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Allelic variants of CYP2C9 and VKORC1 genes and their relation to Warfarin metabolism in patients under Warfarin therapy in Gaza Strip

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Declaration

I declare that this research paper submitted to the Islamic University for the Degree of Master of Science in Biological Sciences-Medical Technology has not been submitted by me or anyone else for a degree at this or any other university. That it is my own work and all materials that consulted have been properly acknowledged.

Signature **Date**

Ahmed Shaker Abu Shaaban Dec. 2013

Dedication

This work is dedicated

To

My father **Shaker Abu Shaaban**

Who always supporting me,

To my mother **Mervat**

To my wife **Sendrella**

To my brothers **Eng. Mohammed, Eng. Hazem and Eng. Waseem.**

To my daughters **Yara and Farah**

To all **Palestinian people…**

Ahmed Shaker Abu Shaaban

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Abstract

Background: Warfarin is the most widely prescribed oral anticoagulant for thromboembolic therapy, patient management is difficult because of significant differences in metabolic rates as a result of allelic variation in its metabolizing enzyme. CYP2C9 gene encoding the enzyme that catalyzes the conversion of Warfarin to inactive metabolites, and the VKORC1 gene encoding the enzyme responsible for reducing vitamin K 2,3-epoxide to the enzymatically activated form.

Three single nucleotide polymorphisms (SNPs), two in the CYP2C9 gene and one in the VKORC1 gene, have been found to play key roles in determining the effect of Warfarin therapy on coagulation.

The presence of CYP2C9*2 and CYP2C9*3 variant alleles decrease the enzymatic activity which cause the elongation of half-life and reduced rate of clearance of Warfarin.

Objectives: The aim of this study was to determine the allelic varints of CYP2C9 gene (alleles *1, *2 and *3) and VKORC1 (alleles 1639-G and 1639-A) in Gaza strip, and to determine the relation between the Warfarin anticoagulation and the genotype of the two genes CYP2C9 and VKORC1.

Methods: Whole blood samples were collected from 101 patients under Warfarin therapy who visited anti-coagulation clinics of Alshifa and Gaza European hospitals then subjected to DNA extraction.

Allelic variants of CYP2C9 and VKORC1 genes were determined by PCR-RFLP technique. Also sodium citrated whole blood samples were collected and plasma were separated for the determination of their Prothrombin time/international normalized ratio (PT/INR) level in order to establish a relationship between the CYP2C9 and VKORC1 allelic variants and the Warfarin anticoagulation.

Results: It was found that none of the patients who are receiving the Warfarin has achieved the therapeutic range during the first month of Warfarin medication. There is a statistically significant difference in average INR between patients who have homozygote or heterozygote CYP2C9*2 and that who have the wild type CYP2C9*1/*1 and VKORC1(G/G) genotypes (3.1±1.29) and (2.05±0.92) respectively (P-Value=0.02, 95% C.I.), but when comparing the average actual weekly dose of Warfarin between the two groups it was found that there is no

statistically significant difference (49.38±20.99 mg/week) and (48.45±14.22) respectively (P-Value=0.89, 95% C.I.).

According to VKORC1 gene, it was found that there is no statistically significant difference in the average INR between patients who have homozygote or heterozygote VKORC1-1639A and that who have VKORC1(G/G) genotype (2.48±1.61) and (2.34±1.12) respectively (P-Value= 0.67, 95% C.I.), but there is a statistically significant difference in the average weekly dose between the two groups (40.36±14.04 mg/week) and (48.71±15.96 mg/week) respectively (P-Value=0.01, 95% C.I.). Also when averages of INR were compared between patients who have at least one copy of VKORC1-1639A allele and those who have CYP2C9*1/*1 and VKORC1(G/G), it was found that there is no statistically significant difference between the two means (2.4±1.23) and (2.05±0.92) respectively (P-Value=0.24, 95% C.I.), but there is a statistically significant difference in the average weekly dose of Warfarin between the two groups (40.63±14.13) and (48.45±14.22) respectively (P-Value=0.04, 95% C.I.).

Conclusion: We conclude that the presence of at least one copy of CYP2C9*2 or VKORC1-1639A alleles or both is observed to be a cause for an increased sensitivity to Warfarin therapy and increased INR level. Therefore we recommend testing for these alleles as a part of the patient management.

Key words: CYP2C9, VKORC1, Warfarin, Thromboembolism, Deep venous thrombosis, Prothrombin time, International Normalized Ratio, Polymorphism, Gaza strip.

Arabic Abstract

ملخص الدراسة باللغة العربية

الأنماط الأليلية للجينين CYP2C9 و VKORC1 وعلاقتها بأيض علاج الوارفارين لدى المرضى الخاضعين لعلاج الوارفارين في قطاع غزة

مقدمة: الوارفارين هو العلاج الأوسع استخداما في علاج الجلطات الدموية ، التحكم في العلاج لدى المرضى يعتبر صعباً لاختلاف الاستجابة للجرعة القياسية من شخص لأخر، وذلك لاختلاف معدل الأيض من شخص لأخر. أيض الوارفاراين يتم التحكم به جينياً بشكل أساسي عن طريق جين CYP2C9 والذي يحمل شفرة الإنزيم الذي يعمل على تحويل الوارفارين إلى متحللات غير نشطة، فيما يعتبر الإنزيم الناتج من جين VKORC1 مسئول عن اختزال Vit. K 2,3 epoxide للشكل النشط من الإنزيم.

نقص فيتامين ك (Vit. K) ممكن أن يتسبب في نزيف حاد وكذلك علاج الوارفارين المضاد لفيتامين ك بجرعة مرتفعة، ويعتبر تغير الأنماط الأليلية للجين VKORC1 سبباً في نقص عوامل التجلط المعتمدة على فيتامين ك.

يتم تحلل الوارفارين وبشكل أساسي معتمدا على CYP2C9 ويعمل نشاطه المانع للتجلط من خلال تثبيط البروتين Vitamin K Epoxide Reductase Complex Subunit 1.

ثلاثة أنماط أليلية وحيدة النيوكليتيدة تعتبر مسئولة عن تحديد الفعالية العلاجية للوارفارين، اثنتين في CYP2C9 وواحدة في VKORC1.

كشفت دراسات سابقة عن وجود الأنماط الأليلة CYP2C9*2 و CYP2C9*3 والتي تعمل على تقليل نشاط الإنزيم وإطالة فترة بقاء الوارفارين في الجسم وإبطاء عملية استخلاصه.

هدف الدراسة: تحديد الأنماط الأليلية CYP2C9*1 و CYP2C9*2 و CYP2C9*3 والأنماط الأليلية VKORC1-1639G و VKORC1-1639A في قطاع غزة وتحديد العلاقة بين وجود الطراز الجيني لكلا الجينين وتأثير الوارفارين المانع للتجلط.

المواد والخطوات: عينات دم خام تم جمعها من المرضى الذين يخضعون لعلاج الوارفارين وقد قاموا بمراجعة عيادات القلب والشرايين والجلطات الدموية في مستشفى الشفاء ومستشفى غزة الأوروبي أثناء فترة الدراسة، ثم خضعت العينات لعملية استخلاص الحمض النووي (DNA) وتم تحديد الأنماط الأليلية لـ CYP2C9 و VKORC1 بواسطة تقنية الـ PCR-RFLP. كذلك تم جمع عينات من المرضى وفصل البلازما لقياس وقت التخثر (Prothrombin time) ونسبة المعايرة الدولية (INR) وذلك لإيجاد العلاقة بين جيني CYP2C9 و VKORC1 وعلاج الوارفارين.

النتائج: ارتفاع معدل الـ INR بدلالة إحصائية عند المرضى اللذين يحملون على الأقل نسخة واحدة من النمط الأليلي CYP2C9*2 بمتوسط حسابي (3.1±1.29) عن المرضى الذين يحملون الطرز الجينية

CYP2C9*1/*1 و VKORC1(G/G) بمتوسط حسابي (2.05±0.92) (P-Value=0.02, 95% C.I.) ولكن لم يكن هناك اختلاف ذو دلالة إحصائية في معدل جرعة الوارفارين بين المجموعتين بمتوسط حسابي (49.38±20.99) و (48.45±14.22) مليجرام في الأسبوع على التوالي (P-Value=0.89, 95% C.I.) .

عدم وجود اختلاف ذو دلالة إحصائية في معدل الـ INR بين المرضى الذين يحملون على الأقل نسخة واحدة من النمط الأليلي VKORC1-1639A بمتوسط حسابي (2.48±1.61) عن أولئك الذين يحملون الطراز الجيني VKORC1(G/G) بمتوسط حسابي (2.34±1.12) (P-Value=0.67, 95% C.I.) ولكن كان هناك اختلاف ذو دلالة إحصائية في معدل الجرعة الأسبوعية بمتوسط حسابي (40.36±14.04) و (48.71±15.96) مليجرام في الأسبوع على التوالي (P-Value=0.01, 95% C.I.) .

عدم وجود اختلاف ذو دلالة إحصائية بين معدل الـ INR في المرضى الذين يحملون النمط الأليلي VKORC1-1639A وأولئك الذين يحملون الطرز الجينية CYP2C9*1/*1 و VKORC1(G/G)، بمتوسط حسابي (2.4±1.23) و (2.05±0.92) على التوالي (P-Value=0.24, 95% C.I.). ولكن كان هناك اختلاف ذو دلالة إحصائية في معدل الجرعة الأسبوعية بمتوسط حسابي (40.63±14.13) و (48.45±14.22) مليجرام في الأسبوع على التوالي (P-Value=0.04, 95% C.I.).

الاستنتاج: وجود نسخة واحدة على الأقل من الأنماط الأليلية CYP2C9*2 أو VKORC1-1639A أو كلاهما ذلك يعتبر عاملا مهما من أسباب ارتفاع الحساسية لعلاج الوارفارين وزيادة معدل الـ INR، لذلك ننصح بالكشف عن هذه الأليلات كجزء من عملية العلاج.

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Abbreviation	Term
ACCP	The American College of Chest Physicians
ADR	Adverse reaction
Arg	Arginine
BMI	Body mass index
bp	Base pair
cDNA	Complementary DNA
CO2	Carbon dioxide
CYP2C9	Cytochrome P450, family 2, subfamily C, polypeptide 9
Cys	Cystine
DNA	Deoxyribonucleic acid
DVT	Deep venous thrombosis
factor VIIa	Activated factor VII
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GGCX	γ-glutamyl carboxylase enzyme
gla	Gamma-carboxyl glutamic acid
Glu	Glutamate
Ile	Isoleucine
INR	International normalized ratio
ISI	International sensitivity index
IWPC	International Warfarin Pharmacogenetics Consortium
K1H2	Active hydroquinone form of vitamin K
K3 EDTA	Ethylenediaminetetraacetic acid
kDa	Kilo dalton

Leu	Leucine
mg	Milligram
MNPT	Mean normal prothrombin time
MOH	Ministry of health
mRNA	Messenger Ribonucleic acid
NAD+	Oxidized form of nicotinamide adenine dinucleotide
NADH	Reduced form of nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
NCCLS	National Committee for Clinical Laboratory Standards
O2	Oxygen
OH	Hydroxy
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
Pmol	Pico mole
PCBS	Palestinian Central Bureau of Statistics
PT	Prothrombin time
PT/INR	Prothrombin time/international normalized ratio
PTT	Activated partial thromboplastin time
RBCs	Red blood cells
RefSeq	Reference Sequence
rpm	Round per minute
SD	Standard deviation
SNPs	Single nucleotide polymorphisms
SPSS	Statistical package for social science
TF	Tissue factor
VitKH2	Reduced form vitamin K hydroquinone
VKA	Vitamin K antagonists
VKOR	Vitamin K epoxide reductase

VKORC1	Vitamin K epoxide reductase complex Subunit 1gene
WBCs	White blood cells
WHO	World Health Organization

Chapter 1

Introduction

1.1. Overview

Warfarin is the most commonly prescribed oral anticoagulant drug for the prophylaxis and treatment of venous and arterial thromboembolic disorders in many countries and is being prescribed to an increasing number of patients (Dipiro et al., 2008). Because of its narrow therapeutic index, predisposition to drug and food interactions, and tendency to cause hemorrhage, Warfarin requires continuous patient monitoring and education to achieve optimal outcomes (Ansell et al., 2004).

Oral Warfarin anticoagulant dosages are adjusted by the healthcare professional according to the results of the Prothrombin time/International normalized ratio (PT/INR) test, which must fit the therapeutic target range of (2.0 – 3.0) (Holbrook et al., 1996; Ansell et al., 2004 and Roche, 2006). Overdosing or under dosing of Warfarin are life threatening due to bleeding or thrombosis respectively.

Warfarin is nearly 100% bioavailable when taken orally and both oral and intravenous formulations display similar pharmacokinetic characteristics (Porter and Sawyer, 1992, and Warrell et al., 2003). Warfarin is rapidly absorbed from the gastrointestinal tract, reaching maximum concentration 90 minutes after administration, with a half-life of 36 to 42 hours. However, the pharmacokinetics of Warfarin can be highly variable. It circulates bound to albumin and is metabolized in the liver (Kaminsky and Zhang, 1997 and Hirsh, 2001). The interindividual variability in anticoagulant response is multifactorial. Patient and environmental factors including genetics, body size, dietary vitamin K status, concurrent diseases and medication have been shown that affect on anticoagulant response to Warfarin (Kamali, 2006). Among the genetic causes of variability, CYP2C9 polymorphisms influences Warfarin pharmacokinetics (Scordo et al., 2002 and Kirchheiner et al., 2005), whereas variants in the gene encoding the vitamin K epoxide reductase complex 1 (VKORC1) affect the drug pharmacodynamics (Rieder et al., 2005 and Wadelius et al., 2005).

Also Low responsiveness to Warfarin (defined as a failure to achieve a target international normalized ratio [INR]) is associated with polymorphisms of the vitamin K epoxide reductase-oxidase complex gene (VKORC1). A highly prevalent promoter single-nucleotide polymorphism (VKORC1-1639 G>A) impairs VKORC1 expression and determines the interindividual variability of the target INR (Stepien et al., 2009).

In general, studies suggest that the CYP2C9 genotype contributes between 10 and 20% of the variability in Warfarin dose requirement compared with a 20–30% contribution from the VKORC1 (vitamin K epoxide reductase complex 1) genotype (klein et al., 2009).

The CYP2C9 gene (cytochrome P450, family 2, subfamily C, polypeptide 9), located on the long arm of human chromosome 10 (10q24), plays a key role in the metabolism of the S-isomer of Warfarin (Mark et al., 2005). CYP2C9 is the main enzyme responsible for Warfarin metabolism, the wild type of the allele (CYP2C9*1), has two other allelic variants (CYP2C9*2 and *3) identified, which result from point mutations of CYP2C9 gene (Lee et al., 2002). CYP2C9*2 and CYP2C9*3 allelic variants differ from the wild type allele by single nucleotide substitution. This leads to an amino acid substitution of arginine by cysteine at position 144 and isoleucine by leucine at position 359 of the enzyme encoded by CYP2C9*2 and CYP2C9*3 respectively (Figure 1.1) (Aithal et al., 1999; Yoon et al., 2001; Loebstein et al., 2001; Lee et al., 2002; Goldstein, 2001 and Ablin et al., 2002).

Recently a number of studies in different countries show that there is a genetic polymorphism to the CYP2C9 gene, variant alleles CYP2C9*1 , CYP2C9*2 and CYP2C9*3 are the most common allelic variants can affect the Warfarin metabolism. (Ghadam et al., 2009; Ngow et al., 2009 and Daly, 2010). The frequency of these allelic variants is dependent on the ethnical groups (Takahashi et al., 2006), CYP2C9 which metabolizes a range of drugs including Warfarin has a significant and meaningful association between CYP2C9 genotype and sensitivity to Warfarin, high international normalized ratio (INR) and bleeding complications are result from the association between CYP2C9 variant genotypes and warfain sensitivity (Ghadam et al., 2009). The sensitivity to Warfarin medication and the INR is dependent on the genotype of CYP2C9 as the CYP2C9*1 allelic form is the wild-type showing the

higher metabolism and lower sensitivity to Warfarin whereas the allelic variants CYP2C9*2 and CYP2C9*3 show the lower metabolism and the higher sensitivity to Warfarin. (Ghadam et al., 2009; Ngow et al., 2009 and Daly, 2010).

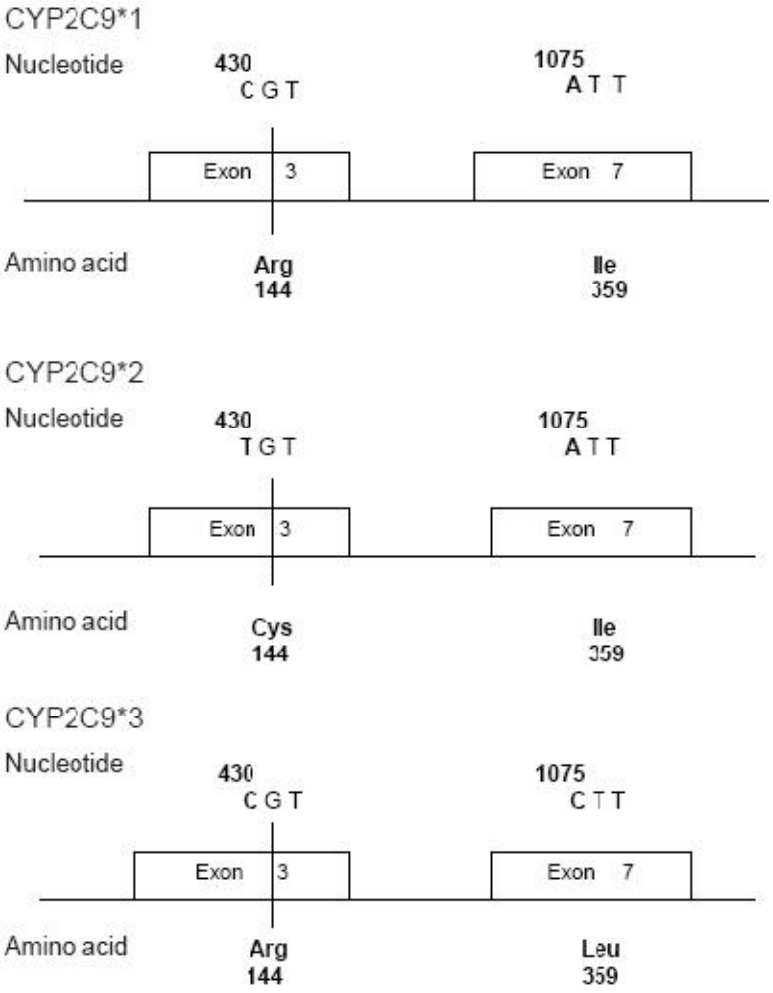

Figure 1.1: The location of SNPs and their amino acid substitution of CYP2C9*2 and CYP2C9*3 compared to CYP2C9*1 (Aithal et al., 1999; Yoon et al., 2001; Loebstein et al., 2001; Goldstein, 2001; Lee et al., 2002 and Ablin et al., 2002).

Vitamin K epoxide reductase subunit 1 (VKORC1) is the enzyme that recycles vitamin K 2,3-epoxide to reduced vitamin K required for the γ-carboxylation of vitamin K–dependent clotting factors (factors II, VII, IX, and X). Warfarin inhibits VKORC1 activity by reducing the regeneration of vitamin K and thus exerting its anticoagulation effect (Bell and Matschiner, 1972 and Wallin and Martin, 1985).

GGCX is an enzyme which catalyses the γ-carboxylation of glutamic acid residues of clotting factors and proteins C, S and Z (Li et al., 2004 and Rost et al., 2004).

Warfarin exerts an anticoagulant effect through its inhibition of the VKORC1 gene product (Suttie, 1987; Rost et al., 2004 and Li et al., 2004). Patients who are carriers of a common polymorphism in the VKORC1 promoter sequence (− 1639 G>A) require a lower Warfarin maintenance dosage (Yuan et al., 2005 and Rieder et al., 2005). The − 1639 G>A genotype and related haplotype can independently determine 20%–25% of Warfarin dose variance (Rieder et al., 2005 and Wadelius et al., 2007). As a result of the reduced expression of VKORC1, less Warfarin is needed in patients carrying the –1639A promoter variant to maintain the target INR.

Warfarin in Gaza strip is widely administered to a large number of patients but the international normalized ratio (INR) is just difficult to be optimized to the therapeutic range, therefore the risk of bleeding further more death is high. Despite that there is no study in Gaza strip to assess the Warfarin therapy and its adverse action, also there is no criterion can adopt the international normalized ratio (INR). The CYP2C9 and VKORC1 genes weren't studied in Gaza strip previously and their allelic variants are unknown so this study aims to find out allelic variants of CYP2C9 and VKORC1 and their relation to Warfarin metabolism in patients under Warfarin therapy in Gaza strip.

1.2. Aim of the study

To assess the allelic variants of CYP2C9 and VKORC1 genes and their relations to Warfarin metabolism in patients under Warfarin therapy in Gaza strip.

1.3. Specific objectives

1- To determine the frequency of CYP2C9*1,*2,*3 alleles among Palestinian population of Gaza strip.
2- To reveal any relationship between the allelic variants of CYP2C9 gene and the Warfarin metabolism among patients under Warfarin treatment.
3- To determine the effect of CYP2C9 and VKORC1 alleles on the international normalized ratio (INR) and the prothrombin time (PT).
4- To help in optimizing the Warfarin dose to minimize the adverse action of Warfarin (Bleeding or thrombosis).

1.4. Significance of the study

- The optimization of Warfarin dosage to match the needs of patients is very critical and aims to protect the patients from any adverse effect of increased or decreased sensitivity to Warfarin.
- The inheritance of the allelic variants CYP2C9*2 and CYP2C9*3 were shown to be a main cause of the increased sensitivity to Warfarin and decreased metabolism which allow the Warfarin to remain longer time in the body, also the inheritance of the VKORC1-1639A mutant allele is an important factor retards the availability of an active coagualation factors. Therefore, detection of allelic variants CYP2C9*2,*3 besides VKORC1-1639A will be important in Warfarin dose adjustment.
- To the best of researcher's knowledge, this is the first study to investigate this issue in the Palestinian population.

