Pharmacogenetic, clinical and demographic factors in the management of warfarin therapy.

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A doctoral project report submitted in partial fulfilment of the requirements of
Sheffield Hallam University
for the degree of Doctor of Professional Studies

September 2012

"All things are poison and nothing is without poison; only the dose permits something not to be poisonous."

Paracelsus (1493-1541)

Abstract

It has been estimated that, at any one time, more than one million people in the U.K. are taking the anticoagulant drug warfarin, for the treatment or prevention of venous thromboembolism. The incidence of life threatening haemorrhage, due to overdose, is approximately two per one hundred patient years. It is well known that there is great inter-individual variability in reaching and maintaining a therapeutic level of oral anticoagulants, in part, due to the combined effect of gender, age, body size and drug interactions.

In recent years, single nucleotide polymorphisms (SNPs) have been identified, which significantly reduce the amount of warfarin required for an individual to reach a therapeutic level. Consequently, the optimum dose of warfarin can be predicted in a higher percentage of patients using an algorithm, which includes pharmacogenomic information rather than one with clinical and demographic data alone.

The aim of this study was to create two rigorous, composite algorithms, one clinical and one pharmacogenetic, which combined as many influencing factors as possible, in an effort to improve the predictability of warfarin dosing beyond that of other published studies to date. The study was carried out using three groups of subjects, after obtaining ethical committee approval and informed consent. Group 1 subjects (n=12) were healthy, non-warfarin treated laboratory staff, whose DNA was used to optimise the DNA extraction procedure. Group 2 subjects (n=207) consisted of warfarin patients, who had had a stable therapeutic International Normalised Ratio (INR) for at least two months. A comprehensive list of clinical and demographic data was obtained from each patient, as well as DNA samples for SNP analysis of the VKORC1, CYP2C9 and CYP4F2 genes. Group 3 subjects (n=20) comprised of pre-warfarinised patients who provided the same data as in group 2. In addition, venous blood samples were obtained for the measurement of the baseline levels of the vitamin K dependent coagulation factors and albumin. The stable warfarin dose for each of the group 3 patients was obtained retrospectively, after several weeks of warfarin therapy.

The two algorithms were then constructed using the data from a random selection of group 2 patients (n=160). These were then used to predict the warfarin dose of the remaining patients in the group (n=47). By plotting the predicted dose against the actual stable dose, the percentage predictability of the new algorithms was calculated. In addition, the predictability of eleven previously published algorithms, eight pharmacogenetic and three clinical, was calculated using the same 47 patients. The clinical algorithm from this study showed the lowest predictability (R^2=0.188) when compared to the three published algorithms (R^2=0.203-0.268). However, the pharmacogenetic algorithm was able to account for a higher proportion of the warfarin dose (R^2=0.553) than any of the other eight published algorithms (R^2=0.383-0.525).

In the group 3 patients, no relationship was demonstrated between the warfarin dose and either the albumin levels or the baseline levels of the vitamin K-dependent coagulation factors, with the exception of factor IX, which showed a negative correlation.

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Abbreviations

AF	Atrial fibrillation
APC	Activated protein C
APOE	Apolipoprotein E
BCSH	British Committee for Standards in Haematology
BMI	Body mass index
BSA	Body surface area
CYP	Cytochrome P450
DNA	Deoxyribonucleic acid
DVT	Deep vein thrombosis
FWS	Foetal warfarin syndrome
GGCX	Gamma glutamyl carboxylase
HMWK	High molecular weight kininogen
INR	International Normalised Ratio
ISI	International Sensitivity Index
LFT	Liver function tests
LMWH	Low molecular weight heparin
LVF	Left ventricular fibrillation
MNPT	Mean normal prothrombin time
OAT	Oral anticoagulant therapy
PC	Protein C
PCR	Polymerase chain reaction
PE	Pulmonary embolism
PIVKA	Proteins induced in vitamin K absence
PS	Protein S
PT	Prothrombin time
SNP	Single nucleotide polymorphism
TF	Tissue factor
TIA	Transient ischaemic attacks
U & E	Urea and electrolytes
VKOR	Vitamin K epoxide reductase

VKORC1	Vitamin K epoxide reductase complex subunit 1
VTE	Venous thromboembolism

Acknowledgements

I would firstly like to express my sincere gratitude to my university supervisor, Prof. Nicola Woodroofe, for all her time and support over the past seven years leading up to the completion of this thesis. Without her input and encouragement it probably wouldn't have seen the light of day. Similarly, I would like to express my appreciation to Dr. Sylvia Feyler, Huddersfield Royal Infirmary, for obtaining the funding from the British Society for Haematology start up grant, without which the PCR analyses would have been more of a problem than they turned out to be.

In that vein, I am indebted to Sheridan Copley for his assistance in the DNA extraction methods and to Dr Rob Widdowson for all his work and expertise with the PCR analyses. Without either of these two, the practical work would have taken significantly longer than they did.

I would like to thank Dr Adrian Hall for his invaluable input with the PCR methodology and in helping to explain the principles of a subject which hadn't been invented when I went to Haematology school. In addition, I am indebted to Dr Karen Kilner for her statistical advice and for making such a complex subject much clearer to someone who abandoned mathematics at O level.

Finally, it would not have been possible to even start this doctoral journey without having been raised in appreciation of hard work and its benefits by my mother, Jean, and my late father, Trevor. It is to them that I owe so much, as well as to my partner, Joan, for his understanding and support over the past seven years.

Chapter 1

1.1 Introduction

It is estimated that between 500,000 and 1 million people in the United Kingdom are currently being prescribed oral anticoagulant therapy (OAT) and of these warfarin is the main drug of choice among clinicians at the present time in the treatment of venous thromboembolism (VTE) (Greaves, 2005; Baglin *et al*, 2006).

Despite having few side effects, the commonest complication by far is the risk of haemorrhage, due to over-anticoagulation and which, if severe enough, can be life threatening. The rate of these occurrences is estimated to be in the order of 2 per 100 patient years, with minor bleeding being more common. Conversely, under-anticoagulation may lead to the development of thrombosis or propagation of an existing thrombus, either of which may also be life threatening (Palareti *et al*, 1996). There are several causes for such serious adverse events; numerous factors are responsible for either potentiating or attenuating the pharmacological effects of OAT. The therapeutic range between over- and under-anticoagulation is narrow and compliance can be problematic, especially in elderly patients. Consequently, it has been estimated that patients are only within the target range between 50-70% of the time, even under the best available management (Greaves, 2005).

In order to fully understand the reasons behind the design and implementation of this study, the thesis will begin with an overview of the haemostatic mechanism and the pathophysiology of thromboembolism. After this, there will be a detailed review of warfarin, firstly through its history, pharmacology and dose monitoring, followed by a comprehensive evaluation of the clinical, demographic and genetic factors that affect OAT. Finally, the aims and objectives of this study will be discussed, in order to explain the design and methodology used in the ultimate development of one clinical and one pharmacogenetic algorithm for the prediction of the warfarin dose in patients prior to the commencement of treatment.

1.2 The haemostatic mechanism

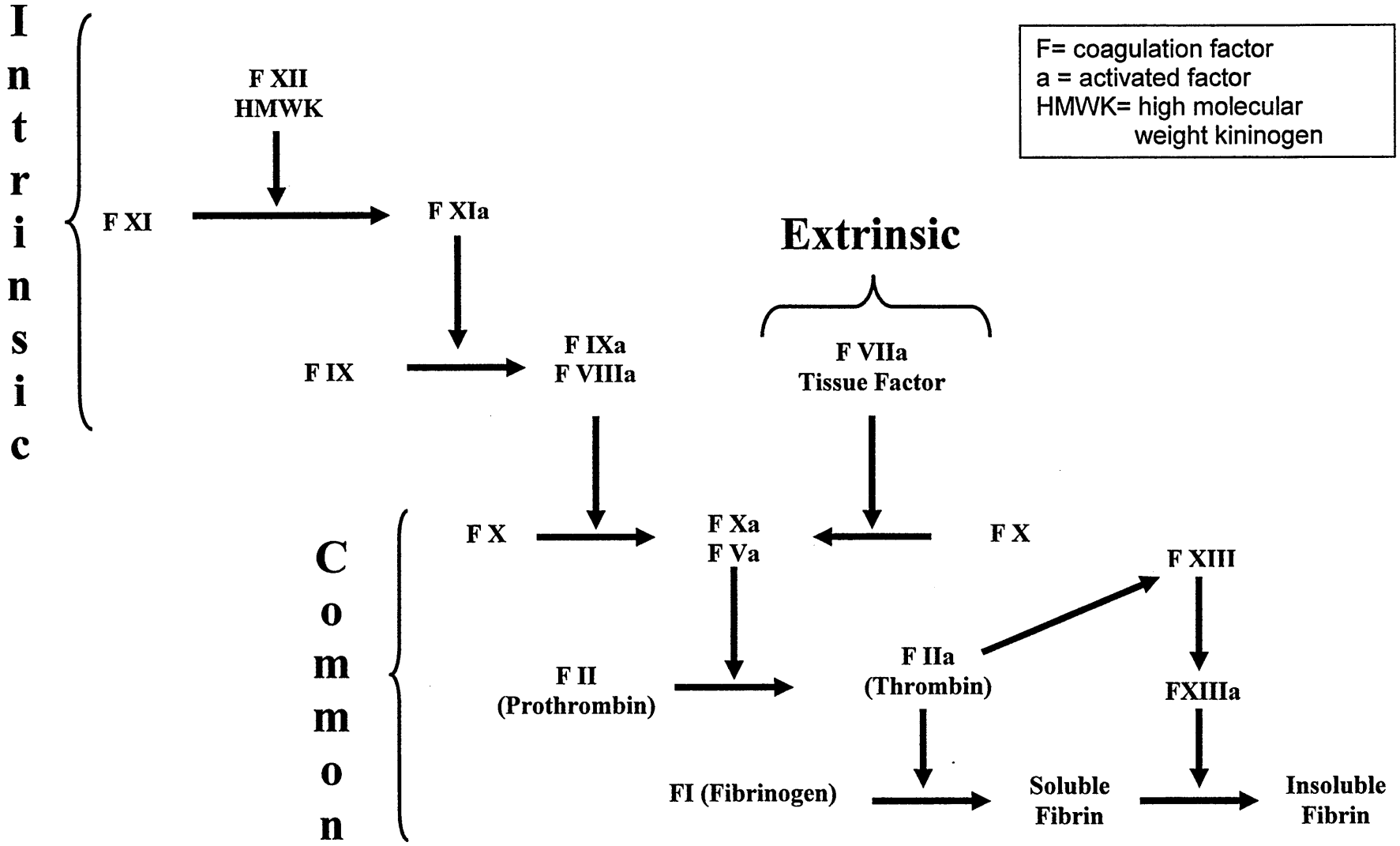
The coagulation cascade and platelets are vital mechanisms for the prevention of haemorrhage. Injury to the blood vessel endothelium results in the exposure of extravascular structures, with the consequent activation of platelets and the coagulation pathway. This ultimately forms a stable thrombus, consisting mainly of an insoluble fibrin mesh and platelets, which occludes the vascular damage (Dahlbäck, 2000).

The coagulation cascade involves a series of enzymatic reactions involving inactive zymogens and other cofactors, which are activated sequentially by proteolytic cleavage and result in an amplified chain reaction leading to the production of fibrin (Norris, 2003). This pathway is controlled simultaneously by the fibrinolytic system, whereby a number of negative feedback reactions involving natural anticoagulants prevent further activation of the cascade and therefore limit the propagation of the thrombus. Thrombosis occurs when one or more stages in the coagulation mechanism and/or the fibrinolytic system are affected by genetic or clinical factors, leading to the production of a thrombus, either in the absence of vascular injury or an inability to limit thrombus propagation as a result of vessel wall damage (Kroegel & Reissig, 2003). Traditionally, the coagulation cascade has been described by dividing it into three sections; the extrinsic (or tissue factor) pathway, the intrinsic (or contact factor) pathway and the common pathway and all the proteins involved are denoted by Roman numerals. Most of these proteins are serine proteases, with the exception of three cofactors, [factor V, factor VIII and high molecular weight kininogen (HMWK)] and one transglutaminase, factor XIII (Norris, 2003).

The intrinsic pathway involves the conversion of inactive factor XII to its active form (XIIa) by contact with a negatively charged surface, particularly collagen, which is exposed during vascular injury. This in turn causes the activation of factor XI to XIa and, together with the cleavage of HMWK, results in the formation of factor IXa (Fig1.1) (Norris, 2003).

Fig. 1.1 The Coagulation Cascade
(adapted from Hoffbrand & Pettit, 1993)

The cascade is an amplification process, which is initiated by the exposure of tissue factor and collagen from damaged blood vessels, as well as platelet phospholipid. Circulating, precursor proteins become activated in sequence, ultimately leading to the production of insoluble fibrin, the main constituent of a venous thrombus. In order to explain the *in vitro* measurement of the factors involved, the cascade is divided into the intrinsic, extrinsic and common pathways


Intrinsic
F XII
HMWK
F XI
F XIa
F IX
F IXa
F VIIIa
Extrinsic
F VIIa
Tissue Factor
Common
F X
F Xa
F Va
F X
F XIII
F II
(Prothrombin)
F IIa
(Thrombin)
FXIIIa
FI (Fibrinogen)
Soluble
Fibrin
Insoluble
Fibrin
F= coagulation factor
a = activated factor
HMWK= high molecular
weight kininogen

The extrinsic pathway is initiated when tissue factor (TF), a cellular membrane-bound protein, becomes exposed to the plasma following vascular damage and binds to factor VII, forming the TF-VIIa complex, the most potent activator of the coagulation system. This complex then activates factor X to Xa, converging with the product of the intrinsic pathway (Norris, 2003).

The overall result of both pathways is to produce what is known as the 'tenase' complex, in which IXa, together with VIIIa, calcium and phospholipid, combine to activate factor X to Xa, (which is also produced by TF-VIIa). The 'tenase' complex is fundamental to the overall function of the coagulation cascade and deficiency in either factor VIII or factor IX results in one of two severe haemorrhagic disorders (haemophilia A and B respectively) (Norris, 2003). The 'tenase' complex is responsible for the formation of the prothrombinase complex, which consists of Xa, the non-enzymatic cofactor Va, calcium and phospholipid, which in turn activates factor II (prothrombin) to IIa (thrombin). Thrombin has a central role in the whole haemostatic mechanism, being responsible for the upstream activation of factors V, VIII, IX and XI, as well as platelets and the fibrinolytic protein C (Norris, 2003). Nonetheless, a pivotal function of thrombin is at the end of the coagulation cascade, involving the conversion of factor I (fibrinogen) to factor Ia (fibrin). However, fibrin is produced as soluble monomers and consequently forms an unstable thrombus. The monomers are cross-linked to form insoluble fibrin by the action of factor XIIIa, which itself is activated by thrombin.

Although there are negative feedback loops within the coagulation cascade, mainly mediated by thrombin, the antithesis of fibrin clot formation is the fibrinolytic system. Using a similar notation to the coagulation pathway, the inactive zymogen plasminogen is activated either intrinsically (by FXIIa and kallikrein) or extrinsically by tissue plasminogen activator (t-PA) from the endothelium. The activated product, plasmin, is a serine protease which hydrolyses peptide bonds not only in fibrin, thereby degrading a thrombus, but also in fibrinogen, FV and FVIII, leading to their inactivation (Hoffbrand and Pettit, 1993).

A second pivotal pathway in fibrinolysis is the formation, by thrombin and thrombomodulin, of the activated protein C/protein S (PC/PS) complex, which not only inactivates t-PA inhibitors but more importantly it is responsible for the inactivation of the coagulation cofactors FVa and FVIIIa, resulting in further suppression of thrombus formation. In addition, activated FX (FXa) is inhibited by a glycoprotein, protein Z (PZ), which has a similar structure to PC and PS (Broze & Miletich, 1984).

1.3 The vitamin K cycle

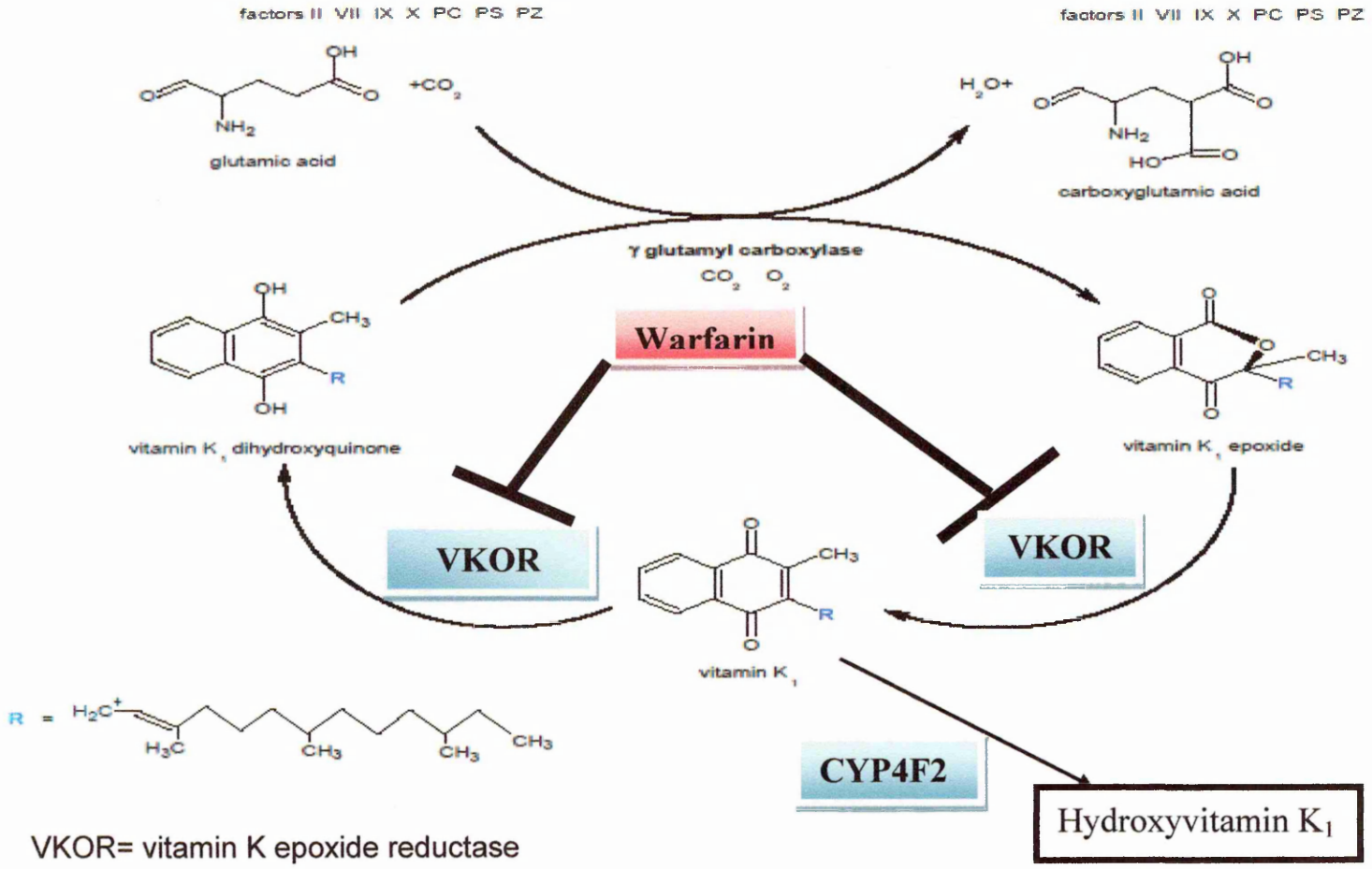
Vitamin K was first suggested as being involved in the coagulation mechanism by Henrik Dam in the 1930's, who noticed that chickens fed on a fat-free diet developed spontaneous haemorrhages (Lindh, 2009). Dam postulated the existence of a fat-soluble vitamin (designated vitamin K, from the German word '*Koagulation*') and, following its subsequent purification and the determination of its molecular structure, Dam was awarded the Nobel Prize for medicine with Edward Doisy in 1943.

Within the haemostatic mechanism, several factors produced in the liver (II, VII, IX, X, PC, PS and PZ) require the presence of vitamin K in order to become functional molecules and are known as the vitamin K-dependent factors (Hoffbrand and Pettit, 1993). A reduced form of vitamin K (vitamin K_1 dihydroxyquinone) acts as an electron donor to the enzyme γ-glutamyl carboxylase (GGCX) and, together with carbon dioxide and oxygen, causes the carboxylation of glutamic acid residues in the active site of the haemostatic proteins, producing a negatively-charged complex (fig. 1.2). This enables them to undergo conformational changes when activated by calcium cations, thereby allowing them to bind to co-factors present on phospholipid surfaces (Ansell *et al*, 2008). As a result, the reduced vitamin K is oxidised to vitamin K epoxide and must undergo two electron reductions in order to be regenerated (McDonald *et al*, 2009). This is mediated by the enzyme vitamin K epoxide reductase (VKOR), which is encoded for by the gene vitamin K epoxide reductase complex subunit 1 (VKORC1), and is the target molecule for the action of warfarin.

Fig 1.2 The vitamin K cycle
(adapted from Krynetskiy & McDonnell, 2007)

Oxidised vitamin K (vitamin K_1 epoxide) is reduced by the enzyme vitamin K epoxide reductase (VKOR) to form the dihydroxyquinone derivative. This is then responsible, via the enzyme γ-glutamyl carboxylase, for the gamma carboxylation of terminal glutamic acid residues of the vitamin K-dependent coagulation factors II, VII, IX and X and the fibrinolytic proteins C, S and Z.

Warfarin inhibits the activity of VKOR, leading to the presence of dysfunctional coagulation molecules in the circulation.



VKOR= vitamin K epoxide reductase

CYP4F2= cytochrome P450 enzyme 4F2

1.4 The pathophysiology of venous thromboembolism (VTE)

In 1856, Virchov proposed three major causes of VTE, which came to be known as Virchov's triad (Virchov, 1856). These were hypercoagulability, changes in the vessel wall and haemodynamic defects resulting in stasis. Although it has been modified over the years, the triad is still the basis of our understanding of VTE today (Sevitt, 1974).

Two distinct but interrelated aspects of VTE are pulmonary embolism (PE) and deep vein thrombosis (DVT), which are major causes of morbidity and mortality within the developed world. The incidence of these two diseases has not decreased significantly over several decades, despite improvements in diagnosis and treatment (Kroegel and Reissig, 2003) and it is estimated that 1 in 1000 people are affected every year in the UK alone (Dahlbäck, 2000). The thrombus in VTE is mainly composed of fibrin, derived ultimately from activation of the coagulation cascade, some trapped erythrocytes and a small layer of platelets (Lopez *et al*, 2004), which is attached to the vessel wall. However, VTE is seldom associated with vessel wall damage (as occurs in arterial thrombosis), indicating that the coagulation mechanism must be activated by other means (Lopez & Chen, 2009). There is general agreement that VTE involves the initiation of coagulation via TF, but most research has involved the use of animal models with vessel wall damage (Esmon, 2009). However, other studies have shown that TF can originate from blood cells, especially monocytes, or from leukocyte-derived microparticles, which not only contain TF but also the cell-cell adhesion molecule P-selectin (Myers *et al*, 2003). Inhibition of P-selectin has been shown to inhibit thrombus formation (Myers *et al*, 2005) and, as it is important for platelet function and platelet-leukocyte interaction, it has been postulated that it is important for the production of the platelet component in the fibrin-dominant layer of a thrombus and therefore may confer stability to the clot (Esmon, 2009). Inflammation has also been implicated in thrombus formation, whereby there is localized activation of the vessel wall endothelium, causing the release of von Willebrand factor and P-selectin from Weibel-Palade bodies. However, both molecules remain attached to the endothelial surface and attract leukocytes, which in turn shed microparticles

from their membrane and result in activation of the coagulation pathway (Lopez and Chen, 2009).

Two major contributors to the occurrence of VTE are stasis and hypoxia, due to either dysfunctional venous valves (Esmon, 2009), an increase in the haematocrit (Hamer *et al*, 1981) or possibly the down regulation of two fibrinolytic proteins, thrombomodulin and the protein C receptor (Brooks *et al*, 2009). Endothelial cell components such as these are important in many haemostatic pathways and, when blood moves from larger to smaller vessels, there is a dramatic increase in the efficacy of these proteins, including TF pathway inhibitor (Esmon, 1989). This is partly due to an increase in the endothelial cell surface exposed to the blood. However, if venous stasis occurs, the blood stays in the large vessels for longer, where the natural anticoagulant properties are not as efficient as in the microcirculation, due to a reduced ratio of the endothelial surface to blood volume (Esmon, 2009). It has also been postulated that venous stasis results in the oxygen desaturation of haemoglobin, resulting in an hypoxic insult to the local endothelium and the spontaneous expression of P-selectin (Closse *et al*, 1997).

The third part of Virchov's triad, hypercoagulability, has been the area of greatest advances in the past two decades. The discovery of the factor V Leiden genetic mutation (Dahlbäck *et al*, 1993) and activated protein C (APC) resistance has helped to explain 20-40% of all cases of hereditary VTE, predominantly in populations of Caucasian origin (Dahlbäck, 2000). In these cases, the FV molecule produced by a single point mutation has arginine 506 replaced by glutamine, resulting in the loss of one of the activated protein C cleavage sites. As a consequence, the mutated FV is not sufficiently deactivated and continues with full procoagulant activity. The risk of VTE in cases of heterozygous APC resistance is approximately 5-10 fold, but increases dramatically in homozygotes to 50-100 fold (Dahlbäck, 2000).

The second commonest genetic factor that confers hypercoagulability is the prothrombin 20210 gene, which was first described in 1996 by Poort *et al*. The mutation causes an increase in the plasma prothrombin concentration, which therefore not only allows for an increased production of thrombin, but also acts as an inhibitor of protein C. The 20210 gene is found in 2% of Caucasians and increases the risk of VTE by 3-5 fold (Dahlbäck, 2000). Changes in the levels of other haemostatic proteins have also been implicated in an increased risk of VTE; increased concentrations of factors VIII, VII and von Willebrand factor (Bertina, 2004) and decreases in protein C, protein S and antithrombin III (Dahlbäck, 2000) have all been shown to cause variable increases in the risk of thrombogenesis. Many acquired disorders and demographic factors are associated with an increased propensity for VTE. In the antiphospholipid syndrome, autoantibodies, or lupus anticoagulant, can cause both arterial and venous thrombosis. Antibodies are produced which target β2-glycoprotein 1 and occasionally prothrombin, and can also be responsible for an increased risk of miscarriage by forming thrombi in the placental circulation (Dahlbäck, 2000).

