DEVELOPMENT OF A LOCAL WARFARIN DOSAGE GUIDELINE BASED ON PHARMACOGENOMICS AND HAEMOSTATIC MARKERS

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DEVELOPMENT OF A LOCAL WARFARIN DOSAGE GUIDELINE BASED ON PHARMACOGENOMICS AND HAEMOSTATIC MARKERS

by

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DEDICATION

For all good people who died due to illness, may their names be written in the book of remembrance of God.

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ACN	Acetonitrile
AGP	α_1-acid glycoprotein
AF	Atrial fibrillation
APOE	Apolipoprotein E gene
aPTT	Activated partial thromboplastin time
AUC	Area under the curve
BMI	Body mass index
BP	Base pair
CALU	Calumenin gene
CHF	Congestive heart failure
CL	Clearance
C_{max}	Maximum concentration
CV	Coefficient of variation
COPD	Chronic obstructive pulmonary disease
CYP	Cytochrome P450 gene
dH_2O	Distilled water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DVT	Deep vein thrombosis
FDA	Food and Drug Administration
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
EPHX1	Microsomal epoxide hydrolase gene

GGCX	γ-carboxyglutamic acid gene
GSTA1	Glutathione S-transferase gene
HDL	High-density lipoprotein
HPLC	High performance liquid chromatography
hr	Hour(s)
IHD	Ischaemic heart disease
INR	International normalised ratio
IQR	Interquartile range
ISI	International sensitivity index
K_a	Absorption rate constant
k_{el}	Elimination rate constant
LDL	Low-density lipoprotein
LOD	Limit of detection
LLE	Liquid-liquid extraction
LLOQ	Lowest limit of quantification
MeOH	Methanol
MI	Myocardial infarction
min	Minute(s)
NaOH	Sodium hydroxide
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
ORM1	Orosomucoid 1 gene
PCR	Polymerase chain reaction

PE	Pulmonary embolism
pH	$-\log_{10}$ of hydrogen ion concentration
pK_a	$-\log_{10}$ of acid dissociation constant
PROC	Protein C gene
PT	Prothrombin time
QC	Quality control
r^2	Correlation coefficient
RF	Response factor
RFLP	Restriction fragment length polymorphism
ROS	Reactive oxygen species
RSD	Relative standard deviation
sec	Second(s)
SD	Standard deviation
Taq	*Thermus aquaticus*
TF	Tissue factor
$t_{1/2}$	Half-life
t_{max}	Time to maximum concentration
UV	Ultraviolet
VKCFD	Vitamin K-dependant clotting factor deficiency
VKD	Vitamin K-dependent
VKOR	Vitamin K epoxide reductase
VKORC1	Vitamin K epoxide reductase complex subunit 1gene
vs	Versus
VTE	Venous thromboembolism
vWF	von Willebrand factor

PEMBANGUNAN GARIS PANDUAN DOS WARFARIN TEMPATAN BERASASKAN FARMAKOGENOMIK DAN PENANDA-PENANDA HEMOSTASIS

ABSTRAK

Warfarin merupakan antikoagulan oral utama yang mempunyai indeks terapeutik yang sempit dan perbezaan dos antara individu yang luas, menyebabkan kerumitan pengekalan dos optimumnya untuk setiap individu. Banyak model pengedosan warfarin telah dibangunkan di seluruh dunia bertujuan untuk menambahbaik ketepatan kaedah pengedosan nisbah ternormal antarabangsa (INR) yang digunakan sekarang. Walau bagaimanapun, model-model pengedosan berkenaan tidak praktikal untuk digunakan kerana memerlukan banyak data tambahan untuk pengiraan dos. Di dalam kajian ini, sebuah model pengedosan warfarin untuk populasi tempatan yang lebih mudah telah dikaji untuk permulaan semula rawatan warfarin berasaskan data klinikal, makmal dan genetik.

Sejumlah 130 pesakit telah direkrut daripada Hospital Universiti Sains Malaysia untuk pembinaan model tersebut. Data klinikal pesakit diambil daripada pengkalan data hospital. Kaedah tindak balas berantai polimerase - polimorfisme pemotongan panjang cebisan (PCR-RFLP) telah digunakan untuk penjenisan *CYP2C9*2, *3* dan *VKORC1* -1639G>A, manakala sebuah kaedah PCR multipleks spesifik alel tersarang yang baharu dibangunkan telah digunakan untuk penjenisan *VKORC1* 381, 861, 5808 dan 9041. Data-data genotip *VKORC1* 381, 861, 5808 dan 9041 telah digunakan untuk mengiferensikan haplotip *VKORC1*. Aktiviti faktor koagulasi II, VII, IX dan X bergantung vitamin K (VKD) telah diukur dengan menggunakan mesin penganalisis hemostatik. Sebuah kaedah baharu kromatografi cecair berprestasi tinggi (HPLC) dengan pengesan UV dibangunkan dan disahkan untuk digunakan bagi mengukur aras warfarin serum yang kemudiannya digunakan untuk pengiraan data farmakokinetik di dalam 24 orang pesakit. Model pengedosan warfarin telah dibangunkan dengan menggunakan kaedah regresi linear berbilang ke depan.

Genotip *CYP2C9*2* dan **3* mutan heterozigot adalah jarang (kedua-duanya dengan kekerapan 3.8%), manakala genotip mutan homozigot langsung tidak dikesan. Kekerapan *VKORC1* -1639G>A dan *VKORC1* 381 adalah sama. Genotip dengan kekerapan paling tinggi adalah genotip yang berkeperluan dos warfarin rendah (GG: 54.6%). *VKORC1* H1H1, H1H7 atau H1H9 dan H7H7 adalah pasangan-pasangan haplotip yang paling lazim (53.1, 32.5 dan 10.0%). Kesemua aktiviti faktor koagulasi VKD tidak berhubungkait secara ketara dengan keperluan dos warfarin INR. Kepekatan maksimum serum, separuh hayat dan pembersihan warfarin tidak berhubungkait secara ketara dengan mana-mana data genetik atau keperluan dos warfarin. Model pengedosan warfarin muktamad adalah terdiri daripada umur, jumlah alel *VKORC1* 381, min INR dan sejarah penggantian injap mitral sebagai faktor-faktor peramal. Faktor-faktor peramal tersebut dapat menerangkan 45.6% kebolehubahan dos warfarin.

