DEVELOPMENT OF WARFARIN DOSING FORMULA BASED ON PHARMACOGENOMICS AND CLINICAL FACTORS IN PATIENTS WITH MECHANICAL HEART VALVE

AJJIMA SARAPAKDI

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
(CLINICAL PHARMACY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2012

Thesis
entitled

DEVELOPMENT OF WARFARIN DOSING FORMULA BASED ON PHARMACOGENOMICS AND CLINICAL FACTORS IN PATIENTS WITH MECHANICAL HEART VALVE

Ajjima Sarapakdi
Miss Ajjima Sarapakdi
Candidate

Lect. Aroonrut Lucksiri, Ph.D.
Co-advisor

Assist. Prof. Surakit Nathisuwan, Pharm.D., BCPS.
Major advisor

Assist. Prof. Chulaluk Komoltri, Dr.PH., (Biostatistics)
Co-advisor

Assist. Prof. Punnarerk Thongcharoen, M.D., Dip. Clinical Science (Surgery), Dip. Thai Board of General Surgery, Dip. Thai Board of Cardiothoracic surgery
Co-advisor

Prof. Banchong Mahaisavariya, M.D.,Dip. Thai Board of Orthopedics
Dean
Faculty of Graduate Studies
Mahidol University

Assoc. Prof. Busba Chindavijak, Ph.D. (Clinical Pharmacokinetics)
Program Director
Master of Science in Pharmacy
Program in Clinical Pharmacy
Faculty of Pharmacy
Mahidol University

Thesis
entitled

DEVELOPMENT OF WARFARIN DOSING FORMULA BASED ON PHARMACOGENOMICS AND CLINICAL FACTORS IN PATIENTS WITH MECHANICAL HEART VALVE

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science in Pharmacy (Clinical Pharmacy)
on
January 17, 2012

Ajjima Sarapakdi
Miss Ajjima Sarapakdi
Candidate

Kanokporn Niwatananun
Assoc. Prof. Kanokporn Niwatananun
Ph.D.
Chair

Assist. Prof. Surakit Nathisuwan,
Pharm.D., BCPS.
Member

C. Komoltri
Assist. Prof. Chulaluk Komoltri,
Dr.PH., (Biostatistics)
Member

Lect. Aroonrut Lucksiri, Ph.D.
Member

Assist. Prof. Punnarerk Thongcharoen,
M.D., Dip. Clinical Science (Surgery),
Dip. Thai Board of General Surgery,
Dip. Thai Board of Cardiothoracic
surgery
Member

B. Mahaisavariya
Prof. Banchong Mahaisavariya,
M.D.,Dip. Thai Board of Orthopedics
Dean
Faculty of Graduate Studies
Mahidol University

C. Suthisisang
Assoc. Prof. Chuthamanee Suthisisang,
Ph.D.
Dean
Faculty of Pharmacy
Mahidol University

ACKNOWLEDGEMENTS

This work would not have been possible without the attentive support from my major advisor, Assist. Prof. Surakit Nathisuwan for his support, valuable advice, guidance, great attention and patience in a long and winding pathway to the completion of my degree. I am deeply grateful to Assist. Prof. Punnarerk Thongcharoen for the helpful guidance, suggestion, comment and recommendation. I would like to thank Assist. Prof. Chulaluk Komoltri and Dr. Aroonrut Lucksiri who provided me with statistical advice at times of critical need. My special thanks are sincerely extended to Assoc. Prof. Kanokporn Niwatananun as the external examiner of thesis defense for her kindness and helpful suggestions. I would also like to thank Prof. Dr. Pilaipan Puthavathana and Mr. Worawat Dangsagul for providing facility support during DNA extraction process. I also would like to thank Roche Diagnostic, Thailand for their technical support and necessary equipment and reagents during my study.

I cannot end without thanking my family, whose constant encouragement and understanding throughout my time at the study is crucial. I am grateful also to my boyfriend, Mr. Sumate Polpukdee, for his unflinching courage and conviction which always inspire me throughout my academic journey.

Ajjima Sarapakdi

DEVELOPMENT OF WARFARIN DOSING FORMULA BASED ON PHARMACOGENOMICS AND CLINICAL FACTORS IN PATIENTS WITH MECHANICAL HEART VALVE

AJJIMA SARAPAKDI 5036284 PYCP/M

M.Sc. in Pharm. (CLINICAL PHARMACY)

THESIS ADVISORY COMMITTEE : SURAKIT NATHISUWAN, Pharm.D., BCPS., PUNNARERK THONGCHAROEN, M.D., DIP. THAI BOARD OF CARDIOTHORACIC SURGERY, CHULALUK KOMOLTRI, Dr.PH., (BIOSTATISTICS), AROONRUT LUCKSIRI, Ph.D.

ABSTRACT

Warfarin, the most frequently used oral anticoagulant, has wide inter-patient variability and is difficult to manage clinically. Several variables affect warfarin dose requirements, such as age, drug interactions, co-morbidities, consumption of vitamin-K containing food supplements, alcohol intake, body size and ethnicity. Recently, genetic variations have been shown to affect warfarin response and dosage requirements to a larger extent than the clinical factors mentioned above. The two most important genes are those encoding the enzyme cytochrome P450 2C9 (*CYP2C9*) and vitamin K epoxide reductase complex 1 (*VKORC1*). The aim of our study was to determine the relationship between *CYP2C9*, *VKORC1* genotype and the clinical factors determining the warfarin dose requirements. These factors were subsequently used to develop a warfarin dosing formula. A retrospective study was conducted in 197 patients at the Cardiovascular and Thoracic Surgical unit, Siriraj Hospital. Patients who were receiving stable warfarin maintenance doses with therapeutic international normalized ratio (INR) 2.0-3.0 were included in the study. Blood samples were collected for DNA extraction using the High Pure PCR Template Preparation Kit protocol (Roche Applied Science, Mannheim, Germany). Subsequently, genotyping for *CYP2C9*2,*3* and *VKORC1 C1173T, G-1639A* was performed using a mutation detection kit and analyzed with LightCycler®480.

Of the 197 patients, 182 patients were found to have *CYP2C9*1/*1* (92.4%) and 15 were found to have *CYP2C9*1/*3* (7.6%). *VKORC1 C1173T* genotyping showed that 114 patients (57.9%) were homozygous TT (low dose group, AA), 69 patients (35%) were heterozygous CT (intermediate dose group, AB), and 14 patients (7.1%) were homozygous for the CC genotype (high dose group, BB). The mean warfarin daily dose requirement in patients with *CYPC9*1/*1* was significantly higher than *CYP2C9*1/*3* (4.07 ± 1.67 vs 2.42 ± 0.75 mg, $p = 0.004$). Regarding *VKORC1*, the mean warfarin daily dose was highest in patients with BB haplotype (7.03 ± 1.58 mg) compared with those with the AB haplotype (4.81 ± 1.43 mg) and AA haplotype (3.04 ± 0.94 mg). There were significant differences between warfarin maintenance doses among different groups of *VKORC1* haplotypes ($p<0.001$). Clinical factors, age, and body weight were significantly correlated with the daily warfarin dose ($p = 0.013$ and $p < 0.001$, respectively). A warfarin dosing formula based on genotypes, age, and body weight was found to explain 60.6 % of the variability in warfarin doses. Subsequently, a warfarin dosing formula was successfully developed and found to have high potential for clinical applications to guide warfarin dosing selection in Thai patients. However, further studies are needed to confirm the clinical utility of such a formula in practice.

KEY WORDS: CYP2C9 / VKORC1 / POLYMORPHISM / GENOTYPE / WARFARIN

113 pages

การพัฒนาสูตรคำนวณขนาดยาวาร์ฟารินโดยใช้เภสัชพันธุศาสตร์ร่วมกับปัจจัยทางคลินิกในผู้ป่วยเปลี่ยนลิ้นหัวใจเทียมแบบโลหะ

DEVELOPMENT OF WARFARIN DOSING FORMULAR BASED ON PHARMACOGENOMICS AND CLINICAL FACTORS IN PATIENTS WITH MECHANICAL HEART VALVE

อัจจิมา สระภักดิ์ 5036284 PYCP/M

ภ.ม. (เภสัชกรรมคลินิก)

คณะกรรมการที่ปรึกษาวิทยานิพนธ์ : สุรกิจ นาฑีสุวรรณ, Pharm.D., BCPS., ปุณณฤกษ์ ทองเจริญ, พ.บ., วว. (ศัลยกรรมหัวใจและทรวงอก), จุฬาลักษณ์ โกมลตรี, Dr.PH., อรุณรัตน์ ลักษณ์ศิริ, Ph.D.

บทคัดย่อ

วาร์ฟารินเป็นยาต้านการแข็งตัวของเลือดชนิดรับประทานที่มีขนาดยาแตกต่างระหว่างบุคคลมากทำให้การใช้ยาในทางคลินิกเป็นเรื่องยาก รวมทั้งมีปัจจัยหลายอย่างที่ส่งผลต่อการตอบสนองของยาในแต่ละบุคคล ได้แก่ อายุ การเกิดอันตรกิริยาระหว่างยากับยา การมีโรคร่วม การรับประทานอาหารที่มีวิตามินเค การดื่มเครื่องดื่มแอลกอฮอล์ ขนาดตัว และเชื้อชาติ ในปัจจุบันพบว่าความผันแปรของยีนมีผลอย่างมากต่อขนาดยาและการตอบสนองของยาวาร์ฟารินได้แก่ยีน cytochrome P450 2C9 (*CYP2C9*) และ vitamin K epoxide reductase complex 1 (*VKORC1*) การศึกษานี้มีวัตถุประสงค์เพื่อหาความสัมพันธ์ระหว่างยีน *CYP2C9, VKORC1* และปัจจัยทางคลินิกกับขนาดยาวาร์ฟาริน นำไปสู่การพัฒนาสูตรคำนวณหาขนาดยาวาร์ฟารินที่เหมาะสมในผู้ป่วยไทย การศึกษานี้เป็นการวิจัยแบบย้อนหลังในผู้ป่วยเปลี่ยนลิ้นหัวใจเทียมแบบโลหะ จำนวน 197 ราย จากหน่วยตรวจโรคศัลยกรรมหัวใจและทรวงอก โรงพยาบาลศิริราช โดยมีการใช้ขนาดยาวาร์ฟารินคงที่และมีค่าการแข็งตัวของเลือด (International Normalized Ratio; INR) อยู่ในช่วงเป้าหมายการรักษา (2.0-3.0) เลือดของผู้ป่วยจะถูกนำไปสกัดดีเอ็นเอและวิเคราะห์หายีน *CYP2C9* และ *VKORC1* จีโนไทป์ ด้วยเครื่อง LightCycler®480

ผลการศึกษาพบว่า ผู้ป่วย 182 ราย (ร้อยละ 92.4) มี *CYP2C9*1/*1* และ 15 ราย (ร้อยละ 7.6) มี *CYP2C9*1/*3* ในส่วนของ *VKORC1* ยีนพบ haplotype AA 114 ราย (ร้อยละ 57.9) haplotype AB 69 ราย (ร้อยละ 35) และ haplotype BB 14 ราย (ร้อยละ 7.1) ขนาดยาวาร์ฟารินเฉลี่ยในกลุ่มผู้ป่วยที่มี *CYP2C9*1/*1* สูงกว่ากลุ่มผู้ป่วยที่มี *CYP2C9*1/*3* อย่างมีนัยสำคัญทางสถิติ 4.07 ± 1.67 มิลลิกรัม เปรียบเทียบกับ 2.42 ± 0.75 มิลลิกรัม ($p = 0.004$) ในส่วน *VKORC1* ยีนพบว่า ขนาดยาวาร์ฟารินเฉลี่ยในกลุ่มผู้ป่วย haplotype BB มากที่สุดคือ 7.03 ± 1.58 มิลลิกรัม รองลงมาเป็นกลุ่มผู้ป่วย haplotype AB มีขนาดยาวาร์ฟารินเฉลี่ย 4.81 ± 1.43 มิลลิกรัม และขนาดยาวาร์ฟารินเฉลี่ยน้อยที่สุดในกลุ่มผู้ป่วย haplotype AA คือ 3.04 ± 0.94 มิลลิกรัม ซึ่งทั้งสามกลุ่มมีขนาดยาวาร์ฟารินเฉลี่ยแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($p < 0.001$) ในส่วนของปัจจัยทางคลินิก พบว่า อายุ และน้ำหนัก มีความสัมพันธ์กับขนาดยาวาร์ฟารินอย่างมีนัยสำคัญทางสถิติ ($p = 0.013$) และ ($p <0.001$) ตามลำดับ เมื่อนำปัจจัยทางพันธุศาสตร์และปัจจัยทางคลินิกมาพัฒนาเป็นสูตรในการคำนวณหาขนาดยาวาร์ฟารินพบว่าสามารถทำนายขนาดยาวาร์ฟารินได้สูงสุดร้อยละ 60.6 ซึ่งสามารถนำไปใช้ในการเลือกขนาดยาวาร์ฟารินที่เหมาะสมในผู้ป่วยไทย อย่างไรก็ตามการนำสูตรคำนวณหาขนาดยาวาร์ฟารินที่เหมาะสมไปใช้ประโยชน์ในทางปฏิบัตินั้นควรมีการศึกษาขั้นต่อไป

113 หน้า

CONTENTS

CONTENTS (cont.)

CONTENTS (cont.)

LIST OF TABLES

LIST OF TABLES (cont.)

LIST OF FIGURES

LIST OF FIGURES (cont.)

LIST OF ABBREVIATIONS

AF	Atrial Fibrillation
ALT	Alanine aminotransferase
APOE	Apolipoprotein E
AST	Aspartate aminotransferase
AVR	Aortic valve replacement
CVT	Cardiovascular and Thoracic
CYP	Cytochrome P450
DNA	Deoxyribonucleic acid
DVT	Deep vein thrombosis
EM	Extensive metabolizer
FDA	Food and drug administration
GGCX	γ- glutamyl carboxylase
INR	International normalize ratio
IM	Immediate metabolizer
ISI	International sensitivity index
LMWH	Low molecular weight heparin
MI	Myocardial infarction
MVR	Mitral valve replacement
PCR	Polymerase chain reaction
PE	Pulmonary embolism
PM	Poor metabolizer
PT	Prothrombin time
SNP	Single nucleotide polymorphism
UM	Ultrarapid metabolizer
VKOR	Vitamin K epoxide reductase
VKORC1	Vitamin K epoxide reductase subunit complex 1
WHO	World health organization

CHAPTER I
INTRODUCTION

Warfarin, a vitamin K antagonist, is the commonest oral anticoagulant. It is indicated for the prevention and treatment of several thromboembolic disorders, such as deep vein thrombosis (DVT), pulmonary embolism (PE), to reduce the risk of embolism in atrial fibrillation (AF), prosthetic heart valve, and after myocardial infarction (MI).[1]

The average daily dose of warfarin is usually initiated at five or ten milligrams in Caucasians and three milligrams in Asians. Then adjusted several weeks to reach optimal therapeutic rage for each patient as followed by the International Normalized Ratio (INR) [2, 3]. Achieving a safe and effective warfarin maintenance dose can take weeks or months after the beginning of therapy. The final maintenance dose has wide interindividual variability, with some patients requiring a sixteen-fold higher dose than other patients [3, 4].

During this dose adjustment period the patients have not only the risk of undesirable clotting due to lower anticoagulation, but also excessive bleeding due to the over anticoagulation [5]. Adverse events from warfarin are more common during the initial months of treatment before the optimal dose is determined [6-8]. The risk of bleeding during the first month of therapy is a significant concern, especially for outpatients not having daily INR testing [2, 9]. With its narrow therapeutic index, the management of the anticoagulated patient is still complicated to maintain target INR level.

Numerous variables have been associated with warfarin dose requirements including age, drug interactions (e.g., amiodarone), co-morbidities (e.g., liver disease), consumption of vitamin-K containing food supplements, alcohol intake, factors related to body mass (body mass index, body surface area, height, weight, gender), and ethnicity have been described to this variability [10]. Recently, a number of genetic variations have been shown to have a significant effect on warfarin response and

dosage requirement up to approximately 40 percent [11-13]. The two most studied genes are those encoding for the enzymes cytochrome P450 2C9 (*CYP2C9*) and vitamin K epoxide reductase complex 1 (*VKORC1*) [3].

CYP2C9 is the major enzyme responsible for metabolize S-warfarin, the enantiomer with the majority of warfarin's therapeutic effect. Numerous single nucleotide polymorphisms (SNPs) in the gene encoding for CYP2C9 have been correlated with reduced enzyme activity, and decreased clearance of S-warfarin [14].

The most common allele for *CYP2C9* is *CYP2C9*1* (wild-type genotype). Patients with a *CYP2C9*1* are classified as extensive metabolizer of warfarin. Whereas two common variant alleles, *CYP2C9*2* and *CYP2C9*3* are associated with a poor metabolizer of warfarin. Studies conducted in Asian population including Thais indicate that frequencies of these variants are low, only less than 5% for *CYP2C9*3* and non-existent for *CYP2C9*2* [15].

The pharmacological target for warfarin is inhibition of the enzyme vitamin K epoxide reductase, VKOR. The enzyme VKOR is encoded by a gene known as the VKOR complex subunit 1 (*VKORC1*). Three haplotypes (A/A, A/B, and B/B) are associated with varying degrees of VKOR enzyme activity. Haplotype A is associated with low dose phenotype, and haplotype B is associated with high dose phenotype. Studies indicate that a wide variation in *VKORC1* haplotypes exists among each ethnic group. For Asian population, haplotype A is found at much higher frequency than other races [15]. This may partly explain why Asian population tends to require much lower warfarin dose compared to other races. Studies conducted among Thai patients confirm this finding.

In the past, several attempts have been made to develop dosing algorithms to promote accurate warfarin dosing. However, such algorithms rely only on clinical factors (such as age, weight, height and sex) which lead to poor results. Recently, a number of researchers have developed new dosing algorithms by incorporating *CYP2C9* and *VKORC1* genotype along with clinical factors which lead to a more accurate warfarin dose prediction [11, 12, 16-24]. However, several barriers exist for the adoption of such dosing algorithms to a different population. According to the results from the retrospective data conducted as a preliminary study in Thai patients, such dosing algorithms were evaluated. The results show much lower accuracy of

warfarin dosing than the original studies and may be unreliable to be used locally [25]. Several factors may help explain such discordance including the validity of retrospective clinical data, differences in races which algorithms were derived, differences in environmental factors and low frequency of some genotypes.

In conclusion, the correct maintenance dose of warfarin for a given patient is difficult to predict, the drug carries a high risk of bleeding, and variability among patients. Moreover, pharmacogenomic warfarin dosing has been suggested to produce more accurate dosing and an improved patient safety profile; however, very few models have been derived in Thai patients. So, the development of algorithms that combine all of the genetic and clinical factors into individualized predictive models for warfarin dose could be used to explain the results of pharmacogenetic testing into actionable clinical application.

As a result, the objective of this study was to determine the relationship between *CYP2C9, VKORC1* genotypes and clinical factors on the warfarin dose requirements followed by develop the warfarin dosing formula in Thai patients with mechanical heart valve at the cardiovascular and thoracic unit (CVT unit). The result will be used as a clinical support tool that can guide individualized dosage regimen and predict dosage of warfarin better than trial and error dosing based on INR evaluation. In addition to increase the efficacy and safety of warfarin therapy and to reduce the cost of adverse drug reaction of this medicine in the future.

CHAPPTER II
LITERATURE REVIEW

2.1 Pharmacogenetics and pharmacogenomics [26, 27]

The term pharmacogenetics/pharmacogenomics was formally defined by Friedrich Vogel in 1959 as “the role of genetics in drug responses”. It refers to the study of genes that alter drug action was published by Werner Kalow [28]. Pharmacogenetics generally refers to the study of variations in a single gene, whereas pharmacogenomics is the study of variations in multiple genes. DNA, or deoxyribonucleic acid, sequence variation occurs when a single nucleotide (A, C, G or T) in the genome sequence is altered (single nucleotide polymorphisms or SNPs). A mutation in the DNA sequence or genetic polymorphism is present in at least 1% of the population. These genetic polymorphisms may affect the pharmacokinetics due to the defect in the molecule of a transporter that metabolizes the enzyme or other factor of absorption, distribution, interaction with target structure and finally alteration with excretion, resulting in high or low concentrations of the drug in the human. Based on these polymorphic enzymes there are four characteristic phenotypes: extensive (normal) metabolizers (EMs), intermediate metabolizers (IMs), poor metabolizers (PMs) and ultrarapid metabolizers (UMs). With the completion of the human genome project, about 12 million true SNPs have been identified to date. SNPs can be present in the coding part (cSNPs) but also in the non-coding part of the genome. Testing and identifying SNPs would help to establish a personalized therapy, to avoid some side effects and to administer the right drug and the right dose.

Recent advances in the field of pharmacogenomics have resulted in a focus on the development of warfarin dosing algorithms that include genetic and non-genetic information. Pharmacogenetic-based warfarin dosing has the potential to bring individualized therapy. It has the potential to reduce the risk for bleeding, increase dosing accuracy, shorten the time to dose stabilization, and help identify individuals who may require more frequent monitoring with long-term therapy.

2.2 Pharmacology of Warfarin

Warfarin or 3-(α-acetonylbenzyl)-4-hydroxycoumarin) (Figure 2.1) is the most common oral anticoagulant widely used in the treatment and prevention of thromboembolism [15, 29, 30]. It produces an anticoagulant effect by inhibiting two enzymes; vitamin K quinone reductase and vitamin K epoxide reductase. Consequently, the synthesis of vitamin K dependent clotting factors (II, VII, IX, and X), which are all essential for thrombus formation turn into nonfunctional. In the normal coagulation cascade, factors II, VII, IX, and X require carboxylation reaction to become functional. The γ-glutamyl-carboxylase is the enzyme to catalyze this reaction which needs vitamin K hydroquinone as a source of protons to complete carboxylation. This carboxylation due to the addition of carbon dioxide and oxygen to form a carboxyl group to glutamate residues on the N- terminal regions of vitamin K-dependent proteins turn into functional clotting factors. In this process, vitamin K is oxidized to vitamin K 2, 3- epoxide and is recycled by vitamin K epoxide reductase complex subunit 1 (VKORC1). VKORC1 represents the target of anticoagulant therapy which is inhibited by coumadin derivative and converts oxidized vitamin K to reduced form. Mechanism of action of warfarin is shown in Figure 2.2. In addition to their anticoagulant effect, the vitamin K antagonists inhibit carboxylation of protein C and S and therefore have the potential to exert a procoagulant effect [31].

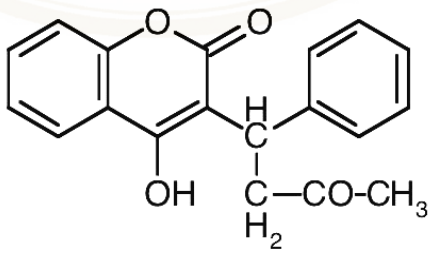


Figure 2.1 Structure of warfarin

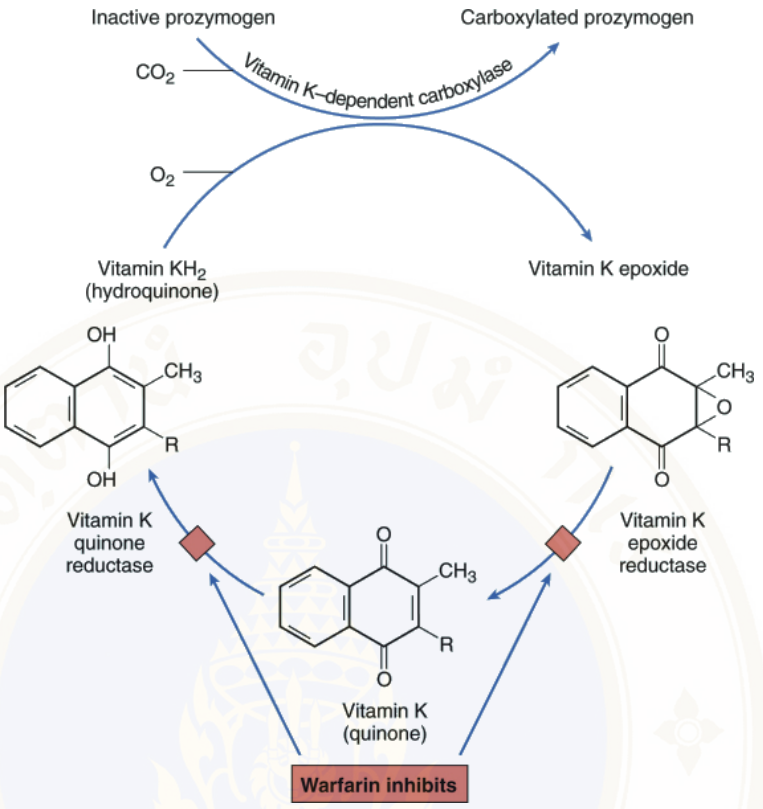


Figure 2.2 Mechanism of action of warfarin

2.3 Pharmacokinetics and pharmacodynamics of warfarin

Warfarin is a racemic mixture of R- and S- enantiomers (Figure 2.3). The S- enantiomer is three to five times more potent than R- enantiomer. At steady state approximately two thirds of anticoagulant response is due to the S- enantiomer. Knowledge of pharmacokinetics of warfarin is helpful in understanding the initial response to therapy. After oral administration, warfarin is rapidly and extensively absorbed by the gut and reaches maximum blood levels within 90 minutes [15]. Its half-life ranges from 36 to 42 hours. Ninety percent of the drug is bound to albumin in the circulation, but only the free form is pharmacologically active. In humans, warfarin is metabolized to five different monohydroxylated metabolites (4′-,6-,7-,8- and 10-hydroxywarfarin). Furthermore, cis- and trans-dehydrowarfarin and two stereoisomeric alcohols have been found (Figure 2.4) [32]. The metabolites of warfarin are excreted in urine and feces eighty percent and twenty percent, respectively. The principle enzyme involved in warfarin metabolism is CYP2C9,

which metabolizes *S*- enantiomer more than ninety percent, while other CYPs, including the isoenzymes CYP1A2, 2C8, 2C19 and 3A4, are more involved in hydroxylation of the *R*-enantiomer [33-35].

R-warfarin S-warfarin

Figure 2.3 Isomer forms of warfarin

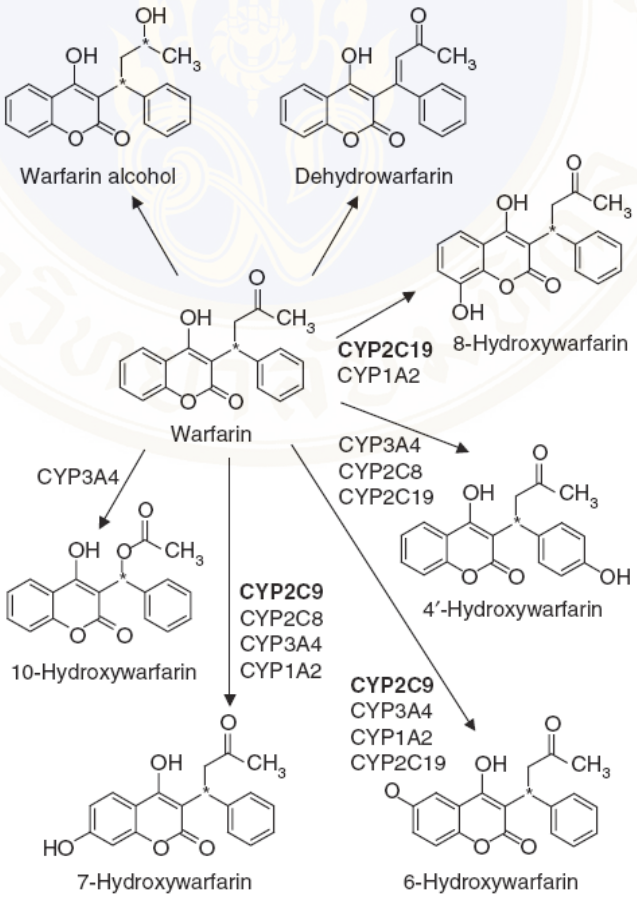


Figure 2.4 Metabolism of warfarin

The relationship between the dose of warfarin and the response is highly variable among individual. In addition to known and unknown genetic factors, environmental factors such as drugs, diet, and various disease states can interfere with the response to warfarin [36].

2.3.1 Drug- drug interactions

Warfarin combines 3 unfavorable properties which make them prone to potentially life threatening drug-drug interactions: (1) high protein binding; (2) cytochrome P450 dependent metabolism; and (3) a narrow therapeutic range. The complex response of warfarin to concomitant drug therapy makes it difficult to predict the occurrence and degree of a deterioration of anticoagulant control in individual patients. For clinical practice, it seems advisable that one should monitor for changes in prothrombin time when adding or deleting any newly approved drug or any drug suspected to cause an interaction to patients on warfarin therapy. The onset of the adverse prothrombin time response might be from between 1 to 2 days up to 3 weeks after starting a concomitant drug regimen [37].

Drug interactions influence the pharmacokinetics of warfarin by reducing gastrointestinal absorption, alteration of protein binding or disrupting metabolic clearance. The inhibition of S-warfarin metabolism is more important clinically, because this isomer is five times more potent than the R-isomer [29, 36, 38, 39]. Other drugs may also influence the pharmacodynamics of warfarin by inhibiting the synthesis of or increasing the clearance of vitamin K-dependent coagulation factors or by interfering with other pathways of haemostasis such as impairment of platelet function [29, 36, 38-42]. The following medications as shown in Table 2.1 may increase or reduce response to warfarin therapy [29, 33, 36, 38, 41, 43].

