PHARMACOGENETICS IN WARFARIN THERAPY

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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DECLARATION

This thesis is the result of my own work. The material contained within this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree of qualification.

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This research was carried out in the Department of Molecular and Clinical Pharmacology, in the Institute of Translational Medicine, at The University of Liverpool

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ABSTRACT

Warfarin is a challenging drug to dose accurately, especially during the initiation phase because of its narrow therapeutic range and large inter-individual variability. Therefore, the aim of this thesis was to investigate the use of pharmacogenetics and clinical data to improve warfarin therapy.

Genetic variants in cytochrome P450 2C9 (*CYP2C9*) and vitamin K epoxide reductase (*VKORC1*) are known to influence warfarin dose. Therefore we developed a pharmacogenetic dosing algorithm to predict warfarin stable dose prospectively in a British population based on 456 patients who started warfarin in a hospital setting and validated it in 262 retrospectively recruited patients from a primary care setting. The pharmacogenetic algorithm which included *CYP2C9*2, CYP2C9*3* and *VKORC1-1693* together with body surface area, age and concomitant amiodarone use, explained 43% of warfarin dose variability. The mean absolute error of the dose predicted by the algorithm was 1.08 mg/day (95% CI 0.95-1.20). 49.6% of patients were predicted accurately (predicted dose fell within 20% of the actual dose).

The HAS-BLED score, a bleeding risk score has recently been suggested for use in the management of patients with atrial fibrillation. We validated HAS-BLED performance in predicting major bleeding using a prospective cohort with 6 months follow-up (n=482) (c-statistic 0.80 95% CI (0.71-0.90). Factors significantly associated with major bleeding in our cohort ($p \leq 0.1$) were concurrent amiodarone use, labile INR, concurrent clopidogrel use, bleeding predisposition, concurrent aspirin use and *CYP2C9*3*. Adding a genetic covariate (*CYP2C9*3*) to the HAS-BLED score did not significantly improve its performance in predicting major bleeding. Considering *CYP2C9*3* is a rare allele, our study was underpowered and requires further investigation in a larger cohort.

A retrospective study of 97 Caucasian children was conducted to gain greater understanding of the factors that affect warfarin anticoagulant control and response in children. Results from multiple regression analysis of genetic and non-genetic factors showed that indication for treatment (Fontan or non-Fontan group), *VKORC1 -1693*, and INR group explained 20.8% of variability in proportion time in which INR measurements fell within the target range (PTTR); *CYP2C9*2* explained 6.8% of the variability in INR exceeding target range within the first week of treatment; *CYP2C9*2, VKORC1 -1693*, age and INR group explained 41.4% of warfarin dose variability and *VKORC1 -1693* explained 8.7% of haemorrhagic events. The contributions of *CYP2C9* and *VKORC1* polymorphism were small in the above outcomes. We therefore went onto explore other genetic markers using genome-wide scanning. Two SNPs on chromosome 5, rs13167496 and rs6882472 were found to be significantly associated at a genome-wide significance level with PTIR. However, none of SNPs were significantly associated with warfarin stable dose, INR values exceeding the target range within the first week of treatment and bleeding complications. Because of our small sample size, these findings will need to be validated in a replication cohort.

Finally, we have validated and evaluated the performance of Genie HyBeacon®, a point of care therapy (POCT) instrument to genotype 135 samples for *CYP2C9*2*, *CYP2C9*3* and *VKORC1 -1693*. We showed that the instrument accuracy was >98% (agreement with ABI Taqman® genotyping), it was relatively simple to use and had a good turn-around time (1.6 hours) making it suitable for clinical use.

In conclusion, the results presented in this thesis demonstrate how knowledge of pharmacogenetics may help in assessing improvement in the quality of care of patients on warfarin. However, for personalized medicine to be widely adopted in clinical practice, payers need evidence of clinical- and cost-effectiveness. How such evidence is produced and evaluated varies in different healthcare settings, which further increases the challenge of implementing personalised medicine into the clinic.

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ABBREVIATION

ABCB1	ATP binding cassette, subfamily B, member 1
AF	atrial fibrillation
bp	base pairs
CALU	calumenin
CEU	Caucasians in Utah, USA
CHB	Han Chinese in Beijing, China
CI	confidence interval
CIVC	cysteine132-isoleucine-valine-cysteine135
CPIC	Clinical Pharmacogenetics Implementation Consortium
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1
CYP1A2	cytochrome P450, family 1, subfamily A, polypeptide 2
CYP2C18	cytochrome P450, family 2, subfamily C, polypeptide 18
CYP2C19	cytochrome P450, family 2, subfamily C, polypeptide 19
CYP2C8	cytochrome P450, family 2, subfamily C, polypeptide 8
CYP2C9	cytochrome P450, family 2, subfamily C, polypeptide 9
CYP3A4	cytochrome P450, family 3, subfamily A, polypeptide 4
CYP3A5	cytochrome P450, family 3, subfamily A, polypeptide 5
CYP4F2	cytochrome P450, family 4, subfamily F, polypeptide 12
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EPHX1	epoxide hydrolase 1
Factor II/FII	coagulation factor 2
Factor IX/FIX	coagulation factor 9
Factor VII/FVII	coagulation factor 7
Factor X/FX	coagulation factor 10
FDA	Food and Drug Administration
GGCX	γ-glutamyl carboxylase
GGCX	gamma-glutamyl carboxylase
gla	gamma-carboxyglutamic acid
glu	glutamic acid

GWAS	genome-wide association study
HWE	hardy–weinberg equilibrium
IBD	identity by descent
INR	international normalised ratio - ratio
IWPC	International Warfarin Pharmacogenetics Consortium
JPT	Japanese in Tokyo, Japan
K1	vitamin K1 (phyloquinone)
KH2	reduced vitamin K, or hydroquinone
KH_2	reduced vitamin K or hydroquinone
KO	vitamin K 2,3 epoxide (oxidised vitamin K)
LD	linkage disequilibrium
MAF	minor allele frequency
MK-4	vitamin K2 (Menoquinone or MK-n) homologous. MK-n has a variables side chain length of isoprene units an ‘n’ stands for the number of isoprenoid residues in the chain
M-PVA	polyvinyl alcohol particles
NCBI	National Center for Biotechnology Information
NSAIDs	nonsteriodal anti-inflammatory drugs
OAC	oral anticoagulant
PBX3	Pre-B-Cell Leukemia Homeobox 3
PCR	polymerase chain reaction
POCT	point of care therapy
PTTR	percentage or proportion time that a patient was within targeted therapeutic range
RCT	randomised control trial
RCT	randomised controlled trial
ROC	receiver operating curves
SNPs	single polymorphisms
ST18	Suppression of tumorigenicity 18
TF	tissue factor
TIFAB	TRAF-Interacting Protein With Forkhead-Associated Domain, Family Member B
VKA	vitamin K anticoagulant

VKORC	vitamin K epoxide reductase complex
YRI	Yoruba in Ibadan, Nigeria

Chapter 1

General Introduction

CONTENTS

1.1 The genetic basis of inter-individual differences in drug response

One of the major challenges in prescribing medicines is to give the right medicine to the right patients at the right dose. There are often large differences among individuals in the way they respond to medications, in term of toxicity, treatment efficacy or both. Potential causes for variability in drug response include age, weight, organ function and drug interaction. In addition, inheritance differences in the metabolism and drug disposition, and genetic polymorphisms in the target of drug therapy (e.g. receptor, target protein) also have an influence on the efficacy and toxicity of medications.

An adverse drug reaction is one example of a toxic response, contributing significantly to morbidity and mortality, which imposes a considerable financial burden on the healthcare system. For example, a meta-analysis of 39 prospective studies in the US revealed that adverse drug reactions (toxicity effects) accounted for more than 2.2 million serious cases and over 100,000 deaths, and are one of the leading causes of hospitalisation and death (Lazarou *et al.* 1998).

1.1.1 Genetic variants

The human genome consists of approximately 3 billion base pairs (bp) which reside in the 23 pairs of chromosomes within the nucleus of all our cells (Venter 2001). Variation within the human genome occurs approximately once every 300-3,000 bp if the genome of two unrelated individuals are compared, which is less than 1% of the entire human genome (Sachidanandam *et al.* 2001, Belmont *et al.* 2005). Variants that are present in at least 5% of the population are called common variants, the variant which frequency lower than these (1-4%) are called low frequency variant and variants with frequency ≤1% are called very rare variants (Abecasis *et al.* 2012).

Genetic variations include insertions or deletions, copy number variations, variable numbers of tandem repeats and single nucleotide polymorphisms. Of these, the most common variations are single nucleotide polymorphism (SNPs). The SNP can occur in coding or non-coding regions of the genome. Nonsynonymous SNPs that occur in a coding region may alter the amino acid sequence and therefore, change protein structure and/or function. Some DNA sequences do not encode proteins but may have a regulatory role in influencing the gene expression level, timing, or tissue specificity. The function of the remainder, and the vast majority, of the DNA sequence is not yet known and is the subject of many investigations.

1.1.2 Pharmacogenetics and pharmacogenomics

The first example of a pharmacogenetic trait was described by Pythagoras and dates back to 510 BC, when he noted that certain individuals who ate fava beans (broad beans) developed red blood cell haemolysis (Rowan 1859). This condition, now known as favism, and it it is known to be affected in people of Mediterranean origins. Susceptibility to favism is inherited as a sex-linked trait and appears to be closely related to deficiency of the enzyme glucose-6-phosphate dehydrogenase (G6PD). (Meletis and Konstantopoulos 2004). Genetic variation in human was recognised as important predictors of drugs response variability in 1950's when researchers found that variability of drug concentration in plasma or urine correspond to a specific inherited phenotype of drug response (Kalow and Genest 1957, Kalow and Gunn 1957, Kalow and Staron 1957).

The term 'pharmacogenetics' was first coined in 1959 (Vogel 1959) and can be defined as the study of the variability in drug response due to heredity. Pharmacogenetics focusing on genome, particularly the variation in nucleotide sequence of candidate genes with respect to drug action. In 1977, the newer term of 'pharmacogenomics' was introduced (Marshall 1997). Both terms are used

interchangeably; however, pharmacogenomic signifies the availability of the knowledge and technology advances in high-throughput DNA and mRNA analysis to elucidate genetic determinants of drug effect and toxicity, and to study the effect of therapeutic agents on the pattern of gene expression in the tissue. Therefore pharmacogenomics focuses on gene, RNA transcripts, and their encoded protein (example: the genome, transcriptome and proteome) and seek to define the effect of drugs on gene expression patterns and protein synthesis in cells, tissue and organ systems (Winkelmann *et al.* 2003).

Advances in pharmacogenetics truly began in the last decade following the completion of the Human Genome Project (Craig Venter *et al.* 2001) and the International HapMap Project (Thorisson *et al.* 2005), along with the rapid development of genotyping and sequencing technologies which have facilitated the assessment of the whole genome and have greatly affected pharmacogenetic discoveries (Pirmohamed 2011). Recently, the genomes of 1,092 individuals representing 14 populations across Europe, Africa, Asia and Americas were published and 38 million validated SNPs, 1.4 million short insertions and deletions, and more than 14,000 larger deletions have been identified in the human genome (Abecasis *et al.* 2012). This has enabled a deep characterisation of human genome sequence variations and, therefore, serves as a foundation for investigating the relationship between genotype and phenotype.

Results from a systematic review suggest that adverse drug reactions could be reduced through the use of pharmacogenomics knowledge (Phillips *et al.* 2001). To date, few tests (genotype of phenotype) have made it to clinical trials (Roden and Tyndale 2011). However, it is possible that a patient's genetic make-up can be used to predict patient's response to a specific drug, enabling the best possible treatment to be delivered. But, it is also important to note that many of these variants will interact

with environmental factors. Hence, a holistic approach that takes into account both environmental and genetic factors is needed to ensure that patients receive the right drug, at the right dosage, at the right time to maximise efficacy and minimise toxicity.

1.2 Warfarin

The discovery of warfarin began 90 years ago when a new cattle disease characterised by fatal bleeding following the consumption of spoiled sweet clover was reported in North America. Later, Link identified dicoumarin as a haemorrhagic agent that is produced by spoiled sweet clover. In 1948 warfarin was patented and promoted as a rodenticide (Link 1959). Three years later, it was reported that a patient who took a high dose of warfarin in a suicide attempt was treated without any complication by blood transfusion and an injection of vitamin K. This incident acted as a catalyst for studying the effect of this drug in humans.

Today, warfarin is the most commonly used oral anticoagulant (OAC) world-wide for the prevention of thromboembolic events in high-risk patients (Fuster *et al.* 2006, NICE 2006, Singer *et al.* 2008, Wallentin *et al.* 2010). The drug is now prescribed annually to 0.5-1.5% of the world-wide population (Johnson *et al.* 2011). The effectiveness of this drug has been demonstrated in a recent pooled analysis of studies, showing that warfarin reduces the risk of stroke by 64% compared with a placebo (Hart *et al.* 2007). Although it is highly efficacious, warfarin's narrow therapeutic index and wide inter-individual variability makes its dosing notoriously challenging (Jacobs 2006, Kimmel 2008). Inappropriate warfarin dosing has been reported to the US Food and Drug Administration (FDA) as one of most frequent reasons for emergency room visits (Shehab *et al.* 2010). Perhaps the most serious complication is a major haemorrhage and the rate of major haemorrhages in patients treated with warfarin has been reported as 2.5–3.4% in a recent study (Sportif

Executive Steering Committee for the SPORTIF V Investigators 2005, Wallentin *et al.* 2010).

1.2.1 Warfarin chemistry

Warfarin is a coumarin derivative. The structures of coumarin and its derivatives are as shown in Figure 1-1. The enolic benzopyrene structure is essential to their common pharmacological action as vitamin K antagonists (VKA).

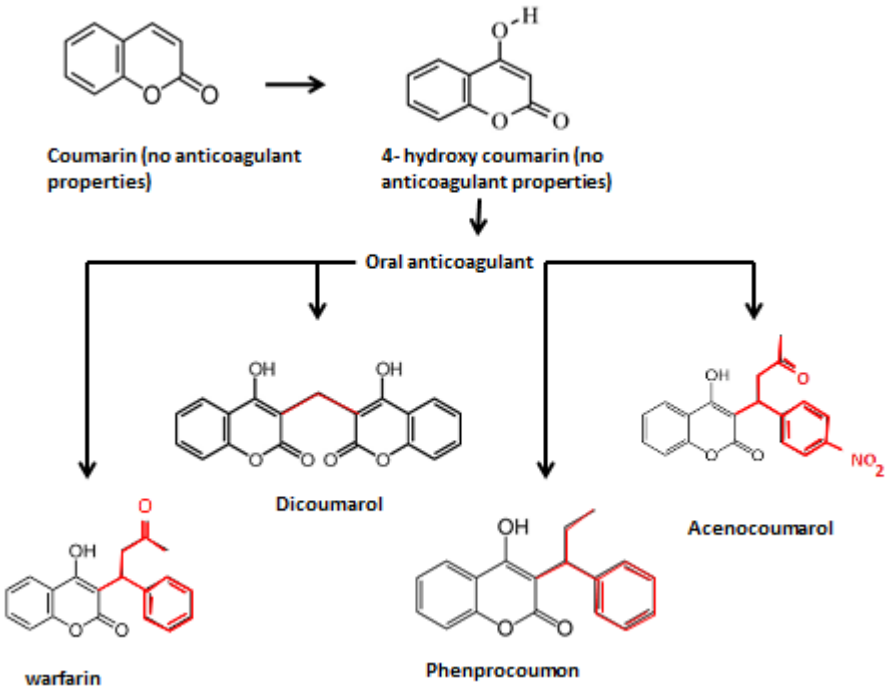


Figure 1-1. The structure of coumarin and derivatives adapted from Au and Rettie (2008). The enolic benzopyrene structure (shown in red) which is important for the molecule's property as a vitamin K antagonist anticoagulant.

The empirical formula for warfarin is $C_{19}H_{15}NaO_4$. Coumarin is insoluble in water but a 4-hydroxy substitution confers weak acidic properties on the molecule making it slightly soluble under weak alkaline conditions. Warfarin contains a single chiral centre at the C9 carbon which gives two enantiomers, 'R' and 'S' warfarin (West *et al.* 1961). In stable anticoagulated patients, free concentrations of S-warfarin range from 0.29-0.82% with the concentration of R-warfarin ranging from 0.26 to 0.96%

(Chan *et al.* 1994). Despite its free concentration being almost the same as that of R-warfarin, S-warfarin is three to five times more potent than its R-enantiomer in inhibiting vitamin K epoxide reductase complex (VKORC) activity. Thus the anticoagulation effects of warfarin are mainly attributed to S-warfarin.

1.2.2 Pharmacokinetics of warfarin and the genes involved

1.2.2.1 Absorptions and distribution

Warfarin is completely absorbed after oral administration and reaches peak concentration in the blood within four hours (Pyörälä *et al.* 1971). In the blood, it binds extensively to plasma proteins, primarily albumin (O'Reilly 1969). The genes involved are presented in Table 1-1 with their known function.

Table 1-1: Genes involved in the transport of warfarin.

Protein Name	Gene	Function
Alpha-1-acid glycoprotein 1, Orosomucoid 1	*ORM1*	A plasma glycoprotein that functions as a carrier in the blood (Otagiri *et al.* 1987, Nakagawa *et al.* 2003)
Alpha-1-acid glycoprotein 2, Orosomucoid 2	*ORM2*	A plasma glycoprotein that functions as a carrier in the blood (Otagiri *et al.* 1987, Nakagawa *et al.* 2003)
P-glycoprotein, Multidrug resistance protein 1	*ABCB1 (MDR1)*	A cellular efflux for xenobiotics (Kroetz *et al.* 2003). Warfarin is a weak inhibitor and may be a substrate (Sussman *et al.* 2002)

Table adapted from Wadelius and Pirmohamed, 2007.

1.2.2.2 Metabolism and excretion

Warfarin is predominantly oxidised by cytochrome P450 in the liver to inactivated hydroxylated metabolites. S-warfarin is oxidised by CYP2C9 (primarily, ~90%), CYP2C8, CYP2C19 and CYP2C18, whereas R-warfarin is oxidised by CYP1A2 (primarily, ~60%), CYP3A4, CYP2C8, CYP2C18, CYP2C19 and CYP3A5 (Wittkowsky 2003). Genes associated with these enzymes are listed in Table 1-2.

Table 1-2: Genes associated with the warfarin metabolising enzymes, cytochrome P450.

Protein Name	Gene	Function of Protein
Cytochrome P450 1A1	*CYP1A1*	Metabolism of R-warfarin (Grossman *et al.* 1993, Zhang *et al.* 1995, Kaminsky and Zhang 1997)
Cytochrome P450 1A2	*CYP1A2*	Metabolism of R-warfarin (Zhang *et al.* 1995, Kaminsky and Zhang 1997)
Cytochrome P450 2A6	*CYP2A6*	Metabolism of S-warfarin (Freeman *et al.* 2000)
Cytochrome P450 2C8	*CYP2C8*	Metabolism of R- and S-warfarin (Rettie *et al.* 1992, Kaminsky and Zhang 1997)
Cytochrome P450 2C18	*CYP2C18*	Metabolism of R- and S-warfarin (Kaminsky *et al.* 1993, Kaminsky and Zhang 1997)
Cytochrome P450 2C19	*CYP2C19*	Metabolism of R- and S-warfarin (Kaminsky *et al.* 1993, Kaminsky and Zhang 1997)
Cytochrome P450 3A4	*CYP3A4*	Metabolism of R-warfarin (Kaminsky and Zhang 1997)
Cytochrome P450 3A5	*CYP3A5*	Polymorphic hepatic and extrahepatic oxidation metabolism of R-warfarin? (Huang *et al.* 2004)
Pregnane X receptor (PXR)	*NR1/2*	Mediates induction of CYP2C9, CYP3A4, other CYP enzymes and ABCB1(Lehmann *et al.* 1998, Geick *et al.* 2001, Chen *et al.* 2004, Yuping *et al.* 2004)
Constitutive androstane receptor (CAR)	*NR1/3*	Transcriptional regulation of a number of genes including CYP2C9 and CYP3A4 (Assenat *et al.* 2004)

Table adapted from Wadelius and Pirmohamed, 2007.

1.2.3 Pharmacodynamics of warfarin and the genes involved

Warfarin exerts its anticoagulant effect by inhibiting the recycling of vitamin K which is important in the blood clotting cascade. The recycling of vitamin K process is particularly important because the amount of vitamin K in the diet and its levels in the body are limited (Stafford 2005).

1.2.3.1 Effect of warfarin on the blood clotting cascade

The classic theory of blood coagulation was described using the Cascade and Waterfall model in the 1960s (Davie and Ratnoff 1964, Macfarlane 1964), as portrayed by Figure 1-2.

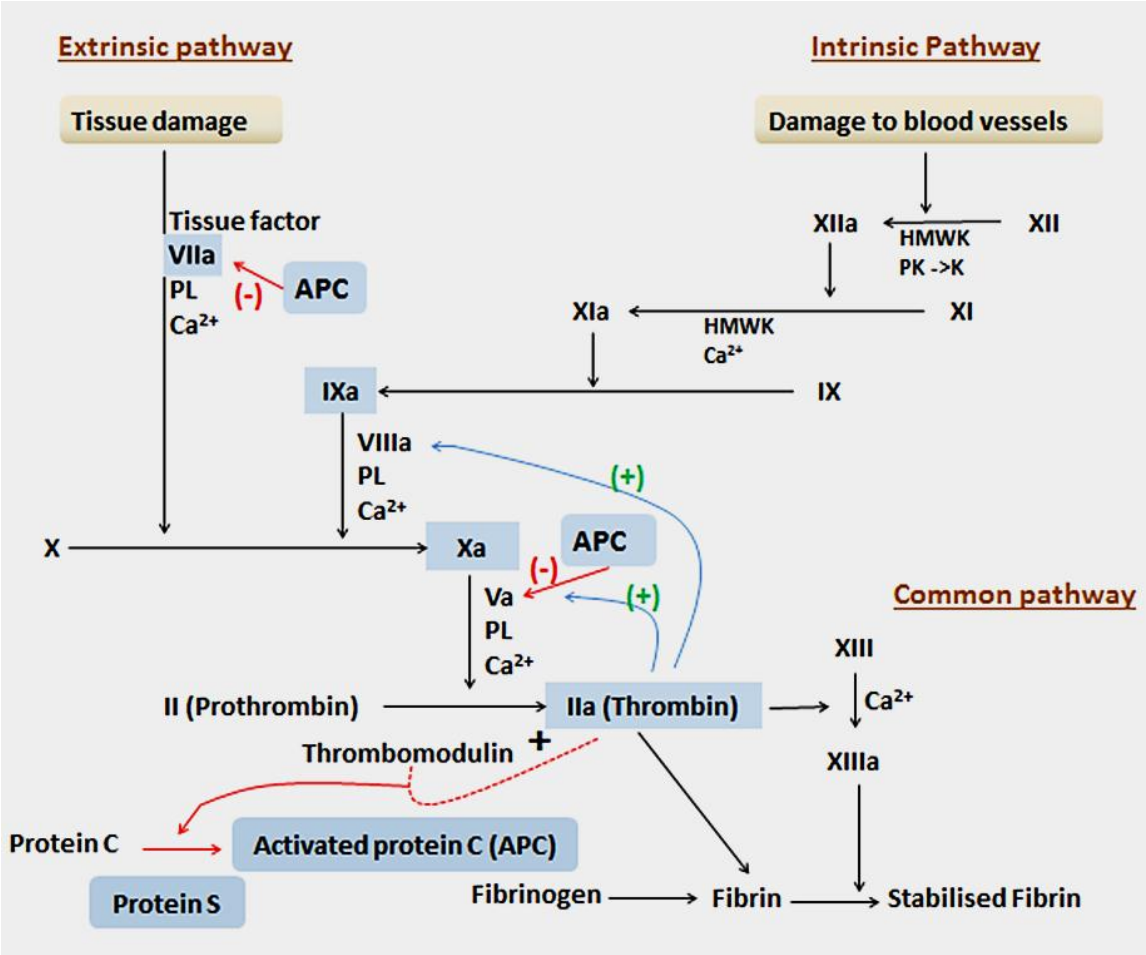


Figure 1-2. . Blood clotting cascade (adapted from Rang and Dale (2012)).). *Extrinsic pathway*: Any trauma to the tissue-activated endothelial cell leads to exposure of the Tissue Factor. This is a cellular receptor for factor VII, which, in the presence of Ca2+, undergoes an active site transition. This results in rapid autocatalytic activation of factor VII to VIIa. The tissue factor, VIIa and Ca2+ formed an extrinsic tenase complex. This compex convert Factor X (**X**) to Factor Xa (**Xa**). In the process acidic phospholipids (**PL**) function as surface catalyst. *Intrinsic pathway*: This pathway is initiated when Factor XII (**XII**) (from the blood) makes contact with a negatively charged surface. Once a small amount of Factor XIIa accumulates, it will convert prekallikrein (**PK**) to kallikrein (**K**) which in turn, accelerates the production of Factor XIIa. Factor XIIa cleaves Factor XI (**XI**) to form Factor XIa (**XIa**). Next, Factor X1a cleaves Factor IX (**IX**) to Factor IXa (**IXa**). Finally, Factor IXa and Factor VIIa, together with Ca^{2+} and negatively charged phospholipids formed an intrinsic tenase complex. Tenase complex subsequently converts Factor X to Factor Xa. *Common pathway*: Factor Xa (from the extrinsic and intrinsic pathways) activates prothrombin (**II**) to thrombin (**IIa**). Thrombin then activates factor V and VIII (as indicated by blue arrow and, furthering the cascade **(+)**. Ultimately, thrombin cleaves fibrinogen to form soluble fibrin monomers. It also activates factor XIII, which strengthens fibrin-to-fibrin links, thereby stabilising the fibrin. *Coagulation pathways regulators indicated (-) by red arrow*: Thrombin binds to thrombomodulin leading to activation of protein C (**APC**) and protein S serves as cofactor in the process. APC inactivates Va and VIIIa which limit thrombin generation. Warfarin interferes with post- translational gamma carboxylation of factors II, VII, IX and X, protein S and protein C (shown in blue box). Legends: HMWK: high molecular weight kininogen.

This model proposed that the process of coagulation can be divided into three distinct parts: extrinsic (so called because some components come from outside circulating blood), intrinsic (so called because all the components were present in circulating blood) and common pathway (process that initiate factor Xa by either pathway and eventually leads to generation of a fibrin clot). When a blood vessel is injured a cascade of reactions aimed at forming fibrin is initiated. The components (called factors) are present in the blood as inactive precursors (zymogen) of proteolytic enzymes and co-factors. They are activated by proteolysis, the active forms being designated by the suffix 'a'. This including vitamin K-dependent coagulation factors:- Factor II, VII, IX, X, protein C and protein S. However, they require vitamin K in reduced form for their biological activity. Factor II, VII, IX, X plays a role to activate blood clotting process whole protein C and S plays roles as regulators in the blood clotting process. The anticoagulant effect of warfarin is due to the sequential depression of Factor VII ($t_{½}$ = 4-6 hours), Protein C ($t_{½}$ = 8 hours), Factor IX ($t_{½}$ = 24 hours), Protein S ($t_{½}$ =30 hours), Factor X ($t_{½}$ = 48-72 hours) and Factor II ($t_{½}$ = 60 hours). The genes that are associated with vitamin K-dependent clotting factors are described in Table 1-3.

Table 1-3. Genes associated with vitamin K-dependent clotting factors.

Protein Name	Gene	Protein Function
Coagulation Factor II, prothrombin	*F7*	Converts fibrinogen to fibrin; activates FV, FVIII, FXI, FXIII, protein C (Berkner 2000, Dahlback 2005)
Coagulation Factor VII	*F7*	Is converted to FIX and then to FXa (Berkner 2000, Dahlback 2005)
Coagulation Factor IX	*F9*	Makes a complex with FVIIIa and then converts FX to its active form (Berkner 2000, Dahlback 2005)
Coagulation Factor X	*F10*	Converts FII to FIIa in the presence of factor Va (Berkner 2000, Dahlback 2005)
Protein C	*PROC*	Activated protein C counteracts coagulation together with protein S by inactivating FVa and VIIIa (Berkner 2000, Dahlback 2005)
Protein S	*PROS1*	Participates in many processes, for example, potentiation of agonist-induced platelet aggregation (Berkner 2000, Dahlback 2005)

Table adapted from Wadelius and Pirmohamed, 2007.

1.2.3.2 The Vitamin K Cycle and the mechanism of warfarin action

Reduced vitamin K, or hydroquinone (KH2), plays an important role as a cofactor for γ-glutamyl carboxylase (GGCX), which catalyses the post-translational carboxylation of a specific glutamic acid residue to γ-carboxyglutamic acid (gla) in a variety of vitamin K-dependent proteins (Presnell and Stafford 2002). The carboxylation process is essential for the biologic functions of vitamin K-dependent proteins involved in blood coagulation.

During the carboxylation process, KH2 is oxidised to vitamin K 2,3 epoxide (KO). KO then undergoes electron reduction to give the reduced forms K1 and KH2. The reduction and subsequent re-oxidation of vitamin K, coupled with carboxylation, is known as the vitamin K cycle Figure 1-3.

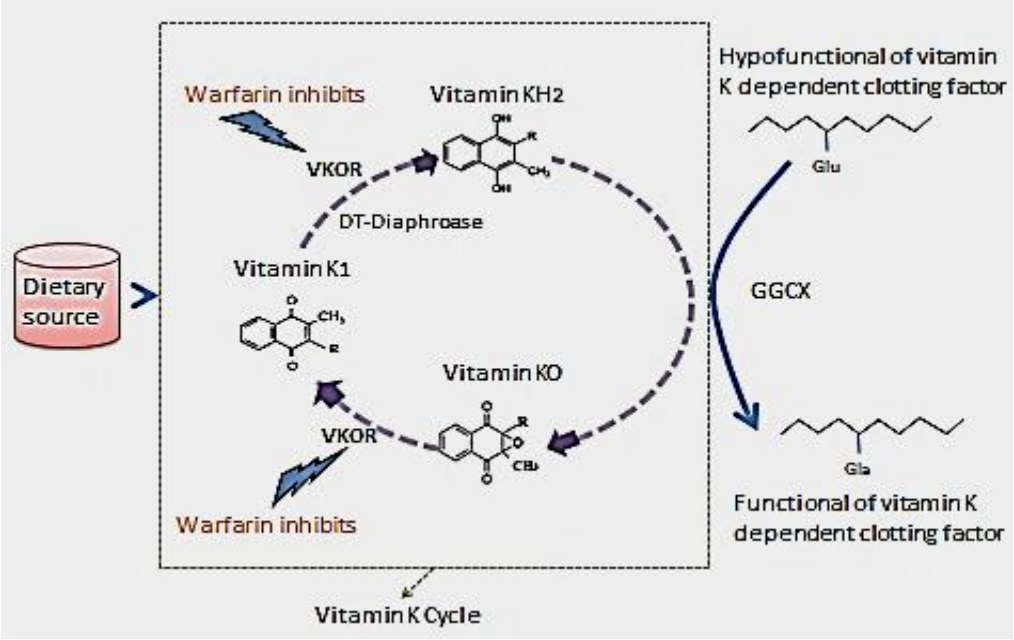


Figure 1-3. The vitamin K cycle. Vitamin K1, which is obtained from dietary sources, is reduced to vitamin KH2 by vitamin K epoxide reductase (VKOR) in a reaction that is coupled to the carboxylation of glutamic acid residues on coagulation factors. By inhibiting VKOR, warfarin blocks the formation of vitamin K1 and vitamin KH2, thus removing the substrate (vitamin KH2) for the carboxylation of glutamic acid.

Warfarin binds to the vitamin K epoxide reductase enzyme (VKOR) and prevents VKOR from recycling vitamins KO and K1 to vitamin KH2 (Silverman 1981). The binding is tight and seems irreversible because of the structural similarity between warfarin and vitamin K (Fasco and Principe 1982). Due to depletion of KH2, vitamin K dependent clotting factor is not activated. As a consequence, the coagulation is reduced due to a decrease in thrombin generation. The genes that are involved in the vitamin K cycle are presented in Table 1-4.

Table 1-4. Genes involved in the vitamin K cycle.

Protein Name	Gene	Protein Function
Vitamin K epoxide reductase	*VKORC1*	A hepatic epoxide hydrolase that catalyses the reduction of vitamin K. The target of warfarin (Bell *et al.* 1972, Li *et al.* 2004, Rost *et al.* 2004a)
Apolipoprotein E	*APOE*	Serves as a ligand for a receptor that mediates the uptake of vitamin K (Saupe *et al.* 1993, Kohlmeier *et al.* 1996)
Epoxide hydrolase, microsomal	*EPHX1*	A hepatic hydrolase in the endoplasmic reticulum that may be complexed with VKOR (Cain *et al.* 1997, Loebstein *et al.* 2005, Morisseau and Hammock 2005)
NAD(P)H dehydrogenase, quinine 1	*NQO1*	A detoxifying enzyme that has the potential to reduce the quinine form of vitamin K (Wallin and Hutson 1982, Berkner and Runge 2004, Ross and Siegel 2004)
Calumenin	*CALU*	Binds to vitamin K epoxide reductase complex and inhibits the effect of warfarin (Wallin *et al.* 2001, Wajih *et al.* 2004)
Gamma-glutamyl carboxylase	*GGCX*	Carboxylates vitamin K-dependent coagulation factors and protein in the vitamin K cycle (Wu *et al.* 1997, Rost *et al.* 2004b)
Vitamin K oxidase	*CYP4F2*	Metabolising vitamin K1 (McDonald *et al.* 2009)

Table adapted from Wadelius and Pirmohamed, 2007

1.3 Indication and monitoring warfarin responses

Warfarin is widely used for the treatment and prevention of thrombosis and thromboembolism, the formation of blood clots in blood vessels and their migration elsewhere in the body. The aim of warfarin treatment is to maintain a patient's international normalised ratio (INR) within the therapeutic range, as recommended by the British Committee for Standards in Haematology fourth edition and shown in Table 1-5 (Keeling *et al.* 2011).

Table 1-5. Indications and recommended target INR (adults).

Indication	Target INR (range)
1. Venous thromboembolism	
First episode	2.5 (2.0-3.0)
Recurrent whilst anticoagulated and within the therapeutic range	3.5 (3.0-4.0)
2. Antiphospholipid	2.5 (2.0-3.0)
3. Atrial fibrillation	2.5 (2.0-3.0)
4. Cardioversion	2.5 (2.0-3.0)
5. Mitral stenosis or regurgitation	2.5 (2.0-3.0)
6. Mechanical prosthetic heart valves	
a) *Patient without risk*†	
*Prothesis thrombogenicity: Low	2.5 (2.0-3.0)
Medium	3.0 (2.5-3.5)
High	3.5 (3.0-4.0)
b) *Patient with risk*†	
*Prothesis thrombogenicity: Low	3.0 (2.5-3.5)
Medium	3.5 (3.0-4.0)
High	3.5 (3.0-4.0)
7. Bioprosthetic heart valves	2.5 (2.0-3.0)
8. Myocardial infarction and cardiomyopathy	2.5 (2.0-3.0)

*Prosthesis thrombogenicity: Low: Carbomedics (aortic position), Medtronic Hall, St Jude Medical (without silzone); Medium: Bjork-Shiley, other bileaflet valves; High: Starr-Edwards, Omniscience, Lillehei-Kaster.

†Patient-related risk factors for thrombosis: Mitral, tricuspid or pulmonary position; Previous arterial thromboembolism; Atrial fibrillation; Left atrium diameter >50 mm; Mitral stenosis of any degree; Left ventricular ejection fraction <35%; Left atrial dense spontaneous echo contrast.

The INR is the ratio of patient's prothrombin time to a normal (control) sample, raised to the power of International Sensitivity Index (ISI) value. It measures the time it takes for the blood to clot compared to an international standard. It has been established by the World Health Organization (World Health Organization) and the International Committee on Thrombosis and Hemostasis for monitoring the effect of VKA (International Committee on Thrombosis and Haemostasis 1985). Values above the 'therapeutic range' will place the patient at an increased risk of haemorrhagic complication, while low values may lead to thrombosis; both scenarios have potentially dangerous consequences, including serious morbidity and death (Baglin *et al.* 2007).

Although the majority of warfarin usage occurs in adults, it is also the mainstay of oral anticoagulation therapy in children and adolescents. Warfarin is recommended as thrombo-prophylaxis for heart valve replacement, cardiac catheterization, post-surgical correction of congenital cardiac defects (e.g. shunt insertion), and haemodialysis (Monagle *et al.* 2012). The recommended INR for children with different condition are shown in Table 1-6 (Keeling *et al.* 2011).

Table 1-6. Indications and recommended target INR (children).

Indication	Target INR (Range)
Dialysis	2.0 (1.5-2.5)
Fontan's Circulation, Cavopulmonary anastomosis, Central Venous line thrombosis, Pulmonary embolus, Proximal DVT, Calf Vein Thrombosis, Recurrence of Venous Thromboembolism, Non-rheumatic Atrial Fibrillation, Mural Thrombus, Cardiomyopathy, Cardioversion, Symptomatic Inherited Thrombophilia	2.5 (2.0-3.0)
Recurrence of venous thromboembolism whilst on Warfarin therapy, Mechanical Prosthetic Valve, Unfenestrated Fontan Circulation	3.5 (3.0-4.0)

After treatment is started, the INR response is monitored frequently until a stable dose-response relationship is obtained; thereafter, the frequency of INR testing is reduced. It has been recommended that patients have their INR measured at least every 12 weeks (Baglin *et al.* 2006). Studies have shown that more frequent testing leads to tighter anticoagulant control and reduces bleeding and antithrombotic complications in patients on OAC (Cannegieter *et al.* 1995, Palareti *et al.* 1996). However, it would be inconvenient and expensive to monitor patients this frequently in primary or secondary care.

Today, many point of care (POC) devices such as CoaguChek® XS (Roche Diagnostics), INRatio® (Hemosense) and ProTime®/ProTime 3 (International Technidyne Corporation) are available commercially which enables patients to test their own INR at home using a finger prick sample of blood. These devices are easy to use and can generate immediate results. Because patients can test their INR at home, this leads to greater convenience, especially for those living in remote rural areas or depend on carers to get them to and from the hospital. Research has shown that home-testing of the INR leads to better anticoagulation control and improved quality-of-life (Newall *et al.* 2006, Smith *et al.* 2012, Ansell 2013, Bereznicki *et al.* 2013, Gaw *et al.* 2013).

1.4 Current warfarin dosing algorithm

In current clinical practice, there is no standardised warfarin dosing algorithm (Ansell *et al.* 2008, Keeling *et al.* 2011). Adult patients are typically initiated with 5mg for 1-2 days but the dose can range from 3-10mg of warfarin depending on age and disease (Harrison *et al.* 1997, Crowther *et al.* 1999, Ansell *et al.* 2008). Then, the subsequent dosing is based on the INR response (Table 1-5 and Table 1-6). For example, the targeted INR for atrial fibrillation is 2-3 whereas for mechanical heart

valves it is 2.5-3.5 (depending on thrombosis prosthesis thrombogenicity). Prosthesis thrombogenicity is related to the biomaterial and valve design features (Edmunds Jr 1996). If the INR result is higher than the targeted value the dose will be decreased, whereas if it is lower the dose will be increased. In patients with a stable dose the INR will be monitored every two to six weeks, although practice varies widely.

There is no generally accepted method designed for increasing, decreasing, or maintaining the weekly warfarin dose based on the current INR. In the Randomized Evaluation of Long-term Anticoagulation Therapy (RE-LY) trials, the algorithm dose recommendations for atrial fibrillation were as follows (Van Spall *et al.* 2012): no change for INR 2.00 to 3.00; increase 15% change for INR ≤1.50, increase 10% for INR 1.51 to 1.99, decrease 10% for INR 3.01 to 4.00. For INR 4.00 to 4.99, the recommendation was to hold the dose for 1 day and then to reduce it by 10%. For INR 5.00 to 8.99, the dose was to be held until the INR was therapeutic and then decreased by 15% per week. It was suggested that the dose be calculated on a weekly, rather than daily basis, because the recommended dose changes were small and difficult to achieve with a daily dosing regimen. Subsequently, weekly INR monitoring was also recommended for out-of-range INR values.

There is lack of specific evidence regarding the safety and efficacy of OAC drugs in children, especially new-borns, as they are physiologically different to adults (Andrew *et al.* 1988, Andrew *et al.* 1992, Andrew 1995). Current guidelines for anti-thrombotic therapy in children have recommended an initial warfarin dose of 0.2 mg/kg, with more frequent monitoring of INR than with adults (Monagle *et al.* 2008). This dosing recommendation was proposed by Michelson *et al.* (1998), based on six publications (Carpentieri *et al.* 1976, Hathaway 1984, Bradley *et al.* 1985, Woods *et al.* 1986, Doyle *et al.* 1988, Andrew *et al.* 1994) and has been evaluated in a prospective cohort (n=115) (Andrew *et al.* 1994). The largest cohort study (n =319)

found that infants required an average of 0.33 (±0.20) mg/kg and teenagers 0.09 (±0.05) mg/kg of warfarin to maintain an INR of 2.0 –3.0 (Streif *et al.* 1999).

1.5 Anticoagulation control in patients receiving warfarin

The safety and efficacy of warfarin therapy are dependent on maintaining the INR within the target range. To attain INR values within the target range, patients are routinely monitored and their therapeutic dosage adjusted when necessary. Regardless of the INR within the therapeutic range (at a level at which the incidence of both thromboembolic and bleeding complications is lowest), adverse events are still reported, but in smaller percentages than for those with time out of range (Cannegieter *et al.* 1995).

Percentage or proportion of time that the patient was within the targeted therapeutic range (PTTR) is used to summarise INR control over time. It has been suggested that this is also an evaluation of the effectiveness of anticoagulant therapies, including warfarin (Rosendaal *et al.* 1993). A high amount of time spent in the therapeutic range is associated with a lower risk of thromboembolic events and bleeding events (Jones *et al.* 2005, Rose *et al.* 2009b).

The relationship between PTTR and the benefit of OAC was examined by Connolly *et al.* (2008), using the PTTRs of patients from 526 centres, within 15 countries involved in the ACTIVE W trial (Atrial Fibrillation Clopidogrel Trial With Irbesartan for Prevention of Vascular Events) and who were randomised to OAC. In patients with a PTTR above 65%, OAC had a marked benefit, reducing vascular events by >2-fold (relative risk, 2.14; 95% confidence interval, 1.61 to 2.85; P<0.0001). A population-average model predicted that a PTTR of 58% would be needed to confidently predict that patients would benefit from being on OAC therapy. Similar findings were observed in a randomised trial evaluating the oral thrombin inhibitor,

ximelagatran, among individuals with atrial fibrillation assigned to warfarin (White *et al.* 2007). Those individuals with poor control (defined as PTTR less than 60%), had higher rates of mortality (4.20% vs. 1.69%) and major bleeding (3.85% vs. 1.58%) compared with the good control group (defined as PTTR greater than 75 %), ($P<0.01$). The poor control group also had higher rates of myocardial infarction (1.38 vs. .62 %, $P = 0.04$) and of stroke or systemic embolic event (2.10 vs. 1.07 %, $P = 0.02$).

In practice, it is recognised that long-term INR stability is difficult to achieve because of unexpected INR fluctuations in patients. For example, a study conducted with a British population (n=2223) reported that patients who were treated with warfarin were inside in the target range only for 67.9% of the time. (Jones *et al.* 2005). This finding is supported by a further meta-regression analysis which reported a mean PTTR in all studies of 64% (van Walraven *et al.* 2006), although PTTR has been reported as low as 29% in some studies (Samsa *et al.* 2000, Sarawate *et al.* 2006).

The PTTR also increases as the duration of INR monitoring increases. In the above British population, during the first three months of warfarin therapy, the PTTR was 48%; after two years, the PTTR increased to 70% (Jones *et al.* 2005). Similarly, in a systematic review and meta-analysis of forty studies reporting the PTTR in patients with VKA for treatment of venous thromboembolism, the mean PTTR was 54.0% in the first three months of treatment and increased to 72% in months four to twelve and over (Erkens *et al.* 2012).

Rose *et al.* (2010) demonstrated that younger age, female sex, lower income, black race, frequent hospitalisations, poly-pharmacy, active cancer, substance abuse, psychiatric disorders, dementia, and chronic liver disease were all associated with lower PTTR. Processes related to warfarin management have also been shown to affect PTTR e.g. INR triggers for dose change, timing of repeat testing following an

out of range INR, selection of INR target and loss to follow-up (Rose *et al.* 2009a, Rose *et al.* 2011, Rose *et al.* 2012, Rose *et al.* 2013).

1.6 Over-anticoagulation and bleeding risk in patients receiving warfarin

Over-anticoagulation is a common problem in warfarin therapy and can lead to a major or life-threatening bleed. It is a measurable parameter for the analysis of bleeding risk in a modestly-sized population. There is strong evidence that higher INR values are associated with bleeding risk (Hull *et al.* 1982, Saour *et al.* 1990, Altman *et al.* 1991, Kearon *et al.* 2003, Finazzi *et al.* 2005) (Table 1-7). An INR higher than 4 places a patient at a greater risk of bleeding (Cannegieter *et al.* 1995, Palareti *et al.* 1996, Hylek *et al.* 2000, Hirsh *et al.* 2003, Pagano and Chandler 2012) and the risk of intracranial hemorrhage increases approximately 2-fold for every 1 unit rise in INR (Hylek *et al.* 1994).

Table 1-7. Relationship between INR and bleeding risk.

Authors, studied population, anticoagulant used	Target INR Range (n)	Duration of Therapy	Incidence of bleeding (%):
Hull *et al.* (1982), deep vein thrombosis, warfarin	3.0–4.5 (96) vs. 2.0–2.5 (96)	3 months	22.4 vs 4.3 P=0.015
Saour *et al.* (1990), mechanical prosthetic heart valves, warfarin	2.3–2.7 (125) vs. 1.3-2.7 (122)	3.4 years	42.4 vs 21.3 P<0.002
Altman *et al.* (1991), mechanical prosthetic heart valves, acenocoumarol	3.0–4.5 (99) vs. 2.0–2.9 (99)	11.2 months	24.0 vs 6.0 P<0.02
Kearon *et al.* (2003), recurrent venous thrombosis, warfarin	1.5-1.9 (369) vs. 2.0-3.0 (36)	2.4 years	8.4 vs 10.6 P=0.26
Finnazi *et al.* (2005), antiphospholipid syndrome, warfarin	3.0–4.0 (54) vs. 2.0–3.0 (55)	12 months	27.8 vs 14.6 P=0.07

1.7 Under-anticoagulation and thromboembolism risk in patients receiving warfarin

In contrast to the bleeding risk associated with VKA, there has been much less research on the thromboembolism risk with under-anticoagulation in patients receiving warfarin. It is known that that under-anticoagulation (INR ≤ 1.5) has been associated with a 16-fold increase in the rate of thromboembolism (Rose *et al.* 2009b).

