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# Genotypes Frequencies of Rs9923231 and Rs7294 Snps in the Vkorci Gene among Emiratis and Their Implications for Warfarin Dosage

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# GENOTYPES FREQUENCIES OF RS9923231 AND RS7294 SNPS IN THE *VKORC1* GENE AMONG EMIRATIS AND THEIR IMPLICATIONS FOR WARFARIN DOSAGE

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This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Medical Sciences (Pharmacology and Toxicology)

Under the Supervision of Professor Bassam R. Ali

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**Declaration of Original Work**

I, Hayat Saad. Al-Jaibeji, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Genotypes Frequencies of rs9923231 and rs7294 SNPs in the VKORC1 gene among Emiratis and their Implications for Warfarin Dosage*", hereby, solemnly declare that this thesis is an original research work that has been done and prepared by me under the supervision of Professor Bassam R. Ali in the College of Medicine and Health Sciences at UAEU. This work has not been previously formed as the basis for the award of any academic degree, diploma or a similar title at this or any other university. The materials borrowed from other sources and included in my thesis have been properly cited and acknowledged.

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## Abstract

Warfarin is the most commonly used oral anticoagulant medication given as a prophylaxis and/or treatment of venous and arterial thromboembolic disorders. Warfarin doses vary up to 10-fold among patients due to pharmacokinetics, pharmacodynamics and pharmacogenomics factors. In addition, Warfarin has a low therapeutic index with the risk of developing serious side effects such as severe bleeding or failure of therapy. Therefore, the main challenge to achieve the therapeutic goal in Warfarin treatment is estimating the appropriate dose for each patient. It is estimated that pharmacogenomic factors contribute to more than 60% of dose variability. The gene encoding for the target enzyme of Warfarin, vitamin K epoxide reductase complex 1 (*VKORC1*), is a highly polymorphic gene and contributes to about 30% of this variability. The US Food and Drug Administration (FDA) recommends genetic testing to determine the *VKORC1* genotype prior to using Warfarin. However, there are no data on *VKORC1* alleles and genotypes or their frequencies among Emiratis. Therefore, the current approach is trial and error with warfarin doses which might lead to some serious complications for patients receiving this medication. In this thesis, we used PCR amplification and direct DNA Sanger sequencing to genotype the two most important variants in *VKORC1* genes (namely, rs9923231 and rs7294). The sample consisted of 117 healthy Emirati nationals as control and 96 patients on stable Warfarin therapy. The alleles and genotypes frequencies were determined for both groups. In addition, the daily Warfarin maintenance dose for patients was examined for associations with the *VKORC1* genotypes at the rs9923231 and rs7294positions. There was no significant difference in allele frequencies between the controls and patients for either SNP. The

genotypes frequencies for rs9923231 were 25%, 48.4%, 26% for GG, GA, AA genotypes, respectively. In addition, genotypes frequencies for rs7294 variant were 44%, 44%, 12% for GG, GA, AA genotypes, respectively. Crucially, both *VKORC1* polymorphisms were found to be strongly associated with the Warfarin doses required to achieve the target international normalized ratio INR ($p < 0.0001$). The results of this study confirm the suitability of *VKORC1* genotyping to guide the use of the appropriate Warfarin dosage for Emiratis.

**Keywords:** Warfarin dosage, rs9923231, rs7294, *VKORC1*, pharmacogenetics of Emiratis, Pharmacogenomics

## Title and Abstract (in Arabic)

**تعدد الانماط الجينية لجين *VKORC1* على الموقعين rs9923231 و rs7294 بين مواطني الامارات وتاثيرات ذلك على جرعة عقار الوارفرين**

هذه الدراسة تعنى بالصيدلة الجينية وذلك بدراسة تاثير المتغيرات الجينية في انزيم VKORC1 على جرعة عقار الوارفرين وهو دواء شائع لعلاج التخثرات الدموية وتسعى هذه الرسالة لمعرفة تردد أهم المتغيرات الجينية في هذا الانزيم في مواطني دولة الامارات ودراسة حقيقة تأثير هذه الانماط الجينية في كل من موقع rs9923231 و rs7294 وطريقة عمل البحث تمت بجمع عينات لامارتين اصحاء عدد 117 ومرضى يتم علاجهم بعقار الوارفرين عدد 96 وذلك بعد موافقة الافراد المشاركين بالدراسة وبعد جمع عينات الدم تم استخلاص مادة الدي ان اي وعمل دراسة جينية عن طريق معرفة السلسلة الجينينه في كل من الموقع rs9923231 و rs7294 واخذ معلومات عن جرعة المرضى اليومية من خلال ملفهم الطبي أدت نتائج هذه الرسالة بمعرفة تردد الانماط الجينية في موقع rs9923231 و كان تردد النمط الجيني ل GG هو 25.3 % و تردد GA 49% وتردد AA هو 26 % ولم يلاحظ اي فرق بين تردد الانماط الجينية بين المرضى والاشخاص الاحصاء وفي موقع rs7294 كان تردد النمط الجيني GG هو 44% وهو نفس تردد النمط الجيني GAوأما النمط الجيني AAكان الاقل بتردد 12 % و للموقعين الجيني اثر كبير في تحديد الجرعة حسب التحليل الاحصائي للدراسة الا ان الموقع rs9923231 كان له اثر اقوى بالتنبؤ بالجرعة مقارنة بالموقع الجيني rs7294 وتعد هذه اول دراسة لتحديد تردد الانماط الجينيةلانزيم VKORC1 في دولة الامارات العربية ودراسة اهمية هذا التغير في الانماط الجينية على المرضى الذين يتناولون عقار الوارفرين .

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# Dedication

*To my beloved family*

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## Abbreviations

| | |
|---|---|
| ADR | Adverse drug reaction |
| DME | Drug metabolizing enzyme |
| dNTP | Deoxyribonucleotide triphosphate |
| SNP | Single nucleotide polymorphism |
| PCR | Polymerase chain reaction |
| MIM | Mendelian inheritance in man |
| WR | Warfarin resistance |
| CYP2C9 | Cytochrome P450 family 2 subfamily C polypeptide 9 |
| VKORC1 | Vitamin K epoxide reductase complex subunit 1 |
| MDR1 | Human multidrug resistance 1 |
| EDTA | Ethylenediaminetetraacetic acid |
| EM | Extensive metabolizer |
| IM | Intermedier metabolizer |
| PM | Poor metabolizer |
| UTR | Untranslated region |

# CHAPTER 1: INTRODUCTION

## 1.1 Human Genetic Variation and Drug Response

Inter-individual variation in drug response and the development of drug side effects among patients are major challenges in clinical pharmacology. Several factors usually contribute to this variability including age, weight, level of organ function, co-medications, disease itself, concomitant diseases, cultural and racial factors including genetics, smoking, alcohol consumption and dietary preferences [1]. However, for some medications, the genetic variation is the major contributor with up to 95% in some cases [2].

All humans are genetically identical in about 99% of their genomes. The approximately 1% differences from person to person are mainly in the forms of single nucleotide polymorphisms (SNPs), copy number variations (CNVs) and variation in the number of tandem repeats [3]. A SNP is a change in the sequence of a single nucleotide base[4]. On average, a SNP appears every 1250 bp of DNA in the human genome and they account for about 90% of human genetic variation [3]. Generally, there are numerous polymorphic variants for most genes among the total world population and consequently it is often hard to know which variant is the "true" wild type sequence [5].

SNPs in coding genes of drug metabolizing enzymes, drug receptors, drug binding proteins and drug transporters may cause variability in the structures and/or activities of the encoded proteins [4]. In addition, in some instances SNPs in genes encoding drug-metabolizing enzymes or proteins involved in handling medications may result

in lower mRNA expressions and hence may cause lower enzymic activity or alteration in receptor function [6]. On the other hand, duplications or multiplications of active genes results in higher mRNA levels and thus higher enzyme expression that lead to an increase in enzymic activity and faster metabolism of the medications handled by that particular enzyme [6].

SNPs are responsible for a large proportion of the genetic contribution to variation in drug response and therefore they are key targets for pharmacogenetics and pharmacogenomics studies [4].

### 1.2 Pharmacogenetics and Pharmacogenomics

Pharmacogenetics is the inherent response or the genetically determined variation in drug response. According to the FDA definition, it is the study of variation in DNA sequences that can affect drug response. Recently, the discipline of "pharmacogenomics" has been defined as the study of variation in DNA sequences and RNA characteristics at the whole genome level that can affect drug response. In other words, pharmacogenomics deals with inter-individual variation at whole genome level with specific expression products and functions that can be used to predict the appropriate treatment for individual patients [7]. To simplify the difference between pharmacogenetics and pharmacogenomics, pharmacogenetics can be defined as the effect of inter-indvidual differences caused by variation in a single gene whereas pharmacogenomics relates to the involvement of many genes or the whole genome in the variability in drug response [7]

To reiterate, genetic variation in genes encoding drug metabolizing enzymes, drug transporters, drug-binding proteins and receptors can all cause variation in the phenotypic response and thus are key targets for studying the pharmacogenetics and pharmacogenomics of most medications [8

Most efforts to study the involvement of genetic variation on dose requirements and responses of patients to drugs were carried out as a result of adverse drug reactions and side effects. These adverse drug reactions are estimated to be the 4$^{th}$ or 6$^{th}$ leading cause of death in the USA and to be the underlying cause of approximately 10% of inpatient admissions to hospitals. It has been estimated that approximately 2,216,000 patients suffer from serious side effects of medication leading to an estimated 106,000 patient deaths in the USA alone [9]. In the UK, adverse drug reactions cost approximately £380 million a year. In addition, an NIH report indicates that one out of ten inpatient admissions are for cases of adverse drug reaction [10].

Study carried out in Saudi Arabia showed that healthcare professionals lack the awareness of the importance of recording adverse drug reactions and analyze related the outcomes [11]. Another Study carried out in the United Arab Emirates (UAE) concluded the prevalence of inadequate knowledge of adverse drug reaction reporting procedures as a major barrier in recording adverse drug reactions [12].

More than 50% of adverse drug reactions relate to an administered dose [1] and 59% of drugs metabolized by polymorphic enzymes have been reported to have serious adverse effects [13].

Therefore, pharmacogenetic and pharmacogenomics studies aim to reduce the risk of medication usage by predicting the optimal dose and the risk of developing side effects associated with particular medication in individual patients. In other words, it aims to identify patients at risk of developing adverse side effects by determining their genetic variants in the genes relevant to the proscribed medication [14].

Over the years, the genetically determined variation in drug response has become increasingly recognized by physicians, geneticists and pharmaceutical researchers. Therefore, there is an urgent need for pharmacogenetics and pharmacogenomics research to determine the appropriate dose for each patient. Consequently, pharmacogenetic testing may open the door for physicians to predict optimal doses and responses to individual medications given to patients.

### 1.3 Warfarin: Historical Overview, Action Mechanism, Clinical Applications and Associated Side Effects

In the early 1920s, an outbreak of unknown cattle disease was observed in the United States and Canada. Severe spontaneous hemorrhages occurred to cattle and some died after castration or dehorning. Prior to that, these procedures had been performed with no of excessive bleeding. Investigation autopsies of dead animals revealed that the animals bled to death [15]. One year later, a Canadian veterinary pathologist called Frank Schofield attributed the cause of death to the ingestion of hay made from spoiled sweet clover. Subsequent experimental testing on rabbits determined that spoiled clover worked as a potent anticoagulant [15].

In 1929, another veterinarian called L.M. Roderick investigated the cause of bleeding and suggested that it was caused by the decrease in the function of prothrombin [16]. Subsequently, Henrik Dam from Denmark discovered that vitamin K deficiency can cause a hemorrhagic disease in chickens with lower prothrombin levels similar to the cases reported in cattle in North America [17]. Several years later, a trial was performed to make potent toxins out of spoiled sweet clover at Wisconsin University [18]. In particular, in 1933 Karl Paul Link and his team were able to isolate the toxic hemorrhagic substance [19]. Link and his group also identified the chemical structure of this compound as 3, 3,-Methylene-Bis-(4-Hydroxycoumarin) [20]. Link later named this compound Dicoumarol, which is a fermentation of the plant molecule Coumarin [21], that is present in many plants and gives the sweet smell of fresh grass and hay. Interestingly, the name "sweet clover" comes from its high content of Coumarin [18].

The Coumarin molecule itself has no anticoagulant properties but this activity results from its fermentation by fungi. Link donated the patents for Dicoumarol to the Wisconsin Alumni Research Foundation (WARF) in 1939 and continued his work on developing potent anticoagulants to be used as rat poison [21]. In 1940, he was able to achieve his goal with the most potent rodenticide which he named "Warfarin" [21]. It was believed that Warfarin would be extremely toxic to humans as well as rats until suicidal attempts in 1952 ended up with hospitalization followed by full recovery of the patient. Crucially, the patient was treated with Vitamin K to antagonize the effect of Warfarin [22]. Further studies demonstrated the possible use

of Warfarin as a treatment in coagulation conditions [21]. Numerous studies were carried out to establish the exact effects of Warfarin on humans [22].

Reports of variable responses by humans to fixed doses of Warfarin were documented very early after its initial use [23]. Today Warfarin is the most widely prescribed oral anticoagulant worldwide due to its effectiveness, low cost and convenient oral dose [24].

Vitamin K play a role in the coagulation cascade since the reduction of vitamin K leads to the activation of clotting factors through the gama carboxylation of these coagulation proteins mostly factors II (Prothrombin), VII, IX, X, protein C and Protein S. Understandably, these proteins are called Vitamin K dependent proteins. Once they are carboxylated, they get transformed into their active forms that lead to a coagulation cascade [25] .

Vitamin K reduced by Vitamin K expoxide reductase (VKOR) functions as a recycling enzyme for Vitamin K. The basic action mechanism of Warfarin is the inhibition of Vitamin K expoxide reductase (VKOR) enzyme and thus the impairing of the synthesis of clotting factors [26], as illustrated in Figure 1.