Table 2.1 Common drug interactions with warfarin

Warfarin effect increased	Warfarin effect decreased	Little or no effect
Acetaminophen	Barbiturates	Alcohol
Acetylsalicylic acid	Carbamazepine	Antacids
Alcohol (if concomitant liver disease)	Cholestyramine	Atenolol
Amiodarone	Dicloxacillin / Nafcillin	Bumetadine
Antibiotics:	Griseofulvin	Diltiazem
▪ Cephalosporins (Cefamandole, Cefazolin, Cefoperazone)	Rifampin	Famotidine
	Sucralfate	Fluoxetine
	Trazodone	Ketorolac
▪ Cotrimoxazole		Metoprolol
▪ Erythromycin		Nizatidine
▪ Isoniazid (600 mg daily)		Psyllium
▪ Metronidazole		Ranitidine
▪ Quinolones (Ciprofloxacin Nalidixic acid, Norfloxacin, Ofloxacin)		Vancomycin
▪ Tetracycline		
Antifungals:		
▪ Fluconazole		
▪ Itraconazole		
▪ Ketoconazole		
▪ Miconazole		
Antineoplastics		
▪ Fluorouracil		
▪ Ifosphamide		
Cimetidine		
Clofibrate / Gemfibrozil		
Disulfiram		
Heparin		
HMG-CoA reductase inhibitors		
▪ Fluvastatin		
▪ Lovastatin		
▪ Simvastatin		

Table 2.1 Common drug interactions with warfarin (continued.)

Warfarin effect increased	Warfarin effect decreased	Little or no effect
NSAIDs: ▪ Ibuprofen ▪ Indomethacin ▪ Ketoprofen ▪ Naproxen ▪ Phenylbutazone ▪ Piroxicam ▪ Sulindac ▪ Sulfinpyrazone Omeprazole Quinidine Sulfisoxazole		

HMG-CoA = 3-hydroxy-3-methylglutaryl-coenzyme A

NSAIDs = Non-steroidal anti-inflammatory drugs

In general, enzyme inducers are relatively slow in onset. This rises in metabolic enzymes and increases the metabolism of drugs, resulting in lower plasma concentrations with the risk of therapeutic failure. Inducers also have a long offset of action for several weeks, so it is important to monitor the patient and gradually reduce the dose of the target drug when inducer is withdrawn. The primary determinant of the onset and offset is the half-life of the inducing agent. In contrast, enzyme inhibitors have a rapid onset of action, between 24 to 48 hours. They decrease the metabolism of target drugs resulting in increased blood levels, which may lead to toxicity. The extent of the enzyme inhibition is often dose related and there is a rapid offset of action for 48 to 96 hours. Drug interactions due to enzyme inhibition are probably the most common and clinically significant drug interactions [44].

Herb-drug interactions are based on the same pharmacokinetics and pharmacodynamics mechanisms as drug-drug interactions. Because all herbal medicines are mixtures of more than one active ingredient, they obviously increase the likelihood of herb-drug interactions [45]. Interactions between warfarin and herbal medicines are summarized in Table 2.2 [33, 45-47].

Table 2.2 Warfarin - herb interactions

Herb	Mechanism of potential interaction	Clinical effects
Boldo [*Penmus boldus*]	Boldo contain anticoagulant coumadins.	Increase INR
Coenzyme Q_{10} (ubiquinone or ubidecarenone)	Ubidecarenone, structurally related to menaquinone (vitamion K_2) and may have procoagulant effects.	Decrease INR
Danshen [*Salvia miltiorrhiza*]	Inhibits absorption rate and metabolism of warfarin, as well as reduce the elimination half-life and inhibits platelet aggregation.	Increase INR Prolonged PT/PTT
Devil's Claw [*Harpagophytum procumbens*]	Unknown of mechanism.	Increase INR and risk of bleeding
Dong Quai [*Angelica sinensis*]	Contains at least 6 coumarin derivatives, may exert an antithrombotic effect by inhibits platelet activation and aggregation.	Increase INR and risk of bleeding
Feverfew [*Tanacetum parthenium*]	Inhibit platelet activity by neutralizing platelet sulfhydryl groups, as well as preventing prostaglandin synthesis.	Increase INR and risk of bleeding
Fish oil	Inhibit platelet aggregation.	Increase INR
Garlic [*Allium sativum*]	Interrupt thromboxane synthesis and inhibit platelet function.	Increase INR and risk of bleeding
Ginger [*Zingiber officinale*]	Potent inhibitor of thromboxane synthetase, which prolongs bleeding time.	Increase INR and risk of bleeding
Ginseng [*Panax ginseng*]	Unknown of mechanism.	Decrease INR
Ginkgo [*Ginkgo biloba*]	Ginkgolides B from ginkgo have antiplatelet activity and inhibits platelet-activating factor (PAF) by displacing it from its receptor-binding site, resulting in reduced platelet aggregation.	Increase INR and risk of bleeding
Green tea [*Camellia sinensis*]	Dried green tea leaves contain substantial amounts of vitamin K. Brewed green tea is generally not considered a significant source of the vitamin. However, large amounts may be potentially antagonizing effects of warfarin.	Decrease INR

Table 2.2 Warfarin - herb interactions (continued.)

Herb	Mechanism of potential interaction	Clinical effects
Mango [*Mangifera indica*]	Hepatic enzyme inhibition. Mango contains high amounts of vitamin A and human studies have shown that vitamin A (retinol) inhibits CYP2C19 enzymes.	Increase INR
Papaya [*Carica papaya*]	Unknown of mechanism.	Increase INR
Soy [*Glycine max*]	Unknown of mechanism.	Decrease INR
St. John's wort [*Hypericum perforatum*]	Hepatic enzyme induction. Warfarin is metabolized by CYP 3A4 which is induced by St. John's wort.	Decrease INR
Vitamin E (more likely if dose > 400 IU/day)	May inhibit the oxidation of reduced vitamin K which is necessary for carboxylation of vitamin K-dependent clotting factors.	Increase INR
Bromelain Cassio Clove Onion	Inhibition of platelet aggregation.	Increase risk of bleeding
Meadowsweet Poplar Willow bark	Contain salicylate derivatives and may interfere platelet aggregation.	Increase risk of bleeding
Borage seed	Contain γ-linoleic acid, which may increase coagulation time.	Increase risk of bleeding
Bogbean	Noted to demonstrate hemolytic activity.	Increase risk of bleeding
Capsicum	Cause hypocoagulability.	Increase risk of bleeding

Table 2.2 Warfarin - herb interactions (continued.)

Herb	Mechanism of potential interaction	Clinical effects
Alfalfa Angelica root Anise Arnica flower Artemesia Asafoetida Celery Chamomile Fenugreek Horse chestnut Licorice root Lovage root Parsley Passionflower Quassia Red clover Sweet woodruff Tonka beans	Contain coumarin derivatives	Increase risk of bleeding

Theoretically, increased anticoagulant effects could be expected when warfarin is combined with coumarin-containing herbal medicines (e.g. boldo, fenugreek and don quai) but other compounds may interact with warfarin by increasing the risk of bleeding without interfering with the anticoagulant effect. Garlic and ginkgo both inhibit platelet aggregation. Other herbal products, including extracts from cassio, clove, feverfew, ginger, and onion may cause a similar interaction. Similarly, bleeding complications may result from use of meadowsweet, poplar, and willowbark, as these compounds contain salicylate derivatives which may interfere with platelet aggregation [39]. Vitamin A inhibits enzyme CYP2C19 which causes an increased anticoagulant effect [45]. Conversely, vitamin K-containing herbs (e.g. green tea) can antagonize the anticoagulant effect of warfarin [46].

2.3.2 Dietary intake

Dietary intake of vitamin K is an important source of intraindividual variability over time in response to warfarin [40]. Excessive vitamin K consumption can promote increased production of the vitamin K clotting factors, decreasing the anticoagulant response to warfarin. Alternatively, decreased vitamin K consumption can increase the anticoagulant response to warfarin [29, 33, 36, 38, 40, 41, 43]. Foods such as green, leafy vegetables and certain vegetable oils appear to have the highest content, but foods that contain lower amounts may become appreciable sources of vitamin K if large amounts are ingested [40]. The content of vitamin K in common foods are shown in Table 2.3 [33].

Patients receiving warfarin are routinely counseled as to which foods may contain amounts of vitamin K that may antagonize their warfarin therapy. To maintain coagulation stability, patients need to maintain vitamin K intake patterns with as little variation as possible [40, 44]. Moreover alternative sources of vitamin K, such as multivitamins and nutritional supplements should also be considered [33].

Table 2.3 Vitamin K content of common foods

Very high (>200 μg)	**High (100-200 μg)**	**Medium (50-100 μg)**	**Low (<50 μg)**
Brussels sprouts	Basil	Apple	Avocado
Chick pea	Broccoli	Asparagus	Beans
Collard greens	Chive	Cabbage	Breads
Lettuce	Coleslaw	Mayonnaise	Carrot
Liver	Cucumber with peel	Pistachio nuts	Celery
Parsley	Mustard greens	Squash	Cereal
Spinach	Soybean oil		Coffee
Tea (black/green)			Corn
Turnip greens			Cucumber without peel
Watercress			Dairy products
			Eggs
			Peanuts
			Peas
			Potato
			Tomato

2.3.3 Concomitant diseases

Several illness or co-morbid diseases can influence anticoagulation response to warfarin therapy such as congestive heart failure can cause hepatic congestion of blood flow and inhibit warfarin metabolism. This can be troublesome in patients with frequent exacerbations of heart failure. Hepatic dysfunction potentiates the response to warfarin through the impaired synthesis of coagulation factors. For patients with renal dysfunction, the activity of warfarin is increase because of the total protein and albumin levels are decreased. Hypermetabolic states such as fever is also affect warfarin's response as same to hyperthyroidism. Hypothyroidism decreases the catabolism of the vitamin K clotting factors. Therefore, hypothyroidism of new onset or due to inadequate replacement therapy could be suspected if there is a general trend toward decreased INR values with a need for increased warfarin doses. In contrast, hyperthyroidism increases the catabolism of the vitamin K clotting factors and could be suspected if there is a general trend toward increased INR values with a need for decreased warfarin doses. In patient with diarrhea, warfarin responsiveness increases because the intestine normal flora, that produces vitamin K, is eliminated [29, 33, 36, 38, 42, 44, 48].

Table 2.4 Drug-disease interactions of warfarin [49]

Diseases	Warfarin effect increased	Warfarin effect decreased
Cancer	✓	
Collagen vascular disease	✓	
Diarrhea	✓	
Fever	✓	
Hyperthyroidism	✓	
Hypothyroidism		✓
Heart failure	✓	
Hepatic disease	✓	
Malnutrition	✓	
Nephrotic syndrome		✓
Steatorrhea	✓	
High vitamin K intake		✓
Vitamin K deficiency	✓	

Common drug-disease interactions are summarized in Table 2.4. In addition, variability in anticoagulant response also results from inaccuracies in laboratory testing, patient noncompliance, and miscommunication between the patient and physician [29, 36].

2.4 Laboratory monitoring

The prothrombin time (PT) is the common test used in monitoring warfarin therapy [29]. This test is performed by measuring the time required for clot formation after calcium and a thromboplastin have been added to citrate plasma [50]. Typical sources of thromboplastin are rabbit brain and human placenta. Prolongation of PT depends on reductions in three of the vitamin K-dependent clotting factors (II, VII, and X) that are reduced by warfarin at a rate proportional to their respective half-lives. During the first few days of warfarin therapy the PT reflects mainly a reduction of factor VII, the half-life of which is approximately 6 hours. Subsequently, the reduction of factors X and II contributes to prolongation of the PT [29, 36].

The PT assay is problematic because of the variations in thromboplastin sensitivity and the different ways of reporting PT [29, 33, 36]. Given the same blood sample, different thromboplastins will produce substantially different results [33, 50]. Consequently, the World Health Organization (WHO) addressed the need for standardization in 1970s when it developed reference thromboplastins and recommends the use of the International Normalized Ratio (INR) to monitor warfarin therapy. The INR value can be calculated by the following equation [29, 36, 38, 50]:

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

Where the patient's PT is divided by the standard normal mean PT and this ratio is raised to power of the International Sensitivity Index (ISI) of the thromboplastin. ISI is a correction factor assigned by the manufacturer, which is used

to measure and compare the variability in thromboplastin responsiveness [29, 36]. Thus the INR value is more reliable than the unconverted PT ratio, and is recommended during both initial and maintenance of warfarin treatment [38, 50].

2.5 Dosing and monitoring of warfarin therapy

For the initiation and maintenance dosing of warfarin, the dose of 5 mg is recommended. Starting dose of less than 5 mg might be appropriate for patients who sensitive to warfarin, including the elderly, patients with liver disease or impaired nutrition and patients who are at high risk of bleeding [29, 48]. In addition, a small study showed that 2.5 mg was suitable for starting dose while another study suggested that the dose of 2.5-3.75 mg daily for Thai people were most likely to provide therapeutic INRs [51, 52].

Administration of a loading dose, although common, does not appear to offer any advantage. Loading doses of warfarin (i.e., 10 mg or more per day) may increase the patient's risk of bleeding episodes early in therapy by eliminating or severely reducing the production of functional factor VII. The administration of loading doses is a possible source of prolonged hospitalization secondary to dramatic rises in INR that necessitate increased monitoring. Administration of loading doses has also been hypothesized to potentiate a hypercoagulable state because of severe depletion of protein C. The practice of using loading doses should be abandoned because it has no effect on the inhibition of thrombosis. A potential paradoxical consequence of loading doses is the development of a hypercoagulable state because of a precipitous reduction in the concentration of protein C (approximate half-life of eight hours) during the first 36 hours of warfarin therapy. Thus, loading doses theoretically may cause clot formation and/or expansion by limiting the production of proteins C and S, which have shorter half-life than prothrombin [38, 53].

After initiation of warfarin therapy, INR monitoring should be performed on a daily basis until the INR is within the therapeutic range for at least 2 consecutive days. Then INR monitoring should be performed 2 to 3 times a week for 1 to 2 weeks. If the patient remains stable, this interval can be widened to a monitoring frequency of once every 4 to 6 weeks, depending on the individual patient’s stability. If dosage

adjustments are necessary, INR monitoring should be performed more often until a new state of stability is achieved [36, 38, 44, 53, 54]. Monitoring of warfarin therapy is summarized in Table 2.5.

Patients should take their warfarin once a day, preferably at the same time, and have their INR test performed in the morning. This limits diurnal variations and provides the physician with a same day window for dosage adjustment in the event of an unanticipated INR change. The anticoagulant effect of warfarin persists beyond 24 hours. If patients forget to take the prescribed dose of warfarin at the scheduled time, the dose should be taken as soon as possible on the same day. The patient should not take the missed dose by doubling the daily dose to make up for missed doses.

Table 2.5 Monitoring frequency based on treatment phase [54]

Patient treatment phase	Monitoring frequency
Warfarin commencement	Daily until two consecutive therapeutic measurements
Initial therapeutic range	2-3 times/week for 1-2 weeks
Stable patient	Every 4-6 weeks
Potentially unstable/dosing change	2-3 times/week until therapeutic on two measurements

During the maintenance phase, dose changes may not be reflected in INR for 4-5 days. For these reasons, frequent dose changes should be avoided. The recent trend is to change the total weekly warfarin dose (TWD), rather than a daily dose. Adjusting a warfarin dose depends on the measured INR values and clinical factors. The dose does not to be adjusted for a single INR that is slightly out of range and most changes should alter the total weekly dose by 5% to 20% [29, 35, 36, 38, 53].

Unexpected fluctuations of the INR in an otherwise stable patient should be investigated. Often, it is possible to identify one or more causes, such as change in diet, poor compliance, undisclosed drug use, alcohol consumption and/or self-medication. If none of these causes can be identified, laboratory error should be

considered. When no cause for INR fluctuations can be determined, weekly dosage adjustment should be tried. The reduction or withholding of a single dose or an increase in that day's dose is often sufficient to restore a therapeutic INR in a patient who is otherwise medically stable [35, 38, 53].

2.6 Anticoagulant and antithrombotic effect of warfarin

The initial increase in the INR, anticoagulant activity, depends on the clearance of functional clotting factors from the systemic circulation after administration. This is dependent on the half-lives of the clotting factors (Table 2.6). On the other hand, the antithrombotic effect (inability to expand or form clots) of warfarin depends on the clearance of functional factor II, which has a half-life of 60 to 100 hours in patients with normal hepatic function. Because prothrombin has a half-life for long time, loading dose of warfarin are of limited value. In clinical practice, loading dose of warfarin may increase the patient's risk of bleeding complications early in therapy by eliminating the production of functional factor VII. Administration of loading doses may place a patient in a hypercoagulable state due to a severe depletion of protein C.

Table 2.6 Biologic half-life of vitamin K dependent coagulation proteins [33]

Coagulation proteins	Half-life (hours)
Factor II (Prothrombin)	60-100
Factor VII	4-6
Factor IX	20-30
Factor X	24-40
Protein C	8-10
Protein S	40-60

The safety and efficacy of warfarin therapy are dependent on maintaining the INR within the therapeutic range for the indication (Table 2.7). In most cases the target INR is 2.5 with an acceptable range of 2.0 to 3.0. The target INR is higher in

patients with mechanical prosthetic heart valves (target = 3.0, range 2.5 to 3.5) [55]. The desirable target range for warfarin is not the same for all indications. This is not only likely to be influenced by indications for its use but also by patient characteristics.

Table 2.7 Recommended therapeutic range for oral anticoagulant therapy

Indication	INR
Treatment of venous thrombosis	2.0-3.0
Treatment of pulmonary embolism	2.0-3.0
Prophylaxis of venous thrombosis	2.0-3.0
Prevention of systemic embolism	2.0-3.0
Tissue heart valves	2.0-3.0
After myocardial infarction	2.0-3.0
Valvular heart disease	2.0-3.0
Atrial fibrillation	2.0-3.0
Bileaflet mechanical valve in aortic position	2.0-3.0
Mechanical prosthetic valves (high risk)	2.5-3.5
Systemic recurrent emboli	2.5-3.5

2.7 Anticoagulation during pregnancy and lactation

Pregnancy is classically thought to be a hypercoagulable state. The hypercoagulable state during pregnancy is the physiological preparation for the haemostatic challenge of delivery. Factors responsible for the hypercoagulable state are increased levels of procoagulant factors, such as v. Willebrand factor, factor VIII, factor V, and fibrinogen, in addition to acquired resistance to endogenous anticoagulation system, activated protein C and a reduction in protein S. Furthermore, the activity of the fibrinolytic system is reduced through an increase in plasminogen activator inhibitors 1 and 2. As a consequence of the hypercoagulability, pregnant women are at risk for thromboembolic events [56-59].

Warfarin is contraindicated during pregnancy because it crosses the placenta and can produce a characteristic embryopathy (nasal hypoplasia and epiphyseal stippling) with first-trimester exposure and, less commonly, central

nervous system abnormalities and fetal bleeding with exposure after the first trimester. For this reason, it has been recommended that warfarin therapy be avoided during the first trimester of pregnancy and, except in special circumstances, avoided entirely throughout pregnancy [36, 56, 57].

Because heparin or low molecular weight heparin (LMWH) does not cross the placenta, it is the preferred anticoagulant in pregnant women. Several reports of heparin failure resulting in serious maternal consequences involving patients with mechanical heart valves, however, have caused some authorities to recommend that warfarin be used preferentially in women with mechanical prosthetic valves during the second and third trimesters of pregnancy. It even has been suggested that the inadequacy of heparin for prevention of maternal thromboembolism might outweigh the risk of warfarin embryopathy during the first trimester [36, 56-59]. Options for management of warfarin in pregnancy are available as following [36]:

(1) heparin or LMWH throughout pregnancy; or

(2) warfarin throughout pregnancy, changing to heparin or LMWH at 38 weeks' gestation with planned labor induction at approximately 40 weeks; or

(3) heparin or LMWH in the first trimester of pregnancy, switching to warfarin in the second trimester, continuing it until at 38 weeks' gestation, and then changing to heparin or LMWH at 38 weeks with planned labor induction at approximately 40 weeks

If heparin or LMWH is used in pregnant women with mechanical prosthetic valves, they should be administered in adequate doses and monitored carefully. Heparin should be given subcutaneously twice daily, starting at a total daily dose of 35,000 U. Monitoring should be performed at least twice weekly with either activated partial thromboplastin time or heparin assays, and higher heparin requirements should be anticipated in the third trimester because of an increase in heparin-binding proteins. LMWH should be given subcutaneously in a dose of 100 anti-Xa U/kg twice daily and the dose adjusted to maintain the anti-Xa level between 0.5 and 1.0 U/mL 4 to 6 hours after injection. Heparin or LMWH should be discontinued 12 hours before planned induction of labor. Heparin or LMWH should be started postpartum and overlapped with warfarin for 4 to 5 days [36].

Based on very limited published data, warfarin has not been detected in the breast milk of mothers treated with warfarin and can be safely given to women requiring therapeutic anticoagulation postpartum [36, 60, 61]. Therefore, women who are breastfeeding and anticoagulated with warfarin should be very carefully monitored.

2.8 Adverse drug reaction of warfarin

The main complication of warfarin therapy is bleeding, which occurs in 6 to 39 percent of recipients annually, and risk is closely related to the intensity of anticoagulation. There is a close relation between the INR and risk of bleeding. The risk of bleeding increases when the INR exceeds 4, and the risk rises sharply with values > 5 [36]. If bleeding occurs during warfarin therapy, the physician should immediately consider the severity of bleeding, the intensity of anticoagulation at the time of the bleeding episode and whether the patient has completed most of the prescribed course of therapy [38].

The anticoagulant effect of warfarin may be reversed by a variety of methods [36, 54, 62]. Options include simple dose omission or administration of vitamin K. For serious bleeding, the replacement of coagulation factors is required. The administration of fresh frozen plasma (FFP) has been the most widely used method for coagulation factor replacement. As a result of concern that FFP may not be the most effective way to reverse warfarin rapidly, prothrombin complex concentrates (PCCs) have been increasingly recommended. More recently, it has been suggested that recombinant activated factor VII (rFVIIa) may be effective. Data in Table 2.8 summarizes the different approaches to warfarin reversal.

Table 2.8 Options for warfarin reversal [62]

Type of reversal	Approach
Rapid (complete; within 10–15 minutes)	PCC (immediate replacement of vitamin K dependent coagulation factors) plus IV vitamin K (switch on hepatic synthesis within a few hours)
Fast (partial)	FFP (immediate replacement of vitamin K dependent coagulation factors but the correction of the coagulopathy is partial)
Prompt (within 4–6 hours)	IV vitamin K
Slow (within 24 hours)	Oral vitamin K
Ultraslow (over days)	Omit warfarin dose (no vitamin K)

PCC = prothrombin complex concentrate, IV = intravenous, FFP = fresh frozen plasma

Simple warfarin dose omission results in slow reversal of the effect of warfarin over several days. It has been known that vitamin K administration is an effective method of warfarin reversal [29, 33, 36, 38, 62].

Vitamin K1 (phytonadione) is a fat-soluble vitamin that requires normal pancreatic and small bowel function for absorption following oral intake [63].Vitamin K is available in several formulations that can be administered intravenously, subcutaneously, or orally [29, 36, 62]. Intravenous injection produces a rapid response but may be caused anaphylactoid reactions manifest by hypotension. Larger doses should be avoided, as the time required achieving therapeutic oral anticoagulation after surgery may be markedly delayed. Intramuscular injection should be avoided due to the risk of hematoma. The response to subcutaneous vitamin K1 is unpredictable and sometimes delayed because it has an erratic absorption [29, 54, 63]. In contrast, oral administration is predictably effective, has the advantages of convenience and safety [29], and is unlikely to result in warfarin resistance when the anticoagulant is re-instituted [54]. Administering FFP affords the most rapid correction (within hours) of hypoprothrombinemia but is more expensive, exposes the patient to potential infectious agents, and achieves an effect that is not durable (less than 24 hours) [54, 62]. Recommendations for the reversal of high INR values in patients with or without bleeding are summarized in Table 2.9 [29].

Table 2.9 Recommendations for managing elevated INRs or bleeding

Condition	Description
INR above therapeutic range but < 5.0; no significant bleeding	Lower dose or omit dose; monitor more frequently, and resume at lower dose when INR therapeutic; if only minimally above therapeutic range, no dose reduction may be required.
INR ≥ 5.0 but < 9.0; no significant bleeding	Omit next one or two doses, monitor more frequently and resume at lower dose when INR in therapeutic range. Alternatively, omit dose and give vitamin K1 (≤ 5 mg orally), particularly if at increased risk of bleeding. If more rapid reversal is required because the patient required urgent surgery, vitamin K1 (2 to 4 mg orally) can be given with the expectation that a reduction of the INR will occur in 24 hours. If the INR is still high, additional vitamin K1 (1 to 2 mg orally) can be given
INR ≥ 9.0; no significant bleeding	Hold warfarin therapy and give higher dose of vitamin K1 (5 to 10 mg orally); with the expectation that the INR will be reduced substantially in 24-48 hours. Monitor more frequently and use additional vitamin K1 if necessary. Resume therapy at lower dose when INR therapeutic.
Serious bleeding at any elevation of INR	Hold warfarin therapy and give vitamin K1 (10 mg by slow IV infusion), supplemented with fresh plasma or prothrombin complex concentrate, depending on the urgency of the situation; recombinant factor VIIa may be considered as alternative to prothrombin complex concentrate; vitamin K1 can be repeated every 12 hours
Life-threatening bleeding	Hold warfarin therapy and give prothrombin complex concentrate supplemented with vitamin K1 (10 mg by slow IV infusion); recombinant factor VIIa may be considered as alternative to prothrombin complex concentrate; repeat if necessary, depending on INR

The risk of bleeding is related to the intensity of anticoagulation. Other contributing factors are the underlying clinical disorder and concomitant administration of aspirin, non-steroidal anti-inflammatory drugs (NSAIDs), or other drugs that impair platelet function, produce gastric erosions, and in very high doses impair synthesis of vitamin K-dependent clotting factors [29, 36, 38].

Other than hemorrhage, the most important side effect of warfarin is skin necrosis [29]. Warfarin induced skin necrosis is a rare side-effect and is estimated to occur in only 0.01-0.1% of the patients taking the drug [64]. This uncommon complication usually is observed on the third to eighth day of therapy and is caused by

extensive thrombosis of venules and capillaries within subcutaneous fat [29, 64]. Warfarin-induced skin necrosis is manifested by a painful maculopapular rash and ecchymosis or purpura that progresses to necrotic gangrene. It appears to be associated with local thrombosis and most frequently in areas of the body rich in subcutaneous fat, such as the breasts, thighs, buttocks, and abdomen [33]. In severe cases, surgical intervention with wide local debridement, skin grafting or even amputation may be necessary (Figure 2.5) [64].

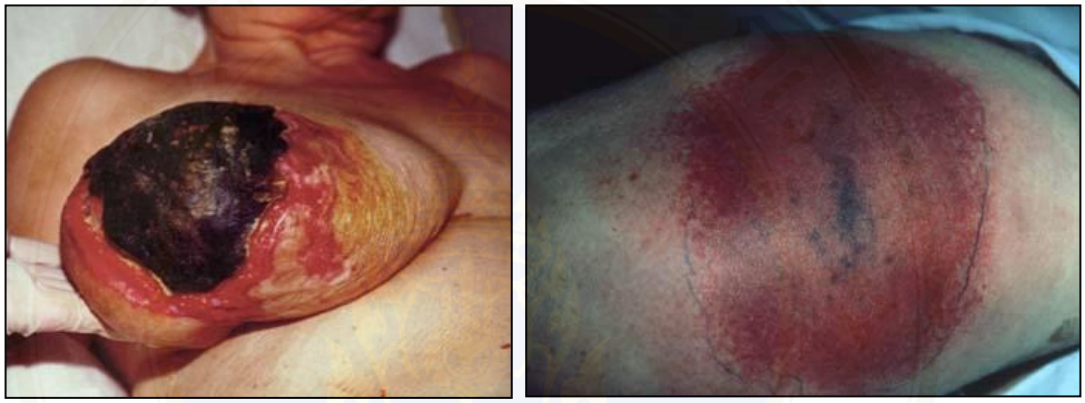


Figure 2.5 Warfarin induced skin necrosis

Non-haemorrhagic adverse effects associated with warfarin are uncommon, but can be serious when occur [33]. Purple toe syndrome is a complication of warfarin therapy characterized by a dark, purplish, or mottled color of the toes, usually occurring between 3 and 10 weeks, or later, after the initiations of therapy with warfarin [65, 66]. Major features of this syndrome includes purple color of plantar surfaces and sides of the toes that blanches on moderate pressure and fades with elevation of the legs, pain and tenderness of the toes, and waxing and waning of the color over time (Figure 2.6) [66]. While the purple toe syndrome is reported to be reversible, some cases progress to gangrene or necrosis that may require debridement of the affected area or may lead to amputation [33].

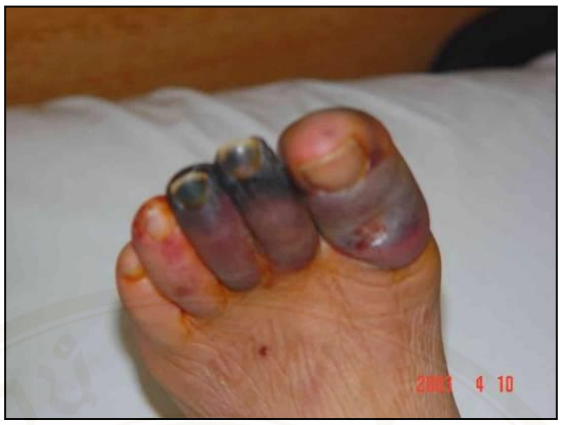


Figure 2.6 Purple toes syndrome related to warfarin therapy [66]

2.9 Warfarin pharmacogenetics pathways [67]

At present time, there are at least 30 genes possibly involved in the mechanism of action of warfarin (Figure 2.7). Biochemical reactions involved in the action of warfarin are the biotransformation of warfarin (transportation, metabolism, and cytochrome P450 inducibility) and biotransformation of vitamin K (transportation, the vitamin K cycle, vitamin K-dependent proteins and other coagulation proteins). Despite the vast number of genes involved in the mode of action of warfarin the *CYP2C9* and *VKORC1* genes are the most important with respect to the pharmacokinetics and pharmcodynamics of warfarin, respectively. The examples of famous gene polymorphism that alter anticoagulant effect of warfarin are *CYP2C9*, apolipoprotein E (*APOE*), *CCGX*, and *VKORC1*.