Hylek *et al.* (1996) demonstrated that atrial fibrillation patients with INRs of 1.7 had nearly twice the risk of stroke compared those with INRs of 2. Those with INRs of 1.5 had nearly three times the risk and those with INRs of 1.3 had a seven-fold greater risk. Similarly, Palareti *et al.* (2005), have shown that the relative risk of recurrence thromboembolism (VTE) was significantly higher in those who spent more time at an INR<1.5 especially in first 90 days of oral anticoagulant therapy.

Dentali *et al.* (2012) conducted a study evaluating the risk of thromboembolic events in patients with isolated subtherapeutic levels of anticoagulant therapy, who were receiving warfarin because of high-risk conditions such as the presence of prosthetic mechanical heart valves or moderate-to-high risk AF (Stroke score CHADS2=3). The sub therapeutic INR value (defined as 0.5–1.0 INR units below the lower limit of the patient-specific target INR range) was associated with a risk of thromboembolic events occurring within 2 weeks of the targeted INR being reached. Based on their observations, patients were exposed to an increased risk of thromboembolism due to a prolong period of inadequate anticoagulation, rather than a single low INR. In contrast, other studies did not replicate this association (Clark *et al.* 2008, Dentali *et al.* 2009), possibly due to the patient's risk profile being unknown (Clark *et al.* 2008) or the inclusion of patients with a single sub-therapeutic INR (Dentali *et al.* 2009).

1.8 Factors influencing the warfarin response

Many clinical factors influence the warfarin dose requirement and response. These include age, weight, height, ethnicity, disease, medications, diet, alcohol, smoking and adherence.

Warfarin dose requirements fall with increasing age (Loebstein *et al.* 2001, Kamali *et al.* 2004, Sconce *et al.* 2005, Gage *et al.* 2008); it has been postulated that this is due to a reduction in liver size with increasing age (Wynne *et al.* 1995). Hillman *et al.* (2004) showed that age, body surface area and the male gender account for 14.6%, 7.5% and 4.7% of variability of warfarin, respectively. Inter-ethnic differences in warfarin dose requirements have been reported between African American, European American and Asian populations. As compared with Caucasians, African-Americans require a higher dose (Gage *et al.* 2004) and Asians require lower doses on average (Dang *et al.* 2005, Lee *et al.* 2006).

The presence of other diseases and medications can affect the pharmacokinetics of warfarin, for example renal failure. Although warfarin is completely metabolised through the liver, chronic renal impairment can alter the degree of protein binding and bioavailability, thereby increasing the risk of adverse events (Grand'Maison *et al.* 2005). Many drugs can inhibit the activity of CYP2C9 enzymes. These include amiodarone, cimetidine, isoniazid and trimetoprim (Smith *et al.* 2004, Greenblatt and Von Moltke 2005, Holbrook *et al.* 2005, Plakogiannis and Ginzburg 2007). Lower warfarin dosing is required to maintain the targeted INR when these drugs are administered together. Conversely, higher warfarin doses are needed when an inducer of CYP2C9 such as rifampicin, phenobarbital or carbamazepine is co-administered with warfarin. Common drugs interactions with warfarin are presented in Table 1-8.

Table 1-8. Common drug interactions with warfarin and proposed mechanism.

INCREASED Effect of warfarin	DECREASED Effect of warfarin
PHARMACOKINETICS **Inhibition of S-warfarin clearance** • By inhibition CYP2C9 enzyme: trimethoprim/sulfamethoxazole, amiodarone, fluvastatin, fluvoxamine, isoniazide, phenylbutazone, sertraline, gemfibrozil, amiodarone, metronidazole, fluconazole **Inhibition of R-warfarin Clearance:** • Inhibition of CYP3A4 enzyme: cimetidine, omeprazole), Clarithromycin, Erythromycin, variconazole, metronidazole, fluconazole • By inhibition CYP1A2 enzyme: Ethanol • By inhibition CYP1A2/CYP3A4 enzymes: Ciprofloxacin • **Increase warfarin absorption:** acarbose	**PHARMACOKINETICS** **Induction of S-warfarin Clearance** • By induction CYP2C9 enzyme: Rifampicin, secobarbital, carbamazepine, phenytoin, phenobarbiturate, primidone, St john's wart **Induction of R-warfarin Clearance** • By induction CYP1A2 enzyme: Cigarette smoking **Alteration of warfarin absorption:** cholestyramine
PHARMACODYNAMIC **Inhibition of synthesis of vitamin K:** Second and third generation cephalosporins **Increase catabolism of clotting factors:** Levothyroxine **Inteference with other pathways of hemostasis:** Acetylsalicylic acid (aspirin) and Nonsteriodal anti-inflammatory drugs (NSAIDs)	**PHARMACODYNAMIC** **Alteration in Dietary Vitamin K Content:** Vitamin K, Vitamin K containing foods

Patient lifestyle also contributes to the warfarin response. As warfarin is a vitamin K antagonist, patients taking warfarin are likely deficient in regenerated vitamin K. Therefore, it is a common practice to administer a supra-physiological doses of vitamin K to reverse the over-anticoagulation effect of warfarin (Hanley *et al.* 2004). Several studies have confirmed an inverse relationship between warfarin maintenance dose requirement and dietary vitamin K intake (Franco *et al.* 2004, Khan *et al.* 2004).

Chronic alcohol intake (long-term) may activate warfarin metabolising enzymes, and as a result, decrease the anticoagulation effect by increasing warfarin metabolism (Weathermon and Crabb 1999). For this reason, chronic alcohol drinkers will need a higher dose of warfarin. Active smokers also need a higher dose of warfarin due to components in tobacco smoke which can induce activation of CYP1A2 enzymes (Ansell *et al.* 2008).

Many studies have validated the contribution of genetic polymorphisms to the warfarin response. *CYP2C9*2*, *CYP2C9*3* and *VKORC1-1639* polymorphisms have been validated in many studies to contribute to warfarin stable doses. These SNPs explain up to 40% of dose variability (Sconce *et al.* 2005, Gage *et al.* 2008, Jonas and McLeod 2009). In 2007, the US FDA updated the warfarin label to include that the CYP2C9 and VKORC1 genotypes may be useful in determining the optimal initial dose of warfarin based on findings regarding pharmacogenetic effects (Thompson 2007).

1.9 Genetics and warfarin dose requirement

As genetic associations with warfarin responses vary between ethnicities, this section will compare the response to warfarin doses in different populations/ ethnicities.

1.9.1 *CYP2C9* gene and SNPs that affect variability in warfarin dose

The *CYP2C9* gene maps to chromosome 10q24.2, contains nine exons, and codes for a 60kDa microsomal protein. Patients expressing the wild-type gene encoding *CYP2C9* have the *CYP2C9*1* genotype (Arg144/Ile359). The most commonly studied SNPs in this gene are *CYP2C9*2* and *CYP2C9*3*. In patients expressing the *CYP2C9*2* variant, arginine is replace by cysteine (Arg144Cys). Arg144 is encoded by exon 3 and is located in helix C, which forms part of the putative P450 reductase binding site of the protein (Rettie and Jones 2005). Any changes are expected to contribute to a change in enzymatic function. In contrast, *CYP2C9*3* is a genotype produced when isoleucine is replace by leucine (Ile359Leu). Ile359 is located in exon 7 and maps close to the active site. Replacement of this amino acid leads to a change in the Vmax and Km of the CYP2C9 substrate (Rettie and Jones 2005). *CYP2C9*2* and *CYP2C9*3* produce enzyme variants with catalytic activities involving S-warfarin that are reduced by 30% and 95% respectively compared with the wild type (Rettie *et al.* 1999).

The minor allele frequency for CYP2C9*2 (rs1799853) is 0.12 in Americans and Europeans and much lower in Africans (0.02) (Abecasis *et al.* 2012). CYP2C9*2 has not been reported in Asian populations except in Malays and Indians in Malaysia (South East Asians) (Gan *et al.* 2004, Ngow *et al.* 2009). The minor allele frequency of CYP2C9*3 (rs1057910) is 0.06 in both Americans and Europeans, 0,04 in Asians and 0.01 in Africans.

Figure 1-4 shows the effect of *CYP2C9* polymorphisms on warfarin dose variation in different populations. One allele, **2*, gives a 6-45% dose reduction compared with wild type. This contributes to a 13-25% dose reduction in Caucasian Americans (Higashi *et al.* 2002, Kealey *et al.* 2007, Limdi *et al.* 2007), 5-14% in Caucasian Europeans (Scordo *et al.* 2002, Topic *et al.* 2004, Sconce *et al.* 2005, Mark *et al.* 2006, Markatos *et al.* 2008) and 26-45% in Asian populations (Gan *et al.* 2004, Tanira *et al.* 2007). Another allele, **3*, gives a 15-48% dose reduction. This contributes to a 28-41% dose reduction in the Caucasian American population (Higashi *et al.* 2002, Kealey *et al.* 2007, Limdi *et al.* 2007), 25-48% in the Caucasian European population (Scordo *et al.* 2002, Topic *et al.* 2004, Sconce *et al.* 2005, Mark *et al.* 2006, Markatos *et al.* 2008) and 12-38% in the Asian population (Gan *et al.* 2004, Tanira *et al.* 2007, Yildirim *et al.* 2008, Huang *et al.* 2009, Ohno *et al.* 2009). Supposedly, the **3* allele has a greater effect on variation in warfarin dosing; in contrast to this, studies by Tanira *et al.* (2007) and Gan *et al.* (2004) revealed that the **2* allele had a greater influence on warfarin dose in the Omani population and the Indian group of the Malaysian population. However, these studies have a small sample sizes and so these findings need to be treated with caution. In addition, there are many other possible factors which can influence warfarin dose such as diet, drugs and lifestyle.

If having one **3* allele gives a great influence on warfarin dosing, homozygosity for the **3* allele gives the greatest influence on warfarin dose (Figure 1-5). Homozygosity for the **3* allele gives a 20-80% dose reduction compared with wild type (Higashi *et al.* 2002, Scordo *et al.* 2002, Gan *et al.* 2004, Topic *et al.* 2004, Huang *et al.* 2009, Ohno *et al.* 2009).

There is no clear association between an individual's genotype and the warfarin maintenance dose for the African American population. Kealy *et al.* (2007) showed

that *CYP2C9* explained the 6% warfarin dose variation in the Caucasian population but had no effect on dose variation in the African American population. Similar findings were observed by Limdi *et al.* (2007). The inconsistent findings might be the result of low prevalence of the variant CYP2C9 genotype in these populations, rather than the lack of effect.

A recent meta-analysis study (Jorgensen *et al.* 2012), has shown that no significant associations were found between CYP2C9*2 and warfarin stable dose requirements for either Asian or white patients butsignificant associations were observed between CYP2C9*3 and stable dose for the White, Chinese, Japanese and a mixed Indian, Chinese and Malaysian populations. Dose reduction observed by comparing the heterozygotes CYP2C9*3 to wild-type showed that white population required the largest dose reduction (approximately 1.80mg/day less), followed by mixed Indian, Chinese and Malaysian populations (1.50mg/day less) and Chinese populations (1.20 mg/day less).

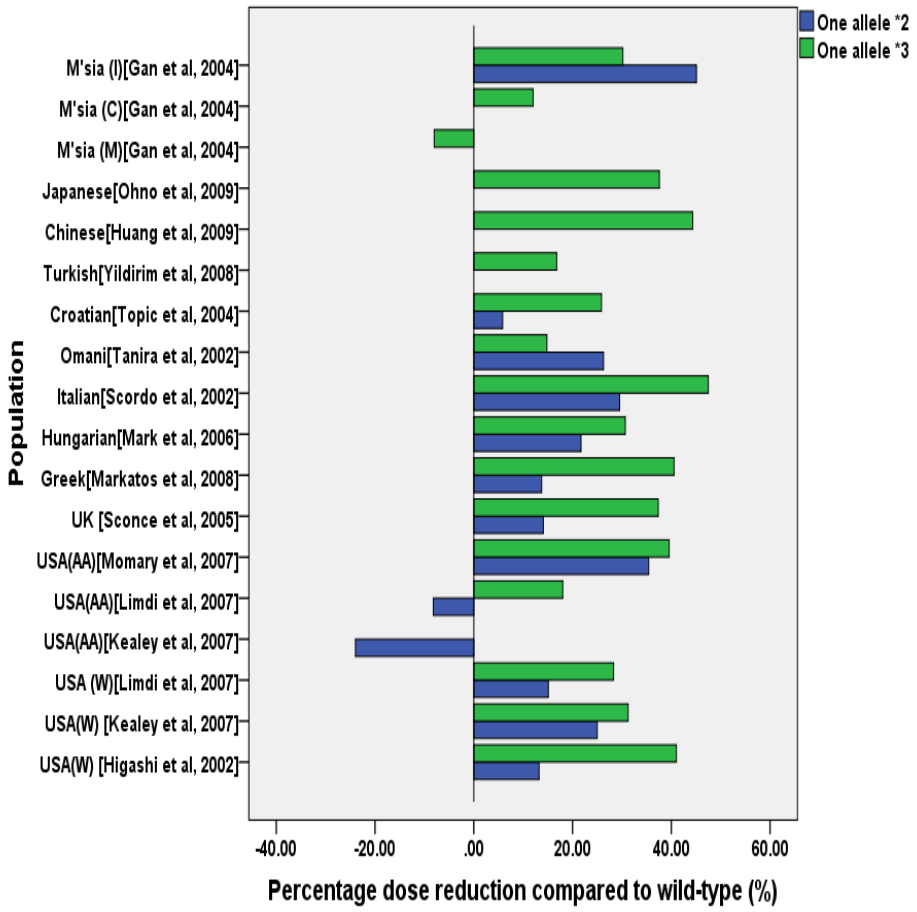
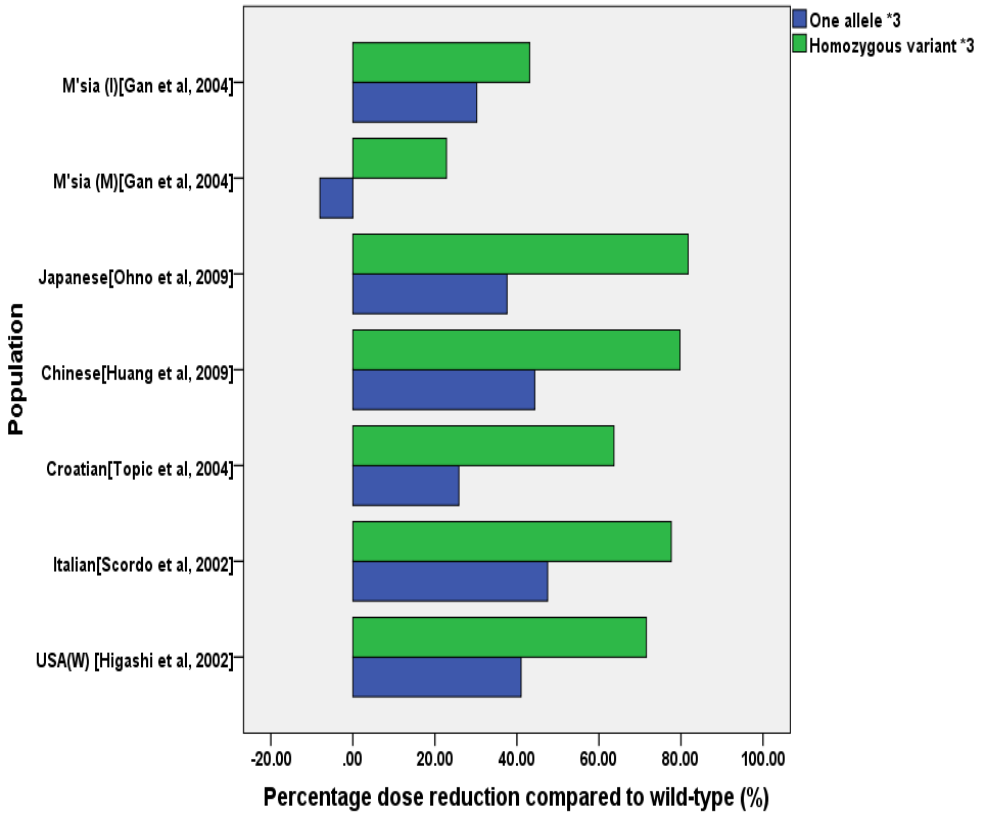


Figure 1-4. Percentage dose reduction by *CYP2C9* polymorphisms (one allele compared with wild-type).



Abreviation:
USA: United state **AA**: African American **W**: White **M'sia**: Malaysia **I**: India
M: Malay **C**: Chinese

Figure 1-5. Percentage dose reduction by *CYP2C9*3* (homozygous and heterozygous compared with one allele).

1.9.2 VKORC1 gene and SNPs that affect variability of warfarin dose

In 2004, two independent study groups discovered the *VKORC1* gene (Li *et al.* 2004, Rost *et al.* 2004a). The gene for human VKORC1, which maps to the short arm of chromosome 16, contains three exons and two introns. The membrane topology of VKORC1 has been investigated by Tie *et al.* (2005), as illustrated in Figure 1-6. The protein is 163 amino acids in length and is an integral membrane protein with three trans-membrane domains.

Alignment of amino acid sequences has identified several conserved amino acids and functional motifs. Five conserved polar amino acids have been proposed to form the active centre of *VKORC1* (Goodstadt and Ponting 2004). These are two conserved cysteine residues located within the cytoplasmic loop (Cys43 and Cys51 in human *VKORC1*), one conserved serine or threonine (Ser57/Thr57) located within the cytoplasmic region and two conserved cysteine residues (Cys 132 and Cys 135) predicted to be partially buried in the ER membrane form a possible CIVC redox motif.

In photo-affinity labelling and site-directed mutagenesis experiments, a hydrophobic sequence motif, Thr-Tyr_{139}-Ala (TYA) was identified as essential for the dicoumarol binding site (Ma *et al.* 1992). This was supported by evidence that mutation at residue Y139 in the rat produces warfarin-resistant phenotypes. Although tyrosine and phenylalanine differ by only one hydroxyl group, substitution led to nearly complete warfarin resistance (Rost *et al.* 2005).

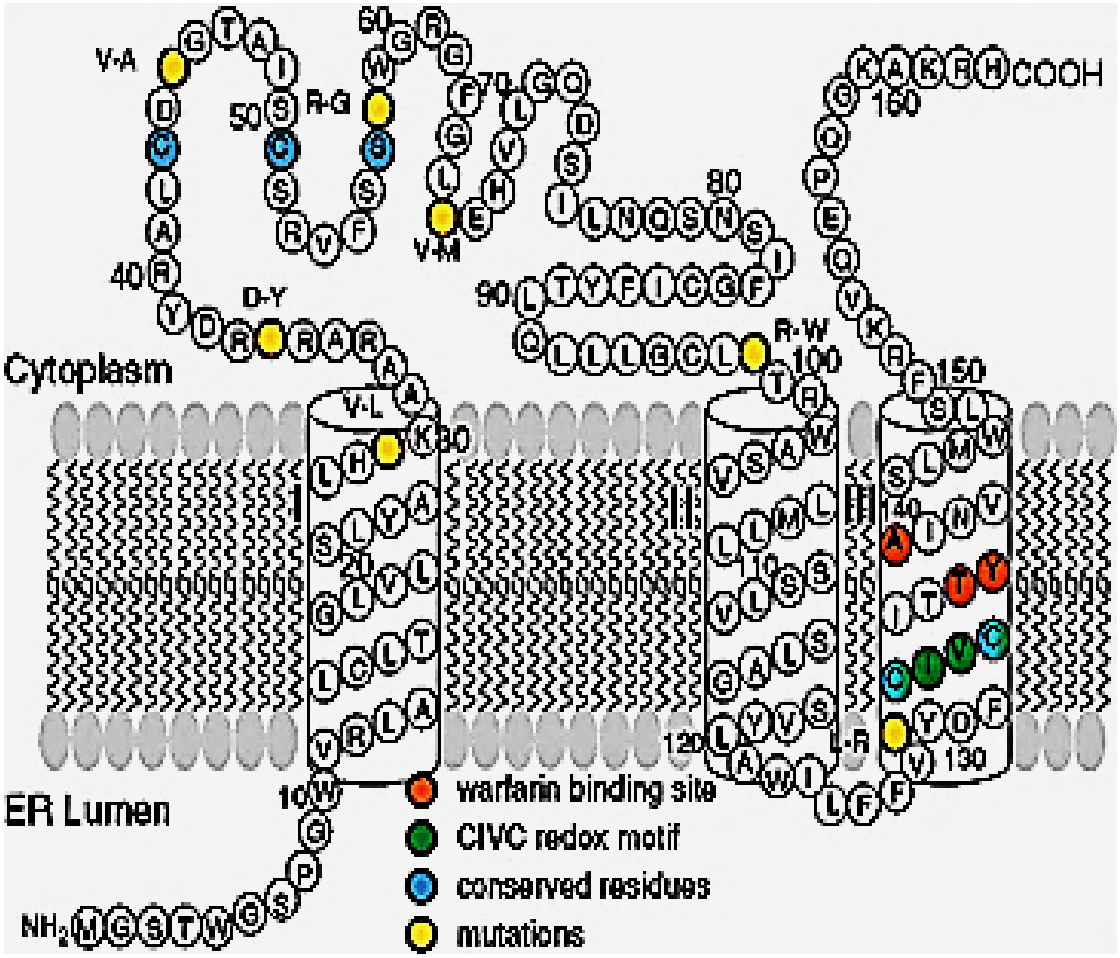


Figure 1-6. Proposed membrane topology of VKORC1. (adapted from Tie *et al.*, 2005).The model predicts three transmembrane α-helices. The amino-terminal part (10aa) of the enzyme is located within the ER-lumen. This is followed by α-helix 1 (aa 11-30), a large cytoplasmic loop of 69 aa, α-helix 2 (aa101-120), and a small ER luminal loop of 7 aa. The third α-helix (aa 128-147) leads over to the the cytoplasmic carboxy terminal comprising 16 aa. Also indicated is the functional motif comprising the TYA warfarin binding site (aa138=aa140, red circles), the CIVC redox motif (aa 132-135, green circles), the ER retention signal (aa159-163) and five amino acids conserved throughout all species (aaCys43, Cys51, Ser/Thr57, Cys132, Cys135, marked by blue circles). Mutations reported to date are indicated by yellow circles with the amino acid exchange given next to the marked circle.

The first identified SNPs in the *VKORC1* gene were reported by D'Andrea *et al*. (2005). Rieder *et al. (2005)* grouped 10 common SNPs to two main haplotypes, A and B, where A required a low dose and B required high-dose warfarin. Similar findings were observed by Giesen *et al.* (2005), who further extended the number of main haplotypes to *VKORC1*1, VKORC1*2, VKORC1*3, VKORC1*4. VKORC1*1* is identified as ancestral, *VKORC1 *2* corresponds to the low-dose group A, as defined by Rieder *et al*., (2005), while *VKORC1*3* and **4* correspond to the high-dose group B.

The effect of VKORC1*2 (the lower dose phenotype), *VKORC1 1173* (rs9934438) and *VKORC1 -1693* (rs9923231) has been extensively studied in Asian populations. Both SNPs are in near perfect linkage disequilibrium (LD). The minor allele frequency is 0.44 in American, 0.40 in European, 0.07 in African and 0.92 in Asian population (Abecasis *et al.* 2012). The effects of polymorphisms are described in Figure 1 7(A-C). In Japanese populations, heterozygosity for these allele gave a ~49% dose reduction whilst homozygosity gave a ~67% dose reduction compared with the wild-type carrier (Kosaki *et al.* 2006, Kimura *et al.* 2007, Ohno *et al.* 2009). In the Chinese population, the heterozygous variant gave ~8-51% dose reduction and the homozygous variant gave ~43-71% dose reduction (Wang *et al.* 2008, Huang *et al.* 2009). The Korean population was shown to be more sensitive to the variant allele; the heterozygous variant led to a 60% dose reduction and the homozygous variant also gave a very similar dose reduction (Cho *et al.* 2007).

In white American Caucasians, both SNPs contributed to a 24-27% dose reduction in heterozygotes and 40-49% in the homozygotes (Li *et al.* 2006, Schelleman *et al.* 2007). However, there were contradictory findings about the association of these SNPs in the African American population. For example, Shellemann *et al.* (2007) studied *VKORC1*2* 1173 and found no significant relationship between genotype and

warfarin dose in African American people. In contrast, a study by Limdi *et al.* (2008) found similar SNP genotypes were associated with a lower warfarin dose ($p<0.005$) among African Americans.

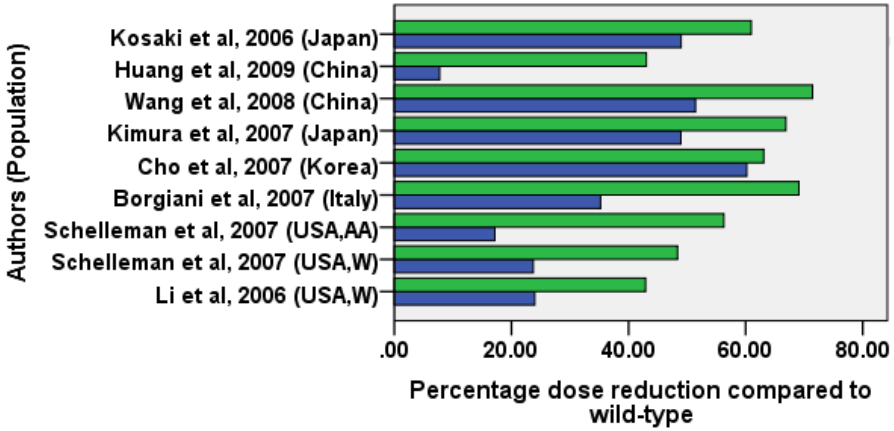
Perini *et al.* (2008) examined the distribution of *VKORC1* -1693 in the Brazilian population. The heterozygous variant for *VKORC1* -1693 contributed to a 20-36% dose reduction while the homozygous variant contributed to 40-46%.

In European populations, studies from Italy on *VKORC1 1173* and Greece on *VKORC1 -1693* demonstrated a 20-35% dose reduction for the heterozygous variant and 60-70% for the homozygous variant (Borgiani *et al.* 2007, Markatos *et al.* 2008).

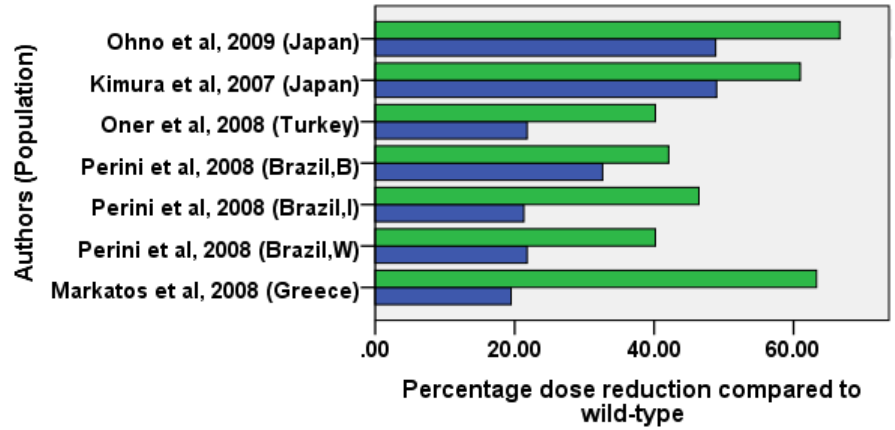
Only *VKORC1*3* (rs 7294), the higher dose phenotype has been studied for its association with warfarin dose. The minor allele frequency is 0.35 in both Americans and Europeans, 0.48 in Africans and 0.07 in Asians (Abecasis *et al.* 2012). In Japanese populations, the heterozygous condition contributed to a 30% dose increment (Kimura *et al.* 2007). In American Caucasians, heterozygosity contributed up to a 19% dosage increment while homozygosity contributed up to 39% (Kimura *et al.* 2007). In European populations, Borgiani, *et al.* (2007) found that the heterozygous variant contributed up to 60% and the homozygous variant contributed up to 90% of the dose increment.

In the same meta-analysis as mentioned earliar (Jorgensen *et al.* 2012), a significant association was observed between the VKORC1 rs9923231 SNP and stable dose in both the white and Chinese ethnic groups, with heterozygotes requiring 1.45 mg/day less than wild-types in both ethnic groups. A significant difference was also observed between VKORC1 rs7294 carriers (heterozygotes) and wild-types the white population. Carriers of VKORC1 rs7294 required 1.80 mg/day more than the wild-type carriers.

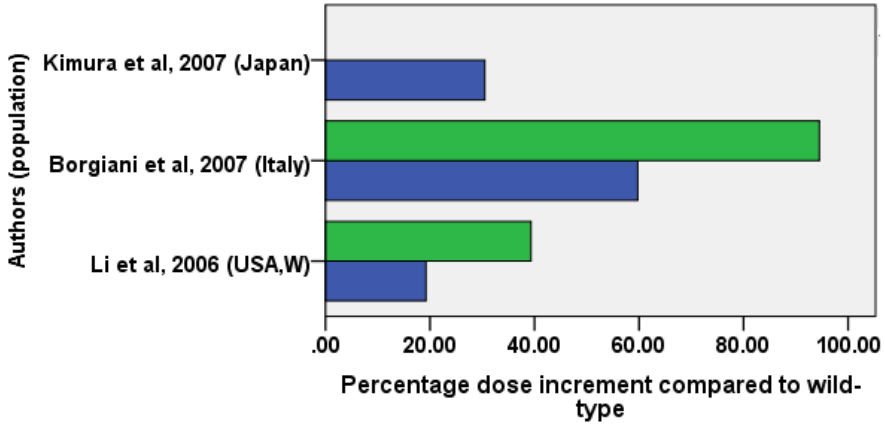
(A) rs9934438 (*VKORC1*2* 1173C>T)



(B) rs9923231 (*VKORC1*2*-1693G>A)



(C) rs7294 (*VKORC1*3)



Legends:

Heterozygous

Homozygous

Figure 1-7 (A-C). The effect of polymorphism of *VKORC1* on warfarin dose. Figure (A)and (B) show the warfarin dose reduction by *VKORC1*2 1173*-rs9934438 and *VKORC1*2* -1693 rs9923231. Carriers of this variant have a lower dose requirement compared with wild-type. Heterozygous carriers have a lower dose requirement (up to 60%) compared with wild-type. Figure (C) shows the percentage dose increment caused by *VKORC1*3* (rs7294). Carriers of this variant have a higher dose requirement compared with wild-type. Heterozygous carriers have a higher dose requirement of up to 60% dose increment compared with wild-type.

In summary, variants of both CYP2C9 and VKORC1 have a significant influence on warfarin dose requirement. Many researchers have evaluated the relationship between genotype and dose to build a statistical model that incorporates a variety of factors (i.e., clinical, demographic and genetic) to estimate the influence of each factor on dose requirement. Such analyses have demonstrated that polymorphisms in VKORC1 account for approximately 40% of the variance in warfarin dose while CYP2C9 explains less than 10% (Schalekamp and De Boer 2010). Together with clinical data, they can explain up to 60% of warfarin variation (Wadelius *et al.* 2009, Lenzini *et al.* 2010). Still, another 40% remains unrecognised and this could result in over or under dosing. Considering the difference of minor allele frequencies in different populations which may contribute to the different effect of the polymorphism to warfarin dose, pharmacogenetics algorithm for a specific population is needed.

1.9.3 Other genes and variability in warfarin dose

A recent study identified a non-synonymous variant (rs2108622) in exon 2 of a cytochrome P450 family 4, subfamily F, polypeptide 2 gene (*CYP4F2*) which led to an increase in warfarin requirement in a European–American cohort of patients (Caldwell *et al.* 2008). *CYP4F2* is a vitamin K1 oxidase and that carriers of the *CYP4F2* V433M allele (rs2108622) have a reduced capacity to metabolize vitamin K1 (McDonald *et al.* 2009). Therefore, patients with the rs2108622 polymorphism are likely to have elevated hepatic levels of vitamin K1, necessitating a higher warfarin dose.

Caldwell *et al.*, (2008) then repeated the study to validate the result in three different sites at Marshfield University, the University of Florida and Washington University in St Louis. The variant predicted an additional 4% (Washington University), 7% (University of Florida) and 12% (Marshfield University) in the

variability of warfarin dose per T allele (minor allele). The effect of this SNP has been confirmed in a Swedish population, where it accounted for 1% of dose variability (Takeuchi *et al.* 2009), Italians where it explained 7.5% of dose variability (Borgiani *et al.* 2009) and North Americans where each copy of the minor allele elevated the dose by ~0.5mg/day (Cooper *et al.* 2008). The effect was not present in African-American populations since the minor allele frequency is low in this population (Cavallari *et al.* 2010).

Contradictory findings were shown in an association study of warfarin dose and polymorphisms of *GGCX, Factor II*, *Factor VII*, *Factor X*, *EPHX1*, *CALU*, *ABCB1*, and *CYP3A4* (D'Ambrosio *et al.* 2004, Wadelius *et al.* 2004, Aquilante *et al.* 2006, Vecsler *et al.* 2006, Rieder *et al.* 2007, Saraeva *et al.* 2007). It was also discovered that the *CYP3A5*3, CYP1A1*1F, CYP2C19* and PROC gene variants did not correlate with warfarin dosage variation (Wadelius *et al.* 2004, Saraeva *et al.* 2007).

1.10 Genetics and other warfarin-related outcomes

Warfarin-related bleeding is the most problematic side effect of this drug. As such, the relationship between genetic variation and bleeding risk has become an area of research interest.

1.10.1 Genetics and over-anticoagulation during warfarin treatment

Carriers of *CYP2C9*2* and *CYP2C9*3* are more frequently over-anticoagulated (INR ≥4) than wild-type carriers (Higashi *et al.* 2002, Wadelius *et al.* 2009, Molden *et al.* 2010, Ma *et al.* 2012). A systematic review and recent meta-analysis have demonstrated that both *CYP2C9*2* and *CYP2C9*3* carriers were susceptible to over anticoagulation (INR ≥4) when compared to the *CYP2C9* wild-type. The hazard ratio was also much higher in carriers of *CYP2C9*3* as the hazard

ratio was 2.37(95%CI 1.46-3.83) for *CYP2C9*3* compared to 1.52 (95%CI 1.11-2.09) for *CYP2C9*2* and 1.90 (95%CI 1.58-2.29) for *CYP2C9*2/*3* (Yang *et al.* 2013). The studies also observed a significantly higher risk for over-anticoagulation (INR ≥4) in both homozygous and heterozygous mutant for *VKORC1* rs 992323231 (compared to wild-type) within 30 days of treatment, but not after 30 days of treatment (Yang *et al.* 2013).

1.10.2 Genetics and major bleeding risk during warfarin treatment

Even though polymorphisms in *VKORC1* and *CYP2C9* have been associated with lower dose and increased risk of over-anticoagulation, only for *CYP2C9* has strong evidence been presented to show that it contributes to major bleeding. For example, the *CYP2C9* polymorphism (*CYP2C9*2* and *CYP2C9*3* combined) influenced major bleeding risk in studies by Higashi *et al.* (2002) and Limdi *et al. (2008b)*. Only three studies have examined the effect of the *VKORC1* genotype on major bleeding risk. Limdi *et al.* (2008b) and Huang *et al., (2009)* found no effect of the *VKORC1* (rs9934438) polymorphism on warfarin-related major bleeding in Caucasian, African-American and Chinese populations. A similar conclusion was drawn by Giansante *et al.* (2012) who studied the *VKORC1* rs9923231 polymorphism in an Italian population. In a recent meta-analysis, *CYP2C9*2* and *CYP2C9*3* were significantly associated with bleeding with *3/*3 carriers showing the highest hazard ratio *(Jorgensen et al. 2012, Yang et al. 2013).* However, there was no association with *VKORC1* rs9923231 and rs9934438. Even though over-anticoagulation is a strong and independent risk factor for hemorrhage, it does not always precede a hemorrhagic complication (Hylek *et al.* 2003, Hylek *et al.* 2008). Therefore, the risk of over-anticoagulation does not associate to the risk of hemorrhagic event (Limdi and Veenstra 2008). This possibly explains why the association between VKORC1 and over-anticoagulation was observed but not with major bleeding.

1.10.3 Genetics and time to stable INR in patients treated with warfarin

This parameter is of interest because achieving a rapid time to stable INR can benefit both patients and care-givers and it also contributes to improving the pharmaco-economic profile and genomic testing. There are contradictory findings regarding the association between *CYP2C9* polymorphism and time to stable INR. *CYP2C9*2* and *CYP2C9 *3* have been reported to increase the time required to reach stable dose in some studies (Higashi *et al.* 2002, Huang *et al.* 2009, Kim *et al.* 2009) but the association was not observed in other studies (Kealey *et al.* 2007, Limdi *et al.* 2008a, Jorgensen *et al.* 2009). Similarly, there are no conclusive findings regarding the association between the *VKORC1* polymorphism and this outcome, with one study observing an association (Huang *et al.* 2009), while three other studies show an association (Schelleman *et al.* 2007, Limdi *et al.* 2008a, Kim *et al.* 2009). In a recent meta-analysis, the association between *CYP2C9*2*, *CYP2C9*3* and *VKORC1* polymorphism and this outcome was found not to be significant (Jorgensen *et al.* 2012).

1.11 Ontogeny which may contribute to changes in warfarin pharmacokinetics

The bioavailability of orally administered drugs such as warfarin may be reduced in infants and young children because the gastric pH is higher in neonates, infants and young children (Van Der Giesen and Janssen 1982). The gastric pH reaches adult values by two years of age (Anderson and Lynn 2009). Unfortunately, there are no bioavailability studies of warfarin in neonates and children.

In neonates and infants, total body water is higher compared with an adult. At birth, the total body water is 80%, decreasing to 60% at two years of age, while in adults the total body water is 54-62% at age 20 (Anderson and Lynn 2009). As a result, the time required to achieve a stable dose is longer in children, especially neonates,

compared with adults. It is possible that these patients require higher loading doses to achieve the targeted concentration in a shorter time.

Koukouritaki *et al.* (2004) undertook the largest study to date to characterise the developmental expression pattern for human hepatic CYP2C9 and determine the overall inter-individual variation in CYP2C9 in 240 foetal and paediatric liver samples. The developmental expression of CYP2C9 is shown in the in scatter plot analysis in Figure 1-8. Relative to mature levels, low CYP2C9 protein levels were detected in many foetal samples between eight and 24 weeks of gestation and increasing values were seen during the remaining gestational period. The levels were significantly greater after birth; however, variability was extensive over a constant range during the first five months. An examination of post-natal samples (day 1 to 18 years, n=166) revealed that most samples from individuals of one to two years age exhibited a mature protein level.

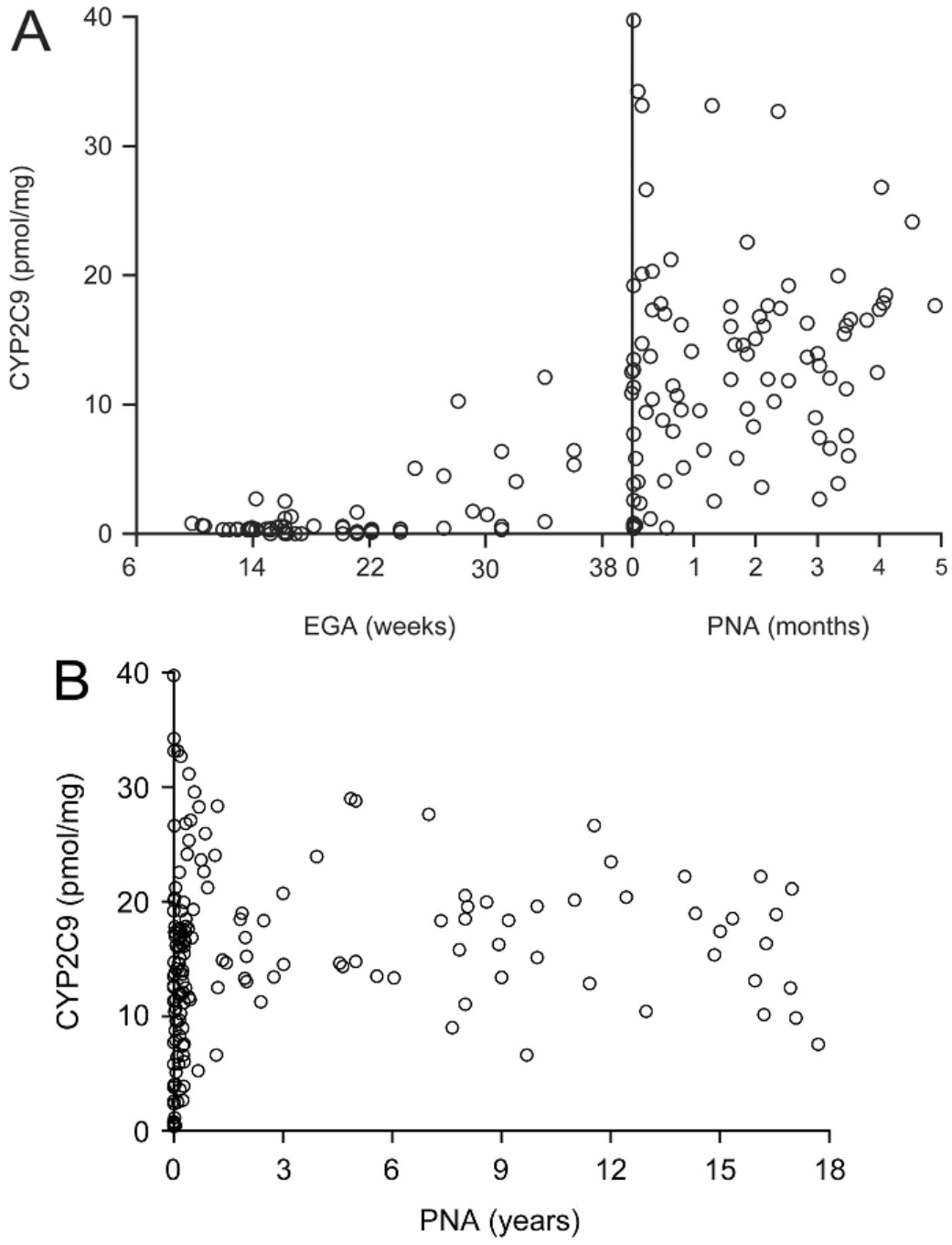


Figure 1-8. A scatter plot analysis showing the development expression pattern of CYP2C9 against age (adapted from Koukouritaki *et al*,.2004). (A) A scatter plot analysis of CYP2C9-specific content as a function of gestational age (weeks) is depicted along with the transition at birth and the neonatal and early infancy periods. (B) A scatter plot analysis of CYP2C9-specific content as a function of postnatal age (years) is depicted **EGA**: Early gestational age, **PNA**: postnatal age.

Results of analyses of variability in expression during different developmental stages have shown that 51% of the samples taken from an early infancy period (up to five months) had protein values that corresponded with adult values but were highly variable (35-fold) compared with the five months to 18 year age group (4-fold variability) (Figure 1-9). It was also shown that CYP2C9 activity (diclofenac 4-hydroxylase metabolic activity) in samples taken from the group aged 0-5 months (median specific activity = 899; n: 88) is not much different compared with samples from the group aged >5 months to 18 years (median-specific activity = 628; n: 71) (Koukouritaki *et al.* 2004).

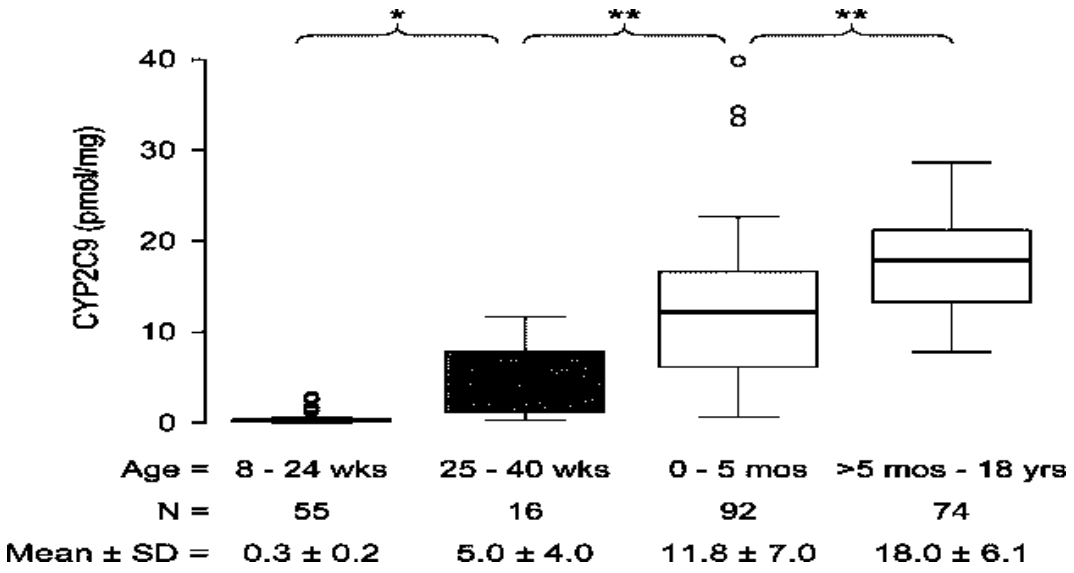


Figure 1-9: Inter-individual variability of CYP2C9 developmental expression within different age groups (adapted from Koukouritaki *et al.* 2004). The boxes represent the 25th to 75th percentiles, and the horizontal bar represents the median of the data. Significant differences between groups, as determined by Kruskal-Wallis nonparametric analysis and Dunn's multiple comparisons test, are shown (*, $p< 0.05$; **, $p< 0.01$).

