EFFECT OF *VKORC1* POLYMORPHISM ON WARFARIN DOSE REQUIREMENT AMONG PATIENTS IN TERTIARY CARE HOSPITAL

BY

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DOCTOR OF MEDICINE
IN
PHARMACOLOGY

Under the guidance of

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April 2017

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Date: Signature of the Candidate

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Dedicated with

LOVE

to

My Daughter

LIST OF ABBREVIATIONS

VKOR	Vitamin K Epoxide Reductase
INR	International Normalised Ratio
BMI	Body mass index
CYP2C9	Cytochrome P450, family 2, subfamily C, polypeptide 9
VKORC1	Vitamin K epoxide reductase complex subunit 1
VKA	Vitamin K antagonists
AF	Atrial fibrillation
GIT	Gastrointestinal tract
Vd	Volume of distribution
PT	Prothrombin time
WHO	World Health Organization
ISI	International sensitivity index
G	Guanine
A	Adenine
Val /V	Valine
Met/M	Methionine

UTR	Untranslated region
C	Cytosine
T	Thymine
SNP	Single nucleotide polymorphism
EDTA	Ethylene diamine tetra acetic acid
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
ssDNA	Single-stranded DNA
Taq	Thermus aquaticus
bp	Basepair
SD	Standard deviation
ANOVA	Analysis of variance
GGCX	Gamma-glutamyl carboxylase

ABSTRACT

INTRODUCTION:

Warfarin, an anticoagulant is used for thromboembolic disorders. Interindividual variation in clinical response to warfarin is due to various factors including polymorphism of vitamin K epoxide reductase complex 1(*VKORC1*) -1639 G > A gene.

OBJECTIVES:

1. To determine the frequency of *VKORC1* polymorphism using polymerase chain reaction (PCR) technique in patients receiving warfarin
2. To evaluate the effect of *VKORC1* polymorphism on maintenance dose of warfarin and INR status in these patients

MATERIALS AND METHODS:

This was a cross-sectional study conducted on 102 patients by Departments of Pharmacology and Cell Biology and Molecular Genetics on patients attending cardiology clinic (both inpatients and out patients), receiving maintenance dose of warfarin for atleast 2 months. Genomic deoxyribonucleic acid was extracted using standard salt extraction method and was stored at -40ºC. Genotyping of *VKORC1* -1639 G > A was performed using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism. Correlation between *VKORC1* gene polymorphism and warfarin maintenance dose were analysed.

RESULTS:

Total of 102 patients with mean age of 47.72±10.31 years of which 58 (56.86%) were male. Frequency of VKORC1 G > A for GG, GA and AA genotypes were 74.51, 19.61 and 5.88% respectively. Minor allele A was less frequent than the major allele G. Mean weekly warfarin dose was 23.12±8.08, 22.93±8.21 and 15.6±5.35 mg in patients with GG, GA and AA genotypes respectively. Patients with GG genotype required therapeutic dose compared to variant type (p= 0.001). Multiple stepwise regression model showed that 26.3% variability in warfarin dose was due to *VKORC1* genotype ($R = 0.513$, $R^2 = 0.263$, adjusted $R^2 = 0.256$, $p = 0.0001$).

CONCLUSION:

VKORC1 polymorphism alone influence 26.3% variability in warfarin dose and AA genotype patients required lower dose. Hence *VKORC1* genotyping at the time of initiating warfarin therapy can improve patients response to treatment.

Key words: Warfarin, *VKORC1* polymorphism, Oral anticoagulant

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Introduction

INTRODUCTION

Anticoagulants interfere with the function of clotting factors, interupt the coagulation cascade and prevent the formation of a stable fibrin meshwork. Warfarin, an oral anticoagulant acts by inhibiting the enzyme Vitamin K Epoxide Reductase (VKOR), is used for the prevention and treatment of thromboembolic conditions.[1] The effective use of warfarin depends on the correct dosing to achieve the therapeutic concentration, because low dose can cause therapeutic failure and increased dose can result in fatal bleeding.[2] Warfarin dose is monitored by estimating prothrombin time which is expressed as International Normalised Ratio (INR). Various factors like age of the patient, lifestyle, body mass index (BMI), body surface area, environmental and genetic factor plays a role in clinical response to the prescribed dose of warfarin.[3,4]

Warfarin exhibits more than 10 fold inter-individual variability in dose requirement to attain therapeutic concentration.[3] Inter-individual variability in warfarin dose requirement is influenced by two genes, *CYP2C9* which encodes for the enzyme cytochrome P450, family 2, subfamily C, polypeptide 9 (CYP2C9) involved in warfarin metabolism and vitamin K epoxide reductase complex subunit 1 (*VKORC1*) which encodes for vitamin K epoxide reductase, the target enzyme for warfarin. Genetic variations in *CYP2C9* gene alters the rate of metabolism of warfarin and *VKORC1* gene results in reduced activity of the target enzyme.[5] By identifying polymorphisms affecting these genes, the oral anticoagulation therapy can be tailored to the patient requirement. These genetic polymorphisms are widely distributed and may vary according to the region and ethnicity.[3,5]

Genetic polymorphism involving *VKORC1* is associated with approximately 25% variability in maintenance dose requirement of warfarin.[6,7] There is limited

information available from Karnataka about the frequency of *VKORC1* polymorphism and its influence on warfarin dose requirement. Hence this study was undertaken which aims at determining the relationship between *VKORC1* polymorphism and warfarin dose requirement and International Normalised Ratio (INR).

Aims

&

Objectives

AIMS AND OBJECTIVES

1. To determine the frequency of *VKORC1* -1639 G > A polymorphism using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique in patients receiving warfarin
2. To evaluate the effect of *VKORC1* -1639 G > A polymorphism on maintenance dose of warfarin and INR status in these patients

Review Of Literature

REVIEW OF LITERATURE

"Variability is the law of life, and as no two faces are the same, so no two bodies are alike, and no two individuals react alike and behave alike under the abnormal conditions which we know as disease"

- sir william Osler (1849-1919)

Hemostasis refers to finely regulated dynamic process that maintains the normal blood fluidity in vessels, thus it avoids vessel occlusion and inadequate perfusion to the vital organs. It also limits the blood loss by forming a hemostatic plug at the site of vascular injury.[8,9] The pathological counterpart of hemostasis is thrombosis which causes blood to clot within the intact vessels either due to damage to the vessel wall or stasis of blood. Hemostasis and thrombosis involves three components- endothelium, platelets and the coagulation cascade. Endothelium plays a key role in maintaining hemostasis by balancing between the anti- and prothrombotic factors. Platelets form the primary hemostatic plug that seals the vessel at the site of injury and activates the coagulation factors leading to the formation of fibrin plaque which results in thromboembolic events.

