The effect of VKORC1 (−1639 G>A) gene polymorphism and its related clotting factor IIa and Xa on warfarin therapy in patients with atrial fibrillation: A multi-ethnic Sarawak population study

MELISSA LIM SIAW HAN

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ABSTRACT

Warfarin, the most frequently prescribed oral anticoagulant therapy management is challenging due to its narrow therapeutic index. It is associated with an increased risk of bleeding when overdose, while an insufficient dose may lead to thromboembolism or stroke. Due to the wide variation of response among patients, frequent monitoring of the efficacy of this drug through INR is essential. Recently emerged Novel Oral Anticoagulants (NOACs) have demonstrated to be non-inferior to warfarin therapy while abolishing the need for frequent blood INR testing. However, these are not commonly prescribed due to their current commercial pricing strategy with relatively limited indications compared to warfarin therapy, scenarios typically encountered in developing countries. Hence, there is a need to delineate a predictive model to determine the appropriate use of anticoagulant therapies in our country. This study aims to investigate the influences of coagulation factors (thrombin and Factor Xa) on poor TTR control patients while taking into account the impact of previously identified VKORC1 (-1639G>A) gene polymorphism. The VKORC1 AA genotype predominates in the multi-ethnic Sarawak population at a 71.5% majority. There was a significant difference in average daily warfarin dose (ADWD) between ethnic groups. Plasma thrombin levels were found to be significantly correlated with the TTR, ADWD, and BMI and were also found to be significantly higher in female patients but not plasma FXa levels. Majority of the patients with poor TTR<60% were on alternate dosing regimen with subtherapeutic INR readings. Multivariate analysis showed that alternate dosing regimen and plasma thrombin levels were independently associated with TTR while the VKORC1 (-1639G>A) gene polymorphism, diabetes mellitus, age and BMI are independent predictors or ADWD. Hence, it is demonstrated that the VKORC1 (-1639G>A) gene polymorphism together with other atrial fibrillation risk factors may be a strong predictive variable for ADWD, while the plasma thrombin levels may be a potential candidate marker in reflecting the quality of anticoagulation control based on TTR. Together, both models may be adopted to delineate a new strategy to determine who may potentially benefit from switching warfarin to the new NOACs.
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Melissa Lim Siaw Han

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DECLARATIONS

This thesis does not contain any material that has been accepted for a degree or diploma by the University or any other institutions, except by the way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due reference is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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2015

As the principal coordinating supervisor, I hereby acknowledge and certify that the above mentioned statements are legitimate to the best of my knowledge.

Dr Hwang Siaw San

2015
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<td>Asymptomatic atrial fibrillation</td>
</tr>
<tr>
<td>ACCP</td>
<td>American College of Chest Physicians</td>
</tr>
<tr>
<td>ACS</td>
<td>Acute Coronary Syndrome</td>
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<tr>
<td>ADWD</td>
<td>Average Daily Warfarin Dose</td>
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<tr>
<td>AF</td>
<td>Atrial Fibrillation</td>
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<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<tr>
<td>CAD</td>
<td>Coronary Artery Disease</td>
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<td>CHF</td>
<td>Chronic Heart Failure</td>
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<tr>
<td>CYP</td>
<td>Cytochrome P450 genes</td>
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<tr>
<td>DM</td>
<td>Diabetes Mellitus</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphates</td>
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<tr>
<td>DVT</td>
<td>Deep Vein Thrombosis</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FIIa</td>
<td>Thrombin</td>
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<tr>
<td>FXa</td>
<td>Factor Xa</td>
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<tr>
<td>GGCX</td>
<td>γ-glutamyl carboxylase</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>INR</td>
<td>International Normalised Ratio</td>
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<tr>
<td>IQR</td>
<td>Interquartile Range</td>
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<td>ISI</td>
<td>International Sensitivity Index</td>
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<td>MLR</td>
<td>Multiple Linear Regression</td>
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NOACs – Novel oral anticoagulants
PCR – Polymerase Chain Reaction
PE – Pulmonary Embolism
PT – Prothrombin Time
RFLP – Restriction fragment length polymorphism
SD – Standard Deviation
SLR – Simple Linear Regression
SNP – Single-nucleotide polymorphism
TF – Tissue Factor
TFPI – tissue factor pathway inhibitor
TMB – Tetramethylbenzidine
TTR – Time in Therapeutic Range
VKA – Vitamin K Antagonist
VKORC1 – Vitamin K epoxide reductase complex subunit 1
VTE – Venous Thromboembolism
WHO – World Health Organisation
CHAPTER 1: INTRODUCTION

1.1 Research Background

The vitamin K antagonist (VKA) Warfarin is the most commonly prescribed oral anticoagulant typically indicated for the prophylaxis and treatment of venous and arterial thromboembolic disorders (Lombardi et al. 2003). However, the management of warfarin therapy is challenging due to its narrow therapeutic index and is associated with increased risk of haemorrhage when above therapeutic range while an insufficient dose may result in thrombosis (Kuruvilla & Gurk-Turner 2001). Even though warfarin has been extensively used in humans for over 5 decades, its most significant side effect, bleeding is still a leading cause of hospitalisation and drug-affiliated death (Pirmohamed et al. 2004). Warfarin’s extended half-life with an indirect way of thrombin inhibition causes adequate anticoagulation to take effect only after several days (Hammwohner & Goette 2008).

Thrombin is located downstream of both the intrinsic and extrinsic pathway of the coagulation cascade (HP, JM & PK 2003). Each pathway consists of tissue factors (TF) and a number of coagulation factors (Factor II, VII, IX and X) which will eventually contribute to thrombin amplification (Walker & Royston 2002). Following endothelial injury, the blood coagulation cascade is activated via the extrinsic pathway where the exposed tissue factors will bind to coagulation factor VII which further activates factor IX then X. The activation of factor X to Xa further drives the pathway for thrombin activation by the conversion of prothrombin to thrombin (FIa). Hence, thrombin is the key protein that promotes the conversion of fibrinogen to fibrin which ultimately, forms the thrombus (Butkowski et al. 1977).

New drugs are now targeting the direct inhibition of thrombin and FXa with promising results demonstrating their non-inferiority to warfarin therapy besides alleviating the need for constant blood monitoring (Connolly et al. 2009; Patel et al. 2011). To date, no study has investigated the clinical role of activated Factor II (thrombin) and FXa in a multi-ethnic Asian population.
Due to the wide variation of response among patients, frequent monitoring of their prothrombin time-international normalised ratio (INR) is compulsory in order to optimise the therapeutic efficacy of warfarin therapy (Connolly et al. 2009). The INR target range for patients with atrial fibrillation is 2.0-3.0 according to The Ninth American College of Chest Physicians (ACCP) guidelines (Ageno et al. 2012). Numerous studies has associated ethnicity to warfarin sensitivity and it was reported that Asian population requires lower doses of warfarin compared to their Caucasian counterpart in order to reach target INR (Buzoianu et al. 2012; Scibona et al. 2012; Yuan et al. 2005). Recently, a variety of ‘optimal’ INR ranges were discovered among the Asian population. A study from China found that a target INR range of 1.8-2.4 to be associated with the lowest rates for major bleeding or thromboembolic events (You et al. 2005). Additionally, one study from Taiwan reported that an INR<2.0 was not associated with an increased thromboembolic events compared to the Caucasians (Yu et al. 2005). In Japan, the Japanese Guidelines for AF management targets an optimal PT-INR range of 1.6-2.6 in patients ≥70 years, whereas the Japanese R-E-L-Y trial pursued a PT-INR range of 2.0-2.6 for the same age group of patients (Mitamura 2011).

The time in therapeutic range (TTR) is used to summarise the INR control over a period of time and has been proposed as a surrogate marker to evaluate clinical outcomes in several studies (Ageno et al. 2012; Schmitt, Speckman & Ansell 2003). Recent large-scale studies reported that an increase in TTR (>60% to 65%) is sufficient to be associated with a reduction in haemorrhage, thromboembolism and risk for stroke events (Connolly et al. 2008; White et al. 2007). However, the TTR status and the recommended therapeutic range for patients in a multi-ethnic Asian population may differ from the Caucasian counterpart and also across centres. It was likely that the most important baseline characteristic associated with variability in TTR in the individual patient was the mean TTR of the institution or country.

What is known in Malaysia in terms of genetic variances, majority of the patients (>90%) were found to have the wild type CYP2C9*1/*1 allele meaning that this genotype is predominant in our population (Ngow et al. 2009). Only a small fraction (<10%) were found to have the heterozygous CYP2C9*1/*3 allele (Ngow et al. 2009; Teh et al. 2012) and none were found to have the homozygous CYP2C9*2/*2
allele. Only the Indian race groups were found to have the *2 and *3 variant in our population. Another study showed that a model which included age and the variants of CYP2C9 and VKORC1 explains for about 37% of the variability in warfarin dose required to achieve and INR of 2-4 (Teh et al. 2012). More than half of the patients have the VKORC1 variant type and the allelic frequency for G:A was 0.16:0.84. The mean (SD) warfarin dose prescribe was 5.25(3.9) mg in patients with heterozygous wild type VKORC1 G-1639G genotype, which was significantly higher than that in the A-1639A genotype at 3.13(1.1); p<0.001 (Teh et al. 2012). A majority (82%) of these patients were indicated for stroke prevention in atrial fibrillation.

Another local study reported the prevalence of asymptomatic atrial fibrillation (AAF) in hypertensive patients was 0.75% with no differences between gender (Wong et al. 2013). This study also reported an increase rate of AAF in patient above 60 years old. Although this finding was consistent with the prevalence rate from other Asian countries (Range: 0.4-2.8%), it is still lower compared to the Caucasian population. In terms of bleeding complications, a local observational study reported a low rate of major bleed and thromboembolic events although a substantial number of INR readings were subtherapeutic (Edwards et al. 2013). It also reported an observation on the clinicians’ perspective that an INR reading of <2.5 will normally deem as beneficial in this region, hence suggesting that a lower intensity of therapy may be beneficial in terms of preventing adverse events in this group of patients as seen in other Asian population studies (Edwards et al. 2013; Mitamura 2011; You et al. 2005).

The current accessibility of human genome sequencing offers promising results in the field of personalised medicine through pharmacogenomics (Anderson et al. 2007). Due to the problematic nature of the warfarin therapy but still being one of the most commonly prescribed drug worldwide, incorporating pharmacogenetic elements to manage this drug would be reasonable. Currently, warfarin is still the mainstay as the most widely prescribed oral anticoagulant, despite the emergence of the NOACs. Hence, the improvement of the safety profile of this drug is vital to maximise the efficacy and minimise its side effects’ magnitude.
In 2007, the Food and Drug Administration (FDA) of the United States has updated the label of warfarin to incorporate the need to initiate lower doses of warfarin in patients with the CYP2C9 and VKORC1 variant alleles (Anderson et al. 2007). One of the reasons for this is that the over-anticoagulation and bleeding risks are generally higher prior to stable anticoagulation state (normally during the first 3 weeks of warfarin therapy) (Ageno et al. 2012). One strategy to reduce this risk is to reduce the time needed to reach steady-state anticoagulation by tailoring the warfarin initiation dose for every patient. The required warfarin dose may vary up to 20-fold among different individuals and can be roughly assessed through analysing certain clinical and patient demographic data like age, BMI, comorbidities, as well as drug-drug and drug-food interactions profile (Roth et al. 2014). A few dosage algorithms incorporating both clinical and patient demographic data have been proven to benefit dosing prediction. Recent discoveries involving the gene variations which encode the main S-warfarin metabolism enzyme (CYP2C9*1*2*3) and the target of warfarin VKORC1 significantly influences dose requirements through the alteration of both pharmacokinetic and pharmacodynamics, respectively. Polymorphisms in these genes are correlated with a higher risk of supratherapeutic INR readings during the initiation of warfarin therapy (Lee & Klein 2013). Hence, adopting the strategy for genotyping both CYP2C9 and VKORC1 genes could avoid warfarin overdosing in warfarin sensitive patients. It is in great hope that the incorporation of pharmacogenetics in dosing algorithms will enhance the safety profile as well as the cost-effectiveness of oral anticoagulant treatments in future.

There is now an increasing trend for certain stakeholders to incorporate pharmacogenetic-based warfarin dose initiation into their conventional clinical practice, hence leading us to anticipate that a more defined, pharmacogenetic-based warfarin dose initiation may lead to a reduction in serious bleeding and thromboembolic side effects (Gaikwad, Ghosh & Shetty 2014). However, until now only 3 small prospective randomised controlled trials were conducted with none adequately powered to detect a difference in major bleeding complications as outcomes. These studies have compared pharmacogenetic-based warfarin therapy initiation to the conventional empiric dosing and the results were not convincing (Lee & Klein 2013; Wadelius & Pirmohamed 2007). Due to the fact that the benefits of pharmacogenetic-based warfarin dosing
strategy currently lacks supporting evidences which are dependent on well designed and substantially powered trials, it would be premature to deduce that the pharmacogenetic-based dosing strategy is more superior compared to the conventional empiric-based dosing strategies employed in majority of the clinical practices. An estimation of the cost vs benefit of routine genotyping prior warfarin therapy initiation is also warranted. It is projected that the adequate information needed will take several more years before arriving to a consensus.

The benefits of pharmacogenetics in individualised patient care are undisputed. However, an argument still exist as whether one should adopt the conventional empiric dosing adjustment strategy or following a pre-genotyped guided warfarin dosing strategy (Wadelius & Pirmohamed 2007). Due to the demanding nature of the healthcare system worldwide, it is not routine to set aside huge resources to this area. Moreover, some areas in the developing countries are severely lacking on their pharmacogenomics data, even on important drugs like warfarin (Gaikwad, Ghosh & Shetty 2014). There is however a need to increase the awareness in this area of pharmacogenetics as it has a major impact in genetically tailored treatment (Roth et al. 2014). It is important that each country has to determine the genotype data of their respective countries as drastic variations do exist even within neighbouring countries which may be attributed to the country’s historical background. In the case of Asian population, more than 90% of the population in the East and South East Asian countries was found to carry the VKORC1 variant genotype and thus belong to the warfarin sensitive category (Gaikwad, Ghosh & Shetty 2014). These findings are extremely important and strongly highlights the need of individualised pharmacogenetic-guided warfarin dosing to avoid warfarin induced bleeding complications.

A recent analysis showed that the prevalence of warfarin sensitive polymorphisms drastically vary from one country to another even in the geographically closely located regions (Gaikwad, Ghosh & Shetty 2014), hence it is important to justify what percentage of the population that will benefit from genotyping-guided warfarin dose. For example, in East and South East Asian countries, genotypes like CYP2C9*1/*2 and CYP2C9*2/*2 are absent and hence in such populations, these genotypes can be omitted in the genotype-guided dosing algorithm for cost-
effectiveness purposes (Gaikwad, Ghosh & Shetty 2014). There is still a fraction of Asian countries that are still not represented in pharmacogenomics research as in the case of Malaysia which consist of a multi-ethnic population system. Hence, data from these underrepresented countries will definitely be beneficial for the safe pharmacogenomics dosing in these cohort.