Since warfarin is a drug with a low hepatic extraction ratio (0.004) (Takahashi and Echizen 2001), the unbound intrinsic hepatic clearance is the sole pharmacokinetic parameter determining its unbound plasma concentration at steady-state. In a study by Takahashi *et al.* (2000), the unbound oral clearance value for S- warfarin in the prepubertal (age 1-11 years) was compared to that of the adult (age 37-76 years) with genotypes *1/*1 and *1/*3 genotypes. The median unbound oral clearance value obtained from the adult patients with the wild-type (CYP2C9*1/*1) genotype (ie, 667 ml/min) was significantly greater than that obtained from the prepubertal patients with the corresponding genotype (ie, 367 ml/min), indicating a substantial development would take place for the activity of CYP2C9 in these subjects. In contrast, there was no significant difference in the median unbound oral clearance value between prepubertal and adult patients with the CYP2C9*1/*3 genotype (ie, 213 and 212 ml/min, respectively), indicating that the development of CYP2C9 activity may be substantially attenuated in carriers of the CYP2C9*3 allele. However, the author concluded that was not a valid findings because the numbers of prepubertal and adult patients with the CYP2C9*1/*3 allele were rather small (n=5 and 4, respectively) and no longitudinal follow-up was made for individual children with CYP2C9*1/*3 genotype.

1.12 Ontogeny which may contribute to changes in warfarin pharmacodynamics

There is disagreement between findings regarding the concentration of vitamin K-dependent proteins in children and in adults. In his review, Andrew (1995) reported that Factor II, Factor VII, Factor IX and Factor X are expressed at approximately 50% of the adult level at birth, and the concentration increases but remains approximately 15-25% lower than the adult value during childhood. With routine vitamin K supplementation, these factors are significantly reduced in the first six months

(Andrew *et al.* 1988). Proteins C and S are also expressed at lower levels at birth and during childhood. A study by Massicotte *et al.* (1998) in paediatric patients (median age, 13 years old) found that plasma concentrations of six vitamin K-dependent clotting factors (Factor II, Factor VII, Factor IX, Factor X, Protein C and free Protein S) were similar for children and adults. The review by Andrew (1995) is likely to be more accurate because he referred to studies which analysed different age groups (1-5 years; 6-10 years, 11-18 years and adult) whilst Massicotte *et al.* analysed children ranging in age from one to 18 years.

In contrast with Vitamin K coagulation factors, expression levels of thrombomodulin (Andrew *et al.* 1994) and α2-microglobulin (Andrew 1995, Massicotte *et al.* 1998) are highest during infancy and early childhood. α2-microglobulin is expressed at approximately twice the adult value at six months of age. Taking everything into account, there is less active thrombin circulating in children's plasma due to an increase in the direct inhibitor, α2-microglobulin, and an increase in thrombomodulin in the Protein S/ Protein C system.

As discussed in 1.2.3.2 (page 13), the role of VKORC1 is to recycle oxidised vitamin K into its hydroquinone form, becoming an essential cofactor for the post translational carboxylation of vitamin K-dependent clotting to its functional form. The action of warfarin is to inhibit the ability of VKORC1 to recycle oxidised vitamin K and, as a result, to reduce the functional vitamin K-dependent clotting factors. A small study (n=46) showed that the activity of VKORC1 is high in the early prenatal period but significantly decreases after birth. However, the study did not observe any significant changes in VKORC1 activity during childhood (Itoh and Onishi 2000).

Dietary vitamin K may influence warfarin response and this has been seen in several studies. Breast-fed infants are more sensitive to the anticoagulant effects of warfarin than formula-fed infants and this is likely to be due to the relatively low

concentration of vitamin K in breast milk compared to vitamin K-supplemented formula feeds (Haroon *et al.* 1982). Children receiving enteral feeding by nasogastric or gastrostomy tube have also been shown to require higher warfarin doses; again this is likely to be due to vitamin K supplementation of the enteral feed (Streif *et al.* 1999).

Recently, Hirai *et al.* (2013) investigated the relationship between warfarin sensitivity and plasma Vitamin K in Japanese children (n=37) who were being treated with warfarin. No significant association was found between warfarin sensitivity and plasma vitamin K1 concentration, but a significant negative correlation was detected with plasma concentrations of MK-4. MK-4 is vitamin K2 homologue and is obtained from dietary Vitamin K1 and vitamin K2 found in various animal tissues. A specific human enzyme which plays a role in converting Vitamin K1 to MK4 has recently been discovered, offering a mechanism for this observation (Nakagawa *et al.* 2010).

1.13 Aims of the thesis

My interest in warfarin pharmacogenomics began when I worked as a pharmacist in a warfarin clinic in my home country (Malaysia). I observed large inter-individual dose variability in different patients. Some patients were very sensitive to warfarin; even a small dose adjustment could result in an unexpected INR and these patients only required less than 0.5mg/day warfarin to achieve a therapeutic INR. In contrast, some patients required >15mg/day of warfarin to achieve a similar target INR.

The primary aim of this work is based on the following two questions: first, would knowledge of genetics be useful in improving warfarin treatment decision? This includes predicting efficacious warfarin doses and at the same time, avoiding intolerable or dangerous side effects. Second, are we ready to use genotyping in a clinical environment? In the clinical setting, the faster we can obtain a genotyping

result the better to help avoid adverse events. Ideally, the genotyping result is needed before warfarin therapy begins, and the result must be accurate.

An outline of my thesis is as follows:

(i) *Literature Review (Chapter 1).* My thesis starts with brief review of the relevant literature on the key concepts of pharmacogenetics of warfarin. This includes basic genetic terminology, the blood coagulation process, the pharmacological properties of warfarin and a review of warfarin pharmacogenetic studies. I have also discussed human ontogeny as it influences the warfarin response.

(ii) *Development and validation of pharmacogenetic algorithms in adult populations (Chapter 2).* It is advantageous in clinical practice if there is an available algorithm to accurately predict the appropriate warfarin dose in given patients. Chapter 2 describes the development and validation of a warfarin dosing algorithm using *CYP2C9* and *VKORC1* polymorphisms together with clinical factors.

(iii) *Assessment of HAS-BLED score and contribution of genetics in predicting bleeding risk (Chapter 3).* Bleeding is the major risk in warfarin treatment. Bleeding assessment will help to determine whether patients will suffer intolerable or dangerous side-effects which are helpful in counselling patients and informing treatment decision. In this chapter, the performance of the novel bleeding score, HAS-BLED, was assessed in our prospective cohort (six months follow-up) and the prediction performance was compared to that incorporating genotype.

(iv) *The role of genetics in predicting warfarin response in children* (*Chapters 4 & 5).* The contribution of *CYP2C9* and *VKORC1* polymorphisms to the warfarin response in children is introduced in the fourth chapter and then extended to an exploration of the Genome Wide Association study (GWAS) in the fifth chapter.

(v) *Validation of Genie – a point of care device to genotype VKORC1 and CYP2C9 polymorphisms (Chapter 6).* Chapter 6 focuses on the application of pharmacogenetic testing in a clinical set-up. The work described in Chapter 6 involves the validation of the Genie, a genotyping instrument used for the European Pharmacogenetics of Anticoagulant Therapy (EU-PACT) trial (Van Schie *et al.* 2009). The aim is to establish whether it is accurate and applicable for use in a clinical situation.

(vi) Finally, Chapter 7 summarises my research in this area to date, and outlines the next phase of my research.

Chapter 2

Development and validation of a warfarin dosing algorithm

CONTENTS

2.1 Introduction

Current warfarin therapy adopts a precarious trial-and error approach where patients are prescribed a standard loading dose during the first 2-3 days of warfarin initiation before a therapeutic dose is titrated according to the patient's INR response (Fennerty *et al.* 1984, Gedge *et al.* 2000, Kovacs *et al.* 2003, Siguret *et al.* 2005). During this dose-titration period, patients are at highest risk of adverse events (Landefeld and Goldman 1989, Fihn *et al.* 1993, Douketis *et al.* 2000). As such, strategies to individualise initial warfarin dose have been sought.

The notion of dosing algorithms utilising clinical variables to improve anticoagulation management has existed for decades (Williams and Karl 1979, Ovesen *et al.* 1989). Studies incorporating clinical variables into computer programs have demonstrated improvement in stable INR maintenance and reduction of complications (Ageno *et al.* 2000, Manotti *et al.* 2001). After the discovery of the *CYP2C9* and *VKORC1* genes, numerous studies have developed pharmacogenetic dosing algorithms that incorporate both genotype and clinical characteristics to predict warfarin dosing requirements. SNPs in *CYP2C9* and *VKORC1* have shown to contribute approximately 30-40% dose variance, and together with clinical variables, they account for nearly up to 60% of dose variability (Sconce *et al.* 2005, Gage *et al.* 2008, Hatch *et al.* 2008, Perini *et al.* 2008). More recently, the International Warfarin Pharmacogenetics Consortium (IWPC) published a study which is the largest to date, where they demonstrated the addition of genetic information (*CYP2C9* and *VKORC1* SNPs) to clinical variables provided dosage prediction that was significantly closer to the actual dosage required than estimates derived from a clinical algorithm or a fixed-dose approach (Klein *et al.* 2009).

Although many research groups throughout the world have developed pharmacogenetic algorithms in different populations, only one study utilising a cohort

of British Caucasians has been reported (2005). Incorporating age, height, *CYP2C9* and *VKORC1* genotypes from a retrospective cohort of 297 British Caucasian patients, Sconce and colleagues (2005) derived a pharmacogenetic algorithm which explained 54.2% of warfarin stable dose variance. However, to the best of our knowledge, no pharmacogenetic algorithm has yet been developed using one large cohort of prospectively recruited British Caucasian patients.

Furthermore, most studies developing warfarin dosing algorithms have assessed their accuracy by referring to the R-squared (R^2) value, which estimates the proportion of variability in the data captured by the model. However, a high R^2 value does not necessarily imply that the model is capable of accurate dose prediction (Jorgensen and Pirmohamed 2011). More recently, studies have referred to the mean or median absolute error (MAE) as a measurement of predictive accuracy (Gage *et al.* 2008, Klein *et al.* 2009, Zambon *et al.* 2011). An alternative measure is the proportion of patients whose predicted dose is within *x* units of actual dose, with *x* appropriately chosen (Klein *et al.* 2009, Finkelman *et al.* 2011, Jorgensen and Pirmohamed 2011).

The goals of this chapter were therefore (1) to develop a pharmacogenetic dosing model utilising our prospective cohort of Caucasian patients being initiated onto warfarin therapy in a secondary care setting, and, (2) to externally validate the predictive capability of our algorithm in an independent cohort of patients recruited retrospectively in a primary care setting.

2.2 Methods

2.2.1 Patient recruitment

The study was approved by the West Midlands Multi-Centre Research Ethics Committee and written informed consent was obtained from each patient prior to study commencement.

Derivation Cohort: 1000 patients were recruited from the Royal Liverpool and Broadgreen University Hospital Trust (RLBUHT) and University Hospital Aintree between November 2004 and March 2006. Patients were followed up prospectively from the start of warfarin treatment for a period up to 26 weeks. The study design was observational. Patients received standard loading doses and dose changes were determined according to in-hospital guidelines. Each patient had four scheduled visits: the first was at the time of warfarin therapy commencement (index visit), then three subsequent follow-up visits at one week, eight weeks and twenty-six weeks of warfarin therapy. At the index visit, several clinical parameters were recorded including baseline INR, concurrent medications, medical history and patient demographics. All INR measurements and dose changes during follow-up were also systematically recorded. This cohort of patients was originally recruited as part of a large prospective study on warfarin pharmacogenetics and the interim analysis has been published (Jorgensen *et al.* 2009). The interim analysis of this cohort has discovered that CYP2C19 (SNP rs3814637) was significantly associated with warfarin stable dose. However, the authors hypothesized that the significant associations found with SNPs in both CYP2C19 could be due to linkage disequilibrium (LD) with CYP2C9.

Validation Cohort: 373 patients undergoing warfarin treatment were recruited retrospectively from 40 primary care practices across the West Midlands between June 2006 and October 2007. Patients' medical records were reviewed and clinical

information including demographic characteristics, indications for warfarin, warfarin dose changes, INR measurements and concurrent medication were collected.

2.2.2 Outcome measure

To develop our dosing algorithm, warfarin stable dose was taken as the main outcome measure. Stable dose was defined as a consistent mean daily dose leading to an individual's INR being within therapeutic range at three or more consecutive clinic visits. Given the anticoagulant effect of warfarin is due to sequential depression of Factor II, Factor VII, Factor IX, Factor X, Protein C and Protein S activities, with the S-warfarin elimination half-life at approximately 24-33 hours (Hirsh *et al.* 1998, Horton and Bushwick 1999), it was assumed that stable dose could only be observed after a period of at least 5 consecutive days of warfarin treatment. If several periods of stable dose were observed, the stable dose at the latest date was taken. The earlier stable dose was not chosen because the early periods of stable dose often associated with a short period of stable dose (example, stable for only 2-3 days).

2.2.3 DNA extraction

DNA was extracted from ethylenediaminetetraacetic acid (EDTA) whole blood using the phenol-chloroform method or the Chemagen Whole Blood DNA Extraction Kit on the Chemagic Magnetic Separation Module 1 (Auto-Q Biosciences, Germany).

Briefly, the phenol-chloroform method involved adding an equal volume of phenol-chloroform to an aqueous solution of lysed red and white blood cells, followed by vigorous mixing and phase separation via centrifugation. Since DNA is polar (because of its negatively charged phosphate backbone), DNA will partition into the aqueous phase (top layer). DNA recovery was subsequently achieved by ethanol precipitation as illustrated in Figure 2-1.

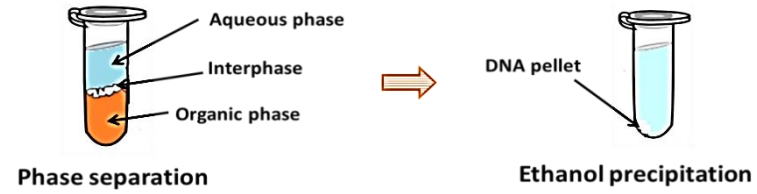


Figure 2-1. DNA extraction by phenol chloroform method.

Figure 2-2 outlines the semi-automated DNA extraction process on the Chemagic Magnetic Separation Module 1. The isolation of DNA is achieved through its capture by magnetic polyvinyl alcohol beads (M-PVA Magnetic Beads). The M-PVA Magnetic Beads exhibit both, a hydrophilic surface and low non-specific protein binding which allows for efficient binding to DNA with high yield and purity. When an electromagnetic field is applied, the beads together with DNA will be attracted to the magnetized metal rods, which then transfer the DNA from one washing buffer to another. At the end of each transfer step, the electromagnet is deactivated and the rotation of the rod is switched on, leading to an efficient and homogenous resuspension of the particles. In the final step, the elution buffer inactivates the interaction between the beads and the DNA. The magnetic beads are then removed, leaving the isolated DNA in suspension.

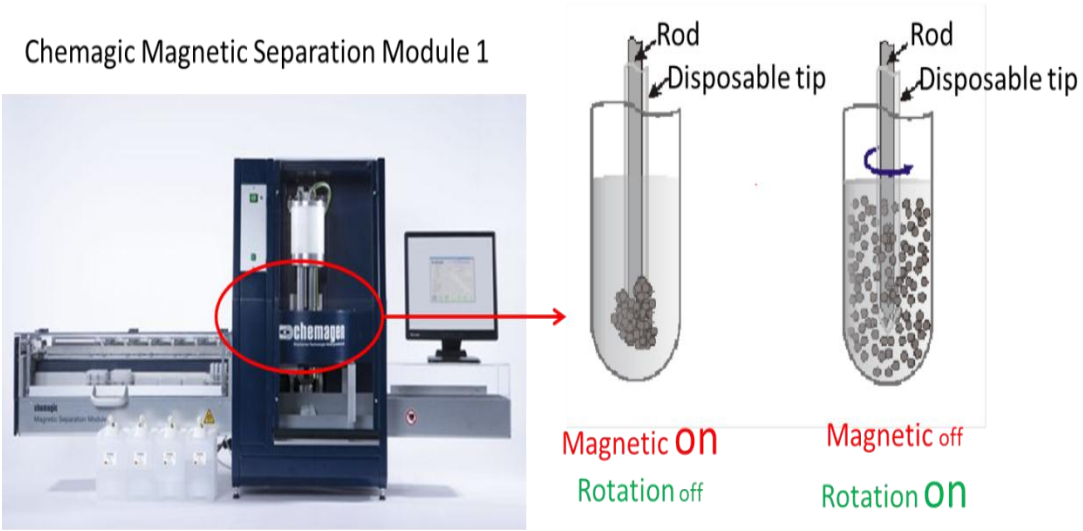


Figure 2-2. DNA extraction via magnetic beads technology. The magnetisable and rotatable rods allow the particles to be homogenously resuspended.

2.2.4 Genotyping

Genotyping of *CYP2C9*2* (rs1799853, C>T), *CYP2C9*3* (rs1057910, A>C) and *VKORC1 -1639* (rs9923231, -1639A>G) were performed on the ABI 7900HT Fast Real-Time polymerase chain reaction (PCR) system (Applied Biosystems). Details of the probes used are listed in Table 2-1.

Table 2-1. Sequences of Taqman probes for CYP2C9*2, CYP2C9*3 and VKORC1 -1639.

SNPs	Context Sequence	Design Strand
*CYP2C9*2*	GATGGGGAAGAGGAGCATTGAGGAC[**C**/**T**]GTGTTCAAGA GGAAGCCCGCTGCCT	Reverse
*CYP2C9*3*	TGTGGTGCACGAGGTCCAGAGATAC[**C**/**A**]TTGACCTTCTCC CCACCAGCCTGCC	Reverse
VKORC1 -1639	GATTATAGGCGTGAGCCACCGCACC[**C**/**T**]GGCCAATGGTT GTTTTTCAGGTCTT	Forward

Figure 2-3 illustrates the Taqman genotyping procedure. Approximately 10 ng of DNA was amplified in a 10 µl reaction mix in a 384–well optical plate containing 1x Taqman drug metabolism genotyping assay mix and 1x Taqman master mix. Thermo-cycling was carried out with an initial activation of the AmpliTaq Gold DNA polymerase at 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 15 sec and extension at 60°C for 90 sec. Allelic discrimination was determined using SDS version 2.2 software (Applied Biosystem). Examples of the allelic discrimination plots and their interpretation are shown in Figure 2-4 to Figure 2-6.

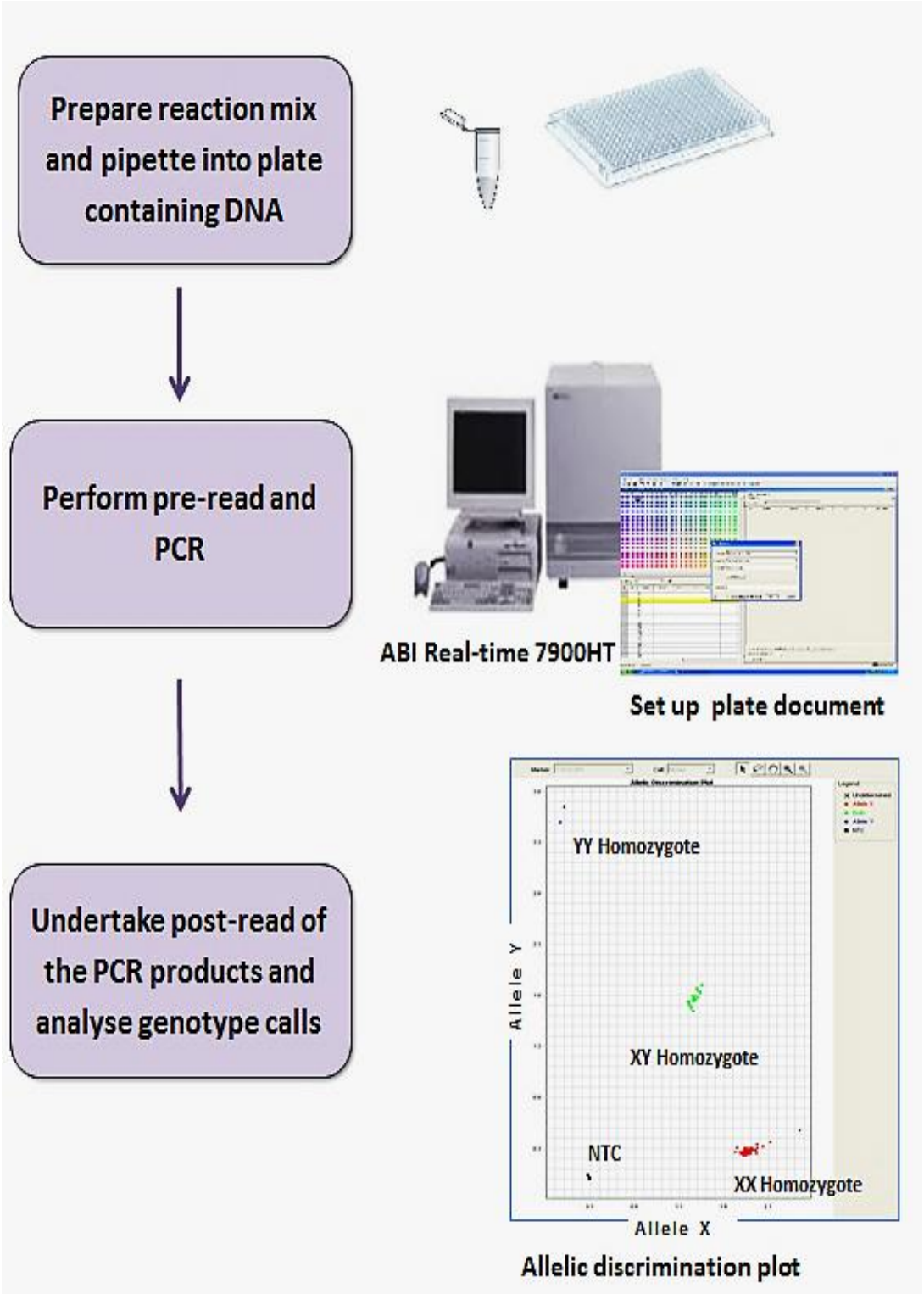


Figure 2-3. Taqman genotyping procedure. The reaction mix was freshly prepared and pipetted to the dried DNA. Two document plates, allelic discrimination and absolute quantification, were set up. Pre- and post-read were performed to ensure accuracy of the results, where any background noise was taken into account when the end point flourescene was determined. On the allelic discrimination plot, the red dot indicates XX homozygote, the green dot indicates XY homozygote, and the blue dot indicates YY heterozygote.

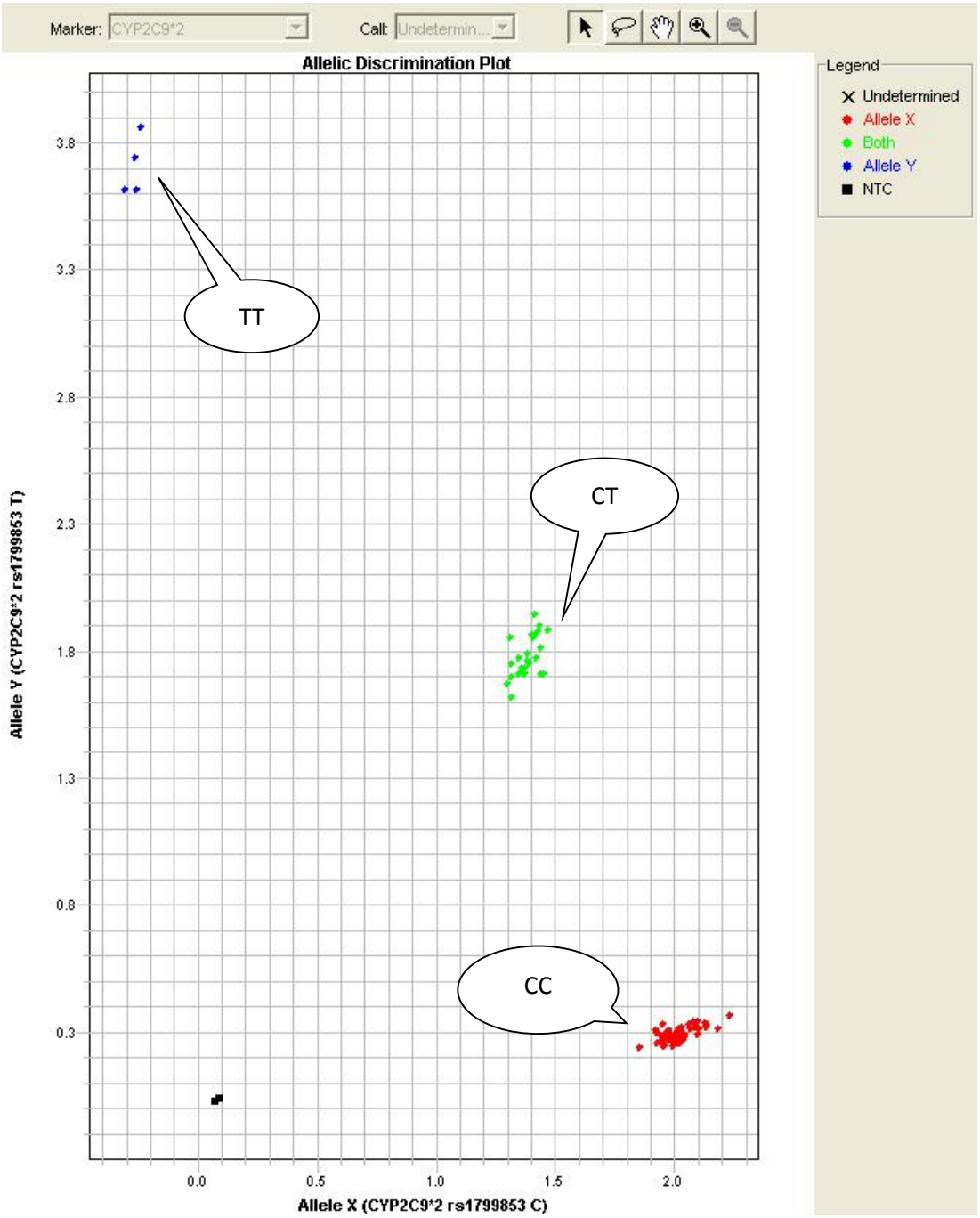


Figure 2-4. Allelic discrimination plot for *CYP2C9*2* genotype using ABI Taqman® Real-Time Polymerase Chain Reaction platform. NTC: No template control.

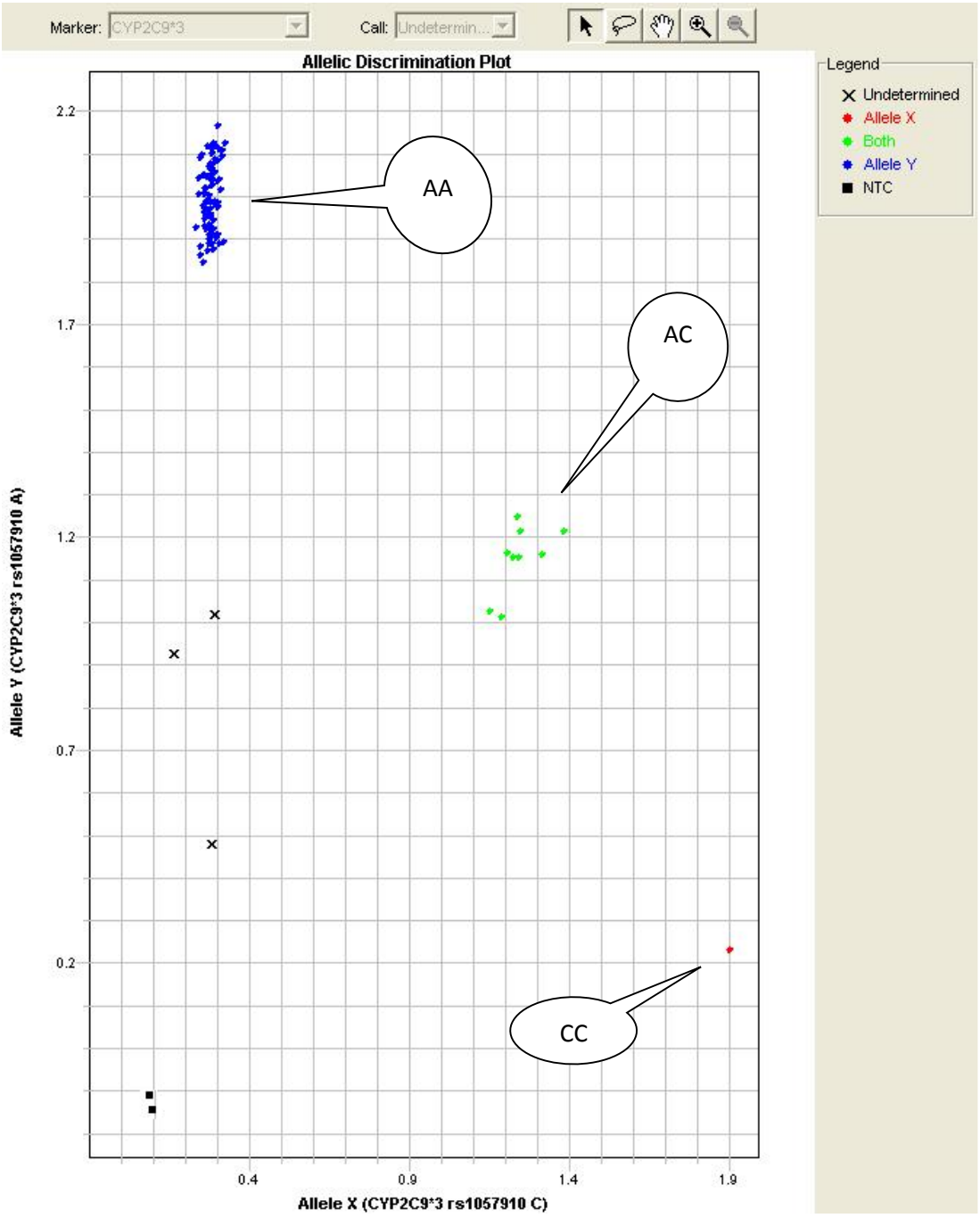


Figure 2-5. Allelic discrimination plot for *CYP2C9*3* genotype using ABI Taqman® Real-Time Polymerase Chain Reaction platform. NTC: No template control.

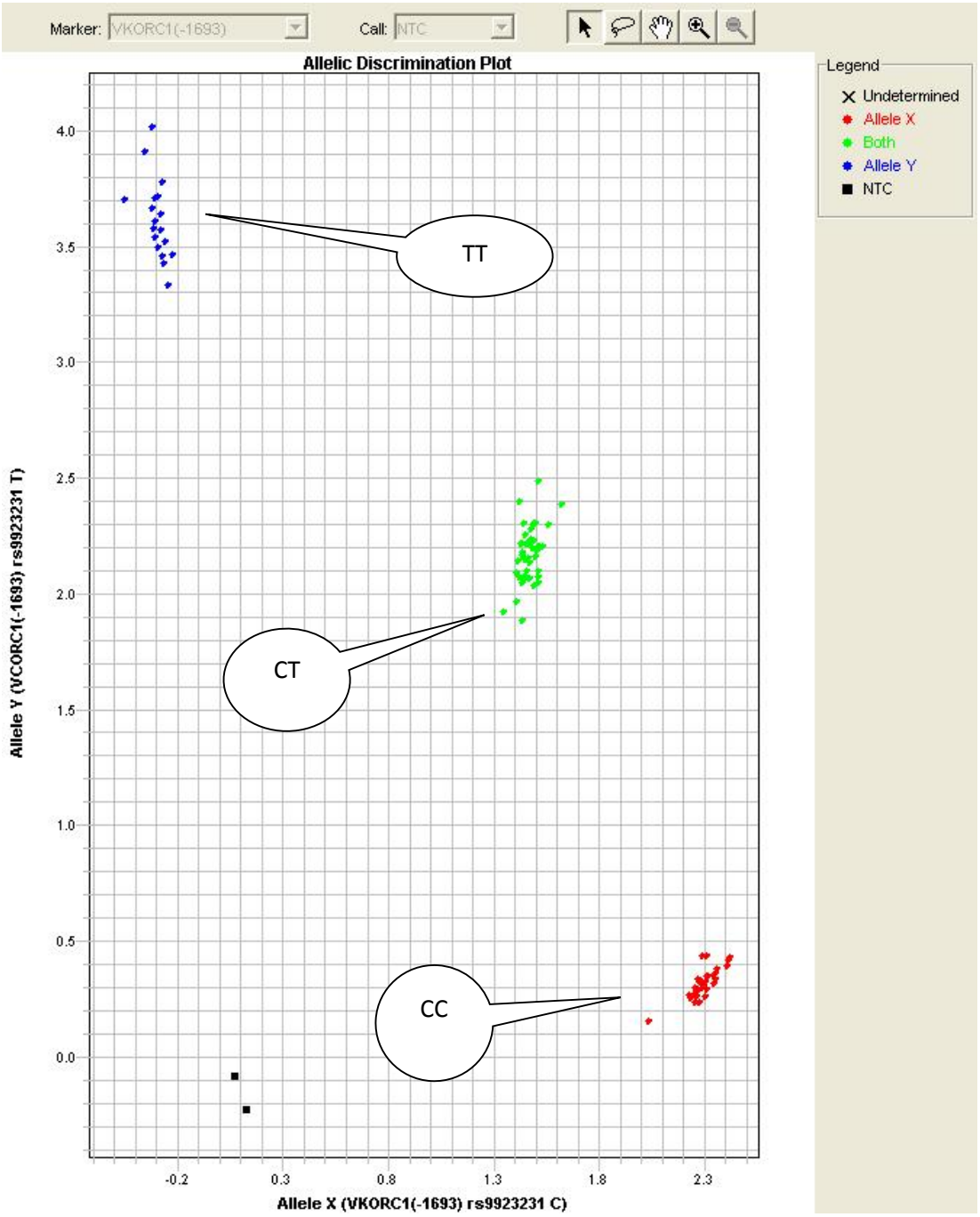
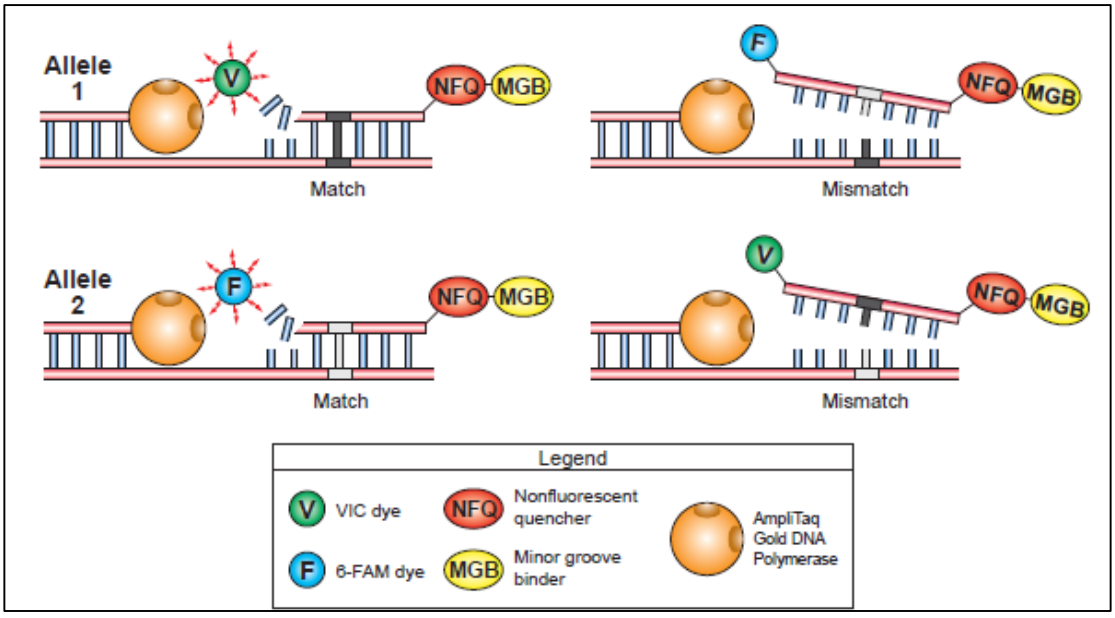


Figure 2-6. Allelic discrimination plot for *VKORC1-1693* genotype using ABI Taqman® Real-Time Polymerase Chain Reaction platform. NTC: No template control.

The Taqman SNP drug metabolism genotyping assay is also called the 5'-nuclease allelic discrimination assay. It requires a pair of forward and reverse PCR primers to amplify the polymorphism of interest, and two Taqman probes for detecting specific alleles. Each Taqman probe has a fluorescent reporter dye (VIC of FAM) and a non-flourescent quencher attached to its 5' and 3' end, respectively. In addition, each probe has a minor groove binder (MGB) at its 3' end which increases the melting temperature (T_m) of a given probe length, allowing the design of shorter probes (Afonina *et al.* 1997, Kutyavin *et al.* 1997). A greater difference in T_m value between the matched and mismatched probes will result in more robust allelic discrimination. Figure 2-7 illustrates the Taqman Probe-based genotyping assay chemistry.



Adapted from TaqMan® Drug Metabolism Genotyping Assays Protocol, Applied Biosystems

Figure 2-7. Taqman probe-based genotyping assay chemistry. During PCR, each Taqman MGB probe anneals specifically to its complementary sequence between the forward and reverse primer sites. Taq DNA polymerase cleaves the reporter dye from the probe and once the probe is cleaved, the reporter dye is free of the quencher dye and emits a fluorescent signal. Therefore, an increase in either FAM or VIC dye fluorescence indicates homozygosity for FAM and VIC specific alleles and an increase in the fluorescence of both dyes indicates heterozygosity.

To ensure quality control, the PCR plate was designed to contain 10% duplicates, two negative controls (using DNase-free water) and two positive controls for each SNP.

2.2.5 Statistical analysis

The assessment of conformity with Hardy Weinberg Equilibrium (HWE) for each SNP was performed using Haploview software (version 4.2). A *p* value of <0.001 was assumed to indicate deviation from HWE. SPSS (version 17) was used to analyse associations between variables and outcomes. As the distribution of warfarin stable dose was skewed, a square-root data transformation was performed to achieve normality (Figure 2-8).

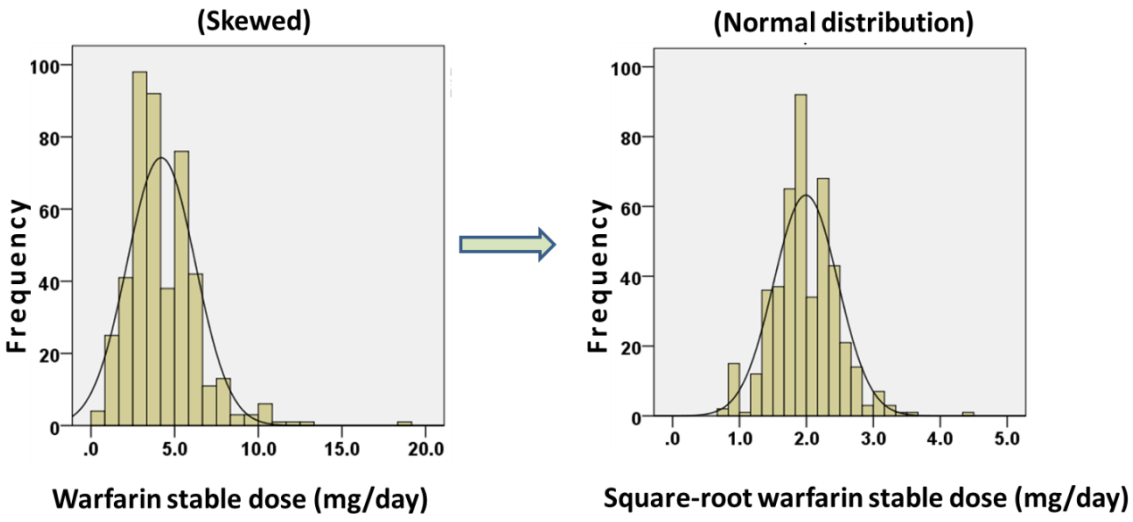


Figure 2-8. Square-root transformation of stable warfarin dose. Normal distribution was achieved by square-root transformation. Normal distribution was achieved by square-root transformation.

2.2.5.1 Univariate analysis

As discussed in section 1.8 (page 23), it is widely known that age, height and weight are significantly correlated with warfarin dose. The following baseline clinical factors were therefore pre-identified as potential predictors of warfarin stable dose in our study: age (Anderson *et al.* 2007), weight (Carlquist *et al.* 2006, Anderson *et al.* 2007), height (Sconce *et al.* 2005), body mass index (BMI) (Wells *et al.* 2010), body surface area (BSA) (Gage and Lesko 2008), gender (Hillman *et al.* 2004) and amiodarone use (Heimark *et al.* 1992). BMI was calculated by dividing weight (kg) over squared height (m) (World Health Organization). To determine BSA, the Mostereller equation was used due to its accuracy and ease of use (Vu 2002) and the equation are: $BSA = \sqrt{((Height\ (cm) \times Weight\ (kg)/3600)}$.

Each genetic and non-genetic factor was univariately assessed for association with warfarin stable dose in the derivation cohort. Continuous variables were examined using univariate linear regression while binary variables were analysed using the Student's T-test. Associations with genotypes were assessed using univariate analysis of variance (ANOVA). The correlations between BMI, weight, height and BSA were tested using Pearson Correlation. Bonferroni correction was used to correct for multiple testing. Bonferroni-corrected p values are denoted as Pc-values. All variables giving a p value <0.05 in the univariate analysis were subsequently included in the multiple regression model.

2.2.5.2 Multiple regression analysis

Using both genetic and clinical factors, a multiple linear regression model was developed to build an algorithm for predicting the stable therapeutic dose of warfarin. A stepwise variable selection method was used, with variables retained in the model if they achieved statistical significance ($p<0.05$). *CYP2C9*2*, *CYP2C9*3* and *VKORC1 -1639* polymorphisms were coded to reflect an additive mode of inheritance (0, wildtype; 1, heterozygous; 2, homozygous).

To assess the predictability and accuracy of our algorithm, the following parameters were investigated:

i) ***R^2derived from the multiple linear regression model.*** R^2 describes the proportion of variance explained by the model. The R^2 value ranges from 0 to 100 where 100 means the model fits the observed dependent variable values perfectly.

ii) ***Mean or Median absolute error*** is calculated as the average absolute values for the difference between algorithm-predicted and observed warfarin stable dose. Lower mean or median absolute error value indicates better accuracy.

iii) ***The proportion of patients whose predicted doses fell within 20% of their actual daily dose***. As described in previous studies, the ideal dose was defined as a predicted dose that differed by no more than 20% from the actual stable therapeutic dose of warfarin (Klein *et al.* 2009, Finkelman *et al.* 2011). These values reflect a difference of 1 mg/day relative to the traditional starting dose of 5 mg/day, which clinicians would likely regard as clinically relevant (Klein *et al.* 2009).

2.3 Results

2.3.1 Clinical and genetic characteristics of participants

Derivation cohort: Out of the 1000 patients recruited, 911 patient were successfully genotyped for *CYP2C9*2*, *CYP2C9*3* and *VKORC1 -1639*. Of these, 468 achieved stable dose in accordance with our definition. Seven non-Caucasians were excluded from the analysis. A further 5 patients with missing data were also excluded. Overall, 456 patients were included in the derivation of our pharmacogenetic algorithm.

Validation cohort: Of the 373 recruited patients, 371 were genotyped successfully. 266 achieved stable dose but 4 patients were excluded due to missing data. As such, only 262 patients were included in the analysis.

The baseline characteristics of both cohorts including demographics, indications for warfarin treatment and warfarin dose requirements are presented in Table 2-2. The majority of patients were prescribed warfarin for the treatment of atrial fibrillation with target INR 2-3, with cardiovascular disease being the most common underlying co-morbidity. No significant differences in clinical characteristics such as age, body weight, sex and indications of warfarin were observed between the two cohorts.

Table 2-2. Baseline characteristics of patients in our derivation and validation cohorts who have achieved warfarin stable dose.

Characteristic	**Derivation cohort n= 456**	**Validation cohort n=262**
Gender – Male *[n, %]*	254 (55.7)	150 (57.3)
Age (years*) [mean, sd]*	68 (13)	74 (10)
Weight (kg) *[mean, sd]*	82 (19)	80 (16)
Height (cm)*[mean, sd]*	169 (10)	169 (10)
BMI [*mean, sd]*	29 (6)	28 (5)
Indications *[n, %]*		
Atrial Fibrillation	302 (66.2)	197 (75.2)
Pulmonary Embolism	76 (16.7)	22 (8.4)
Deep Vein Thrombosis	42 (9.2)	26 (9.9)
Mitral Heart Valve Prosthetic	1 (0.2)	6 (2.3)
Cerebrovascular accident & transient Ischemic diseas	27 (5.9)	10 (3.8)
Others	8 (1.8)	1 (0.4)
Concomitant Disease *[n, %]*		
Cardiovascular	366 (80.3)	189 (72.1)
Respiratory disease	162 (35.5)	33 (12.6)
Hepatic disease	15 (3.3)	0
GI disease	166 (36.4)	5 (1.9)
Renal Disease	44 (9.6%)	50 (19.1)
Neurological Disorder	86 (18.9)	25 (9.0%)
Amiodarone Use *[n, %]*	34 (7.5%)	14 (5.3)
Warfarin dose (mg/day)		
Median [*IQR]*	4.0 (0.38)	4.0 (1.9)
Mean [*sd*]	4.2 (2.04)	4.12 (0.5)
Smoker *[n, %]*	63 (13.8)	14 (5.3)
Alcohol intake (at least 2 or 3 times per week) *[n, %]*	152 (33.3)	162 (61.8)

The genotype frequencies of *CYP2C9*2*, *CYP2C9*3* and *VKORC1 -1639* for both, the derivation and validation cohorts are summarized in Table 2-3. The genotype frequencies for all 3 SNPs conformed to Hardy-Weinberg equilibrium. When the two cohorts were combined, the minor allele frequencies (MAF) for *CYP2C9*2*, *CYP2C9*3* and *VKORC1 -1639* were 0.13, 0.06 and 0.37, respectively, and the MAFs are comparable to previous studies conducted in British populations (Stubbins *et al.* 1996, Aithal *et al.* 1999, Sconce *et al.* 2005).

