

ASSESSMENT OF ADHERENCE TO WARFARIN
ANTICOAGULATION USING THE RATIO OF VITAMIN K
DEPENDENT FACTORS IN A TEACHING HOSPITAL IN SOUTH
AFRICA

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, in partial fulfilment of the degree of Master of Medicine in the branch of Haematology.

Johannesburg, 2017.

DECLARATION

I, Sarisha Naidoo declare that this Research Report is my own, unaided work. It is being submitted for the Degree of Master of Medicine in the branch of Haematology at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



Sarisha Naidoo

Signed in Parktown on 1st day of March 2018

DEDICATION

- *“It is the time that you have wasted for your rose that that makes your rose so important”-Antoinne De Saint-Expury, The Little Prince*

Thank you to my friends, family and colleagues for their unconditional support and encouragement during my studies and research time.

- *“Do not judge me by my successes, judge me by how many times I fell down and got back up again”- Nelson Mandela*

To my husband, Dr Lushen Naidoo for teaching me strength of character, belief in oneself and the ongoing journey toward being a better person.

- *Sound, when stretched is music, movement when stretched is dance, life when stretched is celebration”- Ravi Shankar*

To my parents, Vincent and Jayshree Naidoo for teaching me to strive for the best in life both creatively and academically.

- *“You may forget the one with whom you have laughed, but never the one with whom you have wept” Kahlil Gibran*

To my siblings, Dr Levashni Naidoo and Rayan Naidoo, your unwavering belief in me meant everything.

- *“One can speak poetry just by arranging colours well”-Vincent Van Gogh*

To Professor Mahlangu, thank you for teaching me the art of research. Thank you also for your invaluable mentorship, guidance and support in my years as a registrar.

PRESENTATIONS AND PUBLICATIONS ARISING FROM THIS RESEARCH REPORT

Poster presentations

- 1) Sarisha Naidoo and Johnny Mahlangu. Assessment of Adherence to Warfarin Anticoagulation using the Ratio of Vitamin K Dependent Factors in a Teaching Hospital in South Africa – XXXI World Federation of Haemophilia Congress, Orlando Florida, USA, 24-27 July 2016

- 2) Sarisha Naidoo and Johnny Mahlangu. Assessment of Adherence to Warfarin Anticoagulation using the Ratio of Vitamin K Dependent Factors in a Teaching Hospital in South Africa – Wits Research Day, 18 September 2016

Publications

1. Sarisha Naidoo, Johnny Mahlangu. Assessment of Warfarin Anticoagulation using Vitamin K Dependent Factors- manuscript completed and submitted for peer review
2. Subanalysis manuscript under preparation

ABSTRACT

Background: Warfarin has both anticoagulant and antithrombotic effects, as reflected by the short and long half-lives of the Vitamin K dependent clotting factors. A therapeutic International Normalized Ratio (INR) may reflect the anticoagulant effect, but not the antithrombotic effect with consequent suboptimal assessment of thrombotic risk. A potential approach to monitor adherence to warfarin is to measure the INR in relation to short half-life and long half-life clotting factors.

Objectives: To evaluate adherence to warfarin in participants with a therapeutic INR by measuring clotting factor II and VII levels.

Patients and Methods: This was a prospective cross-sectional study approved by the University ethics committee and participants gave written informed consent. The study included participants ≥ 18 years of age on steady state (≥ 3 months) warfarin anticoagulation. Participants were stratified into a therapeutic INR (study group) and a sub-therapeutic INR group (control group). A 5ml venous blood sample was collected in a trisodium citrate (0.109M, 3.2%) tube and factor II and factor VII plasma levels were measured on a STA-R coagulation analyser in both the study and control groups. Data on patient demographics, concurrent medication and monthly INR results was collected from the Anticoagulation Clinic records.

Results: Of the 350 participants enrolled in the study, 45 (12.8%) had INRs values above target the therapeutic range, whilst 213 (60.8%) had therapeutic INR values, and 92 (26.2%) had a sub-therapeutic INR. Overall non-adherence levels as judged by clotting factor levels were 9.4%. Of the participants with a sub-therapeutic INR, measured factor II and VII levels indicated adequate anticoagulation in 51(64.5%) participants and inadequate anticoagulation in 28 (35.4%) participants. In participants with a therapeutic INR, clotting factor levels indicated inadequate anticoagulation in 2 participants (0.9%). Time in therapeutic range (TTR) was assessed over a 4 month period and overall 71.6 % showed appropriate TTR. Subgroup analysis indicated that Caucasians spent more time in TTR whilst Asians spent the least TTR ($p < 0.0001$; Cramer's $V = 0.24$). In relation to the INR, the clotting factor measurements showed a 79.8% sensitivity and 93.3% specificity in predicting adherence and nonadherence to warfarin anticoagulation respectively.

Conclusions: In this study, clotting factor II and VII levels measured in steady warfarin therapy warfarin did not depict significant levels of non-adherence and are therefore not useful for routine monitoring of warfarin adherence. However, specific indications for clotting factor testing would include patients with a sub-therapeutic INR at increased risk of bleeding, those suspected of being non-adherent on warfarin therapy, those with a hypercoagulable state, and those on drugs that interfere with warfarin metabolism . This study also showed that in a number of patients with a sub-therapeutic INR, anticoagulation may well be adequate based on clotting factor measurements. In the absence of clotting factor levels, the participant's warfarin dose may be inappropriately increased with associated increased bleeding risk.

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LIST OF ABBREVIATIONS

AF	Atrial fibrillation
APLS	Antiphospholipid syndrome
APTT	Activated partial thromboplastin time
ART	Anti-retroviral therapy
CF	Clotting factor
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
CVA	Cerebrovascular accident
CYP P450	Cytochrome P450
DOACs	Direct oral anticoagulants
DVT	Deep vein thrombosis
FRC	Faculty Research Committee
HIT	Heparin induced thrombocytopenia
HRT	Hormone replacement therapy
INR	International normalised ratio
ISI	International sensitivity index

NHLS	National Health Laboratory Service
NSAIDs	Non-steroidal anti-inflammatories
PE	Pulmonary embolus
PT	Prothrombin time
RCF	Relative centrifugal force
RPM	Revolutions per minute
SA	South Africa
SOP	Standard operating procedure
SSRI	Selective serotonin reuptake inhibitor
TF	Tissue factor
TTR	Time in therapeutic range
VKOR	Vitamin K epoxide reductase
VTE	Venous thromboembolism

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The recent introduction of direct oral anticoagulants (DOACs) signalled an important advancement in anticoagulation therapy. However, the associated current high costs and the lack of easily available reversal agents are known barriers to the acceptance and widespread use of DOACs, particularly in resource constrained countries ⁽¹⁾. As a result, warfarin remains the most frequently used oral anticoagulant globally ⁽²⁾. Whilst exact figures are not available in South Africa (SA), in the United Kingdom, warfarin is used by approximately 1% of the total population and 8 % of the elderly people ⁽²⁾. Research into monitoring adherence of warfarin anticoagulation therapy and the implication of these assessments on therapeutic outcomes therefore remain pertinent to effective management of patients on warfarin therapy.

1.2 Warfarin

1.2.1 Background History

Warfarin is a synthetic 4-hydroxycoumarin vitamin K antagonist (VKA) and has been in use since the 1930s after its discovery by Professor Karl Link and his student Harold Campbell from the University of Wisconsin ^(3,4). Coumarins, of which warfarin is a prototype, were isolated from spoiled animal feed, which caused haemorrhagic death in cows ⁽³⁾. The name “warfarin” is derived from the acronym for Wisconsin Alumni Research Foundation (WARF) and the word “coumarin” ⁽⁴⁾

1.2.2 Indications

Warfarin is used to treat or prevent venous thromboembolic (VTE) disease which includes deep vein thrombosis (DVT) and pulmonary embolism (PE) ^(5,6). Thromboprophylaxis is used in patients with cardiac diseases (including atrial fibrillation (AF), valvulopathy, patent foramen ovale, septal wall aneurysms, dilated cardiomyopathy and heart failure) to prevent systemic thromboemboli as well as in patients with vascular diseases to prevent acute

myocardial infarction and cerebrovascular accidents (CVA) ⁽⁶⁻⁹⁾. Warfarin is also indicated as thromboprophylaxis in patients with inherited or acquired thrombophilia including the antiphospholipid syndrome (APLS) ⁽⁶⁾.

1.2.3 Mechanism of Action

The anticoagulant effect of warfarin is achieved through the inhibition of vitamin K epoxide reductase (VKOR) activity in the liver (refer to Figure 1.1) ^(10, 11). Physiologically, vitamin K epoxide is reduced to vitamin K₁ by VKOR. Thereafter vitamin K₁ is reduced to vitamin K_{H₂} by vitamin K₁ reductase in the liver ^(10, 11). Vitamin K_{H₂} acts as a cofactor in the gamma carboxylation reaction of glutamic acid residues on the N-terminal regions of vitamin K dependent proteins ⁽¹²⁾. These proteins include coagulation factors II, VII, IX and X and the natural anticoagulants, protein C and S ^(5, 6). Gamma-carboxylation of these factors promotes their binding to phospholipid surfaces, and thus increases blood procoagulant activity ⁽¹²⁾. Therefore, warfarin affects coagulation indirectly by impeding the effective recycling of vitamin K with consequent decrease in the carboxylation of the vitamin K dependent clotting factors (CF) which are essential for their biological activity ⁽¹²⁾. The effects of warfarin can be overcome by food derived or therapeutic sources of Vitamin K₁ which bypasses the warfarin sensitive VKOR in the formation of vitamin K_{H₂} by vitamin K₁ reductase ⁽⁵⁾.

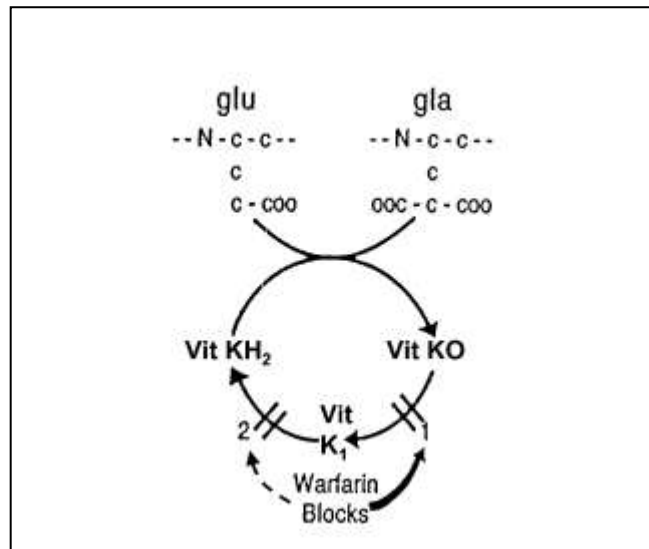


Figure 1.1 The Vitamin K cycle and associated role in the carboxylation of Vitamin K dependent coagulation proteins, 1, Vitamin K epoxide reductase (VKOR), 2, vitamin K1 reductase. Vit KO, Vitamin K epoxide. Adapted from Hirsh et al ⁽⁶⁾

The rate of reduction of the Vitamin K dependent CF's secondary to warfarin therapy is proportionate to their respective half-lives ⁽¹³⁾. The anticoagulant effects of warfarin are delayed until the existing Vitamin K gamma carboxylated CF's are cleared from circulation. The half-life of the CF's VII, IX, X, II are 6, 24, 40 and 60 hours respectively (refer to Table 1.1) ^(5, 13, 14). As a result of these different CF half-life profiles, warfarin has two distinct effects; an anticoagulant effect (which represents a reduction in CF VII and CF IX with shorter half-life profiles) and an antithrombotic effect (which represent a reduction in CF II and CF X with longer half-life profiles) ⁽¹⁵⁻¹⁸⁾. There is therefore a delay in the therapeutic anticoagulant effect of warfarin for four to five days after initiation of treatment when CF II reaches nadir levels ^(5, 16-18). Owing to these reasons and the effect of warfarin on the short half-life natural anticoagulant, Protein C, this necessitates concurrent overlap of warfarin with heparin therapy until a therapeutic International Normalised Ratio (INR) is achieved ^(5, 19).

Table 1.1 Half-lives of the Vitamin K dependent clotting factors and natural anticoagulants. Adapted from Ansell et al, O'Reilly et al and Hoffband et al ^(5, 13, 14).

Vitamin K dependent protein	Half-life (hours)
Clotting Factor II	60
Clotting Factor VII	6
Clotting Factor IX	24
Clotting Factor X	40
Protein C	8
Protein S	60

1.2.4 Pharmacokinetics and Pharmacodynamics

Warfarin is nearly 100% bioavailable when taken as an oral formulation ⁽²⁰⁾. It demonstrates rapid absorption via the gastrointestinal system, primarily through the stomach and proximal small intestine ⁽²¹⁾. Warfarin circulates bound to albumin and reaches maximal concentration 90 minutes post oral administration ⁽²²⁾. Warfarin is comprised of both S and R subtype isomers of which more than 98% are bound to albumin ⁽²³⁾. Only warfarin which is unbound to albumin is pharmacologically active ⁽²³⁾. Warfarin S is approximately 2.7-3.8 times more potent than warfarin R, due to its stronger receptor affinity to VKOR ⁽²⁴⁾. Warfarin is oxidized by cytochrome P450 (CYP P450) enzymes in the liver ⁽²⁵⁾. Warfarin S is 90% oxidised by CYP P450 enzymes primarily the CYP 2C9 isoform. Warfarin R is 60% oxidised by CYP P450, primarily the 1A2 and 3A4 isoforms. Warfarin is eliminated through renal excretion ^(25, 26).