The present research was designed to investigate the effect of the VKORC1 (-1639 G>A) gene polymorphism and its related clotting factor IIa and Xa on warfarin therapy.
1.2 Research Objectives

The general hypothesis of this study is that patients with poor TTR control on warfarin therapy are caused by the over-expression of coagulation Factor IIa (thrombin) and Factor Xa despite the main influences in dose prediction by previous identified warfarin pharmacogenetic factors such as the VKORC1 (-1639G>A) gene polymorphism. Over the recent years, new anticoagulation options have emerged, focusing on the inhibition of thrombin (Dabigatran etexilate) and FXa (Rivaroxaban and Apixaban) for thromboembolic risk prevention. These drugs were found to be non-inferior to warfarin for stroke prevention in patients with non-valvular AF. Although they do not require routine INR testing, their daily costs are 30 times higher than of warfarin therapy. This will boost national healthcare cost in countries with subsidised drug procurement system if all warfarin-treated patients were to be switched to these new medications. Up to date, no study has been conducted to examine the relationship of TTR scores with coagulation factors such as thrombin and FXa. Therefore, this study is conducted, in hope, to generate valuable data and information to help future studies involving predictive models to determine the appropriate use of anticoagulant therapy in our country. The main objectives of this work are:

- To determine the allele frequencies of the VKORC1 (-1639G>A) gene polymorphism in a multi-ethnic Sarawak population and its clinical role in warfarin therapy

- To study the association of protein expression levels of thrombin and FXa with the TTR and risk factors of atrial fibrillation in patients on long-term warfarin therapy

- To investigate the TTR status in a multi-ethnic Sarawakian patients undergoing long-term warfarin therapy and to determine the covariates (genetic biomarkers and clinical factors) that influences the TTR values in this cohort

- To investigate the covariates (genetic, biomarkers and clinical factors) influencing the average daily warfarin dose in this cohort
To give useful information to determine the appropriate uses of anticoagulant therapy in a multi-ethnic population group.
CHAPTER 2: LITERATURE REVIEW

2.1 Warfarin

2.1.1 Pharmacokinetics and Pharmacodynamics

Warfarin consists of a racemic concoction of two optically active isomers which is called the R and S enantiomers. It is highly soluble in water and is rapidly absorbed from the GI tract hence displaying high bioavailability and can reach peak blood concentration levels 90 minutes post oral administration (Breckenridge 1978; Kelly & O'Malley 1979; O'Reilly 1976). The R-warfarin enantiomer has a half-life of 45 hours whereas the S-warfarin enantiomer has a half-life of 29 hours. In total, the racemic warfarin has a half-life of 36 to 42 hours, where it circulates and bound to plasma proteins (albumin) and accumulate in the liver where the two enantiomers are metabolically transformed by different pathways (Miners & Birkett 1998). The S enantiomer is more clinically relevant as it is 2.7 - 3.8 times more potent compared to the R enantiomer. It is subjected to almost 90% oxidative metabolism, mainly by the CYP2C9 enzyme of the cytochrome P450 system and to a lesser degree by the CYP3A4 (Miners & Birkett 1998). On the other hand, the less potent R enantiomer is subjected to a relatively lower 60% oxidative metabolism, mainly the CYP1A2 and CYP3A4 cytochrome P450 enzymes and to a lesser extent by the CYP2C19. The metabolism of what remains of the enantiomers requires their reduction to diastereomeric alcohols. This association between warfarin doses and its response is greatly influenced by genetic, clinical and environmental factors. These factors will alter the absorption, pharmacokinetic and pharmacodynamic of warfarin therapy (Ageno et al. 2012).

The other warfarin derivatives consist of acenocoumarol, phenprocoumon, and fluindione. Both acenocoumarol and phenprocoumon consist also of optical isomers with their stereochemical components being different from warfarin. For acenocoumarol, the R-acenocoumarol is more potent than S-acenocoumarol (Godbillon et al. 1981). The R-acenocoumarol exhibits a 9 hour elimination half-life, and is mainly metabolised by the CYP2C9 and CYP2C19 enzymes whereas the S-acenocoumarol has a faster clearance of a 0.5 hour half-life and is mainly metabolised by CYP2C9.
Alternatively, phenprocoumon has a relatively prolonged half-life, where both R- and S-isomers having an extended 5.5 days elimination half-life (Haustein 1999). Both isomers are metabolised by CYP2C9 with the S-phenprocoumon having a 1.5 - 2.5 times higher potency than R-phenprocoumon (Haustein 1999). Lastly is fluindione, an indandione vitamin K antagonist with an average half-life of 31 hours. Fluindione on the other hand is not a chiral compound (Mentre et al. 1998).

2.1.2 Effective therapeutic dose and adverse effect profile

The optimal INR target range depends heavily on the indications. Due to the fact that bleeding risk is strongly correlated to the anticoagulation intensity, there is a need to establish the lowest effective and safest therapeutic range for every indications (Hull et al. 1982; Hylek & Singer 1994; Landefeld, Rosenblatt & Goldman 1989; Ridker et al. 2003; Turpie et al. 1988; Vink et al. 2003). Comparing the moderate-intensity INR (2.0 - 3.0) to higher-intensity dose-adjusted oral anticoagulation, the former exhibits a reduction in the risk of major bleeding without a reduction in its treatment efficacy (Hull et al. 1982; Turpie et al. 1988). However, reduced treatment intensity (INR of 1.5 - 2.0) portrays to be more inferior in terms of efficacy compared to the moderate-intensity therapy. One randomised trial indicated that an INR of <2.0 (Target INR 1.5 - 2.0) decreases the recurrence rate of venous thromboembolism after undertaking a standard warfarin therapy of 3 to 6 months as compared to placebo (Ridker et al. 2003). However, another trial reported that preserving the INR intensity of 2.0 - 3.0 in a similar clinical setting was more efficacious compared to a reduced intensity of INR 1.5 - 2.0 and this was also not correlated with an increased risk of bleeding (Kearon et al. 2003). In the case of AF patients, a randomised trial concluded that a dose-adjusted warfarin therapy of INR 2.0 - 3.0 was more superior than the fixed-dose warfarin 3mg/day and aspirin combination therapy (‘Adjusted-dose warfarin versus low-intensity, fixed-dose warfarin plus aspirin for high-risk patients with atrial fibrillation: Stroke Prevention in Atrial Fibrillation III randomised clinical trial’ 1996). Various studies have also reported the diminished efficacy of warfarin therapy when the INR plunges below 2.0 in AF patients (Hylek et al. 2003; Indredavik, Rohweder & Lydersen 2005; O'Donnell et al. 2006). There have been numerous reports on the diversity of target INR in the Japanese population indicated for AF patients. For safety reasons, the Japanese
guidelines recommended a PT-INR control of 2.0-3.0 in patients <70 years old and 1.6-2.6 in patients ≥70 years old due to the higher risk of bleeding at this advanced age population (Group 2010; Mitamura 2011; Okumura et al. 2011). On the other hand, the Japanese RE-LY trial suggested a 2.0-2.6 INR target for patients with age ≥70 years (Hori et al. 2011). However, both guidelines showed a more precise and narrow therapeutic target range to be suggested in this cohort of Asian population.

Several studies have reported that the rate of adverse events sharply increases when the INR deviates from the required target ranges (Cannegieter et al. 1995; Hylek et al. 1996; White et al. 2007). A larger retrospective study consisting of more than 3000 AF patients found that a third of the cohort presented with the worst INR control (TTR of 48%) doubled in the rate of stroke (ischaemic and haemorrhagic), MI, major bleeding and death as compared to a third with the best INR control (TTR of 83%) (White et al. 2007). When common adverse effects such as GI and urinary tract bleeding occur, the possibility of the presence of an underlying occult lesion should always be suspected. This is due to the reason that most patients with a history of other bleedings frequently predicts major bleeding due to warfarin, especially those related to the GI tract (Palareti et al. 1996). A few studies have identified that occult lesions were responsible for a majority of the haemorrhage incidences in patients receiving warfarin therapy regardless of their PT and INR status (Coon & Willis 1974; Jaffin, Bliss & LaMont 1987). In terms of haematuria, one study reported no significant differences in the occurrence of haematuria either with therapeutic or elevated INRs (3.2% of anticoagulated patients vs 4.8% in the control group) (Culclasure, Bray & Hasbargen 1994).

There are also uncommon nonhemorrhagic adverse events associated with warfarin therapy. The most significant of these side effects are the acute thromboembolism complications, including skin necrosis and gangrene which usually occurs on the 3rd to 8th day of treatment (Weinberg et al. 1983). An association between warfarin-induced skin necrosis and protein C deficiency and less commonly, protein S deficiency has been reported (Broekmans et al. 1983; Grimaudo et al. 1989). Purple toe syndrome is another rare side effect presented by the sudden appearance of bilateral, painful, purple lesions on the toes and sides of the feet that blanch with pressure. This
adverse effect usually develops 3 to 8 weeks after the start of warfarin therapy (Raj, Collins & Rangarajan 2001). Warfarin therapy also intervenes with the carboxylation of GIa proteins which are generated in the bone and hence contributes to foetal bone abnormalities when mothers are treated with warfarin during pregnancy, hence this drug is contraindicated in pregnancy (Hauschka et al. 1989; Pettifor & Benson 1975)
2.2 VKORC1 (-1639 G>A) Gene Polymorphism

2.2.1 Contribution of VKORC1 (-1639 G>A) Gene Polymorphism in warfarin dose prediction

Warfarin exerts its antithrombotic effects via the inhibition of the vitamin K epoxide reductase complex 1 (VKORC1) and hence causing an interruption of the vitamin K redox cycle (Wittkowsky 2003). VKORC1 catalyses the conversion of vitamin K 2,3 epoxide to vitamin K hydroquinone, which is an essential cofactor for the γ-glutamyl carboxylase (GGCX) (Ageno et al. 2012). Inhibition of VKORC1 results in the synthesis of biologically inactive vitamin K dependent proteins, including the coagulation factors II, VII, IX and X that are responsible for thrombin formation (Hammwohner & Goette 2008; Horton & Bushwick 1999; Schwarz et al. 2008). The gene encoding for the VKOR protein is positioned on chromosome 16 (the short arm) (Li et al. 2004; Rost et al. 2004). This gene encodes numerous isoforms of a protein generally named as the VKORC1 or vitamin K oxide reductase complex 1. The polymorphisms associated with this gene were later discovered generating enzymes exhibiting different degree of sensitivities to warfarin therapy inhibition, subsequently altering the pharmacodynamics of warfarin (Li et al. 2004; Rieder et al. 2005; Sconce et al. 2005).

Genetic mutations which encode the VKORC1 frequently involve several mutations leading to various haplotypes causing the varying responses of warfarin therapy (Ageno et al. 2012). One of the most common SNP that have been extensively studied is the VKORC1 (-1639 G>A) gene polymorphism (King et al. 2008; Rathore et al. 2011; Teh et al. 2012; Wen et al. 2008; Xie et al. 2009; Yuan et al. 2005; Zhu et al. 2007). Individuals with the -1639 G>A single nucleotide polymorphism (SNP) in the VKORC1 gene is correlated with a decreased in VKORC1 expression and reduced level of the VKORC1 enzyme (Flockhart et al. 2008). To maintain the target INR, a relatively lower dose of warfarin is required in patients expressing the -1639A promoter variant due to the reduced in VKOR expression (Wang et al. 2008). On the other hand, patients with the VKORC1-1639GG genotype tend to require higher doses than patients with the GA or AA genotype (Zhu et al. 2007).
Although warfarin is the most commonly prescribed anticoagulant worldwide, it also exhibits large inter-individual and inter-ethnic variability in dose requirement. It has been reported that Asian population, including the Chinese, required a much lower warfarin maintenance dose compared to the Caucasians (Yuan et al. 2005). Besides patient-specific factors such as age, race, comorbidities and concurrent medications, genetic factors influencing warfarin response may explain a significantly higher proportion of variability in term of dosing (Wadelius et al. 2005). Population based studies have demonstrated that Asians usually acquire the VKORC1 -1639A allele and to a lesser extent in white people and those of African descendant (Ageno et al. 2012; Buzoianu et al. 2012; Scibona et al. 2012; Yuan et al. 2005). On the other hand, the VKORC1 -1639G allele is highly acquired by those of African descent and is associated with higher VKORC1 expression and activity and predominates in those of African descent (Kealey et al. 2007; Schelleman et al. 2007). The maintenance dose ranged from as low as 2.7mg daily warfarin dose for the warfarin sensitive haplotypes and up to as high as 6.2mg daily warfarin dose for the warfarin resistant haplotypes (Rieder et al. 2005). Asian American had demonstrated the highest frequency of warfarin sensitive haplotypes while the African Americans regularly demonstrated the warfarin resistant haplotype (Rieder et al. 2005). In terms of distribution of VKORC1 genotypes among the Asian population, the frequency of warfarin sensitive VKORC1 -1639A allele has been found to vary between 0.74 to 0.92 in East and South Asia, whereas the remaining parts of Asia showed variability between 0.14 and 0.56 (Gaikwad, Ghosh & Shetty 2014). Hence, the standard warfarin dose prescribed also varies among these Asian countries, such as China, Oman, Iran, Japan, and India, the standard daily warfarin dosage required is 3.43mg, 4.75mg, 3.79mg, 2.67mg and 4.7mg (±1.0-2.1mg) respectively (Gaikwad, Ghosh & Shetty 2014). This analysis urges the effort for each country to determine the genotype data of their respective population as substantive variations do exist even among neighbouring countries which may be attributed to the country’s historical background.
2.3 Coagulation

2.3.1 The extrinsic and intrinsic coagulation pathway

Thrombosis is the main player in the development of atherosclerosis and its acute vascular complications (Cushman et al. 1996). Referring to the Virchow’s triad, there are three determinants for thrombus formation: (1) circulatory stasis; (2) endothelial injury; and (3) hypercoagulable state (Watson, Shantsila & Lip 2009). There are two main pathways in the coagulation cascade; the intrinsic and extrinsic pathway which leads to the common pathway of coagulation cascade (HP, JM & PK 2003) (Figure 1). Each pathway consist of tissue factors (TF) and a number of coagulation factors (Factor II, VII, IX and X) which eventually contribute to thrombin amplification and fibrin formation (Walker & Royston 2002). Factor II, VII, IX and X as well as regulatory anticoagulant proteins C, S and Z are all the zymogen forms of vitamin K-dependent serine proteases (Becker 2005). Vitamin K is an essential cofactor for a post-translational alteration which attaches the 10 - 12 glutamic acid residues in the amino terminal portion of each proteins to a carboxyl group (Butenas et al. 2007). The vitamin K-dependent proteins use these γ-carboxyl glutamic acid (gla) residues clusters to attach to phospholipid surfaces to agglomerate multi-molecular coagulation complexes (Butenas et al. 2007; Stafford 2005).
Figure 1: Coagulation Cascade (Leftkowitz 2008)

Theoretically, the in vivo haemostasis process is presumed to only be inaugurated by the cell-based tissue factor generated at the point of trauma (Hoffman & Monroe 2005). Tissue factor then binds to factor VII or VIIa in a 1:1 ratio, leading to a tissue factor/factor VIIa complex which activates either factor X or factor IX which subsequently activates serine proteases through the cleavage process of a peptide activation (Hoffman 2003). Once this pathway is activated, the tissue factor/factor VIIa which activates factor X is expeditiously shut down by the endothelial cell produced inhibitor called the TFPI or tissue factor pathway inhibitor. This newly activated factor IXa then attaches to VIIIa, its cofactor on a phospholipid surface to generate the complex that involves in the activation of factor X to Xa (Hoffman 2003). The factor X to Xa activation process hence initiates the common and last pathway for the activation of thrombin. Factor Xa then binds to a cofactor, factor Va and calcium atop phospholipid surfaces for the establishment of a prothrombinase complex. This prothrombinase complex hence influences the conversion of factor II (prothrombin) to factor IIa (thrombin) by means of cleavage of prothrombin (Butkowski et al. 1977;
Hoffman 2003; Mann 2003). Even a minute quantity of thrombin generated by the extrinsic pathway is sufficient to initiate the coagulation pathway and once induced by the appropriate conditions, an amplification of thrombin generation via an intrinsic mechanism will take part (Roberts, Hoffman & Monroe 2006).

The intrinsic arm of the coagulation pathway incorporates the activation of factor XI to XIa by thrombin, with the final intention of generating more thrombin with the help of both factor IXa and factor VIIIa for factor Xa generation (Mann 2003; Stassen, Arnout & Deckmyn 2004). This pathway model illustrates the operation of how the key cofactors, factor V and factor VIII are activated by thrombin (Hoffman 2003; Stassen, Arnout & Deckmyn 2004).