Table 2-3. Genotype frequencies of *CYP2C9*2*, *CYP2C9*3* and *VKORC1-1639* polymorphisms in patients from our derivation and validation cohorts.

Genotypes	Derivation cohort (n= 456) *n*, (%)	Validation cohort (n=262) *n*, (%)
*CYP2C9*2*		
Wild-type (CC)	351 (77.0)	189 (72.1)
Heterozygous mutant (CT)	101 (22.1)	68 (26.0)
Homozygous mutant (TT)	4 (0.9)	5 (1.9)
Hardy-Weinberg p-value	0.9	0.9
*CYP2C9*3*		
Wild-type (AA)	402 (88.2)	233 (88.9)
Heterozygous mutant (AC)	51 (11.2)	29 (11.1)
Homozygous mutant (CC)	3 (0.7)	0
Hardy-Weinberg p-value	0.5	0.9
VKORC1 -1639		
Wild-type (CC)	183 (40.1)	100 (38.2)
Heterozygous mutant (CT)	210 (46.1)	123 (46.9)
Homozygous mutant (TT)	63 (13.8)	39 (14.9)
Hardy-Weinberg p-value	0.9	0.9

2.3.2 Association of clinical and genetic factors with stable warfarin dose

Clinical and genetic factors which showed significant univariate associations ($p<0.05$) in our derivation cohort are listed in Table 2-4. An increase in age was associated with decreased warfarin stable dose while increase in weight, height, BMI and BSA were associated with elevated warfarin stable dose requirements. When compared to female patients, male patients required higher doses of warfarin to achieve stability. Patients who took amiodarone as a concomitant medication showed a significant reduction in warfarin dose requirement.

Table 2-4. Significant variables found in univariate analysis.

Variables	Univariate p-value	Effect on warfarin stable dose	R^2
Age	1.1×10^{-10}	↓	0.09
Weight	7.0×10^{-13}	↑	0.11
Height	4.7×10^{-6}	↑	0.04
BMI	2.3×10^{-7}	↑	0.06
BSA	2.7×10^{-13}	↑	0.11
Sex	5.1×10^{-5}	↑, Male	0.03
Amiodarone use	0.018	↓	0.008
*CYP2C9*2*	2.2×10^{-5}	↓	0.04
*CYP2C9*3*	1.15×10^{-6}	↓	0.04
VKORC1 -1639	3.6×10^{-27}	↓	0.21

Given that weight, height and the derived BMI and BSA indices were all highly inter-correlated ($p<0.001$), BSA which gave the lowest univariate p value was selected to be included in our multiple linear regression analysis.

Figure 2-9 illustrates the distribution of stable dose in our derivation cohort of patients stratified for the *CYP2C9*2*, *CYP2C9*3* and *VKORC1 -1639* genetic polymorphisms. Carriers of the minor alleles for *CYP2C9*2*, *CYP2C9*3* and *VKORC1 -1639* exhibited a significantly reduced warfarin dose requirement when compared to carriers of the major allele.

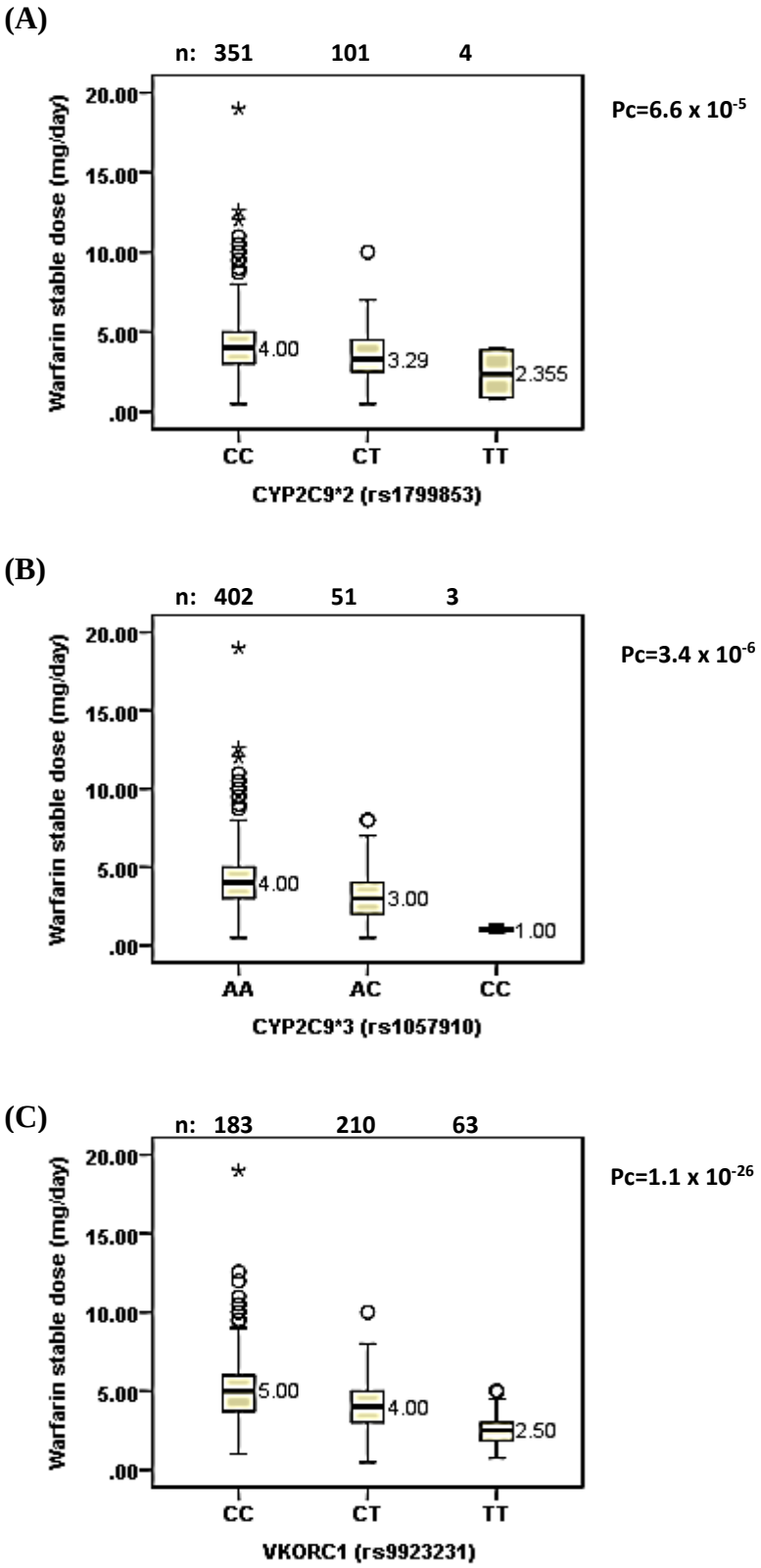


Figure 2-9. Box and whisker plots showing the distribution of daily warfarin doses based on (A) *CYP2C9*2* (B) *CYP2C9*3* and, (C) *VKORC1 -1639* genotypes. Boxes represent 25th-75th percentile (interquartile range) of warfarin dose, whiskers represent 5th-95th percentile, solid lines represent median dose. Open dots denote outliers (a value between 1.5 and 3 times the interquartile range away from the 25th or 75th percentile) and asterisks denote extreme outliers (a value more than 3 times the interquartile range away from the 25th or 75th percentile).

2.3.3 Deriving a pharmacogenetic algorithm for warfarin stable dose

To identify the main clinical and genetic factors affecting warfarin stable dose, multiple linear regression analysis was performed. After stepwise selection, only the predictors age, BSA, amiodarone use, *CYP2C9*2*, *CYP2C9*3* and *VKORC1 -1639* remained significant and were included in our model. Using the coefficients of the regression estimates, the algorithm for predicting daily warfarin stable dose was calculated:

*Predicted daily warfarin stable dose (mg/day) = [2.135 -0.313(VKORC1 -1639) + 0.417(BSA) – 0.009(Age) – 0.309 (CYP2C9*3) – 0.186 (CYP2C9*2) – 0.199 amiodarone]* ^{2}

where BSA is in m^2; age in years; amiodarone use was coded 0 if not prescribed and 1 if prescribed. Our pharmacogenetic model explained 46% of warfarin dose variance and gave a mean (median) absolute error of 1.04 (0.78) (Table 2-5).

Table 2-5. Performance and accuracy comparison of our pharmacogenetic model in the derivation and validation cohorts.

Prediction assessment	Derivation cohort	Validation cohort
Dose variability explained by R^2:		
Genetic and clinical factors	46%	43%
▪ *Clinical factors: (BSA, Age and amiodarone use)*	31%	29%
▪ *Genetic factors: (CYP2C9*2, CYP2C9*3 and VKORC1)*	15%	16%
Mean absolute error (mg/day) (95% Confidence Interval, *CI)*	1.04 (0.94-1.15)	1.08 (0.95-1.20)
Median absolute error (mg/day)	0.78	0.88

2.3.4 Validating the pharmacogenetic algorithm for warfarin stable dose

The performance of our pharmacogenetic algorithm in our validation cohort is shown in Table 2-5. Our algorithm explained 43% of warfarin dose variance in the validation cohort consisting of 262 individuals, and gave a mean (median) absolute error of 1.08 (0.88) mg/day.

To assess the clinical accuracy of our pharmacogenetic algorithm, we calculated the percentage of patients in the validation cohort whose predicted dose was within 20% of the actual therapeutic stable dose. 49.6% of the dose prediction fell within 20% of the actual dose. As shown in Figure 2-10a, our pharmacogenetic algorithm provided accurate dose estimates for the majority of patients requiring more than 2 mg and less than 6mg of warfarin per day. 61.8% of dose predictions for this group of patients fell within the ideal dose range, 15.7% of dose predictions were underestimated, and 22.5% of dose predictions were overestimated (Table 2-6). Our pharmacogenetic algorithm however, did not perform as well for patients who required $\leq$ 2mg/day or $\geq$ 6 mg/day, giving absolute dose differences of $\geq$ 2 mg/day in the majority of the patients (Figure 2-10b). For patients requiring 2 mg or less of warfarin per day, our pharmacogenetic algorithm correctly predicted 26.7% of patients while the remaining 73.3% of dose predictions were overestimated. For patients requiring 6 mg or more of warfarin per day, our pharmacogenetic algorithm accurately predicted 9.7% of patients while the remaining 90.3% of dose estimates were underpredicted.

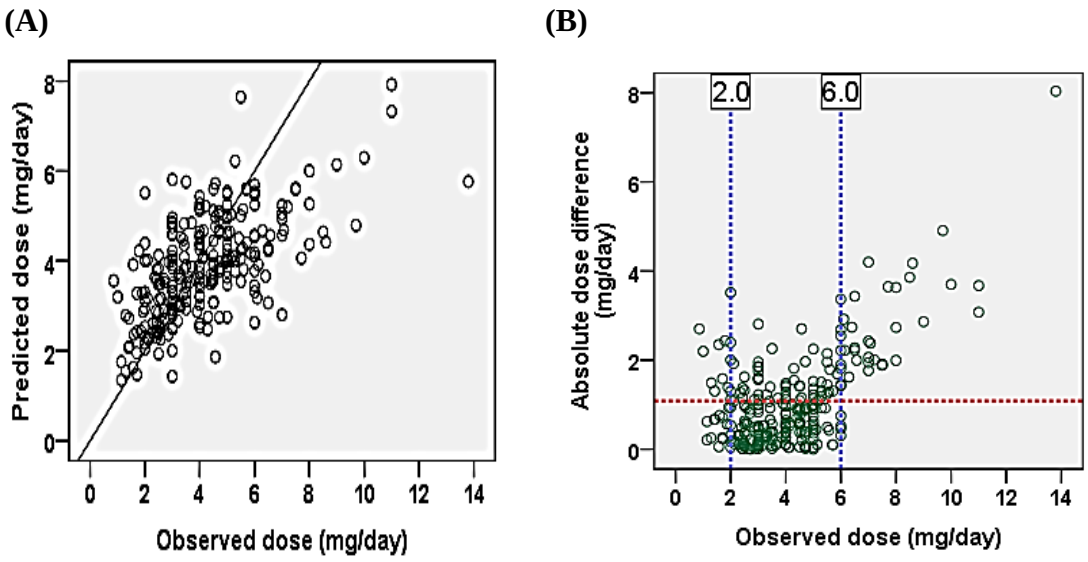


Figure 2-10. Evaluation of pharmacogenetic algorithm-predicted doses versus observed therapeutic doses in the validation cohort (n=262). (A) Scatter plot comparing the observed and predicted warfarin stable doses in our validation cohort. Each black circle represents the predicted versus observed dose for each subject. The solid line represents perfect prediction (y=x). (B) Scatter plot comparing the absolute dose difference versus observed dose in our validation cohort. Each green circle represents the absolute dose difference versus observed dose for each subject. The red dotted line represents mean absolute error and the two blue dotted lines encompass individuals with observed warfarin dose 2-6 mg/day.

Table 2-6. Accuracy of our pharmacogenetic algorithm based on percentage dose estimates within 20% of observed dose in the validation cohort.

Warfarin dose	Patients *[n]*	Ideal dose *[n (%)]*	Underestimation [*n (%)]*	Overestimation [*n (%)]*
Validation cohort				
≤ 2 mg/day	30	8 (26.7)	0 (0)	22 (73.3)
>2 to <6 mg/day	191	118 (61.8)	30 (15.7)	43 (22.5)
≥ 6 mg/day	41	4 (9.7)	37 (90.3)	0 (0)
Total	**262**	**130 (49.6)**	**67 (25.6)**	**65 (24.8)**

Ideal dose: Predicted dose was within 20% of the actual stable therapeutic dose.
Underestimation: Predicted dose was below 20% of the actual stable therapeutic dose.
Overestimation: Predicted dose was more than 20% of the actual stable therapeutic dose.

2.4 Discussion

To date, this is the first study which derived a pharmacogenetic algorithm using one large prospective cohort of British Caucasian patients initiated onto warfarin therapy. Utilising data from 456 prospectively recruited patients, we developed a pharmacogenetic algorithm which incorporated BSA, age, amiodarone use, and the alleles *CYP2C9*2, CYP2C9*3* and *VKORC1*. Using an external cohort of 262 patients recruited retrospectively as a validation cohort, our pharmacogenetic algorithm predicted 43% of warfarin dose variability.

In line with previous studies (Kaminsky *et al.* 1993, Aithal *et al.* 1999, Bodin *et al.* 2005, D'Andrea *et al.* 2005, Herman *et al.* 2005, Sconce *et al.* 2005, Aquilante *et al.* 2006, Carlquist *et al.* 2006, Kosaki *et al.* 2006, Schalekamp *et al.* 2006, Borgiani *et al.* 2007, Cho *et al.* 2007, Limdi *et al.* 2008a, Huang *et al.* 2009), our results replicate the associations of *CYP2C9*2, CYP2C9*3* and *VKORC1 -1639* with warfarin stable dose, where patients carrying the *CYP2C9*2, CYP2C9*3* and *VKORC1-1639* minor allele resulted in lower dose requirements relative to the wild-type carriers.

In agreement with Gage et al, (2008), our results showed that *VKORC1 -1693* is the most important predictor (R^2=21%). This polymorphism alters a VKORC1 transcription factor binding site (Yuan *et al.* 2005) and a *VKORC1* haplotype containing the *VKORC1-1639* A allele was reported to result in lower mRNA expression in human liver (Rieder *et al.* 2005). This change in gene expression may lower the steady-state tissue concentration of vitamin K epoxide reductase, the rate limiting enzyme in vitamin K cycle (Wajih *et al.* 2005). As a result individuals with this allele are most susceptible to inhibition by warfarin.

In the present study, we developed and validated a pharmacogenetic-algorithm incorporating clinical and genetic data for predicting warfarin dose. Our pharmacogenetic algorithm explained less warfarin stable dose variability compared

to the pharmacogenetic algorithm developed by Sconce *et al.* which was validated in a very small cohort of patients (Sconce *et al.* 2005, Hatch *et al.* 2008). The largest prospective warfarin cohort study predicting warfarin dose at initiation of therapy was carried out in Swedish population (Wadelius *et al.* 2009). They derived the pharmacogenetic algorithm which included *CYP2C9*2, CYP2C9*3, VKORC1*, age, sex and number of interacting drugs that potentially increase INR (Appendix A) in 1,496 patients and validated in 181 patients. The pharmacogenetic algorithm explained 52.8% of warfarin stable dose variability which is better compared to our pharmacogenetic algorithm, but the clinical accuracy of the algorithm was not evaluated, thus we cannot establish that their algorithm is better in predicting warfarin stable dose.

For that reason, we calculated the mean and median absolute error of our algorithm. Our mean (median) absolute error is comparable to the study by Gage *et al.* (2008) who used *CYP2C9*2, CYP2C9*3, VKORC1-1639*, target INR, concomitant drug-amiodarone use, smoker, race and indication of warfarin given mean absolute error of 1.0 (1.3) mg/day but better compared to the seminal study by IWPC (Klein *et al.* 2009), where they derived (n=4,043) and validated (n=1,009) pharmacogenetic algorithm from multiple sites worldwide (including patients from our derivation cohort) using *CYP2C9*2*, *CYP2C9*3*, *VKORC1*, age, height, weight, race, concomitants drug – enzyme inducer (carbamazepine, phenytoin, rifampin and rifampicin), concomitant drug – amiodarone given mean absolute error of 1.2mg/day.

We evaluated the potential clinical value of our pharmacogenetic algorithm by calculating the percentage of patients whose predicted dose of warfarin was within 20% of actual dose. In a study by IWPC (Klein *et al.* 2009), their pharmacogenetic algorithm predicted only 35% of patients within 20% of the actual dose. Another retrospective study by Finkelman *et al.* (2011) in 1,378 patients using the

pharmacogenetic algorithm developed by Gage *et al.* (2008) predicted 52% of patients within 20% of the actual dose. Our algorithm has a similar predictability as Gage *et.al* (2008) but the advantage of our pharmacogenetic algorithm eless complicated. Our algorithm only requires VKORC1 and CYP2C9 genotypes, height and weight to calculate BSA and amiodarone use status. While in Gage et al's algorithm, another 5 information need to be collected . In addition, it is difficult to assess accurately for smoking status (Lewis *et al.* 2003, Martínez *et al.* 2004) and race (Foster and Sharp 2002).

Observing the graphs presented in Figure 2-10(a) which compared the observed dose with predicted dose and (b) which compared observed dose with mean absolute error suggesting that our pharmacogenetic algorithm is of most benefit to the group of patients who required a warfarin stable dose range 2-6 mg/day with 62% of prediction within the ideal dose range. Similar predictability accuracy was evidenced in the IWPC study for those patients who required a warfarin stable dose range 4-6 mg/day. Therefore, our pharmacogenetic algorithm benefits the larger group of Caucasian patients.

Our algorithm only explains approximately 43% of warfarin stable dose variability and there still remains 57% to be explained. There are other clinical factors which would contribute to warfarin stable dose which we did not include in our analysis such as vitamin K intake (Reaves *et al.* 2013), diabetes mellitus (Lenzini *et al.* 2010), and alternative medicine (Mousa 2010, Na *et al.* 2011). In addition, we did not include drugs other than amiodarone which have been shown in the literature to affect warfarin dose requirement (example as in Table 1-8, page 24). Despite the available literature review linking warfarin dose/response and smoking and alcohol intake (Havrda *et al.* 2005, Nathisuwan *et al.* 2011, Bazan *et al.* 2013), these factors were not included in our analysis as they are challenging to assess accurately and

objectively. It is also likely that other SNPs influence warfarin stable dose such as polymorphism in *CYP4F2* (McDonald *et al.* 2009, Zhang *et al.* 2009), *APOE* (Kimmel *et al.* 2008), *FII* (D'Ambrosio *et al.* 2004) or some rare variants. Recent publications have reported some rare variants associated with lower requirement of warfarin dose in patients with *CYP2C9*12* (rs9332239) genotypes (O'Brien *et al.* 2013) and higher requirement of warfarin dose in patients with *VKORC1 D36Y* (rs61742245) genotypes (Kurnik *et al.* 2012). The MAF for CYP2C9*12 and VKORC1 D36Y in Caucasians was reported as 0.006 and 0.04 (Kurnik *et al.* 2012, O'Brien *et al.* 2013) respectively.

Whether our pharmacogenetic algorithm can be practiced in a clinical setting is still on open question. Other clinical and genetic factors could be incorporated into a future pharmacogenetic algorithm to improve their accuracy. However, prospective randomised control trials are required to determine whether initial warfarin dosing would improve the outcome of warfarin therapy. At the moment, there are four randomised multicentre controlled trials currently underway with this aim. Three of them are in the United States; the **c**larification of **O**ptimal **A**nticoagulation through **G**enetics trial (COAG), the **G**enetic **Inf**ormatics **T**rial (GIFT) of Warfarin to Prevent Deep Venous Thrombosis and the Clinical and Economic Implication of Genetic Testing for Warfarin Management trial. The other one is in Europe, the European Pharmacogenetic of Anticoagulant trial (EU-PACT). Information about the studies can be view at *www.clinicaltrials.gov*. If such trials show a benefit of using a pharmacogenetic algorithm, then successful implementation of the new algorithm would depend on the availability of a rapid and accurate genotyping method which allows easy genotyping in a clinical setting.

Chapter 3

Incorporating genetic factor into HAS-BLED, a bleeding risk score

CONTENTS

3.1 Introduction

Atrial fibrillation (AF) is the most common heart arrhythmia, with a prevalence of approximately 1.2% in primary care in the UK (NHS Improvement 2009). Stroke is the leading complication of AF and patients with AF have a five-fold higher stroke risk than those without AF (NICE 2006).

There is much evidence that long-term treatment with an oral anticoagulant (OAC) such as warfarin, reduces the risk of stroke associated with AF (Hart *et al.* 1999, Hart *et al.* 2007, Singer *et al.* 2009). However, the main drawback to this approach is a high risk of bleeding (Budnitz *et al.* 2007, Shehab *et al.* 2010). It is estimated that every year, 1-7% of patients on long-term treatment with OAC will suffer a major bleeding event which requires hospitalisation (Levine *et al.* 2004, Mant *et al.* 2007).

As increasing numbers of patients with AF will be treated using this drug class, efforts to develop tools for the estimation of bleeding risk may be helpful in counselling patients and informing them of treatment decisions. Recently, HAS-BLED (acronym for **H**ypertension, **A**bnormal kidney/liver function, **S**troke, **B**leeding disposition, **L**abile INR, **E**lderly and **D**rug/alcohol use) has been recommended by the European Society of Cardiology guidelines (ESC) as a bleeding risk assessment tool to be used before starting anticoagulation treatment for the management of atrial fibrillation (Camm *et al.* 2012).

HAS-BLED is a bleeding risk score to assess the one year risk of major bleeding in AF patients (Pisters *et al.* 2010). It was derived from a European AF cohort participating in the EuroHeart survey, a survey and registry which provide extensive data on the management of heart failure patients from 24 countries (Cleland *et al.* 2003, Komajda *et al.* 2003). In the original study, the prediction ability of HAS-BLED was assessed using the c-statistic. The c-statistic is the area under the curve (AUC) of the

receiver operating characteristic (ROC) graph and it is considered that this can capture the ability of the model to discriminate between those who will develop the event and those who will not (Hanley and McNeil 1982).

Hosmer and Lemeshow (2000) provide general rules to interpret AUCROC values: 0.5 means that discrimination is no better than by chance, 0.7 to 0.8 shows acceptable discrimination, 0.8 to 0.9 indicates excellent discrimination and ≥ 0.9 describes outstanding discrimination. In brief, the ability of HAS-BLED to discriminate was acceptable (good) when tested in patients treated with warfarin (32% of the whole cohort). The value of the c-statistic was better in groups of patients taking warfarin concomitantly with anti-platelet drugs, compared to those who were taking warfarin alone. The c-statistic in the first group (n=239) was 0.78 (95% CI 0.65-0.91), and in the second group (n=1,722), it was 0.69 (95%CI 0.59-0.80).

HAS-BLED has been first externally validated in a trial cohort of 7,329 patients participating in the SPORTIF III and V clinical trials, a randomized controlled trial (RCT) comparing ximelagatran with warfarin for stroke prevention in atrial fibrillation. (Lip *et al.* 2011b). In this validation study, HAS-BLED predictability was compared with the predictability of other contemporary scores as proposed by Shireman *et al.* (2004), Gage *et al.* (2006), Beth *et a*l. (1998), and Kuijer *et al.* (1999). A description of HAS-BLED and the four comparative schemes is presented in Table 3-1. The HAS-BLED scheme showed a marginally better c-statistic value than the other four schemes evaluated (Table 3-2), but there was overlap of the 95% confidence intervals in all cases.

Later, HAS-BLED score has been compared and shown better performance compared to other bleeding scores that have been derived and validated in patients with AF (eg HEMORR2HAGES (Gage et al. 2006) and ATRIA (Fang et al. 2011))

but none of the studies reached statistical significance (Olesen *et al.* 2011b, Apostolakis *et al.* 2012).

Table 3-1. Bleeding Risk Stratification Schemes tested by Lip et al., 2011b.

Schemes	Study design	Calculation of bleeding risk score
Pisters *et al.* (2010)	The score was derived from bleeding risk factors identified from 3,381 atrial fibrillation patients from Euro Heart Survey cohort (12 months retrospective follow-up) together with consistent risk factor for major bleeding identified in systemic review. The score was compared with HEMORR2HAGES scheme (without genetic data) in similar cohort and was found better in prediction	Acronyms: HAS-BLED Hypertension (1 point), Abnormal Renal/Liver Function (1 point each), Stroke, Bleeding History or Predisposition(1 point) , Labile INR(1 point) , Elderly Drugs/ Alcohol concomitantly (1 point each)
Shireman *et al.* (2006)	Retrospective chart review of the National Registry of Atrial Fibrillation (NRAF) cohort and Medicare data of atrial fibrillation patients with 3 months follow-up (n= 26,386) *Derivation cohort:* 19,875 patients *Validation cohort:* 6,511 patients	(0.49 x age >70 years)+(0.32 x female) + (0.58 x remote bleed) +(0.62 x recent bleed) + (0.71 x alcohol/drug abuse) + (0.27 x diabetes) + (0.86 x anaemia) + (0.32 x antiplatelet drug use) with 1 point for presence of each, and 0 if absent
Gage *et al.* (2006)	*Derivation*: The score was derived from systematic review} and PubMed search. *Validation cohort:* Retrospective analysis of 3,791 patients' data from the National Registry of Atrial Fibrillation (NRAF). 1604 were discharged on warfarin (113 of whom also received aspirin), 660 were discharged on aspirin alone and 1,527 were discharged without any anti-thrombotics. However, CYP2C9 genotype was unavailable for the study and was omitted in validation.	Acronyms: $HEMORR_2HAGES$ Liver/renal disease, Ethanol abuse, malignancy, age>75 years, low platelet count or function, re-bleeding risk, uncontrolled hypertension, anaemia, genetic factors (*CYP2C9*), risk of fall or stroke, with 1 point for each factor present with 2 points for previous bleed.
Kuijer *et al.* (1999)l	Use database of Columbus Investigators (prospective cohort). 1021 VTE patients randomly allocated to receive an initial subcutaneous Low Molecular Weight Heparin or continuous fractioned heparin. *Derivation cohort:* 241 patients, 3 months follow-up. *Validation cohort:* 780 patients, 3 months follow-up.	(1.6 x age) +(1.3xsex) +(2.2 x cancer) with 1 point for age≥60 years, female or malignancy, and 0 if none.
Beyth *et al.* (1998)	*Derivation cohort*: 556 patients who started warfarin, 4 years retrospective follow-up. *Validation cohort*: inception cohort of 264 who started warfarin, 4 years prospectively follow-up.	Age≥65 years, GI bleed in the past 2 weeks, previous stroke, comorbidities (recent MI, Hct<30%, diabetes, creatinine>1.5 ml/l) with 1 point for presence of each condition and 0 if absent.

Table 3-2. Comparing performance of published bleeding schemes by Lip et al. (2011b).

Bleeding Risk Score	Warfarin Patients (n = 3,665) (c-statistic (95% CI)	Patients taking warfarin and aspirin (n = 722) (c-statistic (95% CI)
HAS-BLED	0.66 (0.61–0.70)	0.60 (0.53–0.68)
Shireman et al., 2006	0.63 (0.58–0.67)	0.58 (0.31–0.66)
HEMORR$_2$HAGES, 2006	0.61 (0.56–0.65)	0.58 (0.51–0.66)
Kuijer et al. ,1999	0.52 (0.48–0.56)	0.49 (0.45–0.55)
Beyth et al., 1998	0.56 (0.51–0.60)	0.52 (0.46–0.57)

Recently, researchers have suggested calculating IDI (Integrated Discrimination Improvement) and NRI (Net Reclassification Improvement) as statistical method for assessing the performance of newer biomarkers (Pencina *et al.* 2008b). IDI and NRI measure the new model's ability to improve categorization amongst those who do and do not develop the events. Both serve the same purpose, but the NRI method requires patients to be classified into two or more outcome categories based on a model's prediction output. As shown in Table 3-3, IDI and NRI analyses by Lip *et al.*, (Lip *et al.* 2011a) significantly demonstrated that HAS-BLED predicted major bleeding episodes more accurately than the other risk scores, except for the scheme for NRI by Shireman *et al*.(2006).

Table 3-3. NRI and IDI analysis comparing HAS-BLED with other bleeding schemes (Lip et al, 2011a).

HAS-BLED versus	NRI	IDI
Shireman et al, 2006	0.089 (p value 0.25)	0.668 (p value <0.0001)
HEMORR$_2$HAGES, 2006	0.152 (p value 0.05)	0.416 (p value 0.0021)
Kuijer et al., 1999	0.306 (p value <0.0001)	0.883 (p value <0.0001)
Beyth et al., 1998	0.262 (p value <0.0001)	0.828 (p value <0.0001)

P value is a statistical test provided by Pencina et al (2008) to test for null hypothesis of IDI and IDI=0 (refer section 3.2.4).

To date, the usefulness of genetic polymorphisms as biomarkers in predicting bleeding risk score have not been evaluated. HEMORR2HAGES (Gage *et al.* 2006), a bleeding risk score developed based on literature review, included a polymorphism in the *CYP2C9* gene, but the contribution of this polymorphism has never been evaluated in the original study or in any validation cohort (Friberg *et al.* 2012, Poli *et al.* 2013, Seet *et al.* 2013). In recent meta-analyses studies, *CYP2C9*2, CYP2C9*3* have been found significantly associated with major bleeding risk in patients treated with warfarin (Jorgensen *et al.* 2012, Yang *et al.* 2013). Even though *VKORC1* polymorphisms have not been associated with major bleeding they have been associated with over-anticoagulation risk (Yang *et al.* 2013).

On this basis, we decided to further investigate whether the addition of genetic factors could improve the predictive performance of a bleeding risk score. Specifically, our objectives were (i) to identify predictors of bleeding in a cohort of patients during the first 6 months of warfarin treatment, (ii) to investigate the performance of HAS-BLED in this cohort and (iii) to evaluate whether the addition of genetic factors to HAS-BLED schemes could improve the prediction of major bleeding.

3.2 Method

3.2.1 Patient Recruitment and Genotyping

The same cohort of patients as used for analysis in Chapter 2 was used in the current analysis. This cohort of patients was originally recruited as part of a large, prospective study of warfarin pharmacogenetics (Jorgensen *et al.* 2009) Patient recruitment and genotyping methods are described in Chapter 2.

Inclusion criteria: Only atrial fibrillation patients with 6 months' completed follow-up were included in the analysis. The risk of bleeding on anticoagulant therapy is highest during the early phase of treatment (Landefeld and Goldman 1989, Fihn *et al.* 1993, Douketis *et al.* 2000, Linkins *et al.* 2003, Hylek *et al.* 2007) and so we believed that assessing the performance of HAS-BLED on patients during only the initial six months of warfarin therapy would provide important information in decision making during warfarin therapy

3.2.2 Definition of Major Bleeding

Bleeding events were categorised according to classification provided by Fihn *et al.* (1996). The study established three categories to define anticoagulant–related bleeding: (i) minor bleeding (reported but not requiring a further test, referral or visit), (ii) major bleeding (requiring treatment, medical evaluation or ≥ 2 units of blood) and (iii) life-threatening bleeding (leads to cardiac arrest, surgical/angiographic intervention or an irreversible sequel). For each bleeding event, the relationship between warfarin use and the bleeding was recorded as 'unrelated', 'unlikely', 'possible', 'probable' and 'almost certain'. The relationship was determined by the clinical physician. For the purpose of our analyses, patients experiencing either a life threatening bleeding event or a major bleeding event were classified as cases, provided that the event was considered as being 'possible', 'probable' or 'almost certain' in

terms of its relationship with warfarin use. All other patients were classified as controls.

3.2.3 Statistical Analysis

All data analyses were performed with SPSS, version 19. Patients demographics including age, BMI, medical history and genotype polymorphisms were compared across patients who experienced or did not experience bleeding, using percentages to look for any differences between groups. For our analysis, HAS-BLED definitions as described in Table 3-4 were used. Each factor is given a 1 point score if present.

Table 3-4. Definition of HAS-BLED used in our study.

HAS-BLED Score (Acronyms)	Definition in current study
Hypertension (**H**)	Combination therapy with at least two of the following classes of antihypertensive drugs: Adrenergic α-antagonist, non-loop diuretics, vasodilators, beta blockers, calcium channel blockers, and renin angiotensin system inhibitors
Abnormal Kidney Function (**A**)-	presence of chronic dialysis, renal transplant, or serum creatinine ≥ 200 µmol/L.
Abnormal Liver Function (**A**)	The presence of chronic hepatic disease (e.g. cirrhosis) or biochemical evidence of significant hepatic derangement (at least positive in 2 liver function tests)
Stroke (**S**)	Previous history of stroke
Bleeding history or predisposition (anaemia) (**B**)	Haemoglobin<13g/dL for men and <12g/dL in women
Labile INR (**L**)	Labile INR described as <60% time in the therapeutic range, calculated using the Rosendaal method (Rosendaal *et al.* 1993).
Elderly (**E**)	Age >65 years old
Drug (**D**)	Antiplatelet agent or non-steroidal inflammatory drugs conconmitantly prescribed with warfarin during follow-up
Excessive alcohol use (**D**)	AUDIT questionnaire question 3 with an answer of 3 or 4.

We use the same definition of HAS-BLED as in the original study by Pister et al (2010), a part from the following:

(a) *Uncontrolled hypertension:* The original definition is a blood pressure measurement of >160mmHg systolic. Since data of blood pressure were not available in our study, we defined uncontrolled hypertension as combination therapy with at least two of the following classes of antihypertensive drugs: Adrenergic α-antagonist, non-loop diuretics, vasodilators, beta blockers, calcium channel blockers, and renin angiotensin system inhibitors. This definition has been used by Olesen *et al. (2011b)* in their study comparing HAS-BLED Score and HEMORR2HAGES Score. This definition has previously been validated in 75,538 atrial fibrillation patients (Olesen *et al.* 2011a).

(b) *Bleeding history or prediposition:* In the original definition predisposition of bleeding was defined as anemia. We refine the definition as haemoglobin <13 g/dL for men and <12g/dL in women for easiness of diagnosis. We did not include any bleeding history because no record was available.

(c) *Alcohol use:* There is no specific definition in the original study so we used AUDIT questionnaires (Figure 3.1) as a tool to assess excessive use of alcohol in our study. AUDIT is the acronym for the Alcohol Use Disorders Identification Test (Babor et al. 1989). It was developed by the World Health Organization (WHO) as a simple method of screening for excessive drinking and to assist in brief assessment. The purpose of question number 3 in the questionnaire is to evaluate the frequency of heavy drinking. We took an answer of (3) or (4) to represent excessive alcohol drinking. Sensitive analysis was performed to examine excessive use of alcohol using a total AUDIT score >7 (indicators of hazardous and harmful alcohol use) and a total AUDIT score >16 (representing a high level of alcohol problems).

The Alcohol Use Disorders Identification Test: Interview Version

Read questions as written. Record answers carefully. Begin the AUDIT by saying "Now I am going to ask you some questions about your use of alcoholic beverages during this past year." Explain what is meant by "alcoholic beverages" by using local examples of beer, wine, vodka, etc. Code answers in terms of "standard drinks". Place the correct answer number in the box at the right.

1. How often do you have a drink containing alcohol?
   (0) Never [Skip to Qs 9-10]
   (1) Monthly or less
   (2) 2 to 4 times a month
   (3) 2 to 3 times a week
   (4) 4 or more times a week

2. How many drinks containing alcohol do you have on a typical day when you are drinking?
   (0) 1 or 2
   (1) 3 or 4
   (2) 5 or 6
   (3) 7, 8, or 9
   (4) 10 or more

3. How often do you have six or more drinks on one occasion?
   (0) Never
   (1) Less than monthly
   (2) Monthly
   (3) Weekly
   (4) Daily or almost daily
   Skip to Questions 9 and 10 if Total Score for Questions 2 and 3 = 0

4. How often during the last year have you found that you were not able to stop drinking once you had started?
   (0) Never
   (1) Less than monthly
   (2) Monthly
   (3) Weekly
   (4) Daily or almost daily

5. How often during the last year have you failed to do what was normally expected from you because of drinking?
   (0) Never
   (1) Less than monthly
   (2) Monthly
   (3) Weekly
   (4) Daily or almost daily

6. How often during the last year have you needed a first drink in the morning to get yourself going after a heavy drinking session?
   (0) Never
   (1) Less than monthly
   (2) Monthly
   (3) Weekly
   (4) Daily or almost daily

7. How often during the last year have you had a feeling of guilt or remorse after drinking?
   (0) Never
   (1) Less than monthly
   (2) Monthly
   (3) Weekly
   (4) Daily or almost daily

8. How often during the last year have you been unable to remember what happened the night before because you had been drinking?
   (0) Never
   (1) Less than monthly
   (2) Monthly
   (3) Weekly
   (4) Daily or almost daily

9. Have you or someone else been injured as a result of your drinking?
   (0) No
   (2) Yes, but not in the last year
   (4) Yes, during the last year

10. Has a relative or friend or a doctor or another health worker been concerned about your drinking or suggested you cut down?
    (0) No
    (2) Yes, but not in the last year
    (4) Yes, during the last year

Record total of specific items here

If total is greater than recommended cut-off, consult User's Manual.

Figure 3-1. The AUDIT questionnaire.

3.2.3.1 Identifying predictors of bleeding risk in our study

The following baseline clinical and genetic factors which had previously been associated with bleeding risk or with warfarin dose variability were each univariately tested for association with major bleeding.

Clinical Factors*:* height (Sconce *et al.* 2005), weight (De Jaegere *et al.* 1992), age (De Jaegere *et al.* 1992), body surface area (Gage *et al.* 2004), body mass index, abnormal kidney function (Pisters *et al.* 2010), abnormal liver function (Pisters *et al.* 2010), stroke (Pisters *et al.* 2010), bleeding predisposition (Pisters *et al.* 2010), labile INR (Pisters *et al.* 2010), age (Pisters *et al.* 2010, Lip *et al.* 2011b), alcohol abuse (Pisters *et al.* 2010), gender (Hillman *et al.* 2004) and concomitant drug use (aspirin, clopidogrel, dipyridamole, selective serotonin reuptake inhibitors (SSRI), amiodarone, paracetamol 1gm and non-steroidal anti-inflammatory drugs (NSAID) (Hauta-Aho *et al.* 2009, Pisters *et al.* 2010).

Genetic Factors*: CYP2C9*2*, *CYP2C9*3* and *VKORC1* associated with major bleeding or over-anticoagulation in recent meta-analysis were included in the analysis. In the analysis, wild-type homozygotes were coded '0', heterozygous patients were coded as '1' and mutant homozygotes were coded as '2' Prior to analyses of association with these genetic factors, an assessment of conformity with Hardy Weinberg Equilibrium (HWE) for each SNP was performed using Haploview software (version 4.2). A p value of < 0.001 was assumed to indicate deviation from HWE.

Each of these factors were compared between the group of patients who experienced a major bleed (cases), and those that did not (controls). The Fisher exact test or chi-square test (if > 2 categories) was used for categorical factors and the independent sample t-test, was used for continuous factors.

Finally, all factors identified from the univariate analysis as having p value ≤ 0.10 were included together in a logistic regression model.

3.2.3.2 To assess performance of HAS-BLED in our cohort

Prediction performance of HAS-BLED was tested both in the whole cohort and in sub groups of patients, i.e. those who were taking warfarin without antiplatelet drugs and those who were taking warfarin with antiplatelet drugs. All tests were performed 2-tailed, with a p value ≤ 0.05 considered as statistically significant. To test the performance of the bleeding score the following analyses were performed:

ROC and c-statistical analysis *:* Logistic regression analysis including HAS-BLED covariates (uncontrolled hypertension, abnormal kidney function, abnormal liver function, history of stroke, bleeding predisposition, labile INR, age > 65 years old, drug used and alcohol consumed) was undertaken, with discrimination tested on the ROC curve and area under the curve (c-statistic) (Zou *et al.* 2007). For the c-statistic value, we used the Hosmer and Lemeshow rules to interpret the discriminative ability of the model (Hosmer and Lemeshow 2000).

3.2.3.3 To assess improvement made by incorporating genetic variables in HAS-BLED score

To evaluate the improved performance of HAS-BLED when incorporating genetic covariates, (referred to as HAS-BLEDG), the following analyses were performed:

ROC and c-statistical analysis: ROC and c-statistical analysis was performed on both HAS-BLED and HAS-BLEDG as described in 3.2.3.2. Logistic regression analysis was undertaken in the same way as HAS-BLED for HAS-BLEDG, except it also included genotype identified as significant in our univariate analyses.. The ROC curve with the highest rise was the model generally considered to have the best predictive qualities. The log likelihood ratio c-statistic was also calculated to assess the improvement in model fit.

IDI analysis*:* As we did not assign any risk categories for the outcomes based on HAS-BLED or HAS-BLEDG scores (eg: for low, moderate or high risk of bleeding), only IDI was used to assess the discrimination improvement made by the addition of genetic variables to HAS-BLED. The formula to estimate IDI is illustrated below (Pencina *et al.* 2008b):

$$Absolute\ IDI = (P_{new,events} - P_{new,\ nonevents}) - (P_{old,events} - P_{old,nonevents})$$

Where $P_{new,events}$ is the mean of the HAS-BLEDG score predicted probabilities of an event for those who develop events; $P_{old,events}$ is the corresponding quantity, based on the HAS-BLED model; $P_{new,nonevents}$ is the mean of the HAS-BLEDG predicted probabilities of an event for those who do not develop events and $P_{old,nonevents}$ is the corresponding quantity based on the HAS-BLED.

Therefore, $(P_{new,events} - P_{new,\ nonevents})$ is equivalent to the difference in the mean of predicted probabilities for events and non-events by HAS-BLEDG, or the discrimination slope by HAS-BLEDG. Similarly, $(P_{old,events} - P_{old,nonevents})$ is equivalent to the difference in the mean of predicted probabilities for events and non-events by HAS-BLED, or the discrimination slope by HAS-BLED. Thus, IDI is equal to the difference between the discrimination slopes of HAS-BLEDG and HAS-BLED.

Pencina et al. (2008) also provide the following test statistic which tests the null hypothesis of IDI =0:

$$zIDI = \frac{Absolute\ IDI}{\sqrt{(SEevents)^2 + (SEnonevents)^2}}$$

In the equation, *SEevents* is the standard error of paired differences of new (HAS-BLEDG) and old model (HAS-BLED) predicted probabilities across all event subjects, while *SEnonevents* is the standard error of the paired differences of new (HAS-BLEDG) and old model (HAS-BLED) predicted probabilities across all non-event subjects.

The magnitude of IDI is hard to interpret and so we looked at relative IDI (rIDI). rIDI is described as ratio of differences between means of model-based probabilities for events and non-events for model with and without new biomarker minus 1.(Pencina *et al.* 2008a). rIDI represents the percentage relative improvement of the model after this modification.

Rates of major haemorrhage stratified by bleeding score in both HAS-BLED and HAS-BLEDG were also calculated to observe the distribution of percentage patients experiencing bleeding events stratified by score. HAS-BLED was calculated using points given as shown in Table 3-5 . Briefly, score of zero indicated low risk, 2-3 moderate risk and ≥3 high risk (Lip *et al.* 2011b). Previous studies have shown, patients with score of ≥5 have triple time higher risk compared to patients with score 3 (Apostolakis *et al.* 2012, Roldán *et al.* 2013). For HAS-BLEDG the score was calculated in the same way as HAS-BLED, except that the score was adjusted with genotypes identified as significance in univariate analysis, where wild-types, heterozygotes and mutant homozygotes were given scores of 0, 1 and 2 points respectively.

Table 3-5. HAS-BLED calculation

Accronym	Clinical characteristics	Point awarded
H	Hypertension	1
A	Abnormal liver of kidney function (1 point each)	1 or 2
S	Stroke	1
B	Bleeding	1
L	Labile INR	1
E	Elderly	1
D	Drug or alcohol (1 point each)	1 or 2

3.3 Results

Out of 657 AF patients (white European), 526 had complete 6 months follow-up. Of these, 44 patients did not have complete genotyping because of inadequate DNA; therefore a total of 482 patients were included in the analysis. Baseline characteristics of the patients are summarized in Table 3-6.