1.2.5 Dietary Factors

Dietary forms of Vitamin K include phylloquinone (Vitamin K1) which is derived from plant sources and menaquinone (Vitamin K2) which is synthesized by intestinal microflora ^(27, 28). Food sources rich in Vitamin K include spinach, swiss chard, kale, beetroot, brussel sprouts and avocado ^(28, 29). Although food sources high in Vitamin K are able to reduce the effects of warfarin, maintaining an adequate intake of Vitamin K is still advised ^(28, 29). This is owing to the essential role of Vitamin K in the carboxylation of osteocalcin which is required for bone mineralisation. Additionally this is advised due to the increasing evidence that poor Vitamin K status results in increased sensitivity to warfarin with consequent fluctuation of the INR ^(28, 30-32). The daily vitamin K intake recommended for both patients on warfarin therapy and people not on therapy is 100-200mg, as per for non-warfarin users ^(28, 29, 31) . Owing to the impact that diet can have on warfarin, patients presenting with a sub-therapeutic INR, should have contributory dietary factors excluded before non-adherence can be considered.

1.2.6 Smoking and Alcohol

Both polycyclic aromatic hydrocarbons present in cigarettes and ethanol are able to induce CYP P450, thus increasing the clearance of warfarin ⁽³³⁻³⁵⁾. This can result in a sub-therapeutic INR and these patients may require slightly higher doses of warfarin. Exclusion of the latter is therefore pertinent in patients with ongoing fluctuations in the INR or persistent sub-therapeutic INRs, prior to considering non-adherence. Conversely, chronic ethanol-related liver disease can cause coagulopathy secondary to synthetic dysfunction of clotting factors as well as poor nutritional state with decreased albumin production, resulting in more free warfarin available for pharmacologic effect and associated prolonged INR ⁽³⁶⁻³⁹⁾

1.2.7 Drug Interactions

Warfarin is highly susceptible to interactions with prescription, non-prescription drugs, herbal remedies and supplements (Refer to Table 1.2). Medications interact with warfarin through numerous mechanisms. Examples of drugs which potentiate the effect of warfarin include CYP P450 inhibitors (e.g. co-trimoxazole, metronidazole, fluoxetine, fluconazole, simvastatin, amiodarone) and antibiotics (which decrease intestinal flora resulting in decreased production of vitamin K2) ^(40, 41). Drugs that induce CYP P450 (e.g. rifampin) serve to decrease the effects of warfarin ⁽⁴¹⁾. Drugs that cause increased bleeding risk independent of warfarin include anti-platelet agents i.e. acetylsalicylic acid (aspirin), non-steroidal anti-inflammatories (NSAIDs) and P2Y12 inhibitors (clopidogrel) ^(42, 43). Antidepressants, particularly selective serotonin reuptake inhibitors (SSRI's) inhibit platelet aggregation by depleting platelet serotonin levels ⁽⁴⁴⁾. Injury to gastrointestinal mucosa can be caused by NSAIDs or acetylsalicylic acid ^(45, 46).

Table 1.2 Partial list of medications that potentiate or inhibit warfarin. Adapted from Ansell et al, Hollbrook et al, Juurlink et al and Hollbrook et al ^(5, 41, 42, 47).

	Drugs that potentiate warfarin	Drugs that inhibit warfarin
Antibiotic	Co-trimoxazole, doxycycline, penicillin, metronidazole, macrolides (azithromycin, erythromycin, clarithromycin), fluoroquinolones (ciprofloxacin)	
Anti-TB treatment	Isoniazid	Rifampin
Antifungal	Fluconazole, miconazole	Griseofulvin
Antidepressant	SSRI's (fluoxetine, citalopram)	
Anti-arrhythmic/cardiac	Amiodarone, propranol	
Antiplatelet	Acetylsalicylic acid, P2Y12 inhibitors (clopidogrel)	

Anti-inflammatory	NSAIDs	
Analgesia	Tramal, paracetamol	
Gout treatment	Allopurinol	
Cholesterol-lowering agents	Simvastatin	
PUD/anti-reflux	Cimetidine, omeprazole	
Anti-epileptics		Carbamazepine, phenobarbital, phenytoin
ARV's		Protease inhibitors (ritonavir)
Alternative remedies	Gingko biloba, chamomile	Ginseng
	St. John's wort	

PUD, peptic ulcer disease, NSAIDs, non-steroidal anti-inflammatories, SSRI's, selective serotonin reuptake inhibitors, TB, tuberculosis, ARV's , anti-retroviral treatment.

1.2.8 Effects of Comorbid States on Warfarin

The liver is the primary site of warfarin metabolism and is the site of production of the majority of the clotting factors ^(38, 48). Liver disease may thus potentiate the action of warfarin through decreased CYP P450 metabolism or present an increased risk for bleeding through decrease production of the clotting factors, thrombocytopenia or portal hypertension ^(38, 48). However, liver disease may also result in a prothrombotic state by decreased production of activated Protein C ⁽⁴⁹⁾. The co-existing pro-coagulant and anticoagulant states result in labile INR's ⁽⁵⁰⁾. Aside from the warfarin independent mechanisms associated with increased bleeding risk in chronic kidney disease, potentiation of warfarin occurs with decrease in CYP P450 metabolism secondary to inhibition by inflammatory cytokines ⁽⁵¹⁻⁵³⁾. Heart failure has also been associated with decreased stability in INR control. This is owing to decreased warfarin metabolism secondary to liver congestion ^(54, 55).

1.2.9 Genetic Factors

There are many genetic mutations in the gene encoding CYP P450, which is responsible for the metabolism of warfarin in the liver ⁽⁵⁶⁾. The most common mutations are CYP 2C9*2 and CYP 2C9*3 which cause decreased metabolism of warfarin with consequent decreased dose requirements ^(56, 57). Single nucleotide polymorphisms in the gene encoding VKOR1 cause variable sensitivity to the inhibition by warfarin ⁽⁵⁸⁾. The most common of which is VKORC1 1173 CC which is associated with decreased sensitivity to warfarin with increase in dose requirement ⁽⁵⁸⁾.

1.2.10 Side Effects

Aside from an increased risk of bleeding, warfarin is also a known teratogenic drug, which is contraindicated in the first trimester of pregnancy owing to associated bone related foetal abnormalities ^(5, 59). During the initiation of therapy, warfarin-induced skin necrosis can occur secondary to the short half-life of the natural anti-coagulant, Protein C ⁽⁶⁰⁾. Cholesterol embolization “purple toe” syndrome is a rare side effect of warfarin ⁽⁶¹⁾.

1.3 Coagulation in Vivo –The Coagulation Cascade

Coagulation is initiated post vascular injury, by the interaction of tissue factor (TF) and CF VII (refer to Figure 1.2) ⁽⁶²⁾. This complex is able to activate both CF X and CF IX. In the absence of a cofactor, CF Xa forms small amounts of thrombin (CF IIa) from prothrombin (CF II) ⁽¹⁴⁾. Trace amounts of prothrombin consequently activate CF's V, VIII and IX. In the amplification phase of coagulation, CF XIa activates CF IX, which forms a complex with CF VIIIa, (tenase) on the phospholipid surface in presence of calcium. The tenase complex allows activation of CF X, to form a complex with CF Va (prothrombinase) ⁽¹⁴⁾. This results in an explosive thrombin burst in which fibrinogen can be converted to fibrin, in order to form a fibrin clot ⁽⁶²⁾. Thrombin binds to the endothelial cell surface receptor, thrombomodulin, which activates protein C when bound to the endothelial protein C receptor ⁽⁶²⁾. In conjunction with its cofactor, protein S, activated protein C inhibits CF Va and CF VIIIa, which prevents further generation of thrombin ⁽⁶²⁾.

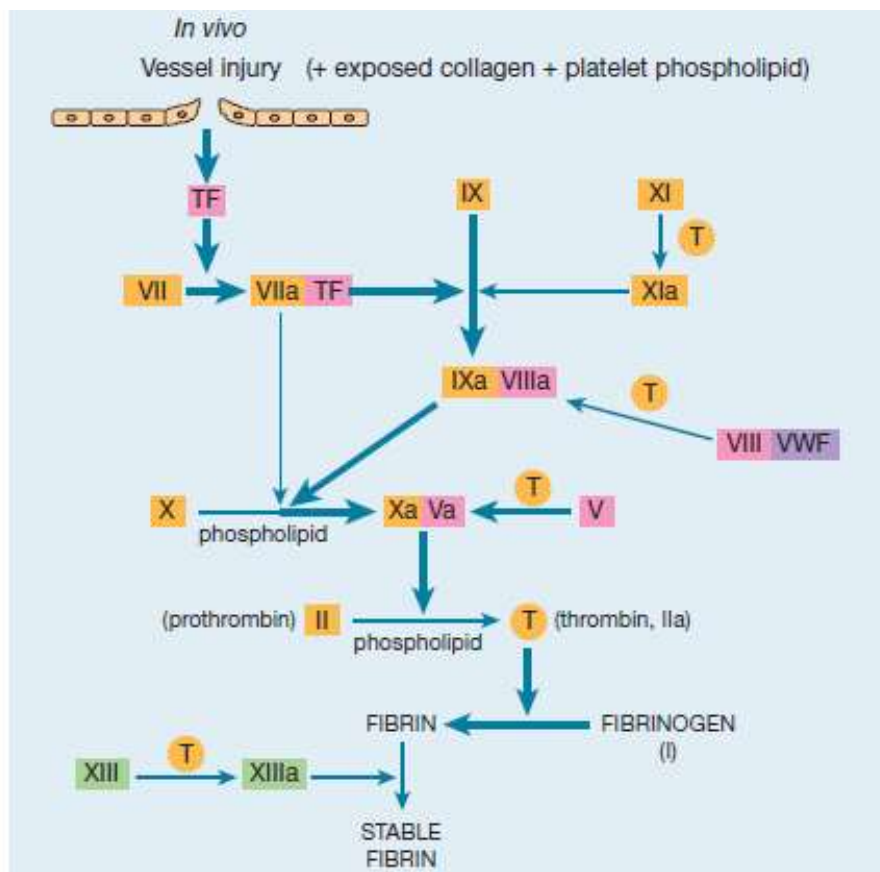


Figure 1.2 The in vivo model of the coagulation cascade. Adapted from Hoffbrand et al. ⁽⁶²⁾

1.4 Coagulation in Vitro

1.4.1 International Normalised Ratio and Prothrombin Time

Patients on warfarin can be monitored through laboratory testing of the prothrombin time (PT) which is a reflection of the extrinsic and common pathway of coagulation as occurs in vitro (shown in Figure 1.3) ⁽¹⁴⁾. Here, the levels of CF V, VII and X are measured, as well as CF II and fibrinogen which form part of the common pathway ⁽¹⁴⁾. Coagulation is initiated using thromboplastin (tissue factor), phospholipid and calcium. The PT will be prolonged by deficiencies of CFs VII, X, V, II or fibrinogen ⁽¹⁴⁾. The PT can be used to monitor warfarin as 3 of the 4 vitamin K dependent factors are tested i.e. CF II, VII and X ⁽¹⁴⁾. Due to variation in the sensitivity of the thromboplastin reagent which is used in PT testing, an international sensitivity index (ISI) number is given to each reagent for standardisation purposes ^(63, 64). The ISI value for a thromboplastin reagent is relative to an international reference tissue factor. Thus the PT is expressed as the International Normalised Ratio (INR) i.e. $INR = (\text{patient PT} / \text{mean normal PT})^{ISI}$ ⁽⁶³⁾. Laboratory related limitations in INR testing include, incorrect ISI of the thromboplastin reagent, varying concentrations of trisodium citrate present in collection tubes, variations in manual testing techniques and errors in sample collection, including insufficient sample material, incorrect storage time and temperature ⁽⁵⁾.

1.4.2 Activated Partial Thromboplastin Time

The activated partial thromboplastin time (APTT) measures the intrinsic and common pathway of coagulation as occurs in vitro (refer to Figure 1.3) ⁽¹⁴⁾. In this pathway coagulation is triggered using a negatively charged surface, such as kaolin or silica to initiate contact activation. Further progression requires the addition of phospholipid and calcium ⁽¹⁴⁾. The APTT will be prolonged by deficiencies in CF's XII, XI, IX, VIII, X, V, II, fibrinogen and high molecular weight kinogen ⁽¹⁴⁾. As factor CF IX forms part of the intrinsic pathway, it is the only Vitamin K dependent CF which is tested via the APTT alone.

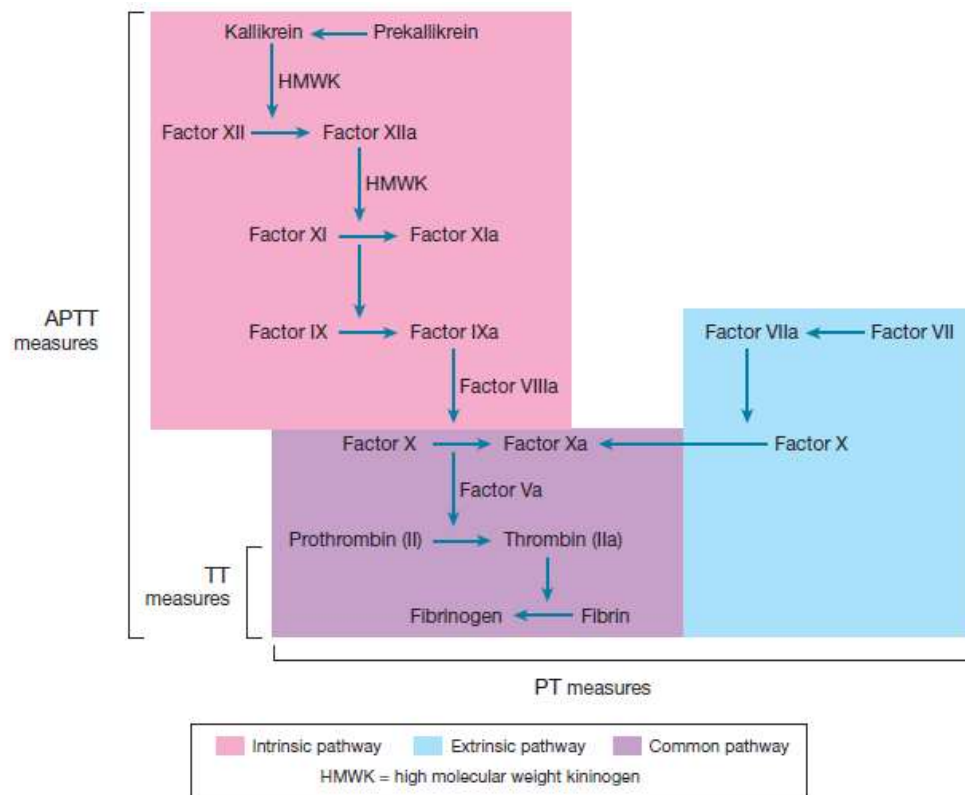


Figure 1.3 The in vitro model of the coagulation cascade. Adapted from Hoffbrand et al ⁽⁶²⁾.
APTT, activated partial thromboplastin time, PT, prothrombin time, TT, thrombin time

1.4.3 Factor Assay Principle

For the testing of CF II and CF VII, the extrinsic pathway of coagulation is tested. The PT factor assay consists of the measurement of clotting time, in the presence of a thromboplastin reagent, factor deficient plasma and patient's plasma; in a system in which all factors are present, and in abundance, except for the CF that is being tested which only originates from the test plasma. Patient plasma deficient in a specific factor will not be able to accommodate the absence of a factor in the corresponding factor deficient plasma and produce a prolonged PT assay time.