The risk of VTE increases with age but the exact reason is uncertain (Esmon, 2009). However it is known that, in the elderly, there is an increase in the concentrations of some coagulation factors without a concomitant increase in anticoagulant proteins, as well as an increase in D-dimer levels, which suggests hypercoagulability (Lowe *et al*, 1997). Furthermore, there is an increased risk of immobility due to frailty and illness, leading to venous stasis (Esmon, 2009) as well as an increase in the number of co-morbidities (Silverstein *et al*, 2007). Malignancy increases the danger of venous thrombosis and approximately 20% of cases presenting with VTE have an underlying tumour (Lopez *et al*, 2004). In these cases, VTE risk is increased 6-10 fold (Bick, 2003) and is thought to be due to procoagulant particles containing TF being produced by tumour cells and initiating a coagulation response (Dvorak *et al*, 1981). Additionally, some tumours compress blood vessels and may lead to an increased risk of stasis (Esmon, 2009).

In summary, VTE is considered to be a multigenic disease (Dahlbäck, 2000) and the risk factors are known to act synergistically. For example, obesity and oral contraceptives are both associated with an increased risk of thrombosis (2

fold and 4 fold, respectively), however, when present together, the increased risk is far greater than that of the two combined (Abdollahi *et al*, 2003).

1.5 The anticoagulant drug warfarin

Warfarin [(RS)-4-hydroxy-3(-3-oxo-1-phenylbutyl)-2H-chromen-2-one] is a synthetic derivative of dicoumarol (4-hydroxycoumarin), which is in turn derived from coumarin, found naturally in many plants. It is one of a number of coumarin-based drugs, which has been used for many years in the field of antithrombotic therapy. Warfarin is prescribed both as a treatment for existing DVT and PE, in order to prevent further propagation and/or embolism of the thrombus, and prophylactically in cases where thrombosis is likely to occur, such as in patients with atrial fibrillation (AF), mechanical heart valves or with a genetic propensity for thrombus formation, also known as hereditary thrombophilia (Kamali *et al*, 2000b). At sub-therapeutic levels, the patient is at risk from propagation of the existing thrombus or the formation of new thrombi; at super-therapeutic levels, there is a high risk of haemorrhage and the rate of life-threatening bleeding is approximately two per one hundred patient years, with minor bleeding being more common (Palareti *et al*, 1996).

1.5.1 The history of warfarin

The serendipitous findings that led ultimately to the introduction of oral anticoagulants began in 1924, when Frank Schofield described a new fatal haemorrhagic disease in cattle, which had occurred almost simultaneously in North Dakota, USA and Alberta, Canada (Schofield, 1924). He traced the cause to the ingestion of sweet clover hay spoiled by the mould *Aspergillus* and the disease became known as 'sweet clover disease'. Schofield also showed that it wasn't the *Aspergillus* itself that caused either the bleeding or the prolonged blood clotting time and that the disease manifested itself as a slow decrease in clotting ability over approximately 15 days, resulting in fatal internal haemorrhage between 30-50 days (Copeland & Six, 2009).

In 1931, Lee Roderick demonstrated that the problem was caused by a marked reduction in one of the coagulation proteins, prothrombin but that the effects could be corrected with a bovine blood transfusion (Roderick, 1931). Moreover,

there was no effect on any of the other coagulation constituents known at that time, namely calcium, fibrinogen and platelets. Late in 1932, Karl Link was invited to set up a research team at the University of Minnesota, in order to study Roderick's findings in more detail. Within a few weeks, Link was visited by a farmer, Ed Carlson, who arrived with a dead heifer, a milk churn full of non-clotted blood and a large sample of spoilt sweet clover. By 1939, one of the team, Harold Campbell, had isolated and purified 6mg of crystalline dicoumarin from the samples (Campbell, 1941). Two years later, Mark Stahman purified 1.8g of dicoumarin and showed that spoilage of sweet clover by *Aspergillus* mould resulted in the oxidation of coumarin to 4-hydroxycoumarin (Link, 1959). Furthermore, in the presence of formalin, a product of additional decay, the 4-hydroxycoumarin was converted to 3,3'methylenebis-(4 hydroxycoumarin) or dicoumarin. Link patented the final product under the trade name dicoumarol.

The team tested dicoumarol on various animal species and made several observations, which still hold relevance for anticoagulant therapy today (Link, 1943). Firstly, they noted that there was a considerable delay between ingestion and effect on the clotting mechanism and that repeated administration produced a cumulative response. Secondly, they found that reduced hepatic and renal function significantly influenced the intensity and duration of action. Furthermore, other drugs such as salicylates potentiated the action of dicoumarol and dietary vitamin K was shown to affect the intensity and duration of its action. Within days of publishing these findings, the Mayo Clinic requested samples of dicoumarol and, within 3 months, trials had shown its effect of increasing the blood clotting time in 6 human subjects (reviewed by Copeland and Six, 2009).

During this time, Link had also become aware of the similarities in biochemical structure between dicoumarol and vitamin K, a compound discovered by Henrik Dam in 1935 and synthesized by Edward Dosey in 1939. In a series of animal experiments, he demonstrated that vitamin K could reverse the anticoagulant effect of dicoumarol (Link, 1943). In 1942, field trials were conducted on dicoumarol for its use as a rodenticide, but it was found not to be potent enough (Link, 1959), so Link assembled a team to reappraise dicoumarol analogues and, in 1948, patented a more potent alternative with Mark Stahman and Lester

Scheel (Link, 1959). This was named warfarin, an acronym derived from the Wisconsin Alumni Research Foundation (WARF) and the ending "-arin", denoting its link with coumarins and it was promoted as a rodent poison.

In 1950, Link recommended the use of the sodium salt of warfarin for use in clinical medicine, as it was at least five-times more potent than dicoumarol. Understandably, there was great reluctance from the medical profession to use a substance that had been promoted as a rat poison on human patients; however two events occurred soon after which would change the situation. In 1955, a US navy inductee attempted suicide by ingesting 567mg of warfarin over a period of 6 days. He was subsequently treated with blood transfusions and intravenous vitamin K, and made a full recovery (Holmes & Love, 1952). In the same year, Colonel Byron Pollock, who was stationed at the Fitzsimons Army Hospital in Denver, presented a paper describing his use of warfarin on 100 patients suffering from either myocardial infarction or DVT (Pollock, 1955). One of the patients was the then President, Dwight D. Eisenhower, following a heart attack and, within a year, warfarin was declared the anticoagulant of choice in American hospitals (reviewed by Lindh, 2009).

1.5.2 The pharmacology of warfarin

From a pharmacological perspective, warfarin is more complicated than many other drugs in use today. Its mode of action and elimination, interaction with other drugs, narrow therapeutic range, susceptibility to genetic influences and severe consequences of over- and under-dosing make it difficult to manage clinically. As a result, patients taking warfarin require frequent laboratory monitoring (see section 1.5.3) as well as clinical experience and a sound theoretical knowledge of the drug's actions (Lindh, 2009).

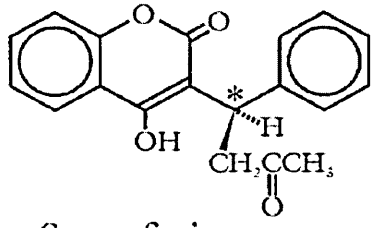
Warfarin consists of a racemic mixture of two enantiomers, R and S (Figure 1.3). S-warfarin is three to five times more potent in its anticoagulant effect and has a plasma half-life of approximately 29 hours, compared to R-warfarin, which has a half-life of 45 hours (Ansell *et al*, 2008). Warfarin is administered orally, usually as a sodium salt, and is readily absorbed from the GI tract, reaching maximum plasma levels within two hours (Gage & Milligan, 2005). Here, it is 99% bound to plasma proteins, mainly albumin, and it is the unbound drug that

is pharmacologically active (Beinema *et al*, 2008). The maximum effect of any one dose may occur up to 48 hours after administration and may continue for several days (D'Andrea *et al*, 2008).

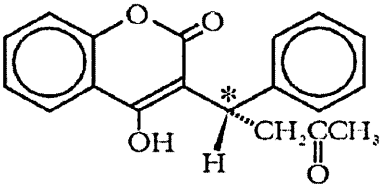
The two enantiomers are metabolised by different pathways in the liver. S-warfarin is metabolised to S-7 hydroxywarfarin by the CYP2C9 enzyme of the cytochrome P450 system (Rettie *et al*, 1992) and is excreted in the bile (Ansell *et al*, 2008). R-warfarin, on the other hand, is metabolised by three different cytochrome enzymes, CYP1A1, CYP1A2 and CYP3A4, and is excreted via the kidneys (Gage & Milligan, 2005).

Figure 1.3 S- and R-enantiomers of warfarin.

* denotes the chiral carbon atom (adapted from Lindh, 2009)



S-warfarin



R-warfarin

The mode of action of warfarin is the inhibition of the vitamin K epoxide reductase enzyme (VKOR) in the vitamin K cycle (see fig 1.2), thus preventing the reduction of vitamin K_1 epoxide to vitamin K_1 dihydroxyquinone (McDonald *et al*, 2009). When approximately 70% of the VKOR molecules have been inactivated in the liver by warfarin, the supply of vitamin K_1 dihydroxyquinone available to γ glutamyl carboxylase (GGCX) is no longer sufficient for the carboxylation of the coagulation factors to take place (Lindh, 2009). This results in the secretion into the circulation of large amounts of uncarboxylated factors II, VII, IX and X, which are unable to participate in the coagulation cascade and are known as PIVKA proteins (proteins induced by vitamin K absence or antagonism) (Hoffbrand and Pettit, 1993). The overall reduction in the activity of the coagulation mechanism takes place over several days following initiation of warfarin therapy, depending on the half-life of the clotting factors affected. Factor VII shows low levels of functionality within 48 hours, due to a half-life of 6 hours, whereas the potency of factors II and X may take several more days to

be sufficiently suppressed. In addition, warfarin rapidly reduces the carboxylation of the fibrinolytic proteins C, S and Z, which leads to the paradoxical state of *hypercoagulability* in the first few days of therapy (Reynolds *et al*, 2007).

1.5.3 The laboratory control of warfarin therapy

The laboratory test for the monitoring of oral anticoagulant therapy is based on the prothrombin time (PT), which measures the activity of factors I, II, V, VII and X (Hall & Malia, 1991). The patient's plasma is incubated with tissue thromboplastin *in vitro*, in order to simulate the activation of the coagulation cascade *in vivo.* Calcium ions, in the form of $CaCl_2$, are then added and the time taken for a fibrin clot to form is measured. In order to standardise the control of anticoagulant therapy, it is necessary to be able to compare results between different laboratories, not only because of the wide variety of automated methods used, but also because of the numerous types and batches of thromboplastin available, each with differences in their sensitivity. Consequently, results are expressed as the International Normalised Ratio or INR (WHO, 1983). All thromboplastins are now calibrated against a WHO standard and their sensitivity is expressed as the International Sensitivity Index (ISI), where the primary standard has an ISI of 1.0. The manufacturer must calibrate every batch of thromboplastin and mark the ISI value clearly on the reagent label.

Within each laboratory, a normal range for the PT must be established every time a new batch or brand of thromboplastin is used, or when an automated system is replaced. This is determined by measuring the PT of at least 20 healthy adults and calculating the geometric mean or Mean Normal Prothrombin Time (MNPT) (Hall and Malia, 1991). This is used for the calculation of all subsequent INRs using the formula:

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

1.5.4 Clinical indications for anticoagulation

In the UK, indications for the commencement and maintenance of OAT have been proposed by the British Committee for Standards in Haematology (BCSH, 1998) and revised (BCSH, 2006) and broadly fall into two categories. Firstly, OAT is indicated in patients who have an increased risk of thrombosis i.e. AF and/or mechanical prosthetic heart valves, as well as patients with an inherited thrombophilia or following orthopaedic surgery (Baglin *et al*, 2006). Secondly, patients require OAT if they have an existing thromboembolic event, such as PE or DVT, in order to prevent further propagation of the thrombus and also to reduce the risk of embolisation.

In either case, therapy may be maintained for a short period of time, usually 3-6 months, or life-long, depending on the risk of thrombus development. In addition, the level of anticoagulation needed for prevention or prophylaxis, as determined by the target INR, needs to be balanced against an individual's risk of developing haemorrhagic complications (Dahlbäck, 2000).

Therapeutic monitoring is guided by maintaining the INR within +/- 0.5 units of the target (i.e. target 2.5, range 2.0-3.0 or target 3.5, range 3.0-4.0), as recommended by the BCSH (BCSH, 1998) and is summarised in Table 1.1.

1.5.5 Adverse effects of warfarin

Warfarin is one of many common drugs that has been linked to fatal medication errors in primary care (Baglin *et al*, 2006) and is near the top of a list of ten drugs associated with prescribing or dispensing errors in secondary care. In the UK, between 1990 and 2002, there were 480 cases of harm or near harm involving anticoagulant therapy, including 92 deaths directly attributable to warfarin; in addition, warfarin is in the top ten of drugs cited in errors leading to litigation against NHS Trusts (Baglin *et al*, 2006). The commonest cause of morbidity and mortality during warfarin therapy is due to haemorrhage, mainly due to accidental overdose (especially in the elderly), drug interactions or a high initiation dose and bleeding may manifest as bruising, haemoptysis, epistaxis, haematuria or melaena (Reardon *et al*, 1995).

Table 1.1 Indications for oral anticoagulant therapy, target INR and recommended duration of therapy.

Indication	Target INR	Duration
PE DVT	2.5	3-6 months
Recurrent PE/DVT Inherited thrombophilia Atrial fibrillation Cardiomyopathy Antiphospholipid syndrome Arterial grafts Coronary artery thrombosis	2.5	lifelong
DVT/PE whilst on OAT	3.5	lifelong
Aortic mechanical heart valves Mitral mechanical heart valves	2.5 or 3.0 3.0 or 3.5	lifelong

BCSH, 2006.

PE = pulmonary embolism

INR = International Normalised Ratio

OAT = oral anticoagulant therapy

DVT = deep vein thrombosis

The risk of haemorrhage during long term OAT has been estimated to be between 1-15% per year (Oden and Fahlen, 2002); however, the risk of major bleeding is greatest at the start of treatment rather than during maintenance, mainly due to the fact that the patient's stable therapeutic dose is unknown (Gage and Eby, 2003; Linkins *et al*, 2003). In one study, 7% of patients (n=162) had some form of bleeding episode within one month of commencing warfarin therapy, which increased to 12% within two months (Beyth *et al*, 2000).

Heparin is administered when anticoagulation is required during pregnancy, due to the adverse effects of warfarin on the foetus. Firstly, warfarin is able to pass through the placental barrier and may cause foetal haemorrhage due to the immaturity of the foetal liver (BCSH Guidelines, 1998). This has been associated with spontaneous abortion and neonatal death (Schardein & Macina, 2007). Secondly, warfarin is a teratogen and the incidence of birth defects in foetuses exposed to it *in utero* has been estimated to be about 5% (Schardein & Macina, 2007). Known as foetal warfarin syndrome (FWS), these defects include skeletal abnormalities (particularly following exposure in the first trimester) and central nervous system disorders, such as seizures and eye defects, in the second and third trimesters. FWS has also been associated with a decrease in birth weight and developmental retardation (Loftus, 1996).

1.5.6 Initiation of OAT

There are several initiation dosing regimens and the decision as to which is used can depend on multiple factors. These include the age, gender and body size of the patient, as well as concomitant drugs and the morbidity and mortality implications of the thrombotic event. The positive diagnosis of a thrombotic event, such as PE or DVT, usually requires higher initial doses of OAT than in cases of prevention (i.e. AF), with concomitant once-daily administration of subcutaneous low molecular-weight heparin (LMWH) (BCSH, 1998).

Fennerty *et al* (1984) proposed a dosing schedule, tailored to each patient's INR on days 2 and 3, using an initial dose of 10mg on the first day (Appendix IX). A prediction of the final maintenance dose was made according to the INR response on day 4 and all the patients in the study (n=50) were within the

therapeutic range by day 6. The study showed a close correlation between the predicted and actual maintenance dose ($r=0.867$ $p<0.001$).

Similarly, a study by Kovacs *et al* (2003) showed that a 10mg initiation protocol not only resulted in patients achieving the therapeutic INR 1.4 days earlier than 5mg ($p<0.001$), but that 83% reached the therapeutic range by day 5 ($n=104$) as opposed to 46% with the 5mg dose ($n=97$, $p<0.001$). There was no significant difference between the two groups in the number of INRs >5.0 and the study concluded that, like Fennerty *et al* (1984), a 10mg dosing protocol was superior to 5mg due to the fact that the therapeutic INR can be achieved more quickly.

While the Fennerty and Kovacs regimens proved to be useful for quickly reaching a stable therapeutic INR for in-patients with an existing thrombus, Tait and Sefcick (1997) designed an initiation protocol which was less intense, due to the fact that in many cases anticoagulant therapy is prophylactic with no immediate threat of thrombosis, especially in patients with AF. Moreover, a rapid induction protocol in elderly patients, using high doses of warfarin, has a high risk of over-anticoagulation and therefore haemorrhage. The Tait protocol (Appendix X), using an induction warfarin dose of 5mg for 4 days ($n=36$), was compared with that of Fennerty *et al,* with a loading dose of 10mg ($n=33$). The time taken to reach the therapeutic INR was similar in both groups; however, with the new regimen, there were fewer INRs >4.5 (2/36 vs. 9/33) as well as a more accurate prediction of the final maintenance dose ($r=0.985$). Similarly, Crowther *et al* (1999) found that more patients achieved a therapeutic INR within five days (21/32) on the 5mg induction dose than those on the 10mg dose (5/21, $p<0.003$).

The original guidance for the initiation of warfarin therapy in the UK (BCSH, 1998) recommended an initial oral dose of 10mg (Fennerty *et al*, 1984), with a subsequent change in dose for days 2, 3, and 4, depending on the INR response. This has been superseded (BCSH, 2006) by taking a more cautious approach; for outpatients not requiring rapid anticoagulation, a smaller loading dose of 2mg (Oates *et al*, 1998) or 3mg (Janes *et al*, 2004) is recommended, as these achieve the therapeutic INR within 3-4 weeks, while significantly reducing

the risks of haemorrhage through over-anticoagulation. In cases where more rapid anticoagulation is required, 5mg doses are recommended, as compared to 10mg, especially in cases of liver disease, cardiac failure, patients over 60 years old and those at risk from haemorrhage (BCSH, 2006).

1.5.7 Reversal of OAT

The risk of haemorrhage with OAT has been shown to significantly increase when the INR reaches 5.0 or higher in a study of patients with mechanical heart valves, with 2 adverse events per 100 patient years for INRs between 2.5-4.9, rising sharply to 75 events per 100 patient years for INRs ≥6.5 (Cannegeiter *et al*, 1995). The mode of intervention for the reversal of excessive anticoagulation is dependent on the INR and the presence or absence of bleeding and its severity. The initial recommendations regarding the management of hypercoagulable states (BCSH, 1998) have since been revised and modified (BCSH, 2006) and these are summarised in Table 1.2.

1.6 Factors affecting warfarin therapy

1.6.1 The pharmacogenetics of warfarin treatment

Pharmacogenetics is the study of variations in the DNA sequence of a patient in relation to their response to a particular drug, and therefore its safety and efficacy (French *et al*, 2010). It is a rapidly developing field of science for predicting both the dose requirements and patients' responsiveness to various drugs and can ultimately help clinicians make informed decisions with regard to dosing and improve patient outcomes (Gage & Lesko, 2008). The eventual aim is to develop personalised drug therapies and progress is becoming more widespread following recently available genetic testing (French *et al*, 2010).

Pharmacogenetics is the molecular basis that drives the processes of pharmacokinetics (the effect of the body on a drug) and pharmacodynamics (the effect of drugs on the body) (French *et al*, 2010). Fundamentally, it is the detection of single nucleotide polymorphisms (SNPs), in which the nucleotide sequence at a particular position is changed from the most common one (wild type) by substitution, deletion, insertion or translocation (Al-Ghoul & Valdes, 2008).

Table 1.2 Recommendations for the treatment of excessive anticoagulation

INR	Action
INR >3.0<6.0 (target 2.5) INR >4.0<6.0 (target 3.5)	Reduce warfarin dose or stop Restart when INR<5.0
INR >6.0<8.0 Bleeding absent or minor	Stop warfarin Restart when INR<5.0
INR >8.0 Bleeding absent or minor	Stop warfarin Restart when INR<5.0 If risk factors for bleeding are present, administer 0.5-2.5mg vitamin K orally
Major bleeding	Stop warfarin Give prothrombin complex concentrate (50U/kg) Give 5mg vitamin K (orally or i.v.)

BCSH 2006

Several SNPs have been detected in recent years that appear to confer a greater sensitivity to warfarin and therefore such patients require a much lower daily dose than those exhibiting the wild-type gene (Elias and Topol, 2008).

1.6.1.1 Vitamin K epoxide reductase (VKOR) polymorphisms

The enzyme VKOR causes the reduction of vitamin K (see Fig 1.2), which is then responsible for the production of functionally active clotting factors II, VII, IX and X and it is this enzyme that is inhibited by warfarin (Reynolds *et al*, 2007). Measurement of VKOR activity has been available since the 1970s, but it was not until 2004 that the gene, located on chromosome 16, was identified and designated VKORC1 (vitamin K epoxide reductase complex subunit 1) (Li *et al,* 2004).

In recent years, several SNPs in VKORC1 have been shown to have a significant effect on warfarin dose requirements. However, complications have arisen in identifying which are responsible for warfarin sensitivity, due to the fact that the majority of VKORC1 SNPs are in linkage disequilibrium, i.e. they are located very close to one another on chromosome 16 and are therefore believed to be inherited together more often than would be expected by chance. Consequently, it is difficult to determine which individual SNP is responsible for the associated trait (Lindh, 2009). Further complications have occurred because several different notations have been used in the literature to designate the various VKORC1 polymorphisms (D'Andrea *et al.*, 2005; Geisen at al., 2005; Reider *et al.*, 2005) and these are shown in Table 1.3. Reider *et al* (2005) divided the VKORC1 SNPs into two main groups of jointly inherited polymorphisms, designated A and B, where group A is associated with a decrease in warfarin requirement and group B individuals show a requirement for higher warfarin doses. Geisen *et al* (2005) subdivided the VKORC1 variants into four groups, VKORC1 *1, *2, *3 and *4. VKORC1*1 is only found in African populations and VKORC1*2 is the equivalent of Reider's group A and therefore confers warfarin sensitivity. Both VKORC1*3 and *4 relate to Reider's group B and require much higher doses of warfarin to remain in the INR therapeutic range (Lindh, 2009). D'Andrea *et al* (2005) simply used the conventional nomenclature of the nucleotide change and its position.

In order to maintain consistency, all further references in this thesis will refer to the VKORC1 polymorphisms using the D'Andrea notation.

1.6.1.2 VKORC1 polymorphisms in various populations

Many studies of VKORC1 polymorphisms and their effect on warfarin dosing have concentrated on non-Caucasian populations, especially Chinese and Japanese, whereas several North American groups have included Afro-Americans in their cohort (Yin & Miyata, 2007). Consequently, the results have shown a wide variation in SNPs, due to the genetic variations in the different ethnic groups. As the cohort for this present study does not include non-Caucasian warfarin patients (see Methods section 2.2.), the remainder of this section will give an overview of research performed on Caucasian subjects.

D'Andrea *et al* (2005) showed that in a group of male and female Southern Italians (n=147), the wild type (CC) of VKORC1 1173 C>T resulted in a mean optimum warfarin dose of 6.2mg/day, whereas two polymorphisms (CT and TT) reduced the dose significantly to 4.8mg/day ($p=0.002$) and 3.5mg/day ($p<0.001$) respectively. No other polymorphisms of VKORC1 showed any correlation to the warfarin dose. In a study of North Americans of southern German decent, Caldwell *et al* (2007), using a multiple regression model, showed that SNPs in VKORC1 1639 G>A resulted in a reduced warfarin dose requirement and made a significant contribution to predictive dosing when combined with the CYP2C9 gene (see section 1.6.1.3), age, gender and body surface area (BSA).

Carlquist *et al* (2006), while studying 213 Swedish patients, found that VKORC1 1173 C>T variants reduced the warfarin dose requirements by 65% ($p<0.001$) and that, together with CYP2C9, genetic testing explained 33% of dose variance, compared to 12% for clinical data alone. Similarly, Wadelius *et al* (2005) looked at three VKORC1 SNPs (1639 G>A, 1173 C>T and 2255 C>T) in 201 Swedish subjects and showed that all had an effect on the maintenance dose required to keep the patients' INR in the required therapeutic range. A study on British Caucasian patients was carried out by Sconce *et al* (2005), which showed that, in 297 subjects, the SNP in VKORC1 1639 G>A had a significant impact on dose requirements ($p<0.001$).

Table 1.3 Nomenclature of the various VKORC1 polymorphisms (adapted from Lindh, 2009)

dbSNP	Nucleotide exchange	D'Andrea	Reider		Geisen			
			A	B	*1	*2	*3	*4
rs9934438	C→T	1173 C>T	●			●		
rs9923231	G→A	1639 G>A	●			●		
rs8050894	G→C	1542 G>C	●			●		
rs2359612	C→T	2255 C>T	●			●		
rs7294	G→A	3730 G>A		●			●	
rs7200749	C→T	3462 C>T		●			●	
rs17708472	C→T	698 C>T		●				●

Nomenclature as described by D'Andrea *et al* (2005), Reider *et al* (2005) and Geisen *et al* (2005).

dbSNP = the Single Nucleotide Polymorphism Database
(http://www.ncbi.nlm.nih.gov/projects/SNP/)

Nucleotides: A=adenosine, C=cytosine, G=guanine, T=thymidine

1.6.1.3 Cytochrome P450 (CYP) polymorphisms

Cytochrome P450 (CYP) enzymes are a group of haem proteins, found in hepatocytes, lung and kidney (Gardiner and Begg, 2006). One isoform,

CYP2C9 located on chromosome 10, is responsible for the metabolism of weakly acidic compounds such as non-steroidal anti-inflammatory drugs, oral antidiabetics and S-warfarin (Lal *et al*, 2006). SNPs of the gene for CYP2C9 were the first to be identified as having a significant effect on the therapeutic dose of warfarin (Scordo *et al*, 2002) and have been designated CYP2C9*2 and CYP2C9*3. Both are relatively common in Caucasians, with frequencies of 11-16% and 7-10% respectively (Lindh, 2009) and are responsible for amino acid substitutions in the CYP2C9 molecule (Arg144Cys with CYP2C9*2 and Ile359Leu with CYP2C9*3). In each case, the altered molecule remains functional, but has a reduced S-warfarin clearance compared to the wild-type CYP2C9*1 (Lindh, 2009). S-warfarin clearance is lower by approximately 20% and 45% in heterozygotes for CYP2C9*2 and CYP2C9*3 respectively (*1/*2 and *1/*3) when compared with the wild-type (*1/*1). In contrast, homozygotes for CYP2C9*3 (*3/*3) have a 90% reduced clearance of S-warfarin (Lindh, 2009). Therefore, CYP2C9*2 and CYP2C9*3 patients have an increased risk of over-anticoagulation, especially during the initiation stage, with a concomitant increase in the risk of haemorrhagic complications. Margaglione *et al* (2000) found that CYP2C9 variants were responsible for a 2.6 fold increase in the odds of bleeding when compared to the wild-type.