Table 3-6. Patient demographics

Characteristic	Bleeding event (n=15)	No Bleed (n=467)	P values
Age, years, mean (SD)	71.60 (6.74)	71.53(10.07)	0.980*
Age >65 (%)	13(86.7)	353(75.6)	0.258**
Weight, kg , mean (SD)	77.47 (20.22)	81.02 (18.78)	0.512*
BMI, mean (SD)	27.76 (6.16)	28.24 (5.81)	0.768*
Height, cm, mean (SD)	166.73 (10.27)	169.15 (10.55)	0.383*
Gender, male (%)	9 (60)	275 (58.9)	0.999**
Medical History			
Cardiovascular disease, n (%)	15 (100%)	430 (92.1)	0.619**
Respiratory disease, n (%)	6 (40%)	149 (31.9)	0.516**
Hepatic disease, n (%)	1(6.7%)	17 (3.6)	0.440**
Renal disease, n (%)	2(13.3%)	49 (10.2)	0.905**
Gastrointestinal disease, n (%)	4(26.7)	165 (35.3)	0.591**
Polymorphism			
*CYP2C9*2*, n (%)			
Wild-type (CC)	12 (80)	365 (78.2)	0.945**
Heterozygous variant (CT)	3 (20)	99 (21.2)	
Homozygous variant (TT)	0	3 (6)	
*CYP2C9*3*, n (%)			
Wild-type (AA)	12 (80)	412 (88.2)	0.038**
Heterozygous variant (AC)	2 (13.3)	52 (11.1)	
Homozygous variant (CC)	1 (6.7)	3 (0.7)	
VKORC1, n (%)			
Wild-type (CC)	5 (33.3)	198 (42.4)	0.446**
Heterozygous variant (CT)	9 (60)	206 (44.1)	
Homozygous variant (TT)	1 (6.7)	63 (13.5)	

*independent sample t-test p value

** fischer exact test p value

During their first 6 months of follow-up, 3.1% (95%CI 1.55-4.65) patients (n=15) presented with a major bleeding event. The percentage of bleeding events in the sub group of patients taking warfarin alone (n=405), patients concomitantly taking aspirin (n=55), clopidogrel (n=18) and aspirin together with clopidogrel (n=4) were 2.5% (95%CI 0.98-4.02), 3.6% (95%CI 0-8.52), 5.9% (95%CI 0-17.1) and 50% (95%CI 1-99) respectively.

3.3.1 Identification of Risk Factors for Major Bleeding in Our Cohort

Results of the univariate analyses of association are given in Table 3-7. Factors significantly associated with major bleeding ($p \leq 0.1$) were concurrent amiodarone use ($p<0.05$), labile INR ($p<0.01$), concurrent clopidogrel use ($p<0.1$), bleeding predisposition ($p<0.1$), aspirin (p 0.1), *CYP2C9*3* (additive mode inheritance) $p<0.05$.

Multivariate analyses of risk factors predictive of major bleeding are shown in Table 3-8. Inclusion of all factors found significant univariately in a multiple regression model, resulted in a c-statistic of 0.86 (95%CI 0.787-0.944).

Table 3-7. Risk factors for major bleeding by univariate analysis using our cohort (n=482).

Bleeding risk factor	P value
Clinical covariates:	
Amiodarone use	0.002
Labile INR	0.007
Clopidogrel	0.023
Bleeding predisposition	0.070
Aspirin use	0.098
Height	0.383
Weight	0.512
Body surface area	0.393
Age >65 years old	0.258
Body mass index	0.768
Abnormal kidney function	0.999
Abnormal liver function	0.999
Stroke	0.249
#Alcohol use (AUDIT question 3)	0.484
Dypiridamole use	0.939
Selective serotonin re-uptake inhibitors use	0.999
Paracetamo 1000 mg use	0.397
Non-steroidal anti-inflammatory drugs use	0.999
Hypertension	0.309
Genetic covariates:	
Analysis with no underlying assumption	
*CYP2C9 *2*	0.827
*CYP2C9*3*	0.130
VKORC1-1693	0.901
Analysis assuming an additive mode of inheritance	
*CYP2C9 *2*	0.945
*CYP2C9*3*	0.038
VKORC1-1693	0.446

In Sensitivity analysis using a total AUDIT score >7 and a total AUDIT score >16 , the p-values were both 0.9 and hence conclusion remain the same.

Table 3-8. Multivariate analyses of bleeding risk using our cohort (n=482).

Bleeding risk factor	Coefficients (B) of the final regression model
Constant	-6.090
Amiodarone	2.051
Labile INR	2.079
Clopidogrel	1.706
Bleeding predisposition	0.34
*CYP2C9*3* (additive)	0.699
Aspirin	0.767

3.3.2 Assessing performance of HAS-BLED in our cohort

HAS-BLED performed well in predicting major bleeding in our whole cohort with a c-statistic 0.80 95%CI (0.707-0.899) (Table 3-9). In the subgroup analyses of patients taking antiplatelet drugs and those who did not, four patients were excluded because they were prescribed both aspirin and clopidogrel concomitantly. The c-statistic in the group of patients concomitantly taking anti-platelet drugs was 0.82 (95% CI: 0.660-0.978) and in the group of patients not on anti-platelet drugs was 0.79 (95% CI: 0.657-0.914).The difference was not statistically significant.

3.3.3 Assessing improvement made by adding genetic variables to HAS-BLED score

There was no evidence of statistically significant improvement in prediction of HAS-BLEDG compared to HAS-BLED either in our c-statistic analysis (Table 3-9), ROC analysis (Figure 3.2) or IDI analysis (Table 3-10). Further, the addition of genetic covariates into the HAS-BLED score only made a small improvement of the prediction as showed by relative IDI of 10% (95%C:7-12) in all patients but decrease by 4% (95%CI: 2-4) in patients not on antiplatelet drugs. The relative IDI for patients on antiplatelet drugs was 58% (95%CI: 47-69) although this result needs to be treated with caution since the number of patients in this group is small (n=73).

Table 3-9. Comparison of c-statistic (95%CI) for HASBLED and HAS-BLED G in whole study cohort and subgroup of patients those who were taking warfarin without antiplatelet drugs and those taking warfarin concomitantly with antiplatelet drugs.

Bleeding risk score	c-statistic (95% CI)		
	Whole cohort (n=482)	Patients without antiplatelet (n=406)	Patients with anti-platelet (n=72)
HAS-BLED	0.80 (0.707-0.899)	0.79 (0.657-0.914)	0.82 (0.660-0.978)
HAS-BLEDG	0.81 (0.717-0.912	0.785 (0.664-0.907)	0.805 (0.627-0.982)
P value c-statistic	0.2	0.7	0.4

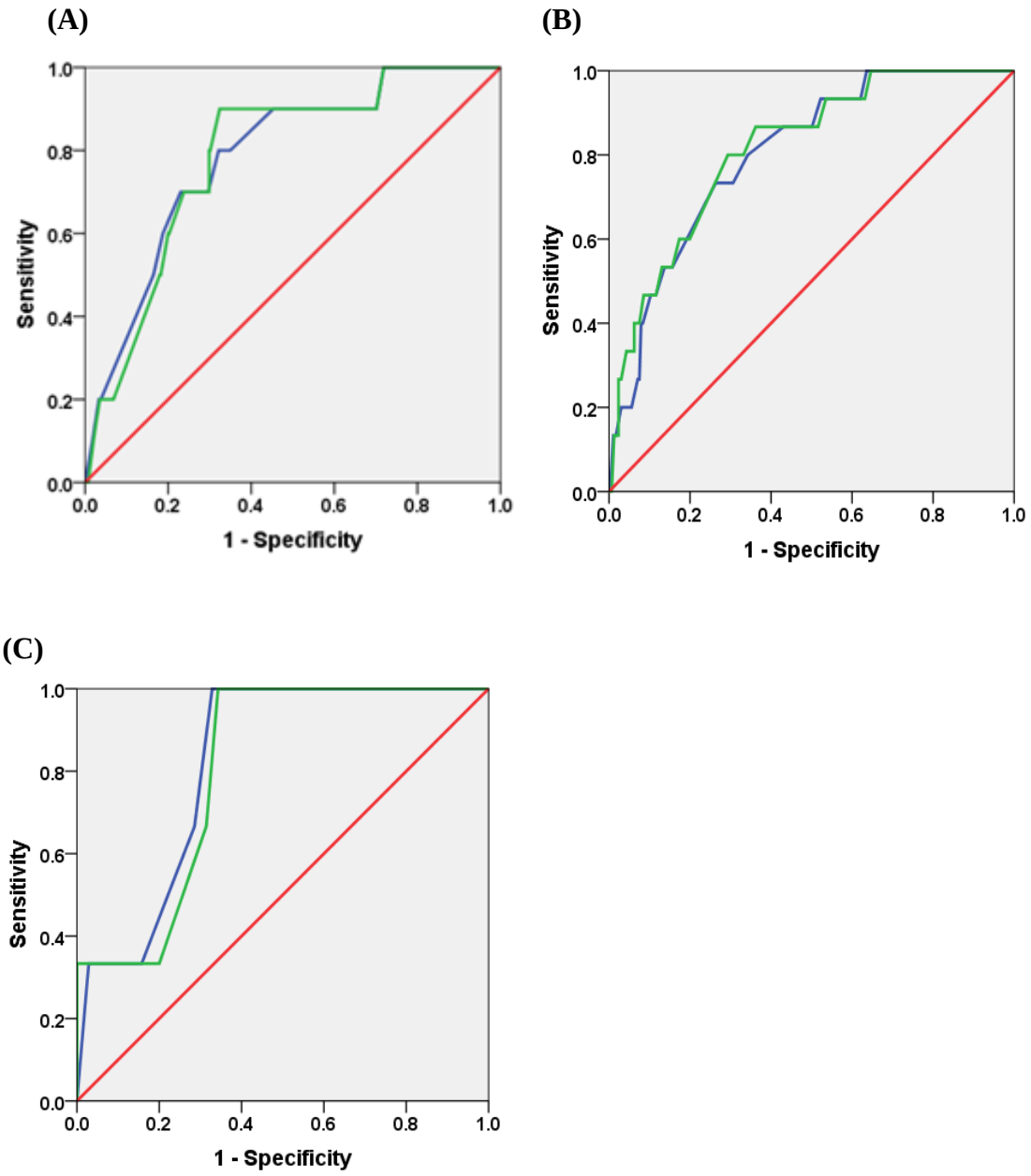


Figure 3-2. ROC curves for the model with and without genetic factors in analyses of three sub groups. (A) Whole cohort (n=482) (B) patients without antiplatelet drugs (n= 406) and (C) patients on antiplatelet drugs (n=72). The blue line represents mean predicted probability with HAS-BLED, the green line represents mean predicted probability with HASBLEDG and the red line represents the reference line where prediction is no better than by chance. Sensitivity is the probability of an individual with the bleeding being predicted as having it, while specificity is the probability of an individual not bleeding and being predicted as not having it.

Table 3-10. IDI analysis to quantify improvement in the performance of HAS-BLEDG (new model) compared to HAS-BLED (old model).

HAS-BLEDG vs. HAS-BLED	Whole cohort (n=482)	Patients without antiplatelet (n=405)	Patients with antiplatelet (n=73)
*Slope for HAS-BLEDG	0.0513	0.0321	0.1158
†Slope for HAS-BLED	0.0465	0.0335	0.0731
IDI	0.0048	(-)0.0013	0.0427
rIDI (95%CI)	0.10 (0.07-0.12)	(-)0.04 (0.02-0.06)	0.58 (0.47-0.69)
SE_{events}	0.0068	0.0014	0.0029
$SE_{non\text{-}events}$	0.0006	0.0002	0.0560
z-score	0.7	-0.9	0.8
**P value for IDI(2 tail)	0.5	0.9	0.4

*Discrimination slope for new model (HAS-BLEDG)= $P_{new,events} - P_{new,\ nonevents}$

†Discrimination slope for old model (HAS-BLED)= $P_{old,events} - P_{old,nonevents}$

rIDI = (Slope for HAS-BLEDG/slope for HAS-BLED) -1

SE_{events} = standard error of paired differences of HAS-BLEDG and HAS-BLED predicted probabilities across all event subjects.

$SE_{non\text{-}events}$= standard error of paired differences of HAS-BLEDG and HAS-BLED predicted probabilities across all non-event subjects.

**P value is a statistical test provided by Pencina et al (2008) to test for null hypothesis of IDI and IDI=0 (refer section 3.2.4).

The rate of major bleeding stratified by HAS-BLED score and HAS-BLEDG are presented in Table 3-11 and Figure 3-3. The median HAS-BLED score was 3 (interquartile range 1). The median HAS-BLEDG score was also 3 (interquartile range 2). Approximately only 4 % of patients who were identified by HAS-BLED and HAS-BLEDG as having a score of ≥3 experienced major bleeding events. Even though the figure shows no difference in rates of haemorrhage between HAS-BLED and HAS-BLEDG score, but interestingly, one patient in bleeding group with HAS-BLED score of 3 and was scored 5 by HAS-BLEDG.

Table 3-11. Rates of major bleeding stratified by HAS-BLED and HAS-BLEDG Score.

Bleeding risk score	HAS-BLED		HAS-BLED G	
	n	Rates of major bleeding *[n, (%; 95%CI)]*	*(n)*	Rates of major bleeding *[n, (%, 95%CI)]*
0	12	0 (0%)	11	0 (0%)
1	84	1 (1.2%; 0 - 3.5)	77	1 (1.3%; 0- 3.8)
2	142	4 (2.8%; 0.1 - 5.5)	137	4 (2.9%; 0.1 - 5.7)
3	143	4 (2.8%; 0.1 - 5.5)	133	3 (2.2%; 0-10.2)
4	75	4 (5.3%; 0.2 - 10.4)	85	4 (4.7%; 0.2- 9.2)
≥5	26	2 (7.7%; 0 - 17.9)	39	4(10.2%; 0 - 19.8)

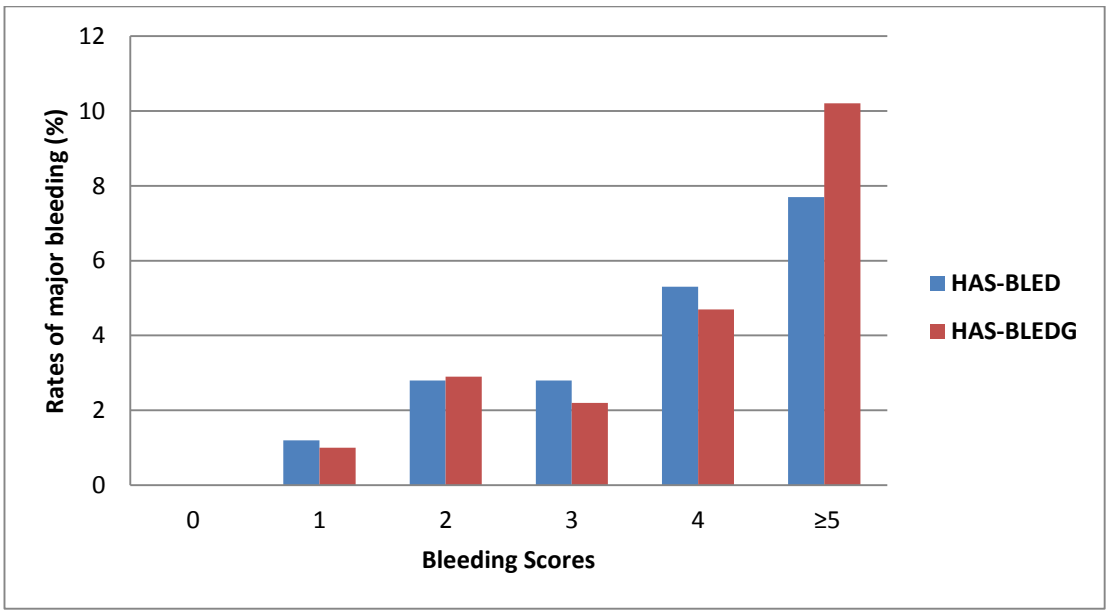


Figure 3-3. Rate of bleeding (%/6-months) of major bleeding events according to the HAS-BLED and HAS-BLEDG scores.

3.4 Discussion

We have validated the HAS-BLED score as a successful predictor of major bleeding, as assessed by the c-statistic, in agreement with previous reports (Pisters *et al.* 2010, Lip *et al.* 2011b, Olesen *et al.* 2011b, Apostolakis *et al.* 2012). Therefore, it appears that HAS-BLED score can be seen to be a useful tool for predicting major bleeding during initial treatment with warfarin.

When using our own dataset to identify predictors of major bleeding, we identified the *CYP2C9*3* (additive mode inheritance), amiodarone, labile INR, concomitant use with clopidogrel, concomitant use with aspirin, and bleeding disposition as significant predictors. The only HAS-BLED covariates which were significant were labile INR, concomitant use of clopidogrel, concomitant use of aspirin and bleeding disposition. This study also replicates the contribution of *CYP2C9*3* (number of variant alleles) to bleeding risk in patients taking warfarin (Jorgensen *et al.* 2012, Yang *et al.* 2013). Multivariate analysis of the predictive risk factors found significant in our cohort gave a c-statistic of 0.86 (95%CI 0.787-0.944). However, this needs an external validation in order to compare it with HAS-BLED or other bleeding scores.

In this present study, we also examined whether addition of genetic covariates (HAS-BLEDG) would improve the discriminative capability of HASBLED. *CYP2C9*3* was found to be a significant predictor of bleeding risk, so was incorporated with HAS-BLED score. Adding these covariates to HAS-BLED score, did not significantly improve HAS-BLED prediction in all analyses.

In addition to the list of drugs included as risk factors for bleeding in previous studies, we found that amiodarone was also a significant contributor to major bleeding in the univariate analysis. The interaction of warfarin and amiodarone has been widely reported. Amiodarone reduces the clearance of R- and S- warfarin by inhibition of

CYP2C9 and CYP1A2 (O'Reilly *et al.* 1987, Heimark *et al.* 1992, Kumar *et al.* 2006), the enzymes responsible for metabolising warfarin in the liver. The interaction that always occurs between these two drugs necessitates a 25-40% warfarin dose reduction (Kerin *et al.* 1988, Sanoski and Bauman 2002). However, the study by Olesen *et al.* (2011b) in a larger cohort of atrial fibrillation patients (n=44,771 patients treated with vitamin K antagonists) did not produce an association between amiodarone use and major bleeding (p=0.71). The number of patients taking amiodarone in that cohort was 86 (4.3%) compared with 50 (10.4%) in our study. The smaller percentages of patients on amiodarone in the Olesen *et al*. (2011b) study possibly influenced the significance of the association. In our view, concomitant use of amiodarone should be considered as one of the more important risk factors for major bleeding in a patient treated with warfarin.

Based on the c-statistic value, performance of HAS-BLED in patients taking warfarin, the current study found that the model prediction was stronger (c-statistic 0.80), when compared to a previous studies with one year follow-up by Pister *et al.* (2010), Lip *et al* (2011b) and recently by Apostolakis et al (2012) where the c-statistic was all 0.65. In the study by Olesen *et al.* (2011b), data for labile INR were not included because the information was unavailable, but they showed similar performance (c-statistic 0.79) to our findings.

The original definition of uncontrolled hypertension used for the HAS-BLED score was a systolic blood pressure >160 mmHg. This definition is problematic because there are many contributing factors to uncontrolled blood pressure, including treatment compliance, disease and lifestyle. The time that blood pressure is taken also influences measurement. Systematic reviews suggest that poorly controlled blood pressure is an independent risk factor for bleeding (Hughes and Lip 2007). In our definition, as suggested by many guidelines, patients were considered as showing

uncontrolled hypertension if they were not responding to initial antihypertensive treatment (Huntzinger 2008, Cheung and Cheung 2012, Schäfer *et al.* 2012).

Thus, since both our analyses and that of Olesen *et al.*(2011b) used similar definitions of uncontrolled hypertension and produced better values of c-statistic, we suggest that the definition we used to define uncontrolled hypertension is used in future applications of the score.

Limitations in our study includes inability to compare our newly developed regression model with HAS-BLED or HAS-BLEDG since we only had one dataset. We cannot use our retrospective cohort used in previous chapter because it will introduce bias in selection of patients as some patients are eliminated because of bleeding. In addition too few people had *CYP2C9*3*, therefore we may require a larger sample size to show that adding genetics to HAS-BLED improved prediction. Furthermore, we did not investigate the contribution of rs6018 in the clotting factor V gene, which was significantly associated with major bleeding events in our interim analysis of our prospective cohort (Jorgensen *et al.* 2012).

We only included patients who was classified as 'possible','probable' or almost certain' related to bleeding due to warfarin as cases. Some patients who are bleeding but classified as 'unrelated', 'unlikely' are referred to as control. Thererefore, some true cases might be eliminated and contaminating the control group with some cases and lessening the chances of finding real exposure differences between cases and controls.

The prospective study design that we chose may reduce the potential for incomplete recording of data whilst enhancing completeness and robustness of the statistical analysis. Moreover, prospective design allowed us to avoid any selection bias due to some patients being excluded because of bleeding during therapy.

None of the prospective studies so far extended the clinical use of pharmacogenetics beyond initial stable warfarin dose prediction (Gage *et al.* 2008, Wadelius *et al.* 2009). Therefore, this study added new knowledge of using pharmacogenetics in predicting the adverse effect of warfarin during initiating the dose. This study is of practical importance because the most dangerous side-effect of warfarin treatment is major bleeding, which occurs most commonly during the early months of therapy (Landefeld and Goldman 1989, Fihn *et al.* 1993, Douketis *et al.* 2000, Linkins *et al.* 2003, Hylek *et al.* 2007).

The primary advantage of the HAS-BLED score is that it is simple and easy to remember, yet offers good prediction of major bleeding risk. It supports physicians in making an informed decision when giving warfarin therapy, rather than guessing. For example, if the patient's bleeding score is sufficiently high, caution should be exercised by ensuring more regular reviews for that patient. Additionally, the bleeding risk score can be modified, by stopping aspirin or by counselling on alcohol intake. Therefore the HAS-BLED score not only gives the option to stop warfarin therapy if the score is sufficiently high, but it also provides alternative management strategies for those patients who would really benefit from warfarin therapy.

Genotyping for *CYP2C9* and *VKORC1* may possibly improve the predictive capability of HAS-BLED, but we were unable to demonstrate this in our study cohort. For example, one patient in bleeding group with HAS-BLED score of 3 and having homozygous *CYP2C9*3* was scored as 5 by HAS-BLEDG. HAS-BLEDG score indicated that patient has higher risk of major bleeding compared by HAS-BLED. Therefore, caution should be taken when prescribing warfarin to this patient, or newer oral anticoagulant such as dabigatran, a direct thrombin inhibitor should be prescribed to this patient. (Apostolakis *et al.* 2012, Roldán *et al.* 2013). However, it would be worthwhile investigating this further in a larger cohort of patients. Of course, if the

genotype of an individual patient is already known, there is no harm in using HAS-BLEDG rather than HAS-BLED. Although genotypes are not available for most patients at present, this may change in the future through patients obtaining their own genome-wide SNP data (eg by sending samples to 23anMe), or as the cost of genotyping/sequencing falls.

Chapter 4

Pharmacogenetics of warfarin in a paediatric population

CONTENTS

4.1 Introduction

The incidence of venous and arterial thrombosis is rare in children, although recent studies have revealed that the incidence has been increasing over the last decade, specifically for venous thromboembolism (VTE) (Vu *et al.* 2008, Raffini *et al.* 2009, Setty *et al.* 2012). As an example, a study in the Children's Hospital of Philadelphia has shown that the annual rates of VTE increased by 70% during the 7-year study period from 2001 (Raffini *et al.* 2009). The increasing incidence of VTE in this population is due to the medical progress in the treatment of critically ill patients. This can be explained by the increasing use of central catheters and innovative interventional procedures in the treatment of premature infants, neonates and older children who are critically ill, suffering from complex cardiac defects, and from malignant diseases.

Warfarin is the most frequently prescribed oral anticoagulant for both the treatment and prevention of thromboembolic disease, and has been used in paediatrics since the 1970s (Carpentieri *et al.* 1976). The American College of Chest Physicians' Guidelines has recommended warfarin as thromboprophylaxis in many diseases including in patients with a prosthetic heart valves, after deep vein thrombosis and after Fontan palliation for congenital heart disease (Monagle *et al.* 2012).

As with adults, warfarin is a difficult drug to prescribe because of unpredictable responses and has a narrow therapeutic range. Similar to adults, age, disease, drugs and dietary factors can interfere with the response to warfarin. Dosing algorithms have been published for paediatric populations, but none of them include pharmacogenetic information (Andrew *et al.* 1998, Paediatric Stroke Working *et al.* 2004, Roach *et al.* 2008, Monagle *et al.* 2012). These warfarin dosing algorithms are extrapolated from adult algorithms, using the patient's age, weight and serial INR, to dictate initial

warfarin dose and dose adjustment to therapeutic levels. While these algorithms are relatively effective in clinical practice, they do not take into consideration differences between adult and paediatric haemostatic systems, which affect the pathophysiology of thrombosis and response to anticoagulation. In an American survey using an on-line questionnaire, paediatric haematologists identified a need for a better warfarin dosing algorithms and expressed an interest in updated algorithms that potentially included genetic factors (Thornburg *et al.* 2010).

Polymorphisms in *CYP2C9* and *VKORC1* have consistently been associated with warfarin dose requirements in adult populations around the world; however the impact of pharmacogenetics (e.g., *CYP2C9* and *VKORC1*) on paediatric therapy outcomes has not been fully explained. A literature search on warfarin pharmacogenetics in Caucasian children led to three small cohort studies evaluating the influence of *CYP2C9* and *VKORC1* polymorphisms on warfarin response (Table 4-1). The studies showed conflicting results, with two studies (Biss *et al.* 2012, Moreau *et al.* 2012) suggesting *CYP2C9* and *VKORC1* explain approximately 40% of warfarin dose variation and one study (Nowak-Göttl *et al.* 2010) showing only a small contribution from these polymorphisms (~4%). A further outcome being studied that relates to the efficacy of warfarin therapy is the time spent within INR range (Moreau *et al.* 2012) and time to therapeutic range (Nowak-Göttl *et al.* 2010); however neither of these were associated with *CYP2C9* and *VKORC1* polymorphisms.

The aims of this study were firstly to examine the association between genetic (*VKORC1, CYP2C9)* and non-genetic factors and the response to warfarin in children at the onset of therapy and during stable maintenance dosing. Secondly, the study aims to quantify the proportion of variability explained by the factors mentioned above. The response to warfarin was measured as the proportion of time in which INR measurement fell within the target range (PTTR), INR exceeding the target range within the first week of treatment, warfarin stable dose and bleeding complications.

Table 4-1 Results of literature search of warfarin pharmacogenetics in children .

Author	Study design	Sample size and study characteristics	Definition of stable dose	Statistical methodology	Multivariate analysis association with the outcomes (R^2)
Moreau *et al.,* (2012)	• Inclusion criteria - VKA therapy for at least the past 2 months -Age: 3 months to 18 years - Targeted INR 1.5-4 • Retrospective cohort	• 83 patients received warfarin, 35 patients received flunidione • Mean age: 8.4 years (warfarin) • 32% patients with indication for Fontan procedure and 39% with indication for mitral/aortic valve replacement • >90% Caucasian	Mean dose required to achieve INRs within the therapeutic range for 3 consecutive INR determinations at 2-week intervals	• Univariate analysis: -- 2-sample Wilcoxon test or nonparametric ANOVA (Kruskal-Wallis test) for qualitative variables -The Spearman rank correlation coefficient for quantitative variables • Multivariate analysis -including covariated with p<0.2 in univariate analysis -Backward selection method -Retain covariates with p <0.05	**Maintenance dose:** • height (48.1%) • number of variant alleles of *VKORC1 -1693* (18.2%) • target INR (4.4%) • number of variant allele *CYP2C9*2*/*CYP2C9*3*(20%) **Time spent within INR range** • Target INR group (the only factors that associated in univariate analysis

Author	Study design	Sample size and study characteristics	Definition of stable dose	Statistical methodology	Multivariate analysis association with the outcomes (R^2)
Biss *et al.,* (2012)	• Inclusion criteria - VKA therapy for at least the past 3 months - Age: 3 months to 18 years - Targeted INR 2-3.5 • Cross sectional cohort	• All received warfarin (n=120) • Median age is 11 years • Majority of patients were prescribed warfarin for Fontan (53%) • 75.8% Caucasians	no change of dose for at least the previous 3 consecutive INR measurements over a minimum period of 4 weeks	• Univariate analysis: -Pearson correlation test for continuous variables -Student t-test or ANOVA for categorical variables • Multivariate analysis - did not mention univariate p value included covariates -retain covariates with p<0.05	**Maintenance dose:** • height (29.8%) • indication (3.2%) • Number of variant allele of *VKORC1-1693* (26.6%) • Number of variant alleles for *CYP2C9*2* and *CYP2C9*3* (12.8%)
Nowak-Göttl *et al.* (2010)	• Inclusion criteria - VKA therapy for at least the past 3 months -Age: 3 months to 18 years - Targeted INR 2-3 • Prospective cohort	• 43 patients received warfarin, 26 received phenprocoumon • Mean age: 2.3 years (both group) • Indication warfarin for the target INR 2-3 with majority are deep vein thrombosis (33%) and pulmonary embolism (32%) • 100% Caucasians	median of 3 consecutive dosages when the patient had achieved stable anticoagulation where stable anticoagulation was defined by VKA requirement remaining constant for 3 consecutive days after achieving the target INR	• Univariate analysis: -Did not mention types of test performed - *VKORC1* genotype was categorized into 3 groups (AA, GA, and GG) and *CYP2C9* genotype into 2 groups for (any mutation 1.2, 1.3 or 2.2, and wild-type 1.1). • Multivariate analysis -including covariates with p<0.2 in a univariate analysis	**Maintenance dose** • age (28.3%) • *VKORC1 -1693*(2.8%) • Variant *CYP2C9*2 or CYP2C9*3* (0.5%) **Time to therapeutic range** None

Author	Study design	Sample size and study characteristics	Definition of stable dose	Statistical methodology	Multivariate analysis association with the outcomes (R^2)
Biss et al (2013)	• Inclusion criteria • - VKA therapy for at least the past 3 months • -Age: 3 months to 18 years • - Targeted INR 2-3.5 • Cross sectional cohort • Same cohort as Biss *et al,*. 2012	• All received warfarin (n=51) • Median age is 4 years • Majority of patients were prescribed warfarin for Fontan (54%)	NA	• The association between the *CYP2C9* genotype and peak INR was examined using the t-test and between the *VKORC1* genotype and peak INR using linear regression analysis • The association between the incidence of supra-therapeutic INR during the first month of warfarin therapy and *CYP2C9* genotype was evaluated using a Mann–Whitney test and the *VKORC1* genotype using regression analysis.	**Over-anticoagulation during <u>initiation therapy</u>** *In univariate analysis (p<0.05): • Children with a variant *CYP2C9* allele (*2 or *3) had a higher mean peak INR during week 1 than those with wild-type • Children with the *VKORC1 AA* genotype had a higher mean peak INR (5.1 ± 2.1) than those with the *GA* (3.5 ± 1.4) or *GG* (3.0 ± 1.3) • Children with a variant *CYP2C9* allele had a greater proportion of INR values above the target therapeutic range during month 1 of warfarin therapy than those with wild-type *CYP2C9*; 19.3% vs. 15.9%, respectively

4.2 Method

4.2.1 Study design and setting

We conducted a retrospective cohort study in Alder Hey Children's Hospital, Liverpool, between November 2009 and January 2011. Alder Hey Children's Hospital is one of Europe's biggest and busiest children's hospitals, providing care for over 200,000 children and young people each year. The majority of patients on warfarin are cared for in the community using point of care therapy (POCT). In POCT, equipment and training for INR monitoring are provided to patients or their families/carers. Patients and families inform the cardiac liaison nurse team after INRs and dose adjustment is carried out accordingly. All INR data is provided and dose adjustments are recorded by the cardiac liaison nurse team.

Patients were identified by the cardiac liaison nurse team or the relevant clinical team within the hospital and approached about participating in the study. This study received full ethical approval from North West 3 Research Ethics Committee.

4.2.2 Patient eligibility

Inclusion criteria were: warfarin prescribed for ≥ 3 months, age ≤18 years and therapeutic drug monitoring of INR performed by Alder Hey Children's Hospital. Written informed consent was sought from children and young people (each child was assessed on a case by case basis for level of understanding). The written informed consent was signed by a parent/guardian for patients aged <16 years and by the patient if aged ≥ 16 years old. Prior to the consent/assent process, information was provided to the parents/guardians and the participants about the nature of the study and the information it was expected to produce. Refusal to give assent was an exclusion criterion.

4.2.3 Data collection

Age, gender, height, weight, BMI, albumin, target INR group and indication for treatment were collected from medical notes or information on the Alder Hey children's hospital computerised results system (Meditech). The data were collected from date of initiation of warfarin therapy to the date of recruitment. Any evidence of bleeding (either verbally reported by parents or recorded in medical notes) was also recorded and graded as mild/severe/life threatening/fatal as classified Fihn et al.(1996) and Streif et al.(1999).

Following the data collection, the coded samples were completely anonymized and could not be traced to any individual. All variables were checked for completeness and accuracy. Where missing data were noted, an attempt was made to retrieve the data. To check data accuracy, range and consistency checks were undertaken.

4.2.4 DNA collection and extraction

Saliva samples were collected using the Oragene® DNA kit (information is available at www.dnagenotek.com). Patients were required to spit 1-2ml of saliva into an Oragene® DNA vial. When the lid was closed, the saliva was automatically mixed with Oragene® DNA preserving solution. If the child was unable to produce a saliva sample, then a buccal swab was used to collect the saliva. The resulting sample solutions were stored at -20°C and then thawed at room temperature before processing. Samples were then incubated at 50°C, to maximize DNA yield and to ensure that nucleases were permanently inactivated.

Purification was conducted using the Oragene DNA purification protocol. In this protocol, Oragene DNA Purifier was added to each sample in the ratio of 1:25 (e.g. 80 µl of purifier was added to 2 ml of sample). To increase the effectiveness of impurity removal, the mixture was then incubated on ice for 10 minutes. The mixture was then centrifuged at 4600 x g and the supernatant transferred to a fresh tube. An

equal volume of 95% ethanol (at room temperature) was added to the clear supernatant to precipitate DNA. The mixture was then centrifuged again at 4600 x g and the supernatant was removed as completely as possible. Subsequently 1 ml of 70% ethanol was added, without disturbing the smear or the pellet. After gentle swirling, the ethanol was discarded from the tube. This process ensured that any residual inhibitors were removed. The DNA pellet was rehydrated in TE buffer and stored in a cold room for a week, before quantification using Nanodrop.

4.2.5 Genotyping

Genotyping of *CYP2C9*2* (rs 1799853), *CYP2C9*3* (rs 1057910) and *VKORC1-1693* (rs 9923231) was performed on an ABI 7900HT Real time PCR using Taqman chemistry. These SNPs have been replicated in many studies associated with warfarin dose in the adult population. PCR was carried out using Taqman® Drug metabolism Genotyping Assays C-30403261_20, C-25625805_10 and C-30403261_20). A reaction volume of 10 µl contained 10 ng DNA, 1X Taqman Universal PCR Master Mix (without AmpErase) and 1X Taqman®Drug Metabolising Genotyping Assays. The genotyping procedure is discussed in detail in Chapter 2.2.4.

4.2.6 Study outcomes

Study outcomes were specified in the protocol, as follows:

a) **Primary outcome measure**: *The proportion of time in which INR measurements fell within the target range (PTTR) within the first six months.* The method of linear interpolation (Rosendaal *et al.* 1993) was used to estimate the proportion of time patients spent in therapeutic range (PTTR) between two test days. The PTTR is used to interpret the relative efficacy and safety of anticoagulant control in children (Streif *et al.* 1999, Bradbury *et al.* 2008, Bhat *et al.* 2010). We chose this as a primary outcome because the value explained the proportion of time patients were adequately protected against thromboembolism without a risk of bleeding. The target

range was defined as recommended by the British Society of Haematology Guidelines (Keeling *et al.* 2011).

b) **Secondary outcome measures**: Five secondary outcomes were evaluated:

i. *INR exceeding the target range within the first week of treatment*. This outcome was chosen because over-anticoagulation could result to bleeding and the bleeding risk is greatest during the first week of therapy (Fihn *et al.* 1993, Landefeld and Beyth 1993, Douketis *et al.* 2000).

ii. *Stable dose (mg/day)*, defined as the mean daily dose required to achieve three consecutive INR measurements within the individual's target range, over a minimum period of four weeks, at the same daily dose. If several periods of stable dose were observed, the stable dose at the earliest date was taken.

iii. *Bleeding complications*, classified according to both Fihn *et al.* (1996) and Streif *et al. (1999).* A summary of bleeding definitions used for this study is provided in Table 4-2.

Table 4-2 Definition of Bleeding Complications

Bleeding grade	Definition
Minor	Bleeding events not meeting the criteria for a major bleed.
Major	Clinically overt bleeding causing admission to hospital, OR a decrease of >20 g/L in haemoglobin in 24 hours OR need for transfusion of red blood cells, OR any CNS or retroperitoneal bleed.
Life Threatening	Leading to cardiopulmonary arrest; surgical or angiographic intervention, or irreversible sequelae, such as myocardial infarction, neurologic deficit consequent to intracerebral haemorrhage, or massive haemothorax.
Fatal	Leading directly to the death of the patient

The time taken to achieve therapeutic INR and a stable warfarin dose were included as secondary outcome measures in the study protocol; however, there was insufficient follow-up data of patient demographics such as weight, height at the time the outcomes were reached to allow accurate assessment of these outcomes.

4.3 Statistical Analysis

All analyses were performed in R version 2.13 by a statistician, Laura Sutton. R is a free software environment for statistical computing and graphics (R Core Team 2011). The conformity of each SNPs with Hardy Weinberg Equilibrium (HWE) was tested ($p<0.01$ indicated deviation).

4.3.1 Non-genetic variables

Non genetic variables including age, gender, height, weight, BMI, albumin, target INR group and indication for treatment, were recognised as being potentially important variables which may influence warfarin response.

Age, height and target INR group was associated with warfarin response in a previous study in a paediatric population (see Table 4-1). Weight, BMI and gender are also very interesting variables to explore, as studies have found them to be associated with warfarin response in adults (as discussed in section 1.2.4).

Warfarin is highly bound to plasma albumin, with only 0.7% free circulating in the blood (Johnson *et al.* 2006). Many children who take warfarin commence on the drug at a time when they may be hypoalbuminaemic (e.g. after cardiac surgery or nephritic syndrome) and therefore the effect of plasma albumin levels needs to be considered.

The indication for warfarin therapy also affects warfarin response. Evidence has shown children who are anticoagulated for prevention of thromboembolic events following a Fontan procedure, have a lower warfarin dose requirement than those who are anticoagulated for other indications (Streif *et al.* 1999, Biss *et al.* 2012). The likely explanations are associated with right-sided cardiac failure, which causes liver dysfunction. As a result, warfarin metabolism is reduced and warfarin sensitivity is increased.

For the variable 'INR group', patients were grouped according to the lower limit of their referral therapeutic range. They were classified as either 1.5, 2.0, 2.5 or 3.0. For example if a patient's target INR was 1.5-2.0, they were grouped to an INR target group as 1.5, but if a patient's INR was 2.0-3.0, they were grouped as 2.0.

For the variable 'treatment indication', patients were divided into non-Fontan and Fontan groups. A second classification was also used and tested in a sensitivity analysis, where patients were divided into non-Fontan cardiac, Fontan cardiac and non-cardiac groups.

Considerable volumes of data for the non-genetic variables were missing (Table 4-3). In order to increase power and reduce bias , multiple imputation using chain equations (van Buuren and Groothuis-Oudshoorn 2011), specifically the predictive mean matching method, was used to impute data which had <30% missing observations. With a rate of missingness of 30%, the efficiency of estimates based on 3 imputations is approximately 91% (Rubin 2008). Multiple imputation was not used for variables which had ≥30% missingness. Instead, these variables were excluded from the list of potential covariates.

4.3.2 Genetic variables

The SNPs *CYP2C9*2* (rs 1799853), *CYP2C9*3* (rs 1057910) and *VKORC1-1693* (rs 9923231) were selected for the analysis as these have been shown to affect warfarin response in adult and children populations using warfarin (Biss *et al.* 2012, Moreau *et al.* 2012, Yang *et al.* 2013).

4.3.3 Association of non-genetic variables with the outcomes

First, the non-genetic variables were each individually tested for association with each outcome. Variables giving a p-value <0.10 were adjusted for, when testing for association with individual SNPs. If a pair of variables was found to be collinear, only the variable with the lowest p-value was adjusted for in the analysis.

For the outcome of proportion of time in which INR measurements fell within the target range (PTTR) within the first six months and INR that exceeded the target range during the first week, the value of non-genetic variables at the start of warfarin treatment were used. For the outcome of stable dose, values of non-genetic variables at the time stable dose was achieved were used. For the outcome of bleeding complications, no tests for association with non-genetic variables were undertaken, as it was not possible to determine which time point should be referred to for obtaining the value of these variables in those not experiencing the complication.

4.3.4 Analysis of association with each SNPs

We tested for associations with each individual SNP. Two regression models were fitted for each SNP. The first (the 'baseline model') included all non-genetic factors giving a p value <0.10 univariately and second (the 'genetic model') was the same as the first but also included covariates to represent the SNP of interest. The likelihood ratio test was used to compare both models. Two analyses were carried out for each SNP; one with no underlying assumptions regarding the mode of inheritance and another assuming an additive mode of inheritance. In the analysis with no underlying assumption, two variables were used to represent the SNP which is one representing heterozygotes and one representing mutant homozygotes. In the analysis assuming an additive mode of inheritance, a single variable to represent the SNP was used. Wild-type homozygotes were coded '0', heterozygous patients were coded as '1' and mutant homozygotes were coded as '2'. The false discovery rate (FDR) was calculated for each SNP association tested (Benjamini and Hochberg 1995).

4.3.5 Multiple regression analysis

Finally, we investigated the total variation explained by all significant non-genetic and genetic factors. A multiple regression model was fitted for each outcome which included covariates with a p value <0.10 in the univariate analysis and all SNPs that were significant under FDR control. For continuous outcomes, multiple linear regression models were fitted and for binary outcomes, logistic regression models were fitted.

4.4 Results

4.4.1 DNA yield and quality

The estimated amount of total DNA extracted from 2 mL saliva samples varied between 0.05 and 619.5 μg with a mean of 85.2 μg. The mean values of the 260/280 and 260/230 nm ratios were 1.7 (range 1.4-2.78) and 0.9 (range 0-4.9), respectively. DNA which did not meet the 260/280 in the range of 1.7-2 was purified by centrifuging and pippette out the cloudy portion. If the 260/280 was still out of target range, EZ-DNA extraction column kit was used to purify the DNA.

4.4.2 Patient Characteristics

A total of 100 patients were recruited (male=55, female=45). Ninety-seven were Caucasian while the others were black (n=1), Indian (n=1) and Afghan (n=1). The three non-Caucasians were excluded from the analysis to minimise the effect of ethnic differences in warfarin response (see chapter 1.9).

Patient characteristics are shown in Table 4-3. The majority of our patients were very young during the start of warfarin therapy: 75.3 % were below 5 years old at the time of start of therapy. Out of 85 patients who reached stable dose, 53% were below 5 years of age.

Of the 97 children, 85 were anticoagulated following surgery for congenital heart disease and 12 were non-cardiac (Table 4-4). The primary indication for warfarin was the Fontan procedure. Genotype distribution for all four SNPs met Hardy-Weinberg equilibrium ($p>0.60$) (Table 4-5). Allele frequencies for *CYP2C9*2* (0.11), *CYP2C9*3* (0.09) and *VKORC1-1693* (0.36) were similar to those previously reported in adult populations (Stubbins *et al.* 1996, Aithal *et al.* 1999, Sconce *et al.* 2005).

Table 4-3. Summary of participant characteristic at the start of warfarin therapy and at the time stable dose was achieved.

Variable	Start of therapy (n=97)			Time of stable dose (n=85)*		
	Mean	SD	Missing (n (%))	Mean	SD	Missing (n (%))
Age (decimal yrs.)	3.9	3.9	0	6.9	4.5	0
Height (m)	0.94	0.28	26 (26%)	1.06	0.28	40 (47%)
Weight (kg)	15.7	14.5	12 (12%)	20.6	15.7	35 (43%)
BMI ($kg.m^{-2}$)†	16.1	3.1	26 (26%)	16.5	2.5	40 (47%)
Albumin ($g.L^{-1}$)	38.3	5.3	30 (30%)	39.2	6.2	62 (64%)

✳12 patients did not achieve stable dose during follow-up

Table 4-4. Indication for warfarin treatment.

Indication for warfarin treatment		Number of patients (%)
Surgery for congenital heart disease	Fontan procedure	61 (62.9%)
	Other cardiac procedures: (e.g. Mitral valve replacement, post shunt, modified Norwood procedure)	24 (24.7%)
Non-cardiac patients	Pulmonary embolism	3 (3.1%)
	Deep Vein Thrombosis	2 (2.1%)
	Prophylaxis of line/catheter clotting or post-surgery	6 (6.2%)
	Factor V Leiden Syndrome	1 (1.0%)

Table 4-5 Genotype frequencies for the four SNPs.

Genotype	Homozygous wild type	Heterozygote	Homozygous mutant
*CYP2C9*2*	76 (78.4%)	19 (19.6%)	2 (2.1%)
*CYP2C9*3*	79 (81.4%)	18 (18.6%)	0 (0%)
VKORC1-1693	40 (41.2%)	45 (46.4%)	12 (12.4%)

4.4.3 Summary of the outcomes

The mean proportion of time spent within the target INR range in the first six months of treatment was 49% (SD 27%) and the mean stable warfarin dose was 2.70 mg/day (SD 1.65) (Table 4-6). 40.2% patients experienced an INR above range in the first week and 50.5% did not. The remainder (9.3%, n=9) were not categorized because insufficient data was available. There were no reported major or severe bleeding events (e.g. life threatening or fatal). Sixty-nine patients experienced a minor (as per Fihn classification) or mild (as Streif classification) bleed. The remaining 28 patients did not experience any bleeding complications.

Table 4-6 Summary of outcome variables.

Variable	Mean	Median	SD	Min	Max	NAs
Proportion of time in INR range (first 6 months)	0.50	0.51	0.27	0.00	1.00	7
Stable dose (mg)	2.7	2.4	1.8	0.0	10.0	8

4.4.4 Association of non- genetic variables with outcomes

Table 4-7 shows the association between the non-genetic factors and the outcome variables. It was not possible to test for association between height, weight, BMI or albumin and stable dose due to a significant amount of missing observations (>30%) for these variables at the time stable dose was achieved.

a) For the outcomes of the proportion of time in the INR range (PTTR) during six months follow-up, target INR group and indication for treatment (2 groups) were both associated ($p<0.10$) with this outcome. The distribution of PTTR stratified by INR group and indication for treatment are shown in Figure 4-1 (a) and (b)

b) For the outcome of INR exceeding target range during the first week, none of the variables were associated.

c) For the outcome of stable dose, age and target INR group were significantly associated ($p<0.10$) with this outcome. The scatter plot in

d) Figure 4-2 shows the relationship between warfarin daily dose and age and Box plot in Figure 4-3 shows the distribution of daily warfarin dose stratified by target INR group.