1.5 Time in Therapeutic Range and Non-adherence to Warfarin

The goal of warfarin anticoagulation is to achieve and maintain a therapeutic target INR for a given indication ⁽⁶⁵⁾. In most instances the range of a therapeutic INR is between 2-3, but can be up to 3.5 in patients with valvulopathy ⁽⁶⁾. An INR above therapeutic range is associated with an increased risk of bleeding and an INR below a therapeutic range is associated with an increased risk of venous thromboembolism (VTE) ^(5, 65). To achieve an ongoing desired therapeutic effect, regular INR monitoring and warfarin dose adjustments are required.

The quality of the INR control is assessed by determining the percentage of time in therapeutic range (TTR), which is a measurement of an INR in therapeutic range over a definite period of time. A TTR of ~65-70% is considered to be good quality control ^(66, 67). The TTR is influenced by a number of factors that include age, non-adherence, diet, medication, alcohol, smoking, genetic aberrations and many other disease states ⁽⁶⁸⁻⁷⁰⁾.

In a number of studies, non-adherence was shown to be a major contributor to poor anticoagulation control with reported rates of up to 92% ⁽⁷¹⁻⁷⁶⁾. Results of the “In-Range” study showed that factors associated with poor adherence included education beyond high school, current employment, lower mental health functioning, and poor cognition ⁽⁶⁸⁾. Other reasons for poor adherence include complexity of dosing regimen, high medication costs, suboptimal communication between healthcare provider and patient, dietary and alcohol restrictions and co-existing comorbidities ⁽⁷⁷⁻⁷⁹⁾. Despite the many variables described, up to 30% of sub-therapeutic INR’s cannot be explained after extensive patient assessment ⁽⁷⁴⁾.

Apostolakis et al. proposed and validated a score to predict quality of warfarin anticoagulation as measured by TTR (SAME-TT2R2 score) (refer to Table 1.3) ⁽⁶⁹⁾. This score was derived from the AFFIRM (Atrial Fibrillation Follow up Investigation of Rhythm Management). The score is comprised of gender, medical history, interacting drugs, current tobacco use and race ⁽⁶⁹⁾. A score of 0-2, suggests that a patient is likely to achieve a high TTR >65% and is a suitable candidate for warfarin. A score of ≥ 3 suggests that either the patient should receive additional education regarding anticoagulation control or should be considered as a more

suitable candidate for a DOAC. This is owing to the decreased drug, dietary, and disease interactions offered by the DOACs which are prescribed at fixed doses and do not require routine monitoring. When comparing the DOAC's, Edoxaban has shown the best TTR, and has been proven to be non-inferior to warfarin.⁽⁸⁰⁾

Table 1.3 The SAME-TT₂R₂ score to predict time in therapeutic range, adapted from Apostolakis et al ⁽⁶⁹⁾

Acronym	Variable	Score
S	Sex (female)	1
A	Age (<60years)	1
M	Medical history*	1
E		
T	Treatment with interacting drugs	1
T	Tobacco	2
R	Race (Non-Caucasian)	2
Maximum points		8

*Medical history (≥ 2 of the following); hypertension, diabetes, coronary artery disease/myocardial infarction, peripheral arterial disease, congestive heart failure, previous stroke, pulmonary disease, hepatic or renal disease.

CHAPTER 2. RATIONALE, AIM AND OBJECTIVES

2.1 Rationale

Poor time in therapeutic range with an ongoing sub-therapeutic INR may raise suspicion for non-adherence, once other confounding variables have been excluded. However, in non-adherence states, participants may still have a therapeutic INR owing to the short half-life of CF VII^(15, 81). As rates of non-adherence to warfarin are reportedly as high as 92%, determination of true non-adherence in a supposedly adherent group with a therapeutic INR would be of interest. Clinical suspicion of poor adherence may be further investigated by measurement of the vitamin K dependent CF's^(81, 82). A concurrent appropriate decrease in plasma levels of both CF VII and II would indicate adherence to warfarin therapy. A result which shows a therapeutic INR, but discrepant CF II and CF VII plasma levels is essentially the same as a sub-therapeutic INR which places a participant at increased risk for VTE⁽⁸²⁾. This study sought to test the hypothesis that in participants on warfarin with a therapeutic INR, plasma levels of CF II and CF VII may be used as a proxy for assessment of adherence to warfarin therapy. Despite previous studies which have shown the efficacy in monitoring vitamin K dependent factors as a means of monitoring warfarin therapy^(17, 83, 84); no studies have been done to date to determine non adherence to warfarin by testing of the vitamin K dependent CF's. This study would be the first of its kind to investigate the incidence of non-adherence in a patient group with a therapeutic INR.

2.2 Aim

This study sought to test the hypothesis that CF II and CF VII in patients with a therapeutic INR may be used as a proxy to test for adherence to warfarin therapy.

2.3 Objectives

- 1) To correlate the CF II and CF VII levels to the INR in the study group (participants with a therapeutic INR) and control group (participants with a sub-therapeutic INR).
- 2) To determine the sensitivity and the specificity of the INR, using the CF levels in the study group and the control group.
- 3) To compare and correlate demographic information (age, gender, race), indication for anticoagulation, duration of treatment on warfarin, dose, previous INR values and concomitant medication.
- 4) To make recommendations regarding the usefulness of using the vitamin K dependent CF's in monitoring adherence to warfarin therapy.

CHAPTER 3. METHODS AND MATERIALS

3.1 Study Design and Site

This was a single centre, prospective, cross-sectional study which was undertaken at the Anticoagulation Clinic at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), a quaternary level hospital in Johannesburg, South Africa, from September to December 2015. All laboratory analysis was undertaken at National Health Laboratory Services (NHLS), CMJAH. The Anticoagulation Clinic is an outpatient clinic, located in the green block, area 454, CMJAH and is dedicated to monitoring patients on warfarin. On average ~50 patients are booked daily. Permission to conduct this study, including the collection of blood samples and patient history was obtained from the Chief Executive Officer of CMJAH (Appendix A for the permission letter) and the Human Research Ethics Committee (HREC) of the University of Witwatersrand, Johannesburg (Clearance No: M150213 Appendix C). The study was conducted in compliance with the Declaration of Helsinki and all participants gave written informed consent (Appendix B for the information leaflet and informed consent)

3.2 Study Population

Patients included in the study were those attending the Anticoagulation Clinic at CMJAH who had been booked for a routine clinic visit. Study participants were ≥ 18 years of age, attending the Anticoagulation Clinic at CMJAH on steady state warfarin therapy for ≥ 3 months for either treatment or prophylaxis of VTE with a target INR (2-3.5). Clinical data including age, sex, race, anticoagulation indication, dose, duration of time on warfarin, concomitant medication, and previous INR values were extracted from Anticoagulation Clinic outpatient records. A control group comprising patients with a sub-therapeutic INR was also obtained.

3.2.1 Eligibility Criteria

3.2.1.1 Inclusion Criteria

- Patients attending the Anticoagulation Clinic at the CMJAH.
- Patients ≥ 18 years.
- All patients on steady state warfarin (>3 months) for any indication including prevention and treatment of VTE, those with AF and vascular diseases.
- Patients with an INR which is within therapeutic range which is defined as 2-3.5.
- Those patients who have given written informed consent

3.2.1.2 Exclusion Criteria

- Patients younger than 18 years of age.
- Patients with an INR out of therapeutic range (sub-therapeutic (<2) and supra-therapeutic INR (>3.5))
- Those patients who had not given informed consent.

3.3 Specimen Collection and Storage

All participants were informed verbally by the primary researchers about the study and thereafter were given time to read the information leaflet prior to signing informed consent. As participants were those already booked for the Anticoagulation Clinic for the day, they all required a sample of blood to be drawn for an INR test. For the study, a second tube of blood was collected in the same blood draw and second venepuncture was not required. The blood samples were taken by trained phlebotomists in area 358, orange block, CMJAH. Blood was collected in 0.109M, 3.2% buffered sodium citrate tubes and reached the laboratory within 30 minutes of collection.

On receipt in the laboratory, the samples were taken to the coagulation bench of the haematology laboratory, NHLS, CMJAH to be run. Samples were centrifuged within one

hour of collection and the INR measured as per routine procedure. For factor analysis, the samples were processed within one hour and were centrifuged at 3500rpm (rev per minute)/ (2000-2500g relative centrifugal force (RCF)) at ambient room temperature (18-25 °C) for 15 minutes to obtain platelet poor plasma. Aliquots for factor testing were frozen at -80°C and maintained at this temperature for a maximum period of one month before the samples could be run. Factor analysis was performed on the STA-R Evolution® II (Diagnostica Stago, Asnières sur Seine, France). Clotted samples and those with insufficient volume were not accepted for analysis as per the NHLS standard operating procedure (SOP) GPL2849.

3.4 Prothrombin Factor Assay Principle

The prothombin (PT) factor assay consists of the measurement of clotting time, in the presence of the thromboplastin reagent, STA® neoplastine (Diagnostica Stago, Asnières sur Seine, France), STA® factor deficient plasma (Diagnostica Stago, Asnières sur Seine, France) and the patient's plasma; in a system in which all factors are present, constant and in excess, except that factor which is derived from the sample being tested. Patient plasma deficient in a specific factor will not be able to accommodate the absence of a factor in the corresponding factor deficient plasma and produce a prolonged PT assay time.

- STA® neoplastine: contains lyophilized thromboplastin prepared from human recombinant tissue factor and from phospholipid.
- The STA® II factor deficient plasma (Diagnostica Stago, Asnières sur Seine, France) contains lyophilized citrated human plasma from which FII has been removed by selective immuno-adsorption.
- The STA® VII factor deficient plasma (Diagnostica Stago, Asnières sur Seine, France) contains lyophilized citrated human plasma from which FVII has been removed by selective immuno-adsorption.

3.5 Reagent Preparation and Storage

The storage and preparation of the reagents were carried out according to the corresponding package inserts and the NHLS SOP GPL2849. The storage, reconstitution, stabilisation and on board stability of the reagents used in the factor testing are shown in Table 3.1. STA® neoplastine, STA® factor deficient II and VII, STA® universal calibrator (Diagnostica Stago, Asnières sur Seine, France), STA® system control N and P (Diagnostica Stago, Asnières sur Seine, France) are available as lyophilised reagents and were reconstituted. The reagents were stored at a temperature of 2-8°C and remained viable until the date of expiration stated on the box. The stabilisation time refers to the amount of time required once the reagents had been reconstituted before they are allowed to be loaded on the coagulation analyser. Beside the STA® Owren-Koller (Diagnostica Stago, Asnières sur Seine, France) which is ready to use, all other reagents required 30 minutes of reconstitution time at room temperature (18-25°C). On board stability refers to the time post reconstitution and stabilisation of the reagents which varied for each of the reagents. For CF II testing, STA® factor II deficient plasma was used and for CF VII testing, STA® factor VII deficient plasma was used.

Table 3.1. Storage, reconstitution, stabilisation time and on board stability of reagents

Reagent	Storage Temp	Reconstitution	Stabilisation time	On board stability
STA® Neoplastine	2-8°C	15ml (1 vial) of calcium containing solvent. Shake vigorously to obtain a homogeneous solution.	30 min (room temperature)	5 days capped
STA® Factor Deficient II and VII.	2-8°C	1ml sterile water	30 min (room temperature)	8 hours
STA® Owren-Koller	2-8°C	Ready for use.	Not required	72 hours
STA® Universal	2-8°C	1ml sterile water		8 hours

Calibrator			30 min (room temperature).	
STA® System	2-8°C	1ml sterile water	30 min (room temperature).	8 hours
Control N and P				

Abbreviations temp, temperature, min, minutes

3.6 Sample Testing

The procedure for factor analysis is described in the NHLS SOP GPL2849. Apart from the specific factor deficient plasma reagent used, the procedure for measurement of both CF II and VII testing is the same. All factor analysis were performed on the STA-R Evolution® II (Diagnostica Stago, Asnières sur Seine, France) instrument in the coagulation bench of the haematology laboratory at the NHLS, CMJAH. The quality control and maintenance steps of the instruments were performed according to the NHLS SOPs NJHH0047. Patient samples were allowed to thaw at room temperate (18-25°C) and then warmed in a water bath at 37°C. Once thawed, samples were not refrozen for later analysis. Samples were run in batches of ~25-50 samples per a run. Factor analysis was done on both the study group (participants with a therapeutic INR) and the control group (participants with a sub-therapeutic INR).

