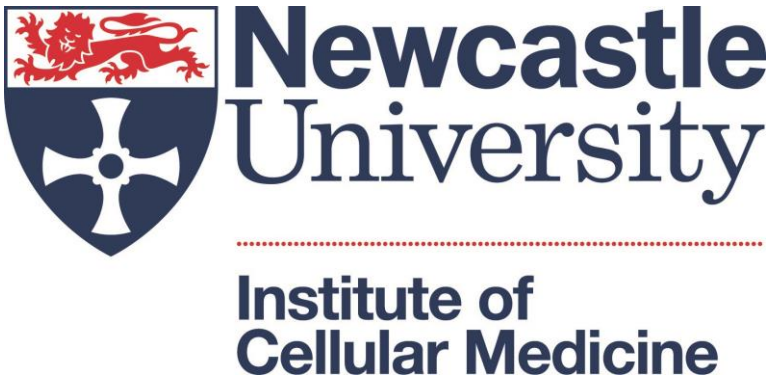

Factors Affecting the Safe Use of Oral Anticoagulants

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Abstract

The aim of this PhD was to elucidate the factors that influence anticoagulation response to the oral anticoagulants, warfarin and non-vitamin K antagonist oral anticoagulants (NOACs). Identification of the factors which influence clinical response to these agents could lead to improvements in patient management and treatment safety.

In a cross-sectional study, the mean TTR over a 12 months period was significantly higher in hospital monitored patients (78%) (P=0.001) compared to those monitored in general practice (71%) or at their homes (68%). Domiciliary monitored patients had the least TTR among the three groups although they had the highest numbers of dose changes and INR monitoring events. In a further longitudinal study of patients on warfarin for up to 14 years, the TTR according to age was significantly lower in home monitored patients, with this group having a higher probability of having poor anticoagulation control (TTR ≤65%).

In an ex-vivo study, rivaroxaban in vitamin K deficient older subjects produced a greater prolongation of both PT and modified PT, and a greater suppression in the rate and amount of thrombin generation compared to vitamin K replete younger subjects. Therefore, poor vitamin K intake could play a role in the reported incidences of bleeding associated with NOACs

We had previously demonstrated that daily vitamin K supplementation causes an increase in warfarin daily dose requirement which varies between different patients and is related to *VKORC1* genotype. Based upon these observations we set out to test our hypothesis that patients on warfarin therapy with the *VKORC1(-1639)GG* polymorphism could have poorer anticoagulation control than those with *GA* or *AA* genotypes as a result of variable dietary intake of vitamin K. However, the study results failed to confirm this hypothesis.

Patients on warfarin therapy scheduled for an invasive surgery have to stop taking the drug for a fixed number of days to avoid peri-operative bleeding. However, there is variance in the rate by which INR falls after stopping warfarin. I found that patients with *CYP2C9* variant alleles (*CYP2C9*2*2 or CYP2C9*2*3*) were >8 times (95% CI = 2.25–33.25) more likely to have INR of ≥ 1.5 before the planned day of surgery than those with wild-type genotype. In a further study, patient age and *CYP2C9 *2*2 & *2*3*

genotype were found to significantly influence the time required to reach an INR of 1.5 following warfarin cessation. *VKORC1* genotype had no effect on the rate of INR decline.

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.

Salah Abohelaika

July 2017

DEDICATION

I dedicate this piece of work to my parents who gave me more than I realized, long may they live. “My Lord! Bestow on them Your mercy even as they cherished me in childhood”. I also dedicate it to my wife and my children, and for every member in my family.

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LIST OF ABBREVIATIONS

µl	Microliter
95% CI	95% confidence interval
ACCP	American College of Chest Physicians
ADVANCE	Apixaban Dose Orally vs. Anticoagulation with Enoxaparin trials
AF	Atrial fibrillation
AMPLIFY	Apixaban for the Initial Management of Pulmonary Embolism and Deep-Vein Thrombosis as First-Line Therapy trial
AMPLIFY-Extension	Apixaban after the Initial Management of Pulmonary Embolism and Deep Vein Thrombosis with First-Line Therapy–Extended Treatment trial
aPCC	Activated prothrombin complex concentrates
APTT	Activated partial thromboplastin time
ARISTOTLE study	Apixaban for Reduction In Stroke And Other Thromboembolic Events In Atrial Fibrillation Trial
BMI	Body Mass Index
COAG	Clarification of Optimal Anticoagulation through Genetics study
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOACs	Direct oral anticoagulants
DTIs	Direct thrombin inhibitors
DVT	Deep venous thrombosis
EDTA	Ethylene diamine tetra acetic acid
EINSTEIN-DVT	Oral, direct Factor Xa inhibitor rivaroxaban in patients with acute symptomatic deep vein thrombosis trial
EINSTEIN-Extension	Once-daily oral rivaroxaban versus placebo in the long-term prevention of recurrent symptomatic venous thromboembolism trial
EINSTEIN-PE	Oral, direct Factor Xa inhibitor rivaroxaban in patients with acute symptomatic pulmonary embolism trial

EMA	European Medicines Agency
ENGAGE study	Effective Anticoagulation with Factor Xa Next Generation in Atrial Fibrillation
ESC	The European Society of Cardiology
ETP	Endogenous thrombin potential
EU-PACT	European trial is called Pharmacogenomic Approach to Coumarin Therapy study
FDA	Food and Drug Administration
FEIBA	Factor eight inhibitor bypassing activity
FFQ	Food frequency questionnaire
FXIs	Factor Xa Inhibitors
gm	Gram
H_2O	Water
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 and/or Lung Blood Clots trial
HPLC	High pressure liquid chromatography
INR	International Normalized Ratio
ISI	International sensitivity index
IWPC	International Warfarin Pharmacogenetics Consortium study
LMWH	Low molecular weight heparin
min	Minute
ml	Millilitre
mPT	Modified Prothrombin time
mTGR	Mean thrombin generation rate
ng	Nano gram
NICE	National Institute of Clinical Excellence in England and Wales
NOACs	New Oral Anticoagulants
PCC	Prothrombin complex concentrates
PE	Pulmonary Embolism
pg	Pico gram
PPPH	Platelet-poor plasma high

PROBE	Prospective, randomised, open-label, blinded end point evaluation design
PT	Prothrombin time
PTR	Prothrombin time ratio
QALY	Quality-adjusted-life-year
RDA	Recommended Dietary Allowance
RECORD	REgulation of Coagulation in major Orthopaedic surgery Reducing risk of DVT and pulmonary embolism trials
RE-COVER I	Efficacy and Safety of Dabigatran Compared with Warfarin for 6 Month Treatment of Acute Symptomatic Venous Thromboembolism trial
RE-COVER II	Phase III Study Testing Efficacy & Safety of Oral Dabigatran Etexilate vs Warfarin for 6 m Treatment of Acute Symptomatic Venous Thromboembolism trial
RE-LY study	Randomized Evaluation of Long-Term Anticoagulation Therapy trial
RE-MEDY	A Phase III, Randomized, Multicenter, Double-blind, Parallel-group, Active Controlled Study to Evaluate the Efficacy and Safety of Oral Dabigatran Etexilate Compared with Warfarin for the Secondary Prevention of Venous Thromboembolism trial
RE-MOBILIZE	Oral thrombin inhibitor dabigatran etexilate vs North American enoxaparin regimen for the prevention of venous thromboembolism after knee replacement surgery study
RE-MODEL	Oral dabigatran etexilate vs subcutaneous enoxaparin for the prevention of venous thromboembolism after total knee replacement
RE-NOVATE	Dabigatran etexilate versus enoxaparin for prevention of venous thromboembolism after total hip replacement
RE-NOVATE II	Oral dabigatran versus enoxaparin for thromboprophylaxis after primary total hip arthroplasty

RE-SONATE	Twice daily Oral Direct Thrombin Inhibitor Dabigatran Etexilate in the Long Term Prevention of Recurrent Symptomatic VTE trial
ROCKET AF study	Rivaroxaban Once Daily Oral Direct Factor Xa Inhibition Compared With Vitamin K Antagonism For Prevention Of Stroke and Embolism Trial in Atrial Fibrillation
rpm	Round per minute
rVII	Recombinant activated factor VII
SNP	Single nucleotide polymorphism
SPE	Solid-phase extraction
TT	Thrombin time
ttPeak	Time to peak
TTR	Time-in-therapeutic range
UFH	Unfractionated heparin
USDA	United States Department of Agriculture
VKA	Vitamin K Antagonists
VKOR	Vitamin K 2,3-epoxide reductase enzyme
VKORC1	Vitamin K Epoxide Reductase Complex Sub Unit 1
VTE	Venous thromboembolism
WHO	World Health Organization

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pharmacodynamics following warfarin withdrawal: implications for patients undergoing surgery (submitted).

CONFERENCE PRESENTATIONS

North East Postgraduate Conference 2012, October 2012, Newcastle upon Tyne, UK

Poster presentation: A Study to Evaluate Patients' Anticoagulation Stability in Three Different Monitoring Settings in Newcastle upon Tyne

British Pharmacological Society, December 2012, Pharmacology 2012, London, UK

Poster presentation: A Study to Evaluate Patients' Anticoagulation Stability in Three Different Monitoring Settings in Newcastle upon Tyne

54th Annual Scientific Meeting of the British Society for Haematology at the International Convention Center (ICC), April 2014, Birmingham, UK

Poster presentation: The Impact of Dietary Vitamin K on the Pharmacological Activity of FXa Inhibitor Rivaroxaban in Man

23rd Biennial International Congress on Thrombosis, the Mediterranean League Against Thromboembolic Diseases, May 2014, Valencia, Spain

Poster presentation: The Impact of Dietary Vitamin K on the Pharmacological Activity of FXa Inhibitor Rivaroxaban in Man

British Pharmacological Society, December 2014, Pharmacology 2014, London, UK

Poster presentation: VKORC1 (-1639) Polymorphism Do Not Affect Long-term Stability of Anticoagulation

VVX Congress of the International Society on Thrombosis and Haemostasis & 61th Annual SSC Meeting, June 2015, Toronto, Canada

Oral presentation: The Effect of CYP2C9 Polymorphism on INR Decline in Patients Stopping Warfarin Before Surgery

26th European Stroke Conference, May 2017, Berlin, Germany

Poster presentation: A longitudinal UK-based Multi-centre Study of Warfarin Anticoagulation Control

CHAPTER. 1 INTRODUCTION

1.1. Vitamin K Antagonists

1.1.1. Introduction

The ideal anticoagulant (blood thinning drug) would be one which abolishes blood clotting where it is not wanted without causing bleeding. Such an agent does not yet exist in clinical practice. For decades coumarins have been used for the treatment and prevention of venous thromboembolism, including deep vein thrombosis, pulmonary embolism and thromboembolic complications of cardiac valve replacement or atrial fibrillation. Meeting a stable and therapeutic effect is clinically important to the safety of warfarin. However, the clinical management of patients on warfarin therapy is complicated by the fact that the drug has a narrow therapeutic window, interacts with many co-administered drugs and food and there is wide intra- and inter-individual variability in anticoagulation response, with the dose required to reach therapeutic anticoagulation in individual patients differing by a factor of 10 or more [1].

1.1.1.1. Definition

Coumarins are vitamin K antagonists (VKAs). They cause anticoagulation by inhibiting the recycling of vitamin K. This leads to depletion of vitamin K-dependent coagulation factors II, VII, IX and X. Warfarin is the most commonly used coumarin worldwide. Acenocoumarol and phenprocoumon are other close derivatives of warfarin which are used in several European countries [2]. The chemical structures of VKAs are shown in Figure 1-1.

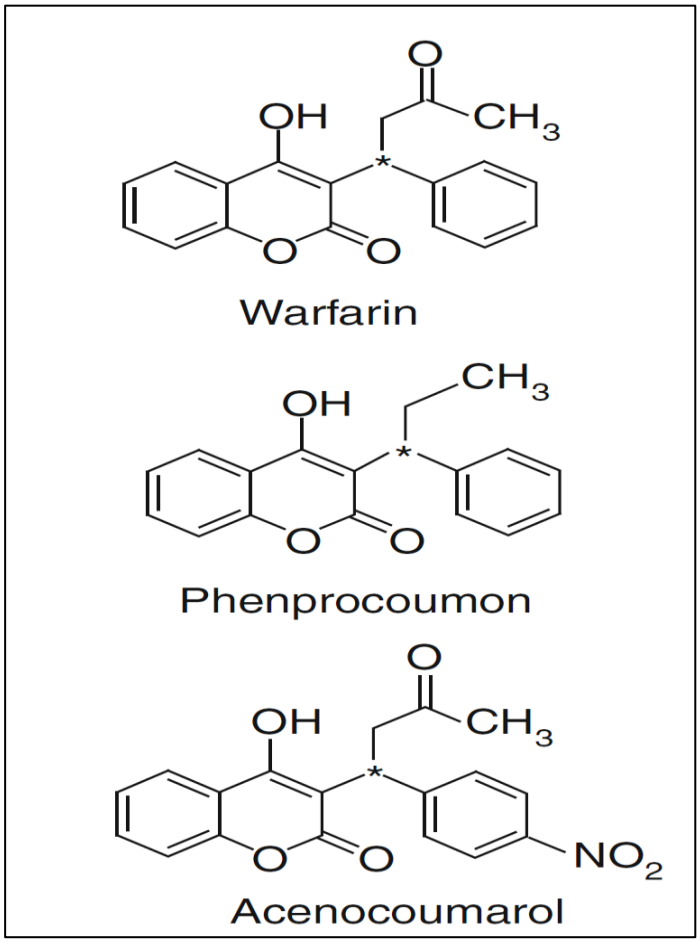


Figure 1-1: Chemical structure of vitamin K antagonists

1.1.1.2. History of coumarin use

At the turn of the twentieth century the high demand for new housing and farmland in Northern America led to the destruction of natural grasses used by cattle for grazing. Sweet clover was grown extensively as an alternative animal feed. During the 1920s, in Canada and northern parts of America, veterinarians observed that cattle were dying of internal haemorrhage but the cause for this was unknown. It was found that haemorrhage took place when wet and spoiled sweet clover was fed to cattle. The symptoms of this "sweet clover disease" appeared within 15 days of ingestion, and the animal died within 30 to 50 days thereafter. The cause for bleeding in cattle which were fed on spoilt sweet clover was identified ten years later by Karl Link (1901-1978) and his senior student Wilhelm Schoeffel. Link identified the responsible substance as dicoumarol (3, 3'-methylene-bis [4-hydroxycoumarin]), a naturally occurring coumarin in mouldy hay (*Melilotus alba* and *M. officinalis*). Moreover, in 1939, Link found that

administration of vitamin K1 reversed dicoumarol action. The (3-a-acetonylbenzyl-4-hydroxycoumarin) product was given the name of Warfarin, (warf-) since it was brought to light by Wisconsin Alumni Research Foundation, and (-arin) from the coumarin group. In 1948, warfarin was initially promoted as a rodenticide, then in 1955, introduced clinically under the name “Coumadin”. The main benefits of warfarin compared to other anticoagulants, heparin and dicoumarol, are its high oral bioavailability and high water solubility. The benefits of coumarins as oral anticoagulants were successfully demonstrated in a randomized clinical trial for the first time in 1960 for patients with pulmonary embolism [3, 4].

1.1.2. Blood coagulation

When an animal is wounded a blood clotting process takes place rapidly at the site of injury to prevent bleeding. Blood clotting through the coagulation cascade is a process that encompasses a series of reactions known as the extrinsic (tissue factor) pathway, intrinsic (contact) pathway, and common pathway.

The extrinsic pathway is so called as one of the coagulation factors, Factor III thromboplastin, does not exist in circulating blood. The extrinsic pathway includes also factors IV calcium and VII proconvertin. The required coagulation factors in the intrinsic pathway are all contained in circulating blood, hence its name. Factors included in the intrinsic pathway are VIII antihemophilic, IX Christmas, XI plasma thromboplastin antecedent, and XII Hageman. Coagulation factors I fibrinogen, II prothrombin, V prothrombin accelerator, X Stuart (Power), and XIII fibrin-stabilizing factor are common factors across the two pathways[5]. The various coagulation factors and their prominence in the coagulation cascade are listed in Table 1-1.

Table 1-1: Coagulation factors

Factor number	Factor name
Extrinsic pathway	
III	Thromboplastin, tissue factor
IV	Calcium
VII	Proconvertin
Intrinsic pathway	
VIII	Antihemophilic factor
IX	Christmas factor
XI	Plasma thromboplastin antecedent
XII	Hageman factor
Common pathway	
I	Fibrinogen
II	Prothrombin
V	Prothrombin accelerator
X	Stuart (Power)
XIII	Fibrin-stabilizing factor

After vascular injury and blood exposure to tissue factor, the coagulation cascade is initiated. This stimulates the extrinsic pathway (heavy arrows in Figure 1-2). Once thrombin is generated, the intrinsic pathway (small arrows in Figure 1-2) is stimulated, which leads to factor XI activation. Both of the two pathways are joined by factor Xa formation (common pathway). Then, factor Xa triggers prothrombinase enzyme which converts prothrombin to thrombin. Fibrinogen is converted by thrombin to insoluble fibrin to create a clot [6, 7]

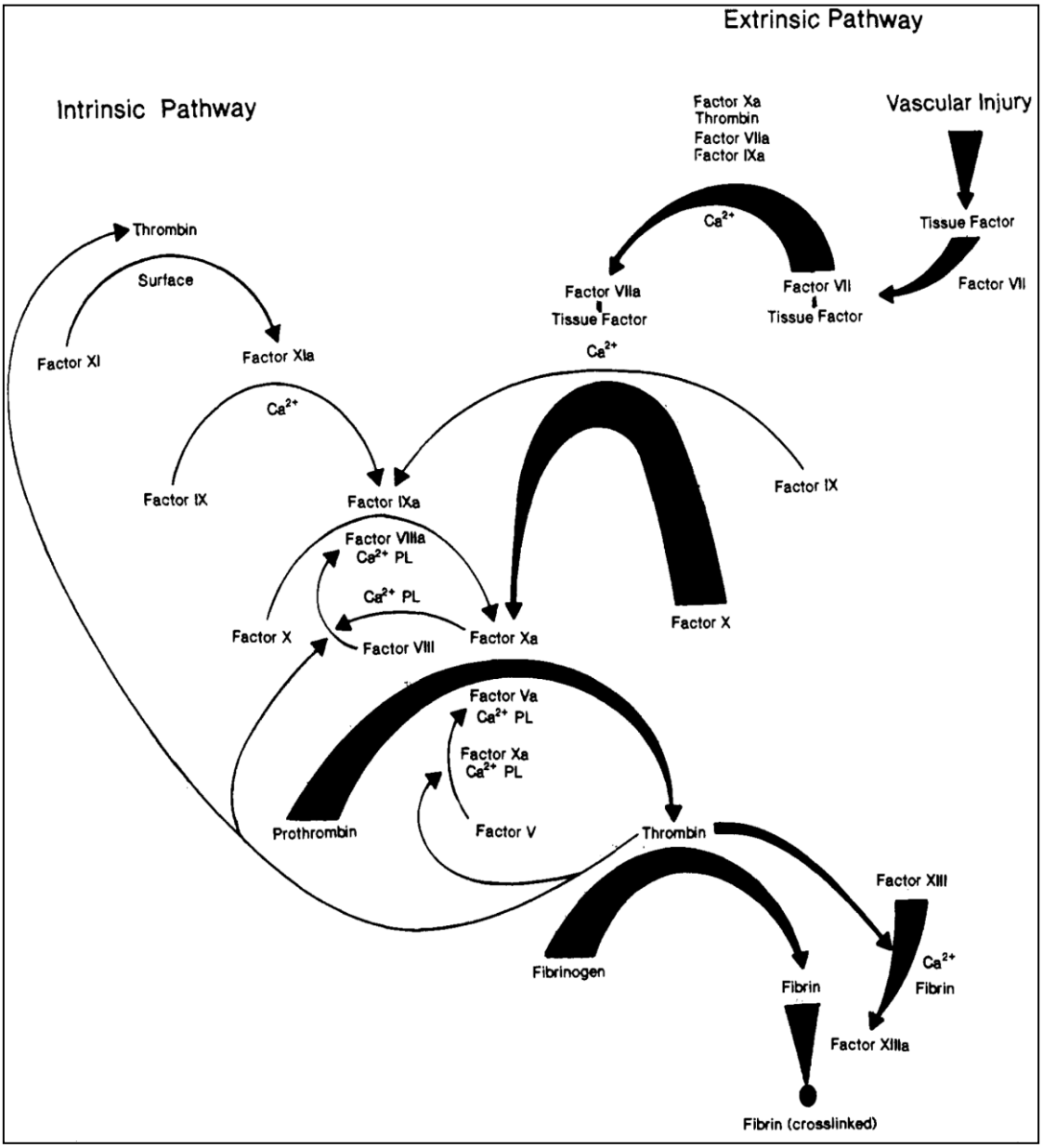


Figure 1-2: Intrinsic (small arrows) and extrinsic (heavy arrows) pathways involved in blood coagulation cascade. PL=phospholipid [6].

1.1.3. Clinical indications for warfarin

In well-designed clinical trials, warfarin has proved its efficacy in the treatment and prevention of thromboembolism. The three indications for which warfarin is most commonly used are:

- Atrial fibrillation
- Venous thrombosis
- Metallic prosthetic heart valves

Atrial fibrillation

Atrial fibrillation (AF) is a major health problem as patients with atrial fibrillation are susceptible to developing atrial thrombi, and the risk of stroke from cardioembolism is increased 4-5 fold compared with those in sinus rhythm [8, 9]. In the presence of AF, strokes are more often lethal or result in more disability than in its absence [10]. In developed countries, AF prevalence affects at least 1% of the population; however, across the world this prevalence varies [11]. A US study has shown that blacks, Asians, and Hispanic populations have a significantly lower AF prevalence compared to white people [12, 13]. Since the prevalence of AF rises with ageing, the prevalence of stroke also rises in older subjects [12, 13]. Atrial fibrillation is associated with up to 15% of all strokes, and 25% of strokes in individuals above 80 years.

Two meta-analyses have demonstrated that stroke was reduced by around 62-64% and 22% in AF patients treated with adjusted-dose warfarin and antiplatelet agents respectively; relative risk reduction for warfarin compared with antiplatelet agents was around 36-40% [14, 15]. In another meta-analysis study, in patients with AF treated with warfarin, risk of systemic embolism was halved compared to those treated with antiplatelet agents (OR=0.50, 95%CI=0.33-0.75), with risk of major bleeding being equal in the two groups (OR=1.07, 95% CI=0.85-1.34). Compared with placebo, warfarin reduced the risk of stroke by 71% (OR=0.29, 95% CI=0.08-1.07), outweighing absolute risk of harm, although relative risk of major bleeding was increased 3-fold (OR=3.01, 95% CI=1.31-6.92) [16]. As ischaemic stroke risk can be reduced by around two thirds by anticoagulation, patients with atrial fibrillation should be considered for treatment with oral anticoagulants [17, 18].

The original $CHADS_2$ scoring scheme, which combines several risk predictors into a 7-point scale (0–6), was developed to help in decision making about anticoagulation for patients with AF by estimating an individual's risk of stroke. Patients receive one point for having any of the following: **C**ardiac failure, **H**ypertension, **A**ge 75 years or above, **D**iabetes mellitus and two points for previous history of **S**troke or transient ischaemic attack [19]. According to ACCP guidelines 9th edition 2012, those scoring zero on $CHADS_2$ (low risk) are considered as AF individuals who should be treated with or without aspirin. AF individuals with $CHADS_2$ score of one (intermediate risk) should be treated with warfarin rather than aspirin or a combination of aspirin and clopidogrel; whereas warfarin should be used with $CHADS_2$ score of two or higher (high risk) if there are no contraindications [18]. However, the $CHADS_2$ scoring scheme has been recently refined to include other risk factors for stroke in AF. Data from the Euro Heart Survey on Atrial Fibrillation were used to generate the 10-point (0-9) CHA_2DS_2-VASc score [20]. Scoring determinants with their assigned points are as follows:

- **C**ongestive heart failure or left ventricular ejection fraction ≤40% (1 point);
- **H**ypertension (1 point);
- **A**ge ≥75 years (2 points);
- **D**iabetes (1 point);
- **S**troke/Transient ischemic attack (TIA)/thromboembolism (2 points);
- **V**ascular disease (myocardial infarction, peripheral arterial disease, or aortic plaque) (1 point);
- **A**ge 65 to 74 years (1 point); and
- **S**ex category female (1 point)

A CHA_2DS_2-VASc score of zero is categorized as “low” risk and may not require anticoagulation, a score of 1 as “low-moderate” risk and should consider antiplatelet or anticoagulation therapy, and a score of equal or more than 2 as “moderate-high” risk and should consider anticoagulation therapy [20]. According to latest NICE guidelines about AF management, patients with a CHA_2DS_2-VASc score of 1 or more should be treated with anticoagulants (a vitamin K antagonist or a NOAC); aspirin as monotherapy is not recommended for stroke prevention for people with atrial fibrillation [21, 22]. Table 1-2 shows the rate of thromboembolism according to CHA_2DS_2-VASc scoring system.

Table 1-2: Thromboembolism frequency at one year based on CHA_2DS_2-VASc Scoring System

CHA_2DS_2-VASc Score	Number of patients	Number of TE Events	TE Rate During one year (95% CI)
0	103	0	0% (0-0)
1	162	1	0.6% (0.0-3.4)
2	184	3	1.6% (0.3-4.7)
3	203	8	3.9% (1.7-7.6)
4	208	4	1.9% (0.5-4.9)
5	95	3	3.2% (0.7-9.0)
6	57	2	3.6% (0.4-12.3)
7	25	2	8.0% (1.0-26.0)
8	9	1	11.1% (0.3-48.3)
9	1	1	100% (2.5-100)
Total	1,084	25	*P* Value for trend 0.003

TE = thromboembolism, CI= confidence interval (Adapted from Lip, 2010 [20])

Deep venous thrombosis (DVT) and pulmonary embolism (PE)

Venous thromboembolism (VTE), which encompasses DVT and PE, is a common disease which is associated with high morbidity and mortality. Incidence of first VTE is around 1 per 1000 persons annually; and for those aged 75 years or older it increases to at least 5 per 1000 persons annually [23]. Around 10-20% of hospital medical inpatients could be expected to develop a VTE [24]. At initial VTE diagnosis, 42% of the patients present with DVT, 44% with PE, and 14% with both [25]. In around 10% of patients, clinically diagnosed PEs are rapidly fatal (within one hour), with an additional 5% mortality subsequently. Thrombosis will recur within 3 months in around 50% of non-treated patients with symptomatic DVT or PE. With recurrent VTE, the mortality rate is 2-3 times higher with PE compared to DVT [26]. The mortality rate within one month of diagnosis is around 6% for patients with DVT [27], and 12% for individuals with PE [28]. The mortality rate in patients with PE has been estimated to be twice that of DVT [29]. Generally, around 25-50% of patients presenting with first-time VTE have an idiopathic condition, without a recognizable risk factor [29]. When no anticoagulant is used as prophylaxis, the incidence of DVT is estimated to be 42-

57% and 41-85% with total hip arthroplasty (THA) and total knee arthroplasty (TKA), respectively, with a 0.1-2% and 0.1-1.7% incidence of fatal PE respectively [30].

Anticoagulant therapy in patients with DVT or PE has two aims. First, anticoagulation used for treating the thrombotic event limits the extension of the thrombus, hence, permitting the fibrinolytic system to resolve the thrombus, a process which might take a total of 6 weeks. Second, it prevents, to a large extent, the development of additional thrombi. Therefore, for thrombus resolution, anticoagulation is required for 3 months or more, according to the clinical conditions. Whether to stop anticoagulants at 3 months or continue the therapy indefinitely is determined by the risk of recurrence, and secondarily affected by bleeding risk and patient preferences [31-33]. Therefore, treatment of VTE with anticoagulants has been classified into three stages: initial treatment, long-term treatment, and extended anticoagulation.

During the initiation of warfarin treatment, overlapping (bridging) treatment with rapid-onset injectable anticoagulants (such as heparin or low molecular weight heparin) is recommended because of warfarin's slow onset of action [34]. However, with the new oral anticoagulants (NOACs), which have been introduced for clinical use recently, bridging treatment is not required owing to their quick onset of anticoagulant effect. The use of new oral anticoagulants are recommended as long-term anticoagulant therapy over warfarin according to recent antithrombotic therapy for venous thromboembolism (VTE) disease guidelines published in Feb 2016 [33]. Further detailed information on NOACs is provided later in this chapter.

Mechanical heart valve prostheses

Prevention of valve thrombosis and systemic embolism is the main goal of anticoagulation in the presence of a mechanical prosthetic heart valve. Without antithrombotic treatment, the risk of major embolism has been estimated as 4.0 per 100 patient-years, while the risk is decreased to 1.0 per 100 patient-years when oral anticoagulation is used [35]. Moreover, by using oral anticoagulation, the risk of valve thrombosis was decreased by 80% when compared with no treatment [35]. Thus, patients with mechanical prosthetic heart valves should be anticoagulated indefinitely. Aspirin is recommended in addition to warfarin in patients with mechanical heart valves who have additional risk factors which include atrial fibrillation, previous

thromboembolism, left ventricular dysfunction, and hypercoagulable condition [36]. The use of NOACs in patients with prosthetic heart valve is not recommended because of the increased risk of thromboembolism [37].

1.1.4. Determination of anticoagulation activity

Prothrombin time (PT) is a laboratory test used for warfarin dosing and monitoring. PT varies depending on the thromboplastin used to measure how long the blood takes to clot. The result, expressed as prothrombin time ratio (PTR), was initially used for monitoring warfarin dosing, but the non-standardization of thromboplastin led to problems. Thus, if the commercial thromboplastin was less responsive, larger doses of warfarin were given to reach the target PTR. This led to over anticoagulation and a high incidence of bleeding. Therefore, a model was adopted by the World Health Organization (WHO) in 1982 to convert the PTR obtained by any reagent to an International Normalized Ratio (INR). This made a uniformity of anticoagulation control worldwide.

The INR is calculated using the following formula:

INR=$\left\{\frac{PT\ (patient)}{PT\ (control}\right\}^{ISI}=PTR^{ISI}$, where ISI is the international sensitivity index. ISI is quantified by commercial manufacturers for the specific thromboplastin reagent used in each batch and reported in the product package insert.

Levels of three of the four vitamin K-dependent clotting factors, II, VII, and X, are reflected by the INR status. At the initiation or discontinuation of warfarin, PT/INR changes will reflect changes in the concentration of FVII, then FX, and finally FII; that is attributed to the different half-lives of these clotting factors (FVII=2-9 hours, FX=17-44 hours, and FII=60-72 hours) [38]. After discontinuation of warfarin and without vitamin K administration, the INR level should normalize within five to ten days as the mean effective warfarin half-life is around 40 hours [39].

For prevention of thromboembolism due to mechanical heart valve, the recommended INR is between 2.5 and 3.5. For most other conditions an INR target range of 2.0 to 3.0 is recommended (Table 1-3).

Table 1-3: Clinical indications for warfarin

Indication	INR target range
VTE and pulmonary embolus Atrial fibrillation Coronary artery disease (controversial) Mechanical heart valves in aortic position Bioprosthetic heart valves in patients with risk factors* Secondary prevention of cerebrovascular accident (CVA) after failing antiplatelet therapy Secondary prophylaxis in antiphospholipid syndrome (APS)	2.0 - 3.0
APS patients who have VTE on therapeutic VKA Mechanical heart valves in mitral position Mechanical heart valves in aortic position with high risk factors*	2.5 - 3.5

*high risk patients include those with atrial fibrillation, previous VTE, left ventricular dysfunction, and hypercoagulable conditions. (Adapted from Moualla, 2011) [4]

Recommendations for INR targets come mainly from clinical trials carried out in white/western, mainly Caucasian, people. These recommendations have been traditionally used to manage anticoagulation treatment in all ethnic groups. More recently, it has been advised that an individualized target INR should be used to take into account the differences in ethnicity [40, 41].

1.1.5. Epidemiology of anticoagulation use

It is calculated that, in the UK, over 1% of the population (around 600,000 individuals) are prescribed warfarin [42]; about 8% of those above 80 years are taking warfarin, with the number of people requiring anticoagulation therapy rising due to a greater prevalence of AF associated with population longevity[3]. In the developed countries around 1-2% of the population are prescribed anticoagulants [43], and there were more than 33 million prescriptions dispensed in the US in 2011 [44].

The use of new oral anticoagulants (NOACs) is increasing; however, warfarin currently remains the most commonly prescribed oral anticoagulant agent, accounting for more than 75% of usage[45].

1.1.6. Pharmacology and mechanism of action of warfarin

The post ribosomal activation of clotting factors II,VII, IX, and X takes place by γ-carboxylation of their N-terminals in the liver. The carboxylation reaction is catalyzed by the γ-glutamyl carboxylase enzyme which requires reduced vitamin K (vitamin K hydroquinone) as a cofactor. Vitamin K-hydroquinone is oxidized by γ-glutamyl carboxylase to 2,3 vitamin K epoxide. Anticoagulation by warfarin (which has a similar chemical structure to that of vitamin K) is accomplished by competitive inhibition of the vitamin K 2,3-epoxide reductase enzyme (VKOR) which is responsible for the repeated interconversion of the reduced form of vitamin K and its 2,3 epoxide in conjunction with γ-glutamyl carboxylase in the vitamin K cycle. Eventually, by the effect of warfarin on the vitamin K epoxide reductase complex 1 (VKORC1), regeneration of the reduced form of vitamin K is blocked. The pharmacological action of warfarin is shown in Figure 1-3.

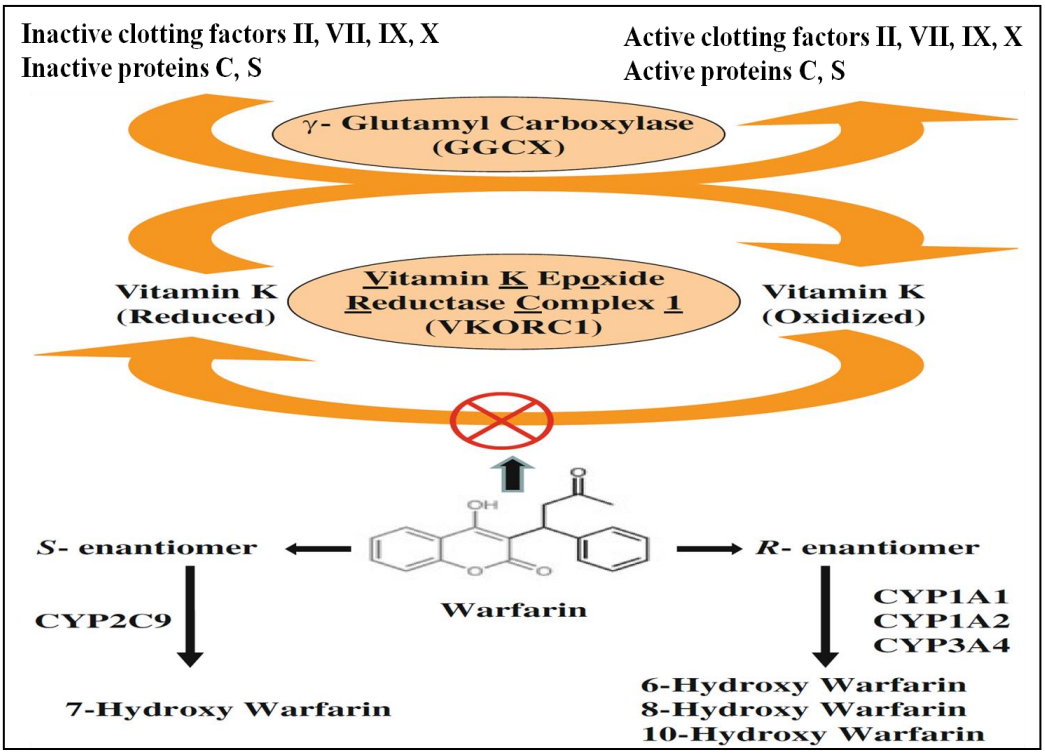


Figure 1-3: Warfarin mode of action and metabolism [1]

1.1.7. Pharmacokinetics and metabolism of warfarin

Warfarin, which is marketed as a racemic mixture (50/50) of S and R enantiomers, is rapidly and completely absorbed from the gastrointestinal tract. S- and R- warfarin result from asymmetry in warfarin carbon number 9. The S-enantiomer is pharmacologically three to five times more potent and has a shorter elimination half-life (24-33 hours) than the R-enantiomer (35-58 hours) [46, 47]. Cytochromes P450 (CYP), part of the microsomal mono oxygenase group of enzymes, are responsible for the oxidation of many exogenous chemicals and drugs, in addition to numerous endogenous substances. S-warfarin is metabolized mainly by the CYP2C9 enzyme. Genetic polymorphism, which influences both S- and R-warfarin metabolism, will be covered in section 1.1.18. R-warfarin is metabolized by more than one CYP enzyme, as shown in Figure 1-3. Racemic warfarin has a half-life of around 36-42 hours. The elimination half-lives of the other VKAs acenocoumarol and phenprocoumon are 1.8–6.6 and 110–130 hours, respectively [47]. Warfarin pharmacokinetic parameters are summarized in Table 1-4.

Table 1-4: Pharmacokinetic parameters of warfarin

	Warfarin
Peak serum concentration	0.3–4 hours
Bioavailability (%)	~ 100
Protein binding (%)	>99
Elimination kinetics	First-order
Metabolism	Major: hydroxylation by hepatic cytochrome p450 enzymes Minor: keto reduction
Excretion (%)	Renal: 80 Faeces: 20
Half-life (hours)	R enantiomer: 35–58 S enantiomer: 24–33

(Adapted from Lee, 2011) [48]

1.1.8. Warfarin pharmacodynamics

Warfarin is a specific inhibitor of the vitamin K epoxide reductase enzyme (VKOR), which was identified in 1974 [49]. The VKOR enzyme is encoded by the vitamin K epoxide reductase complex subunit 1 (VKORC1) [50], which will be covered in section 1.1.18. Warfarin exerts its anticoagulation activity by preventing the ability of VKORC1

to regenerate the active, reduced form of vitamin K (vitamin K hydroquinone) from its epoxide form. The main target step in the coagulation cascade for warfarin is the conversion of prothrombin to thrombin, with the latter having an approximate half-life of 96 hours. There is a time lag for the full anticoagulant effect of warfarin. The anticoagulant effect of warfarin during the first 48 hours following warfarin initiation is dependent on the reduction of factors VII and IX (with half-lives of 5 and 24 hours, respectively). For acute anticoagulation four to five days overlap treatment with unfractionated heparin (UFH) or low molecular weight heparin (LMWH) is recommended when starting warfarin therapy, as the activities of factors II (half-life = 30-40 hours) and X (half-life = 60-70 hours) decrease slowly due to their long half-lives. Warfarin has no role in factors II, VII, IX, and X catabolism, lowering their plasma concentrations indirectly.

1.1.9. Time-in-therapeutic range (TTR)

TTR is a method of estimating quality of anticoagulation management and is defined as the estimated total percentage of time that the INR is within a pre-determined therapeutic range [46]. TTR is a key method used to gauge the quality of VKA anticoagulation treatment [51, 52]. TTR is mainly calculated using the linear interpolation model (assuming changes between consecutive INR readings are linear over time), also known as the Rosendaal method [53]. The Rosendaal method has been recommended by the American College of Chest Physicians (ACCP) for assessing sufficiency and quality of anticoagulation. It has been established that risk of major bleeding, ischaemic stroke, and all-cause mortality can be significantly predicted by TTR [54]. Moreover, it has been demonstrated that anticoagulation quality broadly differs between different countries. For example, as reported in RELY trial in the warfarin treated group, Taiwan had the lowest mean TTR of 44% and the highest was in Sweden at 77% (rank 1st), whereas the mean TTR in the UK was 72% (rank 4th) [55]. An example of TTR calculation is shown in Appendix (A) page 213.

1.1.10. Frequency in range (FIR)

FIR is another method of assessing the quality of anticoagulation control, it is also known as proportion of INRs in the therapeutic range and number of tests in range. It is estimated as the percentage of the total INR readings that are in the therapeutic range during the selected time interval. FIR has the advantage of being easier to estimate and can effectively be done manually compared to TTR which is relatively difficult measure to estimate and need a software programme. A recent study reported that the TTR and FIR showed widespread variability and disagreement, in which TTR values were higher than FIR at the patient level. The study suggested that TTR and FIR are not equivalent and cannot be used interchangeably [56]. Table 1-5 shows the advantages and disadvantages of TTR and FIR in assessing anticoagulation control.

Table 1-5 Advantages and disadvantages of frequency in range and time in therapeutic range methods

Methodology	Advantage	Disadvantage
Frequency of INR in range (FIR)	• Simple to calculate • Requires only one INR value per patient in clinic population • Not influenced by extent of INR out-of-range	• More frequent testing in unstable patients may bias overall results (will under-estimate TTR of group) • Does not take into account actual days within target range • Does not consider individual patients
Time in therapeutic range (Rosendaal linear interpolation)	• Takes into account actual days in target range • Allows one to calculate INR specific incidence rates of adverse events	• Calculation more difficult • Makes assumptions about INR between actual tests • Does not consider individual patients • Extreme out-of-range INR values may bias overall results

(Adapted from Schmitt, 2003 [57])

1.1.11. Warfarin initiation and maintenance therapy

Warfarin is prescribed usually empirically, in which an initial dose is given, followed by at least weekly INR measurement, and further dose adjustment. The initial dose may depend on population average, e.g. 3-5 mg/day in Asians or 5-10 mg/day in Caucasians [49, 58]; however, stable doses to reach the target INR range of 2.0-3.0 range between 0.5-20 mg per day. The process to achieve the desired dose by "trial and error" adjustments could take weeks to months, during which patients are at particular risk of over- or under- anticoagulation and hence at risk of thrombosis or bleeding [59].

Warfarin is characterized by many drug and food interactions, as well as genetic polymorphisms leading to variability in its metabolism and pharmacological activity. These, together with patient age and body size and some as yet unestablished factors, give rise to a 40-fold inter-individual variability in warfarin maintenance dose requirement (0.5 to 20 mg daily) to attain the same degree of anticoagulant intensity. Currently, there is no other commonly prescribed drug which resembles warfarin in this regard [60].

The risk of bleeding caused by excessive anticoagulation is greatest during the initiation phase of warfarin therapy. Figure 1-4 shows the risk of bleeding events with increasing INR. Several warfarin dosing regimens have been developed and tested in an attempt to improve warfarin safety. There is debate as to whether a pharmacogenetics-based loading dose improves the safety of warfarin (please refer to section 1.1.18 for further detail). In addition, there is debate about the optimal frequency of monitoring during maintenance therapy in order to keep patients within their target therapeutic INR range as frequent monitoring is expensive for health care systems and burdensome for patients and physicians [61].

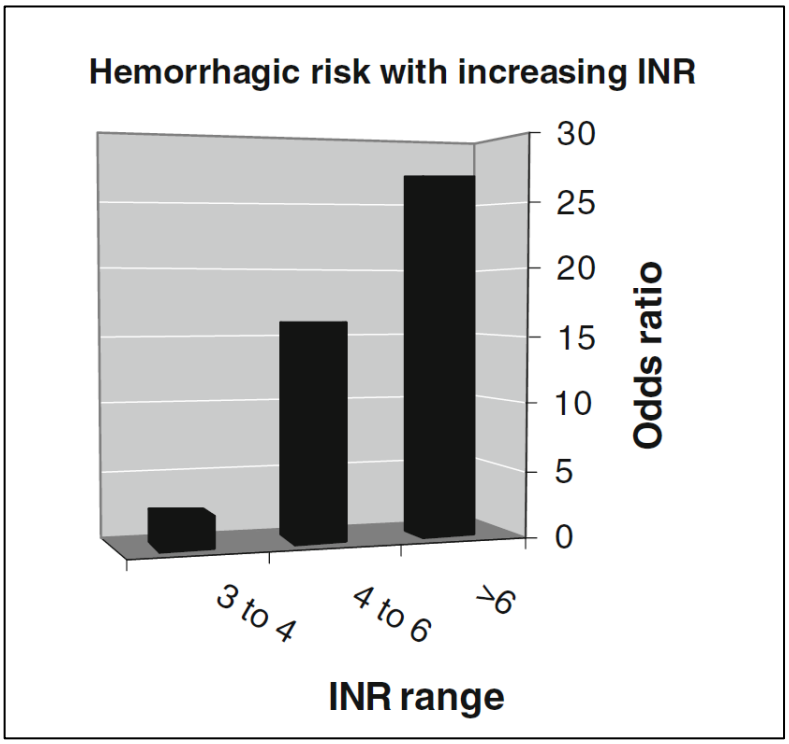


Figure 1-4: The influence of INR on bleeding risk[40]

It has been noted that evidence-based models can be underused for warfarin maintenance dosing by primary care physicians and specialists [62]. The American College of Chest Physicians (ACCP) recommended the commencement of warfarin with doses between 5 mg and 10 mg for the first 1 or 2 days for most patients, with subsequent dosing based on INR value [38, 63]. A systematic review has shown that there is no advantage of using the traditional 10 mg loading dose over 5 mg, but clinical practice has been slow to adopt the new recommendation. There is evidence that lower starting doses or doses adjusted by age might be even more beneficial, leading to fewer supratherapeutic INR values [64].

The maximum acceptable follow-up interval to measure INR in some services seems to be dependent on prescriber discretion and experience, in spite of the existence of evidence-based dosing guidelines. For example, Rose, et al, 2011, studied 100 Veterans' Health Administration sites of care; these sites managed 104,451 patients taking warfarin. Each site had a specialized anticoagulation clinic.

The study estimated the mean follow-up interval after low (≤1.5) or high (≥4.0) INR. 57% of the patients were assigned to the low INR analysis, and 36% of the patients were assigned to the high INR analysis. The average interval until the next INR reading was 6-18 days after a high INR, and 10-24 days after a low INR. The study suggested that, after getting the first or second therapeutic INR reading, the maximum interval should be 28 days. However, after getting the third or higher consecutive INR therapeutic reading, a longer interval might be acceptable [65, 66].

On the other hand, Schulman, et al, (2011), in a non-inferiority randomized study recruited 250 patients and monitored their warfarin dose every 12 or 4 weeks. Every 12 weeks monitoring of warfarin dosing was found efficient and comparable to every 4 weeks monitoring. However, the study was done in one centre, and patients in the 12-week group were seen and examined by clinic staff regularly every 4 weeks [67].