Coagulation cascade consists of extrinsic and intrinsic pathway which in reality interacts with each other constantly.[8] Both the pathways consist of different plasma proteins in their inactive forms called as blood clotting factors. The activation of these clotting factors will cause cascading reactions to form thrombus. Damage to tissue exposes the blood to tissue factor or tissue thromboplastin which complexes with factor VII and activates factor X. The activated factor X in the presence of calcium and factor V binds to form a complex, prothrombin activator. This complex cleaves prothrombin to thrombin within seconds, thrombin then acts as an enzyme to

convert fibrinogen to fibrin that enmesh platelets, red blood cells and plasma to form a thrombus (Figure 1).[10,11]

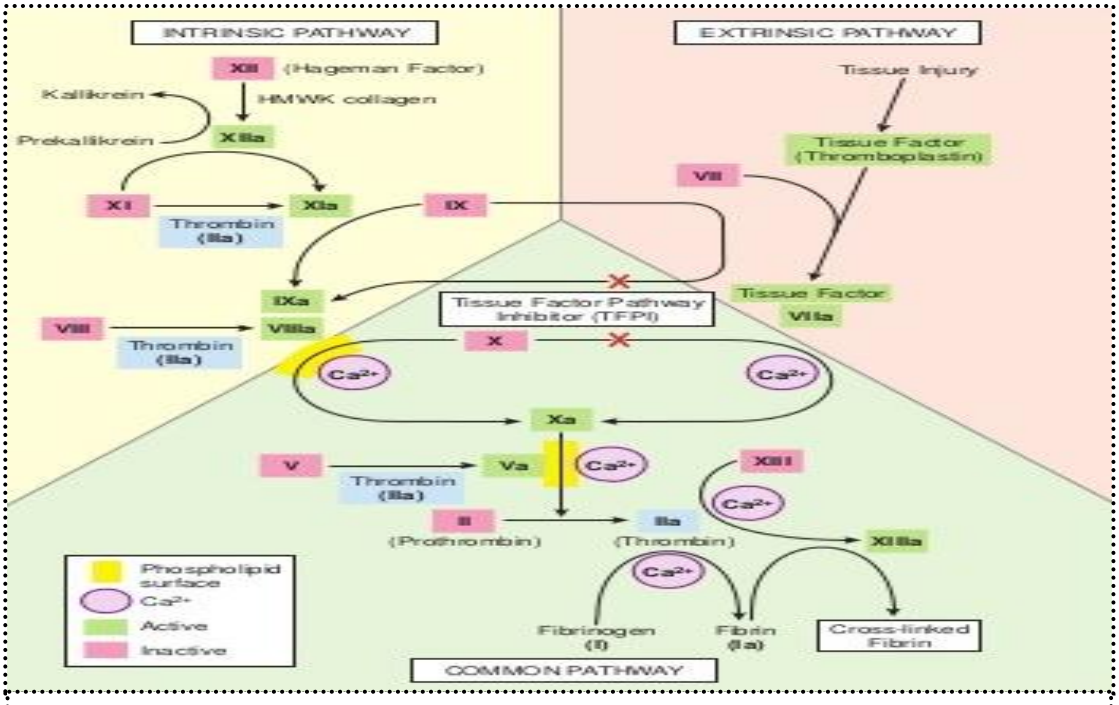

Figure 1: Coagulation cascade

ORAL ANTICOAGULANTS

Oral anticoagulants are effective for lowering the risk of thromboembolic events associated with prosthetic heart valves, atrial fibrillation (AF), cerebrovascular accidents, recurrent infarction in patients with myocardial infarction, deep venous thrombosis, pulmonary embolism, peripheral arterial disease, post hip replacement and major gynaecologic surgeries. Among them, AF is the most common indication for oral anticoagulation.[12]

Warfarin and other coumarin derivatives like acenocoumarol and phenprocoumon are collectively called vitamin K antagonists (VKA) or coumarins.[2,8,13] Among them warfarin is the most commonly used VKA. Until the last few years, VKA remained the mainstay of treatment for nearly 60 years.[14] VKA targets the vitamin K cycle in the liver, thereby inhibiting production of the coagulation factors II, VII, IX and X .[15]

WARFARIN

History of warfarin discovery

Early 1920's: An outbreak of cattle deaths due to bleeding in North Dakota and Canada were reported and they were found dead within the pool of unclotted blood. Epidemiological studies conducted by veterinarians, Dr.Frank.W.Schofield (Figure 2)[16] and Dr. Roderick traced the cause of death to be due to consumption of mold infected sweet clover hay as a result of improper curing, hence was named "sweet clover disease".[17,18]

Figure 2: Dr. Frank.W.Schofield

Sweet clover (Melilotus alba and Melilotus officinalis) was grown as a hay crop to feed cattles in the Northern U.S and Canada (Figure 3).[19] It has a distinctive sweet odour similar to vanilla and its use as hay was widespread in 1920s.[17] They concluded that this disease is reversible and manifests within 15 days after cattle or sheep feeds on the spoiled hay and death occurred due to reduced clotting ability of blood resulting in fatal internal

Figure 3: Sweet clover plant

hemorrhages. They also showed that this fatal disease can be treated by transfusion of freshly drawn blood from normal cattle and by changing their diet which should be free from sweet clover hay.

1931: Dr.Roderick studied the pathophysiology of the disease and concluded that sweet clover disease was due to deficiency of prothrombin in the blood. He also showed that severity of hemorrhagic condition was proportional to the reduction in prothrombin content or the activity using a technique developed by great American pioneer of blood coagulation, the late Professor W. H. Howell.[18]

December 1932: Dr Karl Paul Link (Figure 4)[20] had heard about the sweet clover disease from Ross A Gortner, Head of Department of Biochemistry at Minnesota university who suggested Dr. Link to continue the project on sweet clover disease as they had failed in their attempts about this disease.[18,21] Dr. Link started working on sweet clover disease from January 1933 and he tried to develop a strain of sweet clover suitable for Winconsin climate which was low in or had no coumarin content.

