Identification of the factors influencing anticoagulation response to warfarin in children

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Abstract

The requirement for anticoagulant therapy in children is increasing and warfarin remains the long-term anticoagulant agent of choice. However, little is known about the factors that influence inter-individual variability in response to warfarin among children. The aim of this MD was to gain a greater understanding of the factors that affect warfarin anticoagulant control and response in children. A retrospective study of a cohort of anticoagulated children identified factors contributing to poor anticoagulant control. It also highlighted the importance of the way in which anticoagulant control is assessed in children, with the study results showing that the use of a linear interpolation method may be more appropriate than the proportion of INRs within target range during intermittent periods of instability when INR measurements are carried out more frequently. A multi-centre, cross-sectional study of 120 children with stable anticoagulation with warfarin showed that 72% of the inter-individual variability in warfarin maintenance dose is accounted for by height, *VKORC1* and *CYP2C9* genotype, and indication for warfarin. The study results were used to develop a pharmacogenetics-based warfarin-dosing algorithm. The latter was demonstrated to have the power to accurately predict maintenance warfarin dose in an unrelated cohort of 23 children. Analysis of data for a subgroup of 51 children showed that *VKORC1* and *CYP2C9* genotype influence outcome variables during initiation of warfarin therapy, including peak INR response during week 1 and the proportion of supratherapeutic INRs during month 1 of therapy. The above findings have provided us with an insight into the factors influencing anticoagulant control and variability in response to warfarin in children. Application of a pharmacogenetics-based approach to initiation and maintenance warfarin therapy in children has the potential to improve efficacy and safety of warfarin therapy in this challenging patient population.

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Finally, I dedicate this thesis to my 3 wonderful children, Mili, Jai and Hari.

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BISS, T., HAMBERG, A-K., AVERY, P., WADELIUS, M. & KAMALI F. Warfarin dose prediction in children using pharmacogenetics information. Accepted for publication in *British Journal of Haematology*

BISS, T.T., AVERY, P.J., BRANDAO, L.R., CHALMERS, E.A., WILLIAMS, M.D., GRAINGER, J.D., LEATHART, J.B.S., HANLEY, J.P., DALY, A.K. & KAMALI, F. 2012. *VKORC1* and *CYP2C9* genotype and patient characteristics explain a large proportion of the variability in warfarin dose requirement among children. *Blood,* 119, 868-873 (see Appendix G).

BISS, T.T., AVERY, P.J., WALSH, P.M., KAMALI, F. 2011. Comparison of 'time within therapeutic INR range' with 'percentage INR within target therapeutic range' for assessing long-term anticoagulation control in children: reply to a rebuttal. *Journal of Thrombosis and Haemostasis*, 9, 2332-2333.

BISS, T.T., AVERY, P.J., WALSH, P.M., KAMALI, F. 2011. Comparison of 'time within therapeutic INR range' with 'percentage INR within target therapeutic range' for assessing long-term anticoagulation control in children. *Journal of Thrombosis and Haemostasis,* 9, 1090-1092.

BISS, T.T., AVERY, P.J., WALSH, P.M., KAMALI, F. 2011. Warfarin treatment outcomes in children monitored at home with a point-of-care device. *Thrombosis and Haemostasis,* 105, 1103-1105.

BISS, T.T., ADAMSON, A.J., SEAL, C.J., KAMALI, F. 2011. The potential impact of dietary vitamin K status on anticoagulation control in children receiving warfarin. *Pediatric Hematology and Oncology*, 28, 425-427.

Presentations

British Society for Haematology 52nd Annual Scientific Meeting, Glasgow, April 2012

Oral presentation: Warfarin dose prediction in children using pharmacogenetics information (see Appendix C)

British Society for Haematology 52nd Annual Scientific Meeting, Glasgow, April 2012

Poster presentation: *VKORC1* and *CYP2C9* genotype is associated with over-anticoagulation during initiation of warfarin therapy in children (see Appendix D)

XXIIIrd Congress of the International Society on Thrombosis & Haemostasis, Japan, July 2011

Oral presentation: Inter-individual variability in warfarin dose requirement in children can be explained by *VKORC1* and *CYP2C9* genotype and patient characteristics (see Appendix E)

British Society for Haemostasis & Thrombosis, Newcastle upon Tyne, October 2009

Poster presentation: Anticoagulation control in a cohort of children on chronic therapy with warfarin (see Appendix F)

List of Abbreviations

ANOVA	Analysis of variance
APOE	Apolipoprotein E
CALU	Calumenin
CT	Computed tomography
CI	Confidence interval
CYP1A2	Cytochrome P450 1A2
CYP2C9	Cytochrome P450 2C9
CYP2C19	Cytochrome P450 2C19
CYP3A4	Cytochrome P450 3A4
CYP3A5	Cytochrome P450 3A5
CYP4F2	Cytochrome P450 4F2
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DVT	Deep vein thrombosis
EDTA	Ethylenediaminetetraacetic acid
EPHX1	Epoxide hydrolase 1
GGCX	Gamma-glutamyl carboxylase
INR	International normalised ratio
%ITTR	Percentage of international normalised ratio measurements within therapeutic range
LMWH	Low molecular weight heparin
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
NICE	National Institute for Health and Clinical Excellence
PE	Pulmonary embolism
PCR	Polymerase chain reaction
POC	Point-of-care
PROC	Protein C
RFLP	Restriction fragment length polymorphism
SD	Standard deviation
SNP	Single Nucleotide Polymorphism
TIA	Transient ischaemic attack
TIR	Time in therapeutic range
TTR	Target therapeutic range

VKA	Vitamin K antagonist
VKOR	Vitamin K epoxide reductase
VKORC1	Vitamin K epoxide reductase complex subunit 1
VK1	Vitamin K1

Chapter 1. General Introduction

1.1 Oral anticoagulant therapy

Oral anticoagulant agents are used to treat thrombosis or to prevent thrombosis in individuals who are at risk. Indications for oral anticoagulant therapy include the treatment of deep vein thrombosis (DVT), pulmonary embolism (PE), and thrombosis occurring in more unusual sites such as the cerebral venous sinuses, hepatic or renal veins. Oral anticoagulant therapy may also be indicated in the management of arterial thrombotic events, such as peripheral vascular occlusion, myocardial infarction and stroke, which have failed to respond to anti-platelet therapy. The use of oral anticoagulants for primary prevention of systemic embolism includes stroke prevention in individuals with atrial fibrillation, a prosthetic heart valve or cardiomyopathy.

The most frequently prescribed class of oral anticoagulants are the vitamin K antagonists (VKAs). Many of these agents are derived from coumarin and include warfarin, dicoumarol, acenocoumarol and phenprocoumon. Other vitamin K antagonists that are not derived from coumarin include fluindione and phenindione.

1.2 Warfarin

Warfarin is the most widely prescribed oral anticoagulant agent in the UK and North America and is the agent for which there is the most published data of its' use in childhood.

1.2.1 *History of Warfarin*

The name 'warfarin' is derived from the group at the University of Wisconsin who discovered it, the Warfarin Alumni Research Foundation (WARF), with the ending of '-arin' indicating its' link to coumarin. Coumarin is a chemical that is found in sweetclover hay. Coumarin does not affect the coagulation system but is converted to dicoumarol, which is a powerful anticoagulant, in spoiled animal feeds. This had led to the death of many cattle due to internal haemorrhage during a particularly warm year in the 1920's. Warfarin is a synthetic derivative of dicoumarol which was developed in 1948 as a rodenticide and in the 1950s

was found to be effective in the prevention of thrombosis. It has been used as an anticoagulant in clinical practice since 1954.

1.2.2 *Mechanism of action*

VKAs produce an anticoagulant effect by interfering with the cyclic conversion of vitamin K to its reduced form (vitamin K hydroquinone) (Figure 1-1). Warfarin inhibits the regeneration of vitamin K hydroquinone from vitamin K epoxide by inhibiting the vitamin K epoxide reductase (VKOR) enzyme in the vitamin K cycle. Vitamin K hydroquinone is an essential co-factor for the post-ribosomal activation (γ-carboxylation) of coagulation factors II, VII, IX and X without which they are unable to bind calcium and become active in the coagulation cascade.

1.2.3 *Pharmacokinetics*

Warfarin is given orally and is rapidly absorbed from the gastrointestinal tract. Although the maximum plasma concentration of warfarin is reached within 90 minutes in adults the anticoagulant effect takes several days to develop. This is due to the time taken for the circulating γ-carboxylated coagulation factors to undergo degradation, the onset of action of warfarin therefore being dependant on the half lives of the relevant coagulation factors. The half-life of warfarin is approximately 40 hours and its anticoagulant effect lasts for 4-5 days. Warfarin is given as a once daily dose, usually during the evening.

Warfarin is 97% bound to albumin and is therefore distributed in the plasma compartment. It is only the remaining 3% of unbound warfarin that is pharmacologically active and can be eliminated. Changes in the unbound fraction of warfarin, which may occur due to competition for protein binding sites with other drugs, has a major effect on its' elimination and on warfarin dose requirements.

Commercially available warfarin is a 50:50 racemic mixture of R- and S-enantiomers, with the S-enantiomer being three times more potent than the R-enantiomer in its inhibitory effect on the VKOR enzyme.

There are several different cytochrome P450 enzymes that contribute to the metabolism of warfarin. The main enzyme responsible for the metabolism of the

S-enantiomer of warfarin is cytochrome P450 *CYP2C9*. The R-enantiomer of warfarin is metabolised primarily by *CYP1A2*, with *CYP3A4* and *CYP2C19* providing a lesser contribution (Figure 1-2).

Other VKAs differ to warfarin in terms of their half-life, acenocoumarol having a shorter half-life and phenprocoumon a longer half-life compared to warfarin. Dosing of these agents differs and the stability of the anticoagulation achieved can differ, those agents with a longer half-life resulting in a greater stability.

Figure 1-1. The action of warfarin on the vitamin K cycle. VKOR, vitamin K epoxide reductase.

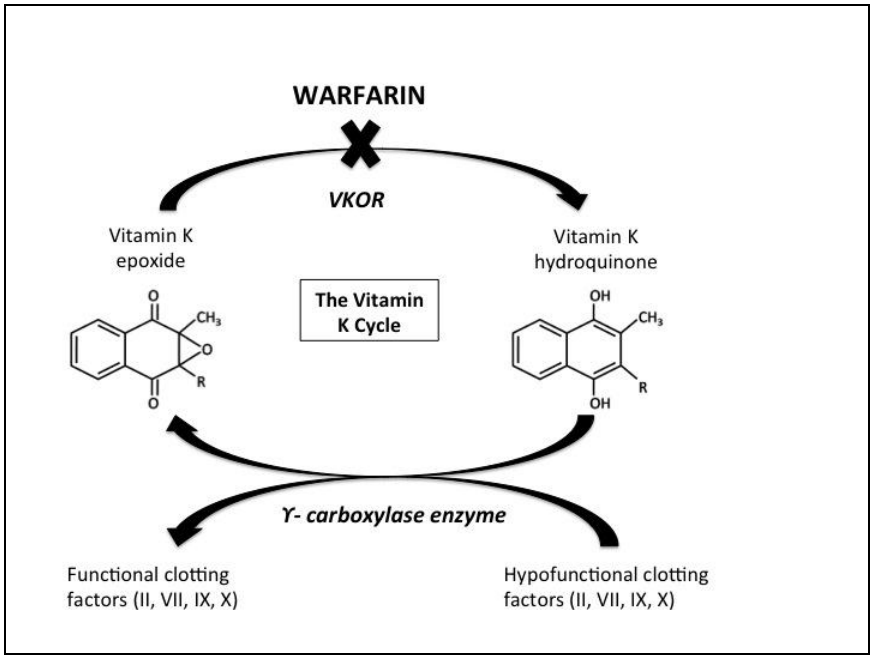
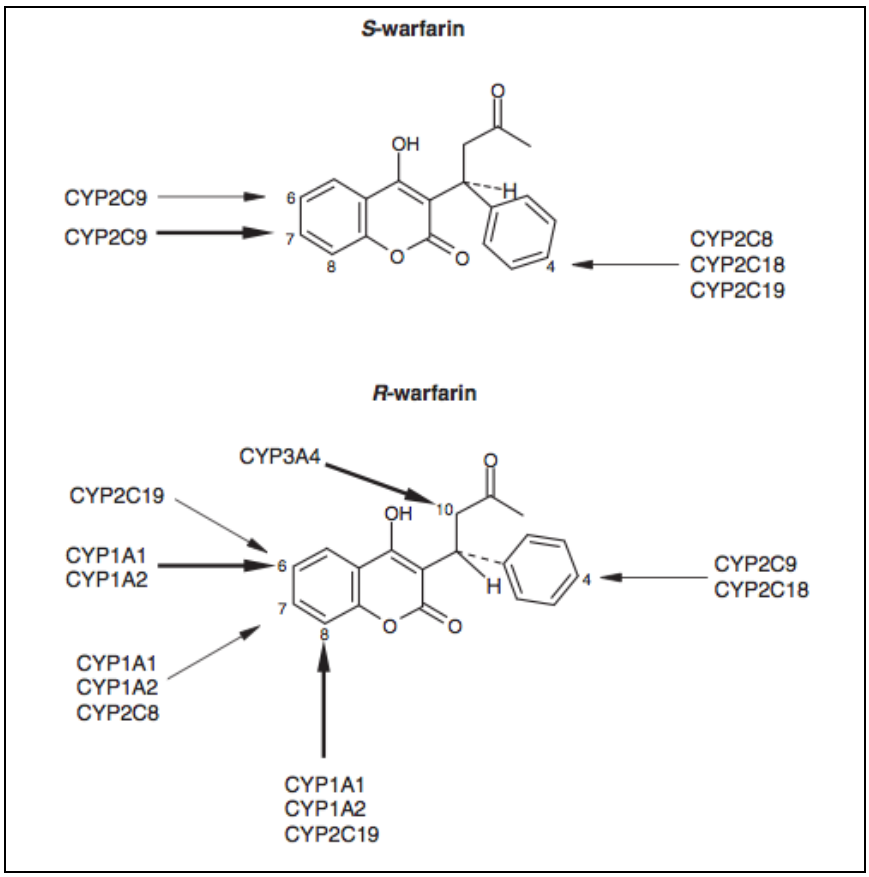


Figure 1-2. The metabolism of the S- and R-enantiomers of warfarin by cytochrome P450 enzymes (Taken from Kaminsky & Zhang (Kaminsky and Zhang, 1997)). The major metabolic pathways are shown by the thicker arrows.



1.2.4 *Monitoring of anticoagulant response*

The anticoagulant effect of the VKAs is monitored using the prothrombin time, which is a measure of the time taken for citrated blood to clot after the addition of calcium and thromboplastin (a reagent composed of tissue factor and phospholipids). The prothrombin time becomes prolonged when the levels of active coagulation factors II, VII and X fall and the degree of prolongation is proportional to the degree of anticoagulant effect. When used for monitoring therapy with a VKA, the prothrombin time is expressed as the International Normalised Ratio (INR), which is a means of standardising results obtained from different laboratories that may use different thromboplastin reagents and equipment. The INR is maintained within a desired therapeutic range, that is dependent upon the clinical indication for anticoagulation, by regular monitoring and adjustment of the dose of VKA (Keeling et al., 2011).

The majority of patients receiving warfarin regularly attend an anticoagulant clinic, in the hospital or their general practice surgery, for monitoring of their INR. Warfarin dose is adjusted if necessary by a trained healthcare professional, usually a pharmacist or an anticoagulant nurse practitioner. Portable coagulometers, such as the CoaguChek®XS, provide some patients with the opportunity of self-testing or self-management of their anticoagulant therapy (Fitzmaurice et al., 2005). For those who self-test, the INR is reported to a healthcare professional who then makes a warfarin dose adjustment if necessary. Those who self-manage make decisions about their own warfarin dosing with the aid of a protocol written by the responsible clinical team. This point-of-care (POC) management of warfarin anticoagulation is suitable for motivated, compliant individuals who are unable to attend an anticoagulant clinic for reasons such as employment. It is also suitable for selected patients with poor stability of anticoagulant control, including children, who require frequent INR testing (Fitzmaurice et al., 2005).

1.2.5 *Adverse effects and reversal*

Warfarin has a narrow therapeutic window and a deviation from the desired target INR range can result in a reduction in efficacy or an adverse event. Under-anticoagulation, as detected by an INR that is below the target range, carries a risk of thromboembolism whereas over-anticoagulation, with an INR

above target range, can result in haemorrhage. Under-anticoagulation is managed by increasing the dose of prescribed warfarin with the addition of an alternative form of anticoagulant therapy, such as heparin, if the perceived risk of thromboembolism is sufficiently high. The management of over-anticoagulation depends upon the degree of elevation of INR, the presence or absence of bleeding symptoms and the indication for anticoagulant therapy. If necessary, in addition to withholding further warfarin doses, the anticoagulant effect of warfarin can be reversed by the administration of vitamin K (either orally or intravenously, depending on the speed of reversal required) and/or fresh frozen plasma or prothrombin complex concentrate which replenishes the absent coagulation factors (Keeling et al., 2011, Hanley, 2004).

1.3 Warfarin anticoagulation in children

1.3.1 *Indications for anticoagulation in children*

Due to significant advances in paediatric medical care previously fatal conditions of childhood, such as congenital cardiac defects, prematurity and malignancy, are now being successfully managed. This has been at the expense of a rising incidence of thromboembolic complications (Stein et al., 2004). The requirement for anticoagulation to treat thrombotic events, as well as for prophylaxis in at risk patients, is therefore increasing (Monagle et al., 2008).

Table 1-1 shows the most frequent indications for anticoagulant therapy in children. The majority of long-term anticoagulant therapy is for those with cardiac defects.

Although the use of the low molecular weight heparins is increasing for short-term anticoagulation in children, warfarin remains the most common anticoagulant agent for long-term anticoagulant therapy in this patient group.

Table 1-1. Indications for anticoagulant therapy in children

Treatment of thrombotic events
- Venous thrombosis, including
  • Central venous line-related thrombosis
  • Deep vein thrombosis
  • Pulmonary embolism
  • Thrombosis at unusual sites: Cerebral venous sinus thrombosis; Renal vein thrombosis; Portal vein thrombosis
- Thrombotic stroke
- Intra-cardiac thrombus
- Arterial thrombosis, including
  • Ischaemic limb
  • Aortic thrombosis

Prevention of thrombotic events - Surgery for congenital heart disease (e.g. Fontan procedure) - Heart valve replacement - Severe cardiomyopathy - Giant coronary aneurysm - Idiopathic pulmonary hypertension - Ventricular assist device - Long-term central venous access for total parenteral nutrition

1.3.2 *Anticoagulation control in children receiving warfarin*

1.3.2.1 Difficulties in anticoagulation control in children. Oral anticoagulant therapy in children is complicated by numerous factors that make control of anticoagulant therapy difficult, including: variable age-related dose-response rates; complex underlying health problems; multiple intercurrent viral illnesses; polypharmacy; and, variation in diet (Table 1-2). Monitoring of anticoagulant therapy is also problematic in children due to the difficulties of obtaining blood by venepuncture. In infants, additional problems include the challenge of accurate dosing (warfarin is only available in tablet form, with 0.5mg tablet being the smallest dose commercially available), increased sensitivity due to physiologically low levels of vitamin K-dependant clotting factors (Andrew et al., 1987), and differing amounts of dietary intake of vitamin K (formula feeds containing varying amounts and breast milk containing very little vitamin K (Bonduel, 2006)).

Previous studies of children anticoagulated with warfarin have reported that around 50% of INR values are within target therapeutic range (TTR) (Newall et al., 2004, Bradbury et al., 2008, Newall et al., 2006, Bhat et al., 2010) and that the mean time interval between INR tests is less than one week (Streif et al., 1999). This compares poorly to adult studies which show achievement of TTR in 60-80% of tests and a longer time interval between INR tests (Palareti et al., 1996), thus confirming that control of oral anticoagulant therapy is difficult in children.

Table 1-2. Factors contributing to poor control of oral anticoagulant therapy in children

Patient-related factors
- Variable age-related dose-response
- Physiological reduction in levels of vitamin K-dependant factors¶
- Poor compliance, particularly in adolescents
- Adolescent alcohol/recreational drug use
- Difficulty in venepuncture
Disease-related factors
- Underlying health problems
- Frequent intercurrent viral illness
Dietary factors
- Variable vitamin K content of diet
- Vitamin K-containing enteral feeds/formula feeds
External factors
- Polypharmacy
- Frequent intermittent antibiotic therapy
Social factors
- Missed school days for INR monitoring (work days for parent/carer)
- Restrictions placed on physical activities
Other
- Difficulty in accurate dosing due to lack of suspension/liquid formulation

¶Relevant to children <1 year of age

1.3.2.2 Assessment of quality of anticoagulation control in children.

Anticoagulation control in children is usually reported as the 'percentage of INR measurements within therapeutic range' (%ITTR). However the advent of point-of-care testing devices, which has allowed INR testing to occur more frequently during periods of instability, means that this measure could potentially underestimate the quality of anticoagulation control in this patient group in whom longer periods of stable control are often interspersed with shorter periods of poor stability. An alternative method, described by Rosendaal et al (Rosendaal et al., 1993), measures 'percentage time within therapeutic range' (%TIR). This method uses linear interpolation to allocate an INR value to each day, including days between INR tests. This is likely to minimise the impact of multiple ('out of range') INR values over a short period of time and places more emphasis on the longer periods of stability during which INR tests are less frequently performed. It should be noted, however, that the TIR method was not developed as a means of assessing quality of anticoagulation control but rather to determine the optimal intensity of anticoagulant therapy (Rosendaal et al., 1993). Cross-section-of-the-files is a further method using which a percentage is obtained by selecting a single time-point and calculating the fraction of patients whose INR value is in range divided by the total number of INRs measured in the patient population at that point in time (Loelinger, 1985). The best measure of quality of anticoagulation control would be one that correlates closely with clinical outcomes in terms of efficacy and safety (Schmitt et al., 2003) and this has not been examined in a paediatric population.

1.3.3 *Factors contributing to inter- and intra-individual differences in response to warfarin in children*

1.3.3.1 Age. Age is a major factor determining dose response in infants and children. The largest cohort study of children anticoagulated with warfarin showed that younger patients required higher weight-adjusted doses to achieve an equivalent anticoagulant effect with infants requiring a mean daily dose of 0.32 mg/kg and teenagers 0.09 mg/kg warfarin to maintain a target INR of 2.0 to 3.0 (Streif et al., 1999). This is in comparison to adult doses that are in the range of 0.04 to 0.08 mg/kg/day for a similar level of anticoagulation (Hirsh, 1991). The influence of age on warfarin dose requirement in children is so great that it negates the effects of differing target INR ranges, concurrent

medications, underlying disorder and diet (Streif et al., 1999). The higher dose requirement by younger children suggests that they may have reduced or underdeveloped activity of the enzymes that mediate the disposition and pharmacological activity of warfarin compared to older children.

1.3.3.2 Diet. Breast-fed infants are more sensitive to the anticoagulant effect of warfarin than formula-fed infants and this is likely to be due to the relatively low concentration of vitamin K (see Section 1.3 'Dietary vitamin K status and response to warfarin') in breast milk compared to vitamin K-supplemented formula feed (Haroon et al., 1982). Children receiving enteral feeding by nasogastric or gastrostomy tube have also been shown to require higher warfarin doses which is again likely to be due to vitamin K supplementation of the enteral feed (Streif et al., 1999). However, a direct association between vitamin K in enteral feed and warfarin dose requirement could not be established as plasma vitamin K concentrations were not determined and this effect may be, at least in part, due to reduced absorption of warfarin as a result of the underlying condition.

1.3.3.3 Indication for anticoagulant therapy. The indication for warfarin therapy in a child has an effect on warfarin dose requirement that is independent from the target INR range. Children who are anticoagulated following a Fontan procedure (which is a form of palliative heart surgery for a severe congenital heart defect) have a significantly lower warfarin dose requirement than those who are anticoagulated for other cardiac indications (Streif et al., 1999). The mechanism for this has not been explored but may be related to liver dysfunction and cholestasis both of which reduce the availability of the vitamin K-dependant coagulation factors (Kaulitz et al., 1997).

1.3.3.4 Medication. Medications that are known to alter the anticoagulant response to warfarin in adults affect the warfarin dose requirement in children in a similar way (Hirsh, 1991). The effects of corticosteroids, phenobarbital, carbamazepine and antibiotics have been specifically confirmed by cohort studies in children anticoagulated with warfarin. Corticosteroids and most of the commonly used antibiotics reduce warfarin dose requirement and raise INR while phenobarbital and carbamazepine increase dose requirement and lower

INR (Streif et al., 1999, Johnson et al., 2005, Ruud et al., 2008). The pharmacokinetic and pharmacodynamic mechanisms for these interactions have been extensively described in adults (Wells et al., 1994).

1.3.3.5 Genetic polymorphisms. There have been three small cohort studies evaluating the influence of polymorphisms in the genes that mediate the pharmacological action and disposition of warfarin on dose requirement in children and these are described in Section 1.4.5 'Polymorphisms in the genes for *CYP2C9* and *VKORC1* in children' below.

1.4 Dietary vitamin K status and response to warfarin

The role of vitamin K in determining the anticoagulant response to warfarin is shown in Section 1.1.1 'Mechanism of action' and Figure 1-1 above. The anticoagulant effect of warfarin, and the other coumarin derivatives, can be antagonised by vitamin K. The administration of supra-physiological doses of vitamin K, either orally, intramuscularly or intravenously is common practice in the reversal of over-anticoagulation with warfarin (Hanley, 2004).

In adults, several studies have confirmed the impact of dietary vitamin K on warfarin dose requirement and the stability of anticoagulant control. These studies have confirmed an inverse relationship between warfarin maintenance dose requirement and dietary vitamin K intake (Khan et al., 2004, Franco et al., 2004), the contribution of low dietary vitamin K intake to poor stability of anticoagulant control (Sconce et al., 2005a) and the positive impact of dietary supplementation with daily low dose vitamin K on the stability of anticoagulant control in previously unstable adult patients (Sconce et al., 2007). None of these studies have been repeated in a paediatric population.

1.5 Pharmacogenetic factors and response to warfarin

In adults genetic polymorphisms in the genes that mediate the pharmacological action and disposition of warfarin have been demonstrated to make significant contributions to warfarin dose requirement.

1.5.1 *Polymorphisms in the gene for CYP2C9*

The cytochrome P450 2C9 *(CYP2C9)* is a liver enzyme required for the oxidative metabolism of the S-enantiomer of warfarin. A number of genetic polymorphisms have been described within the *CYP2C9* locus (Lee et al., 2002).

The two most common variants are *CYP2C9**2 (R144C) and *CYP2C9**3 (I359L) and approximately 35% of Caucasians carry at least one of these variant alleles, although they occur less frequently in some of the other ethnic groups (Sullivan-Klose et al., 1996, Rettie et al., 1994). These variant alleles encode enzymes that have 12% (*CYP2C9**2) and 5% (*CYP2C9**3) of the activity of the wild-type genotype *CYP2C9**1 (Crespi and Miller, 1997, Takanashi et al., 2000). The presence of one or both of these alleles results in impaired hydroxylation of S-warfarin *in vitro* (Sullivan-Klose et al., 1996, Lee et al., 2002, Rettie et al., 1994) resulting in increased sensitivity to warfarin within the individual.

The influence of *CYP2C9* polymorphisms on warfarin dose requirement and the risk of adverse events has been extensively studied. In one of the earliest of these studies, Aithal et al investigated a cohort of anticoagulated patients with a low warfarin dose requirement and found that their odds ratio of having one of the variant *CYP2C9* alleles compared to the general population was 6.21 (95% confidence interval (CI) 2.48-15.6). In addition, they were more likely to have had an INR > 4.0 during induction therapy and they were four times more likely to have had major bleeding complications than an unselected cohort of anticoagulated patients (Aithal et al., 1999). A study of 121 patients anticoagulated with warfarin showed that warfarin dose requirements were highest in those individuals who were homozygous wild-type for CYP2C9 (*1/*1), lower for those who were heterozygous *1/*2, and were lowest for those who were heterozygous *1/*3. This study showed that individuals with variant alleles had a significantly reduced clearance of S-warfarin, but not of R-warfarin, therefore identifying the *in vivo* mechanism of this effect (Kamali et al., 2004).

Several further studies, in a range of ethnic groups, have confirmed that patients with variant alleles of *CYP2C9* have a lower warfarin dose requirement (Ogg et al., 1999, Margaglione et al., 2000, Taube et al., 2000, Higashi et al.,

2002, Scordo et al., 2002, Gage et al., 2004, Joffe et al., 2004, Peyvandi et al., 2004, Wadelius et al., 2004) and have a greater risk of supratherapeutic INR (Taube et al., 2000, Higashi et al., 2002, Joffe et al., 2004, Peyvandi et al., 2004) and bleeding (Ogg et al., 1999, Margaglione et al., 2000, Higashi et al., 2002) during the initiation phase of therapy.

1.5.2 *Polymorphisms in the gene for VKORC1*

The gene encoding vitamin K epoxide reductase (VKOR), the target enzyme for the coumarins, was identified on human chromosome 16 in 2004 (Li et al., 2004). The anticoagulant effect of warfarin and the other coumarins is due to inhibition of this enzyme which results in a failure to regenerate the reduced form of vitamin K (vitamin K hydroquinone) from vitamin K 2,3-epoxide in the vitamin K cycle (see Section 1.1.1 'Mechanism of action' and Figure 1-1). Vitamin K hydroquinone is an essential co-factor for the post-ribosomal activation (γ-carboxylation) of the vitamin K-dependent coagulation factors (II, VII, IX and X) without which they are unable to bind calcium and become active in the coagulation cascade.

The vitamin K epoxide reductase complex subunit 1 (*VKORC1*) gene is one component of the VKOR complex which encodes a small trans-membrane protein of the endoplasmic reticulum. Missense mutations within the *VKORC1* gene resulting in increased activity of VKOR have been shown to be associated with warfarin resistance and rarer mutations are associated with inherited deficiencies of the vitamin K-dependent coagulation factors (Rost et al., 2004, Bodin et al., 2008). More recently, a number of common polymorphisms in non-coding sequences of the *VKORC1* gene have been identified. One of these, C1173T in intron 1 (shown to be in complete linkage disequilibrium with -1639G > A within the 3' untranslated region of the gene), is associated with a lower warfarin dose requirement. This was first described by D'Andrea *et al* (D'Andrea et al., 2005) and confirmed in several subsequent studies as accounting for 15-30% of variance in warfarin dose between individuals (Bodin et al., 2005, Wadelius et al., 2005, Rieder et al., 2005).

1.5.3 *Polymorphisms in other genes*

Cytochrome P450 *4F2 (CYP4F2*) is a vitamin K1 (VK1) oxidase enzyme. Carriers of the V433M polymorphism (rs2108622:C>T nucleotide substitution) have lower hepatic concentrations of the enzyme, resulting in a reduced capacity to metabolise VK1. Elevated hepatic levels VK1 are thought to render these individuals less sensitive to the anticoagulant effects of warfarin (McDonald et al., 2009). This effect was first described by Caldwell et al (Caldwell et al., 2008) who identified an association between warfarin dose and presence of the *CYP4F2* variant allele (V433M: rs2108622) in 3 independent cohorts (total n=1051) of White patients. The presence of a variant allele resulted in a significantly higher warfarin dose requirement, patients who were homozygous TT requiring 1mg/day more warfarin than those with genotype CC, the heterozygous patients having an intermediate dose requirement (equating to a 4-12% increase in warfarin dose per T allele). This effect was consistent when *VKORC1* and *CYP2C9* genotypes were accounted for (Caldwell et al., 2008). Frequency of the *CYP4F2* variant allele varies between ethnic groups explaining why these findings have been replicated in studies of White and Asian populations in whom 30% carry a variant allele (Cen et al., 2010, Singh et al., 2011, Carlquist et al., 2010, Perez-Andreu et al., 2009, Borgiani et al., 2009) but not confirmed in African-Americans in whom only 7% carry a variant allele (Scott et al., 2010, Perini et al., 2010).

Apolipoprotein E (APOE) mediates the uptake of vitamin K-rich lipoproteins from the circulation into liver cells. There are six APOE variants, encoded by the alleles ε2, ε3 and ε4. The ε2, ε3 and ε4 alleles differ according to their amino acid sequence at two sites, which are cysteine/cysteine, cysteine/arginine, and arginine/arginine, respectively (Weisgraber et al., 1982). Individuals who carry the APOE ε4 allele clear the vitamin K-rich lipoproteins from the circulation more efficiently than those who do not and the increased availability of vitamin K in the liver is thought to result in a relative resistance to the anticoagulant effect of warfarin (Kohlmeier et al., 1996). A few studies have shown that APOE genotype influences warfarin dose requirement, adults carrying one or more ε4 alleles requiring a higher warfarin maintenance dose than those without (Kimmel et al., 2008, Kohnke et al., 2005). However, APOE genotype was shown to have a relatively minor contribution to variability in warfarin dose (6%)

(Kohnke et al., 2005) and its effect has not been demonstrated in populations that have a low frequency of ε4 allele (Lal et al., 2008).

