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Pharmacogenetics of Oral Anticoagulants and Antiplatelets

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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by

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Abstract

Thromboembolic disorders are a major cause of morbidity and mortality. Therapeutic intervention with anticoagulants and antiplatelets greatly reduces the risk of arterial and venous thrombosis. However, the observed large interindividual variation in responsiveness to these drugs indicates that subsets of patients are not attaining optimal therapy, resulting in either lack of antithrombotic effect or elevated bleeding risk. Recently, single nucleotide polymorphisms (SNPs) have been linked to the variation observed in efficacy and toxicity for many cardiovascular drugs.

Warfarin has been the gold standard anticoagulant for prevention of stroke and thromboembolism in atrial fibrillation (AF) and venous thromboembolism (VTE) patients. SNPs in genes that affect warfarin metabolism (*CYP2C9*) and response (*VKORC1*) have an important influence on response and dose, particularly during initiation. Accordingly, we developed and evaluated the clinical utility of a pharmacogenetics-based initiation nomogram in AF and VTE patients which provides safe and optimal anticoagulation therapy irrespective of genetic variation.

The new oral anticoagulant (NOAC) rivaroxaban is highly dependent on the kidney for elimination through glomerular filtration and active tubular secretion. Importantly, interindividual variation in exposure and response to rivaroxaban has been reported. Using cell-based and animal models, we demonstrated that rivaroxaban is a dual substrate of the

efflux transporters MDR1 and BCRP, which played a synergistic role in modulating rivaroxaban clearance and brain accumulation. The contribution of interindividual variation in transport and metabolism to the efficacy of rivaroxaban as well as other NOACs requires to be addressed in patients.

Clopidogrel has been the gold standard antiplatelet for prevention of acute coronary syndromes and stent thrombosis following percutaneous coronary intervention. Two enzymes, CYP2C19 and PON1, have been proposed to affect clopidogrel bioactivation and efficacy. We showed that CYP2C19 but not PON1 is capable of bioactivating clopidogrel to its active metabolite. This is in line our finding that *CYP2C19* genetic variation is a predictor of clopidogrel pharmacokinetics and antiplatelet response while PON1 is not.

Taken together, these studies demonstrate the contribution of SNPs to the variation in efficacy and toxicity of cardiovascular drugs, enabling personalized medicine for patients, where an individual's genetic makeup is used to guide drug selection and dosing.

Keywords

Oral anticoagulant therapy, antiplatelet therapy, warfarin, clopidogrel, rivaroxaban, cytochrome P450 enzymes, single nucleotide polymorphisms, pharmacogenomics, pharmacogenetics, metabolism, pharmacokinetics, pharmacodynamics, thromboembolic disorders, efflux drug transporters

Co-Authorship Statement

Chapter Four:
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IYG, RGT, UIS, NC, GKD, AL, PSW, and RBK designed the research study. IYG, NC, and SL participated in patient enrolment and data acquisition. IYG, RGT, UIS, NC, GKD, PSW, and RBK analyzed and interpreted of data. IYG and GZ conducted the statistical analysis. IYG and RBK wrote the manuscript. All authors provided feedback on the manuscript for important intellectual content. All authors approved the final version of the manuscript.

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IYG, UIS, NC, GKD, AL, PSW, RBK, and RGT designed the research study. IYG measured warfarin and biomarker concentrations. IYG and RGT conducted the mathematical modeling. IYG, RBK, and RGT analyzed and interpreted of data. IYG and RGT wrote the manuscript. All authors provided feedback on the manuscript for important intellectual content. All authors approved the final version of the manuscript.

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IYG, and RBK designed the research study. IYG and SE conducted the experiments. IYG and RBK analyzed and interpreted the data. IYG and RBK wrote the manuscript. All authors approved the final version of the manuscript.

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IYG, CS, NC, RGT, UIS, and RBK designed the research study. IYG, NC, CS, and GKD participated in healthy volunteer enrolment and data acquisition. IYG measured clopidogrel and biomarker concentrations. IYG, MJK, MKD, DS, and SW conducted metabolomics experiments. IYG conducted the statistical analysis. IYG, UIS, RGT, and RBK analyzed and interpreted of data. IYG and RBK wrote the manuscript. All authors approved the final version of the manuscript.

Dedication

To Mom, Dad, and my sister Michelle

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Abbreviations

ABCB1	ATP-binding cassette subfamily B member 1
ABCG2	ATP-binding cassette subfamily G member 2
ACS	acute coronary syndromes
ADP	adenosine diphosphate
ADR	adverse drug reaction
AF	atrial fibrillation
anti-Fxa	anti factor Xa
API	apixaban
ApoE	apolipoprotein E
aPTT	activated partial thromboplastin time
AUC	area under the plasma concentration curve
BBB	blood brain barrier
BCRP	breast cancer resistant protein
BID	twice daily
BMS	bare-metal stent
CAD	coronary artery disease
CALU	calumenin
CES1	carboxylesterase 1

CI	confidence interval
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CL	clearance
CL/F	apparent clearance
CL_{int}	intrinsic clearance
C_{max}	maximum plasma concentration
CNS	central nervous system
COX	cyclooxygenase
C_p	plasma concentration
CrCl	creatinine clearance
CYP	cytochrome P450
CYP1A2	cytochrome P450 1A2
CYP2B6	cytochrome P450 2B6
CYP2C19	cytochrome P450 2C19
CYP2C9	cytochrome P450 2C9
CYP3A4	cytochrome P450 3A4
CYP4F2	cytochrome P450 4F2
DAB	dabigatran
DDI	drug drug interaction

DES	drug-eluting stent
DTI	direct thrombin inhibitor
DVT	deep vein thrombosis
ECG	electrocardiogram
ECT	ecarin clotting time
eGFR	estimated glomerulus filtration rate
EM	extensive metabolizer
ESRD	end stage renal disease
FDA	Food and Drug Administration
Fxa	factor Xa
GGCX	gamma-glutamyl carboxylase
GI	gastrointestinal
GSH	glutathione
GWAS	genome-wide association study
Hemoclot	diluted version of thrombin time
HR	hazard ratio
IC_{50}	drug affinity
Imax	maximum inhibitory factor
Imax	maximal inhibitory factor

INR	international normalized ratio
K	zero-order
k_a	absorption constant
k_e	elimination constant
keto	ketoconazole
K_m	enzyme affinity
k_{out}	first-order
LC-MS/MS	liquid chromatography tandem mass spectrometry
LHSC	London Health Sciences Centre
LOWESS	locally weighted scatterplot smoothing regression
MDR1	P-glycoprotein; multi drug resistance protein 1
Mdr1a	multi drug resistance protein isoform 1a
$Mdr1a^{def}$	multie drug resistance protein isoform 1a deficient
Mdr1b	multi drug resistance protein isoform 1b
MI	myocardial infarction
MPB	2-bromo-3-methoxyacetophenone
NOAC	new oral anticoagulant
NSAIDs	nonsteroidal anti-inflammatory drugs
NSTEMI	non-ST segment elevation myocardial infarction

OAC	oral anticoagulant
OD	once daily
P-gp	P-glycoprotein
PCI	percutaneous coronary intervention
PD	pharmacodynamics
PE	pulmonary embolism
PGE1	prostaglandin E1
PIVKA-II	proteins induced by vitamin K absence factor II
PK	pharmacokinetics
PK-PD	pharmacokinetics-pharmacodynamics
PON1	paraoxonase 1
PRU	platelet reactive units
PT	prothrombin time
QALY	quality adjusted life years
RCT	randomized clinical trial
rif	rifampicin
RIV	rivaroxaban
RM	reduced metabolizer
SD	standard deviation

SEM	standard error of the mean
SNP	single nucleotide polymorphisms
SSE	systemic emoblism
ST	stent thrombosis
STEMI	ST segment elevation myocardial infarction
$t_{1/2}$	half-life
$t_{1/2\beta}$	terminal half-life
TF	tissue factor
TIA	transient ishemic attack
TOH	The Ottawa Hospital
TT	thrombin time
TTR	time in therapeutic range
V	volume of distribution
VASP	vasodilator-stimulated phosphoprotein
Vd	volume of distribution
VKOR	vitamin K epoxide reductase
VKORC1	vitamin K epoxide reductase subunit 1
Vmax	maximum rate achieved by enzymatic system
Vmax/Km	intrinsic clearance

VTE	venous thromboembolism
vWF	von Willebrand factor
WRAPID	Warfarin Regimen using A Pharmacogenetics-guided Initiation Dosing

1 THROMBOEMBOLIC DISORDERS: PATHOGENESIS AND RATIONALE FOR ANTICOAGULANT AND ANTIPLATELET THERAPY

1.1 Introduction

Maintenance of blood fluidity within the vasculature is an important human physiological process. Under normal conditions, there is a fine equilibrium between pathological states of hypocoagulability and hypercoagulability. Hemostasis refers to a series of normal physiological processes that confine blood to the vascular spaces, maintain blood fluidity, and importantly, clot formation to limit hemorrhage following vascular injury. Thrombosis is pathological clot formation when hemostasis is inappropriately activated in the absence of a bleeding event. In the 1800s, Rudolf Virchow postulated a triad of causes for thrombosis formation: changes in the composition of blood, alterations in the vessel wall, and disruption of blood flow (1). Indeed, in the event of a vascular vessel injury, a sequence of events is generated in response: vessel constriction to reduce blood flow, hemostatic platelet plug formation at the trauma site following platelet adhesion, activation, and aggregation, formation of a fibrin clot to stabilize the platelet plug by activation of a series of proteins in the coagulation cascade. As such, a thrombus forms in the presence of alterations in the hemostatic system leading to inappropriate platelet aggregation and coagulation. A thrombus in a large blood vessel will decrease blood flow through that vessel while a thrombus in a small blood vessel may completely cut-off blood flow resulting in an occlusive thrombus. An embolism is the dislodging of the thrombus from the site of formation that travels to a distal vessel leading to blood flow blockage in a distant part of the body.

Thromboembolic disorders are a significant source of mortality and morbidity. There are two types of thrombosis, arterial and venous thrombosis, whereby the clinical and therapeutic management differs, reflecting the distinct pathogenesis of the two classifications. Arterial thrombosis usually occurs after the erosion or rupture of an atherosclerotic plaque, potentially leading to ischemic events. Cardiac ischemia and stroke are the most devastating clinical manifestations of atherothrombosis. Venous thromboembolism (VTE) is represented by two main manifestations, deep venous thrombosis (DVT) and pulmonary embolism (PE). The most devastating clinical consequence of VTE is PE where majority of PEs result from DVT that have dislodged from site of formation in the lower extremities and traveled to the pulmonary circulation.

Understanding the pathogenic processes leading to either arterial or venous thrombosis is crucial for selecting effective and safe antithrombotic agents for management of patients with thromboembolic disorders. While arterial thrombosis undoubtedly involves the coagulation cascade, platelet activation and aggregation plays a more prominent role in the rapidly flowing arteries. As such, arterial thrombosis is often referred to as white clot, rich in platelets and little red blood cells. On the other hand, venous thrombosis is associated with venous stasis and hypercoagulability. Thus, the coagulation cascade plays a prominent role in formation of a venous thrombus, often referred to as red clot due to the abundance of red blood cells and little platelets.

1.2 Hemostasis

The endothelium of blood vessel walls plays an important role in maintaining vasculature integrity. Vessel wall damage or disruption of the endothelium leads to exposing the collagen present in the subendothelial matrix, resulting in platelet activation, aggregation, and formation of a primary platelet plug. Concurrently, subendothelial tissue factor (TF) is also exposed on the damaged endothelium leading to activation of the coagulation cascade, resulting in formation of a fibrin mesh to stabilize the platelet plug (2).

1.2.1 Platelet activation

Platelets are small disk shaped colorless cells present in the blood that play a vital role in hemostasis and are the key factor in the pathogenesis of arterial thrombosis. Vascular injury may cause the rupture of an atherosclerotic plaque and denudation of the endothelium (Figure 1.1). The exposure of subendothelial collagen bound to a protein known as von Willebrand factor (vWF) facilitates platelet adhesion to the vessel wall. At the site of injury, vWF binds platelets via the glycoprotein Ib/IX/V receptor complex on the platelet membrane (3). At low shear rates, platelets can also bind to subendothelial collagen through other receptors such as the glycoprotein IV, VI, and Ia/IIa. Upon adhering to the vessel wall, platelets release the granules of platelet agonist thromboxane A_2 and adenosine diphosphate (ADP), initiating an autocrine/paracrine signaling cascade of platelet activation. Activated platelets undergo a change in morphology from smooth disks to irregular spheroids with filopodia, allowing platelets to bind to one another for

platelet aggregation. The formation of this hemostatic plug is essential for wound healing. However, formation of a thrombotic occlusion in coronary arteries can cause tissue ischemia leading to conditions known as acute coronary syndromes (ACS), in the form of either unstable angina or myocardial infarction (MI).

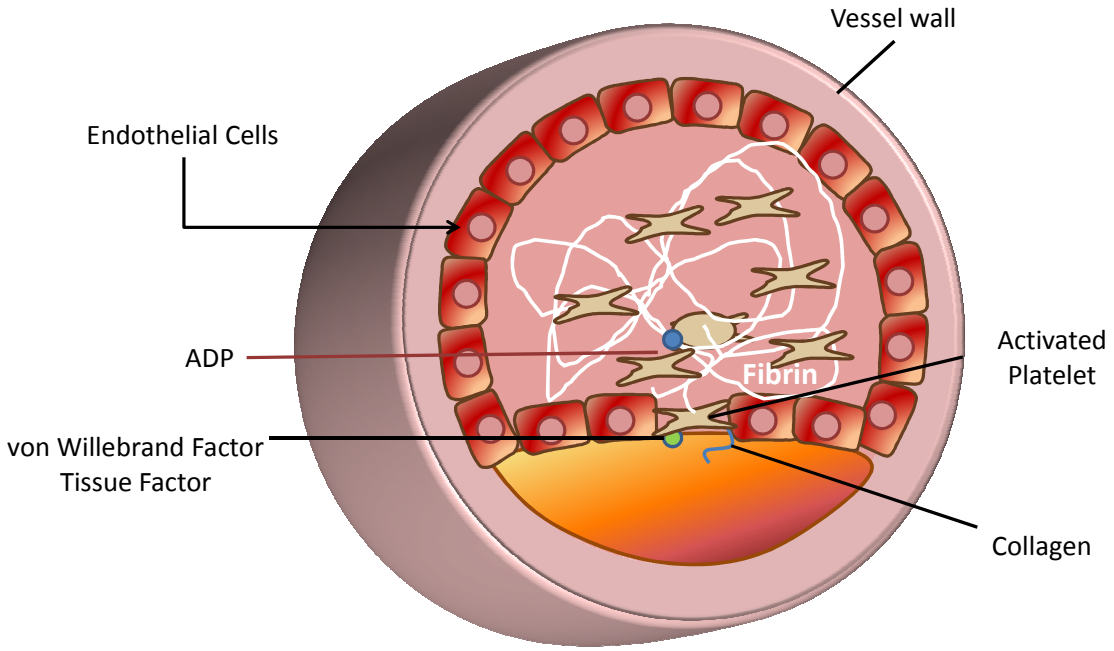

Figure 1.1 Schematic representation of arterial thrombosis.

An atherosclerosis plaque develops through the accumulation of lipid deposits. The rupture of an atherosclerotic plaque is the primary trigger for arterial thrombosis by promoting platelet activation and fibrin generation.

1.2.2 Coagulation cascade

The coagulation cascade plays a pivotal role in forming the fibrin mesh to reinforce the hemostatic platelet plug. The ultimate formation of fibrin is dependent on two distinct pathways: the extrinsic and intrinsic pathway. It was previously thought that both pathways were equally crucial to hemostasis. However, it is evident now that the extrinsic pathway is the predominant pathway for initiation and activation of the coagulation cascade during hemostasis and response to vascular injury. The intrinsic and extrinsic pathways are activated by distinct factors but merge into a common pathway to activate thrombin and form fibrin. The ultimate generation of fibrin is governed by initiation, amplification, and propagation of coagulation (4). The ability to form a fibrin clot is dependent on a series of serine proteases that are clotting factors and/or cofactors (Figure 1.2). These clotting factors are chronologically activated producing thrombin by stepwise activation of a series of proenzymes.

During initiation phase, the extrinsic cascade is activated by tissue injury or trauma triggering the exposure of TF on subendothelial cells, an essential cofactor for factor VII (4). Activated factor VII, a serine protease will subsequently cleave factor X and factor V to Xa and Va, respectively, to combine with phospholipid and calcium to form the prothrombinase complex. The complex then cleaves a small amount of prothrombin (factor II) to active thrombin (factor IIa). In addition to activating platelets, this small amount of thrombin amplifies the coagulation reactions by positive feedback. Thrombin activates factor V and VIII on the platelet surface leading to a burst of activated clotting

factors ready to generate thrombin. On activated platelets, factor XIa activates IX to IXa, which forms the tenase complex with factor VIIIa to activate factor X. Finally, factor Xa and factor Va forms the prothrombinase complex in abundance to produce the thrombin burst. The propagation of thrombin generation converts fibrinogen to fibrin for forming the mesh.

The intrinsic pathway begins with the formation of a primary complex of coagulation factors, including high molecular weight kininogen, prekallikrein, and factor XII, driven by the collagen exposed on the damaged blood vessel wall (4). Prekallikrein is converted to kallikrein and factor XII is activated to factor XIIa through a process called autoactivation, resulting in the activation of factor XI to factor XIa. Subsequently, factor XIa activates factor IX to factor IXa, which associates with factor VIIIa to form the tenase complex to activate factor X to factor Xa. Lastly, the intrinsic pathway merges with the extrinsic pathway in the common pathway where factor Xa activates prothrombin to thrombin to form fibrin. The minor role that the intrinsic pathway plays in hemostasis is evident in the fact that patients with deficiencies in primary proteins prekallikrein, high molecular weight kininogen, and factor XII do not have life-threatening bleeding disorders.

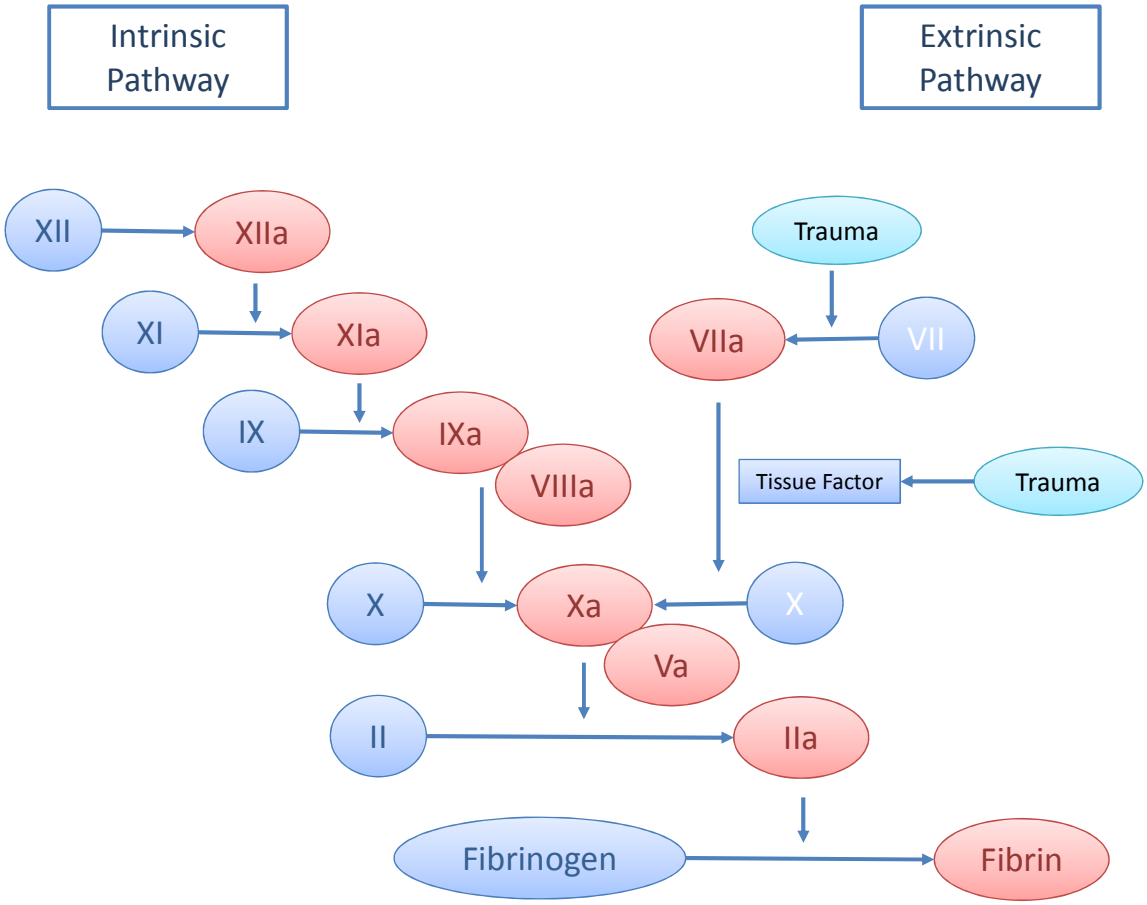

Figure 1.2 Schematic representation of the coagulation cascade.

The coagulation cascade can be activated by intrinsic and extrinsic factors to ultimately generate thrombin and fibrin.

1.3 Thrombosis

1.3.1 Arterial thrombosis

Arterial thrombosis (Table 1.1) typically occurs in conjunction with vascular abnormalities that are the result of atherosclerosis (buildup of cholesterol and fatty plaques on the inner walls of arteries), leading to coronary artery disease (CAD). ACS reflects the clinical manifestations attributed to CAD, thrombus formation, and occlusion of the coronary arteries. It has been suggested that thrombotic coronary occlusion accounts for 50 - 70% of sudden deaths caused by ischemic heart disease (5). The three types of ACS are unstable angina, non-ST segment elevation MI (NSTEMI), and ST segment elevation MI (STEMI). An arterial thrombus that is fibrin-rich is often fully occlusive, results in STEMI as characterized by clinically significant changes on an electrocardiogram (ECG). Platelet-rich thrombus is likely partially occlusive, resulting in unstable angina and NSTEMI, with little change on an ECG. Antiplatelet therapy is the primary therapy for prevention of ACS recurrence.

1.3.2 Atrial fibrillation

Atrial fibrillation (AF) is the most common cardiac arrhythmia, affecting a significant portion of the elderly population. Statistics show that there are currently 5.2 million individuals with AF in North America and this number is expected to increase substantially by 2050 as a consequence of the aging population (6). The incidence of AF

increases from 2.3% among individuals over age 40 to 5.9% among individuals over 65. The most devastating complication of AF is stroke, resulting from cardioembolization to the central nervous system. In all age groups of AF patients, the incidence of stroke is increased by four to five fold with a high mortality rate (7). Furthermore, stroke risk may increase up to seven fold in presence of additional risk factors such as hypertension, diabetes, and heart failure.

The pathophysiology of AF is such that the abnormal electrical charges from the atria reduce the ability to pump blood into the ventricles resulting in stagnant blood flow particularly in the left atrial appendage. The stasis in this area in individuals with prolonged or insufficiently treated AF is a significant contributing factor to thrombus formation. Indeed, more than 90% of the thrombi associated with nonvalvular AF (absence of mitral valve disease) have been found in the left appendage (8). Emboli carried away from thrombus formation site to the brain cerebral vessels may result in an ischemic stroke or a transient ischemic attack (TIA).

AF has been associated with markers of coagulation and platelet activation that reflects its hypercoagulable state. It has been shown that both vWF and TF are overexpressed in the atrial endothelium of AF patients (9). Based on available evidence, pathogenesis of thromboembolism in AF patients follows Virchow's triad for thrombogenesis (9). Although a thrombus formed in the left atrial appendage is considered as an arterial

thrombus, the characteristics of an AF thrombus appear to be more similar to a venous clot. Therefore, anticoagulation therapy is recommended as the primary therapy for stroke risk reduction.

$CHADS_2$ Score

The risk of stroke in AF patients is compounded with co-existing risk factors. The $CHADS_2$ score is a clinical prediction tool for estimating the risk of stroke in patients with nonvalvular AF, where the annual stroke risk significantly increases in individuals with higher $CHADS_2$ scores (10). This system also allows for determining the recommended treatment regimen, in terms of whether to initiate therapy with an antiplatelet or oral anticoagulant. Components of the $CHADS_2$ score include congestive heart failure, hypertension, age $\geq$ 75, diabetes, and prior history of stroke or TIA (Table 1.3.2).

Table 1.3.1 Risk factor and characteristic differences between arterial thrombosis and venous thrombosis.

Arterial Thrombosis	Venous Thrombosis
Artery	Vein
Atherosclerosis	Stasis
Underlying cardiovascular disease	Irregular thrombin generation
Platelet rich	Red blood cell rich
Platelet aggregation dominates	Coagulation cascade dominates

Table 1.3.2 $CHADS_2$ scoring system for determining stroke risk in atrial fibrillation patients.

	Condition	Points
C	Congestive heart failure	1
H	Hypertension	1
A	Age ≥ 75	1
D	Diabetes mellitus	1
S_2	Prior stroke or transient ischemic attack	2

1.3.3 Percutaneous coronary intervention

Percutaneous coronary intervention (PCI) is often part of the standard of care for ACS patients, particularly those presented with STEMI or NSTEMI. The PCI procedure has evolved dramatically since its introduction with bare-metal stents (BMS) to the availability of drug-eluting stents (DES). The development of DES was to incorporate the release of pharmacological agents in the stent design to inhibit responses to injury, a primary contributor to restenosis after BMS implantation (11). However, although restenosis rates are evidently reduced with DES, stent thrombosis (ST) rate has not decreased. ST is the sudden occlusion of a stented coronary artery as a result of thrombus formation, a severe complication after implantation owing to its high mortality (12). A number of trials have observed occurrence of acute, subacute, and late ST following DES implantation, and the rate is suspected to be substantially greater in the real-world population (12). Moreover, ST as long as three years after stent implantation has been noted with DES, which was rarely seen with BMS.

The pathogenesis of ST has not been completely delineated; however, factors increasing risk of ST include the procedure itself, patient and lesion characteristics, and premature cessfation of antiplatelet drugs (13). Stent implantation itself induces platelet adhesion and activation of the coagulation cascade, as it is the introduction of a foreign object in the vessel wall. DES were developed to prevent restenosis by reducing vascular smooth muscle cell proliferation and migration (13). However, they also impair reendothelialization, which prolong arterial healing and in fact, have been found to

induce TF expression and thereby thrombogenesis (14). Lastly, DES impair the endothelial function of coronary arteries, promoting risk of ischemia and coronary occlusion (13).

1.3.4 Venous thrombosis

In contrast to arterial thrombosis, venous thrombosis is typically not associated with underlying vascular pathology (Table 1.3.1). VTE comprises of DVT and PE; DVT occurs most often in the large veins of the lower extremities and when part of the thrombus dislodges, the embolism can travel to the lungs to block blood flow to the pulmonary artery resulting in PE. Venous stasis is an important risk factor for VTE by promoting thrombus formation with reduced ability to clear activated coagulation factors away from the site of injury (15). In fact, majority of venous thrombi is formed in regions of slow blood flow (15). Stasis may be caused by immobility, orthopaedic surgery, and increased venous pressure. Overall, prolonged impairment of venous function, sustained hypercoagulability, and imbalance in the endogenous anticoagulant and fibrinolytic systems all contribute to the risk of developing a clinically significant thrombus.

As previously noted, venous thrombi mostly consist of red blood cells and fibrin and activation of the coagulation cascade is the primary contributor to venous thrombosis. Thus, anticoagulant therapy is the primary therapy for prevention and management of VTE.

1.4 Therapeutic interventions

1.4.1 Oral anticoagulant therapy

For many decades, the vitamin K antagonist warfarin has been the gold standard of therapy for prevention of ischemic stroke in AF patients. The mechanism of action of warfarin is inhibition of vitamin K dependent clotting factor (II, VII, IX, X) activation in the liver. In 1994, it was demonstrated in a group 3,692 patients that warfarin treatment reduced stroke risk by 68% compared to individuals without treatment, along with minimal bleeding risk (16). Indeed, pooled-analysis of six large randomized clinical trials (RCTs) comparing aspirin with warfarin demonstrated superiority of warfarin in reduction of ischemic stroke rate compared to aspirin (17). Furthermore, a meta-analysis of 29 trials incorporating 28,044 AF patients demonstrated that warfarin improved the incidence of stroke outcomes by 40% compared to antiplatelet therapy (18).

Anticoagulation therapy is also crucial for prevention of recurrent DVT and PE. Oral anticoagulant therapy with warfarin has been the mainstay of long-term treatment and prophylaxis of VTE (19). However, warfarin has an indirect pharmacological mode of action whereby it inhibits the vitamin K epoxide reductase (VKOR) enzyme to prevent the formation of coagulation factors in the liver (20). Thus, the onset of warfarin's anticoagulation activity is typically delayed up to 48 hours following initiation. In the

setting of VTE, immediate anticoagulantion is necessary for minimizing risk of recurrence. Accordingly, subcutaneous low-molecular-weight heparin (factor Xa inhibitor) is the anticoagulant of choice to be used concurrently with warfarin during the initial days of overlapping therapy.

We note here that although warfarin has been the gold standard of therapy for the past 60 years, its shortcomings (including the wide interindividual variation in responsiveness and the need to titrate dose to therapeutic response using continuous monitoring) have led to the development and market approval of several new oral anticoagulants. These new agents exert therapeutic efficacy by directly inhibiting the coagulation cascade in the systemic circulation. The new oral anticoagulants include direct thrombin inhibitors such as dabigatran, and factor Xa inhibitors such as rivaroxaban and apixaban. The clinical implications of the new agents to oral anticoagulation therapy will be discussed in the forthcoming chapters.

1.4.2 Antiplatelet therapy

Antiplatelet therapy has emerged as a major success in reducing the risk for MI associated with CAD. Currently, dual antiplatelet therapy with clopidogrel and aspirin is the mainstay of therapy for prevention of recurrent cardiovascular events in patients with ACS, and prevention of ST following PCI (21). The rationale for the dual therapy is to block two important pathways involved in platelet aggregation. Clopidogrel is a

thienopyridine drug, which inhibits ADP-mediated platelet activation by irreversibly binding to $P2Y_{12}$ G-protein coupled receptors on platelets (22). Aspirin exerts its antiplatelet effect by inhibiting the activity of cyclooxygenase-1 (COX-1), preventing the production of thromboxane A_2 from arachidonic acid (22). Dual antiplatelet therapy has been demonstrated to be effective in reducing arterial thrombosis and ischemic events in large RCTs such as CURE and PCI-CURE (23, 24).

1.5 Conclusions

Thromboembolic disorders are major causes of morbidity and mortality. Failure to provide optimal therapeutic intervention to reduce risks of stroke, ACS, and recurrent VTE will undoubtedly be a significant health burden. As outlined earlier, the therapeutic treatment of thromboembolic disorders heavily depends on the pathogenesis of the disease. In conditions of high blood flow (arterial thrombosis), the dominant role of platelets in the formation of a thrombus is the basis for use of antiplatelets in primary treatment. On the other hand, the coagulation cascade plays a more prominent role in low blood flow conditions (venous thrombosis) and anticoagulant therapy is more appropriate. However, review of hemostasis demonstrates that platelet activation and coagulation cascade play a synergistic role in thromboembolic events. Accordingly, combination therapy with anticoagulants and antiplatelets cannot be ruled out for better prevention of thromboembolic events compared to each therapy alone for high-risk patients. Finally, it is important to note that antithrombotic therapy represents a double-

edged sword such that greater prevention of thrombosis is also associated with greater risk of bleeding. Thus, delivering optimal antithrombotic therapy to individual patients at risk for thrombosis will not only augment therapeutic prevention of thromboembolic events but also minimize drug-related hemorrhagic risk.

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2 PHARMACOGENETIC ADVANCES IN CARDIOVASCULAR MEDICINE: RELEVANCE TO PERSONALIZED MEDICINE[1]

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2.1 Introduction

Clinical trials have clearly demonstrated the therapeutic benefit for many cardiovascular agents; however, some patients, even on the same dose, exhibit loss of efficacy or higher risk of toxicity. We know that while an average dose of a medication can benefit a large proportion of the patient population, the one-size-fits-all dosing regimen disregards the importance of identified variation in drug metabolism, transport, and response pathways known to exist in any given population. Not surprisingly, preventable adverse drug reactions (ADRs) often occur in subsets of susceptible patients. Interindividual differences in drug response are multifactorial and may be explained by an array of factors including environmental, genetic, comorbidities, and drug-drug interactions.

Advances in the field of pharmacogenetics over the past decade have dramatically improved our understanding of the impact of genetic variability to observed variation in drug response and toxicity. Accordingly, we now have the capability to use an individual genetic makeup in combination with clinical variables to choose appropriate drug therapy and dosages in an *a priori* fashion. Therefore, the field of pharmacogenetics has significant implication for optimizing cardiovascular drug therapy, considering the large proportion of patients with cardiovascular disease requiring pharmacotherapy.

Associations of genetic variations with drug response largely fall within two categories (Figure 2.1): and polymorphisms that affect drug pharmacokinetics by introducing variability in systemic drug exposure; polymorphisms that affect pharmacodynamics by affecting the drug's ability to act at the target site. Not surprisingly, for the most part, candidate gene approaches have been used to identify single nucleotide polymorphisms (SNPs) in genes suspected to influence variation in response. On the other hand, genome-wide association studies (GWAS) for drug response represent an unbiased screen across the entire genome and may inform novel pathways of relevance to ADRs or therapeutic response.

We now see a number of pharmacogenetic linkages for a number of medications, some of which have resulted in FDA label changes to incorporate guidance on consideration of genetic information during treatment. The present review highlights the genetic determinants of commonly used cardiovascular drugs (Table 2.1), and how the use of such information may enhance patient care in a clinical setting.

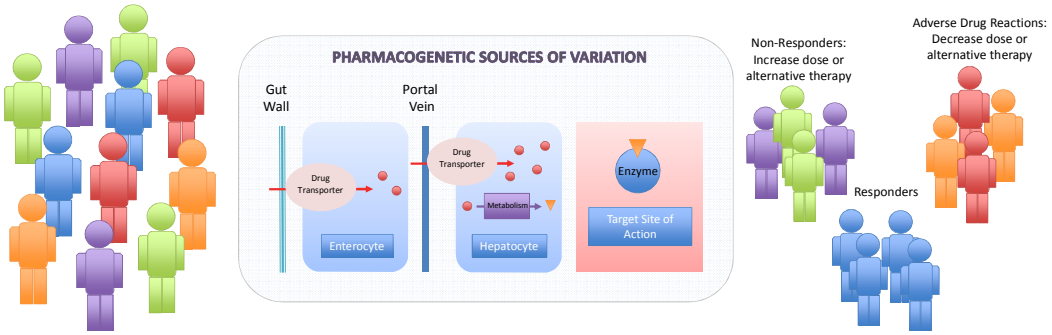

Figure 2.1 Pharmacogenetic determinants of interindividual variability in cardiovascular therapy.

Genetic variability in drug transporter and metabolizing enzymes expressed at the level of enterocytes and hepatocytes affect pharmacokinetics and overall disposition. Genetic variability in the gene(s) encoding the drug's pharmacological target affects pharmacodynamics. Identified genetic variants confer altered drug sensitivity, response and toxicity, allowing classification of patients as responders or nonresponders.

Table 2.1.1 Summary of current evidence in cardiovascular pharmacogenetics.

Drug	Drug Class	Description	Gene(s)	Category	Problem	Clinical Implementation
Warfarin	Anti-coagulant	Variation in dose requirement for achieving and maintaining INR in therapeutic range	CYP2C9, VKORC1	PK, PD	CYP2C9*2, CYP2C9*3, VKORC1 -1639G>A carriers have increased sensitivity to warfarin	Dose reduction for loss-of-function variant carriers
Clopidogrel	Anti-platelet	Variation in antiplatelet response leading to resistance in a subset of patients	CYP2C19	PK	CYP2C19*2 and CYP2C19*3 carriers have insufficient active metabolite formation leading to resistance	Dose increase or consider newer antiplatelet agents for loss-of-function variant carriers

CYP, cytochrome P450; PD, pharmacodynamics; PK, pharmacokinetics; VKORC1, vitamin K epoxide reductase subunit 1.

2.2 Oral Anticoagulants

2.2.1 Warfarin

The vitamin K antagonist warfarin is a common oral anticoagulant prescribed in North America for stroke prevention in atrial fibrillation (AF) patients and thromboembolism prophylaxis in venous thromboembolism (VTE) (1). However, warfarin therapy is particularly challenging due to marked and often unpredictable interindividual dosing variation (up to 20-fold) to reach and maintain adequate anticoagulation. Not surprisingly, its clinical use is associated with ADRs, mainly in the form of bleeding events; in fact, warfarin was recently reported to account for one-third of hospitalizations in the elderly (2). For most indications, optimal warfarin therapy is achieved by maintaining the international normalized ratio (INR) within a narrow therapeutic range of 2-3. An insufficient warfarin dose leads to a lack of antithrombotic effect while over-anticoagulation is associated with elevated bleeding risk. Aside from demographic (age, gender, weight) and clinical variables (renal or hepatic disease, diet, drug-drug interactions), pharmacogenetics explains a large portion of the observed variability in warfarin dose requirement (3).

2.2.1.1 CYP2C9

Warfarin is administered as a racemic drug; *S*-warfarin is 3-5 times more potent than *R*-warfarin. The clearance and thus pharmacokinetics of warfarin is largely dependent on the metabolic pathways of each enantiomer. While CYP1A2, CYP3A4, and CYP2C19

are enzymes that convert the *R*-warfarin to its inactive metabolite in varying extents, CYP2C9 is the primary enzyme responsible for metabolism of *S*-warfarin. As such, candidate gene studies have consistently shown that *CYP2C9* polymorphisms significantly affect warfarin sensitivity (4). In particular, common *CYP2C9**2 and *3 variant alleles result in decreased enzymatic activity (30 and 90 % reduction, respectively) compared to the wild-type allele (5). The clinical implication for these SNPs is lower therapeutic dose requirement, increased time to stable anticoagulation and increased bleeding risk due to greater rate of over-anticoagulation (6). A large meta-analysis of 39 studies (n = 7,907) demonstrated that maintenance dose for *CYP2C9**2 and *3 homozygous patients were 36 and 78 % lower, respectively, as compared to wild-type patients (7).

2.2.1.2 VKORC1

S-warfarin inhibits the vitamin K epoxide reductase, encoded by *VKORC1*, the enzyme responsible for recycling oxidized vitamin K to the reduced form, an essential cofactor for γ-glutamyl-carboxylase carboxylation (GGCX) in clotting factor II, VII, IX and X activation (8). Common genetic variants in *VKORC1* result in altered warfarin sensitivity while rare polymorphisms result in warfarin resistance (9). Of note, the common promoter SNP (*VKORC1* -1639G>A, rs9923231) is thought to be the causative variation responsible for the greater warfarin sensitivity, resulting in lowered dose requirement (10).

2.2.1.3 Other SNPs

In addition to *CYP2C9* and *VKORC1* polymorphisms, SNPs in other genes have been studied as potential contributors to warfarin response. These genes include *GGCX*, calumenin (*CALU*), apolipoprotein E (*ApoE*) as well as multidrug resistance protein (*ABCB1*) (11). However, the impact of these polymorphisms on warfarin response has generally been minimal or none at all. An exception to this is the growing importance of the *CYP4F2* 1297C>T genotype, whereby several retrospective studies have demonstrated that variant T carriers (rs2108622) require 1 mg more than wild-type C carriers (12). Moreover, CYP4F2 is the metabolizing enzyme of vitamin K accounting for the pharmacological basis of the dose difference (13). However, *CYP4F2* genotype only accounts for a small portion of the observed maintenance dose variability (0-4%) (14). These findings were confirmed by recent GWASs, where polymorphisms in *CYP2C9* and *VKORC1* were the only genetic markers identified to influence warfarin dosing, while the *CYP4F2* genotype was only significant after adjusting for *CYP2C9* and *VKORC1* (15).

2.2.1.4 Clinical Applicability of SNPs

*CYP2C9**2, *CYP2C9**3 and *VKORC1* -1639G>A variant carriers are at an increased risk of over anticoagulation (INR > 4) and bleeding as well as delayed time to therapeutic efficacy. Based on these findings, the FDA approved a new label for warfarin in 2007 advising physicians to consider pharmacogenetic testing for patients requiring warfarin. Many dosing algorithms have been developed to predict warfarin dose requirement using

CYP2C9, *VKORC1*, and *CYP4F2* SNPs and clinical parameters (16). The majority of these studies have focused on the effect of genetic variation on warfarin dose during maintenance phase of anticoagulation. However, warfarin initiation is arguably the most challenging therapeutic phase where risk of hemorrhage and recurrent VTE are greatest (17). Moreover, we and others demonstrated that *CYP2C9* and *VKORC1* modulate wafarin response even during early anticoagulation (18).

2.2.1.5 Clinical Implementation

There is a strong association between *CYP2C9* and *VKORC1* SNPs with warfarin response and dose. However, the widespread use of pharmacogenetics-based warfarin dosing remains has not yet been achieved, in part related to concerns regarding costs associated with pharmacogenetic testing and additional clinical evidence to support superiority of a pharmacogenetics-based approach. Recently, the COUMAGEN-II trial showed clear superiority of pharmacogenetics-based warfarin dosing over standard care with respective to time spent in therapeutic range and reduced occurrence of ADRs (19). Additionally, a recent large scale community-based study found that genotyping during warfarin therapy reduced the hospitalization rate for bleeding or thromboembolic event by 30 % compared to a historical control group (20). A number of RCTs (www.clinicaltrials.gov; COAG, GIFT, EU-PACT) involving larger sample sizes are currently underway to more fully confirm such findings focusing on safety and efficacy of pharmacogenetics-based dosing compared to standard dosing. Given the extent of supportive evidence to date for warfarin, pharmacogenetics-based warfarin dosing is

likely to be widely implemented, particularly given the rapid improvement in genotyping technologies that has resulted in greater accuracy, turn-around time, and lower cost.

2.2.2 New oral anticoagulants: dabigatran, rivaroxaban, and apixaban

Dabigatran, a direct thrombin inhibitor, and factor Xa inhibitors rivaroxaban and apixaban, are new oral anticoagulants recently approved for AF and VTE. Dabigatran etexilate is a pro-drug, requiring bioactivation to active dabigatran by esterases while rivaroxaban undergoes metabolism predominantly by CYP2J2 and CYP3A4 (21). Interestingly, all agents are substrates of P-glycoprotein (ABCB1) while rivaroxaban and apixaban are also substrates of breast cancer resistance protein (BCRP) (22). Very recently, a genome-wide subanalysis of the RE-LY trial demonstrated that a common variant in the carboxylesterase 1 (*CES1*) gene (rs2244613) was associated with dabigatran-related bleeding events. The polymorphism is thought to attenuate dabigatran formation resulting in lowered systemic exposure and reduced bleeding risk, indicating the potential for pharmacogenetics-based dosing. Further studies are needed to confirm these findings in addition to identification of additional genetic variations in candidate genes such as *ABCB1* and *BCRP* to determine SNPs capable of modulating the efficacy and toxicity of these new agents.