1.1.12. Warfarin failure and resistance

Warfarin resistance should be differentiated from warfarin failure. Warfarin failure is defined as a new thrombotic episode in the presence of a therapeutic INR. Around 5% of patients will have had a recurrent thrombotic episode by 3 months after starting treatment. Risk factors for recurrent thrombosis include previous history of an idiopathic VTE, sub-therapeutic anticoagulation, malignancy, antiphospholipid antibody syndrome (APLAS), and rare inherited thrombophilia such as antithrombin, protein C and S deficiency [46]. Warfarin resistance is defined as “the inability to prolong the prothrombin time or raise the INR into the therapeutic range when the drug is given at normally prescribed doses”. Warfarin resistant patients are defined as those requiring more than 15 mg daily. Warfarin resistance might be due to extrinsic factors such as low treatment compliance (the most common cause), drug interactions, food interactions, or hereditary factors. Warfarin resistance related to genetic factors is not well characterized. Osinbowale, has suggested an algorithm to evaluate and manage suspected warfarin resistance [68].

1.1.13. Warfarin therapy complications

Since warfarin is a difficult-to-control medication, the main complications occur from supra-therapeutic (the most common) or sub-therapeutic status, which results in haemorrhage or thrombosis, respectively. It has been demonstrated in the USA that the most common drug-related cause of hospitalisation for adverse events among older adults is related to warfarin use, which accounted for 33% of cases requiring hospitalisation. From 2007-2009, it was estimated that 21,010 hospitalisation cases were due to warfarin related bleeds [69].

Major bleeding episodes related to warfarin usage have been estimated to be between 1.3% and 4.2% per annum [70]. In those bleeding as a result of warfarin toxicity, 0.4-2.0% need blood transfusion, hospitalization, or surgery [39]. A study has shown that around 1 in 10 major haemorrhages were fatal, and 1 in 12 patients re-bled after warfarin was restarted [71]. Intracranial haemorrhage is the most serious adverse effect caused by excessive anticoagulation, affecting about 4 per 1000 patients on warfarin annually [72], with a mortality rate approaching 50% [73]. Warfarin is one of the ten drugs most regularly responsible for elderly patients visiting the emergency department because of an adverse drug reaction [74]. It has been demonstrated that the risk of bleeding in patients aged 80 years or above and having an INR of ≥4.0 is increased by more than 22 times [75]. Acute bleeding events requiring a visit to the emergency department associated with the use of antiplatelet drugs, clopidogrel and aspirin, have been calculated to be 12 per 10,000 outpatient prescription visits whereas twice as many number of cases were recorded, 25 per 10,000 outpatient prescription visits, for warfarin usage (Risk Ratio = 0.49, 95% CI, 0.15-0.83) [76]. In the UK, absolute risk of harm is high as between 10-24 cases of bleeding occur per 100 patients using warfarin per annum [42]. A systematic review of the medical literature has advised that there is no association between covariates described in the warfarin prescribing information and enhanced bleeding risk, except possibly for those suffering from renal disease, and malignancy [77].

For all these reasons and others, it has been estimated that warfarin is underutilized in AF patients by 60-70% [8]. Data predating NOAC availability suggested that, of AF patients with indications for anticoagulation for stroke prevention, only about 50% are treated with warfarin [78]. Physicians are hesitant to prescribe it

[79], and patients prefer to stop taking it [80]. A study found that 26% of patients aged 80 years or over discontinued warfarin within the first year of treatment due to safety issues [75]. The outcome is that high-risk patients eligible for warfarin therapy who are not treated with it are at greater risk of stroke [81].

Because of the difficulties with warfarin therapy, various methods have been implemented to enhance anticoagulation management, such as initiating specialised anticoagulation clinics for routine monitoring, launching of dosing algorithms and dosing software programmes to improve dosing efficiency, and improving treatment compliance among patients through education programmes [82].

1.1.14. Other non-bleeding adverse reactions associated with warfarin treatment

- Skin necrosis is an unusual complication occurring in the first few days of warfarin treatment, more commonly in women. It is associated with a high warfarin dose of more than 10 mg daily. Lesions are typically seen in the extremities [83].
- Purple toes syndrome is another rare complication. It occurs from 3 to 10 weeks after starting warfarin therapy, and is characterized by dark, purplish or mottled colour of the toes. This syndrome is reversible. However, in some instances, it progresses to necrosis, which requires debridement or amputation [83].
- Patients with frail skin (e.g., elderly, or those using steroids long term), or with over anticoagulation, may develop ecchymosis and purpura [84].
- Macular-papular, vesicular, or urticarial itchy rashes may occasionally occur after several weeks or months from commencement of treatment [84].

1.1.15. Management of supratherapeutic INRs with and without bleeding

Reversal of excessive anticoagulation is commonly required in patients using warfarin. Variations in INR might be attributed to one or more of the following situations: (a) incorrectness in INR measurements; (b) changes in vitamin K intake; (c) changes in vitamin K or warfarin absorption; (d) changes in warfarin metabolism; (e) changes in vitamin K-dependent coagulation factor synthesis or metabolism; (f) some

effects related to concomitant drug use or comorbidities; or (g) patient noncompliance [38].

1.1.15.1. Warfarin antidotes

Patients on warfarin have decreased levels of functional coagulation factors II, VII, IX, and X. Different modalities are available to treat supra-therapeutic INR and warfarin induced bleeding resulting from clotting factors II, VII, IX, and X deficiency. These include the administration of vitamin K, prothrombin complex concentrate (PCC), recombinant activated FVII (rFVIIa), and fresh frozen plasma (FFP).

Vitamin K

Where anticoagulation by warfarin does not require immediate reversal (where the risk of bleeding in the patient is low), vitamin K is a cheap and effective antidote; it works by enhancing endogenous production of functional vitamin K-dependent clotting factors. Vitamin K is administered intravenously for the swift reversal of excessive INR in patients deemed to be at significant risk of bleeding [39, 85]. Anticoagulation reversal is attained within 6-12 hours if vitamin K is given intravenously, compared to 18-24 hours when it is given orally [45]. The subcutaneous route is no more effective than placebo, and the intramuscular route may lead to haematoma and bleeding in an over-anticoagulated patient, in addition to having unpredictable absorption so these routes should not be used [85].

Prothrombin complex concentrate (PCC)

There are two kinds of PCCs, 3-factor PCC which contains factors II, IX, and X, and 4-factor PCC which contains factors II, VII, IX, and X. Advantages of PCC compared to FFP comprise its fast onset of action, quick reconstitution into a small volume for infusion over 20-30 minutes, no need for determination of blood group of the recipient, minimal risk of cross contamination, and lower risk of other adverse effects such as volume overload [85]. Moreover, clotting factor concentration in PCC is about 25 times higher than that available in plasma [45]. A systematic review, comparing 3-factor against 4-factor PCC, showed that the INR was normalised (≤1.5

within hour of administration) more effectively with 4-factor [86]. However, the systematic review lacked direct comparison studies between 3-factor and 4-factor PCC. In a prospective observational study, 4-factor PCC was compared against FFP for treatment of intracranial bleeding. 4-factor PCC reversed the anticoagulation significantly in a shorter time compared to FFP (65 minutes vs 256 minutes, $P<0.05$) [87]. 3-factor PCC is not recommended as it is poor at correcting INR compared to 4-factor PCC which is able to totally antagonize the warfarin-induced anticoagulation within 10 min [38]. 5-10 mg intravenous vitamin K should be administered with the 4-factor PCC because the infused clotting factors in the PCC have a limited half-life, the shortest of which is FVII at 6 hours, to stimulate clotting factor production. The only PCCs approved for warfarin reversal in the UK are 4-factor PCCs [38].

Recombinant activated FVII (rFVIIa)

It has been shown that rFVIIa rapidly corrects the INR; however, its impact on stopping bleeding is unclear and its use is not approved for warfarin reversal in the UK [38].

Fresh frozen plasma (FFP)

FFP is administered as a blood group specific product, and has to be thawed before use. The blood group of the recipient needs to be determined before administration. Theoretically, one unit of each clotting factor is present in each milliliter of FFP. FFP possesses exogenous clotting factors and has a quick onset of action, but because of its short half-life, it has a temporary effect on INR. As it offers a diluted form of clotting factors more than one liter of FFP may be required to normalise the levels of clotting factors. As it may not be feasible to infuse very large volumes of plasma (15–30 ml/kg) rapidly (the FFP typical dose is 10-15 ml/kg), this is a major obstacle to FFP efficacy. Other disadvantages of FFP are the risk of volume overload in frail and elderly recipients (mitigated if they are losing blood), and increased risk of infectious disease transmission. Moreover, as FFP produces poorer correction of INR compared to other agents, it cannot be recommended for life-threatening bleeding.

Anticoagulation management prior to surgery

Anticoagulation has to be reversed before most surgical interventions in order to avoid serious bleeding intra-operatively. In consequence, stopping warfarin 5 days prior to elective surgery is recommended [88].

The optimal choice for warfarin reversal depends on the time available before surgery, the capability of estimating and managing blood loss, injury risk, and whether anticoagulation is needed after surgery. Vitamin K should be administered intravenously for procedures that could be delayed for 6-12 hours when urgent surgery is required. PCC should be used when the surgical procedure cannot be delayed, accompanied by intravenous vitamin K to provide sustained anticoagulation reversal [45]. As shown in Table 1-6, many minor surgical procedures have a low risk of bleeding, and warfarin reversal might not be needed although this can be controversial [39].

Table 1-6: Bleeding risk and type of surgery

Low risk of bleeding - minor surgery - percutaneous needle procedures in readily compressible sites - dermatological procedures - routine dental procedures - cataract procedures	-Ideal target is INR of 2.0 - If INR<2.5, the patient can proceed to surgery - If INR is >2.5, decide whether the level of anticoagulation is safe for surgery to proceed.
High risk of bleeding - all major surgery/surgery in which a body cavity is entered - percutaneous needle procedures in non-compressible sites - organ biopsies - prostatic surgery - sites where bleeding can be catastrophic, e.g., intracranial and some opthalmological procedures	-Target INR<1.5 - Supplement with heparin if required, e.g., patients with prosthetic valves.

(Adapted from Grobler, 2010) [39]

Anticoagulation reversal according to guidelines (4th edition), published in the British Journal of Haematology 2011 [89]

Major bleeding

If the reversal of warfarin anticoagulation is urgently needed due to major bleeding, 25–50 u/kg of 4-factor PCC should be used with 5 mg intravenous vitamin K. rVIIa is not recommended. If PCC is not available, FFP should be used as it produces suboptimal anticoagulation reversal.

Non-major bleeding

1–3 mg intravenous vitamin K should be used for reversal of anticoagulation for non-major bleeding.

Head injury in patients on warfarin

INR should be measured as soon as possible, and a head CT scan should be done. If a strong possibility of intracerebral bleed is present, the anticoagulation should reversed before the results of any investigations are known.

Non-bleeding with INR>5.0 and <8.0

For patients without bleeding, if their INR is >5.0, they should stop warfarin for 1-2 doses, and their maintenance dose should be reduced also; and if their INR is >8.0, they should also receive vitamin K 1-5 mg orally.

It is feasible to stop warfarin and wait until the INR returns to the required therapeutic range in case of asymptomatic supra-therapeutic INR without administration of vitamin K. Vitamin K may be used to lower a high INR in such a case; however, lowering INR too much in asymptomatic patients might increase the thrombotic propensity, or warfarin resistance may develop once warfarin is restarted [90].

Anticoagulation reversal according to guidelines published by the American College of Chest Physicians 2012, 9th edition [63]

The guidelines are summarised in Table 1-7.

There are many similarities between the American and British guidelines; however, rVIIa is not recommend by the British guidelines for use in major bleeding, whereas it is recommended by the American ones.

Table 1-7: Recommendations for managing high INRs or bleeding in patients receiving VKAs according to ACCP guidelines

Condition	Intervention
INR more than therapeutic range but < 5.0; no significant bleeding	Lower dose or omit dose; monitor more frequently and resume at lower dose when INR therapeutic; if only minimally above therapeutic range, no dose reduction may be required
INR≥ 5.0, but< 9.0; no significant bleeding	Omit next one or two doses, monitor more frequently, and resume at an appropriately adjusted dose when INR in therapeutic range. Alternatively, omit dose and give vitamin K (1–2.5 mg po), particularly if at increased risk of bleeding. If more rapid reversal is required because the patient requires urgent surgery, vitamin K (≤ 5 mg po) can be given with the expectation that a reduction of the INR will occur in 24 h. If the INR is still high, additional vitamin K (1–2 mg po) can be given
INR ≥ 9.0; no significant bleeding	Hold warfarin therapy and give higher dose of vitamin K (2.5–5 mg po) with the expectation that the INR will be reduced substantially in 24–48 h. Monitor more frequently and use additional vitamin K if necessary. Resume therapy at an appropriately adjusted dose when INR is therapeutic.
Serious bleeding at any elevation of INR	Hold warfarin therapy and give vitamin K (10 mg by slow IV infusion), supplemented with FFP, PCC, or rVIIa, depending on the urgency of the situation; vitamin K can be repeated q12h.
Life-threatening bleeding	Hold warfarin therapy and give FFP, PCC, or rVIIa supplemented with vitamin K (10 mg by slow IV infusion). Repeat, if necessary, depending on INR.
Administration of vitamin K	In patients with mild to moderately elevated INRs without major bleeding, give vitamin K orally rather than subcutaneously

(Adapted from Ansell, 2008[38])

1.1.16. Patient factors which influence warfarin sensitivity

1.1.16.1. Age

Higher warfarin sensitivity and lower warfarin dose requirements are associated with advancing age [91], with older women requiring the lowest warfarin doses to achieve the same degree of anticoagulation intensity [92]. This is linked to liver mass shrinkage in elderly people which leads to decrease in liver capacity to eliminate warfarin and also a reduction in the synthesis of vitamin K-dependent clotting factors [93]. Plasma protein binding is decreased by 15%-25% which leads to an increase in the free warfarin concentration initially [94]. It has been shown that mean warfarin daily dose requirement deceased by 0.5 to 0.7 mg every 10 years between the ages of 20 to 90 years [95]. Age has been consistently identified as a significant contributor to warfarin dose requirements in multiple linear regression models used to develop warfarin dosing algorithms [95-97].

1.1.16.2. Body Mass Index (BMI)

As the effect of body size on warfarin dosing is confounded by other variables, such as age, polypharmacy, comorbid diseases, and presence of genetic polymorphisms, the influence of BMI on warfarin has not been firmly established [91, 94, 98, 99]. An association between body size and warfarin dose is likely to be indirect and reflect the link between liver size and dose, body size being positively correlated with liver size. Although the association between body weight and VKA dosing was not confirmed in a recent review of studies due to the variability in the methods of the 32 studies included, evidence suggests that at the time of VKA commencement, obese or morbidly obese patients require a 30-50% larger dose of warfarin than do people of standard weight[99].

1.1.16.3. Alcohol consumption

The anticoagulation activity of warfarin is affected by consumption of even small amounts of alcohol. Free unbound warfarin has been estimated to be increased by 3-34% with alcohol consumption [100]. The anticoagulation effect of warfarin might

increase after consumption of a few drinks in one sitting, putting the individual at a higher risk of bleeding. Alcohol enhances the anticoagulant effect of warfarin by inhibiting its metabolism by cytochrome P450 enzymes in the liver. In contrast, long-term ingestion of alcohol by chronic drinkers induces cytochrome P450, and hence warfarin metabolism; thus larger warfarin doses are needed to attain the required anticoagulation effect. As alcohol can both inhibit and promote warfarin's metabolism, it is advisable to check the INR if the patient has a sudden change in the amount of alcohol consumed, for example, stopping or ingesting more than 3 alcoholic drinks daily [101-103].

1.1.16.4. Nutritional Status

Warfarin interacts with certain foods and supplements which can lead, unintentionally, to a reduction or increase in its anticoagulant effect. Ingestion of regular meals has no effect on warfarin bioavailability; however, fluctuations in consumption of vitamin K containing foods such as green leafy vegetables or supplements containing vitamin K, in particular, affect anticoagulation response [104-106].

1.1.16.4.1. Vitamin K

In 1929, vitamin K was discovered accidentally by Henrik Dam (1895-1976), the Danish nutritional biochemist, as part of sterol metabolic experiments, and it was linked to blood coagulation. He found that chickens had a bleeding diathesis when they ate a fat-free diet. In 1935, Dam was able to rule out cholesterol as the missing agent and named this fat soluble compound as vitamin K for “Koagulations vitamin”. Subsequently, vitamin K_1 was isolated from alfalfa, and vitamin K_2 from putrefied fish. Edward Doisy (1893–1986) was able to identify and synthesize the chemical structure of vitamin K_1 (2-methyl-3-phytyl-1,4-naphtoquinone), and vitamin K_2 (2-methyl-3-(all trans-farnesylfarnesyl)-1,4-naphthoquinone) to have an unsaturated polyprenyl side chain at the 3-position of the naphthoquinone ring [107, 108]. The Nobel Prize for Physiology or Medicine in 1943 was granted to Henrik Dam of the Polytechnic Institute of Copenhagen, Denmark, for his “discovery of vitamin K” and to Edward Adelbert

Doisy of the Saint Louis University at St. Louis, Missouri, for his “discovery of the chemical nature of vitamin K” [109].

1.1.16.4.2. Chemical structures of vitamin K compounds

Products present in nature with vitamin K activity have in common a functional methylated naphthoquinone (2-methyl-1,4-naphthoquinone) ring system, and an aliphatic side chain consisting of isoprenoid residues [110]. Vitamin K compounds differ in the structure of their isoprenoid side chain at position-3, and are classified into vitamin K1 to 3. Vitamin K exists naturally in food as phylloquinone with a phytyl side chain (or vitamin K1, 2-Methyl-3-phytyl-1, 4-naphthoquinone) and as bacterially synthesized menaquinones (MK-n, or collectively vitamin K2, 2-Methyl-3-multiprenyl-1, 4-naphthoquinone) with unsaturated multi-prenyl side chains. Menadione (or vitamin K3, 2-Methyl-1, 4-naphthoquinone) is a synthetic water soluble vitamin K present in animal feed, and hence enters the human food chain indirectly. Menaquinone-4 (MK-4, 2-Methyl-3-geranyl-geranyl-1,4-naphthoquinone), a short chain vitamin K2, is converted from phylloquinone or produced endogenously from menadione. Menaquinones (MK-7 to MK-11), the long chain vitamin K2, are synthesized in the intestine by micro-flora, for example, MK-10 and MK-11 produced by bacteroides, MK-8 by enterobacteria, MK-7 by veillonella species [111]. Vitamin K as a supplement comes only from either phylloquinone, MK-4, or MK-7. Only synthetic phylloquinone and MK-4 are used therapeutically; phylloquinone is used in the treatment of haemorrhagic disease of the new-born and as an antidote to vitamin K antagonists (VKA), whereas MK-4 is used for osteoporosis. MK-7, a naturally fermented product, is used in dietary supplements for maintaining bone health [112].

Figure 1-5 shows the chemical structures of the vitamin K compounds. All forms of vitamin K serve one major function which is as a cofactor for the post-translational enzyme γ-glutamate carboxylase for activation of clotting proteins.

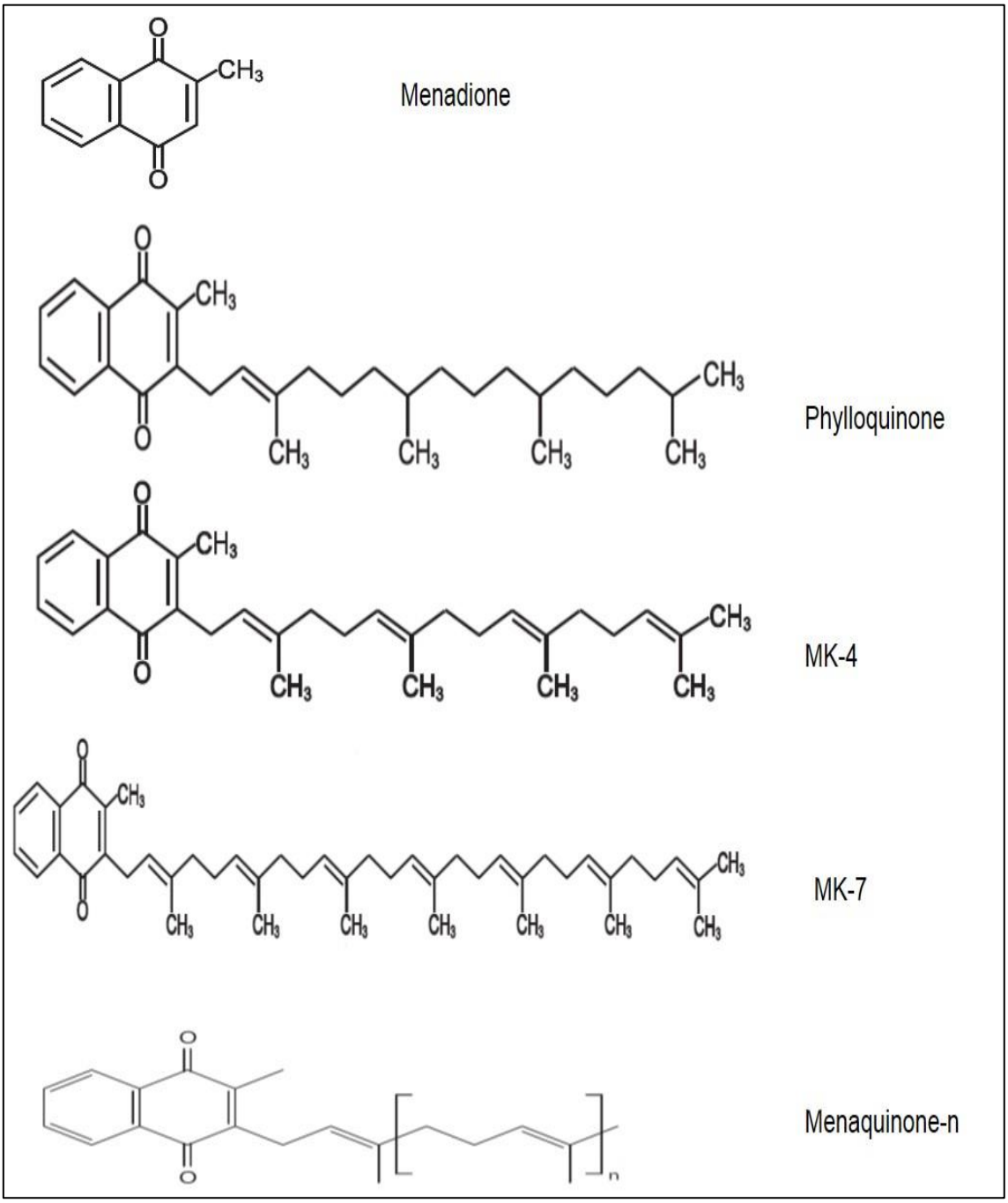


Figure 1-5: Chemical structure of vitamin K compounds

1.1.16.4.3. Dietary sources

Green leafy vegetables are the main dietary source for phylloquinone which is present in concentrations ranging between 400 to 700 µg/100 gm, confirming the established association between phylloquinone and photosynthetic tissues, the darker green the leaf the higher the phylloquinone concentration. The second best sources are certain vegetable oils (such as soybean, rapeseed and olive oils) with varying concentrations of 50 to 200 µg/100 gm. When these oils are regularly consumed as part of a daily diet, they make a significant contribution towards the daily intake of vitamin K. Other vegetable oils, such as peanut, corn, sunflower and safflower oils have lower phylloquinone concentrations (1 to 10 µg/100 gm). A comprehensive list of common foods containing vitamin K compiled by the United States Department of Agriculture (USDA) National Nutrient Database, can be accessed online from the following link (http://www.nal.usda.gov/fnic/foodcomp/Data/SR18/nutrlist/sr18w430.pdf) [94]. Table 1-8 provides vitamin K content of some commonly eaten foods.

Table 1-8: Common foods containing Vitamin K, and its amounts*

Moderate (50-99 µg)	High (100-400 µg)	Very High (401-1800 µg)
Black-eyed peas (cooked) 63 Broccoli (raw) 89 Cabbage (cooked) 73 Lettuce- iceberg, green leaf, romaine (raw) 50-97 Okra (cooked) 88 Prunes 65 Rhubarb (frozen) 71	Broccoli (cooked) 220 Brussels sprouts (cooked) 219 Dandelion greens (cooked) 203 Endive (raw) 115 Lettuce- butter head, red leaf (raw) 130-166 Parsley (raw), 10 sprigs, 164 Scallions (raw) 207 Sauerkraut (canned) 135 Spinach (raw) 144	Beet greens (cooked) 697 Collard greens (cooked) 1059 Kale (cooked) 1146 Mustard greens (cooked) 419 Spinach (cooked) 888 Turnip greens (cooked) 529

*Serving size: one cup unless otherwise specified. (Adapted from White, 2010 [94])

Long-chain menaquinones (MK-6 to MK-13) are present in various amounts in animal livers. According to quantitative HPLC analysis, livers from ruminant species (such as cow) contain values of some menaquinones (e.g., MK-7, MK-11, MK-12, and MK-13) in a range of 10-20 µg/100 gm. Other animal organs (such as kidney, heart, and muscle) are nutritionally not significant sources of menaquinones as they contain very low concentrations. Menaquinones are present in cheese (10-20 µg/100), other fermented dairy products and eggs (≤2 µg/100 gm) and fermented soya-containing

food [113, 114]. Table 1-9 shows menaquinone concentrations in dairy foods and fermented food products. Phylloquinone consumption has been reported to vary greatly between populations (70 to 250 µg per day), mirroring different patterns of food consumption in different geographical regions [115-117].

Table 1-9: Menaquinones concentrations in dairy foods and fermented food products

Food	Menaquinones						
	MK-4	MK-5	MK-6	MK-7	MK-8	MK-9	MK-10
Milk (µg/100ml)							
Whole	0.8	0.1	ND	ND–2.0	ND	ND	ND
Buttermilk	0.2	0.1	0.1	0.1	0.6	1.4	ND
Yogurt							
Whole	0.6–1.0	0.1–0.3	ND–0.2	ND–0.4	0.2–2.0	ND–4.7	ND
Skimmed	ND	ND	ND	ND	ND–0.1	ND	ND
Cheese(µg/100ml)							
Curd	0.4	0.1	0.2	0.3	5.1	18.7	ND
Hard	4.7–10.2	1.5	ND–3.0	ND–2.3	ND–16.9	ND–51.1	ND–6.5
Semihard	NR	NR	1.0–3.5	ND–2.1	2.5–7.3	10.0–32.1	ND–13.8
Soft	3.7	0.3	0.4–2.6	ND–1.7	2.1–14.0	6.6–94.0	ND–5.7
Other(µg/100ml)							
Salami	9.0	ND	ND	ND	ND	ND	ND
Sauerkraut	0.4	0.8	1.5	0.2	0.8	1.1	ND
Natto	ND–2.0	7.5	13.8	939–998	84.1	ND	ND

ND=not determined (Adapted from Walther, 2013 [118])

1.1.16.4.4. Vitamin K pharmacokinetics

Vitamin K as phylloquinone, among all fat-soluble vitamins, has the highest inter- and intra- individual variability ratios for fasting plasma and diet concentrations, even after taking into consideration the effect of the different food matrices. Most of the circulating vitamin K is believed to be obtained from the diet, and not from other vitamin K forms by metabolic conversion. Dietary vitamin K is absorbed in the proximal intestine. Around 80% of an oral phylloquinone dose is absorbed as free form when administered to healthy adults. However, the efficiency of phylloquinone absorption from natural food is affected by the types of the foods eaten; for example, phylloquinone absorption is increased in the presence of fatty food. As no specific plasma carrier protein has been identified for vitamin K, it is probably transported completely by lipoproteins. Dietary vitamin K is integrated, chemically unchanged, into

chylomicrons in the intestinal mucosa, secreted into the lymph, and finally enters the liver via chylomicron remnant particles. Ingested vitamin K is taken to the liver and possibly other body parts, including bone marrow, in the form of chylomicron remnants. After reaching plasma, vitamin K levels show temporal fluctuations, with a mean maximum at 10 pm and mean minimum at 10 am, and the levels of plasma triglyceride reflect vitamin K levels changes. Phylloquinone absorption is reduced in subjects with biliary insufficiency or malabsorption syndromes.

Although the majority of vitamin K is retained in the liver, the capacity for liver storage is limited, with a half-life of 10-15 hours. In humans, vitamin K is stored in the liver as around 90% menaquinones and 10% phylloquinone. Around 75% of the original stored amount will have been lost after 3 days of severe dietary deprivation. As phylloquinone dietary consumption is significantly correlated with fasting plasma levels [113, 119], vitamin K plasma concentration closely reflects hepatic reserves. The median hepatic concentration of phylloquinone in adults is about 5 ng/gm of liver.

Compared to long-chain menaquinones, phylloquinone is the main circulating form of vitamin K, and its level is considerably lower compared to other fat-soluble vitamins. MK-4 has been found predominantly in certain extra-hepatic sites, such as pancreas, salivary gland and brain. Independently of intestinal bacterial action, MK-3 has been shown to be a metabolite of vitamin K1, MK-4 and MK-7. The common hepatic forms MK-9 and MK-13 are not detectable in plasma (which might be attributed to their different route of absorption), whereas MK-7 and possibly MK-8 are present.

Vitamin K is mainly metabolized in the liver. Regardless of the amount of the dose, around 20% is excreted in the urine, and around 40-50% is excreted into bile and excreted via faeces. Phylloquinone is extensively catabolized by the liver to water-soluble metabolites which rapidly appear in the plasma, urine, and bile. Thus, around 60-70% of the amounts of phylloquinone absorbed from each meal will eventually be lost to the body by excretion. The Recommended Dietary Allowance (RDA) of vitamin K is ≥1µg/kg/day (65-80 µg for adult males and 55-65 µg for adult females), which has been suggested to be adequate to maintain a normal prothrombin time; however, the National Academy of Science recommends a higher daily intake of vitamin K (90µg for women and 120µg for men) for maintaining healthy bones and vascular integrity [100, 112, 114, 120-123].

1.1.16.4.5. Safety of excessive vitamin K intake

Cases of toxicity from phylloquinone or menaquinones have not been reported. Whilst it has been claimed that high amounts of vitamin K could cause over-coagulation, increasing risk of thrombosis, in reality, more γ-carboxylation or extreme coagulation could not be achieved as vitamin K dependent clotting factors have a limited number of glutamate residues capable of γ-carboxylation per molecule. Therefore, the use of menaquinones as a source of vitamin K added for nutritional purposes presents no safety concerns when used as low doses, as concluded by the European Food Safety Authority [117, 124].

1.1.16.4.6. Warfarin and dietary vitamin K interaction

Vitamin K status influences warfarin sensitivity both during the initiation and maintenance therapy. Sconce, et al, in 2007 [125] hypothesized that variability in the consumption of food containing vitamin K would lead to a larger impact on anticoagulation outcome in patients with low vitamin K status compared with those with higher vitamin K status. They found that patients with low vitamin K intakes spent a lower amount of time in their therapeutic range (TTR) than patients with high vitamin K intake. Subsequently, in a randomized controlled study, Sconce et al, demonstrated that unstable patients who were given daily oral vitamin K supplementation experienced more stable control of anticoagulation, with a significantly higher reduction in standard deviation of INR versus their counterparts who received placebo (-0.24±0.14 vs. -0.11 ±0.18; $P<0.001$). Moreover, those given vitamin K had a significantly higher increase in the percentage TTR (28%±20% vs. 15%±20%; $P<0.01$) [125]. With regard to improving anticoagulation control, patients should be educated to consume consistent daily amounts of vitamin K. There is now sufficient evidence to indicate that daily vitamin K supplementation improves stability of anticoagulation control in previously unstable patients [70, 126-128].

1.1.16.5. Comorbidity

1.1.16.5.1. Liver disease

Production of coagulation factors V, VII, X, and prothrombin formation are decreased in liver disease, which is reflected by increased sensitivity to warfarin [129]. Contributory factors are hypoalbuminaemia, decreased vitamin K dietary intake, vitamin K malabsorption and a dysfunction in the carboxylation process, which is the essential step in the synthesis of vitamin K-dependent clotting factors, and a reduction in warfarin metabolism [94].

1.1.16.5.2. Renal disease

Warfarin is metabolised by the liver and excreted renally, with a very small amount excreted unchanged; therefore, warfarin dose adjustment in chronic renal failure is not required. Renal insufficiency can influence bleeding risk in warfarin users related to uraemic state and simultaneous use of heparin in dialysis sessions. A study reported that the mortality rate increased by 27% (HR=1.27, 95% CI=1.18-1.37) in patients on warfarin undergoing hemodialysis [130]. Another retrospective large study, in which data from 132,372 patients were analysed, showed that among patients with renal disease, the risk of bleeding increased by 33% (HR=1.33, 95% CI= 1.16 to 1.53, P<0.001) compared to those with no renal disease [131]. Patients with renal insufficiency receiving warfarin therapy should therefore be closely monitored.

1.1.16.5.3. Thyroid disease

Warfarin clearance does not appear to be affected by thyroid dysfunction [132]. However, warfarin sensitivity increases in hyperthyroidism, and this might be related to enhanced prothrombin and factor VII catabolism, or vitamin K-dependent clotting factor reduction. Consequently, due to the interaction between warfarin and drugs used to treat hyperthyroidism which affect thyroid hormones concentrations, a lower warfarin dose is required at the time of initiation, which should be increased later once euthyroidism is achieved [133]. In hypothyroidism, warfarin dose requirement is increased, and INR should be monitored more frequently, especially at the time of

starting thyroid replacement treatment; upon achieving euthyroidism, warfarin dose should be reduced accordingly [94, 133].

1.1.16.5.4. Fever

There are several anecdotal reports suggesting that fever increases sensitivity to VKAs. A very recent study reviewed data published from 1943 to 2014 about the effect of fever on the response to VKAs. The study showed that the extent by which fever potentiates anticoagulation response to VKAs is variable and is possibly contributed to by enhancement of vitamin K-dependent clotting proteins catabolism, reduction in vitamin K intake, and VKA metabolism inhibition [134].

1.1.17. Drug interactions

There are many drugs which interact with warfarin, sometimes resulting in serious adverse events [3, 30]. Interactions which enhance or inhibit the activity of warfarin by pharmacokinetic or pharmacodynamic mechanisms are shown in Table 1-10.

Table 1-10 High-quality evidence of interactions of warfarin with commonly prescribed medications

	Potentiate warfarin (increase INR)	**Inhibit warfarin (decrease INR)**	**No interaction**
Medications with high quality data for interaction	Amiodarone, clofibrate, fluconazole, metronidazole, omeprazole, propafenone, ciprofloxacin, quinidine, tamoxifen, phenytoin (initial phase)	Barbiturates, carbamazepine, cholestyramine, nafcillin, rifampin,	Atenolol, fluoxetine, famotidine, metoprolol, ibuprofen, ketoconazole

(Adapted from Moualla, 2011 [4])

1.1.18. Pharmacogenetics

The term pharmacogenetics was first used in the late 1950s [135], and is defined as the study of how genetic differences between people affect drug response [94]. There is a large overlap between pharmacogenetics and the recent discipline of pharmacogenomics, which uses the whole genome-approach to elucidate the inherited basis of differences between persons and their response to drugs [136], and the two terms are often used interchangeably. The main objective of pharmacogenetics and personalized medicine is to introduce the right dose of the right drug to the right individual. Genetic variants are used in pharmacogenetics fundamentally to classify subgroups of patients who may respond differently to a specific group of drugs. Anticoagulation response to warfarin is influenced by a number of factors including genetics. I will hereon describe polymorphisms in the genes that express enzymes which mediate both warfarin pharmacokinetics and pharmacodynamics.

1.1.18.1. CYP2C9 Polymorphism

As mentioned in section 1.1.7, warfarin is prescribed as a 50:50 mixture of R- and S- enantiomers. CYP2C9 is the primary metabolizing enzyme for S-warfarin. Body weight, age, sex, and specifically *CYP2C9* genotype have a strong influence on S-warfarin clearance; factors which significantly affect R-isomer clearance are body weight, age, and genotypes of *CYP2C19*, and *CYP3A4* [137].

Coding variation in the *CYP2C9* gene, which is located on chromosome 10, leads to the presence of proteins with altered catalytic actions. A minimum of 34 allelic variants of *CYP2C9* as markers related to warfarin dosing have been reported [46]. *CYP2C9*1* is the primary wild-type allele (most common). All other *CYP2C9* genotypes are compared with *CYP2C9*1*, since it is considered the reference. Each named CYP2C9 star (*) allele is defined by a genotype at one or more specific single-nucleotide polymorphisms (SNPs) and is linked with enzyme activity. Variation in the *CYP2C9* gene in exon 3, with substitution of a cysteine for arginine at position 144 produces the *CYP2C9*2* allele (C430T, rs1799853), and substitution of leucine for isoleucine at amino acid position 359 produces the *CYP2C9*3* allele (A1075C, rs1057910) in exon 7. *CYP2C9*2* and *CYP2C9*3* are considered the most common variants. Considering that autosomal chromosomes are paired, persons can be

homozygous for each of the alleles (*CYP2C9 *1*1*, *CYP2CP *2*2*, or *CYP2C9 *3*3*), or heterozygous (*CYP2C9 *1*2*, *CYP2C9 *1*3*, or *CYP2C9 *2*3*). The reduction of S-warfarin metabolism is attributed to enzymes expressed through these alleles. Patients with *CYP2C9*1* (wild type gene / normal metabolizer) metabolize warfarin faster than those with *CYP2C9*2* genotype (30% reduction of enzyme activity), and in turn patients with *CYP2C9*2* metabolize warfarin faster than those with *CYP2C9*3* genotype [43, 138]. CYP2C9 enzyme activity is the lowest in patients who are homozygous for *3*3, in which the activity is decreased by 80%. Enzymatic activity reduction is subsequently associated with slower warfarin metabolism and decreased warfarin clearance [95, 139, 140]. Therefore, a higher maintenance dose is required for persons who are homozygous for the *CYP2C9*1* allele, versus *CYP2C9*2* and *CYP2C9*3* homozygotes, respectively, and for **3*3* carriers the required warfarin dose is the lowest. A study has confirmed that the average warfarin maintenance doses vary across *CYP2C9* variant alleles, compared to wild-type carriers enzyme activity is at 87% in *CYP2C9*1*2*, 72% in *CYP2C9*2*2*, 59% in *CYP2C9*1*3*, 42% in *CYP2C9*2*3*, and 28% in *CYP2C9*3*3* [140]. Warfarin dose in those with single allele of *CYP2C9*2* or *CYP2C9*3* is reduced by 1 and 1.6 mg/day, respectively, as shown in a meta-analysis study [141].

Aithal et al, in 1999 were the first to demonstrate a strong association between *CYP2C9* genotype and warfarin sensitivity, and found that those requiring low warfarin doses (≤1.5 mg per day) were more than six times more likely to carry one or two of *CYP2C9* variant allele (**2* or **3*) than those requiring more than 1.5mg per day[142]. In a meta-analysis study published in 2005, Sanderson et al demonstrated the association between *CYP2C9* genotype and warfarin dosing, and suggested that no further studies are required to confirm this association, at least in Caucasians [143].

In 2002, the association between *CYP2C9* genotype and anticoagulation status or bleeding was firstly reported by Higashi et al [140]. People with one or more alleles of *CYP2C9*2* or **3* are at higher risk of bleeding (around 3-4 times) during warfarin therapy, need lower doses to attain similar anticoagulation levels, and need more time to reach a stable INR, compared with those with *CYP2C9*1* allele, [94, 141, 142, 144-146]; however, the risk of bleeding is decreased once warfarin maintenance dose is achieved during long-term therapy.

In the European-American population, *CYP2C9*2* and *CYP2C9*3* variants are more common than in the African-American population [147]. For the *CYP2C9*2* genotype, 22% of Caucasians are found to be heterozygous (**1*2*), and 1% to be homozygous (**2*2*). Homozygous (**3*3*) and heterozygous (**1*3*) genotypes are found in 0.4% and 15% of Caucasians, respectively. Another 1.4% of Caucasians are found to be (**2*3*) heterozygous.

1.1.18.2. Vitamin K Epoxide Reductase Complex Sub Unit 1 Polymorphism

VKORC1 is the gene that encodes the vitamin K-epoxide reductase enzyme (VKOR), the target protein for warfarin. The conversion of vitamin K-epoxide to vitamin K is the rate limiting step in vitamin K recycling. Two groups independently discovered *VKORC1* in 2004, and described that polymorphism of *VKORC1*, which is located in chromosome 16, is significantly associated with warfarin sensitivity and lower dose requirements [50, 148]. Rieder et al sequenced the *VKROC1* gene from 186 Caucasians and determined ten common SNPs in various regions (also known as haplotypes) of the gene [149]. These SNPs are “in strong linkage disequilibrium” with each other, which means that they are very close on the chromosome, and a variant allele in one position usually suggests a variant allele in the other. Based on linkage disequilibrium values between SNPs, 9 *VKORC1* haplotypes have been identified. Group A haplotypes (H1, H2) were associated with lower warfarin doses, and group B haplotypes (H7, H8, H9) were associated with higher warfarin doses independent of *CYP2C9 *2* and **3* status. SNPs were used to classify haplotype groups; a low-dose haplotype (A), and a high-dose haplotype (B). As with *CYP2C9* allele variations, patients can be classified into *B/B* (homozygous for B) with normal activity which requires higher warfarin dose, *A/A* (homozygous for A) with lowest activity which requires lower warfarin dose, and *A/B* (heterozygous for A and B) with average activity which requires average warfarin dose [150]. Further analyses have demonstrated that genotyping patients for either of the two SNPs in high linkage disequilibrium (*1173C > T* rs9934438 switch in intron 1 and the *-1639G > A* rs9923231 switch in the promoter region, located 1639 nucleotides upstream from the ATG start codon) explains nearly identical percentages in the variability of warfarin dosing and could substitute for haplotype A [151]. Therefore, *VKORC1* allele variations could also be classified as *AA*

(or *TT*) (mutant homozygous with lower enzyme activity), *AG* (or *CT*) (heterozygous with intermediate enzyme activity), and *GG* (or *CC*) (wild homozygous with higher/normal enzyme activity). Lower maintenance doses of warfarin are required for *VKORC1* variants compared to wild-type variants [152]. D'Andrea and colleagues have confirmed this; they found that the mean warfarin dose was higher (6.2 mg/day) in patients with the *VKORC1 1173CC* genotype than that in patients with the *CT* (4.8 mg/day; p=0.002) or *TT* genotype (3.5 mg/day; p<0.001), independently of the presence of confounding variables [153]. As it has been reported in the literature, *VKORC1* polymorphism is associated with variability in warfarin dose requirement and anticoagulation response [50, 149, 153, 154].

VKORC1 complex prevalence varies in different populations. In Caucasians, the prevalence for this mutation is 14% *AA*, 47% *AG*, and 39% for *GG* [155], Table 1-11 shows the frequency of *VKROC1,* and *CYP2C9 *2* and **3* in different ethnic groups.

Table 1-11: *CYP2C9 *2*3* and *VKORC1* haplotypes among different ethnicities

	European–American	**African–American**	**Asian**
*CYP2C9*2*	0.14	0.02	0.0
*CYP2C9*3*	0.06	0.01	0.04
VKORC1 haplotype A group	0.42	0.21	0.85
VKORC1 haplotype B group	0.57	0.58	0.14
VKORC1 haplotype other	0.01	0.21	0.01
Low-dose group	0.55	0.22	0.86
Haplotype A and 2C9 variant	0.18	0.01	0.06

(Adapted from Eby, 2011 [156])

The association between *VKORC1* and *CYP2C9* genotypes and warfarin therapeutic dose has been established in several ethnic groups [69, 157]. For example, patients with wild-type *CYP2C9*1*1* and *VKORC1-1639GG* require doses in the range of 5 to 7 mg daily, whereas those with double variants *CY92C9 *3*3* (the slowest metabolizers) and homozygous *VKORC1 -1639AA* (highly sensitive to warfarin) require therapeutic warfarin doses ranging between 0.5 and 2.0 mg daily [156, 158]. In a Japanese population, the median warfarin daily dose also varied significantly, the lowest at 2.0 mg per day was reported in the *CYP2C9 *3*3* and *VKORC1 1173TT*

group, and the highest at 3.5 mg per day in the *CYP2C9 *1*1* and *VKORC1 1173CC* group ($P=4.4 \times 10^{-13}$) [159].

Table 1-12 shows an example of warfarin dose prediction for an average or typical patient. Although age, weight, gender, and other clinical factors collectively were found to account for about 12% of warfarin dosing variability [160], *CYP2C9* and *VKORC1* genotypes were found to account for 35% of variability [153]. Only around 60% of warfarin dosing variability is explained by pharmacogenetics-based algorithms, whereas the causes for the remaining 40% of the variability are still poorly understood [161].

Table 1-12: Warfarin dose prediction in a typical patient*

	***CYP2C9* Genotype**					
***VKORC1* Genotype**	****1/*1***	****1/*2***	****1/*3***	****2/*2***	****2/*3***	****3/*3***
GG	6	5	4	4	3.5	3
GA	5	4	3	3	2.5	2
AA	3	2.5	2	2	2	1.5

*Typical patient= (65 years old, male, Caucasian, body surface area 2.0, nonsmoker, no other concomitant medications, with atrial fibrillation, target international normalized ratio of 2.5) (Adapted from Kim, 2009 [160])

In 2007, the US Food and Drug Administration (FDA) updated its warfarin product label to contain information on pharmacogenetics. This change did not contain a genotype-based dose modification recommendation; thereafter, from 2010, based on data from around 15,000 patients, the product label has precise advice with tables on how to use a patient's genotype (i.e. *VKORC1*, *CYP2C9*2* and *CYP2C9*3*) to anticipate the therapeutic dose needed in an individual patient [162]. Information about *VKORC1* and *CYP2C9* genotypes have been included in warfarin labels by other medical agencies such as the European Medicines Agency (EMA) [163]. However, none of these agencies mandated using of genotyping information before the initiation of warfarin therapy. In consequence, genotyping is used only in some specialist health centres to aid warfarin dosing [164].