Three additional cytochrome P450 enzymes, *CYP1A2*, *CYP3A4* and *CYP2C19*, are responsible for the metabolism of the R-enantiomer of warfarin. A SNP in intron 1 of *CYP1A2* (C163A; rs762551) has been identified and has been associated with increased enzyme activity (Sachse et al., 1999), although this high inducibility has only been demonstrated amongst tobacco smokers and its effect on warfarin dose requirement has not been examined. *CYP3A4* is a major contributor to R-warfarin metabolism. There is significant inter-individual variability in enzyme levels and several polymorphisms of the gene have been identified. Heterozygotes for the CYP3A4*1G polymorphism (rs2242480) have a higher clearance of R-warfarin in vitro (Lane et al., 2011) but neither the effect of this polymorphism, nor any of the others, on warfarin dose requirement has been examined. *CYP3A5* is an enzyme that has similar substrate specificities to *CYP3A4*. Various polymorphisms encode active and inactive *CYP3A5* enzyme. The *CYP3A5**3 allele (A6986G; rs776746) is the commonest cause of low *CYP3A5* activity in Caucasians but has not been shown to influence warfarin dosing. This may be related to the low frequency of variant alleles in the population (Wadelius et al., 2004).

There are a number of other genes that express mediators involved in the vitamin K cycle and the metabolism of warfarin, including calumenin (CALU) (Gonzalez-Conejero et al., 2007, Hatch et al., 2007), microsomal epoxide hydrolase (EPHX1) (Vecsler et al., 2006, Hatch et al., 2007), gamma-glutamyl carboxylase (GGCX) (Rieder et al., 2007) and protein C (PROC) (Hatch et al.). Allelic variants in these genes have been shown to make minimal or no contribution to warfarin dose requirement in adult populations (Jorgensen et al., 2009). Genome-wide association studies have also failed to identify genetic factors of significant importance other than polymorphisms in the *CYP2C9*, *VKORC1* and *CYP4F2* genes (Cooper et al., 2008, Takeuchi et al., 2009). However, the effect of polymorphisms in these other genes on warfarin dose in the paediatric population may differ.

1.5.4 *Warfarin-dosing algorithms*

Further studies, both retrospective and prospective, have shown that patient genotype for the genes encoding *CYP2C9* and *VKORC1*, age and height collectively account for 55-60% of the variability in warfarin dose requirement in adults. This has informed the development of personalised warfarin-dosing algorithms for estimating maintenance dose (Sconce et al., 2005b, Wadelius et al., 2009, Millican et al., 2007). There are substantial interethnic differences between *CYP2C9* and *VKORC1* genotype frequencies and more recently the International Warfarin Pharmacogenetics Consortium (IWPC) has addressed this by developing an algorithm for estimating warfarin dose based on clinical and genetic data from a total of 4043 patients from 21 centres on four continents (Asia, Europe, North America and South America) (International Warfarin Pharmacogenetics Consortium, 2009). In a validation cohort of 1009 patients the pharmacogenetics-based algorithm was more accurate in identifying those individuals who required low or high doses of warfarin to achieve target INR than an existing clinical algorithm (International Warfarin Pharmacogenetics Consortium, 2009).

Since commercial platforms for rapid genotyping of the polymorphisms relevant to warfarin dosing have been made available (King et al., 2008) it has been feasible to study prospectively the role of pharmacogenetics-based warfarin-dosing algorithms in patients undergoing initiation of warfarin therapy. An early study evaluated an algorithm that incorporated *CYP2C9* genotype in 48 orthopaedic patients. Although patients with a *CYP2C9* variant achieved stable warfarin dosing without excessive delay, they continued to be at increased risk of over-anticoagulation (INR > 4.0) (Voora et al., 2005). A further study by Caraco *et al* examined whether prior knowledge of *CYP2C9* genotype improved outcomes of warfarin therapy in 191 patients. Those treated according to a *CYP2C9* genotype-adjusted algorithm reached therapeutic INR and stable warfarin dosing earlier, spent more time in therapeutic range and experienced less minor bleeding than those treated according to an empirical algorithm (Caraco et al., 2008). The addition of *VKORC1* genotype to a warfarin-dosing model at initiation of warfarin therapy in 187 elderly inpatients showed that, although *VKORC1* genotype was the best predictor of warfarin maintenance dose, the contribution of *VKORC1* and *CYP2C9* to prediction of induction doses

was negligible compared to INR responses measured during the first week of treatment (Moreau et al., 2011). A further randomised controlled trial of a dosing algorithm that incorporated *CYP2C9*, *VKORC1* and *CYP4F2* genotypes in 230 patients showed that genotype-informed dosing improved prediction of therapeutic dose but did not improve time in therapeutic range. Incidence of INR > 4.0 and adverse events were similar for both of the study arms (Burmester et al., 2011). Recent evaluation of a pharmacogenetics-based initiation protocol that incorporated doses based on *VKORC1/CYP2C9* genotype, clinical variables and response in 167 patients during the first 90 days of warfarin therapy resulted in a negligible influence of genotype on risk of over-anticoagulation, time to stable anticoagulation and time spent within therapeutic range (Gong et al., 2011). More recently, the CoumaGen-II study showed that pharmacogenetic-based warfarin-dosing resulted in fewer INRs that were out of range and an earlier achievement of therapeutic INR when compared with a standard empiric dosing protocol (Anderson et al., 2012).

These studies confirm that a pharmacogenetics-guided approach to warfarin initiation is feasible and safe but do not clearly demonstrate a benefit in terms of improvement in anticoagulation control or a reduction in adverse events, an issue that is being addressed by ongoing trials (van Schie et al., 2009, French et al., 2010).

1.5.5 *Polymorphisms in the genes for CYP2C9 and VKORC1 in children*

There have been three small studies to investigate the influence of genetic polymorphisms on warfarin dose requirement in children. A study of 29 children with cancer, treated with low dose warfarin therapy (target INR range: 1.3-1.9), showed that those with a variant *CYP2C9**2 or *3 allele (n=8) achieved target INR sooner and were more likely to have a supratherapeutic INR than children with wild-type *CYP2C9**1 (n=21). Warfarin dose requirements did not differ significantly between children who were heterozygous for *CYP2C9**2 or *3 as compared to those without a variant allele but was significantly lower in the one child who was compound heterozygous for both alleles. There was no adjustment for variables such as age and the number of evaluated patients was small (Ruud et al., 2008).

A later study of 60 children anticoagulated with oral vitamin K antagonists, 34 who were anticoagulated with warfarin and 26 with phenprocoumon, examined the effect of polymorphisms in the *CYP2C9* and *VKORC1* genes on warfarin dose requirement. Carriers of *VKORC1* AA (1639G>A) genotype required significantly lower daily doses than GG or GA genotypes but there was no association between warfarin dose requirement and any mutation in the *CYP2C9* gene. Age was found to be a greater determinant than either of the genetic factors (Nowak-Göttl et al., 2009). The analysis did not consider the difference in dosing between warfarin and phenprocoumon, phenprocoumon doses generally being 2.4 times higher than warfarin doses to achieve the same anticoagulant effect (Van Leeuwen et al., 2008).

A further study identified age and *VKORC1* genotype as the major factors influencing warfarin dose requirement in 48 Japanese children anticoagulated with warfarin. Children with *VKORC1* -1173TT genotype had a 28% lower warfarin dose requirement that those with -1173CT or -1173CC genotype, a difference which was statistically significant. It was not possible to evaluate the effect of *CYP2C9* genotype as only one child possessed a variant *CYP2C9* allele (Kato et al., 2011).

The influence of polymorphisms in other genes has not been studied in a paediatric population and a pharmacogenetics-based warfarin-dosing algorithm has not previously been developed or validated.

1.6 Aims of the research

The aim of this MD is to gain a greater understanding of the factors that affect warfarin anticoagulant control and response in children.

This includes the following studies:

1. A study of anticoagulation control in a cohort of children on chronic anticoagulant therapy with warfarin;
2. A study of the clinical and pharmacogenetic factors affecting inter-individual variability in response to warfarin in children, with particular focus on:
    - The impact of polymorphisms in key genes mediating sensitivity to and metabolism of warfarin on maintenance warfarin dose and outcomes during warfarin initiation;
    - The ability to predict maintenance warfarin dose in children using a dosing algorithm based on clinical and pharmacogenetic factors.

Chapter 2. Anticoagulation control in a cohort of children on chronic therapy with warfarin

2.1 Introduction

Anticoagulant therapy is becoming increasingly used in childhood and warfarin remains the most frequent agent for long-term anticoagulation in this patient group.

Maintenance of anticoagulation with warfarin within the target therapeutic range (TTR) is essential in order to prevent the haemorrhagic complications of over-anticoagulation and the reduction in efficacy seen with under-anticoagulation.

Historically, children have had poor anticoagulant control (Streif et al., 1999) although there has been some improvement with the use of point-of-care (POC) testing devices and patient/parent education programmes (Newall et al., 2004, Bradbury et al., 2008, Newall et al., 2006, Bhat et al., 2010).

Anticoagulation control in children is usually reported as the 'percentage of INR measurements within therapeutic range' (%ITTR). However, with the increasing use of POC testing devices, allowing INR testing to occur more frequently during periods of instability, %ITTR may not accurately reflect the quality of anticoagulation control.

2.2 Aims

The aims of this study were to:

1. Examine anticoagulation control in a cohort of children on chronic therapy with warfarin, monitored at home using a point-of-care device;
2. Identify the factors that are responsible for deviations from target therapeutic range;
3. Compare two measures of quality of anticoagulation control, 'percentage of INR measurements within therapeutic range' (%ITTR) and 'percentage time within therapeutic INR range' (%TIR).

2.3 Methods

2.3.1 *Patient selection and data collection*

Children who had been anticoagulated with warfarin under the care of paediatric cardiology services at Freeman Hospital, Newcastle upon Tyne were identified using a hospital registry. Anticoagulant records and hospital notes for these children were examined. Data were collected from 3 months after the start of anticoagulant therapy. Data collected included: age; gender; weight; indication for anticoagulant therapy; target INR range; warfarin dose; INR values; frequency of INR tests; frequency of warfarin dose changes; deviation from target INR range; factors contributing to deviation from target INR range; management of over- and under-anticoagulation events; and, occurrence of haemorrhagic and thrombotic events. An over-anticoagulation event was defined as an INR > 4.0 and an under-anticoagulation event was defined as an INR > 0.5 below target INR range. A major haemorrhagic event was defined as; (i) fatal bleeding, and/or; (ii) symptomatic bleeding in a critical area or organ, such as intracranial, intraspinal, intraocular, retroperitoneal, intra-articular or pericardial, or intramuscular with compartment syndrome, and/or; (iii) bleeding causing a fall in haemoglobin level of 2g/dL or more, or leading to transfusion of two or more units of whole blood or red cells (Schulman and Kearon, 2005). All other haemorrhagic events were defined as minor.

2.3.2 *Calculation of 'percentage time within therapeutic INR range'*

Data were inputted into 4S Dawn Clinical Software in order to calculate %TIR using the linear interpolation methodology described previously by Rosendaal et al (Rosendaal et al., 1993). The average %ITTR and %TIR was determined for each patient for the duration of their anticoagulant monitoring.

2.3.3 *Statistical analysis*

Advice from a statistician was taken prior to data analysis. Children were divided into two subgroups according to target INR range: 2.0-3.0; and 2.5-3.5, and three subgroups according to the indication for anticoagulant therapy: Fontan circulation; other cardiac indication; and non-cardiac indication. Data from each patient were divided according to their age (in years) at the time of the INR measurement. These data were then combined into age cohorts: ≤ 1 year; 2-5 years; 6-12 years; and 13-17 years, prior to statistical analysis.

Treatment outcome variables were compared between children with different target INR range, different indication for anticoagulant therapy, and between children within each age group. The percent of INR measurements that were within, above and below TTR were compared using t-test or one-way analysis of variance (ANOVA) as the data were approximately normally distributed within groups. Warfarin dose per kg body weight and mean number of dose changes per month were compared following logarithmic transformation to achieve approximate normality within groups. Mean number of INR measurements per month were compared using Mann-Whitney test or Kruskall-Wallis test. Correction for age was done using regression analysis. Correlation of variables with actual age was studied using Pearson correlation coefficient. %ITTR was compared to %TIR using a paired t-test and the differences between %ITTR and %TIR were compared between subgroups. Advice from a statistician was taken prior to data analysis. Statistical analysis was performed using Minitab version 15.0 (Coventry, UK). *P* values < 0.05 were considered significant.

2.4 Results

2.4.1 *Patient characteristics*

Anticoagulation records for 38 consecutive children (21 males) anticoagulated with warfarin between January 1996 and April 2009 were reviewed (Table 2-1). All children were monitored using a point-of-care device (CoaguChek®S or CoaguChek®XS) and their warfarin therapy managed by two paediatric cardiology nurse specialists who were contacted with the INR results by the parent/carer by telephone. The INR test frequency was directed by the nurse specialists although some parents/carers carried out additional tests if they were concerned, e.g. during episodes of illness. Median age was 8.3 years (range: 1.1-17.2 years). The most frequent indications for anticoagulant therapy were: Fontan procedure, 16 patients; prosthetic mitral valve replacement, 8 patients; and, primary pulmonary hypertension, 4 patients (Table 2-1). 29 patients had target INR range 2.0-3.0 and 9 patients had target INR range 2.5-3.5. Median duration of anticoagulant therapy was 29 months (range: 2-115 months) and data were collected for a total of 112 patient years of anticoagulant therapy with warfarin.

Table 2-1. Patient characteristics

Total number of children	38
Age¶: median (range), yrs	8.3 (1.1-17.2)
Sex, no. of children	
- Male	21
- Female	17
Indication for warfarin therapy, no. of children	
- Fontan procedure	16
- Prosthetic mitral valve replacement	8
- Primary pulmonary hypertension	4
- Prosthetic aortic valve replacement	4
- Cardiomyopathy	3
- Other§	3
Target INR range, no. of children	
- 2.0-3.0	29
- 2.5-3.5	9
Duration of warfarin therapy: median (range), months	29 (2-115)

¶at start of data collection period
§giant coronary aneurysm, 1; thrombotic stroke, 1; truncus arteriosus repair, 1

Table 2-2. Treatment outcome variables according to age, target INR range and indication in children anticoagulated with warfarin

	INR measurements within TTR, mean % (range)	INR measurements below TTR, mean % (range)	INR measurements above TTR, mean % (range)	Dose to maintain TTR, mg/kg/day (range)	Mean number of tests per month (range)	Mean number of dose changes per month (range)
Age						
- **≤1 year** (n=2)	63.4 (43.5,83.3)	7.6 (0,15.2)	27.8 (0,41.3)	0.24 (0.19,0.296)	7.6 (5.1, 10)	2.4 (0.5-4.3)
- **2-5 years** (n=23)	60.6 (30.3-100)	28.5 (0-66.7)	10.9 (0-38.5)	0.15 (0.07-0.30)	4.4 (1- 15.3)	2.5 (0-11.7)
- **6-12 years** (n=71)	61.1 (28.6-100)	28.1 (0-71.4)	10.1 (0-50)	0.11 (0.03-0.22)	2.6 (0.5-16)	0.6 (0-4)
- **13-17 years** (n=47)	56.2 (0-100)	30.2 (0-100)	13.5 (0-57.1)	0.089 (0.03-0.18)	2.8 (0.9-15.7)	1.4 (0-9.7)
***P* value**	0.645¶	0.441¶	0.108¶	**<0.001¶**	**0.009§**	0.082¶
Target range						
- **2.0-3.0** (n=29)	59.1 (30.3-81.3)	29 (0-66.7)	12.3 (0-34.5)	0.12 (0.05-0.3)	3.5 (1-10)	1.5 (0.1-9.7)
- **2.5-3.5** (n=9)	51.7 (37.5-63.6)	32.2 (18.2-46.7)	16.1 (0-27.3)	0.14 (0.07-0.22)	4.4 (1.7-13)	2.4 (0.6-9.6)
***P* value**	0.052†	0.554†	0.30†	0.419†	0.264‡	0.175†
Indication						
- **Fontan procedure** (n=16)	56.7 (30.3-81.3)	34.5 (6.3-66.7)	9 (0-24.3)	0.13 (0.05-0.21)	2.8 (1.6-6.6)	1.3 (0.1-5.2)
- **Other cardiac** (n=17)	55.2 (36.4-72.2)	27 (9.5-46.7)	17.7 (0-34.5)	0.12 (0.05-0.22)	4.5 (1.6-15.7)	2.4 (0.4-9.7)
- **Non-cardiac** (n=5)	66.6 (54.3-85.7)	17.6 (0-40)	11.1 (0-17.4)	0.13 (0.06-0.30)	4.3 (1-10)	0.9 (0.4-1.9)
***P* value**	0.212¶	0.182¶	**0.023¶**	0.871¶	0.289§	0.150¶
All patients	57.4 (30.3-85.7)	29.7 (0-66.7)	13.2 (0-34.5)	0.126 (0.03-0.30)	3.8 (0.5-16)	1.7 (0-11.7)

¶one-way ANOVA; §Kruskall-Wallis test; †t-test; ‡Mann-Whitney test

The following variables were logarithmically transformed prior to analysis using one-way ANOVA: dose to maintain TTR, mg/kg/day; mean number of dose changes per month

2.4.2 *Treatment outcome variables*

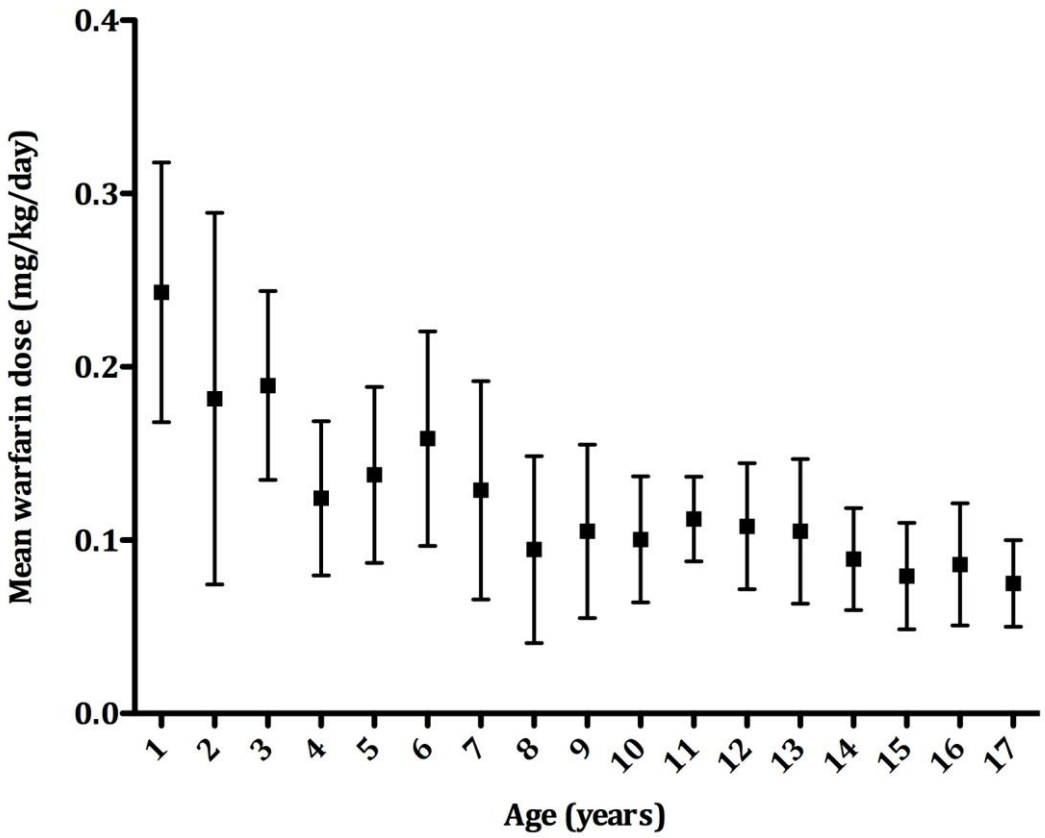
Table 2-2 shows treatment outcome variables according to age, target INR range and indication for anticoagulation. A mean of 57.4% of INR measurements were within TTR, 29.7% were below and 13.2% were above it. INR measurements were more likely to be below TTR than above TTR ($P < 0.0001$, t test). Mean warfarin maintenance dose requirement was 0.13 mg/kg/day (range: 0.03-0.30 mg/kg/day). The mean number of INR tests per month was 3.8 (range: 0.5-16) and the mean number of warfarin dose changes per month was 1.7 (range: 0-11.7).

2.4.2.1 The effect of age on treatment outcome variables. There was no difference in the % of INR measurements that were within TTR between the different age groups (Table 2-2). Children ≤ 1 year of age tended to be more likely to have an INR above TTR than below TTR than older children but the numbers were small. The mean warfarin maintenance dose requirement varied according to age and was highest in the youngest age groups, $P < 0.001$, one-way ANOVA (Table 2-2). Figure 2-1 shows the effect of age on mean warfarin maintenance dose.

Children ≤ 1 year of age had a higher number of INR tests per month (mean: 7.6) than children 2-5 years of age (mean: 4.4) and older children/adolescents (6-12 years, mean: 2.6; 13-17 years, mean: 2.8), $P = 0.009$, Kruskall-Wallis test. Younger children also had a higher number of warfarin dose changes per month (≤1 year, mean: 2.4; 2-5 years, mean: 2.5) than older children/adolescents (6-12 years, mean: 0.6; 13-17 years, mean: 1.4), $P = 0.03$, Pearson correlation test.

Figure 2-1. The effect of age on mean warfarin dose in children anticoagulated with warfarin

Markers indicate the mean values. Vertical lines above and below the markers represent the standard deviations.



2.4.2.2 The effect of target INR range on treatment outcome variables. Children with target INR range 2.0-3.0 had a higher proportion of INRs within TTR than children with target INR range 2.5-3.5 (59.1% vs. 51.7%, respectively). INR values were more likely to be below TTR than above TTR within both groups. There was a difference in age-adjusted warfarin dose requirement between children with target INR 2.0-3.0 and those with target INR 2.5-3.5 (0.14mg/kg vs. 0.17mg/kg, $P < 0.0001$, one-way ANOVA). Children with target INR 2.5-3.5 had a greater number of INR tests per month (mean: 4.4 vs. 3.5) and more warfarin dose changes per month (mean: 2.4 vs. 1.5) than children with target INR 2.0-3.0 (Table 2-2).

2.4.2.3 The effect of indication for anticoagulation on treatment outcome variables. Children anticoagulated with warfarin for non-cardiac reasons (n=5: primary pulmonary hypertension, 4; thrombotic stroke, 1) had a greater proportion of INR values within TTR (mean: 66.6%) than children anticoagulated with warfarin for Fontan procedure (n=16, mean: 56.7%) or other cardiac reasons (n=17, mean: 55.2%). INR values were more likely to be below TTR than above TTR for all groups, particularly for those anticoagulated for Fontan procedure, $P = 0.059$, one-way ANOVA. Children who were anticoagulated for other cardiac reasons were more likely to have an INR that was above TTR (mean: 17.7%) than children who were anticoagulated for Fontan procedure (mean: 9%) or for non-cardiac reasons (mean: 11.1%), $P = 0.023$, one-way ANOVA. There was no difference in mean warfarin maintenance dose requirement between children who were anticoagulated for Fontan procedure (0.13 mg/kg/day), other cardiac reasons (0.12 mg/kg/day) or non-cardiac reasons (0.13 mg/kg/day), even when adjusted for the effect of age. Children who were anticoagulated for Fontan procedure had fewer INR tests per month (mean: 2.8) than those with other cardiac indications (mean: 4.5) or a non-cardiac indication (mean: 4.3) (Table 2-2).

2.4.3 *Episodes of over-anticoagulation*

INR > 4.0 occurred 99 times in 73.7% of children (Table 2-3), i.e. at a rate of 0.88 episodes per patient year of warfarin therapy. Over-anticoagulation with INR > 4.0 was most frequent in children anticoagulated for stroke, truncus arteriosus repair or prosthetic mitral valve replacement and in children

anticoagulated with target INR range 2.5-3.5. INR > 4.0 occurred most frequently in younger children (≤ 5 years of age). The most frequent action taken was to reduce the dose of warfarin (51.5% of events). Vitamin K was administered in 2/99 events, warfarin was omitted in 37/99 events and 9 had no change made to their therapy. INR was tested the following day in the majority of cases (52/99; 52.5% of events) and INR was within TTR after 1-2 days in 42.4% of cases, and within one week in 67.7% of cases. The most frequently reported reasons for over-anticoagulation were antibiotic therapy, illness (diarrhoea/vomiting or fever) and change in regular medication. There were no haemorrhagic events in relation to episodes of over-anticoagulation with INR > 4.0.

Table 2-3. Episodes of over-anticoagulation (INR > 4.0) in children anticoagulated with warfarin

	Number of events	Number of events/Total number of measurements (%)
Total number of over-anticoagulation episodes	99	99/2685 (3.7%)
Indication for anticoagulation		
- Prosthetic mitral valve replacement (n=7)	63	63/1168 (5.4%)
- Fontan procedure (n=9)	9	9/800 (1.1%)
- Stroke (n=2)	6	6/35 (17.1%)
- Truncus arteriosus repair (n=1)	6	6/79 (7.6%)
- Cardiomyopathy (n=3)	6	6/232 (2.6%)
- Primary pulmonary hypertension (n=3)	4	4/179 (2.2%)
- Prosthetic aortic valve replacement (n=3)	1	1/153 (0.7%)
Age		
- ≤ 1 year	4	4/82 (4.9%)
- 2-5 years	39	39/767 (5.1%)
- 6-12 years	27	27/990 (2.7%)
- 13-17 years	29	29/846 (3.4%)
Target INR range		
- 2.0-3.0 (n=14)	35	35/1506 (2.3%)
- 2.5-3.5 (n=7)	64	64/1179 (5.4%)
Action taken		
- No change made	9	
- Dose of warfarin reduced	51	
- Warfarin omitted	37	
- Oral vitamin K given	2	
Time to next INR		
- 1 day	22	
- 2 days	20	
- 3-7 days	25	
- >7 days	32	
Time to next INR within TTR		
- 1 day	22	
- 2 days	20	
- 3-7 days	25	
- >7 days	23	
- Not available	9	
Cause of high INR		
- Antibiotic therapy	9	
- Illness¶	4	
- Change in regular medication	3	
- Took higher dose by mistake	1	
- Alcohol	1	
- Not available	81	

¶Diarrhoea and vomiting, 2; fever, 2

2.4.4 *Episodes of under-anticoagulation*

INR was > 0.5 unit below target range 125 times in 71.1% of children (Table 2-4), i.e. at a rate of 1.04 episodes per patient year of therapy. This accounted for 4.7% of the total of 2685 INR measurements carried out during the intervening 13 years. Under-anticoagulation occurred most frequently in children anticoagulated for primary pulmonary hypertension or a prosthetic mitral valve replacement and in children anticoagulated with target INR range 2.5-3.5. The frequency of under-anticoagulation events did not vary with age. The most frequent action taken was increasing the dose of warfarin (73/125; 58.4% of events). Low molecular weight heparin was administered in 9/125 events (prosthetic mitral valve replacement, 7; prosthetic aortic valve replacement, 1; stroke, 1). INR was tested within 7 days in 77.7% of cases and INR was within TTR in that time period in 50.9% of cases. The most frequently reported causes of under-anticoagulation were doses missed by mistake, rebound after dose omission/reduction for over-anticoagulation and doses missed for a planned procedure. There were no thrombotic events in relation to episodes of under-anticoagulation with INR >0.5 unit below target range.

Table 2-4. Episodes of under-anticoagulation (INR >0.5 unit below lower limit of target INR range) in children anticoagulated with warfarin

	Number of events	Number of events/Total number of measurements (%)
Total number of under-anticoagulation episodes	125	125/2685 (4.7%)
Indication for anticoagulation		
- Prosthetic mitral valve replacement (n=8)	70	70/1168 (6.0%)
- Fontan procedure (n=9)	24	24/800 (3.0%)
- Primary pulmonary hypertension (n=4)	15	15/179 (8.4%)
- Prosthetic aortic valve replacement (n=2)	8	8/153 (5.2%)
- Cardiomyopathy (n=1)	3	3/232 (1.3%)
- Stroke (n=1)	2	2/35 (5.7%)
- Coronary aneurysm (n=1)	2	2/39 (5.1%)
- Truncus arteriosus repair (n=1)	1	1/79 (1.3%)
Age		
- ≤ 1 year	0	0/82 (0%)
- 2-5 years	33	33/767 (4.3%)
- 6-12 years	52	52/990 (5.3%)
- 13-17 years	40	40/846 (4.7%)
Target INR range		
- 2.0-3.0 (n=18)	49	49/1506 (3.3%)
- 2.5-3.5 (n=9)	76	76/1179 (6.4%)
Action taken		
- No change made	43	
- Dose of warfarin increased	73	
- Low molecular weight heparin given	9	
Time to next INR		
- 1 day	39	
- 2 days	15	
- 3-7 days	40	
- >7 days	27	
- Not available	4	
Time to next INR within TTR		
- 1 day	6	
- 2 days	10	
- 3-7 days	42	
- >7 days	56	
- Not available	11	
Cause of low INR		
- Missed dose by mistake	13	
- Rebound after high INR	10	
- Warfarin omitted for planned procedure	8	
- Change in regular medication	6	
- Antibiotic therapy	2	
- Illness¶	1	
- Not available	85	

¶Nature of illness not stated

2.4.5 *Adverse events*

2.4.5.1 Haemorrhagic events. There were no major haemorrhagic events. There were 7 reported minor haemorrhagic events in 6 children: epistaxis, 4; menorrhagia, 1; calf haematoma, 1; haematemesis, 1 (Table 2-5). INR value at the time of haemorrhage was within TTR in 4 cases, below TTR in 2 cases, and above TTR in 1 case. No action was taken in 4 cases, warfarin was omitted in 1, warfarin dose was reduced in 1 and the child with menorrhagia was referred to a family planning clinic and commenced on a progesterone-only contraceptive pill. There was no difference between children who had a haemorrhagic event and those who did not in terms of the percentage of INR measurements that were within, above or below TTR. Haemorrhagic events occurred at a rate of 0.060%, 0.062% and 0.068% per patient year for INRs below, within and above TTR, respectively. There were no episodes of haemorrhage that required admission to hospital and there were no episodes of intracranial haemorrhage.

2.4.5.2 Thrombotic events. There was one reported possible thrombotic event. This occurred in a 15-year-old child who was anticoagulated with a target INR range of 2.5-3.5 for a prosthetic mitral valve replacement. INR was within range (3.5) at the time of presentation with a suspected transient ischaemic attack (TIA). Computed tomography (CT) scan of the head showed evidence of multiple previous ischaemic strokes but the timing of these events could not be determined. No further action was taken.

Table 2-5. Haemorrhagic events reported in children anticoagulated with warfarin

Indication for anticoagulation	Target INR range	Number of haemorrhagic events	Nature of haemorrhagic event(s)	Age at time of event (years)	INR at time of event	Action
Primary pulmonary hypertension	2.0-3.0	1	Epistaxis	7	2.8	None
Fontan circulation	2.0-3.0	1	Epistaxis	5	1.8	None
Prosthetic aortic valve replacement	2.0-3.0	1	Epistaxis	13	3.2	Dose omitted
Prosthetic mitral valve replacement	2.5-3.5	2	Epistaxis Calf haematoma	10 15	3.2 3.2	Dose reduced None
Fontan circulation	2.0-3.0	1	Haematemesis	15	1.8	None
Cardiomyopathy	2.0-3.0	1	Menorrhagia	13	2.1	Referred to family planning clinic-medical therapy

2.4.6 *Comparison of two methods to assess quality of anticoagulation control*

The results of the comparison between two methods of assessing quality of anticoagulation control are shown in Table 2-6 and Figure 2-2. *P* values correspond to the differences between %ITTR and %TIR for the entire cohort and for each of the subgroups. For the entire cohort mean %TIR was higher than the mean %ITTR (63.8% vs. 57.4%; $P = 0.002$). %TIR was higher than %ITTR for each of the two target INR ranges, indication and age cohorts. There were larger differences between %TIR and %ITTR in children with target INR 2.5-3.5 and in those who were anticoagulated for cardiac reasons other than Fontan procedure, the majority of whom had artificial heart valves. These groups of children also had a greater frequency of INR tests and warfarin dose changes per month (Table 2-2) but there was no correlation between the degree of difference between %ITTR and %TIR and the frequency of testing ($r = -0.1$, $P > 0.5$, Pearson correlation coefficient). A difference between %ITTR and %TIR was seen in the older age groups (≥6 years) but not in the younger age groups (<6 years) which may have been due to the smaller numbers of patients.