Figure 4: Professor Karl Paul Link

One Saturday afternoon in February 1933, a farmer named Ed Carlson drove to the Biochemistry building at University of Wisconsin with a dead cow, milk can filled with blood and a truckload of sweet clover that was used as feed for cows. The

farmer met Mr. Eugen Wilheim Shoeffel, a German student of Dr Link who advised him to follow the same measures as advocated by Dr.Roderick and Dr.Schofield which was to stop feeding cattles with the spoiled hay. As the disappointed farmer drove back home Mr.Shoeffel expressed his anger and frustration since he was helpless towards the poor farmer. He along with Dr Link worked on the blood in the can brought by the farmer till 7 pm that day.

February 1933 to June 1939: Dr.Schoeffel, Dr.Roberts and Dr.Campbell developed a bioassay method for identification of the anticoagulant activity in the hay. They also devised a method to estimate the prothrombin concentration or activity in the given sample. Thus they succeeded in chemical extraction, separation and isolation of sweet clover hay.[18,21]

June 1939: Paul Link and his research group involving Dr.Roberts, Dr.Smith and Dr.Campbell managed to extract and crystallize the hemmorhagic agent as anticoagulant bishydroxycoumarin (dicumarol) out of spoiled sweet clover hay which they referred initially as H.A for hemorrhagic agent. The active structure was determined and synthesis of active dicoumarol was done a year later.[18]

1940: They created a synthetic product that was shown to be chemically identical to dicoumarol. During 1941 – 1944 fifty reports on the clinical use of dicoumarol appeared. Later in 1945, Dr Link took a six months sabbatical from his research as he had tuberculosis and spent time at a local sanitorium. While he was recovering from his disease he thought of the use of coumarin derivative as rat poison. During this period he collected chemical and bioassay data from his laboratory and selected candidate relatives of dicoumarol that were synthesized between 1940 and 1944 as potential rodenticides.[21]

1948: More potent analogue of dicoumarol named as warfarin was first promoted as rodenticide.[15,16] Warfarin was used in rats, gopher and ground squirrels as poison and was strong enough to cause death after ingesting many doses in spite of administering vitamin K simultaneously. In 1951, the US army inductee committed suicide by swallowing multiple doses of warfarin present in the rodenticide. He recovered completely after hospitalisation and treatment with vitamin K, which was by then established as specific antidote of warfarin. This incident paved way for studies using warfarin as an anticoagulant to be used clinically.

1954: Warfarin made its appearance in market by Endo-Laboratories (Richmond Hill, New York, United States of America) for use in humans as oral anticoagulant under the trade name "coumandin sodium". Link and his co-workers named it WARFARIN

Figure 5: Exhibition held at Winconsin Alumini Research Foundation in 1954

combining first letters of the sponsor of the project Wisconsin Alumni Research Foundation with "arin" which indicates its link with coumarin (Figure 5).[22] Warfarin was mainly developed for rodent control inferring scepticism among physicians and patients. It is said that until 1955, warfarin use was infrequent. One of the first patient to use warfarin was Dwight.D.Eisenhower, the president of the United States for myocardial infarction while on vacation at his in laws house in Denver.[18] Warfarin was approved by FDA in 1955 for the treatment of thromboembolic complications associated with atrial fibrillation.[17,18,21]

1971: Professor Link retired from the University of Wisconsin after a successful career spanning for about 50 years and passed away in 1978.

PHARMACOLOGY OF WARFARIN

Structure and chemistry

Warfarin is a derivative of 4-hydroxycoumarin and of the related compound indan-1,3-dione (Figure 6).[21] The chiral carbon of warfarin (C9) gives rise to two enantiomers; R-and S-warfarin (Figure 7).[23] These two have similar physical properties except for their direction in which they rotate the plane polarised light. In the biological systems, however R-and S-warfarin enantiomers have distinct pharmacokinetics and are metabolised by different enzymes, although both isomers exert their anticoagulant effect by inhibiting the same target enzymeVKOR. Though R and S-warfarin act on the same target site, the S enantiomer has been reported to have 2–5 times more potency than its R analogue.[2,24,25] This stereospecific potency is believed to be the result of differences in affinity for the target enzyme VKOR. R- and S- enantiomers differs from each other with respect to anticoagulant potency, metabolism, elimination and drug interactions.[2]