D'Andrea *et al* (2005) showed a significant influence of CYP2C9 polymorphisms on dosing in southern Italian Caucasians ($n=147$, $r=0.215$, $p<0.001$), while Carlquist *et al* (2006) found that the CYP2C9 variants reduced the required warfarin dose by 18-72% ($n=213$, $p<0.001$), when compared with the wild-type allele. Gage *et al* (2004) were only able to predict 10% of warfarin dose variability using CYP2C9 testing alone and emphasised the need to combine it with other clinical and pharmacogenetic data for each individual. Similarly, Sconce *et al* (2005), using a large group of 297 subjects, confirmed that the highest warfarin dose was required in those with the wild-type CYP2C9 gene and, together with VKORC1, age and height, was able to predict the correct therapeutic level in 55% of patients.

Kamali *et al* (2004) showed that CYP2C9 SNPs accounted for >20% of warfarin dose variability but suggested that measuring S- and R-warfarin concentrations in blood might be a more useful marker for identifying those patients with CYP2C9 variants, since the enzyme is only responsible for the elimination of S-

warfarin. However, such a technique would be beyond the budget of most laboratories and investing in genetic testing would also allow the measurement of other polymorphisms such as VKORC1.

1.6.1.4 CYP4F2

Cytochrome 4F2 (CYP4F2) is a vitamin K_1 oxidase (Fig 1.2) and therefore its mode of action may be the counterpart to that of VKORC1 in the vitamin K cycle (McDonald *et al*, 2009). A SNP of CYP4F2 (1347C>T) results in an amino acid substitution of valine for methionine at position 433 (Glurich *et al*, 2010), with the wild type being CC. It has been postulated that the presence of a T allele (CT or TT) causes a decrease in enzymatic activity, resulting in a reduced ability to metabolise vitamin K_1 (McDonald *et al*, 2009) and therefore an increase in hepatic levels of the vitamin, as compared to the wild type CC. In the case of anticoagulated patients, this would require higher doses of warfarin in order to inhibit the vitamin K cycle and it has been suggested that CYP4F2 is responsible for 2-7% of warfarin dose requirements (Jonas and McLeod, 2009).

SNPs in CYP4F2 were first suggested as having an influence on warfarin dose requirements by Caldwell *et al* (2008). In the study, three independent cohorts of American Caucasian warfarin patients (total n=625) showed an increase of 4-12% in the required warfarin dose per T allele present ($p=0.023$), which translated to an increase of approximately 1mg/day more in TT subjects when compared to the CC wild type.

The study also showed that, using clinical factors and data for the presence of VKORC1 and CYP2C9 SNPs, warfarin dose predictability from multiple regression analysis was 54%. However, this could be increased to 56% with the addition of CYP4F2 SNP data. The study concluded that, although the inclusion of CYP4F2 data had a significant effect on the ability to improve warfarin dose predictability, the potential benefits depended on race, as the frequency of the T allele decreased from 30% in Caucasians to 7% in African/Afro-Americans.
Other studies have concluded that the homozygous TT allele of CYP4F2 has a significant effect on warfarin dosing. Pautas *et al* (2010), in a study of French Caucasians, demonstrated an actual but statistically insignificant increase in warfarin requirement (mg per day) due to the presence of the T allele for CC,

CT and TT variants of 2.8±1.5, 3.0±1.5 and 3.3±1.7 respectively (n=300 p=0.13). When CYP4F2 was added to the effect of other variables, the warfarin dose predictability increased by 1.4% (p=0.0545).

Carlquist *et al* (2010) in the USA, showed that CYP4F2 increased the predictability from 42% to 47% when added to a regression model (n=170 p<0.001) and Sagrieya *et al* (2010), who also studied the effect of CYP4F2 in the USA, found that the gene added 4% to the variability of warfarin dosing (n=108 p<0.05). However, the ethnicity of the subjects in both studies was not stated. Wells *et al* (2010) also showed a significant increase in the daily warfarin dose in the presence of the TT allele, when compared to CC or CT (p<0.001) in a study of Canadian warfarin patients (n=249), in which 94% were Caucasian, but the actual value in mg per day was not stated.

Conversely, some studies have questioned the significance of CYP4F2 SNPs in warfarin dosing. Lubitz *et al* (2010) did not find any significant effect (n=145 p=0.15) in the USA, but postulated that this could have been due to the inclusion of various ethnic groups in the study. Similarly, Kringen *et al* (2011), in a study of 105 Norwegian Caucasian patients, only found a weak association between warfarin dose and CYP4F2 (p=0.09) and concluded that the contribution of the gene was negligible. However, out of the seven subjects with the homozygous TT allele, four had various combinations of the CYP2C9*3 and the VKORC1 (1173 C>T) TT variant, which have both been shown to confer warfarin sensitivity (see Sections 1.6.1.2 and 1.6.1.3) and which may have had a counteractive effect on the results.

1.6.1.5 Other genetic variants affecting warfarin dosing

The enzyme γ glutamyl carboxylase (GGCX) is an integral part of the vitamin K pathway and is responsible for catalysing the reaction between the vitamin and clotting factors II, VII, IX and X (Lal *et al*, 2006). In one study, Reider *et al* (2007) found that only one SNP of the gene showed a significant correlation with warfarin dose. However, this only accounted for 2% dose variance, as compared with VKORC1 (21%) and CYP2C9 (8%), suggesting that GGCX has little impact on warfarin dose studies.

Apolipoprotein E (APOE) is involved in the hepatic uptake of lipid-soluble vitamin K (Lal *et al*, 2006) and the effect of this gene in warfarin dosing has only been studied by two groups. Sconce *et al* (2006) found only one variant of the APOE gene made a significant but small contribution to the daily warfarin dose (mean +/- SD) when compared to the wild type (3.3mg +/- 1.9 versus 4.0 +/- 1.8, p=0.03). Using an algorithm of age + height + CYP2C9 + VKORC1 + APOE, they were able to make an accurate dosing prediction in 57% of patients, but concluded that APOE polymorphisms are not likely to be clinically significant in predicting dose requirements. A similar conclusion was made by Caldwell *et al* (2007), who found that APOE variants did not significantly contribute to a predictive dose model (n=570).

Genetic variants for the coagulation factors II, VII, IX and X have only been investigated in Japanese patients (Shikata *et al*, 2004). Homozygosity in one FII gene and two FVII genes showed the lowest warfarin maintenance dose requirement (n=45, $p<0.05$) and it was suggested that genotyping for these three variants, together with CYP2C9, could be useful in predicting the warfarin response of an individual.

1.6.2 The influence of age on warfarin dosing

Several groups, when looking at factors affecting warfarin dose, have found a statistically significant, negative correlation with age and have shown close agreement, despite the wide variation in the number of patients studied. Kamali *et al* (2000b) showed that age accounted for 25% of maintenance dose variation ($R^2=0.25$) and a later study equated this to a decrease of approximately 0.6mg of warfarin for each decade increase in age between 55-85 years old (Kamali *et al*, 2004).

This was confirmed by Sconce *et al* (2005), who showed a maintenance dose decrease of 0.5-0.7 mg per decade in patients between 20-90 years old, irrespective of their genotype or height. The largest of these studies (Gage *et al*, 2004) showed that age contributed to an 8% decrease in dose for every decade (n=329). Garcia *et al* (2005) used weekly (as opposed to daily) dosing and estimated that increasing age was responsible for a decrease of 0.4 mg per week every year (95% confidence interval, CI, 0.37-0.44, $p<0.001$). This was

shown to have a marked effect in the initiation phase of therapy, if age was not taken into consideration. Using the loading regime of 5mg once daily for three days resulted in an overdose in 82% of females and 65% of males >70 years of age, leading to a significant risk of haemorrhage in the early stages of treatment.

Many physiological, as well as demographic and clinical factors, have been postulated to be responsible for the significant effect of age with respect to warfarin dosing. Liver size is known to decrease with age and it is possible that a smaller liver volume could cause a decrease in the metabolism of warfarin and therefore a reduced oral requirement (Wynne *et al*, 1995). However, there are other factors that may be responsible, either individually or in synergy. Increasing age has been shown to significantly contribute to the decreased plasma clearance of warfarin (Sconce *et al*, 2005), which could either be as a result of decreased liver volume, renal function or both. As the risk of thrombotic episodes, and therefore the likelihood of warfarin therapy, increases with age, the age distribution of patients on OAT is not normal but skewed. Furthermore, the elderly are more likely to have other interfering co-morbidities and therefore polypharmacy with drugs known to potentiate the effects of warfarin (see section 1.6.6) (Gurwitz *et al*, 1992). In addition, there is an increasing risk of malnutrition with increasing age and consequently a decreased intake of dietary vitamin K, leading to an increased anticoagulant response (Reynolds *et al*, 2007).

1.6.3 The influence of gender on warfarin dosing

Kamali *et al* (2004) showed that gender had no significant effect on the warfarin dose in a cohort of British adults, and that the mean dose for females was 3.49mg and males 3.98mg ($p=0.59$). However, the total number of subjects was relatively low ($n=121$) compared with other studies. Conversely, in a study by Garcia *et al* (2005) in the USA, which examined 4616 patients prospectively and 7586 retrospectively, it was shown that females required 4.5mg per week less than men. On average, the maintenance dose of warfarin for men was 30mg/week, whereas that for women was 25mg/week ($p < 0.0001$) and the lowest doses were prescribed to women over the age of 80 years.

The suggested explanation for this gender difference is that females, on the whole, have a smaller mean body size (measured as body surface area), which is directly related to liver size and therefore a lower hepatic fat content. This could lead to a reduced hepatic clearance of warfarin by cytochrome P450 enzymes. However, data suggested that gender-related differences in warfarin dosing were independent of body weight. Alternatively, it has been suggested that some cytochrome P450 enzymes are regulated by sex steroids (Niemela *et al*, 1999). It appears that gender *per se* is not directly responsible for the difference in mean warfarin dose between men and women, but that it is due to the secondary effects of body and/or liver size and the metabolic effects of the cytochrome enzymes.

1.6.4 The influence of body size on warfarin dosing

Several groups have used some form of body size data in an attempt to ascertain whether or not this influences warfarin dose requirement. Only one group (Sconce *et al*, 2005) used height as a separate variable, showing that it significantly contributed to S-warfarin and total warfarin clearance ($p=0.001$). Using a multivariate regression model including height, age and two genetic variants (VKORC1 and CYP2C9) in 297 British patients, the estimated warfarin dose could be predicted in 55% of cases.

Using body weight as a variable, Carlquist *et al* (2006), in the USA, found it to be a weak parameter for optimum dose prediction ($n=213$ $p=0.021$), forecasting only 12% of patient doses when used together with age and gender as opposed to 33% when including CYP2C9 and VKORC1 genetic variants. Zhu *et al* (2007), also in the USA, increased the predictability to 61% when weight ($p<0.0001$), age ($p=0.0003$) and gender ($p=0.0024$) were considered with CYP2C9 and VKORC1 polymorphisms ($n=65$). A third study in the USA found that increasing weight inversely correlated with warfarin response and therefore required a lower dose ($n=530$ $p<0.001$) (Gurwitz *et al*, 1992).

Some studies have used a combination of weight and height by calculating the body surface area (BSA). Sconce *et al* (2005) found that BSA contributed significantly in a regression model for dose prediction ($r=0.21$, $p=0.005$). Gage *et al* (2004) showed that BSA had a 15% effect on warfarin dose ($r=0.50$,

$p<0.0001$), suggesting that it correlates with liver size and therefore hepatic warfarin clearance. However, Kamali *et al* (2004) demonstrated that, although both body weight ($r=0.25$, $p=0.005$) and BSA ($r=0.21$, $p=0.02$) had an influence on warfarin dose, neither contributed significantly when used in a regression model. Reynolds *et al* (2007) stated that body size, however it is measured, is likely to account for much of the gender based differences in warfarin dose requirements, while Garcia *et al* (2005), in trying to reconcile the differences in significance, compromised by stating that patients whose weight deviates significantly from the norm for their age should have their body mass taken into consideration in any dosing decision.

1.6.5 The influence of vitamin K concentration on warfarin dosing

Kamali and colleagues in the UK have done much of the work regarding vitamin K levels and the effects on warfarin dosage. An initial study (Kamali *et al*, 2000a) showed a significant negative correlation between vitamin K concentration and INR ($r=-0.39$, $p=0.034$) and it was suggested that the variability in dietary intake of the vitamin should be considered in patients with unstable warfarin control. Later in 2000, work by the same group (Kamali *et al*, 2000b) showed a significant correlation between vitamin K levels and both warfarin dose ($p=0.034$) and its metabolite vitamin K epoxide ($p<0.0001$). Further studies showed that there is a diurnal variation in vitamin K concentrations, with a mean maximum at 22.00 hours and a mean minimum (68% reduction of the maximum) at 10.00hrs (Kamali *et al*, 2001). When used in a regression model, vitamin K levels made no significant contribution to the predicted warfarin dose (Kamali *et al*, 2004).

Although these findings have provided further insight into the factors affecting the response to warfarin, they offer no practical solutions in determining the optimum dosage for an individual, due in part to the expense and complexity of the assays, except perhaps in advising patients on the need for a well balanced diet during OAT.

1.6.6 The influence of drugs on warfarin dosing

Several drugs available today are known to affect warfarin dosage, by either attenuating or potentiating its mode of action (BCSH Guidelines, 1998). Some

are prescribed for short-term use e.g. antibiotics, steroids etc. However, others are required for longer periods by anticoagulated patients, due to the fact that many suffer from co-morbidities, and include statins (for the reduction of cholesterol) and amiodarone (an anti-arrhythmic).

Simvastatin has been shown to reduce the daily warfarin dose requirement by approximately 9% (Hickmott *et al*, 2003; Sconce *et al*, 2006) and this is most likely due to its competition with warfarin in its metabolism through the P450 enzymes, CYP3A4 and CYP2C9, which are responsible for the breakdown of R- and S-warfarin respectively. Voora *et al* (2005) created an algorithm in which the effect of simvastatin, fluvastatin and amiodarone were included, together with polymorphisms for CYP2C9, resulting in a 42% dose variance prediction. However, SNPs of the coagulation factors and VKORC1 were not tested for and it was suggested that inclusion of all these factors would improve the accuracy of future dosing algorithms.

Gurwitz *et al* (1992) constructed a multiple linear regression model using medication with either a potentiating (amiodarone, allopurinol and propranolol) or attenuating (corticosteroids and sucralfate) interactive effect with warfarin, on a cohort of 530 warfarin patients. The results showed that only the three drugs that increase the effect of warfarin had a significant effect on the predicted anticoagulant dose.

1.7 The use of mathematical algorithms in warfarin dosing

As stated previously (section 1.5.6), the initiation of warfarin therapy has traditionally been a matter of trial and error, with some studies attempting to develop dosing strategies based on clinical information and/or the dose given in the first few days of therapy (Crowther *et al*, 1999; Fennerty *et al*, 1984; Janes *et al*, 2004; Kovacs *et al*, 2003; Oates *et al*, 1998 and Tait and Sefcick (1997). However, in recent years, with the discovery that certain SNPs (see above) can have a significant effect on the variability of warfarin dosing between patients, several studies have attempted to construct mathematical algorithms for the prediction of an individual's stable maintenance dose, based on a combination of demographic, clinical and pharmacogenetic information (Shaw *et al*, 2010).

Many of these algorithms have been constructed using racially homogenous populations (Lubitz *et al*, 2010), such as Caucasians, African-Americans and southern or south-eastern Asians. Since the frequency of the two main SNPs (CYP2C9 and VKORC1) is significantly different between races (Yin and Miyata, 2007), studies which have attempted to compare the accuracy of the various algorithms have found it difficult without statistically sufficient numbers of all the ethnic groups.

The warfarin patients available for this study in the Anticoagulant out-patient clinic at Huddersfield Royal Infirmary (n=808) consisted of 95.5% Caucasian (n=772), 1.5% Afro-Caribbean (n=12), 2.6% southern Asian (n=21) and 0.4% south-eastern Asians (n=3). Since there were statistically insufficient numbers of non-Caucasians for this study, the published algorithms used for comparison were only selected if either they were constructed from a cohort of Caucasian-only subjects or if race could be removed without any detriment to the final result (Gage *et al*, 2004; Gage *et al*, 2008; IWPC. 2009; Sconce *et al*, 2005; Voora *et al, 2005;* Wadelius *et al*, 2009; Zambon *et al*, 2011 and Zhu *et al*, 2007).

In addition to genetic factors, three of the studies mentioned above also calculated the efficacy of algorithms constructed of clinical and demographic data only (Gage *et al*, 2008; IWPC, 2009 and Zambon *et al*, 2011). Whereas most have used age and a measure of body size (height, weight, BMI or BSA), other parameters such as interacting drugs, target INR, smoking status and gender have been used less frequently. The various parameters used in the published algorithms chosen for testing in this study are shown in Table 1.4.

1.8 Aims and objectives of the study

It is clear that OAT is a vital treatment in the prevention and treatment of thromboembolism. However, finding the correct therapeutic dose for any individual patient is difficult, due to the numerous interacting clinical, demographic and genetic factors that influence warfarin and its action. Achieving a therapeutic INR quickly and safely in the initial stages of treatment is, at the present time, one of trial and error in the vast majority of cases.

Table 1.4 The clinical, demographic and pharmacogenetic parameters used in the construction of the selected algorithms.

- Denotes the parameters used to construct each algorithm.

Algorithms using clinical data only are shown in yellow. Refer to Appendix XI for the full algorithms.

1 The subjects consisted of either Caucasians (C) or a mixture of races (M). The algorithms for the mixed race studies can still be used for single race studies by replacing the non-Caucasian parameter with zero.

2 Body surface area

3 Studies included either groups of drugs, i.e. CYP enzyme-inducers (IWPC 2009), the number of drugs which increase the INR (Wadelius *et al* 2009) or specific drugs, i.e. simvastatin and/or amiodarone (Gage *et al* 2004, Gage *et al* 2008, Voora *et al* 2005).

4 If the initial diagnosis for warfarin therapy was either PE or DVT, this was included in the algorithmic calculation.

5 Polymorphisms for VKORC1 are in linkage disequilibrium and therefore either 1173C>T or 1639G>A were measured.

Author	Race[1]	n	Age	Gender	Weight	Height	BSA[2]	Target INR	Smoke	Drugs[3]	DVT/PE[4]	CYP2C9	VKORC1[5]	CYP4F2	% predictability
Zambon (2011)	C	371	•				•					•	1639G>A	•	52
Gage (2004)	M	329	•	•			•	•		•		•			39
Gage (2008)	M	1015	•				•	•	•	•	•	•	1639G>A		53
IWPC (2009)	M	4043	•		•	•				•		•	1639G>A		43
Sconce (2005)	C	297	•			•						•	1639G>A		55
Voora (2005)	M	48	•	•			•	•		•		•			42
Wadelius (2009)	C	1496	•	•						•		•	1639G>A		59
Zhu (2007)	C	65	•	•	•							•	1639G>A		61
Zambon (2011)	C	371	•				•								31
Gage (2008)	M	1015	•				•	•	•	•	•				22
IWPC (2009)	M	4043	•		•	•				•					26

Consequently, any mechanism by which an individual's therapeutic dose could be predicted with more accuracy should, as a consequence, help in both achieving a safe state of anticoagulation more quickly and maintaining it for longer. The hypothesis is that, by designing a more comprehensive algorithm than has been achieved in previous studies, the warfarin dose requirement can be predicted in a greater number of patients, which can be applied in the future for patients during the initiation stage of warfarin therapy. This will be achieved by the following objectives:

Objective 1.

To test and validate the DNA extraction methodology, fingerprick blood samples will be taken from a small number of healthy adult volunteers.

Objective 2.

Capillary blood samples will be taken from approximately 200 stable warfarinised patients, together with demographic and clinical data. After PCR analysis of the samples, to determine the SNPs of each individual, all the data collected from 160 of these patients will be used to design two comprehensive dosing algorithms, one based on clinical and demographic data alone and one which includes genetic polymorphisms. The data collected on the remaining patients will then be entered into the new algorithms, as well as into published algorithms, to determine which one can most accurately predict the known stable therapeutic warfarin dose in this cohort.

Objective 3.

Venous and capillary blood samples, together with clinical and demographic data will be collected from approximately 40 patients *before* they begin warfarin therapy. The venous samples will be used to assay the baseline coagulation factors II, VII, IX and X, as well as albumin levels. The albumin and factor assay data will be examined by linear regression, in order to determine whether any of the parameters are significant in determining the eventual therapeutic dose. If so, these data could be used to design a future algorithm, because many district general hospitals would find an accurate dosing algorithm useful, and many do not have the facilities for PCR analysis, but do have the capability to assay coagulation factors and albumin. Therefore, it is hoped that the genetic data

may be substituted into the algorithm by albumin and coagulation factor levels and still maintain a high percentage of predictability, thereby producing a useful tool for many hospital anticoagulant departments in the UK.

Chapter 2

Materials and methods

2.1 Ethical approval

Ethical approval was obtained from the Leeds (Central) Research Ethics Committee for the National Research Ethics Service (Appendix I). Further approval was granted by the Research and Development Department, Calderdale & Huddersfield NHS Foundation Trust (Appendix II).

2.2 Subjects

Three separate groups of subjects were selected for the study; each group provided different information with regard to warfarin therapy that was ultimately used to produce the new dosing algorithms. Within each group, every subject was given a patient information sheet at least one week before their next clinic appointment and written consent was obtained prior to inclusion in the study. All subjects were Caucasian, due to the fact that previous studies have shown the absence of the selected SNPs in certain ethnic groups. In addition, less than 10% of the patients attending the Anticoagulant Clinic at Huddersfield Royal (n=800) were non-Caucasian and consequently there were insufficient numbers to allow statistical analysis.

2.2.1 Group 1 - healthy controls

These consisted of healthy members of staff (n=12) from the Haematology Laboratory, Huddersfield Royal Infirmary.

2.2.2 Group 2 – stable warfarin patients

These consisted of 207 individual patients, who regularly attended the out-patient warfarin clinic at Huddersfield Royal Infirmary. The time interval between appointments varied, depending on the stability of their INR result. However, no patient was allowed to go more than 12 weeks between tests. Each patient had

been taking warfarin for several months, if not years, and were only included in the study if both their INR was within the therapeutic range at the time of their visit and there had been no warfarin dose change for at least the previous 8 weeks. Data from 160 of these patients were used to construct the algorithms for determining the most appropriate warfarin dose in future patients. The data of the remaining 47 patients were used to test the predictability of the constructed algorithms.

2.2.3 Group 3 – pre-warfarin treatment patients

Patients were recruited to this group (n=20) after referral to the Anticoagulant out-patient clinic but *before* they had commenced warfarin therapy. Data from this group were used to determine the baseline levels of the vitamin K dependent coagulation factors (FII, FVII, FIX and FX) and serum albumin. The stable warfarin dose was recorded, once the INR had reached the therapeutic range.

2.3 Blood samples

2.3.1 Group 1 subjects

A finger prick blood sample of approximately 100 μl was obtained and placed on an FTA® card (Whatman International Ltd.). This was used to optimise the DNA extraction procedure and the PCR methodology. Samples were randomly numbered from 1 to 12 and anonymized.

2.3.2 Group 2 subjects

As part of their routine clinic appointment, patients have a 50 μl finger prick blood sample taken, in order to measure their INR using a Thrombotrak 2® point-of-care testing machine (Axis-Shield Ltd). For the study, a further 100 μl of blood for DNA analysis was obtained from the same puncture site and this was applied to an FTA® card, which was then labelled with the patient's unique hospital number for identification purposes.

2.3.3 Group 3 subjects

Similarly, each group 3 subject had 100 μl of blood taken for DNA analysis. As is the case for all patients before the commencement of warfarin therapy, routine venous blood samples were taken for full blood count (FBC), liver function tests (LFTs), urea and electrolytes (U&Es) and a coagulation screen. Permission was obtained in the consent form to use any remaining plasma from these samples to assay albumin levels and the vitamin K dependent coagulation factors.

2.4 Demographic information

All the demographic information required for the patients on warfarin therapy was obtained from the DAWN AC anticoagulation software (4S Information Systems Ltd). This software package is used as a tool to enable the Biomedical Scientists at the Calderdale and Huddersfield NHS Foundation Trust Anticoagulant Service to determine the optimum warfarin dose and next test interval on each individual patient, following an INR test result.

From the database, the gender and age were obtained for each patient in groups 2 and 3. The height and weight of all patients in both groups were obtained by a health care assistant at their out-patient clinic visit at the time of blood sampling.

2.5 Clinical information

The following clinical data were also obtained from the DAWN AC software for all patients in group 2:

- Clinical indication for anticoagulant therapy
- Target INR
- Concomitant medications
- Stable warfarin dose

In the case of group 3 patients, the clinical indication for warfarin therapy, concomitant medications and target INR were all obtained from the patients' referral form. The patients' stable INR and warfarin dose were obtained from the

DAWN AC database retrospectively, several weeks after the commencement of therapy.

2.6 DNA analysis

2.6.1 FTA® Elute cards

FTA® filter paper discs are designed for the collection and stabilisation of DNA from biological samples, prior to PCR analysis. They are impregnated with patented chemicals which both lyse cells to release the nucleic acids and also protect the DNA from microbial and fungal degradation. The blood samples are stable for several years, if stored in sealed pouches in a dry environment (www.whatman.com/FTAElute.aspx#OrderingInformation). Approximately 100 µl of capillary whole blood was obtained from each subject in all three groups, which was applied to two separate areas on an FTA® card and allowed to dry at room temperature for over 2 hours. These were then stored in sealed plastic bags at room temperature, containing a desiccant packet.

Before the DNA extraction procedure, four 2mm sample discs were cut from each individual FTA® card, using a 2mm Harris Uni-Core device (Whatman International Ltd) and transferred to a pre-autoclaved 1.5ml PCR grade microcentrifuge tube (VWR International Ltd).

2.6.2 DNA extraction methods

Initial studies using the healthy control spots were performed to optimise the method for DNA extraction for subsequent PCR analysis.

2.6.2.1 QIAamp® DNA mini kit method (QIAGEN)

All the buffers provided by the manufacturer were proprietary with unknown chemical composition and concentration. Using the FTA® card samples from the group 1 subjects (n=12), 180µl buffer ATL was added to each microcentrifuge tube and incubated in a heating block at 85°C for 10 minutes.

After briefly centrifuging at room temperature and 6000g for 30 seconds, 20µl Proteinase K stock solution was added, the samples were vortexed and incubated in a 56°C heating block for 60 minutes. Following a brief

centrifugation as above, 200µl Buffer AL was added and, after immediate vortexing, the samples were incubated for a further 10 minutes at 70°C. The samples were again briefly centrifuged as above, before the addition of 200µl absolute ethanol and then vortexed thoroughly. Each sample was decanted by pouring into an individual Mini Spin Column, supplied in the kit, fitted to a 2ml tube and centrifuged at 6000g for 1 minute at room temperature. The filtrate was then discarded and 500µl Buffer AW1 added without wetting the rim of the column.