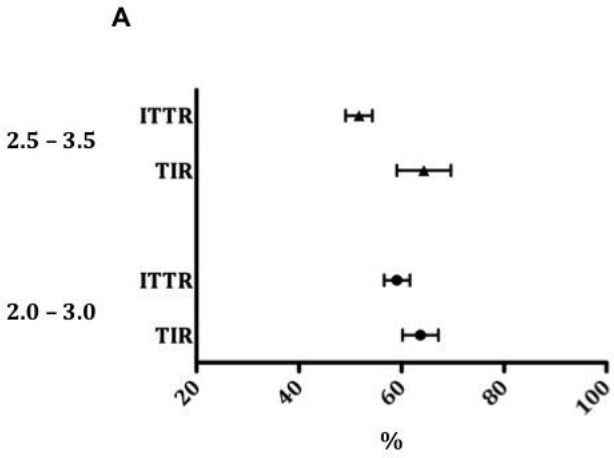
Table 2-6. Comparison of percentage of INR measurements within therapeutic range (%ITTR) with percentage time within therapeutic INR range (%TIR) in children anticoagulated with warfarin

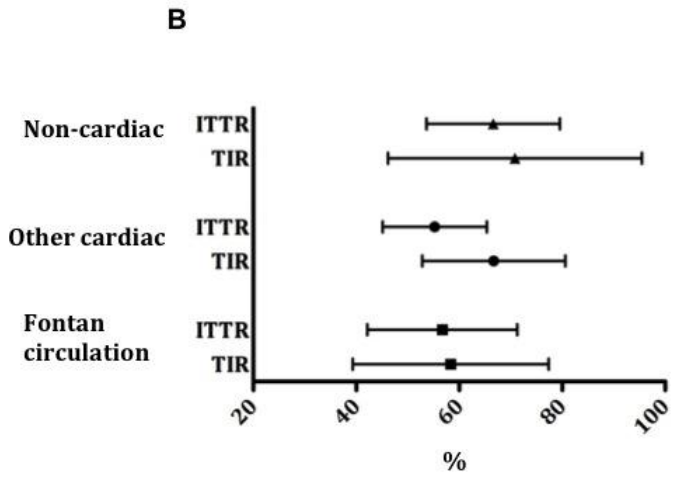
	No. of patients	Duration of monitoring, median number of months (range)	Percentage of INR values within TTR (%ITTR), mean (range)	Percentage time in range (%TIR), mean (range)	*P* value
Target range					
2.0-3.0	29	22 (3.5-90)	59.1 (30.3-81.3)	63.7 (12-95.3)	**0.041**¶
2.5-3.5	9	43 (2-115)	51.7 (37.5-63.6)	64.4 (43.8-94.6)	**0.022**¶
Indication					
Fontan circulation	16	24.5 (5-90)	56.7 (30.3-81.3)	58.3 (12-87.2)	0.512¶
Other cardiac	17	30 (2-115)	55.2 (36.4-72.2)	66.7 (43.8-94.6)	**0.001**¶
Non-cardiac	5	24 (3.5-74)	66.6 (54.3-85.7)	70.8 (38.3-95.3)	0.584¶
Age					
≤1 year	2	6.3 (9, 3.5)	63.4 (43.5,83.3)	79.5 (68.6, 90.3)	0.327¶
2-5 years	23	8.3 (0.5-12)	60.6 (33.3-100)	63.8 (12-100)	0.076¶
6-12 years	71	8 (0.3-12)	61.1 (28.6-100)	67.0 (25.2-100)	**< 0.001**¶
13-17 years	47	10 (1-12)	56.2 (0-100)	65.7 (19.6-100)	**< 0.001**¶
All patients	38	29 (2-115)	57.4 (30.3-85.7)	63.8 (12-100)	**0.002**¶

TIR, Time in therapeutic Range; TTR, target therapeutic range
¶Paired t-test; **§**t-test on percentage TIR minus percentage of INR values within TTR; †One-way ANOVA on percentage TIR minus percentage of INR values within TTR

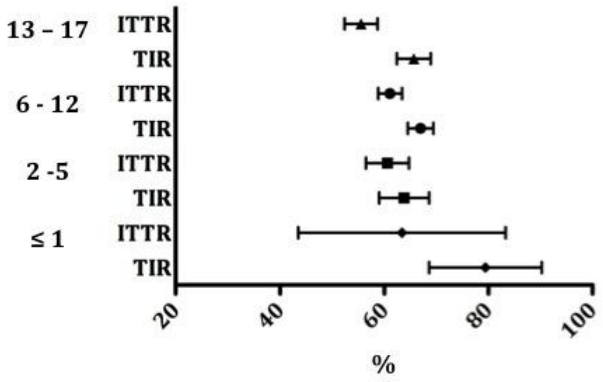
Figure 2-2. Comparison of percentage of INR measurements within therapeutic range (%ITTR) with percentage time within therapeutic INR range (%TIR) according to: A, Target range; B, Indication; C, Age.

Markers indicate the mean values. Horizontal lines to either side of the markers represent the standard deviations.





C



2.5 Discussion

There are a number of factors that contribute to the poor control of oral anticoagulant therapy in children (see Section 1.2.2.1 'Difficulties in anticoagulation control in children' and Table 1-2).

The largest published study of anticoagulated children included 319 children, aged between 1 month and 18 years, followed in a Canadian centre (Streif et al., 1999). Twenty-eight of these children were tested using a POC device at home, the remainder were monitored by attending hospital or outpatient laboratories. The majority of children had underlying cardiac disease. The percentage of INR measurements within target range varied according to age: ≤1 year, 37%; 1-5 years, 45%; 6-12 years, 54%; and, 13-18 years, 53%. INR measurements on average were below target range 32-46% of the time and above target range 13-16% of the time. However, subgroup analysis of the children using a POC device at home showed that 68% of measured INR values were within target range, supporting improved anticoagulant control using this method of monitoring (Streif et al., 1999).

More recent studies in anticoagulated children show that target INR is achieved in 40-75% of measurements (Newall et al., 2004, Bradbury et al., 2008, Newall et al., 2006, Bhat et al., 2010) and that consistently a greater proportion of INR values are below TTR than above TTR (Newall et al., 2004). The overall improvement in control of anticoagulant therapy in children seems to relate to an increasing use of POC testing devices, e.g. CoaguChek®S, which allows testing to occur at a higher frequency during unstable phases of anticoagulant therapy such as might be due to intercurrent illness, antibiotic therapy or a change in regular medication. Further benefits to the patient/parent include reduced trauma of venepuncture, minimal loss of school and workdays, and portability of the device. Comparative studies have shown that there is a good correlation between the INR obtained from using a whole blood testing point-of-care device and that obtained from analysis of citrated blood samples in the laboratory (Marzinotto et al., 2000, Greenway et al., 2009). Recent studies describing the combined use of point-of-care testing and educational programs about warfarin therapy in small cohorts of children and their caregivers show improvements in time in therapeutic range (up to 81.7%) in addition to improved

knowledge about factors affecting warfarin therapy (Newall et al., 2006, Bauman et al., 2009). This is particularly the case for a child-focused educational programme (Bauman et al., 2009).

Our study, which captured data relating to 112 patient years of anticoagulant therapy in children whose anticoagulation was monitored using a point-of-care device, showed that overall only 57.4% of INR values were within TTR, with a greater proportion being below TTR (29.7%) than above it (13.2%). This is likely to reflect a tendency of the physician to have greater concerns about haemorrhagic than thrombotic complications, particularly in children anticoagulated for Fontan procedure who are perceived to be at a relatively low risk of thromboembolism and in whom haemorrhagic complications may be considered less acceptable (Kaulitz et al., 2005). In contrast to the Streif et al study (Streif et al., 1999), which demonstrated that younger children had poorer control than older ones, we found no difference in %TTR in the younger children when compared to the older children and adolescents, which may be due to the greater frequency of testing and dose changes carried out in the younger children leading to improved anticoagulant control. The children with INR target 2.5-3.5 tended to have a lower %TTR than those with INR target 2.0-3.0 despite having a greater number of INR tests and more warfarin dose changes per month. This may be attributed to excessive tinkering with warfarin dose when target INR is close to target INR range. It would therefore be prudent to reserve dose changes only in patients whose INR deviates by 0.3-0.5 units outside the therapeutic range.

Children with a higher target INR range, usually those with artificial heart valves, are likely to be monitored more closely due to a perceived higher risk of thromboembolism. The finding that children anticoagulated for cardiac reasons, other than Fontan circulation, were more likely to have an INR value that was above target range is again likely to relate to the perceived risk of thromboembolism and the tendency that sub-therapeutic INR is avoided in children with artificial heart valves, who accounted for 12 of the 17 children in this group. There were no differences in the warfarin maintenance dose between children with Fontan circulation and those anticoagulated for other reasons, even when allowing for the effect of age. This is in contrast to the

Streif et al study which showed that children with a Fontan circulation had a lower warfarin dose requirement than that in children with other indications (Streif et al., 1999).

It should be noted that treatment outcome variables were compared between the different subgroups using statistical tests and *P* values (Table 2-2). The magnitude of the differences between the groups may have been better represented by stating confidence intervals.

Over-anticoagulation, with an INR > 4.0, occurred at a rate of 0.88 episodes per patient year of warfarin therapy. Over-anticoagulation occurred more frequently in younger children, particularly those with stroke, truncus arteriosus repair or a prosthetic mitral valve replacement, and those with a higher target INR range (2.5-3.5). The risk of bleeding, including intracranial bleeding is significantly increased at INR > 4.0 in anticoagulated adults (Hylek EM, 1994). The routine approach of omitting one or more doses of warfarin or reducing the warfarin dose appears to be a safe and effective way of restoring INR to within the target range: there were no recorded haemorrhagic events as a result of over-anticoagulation and INR was returned to target range within 7 days for the majority of the children. Oral vitamin K was only administered on 3 occasions to reverse high INR. A recent report also showed that management of high INR (≥ 5.0) in non-haemorrhagic children by omission of warfarin, without the administration of vitamin K, was safe and resulted in INR returning to within TTR in 49% after 1 day (Black et al., 2009).

Under-anticoagulation, with an INR < 0.5 below the target INR range, occurred at a rate of 1.04 episodes per patient year of warfarin therapy. This occurred most frequently in children with a prosthetic mitral valve replacement and those anticoagulated with a higher target INR range (2.5-3.5). The routine approach of increasing warfarin dose did not lead to the occurrence of thromboembolic events in our cohort although it took ≥ 3 days for the INR to return to TTR in the majority of children. Low molecular weight heparin was administered to under-anticoagulated children with either a prosthetic heart valve or prior history of stroke.

Serious bleeding has been reported to occur at a frequency ranging from 0.005%- 12.2% per patient year in children receiving warfarin (Massicotte et al., 2003, Newall et al., 2005). There were no major haemorrhagic events seen in our study that reported on 112 patient years of anticoagulant therapy. A gastrointestinal haemorrhage would normally be considered as a major haemorrhagic event. However, the child had only a single episode of haematemesis at home with no evidence of cardiovascular compromise or reduction in haemoglobin level and it was suspected that the reporting of the haematemesis was inaccurate. Minor bleeding occurred at a frequency of 0.063% per patient year but no events were significant enough to require reversal of the anticoagulant effect of warfarin: the bleeding had either stopped by the time of presentation or the INR was not significantly elevated (See Section 1.1.4 'Adverse effects and reversal').

Thromboembolic events in children on warfarin have been reported to occur at a rate of 1.3% per patient year, around half of which are during an episode of under-anticoagulation (Massicotte et al., 2003, Newall et al., 2005). A possible TIA was the only report of a thrombotic event in our study and this occurred at a time when the INR was therapeutic. We can only speculate that the high frequency of under-anticoagulation in children on warfarin may relate to suboptimal dosing due to the responsible physician's concern about the risk of serious bleeding but this practice does place these children at potential risk of thrombotic events.

This study highlights the existent discrepancy between two methods currently used for assessing chronic anticoagulation control in children, the apparent quality of anticoagulation control being significantly better when measured using a linear interpolation method (Rosendaal et al., 1993) than the standard approach of %ITTR. The %TIR method allocates an INR value to each day, including days between INR tests, and is therefore likely to minimise the disproportionate effect of temporary frequent INR testing on the assessment of long-term anticoagulant control. It is likely that %ITTR is a poor measure of control unless INR measurements are taken at regular, predetermined intervals, which is generally not the case for anticoagulated children monitored using a POC testing device. Use of a linear interpolation method reduces the impact of

multiple INR values over a short period of time which are ‘out of range’ and places more emphasis on the longer periods of stability during which the INR is tested less frequently (Schmitt et al., 2003).

Measurement of %TIR, and therefore the actual number of days during which TTR is achieved, may correlate better with adverse clinical events as it would be expected to provide a more accurate indication of the proportion of time that a patient has supra-therapeutic or sub-therapeutic INR and is therefore at risk of haemorrhagic or thromboembolic events. Due to the very low frequency of adverse events in this cohort of children we were unable to evaluate this although a previous study published by Barbui et al found no difference in quality of anticoagulant control as measured by %ITTR or %TIR in a cohort of adult patients who had experienced adverse events (Barbui et al., 1995).

Linear interpolation methodology has some limitations. It can be biased by individual INR values that are far outside of TTR and it assumes that the change in INR over time is linear between each time-point which may not be true. Small departures from target range are considered identical to large departures which may not be correct as a larger deviation from target range is more likely to result in an adverse clinical event. In addition, a more complex calculation is required to determine %TIR whereas the %ITTR is a simple measure of the proportion of the total number of INR values that are within TTR (Samsa and Matchar, 2000).

The cross-section-of-the-files method (Leolinger, 1985) could not be evaluated in this cohort as anticoagulant therapy was recorded over a prolonged period of time,13 years, during which there were no time-points when all of the children were anticoagulated. The use of this method has not yet been evaluated in a paediatric population.

In conclusion, maintenance of anticoagulant control within TTR remains poor in children despite the availability of home monitoring with a point-of-care device and support for warfarin dosing. INR values in children are more likely to be below TTR than above TTR. Age has a major influence on warfarin dose requirement. Despite the frequent occurrence of over- and under-

anticoagulation (in around 5% of INR measurements), haemorrhage related to warfarin occurred infrequently and there were no confirmed thrombotic events. It may be safe to manage over-anticoagulation by omission of warfarin doses. The %ITTR method may underestimate the quality of anticoagulant control in children and the %TIR method may be more appropriate for this patient group. Methods of assessing anticoagulant control in children should be compared in larger numbers of children and should be correlated with adverse clinical outcomes in terms of haemorrhagic and thromboembolic events. Further prospective studies of larger numbers of anticoagulated children are needed in order to confirm these findings.

Chapter 3. Inter-individual variability in response to warfarin in children: Analysis of pharmacogenetic factors

3.1 Introduction

Whilst significant advances have been made in identifying pharmacogenetic factors that contribute to the inter-individual variability in warfarin dose requirement in the adult population, little work has been done in paediatrics. The findings in adults cannot be extrapolated to children because:

1. There are stark physiological and developmental differences between adults and children;
2. There is little or no information available about the activity of the enzymes that mediate the pharmacological activity of warfarin in children and how they compare to adults, and;
3. It is not known whether the activity of each of the enzymes changes, and if so the extent of this change, with increasing age among the paediatric population.

3.2 Aims

This cross-sectional design, multi-centre study in children with stable anticoagulation with warfarin aimed to:

1. Investigate the way in which warfarin dose requirement in children is influenced by polymorphisms in key genes mediating sensitivity to and metabolism of warfarin;
2. Use knowledge of the contribution of these genetic factors to warfarin dose requirement, in addition to demographic factors, to develop a personalised warfarin-dosing algorithm for prediction of maintenance dose in children;
3. Validate the warfarin-dosing algorithm in predicting maintenance dose in a distinct cohort of children established on warfarin therapy.

3.3 Methods

3.3.1 *Study set-up*

Children were recruited from 4 UK sites (Birmingham Children's Hospital; Royal Manchester Children's Hospital; The Newcastle Hospitals NHS Trust; Royal Hospital for Sick Children, Glasgow) and The Hospital for Sick Children, Toronto, Canada. The study was approved by the Regional Ethics Committee, the Medicines and Healthcare products Regulatory Agency and the Institutional review boards at each of the study sites.

3.3.2 *Study protocol*

3.3.2.1 Inclusion and exclusion criteria. The study recruited children, aged 18 years or under, who were anticoagulated for at least 3 months after warfarin initiation and whose target INR range was either 2.0-3.0 or 2.5-3.5. Stability criterion for inclusion was that no change in warfarin dose had been made for at least the previous 3 consecutive INR measurements over a minimum period of 4 weeks. An additional cohort of children and young adults who had received warfarin when aged 18 years or under, and for whom historical data regarding warfarin dose requirement and INR were available, were recruited from one of the participating UK centres.

Children affected by intercurrent illness or temporarily taking a medication known to affect sensitivity to warfarin, e.g. an antibiotic, were temporarily excluded. These children were eligible for recruitment at a later date following recovery from the illness and completion of antibiotic therapy. Children on chronic therapy with medication(s) known to alter response to warfarin were eligible for recruitment but details of their concurrent medication(s) were recorded.

3.3.2.2 Data collection and blood sampling. Written informed consent to take part in the study was obtained from patients aged 16 years or over and from parents/carers of children aged <16 years. Details of diet, indication for anticoagulation with warfarin, target INR range, current warfarin dose and other medication(s) were obtained by questionnaire and review of patients' medical records (see 'Data collection proforma', Appendices A and B). Height, weight and gender were recorded. Ethnicity was reported by the patient or their

parent/carer, according to categories defined by the investigators. For the cohort of children and young adults who had previously been treated with warfarin during childhood, the above details were collected by review of patients' medical records and warfarin-dosing records at a time-point when the child had been stable on warfarin according to the aforementioned criterion. Initiation data, i.e. warfarin doses and INR values during the first 3 months of warfarin therapy, were collected for children for whom they were available. Body surface area was calculated using the Mosteller formula (Mosteller, 1987): Body surface area (m^2) = √([Height (cm) x Weight (kg)]/3600). Body mass index was calculated using the formula: Body mass index = Weight (kg)/ Height $(m)^2$. A venous blood sample (4-8mL) was collected from each patient and stored in ethylenediaminetetraacetic acid (EDTA) tubes at -80^{0}C for later genetic analysis.

3.3.3 *Genetic analysis*

3.3.3.1 DNA extraction from whole blood. Genomic DNA was extracted from whole blood samples according to an established method (Daly et al., 1996). 350µl blood was added to 1150µl cell lysis buffer (10mM Tris-HCL, 320mM Sucrose, 5mM $MgCl_2$, 1% Triton X, pH8), mixed gently, and then centrifuged for 20s at 14,000rpm. The supernatant was discarded and the remaining pellet re-suspended in 200µl nuclear lysis buffer (400mM Tris-HCL, 60mM EDTA, 150mM $MgCl_2$, 1% Sodium Lauryl Sulphate, pH8). For de-proteinisation, 50µl sodium perchlorate was added and the resulting suspension was rotary mixed for 10 minutes and then incubated at 60^{0}C for 15 minutes. 400µl chloroform was added and the solution mixed by inverting by hand and then centrifuged for 1 minute at 14,000rpm. The top layer, containing the DNA, was transferred to a fresh tube and twice the volume of ethanol was added. The solution was inverted by hand until the DNA was visible as a white precipitate. Following centrifugation for 2 minutes at 14,000rpm, the remaining liquid was discarded and the tube inverted and placed onto tissue paper to drain. The DNA was allowed to dry by incubating the open tube at 60^{0}C for 10 minutes, and then re-suspended in 50µl sterile water. The DNA samples were stored at 4^{0}C until analysis.

3.3.3.2 Genotyping using StepOne™ Real-Time Polymerase Chain Reaction System. Genotyping for *VKORC1* (-1639G>A; rs9923231), *CYP2C9**2 allele (R144C; rs1799853) and *CYP2C9**3 allele (I359L; rs1057910), *CYP4F2* (V433M; rs2108622), *APOE* (R158C; rs7412, and C112R; rs429358) and *CYP1A2* (C163A; rs762551) was performed using the StepOne™ Real-Time polymerase chain reaction (PCR) System with Taqman® SNP Genotyping Assays, Applied Biosystems™. This system enables allelic discrimination at a SNP site by using two primer/probe pairs in each reaction. The two primers are able to genotype the two possible variants at the SNP site and the two fluorescent dye detectors target the SNP site, one that is a perfect match to allele 1 and the other that is a perfect match to allele 2. Detection of both fluorescence signals indicates individuals who are heterozygous at the SNP site. Examples of the allelic discrimination plots and their interpretation are shown in Figures 3-1 to 3-7.

The *VKORC1*, *CYP2C9* and *APOE* genotype results were validated using control samples that had been genotyped as part of a previous study (Sconce et al., 2005b, Sconce et al., 2006). *CYP4F2* genotypes were validated by re-genotyping 26 samples in duplicate by PCR-RFLP analysis as described below (Section 3.3.3.4 ‘Genotyping using Polymerase Chain Reaction- Restriction Fragment Length Polymorphism’). The *CYP1A2* genotypes were run with in-house control samples that had been previously genotyped using a PCR-restriction fragment length polymorphism (RFLP) method that was itself validated by DNA sequencing.

Figure 3-1. Allelic discrimination plot for *VKORC1* (-1639G>A; rs9923231) genotype using StepOne™ Real-Time Polymerase Chain Reaction system

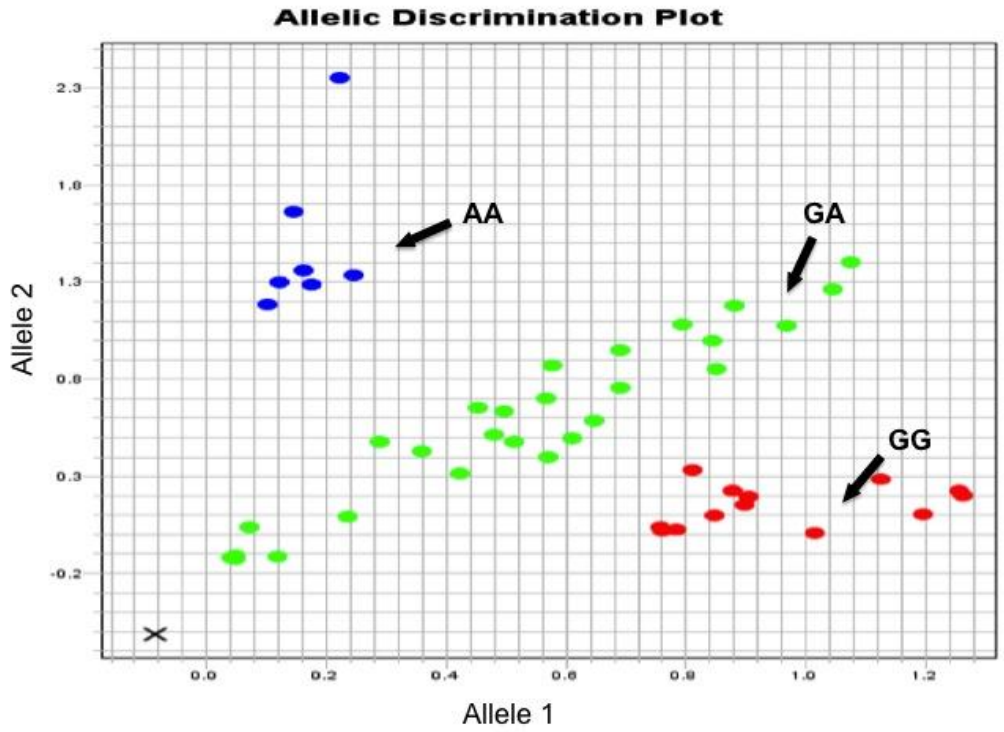


Figure 3-2. Allelic discrimination plot for *CYP2C9*2* (R144C; rs1799853) genotype using StepOne™ Real-Time Polymerase Chain Reaction system

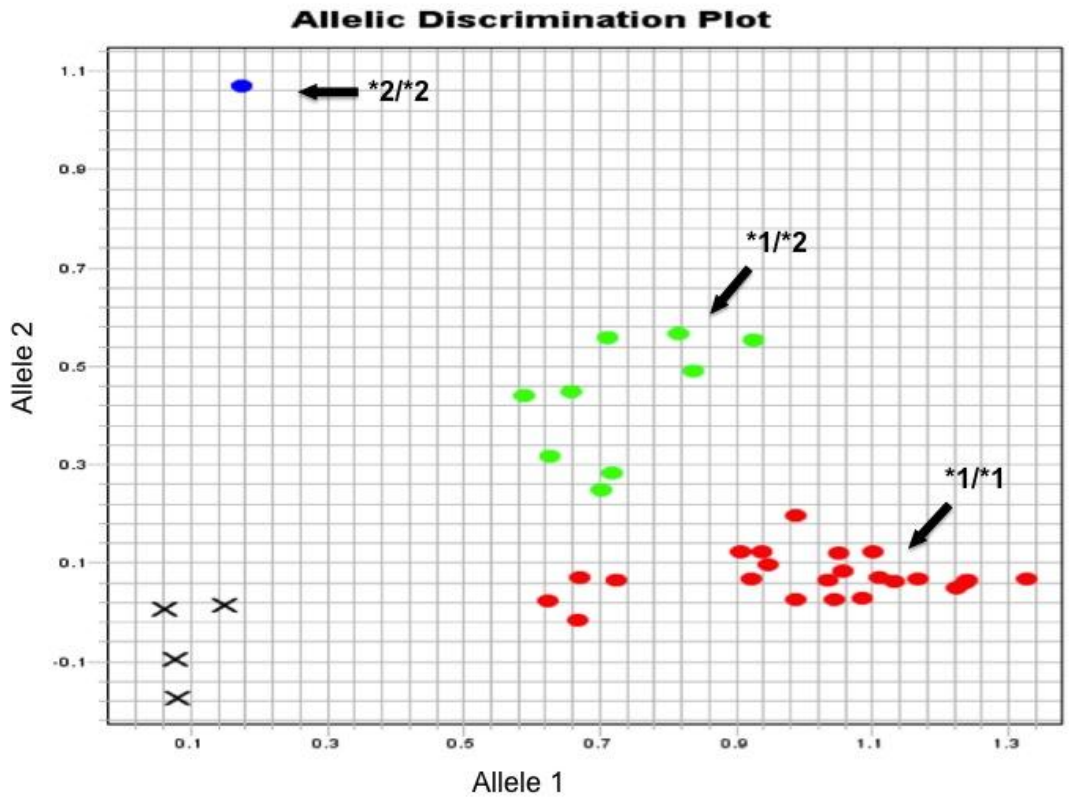


Figure 3-3. Allelic discrimination plot for *CYP2C9*3* (I359L; rs1057910) genotype using StepOne™ Real-Time Polymerase Chain Reaction system

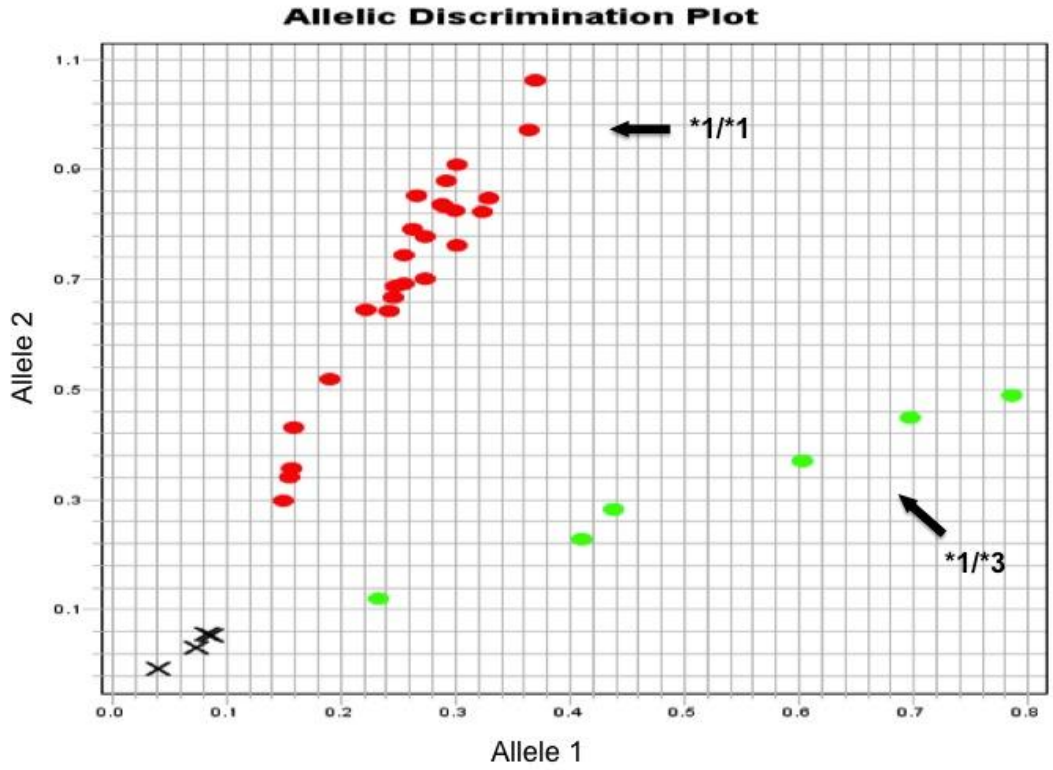


Figure 3-4. Allelic discrimination plot for *CYP4F2* (V433M; rs2108622) genotype using StepOne™ Real-Time Polymerase Chain Reaction system

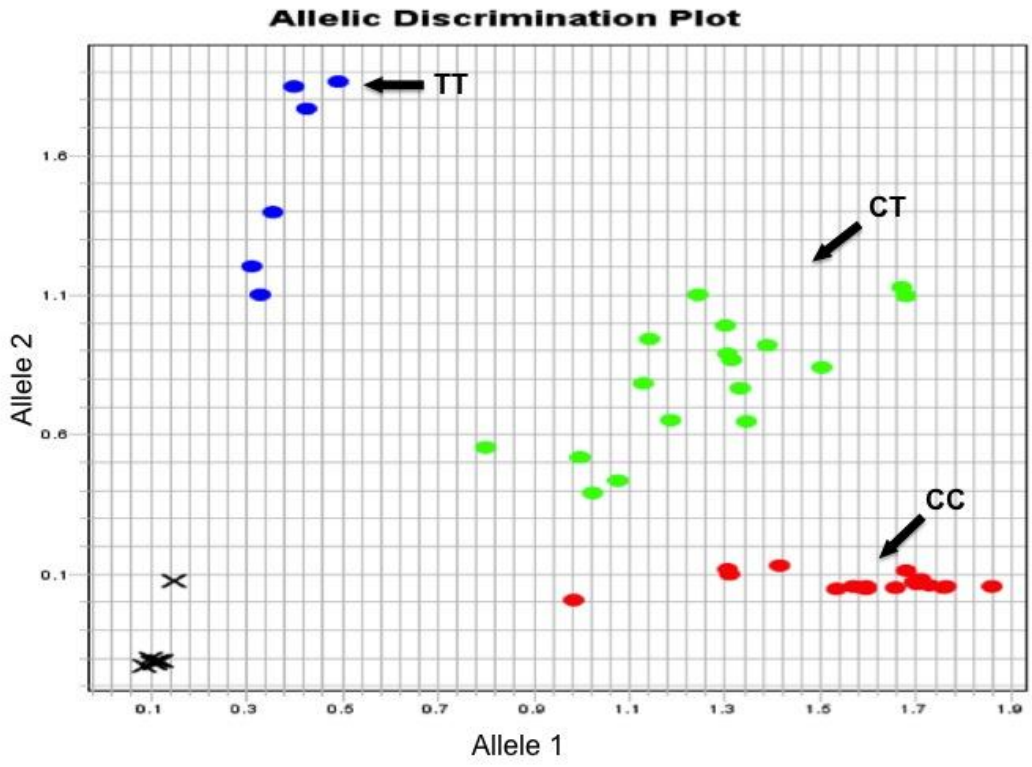


Figure 3-5. Allelic discrimination plot for *APOE* (R158C; rs7412) genotype using StepOne™ Real-Time Polymerase Chain Reaction system