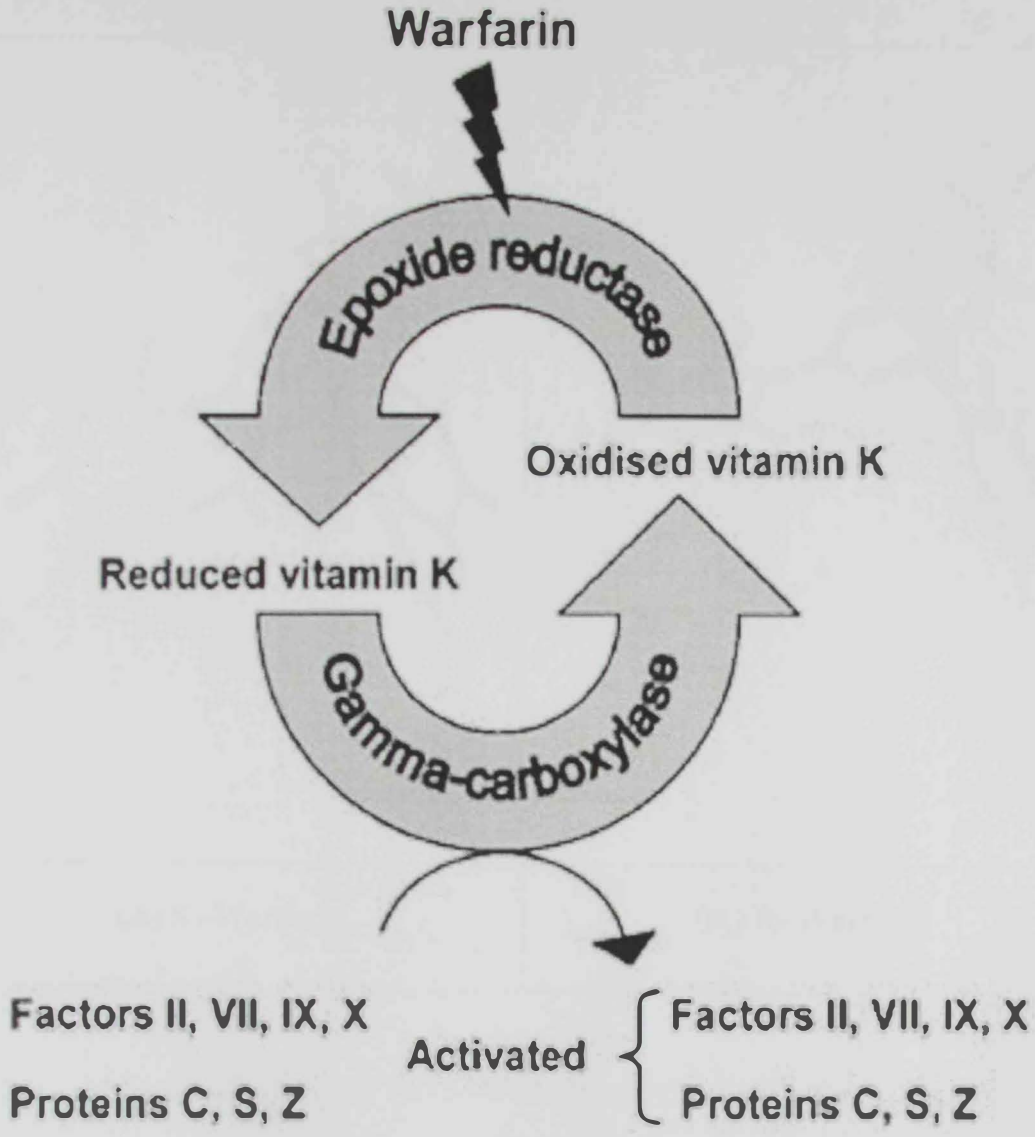
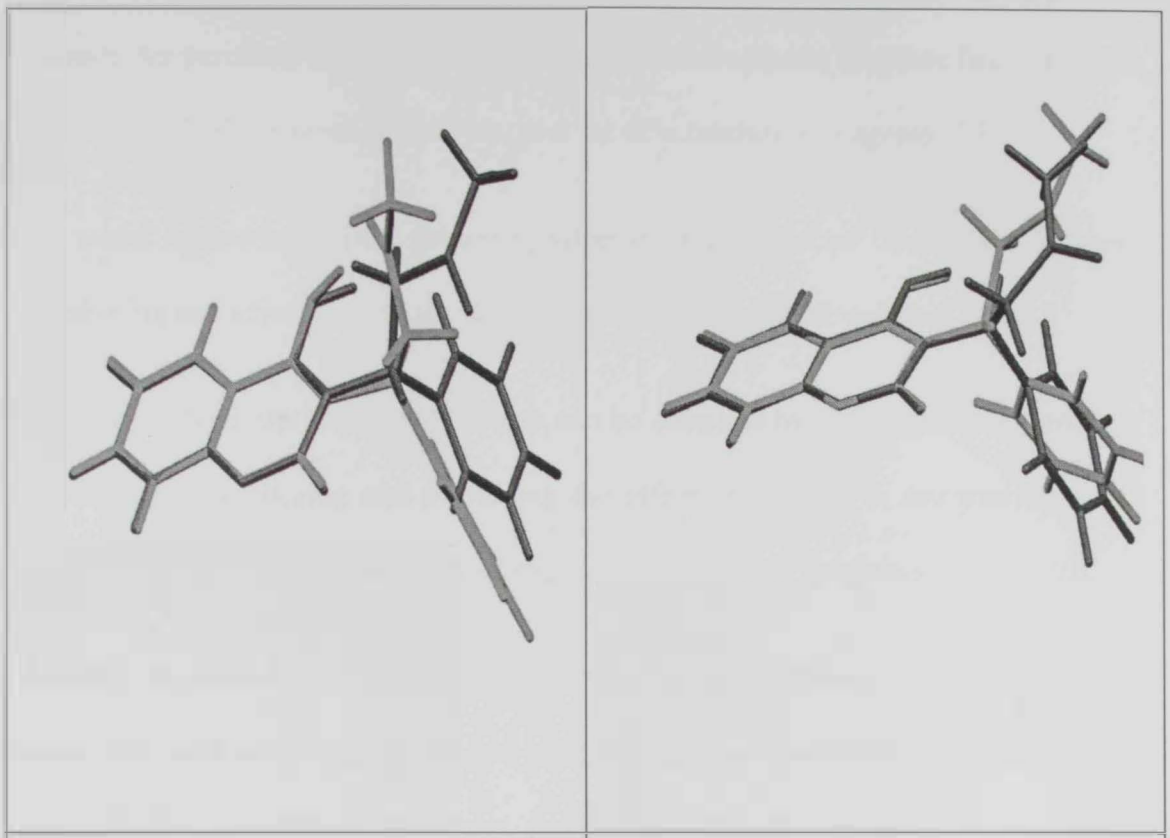



Figure 1. Effect of Warfarin on VKORC activity and Vitamin K cycle. [26].

There are two isomer forms of Warfarin, the S and the R isomers as shown in Figure 2. The S-isomer is five times more potent than the R-isomer in inhibiting VKORC [2



| (A) S –Warfarin | (B ) R- Warfarin |
|---|---|

Figure 2. Molecular Structure of (A) S -Warfarin and (B) R- Warfarin. [27].

The effect of Warfarin therapy on a blood coagulation profile is measured by the International Normalized Ratio (INR) which is a universal conversion of prothrombin time test [28].

In 1990, the INR was adopted as an indicator to monitor Warfarin effects. INR corrects for variation that come with different thromboplastin reagents from different hospitals and laboratories worldwide, and for new batches of reagents [29].

To avoid hemorrhage (the principal adverse side effect of Warfarin), frequent monitoring and adjustment to the anticoagulant effect of the drug is necessary.

This depends on the INR result, which can be obtained by a Prothrombin Time test (PT test) for monitoring and evaluating the effect of vitamin K antagonist (VKA) therapies and is a reliable measure of extrinsic pathways of coagulation [29], [30].

In order to achieve treatment goals with anticoagulation therapy using a Vitamin K antagonist such as Warfarin, patients need to maintain their target INR range. This goal can be achieved with regular monitoring and appropriate adjustment to a Warfarin dose [30]. Target INR intervals vary according to Warfarin indication, but in general an INR value of <2.0 is associated with increased risk of a thrombotic event while INR value of >4.0 is associated with increased risk of a bleeding crisis. Therefore, current recommendations suggesting an INR range of between 2.0 and 3.0 for patients with atrial fibrillation and venous thromboembolism [30], [31] and an INR range of 2.5-3.5 for patients with mechanical heart valves [32].

Warfarin does not exert an instant effect as an antithrombotic/anticoagulant medication as several doses are required to show an effect. The delayed effect is due to pre-existing Vitamin K-dependent clotting factors that must be catabolized by the body while at the same time inhibiting the activation of newly made Vitamin K-dependent clotting factors and for total protection from thrombus formation or

complications. However, the orally administered anticoagulant Warfarin can be given with a rapid acting anticoagulant such as Heparin or Low Molecular Weight Heparin [30].

Warfarin is an oral anticoagulant that is mostly prescribed to prevent the progression or recurrence of acute deep vein thrombosis or pulmonary embolism as a second option after using Heparin. It is also used to prevent venous thromboembolism during surgeries such as orthopaedic or gynecologic surgeries and as a prophylactic treatment in chronic atrial fibrillation, acute myocardial infarction, prosthetic heart valve and stroke [29], [31].

Although it is widely used, Warfarin has one major limitation. It is a drug with a low therapeutic index and is listed among the top 10 agents associated with serious side effects such as bleeding. Approximately 20% of patients using Warfarin suffer from bleeding, thromboembolic events (TEE), inpatient hospitalization and emergency admission or sometimes treatment failure and the complication of coagulation. It should always be given with safety controls as indicated on the drug label [29], [33].

Besides hemorrhages, other minor side effects have been observed such as skin lesions and necrosis. Another problem associated with Warfarin use is the numerous drug-drug interactions and its teratogenic effect. Therefore, Warfarin should never be used during pregnancy due to associated birth defects and abortion [29].

### 1.4 Factors Influencing Variability in Warfarin Dose Requirements

Variability in warfarin doses has been extensively documented. This variability can be attributed to combinations of genetic and non-genetic factors as detailed in the following sections.

#### 1.4.1 Non-Genetic Factors

As already mentioned, Warfarin is a drug with a low therapeutic index and therefore, small increases in dosage may cause serious side effects while a lower than therapeutic dose will be ineffective. Maintenance doses of Warfarin required to safely achieve stable anticoagulation can vary across populations by up to twenty fold with an average dose of 5 mg/day. However, some individuals require doses of 1 mg/day or less whilst others may require up to 20 mg/day or even more to achieve the desired INR target [29]. The identification of the maintenance dose is important to avoid the risk of bleeding that comes with Warfarin use, depending on the co-morbid conditions of the patient [34]. Significant efforts and research have been undertaken to avoid the development of side effects by estimating the exact safe dosage as Warfarin is the most often cited reason for mortality caused by medication [33]. It is also the second leading cause of drug emergency admissions in the United States [35]. It is worth noting that the most often cited life-threatening complications of Warfarin use are gastrointestinal bleeding and intracranial hemorrhage [36].

Many co-medications can change the required dose of Warfarin and the main mechanism is either by induction or inhibition of hepatic CYP2C9 or by competition with the plasma protein binding site for Warfarin [29]. Drugs that can induce the expression of CYP2C9 cause an increase in the enzymic action of CYP2C9 and

possibly the induction of other metabolizing liver enzymes [37]. examples of such drugs include alcohol, barbiturates, glutethimide, grisofulvin, rifampin, carbamazepine, phenobarbital, phenytoin and the herbal antidepressant st. john`s wort. Chronic alcohol consumption has the same action on CYP2C9 enzymic induction [29]. Patients using enzyme inducer drugs along with Warfarin require higher doses of Warfarin [38].

Other medication may cause the opposite effect on the CYP2C9 enzyme as they may inhibit its catalytic activity [38]. For example, azole antifungal medications (e.g. fluconazole and voriconazole), amiodarone, sulfamethoxazole (found in the common combination antibiotic trimethoprim-sulfamethoxazole), statins (e.g. fluvastatin and lovastatin), and some antidepressants (e.g. fluvoxamine and sertraline), cimitdine, chloramaphenicol, cotrimoxazol, disuulfiram, metronidazole, phenylbuazone, and acute alcohol intoxication all have inhibitory effects on CYP2C9. Patients using a CYP2C9 inhibitor medication concomitantly with Warfarin need lower doses of the anticoagulant since the metabolism and the evacuation of Warfarin will be reduced as a result of the decrease in CYP2C9 function and thus increase the risk of an accumulation of Warfarin to toxic levels. In these cases, anticoagulant overdose is a major risk. In terms of drug-drug interactions, among all these medications, the drugs with a strong effect on Warfarin dosage are amiodarone and statins [39], [40], [29].

In order to predict Warfarin dosage the Warfarin dosing website (www.warfarindosing.com) developed an online calculator that takes into account possible factors that can cause changes in Warfarin dosage especially concomitant

therapy with amiodarone, statins, azole antifungals, sulfamethoxazole and trimethoprim as factors to predict the most appropriate Warfarin dose [29].

Vitamin K deficiency, a hepatic disease that impairs a synthesis of clotting factors, any disease or medication that can impair organ function that controls Warfarin metabolism, or a hypermetabolic state that elevates Vitamin K levels depending on clotting factors and catabolism are all factors that can contribute to a hypoprothrombinemic state in patients and therefore they affect the required dose [29].

Warfarin is a water soluble molecule that is quickly absorbed after oral intake with peak plasma concentration obtained from 60 to 90 minutes and 100% bioavailability with little individual variation in bioavailability. However, food may delay absorption without affecting the extent of absorption and 99% is bound to plasma albumin and drugs with a higher affinity to a binding site for albumin like sulfonamides that can displace Warfarin from Plasma albumin leading to an elevation of Warfarin activity [29]. In addition, Lovastatin and Indomethacin compete with Warfarin for plasma protein binding sites and therefore increase the of free activity of Warfarin and potentiate its action [29].