After a second centrifugation at 6000g for 1 minute, the filtrate was discarded and 500µl Buffer AW2 was added to the column. The samples were centrifuged at 20,000g for a further 3 minutes, the filtrate was discarded and the columns were re-centrifuged at 20,000g for 1 minute. The collection tubes were discarded, clean tubes attached to the columns and 150µl Buffer AE was added to each. These were incubated at room temperature for 1 minute before centrifugation at 6000g for a further minute. The columns were discarded, the DNA solution was then pipetted into a sterile Eppendorf tube, labelled and stored at –70°C. The DNA extracted from the samples was quantified using the Nanodrop® technique (section 2.6.2.2).

2.6.2.2 DNA quantification by NanoDrop®

All the samples of extracted DNA were quantified using a NanoDrop® ND-100 Spectrophotometer (NanoDrop Technologies Inc., http://www.nanodrop.com). The absorbance at 260nm, using 1µl of each sample, gave the DNA concentration in ng/ml and the purity was determined from the ratio of the absorbance at 260nm to the absorbance at 280nm.

2.6.2.3 PCR analysis of the extracted DNA from the Group 1 subjects

A Taqman® allele probe for a common SNP unrelated to the study was used in order to assess the quality of the DNA, together with a Universal PCR Master Mix (Applied Biosystems Ltd.). The extracted DNA was analysed by PCR, in order to determine its integrity. The total reaction volume for each well of a 96-well Microamp™ optical plate was 10µl, consisting of 5µl Master Mix, 0.5µl probe and 4.5µl DNA (diluted in sterile pre-DEPC treated water) to a

concentration of 10ng/4.5µl. A negative control was included in two wells of each run which, instead of DNA, consisted of 4.5µl pre-DEPC treated water only. Each sample was run in duplicate.

2.6.2.4 QIAamp® DNA mini kit modified method (QIAGEN)

As poor results were obtained from the initial extraction method above (see results section 3.1), the following modifications to the protocol were made:

a) After the addition of Buffer AW2 and centrifugation, the tube and filter were cleaned thoroughly with a tissue.
b) Following the centrifugation at 20,000g, each filter was cleaned with a tissue after the collection tube had been discarded.
c) The final incubation with Buffer AE at room temperature was extended from 1 to 5 minutes.

The extracted DNA was again quantified using the Nanodrop® technique, as above.

2.6.2.5 Ethanol precipitation of haem

Due to the poor PCR results from section 2.6.2.2 (see results section 3.1), the extracted DNA samples were treated in order to remove any haem contamination derived from the original FTA® cards.

A 1/10 volume (4µl) of sodium acetate (3M, pH 5.2) and 120µl of 100% ethanol was added to 40µl of each extracted DNA sample in a 1.5ml microcentrifuge tube. These were incubated overnight at -20°C and centrifuged at >14,000x g for 30 minutes at room temperature. The supernatant was discarded, taking care not to lose the DNA pellet, which was then rinsed with 70% ethanol and centrifuged as before for 15 minutes. After discarding the supernatant, the DNA was resuspended in 60µl of sterile water for injection. The extracted DNA was again quantified using the Nanodrop® technique.

A total of five samples, covering a range of DNA concentrations (0.6-3.3ng/µl), were chosen for PCR analysis, as in section 2.6.2.2, with each sample being analysed in duplicate.

2.6.2.6 Whatman® method for DNA extraction

Following poor results from the ethanol precipitation samples (see results section 3.1), a third DNA extraction method, as recommended by Whatman®, was performed. As only a small amount of sample remained from the group 1 FTA® cards, discs were used from the first four group 2 subjects.

Four 2mm FTA® sample discs were each placed in a 1.5ml microcentrifuge tube, 500μl of sterile water for injection was added and pulse vortexed three times for a total of five seconds. Excess liquid was removed from the discs by gentle squeezing against the side of the tube with a sterile pipette tip, before transferring the disks to a clean, pre-autoclaved microcentrifuge tube containing 75μl of sterile water for injection. All the tubes were transferred to a heating block at 95°C for 30 minutes, with pulse vortexing half way through the incubation. At the end of the incubation, each tube was pulse vortexed for approximately 60 times and briefly centrifuged for 30 seconds, in order to separate the discs from the eluate containing the eluted DNA. Using a sterile pipette tip, the discs were removed from each tube and the eluted DNA was measured by the Nanodrop® technique before storing at -20°C until analysis.

2.6.3 PCR methodology

2.6.3.1 General principles

The PCR methodology is based on the use of a thermostable DNA polymerase enzyme from the Thermophilus aquaticus bacterium (Taq), which amplifies DNA segments. The process occurs in three stages, with approximately 40-50 cycles at each stage:

1. Denaturation causes the hydrogen bonds between the double strands of DNA to be broken, forming two separate strands. This occurs at approximately 95°C.

2. An annealing phase, at 55-65°C, allows two specific primers, or oligonucleotides, to match and bind to complementary sequences on the DNA strands, resulting in amplification of the region of interest. The primers are

present in excess, compared to the DNA concentration, in order to maximise the chance of encountering the correct template.

3. The extension phase occurs at approximately 72°C, where the Taq polymerase adds a deoxynucleotide triphosphate (present in the reaction mixture) to an exact complementary match on the DNA template. This creates a DNA chain which complements the template.

2.6.3.2 Applied Biosystems TaqMan® Drug Metabolism Genotyping Assays

Each genotyping assay kit contains two specific TaqMan® probes (www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/general documents/cms_040597.pdf) each with a reporter dye at the 5' end. The probe for allele 1 is linked to a VIC® reporter dye and the allele 2 probe to a FAM™ reporter dye. In addition, each probe is attached to a Minor Groove Binder (MGB) and a non-fluorescent quencher. The MGB allows the design of shorter probes by increasing the melting temperature for the specific probe length, while the quencher, at the 3' end, allows detection of the reporter dye. In addition to the probes, each SNP assay requires TaqMan® Universal PCR Master Mix. This contains the DNA polymerase (AmpliTaq Gold®), the deoxynucleotide triphosphates and ROX™ Passive reference, an internal reference dye, which allows the signal from the reporter dye to be normalized, in order to correct for any internal fluctuations in the fluorescence. A graphic representation of the SNP assay is shown in Fig. 2.1.

2.6.3.3 Methodology for the SNPs VKORC1, CYP2C9*2 and *3 & CYP4F2.

All samples from patient groups 2 and 3 were analysed for each of the four selected SNPs. In every run, each sample was assayed in duplicate in a 96-well MicroAmp™ Optical plate. The total reaction mixture for each well was 10µl, consisting of 5µl Master Mix, 0.5µl probe and 4.5µl DNA (diluted in sterile pre-DEPC treated water) to a final concentration of 10ng/4.5µl. A negative control was included in two wells of each run which, instead of DNA, consisted of 4.5µl pre-DEPC treated water. Each plate was covered with MicroAmp™ Optical Adhesive Film before placing in the Applied Biosystems' StepOne™ Real-Time

PCR Thermal Cycler and programming the recommended setting, as given in the assay data sheet (Table 2.1).

Table 2.1 Applied Biosystems PCR thermal cycler conditions

Times and temperatures		
Initial step	Denature	Anneal/extend
Hold	50 cycles	
10 min at 95°C	15 secs at 92°C	90 secs at 60°C

A 2 min HOLD step at 50°C was recommended prior to the initial 10 min at 95°C when using the TaqMan® Universal Master Mix.

2.6.3.4 Nucleotide sequences of the assay probes.

The nucleotide sequence of the forward primer with the SNP in square brackets were as follows:

CYP4F2

CCCCGCACCTCAGGGTCCGGCCACA[C/T]AGCTGGGTTGTGATGGGTTCC GAAA

CYP2C9*2

GATGGGGAAGAGGAGCATTGAGGAC[C/T]GTGTTCAAGAGGAAGCCCGCT GCCT

CYP2C9*3

TGTGGTGCACGAGGTCCAGAGATAC[C/A]TTGACCTTCTCCCCACCAGCCT GCC

VKORC1

GATTATAGGCGTGAGCCACCGCACC[G/A]GGCCAATGGTTGTTTTTCAGGT CTT

2.6.3.5 Reading and analysis of the plates

The StepOne™ software performed a genotype on the whole reaction plate simultaneously by plotting the fluorescent intensity of the reporter dyes from each well as an Allelic Discrimination Plot (Fig. 2.2) and assigned a genotype to each cluster of results according to their position. Fluorescence from the VIC® dye probe indicated the presence of allele 1 and allele 2 was denoted by fluorescence from the FAM™ dye. Heterozygosity for both alleles appeared as a diagonal cluster.

2.7. Coagulation factor assays

Citrated whole blood samples from group 3 subjects were centrifuged at 4000g for 8 minutes at room temperature. The plasma was aliquoted into screw capped polypropylene tubes, which were labelled with the patient's hospital number and stored at –70°C within 2 hours of collection. Coagulation factors have previously been shown to be stable at this temperature for at least 18 months (Woodhams *et al*, 2001). Assay of factors II, VII, IX and X were performed on an ACL TOP® analyser (Instrumentation Laboratory Ltd), using the vitamin K factor assay programme. The assays are based on the principle of turbidimetric clot detection. The coagulation end-point is determined by measuring the change in optical density of the samples during the reaction via transmitted light at 671nm. The light absorption of the sample increases in proportion to the formation of a fibrin clot and consequently the decreasing transmittance through the sample is measured by a photo detector. The signal is then processed via computer software to determine the clot end-point. Before the analysis of the patients' samples, a new calibration curve was constructed for each of the coagulation factors and all subsequent assay runs included a normal and a low control sample (HemosIL® normal control plasma and HemosIL® special test control level II).

Fig. 2.1 Schematic representation of the PCR assay

(Reproduced with kind permission of Applied Biosciences Ltd).

1. Each plate well consists of the target DNA, forward and reverse primers (oligonucleotides) and two probes, specifically designed to detect the polymorphism being assayed.
2. After denaturation, each probe anneals to its specific sequence on the template between the forward and reverse primer locations. The quencher dye quenches the signal from the reporter dye if the probe is intact.
3. The DNA polymerase extends the primers on the template DNA and cleaves the probe or probes which are attached to the target sequence. This separates the reporter and quencher dyes, allowing the reporter dye to emit a fluorescent signal, indicating that either one or both alleles are present.

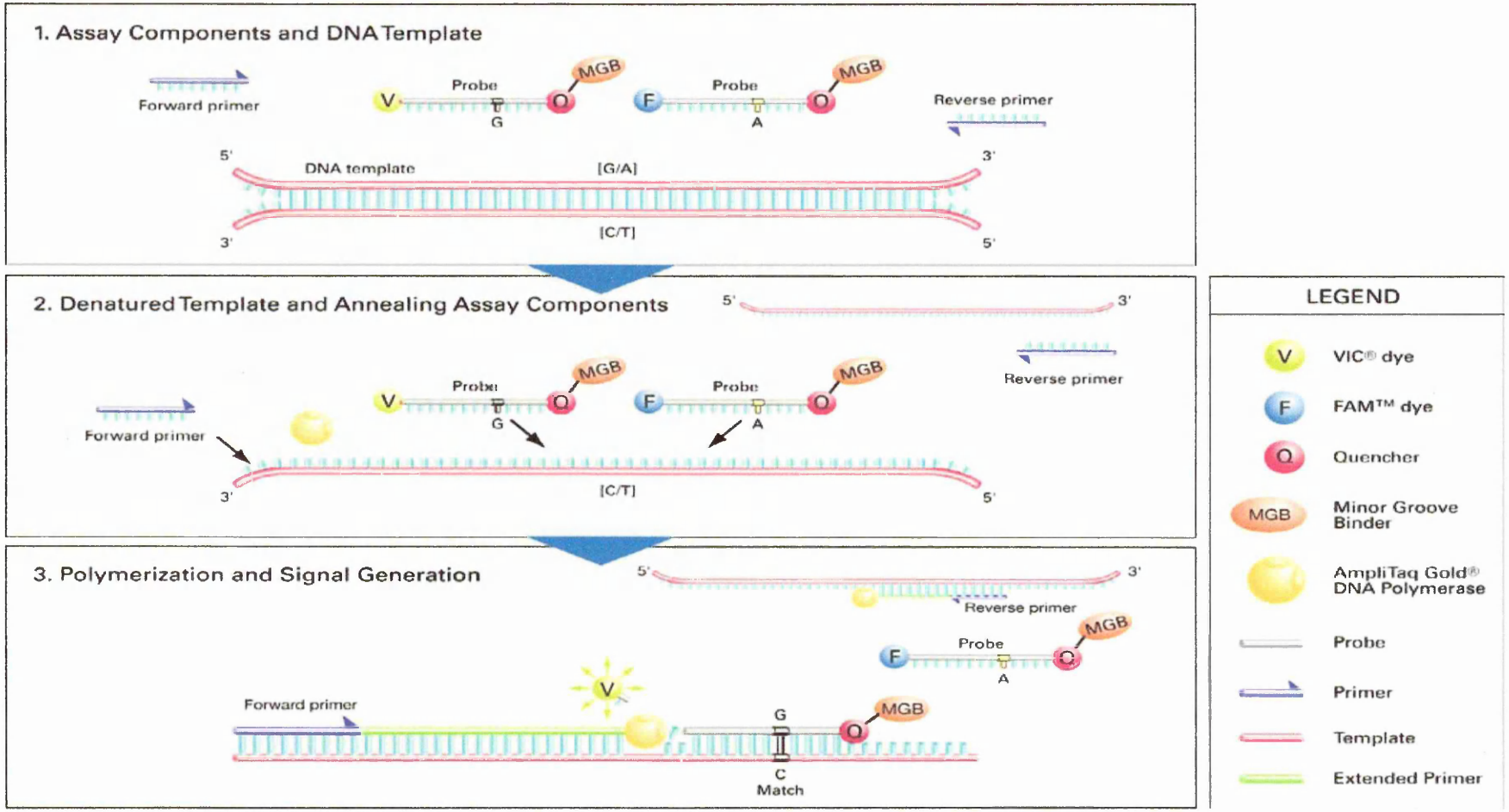
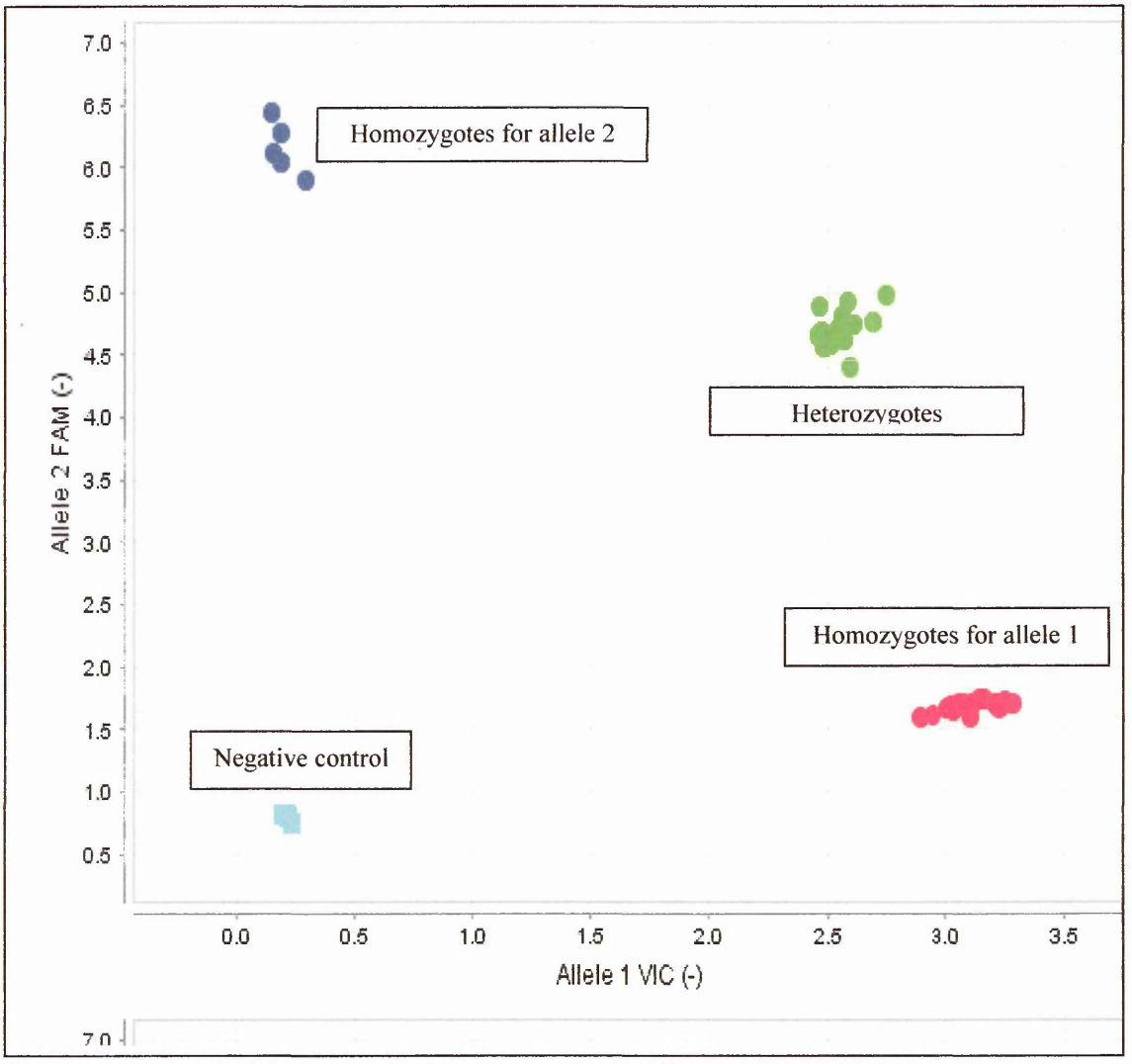

1. Assay Components and DNA Template
Forward primer
Probe
MGB
V
Q
G
F
A
Reverse primer
5'
3'
DNA template
[G/A]
[C/T]
2. Denatured Template and Annealing Assay Components
3. Polymerization and Signal Generation
C
Match
LEGEND
VIC® dye
FAM™ dye
Quencher
Minor Groove Binder
AmpliTaq Gold® DNA Polymerase
Probe
Primer
Template
Extended Primer

Fig. 2.2 Allelic discrimination plot



Reproduced with kind permission from Applied Biosciences.

2.8 Albumin assay

Albumin levels on group 3 patients were performed in the Clinical Chemistry Department, Huddersfield Royal Infirmary, using a Randox Albumin 2 kit (Randox Laboratories Ltd.) and the Siemens Advia 2400 analyser (Siemens Healthcare Diagnostics, IL, USA). Briefly, the assay uses the indicator 5,5-

dibromo-o-cresolsulphonphthalein (bromocresol purple, BCP), which binds quantitatively to serum albumin. The absorbance of the albumin-BCP complex is them measured using a spectrophotometer method at 600nm. The assay was calibrated using Randox Calibration serum Level 3 and controlled daily with Randox Assayed multisera levels 2 and 3. The method is linear up to 60.0 g/L and has a minimum detectable albumin concentration of 5.98g/L.

2.9 Statistical analysis

Statistical analyses were performed using MedCalc for Windows version 9.6.0.0 (MedCalc Software, Mariakerke, Belgium). The Box and Whisker plots were produced using Microsoft® Excel Starter 2010 version 14.0.6112.5000 (Microsoft Corporation). All data were tested for normality using the Kolmogorov Smirnov test and any which proved to be non-parametric were logarithmically transformed before analysis. The Pearson correlation coefficient was used to test the relationship between continuous variables and the warfarin dose; the means and standard deviations of the non-continuous parameters were analysed using the Student t-test. Comparisons between the means of continuous data was performed using the Analysis of Variance (ANOVA) and for non-continuous data by the Chi squared (χ^2) test. In all cases, a p value <0.05 was regarded as significant.

Chapter 3

Results

3.1 Demographic data

The demographic data is shown in Table 3.1. Only age and weight showed a non-parametric distribution, using the Kolmogorov Smirnov test, so these data were logarithmically transformed in order to calculate the means. Of the group 2 subjects (n=207), 111 were male and 96 were female, with a mean age of 66.2 years. The height (mean 1.69m) and weight (mean 85.8Kg) were used to calculate both the BSA (mean 2.00m^2) and the BMI (mean 30.0 Kg/m^2), using the following formulae:

$$BSA = \sqrt{\frac{\text{weight in Kg x height in m}}{36}} \quad \text{(Mosteller, 1987)}$$

$$BMI = \frac{\text{weight (Kg)}}{[\text{Height (m)}]^2} \quad \text{(World Health Organisation)}$$

The subjects were divided into two groups (based on the numerical order of their unique hospital number) consisting of an algorithm construction cohort (n=160) and an algorithm validation cohort (n=47). The analysis of variance (ANOVA) between the two groups showed no significant differences for age (p=0.815), height (p=0.116), weight (p=03821), BSA (p=0.671) and BMI (p=0.230). The pre-warfarinised patients (group 3, n=20) consisted of 15 males and 5 females, with an age range of 42-85 years (mean 68.6).

3.2 Clinical data

The clinical data, shown in Table 3.2, was taken from each subject's entry on the DAWN 4S® dosing software and had been provided initially, at the start of the warfarin therapy, by the referral from the patient's GP or consultant. The majority of all group 2 subjects (n=207) had a target INR of 2.5 (81.2%) and significantly less with a target of 3.0 or 3.5 (6.7% and 12.1% respectively). The commonest clinical indication for warfarin therapy was AF (51.2%), followed by DVT (17.4%), mechanical prosthetic heart valve (13.5%) and PE (9.7%). Other indications totalled 8.4%. Of the long term drugs which are known to interact.

Table 3.1 Demographic data for all group 2 subjects, the construction cohort, the validation cohort and group 3 subjects.

Analysis of variance (ANOVA) was used to compare the means of the data between the construction and the validation cohorts (p significant if <0.05).

	All Group 2 subjects (n=207)		**Group 2 construction cohort (n=160)**		**Group 2 validation cohort (n=47)**		**ANOVA**		**Group 3 (n=20)**	
Male, n (%)	111 (53.6)		79 (49.4)		33 (70.2)				15 (75.0)	
Female, n (%)	96 (46.4)		81 (50.6)		14 (29.8)				5 (25.0)	
Smoke, n (%)	30 (14.5)		23 (14.4)		7 (14.9)				0	
	Range	Mean	Range	Mean	Range	Mean	F	p	Range	Mean
Age (years)	23-90	64.5	23-90	64.3	28-86	65.8	0.0547	0.815	42-85	67.8
Height (m)	1.47-1.96	1.69	1.47-1.89	1.68	1.47-1.96	1.71	2.485	0.116	1.56-1.88	1.76
Weight (Kg)	42-233	83.4	42-233	83.6	50-130	82.8	0.0512	0.821	62-110	91.1
BSA (m^2)	1.33-3.45	2.00	1.33-3.45	1.99	1.47-2.61	2.01	0.181	0.671	1.63-2.37	2.11
BMI (Kg/m^2)	17.9-68.8	30.0	17.9-68.8	30.0	20.1-41.9	28.9	1.447	0.230	24.9-37.5	29.8

Table 3.2 Clinical data for all group 2 subjects, the construction cohort, the validation cohort and group 3 subjects.

Abbreviations for the clinical details included: atrial fibrillation (AF), deep vein thrombosis (DVT), pulmonary embolism (PE), transient ischaemic attacks (TIA) and impaired left ventricular function (LVF).
ANOVA testing (for continuous variables) and Chi squared (for non-continuous variables) was used to compare the data between the construction and validation cohorts.

	All group 2 subjects (n=207)	Group 2 construction cohort (n=160)	Group 2 validation cohort (n=47)	ANOVA		Group 3 subjects (n=20)
Mean warfarin dose, mg/day (range)	3.7 (0.5-11.0)	3.7 (0.5-11.0)	3.4 (1.0-10.0)	F=0.603 p=0.4.38		5.3 (2.3-10.0)
Target INR, n (%):						
2.5	168 (81.2)	131 (81.9)	37 (78.7)	F=1.097 P=0.296		1 (55)
3.0	14 (6.7)	10 (6.2)	4 (8.5)			9 (45)
3.5	25 (12.1)	19 (11.9)	6 (12.8)			0
Clinical details, n (%):				Chi squared		
				χ^2	p	
AF	106 (51.2)	86 (53.8)	20 (42.6)	1.402	0.236	19 (95)
DVT	36 (17.4)	26 (16.3)	10 (21.3)	0.337	0.562	0
PE	20 (9.7)	15 (9.4)	5 (10.6)	0.001	0.982	0
Mechanical heart valve	28 (13.5)	21 (13.1)	7 (14.9)	0.005	0.945	0
Stent	2 (1.0)	1 (0.6)	1 (2.1)	0.006	0.938	0
Arterial thrombus	7 (3.4)	4 (2.5)	3 (6.4)	0.699	0.403	0
Arterial bypass	2 (1.0)	1 (0.6)	1 (2.6)	0.006	0.938	0
Aneurysm	2 (1.0)	2 (1.2)	0	0.006	0.938	0
TIA	1 (0.5)	1 (0.6)	0	0.427	0.514	0
Lupus	0	0	0			1 (5)
LVF	3 (1.5)	3 (1.9)	0	0.063	0.801	0
Drugs, n (%)						
Simvastatin	72 (34.8)	54 (33.8)	18 (38.3)	0.161	0.688	2 (10)
Amiodarone	20 (9.7)	19 (11.9)	1 (2.1)	2.917	0.089	0
Omeprazole	28 (13.5)	23 (14.4)	5 (10.6)	0.173	0.677	1 (5)
Aspirin	24 (11.6)	21 (13.1)	3 (6.4)	1.020	0.312	12 (60)

with warfarin, 72 subjects (34.8%) were taking simvastatin, 28 (13.5%) omeprazole, 24 (11.6%) low dose aspirin and 20 (9.7%) amiodarone.

Using analysis of variance (ANOVA), there was no significant difference between the means of the construction cohort (n=160) and the validation cohort (n=47), with respect to the daily warfarin dose (F=0.603, p=0.4.8) and the target INR (F=1.097, p=0.296). The majority of subjects in both of these sub groups had a target INR of 2.5 (81.9% and 78.7% respectively). Comparison of the non-continuous data between the construction and validation cohorts was done using the Chi squared test (χ^2) and, in all the clinical details and interacting drugs, no significant differences were shown between the two groups.

The serum albumin and vitamin K dependent coagulation factors were assayed on all group 3 (pre-warfarinised) subjects (n=20) and all the parameters showed a normal distribution (Table 3.3).