Table 4-7 P-values from tests of association of non-genetic variables with the outcome.

Variables	**Proportion of time INR within target range in 1st 6 months (n=90)**		**INR exceeding target range in week 1 (Y/N) (n=88)**		**Stable dose (mg/day) (n=85)**	
	P value	n*	P value	n*	**P value**	**n***
Age	0.144	0	0.584	0	**<0.001***	**0**
Height	0.105	26	0.802	26	NA	0
Weight	0.336	12	0.619	12	NA	0
BMI	0.334	26	0.215	26	NA	0
Albumin	0.982	30	0.897	30	NA	0
Gender	0.468	0	0.173	0	0.351	0
INR group*	**0.004***	**0**	0.657	0	**0.015***	**0**
##Indication **	**0.050***	**0**	0.543	0	0.379	0

* INR group: Patients were group into the lower limit of their referral range: i.e 1.5, 2.0, 2.5 or 3.0

**Indication (2 groups): Patients were divided into non-Fontan and Fontan group

In Sensitivity analysis where patients were split to 3 indication groups:-non-Fontan cardiac, Fontan cardiac and non-cardiac, the p-values were 0.144, 0.77 and 0.575 respectively and hence conclusion remain the same

n*number of sample imputated

NA Due to abundant data missing (>30%), the variables was excluded in the analysis

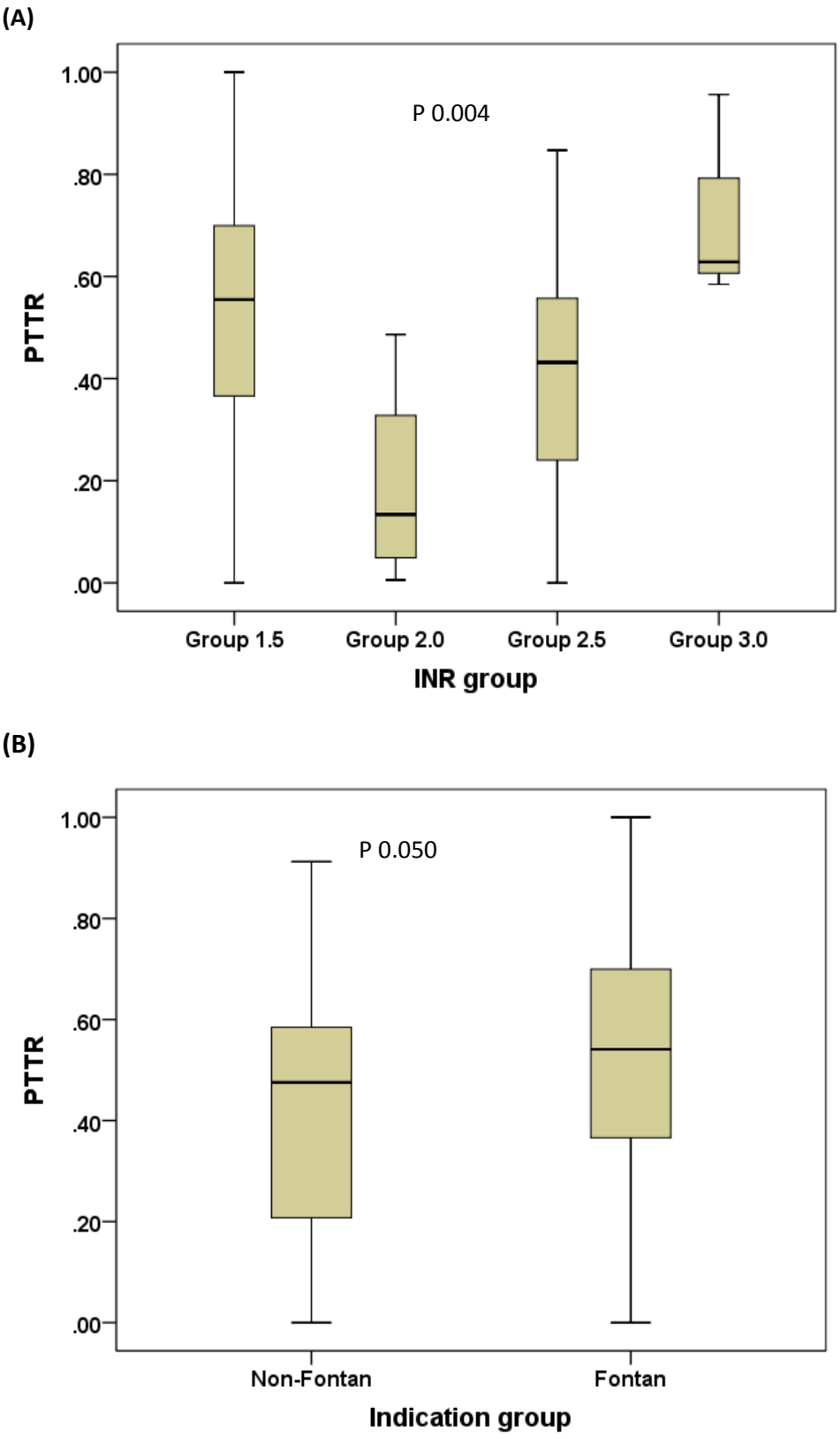


Figure 4-1. Box and whisker plot showing the distribution of PTTR based on (A) target INR group and (B) indication treatment group. Boxes represent 25th-75th percentile (interquartile range) of PTTR, whiskers represent 5th-95th percentile, solid lines represent median dose. Open dots denote outliers (a value between 1.5 and 3 times the interquartile range away from the 25th or 75th percentile).

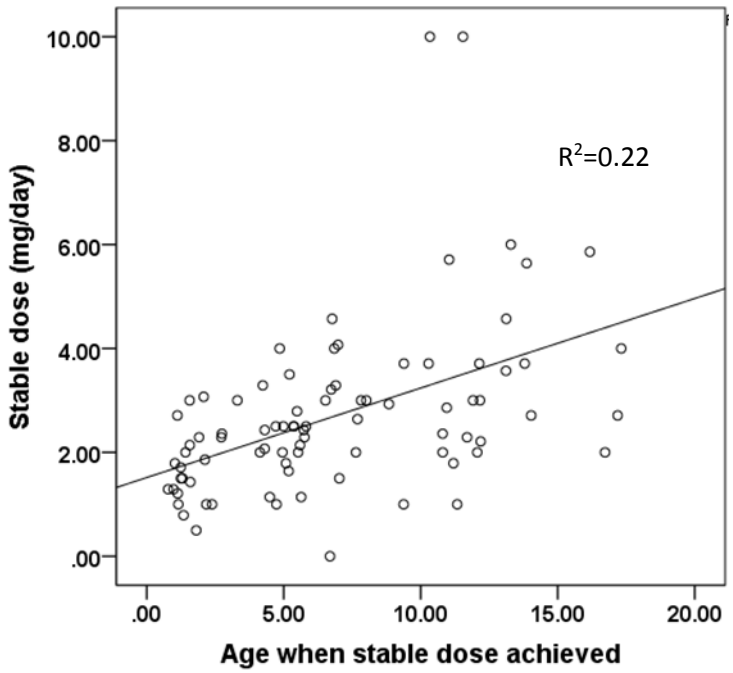


Figure 4-2. Relationship between warfarin daily stable dose and age.

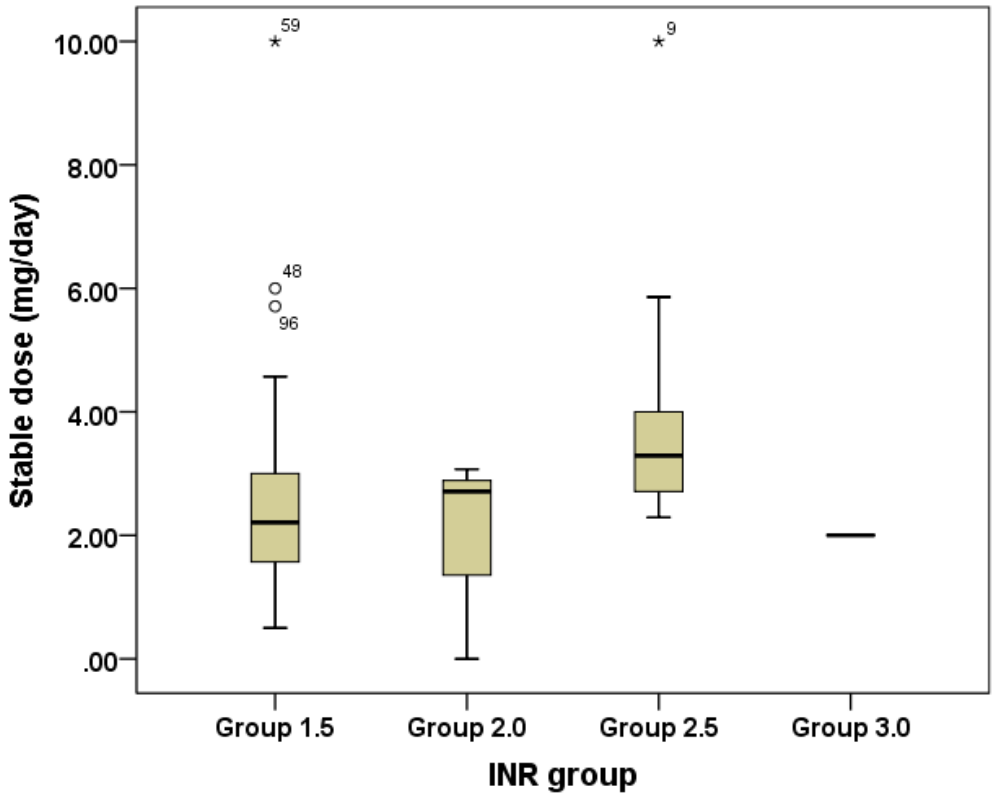


Figure 4-3. Box and whisker plot showing the distribution of PTTR stratified by INR target group. Boxes represent 25th-75th percentile (interquartile range) of PTTR, whiskers represent 5th-95th percentile, solid lines represent median dose. Open dots denote outliers (a value between 1.5 and 3 times the interquartile range away from the 25th or 75th percentile).

4.4.5 Analysis of association with each SNP

Table 4-8 presents the results from undertaking likelihood-ratio tests and comparing the baseline model to the genetic model for each outcome and SNP in turn. *VKORC1-1693* was significantly associated with PTTR and stable dose when assuming an additive mode of inheritance and with bleeding complications when making no assumption regarding mode of inheritance. The distribution of PTTR and stable dose stratified by *VKORC1-1693* genotypes is illustrated in Figure 4-4 and Figure 4-5(b) respectively. *CYP2C9*2* was significantly associated with INR above range in the first week of treatment and warfarin stable dose when assuming an additive mode of inheritance. The distribution of warfarin stable dose stratified by *CYP2C9*2* genotypes showed in Figure 4-5 (a).

Table 4-8 Individual SNP association analyses

Outcome	SNP (assumption)	p-value from LRT	Significant following FDR adjustment
Proportion of time in INR range (PTTR)*	*CYP2C9**2 (none)	0.58	No
	*CYP2C9*2* (additive)	0.53	No
	*CYP2C9*3* (NA†)	0.95	No
	*VKORC1*2* (none)	0.03	No
	*VKORC1**2 (additive)	**0.001**	**Yes**
INR above range in week 1	*CYP2C9**2 (none)	0.04	No
	*CYP2C9*2* (additive)	**0.004,**	**Yes**
	*CYP2C9**3 (NA†)	0.80	No
	*VKORC1*2* (none)	0.23	No
	*VKORC1*2* (additive)	0.020	No
Stable dose**	*CYP2C9*2* (none)	0.09	No
	*CYP2C9*2* (additive)	**0.008**	**Yes**
	*CYP2C9*3* (NA†)	0.049	No
	*VKORC1**2 (none)	0.35	No
	*VKORC1*2* (additive)	**0.003**	**Yes**
Bleeding complications	*CYP2C9*2* (none)	0.819	No
	*CYP2C9**2 (additive)	0.423	No
	*CYP2C9*3* (NA†)	0.482	No
	*VKORC1*2* (none)	**0.006**	**Yes**
	*VKORC1*2* (Additive)	0.288	No

FDR = false discovery rate; LRT = likelihood ratio test.

* Analyses adjusted for indication for treatment and target INR group.

** Analyses adjusted for age and target INR group.

† No mutant homozygotes so assumption regarding mode of inheritance irrelevant.

For non-additive assumption p-value from LRT is for heterozygous and homozygous each SNP.

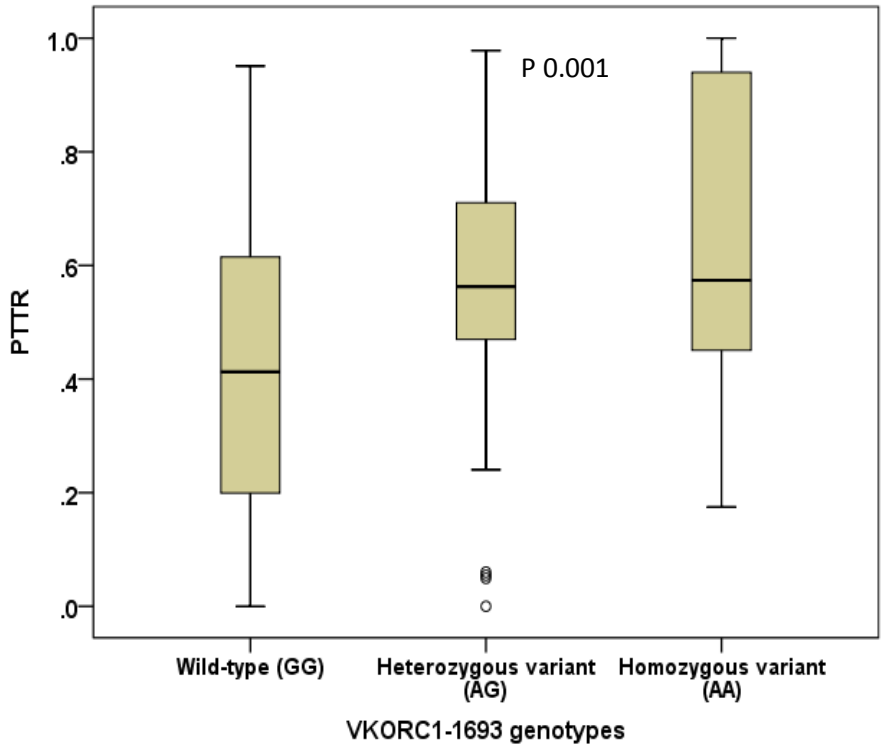
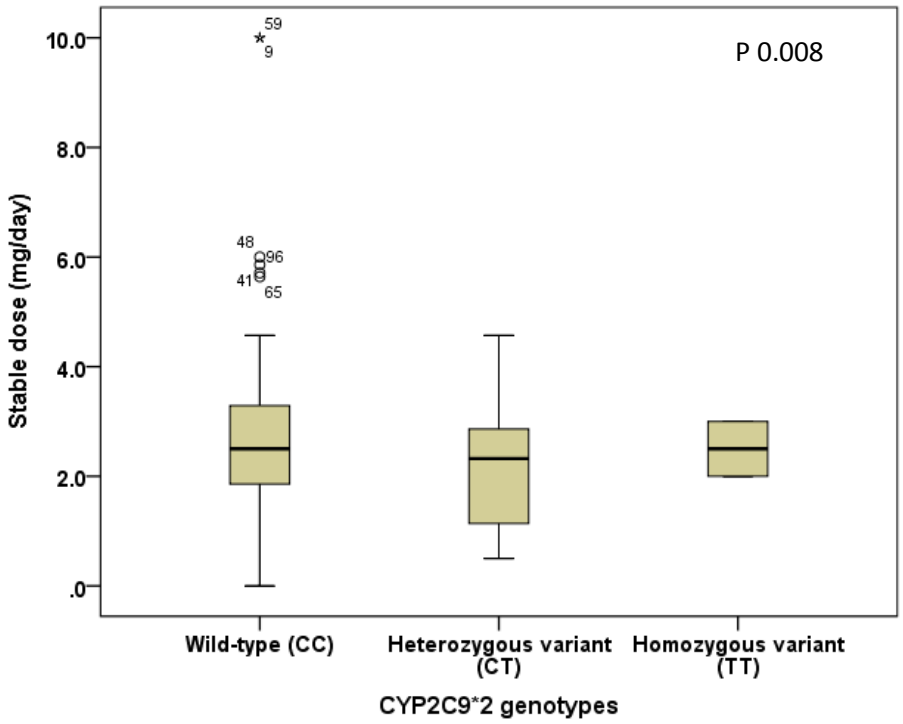


Figure 4-4. Box and whisker plot showing the distribution of PTTR stratified by *VKORC1* genotypes. Boxes represent 25th-75th percentile (interquartile range) of PTTR, whiskers represent 5th-95th percentile, solid lines represent median dose. Open dots denote outliers (a value between 1.5 and 3 times the interquartile range away from the 25th or 75th percentile).

(A)



(B)

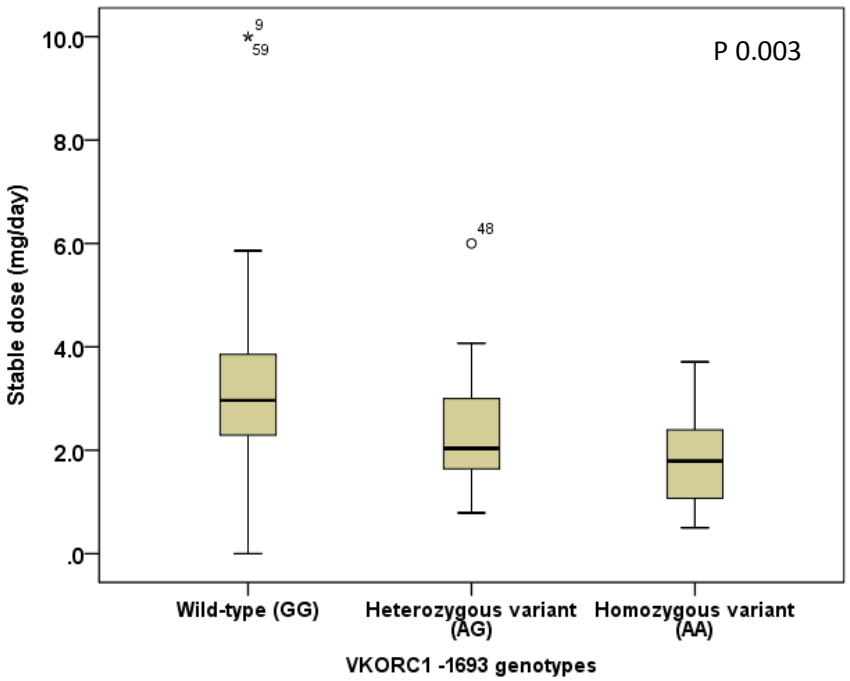


Figure 4-5. Box and whisker plot showing the distribution of warfarin stable daily dose stratified by (A) *CYP2C9*2* and (B) *VKORC1-1693* genotypes. Boxes represent 25th-75th percentile (interquartile range) of PTTR, whiskers represent 5th-95th percentile, solid lines represent median dose. Open dots denote outliers (a value between 1.5 and 3 times the interquartile range away from the 25th or 75th percentile) and and asterisks denote extreme outliers (a value more than 3 times the interquartile range away from the 25th or 75th percentile).

4.4.6 Final multiple regression models

Using the non-genetic variables with $p<0.10$ and genetic variables that were significant after FDR correction, the multiple regression models were built for each outcome. The R^2 values for the models are provided in Table 4-9.

Taken together, the indication for treatment and target INR groupings accounted for approximately 11% of variability in the proportion of the first six months' treatment time spent within target INR range. The presence of variant alleles in *VKORC1-1693* was associated with a greater time spent within range, with each additional variant allele associated with approximately 13% (95% CI 5% to 21%) more time spent within the target INR range in the first six months. *VKORC1-1693* combined with the aforementioned non-genetic factors explained almost 21% variability in proportion of the first six months' treatment time spent within the target INR range.

The variant allele *CYP2C9*2* was associated with an increased likelihood in the INR values exceeding the target range in the first week of therapy. Odds ratio for heterozygotes versus wild-type homozygotes was 4.10 (95% CI: 1.42, 12.34).

Overall, the patient's age, target INR range, *CYP2C9*2* and *VKORC1-1693* genotypes accounted for approximately 41% of the variability in the warfarin dose required to stabilise INR. The presence of variants in *CYP2C9*2* and *VKORC1-1693* were both associated with a lower dosage required to stabilise INR. The *CYP2C9*2* variant allele was associated with an approximate decrease in dose of 0.82 mg/day and the *VKORC1-1693* variant allele was associated with an approximate decrease of 0.66 mg/day. Based on the change in adjusted R^2 values with the addition of the genetic variables, variability in *VKORC1-1693* accounted for approximately 7% and variability in CYP2C9*2 approximately 5% of the overall variance in stable dose.

The presence of one variant allele at *VKORC1-1693* was associated with an increased likelihood of bleeding complications, with the odds ratio for heterozygotes

versus wild-type homozygotes being 4.53 (95% CI: 1.59-12.93). No significant association was found between having two copies of the variant allele and bleeding complications; however bleeding events were both rare, as were patients who were homozygous for variant alleles, and therefore there may be insufficient power to detect a significant association with mutant homozygotes.

Table 4-9 Final multiple regression models.

Outcome	Variables included	Coefficient (95% CI)†	Adjusted/pseudo R^2		
			Non genetic variables	Genetic variables	All variables
Proportion of time INR within target range (PTTR)	Indication for treatment * INR group** *VKORC1-1693* (additive)	0.09 (-0.04, 0.22) 1: -0.33 (-0.54, -0.12) 2: -0.00 (-0.17, 0.17) 3: 0.20 (-0.08, 0.49) 0.13 (0.05, 0.21)	11.3%	9.5%	20.8%
INR exceeding target range in week 1	*CYP2C9*2* (additive)	4.18 (1.42, 12.34)	-	6.8%	6.8%
Stable dose	Age INR group ** *CYP2C9*2* (additive) *VKORC1 -1693* (additive)	0.19 (0.12, 0.25) 1: -1.05 (-2.36, 0.26) 2: 0.93 (0.14, 1.73) 3: -0.27 (-2.81, 2.27) -0.82 (-1.39, -0.25) -0.66 (-1.08, -0.25)	29.2%	11.9%	41.4%
Bleeding complications	*VKORC1-1693* (none)	Het: 4.53 (1.59, 12.93) Homo: 1.13 (0.20, 6.51)	-	8.7%	8.7%

†Regression coefficient for multiple regression models; odds ratio for logistic regression models; CI = confidence interval; INR group: 1 for 1.5, 2 for 2.0, 3 for 2.5; Het=heterozygous; Homo= homozygous.
*Indication for treatment: Patients were divided into non-Fontan and Fontan group (2 group)
**INR group: Patients were group into the lower limit of their referral range i.e 1.5, 2.0,2.5 or 3.0

4.5 Discussion

In this prospective cohort study of 97 Caucasian children treated with warfarin, we have demonstrated that the target INR, indication for treatment and *VKORC1-1693* were significantly associated with the proportion of time spent in INR range (PTTR) explaining approximately 21% of the variation. To date, this is the first study that has demonstrated a significant association between a genetic polymorphism in *VKORC1-1693* and this outcome. We also replicated the previously reported association between *CYP2C9*2* and *VKORC1-1693* polymorphisms and warfarin dose in a paediatric population, together with age and target INR group (which in total explained 41.4% dose variation). This study is also the first to show an association between *CYP2C9*2* and INR exceeding target range during the first week of therapy, and the association between *VKORC1-1693* and minor bleeding events in children treated with warfarin.

The INR is used to monitor warfarin response. It is essential to maintain the INR value within the targeted range to minimize the risk of either thromboembolism (caused by under-dosing) or haemorrhage (caused by over-dosing). It has been suggested (Biss *et al.* 2011) that anticoagulation control in children receiving warfarin should be reported as the proportion (or percentage) of time in which INR measurement fell within the target range (PTTR). Based on a PTTR of 0.49, we reported poor anticoagulation control among children in our cohort compared to control data observed in previous studies (Biss *et al.* 2012, Moreau *et al.* 2012).

Our study corresponds with that of Moreau *et al.* (2012) with regard to the significant association between target INR and PTTR. In their study, three pre-specified INR ranges were used: 2.2 (for INR range 1.5-3.3), 2.5 (for INR range 1.8-3.2) and 3.3 (for INR range 2.5-4). Unlike Moreau *et al.* (2012) however, our study

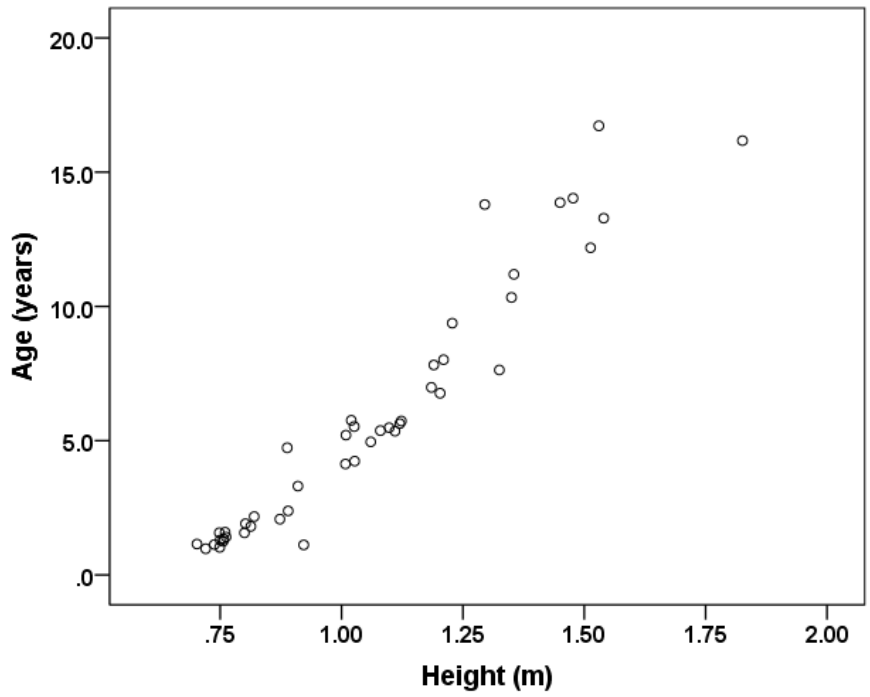
showed that PTTR was also significantly associated with indication of warfarin treatment and *VKORC1-1693* polymorphisms.

Interestingly, the presence of mutant alleles in *VKORC1-1693* was associated with a greater time spent in the target INR range (PTTR), with each additional variant allele associated with approximately 13% (95%CI 5% to 21%) more time spent in range in the first six months. This finding conflicts with a previous study in children (Biss *et al.* 2013) and adults (Schwarz *et al.* 2008). Schwarz et al. (2008) have shown that those who are homozygous for *VKORC1-1693* variant alleles spent significantly more treatment time above their INR therapeutic range. A similar finding was observed in study by Biss *et al* (2013) in children (n=51) given warfarin during the first month of therapy but the findings were not significant. However, Biss *et al.,* (2013) found no association between the *CYP2C9 (*2 and *3)* and *VKORC1-1693* genotypes and the proportion of INR values above the target range beyond the first month of therapy. This suggests that the adjustment of warfarin doses based on INR after the first month of therapy had counteracted the influence of the *CYP2C9 (*2 and *3)* and *VKORC1-1693* genotypes. A possible explanation of what was observed in our analysis is that as carriers of allele A (*VKORC1-1693* variant allele) are more sensitive to warfarin and they reach the target INR in a shorter time, even though they possibly undergo over anti-coagulation, their INR was more frequently monitored by POCT thus leading to greater time spent in the target INR range.

The current recommended dosing regimens for warfarin initiation in children are based solely on patients' weight (Monagle *et al.* 2012). Unfortunately, we had to exclude weight from our analysis because of the high volume of missing data. However, age and height were found to be highly correlated with weight and therefore we believe that omitting weight from our analysis will not unduly affect the quality of

our findings. The relationship of age, height and weight at stable dose in our cohort are illustrated in Figure 4-6.

(A)



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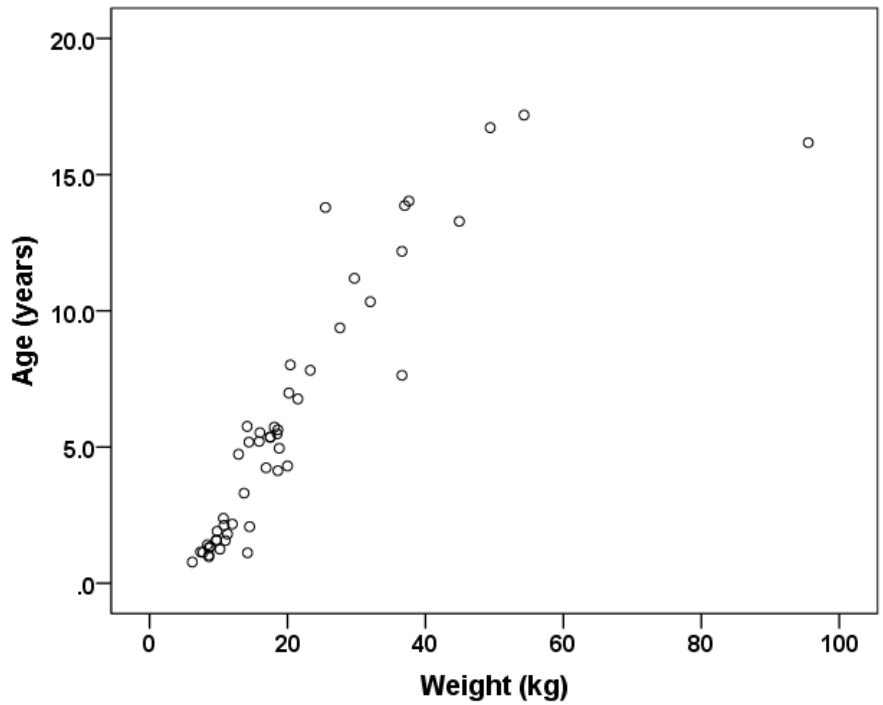


Figure 4-6. Cohort relationship between (A) age and height and (B) age and weight at warfarin stable dose was achieved.

*CYP2C9*2, CYP2C9*3* and *VKORC1-1693* have been shown as important factors in predicting warfarin dose in previous studies (Biss *et al.* 2012, Moreau *et al.* 2012). As described in Table 4-1, the contribution of SNPs in these genes is similar to that explained in the adult population, approximately 38% (except for study by Nowak et al, the findings was not replicated due to small sample size) (Cooper *et al.* 2008, Gage *et al.* 2008, Takeuchi *et al.* 2009). However, this was not reflected in our analysis where these SNPs only explained 11.9% of variation in warfarin dose requirements. One possible reason for this could be that when compared to the other two study groups, our cohort consisted of children of a much younger age. 54% of our study cohort was less than 6 years old when stable dose was reached compared to 23% in the cohort studied by Biss *et al.* (2012). Similarly, in the study by Moreau *et al.* (2012), the mean age was 8.4 years (± 5.6) while in this study, it was 6.9 (± 4.5) years.

There are two possible reasons for the proportion of variability in dose requirements explained by *CYP2C9*2* and *VKORC1-1693* polymorphisms being lower in younger patients. First, the accuracy of dosing taken by patients is questionable. Children aged less than 6 years have difficulty in swallowing warfarin in tablet form, and thus warfarin is given by crushing the tablet or extemporaneously prepared by the pharmacist. There is paucity of data to support the use of extemporaneously prepared suspensions of warfarin (Jackson and Lowey 2010); for example stability issues, uniformity of suspension which is critical for warfarin due to its narrow therapeutic range and interaction with packaging material. In addition, the bitter taste of warfarin possibly caused young patients to spit or vomit the medicine, or refuse to take the medicine. Second, the large variability in CYP2C9 protein expression (approximately 35=fold) in younger children, especially in children below the age of 1 year (Koukouritaki *et al.* 2004, Hines 2007) probably explains the low

contribution of these SNPs in our cohort, where 46% of children were below 1 year old.

In our analysis, we found an association of *CYP2C9*2* with INR above range within the first week therapy and an association between *VKORC1-1693* and bleeding complication. This was supported by recent studies by Biss *et al*. (Biss *et al*. 2013) as they found *CYP2C9*2* and *CYP2C9*3* allele carriers had higher mean INR during the first week of therapy compared to wild-type. These findings provide the possibility to improve warfarin therapy in children because the most dangerous side-effect of warfarin is bleeding, and over-coagulation may lead to severe bleeding. This finding could assist a physician in identifying patients who need extra-care when initiated on warfarin. However, the findings need replication in a large external cohort.

This is a retrospective cohort study. Thus, even though all the data were recorded systematically from a single site, missing data cannot be avoided. We intended to analyse the association as a longitudinal study, so that we could observe the contribution made by variables at different ages, but since we had insufficient data on these variables measured over time, this allowed us only to perform a cross sectional analysis.

As studies have shown large variability in CYP2C9 protein expression in younger children, including patients from newborn to 18 years old as a single studied cohort is not an ideal study design to explore the *CYP2C9* polymorphisms contribution to warfarin dose. In addition, due to the limitations of our sample size, it was not possible to undertake the association analyses stratified by age. Medication adherence of the patients was also not assessed and was also beyond the control of the study team, firstly because medication was administered by family members as these patients are treated as out-patients and secondly because the data was collected retrospectively. This may explain a significant part of the unexplained variability, although the cohort

was closely monitored through the POC testing system and regular education of the patients and their family was undertaken.

We limited our cohort to Caucasian European children in the inclusion criteria, to minimise variability in effect caused by ethnic differences. Consequently, our results may not equally apply to non-white and non-European populations. Further, as a result of our small study samples, we did not identify any children with homozygous *CYP2C9*3* and therefore the effect of homozygous *CYP2C9*3* could not be evaluated in our analysis.

In this chapter, we have replicated the association between *VKORC1-1693* and *CYP2C9*2* polymorphisms and paediatric warfarin dose requirements as seen in previous studies (Nowak-Göttl *et al.* 2010, Biss *et al.* 2012, Moreau *et al.* 2012). Our study also provides new information on the contribution of these SNPs to variations in the proportion of time spent within therapeutic range, INR above range within 1 week of initial warfarin treatment and bleeding complications. This evidence suggests that genetic knowledge could improve the management of warfarin in paediatric populations, but, further work is needed to replicate the study in a larger sample set, in particular with a view to stratify the analyses by age. An alternative approach to achieving a larger sample size would be to organise an individual patient data (IPD) meta-analysis of ours and other similar datasets, and we will be investigating this possibility further.

Chapter 5

Warfarin pharmacogenetic in children: A genome-wide association study

CONTENTS

5.1 Introduction

In addition to *VKORC1* and *CYP2C9,* other genetic factors have been previously reported to have influential effects on warfarin response in adults as discussed in Chapter 1 (section 1.9 and 1.10). These include genes involved in the pharmacokinetics and pharmacodynamics of warfarin such as *CYP4F2, GGCX, Factor II, Factor VII, Factor X, EPHX1, CALU,* and *ABCB1* (Kristensen 2002, D'Ambrosio *et al.* 2004, Shikata *et al.* 2004, van der Heijden *et al.* 2004, Kohnke *et al.* 2005, Loebstein *et al.* 2005, Wadelius *et al.* 2005, Aquilante *et al.* 2006, Kimura *et al.* 2007, Loebstein *et al.* 2007, Caldwell *et al.* 2008, Kimmel *et al.* 2008, Borgiani *et al.* 2009, Takeuchi *et al.* 2009).

In chapter 4, a candidate gene approach was used to investigate the response to warfarin treatment in children, focussing on genetic variants in two genes *CYP2C9* and *VKORC1*. For the outcome 'proportion of time spent in INR range (PTTR)', target INR together with warfarin indication and *VKORC1* -1693 explained only ~21% variability. For warfarin stable dose, ~41.4% of dose variation was explained by age, target INR group, and SNPs in *CYP2C9* and *VKORC1*. A large proportion of missing heritability remains to be accounted for, suggesting that other genetic factors might be involved in warfarin's pharmacological effects in children. Knowledge of additional polymorphisms with large effect sizes would be extremely valuable in improving the safety and efficacy of warfarin therapy specifically in this vulnerable population.

Genome-wide association studies (GWASs) offer a powerful approach to gene function discovery and in understanding the genetic basis of drug response. To date, no GWAS has been reported in any paediatric population on warfarin treatment. Therefore, the aim of this chapter were to perform a GWAS in our cohort of children

prescribed warfarin, and investigate the association of genetic variation across the whole human genome with response to warfarin.

5.2 Methods

5.2.1 Patients and clinical outcomes

The study design, patient recruitment and clinical outcome measures have previously been described in sections 4.2.1, 4.2.2, 4.2.3 and 4.2.6.

5.2.2 Genome-wide genotyping

Of the 100 paediatric patients recruited retrospectively, 1.5-2 µg (quantified by picogreen) DNA samples from 87 patients were shipped to ARK-Genomics (The Roslin Institute, University of Edinburgh) for genome-wide genotyping on the Illumina Human OmniExpressExome-8 v1.0 chip using the Infinium HD Super assay (Illumina, Inc.).

Figure 5-1 summarises the principles and chemistry of genome-wide genotyping on the Illumina platform. In this system, whole-genome amplification is used to increase the amount of DNA up to 1000-fold. The DNA is fragmented and captured on a BeadChip by hybridisation to immobilised SNP-specific primers, stopping one base before the locus of interest. Marker specificity is conferred by enzymatic single-base extension where a fluorescently-labelled nucleotide is incorporated. Subsequent dual-colour florescent staining allows the labelled nucleotide to be detected by Illumina's iScan imaging system, which identifies both colour and signal intensity.

Finally, 951,117 SNPs were mapped to NCBI Build 37 (hg19) using Illumina manifest file Human OmniExpressExome-8v1.0_B.bpm. Bead intensity data were processed and normalized for each sample in GenomeStudio Data Analysis Software (Illumina, Inc.).

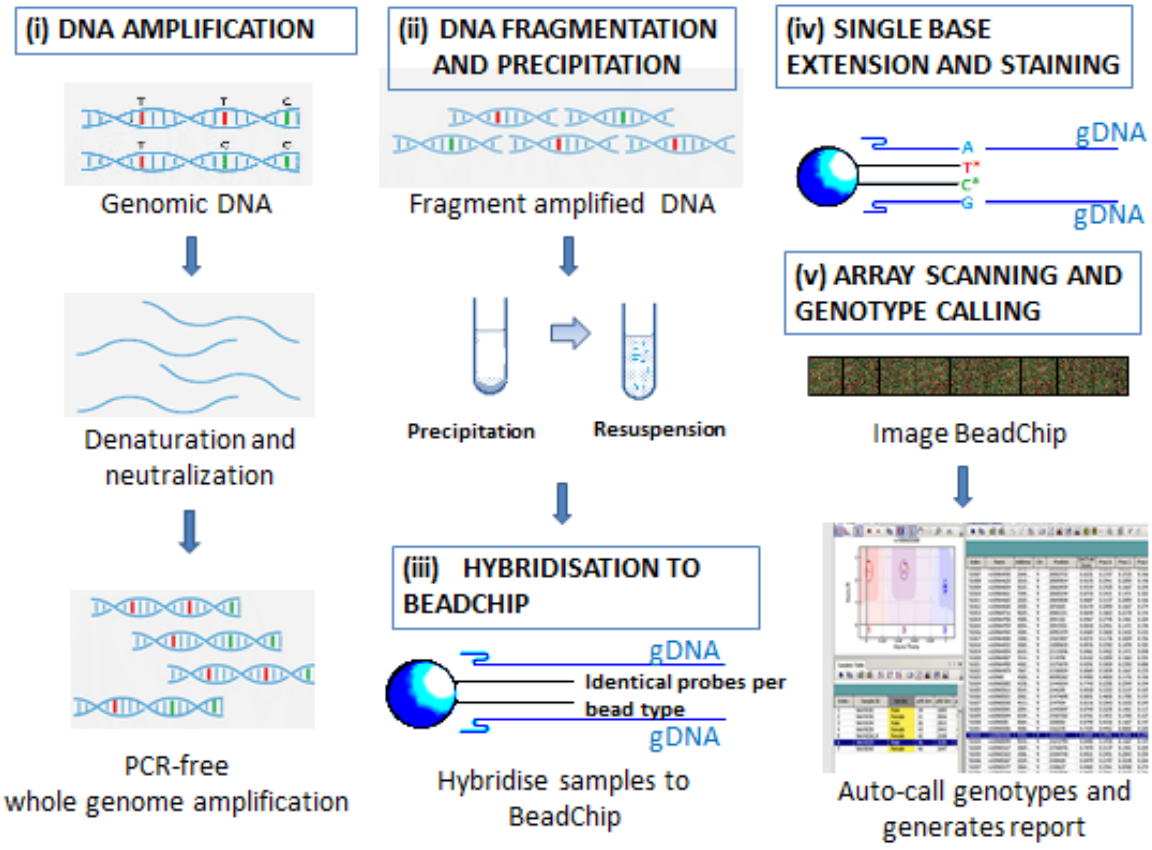


Figure 5-1. Outline of the Infinium HD assay chemistry for whole-genome genotyping. Five modular steps are involved. **(i) DNA Amplification:** After denaturation and neutralisation, the DNA is isothermally amplified whereby whole-genome amplification uniformly increases the amount of DNA by several thousand-fold without introducing large amounts of amplification bias. **(ii) Fragmentation and precipitation:** The amplified product is fragmented by a controlled enzymatic end-point fragmentation process to avoid over-fragmenting the sample. Following isopropanol precipitation, the fragmented DNA is resuspended in hybridisation buffer. **(iii) Hybridisation to BeadChip:** The BeadChip is prepared for hybridization in a capillary flow-through chamber. During hybridisation, the amplified and fragmented DNA samples selectively anneal to locus-specific 50-mer probes, stopping one base before the interrogated marker. Unhybridised and non-specifically hybridised DNA is washed away. **(iv) Single base extension (SBE) and staining:** If there is a perfect match, extension occurs. Extension of the primer incorporates a biotin nucleotide or a dinitrophenyl-labelled nucleotide. C and G nucleotides are biotin-labelled (green); A and T nucleotides are dinitrophenyl labelled (red). Signal amplification of the incorporated label further improves the overall signal-to-noise ratio of the assay. **(v) Array scanning and genotype calling:** The Illumina iScan scans the BeadChip by using a laser to excite the fluorophore of the single-base extension product on the beads, recording high-resolution images of light emitted from the fluorophores. The iScan Control Software then determines the intensity values for each bead type and creates data files for each SNP.

5.2.3 Data quality control

Quality control measures were undertaken using the open-source genome-wide analysis toolset PLINK, v1.07 (Purcell *et al.* 2007).

Sample quality control:

Subjects with genotyping success rate ≤ 95% (n = 3) were excluded from the dataset. Samples that showed gender inconsistencies (n = 2) were also removed. Cryptic relatedness was assessed by calculating identity by descent (IBD) and no kinship was detected. The ethnic origin of our samples was confirmed by principal-component analysis (PCA) using the SNPRelate (Zheng *et al.* 2012) package in R. HumanOmniExpressExome-8 v1.0 chip data were merged with genotype information from four HapMap 2 populations (CEU, YRI, JPT, and CHB), and samples were identified as ethnic outliers on the basis of their projection onto the four principal components of genetic variation (Figure 5-2). Samples of non-European ancestry were removed (n = 4). In total, 78 subjects passed quality control.

SNP quality control:

SNPs were excluded from downstream analysis if they were monomorphic (n = 18), had a MAF < 0.01 (n = 286,940), a HWE *P*-value < 0.0001 (n = 10,656) or a missing call rate ≥ 5% (n = 27,738). A total of 625, 765 SNPs passed quality control.

5.2.4 Imputation

Prior to imputation using the reference genotype data from the 1000 Genomes project (Phase I integrated variant set release v3, NCBI build 37 (hg19))(Altshuler *et al.* 2010), SNP alignment was undertaken. All SNPs with mismatched alleles (n = 125), those not present in the 1000 Genomes project (n = 1,475) and those that gave A>T or G>C or vice versa (n = 5,715) were excluded from the dataset. Haplotype

inference was then carried out by phasing our genotype data (n = 618,450) using the software SHAPEIT v2.r644 (Delaneau *et al.* 2013). Imputation of additional SNPs on chromosomes 1 to 22 throughout the whole genome was performed using the freely available program IMPUTE v2.3.0 (Howie *et al.* 2009). A total of 38,042,669 SNPs were imputed. Using a command line utility program, QCTOOL, imputed variants with low imputation quality ($r^2 < 0.8$), MAF < 1% and HWE *P*-value < 0.0001 were excluded and 7,775,788 SNPs remained for analysis. All the imputation processes were undertaken by Dr Eunice Zhang, a post-doctoral research associate in our research team.