2.9.1 Transportation of warfarin

In the circulating blood, warfarin is 99% bound to albumin and alpha-1-acid glycoproteins. A study conducted by Nakagawa et al [68] shows that warfarin preferentially binds to certain genetic variants of alpha-1-acid-glycoproteins, which are encoded by *ORM1* and *ORM2* (orosomucoid gene 1 and 2 respectively). A recent study shows that polymorphisms and different haplotypes of theses two genes influence warfarin dose. Once warfarin has entered the liver, S-warfarin is metabolized by *CYP2C9* that has many polymorphisms. Polymorphisms in this gene play a significant role in warfarin dosage sensitivity. The induction of these P450

isoforms is dependent on the nuclear hormone receptors, pregnane X receptor (PXR) and the constitutive androstane receptor (CAR), encoded by the *NR1I2* and *NR1I3* genes, respectively. Haplotype analysis of the *NR1I2* gene shows some association with warfarin dosage.

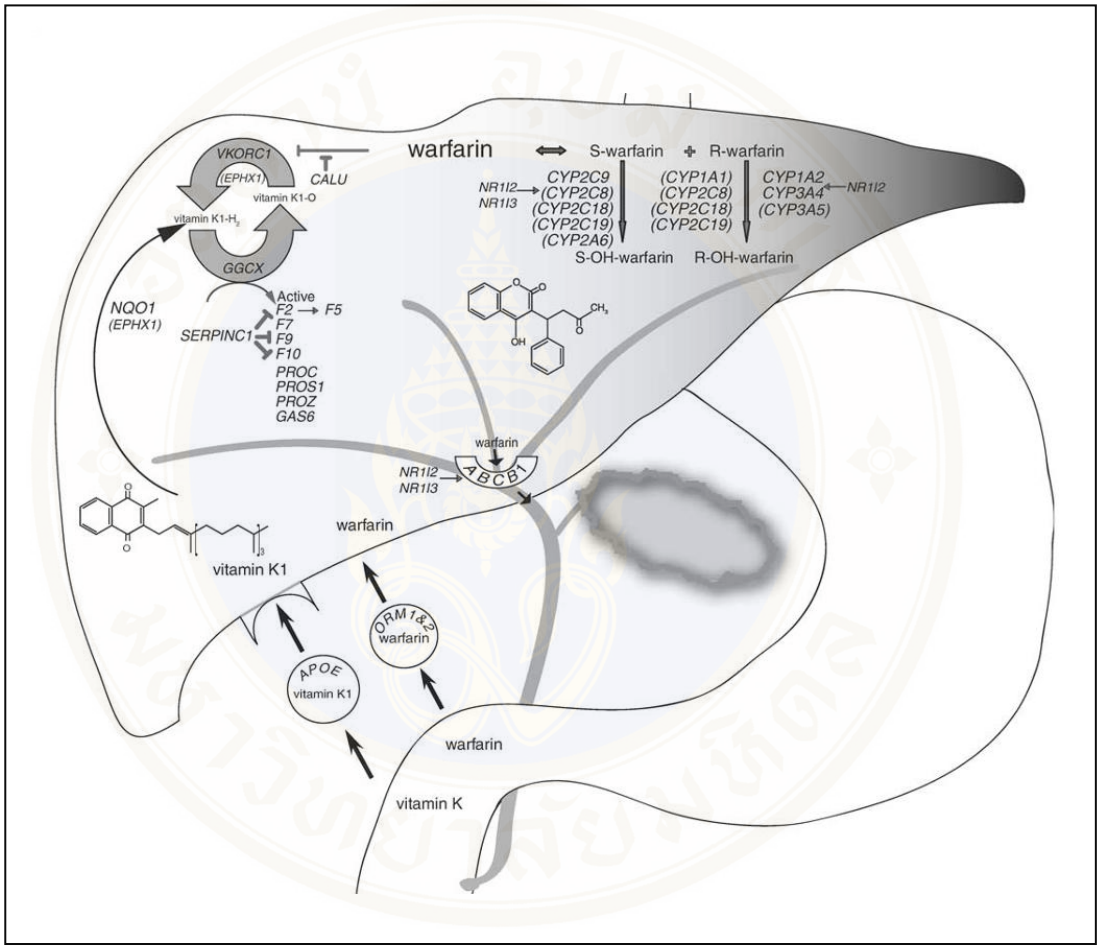


Figure 2.7 An overview of warfarin interactive pathway

2.9.2 Biotransformation of vitamin K

Vitamin K1 is absorbed from the small intestine along with dietary fat. It is transported by chylomicrons in the blood and subsequently cleared by the liver through an APOE (apolipoprotein E) receptor- specific uptake. The uptake of vitamin K1 varies depending on different *APOE* variants. Polymorphisms within *APOE* are significantly associated with warfarin dosage.

Mutations within the vitamin K epoxide reductase gene have been shown to confer warfarin resistance [69, 70]. However, one polymorphism in the promoter

region of this gene decrease warfarin dosage through a reduction in the VKOR (vitamin K epoxide reductase). It has been suggested that this reductase resides in the endoplasmic reticulum and may be complexed with microsomal epoxide hydrolase (encoded by *EPHX1*). It is this multiprotein complex that is responsible for vitamin K epoxide reduction. Polymorphisms in the *EPHX1* gene show a significant association with warfarin dose [67]. Nicotine adenine dinucleotide phosphate (NAD(P)H) dehydrogenase, encoded by *NQO1*, has the potential to reduce dietary vitamin K. The endoplasmic reticulum chaperone protein calumenin (encoded by *CALU*) is able to inhibit the vitamin K cycle. Polymorphisms in *CALU*, and not *NQO1*, are associated with warfarin dose [67].

Very rare autosomal recessive bleeding disorder, caused by mutations in the gamma glutamyl carboxylase gene (*GGCX*), results in the combined deficiency of the vitamin K- dependent coagulation factors II, VII, IX and X, and protein C, S and Z [69]. Mutations within this gene are associated with warfarin dose; however, the effect appears to be modest [67]. Similarly, mutations in the genes that encode these vitamin K-dependent factors and proteins may also influence warfarin dose, but studies have been inconclusive or contradictory [67]. Antithrombin III, a non-vitamin K-dependent protein, inhibits factor II, IX, X, XI and XIII. A deficiency in antithrombin III caused by mutation in its encoding gene *SERPINC1* may create a hypercoagulable state during warfarin induction. List of the genes implicated in the action of warfarin are summarized in Table 2.10.

Table 2.10 List of the genes implicated in the action of warfarin [67]

Protein name	Gene	Function of protein
Biotransformation of warfarin		
Transport		
Alpha-1-acid glycoprotein 1, Orosomucoid 1	*ORM1*	A plasma glycoprotein that functions as a carrier of warfarin in the blood
Alpha-1-acid glycoprotein 2, Orosomucoid 2	*ORM2*	A plasma glycoprotein that functions as a carrier of warfarin in the blood
P-glycoprotein, Multidrug resistance protein 1	*ABCB1 (MDR1)*	A cellular efflux pump for xenobiotics. Warfarin is a weak inhibitor and maybe a substrate

Table 2.110 List of the genes implicated in the action of warfarin [67] (continued.)

Protein name	Gene	Function of protein
Biotransformation of warfarin Metabolism		
Cytochrome P450 2C9	*CYP2C9*	Polymorphic hepatic drug metabolizing enzyme. Metabolism of S-warfarin
Cytochrome P450 1A1	*CYP1A1*	Extrahepatic oxidation, inducible. Metabolism of R-warfarin
Cytochrome P450 1A2	*CYP1A2*	Hepatic oxidation, inducible. Metabolism of R-warfarin
Cytochrome P450 2A6	*CYP 2A6*	Polymorphic hepatic drug metabolizing enzyme. Metabolism of S-warfarin
Cytochrome P450 2C8	*CYP 2C8*	Polymorphic hepatic drug metabolizing enzyme. Minor
Cytochrome P450 2C18	*CYP 2C18*	Found in the liver and lung. Minor pathway for R- and S-warfarin
Cytochrome P450 2C19	*CYP 2C19*	Polymorphic hepatic drug metabolizing enzyme. Minor
Cytochrome P450 3A4	*CYP 3A4*	Hepatic oxidation, inducible. Metabolism of R-warfarin
Cytochrome P450 3A5	*CYP 354*	Polymorphic hepatic and Extrahepatic oxidation. Metabolism of R-warfarin
Cytochrome P450 inducibility		
Pregnane X receptor (PXR)	*NR1I2*	Mediates induction of CYP2C9, CYP3A4, other CYP enzymes and ABCB1
Constitutive androstane receptor (CAR)	*NR1I3*	Transcriptional regulation of a number of genes including CYP2C9 and CYP3A4
Biotransformation of vitamin K Transport		
Apolipoprotein E	*APOE*	Apolipoprotein E serves as a ligand for receptors that mediate the uptake of vitamin K

Table 2.10 List of the genes implicated in the action of warfarin [67] (continued.)

Protein name	Gene	Function of protein
Biotransformation of vitamin K **Vitamin K cycle**		
Vitamin K epoxide reductase	*VKORC1*	A hepatic epoxide hydrolase that catalyses the reduction of vitamin K. The target of warfarin
Epoxide hydrolase 1, microsomal	*EPHX1*	A hepatic epoxide hydrolase in the endoplasmic reticulum that may be complexed with VKOR
NAD(P)H dehydrogenase, quinone 1	*NQO1*	A detoxifying enzyme that has the potential to reduce the quinine form of vitamin K
Calumenin	*CALU*	Bind to the vitamin K epoxide reductase complex and inhibits the effect of warfarin
Gamma-glutamyl carboxylase	*GGCX*	Carboxylates vitamin K-dependent coagulation factors and proteins n the vitamin K cycle
Vitamin K-dependent proteins		
Coagulation factor II, prothrombin	*F2*	Converts fibrinogen to fibrin activates FV, FVIII, FXI, FXIII, protein C
Coagulation factor VII	*F7*	Is converted to FVIIa and then converts FIX to FIXa and FX to FXa
Coagulation factor IX	*F9*	Makes a complex with FVIIIa and then converts FX to its active form
Coagulation factor X	*F10*	Converts FII to FIIa in the presence of factor Va
Protein C	*PROC*	Activated protein C counteracts coagulation together with protein S by inactivating FVa and VIIIa
Protein S	*PROS1*	Cofactor to protein C that degrades coagulation factors Va and VIIIa
Protein Z	*PROZ*	Is together with protein Z dependent protease inhibitor, a cofactor for the inactivation of FXa
Growth-arrest-specific protein 6	*GAS6*	Participates in many processes, for example, potentiation of agonist induced platelet aggregation

Table 2.10 List of the genes implicated in the action of warfarin [67] (continued.)

Protein name	Gene	Function of protein
Biotransformation of vitamin K		
Other coagulation proteins		
Anti-thrombin III	*SERPINC1*	Inhibits FIIa, FIXa, Xa, Xia and XIIa. Anti-thrombin deficiency increases risk of thrombosis
Coagulation factor V	*F5*	A cofactor that activates FII together with FXa, An F5 mutation leads to risk of thrombosis

At this time, there is limited evidence to support testing for only two genes: *CYP2C9*, *VKORC1*, and a limited number of polymorphism that impact warfarin dosing in a significant number of patients. There are few data available to guide the interpretation of finding of other variants.

2.10 Cytochrome P450 (CYP) 2C9 *(CYP2C9)* Gene [71, 72]

The *CYP2C9* gene is mapped to chromosome 10 of genomic DNA. It spans approximately 55 kb in length and codes for a protein consisting of 490 amino acids. To date there are more than 50 described variants within this gene. However, the functional effect of many of these variants is not well established.

The most common and best documented alleles are *CYP2C9*1*, *CYP2C9*2* and *CYP2C9*3*. Wild- type genotype is considered as *CYP2C9*1*. The SNP in exon 3, specifically a cytosine to thymidine conversion at position 430 (C430T), is designated as *CYP2C9*2* that encodes a protein with cysteine instead of arginine at position 144, while the SNP in exon 7, an adenine to cytosine conversion at position 1075 (A1075C), is denoted as *CYP2C9*3* that produces amino acid substitution from an isoleucine to leucine at position 359 (Table 2.11).

The major pharmacokinetic change associated with CYP2C9 SNPs is that the half life of S-warfarin is increased. Patients with homozygous for the wild type allele (*CYP2C9*1*), S-warfarin is metabolized normally, resulting in a normal increase of the INR. In contrast, patients with *CYP2C9*2* or *CYP2C9*3* have impaired ability

to metabolize of S-warfarin, resulting in patients requiring altered doses of warfarin to maintain adequate anticoagulation. This will increase the time to reaching steady- state concentrations of warfarin. Accordingly, the INR determined at a set time in a protocol may not reflect the final steady-state INR on the warfarin dose.

Table 2.11 List of *CYP2C9* described variants [1]

Designation	Protein Change	SNP
*CYP2C9*1*	none	none
*CYP2C9*2*	Arg144Cys	C430T
*CYP2C9*3*	Ile359Leu	A1075C

*CYP2C9*1* is the wild- type allele

The impact on warfarin metabolism can divide into three subgroups. Patients with *CYP2C9*1/*1* are classified as an extensive or normal metabolizer. On the other hand, patients with homozygous variant alleles such as *CYP2C9 *2/*2, *2/*3* and **3/*3* are a poor metabolizer while, patients who have one wild-type and one variant such as *CYP2C9 *1/*2* or **1/*3* are grouped into intermediate metabolizer (Table 2.12).

Table 2.12 The impact of *CYP2C9* variants

Genotypes	Impact on warfarin metabolism
*1/*1	Extensive (normal) metabolism
*1/*2 *1/*3	Intermediate metabolism
*2/*2 *2/*3 *3/*3	Slow metabolism

The allelic frequencies of *CYP2C9*2* and *CYP2C9*3* diverge considerably among different ethnic groups. In Caucasian populations, the allelic frequencies of *CYP2C9*2* and *CYP2C9*3* vary approximately from 8% to 20% and from 6% to 10%, respectively [15]. They are less frequent in Asian and African- American populations.

In fact, *CYP2C9*2* is not present in Asians and only 1-4% of African- Americans carry this allele. In Thai populations, only *CYP2C9*3* is present about 5% of this variant [73]. The prevalence of *CYP2C9* variants correlates with membership of ethnic groups are shown in Table 2.13. Moreover, the frequency of other recently identified *CYP2C9* alleles need to be confirmed in different ethnic groups.

Table 2.13 Frequency of *CYP2C9* [15, 73]

CYP2C9 genetic Alleles point	**CYP2C9*1** Arg_{144}/Ilc_{359}%	**CYP2C9*2** Cys_{144}/Ilc_{359}%	**CYP2C9*3** Arg_{144}/Leu_{351}%	**CYP2C9*4** Arg_{144}/Thr_{359}%	**CYP2C9*5** Arg_{144}/Glu_{360}%
Ethnic group					
White	79-86	8-19.1	6-10	ND	ND
Canadian	91	3	6	ND	ND
African American	98.5	1-3.6	0.5-1.5	ND	ND
Asian	95-98.3	0	1.7-5	0-1.6	0
Thais	95	0	5	ND	ND

ND = not determined

In accordance with this, many studies have shown that patients with the *CYP2C9*2* and *CYP2C9*3* alleles require lower mean warfarin doses, longer times to achieve stable dosing, a higher proportion of individuals with extremely high INR values, and a higher bleeding rate during the induction and maintenance phases [32].

2.10.1 *CYP2C9* variants and dose requirements, bleeding risk and time to reach INR target

Many studies [74-78] showed that polymorphisms in *CYP2C9* gene are associated with increased anticoagulant response resulting in decreased warfarin dosage. Among warfarin taking patients, wild- type genotype individuals required the highest warfarin dose compared with heterozygous of *CYP2C9*1/*2, *1/*3*, and **2/*3* or homozygous of *CYP2C9 *2/*2* and **3/*3*, while homozygous of *CYP2C9 *3/*3* were taking the lowest doses of warfarin.

A systemic review and meta- analysis of nine studies has found that the mean daily warfarin dose was 17% lower in patients with at least one copy of the *CYP2C9*2* allele and 37% lower in patients with at least one copy of the *CYP2C9*3* allele compared with patients who were homozygous for the *CYP2C9*1* allele, respectively [79].

As with most drugs, side effects are often a problem. The most common side effects associated with warfarin treatment are hemorrhagic complications and thrombosis. Major and fatal bleeding events occur at a rate of 7.2 and 1.3/100 patient years, respectively, and are most likely to occur within the first 90 days of therapy [67]. The risk of a bleeding event is higher when the INR is more than 3.0, but also occurs when the INR is in the usual therapeutic range.

The findings about genetic polymorphisms are significant not only because they can predict the warfarin dosing requirement, but also because they have been associated with adverse events. In 1999, Aithal et al [76] investigated an association between *CYP2C9* variant alleles and low warfarin dose requirement. The study was conducted on of three groups of patients, including patients with a daily warfarin dose requirement of 1.5 milligram or less (a low dose group; n=36), patients with a wide range of warfarin dose requirements (a clinic control group; n=52), and 100 healthy control group. The results showed that patients in the low dose group have increased risk of major bleeding complications (rate ratio 3.68; 1.43-9.50) when compared with a clinic control group. In addition, 56% of patients in the low dose group were significantly more likely to experience an INR more than 4.0 within 7 days of therapy initiation when compared with 17% of patients in the normal dose group (OR 5.97; 95% CI 2.26, 15.82).

Therefore, Higashi et al [77] performed the retrospective cohort study to determine the association of *CYP2C9* variants with over anticoagulation and bleeding events during warfarin therapy. The study population consisted of 200 Caucasians patients, 185 of whom were valid for efficacy assessment. Result showed that 58 patients with at least one variant *CYP2C9* genotype had an increased risk of over the target INRs (HR 1.40; 95% CI 1.03, 1.90) and a significantly increased risk of serious or life-threatening bleeding events (HR 2.39; 95% CI 1.18, 4.86). The latter hazard

estimate was apparently underpowered. However, the variant group also required more time to reach stable dosing with a median difference of 95 days (p = 0.004).

Sanderson et al [79] described a meta-analysis of nine different clinical studies consisted of 2,775 patients who were taking warfarin. The investigators examined the relationship of *CYP2C9* variants to warfarin dose and risk of bleeding on warfarin. The study showed that the relative bleeding risk was 1.91 (95% CI 1.16, 3.17) for *CYP2C9*2* and 1.77 (95% CI 1.07, 2.91) for *CYP2C9*3* compared with wild-type. They concluded that differences in treatment and monitoring regimens, selection criteria for studies, and distribution of interacting factors in study populations may lead to an underestimation of bleeding risk or conflicting results between studies. Additionally, the relationship between bleeding in warfarin treated patient and *CYP2C9* polymorphisms have been described in case report [80, 81].

Because of previous studies have rarely been powered to determine the quantitative influence of *CYP2C9* genotypes on warfarin dose requirement, therefore Lindh et al [82] performed a systematic review and a meta-analysis for calculating the warfarin dose reduction that were associated with the five common variant *CYP2C9* genotypes in 2009. The study was conducted on thirty-nine studies. The normalized dose in each of the five genotype groups *CYP2C9*1/*2, *1/*3, *2/*2, *2/*3,* and **3/*3* were compared to the dose in the *CYP2C9*1/*1* group. Results demonstrated that *CYP2C9*1/*2, *1/*3, *2/*2, *2/*3,* and **3/*3* required warfarin dose were 19.6 (95% CI 17.4, 21.9), 33.7 (95% CI 29.5, 38.1), 36 (95% CI 29.9, 42), 56.7 (95% CI 49.1, 64.3), and 78.1 (95% CI 72, 84.3) lower than *CYP2C9*1/*1*, respectively.

Moreover, in study by Higashi et al [77], patients with at least one variant allele had an increased risk of over therapeutic range (HR 1.40; 95% CI 1.03, 1.90). These patients needed a longer time to achieve stable dosing (median difference 95 days) than homozygous wild- type patients (HR 0.65; 95% CI 0.45, 0.94).

In conclusion, patients who have common genetic variants of *CYP2C9* require a lower dose of warfarin and a longer time to reach a stable dose. They are also at higher risk for overanticoagulation and serious bleeding. However, the variation of *CYP2C9* variants could not completely explain the differences in dose requirement between individuals.

2.11 Vitamin K epoxide reductase complex subunit 1 *(VKORC1)* Gene

The *VKORC1* gene, identified in 2004, is located on chromosome 16 and encoded the VKORC1 enzyme. It encodes a 163 amino acid transmembrane protein of the endoplasmic reticulum, known as vitamin K epoxide reductase [71]. This enzyme is responsible for recycling vitamin K. Recycled vitamin K is necessary for the activation of vitamin K- dependent coagulation factors and certain anticoagulant proteins. Later in 2004, the *VKORC1* gene has only recently been identified along with polymorphisms that result in pharmacodynamics effect on warfarin. Most literature focuses on five gene variants, -4931 or 381T>C, -1639 or 3673G>A, 1173 or 6484C>T, 1542 or 6853C>G and 2255 or 7566C>T, which were shown to be highly predictive of differentiating *VKORC1* haplotypes [83]. They found that haplotype A (H1 and H2) were associated with a low-dose warfarin. On the other hand, haplotype B (H7, H8 or H9) were associated with a high-dose warfarin. The degree of suppression depends upon the patient's haplotype. After that, they defined a set of four SNPs that could be used to infer haplotype. There are three haplotypes (AA, AB, and BB) of *VKORC1*, all of which are associated with warfarin dose requirements. *VKORC1* BB represents the presence of two wild- type alleles, AB represents one wild- type allele and one variant allele, and AA represents two variant alleles. Patients with haplotype B require a higher maintenance dose of warfarin than those with haplotype A.

The mutations occur with differing frequencies in various ethnic populations was shown in Table 2.14. The impact of *VKORC1* haplotype on warfarin metabolism was shown in Table 2.15.

Table 2.14 Frequency of *VKORC1* variants among various ethnic groups [15]

VKORC1 genetic Haplotype Sequence	H1 CCGATCTCTG H2 CCGAGCTCTG	H7 TCGGTCCGCA H8 TAGGTCCGCA H9 TACGTTCGCG
Ethnic group %		
European	37	58
African	14	49
Asian	89	10

H1 and H2 represent warfarin-sensitive haplotype. H7, H8, and H9 represent warfarin-resistance haplotype

Table 2.15 Impact of *VKORC1* haplotypes on warfarin metabolism [84]

Haplotypes	**Impact on warfarin metabolism**
AA	High sensitivity
AB	Medium sensitivity
BB	Low sensitivity

In Asian populations, haplotype A is the major haplotype, corresponding to 90% of Chinese, 89% of Japanese and 98% of Thai population. In Europeans, haplotype A and B are dominated with approximately 40% each. In Africans, haplotype A has a frequency of only 14%, while haplotype B are the most frequent haplotype [32, 73].

2.12 Pharmacogenetics-based warfarin dosing algorithms

Several researchers have investigated the individual contribution of various factors to warfarin dose variability. A number of pharmacogenetics- guided warfarin dosing algorithms have been developed with the aim of reducing the risk of overanticoagulation during the initial days of warfarin therapy. The combination of *VKORC1* and/or *CYP2C9* genotype data with non-genetic data including age, weight, height, race, target INR, indication, BSA, sex, prosthetic heart valve presence, and/or

concurrent medications has been shown to explain up to 63% of the variability of warfarin dose.

In 2004, Gage and colleagues [18] developed an algorithm that used genetic, clinical, and demographic factors to estimate the warfarin dose. They collected DNA, demographic data, laboratory values, and medication histories from 369 patients who were taking a stable dose of warfarin. After PCR amplification, they analyzed for *CYP2C9* SNPs. Then using multiple regression to quantify the association between warfarin dose and all factors. Results showed that *CYP2C9* along with gender accounted for 39% of the variance in the maintenance dose of warfarin.

The study conducted by Kamali et al [16] evaluated the contribution of *CYP2C9* genotype, age, body size and vitamin K and lipid status to warfarin dose requirement. 121 patients who were taking a stable warfarin dose and INR within the target 2.0-3.0 were recruited. The experiment showed that both age and CYP2C9 polymorphism affect warfarin dose requirement which accounted for 20% of warfarin dose variability in the regression model.

The study performed by Hillman [17] has determined the relative impact of clinical factors and *CYP2C9* genotype. They conducted a retrospective cohort study in 453 Caucasian patients on a stable warfarin dose. This study found that the relation of age, body surface area, prosthetic heart valve indication, diabetes, and *CYP2C9* genotype in the multiple regression model accounted for 34% of the variability in the warfarin dose versus 23% ignoring genotype. However, prospective studies using warfarin dosing algorithms which included *CYP2C9* genotypes and non-genetic factors could explain about 40% of the warfarin dose variation [85, 86]. The explanation for this may be because *VKORC1* genotypes were not included in these algorithms.

In 2005, Vitamin K epoxide reductase complex subunit 1 (VKORC1) was named and found the association between haplotype and warfarin dose. Several researchers created mathematical model to determine appropriate warfarin dose requirement. British researchers, Sconce et al [11] created a pharmacogenetic equation based on 297 Caucasian patients, which had a stable warfarin dose and INR within target 2.0-3.0. The study showed that the multivariate regression model incorporating the variables of age, *CYP2C9*, *VKORC1* genotype, and height predicted 55% of the

variability in the warfarin dose in the derivation cohort and retrospectively validated the algorithm in 38 patients.

In 2006, The first study in Asian population proposed by Tham et al [12] conducted a retrospective cohort to derive and validate a pharmacogenetics- based dosing model. Data from 107 patients were used to derive the final model. Factors for the multivariate analyses were chosen from the univariate analysis that contributed the factors of age, weight, *CYP2C9*, and *VKORC1*. 108 patients were assigned to the validation model. The study showed that this genotype- guided dosing provided 60% of the variability in daily warfarin dose requirement equal to the study by Herman et al [87], whereas Vescler et al [88] explained 63% of dose variability due to *CYP2C9* and *VKORC1* polymorphism, age, and body weight.

Takahashi et al [19] conducted a retrospective cohort in a large number of patients having different ethnic backgrounds, Caucasians, Japanese and African-Americans. Result showed that the model developed by using the information from Caucasians and Japanese is higher prediction ability than the information from all ethnics (76% vs 57%, respectively). Using retrospective data, algorithms incorporating *VKORC1* have been developed, which could explain as much as 50% of dose variance [11, 19]. However, these algorithms have not yet been validated in prospective clinical trials.

In 2008, Taiwanese investigator, Wen et al [89] conducted a prospective study in 108 Han- Chinese patients without previous history of warfarin therapy. The author evaluated stable dose prediction and time to stabilization. Results showed that the inclusion of *CYP2C9* and *VKORC1*, body surface area, and age explained 62% of warfarin dosing variation.

Recently, Thai researchers [24] investigated the influence of *CYP2C9* and *VKORC1* genotypes on the pharmacokinetics and pharmcodynamics of warfarin and established the formula for predicting the maintenance dose of warfarin in the Thai population. The result revealed that *CYP2C9*, *VKORC1*, and age could explain about 53.8% of the variance of the warfarin maintenance dose. The examples of published warfarin dosing algorithms are shown in Table 2.16.

Table 2.16 Examples of warfarin dosing algorithms

Reference	Published Algorithms
Gage et al 2004 [18]	exp(0.385 – 0.0083 x age in years + 0.498 x BSA – 0.208 x *CYP2C9*2* – 0.350 x *CYP2C9*3* – 0.341 x amiodarone + 0.378 x Target INR – 0.125 x simvastatin – 0.113 x race – 0.075 x female). Exp is the e^x (inverse natural log); BSA is in m^2; the SNPs are coded 0 if absent, 1 if heterozygous, and 2 if homozygous; race is 1 if white (0 otherwise); female is 1 if not male (0 otherwise); target INR is the desired INR; and amiodarone and simvastatin are 1 if the patient is taking that drug (0 otherwise).
Sconce et al. 2005 [11]	√Dose = 0.628 – 0.0135(Age in years) - 0.240 (*CYP2C9*2*) – 0.370 (*CYP2C9*3*) – 0.241 (*VKORC1*) + 0.0162 (Height in cm). *CYP2C9*: input 0, 1, or 2 for the number of *2 and *3 alleles within the patient's genotype; *VKORC1*: input 1 for GG, 2 for GA, and 3 for AA. *VKORC1* =381 T>C.
Tham et al 2006 [12]	10[exp (0.838 – 0.005 x Age in years + 0.003 x Weight in kg – 0.189 x *CYP2C9*3* – 0.283 x *VKORC1*CC – 0.119 x *VKORC1*TC)]. Exp is the exponential function of the warfarin dose and *CYP2C9*3*, *VKORC1*TC are coded as 1 if present and 0 if absent. *VKORC1* = 381 T>C.
Takahashi et al 2006 [19]	Patients with homozygous wild-type genotype for both *CYP2C9* and *VKORC1*: daily dose = 6.6- 0.035 x (age in years) + 0.031 x (weight in kg). For patients with either heterozygous or homozygous variant of *CYP2C9*, the daily dose was reduced by 1.7 or 2.8 mg, respectively. For patients with either heterozygous or homozygous variant of *VKORC1*, the daily dose was further reduced by 1.3 or 2.9 mg, respectively. *CYP2C9* = *2/*3/**1*, *VKORC1* = 1173 C>T.
Miao et al 2007 [20]	Dose (mg/day) = 6.22- 0.011 (age in years) + 0.017 (weight in kg) – 0.775(*CYP*3*) – 3.397(*VKORC1*-x1) - 4.803(*VKORC1*-x2). *CYP*3*: input 1 for *1/*3, input 0 for *1/*1. *VKORC1*-x1: input 1 for GA (0 otherwise). *VKORC1*-x2: input 1 for AA (0 otherwise). *VKORC1* = 1639 G>A.

Table 2.16 Examples of warfarin dosing algorithms (continued.)