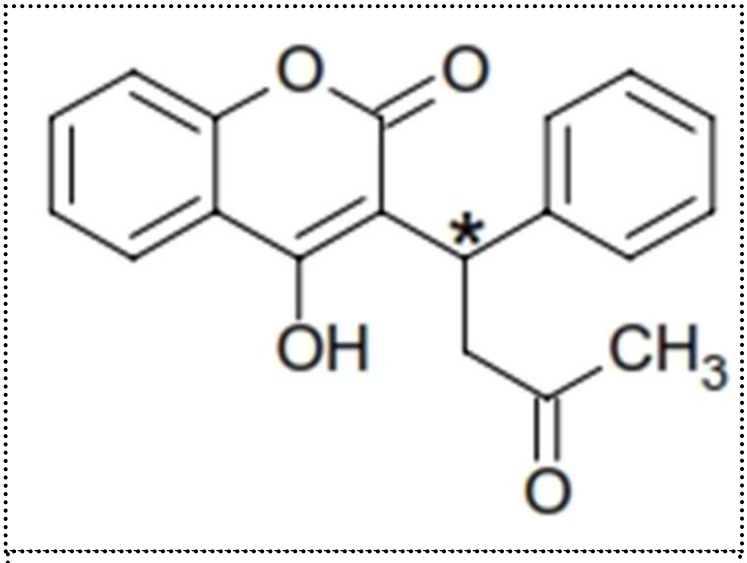

Figure 6: Chemical structure of warfarin

* Indicates chiral centre

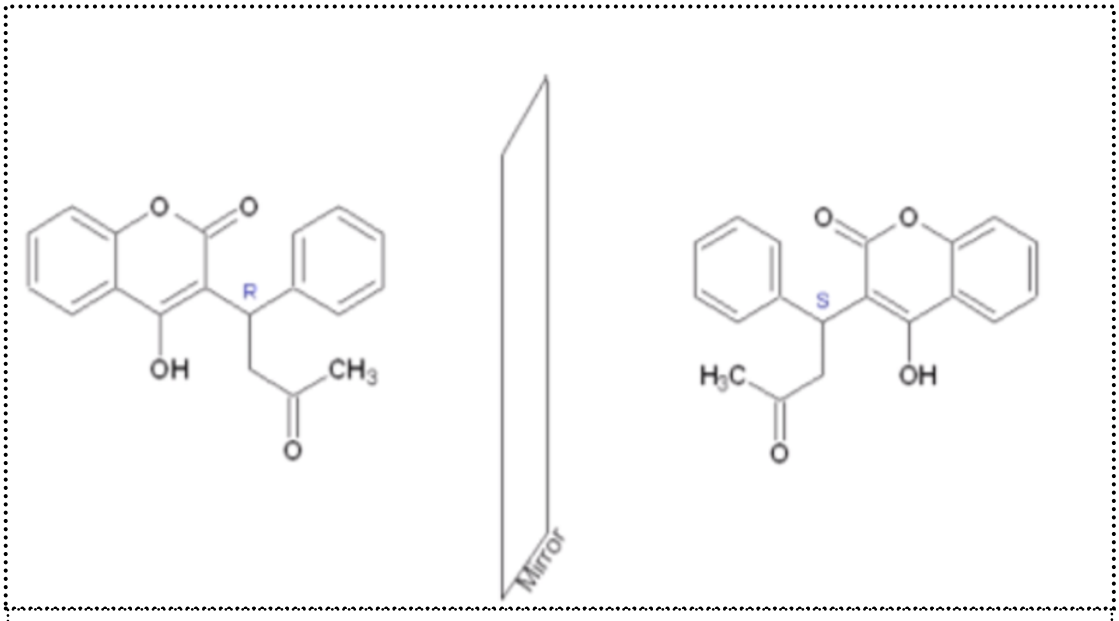

Figure 7: R- and S- enantiomers of warfarin which are mirror images

Mechanism of action

Warfarin inhibits the enzyme vitamin K epoxide reductase, which is involved in the regeneration of reduced form of vitamin K (Figure 8).[2] Reduced vitamin K is required for the post-translational modification (γ carboxylation) of vitamin K dependent clotting factors II (prothrombin), VII, IX, X.[2,7,8,15,26] The inhibition of vitamin K-dependent coagulation factors leads to decrease in thrombin generation. Consequently, the formation of fibrin, the final product of the clotting cascade will decline (Figure 9).[27] The vitamin K-dependent factors have different half-lives in plasma and hence are depressed at different rates during warfarin treatment. Among the four clotting factors, factor VII has the shortest half-life of 6 hours. Thus the pharmacological effect of single dose of warfarin becomes apparent only after about 18-24 hours, whereas suppression of prothrombin occurs slowly and takes several days. Thus, the antithrombotic effect of the drug is delayed until the levels of factors II and X are reduced.[2]

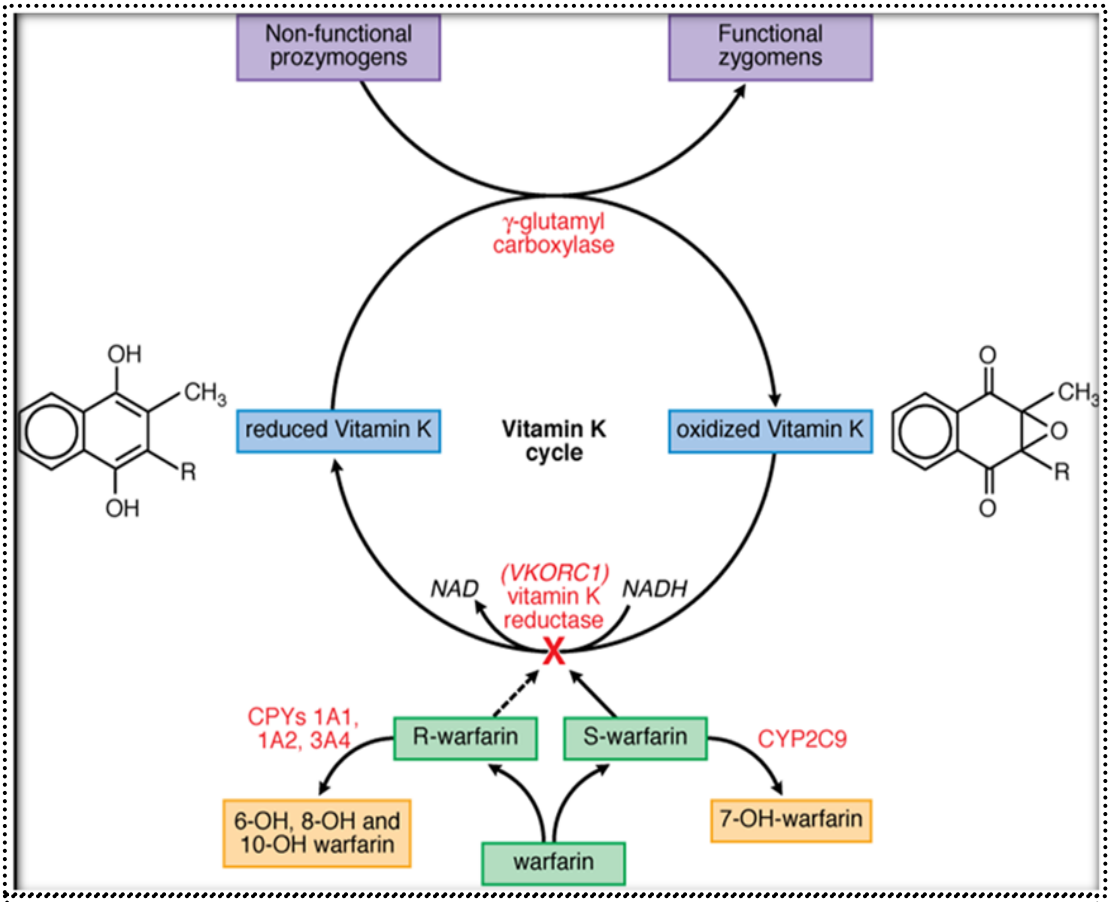

Figure 8: The vitamin K cycle and mechanism of action of warfarin

Endogenous anticoagulants protein C and S synthesis is also dependant on vitamin K. Thus warfarin exhibits concurrent suppression of both coagulation and