Model pengedosan yang dibangunkan adalah sesuai untuk digunakan sebagai garis panduan untuk menentukan dos warfarin pesakit yang memerlukan permulaan semula rawatan warfarin.

DEVELOPMENT OF A LOCAL WARFARIN DOSAGE GUIDELINE BASED ON PHARMACOGENOMICS AND HAEMOSTATIC MARKERS

ABSTRACT

Warfarin, the mainstream oral anticoagulant, has a narrow therapeutic index and wide interindividual dose variability, rendering maintaining its optimal dose in each individual a difficult task. Many warfarin dosing models have been developed worldwide in order to improve the accuracy of currently used international normalised ratio (INR) dosing method. However, those dosing models were not practical to be used due to extensive additional data that are required for dose calculation. In this study, a simpler warfarin's dosing model for local population has been studied for warfarin therapy reinitiation based on clinical, laboratory and genetic data.

A total of 130 of patients on warfarin treatment in Hospital Universiti Sains Malaysia were recruited for the model-building. Patients' clinical data were extracted from the hospital database. Polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) methods were used for genotyping of *CYP2C9*2*, **3* and *VKORC1* -1639G>A while a newly developed nested allele-specific multiplex PCR was used for genotyping of *VKORC1* 381, 861, 5808 and 9041. Genotype data of *VKORC1* 381, 861, 5808 and 9041 were used to infer *VKORC1* haplotype. The activity of vitamin K-dependent (VKD) clotting factors II, VII, IX and X were measured by using a benchtop haemostatic analyser. A newly developed and validated high performance liquid chromatography (HPLC) method with UV detector was used for measurement of serum warfarin levels that were subsequently used for pharmacokinetics data calculation in 24 patients. The warfarin's dosing model was developed by using a forward multiple linear regression.

The heterozygous mutant genotype of *CYP2C9*2* and **3* were rare (both at 3.8%), while the homozygous mutant was not detected. The frequency of *VKORC1* -1639G>A and

VKORC1 381 were similar. The genotype with highest frequency was the low warfarin's dose requirement genotype (GG: 54.6%). The *VKORC1* H1H1, H1H7 or H1H9 and H7H7 were the most common haplotype pairs (53.1, 32.5 and 10.0%). All VKD clotting factor activities were not significantly associated with warfarin's dose requirement or with the INR. Maximum serum concentration, half-life and clearance of warfarin were not significantly associated with any genetic data or warfarin's dose requirement. The final warfarin's dosing model consists of age, the number of *VKORC1* 381 allele, mean INR and history of mitral valve replacement as useful predictor factors. The predictor factors explained 45.6% of warfarin dose variability.

The developed dosing model is suitable to be used as guideline to determine the warfarin dose of patients who need to reinitiate a warfarin therapy.

CHAPTER 1

INTRODUCTION

1.1 Anticoagulation treatment

Warfarin is an oral anticoagulant prescribed to patients to prevent pathological blood clot in circulation, also known as thrombosis. Thrombosis can impose life-threatening health risks such as stroke and pulmonary embolism (PE). However, warfarin has a narrow therapeutic window in which insufficient dose will fail to protect the patient from the disease, while excessive dose can expose patients to uncontrolled bleeding. Therefore, patients are expected to adhere to the prescription and comply with a lower vitamin K diet to prevent food-warfarin interaction. Besides food, concomitant medications, supplements and the presence of other risk factors such as liver impairment and fever can also affect warfarin's sensitivity (Demirkan *et al.*, 2000). Once warfarin is prescribed, the patients are required to regularly visit healthcare providers in order to re-adjust warfarin's dose according to the lifestyle changes.

Although several novel oral anticoagulants require less frequent dose adjustment and show minimum food-drug interactions such as dabigatran, rivaroxaban and apixaban are already commercially available and are superior (or at least not inferior) in their therapeutic outcomes (Ahrens *et al.*, 2011). Warfarin still remains as the mainstay oral anticoagulant due to its cheaper cost and established use especially in the event of overdose.

Nevertheless, dosing of warfarin is not simple because although warfarin is given according to the dosing guideline and patients comply to the therapy, overdosing still occur rather frequently. Numerous studies have indicated that genetic polymorphisms affect the pharmacokinetics and pharmacodynamics of warfarin (Yin and Miyata, 2007; Kamali and Wynne, 2010) thereby rendering patients to be either resistant or sensitive to warfarin treatment. Since warfarin is likely to be incorporated in the clinical settings for years to come,

a better understanding on how genetic polymorphism are associated with warfarin's sensitivity is needed. It is hoped that the integration of genetic data and other coagulation markers into pre-existing warfarin dosing guidelines may improve dosing accuracy thereby reducing adverse effects.

1.2 Thrombosis and thromboembolism

1.2.1 Definition

Blood is a dynamic body fluid which carry various gasses, nutrients, hormones and chemical compounds throughout the body. Escape of blood from the blood vessel need to be immediately stopped to avoid uncontrolled haemorrhage. It has been reported that human can sustain up to 20% of blood loss before experiencing a hypovolaemic shock and getting the risk of reperfusion injury (Mannucci and Levi, 2007; Shults *et al.*, 2008). When the blood vessel is exposed to injury, haemostasis is triggered in which platelet can initiate plasma plug formation to temporarily seal the ruptured blood vessel. Meanwhile, a coagulation cascade will start in which the clotting factors will undergo a series of activations to form blood clot at the injury site to prevent the outflow of blood from the blood vessel. The clot formation is regulated by naturally occurring anticoagulants (antithrombin III, protein C and protein S) to contain the clot to only within the vicinity of the injured blood vessel (High, 1988).