Chapter 2

Literature review

2.1. Coagulation process

Coagulation is the process by which blood forms clots, it is a function of plasma. It depends on interaction of a group of plasma proteins (which are sequentially activated following vascular injury) with some phospholipid (from either damaged tissue or platelets) and calcium. The overall coagulation process involves the formation of the insoluble protein fibrin from the plasma protein fibrinogen through the action of the enzyme thrombin. Fibrin forms a network of fibers which traps blood cells and platelets forming a thrombus or clot (McGill Faculty of Medicine, 2013).

Damaged endothelial cells lining the blood vessel release von Willebrand's Factor which makes the surfaces of the endothelial cells "sticky" and it may be enough to close small damage. In larger blood vessels, platelets adhere to surfaces of endothelial cells. This clumping of platelets provide a surface essential for the clotting process and serves in aggregated platelets release platelet thromboplastin (Factor III) which activates the clotting process. This process depends on the presence in the blood of eleven different clotting factors (proteins) and calcium (Factor IV). Ultimately, these factors will generate the production of prothrombin activator (Factor X). Depending on the initial trigger for the clotting reactions, there are two pathways leading to the formation of the thrombus; the extrinsic pathway and the intrinsic pathway (David, 2007), coagulation process including extrinsic, intrinsic and common pathways is shown in figure 2.1 and how the common pathway join extrinsic and intrinsic pathways.

2.1.1. Extrinsic Pathway

Is initiated with material outside of or "extrinsic to" the blood, in which the damaged tissue releases tissue thromboplastin (Factor III) a tissue factor (TF) represents the primary mechanism of the coagulation pathway in vivo when bound to activated factor VII (factor VIIa), a calcium dependent step (Nemerson and Esnouf,

1973; Nemerson, 1992). TF-Factor VIIa complex activates factors X - prothrombin activator (Calcium dependent step) also TF-factor VIIa complex activates factor IX (Osterud and Rapaport, 1977). Activated factor IX activates more factor X, with cofactors activated factor VIII, anionic phospholipids (from activated platelets) and calcium, activated factor X converts prothrombin to thrombin, with activated factor V, anionic phospholipids (from activated platelets) and calcium as cofactors; prothrombin factor is released merges with extrinsic pathway into common pathway. (Monkovic and Tracy, 1990; Parsons, 2012)

2.1.2. Intrinsic Pathway

Is initiated by the blood coming in contact with exposed collagen in the blood vessel wall. This process results in the formation of larger amounts of thrombin to allow the formation of larger clots (Hoffmana and Monroec, 2007). The contact with collagen underlying the endothelium in the blood vessel wall activates Factor XII to factor XIIa, subsequently it activates factor XI to factor XIa which activates factor IX to Factor IXa depending on calcium presence (Robertset al., 2004) .

Factor IXa activates Factor VIII to Factor VIIIa which is together with calcium ions and factor III from platelets (Platelet Thromboplastin) activate Factor X - prothrombin activator. Since Factor III is released from activated platelets, the completion of the intrinsic pathway depends on there being an adequate number of platelets in circulation. It should be noted that both pathways lead to the same reaction, namely, the activation of factor X - prothrombin activator. From this point on, both pathways follow the same course to fibrin formation. For this reason the steps from Factor X activation to Fibrin formation are referred to as the common Pathway (David, 2007).

2.1.3. Common Pathway

Factor X (active) engages in a series of reactions with Factor V, Calcium ions and phospholipids derived from platelets. This composite of clotting factors and their reactions is referred to as the Factor V Complex or Prothrombin Activator. Factor V complex initiates the conversion of prothrombin to active form of the enzyme

thrombin. Thrombin accelerates the formation of fibrin threads from fibrinogen (Factor I) (David, 2007).

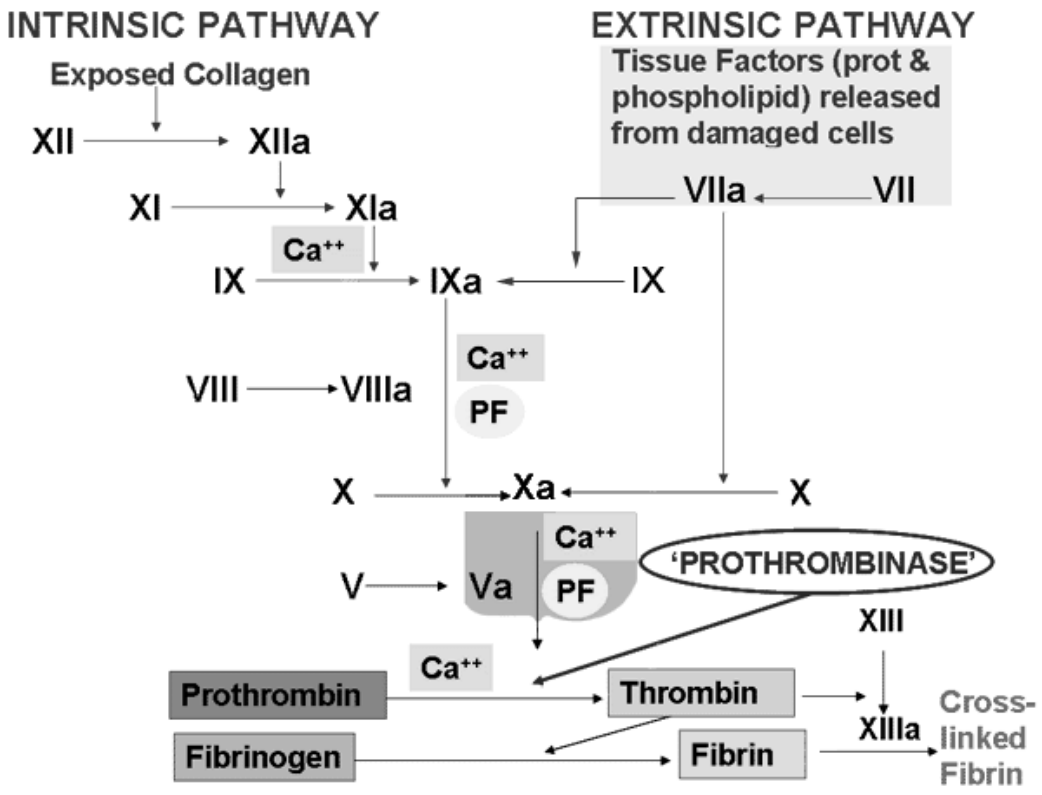

Figure 2.1 Coagulation cascade showing the fibrin clot formation through the activation of clotting factors each other in the presence of calcium and phospholipids, while the extrinsic pathway begins with the activation of factor VII by the help of tissue factor (thromboplastin) released by injured tissue and the process continued to merge the intrinsic pathway with the step of activation of factor X after various steps in intrinsic pathway that begins when the collagene comes from injured blood vessel become in contact to the blood, two pathways joined in the same step of the beginning of the common pathway. McGill Faculty of Medicine (2013).

2.2. Prothrombin time (PT)

The prothrombin time (PT) measures the clotting time from the activation of factor VII, through the formation of fibrin clot (David et al., 2001). The factor VIIa/tissue factor complex activates factor X to factor Xa and through the action of the prothrombinase complex (Figure 2.1), prothrombin is converted to thrombin (Harmening and Bethel, 2009). The time in seconds for the conversion of fibrinogen to soluble (noncross linked) fibrin by thrombin is reported as the prothrombin time. This test measures the integrity of the extrinsic and common pathways of coagulation, whereas the activated partial thromboplastin time (PTT) measures the integrity of the intrinsic and common pathways of coagulation as the figure 2.1. demonstrates each test and its corresponding coagulation process separately. PT prolongations are most commonly caused by factor deficiencies involving fibrinogen (factor I) or factors II, V, VII, or X. Less commonly, PT prolongations are due to an inhibitor, such as therapeutic anticoagulants including heparin, hirudin, or argatroban. Rarely, PT prolongations are caused by lupus anticoagulants or by specific factor inhibitors against fibrinogen or factor II, V, VII, or X (Elizabeth et al., 2001).

Prothrombin time test being responsive to assess the function and deficiencies of many coagulation factors (VII, X, V, II "Prothrombin" and I "fibrinogen") (Moffat et al., 2009). PT became over the years the test of choice to investigate congenital or acquired coagulopathies as well as to monitor the treatment with vitamin K antagonists (VKA) such as Warfarin. The key component for the PT test is thromboplastin (tissue factor) which may be extractive or recombinant. The variable reagent composition makes coagulation times dependent on the reagent used for testing, thus making interlaboratory comparability of results difficult (Ansell et al., 2004).

Early attempts at expressing PT results as either percentage activity, or the PT-ratio (patient-to-normal coagulation time) failed to harmonize results which were still dependent on the thromboplastin⁄coagulometer used for testing (Tripoda et al., 2007).

2.3. Standardization of PT reporting using INR

At the beginning of the 1980's a system of calibration was devised (Kirkwood, 1983) and endorsed by the World Health Organization (WHO) (WHO 1983; Van den Besselaar et al., 1999). Accordingly, commercial PT systems (defined as the combination of thromboplastin ⁄ coagulometer) are now calibrated by their manufacturers against one of the international standards for thromboplastin held by WHO (Ansell et al., 2004).

The international normalized ratio (INR) is used to standardize the reporting of the PT for VKA therapy monitoring. The INR is a mathematically derived value based on the equation: $INR=[patient\ PT/MNPT]^{ISI}$ where the MNPT = mean normal prothrombin time and the ISI = international sensitivity index (Kovacs et al., 1994; Olson et al., 2007; Favaloro et al., 2008; Tripodi, 2009).

A minimum of 20 individual healthy control volunteers should be used to determine the MNPT (Favaloro and Adcock, 2008; Favaloro et al., 2008; Tripodi, 2009). Laboratories need to recognize that different thromboplastins have variable sensitivities to the vitamin K-dependent coagulation factors assessed in the PT (specifically factors VII, X and prothrombin). This variability in sensitivity between thromboplastins is defined by the ISI that is assigned to the reagent (Kovacs et al., 1994; Harmening and Bethel, 2009). To minimize clinically significant variability in the INR, the ISI for the thromboplastin used by the laboratory needs to be verified locally and be specific for the lot number of reagent and the testing procedure or coagulometer used (Olson et al., 2007; Favaloro and Adcock, 2008; Favaloro et al., 2008; Tripodi, 2009).

2.4. International Normalized Ratio (INR)

INR (International Normalized Ratio) which is a standardised PT ratio. Over anticoagulation is common. Over anticoagulation due to Warfarin increasingly treated with small doses of vitamin K (Provan D et al., 2004). The INR is a good indicator of effectiveness and risk of bleeding during Warfarin therapy and is best kept at about 2.5, with a target range of 2.0-3.0, for most clinical indications, although higher levels may be better for certain patients. The lower limit of this target range recognizes a threshold level for effectiveness, while the upper limit is set to minimize bleeding (Holbrook et al., 1996; Ansell et al., 2004 and Roche, 2006).

2.5. International Sensitivity Index (ISI)

PT standardization system calls for the measurement of PT for plasmas from healthy subjects and patients stabilized on vitamin K antagonists. Paired values obtained with the system to be calibrated (working PT) and with the international standard are then plotted on a log-scale and the best-fit orthogonal regression line is drawn through the data points. The slope of the line, called international sensitivity index (ISI), is a measure of the responsiveness of the system to be calibrated relatively to the international standard (Figure 2.2.) (Tripodi et al., 2007).

The sensitivity of the reagent to the VKA is reflected by the ISI. The closer the ISI is to 1.00, the more sensitive the reagent is to the vitamin K-dependent factors as compared to the World Health Organization (WHO) reference thromboplastins. (Kovacs et al., 1994 and Denson et al., 1995; Harmening and Bethel, 2009). The ISI and the conversion of PT results into the INR scale are valid only for patients on VKA. This is because PTs from patients on VKA are inserted in the calibration plot (see Figure 2.2.) (Tripoda et al., 2007).

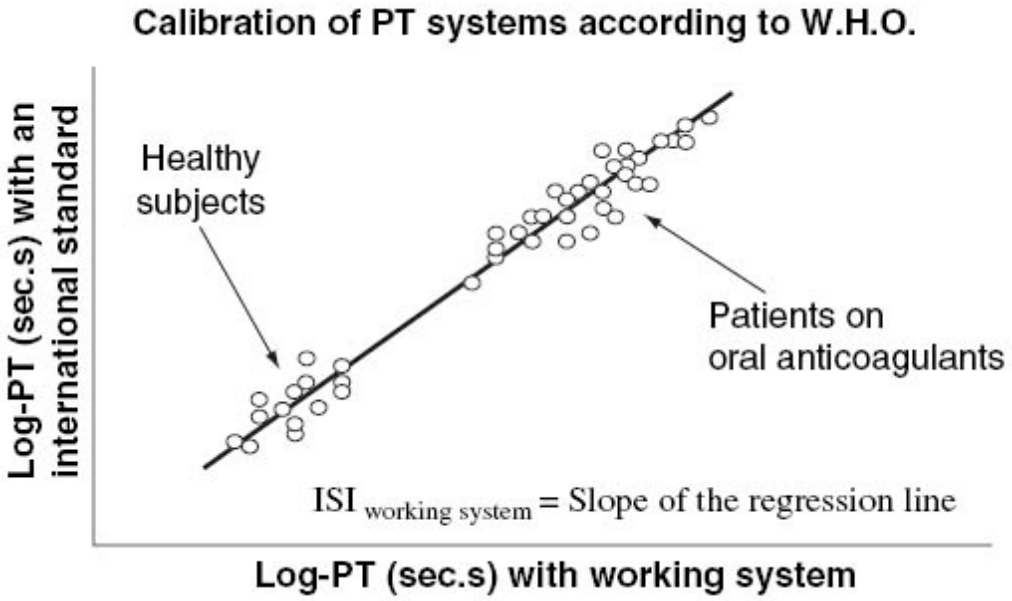

Figure 2.2. Calibration of Prothrombin Time (PT) measuring system according to the World Health Organization (WHO) model (WHO, 1983; Van den Besselar et al., 1999).

2.6. Warfarin

2.6.1. Definition

It is a white crystalline insoluble optically active compound, used as a rodenticide and in the form of its sodium salt as a medical anticoagulant. Warfarin is the drug of choice; few side effects, well tolerated. A vitamin K antagonist, it takes ~72h to be effective; stable state takes 5–7d. t1⁄2 ~35h. Circulates mainly bound to albumin; free Warfarin is active. Many drugs increase Warfarin effect by displacing it from albumin. Monitored by PT using INR (Provan et al., 2004).

2.6.2. Chemical structure

3-(α-phenyl-β-acetylethyl)-4-hydroxycoumarin; 1-(4′-hydroxy-3′-coumarin-3′-yl)-1-phenylbutan-3-one (Meister et al., 1984).

Warfarin chemical structure shown in figure 2.3 $C_{19}H_{16}O_4$.

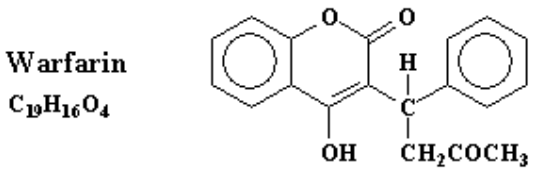

Figure 2.3. The chemical structure of Warfarin (Tomlin, 1994)

2.6.3. Warfarin is a vitamin K antagonist drug

Vitamin K antagonists (VKA) interfere with the function of the vitamin K-dependent factors, specifically factor II, factor VII, factor IX and factor X, as well as the natural occurring anticoagulants protein C and protein S. VKA inhibit the carboxylation of glutamate residues to γ-carboxyglutamate at the N-terminal of the vitamin K-dependent factors (Tripodi, 2009).

2.6.4. Warfarin treatment and clinical use

Warfarin is a well-accepted therapy used for the prevention of stroke (arrhythmia) in patients with atrial fibrillation, for prophylaxis of venous thromboembolism and pulmonary embolism in patients with prosthetic heart valves and myocardial infarction, and for prevention of pulmonary embolism or deep venous thrombosis in patients undergoing orthopedic surgery or with a history of venous or arterial thromboembolism (Baglin et al., 2006; Ziegler and Kher, 2006; Ansell et al, 2008; Flockhart et al., 2008; Husted and Singer et al., 2008).

The effectiveness and safety of Warfarin therapy is critically dependent on maintaining the prothrombin time expressed as INR, which is a ratio of the time required for the patient's blood to coagulate relative to a standardized coagulation time, within the desired therapeutic range (Sconce et al., 2005). Target INR usually 2.5 except for mechanical heart valves in mitral position when target is 3.0 or 3.5 (Provan et al., 2004).

2.6.5. Warfarin initiation

Initiation of Warfarin therapy in a qualifying patient has long been an iterative process, in which, initially a standard dose is prescribed and then adjusted based on observed response. Recognition and incorporation of the influence of patient-specific factors (e.g. age, weight, medications, etc) has facilitated improvements in estimating dose. However despite these refinements, stabilizing therapy may take weeks to months. Even after stabilization, INR is maintained in target range only 40–60% of the time (Chiquette et al., 1998; Wittkowsky, 2004; Wittkowsky and Devine, 2004; Ansell et al., 2007).

Therefore, during the remaining un-protected time periods, especially during initiation of therapy, patients may be at an increased risk of hemorrhagic or thromboembolic complications (Hylek and Singer, 1994; Hylek et al., 1996; Hylek et al., 2003).

After initial dosing of Warfarin, the observed anticoagulant effect is delayed. The antithrombotic effect of Warfarin is not present until approximately the fifth day of therapy, which is dependent on the clearance of prothrombin (Hirsh et al., 1998 and Horton and Bushwick 1999)**.**

The effect of anticoagulation is a function of the half-lives of the clotting factors, specifically factor II, which has the longest half-life (72 hours) (Hirsh et al., 1998). The initial factor affected by Warfarin's anticoagulant effect is factor VII, which has the shortest half-life of the vitamin K dependent clotting factors, six hours (Kovacs et al., 2003). The earliest changes in INR are typically seen 24 to 36 hours after administration of the dose (Hirsh et al., 1998; Horton and Bushwick, 1999). In clinical practice, loading doses (e.g., 7.5 mg or more per day) of Warfarin may increase the patient's risk of bleeding complications early in therapy by eliminating the production of functional factor VII (Horton and Bushwick, 1999).

The American College of Chest Physicians (ACCP) supports an "induction" dose (rather than a large loading dose) for initiation of therapy. This induction dose can range from 2 to 5 mg per day and is adjusted according to the patient's INR (Hirsh et al., 1998). In a recent study comparing the initiation of Warfarin with 5 milligrams versus 10 milligrams it was discovered that the use of 10 milligrams upon initiation lead to a decrease in the time to reach a goal international normalization ratio (INR) of two to three (Kovacs et al., 2003).

In a study for INR monitoring on days three and five after initiation, it was found that 90% of the patients had a therapeutic INR by the fifth day of therapy, which was 1.4 days sooner than 5-milligram Warfarin initiation. However, there was a trend towards an increase bleeding events among patients who received the 10-milligram initiation dose (Michael et al., 2003).

If a rapid anticoagulant effect is required until Warfarin's antithrombotic action takes effect, an initial dose of heparin or a low molecular-weight heparin should be initiated first immediate acting anticoagulant and Warfarin should be started within a day or two. Overlapping with Warfarin for approximately 4 to 5 days to permit Warfarin take anticoagulation action. Once the INR is in the desired range

for at least two consecutive days, the supplemental (heparin) anticoagulation treatment may be discontinued (Burns et al., 1993; Moll and Ortel, 1997; NCCLS, 1998; Hirsh et al., 1998).

2.6.6. Adverse effect of Warfarin initiation dose

Warfarin is a vitamin K antagonist inhibits the synthesis of vitamin K dependent clotting factors, as well as the naturally occurring endogenous anticoagulant proteins C and S (Horton and Bushwick, 1999).

The administration of a loading dose is a possible source of prolonged hospitalization secondary to dramatic rises in the INR value that may necessitate the administration of vitamin K (Lamb, 1997). To treat Warfarin overdose (bleeding), vitamin K or fresh frozen plasma can be administered (Hirsh et al., 1998). If the INR is >5 without bleeding, vitamin K administration can be considered. If large doses of vitamin K are administered, patients can become temporarily Warfarin resistant (Jacobs et al., 2001). It is important to use vitamin K only when recommended, because inappropriate administration of vitamin K is associated with Warfarin resistance. When such resistance develops, it is difficult to achieve a therapeutic INR in a timely manner, which may result in an increased risk of clotting events (Kuruvilla and Gurk-Turner, 2001).

2.6.7. Warfarin monitoring and dose adjustment

The daily maintenance dose of Warfarin differs greatly between individuals, commonly between 0.5 mg/day and 15 mg/day, and often fluctuates over time. The average maintenance dose is about 4.5 mg/day, although this is lower in the elderly. The drug is rapidly and completely absorbed and immediately blocks further hepatic synthesis of the functional vitamin K-dependent haemostasis factors (II, VII, IX, X, protein C, protein S) (Holbrook et al, 1996). However, its impact on the INR is delayed until preformed coagulation factors are removed from the systemic circulation once the drug is administered (Horton and Bushwick, 1999 and Micromedex, 2000) , so dose adjustment must allow for these delayed effects. The plasma half-life of Warfarin is about 36 hours (Holbrook et al., 1996).

During Warfarin initiation, the PT/INR is typically checked daily or at least 4-5 times per week until the dose and INR are therapeutic and stable (Fairweather et al., 1998). The interval between PT/INR tests can then be gradually decreased to as infrequently as every 4 weeks, depending on the stability of the dose and the PT/INR result (Fairweather et al., 1998 and Hirsh et al., 1998).

When dosing adjustments are required, they are generally in the range of 5% to 20% of the daily dose, or 5% to 20% of the total weekly dose (Gage, Fihn and white, 2000), also it was expected that a 15% dose adjustment to result in an approximately 1.0 INR change. Likewise, a 10% dose adjustment will result in an approximate 0.7-0.8 INR change (Maddali et al., 2013).

2.6.8. Warfarin Pharmacogenomics

Pharmacogenomics applies to our understanding of genetic variability in patients' responsiveness to a drug in order to inform clinical decisions about dosing and selection. The anticoagulant Warfarin is a case in point. Genetic variation in CYP2C9 and VKORC1, the two genes that encode the liver proteins required for Warfarin metabolism, explains up to 40% of the differences observed among patients in their responses to the same dose of Warfarin. The Food and Drug Administration has used this information to revise Warfarin labeling in order to allow for genotype-specific dose ranges (Wang et al., 2011).