For factor analysis of 25 samples, the amounts of reagent required were as follows:

- STA® neoplastine (2 bottles lyophilised reagent, reconstituted with 2 bottles of solvent (15ml per bottle).
- STA® factor II/VII deficient plasma (4 bottles lyophilised reagent) reconstituted with 1ml of sterile water per bottle.
- STA® Owren-Koller buffer (2 bottles, 15ml per bottle), ready for use.
- STA® system control plasma N and P (1 bottle each, each reconstituted with 1 ml of sterile water)
- STA® universal calibrator (1 bottle lyophilised reagent reconstituted with 1ml sterile water)

If a batch of 50 samples was run, the reagents used were doubled. The stabilisation time of the reconstituted agents and the on board stability have been described in Table 3.1. The reagent bottles were thereafter scanned onto the STA-R Evolution® II analyser and loaded by placement into the appropriate product draw. Each peripheral blood sample had a unique identity number which was scanned by the analyser.

Calibration was first performed on the instrument. Reference curves were generated by the analyser from dilutions with the calibration reagent, STA® factor deficient plasma and STA® Owren-Kollmer buffer. The curves generated are a measure of time to clot, in a serially diluted sample. An R value of >0.95 was taken as an appropriate calibration.

Thereafter the quality control was run. Each control package insert was scanned onto the system to generate a “LOT” number which provided a reference range that each control should fall within. The controls were run using STA® system control plasma N and P.

After calibration and quality control steps had passed and accuracy and quality of the results could be ensured, the peripheral blood samples were run for CF II and VII levels. Dilutions of patient sample with STA® Owren-Koller buffer were automatically prepared by the instrument. Time to clot from the patient curve was read off from the calibration curve to extrapolate the factor value. Patient factor levels were reported as a percentage in the “test panel/ test status” screen of the coagulation analyser. The reference interval for normal levels of CF II and VII as used by NHLS, CMJAH are both 50-150% (GPL 2849). If any of the results fell outside of this range, the instrument automatically retested the sample at the appropriate dilution.

Where trouble shooting was required, the following steps were followed:

- On-board stability factors of the reagents was checked.
- Stabilisation time of the reagents was checked.
- Expiration of the reagents was checked.
- Appropriate reconstitution of the reagents was checked.
- Daily maintenance of the instrument including needle wash was performed.
- Samples were checked for contamination including use of the same pipette.

3.7 Interpretation of Results

Study Group (Therapeutic INR)

- 1) Those with sufficiently decreased CF II and VII levels
 - CF II and VII levels <50% will indicate appropriate anticoagulation by warfarin and true adherence.
- 2) Those with insufficiently decreased (normal) CF II and VII levels.
 - CF II/VII >50% will indicate inadequate anticoagulation and non-adherence to warfarin.

Control group (Sub-therapeutic INR)

- 1) Those with normal CF II and VII levels.
 - CF II and VII level $\geq$ 50% will indicate inadequate anticoagulation by warfarin and true non-adherence.
- 2) Those with sufficiently decreased CF II and VII levels.
 - CF II and VII <50% will indicate appropriate anticoagulation by warfarin and adherence.

3.8 Limitations of the Assay

The presence of heparins or thrombin inhibitors, factor antibodies and lupus anticoagulants in the sample may result in spurious factor results. This will be minimised by testing only patients who are not hospitalised. Partial activation of the coagulation factors due to incorrect sample handling can lead to falsely elevated factor levels. Sampling was done by trained and very experienced phlebotomists employed by the NHLS to minimise false results.

3.9 Statistical Analysis

Sample size analysis was determined by the key research questions and was based on the chi-square test and shown to be meaningful for analysis ⁽⁸⁵⁾.

Descriptive statistics were performed on the study and control group population using data extrapolation from clinical outpatient records. Categorical variables were summarised by frequency and percentage tabulation and illustrated by means of bar charts. Continuous variables were summarised by the mean, standard deviation, median and interquartile range, and their distribution illustrated by means of histograms.

Time in therapeutic range was calculated in the study group and this was correlated with INR group membership, age, gender, ethnicity, treatment duration, dose and selected medications.

Data analysis was carried out using SAS (version 9.4 for Windows Cary, North Carolina, USA). The X^2 test was used to assess the relationships between categorical variables.

Fisher's exact test was used for 2 x 2 tables or where the requirements for the X^2 test could not be met. The strength of the associations was measured by Cramer's V and the phi coefficient respectively.

The following scale of interpretation was used:

- ≥ 0.50 and above strong association
- 0.30 to 0.49 moderate association
- 0.10 to 0.29 weak association
- < 0.10 little if any association

The relationship between continuous and categorical variables was assessed by the t-test (or ANOVA for more than two categories). Where the data did not meet the assumptions of these tests, a non-parametric alternative, the Wilcoxon rank sum test or the Kruskal-Wallis test for more than two categories was used. The strength of the associations was measured by the Cohen's d for parametric tests and the r-value for the non-parametric tests.

The following scale of interpretation was used:

- ≥ 0.80 large effect
- 0.50 to 0.79 moderate effect
- 0.20 to 0.49 small effect
- < 0.20 near zero effect

The analysis of the relationship between INR group and predictor variables was carried out using multinomial logistic regression, with INR group as the dependent variable and the selected predictor variables as independent variables. Each of the selected independent variables was first examined on its own and variables with p-values < 0.20 were retained for combined analysis. Prior to combined analysis, strong associations between independent variables were identified by chi-squared (or Fisher's exact) test for pairs of categorical variables, ANOVA (or the Kruskal-Wallis test) for categorical-continuous variable pairs, and Spearman's rank correlation coefficient for pairs of continuous variables. A P-value < 0.05 was used to indicate significant results.

CHAPTER 4. RESULTS

Of the 359 patients screened, 350 participants met inclusion criteria and were enrolled in the study (refer to figure 4.1). Nine participants were excluded as they were on warfarin for insufficient time (<3 months). The INR was sub-therapeutic in 92 participants (26.2%), supra-therapeutic in 45 participants (12.9%) and therapeutic in 213 participants (60.6%). Factor II and VII were measured in 204 of the 213 (95.8%) blood samples with therapeutic INRs and in 79 of 92 (85.9%) blood samples with sub-therapeutic INRs. Sample clotting, insufficient sample volume, quality assurance failures and limited reagents were the reasons for not being able to measure CF levels in the 22 participants.

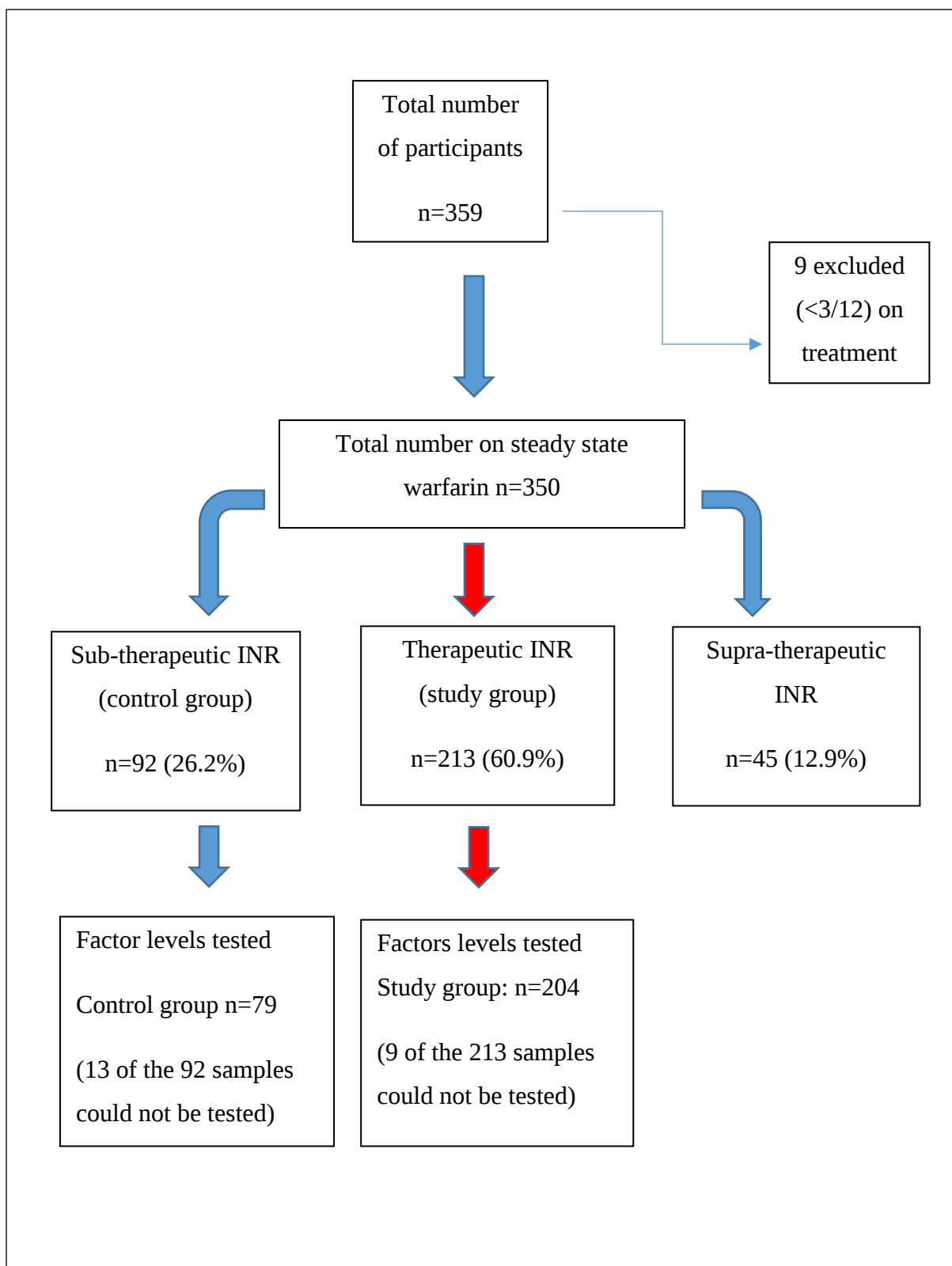


Figure 4.1 Flow diagram showing sample collection

Demographic data for the study population is shown in Table 4.1. In the study group, the median age of the participants was 56 years and the range was 18-91 years (Figure 4.2). The age group with the highest number of participants on warfarin was 61-70 years of age. The majority of the patients were female (62.7%) and the majority of the patients were black (60.8%).

Table 4.1 Demographic information of the study and control group population

	Study group	Control group
Number of participants, n	204	81
Age (years) median, (range)	56 (18-94)	51 (18-86)
Gender, n (%)		
Male	76 (37.3)	16 (19.8)
Female	128 (62.7)	65 (80.2)
Race, n (%)		
Asian	15 (7.4)	4 (4.9)
Black African	124 (60.8)	57 (70.4)
Caucasian	54 (26.5)	14 (17.3)
Mixed race	11 (5.4)	6 (7.4)

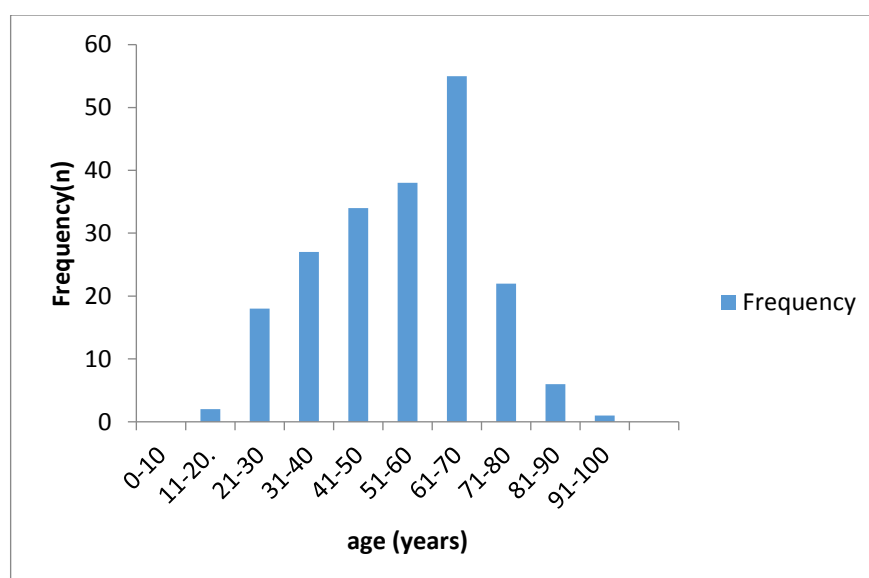


Figure 4.2. Age group distribution of the study population

Anticoagulation for AF, cardiac valve pathology and VTE were the most common indications for the use of warfarin in our study population (Table 4.2). Potassium chloride, simvastatin, acetylsalicylic acid, omeprazole, paracetamol and highly active anti-retroviral therapy (HAART) were the most common concomitant treatments.

Table 4.2 Indications for anticoagulation and concomitant medication in the study and control group population.

	Study group	Control group
Indication for anticoagulation, n (%)		
Atrial fibrillation	62 (30.3)	20 (24.7)
Cardiac valve pathology	69 (33.8)	22 (27.1)
Pulmonary Embolus	37 (18.1)	18 (22.2)
Lower limb deep vein thrombosis	25 (12.3)	15 (18.5)
Thromboses at other sites	13 (6.4)	5 (6.17)
Other indications*	27 (13.2)	10 (12.3)
Concomitant treatment, n (%)		
Potassium Chloride	33 (16.2)	13 (16.0)
Simvastatin	18 (8.8)	6 (7.4)
Acetylsalicylic acid	17 (8.3)	6 (7.4)
Omeprazole	16 (7.8)	10 (12.3)
Paracetamol	11 (5.4)	11 (13.5)
ARV's	11 (5.4)	9 (11.1)
Tramadol hydrochloride	6 (2.9)	5 (6.2)
Tuberculosis treatment	5 (2.5)	3 (3.7)
Allopurinol	4 (2.0)	1 (1.2)
Clopidogrel	4 (2.0)	1 (1.2)
Penicillin VK	3 (1.5)	1 (1.2)
Sodium Valproate	3 (1.5)	2 (2.5)
Fluoxetine	1 (0.5)	0 (0.0)
Hormone therapy	1 (0.5)	0 (0.0)
Methotrexate	1 (0.5)	2 (2.5)
Prednisone	0 (0.0)	2 (2.5)

Abbreviation HAART, highly active anti-retroviral treatment.