1.1.18.3. Polymorphisms in other genes

Both CYP1A2 and CYP3A4, the major metabolizing enzymes for R-warfarin, have been screened for genetic polymorphism. For CYP1A2, a SNP in intron 1 (C163A) has been associated with increased enzyme activity although it has been observed only among tobacco smokers [165]. *CYP1A2* polymorphism does not appear to influence warfarin dose requirements although CYP1A2 contributes to warfarin metabolic clearance [166]. CYP3A4 is a major contributor to the metabolism of many drugs including R-warfarin, and shows a great degree of variability within individuals. Several polymorphisms have been reported for *CYP3A4* gene; however, they are very low in frequency in the general population, and their functional significance is uncertain [166]. *CYP3A5* genotype, another member of CYP3A family, shows no effect on R-warfarin clearance and warfarin dose requirements [166].

Mutation in *CYP4F2* gene (V433M; rs2108622; C>T nucleotide substitution), which metabolises vitamin K, was first reported as a contributor to warfarin dose requirements in Europeans, with those with homozygous variant allele requiring higher warfarin doses [167]. The polymorphisms in CYP4F2 enzyme result in its reduced vitamin K metabolising activity. As a result, patients carrying the polymorphism require larger dose of warfarin to achieve the same level of anticoagulation intensity as those without the *CYP4F2* polymorphism. However, recently, *CYP4F2* polymorphism has been identified as the least contributor (1 to 5%) towards warfarin dose compared to *CYP2C9* and *VKORC1* polymorphisms [163, 168].

It has been identified that apolipoprotein E (APOE) genotype, which is a major component of plasma lipoprotein involved in the transportation of lipids, affect lipoprotein uptake by hepatocyte. Polymorphisms in APOE can affect sensitivity to VKAs because vitamin K is transported bound to lipoprotein including APOE [121], and therefore APOE genotype might influence the rate of hepatic vitamin K uptake. *ε2*, *ε3*, and *ε4* are the three alleles which encode APOE variants with functional differences. Many studies have been conducted examining the effect of APOE genotype as predictor of VKAs dose requirement, with contradictory results; some studies showed positive contribution while the others showed negative contribution towards dose

requirement. In general, all the previous studies have shown that the overall contribution of APOE genotype to warfarin dose requirement is low [43, 168].

In the vitamin K cycle, γ-glutamyl carboxylase enzyme (GGCX) is a vitamin K-dependent enzyme which activates several coagulation proteins by modification of glutamic acid residues by carboxylation. GGCX gene exhibits a single polymorphism. This polymorphism was identified to have a small effect (2%) on warfarin dose requirements in a European population [169].

Calumenin is a calcium binding protein, that when overexpressed in animal model it binds and inhibits VKOR, and hence protects the enzyme from warfarin inhibition. Calumenin was also found to target the carboxylase enzyme and thus prevent the effective transfer of the reduced active form of vitamin K from VKOR to the carboxylase enzyme, which it also inhibits, promoting warfarin resistance.

Two polymorphisms have been reported in microsomal epoxide hydrolase encoded gene. Microsomal epoxide hydrolase is responsible for the hydrolysis of epoxides to trans-hydrodiols and works as vitamin K 2,3-epoxide binding site. Warfarin dose requirements is shown not to be influenced by polymorphisms in either calumenin or microsomal epoxide hydrolase genes [43, 168].

1.1.18.4. Pharmacogenetic-based dosing algorithms

The main aim of warfarin genotype-guided therapy is to decrease the risk of bleeding by avoiding over-anticoagulation (supratherapeutic INR), and the risk of thrombosis by avoiding under anticoagulation (subtherapeutic INR) particularly during the initiation phase of warfarin therapy [170]. Therefore, it would be beneficial to predict an individual's initial warfarin doses accurately by implementing genetic testing, which has been shown to decrease the number of INR tests and number of dose adjustments required to reach the therapeutic INR range [160].

Most of the pharmacogenetics-based dosing algorithms for VKAs have been based on studies involving Caucasian populations, although some algorithms have been developed from data from Asian populations. These algorithms are particularly helpful in identifying those patients who require low doses of warfarin and therefore who might be at increased risk of bleeding if dosed according to conventional dosing algorithms. The most well studied genetic alleles that have an influence on warfarin dosing are

*CYP2C9*2*, *CYP2C9*3*, and *VKORC1 -1639*, whereas several polymorphisms have been found to be associated but are less well studied[171]. Compared to Caucasians, the prevalence of the minor alleles *CYP2C9*2*, *CYP2C9*3*, and *VKORC1 -1639* are much lower among African Americans. These algorithms therefore show more clinical advantages for Caucasians and Asians [44], for whom they predict around 50-60% of warfarin dose variability whereas they predict only 20% of the dosing variability in African Americans [97].

Three separate genome-wide association studies (GWAS) [172-174] have established the association between *CYP2C9*, *VKORC1*, and *CYP4F2* polymorphisms and warfarin dose requirement [163], with *VKORC1* contributing the most and CYP4F2 the least.

The joint effect of polymorphisms in *CYP2C9* and *VKORC1* has been demonstrated to be responsible for about 35% of warfarin dosing variability. Furthermore, around 20% of the interpersonal differences in warfarin dosing has been attributed to the joint effects of age, comorbid diseases, nutrition, sex, smoking, and co-administered drugs [40, 152]. Pharmacogenetic dosing algorithms are available online, such as, http://warfarindosing.org/ and http://www.pharmgkb.org/ [96].

IWPC and Gage algorithms are the two most commonly cited algorithms. Data collected from 4,043 patients (55% Caucasians, 30% Asians, and 9% Blacks) were used to develop pharmacogenetic algorithms in The International Warfarin Pharmacogenetics Consortium (IWPC) study. The study was a collaborative work between 21 research groups from nine countries, including UK (Newcastle and Liverpool) across four continents. The developed algorithms, in which *CYP2C9* and *VKORC1* genetic polymorphism were incorporated, were validated in a group of 1,009 subjects. The study demonstrated that warfarin dosing based on the pharmacogenetic algorithm was more accurate in maintenance dose prediction compared to the clinical or fixed-dose algorithm. It was also observed that patients who required a warfarin dose of ≤ 21 mg/week or ≥49 mg/week benefited greatly from the pharmacogenetic algorithm, with little difference between the predicted and actual dose in those requiring >21 or <49 mg/week [175]. The pharmacogenetic-based dosing algorithm developed in the IWPC study has been used as a basis for warfarin dosing in two recent RCTs [163].

In a US study, data of 1,015 patients on warfarin (83% Caucasians, 15% African Americans, and 2% Hispanics) were used to develop the Gage algorithm [97]. Both *CYP2C9* and *VKORC1* genetic polymorphisms were incorporated in the algorithm, which was validated prospectively in a group of 292 subjects commencing warfarin treatment. The study demonstrated that *VKORC1* was the most important predictor of dose when commencing warfarin. It was found that, by incorporating *CYP2C9* and *VKORC1* genotypes in the pharmacogenetic algorithm, 53-54%, of the variability in warfarin dose could be explained compared to 17-21% when the clinical algorithm was used.

1.1.18.4.1. Pharmacogenetics-based dosing algorithms in ethnic groups

African–Americans are significantly under-represented in pharmacogenetic studies of warfarin, and Hispanics are even less well represented [69], as demonstrated by the IWPC and Gage studies [97, 175].

Whereas *CYP2C9*2* and **3* alleles account for 9-12% of the variability in warfarin dose requirement, the dose variability is significantly lower for African-Americans; and 20-28% of total dose variability is explained by the *VKORC1* allele in Caucasians, whereas for African-Americans it is 5-7% [69]. Since warfarin pharmacogenetic algorithms have largely been developed using Caucasian populations, by comparison African-Americans, for whom accurate algorithms are lacking, might be at greater risk of adverse outcomes as a consequence of sub-therapeutic anticoagulation caused by under-dosing [69]. Hernandez et al 2014, developed a specific warfarin dosing algorithm for African Americans, and found that the use of the algorithm allowed more accurate prediction of the stable therapeutic warfarin dose, which was within 20% of the actual dose, compared to IWPC algorithms [44]. Limdi et al 2015 and Sheth et al 2015 showed that the impact of predictors on warfarin dosing requirements varies by ethnicity, and they recommended that warfarin dosing algorithms should be stratified by ethnicity rather than adjusted for ethnicity [176, 177].

It is worth mentioning that most of commercially available genotyping assays do not detect rare *CYP2C9* and *VKROC1* alleles that may affect warfarin dosing [144]. Specifically, variants in African Americans that are predictive of warfarin dose requirements are not included in most algorithms, even in the FDA-approved warfarin

dosing table or FDA-cleared genotyping platforms [69]. Lack of their inclusion will likely have resulted in lower performance of pharmacogenetics-based dosing algorithms in such populations. Therefore, there is a need to develop appropriate warfarin pharmacogenetics-based dosing algorithms for ethnic groups of patients as genetic structure and genotype frequencies differ between different races. Table 1-13 shows gene alleles frequency, including rare ones, contributing to warfarin response among different ethnicities.

Table 1-13: Gene allele frequency contributing to warfarin response among different ethnicities

Allele	Location	Frequency		
		European Caucasians	***US Hispanics***	***African–Americans***
*CYP2C9*2*	Exon 3	10%	7%	2%
*CYP2C9*3*	Exon 7	6%	5%	1%
*CYP2C9*5*	Exon 7	<1%	<1%	1%
*CYP2C9*6*	Exon 5	<1%	<1%	1%
*CYP2C9*8*	Exon 3	<1%	<1%	6%
*CYP2C9*11*	Exon 7	<1%	<1%	4%
CYP2C9 rs7089580	Intronic	24%	11%	23%
VKORC1 -1639A	5-UTR	40%	46%	11%
VKORC1 rs61162043	5-UTR	Unknown	Unknown	47%
CYP4F2 433M	Exon 2	23%	22%	9%

(Adapted from Cavalalri, 2012) [69]

1.1.18.4.2. EU-PACT and COAG studies

The results of two large prospective multi-centre randomized clinical trials, the US trial Clarification of Optimal Anticoagulation through Genetics (COAG) [178] and the European trial Pharmacogenomic Approach to Coumarin Therapy (EU-PACT) [179] investigating the extent to which pharmacogenetics-guided dosing improves the safety of oral anticoagulation therapy have been recently published. The COAG trial evaluated the merits of pharmacogenetics-based dosing for warfarin whilst EU-PACT, which was made up of 3 separate arms, evaluated the impact of pharmacogenetics-based dosing on the safety of warfarin, acenocoumarol and phenprocoumon. The

warfarin arm of the EU-PACT study was carried out in the UK (Newcastle and Liverpool) and Sweden where newly diagnosed patients with thromboembolism were recruited and randomized to either a genotype-guided dosing regimen or a fixed loading dose regimen, whereas the COAG study compared genotype-guided dosing versus a clinical dosing algorithm. The primary outcome measure was percentage time within target INR (TTR) in both studies; in EU-PACT TTR was assessed over the first 3 months of therapy, and from day 4/5 after treatment initiation to day 28 in COAG. The COAG study failed to show a difference in terms of TTR between those treated with genotype-guided dosing and those treated with a clinical algorithm (45.2% vs 45.4%, P=0.91, mean difference of 0.2%), while EU-PACT demonstrated that genotype-guided dosing significantly improved TTR compared to the fixed-dose regimen (67.4% vs 60.3%, P<0.001, mean difference of 7%).

Of relevance, there was more ethnic variation in COAG (67% White, 27% Black, and 6% Hispanic) compared to EU-PACT (>98% White). In the COAG study, African-American patients experienced significantly lower TTR in the genotype-guided dosing group compared to those in the clinically guided group (35.2% vs 43.5%, P=0.01, mean difference of 8.3%). It has been established that the frequencies of *CYP2C9*2* and *CYP2C9*3* alleles are much higher in European Caucasians (10% and 6%, respectively) than in African Americans (2% and 1%, respectively), and the frequency of the *VKORC1-1639A* allele is 40% in European Caucasians and 11% in African Americans [69]. Other SNPs are found only in African Americans, such as *CYP2C9*8* and *CYP2C9*11*, with a frequency of 6% and 4%, respectively [69]. These genotypes were not considered while dosing African Americans individuals, and this could to some extent explain why there was an 8% deterioration in TTR in African Americans in the genotype-guided dosing group compared to clinically guided group [163].

1.1.19. The effect of ethnicity on warfarin sensitivity

Racial and cultural diversity has a significant impact on warfarin dose requirements. It has been demonstrated that Hong Kong Chinese patients need only 50% of the warfarin maintenance dose of Caucasian patients to achieve the same degree of anticoagulation intensity. The mean warfarin dose for Asian patients resident in southern California is about 40% lower than that for Caucasian or Hispanic patients.

Likewise, the adjusted average weekly warfarin dose needed to sustain INR readings in the range of 2.0 to 3.0 was lowest in Asian Americans, and highest in African Americans, compared to Hispanics and Caucasians [138]. The observed racial differences in warfarin dose requirements were contributed to by racial differences in genotype frequencies between the different ethnicities [69], Table 1-14 and Table 1-15.18% of warfarin dose variability in Caucasians is explained by *CYP2C9* and 30% by *VKORC1* polymorphisms, whereas these variants account for less of the dose variability among Asian and African people [144].

Table 1-14: Warfarin daily dose requirements for *CYP2C9* variants among different ethnicities

	Mean ± S.D. range of warfarin sodium dose by *CYP2C9* variant (mg/day)					
Ethnicity	***1*1**	***1*2**	***1*3**	***2*2**	***2*3**	***3*3**
Caucasian	4.08 ± 2.13– 7.20 ± 3.7	3.56 ± 1.82– 4.88 ± 2.57	2.70 ± 1.38– 3.80 ± 1.6	1.92 ± 1.12– 4.30 ± 1.6	1.58 ± 0.79– 2.58 ± 0.81	0.78 ± 0.2– 1.60 ± 0.81
Japanese	2.50–3.60 ± 1.7	NA	1.80 ± 0.5–2.00	NA	NA	2.00
Chinese/Malay/East Indian	3.9 ± 1.8 for any **1* allele	4.0 for any **2* allele	2.5 ± 1.6 for any **3* allele	See **1/*2*	See **1/*2* and **1/*3*	See **1/*3*

(Adapted from Gulseth, 2009 [180])

Table 1-15: Warfarin daily dose requirements for *VKORC1* variants among different ethnicities

	Mean ± S.D. Range of Warfarin Sodium Dose by *VKORC1* Haplotype (mg/day)		
Ethnicity	***A/A***	***A/B***	***B/B***
Japanese	2.50–3.30	3.50–5.40	4.00–7.00
Chinese	2.93 ± 1.22–3.12 ± 1.20	4.38 ± 1.68–4.96 ± 1.53	6.50 and 6.75
Malay	2.70 ± 1.02	3.90 ±1.2	5.10 ± 1.92
East Indian	1.98 ± 0.90	4.92 ± 1.74	5.58 ± 2.22
Caucasian	2.11–3.77 ± 0.2	3.49–5.20 ± 0.40	5.48–7.30 ± 0.60
African/African American	2.50	4.73	5.71

(Adapted from Gulseth, 2009 [180])

1.2. New oral anticoagulants

1.2.1. Introduction

Vitamin K antagonists as a class, warfarin in particular, have been the cornerstone for the treatment and prophylaxis of thromboembolic disorders for decades. Anticoagulation response to warfarin is difficult to predict because of its wide inter-individual variability in clinical response, as well as interaction with many drugs and foods. As a result significant efforts have been made to develop safer alternative drugs which obviate the need for frequent dose adjustment/monitoring along with minimal food and drug interactions. Initially these were known as “novel” oral anticoagulant (NOACs), but because this designation expires after one year several alternative terms have been suggested. These include Target-Specific Oral antiCoagulants (TSOCs), Direct Oral AntiCoagulants (DOACs), Oral Direct Inhibitors (ODIs), Non-monitored Oral AntiCoagulants (NOACs), Non-warfarin Oral AntiCoagulants (NOACs), Non-vitamin K antagonist Oral AntiCoagulants (NOACs). To avoid confusion, the European Society of Cardiology (ESC) Working Group on Thrombosis Task Force on Anticoagulants in Heart Disease, and others recommends keeping the “NOAC” acronym to indicate “**N**on-vitamin K antagonist **O**ral **A**nti**C**oagulants” [181, 182]. For this reason I will be using the latter throughout my thesis.

Compared to warfarin, NOACs have a more predictable anticoagulation response and a wider therapeutic index, and as such they, do not need frequent monitoring as is the case with warfarin. Moreover, NOACs are characterized by quick onset and offset of action, which avoids the necessity for bridging with heparin in many clinical situations. Table 1-16 shows some of the advantages of NOACs compared to conventional anticoagulants.

Table 1-16: Advantages of newer anticoagulants over conventional anticoagulants

Conventional anticoagulants	Newer anticoagulants
Effective	As or more effective than current
Significant adverse effects	As or safer than current agents
Oral/SC/IV	Oral
Dose variable	Fixed dosing
Significant food and drug interactions	Minimal food and drug interactions
Unpredictable anticoagulant response (needs monitoring)	Predictable anticoagulant response (no monitoring)
Slow onset and offset of action	Rapid onset and offset of action
Irreversible	Reversible

(Adapted from Ranganathan, 2013 [183])

1.2.2. Historical background to NOACs

Ximelagatran (the prodrug of the active agent, melagatran), a direct thrombin inhibitor (DTI), was the first novel oral anticoagulant which was developed by AstraZeneca. It was marketed in 2005, but was withdrawn from the market in 2006 [184] because of hepatic toxicity, despite its initial promising results [185-187]. Ximelagatran was compared with warfarin in patients with AF in the SPORTIF III study [188], and in stroke prophylaxis in atrial fibrillation (SPAF) [60]. It was also studied in other clinical trials versus LMWH for VTE prevention after major orthopaedic surgery [60]. Initially, similar efficacy and haemorrhage rate was observed compared to warfarin and LMWH. Later, it was found that the hepatotoxicity was related to ximelagatran alone and was not generalisable to this class of drugs.

Four NOACs have so far been approved for clinical use; the direct thrombin inhibitor, dabigatran, and FXa inhibitors rivaroxaban, apixaban, and edoxaban, Figure 1-6 shows their chemical structures.

Betrixaban, the direct factor Xa inhibitor, is the latest investigational NOAC that has been shown to have the lowest renal clearance and hepatic metabolism and longest half-life among the NOACs [189], but it will not be discussed here.

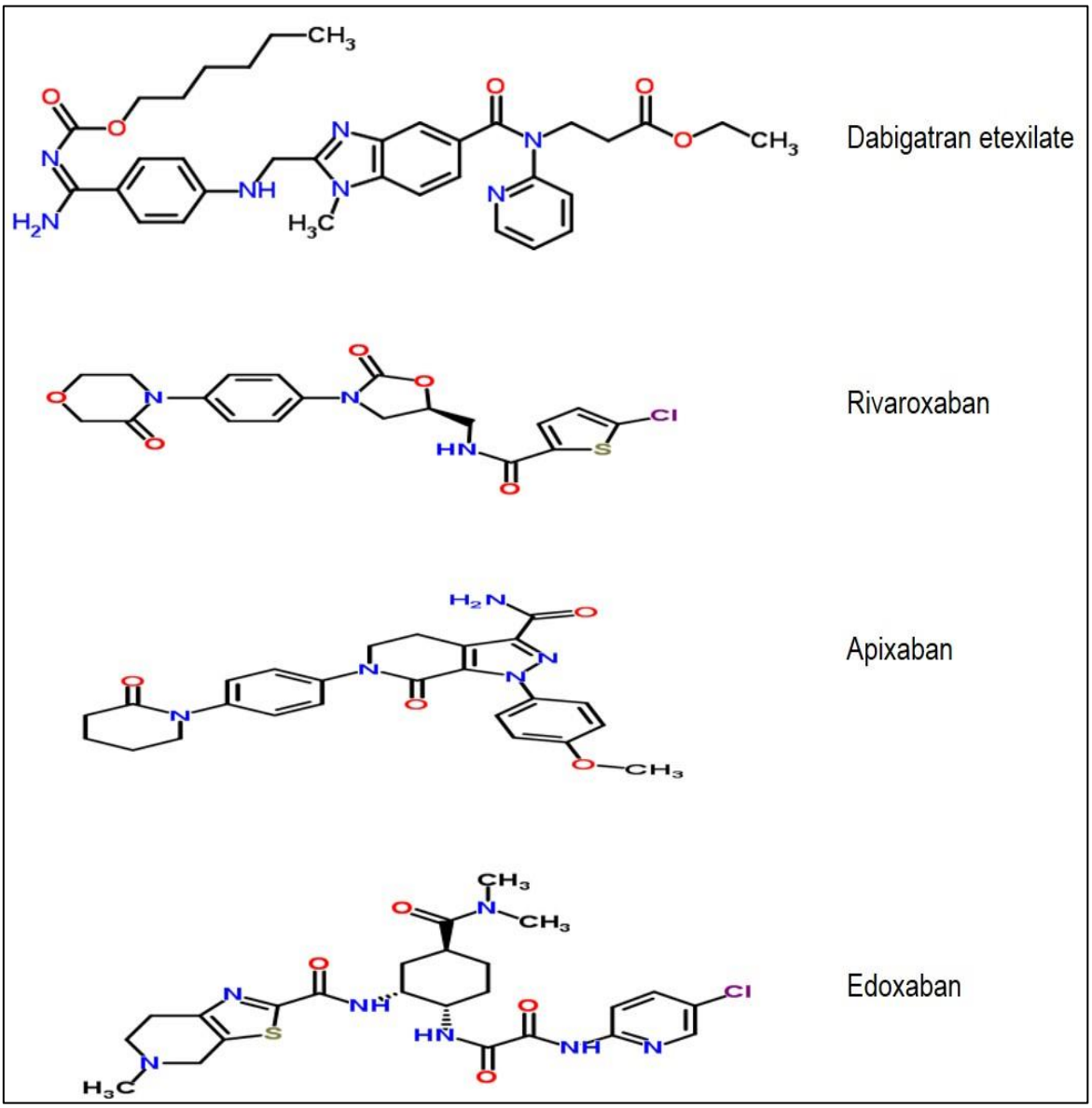


Figure 1-6: NOACs chemical structures (Adopted from http://www.chemspider.com)

1.2.3. Pharmacological properties of NOACs

After oral administration, NOACs have a rapid onset of action with peak plasma levels reached at 1-4 hours [2, 190]. This compares favourably to warfarin where the anticoagulant effect takes at least 4-5 days to reach the therapeutic range [38]. Consequently, initiation of anticoagulation therapy is easier with NOACs than with warfarin.

Warfarin is characterised by having a longer half-life, of around 40 hours [38], compared to the half-lives of NOACs, which range between 5-17 hours [2, 190]. This property makes NOACs management easier, and might reduce the need for specific antidotes in critical bleeding situations, or when an urgent intervention or surgery is required.

Whereas warfarin is minimally excreted renally, all four NOACs are excreted to varying degrees via the kidneys. Dabigatran, rivaroxaban, apixaban, and edoxaban are excreted renally by 77%, 36%, 21-24%, and ~50%, respectively. Therefore, the active drug accumulates in the presence of kidney dysfunction [2, 190], which increases the risk of bleeding.

Both rivaroxaban and apixaban are metabolised by the cytochrome P (CYP) 450 enzyme system, whereas dabigatran and edoxaban are not. All four NOACs are substrates for p-glycoprotein (P-gp). Therefore, drug-drug interactions with dabigatran and edoxaban differ from those with rivaroxaban and apixaban [2, 190].

Table 1-17, Table 1-18 and Table 1-19 summarise NOACs pharmacological properties, drug interactions, and drugs contraindicated with their use, respectively.

Table 1-17: Pharmacological properties of NOACs

	Dabigatran	Rivaroxaban	Apixaban	Edoxaban
Time to Cmax (h)	2	2–4	3–4	1–2
Half-life (h)	12–17	5–9	~12	10–14
Bioavailability (%)	3–7	≥ 80	50	62
Renal elimination (%)	80	66	27	50
Protein binding (%)	35	92–95	87	55
Transporters	P-gp	P-gp/BCRP	P-gp	P-gp
Potential drug interactions	P-gp inhibitors and inducers	Potent dual inhibitors of CYP3A4 and P-gp	Potent dual inhibitors of CYP3A4 and P-gp	Potent P-gp inhibitors and inducers

(Adapted from Weitz, 2016) [190]

Table 1-18: NOACs drug interactions

Mechanism	Dabigatran	Rivaroxaban, apixaban, edoxaban
P-gp inhibition	Interacting drug	Interacting drug
	Ketoconazole	Ketoconazole
	Quinidine	
	Amiodarone	
	Verapamil	
P-gp induction	Rifampicin	Rifampicin
	St. John's Wort	St. John's Wort
CYP3A4 inhibition		Ketoconazole
		Clarithromycin
		Ritonavir
CYP3A4 induction		Rifampicin
		Carbamazepine
		Phenobarbital
		Phenytoin

(Adapted from Schulman, 2013 [191])

Table 1-19: Contraindicated drugs with NOACs

Dabigatran	Rivaroxaban, Apixaban,	Edoxaban
Quinidine	Azole antimycotics	Quinidine (dose should be adjusted)
	Ketoconazole	
	Itrakonazole	
	Vorikonazole	
	Posakonazole	
	HIV protease inhibitors	
	Ritonavir	

(Adapted from Schulman, 2014 and Lip, 2014 [191, 192])

1.2.4. Clinical indications for NOACs

The US Food and Drug Administration (FDA) approved dabigatran, rivaroxaban, apixaban, and edoxaban in 2010, 2011, 2012, and 2015, respectively, for clinical use in adults. They are indicated to reduce the risk of stroke and systemic embolism in patients with non-valvular AF, and for treatment of DVT and PE. In patients undergoing knee or hip replacement surgery, rivaroxaban and apixaban are only indicated for prophylaxis of DVT which might lead to PE. With the exception of edoxaban, they are indicated to reduce the risk of recurrent DVT and PE [193-196]. However, recently (Feb 2016) their use has been recommended for long-term anticoagulant therapy (in patients without cancer) over warfarin, according to antithrombotic therapy for venous thromboembolism (VTE) disease guidelines [33].

In adults, dabigatran, rivaroxaban, apixaban, and edoxaban are recommended in the UK by The National Institute of Health and Clinical Excellence (NICE) for prevention of stroke and systemic embolism in patients with non-valvular AF [197-200], and treatment and prevention of recurrent DVT and PE [201-204]. However, only dabigatran, rivaroxaban, and apixaban are recommended as prophylactic agents against thromboembolism in adults after elective hip or knee replacement surgery [205-207].

1.2.5. Landmark studies

The effectiveness of NOACs was examined in landmark studies for thromboprophylaxis after knee or hip arthroplasty, stroke prevention in AF patients, and for treatment and prophylaxis of VTE. The aim of these studies was to show that NOACs are “as good as” current therapy (i.e. NOACs are non-inferior to warfarin). The primary efficacy result of non-inferiority in comparison with standard therapy was used in most NOAC studies for acute treatment of VTE, stroke prevention in non valvular AF patients, and thromboprophylaxis after knee or hip surgeries. It is worth mentioning that there are no head-to-head comparison studies among NOACs as yet.

1.2.5.1. Thromboprophylaxis after knee or hip arthroplasty studies

During the last 20 years, unfractionated heparin (UFH), or low molecular weight heparin (LMWH) have been used as prophylactic agents for VTE after total knee (TKA) or hip arthroplasty (THA) [208, 209]. Dabigatran, rivaroxaban, and apixaban safety and efficacy after TKA or THA were compared to enoxaparin in phase III double-blind randomized controlled clinical trials. Dabigatran in RE-MODEL [210], RE-MOBILIZE [211], RE-NOVATE [212], and RE-NOVATE II [213] trials; rivaroxaban in RECORD 1-4 trials (REgulation of Coagulation in major Orthopaedic surgery Reducing risk of DVT and pulmonary embolism) [214-217]; and apixaban in ADVANCE 1-3 trials (Apixaban Dose Orally vs. Anticoagulation with Enoxaparin) [218-220]. Patients recruited in the studies were 18 years or above and scheduled for elective total unilateral or same-day bilateral hip or knee arthroplasty in rivaroxaban and apixaban studies, or primary elective unilateral arthroplasty in dabigatran studies.

Primary results of the studies are shown in Table 1-20 and Table 1-21. A pooled analysis for all of the above mentioned studies demonstrated that dabigatran, rivaroxaban, and apixaban compared to enoxaparin had a significantly lower primary efficacy outcome which was reduced by 29% (RR=0.71, 95%CI=0.56-0.90), major VTE was significantly reduced by 41% (RR=0.59, 95%CI=0.41-0.84), and proximal DVT was significantly reduced by 49% (RR=0.51, 95%CI=0.35-0.76). Individually, rivaroxaban showed superiority (RR=0.50, 95% CI=0.34-0.73), then apixaban

(RR=0.63, 95% CI=0.36-1.01), and finally dabigatran (RR=1.02, 95% CI=0.86-1.20), when compared to enoxaparin. The rate of major bleeding (RR=1.04, 95% CI=0.74-1.46) and clinically relevant bleeding (RR=1.03, 95% CI=0.88-1.21) were similar in comparisons between enoxaparin versus dabigatran, rivaroxaban, and apixaban. Compared to enoxaparin, more major bleeding risk was observed with rivaroxaban (RR=1.88, 95% CI=0.92-3.82), whereas the lowest clinically relevant bleeding risk was reported with apixaban (RR=0.81, 95% CI=0.64-1.01) [208].

Table 1-20: Outcomes following hip and knee surgery

Criteria	RE-MODEL [210]			RE-MOBILIZE [211]			RE-NOVATE [212]			RE-NOVATE II [213]		RECORD1 [214]	
Design	RCT			RCT			RCT			RCT		RCT	
Type of Surgery	TKR			TKR			THR			THR		THR	
Drug tested / Comparator	Dabigatran etexilate 150 mg QD(n=703)	Dabigatran etexilate 220 mg QD (n=679)	Enoxaparin 40 mg QD SC (n=694)	Dabigatran etexilate 150 mg QD(n=877)	Dabigatran etexilate 220 mg QD (n=862)	Enoxaparin 30 mg BID SC (n=876)	Dabigatran etexilate 150 mg QD(n=1174)	Dabigatran etexilate 220 mg QD(n=1157)	Enoxaparin 40 mg QD SC (n=1162)	Dabigatran etexilate 220 mg QD (n=1010)	Enoxaparin 40 mg QD SC (n=1003)	Rivaroxaban 10 mg QD (n=2266)	Enoxaparin 40 mg QD SC (n=2275)
Primary efficacy outcome	VTE or death related to VTE			VTE or death related to VTE			VTE or death related to VTE			VTE or death related to VTE		VTE or death related to VTE	
Primary safety outcome	Bleeding			Bleeding			Bleeding			Bleeding		Bleeding	
Days of treatment	6-10			12-15			28-35			28-35		31-39	
Primary efficacy outcome* % **95%CI,** **P-value**	36.4 32.2-40.6 0.0003	40.5 36.3-44.7 0.017		33.7 NR NR	31.1 NR NR	25.3 NR NR	8.6 -0.6-4.4 <0.0001	6.0 4.5-7.6 <0.0001	6.7 5.1-8.3	7.7 5.8-9.6 0.43	8.8 6.8-10.8	1.1 0.7-1.8 <0.001	3.7 2.8-4.8
Major bleeding % **(95% CI)** **P-value**	1.5 (0.7-2.7) NR	1.3 (0.6-2.4) NR	1.3 (0.6-2.4) NR	0.6 NR NR	0.6 NR NR	1.4 NR NR	1.3 0.7-2.1 NR	2.0 1.3-3.0 NR	1.6 0.9-2.5 NR	1.4 0.8-2.3 0.40	0.9 0.4-1.7	0.3 0.1-.6 0.18	0.1 <0.1-0.3
Major or CRNM bleeding % (95% CI) **P value**	5.9 NR NR	6.8 NR NR	5.3 NR NR	0.7 NR NR	0.8 NR NR	0.3 NR NR	4.7 NR NR	4.2 NR NR	3.5 NR NR	3.7 NR 0.33	2.9	2.9 NR NR	

*Primary outcome vs enoxaparin for non-inferiority; RCT= double blind randomised controlled trial; TKR=total knee replacement; THR=total hip replacement; NR= not reported; P-value vs enoxaparin; CRNM=clinically relevant non-major bleeding.

Table 1-21: Outcomes following hip and knee surgery

Criteria	RECORD2 [215]		RECORD3 [216]		RECORD4 [217]		ADVANCE1 [218]		ADVANCE2 [219]		ADVANCE3 [220]	
Design	RCT		RCT		RCT		RCT		RCT		RCT	
Type of Surgery	THR		TKR		TKR		TKR		TKR		THR	
Drug tested / Comparator	Rivaroxaban 10 mg QD (n=1252)	Enoxaparin 40 mg QD SC (n=1257)	Rivaroxaban 10 mg QD (n=1254)	Enoxaparin 40 mg QD SC (n=1277)	Rivaroxaban 10 mg QD (n=1584)	Enoxaparin 30 mg BID SC (n=1564)	Apixaban 2.5 mg BID (n=1599)	Enoxaparin 30 mg BID SC (n=1596)	Apixaban 2.5 mg BID (n=1528)	Enoxaparin 40 mg QD SC (n=1529)	Apixaban 2.5 mg BID	Enoxaparin 40 mg QD SC
Primary efficacy outcome	VTE or death related to VTE		VTE or death related to VTE		VTE or death related to VTE		VTE or death related to VTE		VTE or death related to VTE		VTE or death related to VTE	
Primary safety outcome	Bleeding		Bleeding		Bleeding		Bleeding		Bleeding		Bleeding	
Days of treatment	31-39	10-14	10-14		10-14		10-14		10-14		32-38	
Primary efficacy outcome* % **95%CI,** **P-value**	2.0 1.2-3.1 <0.0001	9.3 7.5-11.5	9.6 7.7-11.8 <0.001	18.9 16.4-21.7	6.9 5.4-8.7 0.01	10.1 8.3-12.2	9.0 7.47-10.79 NR	8.8 7.33-10.66 NR	15.06 12.95-17.46 <0.0001	24.37 21.81-27.14	1.4 NR	3.9 NR
Major bleeding % **(95% CI)** **P-value**	<1 <0.1-0.5 NR	<1 <0.1-0.5 NR	0.6 0.2-1.2 0.77	0.5 0.2-1.1	0.7 0.3-1.2 0.10	0.3 0.1-0.7	0.7 0.4-1.3 0.05	1.4 0.9-2.1	0.6 0.30-1.16 0.30	0.9 0.54-1.57	0.8 0.5-1.3 0.54	0.7 0.4-1.1
Major or CRNM bleeding % **(95% CI)** **P Value**	3.3 NR NR	2.7 NR	2.7 1.9-3.8 NR	2.3 1.5-3.3	2.6 1.8-3.5 NR	2.0 1.4-2.8	2.9 2.2-3.8 0.03	4.3 3.4-5.4	2.9 2.19-3.93 0.16	3.8 2.98-4.95	4.1 3.4-4.9 0.43	4.5 3.8-5.4

*Primary outcome vs enoxaparin for non-inferiority; RCT= double blind randomized controlled trial; TKR=total knee replacement; THR=total hip replacement; NR= not reported; P-value vs enoxaparin; CRNM=clinically relevant non-major bleeding.

1.2.5.2. Stroke prevention in non-valvular AF patients

Stroke prevention in AF patients studies include: dabigatran in Randomized Evaluation of Long-Term Anticoagulation Therapy trial (RE-LY) (Connolly et al 2009) [221], rivaroxaban in 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) (Patel et al, 2011) [222], apixaban in Apixaban for Reduction In Stroke And Other Thromboembolic Events In Atrial Fibrillation Trial (ARISTOTLE) (Lopes et al, 2010) [223], and edoxaban in (Effective Anticoagulation with Factor Xa Next Generation in Atrial Fibrillation–Thrombolysis In Myocardial Infarction study 48) (ENGAGE AF-TIMI 48) (Giugliano et al, 2013) [224].

For the RE-LY and ARISTOTLE trials, AF patients had a minimum CHADS2 [19] (congestive heart failure, hypertension, age ≥75 years, diabetes mellitus, stroke X2) score of one, however, a minimum score of two was required for ROCKET AF and ENGAGE AF trials. The mean CHADS2 scores were about 3.5 in ROCKET AF, and 2.1-2.8 in the other three trials. Median duration of treatment was the longest in the ENGAGE AF trial at 907 days, shortest in the ROCKET AF trial at 590 days, and 730 and 657 days for RE-LY and ARISTOTLE trials, respectively. Median duration of follow-up was longest in the ENGAGE trial at 34 months, and shortest in ARISTOTLE at 22 months, being 24 and 23 months for the RE-LY and ROCKET AF trials, respectively.

Among the four trials, different dosages were assessed with different frequencies of administration, according to patient criteria. Dabigatran was assessed by using two fixed doses (110 or 150 mg twice daily) in the RE-LY study; one dose for rivaroxaban (20 mg once daily, reduced to 15 mg once daily in those with a creatinine clearance of 30-49 ml/min) in the ROCKET AF study; one dose of apixaban (5 mg twice daily, reduced to 2.5 mg twice daily in patients with ≥2 of the following baseline criteria: ≥80 years, a body weight of ≤60 kg, or serum creatinine ≥ 1.5 mg/dl) in the ARISTOTLE study; and two doses for edoxaban (30 or 60 mg once daily, and every dose was reduced by 50% in patients with a creatinine clearance of 30-50 ml/min, body weight ≤60 kg, or simultaneous use of a potent P-gp inhibitor such as verapamil) in the ENGAGE study.

Primary results

As shown in Table 1-22, compared to warfarin for non-inferiority, NOACs decreased stroke or systemic embolic events by 34% with dabigatran 150 mg twice daily, and 21% with rivaroxaban 20 mg once daily, 5 mg apixaban twice daily, or edoxaban 60 mg once daily. Haemorrhagic stroke was decreased by 74% and 69% with dabigatran 150 mg and 110 mg twice daily, respectively; 41% and 49% with rivaroxaban 20 mg once daily and 5 mg apixaban twice daily, respectively; and by 46% and 67% with edoxaban 60 mg and 30 once daily, respectively. Only dabigatran 150 mg twice daily decreased the ischaemic stroke risk by 24%, whereas the others were equal to warfarin. The risk of death by any cause was decreased by 11% and 13% with 5 mg apixaban twice daily, and edoxaban 30 mg once daily, respectively, while the others were equal to warfarin. Major bleeding was decreased by 20% and 31% with dabigatran 110 mg and 5 mg apixaban twice daily, respectively; and by 20% and 53% with edoxaban 60 and 30 mg once daily, respectively. In contrast to warfarin, the risk of gastrointestinal bleeding was increased by 50% and 23% with dabigatran 150 mg twice daily and edoxaban 60 mg once daily, respectively, and the risk decreased by 33% with edoxaban 30 mg once daily, whereas it was the same with apixaban, and not reported with rivaroxaban.

Table 1-22: Stroke prevention in AF landmark studies

	Stroke or SEE		Haemorrhagic stroke		Ischaemic stroke		Death (any cause)		Major bleeding		Gastrointestinal bleeding	
	n (event rate)	HR (95% CI) p-value	n (event rate)	HR (95% CI) p-value	n (event rate)	HR (95% CI) p-value	n (event rate)	HR (95% CI) p-value	n (event rate)	HR (95% CI) p-value	n (event rate)	HR (95% CI) p-value
RE-LY [221]												
150 mg BID (n = 6076)	134 (1.11)	0.66 (0.53–0.82) < 0.001	12 (0.10)	0.26 (0.14–0.49) < 0.001	111 (0.92)	0.76 (0.60–0.98) 0.03	438 (3.64)	0.88 (0.77–1.00) 0.051	375 (3.11)	0.93 (0.81–1.07) 0.31	182 (1.51)	1.50 (1.19–1.89) < 0.001
110 mg BID (n = 6015)	182 (1.53)	0.91 (0.74–1.11) < 0.001	14 (0.12)	0.31 (0.17–0.56) < 0.001	159 (1.34)	1.11 (0.89–1.40) 0.35	446 (3.75)	0.91 (0.80–1.03) 0.13	322 (2.71)	0.80 (0.69–0.93) 0.003	133 (1.12)	1.10 (0.86–1.41) 0.43
Warfarin (n = 6022)	199 (1.69)		45 (0.38)		142 (1.20)		487 (4.13)		397 (3.36)		120 (1.02)	
ROCKET AF [222]												
20 mg QD (n = 6958)	188 (1.7)	0.79 (0.66–0.96) < 0.001	29 (0.26)	0.59 (0.37–0.93) 0.024	149 (1.34)	0.94 (0.75–1.17) 0.581	208 (1.87)	0.85 (0.70–1.02) 0.073	395 (3.6)	1.04 (0.90–1.20) 0.58	224 (3.2)	NR
Warfarin (n = 7004)	241 (2.2)		50 (0.44)		161 (1.42)		250 (2.21)		386 (3.4)		154 (2.2)	
ARISTOTLE [223]												
5 mg BID (n = 9120)	212 (1.27)	0.79 (0.66–0.95) 0.01	40 (0.24)	0.51 (0.35–0.75) < 0.001	162 (0.97)	0.92 (0.74–1.13) 0.42	603 (3.52)	0.89 (0.80–0.998) 0.047	327 (2.13)	0.69 (0.60–0.80) < 0.001	105 (0.76)	0.89 (0.70–1.15) 0.37
Warfarin (n = 9081)	265 (1.60)		78 (0.47)		175 (1.05)		669 (3.94)		462 (3.09)		119 (0.86)	
ENGAGE AF TIMI-48 [224]												
60 mg QD (n = 7012)	182 (1.18)	0.79 (0.63–0.99) < 0.001	49 (0.26)	0.54 (0.38–0.77) < 0.001	236 (1.25)	1.00 (0.83–1.19) 0.97	773 (3.99)	0.92 (0.83–1.01) 0.08	418 (2.75)	0.80 (0.71–0.91) < 0.001	232 (1.51)	1.23 (1.02–1.50) 0.03
30 mg QD (n = 7002)	253 (1.61)	1.07 (0.87–1.31) 0.005	30 (0.16)	0.33 (0.22–0.50) < 0.001	333 (1.77)	1.41 (1.19–1.67) < 0.001	737 (3.80)	0.87 (0.79–0.96) 0.006	254 (1.61)	0.47 (0.41–0.55) < 0.001	129 (0.82)	0.67 (0.53–0.83) < 0.001
Warfarin (n = 7012)	232 (1.50)		90 (0.47)		235 (1.25)		839 (4.35)		524 (3.43)		190 (1.23)	

BID=twice daily; CI=confidence interval; HR=hazard ratio; NR=not reported; QD=once daily; SEE=Systemic embolic events; P value is for noninferiority. All HR values are vs warfarin.

(Adapted from Weitz, 2016[190]

1.2.5.3. Treatment and prophylaxis of VTE

Initial and long-term VTE therapy

Dabigatran in RE-COVER I (Efficacy and Safety of Dabigatran Compared with Warfarin for 6 Month Treatment of Acute Symptomatic Venous Thromboembolism) and RE-COVER II (Phase III Study Testing Efficacy & Safety of Oral Dabigatran Etexilate vs Warfarin for 6 months Treatment of Acute Symptomatic Venous Thromboembolism) trials [225, 226]; rivaroxaban in EINSTEIN-PE (Oral, direct Factor Xa inhibitor rivaroxaban in patients with acute symptomatic pulmonary embolism) and EINSTEIN-DVT (Oral, direct Factor Xa inhibitor rivaroxaban in patients with acute symptomatic deep vein thrombosis) trials [227, 228]; apixaban in AMPLIFY (Apixaban for the Initial Management of Pulmonary Embolism and Deep-Vein Thrombosis as First-Line Therapy) trial [229]; and edoxaban 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) trial [230] were examined for treatment of VTE against conventional treatment. The conventional treatment used was parenteral anticoagulant given alongside a VKA. All of the trials were conducted in a double-blind design, whereas EINSTEIN used a prospective, randomised, open-label, blinded end point evaluation design (PROBE). Recurrent VTE was the end point of these trials. Major bleeding or the composite of major or clinically relevant non-major bleeding was the primary safety outcome in these trials.

The results of rivaroxaban and apixaban, which were obtained from phase 2 studies to treat VTE, helped to decide the dose of these two NOACs to be used in phase 3 studies. Therefore, the rivaroxaban regimen in EINSTEIN was 15 mg twice daily for 3 weeks followed by 20 mg daily, and for apixaban in AMPLIFY, the regimen was 10 mg twice daily for 7 days followed by 5 mg twice daily. On the other hand, neither dabigatran nor edoxaban were examined in phase 2 studies for VTE treatment, but the doses used in RE-COVER and Hokusai were based on the outcomes of phase 2 studies in patients with AF. Therefore, in the absence of evidence supporting the instant use of dabigatran or edoxaban in patient with acute VTE, both RE-COVER and Hokusai used treatment protocols which included at least a 5-day initial course of a parenteral anticoagulant, with patients being switched to warfarin or treated with either dabigatran or edoxaban, respectively thereafter [28].

The trials used different durations of treatment. A fixed duration of 6 months was used for the RE-COVER and AMPLIFY trials. For the EINSTEIN and Hokusai trials, a flexible duration of 3, 6, or 12 months was used. At the time of enrolment, the duration of treatment was determined by the investigators in the EINSTEIN trials, whereas the duration of treatment was selected subsequently in the Hokusai trial.

With the exception of the EINSTEIN trials in which patients with DVT alone were enrolled separately in a trial from those with PE with or without concomitant DVT in another, all the trials were accomplished by recruiting at least 30% of patients who had PE with or without associated DVT.

Rates of recurrent VTE in those treated with NOACs and conventional treatment were similar in patients with acute symptomatic PE and DVT, 2.1-3.2 vs 2.2-3.5, respectively. Across the trials, there is a consistency of the outcomes which suggest that NOACs as a class are non-inferior to conventional treatment of PE and/or DVT. Compared to conventional therapy, rates of major bleeding were significantly lower with rivaroxaban and apixaban (HR=0.55, CI 95%=0.38-0.81, P<0.002) and (HR=0.31, CI 95%=0.17-0.55, P<0.001), respectively; and it was non-significant with dabigatran and edoxaban (HR=0.73, CI=0.48-1.10) and (HR=0.85, CI=0.60-1.21), respectively. On other hand the rates of the composite of major bleeding or clinically relevant non-major bleeding (CRNM) were significantly lower with dabigatran (HR=0.63, CI=0.51-0.77, P<0.001), apixaban (HR=0.44, CI=0.36-0.55, P<0.001), and edoxaban (HR=0.82, CI=0.72-0.94, P<0.004), but was not significant with rivaroxaban (HR=0.94, CI=0.81-1.07). The results are shown in Table 1-23.