2.3.2 Factor IIa (Thrombin) and Factor Xa (FXa)

The antithrombotic effect of warfarin has generally been associated to its anticoagulant nature, which is moderated by the reduction of four vitamin K-dependent coagulation factors (Ageno et al. 2012). Recently, evidences suggest that the anticoagulant and antithrombotic effects can be dissociated and that reduction of prothrombin and possibly factor X are more important than reduction of factors VII and IX for the antithrombotic effect of warfarin (Hirsh et al. 2003). The first observation was recorded 40 years ago utilising a stasis model of thrombosis in rabbits, which demonstrated that the antithrombotic effect of warfarin needed 6 days of treatment whereas an anticoagulant effect may develop within just 2 days (Wessler & Gitel 1984). The warfarin therapy’s antithrombotic effect depends upon the prothrombin (factor II) reduction, which demonstrated a comparatively long half-life of around 60 to 72 hours, compared the other vitamin K-dependent factors which requires only 6-24 hours and are responsible for the more expedited anticoagulant effect (Wessler & Gitel 1984). Another study showed that in a rabbit model of tissue factor-induced intravascular coagulation, there was a protective effect of warfarin therapy which is primarily due to the lowering of prothrombin levels mechanism of this drug (Zivelin, Rao & Rapaport 1993). Using a human model, a study had reported that clots generated from the umbilical cord plasma which contains about half the prothrombin concentration level of an adult control plasma formed significantly less fibrinopeptide A, hence demonstrating
less thrombin activity, compared to the clots formed from maternal plasma (Patel et al. 1996). The theory that warfarin exhibits its antithrombotic effect through the reduction of prothrombin levels has been proven by the observations that clot-bound thrombin is an essential mediator of clot growth (Weitz et al. 1990) and that the reduction in prothrombin levels reduces the amount of thrombin generation bound to fibrin which in turn reduces thrombogenicity (Patel et al. 1996).

Due to the suggestion that the antithrombotic effect of warfarin is reflected in lower levels of prothrombin, overlapping heparin treatment with warfarin until the PT or INR is prolonged into the required therapeutic range when treating patients with thrombosis is essential in clinical practice (Ageno et al. 2012). Since the half-life of prothrombin is about 60 to 72 hours, more than 4 days of heparin-warfarin treatment overlap is necessary. Moreover, the levels of native prothrombin antigen during warfarin therapy reflects antithrombotic activity more accurately than PT (Furie et al. 1990). Due to these reasons, it has been suggested that factor II and factor X determination may present as an alternative to monitoring the efficacy of warfarin therapy due to their long half-lives (approximately 3 and 2 days, respectively) and also their role in the final step of the coagulation cascade (common pathway) (Costa et al. 2000). Both factor II and factor X levels also appears to be major determinants of the therapeutic efficacy of oral anticoagulants (Chan et al. 1987; Lind et al. 1997). In one study, both factor II and factor X levels determination were found to be useful as alternative marker when INR values are not consistent with the therapeutic effect, as occurs when haemorrhagic or thromboembolic events are present (Costa et al. 2000). However, paradoxical results were reported in another where there was a non-linear correlation between INR and both factor II and factor X activities which concluded a negligible role of both markers as alternative response markers (Costa et al. 2010).
2.4 Anticoagulation Monitoring

2.4.1 INR: Monitoring Anticoagulant Intensity

The most common test to monitor the intensity of warfarin therapy is the PT test (Ageno et al. 2012). The PT reflects the three out of the four reduction of the vitamin K-dependent procoagulant clotting factors (i.e., II, VII, and X) which are reduced proportionally by warfarin according to their respective half-lives. At the initial stage of the treatment of warfarin, the PT responds mainly to the reduction of factor VII, which has a relatively shorter half-life of approximately 6 hours (Hirsh et al. 2003). The prolongation of PT was then subsequently influenced by factor X and II reduction which express a relatively longer half-life of 60 to 72 hours. This PT assay is run by adding calcium and thromboplastin to citrated plasma. The term “thromboplastin” usually denotes a phospholipid-protein extract of tissue, usually originating from the brain, lung or placenta that contains both the tissue factor and phospholipid vital in promoting the activation of factor X by factor VII (Ageno et al. 2012). Thromboplastins’ responsiveness heavily depends on the reduction of vitamin K-dependent coagulation factors. The intensity of the activation of factor X by the factor VIIa/tissue factor complex is reflected by the responsiveness of a given thromboplastin to those clotting factors induced by warfarin. A responsive thromboplastin displays higher PT prolongation for a similar vitamin K-dependent clotting factors reduction compared to an unresponsive one. The responsiveness of a thromboplastin can be analysed through the assessment of its international sensitivity index (ISI). Thromboplastins that are very sensitive are usually demonstrated by an ISI of approximately 1.0 and are currently available and are made by human factor manufactured through recombinant technology and intensive phospholipid preparations.

The intensity indicator of warfarin therapy in the form of PT is very inaccurate when expressed in seconds, or simply as a ratio of the patient's plasma value to that of a healthy control subject's plasma, or even as a percentage of diluted thromboplastins towards warfarin (Ageno et al. 2012). The diversity in thromboplastin responsiveness contributes significantly to the variations in oral anticoagulant dosing among different countries leading to an erratic and excessive anticoagulation predicament (Poller & Taberner 1982). These shortcomings in PT monitoring prompted the development of the
standard in the form of INR for monitoring oral anticoagulant therapy. In 1982, a calibration model (Kirkwood 1983) was adopted and is now being used to convert the PT ratio measured with the local thromboplastin time into an INR value, standardising anticoagulation intensity measurement by:

\[ \text{INR} = (\text{patient PT/mean normal PT})^{\text{ISI}} \]

Or

\[ \log \text{INR} = \text{ISI} \log (\text{observed PT ratio}) \]

where ISI denotes the International Sensitivity Index of the thromboplastin used at the local laboratory to perform the PT test. The ISI reflects the responsiveness of a given thromboplastin to the reduction of the vitamin K-dependent coagulation factors compared with the primary World Health Organization (WHO) international reference preparations, hence the more responsive the reagent, the lower the ISI value (Hirsh 1987; Kirkwood 1983; Poller 1987b).

The INR value is based on ISI values that has been derived from patients stabilised with warfarin therapy for at least 6 weeks (Ageno et al. 2012). Hence, during the early course of warfarin therapy, the INR has not been validated and should be interpreted with caution. The precision of INR values may be influenced by the reagents required which display various sensitivities and also by automated clot detectors run in laboratories (D'Angelo et al. 1989; Finck, Doetkott & Miller 2001; Lind et al. 1999; Poggio et al. 1989; Poller, Thomson & Taberner 1989; Ray & Smith 1990; Thomson, Taberner & Poller 1990). As a rule of thumb, it has been recommended that laboratories should use at least moderately responsive thromboplastins reagents, e.g. ISI<1.7 and also by using reagents and instruments where the ISI had been validated and established (Fairweather et al. 1998).

Some of the ISI values given by the manufacturer of thromboplastin reagents may not be appropriate when it comes to local settings; hence local calibrations using plasma samples with valid and certified PT values are required to obtain the ISI specific to the instrument (Kazama et al. 1990; Ng et al. 1993; Poller 1987a). The mean normal plasma PT is not interchangeable with a laboratory control PT, hence the utilisation of a
less than properly established mean normal PT may provide false INR calculations, especially in the presence of less responsive reagents (van den Besselaar et al. 1993). The mean normal PT should be established for every new thromboplastin batch with the same instrument for running the PT assay (van den Besselaar et al. 1993). Another important factor is the citrate concentration used to anticoagulate the plasma which will also affect the INR result (Adcock, Kressin & Marlar 1997; Duncan et al. 1994). Generally, the higher the citrate concentration (e.g. 38%) lead to higher INR values, hence an under filling of the sodium citrate collection tube may prolong the PT due to the excessive citrate component (Duncan et al. 1994). To overcome this problem, carefully filling the tubes to its required volume to ensure a final 3.2% concentration of citrate for blood coagulation is essential (Duncan et al. 1994).

2.4.2 TTR: Evaluating the Quality of Monitoring

Time in therapeutic range or TTR has been used to establish the association between treatment intensity and risk of adverse effects in patients treated with warfarin therapy by evaluating the frequency of such events over time (Forfar 1982; Palareti et al. 2005b; White et al. 2007). Numerous studies involving various patient population adopting different INR target ranges, different scales of anticoagulation intensity measurement (i.e., PT, PT ratio and INR), different methods TTR determination and different algorithms of dose management have demonstrated a strong correlation between TTR and bleeding and thromboembolism incidences (Cannegieter et al. 1995; Charney et al. 1988; Connolly et al. 1991; Connolly et al. 2008; Ezekowitz et al. 1992; Forfar 1982; Palareti et al. 2005b; Petersen et al. 1989; van der Meer et al. 1993; White et al. 2007)

A few retrospective study analyses of patients on long term warfarin therapy reported a higher risk of major bleeding and thromboembolic events during the periods when patients were out of the therapeutic INR rage compared with periods when they were in-range (Cannegieter et al. 1995; Hylek et al. 1996). Another sub study examining the impact of TTR on the efficacy of warfarin vs dual antiplatelet therapy (aspirin and clopidogrel) in nonvalvular AF patients, showed an overall result which favoured
warfarin therapy. The major determinant for warfarin efficacy was the TTR value during the treatment period. (Connolly et al. 2008). However, the superiority of warfarin over antiplatelet therapy was diminished below a TTR threshold of 58-65% (Connolly et al. 2008). The INRs or TTR percentages is majorly dependent on the dose management quality. A poorly managed anticoagulation therapy will lead to an increased ratio in subtherapeutic INRs, especially on the first three months of therapy after an acute DVT event which subsequently leads to a higher chance of recurrence (Palareti et al. 2005a; Palareti et al. 2005b).

TTR can be determined in numerous ways; hence a comparison between these methods can be difficult (Schmitt, Speckman & Ansell 2003). Currently there are three methodologies for TTR calculations: fraction of INR values where the calculation in fractions of all within range INR values are determined (e.g. the amount of in-range INRs divided by the total amount of INR tests); using the “cross-section of the files” method, which looks into the fraction of patients with an in-range INR at one point in time divided by the total count of patients who had their INR measured at that point in time; or using the linear interpolation methodology by Rosendaal et al (Rosendaal et al. 1993), which applies the assumption that a linear correlation exists between two INR values hence enabling the allocation of specific INR values to each day between the INR tests for each patient. There are advantages as well as disadvantages for each method (Schmitt, Speckman & Ansell 2003) as summarised here (Table 1). Moreover, the output of all these methodology highly depends on whether an exact or expanded therapeutic range is being applied, such as when the INRs collected during invasive procedures where the warfarin therapy may be interfered were included, or whether different preparations of oral anticoagulant such as warfarin, phenprocoumon, acenocoumarol, or fluindione were used (Gadisseur et al. 2002; Meier, Seva & Fay 2007; Pattacini et al. 1994). A recent prospective study in Sarawak, Malaysia (Edwards et al. 2013) reported a median (IQR) TTR of 61.6% (44.6-74.1%) using the Rosendaal method. Of the out-of-range readings, there were more subtherapeutic INR readings, 30.0% compared to supratherapeutic readings, 15.4% in this cohort. Due to the low rates of both bleeding and thromboembolic despite a majority of the INR readings being subtherapeutic in this cohort, it has been suggested that a therapy initiation with lower intensity may be more appropriate to adopt (Edwards et al. 2013)
Table 1: Advantages and Disadvantages of TTR methods (Schmitt et al. 2003)

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<tr>
<th>Methodology</th>
<th>Advantage</th>
<th>Disadvantages</th>
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<tr>
<td>Fraction of INR’s</td>
<td>• Simple calculation&lt;br&gt;• ONE INR value per patient in clinic population&lt;br&gt;• Not influenced by extent of INR out-of-range</td>
<td>• Bias (under-estimate TTR of group)&lt;br&gt;• Not calculating actual days within target range&lt;br&gt;• Not consider individual patients</td>
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<tr>
<td>Cross-section-of-the-files</td>
<td>• Simple calculation&lt;br&gt;• Considers individual patients&lt;br&gt;• Not influenced by extent of INR out-of-range</td>
<td>• Not calculating actual days within target range&lt;br&gt;• ONLY considers one point in time</td>
</tr>
<tr>
<td>Rosendaal linear interpolation</td>
<td>• Takes into account actual days in target range&lt;br&gt;• Calculate INR specific incidence rates of adverse events</td>
<td>• Difficult to calculate&lt;br&gt;• Assumptions about INR between actual tests&lt;br&gt;• Not consider individual patients&lt;br&gt;• Bias (extreme out-of-range INR)</td>
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2.5 New oral anticoagulation (NOAC) therapies

2.5.1 Targeted therapies

New oral anticoagulation (NOACs) therapies, targeting the inhibition of thrombin and Factor Xa have emerged over the past few years. Dabigatran is a direct thrombin inhibitor while Rivaroxaban and Apixaban are direct FXa inhibitors were all reported to be noninferior to warfarin in terms of stroke and thromboembolic prevention for patients with non-valvular atrial fibrillation (AF) (Connolly et al. 2009; Ezekowitz et al. 2010; Patel et al. 2011; Potpara & Lip 2013). Furthermore, dabigatran at a higher dose of 150mg twice daily demonstrated superiority for the prevention of thromboembolic events compared to warfarin (Connolly et al. 2009; Hori et al. 2013). Recently published data on the Asian subgroup of the RE-LY trial also concluded that haemorrhagic stroke rates, a potentially fatal condition were much lower on Dabigatran compared to warfarin (Hori et al. 2013).

Dabigatran is a selective, reversible, direct thrombin inhibitor given in the form of dabigatran etexilate, an oral prodrug. The drug is approved by the FDA on October 19, 2010 for the stroke prevention in non-valvular atrial fibrillation patients and has since been used worldwide. This drug is also approved in various countries for the VTE prevention for those undergoing total knee or hip replacement surgery. For AF patients, the dosing schedule consist of either 110mg twice daily or 150mg twice daily depending on their age and creatinine clearance (Connolly et al. 2009; Hori et al. 2013). Rivaroxaban and Apixaban on the other hand are direct factor Xa inhibitors and have just recently been approved in numerous countries, including the United States, for the VTE prevention in patients undergoing total knee or hip replacement surgery and also for non-valvular atrial fibrillation. It has also been indicated for long term DVT and pulmonary embolism (PE) on November 2, 2012. It was reported to be superior to enoxaparin, which is a low-molecular-weight heparin in VTE prevention after total knee or hip replacement surgery. It was also found to be more effective compared to placebo when given to those with a history of DVT or PE after an initiation of a standard warfarin therapy course of 6 to 12 months (Kakkar et al. 2008; Turpie et al. 2009). The dose indicated for AF is 20mg once daily. Apixaban was indicated for non-valvular AF at either 2.5mg or 5mg twice daily dosing (Granger et al. 2011).
2.5.2 Advantages and disadvantages of NOACs

These NOACs display a predictable pharmacodynamics and pharmacokinetic profile with relatively short half-lives, low incidence of food and drug interactions, hence requiring no regular dose adjustments and monitoring (Shameem & Ansell 2013). Dabigatran etexilate display a half-life of 12-17 hours while Rivaroxaban display a half-life of 7-17 hours (Ageno et al. 2012). Despite their advantages over warfarin, these NOACs are not widely prescribed due to their high cost and limited indications, scenarios typically found in developing countries (Cannon & Stecker 2010). Besides that, there are inadequate clinical experiences to confidently guide the management of major bleeding events, drug over dosage, surgery urgency as well as urgent therapeutic or diagnostic procedures in those that are taking these new drugs. Hence, the management of life-threatening bleeding scenarios remains empirical (Ageno et al. 2012). Moreover, dabigatran was also found to be associated with a greater risk of non-haemorrhagic side effects where it remains a possibility that the platelet-activating effect of dabigatran therapy in patients with AF may lead to myocardial infarction events (Connolly et al. 2009). In terms of Rivaroxaban and Apixaban, their use are also limited due to the lack of comprehensive data for its long term safety and efficacy which renders warfarin to remain as the anticoagulant of choice in the coming years (Eikelboom & Weitz 2010).
CHAPTER 3: MATERIALS AND METHODS

3.1 Study Cohort

A total of two hundred and twelve multi-ethnic Sarawakian patients with symptomatic and asymptomatic atrial fibrillation at Sarawak General Hospital Heart Centre were recruited from September 2012 until July 2013 and were followed up retrospectively. Patients treated with long term warfarin therapy for one year and above with at least 10 INR readings, an age of greater than 18 years, and warfarin therapy continuing throughout a 6 month observation period were recruited with prior informed consent. The INR readings within the first 3 weeks of warfarin initiation were excluded for analysis. As the original study approved by the Research Ethics Committee Ministry of Health Malaysia was pre-designed to be a one year outcome clinical study; a total of 10 INR readings for pre and post recruitment were required for standardisation. Patients, who are alcoholic, smokers, have recent stroke or myocardial infarction within 3 months prior enrolment, and having severe kidney or liver impairment were excluded from this study. Among them, a total of two hundred patients were selected as candidate patients for this study.