CYP2C9 polymorphisms are particularly relevant to the metabolism of drug substrates with narrow therapeutic indices, where ADRs are common, such as to Warfarin. In the case of Warfarin, individualization of dose on the basis of drug response is a standard procedure, but a large number of studies have now found a consistent relationship between Warfarin dose requirement and the CYP2C9 genotype (Daly, 2009).

One study summarizes its results to confirm that CYP2C9 genotyping can identify individuals who are poor metabolizers of Warfarin, and hence need lower doses of the drug. Owing to Warfarin's narrow therapeutic range and severe side effects, it would be advantageous if individuals requiring extremely low doses of

Warfarin could be identified before initiating therapy (Iqbal et al., 2001). In general, studies suggest that the CYP2C9 genotype contributes between 10 and 20% of the variability in Warfarin dose requirement (Klein et al., 2009).

In a retrospective cohort study of patients on long-term Warfarin, it was found that the mean maintenance dose varied significantly among the six genotypes of CYP2C9. Compared to patients with the wild type genotype, patients with at least one variant allele required longer time to achieve stable dosing and had a significantly increased risk of a serious or life-threatening bleeding event (Higashi et al., 2002). In the past, it was customary to use a loading dose of 10 mg. However, for most situations, a reduced starting dose of 5 mg per day will achieve an INR of 2.0 in four to five days (Crowther et al., 1999).

2.6.9. Pharmacokinetics and pharmacodynamics of Warfarin

Warfarin consists of two racemic isomers -- an S-isomer and an R-isomer; the S-isomer is 3-5 times more potent than the R-isomer (Rettie et al., 1992). Both individualistic and genetic factors influence a given patient's response to Warfarin, in terms of individualistic factors, a patient's age, body weight or body surface area, diet, concurrent medications, and other factors are all known to affect dose requirements (Krynetskiy and Evans, 2004). In terms of genetic factors, there are both pharmacokinetic and pharmacodynamic effects on Warfarin treatment (See figure 2.4).

The main metabolizing enzymes for Warfarin are members of the cytochrome P450 family, with the S-isomer of Warfarin specifically metabolized by cytochrome P450, subfamily IIc, polypeptide 9 protein (CYP2C9). Genetic variations of CYP2C9 are responsible for the pharmacokinetic effect on Warfarin metabolism (Schwarz and Stein, 2006). The molecular target of Warfarin in vivo is the protein product of the Vitamin K Epoxide Reductase Complex, Subunit 1gene (VKORC1, hereafter) (Li et al. 2004 and Rost et al. 2004), which is inhibited by Warfarin. Genetic variations of VKORC1 are responsible for the pharmacodynamic affect on Warfarin (Wadelius et al. 2005).

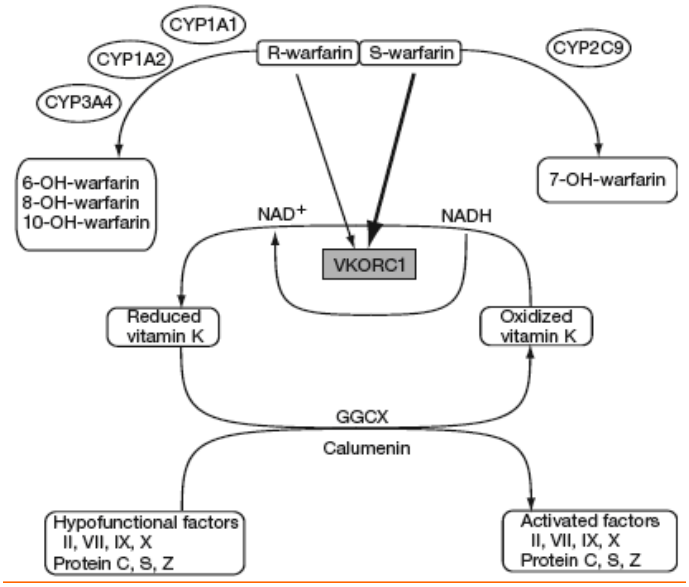

Figure 2.4. Warfarin pharmacokinetic and pharmacodynamics pathway. Warfarin is administered as a racemic admixture of R- and S-enantiomers. The more potent Senantiomer is metabolized principally by cytochrome P450 (CYP) 2C9. The pharmacologic effect of Warfarin is mediated by the inhibition of vitamin K epoxide reductase complex 1 (VKORC1). This results in the decreased concentrations of activated clotting factors (II, VII, IX and X) producing therapeutic anticoagulation. OH = hydroxy; NAD+ = oxidized form of nicotinamide adenine dinucleotide; NADH = reduced form of NAD; GGCX = γ-glutamyl carboxylase. (Nita and david, 2008).

2.6.9.1. Warfarin metabolism (Pharmacokinetics)

Warfarin is nearly 100% bioavailable when taken orally (Stirling et al., 1982). Both oral and intravenous formulations display similar pharmacokinetic characteristics (Breckenridge and Orme, 1973). The drug is rapidly and completely absorbed from the stomach and proximal small intestine with peak blood concentrations within 0.3 to 4 hr (Pyoralak et al., 1971). Food decreases the rate but not the extent of absorption (Musa and Lyons, 1976).

Warfarin is almost completely absorbed after oral administration and is bound extensively (95%) to plasma proteins. Since it is the unbound drug that produces the anticoagulant effect, displacement of albumin-bound Warfarin by other agents may result in bleeding. Although these drugs do not cross the blood-brain barrier, they can cross the placenta and may cause teratogenicity and hemorrhage in the fetus. Warfarin is inactivated by hepatic P450 isozymes; hydroxylated metabolites are excreted into the bile and then into the intestine. Hepatic disease may potentiate the anticoagulant response (Craig and Stitzel, 2004).

2.6.9.2. Mechanism of action of Warfarin (Pharmacodynamics)

Warfarin induces hypocoagulability only in vivo. It is a vitamin K antagonist. This drug thus causes hypocoagulability by inducing the formation of structurally incomplete clotting factors. Commercial Warfarin is a racemic mixture of S- and R-enantiomers; S-Warfarin is more potent than R-Warfarin (Craig and Stitzel, 2004). In the normal coagulation cascade, vitamin K–dependent clotting factors undergo α-glutamyl-carboxylation to become functional (Reynolds et al., 2007; Ansell et al., 2008).

Vitamin K is required to catalyze the conversion of the precursors of vitamin K–dependent clotting factors (Craig and Stitzel, 2004). The enzyme catalyzing this reaction, α glutamylcarboxylase, requires reduced form vitamin K hydroquinone (VitKH2) as a source of protons to complete carboxylation. For this reason, factors II, VII, IX, and X are referred to as the vitamin K– dependent coagulation factors. Vitamin K epoxide is a byproduct of α carboxylation (Reynolds et al., 2007; Ansell et al., 2008). This involves the post translational Gamma-carboxylation of glutamic acid residues at the N-terminal end of the proteins. The Gamma-carboxylation step is linked to a cycle of enzyme reactions involving the active hydroquinone form of vitamin K (K1H2). (Craig and Stitzel, 2004).

In the development of Vitamin K dependent clotting factors the oxidized, inactive form of vitamin K (vitamin K 2,3-epoxide) is converted to the reduced form of vitamin K (VitKH2) by an enzyme called vitamin K epoxide reductase (VKOR); Warfarin inhibits vitamin K epoxide reductase and vitamin K reductase therefore decreasing the conversion of oxidized vitamin K to reduced vitamin K (Figure 2.5). This decrease in functional reduced vitamin K leads to a decline in the production of vitamin K dependent clotting factors (Reynolds et al., 2007; Ansell et al., 2008). Consequently a decrease in production of vitamin K dependent clotting factors, which are all necessary for thrombus formation. The reduced form of vitamin K is necessary for the development of these clotting factors and is achieved in a two-step reduction process (Hirsh et al., 1998; Redman, 2001).

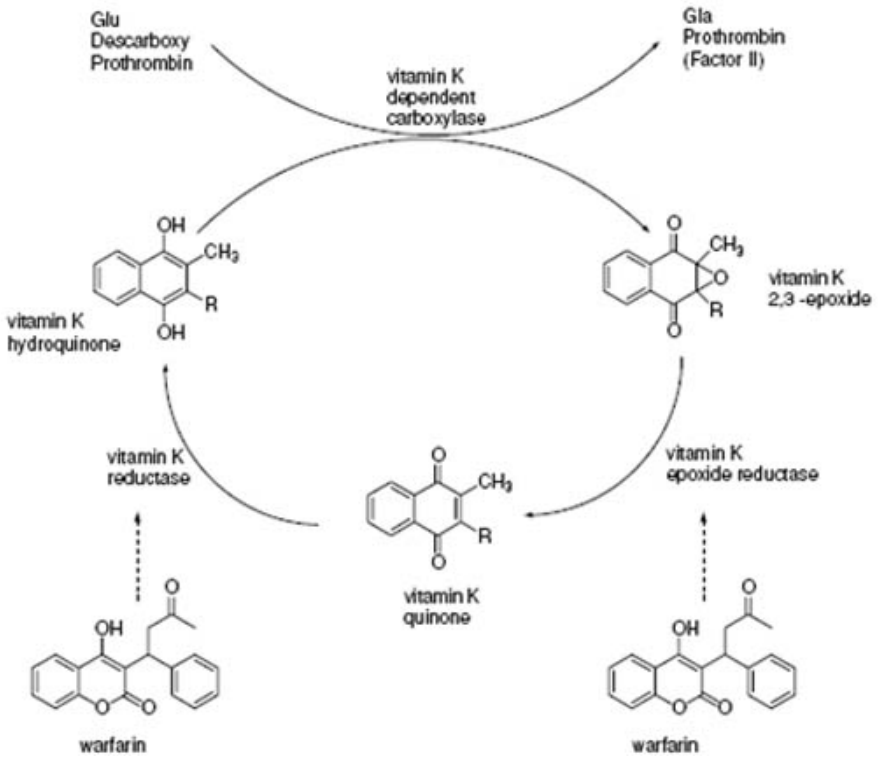

Figure 2.5.: Warfarin blocks the enzymes vitamin K epoxide reductase and vitamin reductase therefore reducing the reduced form of vitamin K available to form clotting factors (Michael et al., 2003).

2.6.10. Warfarin associated adverse events

Warfarin therapy-associated hemorrhage is one of the leading causes of drug-related adverse events, including death, in many Western countries (Levine et al., 1998 and Pirmohamed et al., 2004). Retrospective case studies have shown that the presence of mutant CYP2C9 allele (especially CYP2C9*3 allele) confers a significantly increased risk of bleeding following treatment with Warfarin. Available data, however, indicated that although the CYP2C9*3/ CYP2C9*3 genotype is associated with dramatic over anticoagulation soon after the introduction of oral anticoagulants, overdose during the maintenance period is mostly related to environmental factors (Verstuyft et al., 2003 and Peyyandi et al., 2004).

Previous studies have shown that about 5% of Warfarin-treated patients experience major bleeding (Taghavi et al., 1999). Of the 201 patients recruited from the Uppsala University Hospital anticoagulation clinic, 12 (6%) had at some time experienced a serious bleeding requiring hospital care. Significantly higher PT/INR values were found in connection with the bleeding (Aithal et al., 1999; Ogg et al.,

1999; Margaglione et al 2000; Higashi et al., 2002;). Patients who are given a loading dose of Warfarin often reach a supratherapeutic INR level that can place a patient at risk for bleeding and prolonged hospital stay. This complication has been attributed to excessive depression of factor VII and protein C (Lamb, 1997; Horton and Bushwick, 1999).

2.7. Vitamin K role in coagulation process

Vitamin K is essential for blood clotting but must be enzymatically activated. This enzymatically activated form of vitamin K is the reduced form required for the carboxylation (addition of carboxyl group) to the 10 – 12 of glutamic acid residues in the amino terminal portion in some vitamin K dependent blood-clotting proteins. Fatal bleeding can be caused by vitamin K deficiency and by the vitamin K antagonist Warfarin (RefSeq, 2008).

The four vitamin K-dependent procoagulants (factor II or prothrombin, and factors VII, IX, and X) are serine proteases that are synthesised in the liver and then secreted into the circulation as inactive forms (zymogens) which have a haemostatic role (i.e., they are procoagulants that arrest and prevent bleeding) (FAO/WHO, 2001). Their biologic activity depends on their normal complement of (Gla) gamma-carboxyglutamate residues, which are efficient chelators of calcium ions. In the presence of Gla and calcium ions these proteins bind to the surface membrane phospholipids of platelets and endothelial cells and assemble multimolecular coagulation complexes, where together with other cofactors, they form membrane-bound enzyme complexes. When coagulation is initiated, the zymogens of the four vitamin K dependent clotting factors are cleaved to yield the active protease clotting factors (Furie and Furie, 1990 and Davie, 1995). (Figure 2.6).

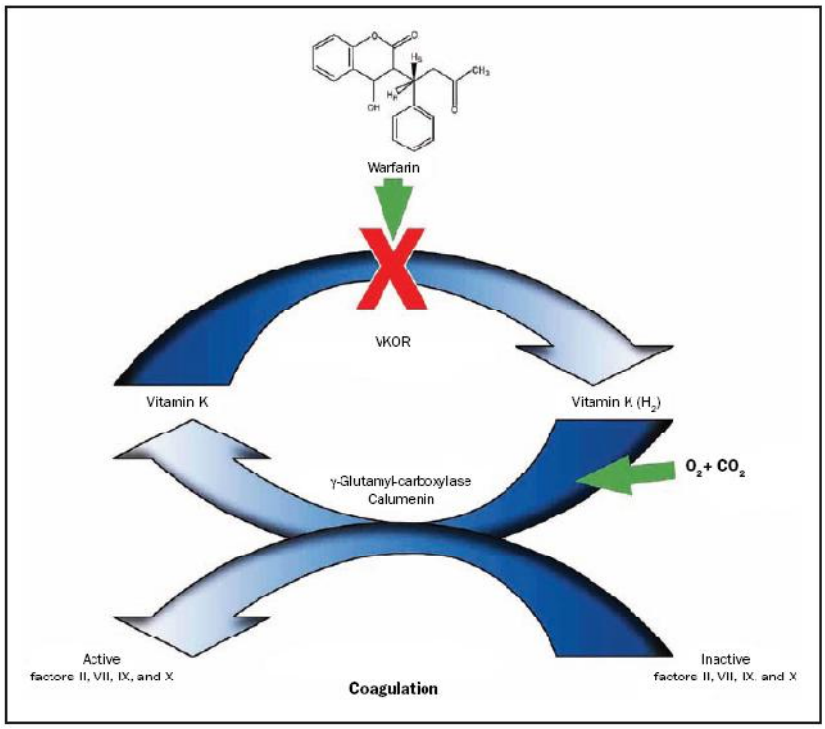

Figure 2.6.: The coagulation cascade requires vitamin K in the reduced form (vitamin K [H2]) as a cofactor for α-glutamyl-carboxylase to convert inactive factors II, VII, IX, and X to the active forms that are required for coagulation. Vitamin K (H2) is oxidized during this process to vitamin K epoxide. To conserve vitamin K (H2), the enzyme vitamin K epoxide reductase (VKOR) converts vitamin K epoxide back into vitamin K (H2). Warfarin inhibits VKOR, decreasing vitamin K (H2) availability, diminishing activatable factors II, VII, IX, and X and thus inhibiting coagulation. Co2 = carbon dioxide; O2 = oxygen. (Thomas et al., 2009)

Without this important post-translational modification (carboxylation process), the assembly of cell-based coagulation complexes is impaired, leading to ineffective fibrin formation. Warfarin, exerts its anticoagulant effect by inhibiting this modification and ultimately producing dysfunctional vitamin K-dependent factors, in turn affecting clot-based PT test in which Warfarin can be monitored.

Two other vitamin K-dependent proteins called protein C and protein S play a regulatory role in the inhibition of coagulation (i.e., they inhibit the clotting process). The function of protein C is to degrade phospholipid-bound activated factors V and VIII in the presence of calcium. Protein S acts as a synergistic cofactor to protein C by enhancing the binding of activated protein C to negatively charged phospholipids (FAO/WHO, 2001).

Briefly the biologic role of vitamin K is to act as a cofactor for a specific carboxylation reaction that transforms selective glutamate (Glu) residues to γ-

carboxyglutamate (Gla) residues (Furie and Furie, 1990). The reaction is catalysed by a microsomal enzyme, γ-glutamyl, or vitamin K – dependent carboxylase, which in turn is linked to a cyclic salvage pathway known as the vitamin K epoxide cycle (Figure 2.6) (FAO/WHO, 2001).

2.8. Wafarin and Vitamin K deficiency with the same effect

Warfarin and vitamin K deficiency share the same molecular basis for their effects. Warfarin is used as a therapeutic anticoagulant because it impairs the regeneration of active vitamin K, thereby decreasing the amount of active vitamin K. Vitamin K in its active form is a cofactor in a reaction which carboxylates glutamic acid residues to form gamma carboxyglutamic acid residues on vitamin K–dependent clotting factors. This carboxylation step is necessary for normal activity of these proteins. As a result, vitamin K deficiency or Warfarin therapy decreases the activity of these proteins and prolongs the PT. Patients with mild vitamin K deficiency or low levels of Warfarin anticoagulation can have a normal PTT (Jacobs et al., 2001).

2.9. Genetics

2.9.1. CYP2C9 gene

2.9.1.1. Definition

The CYP2C9 gene (cytochrome P450, family 2, subfamily C, polypeptide 9) (Tohru et al., 2009), located on the long (q) arm of chromosome 10 at position 24 (10q24), plays a key role in the metabolism of the S-isomer of Warfarin (Figure 2.7) . More precisely, the CYP2C9 gene is located from base pair 96,698,414 to base pair 96,749,147 on chromosome 10 (Genetics Home Reference, 2013a).

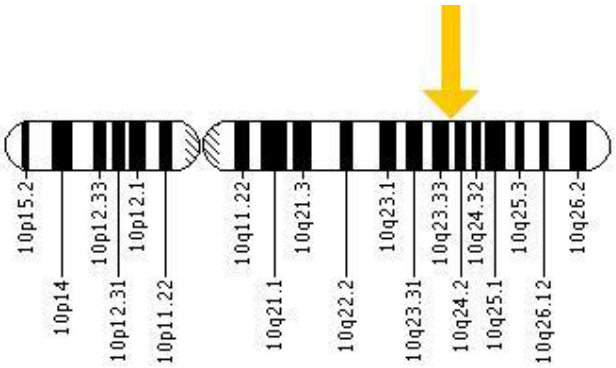

Figure 2.7: CYP2C9 on chromosome 10. (Genetics Home Reference, 2013a)

2.9.1.2. CYP2C9 Polymorphism

A number of cytochrome P450 genes are now known to be subject to functionally significant polymorphisms. In the case of one of these, CYP2C9, the enzyme metabolizes a range of drugs including Warfarin, tolbutamide and non-steroidal anti-inflammatory drugs (Daly, 2010).

Analysis of CYP2C9 cDNA sequences provided evidence for the presence of coding region polymorphisms resulting in amino acid substitutions, with expression studies suggested these were functionally significant (Rettie et al., 1994 and Sullivan-Close et al., 1996). The two most important variants of the CYP2C9 gene shown to have clinical implications for Warfarin dosing and prevention of adverse events are CYP2C9*2 (Chromosome 10, Exon 3, 430 C>T, rs1799853, Arg144Cys) in which a change of cytosine to thymidine at position 430 results in a protein with cysteine instead of arginine at residue 144 with reduced enzymatic activity and CYP2C9*3

(Chromosome 10, Exon 7, 1075 A>C, rs1057910, Ile359Leu) in which a change of adenine to cytosine at position 1075 (1075A>C, rs1057910), results in a protein with leucine instead of isoleucine at residue 359 with minimal enzymatic activity (Aithal et al., 1999 and Higashi et al., 2002; Moyer et al., 2009). Genotyping of patients undergoing treatment with Warfarin, another CYP2C9 substrate, confirmed the functional importance of the two most common coding region CYP2C9 polymorphisms (Furuya et al., 1995 and Aithal et al., 1999).

2.9.1.3 CYP2C9 gene in relation to Warfarin

Many allelic variants of CYP2C9 have been described in the literature, most of which encode dysfunctional or nonfunctional proteins. The two most common alleles of CYP2C9 that affect Warfarin metabolism are CYP2C9*2 (also known as R144C) and CYP2C9*3 (also known as I359L). Polymorphisms at these alleles produce defective CYP2C9 protein that exhibits reduced activity for metabolizing Warfarin. Individuals who are heterozygous or homozygous variant for either CYP2C9*2 or CYP2C9*3 are generally more sensitive to standard doses of Warfarin than similar individuals who lack the variant (Mark et al., 2005).

It is also recognised that inter-individual variability in Warfarin sensitivity also originates from environmental factors. In one study, age and CYP2C9 genotype accounted for 12% and 10% of the variation in Warfarin dose requirements, respectively (Loebstein et al., 2001)

2.9.2. VKORC1 gene

2.9.2.1. Definition

The VKORC1 gene is located on the short (p) arm of chromosome 16 at position 11.2 (16p11.2) (Figure 2.8). More precisely, the VKORC1 gene is located from base pair 31,102,174 to base pair 31,106,275 on chromosome 16. The official name of this gene is "vitamin K epoxide reductase complex, subunit 1" (Genetics Home Reference, 2013b) and encodes a protein of 163 amino acids with a mass of 18.2 kDa (Li et al., 2004).

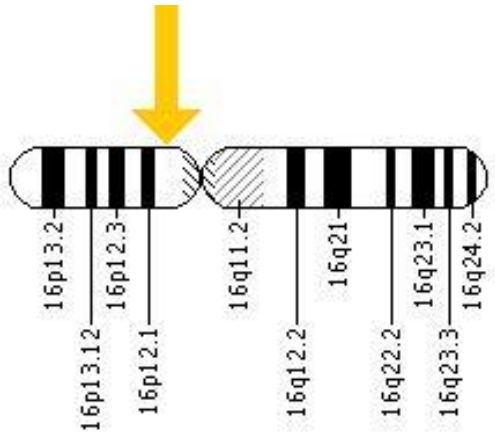

Figure 2.8: VKORC1 on chromosome 16. (Genetics Home Reference, 2013b)

The VKORC1 encodes the VKOR enzyme, a small transmembrane protein of the endoplasmic reticulum. Transcription of VKORC1 occurs primarily in the liver; however, smaller amounts of VKORC1 are present in the heart and pancreas (Moyer et al., 2009). The product of this gene encodes the enzyme that is responsible for reducing vitamin K 2,3-epoxide to the enzymatically activated form. In humans, mutations in this gene can be associated with deficiencies in vitamin-K-dependent clotting factors (RefSeq, 2008).

