THE PHARMACOGENOMICS OF WARFARIN SAFETY AND EFFECTIVENESS IN CHILDREN

by

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Abstract

Use of the anticoagulant warfarin for thromboembolic disease prophylaxis is limited by a large inter-patient dose variability and high risk of adverse drug reactions (ADRs). Polymorphisms in two genes, *CYP2C9* and *VKORC1*, have consistently been shown to be associated with warfarin dose requirements in adults. However, evidence on the importance of genetics in warfarin therapy in children is limited. Further paediatric studies are required to understand the predictive factors contributing to dose variation and to help prevent warfarin-induced ADRs in children.

In this study we aimed to assess the contribution of genotypes and clinical factors to warfarin dose and related outcomes in children. Clinical and genetic data was collected from 93 patients less than 19 years of age who received warfarin therapy. DNA was genotyped for 93 single nucleotide polymorphisms using a custom assay. Associations between *CYP2C9/VKORC1/CYP4F2* genotypes and therapeutic dose, time to therapeutic INR/time to over-anticoagulation, and incidence of adverse drug reactions were analyzed. Additional variants in genes involved in vitamin K and coagulation pathways were tested for an association with warfarin dose.

76.3% of dose variability was explained by weight, indication, *VKORC1* −1639G/A and *CYP2C9*2/*3*, with genotypes accounting for 21.1% of variability. There was a strong correlation (R^2=0.68; p<0.001) between actual and predicted warfarin dose using a pediatric genotype-based dosing model. *VKORC1* genotype also had a significant impact on time to

therapeutic INR (p=0.047) and time to INR >4 (p=0.024) during the initiation of therapy. An additional variant in *CYP2C9* (rs7089580) was significantly associated with warfarin dose in a multivariate model.

This study confirms the importance of *VKORC1/CYP2C9* genotypes for warfarin dose and clinical outcomes in children and validates a pediatric-specific genotype-based dosing algorithm. Furthermore, we identified an additional variant in *CYP2C9* of potential relevance for warfarin dosing in children.

Preface

This study was approved by the University of British Columbia Clinical Research Ethics Board (certificate numbers H04-70538 and H12-00819).

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List of Abbreviations

ACE	Angiotensin Converting Enzyme
ADME	Absorption, Distribution, Metabolism, and Excretion
ADR	Adverse Drug Reaction
AERS	Adverse Event Reporting Systems
ANOVA	Analysis of Variance
ApoE	Apolipoprotein E
BMI	Body Mass Index
BSA	Body Surface Area
BT	Blalock-Taussig
CALU	Calumenin
CEU	Northern and Western European Ancestry (Utah, United States)
CHD	Cardiac Heart Disease
CI	Confidence Interval
CNS	Central Nervous System
COX-2	Cycolooxygenase-2
CPNDS	Canadian Pharmacogenomics Network for Drug Safety
CVL	Central Venous Line
CYP	Cytochrome P450
DNA	Deoxyribonucleic Acid
DVT	Deep Vein Thrombosis

EM	Extensive Metabolizer
EPHX1	Epoxide Hydrolase 1
F2	Factor 2
FDA	U.S. Food and Drug Administration
FFP	Fresh Frozen Plasma
GGCX	Gamma Glutamyl Carboxylase
GI	Gastrointestinal
GST	Glutathione S-Transferase
GWAS	Genome-Wide Association Study
Hb	Hemoglobin
HLA	Human Leukocyte Antigen
HR	Hazard Ratio
HWE	Hardy-Weinberg Equilibrium
INR	International Normalized Ratio
IWPC	International Warfarin Pharmacogenetics Consortium
LD	Linkage Disequilibrium
LDL	Low Density Lipoprotein
MAF	Minor Allele Frequency
mEH	Microsomal Epoxide Hydrolase 1
NSAID	Non-steroidal Anti-inflammatory Drug
OR	Odds Ratio
PD	Pharmacodynamics

PE	Pulmonary Embolism
PK	Pharmacokinetics
PM	Poor Metabolizer
POR	P450 Cytochrome Oxidoreductase
PROC	Protein C
SJS	Stevens-Johnson Syndrome
SLCO1B1	Solute Carrier Organic Anion Transporter Family Member 1B1
SNP	Single Nucleotide Polymorphism
TE	Thromboembolic Event
TEN	Toxic Epidermal Necrosis
TPMT	Thiopurine *S*-methyltransferase
TPN	Total Parenteral Nutrition
UM	Ultrarapid Metabolizer
VKORC1	Vitamin K Epoxide Reductase Complex, Subunit 1
WGA	Whole Genome Amplified
WHO	World Health Organization
YRI	Yoruba African Ancestry (Ibadan, Nigeria)

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Chapter 1: Introduction

1.1 Pharmacogenomics

More than two thousand years ago the concept of personalized medicine was conceived when Hippocrates noted that different patients require different drugs for the same indication[1]. While he attributed this finding to general differences in the constitution of patients, the specific reasoning for inter-patient variability in drug response was far from being understood. It has been well established that multiple clinical factors, including age, body surface area, disease, and concomitant drug interactions can significantly influence an individual's response to a drug[2,3]. However, when only clinical factors are accounted for a large amount of variation in drug response often remains unexplained. In a growing number of cases, genetics is they key to understanding why some patients experience the intended response to a drug while others experience toxic or non-therapeutic responses. Genetic variants in drug metabolizing enzymes, transport systems, and drug targets can alter the pharmacodynamics (PD) or pharmacokinetics (PK) of a drug, leading to variation in response[4]. Approximately 20-95% of drug response variability is attributed to genetics[3]. The field of pharmacogenomics aims to discover genetic variants that influence individual drug efficacy and drug toxicity, in hopes of maximizing both therapeutic potential and drug safety.

Single alterations in the DNA sequence, referred to as single nucleotide polymorphisms (SNPs), are the most commonly investigated genetic variants in pharmacogenomic studies. Targeted pharmacogenomic studies, in which a small number of variants in candidate genes

or target pathways are investigated, are often employed to test a pre-established hypothesis on the effect of changes in drug exposure (PK) or drug effectiveness (PD) on drug response[5]. Drug pharmacokinetics are determined by drug absorption, distribution, metabolism and excretion (ADME). Genetic variation in drug metabolizing enzymes or drug transporters can lead to changes in drug exposure by influencing any of these processes. In contrast, drug pharmacodynamics are dependent on a drug interacting with its target site and producing a biochemical or physiological effect. Polymorphisms that interfere with drug-receptor interactions, such as altered receptor affinity or inhibition of cellular membrane transport, can result in pharmacodynamic alterations[6]. Both drug efficacy and risk of drug toxicity are influenced by genetic variation in PK and PD pathways. Genome-wide association studies (GWAS), whole-exome or whole-genome sequencing may also be used in pharmacogenomic studies for exploratory purposes. This method is often preferred when drug metabolism or ADR pathways are not clearly defined, or a large amount of unexplained variability in drug response remains following candidate gene studies.

1.1.1 Pharmacogenomics and drug effectiveness

If adequate levels of active drug metabolite are not achieved or a drug is unable to reach or bind to its target protein, drug effectiveness may be compromised. Polymorphisms in drug transporters have been shown to influence drug effectiveness by causing changes in tissue-specific or intracellular drug exposure[6]. One example is decreased effectiveness from simvastatin due to a polymorphism in the solute carrier organic anion transporter family member 1B1 (*SLC01B1*) transporter gene. Carriers of the rs4140956 (521T>C) C allele have a significantly smaller reduction in low-density lipoprotein (LDL) levels compared to non-

carriers, implying that carriers of this variant may experience a reduced therapeutic effect from simvastatin[7]. SLCO1B1 is a transporter that mediates the hepatocellular uptake of simvastatin; therefore, polymorphisms in *SLCO1B1* could affect statin efficacy by causing changes in intra-hepatocyte drug exposure. Accordingly, pharmacokinetic analyses have shown that carriers of the 521C>T polymorphism have higher blood statin concentrations and reduced simvastatin uptake, resulting in a decreased amount of drug at the target site and potentially contributing to reduced efficacy[8-10].

Drug efficacy can also be significantly influenced by genetic polymorphisms in drug-metabolizing enzymes. For example, Kim et al. (2008) reported that carriers of the cytochrome P450 (CYP) 2C19 (*CYP2C19*) **2/*2* or **2/*3* genotype (poor metabolizer (PM) phenotype) had higher mean plasma concentrations of the prodrug clopidogrel when compared to homozygous extensive metabolizers (homoEMs) and heterozygous extensive metabolizers (heteroEMs)[11]. Moreover, differences in plasma concentrations were associated with differences in efficacy, with PMs exhibiting a significantly lower antiplatelet effect compared to homoEMs and heteroEMs. This finding may be attributed to an inability by PMs to efficiently metabolize clopidogrel to its active form. Therefore, patients with genetic polymorphisms in enzymes required for drug bioactivation may require alternative therapies in order to achieve a maximal therapeutic effect. In many cases, clinically significant differences in drug efficacy cannot be linked to a single gene or genetic variant, but rather there are a multitude of ADME variants that play a role in individual drug efficacy.

1.1.2 Pharmacogenomics and adverse drug reactions

The World Health Organization (WHO) defines an adverse drug reaction (ADRs) as "a response to a medicinal product that is noxious and unintended"[12]. Over 100,00 deaths in the United States are attributed to ADRs each year, making them one of the top five leading causes of death[13]. Some ADRs develop as an extension of a drug's therapeutic effect and are classified as type A reactions. In contrast, type B reactions are idiosyncratic and occur independent of drug dose and pharmacologic effect. ADRs can vary in severity, with some ADRs presenting as a mild adverse response, while others are life-threatening or produce life-long consequences[14]. Furthermore, ADRs can differ in timing of onset following administration of the causative drug, ranging from just a few minutes to years of exposure. In most cases, a mixture of clinical, environmental and genetic factors contribute to the risk of drug-induced toxicity. It is estimated that genetics play a role in approximately 50% of ADRs[15].

As with drug efficacy, drug toxicity is also influenced by drug concentrations in the plasma and target location, making variation in ADME genes a contributor to many ADRs. One example is codeine-induced central nervous system (CNS) depression in patients with an ultrarapid metabolizer (UM) phenotype. Codeine relies on CYP2D6 for biotransformation to the active metabolite, morphine[16]. A functional duplication of the *CYP2D6* gene, which confers a UM phenotype, has been associated with increased formation of morphine following standard doses of codeine and risk of respiratory depression[17,18]. Associations between variants in additional drug metabolizing enzymes, such as thiopurine *S*-methyltransferases (TPMT) and glutathione *S*-transferases (GST), and drug-toxicity have

also been discovered, as variation in these genes can lead to the accumulation of toxic metabolites and contribute to concentration-dependent toxicities[19,20].

For some ADRs, associations with genes not implicated in drug ADME have been identified. Two examples are the association of human leukocyte antigen (HLA) *B*1502* with carbamazepine-induced Stevens-Johnson syndrome/toxic epidermal necrosis (SJS/TEN) and *HLA-B*5701* with abacavir-induced hypersensitivity[21,22]. Both ADRs are idiosyncratic and are hypothesized to arise from a disruption of the immune system. Therefore, variation in genes involved in the ADR mechanism can also be predictive of drug-induced toxicity.

1.1.3 Importance of pharmacogenomic studies in children

Historically, children have largely been excluded from clinical drug trials. Ethical reasons have prevented children from being used as test subjects in drug trials, resulting in approximately 75% of drugs never being tested in children. Instead, guidelines from adult patients have been extrapolated to account for smaller body mass, resulting in a lack of adequate guidelines on the safety and efficacy of pharmaceuticals in pediatric patients[23]. Optimization of pediatric dosing requires an understanding of age-dependent differences in several pharmacokinetic parameters, including bioavailability, volume of distribution, protein binding, hepatic metabolism, and renal elimination[24]. It is now known that developmental changes in drug transporters and metabolizing enzymes can have a significant impact on the required dosage and risk of toxicity in children. Several severe ADRs occur more frequently in children compared to adults, including valproic acid-induced hepatotoxicity, lamotrigine-induced cutaneous reactions, and Reye's syndrome associated

with aspirin[25-27]. While the reasons for this increased frequency are poorly understood, it has been speculated that developmental changes in drug biotransformation pathways (ex. CYP metabolism) in combination with genetic variation in detoxification pathways (ex. glucuronosyl transferases) may partially contribute to the greater frequency of idiosyncratic ADRs in children[28]. It has been shown that children require a dose that is at least 50% higher than adults for drugs that are primarily metabolized by CYP1A12, CYP2C9, and CYP3A4[24]. This difference in enzyme activity could have implications for both drug efficacy and toxicity, depending on whether the drug requires activation to produce a physiological effect, or detoxification to avoid concentration-dependent ADRs. Age-dependent differences in drug targets, such as ion channels, receptors and downstream signaling pathways, could also have significant effects on the pharmacodynamics of drug response. To date, very little research has been conducted on the ontogeny of drug targets that are relevant to pediatrics[28].

It is largely unknown whether pharmacogenetic biomarkers that have been discovered in adults possess the same predictive value in children, as most of these markers have never been studied in a pediatric population. It is possible that developmental changes in PK and PD pathways, as well as differences in diet, lifestyle, and concomitant medications, may impact the role of genetic variation on drug response in children. Pediatric-specific pharmacogenomic studies are warranted in order to determine the impact of age-dependent developmental changes and to improve therapeutic decision making in children.

1.2 Warfarin

Warfarin is an oral anticoagulant used for the prevention and treatment of thromboembolic events (TEs). It is the most commonly prescribed oral anticoagulant worldwide, with approximately 2 million people started on warfarin annually in the United States[29]. For more than fifty years warfarin has been the mainstay of anticoagulation therapy for patients with cardiac disease or those at risk of TEs. Specific indications for warfarin include atrial fibrillation, prosthetic heart valve, history of vascular thrombosis, or following orthopedic surgery[30]. Warfarin has shown to be effective at reducing the relative risk of stroke in patients with atrial fibrillation and preventing recurrent thrombosis in patients with a history of deep vein thrombosis (DVT)[31,32].

1.2.1 Warfarin mechanism of action

The warfarin target enzyme is vitamin K epoxide reductase complex, subunit 1 (VKORC1). VKORC1 is responsible for producing reduced vitamin via a two-step process, in which vitamin K 2,3-epoxide is converted to vitamin K1 (quinone) and then vitamin K hydroquinone (KH2) (**Figure 1.1**)[33]. The hydroquinone is an essential co-factor in the synthesis of vitamin-K dependent clotting factors II, VII, IX and X, as well as endogenous anticoagulant proteins C and S (**Figure 1.1**)[34]. These factors and proteins are biologically inactive without carboxylation of glutamic acid residues by the enzyme gamma-glutamyl carboxylase (GGCX), which is required for calcium-dependent activation of clotting factors at sites of injury[35]. Inhibition of VKORC1 prevents regeneration of vitamin K that serves as a co-factor for GGCX. Therefore, warfarin acts as an anticoagulant by blocking the production of active clotting factors and reducing blood coagulation. Therapeutic doses of

warfarin reduce the concentration of vitamin-K dependent clotting factors by approximately 30-50%[36].

1.2.2 Warfarin pharmacokinetics

Warfarin is administered as a racemic mixture of R- and S-enantiomers. S-warfarin is approximately 2-5 times more potent than R-warfarin and is almost exclusively metabolized by CYP2C9 in the liver to 7-hydroxywarfarin[37,38]. Conversely, R-warfarin is primarily metabolized by CYP1A2 to 6- hydroxywarfarin, as well as CYP2C19 to 8-hydroxywarfarin and CYP3A4 to 10-hydroxywarfarin (**Figure 1.2**)[39-41]. Hydroxywarfarin metabolites are further reduced to 9S,11S- and 9S,11R-warfarin alcohols, which possess some intrinsic anticoagulant activity[42]. Elimination of hydroxywarfarin metabolites occurs via hepatic metabolism, while warfarin alcohols are eliminated via renal excretion[43]. The rate of elimination for both isomers differs, with the half-life of S-warfarin being approximately 33 hours and R-warfarin being 45.4 hours[37].

The relationship between warfarin's efficacy and plasma drug concentration is indirect. Rather, the time course of warfarin is dependent on the clearance of vitamin-K dependent clotting factors and their associated half-lives. The earliest changes can be seen approximately 24-36 hours following initiation of therapy and are reflected by decreased levels of factor VII, which is the clotting factor with the shortest half-life (~6 hours). Factor IX has the next shortest half-life at ~24 hours, followed by factor X at ~39 hours and factor II at ~50-90 hours. Therefore, the antithrombotic effects of warfarin may not be apparent until approximately the fifth day of therapy when the concentrations of each clotting factor have

decreased to 20-30%[44]. After 10-14 days, the concentrations of all four factors reach equivalent values[45].

1.2.3 Clinical management of warfarin therapy

Clinical management and therapeutic monitoring of warfarin is achieved using the international normalized ratio (INR). INR is a laboratory test that measures the time required for plasma to clot following the addition of calcium and thromboplastin and reflects a decrease in the plasma levels of factors II, VI and X by warfarin[46]. Most patients have a target INR range of 2.0-3.0, but this can vary depending on the clinical indication for anticoagulation and patient-specific risk factors[47]. For example, patients with mechanical prosthetic heart valves typically have a target INR range of 2.5-3.5. Physicians use INRs to determine an appropriate warfarin dose, as INRs that are out of range can indicate that blood is over- or under-anticoagulated. The frequency of INR testing varies depending on numerous factors, including physician preferences, stability of INR results, and amount of time since initiation of therapy. Based on recommendations from the American Heart Association/American College of Cardiology Foundation, the INR should be tested daily until the therapeutic range has been achieved, and then reduced to 2 -3 times weekly for the first few weeks of therapy. Once a stable dose-response relationship is achieved, frequency of testing can be reduced to once every four weeks[46].

1.2.4 Clinical factors and variability in warfarin dose

Warfarin has an approximately 20 fold inter-patient variability in required dose. This high degree of variability can partially be explained by environmental factors, including age, body

surface area, gender, illness, concurrent medications, and dietary intake of vitamin K. For each decade of the life the required warfarin dose decreases by approximately 8%, most likely due to increased warfarin sensitivity in elderly patients or decreased drug clearance[48-50]. The required dose can also be influenced by body surface area, which is reflective of differences in liver size and hepatic clearance rates[51]. Numerous medications have been shown to alter the INR via pharmacokinetic or pharmacodynamic mechanisms. Pharmacokinetic drug interactions mainly involve induction or inhibition of warfarin metabolizing enzymes, including CYP2C9, CYP3A4 and CYP1A2. Amiodarone is an antiarrhythmic commonly prescribed to patients with heart disease and has been shown to potentiate the effect of warfarin via CYP2C9 inhibition, resulting in a lower dose requirement[52]. Drugs that inhibit the clearance of R-warfarin, such as omeprazole, have also been shown to influence the INR but to a lesser degree[53]. Pharmacodynamic drug interactions can alter the INR by inhibiting synthesis of clotting factors, interfering with hemostatic pathways, or altering the physiological control loop for vitamin K metabolism. For example, sulfonamides and broad-spectrum antibiotics may enhance the anticoagulation effect of warfarin by eradicating bacterial flora, contributing to a reduction in vitamin K levels[54]. The amount of dietary vitamin K consumed can also influence the required warfarin dose[55]. Overall, clinical factors account for approximately 12% of inter-patient dose variability[56].

1.2.5 Warfarin use in children

Increasing rates of TEs in children coupled with improvements in management of congenital heart defects has led to an increased use of warfarin in pediatrics for both primary and

secondary prevention of thrombotic episodes[57,58]. While the indications for warfarin therapy in children are similar to adults, the frequency of underlying pathological conditions requiring anticoagulation therapy differs. For example, myocardial infarctions and cerebrovascular accidents are among the most common indications for warfarin use in adults but are the least common indications in children[59]. Warfarin use in children is primarily prophylactic, with the most common indications being prosthetic heart valves and following Fontan surgery[60,61]. Additional indications for warfarin therapy in children include: venous thromboembolic disease, central venous lines (CVLs), congenital prothrombotic conditions, Kawasaki's Disease, and various other congenital heart conditions, or as a result of their surgical treatment (ex. Blalock-Taussig (BT) shunts).

Current recommendations for warfarin therapy in children suggest an initial dose of 0.2 mg/kg, which is considerably higher than the recommended dose of 0.04 to 0.08 mg/kg for adults[62,63]. Evidence suggests that there are limited age-dependent changes in CYP2C9 activity that could account for this difference in dose. Rather, in children >1 year old the functional expression of CYP2C9 per unit weight of liver tissue is comparable to that of adults[64,65]. A study by Takahashi et al. (2000) suggested that age-dependent variation in warfarin dose may be due to age-dependent differences in liver size compared to body weight[64]. In that study, liver weight-normalized unbound oral clearance of S-warfarin in pediatric patients was comparable to adults, suggesting that liver weight may be a better parameter for estimating warfarin dose in pediatric patients compared to body weight. It has also been shown that body weight develops at a slower rate compared to organs that are important for drug metabolism, such as the liver and kidney, further supporting the

hypothesis that weight-dependent dose differences may be attributed to liver size[66,67]. There is no evidence to suggest that VKORC1 activity significantly changes throughout childhood and could account for the high dose requirement in young children.

Compared to adults, children also have significant differences in their coagulation system, which continues to develop until late adolescence[68,69]. At birth, levels of vitamin-K dependent coagulation factors and inhibitors are at 50% of adults values and do not reach the adult range of normal until approximately 6 months of age[68,70]. However, average values of vitamin-K dependent proteins remain 20% lower than adult values until approximately late adolescence[69]. Andrew et al. reported that the mean values of several coagulants in children, including factors II, IV, VII, IX, X, XI and XII, were significantly lower than adult values, while levels of the thrombin inhibitor alpha-2-macroglobulin (α2M) were increased in children[69]. At equivalent INRs, children generate less thrombin than adults due to decreased levels of prothrombin fragment, as well as increased regulation by α2M[71]. Age-related differences in coagulation proteins may contribute to a lower incidence of thrombosis in children, as well as differences in warfarin dose and warfarin-related outcomes between adults and children.

A prospective study by Strief et al. (1999) investigating warfarin outcomes in children concluded that age is the most important variable influencing warfarin therapy during childhood[72]. The weight-adjusted dose to maintain a therapeutic INR was highest in children less than 1 year of age, and continued to decrease with increasing age. Children <1 year old also required a longer time to achieve a therapeutic INR, a longer period of overlap

with heparin therapy, and a higher mean number of dose changes per month when compared to older children. Furthermore, children less than 6 years old had significantly less INR measurements within the target range and significantly more INR measurements below the target range compared to children 6-18 years old. These findings highlight the impact of age on warfarin outcomes in children and the associated complexities of managing children on warfarin therapy.