Table 3.3 Albumin and coagulation factor levels for group 3 subjects (n=20)

	Mean	±SD	Range
Albumin, g/L	39.9	2.8	34-45
F II, %	96.6	12.7	69-131
F VII, %	105.3	20.8	68-151
F IX, %	147.5	20.5	120-212
F X, %	104.3	12.3	75-125

3.3 DNA extraction

The DNA yield obtained by the different extraction methods is shown in Table 3.4. The concentration, obtained from blood spots on the FTA® cards by the QIAamp mini kit (section 2.6.2.1), ranged from 6.2-12.4 ng/µl (mean=9.5 ng/µl, SD±1.71). The PCR analysis of the samples failed to produce any allelic discrimination using the common SNP assay (Fig 3.1). After implementing some modifications to the QIAamp method (section 2.6.2.3), the amount of DNA extracted was significantly lower than the original method, ranging from 2.0-6.9

ng/µl (mean=3.6 ng/µl, SD±1.37). Consequently, PCR analysis of the samples was not performed. The samples were treated in order to remove any haem contamination and produced even lower amounts of DNA
(range 0.4-3.3 ng/µl, mean=1.6 ng/µl, SD±0.82). PCR analysis of samples 4, 7, 8, 9 and 11 (which were chosen as they covered a range of DNA concentrations) using the same SNP assay, did not show any allelic discrimination (Fig 3.2).Table 3.5 shows the DNA yield from a selection of group 2 subjects (n=4), using the Whatman® extraction method (section 2.6.2.5). The amount of DNA obtained was significantly higher than with the previous methods (range 17.0-32.3 ng/µl, mean=21.3 ng/µl, SD 7.33). PCR analysis, using the VKORC1 SNP assay (Fig 3.3) showed good allelic discrimination in three samples. Subjects 2 and 3 were homozygous for each allele (GG and AA respectively) and sample 4 was heterozygous. Sample 1 was undetermined. Therefore, for future genotyping experiments, the Whatman® extraction method was used to extract the DNA from all the patient samples.

Table 3.4 DNA yields using various extraction methods

	DNA (ng/µl)		
Sample number	**QIAamp method**	**Modified QIAamp method**	**After haem extraction**
1	6.2	3.4	1.8
2	7.4	3.5	0.4
3	9.5	2.0	1.2
4	8.5	2.8	1.8
5	9.8	5.4	1.5
6	9.3	2.4	1.7
7	10.6	4.0	1.0
8	10.0	3.3	0.6
9	12.4	3.4	3.3
10	10.4	3.0	1.1
11	8.2	6.9	2.3
12	11.3	2.5	2.4
Mean	9.5	3.6	1.6
SD	1.71	1.37	0.82

Table 3.5 DNA yields of selected group 2 subjects using the Whatman® extraction method

Sample number	DNA (ng/µl
1	18.2
2	32.3
3	17.0
4	17.8
Mean	21.3
±SD	7.33

Fig 3.1 Allelic discrimination plot of group 1 subject DNA (n=12) using the QIAamp extraction method.

All samples were assayed singularly and the sample number is indicated by each allele plot.

Neg ctrl= negative control using water instead of DNA.

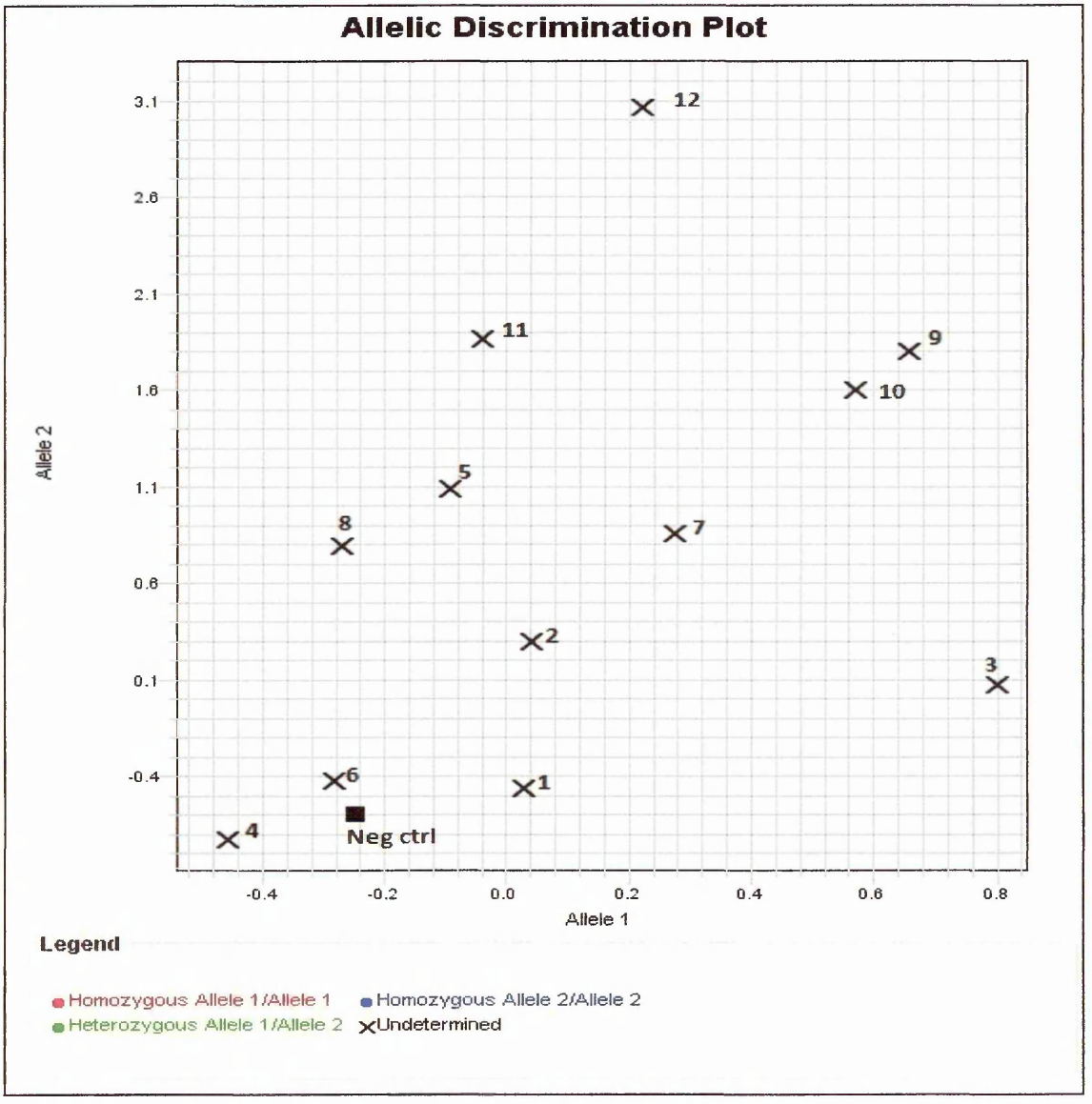


Fig 3.2 Allelic discrimination plot of selected group 1 subjects DNA (n=5) following the precipitation of haem.

All samples were assayed in duplicate and the sample number is indicated by the side of each allele plot.

Neg ctrl= negative control using water instead of DNA.

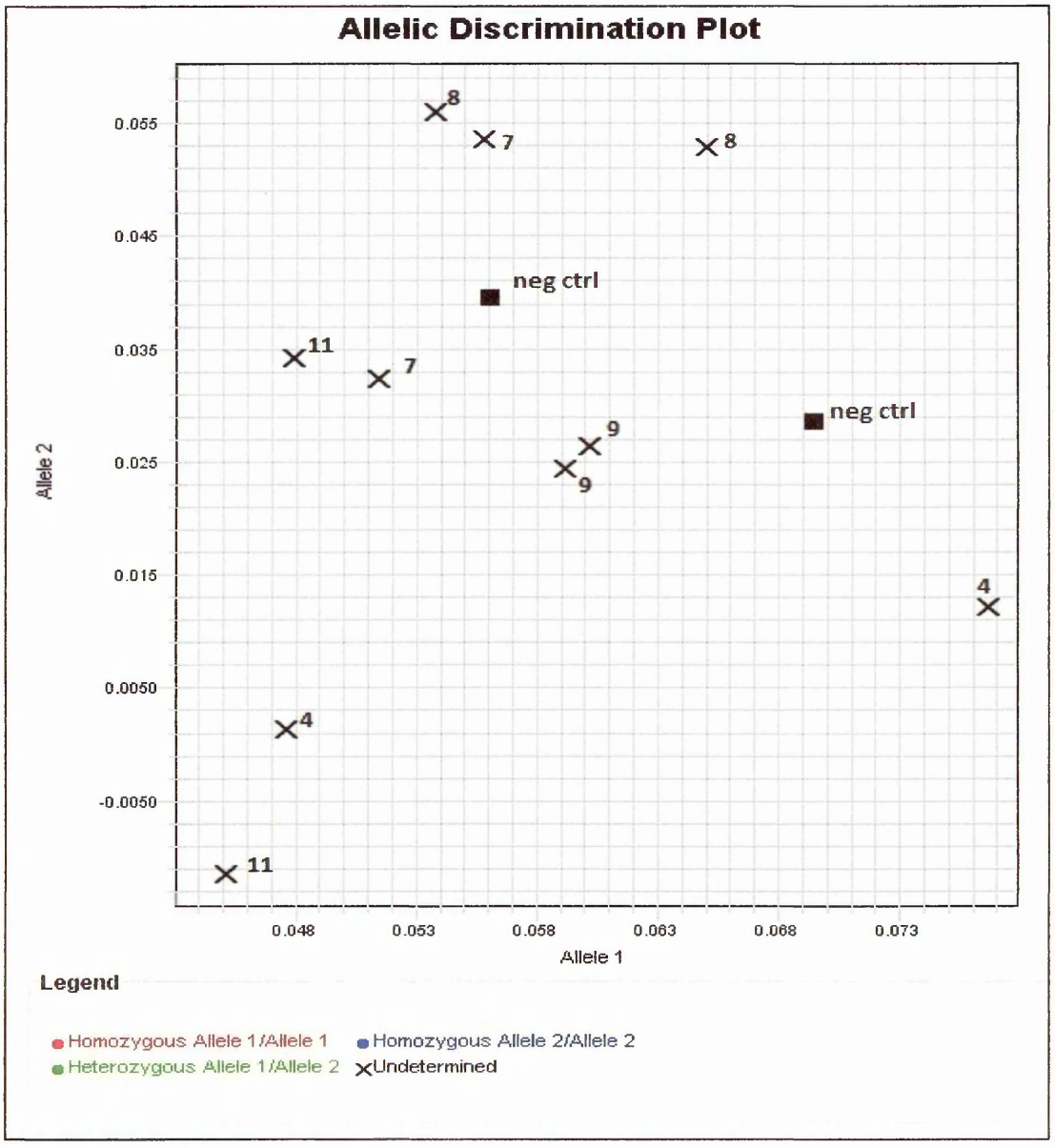
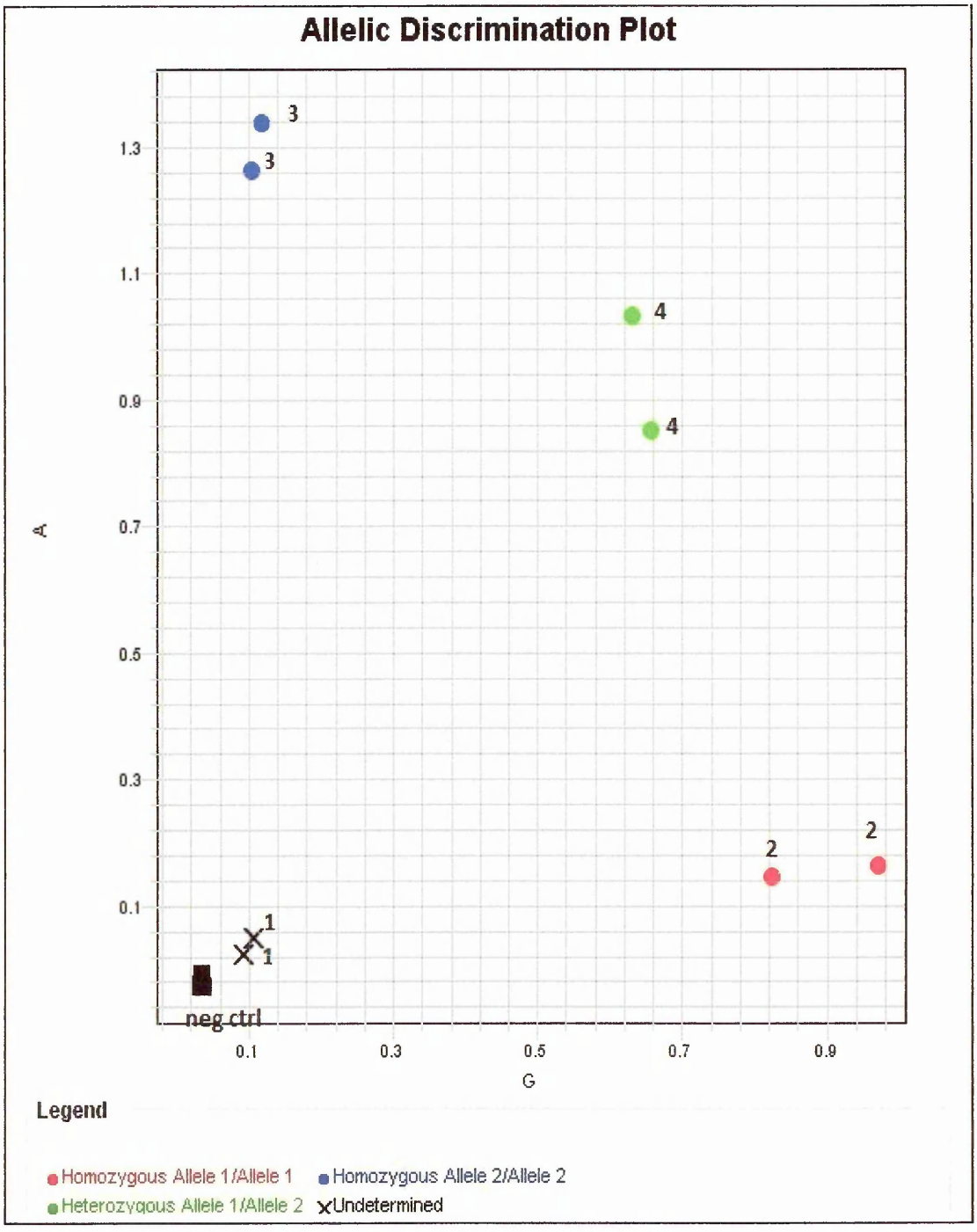


Fig 3.3 VKORC1 SNP assay results of selected group 2 DNA samples (n=4) using the Whatman® extraction method.

All samples were assayed in duplicate and the sample number is indicated by the side of each allele plot.

Neg ctrl= negative control using water instead of DNA



3.4 Pharmacogenetic data

The pharmacogenetic data for all groups of subjects is shown in Table 3.6. In the case of the VKORC1 polymorphisms, there were slightly more GA types (44.9%) compared to the wild-type GG (40.6%) in all group 2 subjects (n=207). For CYP2C9, the frequencies of the *2 and *3 alleles (*2/*2, *2/*3 and *3/*3) (5.8%) were much lower than either the homozygous wild-type (*1/*1) or heterozygous *1 alleles (*1/*2 and *1/*3) (94.2%). The majority of group 2 subjects were homozygote wild-types for the CYP4F2 C allele (59.4%), with only 40.6% possessing one or two T alleles. There were no significant differences in the numbers of the three SNPs between the construction and validation cohorts, as demonstrated by the Chi squared test.

3.4.1 The variation of the warfarin dose within the various SNPs

Figure 3.4 shows the effect of each individual polymorphism on the stable warfarin dose, using data from the construction cohort (n=160). The median daily warfarin dose was 4.0mg in the CYP2C9 homozygous wild type (*1/*1), which was the same as in the *1/*2 genotype. However, this was significantly higher than found in *1/*3 (2.7mg), *2/*2 (2.0mg), *2/*3 (1.0mg) and *3/*3 (0.5mg). The median daily dose was 5.3mg in the VKORC1 wild type (GG), which was higher than that found in the GA (3.5mg) and AA (2.3mg) genotypes. The wild type CYP4F2 (CC) had a median dose of 3.5mg/day, which increased in the presence of the T allele (CT 4.0mg and TT 5.5mg).

3.4.2 The effect of the number of SNPs on the warfarin dose

Using the data from all the group 2 subjects (n=207) in order to obtain a significant number of allele combinations, each subject was categorised depending on the number of polymorphisms they possessed (Figure 3.5). Those who exhibited only the wild type for each of the three genes (VKORC1 GG, CYP2C9 *1/*1 and CYP4F2 CC) had a median daily warfarin dose of 5.8mg. An increase in the number of SNPs present (1, 2 or 3) showed a corresponding decrease in the median dose (3.7mg, 3.5mg and 3.0mg respectively), despite the possible presence of a CYP4F2 SNP which has the effect of increasing the warfarin dose.

Table 3.6 Pharmacogenetic data for all group 2 subjects, the construction cohort, the validation cohort and group 3 subjects.

The Chi squared test was used to compare the numbers of each allele in the construction and validation cohorts.

	All group 2 subjects (n=207)	Group 2 construction cohort (n=160)	Group 2 validation cohort (n=47)	Chi squared		Group 3 subjects (n=20)
VKORC1, n (%)				χ^2	p	
GG	84 (40.6)	64 (40.0)	20 (42.6)	0.021	0.885	9 (45.0)
GA	93 (44.9)	73 (45.6)	20 (42.6)	0.042	0.837	10 (50.0)
AA	30 (14.5)	23 (14.4)	7 (14.8)	0.279	0.598	1 (5.0)
CYP2C9, n (%)						
*1/*1	132 (63.8)	100 (62.3)	32 (68.2)	0.279	0.598	15 (75.0)
*1/*2	41 (19.8)	34 (21.3)	7 (14.9)	0.567	0.451	3 (15.0)
*1/*3	22 (10.6)	17 (10.6)	5 (10.6)	0.071	0.790	2 (10.0)
*2/*2	6 (2.9)	5 (3.1)	1 (2.1)	0.019	0.892	0 (0)
*2/*3	5 (2.4)	3 (1.9)	2 (4.2)	0.155	0.694	0 (0)
*3/*3	1 (0.5)	1 (0.6)	0 (0)	0.427	0.514	0 (0)
CYP4F2, n (%)						
CC	123 (59.4)	94 (58.8)	29 (61.7)	0.037	0.847	8 (40.0)
CT	71 (34.3)	56 (35.0)	15 (31.9)	0.047	0.828	9 (45.0)
TT	13 (6.3)	10 (6.2)	3 (6.4)	0.095	0.757	3 (15.0)

Figure 3.4 Box and Whisker plot to show the change in warfarin dose dependent on the various polymorphisms.

Data are from the construction cohort (n=160).

The boxes indicate the median warfarin dose (mg/day) and the interquartile ranges. The bars above and below indicate the minimum and maximum values.

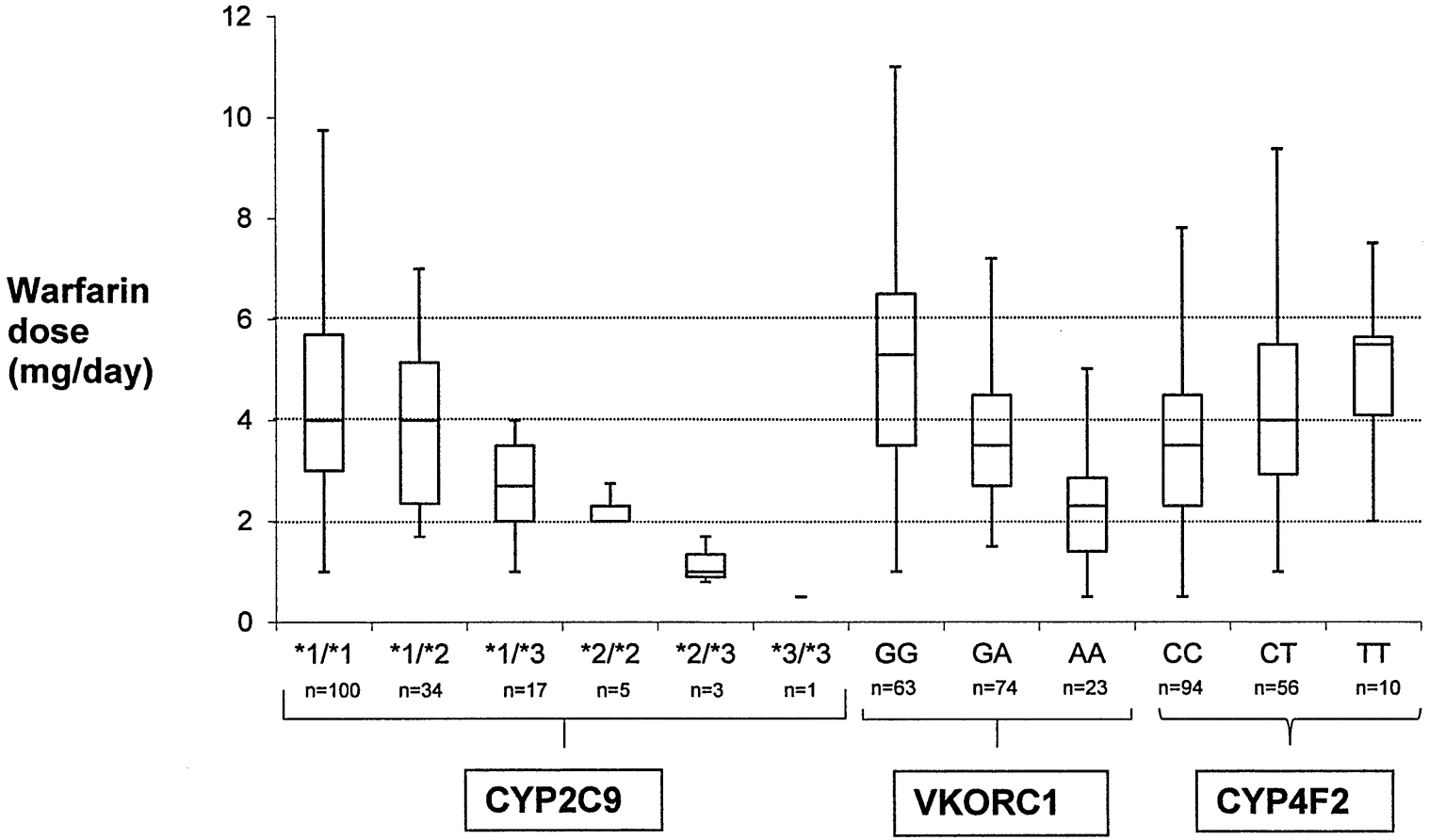
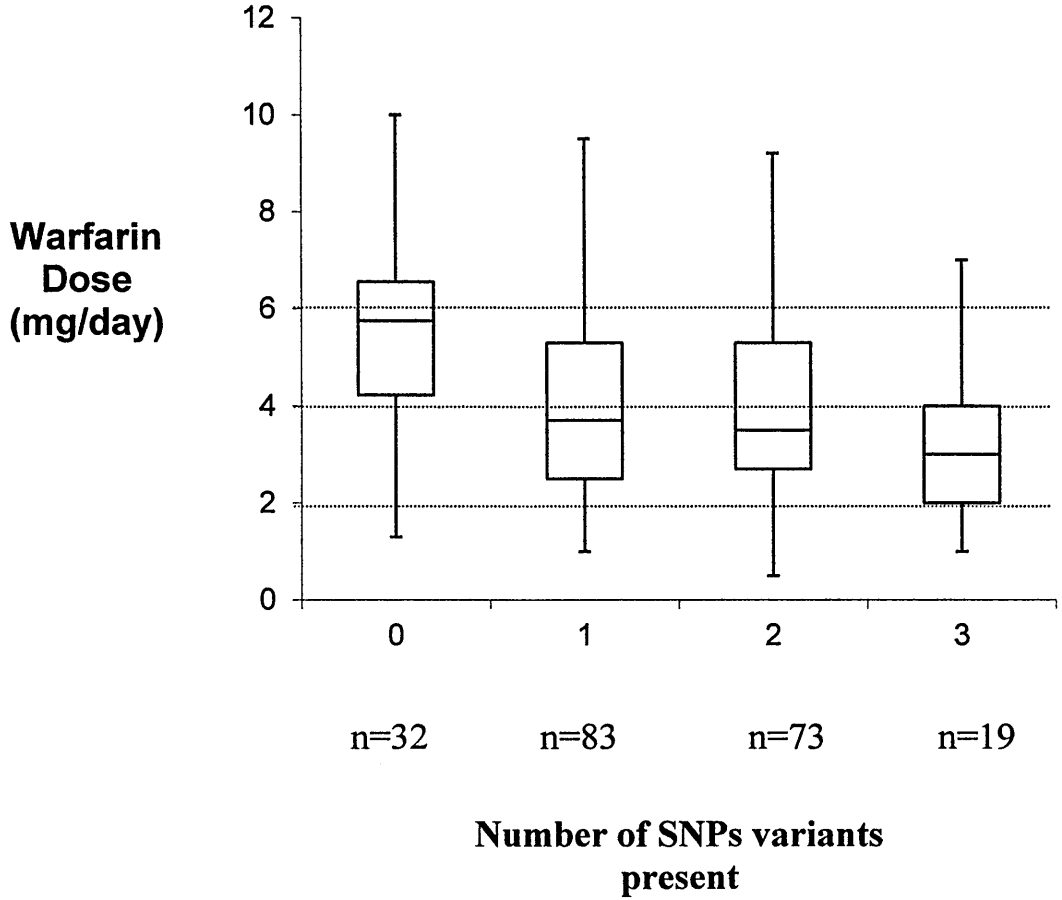

Warfarin dose (mg/day)
12
10
8
6
4
2
0
*1/*1 n=100
*1/*2 n=34
*1/*3 n=17
*2/*2 n=5
*2/*3 n=3
*3/*3 n=1
GG n=63
GA n=74
AA n=23
CC n=94
CT n=56
TT n=10
CYP2C9
VKORC1
CYP4F2

Figure 3.5 Box and Whisker plot to show the effect of one or more SNP variants on the daily warfarin dose.

Data are from all group 2 subjects (n=207).

The boxes indicate the median warfarin dose (mg/day) and the interquartile ranges. The bars above and below indicate the minimum and maximum values. Zero (x axis) represents subjects with the wild type for all three genes.


Warfarin Dose (mg/day)
12
10
8
6
4
2
0
0
1
2
3
n=32
n=83
n=73
n=19
Number of SNPs variants present

3.5 Construction of the clinical/demographic algorithm

Using the data from the construction cohort (n=160), correlation analysis was performed between each continuous variable (age, weight, height, BSA and BMI) and the stable warfarin dose (Table 3.7 a). Since age and weight were shown to be non-parametric, using the Kolmogorov Smirnov test, these data were logarithmically transformed. For each of the non-continuous variables (gender, smoking status and the interacting drugs), a Student-t test was performed to compare the means and SDs of the stable warfarin dose, depending on whether the variable was present or absent, i.e. smoker and non-smoker, male and female etc. These data are shown in Table 3.7 b. There was a strong correlation between age and the warfarin dose ($p<0.001$). Similarly height, weight, BMI and BSA showed significant correlations; however, this would be expected as height and weight are functions of the BSA and BMI. For the non-continuous variables, gender ($p=0.020$), smoking status ($p=0.018$) amiodarone use ($p=0.012$), AF ($p<0.0001$) and DVT ($p=0.030$) appeared to have a significant effect on the stable warfarin dose.