Reference	Published Algorithms
Anderson et al 2007 [21]	Dose (mg/week) = 1.64 + exp_e[3.984 +*1*1(0) + *1*2(-0.197) + *1*3 (-0.360) + *2*3(-0.947) + *2*2(-0.265) + *3*3(-1.892) + Vk-CT(-0.304) + Vk-TT(-0.569) + Vk-CC(0) + age(-0.009) + male sex(0.094) + female sex(0) + weight in kg(0.003)]. Equation yields weekly dose, divide by seven for predicted dose in mg/day. Reduce dose by 25% if on amiodarone. Vk refers to *VKORC1* with variants CT, TT, or CC; exp_e is the exponential to base e; *1, *2, *3 refer to *CYP2C9* wild-type (*1) or variant (*2, *3) genotypes, respectively.
Zhu et al 2007 [22]	ln (dose) = 1.35 – 0.008(age in years) + 0.116(sex) + 0.004(weight in lbs) – 0.376(*VKORC1*-AA) + 0.271(*VKORC1*-GG) – 0.307(*CYP2C9**2) – 0.318(*CYP2C9**3). Sex, input 0 for female and 1 for male; VKORC1(-1639AA), input 0 for GG, 0 for GA, and 1 for AA; VKORC1-1639GG, input 1 for GG, 0 for GA, and 0 for AA; CYP2C9, input 0, 1, or 2 for the number of *2 and *3 alleles.
Ozer et al 2010 [23]	Dose = -14.33-0.009 (age) + 6.228 (BSA) + 1.661 (*VKOR)* +1.079 (*CYP*2*) + 1.555 (*CYP*3*). *CYP2C9* genotype, input 1 or 2 for the number of *2 and *3 alleles within the patient's genotype; *VKORC1* genotype, input 1 for AA, 2 for AG, and 3 for GG.
Sangviroon et al 2010 [24]	mg/wk = exp[1.846 + (0.412 x VKORC1-AB)+(0.559 x VKORC1-BB) + (1.512 x *CYP2C9*1/*1*)+ (1.136 x *CYP2C9*1/*3*)-(0.007 x Age)]

A number of studies were performed to find a new way to guide warfarin dosing using the combination of genetic and clinical factors to develop dosing formula. Despite some success of such formula, their application outside of ethnic groups remains problematic. Although there was a study previously conducted in Thai population to produce a dosing formula, the study was with small sample size and lack adequate representation of certain genes with low frequency. Therefore, a dosing formula developed by Thai patient cohort with large number of patients and adequate representation of all gene types is needed.

CHAPTER III
MATERIALS AND METHODS

Materials

1. Data collecting forms (Appendix A)
2. Whole blood collected by EDTA tube 5 mL
3. HighPure PCR template preparation kit protocol
4. LightCycler® 480 Instruments

Methods

3.1 Definition of terms

The terms used throughout the study were defined as follows:

3.1.1 Allele

Three alleles were identified in this study. *CYP2C9*1* allele encodes the wild- type protein, and the *CYP2C9*2* contains a C-to-T transition, leading to substitution of cysteine by arginine at amino acid position 144. The *CYP2C9*3* allele is defined by an A-to-C nucleotide substitution that leads to exchange of leucine by isoleucine at amino acid position 359. In the *VKORC1* G-1639A (or C1173T) SNP, the B allele is referred to as G-1639 or C1173 allele and the A allele is referred to as -1639A or 1173T allele. The common G allele is replaced by the A allele. Because people with an A allele (or the "A haplotype") produce less *VKORC1* than do those with the G allele (or the "non-A haplotype"), lower warfarin doses are needed to inhibit *VKORC1* and to produce an anticoagulant effect in carriers of the A allele.

3.1.2 Genotype

The genetic formation of an organism or cell; also refers to the set of alleles inherited at a locus. The genotype is expressed when the information encoded in the gene's DNA is used to make protein and RNA molecule. *CYP2C9* genotype in

this study were defined into *CYP2C9*1/*1, CYP2C9*1/*2, CYP2C9*1/*3, CYP2C9*2/*2,* and *CYP2C9*3/*3*. According to *VKORC1*, *VKORC1 G-1639A* and *VKORC1 C1173T* were used in this study. *VKORC1* genotype were classified into three groups (GG or CC), (GA or CT), and (AA or TT) genotype.

3.1.3 Phenotype

Phenotype is defined as an organism's expressed physical trait. *CYP2C9* phenotypes were identified into three types; normal metabolizer (*CYP2C9*1/*1*), intermediate metabolizer (*CYP2C9*1/*2*), and poor metabolizer (*CYP2C9*1/*3, CYP2C9*2/*2,* or *CYP2C9*3/*3*). *VKORC1* phenotypes were identified into group A phenotype (low dose group; H1, H2) and group B phenotype (high dose group; H7, H8, H9) [83].

3.1.4 Haplotype

Haplotype groups A and B are based on classifications from Reider et al [83] where haplotype A represents individuals at risk for excessive anticoagulation with standard warfarin dosing, and haplotype B represents individuals at risk for subtherapeutic anticoagulation from standard warfarin dosing. *VKORC1* refers to the *VKORC1* 1173 C>T genotype, which is in strong linkage disequilibrium with the *VKORC1* -1639 G>A genotype. Individuals with the A allele haplotype (i.e., -1639 A and 1173 T) require less therapeutic warfarin than those with the B allele haplotype (i.e., -1639 G and 1173C). We hence used the haplotype group, A and B, instead of G/A or C/T alleles. Therefore, *VKORC1* in this study can be classified into three haplotypes (AA, AB, and BB).

3.1.5 Single- nucleotide polymorphism (SNP)

A single base change in a DNA sequence which happens when a single nucleotide (A, T, C or G) in the genome sequence is altered.

3.1.6 Time on a stable warfarin dose

The stable warfarin dose was defined as the duration of time being on stable warfarin dose that provided the stable INR at the time of present study.

3.1.7 Smoking [90]

Nonsmokers defined as patients who had never smoked or had stop smoking for at least 1 year. Light smoker defined as smoking ≤ 20 cigarettes/day. Heavy smoker defined as smoking >20 cigarettes/day.

3.1.8 Alcohol consumption [91]

Alcohol drinking status after heart valve replacement was classified into two groups; Non-drinkers and current drinkers. The frequency of drinking reported was: regular drinker (every day or an average of 4-5 times/week), moderate drinker (an average of 1 times/week), and occasional drinker (an average of 1-2 times/month).

3.1.9 Therapeutic INR

This term was defined as the INR values equal to 2.0-3.0. The optimal INR range varied depending on the varying degree of thromboembolism risks of each indication. The American College of Chest Physician recommended that the therapeutic INR range for general indication was 2.0-3.0. However, the INR of 2.5-3.5 was recommended for patients with mechanical prosthetic valves due to the higher risk of thromboembolic complications [15]. Despite recommendations, some studies conducted in Thailand and other Southeast Asia countries suggested that lower INR range may be effective in the prevention of thromboembolism with minimal bleeding complication in Asian patients with mechanical heart valve [52, 92-95]. Therefore, an INR in the range of 2.0-3.0 was selected as the therapeutic INR in this study.

3.1.10 A stable maintenance warfarin dose

A stable weekly maintenance doses is defined as a dose that did not vary by more than 10% between clinic visits, and with stable INR (2.0-3.0). However, because of the intrinsic variability in INR measurement, 1 of the 3 INR values will be allowed to fall 0.2 units above or below the target range. For example, patients with the standard goal INR range of 2.0 to 3.0 will be allowed to have one of the three INR measurements be as low as 1.8 or as high as 3.2. This approach is chosen because in clinical practice a single INR measurement 0.2 units above or below the goal range would not be likely to prompt a warfarin dose change.

3.2 Study design

This study was designed as a retrospective cohort, which was conducted at the cardiovascular and thoracic division of Siriraj Hospital.

3.3 Ethic approval

The study was approved by Siriraj Institutional Review Board of Faculty of Medicine, Siriraj Hospital, Mahidol University on June 17, 2009.

3.4 Study population

The study population was Thai patients with mechanical heart valve replacements who received long term warfarin therapy from the cardiovascular and thoracic outpatient department at the Siriraj Hospital. Patients were recruited according to the following criteria:

3.4.1 Inclusion criteria

1. Thai patients older than 18 years old who underwent mechanical heart valve operation which includes mitral, aortic, pulmonic, and tricuspid valve replacements more than one year.

2. All patients had been taking a stable maintenance dose of warfarin for at least one year with their INR maintained between 2.0 and 3.0 at least three consecutive clinic visits at the time of present study.

3. All patients and his/her parents and paternal and maternal grandparents were Thai or Thai-Chinese ethnic group.

4. Used only preparation of warfarin available in Siriraj hospital.

3.4.2 Exclusion criteria

1. Received warfarin therapy for bioprosthetic valve replacement or valve repaired.

2. Received unstable weekly dose of warfarin.

3. Heavy smoking and regular or moderate alcohol drinking.

4. Received stable weekly dose of warfarin with the INR value out of the patient's goal INR range.

5. Medical records indicated congestive heart failure (NYHA class 3 or greater), abnormal thyroid function (definite hypothyroidism or hyperthyroidism), end- stage renal failure or abnormal liver function test (alanine aminotransferase and aspartate aminotransferase ≥3 times the upper limit of normal) persisting at the time of present study, advanced malignancy, and hospitalization within 4 weeks or febrile/diarrheal illness.

6. Received co-medications known to significant interaction at any level with warfarin therapy but such interaction did not reach steady-state yet during the time of study; to identify such drugs, we used the Drug Interaction Facts [96].

7. Received herbal medicines concurrent with anticoagulant therapy.

8. Stopped their warfarin therapy prior to becoming therapeutic.

9. Pregnant or lactating women.

10. Patients denied to participate in the study.

3.5 Period of study

Study was conducted during June 2009 - May 2011.

3.6 Steps of investigation

3.6.1 Patient screening

- Mechanical heart valve replacement patients, who had follow- up appointment at the outpatient CVT unit, were screened by computer system.

- Patients' medication records were screened using the inclusion and exclusion criteria to identify potential study candidates.

- Potential candidates were asked to participate in the study. Patients who were willing to participate were asked to review and complete consent forms and related materials. All patients who completed this process were then asked to provide blood sample in the next visit. Venous INR measurements were carried out by the Clinical Laboratory department of the Siriraj hospital.

3.6.2 Data collection

Recorded patient's information from the patient's medication record into the data collection form (Appendix A) as following;

- Demographic data: age, gender, weight, height, BMI, native district (geographic region), ethnicity, operation date, position of valve replacement, smoking status and alcohol intake. BMI was calculated in the standard fashion from weight and height. To categorize ethnicity as Thai or Thai-Chinese, each patient and his or her parents and paternal and maternal grandparents had to be of the same ethnic group.

- Clinical data: indication of use, concurrent disease/illness, length of stable warfarin dose, INR, maintenance warfarin daily dose.

- Laboratory data: BUN, SCr, AST, ALT, serum albumin level was measured from laboratory report software computer.

- Medication data: Medication history, concomitant medications during the time of the study.

3.6.3 Blood collection and DNA extraction

Whole blood (5 mL) was drawn from a patient into an EDTA tube and stored at 4 °C until extraction. All DNA samples were extracted from whole blood using the High Pure PCR Template Preparation Kit protocol (Roche Applied Science, Mannheim, Germany). This technique uses various reagents, such as binding buffer and proteinase K, to lyse the membranes of DNA rich cells and remove contaminants such as proteins and cell debris then bind DNA with column by DNA binding buffer. The protein was precipitated out of the solution by heat treating, while the DNA was precipitated from the solution by the addition of 100 percent ethanol and then washed with 70 percent ethanol. The extracted DNA was put off to collecting tube by using

elution buffer and kept in a -20 degree Celsius freezer until the genotyping was performed. Details of this process were provided in Appendix B.

3.6.4 Genotyping for *CYP2C9*2* and *CYP2C9*3*

Genotypes *CYP2C9*1/*1, *1/*2, *2/*2, *1/*3* and *3/*3* were determined using the CYP2C9 Mutation Detection Kit (Roche Diagnostics Corp., Germany) and LightCycler®. This kit is labeled for research purposes only and uses proprietary primers and hybridization probes that have not been released to the general public. The accuracy of the detection assay was verified by including positive and water negative controls. Two point mutations (C/T at base 430 for *CYP2C9*2*, and A/C at base 1075 for *CYP2C9*3*) were simultaneously detected by dual color emission and melting curve analysis.

Briefly, prepare the reaction mix by combining the unlabelled primer, hybridization probes specific for bases 430 and 1075, FastStart Taq DNA polymerase reaction buffer, dNTPs, FastStart Taq DNA polymerase and $MgCl_2$ into a multiwell plate. Detail of this process can be found in Appendix (C). 5 µl (~100 ng) of purified DNA was added to each multiwell plate to give final volume of 20 µl. A positive, heterozygous control (supplied with kit) and a negative control (sterile water) were prepared and analysed with each patient DNA sample. PCR reactions were performed in a Real-time PCR thermal cycle (Appendix E).

The genotypes were identified by running a melting curve with specific melting points (Tm). Melting peaks for the *CYP2C9*2* mutant occur at 50.5 ± 2.5 °C and 58.5 ± 2.5 °C for the wild-type (*CYP2C9**1) in channel 530. Melting peaks for the *CYP2C9*3* mutant occur at 58.3 ± 2.5 °C and 48.3 ± 2.5 °C for the wild-type allele in channel 640 (Figure 3.1). Genotypes were determined by review of the melting curves for individual DNA samples. The negative controls must show no signal.

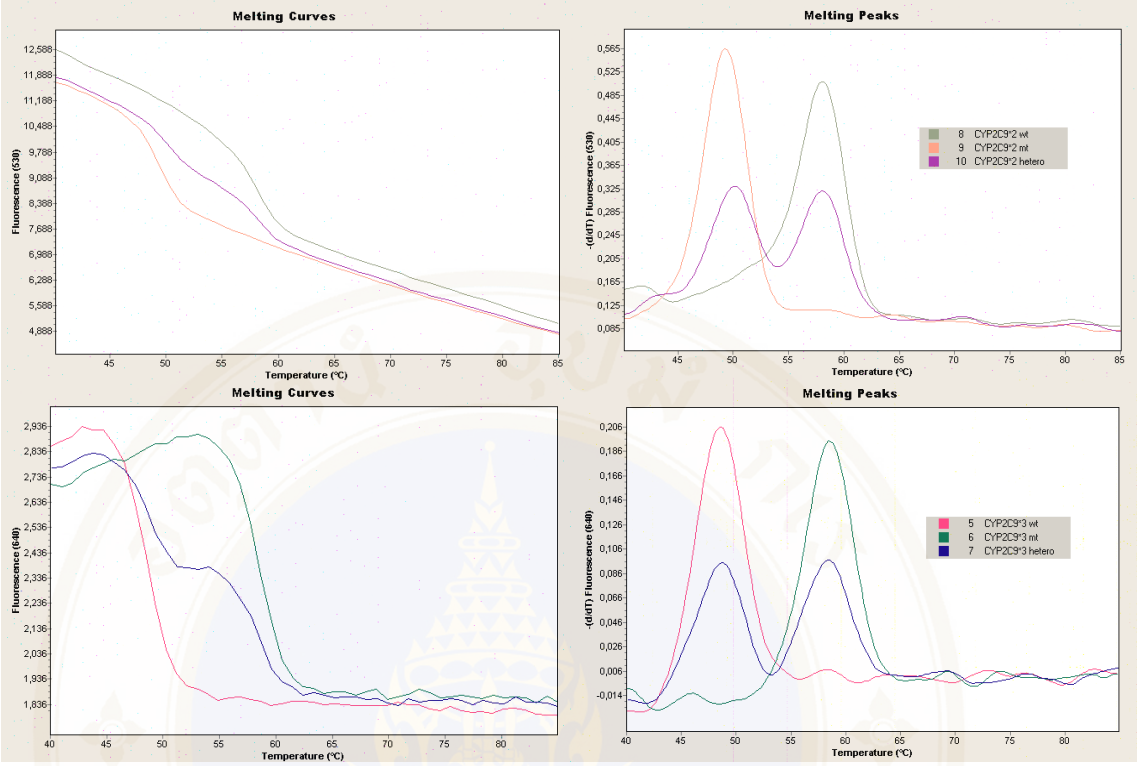


Figure 3.1 Sample data for the human *CYP2C9*2* and *CYP2C9*3* detection system

For upper panels, data was detected from channel 530 and shown melting curves (left panel) and melting peaks (right panel) for human *CYP2C9*2*. Wild-type (wt) corresponding with *CYP2C9*1/*1* was shown in green line. Heterozygous (hetero) corresponding with *CYP2C9*1/*2* was shown in violet line and mutant (mt) corresponding with *CYP2C9*2/*2* was shown in orange line.

For lower panels, data was detected from channel 640 and shown melting curves (left panel) and melting peaks (right panel) for human *CYP2C9*3*. Wild-type (wt) corresponding with *CYP2C9*1/*1* was shown in pink line. Heterozygous (hetero) corresponding with *CYP2C9*1/*3* was shown in blue line and mutant (mt) corresponding with *CYP2C9*3/*3* was shown in green line.

3.6.5 Genotyping for *VKORC1*

The gene variants *C1173T* and *G-1639A* which seems to be in a strong linkage are known to reduce VKORC1 activity and increased the effect of warfarin. Genotypes *VKORC1 C1173T* and *VKORC1 G-1639A* were determined using the

LightMix® Kit (Roche Diagnostics Corp., Germany) and LightCycler®. DNA samples were amplified by PCR in a final volume of 20 µL, each with primers, probes, FastStart DNA Master Hybridization probe and $MgCl_2$. Primers and probes were found to contain DNA human *VKORC1 1173C* (wt), *1173C/*T (hetero), 1173T(mt), *-1639G* (wt), *-1639G/*A (hetero) and -1639A (mt). The accuracy of the detection assay was verified by including positive and water negative controls. PCR reactions were described in Appendix E. The genotypes were identified by running a melting curve with specific melting points (Tm). View *VKORC1 C1173T* data in channel 530 and *VKORC1 G-1639A* data in channel 640. Wild type *VKORC1 C1173* exhibits a Tm of 54.4 ± 2.5 °C while the wild type *VKORC1 G-1639* yields a Tm of 52.9 ± 2.5 °C. The allele variant *VKORC1 1173T* exhibits the Tm of 60.4 ± 2.5 °C and the allele variant *VKORC1 -1639A* exhibits the Tm of 61.7 ± 2.5 °C (Figure 3.2).

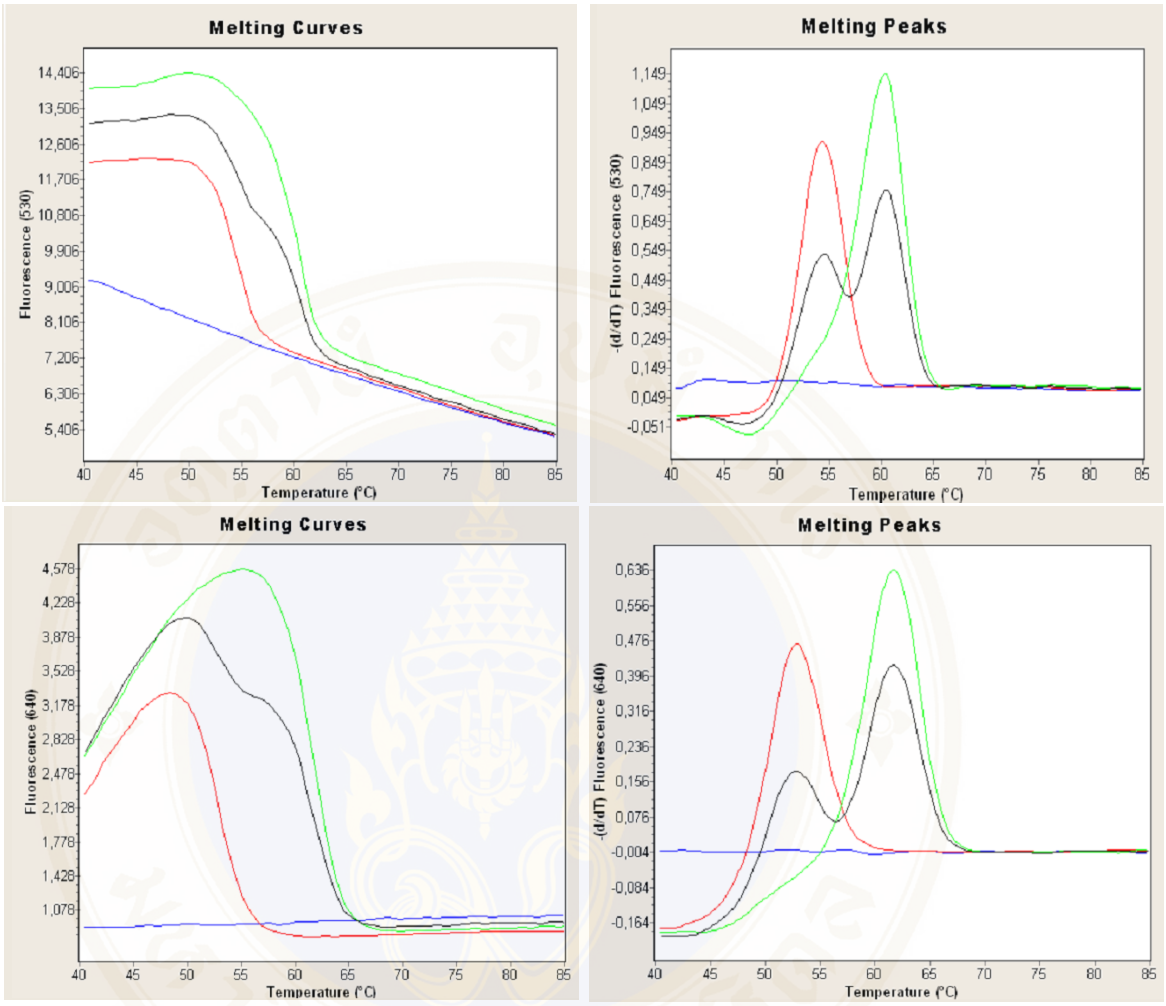


Figure 3.2 Sample data for the *VKORC1 C1173T* and *VKORC1G-1639A* detection system

For upper panels, data was detected from channel 530 and shown melting curves (left panel) and melting peaks (right panel) for human *VKORC1 C1173T*. Wild-type (wt) corresponding with *VKORC1 C1173* was shown in red line. Heterozygous (hetero) corresponding with *VKORC1 C1173T* was shown in violet line and mutant (mt) corresponding with *VKORC1 1173T* was shown in black line.

For lower panels, data was detected from channel 640 and shown melting curves (left panel) and melting peaks (right panel) for human *VKORC1 G-1639A*. Wild-type (wt) corresponding with *VKORC1 G-1639* was shown in red line. Heterozygous (hetero) corresponding with *VKORC1 G-1639A* was shown in black line and mutant (mt) corresponding with *VKORC1 -1639A* was shown in red line.

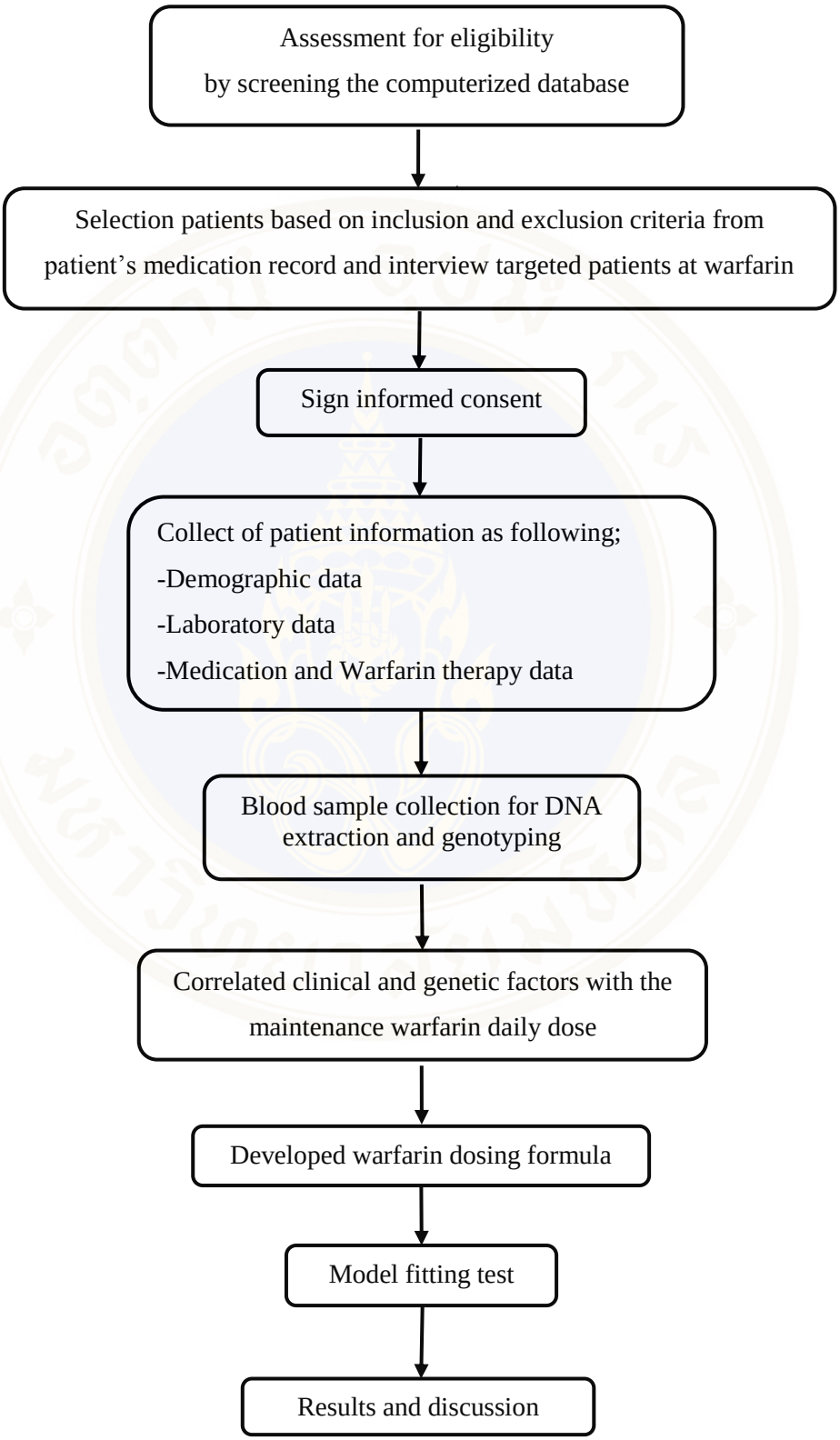


Figure 3.3 Workflow of the study

3.7 Data analysis

The Statistical Package for Social Science (SPSS) version 13.0 was used to analyze the patient's data for descriptive statistics. P-value of less than 0.05 was considered significant for all statistical tests. All data were analyzed as following:

3.7.1 Patient characteristics

Demographic data was presented as descriptive statistics.

3.7.2 Genotype data

The gene counting method was used to estimate allele frequencies. Hardy-Weinberg equilibrium was checked with χ^2 test.

3.7.3 Factors contributing to warfarin dose

The Student's t-test was used to determine the effect of sex, and interacting drugs on the maintenance dose of warfarin. Associations between warfarin dose and the age, body weight, height, and BMI were examined by Pearson correlation test. The relationship between warfarin dose and, *VKORC1, CYP2C9* polymorphisms were tested using Student's t-test.

3.7.4 Warfarin dosing algorithm

Warfarin dose distribution was checked for normality of distribution to complete the criteria for use of linear regression analyses. Univariate association between stable warfarin dose and each potential predictor was assessed using Pearson correlation coefficient or Student's t- test. To reduce the possibility of discarding variables that may not reach obvious statistical significance on univariate analysis but yet may contribute to the model, variables with $p < 0.25$ in the initial analyses were considered to be sufficiently associated with warfarin dose and were retained for further model building. So, to select candidate variables for stepwise multiple regression, we considered statistically significant only those variables with p- value not greater than 0.25. The multiple linear regression analysis, in accordance with the stepwise forward regression, was performed to predict the effect of both genetic and clinical factors. A p-value, 0.05 was considered as significant and the variable was

subsequently entered into the equation; variables included with p-values.0.1 in subsequent models were removed. Predictors were checked for collinearity and required to exhibit statistical significance at $p < 0.05$ to be retained in the final warfarin prediction model. To assess its importance in the final model, the partial R^2 and adjusted R^2 (i.e., the proportion of the variation in the dependent variable accounted for by the independent variable) were calculated for each variable. The partial R^2 of a specific variable allows estimation of the proportion of unexplained variance of the maintenance dose that can be explained by the addition of that variable to the model.

3.7.5 Model fitting test

To assess the predictive power of previously published formulas in Thai patients, correlation coefficient (R^2) was tested for accuracy of such formula and the developed dosing formula from this study. In addition, the extent of a predicted dose deviation from an actual dose was also analyzed.

CHAPTER IV
RESULTS

Complete data were obtained from a total of 197 patients in the cohort study. The results are presented as follows:

4.1 Baseline characteristics data

- Demographic data
- Clinical data

4.2 Genetics data

- Frequency of variant *CYP2C9* and *VKORC1* genotype.
- The correlation of *CYP2C9* and *VKORC1* variants to warfarin maintenance dosage.

4.3 Regression analysis

- The multiple regression model including genetic and clinical factors for estimating warfarin dose.

4.4 Model fitting test

-The relationship between the predicted dose from the pharmacogenetically based warfarin dosing formula and the actual dose requirements in the study cohort.

4.1 Baseline characteristics data

Baseline characteristics data of the study patients are presented in Table 4.1. The gender distribution was 86 males (43.7%) and 111 females (56.3%). The mean age of patients was 50.52 ± 12.25 years. The mean of body weight, height, and BMI were 59.57 ± 11.72 kg, 160.08 ± 10.30 cm, and 23.23 ± 3.95 kg/m^2, respectively. The maintenance dose of warfarin was 3.94 ± 1.67 mg per day (or 27.61 ± 11.72 mg per week). The stable warfarin dose varied over a 8.5-fold interindividual difference in dosage requirement (range 8.75 – 75 mg/week). The distribution of warfarin dosage

amongst patients is shown in Figure 4.1 in milligram per week. Dosage was measured in milligram per week instead of milligram per day because some of the patients alternate their daily dosage. Baseline INR value was 2.43 ± 0.22. Mean duration of time being on stable warfarin dose was 45.59 ± 40.64 months. In term of smoking status, the study found that 189 patients were nonsmokers (96%) and 8 patients were light smokers (4%). In regards to alcohol drinking status, there were 193 patients never drank alcohol after heart valve surgery (98%), 4 patients were occasional drinkers (2%). All patients in the study cohort were Thai nationality. 157 patients (79.7%) were Thai ethnicity and 40 patients (20.3%) were Thai- Chinese ethnicity. For valve position, there were 102 cases of mitral valve position (51.8%), 59 cases of aortic valve position (30%), on case of tricuspid valve position (0.5%), 29 cases of both aortic and mitral (14.7%) positions, 4 cases of both mitral and tricuspid (2%) positions, 2 cases of aortic, mitral and tricuspid positions (1%). Among Central, North-East, West, South, North, and East, the demographic distribution of patient were 15.7%, 9.6%, 7.6%, 6.6%, and 5.1%, respectively.

