Newcastle University

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The influence of age, vitamin K and genetics on anticoagulation outcomes in adults and children

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ABSTRACT

The aim of this PhD was to identify the influence of factors that affect anticoagulation response to warfarin and NOACs on clinical response with the aim to further optimise treatment safety and patient management.

In a randomised controlled trial, 181 patients with INR >6.0 were randomly allocated to receive orally either a tailored dose based upon index INR and BSA, or a fixed-dose (1 or 2mg) of vitamin K. The tailored dose resulted in a greater proportion of patients returning to within target INR range (68.9% v 52.8%; p=0.026), whilst a smaller proportion of patients remained above target INR range (p<0.001).

Studies have confirmed that variant *CYP2C9* and *VKORC1* allele carriers require more time to achieve stable dosing and have a higher risk of supra-therapeutic INR and serious bleeding risk during initiation. In a retrospective cohort study, neither *VKORC1*, *CYP2C9* genotype, nor their total variant allele count was associated with TTR% during warfarin maintenance or induction phase (p>0.05).

A study in children on chronic warfarin therapy showed low levels of plasma vitamin K which are associated with *CYP4F2* genotype with the patients with low plasma vitamin K being more likely to carry one or two variant alleles (76.2% vs 45.4%; p=0.011). Chronic low levels of vitamin K, influenced by *CYP4F2* genotype, might affect bone development and vascular health in children on warfarin.

The link between warfarin treatment and vascular calcification was further explored in the same cohort of children, by assessing plasma uc-MGP concentration, a sensitive biological marker of vascular calcification. No association was found between plasma uc-MGP concentration and either anticoagulation intensity, duration of warfarin therapy, or warfarin daily dose.

Patients on warfarin scheduled for surgery have to withdraw treatment for 5 days to avoid peri-operative bleeding. A previously developed algorithm that included genetic, demographic and clinical data was validated using an independent cohort of patients (n=117) who completed a short course of warfarin. There was a strong and highly significant correlation between the observed and predicted INR values by the algorithm (r=0.949, p<0.001).

In an *ex-vivo* study, rivaroxaban in elderly subjects produced a greater prolongation of PT (p<0.05) and modified PT (p<0.001) and a greater suppression in the rate and amount of thrombin generation compared to young subjects, suggesting an age-related increase in sensitivity to the drug.

DECLARATION OF ORIGINALITY

I hereby declare that all the work presented in this thesis is my own unless stated otherwise within the text or acknowledged accordingly within the references. The data has not been submitted previously for any alternative degrees.

Emmanouela Kampouraki

June 2019

DEDICATION

I dedicate my doctorate thesis to my beloved grandmother Calliope and my late grandfather Manolis, who have always embraced my dreams with their love. I also dedicate it to my parents; Maria and Vasilis and my three siblings; Antonis, Giorgos and Calliope, because family has been the most important blessing I have ever had. Finally, I would like to dedicate it to my fiancé, Giannis, who has been by my side, holding my hand since the beginning of this journey with outstanding patience and care.

"Επιστήμη ίσον αμφισβήτηση"

"Science means challenge"

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

95% CI	95% Confidence Interval
μg	microgram
ABCB1	ATP Binding Cassette Subfamily B Member 1
ACCP	American College Of Chest Physicians
ADR	Adverse Drug Reaction
AF	Atrial Fibrillation
AMPLIFY	Apixaban For The Initial Management Of Pulmonary Embolism And Deep-Vein Thrombosis As First-Line Therapy
ANNEXA-4	Novel Antidote To The Anticoagulation Effects Of FXA Inhibitors
ANOVA	Analysis Of Variance
anti-Xa	Anti-Factor Xa Assay
APTT	Activated Partial Thromboplastin Time
ARISTOTLE	Apixaban For Reduction In Stroke And Other Thromboembolic Events In Atrial Fibrillation
BMD	Bone Mineral Density
BSA	Body Surface Area
CaCl$_2$	Calcium Cloride
CES1	Carboxylesterase 1
CKD	Chronic Kidney Disease
COAG	Clarification Of Optimal Anticoagulation Through Genetics
CT	Computed Tomography
CYP2C9	Cytochrome P450 2C9
CYP4F2	Cytochrome P450 4F2
CYP-	Cytochrome P450 Isoform
DEXA	Dual-Energy X-Ray Absorptiometry
DILI	Drug-Induced Liver Injury
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DOAC	Direct Oral Anticoagulants
DTI	Direct Thrombin Inhibitors

DVT	Deep Vein Thrombosis
ECT	Ecarin Clotting Time
EDTA	Ethylenediaminetetraacetic Acid
EINSTEIN-PE	Oral, Direct Factor Xa Inhibitor Rivaroxaban In Patients With Acute Symptomatic Pulmonary Embolism
EINSTEIN-DVT	Oral, Direct Factor Xa Inhibitor Rivaroxaban In Patients With Acute Symptomatic Deep Vein Thrombosis
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	European Medicine Agency
ENGAGE AF-TIMI 48	Effective Anticoagulation With Factor Xa Next Generation In Atrial Fibrillation–Thrombolysis In Myocardial Infarction 48
ESC	European Society Of Cardiology
ETP	Endogenous Thrombin Potential
EU-PACT	European Trial Pharmacogenomic Approach To Coumarin Therapy
FDA	Food And Drug Administration
FFP	Fresh Frozen Plasma
FII	Factor II
FIX	Factor IX
FVII	Factor VII
FX	Factor X
FXa	Activated factor X
GIFT	GM-CSF For Immunomodulation Following Trauma
Gla	Gamma-Carboxyglutamic Acid
GP	General Practitioner
GWAS	Genome Wide Association Studies
Hokusai-VTE	Comparative Investigation Of Low Molecular Weight Heparin/Edoxaban Tosylate Versus Low Molecular Weight Heparin/Warfarin In The Treatment Of Symptomatic Deep-Vein Blood Clots
HPLC	High Performance Liquid Chromatography
HR	Hazard Ratio
INR	International Normalised Ratio
IQR	Interquartile Range

ISRCTN	International Standard Randomised Controlled Trials Number
ISTH	International Society Of Thrombosis And Haemostasis
KH2	Vitamin K Hydroquinone
LMWH	Low-Molecular-Weight Heparin
MI	Myocardial Infarction
MGP	Matrix-Gla Protein
mPT	Modified Prothrombin Time
MRI	Magnetic Resonance Imaging
NAD(P)H	Nicotinamide Adenine Dinucleotide Phosphate
NCBI	NCBI National Centre For Biotechnology Information Database
NHS	National Health Service
NICE	National Institute For Health And Care Excellence
NOAC	Novel/Non-Vitamin K Antagonist Oral Anticoagulant
NVAF	Non-Valvular Atrial Fibrillation
OR	Odds Ratio
PCC	Prothrombin Complex Concentrate
PCR	Polymerase Chain Reaction
PE	Pulmonary Embolism
P-gp	P-Glycoprotein
PIVKA-II	Protein Induced By Vitamin K Absence Factor Ii
PK/PD	Pharmacokinetics/Pharmacodynamics
PPPH	Platelet Poor Plasma High
PT	Prothrombin Time
RCT	Randomised Controlled Trial
RE-COVER I	Efficacy And Safety Of Dabigatran Compared With Warfarin For 6 Month Treatment Of Acute Symptomatic Venous Thromboembolism
RE-COVER II	Phase III Study Testing Efficacy & Safety Of Oral Dabigatran Etexilate Vs Warfarin For 6 Months Treatment Of Acute Symptomatic Venous Thromboembolism
RE-LY	Long-Term Anticoagulant Therapy
RE-VERSE AD	Reversal Effects Of Idarucizumab On Active Dabigatran

ROCKET-AF	Rivaroxaban Once Daily Oral Direct Factor Xa Inhibition Compared With Vitamin K Antagonism For Prevention Of Stroke And Embolism Trial In Atrial Fibrillation
SD	Standard Deviation
SEM	Standard Error Of Mean
SNP	Single Nucleotide Polymorphisms
SPSS	Statistical Package For Social Sciences
TF	Tissue Factor
ttpeak	Time To Peak
TTR	Time In Therapeutic Range
uc-MGP	Uncarboxylated Matrix-Gla Protein
UFH	Unfractionated Heparin
USDA	United States Department Of Agriculture
VKA	Vitamin K Antagonist
VKDB	Vitamin K Deficiency Bleeding
VKOR	Vitamin K Epoxide Reductase
VKORC1	Vitamin K Epoxide Reductase Complex Subunit 1
VTE	Venous Thromboembolism

PUBLICATIONS LIST

1.   Kampouraki E, Avery PJ, Wynne H, Biss T, Hanley J, Talks K, Kamali F. Assessment of the efficacy of a novel tailored vitamin K dosing regimen in lowering the International Normalised Ratio in over-anticoagulated patients: A randomised clinical trial. (2017) British Journal of Haematology; Sep; 178(5):800-809. doi: 10.1111/bjh.14777. Epub 2017 Aug 2.

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2.   Kampouraki E, Avery P, Biss T, Kamali F. CYP4F2 genotype affects circulating plasma vitamin K concentration in children on chronic warfarin therapy. (Abstract) (2017) Clinical Therapeutics; 39(8), Supplement: e32, ISSN 0149-2918. doi:10.1016/j.clinthera.2017.05.100. Epub 20 June 2017

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3.   Kampouraki E, Avery PJ, Biss T, Kamali F. Association between CYP4F2 genotype and circulating plasma vitamin K concentration in children on chronic warfarin therapy: Possible long-term implications for bone development and vascular health. (2017) Pediatric Blood & Cancer; Dec; 64(12). doi: 10.1002/pbc.26653. Epub 2017 May 18.

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7.   Kampouraki E and Kamali F. Pharmacogenetics of anticoagulants used for stroke prevention in patients with atrial fibrillation. (2019) Expert Opinion on Drug Metabolism & Toxicology, 15:6, 449-458. doi: 10.1080/17425255.2019.1623878

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CONFERENCE PRESENTATIONS

North East Postgraduate (NEPG) conference 2018, November 2018, Newcastle upon Tyne UK

<u>Oral presentation</u>: The impact of age on the pharmacological activity of rivaroxaban

37th World Congress International Society of Hematology, September 2018, Vancouver Canada

<u>Oral presentation</u>: The impact of age on the pharmacological activity of rivaroxaban

Joint Pharmaceutical Analysis Group postgraduate research awards and careers symposium, November 2017, London UK

<u>Poster presentation</u>: *CYP4F2* genotype affects circulating plasma vitamin k concentration in children on chronic warfarin therapy

North East Postgraduate (NEPG) conference 2017, November 2017, Newcastle upon Tyne UK

<u>Oral presentation</u>: Assessment of the efficacy of a novel tailored vitamin K dosing regimen in lowering INR in over-anticoagulated patients

2017 XXVI Congress International Society of Thrombosis and Haemostasis & 63nd Annual SSC, July 2017, Berlin Germany

<u>Poster presentation</u>: Assessment of the efficacy of a novel tailored vitamin K dosing regimen in lowering INR in over-anticoagulated patients

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<u>Invited oral</u> and <u>poster</u> presentation: CYP4F2 genotype affects circulating plasma vitamin K concentration in children on chronic warfarin therapy

North East Postgraduate (NEPG) conference 2016, November 2016, Newcastle upon Tyne UK

<u>Oral</u> and <u>poster</u> presentation: Vitamin K plasma concentration affects anticoagulation response to warfarin in children through association with CYP4F2 genotype

Chapter 1 General introduction

1.1 Blood coagulation

Haemostasis, the arrest of bleeding after vascular insult, is a vital function of human organism and comprises platelets, the vasculature, coagulation factors and the fibrinolytic system (**Figure 1.1**) (Thornton and Douglas, 2010, Chee, 2014). Back in the 1960's, the term coagulation 'cascade' was introduced to describe the activation of downstream serine proteases from their inactive precursor (zymogen), while their glycoprotein co-factor catalyses the next reaction of the cascade (Davie and Ratnoff, 1964, Macfarlane, 1964). Blood coagulation depends on two distinct pathways, the intrinsic and the extrinsic. They converge on a common pathway which leads to the production of fibrin. This protein along with platelets form blood clots over a wound site to stop haemorrhage (Pallister and Watson, 2010).
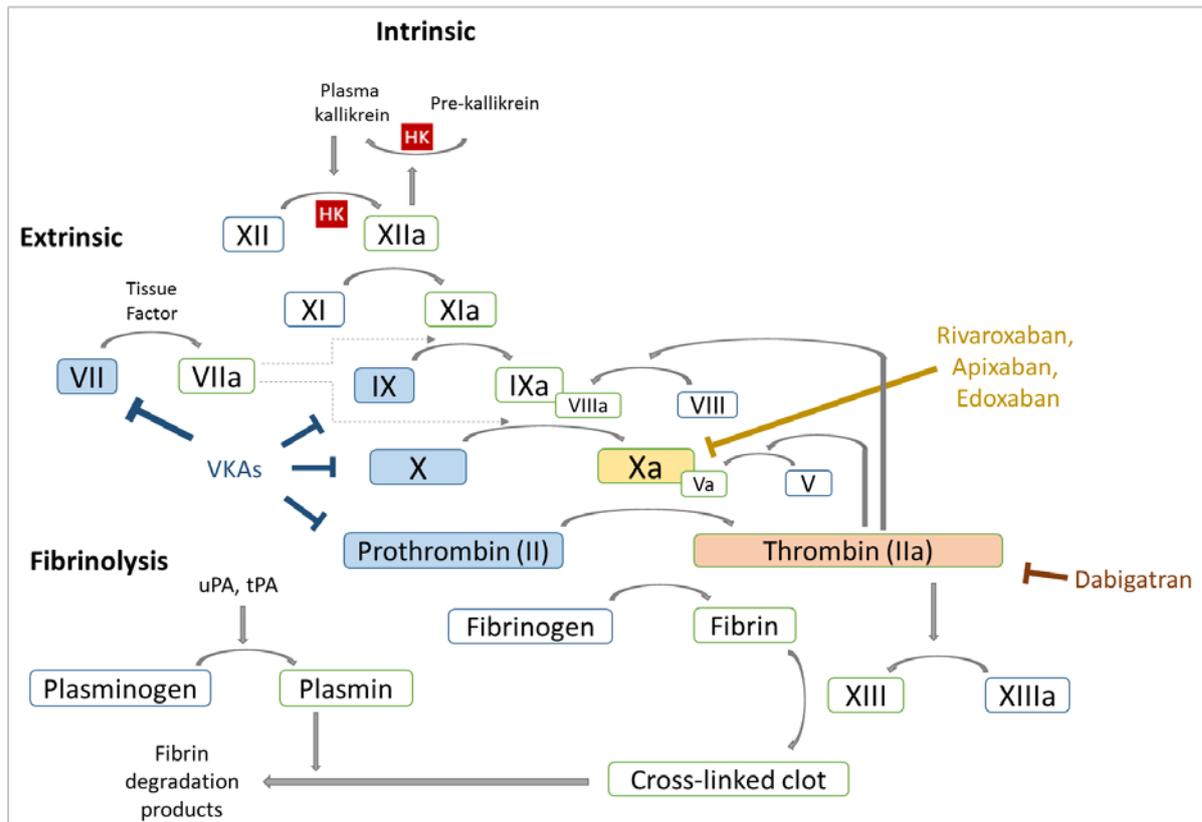
Figure 1.1: The coagulation mechanism in humans

Blood clotting is normally inhibited by endothelial cells in the wall of blood vessels. Although coagulation proteins circulate in the bloodstream, it is their inactive form that is present and therefore clotting does not occur. Tissue injury initiates the blood-clotting cascade.

Physiological haemostasis is achieved through the balance between the two activation pathways. Extrinsic pathway is the initially activated pathway of plasma-mediated haemostasis. As a result of trauma, tissue factor (TF) is exposed to plasma procoagulants, a process that is normally prevented by the normal vascular endothelium. TF then binds with activated coagulation factor VIII (VIIIa) and calcium cations (Ca^{2+}), forming the extrinsic tenase complex that leads to the conversion of factor X to its activated form Xa (Palta et al., 2014).

The contact activation intrinsic pathway begins with the formation of a primary complex consisting of high molecular weight kininogen, pre-kallikrein and coagulation factor XII on the surface of collagen. Pre-kallikrein is converted to kallikrein and factor XII to XIIa, which activates factor XI. Activated factor XI further activates factor IX. IXa along with the cofactor VIIIa form the intrinsic tenase complex on a phospholipid surface which catalyses the conversion to Xa (Palta et al., 2014, Pallister and Watson, 2010).

Factor X acts at the crossroad of the two haemostatic pathways. Both pathways combine to a common route in which factor Xa with its cofactor Va, platelets, tissue phospholipids and Ca^{2+} convert prothrombin (factor II) to thrombin (IIa). Thrombin has numerous functions, with the most important being the conversion of fibrinogen to fibrin, the building block of a haemostatic plug. Factor XIII is also activated by thrombin. Factor XIIIa and activated fibrin monomers form covalent bonds that crosslink the fibrin polymers to create a stabilised clot (thrombus) (Palta et al., 2014).

Factors VIII and V, their inhibitor protein C in the presence of thrombomodulin as well as platelets are all activated by thrombin during the initiation of the extrinsic pathway (Pallister and Watson, 2010). This shows the importance of thrombin for the haemostatic mechanism.

1.2 Thrombosis

1.2.1 *Causes and epidemiology*

Blood clotting (thrombosis) is the most common cause of morbidity and mortality worldwide (Weitz, 2014). It is a disease relevant to ageing and is triggered by three different mechanisms, comprising vessel wall damage, blood composition abnormalities and blood flow disturbances. These mechanisms are known as 'Virchow's triad' (Wolberg et al., 2012).

The predisposition to form a clot (thrombus) inappropriately is called thrombophilia and can arise from either genetic factors, acquired changes in the clotting mechanism such as increased levels of procoagulants, decreased levels of natural anticoagulants and defects in the fibrinolytic system (Heit, 2008) or, more commonly, from the interaction between hereditary and acquired factors.

The most common inherited form of thrombophilia is Factor V Leiden, a mutation in the F5 gene which expresses the coagulation factor V. Among European populations, 3-8% of people are heterozygous and 1 in 5,000 people are homozygous for this mutation. It is less common in other populations (US National Library of Medicine). Due to their increased inherent tendency to form blood clots, these individuals may form a clot without a cause before the age of 45 years. Other inherited causes of blood clots are shown in **Table 1.1** (Indiana Hemophilia and Thrombosis Center).

Decreased level of natural anticoagulants	Increased levels of natural procoagulants	Abnormal fibrinolysis	Other inherited causes
Antithrombin	Factor V Leiden or activated protein C resistance	Decreased levels of tissue plasminogen activator	Paroxysmal nocturnal haemoglobinuria
Protein C			
Protein S	Prothrombin 20210 mutation	Increased levels of plasminogen activator inhibitor	
Thrombomodulin			
Heparin Cofactor II	Hyperhomocysteinemia	Elevated thrombin-activated fibrinolysis inhibitor	
Tissue Factor Pathway Inhibitor	FVII, FIX, FXI, FVII, VWF		

Table 1.1: Inherited causes of blood clots

The most prevalent acquired thrombophilia is antiphospholipid syndrome. It is associated with both venous and arterial thromboses. The risks are even greater during pregnancy, due to the underlying physiological changes (Khare and Nelson-Piercy, 2003, Armstrong et al., 2014).

Arterial thromboembolism is caused when a blood clot blocks the flow of blood to a specific organ. This state normally affects people with atherosclerosis; fatty deposits attach to the arterial wall which narrows, increasing the risk for a blood clot. Embolisation is the process of a blood clot breaking off and travelling to another part of the body. The symptoms of thrombosis are dependent on the part of the body in which the clot is formed. Atherosclerosis and thrombosis in the coronary vessels leads to oxygen starvation to the myocardium leading to cardiac events, while embolization of thrombus in a vein can lead to pulmonary embolism (PE).

The manifestation of venous thrombosis can be either deep vein thrombosis (DVT) or PE. DVT occurs in approximately two thirds of total thromboembolic episodes (Cushman, 2007, Heit, 2008). Recent epidemiological studies estimate that venous thromboembolism affects 133 per 100,000 of the population annually in the USA. Recurrent venous thromboembolism appears in about 30% of individuals within the next 10 years, with serious outcomes for the survival and quality of life of the patient (Heit, 2008, Martinez et al., 2014).

Blood clotting disorders can also arise as a result of surgery, pregnancy, long-term immobilisation, autoimmune diseases, cancer, heart disease and many more situations (Cushman, 2007).

1.2.2 Atrial fibrillation and risk of stroke

Atrial fibrillation (AF) is a common cardiac arrhythmia that affects over 33.5 million people globally (GBD 2013 Mortality and Causes of Death Collaborators, 2015). By 2050, it is predicted to affect 6–12 million people in the USA and 72 million in Asia, while by 2060 it will affect 17.9 million in Europe (Morillo et al., 2017, Chiang et al., 2015). Between 2000 and 2010, AF-related hospitalisations increased by 23% in the US (Tamayo et al., 2015). AF is an abnormality of the heart rhythm, which involves the atria beating irregularly, which causes the blood to pool and eventually may cause the formation of a blood clot ((IQWiG), 2006) (**Figure 1.2**).
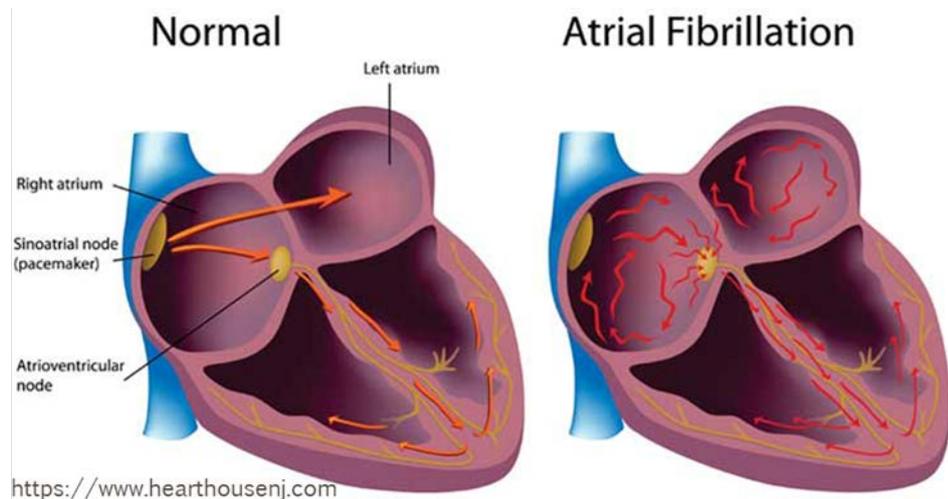
Figure 1.2: Change of normal heart rhythm as a result of atrial fibrillation

AF is the strongest known risk factor (5-fold) for stroke (Singer, 1998) with the association between AF and stroke of cardioembolic origin being widely recognised (Wolf et al., 1991). Stroke in patients with AF accounts for significant morbidity, mortality and economic burden in the developed world. In the presence of AF, strokes are more often lethal or result in more disability than in its absence (McGrath et al., 2013). The percentage of AF-attributed strokes increases with age, with a 16-fold increase between patients aged 50-59 and 80-89 years (1.5% v 23.5% respectively) (Kannel and Benjamin, 2008). The total annual cost for stroke treatment in the USA between 2011 and 2012 was estimated to be $33 billion (Mozaffarian et al., 2015). Stroke is estimated to cost the economy in England around £7 billion per year, which includes direct costs to the NHS, informal care and costs because of lost productivity and disability (Mant et al., 2004). In England, Wales and Northern Ireland between 2015 and 2016 the mean per patient cost of health and social care was £46,039, ranging between £19,101 to £107,336 for patients with acute stroke each year (Xu et al., 2018). In addition, stroke impacts on productivity and employment as 25% of all strokes in the UK happen among people of working age (Patel et al., 2017).

In the UK, scoring systems have been developed and are recommended by the National Institute for Health and Care Excellence (NICE) to calculate the risk of AF-related complications (NICE a). Among many other scores, assessment of the risk of stroke can be performed using $CHADS_2$, which was later refined to create CHA_2DS_2-

VASc (**Figure 1.3**) (Kane a). A CHA$_2$DS$_2$-VASc score of 2 or higher denotes that full oral anticoagulation should be considered, while every increase in the score predicts ischemic stroke rate per year linearly (National Clinical Guideline Centre UK, 2014, O'Neal and Alonso, 2016).

1.2.3 *Mechanical heart valves*

Valvular heart disease is currently well-managed by implanting an artificial metallic or tissue heart valve to either repair or replace the malfunctioning one, based on patient age, type of the valve disease and other medical conditions.

Tissue or bioprosthetic valves are composed of animal or human tissue from donor heart. Despite their limited risk of thrombosis and the short course of treatment to prevent thrombosis, many patients require a second operation within 10 to 20 years from the first valve replacement (Harris et al., 2015).

Criteria		Poss. Point
Congestive heart failure Signs/symptoms of heart failure confirmed with objective evidence of cardiac dysfunction	Yes No	+1
Hypertension Resting BP > 140/90 mmHg on at least 2 occasions or current antihypertensive pharmacologic treatment	Yes No	+1
Age 75 years or older	Yes No	+2
Diabetes mellitus Fasting glucose > 125 mg/dL or treatment with oral hypoglycemic agent and/or insulin	Yes No	+1
Stroke, TIA, or TE Includes any history of cerebral ischemia	Yes No	+2
Vascular disease Prior MI, peripheral arterial disease, or aortic plaque	Yes No	+1
Age 65 to 74 years	Yes No	+1
Sex **C**ategory (female) Female gender confers higher risk	Yes No	+1

Figure 1.3: Atrial fibrillation ischemic stroke CHA$_2$DS$_2$-VASc calculator (Kane a)

Mechanical heart valves are made from titanium and carbon. Several models are available for aortic and mitral replacement. Their main advantage over tissue valves is their durability and their greatest disadvantage is that they dramatically increase the risk of blood clots forming. Preventing the development of blood clots that cause heart attack or stroke is therefore of vital importance. Life-long treatment to prevent blood clots is recommended for mechanical valve replacement patients (Harris et al., 2015, Baumgartner et al., 2017).

1.2.4 *Prevention strategies*

Blood clots and their devastating effects can be prevented with two main strategies. The first includes lifestyle interventions. Wearing compression stockings, regular exercise, maintaining a normal weight, avoiding alcohol abuse and smoking can all reduce the risk of thrombosis (NICE b).

However, in cases where these changes are not adequate to reduce the risk, medical treatment is required. Prevention of blood clots is routinely achieved by taking oral anticoagulant therapy. The success of anticoagulation is demonstrated by a reduction in the incidence of stroke, systemic embolism, myocardial infarction (MI) and death from any cause as well as by an increase in survival.

1.3 Oral anticoagulant therapy

1.3.1 *Introduction*

Anticoagulation can be achieved by targeting different parts of the blood coagulation system and inhibiting clotting factors. Vitamin K antagonism was the first mechanism discovered to promote anticoagulation. In recent years, targeting the coagulation factors directly have resulted in new treatments that have recently become available in clinical practice.

Coumarins, such as warfarin, phenprocoumon and acenocoumarol, also known as vitamin K antagonists (VKAs), have been the mainstay of oral anticoagulation therapy for the treatment of thromboembolic disease for almost 65 years. Randomised controlled studies have demonstrated that anticoagulation therapy with warfarin significantly reduces the overall risk of stroke, its severity and the associated risk of death in patients with AF (van Walraven et al., 2002, Hart et al., 2007).

Consequently, treatment guidelines advocate the use of warfarin prophylaxis in patients with AF at moderate-to-high risk of stroke (Monagle, 2004).

Since 2010, the novel or otherwise known as non-vitamin K oral anticoagulants (NOACs) emerged in the market after receiving approval from the major drug regulatory agencies. The term NOAC is adopted by the European Society of Cardiology (ESC) AF guidelines (Kirchhof et al., 2016), and will be used throughout this thesis, instead of the term DOAC (direct oral anticoagulants) which is preferred in the USA. Drugs with two distinct mechanisms of action have been approved; direct thrombin (factor IIa) inhibitors and factor Xa inhibitors.

1.3.2 *Vitamin K antagonists*

Three VKAs (warfarin, acenocoumarol, phenprocoumon) are currently used in clinical practice with profound structural similarities due to their common structural ancestor, dicoumarol (**Figure 1.4**) (NCBI a). VKAs are still among the most frequently drugs used worldwide. Warfarin is the VKA of choice in North America, the UK and Scandinavian countries, while acenocoumarol and phenprocoumon are mainly used in European countries (Ufer, 2005).

Warfarin Acenocoumarol Phenprocoumon
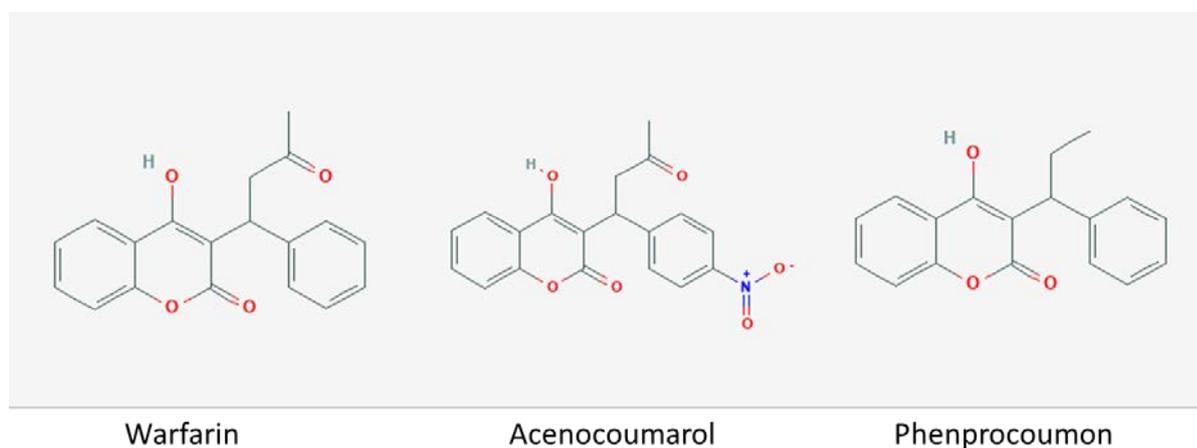
Figure 1.4: Chemical structures of vitamin K antagonists (Adapted from PubChem database)

1.3.2.1 History of warfarin use

With over 65 years of clinical use, warfarin is the first oral anticoagulant drug and one of the most commonly prescribed drugs (Mega and Simon, 2015). Along with other coumarin anticoagulants, warfarin is prescribed for the prophylaxis and treatment of thromboembolic events in patients with AF, PE, MI, artificial heart valves and DVT (Wahl et al., 2015, Kamali and Wynne, 2010).

The discovery of warfarin's action dates back to the 1920s, when veterinarians in Canada noticed the causal relationship between spoiled sweet clover hay and animal deaths that local farmers had observed. Link, an American Biochemist, described the process in detail and reached the conclusion that factors within the spoiled hay reduced the clotting ability of the blood, a process that could be reversed by vitamin K. As a consequence, internal haemorrhage was fatal for cattle and sheep. About a decade later, the active compound called 3,3-methylene bis(4-hydroxycoumarin), was discovered. After the synthesis of multiple derivatives of dicoumarol, one of them was proposed for rodenticidal use. It was named warfarin sodium in 1941 by Link, who combined the first letters of the Wisconsin Alumni Research Foundation with the ending of coumarin, 'arin' (Link, 1959).

1.3.2.2 Mechanism of action

The mechanism of anticoagulant action of warfarin was elucidated in 1978 by Whitlon and colleagues (Whitlon et al., 1978). The hypothesis that vitamin K reverses the action of dicoumarol derivatives and therefore of warfarin, was proven right. Vitamin K (as vitamin K hydroquinone) is an essential cofactor needed for the γ-carboxylation of glutamic acid residues located at the amino-terminals of the vitamin K-dependent clotting proteins II, VII, IX and X, leading to their activation (Kirchhof et al., 2016). Coumarins block the vitamin K epoxide reductase (VKOR) enzyme causing anticoagulation by interfering with the cyclic interconversion of vitamin K hydroquinone (hence also referred to as 'vitamin K antagonists') thus inhibiting the activation of vitamin K-dependent clotting proteins (Whitlon et al., 1978) (**Figure 1.5**).
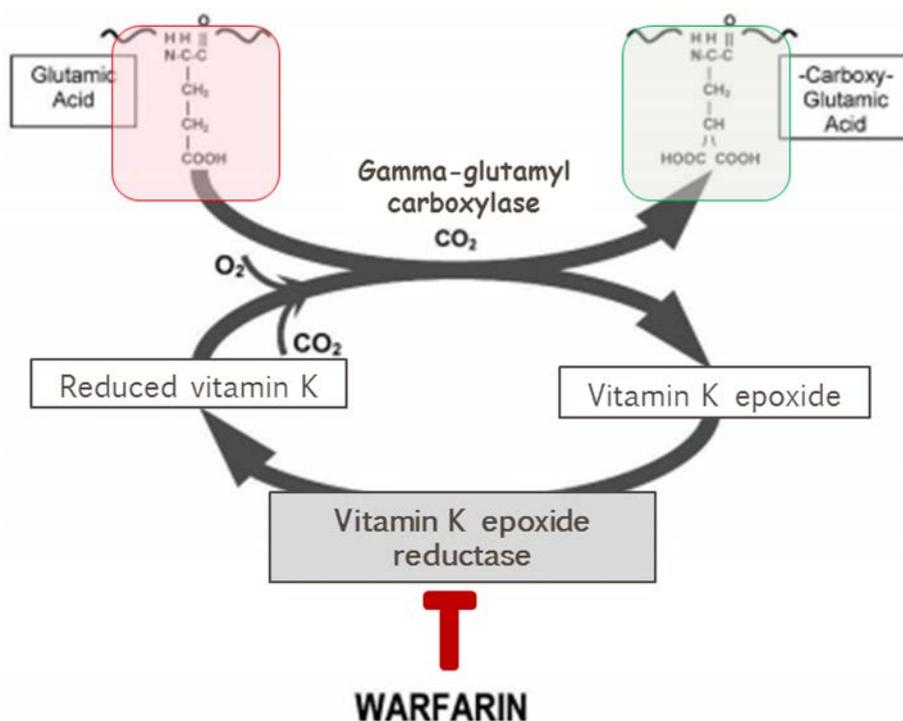
Figure 1.5: Inhibition of cyclic interconversion of vitamin K by warfarin

1.3.2.3 Pharmacokinetics and pharmacodynamics

Warfarin is prescribed as a 50:50 racemic mixture of two stereoisomeric forms, S-warfarin and R-warfarin, as a result of an asymmetric carbon in its structure. Warfarin is metabolised by the liver. S-warfarin is mainly metabolised by the microsomal liver enzyme CYP2C9 (cytochrome P450 isoform 2C9) that belongs to the cytochrome P450 enzyme system. As a result of this metabolic pathway, two inactive metabolites are produced. R-warfarin is metabolised by three different microsomal enzymes; CYP1A2, CYP3A4 and CYP2C19, producing inactive metabolites. S-warfarin is 5 times more potent as inhibitor of VKOR enzyme and is metabolically deactivated 3 times faster than the R enantiomer (Moyer et al., 2009) (**Figure 1.6**).

R-warfarin's clearance is half that of S-warfarin due to the half-life of R-warfarin being longer (37-89 hrs) compared to that of S-warfarin (21-43 hrs), but volume of distribution for both enantiomers is similar (U.S. Food and Drug Association, 2011).

The dose of warfarin normally ranges between 5–10mg daily (Harter et al., 2015), however it can vary from 0.5mg/day to more than 10mg/day (Wadelius et al., 2007).

The mean half-life of warfarin in plasma is approximately 40 hours and after oral administration the anticoagulant action lasts 2-3 days (Mega and Simon, 2015).

Warfarin's concentration in blood reaches its peak within one hour after oral administration. However, the pharmacological effect of warfarin can be observed after about 48 hours. As a result, plasma warfarin concentration is not helpful in monitoring coagulation. For this reason, a standardised measurement of prothrombin time (PT) prolongation is used to monitor anticoagulation response (Monagle et al., 2006).
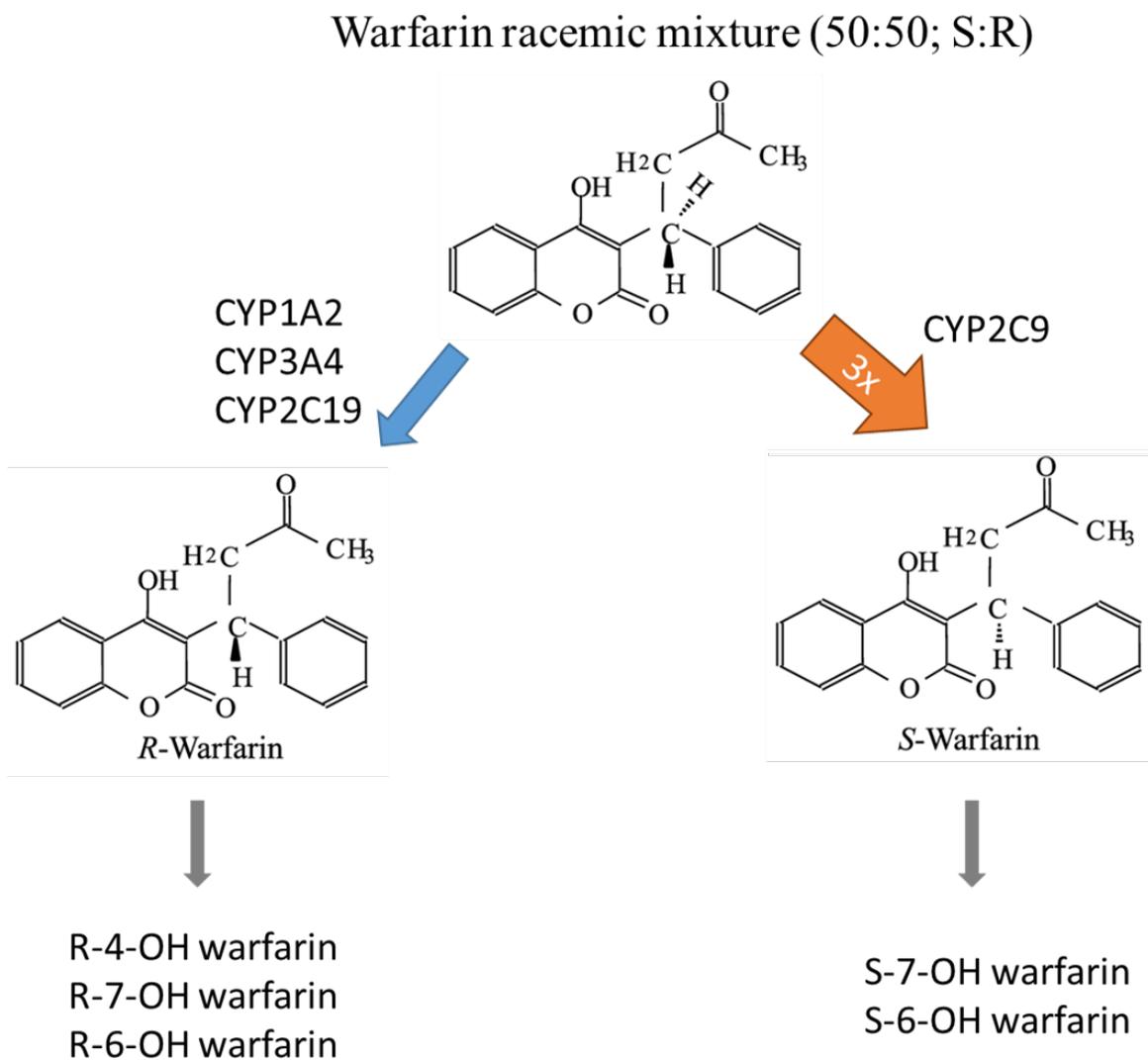
Figure 1.6: Warfarin racemic mixture metabolism by liver enzymes

1.3.2.4 Clinical indications

Warfarin is indicated for the prophylaxis and treatment of venous thromboembolism (VTE), PE, thromboembolic complications such as stroke associated with AF and/or cardiac valve replacement. It is also used for the reduction in the risk of systemic embolisation, recurrent MI or death after MI (2018, Keeling et al., 2011).

Although most indications requiring anticoagulation therapy can also be managed by non-vitamin K oral anticoagulants, currently warfarin is the only treatment available for the prevention of thrombosis in patients with mechanical heart valve replacement (Otto, 2017). However, one *in vivo* preclinical study used a swine surgical model to demonstrate that apixaban had comparable efficacy to warfarin in reducing mechanical valve thrombosis (Lester et al., 2017).

Contraindications for oral anticoagulation therapy in general include haemorrhagic stroke, recent major surgery or postpartum, pregnancy, bleeding disorders and potential bleeding lesions.

1.3.2.5 Warfarin therapy monitoring

1.3.2.5.1 PT/INR

The prothrombin time or prothrombin ratio was previously used to monitor warfarin therapy. The prolongation of PT depends on reductions in three of the vitamin K dependent clotting factors. The PT was replaced in 1982 by the International Normalized Ratio (INR); the big advantage of using INR is that unlike PT the INR value is not affected by variability between reagents used by different laboratories for the measurement of anticoagulation response (Horton and Bushwick, 1999).

The normal range of INR in healthy people is 0.8-1.3 (Horlocker et al., 2001). In patients starting coumarin derivatives, INR measurement is carried out frequently until it reaches the therapeutic range (usually 2.0-3.0 for most indications) for two consecutive days. Then, INR is measured two to three times per week for two weeks and if it remains stable and within the range, the measurement can take place less often but not less than monthly. However, some patients take longer to achieve therapeutic INR and as such require a greater number of INR determinations (Horton and Bushwick, 1999).

Anticoagulation response to warfarin, reflected in the INR value, can fluctuate from time to time in patients on stable maintenance therapy, with consequences in terms of thrombosis or bleeding risk (**Figure 1.7**). This can be caused by environmental factors, such as changes in diet and most importantly in vitamin K intake, poor patient compliance to treatment, alcohol consumption, concurrent illness and medications that interact with warfarin (Kuruvilla and Gurk-Turner, 2001). The quality of anticoagulation control in individual patients can be calculated using the time in therapeutic INR range (TTR).
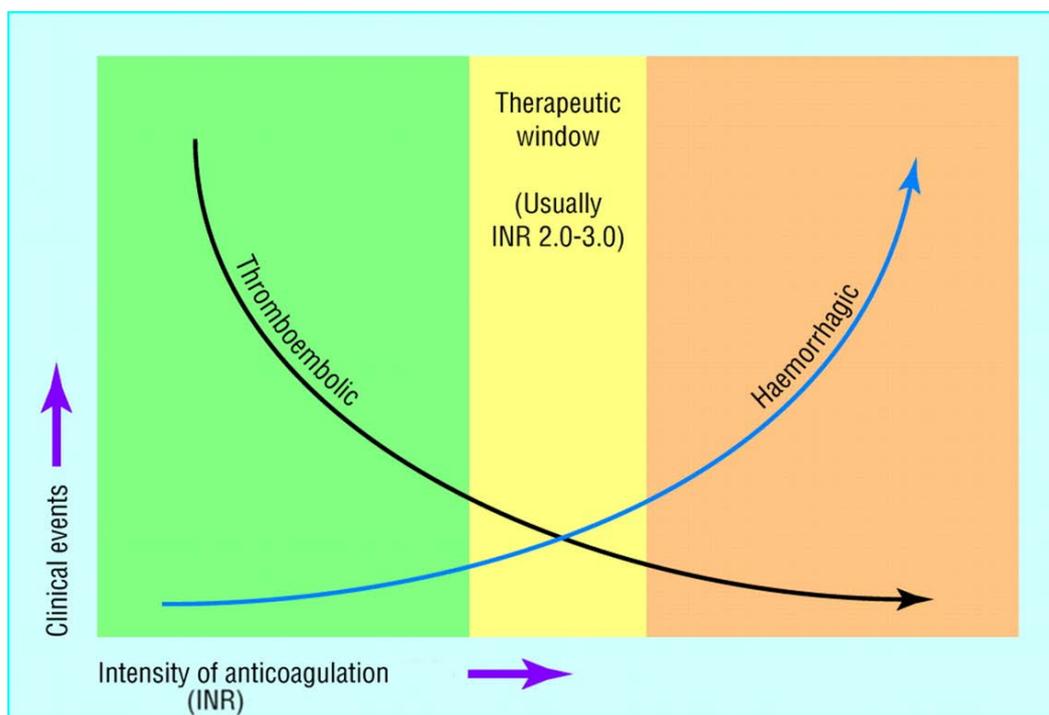
Figure 1.7: INR range and risk of thromboembolic and bleeding events

1.3.2.5.2 Time in therapeutic range

Warfarin is effective and safe for thromboprophylaxis in AF patients when therapy is well managed and frequently monitored. Time in therapeutic range assesses the quality of warfarin treatment management. The higher the TTR, the lower the risk of bleeding or stroke (Connolly et al., 2008).