However, in the presence of a pathological condition, thrombus can block the blood flow to vital organs which can lead to hypoxia and eventually becomes necrotic. The formation of thrombosis in the artery vary from that in the vein (Previtali *et al.*, 2011). Arterial thrombosis is usually an outcome of atherosclerosis. The arterial thrombus, also known as white thrombus, mainly consists of white blood cells and platelets with cardiac muscle being one of the most commonly affected organ. Decreased oxygen supply to cardiac muscles can cause ischaemic heart disease (IHD) and eventually leads to myocardial infarction (MI), while thrombosis in brain artery can cause stroke. On the other hand, venous

thrombosis leads to venous thromboembolism (VTE), where the thrombus itself may dislodge from its original site and travel throughout the circulation as an embolus. The most common VTE is deep vein thrombosis (DVT), where the thrombus is usually formed in the leg veins. The venous thrombus, also known as red thrombus, mainly consist of red blood cells and fibrin. The dislodged embolus from the DVT may stop at the lung blood vessel and lead to PE.

1.2.2 Pathogenesis

Arterial and venous thrombosis have different pathogenesis and risk factors (Table 1.1). In the artery, the thrombus is mainly formed following atherosclerosis. Atherosclerosis is thickening of the artery wall due to the accumulation of monocytes, macrophages, dead cells, cholesterol, calcium and fibrous tissues within the arterial endothelium. One of the major risk factors of atherosclerosis is the presence of metabolic syndrome (Glasser *et al.*, 1996). Individuals with metabolic syndrome are characterised by high level of low-density lipoprotein (LDL), low level of high-density lipoprotein (HDL), hypertension, hyperglycaemia and abdominal obesity (Paoletti *et al.*, 2006). Collectively, these disorders exacerbate the intrusion of macrophages beneath the blood vessel, induce endothelial dysfunction by reducing the level of indigenous vasodilator nitric oxide (NO) (Mehta *et al.*, 2001), produce pro-inflammatory cytokines (Zhu *et al.*, 2005; Fernández-Sánchez *et al.*, 2011) and cell adhesion mediators (Zeiher *et al.*, 1995), increase oxidative stress on the artery (Cosentino *et al.*, 1997; Guzik *et al.*, 2002) ultimately leading to atherosclerosis.

Table 1.1: Risk factors of arterial and venous thrombosis

Arterial	**Venous**	
	Virchow's triad	Contemporary categories
Metabolic syndrome • High LDL level • Low HDL level • Hyperglycaemia • Hypertension • Abdominal obesity **Smoking** **Male** **Cardiac disorders** • AF • Valvular diseases o Ageing o Rheumatic disease o Endocarditis o Congenital heart defect • Prosthetic heart valve	**Blood flow stasis** • Immobilisation • Left atrial appendage coagulation • Left ventricular dilatation • Mitral valve stenosis **Epithelial injury** • Trauma • Endothelial inflammation • Infection **Abnormal blood constituent** • Abnormal platelet level • Abnormal globulin level • Abnormal fibrinogen level • Abnormal calcium level • Dehydration • Pregnancy • Infection • Obesity	**Acquired** • Ageing o Hospitalisation o Dysfunctioned endothelium o Venous insufficiency o CHF o COPD o Increase levels of clotting factors o Increase levels of fibrinolysis inhibitors • Previous thrombosis • Immobilisation • Major surgery • Oral contraceptive • Hormone replacement therapy • Antiphospholipid syndrome • Myeloproliferatide disorders • Cancer • Pregnancy **Inherited** • Decrease expression of antithrombin III • Decrease expression of protein C and S • Factor V Leiden *G1691A* mutation • Prothrombin *G202010* mutation **Mixed** • Ageing with clotting factor genes mutation • Hyperhomocysteinaemia

LDL = low-density lipoprotein; HDL = high-density lipoprotein; CHF = congestive heart failure; COPD = chronic obstructive pulmonary disease

Smoking is a non-metabolic syndrome risk factors of atherosclerosis. Nicotine in tobacco smoke can induce arterial stiffness besides elevating plasma LDL level while lowering plasma HDL levels (Powell, 1998; Villablanca *et al.*, 2000). Male individuals also have increased predisposition to atherosclerosis when compared with females. *In-vitro* study showed that androgen hormone up-regulate atherosclerosis-related genes in macrophages derived from male donors but not in macrophages derived from female donors (Ng *et al.*, 2003).

Non-atherosclerosis-related arterial thromboses caused by cardiac disorders such as atrial fibrillation (AF) leads to irregular heart rhythm that promotes stagnant blood flow at atrial appendage (GalalAzzam, 2013). Valvular heart diseases especially mitral valve stenosis, can cause blood stasis in left atrium (Demir *et al.*, 2001; Misumi *et al.*, 2003; Rost *et al.*, 2008). The stagnant blood eventually leads to thrombosis. Therefore, heart valve replacement as a consequence of valvular disease, especially the use mechanical prosthetic valve, may also increased the risk of thrombosis (Roudaut *et al.*, 2007; Pibarot and Dumesnil, 2009).

Venous thrombosis has different pathogenesis where the thrombus are not formed on an atheromatous plaque. Classically, the pathogenesis of venous thrombosis is related to the risk factors as described in Virchow's Triad, a postulation that relates thrombogenesis with three factors, which are blood flow stasis, epithelial injury and abnormal blood constituent (Lip and Gibbs, 1999). Nevertheless, this postulation is obsolete with a new categorical system used to classify the risk factors of venous thrombosis. Currently, venous thrombosis risk factors are divided into three groups: acquired, inherited and mixed risk factors (Rosendaal, 1997). The strongest acquired risk factor for thrombosis is advanced age, caused by numbers of elderly-related co-morbidities and increase in level of various plasma proteins and clotting factors (Cushman, 2007; Ageno *et al.*, 2008).