Extended VTE treatment

In double-blind controlled designs, rivaroxaban, apixaban, and dabigatran were examined against placebo in the EINSTEIN-Extension [227], AMPLIFY-Extension [231], and RE-SONATE [232] trials, respectively. All the trials examined NOACs against placebo, except the RE-MEDY trial [232] in which dabigatran was compared with warfarin for extended VTE treatment.

In the EINSTEIN-Extension and AMPLIFY-Extension trials, patients recruited were treated with 6 to 12 months of anticoagulation for their primary VTE event, whereas

those enrolled in the RE-SONATE trial were treated for 6 to 18 months. Patients recruited in the RE-MEDY trial were treated with 3 to 12 months of anticoagulation for their primary VTE event.

One dose of rivaroxaban (20 mg once daily) in the EINSTEIN-Extension trial, and one dose of dabigatran (150 mg twice daily) in the RE-SONATE trial were studied; however, in the AMPLIFY-Extension trial two doses of apixaban (2.5 and 5 mg twice daily) were studied.

When compared to warfarin, dabigatran in the RE-MEDY trial showed non-inferiority for the primary efficacy endpoint (HR=1.44, 95% CI=0.78-2.64); and dabigatran showed a significant reduction in the primary endpoint by 92% in the RE-SONATE placebo-controlled study (HR=0.08, 95% CI=0.02-0.25). With rivaroxaban, the primary efficacy result of recurrent symptomatic VTE was significantly decreased by 82% compared to placebo in the EINSTEIN-Extension placebo control study (HR=0.18, 95% CI=0.09-0.39). In the AMPLIFY-EXT trial, apixaban decreased significantly the primary efficacy result of recurrent symptomatic VTE or death from any cause by 67% with the 2.5 mg twice daily dose (HR=0.33, 95%CI=0.22-0.48), and by 64% with the 5 mg twice daily dose (HR=0.36, 95%CI=0.25-0.53).

In the dabigatran group, the rate of major bleeding was decreased by 48% (HR=0.52, 95%CI=0.27-1.02), but it was not significant (P=0.6), whereas major or CRNM bleeding was significantly reduced (HR=0.54, 95% CI=0.41-0.71) when compared to warfarin, in the RE-MEDY trial. However, in the RE-SONATE trial, both major or CRNM bleeding, or any bleeding, was observed to be increased significantly with the use of dabigatran versus placebo (HR=2.92, 95% CI=1.52-5.60) and (HR=1.82, 95% CI=1.23-2.68), respectively; whereas major bleeding only was not increased significantly with dabigatran. In the EINSTEIN-Extension trial, there was a significant increase in major or CRNM bleeding in rivaroxaban treated patients compared to placebo (HR=5.19, 95% CI=2.3-11.7), however, major bleeding was not different between the two groups. It is worth mentioning that the increase in major or CRNM bleeding rate induced by rivaroxaban was mostly related to the increase in haematuria, epistaxis, and rectal bleeding events [233]. For the AMPLIFY-EXT trial, the major bleeding rate was lower, but not significantly so, for both of apixaban 2.5 mg and 5 mg twice daily arms, (HR=0.49, 95% CI=0.09-2.64), (HR=0.25, 95% CI=0.03-

2.24), respectively; and for the rate of major or CRNM bleeding, it was higher and also not significant, in the apixaban 2.5 mg twice daily arm (HR=1.20, 95% CI=0.69-2.10), and in apixaban 5 mg twice daily arm (HR=1.62, 95% CI=0.96-2.73) in comparison with placebo arm. The results are shown in Table 1-24.

Overall, treatment of VTE with NOACs is not inferior to conventional therapy, and it is associated with lower bleeding rates.

Age was not an exclusion criterion in any study. However, for VTE studies (RECOVER I and II, EINSTEIN, AMPLIFY and Hokusai-VTE), the mean age of the patients was 56 years, only 13% being aged ≥75 years.

In the hip and knee surgery studies (REMODEL, REMOBILIZE, RENOVATE I and II, RECORD 1, 2, 3 and 4, and ADVNCE 1, 2, and 3), no reporting of numbers of patients aged ≥75 years was made. The mean age ranged between 61-67years in the studies, and only 5 of 11 studies mentioned the age range, which was between 18-93 years.

For the AF studies (RELY, ARISTOTLE, ROCKET-AF, and ENGAGE), the median age was 72, with those aged ≥75 being 40%, 31%, 44%, and 40%, respectively.

Table 1-23: VTE clinical trials overview

Criteria	RE-COVER I [234]	RE-COVER II [226]	EINSTEIN-DVT [227]	EINSTEIN-PE [228]	AMPLIFY [229]	Hokusai-VTE [230]
Study design	Randomized, double-blind, noninferiority, parallel group		Randomized, open-label, event-driven, non-inferiority, parallel group		Randomized, double blind, parallel group	Randomized, double-blind, non-inferiority, parallel group
Intervention	Dabigatran etexilate 150 mg bid (n = 1,274)	Dabigatran etexilate 150 mg bid (n = 1,279)	Rivaroxaban 15 mg bid for 3 weeks, followed by 20 mg od (n =1,731)	Rivaroxaban 15 mg bid for 3 weeks, followed by 20 mg od (n = 2,419)	Apixaban 10 mg bid for 7 days, followed by 5 mg bid (n = 2,676)	Edoxaban 60 mg od or edoxaban 30 mg od in patients with a CrCl of 30–50 mL/min, body weight B60 kg or receiving strong P-gp inhibitors (n = 4,118)
Comparator	Warfarin dose-adjusted to INR 2.0–3.0 (n = 1,265)	Warfarin dose-adjusted to INR 2.0–3.0 (n = 1,289)	Enoxaparin 1 mg/ kg bid (≥5 days)/VKA (warfarin or acenocoumarol) dose-adjusted to INR 2.0–3.0 (n = 1,718)	Enoxaparin 1 mg/kg bid (≥5 days)/ VKA (warfarin or acenocoumarol) dose-adjusted to INR 2.0–3.0 (n = 2,413)	Enoxaparin 1 mg/kg bid (≥5 days)/warfarin dose-adjusted to INR 2.0–3.0 (n = 2,689)	Warfarin dose-adjusted to INR 2.0–3.0 (n = 4,122)
Parenteral anticoagulation	Mandatory, at least 5 days		Optional, maximum 48 h		Optional, maximum 36 h	Mandatory, at least 5 days
Index event (%) Patients with DVT (%) Patients with PE (%) Patients with PE and DVT (%)	 68.9 21.3 9.6	 68.1 23.2 8.6	 98.7 0 0.1	 0 75.2 24.8	 65.5 25.2 8.8	 59.7 30.4 9.9
Unprovoked VTE (%)	NR	NR	62.0	64.5	89.8	65.7
Patients with active cancer (%)	4.8	3.9	6.0	4.6	2.7	2.5
Primary efficacy endpoint	Recurrent symptomatic VTE or death related to VTE		Recurrent symptomatic VTE	Recurrent symptomatic VTE	Recurrent symptomatic VTE or death related to VTE	Recurrent symptomatic VTE
Primary safety endpoint	Major bleeding		Major or CRMN bleeding		Major bleeding	Major or CRMN bleeding
Treatment duration	6 months		3, 6, or 12 months (pre-specified)		6 months	3–12 months (flexible)
Rate of major bleeding	HR=0.73, CI=0.48-1.10, P>0.05		HR=0.55, CI 95%=0.38-0.81, P<0.002		HR=0.31, CI 95%=0.17-0.55, P<0.001	HR=0.85, CI=0.60-1.21, P>0.05
Rate of major or CRNM bleeding	HR=0.63, CI=0.51-0.77, P<0.001		HR=0.94, CI=0.81-1.07, P>0.05		HR=0.44, CI=0.36-0.55, P<0.001	HR=0.82, CI=0.72-0.94, P<0.004
TTR [%(range)] **% > INR of 3.0** **% < INR of 2.0**	60 (53–66) 19 21	57 (51–62) 19 24	58 (54–66) 16 24	63 (58–73) 16 22	61 (NR) 16 23	64 (NR) 18 19

DVT=deep venous thrombosis; PE=pulmonary embolism; CRNM=Clinical relevant non-major bleeding; HR=hazard ratio; NR=not reported

(Adapted from Dobesh, 2014 ; and Yeh 2014 [28, 233])

Table 1-24: Extended VTE clinical trials overview

Criteria	RE-MEDY [232]	RE-SONATE [232]	AMPLIFY-EXT [231]		EINSTEIN-Extension [227]
Study design	Randomized, double-blind, placebo				
Intervention	Dabigatran etexilate 150 mg bid (n = 1,430)	Dabigatran etexilate 150 mg bid (n = 681)	Apixaban 2.5 mg bid (n =840)	Apixaban 5 mg bid (n =813)	Rivaroxaban 20 mg daily (n = 602)
Comparator	Warfarin dose-adjusted to INR 2.0–3.0 (n = 1,426)	Placebo (n = 662)	Placebo (n = 829)		Placebo (n = 594)
Index event (%)					
Patients with DVT (%)	65.6	63.3	64.8	64.8	64.1
Patients with PE (%)	22.7	26.9	35.2	35.2	35.9
Patients with PE and DVT (%)	11.7	6.9	NR	NR	NR
Unprovoked VTE (%)	NR	NR	93.2	90.7	73.1
Patients with active cancer (%)	4.2	0.1	1.8	1.1	4.7
Primary efficacy endpoint	Recurrent symptomatic VTE or death related to VTE		Recurrent symptomatic VTE or death from any cause		Recurrent symptomatic VTE
Primary safety endpoint	Major or CRMN bleeding		Major or CRMN bleeding		Major or CRMN bleeding
Mean treatment duration before randomization (days)	198	293	NR		VKA pre-treatment 6–12 months: 429 (71.3 %) Rivaroxaban pre-treatment 6–12 months: 173 (28.7 %)
Mean duration of study drug (days)	473	165	NR 2 (0.2%)<6 months 828 (98.6 %) 6–12 months 10 (1.2%)>12 months		NR Median 181 days–6 months Median 264 days–12 months
Rate of major bleeding	HR=0.52 CI 95%=0.27 -1.02 P=0.06	(0.3%) dabigatran (0) placebo	HR=0.49 CI 95%=0.09-2.64 P>0.05	HR=0.25 CI 95%=0.03-2.24 P>0.05	(0.7%) rivaroxaban (0) placebo HR=not estimable
Rate of major or CRNM bleeding	HR=0.54 CI 95%=0.41-0.71 P<0.001	HR=2.92 CI 95%=1.52-5.60 P=0.001	HR=1.20 CI 95%=0.69-2.10 P>0.05	HR=1.62 CI 95%=0.96-2.73 P>0.05	(5.4%) rivaroxaban (1.2%) placebo HR=NR

DVT=deep venous thrombosis; PE=pulmonary embolism; CRNM=Clinical relevant non-major bleeding; HR=hazard ratio; NR=not reported

(Adapted from Dobesh, 2014; and Yeh 2014 [28, 233])

1.2.6. NOACs clinical effectiveness

In a recent meta-analysis, results obtained for all NOACs from 50 randomized clinical trials involving 155,537 patients for all indications were pooled and analysed to evaluate the risk of major bleeding. The study showed that there was no significant difference for major bleeding risk between NOACs and comparators (OR=0.93, 95% CI=0.79–1.09), whereas when NOACs were compared to VKA specifically, major bleeding was significantly reduced by 23% (OR=0.77, 95% CI=0.64–0.91). When NOACs were analysed separately for major bleeding against pharmacologically active comparators, no difference was observed with rivaroxaban (OR=1.10, 95% CI=0.77–1.58), apixaban (OR=0.81, 95% CI=0.56–1.11), or dabigatran (OR=0.96, 95% CI=0.76–1.20). Major bleeding was significantly increased with NOACs after hip surgery (OR=1.43, 95% CI=1.02–1.99), in medically ill patients (OR=2.79, 95% CI=1.69–4.60), and in patients with acute coronary syndrome (ACS) (compared with placebo) (OR=2.89, 95% CI=2.01–4.14). Bleeding was significantly reduced with NOACs when they were used for treatment of acute venous thromboembolism or pulmonary embolism (OR=0.63, 95% CI=0.44–0.90). For treatment of atrial fibrillation and for extended treatment of VTE, no difference was observed between NOACs and comparators (OR=0.89, 95% CI=0.74–1.06), and (OR=0.88, 95% CI=0.27–2.93), respectively [235].

Another meta-analysis of 12 RCTs containing 102,607 patients showed that, compared to VKAs, NOACs significantly decrease the risk of total major bleeding by 28% (RR=0.72, 95% CI=0.62-0.85, P<0.01), fatal bleeding by 47% (RR=0.53, 95% CI=0.43-0.64, P<0.01), intracranial bleeding by 57% (RR=0.43, 95% CI=0.37-0.50, P<0.01), clinically relevant non-major bleeding by 22% (RR=0.78, 95% CI=0.68-0.90, P<0.01), and overall bleeding by 24% (RR=0.76, 95% CI=0.71-0.82, P<0.01). When NOACs were compared to VKAs with respect to major gastrointestinal bleeding, no difference was found (RR=0.94, 95% CI=0.75-1.91, P=0.62) [236].

NOACs for thromboprophylaxis after THR and TKR surgery

A review of six systematic reviews which examined NOACs against LMWH for thromboprophylaxis after THR or TKR showed, with the exception of dabigatran, that the risk of symptomatic DVT was decreased significantly with rivaroxaban and

apixaban (<4 events per 1000 patients, OR=0.46, 95% CI=0.3-0.7), whereas the risk of major bleeding increased (≥2 events per 1000 patients, OR=1.27, 95% CI=0.98-1.65). Among NOACs, compared to dabigatran and apixaban, the lowest risk of VTE (not significant) was observed with rivaroxaban (RR=0.68, 95% CI=0.21-2.23) and edoxaban (RR=0.59, 95% CI=0.26-1.33), respectively; however, rivaroxaban increased non-significantly major bleeding risk (RR=1.59, 95% CI=0.84-3.02) [237].

Another recent systematic review which included the results of 20 trials examined the efficacy and safety of FXa inhibitors versus enoxaparin for thromboprophylaxis after THR or TKR. It revealed that the risk of total VTE was reduced with rivaroxaban (RR= 0.70, 95% CI= 0.60-0.81), apixaban (RR=0.62, 95% CI= 0.47-0.81), and edoxaban (RR=0.62, 95% CI= 0.39 to 0.97) in comparison with enoxaparin. The risk of major or clinically relevant non-major bleeding was higher with rivaroxaban (RR=1.52, 95% CI=1.14-2.02), and similar to apixaban and edoxaban (RR=0.88, 95% CI=0.73-1.05) and (RR=1.30, 95% CI=0.72-2.33), respectively, when compared to enoxaparin. A linear relationship between the risks of major and clinically relevant non-major bleeding was observed with rivaroxaban treatment doses. Rivaroxaban, apixaban, edoxaban, and finally enoxaparin was the low to high ranking of total VTE risk found [238].

NOACs for stroke prevention in AF patients

A meta-analysis study (2014) involved 30,000 AF patients on NOACs previously treated with warfarin. Compared to warfarin, NOACs significantly reduced the composite of stroke or systemic emboli by 20% (RR=0.80; 95%CI, 0.69-0.94), all-cause mortality by 12% (RR=0.88; 95%CI, 0.79-0.98), and intracranial bleeding by 60% (RR=0.40; 95%CI, 0.26-.62). However, no significant difference was observed for major bleeding (RR=0.85; 95%CI, 0.67-1.09) [239].

A meta-analysis study, which used the results of more than 71,000 AF patients treated with either NOACs or warfarin in phase 3 studies, showed that NOACs for prevention of stroke and systemic embolism were at least as effective as warfarin; and also an associated reduction in intracranial bleeding by 50% [240]. Intracranial bleeding is the most critical complication of anticoagulation therapy.

NOACs for treatment and prevention of VTE

The six studies mentioned in section 1.2.5.3 were included in a systematic review. No difference was found between NOACs and standard treatment (parenteral anticoagulant followed by a vitamin K antagonist) for the risk of VTE recurrence (RR=0.91, 95% CI=0.79-1.06). However, compared to standard treatment, NOACs significantly decreased the risk of major bleeding (RR=0.62, 95% CI=0.45-0.85). All-cause mortality was not different between the treatments (RR=0.98, 95% CI=0.84-1.14). The primary efficacy outcome was significantly reduced by 15% with NOACs compared to standard treatment (RR=0.85, 95% CI=0.75-0.97) [241].

Another systematic review included only five of the six studies mentioned in section 1.2.5.3. VTE recurrence and all-cause mortality did not differ with NOACs compared to standard treatment (RR=0.88, 95% CI=0.74-1.05), and (RR=0.97, 95% CI=0.83-1.14), respectively. However, major bleeding and clinically relevant non-major bleeding were significantly reduced by 40% (RR=0.60, 95% CI=0.41-0.88) and 24% (RR=0.76, 95% CI=0.58-0.99) with NOACs compared to standard treatment. Number needed to treat (NNT) with NOACs in place of VKA was calculated to be 149 to prevent one major bleed, which is relatively high, and 56 to prevent one clinically relevant non-major bleeding, whereas NNT calculated for fatal bleeding was 1111 (RR=0.36, 95% CI=0.15-0.87) [242].

1.2.7. Renal function and dosing of NOACs

It has been demonstrated that the Cockcroft-Gault method to calculate renal function is optimal when prescribing the new anticoagulants to the elderly [243]. Kidney function declines with increasing age [244]. The risk of thromboembolism is increased with deteriorating kidney function by 54% independently of any other risk factors, including age, in those not treated with anticoagulants [245]. It has been shown that major bleeding rate was higher in those treated with dabigatran with reduced creatinine clearance compared to those treated with warfarin [246]. For those treated with NOACs with renal impairment, the risk of bleeding is increased as a result of prolongation of their half-lives, and hence prolongation of systemic drug exposure.

Therefore, NOACs should be used cautiously in patients with significant renal impairment.

1.2.8. Choice of NOAC for anticoagulation therapy

As revealed from the landmark studies, NOACs are not inferior to warfarin in terms of anticoagulation effectiveness, and are also associated with a lower rate of intracranial haemorrhage when used to prevent stroke in patients with non-valvular AF, and in the prophylaxis and treatment of VTE in general.

Patients started on dabigatran or rivaroxaban have lower rates of drug stoppage and higher persistence of usage compared to those started on warfarin [247, 248]. NOACs treatment might not be the optimal choice for those who frequently forget their doses in view of their short half-lives. In these circumstances, more consistent anticoagulation could be offered by warfarin's long-half life. However, patients with good adherence and difficulties in achieving a stable INR with warfarin therapy are good candidates for NOACs. NOACs with a single daily dose, i.e. rivaroxaban or edoxaban, are good choices for those with a busy lifestyle. Table 1-25 summarises how to select from the four NOACs for AF patients, and Table 1-26 summarises suggestions on how to choose an anticoagulant for acute VTE therapy. Moreover, for patients who are sensitive to warfarin owing to their genetic polymorphism NOACs may be advantageous.

However, warfarin is still considered as the drug of choice for treatment or prophylaxis of AF patients with mechanical heart valves as studies have shown NOACs to be less effective, for liver disease patients with a coagulopathy, and for those with renal impairment (creatinine clearance < 30 ml/min) [17].

Table 1-25 Selection of NOACs for AF according to patient status

Patient characteristic	Drug choice	Rationale
Not currently anticoagulated	NOAC	NOACs are at least as effective and safe as VKAs, produce less intracranial bleeding and are more convenient because they do not require routine monitoring and have a low propensity for food and drug interactions.
Already receiving warfarin, stable INR and satisfactory TTR	Maintain VKA therapy or consider switching to NOAC	Depends on patient and physician preference
Already receiving warfarin, unsatisfactory TTR	Any NOAC	NOACs produce a more predictable and stable anticoagulant effect and do not require routine coagulation monitoring
CrCl 30–50 ml/min	Apixaban, rivaroxaban or edoxaban	Less affected by renal impairment than dabigatran
CrCl < 15 ml/min	VKA	NOACs are not recommended for use in this patient population
Ischaemic stroke on warfarin, rivaroxaban, apixaban, or edoxaban	Dabigatran	Lower risk of ischaemic stroke with dabigatran (150 mg)
Dyspepsia or upper GI complaints	Rivaroxaban, apixaban, or edoxaban	Dyspepsia with dabigatran in up to 10 % of patients
Recent GI bleed	Apixaban	Dabigatran (150 mg), rivaroxaban, and edoxaban (but not apixaban) produce more GI bleeding than warfarin
Concomitant use of strong inhibitors or inducers of both P-gp and CYP3A4	Dabigatran or edoxaban	Not restricted for concomitant use
Poor compliance with twice-daily dosing	Rivaroxaban or edoxaban	Only agents given once-daily

(Adapted from Weitz, 2016[190])

Table 1-26: Choice of anticoagulant for acute VTE therapy

Characteristic	Drug choice	Rationale
Extensive DVT or massive PE	Heparin	Such patients often require advanced therapy and were excluded from trials with the NOACs
High initial risk of bleeding	Heparin	Enables dose titration; rapid offset and availability of protamine as an antidote simplify management should bleeding occur
Active cancer	LMWH	No trials comparing NOACs with LMWH
Pregnancy	LMWH	Warfarin and NOACs cross the placenta
Liver dysfunction with increased prothrombin time/ INR at baseline	Warfarin	Such patients were excluded from the trials because NOACs undergo hepatic metabolism
Unable to afford NOACs	LMWH followed by warfarin	NOACs cost less than LMWH but are more expensive than warfarin
Limited access to anticoagulation clinic because of impaired mobility or geographical inaccessibility	NOAC	Given in fixed doses without monitoring
All-oral therapy	Rivaroxaban or apixaban	Only NOACs to be evaluated in all-oral regimens
Creatinine clearance <30 mL/min	Warfarin	Such patients were excluded from trials with NOACs
Creatinine clearance 30-50 mL/min	Rivaroxaban, apixaban, or edoxaban	Less affected by renal impairment than dabigatran; if edoxaban is chosen, the 30-mg OD dose should be used
Dyspepsia or upper gastrointestinal symptoms	Rivaroxaban, apixaban, or edoxaban	Dyspepsia in as much as 10% given dabigatran
Recent gastrointestinal bleed	Apixaban	More gastrointestinal bleeding with dabigatran, rivaroxaban, and edoxaban than with warfarin
Recent acute coronary syndrome	Rivaroxaban, apixaban or edoxaban	Small myocardial infarction signal with dabigatran
Poor compliance with long-term twice-daily	Rivaroxaban or edoxaban	OD regimens for long-term use

(Adapted from Yeh, 2014 [28])

1.2.9. NOACs anticoagulation monitoring

One of the advantages of NOACs is that they do not need to be monitored for their anticoagulation response. However, monitoring is required in certain condition, such as bleeding, overdose/toxicity, or in an emergency. It has been suggested that NOACs should be monitored for anticoagulation to determine the correct dose required by each patient [249]. Routine coagulation laboratory test such as INR and aPTT might not correctly mirror NOACs anticoagulation activity.

Currently, there is no specific lab test which could be used as a guide in patients with bleeding induced by NOACs. The INR test is not recommended for NOAC monitoring. It has been reported that those receiving dabigatran and edoxaban showed

an elevation of aPTT, however, no clear dose-response relationship was seen [189]. Although other specialized assays such as thrombin time (TT), dilute thrombin time (dTT), and ecarin-clotting time (ECT) showed sensitivity for measuring dabigatran activity, they are not commercially available in most institutions [250, 251]. Prothrombin time (PT) was found to correlate with plasma rivaroxaban level qualitatively, but unfortunately the sensitivity differs widely between thromboplastin reagents [60, 252, 253]. The anti-factor Xa chromogenic assay gives consistent results with dose-response for rivaroxaban and apixaban, but the results might not be available in a timely manner [189, 254-256]. PT and aPTT showed a linear dose response with edoxaban in healthy volunteers [257]. The need for a precise and commercially available test which could be used quickly and easily in emergency situations to monitor anticoagulation response to NOACs remains.

1.2.10. Reversal of NOACs bleeding

In order to manage the bleeding caused by a NOAC properly, plasma concentration should be determined. An adequately sensitive assay, using the method of liquid chromatography/tandem mass spectrometry (LC-MS/MS), is currently available for routine measurement of plasma drug concentrations [258, 259]. As determined by LC-MS/MS, trough and peak NOACs plasma levels at steady-state are shown in Table 1-27.

Table 1-27: Plasma NOACs concentrations at stead-state

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

(Adapted from Cuker, 2016 [258])

In the presence of minor or non-life threatening bleeding, it can be clinically appropriate to allow NOACs' anticoagulant effect to simply reverse by discontinuation owing to their short half-lives, an advantage of this class of drugs [260]. This may not be appropriate in patients with intracranial bleeding as hematoma may continue to expand with further neurological deterioration [261]. For patients presenting with major bleeding due to NOACs or requiring emergency surgery NOACs should be stopped immediately, supportive measures applied or consideration given to postponing surgery for 12-24 hours after the last NOAC dose, and/or haemostatic agents should be considered.

1.2.10.1. Reversal of NOACs by haemodialysis, and use of activated charcoal

Given that dabigatran has a low protein-bound status and lipophilic profile, as shown in Table 1-17, haemodialysis could be used in bleeding associated with dabigatran [262, 263] although with major bleeding and hemodynamic fluctuation, haemodialysis is challenging. Neither rivaroxaban nor apixaban could be dialyzed effectively owing to their highly protein-bound status [264, 265]. For edoxaban, it is not clear whether it is removed by haemodialysis [189].

In an in vitro study, oral activated charcoal was shown to be effective in preventing dabigatran absorption shortly after drug ingestion [260], Table 1-28.

1.2.10.2. Reversal of anticoagulation by plasma factors

Various concentrated coagulation factors have been used to stem bleeding in those receiving NOACs, such as activated and inactivated prothrombin complex concentrates (PCC), and recombinant FVIIa. Although they have demonstrated efficacy with bleeding induced by NOACs, they have not been evaluated in large randomized controlled trials and most of the results were reported from animal models, in vitro studies or small healthy subject studies [189, 266-270], as shown in Table 1-28.

Table 1-28: Currently used agents for reversing anticoagulation

Intervention	Apixaban	Rivaroxaban	Dabigatran	Edoxaban
Oral activated charcoal	Yes	Yes	Yes	Yes
Hemodialysis	No	No	Yes	No
Hemoperfusion with activated charcoal	Possible	Possible	Yes	?
Fresh frozen plasma	No	No	No	No
Activated recombinant FVIIa	Unclear	Unclear	Unclear	Unclear
3 factor PCC	Unclear	Unclear	Unclear	Unclear
4 factor PCC	Possible	Possible	Possible	Possible

(Adapted from Ansell, 2016[271])

1.2.10.3. Antidotes to NOACs

1.2.10.3.1. Idarucizumab

In 2015, idarucizumab (aDabi-Fab, BI 655075, Boehringer Ingelheim, Ingelheim am Rhein, Germany), a novel humanized mouse monoclonal antibody fragment was approved as antidote for dabigatran by US FDA [272]. The anticoagulation effect of dabigatran is reversed by idarucizumab, which binds to and inactivates free and thrombin-bound dabigatran. The bound complex is then mainly excreted renally. Idarucizumab has a high affinity for dabigatran (around 350 times stronger than the affinity of dabigatran toward thrombin). Idarucizumab administered as a 5 gm intravenous infusion was examined in a recent phase III study. The interim analysis showed that idarucizumab resulted in normalization of ecarin-clotting time in 89% and 88% of the two studied patients groups, one with serious bleeding, and the other requiring an urgent procedure, with normalization of dilute thrombin time in 98% and 93% respectively. The median maximum percentage anticoagulation reversal was 100% in both groups [273]. Both dilute thrombin time and ecarin-clotting time parameters, which were increased by dabigatran, were reversed by idarucizumab, and the reversal was maintained for 72 hours. Idarucizumab is being evaluated in a phase III study, which is expected to be completed in 2017 [189]. Figure 1-7 shows a suggested management chart for NOAC reversal activity.

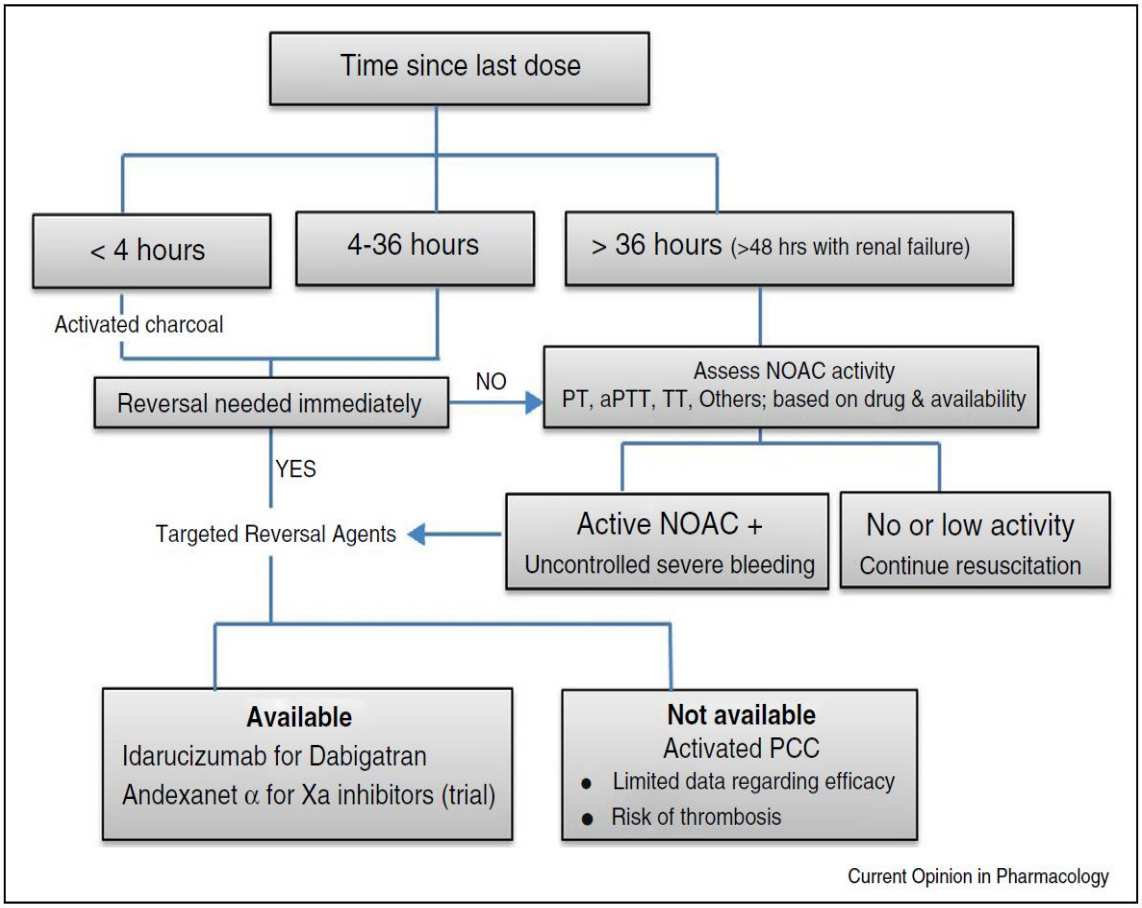


Figure 1-7 A suggested management algorithm for NOAC reversal activity (Adapted from Abo-Salem, 2016 [274])

1.2.10.3.2. Ciraparantag

Ciraparantag (PER977, Perosphere, Danbury, CT, USA) is a small synthetic water soluble and cationic molecule that binds directly to unfractionated (UFH) and low molecular weight heparin (LMWH) and also by forming non-covalent hydrogen bonds and charge–charge interactions. By the same mechanisms, ciraparantag binds to the specific targets of dabigatran, rivaroxaban, apixaban, and edoxaban (Factor IIa and Factor Xa), thus preventing their action. Phase 3 trials of ciraparantag are underway, and it is currently undergoing FDA review [271, 275].

1.2.10.3.3. Andexanet

Andexanet (PRT064445, Portola Pharmaceuticals, San Francisco, CA, USA) is a recombinant human protein made in Chinese hamster ovary cells, and is an inactivated form of FXa designed to be a universal factor Xa inhibitor antidote. Andexanet binds factor Xa inhibitors and removes them from their endogenous specific target factor Xa. It is currently undergoing FDA review [271, 275].

1.2.11. NOACs and food

It is well established that diet can significantly affect anticoagulation response to VKAs. Whilst food delays the absorption of dabigatran and rivaroxaban, it has no effect on the absorption of either apixaban or edoxaban [106, 276-279]. An earlier study demonstrated that anticoagulation response to the direct thrombin inhibitor, ximelagatran, was significantly enhanced in rats receiving a vitamin K deficient diet compared to those receiving a normal diet [280]. However, no study to date has evaluated the potential effect of dietary vitamin K on NOACs pharmacodynamics in man.

1.3. Aims of my PhD research

Attaining a stable anticoagulation response and effective management of patients is critical in achieving safe treatment. As described earlier in this chapter several factors can influence anticoagulation response to warfarin. My PhD research is comprised of a number of studies which explored the factors contributing to the variability in clinical response to warfarin. Dietary vitamin K can have a significant impact on anticoagulation response to warfarin. It is possible that dietary vitamin K also affects response to NOACs since the activation of both factor II and factor X is dependent on vitamin K availability. Examination of the effect of diet on the pharmacological activity of NOACs in older patients is particularly relevant as these patients might be sensitive to these agents because of their generally poor dietary intake and thus be liable to increased risk of bleeding.

The aim of this PhD was to elucidate the factors that influence anticoagulation response to the oral anticoagulants, warfarin and NOACs. Identification of the factors which influence clinical response to these agents could lead to improvements in patient management and treatment safety.

Over the period of my PhD research, the following projects were planned to be undertaken:

1. A cross-sectional study comparing the stability of anticoagulation control and service costs in patients monitored either at home, general practice or hospital clinics.
2. To examine the influence of age and monitoring setting on long-term anticoagulant control in patients with atrial fibrillation.
3. To examine the effect of dietary vitamin K on the pharmacological activity of the direct factor Xa inhibitor, rivaroxaban, in older and younger subjects ex vivo.

4. To evaluate the possible association between *VKORC1* genotype, dietary vitamin K and stability of anticoagulation control in patients on chronic warfarin therapy.

5. To assess the effect of *CYP2C9* genotype on the rate of INR decline following the cessation of warfarin therapy in patients undergoing surgery.
6. To further examine the effect of *CYP2C9* and *VKORC1* polymorphisms and patient demographics on warfarin pharmacokinetics and the rate of INR decline following cessation of warfarin therapy.

CHAPTER. 2 ANTICOAGULATION CONTROL AND COST OF MONITORING OF OLDER PATIENTS ON CHRONIC WARFARIN THERAPY IN THREE SETTINGS IN NORTH EAST ENGLAND

2.1. Introduction

Non-vitamin K antagonist oral anticoagulants, which act directly through inhibition of thrombin or factor Xa, were approved in 2012 by The National Institute of Health and Clinical Excellence (NICE) for use in patients with non-valvular atrial fibrillation (NVAF) aged 75 years or older in accordance with their license, as the incremental cost-effectiveness ratio is plausibly less than £20,000 per QALY gained [281-283]. Doctors now have a choice of oral anticoagulants, but need evidence to guide prescribing decisions. Time spent in therapeutic range (TTR) is an important influence on outcomes of thromboembolic prophylaxis with warfarin, with patients with TTR of 70% or more having a 79% reduced risk of stroke compared with those with a TTR of <30% and lower mortality [284]. A meta-analysis of recent studies concerning thromboembolic and major haemorrhagic events in NVAF in patients receiving warfarin therapy concluded that overall survival is improved where TTR is >40% [285], and a TTR of >58% is needed to be confident that patients will benefit from the treatment [286].

The RE-LY study of patients with NVAF suggested that dabigatran 150 mg bd might be cost-effective in high-risk patients unless INR control with warfarin is excellent (average TTR >72.6%), but warfarin is cost-effective in moderate-risk patients unless INR control is poor (average TTR <57.1%) [287, 288]. In a UK benefit-harm analysis, fewest net potential benefits of dabigatran versus warfarin were seen in centres which achieved a good TTR of ≥65.5% [55]. Non-vitamin K antagonist oral anticoagulants are at least as effective at reducing stroke and systemic embolism, and result in fewer bleeds, in particular intracranial bleeding, when compared with warfarin [289], including in patients aged 80 years and over [290]. Use might be particularly justified in patients exhibiting low TTR, for example, under 50% in spite of good patient adherence to therapy, and in those for whom monitoring is difficult or costly [291].

Difficulties with warfarin therapy are encountered by the very elderly using outpatient anticoagulation monitoring, including the missing of appointments and problems contacting them with dosing instructions [292], and gain from no monitoring requirements for novel anticoagulants has been recognised by NICE [281-283]. While domiciliary monitoring may address this issue, risk of warfarin-related bleeding is associated with dependency and domiciliary monitoring of INR [293].

Novel anticoagulants may be particularly cost-effective compared with warfarin for dependent older people, those whose anticoagulant control is erratic or for whom monitoring is not feasible [294].

2.2. Aim of the study

To examine TTR and service costs in patients housebound due to a high level of dependency using the Newcastle domiciliary service, and to compare these to patients monitored in general practice outreach clinics and patients choosing hospital monitoring for their convenience.

2.3. Materials and Methods

The inclusion criteria were to have atrial fibrillation with a target INR range of 2 – 3, aged 75 years and over and to be on warfarin chronically since before December 2010. INR testing and warfarin dosing are performed throughout the service in accordance with a standard protocol facilitated by the DAWN computer dosing programme (version 6.10) [295] and trained staff. The DAWN software was given the order of inclusion criteria (described below) such that 326 patients were selected from those accessing each of the hospital (n=100), general practice (n=122) and domiciliary (n=104) monitoring services. INR values were collected retrospectively for 12 months (January to December 2011) for each patient, and dose changes and number of INR monitoring events were recorded. Details of age, co-morbidities, chronic drug therapy and duration of warfarin therapy were also recorded. The estimated time within target therapeutic range (TTR) was calculated using the linear extrapolation method of Rosendaal et al [53].

Costs for the monitoring service in 2011 were obtained from the finance department at the Newcastle upon Tyne Hospitals NHS Foundation Trust. Methods followed those set out by the *BMJ* guidelines for economic submissions [296]. Costs of all consumables, reagents, depreciation of INR testing equipment and quality control materials were calculated per test. Staff costs, transport, overheads and computer equipment were calculated per patient treated. Costs which were considered common and equal to all patients (medical advice, consultant supervision, treatment of adverse events) were not included. The price of portable coagulometer and portable computer (assuming a 5 year lifespan) has no impact and the cost of the hospital computer server, being common to all groups, was not included. The price of each domiciliary phlebotomy visit was calculated by dividing salary, car lease and insurance, petrol, consumables and sample transport by the number of tests. No patient relied upon ambulance or other NHS transport assistance as domiciliary visits are used instead. We assumed that patient private travel costs were the same, whether to general practice surgery or to the hospital clinic (all patients were eligible for free public transport) and we did not collect data on these.

2.3.1. TTR Calculation

INR data were manually transposed from DAWN into an Excel spreadsheet. Visual Basic Calculator software (2000) was used to estimate TTR as shown in Figure 2-1. TTR was calculated according to the target INR range 2.0 to 3.0 for all patients.

The TTR result obtained manually was found to be identical to that obtained from the DAWN software, confirming that DAWN software calculation of TTR was both reliable and accurate.

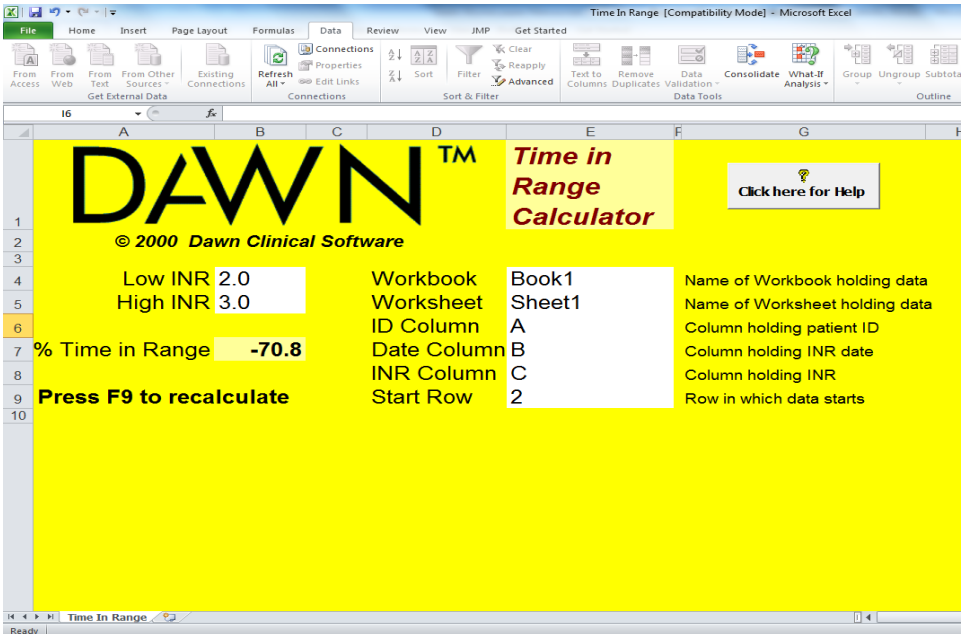


Figure 2-1: DAWN software calculator used to calculate TTR

2.3.2. Statistical analysis

Data were coded and entered using Statistical Package for Social Sciences (SPSS) version 19. A one way analysis of covariance (ANCOVA) model was fitted to test the difference in TTR, number of INR monitoring events and number of dose changes among the three groups. The influences of the other covariates were also examined. Some data were found to be skewed and not normally distributed (number of INR monitoring events, number of dose changes, and years on warfarin); therefore, they were transformed to their square roots to achieve approximate normality. Chi-square was used for the comparison of categorical data.

2.4. Results

The mean age ± SD of the hospital patients (54% male) was 83 ± 5, of the general practice cohort (46% male) was 82 ± 4 and of the domiciliary patients (26% male) was 84 ± 5 years. The domiciliary patients were significantly older ($F(2,323) = 8.61$, $P < 0.0001$) with a larger proportion of females [x^2 (df = 2) =17.65, $P < 0.001$] than the other two groups. The demographics of the anticoagulated population are shown in Table 2-1.

Table 2-1: Demographics of the anticoagulated population studied

	Hospital n(%)	**General Practice n(%)**	**Domiciliary n(%)**
Male	54 (54%)	56 (46%)	27 (26%)
Female	46 (46%)	66 (54 %)	77 (74%)
Total	100	122	104
Ages (years) (mean ± sd)	83 ± 5	82 ± 4	84 ± 5

The important covariates found, and hence allowed for, were age, co-morbidities, years on warfarin and total number of medications. Quality of anticoagulation control is shown in Table 2-2. Mean (95% CI) TTR was 78% (74%, 81%) in hospital monitored patients, 71% (67%, 74%) in general practice monitored patients, and 68% (65%, 72%) in domiciliary monitored patients. After controlling for the covariates, mean TTR means was significantly different ($F (2,277) = 7.60$, $P=0.001$) between the three groups. Patients whose anticoagulation was managed by hospital clinics had a significantly higher TTR compared with both domiciliary and general practice groups (Tukey's HSD test), while the TTR in the latter two groups did not differ significantly from each other. The back-transformed mean number for INR measurements (95% CI) were13.1 (12.2, 14.1) v 11 (10.2, 11.8) v 14.2 (13.2, 15.1) and for warfarin dose changes were 2.4 (1.8, 3.0) v 1.8 (1.3, 2.3) v 3 (2.8, 4.1) in the twelve months for the hospital, general practice and domiciliary monitored patients respectively; the number of INR measurements ($F (2,283) =12.50$, $P<0.0001$), and number of warfarin dose changes ($F (2,283) =7.08$, $P=0.001$) were significantly different among the three groups. Both hospital and

domiciliary monitored based groups had a significantly higher mean number of INR measurements than the general practice based group (Tukey's HSD test), while the former two groups did not differ significantly from each other. Domiciliary monitored patients had a significantly greater mean number of warfarin dose changes than general practice monitored patients with the hospital based patients being intermediate (Tukey's HSD test), whilst the annual number of warfarin dose changes in hospital and general practice monitored groups did not differ significantly. There was a significant difference (F (2,321) = 9.36, P<0.0001) in mean (95% CI) number of years of warfarin usage from the time of commencement for hospital 3.7 (2.9, 4.5), general practice 3.1(2.5, 3.8) and for home 5.2 (4.4, 6.0). Patients monitored at home were on average on warfarin therapy for longer by 1.5 year compared to those monitored at hospital and by 2 years compared to those in general practice settings (Tukey's HSD). The total number of drugs used chronically was significantly negatively related to TTR, (F (1,277) = 7.91, P=0.005), slope = -1.23, whereas age had a marginal negative relationship with TTR (F (1,277) = 3.59, P=0.05), slope = -0.40. Both the number of years on warfarin therapy and co-morbidities were significantly related to the number of INR measurements, (F (1,283) = 6.59, P=0.01), slope = -0.2, (F (1,283) = 8.61, P=0.004), slope = 0.07, respectively; and the number of dose changes, (F (1,283) = 8.72, P=0.003) slope = -0.03, (F (1,283) = 4.65, P=0.03), slope = 0.07, respectively.

Table 2-2: Mean % of time in therapeutic range (TTR), number of INR monitoring events and number of dose changes among the groups

	Hospital Mean(95% CI)	General Practice Mean(95% CI)	Domiciliary Mean(95% CI)	P-Value
TTR %	78 (74, 82)[a]	71 (67, 74)	68 (65, 72)	0.0001
Number of INR monitoring events	13.1 (12.2, 14.1)[b]	11 (10.2, 11.8)	14.2 (13.2, 15.1)[b]	<0.0001
Number of dose changes	2.4 (1.8, 3.0)	1.8 (1.3, 2.3)	3.4 (2.8, 4.1)[c]	0.001
Number of years of warfarin usage	3.7 (2.9, 4.5)	3.1 (2.5, 3.8)	5.2 (4.4, 6.0)[d]	<0.0001

a=hospital patients had higher TTR than GP and domiciliary patients; b=hospital and domiciliary patients had higher INR measurements than GP patients; c=domiciliary patients had higher number of dose changes than hospital and GP patients; d= domiciliary patients had longer years of warfarin usage than hospital and GP patients

Patients used a median of six drugs (five cardiovascular medications including warfarin) and had a median of three chronic diseases (two cardiovascular diseases including atrial fibrillation, plus one other) with no significant difference between the groups. As age and total number of drugs increased, TTR fell (P = 0.005). As years on warfarin increased, the number of INR measurements (P = 0.01) and dose changes (P = 0.003) fell while, as co-morbidities increased, more INR measurements (P = 0.004) and dose changes (P = 0.03) occurred. One (domiciliary monitored) patient on 10 drugs for cardiovascular disease and malignancy, suffered intracranial bleeding, following a fall when his INR was 16. Workload and descriptive costs of monitoring are presented in Table 2-3; being similar in the three groups, it was not possible to calculate confidence intervals around these cost differences as we had no measure of variability of cost.