The time in therapeutic range has typically been assessed by the Rosendaal method (Rosendaal et al., 1993). This linear interpolation method due to its

complexity requires a computerized system for calculation (shown in **Appendix A**). INR values are assigned to days without measured INRs via the drawing of a linear plot from the last measured INR to the next measured INR. Using values that are represented by this line, a value is assigned to each day. Then, all days with measured or assigned values are used to calculate the time in therapeutic range by dividing each patient's time within the therapeutic range by the total time of observation. This assumes that between-test INR varies linearly (2011, Rosendaal et al., 1993) (**Figure 1.8**).
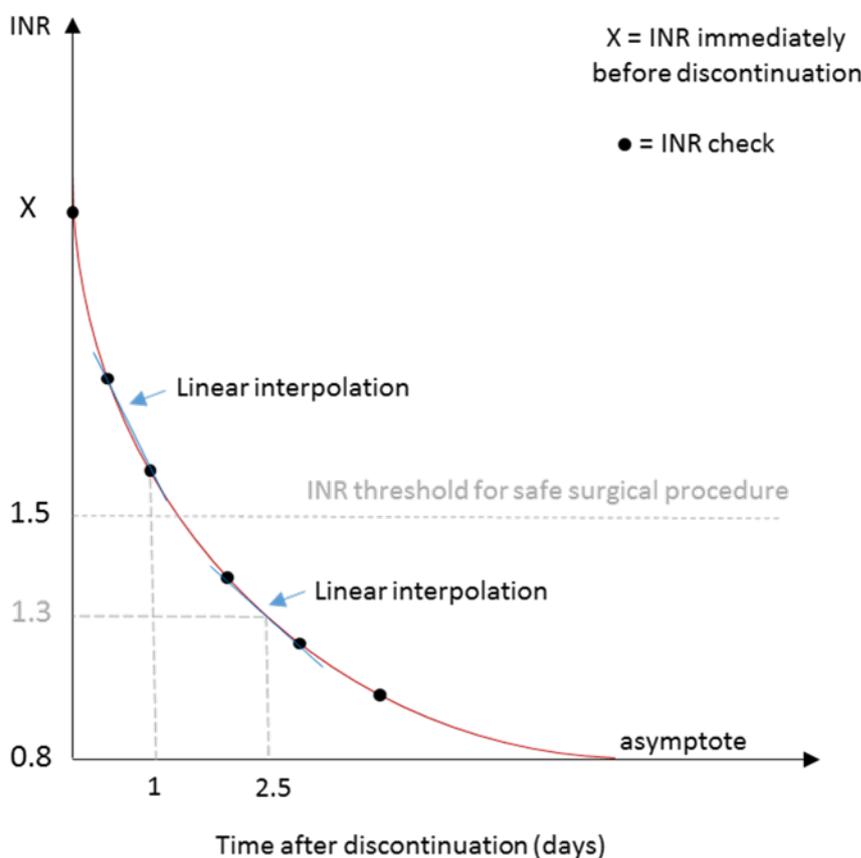
Figure 1.8: Graphical presentation of linear interpolation method of Rosendaal et al, 1993

TTR indicates the days which INR values are within the therapeutic range for the individual patient over a specific period of time. For instance, if a patient has a therapeutic range of 2.0 - 3.0, and on May 1st was tested at 2.5, then tested at 3.5 on May 31st, the number of days the patients was in range can be determined. Since there were 30 days between tests, it is assumed that the patient slowly moved from

2.5 to 3.5 over those 30 days, so around May 15th, the patient was probably over 3.0, and therefore was out of range. Therefore, it is estimated that 15 days the patient was in range, and 15 days was out of range (within the 30 day time period), which means the patient's TTR was 50% within that period of time.

A stable schedule of INR checks is necessary in order to reduce the chance of errors in the calculation of TTR. For example, in the case of having few INR checks over a period of time and one INR measurement out of range, the TTR will be low and will not accurately reflect the quality of warfarin treatment during this time (Reiffel, 2017). According to the results of a multicentre study, overall survival is improved where TTR is >40% and below the TTR threshold of 58% and 65% there is little benefit of oral anticoagulant over antiplatelet therapy (Van Spall et al., 2012, Connolly et al., 2008). A TTR of >58% can provide confidence that patients will benefit from the treatment (Baker et al., 2009).

Variability in the quality of healthcare system leads to great differences in the mean TTR between different geographical regions. Mean TTR was 36% in India and 75% in Sweden in a recent major trial (Daniel et al., 2013).

1.3.2.6 Adverse effects of warfarin

An uncommon adverse event with sudden onset within the first 8 days of treatment is skin necrosis (death of skin tissue), which is not a result of over-anticoagulation. Women are more likely to present with skin necrosis and on average patients are in their 50s (Chan et al., 2000). Treatment includes immediate warfarin discontinuation, warfarin reversal with vitamin K and surgical debridement in over 50% of cases. The suggested mechanism behind this event is underlying protein C, S or antithrombin III deficiencies (Fant and Fant, 2016).

1.3.2.6.1 Bleeding

Bleeding problems are the most common and potentially life threatening complications of warfarin therapy. Risk factors that predispose to bleeding include age over 75, concomitant use of other blood-thinning medications, patients with a previous stroke, cancer, uncontrolled blood pressure, kidney or liver disease, gastrointestinal problems such as stomach ulcers, alcohol excess, and people at increased risk of falls, e.g. elderly.

Bleeding is associated with higher than desired intensity of anticoagulation (over-anticoagulation). Warfarin-associated intracranial haemorrhage is the most feared and devastating, although less likely to occur. Red or brown urine, black or bloody stool, severe bleeding, including heavier menstrual bleeding than normal, coughing up or vomiting of blood and spontaneous bruising are all manifestations of bleeding complications and medical attention is required immediately. Bleeding from the gums or between menstrual periods is less serious but should still be reported (Shoeb and Fang, 2013).

Assessment of the risk of major bleeding can be performed using HAS-BLED (**Figure 1.9**) (Pisters et al., 2010, Kane b). The HAS-BLED scoring system estimates the risk of major bleeding (intracranial bleeding, bleeding requiring hospitalization, a haemoglobin decrease of more than 2 g/dL, or the need for transfusion secondary to bleeding) for patients on anticoagulation for risk assessment in AF care (Kane b). Bleeding risk scores, such as HAS-BLED, should not be used to exclude patients from the use of oral anticoagulation, but rather to identify modifiable bleeding risk factors that can be managed to reduce a patient's risk of bleeding from anticoagulation (Friberg et al., 2012).

Criteria		Poss. Point
Hypertension Uncontrolled hypertension (systolic BP > 160 mmHg)	Yes No	+1
Abnormal renal or liver function **Renal:** Chronic dialysis, renal transplant, serum creatinine ≥ 2.3 mg/dL (200 µmol/L) **Liver:** Cirrhosis, bilirubin > 2x UNL with AST/ALT/AP > 3x UNL	None	+1 or +2
Stroke	Yes No	+1
Bleeding Bleeding history or predisposition (anemia)	Yes No	+1
Labile INR Therapeutic time in range < 60%	Yes No	+1
Elderly Greater than 65 years old	Yes No	+1
Drugs or alcohol **Drugs** - other antiplatelet agents or NSAIDs **Alcohol** - more than 8 drinks per week	None	+1 or +2

Figure 1.9: Bleeding risk HAS-BLED calculator

1.3.2.6.1 Other complications

In addition to bleeding, severe headache, joint pain, swelling especially after an injury, stomach pain, weakness or dizziness and vision changes have been reported as warfarin ADRs (Adverse drug reaction) that require immediate attention. Diarrhoea, inability to eat for more than 24 hours, nausea, vomiting, fever, jaundice, hepatic dysfunction, pancreatitis, pyrexia, alopecia, purpura, and rash are less serious adverse events (FDA, 2011). (FDA, 2011)

1.3.2.6.2 Vascular calcification risk

Warfarin has been suspected to cause vascular and arterial calcifications in patients on long term use. Based on the mechanism of action, warfarin inhibits the cyclic interconversion of vitamin K, blocking its action as a cofactor for the γ-carboxylation of gamma-carboxyglutamic acid (Gla) residues by vitamin-K-dependent gamma-carboxylase and inhibiting calcium binding and normal function. As a result, all vitamin K dependent proteins, ranging from clotting factors II, VII, IX, X, protein C, S and Z to osteocalcin and matrix-Gla protein (MGP), remain inactive.

MGP is mainly secreted by vascular smooth muscle cells and chondrocytes in the arterial tunica media. Its activation requires two posttranslational modifications; γ-glutamate carboxylation and serine phosphorylation. Based on carboxylation and phosphorylation state, different species of MGP have been identified and ELISAs (Enzyme-linked immunosorbent assay) have been made available for the quantification of such species in human plasma (Schurgers et al., 2008). Studies on MGP showed that knockout mouse exhibited extensive and lethal calcification and cartilaginous metaplasia of the media of all elastic arteries. The function of MGP has been considered protective against calcification in human vasculature and is involved in modulating vascular calcium metabolism, although the mechanism is still to be fully understood (Wei et al., 2016, Bjorklund et al., 2018).

Vitamin K antagonism during warfarin therapy has been associated with higher risk of calciphylaxis and arterial calcification in independent patient cohorts (Nigwekar et al., 2017). A systematic review has been published earlier in 2018 which reports conflicting results in terms of specific MGP species associated with local calcification development. Existing studies have looked at different calcification locations and have measured different circulating MGP species. It has become clear that there is a complex relationship between calcification risk and MGP. Further well-designed large

studies will help elucidate the underlying molecular mechanisms that can accelerate local disease progression within the vasculature and the predictive value of the currently defined MGP species (Barrett et al., 2018).

1.3.2.7 Reversal of anticoagulation due to warfarin therapy

Warfarin anticoagulation reversal is required in cases whereby a patient presents in clinic with elevated INR and/or bleeding symptoms. The method of reversal is selected based upon the clinical severity of bleeding, if any, and the balance between bleeding and thrombosis risks (Makris and Watson, 2001).

Immediate warfarin discontinuation is required in most cases. Current guidelines suggest the use of Prothrombin Complex Concentrate (PCC), based on patient weight and INR (Di Fusco et al., 2018) or Fresh Frozen Plasma (FFP) with additional IV administration of vitamin K when rapid reversal of anticoagulation is urgently required, as a result of life-threatening bleeding. Less severe or minor bleeding can be reversed using a lower dose of vitamin K IV, or oral administration (Holbrook et al., 2012, Hanley, 2004).

When no bleeding has occurred and the management of over-anticoagulation is limited on the INR correction, simple omission of warfarin or low dose oral vitamin K in patients with high risk of bleeding is recommended. If INR exceeds a certain value (generally around 8-10), then oral administration of vitamin K is the chosen method for reversal (Holbrook et al., 2012, Hanley, 2004)

1.3.2.8 Variability in anticoagulation response

Achieving effective and safe anticoagulation with warfarin is a major challenge in routine medical care because many factors can influence the pharmacodynamic effect of this drug. Whilst several factors including concurrent disease, drugs and patient compliance have been identified as affecting warfarin requirements in a predictable way, the observed intra-individual variability in anticoagulation response to warfarin cannot be fully explained at present. There is evidence that age and dietary factors can make a significant contribution to unstable control of anticoagulation.

1.3.2.8.1 Ageing

Although the use of warfarin has been steadily declining after the marketing of NOACs, the use of warfarin particularly in elderly patients is still widespread, because of its proven benefits in AF and the increasing prevalence of this condition (Morillo et al., 2017) Vitamin-K antagonists are associated with reduced mortality, even in vulnerable patients with AF over 65 (Bo et al., 2016).

Nevertheless, it has been estimated that a third of patient hospitalizations in the elderly on warfarin are caused by haemorrhages and other adverse drug reactions of warfarin (Budnitz et al., 2011), while another study found that 1.5% of hospital admissions were due to warfarin complications (Hirri and Green, 2002). In the UK, ADRs were responsible for 6.5% of hospital admissions and warfarin alone was responsible for 10.5% of cases (Pirmohamed et al., 2004).

When warfarin is prescribed, elderly people need to be carefully selected based on the ratio of benefit over risks (bleeding and thrombosis). Therapy is initiated in a low dose (1mg daily for 1 week then INR check, followed by 2mg daily for 1 week then INR check) compared to younger adults (usually 5mg daily for first 2 days, then INR check, especially if they have new DVT/PE) and the treatment requires close monitoring of the INR, especially after changes to regular medications or diet, in which case dose adjustments may be necessary (Bereznicki et al., 2006). Studies suggest that the return of the INR to within the individual target range after an over-anticoagulation event is delayed with advanced age (Hylek et al., 2001).

Cognitive decline with age has been associated with frequent haemorrhages and suboptimal therapy (Greg et al., 2010). Additionally, 50% of older patients requiring systemic anticoagulation do not receive it due to the higher risk of falls, which is responsible for discontinuation of warfarin treatment by GPs, however this needs to be properly assessed, and the ongoing review of the appropriateness of therapy as circumstances change is essential (Hagerty and Rich, 2017).

Educating patients and carers can be of paramount importance both for the appropriate management of any ADRs and also the facilitation of self-monitoring, in particular for people who do not have easy access to anticoagulation clinics.

1.3.2.8.2 Diet

The significant impact of diet on adult anticoagulation control has long been reported in the literature (Lurie et al., 2010). Vitamin K intake is a major determinant of warfarin's effect according to its mechanism of action. Prospective observational studies have reported a greater sensitivity of patients with low vitamin K stores to the pharmacodynamic effects of warfarin (Kim et al., 2010, Sconce et al., 2005b). Changes in vitamin K intake can be caused by various syndromes that affect nutrition, such as gastrointestinal ulcers, cancers poor fat absorption caused by celiac disease, cystic fibrosis, an intestinal or biliary tract disorder or part of their intestine removed (Krasinski et al., 1985, Lin et al., 1992). Dietary supplements are another source of vitamin K intake. Warfarin dose adjustments, especially during initiation of therapy, are necessary to balance these changes (Kurnik et al., 2003).

Other food sources interfere with enzymes that metabolise warfarin, as in the example of grapefruit and herbs which can subsequently alter anticoagulation response (Veronese et al., 2003).

The implementation of strategies such as educating patients on anticoagulant therapy to adopt a balanced dietary intake, to report any supplements to their GP or Pharmacist as well as monitoring the INR, intra-patient variability in anticoagulation response can be reduced (White, 2010).

1.3.2.8.2.1 Vitamin K

The discovery of vitamin K dates back to 1929. Henrik Dam, a Danish biochemist, initially named it the "antihaemorrhagic vitamin of the chick" after the observation that muscular and subcutaneous bleeding was common in chicks with lipid-free diet. In 1935, soon after this discovery and because of Dam's work on diets, he proved that vitamin K (from the German and Scandinavian "Koagulation") was a fat soluble vitamin, distinct from other vitamins known at that time (A, C, D and E). Dam's research also paved the way to the discovery of two different forms of vitamin K. The first one, vitamin K_1 or phylloquinone was found to be present in hog liver, vegetable oils and green leafy vegetables, while bacterial activity could lead to the synthesis of vitamin K_2 or menaquinone. Later, in 1939, the group of Edward Doisy synthesised and characterised both forms of vitamin K. Therefore, the 1943 Nobel Prize in Physiology and Medicine was shared between Dan and Doisy for their contributions (O'Reilly, 1976, Azuma et al., 2014).

Due to the antihaemorrhagic effect of vitamin K, it was introduced in clinical practice as treatment or prophylaxis of bleeding conditions. The actual mechanism of action of vitamin K was revealed several years later, in 1974 (Ferland, 2012).

Vitamins K_1 and K_2 share most of their basic chemical structure, which mainly comprises of a naphthoquinone ring and long side chains (**Figure 1.10**). Vitamin K_2, after conversion from vitamin K_1, is the active form in the human body (Okano et al., 2008). Seven major menaquinones (MK-4 to MK-10) have been identified, which contain 4–10 repeating isoprenoid units (Booth, 2012). A third form of vitamin K, called vitamin K_3 or menadione was later synthesised. This form lacks a side chain (**Figure 1.10)** (NCBI b, Booth and Suttie, 1998). Menadione has previously been used as a dietary supplement, however later was prohibited by the Food and Drug Administration of the USA due to its toxicity (Azuma et al., 2014).

Phylloquinone   Menaquinone group   Menadione
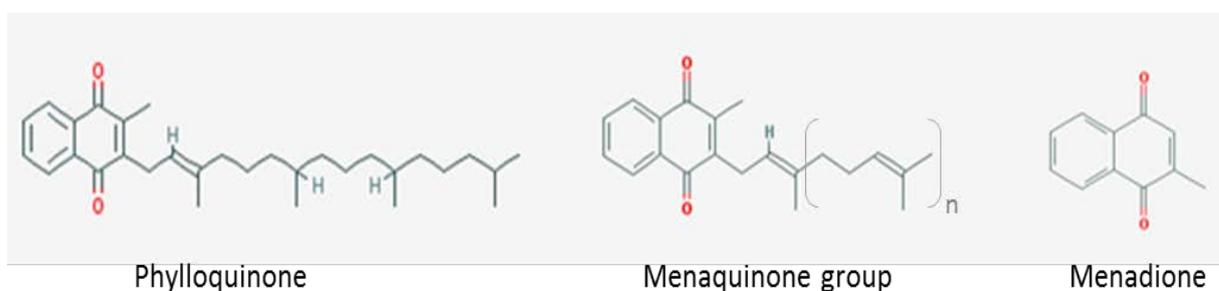
Figure 1.10: Vitamin K forms and structures (Adapted from PubChem database)

As mentioned earlier, blood clotting factors dependent on vitamin K (factors II, VII, IX, X and proteins C, Z and S) are activated following post-translational carboxylation (Kamali and Wynne, 2010). The modification occurs in the N-terminus of the newly formed protein chain of coagulation factors. It consists of carboxylation of glutamic acid residues in the zymogen precursors of coagulation proteins. In 1974, both Stenflo and Nelsestuen with their colleagues, independently showed that the previously unrecognised amino acid, γ-carboxyglutamic acid, was present in functional prothrombin with the property of binding calcium (Suttie, 1993, Stenflo et al., 1974, Nelsestuen et al., 1974). Further investigation of prothrombin's chemical structure revealed that in its amino terminal region between residues 7-33, the 10 previously considered to be glutamic acid residues were modified into Gla. Gla residues are highly conserved among coagulation factors and other vitamin K

dependent proteins. All coagulation factors are homologous to prothrombin in residues 1-40 (Dowd et al., 1995).

The presence of Gla residues enables the proteins to bind calcium, which forms ion bridges between the coagulation enzymes and negatively charged phospholipids on the membrane surfaces of endothelial, vascular cells and blood platelets. This calcium-mediated interaction of these proteins is essential in controlling coagulation protein conformation by enabling internal Gla-Gla binding (Suttie, 1993).

The enzyme that accomplishes the carboxylation, called γ-glutamyl carboxylase (GGCX) was initially discovered by Suttie and colleagues (Suttie, 1993). This enzyme requires certain substrates; CO_2, O_2 and the reduced form of vitamin K (KH_2), hydroquinone for its activity. The levels of KH_2 control the rate of clotting protein carboxylation, which results in the conversion of vitamin K to vitamin K epoxide. The proposed mechanism is the reaction of vitamin K hydroquinone with oxygen resulting in the formation of a strong base which abstracts a proton from the γ-carbon of the glutamic acid residue. An intermediate carbanion is then formed and undergoes carboxylation into Gla. Finally, vitamin K 2,3-epoxide is formed (Suttie, 1993).

Vitamin K epoxide is recycled to vitamin KH_2 via a two-step reaction. VKOR is the enzyme that converts vitamin K epoxide into the quinone form and reduces the quinone into the hydroquinone form of the vitamin. The latter reduction process has an alternative pathway mediated by a NAD(P)H-dependent quinone reductase. The described enzymatic recycling of vitamin K hydroquinone is called "the vitamin K cycle" (Whitlon et al., 1978, Stafford, 2005). Vitamin is rapidly catabolized and from the liver it is excreted mainly in bile. A smaller amount appears in urine (Shearer et al., 1974).

Antagonism or deficiency of vitamin K leads to inadequate γ-carboxylation. Proteins induced by vitamin K absence or antagonism (PIVKA) are released into the blood (Shapiro et al., 1986). Protein Induced By Vitamin K Absence Factor II (PIVKA-II) relates to the under-carboxylated prothrombin due to the absence of vitamin K.

Apart from the coagulation proteins, vitamin K is essential for the synthesis of numerous proteins with different physiological functions, all characterised by the presence of Gla residues. To date, 19 human proteins have been acknowledged as being γ-carboxylated. These include notably the matrix-Gla protein, GAS6, nephrocalcin, osteocalcin, periostin and others. These proteins control a number of

physiological functions as well as being involved in the endothelial function, kidney stone generation and bone formation. The role of vitamin K in osteoporosis has been greatly debated; there are conflicting reports linking vitamin K insufficiency to osteoporosis (Shah et al., 2014).

Nonetheless, it has been shown that certain biochemical mechanisms protect the synthesis of vitamin K dependent coagulation proteins at the expense of other Gla proteins. Based on the triage hypotheis, coagulation factors are essential for short-term survival and therefore acute deficiency of vitamin K can result in life threatening bleeding (Holmes et al., 2012).

1.3.2.8.2.2 Dietary intake

The main source of vitamin K in humans is through the diet. Numerous lists mainly consisting of green leafy vegetables and vegetable oils have been published, along with the vitamin K content of each food (**Table 1.2**) (Suttie, 1992, Booth and Suttie, 1998). The USDA National Nutrient Database for Standard Reference includes a very detailed phylloquinone content in foods (U.S. Department of Agriculture, 2015). Elder et al also published a report of vitamin K content of various foods, with the distinction between vitamin K_1 and K_2 (Elder et al., 2006).

The dietary vitamin K intake varies among different age groups, such as newborn, children and adults. In a U.S. study, the median vitamin K intake from food and supplements was between 82 to 119 µg/day for adult Americans (Institute of Medicine US Panel on Micronutrients, 2001). There is currently no established recommended dietary allowance for phylloquinone due to lack of sufficient data. The current "dietary reference value" of 1 µg/kg (~65-80 µg of phylloquinone/day in an average sized individual) is based on coagulation factor function; substantially higher recommended daily doses have been suggested because of findings that the requirement for vitamin K is greater for the extra-hepatic vitamin K-dependent proteins, including those found in bone (e.g. osteocalcin) (Sokoll et al., 1997). In 2001, the adequate intake level for vitamin K based on consumption levels in healthy individuals was established as shown in **Table 1.3** (Food and Nutrition Board, 2001).

Neither pregnancy nor lactation necessitate additional vitamin K uptake in women (Food and Nutrition Board, 2001). A previous study of dietary intake of vitamin K in a national sample of British elderly people demonstrated that 59% had an intake below 1 µg/kg/day (Thane et al., 2002b).

Food item	Phylloquinone content (µg/100g)	Food item	Phylloquinone content (µg/100g)
Vegetables		*Protein sources*	
Collard greens	440	Dry soybeans	47
Spinach	380	Dry lentils	22
Salad greens	315	Liver	5
Broccoli	180	Eggs	2
Brussels sprouts	177	Fresh meats	<1
Cabbage	145	Fresh fish	<1
Bibb lettuce	122	Whole milk	<1
Asparagus	60	Tuna in oil	24
Okra	40		
Iceberg lettuce	35	Prepared foods[a]	
Green beans	33	Salad dressings	100
Green peas	24	Coleslaw	80
Cucumber	20	Mayonnaise	41
Cauliflower	20	Beef chow mein	31
Carrots	10	Muffins	25
Tomatoes	6	Doughnuts	10
Potatoes	1	Apple pie	11
		Potato chips	15
Fats and oils		French fries	5
Soybean oil	193	Macaroni cheese	5
Canola oil	127	Lasagne	5
Cottonseed oil	60	Pizza	4
Olive oil	55	Hamburger/bun	4
Corn oil	3	Hot dog/bun	3
Margarine[a]	42	Baked beans	3
Butter	7	Bread	3

[a] Phylloquinone content may vary widely according to the source of oil used

Table 1.2: Phylloquinone (vitamin K$_1$) content of foods (Adapted from (Booth and Suttie, 1998))

Life Stage	Age	Vitamin K (µg/day)
Infants	0-6 months	2.0
	7-12 months	2.5
Children	1-3 years	3.0
	4-8 years	5.5
	9-13 years	6.0
Adolescents	14-18 years	7.5
Adults	> 19 years	12.0 (males) 9.0 (females)

Table 1.3: Adequate Intake of vitamin K (Adapted from (Food and Nutrition Board, 2001))

Phylloquinone is used in daily clinical practice for several purposes, for instance as a supplement, antidote and medication for the prevention and treatment of the haemorrhagic disease of the newborn (vitamin K deficiency bleeding; VKDB). Vitamin K deficiency is rare in adults. Malnutrition, malabsorption, medications that can interfere with vitamin K absorption or metabolism, or significant alcohol intake are the common causes of vitamin K deficiency. In cases of vitamin K deficiency, vitamin K is administered orally, intravenously or intramuscularly. In cases of overdose with coumarin, reversal of excessive anticoagulation is achieved through vitamin K, given either orally or intravenously depending on clinical circumstances (Tran and Alderman, 2013, Biss et al., 2012, Hanley, 2004).

Of all the fat-soluble vitamins, vitamin K has the highest individual daily variation in both dietary intake and corresponding plasma concentrations. This is because dark green vegetables, which are the main source of vitamin K, are not consistently consumed on a daily basis. For example, in a study of post-menopausal women in New England, USA, intake ranged from 3-2761µg of phylloquinone per day (Booth et al., 1995). In a UK population, the mean intake was 103 (95% CI 94, 112) µg per day (Yan et al., 2004).

There has been a long-term interest amongst researchers in the detection and quantification of vitamin K in plasma. Detection of vitamin K_1 by fluorimetry was first reported in 1986 and has since been optimised further to achieve better selectivity and sensitivity. Further work resulted in the detection and quantification of vitamin K in plasma using reverse-phase high performance liquid chromatography (HPLC)

technique with fluorescence detection after post-column reduction with zinc (Lambert et al., 1986, Haroon et al., 1986, Wang et al., 2004).

1.3.2.8.2.3   Influence on anticoagulation control

Anticoagulation control during warfarin therapy is highly sensitive to fluctuating levels of vitamin K in the diet because the activation of the main clotting factors (II, VII, IX and X) is dependent on vitamin K availability (Lubetsky et al., 1999, Franco et al., 2004, Booth, 2010). Diet with low vitamin K content is associated with greater susceptibility to INR fluctuations caused by small changes in vitamin K intake (Kurnik et al., 2004).

Sudden changes in dietary vitamin K intake resulting in poor anticoagulation control may put patients at increased risk of potential life-threatening bleeding and thromboembolic complications. Indeed, prosthetic valve thrombosis (Chow et al., 1990) and MI (Walker, 1984) have been reported in patients who increased their vitamin K intake during anticoagulation therapy, and in the case report by Walker (Walker, 1984) this was linked to the patient embarking on a vegetable-rich, weight-reduction diet. Conversely, diffuse bruising (presumably due to enhanced anticoagulation) in one case report was linked to the patient stopping their consumption of large amounts of porcine liver, thereby reducing his vitamin K intake (Chow et al., 1990). Thus, it has been suggested that patients should maintain a consistent dietary vitamin K intake during anticoagulation therapy, generally no more than 250 to 500 µg/day (Pedersen et al., 1991).

An earlier crossover study in anticoagulated patients demonstrated an inverse relationship between vitamin K intake and anticoagulation response, as measured by the International Normalised Ratio (INR) (Franco et al., 2004). Another study in patients on chronic therapy with warfarin and with stable control of anticoagulation demonstrated that for every 100 µg increase in vitamin K intake over the previous 4 days, INR value fell by 0.2 units (Khan et al., 2004), further clarifying the extent of the inter-relationship between dietary vitamin K and the INR. Consistent with these observations, Lubetsky et al. found that, INR values decreased in 32% (16/50) of anticoagulated patients following a vitamin K intake of ≥250 µg/day. The patients had significantly lower median INR values (1.9 and 3.0, respectively; $p<0.001$) and required a higher maintenance steady state warfarin dose (5.7 and 3.5 mg/day,

respectively; p<0.001), compared to those receiving <250 μg/day of vitamin K (Lubetsky et al., 1999).

In order to reduce intra-patient variability in anticoagulation response, patients are advised to maintain an adequate and consistent intake of dietary vitamin K that at least meets the current dietary reference value of 65-80 μg/day. Self-monitoring of vitamin K intake aids, such as the 'K-Card' (Couris et al., 2000), which was designed as a checklist of common foods and beverages providing ≥5 μg vitamin K per serving, may be helpful in this regard (Couris et al., 2000).

1.3.2.8.2.4   Vitamin K supplementation

Low vitamin K levels can cause a patient's warfarin dose requirement to vary with even minor changes in vitamin K intake. Several studies have shown that daily oral supplementation with vitamin K could improve stability of anticoagulation control in patients who are more susceptible to dietary changes, because of their poor and erratic dietary vitamin K intake, such as the elderly (Ford et al., 2007, Sconce E, 2007, Rombouts et al., 2007, Khan et al., 2004). A small study in 2005 demonstrated that vitamin K supplementation improved anticoagulation control, possibly due to the more steady activation of vitamin K–dependent clotting factors (Reese et al., 2005). At the same time, our group demonstrated that patients with variably low dietary intake of vitamin K were more likely to have unstable control of anticoagulation compared to their matched stable counterparts (Sconce et al., 2005b). Kim and colleagues in 2010 confirmed that patients with low vitamin K intake are more sensitive to minor changes in vitamin K intake and thus more likely to have unstable anticoagulation control (Kim et al., 2010). Another prospective study proposed that a vitamin K-guided management strategy is clinically feasible for the maintenance of oral anticoagulation intensity (De Assis et al., 2009).

Schurgers and colleagues investigated the safe initiation of vitamin K supplementation on anticoagulation response in healthy subjects. They studied the dose-response relationship of vitamin K supplementation on anticoagulation control and concluded that the administration of 100 μg of vitamin K daily did not significantly influence INR value (Schurgers et al., 2004). Despite a study showing that 25 μg significantly reduced the INR in patients with low vitamin K status (Kurnik et al., 2004), it was later recommended that supplementation with a 100 μg vitamin K daily

dose is preferable to negate any influence of variability in dietary vitamin K intake (Oldenburg, 2005).

In 2007, three separate studies on the effect of vitamin K supplementation on anticoagulation stability reported similar findings. In a double-blinded placebo controlled study, seventy patients with unstable control of anticoagulation were randomised to receive either 150µg oral vitamin K, or placebo daily for 6 months. The study confirmed that vitamin K supplementation results in significant improvement in anticoagulation stability attributed to a reduction in inter-day variability in dietary vitamin K intake (Sconce E, 2007). A second open label crossover study of 9 patients with unstable anticoagulation control showed a reduction in INR variability after 500µg of oral vitamin K supplementation daily (Ford et al., 2007). Rombouts and colleagues also demonstrated the positive effect on anticoagulation control of 100µg vitamin K supplementation once daily for 24 weeks in an unselected group of 200 patients on chronic therapy with phenprocoumon (Rombouts et al., 2007).

A recent meta-analysis reported no beneficial effect of low-dose vitamin K supplementation on the reduction of clinically relevant adverse events in patients taking VKAs (Lam et al., 2013). However, the meta-analysis lacked sufficient data to show that daily vitamin K supplementation has an impact on the incidence of adverse events in anticoagulated patients. Although the 2008 American College of Chest Physicians (ACCP) guidelines (Ansell J, 2008) recommended the use of low dose vitamin K supplementation in patients with chronically unstable anticoagulation, this was recommended against in the updated 2012 guidelines (Holbrook et al., 2012).

1.3.2.8.2.5   Enteral feeds

Enteral feeds may be an unsuspected source of additional dietary vitamin K in patients receiving warfarin. Although a decrease in the vitamin K content of enteral feedings has been observed over the years (Dickerson, 2008), some feeds may contain for example up to 20 µg vitamin K per 8 fl oz (237ml) serving (**Table 1.4**). This may explain why certain enteral feeds have been reported to lower the anticoagulant effect of warfarin (Penrod et al., 2001, Shearer, 2009a, Klang et al., 2010). Thus, it is important to consider the significant impact of enteral nutrition on anticoagulation response in patients on warfarin therapy.

Product	Kcal Per 100mL serving	Vitamin K content (µg)
Fresubin® original [a]	100	6.7
Fresubin® original fibre [a]	100	6.7
Fresubin® 1000 complete [a]	418	10
Fresubin® 1200 complete [a]	500	10
Fresubin® 1500 complete [a]	420	6.7
Fresubin® 1800 complete [a]	500	10
Fresubin® 2250 complete [a]		
Fresubin® energy [a]	630	6.67
Fresubin® energy fibre [a]		
Fresubin® HP energy [a]		
Ensure TwoCal [b]	200	8.5
Nutrison® [c]	100	5.3
Nutrison® MCT [c]		
Nutrison® Multi Fibre [c]	103	5.3
Nutrison® Energy [c]	150	8
Nutrison® Energy Multi Fibre [c]	153	8
Nutrison® Protein Plus [c]	125	6.6
Nutrison® Protein Plus Multi Fibre [c]	128	6.6
Nutrison® 800 Complete Multi Fibre [c]	83	8
Nutrison® 1000 Complete Multi Fibre [c]	104	8
Resource Energy [d]	151	14
Peptamen® HN [d]	133	9
Vital 1.5kcal [b]	150	7
Novasource® GI Control [d]	106	6
Novasource® GI Control [d]	155	11
Per 8 fl oz serving ^		
Jevity® 1 Cal [b]	250	15
Jevity 1.2 Cal (formerly Jevity Plus) [b]	285	20
Jevity® 1.5 Cal [b]	355	19
Osmolite® 1 Cal [b]	250	15
Osmolite® 1.2 Cal [b]	285	20
Osmolite® 1.5 Cal [b]	355	19
Ensure® [b]	220	20
Ensure® High Protein [b]	160	20
Ensure® Plus [b]	350	20
Ensure® Plus HN [b]	250	20
Ensure® Enlive® Advanced Nutrition Shake [b]	350	20
Two cal® [b]	475	20
Perative® [b]	308	17

[a] Information retrieved from Fresenius Kabi (http://www.fresenius-kabi.co.uk/4727.htm; accessed 14/02/2017)

[b] Information retrieved from Abbott (https://abbottnutrition.com/; accessed 14/02/2017)

[c] Information retrieved from Nutricia Clinical (http://www.nutricia.co.uk/; accessed 15/02/2017)

[d] Information retrieved from Nestlé HealthCare Nutrition (https://www.nestlehealthscience.co.uk; accessed 15/02/2017)

^ 8 fl oz (US) = 237 ml

* Information retrieved from https://www.evidence.nhs.uk/formulary/bnf/current/; accessed 14/02/2017)

Table 1.4: Vitamin K content of various enteral feeds ready-to-drink according to the British National Formulary, 2017*

Vitamin K also plays a key role in anticoagulation control in children. The main sources of phylloquinone in neonates, especially preterm infants, are the prophylactic dose of phylloquinone given at birth, along with the vitamin K intake derived from either parenteral and/or enteral feeding. Breast-fed infants, due to low quantity of vitamin K in human milk, are more sensitive to the anticoagulant effect of warfarin compared to formula-fed infants. Infant formula preparations with high vitamin K content frequently administered to pre-term neonates, lead to higher warfarin dose requirements (Bonduel, 2006, Clarke P., 2015).

1.3.2.8.2.6 Dietary supplements

Vitamin K is a constituent of many multivitamin preparations. The anticoagulant effect of vitamin K antagonists is lessened even by multivitamin formulations containing small amounts of vitamin K. In relation to this, Kurnik et al. reported three cases of patients treated with warfarin who had presented with sub-therapeutic INR after taking multivitamin supplements containing vitamin K, which necessitated an increase in warfarin dose with one patient experiencing major thrombosis (Kurnik et al., 2003). One of the patients, whose warfarin requirements had increased from 45 to 60 mg per week, discontinued the multivitamin preparation without notifying her physician and subsequently experienced haematoma accompanied by an INR of 13.2 (Kurnik et al., 2003). Consequent to these observations, patients are best advised to avoid multivitamin preparations containing vitamin K.

There is evidence that vitamin E interferes with vitamin K metabolism, as demonstrated by an increase in PIVKA-II plasma concentrations (Booth et al., 2004, Heck et al., 2000). Both vitamin K and vitamin E are metabolised by CYP4F2 (cytochrome P450 isoform 4F2) enzyme, which may explain the basis for their interaction (Sontag and Parker, 2002). However, in a randomised double-blind study, vitamin E at relatively high doses (800 or 1200 IU/day) was shown to have no effect on warfarin anticoagulation (Kim and White, 1996).

Vitamin C has been reported to interact with warfarin *in vitro* (Stenton et al., 2001., Harris, 1995). The suggested mechanisms of this interaction include enzyme induction leading to enhanced warfarin metabolism, potentiation of vitamin K activity, influence in either the synthesis or the rate of catabolism of the vitamin K-dependent clotting factors, and capillary fragility (Deckert, 1973). A more recent study in rats *in vivo* demonstrated that the co-administration of vitamin C with warfarin leads to an

increase in the activity of coagulation factors II, VII, IX and X and a decrease in PT and activated partial thromboplastin time (APTT) (Khoshvaghti et al., 2011). Contrary to these findings however, it has been reported that vitamin C does not alter the anticoagulant effect of warfarin in either dogs or guinea pigs (Deckert, 1973, Weintraub and Griner, 1974). In patients on chronic warfarin therapy, we found no association between dietary vitamin C levels (2.2–95.9 µM) and warfarin dose requirement (Wynne et al., 2006).

Low dietary vitamin D intake is shown to be a risk factor for cardiovascular and thrombotic events (Khademvatani et al., 2014, Kojima et al., 2012). Vitamin D also plays an important role in the regulation of bone metabolism and in the prevention of osteomalacia and osteoporosis. There is a suggestion that vitamin D interacts with the mode of action of vitamin K. However, this remains unclear; whilst there are data indicating that vitamin D influences the vitamin K-dependent activation of osteocalcin. (Tsugawa, 2015), there are data showing that vitamin D does not affect levels of serum undercarboxylated osteocalcin, a sensitive measure of vitamin K status (Wynne et al., 2006, O'Connor et al., 2010).

The provitamin coenzyme Q10 (ubidecarenone) is a dietary supplement taken for a range of cardiovascular disorders (Pepping, 1999) and has been linked to decreased anticoagulation when taken with warfarin (Spigset, 1994). Coenzyme Q10 is structurally related to vitamin K and thus may have procoagulant effects in anticoagulated patients (Morton, 1971). There are four case reports of falls in INR values following the co-administration of coenzyme Q10 with warfarin and subsequent restoration of initial INR values after discontinuation of coenzyme Q10 (Combs et al., 1976, Landbo and Almdal, 1998, Spigset, 1994). A study in patients receiving long-term anticoagulation therapy however found that concomitant coenzyme Q10 intake (100 mg/day) had no significant effect on warfarin dose requirements (Engelsen et al., 2003). As, at the present, there are conflicting reports about the effect of coenzyme Q10 on anticoagulation response to warfarin, patients should be advised to, either avoid the concomitant use of coenzyme Q10, or for healthcare providers to perform additional INR tests when patients start or stop taking coenzyme Q10.

Fish oils contain eicosapentanoic and docosahexanoic acids, which may inhibit vitamin K-dependent coagulation factors. One case report described elevated INR values when the patient concerned had taken fish oil supplement with warfarin

(Buckley et al., 2004). However, this contrasts with the findings of an earlier placebo-controlled, randomised, double-blinded study in 16 patients receiving chronic warfarin therapy, in whom fish oil supplementation (3–6 grams/day) was shown to have no significant effect on anticoagulation control (Bender et al., 1998). In the absence of further information, patients are advised to discuss the possible interaction between fish oil supplements and warfarin with their healthcare provider.

Cambria-Kiely reported the case of a 70-year-old man with hypertriglyceridaemia who was stable on long-term warfarin (INR 2.3–2.5) before he started drinking soya milk. Shortly thereafter, his INR dropped below the therapeutic range (1.6); when he stopped taking soya milk, his INR returned to the therapeutic range (Cambria-Kiely, 2002). Interestingly, while soybeans and soybean oil contain high amounts of vitamin K (**Table 1.2, section 1.3.2.8.2.2**), soya milk contains only trace amounts of the vitamin (Cambria-Kiely, 2002). Further confirmation of the existence of an interaction between warfarin and soya milk and the possible mechanism for such interaction is needed. In the absence of further information, patients on warfarin therapy should avoid introducing soya milk into their diet.

Supplements containing L-carnitine have been found to enhance the anticoagulant effect of the vitamin K antagonist, acenocoumarol. The mechanism for such interaction is unclear. Whether L-carnitine also interacts with warfarin or phenprocoumon is unknown at this stage. Therefore, patients on vitamin K antagonists are advised to discuss with their healthcare provider the possible risks before taking L-carnitine (Martinez et al., 1993, Bachmann and Hoffmann, 2004).

1.3.2.8.2.7 Plant materials

Interactions between herbs and other plant material and warfarin are commonly reported, sometimes with serious consequences of major bleeding (Izzo and Ernst, 2009). Theoretically, any herb or plant material that may contain compounds related to coumarin or salicylate can augment the anticoagulant effect of vitamin K antagonists, through inhibition of either blood clotting or platelet aggregation (Hu et al., 2005).

Up until 2014, thirty eight different herbs had been reported in the literature with the potential to interact with warfarin. Evidence suggests that the likelihood of an interaction with warfarin is high (level I) for four of these herbs, probable (level II) for

three, possible (level III) for ten and doubtful (level IV) for twenty one (Ge et al., 2014).

Garlic, ginkgo, St. John's wort, cranberry, grapefruit, cannabis, chamomile and red clover have been identified as having major interaction with warfarin (Ge et al., 2014). Few reports have linked the use of garlic and gingko biloba with bleeding; however, there is no evidence that they directly affect coagulation. Ginger has been shown to inhibit platelet aggregation (Hu et al., 2005), which may potentiate the anticoagulant effect of warfarin. There have been case reports of an interaction between St John's wort (*Hypericum perforatum*) and vitamin K antagonists (warfarin, phenprocoumon), where the use of St John's wort resulted in the lowering of INR and unstable control of anticoagulation (Henderson et al., 2002). A single case of raised INR after concomitant use of *Angelica sinensis* (Dong quai) and warfarin has been reported (Haller, 2006). Enhanced anticoagulation and bleeding events have been reported after patients on warfarin therapy took the traditional Chinese medicine, *Salvia miltiorrhiza* (danshen) (Izzat et al., 1998, Hu et al., 2005).

There have been several reports of interaction between warfarin and cranberry juice (Hamann et al., 2011, Paeng et al., 2007, Mergenhagen and Sherman, 2008). For instance, an elderly patient on warfarin who had a poor appetite following a chest infection consumed very little except cranberry juice and was admitted to hospital 6 weeks later with an INR of >50. He died of gastrointestinal and pericardial haemorrhage (Suvarna et al., 2003). The advice of the UK Committee on Safety of Medicines is to limit the intake of cranberry juice in patients taking warfarin (Suvarna et al., 2003). The mechanism of the interaction between warfarin and cranberry juice is at present unclear. However, it has been suggested that the flavonoids present in cranberry juice, which can inhibit CYP2C9 enzyme responsible for (S)-warfarin metabolism, can explain the interaction between cranberry juice and warfarin (Beckmann-Knopp et al., 2000). It is plausible that vitamin C, also a constituent of cranberry juice, contributes to alteration in anticoagulation response to warfarin, possibly by a number of mechanisms, including alterations in warfarin metabolism, competition with vitamin K in binding to vitamin K epoxide reductase which is the target enzyme for warfarin, and the rate of loss or catabolism of the vitamin K-dependent clotting factors.

It may also be pertinent to avoid the consumption of grapefruit juice given reports of enhanced warfarin anticoagulation after consuming grapefruit juice (Bartle, 1999).

Although an earlier controlled study failed to demonstrate an interaction between warfarin and grapefruit juice (Sullivan et al., 1998), other studies have reported that grapefruit juice enhances the anticoagulant effect of warfarin by inhibiting mainly CYP3A4 and CYP2C9 enzymes involved in warfarin metabolism (Veronese et al., 2003, Guo and Yamazoe, 2004).

Green tea, which is often taken as a dietary supplement because of its reputed health benefits (including antioxidant and antibacterial properties), contains substantial amounts of vitamin K and therefore drinking large amounts may antagonise the anticoagulant effects of warfarin (Booth et al., 1993). Indeed, one report describes a warfarin-treated patient who experienced a marked decrease in INR after daily consumption of large amounts of green tea, with a subsequent INR increase when the beverage was discontinued (without a change in warfarin dosage) (Taylor and Wilt, 1999). In the absence of further information, patients receiving anticoagulation therapy are advised to discuss the consumption of green tea and its possible impact on warfarin anticoagulation response with their healthcare provider.

1.3.2.8.3 Alcohol consumption

Alcohol intake is another important factor in patients receiving warfarin, as this may increase or decrease the anticoagulant effect of coumarins. Acute alcohol intake (e.g. ingestion of a few alcoholic drinks at a single sitting) may increase anticoagulation, via decreased hepatic metabolism of warfarin (Weathermon and Crabb, 1999) or displacement of the drug from albumin with subsequent increase in unbound drug (Ha et al., 2000). Conversely, chronic consumption of alcohol may decrease the anticoagulant effect by activating cytochrome P450, thereby increasing the metabolism of warfarin (Weathermon and Crabb, 1999). However, in an earlier literature review, Wells et al. concluded that alcohol potentiated warfarin anticoagulation only if the patient had concomitant liver disease (Wells et al., 1994). A Dutch study found that although habitual alcohol consumption or heavy drinking ($\geq$6 drinks/day) did not affect anticoagulation control, a recent decrease in alcohol intake increased the risk of having an INR value $\geq$6.0 and thus increasing the risk of bleeding in the patient (Penning-van Beest et al., 2002). A case report of a patient on warfarin with a low-dose beer consumption was found to be the cause of elevated INR. The authors recommended close monitoring of INR when alcohol is taken in combination with warfarin and other concomitant medication (Havrda et al., 2005). In the absence of consistent data, it is clear that patients should either avoid alcohol or

at least avoid erratic consumption of alcohol while receiving warfarin with close INR monitoring.

1.3.2.8.4 Drug interactions

Interactions with other medications are very common for warfarin and evidence shows that NOACs also interact with some drug categories (Hankey and Eikelboom, 2011). Pharmacodynamic as well as pharmacokinetic mechanisms are responsible for these interactions, which either potentiate or inhibit the anticoagulant response.

A distinct example of common interaction of warfarin is antibiotics. Antibiotics generally displace warfarin from albumin, its carrier protein. Hence, there is greater availability of warfarin and increased anticoagulation. Antibiotics that alter gastrointestinal flora also reduce the population of bacteria producing menaquinones. In both instances, a lower warfarin dose is required (White, 2010).

Other medications inhibit or induce cytochrome P450 enzymes, such as CYP2C9. Drugs that interfere with platelet aggregation and increase the risk of bleeding or others that increase the risk of thrombosis interact with anticoagulant therapy. In their review, Holbrook et al summarise the great number of drug interactions with warfarin (Holbrook et al., 2005).