1.3 Warfarin-induced adverse drug reactions

A large therapeutic dose window (20-30 fold) and narrow therapeutic index make safe and effective warfarin dosing difficult to achieve. As a result, warfarin therapy is associated with a high risk of adverse drug reactions. Under-anticoagulation increases the risk of blood clots (affecting 1-5% of patients), while over-anticoagulation puts patients at risk for bleeding complications (affecting 5-15% of patients)[73-75]. Consequently, warfarin is the second-most common drug implicated in emergency room visits and the most often cited cause of drug-related morality[74,76].

Bleeding is the most common ADR associated with warfarin therapy. However, the reported incidence of major and minor bleeding episodes varies between studies. This is mainly due to differences in the definition of bleeding, length of follow-up, and patient-specific factors, such as indication for warfarin and target INR[76]. The rate of major bleeding, defined as intracranial, retroperitoneal, leading directly to death, or resulting in hospitalization or transfusion, ranges from 10-16%, while the rate of fatal bleeding is reported as 0-2.9%[76-78]. The most common type of warfarin-induced bleeding resulting in emergency room visits is

gastrointestinal bleeding[76]. Furthermore, based on data from the Food and Drug Administration's (FDA) Adverse Event Reporting System (AERS), the most commonly reported bleeding events are gastrointestinal tract hemorrhage, hemorrhage not otherwise specified, hematuria, and epistaxis[76].

1.3.1 Clinical risk factors for warfarin-induced ADRs

Many of the clinical factors that contribute to variability in required warfarin dose, such as age, illness, diet, and concomitant medications, also contribute to risk of warfarin-induced ADRs. Specific risk factors for bleeding include: age, history of gastrointestinal (GI) tract bleeding, hypertension, cerebrovascular disease, serious heart disease, anemia, malignancy, trauma, and renal insufficiency[79-82]. Certain concomitant drugs, such as antiplatelet agents and non-steroidal anti-inflammatory agents (NSAIDs), can also increase the risk of bleeding by inhibiting platelet aggregation and prolonging bleeding time[83]. In an observational study by Knijff-Dutmer et al. (2003), the relative risk of bleeding with concomitant NSAID use was 5.8 compared to using warfarin alone[84]. Higher intensity of anticoagulation (INR >4) and longer duration of therapy further contribute to the risk of bleeding complications[76]. Several tools have been developed to more accurately predict patient-specific risk of bleeding based on clinical factors alone. One such model includes the Outpatient Bleeding Risk Index developed by Beyth et al. (1998), which provides a score using 4 criteria: (1) 65 years or older, (2) history of GI bleeding, (3) history of stroke, and (4) one or more comorbid conditions (recent myocardial infarction, anemia, renal impairment, or diabetes mellitus)[81]. Using this scoring system, it was possible to distinguish low-risk from moderate-risk patients

and performed better than physicians when determining patient-specific risk of major bleeding[81,82].

1.3.2 Warfarin-induced ADRs in children

As with adults, the most frequently occurring warfarin-induced ADR in children is bleeding. Significant bleeding has been reported at a rate of 0.5-1.7% per patient year[72,85,86]. The rate of bleeding is higher in children with prosthetic heart valves, estimated as <3.2% events per patient year[86]. Minor bleeding, which includes bruising, nosebleeds, heavy menses, and microscopic hematuria, occurs in approximately 20% of children receiving warfarin[72,87]. The reported incidence of thrombosis in children on warfarin therapy ranges from 0-1.3% per patient year, with all reported episodes occurring in children receiving warfarin for secondary prophylaxis[72,87,88].

Compared to adults, children require more frequent INR monitoring and more dose adjustments to remain stable[72,87]. Primary medical problems, concomitant medications, and fluctuations in diet are a few of the factors that are likely to influence warfarin response in children and contribute to warfarin-induced ADRs. Differences in vitamin K content between solid foods and alternative nutrition sources, such as breast milk, formula, and total parenteral nutrition (TPN), can also render some children more susceptible to warfarin resistance, while making others more sensitive to warfarin's anticoagulation effects[89,90]. The majority of children receiving warfarin therapy also receive concomitant medications to treat their underlying medical problems, which can influence dose requirements and risk of

bleeding[59]. Frequent illnesses and the medications used to treat them can further complicate warfarin therapy in children.

1.3.3 Treatment of warfarin-induced bleeding

The first treatment option in the presence of an elevated INR is temporary cessation of therapy, which will allow the INR to fall within the therapeutic range over several days. In cases of excessive anticoagulation (usually INR >8) without significant bleeding, vitamin K may be administered orally, subcutaneously, or intravenously to cause a rapid decline in the INR (within 24 hours). While vitamin K is an effective treatment option, it may lead to temporary warfarin resistance and result in unsafe subtherapeutic INRs. In the presence of significant bleeding, fresh frozen plasma (FFP) may be administered for immediate reversal of over-anticoagulation[46]. Other immediate treatment options include prothrombin complex concentrates or recombinant factor VIIa[91].

1.4 Pharmacogenomics of warfarin safety and effectiveness

In 1999, the first report was published describing the contribution of genetic variants to inter-patient warfarin dose variability and risk of bleeding complications. Specifically, the authors reported an association between functional variants in the warfarin metabolizing enzyme gene, *CYP2C9*, and low warfarin dose requirement[92]. Since this time, hundreds of papers have been published describing the relationship between genetic polymorphisms and warfarin-specific outcomes in adults.

1.4.1 CYP2C9

CYP2C9 is an isozyme of the cytochrome P450 superfamily of enzymes, which are responsible for the metabolism and elimination of many common prescription drugs. CYP2C9 is one of the most common CYP enzymes in the liver and metabolizes approximately 15% of clinical drugs, including non-steroidal anti-inflammatory drugs (NSAIDS), selective cyclooxygenase-2 (COX-2) inhibitors, diuretics, antiepileptics, angiotensin II receptor inhibitors, and anticoagulants, such as warfarin, phenprocoumon, and acenocumarol[93,94]. The CYP2C9 gene is located on chromosome 10 and is highly polymorphic, with at least 34 variants (**1B* to **35*) identified thus far (http://www.cypalleles.ki.se/cyp2c9.htm, accessed 03 Dec 2012). Approximately 5-30% of the population carry variants in the *CYP2C9* gene that result in an enzyme with reduced or zero activity, contributing to a decreased ability to catalyze the oxidation of S-warfarin and a decreased warfarin dose requirement[18]. In Europeans, the two most common functional *CYP2C9* SNPs that confer reduced enzyme activity are *CYP2C9*2* (rs1799853) and *CYP2C9*3* (rs1057910)[95]. *CYP2C9*2* is an arginine substituted for a cysteine at position 144, while *CYP2C9*3* is a leucine substituted for an isoleucine at residue 359[96]. In vitro, these variants are less than 12% *(*2/*2)* and 5% *(*3/*3)* as efficient as the wild-type enzyme[97,98]. Accordingly, carriers of **3/*3* require an approximately 78.1% lower dose compared to the normal activity allele (**1/*1*) (**Table 1.1**)[99]. Approximately 5-27% of the variability in required warfarin dose is explained by the **2* and **3* variants (**Table 1.2**)[100-102].

The amount of dose variability accounted for by the **2* and **3* variants is less in Africans and Asians compared to Caucasians, most likely due to a lower allele frequency in these

populations (**Table 1.3**). Additional *CYP2C9* gene variants, such as *CYP2C9*5* (rs28371686), **6 (*rs9332131), **8 (*rs7900194), and **11 (*rs28371685) also result in an enzyme with reduced or zero activity and are found almost exclusively in African Americans[103-105]. These variants have been shown to increase the amount of explained dose variability in African Americans when compared to using **2* and **3* alone, accounting for approximately 5-6% of dose variability[106-110].

1.4.2 VKORC1

Vitamin K epoxide reductase complex subunit 1 (VKORC1) is the catalytic subunit of VKOR responsible for the conversion of epoxidized vitamin K to reduced vitamin K for activation of clotting factors (**Figure 1.1**). The *VKORC1* gene is located on chromosome 16 and is approximately 5 KB in length, encoding 163 amino acids. Several polymorphisms in *VKORC1* have been identified that strongly influence warfarin dose requirements. The most commonly cited SNP is -1639G>A (rs992321), which occurs in the functional promoter region and results in decreased gene transcription[111]. Homozygous carriers of the A allele *(*2/*2)* require an approximately 50% lower dose compared to wild-type carriers *(*1/*1)*, with heterozygotes *(*1/*2)* requiring intermediate doses (**Table 1.4**)[112]. Similar effects have been ascribed to the *VKORC1* 1173C>T polymorphism (rs9934438), which is in tight linkage disequilibrium (LD) with the -1639G>A variant[111]. These SNPs can be used interchangeably to differentiate high, moderate, and low sensitivity patients and account for approximately 4-35% of the variability in warfarin dosing[113,114](**Table 1.2**).

Several other *VKORC1* SNPs that are in LD with *VKORC1*-1639 have also been associated with warfarin dose and can be used to further differentiate *VKORC1* sensitivity haplotypes

*(*1, *2, *3, *4)*[115]. However, these variants do not add any predictive power to a dose prediction model that already includes the -1639G>A variant. Alternatively, several rare point mutations in the *VKORC1* gene have been associated with warfarin resistance[116-119]. These variants occur in the coding region of the gene and lead to changes in the VKORC1 protein sequence, potentially altering the warfarin site of action[118]. However, the function of VKORC1 in extreme warfarin resistance is poorly understood.

The frequency of the -1639G>A variant also differs according to ethnicity. Asians possess the highest frequency (89%), which contributes to a lower average dose requirements in Asian patients compared to Europeans (**Table 1.3**)[108,111]. Low allele frequency in Africans also explains why *VKORC1* is less predictive of dose compared to Caucasians, accounting for only 4.2% of dose variation in Africans compared to approximately 22.5% in Caucasians[120]. Several other *VKORC1* SNPs have also been found to be predictive of warfarin dose in Africans independent of the -1639 variant (rs61162043, rs8050894, rs7199949, rs7294); however, these variants possess less predictive ability compared to *VKORC1*-1639[106,110].

1.4.3 Impact of CYP2C9 and VKORC1 variants on warfarin-related outcomes in adults

In addition to being associated with dose, genetic variants in *CYP2C9* and *VKORC1* have been associated with several other warfarin-related clinical outcomes. For example, the time required to reach an INR in or above the therapeutic range is significantly decreased for carriers of the *CYP2C9*2*, **3*, or *VKORC1*-1639 variants[108,121,122]. These variants have also

been associated with a shorter time to first INR greater than 4, a higher incidence of INR greater than 4, and an increased amount of time spent above the therapeutic range[123-125]. These findings can be explained by the increased sensitivity to warfarin that is conferred by these variants, resulting in a higher rate of INR increase during initiation of warfarin therapy and increased risk of over-anticoagulation (INR $\geq$4)[108]. Accordingly, *CYP2C9*2* and **3* have also been associated with an increased time to reach a stable INR, defined as consecutive INRs within the therapeutic range, as carriers of these variants become over-anticoagulated faster and require additional dose adjustments before a stable INR is achieved[124,125].

1.4.4 Genetics and warfarin-induced ADRs

As described previously, the most common ADR attributed to warfarin therapy is bleeding. Few studies have reported a significant association between *CYP2C9* and/or *VKORC1* variants and incidence of bleeding events[124-127]. Lima et al. (2008) reported that the incidence of bleeding events in the first month of therapy was 4.9 times higher for patients possessing a *CYP2C9* variant allele compared to wildtype patients[126]. Similarly, Higashi et al. (2002) found that carriers of the **2* or **3* variant were more likely to experience a serious or life-threatening bleeding event sooner than wild-type carriers during both the initiation phase (HR, 3.94; 95% CI, 1.29-12.06) and entire follow-up period (HR, 2.39; 95% CI, 1.18-4.77)[125]. The risk of hemorrhagic complications has also been found to be higher in carriers of *VKORC1* variants[128]. Thus far, a significant association between genetic variants and thromboembolic episodes has not been identified, most likely due to a low incidence of thrombosis during warfarin therapy.

1.4.5 Pharmacogenetic dosing algorithms

By retrospectively combining clinical and genetic data from patients with known therapeutic doses, researchers have derived algorithms to predict the therapeutic dose for a warfarin-naïve patient. All published algorithms include *VKORC1*, *CYP2C9*2* and **3*, age and body size, while other variables accounted for include: amiodarone use, smoking, indication for anticoagulation, INR target, statin therapy, gender, race, and enzyme-inducing drugs[129-135]. It has consistently been shown that genotype-based dosing algorithms more accurately predict the stable warfarin dose when compared to clinical dosing algorithms, which account for clinical and demographic factors only, or empirical dosing, which requires administering a standard dose to all patients[136-140]. Overall, pharmacogenetics-based dosing models account for approximately 50-60% of dose variation[141]. When various dosing algorithms were compared, it was found that algorithms developed by the International Warfarin Pharmacogenetics Consortium (IWPC) and Gage et al. (2008) possessed the greatest predictive ability and performed very similarly (IWPC: $R^2 = 0.50$, Gage et al.: $R^2 = 0.49$)[129,132,137]. Furthermore, all pharmacogenetic models outperformed a clinical dosing model in terms of R^2 and mean absolute error (MAE) values when predicting the required dose[137]. The FDA has also developed a genetics-based dosing table that is included on the warfarin label (Coumadin, Bristol-Myers Squibb, Princeton, New Jersey) (**Table 1.5**)[142]. However, this table-based dosing approach does not incorporate relevant clinical information and has been shown to be less accurate when predicting a stable dose compared to genetics-based algorithms[139].

As most dosing algorithms have been derived from predominantly Caucasian populations they are generally less predictive of dose in other ethnic populations. One study that compared the performance of five dosing algorithms in Caucasians and African Americans found that three of the algorithms accounted for a greater amount of dose variability in Caucasians compared to African Americans, and that some pharmacogenetic dosing algorithms only performed marginally better than a standard 5 mg dosing nomogram in African patients[138]. However, none of the algorithms tested accounted for African-specific *CYP2C9* variants. One study that investigated genetics-based dosing in Japanese patients found that while the IWPC model was most useful for predicting dose in patients that required low or high doses, the algorithm accounted for only 28% of dose variability[136]. Discovery of genetic variants or clinical factors that are significant for predicting dose in specific ethnic populations would help improve the accuracy of genetics-based dosing models.

Dosing algorithms that incorporate INRs from the first few days of therapy have also been developed to facilitate more accurate dose prediction and dose adjustments. In a prospective study, it was found that a pharmacogenetic dose-refinement algorithm that incorporated INRs from day 4 or 5 was able to account for a larger percentage of dose variability and had a smaller median absolute dosing error when compared to a clinical dose refinement algorithm that did not include genetics[143]. Algorithms that calculate genetics-based loading/initiation doses for the first few days of therapy have also been published[144,145]. One of these algorithms was developed using the IWPC maintenance dose algorithm and *CYP2C9* genotype-based variance in warfarin half-life. When retrospectively evaluating this model it

was found that in patients with mean $INR_{days\ 4–7}$ >4.0 after warfarin initiation, the pharmacogenetics-based initiation dose algorithm predicted a markedly lower dose requirement than the standard regimen, whereas in those with mean $INR_{days\ 4–7}$ <2.0, the predicted dose requirement was very similar to that in the standard regimen[145]. Therefore, a loading-dose algorithm may be beneficial for identifying patients who are particularly sensitive to warfarin and are at risk of over-anticoagulation if administered a standard loading dose.

1.5 Additional genetic variants associated with warfarin therapy in adults

Approximately 40-50% of the variation in warfarin dose still remains unexplained when accounting for *CYP2C9*/*VKORC1* genotypes and known clinical factors. Thus, there is a continuous search to identify additional genetic variants that may be implicated in warfarin dosing and can account for a portion of this unexplained variation. Examples of genetic variants that have been significantly associated with warfarin dose are described below.

1.5.1 CYP4F2

In addition to *CYP2C9* and *VKORC1*, the third gene most commonly associated with warfarin dose variation is *CYP4F2*. CYP4F2 is a vitamin K oxidase that catalyzes the metabolism of vitamin K to hydroxyvitamin K1 and functions as a counterpart to VKOR to prevent accumulation of vitamin K (**Figure 1.1**). A functional polymorphism in the *CYP4F2* gene (rs2108622, C>T) encodes a protein with decreased activity, resulting in increased levels of vitamin K and an increased dose requirement in patients carrying this variant[146]. Homozygous carriers of the variant allele (TT genotype) have an approximately 1 mg/day

increased dose requirement compared to carriers of the CC genotype[146]. When *CYP4F2* was included in models that also accounted for *CYP2C9*, *VKORC1*, and clinical variables, the amount of dose variation explained by *CYP4F2* ranged from approximately 1-11%[122,137,146-157]. Most studies that reported a significant association were conducted in Caucasian patients. This variant is now included in the online dosing algorithm available at www.warfarindosing.org.

1.5.2 APOE

Apolipoprotein E (ApoE) is a class of apolipoprotein that is responsible for transporting vitamin K to the liver. The ApoE gene is polymorphic and has three major isoforms, ApoE-ε2 (dysfunctional), ε3 (normal) and ε4 (dysfunctional), which are discriminated by two SNPs (rs7412 and rs429358). One study found that carriers of the ε4 allele, which causes increased uptake of vitamin K into the liver, required a decreased dose compared to homozygous ε3 carriers[158]. Kimmel et al. (2008) also reported a significant association between ε4 and warfarin dose but the association was in the opposite direction, with ε4 carriers requiring a higher warfarin dose. Furthermore, this association was only evident in African American patients, as Caucasian patients with the ε4 allele did not require a significantly different dose[159]. In contrast, Wadelius et al. (2007) reported a significant association between ε2+ε4 and increased dose in Caucasians after correction for multiple testing[160]. Multiple studies that have examined the role of *APOE* variants in warfarin dosing have not found a significant association, thus further complicating our understanding of the importance of this gene for warfarin dose prediction[148,161,162].

1.5.3 PROC

Protein C is a vitamin-K dependent serine protease that when activated plays a primary role in the regulation of blood clotting via the inactivation of clotting factors Va and VIIIa (**Figure 1.1**)[163,164]. One study reported a significant association between dose and three *PROC* polymorphisms (rs2069919, rs1799809, rs2069901), accounting for 7-9% of the variation in warfarin dose[160]. However, in a dose prediction model that included additional SNPs and clinical factors, only rs2069919 remained significant. When these SNPs were evaluated in additional patient cohorts a significant association was not found[148,162,165,166]. Another SNP in *PROC* (rs5936) was significantly associated with dose in a Han-Chinese population in both univariate and multivariate analyses[162]. This SNP results in a synonymous amino acid change and is hypothesized to be in linkage with a functional SNP that has not yet been identified. The rs1799809 variant has also been associated with protein C concentration and activity in functional studies[160,167,168]. It is hypothesized that variation in the *PROC* gene could affect warfarin dose requirements via changes in protein C expression or activity, thus altering the regulation of clotting factors.

1.5.4 GGCX

Gamma-glutamyl carboxylase (*GGCX*) encodes an enzyme that catalyzes the post-translational modification of vitamin-K dependent clotting factors (**Figure 1.1**). Three variants in *GGCX* (rs11676382, rs10654848, rs12714145) have been significantly associated with warfarin dose. The SNP that has shown the strongest association is rs11676382, which explained 2% of the total dose variation in a multivariate model and was associated with a 6.1% reduction in dose per variant allele[169,170]. This SNP has since been included in the

online dosing algorithm available at www.warfarindosing.org. The rs10654848 variant is a CAA repeat in intron 6 that is a predictor of high dose requirements in multiple ethnic populations. Specifically, the (CAA) 16 or 17 repeat explained 2% of dose variability in African American patients, while the (CAA) 11 or 13 repeat was associated with increased dose in Japanese patients[171,172]. The frequency of this repeat is less common in Caucasians and was only significantly associated with dose in patients who also carried a *CYP2C9*1/*1* genotype[173]. The third *GGCX* SNP, rs12714145, was significantly associated with increased dose in a Caucasian population[174].

1.5.5 POR

Cytochrome P540 oxidoreductase (POR) is as an electron donor for all CYP enzymes, including those that are essential for warfarin metabolism[175]. Polymorphisms that alter POR activity or expression may contribute to individual warfarin dose requirements by affecting warfarin metabolism via CYP pathways. In one study with a predominantly Caucasian population, two SNPs in the proximal promoter region were associated with a lower warfarin dose (rs72553971, rs12537282), while a non-coding tag SNP (rs2868177) was significantly associated with a higher warfarin dose[176]. These SNPs remained significant in a multiple regression analysis, along with SNPs in *CYP2C9*, *VKORC1*, and *CYP4F2*. When combined with the functional *CYP4F2* variant (rs2108622), these *GGCX* SNPs accounted for 6.2% of dose variation. In a study by Tee et al. (2011), neither of the promoter SNPs were associated with transcription of the POR basal promoter, suggesting that these SNPs may be in LD with other unknown variants that can alter POR transcription and expression[177]. Replication of these findings in a separate patient cohort is still required.

1.5.6 CALU

Calumenin is an endoplasmic reticulum chaperone protein that can bind to both GGCX and VKOR to regulate γ-carboxylation and activation of vitamin K-dependent clotting factors (**Figure 1.1**)[178]. Previous studies in rats have identified an association between *CALU* overexpression and warfarin-resistance, as binding of calumenin prevents warfarin inhibition of VKOR activity[179]. A *CALU* intronic SNP, rs339097, was significantly associated with a higher dose requirement in African Americans and conferred an 11-14.5% higher dose requirement than the one predicted using *VKORC1*/*CYP2C9* genotypes and clinical factors[180]. Furthermore, this variant accounted for 5.7% of the dose variation in African Americans and was replicated as a predictor of high dose requirement in two cohorts, including a mixed ethnicity cohort and a second African American cohort. Due to low allele frequency, the influence of this variant on dose requirements in Caucasians could not be determined. This SNP was further replicated in two more studies where it was shown to be significantly associated with increased dose in an African American cohort and an Egyptian cohort, although it did not achieve significance in a multiple regression model in the Egyptian population[181,182]. Interestingly, a review of gene expression data from an immortalized lymphoblastoid cell line revealed that the variant allele is associated with higher *CALU* gene expression, which in turn could explain higher dose requirements in variant allele carriers[180]. As rs339097 is an intronic SNP, the functional significance has yet to be determined. A study by Wadelius et al. (2007) identified additional *CALU* SNPs that were nominally associated with dose but these associations have not yet been replicated[160].