3.5.1 Simple regression analysis of the clinical and demographic variables

As the published algorithms use either the square root ($\sqrt{}$) or log of the warfarin dose (see Appendix XI), these data in the construction cohort were tested using Kolmogorov Smirnov analysis, to determine which transformation produced the best indication of normality. Raw warfarin dose data was rejected as having a normal distribution ($p=0.036$), but both the log and square root of the data were shown to be parametric ($p=0.256$ and $p=0.544$ respectively). It was therefore decided that, in the following regression analyses and the final algorithms, the square root of the warfarin dose would be used, due to it having a higher p value.

3.5.1.1 Simple regression analysis of the continuous clinical and demographic variables versus actual warfarin dose

In order to determine if the continuous variables would require transformation in the final algorithms, regression analyses were performed between the $\sqrt{}$dose and either the raw data, the $\sqrt{}$ data or the log data (Table 3.8).

Table 3.7a Correlation coefficients (r) between the actual warfarin dose and the continuous demographic and clinical variables for the construction cohort (n=160).

Significant p value <0.05

	r	p
Age	-0.4953	<0.0001
Weight	0.3097	0.0001
Height	0.3136	<0.0001
BSA	0.3407	<0.0001
BMI	0.2123	0.0070

Table 3.7b Student t-test for the stable warfarin dose versus the presence or absence of the non-continuous clinical and demographic variables in the construction cohort (n=160).

Significant p value <0.05

	t statistic	p
Male	-2.344	0.020
Female	2.344	0.020
Smoking status	-2.386	0.018
Simvastatin	1.774	0.078
Amiodarone	2.555	0.012
Omeprazole	1.377	0.171
Aspirin	0.361	0.719
AF	4.541	<0.0001
DVT	2.188	0.030
PE	0.719	0.473

Table 3.8 Regression analysis of the actual warfarin dose versus the continuous clinical and demographic variables

a) √dose vs. variable

b) √dose vs. √variable

c) √dose vs. log variable

p values significant if <0.05

a

	R^2	F ratio	p
Age	**0.236**	**48.898**	**<0.001**
Weight	**0.100**	**17.588**	**<0.001**
Height	**0.069**	**11.688**	**0.001**
BSA	**0.120**	**21.505**	**<0.001**
BMI	**0.049**	**8.111**	**0.005**

b

	R^2	F ratio	p
√Age	**0.229**	**46.907**	**<0.001**
√Weight	**0.106**	**18.734**	**<0.001**
√Height	**0.069**	**11.738**	**0.001**
√BSA	**0.121**	**21.708**	**<0.001**
√BMI	**0.050**	**8.370**	**0.004**

c

	R^2	F ratio	p
log Age	**0.218**	**43.991**	**<0.001**
log Weight	**0.107**	**18.998**	**<0.001**
log Height	**0.069**	**11.783**	**0.001**
log BSA	**0.120**	**21.612**	**<0.001**
log BMI	**0.051**	**8.511**	**0.004**

All the variables had similar R^2, F ratio and p values, irrespective of the transformation method, when compared to the raw data. It was therefore decided, for simplicity, to use the raw data in the multiple regression analysis.

3.5.1.2 Simple regression analysis of the non-continuous clinical and demographic variables versus actual warfarin dose

As the non-continuous variables (smoking status, gender, clinical indication for warfarin therapy and use of interacting drugs) could not be transformed, simple regression analysis was performed on these data (Table 3.9), in order to confirm the results of the Student t-test (Table 3.6b).

Table 3.9 Simple regression analysis for the actual warfarin dose versus the non-continuous clinical and demographic variables in the construction cohort (n=160).

Significant p value <0.05

	R^2	F ratio	p
Smoking status	0.028	4.579	0.034
Simvastatin	0.012	1.984	0.161
Amiodarone	0.039	6.414	0.012
Omeprazole	0.013	2.081	0.151
Aspirin	0.001	0.087	0.769
Male	0.027	4.321	0.039
Female	0.027	4.321	0.039
AF	0.124	22.342	<0.001
PE	0.004	0.626	0.430
DVT	0.027	4.398	0.038

The results showed that the variables with a significant effect on the warfarin dose were smoking status (p=0.0.34), amiodarone use (p=0.012), gender (p=0.039), AF (p<0.001) and DVT (p=0.038), confirming the results of the Student t-test.

3.5.1.3 Simple regression analysis of the target INR versus actual warfarin dose

Although the target INR is a non-continuous variable, there are three possible values i.e. 2.5, 3.0 and 3.5. In order to perform the regression analysis, these data were converted into the dummy variables Z_1 and Z_2:

Target INR	2.5	3.0	3.5
Z_1	1	0	0
Z_2	0	1	0

Using these dummy variables in a regression analysis, the target INR showed a significant effect on the warfarin dose (F ratio 16.975, $p<0.001$) and therefore it was included in the final algorithms.

3.5.1.4 Multiple regression analysis of the significant clinical and demographic variables

The multiple regression analysis, for the construction of the algorithms may be performed either forwards or backwards, with the possibility of different results by the two methods. Therefore, the clinical data, which had previously been shown to have a significant effect on the warfarin dose, was entered into the multiple regression calculation by both methods (Table 3.10).

There was no difference in the outcome of the results when using either the forward or backward regression. The same variables (age, BSA, smoking status, amiodarone use and target INR) were included in both methods and showed exactly the same co-efficient and p values. Both methods removed weight, height, BMI, gender, AF and DVT from the regression model. Consequently, the final clinical/demographic algorithm was constructed using a forward multiple regression model.

Table 3.10 Multiple regression analysis of the actual warfarin dose versus the significant clinical and demographic data, by the a) forward and b) backward methods, for the construction cohort (n=160).

Significant p value <0.05

a)

Dependent Y	√warfarin dose			
Method	Forward			
Enter variable if P<	0.05			
Remove variable if P>	0.1			
Sample size				160
Coefficient of determination R^2				0.3595
R^2-adjusted				0.3387
Multiple correlation coefficient				0.5996
Residual standard deviation				0.4061

Regression Equation

Independent variables	Coefficient	Std.Error	t	P
(Constant)	1.3139			
age	-0.01239	0.002627	-4.718	<0.0001
BSA	0.4058	0.1235	3.285	0.0013
smoking status	0.2073	0.09332	2.222	0.0278
amiodarone	-0.3187	0.1030	-3.096	0.0023
target INR	0.2602	0.09412	2.765	0.0064
F-Ratio				17.2855
Significance level				P<0.001

b)

Dependent Y	√warfarin dose			
Method	Backward			
Enter variable if P<	0.05			
Remove variable if P>	0.1			
Sample size				160
Coefficient of determination R^2				0.3595
R^2-adjusted				0.3387
Multiple correlation coefficient				0.5996
Residual standard deviation				0.4061

Regression Equation

Independent variables	Coefficient	Std.Error	t	P
(Constant)	1.3139			
age	-0.01239	0.002627	-4.718	<0.0001
BSA	0.4058	0.1235	3.285	0.0013
smoking status	0.2073	0.09332	2.222	0.0278
amiodarone	-0.3187	0.1030	-3.096	0.0023
target INR	0.2602	0.09412	2.765	0.0064
F-Ratio				17.2855
Significance level				P<0.001

3.5.1.5 The clinical/demographic algorithm

Using the data from the forward multiple regression analysis of the significant clinical and demographic data (Table 3.10a), the following algorithm was constructed:

$$\text{Warfarin dose (mg/day)} = [1.3139 - (0.01239 \times \text{age}) + (0.4058 \times \text{BSA}) + (0.2073 \times \text{smokes}) - (0.03187 \times \text{amiodarone}) + (0.2602 \times \text{target INR})]^2$$

where: age (in years) and BSA (in m^2)

smokes = 1 for a smoker and 0 for a non-smoker

amiodarone = 1 (present) or 0 (absent)

3.6 Simple regression analysis of the genetic polymorphisms versus the actual warfarin dose

A simple regression calculation was performed on each of the polymorphisms (Table 3.11). The results showed that four of the twelve SNPs had no significant effect on the √warfarin dose, CYP2C9 *1/*2 (p=0.862), VKORC1 GA (p=0.346), CYP4F2 CT (p=0.205) and CYP4F2 TT (p=0.053). From the Box and Whisker plot of the various SNPs (Figure 3.4), the median warfarin dose for CYP *1/*2 was the same as for the wild-type *1/*1; however, the other three polymorphisms did display a visible difference in the median dose as compared to the wild-type. Following this discrepancy, it was decided to include all the SNPs in a multiple regression analysis.

3.6.1. Multiple regression analysis of the genetic polymorphisms versus the actual warfarin dose

A multiple regression analysis, both forwards and backwards, was performed on all the polymorphism data against the square root of the warfarin dose, without the clinical or demographic variables, but no results could be calculated. The process was repeated without inputting the wild-type genes (CYP2C9 *1/*1, VKORC1 GG and CYP4F2 CC). The results are shown in Table 3.12.

The forward regression model removed the CYP4F2 CT and TT SNPs and produced an adjusted R^2 of 0.3582 (F ratio 13.675, $p<0.001$). Using the backward regression produced a higher adjusted R^2 of 0.3686 but a lower F ratio of 12.6008 ($p<0.001$). This method also included CYP4F2 TT, but with a high p value of 0.0631. Based on these data, it was decided to use the forward regression method for the final calculation of the pharmacogenetic algorithm, based on the higher F ratio.

Table 3.11 Simple regression analysis for the actual warfarin dose versus the SNPs in the construction cohort (n=160).

Significant p value <0.05

SNP	R^2	F ratio	p
CYP *1/*1	0.0662	10.776	0.001
CYP *1/*2	0.0002	0.030	0.862
CYP *1/*3	0.0378	6.213	0.014
CYP *2/*2	0.0270	4.390	0.038
CYP *2/*3	0.0675	11.437	0.001
CYP *3/*3	0.0419	6.912	0.009
VKORC1 GG	0.1211	21.778	<0.001
VKORC1 GA	0.0056	0.593	0.346
VKORC1 AA	0.1494	22.761	<0.001
CYP4F2 CC	0.0310	5.046	0.026
CYP4F2 CT	0.0101	1.618	0.205
CYP4F2 TT	0.0235	3.804	0.053

Table 3.12 Multiple regression analysis of the actual warfarin dose versus the SNPs in the construction cohort (n=160) by a) forward and b) backward methods.

Significant p value <0.05

a)

Dependent Y	√warfarin dose			
Method	Forward			
Enter variable if P<	0.05			
Remove variable if P>	0.1			
Sample size				160
Coefficient of determination R^2				0.3864
R^2-adjusted				0.3582

Regression Equation

Independent variables	Coefficient	Std.Error	t	P
(Constant)	2.3100			
VKORC1 GA	-0.2336	0.06922	-3.374	0.0009
VKORC1 AA	-0.6978	0.1023	-6.818	<0.0001
CYP2C9 *1/*2	-0.1968	0.08055	-2.444	0.0157
CYP2C9 *1/*3	-0.5586	0.1399	-3.993	0.0001
CYP2C9 *2/*2	-0.5866	0.1845	-3.179	0.0018
CYP2C9 *2/*3	-0.9429	0.2347	-4.017	0.0001
CYP2C9 *3/*3	-0.9051	0.4098	-2.209	0.0287

Variables not included in the model	
CYP4F2 CT	
CYP4F2 TT	
F-Ratio	13.6750
Significance level	P<0.001

b)

Dependent Y	√warfarin dose			
Method	Backward			
Enter variable if P<	0.05			
Remove variable if P>	0.1			
Sample size				160
Coefficient of determination R^2				0.4003
R^2-adjusted				0.3686

Regression Equation

	Coefficient	Std.Error	t	P
(Constant)	2.2811			
VKORC1 GA	-0.2109	0.06972	-3.026	0.0029
VKORC1 AA	-0.7046	0.1016	-6.937	<0.0001
CYP2C9 *1/*2	-0.1909	0.07996	-2.387	0.0182
CYP2C9 *1/*3	-0.5448	0.1390	-3.920	0.0001
CYP2C9 *2/*2	-0.5757	0.1831	-3.145	0.0020
CYP2C9 *2/*3	-0.9193	0.2331	-3.943	0.0001
CYP2C9 *3/*3	-0.8694	0.4069	-2.137	0.0342
CYP4F2 TT	0.2382	0.1273	1.872	0.0631

Variables not included in the model	
CYP4F2 CT	
F-Ratio	12.6008
Significance level	P<0.001

3.6.2 Multiple regression analysis of all the relevant clinical, demographic and genetic data versus the actual warfarin dose.

Using the clinical and demographic data from 3.5.1.5 and the genetic data from 3.6.1, a forward multiple regression analysis was performed (Table 3.13). This gave an adjusted R^2 value of 0.6268 (F ratio 23.250, $p<0.001$). CYP4F2 CT was removed from the model, together with smoking status, which had the highest p value (p=0.0278) in the clinical regression model.

From these data (Table 3.13) the following pharmacogenetic algorithm was produced:

Warfarin dose (mg/day) = $[1.9708 - (0.01145 \times \text{age}) + (0.3119 \times \text{BSA})$
$- (0.3766 \times \text{amiodarone}) + (0.1768 \times \text{target INR})$
$- (0.6863 \times \text{VKORC1 AA}) - (0.2136 \times \text{VKORC1 GA})$
$- (0.1957 \times \text{CYP2C9 *1/*2}) - (0.435 \times \text{CYP2C9 *1/*3})$
$- (0.3524 \times \text{CYP2C9 *2/*2}) - (0.8539 \times \text{CYP2C9 *2/*3})$
$- (0.6471 \times \text{CYP2C9 *3/*3}) + (0.228 \times \text{CYP4F2 TT})]^2$

where: age (in years) and BSA (in m^2)
amiodarone = 1 (present) or 0 (absent)
SNPs = 1 (present) or 0 (absent)

3.7 Correlation and simple regression analyses of the albumin and factor assay data from the group 3 subjects (n=20) versus the actual warfarin dose.

The pre-warfarin levels of albumin and factors II, VII and X showed no correlation with the warfarin dose (Table 3.14). However, there was an inverse correlation between the warfarin dose and factor IX (r=-0.6078, p=0.005). These data were confirmed using a simple regression analysis (Table 3.15), in which factor IX had an R^2 value of 0.3694 (F ratio=10.545, p=0.004).

Factor IX was the only parameter accepted by a forward multiple regression model, giving an adjusted R^2 of 0.3342 (co-efficient = -0.0139 p=0.005, F ratio = 10.539 p = 0.004).

Table 3.13 Forward multiple regression analysis of the actual warfarin dose versus the clinical, demographic and genetic data in the construction cohort (n=160).

Significant p value <0.05

Dependent Y	√warfarin dose
Method	Forward
Enter variable if P<	0.05
Remove variable if P>	0.1
Sample size	160
Coefficient of determination R^2	0.6549
R^2-adjusted	0.6268
Multiple correlation coefficient	0.8093
Residual standard deviation	0.3051

Regression Equation

Independent variables	Coefficient	Std.Error	t	P
(Constant)	1.9708			
CYP2C9 *1/*2	-0.1957	0.06183	-3.165	0.0019
CYP2C9 *1/*3	-0.4350	0.1077	-4.037	0.0001
CYP2C9 *2/*2	-0.3524	0.1429	-2.466	0.0148
CYP2C9 *2/*3	-0.8539	0.1804	-4.732	<0.0001
CYP2C9 *3/*3	-0.6471	0.3140	-2.061	0.0411
VKORC1 AG	-0.2136	0.05467	-3.907	0.0001
VKORC1 AA	-0.6863	0.07892	-8.696	<0.0001
CYP4F2 TT	0.2280	0.09833	2.318	0.0218
Age	-0.01145	0.0020	-5.723	<0.0001
BSA	0.3119	0.09398	3.319	0.0011
Amiodarone	-0.3766	0.07718	-4.880	<0.0001
Target INR	0.1768	0.07281	2.428	0.0164
F-Ratio				23.2500
Significance level				P<0.001

Variables not included in the model
CT
Smoking status

Table 3.14 Correlation of the actual warfarin dose versus albumin and coagulation factors in the group 3 subjects (n=20).

Significant p value <0.05

	r	p
Albumin	-0.3408	0.142
F II	0.1330	0.576
F VII	-0.0072	0.976
F IX	-0.6078	0.005
F X	-0.3802	0.098

Table 3.15 Simple regression analysis of the actual warfarin dose versus albumin and coagulation factors in the group 3 subjects (n=20).

Significant p value <0.05

	R^2	F ratio	p
Albumin	0.1161	2.365	0.141
F II	0.0177	0.324	0.576
F VII	0.0001	0.001	0.976
F IX	0.3694	10.545	0.004
F X	0.1445	3.041	0.098

Chapter 4

Discussion

There is little doubt that the use of warfarin, both therapeutically and prophylactically, has made a major impact on the treatment of thromboembolic disease in the last sixty years or so and countless millions of lives have been saved worldwide during this time. However, the dosing of warfarin is unlike the majority of drug therapies available to clinicians, in so far as there is no linear titration between the dose and its pharmacological effect. In addition, the consequences of under or over estimating the dose in any given patient can have serious outcomes with regard to morbidity and mortality.

It is with these facts in mind that several groups have attempted to formalise a dosing schedule based on frequent, often daily INR testing (Fennerty *et al*, 1984; Tait and Sefcick, 1997). These studies have been shown to reduce the time taken for the INR to become therapeutic at initiation, as well as reducing the number of adverse events when compared to the usual trial and error approach used by many anticoagulant centres. However, with the emergence of pharmacogenetics and the discovery of SNPs which have been shown to have a significant influence on the stable warfarin dose, other studies have attempted to create a dosing regimen from a mathematical algorithm, by combining different combinations of clinical, demographic and genetic factors (Zambon *et al*, 2011; Sconce *et al*, 2005; D'Andrea *et al*, 2005).

It was the aim of this study to create two new algorithms using as many variables as possible, all of which had previously been shown to have a significant effect on the stable warfarin dose. The first algorithm was composed of clinical and demographic data obtained from a cohort of stable anticoagulated patients, in order to show that it is possible to improve the accuracy of warfarin dosing at the beginning of the treatment. If successful in practice, this would lead to a reduction in the number of adverse events, as well as attaining the therapeutic INR range more quickly, in centres which do not have the facilities for DNA analysis.

The second algorithm consisted of the same clinical and demographic data, but also included genotype data on four SNPs which have been shown to have a

significant effect on the pharmacokinetics and pharmacodynamics of warfarin therapy (see section 1.6.1). By combining all these parameters, it was the intention to improve the predictability of warfarin dosing beyond that of previously published algorithms, since many of these used only a selection of demographic, clinical and genetic parameters.

4.1 The demographic and clinical data

From the total number of warfarin patients available for the study (n=808), not all were invited to take part. During the time of recruitment, many had completed their course of therapy and were therefore discharged from the outpatient clinic before they could be asked to participate. In addition, some patients refused (n=25), while others were unsuitable according to the Leeds (Central) Research Ethics Committee criteria, due to varying degrees of dementia.

Further limitations on the number of patients available for the study came from the fact that all subjects were required, at the time of recruitment, to have an INR within the therapeutic range for at least the previous eight weeks. Inevitably, some subjects did not fulfil this criterion during the course of the study. In addition, a decision was made during the design of the study to only include Caucasian subjects. This was primarily done due to the fact that there were statistically insufficient numbers of non-Caucasians available (see 1.7). In addition, it has been shown that some SNPs which have an effect on the stable warfarin dose are more prevalent in non-Caucasians (see 1.6.1.1) and it was not financially possible to include all of these in the study, even if the numbers of non-Caucasians had been higher. In trying to produce an algorithm without these SNPs but including non-Caucasians in the cohort would have meant skewing the final results and therefore decreasing the efficacy of the algorithm produced.

With this in mind, published algorithms were chosen for comparison with the one constructed in this study only if they were created either using Caucasian-only data or a racially heterogeneous group but which allowed for Caucasian subjects to be entered into the final algorithm without any detriment to its predictability.

4.1.1 Demographic factors

In the final regression analysis, age was shown to be a significant factor in determining the stable warfarin dose ($p>0.0001$), confirming the findings of many other studies and which was included in all the chosen algorithms. However, although there was a significant correlation between the stable warfarin dose and gender ($p=0.020$) (see section 3.5), in the final multiple regression analysis it was removed by the Medcalc software. A possible explanation is that, as females generally tend to have a smaller body mass than males, the combination of height, weight, BMI and BSA into the model was sufficient to override the influence of gender. In addition, the correlation coefficients for height and BSA (Table 3.7a) were more highly significant ($p<0.0001$) than those for weight and BMI ($p=0.0001$ and $p=0.007$ respectively), suggesting that the latter were removed in the multiple regression. The eight published algorithms chosen for comparison with this study all use various combinations of gender and/or body mass, so it appears to suggest that, as stated in section 1.6.3, the relationship between the stable warfarin dose and gender is not a direct one but multifactorial and that some provision of either gender or body mass in an algorithm would be an overarching parameter for this particular aspect of warfarin dosing. Furthermore, to include a measure of body mass instead of gender in an algorithm would negate the problem of reducing the warfarin dose for large females or increasing it for small males.

4.1.2 Interacting drugs

The number of drugs which have an effect on an individual's stable warfarin dose is extensive and it would not have been possible to investigate them all in this study. Those which were chosen are the most commonly encountered long term drugs used by patients attending the Huddersfield Royal Infirmary anticoagulant clinic and, although drugs such as antibiotics can also significantly affect warfarin in the short term and therefore require closer INR monitoring at the time, amiodarone, simvastatin and aspirin may cause a permanent reduction in the warfarin dose (Holbrook *et al*, 2005). In the chosen published algorithms, which included interacting drugs, three found amiodarone and statins to have a significant influence (Gage *et al*, 2004; Gage *et al*, 2008; Voora *et al*, 2005) and one found amiodarone alone to have a significant

influence (IWPC, 2009). Interestingly, the two studies by Gage *et al* (2004 & 2008) also found prophylactic aspirin to have a significant effect, but the patient cohorts were much larger than this study (n=329, n=1015 respectively). In this study, 13.1% of patients in the construction cohort were taking aspirin (n=21), but this proved not to be significant in the simple regression analysis R^2=0.001, p=0.769, see Table 3.9)

In this study, although simvastatin was the commonest drug among the construction cohort (33.8%), only amiodarone, taken by 11.9%, was found to have a statistically significant influence on the stable warfarin dose and was included in the final algorithm. This reinforces our current practice in which patients taking amiodarone are initiated on a lower warfarin dose than those who are not, or are monitored more frequently if starting amiodarone while already on warfarin.

4.1.3 Clinical parameters

Simple regression analysis for the clinical indications of warfarin therapy (Table 3.9) showed a significant relationship between AF (p<0.001) and DVT (p<0.038) and the warfarin dose, but not for PE (p=0.430). However, in the construction of the algorithm, the clinical indications for OAT were removed. It is difficult to justify how the clinical condition can have any bearing on the final stable dose; a more obvious related parameter would be the target INR. Gage *et al* (2008) found that the presence of an active VTE (PE or DVT) increased the warfarin dose by 7% (p=0.013) and was significant enough to be included in the final algorithm. But the study also included patients with a range of target INRs, which could have been responsible for the findings. In the other algorithms, clinical details were either not noted (Voora *et al*, 2005; Zhu *et al*, 2007) or were found not to be significant (Sconce *et al*, 2005; Wadelius *et al*, 2009; Zambon *et al*, 2011; IWPC, 2009; Gage *et al*, 2004). It is worth noting however, that the Wadelius and Zambon studies only selected patients with a target INR of 2.5 and not any other therapeutic ranges. It seems logical to assume, therefore, that the target INR, which is determined by the clinical indication for OAT (Table 1.1) is more likely to have a direct influence on an individual's warfarin dose requirement than the clinical condition, due to the fact that the higher the target

INR is, the more warfarin would be needed to reach that level of anticoagulation.

4.1.4 Coagulation factors and albumin levels (Group 3 patients)

Although some studies have investigated the possible effect of the genotypes of the vitamin K dependent coagulation factors II, VII, IX and X (see section 1.6.1.5), there is no literature on whether the actual plasma levels, i.e. the phenotype of these factors, is a possible influencing parameter on the stable warfarin dose before OAT induction. Since these factors are affected once warfarin therapy has commenced, functional assays of factors II, VII, IX and X were performed on a cohort of patients before the initiation of warfarin, to assess whether a higher baseline level in any of the four factors could be reflected in an eventual higher stable warfarin dose and *vice versa*. Similarly, as approximately 99% of warfarin is albumin bound *in vivo* (Gage and Lesko, 2008) and it is only the free drug which is pharmacologically active, samples were taken at warfarin initiation for albumin assay, in order to determine whether the baseline albumin level had any influence on the stable warfarin dose (Table 3.3). This was based on the hypothesis that low albumin levels may not be sufficient to bind 99% of the warfarin and therefore more unbound drug would be available to affect the coagulation factors, leading to a reduced warfarin dose requirement.

Unfortunately the cohort of patients was small (n=20), due partly to the fact that many of the patients were new to the clinic and, unlike the construction and validation cohorts, did not know the staff. This meant that, despite explaining the research project, many felt it would have been a long term commitment and therefore refused. Others did not want their DNA analysed, fearing that the data would be detrimental to them in the future. In addition, as the patients were not known to the clinic staff before attending for their first appointment, many had some form of dementia and were not capable of signing the consent form or of understanding the information sheet, which had been sent to them several days before their clinic visit. The numbers of new patients available for study were also significantly reduced due to the fact that all cases of DVT and PE had been commenced on warfarin as in-patients, before attending the clinic and, as such, the coagulation factor assays could not be performed. Therefore, the remaining

patients available for study were mainly those diagnosed with AF. However, soon after the study began, the Cardiology Department implemented a new treatment pathway in which the Cardiology consultants commenced the AF patients on three days of warfarin, before referral to the Anticoagulant clinic on day 4 for INR monitoring. In all, these problems severely limited the number of subjects suitable for this part of the study.

Nevertheless, a small number of subjects (n=20) were assayed for albumin and the vitamin K dependent factors, to determine if there was any correlation with the eventual stable warfarin dose (Tables 3.14 and 3.15). From the five parameters studied, only FIX showed a significant correlation (r= -0.6078 p= 0.005). Several studies have investigated the possibility of a relationship between the coagulation factors and the warfarin dose (Shikata *et al*, 2004; Caldwell *et al*, 2007; Aquilante *et al*, 2006), but these have involved sequencing SNPs coding for the proteins and it is unclear from the literature what effect any of the different alleles have on either their plasma levels (as measured by immunological assays) or on their activity (as measured by functional assays). Consequently, it is difficult to determine what relationship there may be between the various alleles and the warfarin dose. In the papers mentioned above, no relationship was shown, however, D'Andrea *et al* (2008) reported a mutation in the factor IX propeptide which caused a selective reduction in its activity during warfarin therapy and which occurred in <1.5% of the population studied.

The findings of this study for the factor IX correlation are anomalous. Firstly, the correlation is negative, so that a decrease in factor IX activity suggests an *increase* in the warfarin dose. Secondly, as factor IX is part of the intrinsic coagulation cascade (see Figure 1.1), it does not affect the prothrombin time and therefore the INR. More subjects would need to be studied in order to shed more light on this finding.