1.3.2.9 Warfarin pharmacogenetics

Warfarin dose requirements are mainly determined by the genetic predisposition in adults. Two genes, *CYP2C9* and *VKORC1*, have been identified and well described in terms of their impact on warfarin treatment in adult populations (Nowak-Gottl et al., 2010). *CYP2C9* and *VKORC1* combined with other clinical factors (age, body size, sex etc.) explain more than 50% of interindividual variability in dose requirement in a number of different patient cohorts (Sconce et al., 2005a, Caldwell et al., 2007, Wadelius et al., 2009, Carlquist et al., 2006, Gage et al., 2008 , Pirmohamed et al., 2013).

1.3.2.9.1 *CYP2C9* polymorphisms

Initially observed in 1995, single nucleotide polymorphisms (SNPs) in the coding region of the *CYP2C9* gene impact on the enzymatic activity of CYP2C9. This is clinically important because it is the main cause for variations in the metabolism of warfarin (Furuya et al., 1995, Aithal et al., 1999). *CYP2C9* gene is located on

chromosome 10, cytogenetic band q24 (NCBI a). A mutation in a region of the gene that is important for the formation of the three-dimensional structure of the protein can severely influence the activity and selectivity of the enzyme (**Figure 1.11**). Decreased capacity to metabolise S-warfarin has been demonstrated to lead to increased sensitivity to warfarin and therefore increased risk for bleeding (Kamali and Wynne, 2010).

Figure 1.11: 3D structure of CYP2C9 enzyme and S-warfarin (Adapted from https://www.ncbi.nlm.nih.gov/Structure)

The wild type allele is termed as *CYP2C9 *1*. Among the most widely studied SNPs are c.430C>T rs1799853 (*2 allele), c.1075A>C rs1057910 (*3 allele) and 818delA rs9332131 (*6 allele) (Moyer et al., 2009). The *CYP2C9*2* variant protein is only 12% as potent as the wild-type enzyme in hydroxylating S-warfarin (Rettie et al., 1994), while the *CYP2C9*3* variant enzyme demonstrates only 5% activity compared to *CYP2C9*1* (Linder, 2001).

However, one third of patients carrying wild type *CYP2C9* alleles need a lower daily dose of warfarin. Kamali et al suggested that there are several other factors affecting each patient's warfarin dose requirements (Kamali and Wynne, 2010).

In European Caucasians, frequencies of *CYP2C9*2* and *CYP2C9*3* alleles are 10% and 6%, respectively. Differences in *CYP2C9* genotype distributions among European populations are small (Sánchez-Diz et al., 2009, Cavallari and Perera, 2012). In the Ethiopian population the frequencies are much lower (94, 4 and 2% respectively) and frequencies of **2* and **3* are even lower in African Americans (2% and 1%, respectively). Other SNPs found only in African Americans are *8 and *11 (frequency of 6% and 4%, respectively) (Scordo et al., 2001, Cavallari and Perera, 2012). Interestingly, as we move from East to West Asia, the carrier rate increases for *CYP2C9* *2 and *3 alleles (Gaikwad et al., 2014). In Japanese, warfarin clearance was found to be reduced by up to 90% in *CYP2C9*3* homozygotes (Takahashi and Echizen, 2001).

1.3.2.9.2 VKOR polymorphisms

Polymorphisms in the VKOR enzyme (**Figure 1.12**), the target of warfarin, have a significant effect on warfarin dose requirement. The *VKORC1* gene was located after positional cloning on chromosome 16, cytogenetic band p11.2 (NCBI a). Two SNPs encoding complex subunit 1 of VKOR enzyme (VKORC1) have been found to affect the dose requirement for warfarin. The first variant includes the -1639G>A substitution in the promoter region and results in a reduction of the normal transcription of *VKORC1* gene to a half of it (Carlquist and Anderson, 2011), increasing the sensitivity to warfarin (Yuan et al., 2005). Interestingly, the 1173C>T variant in the intron 1 of *VKORC1* gene is in linkage disequilibrium with the -1639G>A (Kamali and Wynne, 2010). The second SNP is the 3730G>A transition on the 3′ untranslated region. The study of D'Andrea et al showed a significantly lower requirement for warfarin dose in patients being homozygotes for the variant allele in either of these two polymorphisms (D'Andrea et al., 2005).

Large variability has been observed in the prevalence of the different *VKORC1* alleles among different ethnic populations. Associated with low dose warfarin (Yuan et al., 2005), the *VKORC1* -1639 variant allele (A) is found in 90% of East Asians and as we move from East to West Asia, the carrier rate decreases (Gaikwad et al.,

2014). The frequency of the VKORC1-1639A allele is 40% in European Caucasians and 11% in African Americans (Cavallari and Perera, 2012).

Figure 1.12: 3D structure of VKORC1 enzyme (Adapted from https://www.ncbi.nlm.nih.gov/Structure)

1.3.2.9.3 Polymorphisms in other genes

Other SNPs have also been identified by recent meta-analyses to have an impact on warfarin dose requirements. A novel *CYP2C9* polymorphism (rs7089580) has been associated with higher gene expression and faster S-warfarin clearance, thus resulting in higher dose requirements in African Americans (Hernandez et al., 2015).

Additionally, warfarin daily dose requirement is altered in the presence of certain alleles in *CYP4F2* gene (p.V433M, rs2108622). A meta-analysis of thirty studies

showed that T-allele carriers required an 8.3% higher mean daily warfarin dose than CC homozygotes to achieve a stable international normalized ratio (INR). The authors concluded that, although *CYP2C9* and *VKORC1* polymorphisms remain the main genetic causes of inter-individual variability in response to warfarin, this novel *CYP4F2* polymorphism also has a low effect (Danese et al., 2012). Other studies have focused on the mechanism through which *CYP4F2* rs2108622 polymorphism affects warfarin dose (Hirai et al., 2013). *CYP4F2* gene codes for a vitamin K_1 mono-oxidase that catabolises vitamin K as shown by McDonald et al. Thus, a defective oxidase leads to elevated hepatic vitamin K levels and higher warfarin dose requirements (McDonald et al., 2009).

More recent studies have demonstrated the contribution of very rare variants in the observed inter-individual variability in warfarin response. One part of the currently unexplained variability should be attributed to rare variants that have not appeared in previous GWAS, but have been demonstrated as a result of studies with different design (exome array and two-stage extreme phenotype strategies) by Liu and colleagues (Liu et al., 2017) and Luo and colleagues (Luo et al., 2017).

1.3.2.9.4 Combined genotypes effect

Previous research has shed light to the combined effect of *CYP2C9* and *VKORC1* genotypes for calculation of the appropriate warfarin dose (Jorgensen et al., 2012). In 2007, the U.S. Food and Drug Administration (FDA) proposed that *CYP2C9* and *VKORC1* genotypes should be taken into account for dose adjustments (FDA, 2007) and in 2010 specific dosing recommendations for genotype-based dosing ranges (0.5-7mg/day) were published and have been included in warfarin's product label (**Table 1.5**) (FDA, 2010, Johnson et al., 2011).

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

Table 1.5: Ranges of expected maintenance warfarin daily dose based on *CYP2C9* and *VKORC1* genotypes (Taken from (FDA, 2010))

At least four FDA-approved analytical platforms have been available since 2010 for *CYP2C9* and *VKORC1* genotyping. While comparing them, all proved to be accurate for the common alleles with their own strengths and weaknesses (Maurice et al., 2010).

Several small studies on warfarin and acenocoumarol have concluded that carriers of a combination of *CYP2C9* and *VKORC1* polymorphisms have an increased risk for over anticoagulation compared to wild type or single variant carriers (Schalekamp et al., 2006, Tomek et al., 2013). Small pilot studies evaluating the use of pharmacogenomic information to guide initiation of warfarin therapy demonstrated benefits such as decreased frequency of warfarin dose adjustments, less time to stabilise within the target INR therapeutic range and increased time spent within the therapeutic range during initiation (Abohelaika et al., 2016).

In a meta-analysis of nine randomised controlled trials (RCTs) involving in total 2812 patients, a genotype-guided dosing strategy did not demonstrate improvement in TTR, reduction in the number of patients with an INR > 4 or a reduction in major bleeding or thromboembolic events compared with clinical dosing algorithms (Stergiopoulos and Brown, 2014). This finding was replicated in other RCTs, too (Kimmel et al., 2013, Witt et al., 2016).
More recently, the clinical response to warfarin using genetic information was compared with edoxaban as part of Effective Anticoagulation with Factor Xa Next Generation in Atrial Fibrillation–Thrombolysis in Myocardial Infarction 48 (ENGAGE AF-TIMI 48), an RCT in patients with AF. A subgroup of the warfarin-treated group, involving 4833 patients (33.7% of sample size included in the RCT), was genotyped for the common *CYP2C9* and *VKORC1* alleles and patients were categorised in bins according to the FDA guidance, thus validating the genetic binning provided in warfarin label. During the first 90 days, the risk of bleeding with warfarin was 1.3 times higher in the sensitive responders (3-4mg dose, **Table 1.5**) and 2.7 times higher in the highly sensitive (0.5-2mg dose, **Table 1.5**) compared with normal responders. Treatment with edoxaban versus warfarin reduced bleeding risk in all patients, especially so in sensitive and highly sensitive responders (Mega et al., 2015).

1.3.2.9.5 Pharmacogenetics-based dosing algorithms

The extent to which pharmacogenetics-guided dosing can improve the safety of oral anticoagulation therapy has been investigated in two large prospective multi-centre randomized clinical trials; the European trial Pharmacogenomic Approach to Coumarin Therapy (EU-PACT) and the US trial Clarification of Optimal Anticoagulation through Genetics (COAG) (Pirmohamed et al., 2013, Kimmel et al., 2013). Both studies involved patients with an indication for oral anticoagulation therapy with the primary outcome measure being the percentage TTR following the initiation of warfarin therapy.

The double-blinded COAG trial focused on warfarin and evaluated the value of a pharmacogenetics-based dosing as opposed to the single-blinded EU-PACT that studied three vitamin K antagonists in parallel in three arms (warfarin, acenocoumarol and phenprocoumon) (Pirmohamed et al., 2013). COAG study compared genotype-guided dosing versus a clinical dosing algorithm and failed to show a significant difference in the TTR (45.2% vs 45.4%, P=0.91, mean difference of 0.2%). More ethnic variation was observed in COAG (67% White, 27% Black, and 6% Hispanic) compared to EU-PACT (>98% White).

Although the EU-PACT trial found the pharmacogenetic algorithm (which also included clinical data) to be superior over a fixed loading dose regimen in significantly improving TTR (67.4% vs 60.3%, P<0.001, mean difference of 7%), the absence of blinding of clinicians and patients is questioned (Kimmel, 2015).

GM-CSF for Immunomodulation Following Trauma (GIFT) study in 2017 showed that genotype-guided warfarin dosing reduced the combined risk of major bleeding, INR of 4 or greater, venous thromboembolism or death, compared with clinically guided dosing in patients undergoing elective hip or knee arthroplasty and treated with warfarin (Gage et al., 2017).

1.3.3 *Non-vitamin K antagonist oral anticoagulants (NOACs)*

The term NOAC is widely recognized and is recommended by the ESC AF guidelines to describe the newer class of oral anticoagulants. The term DOACs is adopted in the US and both terms refer to the direct factor Xa inhibitors, rivaroxaban, apixaban and edoxaban and the direct thrombin inhibitor (DTI), dabigatran. Solely for

the purpose of consistency with the European guidelines, the term NOAC will be used throughout this thesis.

1.3.3.1 Factor Xa inhibitors

Rivaroxaban, apixaban and edoxaban are new generation oral anticoagulants, which directly target the Xa factor of the coagulation pathway (**Figure 1.13**) (NCBI b). Factor Xa is generated by both the extrinsic and intrinsic coagulation pathways. Factor Xa inhibitors are generally used as prophylaxis in patients having knee and hip replacement surgery, due to their high risk of DVT and PE (Samama, 2011). In 2011, rivaroxaban was also approved for stroke prevention in people with non-valvular atrial fibrillation (NVAF) (U.S. Food and Drug Association, 2011).
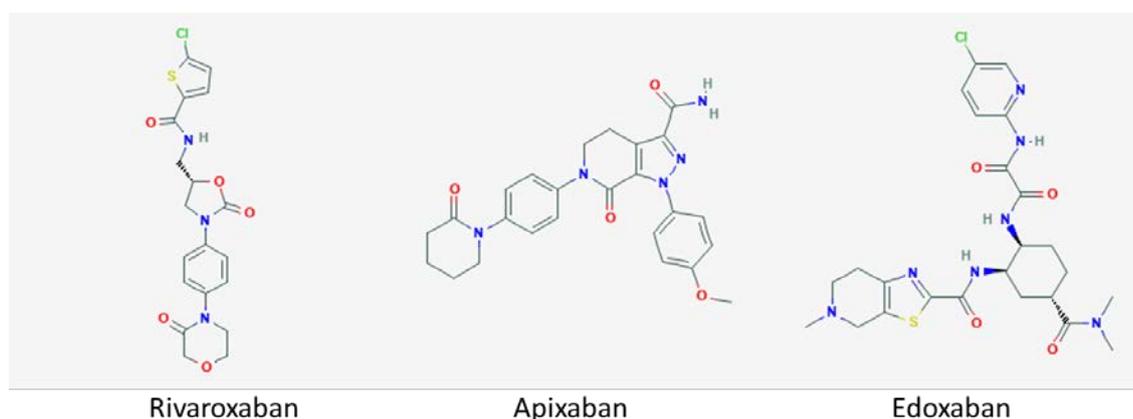
Figure 1.13: Chemical structures of factor Xa inhibitors (Adapted from PubChem database)

Three major trials that proved the non-inferiority of NOACs against warfarin led to the authorisation of factor Xa inhibitors. The Rivaroxaban Once Daily Oral Direct Factor Xa Inhibition Compared with Vitamin K Antagonism for Prevention of Stroke and Embolism Trial in Atrial Fibrillation (ROCKET AF) was a multicentre, randomized, double-blind, double-dummy, event-driven trial that was conducted at 1178 participating sites in 45 countries involving 14,264 patients. The trial compared once-daily oral rivaroxaban with dose-adjusted warfarin for the prevention of stroke and systemic embolism in patients with NVAF who were at moderate-to-high risk for stroke (Patel et al., 2011). Rivaroxaban was also examined for the treatment of DVT in the Oral, direct Factor Xa inhibitor rivaroxaban in patients with acute symptomatic

pulmonary embolism (EINSTEIN-PE) and the Oral, direct Factor Xa inhibitor rivaroxaban in patients with acute symptomatic deep vein thrombosis (EINSTEIN-DVT) trials which demonstrated non-inferior efficacy over subcutaneous enoxaparin followed by a vitamin K antagonist (either warfarin or acenocoumarol) (EINSTEIN Investigators et al., 2010, EINSTEIN–PE Investigators, 2012).

The Apixaban for Reduction in Stroke and Other Thromboembolic Events in Atrial Fibrillation (ARISTOTLE) trial compared apixaban with warfarin for the prevention of stroke or systemic embolism in patients with AF and at least one additional risk factor for stroke. It was a double-blind study which randomly assigned 18,206 patients with AF from about 40 countries in a 1:1 ratio to receive either apixaban or warfarin titrated to a target INR range (2.0-3.0) (Lopes et al., 2010, Granger et al., 2011). Apixaban for the Initial Management of Pulmonary Embolism and Deep-Vein Thrombosis as First-Line Therapy (AMPLIFY) trial showed that apixaban is non-inferior to conventional therapy with subcutaneous enoxaparin and concomitant warfarin for the acute management of patients with symptomatic VTE (Agnelli et al., 2013).

The ENGAGE AF-TIMI 48 trial was a three-group, randomized, double-blind, double-dummy trial comparing two dose regimens of edoxaban with warfarin for the prevention of stroke and systemic embolism. Patients were randomly assigned, in a 1:1:1 ratio, to receive warfarin titrated to achieve an INR of 2.0 to 3.0, or to receive high-dose (60mg) or low-dose (30mg) edoxaban. It was conducted at 1393 centres in 46 countries, involving a total of 21,026 patients (Ruff et al., 2010, Giugliano et al., 2013). Edoxaban administered once daily after initial treatment with heparin in Hokusai-VTE (Comparative Investigation of Low Molecular Weight Heparin/ Edoxaban Tosylate Versus Low Molecular Weight Heparin/ Warfarin in the Treatment of Symptomatic Deep-Vein Blood Clots) was non-inferior to warfarin for the treatment of VTE (The Hokusai-VTE Investigators, 2013).

The advantage of direct factor Xa inhibitors over the VKAs is the lack of the need for monitoring and dose adjustment. Their oral administration, their possible use at fixed dosage in the vast majority of patients, as well as their largely predictable pharmacokinetics have rapidly increased their use worldwide. They have a rapid onset and offset of action, and unlike VKAs have less interactions with food and other medications. Their main disadvantage is currently the absence of a specific reversal agent in the market, unlike dabigatran but this is going to change in early 2020, when

NICE is expected to authorise a new reversal agent for factor Xa inhibitors (NICE d, 2019). Andexanet-alfa is currently being studied and has been shown to decrease factor Xa-activity by 89% for rivaroxaban treated patients and by 95% for apixaban treated patients after a bolus dose (Sartori and Cosmi, 2018).

1.3.3.2 Pharmacokinetics of factor Xa inhibitors

Rivaroxaban has greater bioavailability with concomitant food consumption (almost 100%) than without food (66%). Apixaban is not influenced by food but has a lower bioavailability (50%), while edoxaban is only mildly influenced (6-22% increased absorption) with 62% bioavailability. Plasma drug concentration peaks at 3-4 hours for apixaban and rivaroxaban and 1-2 for edoxaban. Apixaban and edoxaban have similarly long half-life (12h and 10-14h respectively), while rivaroxaban's half-life heavily depends on age, with almost double half-life in elderly (11-13h) compared to young (5-9h) subjects. Unlike edoxaban, liver metabolism of apixaban and rivaroxaban involves the CYP3A4 enzyme with moderate contribution to their elimination. Renal and non-renal clearance is equal for edoxaban (50/50%), while for apixaban and rivaroxaban it is mostly non-renal (73% and 65% respectively) (Heidbuchel et al., 2015, Salmonson et al., 2017).

1.3.3.3 Direct thrombin inhibitors

Ximelagatran (AstraZeneca, Cambridge UK), the first direct thrombin inhibitor, was withdrawn from the market in 2006 after reported hepatotoxicity during clinical trials (**Figure 1.14**) (NCBI b, Keisu and Andersson, 2010). Two years later, in 2008, a new DTI called dabigatran etexilate was approved by the European Medicines Agency (EMA) for the prevention of thromboembolic disease following hip or knee replacement surgery. In 2010, the FDA approved it for the prevention of thromboembolism in AF, DVT and PE. Although the data presented to the authorities were much criticised for selection bias, dabigatran was investigated in the randomized evaluation of Long-Term Anticoagulant Therapy (RE-LY) trial and was found to reduce stroke or systemic embolism by 34% compared to warfarin (Albert, 2014). Dabigatran was examined for treatment of VTE against conventional treatment in Efficacy and Safety of Dabigatran Compared with Warfarin for 6 Month Treatment of Acute Symptomatic Venous Thromboembolism (RE-COVER I) and Phase III Study Testing Efficacy & Safety of Oral Dabigatran Etexilate vs Warfarin for

6 months Treatment of Acute Symptomatic Venous Thromboembolism (RE-COVER II) trials (Schulman et al., 2009, Schulman et al., 2014).

Dabigatran etexilate is a prodrug, which after oral ingestion is activated through esterase-dependent conversion into the active agent, dabigatran (**Figure 1.14**). The bioconversion occurs in the liver, portal vein and small intestine. It causes anticoagulation by directly binding to the active site of thrombin (Hankey and Eikelboom, 2011). Thrombin has a main active site and two additional exosites, which are useful for fibrin and heparin binding respectively. In the presence of dabigatran, both the unbound thrombin and the complex of thrombin-fibrin are inactivated. Hence, there is no route available for the conversion of fibrinogen into fibrin, resulting in the formation of blood clot. In contrast, after heparin administration, the fibrin-binding site is not inhibited (Hankey and Eikelboom, 2011).

Ximelagatran   Dabigatran

Figure 1.14: Chemical structures of direct thrombin inhibitors (Adapted from PubChem database)

Dabigatran is not influenced by food intake and is very poorly absorbed from the gut. Dabigatran's P-glycoprotein dependent absorption can be disrupted by P-glycoprotein inducers or inhibitors (Hankey and Eikelboom, 2011). The oral bioavailability is low, between 6 to 7%. Plasma concentration peaks at 1.5 to 2 hours with a half-life of 12-14 hours, but it increases in patients with renal impairment (Mekaj et al., 2015). All NOACs undergo varying degrees of hepatic metabolism, with much lower hepatic metabolism for dabigatran (Heidbuchel et al., 2015).

The rapid conversion by esterases, particularly liver esterase Carboxylesterase 1 (CES1) has recently been shown to be influenced by genetics. A recent genome-wide association study has brought attention to the polymorphism rs2244613 of the *CES1*. One third (32.8%) of RE-LY participants were identified as carriers of the minor allele, which was associated with lower exposure to active dabigatran metabolite. As a consequence, these patients had lower risk of bleeding due to their genetic background (Paré et al., 2013). *ABCB1* (ATP binding cassette subfamily B member 1) gene encodes p-glycoprotein and its inhibitors have been found to increase bioavailability of dabigatran (Stangier and Clemens, 2009).

1.3.3.4 Clinical indications of NOACs and dosing

The frequency of dosing and dosage with NOACs are governed by the indications the drugs are prescribed for as shown in **Table 1.6.** Dabigatran is preferred for patients with moderate renal impairment, and those with increased risk of bleeding and concomitant treatment with verapamil or amiodarone(NICE c) .

Indication	Drug and dose
Prophylaxis of venous thromboembolism following knee or hip replacement surgery	A*, R◊, D ɟ (total knee or hip replacement surgery)
Treatment of deep-vein thrombosis or pulmonary embolism	R (initialᵠ and continued◊ treatment), Aᵠ, E^, D¢
Prophylaxis of recurrent deep-vein thrombosis or pulmonary embolism	A*, R◊, E^, D¢
Prophylaxis of stroke and systemic embolism in NVAF and at least one risk factor (such as previous stroke or transient ischaemic attack, symptomatic heart failure, diabetes mellitus, hypertension, or age 75 years and over)	A**, R◊, E^, D¢

A: apixaban, R: rivaroxaban, E: edoxaban, * low dose (A=2.5mg, R= 2.5mg bid), ◊ medium dose (A=5mg, R=10/20mg od, E= mg), ᵠ high dose (A=10mg, R=15mg bid), E^ =30mg; body weight < 61kg and 60mg; body weight ≥ 61kg, D ɟ = 110mg, followed by 220mg od for adults 18-74 years and 75mg followed by 150mg after surgery for adults ≥75 years, D¢= 150mg bid (18-74y)/ 110-150mg (75-79y)/ 110mg (≥80y)

Table 1.6: Clinical indications of NOACs (Information obtained from the British National Formulary, 2019)

Contraindications for rivaroxaban mainly include a history of bleeding, previous surgery, cancer and aneurysm. Apixaban is not indicated in the presence of active bleeding or risk factors for bleeding. Edoxaban, apart from bleeding, is

contraindicated in hepatic disease, brain injury, gastrointestinal problems, cancer, aneurysms and uncontrolled hypertension. Dabigatran is not recommended in the presence of active bleeding, prosthetic heart valve, cancer, brain or ophthalmic surgery, gastrointestinal problems and vascular aneurysm (NICE c)(NICE c).

1.3.3.5 Monitoring of NOACs

The great advantage of NOACs is that routine monitoring is not necessary for establishment of anticoagulation. However, laboratory monitoring is recommended in certain cases, as presented in **Table 1.7** (Martin et al., 2016, Moll and Martin, 2018, Favaloro and Lippi, 2015, Baglin et al., 2013).

1. Unexpected bleeding or severe trauma on a NOAC
2. New thromboembolic event on a NOAC
3. Suspicion for NOAC overdose
4. Suspicion for non-adherence
5. Extreme obesity or underweight
6. Status post bariatric surgery or upper intestinal tract resection surgery
7. Concomitant therapy with a drug that may significantly affect NOAC pharmacokinetics
8. Assessment prior to major surgery in patient with recent NOAC intake or significantly impaired renal or liver function
9. Assessment of reversal of anticoagulation
10. Athlete on long-term anticoagulation wishing to engage in contact sports
11. Suspected pregnancy

Table 1.7: Possible reasons for NOAC level testing in clinical practice

No simple first-line test has proven reliable enough for defining the patient's anticoagulation status for all the oral factor Xa inhibitors. Due to the differences in the mechanism of action compared to VKAs, PT/INR test gives misleading results as this is a test for the extrinsic coagulation pathway, mainly affected by VKAs. Other assays have been approved, are commercially available and evaluate coagulation activity in patients on NOACs. Ecarin clotting time (ECT) and in particular APTT are both used to monitor dabigatran as good linear relationship exists between clotting time of

APTT for many reagents and dabigatran plasma concentration (Lippi et al., 2014, Pollack, 2016).

Anti-factor Xa (anti-Xa) assay is used to detect plasma concentration of factor Xa inhibitors (rivaroxaban, apixaban, edoxaban), but had initially been used for the determination of the abilities of unfractionated heparin (UFH) and low-molecular weight heparin (LMWH) to inhibit factor Xa (Newall, 2013, Samama, 2013). However, this assay needs to be specifically calibrated against the individual drug administered, which means calibrators, reagents and controls need to be used to calibrate and monitor coagulation analysers. This complicates the introduction of such assays in clinical practice (Lippi and Favaloro, 2015, Lippi and Favaloro, 2017).

There is still limited information as to whether plasma levels of NOACs correlate with the incidence of bleeding and thrombotic complications. As patients recruited in clinical trials were carefully selected, plasma concentrations reported do not represent a minority of patients with extreme demographics, complicated clinical characteristics and concomitant medications. Clinical trials and post-marketing studies have provided evidence of the wide range of NOAC plasma concentrations after oral dosing. According to recent studies by Reilly et al (Reilly et al., 2014) and Ruff et al (Ruff et al., 2015), trough levels of dabigatran and edoxaban have a strong correlation with haemorrhagic and thrombotic complications. **Table 1.8** summarises the plasma concentration median and ranges of all NOACs at steady-state (Cuker, 2016).

Drug	Dose	Peak concentration (ng/mL)		Trough concentration (ng/mL)	
		Median	5th to 95th percentile	Median	5th to 95th percentile
Dabigatran	150 mg BID	184	64–443	90	31–225
Rivaroxaban	20 mg daily	270	189–419	26	6–87
Apixaban	5 mg BID	171	91–321	103	41–230
Edoxaban	60 mg daily	170	120–250	22	10–40

Table 1.8: Plasma NOACs concentrations at steady-state

1.3.3.6 Bleeding risk of NOACs

Although less frequent, intracranial bleeding is the primary concern due to its devastating rate of mortality and impact on quality of life. Gastrointestinal bleeding is more frequent but less likely to be fatal. Bleeding incidence of NOACs versus warfarin are available from the four phase 3 clinical trials that led to their approval for the prevention of stroke or systemic embolism in NVAF. Compared to warfarin, dabigatran reduced major bleeding risk and had similar gastrointestinal bleeding with low-dose dabigatran (110mg bid), while it had similar major bleeding risk but increased gastrointestinal bleeding risk with high dose (150mg bid). In older patients, major bleeding risk was similar with warfarin after 110mg and higher although not significant after 150mg dabigatran. Apixaban reduced major bleeding risk but had similar gastrointestinal bleeding risk with warfarin. Edoxaban reduced major bleeding risk after both low and high doses. High dose increased gastrointestinal bleeding, in contrast with low dose edoxaban. Rivaroxaban had similar major bleeding risk. In patients with gastrointestinal bleeding history, rivaroxaban had higher gastrointestinal bleeding risk, but similar in patients without history. All NOACs reduced intracranial bleeding risk compared with warfarin (Eikelboom and Merli, 2016)

Despite their limited follow-up period (<1 year) and the potential absence of comparable groups, post-marketing surveillance studies have provided real-world figures regarding the bleeding risks associated with NOACs versus warfarin (Villines and Peacock, 2016). Dabigatran bleeding risks were consistent with the RE-LY study findings overall, with increased gastrointestinal bleeding risk being observed particularly in women aged 75-84 years and elderly aged 85 or over (Lip et al., 2016). Rivaroxaban and apixaban bleeding rates were similar to the ones reported in the ROCKET-AF trial (Tamayo et al., 2015, Peacock, 2015).

Bleeding risk associated with the use of anticoagulants has been attributed to elevated drug exposure on the basis that the latter is increased in anticoagulated patients with renal impairment. Previous studies have demonstrated that renal impairment is common in elderly populations, with a tendency to decline with increasing age (Glassock and Rule, 2012, Imai et al., 2008, Baggio et al., 2005). Dose adjustment is currently considered with dabigatran, rivaroxaban, apixaban and edoxaban, with specific dose recommendations in relation to serum creatinine levels. Age of 80 years or over has been included in the three risk factors that justify a lower dose of apixaban (unlike the other three NOACs), only when at least two risk factors

are present. Unlike the FDA, the EMA suggests using 110 mg dabigatran twice daily for individuals aged 80 years and older (Sardar et al., 2014a).

1.3.3.7 Factors affecting treatment with NOACs

In recent years, four NOACs, which selectively inhibit a single clotting factor (factor Xa or factor IIa (thrombin)) have been developed (Connolly et al., 2009, Patel et al., 2011, Granger et al., 2011, Giugliano et al., 2013) as previously mentioned in sections 1.3.3.1 and 1.3.3.3. It is generally accepted that, unlike coumarins, NOACs do not interact with dietary vitamin K. This is because of the perception that the mode of action of these agents, which exert their pharmacological activity by inhibition of a specific clotting protein, is different to that of coumarins which inhibit the recycling of vitamin K, necessary for the gamma-carboxylation of vitamin K-dependent clotting proteins.

However, since the functionalisation of both factor Xa and thrombin is inextricably linked to vitamin K, there is the possibility that the pharmacological activity of agents that inhibit these proteins could be influenced by alterations in vitamin K availability. Indeed, in an earlier study in rats vitamin K deficiency significantly enhanced the anticoagulation activity of the direct thrombin inhibitor, ximelagatran. Briefly, the anticoagulant activity of ximelagatran (20 mmol/kg, twice daily for 8 days) was significantly greater in rats on vitamin K deficient diet (12.3-fold for PT, 5.1-fold for APTT, and 1.6-fold for ECT) compared to those on normal diet. Factor II activity was reduced to 58% by ximelagatran in rats on normal diet. However, factor II activity was virtually abolished (<1%) by the drug in rats on vitamin K deficient diet (Kamali et al., 2009). Whether dietary vitamin K influences anticoagulation response to NOACs in man remains to be investigated.

Genetic influence on NOAC exposure has been investigated and a number of genetic polymorphisms have been previously identified. Only one genome wide association study (GWAS) has been performed with patients from the RE-LY trial for dabigatran and identified three single SNPs; rs2244613 and rs8192935 in the liver esterase *CES1* gene involved in the rapid conversion of dabigatran etexilate to the active metabolite and rs4148738 in the *ABCB1* gene which codes for P-glycoprotein (P-gp) transporter. ENGAGE-TIMI 48 trial showed no influence of common *VKORC1* and *CYP2C9* polymorphisms on edoxaban pharmacokinetics. No GWAS data are currently available for either rivaroxaban or apixaban (Mega et al., 2015, Vandell et

al., 2017, Paré et al., 2013) . Smaller studies have demonstrated that rivaroxaban and apixaban are influenced by SNPs at *ABCB1* gene (**Figure 1.15**).
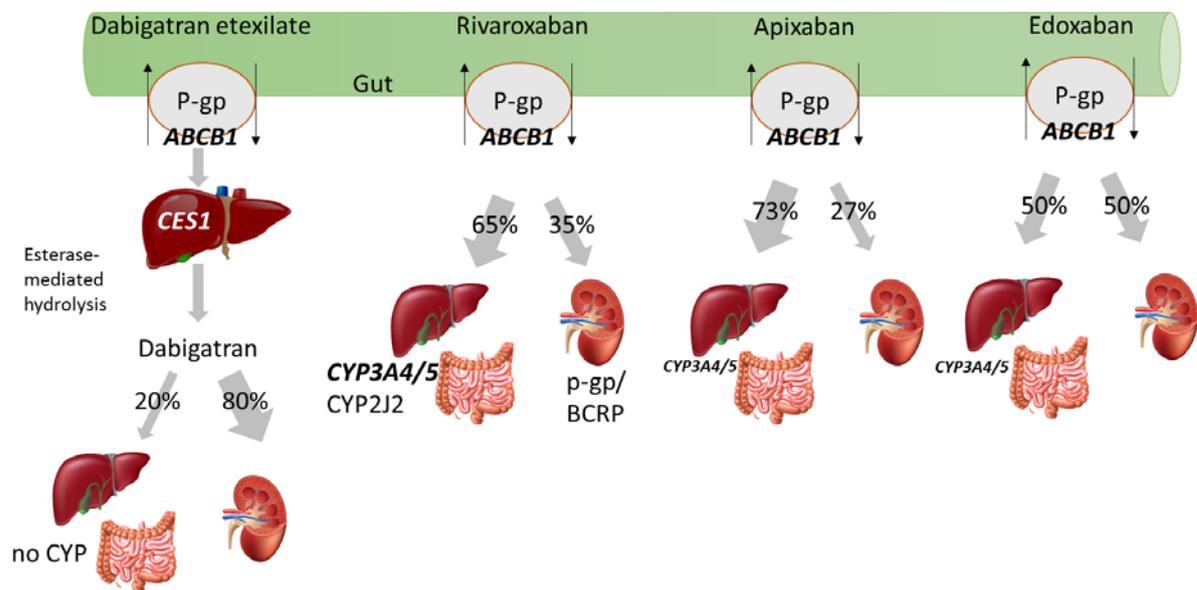
Figure 1.15: Metabolism and genetic influence of NOACs

1.3.3.8 Drug interactions

After absorption in the gut, all NOACs undergo significant gastrointestinal re-secretion by a P-gp transporter, which is also involved in renal clearance (Mekaj et al., 2015). Hence, increased plasma levels will result from competitive P-gp inhibition by drugs commonly prescribed in AF patients (e.g. amiodarone, verapamil, quinidine and dronedarone). Conversely, strong inducers of P-gp reduce NOAC plasma levels, leaving patients at significant risk of thromboembolism. Dabigatran's absorption can be disrupted by both P-glycoprotein inducers or inhibitors (Hankey and Eikelboom, 2011).

Strong CYP3A4 inhibition or induction may affect plasma concentrations and should be evaluated. CYP3A4/3A5 enzymes are involved in the hepatic clearance of rivaroxaban (18%), apixaban (25%) and edoxaban (<4%) (Steffel et al., 2018, Mueck et al., 2014). Strong CYP3A4/3A5 inhibitors like ketoconazole and ritonavir led to 70% and 50% mean inhibition of non-renal (metabolic) clearance of rivaroxaban respectively, while a 44% and more than 80% mean inhibition of active renal

secretion was observed. Apixaban's non-metabolic clearance is diverse, which reduces the potential for drug–drug interaction (Wang et al., 2010).

In general, NOAC use is not recommended in combination with drugs that are strong inhibitors of both CYP3A4/3A5 and P-gp. Combinations with strong inhibitors should be used with great caution or avoided where possible (Steffel et al., 2018).

1.3.3.9 Adverse effects of NOACs

Apart from the increased incidence of bleeds and bruises, NOACs more frequently cause non-bleeding gastrointestinal disorders as well as severe rare toxic liver disease (Maura et al., 2018).

Gastrointestinal complications, other than bleeding, can significantly affect the patient's quality of life leading to reduced compliance or even discontinuation (Beyer-Westendorf et al., 2016, Al-Khalili et al., 2016, Beyer-Westendorf et al., 2015). NOAC therapy is associated with the initiation of drugs for functional gastrointestinal disorders excluding drugs for acid-related disorders. Non-bleeding gastrointestinal disorders have previously been reported to be uncommon (Chan et al., 2016a). Nonetheless, dyspepsia (5-10%) and esophagitis are now increasingly recognized complications of dabigatran (Zhang et al., 2016, Heidbuchel et al., 2015) and rivaroxaban (Cox et al., 2016). Pyrexia, nausea, constipation and vomiting were commonly reported non-bleeding adverse events in rivaroxaban-treated patients (Duggan, 2012). In addition to these, another study that used Danish registries showed an association between NOACs and acute renal failure (Hellfritzsch et al., 2018).

Patients with acute liver disease were excluded from the landmark NOAC randomised controlled trials. However, short-term elevation of transaminase enzymes was reported during these trials (Connolly et al., 2009, Granger et al., 2011, Patel et al., 2011). Although according to a meta-analysis of clinical trials, NOACs were not associated with an increased risk of drug-induced liver injury (DILI) (Caldeira et al., 2014), a range of post-marketing studies have showed that NOACs can cause DILI (Raschi et al., 2016, Rochwerg et al., 2012, Liakoni et al., 2015, Liakoni et al., 2014, Cordeanu et al., 2016, Raschi et al., 2015) with dabigatran new users having the lowest risk compared to factor Xa inhibitors. Close monitoring of liver function after initiation of NOAC treatment is therefore necessary, especially in the presence of

pre-existing liver conditions or concomitant intake of hepatotoxic agents (Alonso et al., 2017).

Generally, hypersensitivity reactions and hair loss are rare. Rivaroxaban causes immunological side effects at a rate of 0.1–1% and few cases with hair loss have been reported (Christopoulou et al., 2017). Cases of skin disorders have also been reported for patients treated with dabigatran and apixaban (Cakmak et al., 2014, NICE c). Reversible neurological reactions have been reported for apixaban (Josyln et al., 2018).

Overall, further studies, hospitalisation data and registry research are needed to investigate the prevalence and impact of adverse events in NOAC-treated patients (Maura et al., 2018).

1.3.3.10   Reversal of NOACs

Despite the great problems they cause and the narrow therapeutic range, VKAs are superior in terms of experience in clinical practice. For most NOACs there is no specific antidote currently available for the reversal of their action in case of over-anticoagulation despite having a less serious bleeding risk than VKAs.

According to the European Heart Rhythm Association Survey in case of a major bleeding from NOACs, 77.4% of centres would use a PCC or activated recombinant factor VII (Larsen et al., 2015). FFP is not recommended as a reversal strategy and is instead considered a plasma expander.

The use of activated charcoal short after ingestion as well as haemodialysis were two options for dabigatran's reversal, with the recent addition of idarucizumab (Praxbind, Boehringer Ingelheim, Germany) in 2015 as a result of the phase 3 multicenter, prospective Reversal Effects of Idarucizumab on Active Dabigatran (RE-VERSE AD) trial (Burness, 2015, Crowther and Crowther, 2015). It is the first NOAC antidote approved by the EMA and the FDA with proven effectiveness in rapid and safe reversal of the anticoagulant activity of dabigatran in the emergency setting. This specific human monoclonal antibody fragment binds dabigatran with an affinity that is 350 times higher than thrombin (Pollack et al., 2015).

For FXa inhibitors, PCC is an anticoagulation reversal strategy. There is lack of evidence about the use of FFP in NOAC-related bleeding (Amish et al., 2017). Andexanet alfa is a recombinant protein with similar structure and high affinity

binding to FXa which has been investigated as a specific antidote for the FXa inhibitors. Andexanet alfa has been shown to reverse the anticoagulant activity of rivaroxaban and apixaban in older healthy subjects. The Novel Antidote to the Anticoagulation Effects of FXA Inhibitors (ANNEXA-4) trial has found a significant reduction in the anticoagulant effect of FXa inhibitors and an effective hemostasis in 79% of the patients with major bleeding treated with andexanet alfa. To date, andexanet alfa is awaiting approval both in the U.S. and in Europe (Connolly et al., 2016, Kaatz et al., 2017, Di Fusco et al., 2018).

A third antidote, called ciraparantag, is a small molecule. It is currently in development with promising evidence showing that it binds direct factor Xa and IIa inhibitors. Available data seem to confirm that it can be considered as a universal reversal agent due to its ability to inhibit the anticoagulant activity of FXa, FIIa, fondaparinux, unfractionated and low-molecular-weight heparins but not VKAs (Milling and Kaatz, 2016).

It can be safely concluded that a specific reversal agent for each NOAC will be available soon. However, due to the risk of a rebound effect which may lead to thromboembolic events, their use should be restricted to ongoing NOAC-induced uncontrolled or life-threatening haemorrhages and in cases of urgent or emergent surgery or invasive procedures associated with a high bleeding risk (Di Fusco et al., 2018).

1.4 Epidemiology of anticoagulant use

VKAs, notably warfarin, has been the only available oral anticoagulant drug for the primary and secondary prevention of venous and arterial thromboembolic events, used by millions of patients worldwide for the past six decades. In 2010, non-vitamin K oral anticoagulants were introduced which have since had dramatic effects on warfarin prescribing. In general, the use of oral anticoagulants have been shown to increase between 2008 and 2014, with the proportion of AF patients receiving oral anticoagulants increasing from 52.4% to 60.7%, NOAC use increasing to 25.8% since their marketing in 2010, while warfarin use decreased from 52.4% to 34.8% (Marzec et al., 2017). In 2014, the most commonly prescribed NOAC for AF was rivaroxaban (47.9% of clinic visits), followed by apixaban (26.5%) and dabigatran (25.5%) (Barnes et al., 2015).

According to the European Heart Rhythm Association Survey conducted in February 2015, in the case of AF, NOACs were used equally (48.5%) or more frequently (33.3%) than VKAs in 38 responding centres (Larsen et al., 2015).

1.5 Anticoagulation in paediatrics

For several years, oral anticoagulation management focused on adult populations that differ in ethnicity, genetic background and medical conditions. A different set of factors influence anticoagulation therapy in paediatric populations making extrapolation from adults unsuccessful at times. This soon led to studies investigating differences in anticoagulation management in both neonates and paediatric patients.

1.5.1 *Physiological differences*

There is a wide age range in children requiring oral anticoagulant treatment (from infants to teenagers) and there is no standardised maintenance dose according to patient age.

Compared to adults, younger children on warfarin have difficulty in swallowing tablets. A limitation in the number of times a tablet can be cut (no more than two) has been recommended (Bonduel, 2006). Recently, in order to overcome the difficulties associated with oral dosing in children and to increase treatment compliance, subcutaneous formulations for LMWH have become available, when oral administration is impossible (Desai and Farrington, 2000).

Differences in other physiological functions among children, such as higher resting heart rate, lower susceptibility of arrhythmias and thromboembolic episodes, emphasise the need for studies evaluating the efficacy and safety of oral anticoagulation therapy in children. Poor absorption and enhanced clearance of warfarin in children have been reported, as well as poor venous access. Frequent infections, therefore increased use of antibiotics, along with higher level of activity and increased possibilities of trauma compared to adults, place children in a more demanding position for proper control of anticoagulation therapy (Desai and Farrington, 2000)].

1.5.2 *Choice of anticoagulant*

Although there are three FDA-approved drugs with paediatric use information (UFH, warfarin and argatroban), the 40-year experience of warfarin use in children

offers clinicians the confidence for its safe use in this patient population (Law C, 2015). On the other hand, as there is limited experience with the use of NOACs in children (Biss and Kamali, 2012), there are currently no recommendations for use of these agents in paediatrics. There are a number of ongoing large multicentre studies currently looking at efficacy. The safety, pharmacokinetics and pharmacodynamics of dabigatran etexilate in adolescents with VTE has previously shown similar PK/PD data to that of adults and good tolerability (Halton et al., 2016).

Well-designed and controlled clinical trials with warfarin have not been conducted in any paediatric population. The current guidelines on the use of oral anticoagulants in children are based upon information extrapolated from clinical studies in adults with slight adjustments guided by studies identifying the differences between adults and children (Law C, 2015, Monagle et al., 2012). No specific recommendations or guidelines have been published by FDA about paediatric population taking warfarin, apart from avoiding any sports activities which may result in injury (FDA, 2011).

1.5.3 *Factors affecting response to warfarin in children*

1.5.3.1 Age

At birth, the coagulation system is immature but dynamic. Infants are generally less susceptible to clots than adults, while being at very high risk of thrombotic and bleeding complications following major haemostatic alterations. The imbalance between pro and anti-thrombotic states observed in the neonatal coagulation system might explain these observations. Both coagulation and haemostatic systems slowly develop over time as a neonate grows, therefore there being significant differences between neonates, older children and adults. Levels of vitamin K-dependent factors II, VII, IX, X and factors XI, XII, pre-kallikrein, high molecular weight kininogen are 70% less than those of adults, while levels of antithrombin, proteins S and C activity are about 20–60% lower in neonates compared with adult levels (Monagle et al., 2006, Andrew et al., 1987). In addition, hepatic immaturity is observed, which results in younger children being more likely to have significantly lower hepatic CYP enzymes expressed (Hakkola et al., 1998). At the age of 6 months, the haemostatic system shows resemblance to that of an adult (Desai and Farrington, 2000).

Age is inversely related to warfarin dose, therefore infants generally receive the highest milligram per kilogram warfarin dose, while adolescents the lowest dose in order to maintain therapeutic anticoagulation.

1.5.3.2 Diet

Diet in infancy plays a key role in anticoagulation management in children. Newborns frequently suffer from VKDB, especially those exclusively breast-fed (Greer, 2001) (Shearer, 2009a). Geer and colleagues showed that plasma phylloquinone concentrations of formula-fed infants were much greater compared with breast-fed infants or adults (Greer et al., 1991). Breast-fed infants, due to low quantity of vitamin K in human milk, are more sensitive to warfarin's anticoagulant effect compared to formula-fed infants. Infant formula preparations with high vitamin K content frequently administered to pre-term neonates, lead to higher warfarin dose requirements (Bonduel, 2006, Clarke P., 2015)

It is recommended that an intramuscular dose of phylloquinone be administered to all newborns, due to the life-threatening consequences of VKDB (American Academy of Pediatrics Committee on Fetus and Newborn, 2003). Due to the very low vitamin K status of neonates, oral anticoagulants are avoided to reduce the risk of bleeding (Bonduel, 2006).