Warfarin exerts it pharmacologic effect through its ability to disrupt the γ-glutamyl carboxylation of vitamin K–dependent clotting factors. The γ-glutamyl carboxylase enzyme requires reduced vitamin K as a cofactor and Warfarin inhibits regeneration of reduced vitamin K by targeting the enzyme vitamin K epoxide reductase. The vitamin K epoxide reductase gene (VKORC1) was cloned in 2004 (Li

et al., 2004 and Rost et al., 2004). Several articles (D'Andrea et al., 2005; Rieder et al., 2005; Sconce et al., 2005; Wadelius et al., 2005; Yuan et al., 2005 and Lee et al., 2006) have studied the VKORC1 gene and found that there is a relationship between the VKORC1 gene and Warfarin dose requirement.

2.9.2.2. VKORC1 polymorphism

A polymorphism within the promoter region of VKORC1, specifically a guanine to adenine conversion at position –1639 (–1639 G>A, rs9923231), is associated with decreased Warfarin dosing in white people and people of Asian descent (Yuan et al., 2005). This may result from decreased production of VKORC1 mRNA by the –1639A allele and reduced expression of the enzyme VKOR (Wang et al., 2008).

As a result of the reduced expression of VKORC1, less Warfarin is needed in patients carrying the –1639A promoter variant to maintain the target INR. Population studies have shown that the VKORCI –1639A allele predominates in people of Asian descent but is less common in white people and those of African descent. The VKORCI –1639G allele associated with greater VKORC1 expression and activity predominates in those of African descent. Several studies have cast doubt that the –1639G allele is responsible for Warfarin dosing variability in people of African origin (Kealey et al., 2007; Kimmel et al., 2008). Other alleles in VKORC1 may affect Warfarin dosing, such as the mild to moderate increase in Warfarin dose associated with the Asp36Tyr amino acid substitution (Loebstein et al., 2007).

2.9.2.3. VKORC1 in relation to Warfarin

Vitamin K epoxide reductase subunit 1 (VKORC1) is the enzyme that recycles vitamin K 2,3-epoxide to reduced vitamin K required for the γ-carboxylation of vitamin K–dependent clotting factors. Warfarin inhibits VKORC1 activity by reducing the regeneration of vitamin K and thus exerting its anticoagulation effect (Bell and Matschiner, 1972; Wallin and Martin, 1985). GGCX is an enzyme which catalyses the γ-carboxylation of glutamic acid residues of clotting factors and proteins C, S and Z (Li et al., 2004 and Rost et al., 2004).

Subunit 1 of the vitamin K epoxide reductase complex (VKORC1), a component of vitamin K epoxide reductase (VKOR), is a chief molecular target of Warfarin (Li et al., 2004) VKOR reportedly is a multisubunit enzyme, but a single peptide, VKORC1, may be responsible for its reductase activity (Chu et al., 2006). This enzyme recycles vitamin K 2,3-epoxide to vitamin K hydroquinone, which is required by Gamma-glutamyl carboxylase (GGCX) for the posttranslational modification of blood coagulation factors II, VII, IX, X, and others. Recent findings have shown that polymorphisms in VKORC1 (Aquilante et al., 2006) (vitamin K epoxide reductase complex, subunit 1) have a large impact on Warfarin dose (Rieder et al., 2005; Wadelius et al., 2005; Veenstra et al., 2005; Sconce et al., 2005; Aquilante et al., 2006; Lee et al., 2006).

2.9.3. CYP2C9 and VKORC1 allelic variants effect on Warfarin function

Functional genetic variants in the VKORC1 gene have been found to affect the pharmacodynamics of Warfarin and influence its dosage requirements in patients. Rieder et al., (2005) have previously identified five haplotypes which are differentiated by five non-coding single nucleotide polymorphisms. These five haplotypes were found to segregate the patients into low- and high- dose groups and account for approximately 25% of the variability in Warfarin doses. In a more recent study in Asian population, the VKORC1 diplotypes were found to contribute to approximately 59.1% of the variability in Warfarin dose requirement. In multivariate analysis, age, weight and genetic polymorphisms presenting CYP2C9 and VKORC1 accounted for 74.2% of the Warfarin dose variability (Sandanaraj et al., 2009). Approximately 25% of the variations in dose requirements still remained unexplained (Saminathan et al., 2010).

Difference in successful outcomes during Warfarin therapy is a multi-factorial issue (Hylek, 2001; Absher et al., 2002; Couris et al., 2006). The individual's unique genetic make-up also plays a cardinal role in the Warfarin response. The first gene to be identified as affecting Warfarin dose-response was CYP2C9. This gene encodes a particular isoform of CYP450 enzyme, responsible for metabolizing S-Warfarin. Approximately 25%–35% of the population have CYP2C9 variants that lead to variably deficient enzyme activity. These variants can cause alterations in initial

Warfarin dose sensitivities, delays in achieving stable maintenance doses and increased bleeding complications (Linder et al., 2002; Scordo et al., 2002 and Sconce et al., 2005). Polymorphisms of CYP2C9 include CYP2C9*2 and CYP2C9*3, which are associated with reduced enzyme activity to 70% and 5% of the normal level respectively (Rettie et al., 1994; Linder et al., 2002; Scordo et al., 2002; Voora et al., 2005; Kamali and Pirmohamed, 2006). The result is Warfarin accumulation and possible hemorrhagic complications (Higashi et al., 2002). The variants have a significant impact on Warfarin metabolism in several populations (Linder et al., 2002; Scordo et al., 2002; Sconce et al., 2005; Wadelius et al., 2005; Reynolds et al., 2007; Zhu et al., 2007). The CYP2C9 status by itself accounts for approximately 15%–20% of the variance in Warfarin dose (Hillman et al., 2004; Voora et al., 2005; Wadelius et al., 2007).

Warfarin exerts an anticoagulant effect through its inhibition of the VKORC1 gene product (Suttie, 1987; Rost et al., 2004; Li et al., 2004). Patients who are carriers of a common polymorphism in the VKORC1 promoter sequence (− 1639 G>A) require a lower Warfarin maintenance dosage (Yuan et al., 2005 and Rieder et al., 2005). The − 1639 G>A genotype and related haplotype can independently determine 20%–25% of Warfarin dose variance (Rieder et al., 2005; Wadelius et al., 2007). Together, the CYP2C9 and VKORC1 combinatorial genotypes may explain up to 45% of Warfarin response variability (Hillman et al., 2004; Sconce et al., 2005; Rieder et al., 2005; Zhu et al., 2007). Combined polymorphisms in VKORC1 and CYP2C9 explain approximately 30% (20%-25% for VKORC1; 5%-10% for CYP2C9) of the variance in the stabilized Warfarin dose distribution (Rieder et al., 2005; Carlquist et al., 2006 and Caldwell et al., 2007) .

The importance of these strong genetic effects was recognized by recent relabeling of Warfarin by the FDA to raise awareness in the clinical community (Gage and Lesko, 2008). However, it is important to note that patient demographics, clinical factors, and genetic variants combined explain only 45% to 55% of the total dose variance (Rieder, 2007 and Crawford, Ritchie and Rieder, 2007). Both VKORC1 and CYP2C9 were identified to be important as a result of their functional relationship to Warfarin pharmacology. Other Warfarin candidate genes that are part of the vitamin K pathway, vitamin K–dependent clotting factors, or minor metabolic

or transport pathways have been systematically screened, with only VKORC1 and CYP2C9 reaching significance (Wadelius et al., 2007). Generally, dose associations with other candidate genes have shown small statistical effects or fail to replicate in independent populations (Aquilante et al., 2006; Caldwell et al., 2007; Rieder, et al., 2007). It remains unclear whether additional common genetic variation outside of these candidate genes contribute significantly to the 45% to 55% of unexplained variance in Warfarin dosing (Gregory et al., 2008).

2.10. Related studies

Higashi et al. (2002) conducted a retrospective cohort study at 2 anticoagulation clinics to study the association between CYP2C9 genetic variants and anticoagulation-related outcomes during Warfarin therapy and they found that, among 185 patients with analyzable data 58 (31.4%) had at least 1 variant CYP2C9 allele and 127 (68.6%) had the wild type CYP2C9 *1/*1 genotype. Mean maintenance dose varied significantly among the 6 genotype groups (*1/*1 n=127, *1/*2 n=28, *1/*3 n=18, *2/*2 n=4 , *2/*3 n=3, *3/*3 n=5). Compared with patients with the wild-type genotype, patients with at least 1 variants allele had an increased risk of above-range INR.the variant group also required more time to achieve stable dosing with a median difference 95 days. Patients with a variant genotype had a significantly increased risk of a serious or life threatening bleeding event.

Bozina et al. (2003) studied the genetic polymorphisms of cytochromes P450: CYP2C9, CYP2C19, and CYP2D6 in Croatian population to determine the most common mutations, CYP genotype was determined in 200 non-related croatian citizens. DNA isolated from blood samples was used for the analysis of the most common allelic variants of CYP2C9, CYP2C19, and CYP2D6 by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method and they found that for 200 subjects genotyped for CYP2C9, the allele frequencies of CYP2C9*1 (wt), CYP2C9*2, and CYP2C9*3 were 0.74, 0.165, and 0.095, respectively. Among them, 3.5% of subjects were predicted to be poor metabolizers.

Sukhyang et al. (2003) studied the relationship between requirement of Warfarin dose and polymorphism in CYP2C9 in Korean population. Ninty Patients on Warfarin therapy for longer than 1 year were included from July 1999 to December 2000 and categorized as one of four groups; regular dose non-bleeding, regular dose bleeding, low dose non-bleeding and low dose bleeding. Blood samples were processed for DNA extraction, genotyping and sequencing to detect polymorphism in CYP2C9. Demographic data, Warfarin dose per week, PT/INR, indications and co-morbid diseases were assessed for each group. Unlike the most of studies made on this topic this study concluded that the polymorphism in CYP2C9 is not a critical

factor for assessing Warfarin dose requirement and risk of bleeding complications in a Korean population.

Adcock et al. (2004) studied the effect of polymorphisms in the cytochrome P450 CYP2C9 gene on Warfarin anticoagulation to investigate the relationship between CYP2C9 genotype and Warfarin anticoagulation in a case of deep vein thrombosis treated with the standard Warfarin dose and investigated the intensity of anticoagulation and CYP2C9 genotype; the case illustrated the relationship between CYP2C9 variant and overanticoagulation with subsequent bleeding complication. They found that the patient's genotype, CYP2C9*1/*3, correlated with an exaggerated anticoagulant response during the initiation of Warfarin therapy at standard dose, and a bleeding episode ensued. Based on heterozygosity for the *3 variant allele, it was recommended that the patient be maintained on a low-dose Warfarin regimen.

Sconce et al. (2005) studied the impact of CYP2C9 and VKORC1 genetic polymorphism and patient characteristics upon Warfarin dose requirements and they investigated the contribution of age, CYP2C9 and VKORC1 genotype, and body size to Warfarin-dose requirements. The study included 297 patients with stable anticoagulation with a target INR of 2.0 to 3.0. Genetic analyses for CYP2C9 (*2 and *3 alleles) and VKORC1 (-1639 polymorphism) were performed and venous INR and plasma R- and S-Warfarin concentrations determined. The mean Warfarin daily dose requirement was highest in CYP2C9 homozygous wild-type patients, compared with those with the variant *2 and *3 alleles and highest in patients with the VKORC1 (position -1639) (G/G) genotype compared with those with the (G/A) genotype and the (A/A) genotype. Mean Warfarin daily dose requirements fell by 0.5 to 0.7 mg per decade between the ages of 20 to 90 years. Age, height, and CYP2C9 genotype significantly contributed to S-Warfarin and total Warfarin clearance, whereas only age and body size significantly contributed to R-Warfarin clearance. The multivariate regression model including the variables of age, CYP2C9 and VKORC1 genotype, and height produced the best model for estimating Warfarin dose.

Zhu et al. (2007) estimated the Warfarin Maintenance Dose Based on VKORC1 (G/A) and CYP2C9 Genotypes. A sample of Sixty-five patients with stable anticoagulation were genotyped for CYP2C9 and VKORC1. Plasma S-Warfarin

concentrations and Warfarin maintenance dose were compared among patients on the basis of the VKORC1 _1639 G>A genotype. They found that eighty percent of CYP2C9*1/*1 patients stabilized on <4.0 mg/day Warfarin had at least 1 VKORC1 _1639A allele. Mean Warfarin doses (SD) were 6.7 (*3/*3), 4.3 (*2/*2), and 2.7 (*1/*2) mg/day for patients with the VKORC1 (G/G), (G/A), and (A/A) genotypes, respectively. Steady-state plasma concentrations of S-Warfarin were lowest in patients with the VKORC1(A/A) genotype and demonstrated a positive association with the VKORC1 _1639G allele copy number (trend P=0.012). A model including VKORC1 and CYP2C9 genotypes, age, sex, and body weight accounted for 61% of the variance in Warfarin daily maintenance dose. They were concluded that the VKORC1 _1639A allele accounts for low dosage requirements of most patients without a CYP2C9 variant. Higher plasma S-Warfarin concentrations corresponding to increased Warfarin maintenance dosages support a hypothesis for increased expression of the VKORC1 _1639G allele. VKORC1 and CYP2C9 genotypes, age, sex, and body weight account for the majority of variance in Warfarin dose among study population.

Samardzija et al. (2008) studied the association of bleeding as a complication of Warfarin therapy with polymorphism of CYP2C9 gene (alleles 1, 2 and 3). The study included 181 patients receiving Warfarin for at least one month. Patients with allele *1 needed significantly higher maintenance Warfarin dose. Those with allele *3 had significantly lower maintenance Warfarin dose and higher prothrombin time (PT) at induction. Bleeding occurred significantly more often in those with lower maintenance Warfarin dose. Patients with allele *3 had increased risk of bleeding. Polymorphism of CYP2C9 could determine dose of Warfarin therapy and thus it could be related to the risk of bleeding complications. Allele *3 carriers need lower Warfarin dose. Therefore, initially reduced Warfarin induction dose in allele *3 carriers could avoid more prolonged PT and decrease the risk of bleeding complication.

Ghadam et al. (2009) studied the pharmacogenetic effects of CYP2C9 polymorphism on Warfarin sensitivity in some Iranian patients who are on Warfarin treatment. The study group consisted of clinically sensitive patients (21 patients) and the control group (37 adult patients). Restriction fragment length polymorphism based

polymerase chain reaction with Eco471 and Kpnl was used for detection of CYP2C9*2 and CYP2C9*3 variants. It is found that among 21 patients who were clinically assigned to be sensitive, 17 patients (81%) finally proved to carry some variant alleles (*1/*2 or *2/*2) and four patients (19%) had normal allele (wild type *1/*1). Among 37 patients with normal response to Warfarin, nine patients (24.3%) had variant alleles (*1/*2 or *2/*2) and 28 patients (75.7%1) had wild type allele (*1/*1). None of their study group or control group patients showed allele *3 variant.

Ngow et al. (2009) was conducted a study to explore the types and frequencies of CYP2C9 alleles in the healthy and Warfarin treated Malays and Chinese. A total of 565 Malay and Chinese subjects, including 191 patients prescribed Warfarin, were recruited into the study. CYP2C9*1,*2 and *3 were detected among the healthy unrelated individuals but only CYP2C9*1 and *3 were found in the diseased patients. Among the healthy Malays, 92.8 percent had CYP2C9*1/*1, 2.6 percent had CYP2C9*1/*2 and 4.6 percent had CYP2C9*1/*3 genotypes. Among the Chinese, 92.3 percent had CYP2C9*1/*1 and 7.7 percent had CYP2C9*1/*3, but CYP2C9*2 and *4 were not found in the Chinese. Among the Warfarin-treated group, only CYP2C9*1 and *3 were detected.

Della Valle et al. (2009) studied the VKORC1 variant genotypes and its relation to influence Warfarin response in patients undergoing total joint arthroplasty in a pilot study, To assess to what extent the VKORC1 variant genotype would alter the likelihood of being a hyperresponder or hyporesponder to Warfarin in patients undergoing total joint arthroplasty. They used INR on the third postoperative day of 3.0 or greater to define Warfarin hyperresponders and 1.07 or less to define hyporesponders. A control group of normal responders was identified. From a cohort of 1125 patients receiving Warfarin thromboprophylaxis, they identified 30 free of predisposing factors that could affect Warfarin response: 10 hyperresponders, eight hyporesponders, and 12 normal responders. Homozygous carriers of the VKORC1 mutant (A/A) genotype were more likely (compared with carriers of (G/A) or (G/G) genotypes) to be hyperresponders (odds ratio, 7.5; 95% confidence interval, 1.04-54.1). Homozygous carriers of the (G/G) (normal) genotype were more likely (compared with carriers of (A/A) or (G/A) genotypes) to be hyporesponders (odds ratio, 9; 95% confidence interval, 1.14-71). It was found that the preoperative

screening for the VKORC-1 genotype could identify patients with a greater potential for being a hyperresponder or hyporesponder to Warfarin and it may allow an adjusted pharmacogenetic-based Warfarin dose to optimize anticoagulation, reducing postoperative risks of bleeding and thrombosis or embolism.

Siddiqi et al. (2010) studied impact of CYP2C9 genetic polymorphism on Warfarin dose requirements in Pakistani population to estimate the frequency of genetic and allelic variants of CYP2C9 in Punjabi population of Pakistan and their effects on Warfarin dose requirement. One hundred and twenty unrelated Pakistani subjects belong to Punjab province, were randomly included from the registry of National Institute of Heart Disease Rawalpindi, Pakistan. The patients had stable INR of 2 to 3 for last 3 months with Warfarin therapy after heart valves replacement. The detection of CYP2C9 variant was done on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. A total of 120 patients (73 males; 47 females) of mean age of 37 years participated in the study. It was found that the genetic variations in the CYP2C9 occur in 14% of Punjabi ethnic group in Pakistan. Presence of CYP2C9*2 or *3 variants is an independent predictor of low Warfarin dose requirement in their patients. CYP2C9 variants assay may be used in high risk groups for appropriate dose adjustment to avoid complications on long term basis.

Wijaya et al. (2012) studied the accuracy of Warfarin dosage based on VKORC1 and CYP2C9 phenotypes in a Chinese population. In a sample of 37 patients they compared the Warfarin dosage obtained from genotype (according to www.Warfarindosing.org) and treatment dosage with INR value within 2.0-3.0. The majority of Chinese people in their study were VKORC1 homozygous (A/A) (89.2%), rarely VKORC1 heterozygous (G/A) and they cannot find a patient with homozygous (G/G). For CYP2C9 genotype, most patients have the wild type variants (CYP2C9*2 CC and CYP2C9*3 AA). The Warfarin dosage for patients with VKORC1(A/A) and CYP2C9*3 AC is lower than for patients with other genotype variants. They were concluded that there is no significant difference between pharmacogenetic algorithm (www.Warfarindosing.org) and their treatment dosage. Therefore, the pharmacogenetic algorithm is accurate to predict the Warfarin dose.

2.11. CYP2C9 and VKORC1 allelic frequency and genotype distribution among some populations (Table 2.1)

Table 2.1 demonstrates the allele frequency and genotype distribution for the genes CYP2C9 and VKORC1 among different populations, which clearly show that the allelic polymorphism is different between populations and ethnically dependent.

Table 2.1: Allelic frequencies and genotype distribution of CYP2C9 and VKORC1 among some populations.

Ethnic group	Alleles			Genotype						Ref.
	*1	*2	*3	*1/*1	*1/*2	*1/*3	*2/*2	*2/*3	*3/*3	
Pakistan	91.6	0.8	7.5	85.8	0	11.7	0.8	0	1.7	Siddiqi et al 2010
Iran	87.2	12.7	0	87.2	10.5	0	7.5	0	0	Zand et al 2007
India	93.7	4.1	2.2	88.3	7.2	3.6	0	0.9	0	Kaur et al 2013
China	97.64	0	2.36	95.28	0	4.72	0	0	0	Seng et al 2003
Malays	94.07	0	5.93	88.98	0	10.17	0	0	0.85	Seng et al 2003
Korea	98.9	0	1.1	97.7	0	2.3	0	0	0	Yoon et al 2001
Japan	97.6	0	2.3	95.0	0	4.0	0	0	1	Mushiroda et al 2006
Egypt	81.7	11.8	6.2	66.3	19	12	2.4	0	0.4	Hamdy et al 2002
UK	84.1	10.6	5.2	69.6	19	0.06	0.003	0.006	0	Sconce et al 2005
Oman	85.8	6.9	6.5	75.0	11.5	8.7	0	2.4	1	Pathare et al 2012

Ethnic group	Alleles		Genotype			Ref.
	-1639A	-1639G	G/G	G/A	A/A	
Oman	36.0	64.0	42.7	42.7	14.6	Pathare et al 2012
India	7.2	92.8	88.3	9	2.7	Kaur et al: 2013
UK	47.3	52.6	25	56	19	Mushiroda Et al 2006
Iran	55.6	44.4	15.9	57.1	27	Azarpira et al 2010
China	94.5	5.5	0	10.8	89.2	Wijaya et al 2012

Chapter 3

Materials and methods

3.1. Study design

Cross sectional study with convenience sample. The study population included all patients under Warfarin therapy attending the anticoagulation clinics of Europian Gaza Hospital and Al-shifa Hospital during the period (1-6-2012) to (1-8-2012).

3.2. Study population

A total of 101 patients recruited to anti-coagulation clinics in Al-shifa hospital and Gaza European hospital were under Warfarin therapy to monitor their Warfarin dosage and INR levels. The sample covered the five main Governorates of Gaza strip; North Gaza, Gaza city, Mid-Zone, Khan Younis and Rafah. The sample consisted of 42 males and 57 females with ages ranging from 16 years to 76 years with mean age of 48.8±14.4 years, while two patients have their personal data lost.

3.3. Ethical consideration

Approval of Helsinki committee was obtained (Appendix 1) to perform this study and collect blood samples from patients. Also the approval of MOH (Appendix 2) was obtained to collect sample from Al-Shifa hospital and Gaza-European hospital. Patients consents were obtained orally to undergo this study and collecting blood samples for analysis, after explaining the aim and objectives of this study.

3.4. Exclusion criteria

Patients who attended to anti-coagulation clinics and have Warfarin medication for less than one week were excluded and those who have still treated with heparin.