Patients’ demographic data were collected from the case notes including age, gender, body weight, body mass index, CHA2DS2-VASc Score, duration of warfarin therapy, average daily warfarin dose, comorbidity and concomitant medications. Baseline INR of each patient were obtained via a portable PT monitor (Coagucheck, Roche Diagnostics, Mannheim, Germany) requiring only a finger prick test. The monitor was internally calibrated so that the mean normal PT was 12.6 seconds. Ten INR readings were collected retrospectively to determine the TTR status of each patient, with each INR and warfarin dose recorded for analyses.

Blood samples of study patients were collected during their regular outpatient clinic visits from 2-5pm on Monday, Wednesday and Friday of the week. Patients recruited had 15mls of venous blood drawn from the antecubital fossa vein. Whole blood was drawn using a 23G needle into three vacuumed blood collection tubes (one of “Tube A” and two of “Tube B”). Tube A is a 5ml tube containing ethylenediaminetetraacetic acid (EDTA), and were used for DNA analysis; Tube B
contains EDTA anticoagulant and was used for plasma protein and plasma ELISA analysis of thrombin and Factor Xa. All blood samples were processed and aliquoted accordingly after centrifugation at 3000g for 10 minutes for plasma separation, and were stored in a secured -80°C freezer for batch analysis.

The study protocol was approved by the Medical and Research Ethics Committee Ministry of Health Malaysia and the SUHREC and Research Ethics Committee. All patients signed informed consent and participated in this study.

3.2 Molecular method for VKORC1 (-1639 G>A) Gene Polymorphism

3.2.1 Genomic DNA Extraction

Blood samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) for genotyping purposes. All blood samples were centrifuged at 3000g for 10 minutes for plasma separation with the buffy coat collected, aliquoted and were stored in a secured -80°C freezer before extraction. The genomic DNA from study patients were extracted using the Puregene™ Blood DNA Extraction Kit (Qiagen, Hilden, Germany) as per manufacturer’s instructions. Concisely, 300µL of buffy coat was added to 900µL Qiagen Red Blood Cell lysis solution, vortexed and incubated at room temperature for 5 minutes. The mixture was then centrifuged at 10,000rpm for 1 minute. The supernatant was discarded and the aforementioned procedure was repeated until the supernatant was clear in colour. The residual was then added with 300µL of Qiagen Cell Lysis solution, vortexed vigorously and incubated in a water bath at 55°C for 1 hour. The mixture was then cooled down to room temperature and was added with 100µL of Qiagen Protein Precipitation solution was added to the mixture and vortexed for 20 seconds then centrifuged at 5000rpm for 1 minute. The clear supernatant was then collected, added with 300µL isopropanol, inverted 50 times until a pellet is seen suspended. The solution was then centrifuged at high speed of 14,000rpm for 1 minute. The supernatant was discarded and 300µL of 70% ethanol was added to the pellet, vortex to suspend the pellet and centrifuged at 14,000rpm for 1 minute. The supernatant was carefully poured out and the pellet was allowed to air dry. Finally, 30µL of Qiagen
DNA hydration solution was added to the pellet and incubated at 65°C for 1 hour in a waterbath to dissolve the DNA. The DNA concentration was then determined using an IMPLEN NanoPhotometer® Pearl before being stored in the -80°C freezer for genotyping.

3.2.2 PCR-RFLP Genotyping

Genotyping of the VKORC1 (-1639 G>A) polymorphism was performed by the PCR-RFLP using the genomic DNA extracted from blood samples of the study patients. The genomic DNA flanking the SNP of interest was amplified with PCR reaction in a final volume of 25µL containing 1.5mmol/L MgCl₂, 200µl/L dNTP, 0.3µmol/L each primer, 10ng genomic DNA as template, and 0.5U PROMEGA Taq polymerase. The forward and reverse primer sequences for VKORC1 (-1639 G>A) is 5’-GCCAGCAGGAGGGAAATA-3’ and 5’-AGTTTGGACTACAGGTGCCT-3’, respectively (Miao, Yang et al. 2007).

The PCR reactions were carried out in a Mastercycler® Gradient thermal cycler (Eppendorf, Hamburg, Germany) with an initial denaturation step of 5 minutes at 94°C, followed by 30 cycles of denaturation at 94°C for 30s, annealing at 59°C for 20s, and extension at 72°C for 30s, with a final extension for 7 minutes at 72°C. Blank tubes without DNA were included in each batch of samples as negative control. The PCR product (8µL) was visualised on 2.5% agarose gels stained with ethidium bromide for confirmation prior to performing the RFLP assay.

After amplification, the 5’-untranslated region PCR product (17µL) was digested with 10U of PROMEGA MspI in a final volume of 19.7µL in the appropriate digestion buffer at 37°C for 4 hours. The digested products was then visualised on 4.5% agarose gels stained with ethidium bromide. After confirming the purity and mobility of each PCR-RFLP product by agarose gel electrophoresis, 10% of samples of each genotype (wild type GG, heterozygous GA, and homozygous AA variant) was randomly selected and sequenced for both strands by direct DNA sequencing by First BASE Laboratories Sdn Bhd for validation purposes.
3.3 ELISA method for Thrombin and FXa Assay

3.3.1 Thrombin Assay

The circulating concentration of thrombin (Factor IIa) in plasma was measured by the ELISA method which adopts the principle of sandwich based enzyme-linked immunosorbent assay (Cusabio Biotech Co. Ltd kit). The upper and lower limit of detection by ELISA for thrombin was 500ng/ml and 7.8ng/ml respectively. Both intra-assay and interassay coefficient variations were less than 8% and 10%, respectively. Plasma was collected using EDTA as an anticoagulant, centrifuged at 3000g for 10 minutes, aliquot and stored at -80°C before batch analysis. A total of 40 samples were performed for each batch within 2 months of sample collection. All samples and assays were analysed in duplicate to account for variation in results. The assay was conducted per Cusabio Biotech Co. manufacturer’s instructions. All reagents and samples were brought to room temperature (18-25°C) for 30 minutes before use. The plasma samples were diluted with the Kit’s Sample Diluent at a 1:2000 ratio prior test. The suggested 2000-fold dilution was achieved by adding 2µL of sample to 98µL of Sample Diluent. The 2000-fold dilution was then completed by adding 6µL of this solution to 234µL of the aforementioned Sample Diluent.

For analysis, 100µL of standard and sample were added to each well, covered with the adhesive strip provided and incubated for 2 hours at 37°C. The liquid was subsequently removed from each well and 100µL of the prepared Biotin-antibody (1x) was then added to each well, covered and incubated for another 1 hour at 37°C. Each well was then aspirated and washed, repeating the process two times for a total of three washes. Each well was washed with the prepared Wash Buffer (200µL) using an autowasher (HydroControl®) and was let stand for 2 minutes prior to the next procedure. The complete removal of liquid in each well was essential for assay accuracy and was ensured by inverting the plate and blotting it against clean paper towels. A total of 100µL of the prepared HRP-avidin (1x) was then added to each well, covered and incubated for 1 hour at 37°C. The aspiration and washing process was then repeated again for a total of 5 washes before adding in 90µL of TMB Substrate to each well, protected from light and incubated for 15-30 minutes at 37°C. Finally, 50µL of Stop Solution was added to each well with gentle agitation to ensure thorough mixing. The
optical density of each well was determined within 5 minutes, using a microplate reader set to 450nm (Sunrise, Tecan®, Switzerland). The wavelength correction was set at 540nm. Readings at 540nm was subtracted from readings at 450nm. These subtractions will correct for optical imperfections in the plate.

### 3.3.2 FXa Assay

The circulating concentration of Factor Xa (FXa) in plasma was measured by the ELISA method which adopts the principle of sandwich based enzyme-linked immunosorbent assay (Cusabio Biotech Co. Ltd kit). The upper and lower limit of detection by ELISA for FXa was 20ng/ml and 0.312ng/ml respectively. Both intra-assay and interassay coefficient variations were less than 8% and 10%, respectively. Plasma was collected using EDTA as an anticoagulant, centrifuged at 3000g for 10 minutes, aliquot and stored at -80°C before batch analysis. A total of 40 samples were analysed for each batch within 2 months of sample collection. All samples and assays were performed in duplicate to account for variation in results. The assay was conducted per manufacturer’s instructions. All reagents and samples were brought to room temperature (18-25°C) for 30 minutes before use. No sample dilution was required for this analysis.

For analysis, 100µL of standard and sample were added to each well, covered with the adhesive strip provided and incubated for 2 hours at 37°C. The liquid was subsequently removed from each well and 100µL of the prepared Biotin-antibody (1x) was then added to each well, covered and incubated for another 1 hour at 37°C. Each well was then aspirated and washed, repeating the process two times for a total of three washes. Each well was washed with the prepared Wash Buffer (200µL) using an autowasher (HydroControl®) and was let stand for 2 minutes prior to the next procedure. The complete removal of liquid in each well was essential for assay accuracy and was ensured by inverting the plate and blotting it against clean paper towels. A total of 100µL of the prepared HRP-avidin (1x) was then added to each well, covered and incubated for 1 hour at 37°C. The aspiration and washing process was then repeated again for a total of 5 washes before adding in 90µL of TMB Substrate to each well, protected from light and incubated for 15-30 minutes at 37°C. Finally, 50µL of Stop Solution was added to each well with gentle agitation to ensure thorough mixing. The
optical density of each well was determined within 5 minutes, using a microplate reader set to 450nm (Tecan®). The wavelength correction was set at 540nm. Readings at 540nm was subtracted from readings at 450nm. These subtractions will correct for optical imperfections in the plate.
3.4 Time in Therapeutic Range

The overall percentage time in therapeutic range (TTR) was calculated based on the method established by Rosendaal et al. which incorporates the linear interpolation theory to estimate the time spent at each INR value. The TTR was also calculated using the traditional method as described in the ACCP guidelines: number of INR measurements within target range divided by the total number of INR measurements.

3.5 Statistical Analysis of Data

All analyses were performed applying Excel for Windows and SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Categorical variables were presented as frequencies with percentages and numerical variables as mean with standard deviation or median with interquartile range. For univariate analyses, the independent-samples t-test and Pearson’s Chi-square test (or Fisher exact test as appropriate) were used to determine the differences of variables between two subgroups to compare. Normally distributed continuous variables are expressed as mean (SD) and were compared using the t test. The non-normal variables are expressed as median (interquartile range; IQR) and were compared using the Mann-Whitney U test. Variables with a p<0.05 and p>0.10 level of significance were entered into multivariate logistic regression using the forward stepwise model analysis to identify independent predictors. All statistical analysis were two-sided with analyses being significant if $\alpha \leq 0.05$. 
CHAPTER 4: RESULTS

4.1 Patients characteristics

4.1.1 Patient demographics

Basic characteristics of the 200 patients with Atrial Fibrillation are shown in Table 2. The average years (SD) that the patients are treated on warfarin was 5.99(3.8) years. There are 54.0% of male patients in this cohort and the mean (SD) age of this cohort was 57.24(8.5) years, ranging from 29 to 74 years. The average duration in years (SD) of them being treated on warfarin therapy was 5.99(3.8) years, ranging from 1.0 to 21.0 years. The mean (SD) INR on admission was 2.24(0.4). Each patient has their INR monitored and recorded for 10 times and the average TTR in this cohort was 61.35(22.0) %. The number of patients with permanent AF, paroxysmal AF, persistent AF and other types of AF were 93(46.5%), 38(19.0%), 18(9.0%) and 51(25.5%), respectively.

In terms of ethnicity, there were 60(30.0%) Malay, 87(43.5%) Chinese and 53(26.5%) non-Malay Bumiputera in this cohort. The mean (SD) daily warfarin dose was 2.81(1.1) mg, ranging from 1mg to 8mg per day. Among the out-of-range readings, there were a total of 582(29.1%) or subtherapeutic INR readings compared to 304(15.2%) of supratherapeutic readings in this cohort. A total of 128(64%) of patients in this cohort receive an alternate dosing regimen at least once with the mean (SD) times of alternate dosing regimen being prescribed at 567(28.4%). The mean (SD) CHA2DS2-VASc score was 2.30(1.3).
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number (%) or mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of total patients</td>
<td>200</td>
</tr>
<tr>
<td>Age</td>
<td>57.24(8.5)</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>108(54.0)</td>
</tr>
<tr>
<td>Female</td>
<td>92(46.0)</td>
</tr>
<tr>
<td><strong>BMI</strong></td>
<td></td>
</tr>
<tr>
<td>26.55(5.1)</td>
<td></td>
</tr>
<tr>
<td><strong>TTR</strong></td>
<td></td>
</tr>
<tr>
<td>61.35(22.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
</tr>
<tr>
<td>Malay</td>
<td>60(30.0)</td>
</tr>
<tr>
<td>Chinese</td>
<td>87(43.5)</td>
</tr>
<tr>
<td>Non-Malay Bumiputera</td>
<td>53(26.5)</td>
</tr>
<tr>
<td><strong>Mean daily warfarin dose(mg)</strong></td>
<td>2.81(1.1)</td>
</tr>
<tr>
<td><strong>Mean years on warfarin</strong></td>
<td>5.99(3.8)</td>
</tr>
<tr>
<td><strong>Risk Factor, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>127(63.5)</td>
</tr>
<tr>
<td>DM</td>
<td>47(23.5)</td>
</tr>
<tr>
<td>CHF</td>
<td>56(28.0)</td>
</tr>
<tr>
<td>CAD</td>
<td>76(38.0)</td>
</tr>
<tr>
<td>History of stroke</td>
<td>36(18.0)</td>
</tr>
<tr>
<td>History of bleeding</td>
<td>41(20.5)</td>
</tr>
<tr>
<td><strong>CHA₂DS₂VASc Score(SD)</strong></td>
<td>2.30(1.3)</td>
</tr>
<tr>
<td>1</td>
<td>67(35.5%)</td>
</tr>
<tr>
<td>2</td>
<td>56(28.0%)</td>
</tr>
<tr>
<td>3</td>
<td>45(22.5%)</td>
</tr>
<tr>
<td>4</td>
<td>18(9.0%)</td>
</tr>
<tr>
<td>5</td>
<td>10(5.0%)</td>
</tr>
<tr>
<td>6</td>
<td>4(2.0%)</td>
</tr>
<tr>
<td><strong>Laboratory Findings</strong></td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (g/100mL)</td>
<td>14.16(1.9)</td>
</tr>
<tr>
<td>Platelet counts (x10⁹/L)</td>
<td>231.29(66.4)</td>
</tr>
<tr>
<td>Creatinine CL(mL/min)</td>
<td>74.35(26.4)</td>
</tr>
<tr>
<td><strong>AF Type</strong></td>
<td></td>
</tr>
<tr>
<td>Permanent</td>
<td>93(46.5)</td>
</tr>
<tr>
<td>Paroxysmal</td>
<td>38(19.0)</td>
</tr>
<tr>
<td>Persistent</td>
<td>18(9.0)</td>
</tr>
<tr>
<td>Others</td>
<td>51(25.4)</td>
</tr>
<tr>
<td><strong>INR (Admission)</strong></td>
<td>2.24(0.4)</td>
</tr>
<tr>
<td><strong>Total concurrent meds</strong></td>
<td>4.22(1.9)</td>
</tr>
<tr>
<td><strong>INR readings, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Subtherapeutic</td>
<td>582(29.1)</td>
</tr>
<tr>
<td>Therapeutic</td>
<td>1114(55.7)</td>
</tr>
<tr>
<td>Supratherapeutic</td>
<td>304(15.2)</td>
</tr>
<tr>
<td><strong>Alternate Dosing</strong></td>
<td>567(28.4)</td>
</tr>
</tbody>
</table>
4.2 Genotyping

4.2.1 The VKORC1 (-1639 G>A) gene polymorphism

The distribution of the VKORC1 (1639 G>A) gene polymorphism, rs9923231 was shown in Figure 2. In our cohort of 200 patients, a majority of 143(71.5%) was found with the AA genotype, 54(27.0%) was found with the GA genotype and only 3(1.5%) was found with the GG genotype. The A:G allelic frequency according to the Hardy-Weinberg Principle is 0.85:0.15. Figure 3 shows the distribution of these genotypes according to ethnic groups. The number of patients with AA genotype in Malay, Chinese and non-Malay Bumiputera patients were 49(34.3%), 58(40.5%) and 36(25.2%), respectively. The number of patients with GA genotype in Malay, Chinese and non-Malay Bumiputera patients were 11(20.4%), 29(53.7%) and 14(25.9%), respectively. All three patients with the GG genotype fell under the non-Malay Bumiputera group.