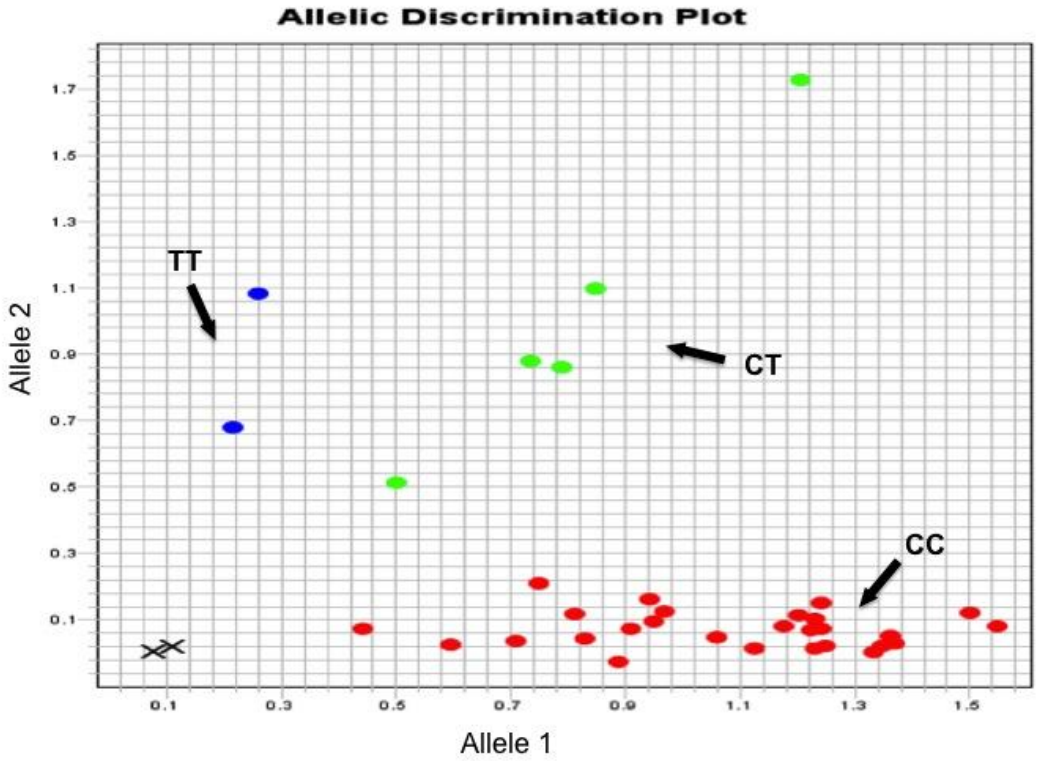


Figure 3-6. Allelic discrimination plot for *APOE* (C112R; rs429358) genotype using StepOne™ Real-Time Polymerase Chain Reaction system

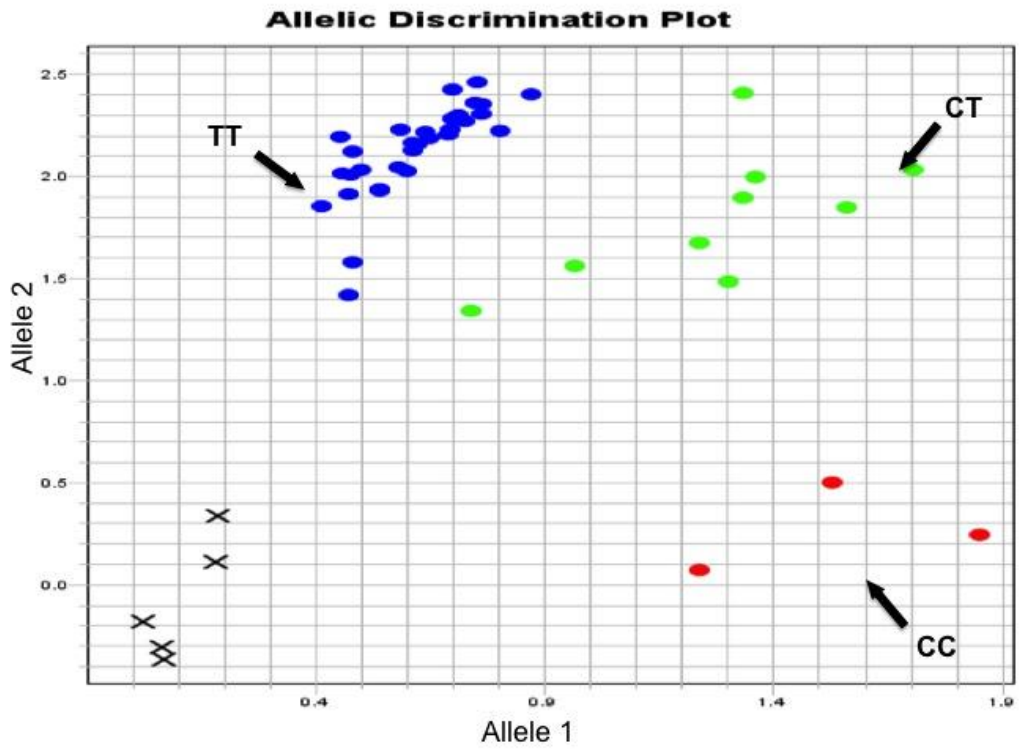
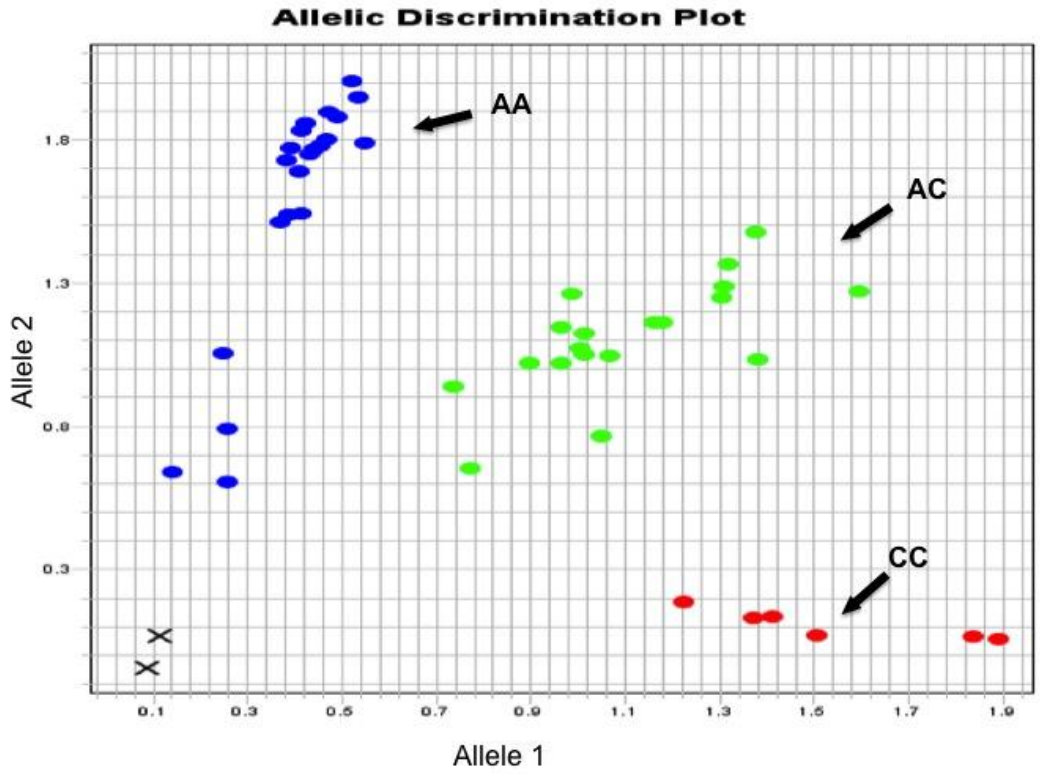


Figure 3-7. Allelic discrimination plot for *CYP1A2* (C163A; rs762551) genotype using StepOne™ Real-Time Polymerase Chain Reaction system



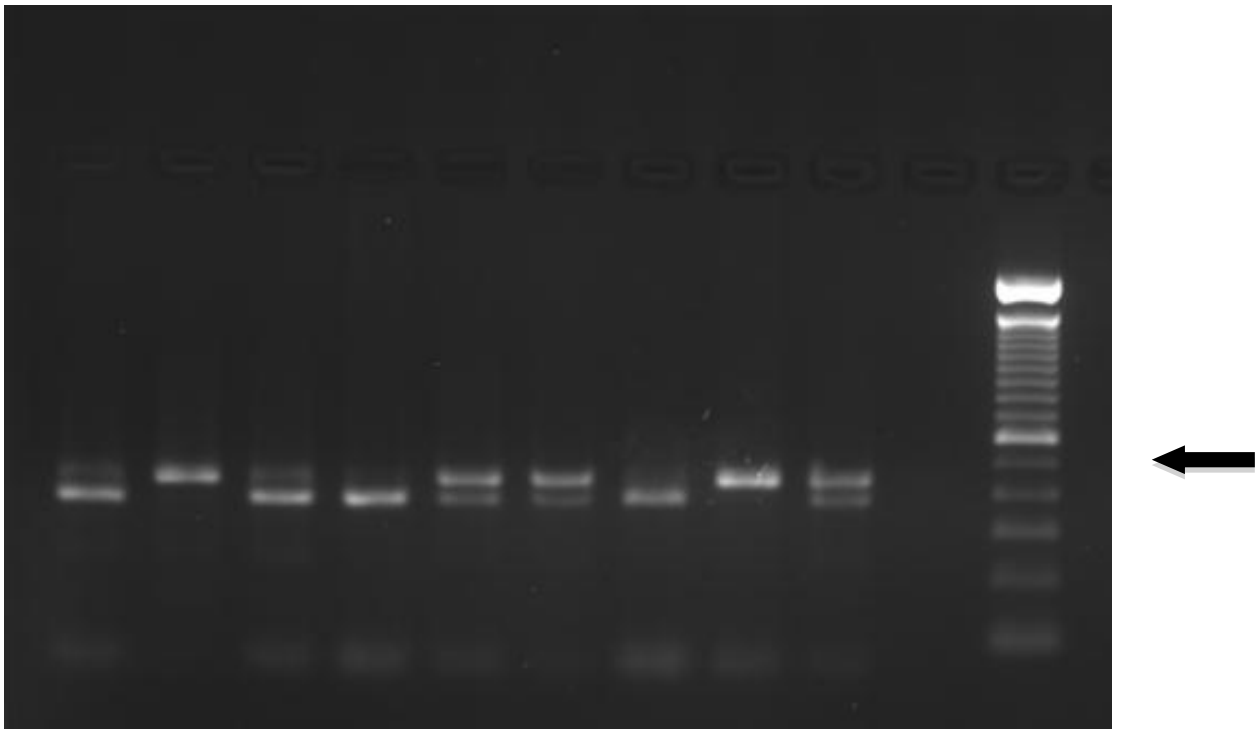
3.3.3.3 Multiplex genotyping using Sequenom® iPLEX™ GOLD assay. Multiplex genotyping was performed at the Bioscience Building, International Centre for Life, Newcastle upon Tyne. SNP Genotyping PCR and extension primers were designed by the Sequenom® software for five 26-plex, one 24-plex, one 19-plex and one single-plex assays with the aim of determining genotype for 174 SNPs in 29 genes as described by Jorgensen et al (Jorgensen et al., 2009). SNP typing was performed using the MassARRAY® platform (Sequenom, Hamburg, Germany). PCR, primer extension and sample clean-up were performed according to a local standard operating procedure. An Autoflex Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometer (Bruker Daltonics) was used to identify the genotypes as described by Whittaker et al (Whittaker et al., 2005). Due to technical problems this method was not successful in identifying genotype in our cohort for the majority of SNPs. Only the genotyping for *CYP3A5**3 allele (A6986G; rs776746) is reported and this was performed in duplicate for verification.

3.3.3.4 Genotyping using Polymerase Chain Reaction- Restriction Fragment Length Polymorphism. *CYP4F2* genotypes were determined using the PCR-RFLP method described by Cen et al (Cen et al., 2010). PCR was performed using the following final concentrations: 0.5 units *Taq* DNA polymerase (New England BioLabs); 0.1mM deoxynucleotide triphosphate (dNTP) (New England BioLabs); 0.25µM forward and reverse primers (Sigma); 1.5mM $MgCl_2$; 1x *Taq* DNA polymerase reaction supplied buffer (New England BioLabs). 20µl of reaction mixture was aliquoted into 0.2ml eppendorf tubes and 1µl of genomic DNA (concentration in excess of 50ng/µl) was added. PCR conditions consisted of the first step being held at 94^0C for 5 minutes, followed by 35 cycles at 94^0C for 30 seconds to denature the DNA, 60^0C for 30 seconds to anneal the primers, and 72^0C for 1 minute to extend the PCR fragment. A further 7 minutes at 72^0C allowed final extension. PCR was performed using an Applied Biosystems 2720 Thermal Cycler. Successful amplification was confirmed by agarose gel electrophoresis (detailed below). The PCR product was digested by the addition of 2units of PvuII (New England BioLabs) to 20µl product followed by incubation in PCR buffer at 37^0C for 3 hours.

Visualisation of the PCR-RFLP product was performed using agarose gel electrophoresis. 2% agarose gels were prepared containing ethidium bromide (0.4µg/ml) for visualisation and 1x TBE (Tris-base, boric acid, EDTA; pH8) as a running buffer. 3µl of loading buffer was added to 10µl of PCR product or digested PCR product. A 100bp DNA ladder (0.1-1.5kb) (New England BioLabs) was run on the gel as a molecular marker. Electrophoresis was for approximately 45 minutes at 75V (Figure 3-8). Fluorescence imaging was done using the GENi gel documentation system (Syngene).

Figure 3-8. PCR-RFLP analysis for *CPY4F2* (V433M; rs2108622) using the restriction enzyme PVUII and 2% agarose gel electrophoresis to visualise

Lanes 1, 3, 4 and 7 contain samples homozygous for the wild-type allele (CC). Lanes 5, 6 and 9 contain heterozygous samples. Lanes 2 and 8 contain samples homozygous for the mutant allele (TT). Lane 10 contains a negative control sample and lane 11 contains a 100bp DNA ladder. The arrow indicates the (denser) band that represents 500bp size.



LANE 1 2 3 4 5 6 7 8 9 10 11

3.3.4 Statistical analysis

3.3.4.1 Sample size. Due to the absence of data on the impact of genetic polymorphisms on warfarin dose in the paediatric population, a power calculation was based on comparable adult data. To detect a difference of approximately 1mg in mean warfarin daily dose between the *CYP2C9* polymorphisms, with significance at the 0.05 level and a power of 80%, it was estimated that a sample size of 120 patients would be required. As the frequency of each of the *VKORC1* genotypes is greater than that of the *CYP2C9* mutant alleles and the effect of *VKORC1* on warfarin dose requirement in the adult population is larger than that of *CYP2C9*, a sample size of 120 was deemed adequate for detecting the effect of *VKORC1* genotype and other significant variables.

3.3.4.2 Data analysis. Advice from a statistician was taken prior to data analysis. Statistical analyses were performed using MiniTab v15.0 (Coventry, UK). Mean warfarin daily dose was transformed by taking the square root of each value to obtain a normal distribution, allowing parametric tests to be performed. Associations between warfarin dose and height, weight, body surface area, body mass index and age were evaluated using Pearson correlation test. The effect of genotype, indication for warfarin, target INR range and ethnicity were evaluated using unpaired t-test or ANOVA. Stepwise regression analysis was used to identify factors contributing to the transformed warfarin dose followed by linear regression to model the relationships of dose with other variables measured. Pearson's correlation analysis was used to compare daily warfarin maintenance doses predicted by the IWPC warfarin-dosing algorithm to the actual daily warfarin doses. Pearson's correlation analysis was used to compare the predicted daily warfarin maintenance doses to the actual daily doses of the validation cohort (see Section 3.3.5 'Recruitment of validation cohort'). Associations between *VKORC1* and *CYP2C9* genotypes and outcome variables during the initiation phase of warfarin therapy were evaluated. The association between the square root of peak INR during the first week of therapy and genotype was examined using linear regression analysis for *VKORC1* genotype and t-test for *CYP2C9* genotype. The association between the incidence of supra-therapeutic INR during warfarin initiation and *VKORC1* genotype was evaluated using regression analysis and *CYP2C9*

genotype using a Mann-Whitney U-test. Results are presented as mean ± SD unless stated otherwise. A *P* value of < 0.05 was taken as statistically significant.

3.3.5 *Recruitment of validation cohort*

For the purposes of a paediatric kinetic pharmacodynamic modelling study, children were recruited from four tertiary care centres in Sweden: Queen Silvia Children's Hospital, Gothenburg; Skane University Hospital, Lund; Uppsala University Children's Hospital, Uppsala; and Astrid Lindgren Children's Hospital, Stockholm. The study was approved by The Regional Ethical Review Board, Uppsala University, Uppsala, Sweden. The study recruited children, aged 18 years or under, who were currently receiving warfarin, and children who had previously received warfarin at the age of 0-18 years provided that historical data regarding warfarin dose requirement and INR were available. Written consent was obtained from patients aged 18 years or over and from parents/guardians of children <18 years. All children provided written or verbal assent. Details of warfarin doses and INR measurements were collected. Patient age, height, weight and warfarin dose were recorded at a time-point when the child was stable on warfarin according to the stability criteria of our previous study (i.e. there had been no change in warfarin dose for at least the previous 3 consecutive INR measurements over a minimum period of 4 weeks.). Indication for warfarin anticoagulation was recorded. 3mls of venous blood or 0.5mls of capillary blood were taken into ethylenediaminetetraacetic acid (EDTA) and stored at -20^{0}C until analysis. Analysis was performed at the Department of Medical Sciences, Clinical Pharmacology, Uppsala University, Sweden. DNA was extracted using the Qiagen blood mini kit. Genotyping was performed using the 7500 Fast Real-Time PCR System with Taqman® SNP Genotyping Assays, Applied Biosystems^{TM} and verified using internal control samples.

3.4 Results

3.4.1 *Patient characteristics*

Recruitment occurred between April 2009 and December 2010. 120 children with a median age of 11.4 years (range: 1-18 years), a median height of 143.5cm (range: 79.0- 195.5cm) and a median duration of warfarin therapy of 49 months (range: 3-199 months) were recruited, including 8 subjects who were recruited retrospectively using historical data. The patients' demographics, indication for anticoagulation and target INR range are shown in Table 3-1. Median warfarin daily dose was 3.4 mg (range: 0.5-12.5mg).

3.4.2 *Genotyping*

Genotype frequencies for *VKORC1* (-1639G>A; rs9923231), *CYP2C9**2 allele (R144C; rs1799853) and *CYP2C9**3 allele (I359L; rs1057910), *CYP4F2* (V433M; rs2108622), *APOE* (C158T; rs7412, and T112C; rs429358), *CYP3A5* (A6986G; rs776746) and *CYP1A2* (C163A; rs762551) for the study population are shown in Table 3-2. All genotypes were in Hardy-Weinberg equilibrium.

Table 3-1. Patient Characteristics

	Number of children (%)
Gender	
- Male	82 (68.3)
- Female	38 (31.7)
Age group	
- 0-3 years	7 (5.8)
- 4-6 years	20 (16.7)
- 7-9 years	20 (16.7)
- 10-12 years	21 (17.5)
- 13-15 years	29 (24.2)
- 16-18 years	23 (19.2)
Ethnic origin	
- White Caucasian	91 (75.8)
- Indian/Pakistani	10 (8.3)
- Chinese	4 (3.3)
- Black Caribbean	3 (2.5)
- Black African	3 (2.5)
- South-East Asian/Filipino	2 (1.7)
- Other¶	7 (5.8)
Indication for anticoagulation with warfarin	
- Fontan procedure	64 (53.3)
- Prosthetic heart valve	18 (15.0)
- Coronary aneurysm	11 (9.2)
- Dilated cardiomyopathy	6 (5.0)
- Deep vein thrombosis/Pulmonary embolism	6 (5.0)
- Pulmonary hypertension	5 (4.2)
- Stroke	2 (1.7)
- Other§	8 (6.7)
Target INR range	
- 2.0-3.0	101 (84.2)
- 2.5-3.5	19 (15.8)
Total number of children	120 (100)

¶Mixed race, 5; Canadian Aboriginal, 1; Middle Eastern, 1
§1 patient each with arrhythmia, Bi-directional Glenn procedure, cerebral sinovenous thrombosis, left coronary artery to right ventricular fistula, recurrent transient ischaemic attack, transposition of the great arteries, truncal valve replacement, ventricular assist device

Table 3-2. Genetic Characteristics of Study Population

	Number of children (%)
***VKORC1* Genotype**	
- GG	43 (35.8)
- GA	55 (45.8)
- AA	22 (18.3)
***CYP2C9* Genotype**	
- *1/*1	84 (70.0)
- *1/*2	17 (14.2)
- *1/*3	17 (14.2)
- *2/*2	1 (0.8)
- *2/*3	1 (0.8)
- *3/*3	0 (0.0)
***CYP4F2* Genotype**	
- CC	61 (50.8)
- CT	49 (40.8)
- TT	10 (8.3)
***APOE* Genotype**	
- ε2ε2	0 (0.0)
- ε2ε3	15 (12.5)
- ε3ε3	79 (65.8)
- ε3ε4	22 (18.3)
- ε4ε4	1 (0.8)
- ε2ε4	3 (2.5)
***CYP3A5* Genotype**	
- GG	107 (89.2)
- GA	3 (2.5)
- AA	1 (0.8)
- Not available	9 (7.5)
***CYP1A2* Genotype**	
- AA	46 (38.3)
- AC	62 (51.7)
- CC	12 (10)

3.4.3 *Association of demographic variables with maintenance warfarin dose*

3.4.3.1 Age*.* The square root of warfarin daily dose was highly significantly correlated with age (r = 0.53, *P* < 0.001, Pearson correlation coefficient). Figure 3-9A shows the relationship between warfarin daily dose in mg and age and Figure 3-9B shows the relationship between warfarin daily dose in mg/kg body weight and age.

3.4.3.2 Body size*.* The square root of warfarin daily dose was highly significantly correlated with body surface area (r = 0.56, *P* < 0.001, Pearson correlation coefficient), height (r = 0.55, *P* < 0.001) and weight (r = 0.53, *P* < 0.001) with body surface area and height being the most accurate predictors of warfarin dose requirement. Body mass index correlated less closely with warfarin daily dose (r = 0.32, *P* < 0.001).

3.4.3.3 Indication for anticoagulant therapy*.* Children who were anticoagulated following a Fontan procedure had a significantly lower mean warfarin daily dose (3.4 ± 1.6mg) than those who were anticoagulated for other indications (4.4 ± 2.5mg), *P* = 0.02, unpaired t-test (Figure 3-10).

Figure 3-9. Relationship between age and: (A) Daily warfarin dose in mg; (B) Daily warfarin dose in mg/kg body weight

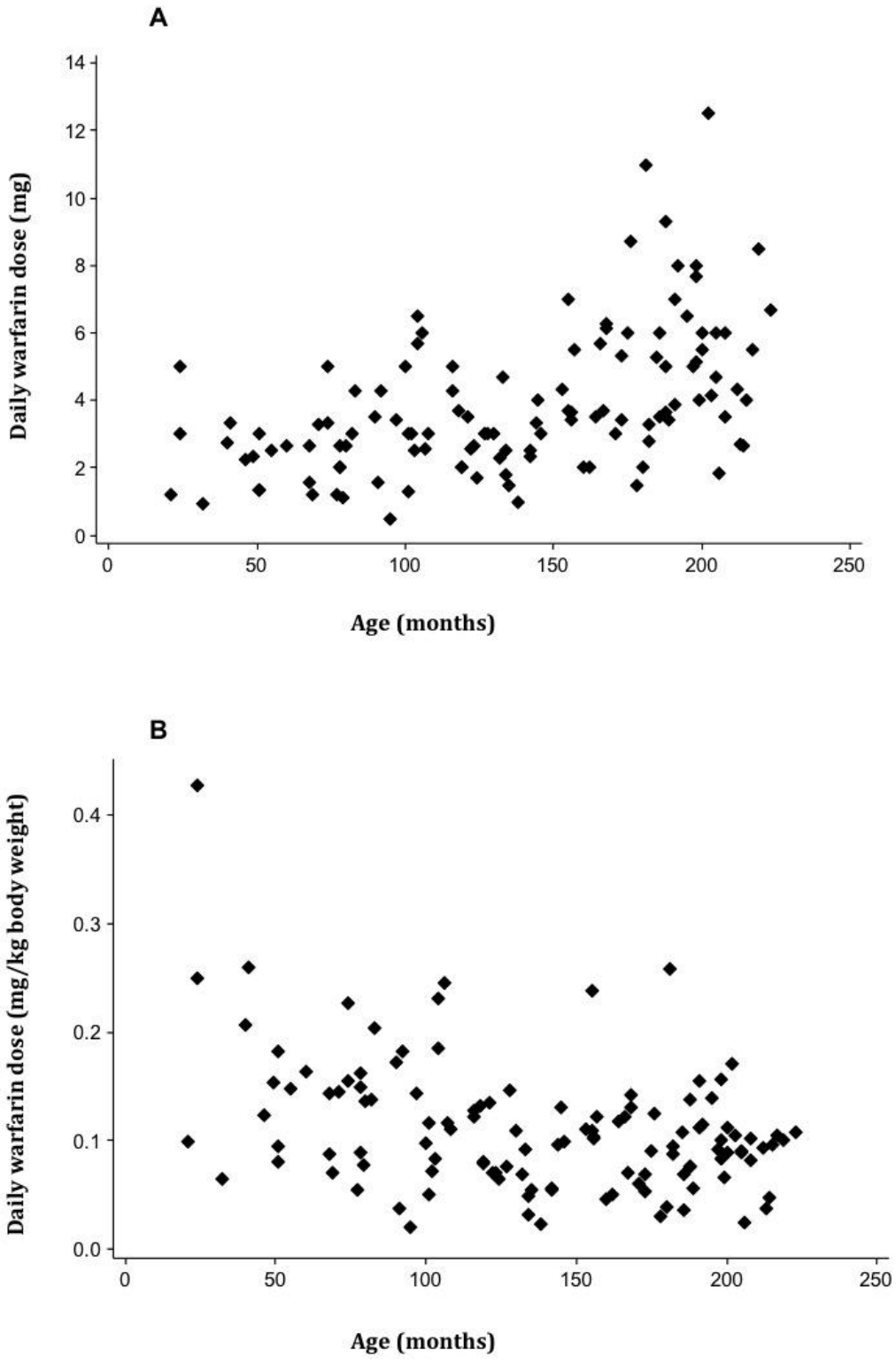
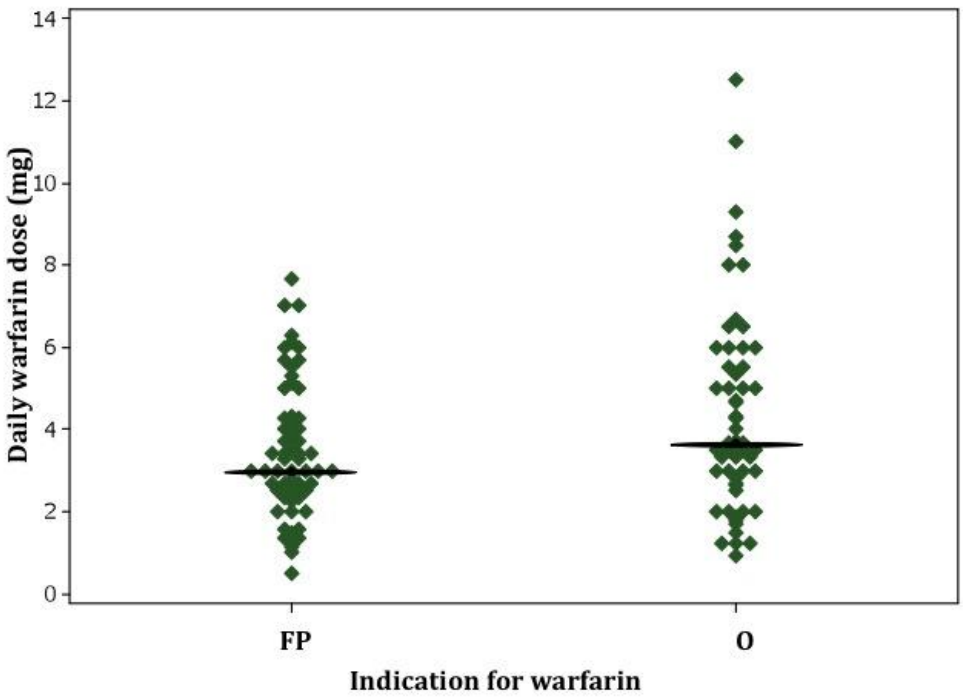


Figure 3-10. Scatter plot showing the relationship between indication for warfarin and warfarin dose

Indicated values are median warfarin doses. FP, Fontan procedure; O, Other.



3.4.3.4 Ethnicity. Children of Indian/Pakistani origin (n = 10) had a higher mean warfarin daily dose requirement (5.1 ± 2.6mg) than children of different ethnic origin (3.8 ± 2.1mg); this was accounted for by the higher frequency of *VKORC1* (-1639) GG genotype in this ethnic group, 8/10 (80%) vs. 35/110 (31.8%) for the rest of the cohort (see Section 3.4.4.1 '*VKORC1* genotype'). However, the difference was not statistically significant (*P* = 0.15, unpaired t-test). There were too few children for other ethnic groups to permit further sub-group analysis.

3.4.3.5 Medication. 83/120 (69.2%) children were taking additional prescribed drug(s). Of these children, 16 (13.3%) were taking one or more drugs known or suspected to have an effect on warfarin metabolism. These included: trimethoprim/co-trimoxazole, 6 patients; omeprazole, 4; erythromycin/azithromycin, 2; amiodarone, 1; imatinib, 1; prednisolone, 1; iloprost, 1; amitryptiline, 1; sodium valproate, 1; carbamazepine, 1. Children receiving trimethoprim or co-trimoxazole required a slightly lower mean warfarin dose than those who were not (3.0 ± 1.6mg vs. 3.9 ± 2.2mg) but this difference was not statistically significant (*P* = 0.25, unpaired t test). There were insufficient numbers of children prescribed other additional medications to permit further subgroup analysis.

3.4.3.6 Gender. The mean warfarin dose requirement in females was slightly higher than in males (female, 4.0 ± 2.3mg vs. male, 3.8 ± 2.1mg) although this difference was not statistically significant (*P* = 0.59, unpaired t-test).

3.4.3.7 Target range. Although children with a higher target INR range required a higher mean warfarin daily dose than those with a lower target INR range the effect of target INR range on warfarin dose requirement was not significant (2.5-3.5, 4.5 ± 2.6mg vs. 2.0-3.0, 3.8 ± 2.0mg, *P* = 0.23, unpaired t test).

3.4.3.8 Diet. 117/120 (97.5%) of the children reported a normal diet, meaning that they were not vegetarian or vegan or receiving enteral, parenteral or infant formula feeds. Of the remainder, one child (4 years old) was receiving enteral feeding, one (14 years old) was vegetarian and one (2 years old) was receiving infant formula feed. When the factors in the regression equation were accounted for (see Section 3.4.5 'Development of a personalised algorithm for

warfarin dosing in children'), none of these children had a warfarin daily dose that was outside of the expected range.

3.4.4 *Association of genotype with maintenance warfarin dose*

3.4.4.1 *VKORC1* genotype. The mean warfarin daily dose requirement in children with the *VKORC1* (-1639) GG genotype (5.0 ± 2.2mg) was significantly higher than in those with GA (3.7 ± 1.9) or AA (2.2 ± 1.1) genotype, $P < 0.001$, ANOVA. This is shown in Figure 3-11.

3.4.4.2 *CYP2C9* genotype. The mean warfarin daily dose requirement in children with homozygous wild-type *CYP2C9* genotype (4.3 ± 2.1mg) was significantly higher than in those with *1/*3 genotype (2.2 ± 1.1mg, $P < 0.001$). Children with *1/*2 genotype also had a lower mean warfarin dose requirement (3.7 ± 2.1mg) than children with homozygous wild-type *CYP2C9* genotype but this difference was not statistically significant ($P = 0.28$). The children with *2/*2 genotype (1.3mg, n = 1) and *2/*3 genotype (1.6mg, n = 1) had low warfarin doses but the numbers were too small to permit statistical analysis. This is shown in Figure 3-12.

3.4.4.3 *CYP4F2* genotype. The mean warfarin daily dose requirement was 5.1 ± 2.8mg in children with *CYP4F2* (rs2108633) TT genotype and was higher than in those with CT (4.0 ± 2.3mg) or CC (3.6 ± 1.8mg) genotype, but the differences were not statistically significant, $P = 0.12$. This is shown in Figure 3-13.

3.4.4.4 *APOE* genotype. To assess the impact of *APOE* genotype children were classified according to their number of ε4 alleles. Mean warfarin daily dose did not vary with number of ε4 alleles, $P = 0.87$. Only one child had *APOE* genotype ε4ε4 and this child had a relatively high warfarin maintenance dose of 5mg daily (Figure 3-14).