3.5. Materials

3.5.1. Instruments (Table 3.1)

Table 3.1 list of instruments used in detection of CYP2C9 and VKORC1 different alleles and INR determination for each patient.

Instrument	Manufacturer
TPersonal thermocycler.	Biometra - Germany
Minispin Microfuge.	Eppendorf - Germany
Power Supply Consort 200V 200mA 20W.	Anachem - UK
Vortex mixer.	Unico - Spain
DRY BLOCK THERMOSTAT TDB-100.	BOECO - Latvia
Water bath.	Gemmy - Taiwan
Centrifuge.	LW Scintific - USA

3.5.2. List of reagents and chemicals (Table 3.2)

Table 3.2 lists the chemicals and reagents used in this study:

Reagents and chemicals	Manufacturer
Primers	Metabion, Germany
Wizard Genomic DNA purification kit	Promega, USA
50 bp DNA ladder	New England Biolabs, England
GoTaq Green Master Mix	Promega, USA
Restriction enzymes AVAII, KpnI, Msp1	New England Biolabs, England
Agarose	Promega, USA
Ethedium promide	Promega, USA
Isopropanol	Sigma-Aldrich, China
Soleplastin Prothrombin or "Quick"test	Dutch Diagnostics, Netherlands
Control Plasma Normal and Pathological	Dutch Diagnostics, Netherlands
70% ethanol	Israel

3.5.3. Primers used in this study (Table 3.3)

Table 3.3 A list of primers and their sequences used in this study.

Primers		Reference
ID	**Sequence (5' to 3')**	
VK-F	GCCAGCAGGAGAGGGAAATA	(Sconce et al., 2005)
VK-R	AGTTTGGACTACAGGTGCCT	(Sconce et al., 2005)
CYP*2-F	GTATTTTGGCCTGAAACCCATA	(Nowak-Göttl et al., 2010)
CYP*2-R	GGCCTTGGTTTTTCTCAACTC	(Nowak-Göttl et al., 2010)
CYP*3-F	TGCACGAGGTCCAGAGGTAC	(Aomori, 2009)
CYP*3-R	ACAAACTTACCTTGGGAATGAGA	(Aomori, 2009)

All primers were reconstituted in normal saline (0.85% NaCl) and adjusted to 100 μM concentration.

3.5.4. Disposables (Table 3.4)

Table 3.4 List of disposables

Item	Manufacturer
Sterile 1.5ml microcentrifuge tubes	Promega – USA
Sterile thin-wall polypropylene PCR tubes with attached caps for labeling	Eppendorf – Germany
Micropipette tips (different sizes from 0.2μ to 1000μ)	Different manufacturers
2.5 K3 EDTA tubes	BD Vacutainer – USA
1.8 ml Sodium Citrate tubes 1:9 (3.2%)	BD Vacutainer – USA
Syringe 5 ml	Homed – China
23 Gauge needle	Homed – China

3.6. Methods

3.6.1. Clinical data collection and assessment

The clinical data were orally collected (weight, height, address, dose of Warfarin, duration under Warfarin treatment, change in Warfarin dose, Vitamin K containing diet, other anticoagulation drugs, cause of Warfarin medication, bleeding complications, other treatment or other chronic disease). The data was obtained by a written form (Appendix 3) provided for each patient after explaining the aim of the study and the agreement to share the study was obtained orally from the participants to perform the study and to draw blood samples.

Daily doses of Warfarin were obtained and the weekly doses were calculated for each patient. Bleeding complications were classified as minor bleeding (mild nose bleeds, bruise, microscopic hematuria, and mild gingival, conjunctival or anal bleeding) and major bleeding (gross hematuria, gastrointestinal bleeding requiring medical evaluation or blood transfusion).

The duration of Warfarin therapy was classified into three classes; less than one month treatment, over one month and less than a year and more than a year Warfarin therapy. Patients were assessed to vitamin K containing foods three days before blood sample collection.

Patients were classified into two groups according to the cause of Warfarin treatmemt; the first who have had a thromboembolic events and the second who have had a cardiovascular disease.

Bleeding complications during Warfarin treatment were classified as minor (once or recurrent) and major (once or recurrent) or combined who experienced minor and major bleeding during the Warfarin treatment.

The standard weekly dose was calculated for each patient as the predicted weekly dose using an algorithmic formula (Appendix 4) designed by The International Warfarin Pharmacogenetics Consortium (IWPC) (2009), depending on CYP2C9, VKORC1 genotypes and patient characteristics (Age, height, weight and

race) (Appendix 5). Also the maximum dose allowed to be used was calculated for each patient, as the maximum dose was calculated by adding 15% to the calculated standard dose for each patient dose and considered the upper limit value for weekly dose, we considered that the 15% of Warfarin dose is the highest elevation might added to the actual dose when the physician need to increase the INR value (Gage et al., 2000).

Body mass index (BMI) was calculated for each patient, it is defined as the weight in kilograms divided by the square of the height in metres (kg/m^2) (WHO, 2013) and patients were classified according to their BMI into four groups (WHO, 2013) as shown in table 3.5.

Table 3.5. The International Classification of adult underweight, overweight and obesity according to BMI (Natural Standard, 2012).

Classification	**BMI (kg/m^2)**
Underweight	<18.50
Normal range	18.5-24.99
Overweight	≥25
Obese	≥30

3.6.2. Blood sample collection

Whole blood samples were collected from 101 patients in EDTA tubes. Approximately 2.5 ml venous blood samples were collected in each tube from each patient, and 1.8 ml of blood was collected in citrated tubes (3.2 % sodium citrate) for coagulation testing PT/INR.

3.6.3. DNA extraction

DNA was extracted from blood samples using the Wizard Genomic DNA Extraction Kit from Promega, USA according to the manufacturer recommendations. Briefly, RBCs from 300 µl blood samples were lysed by 900 µl of cell lysis solution in 1.5 ml microcentrifuge tube then mixed by inversion and let for 10 minutes. Then WBCs were pelleted by centrifugation for 20 seconds at 14000 rpm and the

supernatant were discarded, Hereafter the WBCs were lysed using 300 µl of the nuclei lyses solution and mixed by inversion. Proteins were precipitated by adding 100 µl protein precipitation buffer and mixed by vortex for 20 seconds then centrifuged at 14000 rpm for 3 minutes. The supernatant containing the nucleic acid was transferred to a new tube containing 300 µl of isopropanol and the DNA was pelleted by vortex for 20 seconds then centrifuged at 14000 rpm for 1 minute. The supernatant was discarded and the pelleted DNA was washed by 300 µl of 70 % ethanol and centrifuged at 14000 rpm for 1 minute. The ethanol was aspirated and discarded and air-dried the pellet for 10-15 minutes. Finally the DNA pellet was resuspended in 50 µl of the provided alkaline rehydration solution and rehydrated at 65 ºC for 1 hour.

3.6.4. Detection of VKORC1_1639G>A, CYP2C9*2 AND CYP2C9*3 polymorphisms by PCR-RFLP

The specific nucleotide at the specific position was determined by for each gene. It involved fragmenting a sample of DNA by a restriction enzyme after amplification of a short segment of the region by specific primers for exon 3 or exon 7 of the CYP2C9 gene, and VKORC1 primers at the nucleotide 1639 of promoter region, all flanking the restriction sites. The resulting DNA fragments were separated by length through agarose gel electrophoresis. A RFLP occurred according to the length of a detected fragment variation between individuals. Each fragment length is considered an allele and used in genetic analysis (Table 3.6).

Table 3.6 Allelic variants of CYP2C9 and VKORC1 and product characteristics

	Allelic variant	**PCR product**	**Restriction enzyme**	**Location**
CYP2C9	CYP2C9*2	454 bp	AVAII	Exon 3
	CYP2C9*3	105 bp	KpnI	Exon 7
VKORC1	VKORC1-1639G	290 bp	MspI	Promoter region
	VKORC1-1639A			

(Seng et al., 2003)

3.6.4.1. Procedures of CYP2C9*2 and CYP2C9*3 detection by PCR-RFLP

PCR for the CYP2C9*2 polymorphism, was carried out in a 25 µl volume containing 2 µl of extracted DNA, 10 pmol of each primer CYP*2-F and CYP*2-R Table 3.7, 12.5 µl GoTaq Green master mix (Promega, USA) and 8.5 µl deionized water. The cycling profile consisted of an initial denaturation step at 94 °C for 5 min; followed with 35 cycles of 94 °C for 60 s, 52 °C for 45 s, and 72 °C for 60 s; and a 10-min final extension at 72 °C. The resulting 454-bp product was digested for 1 h with 10 U/µl AvaII at 37 °C in the provided buffer and analyzed by electrophoresis in a 2.5 % agarose gel and identified according to 50 pb DNA ladder. AvaII cut the PCR products containing the 430C allele into 397- and 57-bp fragments and did not cut PCR products containing the mutant 430T allele.

Table 3.7 Specific primer sequences for CYP2C9 and VKORC1

Primer	Detection of allelic variant	Amplicon size
VK-F VK-R	VKORC1-1639A	290
CYP*2-F CYP*2-R	CYP2C9*2	454
CYP*3-F CYP*3-R	CYP2C9*3	105

For the CYP2C9*3 polymorphism, the PCR conditions were the same as that for CYP2C9*2, but the primer sequences were CYP*3F and CYP*3R as shown in (Table 3.7). The resulting 105-bp product was digested for 1 h with 10 U/µl KpnI in the provided buffer at 37 °C and analyzed by electrophoresis on a 3.5% agarose gel and identified according to 50 pb DNA ladder. KpnI did not cut PCR products containing the 1075A allele but did cut PCR products containing the mutant 1075C allele into 85- and 20-bp fragments.

3.6.4.2. Procedure of VKORC1-1639A detection by PCR-RFLP

For the VKORC1-1639G>A polymorphism, PCR was carried out in a 20 µl volume containing 2 µl of extracted DNA, 5 pmol of each primer VK-F and VK-R (Table 3.7), 10 µl GoTaq Green master mix and 6 µl deionized water.

The cycling profile used for all reactions consisted of an initial denaturation step at 94 °C for 5 min; followed with 30 cycles of 94 °C for 60 s, 52 °C for 60 s, and 72 °C for 60 s; and a 5-min final extension at 72 °C. The resulting 290-bp product was digested for 16 h with Msp1 in the provided buffer at 37 °C and analyzed by electrophoresis in a 3.5 % agarose gel and identified according to 50 pb DNA ladder. Msp1 20 U/µl cuts PCR products containing the _1639G allele into 167-bp and 123-bp fragments and PCR products containing the mutant _1639A allele were not cut.

3.6.4.3. Interpretation of CYP2C9 and VKORC1 alleles

Each allele variant for the two genes is caused by a substitution of one nucleotide in turn a substitution of the amino acid, leading to different effect in action or quantity of this gene product according to type of allele. (Table 3.8).

Table 3.8 Interpretation of CYP2C9 and VKORC1 alleles

Gene	Allele	Nucleotide substitution	Amino acid substitution	Enzymatic phenotype
CYP2C9	*1	-	-	Normal
	*2	C_{430}>T	Arg144Cys	Deficiency
	*3	A_{1075}>C	Ile359Leu	~ Null
VKORC1	G	-	-	Normal
	A	_1639G>A	Reduced product	Deficiency

(LaSala et al., 2008)

3.6.4.4. Quality control

Negative control samples were applied in each run of PCR along with patients samples without addition of any DNA material but using a nuclease free water instead, then introduced to the gel electrophoresis to confirm the quality of testing procedure for PCR, and the purity of the reagents used, each negative control was visualized on gel electrophoresis for no band view.

For the restriction enzyme KpnI that used in CYP2C9*3 detection, to confirm the enzyme activity and its ability to cut genomic DNA in specific restriction site, a random sample of genomic DNA is used as positive controls, furthermore restriction analysis products were introduced into gel electrophoresis (0.7% agarose gel) to visualize smear shaped cutting product to confirm the ability of KpnI enzyme to be used in CYP2C9*3 detection.

3.6.5. Prothrombin time and INR measurement

Whole blood specimens were collected in 3.2% sodium citrate tubes for all patients and centrifuged at 1600 - 2000 x g for 10 minutes to obtain Platelet poor plasma. The thromboplastin reagent (Soleplastin, Dutch diagnostics - Netherland) (ISI=1.03) was Prewarmed to 37°C for at least 10 minutes, also 100µl of the patients' plasma and controls were prewarmed for 2-3 minutes at 37°C, then 200µl of working thromboplastin reagent were added to the patients' plasma and controls then the time required to form the clot was observed. The PT was reported in seconds and the INR was calculated using the following formula.

$INR=(PT\ patient/PT\ normal)^{ISI}$

PT patient = patient's measure PT (seconds).

PT normal = laboratory's geometric mean value for normal patients (seconds).

ISI = International Sensitivity Index for the thromboplastin reagent.

The mean value of PT for normal patients was 15.0 second.

Patients were classified into three groups according the INR results, the first is that who their INR was below the lower therapeutic limit (INR < 2.0) and called "Coagulation tendency" group, the second whom the INR is within therapeutic range (

INR= 2.0 – 3.0) and called "therapeutic range" and the last one whom the INR exceeds the upper therapeutic limit (INR > 3.0).

All patients were considered to have the therapeutic range of INR (2.0 – 3.0) (Holbrook et al., 1996, Ansell et al., 2004; Roche 2006) except for three patients who have had mechanical heart valve replacement and need their INR to be (2.5 – 3.5) (Provan et al., 2004), as clinically recommended. Anyway those three patients had their INR out of the needed range and thus they were included in all following analysis like all patients.

3.7. Data analysis

Collected data were reviewed for completeness and accuracy, coded, computed and analyzed using Statistical Package for Social Science (SPSS) program version 17, Epi info TM 7 and appropriate statistical procedures. Data processing also included recording and scoring of some variables. To insure the accuracy and completeness of all questions, frequency distribution and cross tabulation was done for the entire data.

Descriptive statistics were used for summarization of data utilizing frequency distribution tables and graphs. For quantitative variables, mean and standard deviation were calculated.

- T-test was used for comparing the means between the different groups of cases.
- Chi-square (X2) test was used for analysis of categorical data.
- Person's and Sperman Brown correlations were used to test the correlation between two quantitative variable.

Chapter 4

RESULTS

4.1. PCR and RFLP results for CYP2C9 and VKORC1

4.1.1. CYP2C9*2 PCR product and restriction analysis gel electrophoresis

After running on 2.5% agarose gel the PCR product of CYP2C9*2 was identified as 454 bp DNA fragment using 50 bp DNA ladder along with negative control as shown in figure 4.1.

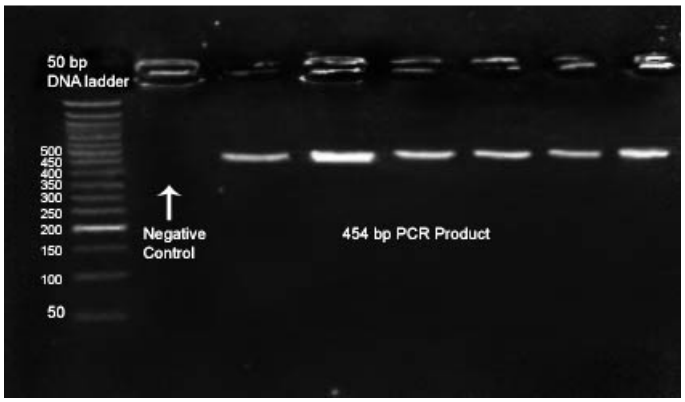

Figure 4.1: CYP2C9*2 PCR product (454 pb) on gel electrophoresis with negative control

PCR product for CYP2C9*2 was introduced to restriction analysis with the restriction enzyme AvaII. It cut the wild type allele containing 430C (CYP2C9*1) into 397- and 57-bp fragments and did not cut PCR products containing the mutant 430T allele (CYP2C9*2), when running on agarose gel CYP2C9*1 shows two bands while CYP2C9*2 shows one band (Figure 4.2).

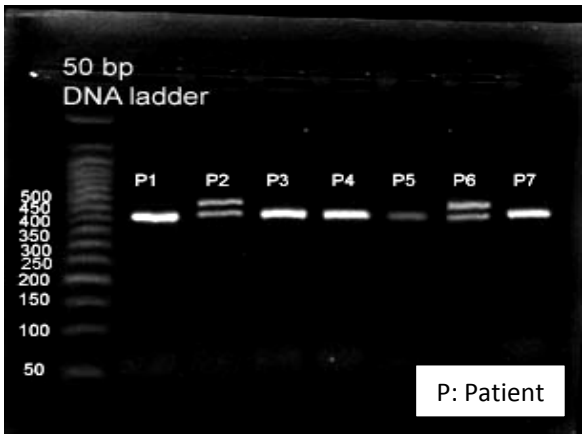

Figure 4.2: Detection of CYP2C9*2 by RFLP with digestion using AVAII restriction enzyme.

4.1.2. CYP2C9*3 PCR product and restriction analysis gel electrophoresis

After running on 2.5% agarose gel the PCR product of CYP2C9*3 was identified as 105 bp DNA fragment using 50 bp DNA ladder along with negative control as shown in figure 4.3.

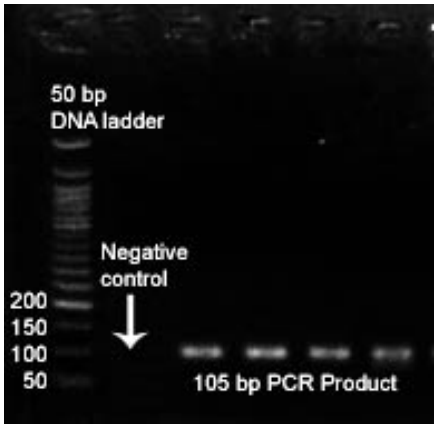

Figure 4.3: CYP2C9*3 PCR product (105 pb) on gel electrophoresis with negative control

PCR product for CYP2C9*3 was introduced to restriction analysis with the restriction enzyme KpnI. It did not cut the wild type allele containing 1075A (CYP2C9*1) and cut the PCR products containing the mutant 1075C allele (CYP2C9*3). When running on agarose gel 3.5% CYP2C9*1 shows one band of 105 bp length, while CYP2C9*3 would show two bands of 85 bp and 20 bp (Figure 4.4) and the CYP2C9*3 allele was not detected in any of sample study subjects.

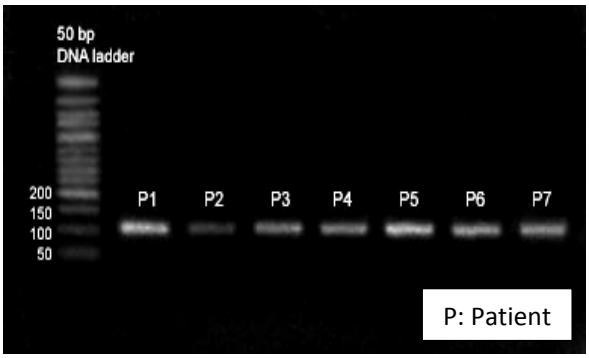

Figure 4.4: Detection of CYP2C9*3 by RFLP with digestion using KpnI restriction enzyme.

4.1.3. VKORC1 PCR product and restriction analysis gel electrophoresis

After running on 2.5% agarose gel the PCR product of VKORC1 was identified as 290 bp DNA fragment using 50 bp DNA ladder along with negative control as shown in figure 4.5.

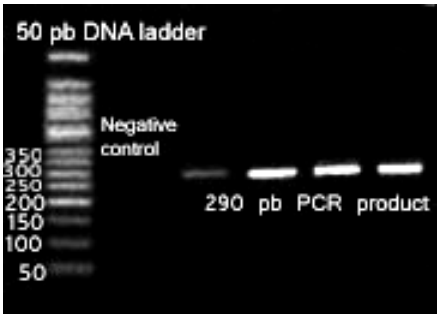

Figure 4.5: VKORC1 PCR product (290 pb) on gel electrophoresis with negative control.

PCR product for VKORC1 was introduced to restriction analysis with the restriction enzyme MspI. It cut the wild type allele containing _1639G allele (VKORC1-1639G) into 167 and 123 bp fragments and did not cut PCR products containing the mutant _1639A allele (VLORC1-1639A). When running on agarose gel VKORC1-1639G shows two bands while VKORC1-1639A shows one band (Figure 4.6).

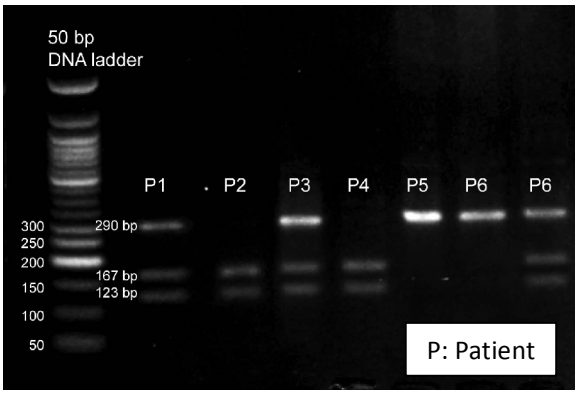

Figure 4.6: Detection of VKORC1-1639A by RFLP with digestion using MspI restriction enzyme.

4.1.4. Positive control for KpnI result on gel electrophoresis

It was needed to confirm the activity of KpnI restriction enzyme to cut the DNA PCR product in detection of CYP2C9*3, because all sample subjects show no cut after enzymatic digestion, though a random genomic DNA sample used as positive control to assess KpnI ability for digestion, as shown in figure 4.7 a smear shaped DNA fragments was visualized on 0.7% agarose gel to confirm the intact activity of KpnI restriction enzyme.

Figure 4.7: KpnI restriction analysis for genomic DNA as a positive control showing smear shaped bands.

4.2. Characteristics of the study population

The study included 101 patients, among which, 2 patients had missing data in some variables. So that the statistical analysis was done for 101 patients according to availability of data and for 99 patients for variables with missed data.

The percentage of males was 42.4% among patients while females were 57.6 %. The average age of patients was 48.9±14.4 years, where that of males was 50.0±13.8 years and that of females was 48.0±15.0 years. More than two thirds of patients were from Gaza Governorate, while 16.2% of them were from Mid-Zone Governorate, also 8.1% were from North Gaza Governorate, and about 9.1% from south Gaza Governorates (Khan-Younis & Rafah) (Table 4.1).