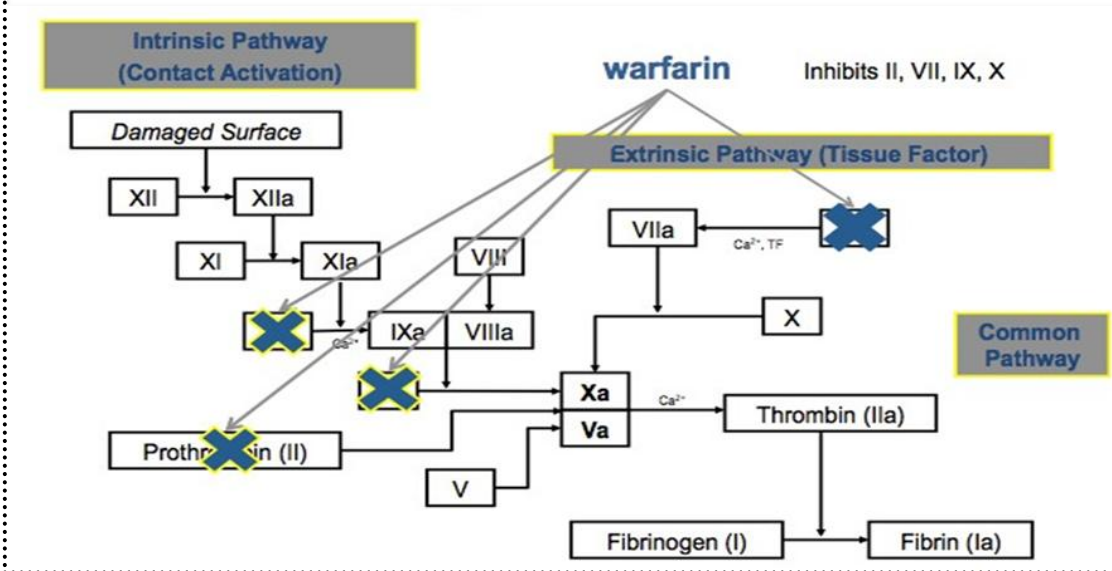

Figure 9: Warfarin action on vitamin K dependent clotting factors

anticoagulation pathway. Therefore, the dose of administered warfarin is important for the balance between coagulation and antithrombotic protection.[26] If the initial warfarin dose is too large the levels of protein C are rapidly depleted, leading to hypercoagulation. The relationship between the dose and response of warfarin is unpredictable. There are no significant correlations between clotting time and maintenance dose or between clotting and plasma concentrations of warfarin.

Pharmacokinetics

Absorption - Warfarin is administered as racemic mixture of R- and S- warfarin with S-warfarin being five times more potent than the R-warfarin. The drug is rapidly and completely absorbed from GIT, the precise location is not identified but proximal duodenum appears to be the most likely part. It has almost 100% bioavailability when administered orally, intravenously and rectally.[26] Warfarin is detectable in plasma within one hour of its oral administration and peak plasma concentration is attained within 2-8 hours.

Distribution - Warfarin is highly plasma protein bound principally to azapropazone binding site on albumin. The R-isomer binds to albumin with higher affinity than the S-enantiomer. The unbound fraction of warfarin only exhibits the pharmacological activity. The plasma half life of R- and S-warfarin is 45 and 29 hours respectively with duration of action of 2-5days.[8,21,28] Both R and S enantiomers have similar volume of distribution (V_d) of about 0.14 L/kg. At steady state, the plasma level of R-warfarin is 1.5 times higher than that of S-enantiomer.

Metabolism and Elimination - It undergoes sterioselective and regioselective metabolism in the liver by cytochrome P450 enzymes principally CYP2C9. Stereoselective metabolism is defined by the existence of a carbon chiral centre,

which in warfarin is at position 9. Thus R- and S-isomers are stereoselectively distinguished by their metabolising enzymes. Regioselectivity on the other hand, refers to the sites on the molecule that are hydroxylated. CYP2C9 enzyme is regioselective mainly for carbon 7 of S-warfarin yielding 7-hydroxywarfarin and to a lesser extent for 6-hydroxywarfarin. The metabolism of R-warfarin is more complex, it undergoes oxidative phase I biotransformation to several monohydroxylated metabolites including 4-, 6-, 7-, 8- and 10-hydroxywarfarin (Table 1).[2,28] Furthermore, cis- and trans-dehydrowarfarin and two diastereomeric alcohols have been found as metabolic products. These hydroxylations have been shown to be the activity of different CYP isoenzymes. CYP1A2 has been suggested to be stereoselective for the formation of 6-,7-and 8-hydroxy R-warfarin with regioselectivity for the 6-position. CYP3A4, CYP2C8, CYP2C18 and CYP2C19 are also involved in the bioconversions of R-warfarin. Warfarin further undergoes phase II metabolism which in humans remains unclarified. Inactive metabolites of warfarin are excreted in urine and stool with 0.045 ml/min/kg average rate of clearance from plasma.

Table 1. Products of warfarin metabolism and the enzymes involved

Enantiomer	Enzymes	Main metabolite	Remarks
S- warfarin	CYP2C9	7-hydroxywarfarin 6-hydroxywarfarin	Main product Minor product
R-warfarin	CYP1A2 CYP3A4 CYP2C8 CYP2C18 CYP2C19	4', 6-, 7-, 8 and 10-hydroxywarfarin	CYP1A2 is regarded to be the principle enzyme for the metabolism of R-warfarin and is regioselective primarily for 6-OH warfarin

Clinical Uses: [2,21,,29,30]

1. Prophylaxis for embolisation in rheumatic heart disease and atrial fibrillation
2. Prevents the progression and recurrence of acute deep vein thrombosis or pulmonary embolism
3. Prevention of venous thromboembolism in patients following major orthopaedic and gynaecological surgeries
4. Prophylaxis for recurrent coronary ischemia in patients with myocardial infarction and prevention of systemic embolisation in patients with prosthetic heart valve
5. Transient ischemic attacks

Route of administration and dosage: [2,29, 30]

Warfarin is administered orally as tablets. Adult dose is 2-5 mg/day for initial 2-4 days followed by 1-10 mg/day as indicated by measurements of INR. It can also be administered intravenously 5mg daily as slow injection over 1 to 2 minutes into the peripheral veins. Intramuscular route is contraindicated due to the risk of hematoma formation.