2.3 Antiplatelets

2.3.1 Aspirin

Aspirin is commonly prescribed for prevention of cardiovascular events. Aspirin irreversibly binds and inactivates cyclooxygenase (COX) 1 and 2 in platelets, and thereby reduce platelet aggregation (23). The term of aspirin resistance has been coined to note the occurrence of cardiovascular events, presumably related to suboptimal antiplatelet inhibitory effect in patients prescribed with therapeutic doses of aspirin. However, classification of aspirin resistance has been highly controversial with prevalence of 5 – 60 % depending on the population and *ex vivo* response measurement used; a patient defined as a non-responder in one test would be normal in another (24). Therefore, it has been challenging to interpret the relevance of genetic markers associated with aspirin response.

2.3.1.1 PTGS1

Since COX1 is the direct pharmacological target of aspirin, considerable amount of focus has been on the COX1 encoding gene, *PTGS1*. However, results of such studies have been variable. Although genetic polymorphisms in this gene have been linked to greater platelet aggregation in some studies, many others failed to replicate such an effect or find an association with cardiovascular events such as myocardial infarction (MI) (25).

2.3.1.2 Other SNPs

Several other genes encoding platelet activation pathway proteins have been linked to aspirin antiplatelet response. The *ITGB3* gene encodes GPIIIa protein and carriers of the risk allele (rs5918) appear to confer elevated risk of MI, arterial and venous thrombosis. However, other studies in this regard have been conflicting (25). A recent study found carriers of an intronic SNP in the *PEAR1* gene (rs12041331) corresponded to higher aspirin response (26). Additionally, a SNP in the *LPA* gene (rs3798220) determined plasma levels of apolipoprotein A and associated with differential aspirin efficacy in a large placebo-controlled trial (27).

2.3.1.3 Clinical Implementation

The extent of clinically relevant genetic markers and their role to aspirin response remain to be defined. The lack of consistent associations indicates it may be premature to include genetic testing for aspirin therapy, at least based on currently published genetic markers. It addition, a key impediment has been the lack of a standardized antiplatelet response measurement assay for aspirin prescribed patients.

2.3.2 Clopidogrel

Antiplatelet therapy with thienopyridine is an important therapeutic intervention for the prevention of ischemic events in patients with high-risk cardiovascular disease, particularly for those undergoing percutaneous coronary intervention (PCI) (28). Clopidogrel is the most widely prescribed thienopyridine known to exert its pharmacological effect by irreversibly binding to $P2Y_{12}$ receptors on platelets, thereby diminishing platelet aggregation (29). Although benefits from clopidogrel has been widely documented in large clinical trials, marked interpatient variation in platelet responsiveness has meant that 21 % of patients (termed nonresponders) remain at risk for coronary artery and stent thrombosis (30).

2.3.2.1 CYP2C19

Clopidogrel is a prodrug and its clinical efficacy is a function of the amount of enzymatically derived active thiol metabolite formed (H4) (31). While most of the prodrug undergoes hydrolysis to an inactive metabolite, clopidogrel bioactivation is a two-step process catalyzed by several CYP isozymes (32). Both metabolic steps leading to H4 formation have been shown to be predominantly dependent on CYP2C19, and to a lesser extent CYP3A4 (32).

A number of studies have examined the influence of genetic variation in CYP enzymes on clopidogrel antiplatelet response. The most consistent finding is that *CYP2C19**2 (rs4244285) and *CYP2C19**3 (rs4986893) loss-of-function SNPs results in lower platelet inhibition, high on-treatment platelet reactivity and consequently, an increased risk of major cardiovascular events, particularly stent thrombosis in PCI patients (33). A meta-analysis including nine studies (n = 9,685) reported a gene-dose effect for variant carriers; one reduced function allele carrier had a hazard ratio of 1.57 (95 % confidence interval, 1.13-2.16) for a composite of cardiovascular endpoints while carriers of two reduced function alleles had an even greater risk (hazard ratio 1.76, 1.24-2.5) (33). Another common *CYP2C19* polymorphism (*17, rs3758581) results in increased enzyme activity, greater efficacy, and better cardiovascular outcomes at the expense of increased bleeding risk (34). These findings prompted the FDA to update the clopidogrel label with a boxed warning for cautious use amongst *CYP2C19* reduced function carriers.

2.3.2.2 ABCB1

Clopidogrel has been demonstrated to be a substrate of P-glycoprotein, which limits the extent of its bioavailability. Not surprisingly, the *ABCB1* 3435C>T SNP (rs1045642) has been shown to result in lower peak concentrations of clopidogrel and its active metabolite (35). However, the definitive role of this SNP on clopidogrel antiplatelet response remains controversial. While Mega et al. reported that the elevated cardiovascular risk of *CYP2C19* variant allele carriers was accentuated in presence of homozygous 3435C>T genotype, other studies could not replicate this finding (36, 37).

2.3.2.3 $P2Y_{12}$

Clopidogrel exerts its pharmacodynamics effects by inhibition of $P2Y_{12}$ receptor on platelets. Recently, Ziegler et al. reported a fourfold increase in cerebrovascular events in patients possessing the 34C>T (rs6809699) variant while another variant, 52G>T (rs6785930), confers clopidogrel resistance following PCI (38). However, other studies have failed to show the same trend for antiplatelet response and cardiovascular events (39).

2.3.2.4 Other SNPs

CYP2C19 undoubtedly predicts cardiovascular outcomes in patients treated with clopidogrel following PCI, yet it is thought to represent only a portion of the observed variation in antiplatelet response. A GWAS demonstrated that clopidogrel platelet aggregation was highly heritable ($r^2 = 0.73$) in an Amish population (40). However, the *CYP2C19**2 SNP only accounted for 12 % of the interindividual variation, indicating that other genetic markers affecting efficacy remain to be identified to explain the large heritable component of clopidogrel response. More recently, the Scripps Clinic conducted whole genome sequencing in 392 patients on clopidogrel and found two novel genes *ATP2B2* and *TIAM2* to predict poor platelet response (unpublished data).

2.3.2.5 Clinical Implementation

Clopidogrel resistance and the lack of adequate therapeutic efficacy is a major concern, thus identification of resistant patients prior to treatment is of great value for favorable outcomes. Although there is ample evidence linking *CYP2C19* with cardiovascular outcomes in PCI patients, RCTs assessing the benefit of genotyping for CYP2C19 over standard care has yet to be conducted. One recent RCT using point-of-care *CYP2C19**2 genotyping showed that such genetic information led to better prediction of antiplatelet response in a real world setting, indicating that personalized clopidogrel therapy may be effective in improving efficacy (41). Accordingly, a number of institutions (Vanderbilt University Medical Centre, Scripps Clinic) adopted routine *CYP2C19* genotyping for patients undergoing PCI (42). Conversely, although several small prospective trials have restored diminished H4 exposure and poor antiplatelet response in *CYP2C19* variant carriers by increasing clopidogrel loading and maintenance dose (43), recent larger studies failed to overcome the reduced antiplatelet response in individuals harboring *CYP2C19**2 variant alleles (44). This indicates that clopidogrel potency at the $P2Y2_{12}$ receptor is likely inadequate for *CYP2C19* variant carriers. Accordingly, the Clinical Pharmacogenetics Implementation Consortium suggested that newer antiplatelet agents with greater affinity and potency (prasugrel and ticagrelor) should be considered for carriers of one or two loss-of-function variant alleles (45). Indeed, *CYP2C19**2 carriers were more likely to switch to prasugrel due to high on-treatment platelet reactivity (46). Current ongoing studies include the GIANT trial which will assess the clinical efficacy of using high-dose clopidogrel for *CYP2C19**2 carriers and the RAPID-STEMI trial that

will evaluate efficacy of either high-dose clopidogrel or switching to prasugrel for *CYP2C19**2 and *ABCB1* 3435TT carriers.

2.3.2.6 New Antiplatelets: Prasugrel and Ticagrelor

Neither agents require extensive bioactivation by CYP2C19. Thus, *CYP2C19* genetic variation has not been demonstrated to affect cardiovascular outcomes in subanalysis of the TRITON-TIMI (prasugrel) or the PLATO (ticagrelor) trial (47). However, we note that CYP3A4 and CYP2B6 are responsible for bioactivation of prasugrel to its active form, and the influence of polymorphisms in these enzymes to prasugrel platelet efficacy remains to be delineated. An important consideration of prasugrel use over clopidogrel is that, although it proved more efficacious than clopidogrel, this is at the price of increased bleeding risk. Similarly, ticagrelor use over clopidogrel is complicated by the finding that ticagrelor was as effect as clopidogrel across all international centres of PLATO trial except for North America.

2.4 Conclusions

Interindividual variation in drug exposure and efficacy lead to subsets of patients experiencing drug-related toxicities or inadequate therapeutic benefit. The one-dose-fits-all paradigm for drug therapy is increasingly recognized as inappropriate for many drugs in clinical use. Alternative paradigm for pharmacotherapy pertains to individualized

therapies. In the past decade, substantial progress has been made linking commonly occurring genetic variants to cardiovascular treatment response. Despite this, pharmacogenetics-guided care is still in its infancy, as most centres have not implemented this approach to routine clinical practice. However, as the cost of genotyping continues to decline while the extent of clinical evidence supporting pharmacogenetics-based approach rises, there is little doubt that implementation of pharmacogenetics-guided personalized medicine will result in reduced incidence of ADRs, and greater likelihood of therapeutic benefit. Indeed, for drugs where pharmacogenetics-guided care has been shown to be noninferior, and potentially superior to standard care, such an approach should be implemented and further studied in a prospective fashion to show the overall merit of pharmacogenetics to enhancing patient care, reducing ADRs and health care costs in the real-world setting. We also recognize that, for many drugs, clinical decision support algorithms that integrate patient-specific pharmacogenetic data along with environmental and clinical variables will be essential to realizing the promise of personalized medicine.

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3 SPECIFIC AIMS AND HYPOTHESES

3.1 Specific aim 1

1. **To develop a novel pharmacogenetics-based initiation protocol that incorporates loading and maintenance doses calculated based on individual patient genetics, clinical variables, and anticoagulation response.**
2. **To evaluate the clinical utility of the novel initiation protocol in a prospective cohort of atrial fibrillation (AF) and venous thromboembolism (VTE) patients.**

Variable warfarin response poses a significant challenge to providing optimal anticoagulation therapy. Single nucleotide polymorphisms in genes that affect warfarin metabolism (cytochrome P450 2C9 gene, *CYP2C9*) and response (vitamin K epoxide reductase complex 1 gene, *VKORC1*) have an important influence on warfarin responsiveness, particularly during initiation. A number of algorithms have been proposed which incorporate genetics as well as clinical parameters to predict individualized maintenance dose with the intent of improving warfarin anticoagulation therapy (1-3). However, there is a paucity of information with respect to optimal pharmacogenetics-based warfarin initiation, arguably the most clinically challenging therapeutic phase where the risk of haemorrhage and recurrent thromboembolism is greatest (4-7). Accordingly, the use of a pharmacogenetics-based warfarin initiation algorithm may prove useful in maximizing therapeutic efficacy while minimizing bleeding risk during this critical period by diminishing interindividual variation in response.

We hypothesized that the use of a novel pharmacogenetics-based dosing algorithm for initiating patients requiring new anticoagulation therapy should effectively eliminate genotype-driven differences in anticoagulation response to provide a safe, rapid, and uniform anticoagulation response in AF and VTE patients. To test this hypothesis, we conducted a prospective cohort study in which patients requiring warfarin therapy for AF or VTE were initiated with a novel pharmacogenetics-initiation protocol (WRAPID, Warfarin Regimen using A Pharmacogenetics-guided Initiation Dosing) that incorporates loading and maintenance doses based on genetics, clinical variables, and response (n = 167, followed up for 90 days), to assess the influence of genetic variations on anticoagulation responses. As described in Chapter Four, application of the WRAPID algorithm resulted in negligible influence of genetic variation in *VKORC1* or *CYP2C9* on time to achievement of therapeutic response as measured by international normalized ratio (INR), risk of overanticoagulation, and time to stable anticoagulation. Overall, we demonstrate the clinical utility of genetics-guided warfarin initiation with the WRAPID protocol to provide safe and optimal anticoagulation therapy for patients with AF or VTE.

3.2 Specific aim 2

To elucidate the genetic and nongenetic determinants of interindividual variability in warfarin pharmacokinetics and pharmacodynamic responses.

Many of the factors influencing the required warfarin maintenance dose such as age, body surface area, drug interactions and importantly, *CYP2C9* genotype relate to their effects on *S*-warfarin pharmacokinetic (PK) parameters, such as volume of distribution and clearance (1, 8-10). However, the influence of genetics and clinical parameters on *S*-warfarin pharmacodynamics (PD) variability is less clear.

We hypothesized that genetic variation in *CYP2C9* and *VKORC1*, as well as clinical variables contribute to interindividual variation in warfarin PK and PD parameters. To test this hypothesis, we investigated the determinants of warfarin kinetics and responses during initiation of warfarin therapy in the cohort of 167 patients. During the first nine days of treatment with pharmacogenetics-guided dosing, *S*-warfarin plasma levels and INR were obtained to serve as inputs to a pharmacokinetic-pharmacodynamic (PK-PD) model. Individual patient PK (*S*-warfarin clearance) and PD (I_{max}) parameter values were estimated. As described in Chapter Five, regression analysis demonstrated that *CYP2C9* genotype, kidney function, and gender were independent determinants of *S*-warfarin clearance while the estimated I_{max} variability was dependent on *VKORC1* and *CYP4F2* genotypes, vitamin K status, indication for warfarin, and weight.

3.3 Specific aim 3

To determine the efflux transporters expressed at the apical side of renal tubular cells capable of secreting rivaroxaban into the urine.

Rivaroxaban is a new oral anticoagulant (factor Xa inhibitor), recently approved for the treatment and prevention of thromboembolic diseases. However, adverse events associated with its use, namely bleeding risk, continue to be an important concern, particularly in patients with renal impairment. Rivaroxaban disposition is governed by hepatic metabolism and renal excretion, whereby 30-40% of the administered rivaroxaban is excreted unchanged through the kidney via a combination of glomerular filtration and active tubular secretion. The renal excretion is greater than glomerular filtration rate, suggesting a significant contribution of active transport processes to rivaroxaban elimination (11). Indeed, tubular secretion is the predominant pathway as the ratio of active tubular secretion to glomerular filtration of unchanged rivaroxaban was estimated to be 4-to-1 in a population pharmacokinetics model (11). Furthermore, there has been a growing appreciation of drug transporters expressed in various tissues in determining the disposition and excretion of a wide range of clinically used drugs.

We hypothesized that the efflux transporters P-glycoprotein (MDR1) and breast cancer resistance protein (BCRP) are capable of transporting rivaroxaban and contribute to the overall disposition of rivaroxaban. As described in Chapter Six, the

ability of MDR1 and BCRP efflux transporters to mediate rivaroxaban transport *in vitro* was assessed in polarized cell monolayers. Indeed, rivaroxaban is a shared substrate of MDR1 and BCRP. Following oral administration of rivaroxaban (2 mg/kg), plasma concentrations did not significantly differ between wild-type and $Mdr1a^{def}$ or $Bcrp^{-/-}$ mice (n = 6 per group). However, rivaroxaban clearance was significantly reduced in $Mdr1a/Mdr1b^{-/-}/Bcrp^{-/-}$ mice. Interestingly, rivaroxaban brain to plasma ratio did not differ in mice lacking only Mdr1a or Bcrp, but was more than two times higher in the $Mdr1a/Mdr1b^{-/-}/Bcrp^{-/-}$ mice. Our results demonstrate that MDR1 and BCRP function synergistically to modulate rivaroxaban disposition and appear to be particularly relevant to limiting its central nervous system entry. As clinically relevant polymorphisms exist in both MDR1 and BCRP, genetic variations in these efflux transporters may play an important role in determining rivaroxaban exposure and anticoagulation efficacy.

3.4 Specific aim 4

To systematically elucidate the mechanism and relative contribution of PON1 in comparison to CYP2C19 to clopidogrel bioactivation and antiplatelet response.

The marked interindividual variation in clopidogrel antiplatelet responsiveness results in a subset of patients at sustained risk for atherothrombosis. It is thought that antiplatelet response to clopidogrel is highly heritable, but the precise genetic determinants of its

metabolism and response remain controversial. Polymorphisms in cytochrome P450 2C19 (*CYP2C19*) have been correlated with clopidogrel antiplatelet response and clinical outcomes in a number of cohort studies and clinical trials, accounting for 12% of interindividual variation. However, a recent study challenged this notion by proposing *CYP2C19* as wholly irrelevant, while identifying paraoxonase-1 (*PON1*) and its Q192R polymorphism as the major driver of clopidogrel bioactivation and efficacy. Given the breadth of data supporting the importance of CYP2C19 to clopidogrel bioactivation and clinical outcomes, additional studies are required to evaluate the validity and relevance of these findings.

We hypothesized that both CYP2C19 and PON1 contribute to clopiodgrel metabolism and antiplatelet response at varying extents. To test this hypothesis, we administered a single 75 mg dose of clopidogrel to a cohort of healthy subject (n = 21) and assessed the influence of *CYP2C19* and *PON1* polymorphisms and plasma paraoxonase activity on clopidogrel active metabolite (H4) plasma levels and antiplatelet response. As described in Chapter Seven, *CYP2C19* but not *PON1* genotype was predictive of H4 levels and antiplatelet response. Moreover, metabolic profiling of clopidogrel *in vitro* confirmed the role of *CYP2C19* in bioactivating clopidogrel to H4. Conversely, PON1 cannot generate H4, but mediates the formation of another thiol metabolite, termed Endo. Our results demonstrate that *PON1* does not contribute to clopidogrel active metabolite formation or antiplatelet action, while *CYP2C19* activity

and genotype remains a predictor of clopidogrel pharmacokinetics and antiplatelet response.

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4 PROSPECTIVE EVALUATION OF A PHARMACOGENETICS-GUIDED WARFARIN LOADING AND MAINTENANCE DOSE REGIMEN FOR INITIATION OF THERAPY[2]

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4.1 Introduction

The vitamin K antagonist warfarin is an oral anticoagulant commonly prescribed in North America to treat venous thromboembolism (VTE) and decrease the risk of stroke in atrial fibrillation (AF) (1). Warfarin therapy is challenging because of marked and often unpredictable interindividual dosing variation to reach and maintain adequate anticoagulation. For most indications, optimal warfarin therapy is achieved by maintaining the international normalized ratio (INR) within a narrow therapeutic range of 2.0 to 3.0. An insufficient warfarin dose leads to a lack of antithrombotic effect, whereas overanticoagulation is associated with elevated bleeding risk (2).

Although warfarin has been in use for the past 60 years, genetic control of warfarin response has only recently been appreciated (3). In this regard, among the most studied genetic determinants are the single-nucleotide polymorphisms (SNPs) in genes that encode cytochrome P450 2C9 (CYP2C9) (4), and vitamin K epoxide reductase complex subunit 1 (VKORC1) (5, 6). CYP2C9 is the primary enzyme responsible for metabolism of the active *S*-enantiomer of warfarin, and its polymorphisms contribute significantly to variability in warfarin response (7). Possession of the common CYP2C9*2 (c.430C>T, rs1799853) and *3 (c.1075A>C, rs1057910) variant alleles results in lower dose requirement, increased time to stability and a higher risk of overanticoagulation (8). VKORC1 is the target of warfarin that recycles oxidized vitamin K to the reduced form, an essential cofactor for activation of clotting factors II, VII, IX, and X, through γ-glutamyl carboxylation (9). Harboring common genetic variants of VKORC1, such as the

functional promoter SNP -1639G>A (rs9923231), results in enhanced warfarin sensitivity, whereas rare mutations have been linked to warfarin resistance (5, 6, 10). In addition to VKORC1 and CYP2C9 polymorphisms, several studies have reported recently that a functional SNP in CYP4F2 (c.1297G>A, rs2108622), the metabolizing enzyme for vitamin K (11), also determines warfarin dose requirement (12).

Since the Food and Drug Administration revised the label for warfarin to note the importance of VKORC1 and CYP2C9 polymorphisms (13), several groups have proposed genotype-guided maintenance dose algorithms that incorporate both genetics and demographic parameters, such as age, weight, and body surface area (14, 15). However, there is a paucity of information with respect to dosing during warfarin initiation, arguably the most clinically challenging therapeutic phase, during which the risk of hemorrhage and recurrent thromboembolism is greatest (16, 17). Standardized loading dose nomograms developed to date have not considered genetics and other patient-specific characteristics and have not been applied to indications other than VTE, such as AF (18-20). We and others have recently shown that VKORC1 and CYP2C9 genetic variations modulate early and stable response to warfarin during initiation when dosing by traditional means is used (21-24). Thus, in the present, a novel and practical VKORC1- and CYP2C9-based loading and maintenance dose algorithm (WRAPID, Warfarin Regimen using A Pharmacogenetics-guided Initiation Dosing protocol) was developed and evaluated in AF and VTE patients with the aim of providing a safe, rapid, and uniform anticoagulation response.

4.2 Experimental Section

4.2.1 Study sample and eligibility

This was a prospective cohort study of outpatients conducted at the London Health Sciences Center (LHSC) and The Ottawa Hospital (TOH). The study was approved by research ethics boards at both institutions. Patients requiring initiation of warfarin therapy were screened for eligibility. The requirement for warfarin therapy was determined on the basis of current American College of Chest Physicians guidelines (1). Patients who met the eligibility criteria were enrolled on provision of written informed consent in accordance with the Declaration of Helsinki. Table 4.1 summarizes patient characteristics.

Study eligibility was determined by the following inclusion criteria: (1) at least 18 years of age, and (2) indication for new warfarin therapy for at least 3 months with a target INR range of 2.0 to 3.0. Exclusion criteria were diagnosis of cancer other than nonmelanoma skin cancer, alcohol or drug abuse, baseline INR > 1.4, known warfarin allergy/intolerance, terminal disease, prior use of warfarin therapy or vitamin K within 2 weeks before study enrolment, and known or suspected pregnancy.

Table 4.2.1 Patient characteristics (n=167).

Demographics	
Age, y	60 ± 17
Sex, male/female, n	96/74
Weight, kg	84 ± 19
Height, cm	171 ± 10
Ethnicity	
White	159 (95.2)
Black	4 (2.4)
Asian	3 (1.8)
Other	1 (0.6)
Prescribed medication	
Amiodarone	2 (1.2)
Statins	45 (26.9)
Antiplatelets	55 (32.9)
Antibiotics	6 (3.6)
Antifungals	1 (0.6)
NSAIDs	12 (7.2)
Indication for warfarin	
Atrial fibrillation	61 (36.6)
Deep vein thrombosis	77 (46.1)
Pulmonary embolism	21 (12.6)
Other	8 (4.7)
Prescribed warfarin dose	
Mean maintenance dose (mg/day)	5.54
CYP2C9	
*1/*1	119 (71.3)

*1/*2	29 (17.4)
*1/*3	14 (8.4)
*2/*2	3 (1.7)
*2/*3	2 (1.2)
*3/*3	0
VKORC1 -1639	
G/G	66 (39.5)
G/A	75 (44.9)
A/A	26 (15.6)
CYP4F2 c.1297	
G/G	80 (48.6)
G/A	68 (40.7)
A/A	19 (11.3)

Values are mean ± SD or n (%), unless otherwise indicated.

NSAIDs indicates nonsteroidal anti-inflammatory drugs.

4.2.2 Clinical data collection and follow-up

Demographic information was obtained at the time of enrolment. Therapy related information was collected by patient interview and review of medical records. A baseline venous blood sample was obtained for DNA extraction and assessment of INR. Subsequent INR measurements, dose adjustments, adverse events, and therapy-related interventions were recorded at both study sites. The study period was September 2008 to August 2010.

4.2.3 Genotyping

Genomic DNA was isolated with Gentra Puregene or DNA Blood Midi extraction kit according to the manufacturer's protocol (Qiagen, Alameda, CA). Genotype analysis included CYP2C9*2, CYP2C9*3, VKORC1 -1639G>A and CYP4F2 c.1297G>A. At LHSC, genotypes were determined by allelic discrimination with TaqMan drug metabolism genotyping assays using the 7500 RT-PCR System (assay IDs: C__25625805_10, C__27104892_10, C__1329189_10, C__16179493_40; Applied Biosystems, Foster City, CA). At TOH Research Institute, genotypes were determined with the Luminex 200 system (Luminex Corp). Briefly, forward and reverse primers for SNPs of interest were designed to amplify regions surrounding each SNP by standard multiplex PCR protocols. The PCR products were then hybridized with appropriate xMAP carboxylated microspheres at 52°C, followed by analysis with the Luminex 200. Genotyping was generally performed within 24 hours of receiving the baseline blood

sample and used prospectively to determine individualized initiation doses before warfarin commencement for all study subjects.

4.2.4 Mathematical foundation for a novel pharmacogenetics-based initiation protocol

Using historical datasets compiled from Vanderbilt University (n = 297) (24) and TOH (n = 63) (25), we developed a pharmacogenetics-based initiation protocol aimed at providing a uniform anticoagulation response among all patients. The dosing regimen comprises of both a loading and maintenance dose algorithm. Development of the loading and maintenance dose algorithms required the integration of pharmacokinetic (PK) and pharmacodynamic (PD) factors to predict the time-course of warfarin plasma levels and response.

In this mechanistic model, the plasma PK of warfarin was described with a simple 1-compartment model for warfarin distribution of a set volume (V). The time course of estimated plasma *S*-warfarin concentration (C_{plasma}) arose from the interplay between drug absorption in the gut (k_a) and drug elimination via CYP2C9 metabolism (k_e). Values for kinetic parameters for *S*-warfarin were obtained from the literature (26-29). Warfarin metabolism capacity (k_e) is mainly dependent on CYP2C9 genotype; thus, the values for k_e were adjusted based on reported clearance reductions in heterozygous and homozygous variant allele carriers of either CYP2C9*2 or *3 (29). The pooling of

genotypes into 3 subgroups was performed because of the lack of confidence in the accuracy of limited available clearance rates reported in literature for the CYP2C9*2/*2, *2/*3, and *3/*3 genotype groups as a result of low allelic frequencies (29).

Warfarin PD was described by an indirect response model that incorporates the known delay and magnitude of anticoagulation effects after achieving the required plasma concentration (30). In this model, the degree of suppression of vitamin K-dependent clotting factor production is related to the effectiveness of warfarin concentrations to inhibit vitamin K epoxide reductase. Here, the rate of change in INR is modeled with zero-order input (K) and first-order output (k_o) variables. Plasma warfarin levels dictate the inhibition of output response according to classic competitive enzyme inhibition kinetics described by the parameters I_{max} (enzyme content and intrinsic activity) and IC_{50} (drug affinity) (31). I_{max} values corresponding to VKORC1 genotypes were determined on simulation of maintenance drug administration to stable therapeutic coagulation. Depiction and parameters of the PK-PD model are shown in supplemental material, Figure 4.5.

The PK-PD model and corresponding parameter values presented here are preliminary and served only to guide the establishment of a practical WRAPID dosing protocol for various VKORC1 and CYP2C9 genotype combinations. A finalized version of the PK-PD model with data-derived parameter values based on formal modeling of *R/S*-warfarin concentrations and INR measurements obtained in this patient cohort, along with

identification of key nongenetic and genetic determinants of warfarin kinetics and responses, will be published elsewhere.

4.2.5 Loading doses

Practical (5, 7.5, and 10 mg) daily loading doses were prescribed for 2 days and were dependent on VKORC1 and CYP2C9 genotypes (Table 4.2). These doses were used to obtain sufficient warfarin plasma concentrations to reach and maintain optimal anticoagulation response (INR 2.0 – 3.0) with similar rapid initial time-course for all genotype groups (supplemental material, Figure 4.6).

4.2.6 Maintenance doses

To obtain the maintenance dose, key patient clinical parameters that are known to influence warfarin dose requirement along with genetics were combined in a generalized linear regression model (Table 4.3). Briefly, warfarin dose was the dependent variable, the VKORC1 and CYP2C9 genetics-based dose was a constant variable (Table 4.4), and coefficients of independent variables were varied according to the least-squares linear regression method.

After the 2-day loading dose, patients were prescribed the calculated maintenance dose to begin on day 3. On 3 occasions within the first 9 days of therapy (initiation), INR

measurements were obtained (typically days 3, 5/6 and 7/8/9), because this frequency of INR measurements has been shown to be practical and efficacious for anticoagulation management (19). When the INR response at each measurement did not conform to the predicted trajectory based on the mathematical model, the daily maintenance dose was further adjusted according to a treatment day specific dose adjustment nomogram (Table 4.5). Adjusted doses were rounded to either the nearest whole number or 0.5.

To make dosing practical, we devised an automated dose calculator for the initiation phase (supplemental material, Figure 4.7). After initiation, dosing was adjusted by pharmacists at both centers on the basis of a standardized post initiation nomogram. Once patients had obtained 2 therapeutic INRs, dosing was assisted by Dawn AC anticoagulation software (4-S Information Systems Ltd).

Table 4.2.2 Pharmacogenetics-based loading dose grid according to VKORC1 and CYP2C9 genotype.

VKORC1	CYP2C9		
	*1/*1	*1/*2 or *1/*3	*2/*2, *2/*3, *3/*3
G/G	10 mg*	10 mg*	7.5 mg†
G/A	10 mg*	7.5 mg†	5 mg†
A/A	5 mg†	5 mg†	5 mg†

Loading doses are in milligrams.

* Loading dose was adjusted to 7.5 mg for patients with weight < 60 kg.

† Loading dose was decreased by 2.5 mg for patients with weight < 45 kg.

Table 4.2.3 Final multiple linear regression for estimation of maintenance dose.

Predictor Variable	B	Standard error	P in final model
Intercept	-1.46	1.23	0.235
Weight (kg)	0.06	0.01	<0.0001
Genetics-based dose grid (Table 4.4)	1	-	<0.0001
Age, y	-0.05	0.01	<0.0001
Sex, female	-0.90	0.34	<0.01
Amiodarone use, yes	-1.97	1.1	0.07
CYP4F2 c.1297G>A, per allele	0.33	0.25	0.199

Table 4.2.4 Genetics-dependent dose grid for maintenance dose regression.

	CYP2C9		
VKORC1	*1/*1	*1/*2 or *1/*3	*2/*2, *2/*3, *3/*3
G/G	7 mg	5 mg	3.5 mg
G/A	5 mg	4 mg	2.5 mg
A/A	3.5 mg	2.5 mg	1.5 mg

Doses are in milligrams.

Table 4.2.5 Dose adjustment nomogram during initiation.

	INR	Warfarin dose adjustment
Day 3	<1.3	↑ 10%
	1.3 – 1.5	No change
	1.6 – 1.8	↓ 10%
	1.9 - 2.1	↓ 20%
	2.2 – 2.5	↓ 50%
	> 2.5	Hold dose for 1 day, then ↓ 50%
Day 5/6	<1.3	↑ 50%
	1.4– 1.7	↑ 20%
	1.8 – 2.5	No change
	2.6 – 3.0	↓ 20%
	3.1 – 3.9	↓ 50%
	≥ 4.0	Hold dose for 1 day, then ↓ 50%
Day 7/8/9	<1.5	↑ 20%
	1.5– 1.9	↑ 10%
	2.0 – 2.8	No change
	2.9 – 3.5	↓ 10%
	3.6 – 4.0	Hold dose for 1 day, then ↓ 15%
	≥ 4.0	Hold dose, test INR daily until in range (2.0-3.0), then ↓ 25%

↑ indicates increase; and ↓, decrease.

4.2.7 Refinement of loading and maintenance dose algorithm

It was our objective to refine the loading and maintenance dose algorithm after monitoring an initial cohort of patients (n = 87) for application in a final cohort. After the initial cohort, we observed a disproportionate number of out-of-range INR responses in those patients with a high loading dose-to-weight ratio. Thus, we modified the loading dose algorithm to consider weight after the first cohort. For patients who weighed 60 kg who had been given a 10 mg load according to the original loading algorithm, we decreased the dose to 7.5 mg. For patients who weighed 45 kg, all loading doses were decreased by 2.5 mg (7.5 - 5 mg, and 5 mg - 2.5 mg).

Planned optimization of the maintenance dose regression was performed by slight modification of the contribution of clinical parameters to dose, whereas the impact of VKORC1 and CYP2C9 remained unchanged. In univariate analysis, we observed a significant relationship between CYP4F2 c.1297G>A genotype and dose, in which A/A carriers required a 1 mg higher warfarin dose than the wild-type group ($P < 0.05$). Thus, we included CYP4F2 genotype in the final regression model to determine maintenance doses of the final cohort.

4.2.8 Sample size

The WRAPID study was powered to assess the effect of VKORC1 -1639G>A genotype on anticoagulation response, after pharmacogenetics-guided initiation. A study size of

150 patients was estimated to have 80% power to detect a response hazard of 2 at a 2-sided significance level of 0.05, which allowed for a dropout rate of 10%. A hazard ratio (HR) of 2 was chosen on the basis of previously published VKORC1-carrier status hazard risk for primary outcomes.(24) Power analysis was performed with SAS Version 9.2 (SAS Institute).

4.2.9 Primary and secondary outcomes

The primary outcomes of the present study were time to first therapeutic INR and time to first overanticoagulation (INR $\geq$ 4). We choose these primary outcomes because they are critical markers of anticoagulation pace and quality of control. Therapeutic INR was defined as 2.0 – 3.0 for all patients.

Secondary outcomes were time to first stable anticoagulation, time spent in therapeutic range, and time spent above therapeutic range during the first 30 days (prestabilization phase) and after 30 days (stabilization phase). Stable anticoagulation was defined as 2 consecutive in-range INRs, at least 7 days apart, with no dose adjustments. For estimation of time spent in or out of range, we adopted the Rosendaal linear interpolation method to calculate the percentage of time each patient spent within and out of the therapeutic range (32). The difference between one INR value and the subsequent INR value was divided by the number of days elapsed between the 2 measurements to produce the average daily increment or decrement of INR.

Additional secondary outcomes included percentage of patients within range during initiation, percentage of patients with an INR ≥ 5, percentage of time spent above INR ≥ 4, percentage of time spent in therapeutic range, and extended therapeutic range (1.8 - 3.2), as well as average maintenance dose.

The outcomes were selected to assess the influence of VKORC1 genotype on anticoagulation response. Although the present study was not powered to evaluate the individual effects of CYP2C9*2 and *3 genotypes on response, analysis was conducted to compare wild-type and any CYP2C9 variant-carrier status.

4.2.10 Statistical analysis

Patients were divided into 3 groups for VKORC1 and 2 groups for CYP2C9: VKORC1 wild-type, heterozygous, and homozygous carriers of -1639G>A; CYP2C9 wild-type (*1/*1) and a CYP2C9 variant group that included 1 or 2 variant allele carriers (*1/*2, *1/*3; *2/*2, *3/*3 or *2/*3). Hardy-Weinberg equilibrium was assessed for each genotype with the chi-square goodness-of-fit test.

The influence of VKORC1 and CYP2C9 genotype on the primary outcomes was evaluated with survival analysis techniques. Kaplan-Meier plots were used to depict the proportion of subjects without events over time. Comparison between survival curves was conducted by the log-rank test. Unadjusted HR and its 95% confidence interval (CI) between genotype groups were computed. The Cox proportional hazard model was

adopted to adjust for potential confounding effects of age, sex, weight, warfarin dose, amiodarone use, indication, patient cohort, and VKORC1, CYP2C9 and CYP4F2 genotype to obtain adjusted HRs and their 95% CIs. For comparison of differences in outcomes between patients with various VKORC1 statuses, G/G genotype was considered as the reference group, because it is the most warfarin-resistant group. Statistical analysis of Schoenfeld residuals and visual inspection of log-minus-log plots revealed no significant variation from the proportional hazards assumption.

Percentages of time spent in therapeutic range and time spent above the therapeutic range were compared among VKORC1 and CYP2C9 genotype groups with the use of Kruskal-Wallis test followed by Tukey-Kramer posttest or Mann-Whitney test, as appropriate.

All enrolled patients (initial and second cohort) were included for outcome analysis to obtain at least 80% power to detect the association of VKORC1 genotype and response. This was acceptable because there were no statistical differences between the 2 cohorts with respect to primary outcomes of time to first therapeutic INR and time to first INR $\geq$ 4. In addition, potential variations between cohorts because of dosing-regimen modifications were accounted for as a confounding variable in the Cox regression analysis of primary survival data and should not interfere with assessment of genetic variation effects on rate of INR anticoagulation responses. Because no significant differences were observed between the initial and second cohorts for secondary outcomes, both patient groups were combined for secondary analysis.

To assess and compare the predictability of our dosing model, we determined the association between maintenance dose and model-derived dose. The proportion of variance explained was calculated as the R^2 statistic. In addition, we determined the mean absolute error of each model for the same purpose.

A 2-tailed P-value < 0.05 was considered significant for all analyses. Statistical analysis was performed with the use of GraphPad Prism Version 5.0 or SPSS Version 17.0.

4.3 Results

4.3.1 Population characteristics

Of the 196 patients enrolled, 29 were excluded from analysis for the following reasons: 3 because of entry error, 4 because they self-administered the wrong dose, 1 for failure to comply with INR measurements, 14 dropouts, 6 because of incomplete follow-up and 1 because of death (cause not attributed to study participation). Of those included for outcome analysis, 61 and 96 patients were enrolled at the LHSC and TOH, respectively.

The allelic frequencies for VKORC1 -1639G>A and CYP4F2 c.1297G>A were 38.0% and 31.7%, respectively. The CYP2C9*2 and *3 allelic frequencies were 11.1% and 4.8%, respectively. There were no deviations from the Hardy-Weinberg equilibrium.

4.3.2 Time to first therapeutic INR (2.0-3.0) and overanticoagulation (INR ≥ 4)

The primary outcomes were compared in terms of VKORC1 and CYP2C9 genotypes. VKORC1 genotype had no significant effect on time required to reach the first INR within the therapeutic range (P = 0.52) or time required to obtain an INR ≥ 4, according to log-rank test (P = 0.64; Figure 4.1A, 4.1C). Similarly, there was no significant difference between CYP2C9 wild-type and variant genotype for either of these outcomes (P = 0.28 for first INR, P = 0.96 for first INR ≥ 4; Figure 4.1B, 4.1D). Concordant with

these findings, HR estimates for the VKORC1 and CYP2C9 genotype groups were not significantly different from unity before or after adjustment for covariates by Cox regression analysis (Table 4.6). Because outcomes during the first 30 days would be most sensitive to the initiation protocol, we compared the time to first INR ≥ 4 during the first month of therapy among genotype groups.

VKORC1 and CYP2C9 polymorphisms were without influence on this outcome (data not shown). Importantly, these outcomes were not associated with genotype when we considered the first and second cohorts of patients independently (data not shown).

4.3.3 Time to stable anticoagulation

The time to first stable anticoagulation was significantly different between VKORC1 ($P < 0.05$) genotype groups, whereas there were no differences between CYP2C9 groups ($P = 0.37$; Figure 4.2). However, when adjusted for confounding covariates, neither VKORC1 nor CYP2C9 showed a significant influence on time to stability (Table 4.6).

Figure 4.1 The effect of pharmacogenetics-guided dosing on time to primary events. Kaplan-Meier plots represent the lack of association for attainment of first international normalized ratio (INR) within therapeutic range (2.0-3.0) and first above-range INR (INR $\geq$ 4) among VKORC1 (A, C) and CYP2C9 (B, D) genotype groups after initiation with WRAPID nomogram. The statistic in each panel represents the log-rank *P* value for testing the equality of survival functions. WT indicates wild type.

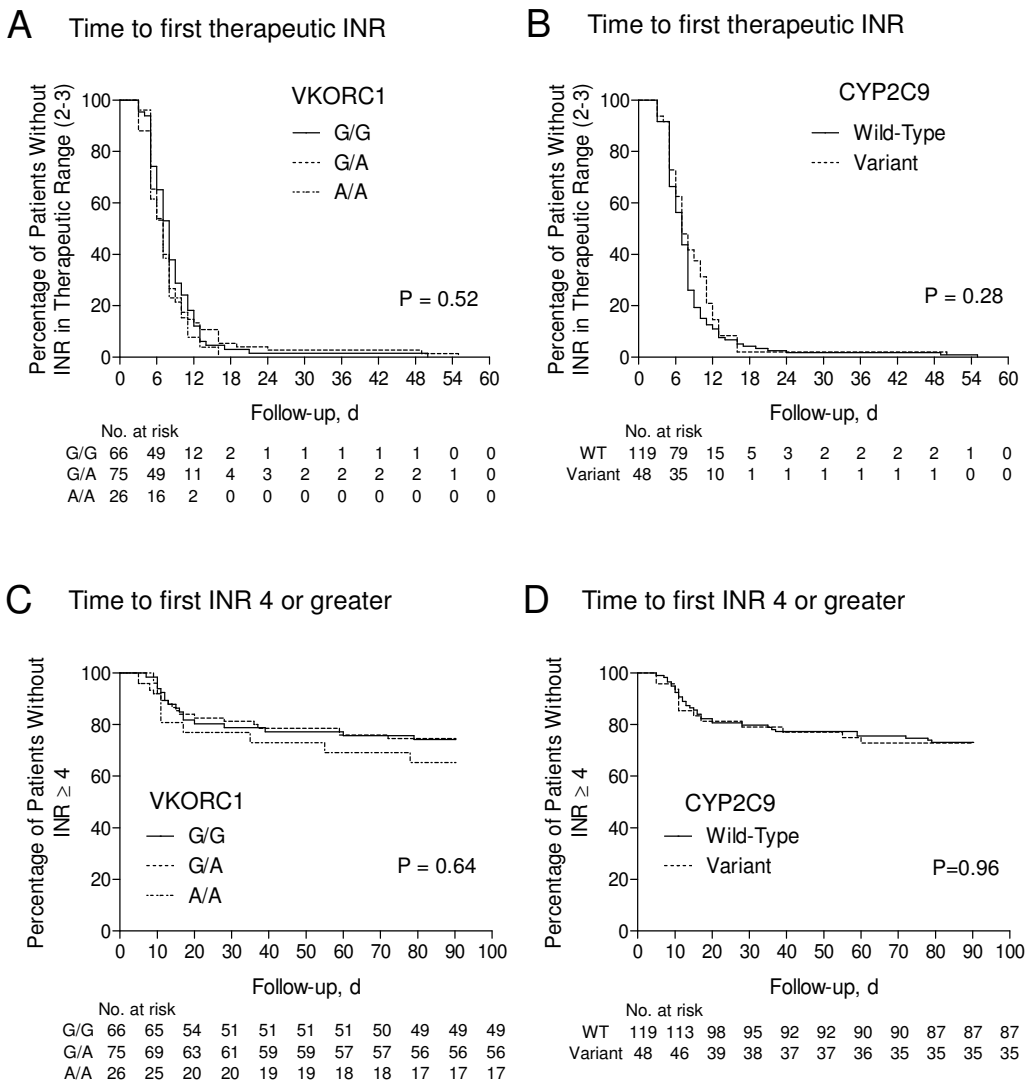

Figure 4.1 The effect of pharmacogenetics-guided dosing on time to primary events.