Table 4.1 Summary table for characteristics of patients

Characteristics	**Patients No.**	**Value**
Gender		
Male	42	42.4%
Female	57	57.6%
Age (Years)	99	48.9
Governorate of residence		
North Gaza	8	8.1%
Gaza City	66	66.7%
Mid-Zone	16	16.2%
Khan-Younis	5	5.1%
Rafah	4	4.0%
Type of Disease		
DVT*	50	50.5
Cardiovascular	41	41.4
Combined	8	8.1
Body Mass Index	99	27.8
BMI Classes		
Obese	34	33.7%
Overweight	30	29.7%
Normal weight	33	32.7%
Underweight	4	4.0%
Average of INR**	99	2.4

* DVT: Deep Venous Thrombosis.
** INR: International Normalized Ratio.

`4.2.1. Classification of patients according to BMI

Our sample study was classified according to the classification of WHO (2013) and it was found that about one third of cases were obese, the other one third were overweight, and the last one third were normal weight. Only 4.0% were underweight (Figure 4.8). The average of body mass index of patients was 27.8±5.2 kg/m^2 where that of males was 27.2±5.0 kg/m^2 and that of females was 28.2±5.3 kg/m^2.

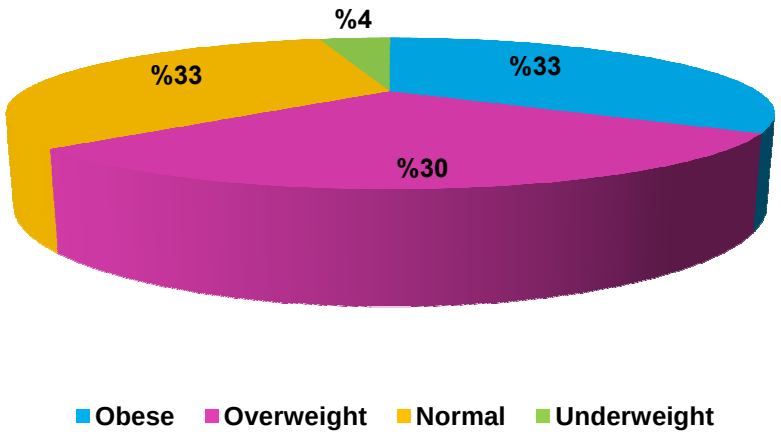

Figure 4.8 Distribution of patients by body mass index.

4.2.2. Classification of patients according to type of disease

About half of patients were complaining from deep venous thrombosis (DVT), while 41.4% of them were complaining from cardiovascular diseases, and 8.1% of them were complaining from both DVT and cardiac diseases (Figure 4.9). It is important to mention that 51.5% of patients were complaining from other chronic diseases as well. The average of INR of patients was 2.4±1.5 (Table 4.1).

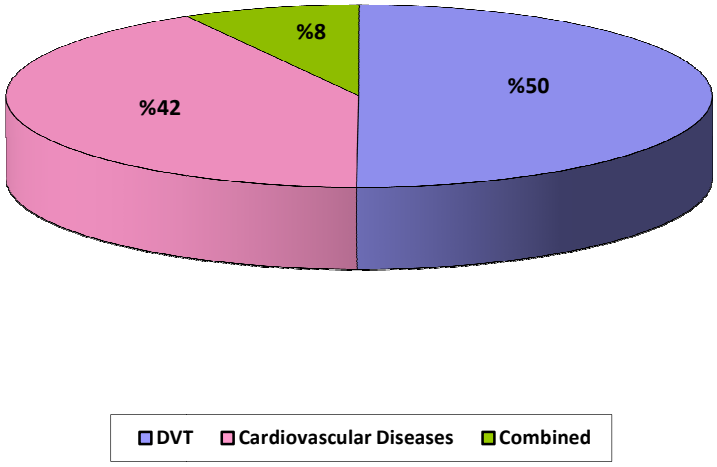

Figure 4.9 Distribution of patients by type of disease.

4.2.3. Classification of patients according to complications

There were 45.5% of patients suffering from Warfarin associated adverse effect and 54.5% were not (Figure 4.10).

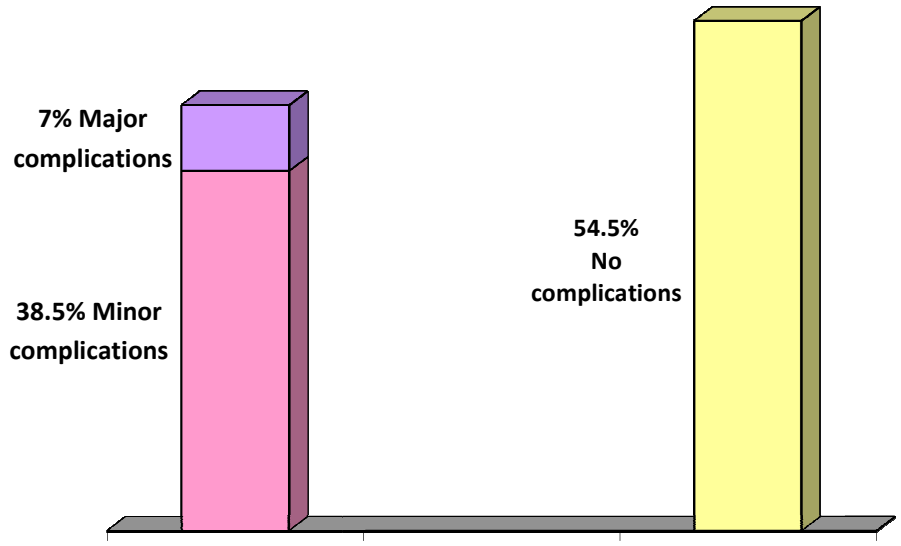

Figure 4.10 Distribution of patients by complications of Warfarin therapy.

4.3. CYP2C9 genotype distribution and allelic frequency of CYP2C9*1, CYP2C9*2 and CYP2C9*3 among patients in Gaza Strip

4.3.1. Genotype distribution of CYP2C9

The results of the study demonstrate that 74.2% of study population have two copies of the wild type allele CYP2C9*1/*1 (Figure 4.11). The remaining patients were either homozygotes for CYP2C9*2 allele 1% or heterozygotes 24.8%. None of patients had the CYP2C9*3 allele.

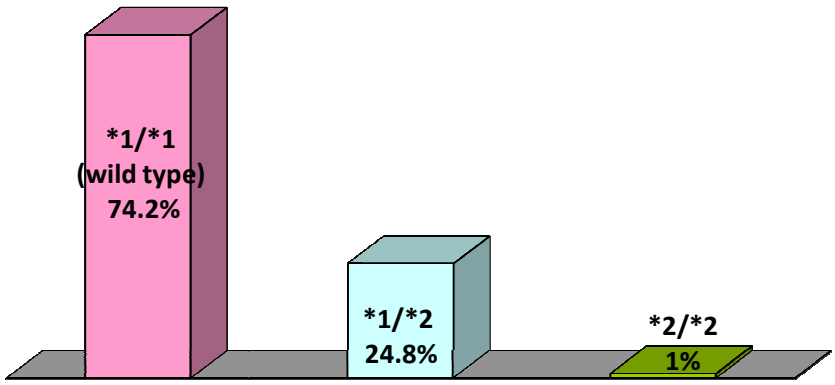

Figure 4.11 Distribution of patients by genotype of CYP2C9.

4.3.2. Over all allelic frequency of CYP2C9*1 and CYP2C9*2 alleles

It is important to mention that for 101 subjects the overall allelic frequency of CYP2C9*1 allele was 86.6%, while that of CYP2C9*2 allele was 13.4% , and none of patient presented with CYP2C9*3 allele (Figure 4.12).

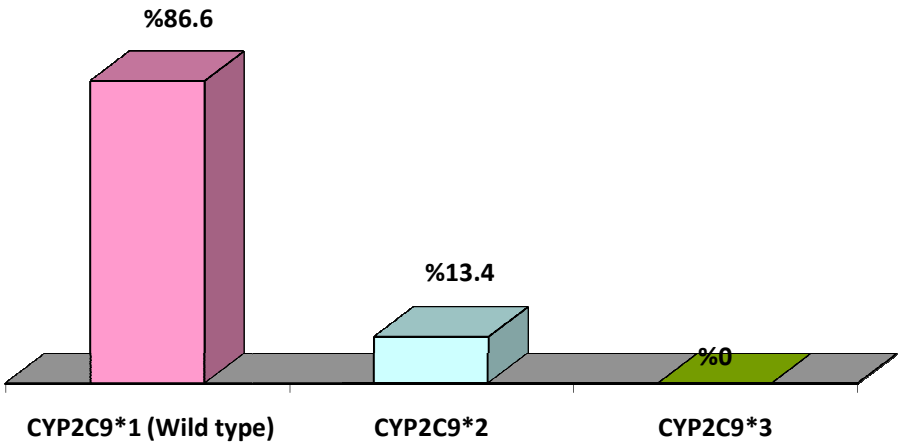

Figure 4.12 Distribution of patients by allelic frequency of CYP2C9.

4.3.3. Distribution of CYP2C9 genotypes and allelic frequency by gender

Table 4.2 showes that there is no significant difference in the prevalence of heterozygous CYP2C9 (*1/*2) according to gender (X2= 0.00, P-value= 0.99%), as about a quarter of males 26.2% and females 24.6% have heterozygous CYP2C9 (1*/2*), and 1.8% of females and none of the males have homozygous CYP2C9 (*2/*2). Allelic frequency of CYP2C9*1 allele among males was 86.9% and CYP2C9*2 allele was 13.1%, while the allelic frequency of CYP2C9*1 among females was 86.0% and CYP2C9*2 was 14.0%.

Table 4.2 Summary table for distribution of patients (n=99) by CYP2C9 genotype and gender.

Gender	CYP2C9					
	*1/*1		*1/*2		*2/*2	
	No.	%	No.	%	No.	%
Male	31	73.8	11	26.2	0	0
Female	42	73.7	14	24.6	1	1.8

*Inspite of known genotypes for 101 subjects, there were two subjects have their data were missed and the gender is unspecified, so the distribution of CYP2C9 according to gender was determined for 99 subjects of known gender.

4.3.4. Distribution of CYP2C9 genotypes among governorates

Figure 4.13 illustrates that heterozygous CYP2C9 (*1/*2) was distributed among Gaza governorates' patients as the following (3 cases in Khanyounis, 2 cases in Rafah, 2 cases in North Gaza, 15 cases in Gaza city, and 3 cases in Mid-Zone), while the only presented case of homozygous CYP2C9 (*2/*2) was in Gaza city.

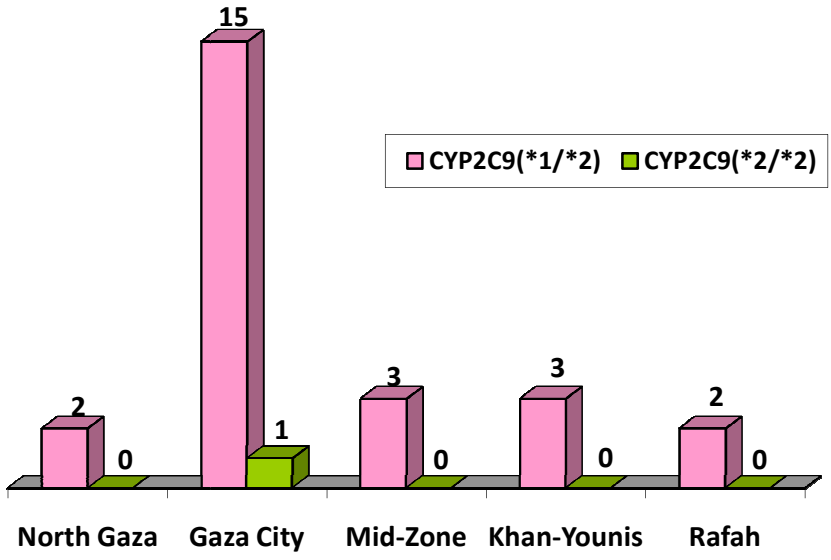

Figure 4.13 Distribution of patients by CYP2C9 genotype and governorates.

4.3.5. Ditribution of CYP2C9 genotypes according to the type of disease

Figure 4.14 reveals that 30% of all DVT patients have CYP2C9*2 allele, where 28% of them have heterozygous CYP2C9 (*1/*2) and 2% of them have homozygous CYP2C9 (*2/*2). The rest 70% of DVT patients have the homozygous wild type CYP2C9 (*1/*1), while about quarter of cardiovascular patients have heterozygous CYP2C9 (*1/*2) and 12.5% of patients with combined DVT and cardiovascular disease have that heterozygous CYP2C9 (*1/*2) (X2= 1.98, P-value= 0.74%).

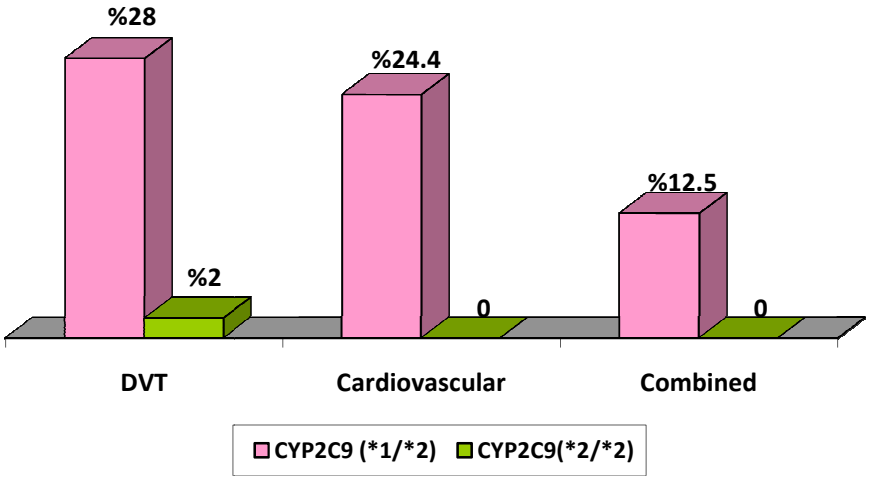

Figure 4.14 Distribution of patients by CYP2C9 genotype and type of disease.

4.4. VKORC1 genotype distribution and allelic frequency of VKORC1-1639G and VKORC1-1639A among Patients in Gaza Strip.

4.4.1. Genotype distribution of VKORC1

The results from this study of 101 subjects demonstrate that 70.3% of the study population were carries of at least one copy of the polymorphic allele VKORC1-1639A (48.5% VKORC1 (G/A) and 21.8% VKORC1 (A/A)), while 29.7% of the study population have two copies of the wild type allele VKORC1-1639G (Figure 4.15). Among patients who carry the VKORC1-1639A allele there were 69.0% heterozygous VKORC1 (G/A), while 31.0% were homozygous VKORC1 (A/A).

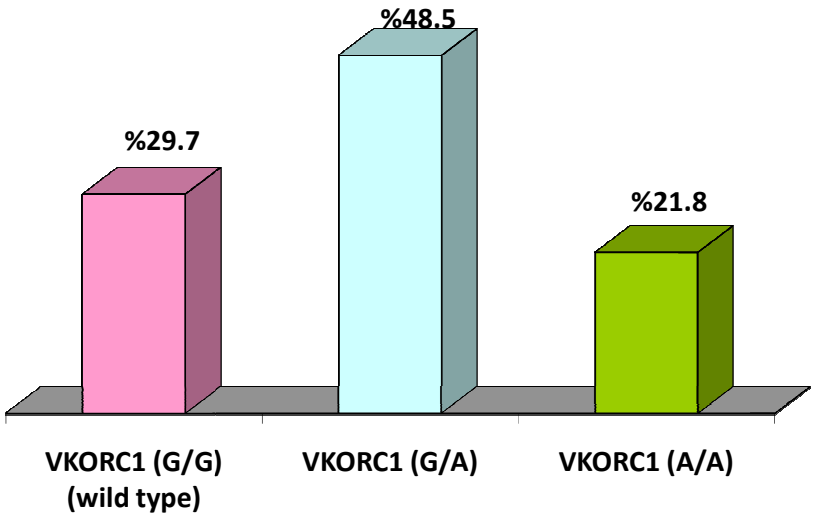

Figure 4.15 Distribution of patients by genotype of VKORC1.

4.4.2. Genotype distribution and allelic frequency of VKORC1-1639G and VKORC1-1639A

It is important to mention that the over all allelic frequency of VKORC1-1639G allele was 54.0%, and that of VKORC1-1639A allele was 46.0% (Figure 4.16). Table 4.3 shows that 69.0% of males have VKORC1-1639A allele (40.5% heterozygous VKORC1 (G/A) and 28.6% homozygous VKORC1 (A/A)) with allelic frequency of VKORC1-1639A allele among males was 48.8%, while 71.9% of females have VKORC1-1639A allele (56.1% heterozygous VKORC1 (G/A) and 15.8% Homozygous VKORC1 (A/A)) with allelic frequency of VKORC1-1639A allele among females was 43.9%.

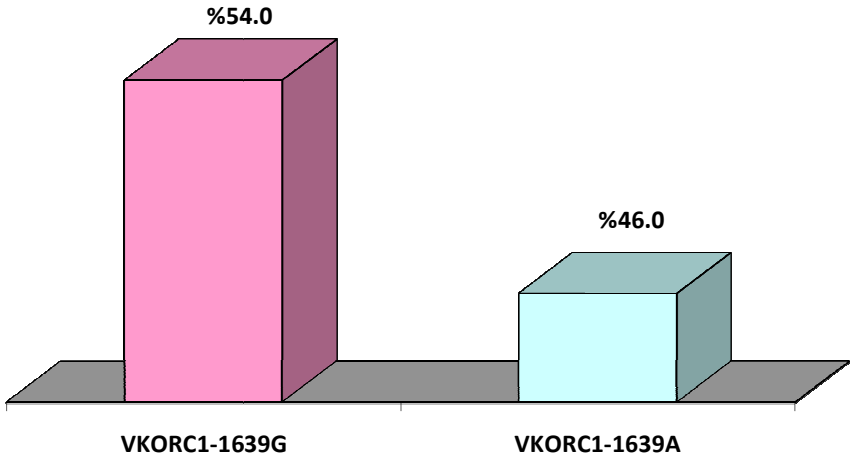

Figure 4.16 Distribution of patients by allelic frequency of VKORC1.

Table 4.3 Distribution of patients (n=99) by VKORC1 genotype and gender.

Gender	VKORC1					
	G/G		G/A		A/A	
	No.	%	No.	%	No.	%
Male	13	31	17	40.5	12	28.6
Female	16	28.1	32	56.1	9	15.8

*Inspite of known genotypes for 101 subjects, there were two subjects have their data were missed and the gender is unspecified, so the distribution of CYP2C9 according to gender was determined for 99 subjects of known gender.

4.4.3. Distribution of VKORC1 genotypes among governorates

Figure 4.17 illustrates that heterozygous VKORC1(G/A) genotype was presented more among south Gaza governorates' patients compared to other governorates' patinets (80% of Khanyounis, 75% of Rafah, 50% of North Gaza, 45.5% of Gaza City, and 50% of Mid-Zone). None of patients from south governorates have homozygous VKORC1 (A/A) genotype, while about the fifth of Gaza City patients have VKORC1(A/A) genotype compared to about one quarter of North Gaza patients and one third of Mid-Zone patients have the homozygous VKORC1 (A/A) (X2=8.18, P-value=0.42).

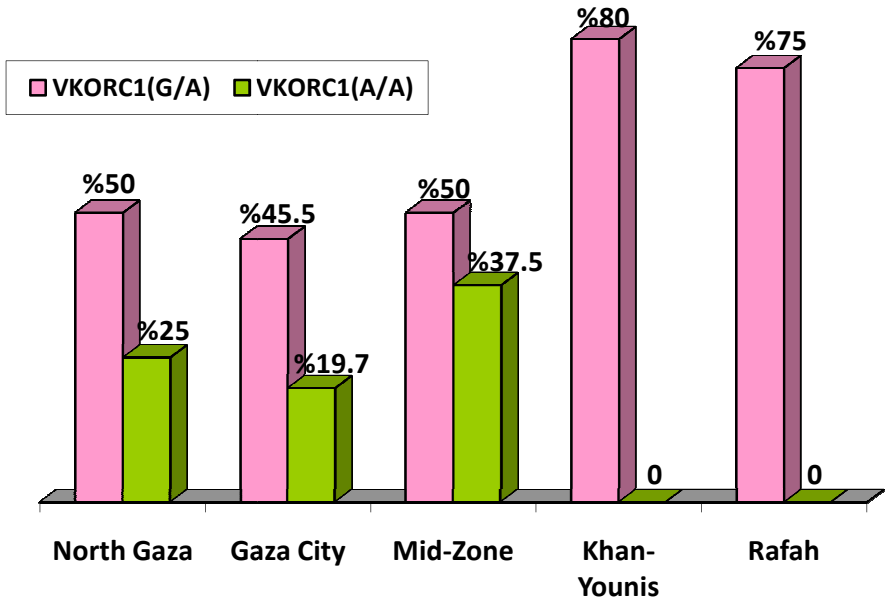

Figure 4.17 Distribution of patients by VKORC1 polymorphism and governorates.

4.4.4. Ditribution of VKORC1 genotypes according to the type of disease

Figure 4.18 reveales that 72% of DVT patients have VKORC1-1639A, where 54% of them were heterozygous VKORC1(G/A) and 18% of them were homozygous VKORC1(A/A), while 68.3% of cardiovascular patients have VKORC1-1639A (41.5% heterozygous VKORC1(G/A) and 26.8% homozygous VKORC1(A/A)) and 75.0% of patients with combined DVT and cardiovascular disease have VKORC1-1639A (62.5 % heterozygous VKORC1(G/A) and 12.5% homozygous VKORC1(A/A)) (X^2= 2.31, P-value= 0.68).

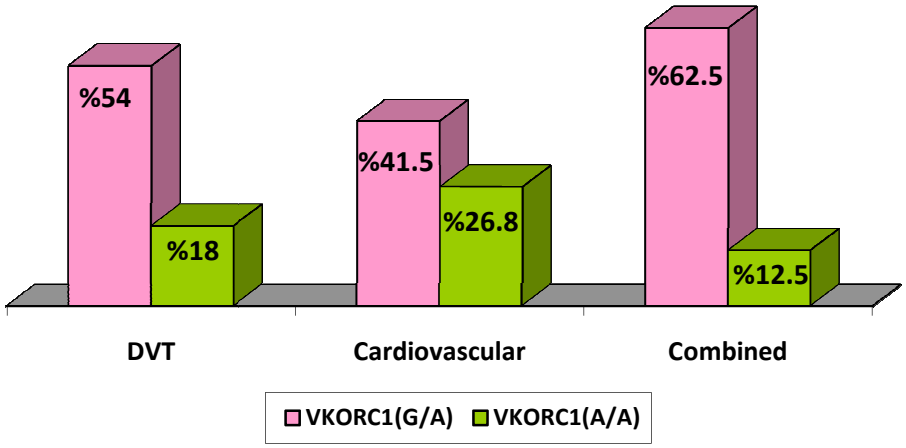

Figure 4.18 Distribution of patients by VKORC1 polymorphism and type of disease.