Table 2-3: Actual workload and costs (pounds sterling) of the anticoagulant monitoring service (adjusted for year 2011)

		Hospital	**General practice**	**Domiciliary**
Total number of patients monitored		1,430	2,150	520
Total INR tests performed		18,105	25,663	6,415
Cost (£)	INR tests	5,612	7,955	20,996
	Staff	134,848	195,410	19,916
	Transport	-	4,196	59,050
	Computers and Software	2,615	3,933	951
Total cost (£)		**143,075**	**211,494**	**100,903**
Warfarin cost (£) /pt/yr (% of total cost)		28.2 (21.8)	28.2 (22.2)	28.2 (12.6)
Cost of warfarin monitoring per patient per annum including medication cost (£)		128	126	222

2.1. Discussion

In our population, TTR results for hospital and general practice patients were both in the upper quartile of patients in the RE-LY study, and even the domiciliary patients at 68% were in the second best quartile, consistent with the observation that the adequacy of anticoagulant control in routine practice can be broadly comparable to that reported in clinical trials [297], and our costs for the ambulatory services were similar to those estimated by NICE [281].

The limited long-term safety data, the increased bleeding risk for those of 80 years or older with impaired renal function or low body weight [298], drug interactions, and the lack of a direct antidote or laboratory measure of adherence and response and the need for good compliance in view of their short half-lives mean that warfarin will continue to have a role for thromboembolic prophylaxis in AF, including in patients with cardiac valve replacements for whom outcomes with novel anticoagulants can be significantly poorer [299]. Further exploration of effective interventions to increase the proportion of INR tests within target range would therefore be worthwhile.

Patients can move from ambulatory to domiciliary monitoring as their physical or mental condition deteriorates, the longer time that our home-monitored patients had been taking warfarin reflecting this. Although which patient characteristics influence outcomes is not known, domiciliary monitoring and greater chronic disease burden are associated with risk of warfarin-related complications [288, 300-302]. The similar number of co-morbidities and prescribed drugs in our general practice and home-monitored patients is perhaps surprising, but it is likely that, as the risk–benefit profile of warfarin changed over time, for more dependent patients their warfarin was discontinued. Although, as this patient cohort was studied before publication of NICE guidance [281-283] no transfers to novel anticoagulants were made, the additional availability of this treatment option makes further exploration of their clinical effectiveness in this patient group of great importance.

2.2. Conclusion

The novel finding is that domiciliary-based patients had poorer anticoagulant control, compared with clinic monitored patients, in spite of having the highest number of INR measurements and dose changes, in a service which costs more than the clinic service per patient year to finance. As INR testing and warfarin dosing are performed throughout the service in accordance with a standard protocol facilitated by the DAWN computer dosing programme and trained staff, differences in stability of control are likely to be attributable to differences in patient characteristics.

The findings of this small retrospective study may not be applicable to a wide population and a longitudinal study is required to better establish the relationship between patient characteristics and anticoagulation stability control.

CHAPTER. 3 IMPACT OF AGE ON LONG-TERM ANTICOAGULATION AND HOW GENDER AND MONITORING SETTING AFFECT IT: IMPLICATIONS FOR DECISION MAKING AND PATIENT MANAGEMENT

3.1. Introduction

Time in therapeutic INR range (TTR) [53] is an important quality measure of anticoagulation control with vitamin K antagonists (VKAs). Optimising anticoagulation control is important as TTR correlates inversely with bleeding and thromboembolic complications [303, 304].

In an earlier cross-sectional study we established that housebound AF patients requiring domiciliary monitoring of INR have poorer anticoagulation control than those attending hospital- or general practice- based clinics [305]. Anticoagulation control was also poorest in the oldest patients, which may explain their higher risk of warfarin related complications than the more independent patients who attend clinic for monitoring [293]. Cross-sectional studies, however, are limited by the design, providing only a “snapshot” of the outcome and the characteristics associated with it at a specific time point and, as such, it is impossible to infer causality. Only a longitudinal design study can identify true age-related changes in anticoagulation control, and their possible implications for treatment outcomes. We therefore set out to investigate the impact of ageing, longitudinally, on anticoagulant control in patients with atrial fibrillation on warfarin therapy, and the extent to which gender and different patient settings of monitoring influence this. In patients being managed in a standard way by staff of the unified Newcastle upon Tyne Hospitals Trust Anticoagulation Monitoring Service, for whom dosing and testing is guided by the DAWN computer dosing programme (version 6.10), Milnthorpe, Cumbria, UK [295]. I audited INR and dosage data for mobile patients who attended either the hospital- or general practice-based clinic based on personal preference, and for patients housebound by physical dependency or limited mobility who were monitored through the domiciliary service whereby trained staff visited them at their place of residence for venous INR checks.

3.2. Aim of the study

To investigate the impact of ageing, longitudinally, on anticoagulant control in patients with atrial fibrillation on warfarin therapy, comparing patients who require domiciliary monitoring of INR due to physical dependency and immobility with the more independent patients who travel either to hospital- or general practice- based clinics for monitoring.

3.3. Materials and Methods

This study involved the audit of anonymised anticoagulant control data held within the secondary healthcare Trust providing and managing the monitoring service and as such it was deemed not to require prior institutional board approval. Inclusion criteria were to have AF with a target INR of 2.0-3.0 and to have been on warfarin for at least 5 years, after excluding a six month initial stabilisation period. Only patients anticoagulated for stroke and systemic embolism prevention in AF were selected to reduce bias related to different indications or target INR ranges

As part of the unified service all patients prior to commencement of therapy received a standard 2-3 hour education session led by either a doctor or trained nurse at their local general practice, according to the UK National Patient Safety Agency (NPSA) educational material [306]. Patients were taught about atrial fibrillation and the clinical benefits and risks of anticoagulation. Information was given about the pharmacology of warfarin, and factors that affect the INR, particularly adherence, drug interactions and diet. Written information and a modified educational session were delivered to patients entering the domiciliary service. For clinic attending patients, at each monitoring visit, potential reasons for any deviation outside the target range is discussed, and education about these and the importance of good adherence stressed where appropriate. For domiciliary patients, this is done when the result from the venous INR sample is available either by phone or at the next monitoring visit.

Data mining was facilitated by DAWN which allowed extraction of information on individual patients, including a DAWN coded patient ID (in order to preserve patient anonymity for data analysis), age, sex, indication for anticoagulation therapy, target INR range, date commencing warfarin treatment and warfarin starting dose, duration

of warfarin therapy, mean yearly warfarin dose, yearly number of INR monitoring events and warfarin dose changes. The raw data were extracted from DAWN as separate text files, and then transferred to Excel spread sheets. Excel pivot-tables tool was used to clean up and rearrange the data before merging them all in one Excel file for statistical analysis. Information on co-morbidity and concurrent therapy was not available. Between 2000 and 2013, based on the study inclusion criteria, 1,490 AF patients starting warfarin therapy accessing hospital and general practice clinic and 627 the domiciliary service were identified. Of these patients 23 were identified as having switched setting during the course of warfarin therapy with 10 switching from hospital to domiciliary, 7 from hospital to GP, 5 from GP to hospital and 1 from GP to domiciliary monitoring. The 23 patients were subsequently omitted from further analysis. TTR and time spent below and above the therapeutic INR range were established using the linear extrapolation method of Rosendaal et al [53]. Time in therapeutic range is the estimated total percentage of time that the INR is within a predetermined therapeutic range which, for AF patients, is between 2.0 and 3.0, with time above and below being estimated as total percentage time above an INR of 3.0 and below an INR of 2.0 respectively.

The INR at both hospital and GP clinics was determined using the KC1 capillary technique according to the manufacturer's instruction (Trinity Biotech, Bray, Eire). For home monitored patients venous INR was determined by Instrument Laboratories (their machine and reagents) IL (UK) Ltd, Warrington, and Cheshire. Internal quality check was performed daily and external quality assurance undertaken monthly through National External Quality Assessment Service (NEQAS), Sheffield, UK.

3.3.1. Statistical analysis

The mean of the individual variables was determined according to each year of age. On examination of the data it was clear that INR control was less good in the youngest and oldest patients. We decided to fit a quadratic model with age to the means to describe this effect in a fairly simple way and to examine how the covariates (setting and gender) affected this model. The numbers of warfarin dose changes were transformed into their square roots to approach normality. Quadratic (curvilinear) and linear regression models were used to examine the effect of age for all the variables

tested. Weighted analysis was used in all statistical calculations because of the large variation in sample sizes across age groups. Individual observations were also analysed using random effects to take account of the longitudinal nature of the data. This form of analysis was more complex due to the non-normal nature of the data. The same conclusions were obtained and so the analysis of means is presented here as the interpretation of the analysis is much more straightforward. Data were analysed using Minitab statistical software (version 17).

3.4. Results

Data belonging to 2,094 AF patients [938 (44.8%) in general practice (GP) and 531(25.4%) in hospital (H) based clinics and 625 (29.8%) through the domiciliary service (D); altogether 891(43%) females, and 1,203 (57%) males] on warfarin therapy for 5-14 years, extracted from DAWN software were analysed, which constituted a total of 16,604 patient years of INR monitoring. Demographic data according to patients' gender, age and setting are shown in Table 3-1. As over 99% of the study population were white Caucasians, no separate analysis by race or ethnicity was possible. The number of patients for every year of age for the whole population, patients monitored in clinic (hospital and GP) and those monitored at home are shown in Figure 3-1.

Table 3-1: Patients' demographic data

	Home	GP & Hospital	Total
Male N (%)	259 (22)	944 (78)	1203
***Age mean(SD)**	74(7)	67(9)	
Female N (%)	366 (41)	525 (59)	891
***Age mean(SD)**	76(8)	70(9)	
Total N (%)	625 (30)	1,469 (70)	2,094

*Age at 6 months after starting warfarin therapy

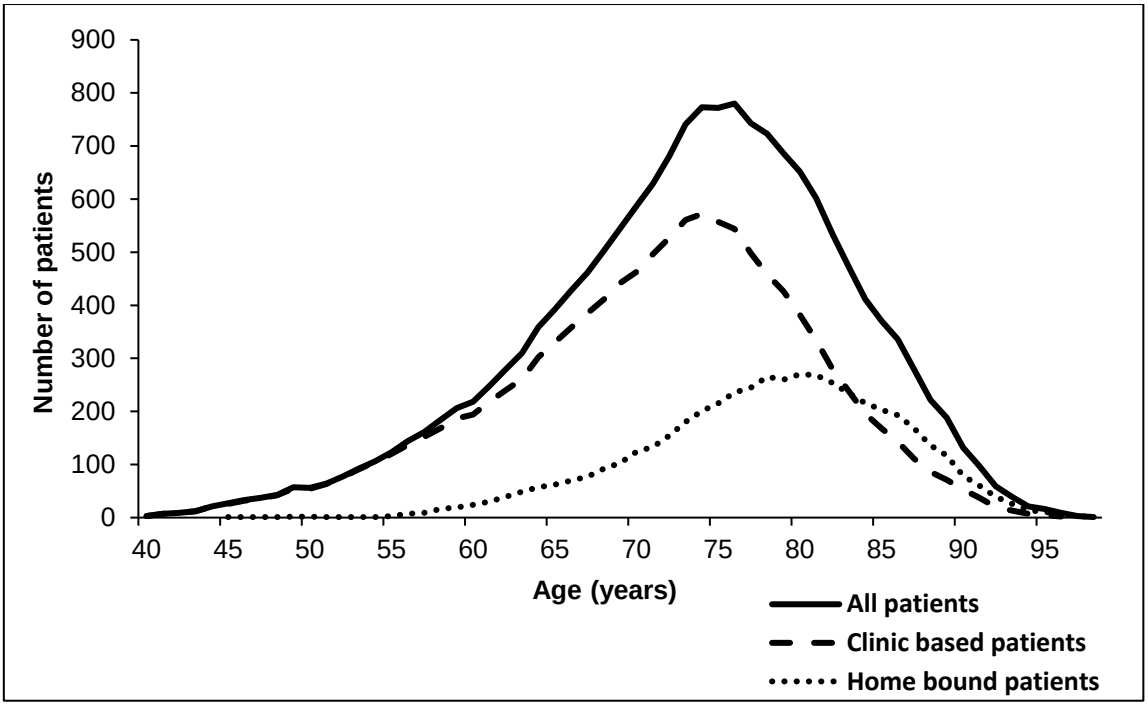


Figure 3-1 Number of patients in every age year

There was a significant relationship between TTR, time spent above and below target INR range and age for the whole population (P<0.0001 in each case; quadratic regression analysis) as shown in Figure 3-2. For the whole patient population, age accounted for 70%, 48% and 53% of the variability in mean TTR, time spent above and below target INR range, respectively. TTR showed a biphasic relationship with age, increasing to about 67 years of age, declining thereafter. Unsurprisingly, time spent above and below the target INR range showed the opposite pattern (Figure 3-2). This relationship held when considering patient setting; thus a significant relationship was found between mean TTR, time spent above and below therapeutic range INR range and age for both home-monitored patients (P<0.001, R^2=0.52; P=0.001, R^2=0.24; P<0.001, R^2=0.36, respectively) and clinic-monitored patients (P<0.001, R^2=0.36; P=0.009, R^2=0.16; P=0.001, R^2=0.21, respectively) (Figure 3-3). Only a small proportion of the domiciliary monitored patients were in 24 hour care with no difference noted in their TTR versus the cohort as a whole.

Mean TTR (determined by age) was significantly lower (P<0.001) and the mean time spent below therapeutic INR range significantly higher (P<0.05), for patients monitored at home than for patients monitored at hospital or general practice clinics (Figure 3-3). There was no significant difference in mean time spent above target INR range between patients monitored at home and those monitored in clinic.

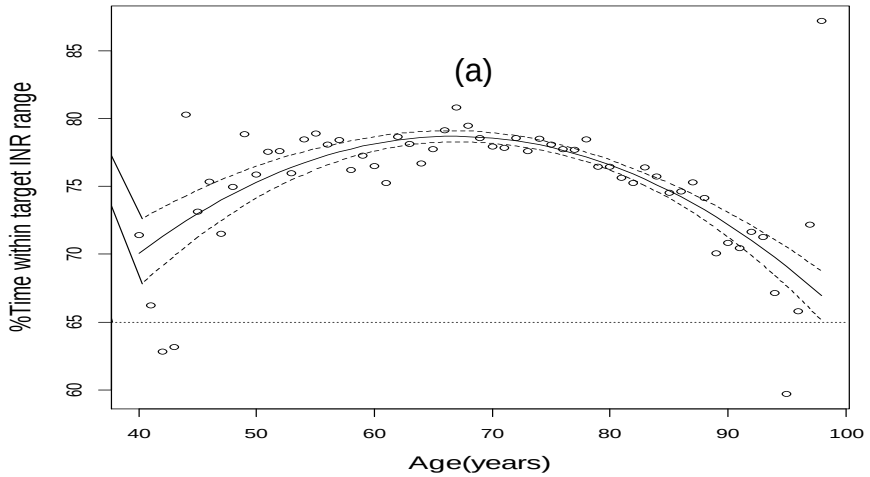
In females the mean TTR was marginally lower [by 1.3% (P<0.001)] and the mean time below range marginally higher [by 1.0% (P<0.001)] than those in males. Sex had no significant effect on mean time spent above target INR range.

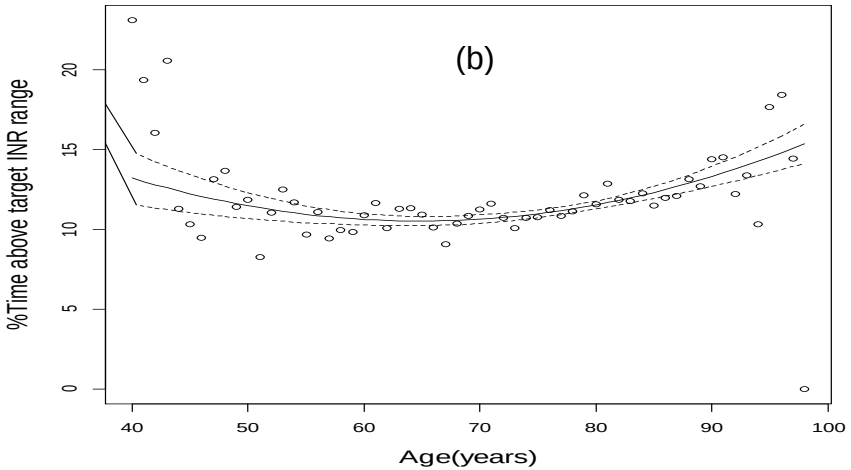
For the whole patient population both the number of dose changes (as mean square root) (P<0.0001, R^2=0.65) and the mean number of INR monitoring events (P=0.0001, R^2=0.44; quadratic regression) were significantly related to age. The number of warfarin dose changes and INR monitoring events were shown to decline until about age 67 years and then increase as patients got older. Similar findings were also noted for both home-monitored patients (square root of number of warfarin dose changes: P<0.001, R^2=0.40; mean number of INR monitoring events: P<0.001, R^2=0.31) and clinic-monitored patients (square root of number of dose changes: P<0.001, R^2=0.31; square root of the number of INR monitoring event: P=0.005, R^2=0.17). The homebound patients had a higher number of warfarin dose changes

and INR monitoring events compared to clinic-monitored patients for all ages, with the difference increasing with increasing age.

There was a strong and highly significant negative relationship between mean TTR and both the mean number of warfarin dose changes and INR monitoring events (determined as the square root) ($P<0.0001$, $R^2=0.70$ and $P<0.0001$, $R^2=0.61$, respectively; linear regression). Although the general pattern did not differ between the sexes, females had a higher age-adjusted frequency of dose changes and monitoring events [by 0.15 for the square root of the number of warfarin dose changes ($P<0.001$) and by 1.1 for the square root of the number of monitoring events ($P<0.001$)]. Age accounted for 97% of the variability in mean warfarin dose requirement, which fell with increasing age ($P<0.0001$; quadratic regression). Females required significantly lower warfarin doses ($P<0.001$) than their male counterparts, reflecting a smaller body weight (Figure 3-4a). Similarly, home monitored patients needed significantly lower warfarin doses compared to clinic monitored patients. However, the size of the difference in warfarin dose requirements between the two groups fell with increasing age (Figure 3-4b).

A logistic regression analysis was deployed to evaluate the probability of having a TTR of ≤65% according to age. A quadratic regression model was found to best fit the data ($P<0.001$). According to the model, home monitored patients had a higher probability of having a TTR ≤65% ($P<0.001$), compared to clinic monitored patients (Figure 3-5a), as did females compared to age-matched males ($P<0.001$) (Figure 3-5b).





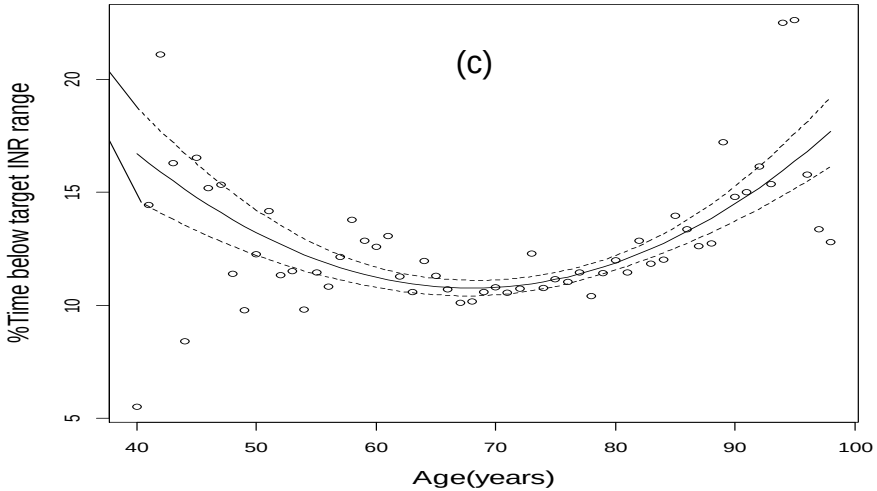


Figure 3-2: (a)TTR, (b) time above therapeutic range and (c) time below therapeutic range for the whole patient population. The solid lines are the fitted curves and the dashed lines are 95% confidence limits based on the observed sample sizes.

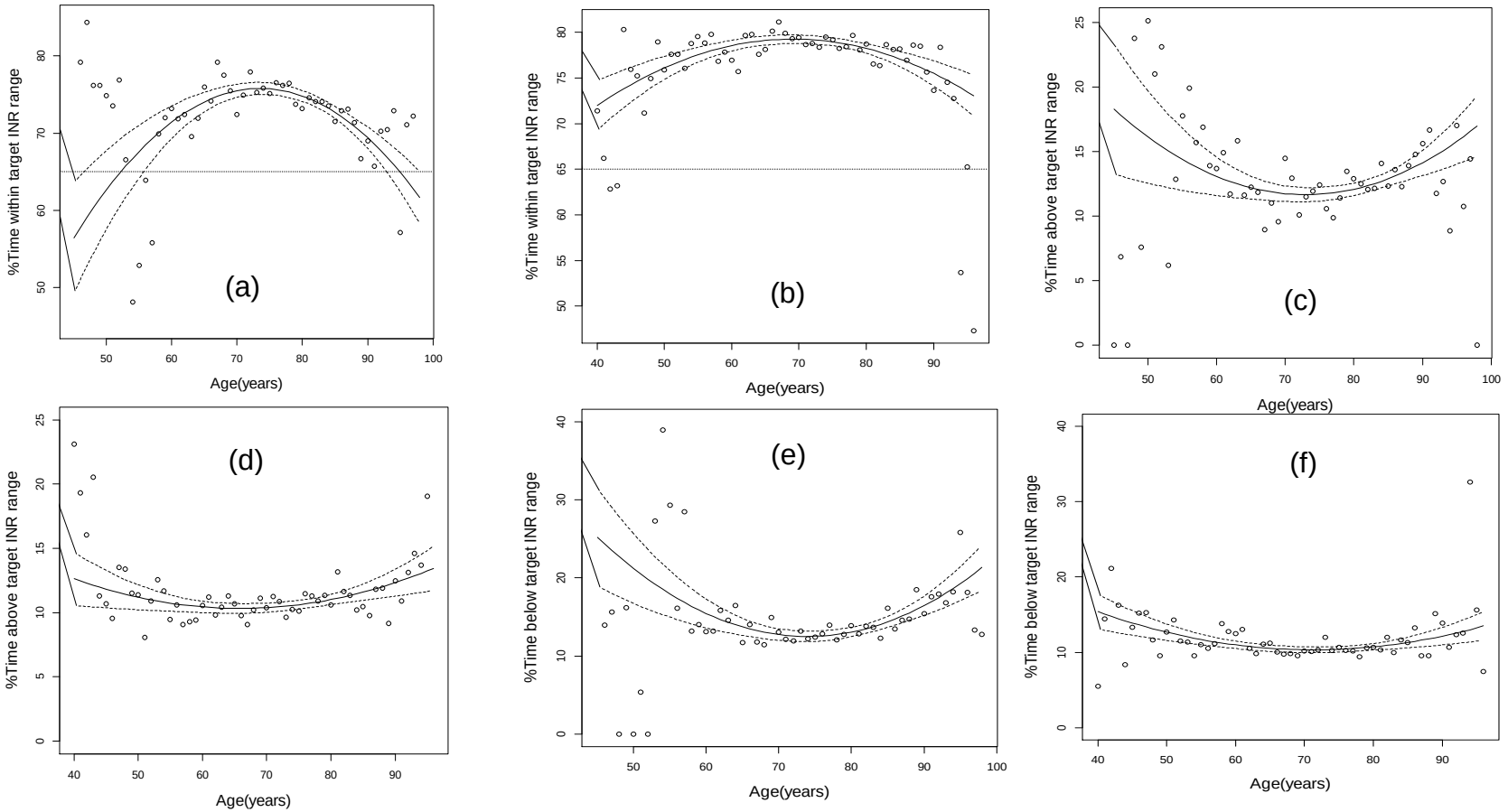


Figure 3-3: TTR for (a) home based and (b) clinic-based patients; time spent above target INR range for (c) home based and (d) clinic-based patients; time spent below target INR range for (e) home based and (f) clinic-based patients. The solid lines are the fitted curves and the dashed lines are 95% confidence limits based on the observed sample sizes.

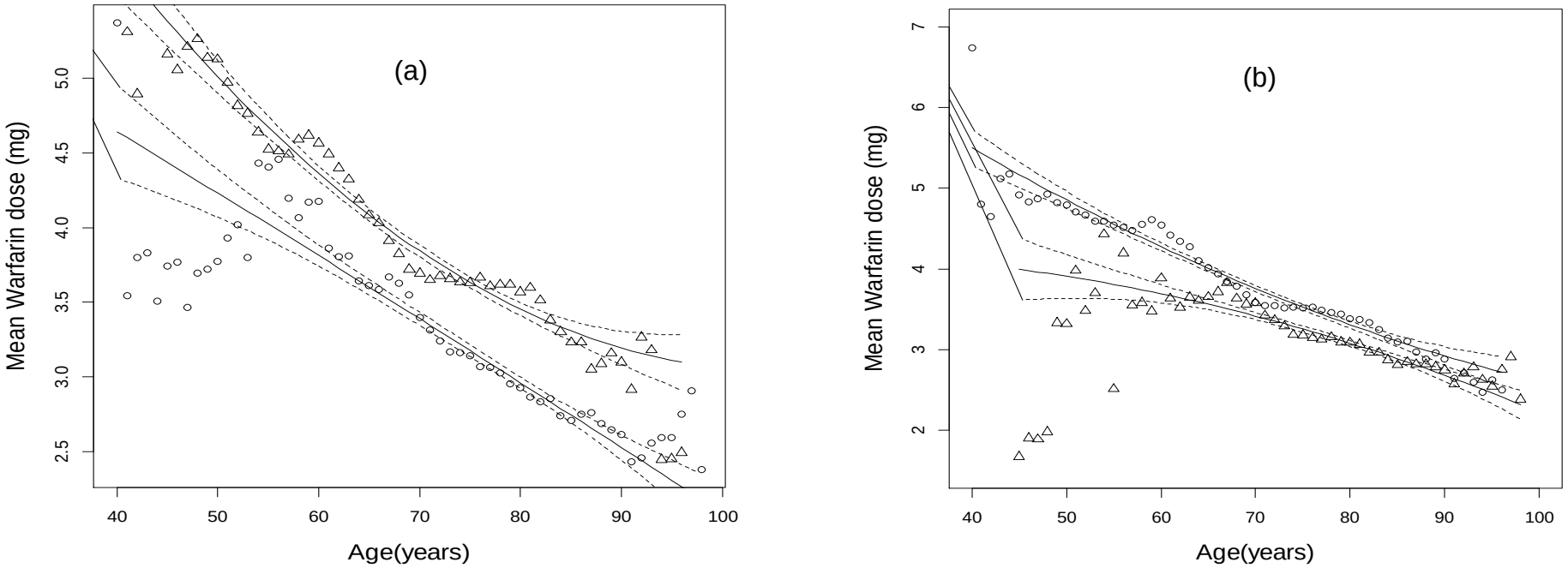


Figure 3-4: (a) Mean warfarin dose in females (circles) and males (triangles) with lines of best fit (solid lines) and 95% confidence limits (dashed lines); (b) Mean warfarin dose in clinic-monitored (circles) and home-monitored patients (triangles) with lines of best fit (solid lines) and 95% confidence limits (dashed lines)

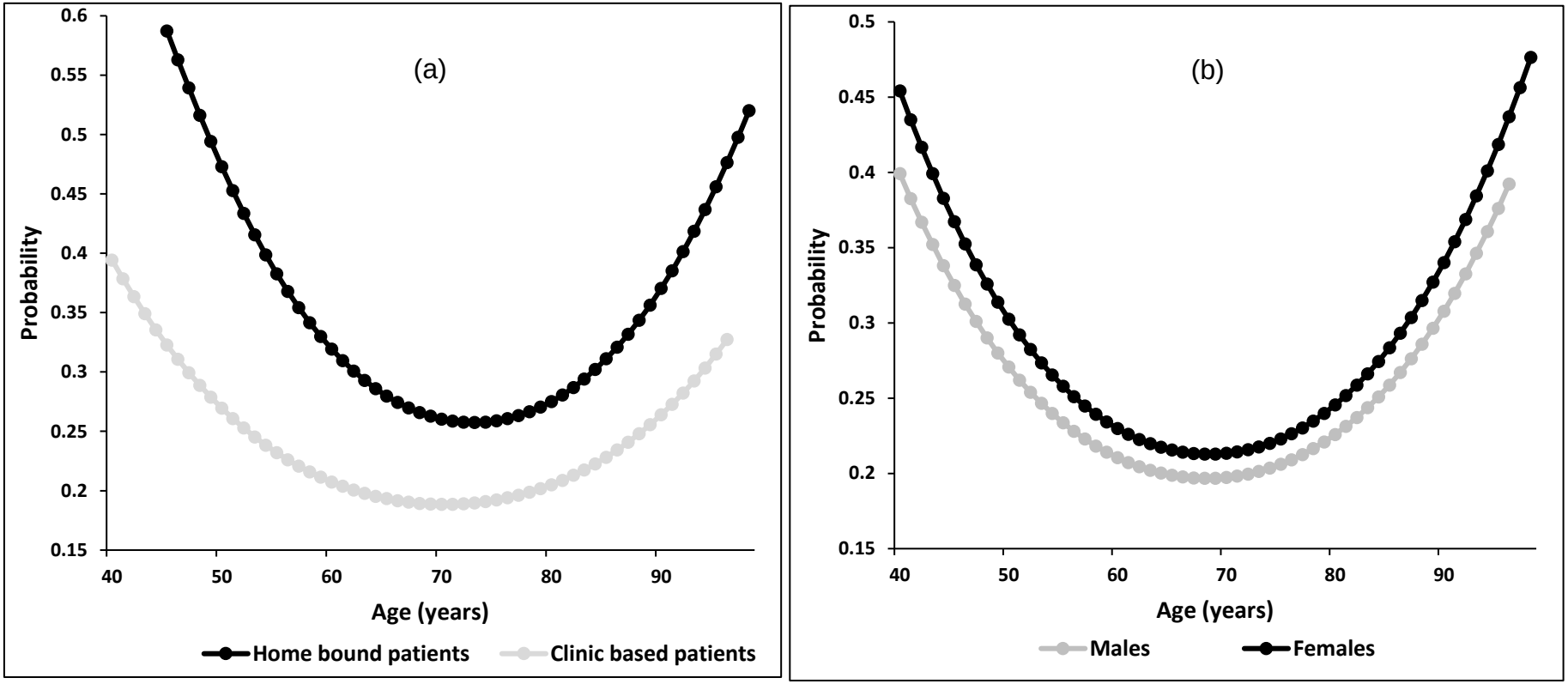


Figure 3-5: Probability of TTR ≤65% by (a) patient setting and (b) sex

3.5. Discussion

In spite of evidence to support use of oral anticoagulants to prevent stroke and systemic embolism in patients with AF [307-309] and the high prevalence of AF of >5% in over 70 year olds and >10% in over 85 year olds, many patients do not get optimal prophylaxis [310, 311], partly because of concerns about the risk of bleeding. Benefit from VKA therapy is greatest for patients remaining within their target therapeutic range; bleeding risk is increased when INR exceeds target range and thromboembolic events increase when INR is below target range. Stable anticoagulation in patients receiving VKA is influenced by anticoagulant service provision, with patients in Europe and the UK having better INR control than those managed in North America [312] and patients managed by individual clinicians having poorer control than cohorts managed in anticoagulant clinics [312]. The anticoagulated patients in our area were well managed with a median TTR value of 78.6% at 69 years and 75.8% at 74 years, compatible with values reported in Sweden which has a similar anticoagulant management system to the UK [55].

A comprehensive examination of patient level characteristics predicting TTR in a veteran population reported that older age was associated with better TTR, although the population studied differed considerably from ours, as it had an upper age band of ≥ 75, lower average TTR of 61%, a higher mental and physical health burden and only 2.7% were female [313]. Through our large dataset which allowed a detailed examination of ageing effect, we found that the likelihood of having a TTR ≤65% increases over the age of 70 years and that not only is anticoagulation control better overall in clinic attending than in domiciliary monitored patients, but that in the latter, anticoagulation control declines more rapidly with age. This is in spite of more warfarin dose changes and INR monitoring events, and a greater age-related increase in these in the home-monitored patients compared to clinic-monitored patients, in an attempt to improve control. It is worth mentioning that TTR and dose changes may have a complex relationship such that too frequent changes my impair TTR. This indicates the importance of the need for ongoing evaluation of patients once a decision to anticoagulate with a VKA is made.

Similar to previous reports [314-316], we found that patients at a younger age (below 60 years) had poorer anticoagulation control compared to the older patients. Possible explanations include poor adherence, social factors such as employment and alcohol use, and clinical factors as, at an age below 65 years, in contrast to older people, they will have had to have additional clinical morbidities to justify anticoagulation.

The 1.3% lower age-adjusted TTR of women, in spite of more dose changes and monitoring events, is consistent with other previous studies reporting on the predictors of anticoagulation control [313, 314]. Poor and erratic dietary vitamin K intake in women has been suggested as one possible explanation for their poorer anticoagulation [315]. The greater proportion of women than men in our domiciliary monitored group (59% v 41%) compared to the clinic monitored group (36% v 64%) may be one factor contributing to the difference in TTR between them.

Although identifying modifiable factors contributing to anticoagulation control is required for improving care of patients receiving VKAs, the evidence base is very limited. Whilst taking more than 16 medications, and four or more hospitalisations, are associated with erratic patterns of INR control, predictors like cancer and dementia are associated with directional poor control [317]. In our study domiciliary monitoring and female gender were associated with more time spent below target range, which could be due to patient factors, especially poor adherence. Whilst dosing decisions were computer driven, and adhered to in the vast majority of monitoring events, staff could override these based on clinical circumstances which would also have had the potential to influence outcomes.

In a French study of patients over 80 years, in rehabilitation or institutionalised care, poorer anticoagulation control was associated with being in hospital, antibiotic use and falls [318]. The conclusions from that study that frequent falls may be a marker of frailty and sacropenia, and antibiotic use a marker of acute illness which may result in deterioration in chronic co-morbidities and changes in drug use, leading to INR instability, may also be relevant to the steeper decline in anticoagulation control observed in our domiciliary group. Whilst our ambulatory patients who attend clinics are a less dependent population, in our previous work in a cross-section of this population [305] we noted no significant difference in either the number of drugs taken

(5 cardiovascular medications including warfarin), or the number of chronic co-morbidities (2 cardiovascular diseases including AF plus one other), between the groups, perhaps because warfarin is discontinued for more dependent patients as the risk/benefit balance changes. This suggests that the poorer stability of anticoagulation noted in the domiciliary monitored patients in both our cross-sectional and longitudinal study of similar populations is the result of diverse factors which may include dietary intake of vitamin K, alcohol intake, weight differences, female gender, cognitive impairment, adherence, social support, attitudes and frailty, and not simply co-morbidities and concurrent use of drugs. Disease and drug therapy, dietary variations and barriers to adherence which include lower cognitive function, poorer physical function, living alone and a higher perceived illness burden, require elucidation as any influence might also extend to outcomes with the use of new oral anticoagulants (NOACs) [319]. The licensing of NOACs presents clinicians with a choice of oral anticoagulants, both for newly diagnosed patients and for those who are currently taking VKAs. The latter is particularly relevant if anticoagulation control is poor as cost-effectiveness of NOACs compared to VKAs is highly dependent on anticoagulant control [320]. Our results indicate that, for some patients as they age, maintaining TTR becomes more difficult, particularly for people who cannot attend a monitoring clinic, which raises the question as to whether a NOAC would be a better option for them as NOACs offer benefit in terms of risk reduction in stroke, largely because of reduced incidence of haemorrhagic stroke and a greater relative risk reduction in major bleeding, when centre-based TTR in VKA treated patients is <66% [240].

It could be argued that the present study was limited by its retrospective nature. However, a retrospective design study for investigating stability of anticoagulation control is appropriate given the longitudinal nature of the investigation in a large cohort of AF patients, all of whom were anticoagulated with warfarin through a unified monitoring service using the same method of dosing. Further, any selection and observational biases were minimised given that information on INR values and warfarin doses for individual patients was obtained directly from electronic clinic records through the DAWN programme, within the confines of the study inclusion criteria. The effects of co-morbidity and concurrent therapy were not assessed, because data on these co-variates were not available through the DAWN programme [295], nor were any potential contributions from variances in patient education at initiation of warfarin

and at monitoring visits which were inevitable between the clinic and domiciliary monitored groups. Nonetheless, the primary aim of the present study was to examine whether age in the context of patient setting per se influences anticoagulation control with warfarin rather than identifying the factors which contribute to the variance in anticoagulant control and we were able to achieve this in this large cohort studied for up to 14 years. This information is relevant as warfarin remains a cost-effective option for anticoagulation, with informed decision making between this and a NOAC being appropriately made by patient and clinician based on the patient's clinical features and preferences.

3.6. Conclusion

I have demonstrated that there are both inter- and intra-individual differences in anticoagulation control achieved with warfarin, influenced by age and gender. In view of the poorer stability of anticoagulation control in older, homebound patients, the importance of reviewing patients' anticoagulant management at least annually is confirmed. Exploration of factors affecting anticoagulation control with warfarin, and whether such factors might also affect response to NOACs, is warranted.

There is ample evidence to show that dietary vitamin K affects anticoagulation response to vitamin K antagonists including warfarin. However, it is not clear whether dietary vitamin K affects the pharmacological activity of NOACs which needs to be investigated.

CHAPTER. 4 THE IMPACT OF VITAMIN K INSUFFICIENCY ON THE PHARMACOLOGICAL ACTIVITY OF RIVAROXABAN IN ELDERLY SUBJECTS

4.1. Introduction

In recent years several Non-vitamin K antagonist oral anticoagulants (NOACs), which selectively inhibit a single clotting factor [factor Xa or factor IIa (thrombin)] have been developed with the aim of providing more predictable pharmacokinetics and pharmacodynamics and obviating the need for patient monitoring. The factor IIa inhibitor, dabigatran, and factor Xa inhibitors, rivaroxaban, apixaban and edoxaban, have been approved for the treatment of thrombosis as well as prevention of stroke in patients with atrial fibrillation (AF) according to their licensed indications, having demonstrated non-inferiority to warfarin in their clinical effectiveness for stroke prophylaxis in patients with atrial fibrillation in the RE-LY, ROCKET-AF, ARISTOTLE and ENGAGE AF-TIMI 48 studies [221, 222, 224, 321]. Prescription numbers for NOACs have grown rapidly since their launch for newly diagnosed patients requiring initiation of anticoagulant therapy and for existing patients on coumarin therapy who are switched to a NOAC because of concerns about treatment safety due to poor anticoagulation control [322-325].

Vitamin K, in its hydroquinone form, is an essential co-factor needed for the γ-carboxylation of the glutamic acid residues of the amino-terminals of the coagulation proteins II, VII, IX and X, resulting in their activation. Coumarin derivatives, also known as vitamin K antagonists (VKAs), cause anticoagulation by inhibiting the recycling of vitamin K hydroquinone in the vitamin K cycle, thus inhibiting the activation of the above mentioned vitamin K-dependent coagulation proteins [326]. Alterations in dietary vitamin K intake affect anticoagulation response to coumarins [327, 328]. People with low dietary vitamin K intake require lower warfarin doses to achieve their anticoagulation target range and have more unstable control than those with vitamin K rich diets [327, 328].

It is possible that dietary vitamin K intake not only influences anticoagulation response to coumarins but also that to NOACs since the activation of both factor II and factor X is dependent on vitamin K availability. The reported incidences of major bleeding in trials of NOACs are contributed to by patient age, with the incidence of

bleeding being greater in patients aged 80 years and over [222, 321, 329]. The bleeding adverse reactions for NOACs may be contributed to by low dietary vitamin K intake, of which older patients could be particularly at risk because of their generally low dietary vitamin K intake [330, 331] compared to younger patients. The idea that dietary vitamin K can affect the pharmacological activity of NOACs in man emanated from the earlier findings of the research group that vitamin K insufficiency in rats significantly enhanced anticoagulation response to the direct thrombin inhibitor, ximelagatran [280].

4.2. The aim of the study

To examine the pharmacological activity of the direct factor Xa inhibitor, rivaroxaban, in a group of older subjects, with a similar age and characteristics to those of older subjects with AF requiring anticoagulation therapy, who were deemed to have poor dietary vitamin K intake, compared to a group of younger subjects with adequate diets.

4.3. Materials and methods

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

4.3.1. Sample size calculation

It was hypothesized that poor dietary vitamin K intake enhances the pharmacological activity of rivaroxaban. Previously we reported significant differences in anticoagulation response between two groups of 15 vitamin K-replete and 15 vitamin K-deplete rats [280]. However, as there is no *priori* information available on the extent to which dietary vitamin K affects haematological measures to rivaroxaban ex-vivo in man a sample size of 60 subjects (30 with poor dietary vitamin K intake and 30 with adequate diets) was deemed to be sufficient to test our hypothesis.

4.3.2. Study subjects

Subjects with liver dysfunction, or a disease requiring drug therapy which affects haemostasis, were excluded from taking part in the study. Following written informed consent, 31 medically stable older inpatients (12 males), not on oral or parenteral anticoagulants, with suspected poor dietary intake in terms of calories and nutrients including vitamin K, and 28 healthy younger subjects (7 males) with adequate diets, took part in the study. The older subjects had been admitted to hospital for one of the following reasons; fall at home (n=6), infection [urinary tract (3), respiratory (4)], exacerbation of chronic obstructive pulmonary disease (4), bone fracture (3), dizziness (2), peripheral vascular disease (2), aortic valve implantation (1), haematuria (1), pacemaker insertion (1), tinnitus (1), and general poor health (3). Information on co-morbidities and drug therapy was recorded. Each subject completed a dietary questionnaire and provided an overnight-fasted venous blood sample (20ml) collected in citrated tubes. Fasted plasma vitamin K concentration was chosen as it is representative of vitamin K store in the liver and is a good indicator of vitamin K dietary status, and also to decrease post-prandial lipidaemia effect as it is a lipid soluble vitamin [110]. Following centrifugation (at 4000 rpm for 7 minutes) the resultant plasma samples were aliquoted prior to storage at -80 ^{o}C for later analyses.

4.3.3. Assessment of dietary vitamin K

4.3.3.1. Dietary questionnaire

Each subject completed a validated food frequency questionnaire (FFQ), based on the European Prospective Investigations into Cancer and Nutrition (EPIC) FFQ, for quantification of vitamin K content of foods eaten in the previous week [332, 333], but modified to expand the sections of high vitamin K-containing foods, Appendix (B) page 215. In order to increase accuracy of participant recall, the timeframe was restricted for participants answering questions on food intake by removing the ‘once a fortnight’ and ‘once a month’ options and replacing them with recall only for the previous week. The dietary data captured in the questionnaire were analysed using a computerized programme which contains the vitamin K values for approximately 1000 food items collated from a number of published sources [334]. Vitamin K contents were not

available for some food items from these sources; therefore, USDA National Nutrient Database for Standard Reference, release 28 [335], was used in addition.

4.3.3.2. Measurement of plasma vitamin K_1 concentration

Plasma vitamin K concentrations were determined using an established high pressure liquid chromatography (HPLC) method using post-column derivatization with zinc metal powder [336].

4.3.3.2.1. Samples preparation

Vitamin K_1 was isolated from plasma by a two-step process involving liquid phase extraction followed by solid-phase extraction (SPE).

4.3.3.2.1.1. Liquid-phase extraction

All samples were analysed in duplicate as follows. To suitably marked glass tubes 0.5 ml of plasma sample was transferred, and then 40 µl of internal standard solution (MK-6, 120 pg/µl) was added. 1ml HPLC water was added followed by 2ml ethanol to precipitate proteins in the plasma. After vortexing for about 15-20 seconds, 6 ml of hexane was added. The tubes were capped and vortexed 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 45ºC under a stream of air, and the residue reconstituted with 1ml hexane.

4.3.3.2.1.2. Solid-phase extraction

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 45ºC under a steam of air. The residue was reconstituted initially with 30 µl dichloromethane, vortexed for two seconds, and then with 100 µl mobile phase, and vortexed for a further 10-15 seconds. The reconstituted samples were transferred into 1.5 ml Eppendorf tubes, and spun on a benchtop centrifuge for 2-3 minutes at maximum speed to precipitate any particulate matter, prior to HPLC analysis.

4.3.3.2.2. Vitamin K free plasma

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

4.3.3.2.3. Preparation of standard curve and quality control

Pure vitamin K_1 was obtained from SIGMA-ALRICH, UK. Hexane (HPLC grade) was used to prepare primary vitamin K_1 stock solution. The primary solution was then diluted with methanol (HPLC grade) to prepare a secondary working stock solution which was used for constructing a vitamin K_1 standard curve (range 0-1620 pg/ml). Appropriate volumes of methanolic vitamin K solution together with 40 µl of internal standard (MK-6) were added to Eppendorf 1.5 ml tubes (in duplicate). After evaporating under a stream of air, the residue was reconstituted and injected as described in section 4.3.3.2.1.2. Quality control samples were extracted in duplicate in the same way as mentioned in section 4.3.3.2.1 and tested for with every assay run.

4.3.3.2.4. HPLC instrumentation and conditions

A Shimadzu VP HPLC machine was used for measuring plasma vitamin K_1 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. 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). The mobile phase was run at a flow rate of 0.5 ml/min. The run time for each sample was 12 minutes. Vitamin K_1 and MK-6 were eluted on average at 5.82 and 7.11 minutes, respectively, as shown in Figure 4-1.