Table 4.1 Baseline characteristics of the study patients

Characteristic	**(N = 197)**
Gender (%)	
Male	86 (43.7)
Female	111 (56.3)
Age (years)	
Mean ± SD	50.52 ± 12.25
Range	21 – 82
Body weight (kg)	
Mean ± SD	59.57 ± 11.72
Range	39.80 – 105.60
Height (cm)	
Mean ± SD	160.08 ± 10.30
Range	130 – 210

Table 4.1 Baseline characteristics of the study patients (continued.)

Characteristic	**(N = 197)**
BMI (kg/m^2)	
Mean ± SD	23.23 ± 3.95
Range	13.61 – 42.58
Maintenance dose of warfarin (mg/day);	
Mean ± SD	3.94 ± 1.67
Range	1.25 – 10.71
Baseline INR; Mean ± SD	2.43 ± 0.22
Time on stable warfarin dose (month); Mean ± SD	45.69 ± 40.72
Smoking status (%)	
Nonsmokers	189 (96)
Light smokers	8 (4)
Alcohol drinking (%)	
Non-drinker	
Never	193(98)
Occasional	4 (2)
Ethnicity (%)	
Thai	157 (79.7)
Thai - Chinese	40 (20.3)
Position of valve replacement (%)	
Mitral	102 (51.8)
Aortic	59 (30.0)
Tricuspid	1 (0.5)
Aortic + Mitral	29 (14.7)
Mitral + Tricuspid	4 (2.0)
Aortic + Mitral + Tricuspid	2 (1.0)
Demographic distribution	
Central	109(55.3)
North-East	31(15.7)
West	19 (9.6)
South	15(7.6)
North	13(6.6)
East	10(5.1)

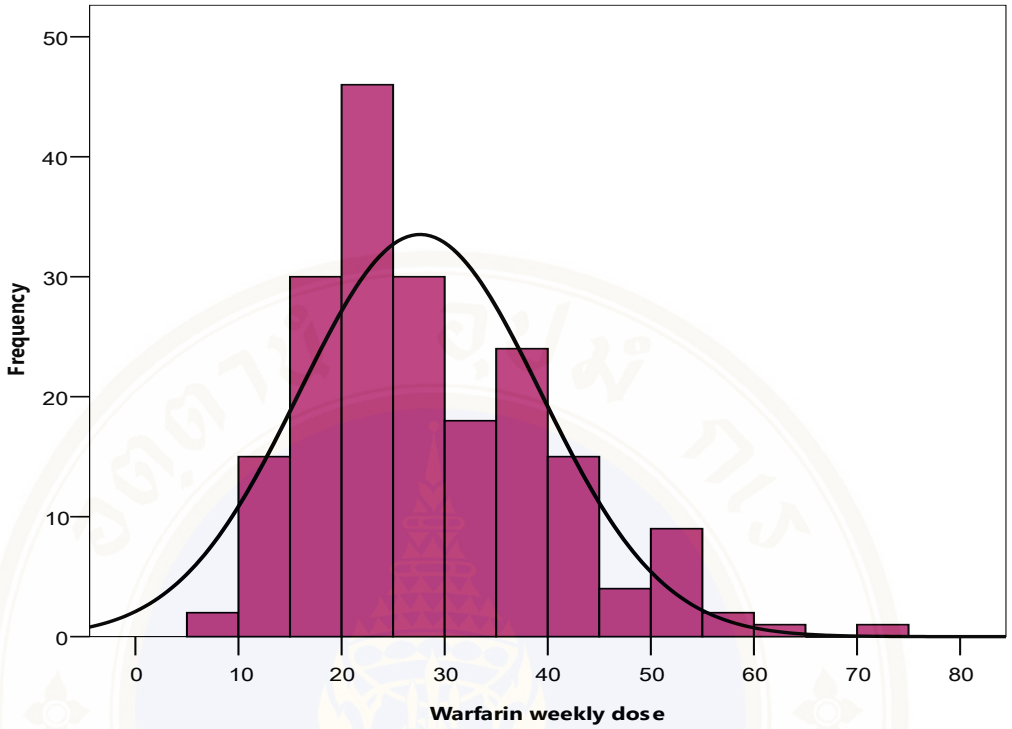


Figure 4.1 Distribution histogram of the therapeutic maintenance doses of warfarin among study patients. The x axis represents the warfarin dose in mg/week. The y axis represents the number of patients for the respective dosing range.

The underlying disease and concomitant medications in the study patients with stable warfarin dose are reported in Table 4.2-4.3. The interacting drugs of patients included aspirin, gemfibrozil, omeprazole, simvastatin and rosuvastatin. The most common interacting drugs taken by patients in this study were simvastatin (4.14%) followed by aspirin (2.36%) and gemfibrozil (2%). Drug interactions that were significantly interact with warfarin are indicated with an asterisk.

Table 4.2 Underlying diseases

Underlying disease*	Frequency
Atrial fibrillation	100
Hypertension	62
Hyperlipidemia	17
Stroke	11
Diabetes mellitus	6
CABG	4
Marfan syndrome	3

*192 patients

Table 4.3 Concomitant medications in the study patient with stable warfarin doses[a]

Drug	Number of patient (%)
Digoxin	99(29.30)
Furosemide	93(27.5)
Enalapril	32(9.47)
Spironolactone	21(6.22)
Atenolol	17(5.03)
Propranolol	16(4.73)
Simvastatin*	14(4.14)
Aspirin*	8(2.36)
HCTZ	8(2.36)
Gemfibrozil*	4(2.0)
Losartan	6(1.77)
Isosorbidedinitrate	5(1.48)
Diltiazem	5(1.48)
Glibenclamide	3(0.89)
Metformin	3(0.89)
Rosuvastatin*	1(0.3)
Omeprazole*	1(0.3)

HCTZ= Hydrochlorothiazide

[a] Patients might have taken more than one concomitant medications

4.2 Genetics data

The prevalence of *CYP2C9* genotypes and the association of *CYP2C9* with mean daily warfarin doses are presented in Table 4.4.

Table 4.4 The prevalence and mean daily warfarin dose of *CYP2C9* genotypes

***CYP2C9* genotypes**	**Prevalence No. (%)**	**Mean Daily Warfarin Dose (mg) ± SD**	**p-value[a]**
*1/*3	15 (7.6)	2.42 ± 0.75	<0.001
*1/*1	182 (92.4)	4.07 ± 1.67	

[a] Student's t-test was used to compare the means between groups

Of the 197 patients, 182 patients were found to have *CYP2C9*1/*1* (92.4%) and 15 were found to have *CYP2C9*1/*3* (7.6%). The results revealed that patients with *CYP2C9*1/*1* received significantly higher dose of warfarin (4.07 ± 1.67 mg/day; $p<0.001$) than patients with *CYP2C9*1/*3* (2.42 ± 0.75 mg/day). The distributions of warfarin dose within the different *CYP2C9* genotypes are illustrated in Figure 4.2. In this result we were able to demonstrate significant differences in warfarin dose requirements based on *CYP2C9* genotype; however, 2 patients who carried *CYP2C9*1/*1* genotype were found to have daily warfarin dose requirement 8.75 and 10.71 milligram per day, respectively. Reanalysis of these patients including the *VKORC1* haplotype revealed that they were *VKORC1*- BB haplotype, a finding that likely accounts for their high dose requirement. The outliers are marked by open circles.

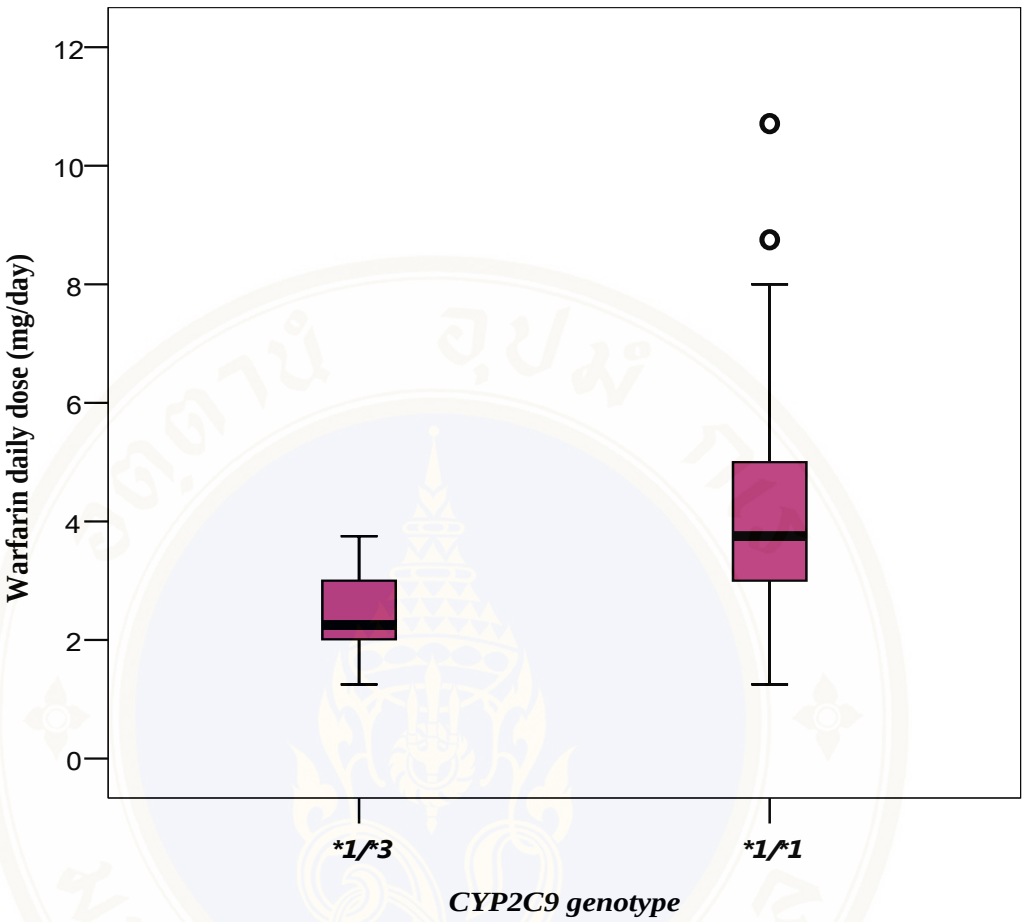


Figure 4.2 Boxplot showing mean daily warfarin dose by *CYP2C9* in the study population

Regarding *VKORC1,* patients were then assigned into two *VKORC1* haplotype groups based on *VKORC1* 1173 or -1639 genotypes. The *VKORC1* Group BB was comprised of patients who possessed two wild-type alleles at position 1173 or -1639. The *VKORC1* Group AA was comprised of patients who possessed two variant alleles at both positions 1173 or -1639. Finally, *VKORC1* Group AB was comprised of patients who possessed on variant allele at position 1173 or -1639.

VKORC1 C1173T genotyping showed that 114 patients (57.9%) were homozygous TT (low dose group, AA), 69 patients (35%) were heterozygous CT (intermediate dose group, AB), and 14 patients (7.1%) were homozygous for the CC genotype (high dose group, BB). All samples were genotyped for the *G-1639A* and *C1173T*. Analysis of genotypes for 2 polymorphisms found to be in linkage

disequilibrium (r^2 =1). The mean warfarin daily dose requirement was highest in patients with BB (7.03 ± 1.58 mg) haplotype compared with those with the AB (4.81 ± 1.43 mg) haplotype and AA (3.04 ± 0.94 mg) haplotype. There were significant differences between warfarin maintenance doses among different groups of *VKORC1* haplotype, p< 0.001 (Table 4.5). The distributions of warfarin doses within the different *VKORC1* haplotypes groups are shown in figure 4.3.

Table 4.5 The prevalence and mean daily warfarin dose of *VKORC1* haplotypes

***VKORC1* haplotypes**	**Prevalence No. (%)**	**Mean Daily Warfarin Dose (mg) ± SD**
AA	114 (57.9)	3.04 ± 0.94
AB	69 (35.0)	4.81 ± 1.43
BB	14 (7.1)	7.03 ± 1.58

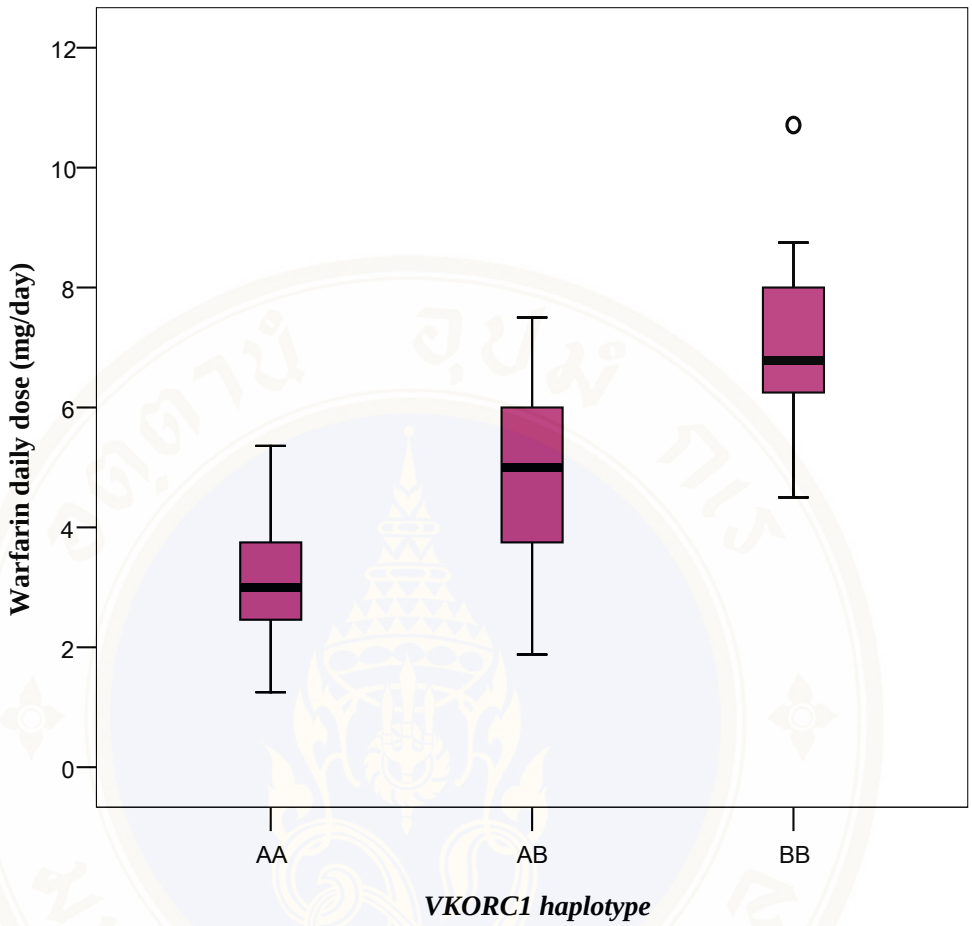


Figure 4.3 Boxplot showing mean daily warfarin dose by *VKORC1* in the study population

Genotypes of *CYP2C9* and *VKORC1* of these patients are shown in Table 4.6. *CYP2C9*2* genotype was not found in the studied population (0%). Allele frequency of *CYP2C9*1* and *CYP2C9*3* observed in these patients were 0.962 and 0.038, respectively. The alleles frequencies of *VKORC1 1173T* and *-1639A* were both equaled to 0.754 indicated that *VKORC1 C>T* was completely correlated with *VKORC1 -1639G>A* (Pearson's correlation =1.0, $p<0.0001$). Allele frequencies of both genotypes were in Hardy-Weinberg Equilibrium.

Table 4.6 Alleles frequencies of *CYP2C9* and *VKORC1* of the study population

Genotypes	**Alleles**	**(197 patients x 2 alleles) N = 394 (%)**
CYP2C9	*1	379 (96.2)
	*2	0
	*3	15 (3.8)
VKORC1		
C1173T	*C*	97 (24.6)
	T	297 (75.4)
VKORC1		
G-1639A	*G*	97 (24.6)
	A	297 (75.4)
		r = 1.0*

*Significant at $p < 0.001$.

For combined *VKORC1* and *CYP2C9* genotypes, numbers of patients within each combination of genotypes and mean weekly doses are summarized in Table 4.7. The lowest warfarin daily dose was found in patients with *CYP2C9*1/*3*/AA (2.21 ± 0.66 mg), whereas *CYP2C9*1/*3*/AB and *CYP2C9*1/*1*/AA were nearly dose equivalent (3.25 ± 0.43 mg and 3.14 ± 0.92 mg, respectively). Conversely, patients with *CYP2C9*1/*1*/AB and *CYP2C9*1/*1*/BB required highly dose warfarin (4.88 ±1.42 mg and 7.03 ± 1.58 mg, respectively). The distributions of warfarin dose by *CYP2C9* and *VKORC1* in the study cohort are illustrated in Figure 4.4. Two patients of 102 in low dose warfarin group (*CYP2C9*1/*3*/AA) are marked as outliers. They received warfarin 3 mg and 3.75 mg per day, respectively. One patient of 14 in high dose warfarin group (*CYP2C9*1/*1*/BB) received warfarin 10.71 mg per day. The outliers are marked as open circle.

Table 4.7 Mean daily warfarin dose by *CYP2C9* genotypes and *VKORC1* haplotypes

Combined *CYP2C9, VKORC1*		Dose (Mean ± SD)
*1/*1,	AA	3.14 ± 0.92 (n = 102)
	AB	4.88 ± 1.42 (n = 66)
	BB	7.03 ± 1.58 (n = 14)
*1/*3,	AA	2.21 ± 0.66 (n = 12)
	AB	3.25 ± 0.43 (n = 3)

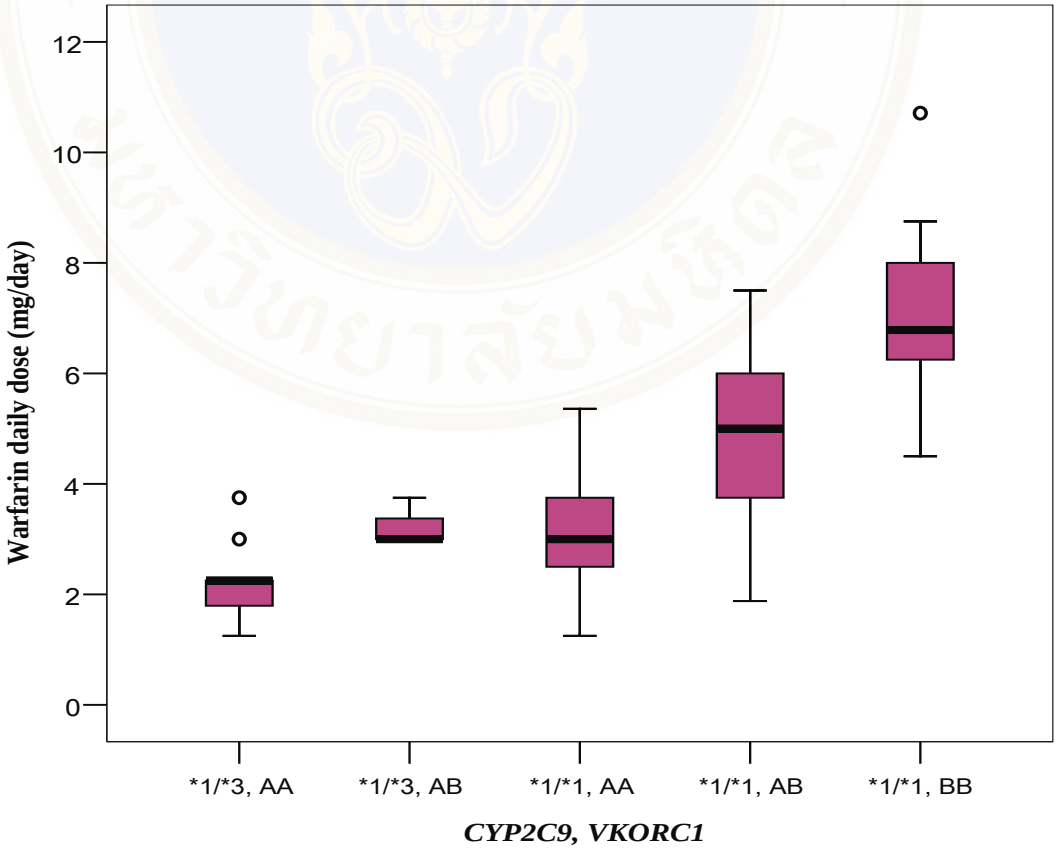


Figure 4.4 Boxplot showing the distribution of warfarin doses by *CYP2C9* and *VKORC1* in the study cohort

Table 4.8 Factors affecting warfarin dose requirements in the study cohort

Variables	N	mg/day (Mean ± SD)	r (Pearson correlation)	p- value
CYP2C9				
**1/*3*	15	2.42 ± 0.75		0.004[a]
**1/*1*	182	4.07 ± 1.67		
VKORC1				
AA	114	3.04 ± 0.94		<0.001[b]
AB	69	4.81 ± 1.43		
BB	14	7.03 ± 1.58		
Gender				
Male	86	3.92 ± 1.74		0.842[a]
Female	111	3.97 ± 1.63		
Age (years)	197		-0.158	0.013[c]
Weight (kg)	197		0.249	<0.001[c]
Height (cm)	197		0.113	0.058[c]
BMI (kg/m^2)	197		0.197	0.003[c]
Statins				
No	181	3.90 ± 1.61		0.203[a]
Yes	16	4.46 ± 2.28		

[a] Student t-test was used to compare the means between groups.

[b] ANOVA was used to compare the means between groups.

[c] Pearson-correlation was used to correlate clinical factors and maintenance warfarin dose.

We examined the distribution of the maintenance doses of our cohort and found that they were approximately normally distributed with *CYP2C9*, *VKORC1*, and gender groups (Figure 4.5-4.8). Univariate analysis was conducted to evaluate the association of all the clinical and genetic variables (in Table 4.8) to the stable warfarin dose an variables with p-value < 0.25 were included in the stepwise multivariate analysis. According to Pearson correlation coefficient or Student's t-test, 7 variables were shown to be significantly ($p < 0.25$) associated with the stable warfarin dose in univariate analysis: *CYP2C9* genotype, *VKORC1* haplotype, age, body weight, height, BMI, and statins use. Age ($r = -0.158$; $p = 0.013$), body weight ($r = 0.249$; $p < 0.001$), height ($r = 0.113$; $p = 0.058$), BMI ($r = 0.197$; $p = 0.003$), statins use (4.46 ± 2.28 mg/day for statins use and 3.90 ± 1.61 mg/day for no statins use; $p = 0.203$), *CYP2C9* genotype (4.07 ± 1.67 mg/day for wild type and 2.42 ± 0.75 mg/day for heterozygous; $p = 0.004$), and *VKORC1* haplotype (3.04 ± 0.94 mg/day for AA, 4.81 ± 1.43 mg/day for AB, and 7.03 ± 1.58 mg/day for BB; $p < 0.001$) (Figure 4.9-4.12). Conversely, there were no significant differences in warfarin dose between male and female patients (3.92 ± 1.74 mg/day for male and 3.97 ± 1.63 mg/day for female; $p = 0.842$).

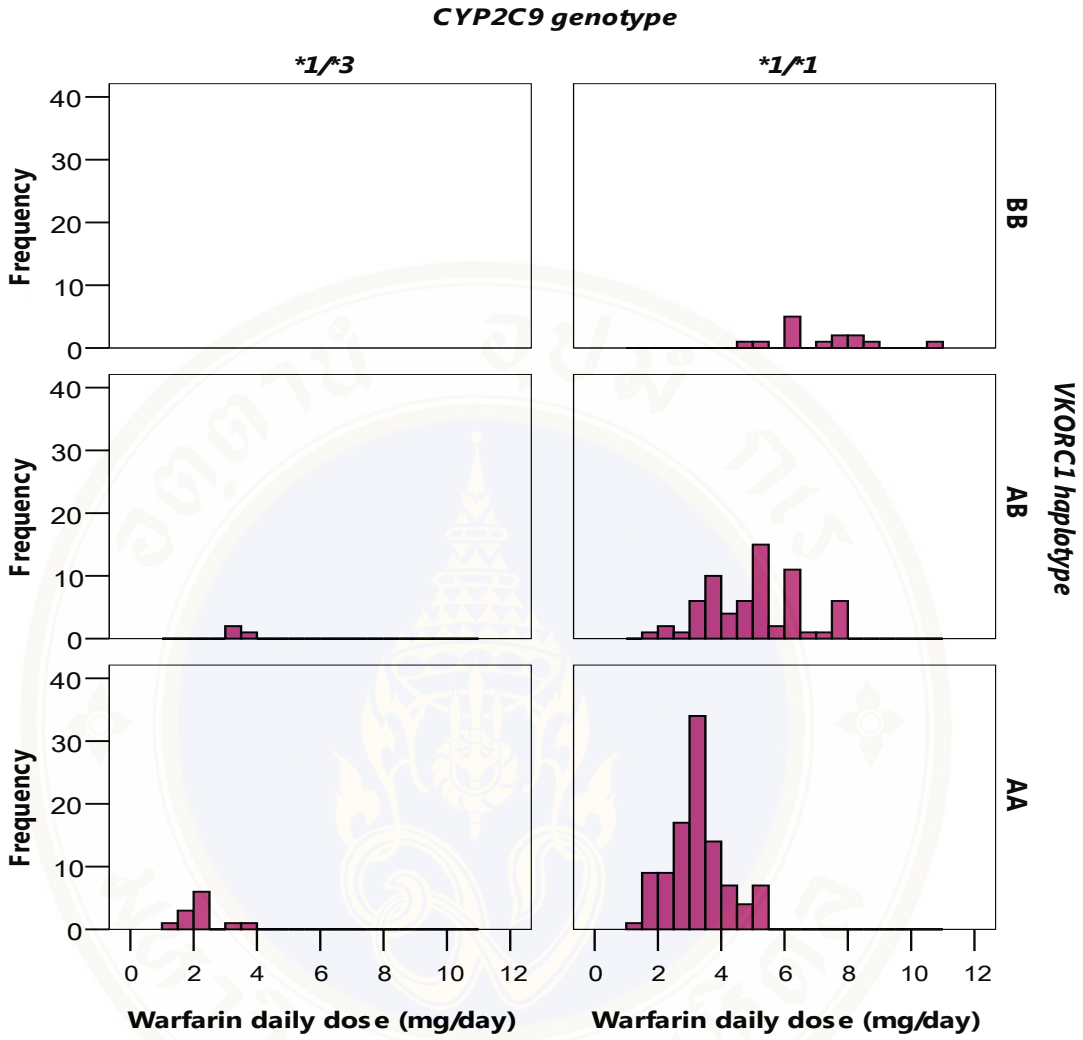


Figure 4.5 Histogram representing the *CYP2C9* genotypes and *VKORC1* haplotypes and warfarin maintenance dose requirements of the study population

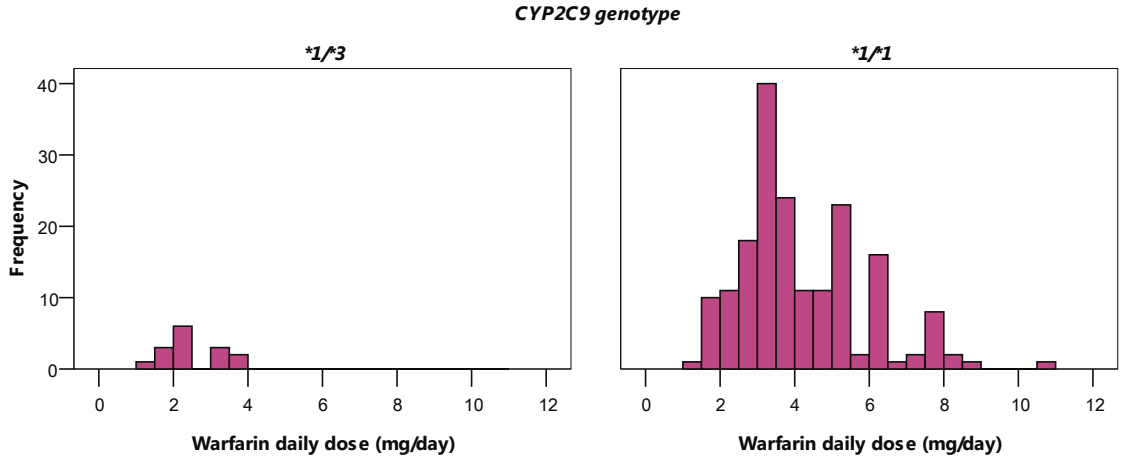


Figure 4.6 Distribution of warfarin daily dose in *CYP2C9* genotypes

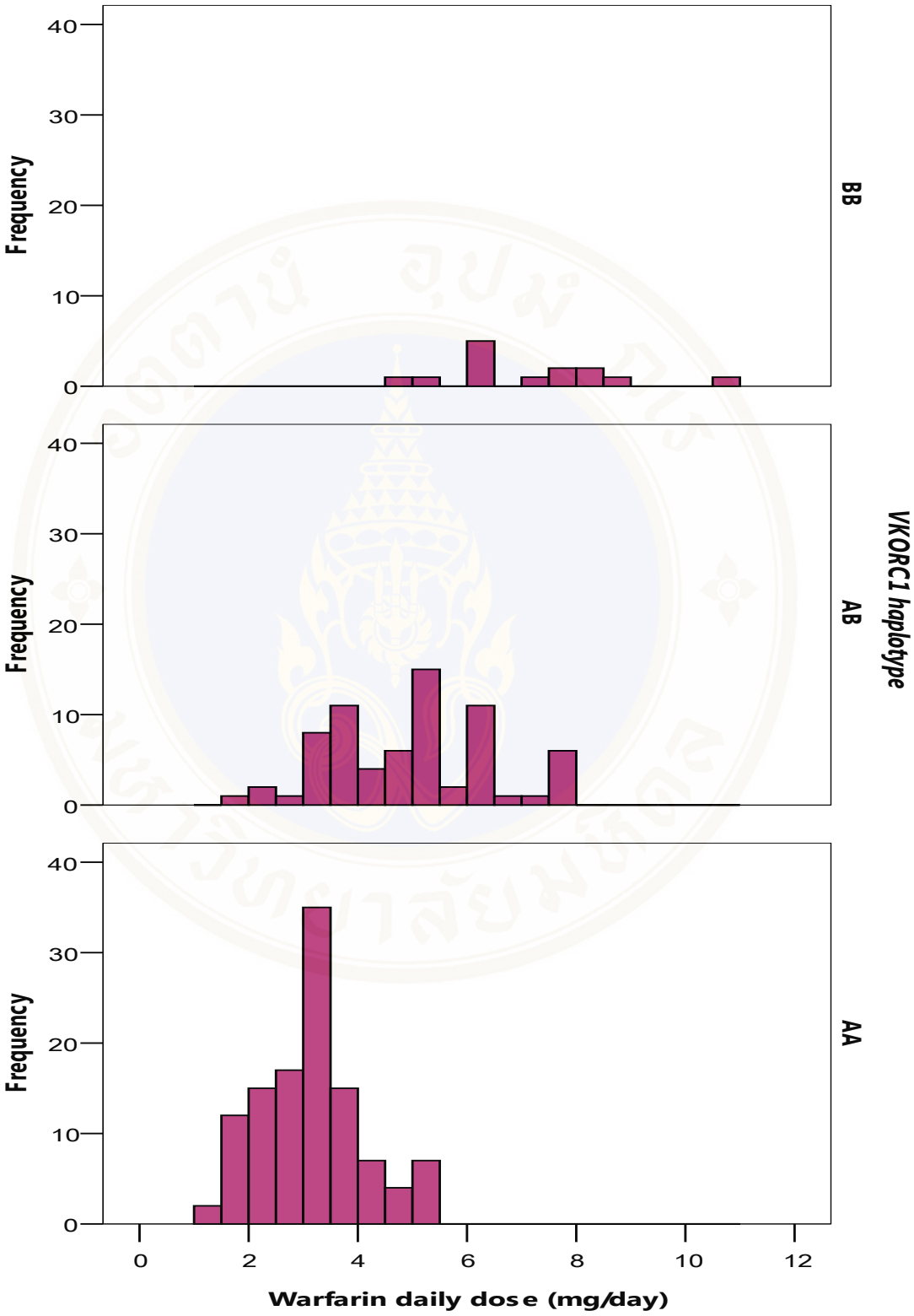


Figure 4.7 Distribution of warfarin daily dose in *VKORC1* haplotypes

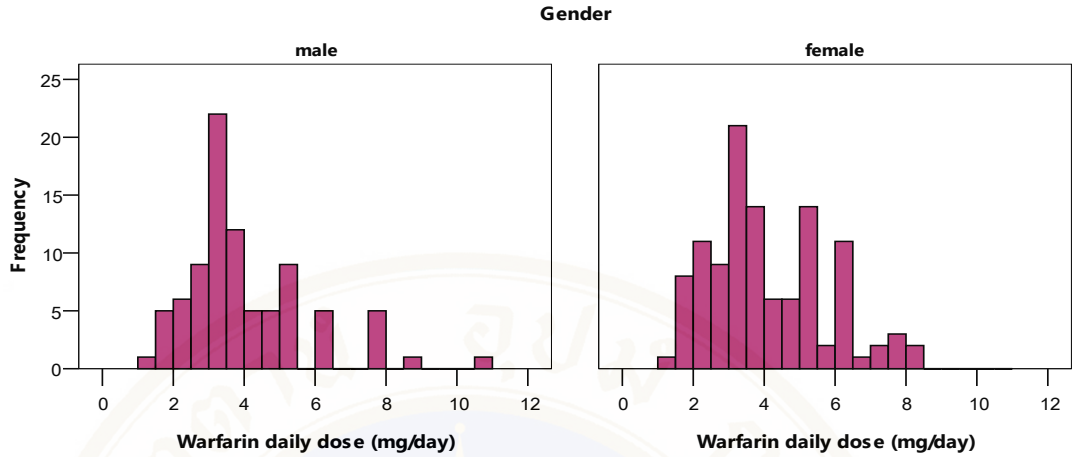


Figure 4.8 Distribution of warfarin daily dose between gender groups.