The remaining acquired risk factors include exposure to previous thrombosis, immobilisation, major surgeries, oral contraceptive-induced activated protein C resistant, hormone replacement therapy, antiphospholipid syndrome and myeloproliferative disorders (Rosing *et al.*, 2001; Ortel, 2005; Tefferi and Elliott, 2007; Pottier *et al.*, 2009; Healy *et al.*, 2010). Cancer patients also have a high risk of thrombosis due to the production of tissue factor (TF) by affected organs and cancer cells (Prandoni *et al.*, 2005; Hron *et al.*, 2007). Pregnancy is also closely related to increased level of thrombosis clotting factors VII, X, and VIII, fibrinogen and vWF levels with decreased the natural anticoagulant protein levels. (Brenner, 2004).

Thrombosis can also be an inherited genetic disorder. Mutations may not only decrease the expression of certain naturally occurring anticoagulants such as antithrombin III, protein C and protein S (Pabinger *et al.*, 1992), but also cause gain-of-function of certain clotting factors. Factor V Leiden G1691A and prothrombin G202010 are the most common thrombophilic mutations, which can cause gain-of-function by making factor V able to resist Protein C inactivation and upregulating the production of prothrombin, respectively (Bertina *et al.*, 1994; Castoldi *et al.*, 2007). It has been reported that family members from a person with a history of thrombophilia are more susceptible to thrombosis (Ben-Tal *et al.*, 1989; Allaart *et al.*, 1993) and may experience thrombosis in earlier age (Lensen *et al.*, 1996) when compared with individuals who do not have such family history.

Non-genetic risk factors may also play a role in thrombosis along with genetic factors. Although there are evidences that increased levels of clotting factors VIII, IX, XI and fibrinogen are familial disorders (Lane and Grant, 2000; Jenkins *et al.*, 2012), other factors like ageing and inflammation also contribute to thrombogenesis (Previtali *et al.*, 2011). Hyperhomocysteinaemia, a disorder that cause endothelial dysfunction, is contributed by both genetic mutation in methylenetetrahydrofolate reductase gene and deficiency in vitamins B_{12}, B_6 and folate levels (Selhub and D'Angelo, 1998).

Despite the discrete categories, venous thrombosis is often multicausal. A study found that 84% of children with DVT have more than a single risk factor for thrombosis (Andrew *et al.*, 1994). Except for some major surgeries and traumas, most single risk factors of thrombosis are unlikely to cause thrombosis hence requiring no prophylaxis (Anderson and Spencer, 2003). However, the interaction between the risk factors for thrombosis is synergistic. For example, individuals with factor V Leiden suffering from leg injury are 50-fold more likely to develop thrombosis when compared with individuals without both risk factors, while individuals with only leg injury and factor V Leiden had only 5 and 6.8-fold higher likelihood, respectively (van Stralen *et al.*, 2008).

Individuals with congestive heart failure (CHF) are known to be more susceptible to thrombosis due to low cardiac output, endothelial dysfunction and increased platelet aggregation. However, the thromboembolic outcome of CHF was formerly overlooked due to the understanding that the primary mortality in CHF is contributed by a sudden cardiac death rather than thrombosis *per se* (Sosin *et al.*, 2003). The role of CHF as an important risk factor for thrombosis was reiterated only after some studies confirmed that CHF patients who receive thromboprophylaxis have better outcomes (less mortality, MI and stroke events) than patients who do not receive the treatments (Cleland *et al.*, 2004; Cokkinos *et al.*, 2006; Massie *et al.*, 2009).

1.2.3 Prevalence

Although heart valve replacement is the main indication for warfarin in other countries (White *et al.*, 1996), warfarin is mainly prescribed for AF in Malaysia. The worldwide prevalence of AF is reported to be 1.5 - 2.0% (Camm *et al.*, 2012). According to a study conducted in an urban hospital in Malaysia, 2.8% from overall acute medication prescription was for patients with AF, while 20% of AF patients were on warfarin treatment (Freestone *et al.*, 2003). However, oral anticoagulant use among AF patients in Malaysia is lower than that in the United States (65.0%) (Kirley *et al.*, 2012) and Europe nations (64.8%) (Pisters *et al.*, 2010). Nevertheless, warfarin remains the top-ranking antithrombotic drug in Malaysia just after aspirin, ticlopidine and clopidogrel (MOH, 2013b). Furthermore, although adverse drug reactions from warfarin use in Malaysia is not extensively reported, it cannot be denied that warfarin is the top-10 leading drugs which cause adverse effects in United States (Wysowski *et al.*, 2007) with the incidence of serious bleeding among patients on warfarin treatment to be as high as 16% while 2.9% was reported to be fatal (Da Silva and Sobel, 2002).

1.3 Warfarin

1.3.1 History

In the early 1920s, a group of farmers in North America reported a strange fatal haemorrhagic disease seen among their cattle following consumption of the molded sweet clover (*Melilotus officinalis*) hay (Schofield, 1924). After an investigation, a veterinarian named Roderick found that the sweet clover contains an unknown anticoagulant which caused 'plasma prothrombin defect' (Roderick, 1929). In 1933, Karl P. Link began an investigation to identify the type of the agent present in the sweet clover. Six years later, his co-workers successfully isolated the agent and began to synthesise it. The anticoagulant was named as dicoumarol (Stahmann *et al.*, 1940; Link, 1959) which inhibits prothrombin, a clotting factor in human that plays a main role in the coagulation cascade (Overman *et al.*, 1941).

In 1948, Link introduced a potent analogue of dicoumarol, named warfarin, as a commercial rodenticide (Last, 2002). Due to its better potency than dicoumarol, warfarin was later proposed as a prophylaxis drug for thromboembolism instead of dicoumarol. In 1951, a US Army inductee used the rodenticide warfarin in an attempt to commit suicide. However, the toxicity effect was not fast enough to kill him instantly and he was successfully rescued from fatal haemorrhage after being given vitamin K as an antidote. This fateful event convinced clinicians that warfarin is indeed a potent anticoagulant with a reliable safety index (Link, 1959). Eventually, warfarin was marketed as Coumadin Sodium in 1954 to replace dicoumarol as an oral anticoagulant.