### 1.4.2 Genetic Factors

There are several genetic factors that can affect a patient's Warfarin dose. Polymorphisms in cytochrome P-450 C9 (CYP2C9), the enzyme responsible for Warfarin metabolism, are major contributors. However, it was estimated that genetic variation in the gene encoding for this enzyme account for approximately 12% of the

changes in the maintenance dose of Warfarin [41],[42]. In addition, genetic variation of *VKORC1*, the gene encoding Warfarin site of action (Vitamin K Epoxide Reductase), account for up to 30% of the inter-individual variation in maintenance doses [43], [44]. More details on the contribution of *VKORC1* and *CYP2C9* will be provided in the next section (1.5.).

Researchers attempted to identify other genes involved in the dose variation of Warfarin among individuals identified *CYP4F2* as a contributory factor to variations in dose. The CYP4F2 enzyme is widely distributed in human livers and other tissues. Its function is to catalyze the hydroxylation of Lipoxygenase-derived eicosanoids as well as the oxidation of Vitamin K [45]. A genetic variation in *CYP4F2* that results in the replacement of a valine amino acid at position 433 by methionine (p.V433M) leads to a decrease in the oxidation of Vitamin K. This in turn causes an elevation in the concentration of vitamin K in hepatic cells and therefore patients on Warfarin with this variant needs higher doses [46]. The total effects of *CYP4F2* on Warfarin dosage accounts for about 1-2% of the change in dose among individuals [47]–[49]. Studies on *GGCX* gene found a correlation of a microsatellite variant with Warfarin sensitivity [26]. This enzyme has a critical role in the vitamin K cycle as it catalyzes the gamma carboxylation of vitamin K dependent proteins in the coagulation cascade as shown in Figure 3.

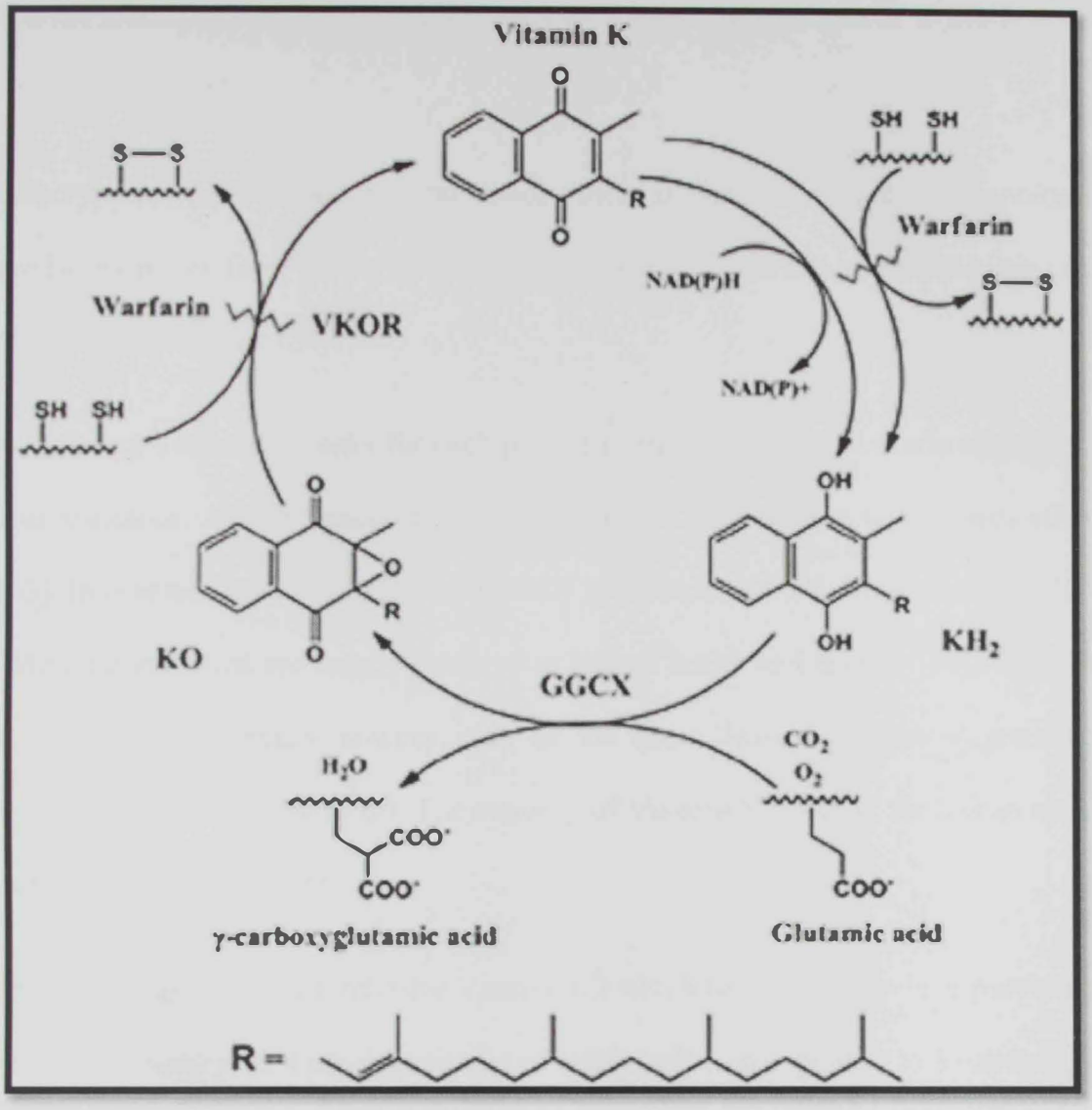



Figure 3. Role of γ-glutamyl carboxylase (GGCX) in Vitamin K cycle [50].

GGCX catalyzes the posttranslational carboxylation of specific glutamic acid residues to γ-carboxyglutamic acid (gla) in a variety of vitamin K–dependent proteins [50].

This gene is located on chromosome 2 and encodes for gamma-glutamyl carboxylase enzyme (GGCX). Variants in *GGCX* require about 6% reduction in Warfarin dosage

and the total effect of *GCCX* gene accounts for <1% of the variation in Warfarin dose requirements [51].

Genotype information and clinical factors such as age and weight are important predictors in Warfarin dosing calculations to select the optimal dose for the patients at the start of therapy [52].

A different Vitamin K intake for each patient is also a factor in Warfarin sensitivity and resistance. A high intake of vitamin K reduces the response to the medication [53]. In general, Vitamin K refers to a family of vitamins K1 (Phylloquinone) and K2 (Menaquinone) that are mainly involved in human health and disease [54]. Vitamin K1 comes from dietary sources such as the green leaves of some vegetables, vegetable oils and supplements. The majority of Vitamin K stores in the human body consist of Vitamin K1 [55].

In addition, gut bacteria synthesize Vitamin K2 which has a limited role in providing the human body with daily requirement of Vitamin K in comparison to Vitamin K1. There are four main fat soluble vitamins (A, D, E and K). Vitamin K has the lowest total stores in the body hence several weeks of restricted dietary intake of Vitamin K can completely deplete all the Vitamin K stores in the body [53]. Studies on the effects of different Vitamin K intake levels on Warfarin requirements found that it is a significant contributor to variation in the initial Warfarin dose [56].

## 1.5 The Pharmacogenetics and Pharmacogenomics of Warfarin

### 1.5.1 Cytochrome P450 Enzymes

Metabolic elimination of most current drugs is largely mediated by the function of cytochrome P450 proteins. This enzyme system is encoded by many genes. The enzymes expressed are mainly found in the liver and they play key roles in drug and other xenobiotic metabolisms [57]. The CYP system is very important for the detoxification process as it eliminates foreign substances. In addition to their involvement in the metabolism of lipophilic compounds involving oxidation, the CYP system is involved in the metabolism of endogenous substrates such as sterols, steroids, eicosanoids, serotonin, bile acids, Vitamin D, retinoid, fatty acids and uroporphyrogens [57].

In every species there are 270 cytochrome P450s (CYPs). However, only 57 CYP genes and 33 pseudogenes are found in the human genome. These are organized in 18 families and 42 subfamilies. of all the CYP families only 3 families (families 1, 2 and 3) are responsible for drug metabolism. CYP2C9 constitutes approximately 18% of the total CYP protein content in the human liver [38], [58].

CYP2C9 is one of the most important enzymes in drug metabolism as it metabolizes approximately 60 drugs. There are inter-ethnic and inter-individual variations in drug metabolisms with the occurrence of genetic polymorphisms in the *CYP2C9* gene [59]–[61]. CYP2C9 is involved in the metabolism of 10-20% of all the clinically important drugs worldwide such as Warfarin and other coumarin anticoagulants, losartan, tolbutamide, sulfonylurea drugs, angiotensin ii blockers, nonsteroidal anti-inflammatory drugs (NSAIDS), Phenytoin and glipizide [47], [60], [61].

There are four distinctive groups of CYP phenotypes arranged according to their metabolic capacity. Extensive metabolizers (EM) with regular metabolic capacity, low metabolizers (LM) with low metabolic activity, intermediate metabolizers (IM) that shows intermediate activity, while the ultra-rapid metabolizers (UM) show the highest metabolic activity group [62].

*CYP2C9* polymorphisms are important determinants of the Warfarin dosage as the presence of *CYP2C9* polymorphisms is associated with a reduction in the metabolism of S-Warfarin [63], [64]. Thirty four CYP2C9 variant alleles have been reported in the world population so far (http://www.cypalleles.ki.se/). *CYP2C9**1 is a wild-type allele, and there are two important single nucleotide variations that result in missense mutations in the coding region of the *CYP2C9* gene. The *CYP2C9**2 allele (C430T in exon 3) results in the replacement of an arginine at the amino acid position 144 by cysteine (p.R144C).

This is a functionally important substitution as this mutation leads to the reduction of the catalytic activity to 12% compared to the wild-type enzyme. Additionally, the *CYP2C9**3 allele (A1075C of exon 7) leads to the substitution of a leucine residue by isoleucine at position 359 (p.L359I). This mutation is associated with a reduction in the catalytic activity to about 5% of the wild type. Studies have shown that the *CYP2C9**3 allele is associated with lower intrinsic clearance of the Warfarin substrate and a lesser enzyme activity (<5%) than CYP2C9*2. Both *CYP2C9**2 and *3 genetic variants are widespread in caucasian populations, with allele frequencies varying from 3.3% to 18% [65], [66].

As above, both *CYP2C9**2 and *3 variants encode for enzymes with reduced catalytic activities that result in impaired hydroxylation of Warfarin compared to the wild-type CYP2C9 enzyme. Therefore, lower doses of Warfarin are required in individuals with these alleles. It was observed that a longer time is required to achieve a stable Warfarin dosage and therefore these patients are at higher risk during the initial phase of Warfarin therapy [47], [63].

In caucasian populations the frequency of the *CYP2C9**1/*1 genotype is 65-70%, the *CYP2C9**1/*2 genotype is 15-20%, the *CYP2C9**1/*3 genotype is 8-10%. Poor metabolizer genotype frequencies for CYP2C9*2/*2, *CYP2C9**3/*3 and *CYP2C9**2/*3 are between 1 and 2% each [63], [64].

### 1.5.2 Vitamin K Epoxide Reductase (VKORC1)

The Vitamin K epoxide reductase VKOC1 enzyme is involved in the biosynthesis of Vitamin K dependent coagulation factors through the Vitamin K regeneration process. On the other hand, Vitamin K plays a key role as a cofactor in gamma carboxylation of several clotting factors (II, VII, IX and X) and proteins C and S that are all involved in coagulation. The Vitamin K epoxide reductase VKOC1 enzyme is Warfarin sensitive and can be inhibited by preventing the Vitamin K recycling process [67]–[69] as shown in Figure 4.

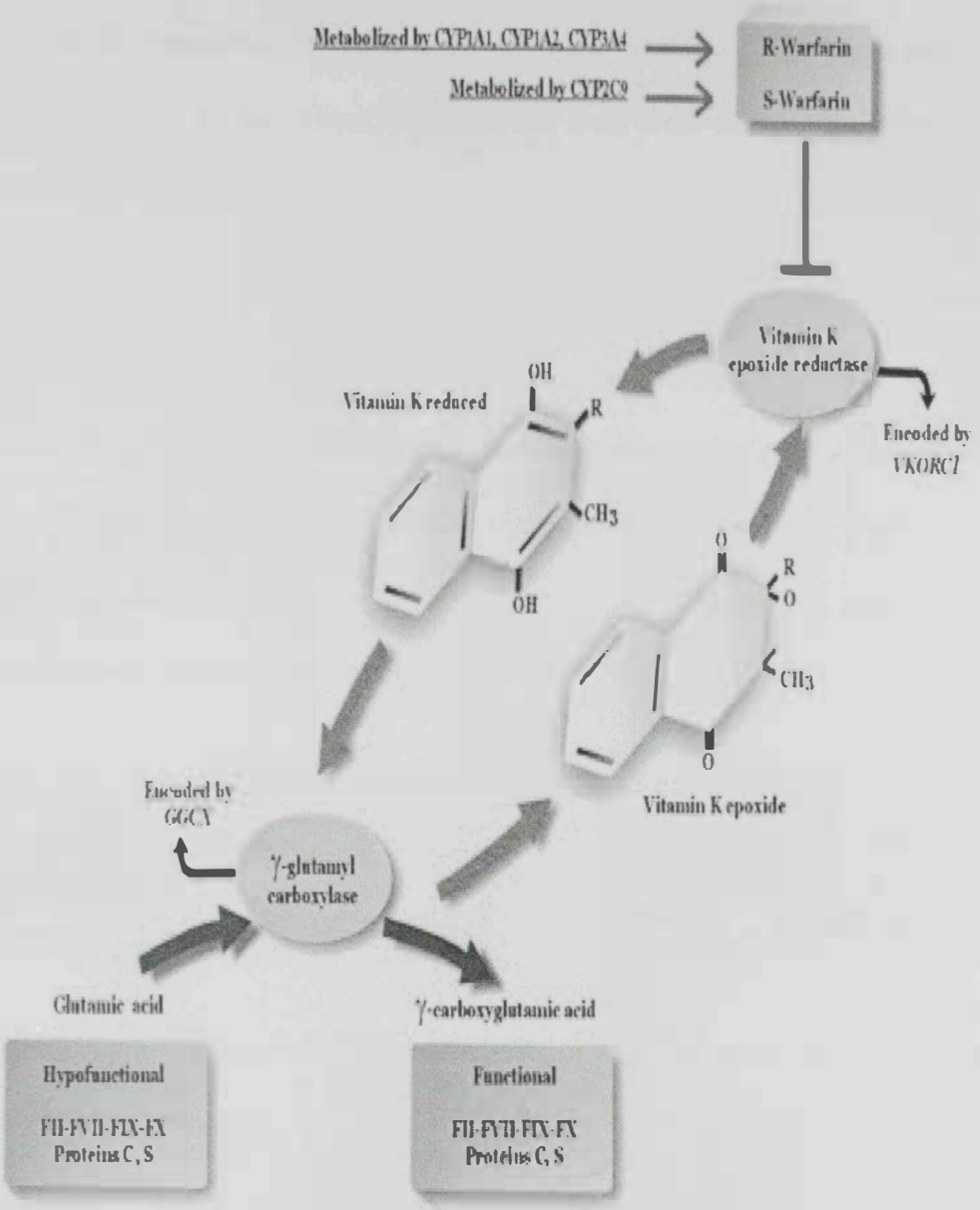



Figure 4. Warfarin effect as Vitamin K anticoagulant. [44]

Warfarin inhibits Vitamin K recycling and thus preventing the formation of more Vitamin K dependent coagulation factors [44].