![Figure 2: Distribution of the VKORC1 (-1639G>A) Gene Polymorphism](image)
4.2.2 Clinical role

The clinical role of this particular genotype is summarised in Table 4. Using One-Way ANOVA to compare between genotype groups, patients with the VKORC1 GG genotype are significantly younger than the AA and GA genotype groups (p=0.005). Bonferroni Post-hoc analysis showed that both AA and GA age groups are significantly higher than the GG age group. The mean daily warfarin dose was also significantly different between each genotype. The mean (SD) daily warfarin dose for patients with the VKORC1 variant AA, GA and GG were 2.49(0.9) mg, 3.53(1.0) mg and 5.26(2.7) mg, respectively (p<0.001). There were no significant differences in gender distribution between all genotype groups (p=0.185). There were also no differences in genotype distribution between ethnic groups (p=0.112). When taking into account the extent and quality of anticoagulation, there were no significant differences in both INR and TTR between genotype groups; p=0.306 and p=0.924, respectively. Both biomarkers involved in this study, Factor IIa and Factor Xa also did not show any significant differences between genotype groups; p=0.210 and p=0.456, respectively.
<table>
<thead>
<tr>
<th>Group</th>
<th>VKORC1 AA</th>
<th>VKORC1 GA</th>
<th>VKORC1 GG</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (SD)</td>
<td>57.12(8.0)</td>
<td>58.39(8.8)</td>
<td>42.33(11.9)</td>
<td>0.005</td>
</tr>
<tr>
<td>Gender (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>73(67.6)</td>
<td>35(32.4)</td>
<td></td>
<td>0.185</td>
</tr>
<tr>
<td>Female</td>
<td>70(76.1)</td>
<td>22(23.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daily Warfarin dose (SD)</td>
<td>2.49(0.9)</td>
<td>3.53(1.0)</td>
<td>5.26(2.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ethnicity (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malay</td>
<td>49(81.7)</td>
<td>11(18.3)</td>
<td></td>
<td>0.112</td>
</tr>
<tr>
<td>Chinese</td>
<td>58(66.7)</td>
<td>29(33.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Malay Bumiputera</td>
<td>36(67.9)</td>
<td>17(32.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>INR(SD)</td>
<td>2.27(0.5)</td>
<td>2.19(0.4)</td>
<td>1.98(0.2)</td>
<td>0.306</td>
</tr>
<tr>
<td>TTR(SD)</td>
<td>61.21(21.6)</td>
<td>61.44(23.3)</td>
<td>66.33(28.7)</td>
<td>0.924</td>
</tr>
<tr>
<td>Factor IIa(SD)</td>
<td>612.62(468.2)</td>
<td>672.7(589.7)</td>
<td>1102.2(583.0)</td>
<td>0.210</td>
</tr>
<tr>
<td>Factor Xa(SD)</td>
<td>1.02(0.7)</td>
<td>1.15(0.9)</td>
<td>0.78(0.35)</td>
<td>0.456</td>
</tr>
</tbody>
</table>

Post Hoc analysis for Age variable; GA vs GG (p=0.004) and AA vs GG (p=0.008)
Post Hoc analysis for daily warfarin dose variable; AA vs GA (p<0.001); AA vs GG (p=0.001) and GA vs GG (p=0.007)
4.3 Thrombin and FXa

4.3.1 Clotting factor levels and its clinical role

The distribution of thrombin and FXa is shown in Figure 4 and 5. The mean (SD) of thrombin and FXa plasma concentrations in our cohort of patients are 636.19(506.4)µg/ml and 1.05(0.7)ng/ml, respectively. Plasma thrombin levels were found to be associated with the average daily warfarin dose, TTR and also BMI as shown in Figure 6, 7 and 8. Spearman’s correlation analysis shows that plasma thrombin levels has a negative and significant correlation with dose (r=-0.180, p=0.011) and TTR (r=-0.171, p=0.015). Using Pearson’s analysis, we also found a moderate and negative correlation between plasma thrombin levels and BMI (r=-0.251, p<0.001). There was however no significant correlation between plasma FXa levels with average daily warfarin dose, TTR and BMI (r=-0.071, p=0.318; r=-0.064, p=0.370 and r=0.049, p=0.490), respectively.

Figure 4: Distribution of Thrombin levels (ug/ml)
Figure 5: Distribution of FXa levels (ng/ml)

Figure 6: Correlation of Thrombin (µg/ml) and average daily warfarin dose (mg)
Figure 7: Correlation of Thrombin (µg/ml) and TTR

Figure 8: Correlation of Thrombin (µg/ml) and BMI
4.3.2 The association of thrombin and FXa with risk factors of atrial fibrillation

Additional analyses were performed to ascertain the correlation of plasma thrombin and FXa levels with known risk factors or atrial fibrillation (Table 4). The plasma thrombin levels were significantly higher in females (762.18 vs 528.87 µg/ml; p=0.001). However, significantly lower levels of plasma thrombin were found in those with hypertension, diabetes mellitus and coronary artery disease (all p<0.05). Previously, we found a correlation between plasma thrombin levels and BMI score. Now, when we analyse the thrombin levels between BMI groups, we found a significant increase in thrombin levels in patients with normal BMI (<25) compared to overweight patients (BMI≥25) (788.71 vs 552.24 µg/ml; p=0.001). There were no correlation between plasma thrombin levels with chronic heart failure, history of stroke and history of bleeding (p=0.424, p=0.193 and p=0.859), respectively. For FXa, no significant difference in plasma FXa levels were found in those with hypertension, diabetes mellitus, coronary artery disease, chronic heart failure, BMI groups, history or stroke and history of bleeding.
<table>
<thead>
<tr>
<th>CVD Risk Factor (n=200)</th>
<th>Thrombin conc (ug/ml) (SD)</th>
<th>P Value</th>
<th>Factor Xa conc (ng/ml) (SD)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hypertension</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (127)</td>
<td>564.55(489.1)</td>
<td>0.008</td>
<td>1.06(0.7)</td>
<td>0.872</td>
</tr>
<tr>
<td>No (73)</td>
<td>760.82(515.3)</td>
<td></td>
<td>1.03(0.8)</td>
<td></td>
</tr>
<tr>
<td><strong>DM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (47)</td>
<td>504.51(590.3)</td>
<td>0.041</td>
<td>0.97(0.6)</td>
<td>0.379</td>
</tr>
<tr>
<td>No (153)</td>
<td>676.64(472.6)</td>
<td></td>
<td>1.08(0.8)</td>
<td></td>
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<tr>
<td><strong>CHF</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (56)</td>
<td>682.19(476.6)</td>
<td>0.424</td>
<td>1.10(0.7)</td>
<td>0.536</td>
</tr>
<tr>
<td>No (144)</td>
<td>618.30(518.1)</td>
<td></td>
<td>1.03(0.8)</td>
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</tr>
<tr>
<td><strong>CAD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (76)</td>
<td>506.26(410.9)</td>
<td>0.004</td>
<td>1.15(0.8)</td>
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<td>No (124)</td>
<td>715.82(543.3)</td>
<td></td>
<td>0.99(0.7)</td>
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</tr>
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<td><strong>Stroke History</strong></td>
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<td></td>
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<tr>
<td>Yes (36)</td>
<td>735.95(650.1)</td>
<td>0.193</td>
<td>0.98(0.7)</td>
<td>0.515</td>
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<tr>
<td>No (164)</td>
<td>614.29(468.7)</td>
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<td>1.07(0.7)</td>
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</tr>
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<td><strong>Bleeding History</strong></td>
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<td></td>
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<tr>
<td>Yes (41)</td>
<td>623.6(443.8)</td>
<td>0.859</td>
<td>1.09(0.8)</td>
<td>0.715</td>
</tr>
<tr>
<td>No (159)</td>
<td>639.44(522.6)</td>
<td></td>
<td>1.04(0.7)</td>
<td></td>
</tr>
<tr>
<td><strong>BMI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (71)</td>
<td>788.71(525.7)</td>
<td>0.001</td>
<td>1.06(0.8)</td>
<td>0.874</td>
</tr>
<tr>
<td>Overweight (129)</td>
<td>552.24(477.1)</td>
<td></td>
<td>1.04(0.7)</td>
<td></td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female (92)</td>
<td>762.18(478.3)</td>
<td>0.001</td>
<td>1.09(0.8)</td>
<td>0.966</td>
</tr>
<tr>
<td>Male (108)</td>
<td>528.87(507.1)</td>
<td></td>
<td>1.01(0.7)</td>
<td></td>
</tr>
</tbody>
</table>
4.4 Time in Therapeutic Range

4.4.1 TTR Status in Sarawak population

The mean (SD) TTR in this cohort using Rosendaal method was 61.35(22.0)%. The TTR distribution was shown in Figure 9. Patients that were on alternate dosing regimen at least once in the 10 INR readings taken were distributed according to TTR category of <60%, 60-75% and >75% as shown in Figure 10. Patients grouped into TTR<60%, 60-75% and >75% were 86, 51 and 63, respectively. A fraction of 76.7% of patients in the TTR<60% group were on alternate dosing regimen for their warfarin treatment at least once, compared to only a fraction of 49.2% of patients in the higher TTR>75% group. The intermediate TTR group of 60-75% reported a fraction of 60.8% of patients on alternate dosing. From the out-of-range readings, 29.1% were subtherapeutic and 15.2% were supratherapeutic (Table 2). Grouping into three TTR categories, there were more subtherapeutic out-of-range readings in all three groups compared to supratherapeutic readings.

Figure 9: TTR Distribution
The lower TTR<60% group showed the largest number of subtherapeutic readings (366) compared to the other higher TTR groups. Figure 11 showed the distribution of INR readings according to TTR categories. One-Way ANOVA showed a significant difference in mean subtherapeutic readings between TTR<60%, 60-75%, and >75% (4.26 vs 2.51 vs 1.40; p<0.001), respectively. Post-hoc analysis showed a significant difference between all TTR groups (all p<0.001). There was also a strong negative correlation between the number of subtherapeutic range and TTR values (r=-0.761, p<0.001) as shown in Figure 12. One-Way ANOVA showed a significant difference in mean supratherapeutic readings between TTR<60%, 60-75%, and >75% (1.78 vs 1.45 vs 1.22; p<0.023), respectively. Post-hoc analysis showed a significant difference between TTR<60% and TTR>75% (p=0.020). However, only a weak negative correlation was found between supratherapeutic INR readings and TTR values (r=-0.198, p=0.005) as shown in Figure 13.
Figure 11: INR Distribution according to TTR categories

Figure 12: Correlation of Subtherapeutic INR and TTR (%)
4.4.2 Covariates associated with TTR

Table 7 shows the covariates that associates with TTR. TTR values were significantly higher in patients were never on alternate dosing regimen (69.11 vs 56.98%; \( p<0.001 \)) and also in those that doesn’t have chronic heart failure comorbidities (63.55 vs 55.70%; \( p=0.023 \)). There were no significant differences in TTR values between races and between genders (\( p=0.121 \) and \( p=0.116 \)), respectively. Patients with comorbidities such as hypertension, diabetes mellitus, and coronary artery disease did not show a significant difference in TTR value compared to those without (all \( p>0.05 \)). TTR values did not correlate with both BMI (\( r=-0.012, p=0.861 \)) and BMI groups (normal and overweight); \( p=0.935 \). A multivariate analysis was performed to identify covariates associated with determining TTR score. Summarised in Table 5, only plasma thrombin levels and alternate dosing status regimen are independently associated with TTR values.
Table 5: Multiple linear regression analysis for factors independently associated with TTR

<table>
<thead>
<tr>
<th>Variables</th>
<th>SLR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MLR&lt;sup&gt;b&lt;/sup&gt;</th>
<th>t-stat</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>b&lt;sup&gt;c&lt;/sup&gt; (95% CI)</td>
<td>Adj.b&lt;sup&gt;d&lt;/sup&gt; (95% CI)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alternate dose</td>
<td>-12.13 (-18.32,-5.94)</td>
<td>-11.64(-17.79,-5.49)</td>
<td>-3.73</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Thrombin CHF</td>
<td>-0.007 (-0.013,-0.001)</td>
<td>-0.006 (-0.012,0.001)</td>
<td>-2.19</td>
<td>0.030</td>
</tr>
</tbody>
</table>

<sup>a</sup> Simple linear regression
<sup>b</sup> Multiple linear regression
<sup>c</sup> Crude regression coefficient
<sup>d</sup> Adjusted regression coefficient

R² = 0.092 meaning that with the 2 significant variables, the model explains 9.2% of variation of TTR in the study sample.
4.5 Warfarin Dose

4.5.1 Covariates associated with warfarin dose

Table 7 shows the covariates that associates with average daily warfarin dose (mg). There was a significant difference in warfarin dose between Malay, Chinese and non-Malay Bumiputera race (2.45mg, 3.00mg and 2.92mg; p=0.008), respectively. Male gender were on significantly higher average daily warfarin dose compared to female gender (3.04mg vs 2.55mg; p=0.002), respectively. Patients that are overweight also showed significantly higher dose of average daily warfarin dose compared to those with normal BMI (2.97mg vs 2.54mg; p=0.013), respectively. There were significantly higher average daily warfarin dose in patients with diabetes mellitus comorbidity (p<0.001) but no differences in dosing were found in those with hypertension, chronic heart failure and coronary artery disease comorbitides (all p>0.05). Patients that were on alternate dosing regimen for at least once were found to be on significantly lower average daily warfarin compared to those that weren’t (2.7mg vs 3.02mg; p=0.046).