1.5.4 *Indication*

Indications for anticoagulation in children include the prevention or treatment of venous and/or arterial thrombosis, mechanical prosthetic heart valves, central venous access devices (CVAD), endovascular stents and Fontan operation (Bonduel, 2006).

Monagle et al reported that a multicenter international RCT of primary prophylactic anticoagulation after Fontan procedure found no significant difference between 5 mg/kg/day aspirin and warfarin (started within 24 h of heparin lead-in; target INR 2.0-3.0) as primary thromboprophylaxis in the first 2 years (Monagle et al., 2011).

Indication influences warfarin dose requirements in children (Biss and Kamali, 2012). Following Fontan procedure, children receive a lower dose than those who are anticoagulated for other indications, as they present increased sensitivity in warfarin (Kaulitz et al., 1997, Streif W, 1999).

1.5.4.1 Genetics

The same *CYP2C9* and *VKORC1* genotypes that have been studied in adults, have also been investigated in children populations. Despite studies showing that *VKORC1* and *CYP2C9* genotype accounted for only 4% of variability in warfarin dose (3.7% and 0.4% respectively), further larger studies have confirmed a similar influence of genotype to that seen in adult dosing (Nowak-Gottl et al., 2010). In a cohort of 120 anticoagulated children, 73% of variability in warfarin dose could be accounted for by patient height (30%), *VKORC1* (27%) and *CYP2C9* genotype (13%) and indication for warfarin therapy (3% lower dose for Fontan procedure) (Biss et al., 2012). The finding was replicated in another study which found target INR to be a significant determinant instead of indication (Moreau et al., 2012).

1.5.5 *Warfarin dosing and anticoagulation control*

There is wide interindividual variability in warfarin dose requirement in children. Current dosing recommendations are based on age and weight (Nowak-Gottl et al., 2010, Desai and Farrington, 2000, Biss and Kamali, 2012), however recent studies have highlighted the importance of genetic polymorphisms in *VKORC1* and *CYP2C9* (Kato et al., 2011) A number of studies have showed that age and genetic variants account for between 38% and 82% of warfarin dose variability (Vear et al., 2014, Moreau et al., 2012, Nguyen et al., 2013).

Vitamin K intake, serious underlying illness and use of other medication are considered in dose calculations, as well (Law C, 2015). Current recommended dosing regimens for children younger than 1 year old are based on body size, mainly represented by weight. Also, dose requirement in younger children is higher due to physiological and dietary differences influencing warfarin's effect. Because of these changes, more frequent determinations of INR are recommended to adjust the dose with increasing age (Desai and Farrington, 2000).

The starting dose of VKA is calculated based on patient weight and varies between 0.33 mg/kg in infant and 0.09 mg/kg in teenage to maintain an INR of 2.0-3.0 (Streif et al., 1999), except in prosthetic mechanical heart valves and recurrent thrombotic episodes where target INR is 2.5-3.5. (Bonduel, 2006). INR checks to monitor warfarin are performed once or 4 times monthly according to the age, dose change, concomitant medication and patient compliance (Andrew et al., 1994). INR

monitoring with point-of-care monitors should be made available where resources make this possible (Monagle et al., 2012).

Most common adverse events encountered with VKA in children are bleeding, skin necrosis and osteoporosis (Rezaieyazdi et al., 2009 , Gatti et al., 2003, Streif et al., 1999). Vitamin K, FFP or PCC may be administered to reverse bleeding (Bonduel, 2006). According to a recent review from Von Vajna and colleagues, a number of studies are currently ongoing to evaluate the use of NOACs in paediatrics (von Vajna et al., 2016). In vitro phase I studies have been completed assessing oral rivaroxaban and dabigatran, while the only apixaban study was terminated early with no published results. Pharmacodynamics and pharmacokinetics of rivaroxaban were similar among different age groups, however adult dosing may not be appropriate for children. Reduced concentrations were found in serum for children less than 40kg of weight (Willmann et al., 2014, Attard et al., 2012). Upon completion, further studies will inform about appropriate paediatric dosing and response to NOACs.

1.6   Aims of my PhD research

Identification of the factors which influence clinical response to oral anticoagulants could lead to improvements in patient management and treatment safety. The aim of my research was to investigate the impact of several factors on patient anticoagulation response to warfarin in paediatric and adult populations and the impact of ageing on the response to two factor Xa inhibitors *ex-vivo* in adult populations.

More specifically, the projects I undertook aimed to explore:

- the performance of a "tailored" vitamin K dosing regimen for the reversal of INR in over-anticoagulated patients at risk of bleeding,
- the potential influence of *CYP2C9* and *VKORC1* genotypes on anticoagulation stability during warfarin induction and maintenance warfarin therapy,
- the effect of *CYP2C9* and *VKORC1* genotypes on the rate of INR fall following warfarin cessation in patients awaiting surgery in order to validate a pharmacogenetics-based algorithm,
- the association between *CYP2C9, VKORC1 and CYP4F2* genotype and plasma vitamin K concentration in children treated with warfarin and any implication this has on development,
- the use of uncarboxylated matrix-Gla protein as a marker of vascular calcification to assess vascular health in children on warfarin.
- the effect of ageing on the pharmacological activity of the factor Xa inhibitors, rivaroxaban and apixaban.

Chapter 2   Reversal of INR using a "tailored" vitamin K dosing regimen in over-anticoagulated adult patients at risk of bleeding

2.1 Introduction

Despite improvements in the management of warfarin therapy, 50% of patients fail to stabilise within their target INR range and are thus prone to the drug's adverse effect of bleeding (Watson et al., 2001). Subgroup analyses of several studies show a sharp increase in the risk of bleeding when the INR exceeds the therapeutic range and increased risk of thrombosis when the INR falls below it (Fihn et al., 1996, No author, 1996a, No author, 1996b). The annual frequency of major bleeding events associated with excessive anticoagulation with warfarin ranges from 2.0 to 3.5% (No author, 1994, Lip et al., 2011), with the absolute risk of thrombosis associated with overcorrection being in the same range (Hanslik and Prinseau, 2004).

The current guidelines for treating over-anticoagulated patients advocate restoration of the INR to the target range quickly in order to prevent bleeding whilst avoiding over-correction which increases the patient's risk of thromboembolism. Apart from warfarin discontinuation, non-urgent correction of over-anticoagulation is achieved through oral vitamin K administration (Makris and Watson, 2001). Although oral vitamin K is effective in lowering INR in over-anticoagulated patients (Watson et al., 2001, Crowther et al., 2000), the currently used fixed-dose regimens do not adequately allow for differences in individual patient INR values. Whilst in excessively anticoagulated patients a particular dose may fail to sufficiently lower the INR, it can lead to over-correction in those who are only mildly over-anticoagulated (Sconce and Kamali, 2006, Denas et al., 2009).

In an earlier pilot study, we demonstrated that individualized vitamin K dosing, based upon index INR, resulted in a greater proportion of over-anticoagulated patients having INR 1.8-4.0 at 24h compared to data held in clinic on patients receiving a fixed-dose regimen (71% v 39%; p<0.001) (Briz et al., 2010). The data generated from this pilot study helped us to develop a new improved individualized vitamin K dosing algorithm, based upon index INR and body surface area (BSA), which was estimated to have a greater power of accuracy in computing vitamin K dose for lowering INR to within therapeutic target range in over-anticoagulated patients than the original dosing algorithm (Briz et al., 2010).

2.2 Aim of the study

The aim of this study was to assess the performance of the improved 'tailored' vitamin K dosing regimen against a fixed-dose regimen currently used in our local hospitals for the reversal of warfarin-induced over-anticoagulation in a randomized controlled clinical trial. As part of the trial, I also investigated retrospectively the potential impact of patient factors, as well as genetic factors which influence warfarin pharmacology and metabolism and vitamin K disposition, on the extent of INR reversal.

2.3 Patients, materials and methods

2.3.1 *Sample size calculation*

Based on our clinic data for a 12-month period, just over 30% of non-urgent cases over-anticoagulated with warfarin achieved an INR within their target range 24 hours after oral vitamin K_1 (1 or 2 mg p.o.) administration. To detect a 20% increase (i.e. from 30 to 50%) in the proportion of over-anticoagulated patients achieving their target INR 24 hours after vitamin K administration using the new tailored dosing regimen, with 80% power and at the 5% significance level, required 93 patients in each of the two study groups.

2.3.2 *Study subjects*

This single-centre, open-label, randomized controlled study had approval by the National Research Ethics Service-Committee North East, Newcastle & North Tyneside (ref: 11/NE/0249). The trial was registered with the ISRCTN Registry under the name 'Reversal of over-anticoagulation with vitamin K' (trial registration no: ISRCTN79841657).

Over-anticoagulated patients presenting to the anticoagulant clinics at the Royal Victoria Infirmary and the Freeman Hospital, Newcastle upon Tyne were recruited into the study. Patients on warfarin therapy (irrespective of the duration) referred to our clinics with supra-therapeutic INR and either asymptomatic or with minor bleeds, according to the classification of Fihn et al (Fihn et al., 1996), were eligible to take part. As in our earlier pilot study, only patients with a confirmed venous INR ≥6.0 were eligible, based on evidence that such patients are at significant risk of imminent harm, with a recorded risk of 8.8% for abnormal bleeding and 4.4% for a major bleed

in the following 14 days compared to no bleeds occurring in a control population within target INR range over the same period (Hylek et al., 2000). Patients were excluded if they met one or more of the following criteria: younger than 18 years of age, life expectancy less than 10 days, presence of major bleeding requiring immediate and complete correction of anticoagulation, a history of thromboembolic or major haemorrhagic event in the previous 3 months, known sensitivity to vitamin K, liver disease, indication for acute normalisation of INR (such as imminent surgery), scheduled discontinuation of warfarin therapy, receiving thrombolytic therapy within 48 hours, a platelet count less than 50×10^9 cells/L, unable to return to the clinic the next day for laboratory/clinical assessment.

The primary outcome was the proportion of patients reaching to within ± 0.2 units of their absolute therapeutic INR range (reported as 'within target range') 24 hours after receiving oral vitamin K in the 'tailored dose' group compared to the 'control' group. An INR value of >0.2 units below or above the individual target INR range at 24 hours was considered as over- and under-correction of over-anticoagulation, respectively, as these ranges (according to our local practice) are deemed to be clinically relevant thus prompting the personnel in charge of patient anticoagulation management to consider the possible need for intervention.

Secondary outcomes included the need for repeated doses of vitamin K_1, the need for heparin administration and the presence of any thromboembolic and haemorrhagic events during the study period.

2.3.3 *Sample preparation*

On presentation, after confirmation of eligibility and having obtained informed consent, 5ml venous blood was taken from each patient and transferred into EDTA tubes for later DNA extraction and genotyping for the *VKORC1*, *CYP2C9* and *CYP4F2* genes. Information on patient age, sex, body weight, height, co-morbidities and concurrent therapy was collected. Possible reasons for over-anticoagulation were ascertained (e.g. increased alcohol consumption, intercurrent illness, change in medication and change in dietary habits).

Patients were allocated using block randomisation (block size=10) to two groups ('control' and 'tailored dose') to receive oral vitamin K_1 (phytomenadione; Konakion®, Roche). The control group received vitamin K_1, either 1 or 2 mg p.o. based on index INR and bleeding risk according to the current guidelines in place in the Northern

Region Hospitals for reversal of non-urgent over-anticoagulation (Hanley, 2004, Keeling et al., 2011). The vitamin K_1 dose for those patients who were randomised to the 'tailored dose' arm was calculated in the clinic using a simple calculator generated within a Microsoft® Excel file which employed the following formula: Vitamin K_1 dose (mg) = (0.247 x index INR on entry- target INR -0.1320+1.417 x BSA (m^2))/1.0135 (Briz et al., 2010). This algorithm was developed by regressing the target INR to index INR, BSA and vitamin K dose received. Body surface area was calculated using the Du Bois formula: BSA = [0.007184 x height $(cm)^{0.725}$ x weight $(kg)^{0.425}$] (Du Bois and Du Bois, 1916).

A further venous sample for INR determination was taken at 24 (+/- 3) hours. The INR check was performed by personnel blinded to the study treatment, thus preserving the study blinding. The individual patient's warfarin therapy was re-started 24 hours after vitamin K ingestion, as instructed by the responsible physician/ pharmacist, based on the 4S DAWN AC dosing program (Poller et al., 1998), providing that the patient was no longer over-anticoagulated.

The need for additional doses of vitamin K at ≥24h was considered if the patient's INR was >6.0 outside of the study following the local guidelines (Keeling et al., 2016). The need for LMWH administration at ≥24h was considered if the patient's INR was sub-therapeutic and he/she was clinically adjudged to be at significant risk of thrombosis. The decision for bridging with LMWH in all patients with sub-therapeutic INR results was made following an assessment of the patient's thrombotic risk according to the following clinical scenarios: (a) history of previous VTE whilst INR was within therapeutic range with subsequent re-assignment to a higher target INR of 3.5; (b) mechanical heart valve (other than a bileaflet aortic valve) (Keeling et al., 2016). LMWH administration was also considered if INR value was below the lower limit of the therapeutic range by ≥0.3 unit.

Patients were followed-up at two further clinic visits (day 7 (+/-2 days) & day 14 (+/-2 days)) to check for anticoagulation control and possible adverse effects of thromboembolism or haemorrhage using standard proforma of questions. Further observational information on thromboembolism/bleeding events was collected from patients' notes up to 30 days after the administration of the first dose of vitamin K. Causality assessment of adverse drug reactions was performed by the clinicians monitoring the study subjects.

The study protocol was amended later during the trial to make the visits on days 7 and 14 optional for patients in an attempt to boost recruitment. The amendment also involved inclusion of patients with repeated admissions to clinic for excessive anticoagulation. We allowed for double inclusion of patients with supra-therapeutic INR provided that there was sufficient length of time (minimum 30 days) between each episode, which would not bias the conclusions of the study.

2.3.4 *Genotyping assay*

Leukocyte DNA was extracted from blood samples according to an established in-house method (Daly et al., 1996). Genotyping for *VKORC1* (-1639G>A; rs9923231), *CYP2C9* (*2 and *3 alleles; rs1799853 and rs1057910) and *CYP4F2* (V433M; rs2108622) was performed using the StepOneTM Real-Time Polymerase Chain Reaction (PCR) System with TaqMan® SNP Genotyping Assays, Applied BiosystemsTM. Genotype results for all polymorphisms were validated using control samples that had been genotyped as part of a previous study (Biss et al., 2012)

2.3.5 *Statistical analysis*

Statistical analyses were performed using Minitab 17.0 (Coventry, UK). Data were checked for normality. Where data were not normally distributed, logarithmic transformation was applied in order to achieve normality. Two sample t-tests were used if approximate normality was achieved. Otherwise, Mann-Whitney U-tests were used. Chi-squared tests were performed for comparing categorical data across groups. Pearson correlation test was used to evaluate relationships between variables. Stepwise regression analysis of data was used to evaluate the impact of patient characteristics (age, weight, height) and genetic polymorphisms in *VKORC1*, *CYP2C9* and *CYP4F2* on INR reversal. Results are presented as mean ± SD (range) unless otherwise stated. A p value of <0.05 was taken as statistically significant.

2.4 Results

Between June 2012 and February 2016, 186 patients (10 repeat cases) were recruited into the study. Five patients were later withdrawn; 3 due to administrative problems associated with screening, one lost to follow-up and one failed screening. Of the remaining 181 cases, 91 (including 2 repeat cases) were allocated to the

control group and 90 (including 6 repeat cases) to the tailored dose group. (**Figure 2.1**)

CONSORT 2010 Flow Diagram

```
Enrolment          Assessed for eligibility (n=186)

                                    Excluded (n=5)
                                    ♦ Not meeting inclusion criteria (n=1)
                                    ♦ Lost to follow-up before day 1 (n=1)
                                    ♦ Administrative reasons (n=3)

                   Randomized (n=181)

Allocation

Allocated to fixed-dose vitamin K regimen, n=91        Allocated to tailored-dose vitamin K regimen, n=90

♦ Received allocated intervention on day 1, n=91        ♦ Received allocated intervention on day 1, n=90

Follow-Up

Lost to follow-up after day 1 (n=14)                    Lost to follow-up after day 1, n=19
Lost to follow-up after day 7 (n=5)                     Lost to follow-up after day 7, n=8

Analysis

Analysed for day 1, n=91                                Analysed for day 1, n=90
Analysed for day 7, n=77                                Analysed for day 7, n=71
Analysed for day 14, n=72                               Analysed for day 14, n=63
```

Figure 2.1: CONSORT flow diagram of the progress through the phases of a parallel randomised trial of two groups

The two groups were well matched with respect to demographic and clinical characteristics (**Table 2.1**). Index INR on presentation for the whole study population was 8.0 ± 2.0 (5.3-21.8). Patients included in the study had different target INR ranges based on the indication for which they were being treated with warfarin.

	Total	Control	Tailored	p-value / 95%CI*
Sample size, n	*181*	91	90	
Age (years), mean ± SD (range)	71 ± 14 (19-94)	72 ± 14 (19-92)	71 ± 15 (22-94)	(-3.26, 5.26)
Sex, n (%)				
Female	96 (53)	50 (55)	46 (51)	0.605
Male	85 (47)	41 (45)	44 (49)	
Weight, mean ± SD (range) ⱡ	75.8 ± 20.7 (37.0-163.0)	77.0 ± 19.8 (43.0-130.9)	74.6 ± 21.5 (37.0-163.0)	(-3.66, 8.46)
BSA, mean ± SD (range) ⱡ	1.81±0.27 (1.21-2.81)	1.82±0.25 (1.30-2.39)	1.81±0.29 (1.21-2.81)	(-0.07, 0.09)
Indication for anticoagulation, n (%)				
Atrial fibrillation	81 (44.75)	40 (43.9)	41 (45.6)	0.878
Venous thromboembolism	29 (16)	17 (18.7)	12 (13.3)	
Heart Valve replacement	26 (14.45)	13 (14.3)	13 (14.4)	
Pulmonary embolism	18 (9.9)	9 (9.9)	9 (10)	
Other	27 (14.9)	12 (13.2)	15 (16.7)	
Target INR range, n (%) ◊				
2.0-3.0	145 (80.2)	70 (76.9)	75 (83.3)	0.338
2.5-3.5	18 (9.9)	9 (9.9)	9 (10)	
3.0-4.0	18 (9.9)	12 (13.2)	6 (6.7)	
Frequency of concomitant medications, n ¥				
No interaction		131	145	0.579
Minor interaction		19	14	
Moderate interaction		35	38	
Major interaction		17	13	

* p-value reported for chi-square test and 95% confidence interval for Student's t-test
ⱡ Based on data for 175 patients
◊ Based on data for 180 patients
¥ According to the degree of interaction with warfarin and based on the 'Drug Interactions Checker' featured on Drugs.com (Drug Information Database) (Drugs.com, c1996-2018)

Table 2.1: Patient Characteristics

The possible reasons for excessive anticoagulation (if recorded) were extracted from the patients' clinical records. These included change in diet (41 patients), excessive alcohol consumption (7 patients) and change in medication (41 patients). Of the latter group of patients, 12 had stopped taking a medication and 29 had started a new medication shortly prior to the event. Of the 12 patients, the medication was identified as having either major (in 6 patients), moderate (in 4 patients) or minor (in 2 patients) interaction with warfarin. Of the 29 patients the medication was identified as having either major (in 6 patients), moderate (in 9 patients) or minor (in 2 patients) interaction with warfarin; in the remaining 12 patients the medication was not deemed to interact with warfarin.

In the control group, 81 patients received 1mg of vitamin K and 9 patients received 2mg of vitamin K, while for 1 patient the vitamin K dose administered was not recorded. The tailored dose group on average received a higher dose of vitamin K than the control group (1.1±0.3, (1.0-2.0) vs 1.8±0.8 (0.4-6.0), 95% CI for difference: -0.8783, -0.5217; t-test) (**Figure 2.2**). Interestingly, one male patient aged 66 years, weighing 113kg, with index INR of 21.8, target INR of 2-3, but no genotypes available, required 6mg of oral vitamin K. Male patients required greater doses of vitamin K than their female counterparts (95% CI for difference: 0.299-0.933; t-test) (**Figure 2.3**).
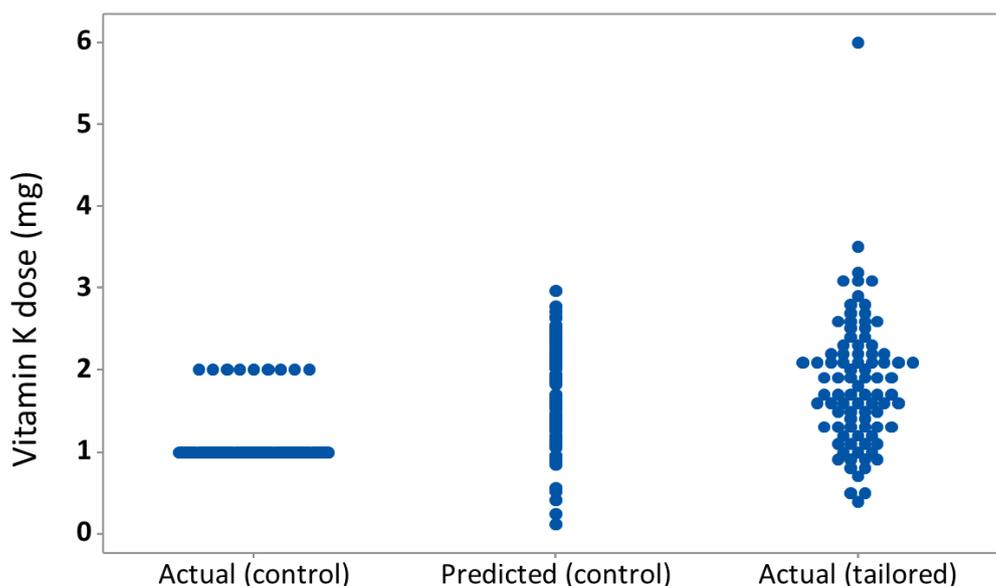
Figure 2.2: Vitamin K dose distribution (actual and predicted dose) per patient group
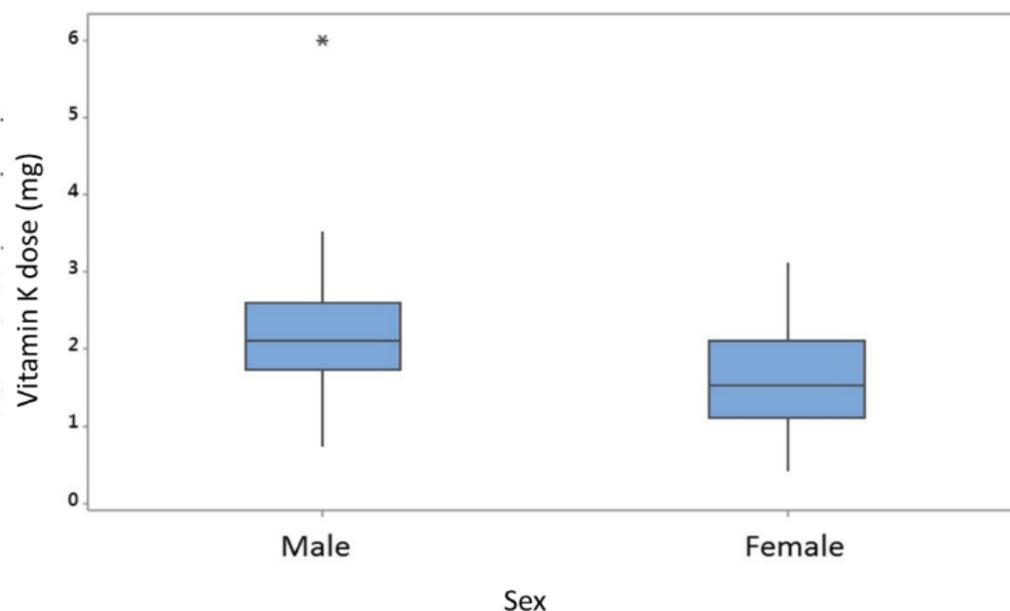
* Outlier value of vitamin K dose (6mg)

Figure 2.3: Boxplot showing the median and interquartile range of vitamin K dose administered according to patient sex in the tailored group

2.4.1 *Primary outcome*

Mean INR at 24 hours was different between the two study groups (95% CI of means: (0.1167- 0.3441; t-test)). (**Table 2.2**) Overall, the tailored dose vitamin K regimen performed better than the fixed-dose regimen, in terms of the proportion of cases being within, above or below the target INR range at 24h (p=0.002; chi-squared test). (**Table 2.2**)

	Control	Tailored	95 % CI^
	Mean INR ± SD (range)		
Index INR	7.9 ±1.8 (5.3-17.0)	8.3 ± 2.2 (6.0-21.8)	(-0.7002,0.2001)*
24h	3.3 ± 2.0 (1.2-16.3)	2.5 ± 0.9 (1.1-5.4)	(0.1167, 0.3441)†
Day 7	3.2 ± 1.3 (1.4-8.0)	3.0 ± 1.4 (1.3-7.0)	(-0.0590, 0.2034)†
Day 14	3.4 ± 1.7 (1.1-9.8)	3.1 ± 1.42 (1.3-8.0)	(-0.0638, 0.2290)†

* Mann-Whitney test
† Log transformed for data analysis

Table 2.2: INR values on admission, at 24hours and on days 7 and 14 after vitamin K administration

A greater proportion of cases in the tailored dose group returned to within their individual target anticoagulation 24h after vitamin K dosing than the control group (68.9% v 52.8%, p=0.026; chi-squared test) (**Table 2.3**). INR value at 24h was above target therapeutic range in a greater proportion of cases in the control group compared to those in the tailored dose group (34.0% v 12.2%, p<0.001; chi-squared test). The proportion of cases with over-corrected INR was not different between the tailored dose group (18.9%) and the fixed-dose group (13.2%) (**Table 2.3**). The median (range) INR at 24h for the under- and over-corrected cases did not differ between the fixed-dose and tailored dose regimens (for undercorrected cases 1.7 (1.2-2.6) vs 1.7 (1.1-2.6), for overcorrected cases 4.5 (3.3-16.3) vs 4.1 (3.3-5.4); Mann-Whitney U test).

	Control	**Tailored**		
Frequency of INR values	n, (%)		**p-value**	
INR-24h				
Below target range	12 (13.2)	17 (18.9)	0.296	
Within target range	48 (52.8)	62 (68.9)	0.026	0.002
Above target range	31 (34.0)	11 (12.2)	<0.001	
Day 7				
Below target range	9 (11.7)	13 (18.3)		
Within target range	42 (54.5)	38 (53.5)	0.480	
Above target range	26 (33.8)	20 (28.2)		
Day 14				
Below target range	6 (8.3)	8 (12.7)		
Within target range	42 (58.3)	33 (52.4)	0.652	
Above target range	24 (33.4)	22 (34.9)		

Table 2.3: Proportion of INR values below, within and above target range at 24 hours and on days 7 and 14 following vitamin K administration

2.4.2 *Secondary outcomes*

For a subset of cases, INR values on day 7 (71 tailored dose and 77 fixed-dose) and day 14 (63 tailored dose and 72 fixed-dose) following vitamin K administration were available. There was no difference in mean INR between both groups at either time-point (54.5% v 53.5% for day 7; 58.3% v 52.4% for day 14) (**Table 2.3**).

Four cases in the control group had an INR >6.0 at 24h and subsequently received an oral dose of vitamin K, according to the study protocol. Of the 4 cases, 2 received a second additional dose of vitamin K 24 and 96 hours later. One case in the tailored dose group admitted with a urinary tract infection and raised INR of 7.5 received a second dose of vitamin K at 48h, following a rise in INR from 5.4 at 24 hours to 6.9 at 48 hours. On day 7, two cases in the control and two in the tailored group had an INR>6.0 and thus received a dose of vitamin K. Similarly, on day 14, three cases in the control and two in the tailored group received a dose of vitamin K. According to the study protocol, the remaining cases with INR <6.0 at 24h were deemed not to require additional dose of vitamin K. One case with INR=6.7 at 24h was not deemed by the reviewing clinician to be at high risk of bleeding and hence did not receive an additional dose of vitamin K.

Twenty nine cases (12 in the control and 17 in the tailored group) had a sub-therapeutic INR (where the INR value was below the lower limit of the therapeutic range by ≥0.3 unit) at 24 hours. Of these LMWH was administered to only two subjects (one in each of the fixed-dose and tailored vitamin K dose group); both were on warfarin therapy for antiphospholipid syndrome and were deemed to be at increased risk of thrombosis. Twenty cases were administered heparin between 48 hours and 16 days after vitamin K administration. However, the decision to administer heparin to these subjects was not study-related and reflected alteration of anticoagulation during admission with the use of therapeutic or prophylactic LMWH while warfarin was interrupted (**Table 2.4**).

Vitamin K has a short half-life of 1.5 to 3 hours, with main effect on anticoagulation appearing within 12-24 hours after its administration (Roche Products Limited). Therefore, events reported during the first 24-48 h after vitamin K administration, were considered as being related to study intervention (i.e. either vitamin K dose was insufficient to return INR to within target range or it was in excess of that required leading to over-correction of INR).

	Total patients analysed, n	Control	Tailored
Warfarin withheld/changed on admission, n (%) ¢			
Yes	174	87	87
No	3	2	1
Warfarin administration at 24 h, n (%) ʌ			
Withheld	29	18	11
Resumed	131	63	68
Additional vitamin K administration post initial dose			
At 24h	4	4	0
48 -96h	3	2	1
On day 7	4	2	2
On day 14	5	3	2
Heparin administration			
At 24h	2	1	1
After 24 h	20	15	5

¢ Based on data for 177 patients
ʌ Based on data for 160 patients

Table 2.4: Changes in patients' treatment following study intervention

No major bleeding events according to the ISTH classification (Schulman and Kearon, 2005) were reported in the trial. Of the 10 minor bleeding adverse events recorded during the trial, 4 belonged to the control group and 6 to the tailored dose group (**Table 2.5**). Three of the bleeding episodes (1 in control group and 2 in tailored dose group) were reported within 2 to 7 days after the initial vitamin K dose administration. The remaining 7 minor bleeding events took place within 7 to 23 days following vitamin K administration (3 in control group and 4 in tailored dose group). All these events are in accordance with bleeding symptoms anticipated while on anticoagulation and were unrelated to the initial vitamin K dose administration.

In all, 1 thromboembolic event in the tailored vitamin K dose group was reported 4 days after the study intervention. The recurrence of thrombosis was associated with malignancy, with the patient's INR being in the therapeutic range and was therefore unrelated to vitamin K administration (**Table 2.5**).

	Bleeding events, n		Thromboembolic events, n	
Time of onset	*Control,* 4	*Tailored regimen,* 6	*Control,* 0	*Tailored,* 1
2 -7 days post intervention	Bruising (leg)[2]	Nose bleed[2] Specks of blood in phlegm[2]		Deep vein thrombosis [1]
7-23 days post intervention	Haematuria[2] Spontaneous bruising (eyelid)[2] Prolonged bleeding after cut[3]	Epistaxis, 2[3,4] Bruising (neck)[2] Vaginal spotting[1]		

[1] Unrelated to warfarin
[2] Possibly related to warfarin
[3] Probably related to warfarin
[4] Definitely related to warfarin

Table 2.5: Bleeding and thromboembolic adverse events according to severity and relation to warfarin treatment

2.4.3 *Associations between genotype, patient characteristics and vitamin K dose*

The genotype frequencies of all tested SNPs were checked for their consistency with Hardy–Weinberg Equilibrium compared with the CEU population, comprising Utah residents (CEPH) with Northern and Western European ancestry) (Whirl-Carrillo et al., 2012) (p=0.797 for *CYP2C9*, p= 0.343 for the *VKORC1*, p=0.339 for *CYP4F2*; chi-square test). No differences in genotype frequency for the *VKORC1* (-1639G>A; rs9923231), *CYP2C9* (*2 and *3; rs1799853 and rs1057910) and *CYP4F2* (V433M; rs2108622) genes were observed between the two study groups (**Table 2.6**).

Factors such as age, weight, height, BSA, vitamin K dose, index INR, *CYP2C9*, *VKORC1* and *CYP4F2* genotype were included in the regression model to predict the INR at 24 hours. According to stepwise regression analysis neither age, height, nor *CYP2C9*, *VKORC1* and *CYP4F2* genotype influenced INR at 24h. Index INR and vitamin K dose contributed to INR at 24h (R^2=24.6%; p<0.001). After including the three factors that appeared to be significant in the regression model, summary statistics are presented in **Table 2.7**. Patient weight appeared to be a more significant predictor of INR at 24h compared to BSA, with the latter used in the

tailored dosing algorithm to calculate vitamin K dose. In contrast to the fixed-dose the tailored vitamin K dose was influenced by both index INR and BSA as expected (**Figure 2.4**).

Genotype	No of patients analysed, n (%) *	Control, n (%)	Tailored, n (%)	P-value
CYP2C9				
*1/*1	102 (62.2)	51 (62.2)	51 (62.2)	0.897
*1/*2, *1/*3	57 (34.8)	28 (34.1)	29 (35.4)	
*2/*2, *2/*3, *3/*3	5 (3)	3 (3.7)	2 (2.4)	
VKORC1				
GG	64 (39.0)	28 (34.2)	36 (43.9)	0.377
GA	75 (45.7)	42 (51.2)	33 (40.25)	
AA	25 (15.2)	12 (14.6)	13 (15.85)	
CYP4F2				
CC	83 (50.6)	38 (46.3)	45 (54.9)	0.330
CT	71 (43.3)	40 (48.8)	31 (37.8)	
TT	10 (6.1)	4 (4.9)	6 (7.3)	

* Genotypes available for 164 patients

Table 2.6: Genotype frequencies for the population and the genes studied

Term	Estimate	Standard error	T-Value	P-Value
Constant	-0.645	0.64	-1.01	0.315
Vitamin K Dose	-1.278	0.198	-6.44	<0.001
Index INR	0.4765	0.071	6.71	<0.001
Weight (kg)	0.02109	0.00563	3.75	<0.001

Table 2.7: Summary statistics and results from multiple regression analysis
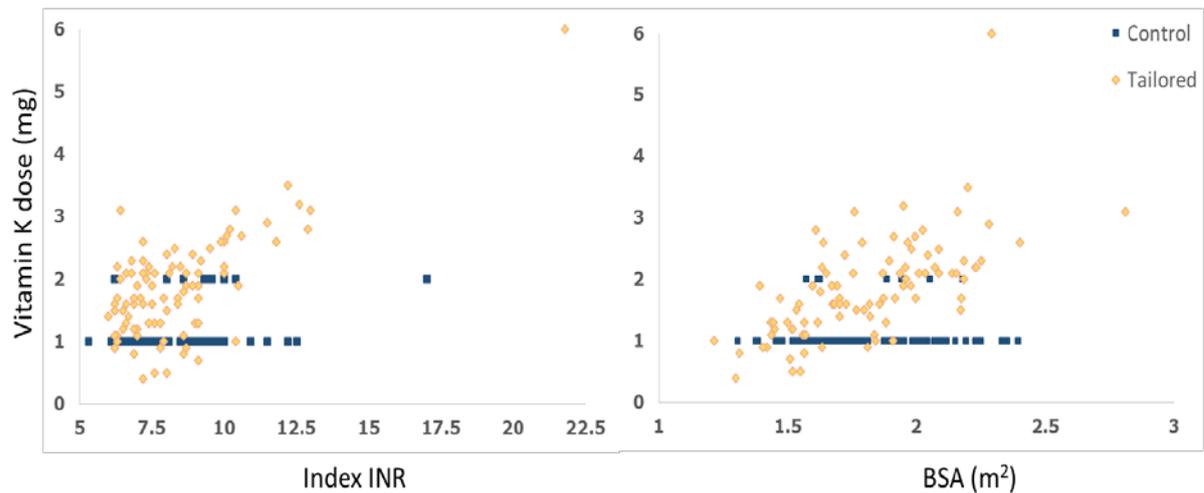
Figure 2.4: Variability in vitamin K dose according to index INR and BSA between the control and tailored vitamin K dose regimen groups

The vitamin K dose for the control group was estimated using the tailored dose equation. According to regression analysis the difference in vitamin K dose (predicted - actual dose) was associated with BSA (R^2=32.7%; $p<0.001$) and index INR (R^2=17.8%; $p<0.001$). The predicted dose of vitamin K increased with increasing BSA and index INR (**Figure 2.5**). The mean (range) predicted vitamin K dose was higher than the actual dose administered [1.7 (0.1 – 3.0) v 1.1 (1.0 – 2.0) mg; difference in dose: 0.6 (-1.0 – 2.0) mg]. For patients who had received the 1mg vitamin K dose (n=77) the predicted mean (range) vitamin K dose was higher [1.7 (0.1 – 3.0) mg; mean difference: 0.7 (-0.9 – 2.0) mg], whilst for those who had received the 2mg dose (n=8) the predicted dose was slightly lower than the actual administered dose [1.9 (1.0 – 2.7); mean difference: -0.1 (-1.0 – 0.7) mg].

The study results were used to produce a new and more refined vitamin K dosing algorithm. By regressing the target INR to index INR, vitamin K dose and weight, the following vitamin K dosing equation was produced:

vitamin K dose = (0.4765 x index INR – target INR + 0.02109 x weight – 0.645) / 1.278; where: vitamin K dose is in milligrams and weight in kilograms.

This formula predicts on average the same vitamin K dose for the group of patients with restored INR to within target range at 24 hours (mean dose change ±SD, 0.06±0.49; range, -1.19 to +1.53 mg). However, for the overcorrected group a reduced dose is predicted (mean dose change ±SD, 0.73±0.80; range, -1.04 to +2.18

mg), while for the under-corrected group a small increase in predicted dose is estimated (mean dose change ±SD, -0.07±0.49; range, -0.91 to +0.95 mg).

A simplified version of the abovementioned formula was also examined for its prediction accuracy. The following formula was employed after appropriate transformations:

vitamin K dose = (index INR / 2.5) – (target INR / 1.3) + (weight / 64) – 0.5

Compared to the original algorithm resulting from the regression model, the simplified algorithm predicted a mean difference of 0.2mg in vitamin K dose, ranging between 0.1 and 0.5mg. While a difference of 0.5mg would have an impact in routine clinical practice, based on the fact that in most hospitals vitamin K dose is calculated in increments of 0.5mg, this difference was only observed in two patients in our cohort with extreme weight and index INR value (one patient weighing 113kg with index INR=21.8 and the second having index INR=17 but missing weight).
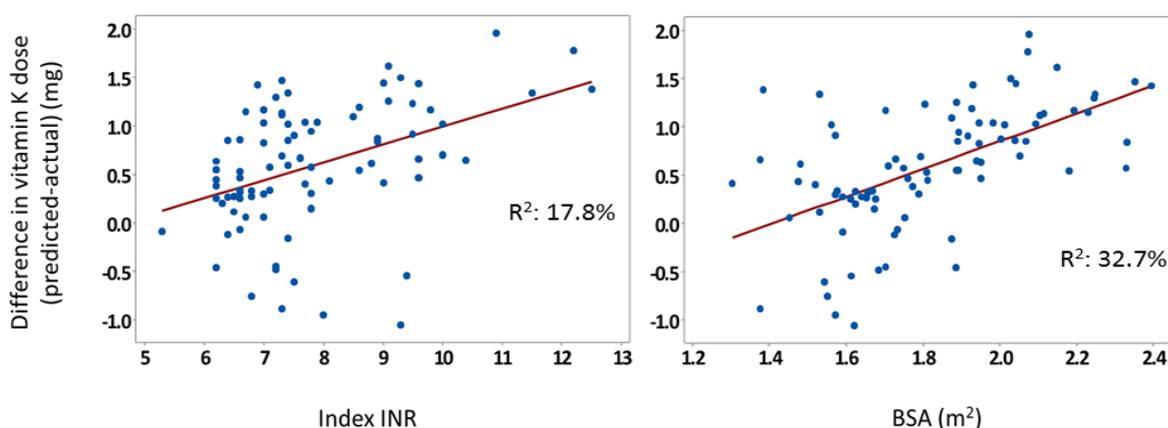
Figure 2.5: Relationship between the difference in vitamin K dose (predicted - actual dose), BSA and index INR

2.5   Discussion

Bleeding is one of the most serious complications associated with warfarin therapy (Palareti et al., 1996). The risk of bleeding increases with excessive anticoagulation which occurs in approximately 0.2-0.3% of outpatients receiving long-term warfarin therapy per annum (Penning-van Beest et al., 2001).

Restoration of the INR towards the target therapeutic range in over-anticoagulated patients is the logical approach, with the choice of treatment depending upon the severity of patient symptoms and the anticipated risks. Evidence from randomized controlled trials supports the use of oral vitamin K in lowering the INR in asymptomatic or mildly bleeding patients (Crowther et al., 2000, Lubetsky et al., 2003, Ageno et al., 2005, Dezee et al., 2006). However, there is no clear consensus about the optimal dose that should be given to patients. The majority of the reported studies have used fixed oral doses of vitamin K (ranging from 1.0 to 5.0 mg), based upon assigning dose according to a dichotomy of elevated INR values to reverse excessive anticoagulation, with a high percentage of patients at 24 hours after vitamin K dosing being either under- or over- anticoagulated, leaving them at risk of either thromboembolism or haemorrhage, respectively (Sconce and Kamali, 2006, Denas et al., 2009). Current guidelines in our locality recommend intervention with 1 or 2mg vitamin K orally to accelerate INR lowering in patients presenting with an INR >8.0 and those with INR of 4.5-7.9 deemed at high risk of bleeding (Hanley, 2004, Keeling et al., 2011).

A tailored approach to vitamin K dosing could ensure a more predictable reversal of over-anticoagulation. In an earlier pilot study of over-anticoagulated patients either asymptomatic or with minor bleeds (87 episodes in total), we demonstrated that an individualized vitamin K dosing regimen whereby vitamin K dose was calculated based upon patient INR on presentation performed better in lowering INR compared to fixed-dose vitamin K (38% v 21% INR within target range at 24 h) (Briz et al., 2010). The results of the pilot study enabled us to refine the original tailored dosing algorithm. The latter can estimate vitamin K dose according to index INR, body surface area and target INR. We have assessed the performance of the new dosing algorithm (measured as the proportion of patients returning to within target INR) against that of the fixed-dose regimen in a randomised parallel design study.

The present study results demonstrate that the tailored vitamin K dosing regimen is superior to the fixed-dose regimen; compared to the fixed-dose regimen, the tailored dose resulted in a greater proportion of patients returning to target INR range, whilst a smaller proportion remained above target INR range at 24h.

The patients in the control group who had received the 1mg vitamin K dose were predicted to require a higher dose of vitamin K whilst those who had received the 2 mg dose require a slightly lower dose. However, there was a wide range in the

predicted vitamin K doses for each of the fixed doses [for 1mg dose: 0.1 – 3.0; mean difference between predicted and actual dose: 0.7 (-0.9 – 2.0) mg; for 2 mg dose: 1.0–2.7 mg; mean difference between predicted and actual dose: -0.1 (-1.0 – 0.7) mg] which further suggests that the fixed-dose regimen is inappropriate for lowering INR for the majority of over-anticoagulated patients.

Anticoagulation response to warfarin is influenced by environmental, clinical and genetic factors (Sconce et al., 2005a, Isbister et al., 2001) which could also influence vitamin K dose requirement in over-anticoagulated patients. We therefore also determined, retrospectively, whether patient age, sex, co-morbidity and genetic polymorphisms in the expression of the vitamin K epoxide reductase, the main target for warfarin (encoded by *VKORC1* gene), cytochrome p450 CYP2C9, the enzyme responsible for the metabolism of S-warfarin (encoded by *CYP2C9* gene) (Sconce et al., 2005a, Kamali and Wynne, 2010), and the mono-oxidase enzyme involved in vitamin K_1 metabolism (encoded by *CYP4F2*) (McDonald et al., 2009), affect vitamin K dose and the extent of INR reversal so that these can be used to further improve the vitamin K dosing algorithm as appropriate. Overcorrection of INR was more frequent in women than men (58.6% v 41.4%) although the differences were not statistically significant, likely due to the small number of patients evaluated. Female sex was found to be independently associated with overcorrection of INR, despite female subjects on average having received lower doses of vitamin K than their male counterparts. Patient factors of age and body size have previously been reported to influence vitamin K dose requirement (Hylek et al., 2001, Kelly et al., 2001). Whilst we found that patient weight and index INR influenced INR at 24h and hence vitamin K dose requirement, patient age had no effect. Similar to our previous pilot study, we found that *CYP2C9* and *VKORC1* polymorphism made no contribution to vitamin K dose requirement, and neither did polymorphisms in *CYP4F2*. However, the present study was not sufficiently powered to detect the influence of genetics on vitamin K dose requirement. Therefore, it remains possible that the influence of these genes on vitamin K dose requirement will become apparent in a purposefully designed study in a larger population of patients.

There were no incidences of major bleeding requiring intervention, or thromboembolic events throughout the trial. There were 10 incidences of minor bleeding; patients in the tailored vitamin K dose group with minor bleeding complications were more likely to be taking drugs that interact with warfarin

(clopidogrel, aspirin) or have chronic comorbidities; factors which are known to increase the risk of bleeding (Tran and Alderman, 2013).

Based on the data of this study, the algorithm predicting the vitamin K dose for lowering the INR in excessively anticoagulated patients on warfarin has been further optimised. The reported simplified version has similar accuracy to the original algorithm resulting from the linear regression model, whilst being more practical for clinicians who don't have easy access to an Excel spreadsheet in clinic. However, as the estimated vitamin K dose is slightly higher compared with the original algorithm, care should be taken to monitor patient INR to prevent over-correction which increases the risk of thrombosis. Adoption of the simple tailored vitamin K dosing algorithm is easy and has the potential to benefit a wide range of patients presenting with excessive anticoagulation with warfarin.

2.6 Limitations

There were missing data in four variables, with the proportion of missing data being no greater than 11% of the total sample size involved in this study. In addition, some patients were lost during the period of follow-up. The proportion of the patients lost in follow-up and randomised to the control and tailored group was 15,4% and 21% respectively at day 7 and 21% and 30% respectively at day 14.