1.3.2 Indication and dosing

Warfarin is used as a prophylaxis for both arterial and venous thromboembolic disorders. Patients who have arterial embolism, AF, coagulopathy, DVT, heart valve replacement, MI with risk of stroke, PE, pulmonary hypertension and transient ischaemic attack can be given warfarin treatment (MOH, 2010; FDA, 2014).

Warfarin is contraindicated in patients a) who are pregnant or may becomes pregnant b) who recently or are planned for surgery c) who experience threatened abortion d) who potentially not compliant [due to senility, alcoholism or psychosis] and d) who are recently or planned for spinal puncture (FDA, 2014).

The decision to prescribe warfarin or otherwise is decided by clinician by assessing the following bleeding risks (MOH, 2010): age≥ 65 years, history of stroke, history of gastrointestinal bleeding, recent MI, haematocrit level (<30%), creatinine level (>133 μmol/L) and diabetes.

Usually, warfarin is administered to a patient based on an individualised dose. The initial dose is usually 5 mg o.d., or 3 mg o.d. in elderly patients, while the subsequent dosage from day-3 onwards are adjusted based on International Normalised Ratio (INR), an index obtained from laboratory test by using patient's plasma sample.

$$\mathrm{INR} = \left(\frac{\mathrm{PT}}{\mathrm{MNPT}}\right)^{\mathrm{ISI}}$$

(Le *et al.*, 1994)

PT = Prothrombin time measured from patient's plasma

MNPT = Mean normal prothrombin time, measured from a pooled plasma of healthy individuals

ISI = International sensitivity index, a coefficient used to measure the sensitivity of thromboplastin provided by the reagent's manufacturer.

Patients on warfarin treatment are expected to achieve a targeted INR (Table 1.2) (MOH, 2013a). An INR below the recommended range indicates that the patient receives insufficient anticoagulation and therefore warfarin dose should be increased. An INR above the recommended range indicates that the patient has received excessive anticoagulation and therefore warfarin dose should be decreased. Once the INR is within the recommended range, the patient is required to visit the clinic monthly in order to determine the INR again and to adjust the dose accordingly. A patient with an INR above 5.0 needs to withhold warfarin immediately and receive vitamin K antidote before restarting the treatment with a lower dose than the previous one (Gallus *et al.*, 2000). Serious bleeding incident suspected due to warfarin treatment in any INR require immediate withholding of warfarin, slow infusion of vitamin K and fresh frozen plasma or prothrombin complex concentrate supplement.

Table 1.2: Recommended INR ranges (MOH, 2010)

Indication	INR
AF	2.0 - 3.0
Bioprosthetic valve replacement	2.0 - 3.0
Mechanical mitral valve replacement	2.5 - 3.5
DVT	2.0 - 3.0
DVT and PE	2.0 - 3.0
High risk surgical patient	2.5 - 3.5

1.3.3 Pharmacokinetics

Warfarin is a racemic mixture of two enantiomers: (S)- and (R)-warfarin (Figure 1.1) which have different pharmacokinetics properties. After oral administration, its bioavailability is almost 100%. The maximum plasma concentration is achieved in 4 hr (FDA, 2014). However, the onset of its anticoagulation effect not immediate and is only begins to be observable in 36-72 hr (Lexi-Comp, 2014).

Warfarin is 99% bound to plasma protein, most of which is human serum albumin, while a small fraction binds to human α_1-acid glycoprotein (AGP), which is also called as orosomucoid (Otagiri *et al.*, 1987). Only 1% of administered warfarin is unbound and manifest its therapeutic effect. The protein-bound warfarin is continuously released into the plasma once the unbound warfarin is depleted (Wilting *et al.*, 1980).

The plasma concentration of (R)-warfarin is 1.5 times higher than the (S)-warfarin at steady state (Osman *et al.*, 2005). However, (S)-warfarin is three to five times more potent than the (R)-warfarin (O'Reilly, 1974). The relatively small volume of distribution of warfarin (0.14 L/kg) (FDA, 2014) conforms with the fact that it is highly plasma protein-bound and is not extensively distributed into other tissues such as muscles or fats.

Warfarin is metabolised by hepatic cytochrome P450 isoenzymes (CYP). Metabolism of warfarin is stereoselective, where in phase I metabolism (R)-warfarin is metabolised by CYP1A2, CYP3A4, CYP2C8 and CYP2C19 isoenzymes, while (S)-warfarin is predominantly metabolised by CYP2C9 isoenzyme (Limdi and Veenstra, 2008). Phase II metabolism of warfarin metabolites is by glucuronidation and sulphonation (Jansing *et al.*, 1992).

Even though (S)-warfarin is more potent than (R)-warfarin, it is eliminated faster than (R)-warfarin [half-life for (S)-warfarin is 29 hr while that for (R)-warfarin is 45 hr]. Elimination of warfarin is mainly through renal excretion (92%), where (S)-warfarin have almost three times faster clearance than (R)-warfarin (Hallak *et al.*, 1993).