1.5.7 EPHX1

The microsomal epoxide hydrolase 1 (mEH) enzyme is encoded by the epoxide hydrolase 1 gene (*EPHX1*). This enzyme possesses a vitamin K 2,3-epoxide binding site and is believed to influence warfarin dose requirements by binding to VKOR and causing changes in the vitamin K cycle, potentially resulting in altered warfarin pharmacodynamics (**Figure 1.1**)[183,184]. Multiple studies have reported a significant association between variants in *EPHX1* and dose, including rs4653436 (*EPHX1*691A>G), which results in a histamine to arginine transition (His139Arg). Two studies in Han Chinese patients found that carriers of the GG or AG genotype required significantly lower doses compared to AA carriers[165,185]. This SNP remained significant in models that also included *CYP2C9*, *VKORC1*, age, and body weight. Interestingly, one study found that *EPHX1* accounted for a greater amount of dose variability than *CYP2C9*, explaining approximately 35% of the dose variation when included in a model with age and body weight, compared to approximately 13% for *CYP2C9*[185]. This SNP was also nominally associated with dose in a Caucasian population but did not reach significance after correction for multiple testing[160]. Two additional studies in Asian cohorts, including an *EPHX1* exon sequencing study, identified intronic SNP rs1877724 as a significant predictor of warfarin dose, accounting for approximately 0.8-<3% of dose variation[162,186]. This SNP resides in the same haplotype block as rs4653436 and is also linked to polymorphisms in the promoter region that are known to alter *EPHX1* transcription rates[187]. Thus, it is hypothesized that rs1877724 and rs4653436 may tag variants that alter protein expression[186]. A coding SNP in *EPHX1* (rs1051740) has also been associated with increased dose requirements but the association was not significant in a multiple regression model that also included *CYP2C9*[184]. Additional replication of *EPHX1*

SNPs that have been shown to alter warfarin dose requirements would help to further elucidate the influence of *EPHX1* on dose variation.

1.5.8 CYP2C18

CYP2C18 belongs to the cytochrome P450 superfamily of enzymes and is thought to play a minor role in the metabolism of warfarin (**Figure 1.2**)[188]. An intronic SNP, rs7896133, was previously associated with therapeutic warfarin dose in a population of Caucasian patients after correcting for multiple testing[160]. This SNP was also significant in a Han-Chinese population, where homozygous carriers required a significantly higher weekly warfarin dose compared to wildtype carriers[162]. However, this SNP did not remain significant in a multiple regression model. It is hypothesized that this SNP is in linkage disequilibrium with *CYP2C9*3* (rs1057910), which is known to be a significant predictor of required warfarin dose[160]. A genome-wide association study in 546 African American patients found that a SNP upstream of *CYP2C18* (rs12777823) was associated with stable dose when conditioned on *VKORC1*-1639, *CYP2C9*2*, and **3*[189]. This SNP was replicated in a separate African American cohort and explained 5% of dose variability[189].

1.6 Pharmacogenetic studies in children on warfarin therapy

As previously described, significant differences in warfarin-related outcomes, such as required dose (mg/kg) and time to achieve a therapeutic INR, exist between pediatric patients of varying ages[72]. Based on a prospective analysis of 319 pediatric patients, Streif et al. (1999) concluded that age was the most important factor influencing warfarin outcomes in children[72]. However, this conclusion was based on a comparison of clinical factors alone. It

is known that children respond differently to warfarin compared to adults and that multiple patient-specific factors, such as age-related dose-response, diet, illness, and underlying health problems can impact warfarin response in children. As such, the question arises of whether genetic variants also play a significant role in warfarin dosing and clinical outcomes in pediatric patients.

To date, six pharmacogenetic studies on warfarin therapy in children have been published. A study with the largest pediatric population (n=120) found that *VKORC1* and *CYP2C9* explained 26.6% and 12.8% of dose variation, respectively, which is comparable to values reported in adult populations[190]. Furthermore, when height and indication for warfarin use were added to a predictive dosing model, 72.4% of the variation in warfarin dose was explained by clinical and genetic factors. Four additional studies in children reported a significant difference in warfarin dose requirement between carriers and non-carriers of the *VKORC1*-1639 variant. However, three of these studies did not find a significant difference in dose across *CYP2C9* genotypes, while the fourth study excluded *CYP2C9* from all analyses due to low allele frequency[88,100,191,192]. Finally, a study by Ruud et al. (2008) in 29 patients did not find a significant difference in dose requirement between carriers of *CYP2C9* low activity variants and the **1/*1* normal activity genotype. However, this finding may have been due to small sample size and concomitant high-dose steroid use in the majority of patients[193]. Overall, the amount of warfarin dose variability that is explained by genetics in children ranges from 0.4-12.8% and 3.7-26.6% for *CYP2C9* and *VKORC1*, respectively.

Two of the aforementioned studies also investigated the association between dose and *CYP4F2* (rs2108622), with both studies reporting a non-significant association[100,190]. Biss et al. (2011) found that carriers of the *CYP4F2* V433M variant required a higher mean daily warfarin dose compared to wildtype carriers but this difference was not statistically significant [190]. *CYP4F2* genotype was also not significant in a dosing model that already included *CYP2C9* and *VKORC1* genotypes.

Aside from dose variability and prediction models, there has been limited investigation of additional warfarin-related outcomes in children. One study reported a significant difference in the number of days needed to achieve the target INR and the number of INR measurements above the therapeutic range when comparing *CYP2C9* low activity variants to normal activity variants, suggesting that *CYP2C9* variants represent a risk factor for over-anticoagulation and potential bleeding complications in children[193]. However, a separate study reported that there was no association between *CYP2C9* and *VKORC1* genotype and time to therapeutic INR[88]. Furthermore, a study by Moreau et al. (2012) did not find an association between genetic factors and time spent within, above, or below the therapeutic range[100].

Based on the described findings, the relevance of genetics in warfarin dosing and clinical outcomes in children remains poorly understood. While *VKORC1* (rs9923231) appears to influence dose requirements in children, the amount of dose variability explained by this variant, as well as the impact of *CYP2C9* variants on dose requirements in children, remains conflicting. The influence of pharmacogenetic markers on clinically relevant warfarin-

related outcomes, such as risk of over-anticoagulation and warfarin-induced bleeding, has also not been studied in children. Accordingly, it is unknown whether variation in key genes involved in warfarin biotransformation and vitamin K pathways contribute to dose variability in children independent of previously identified associations. Further examination of the influence of genetic variants on warfarin dosing and severe outcomes in children is warranted.

1.7 Hypothesis and thesis objectives

This study hypothesizes that variants in genes involved in the metabolism and mechanism of warfarin, as well as vitamin K and coagulation pathways, impact warfarin dosing and are predictive of warfarin-related outcomes in children. The specific objectives of the study were: (1) to assess the impact of *CYP2C9*, *VKORC1* and *CYP4F2* variants on warfarin outcomes in children, including: (a) therapeutic warfarin dose, (b) time to INR events, and (c) warfarin-induced ADRs; (2) to validate a previously published pediatric-specific pharmacogenetic predictive dosing model in an independent cohort of children and compare its performance to an adult-derived dosing model when predicting therapeutic dose; and (3) to investigate the association of additional polymorphisms in genes involved in warfarin biotransformation and coagulation pathways to assess their impact on dose variation in children.

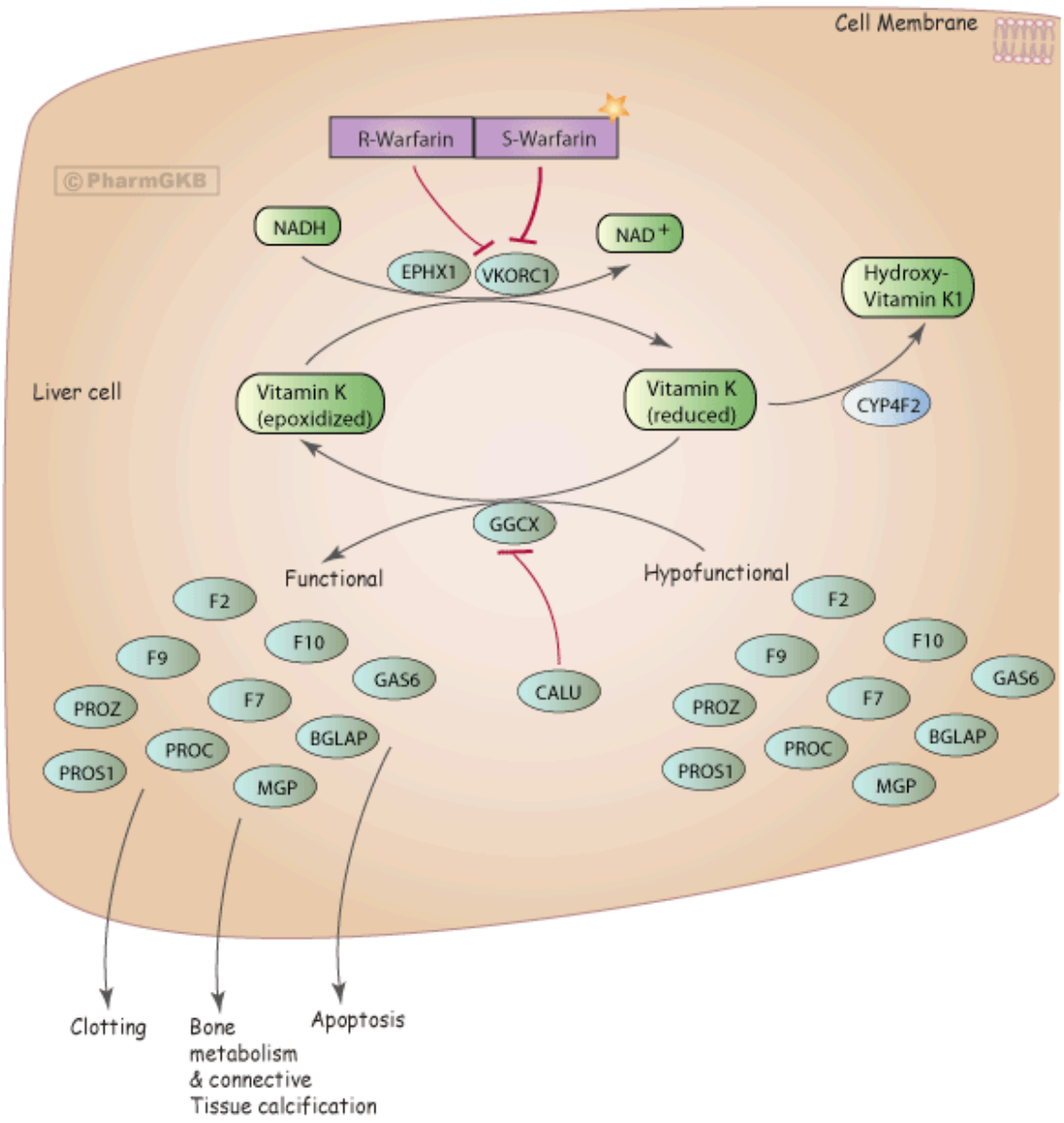

Figure 1.1. Interaction of warfarin with its target enzyme and downstream pathways
This diagram illustrates the interaction between warfarin and its target VKORC1. Inhibition of VKORC1 prevents the regeneration of reduced vitamin K and leads to the accumulation of hypofunctional clotting factors and proteins. Polymorphisms in several enzymes, including GGCX, CALU, EPHX1, and CYP4F2, may disrupt the warfarin pharmacodynamic pathway by interfering with the vitamin K cycle or the activation of clotting factors. This figure is reprinted with permission from PharmGKB and Stanford University[194]. Copyright PharmGKB.

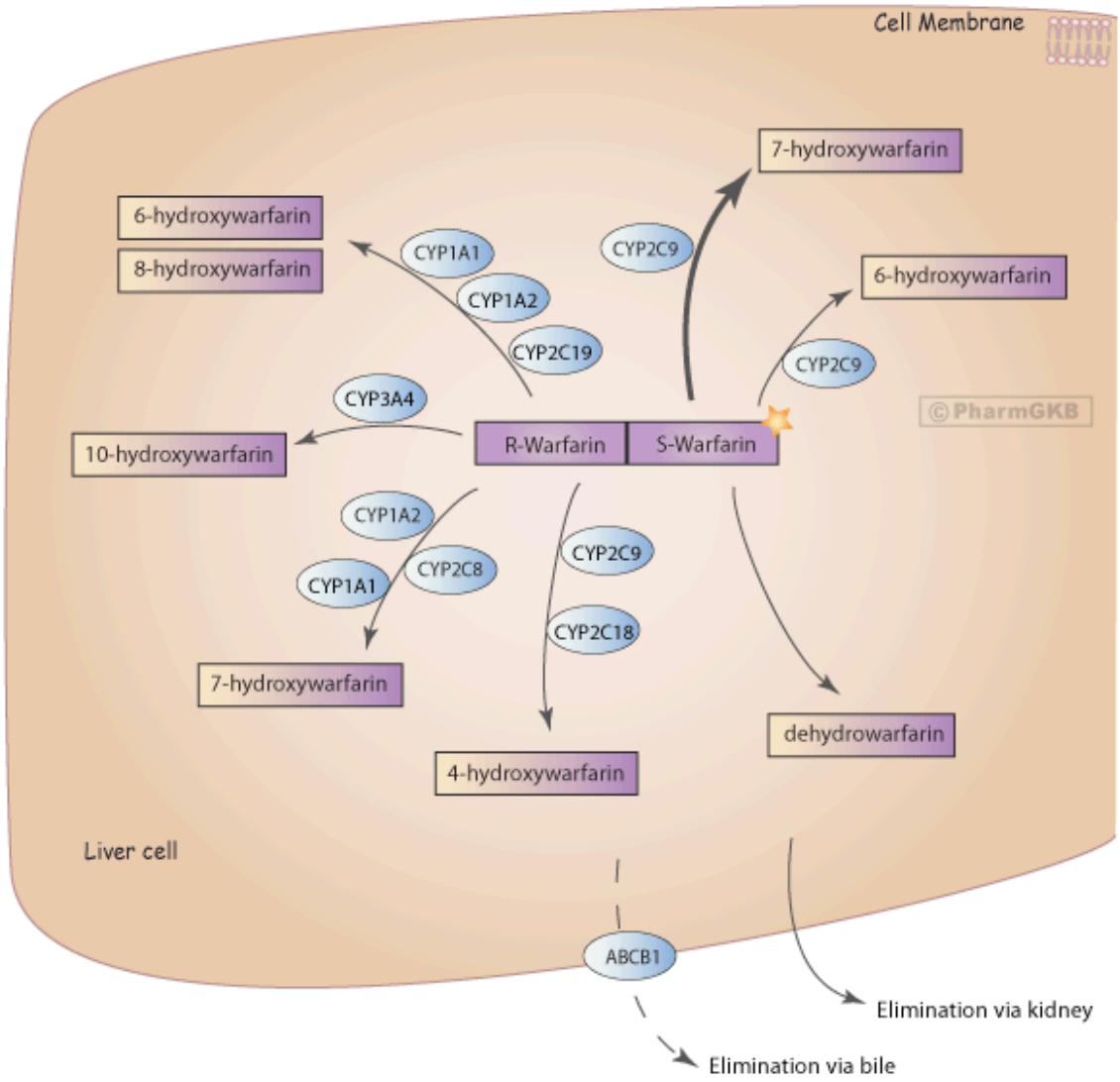

Figure 1.2. Candidate genes involved in the metabolism of warfarin
Several cytochrome P450 genes are involved in the phase 1 metabolism of R-warfarin and S-warfarin isomers to their inactive metabolites. CYP2C9 is the main metabolizer of S-warfarin while R-warfarin is predominantly metabolized via CYP3A4. Warfarin is primarily eliminated via the kidney but has also been shown to undergo hepatic elimination via interaction with the efflux transporter ABCB1 in the liver. This figure is reprinted with permission from PharmGKB and Stanford University[194]. Copyright PharmGKB.

Table 1.1. Effect of variant *CYP2C9* genotypes on warfarin dose requirements[a]

CYP2C9 Genotype	Reduction in warfarin dose requirement
1/*1	Reference
1/*2	19.6%
1/*3	33.7%
2/*2	36.0%
2/*3	56.7%
3/*3	78.1%

[a]Values taken from Lindh et al. (2009)[99]

Table 1.2. Variability in warfarin dose explained by *VKORC1* and *CYP2C9* genotypes in different ethnic populations

Allele	Ethnic Group		
	Caucasian	**Asian**	**African**
CYP2C9*2* or **3	5 - 27% [101,102]	<4.8 - 17.3% [165,195]	<5.6% [106,107]
CYP2C9*5, *6, *8, *11	N/A	N/A	<5 - 6% [107,110]
***VKORC1* (-1639G>A)**	13.8 - 25% [129,196,197]	19.8 - 35.5% [114,198]	4 - 9% [106,113]

Table 1.3. *CYP2C9* and *VKORC1* variant allele frequencies according to ethnicity

Allele	Ethnic Group		
	Caucasian	**Asian**	**African**
CYP2C9*2[a]	0.9 – 20%	0%	0.8 – 7%
CYP2C9*3[a]	0 – 14.5%	0 – 8.2%	0.4 – 3%
***VKORC1* (-1639G>A)**[b]	37%	89%	14%

[a]Allele frequencies for *CYP2C9* taken from Lee et al. (2002)[95]
[b]Allele frequencies for *VKORC1* taken from Rieder et al. (2005)[111]

Table 1.4. Effect of *VKORC1* variant genotypes on warfarin dose requirements[a]

	Increase in Dose Requirement		
***VKORC1* variant**	**High sensitivity**	**Intermediate sensitivity**	**Low sensitivity**
-1639 G>A	Reference	52%	102%
+1173 T>C	Reference	44%	97%

[a]Values taken from Yang et al. (2010)[112]

Table 1.5. Range of expected therapeutic warfarin doses based on *CYP2C9* and *VKORC1* genotypes as listed on the warfarin label insert[a]

VKORC1	***CYP2C9***					
0	****1/*1***	****1/*2***	****1/*3***	****2/*2***	****2/*3***	****3/*3***
GG	5-7 mg	5-7 mg	3-4 mg	3-4 mg	3-4 mg	0.5-2 mg
GA	5-7 mg	3-4 mg	3-4 mg	3-4 mg	0.5-2 mg	0.5-2 mg
AA	3-4 mg	3-4 mg	0.5-2 mg	0.5-2 mg	0.5-2 mg	0.5-2 mg

[a]Adapted from U.S. Food and Drug Administration label for Coumadin® tablets (warfarin sodium tablets, USP) crystalline; Coumadin® for injection (warfarin sodium for injection, USP). http://www.accessdata.fda.gov/drugsatfda_docs/label/2010/009218s108lbl.pdf. Accessed 09/08/2012.

Chapter 2: Methods

2.1 Patient recruitment and characterization

Patients were recruited through the Canadian Pharmacogenomics Network for Drug Safety (CPNDS), an active ADR surveillance network with multiple pediatric and adult surveillance sites at academic health centres across Canada[199]. Inclusion criteria for the study were patients who were under the age of 19 and had received warfarin. Eighty-six study participants were prospectively recruited from B.C. Children's Hospital (Vancouver, Canada) between March 2011 and August 2012. Seven children were recruited at three CPNDS sites (Hospital for Sick Children (Toronto), Winnipeg Health Services Centre (Winnipeg), and B.C. Children's Hospital (Vancouver)). Informed consent was obtained from participants and/or their parents or legal guardians. This study was approved by the ethics committees of all participating institutions.

Relevant clinical and demographic information was obtained from patient health records, including age, gender, height, weight, concomitant medications, indication for warfarin, target INR range(s), bleeding/thrombotic events, and detailed records of warfarin doses and INR measurements. For all analyses, indication for warfarin was grouped into: (1) Fontan surgical procedure, (2) mechanical heart valve, (3) deep vein thrombosis/pulmonary embolism (DVT/PE), and (4) 'other' for all additional warfarin prescribing indications. Target INR varied according to indication and was grouped into <2.5, 2-3, and >2.5. Self-reported ancestry of maternal and paternal grandparents was obtained from patients or their parents/guardians. Ancestry was classified as: (1) European, (2) Asian, (3) mixed (patients

with at least one grandparent of European or Asian descent), and (4) other. High congruency between self-reported geographic ancestry and genetic structure has previously been shown using principal component analysis[200]. All available health records from time of warfarin treatment were reviewed.

2.2 Definition of outcomes

Three main outcomes were investigated using our cohort of children: (1) therapeutic warfarin dose, (2) time to INR events, and (3) warfarin-induced ADRs. Therapeutic warfarin dose was defined as no change in warfarin dose for at least three previous consecutive INR measurements over a minimum period of four weeks, at least two weeks after the initiation of therapy. This definition was modified from criterion used in a previous prospective, pediatric study, where it was required that children be anticoagulated for a minimum of three months prior to study inclusion[201]. Mean daily warfarin dose was calculated for patients who received alternating daily doses. Time to therapeutic INR was recorded as the time in days from first warfarin administration to first INR measurement in or above the therapeutic range. Time to INR >4 during warfarin initiation was recorded as the number of days from warfarin initiation to the first INR >4, within the first 60 days of therapy. Both major and minor bleeding events were recorded throughout the entire treatment period, for which follow-up information was available. Major bleeding events were defined according to the criteria of the International Society of Thrombosis and Haemostasis (ISTH)[202]. Criteria for major bleeding included: (1) Fatal bleeding, and/or; (2) Symptomatic bleeding in a critical area or organ, such as intracranial, instraspinal, intraocular, retroperitoneal, intraarticular, or pericardial, or intramuscular with compartment syndrome, and/or; (3) Bleeding causing a fall

in hemoglobin level of 20g L-1 (1.24 mmol L-1) or more, or leading to a transfusion of two or more units of whole blood or red cells. Based on consultation with a hematologist, it was required that hemoglobin drops occurred within one week of signs of bleeding in order for the event to be considered warfarin-related. Minor bleeding included all signs of bleeding, including epistaxis, hematoma, and heavy menses. Incidence of over-anticoagulation (INR >4) during the first 60 days of therapy was analyzed as a surrogate outcome for risk of warfarin-induced bleeding. Any evidence of a thrombotic episode while receiving warfarin was also recorded.