2.7 Conclusion

The current RCT showed that 'tailored' vitamin K dosing regimen was superior to the fixed-dose regimen, leading to a more accurate reversal of excessive anticoagulation and a higher percentage of patients returning to within their target INR range. However, for the true clinical benefit of the tailored vitamin K dosing regimen in over-anticoagulated patients to be realised, further larger study is required to establish its clinical utility, as well as its superiority over fixed-dose regimen in terms of its cost-effectiveness. Among other benefits, the tailored vitamin K dosing regimen could reduce the burden of management of patients with excessive anticoagulation through reductions in the number of repeat treatments/follow-ups, and the potential gains in patient quality of life and treatment safety in terms of reductions in the incidences of bleeding and thrombosis.

Chapter 3   Evaluation of the effect of *VKORC1* and *CYP2C9* genotype on anticoagulation stability in patients on chronic warfarin therapy

3.1   Introduction

Although anticoagulation with warfarin has been used in clinical practice for over six decades, warfarin use still creates concern due to the high risk of bleeding as a consequence of over-anticoagulation (Linkins et al., 2003). Stability of anticoagulation with an INR within the individual therapeutic range is necessary to ensure the safety of warfarin therapy. Percentage time within therapeutic range (%TTR) is a standard measure of quality of anticoagulation control and has been used as common primary outcome in studies evaluating the factors that affect anticoagulation control and the risk of bleeding in patients on chronic warfarin therapy.

Genetics contribute to the variable response to warfarin, with the role of common *CYP2C9* (rs1788853 and rs1057910) and *VKORC1* (rs9923231) polymorphisms being well established in this regard. A number of studies worldwide have shown that carriers of *CYP2C9* and *VKORC1* polymorphisms have a greater sensitivity to warfarin, thus requiring both lower doses and more time to achieve stable dosing (Sconce et al., 2005a, Wadelius et al., 2009, Gage et al., 2008). During initiation therapy, *CYP2C9* variant alleles have been associated with higher risk of supra-therapeutic INR and serious bleeding (Higashi et al., 2002). Tomek et al have previously suggested that patients who are carriers of 3 variant *CYP2C9* and *VKORC1* alleles had a significantly higher risk of major haemorrhage during the initiation and maintenance phases of warfarin therapy (Tomek et al., 2013). Higashi et al also found an association between *CYP2C9*2* and *3* and an increased risk of over-anticoagulation (INR≥4) and bleeding events in patients on warfarin therapy (Higashi et al., 2002).

Nonetheless, there are a number of studies that have suggested that either, or both *VKORC1* and *CYP2C9* genotypes may not be predictive of over-anticoagulation in patients on warfarin. Meckley et al reported no significant difference in the proportion of time spent above range in the first month of warfarin therapy for patients carrying *CYP2C9* and *VKORC1* variants compared to wild-type, although both genotypes increased the probability of having an INR>5 (Meckley et al., 2008).

Limdi et al found that *VKORC1* variant genotype did not confer a significant increase in risk for major or minor haemorrhage in a mixed-race population, in contrast to *CYP2C9* variant alleles (Limdi et al., 2008b). Another study from the same authors showed that neither of the two genotypes influenced the time to attain target INR or time to stabilization among patients of either European or African Americans ancestry (Limdi et al., 2008a). *CYP2C9* and *VKORC1* polymorphisms were not associated with either sub-therapeutic or super-therapeutic INR in a Jordanian population (Al-Eitan et al., 2019).

3.2 Study aim

It was hypothesised that patients with variant *VKORC1* and *CYP2C9* alleles spend a lower percentage of time within the target therapeutic range and therefore have less stable anticoagulation control. This cohort-design study evaluated the influence of common *CYP2C9* and *VKORC1* genotypes on %TTR during the induction and maintenance phases of warfarin therapy in an adult Caucasian population.

3.3 Patients, materials and methods

3.3.1 *Study subjects*

Patients on warfarin who experienced an over-anticoagulation event (INR≥6.0 and either asymptomatic or with minor bleeds (Fihn et al., 1996)) had been recruited as part of a previous randomised controlled clinical study (Chapter 2).

Demographic characteristics and clinical data relevant to their over-anticoagulation event (indication for anticoagulation with warfarin, individual target INR range and bleeding events) were available. Genotypes for *CYP2C9* (*2 and *3 alleles; rs1799853 and rs1057910) and *VKORC1* (−1639G>A; rs9923231) polymorphisms were also available.

3.3.2 *Stability data collection*

Anticoagulation stability data for 6 months for both the induction and maintenance (before the over-anticoagulation event) phases of warfarin therapy, including INR values and dates of checks were extracted from both 4S DAWN computer dosing programme version 7.0 (4S Information Systems Ltd TS, Cumbria, UK) and the local

medical records system of the Newcastle upon Tyne Hospitals. Patients with less than two INR checks during the 6-month maintenance therapy prior to the over-anticoagulation event and/or during the first 6 months of starting warfarin treatment were excluded from analysis. TTR% was calculated in Visual Basic Calculator software 2000 (4S DAWN, Milnthorpe, Cumbria, UK) using the linear interpolation method of Rosendaal et al (Rosendaal et al., 1993) (**Appendix A**).

3.3.3 *Statistical analysis*

Statistical analysis was carried out using Minitab v17 (Minitab, Inc., Coventry, UK). Analyses with single *CYP2C9* and *VKORC1* genotypes and with total variant allele count for both genotypes were performed. Patients were initially divided into five groups according to the number of variant alleles to allow for all possible combinations, however three groups were used for analysis due to low number of patients carrying three variant alleles (1 patient with genotype rs9923231 G/A and rs1788853/ rs1057910 *2/*3, 3 patients with genotype rs9923231 A/A and rs1788853 *1/*2) and no patients carrying four variant alleles (homozygote for both rs9923231 and rs1788853/ rs1057910). %TTR data followed a normal distribution. The association between single genotypes and variant allele count with TTR% at both maintenance and induction therapy was examined among the genotype groups using ANOVA (analysis of variance). The association between %TTR and genotype will also be examined in a recessive model for the variant allele of both polymorphisms. A p-value of <0.05 was considered as statistically significant.

3.4   Results

One hundred sixty four adult patients, who had previously been genotyped for *CYP2C9* and *VKORC1*, were available for analysis. However, for forty two patients, it was not possible to extract anticoagulation data for either induction or maintenance therapy. One hundred twenty two patients were studied with a mean age of 70 ± 14 years. There were 55 (45.1%) males and 67 (54.9%) females, all of white Caucasian origin. The most frequent indications for anticoagulation were AF (40.9%), VTE (14.8%), heart valve replacement (16.4%), PE (12.3%) and other indications (15.6%). The target INR range was 2.0–3.0 in 95 (77.9%), 2.5–3.5 in 12 (9.8%) and 3.0-4.0 in 15 (12.3%) patients. Genotype frequencies for *CYP2C9* (rs1799853 for *2; rs1057910 for *3) and *VKORC1* (rs9923231) were in Hardy-Weinberg equilibrium,

similar to other larger studies (Mega et al., 2015) (**Table 2.6, section 2.4.3**). Total variant allele count groups are presented in **Table 3.1**.

Number of variant allele groups	VKORC1	CYP2C9	n (%)
0	G/G	*1/*1	35 (28.7)
1	G/A	*1/*1	29 (23.8)
	G/G	*1/*2	7 (5.7)
		*1/*3	4 (3.3)
2	A/A	*1/*1	16 (13.1)
	G/G	*2/*2	0 (0.0)
		*3/*3	1 (0.8)
	G/A	*1/*2	18 (14.8)
		*1/*3	8 (6.6)
3	G/A	*2/*3	1 (0.8)
	A/A	*1/*2	3 (2.4)

Table 3.1: Total variant allele count

3.4.1 *Stability analysis*

Overall, patients had a mean±SD (range) of 9±7 (2-39) INR checks during maintenance and 14±7 (3-28) checks during warfarin induction period. Having excluded patients with insufficient number of INR checks (<2), %TTR was calculated for 54 subjects for induction phase and for 110 for maintenance phase therapy. For 42 patients, data were available both for induction and for maintenance therapy.

Neither *VKORC1*, nor *CYP2C9* genotype were associated with TTR% during warfarin maintenance or induction phase (all p>0.05; ANOVA, **Table 3.2**). There was no association between %TTR and the total variant allele count for both the induction and maintenance phases of therapy (p>0.05; ANOVA, **Table 3.2**) (**Figure 3.1**).

When analysing the %TTR maintenance data using a recessive model, a significantly lower %TTR in patients with two variant *VKORC1* alleles is observed compared with patients who carry only one or no variant *VKORC1* alleles (mean ±SD %TTR: 46.2±28.3 v 32.1±22.1; 95% CI for difference in means: (1.50, 26.65), t-test) (**Figure 3.2**).

	Maintenance therapy	Induction therapy
	Sample size % TTR [mean±SD (range)]	*Sample size* % TTR [mean±SD (range)]
CYP2C9 genotype		
*1*1	*72* 41.2 ± 26.1 (0.0-100.0)	*35* 45.1 ± 26.3 (0.0-94.9)
*1*2 & *1*3	*37* 47.9 ± 29.7 (0.0-96.5)	*18* 43.1 ± 22.7 (0.0-84.1)
*2*2 & *2*3 & *3*3 ^	*1* 100.0	*1* 50.0
p-value	0.151 ^	0.813 ^
VKORC1 genotype		
GG	*41* 47.3 ± 28.8 (0.0-100.0)	*22* 43.9 ± 22.2 (0.0-94.9)
AG	*52* 45.3 ± 28.2 (0.0-100.0)	*25* 44.4 ± 28.0 (0.0-81.8)
AA	*17* 32.1 ± 22.1 (0.0-63.3)	*7* 47.3 ± 23.3 (17.7-81.8)
p-value	0.150	0.950
Total variant allele count		
0	*31* 45.5 ± 28.5 (0.0-100.0)	*15* 41.6 ± 22.9 (0.0-94.9)
1	*37* 43.9 ± 26.1 (0.0-100.0)	*20* 46.9 ± 29.0 (0.0-84.1)
2 ‡	*38* 42.9 ± 29.0 (0.0-96.5)	*18* 44.4 ± 22.2 (0.0-81.8)
3 ‡	*4* 44.7 ± 37.9 (15.9-100)	*1* 22.8
p-value	0.985 ‡	0.767 ‡

^ Data were merged with heterozygote genotype group for analysis due to low sample size of the double variant *CYP2C9* genotype group.

‡ Data were merged with group 2 for analysis due to low sample size of group 3.

Table 3.2: %TTR during maintenance and induction therapy according to genotype group
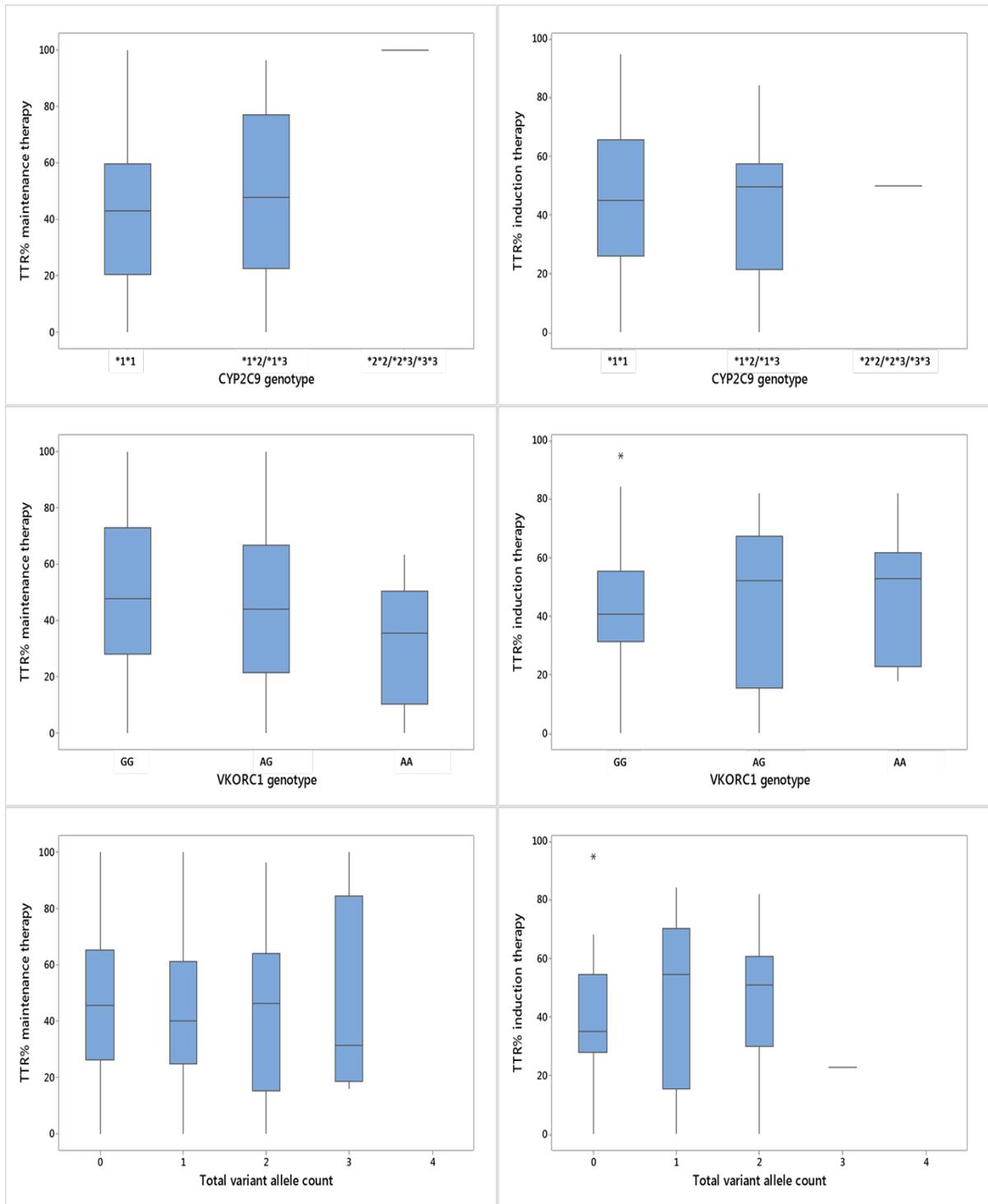
Figure 3.1: Boxplots presenting the TTR% versus *CYP2C9* and *VKORC1* genotype alone and the total variant allele count (*CYP2C9* plus *VKORC1*) during maintenance and induction phase
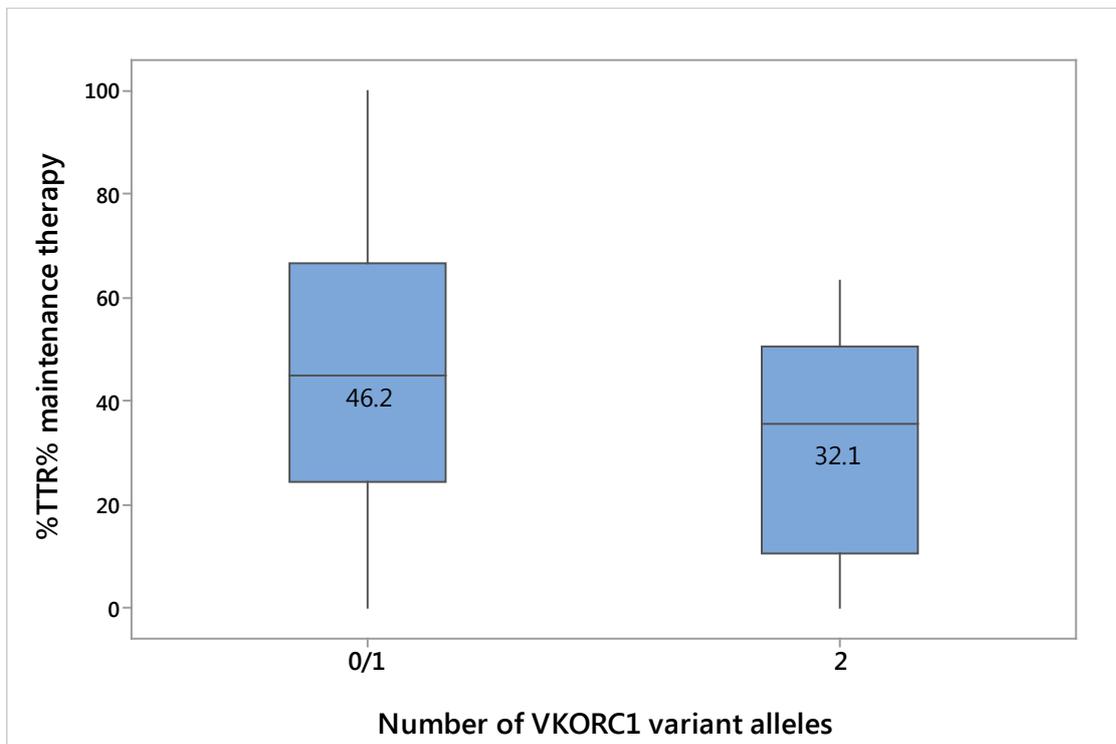
Figure 3.2: Boxplot of %TTR during maintenance therapy between double variant *VKORC1* carriers and carriers of one or no variant alleles

3.4.1 *Bleeding episodes*

Haemorrhagic events for two weeks after excessive anticoagulation were recorded. Nine bleeding episodes occurred in a total of 7 patients (2 patients with two bleeds). For both *VKORC1* and *CYP2C9*, there was no difference in the number of bleedings between the wild-type and carriers of variant alleles (3 and 4 respectively). Considering the total variant allele count for the bleeding episodes, 4 patients (57.1%) had two variant alleles in total, 1 (14.3%) had one *VKORC1* variant allele and the remaining 2 (28.6%) had no variant allele.

3.5 Discussion

Time in therapeutic range is the major determinant of quality of anticoagulation with warfarin, with a lower TTR% being associated with a higher incidence of recurrent thromboembolism and major bleeding (Veeger et al., 2005, Chan et al., 2016b). This study examined the association of %TTR between different genotype groups and showed that *CYP2C9* and *VKORC1* genotypes were not associated with stability of anticoagulation during either induction or maintenance warfarin therapy. Also, there was no deviation from Hardy-Weinberg equilibrium for *CYP2C9* and

VKORC1 genotypes in our over-anticoagulated population, suggesting no immediate causal relationship between genotype and risk of over-anticoagulation. However, when analysing *VKORC1* polymorphism and %TTR during maintenance therapy in a recessive model, patients with two variant VKORC1 alleles appear to have a lower mean %TTR during maintenance compared with wild-type or single variant allele carriers.

The influence of genetic polymorphisms in *CYP2C9* and *VKORC1* genes on the risk of over-anticoagulation and bleeding has been previously examined with conflicting reports. Currently, there is no clear consensus on the predictive value of polymorphisms in these two genes on anticoagulation control. Studies suggest that carriers of *CYP2C9* and/or *VKORC1* polymorphisms have a higher incidence of supra-therapeutic INR (>4.0) during initiation and a higher occurrence of bleeding events during both initiation and maintenance therapy (Meckley et al., 2008, Aithal et al., 1999, Jorgensen et al., 2009).

There is evidence of reduced bleeding risk and shorter time to maintenance dose using genotype-guided warfarin dosing (Kawai et al., 2014, Dahal et al., 2015). However, both EU-PACT and COAG trials have shown that even after consideration of the two genotypes, 20–30% of trial patients experienced an over-anticoagulation response during warfarin induction (Kimmel et al., 2013, Pirmohamed et al., 2013). Taube et al found no increase in the likelihood of over-anticoagulation during long-term therapy caused by the possession of a variant allele (Taube et al., 2000). Neither *CYP2C9*, nor *VKORC1* influenced the time to attain target INR or time to stabilization among patients of either European or African Americans ancestry (Limdi et al., 2008a). Anderson et al found no difference in out-of-range INRs between the standard dosing and pharmacogenetic-guided dosing arms of a randomised trial (Anderson Jeffrey et al., 2007). Chun et al have previously shown that much of the information provided by *CYP2C9* and *VKORC1* genotypes during warfarin initiation is captured by the early INR response, with genotypes having no association with stable warfarin dose (Li et al., 2009).

Current policies on the use of genetic testing support the argument that adjustment of warfarin doses prior to maintenance and during initiation therapy counteract the influence of the *CYP2C9* and *VKORC1* genotypes on the stability of anticoagulation (Lindh et al., 2005). In 2008, the American College of Medical Genetics considered the existing evidence about routine genotyping in warfarin-naive

patients insufficient, followed by the 2008 ACCP guidelines that recommended against genotyping patients until further promising evidence was made available (Flockhart et al., 2008, Ansell J, 2008).

Meta-analyses suggest that bleeding risk is significantly higher in over-anticoagulated patients (OR for INR > 4 compared with the INR 2-3 reference group: 33.23, 95% CI; 9.12-121.07) (Reynolds et al., 2004). It has been recently shown that bleeding risk can be influenced by genetics (Kawai et al., 2014, Tomek et al., 2013). In agreement with this, 71.4% of our patients with bleeding complications had at least one variant allele in either one or both *CYP2C9* and *VKORC1* genes. However, this study was not powered to show an impact of genotype on the incidence of bleeding and to confirm this association a larger sample size is required. The meta-analyses reported to date have looked at different ethnic populations with different allele frequency and with different clinical outcomes, both of which make the results of these studies difficult to interpret (Jorgensen and Williamson, 2008).

Most studies in the literature consider over-anticoagulation as a small deviation (>4) of INR above target therapeutic range, which is 2.0-3.0 for most patients (Dezee et al., 2006). However, guidelines suggest that it is reasonable to offer correction of INR with vitamin K in patients presenting with INR between 5-8 (Keeling et al., 2011), which is further supported by the prospective study of Hylek and colleagues, who showed that patients with INR>6 face a significant short-term risk of major hemorrhage (Hylek et al., 2000). In our study cohort of patients, over-anticoagulated patients at high risk of bleeding exceeding the threshold INR for consideration of correction with oral vitamin K and an INR value above 6 was considered as over-anticoagulation.

3.6 Limitations

Stability data for both induction and maintenance phases of warfarin therapy were available in only a subset of patients (n=42) which is considered as a study limitation. Other study limitations include the retrospective nature of stability data collection and the low number of patients with high total variant allele count and double variant *CYP2C9* alleles, due to the small sample size (n=122). Results for certain variant alleles had to be combined for further analysis due to their low numbers. This might have diluted the potential effect of individual polymorphisms on anticoagulation stability. Overall, sample size and therefore statistical power of this study is low,

despite the fact that the association between the presence of two *VKORC1* alleles and %TTR during maintenance was significant at the statistical level even at this sample size. TTR can be biased by the number of INR checks and the dates performed. The TTR can be very similar between a patient with fairly stable control of anticoagulation with only a few INR checks over a period of time and a patient with very frequent INR checks over the same period of time due to unstable control of anticoagulation, in an attempt to keep the INR within range.

3.7 Conclusion

In this study, the hypothesis that *CYP2C9* and *VKORC1* genotypes have a significant effect on induction and long-term stability of anticoagulation control in patients on chronic warfarin therapy has been disproved. Only when looking at a recessive model, the association between the presence of two *VKORC1* alleles and %TTR during maintenance became significant. The results of this study support published reports suggesting the absence of genetic impact on the anticoagulation response during warfarin maintenance therapy in adults. However, in contrast there are also studies that suggest that common polymorphisms in *CYP2C9* and *VKORC1* are associated with over-anticoagulation particularly during initiation therapy, poor anticoagulation control and bleeding events. In our cohort, bleeding from over-anticoagulation occurred more often in patients with at least one variant allele in *CYP2C9* and/or *VKORC1* genotypes, however the number of events was very low and require a larger sample size and/or prolonged follow-up. Further large, prospective, well designed studies are needed to provide conclusive evidence.

Chapter 4 Validation of a pharmacogenetics-based algorithm to predict INR decline following warfarin cessation

4.1 Introduction

It has been widely demonstrated that genetic factors influence warfarin dose requirements. Polymorphisms in *CYP2C9* and *VKORC1* genes, in particular, are demonstrated to significantly influence sensitivity to warfarin and anticoagulation response following initiation of therapy (Avery et al., 2011, Pirmohamed et al., 2013). The elimination of the pharmacologically more potent enantiomer, S-warfarin, is dependent heavily on *CYP2C9* genotype, with its half-life ranging between 28 and 118 hours according to the absence or presence of variant alleles respectively (Hamberg et al., 2010).

Patients on warfarin are normally required to stop treatment for a fixed number of days (5 days according to local guidelines) prior to an invasive procedure, in order to minimise the risk of peri-operative bleeding (Douketis et al., 2012). However, the anticoagulant activity of warfarin subsides at different rates among different patients, thus a period of 5 days may not result in restoration of normal coagulation in all patients following the interruption of warfarin therapy. Discontinuation of warfarin too early may predispose the patient to thrombosis and stopping it too late may result in peri-operative bleeding or cancellation of the planned procedure. Previous studies have shown that patients with *CYP2C9* variant alleles take a longer period of time (more than the recommended 5 days) after stopping warfarin treatment for their INR to become sub-therapeutic (i.e. INR<1.5) (White et al., 1995, Abohelaika et al., 2015b).

In a recent study in adult patients who interrupted warfarin therapy prior to elective surgery, the rate of INR decline following warfarin cessation was found to be slower in patients with two *CYP2C9* variant alleles, additional to increasing patient age, weight and number of comorbidities. The rate of INR decline was shown to increase with increasing index INR. The aforementioned factors accounted for approximately 90% of the inter-individual variability in the rate of INR decline following cessation of warfarin therapy (Abohelaika et al., 2015a). A genotype-guided protocol to tailor warfarin withdrawal according to individual patient's *CYP2C9* genotype could reduce cancellation or delays of planned procedures and could also be of benefit when transitioning patients from warfarin to a NOAC.

4.2 Aim of the study

Based upon their study data, Abohelaika and colleagues (Abohelaika et al., 2015a) developed a pharmacogenetics-based algorithm to predict the INR decline following warfarin treatment withdrawal in individual patients based upon demographic, clinical and genetic information. This study aimed to validate the algorithm, using data from an independent cohort of patients who had discontinued warfarin therapy.

4.3 Patients, materials and methods

4.3.1 *Study cohorts*

The pharmacogenetics-guided algorithm developed by Abohelaika and colleagues (Abohelaika et al., 2015a) was derived by the multiple regression analysis of data in a cohort of adult patients, who discontinued warfarin for surgery(designated as the algorithm cohort) as shown below:

$$
\begin{aligned}
INR\ decline = -0.195 &- 0.00428\ \big(age\ (years)\big) \\
&- 0.2374\ (presence\ of\ CYP2C9\ double\ variant) \\
&+ 0.9143\ (INR\ value\ 5\ days\ before\ surgery) \\
&- 0.00246\ \big(weight\ (kg)\big) - 0.0306\ (number\ of\ comorbidities)
\end{aligned}
$$

An independent cohort of Caucasian patients aged 18 years and over (designated as the validation cohort), who had completed a course of warfarin for indications of either VTE or AF with a target INR of 2.0-3.0, were available for the algorithm validation. The patients in the validation cohort had been recruited as part of a separate study (Abohelaika et al., 2018). Information on demographics (age, weight, height, and sex), clinical data (medical history, indication for anticoagulation, warfarin daily dose, alcohol intake and concomitant medication) and INR value on the day of warfarin cessation and on alternate days for the following 9 days were available. The patients attended the clinic on 3–4 occasions over the 9 days, with some variation to avoid missed appointments and scheduling conflicts. They had previously been genotyped for *CYP2C9* polymorphisms (*2 and *3) using a real-time PCR technique.

4.3.2 *Data analysis*

The accuracy of the pharmacogenetics-guided algorithm was tested by assessing the correlation between the observed INR decline (calculated by subtracting the INR value on day 5 from the index INR value on the day of warfarin cessation) and the predicted decline in INR according to the algorithm. The performance of the algorithm was evaluated, according to the method of Sheiner and Beal (Sheiner and Beal, 1981), using the root mean squared prediction error (RMSE) as a measure of precision and the mean prediction error (ME) as a measure of bias. The mean squared deviation of prediction errors from their mean (mSDEM) is an estimate of the variance of the prediction error.

The days of INR checks following warfarin cessation varied among the validation cohort for scheduling purposes. For those with an INR check on day 5 after the last dose of warfarin (±10 hours), the INR value was used to calculate the decline which was used as the observed value for comparison with the predicted value derived from the algorithm. For those without an INR check on day 5, a linear approximation model that assumes an exponential INR decline asymptotically over time was used as previously described by White and colleagues (White et al., 1995). This approximation model assumes that following warfarin discontinuation INR will show an exponential decline asymptotically over time to a baseline (warfarin-free) value of 0.8 (Kornberg et al., 1993). Further transformation of INR into the natural logarithm after subtracting the asymptote results in a linear model.

The natural logarithm of INR after subtracting the asymptote 0.8, which is the assumed minimum value of INR, was plotted against the time in hours since warfarin cessation. The linear regression equation was then used to calculate the INR value on day 5 from the point of warfarin withdrawal, which was considered as the observed INR value for comparison (an example of this method is presented in **Appendix B**).

Excel (Microsoft Corp., Redmond, WA, USA) was used for data collation and Minitab (Coventry, UK) was used for statistical analysis. Where necessary, data were transformed to achieve approximate normality. Demographic and clinical data common to both cohorts were used for comparison. Unless otherwise stated, data are presented as mean (range).

4.4   Results

One hundred and thirty one patients were available in the validation cohort for analysis. Seven patients were excluded due to having an index INR of 1.5 or lower. Two patients had no index INR value recorded and for two data on at least two parameters included in the algorithm were missing. Three patients were excluded because their last recorded INR check was performed more than 40 hours before day 5, thus any approximation of their INR value at day 5 was deemed potentially unreliable. Overall, one hundred and seventeen patients were available for analysis, with mean index INR value of 2.5.

Demographics for both patient cohorts are presented in **Table 4.1**. Patients in the algorithm and validation cohorts were significantly different in age (p<0.001 for males and p=0.002 for females; Student's t-test), mean warfarin weekly dose (p=0.004 for males and p=0.036 for females; Student's t-test) and indication for anticoagulation (p<0.001; chi-square test).

All but one patients reached INR<1.5 at day 5. The one patient with INR=1.6 at day 5, which was a predicted value through the linear approximation model in the absence of an INR check close to day 5, was a 71-year old female, weighing 94kg, with index INR of 2.3, 2 comorbidities and with only one variant *CYP2C9* allele.

Seventy out of 117 patients had an INR check on day 5 ($\pm$ 10 hours). The INR on day 5 was 1.1 (0.9-1.4). The decline in the observed INR value (i.e. INR on day 1 – INR on day 5) was 1.4 (0.4-3.5) while the algorithm predicted a decline in INR of 1.6 (0.9-3.4). There was a very strong and highly significant correlation between the observed and predicted INR values (r= 0.969, p<0.001; Pearson test).

To check the accuracy of the linear approximation model, the INR value on day 5 was also estimated using the linear regression equation in this subgroup according to the method of White and colleagues (White et al., 1995) and the observed INR value was compared with the value obtained from the algorithm. Once again, there was a very strong and highly significant correlation (r= 0.905, p<0.001; Spearman test) between the observed INR and the estimated INR on day 5 using the linear regression formula (**Figure 4.1**), with a maximum difference in INR of 0.2 units which is unlikely to be clinically significant.

	Algorithm cohort	Validation cohort	p-value
No of patients	152	117	
Sex, n (%) Male Female	 102 (67) 50 (33)	□ 65 (55.6) 52 (44.4)	□ 0.053
Age (y), mean (range) Male Female	 74 (43-90) 72 (49-93)	* 63 (29-92) 64 (21-88)	♦ <0.001 0.002
Height (cm), mean±SD Male Female	◊ 174 ± 9 160 ± 5	^ 176 ± 7 161 ± 8	♦ 0.052 0.224
Weight (kg), mean±SD Male Female	 88 ± 17 81 ± 19	^ 92 ± 20.3 81 ± 22.6	♦ 0.081 1.000
Warfarin weekly dose (mg), mean±SD Male Female	 28.7 ± 10.3 27.4 ± 14.0	¥ 34.9 ± 15.0 33.4 ± 14.5	♦ 0.004 0.036
Indication for anticoagulation, n (%) Atrial fibrillation Venous thromboembolism Pulmonary embolism Other	 125 (82) 27 (18) 0 0	 12 (10.3) 63 (53.8) 33 (28.2) 9 (7.7)	 □ <0.001
No of comorbidities, mean (range)	3.3 (1-8)	1.7 (0-6)^	<0.001
CYP2C9 genotype *1/*1 *1/*2 *1/*3 *2/*2 *2/*3 *3/*3	 92 35 15 4 6 0	¥ 64 31 14 5 1 0	 □ 0.553
Index INR mean±SD (range)	-	2.5 ± 0.6 (1.6-4.5)	

* Based on 112 patients
^ Based on 116 patients
¥ Based on 115 patients
◊ Based on 140 patients
♦ Student's t-test
□ Chi-squared test

Table 4.1: Patient demographics for the algorithm and validation cohorts
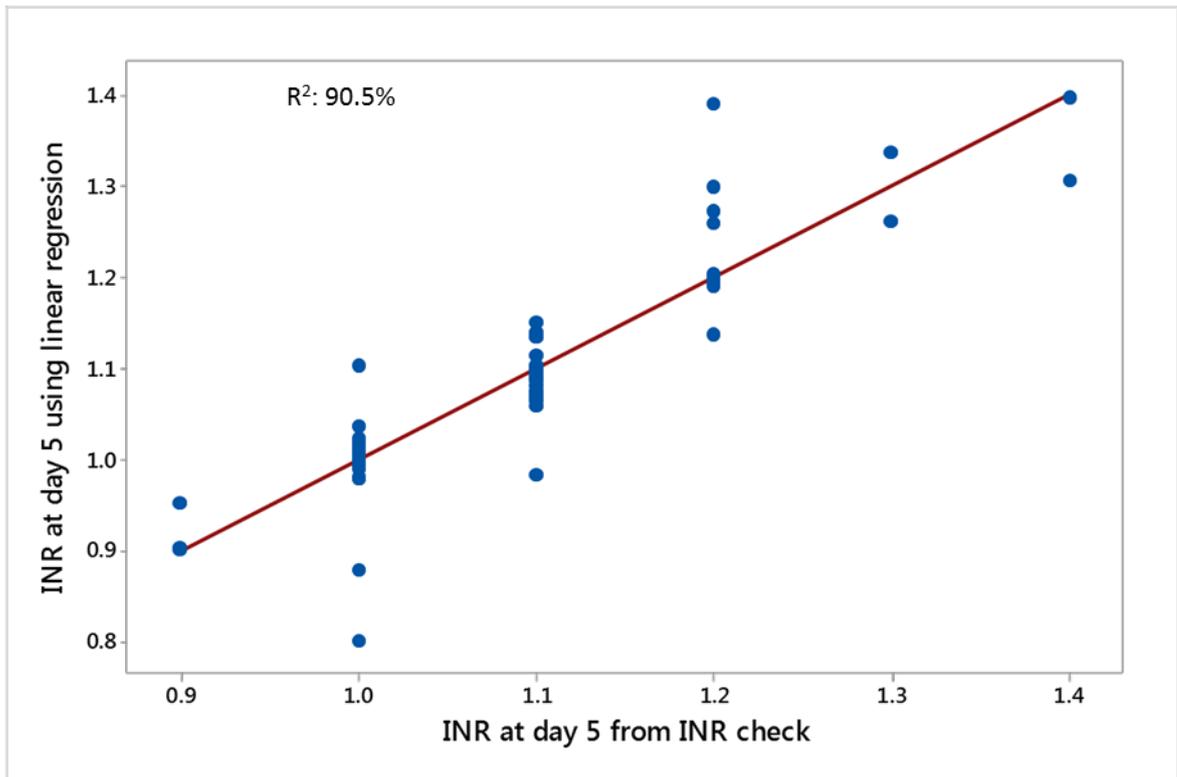
Figure 4.1: Actual INR value on day 5 following warfarin cessation versus INR value obtained from linear regression slope (n=70)

In a subgroup of 20 patients out of the abovementioned 70, with mean index INR 2.4 (1.8-3.6), the INR value was 1.5 or lower at 24 hours and the INR was sub-therapeutic on day 5 (ranging from 1.0 to 1.2). On average, the observed INR decline between day 1 (the day of warfarin withdrawal) and day 5 was 1.3 (0.7-2.5). For this subgroup, according to the algorithm the predicted mean INR decline was 1.5 (0.9-2.8). There was a very strong and highly significant correlation between the observed and the predicted INR values (r= 0.988, p<0.001; Pearson test).

For the remaining 47 out of 117 patients without an INR check on day 5, the INR value was individually estimated using the equation derived from the linear approximation model, with a mean of 1.1 (0.9-1.6) on day 5 and a decline in INR (INR on day 1 – INR on day 5) of 1.3 (0.5-3.3). Based on the algorithm, the predicted decline in INR is 1.5 (0.6-3.3). There was a strong and highly significant correlation between the observed and predicted INR values (r= 0.932, p<0.001; Spearman test).

Overall, there was a strong and highly significant correlation between the observed and predicted INR values for all the 117 patients (r=0.949, p<0.001; Spearman test, **Figure 4.2**).

When removing the effect of genotype from the algorithm, an increase of 0.2 units in the INR decline was predicted only for patients with two variant *CYP2C9* alleles. In the 6 patients with double variant *CYP2C9* genotype, the algorithm based on demographic and clinical characteristics only, without pharmacogenetic information predicted a greater mean (range) INR decline of 0.1 (0-0.2). Removing the presence of *CYP2C9* variant alleles from the INR decline prediction algorithm resulted in an equally strong and highly significant correlation between the observed and predicted INR values for all the 117 patients (r=0.958, p<0.001; Spearman test).
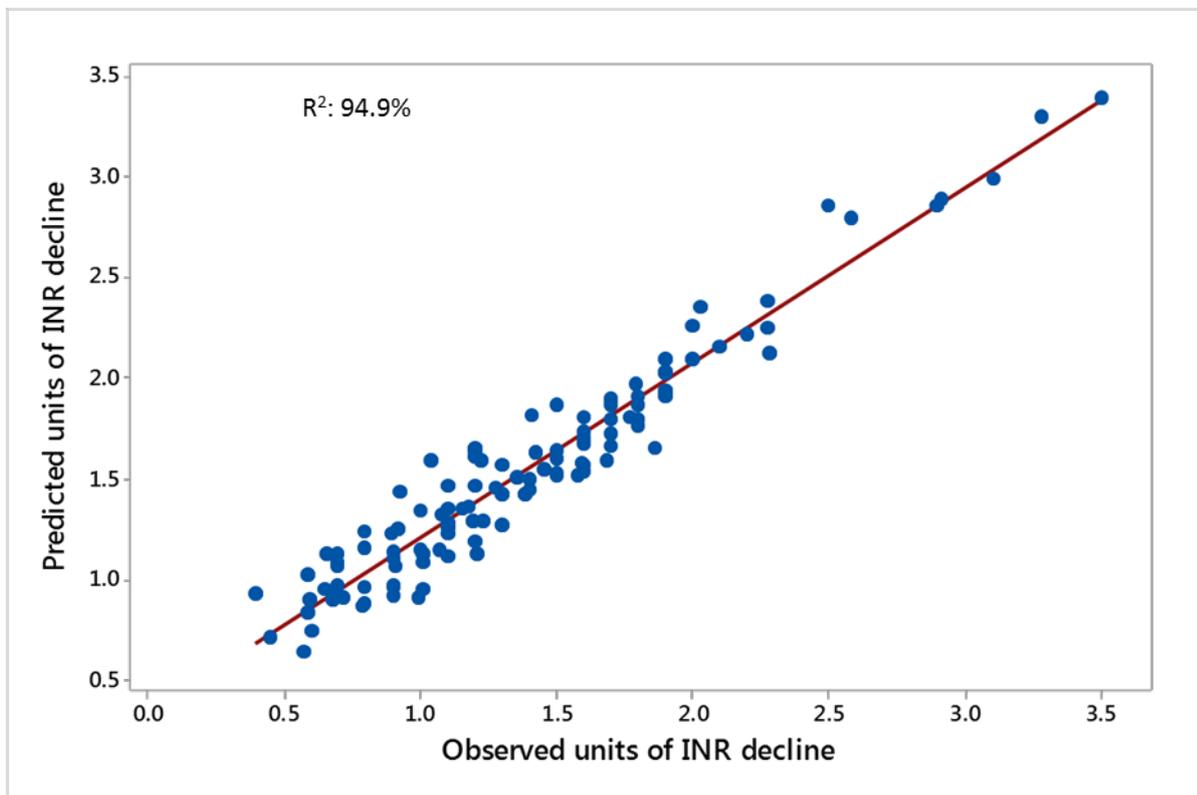
Figure 4.2: Predicted versus observed INR decline (24 hours – day 5)

In terms of prediction performance of the algorithm, the RMSE was 0.22, which means that the algorithm was precise at 0.22 units of INR decline. The ME was 0.15

and the mSDEM was 0.028, which confirm the low degree of bias and variance of the prediction error.

4.5 Discussion

Discontinuation of anticoagulation treatment prior to surgery occurs in approximately 1 in 6 patients per year treated with warfarin (Verdecchia et al., 2016). An accurate method of predicting INR decline is particularly useful for patients with extreme demographics and in frail older patients with many comorbidities and other physiological factors that affect normal coagulation, who may present with therapeutic INR on the day of surgery, as reported in previous studies (Jaffer et al., 2003, Mari et al., 2008). Accurate prediction of INR is also relevant for people withdrawing from warfarin with the aim of switching to treatment with NOACs, in which case there is specific threshold of INR value below which the transitioning to a NOAC can be performed (when INR is ≤3 for rivaroxaban, ≤2.5 for edoxaban, and ≤2 for apixaban and dabigatran) (Heidbuchel et al., 2015).

In order to minimize the risk of perioperative bleeding, guidelines suggest that warfarin therapy is stopped 5 days before an invasive procedure (Douketis et al., 2012, Ortel, 2012). This recommendation is based upon the estimated warfarin clearance and the rate of production of functional coagulation factors II and X after the withdrawal of warfarin treatment (Palareti and Legnani, 1996).

Warfarin elimination is significantly influenced by polymorphisms in the *CYP2C9* gene, which reduce the ability of the CYP2C9 enzyme to metabolise warfarin, as well as other demographic characteristics such as age, which influences both liver size and liver blood flow (Wynne et al., 1995). A significant proportion of patients remain above the INR threshold of 1.5 five days after the cessation of warfarin therapy (White et al., 1995, Kovacs et al., 2004, Steib et al., 2010). Earlier, our group demonstrated that the variability in INR decline following withdrawal from warfarin therapy is significantly influenced by *CYP2C9* genetic polymorphism (Abohelaika et al., 2018). A genotype-guided approach has the potential to reduce treatment costs by reducing the postponement or cancellation of planned invasive procedures in patients on warfarin therapy.

Our research group also recently demonstrated that the variability in the decline in INR over time following warfarin withdrawal is influenced by genetic, demographic and clinical factors. A pharmacogenetic-guided algorithm was subsequently

developed which could predict the length of time needed for individual patients to be off warfarin in order for their INR to reach <1.5. The algorithm included information on patient genetics (presence of *CYP2C9* double variant genotype), demographic (age and weight) and clinical data (index INR, and number of comorbidities). In search of the clinical applicability of this algorithm, I set out to test its accuracy in a second independent cohort of patients on stable maintenance therapy withdrawing from a short course of warfarin treatment. My results showed that the predicted decline in INR on day 5 was very closely correlated with the observed decline in INR value. There was a slight over-estimation of the fall in INR (by 0.2 units) in the subgroup of 70 patients who had an INR check on day 5, and a slight under-estimation of INR in the subgroup of 47 patients who did not have an INR check on day 5. However, this is regarded to be within the expected variability and would not be clinically relevant, but would necessitate another INR check prior to an invasive procedure.

Where INR data on day 5 were missing for some patients due to scheduling arrangements, the linear approximation model (White et al., 1995) was shown to be highly accurate in predicting the INR value.

The results of the present study are in agreement with the well-recognised association of *CYP2C9* genotype and age with the decline in INR following warfarin cessation, however there are also other studies which found no such associations, possibly because of missing data (Burmester et al., 2015).

The findings of this algorithm validation are useful and have practical applicability in patient populations who discontinue warfarin for a surgical procedure (algorithm cohort) and not directly to the validation cohort, which consisted of patients who simply discontinued warfarin after completing a short course of warfarin. The decline of INR and the factors influencing it can be studied in a wide range of patients who discontinue therapy for a number of reasons and results obtained from other patient cohorts could inform clinical practice and benefit patients who discontinue due to a surgical procedure.

The validated algorithm is easy to implement through a simple Excel formula and prior genotyping for *CYP2C9*, which can be available nowadays at a low cost in those healthcare systems with access to such technology. Therefore, a pharmacogenetic-guided approach could be both a safe and cost-effective way of withdrawing patients from warfarin prior to planned invasive procedure.

Considering the lack of genotyping facilities in many healthcare systems worldwide, this study also looked at the predictive ability and accuracy of the same algorithm after removing the requirement for *CYP2C9* genotype. Apart from the fact that out of the 117 patients, only 6 (5.1%) had two variant *CYP2C9* alleles (**Table 4.1**) which is consistent with the observed low frequency in the Caucasian population (Johnson and Cavallari, 2015), the predicted difference in INR decline has a very minor effect on the overall accuracy of the algorithm and would be of little clinical importance for the patient population in this study. Further study in patient populations discontinuing warfarin therapy, either specifically for a surgical procedure or for a different reason, with a greater proportion of patients remaining above the INR threshold of 1.5 at day 5 is necessary for the true clinical benefit of this algorithm to be appreciated.

4.6 Limitations

The two cohorts were significantly different in a number of demographics and clinical data, namely age, warfarin dose and indication for warfarin. The validation cohort included on average younger patients with a higher weekly dose of warfarin and greater variability in indication for anticoagulation, with most patients being treated for VTE and PE instead of AF. While the difference in age and number of comorbidities could have affected the present study outcome, such variability is representative of the wide range of different populations discontinuing warfarin prior to an invasive procedure.

The validation cohort consisted of patients who simply completed a course of warfarin, without an upcoming surgical procedure. However, the study of INR decline after warfarin cessation in such a population resembles the one in patients due for a surgical procedure and therefore, although it is worth clarifying this point, it is unlikely to have affected the study outcomes.