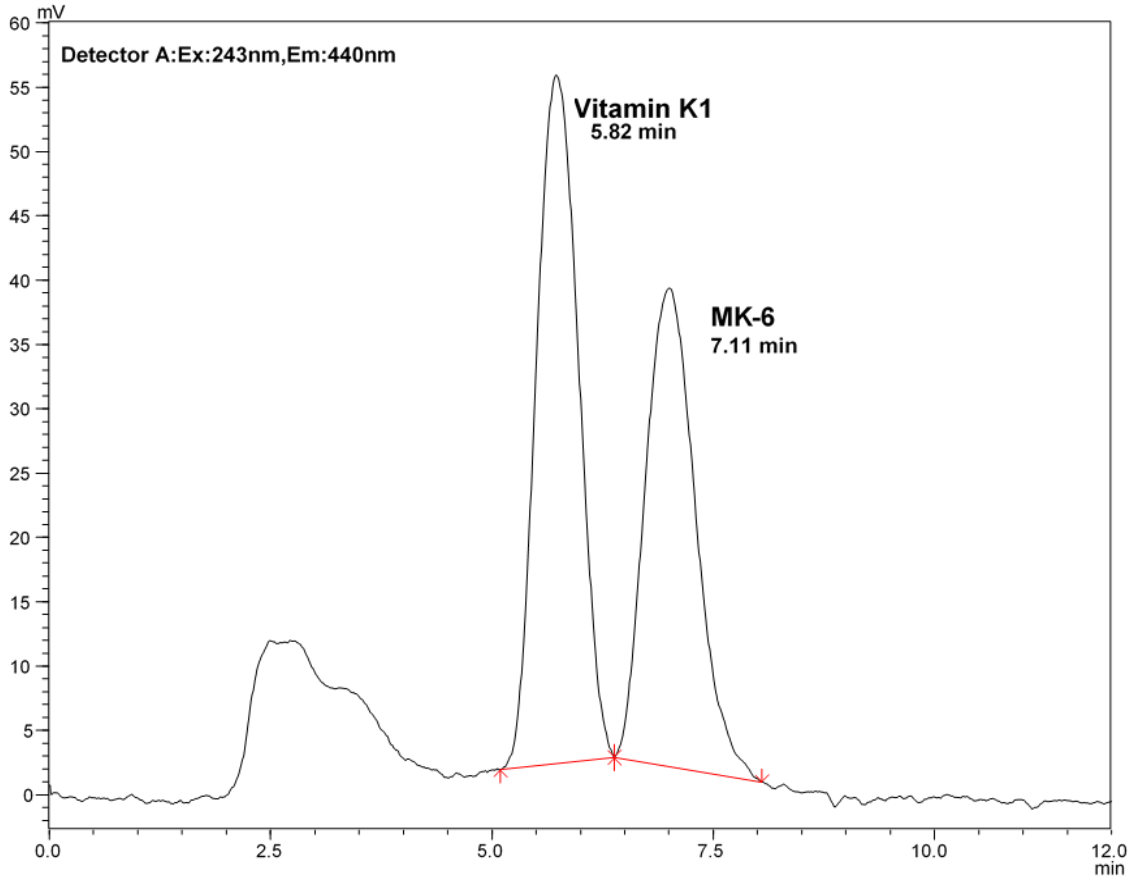


Figure 4-1: The HPLC chromatogram for vitamin K_1 and MK-6

4.3.3.2.5. 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 K_1 concentration.

4.3.3.2.6. Limits of detection and precision

The detection limit of the extracted samples was 10 pg/ml for vitamin K_1 (signal/noise ratio =3). The inter-day coefficient of variation for vitamin K at 720 and 1008 pg/ml was 9.4% and 13.6%, respectively.

4.3.4. Haematological assessments

All tests were carried out at the accredited Haematology Laboratory at the Royal Victoria Infirmary and Freeman Hospital, Newcastle Hospitals NHS Foundation Trust, Newcastle upon Tyne. Rivaroxaban pure powder was kindly provided by Bayer Schering Pharma, (Berlin, Germany). The powder was solubilised in dimethyl sulfoxide solution (DMSO) according to the manufacturer's recommendations, at a final concentration of 10µg/ml.

On the day of each experiment plasma aliquots were allowed to thaw at room temperature and utilized within 30 minutes. Using the method of Gerotziasfas et. al., [337], plasma samples were incubated with rivaroxaban (0, 100, 250, 400, and 500 ng/ml) at concentrations similar to drug plasma concentrations observed following oral dosing [338-340]. Control plasma samples were incubated in parallel with the clinical samples to gauge the accuracy of the measurements.

4.3.4.1. PT, mPT, APTT, and factors II, VII, IX, X

Prothrombin time (PT) was measured using RecombiPlasTin2G®, and activated partial thromboplastin time (APTT) using HemosIL® (Instrumentation Laboratory Company, Bedford, MA, USA). Clotting time was also assessed using modified prothrombin time (mPT) which is a PT assay modified by adding calcium chloride ($CaCl_2$) to the thromboplastin reagent (RecombiPlasTin 2G®) to enhance assay

dynamics and expand sensitivity [341]. For this the thromboplastin reagent was reconstituted with distilled water, and diluted 1:2.25 with $CaCl_2$ [341]. Vitamin K-dependent clotting factors II, VII, IX, and X activities in plasma were measured with standard clotting assays using respective clotting factor deficient plasma (Instrumentation Laboratory Company, Bedford, MA, USA). The IL TOP ACL CTS instrument (Instrumentation Laboratory Company, Bedford, MA, USA) was used for all the assays.

4.3.4.1.1. Method validation for modified prothrombin time (mPT), PT and clotting factor activity

Plasma from healthy volunteers was obtained from the blood bank, Royal Victoria Infirmary (RVI), Newcastle upon Tyne. The plasma was aliquoted into small volumes and stored at -80°C for further analyses. On the day of each experiment plasma was defrosted at room temperature and incubated with rivaroxaban over a range of concentrations (0-2000 ng/ml) for a period of 30 minutes followed by analysis. mPT was checked across a range of $CaCl_2$ concentrations (25, 50, 75, and 100 mmol/L) along with the measurements of PT, APTT, fibrinogen and clotting factors II, VII, IX, and X. To achieve consistent results 1.3 ml plain freeze polyethylene tubes were used throughout all experiments.

Figure 4-2 and Figure 4-3 show typical results for PT and mPT and for PT and APTT, respectively, following the incubation of plasma with different concentrations of rivaroxaban. 100 mmol/L $CaCl_2$ produced the greatest prolongation of PT whilst 25 mmol/L $CaCl_2$ had the least effect. These findings were consistent with the results of Barret et al. [341]. Typical results for clotting factors II, VII, IX, and X activity are shown in Figure 4-4, respectively. Figure 4-5 shows typical results for fibrinogen concentration.

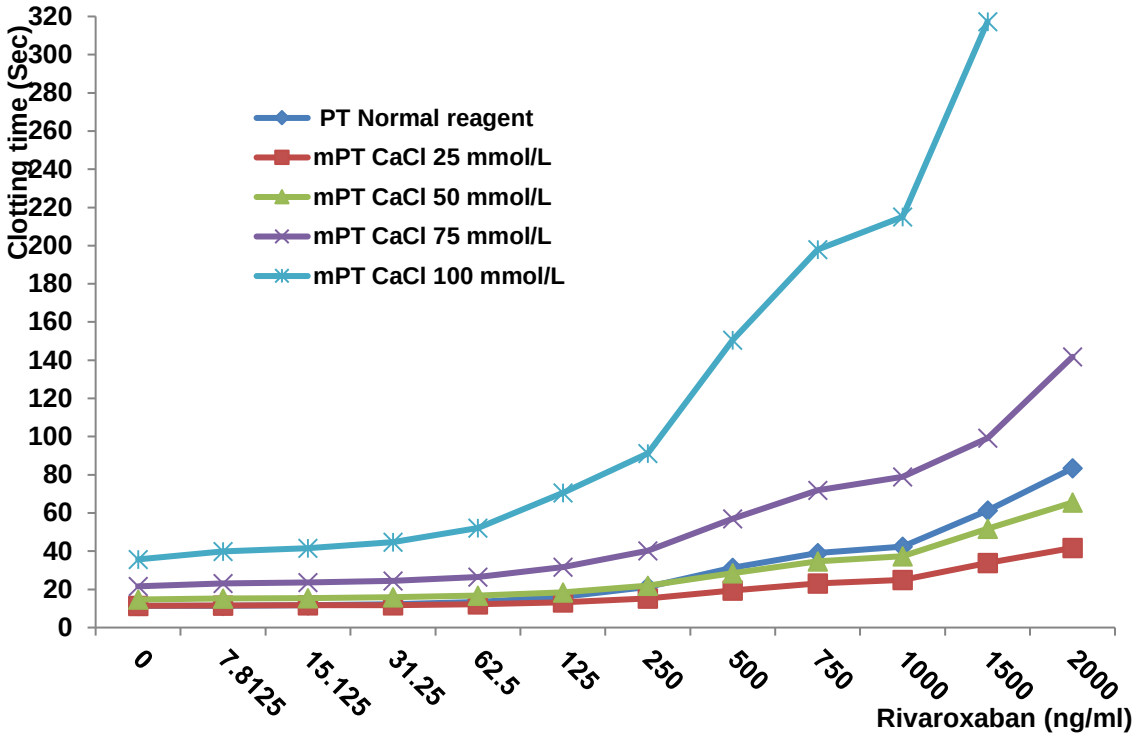


Figure 4-2: PT and mPT (25, 50, 75, 100 mmol/L $CaCl_2$)

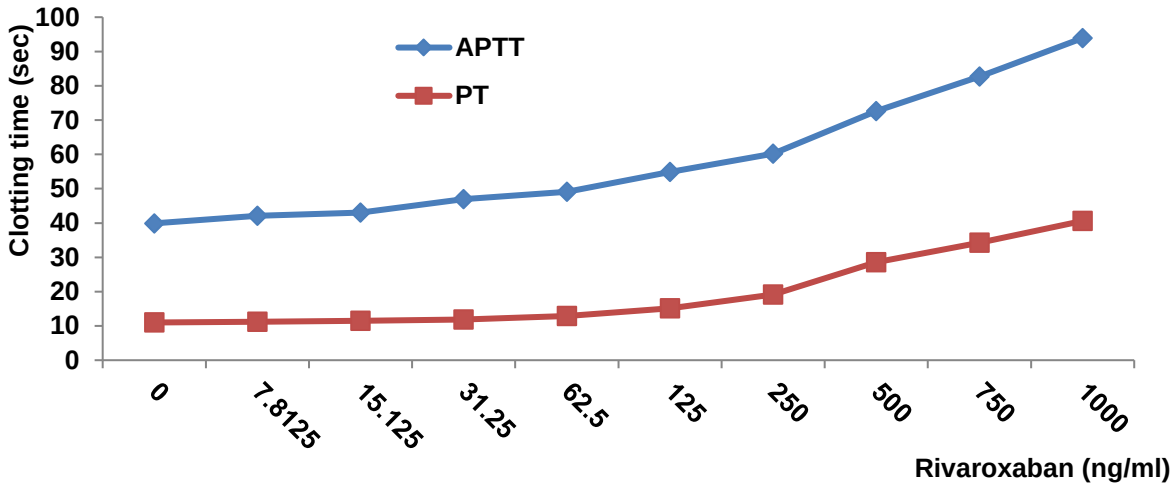


Figure 4-3: PT and APTT for control plasma incubated with different concentrations of rivaroxaban

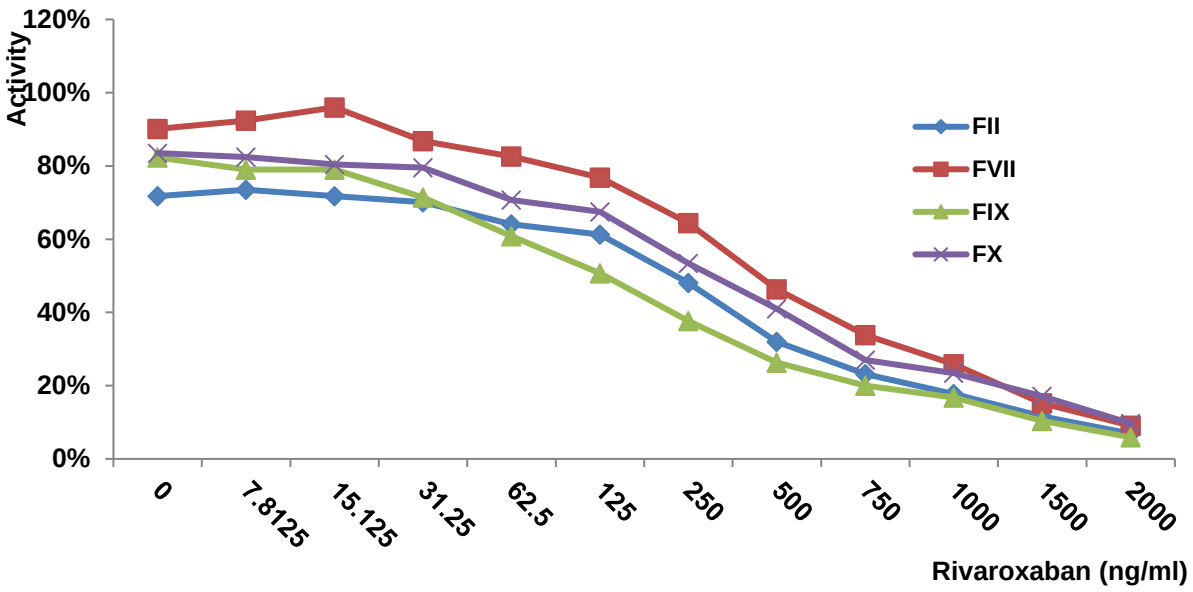


Figure 4-4: Activity of factors II, VII, IX, and X

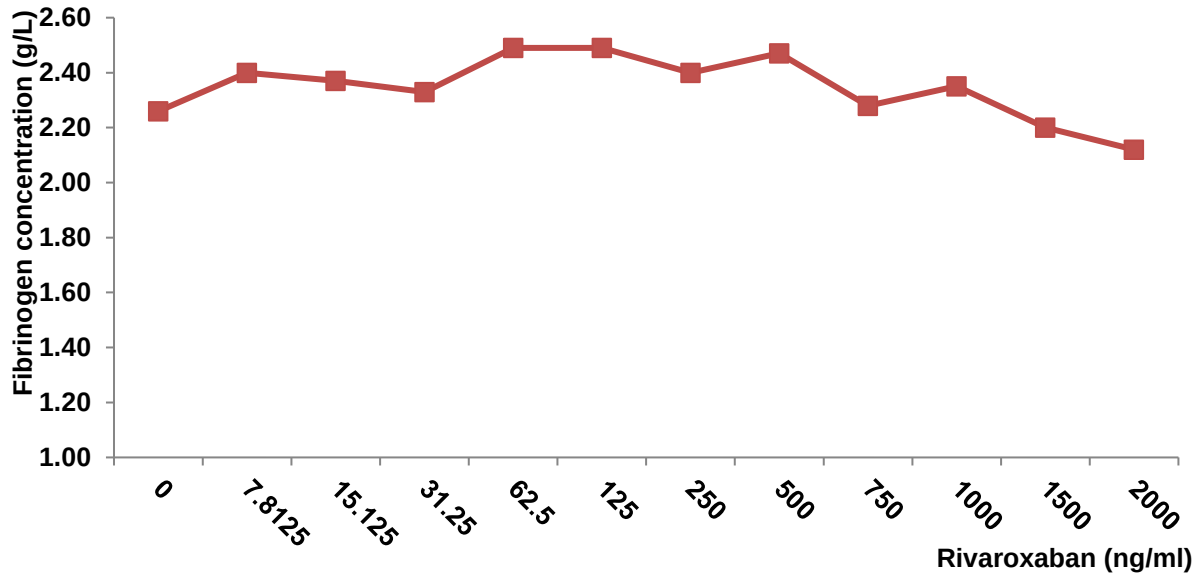


Figure 4-5: Fibrinogen concentration with different rivaroxaban concentrations

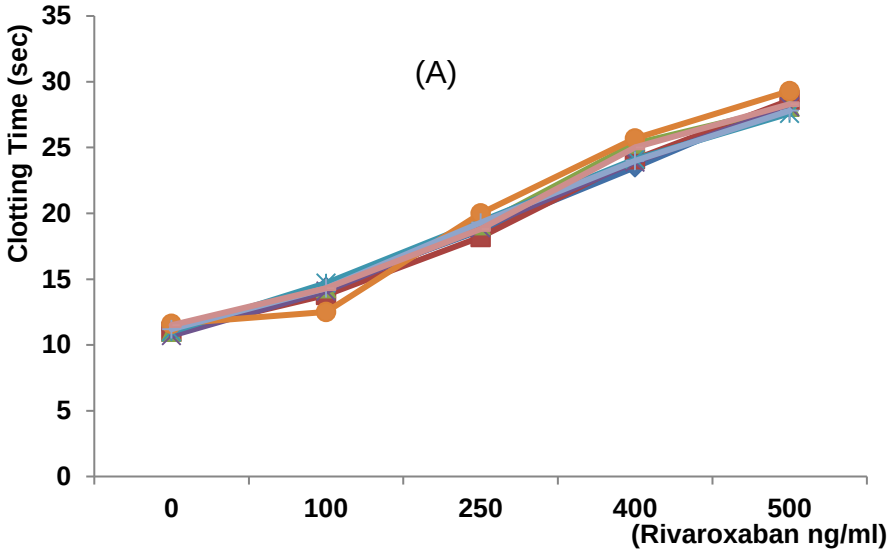
Based upon these results, 100 mmol/L $CaCl_2$ was used for the final analysis for mPT. The same batch of control plasma was used for all analyses for assessing precision and accuracy of the measurements.

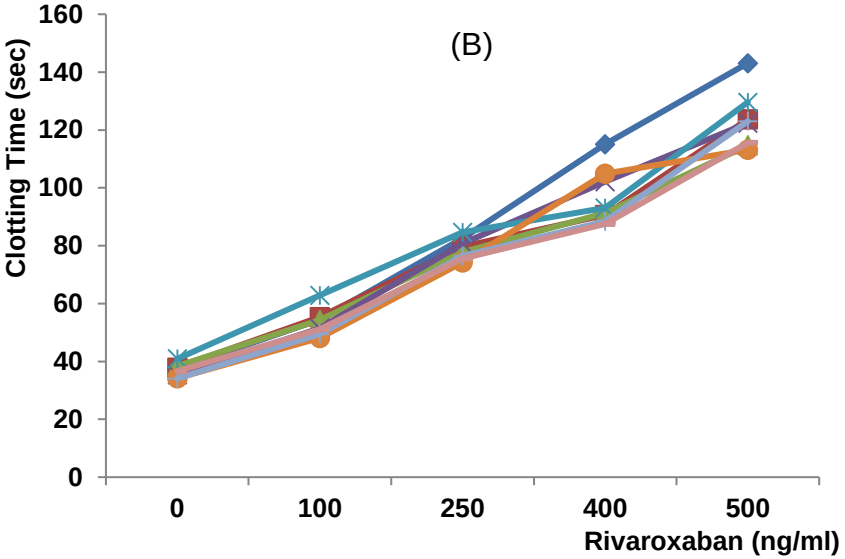
4.3.4.1.1.1. PT, mPT 100 mmol/L $CaCl_2$, and APTT

PT, mPT, and APTT were prolonged following incubation of plasma with rivaroxaban as shown in Figure 4-6, and Figure 4-8. Rivaroxaban had no effect on fibrinogen concentration as shown in Figure 4-8(D). The coefficient of variation for replicate analysis of samples at baseline and at 100, 250, 400 and 500 ng/ml rivaroxaban were as follows; for PT (2.6, 4.8, 2.8, 3.2, 1.9% respectively); and for mPT (6.2, 8.6, 4.6, 10.1, 7.9% respectively).

4.3.4.1.1.2. Clotting factors

Clotting factors II, VII, IX, and X activities were reduced across all rivaroxaban concentrations as shown in Figure 4-7, and Figure 4-8. The coefficients of variation for replicate analysis of samples at baseline and at 100, 250, 400 and 500 ng/ml rivaroxaban were as follows; for FIXa (4.5, 4.3, 6.5, 5.4, 6.6%, respectively) and for FXa (10.4, 11.1, 12.3, 9.1, 8.6%, respectively).





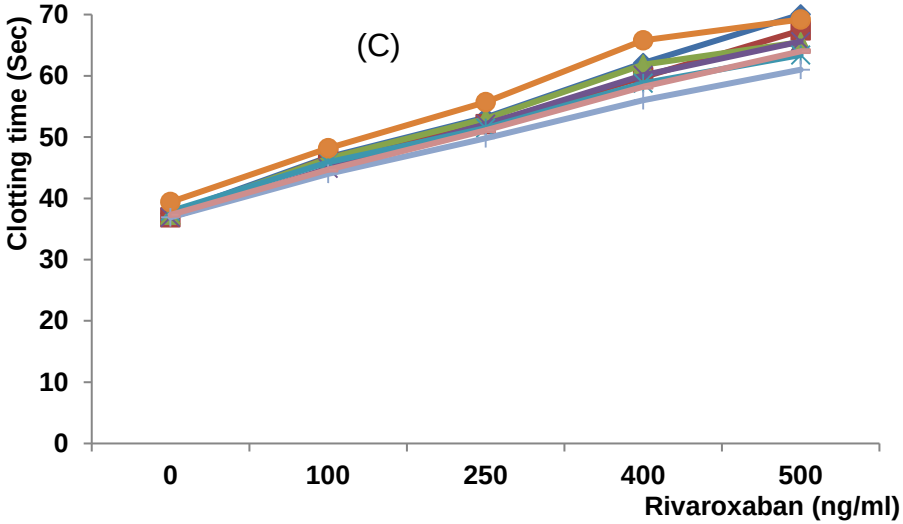


Figure 4-6: PT (A), mPT 100 mmol/L $CaCl_2$ (B), and APTT (C) prolongation with rivaroxaban in control plasma on 8 separate days

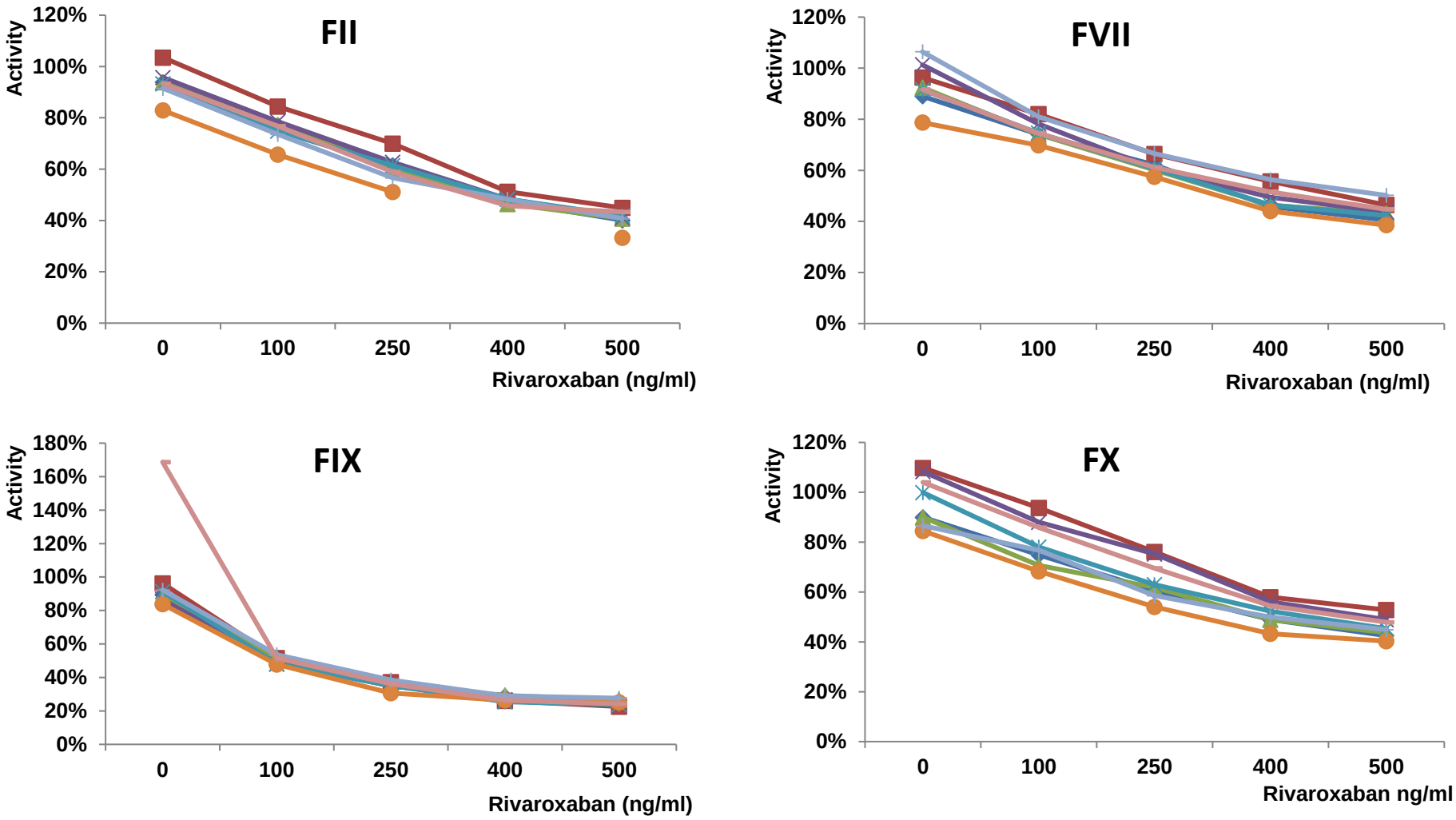


Figure 4-7: The effect of rivaroxaban on clotting factors activity in control plasma on 8 separate days

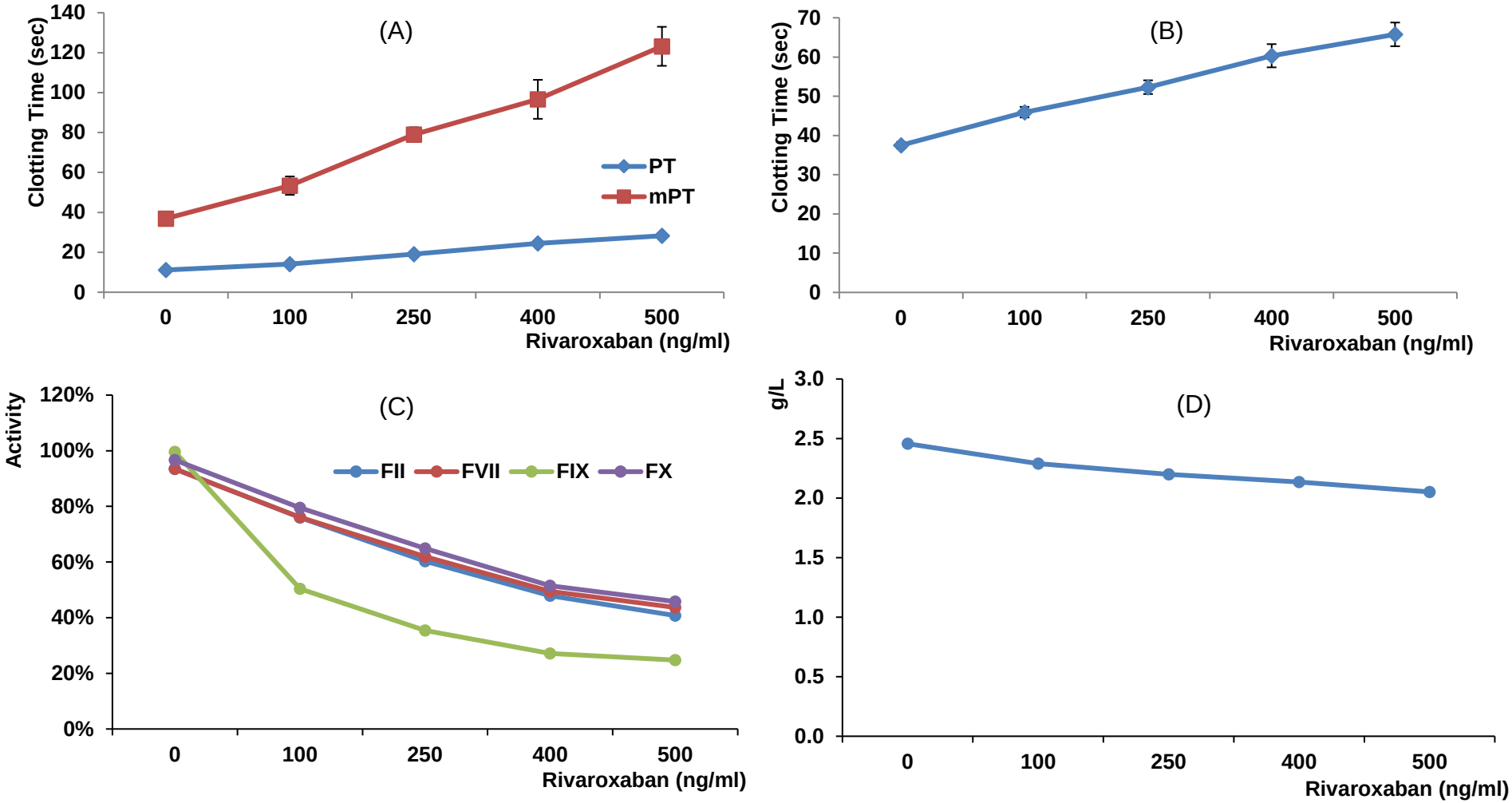


Figure 4-8: Mean PT and mPT (A), APTT (B), clotting factors II, VII, IX, and X activity (C), and mean fibrinogen concentration (D) in control plasma incubated with rivaroxaban on 8 separate days

4.3.4.1.2. The effect of temperature on PT and mPT

Control plasma samples from the same batch were spiked with rivaroxaban (final concentration of 125 and 500 ng/ml) and incubated in a water bath at 37ºC, alongside samples kept at room temperature. PT and mPT ($CaCl_2$ 100 mmol/L) were measured every 10 minutes for 30 minutes. The experiment was done several times on different days. Temperature was found to have no effect on either PT or mPT as shown in Figure 4-9.

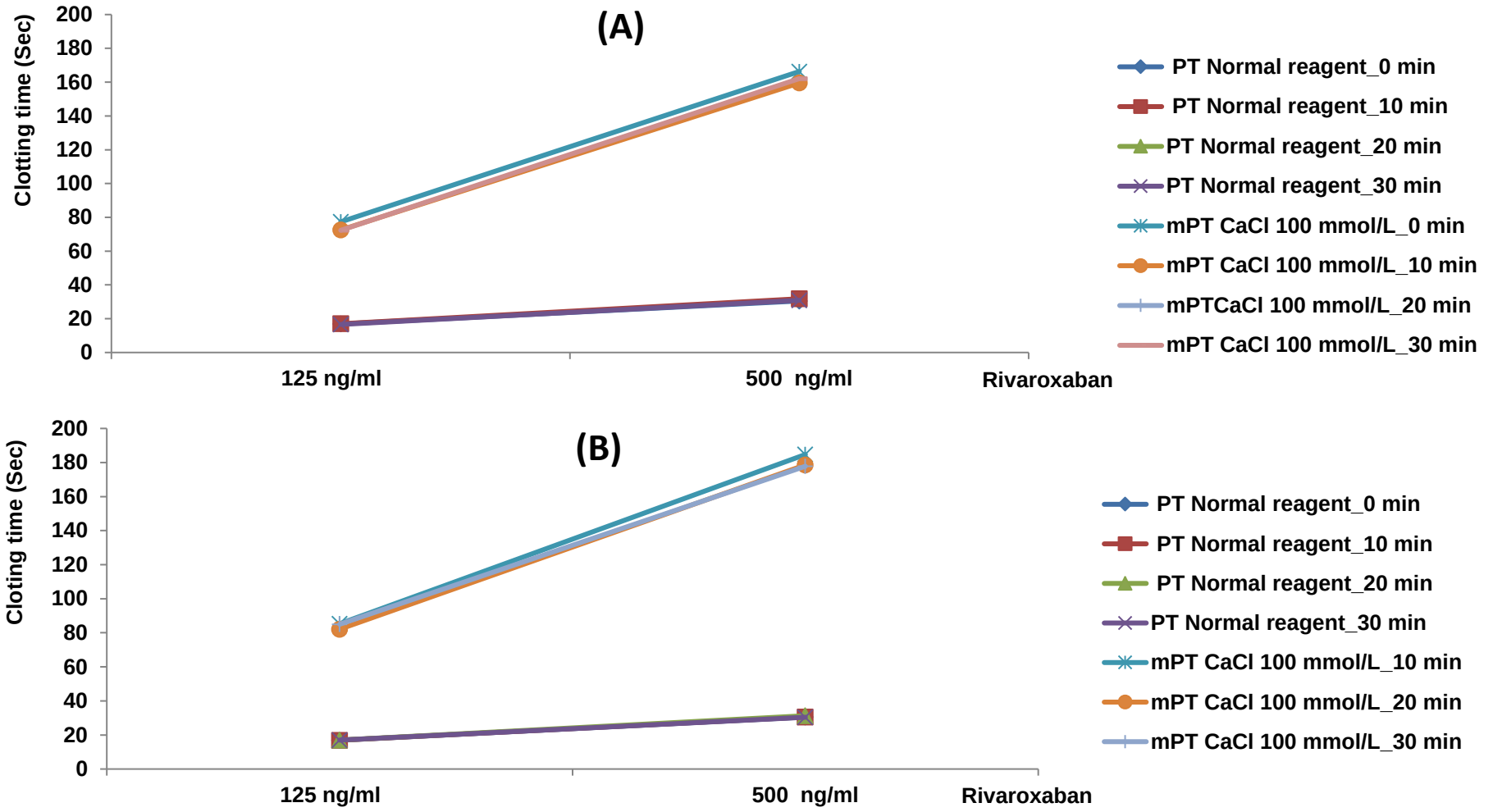


Figure 4-9: The effect of rivaroxaban on PT and mPT at room temperature (A) and at 37ºC (B)

4.3.4.2. FIXa and FXa antigen ELISA analysis

FXa and FIXa antigen levels were measured using a commercially available ELISA kit (Diagnostica Stago, Parsippany, NJ, USA). The manufacturer's protocol used is shown in appendices (D) and (E) (page 229 and 230).

4.3.4.3. Thrombin Generation Assay (ETP)

A modified assay of Perzborn, Morishima, and Douxfils [342-344] was used. 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). The assay was performed as follows: 20µl high platelet-poor plasma (HPPP) reagent (20pM tissue factor and 4 µM phospholipids) and 10µl rivaroxaban solution in DMSO (final concentration in plasma: 200 ng/ml), or 10µl DMSO (for baseline measurement) were pipetted into individual wells of a 96-well microtitre plate, together 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). The following parameters of the thrombin generation assay were analysed; lag time, maximum thrombin generation (peak), time to peak (tt peak), endogenous thrombin potential (ETP), and mean thrombin generation rate (mTGR).

4.3.5. Statistical analyses

Data were checked for normal distribution. Where data were not normally distributed logarithmic transformation was applied to approach normality. Data were compared using Student’s t-test; a p-value <0.05 was considered to be statistically significant. Excel (Microsoft Corp., Redmond, WA, USA), and Statistical Package for Social Sciences (SPSS version 22) were used for data reporting and analyses. Data are presented as mean±SD.

4.4. Results

4.4.1. Study subjects

The mean age and mean weight of the older and younger subjects was 87±6 and 36±10 years ($p<0.0001$) and 51±12 and 74±13 kg ($p<0.0001$), respectively. Whilst none of the healthy subjects was taking prescribed medication, the patients were taking a median of 6 drugs, 3 (47%) of which were classified as affecting the cardiovascular system, 1 (10%) the gastrointestinal tract (usually a proton pump inhibitor or laxative), and 1 (15%) the endocrine system (most commonly calcium and vitamin D or levothyroxine). Their median number of co-morbidities was 4, most commonly cardiovascular disease and cerebrovascular disease (34%).

4.4.1. Dietary assessment

4.4.1.1. Food Frequency Questionnaire & Plasma vitamin K concentrations

During the previous week, the younger subjects had consumed significantly more vitamin K than the older subjects (217±198 µg (range 38 to 760) v 67±98 µg (range <1-363); $p<0.0001$), with a mean difference of 150 µg, as shown in Figure 4-10. The younger subjects had significantly higher plasma vitamin K concentrations than the older ones [383±327 (range 69-1527) pg/ml v 134±82 (range 27-359) pg/ml, $P<0.0001$), as shown in Figure 4-11. The FFQ estimated vitamin K intakes highly significantly correlated with plasma vitamin K concentrations ($r=0.42$; $P=0.001$).

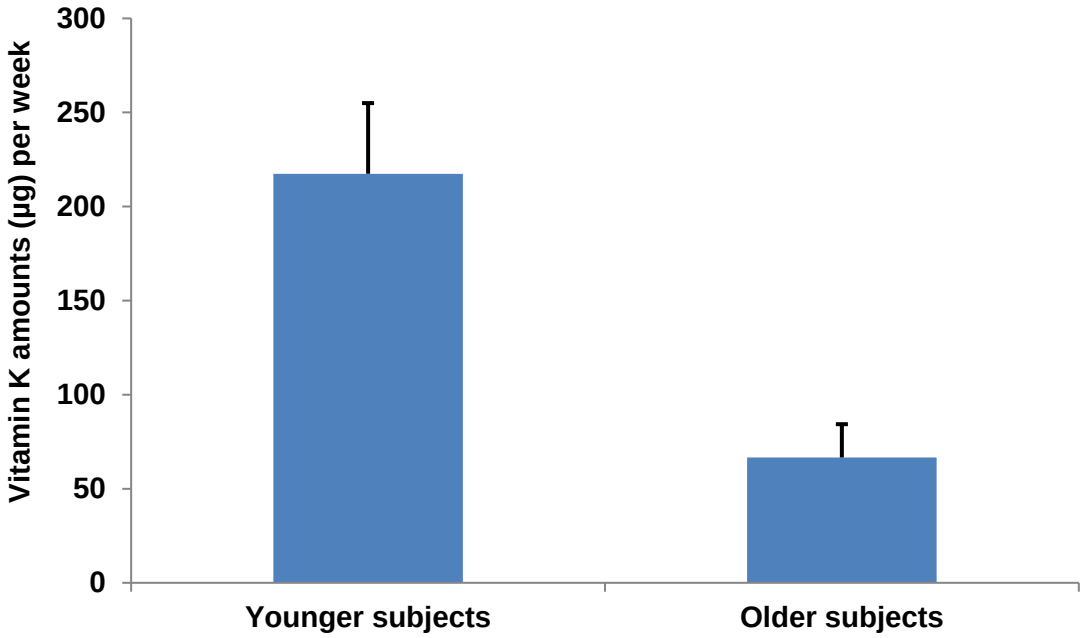


Figure 4-10: Mean weekly amount of vitamin K consumed by the older and younger subjects

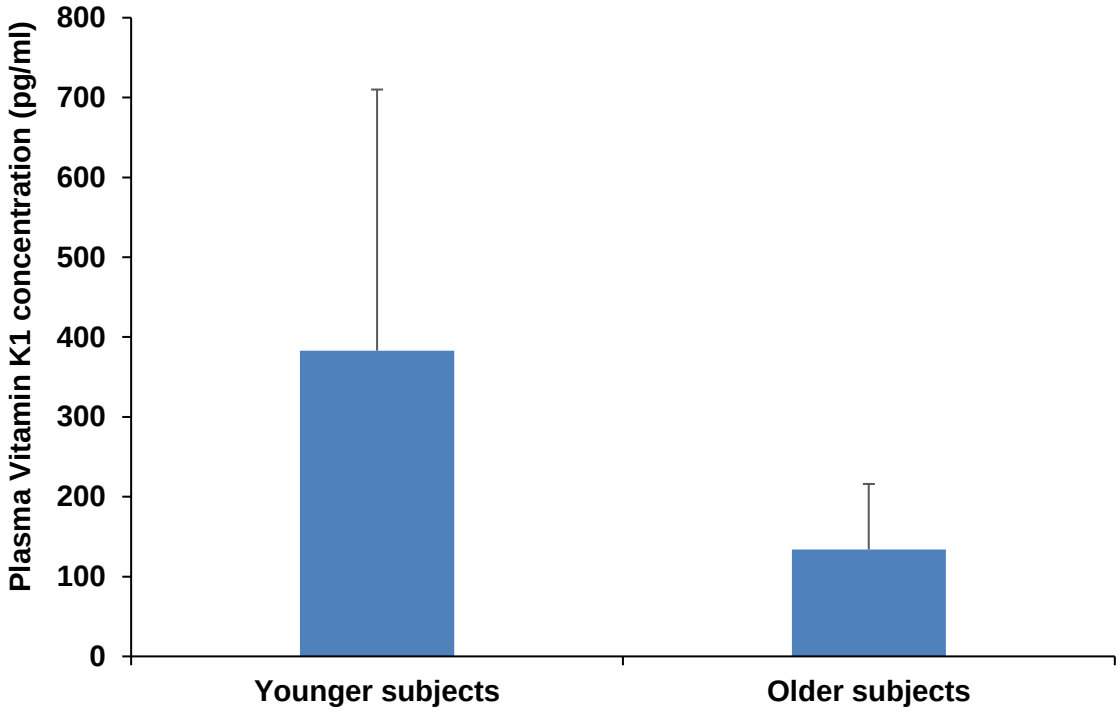


Figure 4-11: Mean plasma vitamin K concentration in the older and younger subjects

4.4.2. Haematological measurements

Rivaroxaban concentration-dependently prolonged PT and mPT in both younger and older subjects, as shown in Figure 4-12. Rivaroxaban caused a significantly higher prolongation of PT and mPT ($p<0.01$ for both) in older subjects compared to younger ones across the rivaroxaban concentration range studied. The mean difference in PT between the two groups ranged from 1.8s to 5.0s at 100 ng/ml and 500 ng/ml plasma rivaroxaban concentrations respectively. The mean difference in mPT between the two groups ranged from 10.0s to 34.0s at 100 ng/ml and 500 ng/ml plasma rivaroxaban concentrations respectively.

The baseline FXa activity was significantly lower (P=0.005) in the older subjects compared to the younger ones, whereas the reverse was the case for FIXa (P=0.01), as shown in Figure 4-13. According to the biogenic assay there was a significantly higher level of functional FX antigen in the younger group compared to the older group of subjects (96.2±8.6% v 85.3±18.1%, P=0.005). However, there was no significant difference in functional F IX antigen levels between the two cohorts. In both older and younger subjects, factor Xa and IXa activity was reduced by rivaroxaban in a concentration-dependent manner; the mean cumulative difference from baseline in factor Xa activity was not significantly different between the two groups. However, rivaroxaban at 500 ng/ml resulted in a significantly greater cumulative difference from baseline in factor IXa activity in the older subjects compared to the younger subjects (96.17 ±19.01 v 85.30 ±15.61; p= 0.02).

There were no significant differences between the two groups in factor II and VII activity, as shown in Figure 4-14; or APTT, or fibrinogen concentration, as shown in Figure 4-15, both with and without rivaroxaban.