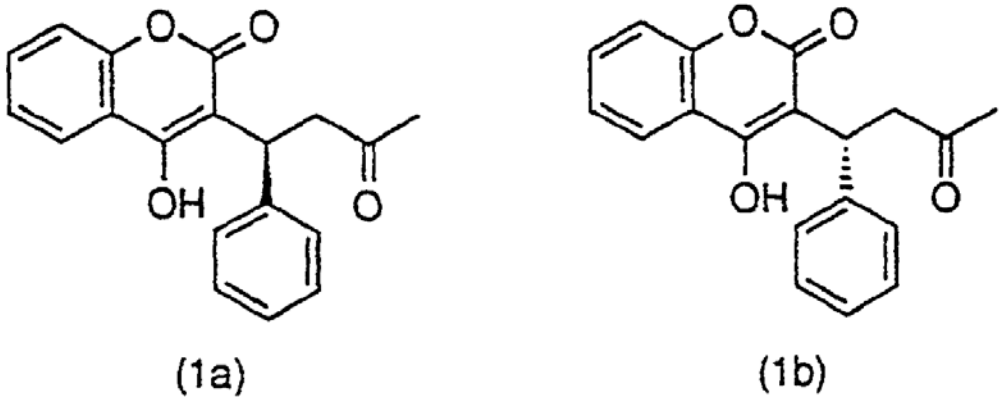

Figure 1.1: (S)-warfarin (1a) and (R)-warfarin (1b) with different orientation of their benzene rings (Li and Robinson, 2001)

1.3.4 Pharmacodynamics

During physiological condition, coagulation occurs when vitamin K-dependent (VKD) clotting factors II, VII, IX and X in the circulation are activated by carboxylation (Figure 1.2). Carboxylation of these VKD clotting factors require vitamin K hydroquinone (reduced vitamin K) as a cofactor. Warfarin however, suppresses the activation of the VKD clotting factors by inhibiting the recycling of vitamin K hydroquinone (reduced vitamin K) from vitamin K epoxide (Fasco and Principe, 1982). The enzyme which is responsible in recycling the reduced vitamin K, vitamin K epoxide (VKOR) is inhibited by both (R)- and (S)-warfarin. However, the latter has higher affinity to VKOR, thus making (S)-warfarin more potent than (R)-warfarin (O'Reilly, 1974). Decrease in activation of VKD clotting factors leads to decreased fibrin formation, therefore reducing the clotting ability of blood.

Each type of activated VKD clotting factors has different half-life before being eliminated (O'Reilly and Aggler, 1968). Clotting factor II has the longest half-life (50 hr). Warfarin's anticoagulation effect will only manifest after all of the activated VKD clotting factors are eliminated from the circulation which explains the slow onset of warfarin after the administration of the first dose. However, large warfarin loading dose is not given to patient because clotting inhibitors protein C and its precursor protein S also require reduced vitamin K for its activation. A sudden decrease in reduced vitamin K can cause warfarin-induced skin necrosis due to the formation of thrombus beneath the skin (Stewart *et al.*, 1999).

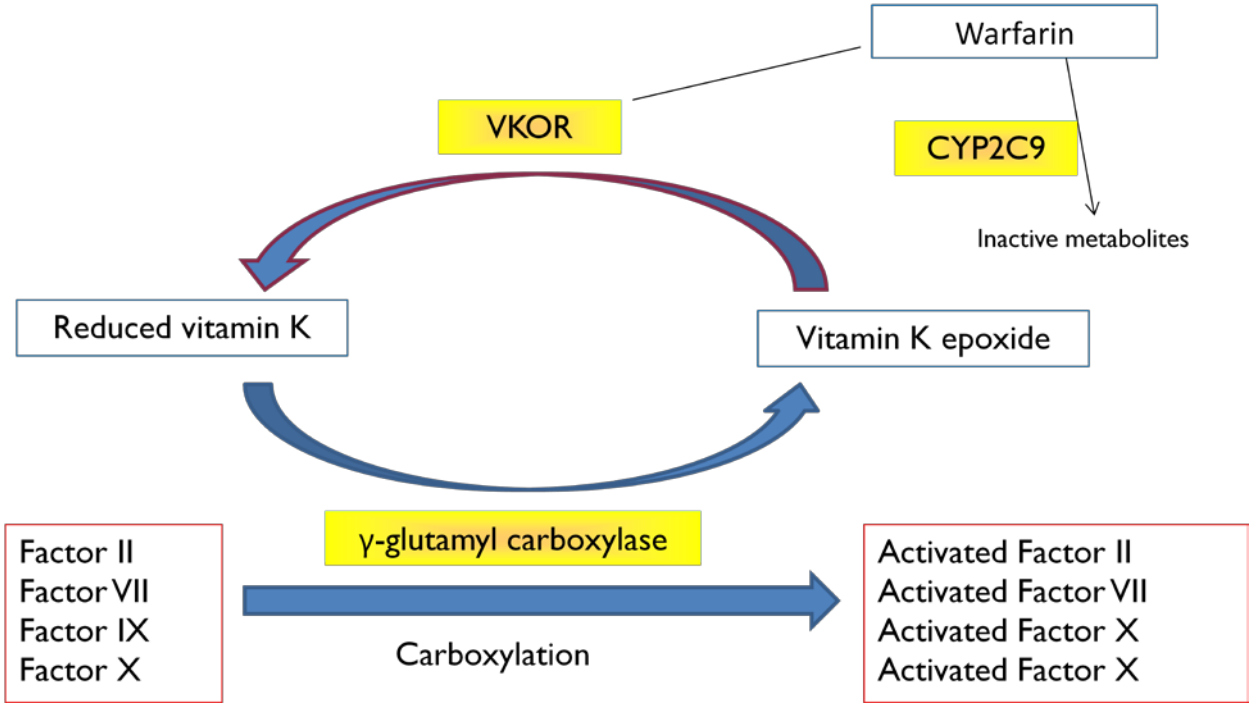

Figure 1.2: Vitamin K cycle and inhibition clotting factors activation by warfarin

1.4 Pharmacogenetics aspects

1.4.1 Cytochrome P450 2C9 genes

Most drugs are metabolised by cytochrome P450 isoenzymes, including warfarin (Kaminsky and Zhang, 1997). Racemic warfarin is metabolised by CYP1A2, CYP2C8, CYP2C9, CYP2C19 and CYP3A4 (Limdi and Veenstra, 2008). The genes that encode for these isoenzymes are polymorphic. Individuals with polymorphic cytochrome P450 gene may have decreased ability to metabolise warfarin and may suffer from adverse drug effect.