Table 4.3.1 Unadjusted and adjusted HRs for anticoagulation outcomes in patients with VKORC1 G/A or A/A and CYP2C9 variant genotype.

Genotype and outcome	Unadjusted		Adjusted	
	HR (95% CI)	P	HR (95% CI)	P
VKORC1 G/A genotype*				
Time to first therapeutic INR	0.79 (0.50-1.25)	0.32	0.62 (0.27-1.39)	0.24
Time to first above-range INR	0.71 (0.32-1.59)	0.40	0.58 (0.11-3.19)	0.53
Time to stable anticoagulation	1.03 (0.65-1.63)	0.91	1.11 (0.64-1.90)	0.72
VKORC1 A/A genotype*				
Time to first therapeutic INR	0.87 (0.55-1.37)	0.54	0.76 (0.34-1.57)	0.43
Time to first above-range INR	0.70 (0.32-1.56)	0.39	0.56 (0.14-3.15)	0.60
Time to stable anticoagulation	0.69 (0.43-1.10)	0.12	0.8 (0.47-1.37)	0.41
CYP2C9 variant genotype†				
Time to first therapeutic INR	0.85 (0.61-1.19)	0.34	1.04 (0.69-1.57)	0.86
Time to first above-range INR	1.02 (0.53-1.94)	0.96	0.91 (0.43-1.92)	0.81
Time to stable anticoagulation	1.17 (0.83-1.65)	0.38	0.88 (0.60-1.29)	0.51

CI, confidence interval; CYP2C9, cytochrome P450 2C9; HR, hazard ratio; INR, international normalized ratio; VKORC1, vitamin K epoxide reductase subunit 1.

All Cox regression models were adjusted for age, gender, weight, warfarin dose, indication for therapy, cohort, VKORC1, CYP2C9, and CYP4F2 genotype.

* Survival function was compared with VKORC1 wild-type G/G genotype group.

† Survival function was compared with CYP2C9 wild-type *1/*1 genotype group.

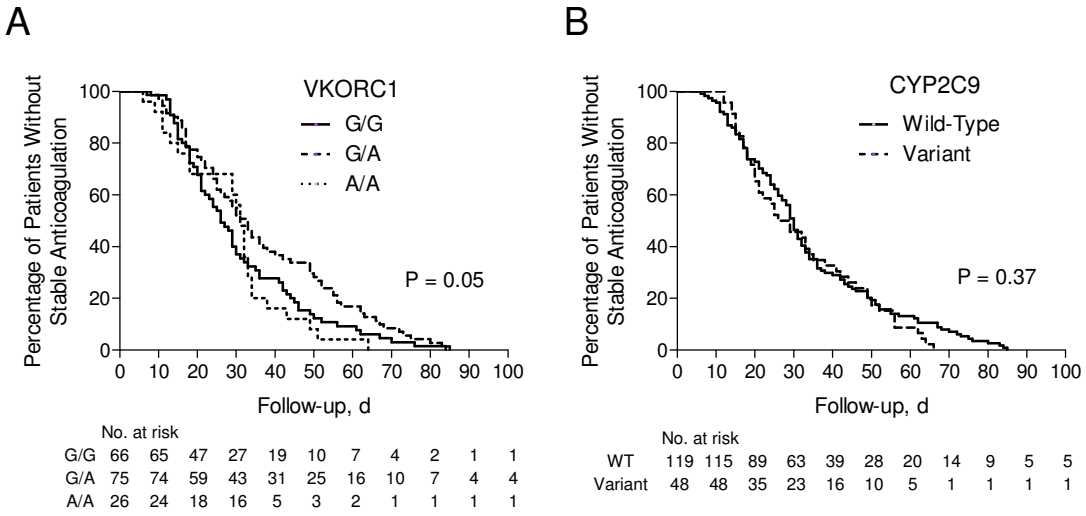

Figure 4.2 The effect of pharmacogenetics-guided dosing on time to stability.

Kaplan-Meier plots representing the time to stable anticoagulation among VKORC1 (A) and CYP2C9 (B) genotype groups. The statistic in each panel represents the log-rank *P* value for testing the equality of survival functions. WT indicates wild type.

4.3.4 Time spent within therapeutic range (INR 2.0-3.0) and above therapeutic range (INR > 3)

To separate the initiation and stabilization phases of therapy, we considered prestabilization as day 1 to 30 and the stabilization phase as day 31 to the end of the study period. We chose 30 days because the median time to stability was 29 days. There was no significant influence of VKORC1 or CYP2C9 genotype on time spent in therapeutic range or above range during prestabilization or stabilization phase (supplemental material, Table 4.8). The present study was not powered to detect secondary outcomes.

4.3.5 INR response time course during first 3 weeks of therapy

With the PK-PD model, the response profiles for various genotype groups were predicted to be similar during the attainment of therapeutic INR. Our algorithm was developed to enable patients to reach the first therapeutic response in a steady and safe manner, with a goal of reaching optimal anticoagulation by the end of initiation. Figure 4.3A illustrates the average INR time course of patients in the present study up to treatment week 3. The time-course observed was similar to the model predicted response profile, particularly during the critical first week. Concordant with our primary outcomes, average INR during initiation rose to the target range in a similar fashion among VKORC1 and CYP2C9 genotype groups, and importantly, anticoagulation stability, as represented by maintenance of INR within range after initiation was comparable (Figure 4.3B, 4.3C).

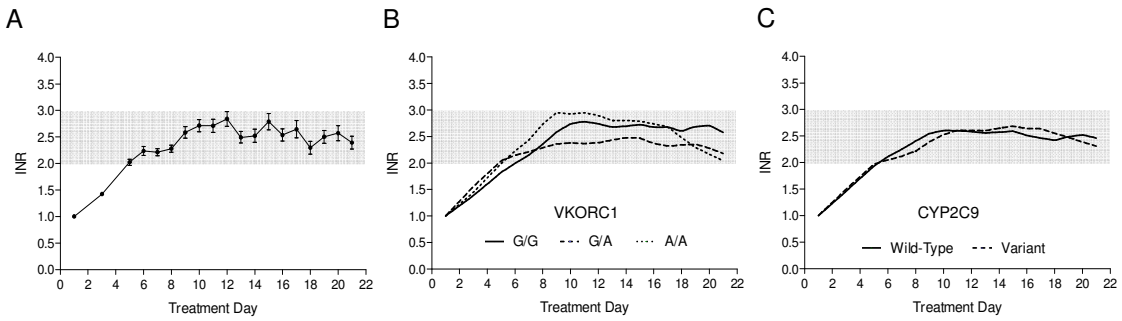

Figure 4.3 The effect of genotype-guided dosing on response time course during the first 3 weeks of warfarin therapy.

(A) The average response observed in patients dosed by the WRAPID nomogram, represented as mean with 95% CI of the SE, is similar to the PK-PD model-predicted anticoagulation response time course. The observed INR time courses among VKORC1 (A) and CYP2C9 (B) genotype groups, presented as LOWESS (locally weighted scatterplot smoothing regression) smoothed plots, rises and is maintained within therapeutic range in a parallel and similar manner.

4.3.6 Secondary outcomes

Secondary efficacy and safety outcomes of anticoagulation are summarized in Table 4.7. By day 6 of therapy, 40.1% of patients enrolled in the present study had an INR within the therapeutic range, whereas 57.5% had an INR within the therapeutic range by day 9. The proportion of patients reaching extended INR range of 1.8-3.2 by days 6 and 9 was 60.5% and 78.4%, respectively. Although approximately 20% of patients reached an INR ≥ 4 during the entire study duration, the percentage of time spent with INR ≥ 4 was only 1.2%. Moreover, only 3.6% of patients experienced excessive overanticoagulation with an INR ≥ 5 during the entire study period. The average maintenance dose was 5.54 mg/d and followed the known gene-dose relationship (supplemental material, Table 4.9).

4.3.7 Dosing algorithm assessment

The association between observed maintenance dose, algorithm predicted dose, and day 7/8/9 dose was determined. The proportion of variance explained by the final maintenance regression was 42% (Figure 4.4A), whereas the variance explained following INR-guided dose adjustments was 70% (Figure 4.4B). In addition, there was less bias between day 7/8/9 dose and maintenance dose than that of the algorithm predicted. The mean absolute error (SE) of the final model was 10.4 (0.1) mg/wk, whereas for the INR-adjusted dose, it was 8.5 (0.9) mg/wk, comparable to that of other pharmacogenetics-based nomograms (33).

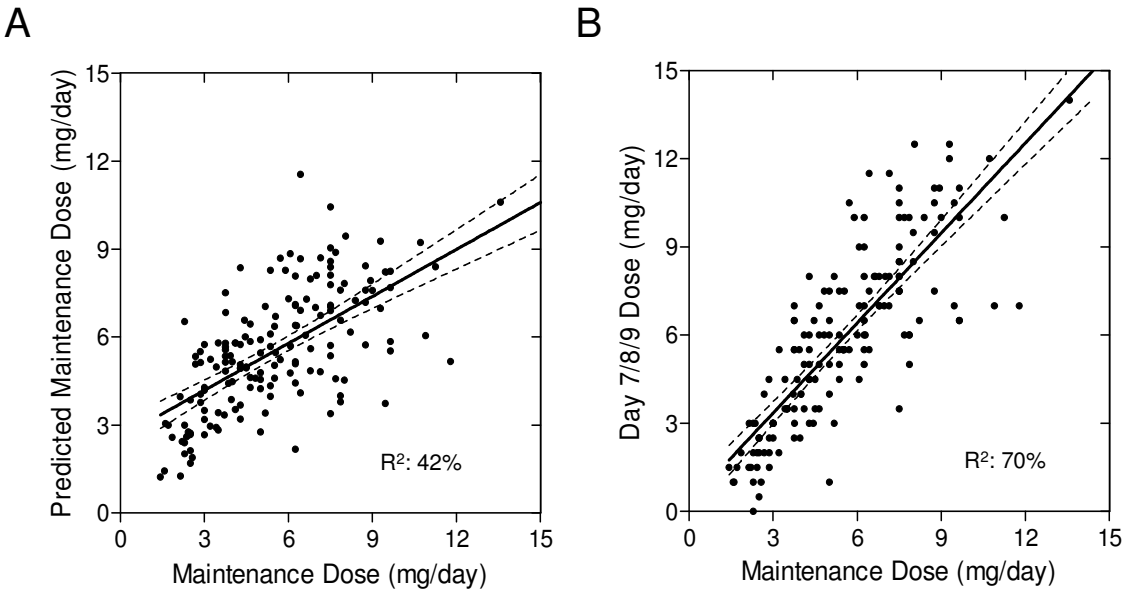

Figure 4.4 Association of predicted maintenance dose to observed maintenance dose.

Scatter plots show the association of algorithm-predicted maintenance dose (A) and day 7/8/9 dose after response-based adjustments (B) with the observed maintenance dose. The solid lines represent the linear regression, and the dashed lines represent its 95% CI.

Table 4.3.2 Secondary outcomes following dosing with pharmacogenetics-based algorithm.

Initiation phase (Day 1-9), n (%)	
INR 2-3 within 3 days	13 (7.8)
INR 2-3 within 6 days	67 (40.1)
INR 2-3 within 9 days	96 (57.5)
INR extended 1.8-3.2 within 3 days	23 (13.8)
INR extended 1.8-3.2 within 6 days	101 (60.5)
INR extended 1.8-3.2 within 9 days	131 (78.4)
INR ≥ 5, No. (%)	0 (0)
90-day follow up period*	
Time spent in range, % (SD)	64.8 (19.8)
Time spent in extended therapeutic INR (1.8-3.2), % (SD)	77.3 (14.4)
Time spent in INR ≥ 4, % (SD)	1.2 (2.9)
Number of INR measurements in 90 days, mean±SD	12.3±2.7
INR ≥ 5, n (%)	6 (3.6)
Post 30 day follow up period	
Time spent in range, % (SD)	68.1 (25.0)

INR, international normalized ratio; SD, standard deviation.

* Ninety-day follow-up period excludes initiation phase (day 1- 9). The outcomes change slightly when initiation is included.

4.4 Discussion

The clinical benefit of warfarin for decreasing stroke risk among AF patients and treating VTE is well established; however, the unpredictable anticoagulation response for a significant proportion of patients poses a substantial clinical challenge to optimal warfarin therapy. Several studies have examined various initiation strategies for treatment of VTE and AF (1, 34-36). Although several of these studies have incorporated loading dose nomograms during initiation, most have been in the setting of VTE (19, 20), and few studies incorporating loading dose strategies for other indications have been reported (34, 37). Pharmacogenomic studies conducted in the last decade have established the contribution of both VKORC1 and CYP2C9 genetic variations to maintenance dose requirements; however, VKORC1 is a more important modulator of early warfarin response than CYP2C9 (24). Not surprisingly, both genes have recently been reported to predict therapeutic doses during the initial weeks of therapy (38, 39). With these considerations, we developed and evaluated a practical and universal pharmacogenetics-based loading dose algorithm for both AF and VTE patients.

In contrast to the findings previously observed with nonpharmacogenetics-based dosing,(24) we show that use of the WRAPID algorithm eliminated VKORC1 and CYP2C9 genotype-related differences in attainment of first therapeutic INR in both AF and VTE patients. This finding did not change after adjustment for confounding variables. Interestingly, subanalysis of patients in the initial cohort, in which CYP4F2 was not included as a predictor of dose, demonstrated that the c.1297G>A SNP did not

significantly influence attainment of therapeutic INR. This was in contrast to a recent study by Zhang et al. that examined the role of CYP4F2 as a genetic determinant during initiation in patients dosed according to standard methods (40). The present findings suggest that dosing according to VKORC1 and CYP2C9 genotype is sufficient. This is consistent with the fact that CYP4F2 genotype accounts for only a small portion of the observed maintenance dose variability (0-4%) (12, 41). However, a caveat here is that the present study was not powered to detect an association between response and CYP4F2 genotype. Thus, the definitive role of CYP4F2 genotype in individualized warfarin therapy requires further assessment in a powered study of sufficient sample size.

With respect to risk for excessive anticoagulation (INR $\geq$ 4), several groups have reported that variant carriers of VKORC1 and CYP2C9 are subject to significantly increased risk of overanticoagulation (22). After initiation with a pharmacogenetics-based dosing algorithm, neither the VKORC1 nor the CYP2C9 variant groups had an elevated risk of supratherapeutic INR during the first month of therapy or throughout the entire study period. The present findings contrast with those of a study conducted by Voora et al. in which patients were dosed prospectively only according to CYP2C9 genotype (42). In that study, carriers of a variant CYP2C9 allele still exhibited an increased risk for excessive anticoagulation. This may be explained by dosing algorithm and adjustment differences during initiation compared with the present study.

An important measure of variability in anticoagulation quality is the time to stability. Higashi et al. reported that variant carriers of CYP2C9 required significantly longer to attain stability in the absence of pharmacogenetics-based dosing (8). The present study demonstrates that differences in time to stability between VKORC1 and CYP2C9 genotype groups can be effectively reduced with the use of pharmacogenetics-guided dosing. Moreover, Wadelius et al. and Limdi et al. recently reported that the INR response profile differed between genotype groups during initiation using a standard-dosing regimen, in which variant carriers had greater warfarin sensitivity (22, 23). In contrast, the increase in INR to therapeutic range in the present study was similar among genotype groups.

It cannot be entirely ruled out that the lack of association observed in the present study may have been caused by insufficient sample size; however, calculations showed that we had ample power (> 80%) to detect the association of a causal VKORC1 -1639G>A SNP, with an allele frequency of 35%, with anticoagulation response, for an HR of 2. Because we observed no evidence of an association between VKORC1 genotype and anticoagulation responses, it would be reasonable to conclude that the WRAPID algorithm eliminated the VKORC1-driven response variation. Although we did not observe a significant association between CYP2C9*2 or *3 genotype and response, this may be because of lack of power. When one considers the small proportion of heterozygous and homozygous carriers of CY2C9*2 and *3 allele, the sample size required to detect such individual associations would be very large (~ 1000). Thus, we

assessed the association of pooled CYP2C9 variant status (at least 1 of *2 or *3 allele), a frequency of 30% in the present study population, with anticoagulation response. In this case, calculation showed that sufficient power was achieved (> 80%) to detect the association of CYP2C9 variant status with anticoagulation responses for an HR of 2. Such pooling of CYP2C9*2 and *3 variants has been used previously by other studies for similar reasons.(8, 43) In particular, these studies demonstrated that among patients whose therapy was initiated with standard dosing protocols, CYP2C9 variant carriers spent more time above therapeutic INR, had an elevated risk of overanticoagulation, and a lower dose requirement overall.

To the best of our knowledge, the WRAPID nomogram is the first warfarin initiation algorithm that incorporates both VKORC1 and CYP2C9 genotype-determined loading doses, which differs from the typical doubling of the maintenance dose. Thus far, there have been 2 prospective randomized clinical trials (RCTs) in which pharmacogenetics-based warfarin initiation with loading doses was compared with standard warfarin loading dose initiation, whereas other studies have not incorporated loading doses. In the first trial, control patients were loaded with 5 mg, whereas study patients were loaded according to CYP2C9 genotype (44). In the second trial, control patients were initiated with 10 mg, whereas study patients were initiated with double the maintenance dose determined with VKORC1 and CYP2C9 genotype (45). Evidently, there is a lack of consensus with respect to warfarin initiation and especially concerning loading dose selection from genetic information. The aforementioned trials, albeit small, indicate that

pharmacogenetics-guided dosing improves warfarin response in terms of more time spent within the therapeutic range, decreased bleeding events, and faster attainment of therapeutic INR, supporting the use of loading doses for initiation. In addition, some studies recommend that loading dose should be age adjusted because of concern about warfarin sensitivity (34). However, we did not observe a disproportional number of elderly patients with excessive anticoagulation with our loading dose regimen, in which some elderly patients were indeed loaded with 10 mg as per genotype. Thus, the present data do not support age-modified loading doses. We did, however, observe an effect of decreased weight on response sensitivity during initiation.

Limitations of the present study include the inability to determine the influence of a pharmacogenetics-guided dosing algorithm on rare bleeding complications because of insufficient sample size; however, we can comment on the general safety of our dosing regimen. The number of INRs over 5 has been used previously as a measure of the safety of a dosing protocol (19). In the present study, only 3.6% of patients experienced such excessive anticoagulation, which is lower than that observed with other initiation protocols (5.6 – 8.6%) (19, 36). Several RCTs involving larger sample sizes are currently under way to evaluate the safety and efficacy of pharmacogenetics-based dosing compared with standard-dosing (www.clinicaltrials.gov; NCT01006733, NCT00839657, and NCT01119300). Interestingly, a proposal has been made for a multicenter trial in Europe that will test pharmacogenetics-guided initiation with the use of a genotype-based loading doses to examine the clinical utility of such dosing methods. The present study

supports the use of genotype-guided loading dose during warfarin initiation. Another limitation is that because the present study lacked a control group (nongenetics-based warfarin initiation), the results may be attributed in part to management of warfarin therapy by anticoagulation clinics. However, the present study was not designed as an RCT; rather, it had the goal of demonstrating the minimization of genotype-dependent differences in early anticoagulation response, because this has not been demonstrated conclusively in the warfarin-pharmacogenetics field. Furthermore, several studies published to date have described the contribution of genetic variations to initial warfarin response variability in patients whose treatment was initiated with respective anticoagulation clinic regimens, likely with similar INR-response monitoring schedules as WRAPID (22, 23, 43). Thus, we believe that pharmacogenetics-based initiation, particularly with the use of loading doses, should result in a safe and similar rise to optimal anticoagulation responses among VKORC1 and CYP2C9 genotype groups. Supportive of the role of genotype-guided initiation for warfarin therapy, a recent study suggested that genotyping for patients in whom therapy was being initiated significantly reduced the hospitalization rate for bleeding or thromboembolic events compared with a control group.(46) Furthermore, the results of 5 small RCTs (range from 38-200 patients) completed thus far largely suggest that pharmacogenetics-guided dosing improves warfarin response, in terms of more time spent within the therapeutic range and decreased bleeding events compared with standard dosing (44, 45, 47, 48), with the exception of 1 study in orthopaedic patients (49). In that RCT, patients were followed up for only 2-4 weeks, and daily INR monitoring in addition to a similar dose adjustment protocol

between the 2 arms may have rendered the effect of genotype-guided dosing nonsignificant. Indeed, larger RCTs that incorporate models such as WRAPID are required to compare adverse event rates between standard and pharmacogenetics-guided dosing of warfarin-based anticoagulation.

To the best of our knowledge, this is the first prospective study to demonstrate the utility of a genotype-guided warfarin initiation algorithm for the minimization of widely recognized VKORC1 and CYP2C9 genotype-associated differences in anticoagulation response for both AF and VTE patients. The pharmacogenetics-based algorithm proposed here is feasible and effective for outpatient management of individuals requiring warfarin-based anticoagulation.

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4.6 Supplemental Material

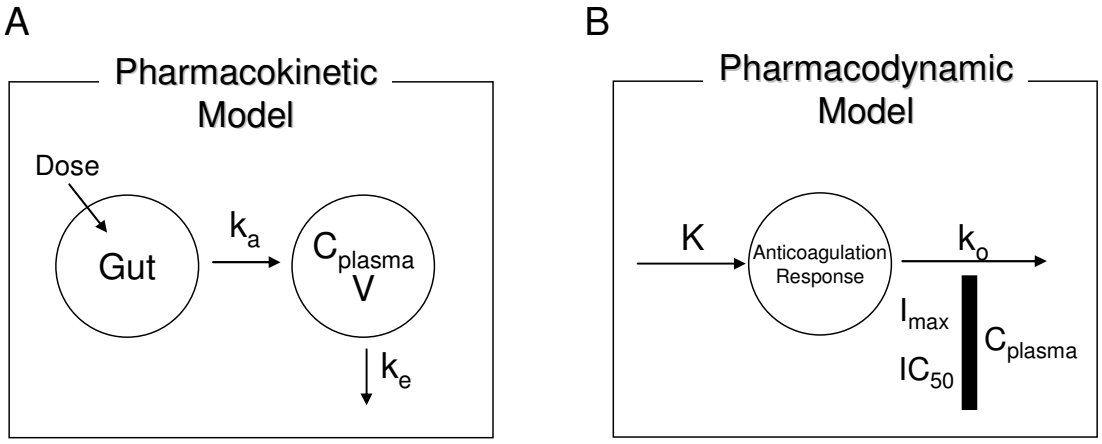
Table 4.6.1 Comparison of percent time spent within therapeutic range (2.0-3.0) and over range (>3) among VKORC1 and CYP2C9 genotype groups.

	VKORC1				CYP2C9		
	G/G	G/A	A/A	P value	Wild-type	Variant	P value
Time within range (day 1-30), %	53.20	46.60	52.40	0.12	49.83	50.24	0.90
Time within range (day 30-90), %	75.04	64.77	68.22	0.19	66.46	70.86	0.30
Time above range (day 1-30), %	15.80	10.18	16.62	0.11	13.14	14.74	0.45
Time above range (day 30-90), %	6.35	5.37	6.97	0.95	5.74	7.18	0.37

Table 4.6.2 Mean prescribed daily maintenance dose (mg) in relation to VKORC1 and CYP2C9 genotype.

	CYP2C9				
VKORC1	*1/*1	*1/*2	*1/*3	*2/*2	*2/*3
G/G	7.10	6.71	4.12	6.5	NA*
G/A	5.59	4.60	3.91	2.50	NA*
A/A	4.10	2.35	2.13	NA	1.93

* Not available



C

Model Parameter Values			
Parameter	Value	Unit	Genotype
k_a	28.32	days-1	
k_e	0.475	days-1	*1*1
	0.322	days-1	*1/*2 or *1/*3)
	0.19	days-1	*2/*2, *2/*3, *3 /*3
V	10	L	
k_o	1		
K	1		
I_{max}	2		G/G
	2.375		G/A
	3.5		A/A
IC_{50}	1500	ng/mL	

Figure 4.5 A schematic representation of the pharmacokinetic-pharmacodynamic (PK-PD) model employed to determine loading doses and dose-adjustment nomogram.

PK of warfarin is described by a one-compartment model with first-order absorption (A) while PD is described by an indirect response model (B), that accounts for delay in anticoagulation response. (C) Parameter values used for simulation of drug concentration and response time course during model development.

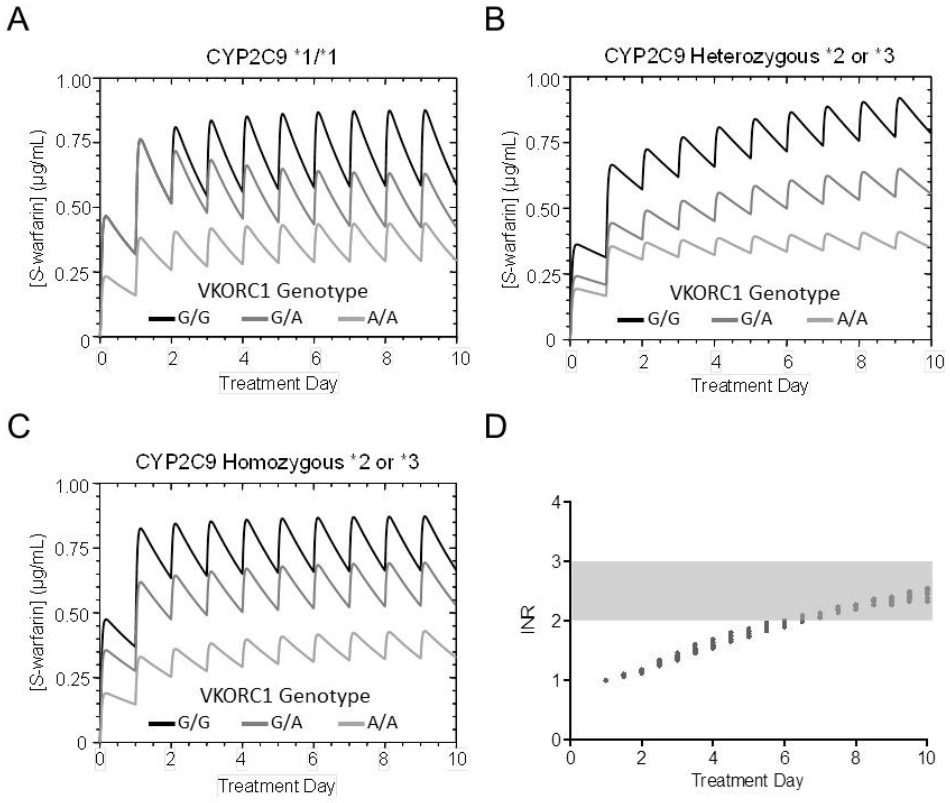


Figure 4.6 Concentration response curves necessary for formulating loading doses.

Loading doses were determined by simulating adequate *S*-warfarin plasma concentrations (A-C) to increase international INR response (D) to therapeutic range (2.0 – 3.0) at a similar trajectory for various VKORC1 and CYP2C9 genotype combinations.

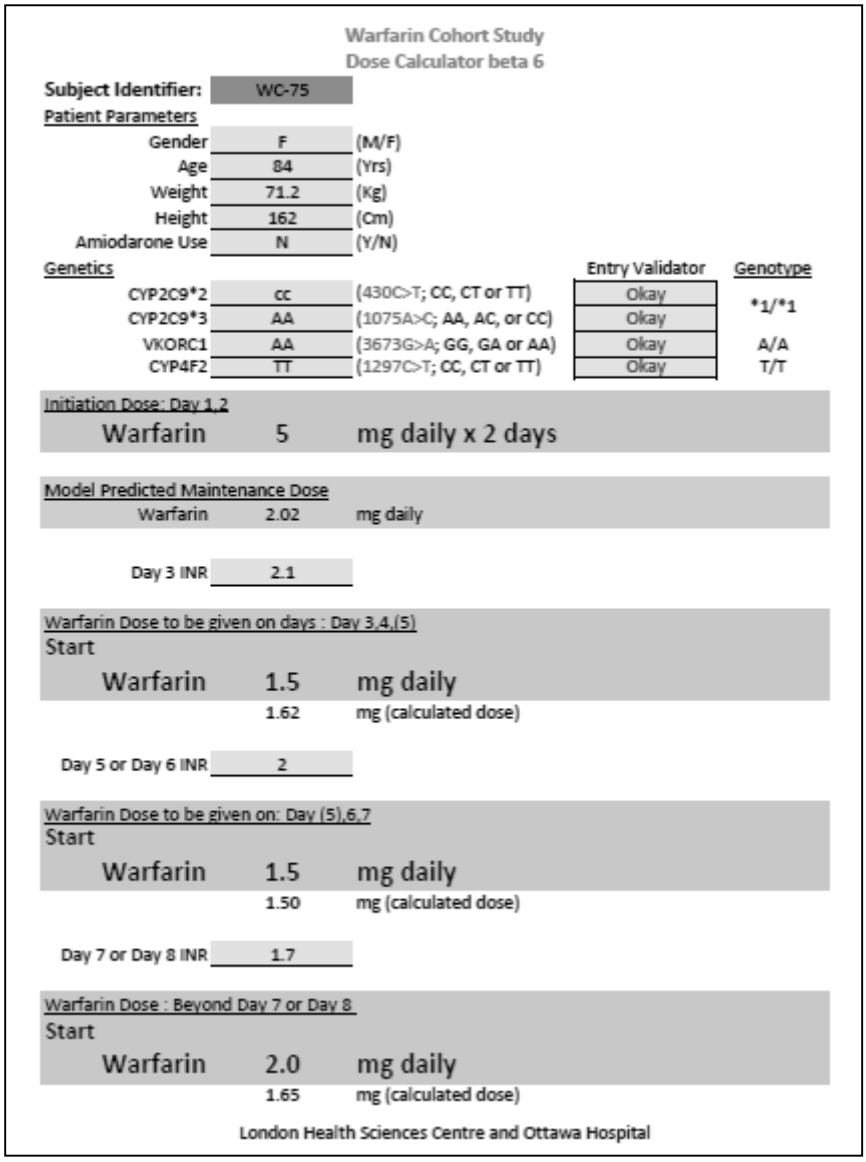


Figure 4.7 An automated dose calculator that incorporates the WRAPID pharmacogenetics-based dosing algorithm and adjustment nomogram for warfarin initiation.

5 CLINICAL AND GENETIC DETERMINANT OF WARFARIN PHARMACOKINETICS AND PHARMACODYNAMICS DURING TREATMENT INITIATION[3]

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5.1 Introduction

The vitamin K antagonist, warfarin, is an oral anticoagulant commonly prescribed to prevent and treat venous thromboembolism (VTE) and decrease the risk of stroke in atrial fibrillation (AF) (1). Warfarin therapy is complicated by the wide interindividual variation in response and dose requirements for adequate anticoagulation. Optimal warfarin therapy is achieved by maintaining the anticoagulation response, international normalized ratio (INR), within a narrow therapeutic range of 2.0 to 3.0 for most indications. Due to the unpredictable pharmacokinetic (PK) and pharmacodynamic (PD) responses to warfarin, initiation of therapy is the most clinically challenging phase as the optimal dose is often determined iteratively, guided by INR (2).

Warfarin is administered as a racemic drug; however, the *S*-warfarin enantiomer is 3-5 times more potent than *R*-warfarin.(3) *CYP2C9* is the primary enzyme responsible for metabolism of *S*-warfarin (4), and studies have consistently shown that *CYP2C9* polymorphisms (*2, c.430C>T, rs1799853; *3, c.1075A>C, rs1057910) significantly contribute to the variable warfarin response (5). Non-genetic factors of warfarin PK variability and dose requirements are also important. For example, age and co-administration with drugs that inhibit or induce *CYP2C9* can alter *S*-warfarin elimination (6-10). Moreover, *S*-warfarin volume of distribution is dependent on weight (11, 12). Taken together, it has been estimated that PK factors determine 26-40% of warfarin maintenance dose variability (10, 13, 14).

Warfarin exerts its anticoagulation effects by inhibiting vitamin K epoxide reductase (VKOR encoded by the *VKORC1* gene), the enzyme responsible for recycling oxidized vitamin K epoxide to its hydroquinone form, an essential cofactor for activation of clotting factors II, VII, IX and X (15). It is appreciated that single nucleotide polymorphisms (SNPs) in *VKORC1* result in altered warfarin sensitivity while rare mutations have been linked to warfarin resistance (8, 16). Of note, the common promoter SNP (*VKORC1* -1639G>A, rs9923231) is likely the causative variation responsible for greater warfarin sensitivity (17, 18). In addition to *CYP2C9* and *VKORC1* polymorphisms, several studies have reported that a functional SNP in *CYP4F2* (c.1297G>A, rs2108622), the metabolizing enzyme for vitamin K (19), also determines dose requirement (20, 21). Furthermore, diet has long been considered an important environmental determinant of warfarin response. Indeed, reduced anticoagulation response was observed in warfarin-stabilized patients with intake of vitamin K-rich foods (22, 23), and vitamin K status was associated with warfarin sensitivity at the onset of treatment (24).

With the intent of improving warfarin anticoagulation therapy, a number of algorithms have been proposed which incorporate genetics as well as clinical parameters to predict individualized maintenance dose (8, 25, 26). Many of the factors influencing required maintenance dose such as age, body surface area, drug interactions and importantly, *CYP2C9* genotype relate to their effects on *S*-warfarin PK parameters, such as volume of distribution and clearance (7-9, 27). The influence of genetics and clinical parameters on *S*-warfarin PD variability is less clear. Although the influence of *VKORC1* genetic

variations and vitamin K intake on dose and anticoagulation response is evident, the quantitative and dynamic influence of these variables on PD parameters, such as drug affinity and maximal inhibition, has not been well established (28). Moreover, there is a paucity of information regarding the influence of other genetic and clinical variables on *S*-warfarin PD variation.

In this study, we aimed to separate warfarin pharmacokinetic factors from intrinsic pharmacodynamic factors to elucidate crucial covariates of each, and their contribution to the overall anticoagulation response variation. To this end, PK-PD modeling was applied to a cohort of patients commencing warfarin therapy using a novel initiation protocol (29).

5.2 Experimental Design

5.2.1 Study subjects and design

Patients with AF (n = 61), VTE (n = 98) or other conditions (n = 8) were prospectively enrolled to evaluate the safety and efficacy of a pharmacogenetics-based warfarin initiation protocol. Patient characteristics and clinical outcomes were described previously in detail (29). The inclusion criteria for study enrolment were minimum of 18 years of age and indication for new warfarin therapy for at least 3 months with a target INR range of 2.0 to 3.0. Patients were excluded on the basis of diagnosis of cancer other than non-melanoma skin cancer, alcohol or drug abuse, baseline INR>1.4, known warfarin allergy/intolerance, terminal disease, prior use of warfarin or vitamin K use within 2 weeks prior to study enrolment, and pregnancy. The majority of patients were Caucasian (95%) with mean age of 60 years (range, 19-88) and mean weight of 84 Kg (43-155). The allelic frequencies for *VKORC1* -1639G>A and *CYP4F2* c.1297G>A were 38.0% and 31.7%, respectively. The *CYP2C9**2 and *3 allelic frequencies were 11.1% and 4.8%, respectively. There was no homozygous *CYP2C9**3 carrier in this population. Amiodarone, statin, antiplatelet, antibiotic, antifungal and NSAID medication use were present in 2%, 45%, 55%, 6%, 1% and 12% of the cohort, respectively.

The Warfarin Regimen using A Pharmacogenetics-guided Initiation Dosing (WRAPID) protocol has been described elsewhere (29). Briefly, a 2-day loading dose (according to *VKORC1* and *CYP2C9* genotype) was administered, followed by a day 3 INR

measurement that was used in combination with the maintenance algorithm to determine the subsequent dose. Two subsequent INR measurements were obtained within the first 9 days of therapy where the maintenance dose was further adjusted accordingly to the dose adjustment nomogram. Simultaneous with INR monitoring, additional blood samples were collected for drug level analysis.

This study was conducted at the London Health Sciences Centre and The Ottawa Hospital upon approval by Research Ethics Boards at the University of Western Ontario and Ottawa Hospital. Patients requiring initiation of warfarin therapy were prospectively screened for study eligibility and informed written consent was acquired.

5.2.2 Genotyping

Genomic DNA was isolated with Gentra Puregene or DNA Blood Midi extraction kit (Qiagen, Valencia, CA). At London Health Sciences Center, genotypes were determined by allelic discrimination using TaqMan Drug Metabolism Genotyping assays with the 7500 RT-PCR System (Applied Biosystems, Carlsbad, CA). At Ottawa Health Research Institute, genotypes were determined using the Luminex 200 system (Luminex, Austin, TX).

5.2.3 Warfarin drug level analysis

Racemic warfarin and internal standard (IS) *R/S-para*-chloro-warfarin were purchased from Sigma-Aldrich. Plasma was extracted from patient blood samples within 1 hour of collection and stored at -80°C until use. Total *S*-warfarin plasma concentration was determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Briefly, 300 µL of acetonitrile and 25 µL of IS was added to 100 µL of plasma and centrifuged at 14,000 rpm for 20 min. The resulting supernatant was added to 5 mM ammonium acetate pH 4 (1:3 v/v). Warfarin and IS enantiomers were separated with the Astec CHIROBIOTIC™ V Chiral Column (5 cm × 4.6 mm, 5 µM particle size) using gradient elution with 5 mM ammonium acetate (pH 4) and acetonitrile (5 to 70%) in a 10 min run time. The MS was set in negative mode for detection of warfarin and IS with transitions 307.2 → 160.0 *m/z* and 340.8 → 160.0 *m/z*, respectively. Calibration curves were prepared by spiking blank plasma with known concentrations of *R/S*-warfarin. The lowest limit of quantification was 1 ng/mL for both enantiomers. The interday coefficient of variation and bias of *S*-warfarin quality controls was 10.5% and 9.3%.

5.2.4 Proteins induced by vitamin K absence factor II (PIVKA-II) assay

PIVKA-II concentrations were analyzed with use of an enzyme-linked immunosorbent assay kit as per manufacturer's protocols (Diagnostica-Stago, Parsippany, NJ).

5.2.5 Kidney function

We measured patient plasma creatinine concentrations by LC-MS/MS. Briefly, creatinine and the IS, creatinine-D3, was purchased from Sigma-Aldrich and Toronto Research Chemicals, respectively. Creatinine and IS were separated with the reverse-phase Hypersil Gold column (50 × 5 mm, 5 µM particle size) using isocratic elution with 25% 1% formic acid in water v/v and 75% acetonitrile with 1% formic acid v/v in a 7 min run time. The MS was set in positive mode for detection of creatinine and IS with transitions 114.1 → 44.3 *m/z* and 117.1 → 47.3 *m/z*, respectively. The lowest limit of quantification was 50 ng/mL. The interday coefficient of variation and bias of creatinine quality controls was 8.7% and 6%. eGFR was estimated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (30). Renal function was categorized according the National Kidney Foundation's classification of chronic kidney disease.

5.2.6 PK-PD modeling

S-warfarin PK was described using a linear one-compartment model with a set volume of distribution (V; 0.14 L/kg) on a per patient basis (12). The time-course of plasma *S*-warfarin concentration (C_p) arose from the interplay between first-order drug absorption (k_a) and drug elimination (k_e) processes. Parameter values for k_a were fixed (28.56 day^{-1}) based on the literature (31). Bioavailability was assumed to be complete (32). Individual k_e values were obtained by least squares fitting (Scientist, Micromath, St. Louis, MO) of the concentration data during the first 9 days with prescribed doses as input. Clearance (CL) was calculated according as the product of V and k_e.

S-warfarin PD was described by an established indirect response model which incorporates the known delay in anticoagulation effects (33). In this model, the rate of change in INR was modeled using zero-order input (K) and first-order output (k_{out}) variables. Plasma *S*-warfarin levels (C_p) modulate the output response according to classical inhibition kinetics, described by parameters maximum inhibitory factor (I_{max}, i.e. inversely related to enzyme content) and drug affinity (IC_{50}) (34). Since *VKORC1* -1639G>A promoter SNP has been correlated with altered mRNA expression levels, I_{max} values were expected to vary with *VKORC1* genotype. R_{max} and k_{out} values in the indirect response model were both fixed at 1. The IC_{50} for *S*-warfarin was fixed at 1500 ng/mL, as reported previously (35). The following equation describes the PD model.

$$\frac{dINR}{dt} = R - INR \cdot k_{out} \left(1 - \frac{I_{max} \cdot C_p}{IC50 + C_p}\right)$$

The response analysis was conducted following estimation of individual *S*-warfarin plasma concentrations. These estimated drug concentrations were used in combination with measured INRs to estimate the individual PD parameter, I_{max}, by least squares fitting.

We note that clearance and I_{max} parameter estimates should be considered independent of the dosing regimen and anticoagulation responses observed in the WRAPID study

because estimations of individual warfarin clearance and individual drug concentration-response profile are unaffected by the doses received.

5.2.7 Vitamin K epoxide reductase protein expression in human liver

The collection and processing of liver samples was described elsewhere (36). In order to obtain a positive control for VKOR protein analysis, the enzyme was over-expressed in cells using previously described protocol (37). For this purpose, human *VKORC1* cDNA was amplified from a human liver cDNA library using primers 5'-TGGAGATAATGGGCAGCACCTGGGGG-3' (forward) and 5'-GTTGAGGGCTCAGTGCCTCTTAGCCTTG-3' (reverse). Samples were separated by SDS-PAGE on 4-10% gels (Invitrogen, Carlsbad, CA) and subsequently transferred onto nitrocellulose membranes. Blots were probed with a custom anti-VKOR antibody (kindly provided by Dr. Kathleen Berkner, Learner Research Institute, Cleveland Clinic (38)) and subsequently probed with anti-rabbit horseradish peroxidise-labeled secondary antibodies (Bio-Rad, Hercules, CA). The bands were detected using the BM Chemiluminescence Western Blotting Substrate (Roche, Indianapolis, IN) and KODAK ImageStation 4000 MM (Carestream, Rochester, NY). Protein expression levels were normalized to a wild-type *VKORC1* sample (HLM100), repeated on all blots.