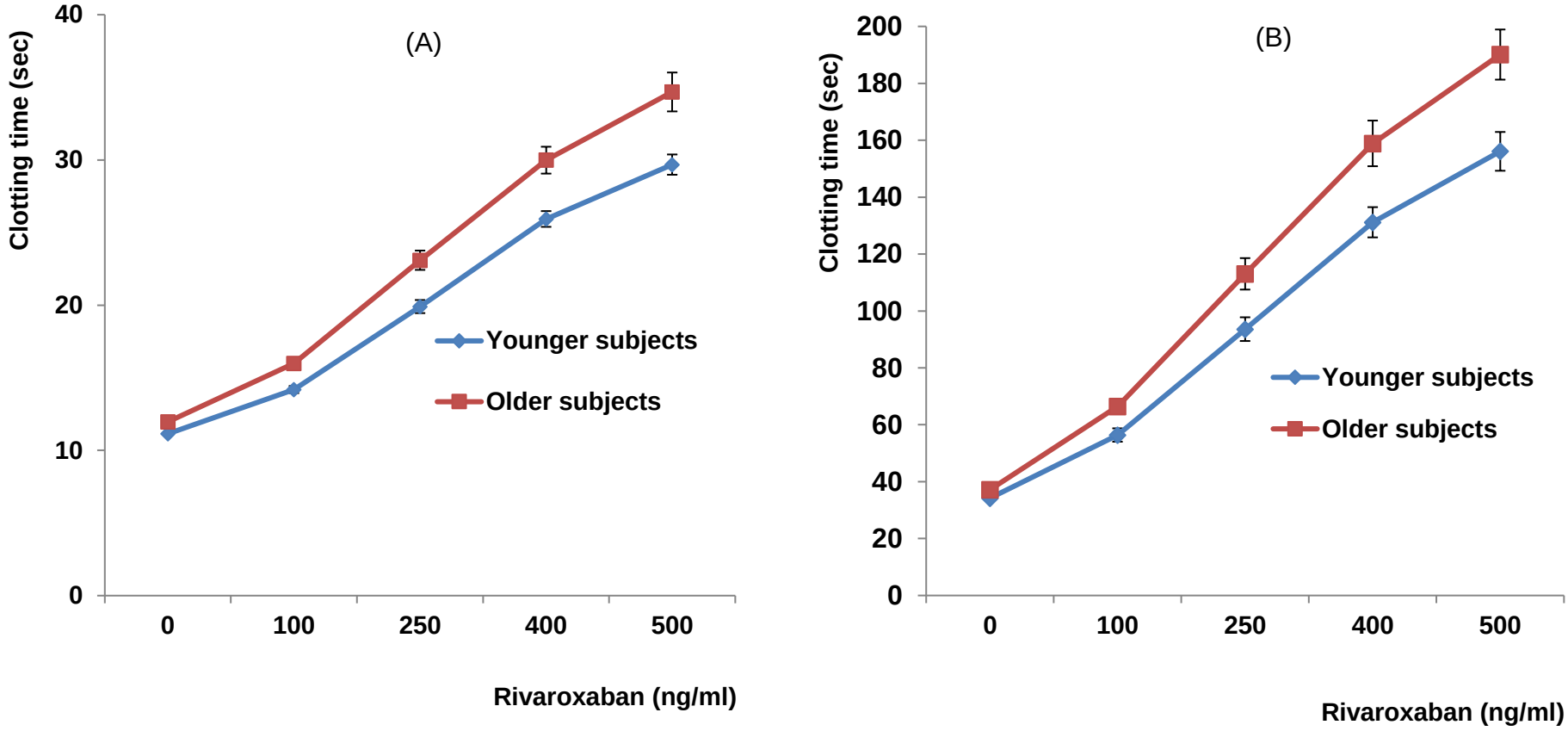


Figure 4-12: The effect of rivaroxaban on mean PT in the younger and older subjects (A), and on mean mPT (B)

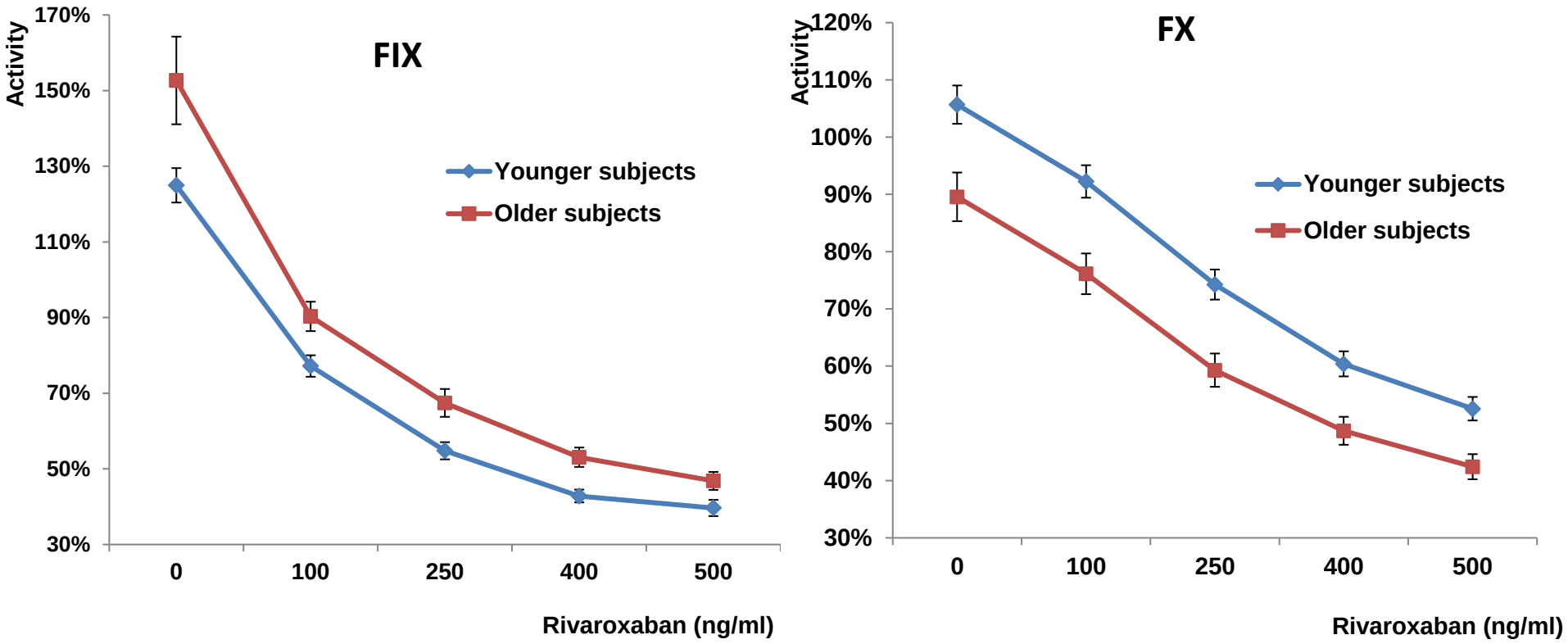


Figure 4-13: The effect of rivaroxaban on mean Factor IX and X mean activity in the younger and older subjects

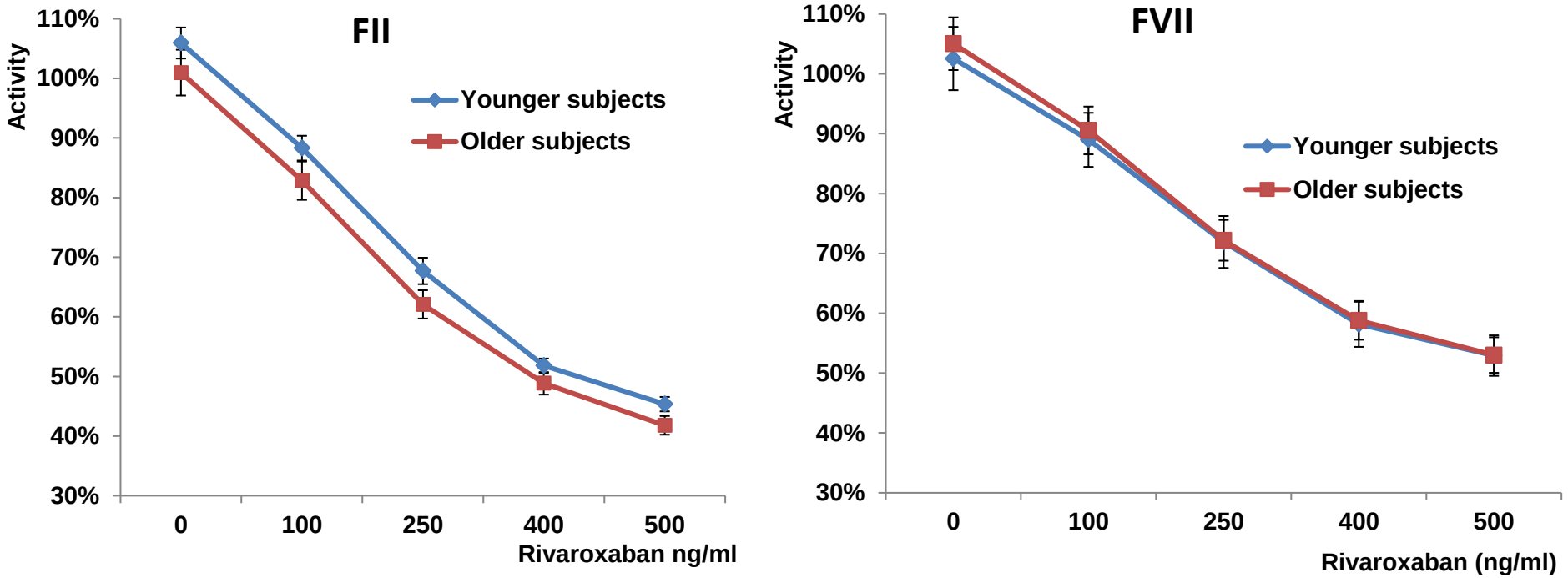


Figure 4-14: The effect of rivaroxaban on Factor II and VII activity in the younger and older subjects

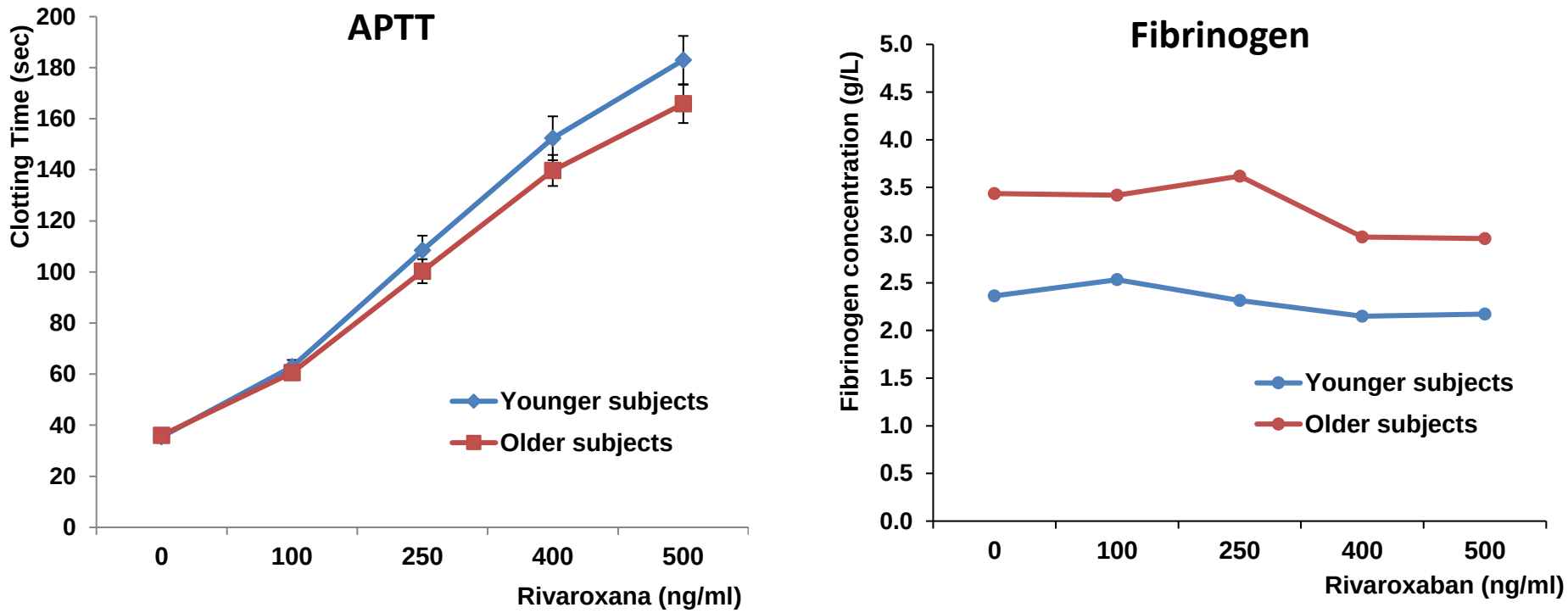


Figure 4-15: The effect of rivaroxaban on APTT and fibrinogen in the younger and older subjects

4.4.3. Thrombin Generation Assay (ETP)

At baseline, younger subjects had a significantly lower lag time (P<0.0001), tt peak (P<0.0001), and significantly higher mTGR (P<0.001) compared to the older subjects. However, there were no significant differences in peak and ETP between the two cohorts Table 4-1.

In both younger and older subjects rivaroxaban (200ng/ml) significantly affected the key parameters of the thrombin generation assay, Table 4-1. Rivaroxaban caused a significantly greater prolongation of the initiation phase of thrombin generation, as demonstrated by a greater prolongation of lag time, in the older subjects compared to the younger subjects (P<0.0001). Rivaroxaban also delayed and suppressed the propagation phase of thrombin generation to a greater extent in older subjects compared to younger ones, as shown by a prolongation of ttPeak (P<0.001) and decreases in both peak (P=0.001), ETP (P=0.001) and mTGR (P=0.001), Table 4-1.

Table 4-1: Thrombin generation assay parameters in human HPPP ((Mean±(SEM))

	Group	Lag time (min)	Peak (nM)	ttPeak (min)	ETP (nM.min)	mTGR (nM/min)
Baseline	Younger subjects	2.83 (0.10)	304.12 (11.27)	5.17 (0.15)	1768.66 (83.21)	133.44 (6.86)
	Older subjects	4.05 (0.18)	274.90 (11.43)	6.85 (0.21)	1836.55 (71.22)	102.06 (5.76)
Rivaroxaban 200 ng/ml	Younger subjects	10.83 (0.49)	93.16 (8.00)	22.47 (1.00)	1860.27 (111.14)	9.25 (1.20)
	Older subjects	16.76 (1.07)	63.82 (4.25)	29.33 (1.19)	1479.23 (78.30)	5.62 (0.53)

4.5. Discussion

Whilst the non-inferiority of NOACs to warfarin has been established in clinical trials without the need for routine laboratory measurement of clotting factor activity, no clinical comparisons between patient cohorts have been performed, and it is unlikely that such information will become available in the near future. It is of concern that very old and malnourished people were not well represented in the clinical trials, and clinical experience is too short to provide models of use which optimise the benefits of therapy and ensure therapy can be safely prescribed.

A meta-analysis of randomized trials has indicated that in patients with renal insufficiency, the recommended doses of NOACs are non-inferior and relatively safe compared to conventional anticoagulants [345], which indicates that the increase in bleeding risk suggested by adverse drug event reporting and case reports may be being contributed to by risk factors of older age and low body weight [298], which may themselves be markers of poor dietary intake in general, and of vitamin K in particular.

As a proof of concept, previously we demonstrated in rats that a reduction in vitamin K intake greatly increased anticoagulation response to the direct thrombin inhibitor, ximelagatran. Briefly, the study results showed that the anticoagulant activity of ximelagatran was significantly greater in rats on a vitamin K deficient diet (by 6.1-fold for PT, 2.6-fold for APTT and 2.9-fold for Ecarin clotting test), in comparison to those on a normal diet. Factor II activity was reduced by ximelagatran to 58% of the control values in rats on normal diet. However, factor II activity was virtually abolished (<0.1%) by the drug in rats on a vitamin K deficient diet [280]. Until now the effect of vitamin K intake in man on the anticoagulation activity of NOACs (direct FXa and FIIa inhibitors) has not been reported. Because of the risk of bleeding it is difficult to examine experimentally the effect of dietary vitamin K on the pharmacological activity of NOACs in man in vivo. To circumvent this we instead examined ex-vivo the pharmacological activity of rivaroxaban in older subjects with poor dietary vitamin K intake in comparison with healthy younger subjects with adequate dietary vitamin K intake.

To determine the effect of vitamin K deficiency on the anticoagulant effect of rivaroxaban, we performed the clotting time assay, PT and the global coagulation assay, thrombin generation assay. PT measures the time taken to initiate clot formation

after the stimulation of the extrinsic coagulation pathway. In contrast, the thrombin generation assay measures not only the time to initiate thrombin generation but also the amount of thrombin formed after stimulation with tissue factor.

Rivaroxaban produced a greater prolongation of prothrombin time (both PT and mPT) in older subjects than in the younger subjects. Currently, there is no specific assay with which rivaroxaban activity can be assessed even though it prolongs all coagulation assays [253, 255]. However, RecombiPlasTin 2G® used in this study appeared to show a good correlation between clotting times and plasma rivaroxaban concentrations as previously indicated [252].

The study results showed that the older subjects had a lower plasma baseline FXa activity compared to the younger subjects and that this was attributable to lower levels of FXa antigen. The inhibition of FXa activity by rivaroxaban was similar between the two groups as expected. However, the net FXa activity in the presence of rivaroxaban was significantly lower in the older subjects because of their lower baseline activity. Paradoxically, baseline FIXa activity was significantly greater in the older subjects compared to the younger ones, which is consistent with the previously reported effect of ageing on FXa activity [346].

Rivaroxaban prolonged time-based parameters of thrombin generation (lag time and ttPeak) and suppressed the rate and amount of thrombin generation (mTGR, peak, and ETP) to a significantly greater extent in older subjects compared to younger ones.

The current study results support our hypothesis that consuming low amounts of vitamin K can affect the functional levels of FX, and as such, it enhances the pharmacological activity of the factor Xa inhibitor, rivaroxaban. Our observation of enhanced anticoagulant response to rivaroxaban in plasma from older malnourished subjects with low vitamin K intake ex-vivo indicates the importance of identifying factors which influence the pharmacodynamics of these drugs through further study in frail, vitamin K deficient older people in vivo, to establish whether the therapeutic outcomes of using fixed doses without laboratory guidance supported by randomized trial results could be improved upon by laboratory-guided dose adjustment in this cohort.

We purposefully examined the effect of poor dietary vitamin K status on the pharmacological activity of rivaroxaban in a group of older subjects, who in general had similar characteristics to those of the older population with AF requiring

anticoagulation therapy. The two study groups differed in age, co-morbidities, drug therapy and dietary vitamin K intake. However, none of the older subjects used any drug which affects haemostasis or interacts with rivaroxaban mode of action. The age difference and the presence of comorbidities are unlikely to have contributed to the significant potentiation of the effects of rivaroxaban in older subjects ex-vivo given that baseline clotting parameters (PT and mPT) in the older subjects were similar to those of the younger subjects.

The amount of vitamin K consumed by the older subjects was less than a third of that of the younger subjects. The differences in vitamin K dietary status between the two cohorts of subjects were further confirmed through the measurement of plasma vitamin K concentrations. The observed highly significant correlation between the estimated vitamin K intake according to the FFQ and plasma vitamin K concentrations indicates that older patients with vitamin K deficiency, and therefore potentially increased sensitivity to rivaroxaban, could be identified in the clinic through the use of the FFQ alone without the need for using a complicated assay to measure plasma vitamin K concentrations.

The poor nutritional status of the older subjects established through the FFQ in the current study is consistent with reports that older people living in Scotland and northern England consume significantly lower amounts of foods containing vitamin K, averaging under 60 µg per day compared to those living in southern England at around 80 µg per day [330, 347]. Moreover, older people living in private houses consume higher amounts of vitamin K containing foods, with those in residential and nursing homes having significantly lower vitamin K levels than free-living British people [331]. It is of concern that older patients requiring anticoagulation therapy were not well represented in the ROCKET-AF trial; the median age and BMI of the patients on rivaroxaban in that trial were 73 years and 28.3 (IQR 25.2-32.1) respectively with a median creatinine clearance of 67 (IQR 52-88) ml/min. Only “less than a quarter” of the patients in ROCKET-AF trial were over 80 years of age or older and a quarter of patients had a BMI <25.0 [222]. In contrast 84% of our patients were over the age of 80, and 81% weighed less than 60 kg (BMI median 19.5 (IQR 16.8-22.5). Because of the limitations of extrapolating trial data based on a younger heavier population into the older, frailer population with low body weight, in particular because of the risk of a major bleed,

clinicians must make a careful evaluation of the risk and benefits of treatment before starting rivaroxaban.

4.6. Conclusion

The study suggests possible augmentation of the rivaroxaban anticoagulation response in older patients with poor dietary vitamin K intake. In-vivo studies to evaluate this further are required.

Polymorphisms in the genes for the vitamin K reductase (the main target for warfarin) and CYP2C9 (the main metabolizing enzyme for S-warfarin) have been shown to influence warfarin sensitivity and dose requirement. However, it is not clear whether genetic polymorphism in these genes in association with diet influence stability of anticoagulation control, or whether the polymorphisms influence the speed by which anticoagulation declines following cessation of warfarin as is required prior to conducting surgical procedure in warfarinised patients. I therefore undertook further studies (which are described in chapters 5-7 of my thesis to address these concerns.

CHAPTER. 5 VKORC1 (-1639) POLYMORPHISMS DO NOT AFFECT LONG-TERM STABILITY OF ANTICOAGULATION WITH WARFARIN

5.1. Introduction

In man, vitamin K is obtained primarily from the diet in the form of phylloquinone which are found in greatest concentration in green leafy vegetables [334], intake of which varies from day to day. Generally people enjoy having varied diets over different seasons of the year. The amount of dietary intake of vitamin K influences warfarin sensitivity and variability in INR [348]. Patients with poor dietary vitamin K intake are more susceptible to fluctuations in anticoagulation control [349], being more sensitive to day to day changes in vitamin K intake because of limited liver stores to stabilise the production of the functional clotting factors II, VII, IX and X, for which vitamin K is an essential co-factor [111]. Warfarin reduces the regeneration of vitamin K hydroquinone from vitamin K 2,3-epoxide by inhibiting vitamin K epoxide reductase (VKOR) in the vitamin K cycle [326]. The VKOR enzyme is expressed by the vitamin K epoxide reductase gene, *VKORC1*, in which a number of common polymorphisms have been identified. These polymorphisms, which include a *G>A* polymorphism at *VKORC1* position *-1639* of Intron 1, are associated with altered hepatic VKOR expression and lower warfarin dose requirements [149, 350].

Stability of anticoagulation control can be improved by daily vitamin K supplementation [348, 351], although an initial increase in warfarin dose may be required because of the antagonist effect of vitamin K on the pharmacological activity of warfarin. This varies between different patients and is related to *VKORC1-1639G>A* genotype, with those carrying the *GG* genotype demonstrating a significantly larger fall in INR, and requiring a significantly greater increase in warfarin dose, by an average of 33%, compared with 11% for those carrying the *GA*, and <1% for those with the AA genotype, following vitamin K supplementation [352]. This is because patients with *GG* genotype are more efficient at converting vitamin K epoxide to vitamin K, leading to a greater regeneration of vitamin K hydroquinone and subsequently a larger increase in the activation of vitamin K dependent clotting factors compared to patients with the *GA* and *AA* genotype [352].

5.2. Aim of the study

Based upon the above observation, it was hypothesized that patients on warfarin therapy with the *VKORC1(-1639)GG* polymorphism could have less stable control of anticoagulation than those with *GA* or *AA* genotypes as a result of variable dietary intake of vitamin K. We set out to examine this possibility in a cohort of patients on chronic warfarin therapy.

5.3. Materials and Methods

The INR values and dose changes of atrial fibrillation patients taking warfarin who had attended the Newcastle upon Tyne Anticoagulation Monitoring Service for at least 18 months for INR testing and dosage advice by staff guided by the DAWN dosing programme [295], and had *VKORC1–1639G>A* established in our previous studies of effect of *VKORC1* on warfarin dose requirements, were analysed. Since patients commencing warfarin require an initial dose titration period following a loading dose regimen to reach anticoagulation stability, the first six months from the time of warfarin commencement were excluded and the subsequent 12 months data analysed. TTR was calculated by the method of Rosendaal et al [53]. The study was approved by the Regional Ethics Committee and was in accordance with the Helsinki Declaration of 1975.

The software used, and INR data entry to calculate TTR were as mentioned in section 2.3.1 page 85.

5.3.1. Statistical Analysis

Statistical analysis was conducted by Statistical Package for Social Sciences version 21 (SPSS). One way analysis of covariance (ANCOVA) was used to examine the difference in TTR, number of INR measurements, mean dose, and number of dose changes among the three *VKORC1* variants (*GG/GA/AA*) taking into account the effect of age and sex covariates.

5.4. Results

234 patients [106 (45%) female] were included in the study. The prevalence of *VKORC1 AA*, *GA*, and *GG* variants was 18.4%, 56.4%, and 25.2% respectively. The mean age ± SD between *AA*, *GA*, and *GG* variants groups were similar, 64±18, 63±15, and 63±13, respectively. No statistical differences were observed between the three variants (*GG GA AA*) in mean TTR (66%, 61%, 68%, respectively), mean number of INR monitoring events (12.4, 13.5, 13.0, respectively), and mean dose changes (3.9, 4.0, 3.3, respectively). Similarly there were no significant differences in these measurements between *AA* and *GG+GA* genotypes. However, patients with *GG* variant required a significantly higher mean warfarin dose compared to those with *GA* variant (4.0 mg v 3.3 mg; P=0.02) with a mean dose difference of 0.7mg. However, no difference in dose requirement was observed between *AA* (mean dose=3.2 mg) and *GA* variants and between *AA* and *GG* variants. No influence of age and sex covariates was detected.

5.5. Discussion

Identifying and controlling factors which contribute to intra-individual variability in response to vitamin K antagonists would improve the average time patients stay within their therapeutic INR range, lowering the risk of bleeding or thromboembolic complications associated with over and under-anticoagulation, helping individual patients, especially the very elderly, who have difficulty attending for monitoring and receiving dosing instructions [292]. Polymorphisms in *CYP2C9* and *VKORC1* are relevant to this in clinical practice as both contribute to the inter-individual variability in coumarin dose requirement and stability of anticoagulation control during the initiation phase of therapy [43]. Excessive anticoagulation occurs more frequently in the carriers of *CYP2C9*3* variants (slow S-warfarin metabolisers) at the initiation of treatment and carriers have a lower TTR than those with the wild type *CYP2C9* genotype [353, 354]. Compared to the *VKORC1-GG* genotype, the *GA* genotype results in less expression of *VKOR* enzyme, so that a lower dose of vitamin K antagonist is needed to achieve the same anticoagulant effect [353]. Our results, which show that *GG* genotype patients required a significantly higher mean warfarin dose compared to *GA* genotype patients, is consistent with this observation. Results of a study which employed a

questionnaire assisted estimation to identify patients with low, medium and high vitamin K intake indicated that the influence of vitamin K intake on warfarin anticoagulation might be *VKORC1* genotype dependent as, in subjects with a variant *VKORC1 – 1639G* allele, the mean daily dose of warfarin was significantly attenuated by low vitamin K intake compared with medium and high whilst no such effects were observed in homozygous patients for the *VKORC1-1639A* allele [352, 355]. As retrospective data were used for this study, limitations for the study include lack of information on patients' comorbidities and concurrent drug therapy, weight and a direct assessment of dietary vitamin K consumption; in consequence, any correlation between warfarin dose requirement and vitamin K consumption could not be evaluated. However, these appear not to have made any significant contribution within the context of this study as the results revealed no difference in % TTR between the three *VKORC1* variants. It should be noted that the main objective of the current study was to investigate the long-term stability of anticoagulation control in association with *VKORC1* polymorphism with the background of a variable diet. Therefore polymorphisms in genes which either affect warfarin (*CYP2C9*) or vitamin K metabolism (*CYP4F2*) would have no effect on anticoagulation control in patients who are on chronic maintenance warfarin therapy.

5.6. Conclusion

The study results, which failed to confirm the hypothesis that longterm anticoagulation control is affected more in patients with the *VKORC1(-1639)GG* variant, in association with the expected variability in dietary vitamin K, indicate that the effect of any such influence at the pharmacological level is too small to influence clinically relevant outcomes in our population. However, it remains possible that a larger prospective study, in which dietary intakes of vitamin K were measured, might yet establish a statistically significant link between diet, *VKORC1* polymorphism and anticoagulation control.

CHAPTER. 6 INFLUENCE OF CYP2C9 POLYMORPHISM ON THE FALL IN INTERNATIONAL NORMALIZED RATIO IN PATIENTS INTERRUPTING WARFARIN THERAPY BEFORE ELECTIVE SURGERY

6.1. Introduction

Warfarin is prescribed as a racemic mixture of R- and S- enantiomers, with the S-enantiomer being approximately 3-5 times as potent as the R- enantiomer [356]. S-warfarin is hydroxylated by the CYP2C9 enzyme to its inactive metabolite [356]. Mutations in the CYP2C9 gene result in the expression of two common allelic variants, *CYP2C9*2* and *CYP2C9*3*. Both CYP2C9*2 and CYP2C9*3 enzymes exhibit reduced catalytic activity compared to the wild-type CYP2C9*1 enzyme, to 12% [357] and 5% respectively [358]. 30% of Europeans carry at least one of these variant alleles [357], with heterozygotes for CYP2C9*2 requiring on average almost 2 mg/day and CYP2C9*3 heterozygotes requiring almost 2.3 mg/day less warfarin to achieve therapeutic anticoagulation, than those without a variant allele [141].

S-warfarin elimination half-life ranges from ~28 h to ~118 h depending on *CYP2C9* genotype [359]. In recognition of this, genotype-guided dosing algorithms have been introduced to improve the accuracy and efficiency of warfarin dosing during the initiation of therapy [179, 360, 361]. Patients on anticoagulation therapy with warfarin who are due for invasive surgery are required to withdraw from the drug for a recommended period of 5 days to minimise risk of perioperative bleeding. It can also be predicted that normalisation of coagulation after interruption of warfarin could be more than the expected recommended 5 days before elective surgery in patients carrying the *CYP2C9* variant alleles [88]. Several non-vitamin K antagonist oral anticoagulants (NOACs) have recently been approved which offer alternatives to warfarin and knowledge of how long to withdraw warfarin is important not only for patients undergoing planned surgical procedures, but also for when transitioning patients from warfarin to one of these drugs.

6.2. Aim of the study

To establish whether there is a clinically relevant genetic influence upon INR decay and therefore a potential role for a genotype-based personalised algorithm to predict the time required for dissipation of anticoagulant effect of warfarin after its withdrawal.

6.3. Materials and Methods

6.3.1. Patients and materials

The study had approval from the South West Frenchay Research Ethics Committee. All patients provided written informed consent according to the Declaration of Helsinki. Patients aged ≥ 18 years, receiving warfarin therapy for venous thromboembolism or atrial fibrillation, with a target INR range of 2.0–3.0, who were to interrupt warfarin therapy prior to elective surgery according to our local hospital protocol, were identified through the surgical preadmission clinic in the Newcastle upon Tyne Hospitals NHS Foundation Trust and invited to take part. Exclusion criteria were having a chronic condition that might affect warfarin metabolism, especially hepatobiliary disease, having acute illness prior to surgery, and taking any medication or excess alcohol intake that could have affected the anticoagulation response. After informed consent had been obtained, demographic data on age, height, weight, gender, daily warfarin dose, indication for anticoagulation therapy, medical diagnosis, surgical operation planned and concomitant medication were recorded. A blood sample was taken for later CYP2C9 genotyping [362].

The current local hospital protocol is to withhold warfarin for 5 days before any high-bleeding-risk invasive procedures can take place. For elective procedures, the patient's INR is checked 1 day before the procedure, and, if the INR is ≥ 1.5, 0.5 mg of oral vitamin K may be given, and the INR checked again on the day of the procedure. The INR at the pre-assessment clinic and on the day before planned surgery were recorded, as was whether vitamin K was given to reverse anticoagulation.

6.3.2. Sample size calculation

Approximately two-thirds of the general population have the *CYP2C9*1*1* genotype, and one-third have at least one *CYP2C9*2* or *CYP2C9*3* allele. If the proportion of patients having an INR of > 1.5 is 0.05 among the *CYP2C9*1*1* patients and 0.25 among those with at least one *CYP2C9*2* or *CYP2C9*3* allele, then a study of 150 patients will have an approximately 80% power for detecting a difference in the incidence of having an INR of ≥ 1.5 between the two groups.

6.3.3. Statistical analysis of data

IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, NY, USA) was used to carry out statistical analysis. The proportions of individuals with the *CYP2C9*1*1* genotype and either *CYP2C9*2* or *CYP2C9*3* variant allele were compared with the proportion of individuals with two variant alleles for those with an INR of < 1.5 versus ≥ 1.5 on the day before planned surgery, by use of the chi-square test. One-way ANOVA was used for comparison of continuous variables. Mean warfarin daily doses were transformed by taking the logarithm of each value to obtain a normal distribution, thus allowing for parametric tests to be performed. The effects of genotype and other variables on the fall in INR were investigated with both logistic regression and multiple regression models. Data are presented as mean ± standard deviation, unless stated otherwise. A *P*-value of < 0.05 was considered to be statistically significant.

6.3.4. Samples preparation and genotyping assay

6.3.4.1. DNA extraction from whole blood

Sodium ethylene diamine tetra acetic acid (EDTA) tubes were used to collect blood samples, and were stored at -80ºC until DNA extraction by using a modified method described by Daly et al [363]. Initially, preparation of nuclei was done by adding 1 ml of blood to 4 ml of cell lysis buffer (10mM Tris-HCL, 320 mM sucrose, 5mM $MgCl_2$, 1% Triton X, pH8) followed by mixing gently for 2 minutes, and then centrifugation at 3000 for 10min at 4º C. After discarding the supernatant, the remaining pellet was re-suspended with 350 µl of nuclear lysis buffer (400mM Tris-HCl, 60 mM EDTA, 150 mM

NaCl, 1% Sodium Lauryl Sulphate, pH8) and finally 0.5 ml (5M) of sodium perchlorate was added to denature plasma proteins. The resulting suspension was rotary mixed for 15 minutes and then incubated for 30 minutes at 65ºC. 0.5 ml of chloroform was added and the mixture was rotary mixed for 10 minutes followed by centrifugation at 1500 for 5 minutes. The aqueous uppermost DNA-containing phase was transferred to a new tube and 1ml of ethanol was added. The DNA was precipitated out of the solution by immediately inverting the tube, and then centrifuged at 14000 for 3 minutes. The ethanol was discarded gently and the DNA was allowed to dry at room temperature for 10-15 minutes. Finally, the DNA was re-suspended with 75 µl 5mM Tris buffer, pH8, and incubated overnight at 60ºC, and subsequently stored at 4 ºC until required.

6.3.4.2. CYP2C9 Genotyping using TaqMan assay

All samples were genotyped for *CYP2C9 *2* and **3* alleles separately using TaqMan® SNP genotyping assay manufactured by Applied Biosystems. Each TaqMan® SNP genotyping assay is composed of a specific, ready-to-use tube which contains (a) 2 sequence-specific primers for amplifying the polymorphism of interest, and (b) TaqMan MGB allele-specific probes for identifying the alleles for the specific SNP of interest. The assays are designed as 20X single tube mixtures (187.5 µl) of forward and reverse primers (900 µM) in addition to two reporter probes (200 µM). The 5' end of each probe is connected to different fluorescent allele-specific dyes; a Fluorescein amidite (FAM) is allele 2 specific while VIC is the reporter for allele 1. The 2X TaqMan universal PCR MasterMix® (Applied Biosystems) used encompasses AmpliTaq Gold® DNA polymerase, dNTP and passive internal reference based on proprietary ROX dye.

20 µl of either *2 or *3 probes was added to 250 µl of universal MasterMix®, and mixed properly by vortexing. Using a (8X6) 48-well plate, 5µl was transferred from the mixture into each of the required wells, followed by 5µl (=50ng) from each diluted DNA sample. Control samples (positive and no template controls) were used in each plate also. The plate was covered with a sticker, and centrifuged at 100 rpm for one minute, and finally analysed using the One Step Applied Biosystems real time PCR machine. PCR temperature was retained on hold for 10 min at 95 °C then decreased to 92 °C

for 15 sec (denaturation) and further decreased in annealing and extension stages to 60 °C for 1 min each for 40 cycles.

The allelic fluorescence detection system which determines and plots different fluorescence signals on a partitioning chart (X or Y axis) is linked to the StepOne® software v2.1. Upon finishing the experiment, distinct groups with different colours (red, green, or blue) are usually displayed with wide separation based on their defined genotypes in an allelic discrimination plot. Signals which located horizontally to the end side towards the X axis was described as homozygous of one allele (XX); signals which located upward vertically towards the Y axis were represented homozygosity for the other allele (YY) whereas those which moved in between the X and Y axis showed the heterozygous genotype (XY), as shown in Figure 6-1.

As two CYP2C9 variants (*2 and *3) were examined for each subject, the result from each variant was combined with the other, resulting in combinations mentioned in Table 6-1.

Table 6-1: Subjects' genotypes according to CYP2C9 *2 and *3 variants results

Subject	*1	*2 Variant	*3 Variant	Genotype
1	+ve	-ve	-ve	*1*1
2	+ve	-ve	+ve	*1*3
3	+ve	+ve	-ve	*1*2
4	-ve	+ve	+ve	*2*3
5	-ve	+ve	-ve	*2*2

N.B. No CYP2C9 *3*3 genotype was detected in our study population

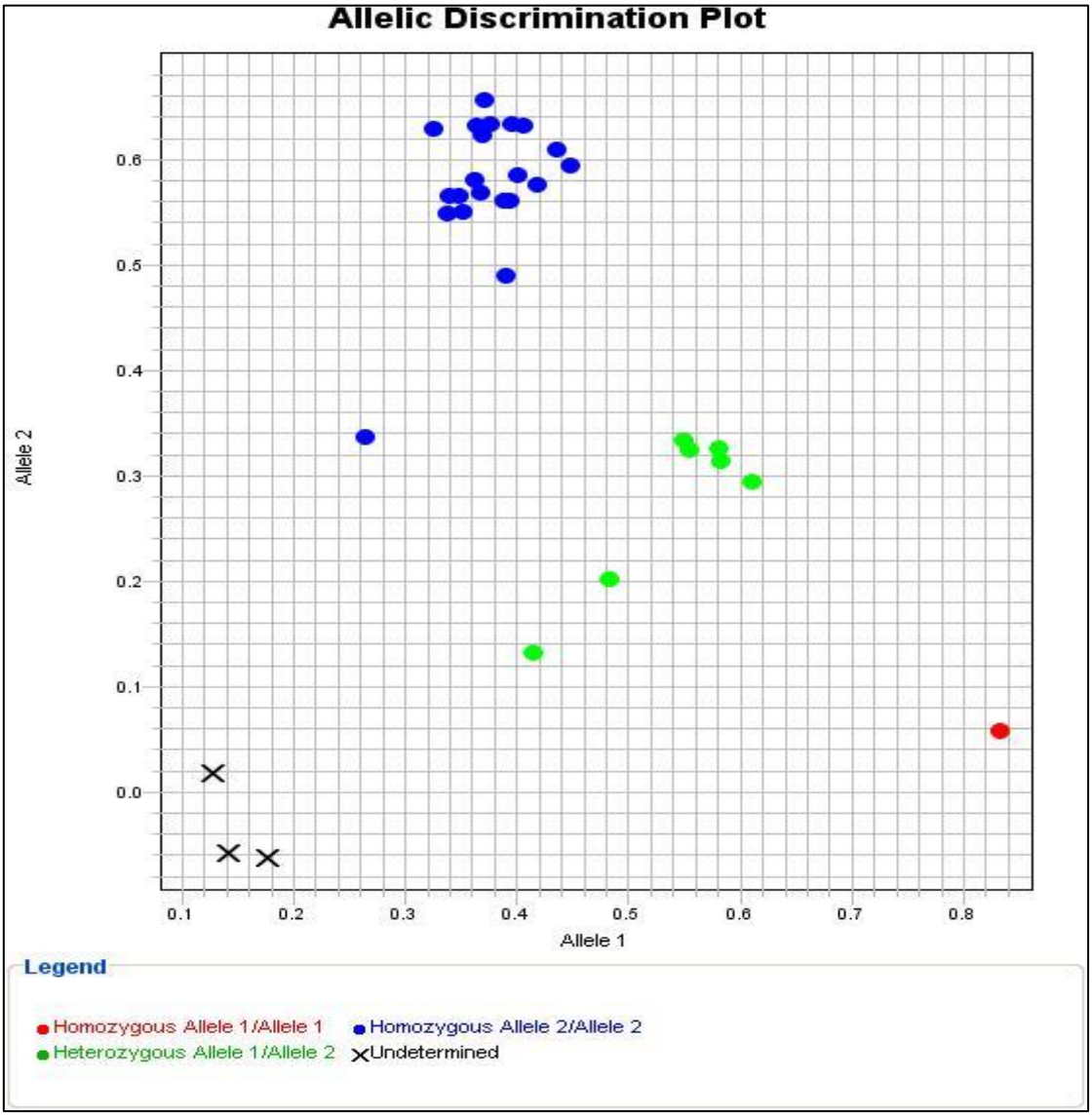


Figure 6-1: Allelic discrimination plot of TaqMan SNP genotyping assay. Blue cluster is homozygous wild type (*1*1 = XX), red cluster is homozygous mutant (*2*2 or *3*3 = YY), and the green one is heterozygous (*1*2 or *1*3 =XY). Black cluster shows the no template control and samples that failed to amplify.

6.4. Results

One hundred and fifty-two patients (50 [33%] females, and 102 [67%] males) were studied. Patient characteristics, reasons for anticoagulation and type of surgery are shown in Table 6-2.

Table 6-2: Patient characteristics, reasons for anticoagulation and type of surgery undergone

	Male	Female
N (%)	102 (67)	50 (33)
Age (years) [mean (range)]	74 (43-90)	72 (49-93)
Weight (Kg) (mean±SD)	88±17	81± 19
Warfarin weekly dose (mg) (mean±SD)	28.7±10.3	27.4± 14.0
Indication for anticoagulation	N	%
Atrial fibrillation	125	82
Venous thromboembolism	27	18
Type of Surgery	N	%
Musculoskeletal	58	38
Genitourinary	55	36
Renal	17	11
Cardiovascular	11	7
ENT	8	5
Other	3	2

The patients were of white British ethnic background, had a median of two chronic diseases (interquartile range [IQR] 1–3), one of which was a cardiovascular disease, and received chronically a median of four drugs (IQR 3–6); three cardiovascular drugs, including warfarin, and one from another pharmacologic class. None of the concurrently taken drugs was deemed to interfere with warfarin disposition or its pharmacologic activity, as per the study exclusion criteria.

The proportion of individuals with *CYP2C9* genotypes, their INR status before surgery and whether they received vitamin K are shown in Table 6-3. In total, INRs were ≥ 1.5 by day 5 of warfarin interruption in 27 patients. Of these, 14 (52%) were given vitamin K on day 5, according to surgical preference. For 10 patients, the INR was < 1.5 on the following day, and they underwent surgery; for four patients, surgery was postponed. Haemoglobin levels were significantly lower in the patients after surgery (mean difference of 1.54 g dL^{-1}, P < 0.0005), with no difference being seen between the groups who did and did not receive vitamin K, and no patient in the latter group had a clinically significant bleed.

Table 6-3: The proportion of individuals with *CYP2C9* variant alleles, their INR status before surgery, and whether they received vitamin K

Genotype	Status of INR		Total N(%)	% INR ≥ 1.5	Patients with INR ≥1.5 who received vitamin K N(%)
	<1.5 (N)	≥1.5 (N)			
*1*1	78	14	92 (61)	15	6 (43)
*1*2	30	5	35 (23)	14	4 (80)
*1*3	13	2	15 (10)	13	1 (50)
*2*2	1	3	4 (3)	75	0 (0)
*2*3	3	3	6 (4)	50	3 (100)
Total	125	27	152	18	14 (52)

A forward selection and backward elimination logistic regression analysis indicated evidence of a significant relationship between genotype and INR status on the day before surgery (logistic regression chi-square = 9.74, d.f. = 1, P = 0.002). For those with two *CYP2C9* variant alleles (*CYP2C9*2*2* or *CYP2C9*2*3*), the odds of having an INR of ≥ 1.5 on the day before planned surgery were 8.64 times greater (95% confidence interval [CI] 2.25–33.25) than for other patients. There was no significant correlation between warfarin dose and the probability of an INR of ≥ 1.5, or < 1.5, after withdrawal, and no influence of other variables on the fall in INR was detected with the logistic regression model. Multiple regression analysis revealed that the rate of the fall in INR was significantly influenced by *CYP2C9*, age, weight, index INR value (the INR at the preadmission clinic), and number of comorbidities ($F_{5,132}$ = 242.9, P < 0.0001), which accounted for ~ 90% of the interindividual variability in the fall in INR, as shown

in Table 6-4. The results show that the rate of the fall in INR is reduced in the presence of two *CYP2C9* variant alleles, as well as increasing age, weight, and number of comorbidities, and is increased with increasing index INR.

As expected, patients with two variant alleles (*CYP2C9*2*2* or *CYP2C9*2*3*) required significantly lower mean weekly warfarin doses (20 ± 6.2 mg; $F_{2,138}$ = 3.7, *P* = 0.03) than those with homozygous *CYP2C9*1*1* alleles (29.4 ± 10.4 mg), with those with a single variant allele, i.e. *CYP2C9*1*2* or *CYP2C9*1*3* (28.0 ± 13.9 mg) being intermediate. The algorithm for the rate of INR decline is as follows:

$$INR\ decline = -0.195 - 0.00428\big(Age(years)\big) - 0.2374(CYP2C9\ double\ variant) + 0.9143\ (INR\ vlaue\ 5\ days\ before\ surgery) - 0.00246\ (Weight\ (kg)) - 0.0306\ (number\ of\ comorbidities)$$

Table 6-4: Summary statistics and results from multiple regression analysis

					95% CI	
Term	**Estimate**	**Std. Error**	**t**	**P-value**	**Lower**	**Upper**
(Constant)	-0.195	0.234	-0.84	0.4040	-0.616	0.238
Age	-0.00428	0.00211	-2.03	0.0450	-0.008	-0.001
CYP2C9 variants	-0.2374	0.0680	-3.49	0.0010	-0.365	-0.103
Weight (Kg)	-0.00246	0.00102	-2.42	0.0170	-0.004	-0.001
Number of comorbidities	-0.0306	0.0130	-2.36	0.0200	-0.048	0.000
Index INR	0.9143	0.0291	31.37	<0.0001	0.851	0.958

CI=confidence interval; INR=International Normalized Ratio. R^2 = 89.9%, adjusted R^2 = 89.5%

6.5. Discussion

Coagulation returns to normal over several days after warfarin withdrawal. In consequence, for patients on chronic therapy with warfarin awaiting invasive surgery, guidelines suggest that warfarin is stopped 5 days before the event to minimise the risk of perioperative bleeding [88]. Whilst evidence suggests that INR values average 1.24 (95% CI 1.19-1.29) after a five day warfarin interruption [364], some patients have an INR >1.5 on the day of planned surgery.

In one study of 22 patients with a mean steady-state INR of 2.6, although their mean INR 115 hours (4.7 days) after discontinuation of warfarin therapy was 1.1, 5 of the 22 patients (23%) had an INR of 1.2 or greater. In the total cohort, and consistent with our findings, age was a significant independent predictor of smaller decreases in the INR between day 1 and day 3 [365]. Warfarin dose requirements fall with increasing age and there is a significant positive correlation between liver volume and dosage [93]. The age related fall in dose requirements and the slower decline in INR indicating slower warfarin metabolism is explainable by the fall in liver volume with age with resultant fall in the absolute content of the enzymes involved in its metabolism. In a larger study involving 224 outpatients with high risk of arterial embolism (prosthetic heart valve or atrial fibrillation), 15 patients (7%) had a preoperative INR >1.5 five days after warfarin withdrawal and needed vitamin K administration, although the factors influencing this were not investigated [366]. Because some patients, despite withholding warfarin, remain anticoagulated for surgery, many hospitals admit anticoagulated patients on the day before elective surgery to check their INR and administer vitamin K if needed, in order to avoid surgery being postponed. This adds costs to the admission and risks a potentially wasted operation slot if the INR is >1.5 on the day of surgery in spite of vitamin K administration.

A personalized genotype-based approach in routine care could facilitate the ongoing management of patients scheduled for warfarin withdrawal prior to an invasive procedure. This might bring benefits in terms of reducing potential harm caused either by discontinuing warfarin too early (thus predisposing the patient to thrombosis) or stopping it too late (resulting in possible perioperative bleeding).