What could have biased these results is the fact that all but one patient in the validation cohort reached INR≤1.5 on or before day 5. Therefore, the results of this algorithm validation study are limited to patients that will reach INR of 1.5 or lower on day 5 after warfarin discontinuation. There is no information about the validity of the algorithm in patients that will remain above the INR threshold at day 5, which requires further study.

4.7 Conclusion

The algorithm generated from a previous study in patients undergoing elective surgery and therefore withdrawing warfarin 5 days prior to the event was validated. The results showed that the algorithm is highly accurate in predicting the decline in INR following cessation of warfarin therapy in an unrelated cohort of patients. In healthcare systems where pharmacogenetic information is not available, the algorithm is still valid and can be used for the accurate prediction of INR decline.

Chapter 5   Association between *CYP2C9, VKORC1 and CYP4F2* genotype and circulating plasma vitamin K concentration in children on chronic warfarin therapy: possible long-term implications for bone development and vascular health

5.1   Introduction

The vitamin K antagonist, warfarin, is commonly prescribed as an oral anticoagulant for the prophylaxis and treatment of thromboembolic disease. In children, it has been estimated that more than 70% of the variability in warfarin dose requirement is attributed to by genetic polymorphisms in *VKORC1* and *CYP2C9*, height and indication for warfarin.

Polymorphism in another member of the cytochrome P450 family, CYP4F2, is also associated with the variability in warfarin dose requirement. Genome-wide association studies have shown that *CYP4F2* variant allele (V433M; *TT, CT*) carriers require higher warfarin doses (Takeuchi et al., 2009). The enzyme expressed by the *TT* and *CT* variant alleles of the *CYP4F2* gene has lower biological activity than that expressed by the *CC* wild-type genotype. CYP4F2 has an important physiological role (Danese et al., 2012). The enzyme is highly expressed in kidney and liver and has an ω-hydroxylase activity towards long-chain (C_{16}-C_{26}) fatty acids, tocopherol and vitamin K, all of which share similar structural characteristics. CYP4F2 is thus important in the activation of signalling compounds, the initiation and resolution phases of inflammation and the regulation of vitamin E (Sontag and Parker, 2002, Johnson et al., 2015). High levels of CYP4F2 protein have also been reported to be present in several forms of cancer (Johnson et al., 2015).

Many of the physiological states or functions dependent upon vitamin K availability can be affected by exposure to warfarin and further compounded by *CYP4F2* genotype (Johnson et al., 2015). The vitamin K-mediated γ-carboxylation, apart from activating coagulation factors, is essential for the biosynthesis of osteocalcin in bones and the functionalisation of MGP which inhibits soft tissue calcification (Villa et al., 2016).

Several studies have shown that vitamin K deficiency can lead to reduced bone mineral density (BMD) and increased risk of fractures (Kuwabara et al., 2011). Furthermore, low BMD seen in children with cardiac disease is likely to be multifactorial; poor nutrition due to cardiac cachexia, malabsorption due to heart

failure, liver congestion and reduced bone loading due to poor exercise tolerance (Laura Gabriela et al., 2012).

Low vitamin K availability can be detrimental to bone formation in children. Achieving adequate peak bone mass in childhood is considered important in preventing the development of osteoporosis later in adulthood (Rezaieyazdi et al., 2009). The prevalence of the "calcification paradox" which refers to concurrent osteoporosis and arterial calcification is shown to be particularly high in postmenopausal women on chronic warfarin therapy (Persy and D'Haese, 2009).

Warfarin through its inhibitory effect on the recycling of vitamin K, can lower vitamin K availability which might, in the long-term, have detrimental effects on bone formation and vascular calcification in children. As vitamin K undergoes CYP4F2-mediated ω-hydroxylation during its catabolism (Danese et al., 2012), we hypothesised that vitamin K systemic availability in children on chronic therapy with warfarin is influenced by *CYP4F2* genotype.

5.2   Aim of the study

This study aimed to assess the effect of dietary vitamin K status, in association with *CYP2C9*, *VKORC1* and *CYP4F2* genotype, on anticoagulation response and long-term stability in children receiving chronic warfarin therapy.

5.3   Patients, materials and methods

5.3.1 *Study subjects*

Plasma samples from a cohort of children recruited as part of a previous study (defined as whole cohort) (Biss et al., 2012), which had been granted ethical approval, were available for this study (defined as sub-cohort). The children were aged 18 years or under and had stable control of anticoagulation with no change in warfarin dose for at least the previous 3 consecutive INR measurements over a minimum period of 4 weeks. Demographic characteristics such as height, weight, age and gender, as well as indication for warfarin anticoagulation, target INR range, warfarin dose, duration of therapy and genotypes for *VKORC1* (-1639G>A; rs9923231), *CYP2C9* (*2 and *3 alleles; rs1799853 and rs1057910) and *CYP4F2* (V433M; rs2108622) for the sub-cohort were available (Biss et al., 2012)

5.3.2 *Plasma vitamin K determination using HPLC*

Plasma vitamin K concentrations were determined by HPLC according to an established high performance liquid chromatography (HPLC) method using post-column derivatization with zinc metal powder (Wang et al., 2004). The detection limit of the assay (signal/noise ratio=3) was 10 pg/ml. The coefficient of variation for quality control samples was 8.4% at 620 pg/ml and 15.0% at 372 pg/ml.

5.3.2.1 Sample preparation

Vitamin K1 was isolated from patient plasma by a two-step process involving liquid phase extraction followed by solid-phase extraction (SPE). All solvents were of HPLC grade. All samples were analysed in duplicate as follows.

For the liquid-phase extraction 0.5 ml of plasma sample was transferred to suitably marked glass tubes with 50 µl of internal standard solution (MK-6, 120 pg/µl) was added. 1ml water was added followed by 2ml ethanol to precipitate proteins in the plasma. After vortex mixing for about 15-20 seconds, 6 ml of hexane was added. The tubes were capped and vortex mixed for about 30 seconds, and then centrifuged at 2500 rpm for 5 minutes to allow separation of the aqueous and organic phases. The uppermost organic phase was transferred to a new glass tube, evaporated to dryness at 45°C under a stream of air, and the residue was reconstituted with 1ml hexane.

The solid-phase extraction followed the liquid one. Silica SPE cartridges (Sep-Pak® Vac 3 cc (200mg), Waters®, USA) were placed in an SPE manifold under vacuum and conditioned with 3ml of 3% diethyl ether in hexane followed by 6 ml of hexane. The samples extracted from the liquid phase extraction step were loaded onto SPE cartridges and washed with a further volume (6ml) of hexane. The retained vitamin K and MK6 (internal standard) were then eluted with 6ml 3% diethyl ether in hexane into a new tube.

The eluted samples were evaporated to dryness at 45°C under a steam of air. The residue was reconstituted with 30 µl dichloromethane and 100 µl mobile phase, and vortex mixed for a further 10-15 seconds. The reconstituted samples were transferred into 1.5 ml tubes, and spun on a benchtop centrifuge for 2-3 minutes at maximum speed to precipitate any particulate matter, prior to HPLC analysis.

5.3.2.2 Preparation of standard curve and quality control

Pure vitamin K1 was obtained from Sigma-Aldrich, UK. A primary vitamin K1 stock solution was prepared in hexane. This solution was then diluted with methanol to prepare a secondary working solution, which was used for constructing a vitamin K1 standard curve (range 0-880 pg/ml). Appropriate volumes of methanolic vitamin K solution together with 50 µl of internal standard (MK-6) were added to labelled 1.5 ml tubes. After evaporating under a stream of air, the residue was reconstituted and injected to the column.

A pool of 50ml plasma was obtained from the hospital blood bank for quality control sample preparation and treated with direct sun light for 24-48 hours to obtain vitamin K free plasma. The sun-treated plasma was then injected to HPLC to confirm that no vitamin K was present. It was subsequently aliquoted into smaller volumes and frozen at -20ºC for later use for the preparation of quality control samples.

Quality control samples were extracted in duplicate in the same way as previously mentioned and tested for with every assay run.

5.3.2.3 HPLC instrumentation and conditions

A Shimadzu VP HPLC machine was used for measuring plasma vitamin K1 concentration. The system was fitted with a RF-10AXL fluorescence detector (emission wavelength 440 nm and excitation wavelength 243 nm). The analytical column was a 3 µm C18 BDS Hypersil, with dimensions of 150 X 3 mm (Thermo, Runcorn) to which a post-column zinc reducer was attached. A degasser was fitted to ensure the flow rate and the function of the pump is not influenced by air bubbles present in solvents. The mobile phase was composed of methanol and dichloromethane [90:10 (v/v)], and 5 ml per litre zinc acid solution (2M zinc chloride, 1M sodium acetate, and 2M acetic acid) and it was run at a flow rate of 0.5 ml/min. The run time for each sample was 12 minutes. Vitamin K1 and MK-6 were eluted on average at 5.9 and 7.2 minutes, respectively (**Figure 5.1**).
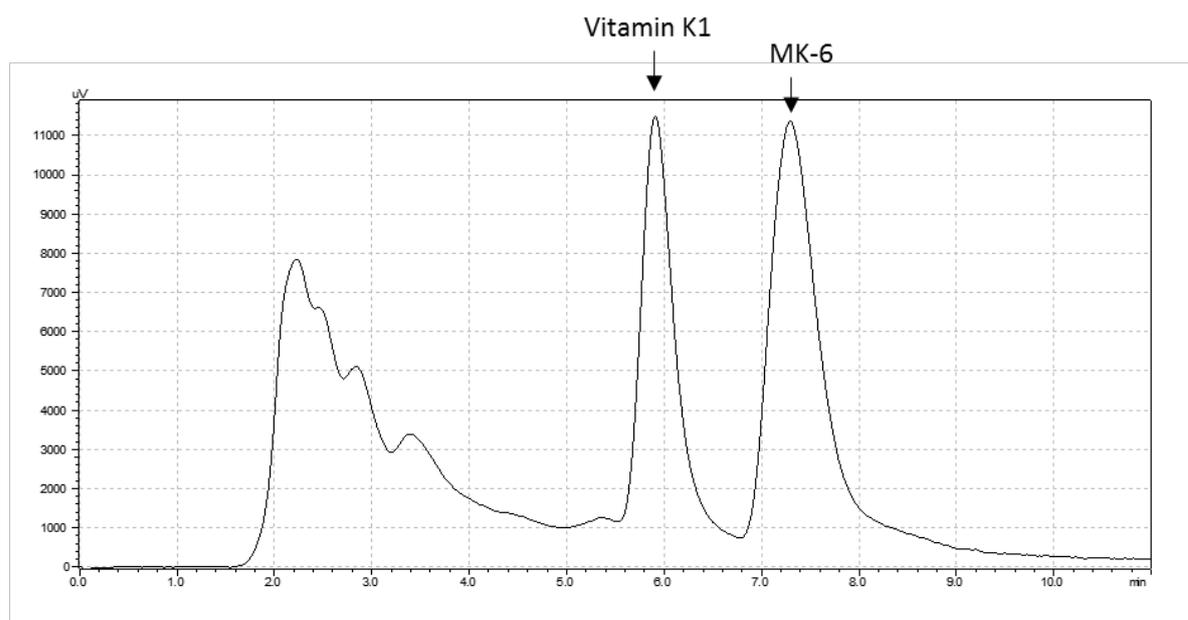
Figure 5.1: Chromatogram of vitamin K1 and MK-6 showing retention times

5.3.2.4 Data analysis

The chromatograms were analysed using Shimadzu LC Solutions Chromatographic software. The standard curve was created by plotting vitamin K to internal standard peak height ratio versus vitamin K1 concentration.

5.3.3 *Statistical analysis*

Statistical analysis was performed using Minitab 17 Statistical Software (Coventry, UK). Where necessary, data were transformed to achieve normal distribution. Between-group comparisons for all demographic, clinical and genetic variables were performed using parametric and non-parametric tests based on data distribution. A p-value <0.05 was considered statistically significant.

5.4 Results

One hundred and seven children on warfarin (74 males) with a median age of 11 years (range: 2-18 years) and a median duration of warfarin therapy of 56 months (range: 2-199 months) were available for analysis. Demographics and genotype results for the cohort are shown in **Table 5.1**. There was no significant difference in demographic and clinical characteristics between the two groups apart from the

target INR range, which depends on the indication for anticoagulation with warfarin (**Table 5.2**).

All genotypes were tested for consistency with the Hardy-Weinberg equilibrium against the CEU population as previously in section **2.4.3** ($p=0.537$ for *CYP2C9*, $p=0.528$ for the *VKORC1*, $p=0.371$ for *CYP4F2*; chi-square test).

Variables	N (%)
Indication for warfarin, n (%)	
Fontan procedure	61 (57.0)
Other (prosthetic heart valve, coronary aneurism, dilated cardiomyopathy, DVT, PE, pulmonary hypertension, stroke)	46 (43.0)
CYP2C9 genotype, n (%)	
*1/*1	75 (70.1)
*1/*2, *1/*3	30 (28.0)
*2/*2, *2/*3	2 (1.9)
VKORC1 genotype, n (%)	
GG	39 (36.4)
AG	46 (43.0)
AA	22 (20.6)
CYP4F2 genotype, n (%)	
CC	52 (48.6)
CT	46 (43.0)
TT	9 (8.4)

Table 5.1: Patient demographic and genotype

Plasma vitamin K was detected in only 21 children (19.6%), with a mean ± SD (range) of 165.7 ± 202.4 (10.1-765.0) pg/ml; in the remaining 86 children (80.4%) plasma vitamin K was below the assay limit of detection. For data analysis, the study subjects were allocated into two groups; children with plasma vitamin K concentration either above or below 10pg/ml. Patient demographic and genetic data for the two groups are shown in **Table 5.2**.

Patient group	Vitamin K levels <10pg/ml	Vitamin K levels >10pg/ml	95% CI
Age (years), mean ± SD (range)	11 ± 4 (2-18)	12 ± 4 (6-18)	(-2.988, 0.988)
Height (cm), mean ± SD (range)	140.2 ± 25.8 (82.0-195.5)	142.7 ± 23.0 (87.6-178.6)	(-14.18, 9.18)
Weight (kg), mean ± SD (range)	38.6 ± 19.1 (11.7-100.1)	44.1 ± 21.5 (12.3-94.6)	(-16.00, 5.00)
			p-value
Warfarin daily dose, mean ± SD (range)	4.0 ± 2.2 (0.9-12.5)	3.8 ± 1.7 (0.5-6.7)	0.790^
Duration on warfarin therapy (months), mean ± SD (range)	61 ± 53 (2-199)	84 ± 60 (10-195)	0.089^

Gender, n (%)
  Male
  Female
	57 (66.3)
	29 (33.7)
		17 (81)
		4 (19)
			0.192

Indication for warfarin, n (%) Fontan procedure Other	51 (59.3) 35 (40.7)	10 (47.6) 11 (52.4)	0.332

Target INR range, n (%)
  2.0-3.0
  2.5-3.5
	77 (89.5)
	9 (10.5)
		15 (71.4)
		6 (28.6)
			0.032

CYP2C9 genotype, n (%)
  *1/*1
  *1/*2, *1/*3
  *2/*2, *2/*3
	63 (73.3)
	21 (24.4)
	2 (2.3)
		12 (57.1)
		9 (42.9)
		0 (0.0)
			0.148*

VKORC1 genotype, n (%)
  AA
  AG
  GG
	20 (23.2)
	33 (38.4)
	33 (38.4)
		2 (9.5)
		13 (61.9)
		6 (28.6)
			0.124

CYP4F2 genotype, n (%)
  CC
  CT
  TT
	47 (54.6)
	33 (38.4)
	6 (7.0)
		5 (23.8)
		13 (61.9)
		3 (14.3)
			0.038

^ Kruskal-Wallis test

* Chi squared test performed between wild-type and variant allele carriers

Table 5.2: Paediatric patient demographic and genetic data

There was a significant difference in the frequency of *CYP4F2* genotypes between the two groups of children (p=0.038; chi-square test). When considering a recessive model for the T allele (*CC* wild-type expresses enzyme with maximal activity), the frequency of *CYP4F2* variant allele carriers *(TT+CT* genotypes) is significantly higher in the group of children with plasma vitamin K levels greater than 10pg/ml (76.2% vs 45.4%, p=0.011; chi square test). After Bonferroni correction for multiple testing (Simes, 1986), there is a significant difference between CC and CT genotypes between the groups (p=0.016). There was no difference in the mean plasma vitamin K concentration among subjects with (n=16) or without (n=5) *CYP4F2* variant alleles in the group of children with detectable plasma vitamin K levels (**Figure 5.2**).
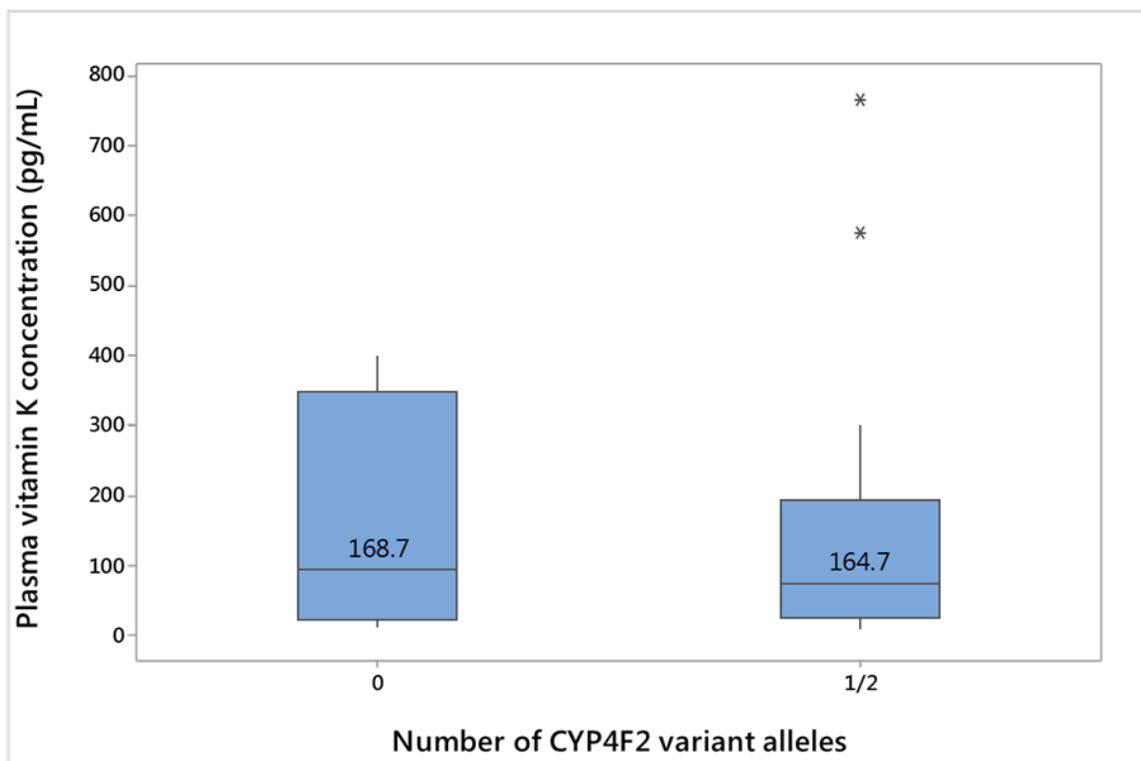
Figure 5.2: Boxplot of median, quartiles and outliers of plasma vitamin K concentration according to the number of variant *CYP4F2* alleles

Although there is a trend towards significance in the frequencies of both *CYP2C9* and *VKORC1* genotypes, this is not significant at the statistical level (**Table 5.2**). As expected, there was no significant difference in warfarin dose requirement between the two groups given that warfarin dose requirement in both adults and children is

mainly governed by *CYP2C9* and *VKORC1* genotype as well as factors of age, body size and intensity of anticoagulation, whilst by comparison *CYP4F2* has a smaller effect on warfarin dose (Cooper et al., 2008, Biss et al., 2012)

5.5 Discussion

Plasma vitamin K concentration is a marker of dietary vitamin K status (Thane et al., 2002a, McKeown et al., 2002, Booth et al., 1997). In healthy adults, reference fasting values of plasma vitamin K concentration range between 0.1-1.2ng/ml with a median of 0.5ng/ml (Sadowski et al., 1989, Shearer, 2009b). In adult hospitalised patients, the prevalence of low vitamin K concentration is high (O'Shaughnessy et al., 2003). In anticoagulated adults, vitamin K concentrations measured prior to warfarin treatment were significantly different from those after receiving warfarin treatment (median 1.72 vs 0.59 ng/ml) (Kim et al., 2015).

The mean observed plasma vitamin K concentration in this cohort of anticoagulated children (in 86 of whom plasma vitamin K concentration was below 10pg/ml) is consistent with the finding in fasting adults and towards the lower limit of the adult range [mean±SD (range): 0.165±0.202 (0.010-0.765) ng/ml). However, this value is lower than those previously reported in healthy children (Kalkwarf et al., 2004). A previous study in anticoagulated children reported non-fasting plasma vitamin K concentration of 670 (20-2810) ng/ml, which is much higher compared to our levels as well as much less variable despite a larger sample size (37 versus 21 children on warfarin with detectable levels) (Hirai et al., 2013). In addition, non-anticoagulated children receiving parenteral nutrition have been shown to have 10 times higher circulating vitamin K concentrations compared with non-anticoagulated control children, whose levels ranged between 0.5 and 5ng/ml (Pettei et al., 1993). Hence, a great variability in reported vitamin K levels in plasma between adults and children is observed. Factors such as young age, fasting status and warfarin therapy appear to be associated with reduced and parenteral nutrition with increased plasma vitamin K concentration.

It is possible that the observed low and undetectable plasma vitamin K levels in the anticoagulated children were caused by either poor nutrition possibly due to ill-health (as most of the children had undergone cardiac surgery), and/or the use of medications which affect vitamin K absorption or disposition. Significantly, though, warfarin through its inhibitory effect on the recycling of vitamin K, can also lower

vitamin K availability which could have detrimental effects on bone formation and vascular calcification. Uncontrolled cohort studies have described reduced bone density in children on long-term warfarin treatment (Boris and Harris, 2003, Monagle et al., 2002). Children with congenital heart disease receiving warfarin have lower BMD than those not receiving warfarin (Bendaly et al., 2015). This however is also likely to be due to a number of other factors, including poor nutrition due to cardiac cachexia, malabsorption due to heart failure and liver congestion and reduced bone loading due to poor exercise tolerance (Laura Gabriela et al., 2012). MGP is a vitamin K-dependent inhibitor of soft tissue calcification, and the risk of cardiovascular disease is increased with decreasing levels of the functional MGP (Shea and Holden, 2012). In relation to this, children on warfarin have been demonstrated to be at an increased risk of arterial and vascular calcification. Tracheobronchial calcification is another reported complication of anticoagulant treatment in children, observed in particular after Fontan procedure (Eckersley et al., 2014).

5.6 Limitations

In this study, vitamin K concentration measurement of a single plasma sample was used as a surrogate marker of dietary vitamin K intake. Additional plasma samples would have provided a more accurate representation of plasma vitamin K status of the study cohort, however the retrospective nature of this study did not allow for further plasma sample collection. There was no direct measure of vitamin K through a validated questionnaire. Also, as the study subjects were previously recruited for a warfarin pharmacogenetics study, there was no information recorded about the possible fasting status of the subjects at the time of blood sampling, variability in which could have affected the plasma vitamin K levels.

Although the detection limit of the HPLC assay used to measure plasma vitamin K levels (10pg/ml) is lower compared to the reported limits of the same assay in the literature, it was not sensitive enough to measure vitamin K levels below 10 pg/ml.

Another limitation is that paediatric plasma volumes were low. Hence, for a few patients only a fraction of the plasma volume required by the protocol was analysed and the results were obtained through extrapolation.

A larger sample size in the group with detectable levels of plasma vitamin K would have the potential to allow for a more robust analysis of the association between

vitamin K concentration and patient genotype groups. This underpowered study is likely to have diluted the effect of *CYP2C9* and *VKORC1* genotypes, therefore further study is necessary to address any existing contribution of these genotypes to plasma vitamin K levels.

5.7   Conclusion

The findings of this study suggest that *CYP4F2* genotype influences vitamin K availability in anticoagulated children. Chronic low vitamin K availability in children on warfarin may have important implications for physiological functions beyond the blood coagulation system. In general, children with low vitamin K levels, compounded by *CYP4F2* genotype and chronic exposure to warfarin, might be at increased risk of developing osteoporosis and cardiovascular disease later in life which need further investigation (Barnes et al., 2005).

Chapter 6 Assessment of vascular health in children on warfarin using uncarboxylated matrix-Gla protein as a marker of vascular calcification

6.1 Introduction

There are concerns about the long-term effect of warfarin therapy on vascular calcification and bone formation in children (Kampouraki et al., 2017). The deleterious effects of warfarin on vascular health have been demonstrated in a number of case reports, cross-sectional and retrospective observational studies showing that warfarin therapy increases the risk of vascular calcification in adults, notably elderly patients (Poterucha and Goldhaber, 2016, Palaniswamy et al., 2011). Until recently vascular calcification was thought to be irreversible. However, the current viewpoint is that vascular calcification is an active process modulated by both inhibitors and stimulators of the calcification process and is reversible. In relation to this, warfarin cessation has been shown to help reverse warfarin-induced vascular calcification in patients with calcific uraemic arteriolopathy (Coates et al., 1998).

Vascular calcification has previously been studied in children with chronic kidney disease (CKD) because coronary calcification is one of the most frequently observed cardiovascular adverse outcomes in children with CKD, with 15–30% of children on chronic dialysis presenting with coronary calcification (Shroff et al., 2011). To date, there is no firm evidence whether long-term warfarin treatment increases the risk of vascular calcification in children on oral anticoagulant therapy.

In humans, the main source of vitamin K is obtained through the diet (as vitamin K_1; phylloquinone) which is particularly abundant in green leafy vegetables (Booth, 2012). Vitamin K in its hydroquinone form is an essential cofactor for the functionalisation, through post-translational γ-carboxylation of glutamic acid residues located at the N-terminal of vitamin-K dependent proteins, such as clotting proteins, osteocalcin and MGP (Kamali and Wynne, 2010).

The vitamin K-dependent clotting proteins are synthesized in the liver, whereas osteocalcin and MGP are synthesised in extrahepatic tissues. The transport system for vitamin K ensures adequate distribution of vitamin K to extrahepatic tissues, whilst maintaining the preferential distribution to the liver when vitamin K availability is limited. This explains the observation that vitamin K insufficiency firstly leads to

under-carboxylation of the extrahepatic Gla proteins, while the clotting mechanism remains intact (Theuwissen et al., 2012).

MGP is a small vitamin K-dependent protein, which is synthesized by vascular smooth muscle cells and chondrocytes and acts as a local inhibitor of vascular mineralization (Shea and Holden, 2012). It has a protective role against tissue calcification and was the first recognised key inhibitory proteins in vivo (Luo et al., 1997). MGP undergoes two types of post-translational modification: carboxylation of five glutamate residues and phosphorylation of three serine residues, both contributing to its function as a calcification inhibitor (Schurgers et al., 2007, Price et al., 1983, Price et al., 1994).

Depending on phosphorylation and/or carboxylation, MGP exists in various states. One form of MGP found in plasma is uncarboxylated MGP (uc-MGP). Uc-MGP concentrations in plasma and serum are inversely associated with the extent of arterial calcification in adults (Cranenburg et al., 2009). The mean plasma uc-MGP concentration in healthy adults is found to be twice as much as that in patients with end-stage renal disease (4704 ± 1053 nM v 2306 ± 706 nM) (Cranenburg et al., 2010, Holden et al., 2010). The latter are at increased risk for cardiovascular complications (Blacher et al., 2001, Schlieper et al., 2016), with a prevalence of vascular calcifications between 60-80% (Cranenburg et al., 2009) and the presence of subclinical vitamin K deficiency (McCabe et al., 2013). It is possible that plasma uc-MGP concentration can be a useful biomarker for assessing the relationship between exposure to warfarin and vascular calcification in children on chronic warfarin therapy.

6.2 Aim of the study

In this post-hoc study in anticoagulated children, we aimed to assess the long-term effect of warfarin therapy on vascular heath in children by measuring plasma uc-MGP concentrations, as an indicator of arterial calcification.

6.3 Study subjects and methods

6.3.1 *Subjects*

Plasma samples from a cohort of anticoagulated children on warfarin recruited as part of a previous study (Biss et al., 2012) were available for laboratory analysis. The

children were aged 18 years or under and with stable anticoagulation control. Anticoagulation stability was considered as no change in warfarin dose for at least the previous 3 consecutive INR measurements over a minimum period of 4 weeks. Demographic and clinical data such as indication for warfarin anticoagulation, target INR range, warfarin dose and duration of therapy were available.

6.3.2 *Plasma MGP concentration determination*

Plasma uc-MGP concentration was determined using a competitive ELISA (Cranenburg et al., 2010). In brief, rabbit polyclonal anti-mouse IgG was bound to the microtitre plate and saturated with a monoclonal antibody directed against uc-MGP. Samples were supplemented with a biotinylated synthetic peptide tracer before being transferred to the plate followed by an overnight incubation at 4 °C. After washing, plates were supplemented with streptavidine-peroxidase and stained with tetramethylbenzidine. The lower limit of detection for the assay was 98 nM. The intra- and inter-assay coefficient of variation were 7 and 11%, respectively.

6.3.3 *Statistical analysis*

Statistical analysis was performed using Minitab 17 Statistical Software. Data were transformed to achieve approximate normality. T-test was used to compare means between the two groups receiving different anticoagulation intensity. Pearson correlation was used to test the associations between plasma uc-MGP concentration, warfarin dose and duration of therapy. Results are presented as mean ± SD (range) unless otherwise stated. A p value of < 0.05 was considered as being statistically significant.

6.4 Results

Ninety one children were included in the study. Demographic characteristics such as height, weight, age and gender, as well as indication for warfarin anticoagulation, warfarin dose and duration of therapy for the study population per INR target group are shown in **Table 6.1**.

There was a wide range in plasma uc-MGP concentration (161 – 2904nM) with a median value of 823nM (**Figure 6.1**, **Table 6.2**). Intensity of anticoagulation, indicated by the target INR range, was 2.0-3.0 for the majority of the children (84.6%), with the remainder (15.4%) having a target INR range between 2.5 to 3.5.

INR target group	2.0-3.0	2.5-3.5	p-value / 95% CI
	n (%)		
n (%)	77 (84.6)	14 (15.4)	
Gender			
Male	52 (85.2)	9 (14.8)	0.813*
Female	25 (83.3)	5 (16.7)	
Indication			
Fontan procedure	48 (98.0)	1 (2.0)	<0.001
Other	29 (69.0)	13 (31.0)	
	mean ± SD (range)		
Age (years)	10.7 ± 4.4 (2.0-18.0)	13.2 ± 4.5 (6.0-18.0)	(-5.25, 0.25)
Height (cm)	139.9 ± 25.0 (82.0-184.0)	147.0 ± 27.3 (87.6-176.5)	(-23.63, 9.43)
Weight (kg)	39.8 ± 20.0 (11.7-100.1)	46.8 ± 20.5 (12.3-73.3)	(-19.52, 5.52)
Warfarin daily dose (mg)	3.9 ± 2.0 (0.9-11.0)	5.0 ± 2.7 (1.9-12.5)	(-2.71, 0.51)
Duration on warfarin (months)	60.1 ± 50.2 (2.0-195.0)	88.0 ± 69.4 (4.0-199.0)	(-69.3, 13.5)

*One cell with expected count less than 5 is present

Table 6.1: Demographic characteristics of the study population by INR target group

There was no difference in mean plasma uc-MGP concentration between children with target INR range 2.0-3.0 and those with target INR range 2.5-3.5 [95% CI: (-0.150, 0.390), Estimate for difference: 0.120; t-test]. There was no association between circulating plasma uc-MGP concentration and either duration of warfarin therapy (p= 0.281, r= -0.114; Pearson correlation) or warfarin dose (p= 0.897, r= -0.014; Pearson correlation), irrespective of age (**Table 6.2**). As expected, there was a significant positive correlation between age and both duration and dose of warfarin (r= 0.521, p< 0.001 and r= 0.496, p< 0.001 respectively; Pearson correlation).
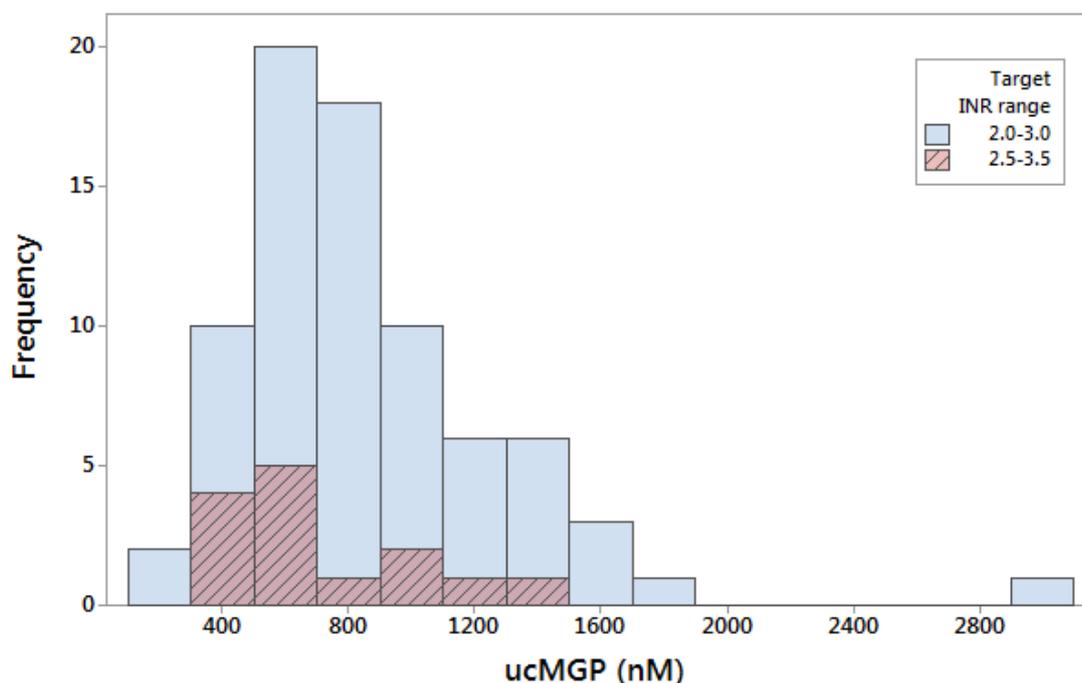
Figure 6.1: Histogram of plasma uc-MGP concentration per INR target group

		n	Mean ± SD (range)	95% CI for difference
uc-MGP (nM) [a]	Total	91	823 ± 408 (161 – 2904)	
	INR target group 2.0-3.0	77	843 ± 425 (161 – 2904)	(-0.150, 0.390)
	INR target group 2.5-3.5	14	714 ± 284 (422 – 1322)	
				p-value/ r
	Warfarin daily dose (mg) [b]	91	4.1 ± 2.1 (0.9 – 12.5)	0.897/ -0.014
	Duration of warfarin therapy (months)	91	64 ± 54 (2 – 199)	0.281/ -0.114

[a] log transformed for data analysis
[b] sqrt transformed for data analysis

Table 6.2: Plasma uc-MGP concentration according to target INR range and associations between plasma uc-MGP and warfarin dose and duration of therapy

There was no correlation between uc-MGP and the previously detected plasma vitamin K concentration, as described and analysed in Chapter 5 (p=0.306, r=0.264; Pearson correlation), nor there was a difference in mean uc-MGP between the two groups with detectable and undetectable plasma vitamin K levels [95% CI: (-0.360, 0.167), Estimate for difference: -0.096; t-test].

6.5 Discussion

Anticoagulation therapy with warfarin is an important modality for the treatment and prevention of thromboembolic disorders in children. There is increasing evidence that long-term warfarin administration can have detrimental effects on bone mineralisation and vascular health as demonstrated in adult patients (Han and O'Neill, 2016, Mayer O Jr et al., 2016). Although such adverse effects of warfarin therapy have not been previously assessed in children, tracheobronchial calcifications are common in children with congenital heart disease on warfarin without firm evidence of a causal relationship (Golding et al., 2013). Cases of warfarin-induced calcification have been reported after both mitral valve replacement and Fontan surgery (Eckersley et al., 2014, Rifkin and Pritzker, 1984). Cardiac vessel calcification is associated with congenital heart disease in both adults and children (Topaz, 1986, Halpern et al., 2015, Nance et al., 2015). Underlying heart condition, immunosuppressive therapy and warfarin therapy have been identified as potential risk factors for calcifications (Nance et al., 2015).

Lately, quantification of a specific form of extrahepatic MGP, namely uc-MGP, a biomarker associated with vascular calcification, has become possible through the use of a conformation-specific ELISA (Cranenburg et al., 2010). The present study thus used the ELISA technique to examine the possible impact of warfarin therapy on arterial calcification in children.

As this is a post-hoc study and the subjects were part of a previous study, a retrospective power calculation was performed to assess what difference could be found with this study population. Regarding the association between log(uc-MGP) levels and target INR, with groups of 14 and 77 children, there is an 80% chance of detecting a difference in means of 0.823 (SD: ±0.385). The actual difference is 0.12. For the correlation between uc-MGP and both warfarin dose and duration of therapy, with 91 observations there is an 80% chance of detecting a correlation of r= ±0.289. Actual correlations were r= -0.014 and r= -0.114.

Anticoagulation intensity was found to have no effect on circulating plasma uc-MGP concentrations in children. Also, we found no association between plasma uc-MGP levels and either duration of warfarin therapy or warfarin daily dose.

The possible association between warfarin therapy and valvular/vascular calcification has mainly been studied in elderly patients with kidney disease and

calcification has been detected using a variety of methods apart from circulating uc-MGP levels [echocardiogram, computed tomography (CT), biopsy, cardiac magnetic resonance imaging (MRI)] (Palaniswamy et al., 2011). As there is no prior information available on plasma uc-MGP concentrations in anticoagulated children, the results cannot be directly compared with the published data in adult cohorts (Koos et al., 2009, Cranenburg et al., 2010, Cranenburg et al., 2009). However, based on a study in anticoagulated adult patients with calcific aortic valve disease (Koos et al., 2009), lower uc-MGP concentrations would be expected in anticoagulated children than those in non-anticoagulated children.

Only one previous study by Shroff et al has investigated plasma uc-MGP concentrations in children (albeit healthy children) using the same ELISA method as that used in our study. In this study the mean±SD uc-MGP concentration in healthy children was 527±185µM, ranging from 220 to 1000µM. Plasma uc-MGP was independent of age (Shroff et al., 2008). Similar to adults, the mean plasma uc-MGP concentration in healthy children is twice as high compared to children on dialysis (232±116µM) (Shroff et al., 2008). Importantly, Shroff et al reported no association between plasma uc-MGP concentration and vascular stiffness or other clinical and vascular measures of calcification assessed in their study (Shroff et al., 2008). However, the plasma uc-MGP concentrations in children reported by these authors are much greater compared to plasma uc-MGP concentrations found both in our cohort of children and those in adults (Cranenburg et al., 2008). There is concern on my part as to whether plasma uc-MGP concentrations reported by Shroff et al has been correctly reported (µm units reported by Shroff et al versus nM reported by Cranenburg et al (Cranenburg et al., 2010)). Notably, the detection limit of the assay is 98nM as reported by Cranenburg et al who first introduced the assay (Cranenburg et al., 2010). It is possible that the vast discrepancy in plasma uc-MGP reported between the abovementioned and other studies (Cranenburg et al., 2008, Dalmeijer et al., 2013, Parker et al., 2010a, Parker et al., 2010b) is a genuine reflection of the vast differences in plasma uc-MGP concentrations that exists between anticoagulated children and healthy children.

6.6   Limitations

Our findings might be mitigated by the fact that the ELISA assay used to measure plasma uc-MGP concentration was originally validated using citrated plasma

whereas the current study used heparinised plasma. Nevertheless, this discrepancy should not affect the overall study findings.

Another consideration is that only a small number of children with the higher target INR range were included in this study (14 children with target INR range of 2.5-3.5 versus 77 children with target INR of 2.0-3.0). In addition, the target INR range between the two groups is overlapping, which was due to the cohort of patients having been recruited as part of a previous study and as a result I had no control over the recruited patients.

Finally, as there is no clear consensus on the reported uc-MGP levels in healthy children, having studied a separate cohort of healthy children and measured their uc-MGP levels in plasma at the same time with the same assay would give the opportunity for direct comparison between uc-MGP plasma levels in anticoagulated and healthy children.

The observed high occurrence of tracheobronchial calcification in children on warfarin could also be due to the poor cardiovascular health of these children. This could only be studied in populations of children with cardiovascular conditions that necessitate anticoagulation with warfarin versus children with other cardiovascular conditions that do not receive warfarin anticoagulation.

6.7 Conclusion

In conclusion, in this preliminary study we found no association between circulating plasma uc-MGP concentration and either the intensity of anticoagulation, warfarin treatment duration, or warfarin daily dose in children. However, to confirm our findings and assess the usefulness of circulating uc-MGP as a biomarker for vascular calcification in children, further prospective studies in larger population of children on chronic warfarin therapy with carefully selected groups of paediatric patients having non-overlapping target INR ranges, and which allow for other potential confounders of vascular calcification, will be needed.

Chapter 7   Investigation of the effect of ageing on the pharmacological activity of the factor Xa inhibitors, rivaroxaban and apixaban

7.1   Introduction

Direct oral anticoagulants (NOACs), including the factor IIa inhibitor, dabigatran and factor Xa inhibitors, rivaroxaban, apixaban and edoxaban, have been approved for the treatment of thrombosis, the prevention of thromboembolism in post-operative patients, as well as the prevention of thromboembolic strokes in patients with AF; in the latter NOACs having been demonstrated to be non-inferior to warfarin in clinical effectiveness (Connolly et al., 2009, Patel et al., 2011, Granger et al., 2011, Giugliano et al., 2013). Prescription numbers for NOACs have grown rapidly both for patients newly diagnosed with AF and for patients taking coumarins, in particular those with unstable anticoagulation control (Abo-Salem and Becker, 2014, Xu et al., 2013, Agnelli et al., 2013, Kirley et al., 2012), such that they now account for 31% of treated patients and around 93% of expenditure on anticoagulant therapy (Lowenstern et al., 2018).

Bleeding associated with anticoagulation therapy remains the major concern for clinicians and patients. Whilst quantification of the risk of bleeding has been inconsistent due to differences in study sample sizes, patient populations and time frames, studies with NOACs in general indicate an increased risk of gastrointestinal haemorrhage and a lower risk of intracranial bleeding compared to warfarin (Tamayo et al., 2015) and with apixaban and edoxaban being superior to rivaroxaban, dabigatran and warfarin in terms of bleeding risk, according to studies reviewing RCT and real-world data (Hellenbart et al., 2017).

Subgroup comparisons of trial data between younger (< 75 years) and older (≥ 75 years) patient groups show higher bleeding rates in the older cohort (Hellenbart et al., 2017), with the most elderly also showing to be at highest risk of bleeding complications in clinical practice (Kirchhof et al., 2016). It is possible that age per se influences anticoagulation response to NOACs. However, it is difficult to evaluate the inherent effect of age on the pharmacological activity of NOACs, being unable to control in vivo for the presence of the potentially confounding influences of age-related changes in body mass, decline in renal function, increasing comorbidities and concurrent medication (Glassock and Rule, 2012, Deedwania, 2013).

7.2 Aim of the study

This study aimed to determine the effect of age on the pharmacological activity of the direct factor Xa inhibitors, rivaroxaban and apixaban, by comparing *ex-vivo*, the effect of each drug on haematological parameters between a group of community dwelling fit elderly people and a group of fit young people.

7.3 Patients, materials and methods

The study was approved by the Newcastle upon Tyne Ethics Committee and was conducted in compliance with the Declaration of Helsinki.

7.3.1 *Sample size calculation*

As there was no *a priori* information available on the extent to which age affects haematological response to rivaroxaban ex-vivo in humans, we deemed that a sample size of 60 subjects (30 elderly and 30 young) would be sufficient to test our hypothesis that ageing enhances the pharmacological activity of factor Xa inhibitors.

7.3.2 *Study subjects*

Following written informed consent, fit elderly subjects were recruited from a local day centre and healthy young subjects were recruited from amongst university staff members. Subjects with liver dysfunction, or other disease, or receiving any medication known to affect haemostasis were excluded. All subjects were generally in good health, had normal diets, were living independently and did not consume alcohol for the last 24 hours before recruitment. Each subject provided a fasted venous blood sample (20ml) collected in citrated tubes. Following double centrifugation at 2000× g for 7 min each, the plasma samples were aliquoted and stored at -80ºC for later analyses.

7.3.3 *Haematological assessments*

All tests were carried out at the accredited Haematology Laboratory at Freeman Hospital, Newcastle upon Tyne Hospitals NHS Foundation Trust. Rivaroxaban and pure powder were kindly provided by Bayer Schering Pharma (Berlin, Germany). Apixaban was commercially available from a local distributor and was initially manufactured from Bristol Myers Squib. Both powders were solubilised in dimethyl

sulfoxide solution (DMSO, Sigma-Aldrich, Missouri USA) according to the manufacturer's recommendations, at a final concentration of 10µg/ml.

Assessment of the factors affecting clotting tests was initially performed. The temperature at which plasma samples were thawed, the presence or absence of centrifugation of the thawed sample as well as the time that the sample remained on the heat block were investigated.

After analysing the impact of these factors, on the day of each experiment plasma aliquots were thawed at $37^{\circ}C$ on heat block for a maximum of 15 minutes and centrifuged at 2000x g for 7 minutes. Plasma samples were incubated with rivaroxaban (0, 100, 250, 400, and 500 ng/ml) and apixaban (0, 75 and 150ng/ml) at a concentration range similar to that observed in plasma following oral dosing (Kubitza et al., 2005a, Kubitza et al., 2005b, Baglin et al., 2012). Control plasma samples were incubated in parallel with the clinical samples to gauge the accuracy of the measurements.