5.2.8 Determinants of warfarin kinetics and response

Regression analysis was performed to determine factors affecting *S*-warfarin clearance and I_{max} . Since the distribution of both of these parameters in our patient population was skewed, square-root transformation was adopted to normalize the data. The variables age, gender, body weight, amiodarone use, other known interacting medications, indication for warfarin therapy, kidney function, vitamin K status as measured by PIVKA-II, *VKORC1* genotype, *CYP2C9**2 and *3 genotype were considered as covariates for both *S*-warfarin clearance and I_{max}. The covariates were added to the model according to the stepwise forward regression. 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. The models with significant covariates were then internally validated through bootstrapping. Bootstrapping was achieved by random sampling with replacements to obtain 1000 samples, allowing estimation of the standard error and the 95% confidence interval (CI) of parameter estimates. Potential collinearity between variables was assessed using condition indices and variance proportions.

The clearance and I_{max} regression equations were then integrated with the PK-PD model in order to predict and compare anticoagulation response profiles following initiation with various nomograms for typical warfarin patients.

5.2.9 Statistical analysis

The Kruskal-Wallis one-way analysis of variance followed by Tukey's test for pairwise comparisons was employed for the following analysis: *S*-warfarin concentration differences with respect to *VKORC1* genotype, influence of *VKORC1* genotype on attainment of therapeutic INR and dose, effect of *VKORC1* genotype on liver protein expression, relationship between *S*-warfarin clearance and *CYP2C9* genotype, effect of kidney function on *S*-warfarin clearance, relationship between *VKORC1* genotype and I_{max}. Mann-Whitney U's test was employed to examine gender effect on *S*-warfarin clearance and warfarin indication effect on I_{max}. A two-tailed P value of less than 0.05 was considered significant for all analyses. Statistical analysis was performed with the use of GraphPad Prism v.5.0 (GraphPad, La Jolla, CA) or SPSS v. 17.0 (SPSS, Chicago, IL).

5.3 Results

5.3.1 PK-PD model performance

We fitted the individual patient *S*-warfarin plasma levels during the first 9 days of therapy to a one-compartment PK model (Figure 5.1A) to furnish estimates of *S*-warfarin clearance. The *S*-warfarin clearance estimated here was similar to that previously observed (39). Moreover, good fits to individual patient levels with the PK model were obtained (Figure 5.1C). Overall, the PK model was sufficiently accurate in describing the data as linear regression analysis for predicted and actual *S*-warfarin concentrations yielded a coefficient of determination (r^2) of 0.91, with a slope of 0.92 (Figure 5.1D). The mean absolute error (MAE) between estimated and actual was 0.04 μg/mL, and 88% of these estimated values were within 25% of actual concentrations.

An indirect response model was used to estimate maximal inhibitory factor (I_{max}), the PD parameter related to the amount of hepatic VKOR enzyme. Here, the *S*-warfarin plasma concentration-INR response relationship is governed by the parameters IC_{50} (related to warfarin affinity to VKOR) and I_{max}, where at constant IC_{50}, increasing I_{max} enhances drug sensitivity (Figure 5.1B). Individual predicted *S*-warfarin concentrations estimated from the PK model in conjunction with observed INR values served as inputs for the PD model. Fits to individual patient INRs over the initiation period were good (Figure 5.1E). Linear regression analysis for predicted and actual INR values of the entire data set

yielded an r^2 of 0.89, with a slope of 0.91 (Figure 5.1F). The MAE was 0.17, and 90% of these estimated values were within 25% of actual INR.

Figure 5.1 PK-PD model performance. (A) Model simulated *S*-warfarin plasma concentration-time profiles after single dose with *CYP2C9* variant alleles. (B) Model simulated steady-state therapeutic INR (2.5) *vs. S*-warfarin plasma concentration with varying I_{max} corresponding to *VKORC1* -1639G>A genotype. (C) Model fit of measured *S*-warfarin concentrations in a single patient. (D) Scatter plot of actual *vs.* predicted *S*-warfarin plasma concentration throughout the initiation phase (coefficient of determination, $r^2 = 0.91$, n = 459). The diagonal line represents the unity line. (E) Model fit of measured anticoagulation INR response values in the same patient as in (C). (F) Scatter plot of actual *vs.* predicted INR during the initiation phase ($r^2 = 0.89$, n = 459). The diagonal line represents the unity line. I_{max}, maximal inhibitory factor; INR, international normalized ratio.

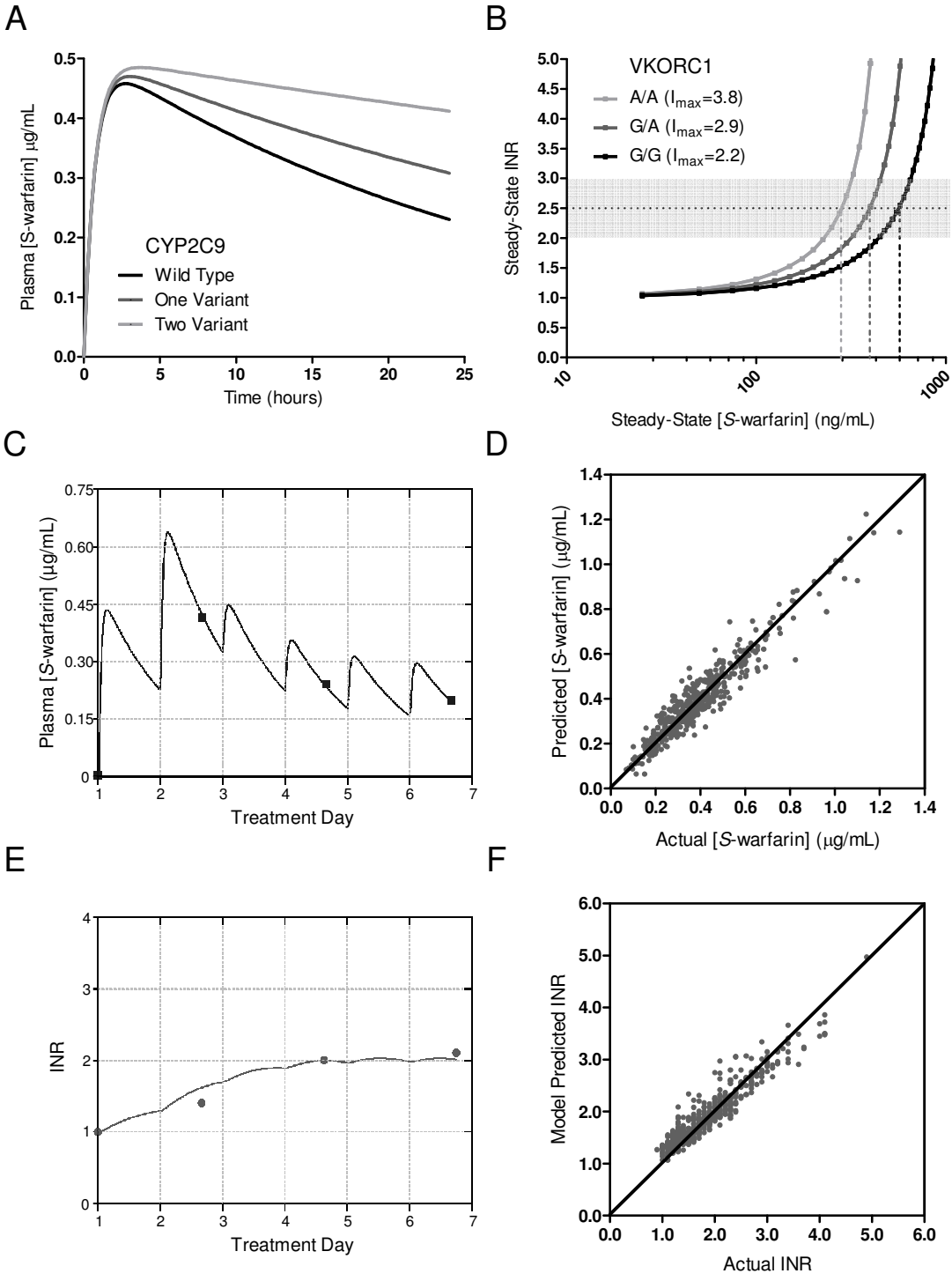

Figure 5.1 PK-PD model performance.

5.3.2 Determinants of *S*-warfarin clearance

Mean *S*-warfarin clearance was 7.5 L/day (SD 3.4) with a range of 0.8 to 20.8, indicating a more than 20-fold interindividual variability in *S*-warfarin PK (Figure 5.2A). *S*-warfarin clearance was significantly associated with *CYP2C9* genotype with mean clearance values of 8.1, 7.0, 4.3, 4.5, and 2 L/day, for *CYP2C9* *1/*1, *1/*2, *1/*3, *2/*2, and *2/*3 genotypes, respectively (Figure 5.2B). Interestingly, lower *S*-warfarin clearance was observed in patients with decreased renal function as estimated by glomerular filtration rate (eGFR) (Figure 5.2C, $P<0.0001$). The cohort average eGFR was 91 mL/min/1.73 m^2 (SD 23) with a range of 22 to 140. Moreover, eGFR was significantly decreased with increase in age ($P<0.0001$, data not shown). Gender also had an influence on *S*-warfarin clearance, where on average, females had significantly lower clearance compared to males (Figure 5.2D, $P<0.001$). With stepwise regression, clearance was found to be dependent on *CYP2C9**3 allele, kidney function, gender, and *CYP2C9**2 allele, in order of covariate entry into the regression equation. *VKORC1* genotype was without influence on *S*-warfarin clearance. The r^2 of the final model for clearance estimation was 36.5%. Parameter estimates of the final clearance model and bootstrap validation results are given in Table 5.1.

Figure 5.2 Determinants of *S*-warfarin clearance. (A) Frequency distribution of estimated *S*-warfarin clearance, shown as percent of total patients for each bin. (B) Relationship between *CYP2C9* genotype and *S*-warfarin clearance. Lines represent mean clearance. (C) *S*-warfarin clearance is significantly correlated with kidney function, as defined by eGFR. (D) Observed *S*-warfarin clearance segregated by gender. Lines represent mean clearance. eGFR, estimated glomerular filtration rate. * $P < 0.05$, ** $P < 0.005$, ***$P < 0.0005$

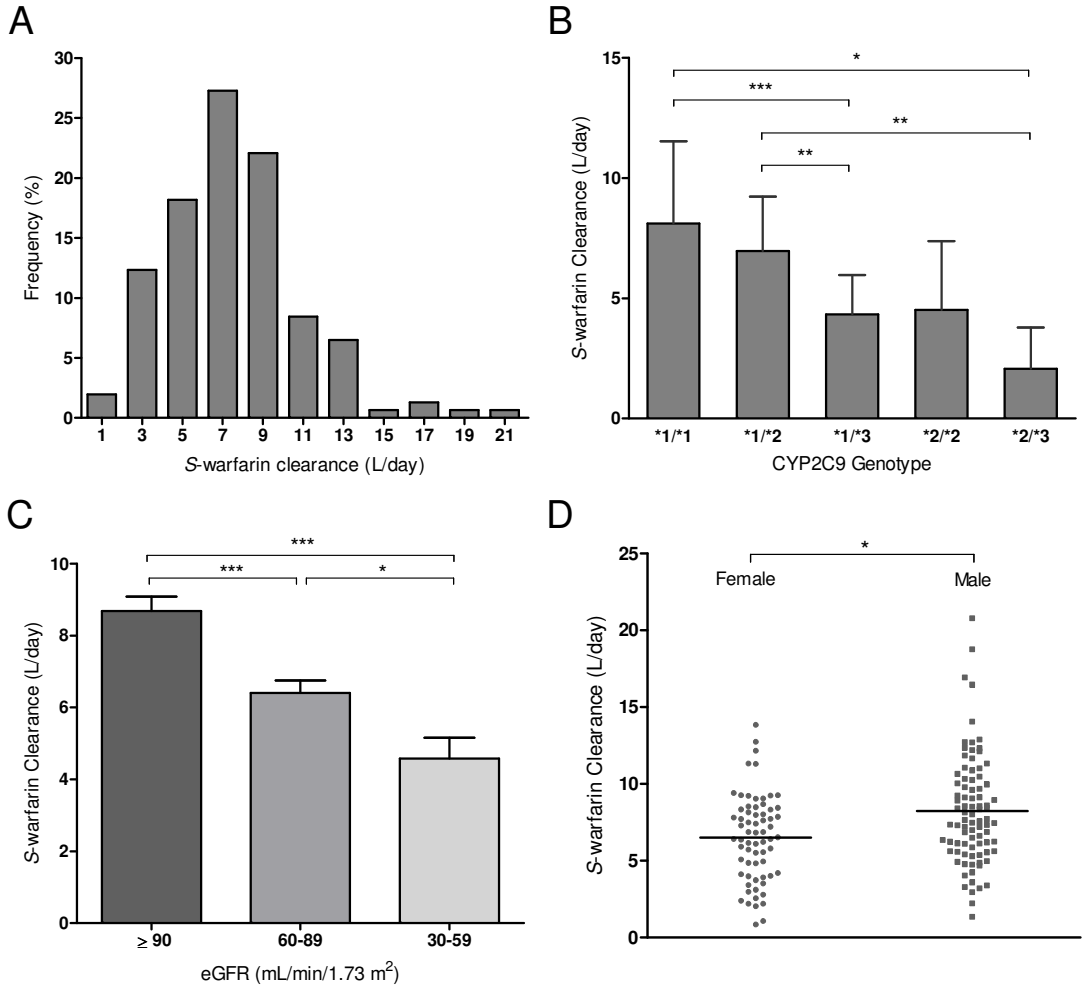

Figure 5.2 Determinants of *S*-warfarin clearance.

Table 5.3.1 Multiple linear regression analysis of independent predictors of S-warfarin clearance (L/day).

Entry into model	Predictor Variable	B	Standard error	95% CI	R^2 after entry (%)	P in final model
-	Intercept	3.105	0.072	2.966, 3.248	-	<0.0001
1	CYP2C9*3, per allele	-0.812	0.151	-1.093, -0.510	14.3	<0.0001
2	eGFR[a]	-0.278	0.080	-0.417, -0.145	24.9	<0.0001
3	Gender (F)	-0.357	0.081	-0.511, -0.186	32.3	<0.0001
4	CYP2C9*2, per allele	-0.274	0.080	-0.427, -0.113	36.5	<0.0001

CI, confidence interval; CYP2C9, cytochrome P450 2C9; eGFR, estimated glomerular filtration rate in mL/min/1.73m^2; F, female.

[a] Coded as follows: ≥ 90 mL/min/1.73 m^2, 0; 60-89, 1; 30-59, 2; 15-29, 3; ≤ 15, 4.

5.3.3 Therapeutic *S*-warfarin plasma concentration correlates with *VKORC1* genotype

S-warfarin plasma concentrations on day 7/8/9 were statistically different between *VKORC1* -1639G>A genotype groups (P<0.0001) despite similar INR (Figure 5.3A). Patients carrying at least one -1639G>A allele required lower plasma concentrations than wild-type patients for similar therapeutic efficacy, and this was gene-dose dependent (Figure 5.3A, 5.3B). The mean plasma *S*-warfarin concentrations for *VKORC1* A/A, G/A, and G/G genotype groups were 0.291 ng/mL (SD 0.157), 0.347 ng/mL (0.170), and 0.503 ng/mL (0.217), corresponding to mean warfarin daily doses of 4.4 mg (2.7), 5.0 mg (2.5) and 7.6 mg (3.0), respectively.

5.3.4 Determinants of *S*-warfarin PD

The mean I_{max} value for subjects was 2.7 (SD 1.0), with a range of 0.3 to 6.9, demonstrating a more than 20-fold interindividual variability in *S*-warfarin PD (Figure 5.3C). A significant relationship between *VKORC1* -1639G>A genotype and I_{max} (Figure 5.3D, P<0.0001) was observed. The mean I_{max} values for *VKORC1* A/A, G/A, and G/G genotypes were 3.7 (SD 1.2), 2.8 (1.0) and 2.2 (0.7), respectively.

Stepwise regression analysis indicated that I_{max} was dependent on *VKORC1* genotype, indication for warfarin, pre-treatment plasma proteins induced by vitamin K absence

(PIVKA-II) concentration, *CYP4F2* 1297C>T genotype, and weight, in order of covariate entry into the regression equation. The r^2 of the final model for I_{max} estimation was 41%. Parameter estimates of the final I_{max} model and the bootstrap validation results are given in Table 5.2. The mean baseline PIVKA-II concentration was 7.1 ng/mL (SD 4.8), with a range of 1.8 to 30.6, indicating that majority of our patients did not exhibit vitamin K deficiency. I_{max} was greater in patients with AF than VTE, denoting that VTE patients were more resistant to warfarin's therapeutic effect. Moreover, there was an additive effect of *VKORC1* genotype and indication, where VTE patients had lower I_{max} than AF patients irrespective of *VKORC1* genotype (Figure 5.3E). VTE patients who were *VKORC1* G/G carriers had the lowest average I_{max} (2.1) while AF and A/A carriers had the highest I_{max} (4.4). These findings imply that differences in INR response would be evident between patients with AF and VTE when warfarin is initiated by a common dosing protocol. Indeed, we found a more rapid response in patients with AF in comparison to VTE over the first week of therapy (Figure 5.3F), despite that the WRAPID protocol eliminated the previously known genetic and clinical contributors to early response variability (29).

Figure 5.3 Determinants of maximal inhibitory factor, I_{max}. (A) Box-and-whisker plots of *S*-warfarin plasma concentration and INR on days 7/8/9 segregated by *VKORC1* -1639G>A genotype. Box-and-whisker plots representing *VKORC1* gene-dose effect during initiation. The top and bottom of the boxes represents 25^{th} and 75^{th} percentile, respectively; median is represented by the middle line, whiskers are the 95% CI, and outliers are identified as closed circles. (B) Warfarin daily dose on days 7/8/9 with respect to *VKORC1* genotype. (C) Frequency distribution of estimated I_{max}, shown as percent of total patients for each bin. (D) Association between *VKORC1* genotype and I_{max}. Results are represented as mean with standard deviation. (E) Additive effect of indication for warfarin therapy and *VKORC1* genotype on I_{max}. (F) INR time course for patients with AF and VTE over the initial 10 days of therapy with common genetics-guided dosing protocol. Results are represented as mean with 95% CI of the standard error. AF, atrial fibrillation; INR, international normalized ratio; VTE, venous thromboembolism. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

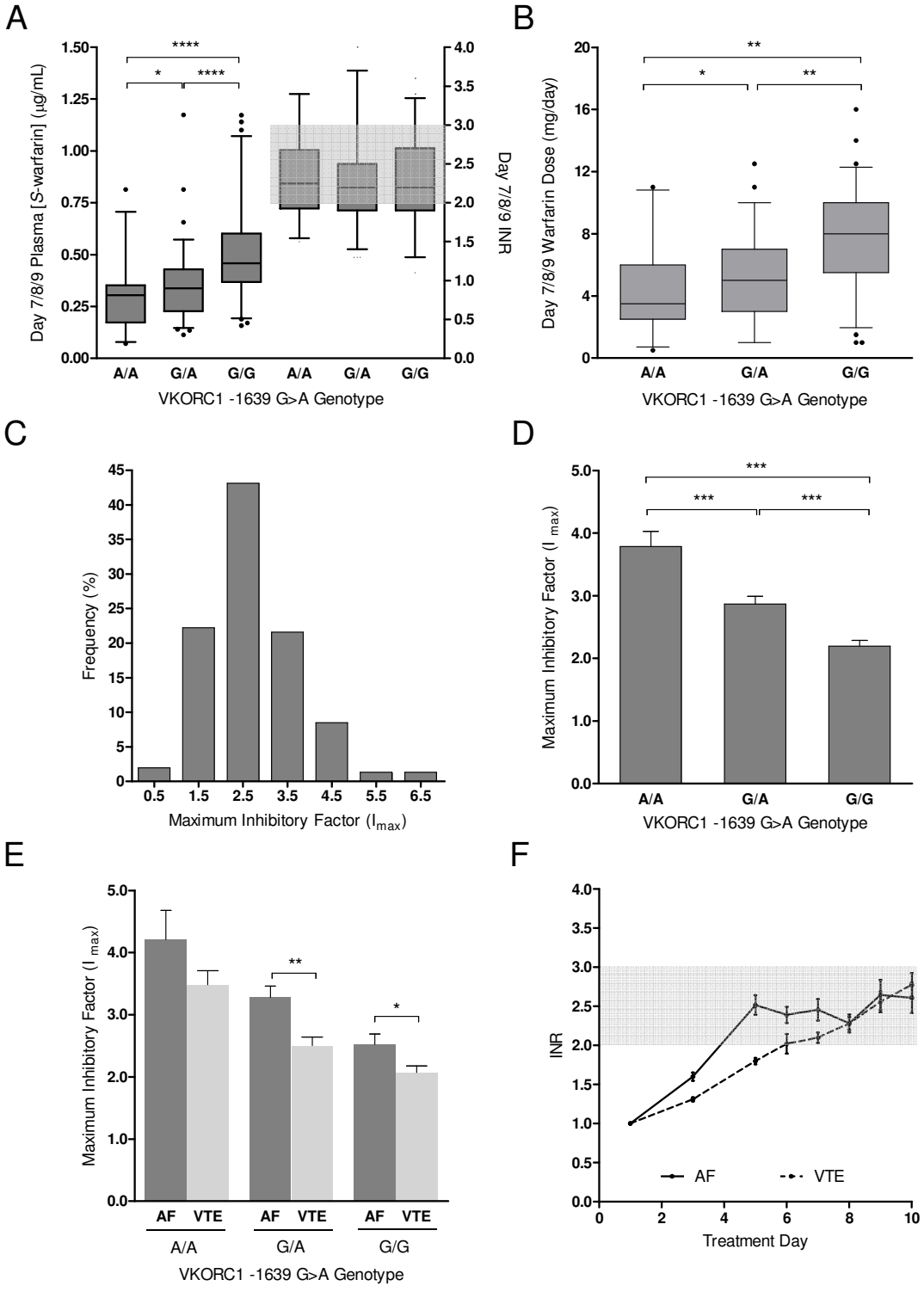

Figure 5.3 Determinants of maximal inhibitory factor, Imax.

Table 5.3.2 Multiple linear regression analysis of independent predictors of I_{max}.

Entry into model	Predictor Variable	B	Standard error	95% CI	R^2 after entry (%)	P in final model
-	Intercept	1.383	0.101	1.156, 1.570	-	<0.0001
1	VKORC1, per allele	0.211	0.030	0.148, 0.267	26.3	<0.0001
2	Indication (VTE)	-0.281	0.049	-0.380, -0.190	34.9	<0.0001
3	PIVKA-II (ng/mL)	0.017	0.007	0.005, 0.033	37.9	<0.05
4	CYP4F2, per allele	-0.072	0.031	-0.133, -0.009	39.6	<0.05
5	Weight (Kg)	0.002	0.001	0.000, 0.004	41.0	<0.05

CI, confidence interval; CYP4F2, cytochrome P450 4F2; I_{max}, maximal inhibitory factor; PIVKA-II, proteins induced by vitamin K absence; VKORC1, vitamin K epoxide reductase complex subunit 1; VTE, venous thromboembolism.

5.3.5 Correlation of VKORC1 genotype to hepatic VKOR protein levels

With Western blot analysis, hepatic microsomal VKOR had electrophoretic mobility consistent with an 18 kDa protein while the two immunoreactive bands observed in over-expressed VKOR control samples likely represent differentially glycosylated forms of the protein (Figure 5.4A, 5.4B, 5.4C). VKOR protein level was significantly correlated to *VKORC1* genotype (Figure 5.4D, $P<0.05$), where the *VKORC1* G allele was associated with higher liver enzyme level than the A allele.

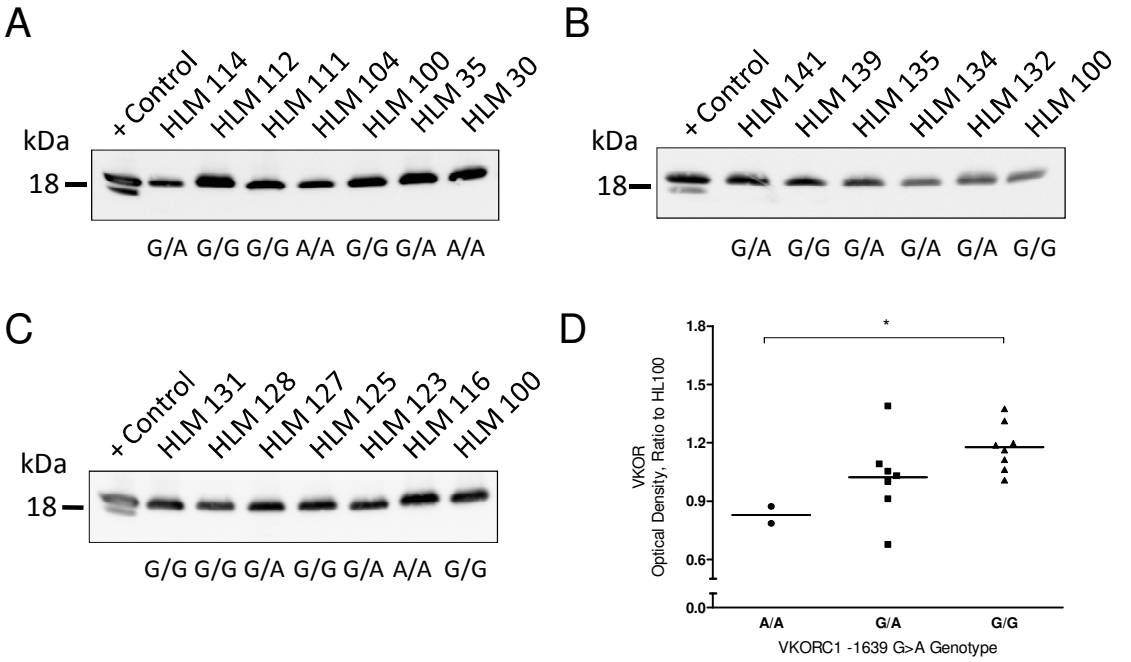

Figure 5.4 The influence of VKORC1 -1639G>A promoter genotype on hepatic VKOR protein expression levels.

(A, B, C) VKOR expression determined in 17 healthy human livers by Western blot analysis. The band intensity was normalized to HLM100. A positive control sample was included on each blot. (D) Semiquantitative measurement of hepatic expression in relation to *VKORC1* genotype. * $P < 0.01$

5.3.6 Simulated anticoagulation response with different warfarin initiation protocols

To demonstrate the utility of the PK-PD model, we simulated and compared INR response profiles of individuals with different combinations of covariates using different published dose initiation schemes (25, 29, 40-42). Specifically, we compared response-time curves of typical patients following initiation with our WRAPID protocol (29), the Kovacs nomogram (non-pharmacogenetics, validated in VTE) with the day 8 dose refinement algorithm (42), and finally, initiation with the pharmacogenetics-based as well as clinical-only maintenance dose algorithms available at www.warfarindosing.org incorporating the recently published day 4 dose refinement algorithm (25, 40). Doses were adjusted according to simulated INR values on days 3, 5, and 8 for WRAPID and Kovacs and on day 4 for warfarindosing.org as per nomogram. Clearance and I_{max} values were calculated based on regression equations (Tables 5.1 and 5.2) for various *VKORC1* and *CYP2C9* genotype combinations in typical AF and VTE patients. Homozygous *CYP2C9**3 patients were not considered in the simulations as we did not encounter such individuals in our population. We used *CYP4F2* wild-type C/C genotype (increased sensitivity) for all calculations of I_{max}. Figure 5.5 illustrates the predicted effect of *VKORC1* or *CYP2C9* variant allele burden on responses of AF and VTE patients initiated with WRAPID nomogram (Figure 5.5A, 5.5B), Kovacs nomogram (Figure 5.5C, 5.5D), warfarindosing.org genetics (Figure 5.5E, 5.5F), and clinical nomogram (Figure 5.5G, 5.5H), respectively. The simulated response curves indicate that increased possession of variant alleles is associated with slightly greater time above therapeutic INR with fixed

10 mg loading doses and iterative response-based Kovacs nomogram than initiation strategies which incorporate genetic and patient factors. In contrast, pharmacogenetics-guided initiation schemes eliminated the genotype-dependent response differences. Furthermore, pharmacogenetics-guided dosing nomograms resulted in comparable rise to optimal anticoagulation response among different genotypes within groups of AF and VTE patients. Evidently, loading dose was not used in simulations of patients initiated with warfarindosing.org and thus, the time to reach optimal anticoagulation was approximately 3 days slower as compared to the WRAPID nomogram. Simulations with the warfarindosing.org clinical algorithm indicate that there would be significant differences in initial INR responses as the burden of genetic variants increases.

Figure 5.5 Model predicted response curves following warfarin initiation using various initiation protocols. Simulations were performed using non-genetics and genetics-based nomograms for typical AF and VTE patients harbouring variable number of variant alleles. The genotype of zero-variant patients is *VKORC1*G/G-*CYP2C9**1/*1. Patients carrying 1 variant allele have one of the following genotype combinations: *VKORC1*G/A-*CYP2C9**1/*1, *VKORC1*G/G-*CYP2C9**1/*2, or *VKORC1*G/G-*CYP2C9**1/*3. Patients carrying 2 variant alleles have one of the following genotype combinations: *VKORC1*A/A-*CYP2C9**1/*1, *VKORC1*G/A-*CYP2C9**1/*2, *VKORC1*G/A-*CYP2C9**1/*3, or *VKORC1*G/G-*CYP2C9**2/*2. Patients carrying 3 variant alleles have one of the following genotype combinations: *VKORC1*A/A-*CYP2C9**1/*2, *VKORC1*A/A-*CYP2C9**1/*3, *VKORC1*G/A-*CYP2C9**2/*2, or *VKORC1*G/A-*CYP2C9**2/*3. Patients carrying 4 variant alleles have one of the following genotype combinations: *VKORC1*A/A-*CYP2C9**2/*2, or *VKORC1*A/A-*CYP2C9**2/*3. AF, atrial fibrillation; VTE, venous thromboembolism.

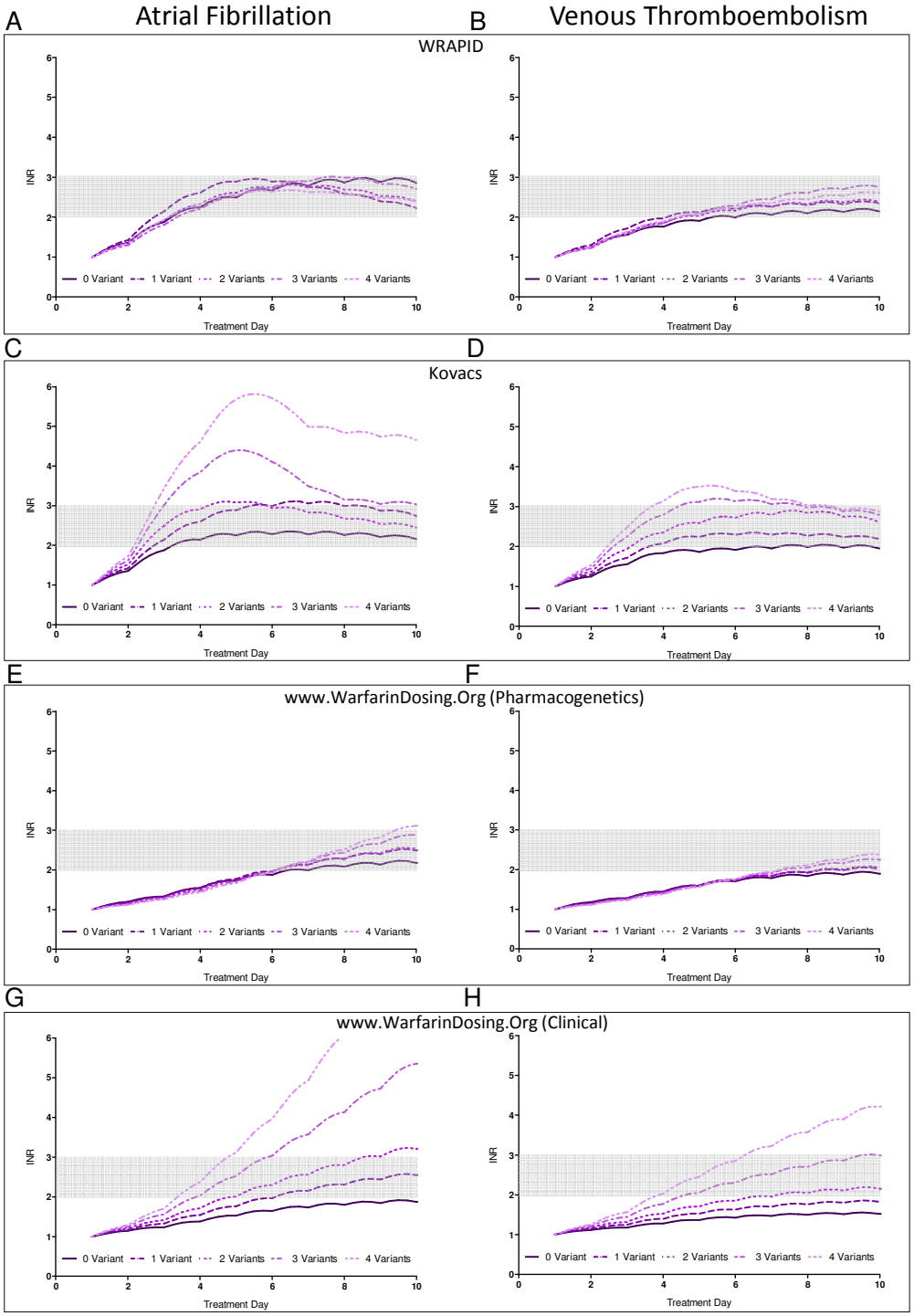

Figure 5.5 Model predicted response curves following warfarin initiation using various initiation protocols.

5.4 Discussion

Warfarin initiation is a challenging therapeutic phase, associated with the highest occurrence of major bleeding events and thromboembolism (43-46). Thus, effective initiation protocols that pre-emptively account and adjust for interindividual variability have significant potential to improve warfarin anticoagulation therapy.

A major contributor to dose requirement and response is *S*-warfarin clearance. The analysis demonstrates that kidney function, gender, *CYP2C9**2 and *3 genotype are major determinants of *S*-warfarin clearance. The finding that *S*-warfarin clearance is reduced in renal impairment supports recent studies that found relationships between both warfarin dose requirement and propensity for over-anticoagulation with kidney function (47, 48). Although age has been correlated with decreased warfarin clearance, we failed to observe this relationship after multivariate regression that included both age and eGFR (7-9, 27). It is plausible that age, as a contributor to clearance, somewhat reflects age-related decline in renal function. Indeed, we note that including eGFR as an additional factor into the regression analysis resulted in 36.5% of clearance variation explained, while only 27.6% of this variation was accounted for when eGFR was absent and age included in the analysis. Interestingly, gender was a significant independent contributor to *S*-warfarin clearance in this study, with females having 22% lower *S*-warfarin clearance than males. While females require lower doses than males for similar anticoagulation quality and efficacy (49), there remain conflicting reports on the role of

gender on *S*-warfarin pharmacokinetics (7, 8, 27). Drug interactions, particularly with amiodarone and antifungals, are significant contributors to variable warfarin response (50-52). Because of the limited number of patients in this cohort taking these medications, we did not find associations between concomitant drugs and warfarin clearance. Larger studies in patients are required to better characterize the quantitative influence of co-administered drugs on warfarin clearance.

While determinants of *S*-warfarin PK have been studied, less is known regarding determinants of the *S*-warfarin plasma concentration-response relationship. We identified *VKORC1*, weight, indication for warfarin, PIVKA-II and *CYP4F2* genotype as significant predictors of *S*-warfarin I_{max}, the PD parameter that governs the magnitude of observed anticoagulation INR response. In addition, we demonstrate that promoter -1639G>A SNP results in lower hepatic VKOR protein expression. In concordance with that previously observed for warfarin-stabilized patients (53), there was a significant relationship between *VKORC1* genotype and *S*-warfarin plasma concentrations at the end of the initiation phase where therapeutic INR was achieved. Taken together, the *VKORC1* -1639G>A promoter SNP confers lower hepatic expression, thus lower plasma *S*-warfarin concentrations and dose required for optimal anticoagulation.

The finding that I_{max} differences exist between AF and VTE patients, following adjustment of confounding variables such as age and weight, suggests that indication for

treatment maybe a prominent contributor to response variability during initiation. This may be attributed to different coaguability states among patients during therapy initiation, in addition to inherent disease differences between the two subsets of patients. Further studies are required to investigate the physiological basis mediating the PD differences between AF and VTE patients. It is also of interest to know whether this dynamic difference would diminish or be maintained throughout the course of anticoagulation therapy. Indeed, indication for anticoagulation was an independent determinant of maintenance dose in the present cohort and one dose algorithm (warfarindosing.org) incorporates VTE as a factor requiring higher warfarin maintenance dose (25). In the present study population, we find a 2.3 mg/day difference in mean maintenance dose between AF and VTE patients. This value is greater than the 0.7 mg/day difference predicted by the WRAPID maintenance dose algorithm when accounting for the average age (21 yrs) and weight (6.5 kg) differences between the AF and VTE groups. While a component of this maintenance dose difference is likely related to the lack of consideration of renal function differences among the disease groups with the WRAPID algorithm, the current PK-PD analysis suggests that indication for anticoagulation remains a contributor to warfarin dose.

In agreement with previous studies linking *CYP4F2* genotype and vitamin K intake to warfarin dose requirement (18, 23, 24), our data demonstrate that pre-treatment plasma PIVKA-II levels and *CYP4F2* 1297G>A genotype affects *S*-warfarin PD sensitivity during initiation. PIVKA-II, a des-carboxylated form of prothrombin, is a direct

biomarker for liver vitamin K status and dietary intake (54). We did not find a relationship between PIVKA-II level and *CYP4F2* genotype during statistical analysis or collinearity during stepwise regression of I_{max} for this cohort of patients. Taken together, our findings highlight the importance of the balance between the VKOR agonist (vitamin K) and antagonistic (warfarin) concentrations in achievement and maintenance of optimal anticoagulation, particularly during initiation.

An important outcome of this study was the formulation of an overall PK-PD model that incorporates the determinants of warfarin kinetics and response. We demonstrate the utility of the model to predict *S*-warfarin concentrations and INR response curves in simulated individuals initiated with different protocols. The simulations predict substantial differences in initial anticoagulation responses depending on the initiation scheme, indication for warfarin treatment and burden of genetic variation in *CYP2C9* and *VKORC1*. Within all of the initiation protocols examined, AF patients would be predicted to have greater initial response than VTE. The indication difference exaggerates the effect of genetic polymorphisms on response especially for initiation protocols that do not consider *CYP2C9* and *VKORC1* genotypes. Interestingly, the simulations indicate that the Kovacs nomogram results in a safe and rapid initiation in VTE patients, consistent with the observed good safety profile in real-world patients (41, 55). On the other hand, the Kovacs nomogram is predicted to be less optimal for in AF as it may pose an over-anticoagulation risk in these patients. Simulations in patients initiated with pharmacogenetics-based dosing algorithms suggest they would be safe and effective

for both patient populations and that the response curves of individuals possessing variant alleles were similar. In comparing initiation with WRAPID and warfarindosing.org, time to therapeutic range was delayed without the use of loading dose. In the case for VTE, delayed attainment of therapeutic INR may have economic consequences as bridging therapy with low molecular weight heparins would need to be extended when loading doses are not administered. The simulations also forecast the time-course of initial INR responses in the large multi-centered randomized clinical trial comparing outcomes between pharmacogenetic and clinical-based warfarin dosing (Clarification of Optimal Anticoagulation Through Genetics, COAG trial) as defined by warfarindosing.org algorithms. The model predicts significant differences in INR response between the two dosing methods during initiation, particularly for patients harbouring variant alleles.

While maintenance dose prediction algorithms typically utilize a number of clinical and genetic parameters, these models are not designed to delineate how each parameter affects warfarin PK, PD or both. The formal PK-PD analysis described herein demonstrates that the interindividual variation in both components of the overall warfarin response can be separated and quantitatively ascribed to respective combinations of non-genetic and genetic factors. Moreover, our integrated PK-PD model allows for robust prediction of INR response profiles particularly during initiation phase of therapy following any initiation-dose scheme, in addition to assessment of covariate effect on responses by altering PK or PD estimates. It should be noted that based on the current model form and input parameters, the PK determinants only account for 36.5% of the

variability observed in *S*-warfarin clearance, while PD determinants accounted for 41% of the I_{max} variation. For this reason, it is expected that the current model would not provide precise response estimations on an individual patient basis due to the large variation still unaccounted for.

In conclusion, the data presented here provides additional insight into the combination of patient characteristics contributing to warfarin PK and PD variability, in turn allowing better prediction of anticoagulation response outcomes without the need for intensive sampling of drug concentrations. Until there is a better understanding of additional determinants of PK and PD variation, and better quantitative characterization of drug-drug interactions, the current model may be useful for predicting outcomes in populations of patients within the context of comparing the effectiveness of various dosing algorithms in early attainment and maintenance of therapeutic INR responses and in guiding future study designs.

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6 ABSENCE OF BOTH MDR1 (ABCB1) AND BCRP (ABCG2) TRANSPORTERS SIGNIFICANTLY ALTER RIVAROXABAN DISPOSITION AND CNS ENTRY[4]

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6.1 Introduction

Thromboembolic events resulting from blood clotting disorders are a significant source of mortality and morbidity and thus often require life-long anticoagulation therapy (1). Although warfarin has been the mainstay of therapy, important limitations in its use have prompted development of newer agents (2). Rivaroxaban is a reversible factor Xa inhibitor recently approved for stroke prevention in atrial fibrillation (AF) patients and treatment of venous thromboembolism (VTE) (3).

Rivaroxaban oral bioavailability was reported to be over 80% and achieves maximal anticoagulation 2-4 hr after administration (4). Excretion of rivaroxaban occurs through two main pathways; cytochrome P450 (CYP) 2J2 and CYP3A4-dependent metabolism are responsible for two-thirds of its elimination while one-third is renally excreted unchanged (4). However, renal elimination may be greater than currently assumed as rivaroxaban exposure is 50% higher in patients with renal impairment (4). Furthermore, bleeding complications were higher in patients with poor renal function (2, 5). Renal excretion appears to be greater than glomerular filtration rate, suggesting a significant contribution of active secretory processes to rivaroxaban elimination (2).