2.3 Designing a custom genotyping panel

A custom-made VeraCode GoldenGate Genotyping Assay (Illumina, San Diego, USA) was designed to capture genetic variation in genes that are known to influence warfarin outcomes in adults, as well as genes involved in the coagulation pathway. Based on a literature search twelve genes were chosen for investigation: *VKORC1*, *CYP2C9*, *CYP4F2*, *CYP2C18*, *EPHX1*, *CALU*, *GGCX*, *PROC*, *APOE*, *POR*, *F2*, and *A2M*. Variation in the first ten genes has previously been shown to influence warfarin dose in adults and thus were chosen for investigation in children. The latter two genes, *F2* and *A2M,* play a role in the coagulation cascade and may affect pediatric-specific outcomes, as levels of proteins encoded by these genes are significantly different between children adults[69]. All variants included on the panel are listed in **Table 2.1**.

Seven polymorphisms in *VKORC1*, *CYP2C9*, and *CYP4F2* that have repeatedly been associated with warfarin dose variability in adults were included on the panel, as it was

hypothesized that genetic variants known to influence warfarin outcomes in adults are also associated with warfarin outcomes in children. In addition, three *VKORC1* variants associated with warfarin resistance, three *CYP2C9* variants associated with dose requirements in African Americans, and one *CYP4F2* variant associated with time to therapeutic INR were included on the panel (**Table 2.1**). Since variation in these key genes has been thoroughly investigated in adults receiving warfarin therapy, no additional tagging or functional SNPs were included for investigation. A single SNP located upstream of *CYP2C18* that has been shown to influence warfarin dose independent of previously identified *CYP2C9* coding SNPs was also included for analysis; however, due to clustering of cytochrome P450 genes and a high potential for linkage disequilibrium between *CYP2C9* and *CYP2C18* variants, no additional *CYP2C18* variants were included on the custom panel.

For the remaining eight genes, SNPs that have previously been shown to influence warfarin outcomes in adults (15 SNPs), as well as coding SNPs (9 SNPs) and haplotype tagging SNPs (54 SNPs) were chosen for investigation (**Table 2.1**). A set of known coding SNPs were chosen due to their increased potential to influence biological pathways and warfarin-related phenotypes. Five coding SNPs with a minor allele frequency (MAF) greater than 1% in the Utah residents with ancestry from northern and western Europe population (CEU) and four coding SNPs with an MAF greater than 1% in the Yoruba in Ibadan, Nigeria population (YRI) were selected (http://hapmap.ncbi.nlm.nih.gov/). A tagging SNP design was also employed to capture sufficient genetic variation while also limiting the number of SNPs tested. Selection of tagging SNPs was performed using data from the International HapMap Project (http://hapmap.ncbi.nlm.nih.gov/) and the Haploview software[203]. Selection criteria

were an MAF greater than 1% in CEU and Toscani in Itali (TSI) populations and an r^2 threshold of 0.8. All SNPs were located within +/- 5kb of the gene of interest. In total, ninety-three SNPs were included for analysis on the custom genotyping panel.

2.4 Genotyping and quality control

Blood or saliva samples were collected from patients (Oragene, DNA Genotek, Ottawa, Canada) and labeled with a unique identifier. Patient DNA was extracted using the QiaSymphony purification system (Qiagen Inc., Toronto, Canada) according to the manufacturer's protocol. DNA concentration was determined using PicoGreen fluorometric assay (Quant-iT PicoGreen dsDNA reagent) and samples with a concentration less than 20 ng/µl were whole genome amplified (WGA) with multiple displacement amplification (Qiagen Inc., Toronto, Canada). DNA was normalized to a concentration of 20-50 ng/µl, while WGA was normalized to a maximum concentration of 100 ng/µl. Samples were genotyped using a custom Illumina GoldenGate Genotyping Assay for VeraCode and plates were scanned using an Illumina BeadXpress Reader (Illumina, San Diego, USA). All plates included a negative control (1x Tris-EDTA buffer) and a positive control with known genotype.

SNP genotype data were clustered manually using GenomeStudio software (Illumina, San Diego, USA). All samples achieved a genotyping call rate >95%. Six SNPs with a call rate <90% and six non-polymorphic SNPs were removed from the analysis. Two SNPs were removed due to Hardy-Weinberg disequilibrium (p<0.01), leaving 79 SNPs for analysis.

CYP4F2 (rs2108622) was genotyped separately using TaqMan Genotyping Assay (Applied Biosystems, Foster City, USA; Assay ID: C__16179493_40).

2.5 Statistical analysis

Analyses were conducted in two phases. In the first phase, *a priori* selected key candidate SNPs in *VKORC1* (-1639G>A; rs9923231), *CYP2C9* (**2* and **3*; rs1799853 and rs1057910), and *CYP4F2* (rs2108622) were analyzed for associations with primary outcomes. In the secondary exploratory phase, all genotyped SNPs were analyzed for an association with therapeutic warfarin dose.

2.5.1 Dose association analysis

Normal distribution of mean daily warfarin dose (mg) was achieved by taking the square root. Associations between continuous variables (age, height, weight, body surface area (BSA), and body mass index (BMI)) and dose were assessed using the Pearson correlation test, while categorical variables (gender, ancestry, indication, target INR, concomitant drugs and genotype) were assessed using unpaired *t*-test or analysis of variance (ANOVA). Concomitant drugs of interest were chosen using the FDA warfarin package insert[142]. All drugs that were listed as either inhibitors or inducers of CYP2C9, CYP1A2, or CYP3A4 were analyzed for an association with dose. Due to small numbers of patients taking each drug, concomitant drugs were also separated according to their potentiating or attenuating effect on warfarin. Potentiating drugs included amiodarone, fluoxetine, propafenone, propranolol, ranitidine, and co-trimoxazole, while attenuating drugs included omeprazole, phenobarbital, and prednisone. Variables with a *p*-value <0.20 in univariate analyses were

entered into a stepwise linear regression model and variables with a *p*-value <0.05 were retained in the model. *VKORC1* (-1639G>A), *CYP2C9*2* and **3* were included in all final regression models. An additive genetic model was used for regression analyses and genotypes were coded as: 0 for homozygous major allele, 1 for heterozygous, and 2 for homozygous minor allele.

Model accuracy was assessed by determining the number of children for which the model over- or under-predicted the actual required dose. The ability of a previously published pediatric-specific warfarin pharmacogenetic dosing model[201] as well as an adult-derived dosing model[132] to predict therapeutic warfarin dose in our cohort of children was also evaluated. Pearson correlation coefficients for predicted dose vs. actual therapeutic dose were compared using methods developed by Meng et al.[204].

2.5.2 Survival analysis and time to INR events

Time-dependent outcomes (time to first INR in or above the therapeutic range and time to first INR >4) were compared among genotype groups using the log-rank test. *VKORC1* was separated into homozygous major allele (GG), heterozygous (AG), and homozygous variant allele (AA) genotype groups. Similarly, *CYP2C9* genotype was analyzed as homozygous major allele (**1/*1*), heterozygous (**1/*2* or **1/*3*), and homozygous variant allele (**2/*2*) genotypes. Hazard ratios (HRs) for genotypes for both time-to-INR outcomes were determined using cox regression analyses. Associations with quantitative (age, height, weight, initiation dose) and qualitative variables (gender, ancestry, target INR, indication for warfarin, potentiating/attenuating drugs) were analyzed using univariate cox regression and

log-rank tests, respectively. Potentiating drugs included amiodarone, clarithromycin, fluoxetine, propafenone, propranolol, ranitidine, and co-trimoxazole, while attenuating drugs included omeprazole, phenobarbital, prednisone, and multivitamins containing vitamin K. Clinical variables with a *p*-value <0.20 were entered into a stepwise model and variables with a *p*-value <0.05 were used as covariates in cox regression analyses to determine adjusted HRs for genotype groups. For all analyses, homozygous major allele genotype was used as the reference genotype.

2.5.3 Case-control analyses

For the investigation of warfarin-induced bleeding, a case-control analysis using Fisher's exact test was performed and odds ratios (ORs) were calculated, with the homozygous major allele genotype as the reference genotype. Fisher's exact test was also used to test for associations with gender, ancestry, and indication for warfarin. This statistical test was chosen due to the small sample size and small number of cases. The non-parametric Wilcoxon-Mann-Whitney test was used to test for associations with age at initiation of therapy and amount of time on therapy that was captured. Target INR and concomitant medications were not analyzed as covariates because these variables did not remain constant over the course of therapy. Variables with a *p*-value <0.20 in univariate analyses were entered into a stepwise model and variables with a *p*-value <0.05 were included as covariates in logistic regression models to determine adjusted ORs for bleeding events.

Risk of over-anticoagulation (INR >4) in the first 60 days of therapy was also analyzed using the case-control statistical methods described above. Target INR and use of potentiating or attenuating drugs were assessed using Fisher's exact test.

2.5.4 Exploratory SNP analyses

In the second analysis phase, an exploratory analysis of the larger genotyping panel was performed. All genotyped SNPs that passed quality control (79 SNPs) were analyzed for an association with the square root of the daily warfarin dose, in order to identify additional SNPs that may impact warfarin dosing in children. An additive allele model was used and genotypes were coded as: 0 for homozygous major allele, 1 for heterozygous, and 2 for mutant homozygous. SNPs with a *p*-value <0.10 using simple linear regression were entered into a stepwise regression model with clinical covariates and SNPs with *p*-values <0.05 were retained in the final model. Due to the limited sample size and exploratory nature of the analysis, reported *p*-values were not corrected for multiple testing.

2.6 Patient cohort inclusion and exclusion

Analyses for therapeutic dose were restricted to patients who achieved this outcome according to our definition (n=77). Patients were removed from time-to-INR event analyses due to missing information following initiation of therapy (n=1) or for reasons that are known to prolong the prothrombin time (ex. concomitant use of argatroban (n=2) or presence of an anti-bovine thrombin inhibitor (n=1))[205-207]. All 93 patients were included in the case/control analyses investigating risk of major and minor bleeding. Patients were removed from INR >4 case/control analyses due to missing information during the first 60 days of

therapy (n=6), duration of therapy less than 60 days (n=2), or factors that prolong the prothrombin time (n=2).

For all tests, two-sided *p*-values <0.05 were considered statistically significant. Statistical analyses were performed using SPSS Statistics 20 (SPSS Inc., Chicago, USA), R 2.15.1 (R Development Core Team,) and SNP and Variation Suite 7.6.11 (Golden Helix, Bozeman, USA).

Table 2.1. List of SNPs included on custom genotyping panel

Gene	Variant	Alleles	Amino Acid Change	Reason for including SNP on custom panel	References
VKORC1	rs9923231	A/C/G/T	N/A	Tagging SNP for 1*2 haplotype	115
VKORC1	rs9934438	A/G	N/A	Tagging SNP for 1*2 haplotype	115
VKORC1	rs7294	A/G	N/A	Tagging SNP for 1*3 haplotype	115
VKORC1	rs17708472	A/G	N/A	Tagging SNP for 1*4 haplotype	115
VKORC1	rs104894542	G/T	Leu1128Arg	Rare point mutation in coding region, associated with warfarin resistance	208
VKORC1	rs61742245	A/C	Asp36Tyr	Rare point mutation in coding region, associated with warfarin resistance	119
VKORC1	rs72547529	C/T	Val66Met	Rare point mutation in coding region, associated with warfarin resistance	118
CYP2C9	rs1799853	C/T	Arg144Cys	CYP2C9*2; Decreased CYP2C9 activity	92
CYP2C9	rs1057910	A/C	Ile359Leu	CYP2C9*3; Decreased CYP2C9 activity	92
CYP2C9	rs9332131	A	Lys273Argfs	CYP2C9*6; Complete loss of CYP2C9 activity	107
CYP2C9	rs28371685	C/T	Arg335Trp	CYP2C9*11; Complete loss of CYP2C9 activity	107
CYP2C9	rs7089580	A/T	N/A	Associated with higher dose in African Americans	106
CYP4F2	rs2108622	C/T	Val433Met	Increased dose requirement in patients carrying the T allele	146
CYP4F2	rs2189784	A/G	N/A	Located upstream of CYP4F2; Associated with increased time to INR	209
CYP2C18	rs12777823	A/G	N/A	Located upstream of CYP2C18; Associated with dose in African Americans	189
EPHX1	rs4653436	A/G	N/A	Associated with lower dose in Han Chinese patients	165,185
EPHX1	rs1877724	C/T	N/A	Associated with lower dose in Asian populations	162,186
EPHX1	rs1051740	C/T	Tyr113His	Associated with dose when comparing high dose vs. low dose requirement	184
EPHX1	rs2292568	C/T	N/A	Tag SNP	
EPHX1	rs6426089	A/G	N/A	Tag SNP	
EPHX1	rs2740174	A/G	N/A	Tag SNP	
EPHX1	rs3753661	G/T	N/A	Tag SNP	
EPHX1	rs2671272	C/T	N/A	Tag SNP	
EPHX1	rs2740170	C/T	N/A	Tag SNP	
EPHX1	rs10799324	A/C	N/A	Tag SNP	
EPHX1	rs10753410	C/T	N/A	Tag SNP	
EPHX1	rs2234922	A/G	His139Arg	Tag SNP	

Gene	Variant	Alleles	Amino Acid Change	Reason for including SNP on custom panel	References
EPHX1	rs10915884	C/T	N/A	Tag SNP	
EPHX1	rs6965	C/T	N/A	Tag SNP	
EPHX1	rs3753663	A/T	N/A	Tag SNP	
EPHX1	rs360063	A/G	N/A	Tag SNP	
EPHX1	rs868966	A/G	N/A	Tag SNP	
EPHX1	rs2260863	C/G	N/A	Tag SNP	
CALU	rs339097	C/T	N/A	G allele associated with increased dose requirement	180-182
CALU	rs2307040	A/G	Ala82Val	Common coding SNP in Caucasians; Nominally associated with dose before correction for multiple testing	160
CALU	rs339099	A/G	N/A	Tag SNP	
CALU	rs17164371	C/T	N/A	Tag SNP	
CALU	rs339057	C/T	N/A	Tag SNP	
CALU	rs2290228	C/T	Arg4Gln	Tag SNP	
CALU	rs2060717	C/T	N/A	Tag SNP	
CALU	rs7776641	C/T	N/A	Tag SNP	
CALU	rs109829	C/T	N/A	Tag SNP	
CALU	rs4731513	A/G	N/A	Tag SNP	
CALU	rs1043595	A/G	N/A	Tag SNP	
GGCX	rs12714145	C/T	N/A	Associated with increased dose	174
GGCX	rs11676382	C/G	N/A	Associated with decreased dose	169,210
GGCX	rs74843621	A/G	Asp31Asn	Common coding SNP in Africans	
GGCX	rs75294795	C/G	Ser284Cys	Common coding SNP in Africans	
GGCX	rs2592551	C/T	Arg406Arg	Tag SNP	
GGCX	rs699664	A/G	Arg268Gln	Tag SNP	
GGCX	rs10179904	A/G	Thr414Thr	Tag SNP	
PROC	rs5936	G/T	Ser141Ser	Associated with warfarin dose in Han Chinese	162
PROC	rs2069919	A/G	N/A	Associated with dose in a multivariate model	160
PROC	rs1799809	A/G	N/A	Associated with decreased dose in univariate regression	160
PROC	rs2069901	C/T	N/A	Associated with dose in univariate regression	160
PROC	rs2069927	A/G	Pro210Pro	Common coding SNP in Africans	
PROC	rs5937	C/T	Asp256Asp	Common coding SNP in Caucasians	
PROC	rs6710535	C/G	N/A	Tag SNP	
PROC	rs1568277	C/T	N/A	Tag SNP	
PROC	rs878461	C/T	N/A	Tag SNP	
PROC	rs1158867	C/T	N/A	Tag SNP	
APOE	rs7412	C/T	Arg176Cys	Associated with both increased and decreased dose requirements	158-160
APOE	rs429358	C/T	Cys130Arg	Associated with both increased and decreased dose requirements	158-160
APOE	rs439401	C/T	N/A	Tag SNP	

Gene	Variant	Alleles	Amino Acid Change	Reason for including SNP on custom panel	References
APOE	rs405697	C/T	N/A	Tag SNP	
APOE	rs405509	A/C	N/A	Tag SNP	
APOE	rs1160985	C/T	N/A	Tag SNP	
APOE	rs445925	C/T	N/A	Tag SNP	
POR	rs12537282	C/G/T	N/A	Associated with decreased dose	[176]
POR	rs72553971	A/C	N/A	Associated with decreased dose	[176]
POR	rs2868177	A/G	N/A	Associated with increased dose	[176]
POR	rs11766772	A/G	N/A	Tag SNP	
POR	rs17148944	A/G	N/A	Tag SNP	
POR	rs6965343	C/T	N/A	Tag SNP	
POR	rs12537277	A/G	N/A	Tag SNP	
POR	rs6953065	A/G	N/A	Tag SNP	
POR	rs7796654	A/G	N/A	Tag SNP	
POR	rs4728533	C/T	N/A	Tag SNP	
POR	rs3898649	A/G	N/A	Tag SNP	
POR	rs1362234	A/G	N/A	Tag SNP	
F2	rs5896	C/T	Thr165Met	Common coding SNP in Caucasians	
F2	rs5899	C/T	Gly271Gly	Common coding SNP in Caucasians	
F2	rs5898	A/G	Pro411Pro	Common coding SNP in Caucasians	
F2	rs3136456	A/C	N/A	Tag SNP	
F2	rs3136520	C/T	N/A	Tag SNP	
F2	rs2070852	C/G	N/A	Tag SNP	
F2	rs3136516	A/G	N/A	Tag SNP	
A2M	rs226405	A/G	Asp639Ter	Common coding SNP in Africans	
A2M	rs669	A/G	Ile1000Val	Tag SNP	
A2M	rs4882978	C/T	N/A	Tag SNP	
A2M	rs10743598	C/T	N/A	Tag SNP	
A2M	rs226397	A/G	N/A	Tag SNP	
A2M	rs1805661	C/T	N/A	Tag SNP	

Chapter 3: Results

3.1 Study population

Ninety-three children were included in the study. The study population was comprised of 52 males and 41 females. The median age at initiation of therapy was 4.8 years (range, 2 months-17.8 years; **Table 3.1**). The main indications for warfarin were following Fontan procedures (n=35), mechanical heart valves (n=22), and deep vein thrombosis or pulmonary embolism (DVT/PE; n=15). The majority of patients were of European descent (n=61), followed by Asian (n=16), mixed (n=12), and other (n=4; First Nations, n=2; African, n=1; Fijan, n=1). The average length of time on warfarin therapy that was captured was 3.91 years (range, <1 month -14.4 years). Patient characteristics and genotyping frequencies for *VKORC1* (-1639G>A, rs9923231), *CYP2C9* (*2 and *3; rs1799853 and rs1057910), and *CYP4F2* (rs2108622) are displayed in **Table 3.1**. Genotype frequencies for the four candidate SNPs were similar to those reported in the literature[95,111,146].

3.2 Association of genetic and non-genetic factors with therapeutic dose

Seventy-seven patients reached a stable therapeutic dose and were included in this analysis. The sixteen excluded patients never achieved a stable dose after a mean of approximately 4.5 months following initiation of therapy. These patients are included in additional analyses described below. The median daily therapeutic warfarin dose was 3 mg (range, 0.75-10 mg) and the median age at time of therapeutic dose was 4.7 years (range, 8 months-18 years). In univariate analyses, age (r=0.59, p<0.001), height (r=0.65, p<0.001), weight (r=0.73, p<0.001), body surface area (BSA) (r=0.71, p<0.001), and body mass index (BMI) (r=0.63,

p<0.001) calculated at time of therapeutic dose were significantly correlated with the square root of the daily dose (**Table 3.2**). A positive correlation was observed between age and warfarin dose expressed in milligrams. However, when warfarin dose was normalized to body weight (mg/kg) there was a negative correlation between age and dose (**Figure 3.1**).

There was a significant difference in dose requirements among indications for warfarin (p<0.001; **Table 3.2**). Patients who were anticoagulated for a Fontan procedure required a significantly lower mean daily warfarin dose (2.5+/-1.2 mg) than patients who were anticoagulated for a DVT/PE (5.0+/-2.6 mg; p=0.002) or for an indication classified as 'other' (4.7+/-2.4 mg; p=0.001). Patients with Fontan procedures also required lower mean daily warfarin doses than patients who were anticoagulated for mechanical heart valves (3.4+/-1.5 mg) but the difference was not statistically significant (p=0.17). Children with a lower target INR (INR <2.5) also required a lower mean daily warfarin dose (3.0+/-1.2 mg) than children with a target INR of 2-3 (3.8+/-2.8 mg) or a target INR >2.5 (3.4+/-1.5 mg). However, dose requirements did not differ significantly among INR target ranges (p=0.46; **Table 3.2**). Gender (p=0.23), ancestry (p=0.69), and use of potentiating (p=0.81) or attenuating drugs (p=0.77) were not associated with dose (**Table 3.2**). Furthermore, no concomitant medications were significantly associated with dose when assessed independently.

Children with the *VKORC1* (-1639) AA genotype required a significantly lower mean daily warfarin dose (2.0+/-1.4 mg) than those with the AG (3.3+/-1.6 mg) or GG (4.1+/-2.2 mg) genotype (p<0.001, ANOVA; **Table 3.3**). Carriers of the AG genotype also required a lower

warfarin dose than GG carriers but this difference was not significant (p=0.274). A gene-dose effect, whereby daily dose requirements decreased with the addition of each variant allele, is displayed in **Figure 3.2**. The mean daily warfarin dose requirement for carriers of the *CYP2C9*1/*3* genotype (2.2+/-1.5 mg) was also significantly lower than in patients with the *CYP2C9*1/*1* genotype (3.7+/-2.1 mg, p=0.013; **Table 3.3**). There was a non-significant trend towards a lower dose requirement in *CYP2C9*1/*2* carriers (3.2+/-1.6 mg) compared to **1/*1* patients (p=0.51). Only one patient carried the *CYP2C9*2/*2* genotype (3.25 mg). When comparing dose requirements among *CYP2C9*1/*1*, **1/*2*, and **1/*3* carriers, a significant difference was observed (p=0.041; **Figure 3.2**). Similarly, when all *CYP2C9* variant allele carriers were grouped together, these patients required a significantly lower dose compared to **1/*1* patients (p=0.049).

Homozygous carriers of the *CYP4F2* (rs2108622) T variant required higher mean daily warfarin doses (3.7+/-1.3 mg) than homozygous CC (3.6+/-2.1 mg) or heterozygous CT patients (3.2+/-1.9 mg) but the difference in dose requirements between genotype groups was not significant (p=0.66, ANOVA; **Table 3.3**). Heterozygous CT patients required the lowest warfarin dose, eliminating a gene-dose effect.