A multivariate analysis was performed to identify covariates associated with determining stable warfarin dose. After adjustment for covariates that were significant in bivariate analysis (Table 6), the VKORC1 (-1639G>A) polymorphism, DM, Age and BMI were found have significant correlations with ADWD. With the 4 significant variables, the model explains 41.6% of variation in the ADWD in this study sample (R²=0.416).
Table 6: Multiple linear regression analysis for factors independently associated with average daily warfarin dose

<table>
<thead>
<tr>
<th>Variables</th>
<th>SLR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MLR&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( b^* (95% \text{ CI}) )</td>
<td>( P \text{ value} )</td>
</tr>
<tr>
<td>VKORC1</td>
<td>( 1.12 (0.85,1.38) )</td>
<td>(&lt;0.001 )</td>
</tr>
<tr>
<td>DM</td>
<td>(-0.69 (-1.04,-0.35) )</td>
<td>(&lt;0.001 )</td>
</tr>
<tr>
<td>Age</td>
<td>(-0.026 (-0.043,-0.008) )</td>
<td>(0.005 )</td>
</tr>
<tr>
<td>BMI</td>
<td>( 0.053 (0.024,0.082) )</td>
<td>(&lt;0.001 )</td>
</tr>
<tr>
<td>Gender</td>
<td>(-0.49 (-0.78,-0.19) )</td>
<td>(0.002 )</td>
</tr>
<tr>
<td>Race</td>
<td>( 0.24(0.04,0.44) )</td>
<td>(0.018 )</td>
</tr>
<tr>
<td>Alt Dose</td>
<td>(-0.32(-0.63,-0.01) )</td>
<td>(0.046 )</td>
</tr>
</tbody>
</table>

<sup>a</sup> Simple linear regression  
<sup>b</sup> Multiple linear regression  
<sup>c</sup> Crude regression coefficient  
<sup>d</sup> Adjusted regression coefficient  
\( R^2 = 0.416 \) meaning that with the 4 significant variables, the model explains 41.6% of variation of average daily warfarin dose in the study sample.
<table>
<thead>
<tr>
<th>Factor (n=200)</th>
<th>TTR (%) (SD)</th>
<th>P Value</th>
<th>Average dose (mg) (SD)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hypertension</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (127)</td>
<td>61.36(21.8)</td>
<td>0.992</td>
<td>2.88(1.1)</td>
<td>0.285</td>
</tr>
<tr>
<td>No (73)</td>
<td>61.33(22.6)</td>
<td></td>
<td>2.71(1.1)</td>
<td></td>
</tr>
<tr>
<td><strong>DM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (47)</td>
<td>60.94(23.3)</td>
<td>0.883</td>
<td>3.34(1.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No (153)</td>
<td>61.48(21.7)</td>
<td></td>
<td>2.65(1.0)</td>
<td></td>
</tr>
<tr>
<td><strong>CHF</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (56)</td>
<td>55.7(21.3)</td>
<td>0.023</td>
<td>2.77(1.0)</td>
<td>0.710</td>
</tr>
<tr>
<td>No (144)</td>
<td>63.55(22.0)</td>
<td></td>
<td>2.83(1.1)</td>
<td></td>
</tr>
<tr>
<td><strong>CAD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (76)</td>
<td>60.79(23.5)</td>
<td>0.779</td>
<td>2.85(1.1)</td>
<td>0.695</td>
</tr>
<tr>
<td>No (124)</td>
<td>61.69(21.2)</td>
<td></td>
<td>2.79(1.1)</td>
<td></td>
</tr>
<tr>
<td><strong>VKORC1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA(143)</td>
<td>61.21(21.6)</td>
<td></td>
<td>2.49(0.9)</td>
<td></td>
</tr>
<tr>
<td>GA(54)</td>
<td>61.44(23.3)</td>
<td>0.924</td>
<td>3.53(1.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GG(3)</td>
<td>66.33(28.7)</td>
<td></td>
<td>5.26(2.7)</td>
<td></td>
</tr>
<tr>
<td><strong>Race</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malay (60)</td>
<td>56.75(22.4)</td>
<td>0.121</td>
<td>2.45(0.9)</td>
<td>0.008</td>
</tr>
<tr>
<td>Chinese(87)</td>
<td>64.33(21.9)</td>
<td></td>
<td>3.00(1.0)</td>
<td></td>
</tr>
<tr>
<td>Non-Malay</td>
<td>61.66(21.3)</td>
<td></td>
<td>2.92(1.3)</td>
<td></td>
</tr>
<tr>
<td>Bumiputera(53)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Alternate Dose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (128)</td>
<td>56.98(22.0)</td>
<td>&lt;0.001</td>
<td>2.70(1.1)</td>
<td>0.046</td>
</tr>
<tr>
<td>No (72)</td>
<td>69.11(19.97)</td>
<td></td>
<td>3.02(1.1)</td>
<td></td>
</tr>
<tr>
<td><strong>BMI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (71)</td>
<td>61.52(20.6)</td>
<td>0.935</td>
<td>2.54(0.8)</td>
<td>0.013</td>
</tr>
<tr>
<td>Overweight (129)</td>
<td>61.26(22.9)</td>
<td></td>
<td>2.97(1.2)</td>
<td></td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female (92)</td>
<td>58.70(20.9)</td>
<td>0.116</td>
<td>2.55(1.0)</td>
<td>0.002</td>
</tr>
<tr>
<td>Male (108)</td>
<td>63.61(22.8)</td>
<td></td>
<td>3.04(1.1)</td>
<td></td>
</tr>
</tbody>
</table>

Post Hoc analysis for dose according to VKORC1 variable; AA vs GA (p<0.001); AAA vs GG (p<0.001) and GA vs GG (p=0.007)
Atrial fibrillation (AF) is a common heart condition which bestow a significant risk of stroke and systemic thromboembolism thus causing oral anticoagulations such as warfarin to be a vital role in stroke prevention (Potpara & Lip 2013). Thrombosis is the main player in the development of atherosclerosis and its acute vascular complications (Cushman et al. 1996). Referring to the Virchow’s triad, there are three determinants for thrombus formation: (1) circulatory stasis; (2) endothelial injury; and (3) hypercoagulable state (Watson, Shantsila & Lip 2009). Of these, the clotting cascade is particularly important and is a common target by new drug therapies to interfere with arterial thrombogenesis (Klement & Rak 2006). Thrombin is located downstream of both the intrinsic and extrinsic amplification pathway of the anticoagulation cascade (HP, JM & PK 2003).

The NOACs targeting the inhibition of activated FIIa and FXa display a predictable pharmacodynamics and pharmacokinetic profile with short half-lives, low incidence for food and drug interactions, requiring no regular dose adjustments and monitoring(Ageno et al. 2012; Shameem & Ansell 2013). However, their usage is restricted by their relatively higher costs and limited indications, scenarios typically encountered in developing countries(Cannon & Stecker 2010; Edwards et al. 2013). A growing suggestion indicates that warfarin may remain as the anticoagulant of choice for the next few decades. Although warfarin has been available in the market for the past 50 years, large inter-individual dose variability and the wide variation of response among patients remains a major predicament. Numerous studies incorporating genetic and clinical factors have been conducted in heightened hope to bridge the gap in this drug-response variation in different populations.

In this 200 cohort of patients, there were 60(30.0%) Malay, 87(43.5%) Chinese and 53(26.5%) non-Malay Bumiputera. As there are around 40 sub-ethnic groups (Iban, Bidayuh, Melanau, Indians, Orang Ulu and others) in our population, we categorised these ethnicities into one group (non-Malay Bumiputeras). The mean (SD) daily warfarin dose was 2.81(1.1) mg which was consistent with the mean dose from other Asian populations such as Japan(Okumura et al. 2011), but not China (3.4mg), Oman
Our mean daily warfarin dose also differs greatly from our Korean counterpart which shows a mean (SD) stable dose requirement of 5.5(1.9)mg (Jeong et al. 2015; Lee et al. 2015). The mean dose in our population is relatively lower compared to our Caucasian counterpart, which is again a consistency in our findings with the other Asian populations. The target INR range for AF patients in this cohort was 2-3. The total INR readings recorded was 2000 (10 for each patient). Of the out-of-range readings, the total INR readings that were below 2 or subtherapeutic were almost double compared to INR readings above 3 or supratherapeutic (29.1% vs 15.2%). This leads to the impression that the majority of our patients, when poorly controlled, are underwarfarinised. This reason could be due to the clinicians in our settings who are less aggressive when it comes to warfarin dose increment especially in warfarin sensitive patients with a relatively lower warfarin dose. More than half of the patients in this cohort (64%) have received an alternate dosing regimen at least once during the study period. Frequent alternate dosing implementation reflects the warfarin sensitivity nature of our multi-ethnic population as dosing adjustments need to be more stringent and careful to avoid haemorrhagic side effects.

In our cohort of 200 multi-ethnic patients, we found a majority of 143(71.5%) with the VKORC1 AA genotype, 54(27.0%) was found with GA genotype and only 3(1.5%) was found with GG genotype. The A:G allelic frequency was 0.85:0.15 which means that a majority of our patients (85%) had at least one A allele in their genotype. The MAF (Minor Allele Frequency) found in this cohort was also similar to other population studies such as the Chinese, Korean and Japanese population (Kimura et al. 2007; Lee et al. 2015; Yuan et al. 2005) There was a significant difference in the mean (SD) daily warfarin dose for patients with the AA, GA and GG genotype at 2.49(0.9) mg, 3.53(1.0) mg and 5.26(2.7) mg, respectively (p<0.001) which is consistent with previous studies. Although there were no significant differences in genotype distribution between the three ethnic groups (p=0.112), there were however a significant difference in the average daily warfarin dose between ethnic groups (p=0.008) as shown in Table 7. These findings further suggest that race may be a potential predicting factor in average daily warfarin dose in our population.
There were only three patients identified with GG genotype (two male and one female). The ethnicity for both males is Bidayuh while the female is of Iban origin. The male patients with GG genotypes have significantly higher ADWD of 5.0mg and 8.06mg, whereas the ADWD of the GG genotype female patient was only 2.73mg. Although the ADWD for male patients with the GG genotype were consistent with previous findings (Zhu et al. 2007), the reason for the lower dose in the lone GG genotype female patient remains unknown. However, patients with the GG genotype were found to be significantly younger compared to those with the AA and GA genotype (p=0.005), suggesting also that age might be a contributory factor in warfarin dose variation besides genetic factors. No relationships were found between the genotype groups and its related plasma thrombin and FXa concentration (p=0.210 and p=0.456), respectively.

There was a negative and significant correlation between plasma thrombin levels and ADWD suggesting that the ADWD reduces the thrombin levels in a non-linear behaviour. A negative and significant correlation was also found between plasma thrombin levels with TTR values suggesting that thrombin may be a potential biomarker in reflecting the quality of anticoagulation of warfarin therapy. There was however no association found between both thrombin and FXa with baseline INR as opposed to previous similar studies (Costa et al. 2010). FXa was not associated with either TTR or ADWD which further suggests that plasma levels of FXa does not fall proportionally with plasma thrombin levels, although both biomarkers are interconnected in the blood coagulation cascade. These findings were also consistent with previous similar studies reporting that a relatively lower activity level in Factor X was found in comparison with the Factor II, VII and IX levels at the same INR value (Gulati et al. 2011; Lind et al. 1997).

We also found that plasma thrombin levels showed a moderate, negative and significant correlation with BMI (Figure 8), where patients with normal BMI (<25) have significantly higher plasma thrombin levels compared to those with overweight BMI (>25) as show in Table 4. The reason for this remains unclear. Patients associated with hypertension, diabetes mellitus and coronary artery disease risk factors have significantly lower plasma thrombin levels compared to those without these risk factors. One possibility for this finding is that those patients that were identified with these risk
factors were being treated aggressively for these, and hence could have led to a reduction in plasma thrombin levels. One study has demonstrated a correlation of thrombin levels with low levels of glucose where patients with lower glucose levels have significantly lower plasma thrombin levels (Cushman et al. 1996) and hence, can be used to relate to the findings in this study. Again, one of the possible explanations for the significantly lower plasma thrombin levels in patients with Type 2 diabetes mellitus compared to those without this comorbidity was that these patients were successfully treated for these and thus have lower blood glucose level.

Female patients were associated with significantly higher thrombin levels (Table 4), regardless of their TTR status and cardiovascular risk factors (Table 7). A theoretical reason for this may be that the female gender itself can be an independent predicting factor for elevated thrombin levels and the consequences of these remains unclear. No correlations were found between plasma thrombin levels with history of stroke and bleeding. The reasons for this may be due to the small number of such events in this cohort (Table 7).

On the other hand, FXa plasma levels in this study did not show any correlation with BMI, hypertension, diabetes mellitus, coronary heart disease, chronic heart failure, history of stroke, and history of bleeding which suggests that it may be a less valuable predictive marker compared to thrombin. One of the possible explanations for this observation is that Factor II and thrombin have the longest half-life compared to the other clotting factors (60-72 hours) (D'Angelo et al. 2002; Franchini & Lippi 2010). Even Factor X has a half-life of approximately 20-42 hours (Franchini & Lippi 2010). As previous studies conducted only evaluated the plasma levels of coagulation factors in patients receiving long term warfarin therapy without taking into account the patients’ cardiovascular risk factors (Costa et al. 2010; Gulati et al. 2011; Paul et al. 1987; Watala, Golanski & Kardas 2003), this is the first study that measures the activated form of Factor II and X while taking into account its clinical role in AF patients on long term warfarin therapy.

The mean(SD) TTR of 61.35(22.0)% found in this study is comparable with previously reported studies (Edwards et al. 2013) and other international publications (Connolly et al. 2008; Mitamura 2011; Okumura et al. 2011). We grouped the patients into three TTR groups and found that a majority of them falls under the poorer
TTR<60% group, with a large fraction of these (76.7%) being on an alternate dosing regimen at least once in this study (Figure 10). Whereas, less than half (49.2%) of the total patients in the excellent TTR>75% group were on alternate dosing regimen. This may indicate that patients that were on alternate dosing strategies had poorer INR control over time compared to those that weren’t. And because more than half of the cohort had experience an alternate dosing regimen at least once, it makes sense that most of them would fall under the poorer TTR<60% group. The reason for this may be due to the confusion in those patients when taking alternate dosing regimens rendering them to either miss or doubling the warfarin dose, causing either a subtherapeutic INR or supratherapeutic INR, respectively. Examples of alternate dosing regimen are as below:

<table>
<thead>
<tr>
<th>Monday</th>
<th>Tuesday</th>
<th>Wednesday</th>
<th>Thursday</th>
<th>Friday</th>
<th>Saturday</th>
<th>Sunday</th>
</tr>
</thead>
<tbody>
<tr>
<td>2mg</td>
<td>2.5mg</td>
<td>2mg</td>
<td>2.5mg</td>
<td>2mg</td>
<td>2.5mg</td>
<td>2mg</td>
</tr>
</tbody>
</table>

Or

<table>
<thead>
<tr>
<th>Monday</th>
<th>Tuesday</th>
<th>Wednesday</th>
<th>Thursday</th>
<th>Friday</th>
<th>Saturday</th>
<th>Sunday</th>
</tr>
</thead>
<tbody>
<tr>
<td>2mg</td>
<td>2mg</td>
<td>2mg</td>
<td>2mg</td>
<td>2mg</td>
<td>2.5mg</td>
<td>2.5mg</td>
</tr>
</tbody>
</table>

Further distributing the INR readings according to three TTR categories, we found that regardless of the TTR status, all three groups showed more subtherapeutic INR readings compared to supratherapeutic INR reading, with the highest number found in the poorer TTR<60% group (Figure 11). There was also a strong correlation between subtherapeutic INR readings and TTR compared to only a week correlation between supratherapeutic INR readings and TTR (Figure 12 and 13, respectively). This suggests that subtherapeutic INR readings contributed more to the poor TTR status in our cohort, hence leading to the assumption that most poorly controlled patients are typically underwarfarinised in our cohort. Whether this condition is correlated to stroke or bleeding outcomes is unknown as the number of prior stroke and bleeding events in this cohort is very small (Table 4).
In terms of comorbidities, only patients without CHF were found to have a significantly higher TTR value compared to those with CHF (Table 7). The reason for this may be due to the extreme fluctuations in body weight due to fluid retention in patients with CHF which may lead to unpredictable and erratic INR results. Other comorbidities such as hypertension, diabetes mellitus, CAD and BMI did not associate with TTR control. There were no significant differences in TTR between genders and between race groups, suggesting that genetic factors may play very little role in predicting the quality of anticoagulation. Multiple linear regression analysis shows that alternate dosing regimen and plasma thrombin levels but not CHF were independent predictors of TTR values (Table 5). This leads us to the assumption that patients that are on alternate dosing regimen has a higher chance of having poorer INR control and hence lower TTR value while plasma thrombin levels on the other hand may be a candidate marker to reflect the quality of anticoagulation based on TTR values.