The *VKORC1* gene encodes the vitamin K epoxide reductase complex subunit-1, a small transmembrane protein of the endoplasmic reticulum. VKORC1 [MIM

608547] is the key enzyme of the Vitamin K cycle and the molecular site for the action of Vitamin K antagonists (Coumarins) including Warfarin, Acenocoumarol and Phenprocoumon, which are used for long-term treatment of thromboembolic diseases [44], [70].

It has been known for many years that the action of Warfarin involved Vitamin K yet without full understanding of the exact nature of its involvement until 2004. The exact molecular target of Warfarin was discovered by two independent research groups when they found a protein that was later named as Vitamin K epoxide reductase complex subunit 1 (VKORC1) [68], [71]. This gene is located on chromosome 16p11 as shown in figure5.


Figure 5. Chromosomal location of *VKORC1* gene on the short arm of chromosome 16. [72].

The first group of researchers, Li and colleagues tested the ability of candidate genes to inhibit vitamin K epoxide reductase (VKOR) activity in human cells by using short interfering (siRNA) pools against these genes to observe gene activity. Li and colleagues expressed the gene in insect cells and described the gene as coding for a 163-amino acid protein with 3 exons. They also showed that the protein contained 1 to 3 transmembrane domains and 7 cysteine residues, 5 of which are conserved among human, mouse, rat, zebrafish, Xenopus, and Anopheles [68].

In same the year, Rost and colleagues cloned the *VKORC1* gene and described it as a gene encoding a protein of 163 amino acids with a mass of 18KDa [69]. It was mainly expressed in fetal and adult livers and to a lesser extents in fetal hearts, kidneys, lungs, adult hearts and the pancreas. The enzyme is associated with the endoplasmic reticulum [71].

Rost colleagues also studied the involvement of *VKORC1* in the combined deficiency of Vitamin-K-dependent clotting factor type 2 (VKCFD2; OMIM 607473), and resistance to Coumarin oral anticoagulant drugs (Warfarin resistance, WR; OMIM 122700). They found that it correlated with pathological conditions and Warfarin resistance [69]. In the rat, four heterozygous missense mutations in *VKORC1* were reported in Warfarin resistant cases. In addition, one homozygous point mutation in exon 3 has been found in two unrelated index cases of a combined deficiency of vitamin K-dependent clotting factor type-2 (VKCFD2) [69].

They also studied the over expression of wild-type *VKORC1* and the form carrying the VKCFD2 mutation and noticed a marked increase in VKOR activity and increasing Vitamin K production by approximately 14 to 21 times compared with untreated or mock-transfected cells [68], [69]. These discoveries helped in the understanding of Warfarin resistance in humans and rodents.

The purification of recombinant human VKORC1 protein from baculovirus-infected insect cells was reported in by Chu who demonstrated that it was possible to convert Vitamin K epoxide to Vitamin K; and Vitamin K to reduced Vitamin K through the enzymatic function of a single *VKORC1* polypeptide [73]. It was thought that

VKORC1 was subunit 1 of the enzymatically functional complex involved in Vitamin K recycling. However, it was found that the gene product of VKORC1 was enzymatically active on its own which led to the informal shorting of the name to VKOR [74].

Genetic variations in *VKORC1* was associated with significant inter-individual and inter-ethnic variability in the dosage of Vitamin K antagonists (Coumarin) [75]. Studies on *VKORC1* enhanced our understanding of the Vitamin K cycle and led to improved clinical practice [76].

## 1.6 The Global Prevalence of the Clinically Relevant *VKORC1* Alleles and Genotypes

It is well known that Warfarin dosage and responses are greatly affected by genetic variation in the *VKORC1* gene. In addition, genetic variation in this gene results in different phenotypes such as resistance to Warfarin (Warfarin resistance, WR) or pathological conditions known as multiple coagulation factors deficiency type 2 (VKCFD2) that can cause a rare bleeding disorder [69], [77].

The discovery of a haplotype structure in the human genome and the ability to define SNPs, facilitated genotyping studies at significantly reduced costs. There is a need to take into account not only differences between the genotypes of individuals but the differences in genotypes between different populations to improve health care [78].

In Warfarin resistant patients, several rare mutations in the *VKORC1* gene lead to amino acid changes in VKORC1 proteins. These rare mutations are not reported in the general population [69].

The genetic basis of the large inter-individual variability among Warfarin patients investigated by Rieder, who described the coding region variants of *VORC1* that are highly detrimental and cannot explain Warfarin dosage variation [79] .

In addition, a study was conducted to determine whether polymorphisms in non-coding regions of the *VKORC1* contribute to variability in the maintenance dose of Warfarin and found that SNPs in non-coding regions are associated with Warfarin dosages across the normal dose range (2 to 10 mg per day) [79]. This opened the door to further study other regulatory polymorphisms in the *VKORC1* that could possibly change Warfarin dosage requirements. Consequently, SNPs in the *VKORC1* non-coding region were found in various populations [79], as illustrated in figure 6.

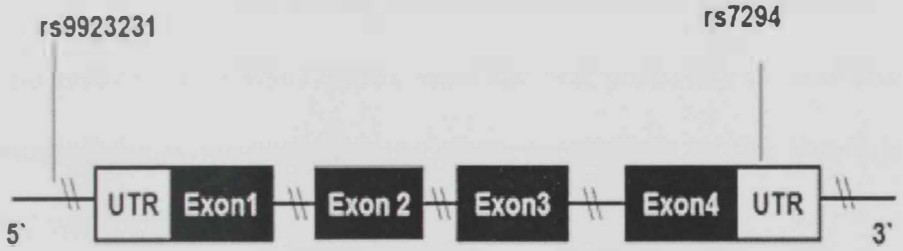



Figure 6. *VKORC1* gene structure with the relevant non-coding variants.

The mechanism of how these SNPs in non-coding affect Warfarin dosage variability were thought to be through the effect on the mRNA levels [79].

The first SNP (C6484T, rs9934438 (608547.0008) or 1173C>T) was reported to associate with low Warfarin doses. This SNP is located in the first intronic region of the *VKORC1* gene and the wild type C/C genotype requires significantly higher doses compared to the T/T genotype [43].

This variant is in near perfect linkage disequilibrium with promoter region variant G3673A (rs9923231) [43]. Since the frequency of C6484T is approximately similar, if not identical, to the frequency of G3673A, some researchers consider this variant inert but it is still used frequently in haplotype determination of the *VKORC1* gene as marker of SNP [52].

Warfarin sensitivity in patients with lower doses is frequently associated with promoter region single nucleotide polymorphism -1639G-A (608547.0006). On the other hand, *VKORC1* variant rs7294 (G9041A), or 3730G>A are reported to be associated with Warfarin resistance in patients who need higher doses compare to the wild type [66].

Two SNPs in the non-coding region of the *VKORC1* gene, rs9934438 (608547.0008) and rs9923231 (608547.0006), were the best predictors of Warfarin dosage through transcriptional mechanisms that alters expressions of the Warfarin target receptor *VKORC1* [79]–[81]

The-1639G-A (rs9923231) variation is associated with lower Warfarin dosages, Warfarin sensitivity and higher risks of bleeding [66]. Individuals with the A/A genotype (group A, haplotype 2* mainly) required a lower dose than those with the A/G or G/G genotypes. The G allele comes with a 44% increase in activity when compared with the A allele [82].

The rs7294 (G9041A), is a variant associated with high warfarin dosages that occurs at the 3'UTR of *VKORC1* gene [43], [83]. It does not usually occur with the 1173C>T and 3673G>A variant allele haplotype [43].

A patients with (rs9923231) and 1173T (rs9934438) variant allele was reported to have a low dose of about 24-26 mg/week while patients with wild type required a dose of 36mg/week [26].

A high dosage reported for patients with (rs7294) allele variants of approximately 40 mg/week as a mean dose [26], [83]. This indicates the effect of *VKORC1* in non-coding variants on Warfarin pharmacodynamics studies that concluded that *VKORC1* variation can explain 25% of the variability in Warfarin dosage [79].

A significant correlation of the relevant SNPs in the *VKORC1* with approximately 30% of Warfarin dosage variation has been determined in a genotyping study of 201 patients using Warfarin. These findings indicate that the *VKORC1* genetic variation plays a greater role in dose requirements compared to *CYP2C9* which explains only 12% of the variation in dose requirements [26]. SNPs in non-coding regions of the *VKORC1* gene classify Warfarin patients according to their required dose of Warfarin to safely achieve the target INR [79].

According to this classification, patients with SNP (rs9923231) have low Warfarin dose-requirement with approximately 2.7 mg per day for the A/A genotype that is mostly distributed in Asian Americans. High dosage groups with rs7294, A/A genotype need 6.2 mg per day which is widely distributed among African Americans and an intermediate dose of 4.9 mg per day for patients with heterozygosity in both SNPs (rs7294 and rs9923231) [79]. There are continuous efforts to identify *VKORC1* haplotypes but the nomenclature is still varied [79].

### 1.7 Warfarin Usage in the UAE

There are a limited number of clinical research studies on Warfarin usage in the UAE and no pharmacogenetics studies so far. There was a study in 2012 conducted in Al-Ain to evaluate patient and staff compliance with Warfarin usage guidelines and to assess the overall practice and patient outcome [84]. The authors reported that at Al-Ain Hospital, each year there are 800 cases (~27 new) of patients on Warfarin including Emiratis and expatriates. Indications for Warfarin usage among those patients were 35% for atrial fibrillation, 28% for deep vein thrombosis, 20% for prosthetic heart valves and 12% for stroke and dilated cardiomyopathy.

The authors found that regular INR monitoring was carried out for only 23% of the patients. They also found that 73% of the patients had a stable INR while 20% had a high INR and 7% had lower values than the therapeutic target. 22% of patients have been admitted to the emergency ward from Warfarin patients with 9% of the cases unrelated to Warfarin use. In addition, side effects such as abnormal bleeding have been frequently reported among patients with a high INR. Furthermore, hematemesis was reported for 25.3%, shaving cuts for 21.8%, bleeding gums for 19.9%, bleeding wound for 14.6% and melena in 11.3% of the patients [84].

A lack of genetic studies related to Emiratis on Warfarin prompted us to carry out this project.

# CHAPTER 2: STUDY RATIONALE, AIM AND OBJECTIVES

## 2.1 Rationale

Warfarin is a drug with a low therapeutic index where small dose changes may result in serious side effects or treatment failure. It is well known that the genetic makeup of the individual contributes to their response to medication including Warfarin. A significant proportion of patients receiving the standard dose of Warfarin may either not respond to treatment or exhibit bleeding as a side effects. Genetic variations in the *VKORC1* gene play a major role in Warfarin dosages and are responsible for about 30% of the dose variation. Therefore, it can be considered to be the major genetic determinant. Despite their importance for determining the optimal starting dose for patients, no genetic studies have been carried out on Emiratis in relations to Warfarin use. Therefore, the purpose of this thesis is to start to fill this gap in our knowledge for the local Emirati population.

## 2.2 Aim and Objectives

### 2.2.1 Aim

Identify the alleles and genotypes frequencies among Emiratis for two important SNPs (rs9923231 and rs7294) in the *VKORC1*, the gene encoding the cellular target for Warfarin treatment.

### 2.2.2 Objectives

To achieve the above aim, the thesis objectives are:

1. Recruit and obtain blood samples from healthy Emirati donors and patients receiving Warfarin as part of their healthcare.

2. Isolate and quantify genomic DNA from each subject using standard molecular techniques.

3. Genotype those samples for two SNPs, rs9923231 and rs7294 SNPs, in the *VKORC1* gene using PCR and direct Sanger DNA sequencing.

4. Determine the SNPs allele and genotype frequencies among healthy Emiratis subjects and patients receiving Warfarin.

5. Correlate the determined allele and genotype frequencies with drug response in patients.

6. Generate genetic data that might be deposited/ shared with health care providers in the UAE and/or with international databases.

# CHAPTER 3: MATERIALS AND METHODS

## 3.1 Blood Sampling

Blood samples were collected from two groups: the first group consisted of 96 unrelated UAE nationals who suffered from a coagulation disorder and are being treated with Warfarin as part of their care. This group of subjects was recruited from patients attending the INR clinic at Tawam Hospital, Al-Ain, UAE. The second group consisted of 117 healthy Emirati subjects from Al-Ain and Abu Dhabi.

All the individuals had read the information sheet and signed informed consent forms to participate in the study. About 10ml of peripheral venous blood was collected. Five ml of the blood sample was placed in an EDTA tube (containing an anticoagulant) to be genotyped. The subjects also completed a questionnaire and their medical files were reviewed to examine the fulfillment of the inclusion/exclusion criteria. This study protocol was carried out at the laboratories of the College of Medicine and Health Sciences at UAE University. In addition, it was approved by the ethical committee for human research in the Al-Ain District (CRD 261-Protocol No. 13/38).