4.2 The clinical/demographic algorithm

Regression analysis, using the clinical and demographic data from the validation cohort (n=47), was performed using the clinical algorithm from this study (see section 3.5.1.5), together with three published clinical algorithms (Gage *et* al, 2008, IWPC, 2010 and Zambon *et* al,2011) and the results are shown in Table 4.1.

Table 4.1 Comparison of the four clinical algorithms using data from the validation cohort (n=47)

NB R^2 represents the amount of variation in the data which is explained by the regression; i.e. R^2=0.50 means that 50% of the variation in warfarin dose is explained by the algorithm.

	This study	Gage *et al* (2008)	IWPC (2010)	Zambon *et al* (2011)
R^2	0.188	0.263	0.268	0.203
F ratio	10.413	16.084	16.469	11.138
p	0.002	<0.001	<0.001	0.001

Using the data from the validation cohort (n=47) for each of the algorithms, the one produced in this study gave the lowest predictability of the stable warfarin dose (R^2=0.188). However, there is a wide variation between the results from the other three formulae and those stated in their individual papers. Gage *et al* (2008) had a 17% predictability (n=292), IWPC (2010) 26% (n=1009) and Zabon *et al* (2011) 31% (n=97). The overall results appear to confirm the difficulty in comparing the inter-algorithm validity due to the different parameters included in the formulae and the numbers of subjects in each validation cohort. Furthermore, whereas Gage *et al* (2008) calculated the predictability of the clinical algorithm using the whole of their validation cohort data, the Zabon *et al* (2011) cohort was divided into three groups, <3.6mg/day, >3.6<6.4mg/day and >6.4mg/day (low, medium and high doses respectively), based on 1.4mg/day below or above the standard daily loading dose of 5mg. The predictability for each group (16.8%, 56.8% and 9.6% respectively) was used to determine whether using the clinical algorithm would have under- or overdosed the patients when compared to the standard 5mg daily trial and error approach. Similar results were produced by the IWPC (2010) of 24%, 53% and 13% for low, medium and high stable doses (<3mg/day, >3<7mg/day and >7mg/day respectively. Both studies show that the predictability of their respective clinical

algorithms was highest in the intermediate dose group. In order to do the same, the validation cohort for this study was divided into three groups according to the interquartile ranges of the stable warfarin dose and the data is shown in Table 4.2.

Table 4.2 Predictability of the clinical algorithm in low, medium and high warfarin doses in the validation cohort (n=47).

25th quartile = 2.8mg/day

75th quartile = 5.0mg/day

	<2.8mg/day (n=12)	>2.7<5.0mg/day (n=24)	>5.0mg/day (n=11)
R^2	0.009	0.077	0.016
F Ratio	0.095	2.075	0.150
p	0.764	0.162	0.708

Although the results show no statistical significance, it is interesting to note that, as with Zambon *et al* (2011) and IWPC (2010), the clinical algorithm has a higher predictability in patients who require an intermediate warfarin dose but is less effective with the low and high dose groups. This could be explained, at least in part, due to the absence in the algorithms of warfarin sensitive or resistant SNPs, although a much larger cohort of patients would probably have increased the predictability to a level more comparable with the other studies.

4.3 DNA extraction problems

Blood samples were obtained from healthy laboratory staff (Group 1 subjects, n=12), for optimisation of the DNA extraction procedure and PCR analysis, in order not to use up samples from the warfarin patients. The first extraction method, using the QIAamp® DNA mini kit (section 2.6.2.1) produced low yields of DNA (mean=9.5 ng/µl, ±SD 1.71), which failed to show any allelic discrimination with a common SNP assay. Various modifications to the

methodology were performed, including the precipitation of any haem contamination, but the DNA yields and PCR results did not improve. The results were unexplainable, even though the extraction method provided by the QIAamp® kit was followed implicitly.

Because a large amount of the healthy control samples had been used, a small number of group 1 samples (n=4) were selected for the Whatman® extraction method (Table 3.5). These produced a much higher DNA yield (mean=21.3 ng/µl, ±SD 7.33) and all but one showed clear allelic discrimination (Figure 3.3); the negative result for sample 1 may have been due to a pipetting error. This method was then used for the DNA extraction on all the warfarin patient samples (groups 2 and 3 subjects). The final Whatman ® extraction procedure proved to be the cheapest and quickest method for isolating a substantial yield of DNA from the FTA® Elute cards.

4.4 Pharmacogenetic data; a comparison of the frequencies with the other published algorithm studies

The SNPs which have been shown beyond doubt to cause a significant reduction of the stable warfarin dose (Gage, 2006) are VKORC1 and CYP2C9 (see sections 1.6.1.1-1.6.1.3). In addition, Caldwell *et al*, (2008) discovered that the CYP4F2 SNPs have the effect of *increasing* the warfarin requirement. The majority of these studies have simply investigated the relationship between the warfarin dose and the presence of the polymorphisms, but a few have used the data in order to design algorithms which, together with clinical and demographic data, have been able to improve the predictability of warfarin dosing beyond that of simple clinical algorithms (Gage *et al*, 2004; Sconce *et al*, 2005; Voora *et al*, 2005; Zhu *et al*, 2007; Gage *et al*, 2008; IWPC, 2009; Wadelius *et al*, 2009; Zambon *et al*, 2011). Although there have been more published algorithms, these ones were chosen for comparison with the formula produced in this study (section 3.6.2) as the ethnicity of the subjects in the data used had no effect on the final outcome.

4.4.1 Comparison of the VKORC1 and CYP2C9 SNP frequencies from this study with the chosen algorithms.

The frequencies of the VKORC1 and CYP2C9 SNPs from this study and some of the chosen algorithm papers are shown in Table 4.3.

Table 4.3 Comparison of the SNP frequencies for VKORC1 and CYP2C9 with those from six published algorithm studies.

N/A= not applicable

	This study n=207 Caucasian	IWPC (2009) n=4043 Mixed race	Sconce *et al* (2005) n=297 Caucasian	Wadelius *et al* (2009) n=1496 Caucasian	Zambon *et al* (2011) n=437 Caucasian	Zhu *et al* (2007) n=65 Caucasian	Voora *et al* (2005) n=48 Caucasian
VKORC1, %							Not studied
GG	40.6	29.7	25	36	32.2	52.3	
GA	44.9	35.7	56	49	46.9	35.4	
AA	14.5	32.5	19	15	20.8	12.3	
CYP2C9, %						N/A	
*1/*1	63.8	73.5	56	66	61.1		66.6
*1/*2	19.8	12.6	22	19	23.3		20.8
*1/*3	10.6	9.3	14	12	11.9		10.4
*2/*2	2.9	0.9	3	1.4	2.1		0
*2/*3	2.4	1.3	4	1.3	1.1		2.1
*3/*3	0.5	0.4	<1	0.5	0.5		0

The frequencies of some SNPs are not shown; Gage *et al* (2008) did not state the frequency of either gene, Gage *et al* (2004) and Voora *et al* (2005) did not analyse the VKORC1 gene and Gage *et al* (2004) and Zhu *et al* (2007) did not subdivide the frequencies of the individual CYP2C9 SNP combinations. Those studies which did state the individual SNP frequencies compare favourably to those from this study. With the exception of Zhu *et al* (2007), who only used a small cohort of patients (n=65), all the algorithms showed that the heterozygous GA allele for VKORC1 was more prevalent than either of the homozygous alleles GG and AA.

For the CYP2C9 alleles, all the studies demonstrated a decrease in the frequency from the wild type (*1/*1) through to the rare *3/*3 SNP, except Voora *et al* (2005) with a study consisting of 48 subjects.

4.4.2 Comparison of the CYP4F2 SNP frequencies from this study with the chosen algorithms

Since its discovery by Caldwell *et al* (2008), several studies have recently been published which have examined the effect of the CYP4F2 SNPs on warfarin dosing (Carlquist *et al*, 2010; Pautas *et al*, 2010; Wells *et al*, 2010; Kringen *et al*, 2011; Zambon *et al*, 2011). The frequencies of the three alleles (CC-wild type, CT and TT) from these papers and from this study are compared in Table 4.4. All the studies, with the exception of Carlquist *et al* (2010), were conducted on Caucasian subjects and a comparison of the frequencies shows a distinct similarity for each allele, with CC having the highest occurrence and TT the lowest.

In summary, the frequencies for both the wild type and the variant alleles of VKORC1, CYP2C9 and CYP4F2 from this study are of comparable levels to those found in the chosen published algorithms.

Table 4.4 Comparison of the SNP frequencies for CYP4F2 with those from other published studies.

	This study n=207 Caucasian	Caldwell *et al* (2008) n=1051 Caucasian	Pautas *et al* (2010) n=300 Caucasian	Carlquist *et al* (2010) n=170 Race unknown	Wells *et al* (2010) n=249 Caucasian	Kringen *et al* (2011) n=105 Caucasian	Zambon *et al* (2011) N=437 Caucasian
CYP4F2, %							
CC	59.4	49.9	48.8	48.5	50.0	58.0	44.4
CT	34.3	42.3	43.6	40.7	40.0	34.9	46.2
TT	6.3	7.8	7.6	10.8	10.0	7.1	9.4

4.5 The pharmacogenetic algorithm

The chosen published pharmacogenetic algorithms and the one constructed in this study were used to calculate a predicted warfarin dose, using the relevant data from the validation patient cohort (n=47). Regression analysis was then performed on the resultant doses for each algorithm in order to compare the predicted and actual warfarin doses and the data is shown in Table 4.5.

Each individual paper containing the chosen algorithms states a different predictability with their own patient cohort than was found with the validation cohort from this study. However, the data shown in Table 4.5 compares all the algorithms with the same data set; all formulae show a high level of significance ($p<0.001$) with varying degrees of predictability (R^2 0.309-0.553). The algorithm from this study produced the highest R^2 value (0.553), indicating that 55.3% of the warfarin dose variability can be explained by using this formula. This suggests that, by including as many clinical, demographic and genetic parameters as can be shown to have a significant influence on the warfarin dose, the efficacy of the algorithm can be improved.

4.6 Limitations of the study

As with many projects of this type, increasing the number of participants would have been expected to consolidate the outcomes reported here. Despite the fact that the pharmacogenetic algorithm from this study showed the highest warfarin dose predictability among all the chosen algorithms, there still remains approximately 45% of the dose variability which is unaccounted for. By increasing the number of patients in both the construction and validation cohorts, more examples of the long term drugs which interact with warfarin (i.e. simvastatin, omeprazole and aspirin) may have shown a significant correlation.

An increase in both cohorts may also have led to an improvement in the predictability of the clinical algorithm, which would be more useful to the majority of district general hospitals, as these usually do not have the capability for PCR SNP analysis. Furthermore, a much larger number of group 3 pre-warfarin patients would be necessary to definitively prove whether or not the baseline levels of the vitamin K dependent clotting factors and albumin were an

Table 4.5 Regression analysis comparison of the chosen pharmacogenetic algorithms with the one from this study using the validation cohort data (n=47)

	This study	Gage *et al* (2004)	Sconce *et al* (2005)	Voora *et al* (2005)	Zhu *et al* (2007)	Gage *et al* (2008)	IWPC (2009)	Wadelius *et al* (2009)	Zambon *et al* (2011)
R^2	0.553	0.383	0.515	0.383	0.508	0.309	0.525	0.483	0.436
F ratio	55.627	27.928	47.699	27.928	46.367	20.095	49.676	42.026	34.753
p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Influence on the eventual stable warfarin dose, thereby improving both algorithms. This would also have helped to determine if the correlation between the warfarin dose and the factor IX levels was an anomaly or a true relationship.

Only Caucasian subjects were chosen for this study as previously stated (section 2.2). A larger study, which included a statistically significant number of ethnic groups, would have been preferable. However, in order to include other races and therefore a wider genetic variation, it would have been necessary to include many more SNPs in the study.

This study did not include any measurement of either alcohol or vitamin K intake. Trying to evaluate these parameters by patient self-assessment is unreliable, subjective and has been shown to have no significant correlation with the stable warfarin dose (Aquilante *et al*, 2006). However, Kamali *et al* (2000b) assayed plasma vitamin K levels and did find a significant relationship; however, the cost of establishing a vitamin K assay in most hospitals would be prohibitive.

4.7 The future of warfarin

Since this study was started, two new anticoagulant drugs have become available which have the potential to replace warfarin for a large number of patients. Dabigatran etexilate (Pradaxa®, Boehringer Ingelheim) is an oral anti-thrombin drug and has been approved for the prevention of stroke in non-valvular AF patients (National Institute for Clinical Excellence Guidelines, March 2012), with a dose of 110mg or 150mg twice daily. The second drug, Rivaroxaban (Xarelto®, Bayer) is an anti-Xa drug which is administered orally once daily and has been approved for the treatment and prevention of DVT and PE in patients requiring anticoagulation for less than twelve months (National Institute for Clinical Excellence Guidelines, July 2012).

Both drugs have several advantages and disadvantages when compared to warfarin. Firstly, neither Dabigatran nor Rivaroxaban require any laboratory monitoring, as there is no definitive assay at the present time which correlates with the dose in a similar way to the INR and warfarin therapy. This has significant advantages for the patients, who do not have to arrange their working or social lives around frequent blood tests. However, this also means

that any severe suppression of the coagulation mechanism cannot be detected until the patient presents with some evidence of haemorrhage. Secondly, non-compliance is not uncommon among warfarin patients, especially in the elderly with memory problems, which is only required to be taken once daily; in the case of Dabigatran, which is administered twice daily, there may be an increase in non-compliant events.

At the present time, Dabigatran and Rivaroxaban are much more expensive, at approximately £2.52 per day (National Institute for Clinical Excellence Guidelines, March 2012), when compared to a 5mg/day warfarin dose which costs £0.03 (British National Formulary. December 2011). The cost differential should decrease in time with an increase in use of the new anticoagulants but, in the present economic climate, clinicians may be reticent to prescribe them in order to prevent a sharp increase in their drug budget spending.

Despite the pros and cons, Dabigatran and Rivaroxaban are the closest that the pharmaceutical industry has come to replacing warfarin in over fifty years and they offer a real alternative which may irrevocably alter the way patients are anticoagulated in the future.

4.8 Future study

Despite the advent of the new anticoagulant drugs, warfarin will most likely be the drug of choice for many clinicians for the foreseeable future. In this case, any protocol which has the potential to minimise the occurrence of adverse events and maximise the time in the therapeutic range for warfarin patients has to be beneficial for their safety and wellbeing. Therefore, the next step from this study should be an attempt to increase the predictability of both the clinical and pharmacogenetic algorithms, using a much larger cohort of subjects; this would also help in determining whether the baseline coagulation factors and albumin have any significant effect on the stable warfarin dose. Once the algorithms have been constructed, they should both be used in a randomised clinical trial, together with the present trial-and-error dosing approach, to determine which method produces the least number of adverse events as well as increasing the time spent in the therapeutic range and decreasing the time taken to reach it. Such a study would need to include as many representatives of ethnic groups as well as SNP variations as possible, especially the newly reported VKOR

3730G>A SNP, which has been associated with an increase in the warfarin dose requirement (Carlquist *et al*, 2010; Cini *et al*, 2012).

4.9 Summary

For almost sixty years, warfarin has been the principle drug of choice for the long term prevention and treatment of VTE and it has been estimated that approximately 500,000 people are taking oral anticoagulant therapy in the UK alone (Baglin *et al*, 2006). Although many of the demographic and clinical factors which affect the pharmacological actions of warfarin have been known for many years, it is only recently that the area of pharmacogenetics has opened up a whole new field of anticoagulant study. As a consequence, our understanding of the mechanisms of warfarin action has improved and the subsequent development of pharmacogenetic algorithms has, for the first time, provided clinicians and scientists with a tool for increasing the safety of warfarin dosing. However, there is still an on-going debate as to the value of such algorithms, not only due to the emergence of new therapies which do not require laboratory monitoring or dose titration, but also because of the expense of PCR analysis and the time taken to obtain a result.

Nevertheless, these algorithms do provide a clearer guideline as to the stable warfarin dose for an individual patient than either the trial and error approach or the protocols devised by studies such as Fennerty *et al* (1984) and Tate and Sefcick (1997). With advances in technology, it is possible that the time taken to perform the SNP assays may be reduced to hours instead of days in the near future, but this idea must be tempered with the fact that the new oral anticoagulant therapies would completely negate the need for algorithms altogether if and when they become the drugs of choice. In the meantime, the number of people worldwide who are taking warfarin remains very large and any system which can be proven to increase the effectiveness and safety of its use can only be beneficial to those patients.

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Appendix I Ethical approval

Leeds (Central) Research Ethics Committee
Room 23
Floor CD
Block 40 King Edward Home
Leeds General Infirmary
LS1 3EX

Telephone: 0113 3923772
Facsimile: 0113 3922863

17 September 2009

Mr John Barraclough
Specialist Biomedical Scientist
Calderdale & Huddersfield NHS Foundation Trust
Dept. of Haematology,
The Royal Infirmary, Acre St.,
Lindley, Huddersfield.
HD3 3EA

Dear Mr Barraclough

Study Title:	**Pharmacogenetic, clinical and demographic factors in the management of warfarin therapy.**
REC reference number:	**09/H1313/54**
Protocol number:	**1.0**

Thank you for your letter of 11 September 2009, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Vice-Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk. *Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.*

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Evidence of insurance or indemnity		01 August 2009
Approval letter from University		09 July 2009
Supervisor CV		
Statistician Comments		27 April 2009
Peer Review		12 February 2009
Letter from Sponsor		17 August 2009
Protocol	1.0	22 July 2009
Investigator CV		
REC application		17 July 2009
Participant Information Sheet: Staff	1.2	31 July 2009
Participant Information Sheet: Patient	1.2	05 September 2009
Participant Information Sheet: Patient	2.2	05 September 2009
Participant Consent Form: Staff	1.2	05 September 2009
Participant Consent Form: Patient	1.2	05 September 2009
Response to Request for Further Information		11 September 2009
Participant Consent Form: Patient	2.2	05 September 2009

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

09/H1313/54	**Please quote this number on all correspondence**

Yours sincerely

Dr Margaret L Faull
Chair

Email: rachelt.bell@leedsth.nhs.uk

Enclosures: "After ethical review – guidance for researchers"
Copy to: *Mr Brian Littlejohn*
R&D office for Calderdale and Huddersfield NHS Foundation Trust

Calderdale and Huddersfield NHS

NHS Trust

Please reply to: Research & Development Department
3rd floor, Learning Centre
Huddersfield Royal Infirmary
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Mr John Barraclough
Specialist Biomedical Scientist
Dept Haematology
Huddersfield Royal Infirmary
Lindley
Huddersfield
HD3 3EA

17th September 2009

Dear Mr Barraclough

ID: 775 Pharmacogenetic, clinical and demographic factors in the management of warfarin therapy.

The Research and Development department has considered the following documents in support of your application for approval to undertake the study on the premises of Calderdale and Huddersfield NHS Foundation Trust:

Document	Version	dated
Proposal Document	**15033443**	14/05/2008
Signed SSI - 2313/51358/6/72/25102/146763		
Signed R&D application - 2313/50434/14/418		
Ethic provisional approval 09/H1313/54		02/09/2009
Protocol	**1**	22/07/2009
Consent form-patient	**1.1**	22/07/2009
PIS - patient	**2.1**	22/07/09
NRES submission	09/H1313/54	
PIS - patient	**1.1**	22/07/09
PIS - staff	**1.1**	22/07/09
Consent form-patient	**2.1**	22/07/2009
Consent form-staff	**1.1**	22/07/2009
Evidence of insurance/indemnity		01/08/2008
NRES final Approval	**09/H1313/54**	17/09/2009

Your study now has R&D approval on the understanding and provision that you will adhere to the following conditions:

That the research should:

- Comply with the requirements of The Research Governance Framework for Health and Social Care (2nd DH 2005);
- Comply with regulatory requirements and legislation relating to: Clinical Trials, Data Protection, Health and Safety, Trust Caldicott Guidelines, and the use of Human Tissue for research purposes;
- Be conducted in accordance with: ICH Good Clinical Practice and/or the MRC guidelines for good clinical practice (as appropriate);
- Not commence until it has received written approval from a UKECA recognised Research Ethics Committee (REC) and that any REC imposed conditions of that approval are implemented;

You must also:

- Request written approval for any change to the approved protocol/study documents that you or the Chief Investigator wish to implement;
- Ensure that all study personnel, not employed by Calderdale and Huddersfield NHS Foundation Trust, hold either an honorary contract with the Trust or a letter of access issued by the Trust, before they have access to any facilities, patients, staff, their data, tissue or organs;
- Complete the Research Governance interim and final reports as requested;
- Submit monthly recruitment and screening data to R&D (if applicable).
- Comply with our audit and monitoring procedures as required.

This approval letter constitutes a favourable Site Specific Assessment (SSA) for this site.

Please be aware that the R&D department has a database containing study related information, and personal information about individual investigators e.g. name address, contact details etc. This information will be managed according to the principles established in The Data Protection Act

yours sincerely

D Birkenhead

Dr David Birkenhead
Director of Research and Development

Calderdale and Huddersfield NHS

NHS Foundation Trust

Anticoagulant Dept
Huddersfield Royal Infirmary
Lindley,
Huddersfield HD3 3EA
Tel 01484 347258
Fax 01484 342843

Staff Volunteer Information Sheet Version Staff 1.2

Pharmacogenetic, clinical and demographic factors in the management of warfarin therapy

You are being invited to take part in a research study as part of a Professional Doctorate degree. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with your colleagues if you wish. Please ask if there is anything that is not clear or if you would like more information.

What is the purpose of the study?

As you may be aware, finding the optimum dose of warfarin for a given patient, in order to maintain the INR in the therapeutic range, is not straightforward; there are many variables which influence oral anticoagulant dosing, including age, body size, gender etc. In recent years, three single nucleotide polymorphisms (SNPs) have been identified in two genes that are involved in the action and metabolism of warfarin. These SNPs, if present, significantly increase the response to warfarin and so these patients require a lower dose than the majority of the population in order to reach a therapeutic level. As a consequence several researchers have developed mathematical formulae, or algorithms, which take into account many of the variables associated with warfarin dosing, including these SNPs, thereby allowing clinicians to predict an individual's optimum dose *before* commencing treatment.

The aim of this study is to collect data on many of the known variables from existing, warfarin patients and to develop a new algorithm, which will give a higher prediction rate than the existing ones. Part of the research will be to test patients for the three SNPs using polymerase chain reaction (PCR).
However, in order to ensure that the PCR technique works correctly, I would like to perform a pilot study using a small number of staff before collecting samples on patients.

What will it involve if I agree?
If you agree to take part you will be asked to sign a consent form and one or two drops of blood will be taken from a fingerprick for PCR analysis.

What will happen to the blood sample?
The fingerprick sample will be spotted onto a card, which will release the DNA from the leucocytes and maintain it in a stable condition prior to analysis. Once the PCR technique has been verified, all remaining samples will be destroyed according to normal laboratory procedures. Your blood will not be tested for any other genes, such as those associated with other diseases.

When will the pilot study take place?
It is hoped that sample collection will begin early in 2010.

Will my results be kept confidential?
Yes. All samples and the information obtained from them will be identified by a random number only and will not be traceable back to the participant.

What will happen to the results of the pilot study?
As stated above, your individual results will not be passed directly on to you or anyone else. The collective results will only be used to verify the PCR technique. These data will be included in the final thesis and any scientific publications that arise from it. No names will be used and it will not be possible to identify you from any publication of the results.

Thank you for your time
John Barraclough MPhil. CSci. FIBMS Tel ext.7258

05/09/09 version Staff 1.2

Calderdale and Huddersfield NHS
NHS Foundation Trust

Anticoagulant Dept
Dept. of Haematology,
Huddersfield Royal Infirmary,
Lindley, Huddersfield, HD3 3EA.
Tel 01484 347258
Fax 01484 342843

Patient Information Sheet Version Patient 1.2

Pharmacogenetic, clinical and demographic factors in the management of warfarin therapy

You are being invited to take part in a study, which is part of a Professional Doctorate degree. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

As you may be aware, finding the correct dose of warfarin for a patient is not straightforward. There are many factors which influence this, including age, body size, gender and genetics etc.
The aim of this study will be to collect data from existing warfarin patients in order to develop a better way of finding out the correct warfarin dose in new patients much more quickly than at present.

Why have I been chosen?

You have been chosen because you have been taking warfarin long enough for your dose to be relatively stable.

What will it involve if I agree to take part in the study?

If you agree to take part you will be asked to donate an extra drop or two of blood from the fingerprick site, after enough has been taken for your routine test. You will not have to attend a separate appointment to donate this blood and you will not have to have more than the usual single fingerprick. If you do agree to take part, this blood sample will be taken the next time you are seen in clinic.

In addition, we will ask one of the nurses to measure your height and weight, while you are waiting for your usual blood result. We will also ask you to bring an up-to–date list of all your other medications, as these may have an effect on your warfarin treatment.

Do I have to take part?
It is up to you to decide whether or not to take part. If you decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. By signing the consent form you are saying that you are happy to take part in the study and that you understand what has been explained to you.

What will happen to my blood?
Your blood will be processed in the laboratory at a later date. Your genetic material, or DNA, will be analysed to see if you possess one of the genes responsible for making people more sensitive to warfarin than others. Your blood will not be tested for any other genes, such as those associated with other diseases. Once the test has been done, any sample left over will be destroyed using normal laboratory procedures.

What are the possible disadvantages and risks of taking part?
There will be no risks or disadvantages. Apart from measuring your weight and height and taking a drop or two more of blood from the fingerprick, the clinic visit will be exactly the same as usual.

What are the possible benefits of taking part?
There are no direct benefits to you from taking part in this study at this stage. However, it is hoped that, once the study has been completed, we will be able to dose patients who are starting warfarin therapy much more accurately.

When will the study be completed?
The study should be completed late in 2010.

Will my taking part in this study be kept confidential?
Yes. All information we obtain from you will be kept on a computer database, using only your hospital number as identification. No names, addresses or dates of birth will be used. When the study has been completed, the database will be destroyed.

What will happen to the results of this study?
Your individual results will not be directly passed on to you. The collective results from all the patients who are included in the study may be presented in a scientific publication to aid other doctors and scientists

managing patients on warfarin. No patient names will be used and it will not be possible to identify you from any publication of the results of the study.

Will I be paid for taking part?
We regret you will not be paid for taking part.

Who has reviewed this study?
This study has been reviewed and approved by the Leeds Central Research Ethics Committee and the Sheffield Hallam University Ethics Committee.

What if something goes wrong?
If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanism may be available to you.

Contact for further information
Further information about this study can be obtained by contacting Mr. John Barraclough, Anticoagulation Department, Huddersfield Royal Infirmary, Acre, St., Lindley, Huddersfield, HD3 3EA, telephone number 01484 347258.

What if I wish to complain about the way in which the study has been conducted?
If you have any cause to complain about any aspect of the way in which you have been approached or treated during the course of the study, the normal National Health Service complaints mechanisms are available to you and are not compromised in any way because you have taken part in a research study.

If you have any complaints or concerns please contact the project co-ordinator: Mr. John Barraclough, telephone number 01484 347258.