4.5. Averages of Warfarin Dose and INR among Patients

4.5.1. Average of actual weekly dose of Warfarin and calculated standard weekly dose

It was observed that there was statistically significant difference between the actual weekly dose of Warfarin administered to the patients and the calculated standard weekly dose of Warfarin for the sample patients according to their body characteristics and genotypes. The average actual weekly administered dose was 42.8± 15.0 mg/week while the average standard weekly dose that should been administered was 36.1 mg/week (t=4.42, p-value= 0.00 with 95% C.I. = 3.68- 9.68). (Table 4.4)

Table 4.4 Comparison between actual and calculated standard weekly doses of Warfarin of sample patients.

Warfarin Dose	Actual Dose (mg/week) (n=99)	Standard Dose (mg/week) (n=99)	t	P-value	95% C.I.
Average	42.8±15.0	36.1±9.8	4.42	0.00*	3.68-9.68

*(P<0.05) Significant.

Moreover 50.5% of patients were receiving high dose of Warfarin, also 32.3% of them were receiving low dose of Warfarin, while 17.2% of them were receiving the standard dose.

4.5.2. Average of actual weekly dose between males and females

According to gender there was no statistically significant difference between male and female weekly dose of Warfarin (t= -1.36, p-value= 0.18, 95%C.I. = -10.18-1.90), where male weekly dose was 40.4± 15.0 mg, and female weekly dose was 44.6±15.0 mg as shown in Table 4.5. Also about two-third of patients who received high dose were females 64%, and 58.8% of those who received the target dose were females, but about more than half of patients who received low dose of Warfarin were males 53% as illustrated in Figure 4.19 (X^2= 2.36, P-value= 0.31%).

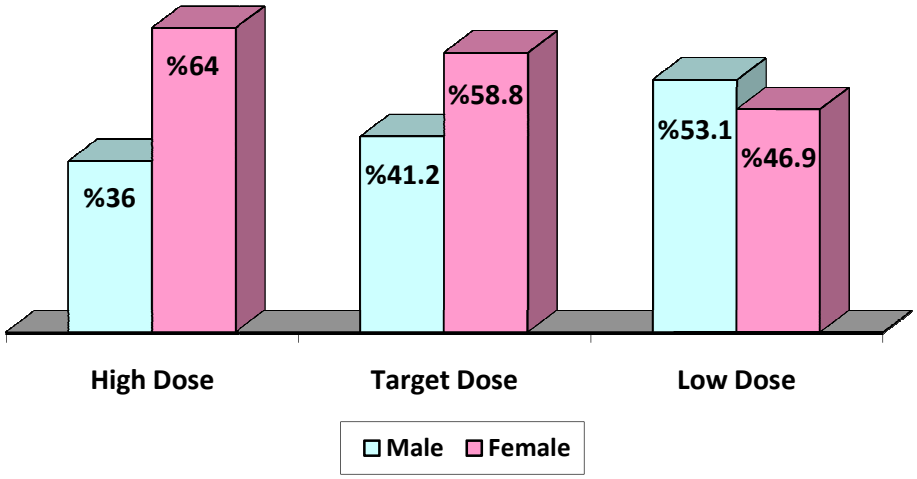

Figure 4.19 Distribution of patients by gender and classes of weekly Warfarin dose.

Table 4.5 Comparison between male and female actual weekly doses of Warfarin of sample patients.

Warfarin Dose	Male Dose (mg/week) (N=42)	Female Dose (mg/week) (N=57)	t	P-value	95% C.I.
Average	40.4±14.9	44.6±15.0	-1.36	0.18	-10.18-1.90

4.5.3. Association between the class of Warfarin dose and patients complications

There was statistically significant association between classes of Warfarin dose weekly received by the patients and the complications that the patients complained from (X^2= 7.48, p-value= 0.02). Table 4.6 shows that the percentage of patients who complained from any type of complications was the highest among the patients who received high dose of Warfarin weekly 59.1%, whereas the lowest percentage was among those who received low dose of Warfarin weekly 18.2%. It was observed that the percentage of patients who had complained of major complications is significantly higher than those who complained of minor complications among those who received high dose of Warfarin (X^2= 16.54, p-value= 0.00) as shown in figure 4.20.

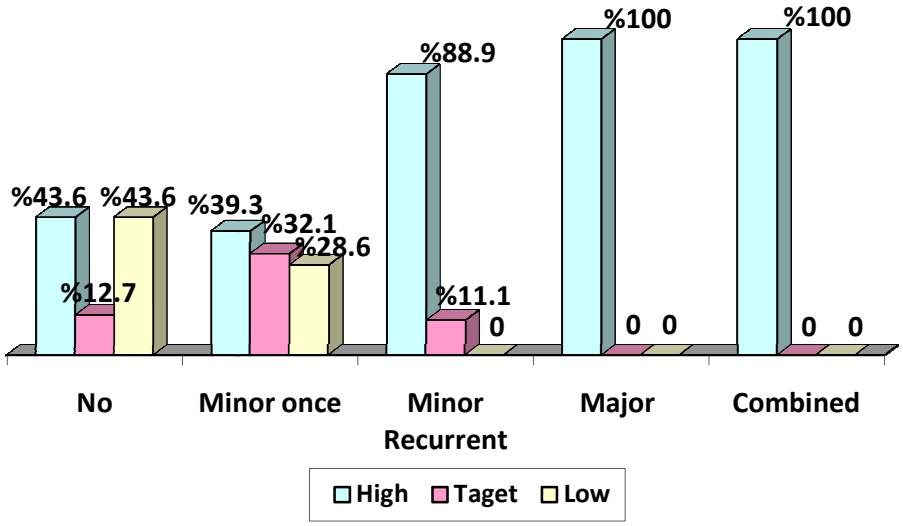

Figure 4.20 Distribution of patients by severity of complications and classes of weekly Warfarin dose.

Table 4.6 Distribution of sample patients by complications and classes of weekly Warfarin dose

Warfarin Dose Classes	Complications						X^2	P-value
	Yes		No		Total			
	No.	%	No.	%	No.	%		
High dose	26	59.1	24	43.6	50	50.5	7.48	0.02*
Target dose	10	22.7	7	12.7	17	17.2		
Low dose	8	18.2	24	43.6	32	32.3		

*(P<0.05) significant

4.5.4. Association of Warfarin actual weekly dose classes and duration of therapy

The results of the study revealed that the correlation between the duration of therapy under Warfarin and the actual weekly dose classes was not statistically significant (X^2= 2.93, p-value= 0.57), where it was observed that there was no difference in the weekly dose of Warfarin among the sample patients according to duration of therapy. As shown in table 4.7 the percentages of patients who were receiving high dose of Warfarin were approximately equals among the different three classes of therapy duration (<1 month= 63.6%, 1- 12 months= 46.3%, and > 12 months= 50%). The same finding was observed among patients who were receiving low dose of Warfarin (<1 month= 36.4%, 1- 12 months= 34.2%, and > 12 months= 29.5%). Generally none of patients was receiving the target dose during the first month, while the percentage of patients who were receiving the target dose during the first year approximately equal that of who were receiving the target dose after the first year from therapy.

Table 4.7 Distribution of sample patients by Warfarin therapy duration and classes of weekly Warfarin dose.

Warfarin Dose Classes	Therapy Duration								**X^2**	**P-value**
	<1 month		1-12 months		>12 months		Total			
	No.	%	No.	%	No.	%	No.	%		
High dose	7	63.6	19	46.3	22	50	48	50	2.93	0.57
Target dose	0	0	8	19.5	9	20.5	17	17.7		
Low dose	4	36.4	14	34.2	13	29.5	31	32.3		

4.5.5. Correlation between actual weekly dose and calculated standard dose of Warfarin

There was a statistically significant correlation between actual weekly dose and calculated standard dose of Warfarin of the patients (R= 0.37 , P-value= 0.00) (Figure 4.21).

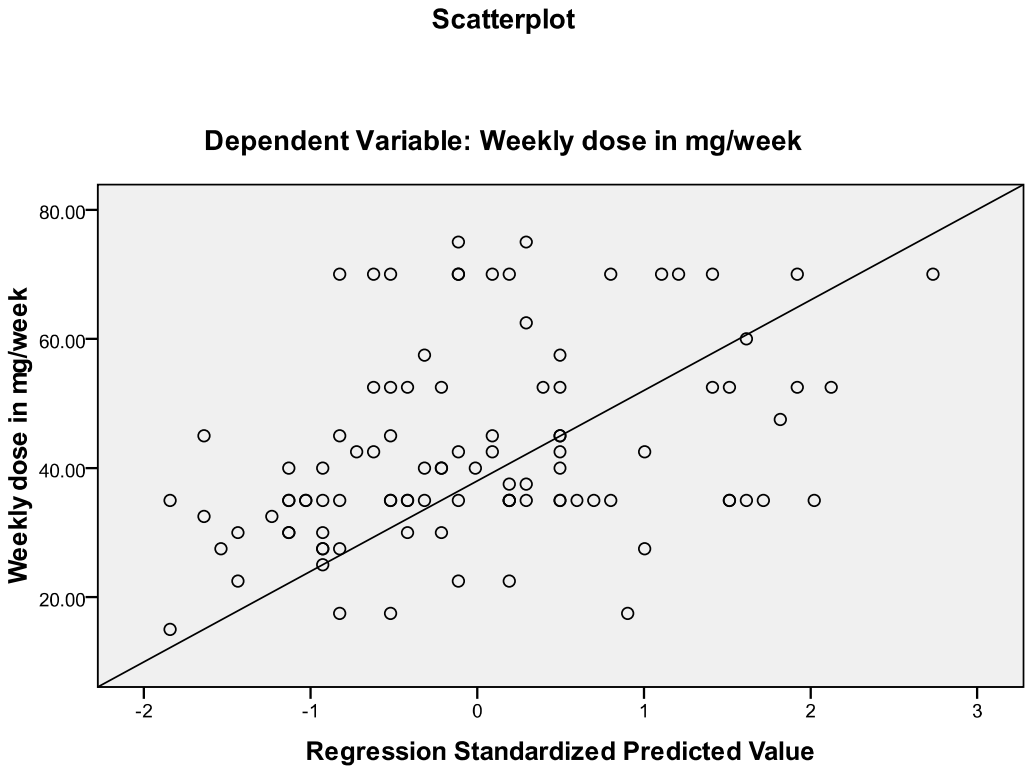

Figure 4.21 Scatterplot of the correlation and regression between the actual weekly dose and the calculated standard dose of Warfarin among the sample patients.

4.5.6. Classes and average of INR and its association with gender, age and BMI

The average of INR of sample was 2.4±1.5, whereas 33.7% of patients were of therapeutic range of INR, 20.8% of them were of bleeding tendency group, and 45.5% were of coagulation tendency group (Figure 4.22). As illustrated in figure 4.23 more than half of patients within therapeutic range of INR were females 58.8% and 41.2% were males. About two-thirds of patients of bleeding tendency were males 61.9% and 38.1% were females. Two-thirds of patients of coagulation tendency were females 65.9% and about one-third were males 34.1%. There was statistically no significant difference (t= 0.53, p= 0.60, 95% C.I.=-0.44- 0.76) in the average of INR of males 2.5±1.0 and that of females 2.4±1.7 as shown in table 4.8. It was observed that there was no significant correlation between INR and BMI (R= -0.16, P-value= 0.12). Also there was no significant correlation between INR and age of patients (R=-0.02, p-value= 0.88).

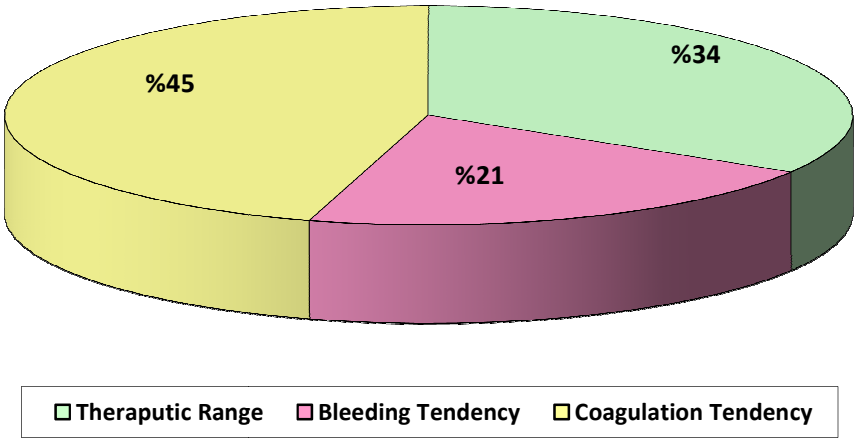

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Figure 4.22 Distribution of patients by classes of INR range.

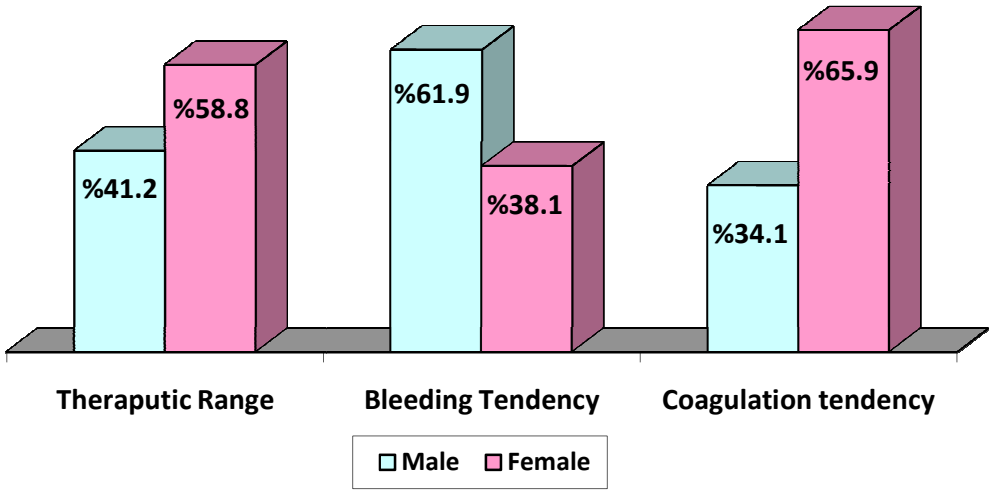

Figure 4.23 Distribution of patients by classes of INR range and gender.

Table 4.8 Comparison between male and female INR range of patients.

Warfarin Dose	**Male INR (N=42)**	**Female INR (N=57)**	**t**	**P-value**	**95% C.I.**
Average	2.5±1.0	2.4±1.7	0.53	0.60	-0.44-0.76

4.6. Relationships between CYP2C9 & VKORC1 Polymorphism and INR.

According to CYP2C9 gene only and exclusion of VKORC1 genotype, there was difference between the average of INR of patients who have at least one copy of CYP2C9*2 allele 2.8±2.1 and that of patients who have homozygous CYP2C9*1/*1 (wild type) 2.3± 1.2, but this difference did not reach the statistically significant level (t= -1.58. P-value= 0.12 with 95% C.I. = -1.19-0.14) as shown in table 4.9.

Table 4.9 Comparison between averages of INR and weekly dose of Warfarin according to CYP2C9 gene only among sample patients.

Variables	CYP2 C9*2		t	P-value	95% C.I.
	Yes (N=26)	NO (N=73)			
Average of INR	2.8±2.1	2.3±1.2	-1.58	0.12	-1.19-0.14
Average of Weekly Dose of Warfarin (mg)	42.6±16.8	42.9±14.5	0.08	0.94	-6.57-7.13

But by stratification of patients according to CYP2C9 genetic polymorphism, the comparison between averages of INR between the patients with wild type alleles of both genes (CYP2C9 and VKORC1) and patients carrying at least one CYP2C9*2 allele, there was statistically significant difference between the both averages of INR (wild type alleles in both genes= 2.1±0.9, that carrying at least one copy of CYP2C9*2= 3.1±1.3, (t= -2.46, P-Value= 0.02 with 95% C.I. = -1.92- -0.17) as shown in table 4.10.

Table 4.10 Comparison between averages of INR and weekly dose of Warfarin among patients who have wild type alleles only of (VKORC1 and CYP2C9) and those carrying at least one copy of CYP2C9*2.

Variables	At least one copy of CYP2C9*2 (n=8)	CYP2C9(*1/*1) and VKORC1(G/G) (n=21)	t	P-value	95% C.I.
Average of INR	3.1±1.3	2.1±0.9	-2.46	0.02*	-1.92- -0.17
Average of Weekly Dose of Warfarin (mg)	49.4±20.1	48.5±14.2	-0.14	0.89	-14.77-12.93

*(P<0.05) significant

Also table 4.9 revealed that there was no significant difference (t= 0.08, P-value= 0.94 with 95% C.I. = -6.57-7.13) between the average dose of Warfarin of patients of wild type allele CYP2C9*1/*1 42.9±14.5 mg and that of patients who carrying at least one copy of CYP2C9*2 allele 42.6±16.8 mg.

Furthermore according to VKORC1 gene, there was no significant difference (t=- 0.43, P-value= 0.67 with 95% C.I. = -0.79- 0.51) between the mean of INR of patients who have homozygous VKORC1(G/G) (wild type) 2.3±1.1 and that of patients carrying at least one copy of VKORC1-1639A allele 2.5±1.6, (Table 4.11). On the other hand, there was statistically significant difference (t=2.59, P-value= 0.01 with 95% C.I. = 1.94-14.76) between the average dose of Warfarin of patients who have homozygous VKORC1(G/G) (wild type) 48.7±16.0 mg and that of patients carrying at least one copy of VKORC1-1639A allele 40.4±14.0 mg.

Table 4.12 demonstrates that by stratification of patients according to VKORC1, there was significant difference between the average of weekly dose of Warfarin of patients of wild type alleles of both genes (CYP2C9*1/*1 and VKORC1(G/G) genotypes) and those with at least one copy of VKORC1-1639A (wild type alleles of both genes= 48.5±14.2 mg, carrying at least one copy of VKORC1-1639A= 40.6±14.1 mg, t=2.14, P-value=0.04 with 95% C.I. = 0.53-15.13). However, there was no significant difference in the average of INR of both groups

(wild type alleles of both genes = 2.1±0.9, carrying at least one copy of VKORC1-1639A = 2.4±1.2, t=-1.18, P-value=0.24 with 95% C.I. = -0.94-0.24).

Table 4.11 Comparison between averages of INR and weekly dose of Warfarin according to VKORC1 gene only among sample patients.

Variables	VKORC1-1639A		t	P-value	95% C.I.
	Yes (n=26)	NO (n=73)			
Average of INR	2.5±1.6	2.3±1.1	-0.43	0.67	-0.79-0.51
Average of Weekly Dose of Warfarin (mg)	40.4±14.0	48.7±16.0	2.59	0.01*	1.94-14.76

*(P<0.05) significant

Table 4.12 Comparison between averages of INR and weekly dose of Warfarin among patients who have wild type alleles only of (VKORC1 and CYP2C9) and those carrying at least one copy of VKORC1-1639A

Variables	At least one copy of VKORC1-1639A (n=52)	CYP2C9*1/*1 and VKORC1(G/G) (n=21)	t	P-value	95% C.I.
Average of INR	2.4±1.2	2.1±0.9	-1.18	0.24	-0.94-0.24
Average of Weekly Dose of Warfarin (mg)	40.6±14.1	48.5±14.2	2.14	0.04*	0.53-15.13

*significant

Here we can predict that the presence of CYP2C9*1/*2 - CYP2C9*2/*2 genotype seems to be a cause of the elevation of the INR as there is no significantly difference between the Warfarin doses of two patient groups (first group of the genotype CYP2C9*1/*2 - CYP2C9*2/*2 with VKORC1(G/G) and the other group of CYP2C9*1/*1 with VKORC1(G/G)).

Moreover it was found that there was no significant difference in the averages of INR of patients carrying VKORC1-1639A allele and those of wild type alleles (VKORC1(G/G)), and this is because there was significant difference in the averages of the weekly doses of Warfarin of both groups, where the average dose of those of wild type group (VKORC1(G/G)) was significantly higher than that of those carrying VKORC1-1639A allele.

Here we can predict that the presence of VKORC1(G/A) - VKORC1(A/A) genotype seems to be a cause of the lower dose of Warfarin as there is no significant difference between the averages of INR of two patient groups (first group of the genotype VKORC1(G/A) - VKORC1(A/A) with CYP2C9*1/*1 and the other group of CYP2C9*1/*1 with VKORC1(G/G)).

This revealed that CYP2C9 and VKORC1 genes polymorphisms play an important role in adjusting the dose of Warfarin and therefore the INR of DVT and cardiac patients under Warfarin therapy.

Chapter 5

Discussion

Different studies have shown that there is a genetic polymorphism in the CYP2C9 gene. Variant alleles CYP2C9*1 , CYP2C9*2 and CYP2C9*3 are the most common allelic variants that can affect the Warfarin metabolism (Ghadam et al., 2009; Ngow et al., 2009 Daly, 2010). The frequency of these allelic variants is dependent on the ethnical groups (Takahashi et al., 2006). The sensitivity to Warfarin medication and the INR is dependent on the genotype of CYP2C9 as the CYP2C9*1 allelic form is the wild-type showing the higher metabolism and lower sensitivity to Warfarin whereas the allelic variants CYP2C9*2 and CYP2C9*3 show the lower metabolism and the higher sensitivity to Warfarin (Ghadam et al., 2009; Ngow et al., 2009; Daly, 2010).

Also Low responsiveness to Warfarin (defined as a failure to achieve a target INR is associated with polymorphisms of the vitamin K epoxide reductase-oxidase complex gene (VKORC1). A highly prevalent promoter single-nucleotide polymorphism (VKORC1-1639 G>A) impairs VKORC1 expression and determines the interindividual variability of the target INR (Stepien et al., 2009).
Patients who are carriers of a common polymorphism in the VKORC1 promoter sequence (− 1639 G>A) require a lower Warfarin maintenance dosage (Yuan et al., 2005 and Rieder et al., 2005).