## 3.2. Extraction of DNA from Peripheral Leukocytes

Deoxyribonucleic acid (DNA) was extracted from an ethylene-diamine-tetra- acetic acid (EDTA)-anticoagulated blood using a whole blood extraction kit (Flexigene DNA isolation kit (250)- Applied Biosystems). The isolated genomic DNA was

stored in sterile vials at 4 °C until analyzed. DNA samples were kept at – 20 °C for long term storage.

### 3.3. Primers Design

Primers for amplifying the relevant parts of the *VKORC1* gene were designed using primer3 http://primer3.ut.ee and were custom made by Metabion Inc (http://www.metabion.com). The list of PCR and sequencing primers are listed in Table 1.

| SNP | Forward Primer | Reverse Primer |
|---|---|---|
| rs9923231 | 3'GCCAGCAGGAGAGGGAAATA5 | 3'AGTTTGGACTACAGGTGCCT5' |
| rs7294 | 3'GGCTTACGCACGTATTCC5' | 3'GGTTCAGACTTGGCTGATTG5' |

Table 1. Sequences of the oligonuceloetide primers used for the PCR amplification and genotyping of the two *VKORC1* SNPs

### 3.4 Polymerase Chain Reaction (PCR)

PCR amplification (thermal cycling) conditions were as follows: a single denaturation cycle of 5 minutes at 95 °C followed by 40 cycles of 95 °C for 2 minutes. The exact annealing temperatures and times are listed in table 2 and then at 72 °C for 2 minutes. This was followed by a single cycle of 7 minutes at 72 °C.

| Primer Name | Annealing Temperature (°C) | Annealing Time (Sec) | Product Size (pb) |
|---|---|---|---|
| rs9923231 | 58 | 30 | 290 |
| rs7294 | 60 | 45 | 659 |

Table 2. Annealing temperatures optimization and expected product sizes.

**3.5 Analysis of PCR Amplicons by Agarose Gel Electrophoresis .**

The two VKORC1 specific products (amplicons) from each subject were separated by 0.6% agarose gel electrophoresis to ensure a good DNA quality was produced before sequencing. The promoter region SNP (rs9923231) is expected to produce DNA band (A)of 290 bp whereas SNP (rs7294) will give a Size of 659 bp (B) as presented in Figure 7

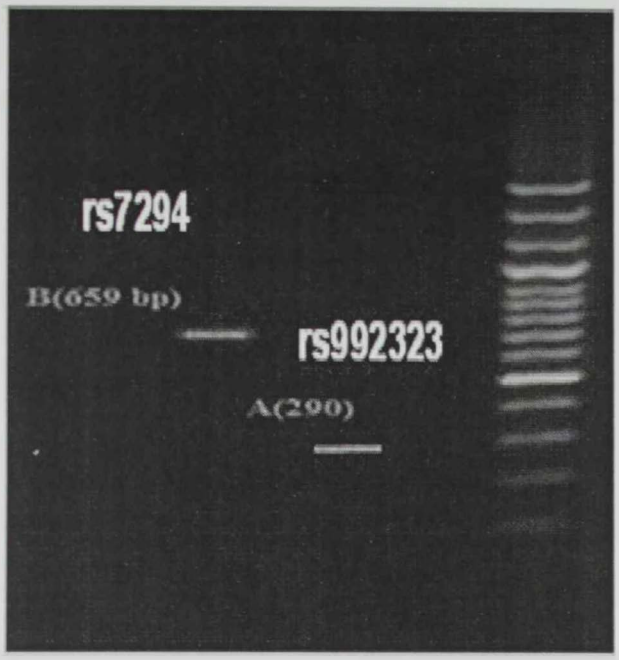



Figure 7. Agarose electrophoresis analysis of representative PCR reactions for the two SNPS and primers of experiment and their approximate base pairs sizes.

### 3.6 DNA Sequencing

DNA was sequenced using a dideoxy sequencing (chain termination of Sanger) method with a 3130Xl Genetic Analyzer (applied Biosystems). The procedure involved cleaning up the PCR reactions using an Exo-SAP-IT treatment prior to Sanger DNA sequencing.

#### 3.6.1 ExoSap-IT Treatment

The ExoSAP–IT was used for cleaning up the PCR products. This technique has been shown to be suitable for cleaning up PCR products of less than 100 bp to over than 20kb without any sample loss, by removing the unused excess primers and nucleotides through a degradation by enzyme action. The method in this experiment was carried out by 1.25 µl of purified PCR product to undergo enzymatic purification

with 0.5 µl of an ExoSAP –IT enzyme mixture at 37 °C for 15 minutes. This was followed by heating at 80 °C for 15 minutes to inactivate the exonuclease and SAP enzymes.

### 3.6.2 DNA Cycle Sanger Sequencing

The cycle sequencing was carried out by preparing a sequencing mix containing 1µl (3.2 pmol) of sequencing primers, 4µl of ready reaction premix 2.5X (Big Dye Terminator Cycle Sequencing Kit Applied Biosystems), 1 µl of BigDye Terminator v.3.1 sequencing buffer (5X) and 20ng/ ml template, miliQ water was added to form 20µl reaction. The tubes were placed in a thermal cycler and an initial denaturation step was performed with a rapid thermal ramp to 96 °C for 1 minute, then the following steps were repeated up to 25 cycles:

- A.rapid thermal ramp to 96 °c (96 °c for 10 seconds).
- A rapid thermal ramp to 50 °c (50 °c for 5 seconds).
- A rapid thermal ramp to 60 °c (60 °c for 4 minutes).
- A rapid thermal ramp to 4°c.
- Purification of the sequencing products.

### 3.6.3 DNA Sequencing Purification Step

If the dye terminators are not removed completely before the electrophoresis step, the signal from the dyes will be obscured due to an excess of DNTPs. This method is suitable when using Big Dye Terminators v3.1 as it produces consistent signaling and it is good at getting a 20µl sequencing reaction in 96-well reaction plates following a brief spinning after removing the reaction from the thermal cycler. 5µl of

125 mm EDTA is added to the 60 µl of 100% ethanol in each well and the mixture is sealed and inverted 4 times before being incubated for 15 minutes at room temperature. This was then immediately centrifuged at 3000 xg for 30 minutes (Sigma 2-16 pk) and the plate was inverted and spun at up to 185 xg then removed from the centrifuge. A 60 µl of 70% ethanol was also added to each well then spun again at 1650 x g for 15 minutes with the centrifuge set at 4 °C. This was followed by inversion and spinning up to 185xg for 1 minutes before removal from the centrifuge.

### 3.6.4 Formamide Treatment

Hi-Di Formamide is a highly deionized formamide formulated with stabilizers and ready for use as an injecting solvent in DNA analysis methods. The samples were resuspended in 15 µl formamide (Hi-Di TM formamide, Applied Biosystems), denaturated at 95 °C for 2 minutes and then kept on ice for 2 minutes until sequencing.

### 3.6.5 Sequencing Data Interpretation and Genotype Calling

For each patient the chromatograms of the nucleotide sequence were aligned with the original *VKORC1* sequence (ENST00000394975) using the chromas lite program in order to detect the presence of SNPS/mutations as well as to define each patient's genotype.

### 3.7 Statistical Analysis

SSPS and Graph Pad programs were used to apply selected statistical analysis; we used chi-square tests for non-parametric variables to compare between frequency results, while multiple linear regression models, ANOVA and Spearman analysis were used to study dose-genotype relations.

# CHAPTER 4: RESULTS

## 4.1 Description of Study Sample

This study involved two groups of UAE nationals. The first group included 117 unrelated healthy subjects as controls and the second group consisted of 96 patients who were on Warfarin treatment for at least two months. Patient characteristics are summarized in table 3.

| | | |
|---|---|---|
| Age | Median Age | 64 year |
| | Average Age | 59 year |
| | Standard Deviation (SD) | 19.73 year |
| Gender | Male | 48(50%) |
| | Female | 48(50%) |
| Indication of Warfarin | Atrial Fibrillation | 46(48%) |
| | Deep Vein Thrombosis | 15(16%) |
| | Valve Replacement | 26(27%) |
| | Antiphospholipid Syndrome | 3(3%) |
| | Cardio Vascular Shunt | 1(1%) |
| | Cerebella Ataxia | 1(1%) |
| | Artery Thrombosis | 1(1%) |
| | Pulmonary Embolism | 2(2%) |
| | Valve Disease | 1(1%) |

Table 3. Characteristics of the recruited patients

Informed consent was obtained from each participant. This study was approved by the ethics committees of each participating institution. Patient information (indicators of Warfarin use, maintenance doses and INR values etc.) were collected from Tawam Hospital medical records as of 23/09/2014.

In this study we recruited equal numbers of patients of both genders: 48 females and 48 males. The mean age was 64 and the median age was 59. The age ranged from 17 to 99 years with a standard deviation of 19.73 years.

Warfarin was prescribed for patients for different pathological conditions with Atrial Fibrillation (AFIB) at 48%; Deep Vein Thrombosis (DVT) at 15%; Antiphosholipid Syndrome (APS) at 3%; After Mitral Valve Replacement (MVR) and Aortic Valve Replacement (AVR) for 27%; 2% for Pulmonary Embolism (PE), while Valve Disease, Arterial Thrombosis, Cerebella Ataxia, Cardio Vascular Shunt each constituted 1% of the total use of Warfarin.

The target INR range for 67% of the patients was 2.5-3.5; 32% with an INR target range of 2 -3 where only 1% have a target range of 1.5-2.

The average Warfarin dosage of patients was 4.7 mg/day with an SD of 2.47 and a median dose of 4.5 mg/day. The highest dosage was 15 mg/day and the minimum dose was 0.5 mg/day.

### 4.2. *VKORC1* Genotype and Allele Frequencies

Using PCR amplification and direct DNA sequencing, we genotyped each subject from both groups for two *VKORC1* gene variants relevant to Warfarin dosage. The two variants are the promoter region variant rs9923231 and the 3' UTR region variant rs7294, as listed in table 4.

| SNP | Nucleotide position | | Nucleotide change | Gene region |
|---|---|---|---|---|
| | Genomic | cDNA | | |
| rs9923231 | 3673 | c. -1639 | G>A | Promoter |
| rs7294 | 9041 | 492+134 | G>A | 3'-region |

Table 4. Description of the studied polymorphisms (SNPs) of the *VKORC1* gene. (cDNA: coding DNA, rs: reference subgroup)

Rs7294 was selected as per the literature due to its key role in Warfarin resistance cases, while variant SNP rs9923231 was selected as per the USA Food and Drug Administration (FDA) recommendations [85]; as shown in table 5.

| Drug Link in Drugs@FDA | Referenced Subgroup | Labeling Sections with Pharmacogenomic Information |
|---|---|---|
| Warfarin | *VKORC1* rs9923231 A allele carriers | Dosage and Administration<br>Clinical Pharmacology |

Table 5. Food and Drug Administration (FDA) labeling recommendations for Warfarin

### 4.2.1 Determination of the *VKORC1* rs9923231 Genotypes and Alleles Frequencies

*VKORC1* rs9923231 SNP genotype and allele frequencies among patients and controls are presented in table 6.

| | | Controls N=117 (%) | Patients N=96 (%) | UAE N=213 (%) |
|---|---|---|---|---|
| Genotypes | GG | 30(25.6) | 24(25) | 54(25) |
| | GA | 58(49.6) | 46(47.9) | 104(49) |
| | AA | 29(24.8) | 26(27.1) | 55(26) |
| Alleles | G | 118(50.4) | 94(49) | 212(49.8) |
| | A | 116(49.6) | 98(51) | 214(50.2) |

Table 6. Genotypes and alleles frequencies among patients and controls for rs9923231 .(N indicates the total number of study subjects in both controls and patients)

We compared the percentage of the three genotypes (GG, GA and AA) between both groups (control and patients) as listed in table 7

| Genotype | Controls n=117 | Patients n=96 |
|---|---|---|
| GG | 55.6% | 44.4% |
| GA | 55.8% | 44.2% |
| AA | 52.7% | 47.3% |

Table 7. Percentage of rs9923231 SNP Genotypes among both groups, controls and patients.

(N indicates the total number of study subjects in both controls and patients)

There are no statistically significant differences in the prevalence of GG, GA, AA genotypes for rs9923231 between the control group and the patients. We used a chi-square test to investigate (chi-square= 0.1459, df = 2, p-value = 0.9296). The aim of this test was to investigate if there was a difference in genotype frequencies between healthy control groups and patients.

The genotype frequencies of rs9923231 in the 213 UAE participants are presented in (table 6). They show that the genotypes GG and AA have similar distributions of 25% and 26% respectively. On the other hand, the heterozygous genotype GA frequency was 49 %. The allele frequencies for rs9923231 variants are presented in Table 6 with almost equal frequencies of 49.8% for the G allele and 50.2% for the A alleles.*VKORC1* rs9923231 SNP genotype frequencies among patients and control subjects are presented graphically in Figure 8.

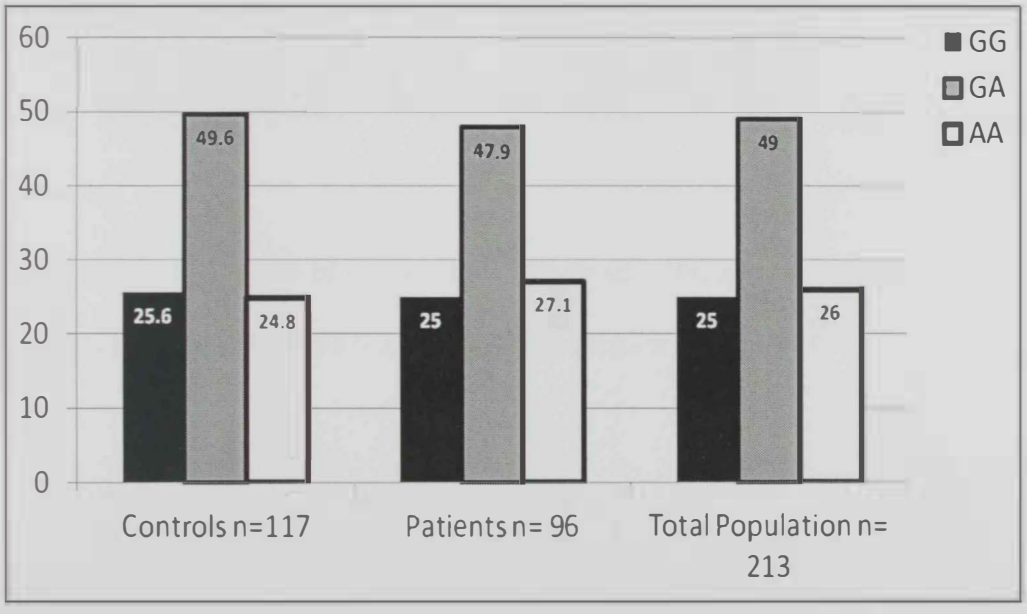



Figure 8. Graphical presentation of genotype frequencies among patients and control for rs9923231.