There has been growing appreciation of drug transporters expressed in various tissues in determining the disposition and excretion of a wide range of xenobiotics (6). P-glycoprotein (P-gp), or multi-drug resistance protein 1 (MDR1, ABCB1) and breast cancer resistance protein (BCRP, ABCG2) belong to the ATP-binding cassette family of

efflux transporters. Both are expressed in organs important for drug elimination such as the intestine, liver, and kidney. Moreover, their expression in organs such as the brain and placenta prevent the entry of substrate drugs (7, 8).

Rivaroxaban was recently reported to be a substrate of P-gp (9), and correspondingly, concomitant administration of potent P-gp inhibitors in human beings increases plasma levels by over two times (http://www.ema.europa.eu.proxy1.lib.uwo.ca/docs/en_GB/document_library/EPAR_Product_Information/human/000944/WC500057108.pdf). However, rivaroxaban pharmacokinetics (PK) was not altered in P-gp knockout mice, likely due to involvement of other efflux transporters (9). It is known that P-gp and BCRP exhibit overlapping substrate specificity and tissue expression (10). For common drug substrates, the transporters tend to exhibit a synergistic effect at the blood brain barrier (BBB) (11). Thus, we suggested that BCRP may be the key compensatory transporter for rivaroxaban. Accordingly, we investigated the transcellular transport of rivaroxaban in polarized cell monolayers followed by assessment of the *in vivo* relevance of P-gp and BCRP to rivaroxaban disposition using knockout mouse models.

6.2 Methods

6.2.1 Rivaroxaban permeability in polarized LLCPK, LMDR1 and Caco-2 monolayers

LLCPK, LMDR1 (LLCPK cells over-expressing MDR1) and Caco-2 cells were grown in DMEM (Lonza, Walkersville, MD, USA) supplemented with 10% FBS (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 50 U/mL penicillin (Invitrogen), 50 μg/mL streptomycin (Invitrogen), and 1% non-essential amino acids (Invitrogen) and incubated at 37°C in 5% CO2. LMDR1 cells were cultured with 640 nM vincristine (Sigma-Aldrich, St. Louis, MO, USA) to maintain MDR1 expression. LLCPK and LMDR1 cells were seeded at a density of 2 x 10^5 cells per 12 mm well while Caco-2 cells were seeded at 3 x 10^5 cells/mL onto 0.4 μm porous membrane inserts (BD, Franklin Lakes, NJ, USA). LLCPK and LMDR1 cells were allowed to polarize over a 7-day incubation period while Caco-2 cells were allowed to polarize for 12 days, with media change every 2 days. Prior to commencement of the transport experiment, cells were washed and incubated with Opti-MEM (Invitrogen) for 1 hr. To initiate transport, media from either the basal or apical compartment were replaced with 700 μL Opti-MEM with or without 5 μM rivaroxaban (Toronto Research Chemicals, Toronto, ON, Canada), as appropriate. Cells were incubated at 37°C in 5% CO_2 and transcellular flux was measured in the apical-basal or basal-apical direction over 4 hr by liquid chromatography-tandem mass spectrometry (LC-MS/MS). In LLCPK and LMDR1 monolayers, rivaroxaban transport was also conducted in the presence of the selective P-gp inhibitor LY335979 (12) (1 μM;

Eli Lilly Pharmaceuticals, Indianapolis, IN, USA). In Caco-2 monolayers, rivaroxaban transport was also conducted in the presence of LY335979, the selective BCRP inhibitor, fumitremorgan C (13, 14) (1 μM; Sigma-Aldrich) or both. Digoxin ([^{3}H]-radiolabeled, 1 μM) was used as the positive control. Apparent permeability (P_{app}) was calculated from the following equation: $P_{app} = dQ/dt*1/(A*C_0)$, where dQ/dt is the rate of transport over 4 hr, C_0 is the resultant rivaroxaban concentration, and A is the surface area of the membrane insert.

6.2.2 *In vivo* disposition of rivaroxaban disposition in transporter knockout mice models

Male 6-12 week old Mdr1a-deficient mice ($Mdr1a^{def}$), a subpopulation of CF-1 mouse strain lacking expression of P-glp in the intestine and brain, and age matched male wild-type CF-1 mice were obtained from Charles River Laboratories (Wilmington, MA, USA). Bcrp knockout ($Bcrp^{-/-}$), Mdr1a/Mdr1b/Bcrp triple knockout ($Mdr1a/Mdr1b^{-/-}/Bcrp^{-/-}$) mice, and age-matched (6-12 weeks old) wild-type FVB mice were obtained from Taconic Farms (Hudson, NY, USA). Mice were administered 2 mg/kg of rivaroxaban via oral gavage. Doses were prepared by dissolving rivaroxaban in DMSO (2%) and (1:1 v/v) PEG-400/sterile water. Serial blood samples (60 – 100 μL) were obtained over 4 hr from the saphenous vein and centrifuged to obtain plasma. At 4 hr, mice were killed using isoflurane, blood was collected via cardiac puncture and liver, kidney, and brain tissues were harvested and flash-frozen. Tissues were weighed and homogenized in 5 mM ammonium acetate, pH 4.

6.2.3 Rivaroxaban drug level analysis by LC-MS/MS

Samples from *in vitro* permeability studies, plasma and tissue homogenates were spiked with 15 µL of internal standard (D3-rivaroxaban, 500 ng/mL; Toronto Research Chemicals). Subsequently, acetonitrile was added to samples (3:1 v/v) and centrifuged at 9000 x *g* for 20 min to precipitate protein. The resulting supernatant was added to two volumes of 5 mM ammonium acetate pH 4 and injected into the liquid chromatograph. Analytes were separated with the reverse-phase Hypersil Gold column (50 × 5 mm, 5 µM particle size) using gradient elution with 5 mM ammonium acetate pH 4 and acetonitrile (20 to 80%) in a 6 min run time. The MS was set in positive mode for detection of rivaroxaban and IS with transitions 437 → 144 m/z and 440 → 144 m/z, respectively. The lowest limit of quantification was 1 ng/mL. The interday coefficient of variation and bias of rivaroxaban quality controls were 10.5% and 9.3%, respectively.

6.2.4 Statistical analysis

The Mann-Whitney non-parametric t-test was used to analyse the statistical difference between two groups (Graphpad Prism v5.0, La Jolla, CA, USA). Rivaroxaban clearance was determined by non-compartmental analysis of plasma concentration curves in mice.

6.3 Results

6.3.1 Permeability of rivaroxaban in LLCPK and LMDR1 cells

The ability of P-gp to transport rivaroxaban was assessed using the parental LLCPK and LMDR1 cells. Rivaroxaban flux was markedly greater in the basal-apical direction, where the permeability difference was 5.46 (Figure 6.1A). Modest basal-apical transport was observed in LLCPK cells (1.68), suggesting transport by an endogenously expressed transporter. In the presence of a specific P-gp inhibitor, LY335979, the basal-apical flux in LMDR1 monolayers was attenuated, whereas apical-basal flux was enhanced, abrogating the net efflux (Figure 6.1B). In contrast, little change was observed in the net efflux of rivaroxaban in LLCPK cells in the presence of LY335979. As expected, net flux of digoxin, a well-recognized P-gp substrate, was in the basal-apical direction in LMDR1 cells, which was abrogated with LY335979.

6.3.2 Permeability of rivaroxaban across intestinal Caco-2 cells

Vectorial transport of rivaroxaban was also assessed in the intestinal Caco-2 model, known to express both MDR1 and BCRP. There was significantly greater permeability of rivaroxaban in the basal-apical direction, leading to an efflux ratio of 2.82 (Figure 6.1C). In the presence of either LY335979 or the specific BCRP inhibitor, fumitremorgan C, rivaroxaban net flux was attenuated, reducing the efflux ratio to 1.61 and 1.88, respectively (Figure 6.1D, 6.1E). Moreover, co-administration of both LY335979 and fumitremorgan C with rivaroxaban lead to near complete loss of directional transport difference (1.29) (Figure 6.1F). The permeability data are summarized in Table 6.1.

Figure 6.1 *In vitro* transport of 5 μM rivaroxaban across monolayers. Rivaroxaban transport was assessed in LMDR1 (A) and Caco-2 cells (C). The flux in LMDR1 cells was also evaluated with co-administration of 1 μM of the specific MDR1 inhibitor, LY335979 (B). The flux in Caco-2 cells was also evaluated with co-administration of 1 μM of the specific BCRP inhibitor, fumetrimorgan C (D), 1 μM LY335979 (E) or both (F). Symbols and bars represent the mean and standard error. All transport experiments were conducted in triplicates. ** $P < 0.01$, *** $P < 0.001$

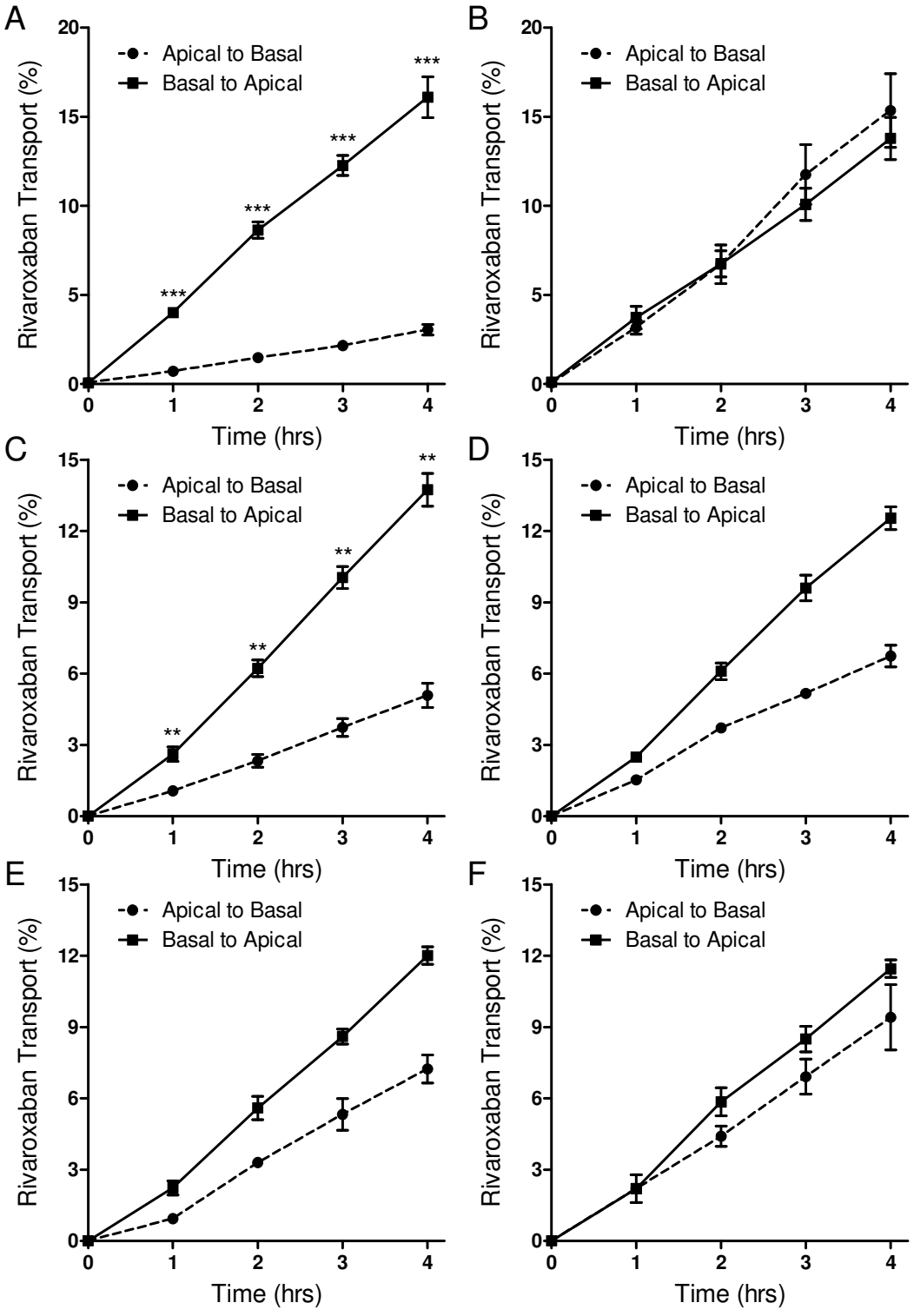

Figure 6.1 In vitro transport of 5 µM rivaroxaban across monolayers.

Table 6.3.1 Apparent permeability of rivaroxaban across cell monolayers.

	P_{app} A-B (SEM)	**P_{app} B-A (SEM)**	**Efflux Ratio (P_{app}B-A/P_{app}A-B)**
	10^{-6} cm/s		
LLCPK			
Rivaroxaban	5.55 (0.49)	9.35 (0.20)	1.68
Rivaroxaban + LY	6.72 (1.16)	8.88 (1.15)	1.32
LMDR1			
Rivaroxaban	2.28 (0.67)	12.44 (2.48)	5.46
Rivaroxaban + LY	12.10 (4.61)	10.40 (1.84)	0.86
[^{3}H]Digoxin	2.59 (0.30)	7.01 (0.50)	2.70
[^{3}H]Digoxin + LY	4.26 (0.29)	4.86 (0.28)	1.14
Caco-2			
Rivaroxaban	3.85 (0.44)	10.85 (0.15)	2.82
Rivaroxaban + LY	5.82 (0.56)	9.37 (0.22)	1.61
Rivaroxaban + FTC	5.29 (0.28)	9.93 (0.27)	1.88
Rivaroxaban + LY + FTC	7.26 (0.90)	9.38 (0.76)	1.29
[^{3}H]Digoxin	2.44 (0.12)	6.16 (0.28)	2.52
[^{3}H]Digoxin + LY	3.57 (0.31)	4.13 (0.45)	1.16

BCRP, breast cancer resistance protein; FTC, fumitremorgan C; LY, LY335979; MDR1, multi-drug resistance protein 1; P_{app}, apparent permeability; S.E.M., standard error of the mean.

6.3.3 Rivaroxaban in vivo disposition in wildtype and knockout mice

To determine the relevance of MDR1 and BCRP *in vivo*, plasma (Table 6.2) and tissue (Table 6.3) concentrations were assessed after rivaroxaban oral administration (2 mg/kg) to wild-type, Mdr1a^{def}, Bcrp$^{-/-}$, and Mdr1a/Mdr1b$^{-/-}$/Bcrp$^{-/-}$ mice (n = 6 per group). Plasma concentrations did not significantly differ between wild-type and Mdr1a^{def} or Bcrp$^{-/-}$ mice (Figure 6.2A, 6.2B). However, the Mdr1a/Mdr1b$^{-/-}$/Bcrp$^{-/-}$ mice appeared to exhibit higher rivaroxaban plasma levels (Figure 6.2C). At 4 hr, rivaroxaban liver-to-plasma ratio was significantly lower in the absence of Mdr1a, while no difference was observed in Bcrp$^{-/-}$ or Mdr1a/Mdr1b$^{-/-}$/Bcrp$^{-/-}$ mice compared with wild-type (Figure 6.3A-C). Rivaroxaban kidney-to-plasma ratio did not differ across the various knockout mice assessed (Figure 6.3D-F). However, kidney rivaroxaban accumulation was significantly higher in Mdr1a/Mdr1b$^{-/-}$/Bcrp$^{-/-}$ mice, likely due to the higher plasma concentration observed in these mice (data not shown). Importantly, rivaroxaban total apparent clearance was only significantly reduced in the absence of both Mdr1 and Bcrp compared with wild-type (Figure 6.2D). Interestingly, rivaroxaban brain-to-plasma ratio was not increased in Mdr1a^{def} or Bcrp$^{-/-}$ mice, but was significantly elevated in Mdr1a/Mdr1b$^{-/-}$ /Bcrp$^{-/-}$ mice by over two times (Figure 6.3G-I).

Table 6.3.2 Mean plasma tissue concentrations of rivaroxaban (ng/mL) after oral administration of 2 mg/kg rivaroxaban (n = 6) in knockout and wild-type mice.

	Time (hr)			
	0.5	1	2	4
$Mdr1a^{def}$				
Wildtype	29.3 (14.7)	20.5 (8.5)	12.1 (3.20	9.7 (2.6)
$Mdr1a^{def}$	32.4 (24.5)	16.8 (7.6)	13.0 (6.2)	7.5 (3.5)
$Bcrp^{-/-}$				
Wildtype	182 (28)	94 (15)	31 (8)	11 (4)
$Bcrp^{-/-}$	133 (38)	85 (30)	26 (6)	18 (10)
$Mdr1a/Mdr1b^{-/-}/Bcrp^{-/-}$				
Wildtype	45.8 (23.7)	27.1 (3.4)	12.3 (3.2)	5.0 (2.2)
$Mdr1a/Mdr1b^{-/-}/Bcrp^{-/-}$	31.6 (12.8)	27.0 (9.4)	19.8 (8.0)	8.5 (2.8)

Bcrp, breast cancer resistance protein; Mdr1a, multi-drug resistance protein isoform 1a; Mdr1a, multi-drug resistance protein isoform 1b.

Table 6.3.3 Mean tissue concentrations of rivaroxaban (ng/mL) 4 hr after oral administration of 2 mg/kg rivaroxaban (n = 6) in knockout and wild-type mice.

	Organ		
	Liver	Kidney	Brain
$Mdr1a^{def}$			
Wildtype	12.5 (3.5)	2.9 (2.1)	0.14 (0.05)
$Mdr1a^{def}$	6.6 (3.0)	4.0 (3.9)	0.18 (0.05)
$Bcrp^{-/-}$			
Wildtype	19.7 (8.2)	3.1 (2.2)	0.7 (0.3)
$Bcrp^{-/-}$	38.6 (18.0)	4.8 (2.3)	0.7 (0.3)
$Mdr1a/Mdr1b^{-/-}/Bcrp^{-/-}$			
Wildtype	4.6 (1.7)	2.5 (0.8)	0.05 (0.01)
$Mdr1a/Mdr1b^{-/-}/Bcrp^{-/-}$	7.7 (1.8)	4.0 (1.0)	0.22 (0.07)

Bcrp, breast cancer resistance protein; Mdr1a, multi-drug resistance protein isoform 1a; Mdr1a, multi-drug resistance protein isoform 1b.

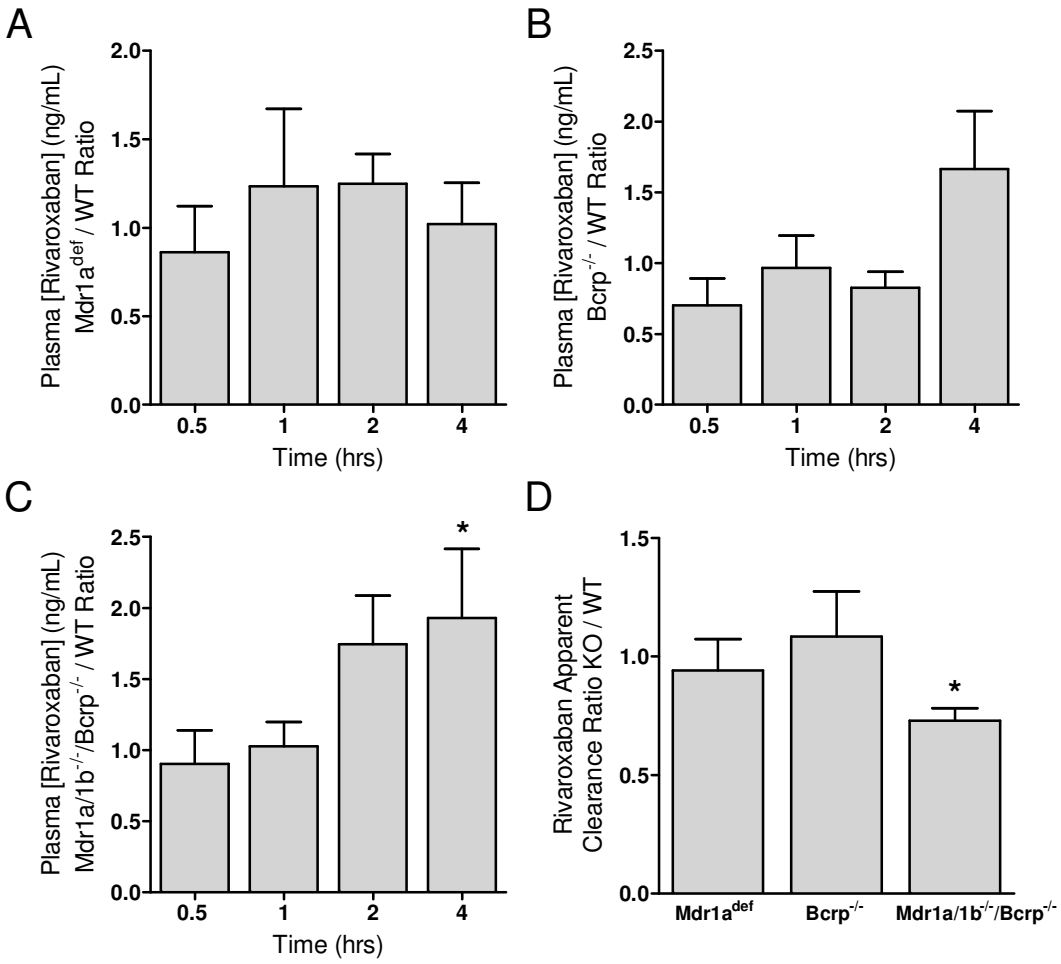

Figure 6.2 Rivaroxaban pharmacokinetics in mice.

Rivaroxaban was administered by oral gavage to wildt-ype, Mdr1a deficient (Mdr1a^{def}), Bcrp$^{-/-}$, and Mdr1a/Mdr1b$^{-/-}$/Bcrp$^{-/-}$ mice (n = 6 for each group). The ratio of rivaroxaban concentration and apparent systemic clearance in knockout and wild-type mice over 4 hr was determined. Data are represented as mean with standard error of the mean. * $P < 0.05$

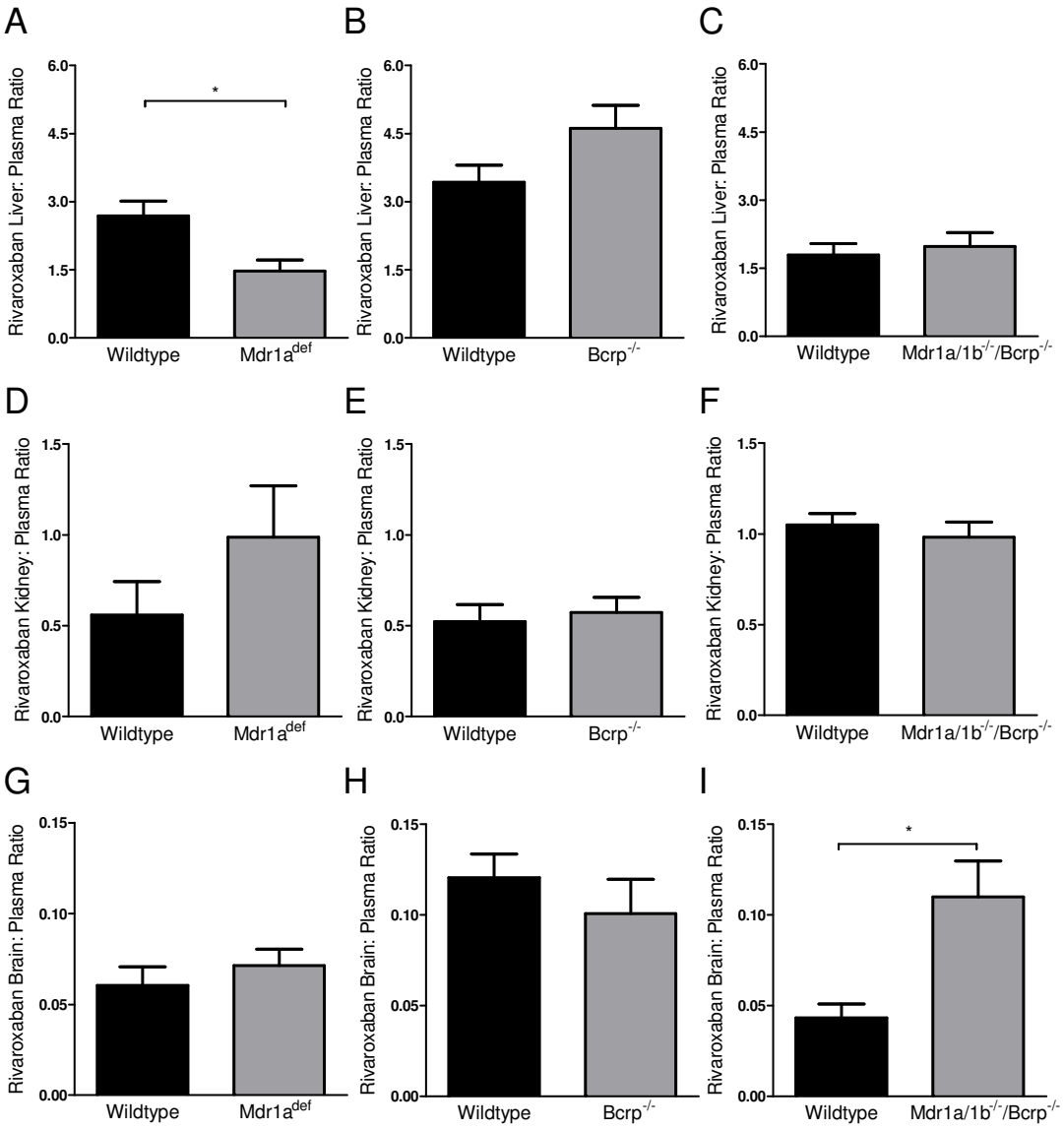

Figure 6.3 Rivaroxaban liver, kidney and brain distribution in mice.

Liver to plasma ratios in Mdr1a^{def} (A), Bcrp$^{-/-}$ (B), Mdr1a/Mdr1b$^{-/-}$/Bcrp$^{-/-}$(C) are compared to wild-type. Kidney to plasma ratios in Mdr1a^{def} (D), Bcrp$^{-/-}$ (E), Mdr1a/Mdr1b$^{-/-}$/Bcrp$^{-/-}$(F) are compared to wild-type. Brain to plasma ratios in Mdr1a^{def} (G), Bcrp$^{-/-}$ (H), Mdr1a/Mdr1b$^{-/-}$/Bcrp$^{-/-}$(I) are compared to wild-type. Data are represented as mean with standard deviation. * $P < 0.05$

6.4 Discussion

In this report, we demonstrate that rivaroxaban is a shared substrate of both P-gp and BCRP. *In vivo* relevance and interplay of both transporters are demonstrated through the observation of a lack of any impact to rivaroxaban clearance in the individual transporter knockout mice, but significantly reduced in the Mdr1a/Mdr1b$^{-/-}$/Bcrp$^{-/-}$ mice. Similar to certain tyrosine kinase inhibitors (11), rivaroxaban accumulation in the brain was considerably higher when both transporters were absent than either one alone, suggesting the collective effect of P-gp and Bcrp in determining rivaroxaban brain exposure in brain.

Although drug interaction studies have traditionally been focused on metabolizing enzyme-mediated interactions, emerging evidence in recent years indicates the important role of drug transporters in modulating pharmacokinetics and hence, efficacy and toxicity (6, 15). Therefore, identification of drug transporters involved in rivaroxaban disposition in addition to metabolizing enzymes is crucial for optimal efficacy while reducing side effects. Accordingly, our findings are of particular importance in predicting rivaroxaban exposure and certain drug drug interactions (DDIs) involving P-gp and BCRP. While anticoagulation therapy effectively reduces ischaemic strokes, the elevated risk of haemorrhage complications paradoxically causes intracranial bleeding, including the life-threatening haemorrhagic stroke in some patients (16). Moreover, the risk of rivaroxaban-associated adverse events was compounded by poor renal function, driven by increased systemic levels of the drug (5). Thus, exposure to rivaroxaban is expected to modulate the extent of bleeding risk in patients. Our findings would suggest that patients at the highest

bleeding risk likely have not only poor renal function but also reduced P-gp and BCRP function, where the net effect would be increased gastrointestinal rivaroxaban absorption leading to increased bioavailability, made worse through reduced rivaroxaban renal secretion.

The lack of dramatic change in rivaroxaban disposition *in vivo* in mice lacking P-gp function is in concordance with a previous report (9). Moreover, there may be rodent and human differences in substrate specificity of P-gp, leading to differential P-gp-mediated transport efficiency (17). Consequently, caution should be used for translating results found in animal models to human beings, particularly for weak substrates of P-gp. Nonetheless, mice deficient in P-gp has been used as a powerful tool for assessing the role of P-gp *in vivo*, likely attributed to the overlapping expression pattern and function between rodents and human P-gp (18). Despite this, the role of P-gp in modulating rivaroxaban efficacy and risk of bleeding complications in human beings should not be overlooked. In fact, concurrent use of rivaroxaban and P-gp inhibitors is contraindicated due to prolonged anticoagulation and increased bleeding risk as a result of elevated plasma levels, as observed with ketoconazole and ritonavir. Given that the bioavailability of rivaroxaban is high, it is likely that the observed clinical interaction is owing to inhibition of metabolism and excretion of rivaroxaban rather than affecting its absorption. We know that P-gp and CYP3A4 share a large number of common inhibitors (19), and thus the over two times increase in rivaroxaban exposure in human beings with co-administration of ketoconazole and ritonavir is likely a result of dual inhibition of P-gp

and CYP3A4. In addition, it is possible that the inability to translate from deficient mouse model to the observed P-gp interaction in human beings is due to the less prominent role of renal excretion to overall rivaroxaban disposition in rodents (20). Thus, although our data indicated that the overall clearance was only modestly reduced even in the Mdr1a/Mdr1b$^{-/-}$/Bcrp$^{-/-}$ knockout mice, the relevance of P-gp and BCRP-mediated rivaroxaban excretion in humans should not be disregarded and requires further assessment.

Drug interactions with BCRP are less likely as a large fraction of BCRP substrates and inhibitors are chemotherapeutic agents (21). However, there are clinically relevant polymorphisms that exist in MDR1 and BCRP, particularly the common reduced function polymorphisms in BCRP (c.34 G>A, c.421 C>T) recognized for affecting the PK of its substrates (22, 23). Thus, genetic variations in efflux transporters may play an important role in determining rivaroxaban exposure, efficacy, and toxicity. Collectively, we postulate that homozygous carriers of BCRP variants concomitantly taking P-gp/CYP3A4 inhibitors likely possess the greatest risk for haemorrhage. This is especially a concern for AF patients, the predominant disease of the elderly with declining renal function, whereby comorbidities and concomitant use of P-gp/CYP3A4 inhibiting medications may be difficult to avoid. Our findings would suggest that the highest at risk subset of such patients would be those who carry loss of function polymorphisms in BCRP.

Additionally, higher rivaroxaban entry in central nervous system (CNS) in Mdr1a/Mdr1b$^{-/-}$/Bcrp$^{-/-}$ mice suggests that patients with attenuated P-gp and BCRP transport function could potentially be at greater risk of intracranial haemorrhage. Modulation of P-gp function with use of inhibitors has indeed been shown to allow greater CNS access for P-gp substrates, such as HIV protease inhibitors and loperamide (12, 24). The mechanisms underlying haemorrhagic strokes have not been clearly delineated; however, oral anticoagulant-associated haemorrhage has been suspected to be due to increased haematoma expansion, particularly in patients with over-anticoagulation as a result of elevated systemic exposure of the drug (25). Moreover, rivaroxaban was developed with the intent of eliminating routine response monitoring. Hence, evaluation of anticoagulation efficacy and bleeding risk in patients will likely be problematic in real-world patients without the use of a standardized test. Furthermore, the lack of a validated antidote for reversing rivaroxaban overdose will likely potentiate the danger of bleeding events (26). Thus, similar to other new anticoagulant agents (27), there is a need for post-marketing surveillance of adverse events, including analysis of bleeding events within the context of drug transporter pharmacogenetics, to more fully delineate rivaroxaban safety and efficacy. Future clinical studies are required to elucidate the combined effect of P-gp and BCRP in modulating rivaroxaban exposure in plasma, bleeding complications, and anticoagulation efficacy.

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7 CLARIFYING THE IMPORTANCE OF CYP2C19 AND PON1 IN THE MECHANISM OF CLOPIDOGREL BIOACTIVATION AND IN VIVO ANTIPLATELET RESPONSE[5]

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7.1 Introduction

Antiplatelet therapy is an important therapeutic intervention for prevention of ischaemic events in patients with high-risk cardiovascular disease, particularly for those who undergo percutaneous coronary intervention (PCI) (1). Currently, the standard of care for managing such patients is dual antiplatelet therapy with a $P2Y_{12}$ receptor antagonist and the cyclooxygenase I inhibitor aspirin. Clopidogrel is the most widely prescribed thienopyridine that exerts its pharmacological effect by irreversibly binding to $P2Y_{12}$ receptors on platelets, thereby diminishing adenosine diphosphate (ADP)-mediated platelet aggregation (2). Although benefits from clopidogrel have been widely documented in large clinical trials, marked interpatient variation in platelet responsiveness has meant that 21% of the patients remain at risk for coronary artery and stent thrombosis (3).

Clopidogrel is a prodrug and its clinical efficacy appears to be a function of the amount of enzymatically derived active thiol metabolite formed (4, 5). Previous *in vitro* studies have delineated that this bioactivation is a two-step process, catalysed by several cytochrome P450 (CYP) isozymes (6-8). Clopidogrel is first metabolized to the intermediate metabolite, 2-oxo-clopidogrel, followed by metabolism to a number of thiol metabolite stereoisomers, only one of which (H4) is active *in vivo* (2, 5, 9). Notably, both metabolic steps leading to H4 formation have been shown to be predominantly dependent on CYP2C19 and to a lesser extent CYP3A4 (6). Importantly, in large clinical trials, *CYP2C19**2 or *3 loss-of-function single nucleotide polymorphisms (SNPs) have been

associated with lower platelet inhibition and consequently, an increased risk of major cardiovascular events (10-12).

In contrast, Bouman *et al.* recently demonstrated that a non-CYP enzyme, paraoxonase-1 (PON1) was the key determinant of clopidogrel active metabolite formation (13). Importantly, they showed that plasma PON1 activity as well as the Q192R SNP (rs662) in *PON1*, but not *CYP2C19* SNPs was predictive of antiplatelet response and risk for stent thrombosis in clopidogrel-treated patients. These findings fundamentally challenged our prior understanding of clopidogrel metabolism and efficacy. In the report, we set out to define a mechanistic link between clopidogrel metabolism and antiplatelet action to clarify the clinical relevance of *PON1* and *CYP2C19* to clopidogrel response.

7.2 Methods

7.2.1 Clinical study design

The study was approved by the Research Ethics Board at the University of Western Ontario. All subjects (age 18-65) were non-smokers, not taking concomitant medications, without previous exposure to clopidogrel, and deemed healthy per medical exam. Healthy volunteers who met the eligibility criteria were enrolled upon provision of written informed consent (n = 21; supplemental material, Table 7.3).

Overnight fasted subjects received a single oral dose of clopidogrel (75 mg). In addition, 100 µg of midazolam was administered orally as an *in vivo* probe for CYP3A4 activity. For pharmacokinetic analysis, blood samples were collected over an 8 h period. Clopidogrel thiol metabolites were stabilized for analysis using EDTA tubes containing 50 µL of 125 mM 2-bromo-3-methoxyacetophenone (MPB) (Sigma-Aldrich, Oakville, Canada).

To determine antiplatelet response, blood was collected using a 1.8 ml sodium citrate (3.2%) tube at baseline and 4 hours post-clopidogrel dose and subjected to the VerifyNow $P2Y_{12}$ assay (Accumetrics, San Diego, CA, USA), as per the manufacturer's protocol.

Genotype analysis and plasma paraoxonase activity were determined as described in supplemental materials.

7.2.2 Clopidogrel bioactivation

The *in vitro* metabolic profiling of clopidogrel metabolism was conducted in microsomes (supplemental material).

7.2.3 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) analysis

Quantitation of midazolam and clopidogrel metabolites (*in vivo* and *in vitro*) was performed as described in supplemental materials.

7.2.4 Data analysis

All data analyses are as described in supplemental material.

7.3 Results

7.3.1 Influence of *CYP2C19*, *PON1,* and CYP3A4 on clopidogrel kinetics and response

Following a 75 mg dose, carriers of at least one reduced function *CYP2C19* allele [*CYP2C19**2 or *3 allele, reduced metabolizers (RMs); 38% of the study population] had significantly decreased total plasma exposure (area under the plasma concentration curve, AUC) of the H4 active metabolite when compared with the non-carrier extensive metabolizers (EMs) (Figure 7.1A; supplemental material, Figure 7.6). Similarly, the maximum plasma concentration (C_{max}) was higher in EMs than RMs (Table 7.1). Prior to administration of clopidogrel, the mean platelet responsiveness [platelet reactive units (PRU)] induced by 20 µmol/L ADP and 22 nM prostaglandin E1 (PGE1) was similar between EMs and RMs (VerifyNow $P2Y_{12}$ assay; Table 7.2). Four hours following clopidogrel administration, the absolute percentage change in PRU was significantly lower in RMs when compared with EMs (Figure 7.1B). In fact, we observed a strong correlation between H4 plasma exposure and platelet inhibition, demonstrating that individuals with highest exposure to H4 active metabolite have the greatest antiplatelet response (Figure 7.1C). Interestingly, we did not observe any correlation between gain-of-function *PON1* Q192R polymorphism and clopidogrel pharmacokinetics or response, despite the fact that PON1 plasma activity, assessed ex vivo using paraoxon as the prototypical substrate, in the same healthy volunteers correlated well with the *PON1* Q192R genotype (Figure 7.1D, 7.1E; supplemental material, Figure 7.6). In addition, no significant correlation was found between paraoxonase plasma activity and antiplatelet

response (Figure 7.1F). Of note, exclusion of non-Caucasian participants in these analyses does not modify the above findings (data not shown).

Midazolam plasma AUC was not related to H4 AUC or antiplatelet response (P = 0.91, 0.65) (Figure 7.2).

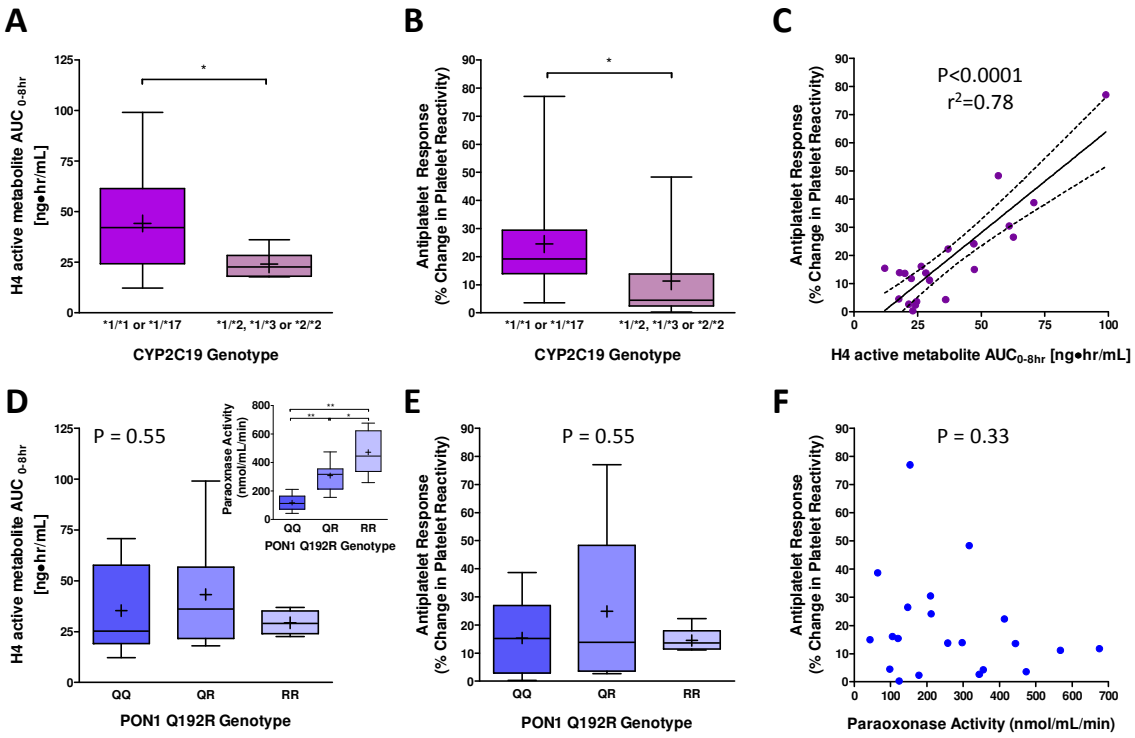

Figure 7.1 The role of CYP2C19 and PON1 genetic polymorphisms in clopidogrel pharmacokinetic and pharmacodynamic responses.

(A and B) Box-and-whisker plots of total active metabolite H4 plasma exposure (AUC) and antiplatelet response according to *CYP2C19* genotype. (C) Scatter plot of H4 AUC and antiplatelet response (r^2 = 0.78). (D and E) Box-and-whisker plots of H4 AUC, paraoxonase plasma activity, and antiplatelet response according to the *PON1* genotype. (F) Scatter plot of plasma paraoxonase activity and antiplatelet response. Boxes indicate 25th and 75th percentile, whiskers denote the 95% confidence interval, and '+' represents the mean. *$P < 0.05$; **$P < 0.01$.

Table 7.3.1 H4 active metabolite pharmacokinetic parameters following administration of a single 75 mg oral dose of clopidogrel.

C_{max}, (ng/mL)	33.9 (21.0)	P-Value
CYP2C19		
EM	40.2 (23.3)	
RM	25.5 (13.7)	0.05
PON1		
Q192Q	30.34 (19.80)	
Q192R	41.84 (30.67)	
R192R	27.70 (6.08)	0.55
t_{max}, (h)	0.73 (0.18)	
CYP2C19		
EM	0.77 (0.16)	
RM	0.67 (0.17)	0.18
PON1		
Q192Q	0.78 (0.16)	
Q192R	0.68 (0.19)	
R192R	0.65 (0.13)	0.33
$t_{1/2}$ (h)	0.67 (0.19)	
CYP2C19		
EM	0.67 (0.20)	
RM	0.68 (0.19)	0.91
PON1		

Q192Q	0.63 (0.25)	
Q192R	0.69 (0.20)	
R192R	0.67 (0.16)	0.77
$AUC_{0\text{-}8\ hr}$ (ng h/mL)	37.40 (21.97)	
CYP2C19		
EM	44.79 (25.09)	
RM	27.54 (12.27)	0.03
PON1		
Q192Q	35.31 (21.57)	
Q192R	43.27 (28.33)	
R192R	27.47 (6.64)	0.66

C_{max}, maximum plasma concentration; EM, extensive metabolizers (CYP2C19 *1/*1 or *1/*17); RM, reduced metabolizers (*1/*2, *2/*2 or *1/*3); t_{max}, time to C_{max}; $t_{1/2}$, half-life; $AUC_{0\rightarrow 8\ hr}$, area under the plasma concentration curve. Data are represented as mean with standard deviation.