In univariate analysis using simple linear regression, *VKORC1* accounted for 17.2% of the variability in required warfarin dose. Alternatively, *CYP2C9*2* and **3* genotypes accounted for 0.04% and 7.8% of dose variability, respectively.

3.2.1 Dose prediction models

In multivariate analysis, body weight, indication for warfarin, *VKORC1* and *CYP2C9* genotypes explained 76.3% of the variability in warfarin dose (**Table 3.4**). Weight (kg) was the most important contributor to the model, explaining 52.8% of dose variability. *VKORC1* accounted for 12.2% of variability, *CYP2C9* (**2* and **3*) for 8.9%, and indication for 2.4%. *CYP2C9*2* alone explained 1% of dose variability and was not significant in the overall model (p=0.078). However, excluding *CYP2C9*2* from the model did not change the contribution of the other covariates towards warfarin dose variability. The final regression equation was: square root of daily dose (mg) = 1.711 + 0.014 (weight, kg) -0.257 (number of VKORC1 variant alleles) – 0.127 (number of *CYP2C9*2* alleles) – 0.463 (number of *CYP2C9*3* alleles) – 0.161 (Fontan procedure). **Figure 3.3** shows the distribution of observed versus predicted dose when applying the model to our cohort of children. In 92.2% of patients, the predicted dose (mg/day) was within +/-1 mg of the actual therapeutic dose.

In a subgroup analysis of patients less than 6 years old (n=42), or approximately half the patient cohort, the contribution of both *VKORC1* and *CYP2C9* genotypes to dose variability increased (**Table 3.5**). This was especially apparent for *VKORC1,* which increased from 12.2% in the original model to 44.4%. The contribution of *CYP2C9*3* to dose variability increased slightly compared to the original model, from 7.9% to 9% (**Table 3.5**). No additional genetic or clinical covariates were significantly associated with dose in a multivariate model. The majority of patients less than 6 years old were receiving warfarin for a Fontan procedure (n=27). When analyzing all Fontan patients who achieved a therapeutic dose (n=34) the amount of dose variability explained by *VKORC1* increased even

further, to 47.7%. Body surface area accounted for the second greatest amount of dose variability (20.4%), followed by *CYP2C9*3* (5.3%) and *CYP2C9*2* (2.5%).

In contrast, the dose prediction model developed using children ≥6 years of age (n=35) was similar to the original model. Weight remained the most important contributor to dose variability, accounting for 48% (**Table 3.6**). The contribution of *VKORC1*, *CYP2C9*2* and **3* genotypes to dose variability changed only slightly from the original model (11.1%, 2.1% and 7.3%, respectively; **Table 3.6**). Indication was removed from the model and target INR became significantly associated with dose.

3.2.2 Association of genetic factors using alternative units of dose measurement

Multivariate regression analyses using alternative units of therapeutic dose were also performed. The overall contribution of *VKORC1* and *CYP2C9* genotypes to body weight-adjusted (mg/kg) dose variability when adjusting for weight and indication was similar to using unadjusted doses (mg). *VKORC1* accounted for 12.3% of dose variability, while *CYP2C9* (**2* and **3*) accounted for 8%. A similar trend was observed when using body-size adjusted doses (mg/m^2). However, the contribution of both *VKORC1* and *CYP2C9* genotypes to dose variability increased slightly compared to using unadjusted doses (13.6% and 13.7%, respectively).

3.2.3 Evaluation of existing pharmacogenetic dosing models

A previously published pediatric-specific pharmacogenetic dosing model[201] for predicting daily therapeutic warfarin dose was strongly correlated with actual therapeutic warfarin dose

in our cohort of children (R^2=0.68, p<0.001), confirming the validity of the pediatric dosing model (**Figure 3.4**). When comparing the correlation between predicted dose and therapeutic dose using the pediatric-derived dosing model to an adult-derived dosing model developed by the International Warfarin Pharmacogenetics Consortium (IWPC)[132] (R^2=0.57, p<0.001; **Figure 3.4**), the pediatric model was significantly more accurate (p=0.023). The IWPC model overestimated the required daily dose by an average of 1.8+/-1.3 mg, while the pediatric model slightly underestimated the required daily dose (-0.22+/-1.1 mg) (**Figure 3.5**).

3.3 Time to INR events

The influence of genetic and clinical factors on (1) time to first INR in or above the therapeutic range, and (2) time to over-anticoagulation (INR >4) was analyzed. Eighty-nine patients were included in both analyses. The median time to therapeutic INR was 5 days and the median time to INR >4 was 5.5 days.

3.3.1 Influence of genetic factors on time to therapeutic INR

The median time required to achieve an INR in or above the therapeutic range was longest for *VKORC1* GG patients (8 days) (**Table 3.7**). *VKORC1* AG heterozygous and AA homozygous patients had a shorter median time to therapeutic INR, requiring 4 and 3 days to reach this outcome, respectively. *VKORC1* had a significant influence on time to first INR in or above the therapeutic range (p=0.047; **Figure 3.6A**). Concordant with these findings, the rate of achieving a therapeutic INR was significantly higher in *VKORC1* homozygous variant patients compared to GG patients (HR, 2.22; **Table 3.8**). There was a trend towards

decreased time to therapeutic INR in *VKORC1* heterozygous patients compared to GG patients but the difference was not significant (HR, 1.32; p=0.252) (**Table 3.8**).

Among *CYP2C9* genotype groups, carriers of **1/*1* genotype required the longest median time to achieve a therapeutic or supratherapeutic INR (5 days) (**Table 3.7**). In contrast, heterozygous patients *(*1/*2* or **1/*3*) required a median of 2 days to achieve a therapeutic INR, while **2/*2* patients required a median of 2.5 days (n=2) (**Table 3.7**). When comparing time to first INR in or above the therapeutic range among *CYP2C9* genotype groups there was no significant association (p=0.152; **Figure 3.6C**). Accordingly, there was no significant difference in the rate of achieving a therapeutic INR in *CYP2C9* heterozygous (HR, 1.33; p=0.255) or homozygous variant allele carriers (HR, 3.53; p=0.090) compared to **1/*1* patients (**Table 3.8**). *CYP4F2* genotype also did not significantly influence time to first INR in or above the therapeutic range (log rank, p=0.701).

Regarding clinical covariates, high target INR range (INR >2.5) (p=0.006), indication for a mechanical heart valve (p=0.007), indication for 'other' (p=0.023), and use of a warfarin-attenuating drug (p=0.095) showed an association with time to therapeutic INR. When these variables were entered into a stepwise cox regression model, only high target INR range remained significant (p<0.05). Therefore, high target INR was used as a covariate to determine adjusted hazard ratios for genotype groups. In adjusted analyses, patients carrying the *VKORC1* AA genotype achieved an INR in or above the therapeutic range at a significantly higher rate compared to GG patients (adjusted HR, 2.21; p=0.020) (**Table 3.8**). No additional *VKORC1* or *CYP2C9* variant genotype groups showed a significant difference

in the rate of achieving a therapeutic INR compared to wildtype patients when adjusting for target INR (**Table 3.8**). However, there was a consistent trend towards decreased time to therapeutic INR in variant allele carriers (**Table 3.8**). There was no significant difference in time to therapeutic INR in *CYP4F2* variant allele carriers compared to CC patients when adjusting for target INR (adjusted HR, 1.14; p=0.551).

3.3.2 Influence of genetic factors on time to over-anticoagulation

In total, thirty-eight patients achieved an INR >4 in the first 60 days of therapy. Unlike the previous analysis, the median time to INR >4 was shortest in *VKORC1* GG patients, who achieved this outcome in a median of 2.5 days (**Table 3.7**). In comparison, *VKORC1* heterozygotes reached an INR >4 in a median of 7.5 days, while homozygous variant allele carriers reached this outcome in a median of 4.5 days (**Table 3.7**). Reported medians are only for patients who reached this outcome in the first 60 days of therapy and therefore are likely to be shorter than the true medians.

VKORC1 genotype had a significant influence on time to first INR >4 (p=0.024; **Figure 3.6B**). Accordingly, homozygous variant allele carriers reached an INR >4 at a significantly faster rate than GG patients (HR, 2.92; p=0.018) (**Table 3.8**). There was no significant difference in the rate of achieving an INR >4 between *VKORC1* AG and GG patients (HR, 1.99; p=0.081). However, when all *VKORC1* variant allele carriers were grouped together (A/_) these patients reached an INR >4 significantly faster than GG carriers (HR, 2.29; p=0.025) (**Table 3.8**).

The median time to over-anticoagulation (INR >4) was shortest in *CYP2C9* heterozygous patients (3 days), with **1/*1* and **2/*2* patients achieving this outcome in a median of 10 and 15.5 days, respectively (**Table 3.7**). As with the previous outcome, *CYP2C9* did not significantly influence time to INR >4 (p=0.071; **Figure 3.6D**). There was also no significant difference in the rate of achieving an INR >4 in heterozygous patients (HR, 1.78; p=0.099) or homozygous variant patients (HR, 3.88; p=0.068) compared to **1/*1* carriers (**Table 3.8**). When analyzing all *CYP2C9* variant allele carriers, the difference in the rate of achieving an INR >4 compared **1/*1* carriers approached significance (HR, 1.92; p=0.050). Similar to previous analyses, *CYP4F2* did not significantly influence time to INR >4 (log rank, p=0.688).

Low target INR (INR <2.5) (p=0.076), high target INR (INR >2.5) (p=0.003), indication for a mechanical heart valve (p=0.006), and indication for 'other' (p=0.026) were also associated with time to INR >4. When these variables were entered into a stepwise cox regression model, high target INR was again the only clinical covariate that remained significant ($p<0.05$). Adjusting for this covariate revealed that the rate of achieving an INR >4 was 2.7 times higher in *VKORC1* AA carriers compared to wildtype patients (adjusted HR, 2.70; p=0.029) (**Table 3.8**). There was no significant difference in the rate of achieving an INR >4 between *VKORC1* heterozygous variant allele carriers and homozygous GG patients (adjusted HR, 1.70; p=0.185). Furthermore, when all *VKORC1* variant allele patients were analyzed together there was no significant difference compared to GG patients (adjusted HR, 2.06; p=0.054). In contrast, the rate of achieving an INR >4 was higher by more than a factor of 2.5 in *CYP2C9* heterozygous patients compared to **1/*1* patients (adjusted HR,

2.57; *p*=0.014) (**Table 3.8**). The rate of achieving an INR >4 was also significantly higher when analyzing all *CYP2C9* variant allele carriers together (HR, 2,40; *p*=0.011). There was a non-significant trend towards decreased time to INR >4 in *CYP2C9*2/*2* carriers (HR, 2.40; *p*=0.255). When analyzing *CYP4F2* genotypes, the rate of achieving an INR >4 was not significantly different between variant allele carriers and wildtype carriers (adjusted HR, 1.11; *p*=0.752).

3.4 Warfarin-induced adverse drug reactions

Bleeding and thrombosis events were captured using patient health records. Bleeding was common among patients receiving warfarin while thrombosis occurred much less frequently, affecting only three patients in the study cohort. Therefore, subsequent analyses on warfarin-induced ADRs were limited to bleeding events.

3.4.1 Bleeding

Overall, 39.8% of patients (n=37) experienced a bleeding event while receiving warfarin therapy. The most common sign of bleeding was epistaxis, occurring in a minimum of 20.4% patients (n=19). Other signs of bleeding included heavy menses, hematemesis, hemarthrosis, and bloody stools. The median time to first bleeding event was 259 days (range, 1-3697 days) and the median age at time of reaction was 9.9 years. Bleeding events were more likely to occur in patients with mechanical heart valves (*p*=0.046) and in patients for whom a longer amount of time on warfarin therapy was captured (*p*=0.027). When analyzing risk of bleeding in patients carrying *VKORC1* or *CYP2C9* variant alleles, no significant observations were observed (**Table 3.9**). Carriers of the **1/*3* genotype had the

highest odds of bleeding (OR, 2.22; p=0.220), though this finding was not significant (**Table 3.9).** For all additional *VKORC1* and *CYP2C9* variant genotype groups the odds ratios centered around 1, indicating no evidence of increased risk of bleeding compared to wildtype patients (**Table 3.9**). Interestingly, carriers of the *CYP4F2* variant allele, which confers decreased sensitivity to warfarin, were at greater risk of bleeding compared to CC patients (OR, 1.63; p=0.291) (Table 3.9). This was mainly attributed to the higher risk of bleeding in heterozygous patients (OR, 1.74; p=0.275). However, the difference in risk of bleeding between wildtype patients and *CYP4F2* variant allele carriers was not significant (**Table 3.9**).

In addition to length of time captured on warfarin therapy and indication for a mechanical heart valve, indication for a DVT/PE was also marginally associated with risk of bleeding (p=0.148). When these variables were entered into a stepwise regression model, only length of time captured on therapy remained significant (p<0.05). Adjusting for this covariate increased the odds ratio for risk of bleeding in *CYP2C9*1/*3* carriers (OR, 2.81; p=0.125) (**Table 3.9**). Once again, however, there was no evidence of significantly increased risk of bleeding in *VKORC1*, *CYP2C9*, or *CYP4F2* variant allele carriers (**Table 3.9**).

3.4.2 Major bleeding

Seven patients (7.5%) experienced a major bleeding event according to the criteria of the International Society of Thrombosis and Haemostasis[202]. Bleeding events included: subdural hematoma, hemorrhagic stroke, hemorrhage into tonsillar abscess, intracerebral hemorrhage, gross hematuria, hematemesis, and menorrhagia. More than half of these events (n=4) occurred within the first six weeks after initiation of therapy (range, 1 day to 8.7 years) and

the majority of bleeding events occurred in patients with mechanical heart valves (n=4; p=0.052). Length of time captured on therapy was also marginally associated with risk of major bleeding (p=0.069).

Strikingly, six of the patients with major bleeding carried at least one *VKORC1* variant A allele, conferring an odds ratio of 4.75 in variant allele carriers compared to homozygous GG patients, even though the association was not statistically significant (p=0.157; **Table 3.10**). Four patients were homozygous for the *CYP2C9*1* allele while three patients were variant allele carriers. Interestingly, all three patients were heterozygous for **1/*3*, conferring an odds ratio of 4.93 (p=0.072; **Table 3.10**).

As with the previous analysis, length of time captured on therapy was the only clinical variable that was significantly associated with incidence of major bleeding in a stepwise regression model. When adjusting for this confounder there was a non-significant trend towards increased risk of major bleeding in both *CYP2C9* and *VKORC1* variant allele carriers, conferring odds ratios greater than 3 for both variant genotypes (**Table 3.10**). When analyzing the high sensitivity *CYP2C9*1/*3* genotype, there was a significantly increased risk of major bleeding (OR, 10.21; p=0.029) (**Table 3.10**). No additional variant genotype groups were at a significantly greater risk of major bleeding compared to wildtype carriers. Furthermore, there was no association between risk of major bleeding and *CYP4F2* variant genotype (adjusted OR, 1.01; p=0.991) (**Table 3.10**).

3.4.3 Risk of over-anticoagulation

As a supplementary analysis the risk of over-anticoagulation (INR >4) in the first 60 days of therapy was also investigated. Thirty-nine of 83 patients (47%) who were included in the analysis had at least one INR measurement greater than 4 in the initiation of therapy, with the majority of events occurring in the first 7 days of therapy (54%). Compared to wildtype patients, *VKORC1* variant allele carriers were at significantly greater risk of over-anticoagulation (OR, 3.30; p=0.014) (**Table 3.11**). The risk was highest in homozygous *VKORC1* variant allele carriers, conferring an odds ratio of 5.47 (p=0.012). There was a trend towards increased risk of over-anticoagulation in *CYP2C9* variant allele carriers but the results were not significant (**Table 3.11**).

When adjusting for high target INR (INR >2.5), which was the only clinical variable that was significant in a stepwise regression model, the risk of over-anticoagulation in *VKORC1* homozygous variant allele carriers increased further (OR, 6.95; p=0.008) (**Table 3.11**). When all *VKORC1* variant allele carriers were analyzed, the risk of over-anticoagulation remained significant (OR, 3.63; p=0.010). There was also a non-significant trend towards increased risk of over-anticoagulation in *CYP2C9* variant allele carriers compared to **1/*1* patients (**Table 3.11**). The odds ratio was highest when all *CYP2C9* variant allele carriers were grouped together (OR, 2.69; p=0.057). *CYP4F2* variant genotype was not significantly associated with risk of INR >4 in univariate or adjusted analyses (**Table 3.11**).

3.5 Exploratory analysis of additional genetic variation in candidate genes

Overall, thirteen SNPs of the full genotyping panel had an unadjusted *p*-value <0.10 in univariate analysis, with R^2 values ranging from 3.7-17.3% (**Table 3.12**). *VKORC1*-1639G>A (rs9923231) showed the strongest association with warfarin dose ($p=1.7x10^{-4}$), followed by *CYP2C9**3 (rs1057910; $p=0.014$), thus confirming the importance of these SNPs for warfarin dosing in children (**Table 3.12**). A *VKORC1* SNP (rs9934438) that is in complete linkage disequilibrium with *VKORC1*-1639G>A was also significantly associated with dose ($p=1.7x10^{-4}$). The remaining top ten SNPs were non-coding variants in *CYP2C9*, *PROC, POR, GGCX, CALU*, and *APOE* (**Table 3.12**). Of these, five SNPs had previously been associated with warfarin dose in adults. Specifically, rs7294 in *VKORC1,* rs7089580 in *CYP2C9*, rs72553971 in *POR*, rs11676382 in *GGCX*, and rs2069901 in *PROC*[106,115,160,169,176]. The remaining five SNPs were tagging SNPs. Neither the *CYP2C9*2* variant (rs1799853) nor the *CYP4F2* rs2108622 variant were associated with dose in univariate analyses ($p=0.86$, $p=0.78$, respectively).

When all SNPs with *p*-values <0.10 were entered into a stepwise regression model including weight and indication for warfarin, one additional SNP in *CYP2C9* (rs7089580, A>T) remained significantly associated with dose ($p=0.020$). This SNP explained 2.5% of the variability in warfarin dose, in a model that also included weight (52%), *VKORC1* -1639G>A (12.5%), *CYP2C9*3* (7.8%), indication (2.6%), and *CYP2C9*2* (0.4%) (**Table 3.13**). Compared to the original model, there were slight differences in R^2 values for all variables due to undetermined rs7089580 genotype in two patients. Once again *CYP2C9**2 was not significantly associated with dose ($p=0.274$) and explained less variability in

warfarin dose when rs7089580 was added to the model (R^2=0.4%) compared to a model that did not include this SNP (R^2=1.1%). The contribution of *VKORC1* and *CYP2C9*3* to dose variability did not change with the addition of rs7089580. The overall amount of dose variability explained by clinical and genetic factors increased from 75.9% to 77.8% (**Table 3.13**). Upon further analysis of rs7089580 there was no significant difference in dose requirements across genotypes (ANOVA, *p*=0.112). Nevertheless a gene-dose trend was observed, with homozygous variant allele carriers requiring higher mean daily warfarin doses (5.0+/-1.8 mg) compared to AT heterozygous (3.6+/-2.4 mg) and AA homozygous (3.1+/-1.6 mg) patients (**Figure 3.7**).

When analyzing all SNPs with a *p*-value <0.10 and adjusting for weight, indication, *VKORC1* (-1639G>A) and *CYP2C9*3*, an intronic SNP in *GGCX* (rs11676382) was also significantly associated with dose (*p*=0.042). However, when rs7089580 was added to the model this *GGCX* SNP was no longer significant (*p*=0.089).

Table 3.1. Clinical and genetic characteristics of study cohort

Variable	All patients
Median age at initiation of therapy, yrs (range)	4.8 (0.2-17.8)
Sex, no. (%)	
Male	52 (55.9)
Female	41 (44.1)
Ancestry, no. (%)	
European	61 (65.6)
Asian	16 (17.2)
Mixed European	12 (12.9)
Other[a]	4 (4.3)
Target INR, no. (%)	
Less than 2.5	36 (38.7)
2.0-3.0	37 (39.8)
Greater than 2.5	20 (21.5)
Indication, no. (%)	
Fontan procedure	35 (37.6)
Mechanical heart valve	22 (23.7)
Deep vein thrombosis/pulmonary embolism	15 (16.1)
Dilated cardiomyopathy	3 (3.2)
Stroke	3 (3.2)
Coronary aneurysm	2 (2.2)
Other[b]	13 (14.0)
VKORC1	
GG	39 (41.9)
AG	37 (39.8)
AA	17 (18.3)
CYP2C9	
**1/*1*	65 (69.9)
**1/*2*	14 (15.0)
**1/*3*	12 (12.9)
**2/*2*	2 (2.2)
**2/*3, *3/*3*	0 (0.0)
CYP4F2	
CC	42 (45.2)
CT	45 (48.4)
TT	6 (6.4)
Total no. of children	**93**

[a]First Nations, n=2; African, n=1; Fijan, n=1.

[b]One patient each with pulmonary hypertension, arrhythmia, clot in right atrium, clot in left ventricle, clot in coronary vein, clot in anterior descending coronary artery, anomalous left coronary artery from the pulmonary artery, atrioventricular septal defect, middle cerebral artery aneurysm, partial anomalous pulmonary venous return, superior vena cava stent, necrosis to extremities, and chemotherapy prophylaxis.

Table 3.2. Association between therapeutic warfarin dose and patient characteristics

Variable	No. (%) or mean ± SD	*p*-value[a]
Age (y)	7.6 ± 5.5	**<0.001**
Height (cm)	116.8 ± 32.5	**<0.001**
Weight (kg)	28.8 ± 22.1	**<0.001**
Body surface area (m2)	0.94 ± 0.5	**<0.001**
Body mass index (kg/m2)	18.1 ± 4.5	**<0.001**
Gender		0.233
Male	42 (54.5)	
Female	35 (45.5)	
Ancestry		0.689
European	50 (64.9)	
Asian	14 (18.2)	
Mixed European	10 (13.0)	
Other	3 (3.9)	
Target INR		0.464
Less than 2.5	31 (40.3)	
2.0-3.0	26 (33.8)	
Greater than 2.5	20 (26.0)	
Indication		**<0.001**
Fontan procedure	34 (44.2)	
Mechanical heart valve	21 (27.3)	
DVT/PE	9 (11.7)	
Other	13 (16.9)	
Use of potentiating drug(s)		0.805
Yes	7 (9.1)	
No	70 (90.9)	
Use of attenuating drug(s)		0.768
Yes	8 (10.4)	
No	69 (89.6)	

[a] *p*-values determined using square root of mean daily dose.