In addition, we compared the results from this study to data from other populations as listed in table 8

| *VKORC1* SNP | Genotype | UAE n=213 | Saudi n=131 [86] | Iran n=81 [87] | China n=178 [88] | Caucasian n=22 [89] | Indian n=43 [90] |
|---|---|---|---|---|---|---|---|
| G-1639A | GG | 25 | 37.4 | 15.87 | 0.6 | 31.8 | 83.8 |
| | GA | 49 | 39.7 | 57.14 | 15.7 | 50.0 | 9.50 |
| | AA | 26 | 22.9 | 26.98 | 83.7 | 18.2 | 6.80 |
| Alleles Frequency | G | 49.8 | 57 | 44.4 | 8.42 | 56.82 | 88.4 |
| | A | 50.2 | 42.7 | 55.6 | 91.57 | 43.18 | 11.6 |

Table 8. Ethnic distribution of rs9923231 Genotypes and alleles in various populations.

The graphic representation of ethnic distributions of rs9923231 genotype frequencies is shown in figure 9 and allele frequencies in figure 10

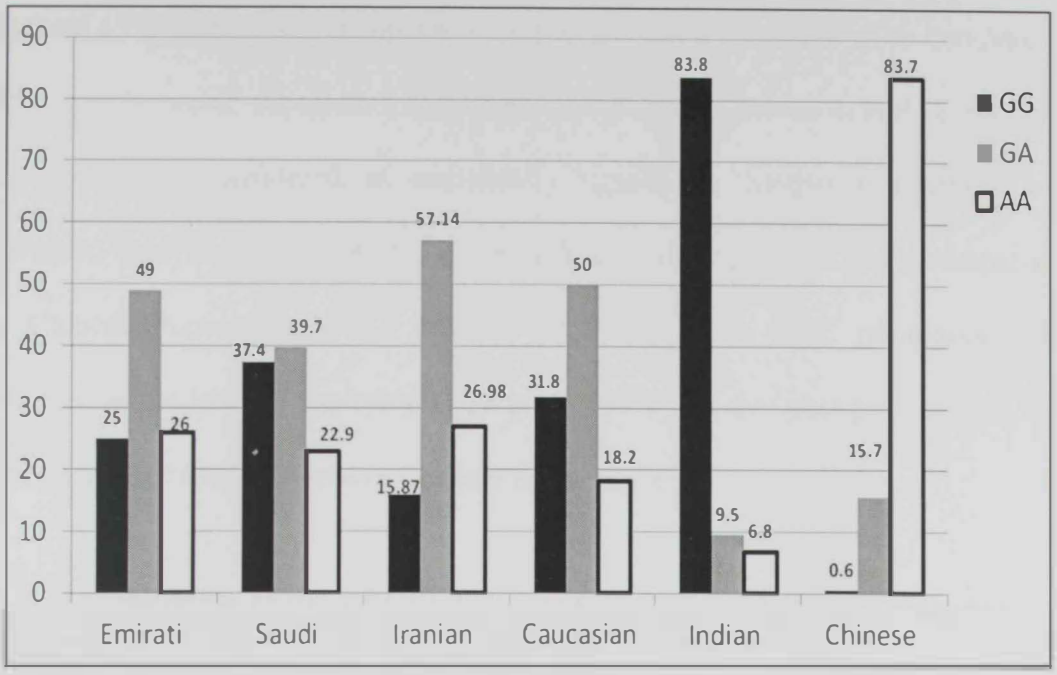



Figure 9. Ethnic distribution of rs9923231 genotypes

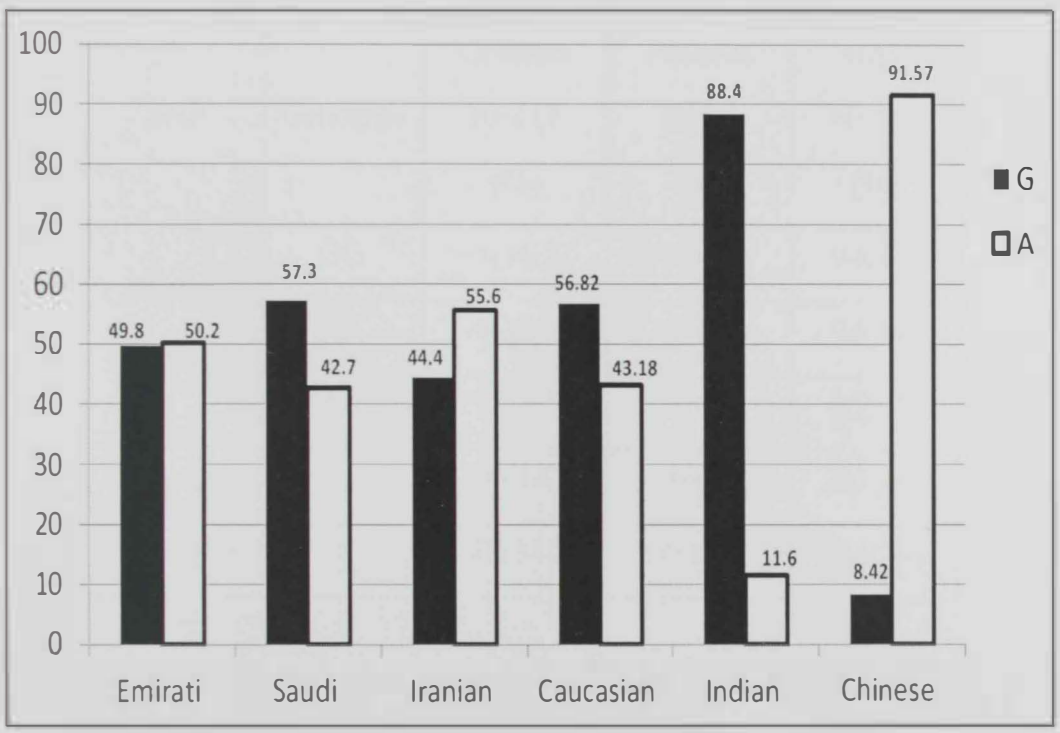



Figure 10. Ethnic distribution of the rs9923231 alleles

We used a chi-square test (nonparametric test for discrete variables) to compare the differences between the allele frequencies in populations shown in table 8; the value of $p<0.05$ was considered as statistically significant. Statistical analyses were performed applying the Graph Pad program.The UAE population when compared to the Chinese Population had a p value of <0.001. The UAE population when compared to the Indian Population had a p of <0.001. For the other populations, there were no significance differences in allele frequencies

### 4.2.2 Determination of the *VKORC1* rs7294 Genotype and Allele Frequencies

*VKORC1* rs7294 SNP genotype and allele frequencies among patients and control subjects are presented in table 9..

| SNP | Genotype | Controls N=117 (%) | Patients N=96 (%) | UAE N=213 (%) |
|---|---|---|---|---|
| G9041A rs7294 | GG | 54(46.2) | 40(41.7) | 94(44) |
| | GA | 46(39.3) | 48(50) | 94(44) |
| | AA | 17(14.5) | 8(8.3) | 25(12) |
| Alleles Frequency | G | 154(66) | 128(66.7) | 282(66) |
| | A | 80(34) | 64(33.3) | 144(34) |

Table 9. Genotype and Allele frequency for rs7294 for controls and patients.

We compared the percentage of the three genotypes (GG, GA and AA) for rs7294 between both groups (control and patients) as listed in table 10.

| Genotype | Controls n=117 | Patients n=96 |
|---|---|---|
| GG | 57.4 % | 42.6 % |
| GA | 48.9 % | 51.1 % |
| AA | 68.0 % | 32.0 % |

Table 10. Percentage of rs7294 SNP genotypes among both control and patient groups.

(N= total number of subjects in both control and patient groups)

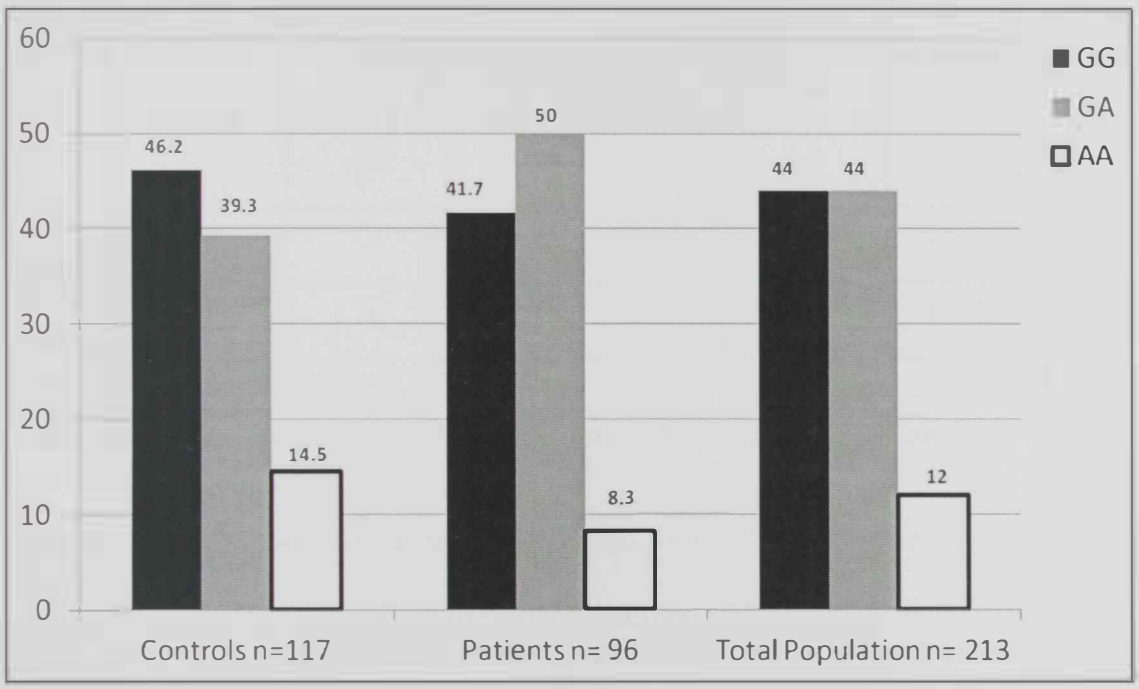



Figure 11. Graphic presentation of genotype frequencies among patients and control subjects for rs7294.

The genotype frequency of rs7294 for the total study sample of 213 UAE locals is shown in table 9. It shows differences in the distribution of the homozygous GG and AA genotypes with frequencies of 44% and 12%, respectively. On the other hand,

the heterozygous GA genotype is in equal distribution with the homozygous genotype 44 %. Allele frequencies are shown in table 9 with the G allele being present at frequency of 66% ,while the A allele is present at a frequency of 34%. We compared results from this study to data from other populations as listed in table 11.

| *VKORC1* SNP | Genotype | UAE n=213 | Chinese n=551 [91] | Caucasian n=23 [89] | Indian n=43 [90] |
|---|---|---|---|---|---|
| G9041A rs7294 | GG | 44 | 81.3 | 43.5 | 8.1 |
| | GA | 44 | 18.3 | 47.8 | 18.9 |
| | AA | 12 | 0.4 | 8.7 | 73 |
| Alleles Frequency | G | 66 | 90.5 | 67.4 | 18.6 |
| | A | 34 | 9.5 | 32.6 | 81.4 |

Table 11. Ethnic distribution of the rs7294 genotypes and alleles frequencies.

Graphic representation of ethnic distribution of rs7294 genotype frequencies is shown in figure 12 and the allele frequencies in figure 13.

