additions to the original submitted protocol should be reported to the committee to seek approval prior to continuation.

Your Research Ethics Approval No. is: **QU-IRB 652-E/16**

Kindly refer to this number in all your future correspondence pertaining to this project.

Best wishes,

Dr. Khalid Al-Ali
Chairperson, QU-IRB

Institutional Review Board
(IRB)
Office Of Academic Research

Qatar University-Institutional Review Board (QU-IRB), P.O. Box 2713 Doha, Qatar
Tel +974 4403-5307 (GMT +3hrs) email: QU-IRB@qu.edu.qa

Qatar University
Institutional Bio-safety Committee

To: Dr. Hazem Elewa
College of Pharmacy

26ᵗʰ September, 2016

Qatar University, PO Box 2713

Dear Dr. Hazem Elewa

Subject: Research grant #QUUG-CPH-CPH-14/15-4

Ref: Project Titled "The Effect Of Genetic Variants On Appropriate Warfarin Dosing To Achieve Optimal Therapeutic Outcome"

We would like to inform you that your application along with supporting documents provided for the above proposal have been reviewed by QU-IBC, and having met all the requirements, has been granted approval for a period of one year and renewable for each year thereafter, should be sought and approved by QU-IBC period to continue.

Please note that QU-IBC approval is contingent upon your adherence to the following QU-IBC Guidelines:

- Ensuring compliance with QU Safety Plans and applicable national and international regulations.
- Ensuring experiments that require prior IBC approval are not conducted until IBC approval is obtained and making initial determination of containment levels required for experiments.
- Notifying the IBC of any changes to other hazardous material experiments previously approved by the IBC.
- Reporting any significant problems, violations of QU Safety Plans and applicable regulations/guidelines, or any significant research-related accidents and illnesses to the QU-IBC. Also, ensuring personnel receive appropriate orientation and specific training for the safe performance of the work.

Your research approval No. is: **QU-IBC 5/16-17**. Please refer to this approval number in all your future correspondence pertaining to this research.

Best wishes.

Dr. Marawan Abu-Madi PhD, MLS (ASCP)ᶜᵐ
Chairperson, QU-IBC
Department of Health Sciences
College of Arts & Sciences
Qatar University
Tel: +974 4403 4791
abumadi@qu.edu.qa

Institutional Bio-safety Committee
(IBC)
Office Of Academic Research

Appendix 5 Data Collection Form

DATA COLLECTION FORM – Warfarin Pharmacogenetics Study

Gender	Age	Weight	Height	Origin of patient	Origin of parents
___male ___female					

Past Medical History:

_____Diabetes
_____CHF
_____HTN
_____CHF
_____Cancer
_____Smoker (if yes, how much? _____)
_____EtOH (if yes, how much? _____)
_____#VitK servings/week (salads/ dark leafy greens/liver)
_____Interacting meds (Amiodarone, fluconazole, metronidazole, SMX/TM, Fluvastatin, Ciprofloxacin, rifampin, others

Other medical conditions:

Outpatient Meds:

Indication(s) for anticoagulation:

INR goal: __ 2-3   __ 2.5-3.5   __ Other (specify)

Duration of anticoagulation:

Genotyping results:

VKORC1-1639/3673:	GG	AG	AA
CYP2C9*2:	CC	CT	TT
CYP2C9*3:	AA	AC	CC

Visit date	INR	Pre-visit weekly dose	New weekly dose	Minor Bleeding	Major bleeding	Thromboe mbolism	ER visit	Hospitaliza tion	New meds added or d/c

Appendix 6 Supplementary Data Analysis and Results

Section S1 Correlation between CHF and Age, and the Effect of this Correlation on

Warfarin Dose

Pearson's correlation test was done to test for the association between age and CHF. It

showed significant correlation between both variables, Pearson's correlation=0.256;

p=0.009. Independent sample t-test showed significant difference in mean age between

patients who have CHF and those who don't. Patients with CHF had a significantly higher

mean age compared to the other group, 71.50±9.76 vs. 61.29±12.72, p=0.009.

We re-ran the multivariate analysis including $CYP2C9$, smoking, and age (but not CHF).

Age did not show significant association in the final model (p=0.06), and excluding CHF

from the model resulted in a great decrease in the adjusted-R^2 value from 24.1% to 19%.

After re-running the multivariate analysis including CYP2C9; smoking; CHF; and age, age

remained not a significant predictor (p=0.26) and adjusted-R^2 was only enhanced by 0.2%.

Section S2 Association between $VKORC1$ and $CYP2C9$ Variants

Cross-tabulation showed no significant difference in the frequencies of the $CYP2C9$

variants between $VKORC1$-1639G>A carriers and non-carriers, Pearson Chi-Square was

0.27, p=0.86.

Section S3 Association between Age and VKORC1

We ran independent sample t-test to estimate the mean difference in age between VKORC1 carriers and non-carriers. No significant difference in mean age was found between both groups, 61.41±12.17 vs. 65.06±14.13, p=0.19.

Section S4 Association between Body Mass Index and VKORC1

Pearson's correlation was done to test for the association between mean BMI and *VKORC1*. It showed no significant association between both variables, Pearson correlation=0.06, p=0.52. Independent sample t-test showed no significant difference in mean BMI between carriers and non-carriers of *VKORC1*-1639G>A, 31.68±6.6 vs. 32.71±7.6, p=0.52.

Section S5 Probes Manufacturer, Catalog Number and Assay ID

All probes were purchased at ThermoFisher Scientific (Table 15).

Table 15 *SNP ID's and Catalog Numbers*

SNP ID	Assay ID	Catalog number
CYP2C9*2	C__25625805_10	4362691
CYP2C9*3	C__27104892_10	4362691
VKORC1-1639G>A	C__30204875_10	4362691
CYP4F2*3	C__30204875_10	4362691

Section S6 Assessing the Quantity and Quality of Saliva DNA vs. Blood DNA

To assess the quality and quantity of saliva DNA versus blood DNA, we compared the amount of DNA derived from saliva and blood using different starting volumes and 2 different techniques of DNA extraction.

Sample size: we recruited 3 healthy volunteers.

Genetic sample collection: Saliva samples were collected using the Oragene•DNA (OG-500) self-collection kit (DNA genotek, USA). While blood samples were collected by a trained nurse using BD Vacutainer® K3 EDTA 12.15 mg (15% Sol, 0.081 mL) glass collection tubes (Reference number 366450).

DNA extraction and purification: genomic DNA was extracted from 200 µl of fresh frozen whole blood using the PureLink® Genomic DNA mini kits, Invitrogen™, following the manufacturer protocol. While for saliva, genomic DNA was extracted using the same previous kit with two different starting volumes: 200 and 500 µl. Genomic DNA was also extracted from saliva using the prepIT®•L2P manual protocol for the purification of DNA from 0.5 mL sample.

DNA quantification: To assess the quality of the purified DNA and quantify it, we used the Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific). A volume of 2 µl was used.

Results: DNA extracted from saliva was of good quality with an average 260/280 measurement of 1.86. When comparing DNA yield we found that amount of DNA

extracted from saliva was significantly higher than DNA extracted from blood, p<0.001

(Table 16).

Table 16 *Mean DNA Concentration between Saliva and Blood*

	Blood-200 µl PureLink	Saliva-500µl PureLink	Saliva-200µl PureLink	Saliva-500 µl PrepIT L2P
Mean DNA yield (ng/µl)	10.08 ± 2.82	5.68 ± 0.7	10.46 ± 6.26	83 ± 8.02