In general, studies suggested that the CYP2C9 genotype contributes to between 10 and 20% of the variability in Warfarin dose requirement compared with a 20–30% contribution from the VKORC1 (vitamin K epoxide reductase complex 1) genotype (klein et al., 2009).

In spite of the importance of the determination of allelic polymorphisms of the two genes in optimizing the more accurate Warfarin dose to maintain the INR in the narrow therapeutic range, till now there is no study in Palestine to assess the importance of these two genes in Warfarin dose prescription, therefore this is the first

study to determine the allelic variants present in Gaza people and the allelic frequency of each variant.

In this study we found that the average INR of patients who have at least one copy of CYP2C9*2 allele is 2.8±2.1 and that of patients who have homozygous CYP2C9*1/*1 (wild type) is 2.3± 1.2, this difference show slightly increased INR in patients carrying the CYP2C9*2 allele than those of homozygous CYP2C9*1/*1 genotype, but this difference did not reach the statistically significant level (t= -1.58. P-value= 0.12 with 95% C.I. = -1.19-0.14).

When the averages of INR between patients with wild type alleles of both genes (CYP2C9 and VKORC1) were compared with that of patients carrying at least one CYP2C9*2 allele, there was statistically significant difference between both averages of INR (in patients of CYP2C9*1/*1 and VKORC1(G/G) = 2.1±0.9, carrying at least one copy of CYP2C9*2= 3.1±1.3, t= -2.46, P-Value= 0.02 with 95% C.I. = -1.92- -0.17).

Moreover 50.5% of patients were receiving high dose of Warfarin, also 32.3% of them were receiving low dose of Warfarin, while 17.2% of them were receiving the standard dose. This reveals that the adjustment of dose of Warfarin for sample patients was according to multiple factors rather than the factors of standard dose (age, height, weight, VKORC1, CYP2C9 and race).

The present data showed no significant difference (t= 0.08, P-value= 0.94 with 95%C.I. = -6.57-7.13) in Warfarin dose between the two groups (carrying CYP2C9*2 allele and those of (wild type) CYP2C9*1/*1 and VKORC1(G/G) genotypes 42.9±14.5 mg and 42.6±16.8 mg respectively. This which explains that the CYP2C9*2 allele has increased the Warfarin sensitivity, therefore increased the average of the INR between patients carrying CYP2C9*2 allele and not VKORC1-1639A when the Warfarin dose approximately equals.

This study found that there is no statistically significant difference (t=- 0.43, P-value= 0.67 with 95% C.I. = -0.79- 0.51) between the mean of INR of patients who have homozygous VKORC1(G/G) (wild type) 2.3±1.1 and that of patients carrying

least one copy of VKORC1-1639A allele 2.5±1.6. This may predict that the VKORC1-1639A allele has no effect on INR level but in fact this results of average INR level between two groups clarify that the presence of this allele has its effect on the Warfarin dose and lowered the average dose, as it was found in this study, there is a statistically significant difference (t=2.59, P-value= 0.01 with 95% C.I. = 1.94-14.76) between the average dose of Warfarin of patients who have homozygous VKORC1(G/G) (wild type) 48.7±16.0 mg and that of patients carrying least one copy of VKORC1-1639A allele 40.4±14.0 mg.

Also when the averages of INR between patients with wild type genotypes (CYP2C9 and VKORC1) were compared with that of patients carrying at least one VKORC1-1639 allele there was statistically significant difference between the average of weekly dose of Warfarin (wild type alleles of both genes= 48.5±14.2 mg, carrying at least one copy of VKORC1-1639A= 40.6±14.1 mg, t=2.14, P-value=0.04 with 95% C.I. = 0.53-15.13), where there was statistically no significant difference in the average of INR of both groups (wild type alleles of both genes = 2.1±0.9, carrying at least one copy of VKORC1-1639A = 2.4±1.2, t=-1.18, P-value=0.24 with 95% C.I. = -0.94-0.24).

The strong correlation between the actual weekly dose and standard predicted dose (Figure 4.21) indicates that the predicted dose could have been used as a starting management and to save the patients the burden of empirically adjusting the dose to be taken. This may be supported by the fact that all patients participating in this study didn't reach their target dose before at least one month from the start of treatment (see Table 4.7)

These findings confirm the significance of pharmacogenomics to be applied for personalized medicine and Warfarin dose requirement, CYP2C9 and VKORC1 genes seems to be important factors affecting INR level and Warfarin dosing. The recent report by Schwarz et al. (2008) indicates that both the CYP2C9 and VKORC1 had a significant influence on the required Warfarin dose after the first 2 weeks of therapy.

Comparing this study with a study made by Alzahrani et al. (2013) for genotyping of CYP2C9 and VKORC1 in Arabic population of Al-Ahsa in Saudi Arabia, it was found that this study was reported that the allelic frequencies of CYP2C9*2, CYP2C9*3 and VKORC1-1639A were 13.4%, 0.0% and 46.0% respectively, it was found that the allelic frequency of CYP2C9*2 allele in our study equals that of Al-Ahsa population (13.3%) in Saudi Arabia in a study made there for genotyping of CYP2C9 and VKORC1, while the CYP2C9*3 allelic variant didn't found in our study population and it was 2.3% in Al-Ahsa people. Also the allelic frequency of VKORC1-1639A allele was similar between our study and Al-Ahsa people as it was 46.0% of our study and 42.7 in Al-Ahsa people. Regarding the genotype distribution of CYP2C9 and VKORC1 it was found that in this study the CYP2C9*1/*2 24.8% and CYP2C9*2/*2 1.0% while it was 26.7% and 0% respectively in Al-Ahsa population. This study recorded 0% CYP2C9*3 allele but the CYP2C9*1/*3 genotype was 4.6% in Saudi Arabi in Al-Ahsa region while CYP2C9*3/*3 was not recorded in both populations.

The VKORC1(A/A) genotype distribution in this study and Al-Ahsa population were 21.8% and 22.9% respectively, while VKORC1(G/A) was 48.5% and 39.7% respectively. For the VKORC1(G/G) genotype in our study and Al-Ahsa it was 29.7% and 37.4% respectively.

Also a Romanian study by Anca et al. (2012) agrees with our finding 46.0% about the VKORC1-1639A allelic frequency which is found in Romanian population to be 42.2%, but there is a noticed difference in the VKORC1(G/A) genotype distribution between ours 48.5% and Romanian which reached 67.8%, while the VKORC1(A/A) genotype distribution in Romanian population was 16.6% and ours was 21.8%.

Romanian population recorded the CYP2C9*2 heterozygous distribution with 18.7% compared to our study 24.8% and a large increase in CYP2C9*3 heterozygous genotype distribution 14.1% which is not recorded in our study population, while the Romanian study was found 4.2% included all CYP2C9*2 homozygous, CYP2C9*3 homozygous or CYP2C9*2/CYP2C9*3 compound heterozygous genotypes, also

allele frequencies of the CYP2C9*2 and CYP2C9*3 polymorphisms in Romanian study were 11.3% and 9.3% respectively.

Esmerian et al. (2011) published a study for CYP2C9 and VKORC1 polymorphisms among Lebanese population and there is a difference in allelic frequencies between their and our study. They found that the CYP2C9*2 allelic frequency 15.4% about a half of our study observation 24.8, and they have a large frequency of the CYP2C9*3 allele of 7.8% which is absent in our study. Compared to our study the closest allelic frequency they have observed was seen in VKORC1-1639A allele of 52.4% which is 46.0% in Gaza population.

A study by Efrati et al. (2010) on Israeli population showed that Arab Moslems had a higher frequency of Warfarin "sensitive" CYP2C9*2, CYP2C9*3 and VKROC1 -1639G>A alleles 21.0%, 7.0% and 58.0%, respectively than both Jews 13.0%, 11.0% and 57.0%, respectively and Druze 12.0%, 6.0% and 53.0%, respectively populations.

As seen in table 2.1 the frequency of different alleles of CYP2C9 and VKORC1 was different among different populations also the genotype distribution, which shows that the allelic polymorphism of these genes is ethnically dependent. As the vast majority of studies published in these populations support that the importance of personalized medicine theory and the use of pharmacogenomics to apply an accurate dose prescription of Warfarin according the genotype of CYP2C9 and VKORC1 the most effective genes on Warfarin metabolism and sensitivity.

our findings were similar to those reported in some populations, thus the allelic frequency of CYP2C9*2 and VKORC1-1639A alleles must be taken in consideration to improve the genotypeguided dosing to decrease the risk of over-anticoagulation and enhancing the patient safety.

Chapter 6

Conclusions and recommendations

6.1. Conclusions

In our population only the CYP2C9*1 and CYP2C9*2 alleles were found with allelic frequency 86.6% and 13.4% respectively, while no CYP2C9*3 allele was found. Both VKORC1-1639G and VKORC1-1639A alleles were found in our population with allelic frequency (54.0% and 46.0%) respectively.

It was observed that among patients carrying CYP2C9*2 allele, the INR was significantly higher than those of wild type alleles (CYP2C9*1/*1 and VKORC1-GG) and there was no significant difference in the weekly dose of Warfarin between the two groups.

Probably because the dose was adjusted by the physician before the start of the study it was found that patients who have the VKORC1-1639A allele having lower warfarin dose than those who have the wild type alleles of both genes CYP2C9 and VKORC1 with no significant difference in the INR between the two groups.

Finally we can conclude that the presence of at least one copy of CYP2C9*2 or VKORC1-1639A alleles or both can increase the sensitivity to Warfarin therapy and INR level which need to decrease the Warfarin dose to optimize the INR in needed range.

The study highlights the importance of pharmacogenomics as a modifier in the clinical practice and it is important to determine the genotype for CYP2C9 and VKORC1 before determining the weekly dose of Warfarin for patients with indicated therapy to save the patients and physicians the burden of Warfarin dose adjustment.

6.2. Recommendations

1- Determination of CYP2C9 genotype, and VKORC1 genotype as a prerequisite before the initiation and calculation of Warfain dose.

2- This study represents a model for the application of genetic testing in clinical practice that must be adapted in other drugs and genes.

3- Further studies must be performed to evaluate the role of other alleles in the both genes as possible determinants of initiation Warfarin dose.

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Appendices

Appendix 1

Palestinian National Authority
Ministry of Health
Helsinki Committee

السلطة الوطنية الفلسطينية
وزارة الصحة
لجنة هلسنكي

التاريخ: 4/6/2012

Name: **Ahmed Abu Shaaban**

الاسم: احمد أبو شعبان

I would like to inform you that the committee has discussed your application about:

نفيدكم علماً بأن اللجنة قد ناقشت مقترح دراستكم حول:-

"Allelic Variants of CYP2C9 gene and their relation to warfarin metabolism in patients under warfarin therapy in Gaza Strip".

In its meeting on **June 2012**
and decided the Following:-
To approve the above mention research study.

و ذلك في جلستها المنعقدة لشهر 6 2012
و قد قررت ما يلي:-
الموافقة على البحث المذكور عاليه.

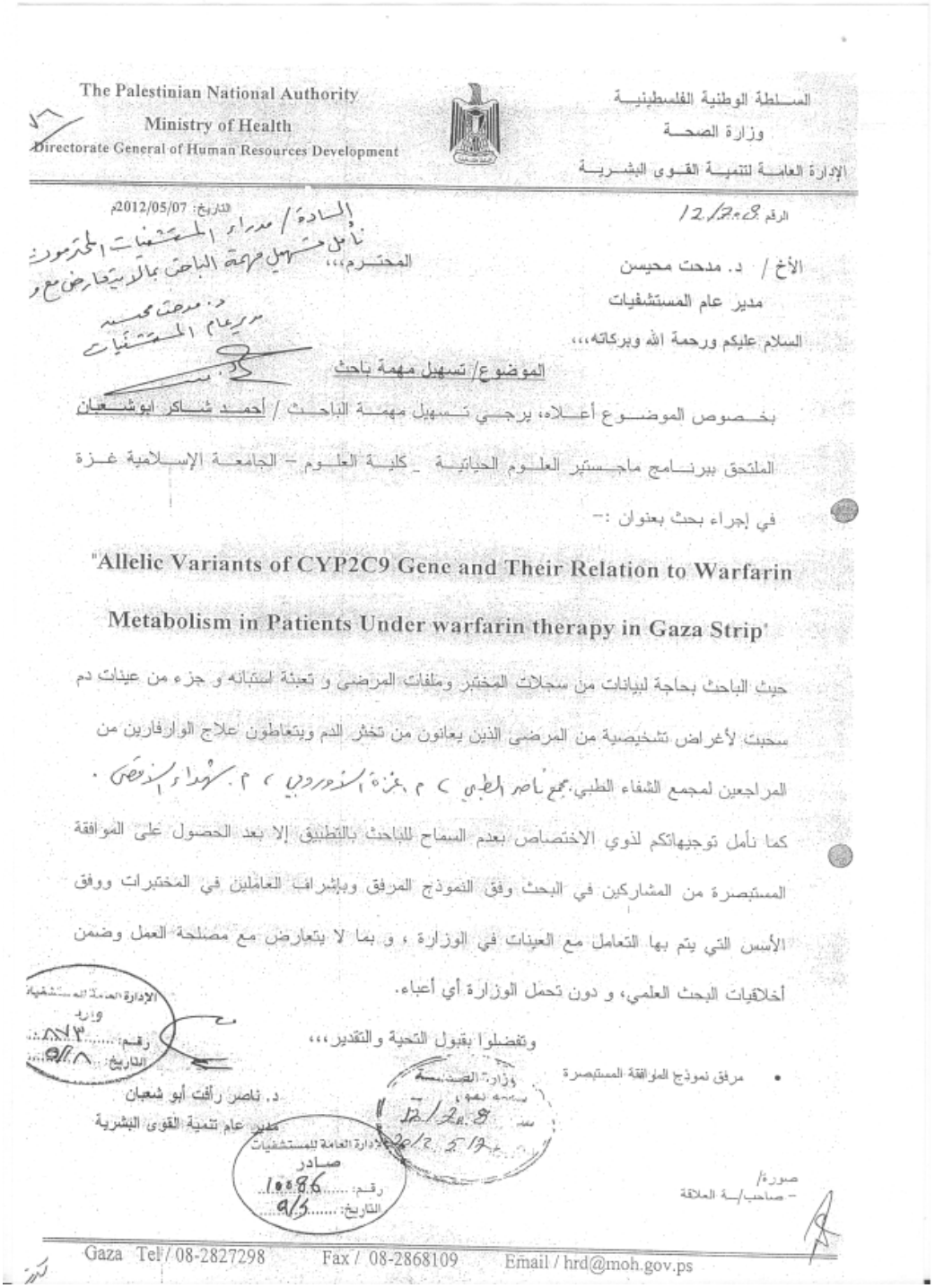
Signature
توقيع

Member عضو | Member عضو | Chairperson

Conditions:-

- Valid for 2 years from the date of approval to start
- It is necessary to notify the committee in any change in the admitted study protocol.
- The committee appreciate receiving one copy of your final research when it is completed.

Appendix 2



The Palestinian National Authority
Ministry of Health
Directorate General of Human Resources Development

السلطة الوطنية الفلسطينية
وزارة الصحة
الإدارة العامة لتنمية القوى البشرية

الرقم: 12/2008

التاريخ: 2012/05/07م

الأخ / د. مدحت محيسن
مدير عام المستشفيات

السلام عليكم ورحمة الله وبركاته،،،

الموضوع/ تسهيل مهمة باحث

بخصوص الموضوع أعلاه، يرجى تسهيل مهمة الباحث / أحمد شاكر ابوشعبان الملتحق ببرنامج ماجستير العلوم الحياتية _ كلية العلوم – الجامعة الإسلامية غزة في إجراء بحث بعنوان :-

"Allelic Variants of CYP2C9 Gene and Their Relation to Warfarin Metabolism in Patients Under warfarin therapy in Gaza Strip"

حيث الباحث بحاجة لبيانات من سجلات المختبر وملفات المرضى و تعبئة استبانه و جزء من عينات دم سحبت لأغراض تشخيصية من المرضى الذين يعانون من تخثر الدم ويتعاطون علاج الوارفارين من المراجعين لمجمع الشفاء الطبي، مجمع ناصر الطبي ، م. غزة الأوروبي ، م. شهداء الأقصى .

كما نأمل توجيهاتكم لذوي الاختصاص بعدم السماح للباحث بالتطبيق إلا بعد الحصول على الموافقة المستبصرة من المشاركين في البحث وفق النموذج المرفق وبإشراف العاملين في المختبرات ووفق الأسس التي يتم بها التعامل مع العينات في الوزارة ، و بما لا يتعارض مع مصلحة العمل وضمن أخلاقيات البحث العلمي، و دون تحمل الوزارة أي أعباء.

وتفضلوا بقبول التحية والتقدير،،،

- مرفق نموذج الموافقة المستبصرة

د. ناصر رأفت أبو شعبان
مدير عام تنمية القوى البشرية

صورة/
- صاحب/ـة العلاقة

الإدارة العامة للمستشفيات
وارد
رقم: ٢٧٧٣
التاريخ: ٩/٥

وزارة الصحة
الإدارة العامة للمستشفيات
صادر
رقم: 10086
التاريخ: 9/5

Gaza Tel / 08-2827298 Fax / 08-2868109 Email / hrd@moh.gov.ps

Appendix 3

Data collection form

Name:_____

Age: _____

Gender: _____

Weight: _____

Height: _____

Tell/Cell: _____

- Vitamin K containing food three days before: _____
- Duration of Warfarin therapy "Coumadin": _____
- Dose of Warfarin "Coumadin" (Daily): _____
- Change in Warfarin dose increase or decrease : _____
- Bleeding complication during therapy:

Minor bleeding:

  - Nose
  - Bruise
  - Hematuria
  - Gingival
  - Conjunctival
  - Anal

Major bleeding:

  - Gross hematuria.
  - Gastrointistinal.
- Diagnosis (Cause of Warfarin therapy): _____
- Other treatment: _____
- Other chronic disease: _____
- INR/PT result: _____

Appendix 4

This calculator provides a predicted starting dose for Warfarin based on a pharmacogenetic algorithm developed by the IWPC (International Warfarin Pharmacogenetics Consortium), as described in N. Engl. J. Med. **360:** 753-764 (2009).
To obtain a predicted starting dose, enter data for your patient in the column labeled **Enter Value** on the **Calculator** tab of this Excel Workbook. An entry is required for each variable listed in the Calculator. The predicted dose is given below the last line in the data entry table. Please note that this is a **Weekly** dose, not a daily dose. Divide this dose as evenly as possible into 7 parts to obtain daily doses.

Variables required for the computation are as follows:

Variable	**Units or Allowed Values**	**Comments**
Age	Years	The algorithm only uses age at the granularity of decades, but you must enter age in years and the calculator will convert to decades
Height	Centimeters (cm)	Height must be entered in centimeters, not feet or inches. Very large or very small values of height will result in a warning, as these may indicate data entry errors, but they will not stop the computation. If a warning appears, please check that height was entered correctly.
Weight	Kilograms (kg)	Weight must be entered in kilograms, not pounds. To flag potential data entry errors, Height and Weight are used to compute BMI, and very large or very small values of BMI result in a warning, but will not stop the computation. If a warning appears, please check that weight was entered correctly.
VKORC1 genotype	A/A A/G G/G U ((for Unknown)	This is the genotype at the -1639 A>G SNP in the VKORC1 promoter. Only the values shown in the exact format shown are allowed; any other values will stop the computation and must be corrected. If genotype is unknown, enter the single letter "U." If you have genotyped a different VKORC! SNP which you know to be in high Linkage Disequilibrium with the -1639 SNP in the racial group to which your patient belongs, you may wish to substitute an allowed value that represents the haplotypes indicated by the SNP you typed. Consult the literature for appropriate substitutions.
CYP2C9 genotype	*1/*1 *1/*2 *1/*3 *2/*2 *2/*3 *3/*3 U (for Unknown)	This is the genotype for CYP2C9. Only genotypes composed of combinations of the *1, *2 and *3 alleles in the exact format shown are allowed. These were the only alleles that occurred in the IWPC patient population at high enough frequencies to reliably estimate their contribution to Warfarin dose. If you have genotyped other alleles, you may wish to enter an allowed genotype that is closest in enzyme activity to your actual genotype, based on the pharmacologic literature. If genotype is unknown, enter the single letter "U." Any value other than the allowed values will stop the computation and must be corrected.
Race	A (for Asian) B (for Black or African American) C (for Caucasian or White) U (for Unknown or Mixed Race)	Enter single letter codes (A, B or C) for one of the three broad racial groupings that were used in the IWPC algorithm (Asian, Black, and Caucasian). These largely correspond to the US OMB racial categories, although Hispanics were included in the Caucasian category in the IWPC data set. Other racial groups occurred in the IWPC data set in numbers too small to provide reliable estimates of effect on Warfarin dose, and were included in the Unknown group. Enter the single letter "U" for unknown or mixed race. Any other value will stop the computation and must be corrected.
Taking Enzyme Inducer	Y or N	Enter Y for patients taking a CYP2C9 inducer, N for patients not taking or not known to be taking a CYP2C9 inducer. Inducers considered in the development of the IWPC algorithm were rifampin or rifampicin (Rifadin, Rimactane), phenytoin (Dilantin), and carbamazepine (Tegretol).

Appendix 5

Example calculations

In the first example, all data are entered correctly and a predicted value for weekly Warfarin dose is returned. There are no error messages or warnings. The patient is a 70 year old Caucasian, 180 cm tall and weighing 75 kilograms, heterozygous at the VKORC1 -1639 SNP and wild type for CYP2C9, who is not taking CYP2C9 enzyme inducers.

Variable	Units or Allowed Values	Enter Value	Error Messages/Warnings
Age	Years	70	
Height	Centimeters (cm)	180	
Weight	Kilograms (kg)	75	
VKORC1 genotype	A/A A/G G/G U ((for Unknown)	A/G	
CYP2C9 genotype	*1/*1 *1/*2 *1/*3 *2/*2 *2/*3 *3/*3 U ((for Unknown)	*1/*1	
Race	A (for Asian) B (for Black or African American) C (for Caucasian or White) U (for Unknown or Mixed Race)	C	
Taking Enzyme Inducer	Y or N	N	
Taking Amiodarone	Y or N	N	
Computed Weekly Starting Dose (mg/week):		30	