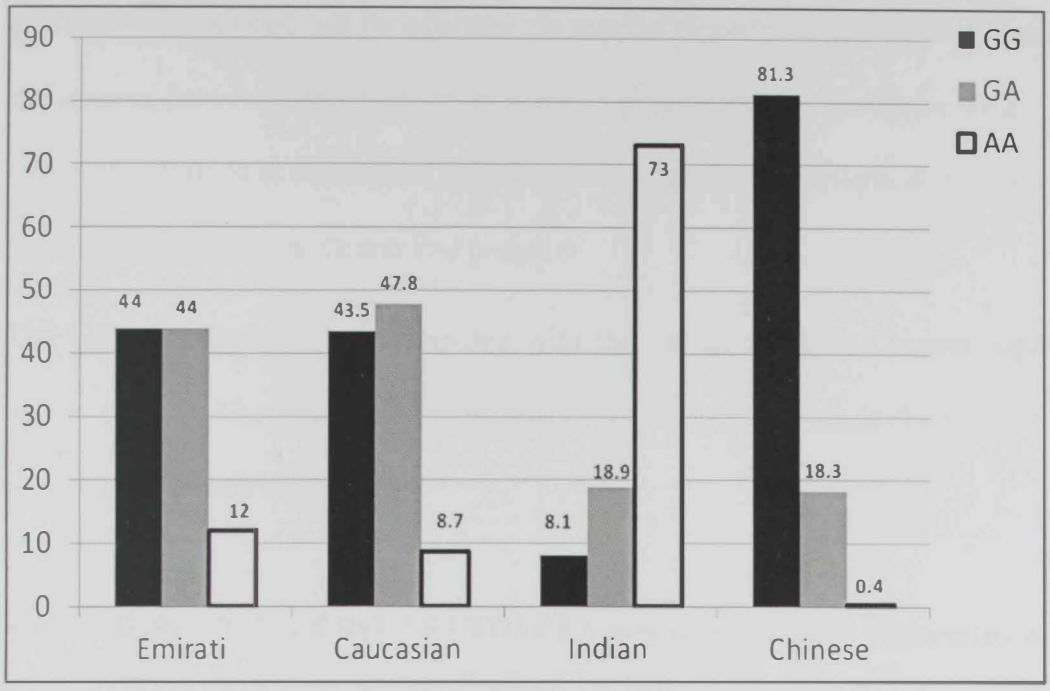



Figure 12. Ethnic distribution of rs7294 genotypes

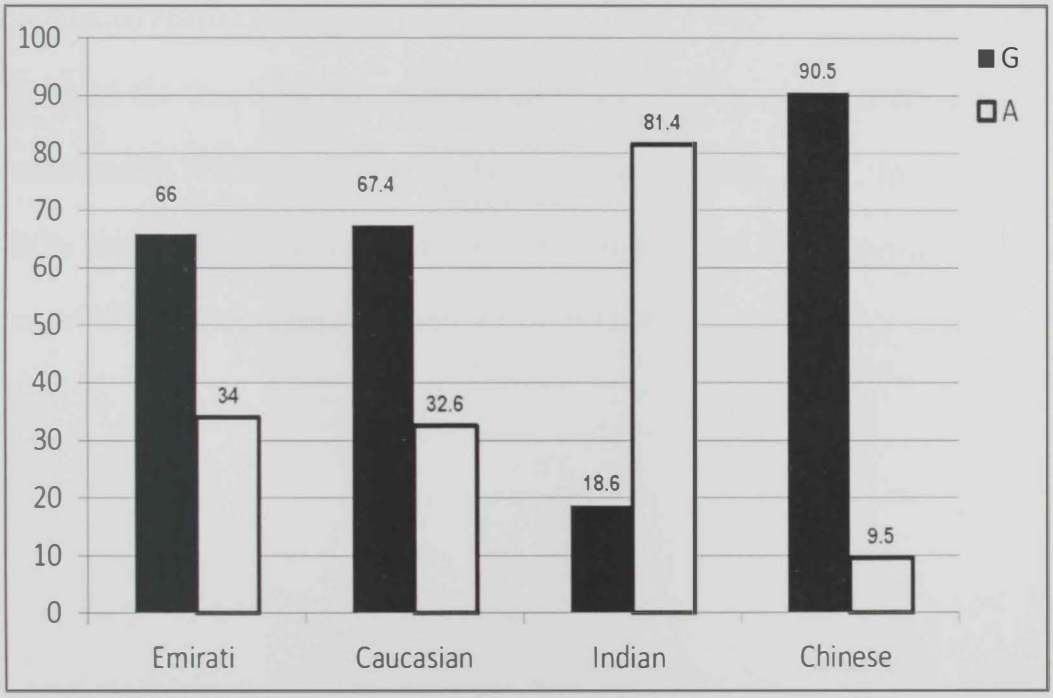



Figure 13. Ethnic distribution of rs7294 alleles.

We used a chi-square test (nonparametric test for discrete variables) to compare the differences between the allele frequencies in populations as shown in table 11.The value of $p<0.05$ was considered as statistically significant. Statistical analyses were performed applying the Graph Pad program.

The UAE population when compared with the Indian and Han-Chinese population had a $p<0.001$.There were no significance differences in allele frequencies with European populations.

### 4.3 The Rs9923231 and Rs7294 *VKORC1* Variants Genotypes Associated with Warfarin Doses Among Emirati Patients

A Spearman correlation coefficient statistical analysis was selected to test the correlation coefficient in each SNP.

We used the Graph Pad to study the correlation between each SNPs and the daily maintenance Warfarin dose for patients included in this study. In summary, both SNPs shows significant correlations with dosages ($P<0.05$) as shown in figures 14 for rs9923231 genotypes and figure 15 for rs7294 genotypes.

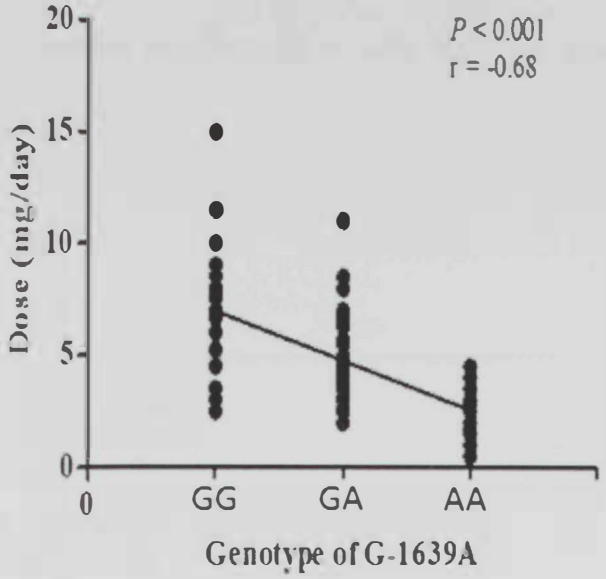



Figure 14. Spearman Correlation Coefficient between rs9923231 genotypes and daily Warfarin dosage.

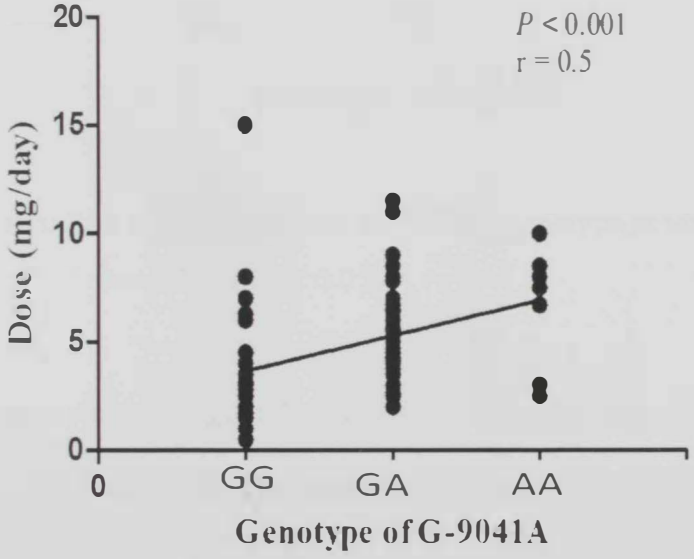



Figure 15. Spearman Correlation Coefficient between rs7294 genotypes and daily Warfarin dose.

We used a Scatter Plot to graphically represent each genotypes relation to dosage. Both SNPs were correlated significantly to daily Warfarin dosage (P-value<0.05) as shown in figures 16 and 17.

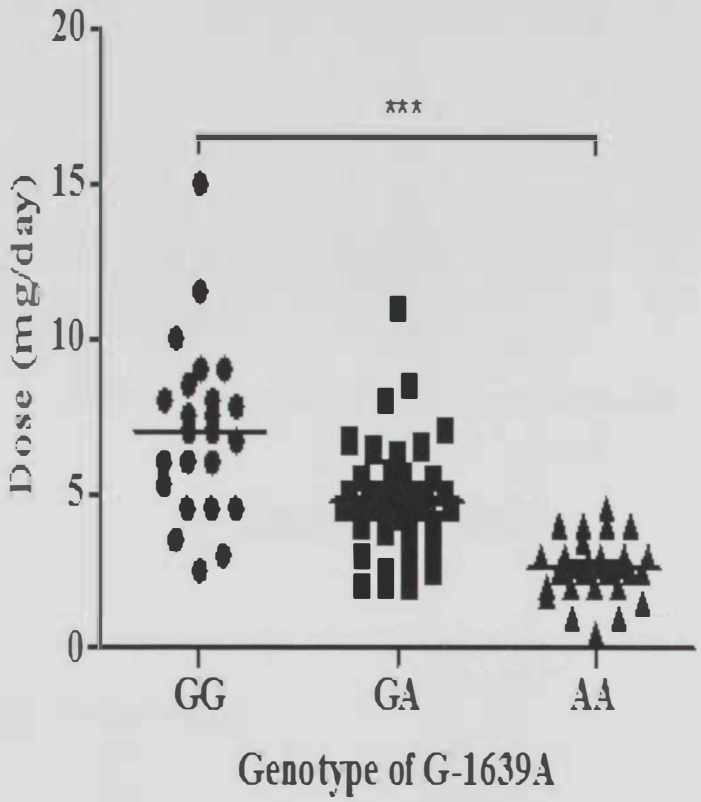



Figure 16. Scatter Plot representation of rs9923231 genotype relation to daily Warfarin dose (*** means P-value < 0.05).

The GG genotype shows a higher dosage compared to GA and a lesser dosage to the AA genotype. While for rs7294 the higher dose is associated with the AA genotype.

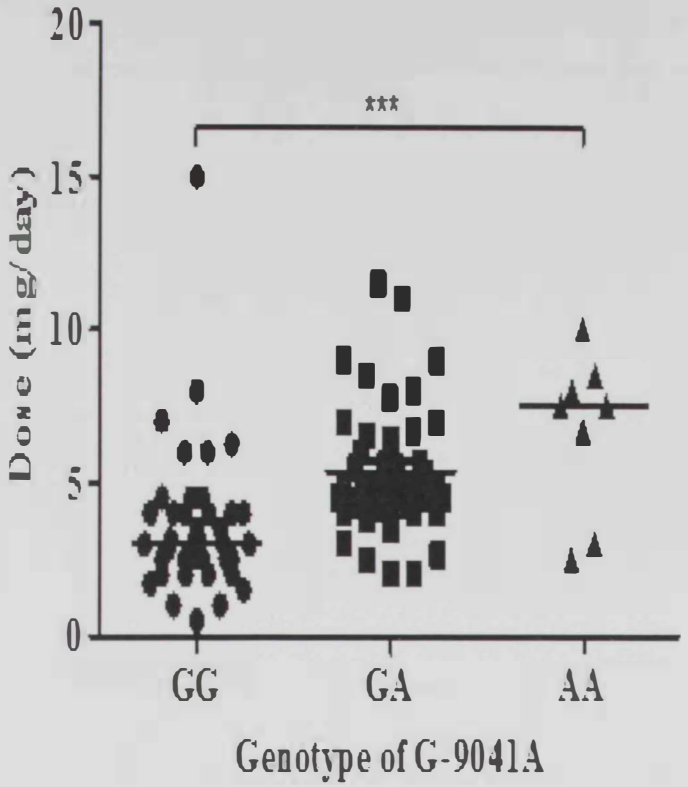



Figure 17. Scatter Plot representation of rs7294 genotype relation to daily Warfarin doses (*** means P-value < 0.05).

In addition, we used a Boxplot method to represent patient dose variation in relation to genotype and to show the dispersion (the spread) of doses among each genotype for SNPs. This data is shown in figures 18 and 19.

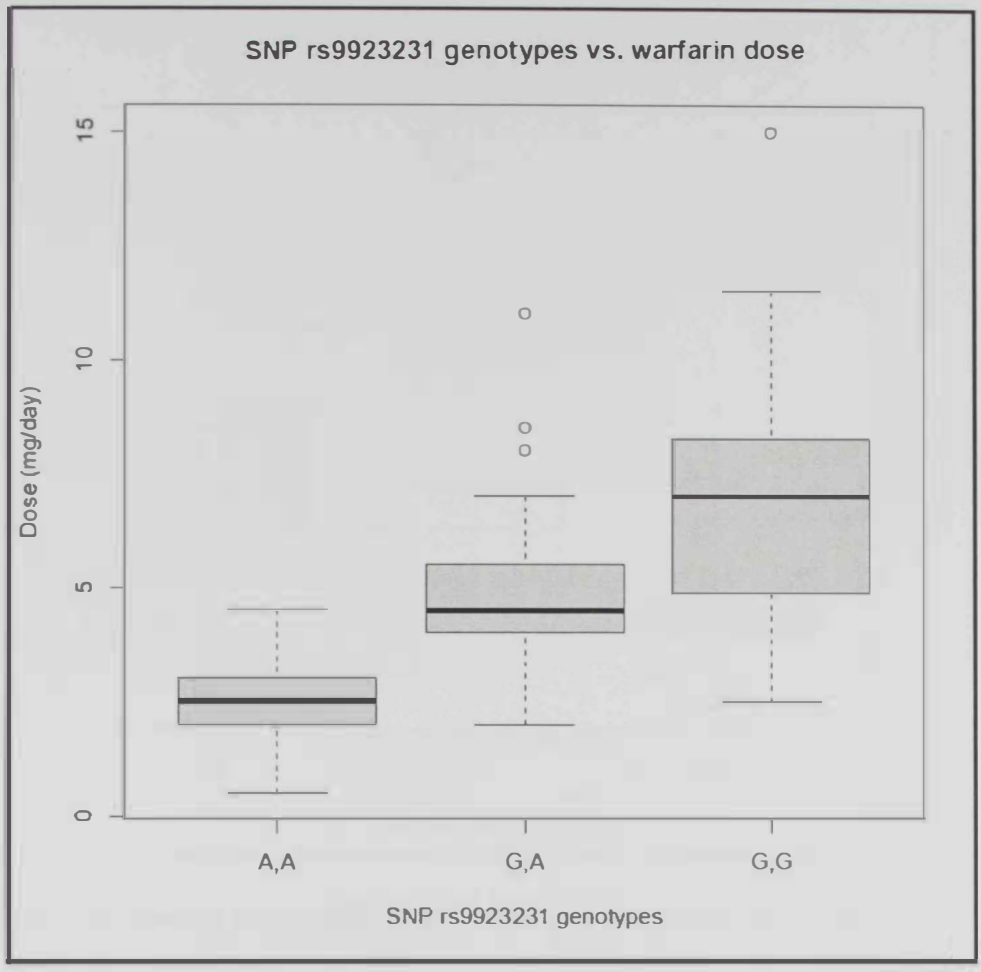



Figure 18. Boxplot of mean daily Warfarin doses for different *VKORC1* genotypes (SNP rs9923231).

The horizontal line indicates the median; the box covers 25–75% percentiles. Points outside this show up as outliers. In all 96 individuals were genotyped for rs9923231 genotyping. The maintenance dose of Warfarin was related significantly to genotype since AA genotype required a lesser dose compared to the intermediate dose for a GA genotype and a relatively higher dose compared to the GG genotype.