Due to the evidences that (S)-warfarin is more potent than (R)-warfarin, most researchers only investigate the gene the encodes for the isoenzyme that metabolise (S)-warfarin, which is *CYP2C9*. *CYP2C9*2* retains approximately 12% of the enzymatic activity of the wild-type variant, while *CYP2C9*3* retains only 5% (Rettie *et al.*, 1994; Haining *et al.*, 1996). Significant association between *CYP2C9*2* and **3* polymorphisms with warfarin's dose requirement and bleeding complication have been reported (Aithal *et al.*, 1999; Higashi *et al.*, 2002; Hillman *et al.*, 2004; Yildirm *et al.*, 2012). Due to the strong findings on the influence of the pharmacogenetics effect of *CYP2C9*, the recommendation to use *CYP2C9* genetic data was integrated into warfarin dosing label by the FDA starting from 2007 (FDA, 2007).

On the other hand, CYP3A4 is the major isoenzyme which metabolises (R)-warfarin. However, a study which investigated the association of *CYP3A4* polymorphisms and the dose requirement for a warfarin analogue (acenocoumarol) failed to establish any significant association (Saraeva *et al.*, 2007). Unlike other cytochrome P450 isoenzymes which metabolise warfarin, CYP4F2 is responsible in metabolising reduced vitamin K (McDonald *et al.*, 2009). Therefore, individuals with *CYP4F2* polymorphism tend to require higher warfarin dose due to high level of vitamin K in their body. There are evidences of association of *CYP4F2* with warfarin's dose requirement (Caldwell *et al.*, 2008; Pautas *et al.*,

2010; Perini *et al.*, 2010). Nevertheless, it was finally concluded that the association is not as clinically important as *CYP2C9* in actual practice (Kringen *et al.*, 2011).

1.4.2 Vitamin K epoxide reductase complex subunit 1

CYP2C9 was generally accepted as the main genetic determinant for the sensitivity to warfarin treatment, until the role of vitamin K epoxide reductase complex-1 gene (*VKORC1*) polymorphism was extensively investigated. The gene itself was relatively newly discovered in 2004 (Li *et al.*, 2004; Rost *et al.*, 2004). Unlike *CYP2C9* whose single nucleotide polymorphisms (SNP) were located at the coding region, the common human *VKORC1* polymorphisms were at the intronic region, thus affecting the expression of VKORC1 enzyme instead of changing the conformation of the enzyme. Depending on the type of *VKORC1* variant, it may render an individual to be more sensitive or resistant to warfarin treatment (Table 1.3).

Unlike other studies where a single *VKORC1* SNP is reported to be independently correlated to a phenotype (Carlquist *et al.*, 2006; Caldwell *et al.*, 2007), Rieder and colleagues identified 10 SNPs and combined them in a sequence called 'haplotype sequence'. Depending on the type of combinations, the haplotype sequences are designated with different types of 'haplotype identification code': H1, H2, H7 (for low dose requirement), H8 and H9 (for high dose requirement). Due to the fact that human DNA allele is double-stranded, two haplotype identification codes are required to determine the phenotype of an individual (Rieder *et al.*, 2005).

Stratification of warfarin's sensitivity based on the haplotype method is believed to be more accurate than when only a single *VKORC1* SNP is used. However, this fact may not be true in a population where most of the *VKORC1* SNPs are uncommon. It has been suggested that identification of only *VKORC1* 381 alone in an Asian population is sufficient to determine whether an individual is resistant or sensitive to warfarin treatment (Lee *et al.*, 2006). The *VKORC1* 381 alone were also theoretically useful because it is closely linked to

VKORC1 -1639G>A (Rieder *et al.*, 2005), which is the most extensively studied single *VKORC1* genotype (Sconce *et al.*, 2005; Aquilante *et al.*, 2006; Gan *et al.*, 2012). However, a retrospective study which apply this suggestion found that approximately 10% of the study population are still inaccurately dosed (Tham *et al.*, 2006). Therefore, haplotyping method may still be useful to accurately stratify patients sensitivity to warfarin treatment.

In terms of usefulness in predicting sensitivity to warfarin, *VKORC1* is considered a stronger predictor than *CYP2C9*. When combined with other data such as age, weight and *CYP2C9* genotype, *VKORC1* is able to explain as high as 68% of the dose variability (Table 1.4). Along with *CYP2C9*2* and **3*, *VKORC1* -1639G>A genotypic data is therefore recommended by the FDA to be used for warfarin's dose determination (FDA, 2007).

Table 1.3: *VKORC1* SNP variants used by Rieder *et al.* (2005) to delineate the haplotype of sensitivity to warfarin treatment

Position	**RefSNP**	**Variant**		**Variant**	
		Nucleotide	Warfarin dose requirement	Nucleotide	Warfarin dose requirement
381	rs7196161	T	High	C	Low
861	rs17880887	C	Low	A	High
2653	rs17881535	G	Low	C	High
3673	rs9923231	G	High	A	Low
5808	rs2884737	T	High	G	Low
6009	rs17708472	C	Low	T	High
6484	rs9934438	C	High	T	Low
6853	rs8050894	G	High	C	Low
7566	rs2359612	C	High	T	Low
9041	rs17880624/ rs7294	G	High	A	Low

RefSNP = reference SNP

Table 1.4: Application of *VKORC1*, other genetic and demographic data and their reliability to explained warfarin dose variability

Investigators	**% of dosing variability explained when *VKORC1* and other predictors were used**	**% of dose variability explained when *CYP2C9* alone was used**
Rieder *et al.* (2005)	*VKORC1* haplotypes: 25%	*CYP2C9* variants: 10.0%
Bodin *et al.* (2005)^	*VKORC1* -1639G>A: 50%	*CYP2C9*3*: 14.0%
	VKORC1 -1639G>A, *CYP2C9* and body weight: 54%	
Sconce *et al.* (2005)	Age, height, *CYP2C9**2 & *3 and *VKORC1* -1639G>A: 54.2%	*CYP2C9* variants: 17.5%
Veenstra *et al.* (2005)	*VKORC1* genotypes: 31%	*CYP2C9* variants: 7.9%.
Lee *et al.* (2006)	*VKORC1* haplotypes: 30%	*CYP2C9*3*: 29.0%
	VKORC1 haplotypes, age, *CYP2C9**3, gender and race: 48 %	
Tham *et al.* (2006)	Age, weight, *CYP2C9*3, VKORC1* 381: 60.2%	*CYP2C9*3*: 17.5%
Caldwell *et al.* (2007)	*VKORC1* 6853 (rs 8050894), *CYP2C9*2,*3*, age, gender, body surface area, history of diabetes, history of heart valve replacement: 56%	*CYP2C9* variants: 28.2%

^ The study was to observes the effect of *VKORC1* SNPs on acenocoumarol.