Table 3.3. Genotype frequencies and association between genotypes and therapeutic warfarin dose

Genotype	No. (%) of children	Mean daily dose (SD), mg	*p*-value[a]
VKORC1			**0.0005**
GG	31 (40.3)	4.1 (2.23)	
AG	32 (41.5)	3.3 (1.55)	
AA	14 (18.2)	2.0 (1.44)	
CYP2C9			
**1/*1*	54 (70.1)	3.7 (2.07)	
**1/*2*	12 (15.6)	3.2 (1.57)	0.507[b]
**1/*3*	10 (13.0)	2.2 (1.49)	**0.013**[b]
**2/*2*	1 (1.3)	3.25	
CYP4F2			0.656
CC	36 (46.8)	3.6 (2.12)	
CT	36 (46.8)	3.2 (1.90)	
TT	5 (6.4)	3.7 (1.32)	

[a]*p*-values determined using square root of mean daily dose.
[b]Dose requirements compared to *CYP2C9*1/*1* patients.

Table 3.4. Contribution of *VKORC1* and *CYP2C9* genotypes and clinical factors to multivariate regression model for predicting therapeutic warfarin dose in children

x variable	*p*-value	Contribution to model, %
Weight	<0.001	52.8
VKORC1 (-1639G>A)	<0.001	12.2
*CYP2C9*3*	<0.001	7.9
Indication	0.015	2.4
*CYP2C9*2*	0.078	1.0
TOTAL	<0.001	**76.3**

Regression equation: Square root of daily dose (mg) = 1.711 + 0.014 (weight, kg) -0.257 (number of *VKORC1* variant alleles) – 0.127 (number of *CYP2C9*2* alleles) – 0.463 (number of *CYP2C9*3* alleles) – 0.161 (Indication). Indication: input 1 for Fontan procedure, 0 for other indication.

Table 3.5. Regression equation for modeling warfarin dose requirements in children <6 years old

x variable	*p*-value	Contribution to model, %
VKORC1	<0.001	44.4
*CYP2C9*3*	<0.001	9.0
*CYP2C9*2*	0.142	1.8
TOTAL	<0.001	**55.2**

Table 3.6. Regression equation for modeling warfarin dose requirements in children ≥6 years old

x variable	*p*-value	Contribution to model, %
Weight	<0.001	48.0
VKORC1	<0.001	11.1
*CYP2C9*3*	<0.001	7.3
Target INR <2.5	0.032	4.7
*CYP2C9*2*	0.142	2.1
TOTAL	<0.001	**73.2**

Table 3.7. Median number of days to achieve first INR in or above the therapeutic range and first INR greater than 4 according to *VKORC1* and *CYP2C9* genotypes[a]

Variant	Median time (days) to first INR in or above therapeutic range	Median time (days) to first INR >4
VKORC1		
GG	8	2.5
AG	4	7.5
AA	3	4.5
CYP2C9		
**1/*1*	5	10
**1/*x*[b]	2	3
**2/*2*	2.5	15.5

[a]Medians are reported for patients who reached the specified outcomes only.
[b]**1/*x*, *CYP2C9*2* or *CYP2C9*3* heterozygotes.

Table 3.8. Hazard ratios (HR) for time to achieve first INR in or above the therapeutic range and first INR greater than 4 according to *VKORC1* and *CYP2C9* genotype[a]

Outcome	n	Unadjusted HR (95% CI)	*p*-value	Adjusted HR (95% CI)[b]	*p*-value
Time to first INR in or above therapeutic range:					
VKORC1					
AG	36	1.32 (0.82-2.11)	0.252	1.27 (0.79-2.04)	0.326
AA	16	2.22 (1.14-4.31)	**0.019**	2.21 (1.13-4.31)	**0.020**
A/_	42	1.47 (0.95-2.28)	0.085	1.44 (0.92-2.23)	0.108
CYP2C9					
**1/*x*	26	1.33 (0.81-2.17)	0.255	1.43 (0.87-2.35)	0.155
**2/*2*	2	3.53 (0.82-15.15)	0.090	3.26 (0.75-14.15)	0.114
Any variant	28	1.39 (0.86-2.24)	0.175	1.49 (0.92-2.41)	0.105
Time to first INR >4:					
VKORC1					
AG	36	1.99 (0.92-4.32)	0.081	1.70 (0.78-3.74)	0.185
AA	16	2.92 (1.21-7.07)	**0.018**	2.70 (1.11-6.59)	**0.029**
A/_	42	2.29 (1.11-4.72)	**0.025**	2.06 (0.99-4.28)	0.054
CYP2C9					
**1/*x*	26	1.78 (0.90-3.52)	0.099	2.57 (1.22-5.42)	**0.014**
**2/*2*	2	3.88 (0.90-16.65)	0.068	2.40 (0.53-10.81)	0.255
Any variant	28	1.92 (1.00-3.70)	0.050	2.40 (1.22-4.73)	**0.011**

[a]Hazard ratios are for *VKORC1* variant allele carriers as compared with *VKORC1* GG genotype, *CYP2C9* variant allele carriers as compared with **1/*1* genotype (A/_, AG or AA carriers; **1/*x*, *CYP2C9*2* or *CYP2C9*3* heterozygotes).
[b]Adjusted for target INR range (target INR >2.5).

Table 3.9. Odds ratios (OR) for risk of bleeding according to genotype[a]

Genotype	Cases (n=37)	Controls (n=56)	Unadjusted OR (95% CI)	*p*-value	Adjusted OR (95% CI)[b]	*p*-value
VKORC1						
AG	17	20	1.36 (0.50-3.74)	0.643	1.06 (0.40-2.80)	0.906
AA	5	12	0.67 (0.15-2.59)	0.671	0.68 (0.20-2.35)	0.539
A/_	22	32	1.10 (0.44-2.80)	1	0.92 (0.38-2.22)	0.847
CYP2C9						
**1/*2*	4	10	0.64 (0.13-2.54)	0.556	0.76 (0.21-2.81)	0.679
**1/*3*	7	5	2.22 (0.54-9.90)	0.220	2.81 (0.75-10.52)	0.125
**2/*2*	1	1	1.59 (0.02-128.55)	1	1.90 (0.10-35.57)	0.666
Any variant	12	16	1.20 (0.44-3.23)	0.818	1.41 (0.55-3.60)	0.478
CYP4F2						
CT	21	24	1.74 (0.67-4.58)	0.275	1.68 (0.68-4.17)	0.261
TT	2	4	1.00 (0.08-8.00)	1	0.82 (0.12-5.63)	0.837
T/_	23	28	1.63 (0.65-4.20)	0.291	1.56 (0.65-3.76)	0.323

[a]Odds ratios are for *VKORC1* variant allele carriers as compared with *VKORC1* GG genotype, *CYP2C9* variant allele carriers as compared with **1/*1* genotype, and *CYP4F2* variant allele carriers as compared with *CYP4F2* CC genotype (A/_, AG or AA carriers; T/_, CT or TT carriers).
[b]Adjusted for length of time on therapy captured.

Table 3.10. Odds ratios (OR) for risk of major bleeding according to genotype[a]

Genotype	Cases (n=7)	Controls (n=86)	Unadjusted OR (95% CI)	*p*-value	Adjusted OR (95% CI)[b]	*p*-value
VKORC1						
AG	5	32	5.82 (0.61-287.62)	0.103	3.56 (0.35-35.87)	0.283
AA	1	16	2.33 (0.03-190.90)	0.519	2.37 (0.14-40.33)	0.552
A/_	6	48	4.75 (0.54-41.16)	0.157	3.43 (0.37-31.77)	0.278
CYP2C9						
**1/*2*	0	14	0	1	0	1
**1/*3*	3	9	4.93 (0.62-34.81)	0.072	10.21 (1.27-82.01)	**0.029**
**2/*2*	0	2	0	1	0	1
Any variant	3	25	1.83 (0.38-8.78)	0.450	3.12 (0.52-19.21)	0.210
CYP4F2						
CT	4	41	1.26 (0.20-9.20)	1	1.20 (0.23-6.30)	0.834
TT	0	6	0	1	0	1
T/_	4	47	1.10 (0.23-5.24)	1	1.01 (0.19-5.26)	0.991

[a]Odds ratios are for *VKORC1* variant allele carriers as compared with *VKORC1* GG genotype, *CYP2C9* variant allele carriers as compared with **1/*1* genotype, and *CYP4F2* variant allele carriers as compared with *CYP4F2* CC genotype (A/_, AG or AA carriers; T/_, CT or TT carriers).
[b]Adjusted for length of time on therapy captured.

Table 3.11. Odds ratios (OR) for risk of over-anticoagulation (INR >4) during the initiation of therapy according to genotype[a]

Genotype	Cases (n=39)	Controls (n=44)	Unadjusted OR (95% CI)	*p*-value	Adjusted OR (95% CI)[b]	*p*-value
VKORC1						
AG	18	15	2.69 (0.92-8.24)	0.053	2.71 (0.94-7.82)	0.065
AA	10	4	5.47 (1.24-29.40)	**0.012**	6.95 (1.67-28.98)	**0.008**
A/_	28	19	3.30 (1.22-9.35)	**0.014**	3.63 (1.36-9.74)	**0.010**
CYP2C9						
**1/*2*	6	6	1.41 (0.33-5.99)	0.750	2.14 (0.59-7.84)	0.250
**1/*3*	7	4	2.45 (0.55-12.72)	0.202	2.65 (0.64-10.92)	0.177
**2/*2*	2	0	1.08 (0.97-1.21)	0.184	Inf.	0
Any variant	15	10	2.43 (0.82-5.53)	0.152	2.69 (0.97-7.47)	0.057
CYP4F2						
CT	19	23	0.97 (0.37-2.59)	1	0.78 (0.31-2.01)	0.612
TT	3	1	3.43 (0.25-194.14)	0.343	2.62 (0.23-30.26)	0.441
T/_	22	24	1.08 (0.42-2.81)	1	0.84 (0.33-2.12)	0.704

[a]Odds ratios are for *VKORC1* variant allele carriers as compared with *VKORC1* GG genotype, *CYP2C9* variant allele carriers as compared with **1/*1* genotype, and *CYP4F2* variant allele carriers as compared with *CYP4F2* CC genotype (A/_, AG or AA carriers; T/_, CT or TT carriers).
[b]Adjusted for target INR range (target INR >2.5).

Table 3.12. SNPs from full genotyping panel with *p*-value <0.10 in univariate dose analysis

Gene	SNP	Unadjusted *p*-value	R^2
VKORC1	rs9923231	0.00017	0.173
VKORC1	rs9934438	0.00017	0.173
CYP2C9	rs1057910 (*3)	0.014	0.078
PROC	rs6710535	0.016	0.075
VKORC1	rs7294	0.022	0.068
CALU	rs7776641	0.040	0.055
PROC	rs878461	0.054	0.048
CYP2C9	rs7089580	0.056	0.048
POR	rs72553971	0.062	0.046
GGCX	rs11676382	0.066	0.044
APOE	rs405697	0.075	0.042
CALU	rs2060717	0.094	0.039
PROC	rs2069901	0.094	0.037

Table 3.13. Contribution of *CYP2C9* rs7089580 genotype to therapeutic warfarin dose in children

x variable	*p*-value	Contribution to model, %
Weight	<0.001	52.0
VKORC1 (-1639G>A)	<0.001	12.5
*CYP2C9*3*	<0.001	7.8
Fontan	0.010	2.6
CYP2C9 rs7089580	0.020	2.5
*CYP2C9*2*	0.274	0.4
TOTAL	<0.001	**77.8**

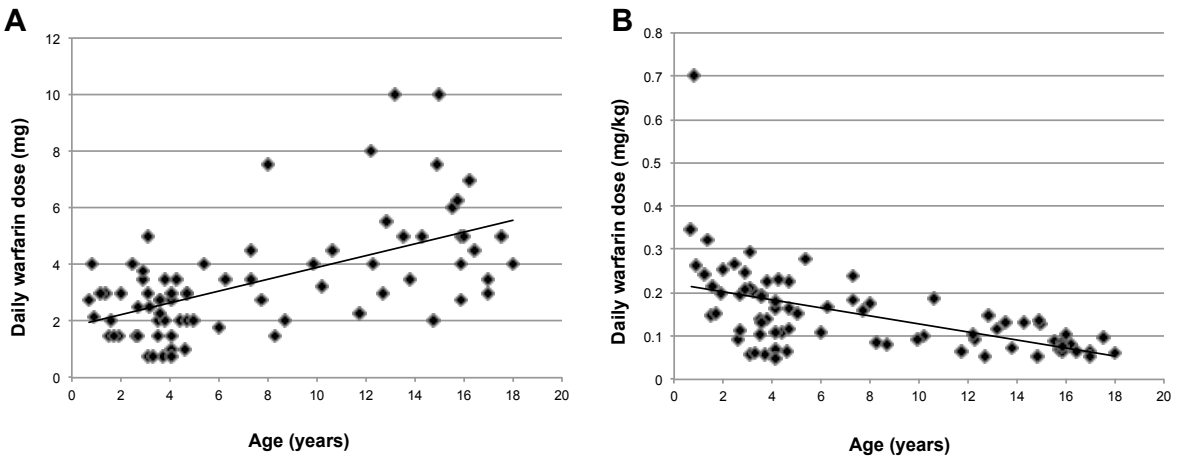

Figure 3.1. Correlation between age and warfarin dose in milligrams (A) and milligrams per kilogram of body weight (B)

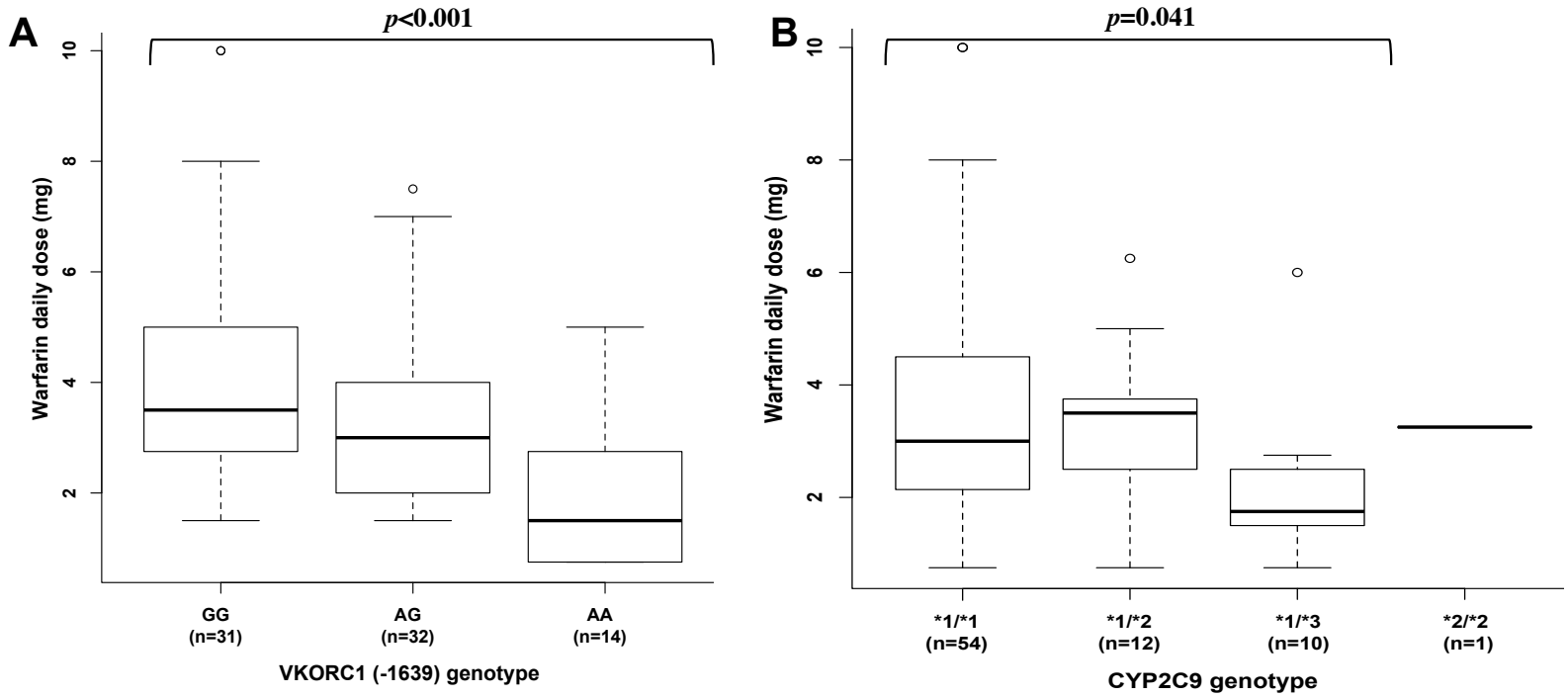

Figure 3.2. Warfarin dose requirement by *VKORC1* (A) and *CYP2C9* (B) genotype. Boxes display median and interquartile ranges (IQR). Whiskers indicate data points within 1.5 times the IQR. Outliers and extreme outliers are represented by circles and asterisks.

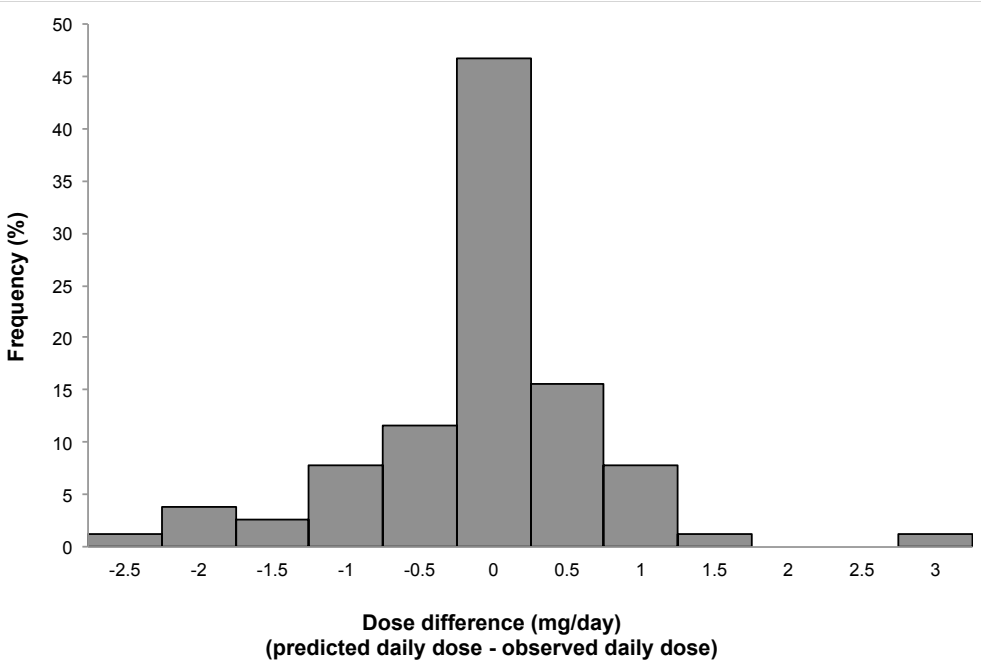

Figure 3.3. Difference between observed daily dose and predicted dose using our genotype-based dosing model

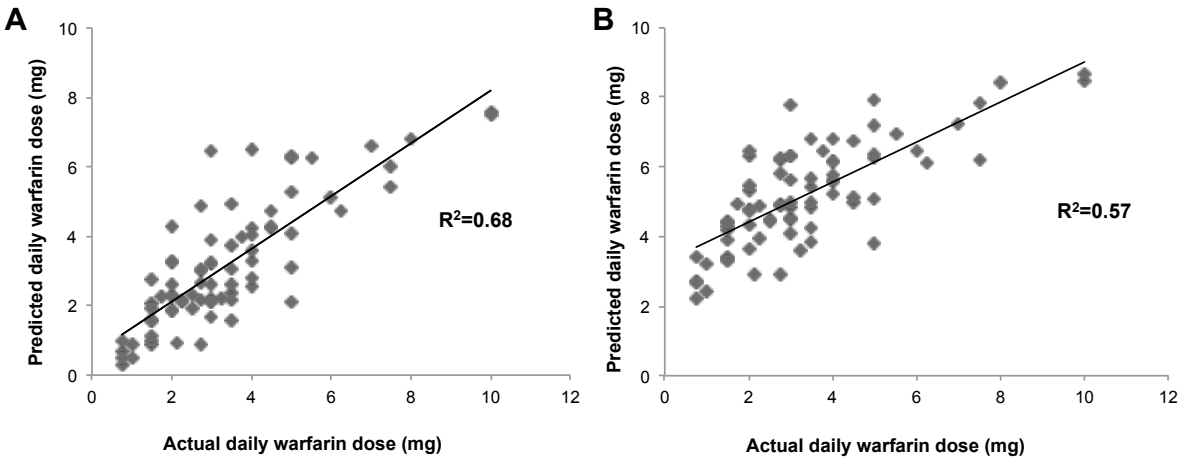

Figure 3.4. Relationship between actual daily warfarin dose (mg) and predicted dose (mg) using a pediatric derived pharmacogenetic dosing model developed by Biss et al. (A) and an adult-derived dosing model developed by the IWPC (B)

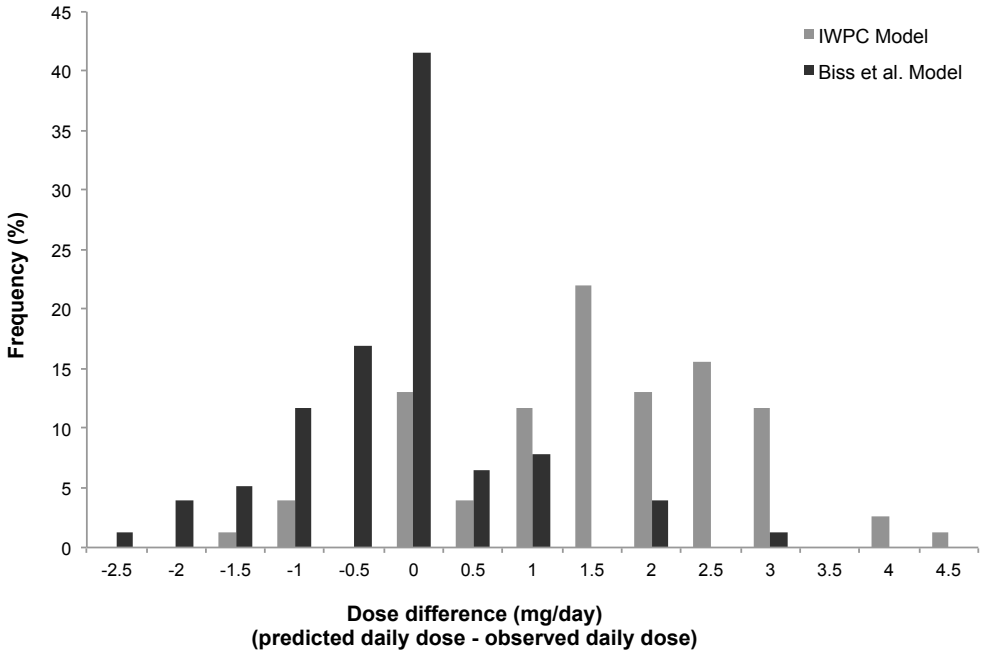

Figure 3.5. Comparison of difference between observed daily dose and predicted dose using pharmacogenetic dose prediction models developed by Biss et al. and the IWPC