Thank you very much for reading through this information sheet and please remember to bring an up-to-date list of all your current medications.
John Barraclough MPhil. CSci. FIBMS

Appendix V Group 3 subject information sheet

Calderdale and Huddersfield NHS
NHS Foundation Trust

Anticoagulant Dept
Dept. of Haematology,
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Tel 01484 347258
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Patient Information Sheet Version Patient 2.2

Pharmacogenetic, clinical and demographic factors in the management of warfarin therapy

You are being invited to take part in a study, which is part of a Professional Doctorate degree. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?
As you may be aware, finding the correct dose of warfarin for a patient is not straightforward. There are many factors which influence this, including age, body size, gender and genetics etc.
The aim of this study will be to collect data from new patients in order to develop a better way of finding out the correct warfarin dose in new patients much more quickly than at present.

Why have I been chosen?
You have been chosen because you are about to start warfarin treatment and therefore your clotting mechanism has not yet been affected.

What will it involve if I agree to take part in the study?
If you agree to take part you will be asked to donate an extra drop or two of blood from the fingerprick site, after enough has been taken for your routine test. You will not have to attend a separate appointment to donate this blood and you will not have to have more than the usual single fingerprick. If you do agree to take part, this blood sample will be taken the first time you are seen in clinic.

Patients new to warfarin are normally asked to have a blood sample taken from their arm, in order to check their general health. We need to ask your permission to use some of the blood left over for this research project. You will not be asked to have any more blood tests than is usual. In addition, we will ask one of the nurses to measure your height and weight, while you are waiting for your usual blood result. We will also ask you to bring an up-to–date list of all your other medications, as these may have an effect on your warfarin treatment.

Do I have to take part?
It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. By signing the consent form you are saying that you are happy to take part in the study and that you understand what has been explained to you.

What will happen to my blood?
Your blood will be processed in the laboratory at a later date. Your genetic material, or DNA, will be analysed to see if you possess one of the genes responsible for making people more sensitive to warfarin than others. Your blood will not be tested for any other genes, such as those associated with other diseases. Once the test has been done, any sample left over will be destroyed using normal laboratory procedures.

What are the possible disadvantages and risks of taking part?
There will be no risks or disadvantages. Apart from measuring your weight and height and taking a drop or two more of blood from the fingerprick, the clinic visit will be exactly the same as for all patients.

What are the possible benefits of taking part?
There are no direct benefits to you from taking part in this study at this stage. However, it is hoped that, once the study has been completed, we will be able to dose patients who are starting warfarin therapy much more accurately.

When will the study be completed?
The study should be completed early in 2011.

Will my taking part in this study be kept confidential?
Yes. All information we obtain from you will be kept on a computer database, using only your hospital number as identification. No names, addresses or dates of birth will be used. When the study has been completed, the database will be destroyed.

What will happen to the results of this study?
Your individual results will not be directly passed on to you. The collective results from all the patients who are included in the study may be presented in a scientific publication to aid other doctors and scientists managing patients on warfarin. No patient names will be used and it will not be possible to identify you from any publication of the results of the study.

Will I be paid for taking part?
We regret you will not be paid for taking part.

Who has reviewed this study?
This study has been reviewed and approved by the Leeds Central Research Ethics Committee and Sheffield Hallam University Ethics Committee.

What if something goes wrong?
If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanism may be available to you.

Contact for further information
Further information about this study can be obtained by contacting Mr. John Barraclough, Anticoagulation Department, Huddersfield Royal Infirmary, Acre, St., Lindley, Huddersfield, HD3 3EA, telephone number 01484 347258.

What if I wish to complain about the way in which the study has been conducted?
If you have any cause to complain about any aspect of the way in which you have been approached or treated during the course of the study, the normal National Health Service complaints mechanisms are available to you and are not compromised in any way because you have taken part in a research study.
If you have any complaints or concerns please contact the project co-ordinator: Mr. John Barraclough, telephone number 01484 347258.

Thank you very much for reading through this information sheet.
Thank you for your time
John Barraclough MPhil. CSci. FIBMS Tel Hudds. 347258

Appendix VI Group 1 subject consent form

Calderdale and Huddersfield NHS

NHS Foundation Trust

Anticoagulant Dept.,
Dept. of Haematology,
Huddersfield Royal Infirmary,
Lindley, Huddersfield,

HD3 3EA.

Tel 01484 347258
Fax 01484 342843

Study Number: 09/H1313/54

Staff Identification Number for this project:

CONSENT FORM

Title of Project: Pharmacogenetic, clinical and demographic factors in the management of warfarin therapy.

Name of Researcher: Mr. John Barraclough

1. I confirm that I have read and understand the information sheet dated 22/07/09 (version Staff.1.2) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from Calderdale & Huddersfield NHS Foundation Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to take part in the above study. ☐

Name of Staff Member _____ Date _____ Signature _____

Name of Person Taking consent _____ Date _____ Signature _____

Copies: 1 for staff member, 1 for researcher site file.

05/09/09 version Staff 1.2

Appendix VII Group 2 subject consent form

Calderdale and Huddersfield NHS

NHS Foundation Trust

Anticoagulant Dept.,
Dept. of Haematology
Huddersfield Royal Infirmary,
Lindley, Huddersfield, HD3 3EA.
Tel 01484 347258
Fax 01484 342843

Study Number: 09/H1313/54

Patient Identification Number for this project:

CONSENT FORM

Title of Project: Pharmacogenetic, clinical and demographic factors in the management of warfarin therapy.

Name of Researcher: Mr. John Barraclough

1. I confirm that I have read and understand the information sheet dated 05/09/09 (version Patient 1.2) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from Calderdale & Huddersfield NHS Foundation Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
Name of Person Taking consent	Date	Signature

Copies: 1 for patient, 1 for researcher site file, 1 (original) to be kept in medical notes

Appendix VIII Group 3 subject consent form

Calderdale and Huddersfield NHS

NHS Foundation Trust

Anticoagulant Dept.,
Dept. of Haematology,
Huddersfield Royal Infirmary,
Lindley, Huddersfield,
HD3 3EA.

Tel 01484 347258
Fax 01484

342843

Study Number: 09/H1313/54

Patient Identification Number for this project:

CONSENT FORM

Title of Project: Pharmacogenetic, clinical and demographic factors in the management of warfarin therapy.

Name of Researcher: Mr. John Barraclough

1. I confirm that I have read and understand the information sheet dated 05/09/09 (version Patient 2.2) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from Calderdale & Huddersfield NHS Foundation Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
Name of Person Taking consent	Date	Signature

Copies: 1 for patient, 1 for researcher site file, 1 (original) to be kept in medical notes

05/09/09 version Patient 2.2

Appendix IX Fennerty *et al (1984)* dosing schedule

Day	INR	Warfarin Dose (mg)
1	<1.4	10.0
2 (16hrs post 1st dose)	<1.8	10.0
	1.8	1.0
	>1.8	0.5
3 (16hrs post 2nd dose)	<2.0	10.0
	2.0-2.1	5.0
	2.2-2.3	4.5
	2.4-2.5	4.0
	2.6-2.7	3.5
	2.8-2.9	3.0
	3.0-3.1	2.5
	3.2-3.3	2.0
	3.4	1.5
	3.5	1.0
	3.6-4.0	0.5
	>4.0	Nil
		Predicted dose
4 (16hrs post 3rd dose)	<1.4	>8.0
	1.4	8.0
	1.5	7.5
	1.6-1.7	7.0
	1.8	6.5
	1.9	6.0
	2.0-2.1	5.5
	2.2-2.3	5.0
	2.4-2.6	4.5
	2.7-3.0	4.0
	3.1-3.5	3.5
	3.6-4.0	3.0
	4.1-4.5	Miss 1 day then 2mg
	>4.5	Miss 2 days then 1mg

Appendix X Tait and Sefcick *(1997)* dosing schedule

Day 5 INR	Dose for days 5-8	Day 8 INR	Dose from day 8
		≤1.7	6mg
≤1.7	5mg	1.8-2.4	5mg
		2.5-3.0	4mg
		>3.0	3mg for 4 days
		≤1.7	5mg
		1.8-2.4	4mg
1.8-2.2	4mg	2.5-3.0	3.5mg
		3.1-3.5	3mg for 4 days
		>3.5	2.5mg for 4 days
		≤1.7	4mg
		1.8-2.4	3.5mg
2.3-2.7	3mg	2.5-3.0	3mg
		3.1-3.5	2.5mg for 4 days
		>3.5	2mg for 4 days
		≤1.7	3mg
		1.8-2.4	2.5mg
2.8-3.2	2mg	2.5-3.0	2mg
		3.1-3.5	1.5mg for 4 days
		>3.5	1mg for 4 days
		≤1.7	2mg
		1.8-2.4	1.5mg
3.3-3.7	1mg	2.5-3.0	1mg
		3.1-3.5	0.5mg for 4 days
		>3.5	omit for 4 days
		<2.0	1.5mg for 4 days
>3.7	0mg	2.0-2.9	1mg for 4 days
		3.0-3.5	0.5mg for 4 days

All patients with a baseline INR ≤1.4 were given 5mg/day for 4 days

Appendix XI Chosen published algorithms

Zambon *et al*, 2011:

Dose (mg/week) = [7.39764 - (0.02734 x age) + (1.06287 x BSA)
- (1.04468 x VKORC1 AG) - (2.12117 x VKORC1 AA)
- (0.78983 x CYP2C9*1/*2) - (1.17138 x CYP2C9*1/*3)
- (1.81292 x CYP2C9*2/*2 or *2/*3 or *3/*3)
- (0.46723 x CYP4F2 CT) - (0.71528 x CYP4F2 CC)]^2

where: age (in years) and BSA (in m^2)

Gage *et al*, 2004:

Dose (mg/day) = exp[0.385 - (0.0083 x age) + (0.498 x BSA)
- (0.208 x CYP2C9*2) - (0.350 x CYP2C9*3)
- (0.341 x amiodarone) + (0.378 x target INR)
- (0.126 x simvastatin) - (0.113 x race)
- (0.075 x female)]

where: exp = exponential function
age (in years) and BSA (in m^2)
CYP2C9 = 0 (absent), 1(heterozygous) or 2 (homozygous)
amiodarone/simvastatin = 1 (present) or 0 (absent)
race = 1 (Caucasian) or 2 (other)
female = 1, male = 0

Gage *et al*, 2008:

Dose (mg/day) = exp[0.9751- (0.3238 x VKORC1 AA) + (0.4317 x BSA)
- (0.4008 x CYP2C9*3) - (0.00745 x age)
- (0.2066 x CYP2C9*2) + (0.2029 x target INR)
- (0.2538 x amiodarone) + (0.0922 x smokes)
- (0.0901 x Afro American) + (0.0664 x DVT/PE)]

where: exp = exponential function
age (in years) and BSA (in m^2)
Afro American = 1 other = 0
amiodarone = 1 (present) or 0 (absent)

smokes = 1 for a smoker or 0 for a non-smoker

DVT/PE = 1 (if initial diagnosis was DVT or PE), others = 0

IWPC, 2009:

Dose (mg/week) = [5.6044 - (0.2614 x age) + (0.0087 x height)
+ (0.0128 x weight) - (0.8677 x VKORC1 GA)
- (1.6974 x VKORC1 AA) - (0.5211 x CYP2C9*1/*2)
- (0.9357 x CYP2C9*1*3) - (1.0616 x CYP2C9*2*2)
- (1.9206 x CYP2C9*2*3) - (2.3312 x CYP2C9*3*3)
- (0.1092 x Asian) - (0.2760x Black) - (0.1032 x mixed race)
+ (1.1816 x CYP enzyme inducer drugs)
- (0.5503 x amiodarone)]^2

where: age (in decades), weight (in kilos) and height (in centimetres)

Asian, Black or mixed race = 1, others = 0

CYP enzyme inducers = phenytoin, carbamazepine or rifampicin

amiodarone = 1 (present) or 0 (absent)

Sconce *et al*, 2005:

√Dose (mg/day) = [0.628 - (0.0135 x age) - (0.240 x CYP2C9*2)
- (0.370xCYP2C9*3) - (0.241 x VKORC1)
+ (0.0162 x Height)]

where: age (in years)

CYP2C9 *2 and *3 = 0, 1 or 2 for the number of alleles present

VKORC1 = 1 (for GG), 2 (for GA) and 3 (for AA)

Height = centimetres

Voora *et al*, 2005:

Dose (mg/week) = exp [0.385 - (0.0083 x age) + (0.498 x BSA)
- (0.208 x CYP2C9*2) - (0.350 x CYP2C9*3)
- (0.341x amiodarone) + (0.378 x target INR)
- (0.125 x statin) - (0.0113 x race) - (0.075 x female)]

where: exp = exponential function

age (in years) and BSA (in m^2)

CYP2C9 *2 and *3 = 0, 1 or 2 for the number of alleles present

amiodarone/simvastatin/fluvastatin = 1 (present) or 0 (absent)

race = 1 (Caucasian) or 0 (other)

female = 1, male = 0

Wadelius *et al*, 2009:

Dose (mg/week) = [9.46832 - (0.90112 x VKORC1 GA)
- (2.01863 x VKORC1 AA) - (0.50836 x CYP2C9*1/*2)
- (0.97546 x CYP2C9*1/*3) - (1.10204 x CYP2C9*2/*2)
- (1.74761 x CYP2C9*2/*3) - (3.40061 x CYP2C9*3/*3)
- (0.03686x age) - (0.27698 x female)
- (0.06992 x potentiating drugs)]^2

where: age (in years)

female = 1, male = 0

potentiating drugs = the number of drugs taken which can increase the INR

Zhu *et al*, 2007:

ln dose (mg/day) = [1.35 - (0.008 x age) + (0.116 x gender) + (0.004 x weight)
- (0.376 x VKORC1 AA) + (0.271 x VKORC1 GG)
- (0.307 x CYP2C9*2) - (0.318 x CYP2C9"3)]

where: ln = log of dose

age (in years) and weight (in pounds)

gender = 0 for female and 1 for male

CYP2C9 = input 0, 1 or 2 for the number of *2 and *3 alleles

VKORC1 AA = input 0 for GG, 0 for GA and 1 for AA

VKORC1GG = input 1 for GG, 0 for GA and 0 for AA

Gage *et al*, 2008 (clinical algorithm):

Dose (mg/day) = exp [0.613 + (0.425 x BSA) - (0.0075 x age)
+ (0.156 x Afro American) + (0.216 x target INR)
- (0.257 x amiodarone) + (0.108 x smokes)
+ (0.0784 x DVT/PE)]

where: exp = exponential function

age (in years) and BSA (in m^2)

Afro American = 1 other = 0

amiodarone = 1 (present) or 0 (absent)

smokes = 1 for a smoker or 0 for a non-smoker

DVT/PE = 1 (if initial diagnosis was DVT or PE), others = 0

IWPC, 2009 (clinical algorithm):

$$\text{Dose (mg/week)} = [4.0376 - (0.2546 \times \text{age}) + (0.0118 \times \text{height}) + (0.0134 \times \text{weight}) - (0.6752 \times \text{Asian}) + (0.4060 \times \text{Afro American}) + (0.0443 \times \text{mixed race}) + (1.2799 \times \text{enzyme inducer drugs}) - (0.5695 \times \text{amiodarone})]^2$$

where: age (in decades), weight (in kilos) and height (in centimetres)

Asian, Afro American or mixed race = 1, others = 0

CYP enzyme inducers = phenytoin, carbamazepine or rifampicin

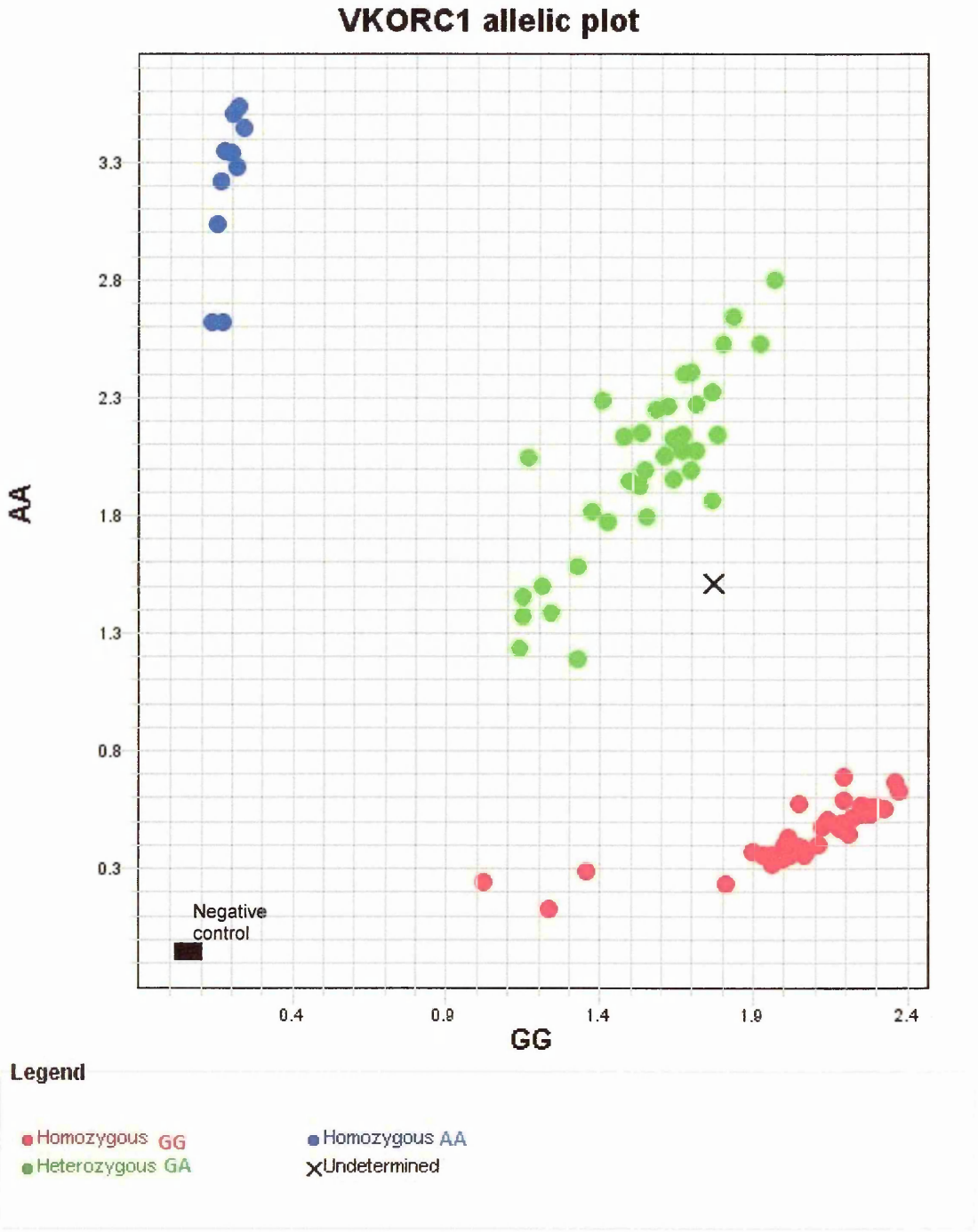
amiodarone = 1 (present) or 0 (absent)

Zambon *et al*, 2011 (clinical algorithm):

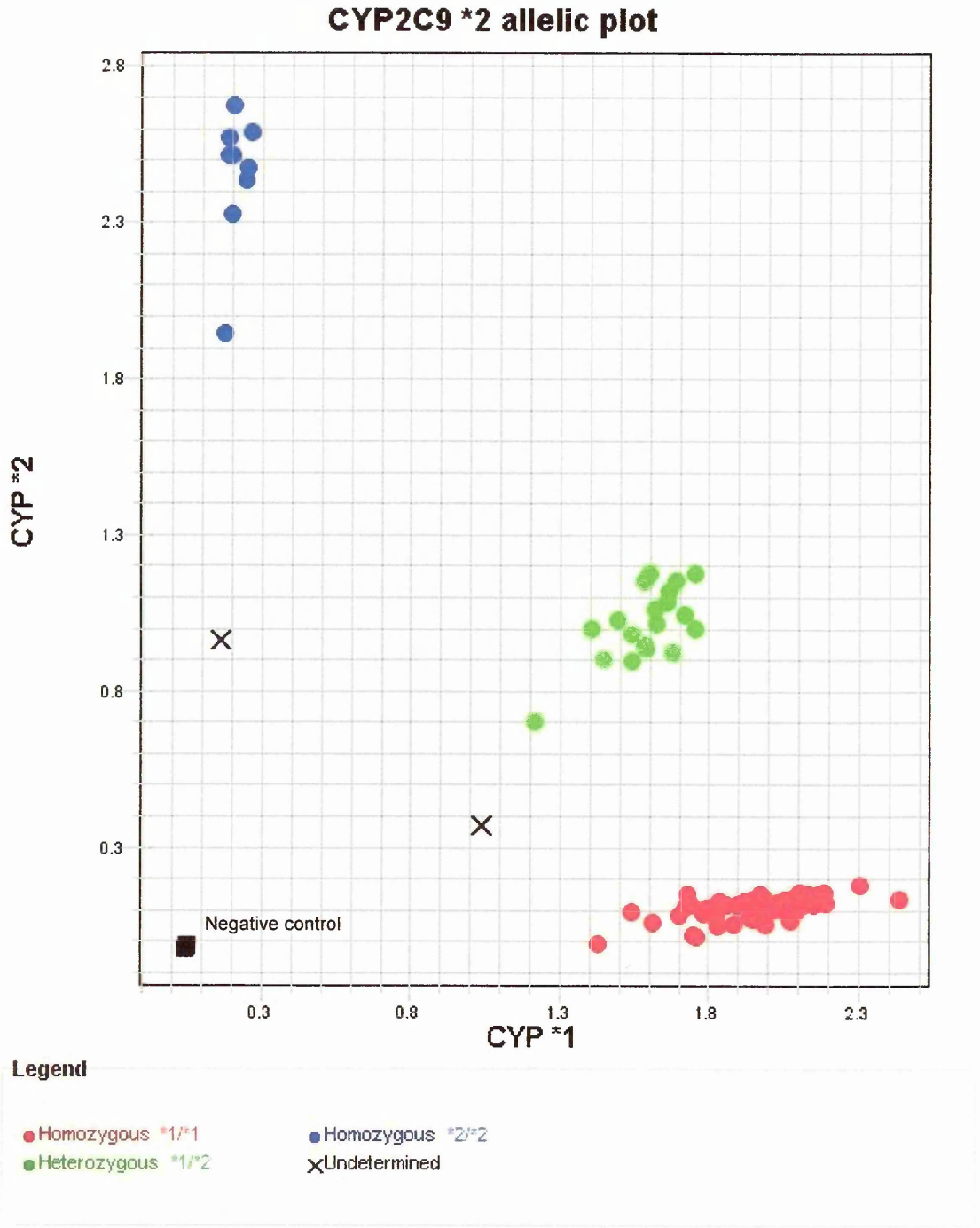
$$\text{Dose (mg/week)} = [2.9241 - (0.01943 \times \text{age}) + (1.94651 \times \text{BSA})]^2$$

where: age (in years) and BSA (in m^2)

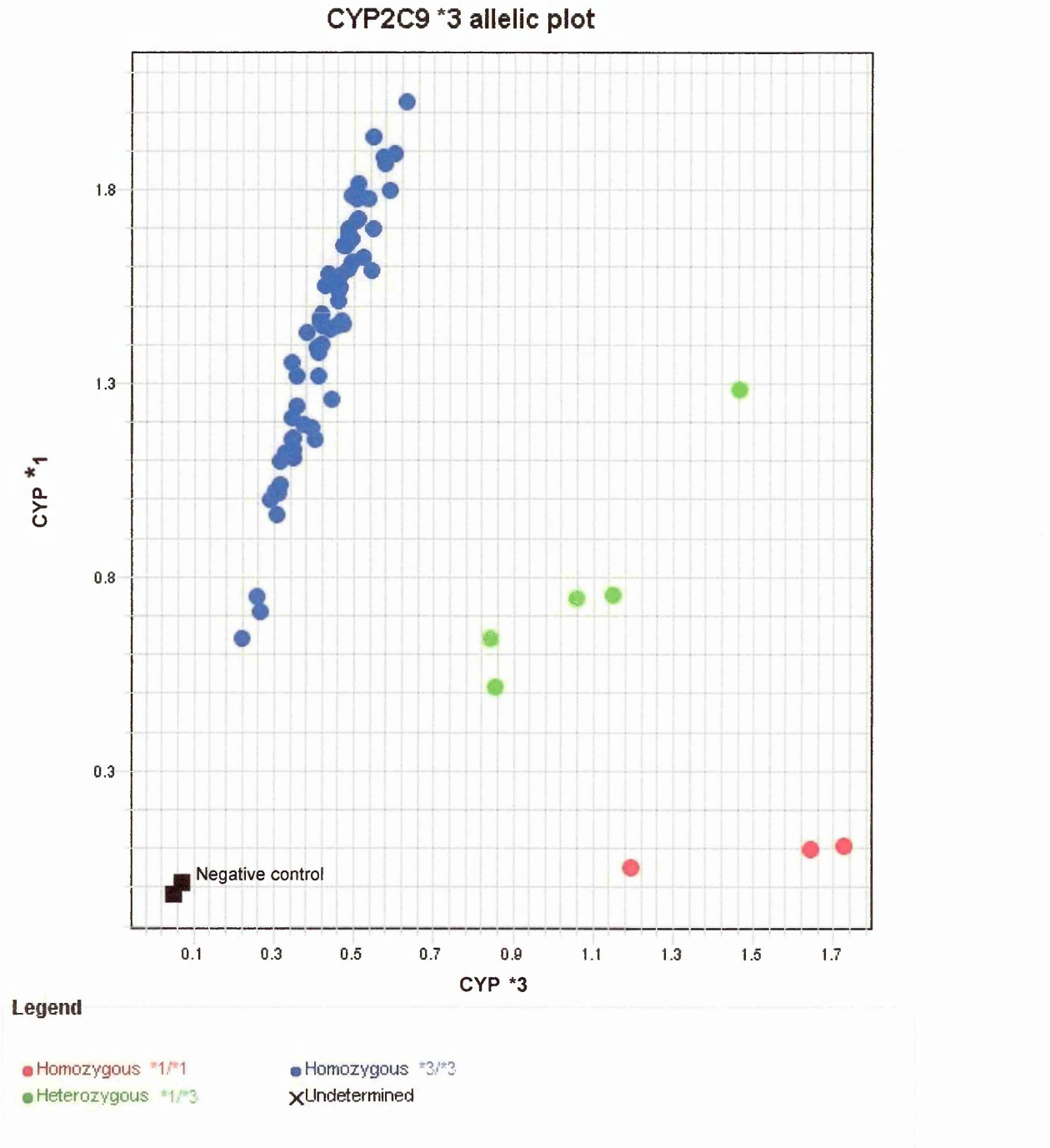
Appendix XII Example of a VKORC1 dot plot



Appendix XIII Example of a CYP2C9 *2 dot plot



Appendix XIV Example of a CYP2C9 *3 dot plot



Appendix XV Example of a CYP4F2 dot plot