Table 7.3.2 Platelet response pre- and 4 h post-clopidogrel administration.

Pre-dose PRU		*P*-value
CYP2C19		
EM	348 (32)	
RM	315 (43)	0.30
PON1		
Q192Q	343 (41)	
Q192R	306 (34)	
R192R	361 (29)	0.07
Antiplatelet response (absolute % change PRU)		
CYP2C19		
EM	24.5 (19.0)	
RM	11.31 (14.82)	0.02
PON1		
Q192Q	15.4 (13.4)	
Q192R	24.8 (28.2)	
R192R	14.51 (4.5)	0.55

EM, extensive metabolizers; RM, reduced metabolizers; PRU, platelet reactive units. Data are represented as mean with standard deviation.

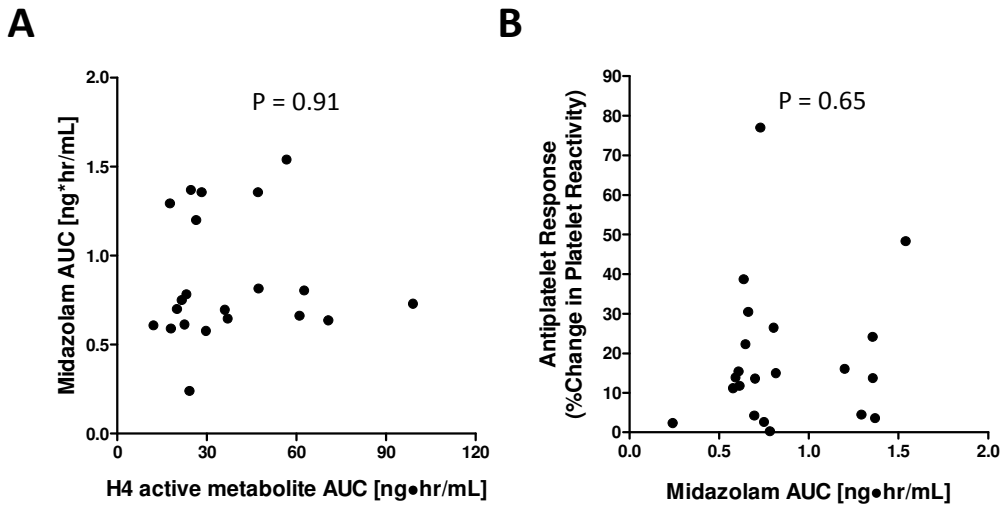

Figure 7.2 The role of CYP3A4 activity in clopidogrel pharmacokinetics and pharmacodynamics.

(A and B) Scatter plot of total midazolam plasma exposure and H4 active metabolite exposure, or antiplatelet response, respectively.

7.3.2 Identification of other clopidogrel thiol metabolites in plasma

Using an ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method, we were able to detect and quantify other clopidogrel thiol metabolites isobaric to H4 in the plasma of study subjects (Figure 7.3A) (9). One metabolite, known to be inactive is termed H3, is stereochemically similar to H4 except being diastereomeric at the carbon 4 position. Another observed thiol metabolite is termed Endo, which differs from H3/H4 in that the carbon double bond is in the endocyclic position. In addition to chromatographic separation of H3, H4 and Endo (Figure 7.3C, 7.3E), the MS fragmentation signatures of the H3/H4 and Endo thiol metabolite were distinct (Figure 7.3D, 7.3F), ensuring analytical specificity of the isomers being analysed.

Figure 7.3 Representative chromatograms of derivatized H4 and Endo metabolite. (A) Chromatogram of a human plasma sample derivatized with 2-bromo-3-methoxyacetophenone. (B) Chromatogram of a sample derived from PON1-mediated hydrolysis of 2-oxo-clopidogrel. (C and D) Chromatogram of Endo standard and its MS/MS fragmentation signature. (E and F) Chromatogram of H4 standard and its MS/MS fragmentation signature.

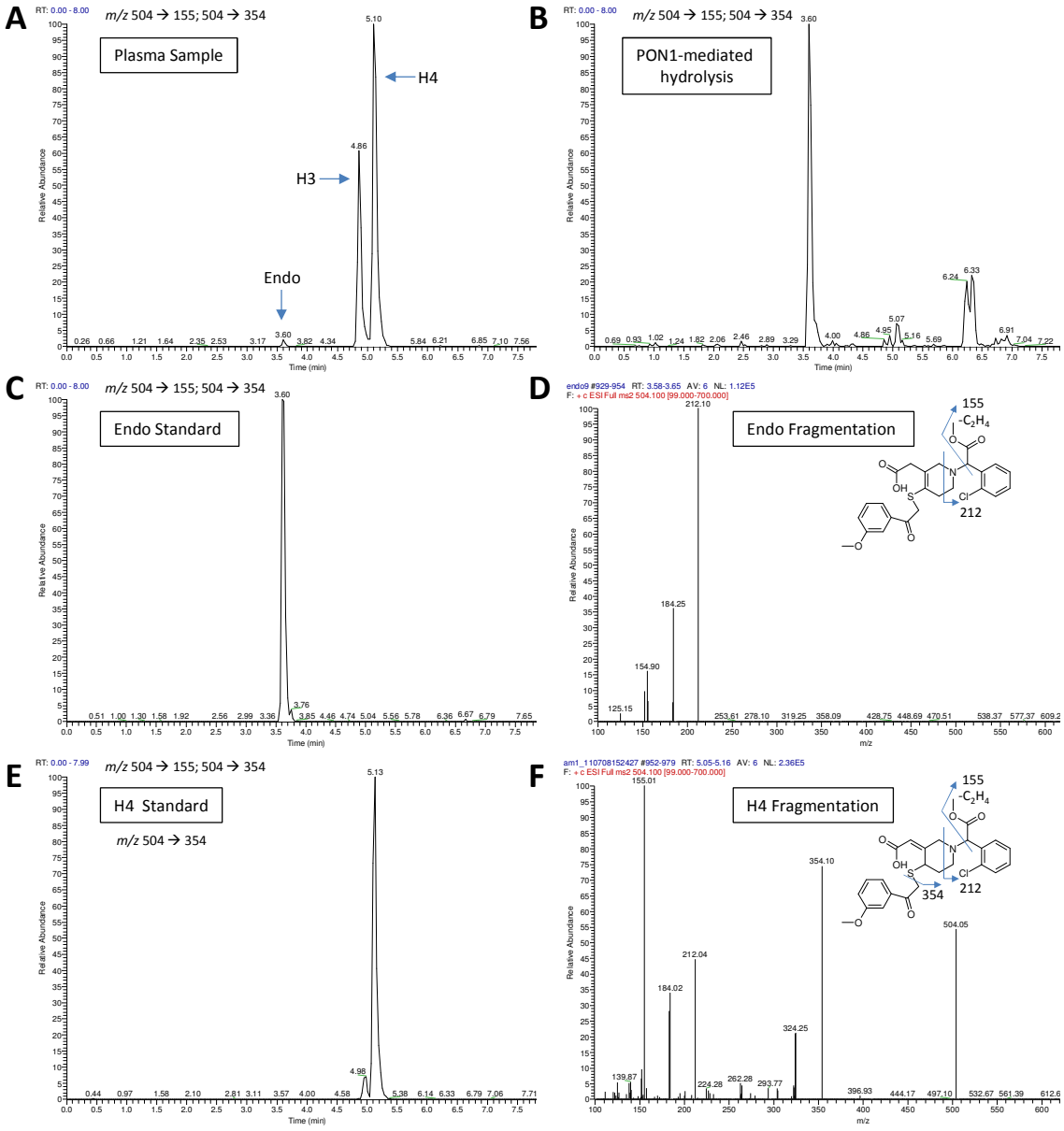

Figure 7.3 Representative chromatograms of derivatized H4 and Endo metabolite.

7.3.3 Biotransformation of clopidogrel to 2-oxo-clopidogrel

In the first step of the bioactivation process, the intrinsic clearances (CL_{int}) calculated from estimated K_m and V_{max} values show that CYP2C19 is more efficient in forming the intermediate metabolite than CYP3A4 *in vitro* (Figure 7.4A; supplemental material, Table 7.4).

7.3.4 Biotransformation of 2-oxo-clopidogrel to H3 and H4 thiol metabolites

In the second step of the bioactivation process, the CL_{int} for H4 formation from 2-oxo-clopidogrel by CYP2C19 was greater than that with CYP3A4 (Figure 7.4B; see Supplemental material, Table 7.4). Notably, the formation of H4 from 2-oxo-clopidogrel was dependent on the presence of 5 mM reduced glutathione (GSH) (data not shown). Based on lower H4 formation compared with 2-oxo-clopidogrel formation, it appears that the second reaction is the rate-limiting step of the overall clopidogrel bioactivation. We observed that the inactive metabolite, H3, was also formed from 2-oxo-clopidogrel by CYP2C19 at a relatively similar efficiency as H4 (supplemental material, Table 7.4).

7.3.5 Biotransformation of 2-oxo-clopidogrel to Endo thiol metabolite

We incubated a range of 2-oxo-clopidogrel concentrations with baculovirus microsomes heterologously expressing CYP2C19 and HeLa cell-derived microsomes constitutively expressing PON1 while lacking any drug-metabolizing CYP enzymes. In the baculovirus microsomes, Endo metabolite formation was greater with CYP2C19 than in control baculovirus microsomes (Figure 7.4C; supplemental material, Table 7.5). In addition, HeLa cell microsomes constitutively expressing PON1 were capable of forming Endo metabolite but not H4 (Figure 7.3B, 7.4D; supplemental material, Table 7.5). We further confirmed the ability of PON1 to hydrolyse 2-oxo-clopidogrel using an adenovirus overexpressing system in HeLa cells, where Endo formation was 3.5-fold higher than vector control (LacZ) and no H4 was detected (Figure 7.4E). Moreover, Endo formation by PON1 was attenuated by the specific PON1 inhibitor 2-hydroxyquinoline (Figure 7.4E). We note that PON1-mediated Endo formation was not dependent on GSH (data not shown). Overall, our data suggests that PON1 can hydrolyse 2-oxo-clopidogrel to form Endo metabolite while unable to mediate H4 formation and that CYP2C19 also catalyses Endo formation. Analysis of total Endo plasma exposure in healthy volunteers demonstrated that its levels were more than 20-fold lower than H4 (data not shown), and unlike H4 (Figure 7.1C), Endo AUC did not correlate with antiplatelet response (Figure 7.4F).

Figure 7.4 Clopidogrel bioactivation *in vitro*. (A) Formation of 2-oxo-clopidogrel by CYP2C19 and CYP3A4. (B) Formation of H4 active metabolite by CYP2C19 and CYP3A4. (C) Formation of the Endo metabolite by CYP2C19 and CYP-lacking baculovirus insect-cell microsomes. (D) 2-oxo-clopidogrel metabolism to Endo metabolite by HeLa cell-line derived microsomes. (e) PON1-mediated hydrolysis of 2-oxo-clopidogrel to the Endo metabolite in the absence or presence of PON1 inhibitor, 2-hydroxyquinoline. Symbols and bars represent means and standard errors. (F) Scatter plot of Endo metabolite plasma exposure and antiplatelet response. $*P < 0.001$.

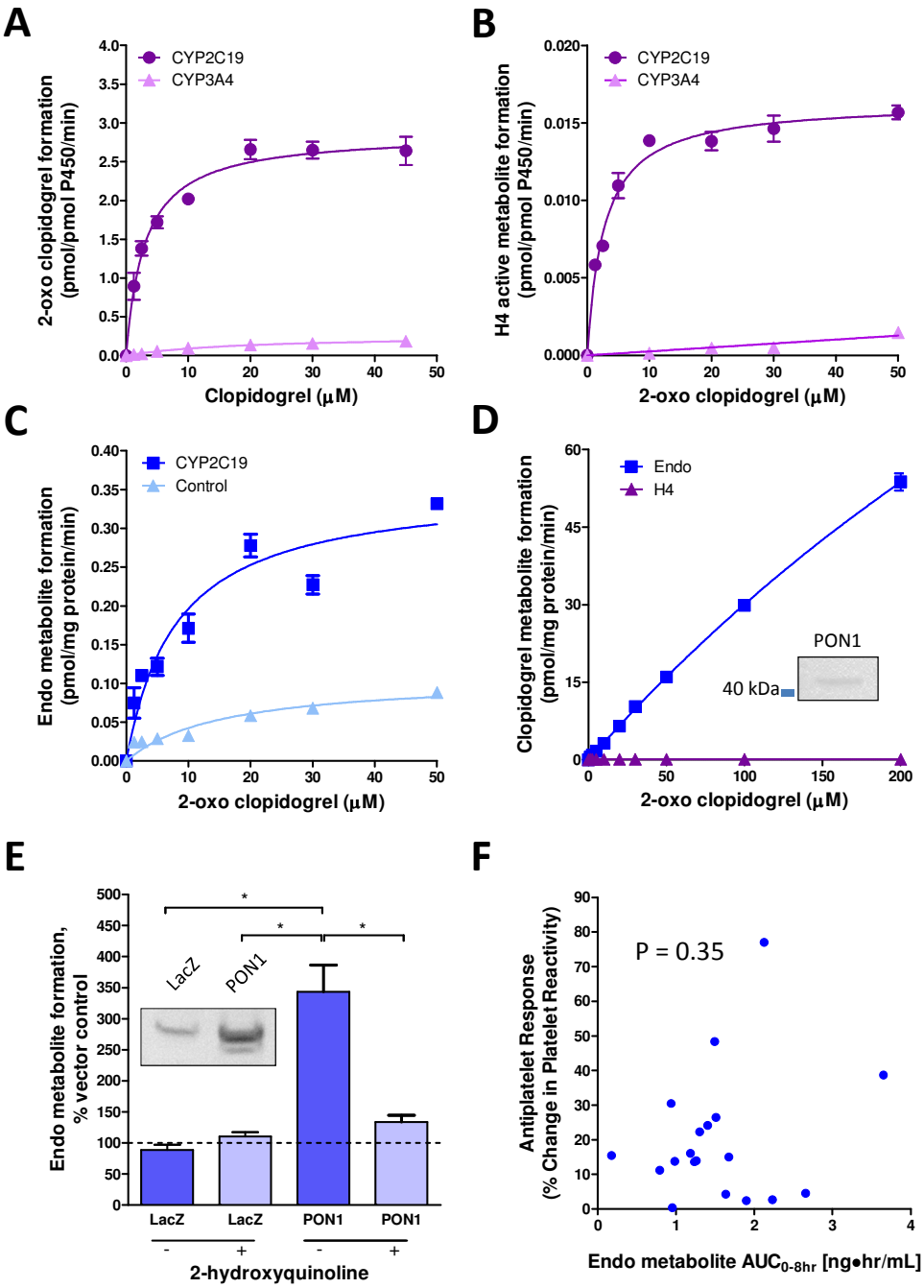

Figure 7.4 Clopidogrel bioactivation in vitro.

7.4 Discussion

The impact of *CYP2C19* genetic variations on clopidogrel antiplatelet response has been documented in a number of studies (11, 14). In fact, a recent meta-analysis reported that carriers of *CYP2C19**2 allele had a higher risk for major adverse cardiovascular events, increased mortality and stent thrombosis compared with non-carriers, independent of baseline cardiovascular risk (14). We note that in contrast to these studies, one trial (CURE) reported similar clopidogrel efficacy irrespective of the *CYP2C19* genotype (15). A potential explanation for the disparate findings is the difference in rate of PCI with stenting, where only 14.5% of the population underwent stenting in the CURE trial while majority of patients underwent stenting in other *CYP2C19*-supportive trials. Indeed, it has been consistently shown that the greatest clinical benefit with clopidogrel use is the reduction in stent thrombosis rate (16). Moreover, several prospective trials have restored diminished H4 exposure and poor antiplatelet response in *CYP2C19* variant carriers by increasing clopidogrel dose, including one recently published multi-centre double-blinded randomized clinical trial (17-19). However, our understanding of clopidogrel response in the context of pharmacogenomics was further complicated when Bouman *et al.* challenged the aforementioned findings by identifying the *PON1* Q192R polymorphism as the only genetic marker associated with stent thrombosis, accounting for 72.5% of the response variation.

In the present study, we aimed to determine the influence of CYP2C19 and PON1 on clopidogrel metabolism and antiplatelet response. In contrast to Bouman *et al.*, our results support the notion that decreased CYP2C19 function reduces the formation of clopidogrel active thiol metabolite, while PON1 showed no effect on active metabolite exposure. It should be noted that there are several challenges to accurate quantification of the active metabolite, H4. First, H4 is one of several diastereomeric thiol metabolites of clopidogrel observed during *in vitro* metabolomic analysis that requires analytical techniques capable of distinguishing the related species (5, 9). Secondly, due to the reactive nature of the free thiol metabolites, derivatization using alkylating agents, such as MPB, has been required to trap the metabolite for quantitation (9). In the current study, we followed a recently published stereoselective UHPLC-MS/MS method to determine plasma concentrations of clopidogrel thiol metabolites (H4, H3, and Endo) in our cohort (9). In the study by Bouman *et al.*, stereo-specific separation of H3, H4, and Endo was not demonstrated, thus resulting in inaccurate clopidogrel pharmacokinetic analysis (13). Furthermore, they used an alternative method of stabilizing the active metabolite for quantitation and thus, these technical discrepancies may in part explain the discordant findings with regards to contribution of CYP2C19 and PON1 to clopidogrel pharmacokinetics.

Similar to a number of recent studies that refuted a clinically relevant role of *PON1* genotypes to clopidogrel response, we show that *CYP2C19* but not *PON1* genotype is related to ADP-induced antiplatelet response (20-22). This agrees well with a recently published genome-wide association study in a healthy Amish population showing that the

*CYP2C19**2 allele had a significant association with clopidogrel platelet aggregation, while the *PON1* genotype did not (23). The strong correlation between H4 exposure and antiplatelet response (r^2=0.78) presented here suggests that known genetic variation in *CYP2C19* as well as interpatient variation in expression and activity of this enzyme likely account for clopidogrel resistance observed in nonresponders.

The role of CYP3A4 in clopidogrel pharmacokinetics and pharmacodynamics remains unclear. While some studies postulate that *CYP3A4* genotype and inhibition modulates clopidogrel pharmacokinetics and action (24), many others do not (25). Midazolam has been well documented as an *in vivo* probe drug for CYP3A4 activity (26). Thus, administration of a microdose of midazolam was used here to measure CYP3A4 phenotype in subjects. To the extent of our knowledge, we demonstrate for the first time that CYP3A4 activity, as measured by midazolam exposure, is not an important driver of clopidogrel pharmacokinetics or antiplatelet response.

Recently, a correspondence from Dansette *et al.* questioned the validity of the original findings of Bouman and colleagues (27). Specifically, they report that the Endo metabolite, but not other thiol metabolites is formed in human liver microsomes incubated with 2-oxo-clopidogrel in the absence of CYP-requiring NADPH. This Endo formation was attenuated in the presence of the PON1 substrate paraoxon but unaffected by the presence of a CYP inhibitor. Moreover, Dansette and colleagues found that the Endo metabolite was generated in serum (devoid of CYP enzymes) upon ex vivo

incubation with 2-oxo-clopidogrel. Those results indicate a role for PON1 in the formation of the Endo metabolite but not in the bioactivation to H4. The results from the current study not only confirm the findings of Dansette *et al.*, but solidify PON1 as the key player in Endo but not H4 generation through systematic and definitive metabolic experiments that include a PON1 overexpression system and recombinant CYP enzymes.

We believe that lack of analytical specificity, followed by misidentification of the synthesized analytical standard used to quantify clopidogrel active metabolite levels may have been the critical missteps which led to the conclusion by Bouman *et al.* that PON1 generates the active metabolites [16]. First, the conditions for the separation of H4 from other structurally similar, but inactive metabolites requires high-resolution chromatographic techniques such as UHPLC coupled with MS/MS. The liquid chromatographic method used by Bouman *et al.* was likely insufficient for discriminating between active H4 from the inactive H3 and Endo metabolites. It is also not certain whether the analytical method used could quantify non-Endo thiol metabolites such as H4. Secondly, Bouman *et al.* obtained their thiol metabolite reference standard from purification of PON1-mediated 2-oxo-clopidogrel hydrolysis [16]. They suggest that this product was active based on platelet reactivity experiments. However, those experiments were performed at purified thiol metabolite concentrations of 2 mg/L, which is 100 times greater than the reported plasma thiol metabolite concentration. Since we clearly show that PON1 can only generate Endo and not H4, it is clear that the thiol metabolite they had used as analytical standard to represent the active metabolite was in fact the inactive Endo metabolite. Bouman and colleagues admit that their analytical methods did not

distinguish between different thiol metabolites derived from PON1, human liver microsomes, or human serum *in vitro* (27). They argue that the ratio of produced active thiol metabolites to total thiol metabolites was constant between enzyme preparations based on platelet reactivity studies of purified thiols, suggesting that PON1 creates a similar degree of active metabolites as human liver microsomes. Again, it is important to note that those platelet incubations were performed at purified thiol metabolite concentrations of 0.5 - 5 mg/L, values that are 25 - 250 times greater than their reported concentration of thiol metabolites patient plasma, bringing to question the relevance of such experiments as an argument for not requiring greater analytical specificity. Without identification and quantitation of these unknown PON1-derived active metabolites in relation to the amount of H4 active metabolite known to be found at significant levels in patient/subject plasma, it would seem that Bouman and colleagues have not clarified a role for PON1 in clopidogrel bioactivation.

Here, the *in vitro* data are consistent with previous studies that demonstrated the importance of CYP isozymes in clopidogrel bioactivation (6, 7, 27). In addition, the mechanism by which the 2-oxo-clopidogrel ring opens to expose the free thiol in H4 metabolite has been shown to be dependent on the presence of a reducing agent such as GSH (7, 8). Consistent with such data, we see a lack of H4 formation by CYP enzymes in the absence of GSH. Overall, we propose that both steps of clopidogrel bioactivation are mainly driven by CYP2C19, ultimately generating active H4, while PON1-mediated hydrolysis of 2-oxo-clopidogrel generates the Endo metabolite (Figure 7.5). Notably, we show that the estimated enzyme affinity (K_m) of 2-oxo-clopidogrel to CYP2C19 is much

higher than that of PON1, concordant with the low 2-oxo-clopidogrel affinity for PON1 demonstrated by Bouman *et al*. Accordingly, in hepatocytes, where clopidogrel bioactivation occurs, CYP2C19-mediated oxidation of 2-oxo-clopiodgrel would be the preferred pathway at therapeutic concentrations of the drug. Importantly, to the extent of our knowledge, this is the first study to quantify Endo and H4 concentrations simultaneously in humans. We demonstrate that Endo levels are nearly 20-fold lower compared with H4; thus, Endo is unlikely to contribute substantially to clopidogrel antiplatelet response, in addition to the lack of association between Endo levels and antiplatelet response. Moreover, these results are further substantiated by our findings of no association between PON1 plasma activity and genotype with H4 levels or antiplatelet activity.

There are several noteworthy limitations to our study. First, the current clinical study was conducted in a relatively small cohort of study subjects. Therefore, we combined carriers of one or two alleles of *CYP2C19**2 or *CYP2C19**3 into one group and cannot comment on the differential influence of these alleles or its gene-dose effect on clopidogrel pharmacokinetics and pharmacodynamics. Such pooling of *CYP2C19* variant carriers has been done previously for similar reasons (28). It should be noted that in terms of sample size, an 80% power was achieved to detect a 40% difference in antiplatelet response between *CYP2C19* EM and RM genotype groups with a two-sided significance level of 0.05, and a standard deviation of 20%. With respect to PON1 analysis, the high interindividual variability observed within *PON1* genotype groups lead to reduction in power below 80%. However, the lack of *PON1* Q192R genetic influence on clopidogrel

response is in agreement with recently published studies of large sample sizes. Secondly, this study used the point-of-care VerifyNow $P2Y_{12}$ assay to measure clopidogrel antiplatelet response. While VerifyNow utilizes ADP and PGE1 to induce and measure global platelet aggregation, it is not as direct a measure of platelet $P2Y_{12}$ signalling activity as the vasodilator-stimulated phosphoprotein (VASP) assay or even direct receptor occupancy assays using ^{33}P-2MeS-ADP (29). Indeed, clopidogrel H4 metabolite plasma exposure tracks with the VASP phosphorylation platelet reactivity index (17). Whether the H4 metabolite level is associated with VerifyNow PRU response has not been well described until now. Although some studies show high correlation between VASP and VerifyNow measured antiplatelet response(19, 30, 31), others did not (32). Nevertheless, clopidogrel response as measured by VerifyNow has consistently been shown to predict therapy resistance and clinical outcomes in a number of large trials and thus represents a feasible alternative technique for monitoring clopidogrel response (30, 33). Indeed, the more pressing issue to address is delineating relevant cut-off thresholds for distinguishing between responders and non-responders, which will then allow clinicians to increase clopidogrel dose as appropriate. A standardized optimal cut-off value for each $P2Y_{12}$ activity assay remains to be evaluated and defined. Lastly, since this is a proof-of-principle study in healthy subjects given a single 75 mg dose of clopidogrel, designed to delineate the role of PON1 relative to that of CYP2C19, we cannot directly extrapolate our findings to clopidogrel responsiveness in patients but it should be noted that the influence of CYP2C19 and PON1 on clopidogrel platelet aggregation has been assessed large patient cohorts in multiple studies (20-22). However, a key advantage of our study population and design is the ability to more clearly delineate the effect of

pharmacogenetic markers on clopidogrel pharmacokinetics and response and elimination of the potentially confounding effects of concomitant medications and existing disease states. In addition, quantification of the active H4 thiol metabolite here allowed for a more precise assessment of the role of genetics on clopidogrel pharmacokinetics.

In conclusion, we show that PON1, unlike CYP2C19, is incapable of generating the clopidogrel active metabolite H4 *in vitro*. In human subjects, the *CYP2C19* loss-of-function genotype is a major driver of H4 exposure, corresponding to lowered ADP-induced antiplatelet response. Furthermore, we demonstrated that PON1 generates the Endo metabolite; however, no correlation existed between plasma paraoxonase activity and Endo levels to antiplatelet response. Accordingly, although it remains likely that there are other genetic and non-genetic determinants of clopidogrel efficacy, our study suggests that CYP2C19 but not PON1 or CYP3A4 is a mechanistic determinant of interpatient antiplatelet response variability to clopidogrel therapy.

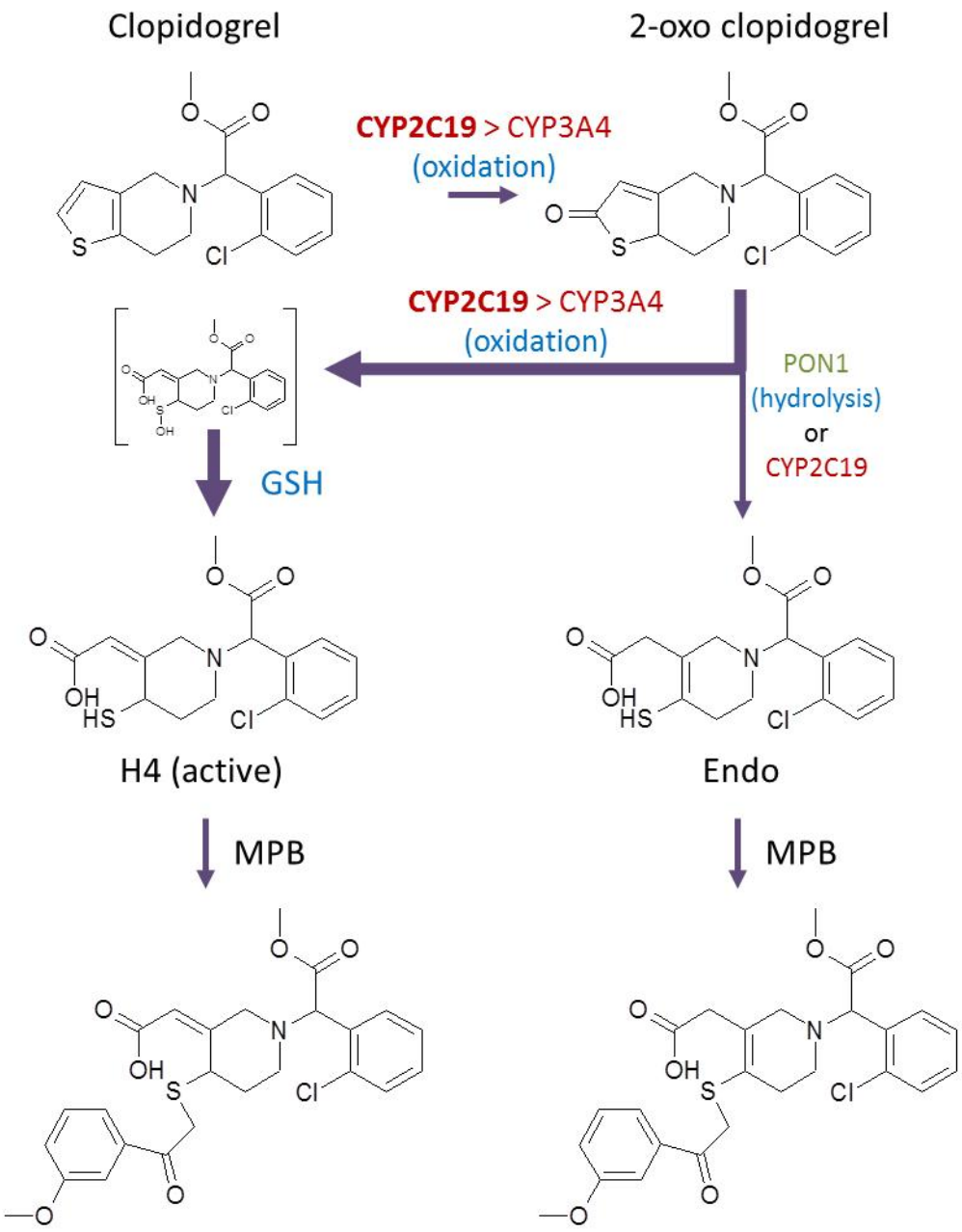

Figure 7.5 Schematic summary of clopidogrel bioactivation.

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7.6 Supplemental material

7.6.1 Genotyping

Genomic DNA was isolated with Gentra Puregene or DNA Blood Midi extraction kit as per manufacturer's protocol (Qiagen, Alameda, CA). Genotype analysis included CYP2C19*2(rs4244285), CYP2C19*3 (rs4986893), CYP2C19*17 (rs12248560), and PON1 Q192R (rs662). Genotypes were determined by allelic discrimination using TaqMan Drug Metabolism Genotyping assays with the 7500 RT-PCR System (assay IDs: C_25986767_70, C_27861809_10, C_469857_10, C_2548962_20; Applied Biosystems, Foster City, CA). Genotype analysis was conducted in parallel with known, sequence-verified wild-type, heterozygous, and variant controls for each SNP.

7.6.2 Kinetics of clopidogrel metabolism

Microsomes from baculovirus cells overexpressing human cytochrome P450 2C19 and 3A4 as well as NADPH CYP Oxidoreductase (BD Biosciences, Mississauga, Ontario) were used to examine the formation of 2-oxo-clopidogrel. Clopidogrel (0 – 45 μM) was incubated with microsomes at 37°C for 5 min, followed by addition of NADPH-regenerating system to start the reaction, yielding a final volume of 100 μL in potassium phosphate buffer (100 mM pH 7.4). After incubation at 37°C for 10 min, the reaction was

terminated by adding 2-fold volume of acetonitrile with 125 mM MPB and 500 ng/mL D4-clopidogrel carboxylic acid as the internal standard (IS).

7.6.3 Kinetics of 2-oxo-clopidogrel metabolism

CYP2C19, CYP3A4 and control baculovirus microsomes were used to characterize the metabolism of 2-oxo-clopidogrel. 2-oxo-clopidogrel (0 – 50 μM) was incubated with microsomes at 37°C for 5 min, followed by addition of NADPH-regenerating system and 5 mM reduced glutathione (GSH) to start the reaction, yielding a final volume of 100 μL in potassium phosphate buffer (100 mM pH 7.4). After incubation at 37°C for 30 min, the reaction was terminated by adding 2-fold volume of acetonitrile with 125 mM MPB and 500 ng/mL IS.

7.6.4 PON1-mediated hydrolysis of 2-oxo-clopidogrel

LacZ control and PON1 overexpressing HeLa microsomes (Supplemental Methods) were pre-incubated with 100 mM Tris buffer (pH 7.4), 1 mM calcium chloride, and 5 mM GSH at 37°C for 5 min, followed by addition of 2-oxo-clopidogrel to start the reaction (total volume of 50 μL) in presence or absence of specific PON1 inhibitor, 2-hydroxyquinoline (400 μM). After incubation at 37°C for 15 min, the reaction was

terminated by adding 2-fold volume of acetonitrile with 125 mM MPB and 500 ng/mL IS.

7.6.5 UHPLC-MS/MS analysis

Clopidogrel active metabolite H4-MPB derivative, Endo-MPB derivative, and internal standard (IS) D4-clopidogrel carboxylic acid were purchased from Toronto Research Chemicals (Toronto, Canada). H4 and Endo concentrations were determined using ultra high pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS, Agilent 1290 UPLC coupled with ThermoScientific TSQ Quantum). Briefly, 200 µL of acetonitrile and 10 µL of IS (0.5 µg/mL) were added to 100 µL of MPB-treated plasma and centrifuged at 14,000 rpm for 20 min. The resulting supernatant was added to water (1:2 v/v) prior to injection into the chromatograph. Analytes were separated **by** reverse-phase chromatography (Hypersil Gold Column 100 × 2 mm, 1.9 µM; or Shimpack XR-ODS II 75 mm x 2.0 mm, 2.2 µM) using gradient elution of 0.1% formic acid in water v/v and 0.1% formic acid in acetonitrile (35 to 70%) with a flow rate of 0.3 mL/min in an 8 min run time. The MS was equipped with heated electrospray ionization in positive mode for detection of H4, Endo, and IS with transitions 504 → 354 *m/z*, 504 → 155 *m/z* and 312 → 212 *m/z*, with collision energies of 44 eV, 22 eV and 20 eV, respectively. The lowest limit of quantification was 1 ng/mL for all analytes. The interday coefficient of variation of H4 and Endo quality controls were 10.3% and 7%, respectively.

7.6.6 Midazolam LC-MS/MS analysis

Midazolam and the IS, alprazolam, were purchased from Diagnostix (ThermoFisher, Mississauga, Canada). Midazolam plasma concentrations were determined using LC-MS/MS (Agilent 1200 coupled with ThermoScientific TSQ Vantage). Midazolam was extracted using solid-phase extraction (SPE), using a Waters Extraction Plate manifold coupled with Oasis Hydrophilic-Lipophilic-Balanced µElution 96-well plates. Briefly, column packings were conditioned with 200 µL of acetonitrile and equilibrated with 200 µL of water, followed by 200 µL of 0.1% formic acid in water. Subsequently, each column was loaded with 500 µL of 0.1% formic acid in water and alprazolam (10 ng/mL) mixed with 500 µL of plasma. The sample was washed with 200 µL of 10 mM ammonium acetate and eluted with 100 µL of 0.1% formic acid in acetonitrile. The resulting elutant was added to 0.1% formic acid in water (1:2 v/v). Upon injection into the liquid chromatograph, analytes were separated **with the** reverse-phase Hypersil Gold Column (50 × 5 mm, 5 µM particle size) using gradient elution of 0.1% formic acid in water and 0.1% formic acid in acetonitrile (20 to 80%) in a 6.5 min run time. The MS was set in positive ionization mode for detection of midazolam and alprazolam of respective transitions 309 → 281 *m/z* and 326 → 291 *m/z*, with collision energy of 30 eV. The lowest limit of quantification was 0.025 ng/mL. The interday coefficient of variation of midazolam quality controls was 12.1%.

7.6.7 Determination of paraoxonase activity

Plasma paraoxonase activity was measured by UV spectrophotometry in a 96-well plate format using paraoxon as the substrate, as previously described.(34) Briefly, the reaction mixture composed of 9 mM Tris hydrochloride pH 8, 1 mM calcium chloride, 1 M sodium chloride, 1.5 mM paraoxon, and 40-fold diluted plasma. The rate of para-nitrophenol generation was monitored at 405 nm over 10 mins at 25°C, in 30 sec intervals. An extinction coefficient of 17000 M^{-1} cm^{-1} was used to calculate units of paraoxonase activity, expressed as nmol/mL/min para-nitrophenol produced.

7.6.8 PON1 overexpression in an adenovirus system

Microsomes from HeLa cells overexpressing human PON1 was used to study PON1-mediated 2-oxo-clopidogrel hydrolysis. Briefly, the full open reading frame of human PON1 cDNA was amplified by PCR from a pooled human liver cDNA library using the oligonucleotide primers 5'- ctatccccgaccatggcgaagctgattg-3' and 5'-catgggtgcaaatcggtctgttagagctc-3', and subsequently cloned into expression vectors pEF and pENT (Invitrogen, Carlsbad, CA). Adenoviral expression vector containing PON1 was generated in pAD/CMV/V5-DEST using the ViraPower adenoviral expression system (Invitrogen, Carlsbad, CA). Adenovirus containing LacZ was used as a negative control. Following a 48-hour adenovirus transduction, HeLa cells were lysed with

hypotonic buffer (5 mM Tris HCl) and subsequently sonicated. Microsomes were prepared using differential centrifugation methods.

7.6.9 Western blot analysis

Microsome samples were separated by SDS-PAGE on 4-10% gels (Invitrogen, Carlsbad, CA) and subsequently transferred onto nitrocellulose membranes. Blots were probed with a mouse anti-PON1 antibody (Abcam, Cambridge, MA) and subsequently probed with anti-mouse horseradish peroxidise-labeled secondary antibodies (Bio-Rad, Hercules, CA). The bands were detected using BM Chemiluminescence Western Blotting Substrate (Roche, Indianapolis, IN).

7.6.10 Data analysis

In the clinical study, the PK parameters maximum observed plasma concentration (C_{max}), time to reach C_{max} (t_{max}), area under the plasma concentration vs time curve from time 0 to 8 hr ($AUC_{0\text{-}8hr}$), and half-life ($t_{1/2}$) were calculated using noncompartmental analysis techniques with GraphPad Prism v5.0 (GraphPad, La Jolla, CA) and PKSolver v2.0. Briefly, $AUC_{0\text{-}8hr}$ was calculated using the linear trapezoidal rule while $t_{1/2}$ was defined as $Ln2/k_e$, where k_e represents the slope of the elimination phase of the log plasma concentration profile.

For evaluation of genetic variation on clopidogrel disposition and response, subjects were divided into 2 groups for *CYP2C19* and 3 groups for *PON1*: *CYP2C19* extensive metabolizers, EMs (*1/*1 or *1/*17) and *CYP2C19* reduced metabolizers, RMs (*1/*2, *1/*3 or *2/*2); *PON1* wild-type, heterozygous, and homozygous carriers of Q192R. The independent effect of CYP2C19*2 and *3 variation could be not be evaluated due to insufficient subjects with heterozygous and homozygous variant status of each. However, a dominant model for *PON1*, comparing *PON1* wild-type Q192Q carriers to those subjects with either Q192R or R192R genotype, was assessed to further evaluate the influence of *PON1* on clopidogrel response. Hardy-Weinberg equilibrium was assessed for each genotype using the chi-square goodness-of-fit test. No genotype deviated from Hardy-Weinberg equilibrium.

For all *in vitro* experiments, the Michaelis-Menten enzyme kinetic parameters K_m and V_{max} were calculated using GraphPad Prism v5.0. The calculated K_m and V_{max} values were expressed as mean ± standard error (SEM). The intrinsic clearance (CL_{int}) was calculated as V_{max}/K_m.

The Mann-Whitney test and Kruskal-Wallis test one-way analysis of variance followed by Tukey's test for pairwise comparisons was employed to test the differences between two or three groups of data, respectively. Linear regression analysis was used to examine the association between two variables. A two-tailed P value of less than 0.05 was

considered significant for all analysis. Statistical analysis was performed with the use of GraphPad Prism v.5.0.

Table 7.6.1 Healthy volunteer baseline demographics (n = 21).

Age, mean (SD) years	35 (13)
Men, n (%)	11 (52)
Ethnicity, n (%)	
Caucasian	17 (81)
Black	1 (5)
Asian	2 (10)
Other	1 (5)
Weight, mean (SD) Kg	70 (15)
Height, mean (SD) cm	169 (10)
CYP2C19, n (%)	
*1/*1	11 (52)
*1/*2	6 (28)
*1/*3	1 (5)
*2/*2	1 (5)
*1/*17	2 (10)
PON1 Q192R, n (%)	
QQ	8 (38)
QR	8 (38)
RR	5 (24)

Table 7.6.2 Kinetic parameters of clopidogrel metabolism determined in vitro.

	V_{max} (SEM)	K_m (SEM)	V_{max}/K_m
Clopidogrel to 2-oxo-clopidogrel			
CYP2C19	2.869 (0.094)	3.008 (0.414)	0.954
CYP3A4	0.263 (0.039)	18.870 (6.366)	0.014
2-oxo-clopidogrel to H4 active metabolite			
CYP2C19	0.016 (0.0005)	2.696 (0.353)	0.006
2-oxo-clopidogrel to H3 metabolite			
CYP2C19	0.012 (0.0004)	1.729 (0.373)	0.007

V_{max} represents maximum rate achieved by enzymatic system; K_m represents substrate concentration required to reach half the value of V_{max}; V_{max}/K_m represents the intrinsic clearance (CL_{int}). Units are as follows: pmol/pmol P450/min for V_{max}; μM for K_m; μL/pmol P450/min for intrinsic clearance. Data represented as mean (standard error, SEM).

Table 7.6.3 Kinetic parameters of 2-oxo-clopidogrel metabolism to Endo metabolite determined in vitro.

	V_{max} (SEM)	K_m (SEM)	V_{max}/K_m
2-oxo-clopidogrel to Endo metabolite			
CYP2C19	0.3544 (0.024)	8.036 (1.631)	0.044
Control (insect cell) microsomes	0.108 (0.009)	15.38 (3.434)	0.007
HeLa cell microsomes	250.5 (30.9)	732.9 (109.7)	0.342

V_{max} represents maximum rate achieved by enzymatic system; K_m represents substrate concentration required to reach half the value of V_{max}; V_{max}/K_m represents the intrinsic clearance. Units are as follows: pmol/mg protein/min for V_{max}; μM for K_m; mL/mg protein/min for intrinsic clearance (CL_{int}). Data represented as mean (standard error, SEM).