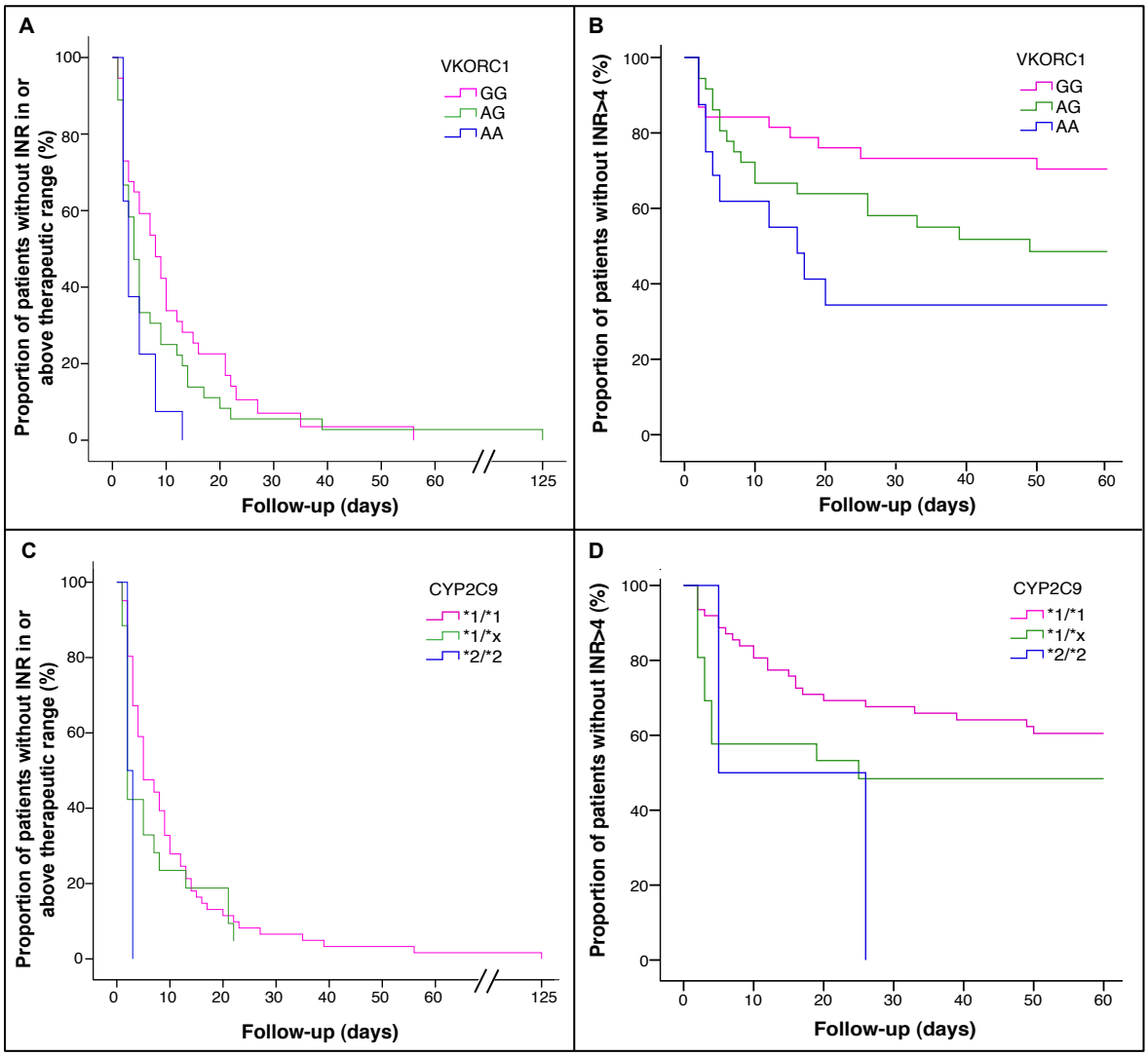

Figure 3.6. Kaplain-Meier curves showing time to study outcomes for *VKORC1* and *CYP2C9* genotypes. *VKORC1* significantly influenced time to first INR in or above the therapeutic range (panel A, p=0.047) and time to first INR >4 (panel B, p=0.024). CYP2C9 was not significantly associated with time to first INR in or above the therapeutic range (panel C, p=0.152) or time to first INR >4 (panel *D*, p=0.071) (1/*x, *CYP2C9*2* or *CYP2C9*3* heterozygotes; and *x/*x, *CYP2C9*2* homozygotes).

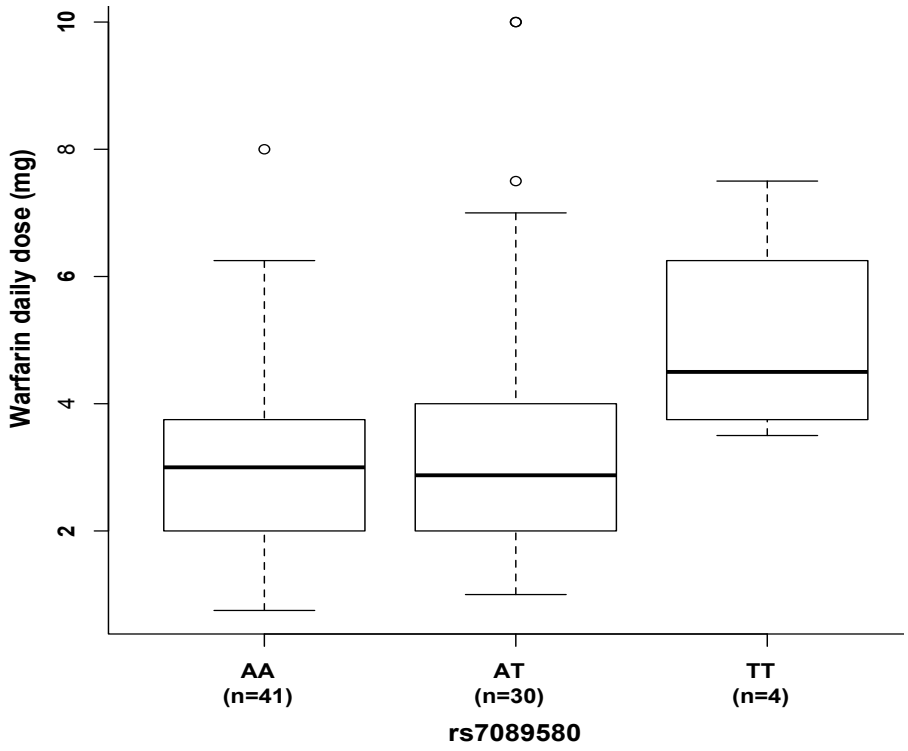

Figure 3.7. Warfarin dose requirement by rs7089580 genotype. Boxes display median and interquartile ranges (IQR). Whiskers indicate data points within 1.5 times the IQR. Outliers are represented by circles.

Chapter 4: Discussion and future directions

Warfarin remains the drug of choice for children requiring anticoagulation therapy. However, its large inter-patient dose variability and high risk of ADRs limits its use in clinical practice. With one of the largest pediatric cohorts studied to date, we have shown that a large proportion of dose variability can be explained by both clinical and genetic factors. Furthermore, we have provided evidence that the same genetic variants known to influence risk of warfarin-induced ADRs and early-phase warfarin response in adults are also relevant in children. Finally, this is the first study to assess the contribution of a larger panel of genetic variants to warfarin dose requirements in children.

4.1 Predictors of warfarin dose in children

Pharmacogenetic-guided dosing in adults receiving warfarin therapy has been shown to aid in achieving safe and effective anticoagulation. This study provides further evidence that genetic factors significantly contribute to warfarin dose variability in children. When combining genetic and clinical factors, 76.3% of inter-patient dose variability was explained by *VKORC1* and *CYP2C9* genotypes, weight, and indication for warfarin.

4.1.1 Clinical variables

The large contribution of body weight to the dose model (52.8%) is in keeping with previous pediatric studies, which found that age-dependent variables contributed to the largest proportion of dose variability[88,100,192,201]. Biss et al. and Moreau et al. found that height was the primary determinant of dose variability in a multivariate model, with both age and weight

also being highly correlated with dose in univariate analyses[100,201]. The inclusion of weight in our model rather than height may be related to the younger median age of our patient cohort (4.7 years). Young children require a higher dose (mg) per kilogram of body weight compared to older children[72,201] and may explain the more marked contribution of weight to dose variability in a young population.

The only age-independent clinical variable that was a significant predictor of warfarin dose was indication for warfarin, specifically those with a Fontan procedure (2.4%). This is in agreement with Biss et al. who also reported that children who were anticoagulated following a Fontan procedure required significantly lower doses than children receiving warfarin for other indications[201]. The lower target INR range (2.0-2.5) and smaller body mass of most children receiving warfarin for a Fontan procedure may contribute to the decreased dose requirement in these patients. However, target INR was not significantly associated with dose and indication remained significant in the model after adjusting for weight, suggesting that underlying physiological differences, such as altered liver function and impaired warfarin metabolism, may also contribute to differences in dose requirements in patients undergoing Fontan procedures[72,201].

4.1.2 Candidate SNPs

VKORC1 was the most significant genetic predictor of dose, contributing to 12.2% of dose variability. This value is slightly less than what has been reported in most adult studies (15-35%) as well as some previous pediatric studies (18-47%)[100,112,133,192]. In univariate analysis, *VKORC1* explained approximately 17% of dose variability. Therefore, the large effect of

body weight on dose variability (52.8%) in our cohort likely decreased the influence of *VKORC1* genotype. Our study further confirms a gene-dose relationship, with homozygous carriers of the *VKORC1* variant allele (AA) requiring lower doses than heterozygous and homozygous GG patients.

Adult studies have shown that *CYP2C9* accounts for approximately 5-22% of total inter-patient dose variability[102,196]. Our findings in children are very similar, with *CYP2C9* accounting for 8.9% of dose variability. This was primarily due to the **3* allele, which explained 7.9% of the total variability and is in agreement with an increased warfarin sensitivity reported for the **3* variant in adult studies[99]. Due to the difference in sensitivity between the *CYP2C9*2* and **3* variant genotypes, both genotypes were entered as separate variables into the model in order to avoid diluting the stronger effect of the **3* allele on dose. Four previous pediatric studies did not find a significant difference in dose requirements across *CYP2C9* genotypes[88,100,192,193]. In the majority of these studies, children carrying the **1/*2* or **1/*3* heterozygous genotype were grouped together for analysis. In contrast, our findings are in line with those of Biss et al., who also reported a significant difference in dose requirements between *CYP2C9 *1/*1* and **1/*3* carriers[201]. In their model, *CYP2C9* accounted for 12.8% of dose variability, while other pediatric studies reported only 2-5%[100,192]. Our study thus provides further evidence that *CYP2C9* is a significant predictor of dose in children and that the amount of variability explained by *CYP2C9* is higher than previously reported in other pediatric studies.

Similar to our own findings, two other studies in children also did not find an association between *CYP4F2* genotype and dose[100,201]. Given the relatively minor contribution of *CYP4F2* genotype to dose in adults, these negative associations are likely due to lack of statistical power given the small sample size of the current study as well as previously published studies. Based on an R^2 of approximately 2% for the *CYP4F2* genotype in adult warfarin dosing studies, and the observed allele frequencies in the current study cohort, a sample size of approximately 390 children was required to adequately detect an association between *CYP4F2* and dose.

4.1.3 Predictors of warfarin dose in subpopulations of patients

To determine whether the predictive dosing model differed between children of varying age, multiple linear regression using children <6 and ≥6 years of age was performed. In children less than 6 years of age, the amount of variability explained by genetics increased substantially. This was mainly attributed to *VKORC1* genotype, which increased in R^2 value in the original model from 12.2% to 44.4%. Unlike the previous model, no clinical factors were significantly associated with dose after adjusting for genetics. In contrast, the overall contribution of genetics to dose variability in children ≥6 years of age remained approximately the same, explaining 20.1% of dose variability overall, compared to 21.1% in the original model. The increased effect of *VKORC1* on dose variability in young children is likely attributed to the decreased heterogeneity of the patient population, as there is less variability in relevant clinical factors, such as body weight and height, in young compared to older children. By decreasing the influence of age-dependent clinical factors, the influence of genetic factors on dose variability thus becomes more apparent.

The findings in children with an indication for a Fontan procedure further supports this hypothesis. When analyzing this subset of patients the influence of *VKORC1* on dose variability increased even further, explaining 47.7% of the observed variability. Once again, this could be attributed to younger age and decreased patient heterogeneity, making age-dependent clinical factors less important for dose variability and significantly increasing the effect of genotype on dose requirements. Furthermore, the significant influence of warfarin indication on dose variability is eliminated, allowing *VKORC1* to explain an even greater percentage of dose variability. Since the majority of children receiving warfarin for a Fontan procedure were less than six years of age, we were are unable to determine whether the increased effect of genetics on dose variability is specific to this indication, or to this age group. Differences in enzyme activity and gene expression, as well as additional developmental pathways, could also contribute to the increased influence of genetics on dose variability in young compared to older children. Further study of specific patient populations and patients of varying ages using a larger cohort would further delineate the contribution of *VKORC1* and *CYP2C9* genotypes to warfarin dosing in children.

4.1.4 Pharmacogenetic predictive dosing models

This study validated the performance of a pediatric-specific pharmacogenetic warfarin-dosing algorithm developed by Biss et al., which in our cohort resulted in an R^2 of 0.68[201]. This algorithm also performed very similarly in a separate validation study using an independent pediatric cohort (R^2=0.69)[211]. Furthermore, a recent study that compared the performance of three genotype-based pediatric dosing models found that the model developed by Biss et al. predicted the ideal dose in the largest percentage of patients, thus

strengthening the evidence for the validity of this dosing model[212]. When the performance of the Biss et al. dosing model and the IWPC adult-derived model were compared, the pediatric model was significantly more accurate when predicting the required dose in children. Similar to findings from Biss et al., the IWPC model consistently over-estimated the required dose in pediatric patients[201]. Taken together, these findings strongly support the prospective evaluation of a pediatric-specific dosing model in order to validate a dose-prediction method developed specifically for children.

The model derived from our cohort was very similar to the Biss et al. model, with the exception of weight being the most significant clinical predictor instead of height. Therefore, this study provides further evidence that age-dependent variables, indication for warfarin, and *VKORC1* and *CYP2C9* genotypes are the most important predictors of warfarin dose in children. Further studies are required to evaluate the performance of our own dosing model in a validation cohort and to compare its performance to previously developed pediatric models.

4.2 Time to INR events in children

By investigating time to therapeutic INR and time to INR >4, this study was able to further elucidate the influence of genotype on early response to warfarin in children. One previous pediatric study reported that *CYP2C9* heterozygous patients required significantly less time to reach a therapeutic INR compared to **1/*1* patients[193]. However, all children in that study had a target INR of 1.3-1.9, making the results less applicable to the general pediatric population receiving warfarin. In contrast, Nowak-Göttl et al. reported no difference in time

to therapeutic INR, defined as three consecutive therapeutic daily INRs, between *VKORC1* and *CYP2C9* genotype groups in children[88]. Furthermore, a recent study in children reported no association between genotype and time to first therapeutic or supra-therapeutic INR[213]. All studies did not use survival analyses when investigating INR outcomes. These findings are in contrast to most adult studies, where carriers of *VKORC1* and *CYP2C9* polymorphisms have been shown to reach INR outcomes earlier[108,122,123,214,215]. In our cohort, *VKORC1* significantly influenced time to the first INR in or above the therapeutic range as well as time to first INR >4, while *CYP2C9* was not significantly associated with either outcome. These findings are in line with a previous study in adults where only *VKORC1* and not *CYP2C9* was associated with time to therapeutic INR, suggesting that *VKORC1* is more important for warfarin response in the early phase of therapy[123]. Accordingly, when adjusting for clinical factors *VKORC1* homozygous variant allele carriers had a decreased time to therapeutic INR, while there was no significant difference between *CYP2C9* genotypes. However, both *VKORC1* homozygous variant and *CYP2C9* variant allele carriers were at significantly greater risk of over-anticoagulation (INR >4) during the first 60 days of therapy, achieving an INR >4 at a rate that was more than twice that of patients carrying low sensitivity variants. Therefore, our findings on the genetics of warfarin sensitivity are more in line with adult studies than with previous pediatric studies.

For both outcomes, the hazard ratios were highest in *VKORC1* AA carriers, whereas a non-significant trend towards decreased time to INR was observed in *VKORC1* heterozygous patients. Thus, while all *VKORC1* variant allele carriers appear to be sensitive to warfarin at the initiation of therapy the effect is greatest in patients carrying two variant alleles. These

findings are in keeping with our previously described dose analysis, where *VKORC1* AA carriers required a lower dose than both heterozygous and wildtype carriers. Therefore, the increased warfarin sensitivity that is conferred by the A variant is reflected by a lower dose requirement as well as a decreased time to INR outcomes during the initiation of therapy.

These findings have important implications for the treatment of children receiving warfarin therapy. Genotyping prior to initiation of therapy can aid in the identification of warfarin-sensitive patients earlier, allowing clinicians to make a more informed decision regarding the most appropriate starting dose. In contrast, children carrying low sensitivity variants are more likely to reach a therapeutic INR slower, potentially delaying discharge from hospital or increasing the amount of time required for bridging with other anticoagulants. Tailoring the starting dose using pharmacogenetic information for these patients may thus decrease hospitalization times during warfarin initiation. Indeed, prospective studies in adults have shown that genotype-guided dosing can decrease the number of days required to reach a therapeutic INR compared to standard dosing practices[216,217]. Similar prospective studies using genotype-based warfarin dosing are now needed in children.

4.3 Warfarin-induced ADRs in children

4.3.1 Incidence of bleeding

This is the first study to investigate the influence of warfarin-sensitivity variants on risk of bleeding in children. The reported rate of major bleeding in children ranges from 0.5-1.7% per patient year[72,85,86]. In children with mechanical heart valves the incidence is higher, estimated to be approximately 3.2% per year[86]. In our cohort, seven patients experienced a

major bleeding episode, conferring an incidence rate of approximately 1.9% per patient year. In accordance with the literature a higher rate of major bleeding in patients with mechanical heart valves was also observed (3.1% per patient year). The incidence of bleeding that is of minor clinical consequence is estimated to be substantially higher, occurring in approximately 20% of patients[72,87]. In our cohort, approximately 40% of patients experienced at least one bleeding event. The two-fold difference between incidence of bleeding in our study and the literature may be due to differences in follow-up length, as the previous studies in children were prospective and captured a shorter duration of therapy. Moreover, including patients in our study who were followed by a dedicated nurse clinician may have increased the number of bleeding events that were captured and recorded in patient health records.

4.3.2 Factors associated with risk of warfarin-induced bleeding

When analyzing risk of bleeding the clinical factors that were most strongly associated were indication for a mechanical heart valve and length of time on therapy that was captured. The higher target INR range (2.5-3.5) in patients with mechanical heart valves most likely contributed to this finding, as intensity of warfarin treatment is highly associated with risk of bleeding[58]. The influence of target INR on risk of bleeding was unable to be assessed as some patients had changes to their target INR range over the course of therapy. Patients with a longer length of time on therapy that was captured were also more likely to experience a bleeding event.

Both *VKORC1* and *CYP2C9* did not significantly influence the risk of all bleeding events. Rather, the odds ratios for all variant genotype groups did not deviate far from 1, indicating no evidence of increased risk. The highest odds ratio was observed in *CYP2C9*1/*3* carriers. This finding is similar to a previous study in adults where only the *CYP2C9*3* allele was significantly associated with increased risk of minor hemorrhage[218]. However, **2* allele was not investigated in that study. One possible explanation for the lack of significant findings in our study may be small sample size. Due to the high frequency of minor bleeding events, low odds ratios for variant genotypes would be expected. Therefore, a large sample size would be needed in order to detect a small effect. Another explanation could be the influence of confounding factors. Frequent illnesses and changes in diet, which were not controlled for in analysis, are known to influence INRs and may have contributed to risk of bleeding. Some bleeding events may have also occurred irrespective of receiving warfarin, thus diluting the influence of genotypes on risk of warfarin-induced bleeding. Finally, the close monitoring of the majority of patients by a dedicated thrombosis nurse may have prevented some variant allele carriers from experiencing a bleeding event, further diluting an association between genotypes and ADRs.

In contrast to the above findings, when adjusting for clinical variables carriers of the *CYP2C9*1/*3* genotype were at significantly greater risk of major bleeding compared to **1/*1* patients. This is in keeping with our finding that the **3* allele is significantly associated with a decreased dose requirement, suggesting an increased warfarin sensitivity in patients who carry this allele. Furthermore, this finding is in accordance with the previous bleeding analysis where **1/*3* carriers had the highest odds ratio among all variant genotype

groups. A similar trend towards increased risk of bleeding was also observed in *VKORC1* variant allele carriers, which may not have reached statistical significance due to small sample size. It has previously been reported that variant allele carriers are at greater risk of major bleeding during the initiation of therapy compared to the entire duration of therapy[125]. The majority of major bleeding events (n=4) occurred within the first six weeks of therapy. However, due to the limited number of cases we were unable to adequately assess this in our study.

It is interesting to note that the risk of bleeding was higher in *CYP4F2* variant allele carriers compared to homozygous CC carriers for both bleeding analyses. This is contradictory to what would be expected, as carriers of the variant T allele are generally less sensitive to warfarin, conferring an increased dose requirement. Thus far, only one study in adults has examined the association between bleeding events and *CYP4F2*[218]. The results of that study were similar to the current study, with variant allele carriers being at a non-significantly higher risk of bleeding. In our cohort heterozygous CT carriers had a decreased dose requirement compared to CC patients, which coincides with an increased sensitivity to warfarin and a higher risk of bleeding. Due to small sample size and non-significant findings, it is possible that this unexpected trend is due to chance.