Figure 3-11. Scatter plot showing the relationship between *VKORC1* genotype and warfarin dose

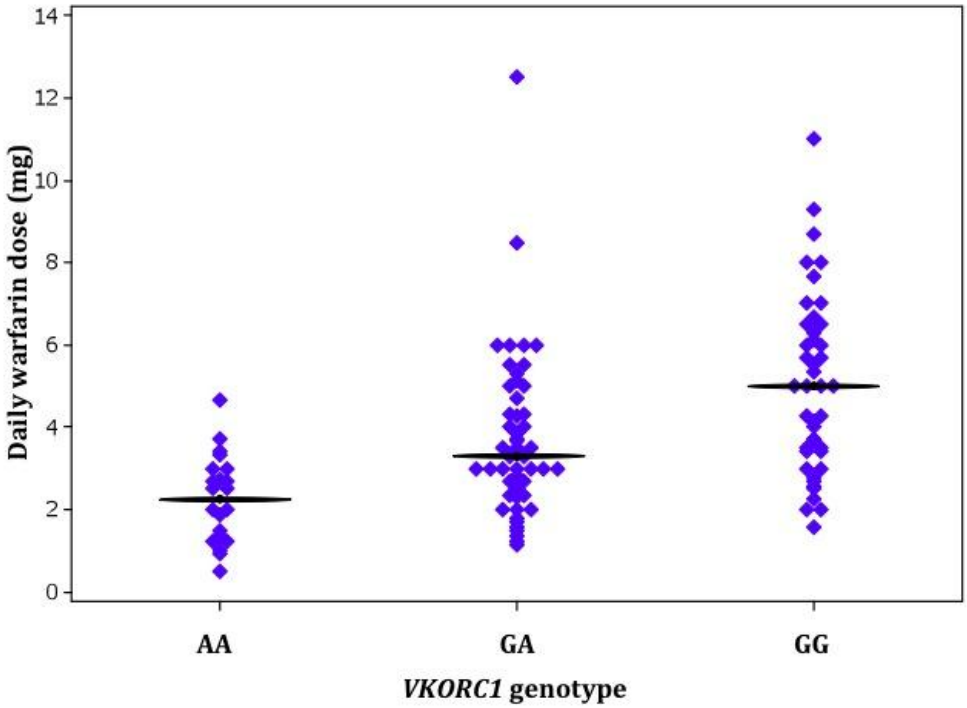


Figure 3-12. Scatter plot showing the relationship between *CYP2C9* genotype and warfarin dose

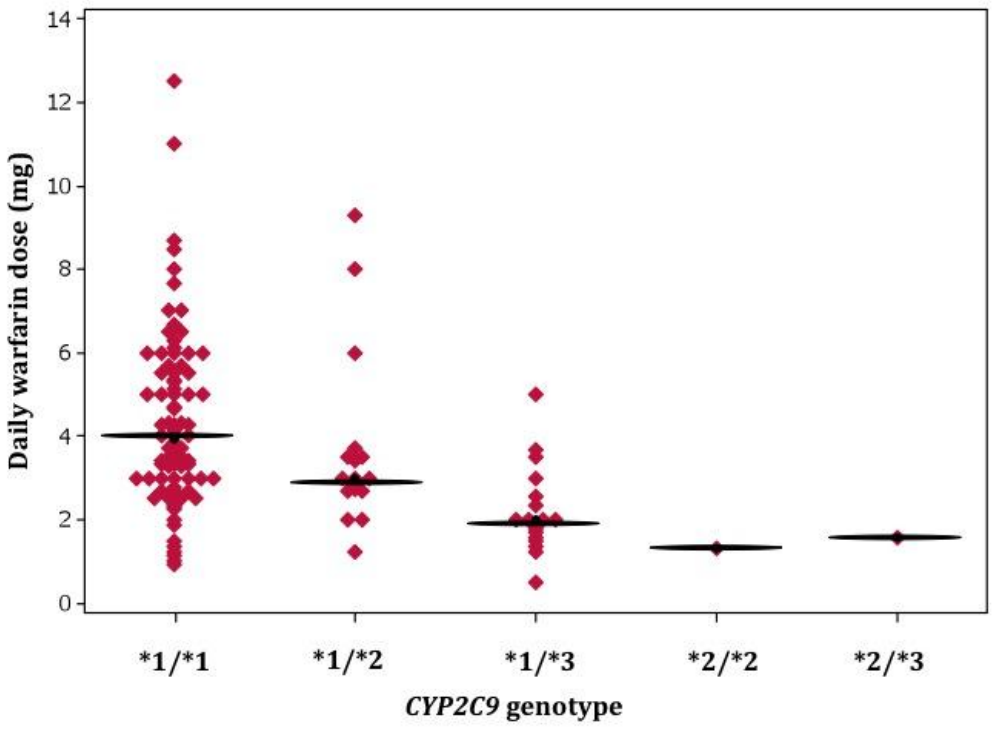


Figure 3-13. Scatter plot showing the relationship between *CYP4F2* genotype and warfarin dose

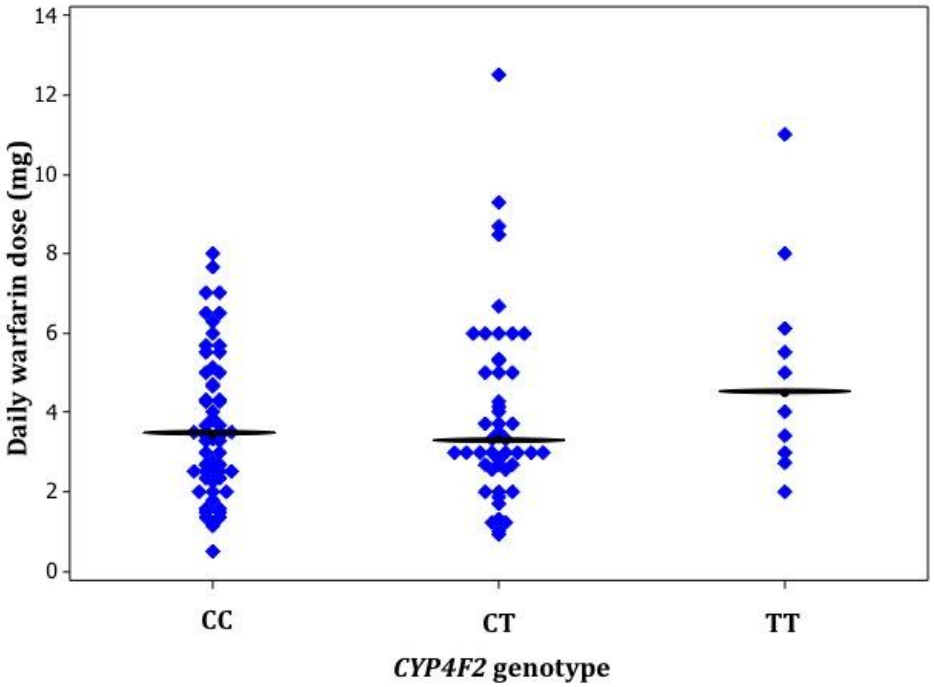
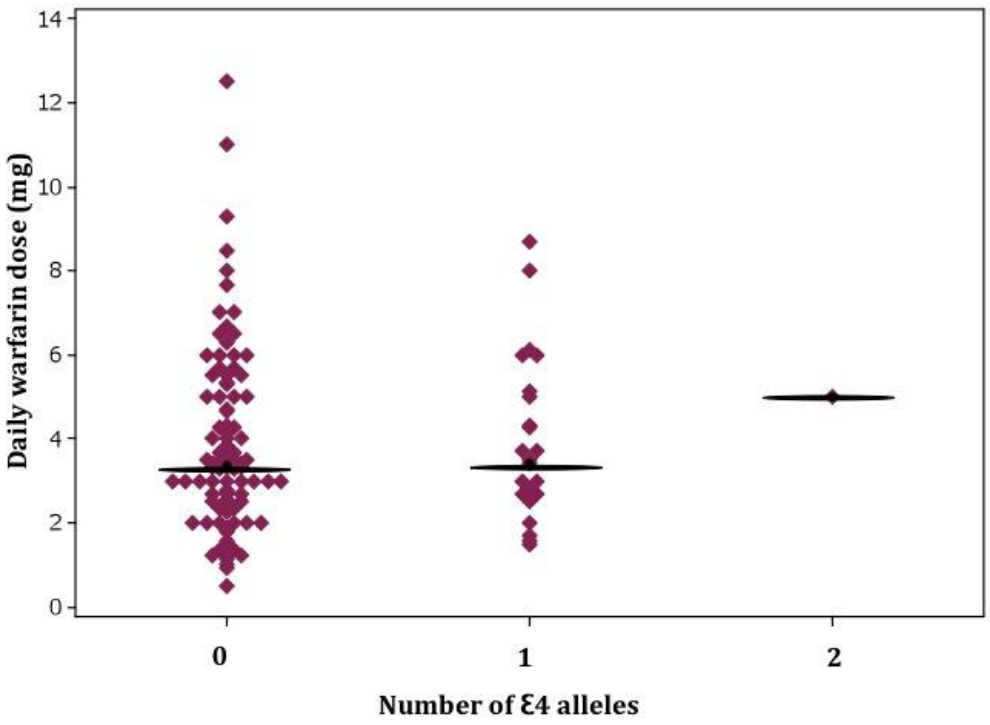


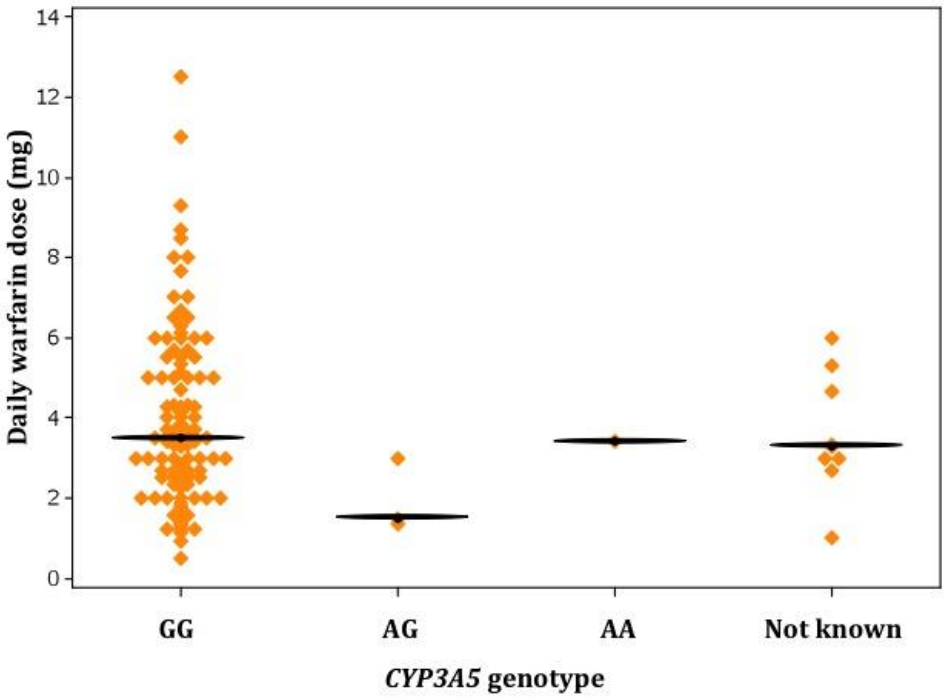
Figure 3-14. Scatter plot showing the relationship between *APOE* genotype and warfarin dose



3.4.4.5 *CYP3A5* genotype. Data were available for *CYP*3A5 (rs776746) genotype for 111 of the 120 children. Children with *CYP*3A5 (rs776746) genotype AG had a lower warfarin dose requirement than those with genotype GG, 1.9 ± 0.9mg vs. 4.0 ± 2.2mg, respectively, but this was not statistically significant (*P* = 0.07, unpaired t test) (Figure 3-15). Using stepwise regression analysis *CYP3A5* genotype had a *P* value of 0.04. However, only 3 children had genotype AG and only one had genotype AA so *CYP3A5* genotype was not included in the final regression equation (see Section 3.4.5 'Development of a personalised algorithm for warfarin dosing in children').

3.4.4.6 *CYP1A2* genotype. There was no significant relationship between *CYP1A2* genotype and warfarin dose requirement, children with *CYP1A2* (rs762551) genotype CC, CA and AA having a mean warfarin dose requirement of 3.6 ± 2.8mg, 4.0 ± 2.0mg and 3.8 ± 2.1mg, respectively.

Figure 3-15. Scatter plot showing the relationship between *CYP3A5* genotype and warfarin dose



3.4.5 *Development of a personalised algorithm for warfarin dosing in children*

According to the regression model, height, indication and *VKORC1* and *CYP2C9* genotypes made a significant contribution to warfarin dose requirement and together explained 72.4% of the inter-individual variability in warfarin dose (Figure 3-16). The individual contributions of each of these variables are shown in Table 3-3. Both height and body surface area were found to be good predictors of the square root of dose but in a model where the genetic parameters were included height was found to be the superior predictor and, once height was included, body surface area did not improve the fit of the model. The regression equation is: √dose= -0.009 + 0.011 (height) + 0.357 (*VKORC1*) – 0.478 (*CYP2C9*3*) – 0.277 (*CYP2C9*2*) + 0.186 (Indication) using the following key: input height in centimetres; *VKORC1* genotype: input 0 for AA, 1 for AG, and 2 for GG; *CYP2C9*3* genotype: input 0, 1, or 2 for the number of *3 alleles; *CYP2C9*2* genotype: input 0, 1 or 2 for the number of *2 alleles; Indication: input 0 for Fontan procedure, 1 for other indications. The 95% confidence intervals for the coefficients are shown in Table 3-3. The regression equation allows for the determination of the square root of the predicted dose which is then squared to provide the actual predicted warfarin dose in mg/day.

Subgroup analysis using two age groups (children <10 years of age and children >/=10 years of age) showed that neither the contribution of each individual factor nor the dosing algorithm differed significantly between children of differing age.

Figure 3-16. Box plots showing the influence of *VKORC1* and *CYP2C9* genotypes, and indication for warfarin, on warfarin dose

Boxes indicate the median and interquartile ranges. Vertical lines above and below boxes show the minimum and maximum values. Indicated values are mean warfarin doses. FP, Fontan procedure; O, Other.

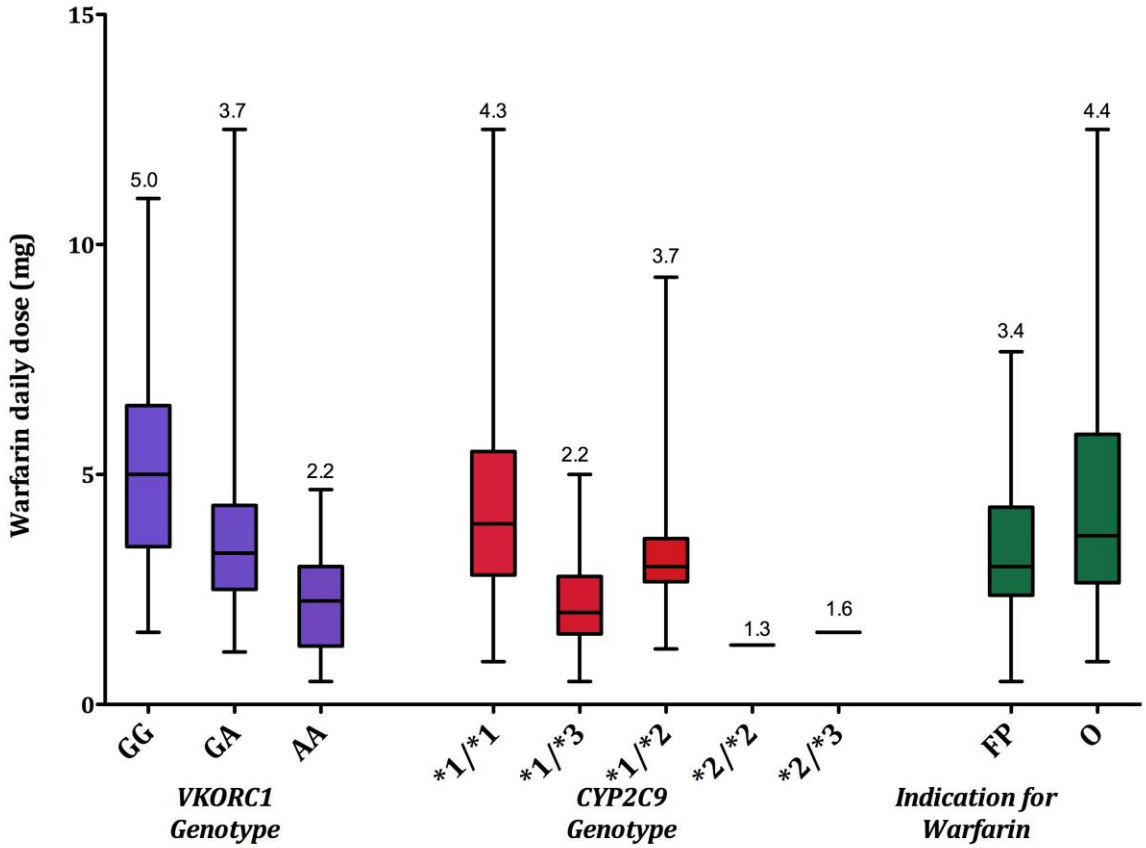


Table 3-3. Contribution of height, *VKORC1*, *CYP2C9*2* and **3* genotypes, and indication for warfarin to regression equation for modelling warfarin daily dose requirements in children

Predictor	Coefficient	95% Confidence Intervals	*P* value	Contribution to model, R^2/%
Intercept	-0.009	-0.313, 0.294	-	-
Height, cm	0.011	0.009, 0.013	< 0.001	29.8
Number of *VKORC1* (-1639) G alleles	0.357	0.284, 0.425	< 0.001	26.6
Number of *CYP2C9*3* variant alleles	-0.478	-0.335, -0.621	< 0.001	12.8
Number of *CYP2C9*2* variant alleles	-0.277	-0.148, -0.407	< 0.001	
Indication: Other than Fontan procedure	0.186	0.085, 0.29	< 0.001	3.2
Height, *VKORC1*, *CYP2C9*2/*3*, Indication	**-**	**-**	**<0.001**	**72.4**

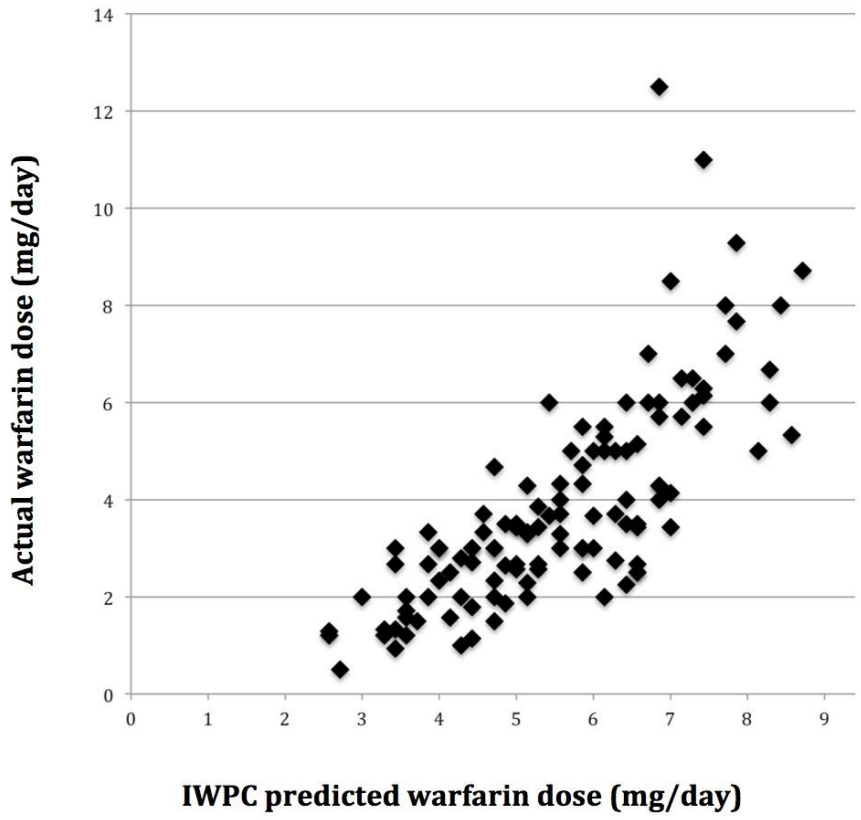
Regression equation: √dose= -0.009 + 0.011 (height) + 0.357 (*VKORC1*) – 0.478 (*CYP2C9*3*) – 0.277 (*CYP2C9*2*) + 0.186 (Indication).
Height: input height in centimetres
VKORC1 genotype: input 0 for AA, 1 for AG, and 2 for GG
*CYP2C9*2/*3* genotype: input 0, 1, or 2 for the number of *2/*3 alleles
Indication: input 0 for Fontan procedure, 1 for other indication.

3.4.6 Predictive value of IWPC algorithm

There was a close and highly significant correlation between the actual warfarin maintenance dose and the predicted maintenance dose according to the International Warfarin Pharmacogenetics Consortium (IWPC) algorithm (r = 0.76, P <0.001) (International Warfarin Pharmacogenetics Consortium, 2009). However, the algorithm consistently over-estimated warfarin dose in our cohort of children by, on average, 1.5mg/day (± 1.4mg/day) (Figure 3-17).

Figure 3-17. Relationship between predicted warfarin dose using IWPC algorithm (mg/day) and actual warfarin dose (mg/day)

IWPC (International Warfarin Pharmacogenetics Consortium, 2009)



3.4.7 *Validation of a personalised algorithm for warfarin dosing in children*

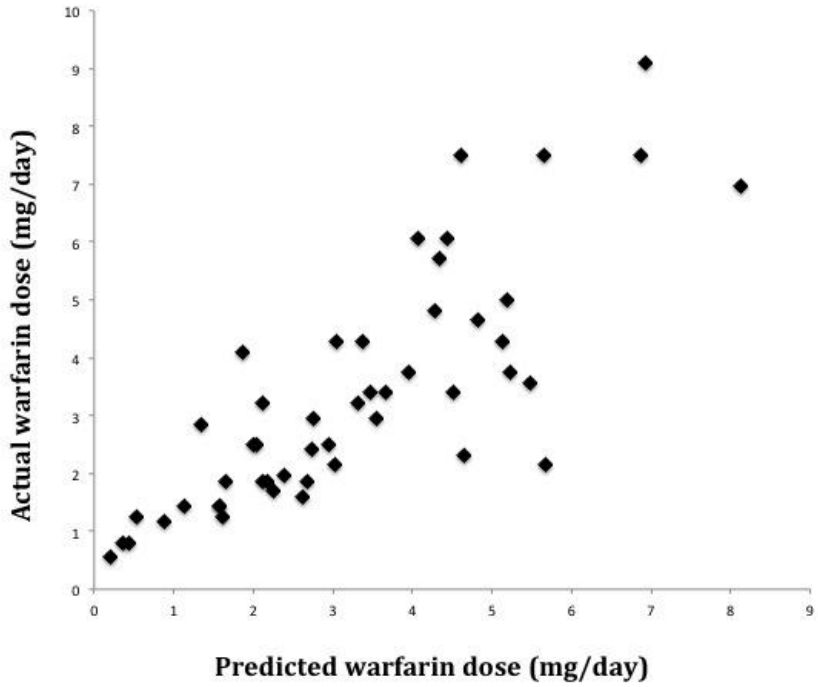
The warfarin-dosing algorithm was assessed in an unrelated population of 49 children (see Section 3.3.5 'Recruitment of validation cohort'). Median age was 7.2 years (range: 0- 17 years) and height 117.0cm (range: 58.0- 182.0cm). A summary of the demographics, indication for treatment, target INR range and genotypes for *VKORC1* and *CYP2C9* for the validation cohort is shown in Table 3-4.

The predicted mean (±SD) daily warfarin dose was 3.3 ± 1.8mg (range: 0.2-8.1mg) and the actual mean daily warfarin dose was 3.3 ± 2.0mg (range: 0.6-9.1mg). Pearson's correlation analysis showed a close and highly significant relationship between the square root of the actual warfarin dose and the square root of the predicted dose ($r = 0.833$, $P < 0.001$) with regression equation: $\sqrt{\text{actual warfarin dose (mg)}} = 0.329 + 0.825\ \sqrt{\text{predicted warfarin dose (mg)}}$. The square roots of the doses provide a better statistical correlation (as this transformation stabilises the variance and gives an approximately Normal distribution). However to aid data interpretation Figure 3-18 shows the relationship between the absolute values of predicted warfarin dose and actual dose.

Table 3-4. Demographic and Genetic Characteristics of the Validation Cohort

	Number of children (%)
Gender	
- Male	26 (53.1)
- Female	23 (46.9)
Ethnic origin	
- White Caucasian	40 (81.6)
- Asian	6 (12.2)
- Other	3 (6.1)
Indication for anticoagulation with warfarin	
- Fontan procedure	13 (26.5)
- Prosthetic heart valve	21 (42.9)
- Cardiomyopathy	7 (14.3)
- Other	8 (16.3)
Target INR range	
- 1.8- 2.5	2 (4.1)
- 2.0- 3.0	29 (59.2)
- 2.5- 3.5	16 (32.7)
- 3.0- 4.0	2 (4.1)
VKORC1 genotype	
- GG	19 (38.8)
- GA	23 (46.9)
- AA	7 (14.3)
CYP2C9 genotype	
- *1/*1	34 (69.4)
- *1/*2	7 (14.3)
- *1/*3	6 (12.2)
- *2/*2	1 (2.0)
- *2/*3	1 (2.0)
- *3/*3	0 (0)

Figure 3-18. Relationship between predicted warfarin dose (mg/day) and actual warfarin dose (mg/day) for the validation cohort



3.4.8 *Association of genotype with initiation of warfarin in children*

Initiation data were collected from 51 patients. There were 39 males and 12 females, median age at initiation of warfarin therapy 4 years (range: 1- 17 years). *VKORC1* and *CYP2C9* genotypes for this subgroup are shown in Table 3-5.

Data were available for patient weight at the time of initiation of warfarin therapy for 48 patients. Warfarin initiation was based on the recommended dose of 0.2mg/kg warfarin (to a maximum of 5mg) (Monagle et al., 2008). Mean dose given on days 1 and 2 of initiation of warfarin therapy was 0.14 mg/kg (± 0.06).

There was a significant correlation between *VKORC1* genotype and square root of peak INR during the first week of warfarin therapy, those with *VKORC1* (-1639) AA genotype having a higher peak INR (mean 5.1 ± 2.2) than those with GA (3.5 ± 1.4) or GG (3.0 ± 1.3) genotype, $P = 0.01$, regression analysis on the number of A alleles, as shown in Figure 3-19. Peak INR during the first week of warfarin therapy was also dependent on *CYP2C9* genotype, children with one variant *CYP2C9* allele having a higher peak INR than those with wild-type *CYP2C9*, mean 4.1 ± 1.7 vs. 3.2 ± 1.4, respectively. The child with *CYP2C9* *2/*3 genotype had a peak INR in week 1 of 5.5 (Figure 3-20). The relationship between *CYP2C9* genotype and peak INR by regression analysis was not established because there was only one child who had two mutant alleles. A more conservative analysis, comparing square root of peak INR in children with wild-type *CYP2C9* to those with one or more variant alleles established a significant difference between the two groups (Student's t-test, $P = 0.04$).

Table 3-5. Genetic Characteristics of the subgroup with available initiation data

	Number of children (%)
***VKORC1* Genotype**	
- GG	19 (37)
- GA	26 (51)
- AA	6 (12)
***CYP2C9* Genotype**	
- Wild-type	35 (69)
- Variant¶	16 (31)

¶*CYP2C9* genotype; *1/*2, 6 children; *1/*3, 9 children; *2/*3, 1 child

Figure 3-19. Box plot showing the relationship between *VKORC1* genotype and peak INR during the first week of warfarin therapy

Boxes indicate the median and interquartile ranges. Vertical lines above and below boxes show the minimum and maximum values. Mean values are indicated.

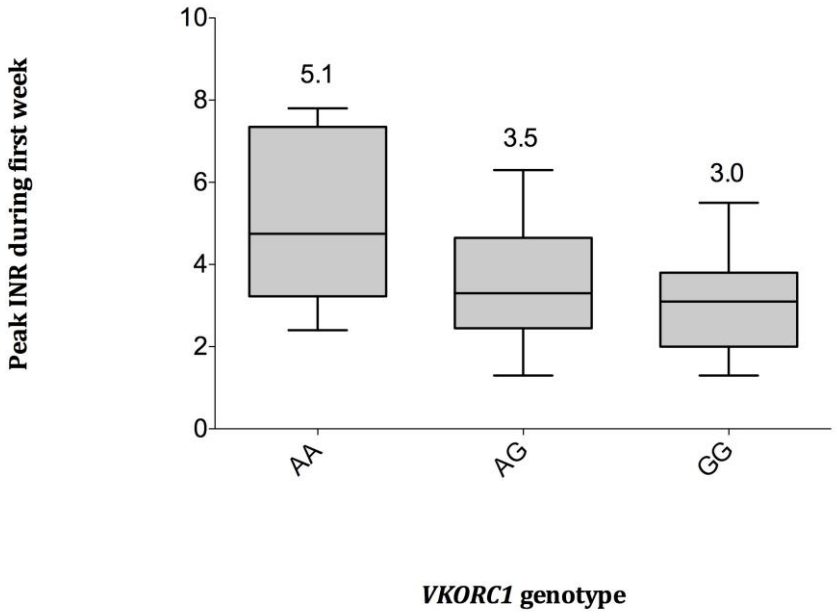
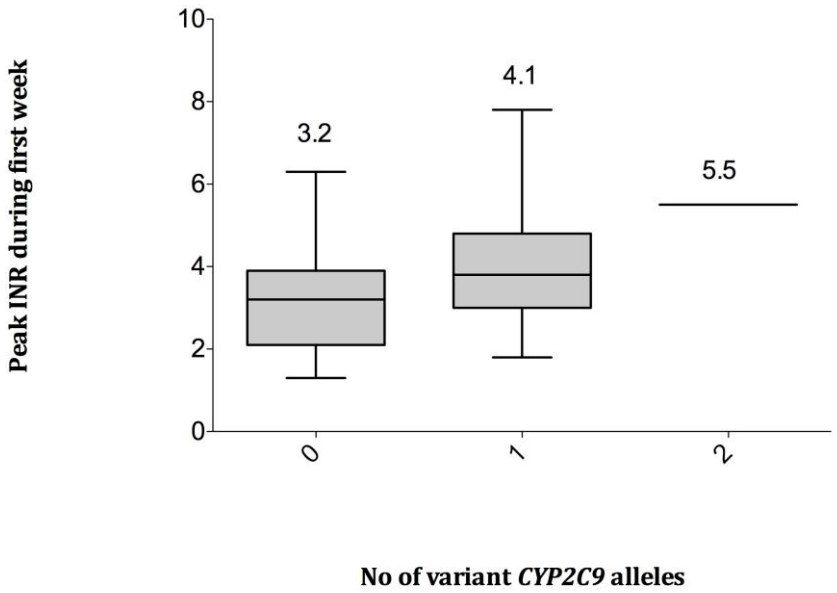


Figure 3-20. Box plot showing the relationship between *CYP2C9* genotype and peak INR during the first week of warfarin therapy

Boxes indicate the median and interquartile ranges. Vertical lines above and below boxes show the minimum and maximum values. Mean values are indicated.



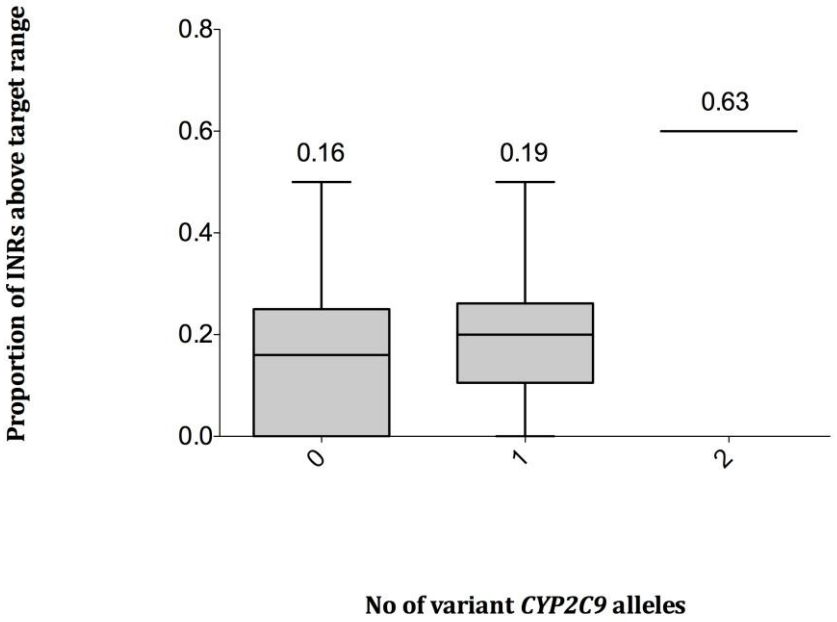
Children with a variant *CYP2C9* allele had a greater proportion of INR values above target range during the first month of warfarin therapy, those with one variant allele having 19.3% vs. 15.9% for those who were wild-type for *CYP2C9* (the single child with two variant *CYP2C9* alleles had 63.1% of the INR values above target range during the first month) as shown in Figure 3-20. There was a marginal effect of *CYP2C9* genotype on the incidence of supra-therapeutic INR during the first month of warfarin therapy ($P = 0.08$, Mann-Whitney U test). Children with *VKORC1* (-1639) AA genotype had a greater proportion of INR values above target range during the first month of warfarin (21.5%) than those with GA (17.6%) or GG (17.2%) genotype but this was not statistically significant.