In recent years several direct oral anticoagulants (NOACs), which selectively inhibit a single clotting factor (factor Xa inhibitions or factor IIa (thrombin) inhibitors), have been developed and approved for the treatment of thrombosis, as well as prevention of stroke in patients with atrial fibrillation (AF) having demonstrated non-inferiority to warfarin in their clinical effectiveness [221, 222, 321]. Prescription numbers of NOACs have grown rapidly since their launch, mainly for newly diagnosed patients requiring initiation of anticoagulant therapy but also for existing patients on warfarin therapy who are switched to a NOAC because of concerns about the safety of warfarin therapy [322-325]. Tailoring the current standard warfarin withdrawal protocols according to each individual patient's *CYP2C9* genotype could bring potential benefit to healthcare

providers in terms of improved delivery of care and patient satisfaction, and reduction in costs, through a reduction in cancellation or delays of planned invasive procedures in patients on warfarin therapy. A genotype-guided protocol could also be beneficial for when transitioning patients from warfarin to one of the new oral anticoagulants.

Polymorphisms in CYP2C9 (encoding CYP2C9, which metabolizes S-warfarin) and VKORC1 (encoding vitamin K epoxide reductase, to which warfarin binds, thereby inhibiting vitamin K recycling in the liver) significantly influence the sensitivity to warfarin and the daily warfarin dose requirement [43]. The study hypothesis was that the rate of fall in the INR following warfarin withdrawal is mainly influenced by polymorphisms in CYP2C9 rather than by polymorphisms in VKORC1. However, because we did not investigate VKORC1 polymorphisms in the study cohort, we are unable to refute or confirm any such influence on the fall in the INR. Although the assumption of 67% of the European general population having the CYP2C9*1*1 genotype was close to the 61% achieved in the study cohort, not all patients with CYP2C9 variant alleles had an INR of ≥ 1.5 and our assumption regarding the probability of an INR of ≥ 1.5 in the different genotype groups turned out to be incorrect.

6.6. Conclusion

This is the first study to establish that rate of fall in the INR on warfarin withdrawal is dependent on CYP2C9 genotype, and that normalization of coagulation takes place more slowly in patients with CYP2C9 mutations, with those with two CYP2C9 variants being more than eight times more likely to have an INR of ≥ 1.5 on the fifth day of warfarin withdrawal. Although the influence of CYP2C9 genotype and age on warfarin elimination is well established, further research is needed to assess whether the relationships noted between the rate of fall in the INR and weight, number of comorbidities and the index INR can be replicated.

CHAPTER. 7 FURTHER EVALUATION OF THE EFFECT OF GENETIC AND PATIENT FACTORS ON INR DECLINE FOLLOWING WARFARIN WITHDRAWAL

7.1. Introduction

In my previous study (0) of the effect of *CYP2C9* genotype upon INR decline, patients with two *CYP2C9* variant alleles (*CYP2C9*2*2* or *CYP2C9*2*3*) were found to be over 8 times more likely (95% [CI] = 2.25–33.25) to have an INR of ≥ 1.5 on the day before planned surgery compared to those with either **1*1*, **1*2* or **1*3* genotypes. However, the possible influence of other factors, including *VKORC1* polymorphism as well as patient and clinical factors, on the rate of INR decay after cessation of warfarin therapy is yet to be determined.

7.2. Aim of the study

To further characterise the contribution of genetic, patient and clinical factors toward warfarin elimination and its changing pharmacodynamics in patients with thromboembolic disease following warfarin withdrawal.

7.3. Patients, materials and methods

The study had the approval of the Newcastle upon Tyne Ethics Committee. All patients provided written informed consent according to the Declaration of Helsinki. 135 Caucasian patients aged over 18 years, completing a course of warfarin for indications of either venous thromboembolism (VTE) or atrial fibrillation (warfarin therapy stopped after successful cardioversion), with a target INR of 2.0-3.0 and stable control of anticoagulation (defined as having a stable warfarin dose for at least the previous 2 clinic visits a minimum of one week apart), were identified through the anticoagulant clinic records at the Newcastle upon Tyne Hospitals NHS Foundation Trust, UK. Any patient with a condition which might affect warfarin metabolism, notably congestive cardiac failure, hepatobiliary or renal disease or active cancer, taking medication known to interact with warfarin, or drinking more than the recommended safe limits in the UK (14 units per week for men and women) [367] was excluded.

Demographic data of age, weight, sex, daily warfarin dose, indication for anticoagulation therapy, medical diagnoses, concomitant medication and alcohol intake, were recorded. Each patient attended the clinic on 3-4 separate occasions, spread over 9 days, beginning the day after their last dose of warfarin, with some variation to avoid scheduling conflicts (e.g. weekends) and missed appointments. At each visit venous blood (10 ml) was collected for determination of INR and plasma warfarin enantiomer concentrations. The blood sample obtained at the first visit was also used for *CYP2C9* and *VKORC1* genotyping.

The rates of decline in INR and plasma warfarin enantiomer concentrations for individual patients were determined from the slope of the plot of INR versus time and plasma S- and R- warfarin concentration versus time, respectively. The rate of decline in plasma S- and R-warfarin concentration was subsequently used to calculate warfarin enantiomer half-life using the formula 0.693/slope. S- and R- warfarin clearance (CL) was calculated according to the formula: D/(2t X Css.av), where D is the daily dose of warfarin (mg), t is the dosing interval (24 hours), and Css.av is the average steady-state plasma warfarin enantiomer concentration (mg/L), assuming that warfarin compliance and bioavailability are 100% [368].

7.3.1. Sample size calculation

According to previous studies INR reached a value of <1.5 in 74±19 hours following warfarin withdrawal [365, 366]. To achieve 80% power and to detect a 15 hour difference in the time needed to reach an INR <1.5 between individuals with the *CYP2C9 *1*1* genotype and either those with at least one variant allele (i.e. either *CYP2C9*2* or *CYP2C9*3*) and allowing for the expected proportion in the three genotype groups, a sample size of 135 patients were needed to be studied.

7.3.2. Genotyping assay and samples preparation

All samples were prepared and genotyped for *CYP2C9 *2* and **3* alleles as mentioned in section 6.3.4 page 145. TaqMan® SNP genotyping assay was also used for *VKORC1* genotyping by using *VKORC1* probe with the same procedure as

mentioned in section 6.3.4.2 page 146. Since both of G-*1639A* and *C1173* are in strong linkage disequilibrium, the samples were genotyped for only *G-1639A*.

7.3.3. Determination of plasma warfarin enantiomer concentration

Blood samples were collected in heparinized tubes; subsequently, plasma was separated by centrifugation at 2500 rpm for 10 minutes and stored at -80ºC until further analysis. Plasma S- and R-warfarin enantiomer concentrations were determined by High Performance Liquid Chromatography (HPLC) using a modified method described by Naidong, et al. [369] as follows.

7.3.3.1. Warfarin enantiomer extraction from plasma

All samples were analysed in duplicate as follows. 200 µl of plasma was transferred into a clean tube. Then 30 µl of internal standard (S-naproxen in methanol, 40 µg/ml), 280 µl 1N sulphuric acid and 2 ml diethylether were added. The mixture was then rotary mixed for 15 minutes followed by centrifugation at 2500 rpm for 10 min. The upper organic phase was transferred into a new tube, and evaporated to dryness under a stream of air at 45ºC. The residue was reconstituted with 200 µl of mobile phase, spun on bench top centrifuge at maximum speed, and finally 50 µl of the mixture was injected onto the HPLC column.

7.3.3.2. Standard curve and quality control preparation

Warfarin racemic primary stock solution was prepared at concentration of 1 mg/ml in acetonitrile (0.5 mg/ml for each warfarin enantiomer). Secondary working stock solution was prepared with HPLC water at final concentration of 5 ng/µl (2.5 ng/ml for each warfarin enantiomer). The standard curve was constructed for *S*- and *R*- warfarin over the concentration range, 0, 125, 250, 380, 500, 750, and 1000 ng/ml. A quality control sample (in duplicate) was run with every assay to check for assay precision and accuracy.

7.3.3.3. HPLC instrumentations and conditions

Shimadzu VP HPLC machine was used with a UV detector (wavelength set at 320). The analytic column used was Astec Cyclobond I 2000 β-cyclodextrin, 5 µm, 25mm x 4.6mm (Supeclo Analytical, USA). The mobile phase was composed of acetonitrile, acetic acid, and trimethylamine (1000/3/2. v/v/v) and was pumped into the column at flow rate of 1.0 ml/min. The run time for each sample was 27 minutes. Before using the mobile phase, it was sonicated for 10-15 minutes in order to remove any traces of oxygen. S- and R-warfarin, and the internal standard (naproxen) were eluted on average at 5.59, 6.10, and 22.95 minutes, respectively, as shown in Figure 7-1.

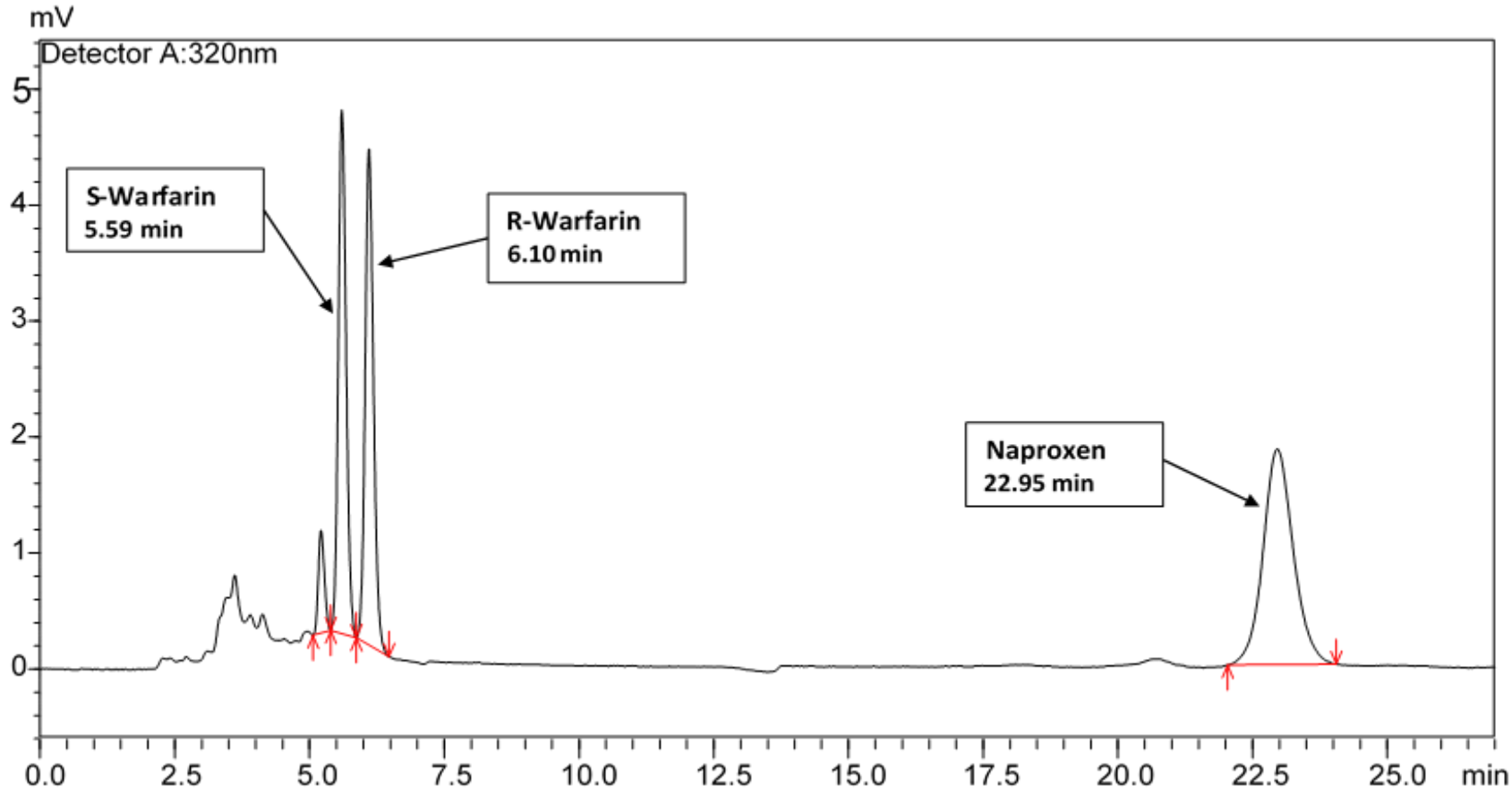


Figure 7-1: The separation and retention times of S- and R- warfarin and naproxen on the chromatogram

7.3.3.4. Data analysis

The chromatograms were analysed using Shimadzu LC solutions chromatographic software. For each of S- and R- warfarin, a separate standard curve was constructed by plotting the warfarin enantiomer to internal standard peak area ratio versus enantiomer concentration. Warfarin enantiomer concentration in patients' plasma and that of the quality control were derived from the standard curve.

7.3.3.5. Limits of detection and precision

The detection limit of the extracted samples was 2 ng/ml (signal/noise ratio =3). The inter-day coefficient of variation for S- and R- warfarin at 690 ng/ml was 4.2% and 3.8%, respectively.

7.3.4. Statistical analysis

Statistical analysis of the data was carried out using Minitab software version 17 (Coventry, UK). The effects of study variables on INR, and decay in plasma S- and R-warfarin enantiomer concentrations were assessed using multiple and logistic regression models. By regressing the logarithm of INR against time for each individual, the time for INR to reach 1.5 was estimated. This estimated time was modelled using *CYP2C9* and *VKORC1* genotypes, and other study variables. When necessary, data were transformed into their logarithmic values to approach normality. One way analysis of variance (ANOVA) was used for comparison of continuous data. A $P<0.05$ was taken as being statistically significant. Data are presented as mean±SD unless otherwise stated.

7.4. Results

135 patients were recruited into the study. However, for statistical analysis data were available for 131 participants [73 (56%) males] as four later withdrew from the study due to inability to attend study visits. The age range for the study cohort was 21-92 years; there was no significant difference in mean age between males and females

(62±15 versus 64±18 years). At the point of recruitment, patients were taking a median of 2 drugs (IQR 1-5), excluding warfarin, 1 of which affected the cardiovascular system. As well as indications for warfarin (atrial fibrillation 11%, deep venous thrombosis 61%, pulmonary embolism 26%, and valvular prosthesis 2%), the median number of comorbidities was 1 (IQR 1-2), most commonly cardiovascular disease (69%). The frequency of *CYP2C9* and *VKORC1* genotypes in the study population are presented in Table 7-1.

Table 7-1: *CYP2C9* and *VKORC1* genotype frequency distribution in the study population

CYP2C9 polymorphism, n (%)	
**1*1*	72 (56.7)
**1*2*	33 (26.0)
**1*3*	15 (11.8)
**2*2*	6 (4.7)
**2*3*	1 (0.8)
VKORC1 polymorphism, n (%)	
GG	49 (38.6)
AG	63 (49.6)
AA	15 (11.8)

N.B. Genotyping data missing for 4 patients

On the screening day following the final warfarin dose (warfarin dose usually taken early evening the day before), mean INR was 2.4±0.6. At the first study visit (16-24 hours after the last warfarin dose), the proportion of INR values ≥1.5 among those with *CYP2C9* wild-type, single variant, and double variant genotype was 78%, 94%, and 100%, respectively; *CYP2C9* genotype was the only predictor of an INR <1.5 (P=0.009) at the first visit; the odds ratio for the single variant genotype relative to the wild-type was 0.22 (95%CI= 0.06, 0.79). The proportion of INR values ≥1.5 at the second visit (mean of 102 hours) among those with *CYP2C9* wild-type, single- and double-variant alleles was 9%, 15%, and 40%, respectively, but these differences were no longer statistically significant; no further genetic influences were identifiable beyond the second visit as most of the INR values were close to 1.0 by the third and fourth study visits.

According to regression analysis, which included the variables of *VKORC1* and *CYP2C9* genotype, age, weight, sex, warfarin dose, comorbidities, concurrent drugs, and alcohol, *CYP2C9* genotype was the only factor which influenced the rate of INR decline (P=0.014); the slope of the regression lines for INR for *CYP2C9* (single and double variants) were significantly shallower than that for *CYP2C9* wild-type. Regression analysis revealed that the time required to reach an INR value of 1.5 was significantly associated with both patient age (P=0.037) and *CYP2C9* genotype (P=0.005) (R^2=15.7%). Using the regression equation, it can be estimated that for example, a 90-year old will take 18 hours longer to reach an INR of 1.5 than a 30-year old, and, for example, at the age of 60, in patients with either **1*1, *1*2, *1*3, *2*2*, or **2*3* genotype, on average it will take 61, 63, 78, 86, and 100 hours, respectively for INR value to reach 1.5.

In patients possessing either a single or double *CYP2C9* variant allele, S-warfarin half-life was longer (mean difference of 11 h for **1*2* and **1*3* and 20.5 h for **2*2* and **2*3*; P=0.004) and its clearance slower (mean difference of 0.1 ml/hour, for both single and double variant alleles; P<0.0001), compared to patients with wild-type genotype. Warfarin clearance results were comparable to results described in literature [370]. Table 7-2 shows S- and R- warfarin half-life and clearance for the various *CYP2C9* genotypes. CYP2C9 genotype had no influence upon R-warfarin half-life or clearance.

According to the regression analysis, neither *VKORC1* genotype, concurrent therapy, co-morbidity, patient weight, nor alcohol consumption influenced either the time taken for INR to reach 1.5, or S- and R- warfarin clearance.

Table 7-2: Influence of *CYP2C9* polymorphism on mean (SD) warfarin dose, half-life and clearance

	Warfarin weekly dose (mg)	Warfarin half-life (hr)		Warfarin clearance (L/hr)		
		S	R	S	R	Racemic
***CYP2C9* polymorphism**						
****1*1***	40 (16)	52.6 (19.5)	67.2 (28.0)	0.20 (0.22)	0.07 (0.07)	0.11 (0.09)
****1*2/*1*3***	29 (14)	63.3 (20.9)	70.6 (33.6)	0.09 (0.04)	0.05 (0.04)	0.07 (0.03)
****2*2/*2*3***	25 (9)	73.1 (12.1)	63.0 (32.9)	0.09 (0.05)	0.07 (0.01)	0.07 (0.02)

N.B. Data based on 127 patients as genotyping information was missing for 4 patients.

As expected, warfarin weekly mean dose was significantly different between patients according to their *CYP2C9* (p=0.0003) and *VKORC1* (P<0.0001) genotype. Patients with *CYP2C9* single- or double-variant alleles had a lower weekly mean dose requirement compared to the wild-type carriers; mean difference of 11mg (P=0.001) and 15 mg (P=0.03), respectively. Patients with *VKORC1 AA* or *AG* genotypes had lower weekly mean dose requirement compared to those with *GG* genotype; mean difference of 21 mg (P<0.0001) and 10 mg (P=0.003), respectively. Similarly, patients with *VKORC1 AA* genotype had a lower weekly mean dose requirement compared to those with *AG* genotype; mean difference 11 mg, P=0.02. Also, as expected, warfarin weekly dose requirement was negatively correlated with age (r= -0.4, P<0.0001).

7.5. Discussion

Patients on warfarin are normally required to interrupt warfarin therapy for a fixed number of days prior to an invasive procedure. However, the anticoagulant activity of warfarin (as measured by INR) subsides at different rates among different patients. Discontinuation of warfarin too early may predispose the patient to thrombosis and stopping it too late may result in peri-operative bleeding.

The results of this prospective study which investigated genetic and non-genetic influences upon INR decline support our hypothesis that the time required for the resumption of normal coagulation to take place following the cessation of warfarin therapy is largely dependent upon S-warfarin clearance, which in turn is influenced by *CYP2C9* polymorphism and patient age [95]. Polymorphisms in CYP2C9 (the main enzyme responsible for S-warfarin metabolism) and differences in patient age (which influences liver size and liver blood flow [93]) thus explain the previously reported variation in INR decay in patients withdrawing from warfarin prior to surgery [365, 366].

We also assessed the potential influence of *VKORC1* polymorphism, based upon the notion that polymorphisms in VKOR, the pharmacological target for warfarin, can affect the fall in warfarin anticoagulant activity following warfarin cessation. However, *VKORC1* polymorphism made no significant contribution to the variability in INR decline in our study population. Two previous studies, one in healthy subjects [371],

and the other in patients prior to surgery [372], found that neither *VKORC1*, nor *CYP2C9*, influenced the normalization of coagulation following warfarin withdrawal. However, neither study was sufficiently powered to detect the influence of either gene on the fall in INR; this was evident from the very small number of patients identified in either study as having variant alleles for *VKORC1* and *CYP2C9*.

In our self-reported Caucasians study population there were too few patients who were of *CYP2C9 *2*2** genotype, whilst there was only one patient who was of **2*3* genotype and no patient was of **3*3* genotype. As such, it was not possible to evaluate the effect of the individual genotypes on S-warfarin clearance and the fall in INR following warfarin withdrawal. Nevertheless, the frequency distribution for *CYP2C9* genotype fits in with that of the Caucasian general population in whom only about 2% are homozygous and ~20% are heterozygous carriers of the *CYP2C9*2* allele, with a significantly smaller proportion being either homozygous or heterozygous carriers of the *CYP2C9*3* allele [373]. In common with the literature we found that warfarin dose requirement was significantly influenced by *CYP2C9* and *VKROC1* genotype [95, 374, 375].

7.6. Conclusion

The study results show that the inter-individual variability in the time needed for normal coagulation to resume following warfarin withdrawal is influenced, in the main, by variance in S-warfarin clearance which in turn is affected by CYP2C9 polymorphism and age. A pharmacogenetics-based algorithm incorporating *CYP2C9* genotype and patient age (which could be easily adopted for routine use) could be clinically useful for estimating the correct length of time needed for individual patients to stop taking warfarin prior to surgery. Such an approach could benefit patients, in terms of reducing potential harm caused either by discontinuing warfarin too early (thus predisposing the patient to thrombosis) or stopping it too late (resulting in possible peri-operative bleeding). A pharmacogenetic-guided intervention could also benefit the health service providers in terms of improved delivery of care. However, whether such approach is cost-effective (in terms of the balance between savings made through reductions in the number of cancelled surgical procedures and the cost of genotyping) needs further investigation.

CHAPTER. 8 GENERAL DISCUSSION

Although warfarin has been used effectively for more than 60 years as an anticoagulant agent for the prophylaxis and treatment of thromboembolic diseases, difficulties with its management remain, owing to its narrow therapeutic index and inter- and intra- individual variability in anticoagulation response, which can be unpredictable. Variability in dietary intake of food, vitamin K in particular, as well as many drugs taken concomitantly with warfarin, affect resultant anticoagulation. In consequence, patients anticoagulated with warfarin require regular monitoring of prothrombin time, expressed as the INR, to ensure adequate anticoagulation and reduce the risk of adverse events, especially the risk of bleeding [376]. With the recent introduction and approval of NOACs for the treatment and prophylaxis of thromboembolic disease, the aim of having safe alternative oral anticoagulant drugs with at least the same efficiency as warfarin is being realised as they are characterised by a wider therapeutic range with lower drug-drug interaction profiles. Improving both warfarin and NOACs' safety by studying factors which affect their pharmacological activity is highly important. Therefore, the overall theme of this thesis was to study factors contributing to both inter- and intra- individual variability in clinical response to warfarin and the impact of diet on the NOACs' pharmacological activity.

I found in my cross-sectional study that warfarin anticoagulation control was more unstable in domiciliary monitored patients, in spite of such patients having the highest number of INR measurements and dose changes, compared to the more mobile patients attending general practice or hospital based clinics for monitoring. Further investigations to identify the clinical and demographic characteristics of housebound patients which contribute to the poorer anticoagulation stability, and exploration of effective interventions which improve anticoagulation control in such patients is indicated. Furthermore, the case for establishing the relative risks and benefits of NOACs as alternatives to warfarin in patients with poor anticoagulation control is strengthened by the results of my study.

Age is an important factor affecting anticoagulation response [91, 93], with some evidence showing that physical dependency can affect anticoagulation control. As only a longitudinal study can accurately identify true age-related changes, I went on to study

the effect of age on warfarin anticoagulation using longitudinal data belonging to more than 2000 patients. I showed that age, gender, and physical dependency all had an effect on anticoagulation stability.

I failed to prove the hypothesis that *VKORC1 (-1639)* genotype has a role in stability of long-term anticoagulation control [377]. Warfarin anticoagulation control as measured by time in therapeutic range was not different between *VKORC1* genotype groups. Owing to the type of data used (retrospective) in this study, and lack of information about patients' comorbidities and concurrent drug therapy, weight and a direct assessment of dietary vitamin K consumption, a prospective study to investigate whether *VKORC1 (-1639)* genotype has an effect on anticoagulation control would allow more scientifically robust conclusions.

Patients on warfarin therapy scheduled for invasive procedures should stop taking the drug (usually 5 days prior to the intervention according to guidelines) in order to minimise the risk of peri-operative bleeding. However, the rate of decline in INR differs between patients. To date the factors which influence the rate of fall in INR had not been identified. In the first study of its kind, I examined the effect of *CYP2C9* genotype on the rate of INR decline after warfarin interruption. I found that normalisation of coagulation occurred more slowly in patients with *CYP2C9* mutations. Patients with two *CYP2C9* variant alleles were more than eight times more likely to have an INR of ≥ 1.5 on the fifth day of warfarin interruption. In a separate study I was able to demonstrate that, in addition to age, the rate of fall in INR was influenced by S-warfarin clearance which in turn was influenced by *CYP2C9* genotype. An algorithm for the rate of INR decline incorporating *CYP2C9* genotype was developed and has the potential to benefit all patients requiring warfarin interruption, as the ideal period off warfarin could be determined for individual patients. Further study is required to validate this algorithm and its clinical utility examined.

With the development of pharmacogenetics-based algorithms guiding initiation of anticoagulation with warfarin, a number of handheld medical devices for *CYP2C9* *2/*3 and *VKORC1 − 1639G>A* or *1173C>T* genotyping, such as Nanosphere (Verigene®), Autogenomics (INFINITI®), GenMark Dx (eSENSOR®), Paragon Dx reagents with Cepheid Smart Cycler®, and TrimGen reagents with Roche Light Cycler® [156], have been developed and approved by the FDA. Genotyping results for CYP2C9 are usually

obtained within 2-3 days if performed in a reference molecular diagnostic laboratory or the next working day if testing is done locally; however, with these devices, genotype results are available in less than 8 hours. If the results of my study are validated within a wider population, such handheld devices could be used to promote the clinical utility of the algorithm on a wide scale.

The clinical effectiveness of NOACs has been established in a number of landmark studies. These studies have shown that, within trial populations, NOACs were at least as effective as warfarin, and were associated with a significantly lower major bleeding risk. Trial populations tend to exclude frail patients, the very elderly and those with multiple comorbidities, many of whom have a diet low in vitamin K, an important factor in blood coagulation. Besides poor dietary vitamin K intake, age might play a role in increasing sensitivity to anticoagulation response to NOACs, as shown by Reilly et al [378] in a recent pharmacokinetic analysis of the RE-LY trial data in which they examined the association between efficacy and safety results and dabigatran plasma concentrations. Dabigatran plasma concentrations increased with age in association with declining renal function, trough plasma drug concentrations increasing by 68% in patients aged 75 years and above compared to those aged less than 65. Moreover, dabigatran plasma concentration was higher by 21% and 53% in those weighing below 50 kg compared to those weighing 50 to 100 kg and ≥ 100 kg, respectively. Lower dabigatran plasma concentrations were positively correlated with thrombotic stroke, and higher trough and post-dose dabigatran concentrations were associated with more major bleeding events. I studied the effect of vitamin K in diet on rivaroxaban pharmacological response in humans ex-vivo, which has not been previously studied. I compared results in older subjects who were frail and underweight and with poor oral intake of vitamin K with those of young healthy controls with vitamin K replete diets. I showed that anticoagulation response to rivaroxaban ex-vivo was increased in the group with poor vitamin K intake, suggesting that the latter are at increased risk of bleeding associated with rivaroxaban treatment. Therefore, as for patients on warfarin therapy who are advised to have a regular dietary intake of vitamin K to maintain adequate anticoagulation stability [125, 349, 379], anticoagulation might be more within the boundaries predicted for NOACs in patients ingesting adequate amounts of vitamin K in their diet. These results could be applicable to all NOACs; therefore, the

malnourished elderly with multiple comorbidities and/or renal dysfunction might be candidates for NOAC dose adjustment; further studies to investigate this are required.

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APPENDICES

Appendix (A) TTR calculations

Here is an example of calculating TTR manually.

1. As shown in Table A-1, calculate number of days between each two dates, e.g. 21 days between 07/02/2006 and 17/01/2006.
2. Calculate the amount of total shift between every two consecutives INR readings, and also that within the specified therapeutic range (i.e. INR of 2-3 in this example), for example:
    a. between 07/02/2006 and 17/01/2006, 2.3-2.7= -(0.4). Since the two INR readings are still within the required range (i.e. 2-3), the TTR =0.4/0.4=1; therefore, the TTR in those 21 days is 100%
    b. 12/04/2006 and 04/04/2006, 8 days are the difference between these two dates. 1.9-2.2=-(0.3) which is the total difference between the two INR readings, and 2.2-2.0= 0.2 is the shift in the required range (INR of 2-3)
        i. $\frac{The\ total\ amount\ within\ therapeutic\ range\ (e.g. 2.2-2)}{The\ total\ amount\ between\ the\ two\ readings} = \frac{0.2}{0.3}$= 0.67, or 67%. 0.67 X 8 days= 5 days. This means that 5 out of 8 days were within the therapeutic range.
        ii. 25/04/2006 and 12/05/2006, 13 days are the difference. INR difference =1.2-1.9=0.7. Both of the two readings are out of the range (i.e. 2-3), and the amount within the therapeutic range is zero.

            Therefore, the TTR =$\frac{0}{0.7}$= 0 % for the 13 days. The same is applied if the two INR readings are above 3, e.g. 3.5 (30/05/2005) and 3.7 (22/05/2006).
3. Complete the above calculation for all INR readings
4. Take the sum of all days within the period required, 133 days in this example Table A-1; and the days within the therapeutic range, 89 days.
5. Divide the days within the therapeutic range by the total days
6. $\frac{89}{133}$ =0.7, which means that the time in therapeutic range within the given period (from 17/01/2006 to 30/05/2006) was 70%.

Table A-1: Example of how to calculate TTR

Visit Date	INR Reading (a)	Days since (b)	INR Difference (c)	% in range (d)	Days in therapeutic range (c X d)
17/01/2006	2.7				
07/02/2006	2.3	21	-0.4	100=1	21
07/03/2006	2.4	28	0.1	100=1	28
04/04/2006	2.2	28	-0.2	100=1	28
12/04/2006	1.9	8	-0.3	67=0.67	5
25/04/2006	1.2	13	-0.7	0	0
02/05/2006	1.8	7	0.6	0	0
15/05/2006	3.9	13	2.1	48=0.48	6
22/05/2006	3.7	7	-0.2	0	0
30/05/2006	3.5	8	-0.2	0	0
Total days number		133			89

Appendix (B) Validated dietary questionnaire

How to answer the questions

There are several types of question in this booklet. Most of them can be answered by ticking one box (ONLY) beside each food types.

For example:

FOODS & AMOUNTS	AVERAGE USE IN THE LAST WEEK							
FISH *(medium serving)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Fried fish in batter, as in fish and chips		✓						

Please put ONE tick in the appropriate box (✓) on each line to indicate how often, **on average**, you have eaten each food **during the past week.**

- Answer every question by putting ONE tick (✓) on every line
- Do not leave **ANY** lines blank.

Another example of questions requiring boxes to be ticked:

Q. Do you usually add salt to food while cooking?
Yes……☑
No……❒

Some of these questions have several boxes and you may be asked to tick ONE only.

For example:

What kind of fat did you most often use for frying, roasting, grilling etc?

Select one only

Butter……❒
Lard/dripping……❒
Solid vegetable fat……❒
Margarine……❒
Vegetable Oil……❒
Olive oil……☑
Walnut Oil……❒
Soya Oil……❒
None……❒
Other……❒

Some of these questions have several boxes and you may be asked to tick all the boxes you think apply to you.

For example:

14. What kind of fat did you use for cooking?

Please tick <u>all</u> that apply

Butter……☑
Lard/dripping……❒
Solid vegetable fat……❒
Margarine……❒
Vegetable oil……☑
Olive oil……❒
Walnut Oil……❒
Soya Oil……❒
None……❒
Other……❒

If “other” selected in question 14, please state …….....

What do I do if I make a mistake?

Cross out the incorrect answer, and put a tick where you think the right answer should be.

For **Questions 1-12**, please put **ONE tick** in the appropriate box (✓) on each line to indicate how often, **on average**, you have eaten each food **during the past week.** Please **DO NOT leave any lines blank.**

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
1. MEAT ***(medium serving)***	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Beef: e.g. roast, steak, mince, stew, casserole, curry, Bolognese								
Beefburgers *(single burger)*								
Corned beef, Spam, luncheon meats *(2 slices – a sandwich's-worth)*								
Lamb: e.g. roast, chops, stew, curry								
Chicken, turkey or other poultry: e.g. casserole, sliced, curry								
Breaded or fried poultry products: e.g. chicken nuggets, deep fried chicken pieces *(1 breaded chicken portion or c.6 nuggets)*								
Pork: e.g. roast, chops, stew, curry								
Bacon and ham *(2 rashers/slices – a sandwich's-worth)*								
Sausages *(one)*								
Savoury pies, e.g. meat pie, pork pie, pasties, steak & kidney pie, sausage rolls, scotch egg *(single pie/savoury)*								
Game and Wild-fowl: e.g. duck, rabbit, grouse								
Kidneys or liver; including liver pate, liver sausage								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
2. FISH and SEAFOOD ***(medium serving)***	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White fish- not coated e.g. cod, halibut, haddock, whiting, plaice, sole, etc *(per portion)*								
White fish- in batter or crumbs e.g. cod, haddock, plaice, etc *(per portion)*								
Oily fish e.g. herring, mackerel, *(tinned)* salmon- not tinned, trout, kippers etc *(per portion)*								
Tinned fish e.g. Sardines, Pichards, Tuna, Salmon etc *(per can, or portion)*								
Prawns, shellfish and other fish *(within dish or one sandwich's-worth)*								
Fish cakes, Fish fingers *(one)*								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
3. BREAD & SAVOURY BISCUITS ***(one slice or biscuit)***	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White bread and rolls, white pitta bread (per slice/roll)								
Scones, teacakes, crumpets, muffins or croissants (each)								
Brown bread and rolls (per slice/roll)								
Wholemeal pitta bread (each)								
Wholemeal bread/rolls (per slice/roll)								
Granary bread (per slice/roll)								
Rye bread (per slice/roll)								
Naan bread, chapatti (each)								
Garlic bread (per serving)								
Cream crackers, cheese biscuits (each)								
Wholemeal crackers (per cracker)								
Crispbreads e.g. Ryvita, Ryvita currant crunch (one)								
Oatcakes (one)								
Other speciality breads (each) (please state and tick for frequency) 1. 2.								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
4. CEREALS ***(one bowl)***	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Porridge, Readybrek								
Sugar coated cereals e.g. Sugar Puffs, Cocoa Pops, Frosties								
Non-sugar coated cereals e.g. Cornflakes, Rice Crispies								
Muesli								
Bran containing cereals e.g. All Bran								
Cheerios								
Branflakes								
Weetabix								
Shredded Wheat, Shreddies								
Wholegrain cereals with fruit e.g. Sultana Bran, Fruit n Fibre								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
5. POTATOES, RICE & PASTA *(medium serving)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Boiled, mashed, instant or jacket potatoes *(about 1/3 of a plate)*								
Chips, potato waffles *(side order with meal – chip-shop portions count as 2)*								
Roast potatoes *(3 – 5 potatoes)*								
Yorkshire pudding, pancakes, dumpling *(each medium)*								
Potato salad *(per small tub, c. 2 tablespoons)*								
White rice *(1/2 plateful, or in a dish e.g. rice salad, risotto etc)*								
Brown rice *(1/2 plateful, or in a dish e.g. rice salad, risotto etc)*								
White or green pasta, e.g. spaghetti, macaroni, noodles, *(1/2 plate)*								
Tinned pasta, e.g. spaghetti, ravioli, macaroni *(1/2 standard tin)*								
Super noodles, pot noodles, pot savouries *(per pot)*								
Wholemeal pasta/spaghetti *(1/2 plate)*								
Pasta dishes e.g. Lasagne, moussaka, cannelloni *(as individual ready-meal)*								
Pizza *(10" = 1, 12" = 2, 12"+ = 3-4)*								
Wholegrain dishes not mentioned (Please state and tick for frequency) 1. 2. 3.								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
6. (a) DAIRY & EGG PRODUCTS	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Single or sour cream *(tablespoon)*								
Double or clotted cream *(tablespoon)*								
Low fat yoghurt, fromage frais *(125g carton)*								
Full fat or Greek yoghurt *(125g carton)*								
Dairy desserts *(125g carton),* e.g. mousse								
Cheese, e.g. Cheddar, Brie, Edam *(medium serving)*								
Cottage cheese, low fat soft cheese *(medium serving)*								
Eggs as boiled, fried, scrambled, omelette etc. *(one)*								
Quiche *(medium serving = 1/6 of pie)*								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
6.(b) DAIRY PRODUCTS & FATS used on bread *(teaspoon/curl)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Butter								
Margarines/spreads (state type and tick for frequency) 1. 2. 3.								
Reduced/Low fat spreads (state type and tick for frequency) 1. 2. 3.								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
6.(c) DAIRY PRODUCTS & FATS used on vegetables *(teaspoon/curl)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Butter								
Margarines/spreads (state type and tick for frequency) 1. 2. 3.								
Reduced/Low fat spreads (state type and tick for frequency) 1. 2. 3.								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
7. SWEETS & SNACKS *(medium serving)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Chocolate coated sweet biscuits, e.g. Penguin, kit-kat, chocolate digestive *(one)*								
Sweet biscuits, plain, e.g. Nice, ginger *(one)*								
Cakes e.g. fruit, sponge, sponge pudding *(medium slice)*								
Sweet buns & pastries e.g. doughnuts, Danish pastries, cream cakes *(each)*								
Flapjacks (*each)*								
Fruit pies, tarts, crumbles (per individual pie/medium serving)								
Milk puddings, e.g. rice, custard, trifle *(medium serving)*								
Ice cream, choc ices *(one)*								
Chocolates,, toffee, sweets and other confectionary *(medium bar of chocolate, one snack bar, one packet)*								
Sugar added to tea, coffee, cereal *(teaspoon)*								
Crisps or other packet snacks e.g. Wotsits *(one packet)*								
Peanuts *(one packet)*								
FOODS & AMOUNTS	**Average Use In LAST WEEK (Tick ONE per line)**							
8. SOUPS, SAUCES AND SPREADS	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Vegetable soups *(medium bowl)*								
Meat soups *(medium bowl)*								
Sauces, e.g. white sauce, cheese sauce, gravy *(1/3 of plate or in dish)*								
Tomato based sauces e.g. pasta sauces *(1/3 of plate or in dish)*								
Tomato ketchup, brown sauce *(per tablespoon)*								
Relishes e.g. pickles, chutney, mustard *(per tablespoon)*								
Salad cream, mayonnaise, other salad dressings *(per tablespoon)* (state type and tick for frequency) 1. 2. 3.								
Marmite, Bovril *(per teaspoon/slices of bread)*								
Jam, marmalade, honey, syrup *(per teaspoon/slices of bread)*								
Peanut butter *(per teaspoon/slices of bread)*								
Chocolate spread, chocolate nut spread *(per teaspoon/slices of bread)*								
Dips e.g. houmous, cheese and chive *(per tablespoon/slices of bread)*								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
9. DRINKS	None	Once A Week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Tea *(cup)*								
Coffee, instant or ground *(cup)*								
Coffee whitener, e.g. Coffee-mate *(teaspoon)*								
Cocoa, hot chocolate *(cup)*								
Horlicks, Ovaltine *(cup)*								
Wine *(glass)*								
Beer, lager or cider *(half pint)*								
Port, sherry, vermouth, liqueurs *(glass)*								
Spirits, e.g. gin, brandy, whisky, vodka *(single)*								
Low calorie or diet fizzy soft drinks *(glass)*								
Fizzy soft drinks, e.g. Coca cola, lemonade *(glass)*								
Pure fruit juice (100%) e.g. orange, apple juice *(glass)*								
Fruit squash or cordial *(glass)*								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
10. FRUIT *(1 fruit or medium serving)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Apples *(each)*								
Pears *(each)*								
Oranges *(1x)*, satsumas, mandarins, tangerines, clementines *(all 2x)*								
Grapefruit *(1/2 a fruit)*								
Bananas *(each)*								
Grapes *(per small handful)*								
Melon *(1 medium slice)*								
Peaches *(1x)*, plums, apricots, nectarines *(2 – 3x)*								
Strawberries, raspberries *(per small handful)*, kiwi fruit *(each)*								
Tinned fruit *(1/2 tin)*								
Dried fruit, e.g. raisins, prunes, figs *(per small handful)*								
Other fruit or fruit dishes / products not mentioned (please state and tick for frequency) 1. 2. 3.								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
11. VEGETABLES Fresh, frozen or tinned *(medium serving)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Carrots *(2-3 table spoonfuls)*								
Cooked Spinach *(major ingredient in dish (e.g. curry) or per 2 – 3 tablespoonfuls)*								
Broccoli *(per 4 – 5 florets)*								
Brussels sprouts *(2-3 tablespoonfuls)*								
Cabbage *(2-3 tablespoonfuls)*								
Peas *(2-3 tablespoonfuls)*								
Green beans, broad beans, runner beans *(2-3 tablespoonfuls)*								
Marrow, courgettes *(major ingredient in dish or 2-3 tablespoonfuls)*								
Cauliflower *(major ingredient in dish (e.g. curry) or 2-3 tablespoonfuls)*								
Parsnips, turnips, Swedes *(2-3 tablespoonfuls)*								
Leeks *(2-3 tablespoonfuls)*								
Onions *(per onion)*								
Garlic *(2 cloves)*								
Mushrooms *(handful of uncooked mushrooms, or 2-3 tablespoonfuls)*								
Sweet peppers *(per ½ pepper)*								
Beansprouts *(major ingredient in dish or (2-3 tablespoonfuls)*								
Mixed salad leaves, lettuce, rocket *(side-salad or per 1/3 plate)*								
Cucumber *(per ¼ cucumber)*								
Mixed vegetables (frozen or tinned) *(2-3 tablespoonfuls)*								
Watercress *(per bunch, or as a major ingredient in salad)*								
Tomatoes *(2 medium tomatoes, ½ can of tomatoes)*								
Sweetcorn *(2-3 tablespoonfuls)*								
Beetroot *(1 medium)*								
Radishes *(3-4 pieces)*								
Coleslaw *(2-3 tablespoonfuls)*								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
11. VEGETABLES Fresh, frozen or tinned *(medium serving)*	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Avocado *(per ½ fruit)*								
Baked Beans *(per ½ tin)*								
Dried lentils, beans, peas *(2-3 tablespoonfuls, or major ingredient)*								
Tofu, soya meat, TVP, (*in dish e.g. curry),* Vegeburger *(each)*								

FOODS & AMOUNTS	Average Use In LAST WEEK (Tick ONE per line)							
11. VEGETABLES Fresh, frozen or tinned (continued)	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Other vegetables or vegetable dishes / products not mentioned (please state and tick for frequency) 1. 2. 3.								

YOUR DIET IN THE LAST WEEK, *continued*

12. What type of milk did you most often use?

Select one only

Full cream……❒
Channel Islands……❒
Dried milk……❒
Semi-skimmed……❒
Skimmed……❒
Soya……❒
Other……❒
None……❒

13. Approximately, how much milk did you drink each day, including milk with tea, coffee, cereals etc?

None .....❒
Quarter of a pint (roughly 125mls) .....❒
Half a pint (roughly 250mls) .....❒
Three quarters of a pint (roughly 375mls) .....❒
One pint (roughly 500mls) .....❒
More than one pint (more than 500mls) .....❒

14. What kind of fat did you use for cooking?

Please tick <u>all</u> that apply

Butter……❒
Lard/dripping……❒
Solid vegetable fat……❒
Margarine……❒
Vegetable oil……❒
Olive oil……❒
Walnut Oil……❒
Soya Oil……❒
None……❒
Other……❒

If “other” selected in question 14, please state…….....

15. Do you usually add salt to food while cooking?

Yes……❒
No……❒

16. Do you usually add salt to any food at the table?

Yes……❒
No…….❒

17. Do you usually eat the fat on cooked meats?

Yes……❒
No…….❒

18. Do you usually eat the skin on cooked meats?

Yes……❒
No……❒

19. Do you usually add sugar to drinks i.e. tea/coffee?

Yes……❒
No……❒

20. On average, in the past week, how many portions of fruit and vegetables did you eat per DAY?

Please estimate:…..……..……

21. On average, in the past week, how many servings of wholegrain foods did you eat per DAY?

Please estimate:……

22. Have you taken any of the following during the past week?

	None	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day
Vitamins (e.g. multivitamins, vitamin B, vitamin C, folic acid)						
Minerals (e.g. iron, calcium, zinc, magnesium)						
Fish oils (e.g. cod liver oil, omega-3)						
Other food supplements (e.g. oil of evening primrose, starflower oil, royal jelly, ginseng)						

- Did you use any other food supplements? Please state below:

1_____
2_____
3_____

Thank you for taking the time to complete this questionnaire!!

ASSERACHROM® IX:Ag

Enzyme Immunoassay for Factor IX

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

(REF 00943) IVD CE

April 2014 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 Ca^{++}, activates factor IX to factor IXa
- tissue factor:factor VIIa complex activates either factor X or factor IX.

Factor IXa forms an enzymatic complex with phospholipids, Ca^{++} 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-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. Whenever 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.

Exercice 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 (< 0.06 %), 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	t:2	t:4	t:8	t:16
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 H_2SO_4	50 µl
Swirl the plate to mix contents after H_2SO_4 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 in 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:

	Intra-Assay Reproducibility		Inter-Assay Reproducibility	
Sample	Sample 1	Sample 2	Sample 3	Sample 4
n	21	21	10	10
X (%)	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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Significant changes are indicated by dotted lines in the margin.

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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 Vials 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-Ca^{++}-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, Ca^{++} 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. Whenever 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 (see paragraph 8.4.) as soon as the strips 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/H_2O_2 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), sensitising 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 H_2O.
  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 H_2O.
  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	t:2	t:4	t:8	t:16
Dilution of starting solution	None	1:2	1:4	1:8	1:16

8.2. Plasma Samples and Control

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).

8.3. 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/H_2O_2 Substrate (Reagents 3a + 3b)	200 µl
	Let incubate at room temperature for **exactly** 2 minutes for each well, then add acid:	
	either 3 M H_2SO_4 **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.

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