Adverse effects: [2]

1. Bleeding, the risk increases with the intensity and duration of anticoagulant therapy
2. Skin necrosis which is a rare complication usually occurs after 3-10 days as typical lesions on the extremities, breast and penis
3. Purple toe syndrome characterised by reversible painful, blue-tinged discolouration of plantar surface and sides of toes which blanches with pressure and on elevation of the legs

4. Alopecia, urticaria, dermatitis, fever, nausea, diarrhoea, abdominal cramps and anorexia
5. Exposure to warfarin during foetal life can lead to intrauterine deaths and foetal warfarin syndrome characterised by nasal hypoplasia, stippled epiphyseal calcifications and foetal hemmorhages

Drug interactions [2,20,23,28]

1. Absorption of warfarin can be reduced when co-administered with drugs like cholestyramine
2. Increased metabolic clearance resulting in therapeutic failure when co-administered with hepatic enzymes inducers like barbiturates, carbamazepine, phenytoin and rifamipicin
3. Drugs which inhibit hepatic enzymes like amiodarone, clopidogrel, alcohol, fluconazole, fluoxetine which results in decreased warfarin metabolism leading to increased incidence of bleeding
4. Aspirin when co-administered can increase the risk of bleeding
5. Broad spectrum antibiotics like tetracyclines, chloramphenicol and sulphonamides decrease the availability of vitamin K by depressing the intestinal flora that is required for synthesis of vitamin K
6. Ingestion of large amounts of food rich in vitamin K like cabbage, green leafy vegetables will reduce the efficacy of warfarin

Monitoring and quality control of warfarin treatment

The effect of warfarin is monitored based on INR value which is a standardized measure of the effects of VKA on clotting activity in the blood.[31] The interindividual variation in the dose needed to reach therapeutic levels of

anticoagulation with warfarin therapy results in more than tenfold variability in dose requirement, ranging from less than 10 mg to over 100 mg per week.[31] Since it has a narrow therapeutic index, the INR has to be maintained between 2 to 3 in order to minimize both bleeding and thromboembolic complications. Frequent INR monitoring and dose alteration is therefore necessary especially during the initiation of warfarin treatment and temporary interruptions in treatment.

Monitoring the warfarin therapy by prothrombin time (PT) expressed as INR was proposed by the World Health Organization (WHO) in 1982.[33] Before this PT was used which was measured in seconds and the test was performed by adding calcium and thromboplastin to citrated plasma. The PT test was developed by Quick in 1935 and it is sensitive to the presence and activity of factors II, V, VII, X and fibrinogen. In the beginning it had some significant drawbacks like the lack of standardization hence results varied with different laboratories. The problem was shown to be caused by the use of different thromboplastins which vary in responsiveness to reduction in the vitamin-K dependent coagulation factors. INR which is also sometimes referred to as PT-INR is standardized by dividing the PT of a patient with the geometric mean of PT for at least 20 healthy subjects with the same test system and adjusting the result according to the international sensitivity index (ISI) of the thromboplastin used in the laboratory (Formula1).[32,33] As a result of the standardization, an INR value of 1.0 is considered to be normal coagulation and an INR of 2.0 means that the time has been prolonged to double the normal time for coagulation. Untreated healthy individuals have an INR of 0.8-1.2.

$$INR = \left(PT_{patient} / PT_{normal} \right)^{ISI} \qquad \text{[Formula 1]}$$

INR target range for anticoagulation varies between countries and clinical indications. The INR target range is between 2.0 - 3.0 for most indications except for patients who are at high risk of thrombosis like heart valve prostheses in whom it is maintained between 2.5-3.5 or even higher.[34] Patients on warfarin require frequent INR monitoring during the induction phase of therapy and once the target range for anticoagulation is achieved the INR is measured once or twice a month.

PHARMACOGENETICS OF WARFARIN

Warfarin is a drug with narrow therapeutic range. The required dosing is highly variable and the treatment is regularly controlled with repeated analysis of PT-INR to ensure the stable coagulation. Number of variables are believed to contribute to the observed inter-individual differences in dosage. Variations in dietary intake of vitamin K, interactions with other drugs involving pharmacodynamic or pharmacokinetic mechanisms, interactions with natural substances and herbal medicines, age, BMI and smoking are all well known factors that affect warfarin treatment. However, over 35-50% of all inter-individual dose variations of warfarin are explained by genetic polymorphisms of two genes, *VKORC1* and *CYP2C9*.[3,5,35,36,37]

VKORC1 polymorphism

Following the identification of the *VKORC1* gene by two separate research conducted by Rost et al and Li et al, it was observed that polymorphisms affecting *VKORC1* would influence the sensitivity of warfarin.[38,39] Rost et al reported warfarin resistant patients with single-nucleotide exchanges in *VKORC1* where three patients required warfarin dose of 220 − 250 mg/ week, which is considered unusually high and two other patients who did not respond to any dose of warfarin. This observation

was followed by the report by Harrington et al who found a Val66Met transition in the *VKORC1* protein causing warfarin resistance. These point mutations were found in the coding regions (exons) of the *VKORC1* gene. They are rarely found in the general population and hence cannot explain the high degree of inter-individual variation in warfarin dose requirement.

The mutations in *VKORC1* that mostly influence warfarin dosage are found in the noncoding regions of the genes. These common single-nucleotide polymorphisms (SNPs) show inter-ethnically different distributions and have a greater impact on dose requirement than do *CYP2C9* variants. The first identified SNPs in the *VKORC1* gene were reported by D'Andrea et al, who studied two SNPs at nucleotide positions 6484 and 9041.[40] The 6484 SNP is located in intron 1 and involves C > T change wherein T is the minor allele, but can be found as CT or TT allelic variants. Patients with the CC genotype were generally found to require a higher (6.2 mg/day) warfarin dose requirement than patients carrying the CT (4.8 mg/day) or TT (3.5 mg/day) genotypes. Three exons code for the primary structure of the protein. Untranslated regions (5' and 3') containing putative regulatory sequences are shown in figure 10.[41] The 9041 polymorphism is found in the 3'untranslated region (UTR) of the gene and is involved in a G > A transition, where the AA genotype is associated with increased warfarin dosage. Rieder et al identified 10 common noncoding SNPs forming two

Figure 10: Graphic representation showing the organisation of *VKORC1* gene

main haplotype groups: A (low dose) and B (high dose).[42] They found that the A haplotype has a frequency of nearly 89% in Asians, 37% in Caucasians, and only 14% in Africans. This difference in haplotype distributions matched the differences in warfarin dosage between the different ethnic populations.