There was no association between *CYP2C9* and *VKORC1* genotype and time to first supra-therapeutic INR, time to first therapeutic INR, number of high INRs (>4.0) during initiation therapy, time to stable warfarin dosing (as defined in Section 3.3.2.1 ‘Inclusion and exclusion criteria’) or number of warfarin dose changes.

No haemorrhagic or thrombotic events occurred during the initiation of warfarin therapy in this cohort.

Figure 3-21. Box plot showing the relationship between *CYP2C9* genotype and proportion of INRs above target range during the first month of warfarin therapy

Boxes indicate the median and interquartile ranges. Vertical lines above and below boxes show the minimum and maximum values. Mean values are indicated.



3.5 Discussion

This study is the largest cross-sectional study of the effect of genetic, clinical and demographic factors on warfarin dose requirement in children reported to date. The results show that a major proportion (72.4%) of the inter-individual variability in warfarin dose requirement in children is attributed to by *VKORC1*, *CYP2C9*, height and indication for warfarin therapy.

The recent study published by Nowak-Göttl et al identified only a minor effect of *VKORC1* (-1639G>A) and *CYP2C9* (*2, *3) polymorphisms on maintenance warfarin dose in children, 3.7% and 0.4% respectively (Nowak-Göttl et al., 2010). However, only 59 children were included in the analysis, not all were anticoagulated with the same oral coumarin derivative (26 were anticoagulated with phenprocoumon), there were few children identified as having *CYP2C9* *2 and *3 alleles and a less robust criterion was used to establish stable anticoagulation status (requiring only three consecutive days of stable warfarin dose prior to subject study participation) (Nowak-Göttl et al., 2010) than that adopted for our study. A further study by Kato *et al* in 48 children of Japanese origin identified a 28% lower warfarin dose requirement in children with *VKORC1* -1173TT genotype when compared to those with -1173CT or -1173CC genotype (Kato et al., 2011). The investigators were unable to assess the impact of *CYP2C9* genotype on warfarin dose requirement as they found only one child to be of *CYP2C9**3 genotype. This study was also limited by a small sample size. Neither Nowak-Göttl et al (Nowak-Göttl et al., 2010) nor Kato *et al* (Kato et al., 2011) evaluated the effect of *CYP4F2* polymorphism on paediatric warfarin dose requirement.

In keeping with the studies by Nowak-Göttl et al (Nowak-Göttl et al., 2010) and Kato et al (Kato et al., 2011) body size, in this case measured by height, is the primary determinant of warfarin dose requirement in a paediatric cohort, contributing 29.8% to inter-individual variability. Age and weight were highly correlated with height but there was a closer correlation between height and warfarin dose. This may be explained by the presence of a positive correlation between liver size and height, resulting in greater warfarin dose requirement due to increased warfarin hepatic clearance (Murry et al., 1995, Takahashi et al., 2000) and possibly greater hepatic availability of coagulation proteins and

stored vitamin K with increasing height. The positive correlation of dose with height overrides the smaller effect of younger children requiring a higher warfarin dose per kg body weight than older children and adolescents, shown by this (Figure 3-9) and previous studies (Streif et al., 1999, Bonduel et al., 2003). Figure 3-9 suggests that there may be an exponential effect rather than a linear relationship between age and dose or dose per kg body weight.

Studies in adults anticoagulated with warfarin have shown that carriers of the common allelic variants (*2 or *3) of the *CYP2C9* gene are associated with a lower warfarin dose requirement accompanied by a greater tendency to experience haemorrhagic complications during warfarin initiation (Aithal et al., 1999, Higashi et al., 2002). Studies have shown that the *CYP2C9* polymorphism accounts for 5.7-17.5% of inter-individual variability in warfarin dose requirement in adults (Sconce et al., 2005b, Wadelius et al., 2009, Kamali et al., 2004). The influence of *CYP2C9* on maintenance warfarin dose requirement was similar in our paediatric cohort (in which it accounted for 12.8% of variability) to adults. Our findings are more in keeping with the adult literature than either those of Nowak-Göttl et al (Nowak-Göttl et al., 2010) or of an earlier study by Ruud et al (Ruud et al., 2008) who found no association between *CYP2C9* genotype and warfarin dose in 29 anticoagulated children although the latter noted that children with a *CYP2C9* variant allele reached target INR sooner and were more likely to be over-anticoagulated than those without (Ruud et al., 2008). In the Ruud et al study warfarin doses were not corrected for either body size or age (Ruud et al., 2008).

Vitamin K epoxide reductase (target enzyme for warfarin) is responsible for the recycling of vitamin K and is encoded by the vitamin K epoxide reductase subunit complex (*VKORC1*). Adults with *VKORC1* (-1639) GG genotype require higher warfarin doses than those with GA or AA genotype (Rieder et al., 2005, D'Andrea et al., 2005). *VKORC1* genotype accounts for 15-30% of inter-individual variability in warfarin dose requirement in adults (Rieder et al., 2005, Sconce et al., 2005b, Wadelius et al., 2009), consistent with that seen in our paediatric population in which it accounted for 26.6%.

Indication for warfarin (Fontan procedure) contributed to 3.2% of inter-individual variability in warfarin dose requirement. A lower warfarin dose requirement in children following a Fontan procedure was previously reported by Streif et al; showing a 25% reduction in dose when compared to patients with the same target INR range (Streif et al., 1999). The mechanism for the lower dose requirement may be related to abnormal liver function in children after Fontan procedure resulting in reduced warfarin metabolism (Kaulitz et al., 1997). Although all of the children in our cohort who had a Fontan procedure were anticoagulated with the lower target INR range of 2.0-3.0, target INR range was not an independent variable in terms of its' effect on warfarin dose requirement. The necessity for long-term anticoagulation of children after Fontan procedure is controversial and many physicians prefer to anticoagulate with warfarin for a defined period of time only (e.g. 6-12 months) or to use anti-platelet therapy as an alternative to warfarin (Monagle et al., 2011). This variation in practice may lead to a tendency to anticoagulate these children at the lower end of the target INR range as they are perceived to be at a lesser risk of thromboembolism, therefore resulting in a lower maintenance warfarin dose requirement.

Recent studies have identified that the rs2108622 SNP in *CYP4F2* accounts for 2-7% of inter-individual variability in warfarin dose requirements in adults (Caldwell et al., 2008, Takeuchi et al., 2009). Although there appears to be no direct role for *CYP4F2* in warfarin metabolism, it has been suggested that it influences vitamin K oxidase activity and therefore hepatic levels of vitamin K (McDonald et al., 2009). The lack of effect of *CYP4F2* on warfarin dose in our cohort of children may relate to differences in dietary vitamin K intake when compared to their adult counterparts. This requires further study of the impact of vitamin K status on warfarin dose requirement in children.

Previous studies have shown that APOE genotype influences warfarin dose requirement, adults carrying one or more ε4 alleles requiring a lower warfarin maintenance dose than those without, although this contribution accounted for only 6% of variability (Kohnke et al., 2005). APOE mediates the uptake of vitamin K-rich lipoproteins into the cells, increasing hepatic availability of vitamin K which may result in a relative resistance to the anticoagulant effect of warfarin (Kohlmeier et al., 1996). Our study did not confirm these findings, children with

one ε4 allele having a warfarin dose that did not differ from those without. As for *CYP4F2* genotype, the lack of evidence for an effect of APOE genotype on warfarin dose requirement in our cohort may relate to a difference in dietary vitamin K intake in children. Again, the role of dietary vitamin K intake in determining warfarin dose requirement in children requires further study.

Analysis of the polymorphisms influencing metabolism of the R-enantiomer of warfarin showed that children who had *CYP3A5**3 genotype AG had a lower warfarin maintenance dose than those who had genotype GG. The difference was not statistically significant and was likely to have been limited by the small number of children who had one or more variant alleles (n = 4).
There was no correlation between the presence of *CYP1A2* allele and maintenance warfarin dose requirement. The smoking history of patients and their co-habitants was not assessed in this cohort.

The influence of each of the genotypes on warfarin dose was evaluated using analysis of variance (ANOVA). This does not take into account the direction of change in dose. Linear regression analysis on the number of variant alleles present may have identified a greater impact of some of these genotypes on stable warfarin dose.

In this study we found that children of Indian/Pakistan origin tended to require a higher daily warfarin dose than other ethnic groups which was accounted for by the higher frequency of *VKORC1* (-1639) GG genotype in this ethnic subgroup. This is consistent with data previously reported in adults (Limdi et al., 2010). Our study was not designed to explore the effect of ethnicity on warfarin dose requirement which should be evaluated in a larger population.

A significant number of our cohort (16 patients: 13.3%) were taking additional medications that are known or suspected to have an effect on warfarin metabolism. In the interests of obtaining an adequate sample size it was not possible to exclude these children from recruitment to the study. Children who were taking additional medications did not have a warfarin dose that differed to children who were not. Children who were taking trimethoprim or co-trimoxazole (trimethoprim-sulphamethoxazole) required a lower warfarin dose than those

who were not, although this difference was not statistically significant. The augmentation of the anticoagulant effect of warfarin by trimethoprim-containing compounds is well documented and it is suggested that this is due to competitive protein binding with displacement of warfarin from albumin binding sites (O'Reilly and Motley, 1979). Concomitant steroids have previously been shown to lower the warfarin dose requirement in children (Streif et al., 1999, Ruud et al., 2008) but this could not be confirmed in our cohort as only one child was prescribed an oral steroid.

The effects of dietary vitamin K intake on warfarin dose requirement and stability of anticoagulant control are well established in adult patient populations, a lower vitamin K intake being associated with increased sensitivity to the anticoagulant effect of warfarin (Khan et al., 2004, Franco et al., 2004) and poorer stability of anticoagulant control (Sconce et al., 2007). Vitamin K supplementation in children, in the form of infant formula feed or enteral/parenteral feeding, has been shown to increase warfarin dose requirement (Streif et al., 1999) but this effect could not be examined in our cohort with only one formula-fed infant and one child receiving enteral feeds.

Based on the study data a pharmacogenetics-based algorithm was developed for predicting warfarin maintenance dose (Table 3-3). This incorporates the four clinical and genetic factors that had the greatest influence on maintenance warfarin dose in our cohort; height; *VKORC1* genotype; *CYP2C9* genotype; indication for warfarin. Using a validation cohort of 49 children, recruited to a separate study, the algorithm was able to accurately predict maintenance warfarin dose, showing a close and highly significant correlation between actual and predicted warfarin doses (Figure 3-17). The derivation and validation cohorts showed some differences, the validation cohort being younger (median age, 7.2 vs. 11.4 years) and having a greater proportion of children with a higher target INR range (range > 2.5, 36.8% vs. 15.8%) when compared to the derivation cohort. However, the two cohorts were similar in ethnic background and this is reflected in the lack of major differences in the distribution of genotypes. The paediatric pharmacogenetics-based warfarin dosing equation performed better in the validation cohort than an adult equation, the IWPC algorithm (International Warfarin Pharmacogenetics Consortium, 2009),

performed in the derivation cohort. Despite a good correlation between the actual and predicted warfarin doses the IWPC algorithm consistently over-estimated warfarin dose by, on average, 1.5mg/day (Figure 3-17). The over-estimation of warfarin dose by the IWPC algorithm was explained by a greater influence of age on the variability in warfarin dose requirement in children than in adults. This indicates that pharmacogenetic-based dosing algorithms for use in children should be developed from data on paediatric populations.

The role of genetic polymorphisms in determining response to warfarin during the initiation phase of therapy is well established in adult patient populations in which studies have shown that individuals with *CYP2C9* variant alleles have a higher incidence of high INR (>4.0) (Higashi et al., 2002, Aithal et al., 1999, Joffe et al., 2004, Meckley et al., 2008) and a higher rate of serious bleeding events (Higashi et al., 2002, Aithal et al., 1999) during the first 3 months of warfarin therapy in addition to taking longer to reach a stable warfarin dose (Higashi et al., 2002, Meckley et al., 2008). A small study of 29 children showed that children who were heterozygous for a variant *CYP2C9* gene achieved target INR sooner and more frequently had an INR above the target level than those who were not (Ruud et al., 2008). In adults, *VKORC1* (-1639) genotype AA has been shown to be associated with a higher incidence of INR >5.0 during warfarin initiation when compared to GA or GG genotypes (Meckley et al., 2008). Our study showed a significant association between *VKORC1* (-1639) genotype and peak INR during the first week of warfarin therapy, children with AA genotype having a higher peak INR than those with GA or GG genotype. Also significant was the finding that children with one or more variant *CYP2C9* allele had a higher peak INR in week 1 compared to those with wild-type *CYP2C9* genotype. Children with a variant *CYP2C9* genotype tended to have a greater proportion of INR values above target range during the first month of warfarin therapy as did those with *VKORC1* (-1639) AA genotype. There was no correlation between genotype and the other initiation outcome measures including time to first therapeutic INR, time to first supra-therapeutic INR, number of high INRs (>4.0), time to stable warfarin dosing and number of warfarin dose changes. There was also no correlation between *CYP2C9* and *VKORC1* genotype and the proportion of INR values above target range beyond the first month of therapy suggesting that adjustment of warfarin doses

prior to months two and three of initiation therapy had counteracted the influence of genotype. The finding that *VKORC1* and *CYP2C9* genotypes were associated with the peak INR in week 1 and the proportion of INRs above range during the first month of warfarin therapy is in keeping with the results of previous studies in adult populations. However, my study was limited by the small patient cohort (n = 51), the lack of standardisation of data collection and the lack of a consistent approach to warfarin dosing during initiation. Most of the recruitment centres from which the study subjects were recruited have a warfarin-dosing protocol which is based on the recommended dose of 0.2mg/kg warfarin (maximum 5mg) on day 1 (Monagle et al., 2008) but the recorded warfarin doses suggested that this recommendation was not always adhered to and that doses were adjusted for reasons such as nutritional status and concomitant medications. There were no haemorrhagic events during initiation therapy despite a peak INR of >5.0 in 10 patients. The effect of *CYP2C9* and *VKORC1* genotype during warfarin initiation therapy in children requires further, prospective study in a larger cohort of children.

In conclusion, this study has shown that the majority (72.4%) of the inter-individual variability in warfarin dose requirement in children is attributed to by height, *VKORC1* and *CYP2C9* genotype and indication for warfarin therapy. Knowledge of the relative contributions of each of these factors has informed the development of a paediatric pharmacogenetics-guided warfarin-dosing algorithm which was shown to accurately predict maintenance warfarin dose when validated using an unrelated cohort of children. Before being used in clinical practice, this algorithm requires prospective evaluation in a large number of children undergoing initiation of anticoagulant therapy with warfarin. This would involve randomisation of children to a pharmacogenetics-guided warfarin-dosing algorithm or to standard empirical dosing. Due to the relatively small numbers of warfarinised children available for study this would likely require an international, multi-centre effort. In addition, this study has demonstrated that *VKORC1* and *CYP2C9* genotype have a modest effect on initiation therapy with warfarin and again this requires further study in a larger cohort of children.

Chapter 4. General Discussion

The anticoagulant response to warfarin is difficult to predict and there is considerable variation in dose requirement between individuals. Warfarin has a narrow therapeutic window and maintaining an INR within the appropriate target therapeutic range (TTR) is essential to optimise efficacy and safety of warfarin therapy. Deviations from TTR render the individual at risk of complications; thromboembolism when the INR is sub therapeutic and haemorrhage when the INR is supratherapeutic. Maintenance of INR within TTR is particularly difficult in anticoagulated children as there are many factors that contribute to poor stability of anticoagulant control in this patient group. The consequences for these children, the majority of whom have congenital cardiac disease and are anticoagulated long-term for the prevention of stroke, can be severe with deviations from TTR resulting in significant morbidity and mortality. In addition, the need for frequent blood sampling for INR monitoring is a significant burden for children and their carers.

The aim of this thesis was to identify the factors that influence anticoagulation outcomes in children and the potential for personalised warfarin therapy in this challenging patient population.

Our retrospective study of a cohort of children anticoagulated with warfarin showed that 57.4% of INR measurements were within target INR range. This finding is in keeping with the results of previous studies (Streif et al., 1999, Newall et al., 2004, Bradbury et al., 2008, Bhat et al., 2010) and highlights the poor stability of warfarin control that occurs in childhood even when INR monitoring occurs frequently with the aid of a home monitoring device. Some studies have shown an improvement in anticoagulation control using point-of-care (POC) monitoring combined with an education programme for parents and carers (Newall et al., 2006, Bauman et al., 2009). Quality control of the home monitoring device is also necessary, usually performed by duplicate testing of blood samples using either a machine that is quality controlled by the laboratory or the laboratory analyser (Fitzmaurice et al., 2005). This should occur at least once per year. However, this was not done routinely for our cohort of children, which may have influenced the INR results. Despite 42.6% of INR

measurements being outside of the TTR, more likely to be below than above target range, adverse events occurred infrequently. However, these findings were limited by the retrospective nature of data collection making it difficult to rule out incomplete and/or inaccurate reporting of adverse clinical events. The conservative management of a high INR (> 4.0), by omission of warfarin or a reduction in dose, ensured a prompt fall in INR in the majority of the cases with no reported haemorrhagic events. However, for the reasons explained above, these findings require verification in a larger cohort of prospectively-studied children.

In recent years there has been substantial progress in the identification of genetic polymorphisms that contribute to warfarin sensitivity in adults. My work, based on data derived from the largest cohort of anticoagulated children (n = 120) studied to date, has shown that 72% of the inter-individual variability in response to warfarin in children can be accounted for by height, *VKORC1* and *CYP2C9* genotype and the indication for warfarin therapy. The study results were used to develop a pharmacogenetics-based dosing algorithm. A study in an unrelated cohort (n = 23) of children further validated the algorithm for accurately predicting warfarin maintenance dose. The influence of polymorphisms in the genes for *VKORC1* and *CYP2C9* in children was shown to be consistent with studies in adults and the extent of the contribution to inter-individual variability in maintenance warfarin dose requirements was similar between adults and children (Wadelius et al., 2009, D'Andrea et al., 2005). Height, as an indicator of body size, made the most significant contribution to warfarin dose. The indication for warfarin therapy made a smaller contribution to warfarin dose requirement which was explained by a lower warfarin dose requirement in children who were anticoagulated following a Fontan procedure, a phenomenon that has been described previously (Streif et al., 1999). A greater proportion of variance in warfarin dose requirement could be explained in this paediatric population than has previously been explained in adult populations. This may in part relate to the observed effect of alcohol on warfarin anticoagulation response and the variation in alcohol intake that is seen amongst adults. An additional factor that is known to influence warfarin response and stability of anticoagulation control is adherence to warfarin therapy which has not been studied in children.

Analysis of data from a subgroup of the studied children (n = 51) showed that *VKORC1* and *CYP2C9* genotype influence outcome variables during initiation of warfarin therapy. These variables include peak INR response during week 1 and the proportion of supratherapeutic INRs during the first month of therapy. These data were limited by the retrospective nature of collection (which prevented the collection of reliable data on haemorrhagic complications that occurred during initiation of warfarin therapy) and a lack of conformity of approach between different centres in terms of the doses prescribed during warfarin loading. However, the results suggest that patient genotype can affect warfarin dose requirement during the initiation of therapy. Therefore information on *VKORC1* and *CYP2C9* genotype in individual patients could enable the identification of those who could be at risk of over-anticoagulation and haemorrhage during initiation of therapy and who may thus benefit from a pharmacogenetics-based personalised approach to warfarin dosing.

Prior to using a pharmacogenetics-based warfarin-dosing algorithm routinely in paediatric practice it will be necessary to prospectively validate such an algorithm in a large cohort of children and to compare efficacy and safety of this approach to that of standard warfarin dosing regimes. Due to variations in distribution of genotypes between different racial groups more data from populations of different ethnic backgrounds is required.

My study results showed that 27.4% of the inter-individual variability in warfarin dose requirement remains unexplained. This may partly be explained by differences in dietary vitamin K intake between children. The effect of vitamin K status on warfarin anticoagulation in adults is well established; a lower dietary vitamin K intake contributing to an increased sensitivity to warfarin (Khan et al., 2004, Franco et al., 2004) and poorer stability of anticoagulation control (Sconce et al., 2005a). To date no studies have looked at the effect of dietary vitamin K on warfarin dose requirement in a paediatric population. However, there is evidence that supplementation of a child's diet with (vitamin K-containing) infant formula or enteral feeds results in a reduced sensitivity to warfarin and a higher warfarin dose being required for the same level of anticoagulant effect (Streif et al., 1999). Variability in dietary vitamin K intake in

children is possible for a number of other reasons. The majority of children receiving warfarin have an underlying cardiac defect and may have a degree of malabsorption of vitamin K resulting from gut congestion due to right-sided cardiac failure (Kaulitz et al., 1997). Some also have ‘cardiac cachexia’, a reduced dietary intake due to breathlessness and fatigue. Warfarinised children also suffer from frequent intercurrent illnesses such as gastrointestinal infection which can have an immediate effect on dietary vitamin K intake and absorption and can quickly result in an over-anticoagulated state. Many children are ‘fussy’ eaters and eat only a limited range of foods. Vitamin K is found in high quantities in only a small number of foodstuffs, predominantly green leafy vegetables such as broccoli, cabbage, lettuce, spinach and Brussels sprouts. Milk and dairy products, cereals, fruit, eggs and potatoes also contain vitamin K but in much lower quantities. Foods that have been prepared with phylloquinone-rich oils, such as cakes, biscuits, potato chips and crisps, also contain a small amount of vitamin K (Shearer et al., 1996). A recent collaborative work with the Human Nutrition Research Centre, Newcastle University, suggests that these foods may contribute to the majority of the vitamin K content of a child’s diet as they are consumed more frequently by children. Using dietary questionnaires in a cohort of healthy children it was also seen that dietary vitamin K intake was low, particularly in younger children, and showed significant inter-individual variability [unpublished data]. Additional fasting blood samples were taken from the children recruited to the Pharmacogenetics study (as described in Chapter 3) for the estimation of serum vitamin K levels at a later date. The results of these analyses will allow us to determine whether a relationship exists between vitamin K status and warfarin maintenance dose and stability in children anticoagulated with warfarin.

There are now several alternative oral anticoagulant agents that are available for use in adult patients. These include the direct thrombin inhibitors, such as dabigatran, and the direct factor Xa inhibitors, such as rivaroxaban. These have a predictable dose-response relationship in adults, when dosed according to body weight, have few drug interactions, and do not require monitoring (Connolly et al., 2009, Eriksson et al., 2008, Lassen et al., 2008). However, as regard to interaction with diet, there is now firm evidence to show that dietary vitamin K affects anticoagulation response to these novel agents despite having

a different target to warfarin. These agents are an attractive alternative to warfarin in adults. National Institute for Health and Clinical Excellence (NICE) has approved dabigatran for extended thromboprophylaxis following hip or knee replacement surgery and for the prevention of stroke in adults with atrial fibrillation and additional risk factors. There are no dosing, efficacy or safety data available for the use of these oral agents in paediatric practice. Trials are being planned but they are likely to be limited by the small numbers of anticoagulated children who are available for study and licensing of these agents in children is likely to take several years or more (Young, 2011). Over recent years there has been an increasing tendency to substitute warfarin with low molecular weight heparins (LMWH) for the short-term treatment of thrombotic events occurring in childhood (Monagle et al., 2008). There are many advantages to this approach. LMWHs are administered subcutaneously, ensuring compliance and bioavailability, and tend to be monitored only monthly once a therapeutic level of activity has been confirmed (Payne, 2010). Dose requirements are more predictable (on a per kg dosing regime) than for warfarin. Anticoagulation response to LMWHs is not affected by intercurrent illness or variations in diet and LMWHs do not interact with other medications. However, warfarin remains the preferred approach for long-term anticoagulant therapy in the majority of children as injection sites become problematic and LMWHs are significantly more expensive than warfarin. For the reasons explained above it is likely that warfarin will remain the preferred option for long-term anticoagulant therapy in children for many years to come.

In summary, this research has provided an increased understanding of the factors that influence the variability in response to warfarin in children. The research led to the development and validation of a pharmacogenetics-based dosing algorithm for use in children. Before being used in clinical practice this algorithm requires prospective evaluation in a large number of children undergoing initiation therapy with warfarin in order to examine its' potential benefits in terms of a reduction in adverse events, reduced frequency of INR monitoring, improved quality of life and a reduction in healthcare-associated costs in this challenging patient population. A study of this nature is likely to require an international, multi-centre collaborative approach in order to have adequate power.

Chapter 5. References

AITHAL, G. P., DAY, C. P., KESTEVEN, P. J. L. & DALY, A. K. 1999. Association of polymorphisms in the cytochrome P450 CYP2C9 with warfarin dose requirement and risk of bleeding complications. *Lancet,* 353**,** 717-719.

ANDERSON, J. L., HORNE, B. D., STEVENS, S. M., WOLLER, S. C., SAMUELSON, K. M., MANSFIELD, J. W., ROBINSON, M., BARTON, S., BRUNISHOLZ, K., MOWER, C.P., HUNTINGHOUSE, J. A., ROLLO, J. S., SILER, D., BLAIR, T.L., KNIGHT, S., MUHLESTEIN, J. B. & CARLQUIST, J. F. 2012. Randomized and clinical effectiveness trial comparing two pharmacogenetic algorithms and standard care for individualizing warfarin dosing: CoumaGen-II. *Circulation,* 125**,** 1997-2005.

ANDREW, M., PAES, B., MILNER, R., JOHNSON, M., MITCHELL, L., TOLLEFSEN, D. M. & POWERS, P. 1987. Development of the human coagulation system in the full-term infant. *Blood,* 70**,** 165-172.

BARBUI, T., FINAZZI, G. & REMUZZI, A. 1995. Clinical coagulation laboratory and oral anticoagulant therapy treatment. Instrumentation and methodology. *Thrombosis and Haemostasis,* 74**,** 511-514.

BAUMAN, M. E., BLACK, K. & KUHLE, S. 2009. KIDCLOT©: The importance of validated educational intervention for optimal long term warfarin management in children. *Thrombosis Research,* 123**,** 707-709.

BHAT, D., UPPONI, A., RAKECHA, A. & THOMSON, J. 2010. Evaluating safety, effectiveness, and user satisfaction of home international normalized ratio monitoring service: Experience from a tertiary pediatric cardiology unit in the United Kingdom. *Pediatric Cardiology,* 31**,** 18-21.

BLACK, K. L., BAUMAN, M. E., KUHLE, S., BAJZAR, L. & MASSICOTTE, M. P. 2009. Warfarin therapy induced coagulopathy in children: assessment of a conservative approach. *Journal of Thrombosis and Haemostasis,* 7 (Suppl.1)**,** OC-TU-066.

BODIN, L., PERDU, J., DIRY, M., HORELLOU, M. H. & LORIOT, M. A. 2008. Multiple genetic alterations in vitamin K epoxide reductase complex subunit 1 (VKORC1) gene can explain high dose requirement during oral anticoagulation in humans. *Journal of Thrombosis and Haemostasis,* 6**,** 1436-1439.

BODIN, L., VERSTUYFT, C., TREGOUET, D.-A., ROBERT, A., DUBERT, L., FUNCK-BRENTANO, C., JAILLON, P., BEAUNE, P., LAURENT-PUIG, P., BECQUEMONT, L. & LORIOT, M.-A. 2005. Cytochrome P450 2C9 (CYP2C9) and vitamin K epoxide reductase (VKORC1) genotypes as determinants of acenocoumarol sensitivity. *Blood,* 106**,** 135-140.

BONDUEL, M., SCIUCCATI, G., HEPNER, M., FELIU TORRES, A., PIERONI, G., FRONTROTH, J. P. & SERVIDDIO, R. M. 2003. Acenocoumarol therapy in pediatric patients. *Journal of Thrombosis and Haemostasis,* 1**,** 1740-1743.

BONDUEL, M. M. 2006. Oral anticoagulation therapy in children. *Thrombosis Research,* 118**,** 85-94.

BORGIANI, P., CICCACCI, C., FORTE, V., SIRIANNI, E., NOVELLI, L., BRAMANTI, P. & NOVELLI, G. 2009. CYP4F2 genetic variant (rs2108622) significantly contributes to warfarin dosing variability in the Italian population. *Pharmacogenetics,* 10**,** 261-266.

BRADBURY, M. J., TAYLOR, G., SHORT, P. & WILLIAMS, M. D. 2008. A comparative study of anticoagulant control in patients on long-term warfarin using home and hospital monitoring of the international normalised ratio. *Archives of Disease in Childhood,* 93**,** 303-306.

BURMESTER, J. K., BERG, R. L., YALE, S. H., ROTTSCHEIT, C. M., GLURICH, I. E., SCHMELZER, J. R. & CALDWELL, M. D. 2011. A randomized controlled trial of genotype-based Coumadin initiation. *Genetic in Medicine,* 13**,** 509-518.

CALDWELL, M. D., AWAD, T., JOHNSON, J. A., GAGE, B. F., FALKOWSKI, M., GARDINA, P., HUBBARD, J., TURPAZ, Y., LANGAEE, T. Y., EBY, C., KING, C., BROWER, A., SCHMELZER, J. R., GLURICH, I., VIDAILLET, H. J., YALE, S. H., ZHANG, K. Q., BERG, R. L. & BURMESTER, J. K. 2008. CYP4F2 genetic variant alters required warfarin dose. *Blood,* 111**,** 4106-4112.

CARACO, Y., BLOTNICK, S. & MUSZKAT, M. 2008. CYP2C9 genotype-guided warfarin prescribing enhances the efficacy and safety of anticoagulation: a prospective randomized controlled study. *Clinical Pharmacology and Therapeutics,* 83**,** 460-470.

CARLQUIST, J. F., HORNE, B. D., MOWER, C., PARK, J., HUNTINGHOUSE, J., MCKINNEY, J. T., MUHLSTEIN, J. B. & ANDERSON, J. L. 2010. An evaluation of nine genetic variants related to metabolism and mechanism of action of warfarin as applied to stable dose prediction. *Journal of Thrombosis and Thrombolysis,* 30**,** 358-364.

CEN, H.-J., ZENG, W.-T., LENG, X.-Y., HUANG, M., CHEN, X., LI, J.-L., HUANG, Z.-Y., BI, H.-C., WANG, X.-D., HE, Y.-L., HE, F., ZHOU, R.-N., ZHENG, Q.-S. & ZHAO, L.-Z. 2010. CYP4F2 rs108622: a minor significant genetic factor of warfarin dose in Han Chinese patients with mechanical heart valve replacement. *British Journal of Clinical Pharmacology,* 70**,** 234-240.

CONNOLLY, S. J., EZEKOWITZ, M. D., YUSUF, S., EIKELBOOM, J., OLDGREN, J., PAREKH, A., POGUE, J., REILLY, P. A., THEMELES, E., VARRONE, J., WANG, S., ALINGS, M., XAVIER, D., ZHU, J., DIAZ, R., LEWIS, B. S., DARIUS, H., DIENER, H.-C., JOYNER, C. D. &

WALLENTIN, L. 2009. Dabigatran versus warfarin in patients with atrial fibrillation. *New England Journal of Medicine,* 361**,** 1139-1151.

COOPER, G. M., JOHNSON, J. A., LANGAEE, T. Y., FENG, H., STANAWAY, I. B., SCHWARZ, U. I., RITCHIE, M. D., STEIN, C. M., RODEN, D. M., SMITH, J. D., VEENSTRA, D. L., RETTIE, A. E. & RIEDER, M. J. 2008. A genome-wide scan for common genetic variants with a large influence on warfarin maintenance dose. *Blood,* 112**,** 1022-1027.

CRESPI, C. L. & MILLER, V. P. 1997. The R144C change in the CYP2C9*2 allele alters interaction of the cytochrome P450 with NADPH:cytochrome P450 oxidoreductase. *Pharmacogenetics,* 7**,** 203-210.

D'ANDREA, G., D'AMBROSIA, R. L., DI PERNA, P., CHETTA, M., SANTACROCE, R., BRANDCACCIO, V., GRANDONE, E. & MARGAGLIONE, M. 2005. A polymorphism in the VKORC1 gene is associated with an interindividual variability in the dose-anticoagulant effect of warfarin. *Blood,* 105**,** 645-649.

DALY, A. K., STEEN, V. M., FAIRBROTHER, K. S. & IDLE, J. R. 1996. CYP2D6 multiallelism. *Methods in Enzymology,* 272**,** 199-210.

ERIKSSON, B. I., BORRIS, L. C., FRIEDMAN, R. J., HAAS, S., HUISMAN, M. V., KAKKAR, A. K., BANDEL, T. J., BECKMANN, H., MUEHLHOFER, E., MISSELWITZ, F. & GEERTS, W. 2008. Rivaroxaban versus enoxaparin for thromboprophylaxis after hip arthroplasty. *New England Journal of Medicine,* 358**,** 2765-2775.