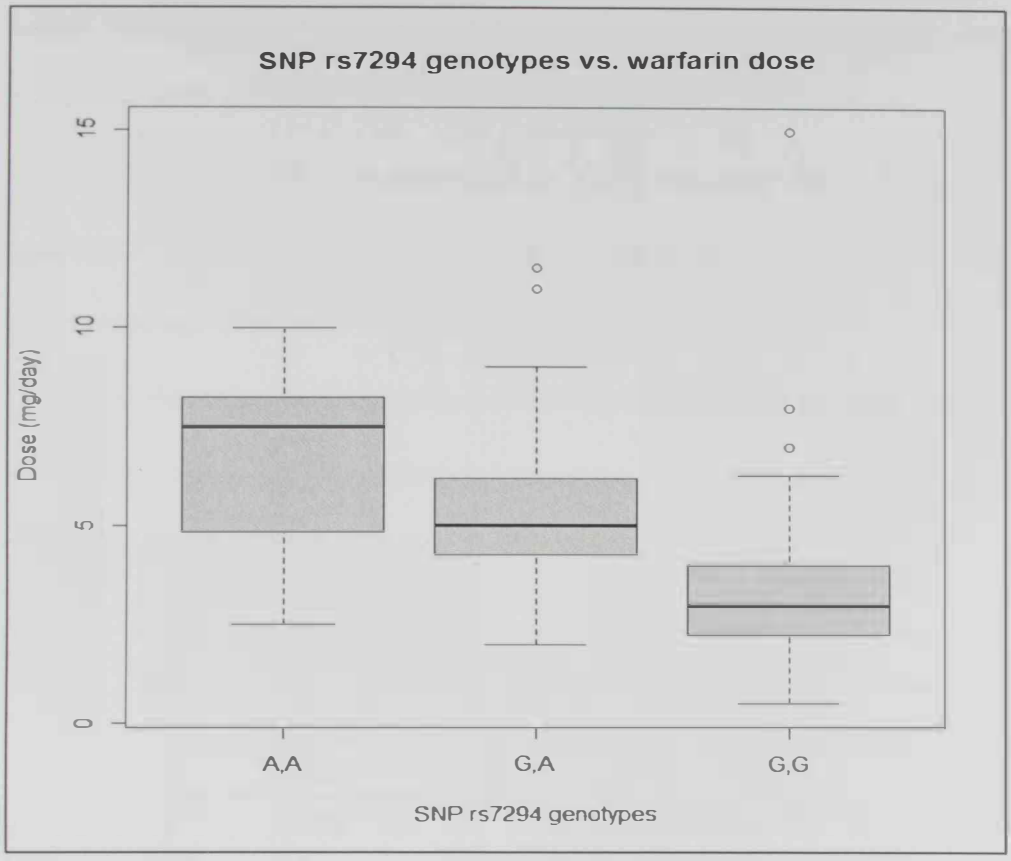



Figure 19. Boxplot of mean daily Warfarin doses for different *VKORC1* genotypes (SNP rs7294).

The horizontal line indicates the median, the box covers 25–75% percentiles. Points outside this show up as outliers. In all 96 individuals were genotyped for rs7294 genotyping. The maintenance dose of Warfarin was related significantly to genotype since GG genotypes required a lesser dose compared to an intermediate dose for GA genotypes and a relatively higher dose for AA genotype carrying patients.

Furthermore, we used a multiple liner regression analysis, where daily Warfarin dosages in milligrams (mg) was the dependent variable and the genotype was an independent variable and controlling for other factors such as age and gender. Using *VKORC1* rs9923231 genotypes, age, and gender.

Linear regression analysis shows that there is significant relationship between the rs9923231 SNP genotypes (AA, GA and GG).

The average dose for AA genotypes is (2.61 mg /day SD 1.022), for the GA genotype it is (4.74 mg/day SD 1.72) and for GG genotype it is (7 mg/day SD 2.796). the average age of patients was 59 (SD 19).

We found that there was a significant relationship between age and dosage (P-value $< 0.01$) but to a lesser extent than for genotype while no relationship with gender to daily Warfarin dose (P-value = 1). This data is shown in table 12.

| Variable | Daily dose P value |
|---|---|
| AA | $< 0.001$ |
| GA | $< 0.001$ |
| GG | $< 0.001$ |
| Age | $< 0.01$ |
| Gender | 1 |

Table 12. Multiple linear regression models with rs9923231 genotypes, age, and gender.

(Multiple R-squared: 0.477, adjusted R-squared: 0.4541, degree of freedom (DF): 91, p-value: 3.518e-12. F-statistic: 20.75)

For rs7294 we also used a multiple linear regression model and results are shown in table 13

| Factors | Daily dose P-Value |
|---|---|
| GG | >0.001 |
| GA | >0.001 |
| AA | >0.001 |
| Age | >0.001 |
| Gender | 1 |

Table 13. Multiple linear regression models with rs2794 genotypes, age, and gender. (Multiple R-squared: 0.2797, adjusted R-squared: 0.248, F-statistic: 8.832 on 4, p-value: 4.525e-06).

Using a linear model where dose is the dependent variable and rs2794 genotypes are the independent variable, and controlling for other factors such as age and gender, we found that there was a significant relationship between the rs7294 SNP genotype and the dose taken (P-value = 0.0003). The average Warfarin dose required for the GG genotype is 3.6 mg/day (SD 2.5), the GA genotype is 5.3 mg/day (SD 2) and for the AA genotype it was 6.7 mg/day (SD 2.6). Age significantly correlated to dose (P-value >0.001) while gender was found not to be correlated (P>1).

# CHAPTER 5: DISCUSSION AND CONCLUSIONS

Allele and genotype frequencies for SNPs rs9923231 and rs7294 in the *VKORC1* gene were found to be in a Hardy-Weinberg equilibrium among the study sample of 213 UAE nationals. There were no significance differences in the prevalence of GG, GA and AA genotypes for rs9923231 variants between the patients and control groups as determined by a chi-square test (p-value = 0.9296). Similarly there was no significant difference between the two group for the rs7294 variant (chi-square test for GG, GA and AA genotypes (p-value = 0.1842). This means, as expected, that both SNPs distributed equally between control and patient groups without any significant difference associated with any pathological condition.

The genotype frequency for the rs9923231 variant in the total study sample of 213 local UAE participants for homozygous GG and AA are presented in Figure 8 and Table 6. They show similar distributions of 25% and 26% respectively while the distribution of the heterozygous GA genotype was higher at 49%. Allele frequencies for rs9923231 are shown in Table 6 and indicate no differences in G and A allele frequencies at 49.8% and 50.2%, respectively.

The genotypes frequency for the rs7294 variant in the total study sample of 213 subjects is shown in table 9 and indicates some differences in the distribution of homozygous genotypes (GG and AA) of 44% and 12%, respectively, while the heterozygous GA genotype is in equal distribution with homozygous genotype at 44%. We found that the allele frequency for G (66%) more than for the A allele (34%) as shown in table 9.

Warfarin dosages vary among Emirati patients with doses ranging from 0.5 mg to 15 mg of Warfarin per day (figure 16, and figure 17). This wide variation in dose

requirements is a major justification for carrying out this type of genetic study among Emiratis. Genetic causes are likely to be contribution factor in a significant proportion of this variability, as demonstrated by many studies on Warfarin dosage [92]. Genotypes for *VKORC1* SNPs correlate significantly with daily Warfarin doses according to the results of this study (figures 14 and15). The rs9923231 and rs7294 are closely associated with dosage with a p-value of < 0.001 for both SNPs (figures 14 and15). Therefore, we can conclude that the rs9923231 and rs2794 are good predictors of Warfarin maintenance dosage as shown in the Boxblot analysis in figures 18 and 19. For the rs9923231 homozygous AA genotype a smaller dose is required (2.6 mg/day, SD 1.022) compared to the heterozygous GA genotype (4.75 mg/day, SD1.7). A higher dose is required for the homozygous GG genotype (7 mg/day, SD 2.7) with a P-value < 0.0001(3.518e-12).

A recent study in Al- Ain evaluated the effects of other factors such as age, gender, body mass index (BMI), indications of Warfarin treatment, patient compliance in maintaining stable INR indicators of correct dosage, etc. They found that gender, and BMI have no effect while age, co-morbid status, and compliance were found to significantly affect the coagulation profile of patients [84]. Although age and gender both have been studied in previous studies, we included it in this study to show have direct effect on dosage but not on INR. In our study age significantly affected Warfarin dosage as demonstrated by the multiple regression model of analysis (tables 12, 13). Gender on the other hand had no significance effect (table 12, 13).

Generally there is a focus on rs9923231 (-1639 G/A) variant which is located at the second nucleotide of an E-box (CANNTG) of the 5` untranslated regions (5`UTR) of the *VKORC1* gene [82]. This variant plays a key role on the activity of the promoter

region of the *VKORC1* gene and it is believed to be a causative SNP for low dose requirement phenotypes in some patients [52]. The activity of the G allele is 44% higher than the A allele in this position [82]. Polymorphism in rs9923231(-1639 G/A) shows an interference with the mRNA expression in liver samples since it is associated with variable levels of mRNA. The A allele disturbs the binding of transcription factors to the *VKORC1* gene promoter region leading to a decrease in active mature copies of the *VKORC1* protein activity [82].

According to the literature, patients with the G allele require a daily dose of 5 mg/day compared to 3.5-3.7 mg/ day for patients with the A allele [26]. In our study daily doses of Warfarin were approximately 2.6 for homozygous patients with the A allele and approximately 7 mg/day for patients with the homozygous G allele (Figures 16 and 18).

Another study has reported that patients with the A allele have a Warfarin dose ranging from 2.9 up to 3 mg per day, while patients with the homozygous G allele range from 5.5 up to 6 mg/day [79].

The frequencies of the rs9923231 (-1639 G/A) genotypes and alleles have been widely studied in different populations [52]. For example, 90% of the Asian population have the A allele and therefore patients of Asian origin require lower doses of Warfarin [52]. On the other hand, 40% of some caucasian populations have the A allele. It was postulated that rs9923231 is the best predictor of Warfarin dose requirements especially in the initial stages of Warfarin therapy [79].

It is worth mentioning that the rs9923231 variant is the only SNP mentioned in FDA recommendations for labeling Warfarin leaflets amongst all the other important SNPs in the *VKORC1* gene. However, many studies have shown the importance of

other non-coding SNPs in the *VKORC1*. However, these SNPs are mostly in perfect linkage disequilibrium with rs9923231 [79]. Based on several studies, the FDA has recommended genetic testing on Warfarin for the *VKORC1*, rs9923231, A allele carriers and for the *CYP2C9* gene haplotype (http://www.fda.gov/drugs/scienceresearch/researchareas/pharmacogenetics/ucm083378.htm) [85].

Another SNP located in the 3'UTR of the*VKORC1* rs7294 or as it called in the literature G9041A, or 3730G>A. It is not correlated with any other SNP in *the*VKORC1* gene [79]. Generally this SNP is not found in the same haplotypes as the G3673A. This is the reason for selecting this SNP in our study although it was not recommended by the FDA. Patients with rs7294 alternative variants may require higher doses of Warfarin [43].

In this current study, rs7294 as shown in figure 15 and figure 17 for the homozygous AA genotype, required higher doses (6.7 mg/day, SD 2.6), while the heterozygous GA genotype required 5.3 mg/day, (SD 2) a lesser dose was required for homozygous GG genotypes (3.61 mg/day SD 2.5) With a P-value of $< 0.0001$ (0.0003). Therefore, findings in this study support previously published data confirming a higher dose requirement for individuals with the homozygous A allele genotype (5.7 mg/ day) [26].

It was suggested that 6-37% of the variation in Warfarin dosage among individuals is due to SNPs in the *VKORC1* gene [79], [81], [85]. It was well documented that the *VKORC1* polymorphism highly influences Warfarin dosage and in some cases may

cause resistance at the A allele in rs7294 variants or sensitivity with A alleles of the rs9923231 variant [79], [81].

This is first genetic study among Emiratis regarding *VKORC1* allele and genotype frequencies. Our data suggests that SNPs are very important for Warfarin maintenance dosage and therefore they are likely to be of clinical significance (Figures 14 and 15).

Different ethnic groups require different Warfarin doses. For instance Chinese individuals, on average, require lower dose compared to African Americans [93], [94].

There have been many trials on new medication to replace Warfarin since its approval in 1954. This is mainly because of the many reports of side effects observed in patients receiving this medication. However, surveillance studies on complications and pharmaco-epidemiology of Warfarin are not currently available in the UAE. There is generally a lack of drug-drug and food-drug interaction studies locally. For instance, it was not easy to find reported cases of side effects associated with Warfarin treatment, such as Inpatient hospitalization due to Warfarin, patient' outcomes in selecting a dosage of Warfarin depending on non-genetic factors in Emiratis. Our data indicates that genotyping will be useful and most probably cost effective when initiating Warfarin treatment.

The two variants tested are useful but a clearer and more accurate picture can be observed if other parts of the *VKORC1* gene and CYP2C9 gene are studied in more depth in Emiratis. It is also important to look at non-genetic factors involved in Warfarin dose requirements among Emiratis such as lifestyle, diet, chronic use of

other medications, food supplements and herbal remedies used by the local population.

Our data indicates that pharmacogenetic testing of rs9923231 and rs7294 variants is highly relevant to the maintenance of Warfarin dosage among Emirati patients on this medication. Implementation of genetic testing, at least for these two variants, is likely to have a direct effect with potential benefits to the patients and healthcare system in UAE. This is also likely to have a direct impact on patient safety and reduce suffering and cost.

The evidence provided here demonstrates that genetic factors in the *VKORC1* gene are significant contributors to Warfarin dosage and, as a result may be employed to reduce the risk of developing side effects such as bleeding or thrombosis.

Having said that, we believe that it is important to sequence the rest of the *VKORC1* gene to study the haplotypes and perhaps examine for the presence of private variants in this gene among Emiratis. It is also important to study the variants of the *CYP2C9* gene.

In conclusion, this is the first study reporting the influence of genetic factors such as *VKORC1* polymorphisms (rs9923231 and rs7294) in Emiratis. We also report the frequencies of alleles and genotypes of these SNPs allowing us to compare with other populations which might be useful for genealogical studies.

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