A more comprehensive haplotype map involving 28 *VKORC1* polymorphisms swas reported by Geisen et al which confirmed previous observation of Rieder et al and further extended the number of main haplotypes to four.[43] They called their haplotypes *VKORC1*2* (corresponding to the low dose A haplotype of Rieder), *VKORC1*3* and *VKORC1*4* (Both correspond to the high dose B haplotype of Rieder). A fourth variant, *VKORC1*1*, was also identified as the ancestral haplotype and was found only in Africans. These main haplotypes were suggested to cover almost all of the *VKORC1* genetic variability. In populations of Asian ethnicity, *VKORC1*2* has been found to be the dominating haplotype, corresponding to 90% in Chinese Americans, 86% in HongKong Chinese and 89% in Japanese. Among Europeans *VKORC1*2* and *VKORC1*3* are the principal variants (about 40% each), whereas Africans have a frequency of 14% for *VKORC1*2*, 31% for *VKORC1*1* and 43% for *VKORC1*4*. Haplotype *VKORC1*4* is rare in Asians but is more common in African and European populations.[39,40,41,44,45] Seven other SNPs of *VKORC1* studied which extensively are 1639G > A, 497T > C, 1173C > T, 1542G > C, 2255C > T and 3730G > A.[4] *VKORC1 -16*39 G > A is the polymorphism in the promoter region of the gene which influences the VKOR enzyme activity.[4,5]

The noncoding polymorphisms in *VKORC1* explain approximately 30% of the variations in warfarin dose requirement when factors such as *CYP2C9* polymorphisms, age, body weight and drug interactions were excluded. Sconce et al proposed a dosing regimen for warfarin where the contribution of age, body size,

CYP2C9 and *VKORC1* polymorphisms were investigated.[44] They found that the total impact of these factors on warfarin dose variability was nearly 55%. Altogether, it is becoming clear that *VKORC1* is an important genetic determinant for warfarin dose requirement.

Materials & Methods

MATERIALS AND METHODS

This study was conducted from January 2015 to June 2016 on patients receiving warfarin for cardiovascular disorders.

Location of study

This was a cross-sectional study conducted by Department of Pharmacology on patients attending Department of Cardiology at R.L.Jalappa Narayana Heart Centre in association with Department of Cell Biology and Molecular Genetics attached to Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar, Karnataka.

Data collection

A proforma containing detailed information of each patient was designed according to the study protocol. Ethical clearance was obtained from Institutional Ethics Committee. Patients who were willing to give the written informed consent were included in the study. A total of 102 patients were recruited for the study.

Inclusion criteria

1. Patients of either gender aged between 18-65 years
2. Body mass index (BMI) 18-29 kg/m^2
3. Patients who had received oral warfarin for atleast 2 months

Exclusion criteria

1. Patients with specific systemic disorders like renal or hepatic insufficiency
2. Patients receiving concomitant medications like phenytoin, rifampicin, isoniazid, tetracycline, erythromycin, metronidazole, cephalosporins, barbiturates and oral contraceptive pills

3. Patients diagnosed to have malignancy or on cancer chemotherapy
4. Patients addicted to smoking and alcoholism
5. Pregnant and lactating women

Method of collection of data

Patients receiving warfarin as maintenance therapy for cardiovascular disorders attending the outpatient department or inpatients of cardiology wards, between January 2015 to June 2016 were recruited. Data from each participating patient was collected using proforma which included age, weight, height, gender, daily warfarin dose, changes in warfarin dose, INR values, diet history and bleeding complications. All patients were advised about diet and to avoid green leafy vegetables, cabbage, cranberry juice and alcohol that can interfere with drug pharmacokinetics. INR was calculated according to the formula INR = (patient PT/ normal PT)^{ISI}

Study protocol was explained and written informed consent was obtained. Three ml of venous blood was collected in vaccutainer with anticoagulant ethylene diamine tetra acetic acid (EDTA) from each patient under strict aseptic precautions. DNA was extracted using standard salt extraction method and was stored at -40ºC.[46] Genotyping was done using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Genotyping of *VKORC1 -1639G > A* poymorphism

The tests were performed by the primary investigator under the guidance of technical expert in molecular biology for all experimental aspects.

Polymerase Chain Reaction (PCR)

PCR is used to enzymatically duplicate DNA which was introduced by Kary Mullis in the mid 1980s and has since become an essential tool in every laboratory in which some form of genetic analysis is carried out. Any region of DNA can be copied using a reagent containing primers which are short single stranded DNA, DNA polymerase enzyme, deoxynucleotide triphosphate (dNTPs), magnesium and buffer and it requires heat recycling.

Genotyping of *VKORC1* was performed as described by Scone et al.[46] Sequences for the forward and reverse primers are: 5'-GCCAGCAGGAGAGGGAAATA-3' and 5'-AGTTTGGACTACAGGTGCCT-3' respectively. The PCR was performed using BIORAD C1000 touch thermal cycles at 25 cycles of 1 min at each of the following: 94°C, 61°C and 72°C. Each cycle contains three different steps: denaturation, annealing and extension. In denaturation, DNA molecules are heated at 94ºC to break the chemical bonds between the two antiparallel strands of DNA resulting in consequent formation of two single-stranded DNA (ssDNA). In annealing, the sample is cooled down to allow a pair of primers to bind, each being complementary to one ssDNA. The anneling temperature was 61ºC. In the last extension step, the heat is increased to 72 ºC to allow the amplification reaction to take place. The Taq polymerase used in PCR is stable at high temperatures. The PCR process was automated and operated by a thermal cycler that heats and cools the reaction tubes at the temperature required for each step. Once the reaction was completed, the copied DNA material containing a selected region of a gene was analysed on 2% agarose gel electrophoresis to estimate the quality of the DNA sample.

PCR product was then used to carry out genotyping using restriction fragment length polymorphism (RFLP) technique. The PCR product (10 μL) was digested with ten units of restriction enzyme Msp1 in a final volume of 20 μL in the appropriate digestion buffer at 37°C for at 16 hours. The digested product was visualized on 2% agarose gel stained with ethidium bromide. RFLP pattern were interpreted as represented in table 2.

Table 2: PCR-RFLP pattern of *VKORC1* -1639 genotype

-1639 *VKORC1* G > A Genotype	**PCR product size = 290bp**
GG	168bp
GA	122bp
AA	290bp

Validation of test:

1. After first round of screening one sample each with GG, GA, AA was repeated at least 3 times
2. 10 % of the total samples were repeated for examining the accuracy and these samples were used as control for each batch of sample testing.