In terms of warfarin doses, patients that were on alternate dosing regimen for at least once a week were found to be in a significantly lower ADWD as shown in Table 7. The reason for this is that patients with lower warfarin maintenance dose need to have more careful and smaller dosing adjustments to reach their target INR, rendering alternate dosing regimen to be inevitable in these situations. There were significant differences in ADWD among the racial groups and between gender groups. Male patients have higher ADWD compared to female patients while patients that have overweight BMI also have higher ADWD compared to those with normal BMI. Significantly higher warfarin doses were also found in patients that have diabetes mellitus comorbidity but not in patients with hypertension, CHF and CAD comorbidities. Reasons for these remains unclear but we can assume that genetic factors, gender and clinical factors such as BMI and diabetes mellitus comorbidities may play a role in warfarin dose prediction. Multiple linear regression analysis shows that the VKORC1 (-1639G>A) polymorphism, Age, BMI and DM are independent predictors of ADWD in this cohort. Table 6 shows that all four variables explaining around 41.6% of the variation in ADWD in this study sample with the most contribution from the genetic factor VKORC1 (-1639G>A) polymorphism which alone can explain 25.4% of ADWD variation in this study.
In conclusion, this study suggests that the VKORC1 (-1639G>A) gene polymorphism, Age, BMI and DM are associated with warfarin dosing (ADWD). Whereas the plasma thrombin levels and alternate dosing status are associated with TTR. This study confirms that the VKORC1 (-1639G>A) gene polymorphism may be a strong predictive variable for ADWD along with other clinical factors, while the plasma thrombin levels may be a potential candidate marker in reflecting the quality of anticoagulation control based on TTR in our multi-ethnic population study on patients with atrial fibrillation. Together, both can be used to delineate a new strategy to determine who may benefit most from switching to the new NOACs. Preliminary data from this study will help to delineate a predictive model to determine the appropriate use of anticoagulant therapy in our country.
5.1 Summary of findings

1. According to our results, there was a significant increase in VKORC1 AA prevalence in our multi-ethnic Sarawak population. We found that the wild type VKORC1 GG genotype patients all fall under the non-Malay Bumiputera category and are younger compared to the AA and GA genotype patients. There was a significant difference in ADWD between the genotypes, a finding which is consistent with previous publications.

2. Our results showed a significant correlation between the plasma thrombin levels with ADWD, TTR and BMI. Plasma thrombin levels were also found to be significantly higher in female patients. Plasma FXa levels however, did not correlate with ADWD, TTR, BMI, gender and other atrial fibrillation risk factors.

3. We presented that more than half of the patients in the TTR<60% group were on alternate dosing regimen. Of the out-of-range readings, the subtherapeutic INR readings taken were almost double the number of supratherapeutic INR readings. Regardless of the TTR status (<60%, 60-75%, >75%), all showed more subtherapeutic compared to supratherapeutic INR readings. Multivariate analysis showed that only alternate dosing regimen status and plasma thrombin levels were independently associated with TTR in this study.

4. We demonstrated there was a significant difference in ADWD between ethnic groups. Male patients were also shown to have significantly higher ADWD compared to their female counterpart. Patients that have BMI and DM comorbidity also showed significantly higher ADWD doses compared to those that doesn’t have. Patients with alternate dosing also have significantly lower ADWD compared to those without alternate dosing regimens. Multivariate analysis showed 4 variables that are independent predictors for ADWD in this study [VKORC1 (1639G>A) gene polymorphism, DM, Age, and BMI].
5.2 Limitations

This is a single centre study involving a relatively small number of patients. The total INR readings taken for each patient was only 10 and hence may not reflect the true TTR over time in this cohort. Only one blood sample was taken for plasma thrombin, FXa and INR level analysis and hence a trend in the reduction of these biomarkers according to INR or TTR could not be obtained. However, this was the methodology we felt best applied for this study. The ELISA method was performed to measure the thrombin and FXa concentration in this study and hence, in future, a more sensitive assay may be considered to measure these biomarkers to compare and further validate these results. The Vitamin K diet and co-medications factor were also not taken into account in this study. Other genes which contribute to the pharmacokinetics of warfarin therapy such as the CYP2C9 polymorphism was also not investigated in this study. Although the effect of the CYP2C9 gene polymorphism on warfarin therapy is low in Asian population due to the low MAF (minor allele frequency), the contribution to warfarin dose variability is around 7% in the Asian population (Takahashi et al. 2006). The number of stroke and bleeding history in this cohort was small. This may be a finding associated with our study which may require future extensive study to investigate into. This current study also did not encompass any clinical outcomes such as bleeding, stroke, myocardial infarction and ACS on follow-up patients, and therefore further studies with larger sample size in this field are warranted.

5.3 Future directions

Future work should aim to explore the impact of various Vitamin K cycle influencing genes, clotting factors and concomitant medications on warfarin dose prediction. Further prospective studies are desired to assess other important factors that improve dosing algorithm for personalising warfarin dose during both the initiation and maintenance therapy to determine the cost-effectiveness of implementing pharmacogenetic in our routine clinical practice.
REFERENCES


Hirsh, J 1987, 'Is the dose of warfarin prescribed by American physicians unnecessarily high?', *Arch Intern Med*, vol. 147, no. 4, Apr, pp. 769-71.


Mann, KG 2003, 'Thrombin formation', *Chest*, vol. 124, no. 3 Suppl, Sep, pp. 4S-10S.


PUBLICATIONS ARISING FROM THIS WORK

Papers

Lim MSH, Anchah L, Tiong WN, Hwang SS, Ong TK, Sim KH, Fong AYY:
*Thrombin and FXa plasma concentration levels in patients with atrial fibrillation on long term warfarin therapy.*

Citable Abstracts

Wen Ni Tiong, Melissa Lim, Ching Ching Wee, Lawrence Anchah, Alan Fong, Ong Tiong Kiam:
*Evaluation of the Time in Therapeutic Range in a Warfarin Anticoagulation Clinic: A Sarawak General Hospital Heart Centre Experience.*
ASEAN Heart Journal. Vol.21, no.1, pg134, 2013

Lim MSH, Anchah L, Tiong WN, Chin FYY, Mejin M, Tiong LL, Kong KL, Hwang SS, Ong TK, Fong AYY
*The Relationship of Thrombin and Factor Xa with The Time in Therapeutic Range in Patients on Long Term Warfarin Therapy*
Global Heart, Volume 9, Issue 1, Supplement pp. e238-239 (March 2014) World Congress of Cardiology Scientific Sessions 2014
JIF: 7.467

Oral Presentations

Melissa Lim, Wen Ni Tiong, Alan Fong, Hwang Siaw San, Kong Khai Liy, Melissa Mejin, Felicia Chin, Henry Gudum, Tay Siow Ping, Ong Tiong Kiam, Lawrence Anchah.
*The Prevalence of VKORC1 (-1639 G>A) Polymorphism and Its Association with Weekly Warfarin Dose Requirement in Multietnic Sarawak Population with Atrial Fibrillation.*
Lawrence Anchah, Wen Ni Tiong, Melissa Lim, Hwang Siaw San, Melissa Mejin, Alan Fong Yean Yip, Tan Siang Kong, Ong Tiong Kiam

*Relationship of Plasma Thrombin Concentration in Patients with Atrial fibrillation to differences in warfarin anticoagulation dosing*

National Heart Association of Malaysia (NHAM) Annual Scientific Meeting (ASM) 2013 12-14th April 2013

Lau Kent Ter, Alan Fong Yean Yip, Melissa Mejin, Kong Khai Liy, Tiong Lee Len, Lana Lai Yin Hui, Tiong Wen Ni, Melissa Lim Siaw Han, Ong Tiong Kiam

*Aspirin Non-Responders in Malaysian Patients undergoing Percutaneous Coronary Intervention*

National Heart Association of Malaysia (NHAM) Annual Scientific Meeting (ASM) 2013 12-14th April 2013

Asri Bin Said, Alan Fong Yean Yip, Melisa Mejin, Kong Khai Liy, Melissa Lim Siaw Han, Ong Tiong Kiam

*Point-of-care Genotyping and Clopidogrel response in patients implanted with drug eluting stents*

National Heart Association of Malaysia (NHAM) Annual Scientific Meeting (ASM) 2013 12-14th April 2013

Gudum H, Tiong WN, Lim MSH, Tay SP, Hwang SS, Fong AYY, Ong TK, Anchah L

*PIVKA-II can be an ideal marker for monitoring the Therapeutic Efficacy of warfarin on Atrial Fibrillation patients*

National Heart Association of Malaysia (NHAM) Annual Scientific Meeting (ASM) 2013 12-14th April 2013

Tan SK, Lim MSH, Tiong WN, Kong KL, Mejin M, Chin FYY, Fong AYY, Hwang SS, Gudum H, Tay SP, Anchah L, Ong TK
**Clinical Outcomes for Activated Factor IIa and Xa Inhibition in Long Term Warfarin Treated Population**

National Heart Association of Malaysia (NHAM) Annual Scientific Meeting (ASM) 2013 12-14th April 2013

Mejin M, Fong AYY, Kong KL, Chin YY, Tiong LL, Gerunsin J, **Lim MSH**, Yew KL, Nor Hanim MA, Chua SK, Tan SK, Cham YL, Khiew NZ, Asri S, Voon CY, Ong TK

*The impact of generic clopidogrel preparations on platelet aggregation and 1-month clinical outcomes in patients with drug-eluting stents*

7th National Conference on Clinical Research 3rd-5th September 2013

**Lim MSH**, Tiong WN, Anchah L, Fong AYY, Hwang SS, Ong TK.

*The association of VKORC1 (-1639 G>A) polymorphism with weekly warfarin dose requirement and its impact on long term warfarin therapy in multiethnic Borneo population*

The 13th Asian Conference on Clinical Pharmacy (ACCP) at Haiphong Vietnam on 13th-15th September 2013

**Lim MSH**, Anchah L, Fong AYY, Chin FYY, Ong TK

*Factors influencing time in therapeutic range in a multiethnic group of patients on long term warfarin anticoagulation*

5th Sarawak State Health Research Day 2013 at Sibu, Sarawak on 11-12th October 2013

**Lim MSH**, Anchah L, Tiong LL, Mejin M, Yanti S, Ong TK, Fong AYY

*The Association of Thrombin and Factor Xa with the Time in Therapeutic Range in Patients on Long Term Warfarin Therapy*

8th Pharmacy Research and Development Conference 2014 at Ipoh, Perak on 10-12th June 2014

Anchah L, **Lim MSH**, Tiong LL, Mejin M, Yanti S, Ong TK

*Factors Influencing Time in Therapeutic Range in Patients on Long Term Warfarin Therapy*
8th Pharmacy Research and Development Conference 2014 at Ipoh, Perak on 10-12th June 2014

Foo DHP, Lim MSH, Anchah L, Tiong WN, Hwang SS, Fong AYY, Mejin M, Chin FYY, Ong TK

*Impact of gender on plasma thrombin levels in patients with atrial fibrillation on long term warfarin therapy*

20th ASEAN Federation of Cardiology Congress 2014 at Kuala Lumpur Convention Centre

on 12-15th June 2014

**Poster Presentations**

Wen Ni Tiong, Melissa Lim, Ching Ching Wee, Lawrence Anchah, Alan Fong, Ong Tiong Kiam:

*Evaluation of the Time in Therapeutic Range in a Warfarin Anticoagulation Clinic: A Sarawak General Hospital Heart Centre Experience.*

Asian Pacific Society of Cardiology Congress 2013 (APSC 2013) PEACH Pattaya Thailand 21-24th February 2013

Lim MSH, Anchah L, Tiong WN, Chin FYY, Mejin M, Tiong LL, Kong KL, Hwang SS, Ong TK, Fong AYY

*The Relationship of Thrombin and Factor Xa with The Time in Therapeutic Range in Patients on Long Term Warfarin Therapy*

The World Congress of Cardiology (WCC 2014) at the Melbourne Convention and Exhibition Centre 4-8th May 2014
APPENDIX 1
Consent Form

PARTICIPANT INFORMATION SHEET AND CONSENT

Title: Thrombin and Activated Coagulation Factor X as Biomarkers to Identify Poor Respondents towards Warfarin among Multiethnic Malaysian patients - A Pilot Study (The Warfarin-Thrombin Study)

Investigator: Dr Lawrence Anchah
Address: Sarawak General Hospital Heart Centre
94300 Kota Samarahan, Sarawak
Malaysia
Telephone: 082-281111
Fax: 082-412240, 281092

Sponsor: KEMENTERIAN KESIHATAN MALAYSIA

Participant’s Name: ________________________________________________________

Participant’s IC No: ________________________________

Date of Birth: _____/_____/______ (hari / bulan / tahun)

Address: _________________________________________________________________

Telephone No : ______________________________

Please read this Information Sheet carefully. You will be given the chance to ask questions. Any question about this study will be explained to you by the study investigators or researchers, until you are properly satisfied that you understand this study.
Background of the study
You are invited to participate in a research study because you underwent warfarin (e.g. Coumarin®) oral anticoagulant therapy for at least 2 years and expected to continue this therapy for at least the next 6 months. Investigators at the Sarawak General Hospital are interested in studying the markers which can help to identify or predict who are poorly controlled warfarin users.

During the period of your warfarin therapy, you had undergone at least TEN times of monthly or bimonthly international normalized ratio (INR) monitoring to maintain the INR between 2.0 and 3.0. Recently, a new tool known as "Time in therapeutic range" (TTR) has been used to assess how often had Warfarin been at the correct dose. Several reports indicate an association between frequently high and/or low TTR and increased rates of both vascular events (eg strokes) and major bleeding events in patients on oral anticoagulant therapy.

It has been suggested that warfarin-treated patients whom had TTR below the minimum threshold (i.e. less than 60%) should switch to new antithrombotic agents, such as dabigatran or rivaroxaban, which could eliminate doctor's reluctance to adjust the dose in a less effective direction. Though these drugs are found non-inferior to warfarin in preventing strokes, their costs are approximately five times more than warfarin. Moreover, the factors known to influence TTR of an individual also include the patients' noncompliance and/or drug resistance toward warfarin.

Study objective
One of the goals of the study is to explore the role of thrombin and activated coagulation factor X (FXa) as biomarker to identify patients who are poor respondents to warfarin. Both thrombin and factor Xa are directly involved in clot formation in the arteries. This sudden clot formation can cause severe blockages of arteries, causing heart attack or stroke.

To date, it is not known if patients having poor TTR are due to the excessive expression of thrombin or FXa. If a patient is found to have high level of thrombin or factor Xa, he or she may be advised to switch from warfarin to new antithrombotic drugs: dabigatran (direct thrombin inhibitor) or rivaroxaban (direct FXa inhibitor), respectively.

Study procedures
A total of 200 patients will be enrolled in this study. If you take part in this study, you will be asked to provide one blood specimen equivalent to 5 teaspoon of blood each (25 ml). Your blood specimen will be processed to obtain your genetic material, plasma and serum. These materials will be used to perform laboratory analysis, and in combination with your other medical information, will be available to researchers to study factors that contribute to poor TTR of warfarin. By agreeing to participate in this research, you authorize the MOH and members of its staff to use your blood specimens for these purposes. The principal investigator and his team will maintain these samples until the end of the study (for no longer than 3 years) from the date signed on your Consent Form. These samples may be reserved for additional analyses if there are new biomarkers may be discovered to help us to improve the current anticoagulation management.