4.3.3 Over-anticoagulation during the initiation of therapy

Risk of over-anticoagulation during the initiation of therapy was used as a surrogate marker for risk of warfarin-induced bleeding. INR >4 is often used as a clinical endpoint in prospective studies, as this represents a risk factor for potential bleeding complications[219].

By examining this endpoint in the first 60 days of therapy, we aimed to eliminate some of the confounding factors that contribute to warfarin-induced bleeding while still identifying genetic risk factors. Studies in adults have reported a significantly higher risk of supra-therapeutic INRs in *CYP2C9* and/or *VKORC1* variant allele carriers[113,124,125,220]. In our cohort, the risk of over-anticoagulation was significantly higher in patients carrying a *VKORC1* variant A allele, while the odds ratio in *CYP2C9* variant allele carriers approached significance. This is in keeping with previous reports from adult studies where the odds ratio for risk of a supra-therapeutic INR was highest for the *VKORC1* sensitivity haplotype[124,220]. Furthermore, these results coincide with our findings on time to over-anticoagulation, where the hazard ratio was highest in *VKORC1* homozygous variant allele carriers. Unlike the time to over-anticoagulation analysis, *CYP2C9* was not significantly associated with increased risk of over-anticoagulation. However, this could be attributed to differences in patient cohorts between the two analyses, as some patients were excluded from this analysis due a treatment length less than 60 days or confounding factors that are known to prolong the prothrombin time. More importantly, the odds ratios trend in the expected direction, with *CYP2C9* variant allele carriers being at greater risk of over-anticoagulation compared to **1/*1* carriers. Once again, risk of over-anticoagulation was unexpectedly higher in *CYP4F2* homozygous variant allele carriers compared to homozygous CC carriers. However, only four patients carried this genotype, suggesting that this finding may be due to chance and that a larger sample size is required to more adequately determine the effect of *CYP4F2* genotype on risk of over-anticoagulation.

As with many of the previous outcomes investigated, this is the first pediatric study to investigate risk of over-anticoagulation during the initiation of therapy. Odds ratios greater than 1 provides further evidence that *CYP2C9* and *VKORC1* variant allele carriers are more sensitive to the anticoagulant effects of warfarin, particularly during the early phase of drug initiation. With a larger patient cohort, the risk of warfarin-induced bleeding during the initiation of therapy could be investigated. However, the current analysis serves as a valuable starting point. These results also suggest that certain precautions, such as a lower starting dose, should be carefully considered when initiating warfarin therapy in high sensitivity patients based on their *CYP2C9* and/or *VKORC1* genotypes.

4.4 Novel genetic associations with warfarin dose in children

An exploratory analysis revealed one additional SNP in *CYP2C9* (rs7089580) that was a significant predictor of dose when adjusting for genetic and clinical covariates. Perera et al. first described this intronic *CYP2C9* variant in a sequencing study where it was associated with increased dose requirements in an African-American population, accounting for 1.4% of overall dose variability[106]. Our findings are very similar, with this SNP explaining 2.5% of overall dose variability. Homozygous variant allele carriers also required significantly higher doses compared to non-carriers (3.1+/-1.6 vs. 5.0+/-1.8 mg/day; p=0.033). The overall R^2 of our model increased by approximately 2% when this SNP was added, which is similar to the contribution of the functional *CYP4F2* SNP (rs2108622) to warfarin dose in adults and is included in warfarin pharmacogenetic dosing algorithms. Interestingly, the minor allele frequency of rs7089580 in our cohort was similar to the reported MAF in the African-American cohort (25% and 23%, respectively), suggesting that this variant is also

common in patients of non-African descent. To our knowledge, this is the first study to investigate the association of this SNP with warfarin dose in a non-African population. The functional impact of this noncoding variant is currently unknown. Perera et al. reported zero or little linkage between this SNP and the *CYP* star variants. When analyzing our own cohort a similar trend was observed, with pairwise r^2 values of 0.042 and 0.006 between rs7089580 and the **2* and **3* variants, respectively. In contrast, Perera et al. hypothesized that the observed association with dose may be due to linkage with a second SNP (*CYP2C9*-25706*;* r^2=0.98) that is in close proximity to a predicted transcriptional binding cluster. These findings suggest that further investigation of this SNP in the context of warfarin dosing in both adult and pediatric patients, as well as its functional impact, is warranted.

When analyzing the full genotyping panel, *VKORC1*-1639 and *CYP2C9*3* were the SNPs most strongly associated with dose. While only a limited number of SNPs were investigated, this finding nevertheless provides further evidence of the significant impact of these SNPs on warfarin dosing in children. When using the bonferroni correction to correct for multiple testing the only SNP that remained significant was *VKORC1*-1639. All remaining SNPs had a non-significant *p*-value when using the adjusted *p*-value of 0.0006 based on 79 tests. A larger sample size is required to more accurately determine whether any additional genotyped SNPs are significantly associated with dose variation in children and emphasizes the importance of replication and validation of this novel association in an independent cohort of children.

4.5 Strengths and limitations

Compared to previous pediatric studies on warfarin pharmacogenetics our study has the youngest patient cohort, with a median age of 4.8 years. This is important because it confirms that genetic and clinical factors that have been shown to influence warfarin outcomes in older children are also important in young children. With numerous developmental changes, physiological and genetic, occurring throughout childhood and up until adulthood, it is inappropriate to assume that findings from a pediatric study are relevant for children of all ages. Compared to previous pediatric studies our cohort also has the largest proportion of non-European patients, suggesting that the impact of genetics on warfarin dose and related outcomes is not specific to one ancestry.

While most studies in children have primarily focused on the influence of genetic factors on therapeutic dose, this is the first study to investigate several clinically relevant outcomes, including risk of warfarin-induced bleeding and risk of/time to over-anticoagulation. These outcomes are important for understanding how children respond to warfarin during the initiation of therapy and whether there are specific children who are at increased risk of experiencing a serious adverse drug reaction based on their genotype. This is also the first study to examine the influence of a broad range of genetic variants on required dose. In doing so, we identified an additional SNP that contributes to warfarin dose variability independent of known genetic variants and can be incorporated into a model to more accurately predict the required dose in individual children.

A key limitation of the study is the large impact of clinical variables on warfarin outcomes. While many clinical confounders were controlled for in analyses, additional factors such as diet and illness, which are known to impact INR measurements and dose requirements, were not accounted for. One previous study found that children receiving enteral nutrition, infant formula feeds, or who were vegetarian, did not require doses outside of the expected range when applying a predictive dosing model[201]. Future studies in children could be used to obtain detailed information regarding dietary intake of vitamin K-rich foods and to determine their impact on warfarin dose and additional warfarin-related outcomes. The variable frequency of INR testing as part of clinical care may have also impacted the time to INR outcomes analyses.

Another limitation is that patients who were still receiving warfarin when enrolled into the study may not be 'true' controls in the case-control analysis, as there is a potential for these patients to experience a warfarin-induced bleeding event in the future. We attempted to account for this confounder by controlling for the amount of time on therapy that was captured. Due to the nature of ADR itself, it is also difficult to determine whether bleeding events were induced by warfarin, or whether these events were likely to occur even in the absence of the drug. Close discussions with clinicians and extensive review of patient records were undertaken to limit this confounder. Prospective studies or randomized controlled trials could be used to more accurately determine the impact of genetics on warfarin-induced bleeding in children by ensuring that all relevant clinical information is collected.

Finally, due to sample size limitations this study did not have enough power to detect alleles of small effect (low odds ratio) when studying warfarin-induced ADRs. Based on the incidence of major bleeding and *CYP2C9/VKORC1* allele frequencies, the lowest odds ratio that could be detected with the current sample size was 6, which is substantially higher than reported odds ratios of approximately 2-3 from adult studies[124,125]. However, it was known *a priori* that adequate numbers of patients would not be recruited and that this would serve as a pilot study for future, larger studies. National or international collaborations would help increase the sample size, while studying only the most severe cases of warfarin-induced toxicity would increase the expected effect size. Both of these options would help to increase the statistical power. Small sample size may have also diluted the association of genetic variants, especially rare variants, with therapeutic warfarin dose when analyzing the full genotyping panel in exploratory analyses. For example, *CYP2C9*6* has been shown to impact warfarin dose in African Americans but was monomorphic in our study cohort. Again, a larger sample size would be required to detect the impact of rare variants on warfarin dosing.

4.6 Future directions

This work replicates and validates previous findings regarding the relevance of both clinical and genetic factors in the determination of optimal warfarin dosing in children. Furthermore, through exploratory analyses an additional SNP that may also be predictive of optimal warfarin dose in children was identified, as well as genetic variants that contribute to warfarin-sensitivity outcomes. The findings of the current study provide support for prospective studies in the investigation of warfarin pharmacogenetics in children.

As an immediate next step in this project, active surveillance could be conducted at CPNDS sites across Canada to recruit additional pediatric patients receiving warfarin therapy. With a larger sample size, a dose prediction model could be further refined to more accurately predict the required warfarin dose in a warfarin-naïve patient. Replication of time to INR outcomes and ADR analyses could also be evaluated using a separate Canadian cohort. Due to the low incidence of major bleeding, increasing the sample size would enable a well-powered analysis and potentially identify additional genetic variants associated with risk of warfarin-induced bleeding. Furthermore, active surveillance for extreme cases of warfarin-resistance or warfarin-sensitivity (ex. major bleeding cases) across the CPNDS network would increase the likelihood of detecting rare variants that confer high odds ratios. Replication of the association between therapeutic dose and *CYP2C9* rs7089580 could also be evaluated using a Canada-wide cohort. Thus far, this SNP has not been studied in a non-African cohort, further emphasizing the importance of replication of this SNP in both adults and children. Finally, patients could be recruited prospectively at the initiation of therapy, allowing relevant information that may impact warfarin dosing but is not captured in patient charts to be collected, including diet and Tanner stage. Description of the data by patient Tanner stage may help better characterize growth/maturation v. genetic contributions to warfarin dose variability.

4.6.1 Remaining unexplained inter-patient variability in warfarin dose

Based on results from our study, as well as previous pediatric studies, approximately 25-30% of inter-individual warfarin dose variability remains unexplained in children[100,201]. It has been hypothesized that differences in dietary vitamin K intake could largely influence

variability in warfarin dose requirements in children[221]. An inverse relationship between vitamin K intake and INR has been reported, suggesting that patients with a lower intake of vitamin K are more sensitive to the anticoagulant effects of warfarin[222]. This is supported by studies in children showing that breastfed infants, who likely consumed lower amounts of vitamin K, were more sensitive to warfarin than infants receiving vitamin K supplemented formula feed. Furthermore, children who received vitamin K supplemented enteral nutrition required higher warfarin doses[72]. Poor absorption of vitamin K due to gut congestion in cardiac patients could also influence warfarin dose requirements[223]. Establishing a quantitative relationship between vitamin K status and therapeutic dose may increase our understanding of warfarin dose variability in children. The influence of concomitant illnesses on dose has also never been investigated and may account for a portion of unexplained variability.

This is the first pediatric study to investigate the association between warfarin dose and a broad panel of SNPs. While one additional SNP in *CYP2C9* was identified as being significantly associated with dose, the number of SNPs investigated was small (79 SNPs). Multi-centre collaborations to increase sample size would likely help to identify additional SNPs associated with warfarin dose variability in children by increasing the power to investigate a larger number of SNPs in exploratory analyses. Furthermore, a larger study would enable a better understanding of the role of *CYP4F2* on warfarin dosing in children, as the current sample size may have been too small to detect a minor effect on dose variability.

With decreasing costs in DNA sequencing, future studies could also focus on whole sequencing of genes implicated in warfarin outcomes to discover additional SNPs that contribute to the current unexplained dose variation. Genome-wide association studies are designed to capture common variation in a large number of genes. Therefore, rare variants are more likely to remain undiscovered using a GWAS. Gene sequencing increases the likelihood of discovering rare variants with a functional impact, as well as SNPs that are associated with severe warfarin phenotypes, such as extreme warfarin-resistance or warfarin sensitivity.

Interestingly, a larger proportion of dose variability is explained by clinical and genetic factors in children than in adults, accounting for approximately 70-75% of variability in children compared to approximately 50-60% in adults[100,201]. This may be explained by differences in lifestyle, as certain factors that are known to influence warfarin dose, such as alcohol consumption, smoking status and compliance, are less likely to influence warfarin dose in children. As children age, the influence of these factors, as well as other changes in patient behavior, may become more relevant for warfarin dosing. Larger pediatric studies are required to discern the contribution of lifestyle habits to dose requirements in children of varying ages.

4.6.2 Genetics-based loading and initiation doses

As previously described, dosing algorithms that calculate both genetics-based loading doses and maintenance doses have been developed for adult patients[144,145]. The risk of bleeding or recurrent thromboembolism is highest during the initiation phase of anticoagulation[224].

Therefore, a genetics-based loading dose algorithm is beneficial for identifying patients who are at risk for over- or under-anticoagulation if administered a standard loading dose. Genotype-based dosing is most likely beneficial at the beginning of therapy when dose titration using INRs cannot be used[214]. Results from randomized clinical trials in adults support the use of genetics-based loading doses as they have been shown to decrease the amount of time required to reach a therapeutic INR and increase the amount of time spent in the therapeutic range[134,225]. To date, no pediatric study has investigated the impact of genetic variants on required dose during the initiation phase of therapy. Rather, all studies have focused on identifying genetic and clinical variables that are predictive of maintenance dose. In this study genetic variants in *CYP2C9* and *VKORC1* were found to significantly influence the early warfarin response in children, with carriers of variant alleles reaching INR outcomes at a faster rate than wildtype carriers. The development and evaluation of a genotype-based loading dose algorithm that builds upon current pediatric dosing algorithms, such as the one presented in this study, and also accounts for early warfarin response in children is warranted.

4.6.3 Replication and validation

Replication of genetic results in an independent cohort of patients is essential for determining the true influence of genetic variants on clinical outcomes and to reduce the number of false positive findings. One study reported that only 6 of 166 published associations between gene variants and common diseases that were studied a minimum of three times consistently replicated[226]. Common causes of lack of replication include small sample sizes, population stratification, and publication bias[227]. Furthermore, inconsistencies in phenotyping limit the

ability to compare results across studies. This study replicated the findings of previous pediatric studies that reported an association between *CYP2C9/VKORC1* variants and therapeutic dose in children. This study also further validated the performance of a pediatric genotype-based dosing algorithm when predicting required dose in an independent cohort of children. This type of validation is crucial for clinical implementation and provides confidence that the dosing model is applicable to a variety of patients. Using the current pediatric cohort a genetics-based dosing model was developed, which requires validation in an independent cohort before the utility of the model can be fully understood. Differences in age, ancestry, and underlying medical conditions between derivation and replication cohorts may influence the accuracy of a dose prediction model. As previously described, replication of time to INR outcomes and ADR analyses, as well as the association between therapeutic dose and *CYP2C9* rs7089580, should also be evaluated in independent cohorts.

In addition to replication, findings of this study can be validated using functional methods. Pharmacokinetic studies describing the relationship between clearance rates of warfarin enantiomers and *CYP2C9* genotypes have been performed in both adults and children and have helped to confirm the influence of *CYP2C9* variants on therapeutic dose[64]. To date, however, no functional studies have examined the expression of *VKORC1* in pediatric livers. Little is currently known about the ontogeny of *VKORC1*. Examining the influence of *VKORC1* SNPs on gene expression and enzyme activity in children of various ages using functional methods would provide a better understanding of age-dependent changes in warfarin response. When an adult-derived pharmacokinetic/pharmacodynamic (PK/PD) dose prediction model was bridged to children using allometric scaling, the required dose

was consistently over-predicted in children less than 2 years of age[212]. Examining changes in warfarin pharmacodynamics between children of varying age would be beneficial for determining the most appropriate methods for predicting warfarin dose in young children. Changes in *CYP2C9* gene expression and enzyme activity conferred by rs7089580 variant could also be explored in both adults and pediatric liver cells as a potential next step towards confirming the importance of this SNP for warfarin dosing. Since this SNP is hypothesized to be in linkage disequilibrium with a putative transcriptional binding cluster SNP (25706)[106], functional studies could also be used to determine the impact of SNP 25706 on CYP2C9 mRNA and protein levels.

4.6.4 Implementing genetic testing

Despite a large body of evidence showing reproducible pharmacogenetic associations, several factors have hindered the use of genetic testing in clinical practice. Some of these barriers include high turnaround times, lack of reimbursement, unclear understanding of the evidence by clinicians, or insufficient evidence showing a therapeutic advantage[228]. Randomized controlled trials (RCTs) have historically been the gold standard for evaluating the effectiveness of any novel treatment or intervention[229]. Results from RCTs in adults receiving warfarin have shown that compared to standard dosing, genotype-guided dosing increases the amount of time spent within the therapeutic range, decreases the number of sub- or supra-therapeutic INRs, and reduces rates of hospitalization[225,230,231]. However, the majority of clinical practice guidelines do not recommend genetic testing in warfarin management for all patient populations[47,232,233]. This is mainly due to a lack of evidence showing a difference in number of thrombotic events, major bleeding events, or survival

between genotype-guided and standard dosing groups. Three RCTs are currently underway to further investigate the impact of genotype-guided dosing on clinical outcomes in adults (www.clinicaltrials.gov; NCT01006733, NCT00839657, and NCT01119300). Results from these trials are expected to largely influence future recommendations on genetic testing.

To date, no prospective studies or randomized controlled trials have investigated the effectiveness of genetics-based dosing in children. A recent retrospective study in children found that a fixed dose of 0.2 mg/kg per day, as recommended for initiation of therapy, over-estimated the required dose much more frequently than genetics-based dosing models[212]. Furthermore, a handful of studies in children, including our own, have provided evidence that clinical and genetic factors explain a large percentage of inter-patient dose variability. Prospective randomized controlled trials in children are also required to comprehensively assess the clinical utility of a genetics-based dosing model for reducing the number of adverse events. However, the small number of pediatric patients receiving warfarin, in combination with the relatively low incidence of serious adverse events, would make a well-powered study difficult to conduct. Multi-centre collaborations could be used to increase the sample size of such a study. Furthermore, a surrogate marker, such as time spent within the therapeutic INR range, could be used to evaluate the clinical utility of genetics-based dosing in children without significantly increasing sample size requirements. By validating a pediatric genotype-based dosing model using retrospective data we have provided a necessary step towards prospective evaluation of a predictive dosing model.

It can also be argued that results from RCTs in adults could be extrapolated to children. In this study we have shown that the influence of *VKORC1* and *CYP2C9* genotypes on therapeutic dose and early warfarin response in children is similar to adults. Therefore, any evidence of a clinical benefit from genetic testing in adults could also be assumed to be true in children. In doing so, we could provide this population with the same pharmacogenetic advantages as adults without the delay of an RCT. Furthermore, some of the barriers that hinder genetic testing in adults, such as long turn around times, are not as relevant in children as the majority of children are initiated on warfarin as hospital in-patients following pre-scheduled surgeries. Therefore, genetic test results could be available prior to the initiation of therapy when genetic testing is presumed to be most beneficial, thus increasing the feasibility of genetic testing in children in clinical practice.

4.6.4.1 Pharmacoeconomic considerations

Another substantial barrier to implementing genetic testing is inconsistent results regarding the cost effectiveness of a genetic test. One study by the U.S. Food and Drug Administration estimated that testing for *CYP2C9* and *VKORC1* variants could result in over a billion dollars saved annually by avoiding approximately 85,000 serious bleeding events and 17,000 strokes[29]. On the other hand, some studies have concluded that genotype-guided warfarin dosing is unlikely to be cost-effective as it provides only minor clinical improvement[234,235]. A major limitation of these studies is the uncertainty regarding number of adverse events avoided when implementing genetic testing. Most clinical trials use surrogate outcomes, such as time spent within the therapeutic INR range, as a substitute for ADRs[236]. Thus far, no clinical trial has been sufficiently powered to detect a difference in hemorrhagic or TE

events when comparing genotype-guided to standard dosing regimens[235]. A review on this topic concluded that genotyping could be cost effective for specific patient populations, such as those who are at high risk of bleeding, or who attend clinics where anticoagulation management is suboptimal[237]. Changes in the cost of genotyping had the largest impact on cost-effectiveness simulation results. With current trends in decreasing genotyping costs, it is likely that future pharmacoeconomic assessments will produce different conclusions.

The lack of prospective clinical trials in children further limits an evidence-based cost-effectiveness analysis of *CYP2C9/VKORC1* genetic testing in pediatric patients. In this study we have provided evidence that *CYP2C9* and *VKORC1* genotypes significantly influence the risk of over-anticoagulation and major bleeding in children. Randomized controlled trials in children would help discern the cost-effectiveness of genetic testing in pediatric patients. Due to the limited number of pediatric patients receiving warfarin and the increased difficulties of managing children on anticoagulation therapy, a cost-effectiveness analysis may produce results that are more favorable of genetic testing in children compared to adults. A prospective trial would also help to determine whether genetic testing reduces the number of days required for post-operative hospitalization due to sub- or supra-therapeutic INRs and the coinciding economic impact.

4.7 Conclusions

As newer anticoagulants, including direct thrombin and factor Xa inhibitors, become available it is possible that the use of warfarin in pediatrics may decrease. Nevertheless, the paucity of information regarding long-term safety and effectiveness of these novel therapies,

as well as unexpected drug-drug and drug-gene interactions, warrants careful consideration before switching patients from warfarin. Warfarin has been the subject of extensive post-market surveillance since its market approval in 1954. This is an especially important consideration in pediatrics where the majority of newer anticoagulants have not yet been tested. As such, the work presented in this thesis is highly relevant for the current treatment of children requiring anticoagulation therapy and provides novel and significant results that can directly impact patient care.

The evidence provided here demonstrates that genetic factors are a significant contributor to warfarin-related outcomes in children. This study replicates findings from previous pediatric studies showing that *VKORC1* and *CYP2C9* genotypes are significant predictors of warfarin dose in children and further validated a pediatric dosing algorithm, which can be used in clinical practice to more accurately predict the required warfarin dose prior to initiation of therapy. Furthermore, substantial evidence for an increased sensitivity to warfarin in *VKORC1* and *CYP2C9* variant allele carriers was observed, reflected by a decreased time to INR outcomes and increased risk of over-anticoagulation and major bleeding, emphasizing the diagnostic significance of predictive genotyping. Finally, this work has identified a novel association between a noncoding SNP in *CYP2C9* and dose in a non-African population, potentially increasing the accuracy of a dose prediction model. Uptake of this knowledge into clinical practice will enable warfarin therapy tailored specifically for children and contribute to safer drug initiation and anticoagulation treatment in this complex and vulnerable patient population.

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