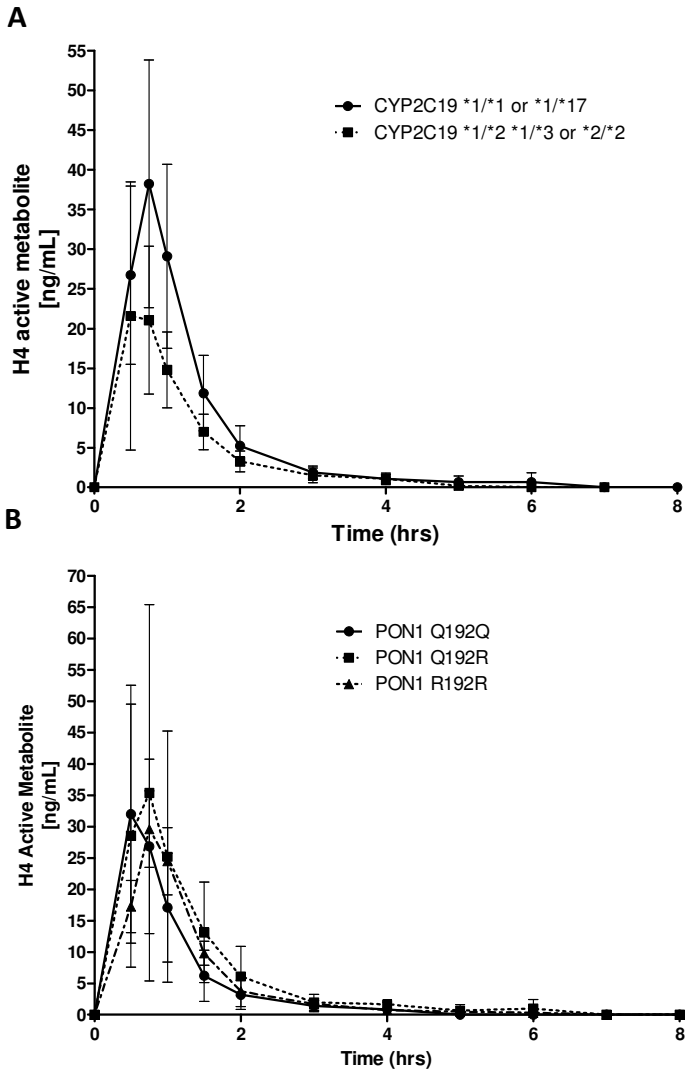

Figure 7.6 Plasma concentration curves of H4 active metabolite measured over 8 hours following 75 mg oral administration of clopidogrel in healthy volunteer study subjects.

(A, B) Curves grouped by *CYP2C19* and *PON1* Q192R genotype respectively. Symbols represent mean and error bars represent the standard deviation.

8 DISCUSSION AND CONCLUSIONS

8.1 Summary and Discussion

8.1.1 Chapter Four

Single nucleotide polymorphisms in genes that affect warfarin metabolism (cytochrome P450 2C9 gene, *CYP2C9*) and response (vitamin K epoxide reductase complex 1 gene, *VKORC1*) have an important influence on warfarin therapy, particularly during initiation; however, there is a lack of consensus regarding the optimal pharmacogenetics-based initiation strategy. The aim of Chapter Four was to develop a novel pharmacogenetics-based initiation protocol that incorporates loading and maintenance doses calculated based on individual patient genetics, clinical variables, and anticoagulation response (WRAPID). Subsequently, the clinical utility of this initiation protocol was evaluated in atrial fibrillation (AF) and venous thromboembolism (VTE) patients. We hypothesized that the use of a pharmacogenetics-based dosing algorithm for initiating patients requiring new anticoagulation therapy should effectively eliminate genotype-driven differences in anticoagulation response to provide a safe, rapid, and uniform anticoagulation response in patients. Indeed, application of the WRAPID algorithm resulted in a negligible influence of genetic variation in *VKORC1* or *CYP2C9* on time to achievement of therapeutic response and risk of overanticoagulation. After adjustment for covariates, time to stable anticoagulation was not influenced by *VKORC1* or *CYP2C9* genotype. Importantly, time spent within or above the therapeutic range did not differ among *VKORC1* and *CYP2C9* genotype groups. Moreover, the overall time course of the anticoagulation response among the genotype groups was similar and predictable. Collectively, these findings demonstrate the clinical utility of genetics-guided warfarin

initiation with the WRAPID protocol to eliminate genotype-driven differences in anticoagulation response, in turn providing safe and optimal anticoagulation therapy for patients with AF or VTE.

8.1.2 Chapter Five

While the influence of factors affecting *S*-warfarin PK parameters have been identified (ie, age, drug interactions, and *CYP2C9* genotype), the influence of genetics and clinical parameters on *S*-warfarin PD variability is less clear. We hypothesized that genetic variations in *CYP2C9* and *VKORC1*, as well as clinical variables contributes to interindividual variation in *S*-warfarin PK and PD parameters. Indeed, regression analysis demonstrated that *CYP2C9* genotype, kidney function, and gender were independent determinants of *S*-warfarin clearance. The values for I_{max} were dependent on *VKORC1* and *CYP4F2* genotypes, vitamin K status (as measured by plasma concentrations of proteins induced by vitamin K absence, PIVKA-II), and weight. Importantly, indication for warfarin was a major independent determinant of I_{max} during initiation, where PD sensitivity was greater in AF than VTE. To demonstrate the utility of the global PK-PD model, we compared the predicted initial anticoagulation responses with previously established warfarin dosing algorithms. These insights and modeling approaches support our hypothesis and have important implications for personalized warfarin therapy.

8.1.3 Chapter Six

Rivaroxaban is a new oral anticoagulant (factor Xa inhibitor), where 30-40% of the administered drug is excreted unchanged through the kidney via a combination of glomerular filtration and active tubular secretion. As such, we hypothesized that the efflux transporters P-glycoprotein (MDR1) and breast cancer resistance protein (BCRP) contributes to the overall disposition of rivaroxaban. The ability of MDR1 and BCRP efflux transporters to mediate rivaroxaban transport *in vitro* was demonstrated in that significantly greater vectorial transport of rivaroxaban was observed in the basal to apical direction in Caco-2 cells, which was attenuated in the presence of the selective inhibitors. Thus, rivaroxaban is a shared substrate of MDR1 and BCRP. Following oral administration of 2 mg/kg rivaroxaban, plasma concentrations did not significantly differ between wild-type and $Mdr1a^{def}$ or $Bcrp^{-/-}$ mice (n = 6 per group). However, rivaroxaban clearance was significantly reduced in the triple knockout mice, $Mdr1a/Mdr1b^{-/-}/Bcrp^{-/-}$. Interestingly, rivaroxaban brain to plasma ratio did not differ in mice lacking only Mdr1a or Bcrp, but more than two times higher in the $Mdr1a/Mdr1b^{-/-}/Bcrp^{-/-}$ mice. Overall, supportive of our hypothesis, MDR and BCRP likely function synergistically to modulate rivaroxaban disposition *in vivo* and appear to be particularly relevant to limiting its central nervous system entry. These data have important implications for safety and efficacy of anticoagulation therapy with rivaroxaban as many drugs in clinical use are known MDR1 inhibitors and loss-of-function polymorphisms in BCRP are common.

8.1.4 Chapter Seven

It is thought that clopidogrel bioactivation and antiplatelet response are related to cytochrome P450 2C19 (*CYP2C19*). However, a recent study challenged this notion by proposing *CYP2C19* as wholly irrelevant, while identifying paraoxonase-1 (*PON1*) and its Q192R polymorphism as the major driver of clopidogrel bioactivation and efficacy. The aim of Chapter Seven was to systematically elucidate the mechanism and relative contribution of PON1 in comparison to CYP2C19 to clopidogrel bioactivation and antiplatelet response. Indeed, we found a remarkably good correlation between clopidogrel active metabolite (H4) area under the plasma concentration curve ($AUC_{0 - 8\ hours}$) and antiplatelet response (r^2 = 0.78). Interestingly, *CYP2C19* but not *PON1* genotype was predictive of H4 levels and antiplatelet response. Furthermore, there was no correlation between plasma paraoxonase activity and H4 levels. Metabolic profiling of clopidogrel *in vitro* confirmed the role of *CYP2C19* in bioactivating clopidogrel to H4. However, heterologous expression of PON1 in cell-based systems revealed that PON1 cannot generate the H4 active metabolite from its parent compound, but mediates the formation of another thiol metabolite, termed Endo. Importantly, Endo plasma levels in humans are nearly 20-fold lower than H4 and were not associated with any antiplatelet response. Our results demonstrate that PON1 does not mediate clopidogrel active metabolite formation or antiplatelet action, while CYP2C19 activity and genotype remains a predictor of clopidogrel pharmacokinetics and antiplatelet response.

8.2 Therapeutic Implications

The occurrence of adverse drug reactions (ADRs) is a prominent matter of concern in medicine and drug therapy today (1). In many cases ADRs are caused by inappropriately or often inadvertently high doses of prescribed drugs, particularly worrisome for drugs with narrow therapeutic window and large interindividual variability in response. Indeed, interindividual variation in drug exposure and efficacy has meant that subsets of patients experience drug-related toxicities and inadequate therapeutic benefit. The one-dose-fits-all paradigm for drug therapy is increasingly recognized as inappropriate for many drugs in clinical use. Alternative dosing models should be considered to provide and ensure therapeutic benefit for the vast majority of patients while reducing ADRs. In the past decade, a substantial amount of pharmacogenomics research has unraveled many genetic variants affecting cardiovascular treatment response. Accordingly, personalized medicine represents an exciting emerging field that integrates genetic makeup with environmental influences and disease states to determine a precise individualized dose regimen.

To the best of our knowledge, the WRAPID nomogram represents the first warfarin initiation algorithm that incorporates both *VKORC1* and *CYP2C9* genotype-determined loading doses, which differs from the typical doubling of the maintenance dose. We demonstrated that the use of such an algorithm for warfarin initiation is not only practical in the ambulatory setting, but also effectively minimized interindividual variation in anticoagulation response to provide similar efficacy in a cohort of genotype-defined patients. A better understanding of pharmacogenomics and *in vivo* mechanisms

responsible for the observed variation in combination with *a priori* predictive modeling constitute the foundation for evaluating optimal pharmacogenetics-based dosing algorithms.

One of the limitations of the study outlined in Chapter Four is that we did not evaluate the use of pharmacogenetics-based warfarin dosing in the randomized clinical trial (RCT) setting. However, the recent COUMAGEN-II RCT showed the superiority of pharmacogenetics-based warfarin dosing over standard care with respective to greater time spent in therapeutic range and reduced occurrence of ADRs (2). Moreover, a recent study in real-world patients found that genotyping during warfarin therapy reduced the hospitalization rate for bleeding or thromboembolic event by 30% compared to a historical control group (3). These results are well aligned with our findings. Indeed, we expect that personalized warfarin therapy will ultimately result in a more cost-effective, safer, and faster treatment outcome to maximize anticoagulation efficacy while minimizing life-threatening bleeding events. A number of additional RCTs involving larger sample sizes are currently underway to confirm these encouraging results.

Although cost-effective analysis for use of warfarin pharmacogenetics has been controversial, there is consensus in that genotyping for *CYP2C9* and *VKORC1* for patients with higher risk of thromboembolic events or bleeding will be cost-effective resulting in increased quality-adjusted life years (QALYs) (4, 5). Indeed, additional cost-effective analysis will be required as more convincing data from RCTs become available.

Nevertheless, given the richness of evidence for warfarin, it will likely be the first widely adopted pharmacogenetic application in cardiovascular medicine.

The limitations of warfarin therapy have prompted the clinical development of novel oral anticoagulants that have more predictable response. The factor Xa inhibitor rivaroxaban is a new oral anticoagulant with direct mechanism of action by inhibiting the clotting cascade. It is known that rivaroxaban depends on the kidney for excretion of unchanged drug as well as its metabolites. Not surprisingly, elevated bleeding risk in patients with renal impairment is of particular concern with rivaroxaban therapy. In the past decade, remarkable progress has been made in the field of drug transporters in elucidating their importance in determining the disposition and efficacy of many drugs in clinical use. Indeed, we demonstrate in Chapter Six that the new oral anticoagulant rivaroxaban is a shared substrate of the efflux transporters MDR1 and BCRP. As functional polymorphisms exist in both MDR1 and BCRP genes, in addition to the wide array of MDR1 drug inhibitors in clinical use, genetic variations as well as drug interactions will likely play an important role in modulating rivaroxaban exposure and thus efficacy. While the clinical experience with rivaroxaban is currently very limited, the full spectrum of factors determining efficacy and safety within the context of pharmacogenetics, demographics, concomitant medications, and comorbidities should be evaluated as it is used more frequently in real-world patients.

The antiplatelet drug clopidogrel is used to treat patients with acute coronary syndrome, particularly for prevention of stent thrombosis in those undergoing percutaneous coronary

intervention. Similar to warfarin, pharmacogenetic studies have demonstrated clinically relevant implications on clopidogrel antiplatelet response and thrombosis event rates. A layer of complexity in defining optimal genetic markers for predicting clopidogrel response is that the mechanistic enzymes potentially involved in bioactivating inactive clopidogrel to its active metabolite has been under scrutiny. In Chapter Seven, we conducted a proof-of-principle healthy volunteer study powered sufficiently to delineate the effect of *PON1* genetic variation in comparison to *CYP2C19* polymorphisms on clopidogrel bioactivation and antiplatelet response. There was a lack of correlation between either *PON1* genotypes or paraoxinase activity to clopidogrel active metabolite formation or antiplatelet effect. Rather, we observed an excellent correlation in antiplatelet effect with *CYP2C19* polymorphisms, the enzyme that has been long appreciated as the key player for clopidogrel bioactivation. Subsequently, systematic *in vitro* metabolism studies confirmed that PON1 does not generate the active metabolite while CYP2C19 does, providing the mechanistic basis for the clinical significance of *CYP2C19* polymorphisms. PON1, on the other hand, generates an inactive metabolite that likely does not confer any measureable antiplatelet effect *in vivo*. This data is in line with analysis of clinical trials data demonstrating the lack of association of *PON1* genetic variation with clopidogrel antiplatelet response or risk of stent thrombosis (6-8). Overall, Chapter Seven highlights the importance of elucidating underlying molecular mechanisms of drug metabolism, disposition, and response for accurate interpretation of the clinical significance of candidate genetic markers. Indeed, recent RCT using point-of-care *CYP2C19**2 genotyping showed that guidance using genetic information led to

better antiplatelet response, setting the stage for personalized clopidogrel therapy to improve efficacy (9).

8.3 Future Directions

Warfarin has been the mainstay oral anticoagulant (OAC) prescribed for stroke prevention in AF patients. However, warfarin therapy is challenging due to marked interindividual variability in dose and response, requiring frequent monitoring and dose titration. These limitations have prompted the clinical development of new OACs (NOACs) that directly target the coagulation cascade with rapid onset/offset of action, lower risk for drug-drug interactions, and more predictable response. Recently, NOACs dabigatran (direct thrombin inhibitor), rivaroxaban and apixaban (factor Xa inhibitors) have gained regulatory approval as alternative therapies to warfarin. While the anticoagulation efficacy of these NOACs has been characterized, differences in their pharmacokinetic and pharmacodynamic profiles have become a significant consideration in terms of drug selection and dosing. Moreover, interindividual variability in plasma exposure/response of NOACs and bleeding risk associated with anticoagulation therapy remains a pertinent question. Indeed, even in a clinical trial setting with stringent enrollment criteria, the one-dose-fits-all dosing regimen strategy did not appear successful for NOACs, likely due to the various clinical covariates that significantly affected extent of drug exposure and response. It is evident that variability in NOAC plasma exposure will have significant impact on anticoagulation efficacy given the direct PK-PD relationship and its association with clinical outcomes. Thus, quantifying NOAC

plasma concentration is likely the most reliable assessment of response and bleeding risk. Collectively, studies in real-world patients treated with NOACs are required to better understand the effect of variables such as age, renal/hepatic function, dosing interval, as well as drug metabolism and transport pharmacogenetics and interactions on the variability in NOAC drug exposure. These proposed studies would allow for prediction of patients at risk for sub- and supra-therapeutic anticoagulation response and individualize OAC selection and dosing. Appendix C highlights in greater detail the clinical implications of the availability of NOACs in treating AF patients.

Antiplatelet therapy with clopidogrel has emerged as a major success in the treatment of cardiovascular disease, particularly in reducing the risk for myocardial infarctions associated with coronary artery disease. Despite the well-known clinical benefit of clopidogrel in prevention of coronary ischemic events and stent thrombosis, the large interindividual variability observed with clopidogrel antiplatelet response results in therapy complexity. According to a recent genome-wide association (GWAS) study, *CYP2C19* genotype only explains 12% of the variation in response (10). Thus, further delineation of additional genetic and nongenetic markers is required to better explain and predict response variation, which may in turn define optimal individualized doses for patients. In addition, the first evidence of a therapeutic window range for clopidogrel antiplatelet efficacy has recently been proposed to classify responders and non-responders, and more importantly, early identification of patients not achieving optimal platelet inhibition (11). Consensus on defining the optimal clopidogrel therapeutic window has been challenging due to the lack of a standardized platelet-function assay.

The point-of-care VerifyNow $P2Y_{12}$ assay has recently been used as a feasible and reliable method of measuring antiplatelet response (12). However, VerifyNow is not as direct a measure of platelet $P2Y_{12}$ signalling activity as the vasodilator-stimulated phosphoprotein (VASP) assay or even direct receptor occupancy assays using ^{33}P-2MeS-ADP (13). Although some studies show high correlation between VASP and VerifyNow measured antiplatelet response, others did not (14). Thus, the relationship between the gold-standard VASP assay and VerifyNow point-of-care assay in relation to optimal therapeutic response remains to be evaluated and defined. Furthermore, the stent thrombosis risk has been demonstrated to be the highest during initiation of clopidogrel therapy (15). Accordingly, the intra-individual variability in platelet response during this critical period is another area to be assessed to evaluate consistency of the responder and non-responder phenotypes. Moreover, quantification of active metabolite H4 plasma levels will aid in defining the relationship between the proposed therapeutic response window and a 'therapeutic H4 concentration window'.

As with warfarin, new antiplatelet agents (prasugrel, ticagrelor) are now available as alternative agents to clopidogrel. Both prasugrel and ticagrelor do not require extensive bioactivation by CYP2C19. However, CYP3A4 and CYP2B6 are responsible for bioactivation of prasugrel to its active metabolite and the influence of polymorphisms in these enzymes to prasugrel platelet efficacy remains to be delineated. Similarly, ticagrelor is a substrate of MDR1 and the influence of drug interactions as well as *ABCB1* polymorphisms remain to be addressed.

8.4 Conclusions

Physicians have long sought over the phenomenon that patients respond variably to the same standard drug treatment. Over half a century ago, Dr Werner Kalow of University of Toronto (1917-2008) was one of the pioneering physicians to recognize interpatient variability in drug responsiveness following standard therapeutic doses (16). Werner Kalow's work along with others set precedence for studying the link between genetic variations and pharmacology. Subsequently, the term pharmacogenetics was coined for describing this phenomenon. In fact, the first monograph of pharmacogenetics was by Werner Kalow in 1962 entitled "Pharmacogenetics: Heredity and Response to Drugs", highlighting his work on the relationship between genetic polymorphisms of butyrylcholinesterase and the risk of prolonged apnea to standard doses of succinylcholine due to differential ability to hydrolyze succinylcholine (17).

The field of pharmacogenetics has evolved significantly since the initial observations; however, the fundamental principles remain the same and that is to delineate the genetic basis of interindividual variation in drug pharmacokinetics, pharmacodynamics and ADRs. Indeed, remarkable process has been made in the past decade using a variety of study designs to unveil the genetic contribution to variable drug responsiveness for a variety of medications. Moreover, pharmacogenetic studies form the foundation and evidence needed to implement personalized medicine, an emerging drug treatment paradigm utilizing individual genetic makeup to guide the appropriate drug therapy and dosages. This in turn will allow for the greatest therapeutic benefit to be seen while

reducing risk for toxicity. Many classes of cardiovascular drugs exemplify how heritable genetic variations influence its efficacy.

The first section of the thesis focused on the development of a novel pharmacogenetics-based initiation protocol for warfarin therapy. We demonstrated its clinical utility in eliminating genotype-driven variability in anticoagulation response during the critical initiation phase, as well as sustaining optimal efficacy throughout maintenance therapy for AF and VTE patients. Additionally, we defined the genetic and non-genetic determinants of warfarin PK and PD parameters by use of mathematical PK-PD modeling to estimate individual clearance and anticoagulation responsiveness. The integration of elucidated variables contributing to PK and PD variability with modeling techniques represents a powerful tool for *a priori* prediction of anticoagulation outcomes in a variety of clinical scenarios. The availability of NOACs has meant that clinicians now have alternatives to warfarin; however, interindividual variation in anticoagulation efficacy remains a concern for NOACs. We determined that both efflux drug transporters MDR1 and BCRP are involved in rivaroxaban active renal secretion, which likely play a synergistic role in modulating rivaroxaban accumulation and elimination in humans. The last part of the thesis involved elucidating the metabolic enzymes involved in clopidogrel bioactivation. We evaluated the impact of relevant genetic variations on the observed active metabolite plasma concentration and antiplatelet response in a healthy volunteer setting. Indeed, individuals harbouring reduced function *CYP2C19* polymorphism have a reduced capacity to generate clopidogrel active metabolite and lower antiplatelet response.

It is evident that many factors contribute to variability in drug responsiveness, including renal and hepatic function, underlying disease mechanisms, drug interactions, and pharmacogenomics. Improvement in our understanding of interindividual variation in drug responsiveness allows for better prediction of patients at risk for lack of therapeutic efficacy or adverse event, setting the stage for personalized therapy. One of the major barriers to the widespread clinical implementation of personalized therapy is the requirement from trialists to demonstrate outcome and safety benefit of pharmacogenetics-directed therapy in randomized clinical trials (RCTs). However, these RCTs are too expensive, not feasible, and unethical in many clinical scenarios. Thus, it is necessary to resort to pragmatic trials and prospective cohort studies where early adopters of pharmacogenetics are needed to develop evidence for better efficacy and safety. As results from pharmacogenetic studies emerge, pharmacoeconomics should be continuously conducted to evaluate the health-economic benefit and cost-effectiveness of adopting pharmacogenetics into standard patient care.

The studies presented herein demonstrate pragmatic translational medicine by bridging the necessary interplay between understanding molecular mechanisms and evaluating their impact on clinically important endpoints in the real-world setting. It is important to note that as we recognize that an individual patient's drug response phenotype is the ultimate clinical endpoint, the use of pharmacogenomics should be integrated in context with other patient-specific parameters such as drug exposure, biomarkers, and other measures of response and toxicity, in the practice of personalized medicine. Overall, our

studies support the use of pharmacogenetics-guided care for delivery of personalized medicine to maximize efficacy while reducing toxicity by selecting the right drug at the right dose for the right patient.

8.5 References

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Appendices

Appendix A: Ethics Approval

Office of Research Ethics

The University of Western Ontario
Room 4180 Support Services Building, London, ON, Canada N6A 5C1
Telephone: (519) 661-3036 Fax: (519) 850-2466 Email: ethics@uwo.ca
Website: www.uwo.ca/research/ethics

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. R.B. Kim
Review Number: **15391** **Review Level:** Full Board
Review Date: August 12, 2008
Protocol Title: Genetics-based warfarin dosing in patients with atrial fibrillation; a prospective cohort study
Department and Institution: Medicine-Dept of, London Health Sciences Centre
Sponsor:
Ethics Approval Date: September 11, 2008 **Expiry Date:** December 31, 2009
Documents Reviewed and Approved: UWO Protocol, Letter of Information and Consent (ver. Aug.29/08)
Documents Received for Information:

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The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

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b) all adverse and unexpected experiences or events that are both serious and unexpected;
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Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. Victor Han

Office of Research Ethics

The University of Western Ontario
Room 4180 Support Services Building, London, ON, Canada N6A 5C1
Telephone: (519) 661-3036 Fax: (519) 850-2466 Email: ethics@uwo.ca
Website: www.uwo.ca/research/ethics

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. R.B. Kim

Review Number: 15391 **Revision Number:** 2

Review Date: August 14, 2009 **Review Level:** Expedited

Protocol Title: Genetics-based warfarin dosing; a prospective cohort study

Department and Institution: Medicine-Dept of, London Health Sciences Centre

Sponsor:

Ethics Approval Date: August 25, 2009 **Expiry Date:** July 31, 2010

Documents Reviewed and Approved: Revised study end date, revised co-investigators, revised study methodology, and revised Letter of Information and Consent Form dated 08/21/2009

Documents Received for Information:

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Office of Research Ethics

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Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. R.B. Kim
Review Number: 16714 **Review Level:** Full Board
Review Date: December 15, 2009 **Approved Local # of Participants:** 36
Protocol Title: Proton Pump Inhibitors and CYP2C19 genotype-dependent reduction in clopidogrel metabolism as a risk factor for myocardial infarction
Department and Institution: Medicine-Dept of, London Health Sciences Centre
Sponsor: AMOSO Innovation Fund
Ethics Approval Date: January 14, 2010 **Expiry Date:** April 30, 2011
Documents Reviewed and Approved: UWO Protocol, Letter of information & consent form dated January 6, 2010
Documents Received for Information:

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Investigators must promptly also report to the HSREB:

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If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

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Chair of HSREB: Dr. Joseph Gilbert
FDA Ref. #: IRB 00000940

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Western

January 12, 2009

This is the Original Approval for this protocol
A Full Protocol submission will be required in 2013

Dear Dr. Kim:

Your Animal Use Protocol form entitled:
Pharmacokinetic analysis of OATP1B substrates in Oatp1b2 knockout and wildtype mice
Funding Agency CIHE - Grant #MOP-89753

has been approved by the University Council on Animal Care. This approval is valid from **Jan. 12th, 2009 to Jasnuary 31, 2010**. The protocol number for this project is **#2008-123**.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED FOR 4 Years

Species	Strain	Other Detail	Pain Level	Animal # Total for 4 Years
Mouse	DBA/lacJ Wildtype	6 weeks-1 yr.	B	280
Mouse	Oatp1b2-/-	6 weeks-1 yr. (designed by Jeff Stock at Pfizer Groton Laboratories and provided by Charles River Laboratories, Wilmington; not commercially available)	B	280

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

c.c. Approved Protocol - R. Kim, S. Lemay, W. Lagerwerf
Approval Letter - R. Kim, S. Lemay, W. Lagerwerf

Western

02.01.2010
*This is the 2nd Renewal of this protocol
*A Full Protocol submission will be required in 01.31.2013

Dear Dr. **Kim**

Your Animal Use Protocol form entitled:

Pharmacokinetic analysis of OATP1B substrates in Oatp1b2 knockout and wildtype mice

has had its yearly renewal approved by the Animal Use Subcommittee.

This approval is valid from **02.01.2010 to 01.31.2011**

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4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.
The holder of this *Animal Use Protocol* is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

c.c. S. Lemay, W. Lagerwerf

The University *of* Western Ontario
Animal Use Subcommittee / University Council on Animal Care
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Appendix B: Copyright Approval

Chapter Two

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Chapter Four

Chapter Five

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Appendix C: Future of oral anticoagulation therapy: Importance of pharmacokinetic profile and variability as determinants of dose and response to dabigatran, rivaroxaban, and apixaban[6]

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Introduction

Atrial fibrillation (AF) is associated with a fivefold increase in risk of disabling stroke (1). Therefore, antithrombotic therapy is required for stroke and systemic embolism (SSE) prophylaxis. Until recently, the vitamin-K antagonist warfarin was the primary treatment choice for long-term oral anticoagulation (OAC) as stroke risk is reduced by 60% in nonvalvular AF patients (1). However, warfarin has a number of limitations including delayed onset of action, large interindividual variability in response, unpredictable pharmacokinetics (PK), drug-drug interactions, and genetic polymorphisms in genes affecting metabolism and pharmacodynamics (PD). Intensive monitoring using international normalized ratio (INR) and frequent dose adjustments are necessary to provide adequate anticoagulation within warfarin's narrow therapeutic window.

Significant effort has been made to develop new OACs (NOACs) with direct mechanisms of action with a sufficiently wide therapeutic window to allow for fixed dose administrations without the need for routine response monitoring. NOACs directly inhibit the coagulation pathway, either factor Xa or thrombin, for rapid onset of antithrombotic effects. The PK and pharmacology of these NOACs are distinct from that of warfarin (Table C.1, Figure C.1). In the present review, we will highlight the PK and PD features of the three NOACs that have recently been approved for stroke prevention in AF patients and provide NOAC selection and dosing recommendations based on their PK-PD profiles.

Table C.1 Comparison of pharmacokinetic features of warfarin, dabigatran, rivaroxaban and apixaban.

Parameter	Warfarin	Dabigatran	Rivaroxaban	Apixaban
Mechanism of action	Inhibition of VKOR	Direct thrombin inhibitor (free or bound), reversible	Factor Xa inhibitor (free or bound), reversible	Factor Xa inhibitor (free or bound), reversible
Onset of action	Slow, indirect inhibition of clotting factor synthesis	Fast	Fast	Fast
Offset of action	Long	Short	Short	Short
Absorption	Rapid	Rapid, acid dependent	Rapid	Rapid
Bioavailability (%)	100	6.5	80[a]	50
t_{max} (hr)	2.0 – 4.0	1.0 - 3.0	2.5 - 4.0	1.0 - 3.0
V_d (L)	10	60 - 70	50 - 55	21
Protein Binding (%)	99	35	95	87
$t_{1/2\beta}$ (hr)	40	12 - 17	9 - 13	8 - 15
Renal excretion	None	80	33	25
Fecal excretion	None	20	28	50 - 70
CL/F (L/hr)	0.35	70 - 140	10	5
Accumulation in plasma	Dependent on CYP2C9 metabolic efficiency	None	None	1.3 - 1.9

Food effect	None	Delayed absorption with food with no influence on bioavailability	Delayed absorption with food with increased bioavailability	None
Age	Yes, lower CL/F as age increases	Yes, lower CL/F as age increases	None	Yes, lower CL/F as age increases
Bodyweight	Yes, higher dose for increased weight	None	None	Yes, higher exposure with increased weight
Gender	Yes, lower CL/F in females	Yes, lower CL/F in females	None	Yes, higher exposure in females
Ethnicity	Lower dose in Asians; Higher dose in African-Americans	None	Lower dose in Japanese patients	None
Drug transporter	None	P-gp	P-gp, BCRP	P-gp, BCRP
CYP-mediated metabolism	CYP2C9, CYP3A4, CYP2C19, CYP1A2	None	CYP3A4/5, CYP2J2 (equal)	CYP3A4/5, CYP2J2 (minor), CYP1A2 (minor)
Drug-drug interactions	Many; affecting metabolism	Potent P-gp inhibitors; affecting absorption	Potent CYP3A4 and P-gp inhibitors;	Potent CYP3A4 and P-gp inhibitors;

			affecting absorption, metabolism, and excretion	affecting absorption, metabolism, and excretion
Dosing for AF	Variable (0.5 – 16 mg OD)	150, 110 mg BID	20, 15 mg OD	5, 2.5 mg BID
Coagulation measurement	INR	TT > Hemoclot > ECT > aPTT	anti-FXa > PT	anti-FXa

AF, atrial fibrillation; aPTT, activated partial thromboplastin test; BCRP, breast cancer resistance protein; BID, twice daily; CL/F, apparent clearance; CYP, cytochrom P450 isozymes; ECT, ecarin clotting time; OD, once daily; P-gp, P-glycoprotein; PD, pharmacodynamics; PT, prothrombin time; t_{max}, time to maximum plasma concentration; $t_{1/2\beta}$, terminal half-life; TT, thrombin time; V_d, volume of distribution; VKOR, vitamin K epoxide reductase enzyme.

[a] Bioavailability is dependent on dose (over 10 mg) and food intake. Thus, rivaroxaban doses over 10 mg OD should be administered with food. However, 10 mg rivaroxaban is only licensed for prophylaxis of thromboembolism following elective hip or knee surgery.

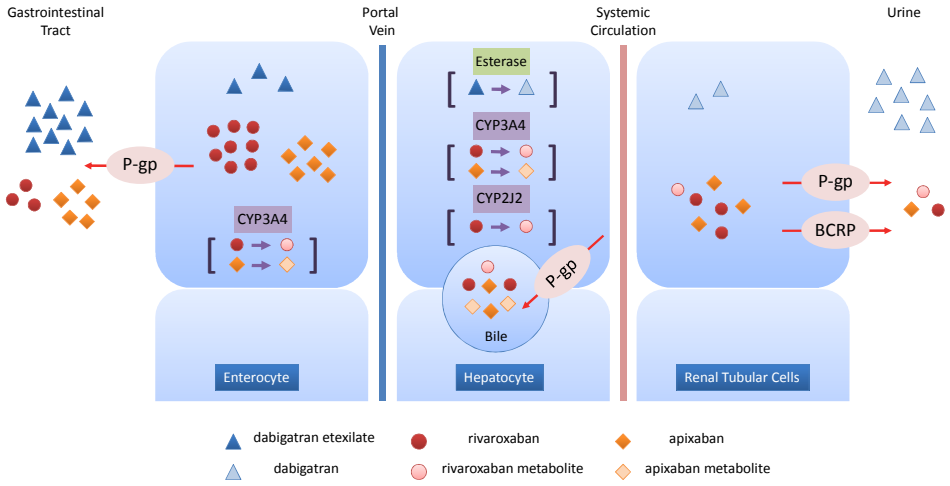

Figure C.1 Summary of absorption, metabolism, and excretion of dabigatran, rivaroxaban, and apixaban.

Clinical outcomes data comparing NOACs to warfarin

Dabigatran etexilate is the first oral direct thrombin inhibitors (DTIs) to be approved for its clinical use in AF patients (2). In the RE-LY trial (open-label; n = 18,133), 150 mg dabigatran twice daily (BID) was associated with lower rates of stroke/SSE, achieving superiority, but a similar rate of major bleeding while 110 mg BID was associated with similar rates of stroke/SSE but fewer bleeds compared to warfarin (3). Rivaroxaban and apixaban are the first and second oral direct factor Xa (FXa) inhibitors approved for AF (4). In the ROCKET-AF trial (double-blinded, n = 14,264), rivaroxaban was noninferior to warfarin where 20 mg rivaroxaban once daily (OD) resulted in similar rates of stroke/SSE and major bleeding (5). In the ARISTOTLE trial (double-blinded; n = 18, 201), the reduction in stroke/SSE by 5 mg BID apixaban was superior to warfarin (both ischemic and haemorrhagic, driven by haemorrhagic) (6). Interestingly, all three NOACs were associated with less intracranial bleeding compared to warfarin irrespective of time spent in therapeutic range (TTR) in the warfarin arm. However, more gastrointestinal (GI) bleeding was observed with dabigatran and rivaroxaban.

Importance of renal function and NOAC clearance

The extent of renal excretion is an important distinguishing feature of NOACs. Up to 80% of circulating dabigatran is eliminated renally, whereas only 33% and 25% of unchanged rivaroxaban and apixaban is cleared by the kidney (7-9). Following 150 mg administration, total dabigatran AUC was increased by 1.5-, 3.1-, and 6.3-fold in

individuals with mild, moderate and severe renal impairment, respectively, compared to healthy individuals, leading to increased terminal half-life ($t_{1/2\beta}$) to 15, 18, and 27 hours (10). For rivaroxaban, subjects with mild, moderate, and severe impairment exhibited an increase in AUC of 44, 52, and 65 %, respectively (11). The coagulation parameter prothrombin time (PT) was affected in a similar fashion. For apixaban, while no changes were observed in apixaban anti-FXa activity, overall AUC increased by 16, 29, and 44 % in individuals with mild, moderate and severe renal impairment compared to individuals with normal renal function (12).

Importance of drug metabolism and transport processes for NOACs

Dabigatran etexilate is the prodrug of dabigatran, and a substrate of efflux transporter P-glycogprotein (P-gp, encoded by *ABCB1*), with an absolute bioavailability of only 6.5% (13, 14). Bioavailable dabigatran etexilate is converted entirely to dabigatran by nonspecific ubiquitously expressed carboxylesterases in the enterocytes, portal circulation, and hepatocytes (7, 13, 15). Very recently, a genome-wide subanalysis of the RE-LY trial demonstrated that a single nucleotide polymorphism (SNP) in the carboxyesterase 1 gene (*CES1*; rs2244613) attenuated dabigatran formation leading to lower trough concentrations while the *ABCB1* SNP rs4148738 and *CES1* SNP rs8192935 were associated with higher and lower peak dabigatran concentrations, respectively (16). Rivaroxaban's bioavailability is dose-dependent; the absolute bioavailability of 10 mg rivaroxaban ranged from 80 to 100 %, while bioavailability of 20 mg under fasting conditions was 66% (17). Coadministration of 15 or 20 mg rivaroxaban with food

increased the AUC by 39% (18). The low bioavailability in fasting conditions may result in risk of inadequate anticoagulation and thus, rivaroxaban should be administered with food. Apixaban's absolute bioavailability is 50% and not affected by food intake (9).

Dabigatran is not metabolized by the cytochrome P450 (CYP) isoenzymes; rather, it is conjugated to acylglucuronides (19). In patients with moderate hepatic impairment, the bioconversion from prodrug to dabigatran was slightly slower when compared to healthy subjects although the AUC and extent of dabigatran glucuronidation was unchanged (20). Conversely, rivaroxaban and apixaban are both subject to CYP-mediated metabolism whereby CYP3A4/5 and CYP2J2 accounts for clearance of two-thirds of rivaroxaban (8, 21), and apixaban metabolism is predominantly driven by CYP3A4/5 (22). Patients with mild (Child-Pugh A) hepatic impairment showed no difference in rivaroxaban PK and PD (23). However, patients with moderate (Child-Pugh B) hepatic impairment exhibited reduced clearance of rivaroxaban. Therefore, rivaroxaban is not recommended in patients with moderate or severe (Child-Pugh C) hepatic impairment. The PK and anti-Fxa activity of 5 mg apixaban was not altered in subjects with either mild or moderate liver impairment when compared to healthy individuals (12).

Both rivaroxaban and apixaban are substrates of efflux transporters P-gp and breast cancer resistance protein (BCRP, encoded by *ABCG2*); thus, active secretion likely contributes significantly to their renal elimination and systemic exposure (24-26).

NOAC drug-drug Interactions result from inhibitors or inducers of CYP3A4 and P-glycoprotein

CYP3A4 located in the intestine and liver plays a pivotal role in governing metabolic pathways of 50% of all drugs (27). Similarly, many drugs are P-gp substrates, one of the most important efflux transporters in modulating drug disposition by preventing absorption and enhancing excretion into the bile and urine (28). Interestingly, there is a high degree of overlap in CYP3A4 and P-gp drug substrates (29), as the case for rivaroxaban and apixaban. In addition, the coexpression of these proteins in the gut, liver, and kidney means that CYP3A4 and P-gp play a concerted role in modulating excretion of NOACs.

When dabigatran was coadministered with amiodarone or ketoconazole (P-gp and strong CYP3A4 inhibitor), AUC was increased by about 50% (30). Similarly, the potent P-gp inhibitor verapamil increased AUC by 2.4-fold when administered 1 hour before dabigatran while AUC increased by 71% when coadministered (31). Interestingly, patients of the RE-LY trial coadministered with amiodarone and verapamil only had a 13% and 23% increased bioavailability, respectively (3). Coadministration of ketoconazole with rivaroxaban increased the AUC by 160% along with similar increases in coagulation measurements (26). The strong P-gp/CYP3A4 inhibitor ritonavir elevated rivaroxaban AUC by 150%. Coadministration of ketoconazole increased apixaban AUC by twofold (32). Overall, coadministration of rivaroxaban and apixaban with P-gp/CYP3A4 inhibitors (namely azoles and protease inhibitors) should be avoided while

coadministration with moderate inhibitors (ie., erythromycin) should be exercised with caution (Table C.2).

Inducers of CYP3A4/P-gp such as rifampin has been show to result in 65% reduction in dabigatran bioavailbility and 50% decrease in AUC and clotting parameters for rivaroxabin and apixaban (26, 32, 33). Thus, concomitant use of rivaroxaban/apxiaban with strong CYP3A4/P-gp inducers is contraindicated due to concern of reduced anticoagulation efficacy.

We note that the clinical significance of concomitant use of multiple moderate inhibitors in the same patient, a particular concern in elderly AF patients where polypharmacy is common, remains to be established as it is unknown whether the effect on NOAC PK-PD would be equivalent to use of a single potent inhibitor. The full spectrum of these interactions remains to be addressed in the real-world population. Until then, dose lowering adjustments in conjunction with anticoagulation monitoring should be used to ensure efficacy and safety.

Table C.2 List of dual substrates, inhibitors, and inducers of CYP3A4 and P-glycoprotein.

Substrates	Inhibitors	Inducers
Apixaban	Amiodarone	Carbamazepine
Atorvastatin	Cimetidine	Phenobarbital
Celiprolol	Clarithromycin	Rifampicin
Cyclosporine	Erythromycin	St. John's wort
Docetaxel	Fluconazole	
Paclitaxel	Ketoconazole	
Rivaroxaban	Itraconazole	
Tacrolimus	Nifedipine	
	Nelfinavir	
	Ritonavir	
	Saquinavir	
	Verapamil	
	Voriconazole	

Why understanding the pharmacokinetic profile of NOACs is essential for predicting anticoagulation efficacy

The predictable PK/PD of NOACs does not necessitate routine monitoring; however, the ability to accurately measure their efficacy is highly desirable in a variety of scenarios including assessment of compliance, and identifying patients at risk for over-anticoagulation/bleeding or lack of efficacy. Although standardized coagulation assays are unavailable, several assays have been evaluated to determine their validity as a surrogate marker of NOAC plasma exposure and anticoagulation response. Generally, a parallel relationship exists between NOACs plasma exposure and anticoagulation effects in a concentration-dependent manner (13, 17, 34-37).

The anticoagulant effect of dabigatran can be measured by activated partial thromboplastin time (aPTT), PT, thrombin time (TT), ecarin clotting time (ECT), and the diluted version of TT (Hemoclot). The TT, Hemoclot and ECT are most sensitive and precise for measuring dabigatran anticoagulation. However, TT is likely too sensitive for dabigatran efficacy and Hemoclot is currently considered more suitable to quantitate dabigatran concentrations (38). Unfortunately, these assays are not widely available and in emergency situations, aPTT may be most accessible for measuring anticoagulation. The clinical relevance of plasma concentrations was demonstrated in the RE-LY trial where dabigatran trough plasma concentrations were significantly associated with risk of stroke/SSE (30). Patients with lower trough plasma levels had substantially greater probability of ischemic stroke/SSE (<50 ng/mL) (39). Similarly, strong association was

found between increased dabigatran trough concentrations and bleeding risk. The mean dabigatran exposure in patients with major or minor bleeding was 50% and 20% greater than patients without. Bleeding risk was also dependent on age and renal impairment owing to modulation of dabigatran PK.