This study will be conducted for 3 years and if it is to be continued after 31 December 2015, formal permission will be requested by the study team to the approved Ethics Committee (MREC). You will be duly informed of this if this event occurs. However, you may withdraw consent for the future use of these samples at any time you see fit.
Participant’s responsibility

Your participation in the study will last for 12 months. You will not be required to return for any further visits, but you will be followed up via telephone contact at 1 month, 6 months and 12 months after your admission to this study. Each phone interview will last less than 15 minutes.

Voluntary participation

If you should disagree to participate in this study, you will still be treated in the standard manner. Participation in this study is absolutely voluntary. You may withdraw consent at any time without giving any reason, and blood samples taken from you will be disposed. There will not be any risk or impact to your current medical treatment.

Confidentiality of your Information

If you agree to participate in this Study, personal information will be collected from your medical records. This data will be used and processed manually or by computer. The data collected is for medical research purposes, to be used within the limits specified in this Study. Any database containing information about you shall refer to a participant identification number which is unique to each participant and will not reveal your identity. You unconditionally accept that this data will also be made available to, or accessed by, the relevant regulatory authorities, the relevant Ethics Committees and monitor(s) and auditor(s) appointed by our organization for verification of the procedures of this Study. Such access shall not violate the confidentiality of your identity and personal information and shall be subject to the extent permitted by the applicable laws and regulations. It is likely that the results of this Study will be published in one or more medical journals. Any such publication will not identify you by name.

Problems or Questions

If you have further questions about this Study, you can contact any doctor working in our Department. (082-276666; ext 2222 or 5925)

If you have further questions regarding your rights as a participant in clinical research, please contact the Medical Research and Ethics Committee, c/o National Institutes of Health Secretariat. (03-22874032)
INFORMED CONSENT FORM

I have read and understood the information of this study. Before signing, information about the contents of this document and the study was explained to me. My physician has answered all my questions regarding the procedure and the study. I have had sufficient time to consider my participation in this study and I am aware that participation in this study is completely voluntary. I realize that I may decide to stop participation at any time without affecting the quality of healthcare provided.

I authorize and instruct my physician(s) and institution to release necessary personal information about me pertaining only to this study. I understand that I am entitled to access the personal information collected about me and to have inaccuracies corrected.

I agree to participate voluntarily in this study. Any testing or research will require permission from an approved Ethics Committee.

**Patient (or if applicable, Legal Representative)**

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**Witness**

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**Investigator or delegated person by the investigator**

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## APPENDIX 2
Ethics Approval Letter from MREC

<table>
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<tr>
<th>MEDICAL RESEARCH &amp; ETHICS COMMITTEE</th>
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<tr>
<td>MINISTRY OF HEALTH MALAYSIA</td>
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<tr>
<td>c/o Institute for Health Management</td>
</tr>
<tr>
<td>Jalan Rumah Sakit, Bangsar</td>
</tr>
<tr>
<td>59000 Kuala Lumpur</td>
</tr>
</tbody>
</table>

Ruj. Kami: (6)dim.KKM/NHSEC/08/0804/P12-167  
Tarikh: 17 August 2012

**Protocol Title:**  
NMRR-12-82-10952  
Thrombin and Activated Coagulation Factor X as Biomarkers to Identify Poor Respondents towards Warfarin among Multietnic Malaysian patients - A Pilot Study

**Principal Investigator:**  
Dr Lawrence Anak Anghah  
Jabatan Farmasi  
Hospital Umum Sarawak

**Documents received and reviewed with reference to the above study:**
1. Study Proposal version 3 dated 05-06-2012
4. Curriculum vitae of investigators

The Medical Research & Ethics Committee, Ministry of Health Malaysia operates in accordance to the International Conference of Harmonization Good Clinical Practice Guidelines.

**Project Sites:** Hospital Umum Sarawak

**Comments (if any):** Please note that the approval is for one year & a completed ‘Continuing Review Form’ has to be submitted to MREC in November every year for renewal of approval.

**Decision by Medical Research & Ethics Committee:**
- ( ) Approved  
- ( ) Disapproved

**Date of Decision:** 17 August 2012

---

**DATO’ DR CHANG KIAN MENG**  
Chairman  
Medical Research & Ethics Committee  
Ministry of Health Malaysia

*Sila catatkan rujukan surat ini apabila menjawab*
APPENDIX 3
Ethics Approval E-mail from SUHREC

To: Dr Hwang Siaw San, Swinburne Sarawak

Dear Dr Hwang Siaw San

SUHREC Project 2012/306 Thrombin and Activated Coagulation Factor X as Biomarkers to Identify Poor Respondents towards Warfarin among Multiethnic Malaysian patients - A Pilot Study
Dr Hwan Siaw San, SUTS; Ms Melissa Lim, Dr Alan Fong et al (Sarawak General Hospital Cl Dr Lawrence Anak Anchah et al. Project ID: NMRR-12-82-10952)

I refer to the proposed Swinburne University of Technology Sarawak (SUTS) involvement in the above project led by Dr Anchah of the Sarawak General Hospital, including supervised student research.

The Swinburne ethical review was undertaken by delegates of Swinburne’s Human Research Ethics Committee (SUHREC) significantly on the basis of the prior ethical review and clearance by the Ministry of Health (MOH) Malaysia Medical Research & Ethics Committee (Protocol NMRR-12-82-10952). The submissions for review were as per your emails of 30 November 2012 and 17 January 2013 both with attachments, the latter email in response to feedback from the SUHREC delegates.

I am pleased to advise that Swinburne Sarawak research involvement in the above project has approval to commence in line with standard ongoing ethics clearance conditions listed below (as applicable). However, this clearance is given on the understanding that, where applicable or required, the Malaysia Ministry of Health Medical Research & Ethics Committee has been or will have been properly notified of the Swinburne ethics clearance and Swinburne Sarawak involvement in relation to the project originally approved by the MOH Medical Research & Ethics Committee. A copy of the notification sent to the MOH Committee should be sent to my office for the record as soon as practicable. In the event of a query or complaint relating to the original project, the notification may prove significant for and protective of Swinburne researchers. You will have received a separate communication today in relation to this particular matter.

- All human research activity undertaken under Swinburne auspices must conform to Swinburne standards, including external regulatory standards such as the current National Statement on Ethical Conduct of Human Research and with respect to secure data use, retention and disposal.

- The named Swinburne Chief Investigator/Supervisor remains responsible for any personnel appointed to or associated with the Swinburne student project being made aware of ethics clearance conditions, including research and consent procedures or instruments approved. Any change in chief investigator/Supervisor requires timely notification and appropriate endorsement.

- The above project has been approved as submitted for ethical review by or on behalf of SUHREC. Amendments to approved procedures or instruments ordinarily require prior ethical appraisal/clearance. SUHREC must be notified immediately or as soon as possible thereafter of (a) any serious or unexpected adverse effects and any redress measures; (b) proposed changes in protocols; and (c) unforeseen events which might affect continued ethical acceptability of the project.
- At a minimum, an annual report on the progress of the project is required as well as at the conclusion (or abandonment) of the project.

- A duly authorised external or internal audit of the project may be undertaken at any time.

Please contact the Research Ethics Office at Swinburne Research if you have any queries about the Swinburne ethical review and if you need a signed ethics clearance certificate, citing the SUHREC project number. Copies of communication emails should be retained as part of project record-keeping.

Best wishes for the project, including to Dr Anchah and Ms Melissa Lim.

Yours sincerely

Keith Wilkins
Secretary, SUHREC

-------------------------------------------------------------------------------------------------

Keith Wilkins
Research Ethics Officer
Swinburne Research (H68)
Swinburne University of Technology
P O Box 218
HAWTHORN VIC 3122
Tel +61 3 9214 5218
Fax +61 3 9214 5267
APPENDIX 4
Notification to MOH Medical Research and Ethics Committee of the SUTS project involvement

From: Keith Wilkins [kwilkins@swin.edu.au]
Sent: Monday, January 28, 2013 12:24 PM
To: SiawSan Hwang
Cc: [email redacted]; RES Ethics; Wallace ShungHu Wong
Subject: Swinburne Ethics Clearance Forthcoming RE: SUHREC Project 2012/306 Swinburne Ethical Review (MOH Project ID: NMRR-12-82-10952)

Dear Hwang SiawSan

SUHREC Project 2012/306 Thrombin and Activated Coagulation Factor X as Biomarkers to Identify Poor Respondents towards Warfarin among Multietnic Malaysian patients - A Pilot Study
Dr Hwan Siaw San, SUTS; Ms Melissa Lim, Dr Alan Fong et al
(Sarawak General Hospital CI: Dr Lawrence Anak Anchah et al. Project ID: NMRR-12-82-10952)

Your email of 17 January 2013 with attachments was put to a SUHREC delegate whose recommendation to approve has now been endorsed by the Acting Chair of SUHREC.

I will separately be issuing the Swinburne ethics approval for the SUTS involvement in the MOH-approved Sarawak General Hospital project (CI: Dr Lawrence Anak Anchah). But the clearance being issued is on the understanding that you strongly suggest to Dr Anchah that he notifies the MOH Medical Research & Ethics Committee of the SUTS involvement in the project, including the supervised student research. A copy of the communication to/from MOH should be forwarded to you by my office for the record here, as well as a copy retained for your records.
The original MOH ethics approval for Protocol NMRR-12-82-10952 does not on the face of it explicitly mention the extent of SUTS or, more particularly, the Swinburne student involvement in the project.

Yours sincerely

Keith

Keith Wilkins
Secretary, SUHREC & Research Ethics Officer
Swinburne Research (H68)
Swinburne University of Technology
P O Box 218
HAWTHORN VIC 3122
Secretariat National Institutes of Health (NIH)
KEMENTERIAN KESIHATAN MALAYSIA
d/a Health Management Institute
Jalan Rumah Sakit, Bangsar
59000 Kuala Lumpur

Dear Sir/Madam,

NMRR-12-92-10952
Thrombin and Activated Coagulation Factor X as Biomarkers to Identify Poor Respondents towards Warfarin among Multiethnic Malaysian Patients – A Pilot Study

With reference to the above subject,

2. I am pleased to inform you that this study is going smoothly with the collaboration work of Dr. Hwang Siaw San as Co-Investigator from the Faculty of Engineering, Computing and Science, Swinburne University of Technology, Sarawak (SUTS).

3. Our Pharmacist, from Clinical Research Centre (CRC), Ms Melissa Lim Siaw Han who is also one of the Co-Investigator of this study had applied for Master Program by research under SUTS, with Dr. Hwang Siaw San as the coordinating supervisor.

4. Please be acknowledged that I, as the Primary Investigator of this study will take full responsibility of the progress and accomplishment of this study.

Sekian Terima kasih.

* PENYAYANG, BERKEJAR BERPAKEKAN DAN PROFESIONALISMA ADALAH BUDAYA KERAJA KITA*

"BERKHIDMAT UNTUK NEGARA"

Saya yang menutup peribah.

(DR. LAWRENCE ANAK ANCHAH)
Pegawai Farmasi U54
Pusat Jantung
Hospital Umum Sarawak, Kota Samarahan,

s.k. Faculty of Engineering, Computing and Science,
Swinburne University of Technology, Sarawak (SUTS).
Dr Lawrence Anak Anchah  
Cardiology Centre  
Sarawak General Hospital

Dear Sir,

NMRR-12-92-10952  
THROMBIN AND ACTIVATED COAGULATION FACTOR X AS BIOMARKERS TO IDENTIFY POOR RESPONDENTS TOWARDS WARFARIN AMONG MULTIETHNIC MALAYSIAN PATIENTS- A PILOT STUDY

With reference to the above subject and your letter dated 7th March and Medical Research and Ethics Committee approval letter dated 17th August 2012.

2. The Secretariat National Institutes of Health acknowledges and re-affirms that Ms Melissa Lim Siaw Han, one of the Co-investigator of this study, has applied for Masters Programme by research with the Swinburne University of Technology, Sarawak (SUTS), under the supervision of Dr Hwang Siaw San.

Thank you.

Your Sincerely,

[Signature]  
[Head, Secretariat National Institutes of Health  
Ministry of Health, Malaysia  
asmaliza@nih.gov.my]
Dear Hwang Siaw San,

SUHREC Project 2012/306 Thrombin and Activated Coagulation Factor X as Biomarkers to Identify Poor Respondents towards Warfarin among Multiethnic Malaysian patients - A Pilot Study
Dr. Hwan Siaw San, SUTS; Ms Melissa Lim, Dr Alan Fong et al
(Sarawak General Hospital CI: Dr Lawrence Anak Anchah et al. Project ID: NMRR-12-82-10952)

Thank you for the information re notification to/acknowledgment by Secretariat NIH Malaysia for the Swinburne Sarawak involvement in Dr Anchah’s Project (NMRR-12-82-10952) which complements previous documentation. The Swinburne ethics clearance issued on Mon 28/01/2013 still holds.

As before, best wishes to the SUTS research team for this project.

Yours sincerely

Keith

Keith Wilkins
Secretary, SUHREC & Research Ethics Officer
Swinburne Research (HE8)
Swinburne University of Technology
P O Box 218
HAWTHORN VIC 3122
Tel +61 3 9224 5218
Fax +61 3 9224 5267
APPENDIX 5
Ethics Extension Approval E-mail from SUHREC

From: Astrid Nordmann on behalf of REO Ethics
To: Siew Geen Tan
Cc: REO Ethics
Subject: RE: Swinburne Ethics Clearance Confirmation RE: SUHREC Project 2012/306 Swinburne Ethical Review (MONU Project ID: NMRR-12-82-10952), Extension/Modification (1)
Date: Friday, 21 March, 2014 4:36:20 AM

Dear Hwang Siaw San,

SUHREC Project 2012/306 Thrombin and Activated Coagulation Factor X as Biomarkers to Identify Poor Respondents towards Warfarin among Multiethnic Malaysian patients - A Pilot Study
Dr Hwan Siaw San, SUTS; Ms Melissa Lim, Dr Alan Fong et al
(Sarawak General Hospital CI: Dr Lawrence Anak Anshah et al. Project ID: NMRR-12-82-10952)
Approval extension: 31/12/2014

I refer to your request for a simple extension of ethics clearance to complete the approved human research activity as per the report form received at Swinburne Research on 11/03/2014.

There being no change to the approved protocol as submitted to date, I am authorised to issue the clearance for the extension to 31/12/2014. The standard ethics clearance conditions previously communicated and reprinted below still apply.

Please contact the Research Ethics Office if you have any queries about on-going ethics clearance, citing the SUHREC project number. Copies of clearance emails should be retained as part of project record-keeping.

As before, best wishes for the project.

Yours sincerely,
Astrid Nordmann

-------------------------------------------------------------------
Dr Astrid Nordmann
Research Ethics Executive Officer
Swinburne Research (H88)
Swinburne University of Technology
PO Box 218, Hawthorn, VIC 3122
Tel: +613 9214 3845
Fax: +613 9214 5267
Email: anordmann@swin.edu.au
-------------------------------------------------------------------
APPENDIX 6
Approval E-mail for Acknowledgement of Final Report from SUHREC

From: resethics@swin.edu.au
To: SiawSan Hwang
Cc: resethics@swin.edu.au
Subject: Acknowledgement of Report for SUHREC Project - 2012/306
Date: Thursday, 23 July, 2015 12:06:56 PM

Dear Siaw San Hwang,

Re: Final Report for the project (Report Date: 23-07-2015)

2012/306 'Thrombin and Activated Coagulation Factor X as Biomarkers to Identify Poor Respondents towards Warfarin among Multiethnic Malaysian patients - A Pilot Study'

The Final report for the above project (Report Date: 23-07-2015) has been processed and satisfies the reporting requirements set under the terms of ethics clearance.

Research Ethics Team
Swinburne Research (H68)
Swinburne University of Technology
PO Box 218
HAWTHORN VIC 3122
Tel: 03 9214 5218
Fax: 03 9214 5267
Email: resethics@swin.edu.au