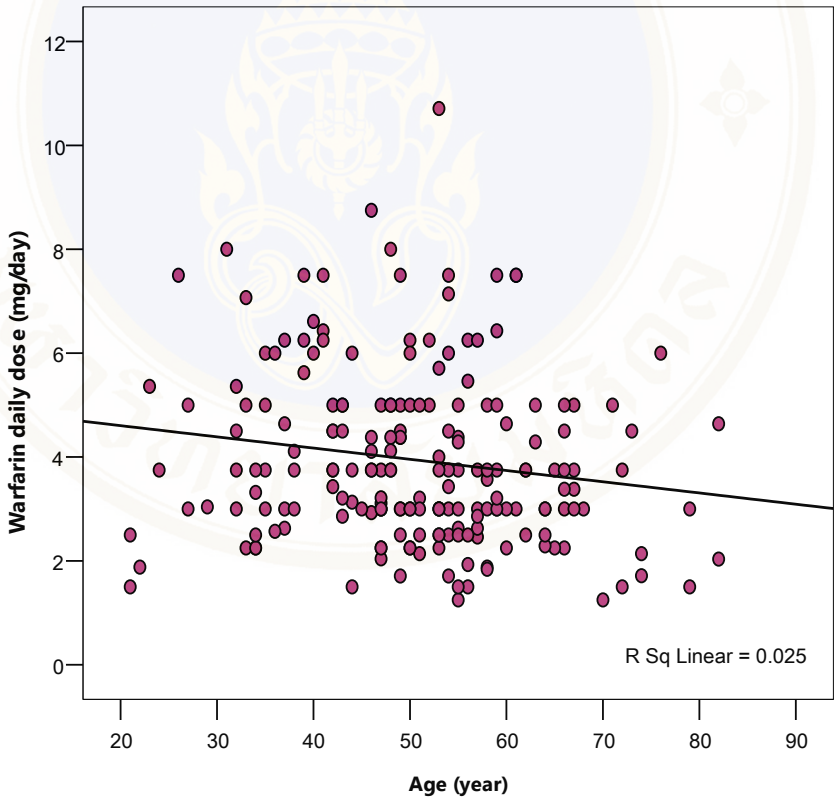


Figure 4.9 Correlation between warfarin daily dose and age (r = - 0.158)

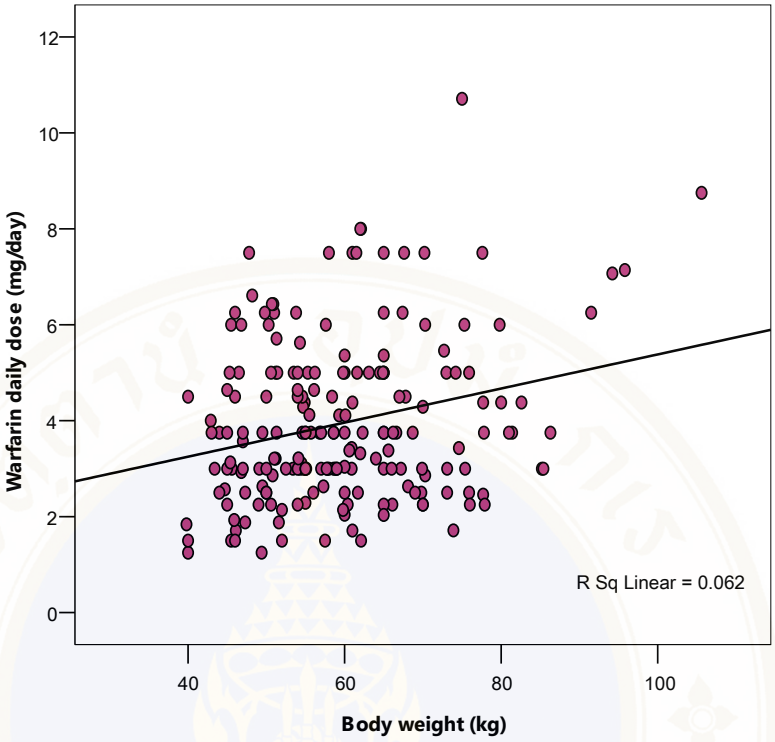


Figure 4.10 Correlation between warfarin daily dose and body weight (r = 0.249)

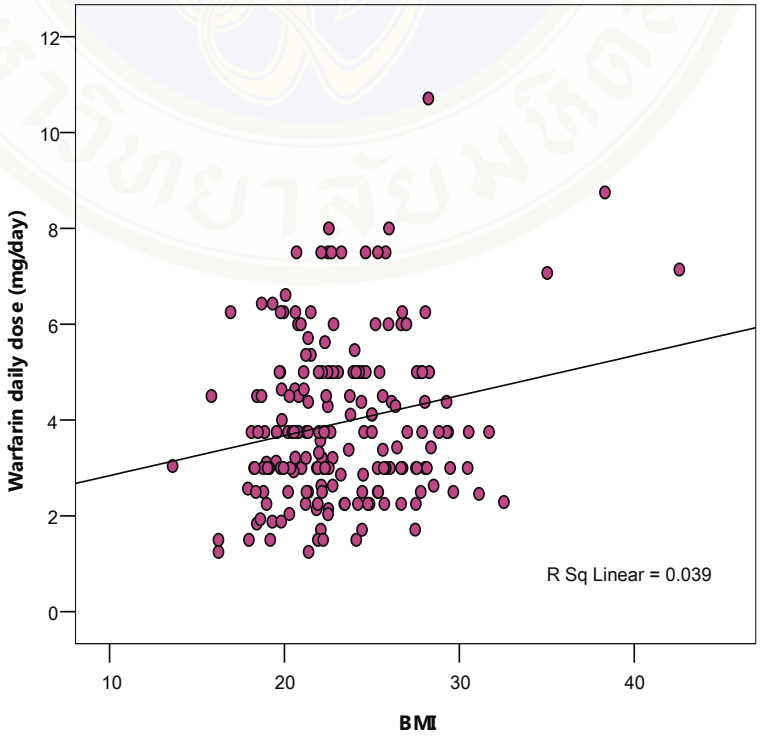


Figure 4.11 Correlation between warfarin daily dose and body mass index (r = 0.197)

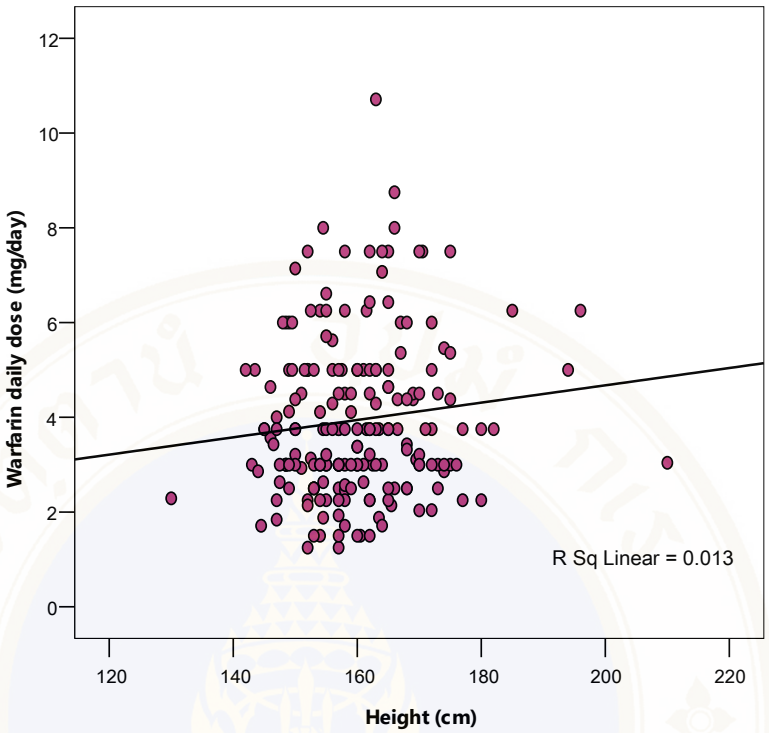


Figure 4.12 Correlation between warfarin daily dose and height (r = 0.113)

4.3 Development of warfarin dosing formula

The initial univariate analysis disclosed the following variables as being associated ($p \leq 0.25$) with weekly warfarin dose: *CYP2C9* genotypes, *VKORC1* haplotypes, statins use, age, weight, height, and BMI. These variables were entered into a stepwise multiple regression model. While we set $p \leq 0.05$ for entry and $p \geq 0.1$ for exit into the stepwise regression, four factors (age, body weight, *CYP2C9* genotype and *VKORC1* haplotype were shown to be significantly ($p < 0.001$) associated with the stable warfarin dose. Excluded variable were height ($p = 0.544$), BMI ($p = 0.474$), and statins use ($p = 0.42$). Table 4.9 depicted the multiple linear regression analysis of variables influencing warfarin dose requirements.

Table 4.9 Multiple linear regression analysis of variables influencing warfarin dose requirements

Model	B	SE	R^2 after entry	Tolerance	VIF	p-value
Constant	2.075	0.590				0.001
VKORC1-BB	3.705	0.308	0.262	0.92	1.087	< 0.001
VKORC1-AB	1.788	0.164	0.506	0.93	1.070	< 0.001
Age	-0.028	0.006	0.551	0.98	1.024	< 0.001
CYP2C9	1.173	0.289	0.583	0.98	1.022	< 0.001
Weight	0.022	0.007	0.606	0.96	1.040	0.001

Dose (mg/day) = 2.075-0.028 x (age) + 0.022 x (weight) + 1.173 x (*CYP2C9*1/*1*) + 1.788 x *VKORC1*-AB + 3.705 x *VKORC1*-BB Input age in years; weight = weight in kg; input 0 for *CYP2C9*1/*3*, 1 for *CYP2C9*1/*1*; input 1 for *VKORC1*-AB, 0 for otherwise; input 1 for *VKORC1*-BB, 0 for otherwise.
R = 0.779, R^2 = 0.606, Adjusted R^2 = 0.596

The collinearity check for this model, which ensured that the assumption of independent covariates in linear regression was not violated, showed tolerance values ranging from 0.92 for *VKORC1*- BB haplotype to 0.98 for *CYP2C9* genotype corresponding variance inflation factors (VIF) values of 1.087 to 1.022. These were within the normal acceptable limits of greater than 0.20 for tolerance and less than 4 for VIF.

Table 4.10 Stepwise regression modeling

Variables	Regression model	R^2	p-value
VKORC1 haplotype	D = 3.042 + 3.991(BB) + 1.77(AB)	0.501	< 0.001
+ Age	D = 4.480 + 4.005(BB) + 1.861(AB) – 0.029(Age)	0.551	< 0.001
+ *CYP2C9* genotype	D = 3.513 + 3.886(BB) + 1.794(AB) – 0.03(Age) + 1.134 (*CYP2C9*1/*3*)	0.583	< 0.001
+ Weight	D = 2.075 + 3.705(BB) + 1.788(AB) – 0.028(Age) + 1.173(*CYP2C9*1/*1*) + 0.022(Weight)	0.606	< 0.001

Input age in years; weight = weight in kg; input 0 for *CYP2C9*1/*3*, 1 for *CYP2C9*1/*1*; input 1 for *VKORC1*-AB, 0 for otherwise; input 1 for *VKORC1*-BB, 0 for otherwise.

Four factors were shown to be significantly associated with the stable warfarin dose in stepwise multivariate analysis (Table 4.10). *VKORC1* haplotype explained 50.1% of the variability in warfarin dose, followed by age (5%), *CYP2C9* genotype (3.2%), and body weight (2.3%). The final formula exhibited an R^2 value of 0.606 ($p < 0.005$).

The final formula (Equation 1) included *CYP2C9* genotype, *VKORC1* haplotype, age and weight for estimating mean daily warfarin dose requirements in milligram per day was as follows:

Dose = 2.075-0.028 x (age) + 0.022 x (weight) + 1.173 x (*CYP2C9*1/*1*) + 1.788 x *VKORC1*-AB + 3.705 x *VKORC1*-BB …..Eq (1)

These variables in this regression model explained 60.6% ($p < 0.001$) of the overall variability in daily warfarin dose in the study cohort as measured by R^2 (Figure 4.13).

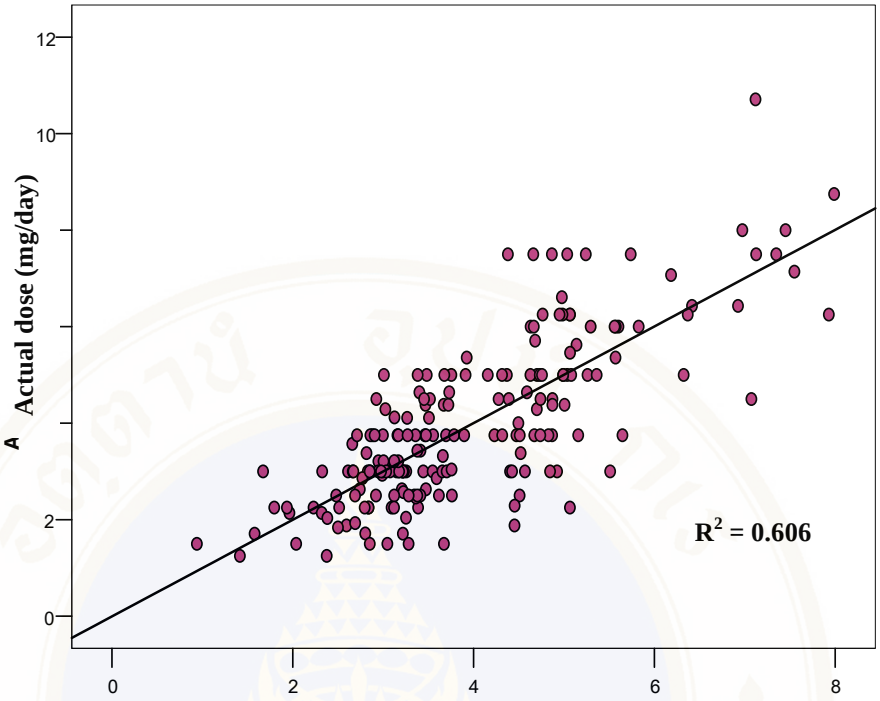


Figure 4.13 Correlation between actual and predicted daily warfarin dose in the study cohort (Equation 1)

The simplified calculation formula (Equation 2) included such variables for estimating mean warfarin dose requirements in milligram per day was as follows:

Dose = 2.1 – 0.03 x (age) + 0.02 x (weight) + 1.2 x (*CYP2C9*1/*1*) + 1.8 x *VKORC1*-AB + 3.7 x *VKORC1*-BB …..Eq (2)

This simplified calculation model explained 60.6% (Figure 4.14) of the variation in the study cohort.

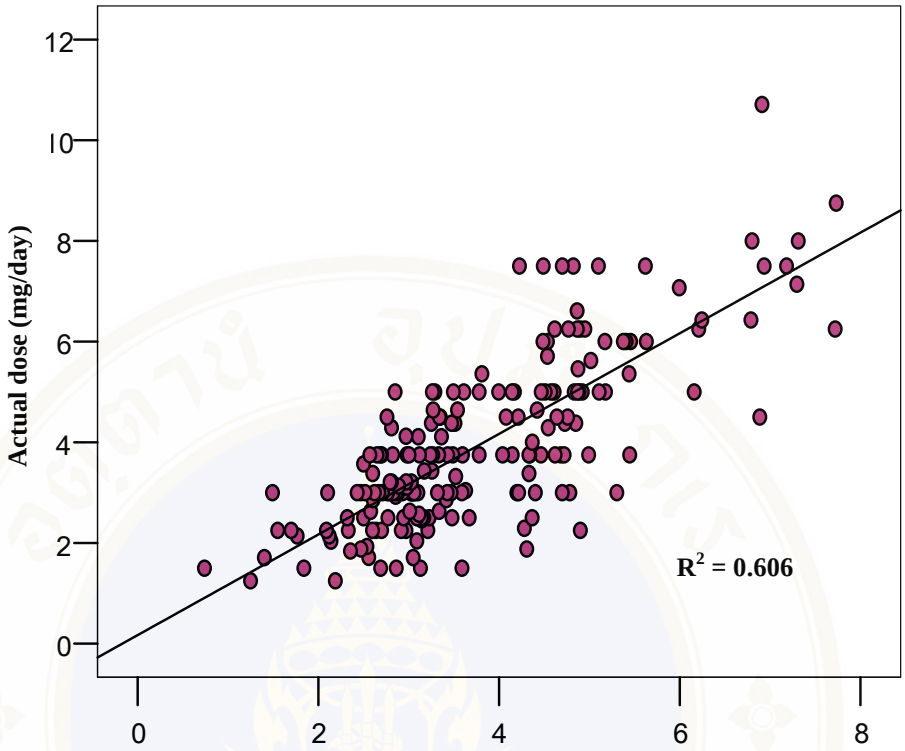


Figure 4.14 Correlation between actual and predicted dose of simplified dosing formula (Equation 2)

If we showed less decimal places of the simplified calculation dosing formula (Equation 3) as followed:

Dose (mg/day) = 2 – 0.03 x (age) + 0.02 x (weight) + (*CYP2C9*1/*1*)
+ 2 x *VKORC1*-AB + 4 x *VKORC1*- BB ….. Eq (3)

An R^2 value decreased to 60.4% (Figure 4.15). In addition, the unstandardized residual of actual dose and predicted dose by using the equation 3 (Figure 4.16) was more than the unstandardized residual of the simplified calculation dosing formula (Figure 4.17).

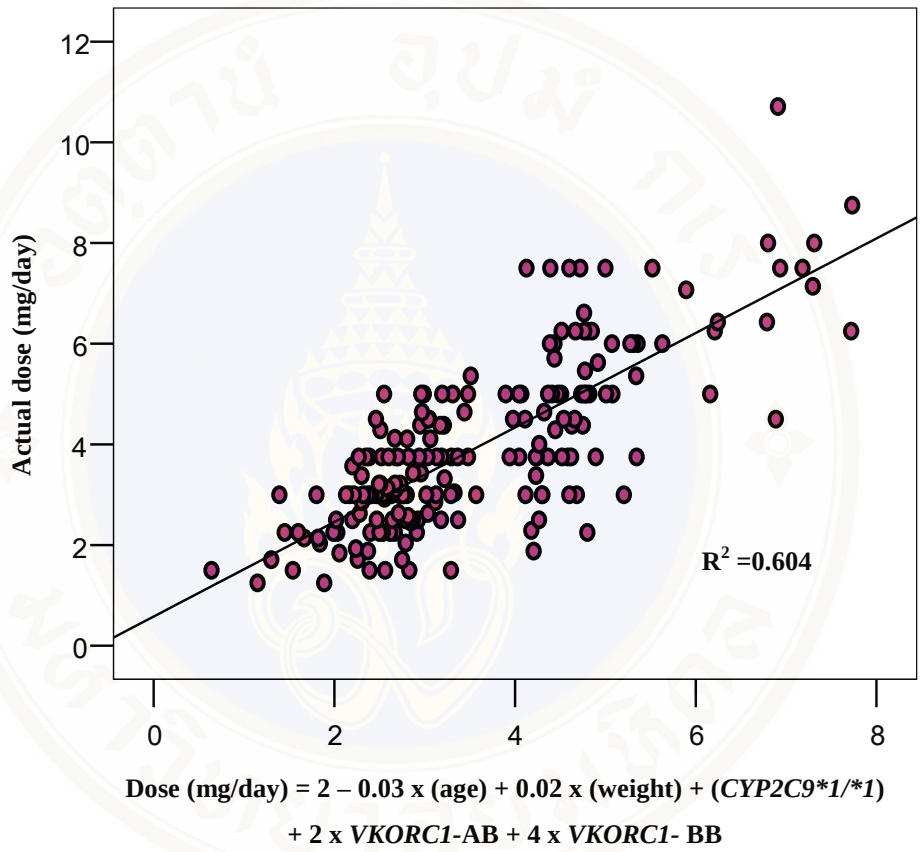


Figure 4.15 Correlation between actual and predicted dose of simplified dosing formula (Equation 3)

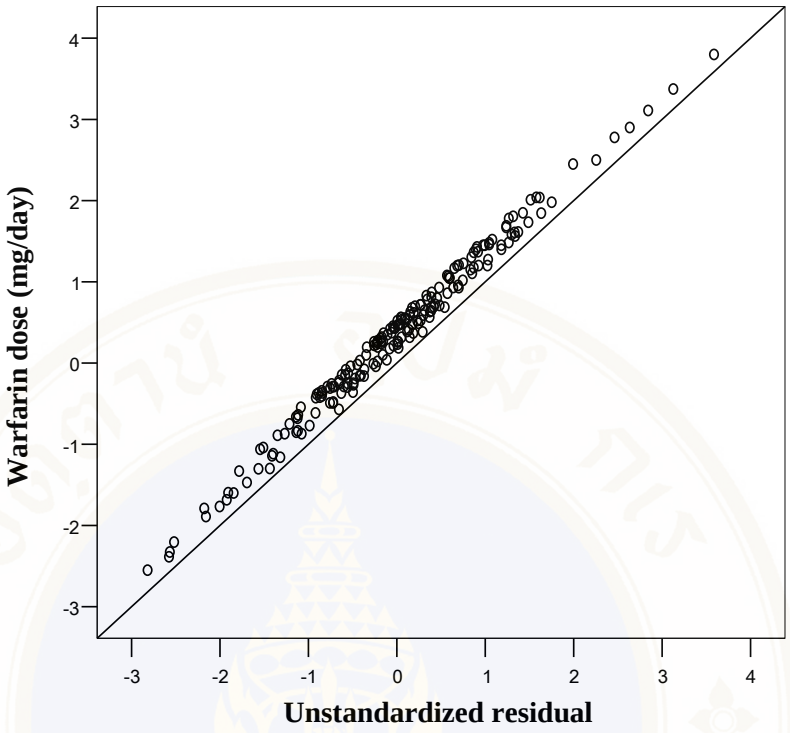


Figure 4.16 The unstandardized residual of the formula that have been reducing the number of decimal point (Equation 3)

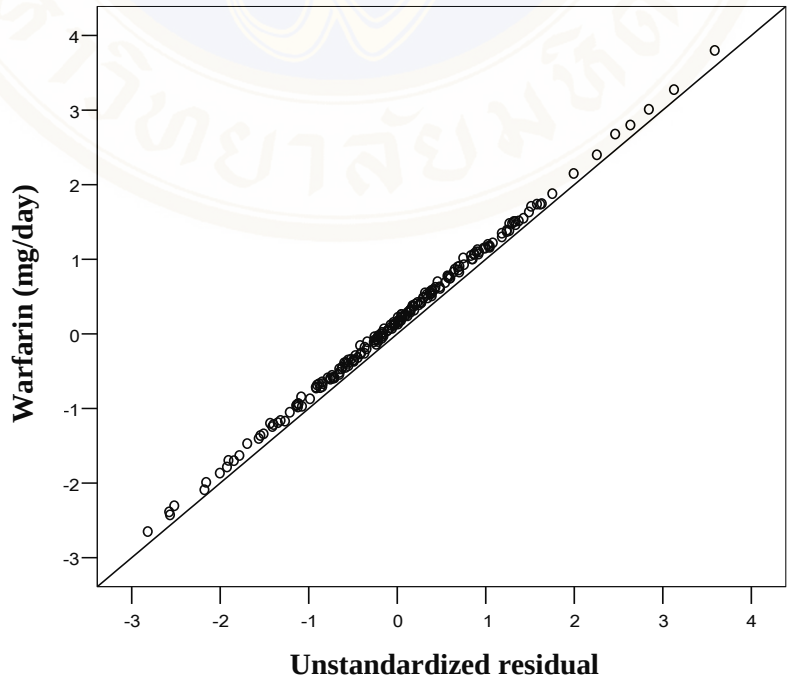


Figure 4.17 The unstandardized residual of the simplified calculation dosing formula (Equation 2)

Several multiple linear regression formulas were designed for patients who had all clinical and genetic variables (Table 4.11). Separate formulas for each combined genotype and haplotype were shown in Table 4.12.

Table 4.11. Regression formulas for warfarin daily dose requirements based on clinical and genetic factors

Variables	Formula	R^2	p-value
Age, Weight	Dose (mg/day) = 2.88 – 0.019 x (age) + 0.034 x (weight)	0.081	< 0.001
+ *CYP2C9*	Does (mg/day) = 1.302 – 0.21 x (age) + 0.035 x (weight) + 1.746 x (*CYP2C9*1/*1*)	0.157	< 0.001
+ *VKORC1*	Does (mg/day) = 2.075 – 0.028 x (age) + 0.022 x (weight) + 1.173 x (*CYP2C9*1/*1*) + 1.788 x (*VKORC1*-AB) + 3.705 x (*VKORC1*-BB)	0.60.6	< 0.001

Input age in years; weight = weight in kg; input 0 for *CYP2C9*1/*3*, 1 for *CYP2C9*1/*1*; input 1 for *VKORC1*-AB, 0 for otherwise; input 1 for *VKORC1*-BB, 0 for otherwise.

Table 4.12 Separated formulas for each combined *CYP2C9* genotype and *VKORC1* haplotypes

Genotypes of patient	Formula
**1/*3, AA*	2.075 – 0.028 x (age) + 0.022 x (weight)
**1/*3, AB*	2.075 + 1.788 – 0.028 x (age) + 0.022 x (weight)
**1/*1, AA*	2.075 + 1.173 – 0.028 x (age) + 0.022 x (weight)
**1/*1, AB*	2.075 + 1.788 + 1.173 – 0.028 x (age) + 0.022 x (weight)
**1/*1, BB*	2.075 + 3.705 + 1.173 – 0.028 x (age) + 0.022 x (weight)

Input age in years; weight = weight in kg

4.4 Model fitting test

Predicted warfarin doses of our patient cohort (N =197) were calculated using the previously published formulas that covered multi- ethnic populations and compared for accuracy with actual daily warfarin dose (Table 4.13). The correlation coefficient (R^2) in Thais population was tested.

Compared with ours, formulas derived from Iran, Turkish, Brazilian and Caucasian performed poorly when applied to our study cohort, with correlation coefficient (R^2) 22% - 52%. While, the correlation coefficient explained were 55% and 56.5% in Miao's and Tham's studies, respectively. In addition, the extent of a predicted dose deviation from an actual dose was also analyzed. The predicted dose of 105 of 197 (53.3%) patients were within ≤ 20% and 148 of 197 (75.1%) were within ≤ 30% of actual dose (Figure 4.18-4.19).