The IL TOP ACL 700 CTS instrument (Instrumentation Laboratory Company, Bedford, MA, USA) was used for all coagulation assays. Prothrombin time (PT) was measured using RecombiPlasTin2G® and APTT using HemosIL® (Instrumentation Laboratory Company, Bedford, MA, USA) (Rodgers et al., 2013). Clotting time was also assessed using modified prothrombin time (mPT), which is a PT assay modified by adding calcium chloride ($CaCl_2$) to the RecombiPlasTin 2G® reagent to expand sensitivity and enhance assay dynamics. After reconstituting the thromboplastin reagent with distilled water, it was diluted 1:2.25 with 100mmol/L $CaCl_2$ solution, prepared with anhydrous powder (VWR, Pennsylvania, USA) (Barrett et al., 2013). Clotting factor II (FII), VII (FVII), IX (FIX), and X (FX) activities in plasma were measured with standard clotting assays using respective clotting factor deficient plasma (Instrumentation Laboratory Company, Bedford, MA, USA). The average coefficient of variation for replicate analysis of samples at baseline and with factor Xa inhibitor using control plasma were as follows; for PT (4.9 and 2.9%); for mPT (5.7 and 4.8%); for APTT (6.4 and 4.3%); for fibrinogen (16.4 and 17%); for FII (7 and 6%); for FVII (17.7 and 15%); for FIX (21.8 and 14%) and for FX (13.6 and 12.3%).

FX and FIX antigen levels were measured using commercially available ELISA kits (Diagnostica Stago, Parsippany, NJ, USA). (**Appendix C and D**)

7.3.4 *Thrombin Generation Assay*

Thrombin generation assay is used to evaluate the endogenous capacity of the overall haemostatic potential and is therefore a measure of coagulability of blood. More specifically, it is useful in measuring the speed in which a clot is formed by assessing thrombin generation and decay. Despite its limitations including the lack of standardisation to date, studies have demonstrated the wide range of applications of this assay in clinical practise, as well as its potential superiority over routine coagulation assays (Duarte et al., 2017, Mullier et al., 2012, Tripodi, 2016).

A number of parameters of thrombin generation assay were analysed. Lag time is the time until thrombin generation commencement; peak height shows the maximum thrombin concentration; time to peak (ttpeak) is the time it takes to reach the maximum amount of thrombin; endogenous thrombin potential (ETP) indicated the total amount of thrombin generated; and velocity index which is the speed of thrombin generation/clot formation. (**Figure 7.1**)
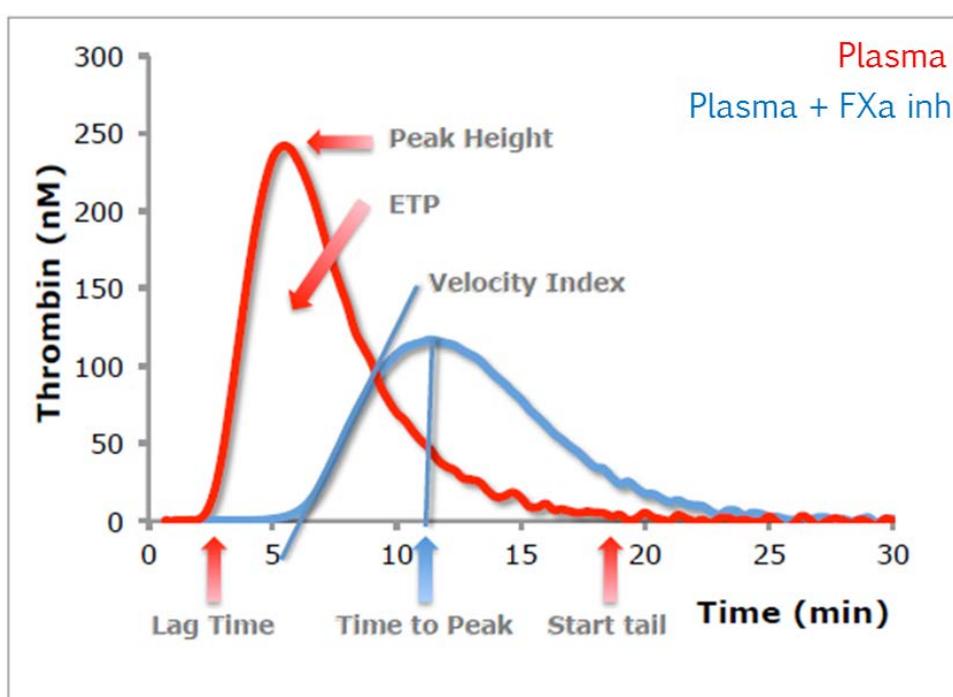
Figure 7.1: Thrombin generation assay parameters in plasma (red) and plasma spiked with a factor Xa inhibitor (blue)

Thrombin generation assay was performed using the Calibrated Automated Thrombogram with Fluoroskan® Ascent Fluorometer (Thermo Fisher Scientific, Waltham, MA) and the thrombinoscope software (Thrombinoscope BV 5.0). The assay was performed by pipetting 20µl platelet-poor plasma (PPPH) reagent (20pmole/L TF) and 10µl factor Xa inhibitor solution in DMSO (final concentration in plasma was 187.5 ng/ml for rivaroxaban and 100 ng/ml for apixaban), or 10µl DMSO (for baseline measurement) into individual wells of a 96-well microtiter plate with 70µl plasma. After 10 minutes of pre-incubation at 37°C, the reaction was started by the addition of 20µl FluCa-kit. The fluorescence was measured for 120 minutes at 37°C (excitation, 390 nm; emission, 460 nm) (detailed protocol in **Appendix E**).

The concentration selected for incubation of subject plasma with both rivaroxaban and apixaban was similar to the mean plasma concentrations after oral dosing (Kubitza et al., 2005a, Kubitza et al., 2005b, Baglin et al., 2012). For rivaroxaban, the concentration selected was 187.5ng/ml and for apixaban, the concentration of 100ng/ml was chosen.

7.3.5 *Statistical Analysis*

Data were checked for normal distribution. Logarithmic transformation was applied to data not normally distributed to approach normality. Parametric (Student's t-test) and non-parametric (Mann-Whitney U) tests were used to compare data; a p-value of < 0.05 was considered to be statistically significant. Microsoft Excel (Microsoft Corp., Redmond, WA, USA) and Statistical Package for Social Sciences (SPSS version 22) were used for data analyses and reporting. Data are presented as mean±SD unless otherwise stated.

7.4 Results

7.4.1 *Rivaroxaban*

7.4.1.1 Study subjects

Two groups of subjects were recruited; 36 fit elderly subjects (16 males) and 30 healthy young subjects (10 males). The median (IQR) age and weight of the elderly and young subjects were [83 (75-87) v 30 (26-38) years; p<0.001] and 77 (69-89) v 72 (64-85) kg, respectively.

7.4.1.2 Factors affecting routine clotting tests

Based on previous findings with control plasma (Abohelaika, 2017), PT as opposed to mPT, APTT and fibrinogen showed no significant variability between different days of analysis. A number of factors were deemed potentially important for the observed variability.

Plasma samples were prepared for analysis using routine clotting tests APTT and fibrinogen as well as mPT. Baseline samples consisted of pure plasma obtained from healthy volunteers, while plasma was also spiked with rivaroxaban (400ng.ml) to create a different set of samples. Results both at baseline and with 400ng/ml rivaroxaban are shown in **Table 7.1**.

In order to gauge the impact of temperature during plasma thawing (room temperature versus 37^0C on heat block), centrifugation after thawing and the time plasma samples stay on the heat block before use, a number of tests were performed in the same pool of control plasma donated from healthy volunteers. Clotting time at baseline (0ng/ml rivaroxaban in plasma) and at 400ng/ml concentration of rivaroxaban was assessed using mPT, APTT and fibrinogen assays. The conditions of sample preparation (thawing at 37^0C on heat block for 15 minutes followed by centrifugation) remained the same for all the samples to reduce the variability in the above-mentioned clotting tests and eliminate the chance of type I or II errors.

The thawing temperature had a significant effect on plasma fibrinogen levels and mPT, although it had a lesser effect on APTT. At 25^0C compared to 37^0C, plasma fibrinogen levels were reduced both at baseline and 400ng/ml rivaroxaban, mPT increased mainly at 400ng/ml rivaroxaban concentration and APTT slightly increased at both rivaroxaban concentrations. The impact of centrifugation was significant only for mPT, mainly at 25^0C, but had no impact on APTT and fibrinogen. The time delay of plasma on heat block did not affect clotting time at baseline. Only mPT value decreased as time delay increased at 400ng/ml.

Tests	Baseline: 0 ng/ml			
	No centrifugation		Centrifugation	
	37⁰C	25⁰C	37⁰C	25⁰C
Time	**0 minutes**			
Fibrinogen	3	2.45	2.89	2.35
APTT	28.6	30.6	28.3	30.6
mPT	21.8	22.9	22.7	24.9
Time	**15 minutes**			
Fibrinogen	2.93	2.42	2.96	2.39
APTT	28.5	30.2	28.5	30.8
mPT	21.5	23.6	22.8	24.1
Time	**30 minutes**			
Fibrinogen	3.06	2.42	3	2.38
APTT	29.2	30	29.3	30.4
mPT	22.7	22.8	23.9	24.6
	Rivaroxaban: 400 ng/ml			
	No centrifugation		Centrifugation	
	37⁰C	25⁰C	37⁰C	25⁰C
Time	**0 minutes**			
Fibrinogen	2.2	1.82	2.21	1.78
APTT	51.4	54.4	51.9	54.4
mPT	63.5	68.3	71.3	76.7
Time	**15 minutes**			
Fibrinogen	2.19	1.83	2.22	1.79
APTT	50.3	53.2	52.8	54
mPT	63	67.9	65.2	74.3
Time	**30 minutes**			
Fibrinogen	2.23	1.82	2.21	1.77
APTT	51	52.9	51.7	53.9
mPT	66.5	62	65.9	70.4

Table 7.1: Impact of temperature, centrifugation and time delay between preparation and time of run on clotting tests (data presented as mean)

7.4.1.3 Haematological measurements

Rivaroxaban concentration-dependently prolonged PT and mPT (**Figure 7.2a, b**). Rivaroxaban caused a significantly greater prolongation of PT (p<0.05) and mPT (p<0.001) in elderly subjects compared to young subjects across the rivaroxaban concentration range studied. The mean difference in PT between the two groups ranged from 1.6s at 100 ng/ml to 6.1s at 500 ng/ml plasma rivaroxaban concentrations. The mean difference in mPT between the two groups ranged from 23.5s at 100 ng/ml to 71.1s at 500 ng/ml plasma rivaroxaban concentrations.
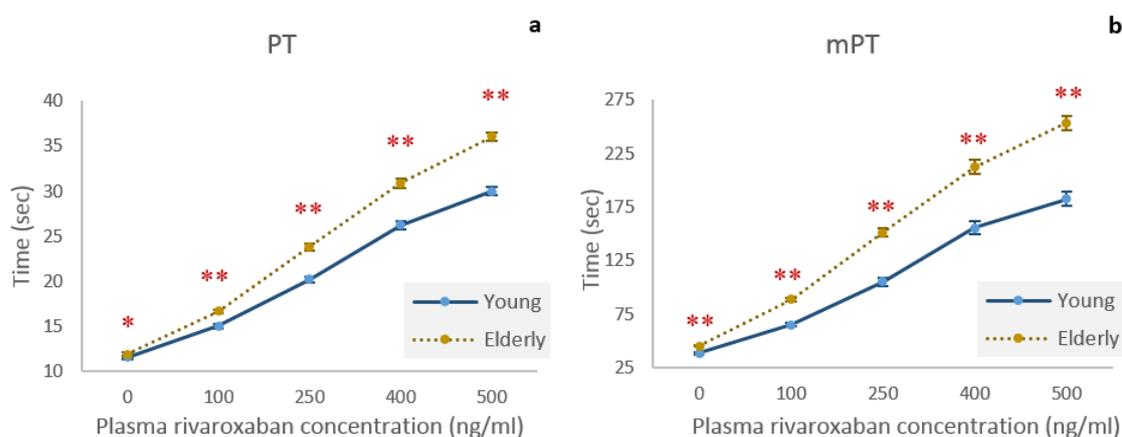
Figure 7.2: Mean (SEM) PT (a) and mPT (b) prolongation by rivaroxaban in young and elderly subjects

The mean baseline FX activity in elderly subjects was similar to that in the young subjects (**Figure 7.3a**). In both elderly and young subjects, FX activity decreased in the presence of rivaroxaban in a concentration-dependent manner. Rivaroxaban caused a significantly greater inhibition of FX activity in the elderly compared to the young subjects (**Figure 7.3b**). There was a close correlation between FX antigen and factor activity levels (r=0.632, p<0.001; **Figure 7.4**). According to the ELISA assay functional FX antigen levels were lower in the elderly compared to the young group of subjects (median±SD: FX:Ag= 70.4±16.3% v 83.3±13.4%, p=0.001, t-test). Calculation of the activity/antigen ratio showed relative suppression of factor X

protein expression in the elderly, but functionality was similar between the two groups (FX:C / FX:Ag =1.25 v 1.03 for young and elderly, respectively).

At baseline, mean FII activity was significantly lower in the elderly compared to young subjects (p=0.002) (**Figure 7.3c**). Although FII activity was significantly lower in the elderly group compared to the young group across the entire rivaroxaban concentration range studied, there were no significant differences in the extent of FII activity inhibition between the two groups (**Figure 7.3d**). In both elderly and young subjects, FIX activity decreased in the presence of rivaroxaban in a concentration-dependent manner. Mean FIX activity at baseline and in the presence of rivaroxaban was significantly greater in the elderly compared to the young subjects (110.2±22.8% v 90.7±18.5%; p=0.001 at baseline, 38.2±10.3% v 29.8±7.5%; p=0.007 at 500ng/ml) (**Figure 7.3e**). FIX was inhibited to a similar extent in both groups.

There were no significant differences between the two groups in FVII activity both at baseline and in the presence of rivaroxaban (88.1±28.8% v 97.0±30.9% at baseline and 45.4±15.8% v 49.6±17.9% at 500ng/ml for young v elderly subjects respectively) (**Figure 7.3f**). Baseline plasma fibrinogen concentration was significantly greater in the elderly compared to the young group (4.34±1.08 v 3.79±0.65 g/L, p=0.02). However, fibrinogen concentrations were similar between the young and the elderly groups in the presence of rivaroxaban (2.92±0.71 v 2.88±0.42 g/L at 150ng/ml for elderly v young subjects) (**Figure 7.3g**).

APTT was prolonged in a concentration-dependent manner in both groups; however, the prolongation was greater in the elderly compared to young subjects across the rivaroxaban concentration range studied (p<0.01 for all comparisons) (**Figure 7.3h**).
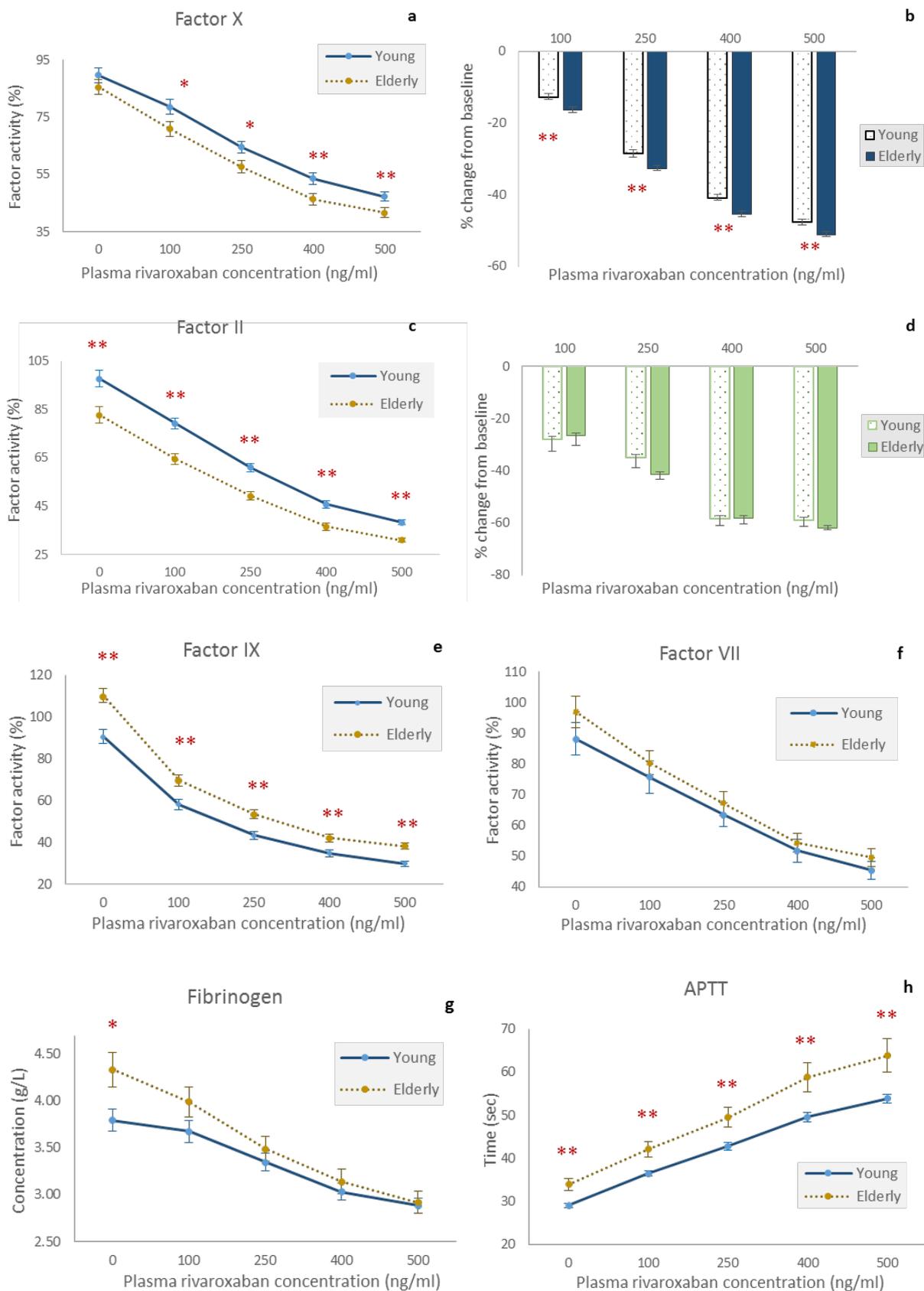
* p<0.05, **p≤0.01 (t-test)

Figure 7.3: Mean (SEM) clotting factor activity (a-g) inhibition and APTT prolongation (h) by rivaroxaban in young and elderly subjects
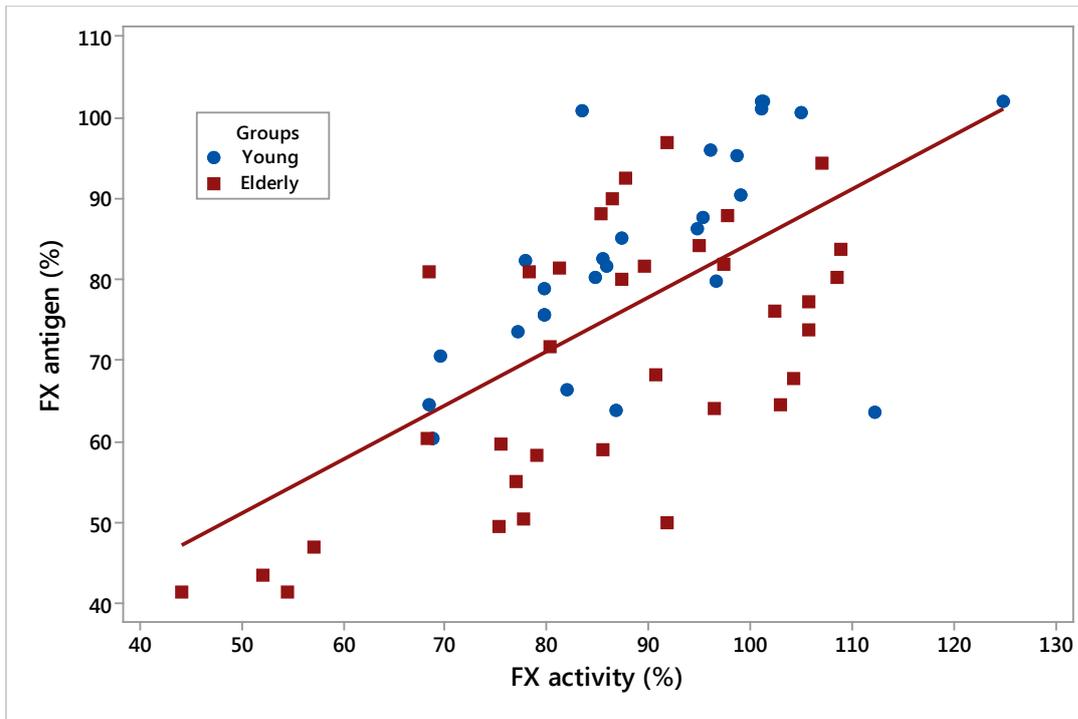
Figure 7.4: Scatterplot of factor X antigen and activity levels at baseline for rivaroxaban cohort by subject group

7.4.1.4 Thrombin generation assay

At baseline, the elderly subjects had a longer initiation phase of thrombin generation, as demonstrated by the longer lag time (p=0.01) and ttpeak (p=0.001), and significantly higher ETP (p=0.004), peak thrombin concentration (p<0.001) and velocity index (p<0.001) compared to the young subjects (**Table 7.2**).

In both young and elderly subjects rivaroxaban (187.5ng/ml) significantly affected the key parameters of the thrombin generation assay (**Table 7.2**). Rivaroxaban caused a greater prolongation of lag time as demonstrated by the greater percentage difference from baseline in the elderly compared to the young subjects (p=0.03). Rivaroxaban also suppressed the propagation phase of thrombin generation to a greater extent in elderly subjects compared to the young, as shown by a greater decrease from baseline in both peak (p<0.001) and ETP (p=0.002).

An example thrombogram from a young and an elderly subject is presented in **Figure 7.5**. The change from baseline for both ttpeak and velocity index were comparable between the two groups (**Figure 7.6**).

	Baseline		Rivaroxaban	
	Young	Elderly	Young	Elderly
Lag time (min)	2.9 (0.1)	3.3 (0.1)	9.6 (0.2)	11.6 (0.6)
Peak (nM)	322.9 (8.2)	263.9 (7.9)	67.0 (4.2)	41.3 (1.8)
ttpeak (min)	5.5 (0.1)	6.4 (0.2)	25.6 (0.7)	29.1 (0.9)
ETP (nM*min)	2396.9 (57.0)	1978.4 (63.9)	1682.2 (51.6)	1279.7 (38.3)
Velocity index (nM/min)	127.7 (5.4)	87.7 (4.8)	4.7 (0.6)	2.6 (0.2)

Table 7.2: Thrombin generation assay parameters, at baseline and with the presence of rivaroxaban. Data are presented as mean ± SEM
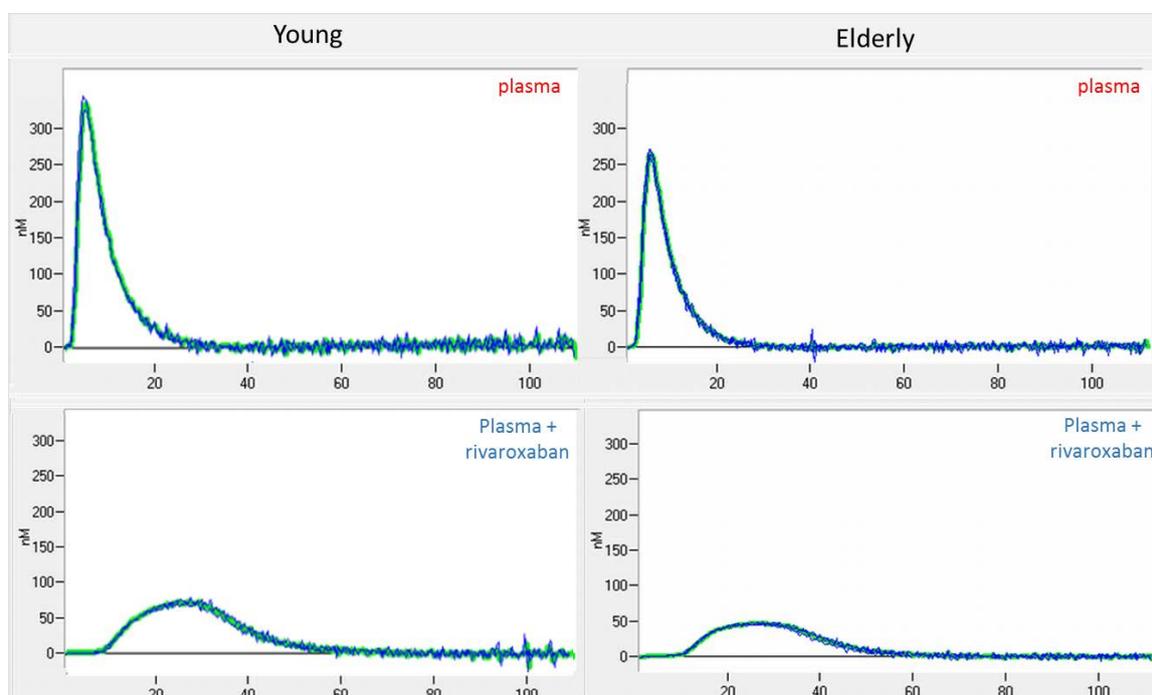
Figure 7.5: Representative thrombogram of a young and an elderly subject
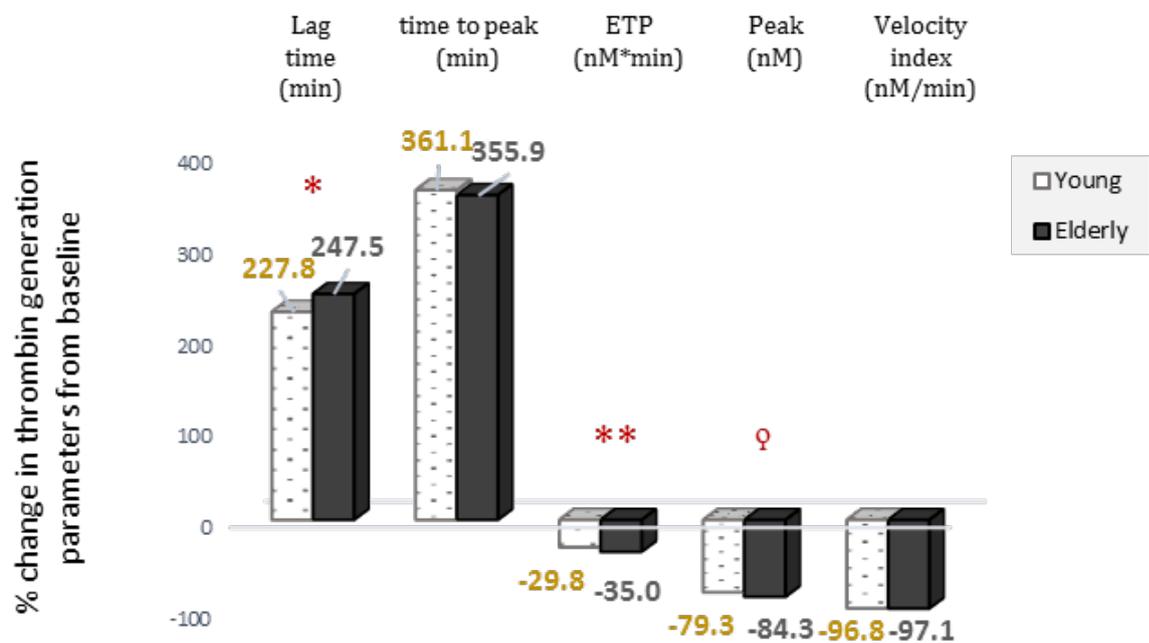
* p<0.05, ** p≤0.01 (t-test), ♀ p≤0.01 (Mann-Whitney U test)

Figure 7.6: Change from baseline in thrombin generation assay parameters for rivaroxaban cohort

7.4.2 *Apixaban*

7.4.2.1 Study subjects

Following the completion of the rivaroxaban experiments there was sufficient plasma remaining from only 17 subjects (7 males) in the elderly cohort to carry out experiments with fewer apixaban concentrations. The median (IQR) age and weight of the elderly and young subjects included for the apixaban study were [83 (79-90) v 30 (26-38) years; p<0.001] and 72 (64-85) v 74 (67-82) kg, respectively.

7.4.2.2 Haematological measurements

Due to the limited volume of plasma available from the elderly cohort, haematological tests were performed at 150ng/ml (n=17) and at the 75ng/ml (n=8) apixaban final concentration. Apixaban concentration-dependently prolonged PT and mPT (**Figure 7.7a**). Apixaban caused a significantly greater prolongation of PT (p<0.001) in elderly subjects compared to young subjects at the highest concentration studied (150ng/ml).
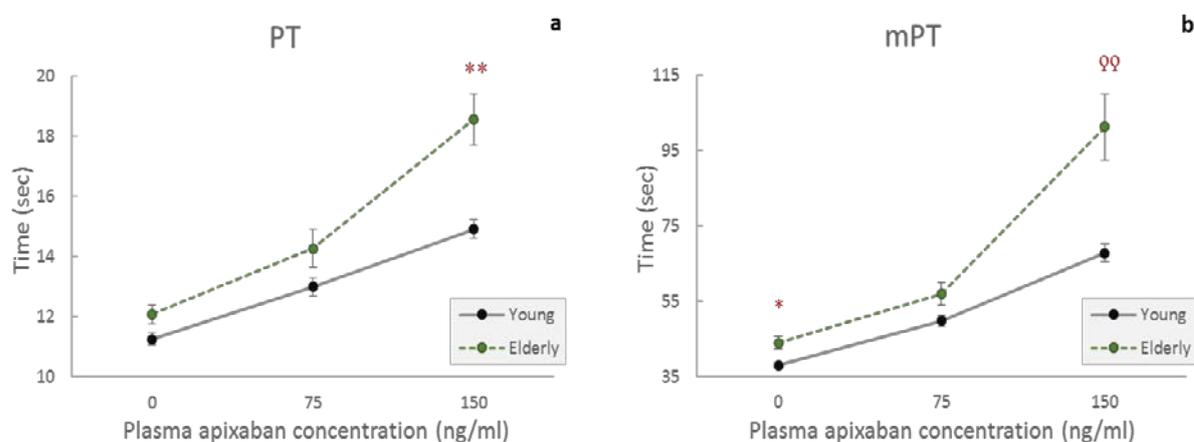
* p<0.05 (t-test), ** p≤0.01 (t-test), ♀♀ p≤0.01 (Mann-Whitney U test)

Figure 7.7: Mean (SEM) PT (a) and mPT (b) prolongation by apixaban in young and elderly subjects

At baseline, mPT was greater in elderly compared to young subjects (p=0.004). Apixaban caused a greater prolongation of mPT (p<0.001) in elderly subjects compared to young subjects at 150ng/ml. At 150 ng/ml plasma apixaban

concentration, the mean difference from baseline between the two groups in PT was 3.6s, which was not statistically significant and in mPT was 33.4s, which was statistically significant (**Figure 7.7b**).

The mean baseline FX activity in elderly subjects was similar to that in the young subjects (**Figures 7.8a**). In both elderly and young subjects, FX activity decreased in the presence of apixaban in a concentration-dependent manner. Apixaban caused a significantly greater inhibition of FX activity (at 150ng/ml) in the elderly compared to the young subjects (**Figure 7.8b**).

At baseline, mean FII activity was significantly lower in the elderly compared to young subjects (p=0.001) (**Figure 7.8c**). Although FII activity was significantly lower in the elderly group compared to the young group across the apixaban concentration range studied, there were no significant differences in the extent of FII activity inhibition between the two groups) (**Figure 7.8d**).

FIX activity was similar both at baseline and in the presence of rivaroxaban between the young and elderly groups (**Figure 7.8e**). There were no significant differences between the two groups in FVII activity both at baseline and in the presence of apixaban (96.0±30.8% v 110.3±63.1% at baseline and 45.4±15.8% v 30.9±17.9% at 500ng/ml for elderly v young subjects respectively) (**Figure 7.8f**). Baseline plasma fibrinogen concentration was significantly greater in the elderly compared to the young group (4.18±0.78 v 3.88±0.54 g/L, p=0.001). However, fibrinogen concentrations were similar between the young and the elderly groups in the presence of apixaban (3.45±0.98 v 3.19±0.52 g/L at 150ng/ml for elderly v young subjects) (**Figure 7.8g**).

APTT was prolonged in a concentration-dependent manner in both groups. Although at baseline, APTT was similar between the two groups, the prolongation was greater in elderly compared to young subjects at 150ng/ml apixaban concentration (p=0.018) (**Figure 7.8h**).
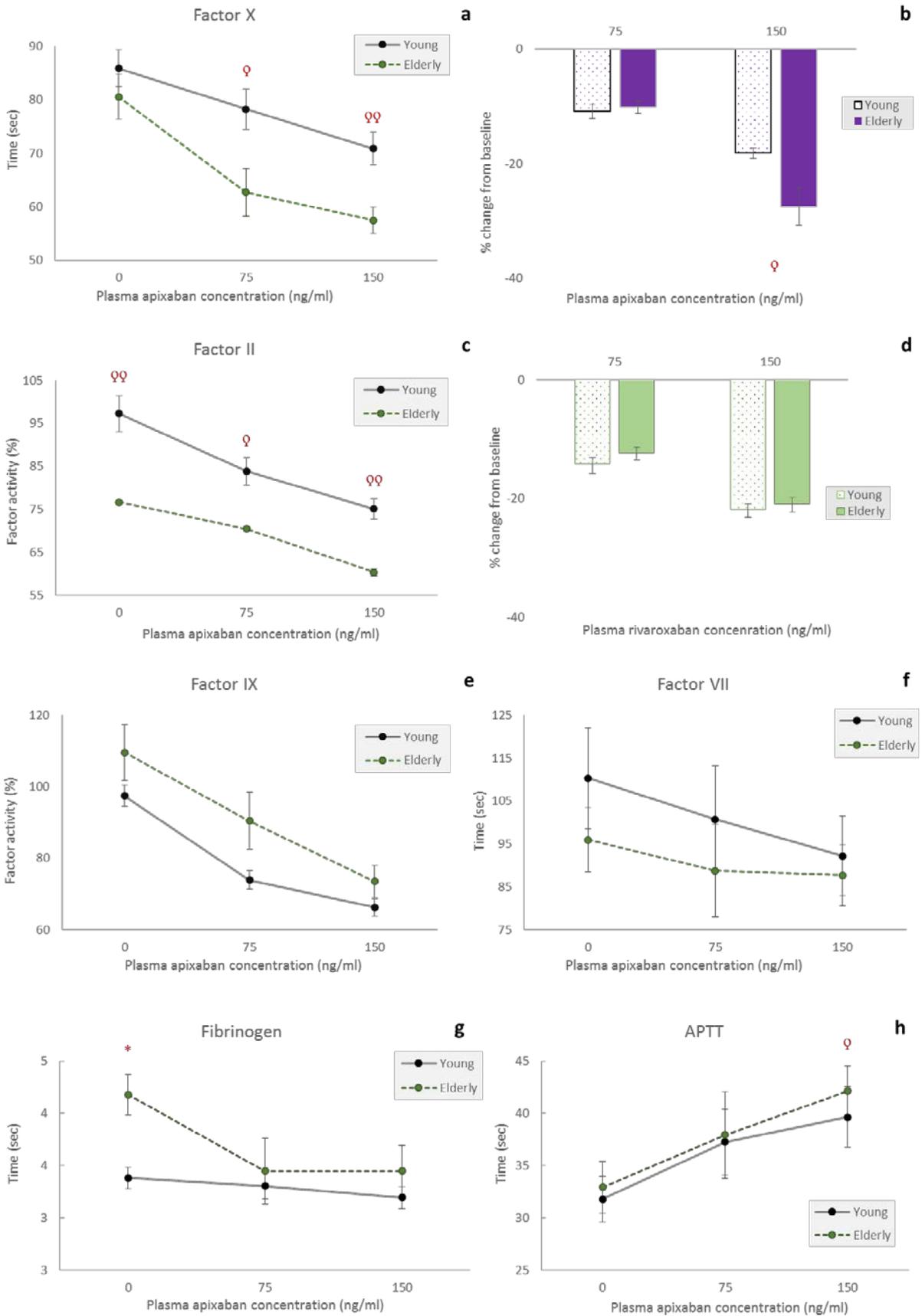
�okp<0.05 (Mann-Whitney U test), ��okp≤0.01 (Mann-Whitney U test)

Figure 7.8: Mean (SEM) clotting factor activity (a-g) inhibition and APTT prolongation (h) by rivaroxaban in young and elderly subjects

7.4.2.3 Thrombin generation assay

At baseline, the elderly subjects had a longer initiation phase of thrombin generation, as demonstrated by the longer lag time (p=0.002) and ttpeak (p=0.003), and significantly higher ETP (p=0.026), peak thrombin concentration (p=0.014) and velocity index (p=0.014) compared to the young subjects (**Table 7.3**).

		Lag time (min)	Peak (nM)	ttpeak (min)	ETP (nM*min)	Velocity index (nM/min)
Baseline	Young	3.1 (0.11)	301.3 (9.30)	5.7 (0.15)	2210.8 (70.51)	117.6 (6.39)
	Elderly	4.1 (0.25)	260.7 (12.21)	7.0 (0.34)	1898.3 (79.23)	91.9 (7.43)
Apixaban	Young	8.1 (0.62)	70.2 (5.81)	22.1 (1.14)	1649.9 (69.26)	6.5 (1.28)
	Elderly	10.5 (0.83)	45.3 (4.05)	23.5 (1.44)	1183.8 (67.27)	3.9 (0.68)

Table 7.3: Thrombin generation assay parameters, at baseline and with the presence of apixaban. Data are presented as mean ± SEM

In both young and elderly subjects, apixaban (100ng/ml) significantly affected the key parameters of the thrombin generation assay (**Table 7.3**). Apixaban suppressed the propagation phase of thrombin generation to a greater extent in elderly subjects compared to the young, as shown by a greater decrease from baseline in both peak (p=0.011), ttpeak (p=0.012) and ETP (p=0.001). The change from baseline for both lag time and velocity index were comparable between the two groups (**Figure 7.9**).
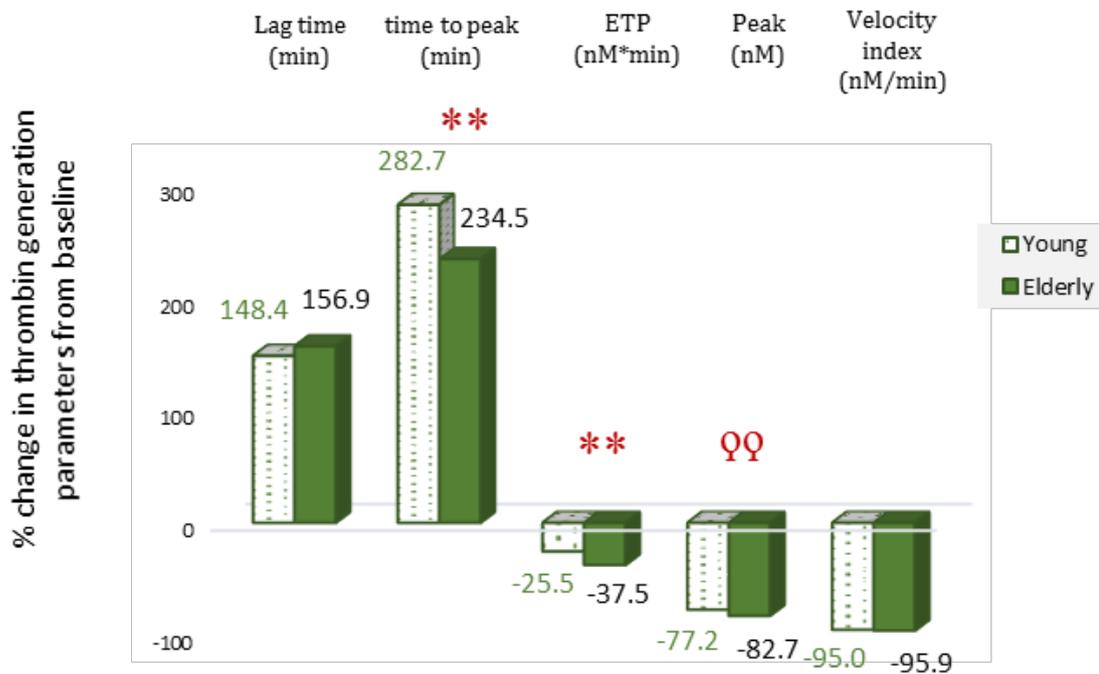
** p≤0.01 (t-test), ϙϙ p≤0.01 (Mann-Whitney U test)

Figure 7.9: Mean (SEM) FIX activity for apixaban cohort

7.5 Discussion

Elderly people were not well represented in the major clinical trials evaluating the safety and efficacy of NOACs and this is of concern for clinicians prescribing them. In the ROCKET-AF trial, for example, the median age of the patients on rivaroxaban therapy was 73 years and only a quarter of the patients were 78 years of age or older (Patel et al., 2011). In the ARISTOTLE trial, the median age of patients on apixaban was 70 years or over, less than a third of patients were 75 years of age or older and only 13% were aged 80 years or over (Granger et al., 2011, Halvorsen et al., 2014). In the AMPLIFY trial, the mean age of patients on apixaban was 57.2 years (Agnelli et al., 2013). Post-marketing surveillance studies have reported bleeding rates associated with rivaroxaban to be similar to those reported in the ROCKET-AF trial (2.86 per 100 person-years (95% CI: 2.61-3.13) and 3.6 per 100 person-years (HR 1.04 (95% CI: 0.90-1.20) respectively) (Tamayo et al., 2015, Eikelboom and Merli, 2016, Villines and Peacock, 2016, Peacock, 2015, Adeboyeje et al., 2017). However, a real-world observational study reported a higher bleeding event rate of 17.2% for rivaroxaban compared to 7% for dabigatran, and 8.7% for apixaban (Al-Khalili et al., 2016). The incidence rate of hospitalization for NOAC-related bleeding has been

reported as 3.44 per 100 person-years (95% CI: 2.35-4.86) with 87% of the admissions being aged ≥75 years, reflecting both the age profile of those prescribed these drugs and their increased risk of bleeding due to age, renal insufficiency, comorbidities and concomitant medication. Of these patients, 19% were receiving the maximum therapeutic daily doses in spite of dose reduction recommendations (Garbayo et al., 2018).

Current guidelines suggest dose adjustment for NOACs in relation to serum creatinine levels on the basis that renal impairment increases systemic drug exposure, with impaired renal function accounting for approximately a 44 to 64% increase in rivaroxaban systemic exposure and 56% higher exposure to apixaban (Byon et al., 2017), and adhering to these guidelines could reduce bleeding episodes. A meta-analysis of randomised trials has indicated that, in patients with renal insufficiency, the recommended doses of NOACs are non-inferior and relatively safe compared to warfarin (Sardar et al., 2014b), which indicates that the increase in bleeding risk suggested by drug adverse event reporting and case reports may be contributed to by other factors, such as old age (Harper et al., 2012). Unlike the guidance for rivaroxaban use, apixaban guidelines suggest that age of 80 years or over is one of the three risk factors that would lead to receiving lower dose of apixaban (2.5mg compared to 5mg twice daily) (Halvorsen et al., 2014).

Evaluation of the true effect of age on anticoagulation response to NOACs *in vivo* is challenging in older people because of the difficulty in separating any such effect from co-factors which influence drug exposure, including declining renal function, change in body mass, concomitant medication and illness. To overcome this, I compared the pharmacological activity of rivaroxaban in groups of fit elderly and young subjects, *ex-vivo*. We purposefully selected the elderly subjects, on the basis of them having similar ages (72% in the rivaroxaban cohort and 82.3% in the apixaban cohort were aged 78 years and over) to those of the elderly population with AF who are prescribed anticoagulation therapy. None of the study subjects used any drugs which affect haemostasis or interact with the mode of action of factor Xa inhibitors. Rivaroxaban and apixaban concentrations chosen for this study were in the range of the plasma concentrations reported following therapeutic dosing (Kubitza et al., 2005a, Kubitza et al., 2005b, Baglin et al., 2012). The clotting time assay, PT and the global coagulation test, thrombin generation assay were performed and clotting protein activity was measured. PT measures the time taken to

initiate the clot formation after the stimulation of the extrinsic coagulation pathway. In contrast, thrombin generation assay measures not only the time to initiate thrombin generation but also the rate and amount of thrombin formed after stimulation with TF.

My results, which demonstrate elderly subjects to be more sensitive to the pharmacological activity of both rivaroxaban and apixaban than younger subjects, may indicate one mechanism for the observed trend to a higher risk of NOAC-associated major bleeding with age in the previous clinical trials (Schulman, 2014). There is evidence that the coagulation system becomes more active with increasing age, while fibrinolysis is impaired. Physiological changes taking place with increasing age can thus affect the production of activated clotting proteins (Palta et al., 2014, Franchini, 2006, Attena et al., 2015, Engbers et al., 2010, Abbate et al., 1993). Both factor Xa inhibitors produced a greater prolongation of prothrombin time (both PT and mPT) in the elderly than in the young subjects. The baseline FXa activity was similar between the two groups in both cohorts. The older subjects had a greater inhibition of FXa activity by rivaroxaban compared to the younger subjects, which was replicated in the apixaban cohort at the highest concentration (150ng/ml). Baseline FIX activity was significantly greater in the older subjects compared to the younger ones, which is consistent with the previously reported effect of ageing on FIX activity (Boland et al., 1995, Mari et al., 2008). Higher FIX levels are suggested to be associated with increased risk of venous thromboembolism (van Hylckama Vlieg et al., 2000) which increases with advancing age. FX and FIX antigen levels for all the study subjects were within the reported assay normal ranges. At baseline, the elderly subjects had significantly lower FII activity levels, but the extent of FII activity inhibition from baseline was similar between the two groups for both cohorts. Rivaroxaban prolonged time-based parameters of thrombin generation (lag time and ttpeak) and suppressed the rate and amount of thrombin generation (velocity index, peak and ETP) to a significantly greater extent in older subjects compared to young ones. Apixaban prolonged the ttpeak instead of lag time, while all other findings were similar to those for rivaroxaban.