*Other indications included antiphospholipid syndrome, systemic lupus erythematosus, cerebrovascular accident, transient ischaemic attack, ischaemic heart disease, coronary bypass, pacemaker, Eisenmenger Syndrome, critical limb ischaemia, femoral bypass, pulmonary hypertension, malignancy, nephrotic syndrome and polycythaemia.

Time on warfarin in the study group ranged from 3 months to 288 months with a median of 43 months (Table 4.3 and Figure 4.3). The group with the most participants were those on warfarin for 3-12 months (Figure 4.3) The dose of warfarin treatment ranged from 2.35mg to 67.5mg with a mean of 32.95mg weekly (Table 4.3 and Figure 4.4.)

Table 4.3 Characteristics of treatment and clotting factor levels in the study and control group population.

	Study group	Control group
Duration (months), median (range)	43 (3-288)	34.0 (3-360)
Dose (mg), median (range)	32.5 (2.35-67.5)	35.0 (0.5-55)
Factor levels(%), median (range)		
Factor II	24.5 (11-62)	37.5 (21-81)
Factor FVII	22.0 (6-58)	38.0 (17-127)

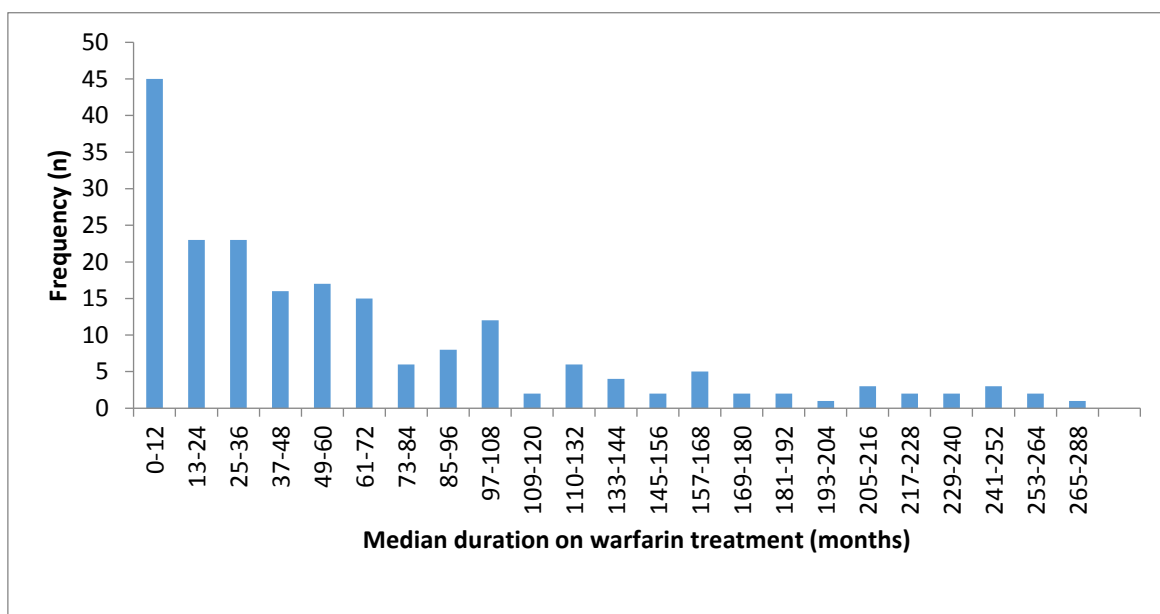


Figure 4.3 Duration of time on warfarin in the study group population.

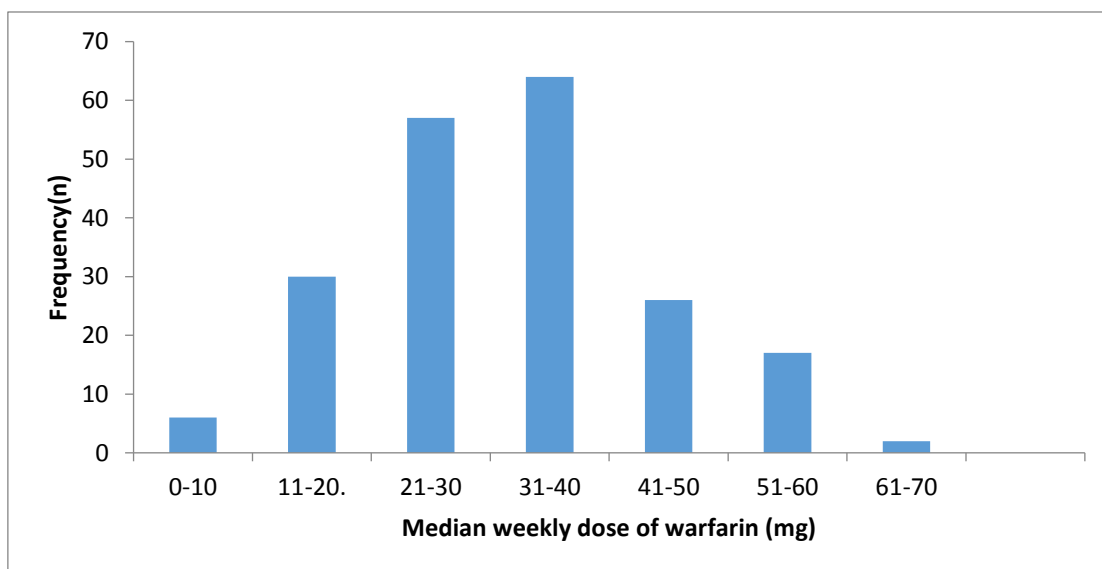


Figure 4.4 Therapeutic dose of warfarin in the study group population.

Based on analysis of 4 consecutive INRS, over a 4 monthly interval (Figure 4.5), 32.4% of the participants had all four INRs in the therapeutic range i.e. 100% TTR, 39.2% had three INRs in range and 19.1% had two therapeutic INR's and 9.3% of the patients had only one INR in range.

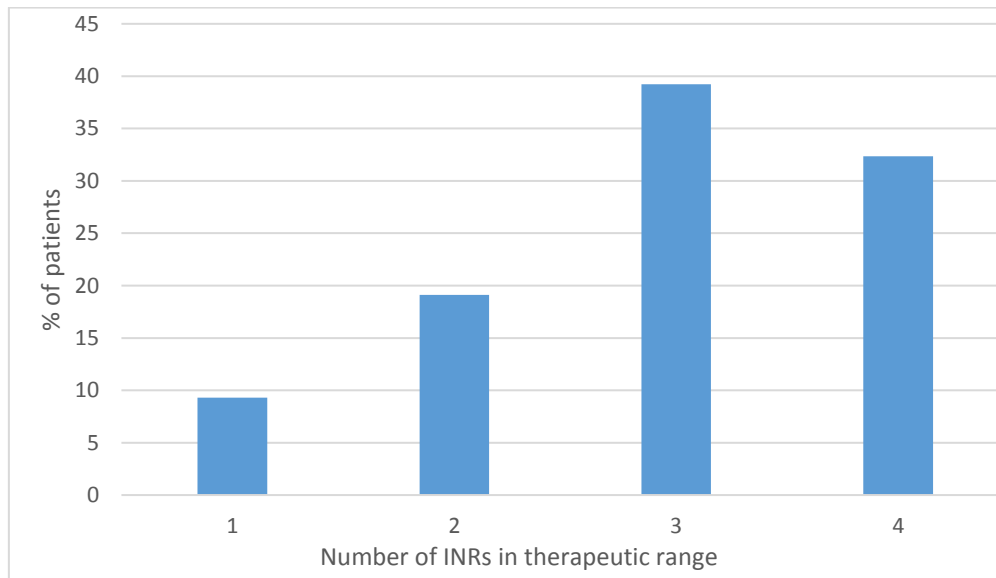


Figure 4.5 Time in therapeutic range shown by the study population

Significant associations between race, concomitant treatment and adherence to warfarin were demonstrated on univariate analysis. A multinomial regression analysis revealed a significant association between race and INR group ($p < 0.0001$; Cramer's $V = 0.24$). As shown in Figure 4.6, Asian participants had the least number of therapeutic INRs whereas Caucasians had the highest number of therapeutic INRs. The odds of an Asian participant with the least TTR was 15 times that of an Asian participant being in the group with the highest TTR (Odds Ratio=15; 95% CI 3.0-73).

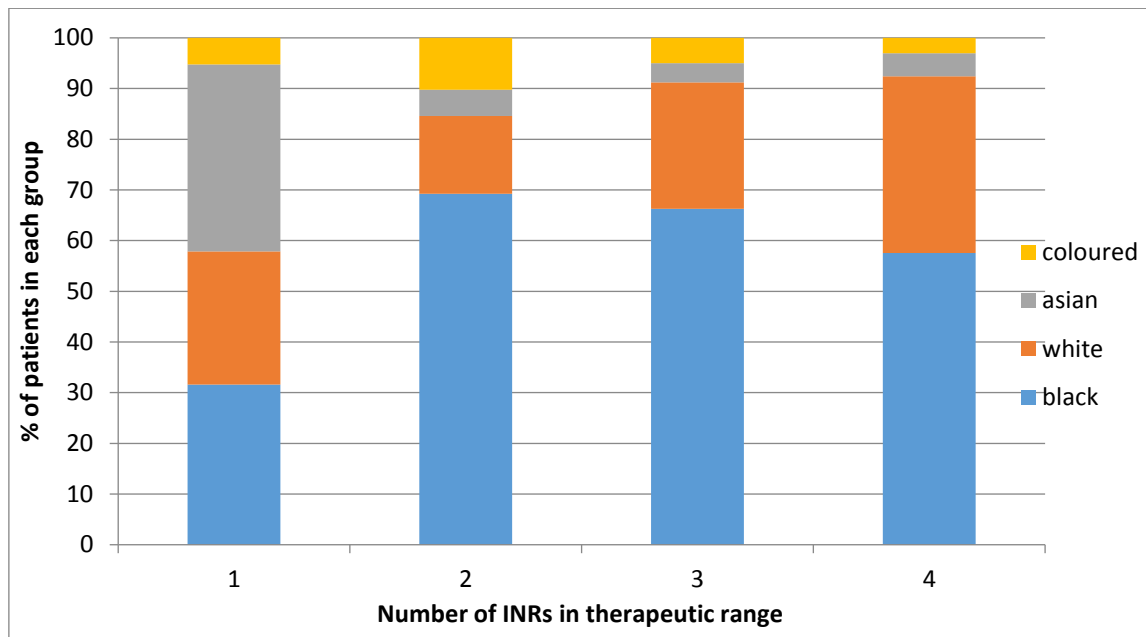


Figure 4.6 Race distribution of participants by number of INRs in the therapeutic range.

There was a statistically significant ($p=0.037$) but weak (ϕ coefficient=0.20) association between the use of potassium chloride and a therapeutic INR (Figure 4.7). The use of potassium chloride was higher in participants with higher number of therapeutic INR values. There was a statistically significant ($p=0.047$) but weak association (ϕ coefficient=0.21) with simvastatin being used less in the group with higher number of therapeutic INRs.

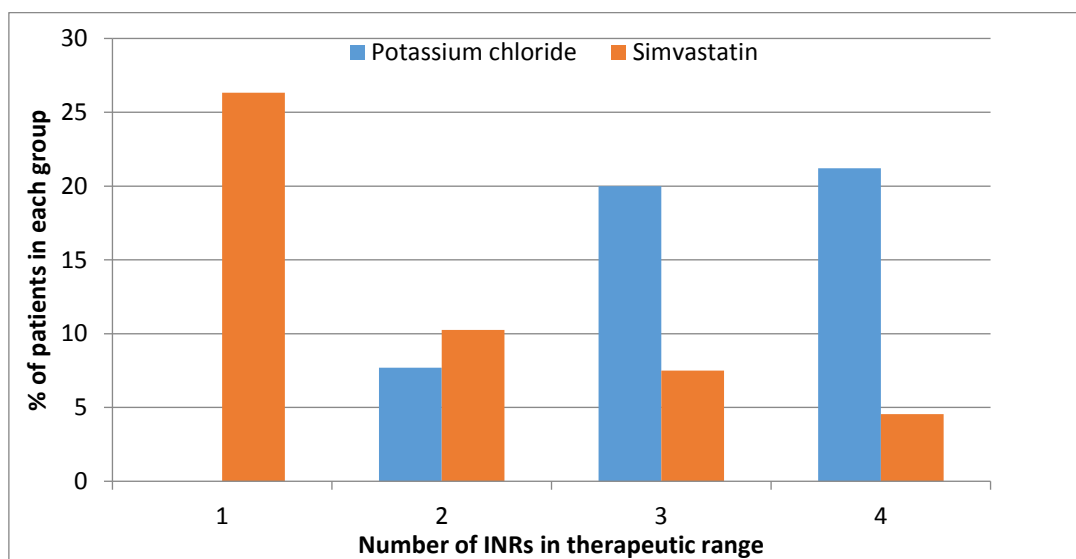


Figure 4.7 Use of simvastatin and potassium chloride in participants by number of INRs in the therapeutic range.