Table 4.13 Application of published formulas in Thai population

Published formula	**Populations**	**R^2 (%) in Thais**
Namazi et al [97]	Iran	22.7
Ozer et al [23]	Turkish	30.6
Sconce et al [11]	Caucasian	32.1
Perini et al [98]	Brazilian	43.8
Miao et al [20]	Chinese	55.0
Tham et al [12]	Chinese/Malay/Indian in Singapore	56.5

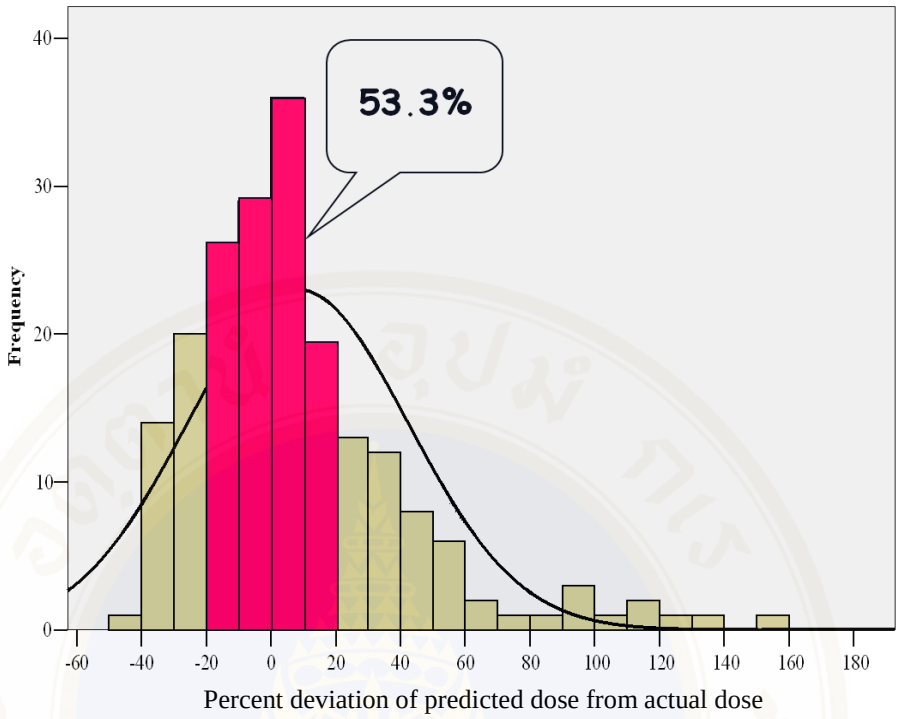


Figure 4.18 Distribution of warfarin predicted dose (percent) from actual dose (≤ 20%)

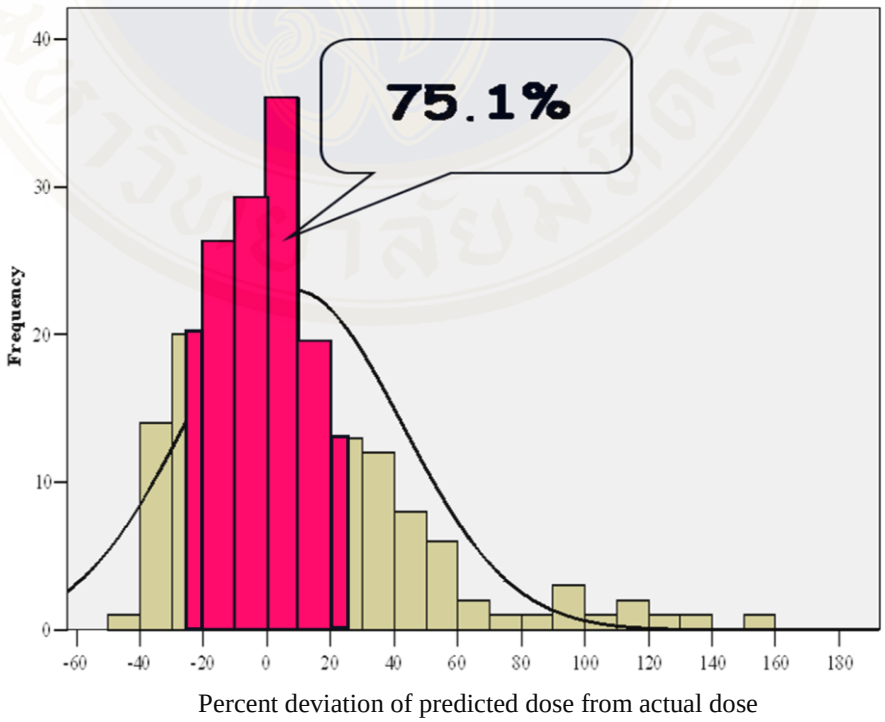


Figure 4.19 Distribution of warfarin predicted dose (percent) from actual dose (≤ 30%)

CHAPTER V
DISCUSSION

Despite major development in healthcare system, valvular heart disease especially rheumatic heart disease remains to be an important burden on Thai healthcare system. At the Siriraj Hospital alone, mechanical valve replacement is performed in approximately 300 cases annually. Mechanical valve was used in two third of total valvular surgery. Consequently, a large number of patients are placed on lifelong oral anticoagulant therapy each year. Despite its effectiveness, managing warfarin therapy is a major challenge. It is now well known that genetic factors play an important role in variation of warfarin dose requirement. A number of studies were performed to find a way to guide warfarin dosing using the combination of genetic and clinical factors to develop dosing formula. Despite some success of such formula, their application outside of ethnic groups remains problematic. Although there was a study previously conducted in Thai population to produce a dosing formula, the study was with small sample size and lack adequate representation of certain genes with low frequency. Therefore, a dosing formula developed by Thai patient cohort with large number of patients and adequate representation of all gene types is needed.

This study was designed to develop the warfarin dosing formula based on pharmacogenomics and clinical factors in patients with mechanical heart valve replacement. The association between genetic and clinical factors with maintenance dose of warfarin requirement was mainly outcome for evaluation. In addition, prevalence of *CYP2C9* and *VKORC1* genotype were also reported.

In this study, an INR in the range of 2-3 was selected as the therapeutic range which was lower than INR of 2.5-3.5, the range recommended by guidelines from western countries. Studies conducted in Thailand and other Asian countries suggest that lower range of INR may be comparably effective in the prevention of thromboembolic complications in this population [93-95, 99-101].

5.1 The prevalence of *CYP2C9* genotype and *VKORC1* haplotype

Genetic polymorphism of *CYP2C9* is one of the major factors responsible for the metabolism of warfarin. In this study, we found that prevalence of *CYP2C9* polymorphisms is mainly *CYP2C9*1/*1* (92.4%). Our study was similar to three studies which were conducted the prevalence of *CYP2C9* genotype in Thai population [24, 73, 102]. No mutant *CYP2C9*2* allele was found in any of 197 Thai patients genotyped in this study. In addition, no patient homozygous for *CYP2C9*3* was found. Several reports have documented the difference in prevalence of the allelic variants of *CYP2C9* among different ethnic group involving Caucasian, African and Asian populations. The allele frequencies of *CYP2C9*2* and *CYP2C9*3* tend to be greater in Caucasian and African-Americans populations but *CYP2C9*2* allelic variant has not been found in Asian population. These results suggest that the *CYP2C9*2* genotype is absent or at least very rare in Thais and other East Asian populations, and routine genotyping for this mutant allele is not necessary to determine the effects of these variant on catalytic activity of *CYP2C9* in these Asian populations. By comparison, the study conducted by Busakornruangrat et al found the highest prevalence of *CYP2C9*1/*3* (8.9%), followed by our study (7.6%), Kuanprasert et al (4.87%), and Sangviroon et al (3.4%). One possible explanation is that the indication of warfarin treatment in Busakornruangrat et al was venous thromboembolism; they may have patients with various warfarin therapy. While Kuanprasert et al. and Sangviroon et al. including this study were patients with valvular heart disease. Study conducted by Sangviroon found the smallest prevalence of *CYP2C9*1/**3, it is likely that sample size was too small (N=89). In addition, the possible reason of Kuanprasert et al may that the cohort evaluated by Kuanprasert et al was entirely from the Northern part of Thailand with some mixture of Burmese descendants and hill-tribe minorities. Supported by Tassaneeyakul et al [103], who studied the pattern of *CYP2C19* polymorphism in three Southeast Asian populations. The subjects were Thai, Burmese, and Karen. They found that the frequency of *CYP2C19*1/*3* genotype in the Karen population (0.8%) was significantly lower than those observed in Thai (3.7%) and Burmese populations (5.5%). Heterogeneity among the Thai ethnic particularly those who reside in the different part of the country is well recognized. They are different in several aspects including living style, diet, culture and their ancestors.

When looking at demographic distribution of our study, we may have a better representation of Thai patients across the country. So, genetic diversity among Thai populations residing in different parts of Thailand is quite apparent. Therefore, data obtaining from one population may not be a good representative of a Thai population as a whole. For *CYP2C9*3/*3,* none of the 197 study patients was homozygous, but the study conducted by Sangviroon et al found 1 patient of 89 carried *CYP2C9*3/*3* (1.1%) [24]. Unlike East Asian populations, several patients homozygous for *CYP2C9*3* have been identified in Caucasian and Turkish populations [104]. In conclusion, frequency of *CYP2C9* polymorphisms in Thais is mainly *CYP2C9*1/*1* and *CYP2C9*1/*3,* but no individual expressing the *CYP2C9*2* which was similar to several previous studies among Japanese [105], Chinese, Taiwanese [106], Korean [107] and Malaysian [108].

Regarding *VKORC1,* haplotype AA was dominated in Thai patients. Klamchuen et al [109] reported the prevalence of *VKORC1* and *CYP450 2C9* in normal Thai healthy volunteers and patients who took warfarin and found the frequencies of *VKORC1* AA, AB, and BB haplotypes to be 61.1%, 33.6% and 5.3% in healthy volunteer and 63.2%, 31.1% and 5.7% in patients taking warfarin, respectively [109]. Our results confirm such findings in previous studies. For *VKORC1* BB, however, our study identified the highest number of such cases compared to previous studies. Kuanprasert et al [73] identified only 2% of *VKORC1* haplotype BB among 242 patients. *VKORC1* haplotype frequencies in Thais were different from other Asians such as Chinese, Japanese, Malaysian and Indian [12, 20, 110]. Mushiroda et al [110] reported in 828 Japanese patients that *VKORC1* BB, AB and AA groups were 0.8%, 15.9% and 83.3%, respectively. Miao et al [20] demonstrated in 178 Chinese patients that *VKORC1* BB, AB and AA groups were 0.6%, 15.7% and 83.7%, respectively.

5.2 Relationships between genetic and clinical factors with warfarin dose requirements

Warfarin has a narrow therapeutic index. Consequently, the dose required to achieve therapeutic anticoagulation is very close to the dose that leads to over-anticoagulation. Furthermore, the maintenance dose varies between different individuals. In our study, warfarin dose range from 1.25-10.71 mg per day. This unpredictability leads to difficulties in maintaining patients within a therapeutic anticoagulation range. The most important genes affecting the pharmacokinetic and pharmacodynamics of warfarin are *CYP2C9* and *VKORC1*. These two genes, together with clinical factors, partly explain the interindividual variation in warfarin dose requirements.

CYP2C9 polymorphism was apparently associated with the clearance of warfarin. Wild type patients (*CYP2C9*1/*1*), S-warfarin is metabolized normally, resulting in a normal increase of the INR. In contrast, patients with *CYP2C9*2* or *CYP2C9*3* have impaired ability to metabolize of S-warfarin, resulting in patients requiring altered doses of warfarin to maintain adequate anticoagulation. The results of our study found that the daily maintenance dose of warfarin in patients having *CYP2C9*1/*3* was significant less than that of patients with *CYP2C9*1/*1* genotype (4.07 ± 1.67 mg vs 2.42 ± 0.75 mg; p = 0.004). In contrast, Busakornruangrat et al [102] showed that the weekly warfarin maintenance dose in patients with *CYP2C9*1/*3* tended to be less than that of patients with *CYP2C9*1/*1* (20.1 ± 5.9 mg vs 28.7 ± 13.0), but the difference was not statistically significant (p = 0.081). One possible explanation is that the sample size in their study was small (N = 58). In conclusion, our results extend the known association between *CYP2C9* genotype and daily warfarin dose. There were significant differences in mean dose requirements between each of the variant alleles compared with the wild type. Unfortunately, there was no one patient with the genotype **3/*3*. Therefore, the predicted doses for this genotype should be viewed with caution.

An association between *VKORC1* haplotype and warfarin daily dose was also observed in our patients. We also confirm that carriers of the *VKORC1*-AA haplotype require a significantly lower daily warfarin dose than those carrying the AB

or BB haplotypes ($p<0.001$). Similarly, in Klamchuen et al [109], they found that those who had AA haplotype required significantly lower warfarin doses (24.0 ± 10.2 mg/week) compared to those with AB (37.6 ± 13 mg/week) and BB (39.5 ± 21.3 mg/week) with the p values of <0.001. From the information of the frequency of *VKORC1* and association between *VKORC1* haplotype, this may be related to the observation that Asian patients often require a lower mean maintenance dose of warfarin compared with white patients. In conclusion, there were significant differences between warfarin maintenance dose among different *VKORC1* haplotype groups, $p <0.001$. Patients with the *VKORC1*- BB haplotype and *CYP2C9*1/*1* required the highest warfarin maintenance dose, while the patient with the *VKORC1*- AA haplotype and *CYP2C9*1/*3* required the lowest warfarin maintenance doses. Most of this Thai population were *VKORC1*- AA haplotype, *CYP2C9*1/*1* and required only about 55% of the warfarin dose required by the highest dose group (*VKORC1*- BB, *CYP2C9*1/*1*).

In the current study, in univariate analysis, age was a clinical factor that significantly affected warfarin dose ($p = 0.013$). We also found that patient with lower warfarin maintenance dosages were older than those with higher maintenance dosage in the univariate regression model. This relationship between age and the warfarin dose requirement have been confirmed by other studies [111, 112]. The exact mechanism of how age influenced the warfarin dosage was not completely understood. One of the suggested mechanisms was the pharmacokinetics (absorption, volume of distribution, metabolism, and excretion) of warfarin are not appreciably affected by advancing age. Pharmacodynamic age -related changes have not been well established but may be related to decrease dietary vitamin K intake, absorption, or changes in the capacity to utilize vitamin K for clotting factor synthesis [113]. Another proposed mechanism was the change in activity of vitamin K epoxide reductase or its affinity for warfarin in elderly [114]. There were no significant differences in warfarin dose between male and female patients and gender made no significant contribution to the regression model. In addition, height and BMI were excluded as predictor because they did not have a sufficient association with daily warfarin dose. The influence of body mass index on maintenance warfarin dose requirements has been examined in small retrospective, multivariate analysis. Two studies showed significant correlation

of BMI with warfarin dose [113]. This study found the positive correlation of BMI and height with warfarin dose requirement but not statistically significant. Unlikely height, body weight was routinely recorded for all patients in clinical practice.

Some studies have reported that cigarette smoking has a significant impact on warfarin dose [97], but Whitley et al [115] did not find a significant relationship between cigarette smoking and warfarin response. Smoking can induce CYP1A2, which may increase *R*-warfarin metabolism in patients who start or stop smoking during warfarin therapy [97]. We did not evaluate the effect of cigarette smoking on warfarin dose requirement in this study. Only 4% of patients were light smokers; therefore, none were selected for regression model. The possible reason for drinking to have an influence on warfarin dosage was ethanol was found to displace warfarin from human serum albumin and affecting the pharmacokinetics of warfarin [116]. However, 98% of patient never drinking after mechanical valve replacement; therefore, none were selected for regression model. Factors of comorbidity, concurrent medication and the target INR were controlled for in our study, as these formed part of the exclusion criteria of the study. The target INR for the patients in our study was 2 to 3; the range was probably too narrow to have much influence on warfarin dose. The effect size of drugs known to have significant interactions (such as statins use, omeprazole) were not factored in the final warfarin dosing formula, because of the small number of patients taking such medications.

5.3 Warfarin dosing model

In previous studies, dosing formulas using genetic factors incorporated with non-genetic factors contained only *CYP2C9* genetic information [18, 117, 118]. Although the formulas showed the potential of pharmacogenetics based dosing, they accounted for less than half of the variability in warfarin dose. A review of published dosing formulas showed that the model could be explained for 50-60% of warfarin dose variability when incorporate *VKORC1* genotype as covariates. As seen in the present study, the stable warfarin dose required to achieve a stable INR are highly variable between patients (1.25 – 10.71 mg/day). Genetic factors; *CYP2C9*, *VKORC1* and non- genetic factors; age and weight, were found to be significantly correlated

with warfarin dose requirements in our study. A warfarin dosing formula was developed using these factors help explaining 60.6 % of the variability in warfarin dose. Our results were in accordance with previous studies. Tham et al [12] reported that a multiple linear regression analysis was developed based on patient age, weight, *VKORC1* and *CYP2C9* can explained warfarin dose variability 60.2%. Gender, height, *CYP2C9**2, serum albumin concentration, and INR were not found to be significantly correlated with warfarin dose requirement. The correlation was similar to Miao et al [20]. They found significant that age and weight were significant predictors of warfarin dose requirement. Kimura et al [13] found that the age, sex, and weight were significantly associated with daily warfarin dose. Similar to the study conducted by Zhu et al [22], a model including *VKORC1,* CYP2C9, age, sex and body weight accounted for 61% of the variance in warfarin daily dose. Sconce et al [11] reported that a multiple linear regression analysis was developed on the basis of patient age, height, *VKORC1* and *CYP2C9* genotype produced the best model accounted for 55% of the variable warfarin dose requirement. The inclusion of height as a variable is unique to the Sconce et al [11] report. However, weight-based dosing of warfarin is a more conventional approach to warfarin dose adjustments in our model. Comparing with a previous study conducted in Thai population, our warfarin dosing model accounted for 60.6% compared with Sangviroon et al [24] that explained about 53.8% of the variance of warfarin maintenance dose. Important differences between Sangviroon et al [24] and this study was that Sangviroon's study developed model using an exponential function while linear regression was conducted in this study. In contrary to our study, Sangviroon et al [24] did not observe significant influence of weight on warfarin dosing. One possible explanation of this discrepancy is the small sample size of Sangviroon's study. Moreover, most studied report that warfarin dose requirements of the study populations do not show a normal distribution. Sconce et al [11] handled this using a square root transformation of data, where as Zhu et al and 4 other groups [12, 21, 24, 119] reported logarithmic transformations. Overall, the model that we developed combines many of the most favorable aspects of prior approaches, specifically using simple regression equation. Table 5.1 depicts comparison of different variables included in dosing formula from various investigators.

Table 5.1 Comparison of different variables included in warfarin dosing formula from published reports

Researcher / Variables	Zhu et al.	Sconce et al.	Tham et al.	Miao et al	Sangviroon et al.	Gage et al.	Our study
VKORC1	✓	✓	✓	✓	✓	✓	✓
CYP2C9	✓	✓	✓	✓	✓	✓	✓
Age	✓	✓	✓	✓	✓	✓	✓
Weight	✓		✓	✓			✓
Height		✓					
Gender	✓						
BSA						✓	
Race						✓	
Target INR						✓	
Indication						✓	
Smoking						✓	
Amiodarone						✓	
R^2 (%)	61%	55%	60.2%	63%	53.8%	53%	60.6%

5.4 Model fitting test

Two tests were performed to evaluate precision of this formula. First, predicted warfarin doses were calculated and compared for accuracy with actual doses. The proportion of variation explained (R^2) was low in the Iran, Turkish, Brazilian and Caucasian but high in the Asian group. Because no formulas could be considered the best for all dosing ranges, it may be important to consider the nature of a local service population in choosing the most appropriate pharmacogenetics-based dosing formula. Several factors may help explain such discordance including the validity of retrospective clinical data, differences in races which algorithms were derived, differences in environmental factors and low frequency of some genotypes. Second, clinical accuracy of the predictions was assessed by computing the proportion of

patients in which the predicted dose within 20% and 30% of actual dose. Results of these two tests indicate a strong potential for clinical application of such formula.

5.5 Limitations of the study

One of the limitations of the present study was the small sample size. Obviously, a larger sample size would have provided a more reliable regression model. It should also be noted that our inclusion and exclusion criteria limited the ability to extrapolate results to other members of the general Thai population. Our proposed warfarin dosing formula should be validated in a second cohort of patients receiving chronic therapy with warfarin. By being retrospective, the associations and dosing algorithm described in our study must be prospectively tested in an appropriately large cohort. By explaining approximately 60% of the variance in warfarin dosing, our formula leaves 40% of this variance to be explained by other variables. Factors of co-morbidities, drug interactions, alcohol consumption, and smoking status were controlled through exclusion criteria of the study. However, due to the study design, the contribution of vitamin K status was not examined. One study that examined the influence of vitamin K upon warfarin anticoagulation found that plasma vitamin K concentration was significantly negatively correlated with patient INR [114]. Other published model examined the contribution of several other predictors such as concurrent interacting drugs, treatment indications, mean and goal INRs, factor X and factor VII genotypes, vitamin K intake, smoking status, calumenin, γ-glutamyl carboxylase, polymorphisms in apolipoprotein E and *CYP4F2*. The inclusion of polymorphisms of additional genes did not significantly increase the predictability of the models compared with simpler models that incorporated only *VKORC1* and *CYP2C9* genotypes. Therefore *VKORC1* and *CYP2C9* polymorphisms remain the most informative genotypic predictors in our population. Moreover, our dosing formula, developed for Thai patient with mechanical heart valve replacement, may not be applicable to other population groups.

CHAPTER VI
CONCLUSION

Overall, the results of this study provided important information on prevalence of *CYP2C9* and *VKORC1* genotype among Thai patients with mechanical heart valves. In consistent with previous findings, the majority of Thai populations carried *VKORC1*-AA and *CYP2C9*1/*1* which help explain lower average dose of warfarin in Thai populations compared to that of Caucasians and Blacks. Our study also confirms findings of previous studies on the importance of *CYP2C9* and *VKORC1* genotype on warfarin dose requirement. Among various clinical factors, age and weight showed significant relationship with warfarin dose but with only minor contribution compared to that of genetic factors. Subsequently, a warfarin dosing formula is successfully developed and found to explain 60.6 % of the variability in warfarin dose. With a simplified calculation dosing formula, it can be made easily accessible for use to select the patient's initial warfarin dose and predict the stable warfarin dose requirement in the clinical practice. Preliminary tests to evaluate accuracy of such dosing formula suggest high potential for clinical application to guide warfarin dosing selection in Thai patients compared to other existing formulas. However, further studies are needed to evaluate precision, clinical benefits, and cost-effectiveness of such formula in clinical practice.

REFERENCES

1 Gage BF. Pharmacogenetics-based coumarin therapy. Hematology Am Soc Hematol Educ Program. 2006:467-73.

2 Ageno W, Squizzato A, Dentali F, Crowther M. Tailoring warfarin induction doses to reflect individual and disease-specific factors. Am J Med. 2005; 118(2):143-4.

3 Yin T, Miyata T. Warfarin dose and the pharmacogenomics of CYP2C9 and VKORC1 - rationale and perspectives. Thromb Res. 2007; 120(1):1-10.

4 Rettie AE, Tai G. The pharmocogenomics of warfarin: closing in on personalized medicine. Mol Interv. 2006; 6(4):223-7.

5 Zhang K, Young C, Berger J. Administrative claims analysis of the relationship between warfarin use and risk of hemorrhage including drug-drug and drug-disease interactions. J Manag Care Pharm. 2006; 12(8):640-8.

6 Fihn SD, McDonell M, Martin D, Henikoff J, Vermes D, Kent D, et al. Risk factors for complications of chronic anticoagulation. A multicenter study. Warfarin Optimized Outpatient Follow-up Study Group. Ann Intern Med. 1993; 118(7):511-20.

7 Gage BF, Eby CS. Pharmacogenetics and anticoagulant therapy. J Thromb Thrombolysis. 2003; 16(1-2):73-8.

8 Landefeld CS, Beyth RJ. Anticoagulant-related bleeding: clinical epidemiology, prediction, and prevention. Am J Med. 1993; 95(3):315-28.

9 Beyth RJ. Management of haemorrhagic complications associated with oral anticoagulant treatment. Expert Opin Drug Saf. 2002; 1(2):129-36.

10 Reynolds KK VRJ, Hartung BR, Linder MW. Individualizing warfarin therapy. Personalized Med. 2007; 4:11-31.

11 Sconce EA, Khan TI, Wynne HA, Avery P, Monkhouse L, King BP, et al. The impact of CYP2C9 and VKORC1 genetic polymorphism and patient

characteristics upon warfarin dose requirements: proposal for a new dosing regimen. Blood. 2005; 106(7):2329-33.

12 Tham LS, Goh BC, Nafziger A, Guo JY, Wang LZ, Soong R, et al. A warfarin-dosing model in Asians that uses single-nucleotide polymorphisms in vitamin K epoxide reductase complex and cytochrome P450 2C9. Clin Pharmacol Ther. 2006; 80(4):346-55.

13 Kimura R, Miyashita K, Kokubo Y, Akaiwa Y, Otsubo R, Nagatsuka K, et al. Genotypes of vitamin K epoxide reductase, gamma-glutamyl carboxylase, and cytochrome P450 2C9 as determinants of daily warfarin dose in Japanese patients. Thromb Res. 2007; 120(2):181-6.

14 Phillips KW, Dobesh PP, Haines ST. Considerations in using anticoagulant therapy in special patient populations. Am J Health Syst Pharm. 2008; 65(15 Suppl 7):S13-21.

15 Ansell J, Hirsh J, Hylek E, Jacobson A, Crowther M, Palareti G. Pharmacology and management of the vitamin K antagonists: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest. 2008; 133(6 Suppl):160S-98S.

16 Kamali F, Khan TI, King BP, Frearson R, Kesteven P, Wood P, et al. Contribution of age, body size, and CYP2C9 genotype to anticoagulant response to warfarin. Clin Pharmacol Ther. 2004; 75(3):204-12.

17 Hillman MA, Wilke RA, Caldwell MD, Berg RL, Glurich I, Burmester JK. Relative impact of covariates in prescribing warfarin according to CYP2C9 genotype. Pharmacogenetics. 2004; 14(8):539-47.

18 Gage BF, Eby C, Milligan PE, Banet GA, Duncan JR, McLeod HL. Use of pharmacogenetics and clinical factors to predict the maintenance dose of warfarin. Thromb Haemost. 2004; 91(1):87-94.

19 Takahashi H, Wilkinson GR, Nutescu EA, Morita T, Ritchie MD, Scordo MG, et al. Different contributions of polymorphisms in VKORC1 and CYP2C9 to intra- and inter-population differences in maintenance dose of warfarin in Japanese, Caucasians and African-Americans. Pharmacogenet Genomics. 2006; 16(2):101-10.

20 Miao L, Yang J, Huang C, Shen Z. Contribution of age, body weight, and CYP2C9 and VKORC1 genotype to the anticoagulant response to warfarin: proposal for a new dosing regimen in Chinese patients. Eur J Clin Pharmacol. 2007; 63(12):1135-41.

21 Anderson JL, Horne BD, Stevens SM, Grove AS, Barton S, Nicholas ZP, et al. Randomized trial of genotype-guided versus standard warfarin dosing in patients initiating oral anticoagulation. Circulation. 2007; 116(22):2563-70.

22 Zhu Y, Shennan M, Reynolds KK, Johnson NA, Herrnberger MR, Valdes R, et al. Estimation of warfarin maintenance dose based on VKORC1 (-1639G>A) and CYP2C9 genotypes. Clin Chem. 2007; 53(7):1199-205.

23 Ozer N, Cam N, Tangurek B, Ozer S, Uyarel H, Oz D, et al. The impact of CYP2C9 and VKORC1 genetic polymorphism and patient characteristics upon warfarin dose requirements in an adult Turkish population. Heart Vessels. 2010; 25(2):155-62.

24 Sangviroon A, Panomvana D, Tassaneeyakul W, Namchaisiri J. Pharmacokinetic and pharmacodynamic variation associated with VKORC1 and CYP2C9 polymorphisms in Thai patients taking warfarin. Drug Metab Pharmacokinet. 2010; 25(6):531-8.

25 Siritatdhamrong J, Assawawongprom, P. Evaluation of warfarin dosing formulas using cytochrome 2C9 and vitamin- K epoxide reductase complex 1 polymorphism. Thailand: Mahidol; 2008. 63p.

26 Evans WE, Johnson JA. Pharmacogenomics: the inherited basis for interindividual differences in drug response. Annu Rev Genomics Hum Genet. 2001; 2:9- 39.

27 Hill CE, Duncan A. Overview of pharmacogenetics in anticoagulation therapy. Clin Lab Med. 2008; 28(4):513-24.

28 Wolf CR, Smith G. Pharmacogenetics. Br Med Bull. 1999; 55(2):366-86.

29 Ansell J, Hirsh J, Poller L, Bussey H, Jacobson A, Hylek E. The pharmacology and management of the vitamin K antagonists: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. Chest. 2004; 126(3 Suppl):204S-33S.

30 Hirsh J, Dalen J, Anderson DR, Poller L, Bussey H, Ansell J, et al. Oral anticoagulants: mechanism of action, clinical effectiveness, and optimal therapeutic range. Chest. 2001; 119(1 Suppl):8S-21S.

31 Ansell J, Hirsh J, Dalen J, Bussey H, Anderson D, Poller L, et al. Managing oral anticoagulant therapy. Chest. 2001; 119(1 Suppl):22S-38S.

32 Stehle S, Kirchheiner J, Lazar A, Fuhr U. Pharmacogenetics of oral anticoagulants: a basis for dose individualization. Clin Pharmacokinet. 2008; 7(9):565-94.

33 Haines ST, Racine E, Zeolla M. Venous thromboembolism. In: Dipiro JT, Talbert RL, Yee GC, Matzke GR, Wells BG, Posey LM, eds. Pharmacotherapy: a pathophysiologic approach. 4 ed. USA: McGraw-Hill, 2002:337-73.