Table 1.4 (Continued)

Investigators	**% of dose variability explained when *VKORC1* and other predictors were used**	**% of dose variability explained when *CYP2C9* alone was used**
Wadelius *et al.* (2007)	*VKORC1, CYP2C9*2* and **3, PROC, EPHX1, GGCX, ORM1-2,* age, body weight and drug interactions: 73%	*CYP2C9* variants: 15.9%
Gage *et al.* (2008)	*VKORC1* -1639, 3730, *CYP2C9*2,*3,* body surface area, INR, amiodarone, smoker status, age, race, current thrombosis: 53-54%	*CYP2C9*3*: 33%
IWPC (2009)	Age, height, weight, VKORC1 -1639G>A, CYP2C9*2, CYP2C9*3, race, enzyme inducers, amiodarone: 47.0%	Not tested
Lenzini *et al.* (2010)	Age, INR, *VKORC1* -1639 G>A, *CYP2C9*2*, *CYP2C9*3*, body surface area, target INR, African origin, stroke, diabetes, amiodarone, fluvastatin, dose of warfarin in 2, 3 and 4 days before INR measurement: 43%	Not stated
You *et al.* (2011)	*CYP2C9*3*, *VKORC1* 1173C>T, age, weight and vitamin K intake: 68%	Not stated
Teh *et al.* (2012)	Age, *CYP2C9*3*, *VKORC1* -1639G>A and 1173 C>T: 37%	Not stated

1.4.3 Clotting factor genes

Although there were cases of congenital vitamin K-dependant clotting factor deficiency (VKCFD) which caused bleeding disorder, the decreased levels of these VKD clotting factors are usually not contributed by the mutation of the genes that encode the VKD clotting factors themselves (Oldenburg *et al.*, 2000). However, mutation of clotting factors VII-related genes are reported to decrease the plasmatic clotting factor level in healthy subjects (Bernardi *et al.*, 1997). Mutation of clotting factor IX also have been documented to cause warfarin hypersensitivity in actual clinical cases (Oldenburg *et al.*, 1997; Kristensen, 2002). The occurrence of such clinical cases were however, rare and the correlation study between VKD clotting factors alone and warfarin's dose requirement in large cohort did not indicate any significant contribution to warfarin dose variability (Aquilante *et al.*, 2006). A combination of certain mutations in clotting factors II and VII with *CYP2C9*3* genotypic data is able to explain 50% of the warfarin dose variability in a study cohort (Shikata *et al.*, 2004) but the utility of VKD clotting factor mutation were never proven in an actual warfarin dosing practice.

1.4.4 Apolipoprotein E gene

Vitamin K from food is bound to chylomicron in the blood circulation and then taken up by the liver. The affinity of chylomicron to be uptaken by the liver is influence by apolipoprotein E (APOE) in the chylomicron (Lamon-Fava *et al.*, 1998). However, the gene that encodes for APOE is polymorphic, with three *APOE* variants (ε2, ε3 and ε4) found. APOE receptor on the hepatocyte has an increasing affinity to *APOE* variants in the order of ε2<ε3<ε4 (Weintraub *et al.*, 1987). Along with *VKORC1, APOE* polymorphism may explain up to 57% of warfarin dose variability (Sconce *et al.*, 2006). However, univariate analyses of APOE usually indicates that *APOE* is not significantly associated with warfarin's dose requirement. Nevertheless, the frequency of the *APOE*, especially ε4, is too low for it to be

clinically practical to help in the determination of warfarin's dose requirement (Kohnke *et al.*, 2005; Kimmel *et al.*, 2008; Lal *et al.*, 2008; Huang *et al.*, 2011).

1.4.5 Gamma-glutamyl carboxylase gene

Propeptide VKD clotting factors contain a glutamic acid residue which requires carboxylation into γ-carboxyglutamic acid before they can bind to the vascular surface at the site of injury. The carboxylation process is catalysed by VKD γ-glutamyl carboxylase (Stenflo and Suttie, 1977). The gene that encodes for γ-glutamyl carboxylase (*GGCX*) is polymorphic. Although as many as 37 *GGCX* SNPs have been discovered in an investigation, only a SNP, *GGCX* 1181T>G, is reported to be marginally associated with warfarin's dose requirement and explains only 2% of warfarin dose variability in the study cohort (Rieder *et al.*, 2007) indicating that it is not a polymorphism that should be investigated in our study.

1.4.6 Calumenin gene

VKOR and γ-glutamyl carboxylase are endoplasmic reticulum transmembrane proteins that resided close to each other in order to facilitate the transfer of vitamin K between the two proteins (Carlisle and Suttie, 1980; Tie *et al.*, 2005). The enzymatic activity of both proteins were believed to be regulated by a chaperone protein called calumenin (Wajih *et al.*, 2004). Polymorphism of calumenin gene (*CALU*) lead to overexpression of calumenin and therefore reduce the activity of both enzymes (Voora *et al.*, 2010). However, multiple investigations showed that the polymorphism of *CALU* was just nominally associated with warfarin's dose requirement (Wadelius *et al.*, 2007; Voora *et al.*, 2010; Zohir *et al.*, 2014).