The median (range) CF II plasma level in the study and control groups were 24.5 % (11-62%) and 37.5 % (21-81%) respectively (Table 4.3). The median (range) CF VII plasma level was 22.0 % (6-58%) in the study group and 38.0 % (17-127%) in the control group. The study group (n=204) had 202 participants with proportionately low CF II and CF VII plasma levels as expected on warfarin therapy. There were 2 participants with discrepant CF II and VII levels. In one of the two, CF VII level was low (14%) and CF II level was normal (62%) with a therapeutic INR of 3.32. In the second participant, CF VII was 58% and CF II was 18% with a therapeutic INR of 2.14.

In the sub-therapeutic INR cohort, 51 participants (64.6%) still showed sufficient anticoagulation while only 28 participants (35.4%) were insufficiently anticoagulated based on the measured CF II and CF VII levels. Based on the CF II and VII levels, there were 253 adherent participants (202 with therapeutic INR and 51 with a sub therapeutic INR) and 30 non-adherent (2 with therapeutic INR and 28 with non-therapeutic INR). The sensitivity and specificity of CF II and CF VII plasma levels to predict adherence were evaluated using a 2x2 table shown Table 4.4. In relation to the INR, the results showed a sensitivity of 79.8% and a specificity of 93.3% of predicting adherence and non-adherence to warfarin respectively.

Table 4.4 Sensitivity and specificity of factor level in predicting therapeutic INR

		Adherence		
		No	Yes	Total
Therapeutic INR	No	28	51	79
	Yes	2	202	204
	Total	30	253	283

CHAPTER 5. DISCUSSION

Adherence to warfarin therapy is of particular importance in achieving a therapeutic INR and avoiding risk of associated thrombotic or bleeding events ^(5, 65). The high incidence of warfarin non-adherence ranging from 10-92% in participants on chronic oral anticoagulation remains a concern necessitating better tools to evaluate adherence, which formed the rationale for exploring the use of vitamin K dependent CF's in predicting adherence to warfarin anticoagulation in this study ⁽⁷¹⁻⁷⁶⁾.

The study population was comprised predominantly of black females (68.4%) with comparable median ages between the study and control groups (56 years versus 51 years) (Table 4.1). The participant gender and race distribution in our study mirrors the demography of attendees in our hospital. The main indications for anticoagulation in the study population were AF, cardiac valve pathology and VTE (Table 4.2). There were 32 young adults (<35 years) (15.7%) in the study population. The main indication for warfarin in this group was valvulopathy including cardiac valve replacements (16 participants). This is most likely secondary to congenital heart disease or complications from rheumatic heart disease. In the study group there were 66 participants (32.4%) that were above the age of 65 years which is a high risk group, associated with increased incidence of comorbidities. The main indication for anticoagulation in this group was lower limb DVT (34 participants) and AF (16 participants). Patients >65 years of age are at increased risk for stroke in accordance with the CHADS-VASC score, as well as at increased risk of bleeding according to the HAS-BLED score ^(86, 87).

The group with the most participants were those on warfarin for 3-12 months (Figure 4.3). This reflects the recommended treatment guidelines of 3 months for VTE, in the absence of an ongoing provoking factor ⁽⁶⁾. A normal dose of warfarin ranges between 2-10mg daily ⁽⁵⁾. In the study group the highest dose of warfarin therapy was 67mg (Figure 4.4). Although this may be suggestive of resistance to warfarin and warrant further monitoring and mutation analysis, warfarin resistance is defined as warfarin dose requirements greater than 70 mg per week to maintain a therapeutic INR ⁽⁸⁸⁾. In the study group there were 12 participants (5.9%) that were on a low dose of warfarin (<14 mg weekly). Although increased sensitivity can

occur in elderly patients and in those with a low BMI, specific mutations, including CYP 2C9*2 and CYP 2C9*3 in the gene encoding CYP P450 can also be causative factors ^(56, 57).

Participants with the least TTR comprised a higher proportion of Asians and a lower proportion of Black African participants (Figure 4.6). Participants with the highest TTR had a higher proportion of Caucasians. The actual reasons for this finding are beyond the scope of this study. However, given the context of this healthcare system setting, the most plausible explanations would include low levels of education amongst participants and possible language barriers between healthcare providers and study participants. Additionally, race (non-Caucasian) is used as one of the criteria in the (SAME-TT2R2 score) (refer to Figure 1.3) validated by *Apostolakis et al* (2013) to predict quality of warfarin anticoagulation ⁽⁶⁹⁾.

Two commonly used concomitant medications in the study population were potassium chloride and simvastatin. In our study, the use of potassium chloride was higher and the use of simvastatin was lower in participants with the most time in the therapeutic range (Figure 4.7). The lower use of simvastatin relates to its known interaction with warfarin resulting in the drug being prescribed less in participants on warfarin anticoagulation ^(82, 89). Potassium chloride has no interaction with warfarin and therefore its prescription was unrestricted.

In our study the majority of participants (61%) had INR values in the therapeutic range, with 26% having sub-therapeutic INR and 13% having supra-therapeutic INR values (Figure 4.1). These results are similar to those of published studies ^(73, 74). None of the participants with supra-therapeutic or sub-therapeutic INR values presented with bleeding or thrombotic events at the time of study conduct. The quality of the INR control is measured by time in therapeutic range (TTR). In this study, INR levels were evaluated in the preceding 3 months prior to study enrolment and the calculated overall TTR was 71.6%. In the literature, a TTR of ~65%-70% is considered an acceptable target ^(66, 67) and our study population exceeded this target TTR.

There was no statistically significant association between INR levels and age, gender, treatment duration, warfarin dose or treatment indication. In other similar studies, a number factors including employment status, low level of education, disability, frailty, complexity of

dosing regimen, high medication cost, dietary and alcohol restrictions, indication for treatment, suboptimal communication between healthcare provider and participant and treatment outside of a dedicated Anticoagulation Clinic provider were determinants to explain non-adherence and decreased TTR^(66, 68-70, 77, 78, 90). In our study, all participants were attendees of the anticoagulant clinic at the CMJAH whose employment status and level of education were not specifically investigated. None of our study participants were disabled, frail or had complex anticoagulation regimens

We measured CF II and CF VII plasma levels in 283 participants of which 204 were in the study group and 79 in the control group. The median CF II and CF VII levels in the study group were 24% and 22% respectively. Higher median CF II and CF VII levels of 37% and 38% were seen in the control group. This difference in median CF II and CFVII levels is consistent with the expected warfarin effect. These results are consistent with a number of studies which reported a decrease in the vitamin K-dependent CF's levels ranging from 10% to 40% following warfarin anticoagulation^(15, 16, 84, 91-93). Two of the 204 study group participants had discrepant CF II and CF VII plasma levels. Whilst these results may suggest non-adherence, other possible confounding variables such as foods, drugs, disease states, dietary stores of vitamin K and cytochrome P-450 gene mutation need to be considered and excluded before making a judgment on adherence.

In the first participant with discrepant CF II and CF VII levels, the CF VII was 58% and CF II was 18% with an INR of 2.14. This scenario may represent short term non-adherence to warfarin due to the short half- life of CF VII (~6 hours). In the second participant the CF VII was 14% and the CF II was 62% with an INR of 3.32. In this patient the discrepancy between CF II and CF VII may represent more prolonged non-adherence to warfarin due to the long half-life of CF II (~60 hours). This would represent an anticoagulant effect rather than a true change in the antithrombotic effect, which places a patient at increased risk of thrombosis. Studies have shown that measuring the CF II activity alone more accurately reflects the antithrombotic activity of the warfarin over the INR^(16, 17, 94). The levels of CF II and CF VII seen in this study were similar to those seen in other studies in patients on anticoagulation with warfarin^(15, 16, 84, 91-93). In a study by *Kumar et al* (1990)⁽⁹³⁾, factor levels >50% were reported despite participant adherence to warfarin therapy. The possible explanation for this may be attributed to the presence of confounding variables such as genetic factors,

environmental factors, pharmacokinetics and pharmacodynamics of warfarin and should also be considered before participant non-adherence can be assumed. More recently Ooi *et al* (2017) evaluated the 6 Vitamin K dependent proteins in participants on warfarin from initiation to steady state level and developed a model to further explain the relationship of the 6 Vitamin K related proteins ⁽⁹⁵⁾. This research provides important information on the use of measuring adequate anticoagulation by vitamin K dependent proteins and lays the foundation for further studies to evaluate warfarin overdose and management of warfarin anticoagulation in the peri-surgical setting ⁽⁹⁵⁾.

In the sub-therapeutic INR group, 51 participants (64.6%) were shown to be adherent by their CF level while 28 participants (35.4%) were truly non-adherent when using the CF levels. In both the study and the control groups, 253 participants were considered adequately anticoagulated (202 with therapeutic INR and 51 with a sub-therapeutic INR), while 30 participants were shown to be truly non-adherent (2 with therapeutic INR and 28 with non-therapeutic INR). When using factor plasma levels, sensitivity (true adherents) and specificity (true non-adherents) were 79.8% and 93.3% respectively. This study showed non-adherence by the INR levels and by factor levels to be 26.2% and 9.4% respectively. These results suggest that there is still evidence of sufficient anticoagulation in a significant number of participants with a sub-therapeutic INR. In clinical practice, this translates to unnecessary dose adjustment of warfarin and may confer an increased risk of bleeding.

There may be benefit in measuring individual CF levels in participants at increased bleeding risk before warfarin dose adjustment. Participants on warfarin are already susceptible to a twofold increase in risk of bleeding which increases with higher doses of warfarin ^(96, 97). Participants with increased risk of thrombosis may also show benefit from individual factor testing, particularly CF II which shows true anti-thrombotic effect ^(16, 17, 94). Acquired prothrombotic states which may benefit from CF measurement are many and include major surgery or trauma, immobilization (e.g. hip/knee replacement, prolonged cast, stroke, bedridden owing to illness), solid or hematologic malignancies, pregnancy, drugs (e.g. oral contraceptives and oestrogen replacement therapies), antiphospholipid antibody syndrome (APLS), heparin-induced thrombocytopenia (HIT), paroxysmal nocturnal haemoglobinuria, obesity and the nephrotic syndrome ⁽⁹⁸⁾. Further investigation of these clinical scenarios will require CF measurement and will be informed by the clinical indication for anticoagulation and the clinical assessment of risk of bleeding or clotting.

Measurement of vitamin K dependent CFs may also be considered in inherited causes of thrombophilia which include Protein C and Protein S deficiency, Factor V Leiden mutation, Prothrombin gene G20210A mutation, antithrombin deficiency and hyperhomocysteinemia⁽⁹⁸⁾. Monitoring should also be considered where drugs are used that increase the metabolism of warfarin through CYP P450 induction (e.g. rifampicin, phenytoin, carbamazepine, protease inhibitors)⁽⁹⁹⁾.

Other areas where individual factor testing may be of benefit include initiation of therapy to assess when concurrent overlap therapy with heparin can be discontinued. This would result in earlier discharge from hospital on adequate oral anticoagulation as well as decreased exposure to heparin with associated reduction in the incidence of HIT. Additionally assessment of true antithrombotic status through factor testing would allow for decreased concern for the development of warfarin induced skin necrosis secondary to decreased protein C levels which occurs on initiation of warfarin therapy⁽¹⁹⁾.

There are also a number of instances where INR testing is unreliable such as in patients with a lupus anticoagulant, as binding of the antiphospholipid antibodies to phospholipid can falsely prolong the clot based assays⁽¹⁰⁰⁾. Testing of the Vitamin K dependent CF's is therefore recommended in this patient group⁽¹⁰¹⁾. Monitoring of CF levels for bridging purposes prior to certain surgical procedures would be reassuring to prove CF levels that were within haemostatic range. Although the INR has helped in the standardisation of the PT, different thromboplastin reagents may still show a degree of variability. Where there is concern about the latter, CF II testing is recommended, as it shows the least sensitivity to thromboplastin reagents when compared with the other Vitamin K dependent CF's⁽¹⁰²⁾.

This study also showed that in a number of patients with a sub-therapeutic INR, anticoagulation may well be adequate based on CF measurements. In the absence of CF knowledge, the participant's warfarin dose may be inappropriately increased with associated increased bleeding risk.

This study has a number of limitations which may curb the generalisability of its results. It is a single centre study whose results will need to be confirmed in a multicentre setting. The study excluded the adolescent and paediatric populations and therefore results may not apply to these populations. All participants in the study were included only if they were on steady state warfarin therapy for at least 3 months. The results of the study would therefore not be applicable to participants who have just started anticoagulation therapy with warfarin. As this

was a cross sectional study the true picture of a longitudinal follow up of anticoagulated participants may not be entirely reflected. And finally, only two vitamin K dependent factors (CF II and CF VII) were measured in this study, therefore these results may not generalisable to other vitamin K dependent factors.

CHAPTER 6. CONCLUSION AND RECOMMENDATIONS

Analysis of the Vitamin K dependent CF's provides a more substantial understanding of the effect of warfarin on the INR, which is required for its monitoring and dose adjustment. In this study population, however, non-adherence on steady state warfarin evaluated by CF II and CF VII levels was very low and may not be of clinical value for use in a routine diagnostic and monitoring setting.

As individual CF testing is more expensive than an INR test, we recommend measuring clotting factor levels in specific circumstances where this may be clinically indicated. These indications include participants with a sub-therapeutic INR at increased risk of bleeding, those suspected of being non-adherent on warfarin therapy, those with a hypercoagulable state, and those on drugs that interfere with warfarin metabolism.