34 Kaminsky LS, Zhang ZY. Human P450 metabolism of warfarin. Pharmacol Ther. 1997; 73(1):67-74.

35 Park BK. Warfarin: metabolism and mode of action. Biochem Pharmacol. 1988; 37(1):19-27.

36 Hirsh J, Fuster V, Ansell J, Halperin JL. American Heart Association / American College of Cardiology Foundation guide to warfarin therapy. Circulation. 2003; 107(12):1692-711.

37 Harder S, Thurmann P. Clinically important drug interactions with anticoagulants. An update. Clin Pharmacokinet. 1996; 30(6):416-44.

38 Horton JD, Bushwick BM. Warfarin therapy: evolving strategies in anticoagulation. Am Fam Physician. 1999; 59(3):635-46.

39 Wittkowsky AK. Drug interactions update: drugs, herbs, and oral anticoagulation. J Thromb Thrombolysis. 2001; 12(1):67-71.

40 Greenblatt DJ, von Moltke LL. Interaction of warfarin with drugs, natural substances, and foods. J Clin Pharmacol. 2005; 45(2):127-32.

41 Holbrook AM, Pereira JA, Labiris R, McDonald H, Douketis JD, Crowther M, et al. Systematic overview of warfarin and its drug and food interactions. Arch Intern Med. 2005; 165(10):1095-106.

42 Demirkan K, Stephens MA, Newman KP, Self TH. Response to warfarin and other oral anticoagulants: effects of disease states. South Med J. 2000; 93(5):448-54.

43 Wells PS, Holbrook AM, Crowther NR, Hirsh J. Interactions of warfarin with drugs and food. Ann Intern Med. 1994; 121(9):676-83.

44 Jaffer A, Bragg L. Practical tips for warfarin dosing and monitoring. Cleve Clin J Med. 2003; 70(4):361-71.

45 Izzo AA, Di Carlo G, Borrelli F, Ernst E. Cardiovascular pharmacotherapy and herbal medicines: the risk of drug interaction. Int J Cardiol. 2005; 98(1):1-14.

46 Heck AM, DeWitt BA, Lukes AL. Potential interactions between alternative therapies and warfarin. Am J Health Syst Pharm. 2000; 57(13):1221- 7.

47 Miller LG. Herbal medicinals: selected clinical considerations focusing on known or potential drug-herb interactions. Arch Intern Med. 1998; 158(20):2200-11.

48 Kayser SR. Practical challenges in the management of oral anticoagulation. Prog Cardiovasc Nurs. 2005; 20(2):80-5.

49 สุรกิจ นาฑีสุวรรณ. ยาที่มีความเสี่ยงสูงที่ใช้ในระบบการแข็งตัวของเลือด: warfarin และ heparins.ใน: บุษบา จินดาวิจักษณ์, สุวัฒนา จุฬาวัฒนทล, เนติ สุขสมบูรณ์, วิมล อนันต์สกุลวัฒน์, สุรกิจ นาฑีสุวรรณ, ปรีชา มนฑกานติกุล, บรรณาธิการ. การบริบาลทางเภสัชกรรมเพื่อความปลอดภัยของผู้ป่วย.กรุงเทพฯ:สมาคมเภสัชกรรมโรงพยาบาล (ประเทศไทย) 2547:126-43.

50 Hirsh J, Poller L. The international normalized ratio. A guide to understanding and correcting its problems. Arch Intern Med. 1994; 154(3):282-8.

51 Ageno W, Turpie AG, Steidl L, Ambrosini F, Cattaneo R, Codari RL, et al. Comparison of a daily fixed 2.5-mg warfarin dose with a 5-mg, international normalized ratio adjusted, warfarin dose initially following heart valve replacement. Am J Cardiol. 2001; 88(1):40-4.

52 สุอาภา พลอยเลื่อมแสง. การหาขนาดยาวาร์ฟารินที่เหมาะสมในผู้ป่วยนอกหลังผ่าตัดเปลี่ยนลิ้นหัวใจที่โรงพยาบาลราชวิถี (วิทยานิพนธ์ปริญญาเภสัชศาสตรมหาบัณฑิต สาขาวิชาเภสัชกรรม). กรุงเทพฯ: บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย; 2541.

53 Gage BF, Fihn SD, White RH. Management and dosing of warfarin therapy. Am J Med. 2000; 109(6):481-8.

54 Glasheen JJ. Preventing warfarin-related bleeding. South Med J. 2005 ; 98(1):96-103.

55 Kuruvilla M, Gurk-Turner C. A review of warfarin dosing and monitoring. Proceedings (Baylor University. 2001; 14(3):305-6.

56 Leyh RT, Fischer S, Ruhparwar A, Haverich A. Anticoagulant therapy in pregnant women with mechanical heart valves. Arch Gynecol Obstet. 2003;268(1):1-4.

57 Frewin R, Chisholm M. Anticoagulation of women with prosthetic heart valves during pregnancy. Br J Obstet Gynaecol. 1998; 105(7):683-6.

58 James AH. Thromboembolism in pregnancy: recurrence risks, prevention and management. Curr Opin Obstet Gynecol. 2008; 20(6):550-6.

59 Greer IA, Thomson AJ. Management of venous thromboembolism in pregnancy. Best Pract Res Clin Obstet Gynaecol. 2001; 15(4):583-603.

60 Clark SL, Porter TF, West FG. Coumarin derivatives and breast-feeding. Obstet Gynecol. 2000; 95(6 Pt 1):938-40.

61 Dardick KR. Warfarin during lactation. Conn Med. 1980; 44(11):693.

62 Hanley JP. Warfarin reversal. J Clin Pathol. 2004; 57(11):1132-9.

63 Heit JA. Perioperative management of the chronically anticoagulated patient. J Thromb Thrombolysis. 2001; 12(1):81-7.

64 Chan YC, Valenti D, Mansfield AO, Stansby G. Warfarin induced skin necrosis. Br J Surg. 2000; 87(3):266-72.

65 Talmadge DB, Spyropoulos AC. Purple toes syndrome associated with warfarin therapy in a patient with antiphospholipid syndrome. Pharmacotherapy. 2003; 23(5):674-7.

66 Reza S-M, Mohamad RK, Kobra G, Mojtaba M. Purple Toes Syndrome Related to Warfarin Therapy: A Case Report. Internet J Anesthesiol. 2005; 9(1).

67 Wadelius M, Pirmohamed M. Pharmacogenetics of warfarin: current status and future challenges. Pharmacogenomics J. 2007; 7(2):99-111.

68 Nakagawa T, Kishino S, Itoh S, Sugawara M, Miyazaki K. Differential binding of disopyramide and warfarin enantiomers to human alpha(1)-acid glycoprotein variants. Br J Clin Pharmacol. 2003; 56(6):664-9.

69 Rost S, Fregin A, Ivaskevicius V, Conzelmann E, Hortnagel K, Pelz HJ, et al. Mutations in VKORC1 cause warfarin resistance and multiple coagulation factor deficiency type 2. Nature. 2004; 427(6974):537-41.

70 Harrington DJ, Underwood S, Morse C, Shearer MJ, Tuddenham EG, Mumford AD. Pharmacodynamic resistance to warfarin associated with a Val66Met substitution in vitamin K epoxide reductase complex subunit 1. Thromb Haemost. 2005; 93(1):23-6.

71 D'Andrea G, D'Ambrosio RL, Di Perna P, Chetta M, Santacroce R, Brancaccio V, et al. A polymorphism in the VKORC1 gene is associated with an interindividual variability in the dose-anticoagulant effect of warfarin. Blood. 2005; 105(2):645-9.

72 Moyer TP, O'Kane DJ, Baudhuin LM, Wiley CL, Fortini A, Fisher PK, et al. Warfarin sensitivity genotyping: a review of the literature and summary of patient experience. Mayo Clin Proc. 2009; 84(12):1079-94.

73 Kuanprasert S, Dettrairat S, Palacajornsuk P, Kunachiwa W, Phrommintikul A. Prevalence of CYP2C9 and VKORC1 mutation in patients with valvular heart disease in Northern Thailand. J Med Assoc Thai. 2009;92(12):1597-601.

74 Freeman BD, Zehnbauer BA, McGrath S, Borecki I, Buchman TG. Cytochrome P450 polymorphisms are associated with reduced warfarin dose. Surgery. 2000; 128(2):281-5.

75 Taube J, Halsall D, Baglin T. Influence of cytochrome P-450 CYP2C9 polymorphisms on warfarin sensitivity and risk of over-anticoagulation in patients on long-term treatment. Blood. 2000; 96(5):1816-9.

76 Aithal GP, Day CP, Kesteven PJ, Daly AK. Association of polymorphisms in the cytochrome P450 CYP2C9 with warfarin dose requirement and risk of bleeding complications. Lancet. 1999; 353(9154):717-9.

77 Higashi MK, Veenstra DL, Kondo LM, Wittkowsky AK, Srinouanprachanh SL, Farin FM, et al. Association between CYP2C9 genetic variants and

anticoagulation-related outcomes during warfarin therapy. JAMA. 2002; 287(13):1690-8.

78 Peyvandi F, Spreafico M, Siboni SM, Moia M, Mannucci PM. CYP2C9 genotypes and dose requirements during the induction phase of oral anticoagulant therapy. Clin Pharmacol Ther. 2004; 75(3):198-203.

79 Sanderson S, Emery J, Higgins J. CYP2C9 gene variants, drug dose, and bleeding risk in warfarin-treated patients: a HuGEnet systematic review and meta- analysis. Genet Med. 2005; 7(2):97-104.

80 Steward DJ, Haining RL, Henne KR, Davis G, Rushmore TH, Trager WF, et al. Genetic association between sensitivity to warfarin and expression of CYP2C9*3. Pharmacogenetics. 1997; 7(5):361-7.

81 Bloch A, Ben-Chetrit E, Muszkat M, Caraco Y. Major bleeding caused by warfarin in a genetically susceptible patient. Pharmacotherapy. 2002; 22(1):97-101.

82 Lindh JD, Holm L, Andersson ML, Rane A. Influence of CYP2C9 genotype on warfarin dose requirements--a systematic review and meta-analysis. Eur J Clin Pharmacol. 200; 65(4):365-75.

83 Rieder MJ, Reiner AP, Gage BF, Nickerson DA, Eby CS, McLeod HL, et al. Effect of VKORC1 haplotypes on transcriptional regulation and warfarin dose. N Engl J Med. 2005; 352(22):2285-93.

84 Flockhart DA, O'Kane D, Williams MS, Watson MS, Flockhart DA, Gage B, et al. Pharmacogenetic testing of CYP2C9 and VKORC1 alleles for warfarin. Genet Med. 2008; 10(2):139-50.

85 Hillman MA, Wilke RA, Yale SH, Vidaillet HJ, Caldwell MD, Glurich I, et al. A prospective, randomized pilot trial of model-based warfarin dose initiation using CYP2C9 genotype and clinical data. Clin Med Res. 2005; 3(3):137-45.

86 Voora D, Eby C, Linder MW, Milligan PE, Bukaveckas BL, McLeod HL, et al. Prospective dosing of warfarin based on cytochrome P-450 2C9 genotype. Thromb Haemost. 2005; 93(4):700-5.

87 Herman D, Peternel P, Stegnar M, Breskvar K, Dolzan V. The influence of sequence variations in factor VII, gamma-glutamyl carboxylase and

vitamin K epoxide reductase complex genes on warfarin dose requirement. Thrombo Haemost. 2006; 95(5):782-7.

88 Vecsler M, Loebstein R, Almog S, Kurnik D, Goldman B, Halkin H, et al. Combined genetic profiles of components and regulators of the vitamin K-dependent gamma-carboxylation system affect individual sensitivity to warfarin. Thromb Haemost. 2006; 95(2):205-11.

89 Wen MS, Lee M, Chen JJ, Chuang HP, Lu LS, Chen CH, et al. Prospective study of warfarin dosage requirements based on CYP2C9 and VKORC1 genotypes. Clin Pharmacol Ther. 2008; 84(1):83-9.

90 Nathisuwan S, Dilokthornsakul P, Chaiyakunapruk N, Morarai T, Yodting T, Piriyachananusorn N. Assessing evidence of interaction between smoking and warfarin: a systematic review and meta-analysis. Chest. 2011; 139(5):1130-9.

91 Ucharattana P, Khaikeow S, Punsakd W, Hoontrakul S. Alcohol Consumption Behavior of Urban Thai: The Banbu Community, Bangkok-Noi District, Bangkok. J Nurs Sci 2011; 29(1):53-62.

92 Sun X, Hu S, Qi G, Zhou Y. Low standard oral anticoagulation therapy for Chinese patients with St. Jude mechanical heart valves. Chin Med J (Engl). 2003; 116(8):1175-8.

93 Tientadakul P SW, Opartkiattikul N, Luenee P, Sakiyalak P. Retrospective evaluation for optimal oral anticoagulant intensity after mechanical heart valve replacement at Siriraj Hospital. Thai Heart J 1994; 7:97-103.

94 Matsuyama K, Matsumoto M, Sugita T, Nishizawa J, Yoshida K, Tokuda Y, et al. Anticoagulant therapy in Japanese patients with mechanical mitral valves. Circ J. 2002; 66(7):668-70.

95 Lee LH. Thrombotic and haemorrhagic complications in patients with mechanical heart valve prostheses attending the Singapore General Hospital Anticoagulation Clinic. Ann Acad Med Singapore. 2000; 29(1):71-4.

96 Tatro DS. Drug interaction facts. St. Louis, Missouri: Facts and Comparisons. 2009.

97 Namazi S, Azarpira N, Hendijani F, Khorshid MB, Vessal G, Mehdipour AR. The impact of genetic polymorphisms and patient characteristics on warfarin dose requirements: a cross-sectional study in Iran. Clin Ther. 2010; 32(6):1050-60.

98 Perini JA, Struchiner CJ, Silva-Assuncao E, Santana IS, Rangel F, Ojopi EB, et al. Pharmacogenetics of warfarin: development of a dosing algorithm for brazilian patients. Clin Pharmacol Ther. 2008; 84(6):722-8.

99 Rachanee H. New dosage regimen for starting warfarin in patients with mechanical prosthetic heart valve replacement at Rajwithi hospital.; 1997.

100 Du X, Zhang K, Hu Z, Lan H, Luo J, Jin Y. The effects of lower intensity anticoagulation therapy on coagulation system in patients with mechanical prosthetic valves. J Tongji Med Univ. 1999; 19(1):56-8.

101 Chenhsu RY, Chiang SC, Chou MH, Lin MF. Long-term treatment with warfarin in Chinese population. Ann Pharmacother. 2000; 34(12):1395-401.

102 Busakornruangrat S, Chuansumrit A, Angchaisuksiri P, Sasanakul W, Kadekasem P. Frequencies of polymorphism associated with cytochrome P450 2C9 in Thais. Thai J Hematol Transf Med. 2006; 16:213-20.

103 Tassaneeyakul W, Mahatthanatrakul W, Niwatananun K, Na-Bangchang K, Tawalee A, Krikreangsak N, et al. CYP2C19 genetic polymorphism in Thai, Burmese and Karen populations. Drug Metab Pharmacokinet. 2006; 21(4):286-90.

104 London SJ, Daly AK, Leathart JB, Navidi WC, Idle JR. Lung cancer risk in relation to the CYP2C9*1/CYP2C9*2 genetic polymorphism among African-Americans and Caucasians in Los Angeles County, California. Pharmacogenetics. 1996; 6(6):527-33.

105 Takahashi H, Wilkinson GR, Caraco Y, Muszkat M, Kim RB, Kashima T, et al. Population differences in S-warfarin metabolism between CYP2C9 genotype-matched Caucasian and Japanese patients. Clin Pharmacol Ther. 2003; 73(3):253-63.

106 Leung AY, Chow HC, Kwong YL, Lie AK, Fung AT, Chow WH, et al. Genetic polymorphism in exon 4 of cytochrome P450 CYP2C9 may be

associated with warfarin sensitivity in Chinese patients. Blood. 2001; 9 8(8):2584-7.

107 Yoon YR SJ, Kim MK, Lim YC, Lee HR, Park IY, et al. Frequency of cytochrome P450 2C9 mutant alleles in a Korean population. Br J Clin Pharmacol. 2001; 51:227-80.

108 Gag G, Phipps M, K u C. Genetic polymorphism of CYP2C9 subfamily of three different races in warfarin maintenance dose. Int J Hematol. 2004; 80:295-6.

109 Klamchuen S, Chuansumrit A, Angchaisuksiri P, Sasanakul W. Frequency of polymorphism associated with VKORC1 gene in Thai blood donors and patients receiving warfarin. J Hematol Transf Med. 2008; 18:307-13.

110 Mushiroda T, Ohnishi Y, Saito S, Takahashi A, Kikuchi Y, Saito S, et al. Association of VKORC1 and CYP2C9 polymorphisms with warfarin dose requirements in Japanese patients. J Hum Genet. 2006; 51(3):249-53.

111 Absher RK, Moore ME, Parker MH. Patient-specific factors predictive of warfarin dosage requirements. Ann Pharmacother. 2002; 36(10):1512-7.

112 Khan T, Kamali F, Daly A, King B, Wynne HA. Warfarin sensitivity: be aware of genetic influence. Age Ageing. 2003; 32(2):226-7.

113 White P. Patient factors that influence warfarin dose response. J Pharm Pract. 2010; 23(3):194-204.

114 Kamali F, Edwards C, Butler TJ, Wynne HA. The influence of (R)- and (S)-warfarin, vitamin K and vitamin K epoxide upon warfarin anticoagulation. Thromb Haemost. 2000; 84(1):39-42.

115 Whitley HP, Fermo JD, Chumney EC, Brzezinski WA. Effect of patient-specific factors on weekly warfarin dose. Ther Clin Risk Manag. 2007; 3(3):499-504.

116 Ha CE, Petersen CE, Park DS, Harohalli K, Bhagavan NV. Investigations of the effects of ethanol on warfarin binding to human serum albumin. J Biomed Sci. 2000; 7(2):114-21.

117 Chern HD, Ueng TH, Fu YP, Cheng CW. CYP2C9 polymorphism and warfarin sensitivity in Taiwan Chinese. Clin Chim Acta. 2006; 367(1-2):108-13.

118 Lee SC, Ng SS, Oldenburg J, Chong PY, Rost S, Guo JY, et al. Interethnic variability of warfarin maintenance requirement is explained by VKORC1 genotype in an Asian population. Clin Pharmacol Thers. 2006; 79(3):197-205.

119 Gage BF, Eby C, Johnson JA, Deych E, Rieder MJ, Ridker PM, et al. Use of pharmacogenetic and clinical factors to predict the therapeutic dose of warfarin. Clin Pharmacol Ther. 2008; 84(3):326-31.

APPENDICES

APPENDIX A

Data Collection Form

Patient ID……..……..Age…..yrs Gender ☐ M ☐ F Ht……cms
Wt……kg BMI……..Native……
District…… Nationality…….. Race……..
Ethnic of parents: Father…….. Mother……
Case No……..

Indication:

Mechanical valve replacements: ○ Mitral valve ○ Aortic valve ○ Tricuspid valve ○ pulmonic valve

Underlying disease

☐ 1 Cardiovascular disease

- ☐ 1.1 Stroke
- ☐ 1.2 Coronary Heart Disease
- ☐ 1.3 Aortic Dissection
- ☐ 1.4 Aortic Aneurism
- ☐ 1.5 Marfan's syndrome
- ☐ 1.6 Behcet's disease
- ☐ 1.7 Atrial fibrillation
- ☐ 1.8 Antiphospholipid syndrome
- ☐ 1.9 Valvular heart disease
- ☐ 2.0 Atrial septum defect
- ☐ 2.1 ventricular septum defect
- ☐ 2.2 Patent ductus arteriosus
- ☐ 2.3 Tetralogy of fallot
- ☐ 2.4 Coarctation of aorta

☐ 2 Pulmonary disease

☐ 3 GI disease

☐ 4 Renal disease

☐ 5 Diabetes mellitus

☐ 6 Hyperthyroidism

☐ 7 Hypothyroidism

☐ 8 CNS disease

☐ 9 Epilepsy

☐ 10 Deep venous thrombosis with pulmonary embolism

☐ 11 Deep venous thrombosis without pulmonary embolism

Smoking status (at the period of stable warfarin dose)

☐ No ☐ Yes……

Alcohol intake (at the period of stable warfarin dose)

☐ No ☐ Yes……

Herbal medicine (at the period of stable warfarin dose)

☐ No ☐ Yes……

Target of INR

☐ 1) 2.0-3.0

☐ 2) Others……

Dosage of warfarin

DATE	WARFARIN DOSE	MG/ WEEK	IN R	CONCOMITTANT MEDICATIONS

Laboratory

DATE	LABORATORY					
	AST	ALT	Albumin	Scr	ClCr	Other

Time within therapeutic range =……day

Mean dose =…….mg/day

Mean INR =…….

Drug interaction: ☐ **No** ☐ **Yes**

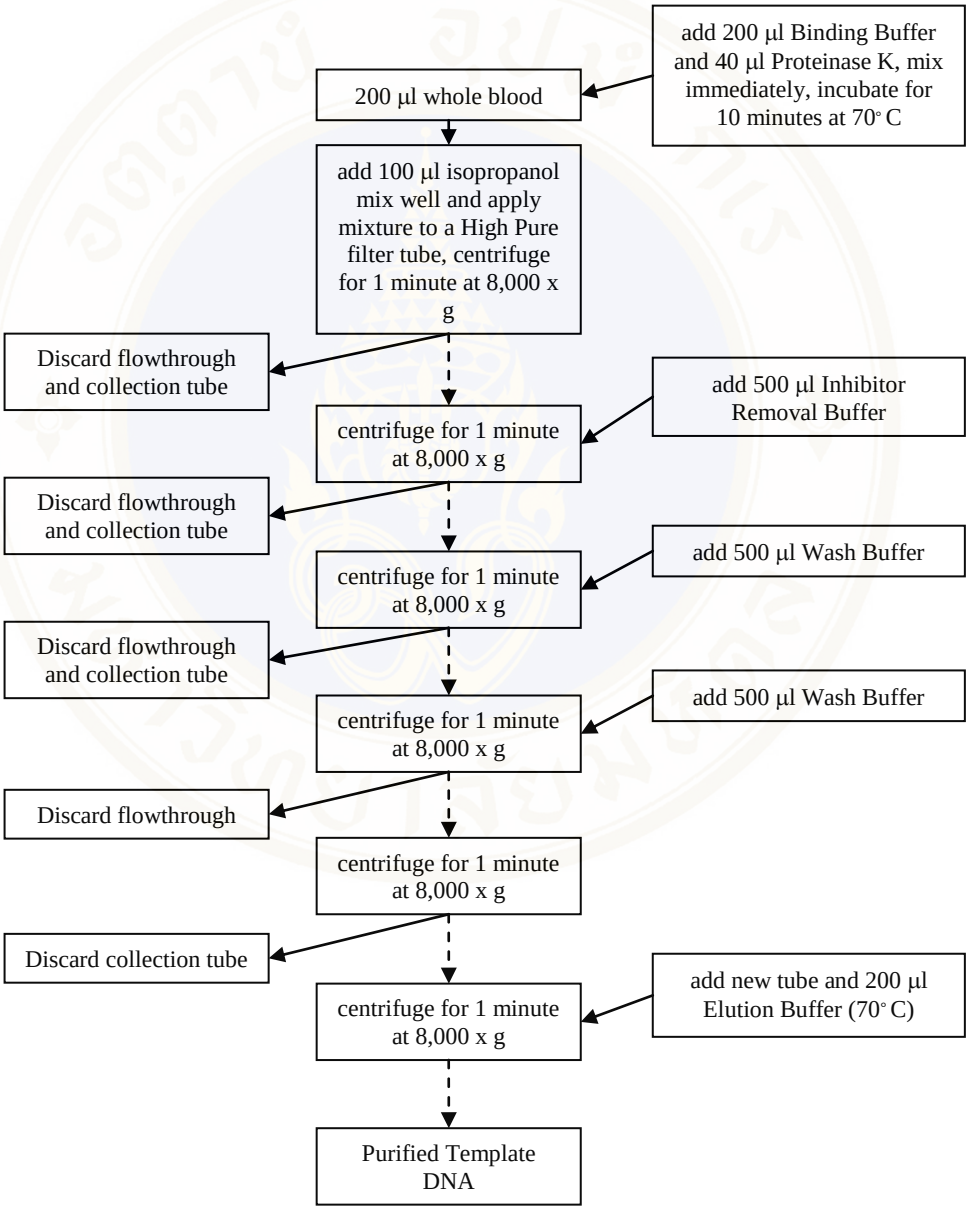
Precipitating drug	Significance level

Genotyping

*CYP2C9*2*	☐ WT	*CYP2C9*3*	☐ WT
	☐ HT		☐ HT
	☐ MT		☐ MT
CYP2C9 genotype	☐1) *1/*1		
	☐2) *1/*3		
	☐3) *3/*3		
	☐4) Others……..		
VKORC1 -1639G>A	☐ GG	*VKORC1 1173 C>T*	☐ CC
	☐ GA		☐ CT
	☐ AA		☐ TT
*VKORC1*haplotype	☐ 1) AA		
	☐ 2) AB		
	☐ 3) BB		

APPENDIX B

DNA extraction process



APPENDIX C

Preparation of parameter-specific reagents for *CYP2C9*2* and *CYP2C9*3*

One reagent vial with a red clip contains all primers and probes to run 16 LightCycler reactions for *CYP2C9*2* and *CYP2C9*3*.

Add 66 µl PCR-grade water to each reagent vial, mix the solution (vortex) and spin down. Use 4 µl reagent for a 20 µl PCR reaction.

This solution is stable for three days or longer if stored refrigerated at 4°C. Avoid prolonged exposure to light.

Preparation of the LightCycler® reaction mix

In a reaction tube cooled below 4°C, prepare the re action mix by multiplying each volume for a single reaction by the number of reactions to be cycled plus one additional reaction.

For use with the Roche FastStart® kit	
Single reaction	**Component**
7.8 µl	water, PCR-grade
1.2 µl	Mg^{2+} solution 25 mM
4.0 µl	reagent mix (parameter specific reagents containing primers and probes)
2.0 µl	Roche master
15.0 µl	Volume of reaction mix

Mix gently, spin down and transfer 15 µl each of the reaction mix to a LightCycler® capillary. Add 5 µl of sample or control DNA to each capillary or multiwell plate to give a final reaction volume of 20 µl.

APPENDIX D

Preparation of parameter-specific reagents (32 reactions) for *VKORC1 C1173T* and *VKORC1 G-1639A*.

One reagent vial with a red cap contains primers and probes to run 32 LightCycler® reactions for *VKORC1 C1173T* and *VKORC1 G-1639A.*

Add 66 µl PCR-grade water to each reagent vial, mix the solution (vortex) and spin down. Use 2 µl reagent for a 20 µl PCR reaction.

Preparation of the LightCycler® reaction mix

In a cooled reaction tube, prepare the reaction mix by multiplying each volume for a single reaction by the number of reactions to be cycled plus one additional reaction.

For use with the Roche FastStart® kit	
Single reaction	**Component**
10.2 µl	water, PCR-grade
0.8 µl	Mg^{2+} solution 25 mM
2.0 µl	reagent mix (parameter specific reagents containing primers and probes)
2.0 µl	Roche master
15.0 µl	Volume of reaction mix

Mix gently, spin down and transfer 15 µl each of the reaction mix to a LightCycler® capillary. Add 5 µl of sample or control DNA to each capillary or multiwell plate to give a final reaction volume of 20 µl.

APPENDIX E

The protocol consists of four program steps for *CYP2C9* and *VKORC1* genotype

LightCycler® 480 instrument

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification of the target DNA
- 3: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 4: Cooling: cooling the instrument

CYP2C9 genotype

Program:		Denaturation	Cycling			Melting			Cooling
	Parameter								
	Analysis Mode	None	Quantification			Melting Curves			None
	Cycles	1	45			1			1
	Segment	1	1	2	3	1	2	3	1
	Target [℃]	95	95	60	72	95	40	85	40
	Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:10	00:00:15	00:00:20	00:00:20	00:00:00	00:00:30
LC1.x/2.0	Ramp Rate [℃/s]	20	20	20	20	20	20	0.2	20
LC 480:	*Ramp Rate [℃/s]* ***96***	*4.4*	*4.4*	*2.2*	*4.4*	*4.4*	*1.5*	-	*1.5*
LC 480:	*Ramp Rate [℃/s]* ***384***	*4.6*	*4.6*	*2.4*	*4.6*	*4.6*	*2.0*	-	*2.0*
	Acquisition Mode	None	None	Single	None	None	None	Continu.	None
LC 480:	Acquisitions [per ℃]							1	

VKORC1 genotype

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	45			1			1
Target [℃]	95	95	60	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:10	00:00:15	00:00:30	00:02:00	00:00:00	00:00:30
Ramp Rate [℃/s] **96**	4.4	4.4	2.2	4.4	4.4	1.5	-	1.5
Ramp Rate [℃/s] **384**	4.6	4.6	2.4	4.6	4.6	2.0	-	2.0
Acquisition Mode	None	None	Single	None	None	None	Continuous	None
Acquisitions [per ℃]	-	-	-	-	-	-	**3**	-

BIOGRAPHY

NAME	Miss Ajjima Sarapakdi
DATE OF BIRTH	January 16, 1981
PLACE OF BIRTH	Chumporn, Thailand
INSTITUTIONS ATTENDED	Mahidol University, 1999-2004: Bachelor of Science in Pharmacy Mahidol University, 2007-2011: Master of Science in Pharmacy (Clinical Pharmacy)
HOME ADDRESS	105/368 Ratchapreuk Rd., Bangkrang, Muang, Nonthaburi, Thailand 11000 Tel. 0802672245 E-mail : ajjima_sa@hotmail.com
EMPLOYMENT ADDRESS	2004-present : Department of Pharmacy, 2 Siriraj Hospital, Bangkok, Thailand Position: Pharmacist Tel. 024197661 E-mail : warfarin_6@yahoo.com
PUBLICATION / PRESENTATION	A. Sarapakdi, S. Nathisuwan, P.Thongcharoen, C. Komoltri and A.Lucksiri. Development of warfarin dosing formula based on pharmacogenomics and clinical factors in patient with mechanical heart valves. In:

Proceedings of the 7 th Indochina Conference on Pharmaceutical Sciences Advancing Pharmacy for ASEAN Community. Faculty of Pharmacy, Mahidol University; 2011 Dec 14-16, Bangkok, Thailand: 2011.