For rivaroxaban and apxiaban, the anti-FXa activity assay is a direct measure of anticoagulation intensity, and thus, the most sensitive and consistent assay for quantitating plasma concentrations (40, 41). The PT and HepTest also demonstrated sensitivity for rivaroxaban response and may be used as a more readily available measure of response (17). Apixaban concentrations was correlated with INR, PT, and aPTT with less sensitivity (36). A dedicated study of 161 patients embedded into the ROCKET-AF trial confirmed the linear relationship between PT and anti-FXa activity to rivaroxaban plasma exposure (42). However, PT did not predict the occurrence of ischemic strokes in the ROCKET-AF trial (42). Conversely, a linear relationship was demonstrated between PT prolongation and risk of major bleeding (42). The linear relationship between apixaban plasma concentrations and anti-FXa activity was confirmed in elective hip/knee replacement patients (12).

It is important to keep in mind that the sensitivity and precision of different reagents and instruments used for these coagulation assays is yet to be established. Further validation of these coagulation assays in measuring NOAC anticoagulation response is required in the real-world clinical setting. Moreover, is it evident that variability in NOAC plasma

exposure will have significant impact on anticoagulation efficacy given the direct PK-PD relationship and its association with clinical outcomes; thus, quantifying NOAC plasma concentration is likely the most reliable assessment of response and bleeding risk.

Selecting the right OAC

Although the NOACs have shown similar or greater efficacy to warfarin, it is unlikely that they will fully replace warfarin. The interindividual variability in exposure/response of NOACs and bleeding risk associated with anticoagulation therapy remains a pertinent issue. Indeed, even in a clinical trial setting with stringent enrollment criteria, the one-dose-fits-all dosing regimen strategy did not appear successful for NOACs, likely due to the various clinical covariates that significantly affected extent of drug exposure and response (Figure C.2) (41, 43-46). Moreover, dabigatran and rivaroxaban use outside of the clinical trial setting has recently been noted to exhibit large interindividual variability in concentration and response (43, 47). The same trend is likely to be observed with apixaban as its clinical use increases.

Nevertheless, the emergence of multiple NOACs has meant greater therapeutic options for treating physicians. However, we are now starting to face the question of how to select the most appropriate NOAC for individual patients. Factors to be assessed for deciding the right anticoagulant include patient bleeding risk (HAS-BLED) and benefit (CHADS2) as recommended by the Canadian Cardiovascular Society (48), lack of an antidote for NOACs, GI bleeding, renal/hepatic function, age, and concomitant

medications, relevant genetic variations, of which all have been demonstrated to influence the PK and PD of OACs.

Figure C.2 Plasma concentration profiles of dabigatran, rivaroxaban, and apixaban in atrial fibrillation patients. (A) Mean steady-state dabigatran plasma concentrations following 150 mg twice-daily administration is represented by the dashed black line (digitized from Dansirikul *et al.*) (49). Colored solid lines represent the predicted effect of various clinical variables and a genetic variation in the carboxylesterase 1 (*CES1*) gene on dabigatran concentration based on known area under the concentration curve (AUC) change (45). The shaded area represents C_{trough} dabigatran concentrations associated with increased antithrombotic efficacy and decreased major bleeding risk according to population pharmacokinetics modeling of the RE-LY trial data. (B) Mean steady-state rivaroxaban plasma concentrations following 20 mg once daily administration is represented by the dashed black line (digitized from Mueck *et al.*) (44). Colored solid lines represent the predicted effect of various clinical variables on rivaroxaban concentration based on known AUC change (44). Although the optimal plasma C_{trough} of rivaroxaban has not been well-defined as of yet, the shaded area represents the 5% - 95% confidence interval (CI) of C_{trough} observed in the ROCKET-AF trial and the dashed line represents the average C_{trough}. (C) Predicted mean steady-state apixaban plasma concentrations following 5 mg twice-daily administration is represented by the dashed black line (digitized from Leil *et al.*) (46). Colored solid lines represent the predicted effect of various clinical variables on apixaban concentration based on known AUC change (12). The shaded area represents the population pharmacokinetics model predicted 5% - 95% CI of C_{trough} in atrial fibrillation patients. AF, atrial fibrillation; CrCl, creatinine clearance; P-gp, P-glycoprotein.

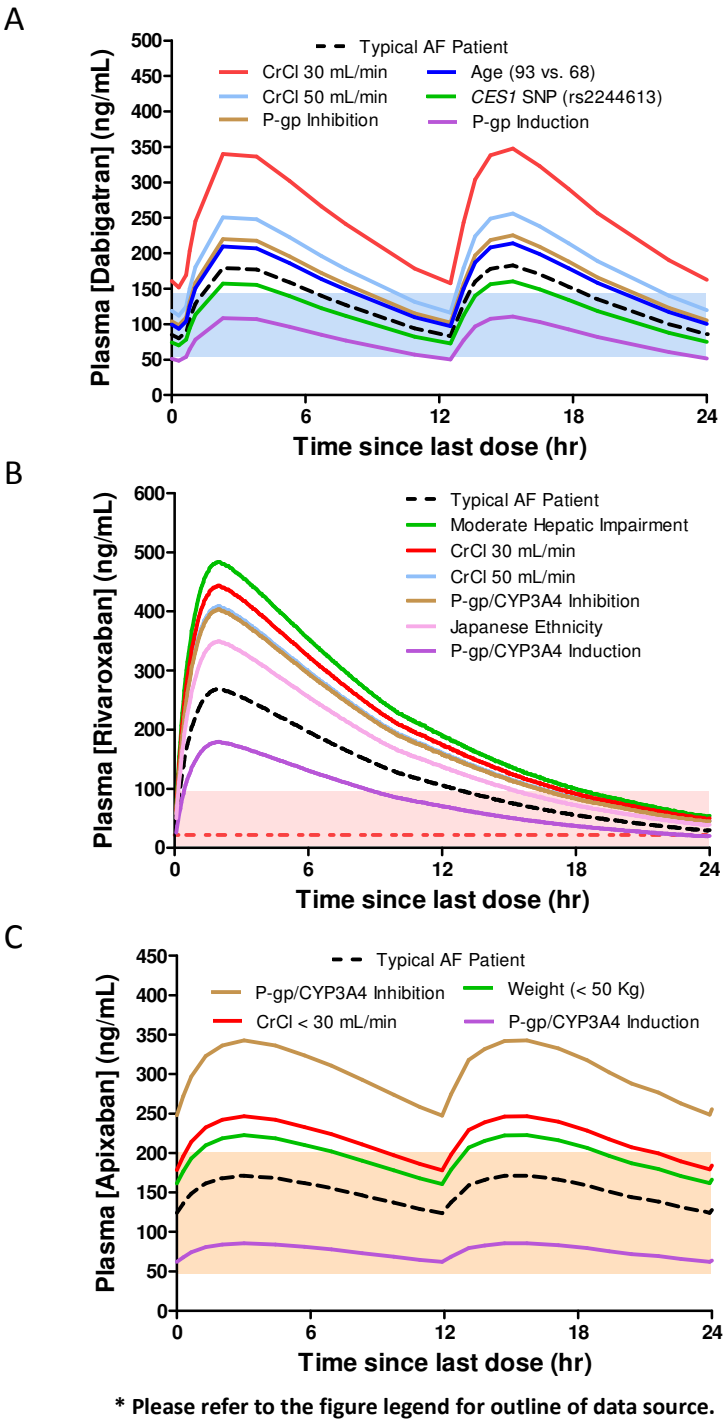

Figure C.2 Plasma concentration profiles of dabigatran, rivaroxaban, and apixaban in atrial fibrillation patients.

A potential decision tree for selecting the right oral anticoagulant based on individual patient characteristics *(summarized in Figure C.3):* The first question to address is whether the patient is a better candidate for NOAC or warfarin. For patients requiring new OAC therapy , the factors to consider in this regard include high cost of NOACs (dependent on patient insurance policy), convenient and accessibility of INR response monitoring, renal function, and potential drug-drug interactions (Table C.3). Patients who are already stably anticoagulated on warfarin achieving optimal TTR need not to be switched, given the familiarity of its use and ability to monitor anticoagulation efficacy. Indeed, the benefits of NOACs over warfarin are less impressive for patients achieving well-controlled TTR (> 65%) as evident in the finding that 150 mg BID dabigatran was no longer superior to warfarin for prevention with stroke (50). However, we note that no significant interaction was found between TTR and stroke/SSE rates in sub-analysis of the RE-LY, ROCKET-AF, and ARISTOTLE trial.

In suspected noncompliant patients, warfarin will likely be the better choice because missed doses of NOACs with short half-lives will be more detrimental to efficacy than missed doses of warfarin. Moreover, adherence to drug therapy decreases by 10% when comparing OD to BID dosing, leading to unknowable consequences, especially without active monitoring of efficacy. Of the NOACs, rivaroxaban is the only one with OD dosing, preferred for suspected noncompliant patients. We note however, based on the $t_{1/2\beta}$ of rivaroxaban (Figure C.2), when strictly considering optimal dosing interval, splitting the total daily dose to BID dosing interval would be predicted to result in a more consistent exposure and response.

Since NOACs are dependent on renal excretion at varying extents, estimated CrCl (creatinine clearance) should be determined in all patients prior to initiating NOACs. Moreover, risk of bleeding associated with use of the new agents is compounded by poor renal function; more than 50% of AF patients over the age of 80 have moderate renal impairment (51). Although dosing adjustments in renal insufficiency has been recommended, a recent report indicated that the dose reduction did not obviate elevated bleeding risk completely (52). Thus, patients with CrCl below 30 mL/min are not suitable for NOACs; however, if NOAC in patients with renal impairment is preferred, apixaban is more appropriate than rivaroxaban and dabigatran, otherwise, warfarin should be used. Elderly patients and those with renal impairment should be assessed regularly for renal function throughout treatment to ensure that CrCl remains greater than 30 mL/min.

Elevated GI bleeding risk is an important concern for dabigatran and rivaroxaban, and apixaban is a better choice in patients with a recent and/or recurrent history of GI bleed as well as patients with disease condition(s) predisposing higher risk of GI bleed (active peptic ulcer disease, recent biopsy or trauma, gastritis). However, in the case that the site of GI bleed is identified and the underlying condition adequately treated without suspected recurrence, any OAC may be considered for therapy if the patient is otherwise deemed suitable. Dose reduction is required for patients > 75 years old on dabigatran due to increased bleeding risk with age. The stability of dabigatran may be compromised

when stored in humid conditions, and once the bottle is opened the tablets should be used within 4 months. Patient education in this regard may be important.

Since all NOACs depend on the liver for hepatic metabolism to some degree, patients with hepatic dysfunction are not candidates for NOACs. Moreover, both rivaoxaban and apixaban are substrates of CYP3A4, rendering these NOACs susceptible to drug-drug interactions whereby dose reduction is required in presence of strong inhibitors. Lastly, for AF patients with recent acute coronary syndrome or percutaneous coronary intervention requiring triple therapy with antiplatelets and OAC (warfarin or NOACs), caution is warranted as the benefit in terms of decreased stroke and myocardial infarction risk with triple therapy may be offset by the near two-fold increase in bleeding risk (53). Warfarin may be the better choice for these patients owing to the ability to conduct routine INR monitoring as well as tailoring dose to a lower anticoagulant intensity (lower INR target) to reduce bleeding risk.

Figure C.3 Guide for choosing an oral anticoagulation based on influence of patient characteristics on drug pharmacokinetics. API, apixaban; DAB, dabigatran; RIV, rivaroxaban. BID, twice daily dosing, OD, once daily dosing. CrCl, creatinine clearance; ERSD, end-stage renal disease; DDI, drug-drug interaction; GI, gastrointestinal; mod, moderate; NOAC, new oral anticoagulant. Renal function classification based on estimated CrCl is in accordance with the product monographs for dabigatran, rivaroxaban, and apixaban.(12, 54, 55) †Moderate renal impairment is defined as CrCl 30 – 50 mL/min for dabigatran and apixaban, while moderate renal impairment is defined as CrCl 30 – 49 mL/min for rivaroxaban. *History of GI bleed refers to recent and/or recurrent GI bleeding events. Disease condition(s) predisposing higher risk of GI bleed include but not exclusive to active peptic ulcer disease, ulcerative gastrointestinal disease, recent biopsy or trauma, and gastritis.

Disclaimer: The information presented herein represents the opinion of the authors based on currently available data and should not serve as substitute for clinical judgment.

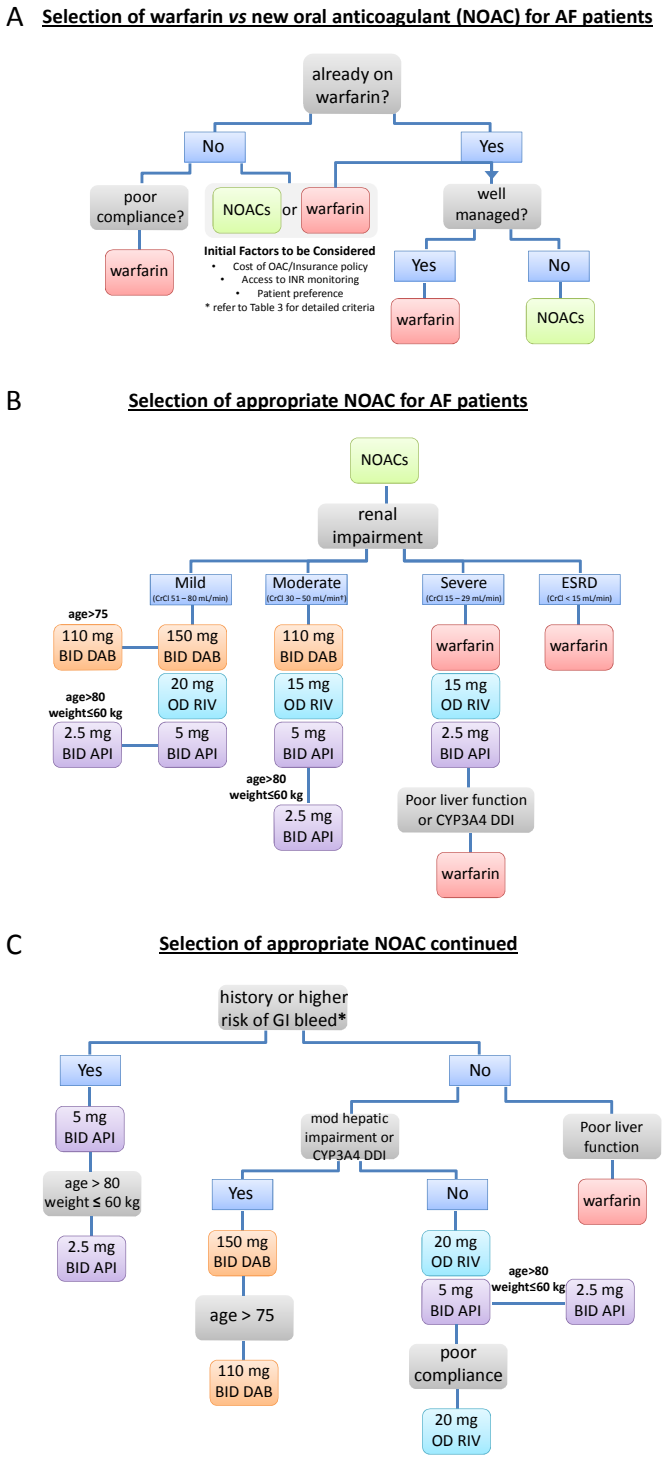

Figure C.3 Guide for choosing an oral anticoagulation based on influence of patient characteristics on drug pharmacokinetics.

Table C.3 Summary of patient criteria for selecting warfarin vs new oral anticoagulants (NOACs).

Select warfarin	Select NOACs
stable and well controlled INR	previously on warfarin with poor INR control
CrCl < 30 mL/min	normal renal function or mild renal dysfunction
low cost to patient/lack of insurance coverage	high cost of drug affordable
good compliance	inadequate access to routine INR monitoring
history of gastrointestinal bleeding	require rapid onset of action
require rapid reversal (antidote)	harboring multiple variant alleles in CYP2C9 and VKORC1 known to confer warfarin sensitivity *
concomitant use of P-gp/CYP3A4 inhibitor or inducer	patient preference

CrCl, creatinine clearance; CYP, cytochrome P450; INR, international normalized ratio; P-gp, P-glycoprotein; VKORC1, vitamin K epoxide reductase enzyme subunit 1.

* particularly CYP2C9 poor metabolizer genotype combined with VKORC1 sensitive genotype.

Dosing adjustments

Dosing adjustments are summarized in Figure C.3 and Table C.4. As noted earlier, kidney is the predominant pathway of excretion for dabigatran while to a lesser extent for rivaroxaban and apixaban. Renal function is an important variable that must be considered carefully in AF patients, particularly because age-related decline in renal function and variation introduced by comorbidities has been well-characterized. The impact of renal function and demographics has been evaluated in RE-LY AF patients. The median CrCl of the trial was 69 mL/min; patients with CrCl of 30 and 50 mL/min had a 1.8- and 1.2- fold increase in dabigatran AUC, respectively (45). Not surprisingly, age reduced apparent clearance (CL/F) by 0.41% per year from median age of 72. Based on these data, age and renal function warrants dose adjustment. The standard dabigatran dose for AF patients with normal renal function is 150 mg BID while approved doses for patients with CrCl < 30 mL/min differs between countries. In the United States, a dose of 75 mg is approved for patients with CrCl 15 - 29 mL/min. However, these recommendations were approved on the basis of PK modeling studies and clinical data supporting this is lacking, limiting the creditability of its use in real-world patients (45). In other countries including Canada, dabigatran use is contraindicated in patients with CrCl < 30 mL/min. For patients over 75 years old at increased bleeding risk, previous history of GI/intracerebral bleeding, or receiving concomitant antiplatelet or P-gp potent inhibitor, 110 mg BID should be used (a dose not available in the United States).

The typical rivaroxaban dose for most AF patients is 20 mg OD. Based on PK modeling,

15 mg OD of rivaroxaban in individuals with moderate or severe renal impairment would have similar plasma levels as individuals with normal renal function taking 20 mg (42, 44). In the ROCKET-AF trial, safety and efficacy of this dosing regimen in patients with moderate renal impairment was demonstrated in the comparable stroke and bleeding rates irrespective of renal impairment (56). Similar to dabigatran, approved doses of rivaroxaban is country-specific. In United States, 20 mg rivaroxaban is approved for patients with CrCl > 50 mL/min while 15 mg is approved for patients with CrCl 15 – 49 mL/min. In all other countries, 15 mg is approved only for those with CrCl 30 – 49 mL/min and contraindicated in those with severe impairment. Unlike dabigatran, patient age doesn't appear to elevate bleeding risk and no dosing adjustments is suggested. Lower dose of 15 mg should be used with coadministration of a strong CYP3A4/P-gp inhibitor or multiple moderate inhibitors.

The dose of apixaban to be administered to the average patient is 5 mg BID. In patients with mild or moderate renal impairment, no dose adjustment is necessary. Patients with severe renal impairment (CrCl 15 - 29 mL/min) require 2.5 mg BID and use in patients with CrCl < 15 mL/min is contraindicated. While no dose adjustment is required in patients with mild or moderate hepatic impairment, apixaban use should be used with caution in such patients. Individuals with body weight < 50 Kg had 30% greater exposure than individuals weighing 65 to 85 kg, whom had 30% lower exposure compared to individuals weighing > 120 kg (12). Elderly patients above 65 years-of-age exhibited 32% greater AUC than younger patients (12). Accordingly, dose reduction to 2.5 mg BID is warranted for patients concomitantly taking CYP3A4/P-gp inhibitors or meeting at

least two of the following criteria: > 80 years old, body weight ≤ 60 kg, and creatinine > 133 μmol/L.

Ethnic differences in dosing requirement have only been found for rivaroxaban. The J-ROCKET trial was conducted to evaluate rivaroxaban use in Japanese patients (57). This was due to the observation that healthy Japanese subjects had a 20-40% increase in rivaroxaban exposure compared to Caucasian subjects (57). Thus, 15 mg rivaroxaban in J-ROCKET patients resulted in similar efficacy and exposures as that observed in non-Japanese patients. Similar reductions in rivaroxaban dose should be considered for patients of Asian ancestry, and not limited to those of Japanese ancestry.

Notably, we are starting to realize the role of genetic variation to interindividual variability of NOAC exposure and clinical outcomes. The *CES1* rs2244613 SNP was not only associated with lower dabigatran exposure but also with reduced bleeding risk, indicating the potential for pharmacogenetics-based dosing adjustments using this intronic SNP (16). As clinically relevant polymorphisms also exist in CYP3A4/5, P-gp, and BCRP, these genetic variations may also play an important role in determining NOAC exposure. Overall, the full spectrum of NOAC efficacy and safety within the context of pharmacogenetics, demographics, concomitant medications, and comorbidities should be characterized as the use of these new agents increase in AF patients outside of the clinical trial setting.

Table C.4 Dosing adjustments based on pharmacokinetic considerations.

	Dabigatran (mg BID)	Rivaroxaban (mg OD)	Apixaban (mg BID)
Renal impairment			
Mild (CrCl 51 – 80 mL/min)	150	20	5
Moderate (CrCl 30 – 50 mL/min)	110	15	5
Severe (CrCl < 30 mL/min)	n.r.	15	2.5
Hepatic impairment			
Mild (Child-Pugh A)	150	20	5
Moderate (Child-Pugh B)	150	n.r.	5
Severe (Child-Pugh C)	n.r.	n.r.	n.r.
Hepatic dysfunction	n.r.	n.r.	n.r.
Demographic variables			
Ethnicity, Asian	150	15	5
Age, > 75 - 80 yr	110	20	2.5
Weight, < 50 kg	150	20	2.5
Drug-drug interactions			
P-gp inhibitor	110	15	2.5
CYP3A4 inhibitor	150	15	2.5
P-gp/CYP3A4 inducer	n.r. *	n.r.	n.r.

n.r., not recommended. CYP, cytochrome P450; P-gp, P-glycoprotein.

* CYP3A4 and P-gp are both transcriptionally regulated by xenobiotic sensing nuclear receptors such as Pregnane X Receptor (PXR) and Constitutive Androstane Receptor (CAR) (58). Therefore, inducers listed in Table C.2 would lower the plasma level of all the NOACs outlined in this review.

Limitations of NOACs

A major limitation of NOAC use is the lack of standardized coagulation tests for monitoring anticoagulation response. The need for monitoring has been heavily debated and regulators recently stated that monitoring is necessary in specific clinical circumstances: renal/liver dysfunction, overdose, prior to invasive surgery, non-compliance, bleeding or thrombosis/stroke, drug-drug interaction and suspected variation in drug exposure (59). Although some assays have been suggested as surrogate markers of exposure, limitations in their use include accuracy and clinical experience/availability. More importantly, meaningful interpretation of the results is difficult owing to lack of guidance on extrapolating coagulation results to bleeding and thrombosis risk. Accordingly, directly measuring plasma NOAC drug concentrations may be more desirable and more accurate for monitoring anticoagulation. An important consideration for measuring either coagulation or NOAC drug exposure is the standardization of sampling time from the last dose. The C_{trough} level is preferred over the C_{max}, avoiding misinterpretation of results due to variability in absorption phase.

The second limitation is the lack of a validated antidote for rapidly reversing NOACs' effects. Hemodialysis of dabigatran may be an option given its relatively low protein binding, but not for rivaroxaban and apixaban which have high protein binding. Potential reversal agents that have shown promise for reversing NOAC anticoagulation include prothrombin complex concentrate and recombinant factor VIIa (60, 61). Until better guidelines are available, NOACs should be used with caution, particularly in patients with elevated bleeding risk.

Switching from warfarin to NOAC and vice versa

Patients without adequate INR control will likely benefit from transitioning from warfarin to one of the NOACs for enhanced efficacy and reduced risk of intracranial bleeding. It has been recommended that NOAC should be started after INR decreases to < 2.3 following warfarin discontinuation (62). For patients unable to continue NOAC therapy and require transitioning to warfarin, it is necessary to consider the delayed onset of warfarin and allow attainment of therapeutic INR prior to discontinuation of NOAC.

Conclusions

NOACs are promising alternatives to warfarin, demonstrating at least similar antithrombotic efficacy and decreased rate of intracranial hemorrhage. However, the availability of multiple NOACs has introduced difficulty in deciding the best agent since head-to-head trials are unavailable and unlikely to be performed. Rather, clinicians are required to make informed decisions in selecting the appropriate agent based on characteristics of the patient and OAC pharmacology. As the clinical use of NOACs increases, surveillance using therapeutic monitoring (measurement of plasma drug concentration or anticoagulation response) throughout the treatment period may be valuable in minimizing risk of bleeding and lack of efficacy. Finally, due to the extent of interindividual variation in the metabolism and clearance of NOACs, it is likely a greater range of NOAC doses will be needed to more precisely treat our patients.

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13. Stangier J, Rathgen K, Stahle H, Gansser D, Roth W. 2007. The pharmacokinetics, pharmacodynamics and tolerability of dabigatran etexilate, a new oral direct thrombin inhibitor, in healthy male subjects. *Br J Clin Pharmacol* 64: 292-303

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17. Kubitza D, Becka M, Wensing G, Voith B, Zuehlsdorf M. 2005. Safety, pharmacodynamics, and pharmacokinetics of BAY 59-7939--an oral, direct Factor Xa inhibitor--after multiple dosing in healthy male subjects. *Eur J Clin Pharmacol* 61: 873-80

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32. Nutescu E, Rhoades R, Bailey C, Zhou S. 2012. Apixaban: a novel oral inhibitor of factor Xa. *Am J Health Syst Pharm* 69: 1113-26

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60. Marlu R, Hodaj E, Paris A, Albaladejo P, Crackowski JL, Pernod G. 2012. Effect of non-specific reversal agents on anticoagulant activity of dabigatran and rivaroxaban: a randomised crossover ex vivo study in healthy volunteers. *Thromb Haemost* 108: 217-24

61. Bauer KA. 2012. Reversal of antithrombotic agents. *Am J Hematol* 87 Suppl 1: S119-26

62. Schulman S, Crowther MA. 2012. How I treat with anticoagulants in 2012: new and old anticoagulants, and when and how to switch. *Blood* 119: 3016-23

Curriculum Vitae

Inna Y Gong

London Health Sciences Centre, University Hospital
339 Windermere Road, BLL-116
London, ON, Canada

Post-secondary Education

Doctor of Philosophy, Pharmacology
2008-2013
The University of Western Ontario
London, Ontario, Canada

Bachelor's of Medical Sciences, Pharmacology and Toxicology
Scholar's Electives
2004-2008
The University of Western Ontario
London, Ontario, Canada

Scholarships

Canadian Institutes of Health Research (CIHR)
Canada Graduate Scholarships: Doctoral Research Award ($86,667)
2011-2013

Canadian Institutes of Health Research (CIHR)
Canada Graduate Scholarships: Master's Award ($17,500)
2010-2011

Government of Ontario
Ontario Graduate Scholarships (OGS) ($30,000)
2008-2010

The University of Western Ontario
Schulich Graduate Scholarship ($32,000)
2008-2013

The University of Western Ontario
Entrance scholarship ($5,000)
2004

Awards

Canadian Society of Pharmacology and Therapeutics
Publication Award
2012
Canadian Society of Pharmacology and Therapeutics
CIHR Drug Safety and Effectiveness Network Presentation Award
2012
Canadian Society of Pharmacology and Therapeutics
Piafsky Travel Award
2010
Department of Physiology and Pharmacology Research Day, The University of Western Ontario
Presentation Award
2010
International Society for the Study of Xenobiotics
Presentation Award
2009
Schulich Margaret Moffat Research Day, The University of Western Ontario
Presentation Award
2009
J. Allyn Taylor Prize in Medicine Symposium, The University of Western Ontario
Presentation Award
2009
21st Charles W. Gowdey Lecture, The University of Western Ontario
Presentation Award
2009

Extra-curricular Activities: Related Work Experience

Research Intern, Department of Clinical Pharmacology
Genentech Inc, South San Francisco, California
Analysis and mathematical modeling of data collected from human trials for proprietary drugs, analysis of drug-drug interaction potential of proprietary compounds in preparation for submission to the FDA, conducted a department seminar, attendance of weekly seminars, interaction and exchange of ideas with world-renowned scientists, drafting of a manuscript.
Sept-Dec 2011

Systematic Review Data Extractor, Department of Epidemiology and Biostatistics
McMaster University, Hamilton, Ontario
Reviewed literature in PubMed, screening of appropriate articles for a systematic review.
2011

Extra-curricular Activities: Teaching Experience

Teaching Assistant, Department of Physiology and Pharmacology
Pharmacology 2060: Introduction to Pharmacology and Therapeutics
Organized and delivered brief lectures covering basic areas of pharmacology and therapeutics, proctoring exams, addressing questions raised by students on the discussion board.
2008-2012

Teaching Assistant, Department of Physiology and Pharmacology
Pharmacology 4350: Clinical Pharmacology
Organized student presentations, held office hours, marking exams, proctoring exams, addressing questions raised by students on the discussion board.
2012-2013

Undergraduate Thesis Project Supervisor, Division of Clinical Pharmacology

Responsible for teaching experimental design and techniques, critical thinking, and written and oral communication skills to a 4th year Honours student.
2010-2011

International Collaborations

Vanderbilt University
Collaborated with Dan Roden, MD, and C Michael Stein, MD, at Vanderbilt University Medical Centre (Tennessee), to interpret and analyze data from their database of atrial fibrillation and venous thromboembolism patients prescribed with warfarin.

Genentech Inc
Collaborated with Edna Choo, PhD, at Genentech Inc. to design and analyze data obtained from pharmacological efficacy studies of endoxifen in preclinical animal models. Subsequently, the collaboration extended to translating the preclinical data to that observed in the patient clinical setting to predict efficacy.

National Collaborations

Ottawa Hospital Research Institute
Collaborated with Philip Wells, MD, at the Thrombosis Clinic to initiate patients with new diagnosis of deep vein thrombosis or pulmonary embolism using warfarin based on individual genetic make-up.

Publications

Published

Reviews

Gong IY, Kim RB. (2013) Importance of pharmacokinetic profile and variability as determinants of dose and response to dabigatran, rivaroxaban, and apixaban. *Canadian Journal of Cardiology,* Jul;29(7 Suppl):S24-33.

Gong IY, Kim RB. (2013) Pharmacogenetic advances in cardiovascular medicine: Relevance to personalized medicine. *Current Genetic Medicine Reports*, in press.

Gong IY, Kim RB. (2012) Impact of genetic variation in OATP transporters to drug disposition and response. *Drug Metab Pharmacokinet*, E-pub Oct 9, 2012.

Original Articles

Gong IY, Teft WA, Ly J, Chen Y, Alicke B, Kim RB, Choo EF. (2013) Determination of clinically therapeutic endoxifen concentrations efficacy from human MCF7 breast cancer xenografts. *Breast Cancer Res Treat,* doi10.1007/s10549-013-2530-1.

Gong IY, Mansell SE, Kim RB. (2013) Absence of both MDR1 (ABCB1) and BCRP (ABCG2) transporters significantly alter rivaroxaban disposition and CNS entry. *Basic Clin Pharmacol Toxicol*, 112(3):164-70.

Gong IY, Crown N, Suen CM, Schwarz UI, Dresser GK, Knauer MJ, Sugiyama D, DeGorter MK, Woolsey S, Tirona RG, Kim RB. (2012) Clarifying the importance of CYP2C19 and PON1 in the mechanism of clopidogrel bioactivation and in vivo antiplatelet response. *Eur Heart J,* 33(22):2856-2464a.

Gong IY, Schwarz UI, Crown N, Dresser GK, Lazo-Langner A, Wells PS, Kim RB, Tirona RG. (2011) Clinical and genetic determinants of warfarin pharmacokinetics and pharmacodynamics during treatment initiation. *PloS One,* 6(11): e27808.

Gong IY, Tirona RG, Schwarz UI, Crown N, LaRue S, Langlois N, Dresser GK, Lazo-Langner A, Zou GY, Rodger M, Carrier M, Forgie M, Wells PS, Kim RB. (2011) Pharmacogenetics-guided warfarin loading and maintenance dosing regimen eliminates VKORC1 and CYP2C9 associated variation in anticoagulation response. *Blood,* 118(11):3163-71.

Teft WA, **Gong IY**, Dingle B, Potvin K, Younus J, Vandenberg TA, Brackstone M, Perera FE, Choi Y, Zou G, Legan RM, Tirona RG, Kim RB. (2013) CYP3A4 and seasonal variation in vitamin D status contribute to therapeutic endoxifen level during tamoxifen therapy. *Breast Cancer Res Treat,* doi10.1007/s10549-013-2511-4.

Clark J, Cutler M, **Gong IY**, Schwarz UI, Freeman D, Dasgupta M. (2011) Cytochrome P450 2D6 phenotyping in an elderly demented population and response to galantamine in dementia. *Am J Geriatr Psychiatry*, Aug;9(4):224-33.

Madadi P, Hildebrandt D, **Gong IY**, Schwarz UI, Ciszkowski C, Ross C, Sistonen J, Carleton BC, Hayden MR, Lauwers AE, Koren G. (2010) Fetal hydrocodone overdose in a child: pharmacogenetics and drug interactions. *Pediatrics,* Oct;126(4):e986-9.

Publications, in preparation for submission

Jin JY, **Gong IY**, Chen Y, Salphati L, Budha N, West DA, Mukadam S, Holden S, Dresser MJ, Ware JA. Prediction of CYP3A4 mediated drug-drug interaction potential between PI3K inhibitor GDC-0941 and ketoconazole through physiologically based pharmacokinetics modeling using Simcyp.

Richardson B, **Gong IY**, Tirona RG, Huang SS, Ledingham D. Case Report: Dabigatran toxicity and clearance with hemodialysis.

Published Abstracts

Involvement of efflux transporters MDR1 (ABCB1) and BCRP (ABCG2) in rivaroxaban disposition. (2012) Gong IY, Mansell SE, Kim RB. *Drug Metab Rev.*

Clarifying the importance of CYP2C19 and PON1 in the mechanism of clopidogrel bioactivation and *in vivo* antiplatelet response. (2012) **Gong IY**, Crown N, Suen CM, Schwarz UI, Dresser GK, Knauer MJ, Sugiyama D, DeGorter MK, Woolsey S, Tirona RG, Kim RB. *Journal of Population Therapeutics and Clinical Pharmacology*.

Regulation of Cytochrome P450 3A4 in Non-Alcoholic Fatty Liver Disease by Fibroblast Growth Factor 21. (2012) Woolsey SJ, **Gong IY**, Stein S, Kim RB, Levstik M, Beaton MD, Tirona RG. *Hepatology*.

Pharmacokinetic-Pharmacodynamic Modeling Reveals Novel Determinants of Warfarin Response During Initiation of Therapy. (2011) **Gong IY**, Schwarz UI, Tirona RG, Crown N, Dresser GK, Roden DM, Stein CM, Lazo-Langner A, Zou GY, LaRue S, Langlose N, Wells P, Kim RB. *Clin Pharmacol Thera*.

Molecular Determinants of Warfarin Pharmacokinetics and Pharmacological Response. (2010) **Gong IY**, Schwarz UI, Tirona RG, Crown N, Dresser GK, Lazo-Langner A, Zou GY, LaRue S, Langlose N, Wells P, Kim RB. *Can J Clin Pharmacol*.

Pharmacokinetic and pharmacodynamic determinants of warfarin anticoagulation response. (2009) **Gong IY**, Schwarz UI, Tirona RG, Crown N, Dresser GK, Lazo-Langner A, Zou GY, LaRue S, Langlose N, Wells P, Kim RB. *Drug Metab Rev.*

Warfarin oral anticoagulation therapy: *R/S*-warfarin and vitamin K levels as determinants of maintenance dose. (2009) **Gong IY**, Schwarz UI, Tirona RG, Kim RB. *Can J Clin Pharmacol*.

Projects in Progress

Antiplatelet therapy:
Role: Lead; recruit patients already on prescribed with clopidogrel
Objectives:
Evaluate the role of genetics in explaining the interpatient variability in pharmacokinetics and antiplatelet response to clopidogrel therapy.
Elucidate the PK-PD relationship of clopidogrel active metabolite to platelet reactivity.

Antiplatelet therapy: (in collaboration with cardiologists)
Role: Lead; enrollment of patients on clopidogrel and new antiplatelet agent ticagrelor
Objective:
Evaluate the interpatient variability in pharmacokinetics of patients on clopidogrel and ticagrelor, as well as delineating biomarkers of drug-related side effects.
Assessment and compare endothelial function in patients treated with clopidogrel and ticagrelor.

Immunosuppressant therapy: (in collaboration with nephrologists)
Role: Lead; enrollment of patients on tacrolimus following organ transplantation
Objective:
Assessment of genetic markers of tacrolimus response, pharmacokinetics, and toxicities to design a precise protocol for individualized loading and maintenance tacrolimus dosing regimen.

Invited Oral Presentations

"Genetic and clinical determinants of CYP3A4 activity in patients using 4β-hydroxycholesterol as an in vivo probe" **Gong IY**, DeGorter MK, Schwarz UI, Choi YH, Yin P, Tirona RG, Kim RB. American Society of Pharmacology and Experimental Therapeutics 2013, Boston, Massachusetts, April 2013.

"Pharmacogenetics of oral anticoagulants and antiplatelets: implications on clinical practice" **Gong IY**. Department of Physiology and Pharmacology Seminar Series, the University of Western Ontario, London, Ontario, January 2013.

"Learn and confirm: Evaluation of CYP3A4 Mediated Drug-Drug Interaction Potential Between PI3K Inhibitor GDC-0941 and Ketoconazole Using Simcyp" **Gong IY**. Department of Clinical Pharmacology Seminar Series, Genentech, South San Francisco, California, December 2011.

"Role of Efflux Transporters MDR1 and BCRP to Rivaroxaban Disposition" **Gong IY**, Kim RB. Canadian Society of Pharmacology and Therapeutics Meeting 2011, Montreal, Quebec, May 2011.

"Warfarin pharmacogenetics and better patient care: the importance of teamwork in patient oriented research" **Gong IY**, Crown N, Dresser GK, Schwarz UI, Tirona RG, Kim RB. Medical Grand Rounds, Department of Medicine, London Health Sciences Centre, London, Ontario, January - February 2011.

"Molecular Determinants of Warfarin Pharmacokinetics and Pharmacological Response" **Gong IY**, Schwarz UI, Tirona RG, Crown N, LaRue S, Langlois N, Dresser G, Lazo-Langner A, Zou GY, Wells P, Kim RB. Canadian Society of Pharmacology and Therapeutics Meeting 2010, Toronto, Ontario, June 2010.

"Drug disposition and dosing strategies of old and new oral anticoagulants" **Gong IY.** Division of Clinical Pharmacology Grand Rounds, University Hospital, London, Ontario, January 2010.

"Liquid chromatography mass spectrometry: Basic Principals and Application to Clinical Pharmacology" **Gong IY**, Urquhart BL. Academic Teaching for Post-Graduate Medical Residents, University Hospital, London, Ontario, September 2009.

"Personalized Medicine and Warfarin Initiation: UWO Experience and Preliminary Data" Kim RB, Tirona RG, Schwarz UI, **Gong IY**. Pharmacogenetics Research Network Webinar, April 2009.

External Poster Presentations

"CYP3A4*22 single nucleotide polymorphism is an important determinant of endoxifen plasma concentration" **Gong IY**, Teft WA, Dingle B, Potvin K, Younus J, Vandenberg TA, Brackstone M, Perera FE, Choi Y, Zou G, Legan RM, Tirona RG, Kim RB. American Society of Clinical Pharmacology and Therapeutics Meeting 2013, Indianapolis, Indiana, March 2013.

"Role of Efflux Transporters MDR1 and BCRP to Rivaroxaban Disposition" **Gong IY**, Kim RB. 19th International Symposium on Microsomes and Drug Oxidations and 12th European International Society for the Study of Xenobiotics Meeting, Noordwijk Aan Zee, Netherlands, June 2012.

"CYP2C19, PON1, and the role of PPIs to clopidogrel bioactivation and *in vivo* antiplatelet response" **Gong IY**, Crown N, Suen CM, Schwarz UI, Dresser GK, Knauer MJ, Sugiyama D, DeGorter MK, Woolsey S, Tirona RG, Kim RB. Canadian Society of Pharmacology and Therapeutics Meeting 2012, Toronto, Ontario, June 2012.

"Pharmacokinetic-Pharmacodynamic Modeling Reveals Novel Determinants of Warfarin Response During Initiation of Therapy" **Gong IY**, Schwarz UI, Crown N, Dresser GK,

Lazo-Langner A, Zou G, Roden DM, Stein CM, Wells PS, Kim RB, Tirona RG. American Society of Clinical Pharmacology and Therapeutics Meeting 2011, Dallas, Texas, March 2011.

"Molecular determinants of warfarin pharmacokinetics and response" **Gong IY**, Schwarz UI, Tirona RG, Crown N, LaRue S, Langlois N, Dresser G, Lazo-Langner A, Zou GY, Wells P, Kim RB. 16th World Congress of Basic and Clinical Pharmacology, Copenhagen, Denmark, July 2010.

"Pharmacokinetic and Pharmacodynamic Determinants of Warfarin Anticoagulation Response" **Gong IY**, Schwarz UI, Tirona RG, Crown N, LaRue S, Langlois N, Dresser G, Lazo-Langner A, Zou GY, Wells P, Kim RB. 16th Annual Meeting of the International Society for the Study of Xenobiotics, Baltimore, Maryland, October 2009.

"Warfarin oral anticoagulation therapy: *R/S*-warfarin and vitamin K levels as determinants of maintenance dose." **Gong IY**, Schwarz UI, Tirona RG, Kim RB. Canadian Society of Pharmacology and Therapeutics Meeting 2009, Saskatoon, Saskatchewan, May 2009.

"Prediction of CYP3A4 mediated drug-drug interaction potential between PI3K inhibitor GDC-0941 and ketoconazole through physiologically-based pharmacokinetics modeling" Jin JY, **Gong IY**, Chen Y, Salphati L, Budha N, West DA, Mukadam S, Holden S, Ware JA, Dresser MJ. American Society of Clinical Pharmacology and Therapeutics Meeting 2012, Washington, Maryland, March 2012.

"Regulation of Cytochrome P450 3A4 in Non-Alcoholic Fatty Liver Disease by Fibroblast Growth Factor 21" Woolsey SJ, **Gong IY**, Stein S, Kim RB, Levstik M, Beaton MD, Tirona RG. American Association for the Study of Liver Diseases Meeting 2012, Boston, Massachusetts, November 2012.