REFERENCES

1. Dalby AJ, Wessels P, Opie LH. Warfarin in non-valvular atrial fibrillation. *S Afr Med J* 2013; 103 (12): 901-4.
2. Pirmohamed M. Warfarin: almost 60 years old and still causing problems. *Br J Clin Pharmacol*. 2006; 62 (5): 509-11.
3. Stahmann MA HC, Link KP. Studies on the hemorrhagic sweet clover disease. Identification and synthesis of the hemorrhagic agent. *J Biol Chem*. 1941; 138: 513-27.
4. Wardrop D, Keeling D. The story of the discovery of heparin and warfarin. *Br J Haematol*. 2008; 141 (6): 757-63.
5. Ansell J, Hirsh J, Hylek E, *et al*. Pharmacology and management of the vitamin K antagonists: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). *Chest*. 2008; 133 (6 Suppl): 160S-98S.
6. Hirsh J, Fuster V, Ansell J, *et al*. American Heart Association/American College of Cardiology Foundation guide to warfarin therapy. *Circulation*. 2003; 107 (12): 1692-711.
7. Mas JL, Arquizan C, Lamy C, *et al*. Recurrent cerebrovascular events associated with patent foramen ovale, atrial septal aneurysm, or both. *N Engl J Med*. 2001; 345 (24): 1740-6.
8. Fuster V, Gersh BJ, Giuliani ER, *et al*. The natural history of idiopathic dilated cardiomyopathy. *Am J Cardiol*. 1981; 47 (3): 525-31.
9. Pullicino PM, Halperin JL, Thompson JL. Stroke in patients with heart failure and reduced left ventricular ejection fraction. *Neurology*. 2000; 54 (2): 288-94.
10. Stafford DW. The vitamin K cycle. *J Thromb Haemost*. 2005; 3 (8): 1873-8.
11. Fasco MJ, Hildebrandt EF, Suttie JW. Evidence that warfarin anticoagulant action involves two distinct reductase activities. *J Biol Chem*. 1982; 257 (19): 11210-2.
12. Nelsestuen GL. Role of gamma-carboxyglutamic acid. An unusual protein transition required for the calcium-dependent binding of prothrombin to phospholipid. *J Biol Chem*. 1976; 251 (18): 5648-56.
13. O'Reilly RA. The pharmacodynamics of the oral anticoagulant drugs. *Prog Hemost Thromb*. 1974; 2 (0): 175-213.

14. Hoffbrand AV, Catovsky D., Tuddenham E.G.D, Green, A.R. Postgraduate Haematology. A John Wiley and Sons,Ltd., 2011.
15. Lind SE, Callas PW, Golden EA, *et al.* Plasma levels of factors II, VII and X and their relationship to the international normalized ratio during chronic warfarin therapy. Blood Coagul Fibrinolysis. 1997; 8 (1): 48-53.
16. Zivelin A, Rao LV, Rapaport SI. Mechanism of the anticoagulant effect of warfarin as evaluated in rabbits by selective depression of individual procoagulant vitamin K-dependent clotting factors. J Clin Invest. 1993; 92 (5): 2131-40.
17. Furie B, Diuguid CF, Jacobs M, *et al.* Randomized prospective trial comparing the native prothrombin antigen with the prothrombin time for monitoring oral anticoagulant therapy. Blood. 1990; 75 (2): 344-9.
18. Wessler S, Gitel SN. Warfarin. From bedside to bench. N Engl J Med. 1984; 311 (10): 645-52.
19. Broekmans AW, Bertina RM, Loeliger EA, *et al.* Protein C and the development of skin necrosis during anticoagulant therapy. Thromb Haemost. 1983; 49 (3): 251.
20. Stirling Y, Howarth DJ, Stockley R, *et al.* Comparison of the bioavailabilities and anticoagulant activities of two warfarin formulations. Br J Haematol. 1982; 51 (1): 37-46.
21. Pyorala K, Jussila J, Mustala O, *et al.* Absorption of warfarin from the stomach and small intestine. Scand J Gastroenterol Suppl. 1971; 9: 95-103.
22. Musa MN, Lyons LL. Effect of food and liquid on the pharmacokinetics of propoxyphene. Curr Ther Res Clin Exp. 1976; 19 (6): 669-74.
23. O'Reilly RA. Interaction of the anticoagulant drug warfarin and its metabolites with human plasma albumin. J Clin Invest. 1969; 48 (1): 193-202.
24. Hignite C, Uetrecht J, Tschanz C, *et al.* Kinetics of R andS warfarin enantiomers. Clin Pharmacol Ther. 1980; 28 (1): 99-105.
25. Lewis RJ, Trager WF. Warfarin metabolism in man: identification of metabolites in urine. J Clin Invest. 1970; 49 (5): 907-13.
26. Lewis RJ, Trager WF, Robinson AJ, *et al.* Warfarin metabolites: the anticoagulant activity and pharmacology of warfarin alcohols. J Lab Clin Med. 1973; 81 (6): 925-31.

27. Booth SL, Al Rajabi A. Determinants of vitamin K status in humans. *Vitam Horm.* 2008; 78: 1-22.
28. Johnson MA. Influence of vitamin K on anticoagulant therapy depends on vitamin K status and the source and chemical forms of vitamin K. *Nutr Rev.* 2005; 63 (3): 91-7.
29. Sato Y, Murata M, Chiba T, *et al.* A Systematic Review of the Acceptable Intake Level of Vitamin K among Warfarin Users. *Shokuhin Eiseigaku Zasshi.* 2015; 56 (4): 157-65.
30. Binkley NC, Krueger DC, Kawahara TN, *et al.* A high phylloquinone intake is required to achieve maximal osteocalcin gamma-carboxylation. *Am J Clin Nutr.* 2002; 76 (5): 1055-60.
31. Lurie Y, Loebstein R, Kurnik D, *et al.* Warfarin and vitamin K intake in the era of pharmacogenetics. *Br J Clin Pharmacol.* 2010; 70 (2): 164-70.
32. Sconce E, Avery P, Wynne H, *et al.* Vitamin K supplementation can improve stability of anticoagulation for patients with unexplained variability in response to warfarin. *Blood.* 2007; 109 (6): 2419-23.
33. Kroon LA. Drug interactions with smoking. *Am J Health Syst Pharm.* 2007; 64 (18): 1917-21.
34. Nathisuwan S, Dilokthornsakul P, Chaiyakunapruk N, *et al.* Assessing evidence of interaction between smoking and warfarin: a systematic review and meta-analysis. *Chest.* 2011; 139 (5): 1130-9.
35. Weathermon R, Crabb DW. Alcohol and medication interactions. *Alcohol Res Health.* 1999; 23 (1): 40-54.
36. Ha CE, Petersen CE, Park DS, *et al.* Investigations of the effects of ethanol on warfarin binding to human serum albumin. *J Biomed Sci.* 2000; 7 (2): 114-21.
37. Roth JA, Bradley K, Thummel KE, *et al.* Alcohol misuse, genetics, and major bleeding among warfarin therapy patients in a community setting. *Pharmacoepidemiol Drug Saf.* 2015; 24 (6): 619-27.
38. Marks PW. Hematologic manifestations of liver disease. *Semin Hematol.* 2013; 50 (3): 216-21.
39. Shah NL, Intagliata NM, Northup PG, *et al.* Procoagulant therapeutics in liver disease: a critique and clinical rationale. *Nat Rev Gastroenterol Hepatol.* 2014; 11 (11): 675-82.

40. Conly JM, Stein K, Worobetz L, *et al.* The contribution of vitamin K2 (menaquinones) produced by the intestinal microflora to human nutritional requirements for vitamin K. *Am J Gastroenterol.* 1994; 89 (6): 915-23.
41. Holbrook AM, Pereira JA, Labiris R, *et al.* Systematic overview of warfarin and its drug and food interactions. *Arch Intern Med.* 2005; 165 (10): 1095-106.
42. Juurlink DN. Drug interactions with warfarin: what clinicians need to know. *CMAJ.* 2007; 177 (4): 369-71.
43. Delaney JA, Opatrny L, Brophy JM, *et al.* Drug drug interactions between antithrombotic medications and the risk of gastrointestinal bleeding. *CMAJ.* 2007; 177 (4): 347-51.
44. Maurer-Spurej E, Pittendreigh C, Solomons K. The influence of selective serotonin reuptake inhibitors on human platelet serotonin. *Thromb Haemost.* 2004; 91 (1): 119-28.
45. Fortun PJ, Hawkey CJ. Nonsteroidal antiinflammatory drugs and the small intestine. *Curr Opin Gastroenterol.* 2007; 23 (2): 134-41.
46. Garcia Rodriguez LA, Lin KJ, Hernandez-Diaz S, *et al.* Risk of upper gastrointestinal bleeding with low-dose acetylsalicylic acid alone and in combination with clopidogrel and other medications. *Circulation.* 2011; 123 (10): 1108-15.
47. Holbrook A, Schulman S, Witt DM, *et al.* Evidence-based management of anticoagulant therapy: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest.* 2012; 141 (2 Suppl): e152S-84S.
48. Tripodi A, Mannucci PM. The coagulopathy of chronic liver disease. *N Engl J Med.* 2011; 365 (2): 147-56.
49. Tripodi A, Anstee QM, Sogaard KK, *et al.* Hypercoagulability in cirrhosis: causes and consequences. *J Thromb Haemost.* 2011; 9 (9): 1713-23.
50. Efird LM, Mishkin DS, Berlowitz DR, *et al.* Stratifying the risks of oral anticoagulation in patients with liver disease. *Circ Cardiovasc Qual Outcomes.* 2014; 7 (3): 461-7.
51. Limdi NA, Limdi MA, Cavallari L, *et al.* Warfarin dosing in patients with impaired kidney function. *Am J Kidney Dis.* 2010; 56 (5): 823-31.

52. Sakaan SA, Hudson JQ, Oliphant CS, *et al.* Evaluation of warfarin dose requirements in patients with chronic kidney disease and end-stage renal disease. *Pharmacotherapy*. 2014; 34 (7): 695-702.
53. Dreisbach AW, Japa S, Gebrekal AB, *et al.* Cytochrome P4502C9 activity in end-stage renal disease. *Clin Pharmacol Ther*. 2003; 73 (5): 475-7.
54. Witt DM, Delate T, Clark NP, *et al.* Outcomes and predictors of very stable INR control during chronic anticoagulation therapy. *Blood*. 2009; 114 (5): 952-6.
55. Witt DM, Delate T, Clark NP, *et al.* Twelve-month outcomes and predictors of very stable INR control in prevalent warfarin users. *J Thromb Haemost*. 2010; 8 (4): 744-9.
56. Aithal GP, Day CP, Kesteven PJ, *et al.* Association of polymorphisms in the cytochrome P450 CYP2C9 with warfarin dose requirement and risk of bleeding complications. *Lancet*. 1999; 353 (9154): 717-9.
57. Higashi MK, Veenstra DL, Kondo LM, *et al.* Association between CYP2C9 genetic variants and anticoagulation-related outcomes during warfarin therapy. *JAMA*. 2002; 287 (13): 1690-8.
58. D'Andrea G, D'Ambrosio RL, Di Perna P, *et al.* A polymorphism in the VKORC1 gene is associated with an interindividual variability in the dose-anticoagulant effect of warfarin. *Blood*. 2005; 105 (2): 645-9.
59. Hall JG, Pauli RM, Wilson KM. Maternal and fetal sequelae of anticoagulation during pregnancy. *Am J Med*. 1980; 68 (1): 122-40.
60. Bauer KA. Coumarin-induced skin necrosis. *Arch Dermatol*. 1993; 129 (6): 766-8.
61. Rauh G, Spengel FA. Blue toe syndrome after initiation of low-dose oral anticoagulation. *Eur J Med Res*. 1998; 3 (6): 278-80.
62. Hoffbrand A. Hoffbrand's Essential Haematology. John Wiley and Sons, 2016.
63. Zucker S, Cathey MH, Sox PJ, *et al.* Standardization of laboratory tests for controlling anticoagulant therapy. *Am J Clin Pathol*. 1970; 53 (3): 348-54.
64. Kirkwood TB. Calibration of reference thromboplastins and standardisation of the prothrombin time ratio. *Thromb Haemost*. 1983; 49 (3): 238-44.

65. Hirsh J, Dalen J, Anderson DR, *et al.* Oral anticoagulants: mechanism of action, clinical effectiveness, and optimal therapeutic range. *Chest*. 2001; 119 (1 Suppl): 8S-21S.
66. Kaatz S. Determinants and measures of quality in oral anticoagulation therapy. *J Thromb Thrombolysis*. 2008; 25 (1): 61-6.
67. Rosendaal FR, Cannegieter SC, van der Meer FJ, *et al.* A method to determine the optimal intensity of oral anticoagulant therapy. *Thromb Haemost*. 1993; 69 (3): 236-9.
68. Platt AB, Localio AR, Brensinger CM, *et al.* Can we predict daily adherence to warfarin?: Results from the International Normalized Ratio Adherence and Genetics (IN-RANGE) Study. *Chest*. 2010; 137 (4): 883-9.
69. Apostolakis S, Sullivan RM, Olshansky B, *et al.* Factors affecting quality of anticoagulation control among patients with atrial fibrillation on warfarin: the SAME-TT(2)R(2) score. *Chest*. 2013; 144 (5): 1555-63.
70. Pokorney SD, Simon DN, Thomas L, *et al.* Patients' time in therapeutic range on warfarin among US patients with atrial fibrillation: Results from ORBIT-AF registry. *Am Heart J*. 2015; 170 (1): 141-8, 8 e1.
71. Kimmel SE, Chen Z, Price M, *et al.* The influence of patient adherence on anticoagulation control with warfarin: results from the International Normalized Ratio Adherence and Genetics (IN-RANGE) Study. *Arch Intern Med*. 2007; 167 (3): 229-35.
72. Parker CS, Chen Z, Price M, *et al.* Adherence to warfarin assessed by electronic pill caps, clinician assessment, and patient reports: results from the IN-RANGE study. *J Gen Intern Med*. 2007; 22 (9): 1254-9.
73. Waterman AD, Milligan PE, Bayer L, *et al.* Effect of warfarin nonadherence on control of the International Normalized Ratio. *Am J Health Syst Pharm*. 2004; 61 (12): 1258-64.
74. Wittkowsky AK, Devine EB. Frequency and causes of overanticoagulation and underanticoagulation in patients treated with warfarin. *Pharmacotherapy*. 2004; 24 (10): 1311-6.
75. Casciano JP, Dotiwala ZJ, Martin BC, *et al.* The costs of warfarin underuse and nonadherence in patients with atrial fibrillation: a commercial insurer perspective. *J Manag Care Pharm*. 2013; 19 (4): 302-16.
76. Chen SY, Wu N, Gulseth M, *et al.* One-year adherence to warfarin treatment for venous thromboembolism in high-risk patients and its association with long-term risk of recurrent events. *J Manag Care Pharm*. 2013; 19 (4): 291-301.