Fibrinogen, apart from its key role in haemostasis, also has a role in acute-phase inflammation and plasma fibrinogen levels are shown to increase in response to interleukin-6, with both being strongly correlated with ageing (Ershler, 1993). The elderly subjects had significantly higher baseline fibrinogen levels compared to the young subjects in both cohorts, consistent with previous observations (Mari et al.,

2008). In the presence of rivaroxaban and apixaban, fibrinogen levels were found to be similar between the two groups. Factor VII plasma levels are also shown to progressively increase with age (Mari et al., 2008). Thrombotic disorders have been shown to be more frequent in subjects with higher plasma FVII levels but this has not been replicated in other studies (Tripodi, 2003). Interestingly, our study results showed that factor VII activity was similar between the two groups both at baseline and in the presence of both rivaroxaban and apixaban.

The results of this study support the hypothesis that elderly subjects are more sensitive to the pharmacological activity of both factor Xa inhibitors studied and that, in addition to factors which increase drug exposure, including impaired renal function, low body weight, concurrent medication and illness, age *per se* could be an important contributor to the reported incidence of bleeding associated with NOAC therapy in older patients.

Accurate, validated and clinically approved assays for use with NOACs are expected to be available in the near future and it is anticipated that they will be of considerable benefit for patients requiring invasive procedures and for the over-anticoagulated bleeding patients. More routine use of these assays before bleeding occurs might also be of benefit to some elderly patients who, because of their increased sensitivity to both rivaroxaban and apixaban, and potentially other NOACs yet to be investigated, may require lower doses of the drug to achieve therapeutic anticoagulation. As clinical experience with NOACs is too short to provide models of use which can take into account the influences of renal function, comorbidities, medication, weight and age to minimise the complication of bleeding and optimise the benefits of therapy, monitoring has the potential to make an important contribution to improving the clinical effectiveness of these already valuable drugs. Prospective studies in patients taking NOACs are needed in order to establish whether the therapeutic outcomes of using fixed doses supported by randomised trial results could be improved upon by NOAC monitoring and dose adjustment, to reduce stroke and bleeding events. Our results indicate that these studies should include the investigation of the effect of age as well as the established confounders of renal function, interacting drugs and body mass.

7.6 Limitations

There was insufficient plasma to carry out a full set of experiments with apixaban as had originally been planned. A small sample size may well be responsible for some of the statistically non-significant data observed with apixaban at 75 ng/ml.

There were some differences between the baseline values for rivaroxaban and apixaban, possibly due to a combination of day-to-day assay variability, reagent batch variability and the impact of multiple freeze-thaw cycles.

7.7 Conclusion

Elderly subjects were more sensitive to rivaroxaban compared to young subjects with the same levels of drug exposure ex-vivo. Both rivaroxaban and apixaban produced a greater prolongation of both PT and modified PT in the elderly compared to young subjects, while factor II and X activity levels were significantly lower in elderly than young subjects in both cohorts. Both factor Xa inhibitors prolonged time-based parameters of thrombin generation and suppressed the rate and amount of thrombin generation to a significantly greater extent in the elderly compared to young subjects. The impending validated assays for use with NOACs will be of considerable benefit for bleeding patients and those requiring urgent invasive procedures. Their more routine use might also benefit some elderly patients who, because of their age-related increase in sensitivity to the factor Xa inhibitors studied, may require lower doses of the drug for safe and therapeutic anticoagulation.

Chapter 8   General discussion

Current guidelines advocate using fixed-doses of oral vitamin K to reverse excessive anticoagulation in warfarinised patients either asymptomatic or with minor bleeds. Over-anticoagulated patients present with a wide range of INR values and response to fixed doses of vitamin K varies. Consequently, a significant proportion remain outside their target INR after vitamin K administration making them prone to either haemorrhage or thromboembolism. In an RCT performed at the Newcastle upon Tyne NHS Foundation Trust, I compared the performance of a novel tailored vitamin K dosing regimen to that of a fixed-dose regimen with the primary measure being the proportion of over-anticoagulated patients returning to their target INR 24h later. A fixed-dose regimen, whereby patients with an index INR >6.0 (asymptomatic or with minor bleeding) received orally either 1 or 2mg of vitamin K, was compared to a tailored dose regimen based upon an individualised dosing algorithm. The algorithm uses patient INR at presentation, target INR and BSA to calculate the vitamin K dose required.

I found that the tailored dose regimen was superior and more accurate to the fixed dose regimen in terms of the proportion of patients returning to within target INR above target INR range. Further refinement of the algorithm showed weight to be a more significant predictor of INR at 24h compared to BSA. This algorithm has already been adopted by the local Trust and used in routine practice for the reversal of non-urgent excessive anticoagulation in patients either asymptomatic or with minor bleeds.

A further, simplified version of this refined algorithm was also tested against its differences in the predicted vitamin K dose compared with the original refined algorithm. I found that the differences in computed vitamin K dose are minor and such differences unlikely to heavily affect the application of the more practical algorithm in clinical practice. This simple algorithm provides a powerful tool to clinicians who may even memorise the formula and who don't always have the opportunity to use an Excel spreadsheet for the calculation of the appropriate, individualised oral vitamin K dose in excessively anticoagulated patients presenting in clinic with high INR and requiring non-urgent reversal of anticoagulation.

Communication of the success of local practices to clinicians through committees and scientific conferences could lead to further refinement of the algorithm using a

larger more diverse sample size and eventually adoption of the individualised algorithm to other Trusts nationally. Further research and adjustment of the algorithm to other countries healthcare systems is also possible.

Stability of anticoagulation with an INR within the individual therapeutic range is a necessary commodity to ensure the safety of warfarin therapy. One measure of quality of anticoagulation control is %TTR. In my cohort study, I examined the association of %TTR between different genotype groups in a subset of patients who previously participated in the RCT and showed that *CYP2C9* and *VKORC1* genotypes were not associated with stability of anticoagulation during either induction or maintenance warfarin therapy. However, when a different type of analysis was employed, *VKORC1* appeared to be associated with %TTR only during maintenance therapy. Consequently, whilst patient genotype may be valuable information in predicting warfarin dose and thus preventing excessive anticoagulation and bleeding during initiation of therapy, combined genetic information from common SNPs at *CYP2C9* and *VKORC1* may not be useful in predicting anticoagulation stability. Further study for the role of VKORC1 is necessary to replicate my results.

It is common for patients on warfarin to discontinue their anticoagulation treatment a fixed number of days prior to surgery. However, studies report that there are still patients who present with therapeutic INR on the day of surgery, making the prediction of INR decline particularly useful for frail older patients with many comorbidities and those with extreme demographics. In a previous study, a pharmacogenetic-guided algorithm was developed including information on patient genetics, demographic and clinical data, which could predict the length of time needed for individual patients to withdraw warfarin in order for their INR to reach <1.5. I used data from an unrelated patient cohort on stable maintenance therapy who completed a short course of warfarin treatment for a reason unrelated to surgery, but providing the same opportunity to study INR decline following warfarin cessation. My results showed that the predicted decline in INR on day 5 was very closely correlated with the observed decline in INR value. In addition, the observed slight over-estimation and under-estimation of the fall in INR (by 0.2 units) in two patient subgroups was within the expected variability and would not be clinically relevant, but would mandate another INR check prior to an invasive procedure. With the cost of genotyping dropping every year, genotyping devices are quickly incorporated into clinical practice. However, even without the availability of

genotyping facilities, a healthcare system would still benefit from adopting the algorithm without pharmacogenetic information. I have showed that the predictive accuracy of the algorithm is only slightly undermined in patients with double variant *CYP2C9* genotype. The algorithm that includes only demographic and clinical characteristics could be a good alternative where genotyping is not easy or available. The algorithm I have validated could be further tested in a wider population as part of an RCT. If my study results are replicated, they could pave the way for the clinical utility of either the existing or a refined version of the algorithm on a wider scale.

Warfarin, a vitamin K antagonist, inhibits the recycling of vitamin K and can also lower vitamin K availability in the body. This has the potential to affect all vitamin k dependent proteins, such as osteocalcin in the bones and matrix-Gla protein which is a natural calcification inhibitor. In my second cohort study in an anticoagulated paediatrics population, I looked at the association between *CYP4F2* genotype and vitamin K availability. Plasma vitamin K concentration was measured by HPLC with plasma vitamin K being detected in only around 20% children. In the remaining children plasma vitamin K was below the assay limit of detection. When comparing the frequency of *CYP4F2* variant allele carriers, there was a significant difference between the two groups of children, suggesting that *CYP4F2* genotype influences vitamin K availability in children on warfarin. Several studies have shown that vitamin K deficiency can lead to reduced BMD and increased risk of fractures. Consequently, chronic low vitamin K availability in anticoagulated children may have important implications for physiological functions beyond the blood coagulation system and might be at increased risk of developing osteoporosis and cardiovascular disease later in life which need further investigation. Further assessment of BMD using DEXA (dual-energy x-ray absorptiometry) scan as well as detection of calcification using imaging techniques such as CT scans, intravascular ultrasound, MRI or angiography (Wang et al., 2018) in anticoagulated and non-anticoagulated (control) children would help identify any links to vitamin K deficiency.

In adult populations, there are studies reporting that warfarin therapy is indeed associated with increased risk of vascular calcification. As there is no such information available in children, I examined the total uncarboxylated form of MGP (uc-MGP) which has been shown to be a sensitive biological marker of vascular calcification and explored the link between warfarin treatment duration and intensity of anticoagulation and vascular calcification in the abovementioned cohort of

children. In this preliminary study, I did not find an association between warfarin therapy and circulating uncarboxylated MGP which would suggest immediate concern about warfarin-induced arterial calcification in children. The absence of a control group of healthy children did not allow for direct comparisons of the uc-MGP values, taking into consideration the large variability in levels reported in the literature. Further studies in larger cohorts of children are required to assess the long-term effects of warfarin therapy on vascular health. Study design can be crucial in revealing any possibly existing associations between warfarin therapy and vascular calcification measures; studying in parallel a group of healthy children, a group of children on chronic warfarin therapy and a group of children with cardiovascular disease that does not necessitate warfarin therapy may provide an insight into the potential causal relationship between warfarin and vascular calcifications. On the other hand, it will provide convincing evidence as to whether calcification is a common complication in children with cardiovascular diseases, irrespective of warfarin therapy. In addition, as already mentioned, direct measures of the extent of calcification using imaging techniques could be used to detect any associations with both the duration and intensity of anticoagulation with warfarin in children. These measures could be used for the verification of existing surrogate markers of calcification, such as uc-MGP or identification of novel, more practical ones that could be used for further research.

In an attempt to identify anticoagulant treatment alternatives to warfarin, a number of landmark studies have proven the safety and clinical effectiveness of NOACs. All four currently available NOACs; dabigatran, rivaroxaban, apixaban and edoxaban are at least as effective as warfarin and were associated with a significantly lower risk of major bleeding. Elderly people were not well represented in the major clinical trials which causes concern for clinicians prescribing them. According to a meta-analysis of randomised trials, the recommended doses of NOACs in patients with renal insufficiency are non-inferior and relatively safe compared to warfarin, which indicates that the increase in bleeding risk may be contributed to by other factors, such as old age. However, evaluation of the true effect of age on anticoagulation response to NOACs in vivo is challenging in older people due to the presence of other co-factors that influence drug exposure, including body mass changes, declining renal function, concomitant medication and illness. To overcome this, I compared the pharmacological activity of rivaroxaban ex-vivo in groups of fit elderly and young subjects.

Rivaroxaban and apixaban concentrations chosen for this study were in the range of the plasma concentrations reported following therapeutic dosing. Both factor Xa inhibitors produced a greater prolongation of both PT and modified PT in the elderly compared to young subjects. Factor II and X activity levels were significantly lower in elderly than young subjects in both cohorts. Both drugs prolonged time-based parameters of thrombin generation and suppressed the rate and amount of thrombin generation to a significantly greater extent in the elderly compared to young subjects. My study results demonstrate that elderly subjects to be more sensitive to the pharmacological activity of both rivaroxaban and apixaban than younger subjects. This provides evidence that in addition to factors that increase drug exposure, age *per se* could be an important contributor to the reported incidence of bleeding associated with NOAC therapy in older patients.

According to the results of all clotting factors studied, a trend towards great differences in clotting factors of the intrinsic pathway can be observed in older subjects compared to the young. The only factor studied from the extrinsic pathway did not show any differences in clotting factor activity. This may provide evidence of a specific impairment of the intrinsic pathway of the coagulation mechanism with age, which is also supported by the literature while comparing individual clotting factors. A second explanation is the presence of higher albumin levels in the plasma of elderly people compared with young subjects. Both factor Xa inhibitors studied have a high rate of protein-binding (primarily in albumin; 93.5% for rivaroxaban and 87% for apixaban). Therefore, it is possible that the augmented pharmacological activity of factor Xa inhibitors is a reflection of protein binding and increased recovery. This warrants further evaluation.

Following further replication of these results, clinicians should consider this when prescribing NOACs to elderly and frail patients, who are generally more susceptible to bleeding complications according to real-world data. A dose modification may be required for this population irrespective of renal function or the presence of concomitant illnesses and medication. Treatment monitoring could also assist with dose adjustments, with the latest development and clinical application of anti-Xa and direct thrombin inhibitor assays for detection of factor Xa inhibitors and dabigatran plasma concentrations respectively.

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APPENDICES

Appendix A. 4S DAWN software calculator for TTR calculation

Appendix B. Example calculation of INR at day 5 using the linear approximation model

Pt No	Day	Time (hr)	INR	INR-0.8	ln (INR-0.8)	(ln INR-0.8) at 120 h	INR AT 120H	INR at 120 hours	Did they reach 1.5 by day 5?	time (hr) to reach INR=1.5
1		0.0	2.2	1.4	0.3365	-1.5967	1.0	1.0		60.4
1	1	24.0	1.6	0.8	-0.2231				Yes	
1	2	122.5	1	0.2	-1.6094					

=(2.72^G2)+0.8

= -0.0154*120 + 0.2513

= -0.0087*120 + 2.0259

=(1.5-2.0259)/-0.0087

ln (INR-0.8) vs time - pt1

y = -0.0154x + 0.2513
R² = 0.9906

INR vs time (Pt 1)

y = -0.0087x + 2.0259
R² = 0.8902
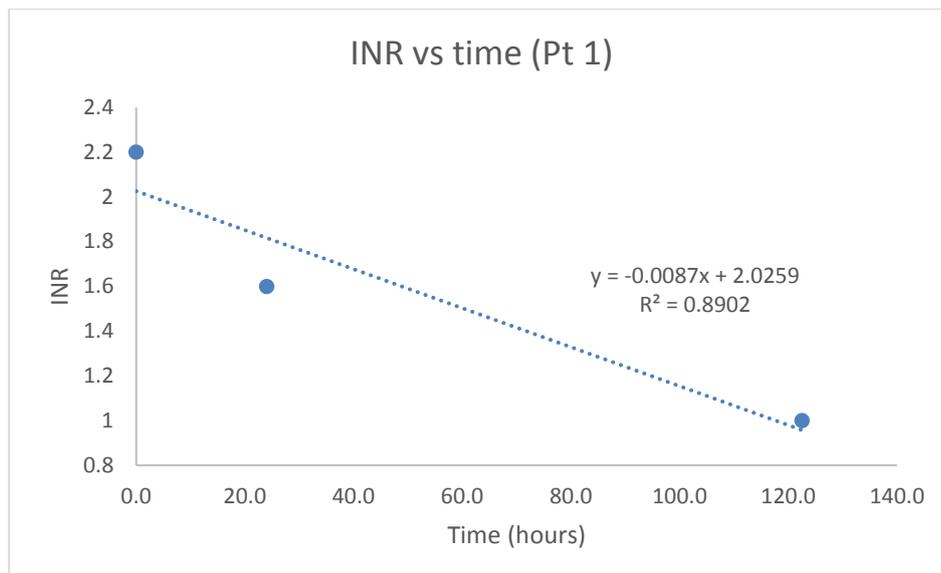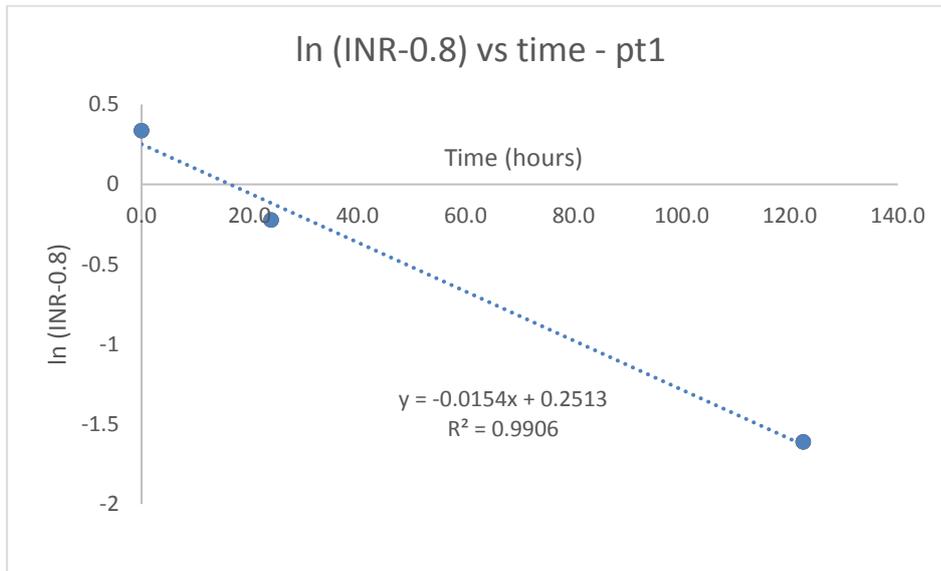
ASSERACHROM® IX:Ag
Enzyme Immunoassay for Factor IX

- 96-Test Kit Containing:
  - 3 × 2 Strips of Reagent 1 (Coated Strip)
  - 3 × 8-ml Vials of Reagent 2 (Anti-IX:Ag-Peroxidase)
  - 3 × 8-ml Vials of Reagent 3 (TMB)
  - 3 × 50-ml Bottles of Reagent 4 (Dilution Buffer)
  - 1 × 50-ml Bottle of Reagent 5 (Washing Solution)
  - 3 × 0.5-ml Vials of Reagent 6 (F. IX:Ag Calibrator)
  - 3 × 0.5-ml Vials of Reagent 7 (F. IX:Ag Control)
  - 1 Plate Frame
  - 1 Plate Cover

(REF 00943)

April 2014   IVD CE   English 2

1/ INTENDED USE

The **Asserachrom® IX:Ag** kit is an antigenic assay for the quantitative determination of factor IX by the enzyme-linked immunosorbent assay (ELISA).

2/ SUMMARY AND EXPLANATION

Biochemistry of Factor IX

Factor IX is a glycoprotein with a molecular weight of approximately 55,000 daltons, present in plasma at a concentration between 3 and 5 mg/l (5). It is synthesized by the liver (5). The synthesis of biologically active factor IX is vitamin K-dependent (5). This vitamin is necessary for the carboxylation of glutamic acid residues, which are essential for the fixation of factor IX on platelet or tissue phospholipids in the presence of calcium ions (5).

Factor IX can be activated in two different ways (6):

- factor XIa, in the presence of Ca²⁺, activates factor IX to factor IXa
- tissue factor VIIa complex activates either factor X or factor IX. Factor IXa forms an enzymatic complex with phospholipids, Ca²⁺ and factor VIIIa; this complex then activates factor X to factor Xa (5).

Pathological or Therapy-Related Variations

- Hemophilia B (3)
  The severity of hemophilia is based on factor IX:C level (7):
  ◊ < 1 %: severe hemophilia,
  ◊ 1-5 %: moderate hemophilia,
  ◊ 5-25 %: mild hemophilia.
- Hypovitaminosis K
  ◊ treatments with vitamin K antagonists (VKA) (2),
  ◊ nutritional intake deficiency; disorders in absorption or metabolism of vitamin K (hemorrhagic disease of the newborn, cholestasis, treatment with antibiotics) (1).
- Liver failure (1)
  ◊ cirrhosis,
  ◊ hepatitis.

3/ TEST PRINCIPLE

The factor IX to be measured is captured by specific rabbit anti-human factor IX antibodies (Reagent 1) coated on the internal walls of a plastic microplate well. Next, rabbit anti-human factor IX antibodies coupled with peroxidase (Reagent 2) bind to the remaining free antigenic determinants of the bound factor IX. The bound enzyme peroxidase is revealed by its action on the TMB substrate (Reagent 3). After stopping the reaction with a strong acid, the intensity of the color is directly proportional to the concentration of factor IX initially present in the sample.

4/ KIT REAGENTS

- **Reagent 1**: 16-well strip coated with rabbit anti-human factor IX F(ab')₂ fragments.
- **Reagent 2**: rabbit anti-human factor IX antibodies coupled with peroxidase, lyophilized.
- **Reagent 3**: ready for use tetramethylbenzidine (TMB < 1 %) solution.
- **Reagent 4**: ready for use phosphate buffer.
- **Reagent 5**: 20-fold concentrated washing solution.
- **Reagent 6**: lyophilized human plasma containing, after reconstitution, a known quantity of factor IX (see the Assay Value insert provided in the kit). This quantity is determined against a secondary standard of the 99/826 International Standard established in 2001.
- **Reagent 7**: lyophilized human plasma containing, after reconstitution, a known quantity of factor IX (see the Assay Value insert provided in the kit). This quantity is determined against a secondary standard of the 99/826 International Standard established in 2001.

WARNING - POTENTIAL BIOHAZARDOUS MATERIAL.
Some reagents provided in this kit contain materials of human and/or animal origin. Wherever human plasma is required for the preparation of these reagents, approved methods are used to test the plasma for the antibodies to HIV 1, HIV 2 and HCV, and for hepatitis B surface antigen; and results are found to be negative. However, no test method can offer complete assurance that infectious agents are absent. Therefore, users of reagents of these types must exercise extreme care in full compliance with safety precautions in the manipulation of these biological materials as if they were infectious.

5/ CAUTION

Store at 2-8 °C. For *in vitro* diagnostic use only. These reagents are to be used only by certified medical laboratory personnel authorized by the laboratory. Take care to use only the reagents from the same kit or the same lot.

Exercise great care in the handling of these reagents and of patient samples. The disposal of waste materials must be carried out according to current local regulations.

In the USA, wherever appropriate, observe CLIA-88 requirements.

6/ SPECIMEN COLLECTION AND TREATMENT

Sample collection must be in conformity with the recommendations for haemostasis tests.

- Blood (9 vol.) is collected in 0.109 M (i.e., 3.2 %) trisodium citrate anticoagulant (1 vol.).
- Centrifugation: 15 minutes at 2000-2500 g.
- Sample storage: 8 hours at 20 ± 5 °C.
  1 month at −20 °C. Thaw the sample at 37 °C, allow sufficient time to obtain complete thawing.

7/ REAGENT PREPARATION AND STORAGE

Stored at 2-8 °C, intact kits and contents are stable until the expiration date indicated on the box label.

- **Reagent 1**
  Allow Reagent 1 to stand at room temperature (18-25 °C) for 30 minutes before opening. The strips are then ready for use. Begin the test (see paragraph 9.4.) as soon as the strips are removed from the packet.
- **Reagent 2**
  Reconstitute each vial of Reagent 2 with 8 ml of Reagent 4 (R4). Allow the solution to stand at room temperature (18-25 °C) for 30 minutes. Then, vortex the vial before use. Due to the characteristics of Reagent 4, Reagent 2 is regarded as sensitising after reconstitution.
  Reconstituted stability: 4 hours at 20 ± 5 °C.
- **Reagent 3**
  Allow Reagent 3 to stand at room temperature (18-25 °C) for 30 minutes. Then use immediately.
  WARNING: AVOID ALL CONTACT WITH METALLIC COMPOUNDS AND OXIDIZING AGENTS.
- **Reagent 4**
  Allow the bottle (R4) to stand at room temperature (18-25 °C) for 30 minutes before use.
  Stability after opening: 15 days at 2-8 °C, when free of any contamination.
  Reagent 4 contains a 3:1 mixture of 5-chloro-2-methyl-2H-isothiazol-3-one and 2-methyl-2H-isothiazol-3-one. At the concentration provided (< 0.05 %), this mixture is classified as sensitising.
  Warning:
  May cause an allergic skin reaction.
  Wear protective gloves/protective clothing/eye protection/face protection.
  IF ON SKIN: Wash with plenty of soap and water.
- **Reagent 5**
  Dilute 1:20 with distilled water before use. For 2 strips (32 wells), use 15 ml of Reagent 5 and add distilled water to a final volume of 300 ml. Stability after dilution: 15 days at 2-8 °C, when free of any contamination. The presence of crystals will not affect the quality of the reagent. If necessary warm at 37 °C until all crystals have dissolved.
- **Reagents 6 and 7**
  Reconstitute each vial of Reagent 6 (R6) and Reagent 7 (R7) with exactly 0.5 ml of distilled water. Allow the solution to stand at room temperature (18-25 °C) for 30 minutes. Then, vortex the vial before use. Reconstituted stability: 4 hours at 20 ± 5 °C.

8/ REAGENT AND EQUIPMENT REQUIRED BUT NOT PROVIDED

- **1 M** sulfuric acid (concentrated sulfuric acid is approx. 18 M).
- Plate washing equipment.
- Plate reader set at **450 nm**.
- Common clinical laboratory equipment and materials (centrifuge, shaker such as Vortex, stopwatch, multichannel pipettes, distilled water…).

9/ PROCEDURE

9.1. Calibration

The assay is calibrated with Reagent 6 diluted 1:51 in a plastic test tube with Reagent 4 (20 µl Reagent 6 + 1 ml Reagent 4). This dilution (called starting solution) contains the highest calibrator of value "t" (see the Assay Value insert provided in the kit). Use Reagent 4 to dilute in plastic test tubes the starting solution according to the following scheme to obtain the other lower calibrator levels:

Calibrator level (%)	t	t2	t4	t8	t16
Reagent 6 (µl)	20				
Starting solution or its dilution (µl)		→500	→500	→500	→500
Reagent 4 (µl)	1000	500	500	500	500

9.2. Plasma Samples

Patients' plasmas are diluted 1:51 in plastic test tubes with Reagent 4 before use (20 µl patient's plasma + 1 ml Reagent 4) and if needed 1:102 (1 vol. 1:51 dilution + 1 vol. Reagent 4).

9.3. Quality Control

Reagent 7 is used for quality control. It is used at two dilutions prepared in plastic test tubes: 1:51 (20 µl Reagent 7 + 1 ml Reagent 4) and 1:102 (1 vol. 1:51 dilution + 1 vol. Reagent 4).

9.4. Assay

- Remove the strips from the packet just before starting the assay.
- Test all samples in duplicate within two hours of their preparation (calibrators, controls and patients' plasmas).
- Test Reagent 4 as the reagent blank in duplicate.
- Complete the pipetting of all samples into the wells within 10 minutes.

	Pipette into each precoated well:	
ANTIGEN IMMOBILIZATION	Test sample	200 µl
	Cover the wells and incubate for 1 hour at room temperature (18-25 °C).	
	Wash all wells 5 times with Reagent 5, then immediately proceed:	
IMMOBILIZATION OF IMMUNOCONJUGATE	Reagent 2	200 µl
	Cover the wells and incubate for 1 hour at room temperature (18-25 °C).	
	Wash all wells 5 times with Reagent 5, then immediately proceed:	
COLOR DEVELOPMENT	TMB (Reagent 3)	200 µl
	Incubate each well at 18-25 °C for **exactly** 5 minutes, then add acid:	
	1 M H₂SO₄	50 µl

Swirl the plate to mix contents after H₂SO₄ has been added to all the wells.

Wait **15 minutes**, then measure the absorbance at **450 nm within one hour**. Adjust reader to zero on reagent blank.

Procedural Notes

- During washing of strips, ensure that each well is completely filled and then completely emptied. The number of washings has to be strictly adhered to.
- Do not leave the strips dry at any time during the washing steps. If interruption cannot be avoided, leave the wells filled with Reagent 5 until ready to resume.
- Do not leave the strips exposed to bright light.
- The color development is temperature-dependent. This procedure has been optimized at 22 ± 2 °C.

10/ RESULTS

Use log-log graph paper to plot the calibrator values (%) on the x-axis and their corresponding absorbance values on the y-axis. Draw the calibration curve (it is non linear). Interpolate the absorbance values of the control and patients' plasma dilutions on this calibration curve to derive their respective factor IX levels. Results for the 1:51 dilutions are read directly from the curve; those for the 1:102 dilutions will have their values multiplied by 2. The result is to be interpreted according to the patient's clinical and biological states.

Ensure that the values obtained for Reagent 7 are within the range indicated on the Assay Value insert. If the control values are outside the stated range, check all components of the test system to ensure that all are functioning correctly, i.e. assay conditions, reagents, calibration, integrity of the plasmas being tested, etc. If necessary, repeat the test-run.

11/ LIMITATIONS

The presence of anti-rabbit antibodies in certain subjects may lead to aberrant results.
The utilization of F(ab')₂ fragments for coating of solid supports eliminates interference by rheumatoid factor (RF).

12/ REFERENCE INTERVAL

The factor IX plasma level determined in the normal adult population is usually in the range of 60-150 % (4). However, each laboratory should determine its own normal range.

13/ PERFORMANCE CHARACTERISTICS

- **Detection Limit - Working Range**
  When performed as described in the package insert, the detection limit of this assay system is 1 % of factor IX.
  The working range is between 1 and 120 % of factor IX. However, for a given lot, the upper limit corresponds to the value given for Reagent 6 in the Assay Value insert.

- **Reproducibility**
  Intra-assay and inter-assay reproducibility results are shown below:

Sample	Intra-Assay Reproducibility			Inter-Assay Reproducibility	
	Sample 1	Sample 2	Sample 3	Sample 4	
n	21	21	10	10
X̄ (%)	25.8	80.6	28.6	82.2
SD (%)	1.00	2.25	1.26	2.86
CV (%)	3.9	2.8	4.4	3.5

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Appendix D. FX antigen ELISA analysis protocol

ASSERACHROM® X:Ag
Enzyme Immunoassay of Factor X

- 96-Test Kit Containing:
  - 3 x 2 Strips of Reagent 1 (Coated Strip)
  - 3 x 8-ml Vials of Reagent 2 (Anti-X:Ag-Peroxidase)
  - 6 Tablets of Reagent 3a (ortho-Phenylenediamine)
  - 6 Tablets of Reagent 3b (Urea Peroxide)
  - 1 x 20-ml Vial of Reagent 4 (Dilution Buffer)
  - 1 x 50-ml Vial of Reagent 5 (Washing Solution)
  - 3 x 0.5-ml Vial of Reagent 6 (F. X Calibrator)
  - 3 x 0.5-ml Vials of Reagent 7 (F. X Control)
  - 1 Plate Frame
  - 1 Plate Cover

(REF 00243)

February 2014   English 2

FOR RESEARCH USE ONLY - NOT FOR USE IN DIAGNOSTIC PROCEDURES

1/ SUMMARY AND EXPLANATION

The **Asserachrom® X:Ag** kit is an enzyme immunoassay (EIA) procedure for the quantitative determination of factor X by the sandwich technique, also known as enzyme-linked immunosorbent assay (ELISA).

Factor X is a vitamin K-dependent glycoprotein of molecular weight approximately 60,000 daltons, synthesized in the liver. It is made up of two polypeptide chains linked to each other by a disulphide bridge. The active site of factor X is located on the heavy chain (1).

Factor X can be activated into factor Xa in different ways:
- by the factor IXa-Ca²⁺-phospholipid-factor VIIIa complex (1)
- by the factor VIIa-tissue factor complex (2).

The prothrombinase is an enzymatic complex made up of factor Xa, factor Va, Ca²⁺ and phospholipids. This complex transforms prothrombin into thrombin (1).

Factor Xa can also activate factor VII into factor VIIa (1).

Factor Xa is inhibited by antithrombin III, associated or not with heparin. This inhibition, which is strong in the liquid medium, is considerably decreased when factor Xa is fixed on phospholipid surfaces (1).

The factor X plasma level in the normal adult population is usually in the range of 70-130 %.

2/ TEST PRINCIPLE

A plastic support coated with specific rabbit anti-human factor X antibodies (Reagent 1) captures the factor X to be measured. Next, rabbit anti-factor X antibody coupled with peroxidase (Reagent 2) binds to the remaining free antigenic determinants of the factor X, forming the "sandwich". The bound enzyme peroxidase is then revealed by its activity in a predetermined time on the substrate ortho-phenylenediamine (Reagent 3a) in the presence of hydrogen peroxide. After stopping the reaction with a strong acid the intensity of the color produced bears a direct relationship with the factor X concentration initially present in the plasma sample.

3/ KIT REAGENTS

- **Reagent 1:** aluminium pouch containing 2 strips, each of 16 wells coated with specific rabbit anti-human factor X F(ab')₂ fragments, hermetically sealed.
- **Reagent 2:** specific rabbit anti-human factor X antibody coupled with peroxidase, lyophilized.
- **Reagent 3a:** tablet containing 2 mg ortho-phenylenediamine (OPD, 2 HCl).
- **Reagent 3b:** tablet containing 5 mg urea peroxide as a source of hydrogen peroxide.
- **Reagent 4:** 10-fold concentrated phosphate buffer.
- **Reagent 5:** 20-fold concentrated washing solution.
- **Reagent 6:** lyophilized human plasma containing factor X at approximately 100 % after reconstitution and dilution (see the Assay Value insert provided in the kit for exact value). This reagent is for assay calibration.
- **Reagent 7:** lyophilized human plasma containing a known factor X level (see the Assay Value insert provided in the kit). This reagent is used for the preparation of assay controls.

WARNING - POTENTIAL BIOHAZARDOUS MATERIAL.
Some reagents provided in this kit contain materials of human and/or animal origin. Wherever human plasma is required for the preparation of these reagents, FDA-approved methods are used to test the plasma for the antibodies to HIV 1, HIV 2 and HCV and for hepatitis B surface antigen, and results are found to be negative. However, no test method can offer complete assurance that infectious agents are absent. Therefore, users of reagents of these types must exercise extreme care in full compliance with regulatory safety precautions in the manipulation of these biological materials as if they were infectious.

4/ CAUTION

Store at 2-8 °C.
For research use only - Not for use in diagnostic procedures.
For *in vitro* use only.
The disposal of waste materials must be carried out according to the current local regulations.

5/ SPECIMEN COLLECTION AND TREATMENT

- Blood (9 vol.) is collected in 0.109 M (i.e., 3.2 %) trisodium citrate anticoagulant (1 vol.).
- Centrifugation: 10 minutes at 2,500 g.
- Plasma storage: 8 hours at 20 ± 5 °C.
  1 month at –20 °C. Thaw the sample at 37 °C, allow sufficient time to obtain complete thawing.

6/ REAGENT PREPARATION AND STORAGE

Intact kit and contents are stable until the expiration date indicated on the box label, when stored at 2-8 °C.

- **Reagent 1**
  Before opening the pouch, allow Reagent 1 to stand at room temperature (18-25 °C) for 30 minutes. The strips are then ready for use. Start the test until the number of necessary wells are removed from the aluminium pouch.

- **Reagent 2**
  Reconstitute each vial of Reagent 2 with 8 ml of diluted Reagent 4 (R4). Allow the solution to stand at room temperature (18-25 °C) for 30 minutes. Then, vortex the vial before use.
  Reconstituted stability: 24 hours at 2-8 °C.

- **Reagents 3a and 3b**
  Prepare the OPD/H₂O₂ substrate solution 5 minutes before use. For 2 strips (32 wells), dissolve **together** 2 tablets of Reagent 3a and 2 tablets of Reagent 3b in 8 ml of distilled water.
  The OPD/urea peroxide solution thus obtained is stable for 1 hour at room temperature (18-25 °C).

  Reagent 3a contains 2 mg ortho-phenylenediamine dihydrochloride. At the concentration provided, this chemical has been classified as carcinogen and mutagen (category 2), sensitizing and very toxic to aquatic life.
  Warning
  Suspected of causing cancer.
  Suspected of causing genetic defects.
  May cause an allergic skin reaction.
  May be harmful if swallowed.
  Very toxic to aquatic life with long lasting effects.
  Wear protective gloves/protective clothing/eye protection/face protection.
  Avoid release to the environment.
  Dispose of contents/container to an approved waste disposal plant.

  Reagent 3b contains 5 mg urea peroxide. At the concentration provided, this chemical has been classified as corrosive.
  Danger
  Causes severe skin burns and eye damage.
  Wear protective gloves/protective clothing/eye protection/face protection.
  IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
  Immediately call a POISON CENTER or doctor/physician.
  Keep the OPD tablets in their packaging and dissolve them only just before use in order to minimize spontaneous hydrolysis. Avoid all contacts with metallic surfaces as well as all contacts with oxidizing agents.

- **Reagent 4***
  Allow the vial (R4) to remain at room temperature (18-25 °C) for 15 minutes before diluting at 1:10 with distilled water.
  For 2 strips (32 wells), add 6 ml of Reagent 4 to 54 ml of H₂O.
  Stability after dilution: 15 days at 2-8 °C, when free of any contamination.

- **Reagent 5***
  It must be diluted (1:20 dilution) with distilled water before use.
  For 2 strips, add 16 ml of Reagent 5 to 304 ml of H₂O.
  Stability after dilution: 15 days at 2-8 °C, when free of any contamination.

- **Reagents 6 and 7**
  Reconstitute each vial of Reagent 6 or Reagent 7 with exactly 0.5 ml of distilled water. Allow the solution to stand at room temperature (18-25 °C) for 30 minutes. Then, vortex the vial before use.
  Reconstituted stability: 4 hours at 20 ± 5 °C.

- The presence of crystals will not affect the quality of Reagents 4 and 5. If necessary warm at 37 °C until all crystals have dissolved.
  Do not use any of the reagents if they show obvious signs of contamination.

7/ REAGENT AND EQUIPMENT REQUIRED BUT NOT PROVIDED

- **3 M** sulphuric acid (the concentrated sulphuric acid is approximately 18 M) or **1 M** hydrochloric acid.
- Plate washing equipment.
- Plate reader set at **492 nm**.
- Common laboratory equipment and materials (centrifuge, shaker such as vortex, stopwatch, multichannel pipettes, distilled water...).

8/ PROCEDURE

8.1. Calibration

The assay calibration is carried out with Reagent 6. Use diluted Reagent 4 to prepare a 1:101 dilution of the reconstituted Reagent 6 (50 µl reconstituted Reagent 6 + 5 ml diluted Reagent 4) to obtain the starting solution containing the highest calibrator of value "t", the latter being indicated in the Assay Value insert provided in the kit. Then, use diluted Reagent 4 to dilute this starting solution according to the following scheme to obtain other lower calibrator levels:

To obtain calibrator value (%)	t	t2	t4	t8	t16
Dilution of starting solution	None	1:2	1:4	1:8	1:16

Plasma samples and Reagent 7 are tested at two dilutions: 1:101 (50 µl plasma or Reagent 7 + 5 ml diluted Reagent 4) and 1:202 (1 vol. 1:101 dilution + 1 vol. diluted Reagent 4).

8.2. Plasma Samples and Control

8.3. Procedural Notes

- As for any ELISA method, use sterile deionized or distilled water to reconstitute or dilute reagents.
- During washing of strips, ensure that each well is completely filled, and then completely emptied. The number of washings (2 x 5 in total) has to be strictly adhered to.
- During the washing steps do not leave the strips dry at any time. If interruption cannot be avoided, leave the wells filled with washing solution until ready to resume.
- Do not leave the strips exposed to bright light.
- The color development is temperature-dependent. This procedure has been optimized for the 18-25 °C temperature range.

8.4. Assay

Cut open the required number of aluminium pouches to remove the precoated strips only just before being ready to start the assay.
- Within two hours of their preparation, test each calibrator, plasma dilution and control in duplicate.
- Test Reagent 4 as reagent blank in duplicate.

	Pipette into each precoated well:	
ANTIGEN IMMOBILIZATION	Test sample	200 µl
	Cover the wells and let incubate 2 hours at room temperature (18-25 °C).	
	Wash all wells 5 times with Reagent 5, then immediately proceed:	
IMMOBILIZATION OF IMMUNOCONJUGATE	Reagent 2	200 µl
	Cover the wells and let incubate 2 hours at room temperature (18-25 °C).	
	Wash all wells 5 times with Reagent 5, then immediately proceed:	
COLOR DEVELOPMENT	OPD/H₂O₂ Substrate (Reagents 3a + 3b)	200 µl
	Let incubate at room temperature for **exactly** 2 minutes for each well, then add acid:	
	either 3 M H₂SO₄ or 1 M HCl	50 µl / 100 µl
	Wait 10 minutes, then measure the absorbance at 492 nm within 2 hours (adjust reader to zero on reagent blank).	

9/ RESULTS

Use log-log graph paper to plot the calibrator values (%) on the abscissa (x-axis) and their corresponding absorbance values on the ordinate (y-axis). Draw the calibration curve (it is non linear). The factor X:Ag levels of the controls and plasmas are deduced from the calibration curve:
- results for the 1:101 dilution are read off directly from the curve
- results for the 1:202 dilution will have their values multiplied by 2.

Ensure that the value obtained for the control is within the range indicated in the box. If the control value is outside the stated range, check all components of the test system to ensure that all are functioning correctly, i.e. assay conditions, reagents, calibration, integrity of the plasmas being tested, etc. If necessary, repeat the test-run.

10/ LIMITATION

The extremely rare presence of anti-rabbit antibodies in certain subjects leads to aberrant results.

11/ CHARACTERISTICS

- **Specificity**
  The utilization of F(ab')₂ fragments for coating of solid supports eliminates interference by rheumatoid factor (RF).

- **Detection Limit**
  When performed as recommended in the package insert, the detection limit of this assay is 0.5 % of factor X.

REFERENCES

1. SAMAMA M, CONARD J, HORELLOU M.H., LECOMPTE T.: "Physiologie et exploration de l'hémostase". Paris: Doin, p. 81-82, 112-118, 1990.

2. SAMPOL J., ARNOUX D., BOUTIERE B.: "Manuel d'hémostase". Paris: Éditions scientifiques et médicales Elsevier, p. 46-48, 364-366, 395-405, 1995.

Appendix E. Thrombin generation assay protocol

A modified assay of Perzborn, Morishima, and Douxfils was used (Morishima and Kamisato, 2015, Perzborn and Harwart, 2009, Douxfils et al., 2012). Fluoroskan® Ascent Fluorometer instrument (Thermo Fisher Scientific, Waltham, MA) and the thrombinoscope software (Thrombinoscope BV) were used. The fluorescence was measured at excitation of 390 nm and emission of 460 nm, which the machine is already programmed for and there is no need to adjust it.

1. Switch on the large, corner waterbath at 37°C
2. Reconstitute Normal Control with 1ml H$_2$O (leave for 30 mins)
3. Switch on PC and Fluoroskan Ascent
4. Place Fluo buffer *(2 for a full plate)* and a universal of distilled water in 37 $^\circ$C waterbath
5. Defrost samples in heat block at 37°C and then centrifuge at 3500rpm for 7 minutes
6. Open Thrombinoscope Software and login.
7. Select "Measure"
8. Click yes to take plate drawer back in.
9. *Check **every time** calibrator activity in settings and change if necessary*
10. Programme plate layout (this can be done at any time before analysis)
    a. Each sample needs 9 wells (3 Thrombin calibrator + DMSO, 3 Thrombin generator + DMSO and 3 Thrombin generator + Rivaroxaban)
    b. Select yellow button for calibrator wells then select A1, A2 and A3
    c. Select red button for thrombin generation wells, then select A4 – A6
    d. Click Add Group and Rename the group with lowercase letters
    e. Add another group with A1-3 (Thrombin calibrator), plus A7-9 (thrombin generator) and name it with capital letters +R (which means the group that contains generator with rivaroxaban and calibrator)
    f. Continue as per plate design, with controls in the very last positions (wells A/B/C 10-12). Controls have the same configuration as patients therefore 9 wells/control.
    g. Ensure temperature is 37°C, check time and intervals
    h. Ensure the running time is set at **120 minutes**
11. Reconstitute **PPPH** (20pM tissue factor and 4 μM phospholipids) and Thrombin Calibrator reagents with 1ml H$_2$O. Swirl and leave for 2mins.

12. Pipette 10µl of **factor Xa inhibitor** (i.e. rivaroxaban or apixaban) 1.5 ng/µl working stock solution (187.5ng/ml final rivaroxaban concentration in plasma or 100ng/ml final apixaban concentration in plasma) in wells 7-9, or 10µl **blank DMSO** in appropriate wells (1-6)

13. Pipette 20µl **thrombin calibrator** using reverse pipetting into appropriate wells (1-3)

14. Pipette 20µl **PPPH** reagent using reverse pipetting into appropriate wells (4-9).

15. Pipette 70µl **subject plasma** into appropriate wells (1-9).

16. Click Plate out, place plate with A1 in top left corner, click Plate in.

17. Click START – 10 minute incubation starts

18. *Fluo Substrate* is precious, take care when handling. It is stored in the second drawer at Paul's bench in a separate box. Prepare the FluCa by adding 40µl *Fluo Substrate* to each warmed vial of Fluo buffer. Vortex until clear.

19. If running a full plate, combine both vials in a glass jar.

20. Follow on screen instructions to prime the dispenser using the warmed universal of H_2O and empty universal.

    - Watch plunger for bubbles and check H_2O is dispensed in a straight line.
    - Fill the system with FluCa as directed on screen. Ensure no bubbles in plunger and FluCa dispensing in a straight line.
    - Place dispenser tip in the hole "M" ensuring aspirating tube remains **in the FluCa** throughout the dispensing step.
    - Place the dispenser tip in a universal of H_2O to avoid blockage.
    - Close lid and let it run.

21. After measurement, place the aspirating tube in a universal of at least 5ml H_2O

    - Place the dispenser in an empty universal
    - Go to "instrument" and select "prime dispenser", enter 5000µl
    - After priming, go to "instrument" and select "empty dispenser", making sure that both the dispenser and aspirating tubes are in the empty universal.