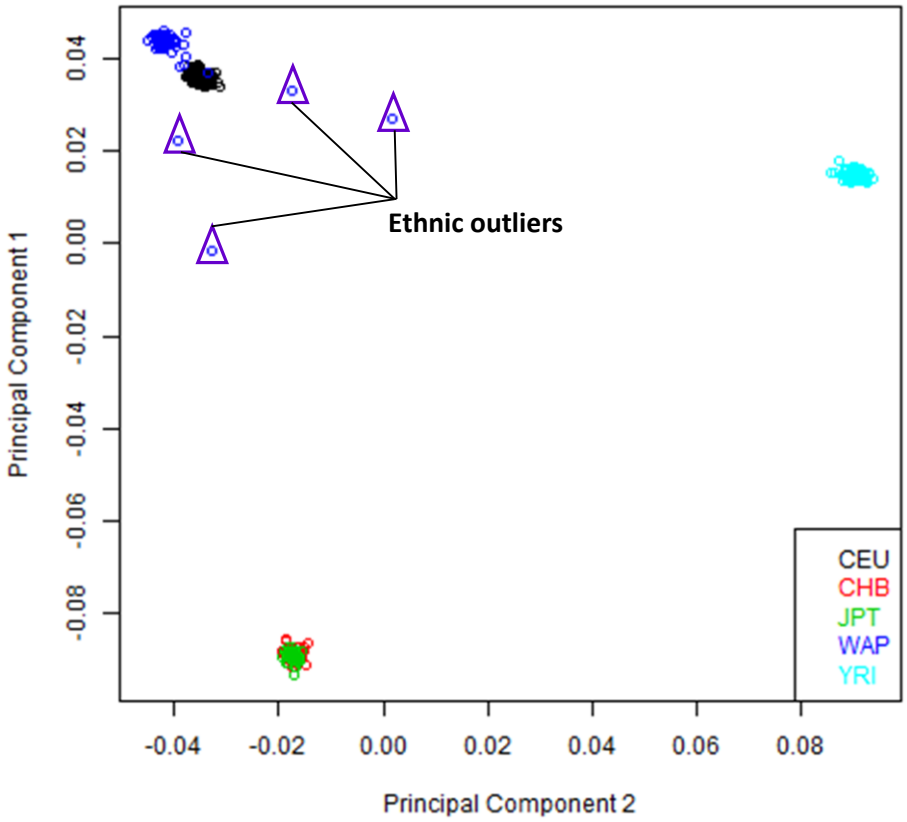


Figure 5-2. Principal-component analysis of subjects in our study. CEU = HapMap Caucasians (n=90); CHB = HapMap Han Chinese (n=45); JPT = Hapmap Japanese (n=45); YRI = HapMap Yoruban (n=90); WAP = Retrospectively recruited children on current or previous warfarin therapy (n = 87). Blue circles enclosed in purple triangles represent ethnic outliers (n = 4).

5.2.5 Statistical analysis

Statistical analyses were conducted in SPSS v21 or in PLINK, v1.07. Clinical variables with *P*-value < 0.05 were included as covariates in the regression models. Using an additive model, the linear regression was employed to test for SNP associations with continuous outcomes (proportion of time in which INR measurements fell within the target range within the first six months, and stable dose) while the logistic regression was utilised for binary outcomes (INR exceeding the target range within first week of treatment, and haemorrhagic complications). SNP showing a *P*-value < 5×10^{-8} was regarded as genome-wide significant. A threshold *P-value* < 1×10^{-5} was suggestive of genome-wide significance. Manhattan plots were generated using the freely available high-performance visualization tool Integrative Genomics Viewer (IGV) (Robinson *et al.* 2011, Thorvaldsdóttir *et al*. 2013). The open-source web-based plotting tool, LocusZoom, was used for regional visualisation of genome-wide results (Pruim *et al.* 2010). The statistical analyses (in PLINK), manhattan and regional plots were performed and generated by Dr Eunice Zhang.

5.3 Results

5.3.1 Patient characteristics

Of the 78 patients who remained after genome-wide genotyping quality control, insufficient clinical data was available for one patient and was therefore excluded from further analysis. The demographics of the remaining 77 paediatrics are summarised in Table 5-1 and Table 5-2. All patients were Caucasians with Fontan procedure being the most common indication for warfarin therapy. Out of the 77 patients included in the analysis, 66 (86%) achieved stable dose during the course of follow-up and 53 (68.8%) experienced a haemorrhagic complication. Complete data on proportion of time in which INR measurements fell within the target range (PTTR) within the first six months were available for 72 patients (93.5%). INR measurements during the first week of warfarin therapy were unavailable for 8 patients. Hence of the remaining 71 patients, 31 (40.3%) had INR values exceeding the target range within first week of treatment.

Table 5-1. Indication of warfarin treatment

Indication of warfarin treatment		**Number of patients (%)**
Surgery for congenital heart disease	Fontan procedure	51 (66.2)
	Other cardiac procedures: (e.g. Mitral valve replacement, post shunt, modified Norwood procedure)	19 (24.7)
Non-cardiac patients	Pulmonary embolism	1(1.3)
	Prophylaxis of line/catheter clotting or post-surgery	5 (6.5)
	Factor V Leiden Syndrome	1 (1.3)

Table 5-2. Patients' characteristics at the start of warfarin therapy and at the time stable

Variable	**Start of therapy (n=77)**			**Time of stable dose (n=66)***		
	Mean	**SD**	**Missing**	**Mean**	**SD**	**Missing**
Age (years)	4.16	4.21	0	7.54	4.70	0
Height (m)	0.96	0.29	20	1.09	0.29	31
Weight (kg)	16.86	16.24	10	22.43	17.05	27
Albumin ($g.L^{-1}$)	38.38	5.72	27	39.67	6.44	48

✳11 patients did not achieve stable dose during follow-up

5.3.2 Univariate analysis of association between clinical variables and outcomes

Table 5-3 summarises the tests of association results from the univariate analyses of clinical variables with each outcome. For PTTR within the first six months, INR group was the only clinical variable that gave a *p*-value < 0.05 and was included as a covariate in subsequent regression analysis. For the outcome warfarin stable dose, age at time of dose stability showed a significant association ($p = 2.7 \times 10^{-5}$) and was accounted for in the GWAS regression analysis. No clinical variables were found significant for outcome INR values exceeding the target range within first week of treatment.

Table 5-3. *p*-values from tests of association of non-genetic variables with outcome measures

Variables	*p*-value		
	Proportion of time INR within target range in 1st 6 months	**Stable dose¥ (mg/day)**	**INR exceeding target range in week 1**
Age	0.257	**2.7×10^{-5}***	0.376
Height	0.329	-	0.517
Weight	0.451	-	0.580
Albumin	0.665	-	0.919
Gender	0.120	0.547	0.476
INR group	**0.010***	0.26	0.744
Indication (2 groups)**	0.121	0.288	0.912
Indication (3 groups)***	0.235	0.571	0.493

*Included as covariate in GWAS regression analysis.
**Indication (2 groups): Patients were divided into non-Fontan and Fontan group.
***Indication (3 groups): Patients were divided into non-Fontan cardiac, Fontan cardiac and non-cardiac group.
¥ log10 stable dose transformation.

5.3.3 Genome-wide association analysis

Proportion of time in which INR measurements fell within the target range (PTTR) within the first six months

GWAS for the outcome PTTR within the first six months identified two genome-wide significant variants in chromosome 5q31.1 (Figure 5.3). One was an imputed SNP rs13167496 and the other a genotyped SNP rs6882472 (Figure 5.4A; $p = 1.12 \times 10^{-8}$). These two SNPs are 985bp away from each other and are in complete LD ($r^2=1$, D'=1). rs13167496 and rs6882472 are located in an intergenic region ~34kb upstream of the gene which encodes for TRAF-Interacting Protein With Forkhead-Associated Domain, Family Member B (TIFAB). Carriers of the mutant allele for rs13167496 or rs6882472 were significantly associated with decreased time spent within target INR range (PTTR: homozygous wild-type = 0.58 ± 0.23, heterozygous = 0.05 ± 0.16). Since rs13167496 tags for rs6882472, their association results were exactly the same. Therefore, only results for rs13167496 were reported in Figure 5.5A.

SNPs with $p < 10^{-5}$ were observed in several other regions. These included a region on chromosome 8q11.23 as well as a region on chromosome 9q33.3. The most significant SNP on chromosome 8q11.23 was *rs4873646* ($p=5.3 \times 10^{-7}$; Figure 5.4B) located ~6.7kb from the 5' end of the transcription factor Suppression of tumorigenicity 18 (*ST18*). Carriers of the minor A-allele for rs4873646 showed increased time spent within therapeutic INR (PTTR: TT = 0.32 ± 0.20, AT = 0.51 ± 0.25, AA = 0.7 ± 0.21; Figure 5.5B). On chromosome 9q33.3, *rs4838313* was the most significant genetic variant ($P=1.6 \times 10^{-6}$; Figure 5.4C) and is located 63kb from the 3' end of Pre-B-Cell Leukemia Homeobox 3 (*PBX3*). In contrast to rs4873646 on chromosome 8q11.23, carriers of the minor A-allele for rs4838313 exhibited decreased time spent within therapeutic INR (PTTR: GG = 0.66 ± 0.19, AG = 0.43 ± 0.27, AA = 0.05 ± 0.24; Figure 5.5C).

Warfarin stable dose

For warfarin stable dose, although no SNPs attained genome-wide significance, a cluster of 4 SNPs on chromosome 10p14 had $p < 10^{-5}$ (Figure 5.6). However, within this cluster, all 4 SNPs were imputed (rs71483712; $P=3.4\times10^{-7}$, rs80049351; $P=4.4\times10^{-7}$, rs116905303; $P=5.7 \times 10^{-7}$, rs78428882; $P=9.9 \times 10^{-6}$) and none of these SNPs are located in any genes (Figure 5.7). Interestingly for all these 4 SNPs, heterozygote patients carrying the minor allele required lower warfarin stable doses compared to patients homozygous for the major allele. Given that the association results for these 4 SNPs were very similar, only results for rs71483712 (the most significant SNP on chromosomal region 10p14) were reported in Figure 5.8. Subjects homozygous for the rs71483712 major T-allele required ~1.7mg more of warfarin per day than heterozygous carriers (mg/week: TT = 2.71 ± 1.55, CT = 1 ± 0.54; Figure 5.8). In addition, rs71483712 was found to account for ~21% of warfarin dose variability after adjusting for stable age (the only significant covariate observed during univariate analysis) in our multiple regression model.

INR values exceeding the target range within first week of treatment and Haemorrhagic complications

None of the SNPs achieved $p<10^{-5}$ for outcomes INR values exceeding the target range within first week of treatment and haemorrhagic complications.

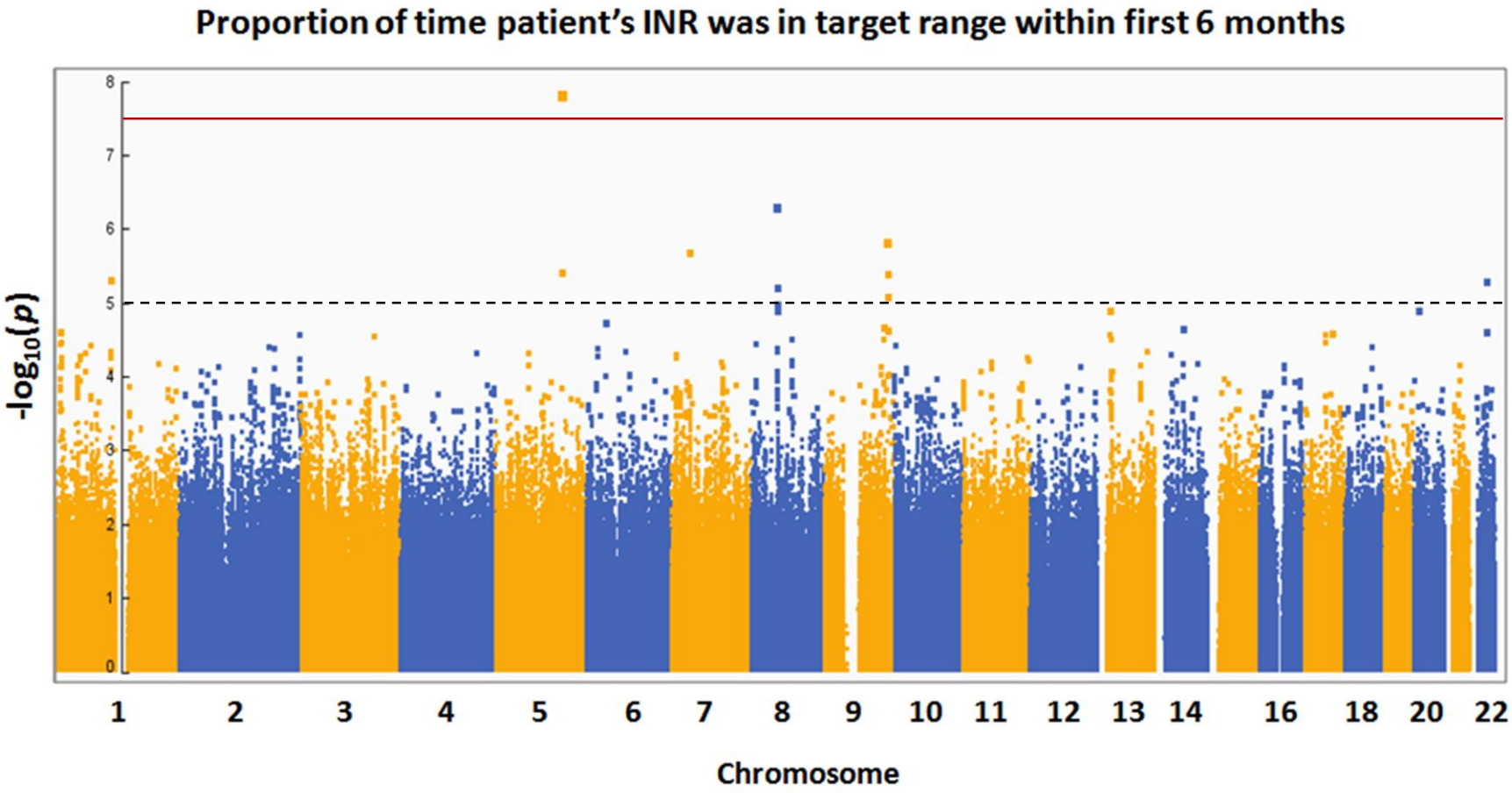
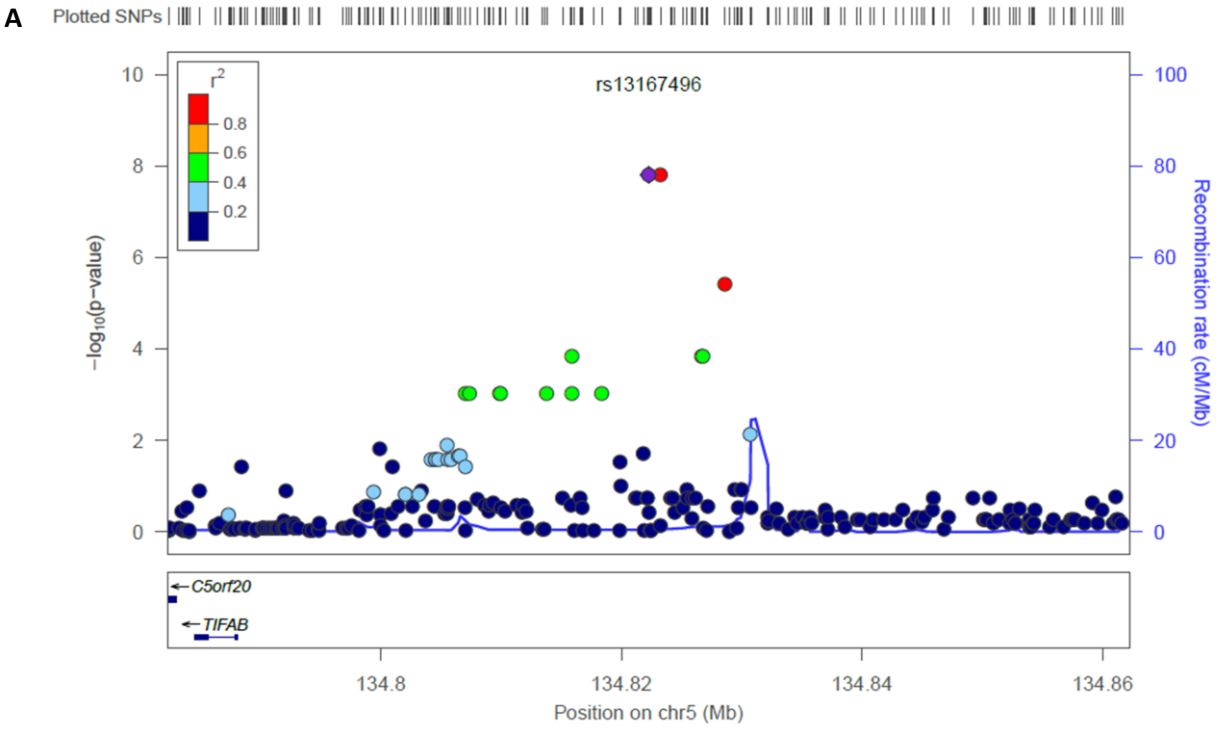
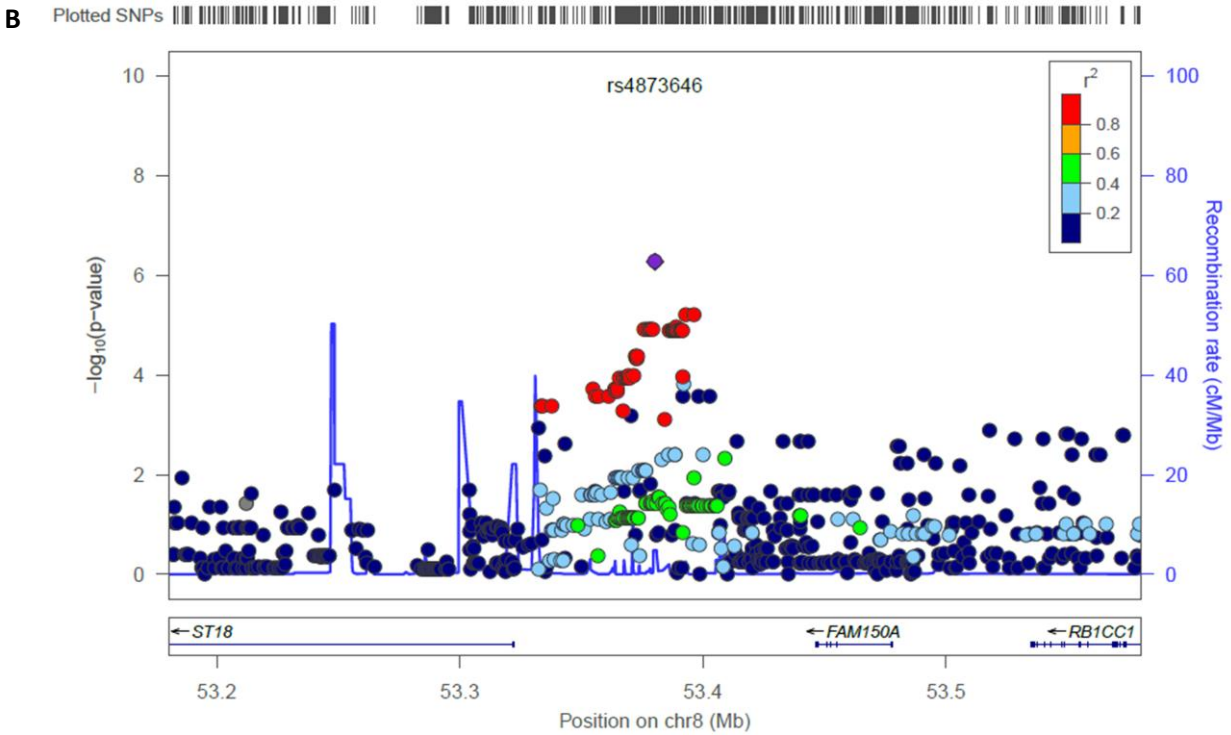


Figure 5-3. Genome-wide Manhattan plot showing the proportion of time in which INR measurements fell within the target range (PTTR) within the first six months in our cohort of warfarin-treated children. INR group was included as a covariate. Individual $-\log_{10} P$ values are plotted against their genomic position by chromosome. The red solid line at 5×10^{-8} marks the genome-wide significance threshold and the dotted line at 10^{-5} marks the threshold for promising SNPs.


A
Plotted SNPs
rs13167496
r²
0.8
0.6
0.4
0.2
−log10(p−value)
Recombination rate (cM/Mb)
C5orf20
TIFAB
134.8
134.82
134.84
134.86
Position on chr5 (Mb)


B
Plotted SNPs
rs4873646
r²
0.8
0.6
0.4
0.2
−log10(p−value)
Recombination rate (cM/Mb)
ST18
FAM150A
RB1CC1
53.2
53.3
53.4
53.5
Position on chr8 (Mb)

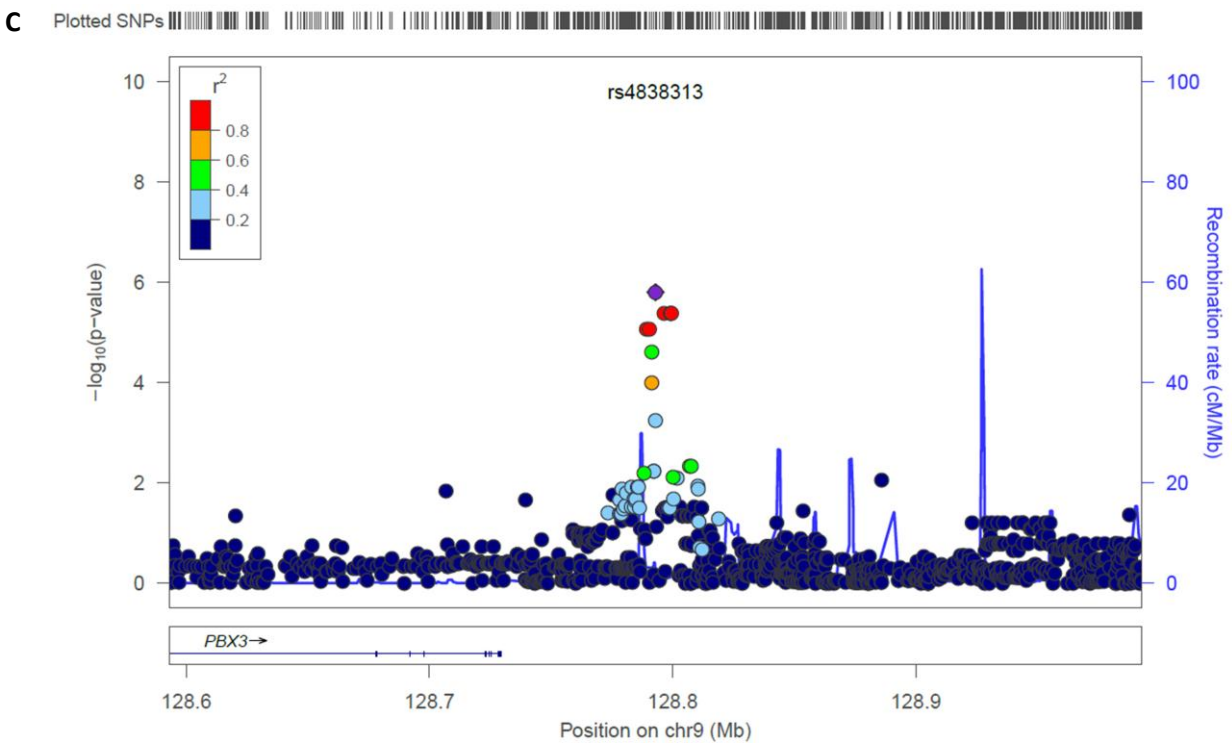


Figure 5-4. Regional association plots and recombination rates for the proportion of time in which INR measurements fell within the target range within the first six months in our cohort of warfarin-treated children. (**A**) Chromosome 5q31.1. (**B**) Chromosome 8q11.23. (**C**) Chromosome 9q33.3. Each coloured circle represents a SNP *P* value, with the colour scale reflecting the extent of LD (r^2) with the target SNP (purple diamond). Genetic recombination rates (cM/Mb), estimated using HapMap CEU samples, are shown with a light blue line. Physical positions are based on build 37 (NCBI) of the human genome. Also shown are the relative positions of genes mapping to the region of association.

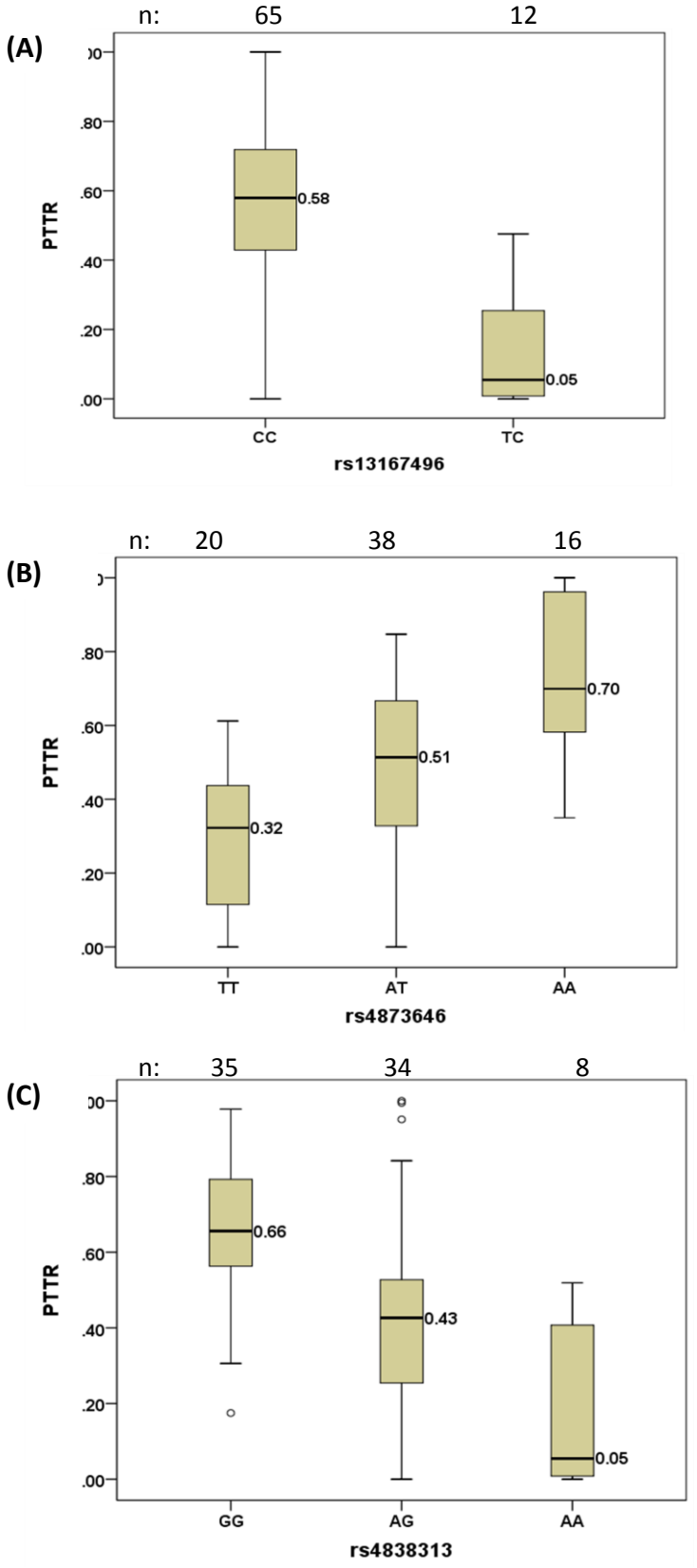


Figure 5-5. Box and whisker plots showing the proportion of time in which INR measurements fell within the target range within the first six months (PTTR) based on genotype groups in (A) rs13167496, (B) rs4873646 and (C) rs4838313. Boxes represent 25th-75th percentiles of PTTR, whiskers represent 5th-95th percentiles, solid lines represent median PTTR in each group, and open dots represent outliers.

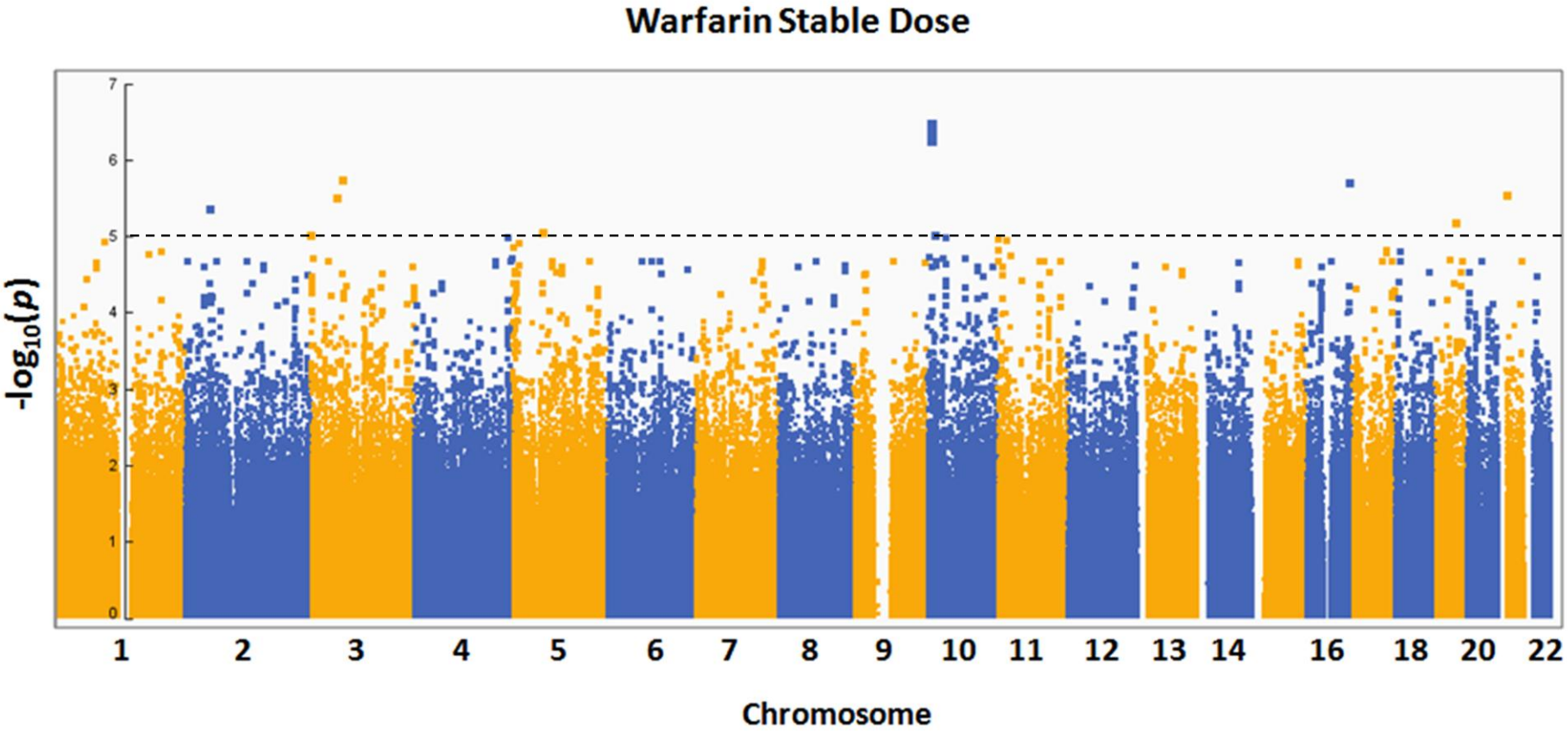


Figure 5-6. Genome-wide Manhattan plot showing warfarin stable dose in our cohort of warfarin-treated children. Age at stable dose was included as a covariate. Individual $-\log_{10} P$ values are plotted against their genomic position by chromosome. The dotted line at 10^{-5} marks the threshold for promising SNPs.

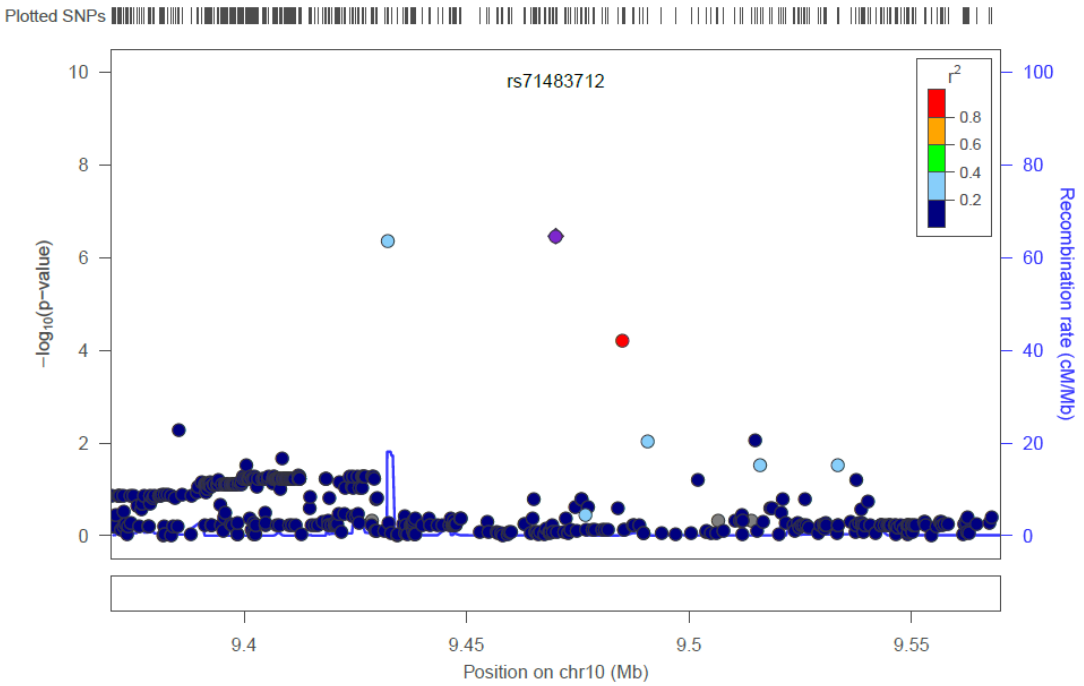


Figure 5-7. Regional association plots and recombination rates on chromosome 10p14 for warfarin stable dose in our cohort of warfarin-treated children. Each coloured circle represents a SNP *P* value, with the colour scale reflecting the extent of LD (r^2) with the target SNP (purple diamond). Genetic recombination rates (cM/Mb), estimated using HapMap CEU samples, are shown with a light blue line. Physical positions are based on build 37 (NCBI) of the human genome. Also shown are the relative positions of genes mapping to the region of association.

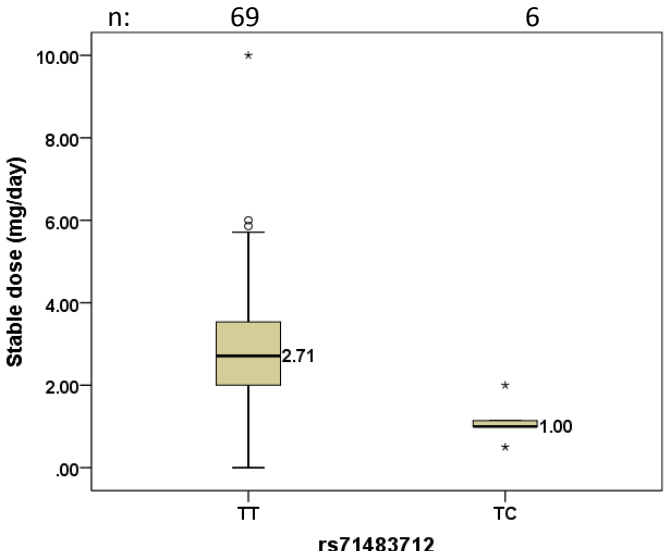


Figure 5-8. Box and whisker plots showing the distribution of stable warfarin daily doses based on genotype groups in rs71483712. Boxes represent 25th-75th percentiles of PTTR, whiskers represent 5th-95th percentiles, solid lines represent median stable warfarin dose in each group, and open dots represent outliers.

5.4 Discussion

To date, we are the first to report a GWAS in a paediatric population on warfarin treatment. Two genetic variants on chromosomal region 5q31.1, rs13167496 and rs6882472, reached genome-wide significance and were associated with ~50% reduction in PTTR during the first six months of warfarin therapy. These two SNPs are in complete LD with each other and are located ~34kb 5' of *TIFAB*. *TIFAB* is a protein-coding gene which associates with TRAF-Interacting protein with Forkhead-Associated domain (TIFA) and inhibits TIFA-mediated activation of the transcription factor, NF-κB (Matsumura *et al.* 2004). Studies have shown that NF-κB mediates the expression of tissue factor (TF) and factor VIII (Mackman *et al.* 1991, Figueiredo and Brownlee 1995), which activate the extrinsic and intrinsic coagulation pathways, leading to thrombin generation and coagulation (Song *et al.* 2009). Thrombin produced during coagulation activates NF-κB–dependent genes, forming a positive feedback loop that further amplifies coagulation (Anrather *et al.* 1997). NF-κB has also been reported to mediate type 1 plasminogen-activator inhibitor (PAI-1) expression, where an increased level of PAI-1 impairs fibrinolysis, promoting coagulation (Hou *et al.* 2004). Therefore, the suppression of NF-κB by TIFAB may lead to reduced coagulation, resulting in decreased proportion of time where patients were adequately protected against thromboembolism or bleeding complications.

We have also identified two other SNPs, rs4873646 and rs4838313**,** which showed association with PTTR during the first six months of warfarin therapy at significance levels of $P < 10^{-5}$. rs4873646 was associated with increased PTTR and is located ~6.7kb upstream of *ST18* on chromosomal region 8q11.23. *ST18* has been reported to play an important role in regulating the mRNA levels of proapoptotic and proinflammatory genes (e.g. TNF-α, IL-1α, IL-6) and when overexpressed, *ST18* significantly enhances apoptosis (Yang *et al.* 2008) but its relation to the coagulation

pathway is unknown. Conversely, rs4838313 on chromosome 9q33.3 (~63kb downstream of *PBX3*) was associated with decreased PTTR. *PBX3* is one member of a group of PBX transcription factors belonging to the TALE (3 amino acid loop extension) homeobox gene family. They seem to play important developmental roles in cancers, particularly of hematolymphoid origin (Ho *et al.* 2013, Li *et al.* 2013), but its role in coagulation is not known.

Although no SNPs attained genome-wide significance with warfarin stable dose, results from our preliminary multiple regression model showed that the most significant SNP located on chromosome 10p14 (rs71483712) explained ~21% of warfarin dose variability. Given that rs71483712 is an imputed SNP, genotyping will need to be performed to ensure that this association result was not an imputation artefact.

Albeit none of the usual suspects *CYP2C9*2*, *CYP2C9*3* and *VKORC1*-1639 showed genome-wide significance for any of the clinical outcomes tested, but statistically significant associations between warfarin stable dose and *CYP2C9*2* (P = 0.0026) and *VKORC1*-1639 ($P = 7.9 \times 10^{-5}$) were observed (confirms results reported in Chapter 4). Our small sample size may be underpowered to detect genome-wide significant differences for the numerous polymorphisms tested.

Despite our small sample size, our findings implicate that other genetic factors may possibly be involved in warfarin clinical outcomes. However, this must be interpreted with caution, and these results need to be validated in a replication cohort.

In addition, to gain further insight into the biologic networks underlying the different warfarin outcomes in children, genes and pathway analyses will need to be performed as potential combined effects of SNPs contributing to warfarin clinical outcomes may be missed through conventional single marker association.

Chapter 6

Validation of a novel point of care HyBeacon® genotyping method on a prototype PCR instrument Genie I for CYP2C9 and VKORC1 SNPs

CONTENTS

6.1 Introduction

In response to the accumulating evidence of the importance of genotyping in guiding warfarin dosing, the FDA has updated the warfarin label stating that genetic information may improve estimation of warfarin dosing (Thompson 2007). Recently a survey conducted by the Clinical Pharmacogenetics Implementation Consortium (CPIC) among members of the American Society for Clinical Pharmacology and Therapeutics has shown that two of the highest ranked gene drug/pairs, based on the perceived importance of the data linking the drug to the gene variation, are *CYP2C9*/warfarin and *VKORC1*/warfarin (Relling and Klein 2011). Despite a CPIC rating of level A (strong evidence) for a recommendation of warfarin dosing based on patients' genotypes (Johnson *et al.* 2011), the genetic dosing of warfarin has not yet been implemented. In contrast, the American College of Chest Physicians recommends against routine pharmacogenetic testing (grade 1B) because of the lack of randomized evidence that it improves clinical end points or that it is cost effective (Holbrook *et al.* 2012).

The translation of warfarin pharmacogenetics into clinical practice has been slow because no large randomized controlled trial has been published that has evaluated clinical outcomes of warfarin dosing based on pharmacogenetic testing. As a result, several clinical trials are currently in progress, including the European Pharmacogenetic of Anticoagulant Therapy (EU-PACT) trial. The EU-PACT trial is a prospective randomised controlled trial to assess the safety and clinical utility of genotyped-guided dosing of the three main coumarins used in Europe: acenocoumarol, phenprocoumon and warfarin (Van Schie *et al.* 2009). This trial aimed to include 3000 patients with atrial fibrillation or venous thromboembolism recruited across 7 European countries: UK, Sweden, the Netherlands, Spain, Greece, Germany and

Australia. Patients were randomised to either genotyping-guided dosing (intervention arm) or to standard dosing (control arm). Patients in the intervention arm were genotyped for *CYP2C9*2* (rs1799853), *CYP2C9*3* (rs1057919) and *VKORC1-1693* (rs9923231) prior to initiation of treatment. They were given a three day loading dose based on the IWPC pharmacogenetic maintenance dose algorithm (Klein *et al.* 2009) and the predicted elimination half-life of warfarin for each *CYP2C9* genotype (Hamberg *et al.* 2010). After the first three days of therapy, patients were dosed according to a modified version of a pharmacogenetic warfarin dose algorithm (Lenzini *et al.* 2010).

The other important barrier to implementing testing is the non-availability of a rapid genotyping platform with properties such as accuracy, testing at point of care and a good turn-around time so that patients' dosing can be based on the genotyping results. A range of genotyping assays for detecting *CYP2C9* and *VKORC1* polymorphisms related to warfarin response have been published previously, including PCR-restriction fragment length polymorphism (RFLP) analysis (Sconce *et al.* 2005), pyrosequencing (King *et al.* 2008), Invader assays (Burmester *et al.* 2011) and several fluorescence based real time PCR methods (Verstuyft *et al.* 2003, Hatch *et al.* 2006, Lefferts *et al.* 2010). At the time of writing of this thesis, seven platforms for *CYP2C9/VKORC1* genotyping have been approved by the FDA as described in Table 6.1. In general, studies comparing the validity of the various methods have shown very high levels of accuracy and good agreement across platforms (King *et al.* 2008, Joshi *et al.* 2009, Lefferts *et al.* 2010). However, all the available technologies require initial DNA extraction from blood and the process takes more than two hours to obtain genotype results.

Table 6.1. Platform for *CYP2C9*/*VKORC1* genotyping approved by FDA.

Test Name/ Manufacturer	Properties
Test name: eSensor Warfarin Sensitivity Saliva Test **Manufacturer:** GenMark Diagnostics	• Requires DNA extraction using ethanol precipitation • HOT approximately 40 minutes (product flyers) • TAT approximately 3.5 hours (product flyers)
Test name: eQ-PCR LC Warfarin Genotyping kit (requires Roche LightCycler) **Manufacturer:** TrimGen Corporation	• Requires DNA extraction • Test time approximately 2 hours including DNA extraction (product flyer)
Test name: eSensor Warfarin Sensitivity Test and XT-8 Instrument **Manufacturer:** Osmetech Molecular Diagnostics	• Requires DNA extraction • HOT approximately 14 minutes per sample (Maurice *et al.* 2010) • TAT approximately 3-4 hours per sample (Joshi *et al.* 2009, Maurice *et al.* 2010) • A simple, touch-screen user interface, easy-to-interpret, customizable reports, and no routine maintenance or instrument calibration required
Test name: INFINITI 2C9 & VKORC1 Multiplex Assay for Warfarin (requires Autogenomic INFINITY Analyzer) **Manufacturer:** AutoGenomics, Inc.	• Requires DNA extraction • TAT approximately 6-8 hours (King *et al.* 2008, Joshi *et al.* 2009)
Test name: Gentris Rapid Genotyping Assay - CYP2C9 & VKORCI (requires Cepheid Smart Cycler Dx) **Manufacturer:** ParagonDx, LLC	• Requires DNA extraction • TAT approximately 4 hours (Babic *et al.* 2009) • Technically complex method compared to eSensor Warfarin Sensitivity Test and XT-8 Instrument or INFINITI 2C9 & VKORC1 Multiplex Assay for Warfarin (Babic *et al.* 2009)
Test name: Verigene Warfarin Metabolism Nucleic Acid Test and Verigene System **Manufacturer:** Nanosphere, Inc.	• Requires DNA extraction and high DNA concentration • HOT approximately 2 minutes per sample (Maurice *et al.* 2010) • TAT approximately 1.7 hours per sample (Maurice *et al.* 2010)

Turn-around time (TAT) was calculated as the time from computer data entry to final results.

Hands on time (HOT) = manual steps involved, i.e. computer data entry, reagent and sample preparation, pipetting and plate handling.

Genotyping using HyBeacon® probes on the prototype Genie I PCR instrument is a new rapid point of care test (POCT) for determining *CYP2C9*2* (rs1799853), *CYP2C9*3* (rs1057910) and *VKORC1 -1639*G>A (rs9923231) genotypes in the EU-PACT trial. The Genie 1 PCR instrument and the HyBeacon® assays were developed by the British company LGC (Teddington, Middlesex). It provides genotype results for the three SNPs within 1.5 hours to enable initial warfarin dosing based on individual genetic make-up. In addition, the procedure is simple because genotyping is performed directly from whole blood.

Therefore, the aim of this study was to validate the performance of this novel HyBeacon® POCT on the Genie I PCR instrument and to compare the accuracy of *CYP2C9*2* (rs1799853), *CYP2C9*3* (rs1057919) and *VKORC1-1693* (rs9923231) genotypes to data obtained using TaqMan® allelic discrimination assays on an ABI 7900HT Real-Time PCR system (Applied Biosystems).

6.2 Method

6.2.1 Sample recruitment

A total of 278 patients were recruited from the Royal Liverpool University Hospital NHS Trust (RLUHT) and the St Helens and Knowsley Teaching Hospital NHS Trust (SKTHT) for the EU-PACT study. The latter NHS Trust covers two sites, which were both used for the study: Whiston Hospital and St Helens Hospital. A minimum of 9mL EDTA-blood was collected from all patients. Patient recruitment started in 2010 and ended in February 2013. Out of the total recruitment, 135 patients (95 patients from the RLUHT and 40 patients from SKTHT) were randomized into the genotype-guided dosing arm. Genotyping for *CYP2C9*2*, *CYP2C9*3* and *VKORC1-1693* was performed using the Genie 1 PCR instrument prior to initiating warfarin dosing. Genie genotyping was carried out by a laboratory research staffs at the University of Liverpool or by nurses at the St Helens & Knowsley Teaching Hospital NHS Trust.