77. Ho PM, Bryson CL, Rumsfeld JS. Medication adherence: its importance in cardiovascular outcomes. *Circulation*. 2009; 119 (23): 3028-35.
78. LaMori JC, Mody SH, Gross HJ, *et al*. Burden of comorbidities among patients with atrial fibrillation. *Ther Adv Cardiovasc Dis*. 2013; 7 (2): 53-62.
79. Williams A, Manias E, Walker R. Interventions to improve medication adherence in people with multiple chronic conditions: a systematic review. *J Adv Nurs*. 2008; 63 (2): 132-43.
80. Giugliano RP, Ruff CT, Braunwald E, *et al*. Edoxaban versus warfarin in patients with atrial fibrillation. *N Engl J Med*. 2013; 369 (22): 2093-104.
81. Sterling T, Bennett CML, George M, Rodgers. *Laboratory Hemostasis: A Practical Guide for Pathologists*. E-Book.: Springer International Publishing, 2015.
82. Andersson ML, Eliasson E, Lindh JD. A clinically significant interaction between warfarin and simvastatin is unique to carriers of the CYP2C9*3 allele. *Pharmacogenomics*. 2012; 13 (7): 757-62.
83. Watala C, Golanski J, Kardas P. Multivariate relationships between international normalized ratio and vitamin K-dependent coagulation-derived parameters in normal healthy donors and oral anticoagulant therapy patients. *Thromb J*. 2003; 1 (1): 7.
84. D'Angelo A, Della Valle P, Crippa L, *et al*. Relationship between international normalized ratio values, vitamin K-dependent clotting factor levels and in vivo prothrombin activation during the early and steady phases of oral anticoagulant treatment. *Haematologica*. 2002; 87 (10): 1074-80.
85. Faul F, Erdfelder E, Lang AG, *et al*. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods*. 2007; 39 (2): 175-91.
86. Pisters R, Lane DA, Nieuwlaat R, *et al*. A novel user-friendly score (HAS-BLED) to assess 1-year risk of major bleeding in patients with atrial fibrillation: the Euro Heart Survey. *Chest*. 2010; 138 (5): 1093-100.
87. Lip GY, Frison L, Halperin JL, *et al*. Comparative validation of a novel risk score for predicting bleeding risk in anticoagulated patients with atrial fibrillation: the HAS-BLED (Hypertension, Abnormal Renal/Liver Function, Stroke, Bleeding History or Predisposition, Labile INR, Elderly, Drugs/Alcohol Concomitantly) score. *J Am Coll Cardiol*. 2011; 57 (2): 173-80.

88. Loebstein R, Dvoskin I, Halkin H, *et al.* A coding VKORC1 Asp36Tyr polymorphism predisposes to warfarin resistance. *Blood*. 2007; 109 (6): 2477-80.
89. Hickmott H, Wynne H, Kamali F. The effect of simvastatin co-medication on warfarin anticoagulation response and dose requirements. *Thromb Haemost*. 2003; 89 (5): 949-50.
90. Cruess DG, Localio AR, Platt AB, *et al.* Patient attitudinal and behavioral factors associated with warfarin non-adherence at outpatient anticoagulation clinics. *Int J Behav Med*. 2010; 17 (1): 33-42.
91. Adam SS, McDuffie JR, Ortel TL, *et al.* Comparative effectiveness of warfarin and new oral anticoagulants for the management of atrial fibrillation and venous thromboembolism: a systematic review. *Ann Intern Med*. 2012; 157 (11): 796-807.
92. Howarth DJ, Brozovic M, Stirling Y, *et al.* Factor VII during warfarin treatment. *Scand J Haematol*. 1974; 12 (5): 346-54.
93. Kumar S, Haight JRM, Tate G, *et al.* . Effect of warfarin on plasma concentrations of vitamin K dependent coagulation factors in patients with stable control and monitored compliance. *Br J Haematol*. 1990; 72: 82-5.
94. Gudmundsdottir BR, Francis CW, Bjornsdottir AM, *et al.* Critical role of factors II and X during coumarin anticoagulation and their combined measurement with a new FiiX-prothrombin time. *Thromb Res*. 2012; 130 (4): 674-81.
95. Ooi QX, Wright DF, Tait RC, *et al.* A Joint Model for Vitamin K-Dependent Clotting Factors and Anticoagulation Proteins. *Clin Pharmacokinet*. 2017.
96. Hart RG, Pearce LA, Aguilar MI. Adjusted-dose warfarin versus aspirin for preventing stroke in patients with atrial fibrillation. *Ann Intern Med*. 2007; 147 (8): 590-2.
97. Schulman S, Beyth RJ, Kearon C, *et al.* Hemorrhagic complications of anticoagulant and thrombolytic treatment: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). *Chest*. 2008; 133 (6 Suppl): 257S-98S.
98. Nakashima MO, Rogers HJ. Hypercoagulable states: an algorithmic approach to laboratory testing and update on monitoring of direct oral anticoagulants. *Blood Res*. 2014; 49 (2): 85-94.
99. Kaminsky LS, Zhang ZY. Human P450 metabolism of warfarin. *Pharmacol Ther*. 1997; 73 (1): 67-74.

- 100.** Fleck RA, Rapaport SI, Rao LV. Anti-prothrombin antibodies and the lupus anticoagulant. *Blood*. 1988; 72 (2): 512-9.
- 101.** Baumann Kreuziger LM, Datta YH, Johnson AD, *et al*. Monitoring anticoagulation in patients with an unreliable prothrombin time/international normalized ratio: factor II versus chromogenic factor X testing. *Blood Coagul Fibrinolysis*. 2014; 25 (3): 232-6.
- 102.** Testa S, Morstabilini G, Fattorini A, *et al*. Discrepant sensitivity of thromboplastin reagents to clotting factor levels explored by the prothrombin time in patients on stable oral anticoagulant treatment: impact on the international normalized ratio system. *Haematologica*. 2002; 87 (12): 1265-73.

APPENDICES

Appendix A. Permission Letter from CMJAH Chief Executive Officer.



Dr Sarisha Naidoo
CMJAH

Dear Dr. S. Naidoo

RE: Assessment of Adherence to Warfarin Anticoagulation using the Ratio of Vitamin K Dependent Factors in a Teaching Hospital.

Permission is granted for you to conduct the above recruitment activities as described in your request provided:

1. Charlotte Maxeke Johannesburg Academic Hospital will not anyway incur or inherit costs as result of the said study.
2. Your study shall not disrupt services at the study sites.
3. Strict confidentiality shall be observed at all times.
4. Informed consent shall be solicited from patients participating in your study.

Please liaise with the HOD and Unit Manager or sister in charge to agree on the dates and time that would suit all parties.

Kindly forward this office this office with the results of your study on completion of the research.

~~Supported / not supported~~

Dr. M.I. Mphokeng
Clinical Director
DATE: 8/05/2015

Approved / not approved

Ms. G. Bogoshi
Chief Executive Officer
DATE: 13/05/2015



Appendix B. Information Leaflet and Informed Consent.

INFORMATION LEAFLET AND INFORMED CONSENT

Study Title: Assessment of Adherence to Warfarin Anticoagulation using the Ratio of Vitamin K Dependent Factors in a Teaching Hospital in South Africa.

Investigators: Prof Johnny Mahlangu

Dr Sarisha Naidoo

Introduction

Good day, we are blood specialist doctors working at the Charlotte Maxeke Johannesburg Academic Hospital Anticoagulation Clinic. We look after all patients on blood thinners (warfarin) for the treatment and prevention of blood clots and patients with heart conditions needing blood thinners. We would like to invite you to consider participating in a research study called **“Assessment of Adherence to Warfarin Anticoagulation using the Ratio of Vitamin K Dependent Factors in a Teaching Hospital in South Africa”**

1. Before agreeing to participate, it is important that you read and understand the following explanation about the purpose of the study, the study procedures, benefits, risks and discomforts.
2. This information leaflet is to help you to decide if you would like to participate in the study. You should fully understand what is involved before agreeing to take part in this study.
3. If you have any questions, do not hesitate to ask any of the study team members.
4. You should not agree to take part unless you are satisfied about all the procedures involved.
5. If you choose to participate in this study, you will be free to withdraw from the study any time without giving reasons for the withdrawal. This will not affect your right to future care at this hospital.
6. If you choose to participate in this study, you will be asked to sign this document to confirm that you understand the study.

The following information describes the study and your role as a possible participant. We will answer any questions you may have about this information sheet and about the study. Please read this information sheet carefully

Why are we doing this study?

The aim of this study is to assess how well patients take the blood thinners that is prescribed. This is called adherence to warfarin anticoagulation.

What do we know already?

Warfarin causes blood thinning by stopping production of vitamin K dependent factors (factors II, FVII, IX, X). We measure how thick or thin the blood is by using a laboratory test called the INR. The goal is to achieve an INR of 2-3.5 to avoid the risks of bleeding or clotting. The INR can be influenced by many factors including non-adherence, diet, diseases, alcohol intake and the properties of the drug itself. We know that with an INR that is below 2 warfarin non-adherence must be excluded, however we also know that in non-adherence a therapeutic INR can be present.

What do we want to study further? We want to determine adherence to warfarin, in patients with a therapeutic INR, by measuring vitamin K clotting factors.

What will happen during the study visit?

You will be given a copy of the information leaflet and the consent form to read, understand and ask questions.

2. If you would like to take part in the study, you will be asked to sign an informed consent form.
3. You will be asked to complete a patient information sheet about your age, gender, race; length of time on warfarin, indication and dose of warfarin.
4. Directly after the phlebotomist/nursing sister has drawn blood for your INR test, a second tube of blood will be drawn as the study sample.
5. Once this is complete, no subsequent visits related to the study are required

What are the risks and discomforts in this study? There are no risks associated with participation in this study.

What are the benefits of participating? By taking part in this study you will assist in generating knowledge we can use to management patients on warfarin better.

What if you do not want to participate in the study? You may choose not to participate in this study. If you decide not to participate in this study, you will continue to receive the standard care you have always been given.

How is confidentiality of the study information protected? Your privacy will be respected at all times. All the information collected from you will be identified with a number which is separated from your identification information. Only the study investigators hold the information that allows the number to be linked to your name.

The information collected will be processed, analysed and reported by the study doctors. The results will also be published in scientific articles.

You will not be identified in person in any published reports/publications.

Ethical Approval

This study protocol has been submitted to the University of the Witwatersrand, Human Research Ethics Committee (HREC) (Medical) and written approval has been granted by this Committee. If you have questions about your rights as a study participant you can contact Professor Cleaton-Jones at 011 717 1234. If you require clarification of more information about this study you can contact Professor Mahlangu (011 489 8413) or Dr Naidoo (011 489 8552)

Voluntary Participation Your participation in this study is voluntary. No study procedures will be performed until you have read, understood, and signed this informed consent form.

You may refuse to participate or may discontinue participation at any time during the study without penalty or loss of benefits to which you are otherwise entitled as a patient.

INFORMED CONSENT FOR PARTICIPANTS 18 YEARS OR OLDER (ADULTS)

1. I have read this information sheet and my questions have been answered to my satisfaction.
2. I voluntarily consent to participate.
3. I understand that if I choose not to participate or to withdraw, my current medical care will not be affected by this decision.
4. I authorize the release of my medical records to the authorized representatives, as specified in this consent form.
5. By signing and dating this consent form, I have not waived any of the legal rights that I would have if I were not a participant in a research study.

1. PARTICIPANT:

Printed Name

Signature / Mark or Thumbprint

Date and Time

2. NURSE/DOCTOR OBTAINING INFORMED CONSENT:

Printed Name

Signature / Mark or Thumbprint

Date and Time

3. WITNESS (ONLY IF APPLICABLE):

Printed Name

Signature / Mark or Thumbprint

Date and Time

Appendix C Ethics Clearance Certificate



R14/49 Dr Sarisha Naidoo

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL) CLEARANCE CERTIFICATE NO. M150213

NAME:
(Principal Investigator)

Dr Sarisha Naidoo

DEPARTMENT:

Haematology and Molecular Medicine
National Health Laboratory Service,
Charlotte Maxeke Johannesburg Academic Hospital
Chris Hani Baragwanath Academic Hospital

PROJECT TITLE:

Assessment of Adherence to Warfarin Anticoagulation using the
Ratio of Vitamin K Dependant Factors in a Teaching Hospital
in South Africa

DATE CONSIDERED:

27/02/2015

DECISION:

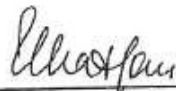
Approved unconditionally

CONDITIONS:

SUPERVISOR:

Prof Johnny Mahlangu

APPROVED BY:


Professor P Cleaton-Jones, Chairperson, HREC (Medical)

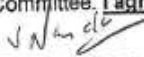
DATE OF APPROVAL: 23/03/2015

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. I agree to submit a yearly progress report


Principal Investigator Signature

Date

14 / 04 / 15

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix D Turnitin Results

0402364w:TURNITIN-
ASSESSMENT_OF_ADHERENCE_TO_WARFARIN_ANTICOA..

ORIGINALITY REPORT

13%

SIMILARITY INDEX

8%

INTERNET SOURCES

7%

PUBLICATIONS

5%

STUDENT PAPERS

MATCH ALL SOURCES (ONLY SELECTED SOURCE PRINTED)

4%

★ Submitted to University of Witwatersrand

Student Paper

Exclude quotes On

Exclude matches Off

Exclude bibliography On