FITZMAURICE, D., GARDINER, C., KITCHEN, S., MACKIE, I., MURRAY, E. T. & MACHIN, S. J. 2005. An evidence-based review and guidelines for patient self-testing and management of oral anticoagulation. *British Journal of Haematology,* 131**,** 156-165.

FRANCO, V., POLANCYZK, C. A., CLAUSELL, N. & ROHDE, L. E. 2004. Role of dietary vitamin K intake in chronic oral anticoagulation: Prospective evidence from observational and randomized protocols. *American Journal of Medicine,* 116**,** 651-656.

FRENCH, B., JOO, J., GELLER, N. L., KIMMEL, S. E., ROSENBERG, Y., ANDERSON, J. L., GAGE, B. F., JOHNSON, J. A. & ELLENBERG, J. H. 2010. Statistical design of personalized medicine interventions: the Clarification of Optimal Anticoagulation through Genetics (COAG) trial. *Trials,* 11**,** 108.

GAGE, B. F., EBY, C., MILLIGAN, P. E., BANET, G. A., DUNCAN, J. R. & MCLEOD, H. L. 2004. Use of pharmacogenetics and clinical factors to predict the maintenance dose of warfarin. *Thrombosis and Haemostasis,* 91, 87-94.

GONG, I. Y., TIRONA, R. G., SCHWARZ, U. I., CROWN, N., DRESSER, G. K., LARUE, S., LANGLOIS, N., LAZO-LANGNER, A., ZOU, G., RODEN, D. M., STEIN, C. M., RODGER, M., CARRIER, M., FORGIE, M., WELLS, P.

S. & KIM, R. B. 2011. Prospective evaluation of a pharmacogenetics-guided warfarin loading and maintenance dose regimen for initiation of therapy. *Blood,* 118**,** 3163-3171.

GONZALEZ-CONEJERO, R., CORRAL, J., ROLDAN, V., FERRER, F., SANCHEZ-SERRANO, I., SANCHEZ-BLANCO, J. J., MARIN, F. & VICENTE, V. 2007. The genetic interaction between VKORC1 c1173t and calumenin a29809g modulates the anticoagulant response of acenocoumarol. *Thrombosis and Haemostasis,* 95**,** 205-211.

GREENWAY, A., IGNJATOVIC, V., SUMMERHAYES, R., NEWALL, F., BURGESS, J., DEROSA, L. & MONAGLE, P. 2009. Point-of-care monitoring of oral anticoagulant therapy in children. Comparison of the CoaguChek XS system with venous INR and venous INR using an Internation Reference Thromboplastin preparation (rTF/95). *Thrombosis and Haemostasis,* 102**,** 159-165.

HANLEY, J. P. 2004. Warfarin reversal. *Journal of Clinical Pathology,* 57**,** 1132-1139.

HAROON, Y., SHEARER, M. J., RAHIM, S., GUNN, W. G., MCENERY, G. & BARKHAN, P. 1982. The content of phylloquinone (vitamin K1) in human milk, cow's milk and infant formula foods determined by high-performance liquid chromatography. *Journal of Nutrition,* 112**,** 1105-1117.

HATCH, E., DARBYSHIRE, S., SCONCE, E., KING, B. P., WYNNE, H., DALY, A. K. & KAMALI, F. 2007. Polymorphisms in the components of the vitamin K epoxide reductase complex do not affect warfarin dose requirement. *British Journal of Clinical Pharmacology,* 63**,** 755.

HATCH, E., DARBYSHIRE, S., SCONCE, E. A., KING, B. P., WYNNE, H., AVERY, P., DALY, A. K. & KAMALI, F. Polymorphisms in the protein C and CYP4F2 genes and genes relevant to the Vitamin K cycle affecting warfarin dose requirement. Newcastle upon Tyne: Newcastle University.

HIGASHI, M. K., VEENSTRA, D. L., KONDO, L. M., WITTOWSKY, A. K., SRINOUANPRACHANH, S. L., FARIN, F. M. & RETTIE, A. E. 2002. Association between CYP2C9 genetic variants and anticoagulation-related outcomes during warfarin therapy. *Journal of the American Medical Association,* 287**,** 1690-1698.

HIRSH, J. 1991. Oral anticoagulant drugs: review article. *New England Journal of Medicine,* 324**,** 1865-1875.

HYLEK EM, S. D. 1994. Risk factors for intracranial hemorrhage in outpatients taking warfarin. *Annals of Internal Medicine,* 120**,** 897-902.

INTERNATIONAL WARFARIN PHARMACOGENETICS CONSORTIUM. 2009. Estimation of the warfarin dose with clinical and pharmacogenetic data. *New England Journal of Medicine,* 360, 753-764.

JOFFE, H. V., XU, R., JOHNSON, F. B., LONGTINE, J., KUCHER, N. & GOLDHABER, S. Z. 2004. Warfarin dosing and cytochrome P450 2C9 polymorphisms. *Thrombosis and Haemostasis,* 91, 1123-1128.

JOHNSON, M. C., WOOD, M., VAUGHN, V., COWAN, L. & SHARKEY, A. M. 2005. Interaction of antibiotics and warfarin in pediatric cardiology patients. . *Pediatric Cardiology,* 26**,** 589-592.

JORGENSEN, A. L., AL-ZUBIEDI, S., ZHANG, J. E., KENIRY, A., HANSON, A., HUGHES, D. A., VAN EKER, D., STEVENS, L., HAWKINS, K., TOH, C. H., KAMALI, F., DALY, A. K., FITZMAURICE, D., COFFEY, A., WILLIAMSON, P. R., PARK, B. K., DELOUKAS, P. & PIRMOHAMED, M. 2009. Genetic and environmental factors determining clinical outcomes and cost of warfarin therapy: a prospective study. *Pharmacogenetics and Genomics,* 19**,** 800-812.

KAMALI, F., KHAN, T. I., KING, B. P., FREARSON, R., KESTEVEN, P., WOOD, P., DALY, A. K. & WYNNE, H. 2004. Contribution of age, body size, and CYP2C9 genotype to anticoagulant response to warfarin. *Clinical Pharmacology and Therapeutics,* 75**,** 204-212.

KAMINSKY, L. S. & ZHANG, Z. Y. 1997. Human P450 metabolism of warfarin. *Pharmacology & Therapeutics,* 73**,** 67-74.

KATO, Y., ICHIDA, F., SAITO, K., WATANABE, K., HIRONO, K., MIYAWAKI, T., YOSHIMURA, N., HORIUCHI, I., TAGUCHI, M. & HASHIMOTO, Y. 2011. Effect of the VKORC1 genotype on warfarin dose requirements in Japanese pediatric patients. *Drug Metabolism and Pharmacokinetics,* 26**,** 295-299.

KAULITZ, R., LUHMER, I., BERGMANN, F., RODECK, B. & HAUSDORF, G. 1997. Sequelae after modified Fontan operation: postoperative haemodynamic data and organ function. *Heart,* 78**,** 154-159.

KAULITZ, R., ZIEMER, G., RAUCH, R., GIRISCH, M., BERTRAM, H., WESSEL, A. & HOFBECK, M. 2005. Prophylaxis of thromboembolic complications after the Fontan operation (total cavopulmonary anastomosis). *Journal of Thoracic and Cardiovascular Surgery,* 129**,** 569-575.

KEELING, D., BAGLIN, T., TAIT, C., WATSON, H., PERRY, D., BAGLIN, C., KITCHEN, S. & MAKRIS, M. 2011. Guidelines on oral anticoagulation with warfarin- fourth edition. *British Journal of Haematology,* 154**,** 311-324.

KHAN, T., WYNNE, H., WOOD, P., TORRANCE, A., HANKEY, C., AVERY, P., KESTEVEN, P. & KAMALI, F. 2004. Dietary vitamin K influences intra-individual variability in anticoagulant response to warfarin. *British Journal of Haematology,* 124**,** 348-354.

KIMMEL, S. E., CHRISTIE, J., KEALEY, C., CHEN, Z., PRICE, M., THORN, C. F., BRENSINGER, C. M., NEWCOMB, C. W. & WHITEHEAD, A. S.

2008. Apolipoprotein E genotype and warfarin dosing among Caucasians and African Americans. *Pharmacogenomics,* 8**,** 53-60.

KING, C. R., PORCH-SORBET, R. M., GAGE, B. F., RIDKER, P. M., RENAUD, Y., PHILLIPS, M. S. & EBY, C. 2008. Performance of commercial platforms for rapid genotyping of polymorphisms affecting warfarin dose. *American Journal of Clinical Pathology,* 129**,** 876-883.

KOHLMEIER, M., SALOMON, A., SAUPE, J. & SHEARER, M. J. 1996. Transport of vitamin K to bone in humans. *Journal of Nutrition,* 126**,** 1192S-1196S.

KOHNKE, H., SORLIN, K., GRANATH, G. & WADELIUS, M. 2005. Warfarin dose related to apolipoprotein E (APOE) genotype. *European Journal of Clinical Pharmacology,* 61**,** 381-388.

LAL, S., SANDANARAJ, E., JADA, S. R., KONG, M. C., LEE, L. H., GOH, B. C., LEE, S. C. & CHOWBAY, B. 2008. Influence of APOE genotypes and VKORC1 haplotypes on warfarin dose requirements in Asian patients. *British Journal of Clinical Pharmacology,* 65**,** 260-264.

LANE, S., AL-ZUBIEDI, S., HATCH, E., MATTHEWS, I., JORGENSEN, A. L., DELOUKAS, P., DALY, A. K., PARK, B. K., AARONS, L., OGUNGBENRO, K., KAMALI, F., HUGHES, D. & PIRMOHAMED, M. 2012. The population pharmacokinetics of R and S-warfarin: effect of genetic and clinical factors. *British Journal of Clinical Pharmacology***,** 73, 66-76.

LASSEN, M. R., AGENO, W., BORRIS, L. C., LIEBERMAN, J. R., ROSENCHER, N., BANDEL, T. J., MISSELWITZ, F. & TURPIE, A. G. G. 2008. Rivaroxaban versus enoxaparin for thromboprophylaxis after total knee arthroplasty. *New England Journal of Medicine,* 358**,** 2776-2786.

LEE, C. R., GOLDSTEIN, J. A. & J.A., P. 2002. Cytochrome P450 2C9 polymorphisms: a comprehensive review of the in-vitro and human data. *Pharmacogenetics,* 12**,** 251-263.

LI, T., CHANG, C. Y., JIN, D. Y., LIN, P. J., KHVOROVA, A. & STAFFORD, D. W. 2004. Identification of the gene for vitamin K epoxide reductase. *Nature,* 427**,** 541-544.

LIMDI, N. A., WADELIUS, M., CAVALLARI, L., ERIKSSON, N., CRAWFORD, D. C., LEE, M. T., CHEN, C. H., MOTSINGER-REIF, A., SAGREIYA, H., LIU, N., WU, A. H., GAGE, B. F., JORGENSEN, A. L., PIRMOHAMED, M., SHIN, J. G., SUAREZ-KURTZ, G., KIMMEL, S. E., JOHNSON, J. A., KLEIN, T. E. & WAGNER, M. J. 2010. Warfarin pharmacogenetics: a single VKORC1 polymorphism is predictive of dose across 3 racial groups. *Blood,* 115**,** 3827-3834.

LOELINGER, E.A. 1985. Laboratory control, optimal therapeutic ranges, and therapeutic quality control in oral anticoagulation. *Acta Haematologica,* 74, 125-131.

MARGAGLIONE, M., COLAIZZO, D., D'ANDREA, G., BRANCACCIO, V., CIAMPA, A., GRANDONE, E. & DI MINNO, G. 2000. Genetic modulation of oral anticoagulation with warfarin. *Thrombosis and Haemostasis,* 84, 775-778.

MARZINOTTO, V., MONAGLE, P., CHAN, A., ADAMS, M., MASSICOTTE, P., LEAKER, M. & ANDREW, M. 2000. Capillary whole blood monitoring of oral anticoagulants in children in outpatient clinics and the home setting. *Pediatric Cardiology,* 21**,** 347-352.

MASSICOTTE, P., JULIAN, J. A., GENT, M., SHIELDS, K., MARZINOTTO, V., SZECHTMAN, B. & ANDREW, M. 2003. An open-label randomized controlled trial of low molecular weight heparin compared to heparin and coumadin for the treatment of venous thromboembolic events in children: the REVIVE trial. *Thrombosis Research,* 109**,** 85-92.

MCDONALD, M. G., RIEDER, M. J., NAKANO, M., HSIA, C. K. & RETTIE, A. E. 2009. CYP4F2 is a vitamin K1 oxidase: An explanation for altered warfarin dose in carriers of the V433M variant. *Molecular Pharmacology,* 75**,** 1337-1346.

MECKLEY, L. M., WITTOWSKY, A. K., RIEDER, M. J., RETTIE, A. E. & VEENSTRA, D. L. 2008. An analysis of the relative effects of VKORC1 and CYP2C9 variants on anticoagulation related outcomes in warfarin-treated patients. *Thrombosis and Haemostasis,* 100**,** 229-239.

MILLICAN, E. A., LENZINI, P. A., MILLIGAN, P. E., GROSSO, L., EBY, C., DEYCH, E., GRICE, G., CLOHISY, J. C., BARRACK, R. L., BURNETT, R. S. J., VOORA, D., GATCHEL, S., TIEMEIER, A. & GAGE, B. F. 2007. Genetic-based dosing in orthopedic patients beginning warfarin therapy. *Blood,* 110**,** 1511-1515.

MONAGLE, P., CHALMERS, E., CHAN, A., DEVEBER, G., KIRKHAM, F., MASSICOTTE, P. & MICHELSON, A. D. 2008. Antithrombotic therapy in neonates and children. *Chest,* 133**,** 887S-968S.

MONAGLE, P., COCHRANE, A., ROBERTS, R., MANLHIOT, C., WEINTRAUB, R., SZECHTMAN, B. A., HUGHES, M., ANDREW, M. & MCCRINDLE, B. W. 2011. A multicenter, randomized trial comparing heparin/warfarin and acetylsalicylic acid as primary thromboprophylaxis for 2 years after the Fontan procedure in children. *Journal of the American College of Cardiology,* 58**,** 645-651.

MOREAU, C., PAUTAS, E., GOUIN-THIBAULT, I., GOLMARD, J. L., MAHE, I., MULOT, C., LORIOT, M. A. & V., S. 2011. Predicting the warfarin maintenance dose in elderly inpatients at treatment initiation: accuracy of dosing algorithms incorporating or not VKORC1/CYP2C9 genotypes. *Journal of Thrombosis and Haemostasis,* 9**,** 711-718.

MOSTELLER, R.D. 1987. Simplified calculation of body-surface area. *New England Journal of Medicine,* 317, 1098.

MURRY, D. J., CROM, W. R., REDDICT, W. E., BHARGAVA, R. & EVANS, W. E. 1995. Liver volume as a determinant of drug clearance in children and adolescents. *Drug Metabolism and Disease,* 23**,** 1110-1116.

NEWALL, F., CAMPBELL, J., SAVOIA, H., BARNES, C. & MONAGLE, P. 2005. Incidence of major bleeding in a large paediatric cohort of patients requiring warfarin therapy. *Journal of Thrombosis and Haemostasis,* 3 (Suppl.1)**,** OR357.

NEWALL, F., MONAGLE, P. & JOHNSTON, L. 2006. Home INR monitoring of oral anticoagulant therapy in children using the CoaguchekTM S point-of-care monitor and a robust education program. *Thrombosis Research,* 118**,** 587-593.

NEWALL, F., SAVOIA, H., CAMPBELL, J. & MONAGLE, P. 2004. Anticoagulation clinics for children acheive improved warfarin management. *Thrombosis Research,* 114, 5-9.

NOWAK-GÖTTL, U., DIETRICH, K., SCHAFFRANEK, D., ELDIN, N.S., YASUI, Y., GEISEN, C. & MITCHELL, L. G. 2010. In pediatric patients, age has more impact on oral anticoagulant dose than VKORC1 or CYP2C9 genotypes. *Blood,* 116, 6101-6105.

OGG, M. S., BRENNAN, P., MEADE, T. & HUMPHRIES, S. E. 1999. CYP2C9*3 allelic variant and bleeding complications. *Lancet,* 354**,** 1124.

O'REILLY, R. A. & MOTLEY, C. H. 1979. Racemic warfarin and trimethoprim-sulfamethoxazole interaction in humans. *Annals of Internal Medicine,* 91**,** 34-36.

PALARETI, G., LEALI, N., COCCHERI, S., POGGI, M., MANOTTI, C., D'ANGELO, A., PENGO, V., ERBA, N., MOIA, M., CIAVARELLA, N., DEVOTO, G., BERRETTINI, M. & MUSOLESI, S. 1996. Bleeding complications of oral anticoagulant treatment: an inception-cohort, prospective collaborative study (ISCOAT). Italian Study on Complications of Oral Anticoagulant Therapy. *Lancet,* 348**,** 423-428.

PAYNE, J. H. 2010. Aspects of anticoagulation in children. *British Journal of Haematology,* 150**,** 259-277.

PEREZ-ANDREU, V., ROLDAN, V., ANTON, A. I., GARCIA-BARBERA, N., CORRAL, J., VICENTE, V. & GONZALEZ-CONEJERO, R. 2009. Pharmacogenetic relevance of CYP4F2 V433M polymorphism on acenocoumarol therapy. *Blood,* 113**,** 4977-4979.

PERINI, J. A., STRUCHINER, C. J., SILVA-ASSUNCAO, E. & SUAREZ-KURTZ, G. 2010. Impact of CYP4F2 rs2108622 on the stable warfarin dose in an admixed patient cohort. *Clinical Pharmacology and Therapeutics,* 87**,** 417-420.

PEYVANDI, F., SPREAFICO, M., SIBONI, S. M., MOIA, M. & MANNUCCI, P. M. 2004. CYP2C9 genotypes and dose requirements during the

induction phase of oral anticoagulant therapy. *Clinical Pharmacology and Therapeutics,* 75, 198-203.

RETTIE, A. E., WIENKERS, L. C., GONZALEZ, F. J., TRAGER, W. F. & KORZEKWA, K. R. 1994. Impaired (S)-warfarin metabolism catalysed by the R144C allele variant of CYP2C9. *Pharmacogenetics,* 4**,** 39-42.

RIEDER, M. J., REINER, A. P., GAGE, B. F., NICKERSON, D. A., EBY, C. S., MCLEOD, H. L., BLOUGH, D. K., THUMMEL, K. E., VEENSTRA, D. L. & RETTIE, A. E. 2005. Effect of VKORC1 haplotypes on transcriptional regulation and warfarin dose. *New England Journal of Medicine,* 352**,** 2285-2293.

RIEDER, M. J., REINER, A. P. & RETTIE, A. E. 2007. Gamma-glutamyl carboxylase (GGCX) tag SNPs have limited utility for predicting warfarin maintenance dose. *Journal of Thrombosis and Haemostasis,* 5**,** 2227-2234.

ROSENDAAL, F., CANNEGIETER, S., VAN DER MEER, F. & BRIET, E. 1993. A method to determine the optimal intensity of oral anticoagulant therapy. *Thrombosis and Haemostasis,* 69**,** 236-239.

ROST, S., FREGIN, A., IVASKEVICIUS, V., CONZELMANN, E., HORTNAGEL, K., PELZ, H.-J., LAPPEGARD, K., SEIFRIED, E., SCHARRER, I., TUDDENHAM, E. G. D., MULLER, C. R., STROM, T. M. & OLDENBURG, J. 2004. Mutations in VKORC1 cause warfarin resistance and multiple coagulation factor deficiency type 2. *Nature,* 427**,** 537-541.

RUUD, E., HOLMSTROM, H., BERGAN, S. & WESENBERG, F. 2008. Oral anticoagulation with warfarin is significantly influenced by steroids and CYP2C9 polymorphisms in children with cancer. *Pediatric Blood and Cancer,* 50**,** 710-713.

SACHSE, C., BROCKMOLLER, J., BAUER, S. & ROOTS, I. 1999. Functional significance of a C-->A polymorphism in intron 1 of the cytochrome P450 CYP1A2 gene tested with caffeine. *British Journal of Clinical Pharmacology,* 47**,** 445-449.

SAMSA, G. P. & MATCHAR, D. B. 2000. Relationship between test frequency and outcomes of anticoagulation: A literature review and commentary with implications for the design of randomized trials of patient self-management. *Journal of Thrombosis and Thrombolysis,* 9**,** 283-292.

SCHMITT, L., SPECKMAN, J. & ANSELL, J. 2003. Quality assessment of anticoagulation dose management: comparative evaluation of measures of time-in-therapeutic range. *Journal of Thrombosis and Thrombolysis,* 15**,** 213-216.

SCHULMAN, S., KEARON, C. 2005. Definition of major bleeding in clinical investigations of antihemostatic medicinal products in non-surgical patients. *Journal of Thrombosis and Haemostasis,* 3, 692-694.

SCONCE, E., AVERY, P., WYNNE, H. & KAMALI, F. 2007. Vitamin K supplementation can improve stability of anticoagulation for patients with unexplained variability in response to warfarin. *Blood,* 109**,** 2419-2423.

SCONCE, E., KHAN, T., MASON, J., NOBLE, F., WYNNE, H. & KAMALI, F. 2005a. Patients with unstable control have a poorer dietary intake of vitamin K compared to patients with stable control of anticoagulation. *Thrombosis and Haemostasis,* 93**,** 872-875.

SCONCE, E. A., KHAN, T. I., WYNNE, H. A., AVERY, P., MONKHOUSE, L., KING, B. P., WOOD, P., KESTEVEN, P., DALY, A. K. & KAMALI, F. 2005b. The impact of CYP2C9 and VKORC1 genetic polymorphism and patient characteristics upon warfarin dose requirements: proposal for a new dosing regimen. *Blood,* 106**,** 2329-2333.

SCORDO, M. G., PENGO, V., SPINA, E., DAHL, M. L., GUSELLA, M. & PADRINI, R. 2002. Influence of CYP2C9 and CYP2C19 genetic polymorphisms on warfarin maintenance dose and metabolic clearance. *Clinical Pharmacology and Therapeutics,* 72, 702-710.

SCOTT, S. A., KHASAWNEH, R., PETER, I., KORNREICH, R. & DESNICK, R. J. 2010. Combined CYP2C9, VKORC1 and CYP4F2 frequencies among racial and ethnic groups. *Pharmacogenetics,* 11**,** 781-791.

SHEARER, M. J., BACH, A. & KOHLMEIER, M. 1996. Chemistry, nutritional sources, tissue distribution and metabolism of vitamin K with special reference to bone health. *Journal of Nutrition,* 126**,** 11815-11865.

SINGH, O., SANDANARAJ, E., SUBRAMANIAN, K., LEE, L. H. & CHOWBAY, B. 2011. Influence of CYP4F2 rs2108622 (V433M) on warfarin dose requirement in Asian patients. *Drug Metabolism and Pharmacokinetics,* 26**,** 130-136.

STEIN, P. D., KAYALI, F. & OLSON, R. E. 2004. Incidence of venous thromboembolism in infants and children: data from the National Hospital Discharge Survey. *Journal of Pediatrics,* 145**,** 563-565.

STREIF, W., ANDREW, M., MARZINOTTO, V., MASSICOTTE, P., CHAN, A. K. C., JULIAN, J. A. & MITCHELL, L. 1999. Analysis of warfarin therapy in pediatric patients. A prospective cohort study of 319 patients. *Blood,* 94**,** 3007-3014.

SULLIVAN-KLOSE, T., GHANAYEM, B. I., BELL, D. A., ZHANG, Z. Y., KAMINSKY, L. S., SHENFIELD, G. M., MINERS, J. O., BIRKETT, D. J. & GOLDSTEIN, J. A. 1996. The role of the CYP2C9-Leu359 allelic variant in the tolbutamide polymorphism. *Pharmacogenetics,* 6**,** 341-349.

TAKAHASHI, H., ISHIKAWA, S., NOMOTO, S., NISHIGAKI, Y., ANDO, F., KASHIMA, T., KIMURA, S., KANAMORI, M. & ECHIZEN, H. 2000. Developmental changes in pharmacokinetics and pharmacodynamics of warfarin enantiomers in Japanese children. *Clinical Pharmacology and Therapeutics,* 68**,** 541-545.

TAKANASHI, K., TAINAKA, H., KOBAYASHI, K., YASUMORI, T., HOSAKAWA, M. & CHIBA, K. 2000. CYP2C9 Ile359 and Leu359 variants: enzyme kinetic study with seven substrates. *Pharmacogenetics,* 10**,** 95-104.

TAKEUCHI, F., MCGINNIS, R., BOURGEOIS, S., BARNES, C., ERIKSSON, N., SORANZO, N., WHITTAKER, P., RANGANATH, V., KUMANDURI, V., MCLAREN, W., HOLM, L., LINDH, J., RANE, A., WADELIUS, M. & DELOUKAS, P. 2009. A genome-wide association study confirms VKORC1, CYP2C9 and CYP4F2 as principal genetic determinants of warfarin dose. *PLoS Genetics*, 5:e1000433.

TAUBE, J., HALSALL, D. & BAGLIN, T. 2000. Influence of cytochrome P-450 CYP2C9 polymorphisms on warfarin sensitivity and risk of over-anticoagulation in patients on long-term treatment. *Blood,* 96, 1816-1819.

VAN LEEUWEN, Y., ROSENDAAL, F. R. & VAN DER MEER, F. J. M. 2008. The relationship between maintenance dosages of three vitamin K antagonists: Acenocoumarol, warfarin and phenprocoumon. *Thrombosis Research,* 123**,** 225-230.

VAN SCHIE, R. M., WADELIUS, M., KAMALI, F., DALY, A. K., MANOLOPOULOS, V. G., DE BOER, A., BARALLON, R., VERHOEF, T. I., KIRCHHEINER, J., HASCHKE-BECHER, E., BRIZ, M., ROSENDAAL, F. R., REDEKOP, W. K., PIRMOHAMED, M. & MAITLAND VAN DER ZEE, A. H. 2009. Genotype-guided dosing of coumarin derivatives: the European pharmacogenetics of anticoagulant therapy (EU-PACT) trial design. *Pharmacogenomics,* 10**,** 1687-1695.

VECSLER, M., LOEBSTEIN, R., ALMOG, S., KURNIK, D., GOLDMAN, B., HALKIN, H. & GAK, E. 2006. Combined genetic profiles of components and regulators of the vitamin K-dependent gamma-carboxylation system affect individual sensitivity to warfarin. *Thrombosis and Haemostasis,* 95**,** 205-211.

VOORA, D., EBY, C., LINDER, M. W., MILLIGAN, P. E., BUKAVECKAS, B. L., MCLEOD, H. L., MALONEY, W., CLOHISY, J., BURNETT, R. S., GROSSO, L., GATCHEL, S. K. & GAGE, B. F. 2005. Prospective dosing of warfarin based on cytochrome P-450 2C9 genotype. *Thrombosis and Haemostasis,* 93**,** 700-705.

WADELIUS, M., CHEN, L. Y., DOWNES, K., GHORI, J., HUNT, S., ERIKSSON, N., WALLERMAN, O., MELHUS, H., WADELIUS, C., BENTLEY, D. & DELOUKAS, P. 2005. Common VKORC1 and GGCX polymorphisms associated with warfarin dose. *Pharmacogenomics Journal,* 4**,** 40-48.

WADELIUS, M., CHEN, L. Y., LINDH, J. D., ERIKSSON, N., GHORI, M. J. R., BUMPSTEAD, S., HOLM, L., MCGINNIS, R., RANE, A. & DELOUKAS, P. 2009. The largest prospective warfarin-treated cohort supports genetic forecasting. *Blood,* 113**,** 784-792.

WADELIUS, M., SORLIN, K., WALLERMAN, O., KARLSSON, J., YUE, Q.-Y., MAGNUSSON, P. K. E., WADELIUS, C. & MELHUS, H. 2004. Warfarin sensitiviey related to CYP2C9, CYP3A5, ABCB1 (MDR1) and other factors. *Pharmacogenomics Journal,* 4**,** 40-48.

WEISGRABER, K. H., INNERARITY, T. L. & MAHLEY, R. W. 1982. Abnormal lipoprotein receptor-binding activity of the human E apolipoprotein due to cysteine-arginine interchange at a single site. *The Journal of Biological Chemistry,* 257**,** 2518-2521.

WELLS, P. S., HOLBROOK, A. M., CROWTHER, N. R. & HIRSH, J. 1994. Interactions of warfarin with drugs and food. *Annals of Internal Medicine,* 121**,** 676-683.

WHITTAKER, P., BUMPSTEAD, S., DOWNES, K., GHORI, J. & DELOUKAS, P. 2005. SNP analysis by MALDI-TOF mass spectrometry. *In:* CELIS, J., CARTER, N., SIMONS, K., SMALL, J. V., HUNTER, T. & SHOTTON, D. (eds.) *Cell Biology: A Laboratory Handbook.* 3rd ed. Amsterdam: Elsevier.

YOUNG, G. 2011. New anticoauglants in children: a review of recent studies and a look to the future. *Thrombosis Research,* 127**,** 70-74.

Appendix A. Data collection proforma (UK)

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

DATA COLLECTION PROFORMA

Inter-individual variability in response to warfarin in children: Analysis of environmental and pharmacogenetic factors

Date: _____

Centre Number: _____ Patient Identification Number: _____

Age: _____ Date of birth (dd/mm/yy): _____

Gender: Male ☐ Female ☐

Height: _____cm Weight: _____kg

Ethnicity: (please indicate overleaf)

Current diet:
Normal ☐ Vegetarian ☐ Vegan ☐ Infant formula ☐ Breastfed ☐ Enteral nutrition ☐
Parenteral nutrition ☐ (please mark all that apply)

For infant formula, enteral and parenteral nutrition please indicate brand, daily volume received and vitamin K content (e.g. X mcg/100 mls) _____

Indication(s) for treatment with warfarin: _____

Other medical problems: _____
_____

Start date of warfarin: _____

Desired INR range: _____

Other medication(s): _____
_____

Current warfarin dose: _____
No change in warfarin dose for the last 3 INR measurements AND at least the last one month

Most recent INR value: _____ Date of result (dd/mm/yy): _____

Please give details of any previous thrombotic or haemorrhagic events occurring whilst receiving treatment with warfarin (include date; other precipitating factors; INR at time of event; management of event; outcome): _____
_____
_____
_____

DETAILS OF SAMPLES PROVIDED:

Date of blood sample: _____ Time of blood sample: _____

Fasting: Y ☐ N ☐ If No, Time last ate: _____ Details of last food eaten: _____
_____

Has the warfarin been stopped, e.g. for a procedure? : Y ☐ N ☐ If Yes, Date of last dose: _____

1. Genotyping- 8mls in EDTA (minimum 4mls): ☐

2. Vitamin K/ Warfarin levels- 4mls in Lithium heparin (minimum 2mls): ☐

Version 2.0- (UK) Date: 28th June 2009

DATA COLLECTION PROFORMA

Ethnicity: (please select from the list below)

White British	☐	
White Irish	☐	
Indian	☐	
Pakistani	☐	
Bangladeshi	☐	
Other Asian	☐	
Black Caribbean	☐	
Black African	☐	
Black Other	☐	
Chinese	☐	
Other ethnic group (please state)	☐	_____
Mixed White and Black Caribbean	☐	
Mixed White and Black African	☐	
Mixed White and Asian	☐	
Other Mixed background (please state)	☐	_____

Appendix B. Data collection proforma (Canada)

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

DATA COLLECTION PROFORMA

Inter-individual variability in response to warfarin in children: Analysis of environmental and pharmacogenetic factors

Date: _____

Centre Number: _____ Patient Identification Number: _____

Age: _____ Date of birth (dd/mm/yy): _____

Gender: Male ☐ Female ☐

Height: _____cm Weight: _____kg

Ethnicity: (please indicate overleaf)

Current diet:
Normal ☐ Vegetarian ☐ Vegan ☐ Infant formula ☐ Breastfed ☐ Enteral nutrition ☐
Parenteral nutrition ☐ (please mark all that apply)

For infant formula, enteral and parenteral nutrition please indicate brand, daily volume received and vitamin K content (e.g. X mcg/100 mls) _____

Indication(s) for treatment with coumadin: _____

Other medical problems: _____
_____

Start date of coumadin: _____

Desired INR range: _____

Other medication(s): _____
_____

Current coumadin dose: _____
No change in coumadin dose for the last 3 INR measurements AND at least the last one month

Most recent INR value: _____ Date of result (dd/mm/yy): _____

Please give details of any previous thrombotic or haemorrhagic events occurring whilst receiving treatment with coumadin (include date; other precipitating factors; INR at time of event; management of event; outcome): _____
_____
_____
_____

DETAILS OF SAMPLES PROVIDED:

Date of blood sample: _____ Time of blood sample: _____

Fasting: Y ☐ N ☐ If No, Time last ate: _____ Details of last food eaten: _____
_____

Has the coumadin been stopped, e.g. for a procedure? : Y ☐ N ☐ If Yes, Date of last dose: _____

1. Genotyping- 8mls in EDTA (minimum 4mls): ☐

2. Vitamin K/ coumadin levels- 4mls in Lithium heparin (minimum 2mls): ☐

Version 2.0- (Canada) Date: 11th January 2010

DATA COLLECTION PROFORMA

Ethnicity: (please select from the list below)

An aboriginal person (eg. North American Indian, Metis, Inuit, Eskimo)	☐	
White	☐	
Chinese	☐	
South Asian (eg. East Indian, Pakistani, Sri Lankan, etc.)	☐	
Black	☐	
Filipino	☐	
Latin American	☐	
Southeast Asian (eg. Cambodian, Indonesian, Laotian, Vietnamese, etc.)	☐	
Arab	☐	
West Asian (eg. Afghan, Iranian, etc.)	☐	
Japanese	☐	
Korean	☐	
Other (please specify)	☐	_____

Appendix C. Oral presentation at British Society for Haematology 52nd Annual Scientific Meeting, Glasgow, April 2012

Warfarin dose prediction in children using pharmacogenetics information

Tina T. Biss[1], Anna-Karin Hamberg[2], Peter J. Avery[3], Mia Wadelius[2], Farhad Kamali[1]

[1]Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, UK; [2]Department of Medical Sciences, Clinical Pharmacology, Uppsala University Hospital, Uppsala, Sweden; [3]School of Mathematics & Statistics, Newcastle University, Newcastle upon Tyne, UK.