6.2.2 Genotyping *CYP2C9**2, *CYP2C9**3 and *VKORC1* SNPs by HyBeacon® POCT on the Genie 1 instrument.

For patients who were randomized to the genotype-guided arm, genotyping was performed on the Genie 1 PCR platform immediately after whole blood samples were received. Details of primers used are listed in Table 6.2.

Table 6.2. Primers for point of care genotyping.

Assay	Primer Name	Primer sequence 5' → 3'
CYP2C9*2	C9*2CF3	CCTCCTAGTTTCGTTTCTCTTCCTGTTAGGAATT
	C9*2CR4	GTAGAGAAGATAGTAGTCCAGTAAGGTCAGTGATATG
CYP2C9*3	C9*3F2	TGCATGCAAGACAGGAGCCACAT
	C9*3R2	GGAGAAACAAACTTACCTTGGXAATGAGA X = Inosine
VKORC1 **For blood only**	VKOF2	GGAGCCAGCAGGAGAGGGAAATA
	VKOR3	CGGCCTCCCAAAATGCTAGGATT
VKORC1 **For DNA only**	VKOF3	CAG AAG GGT AGG TGC AAC AGT AAG GGA TCC
	VKOR4	CTG ACC TCA AGT GAT CCA CCC ACC TCG

(Adapted from Howard et al., 2011)

A novel HyBeacon® probe technology was used to detect and identify *CYP2C9*2*, *CYP2C9*3* and *VKORC1-1639* (Howard *et al.* 2011). HyBeacon® probes comprise of fluorescent dye labels (fluorophore) attached to the internal position of the oligonucleotide and a 3'phosphate (PCR blocker) to prevent PCR extension from probes (French *et al.* 2001). The probes emit greater amounts of fluorescence when bound to complementary target DNA sequences than when the probes are single stranded (Figure 6-1). The quantity change in fluorescence emission occurs as a direct result of target hybridization and therefore permits the detection and discrimination of sequences by melting curve analysis on the Genie 1 PCR instrument. Details of probes used are provided in Table 6.3.

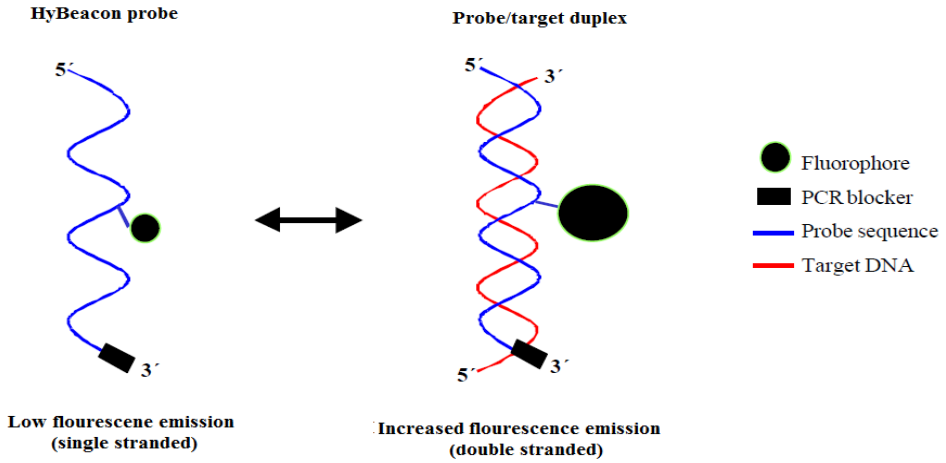


Figure 6-1. Structure of HyBeacon® probe and the method of target detection. HyBeacon probes are single-stranded fluorescence labeled probes complementary to the target DNA sequences. In unhybridised condition, the fluorophore on the HyBeacon® probe cannot emit fluorescence. When bound to complementary target DNA sequences, excitation and fluouresence emission occurs, allowing the detection of target sequences.

Table 6.3. Probes for point of care genotyping.

Assay	Probe Name	Probe sequence 5'→ 3'
*CYP2C9*2*	C9*2C2	2GCAT**F**GAGGACCG**F**GTTCAAG3
*CYP2C9*3*	2C9*3C5	2TCCAGAGATACCT**F**GACCT**F**CTCCC3
VKORC1	VKORC1	2CAT**F**GGCCAGG**F**GCGGT**3**

F = FLUOROSCEIN dT 2= Trimethoxystilbene 3 = PHOSPHATE

All genotyping assays, PCR reagents and lysis buffer were supplied by LGC (Teddington, UK). The PCR mastermix was pre-prepared in strips of 8 low profile 0.2 ml tubes, where each assays was prepared in 17 µl reaction containing 0.7 µl HemoKlen Taq in 1 X HemoKlen buffer (New England Biolabs), 0.2 mM dNTP (New England Biolabs), 0.1 µM forward primer, 10 µM reverse primer and 0.2 µM HyBeacon® probe. The first three tubes (tubes 1 to 3) were the diagnostic PCR master mixes for *CYP2C9*2*, *CYP2C9*3* and *VKORC1-1693*. The next three tubes were control assays (tubes 4 to 6), which included 1.5 ng DNA from known heterozygote for each SNP, and the last two tubes (tubes 7 and 8) were template controls that did not contain any DNA. Prior to use, the strips were stored at -20°C in the dark.

The genotyping process is illustrated in Figure 6-2. To genotype an individual for *CYP2C9*2*, *CYP2C9*3* and *VKORC1 -1693*, a mastermix strip was thawed. 5 µl of blood was mixed with 100 µl of lysis buffer A containing 0.32 M sucrose in 10 mM Tris-HCL pH 7.4 and 1% (v/v) Triton X-100 in a sterile microfuge tube. The mixture of blood and Buffer A was pipetted up and down to ensure a good mix and then left for 5 minutes at room temperature before use. 1 µl of the diluted blood was then transferred into each of the first three tubes (tubes 1 to 3) containing diagnostic PCR master mixes for *CYP2C9*2*, *CYP2C9*3* and *VKORC1-1693*. PCR amplification was performed in the Genie 1 PCR instrument (Optigene Ltd). Thermocyling was carried out with an initial step of 95°C for 4 min followed by 50 cycles of 95°C for 3 s and 64°C for 1 min, using a 5°C/s ramp rate. Following amplification, results were obtained by performing melting curve analysis from 40°C to 75°C at a ramp rate of 0.1°C/s and fluorescence changes were measured as the HyBeacon® probe dissociates from the amplified target DNA were measured. Analysis of the fluorescence derivative profile was performed on a PC connected to the Genie 1 instrument using software developed for this purpose by Optigene Ltd.

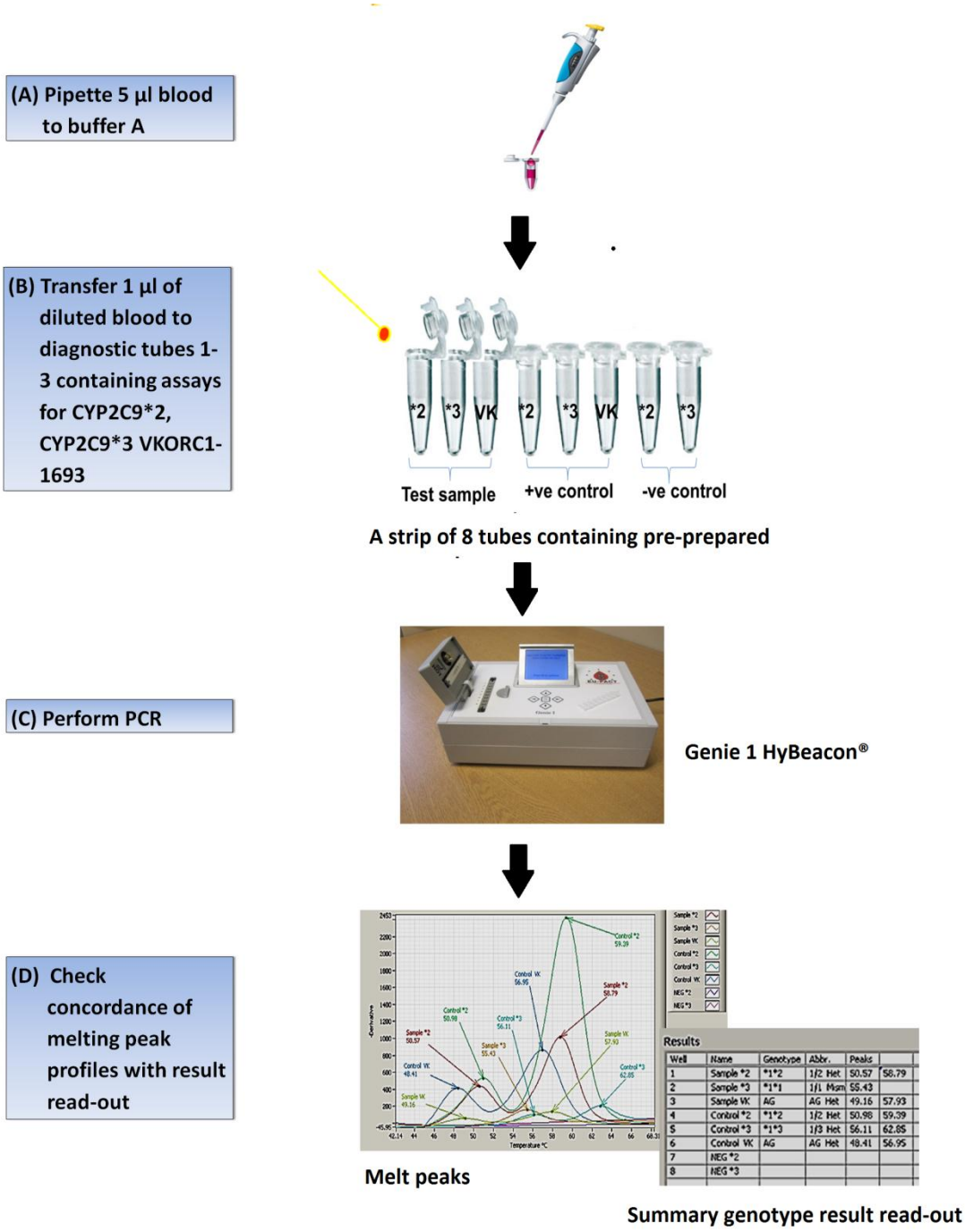


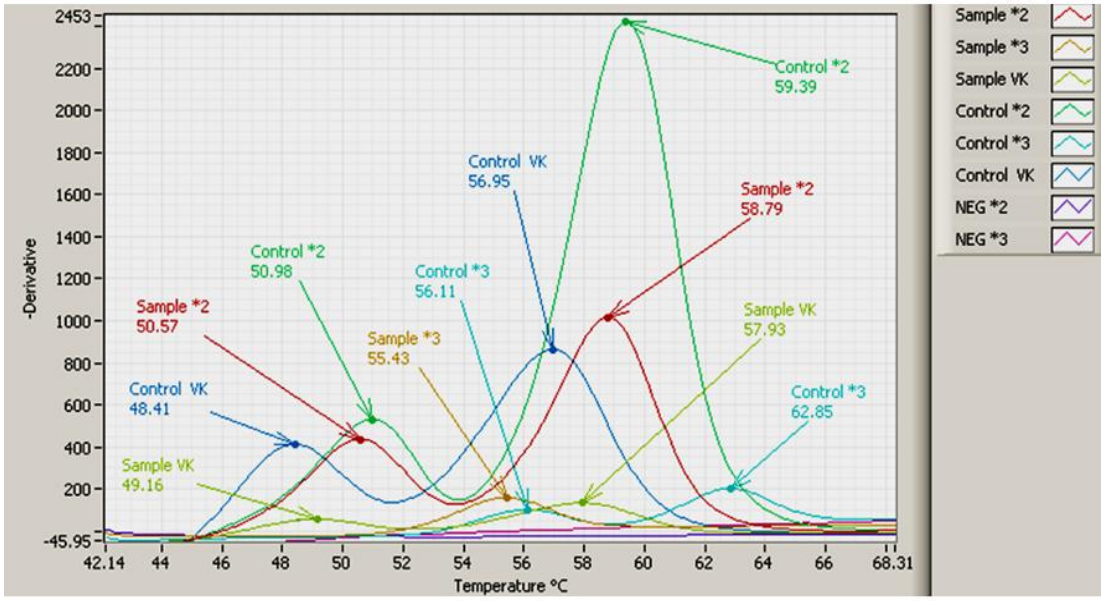
Figure 6-2. Genotyping procedure on the Genie I HyBeacon®. (A) First, 5 µl of blood was mixed with 100 µl of buffer A. Five minutes were allowed for the mixture to complete the lysis process. (B) Then 1 µl of diluted blood was added to the 3 diagnostic PCR tubes (1 to 3). Before placing the tubes into the machine, the tubes were gently flicked to ensure that all of the liquid fell to the bottom of the tubes. (C) PCR was performed on Genie 1 PCR instrument. (D)The analysis of the fluorescence derivative profile was performed on a PC connected to the Genie 1 instrument using software developed for this purpose by Optigene Ltd. Concordances of melting peak profiles with result read out were checked. A clear picture of melting peak profile was shown in figure 6.3.

The typical melting temperature ranges for each assay are shown in Table 6.4. Homozygous samples generate single melt peaks, which are matched or mismatched depending on the identity of target sequences. On the other hand, heterozygous samples produce melting traces possessing both matched and mismatched peaks. The genotype results were considered valid if (i) the melting temperature peak fell within the specified range for each assay, (ii) the melting peak profiles were concordant with that in the result read-out, (iii) positive controls showed heterozygous genotypes and (iv) no peaks in the negative controls were detected. An example of a valid result is shown in Figure 6-3.

Table 6.4. The temperature ranges for each assay peak to determine genotype call.

Assay	Match (wild-type) Tm (°C)	MisMatch (variant) Tm (°C)	Heterozygous Tm (°C)
*CYP2C9*2*	58.9 - 60.4	50.9 - 52.2	50.9 - 52.2; 58.9 - 60.4
*CYP2C9*3*	61.4 - 65.1	54.9 - 58.1	54.9 - 58.1 ;61.4 - 65.1
VKORC1	48.5 - 50.2	57.0 - 59.7	48.5 - 50.2 ;57.0 - 59.7

(A) Melt peaks



(B) Summary genotype result

Results

Well	Name	Genotype	Abbr.	Peaks	
1	Sample *2	*1*2	1/2 Het	50.57	58.79
2	Sample *3	*1*1	1/1 Mism	55.43	
3	Sample VK	AG	AG Het	49.16	57.93
4	Control *2	*1*2	1/2 Het	50.98	59.39
5	Control *3	*1*3	1/3 Het	56.11	62.85
6	Control VK	AG	AG Het	48.41	56.95
7	NEG *2				
8	NEG *3				

Figure 6-3. Example of a valid melting peak profile. Based on the melting peak profiles (A), the *CYP2C9*2, CYP2C9*3* and *VKORC1-1693* genotypes for the sample were *1*2 (heterozygous); **1*1*(homozygous wild-type) *and AG* (heterozygous), respectively. All melt peaks were in the relevant range, the positive controls showed heterozygous genotypes and no peaks were detected in the negative controls. The summary genotype result read-out (B) confirms the detection of the peaks in all 8 tubes.

6.2.3 Genotype validation of *CYP2C9*2*, *CYP2C9*3* and *VKORC1-1693* by Taqman® Allelic Discrimination

To validate the performance reliability of HyBeacon® genotyping on the Genie I instrument, DNA from all 135 patients were extracted using the chemagic module (as described in section 2.2.3). The DNA were genotyped via real-time Taqman® Allelic Discrimination (as described in section 2.2.4). The genotypes results from the two different platforms were compared. The Taqman® genotyping method was chosen because it has previously been validated with results from DNA sequencing (Carlquist *et al.* 2008, Joshi *et al.* 2009, Lefferts *et al.* 2010).

6.2.4 Statistical analysis

To test the reliability of the genotype results from the Genie I HyBeacon® and ABI 7900HT Real-Time Taqman®, the exact agreement and kappa (κ) statistics were calculated. Kappa statistic is thought to be a more robust measure than simple percent agreement calculation since κ takes into account the agreement occurring by chance. Guidelines by Landis and Koch (1977) were used to evaluate simple and weighted kappa statistics: 0.00 to 0.20, poor agreement; 0.21 to 0.40, fair; 0.41 to 0.60, moderate; 0.61 to 0.80, good; and 0.81 to 1.00, almost perfect agreement. An example of the calculations is shown below:

Example of result table:

Genie	*ABI Taqman®*			*Total*
	Wild-Type	*Heterozygous*	*Homozygous*	
Wild-Type	***65***	*3*	*2*	*70*
Heterozygous	*4*	***10***	*6*	*20*
Homozygous	*1*	*2*	***7***	*10*
Total	*70*	*15*	*15*	*100*

Example of calculation:

1) Exact agreement (%)

=65 + 10 +7 =82 out of 100 rating (82%)

2) Kappa (κ)

$$= \frac{\text{proportion in agreement - proportion expected to agree by chance}}{\text{1- proportion expected to agree just by chance}}$$

where:

(a)Proportion in agreement= proportion of exact agreement= 82/100=0.82

(b)Proportion expected to agree just by chance =

= Probabilities (genotype wild-type) + Probabilities* (genotype heterozygous) + Probabilities* (genotype homozygous variant)*

= (70/100x70/100) + (20/100x15/100)+ (10/100x15/100) = 0.535

**probabilities of both platform*

Therefore,

kappa (κ) = (0.82-0.535)/(1-0.535) = 0.61

In addition, we evaluated the speed performance of the Genie I HyBeacon ® and ABI 7900HT Real-Time Taqman® platforms by evaluating hands-on and turn-around times. The percentage of results which gave successful genotypes the first-time were calculated because failure to produce perfect results in the first-time test will contribute to longer turn-around time. Of the samples which gave successful genotypes during the first test, 50% (n=55) of samples were selected randomly and retested to investigate the reproducibility of the Genie I HyBeacon ® platform.

6.3 Results

A total of 135 samples were genotyped for *CYP2C9*2* (Table 6.5), *CYP2C9*3* (Table 6.6) and *VKORC1-1693* (Table 6.7) by each method. Kappa statistic showed strong agreement between Genie I HyBeacon® and ABI Taqman® platforms. The lowest kappa value was obtained when testing the *VKORC1-1693* genotype (κ=0.98), where two samples were discordantly genotyped by these platforms: "AG" was the genotype by ABI Taqman® and "AA" by Genie I HyBeacon®. This is due to the low sensitivity of the *VKORC1* HyBeacon® assay to detect the two heterozygous alleles in *VKORC1-1693*. The peak for G allele (melting peak at~40-50 °C) is very small (Figure 6-4), therefore the heterozygosity of the *VKORC1-1693* genotype was not detected by the software. Furthermore, this small peak could not be spotted by eye due to the overlapping peaks from other assays (*CYP2C9*2* and *CYP2C9*3* assays).

Table 6.5. *CYP2C9*2* genotype comparison between the Genie I HyBeacon® and ABI Taqman® genotyping methods.

Genie	ABI Taqman®			Total
	Wild-Type	Heterozygous	Homozygous	
Wild-Type	**98**	0	0	98
Heterozygous	0	**35**	0	35
Homozygous	0	0	**2**	3
Total	98	35	2	135

Exact agreement: 100% Kappa: 1 (95% CI 1-1)

Table 6.6. *CYP2C9*3* genotype comparison between the Genie I HyBeacon® and ABI Taqman® genotyping methods.

Genie	ABI Taqman®			Total
	Wild-Type	Heterozygous	Homozygous	
Wild-Type	**124**	0	0	124
Heterozygous	0	**11**	0	11
Homozygous	0	0	**0**	0
Total	124	11	0	135

Exact agreement: 100% Kappa: 1 (95% CI 1-1)

Table 6.7. ***VKORC1*** **genotype comparison between the Genie I HyBeacon® and ABI Taqman® genotyping methods.**

Genie	ABI Taqman®			Total
	Wild-Type	Heterozygous	Homozygous	
Wild-Type	**47**	0	0	47
Heterozygous	0	**62**	0	62
Homozygous	0	2	**24**	26
Total	47	64	24	135

Exact agreement: 98.5% Kappa: 0.98 (95% CI 0.94-1.00)

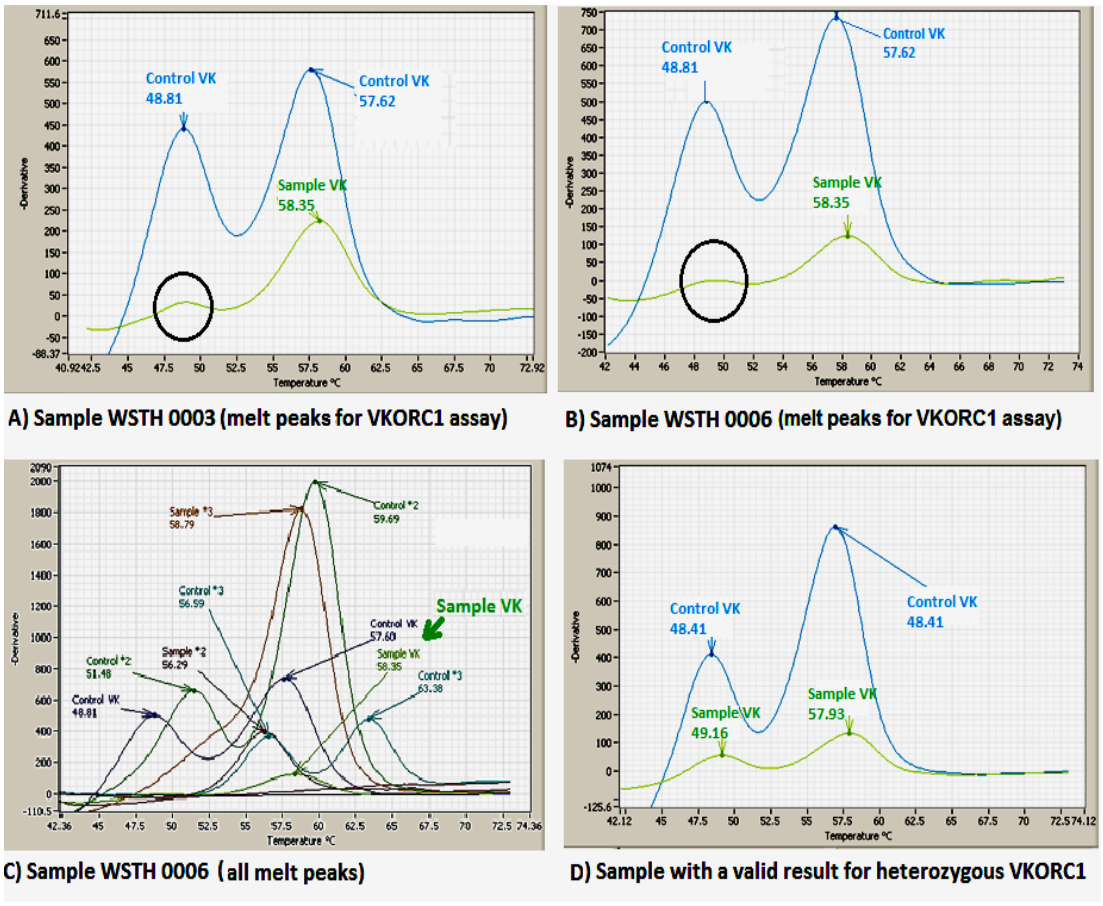


Figure 6-4. ***VKORC1*** **genotyping discordance-results.** Figures (A) and (B) show the melting peaks of the two patients, where Genie I Hybeacon® gave VKORC1 homozygous variant genotypes while ABI Taqman® gave heterozygous genotypes. The black circle shows a very small peak but below the intensity threshold limit. The Genie I Hybeacon® software could not detect this little peak, explaining why the result red outs were *VKORC1* homozygous variant. In figure (C), the peak for allele G could not be spotted by eye due to the overlapping peaks from other assays. Figure (D) shows the melting peak of a valid result where a patient was successfully genotyped as VKORC1 heterozygous by Genie I Hybeacon®.

Out of the 135 samples randomised to genotype-guided arm, 112 (83%) samples were succesfully genotyped on the Genie I HyBeacon®. Of these, 77 were from RLUHT and 35 from the SKTHT. The 55 which were successfully genotyped were randomly selected and tested for a second time on the Genie I HyBeacon® to test for reproducibility and 100% of these were concordant.

The technical problems on the Genie I Hybeacon® are presented in Table 6.8. The total percentage failure for *CYP2C9*2* and *CYP2C9*3* and *VKORC1-1693* were 8.95%, 6.37% and 7.7% respectively. Of which, 4.7%, 4.25% and 5.96% were not successfully genotyped for *CYP2C9*2, CYP2C9*3* and *VKORC1-1693* respectively. The failure rate for positive controls were 4.25%, 2.12%, 1.7% for *CYP2C9*2, CYP2C9*3* and *VKORC1-1693*, respectively.

It is surprising that peaks were observed in negative controls (4.2%) because the tubes were never been opened during genotyping. Possibly the contamination source was from the supplier or during transportation (as the lids of the tubes were not really tight). 4.7% of summary genotype result read-out did not tally with the melting peaks profile.

For the ABI Taqman® platform, 20 samples failed to genotype the first time (6 for *CYP2C9*2*, 9 for *CYP2C9*3*, 5 for *VKORC1-1*693) but were successfully genotyped on the second time. The failure of genotyping was due to pipetting error (human error) during DNA transferred into a 384-well PCR plate.

Table 6.8. Technical problems with Genie

Description of technical problem	RLUHT *(n)*	SKTHT *(n)*	Total failure rate (%)
CYP2C9*2 **Test**: Total number of tests performed	176	59	
Test sample not genotyped	8	3	4.7%
Positive controls not genotyped	10	0	4.25%
Total percentage failure			8.95%
CYP2C9*3 **Test:** Total number of tests performed	176	59	
Test sample not genotyped	6	4	4.25%
Positive controls not genotype	5	0	2.12%
Total percentage failure			6.37%
VKORC1 **Test:** Total number of tests performed	176	59	
Test sample not genotyped/ wrong genotype given	10	4	5.96%
Positive controls not genotype	4	0	1.7%
Total percentage failure			7.7%
Peak detected in negative control			4.2%
CYP2C9*2 negative control	3	0	
CYP2C9*3 negative control	7	0	
Result read-out discordant with peaks observed	11	0	4.7%

RULHT: Royal Liverpool University Hospital NHS Trust.

SKTHT: St Helens & Knowsley Teaching Hospital NHS Trust.

A comparison of hands-on and total turn-around time for both platforms per experiment is shown in Table 6.9. The total turn-around time per run on the Genie I HyBeacon® is 97.5 minutes while that on the ABI Taqman® is 247 minutes. Only one sample can be genotyped for three SNPs by Genie I HyBeacon® and a maximum of 80 samples by ABI Taqman® in each run. Therefore, with regards to hands-on and total turn-around time, our results suggest that the Genie I HyBeacon® is more practical to be use in a clinical setting because it can produce genotype results less in than 2 hours reducing patient waiting time.

Table 6.9. Comparison of hands-on and total turn-around time between Genie 1 HyBeacon® and ABI Taqman®.

Description	Genie 1 HyBeacon®	ABI Taqman®
DNA extraction	0	55 min
Sample preparation	6.5 min	90 min
PCR/Run	90 min	100 min
Result read	1 min	2 min
Turn-around time per run	97.5 min (~1.6 hours)	247 min (~4 hours)

6.4 Discussion

In this chapter we have genotyped 135 samples using the Genie 1 HyBeacon® platform and validated the results by comparing genotypes using the ABI 7900HT Taqman® platform. Genotyping by Genie HyBeacon® agreed strongly with the genotype results using ABI Taqman®. With a turn-around time of 97.5 min per run, it was possible to obtain the genotypes for *CYP2C9*2*, *CYP2C9*3* and *VKORC1-1693* before dosing with warfarin. However, caution must be taken during genotype determination as 4.7% of the summary genotype result read-outs did not tally with observed melt peaks. Checking the concordance of melting peak profiles with summary genotype read-outs is mandatory before the genotype results are used to dose the patients with warfarin.

Despite there being a strong concordance in our study, two patients were discordantly genotyped for *VKORC1-1693* by the platforms and we believe this is due to the low sensitivity of the HyBeacon® assay to detect the polymorphism. For example, two patients who were carriers of the heterozygous *VKORC1-1693* variant were detected as homozygous variants. As demonstrated in Chapter 2.3.2 (Figure 2-9, page 64), a carrier of the heterozygous variant requires an approximately 40% higher dose compared with the homozygous variant. Consequently, one of the patients was not given the therapeutic dose until the 5th visit and the other patients on the 6th visit. During the period of not having the therapeutic dose, the risk of thromboembolism was higher and could result in death. In addition, the failure rate for all SNPs tested was over 7% and with the highest for *VKORC1-1693* test (10.6%) (Table 6.8). Because each assay strip contained assays to test 3 SNPs, repeating the genotyping procedure for any SNP which failed to genotype not only increased the time to dose the patients but it also resulted in the assays being wasted.

The low sensitivity of Genie 1 HyBeacon® assay to detect *VKORC1-1693* allele has been observed in a previous study by Howard *et al.* (2011) as they did not produce a genotyping result for *VKORC1-1693* in one of 28 blood samples (Howard *et al.* 2011). Therefore, the success rate for Genie 1 HyBeacon® compared with LightTyper HyBeacon® was 96% with a 100% concordance. Furthermore, they validated 128 blood samples by comparing results from various different genotyping platforms with a success rate of 100% complete concordance between methods.

Several small studies have produced promising results regarding pharmacogenetic dosing to improve VKA management. For example, one study showed that patients receiving a dose by pharmacogenetic algorithm required fewer and smaller dose adjustments and fewer INR determinations (Anderson *et al.* 2007). Other studies demonstrated that patients in a pharmacogenetic dosing group reached a stable dose faster (Huang *et al.* 2009) and spent more time within the therapeutic range (Caraco *et al.* 2008). Ongoing Randomised Control Trials (RCT) such as EU-PACT should answer the question as to whether pharmacogenetic testing prior to dosing will significantly improve anti-cogulation with warfarin therapy. If the findings are positive, we would recommend Genie HyBeacon® as a practical genotyping platform to be used in the hospital laboratory because the procedure is very easy, as demonstrated in this study where the genotyping for patients from the St Helens & Knowsley Teaching Hospital NHS Trust has been performed by nurses at the hospital sites. However, there would need to be some improvement to the assays design to improve the reliability of *VKORC1* genotyping assay.

The limitation of our analysis is that we did not consider the cost of the instrument and reagents for the platform evaluated. The Genie 1 instrument is predicted to cost in the region of 15,000 US dollars with each assay strip likely to cost under 50 US dollars (Howard *et al.* 2011).

As many studies have validated the usefulness of *CYP2C9*2*, *CYP2C9*3* and *VKORC1* in predicting warfarin responses, it is imperative to have a genotyping assay that is compatible with the clinical environment. The Genie HyBeacon® genotyping platform is being validated in our study as it produces accurate results, the procedure is easy and has a very quick turn-around time. In addition, a new formulation is currently being investigated by the LGC Company to improve total process time (Howard *et al.* 2011). It is expected that by using a new high-performance blood-tolerant enzyme technology, total analysis time will decrease at least 60 minutes. All of these properties are very valuable when optimizing the accuracy of warfarin therapy based on a patient's genetic make-up.

Chapter 7

Final discussion

7.1 Thesis summary

Warfarin, although a difficult drug to dose due to its large inter-individual variability and narrow therapeutic window, is a very effective oral anticoagulant that has saved millions of lives worldwide (Wysowski et al. 2007, Kim et al. 2009). As quoted by Rose A.J (2013), "warfarin is a drug we all love to hate and wish to see replaced. But it is the drug that has been something of a puzzle, a puzzle that we may finally be starting to solve as we truly move towards optimising its management". I truly agree with him; from my experience in warfarin clinics, I have seen large variability in warfarin responses amongst patients. However through medication counselling (one of the roles of a pharmacist in improving drug therapy), where drug adherence and factors influencing warfarin responses are discussed, we try to make warfarin a safe and more effective drug. But given that patients still present with warfarin-related adverse events, this is probably not enough.

Dabigatran, a direct thrombin inhibitor, has recently been shown to be either non-inferior (110mg bd) or superior (150mg bd) to warfarin in stroke prevention (Connolly *et al.* 2009). Its pharmacokinetic and pharmacodynamic characteristics have some advantages when compared with warfarin including more rapid onset of action, reduced food-drug and drug-drug interactions, and a predictable anticoagulant response (Yusuf 2002, Van Ryn *et al.* 2010, Baglin *et al.* 2012, Schulman and Crowther 2012). However, dabigatran is much more expensive and the absence of a reliable reversal agent and the lack of a pharmacodynamic biomarker have limited the safe prescription of dabigatran (Liesenfeld *et al.* 2006, Van Ryn *et al.* 2010, Warkentin *et al.* 2012). As such, warfarin may remain the mainstay of oral anticoagulant therapy for the foreseeable future. Thus, there remains a need to improve warfarin therapy.

The main aim of this thesis was to explore the use of pharmacogenetic data to guide personalised warfarin therapy. Prescribing the correct dose at the start of warfarin therapy is the most challenging. As demonstrated by many studies, clinical factors together with genetic variables (*CYP2C9*2, CYP2C9*3, VKORC1*-1693) explain 50-60% of dose variability (Sconce *et al.* 2005, Gage *et al.* 2008, Hatch *et al.* 2008, Perini *et al.* 2008). Therefore, our study in Chapter 2 sought to answer how accurate a pharmacogenetic algorithm is in predicting warfarin stable dose. Taking into account that our main concern when prescribing warfarin is major bleeding, our research question in Chapter 3 was whether genetic risk factors associated with major bleeding in patients treated with warfarin would be useful in distinguishing individuals with different risks of bleeding. The paediatric population represents a unique pharmacogenetic challenge as children have the additional complexity of ontological phenotypes that impact on drug response. So our next research questions in Chapters 4 and 5 address whether pharmacogenetics can predict the outcome of warfarin therapy in children population. Chapter 6 focused on the application of warfarin pharmacogenetic testing in a clinical setting. This chapter focused on whether a currently available genotyping platform can be used in a clinical setting.

7.1.1 Research Question 1: Can pharmacogenetic algorithm accurately predict warfarin dose?

In Chapter 2, we developed a pharmacogenetic dosing algorithm to predict warfarin stable dose in a large population of prospectively recruited British Caucasians (n=456) initiated and maintained on warfarin therapy in a secondary care setting. Our algorithm was then validated in a cohort of retrospectively recruited patients from a primary care setting. Similar to most published algorithms, we included age, BSA and amiodarone use together with *CYP2C9*2*, *CYP2C9*3* and *VKORC1 -1693* as covariates.

In order to properly assess the accuracy of our algorithm, we examined our algorithm in all aspects including (i) the R^2 statistic to measure the variability of warfarin dosing explained by the prediction model, (ii) the mean absolute error to measure the predictive accuracy and (iii) the percentage of patients whose predicted warfarin dose was within 20% of the actual stable therapeutic dose to evaluate its potential clinical value. The predictability of our algorithm was reproduced in our validation cohort giving a R^2 value of 43% (mean absolute error of 1.08mg/day; 95% CI: 0.95-1.20) while the predicted dose of approximately 50% of patients fell within 20% of the actual dose. The performance of our pharmacogenetic algorithm was comparable to published algorithms, (Gage *et al.* 2008, Klein *et al.* 2009). Furthermore, our algorithm included fewer variables (6 vs. >8 variables), making it easier to use. There is therefore a possibility of implementing our secondary care setting-derived pharmacogenetic algorithm in a primary care setting. However, it is unclear how this algorithm would compare to computerised dosing software programs which are now available in many primary and secondary care clinics.

Our algorithm underestimated the dose for patients who required ≥ 6 mg/day and overestimated for patients who required ≤ 2 mg/day. The same observation has been reported in a seminal study conducted by the IWPC (Klein *et al.* 2009). As a pharmacist, my main concern would be when the dose is underestimated by the algorithm especially in patients suspected of having deep vein thrombosis and pulmonary embolism where immediate full anticoagulation is mandatory (Ansell *et al.* 2008). Therefore, further research in this group of patients should be undertaken to identify other non-genetic and genetic markers which lead to dose requirement in this group of patients, and develop dosing protocols which perform better in the extremes of the dosing range.

7.1.2 Research Question 2: Can pharmacogenetics provide guidance in predicting patients at high risk of major bleeding complications?

In Chapter 3, we assessed the performance of HAS-BLED, a validated bleeding score developed to predict major bleeding. We confirmed that HAS-BLED could be a useful tool for identifying atrial fibrillation patients at risk of major bleeding during warfarin therapy with a c-statistic of 0.80. We also explored the contribution of *CYP2C9*2*, *CYP2C9*3* and *VKORC1* -1693 in predicting major bleeding, but in contrast to previous reports (Jorgensen *et al.* 2012, Yang *et al.* 2013), only *CYP2C9*3* was associated with major bleeding in our population ($p \leq 0.05$). However, including this SNP into HAS-BLED did not improve its prediction. Taking into account the low MAF of *CYP2C9*3* (6%), our study cohort was possibly underpowered to address our research question. Nevertheless, our findings indicate that HAS-BLED scores without the incorporation of genetic factors could be used to identify patients at high risk of major bleeding due to warfarin use. In our analysis, some patients (3 out of 15) who experienced major bleeding during warfarin therapy were predicted at higher risk by HAS-BLEDG compared to HAS-BLED score. Therefore, if genetic information was already available, there is no harm for it to be included in the HAS-BLED score.

7.1.3 Research question 3: Can pharmacogenetics predict the outcome of warfarin therapy in children?

In Chapter 4, we extended our research to a paediatric population as pharmacogenetic studies have the potential to improve the quality of warfarin therapy for this vulnerable population. We evaluated the contribution of *CYP2C9*2*, *CYP2C9*3* and *VKORC1 -1693* to four clinical outcomes that could potentially lead to safer and more effective warfarin therapy. The first outcome was proportion of time in which INR measurements fell within the target range within the first six months (PTTR). This outcome would link pharmacogenetics to the quality of anticoagulation

control. The second outcome was stable dose which would link to warfarin prediction dose. The third and fourth outcomes were INR exceeding target range in week one and bleeding complications, which provided an indication of bleeding risk.

In the 97 retrospectively recruited Caucasian children treated with warfarin, we showed that the mean PTIR was 49%. This finding highlights the poor stability of warfarin control that occurs in childhood during the first six 6 months of therapy, even when INR monitoring occurs frequently with the aid of a home monitoring device. Our work has demonstrated that *VKORC1 -1693,* indication for treatment (Fontan or non-Fontan group) and INR group (according to the lower limit of the referral therapeutic range) explained 20.8% of inter-individual variability in PTTR. *VKORC1*-1693 only contributed to a small proportion of PTIR variability (9.5%). We observed that carriers of the *VKORC1-1693* minor allele and frequent INR monitoring with a POCT device were associated with greater PTTR. Our results emphasize that by identifying and characterizing gene-environment interactions, we have more opportunities to effectively target intervention strategies.

Polymorphisms in *CYP2C9*2* and *VKORC1-1693* together with age and INR target group explained 41% of warfarin stable dose variability. In comparison to previous studies (Biss *et al.* 2012, Moreau *et al.* 2012), *CYP2C9*2* and *VKORC1 -1693* accounted for much smaller warfarin dose variability (12% vs >30%). This could be explained by the younger ages of the children in our cohort. For instance, only 5.8% of children in the study by Biss et al., (2012) were ≤ three years of age compared to 27% in our study. Children less than three years old have greater physiological differences compared to adults (Koukouritaki et al. 2004, Anderson and Lynn 2009). Our observations suggest that *CYP2C9*2,*and *VKORC1 -1693* could be used to predict warfarin dose in children, but further work is needed to replicate our findings in a larger sample set, in particular with a view to stratifying the analyses by age.

We showed that *CYP2C9*2* was significantly associated with INR exceeding target range in the first week of warfarin treatment, explaining 6.8% of the variability. We also found that *VKORC1 -1693* was significantly associated with bleeding complications and explained 8.7% of the variability. Therefore information on *VKORC1-1693* and *CYP2C9*2* genotypes in paediatric patients could enable the identification of those at high risk of developing bleeding complications during the initiation of warfarin therapy and those who may benefit from pharmacogenetic-based personalised approach to warfarin dosing.

Taken together, results from our candidate gene study show that a large proportion of genetic variance contributing to warfarin response in children remains unidentified and, hence we extended our investigations to using the genome wide association (GWA) approach in Chapter 5. To our knowledge, this is the first GWAS investigating the influence of genetic factors on warfarin response in children. Two novel SNPs on chromosome 5, rs13167496 and rs6882472, were found to be significantly associated with PTIR at genome-wide level. These SNPs are located ~34kb from the 5' of *TIFAB*, a protein-coding gene which inhibits the activation of NF-κB, a transcription factor which has previously been reported to be involved in controlling coagulation (Mackman *et al.* 1991, Figueiredo and Brownlee 1995, Anrather *et al.* 1997, Hou *et al.* 2004, Song *et al.* 2009). . We did not identify any SNPs that attained genome-wide significance with warfarin stable dose, INR values exceeding the target range within the first week of treatment and bleeding complications.

In adults, several GWA studies have shown that *CYP2C9*, *VKORC1* and *CYP4F2* were associated with warfarin stable dose in Caucasians at the genome-wide level (Takeuchi *et al.* 2009). However, we cannot elucidate the contribution of these SNPs in children because our study was underpowered due to the small sample size.

An adequate sample size is very important to detect genome-wide significance ($p<5\times10^{-8}$) for polymorphisms that modestly alter warfarin response as shown by Takeuchi et al., (2009) when they failed to demonstrate the significance of *CYP2C9, VKORC1* and *CYP4F2* SNPs with warfarin dose in GWAS of 181 patients but then detected the significance in 1,053 patients (Cooper *et al.* 2008, Takeuchi *et al.* 2009).

In this analysis, we focused on the association of single loci with target phenotypes. Recently, a novel statistical approach has been adopted taking into account the correlations among SNPs in genes within a biological pathway (Luo *et al.* 2010, Yaspan *et al.* 2011). This analytical approach warrants further investigation. A sample size with statistical power is very important in detecting the significance of SNPs with phenotype but recruiting children for a clinical study is difficult. An international multi-centre collaboration would be a good strategy to increase the sample size. Such a collaboration would permit a more accurate representation of the distribution of genotypes amongst children of multiple ethnicities, and may help in determining the relative contributions of both non-genetic and genetic factors to warfarin response in children.

7.1.4 Research question 4: Is the Genie point of care genotyping device usable in a clinical setting?

Considering the large amount of evidence that has accrued which shows the usefulness of genotyping of *CYP2C9*2*, *CYP2C9*3* and *VKORC1-1693* polymorphisms in improving warfarin therapy, there is a need to develop and test suitable genotyping platforms that could be used in clinical settings. In order genotyping result to be used clinically, the test must be performed in a regulated clinical laboratory, have a quick turn-around time and be easy to perform. An alternative to performing genotyping within a clinical laboratory, is to use a point-of-care-test, which can be performed at the "bedside" in a clinical setting. The aim in

chapter 6 was to evaluate such a platform, the Genie HyBeacon®, regarding its accuracy, turn-around time and complexity.

We validated the genotyping performed on the point-of-care platform by comparing the results with Applied Biosystems Taqman® genotyping platform. Very good agreement was observed overall, but further development is needed in the assay design for VKORC1 in particular to increase the reliability of genotyping. Genie HyBeacon® was also performed by hospital staff in anticoagulant clinics. With a turn-around time of 97.5 min per run, it was possible to obtain the genotypes for *CYP2C9*2, CYP2C9**3 and *VKORC1-1693* before warfarin dosing. Therefore, Genie Hybeacon® is suitable to use as point of care device to determine CYP2C9 and VKORC1 genotypes before starting warfarin therapy.

7.2 Pharmacogenetics-guided warfarin therapy: recommendations for future research

From the findings in my thesis and those published in the literature, there is compelling evidence that genetic and clinical factors in combination determine the dose requirements for warfarin. The effectiveness of this has recently been tested in the EU-PACT trial which showed that genotype guided dosing improved the time within range and some secondary outcome measures when compared with standard dosing (Pirmohamed *et al.* 2013). However, the US study (COAG) failed to show any advantage of genotype-guided dosing compared with the use of a clinical algorithm (Kimmel *et al.* 2013). The reasons for the difference between the two studies are related to the design, in particular the algorithmic strategies used. EU-PACT used a loading dose algorithm while COAG used a maintenance dose algorithm to initiate warfarin therapy. Giving a loding dose, a largest dose on the first day of therapy to the patients so they get to a steady state early on might leading to shorter time to achive stable dose. This highlights the importance of ensuring that the design of your clinical

study including randomised trials is as robust as possible. The lessons from this need to be taken forward to further evaluate how the benefit-risk ratio of warfarin therapy can be improved. Specific areas that could potentially be looked at include:

- Refine dosing algorithms to improve accuracy in those at extremes of the dosing range. This include using next-generation sequencing method to sequence individuals who denotes as extreme phenotype (figure 2.9).
- Improve the identification of patients who at high risk of major bleeding complications during warfarin therapy through discovery of novel genetic factors. This could then be incorporated into a revised bleeding score system.
- To further evaluate the contribution(s) of genetic factors to warfarin outcomes in a larger prospective cohort of children undergoing initiation therapy allowing for analyses stratified by age. A multi-centre collaborative approach is needed to achieve adequate power.
- To investigate the contribution of multiple variations in gene or collection of genes to phenotypic outcomes using pathway analysis of genome-wide data.
- To replicate the association of rs13167496 and rs6882472 with PTTR in children population.
- To develop a decision analytic model to examine the cost-effectiveness of a genotype-based dosing strategy compared with standard induction of warfarin.

7.3 Conclusions

This thesis has extended current knowledge on the role of genetic and clinical factors determining daily warfarin dose requirement and the potential for this to cause bleeding in both adults and children. With the recent publication of two trials that have tested the utility of genotype guided dosing, it will be interesting to how this plays out in the research and clinical community. The poor uptake of the new oral anticoagulants in clinical practice further adds to the uncertainty of what anticoagulants will be used and in what settings. In summary, the research in this thesis shows that genetic factors are important in determining individual doses of warfarin. Further work is required to develop strategies to choose the best anticoagulant for individual patients based on both their clinical characteristics and genetic background.

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