We recently reported the development of the first pharmacogenetics-based algorithm for predicting warfarin dose in children, with dose prediction based on height, *VKORC1* and *CYP2C9* genotype and indication for warfarin therapy. This study aimed to validate the algorithm in an unrelated cohort of anticoagulated children. 23 children with a stable warfarin dose were studied, 13 females, median age 7.9 years (range: 10 months- 16 years). Children were genotyped for *VKORC1* (-1639G>A; rs9923231) and *CYP2C9* *2 (R144C; rs1799853) and *3 (I359L; rs1057910) alleles and their clinical and demographic features were recorded. Although younger (median age 7.9 years vs. 11.4 years) and with a higher proportion of children anticoagulated to a higher target therapeutic range (43.4% vs. 15.8% INR >2.5) this patient cohort compared well to the derivation cohort in terms of ethnicity and genotype distribution. *VKORC1* (-1639G>A) genotype was GG in six children, GA in 13 and AA in four. Fifteen children had wild-type *CYP2C9*, three each were heterozygote for *2 or *3 allele, one was homozygous *2/*2 and one was double heterozygote *2/*3. Predicted warfarin dose was compared with the actual warfarin dose using Pearson's correlation analysis. The actual mean daily warfarin dose was 3.0mg (range: 0.9- 5.4mg; SD ± 1.4) which compared well to the mean predicted daily warfarin dose of 2.9mg (range: 0.1- 5.3mg; SD ± 1.6). There was a close and highly significant relationship between the square root of the actual and the predicted warfarin dose ($r = 0.874$, $P < 0.001$). Our pharmacogenetics-based warfarin-dosing algorithm is able to accurately predict maintenance warfarin dose in children. Further prospective studies in children are required in order to evaluate the utility of the pharmacogenetics-based algorithm and its potential benefits in terms of improvements in safety and efficacy of warfarin.

WARFARIN DOSE PREDICTION IN CHILDREN USING PHARMACOGENETICS INFORMATION

Tina T. Biss[1], Anna-Karin Hamberg[2], Peter J. Avery[3], Mia Wadelius[2], Julian B.S. Leathart[1],Farhad Kamali[1]

[1] Institute of Cellular Medicine, Newcastle University, UK; [2]Department of Medical Sciences, Clinical Pharmacology, Uppsala University Hospital, Sweden;[3]School of Mathematics & Statistics, Newcastle University, UK

Newcastle University

Background

- Anticoagulant therapy is being used increasingly in children for the treatment and prevention of thromboembolic events
- Warfarin remains the long-term anticoagulant of choice in children
- Anticoagulation response to a fixed dose of warfarin is difficult to predict in this patient group

Background

- Approximately half of the variability in response to warfarin in adults can be explained by polymorphisms in the *VKORC1* (-1639G>A) and *CYP2C9* (*2/*3) genes
- Pharmacogenetics-guided warfarin-dosing algorithms have been developed and validated for use in adults
- 72% of the variability in maintenance warfarin dose in a cohort of children can be explained by clinical and genetic factors

blood 2012 119: 868-873
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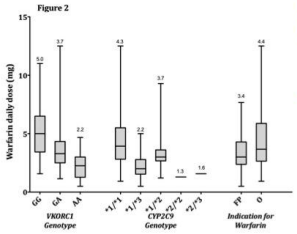
***VKORC1* and *CYP2C9* genotype and patient characteristics explain a large proportion of the variability in warfarin dose requirement among children**

Tina T. Biss, Peter J. Avery, Leonardo R. Brandão, Elizabeth A. Chalmers, Michael D. Williams, John D. Grainger, Julian B. S. Leathart, John P. Hanley, Ann K. Daly and Farhad Kamali

Figure 2



- 120 children
- 4 UK centres, 1 Canadian centre
- Height was the major predictor of maintenance warfarin dose

Regression model

x variable	*P* value	Contribution to model, %
Height	< 0.001	29.8
VKORC1	< 0.001	26.6
CYP2C9	< 0.001	12.8
Indication	< 0.001	3.2
Height, *VKORC1*, *CYP2C9*, Indication	**<0.001**	**72.4**

√dose = -0.009 + 0.011 (height, cm) + 0.357 (*VKORC1*)* – 0.478 (*CYP2C93)** – 0.277 (*CYP2C9**2)*** + 0.186 (Indication)******

* 0 for AA, 1 for AG, 2 for GG
** Input 0, 1 or 2 for the number of *3 alleles
*** Input 0, 1 or 2 for the number of *2 alleles
**** 0 for Fontan procedure, 1 for other indication

Aim of the study

- To evaluate the accuracy of warfarin dose prediction using the regression equation in an unrelated cohort of anticoagulated children

Methods

- Cross-sectional, observational study
- 3 tertiary care centres in Sweden
- Eligibility criteria:
  - Children aged ≤ 18 years
  - Currently or previously anticoagulated with warfarin
  - Accessible history of warfarin dosing and INRs
- Indication for warfarin therapy was recorded
- Patient age, height, weight and warfarin dose were recorded at a time-point when the child was stable on warfarin:
  - No change in dose for at least 3 consecutive INR measurements AND for at least 4 weeks

Methods

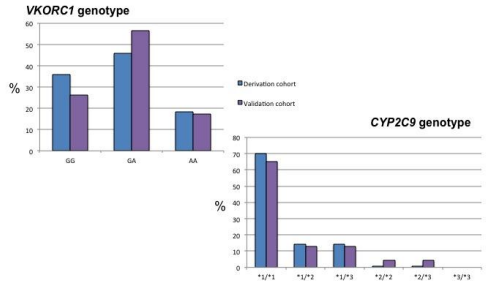
- Venous blood sample taken into EDTA and stored at -80°C
- Genotyped using StepOne™ Real-Time PCR System with Taqman® SNP Genotyping Assays (Applied Biosystems™)
  - *VKORC1*: -1639G>A
  - *CYP2C9*: *2/*3 alleles
- The predicted daily warfarin dose for each patient was calculated using the regression equation
- The square root of the predicted dose was compared to the square root of the actual dose using Pearson's correlation analysis

Patient Characteristics

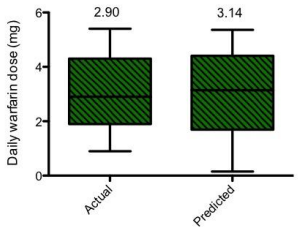
	Derivation Cohort (n = 120)*	Validation Cohort (n = 23)
Median age, yrs	11.4 (1- 18)	7.9 (0-16)
Gender, %		
- **Male**	68.8	43.5
- **Female**	31.7	56.5
Ethnic origin, %		
- **White Caucasian**	75.8	82.6
- **Asian**	8.3	13.0
- **Other**	15.8	4.3
Indication, %		
- **Fontan**	53.3	30.4
- **Prosthetic valve**	15.0	34.8
- **Other**	31.7	34.8
Target INR range, %		
- **1.8 – 2.5**	0	4.3
- **2.0 – 3.0**	84.2	52.2
- **2.5 – 3.5**	15.8	30.4
- **2.7 – 4.0**	0	13.0

*Biss et al., Blood 2012

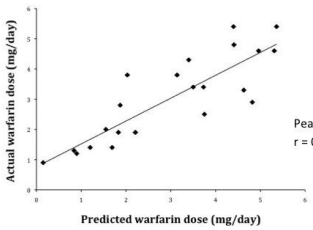
Genetic Characteristics of Study Population



Comparison of Actual vs. Predicted Warfarin Dose



Comparison of Actual vs. Predicted Warfarin Dose



Pearson's correlation analysis
r = 0.874, *P* <0.001

√actual warfarin dose (mg) = 0.511 + 0.717 √predicted warfarin dose (mg)

Conclusions

- Height, *VKORC1* and *CYP2C9* genotype, and indication for warfarin therapy are the major determinants of warfarin dose in children
- A regression equation incorporating these 4 factors performs well in predicting warfarin dose in children
- The results of this study suggest that there is potential for a pharmacogenetic approach to warfarin-dosing in children
- This dosing equation requires prospective evaluation in children undergoing warfarin initiation

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Appendix D. Poster presented at British Society for Haematology 52nd Annual Scientific Meeting, Glasgow, April 2012

VKORC1 and _CYP2C9_ genotype is associated with over-anticoagulation during initiation of warfarin therapy in children

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The role of genetic polymorphism in determining response to warfarin during initiation therapy is well established in adult patient populations. Data in warfarinised children is lacking. Retrospective data on INR and warfarin dose for the first 3 months of therapy were collected for 51 children; 39 males, median age at initiation of warfarin therapy 4 years (range: 1- 17 years). All children were genotyped for *VKORC1* (-1639G>A; rs9923231) and *CYP2C9**2 (R144C; rs1799853) and *3 (I359L; rs1057910) alleles. Associations between genotype and outcome variables during initiation therapy were examined using regression analysis. Children with *VKORC1* (-1639G>A) genotype AA (n = 6) had a higher peak INR during the first week of therapy (mean 5.1 ± 2.2) than those with GA (3.5 ± 1.4, n = 26) or GG (3.0 ± 1.3, n = 19) genotype, $P = 0.008$. Children with a single variant *CYP2C9* allele (*2, 6 children; *3, 9 children) had a higher peak INR (mean 4.1 ± 1.7) than those with wild-type *CYP2C9* (3.2 ± 1.4, n = 35), $P = 0.03$. One child with *CYP2C9* *2/*3 genotype had a peak INR in week 1 of 5.5. Children with a variant *CYP2C9* allele had a greater proportion of INR values above target therapeutic range during the first month of warfarin than those with wild-type for *CYP2C9* (14.3% vs. 8.7%, respectively, $P = 0.007$). Children with *VKORC1* (-1639) AA genotype had a greater proportion of INRs above target range during the first month of warfarin therapy (12.8%) than those with GA (10.3%) or GG (10.8%) genotype but this was not statistically significant. Knowledge of *VKORC1* and *CYP2C9* genotype may identify children who are at risk of over-anticoagulation during initiation of warfarin therapy and who may benefit from pharmacogenetics-based warfarin dosing in order to prevent early complications.

VKORC1 and *CYP2C9* genotype is associated with over-anticoagulation during initiation of warfarin therapy in children

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INTRODUCTION

- There is an increasing need for anticoagulation in children. Warfarin remains the agent of choice for long-term therapy
- Prediction of warfarin dose requirement in children is difficult. Over-anticoagulation during the initiation phase of therapy is not uncommon
- Studies in adults have shown that polymorphisms of the *VKORC1* and *CYP2C9* genes cause increased sensitivity to warfarin and result in high INRs and bleeding during initiation of therapy
- The effect of genetics on anticoagulation outcomes has not been examined in a paediatric population

AIMS

- To determine the effect of *VKORC1* and *CYP2C9* genotype on anticoagulation response during initiation of warfarin therapy in children

PATIENTS & METHODS

- This was a retrospective cohort study
- Children were recruited from 3 UK centres: Birmingham Children's Hospital; The Newcastle upon Tyne Hospitals NHS Foundation Trust; Royal Manchester Children's Hospital: and 1 Canadian centre, The Hospital for Sick Children, Toronto
- Inclusion criteria:
  - Age ≤ 18 years;
  - Anticoagulated with warfarin for at least 3 months;
  - Target range 2.0-3.0 or 2.5-3.5;
  - Retrospective data available for warfarin doses and INRs during the first 3 months of warfarin therapy
- Demographic details were collected from the patient's medical records
- A blood sample was taken. Genotyping for *VKORC1* (-1639G>A; rs9923231) and *CYP2C9* (*2/*3 alleles; rs1799853/ rs1057910) was performed on extracted DNA using the StepOne™ Real-Time PCR System, Applied Biosystems
- Statistical analyses were performed using MiniTab v15.0 (Coventry, UK)

ACKNOWLEDGMENTS

- *Funding*: Baxter; Royal College of Pathologists
- *Study co-ordination and recruitment:* Patricia Walsh, Newcastle; Darlene Castle, Toronto; Gillian Taylor, Birmingham; Anne Littley, Manchester
- *Study participants and their parents/carers*

RESULTS

- 51 children were studied (*Table 1*)
- Median age at warfarin initiation was 4 years (range: 0-17 years)
- Mean dose of warfarin given on days 1 and 2 of warfarin initiation was 0.14 mg/kg (± 0.06)

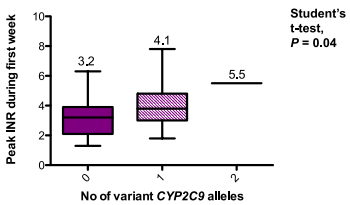
Table 1. Details of study population

	Number of children (%)
Gender	
Male	39 (76.5)
Female	12 (23.5)
Ethnic origin	
White Caucasian	33 (64.7)
Indian/Pakistan	6 (11.8)
Chinese	2 (3.9)
Afro-Caribbean	2 (3.9)
Black African	2 (3.9)
Other	6 (11.8)
Indication for anticoagulation with warfarin	
Fontan procedure	28 (54.9)
Prosthetic heart valve	7 (13.7)
Cardiomyopathy	5 (9.8)
Coronary aneurysm	4 (7.8)
DVT/PE	2 (3.9)
Other	5 (9.8)
Target INR range	
2.0 - 3.0	46 (90.2)
2.5 - 3.5	5 (9.8)
Total number of children	51 (100)

THE EFFECT OF GENOTYPE ON PEAK INR IN WEEK 1

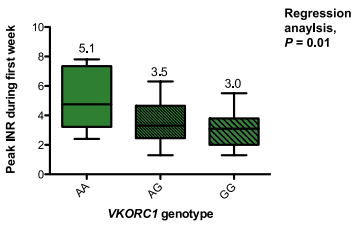
- Mean peak INR was higher in children with one variant *CYP2C9* allele (*2 or *3) than those with wild-type *CYP2C9*
- The single child with two variant alleles (*CYP2C9 *2/*3* genotype) had a peak INR in week 1 of 5.5 (*Figure 1*)

Figure 1. The effect of CYP2C9 genotype on peak INR in week 1



- Mean peak INR was higher in children with *VKORC1 (-1639) AA* genotype than in those with *AG* or *GG* genotype (*Figure 2*)

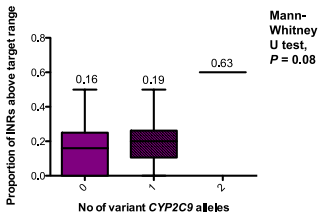
Figure 2. The effect of VKORC1 genotype on peak INR in week 1



THE EFFECT OF GENOTYPE ON INCIDENCE OF SUPRA-THERAPEUTIC INR DURING THE FIRST MONTH OF WARFARIN

- Children with a variant *CYP2C9* allele had a greater percentage of INR values above target range (19.3%) than those with wild-type *CYP2C9* (15.9%) during the first month (marginally significant) (*Figure 3*)
- Children with *VKORC1 AA* genotype had a greater proportion of INR values above target range (21.5%) than those with *GA* (17.6%) or *GG* (17.2%) genotype during the first month (not significant)

Figure 3. The effect of CYP2C9 genotype on the proportion of INRs above target range during the first month



- There was no association between genotype and:
  - Time to first therapeutic INR;
  - Time to first supra-therapeutic INR;
  - Number of INRs > 4.0;
  - Time to stable warfarin dose;
  - Number of warfarin dose changes
- No haemorrhagic or thrombotic events occurred during initiation of warfarin therapy

CONCLUSIONS

- The peak INR during week 1 of warfarin therapy in children is influenced by *CYP2C9* and *VKORC1* genotype
- Children who have polymorphisms that render them sensitive to the anticoagulant effect of warfarin are more likely to be over-anticoagulated during the first month of warfarin therapy
- These children may be at a higher risk of haemorrhage during initiation of warfarin therapy
- The use of warfarin-dosing algorithms that include genetic information may improve the safety of initiation therapy in children

Appendix E. Oral presentation at XXIIIrd Congress of the International Society on Thrombosis & Haemostasis, Japan, July 2011

Inter-individual variability in warfarin dose requirement in children can be explained by VKORC1 and CYP2C9 genotype and patient characteristics

Biss TT, Avery PJ, Brandao LR, Chalmers EA, Williams MD, Grainger JD, Leathart JBS, Hanley JP, Daly AK, Kamali F

Warfarin dose requirements vary among children. The aim of this study was to evaluate the impact of genetic polymorphism, in the enzymes that mediate the pharmacology and disposition of warfarin, and patient characteristics on maintenance warfarin dose requirement in a large cohort of children.

Children with stable warfarin dose requirement were genotyped for VKORC1 (-1639G>A polymorphism), CYP2C9 (*2 and *3 alleles), CYP4F2 (rs2108622;V433M;C>T polymorphism) and APOE ε2, ε3 and ε4 variants.

120 children with median age of 11 years (range: 1-18 years) took part in the study. There were 82 males (68.3%), 91 White Caucasians (75.8%), 101 children with target INR range 2.0-3.0 (84.2%) and 64 anticoagulated following a Fontan procedure (53.3%). Daily warfarin dose significantly correlated with height, weight and age, height being the best predictor of dose ($r = 0.55$, $P < 0.001$). Mean daily warfarin dose was higher in children with VKORC1 GG genotype (5.01 ± 2.17mg) than in those with GA (3.65 ± 1.92mg) or AA (2.23 ± 1.06mg) genotype, $P < 0.001$, and higher in children who were CYP2C9 homozygous wild-type (4.30 ± 2.13mg) than in those with a *2 (3.68 ± 2.13mg) or *3 (2.22 ± 1.08mg) allele, $P < 0.001$. Children anticoagulated following a Fontan procedure required a lower warfarin dose than those anticoagulated for other reasons (3.43 ± 1.62mg vs. 4.38 ± 2.51, $P = 0.02$). Asian children required a higher warfarin dose due to the greater frequency of VKORC1 GG genotype. Gender, CYP4F2 and APOE genotype had no significant impact on warfarin dose requirement. In a multivariate regression analysis the variables of height, VKORC1 and CYP2C9 genotype and indication for warfarin explained 72.4% of inter-individual variability in maintenance warfarin dose requirement.

A pharmacogenomic approach to warfarin dosing has the potential to improve the efficacy and safety of oral anticoagulation in this challenging patient population.

INTER-INDIVIDUAL VARIABILITY IN WARFARIN DOSE REQUIREMENT IN CHILDREN CAN BE EXPLAINED BY *VKORC1* AND *CYP2C9* GENOTYPE AND PATIENT CHARACTERISTICS

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Newcastle University

Background

- Anticoagulant therapy is being increasingly used in children for the treatment and prevention of thromboembolic events
- Warfarin remains the most frequently used agent for long-term anticoagulation therapy in children
- Anticoagulation response to a fixed dose of warfarin is difficult to predict in this patient group

Background

- Studies in anticoagulated adults have identified that warfarin dose requirement is influenced by single nucleotide polymorphisms (SNPs) in the *VKORC1*, *CYP2C9* and *CYP4F2* genes
- The contribution of genetic polymorphism to inter-individual variability in response to warfarin in children has not previously been evaluated in a large cohort of anticoagulated children

Aim of the study

- To evaluate the effects of genetic, clinical and demographic factors on maintenance warfarin dose in children

Methods

- Cross-sectional, multi-centre study of children stable on warfarin
- 1 Canadian and 4 UK tertiary care centres
- Eligibility criteria:
  - Children aged ≤ 18 years
  - Anticoagulated with warfarin for at least 3 months
  - Target INR range 2-3 or 2.5-3.5
  - Stable on warfarin, with no change in dose for at least 3 consecutive INR measurements AND for at least 4 weeks
- Temporary exclusion if acutely unwell or had a recent change (within the prior 4 weeks) in medication known to alter response to warfarin

Methods

- Venous blood sample taken and stored in EDTA at -80°C
- Genotyped using StepOne™ Real-Time PCR System with Taqman® SNP Genotyping Assays (Applied Biosystems™)
  - *VKORC1*: -1639G>A
  - *CYP2C9*: *2/*3 alleles
  - *CYP4F2*: V433M
- *VKORC1* and *CYP2C9* genotypes were validated using control samples genotyped for a prior study. *CYP4F2* genotypes were validated by PCR-RFLP on 26 of the study samples

Patient Characteristics

	Number of children
Gender	
Male	82 (68%)
Female	38
Age group	
0-3 years	7
4-6 years	20
7-9 years	20
10-12 years	21
13-15 years	29
16-18 years	23
Ethnic origin	
White Caucasian	91 (76%)
Other	29
Indication for warfarin	
Fontan procedure	64 (53%)
Prosthetic heart valve	18
Coronary aneurysm	11
Dilated cardiomyopathy	6
DVT/PE	6
Pulmonary hypertension	5
Stroke	2
Other	8
Target INR range	
2.0-3.0	101 (84%)
2.5-3.5	19

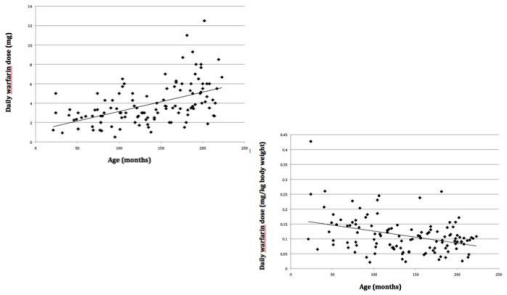
120 children recruited

April 2009- December 2010

Genetic Characteristics of Study Population

	Number of children
***VKORC1* genotype**	
GG	43
GA	55
AA	22
***CYP2C9* genotype**	
*1/*1	84
*1/*2	17
*1/*3	17
*2/*2	1
*2/*3	1
*3/*3	0
***CYP4F2* genotype**	
CC	61
CT	49
TT	10

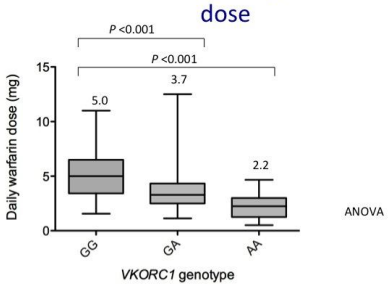
Effect of age on warfarin dose



Effect of body size on warfarin dose

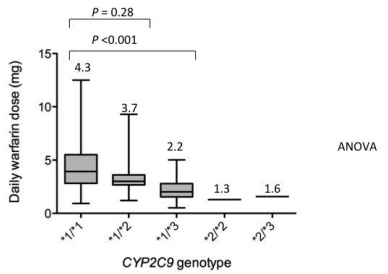
- Pearson correlation test:
  - Body surface area: r = 0.56, *P* < 0.001
  - Height: r = 0.55, *P* < 0.001
  - Weight: r = 0.53, *P* < 0.001
  - Age: r = 0.53, *P* < 0.001
  - BMI: r = 0.32, *P* < 0.001

Effect of *VKORC1* genotype on warfarin dose



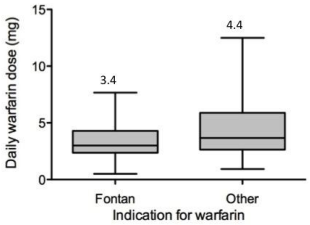
ANOVA

Effect of *CYP2C9* genotype on warfarin dose



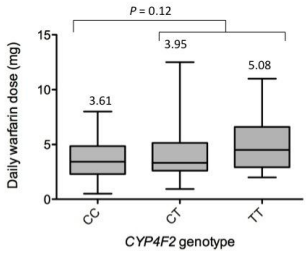
ANOVA

Effect of indication for anticoagulation on warfarin dose



P = 0.02, unpaired t-test

Effect of *CYP4F2* genotype on warfarin dose



ANOVA

Regression model

x variable	*P* value	Contribution to model, %
Height	< 0.001	29.8
VKORC1	< 0.001	26.6
CYP2C9	< 0.001	12.8
Indication	< 0.001	3.2
Height, *VKORC1*, *CYP2C9*, Indication	**<0.001**	**72.4**

√dose = -0.009 + 0.011 (height) + 0.357 (*VKORC1*) – 0.478 (*CYP2C93) – 0.277 (*CYP2C9**2) + 0.186 (Indication)**

Conclusions

- A similar proportion of inter-individual variability in response to warfarin is explained by genetic factors (*VKORC1* and *CYP2C9* genotype) in children compared to adults
- Body size is a greater predictor of warfarin dose in children than in adults
- Children who are anticoagulated for Fontan procedure require a lower warfarin dose
- *CYP4F2* genotype did not have a significant effect on warfarin dose in this cohort

Conclusions

- Our pharmacogenetic paediatric warfarin-dosing algorithm requires validation in an unrelated patient population
- Further study is required to identify the factors that account for the remaining 27.4% of variability in warfarin dose in children
- The results of this study suggest that there is potential for a pharmacogenetic approach to warfarin-dosing in children

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Baxter

The Royal College of Pathologists
Pathology: the science behind the cure

Appendix F. Poster presented at the British Society for Haemostasis & Thrombosis, Newcastle upon Tyne, October 2009

ANTICOAGULATION CONTROL IN A COHORT OF CHILDREN ON CHRONIC THERAPY WITH WARFARIN

T.Biss, P.Walsh*, F.Kamali. Institute of Cellular Medicine, Newcastle University, *Department of Paediatric Cardiology, Freeman Hospital, Newcastle, UK

Children on warfarin therapy generally have poor anticoagulant control although it has been suggested that the use of home monitoring devices can improve it. This study examined anticoagulation control in a cohort of children on chronic therapy with warfarin in Newcastle upon Tyne, monitored at home using a point-of-care device, and identified the factors that were attributed to the deviations from target therapeutic range (TTR). Clinical records on children who were anticoagulated with warfarin for at least 3 months between January 1996 and April 2009 were examined retrospectively. Data on 37 children (20 males) with a median age of 8.3 (range: 1-17) years at start of therapy, for a total of 62 patient-years of warfarin therapy were included in the final analysis. All were monitored by home testing using a Coaguchekâ S device and dosed by a cardiology nurse specialist who was contacted by the parent/carer by telephone. Indications for anticoagulant therapy included Fontan circulation (16 patients), prosthetic mitral valve (8) and primary pulmonary hypertension (4). TTR was 2.0-3.0 in 29 and 2.5-3.5 in 8. Following initiation of warfarin therapy, data was collected from the time that INR values were therapeutic on two consecutive measurements. A mean of 56.1% (range: 30.3-85.7%) of tests were within TTR, 28.9% (range: 8.7-54.5%) were below, and 14.9% (range: 0-37.1%) were above. The most frequent reasons for INR being above TTR were antibiotic therapy, intercurrent illness and medication changes. The most frequent reasons for INR being below TTR were poor compliance, doses omitted for a planned procedure and antibiotic therapy. Children with TTR 2.5-3.5 had fewer INR values within TTR (53.4% vs. 58.1%) and more INR values below TTR (33.6% vs. 28.6%) when compared to children with TTR 2.0-3.0. Maintenance of anticoagulant control within TTR remains poor in children despite the availability of home monitoring and support for warfarin dosing. Further studies should assess the consequences of under- and over-anticoagulation in children in terms of thrombotic and haemorrhagic events.

- There is an increasing need for chronic anticoagulant therapy in childhood
- Oral anticoagulation is difficult to manage in children because of age-related differences in dose response rates, chronic health conditions, frequent intercurrent illness and concomitant medications
- International Normalised Ratio (INR) results obtained with a point-of-care device in children are comparable with venous measurements
- Home monitoring with a point-of-care device may allow closer monitoring and therefore improve anticoagulant control in addition to improving quality of life for the child and their parent or carer

- To examine anticoagulation control in a cohort of children on chronic therapy with warfarin, monitored at home using a point-of-care device;
- To identify the factors that are responsible for deviations from target therapeutic range (TTR)

- All children (< 18 years of age) who were anticoagulated with warfarin for at least 3 months between January 1996 and April 2009 were eligible for study inclusion
- All were monitored at home using a point-of-care device (Coaguchek®S or Coagucheck®XS)
- All had their warfarin dosed by a Cardiology Nurse Specialist who was contacted with the INR results by the parent/carer by telephone
- Records of INR and warfarin dose were examined retrospectively
- Following initiation therapy data were collected from the time that INR values were therapeutic on two consecutive measurements

Table 1: Patient characteristics

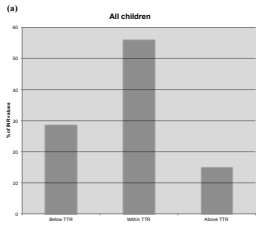
Total number of children	37
Age*: median (range), yrs	8.3 (1-17)
Sex, no. of children	
Male	20
Female	17
Indication for warfarin therapy, no. of children	
Fontan circulation	16
Prosthetic mitral valve replacement	8
Primary pulmonary hypertension	4
Prosthetic aortic valve replacement	3
Cardiomyopathy	3
Other**	3
Target INR range, no. of children	
2.0-3.0	29
2.5-3.5	8
Duration of warfarin therapy: median (range), months	28 (3-115)

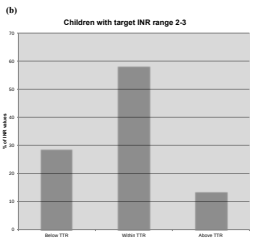
***at start of warfarin therapy**
****giant coronary aneurysm, 1; thrombotic stroke, 1; truncus arteriosus repair, 1**

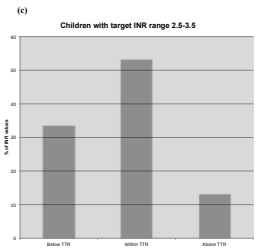
Table 2: Mean % INR values within, below and above target therapeutic range

Target INR range	Mean % of INR values (range)		
	Below TTR	Within TTR	Above TTR
2.0-3.0 *N=29*	28.6 (0- 54.5)	58.1 (30.3- 85.7)	13.3 (0- 37.1)
2.5-3.5 *N=8*	33.6 (23.3- 46.7)	53.4 (37.0- 61.5)	13.0 (10.3- 21.9)
All children *N=37*	28.9	56.1	14.9

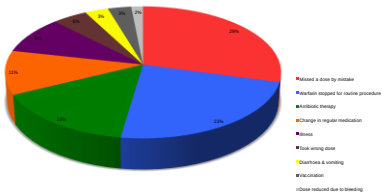
Figure 1: Mean % of INR values for all children (a), those with target range 2.0-3.0 (b) and those with target range 2.5-3.5 (c)



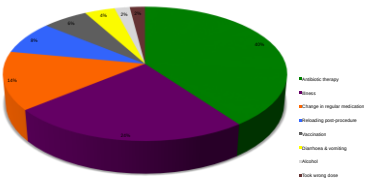




Reasons for a subtherapeutic INR



Reasons for a supratherapeutic INR



- Maintenance of anticoagulant control within TTR remains poor in children despite the availability of home monitoring with a point-of-care device and support for warfarin dosing
- INR values in children are more likely to be below TTR than above TTR, increasing their risk of thrombotic events
- Children with a higher TTR (2.5-3.5) are less likely than those with a lower TTR (2.0-3.0) to have an INR within TTR and more likely to have an INR below TTR
- The most frequent reasons for a subtherapeutic INR are missed doses, cessation of warfarin for a planned procedure and antibiotic therapy
- The most frequent reasons for a supratherapeutic INR are antibiotic therapy, illness and a change in regular medication
- Further studies should assess the consequences of under- and over-anticoagulation in children in terms of thrombotic and haemorrhagic events

Appendix G. *VKORC1* and *CYP2C9* genotype and patient characteristics explain a large proportion of the variability in warfarin dose requirement among children. Paper published in *Blood*