Sample size calculation

A precision of 4%, desired confidence interval of 80%, to have an expected proportion of polymorphism of 0.86, the sample size was calculated to be 92.[47]

Statistical tests

1. Demographic data was analysed using descriptive statistics and expressed as mean ± standard deviation
2. Mean warfarin dose between the groups was compared using ANOVA followed Bonferonni post-hoc test
3. Regression tool was used to study the association of independent variables on warfarin dose
4. p value less than 0.05 was considered statistically significant

Results

RESULTS

A total of 125 patients were screened for the study, among them 102 patients were recruited on whom genotyping was performed using PCR-RFLP method (Figure 11). All the patients of our study were residents of Kolar, Karnataka (South India).

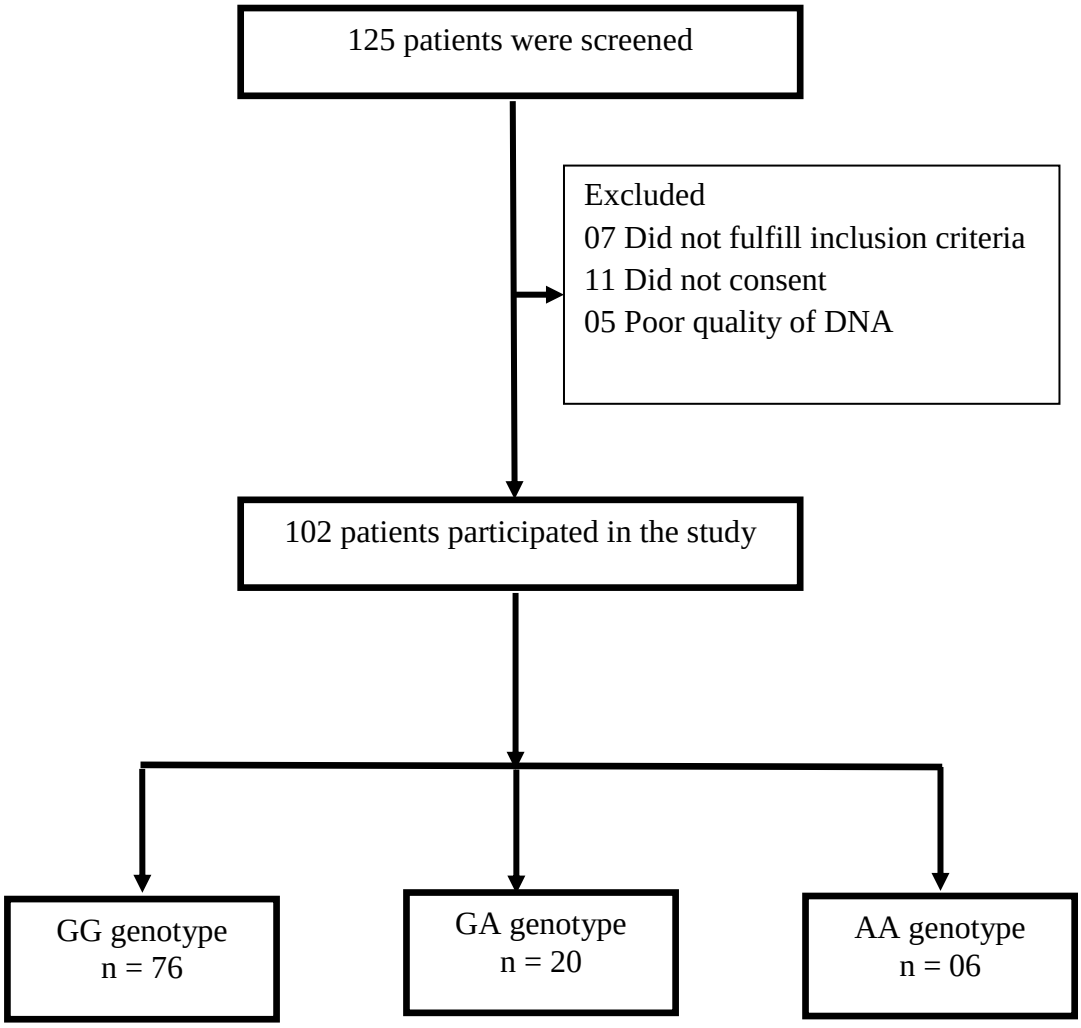

Figure 11:Screening, recruitment and grouping of patients according to genotype

Table 3: Demographic profile of patients

Patient characteristics	*VKORC1* genotype		
	GG (n=76)	**GA (n=20)**	**AA (n=6)**
Age (years)*	40.03±10.78	42.70±12.71	50.83±11.56
Gender, n (%) Male Female	 43 (56.57) 33 (43.42)	 12 (60) 08 (40)	 03 (50) 03 (50)
BMI (kg/m^2)*	23.05±3.42	22.20±2.60	24.27±2.77
Languages spoken, n (%) Kannada Telugu Tamil Urdu	 36 (47.36) 08 (10.52) 24 (31.57) 08 (10.52)	 09 (45) 10 (50) 01 (05) 00 (0)	 02 (33.33) 01 (16.66) 01 (16.66) 02 (33.33)
Indications for warfarin, n (%) Mechanical heart valves Valvular heart disease Atrial fibrillation Venous thrombosis	 37(48.68) 27(35.52) 08(10.52) 04(05.23)	 07(35) 10(50) 02(10) 01(05)	 02(33.33) 01(16.66) 01(16.66) 02(33.33)
Concomitant medications, n Digoxin Furosemide Aldactone Metoprolol Atenolol	 22 15 12 09 04	 08 11 08 05 00	 03 04 01 02 02
Mean INR value*	2.51 ± 0.55	2.37 ± 0.50	2.52 ± 1.66

*values in mean ± SD

There were 58 (56.86%) male and 44 (43.13%) female patients with mean age of 47.72 ±10.31 and 42.95 ±11.98 years respectively. Males formed the majority overall and in all three genotype groups (GG genotype there were 56.57% males). Mechanical heart valves (45.09%) and valvular heart diseases (37.25%) were the most common indications for warfarin therapy. Among the concomitant medications, digoxin (32.35%) followed by furosemide (29.41%) were most commonly used. Duration of warfarin treatment among these patients ranged between three months to 15 years. Although majority of patients (84.31%) had their INR within therapeutic

range, only 15.7% (16) of patients had their INR in the sub-therapeutic range. Among them 10, 4 and 2 patients belonged to GG, GA and AA genotypes respectively.

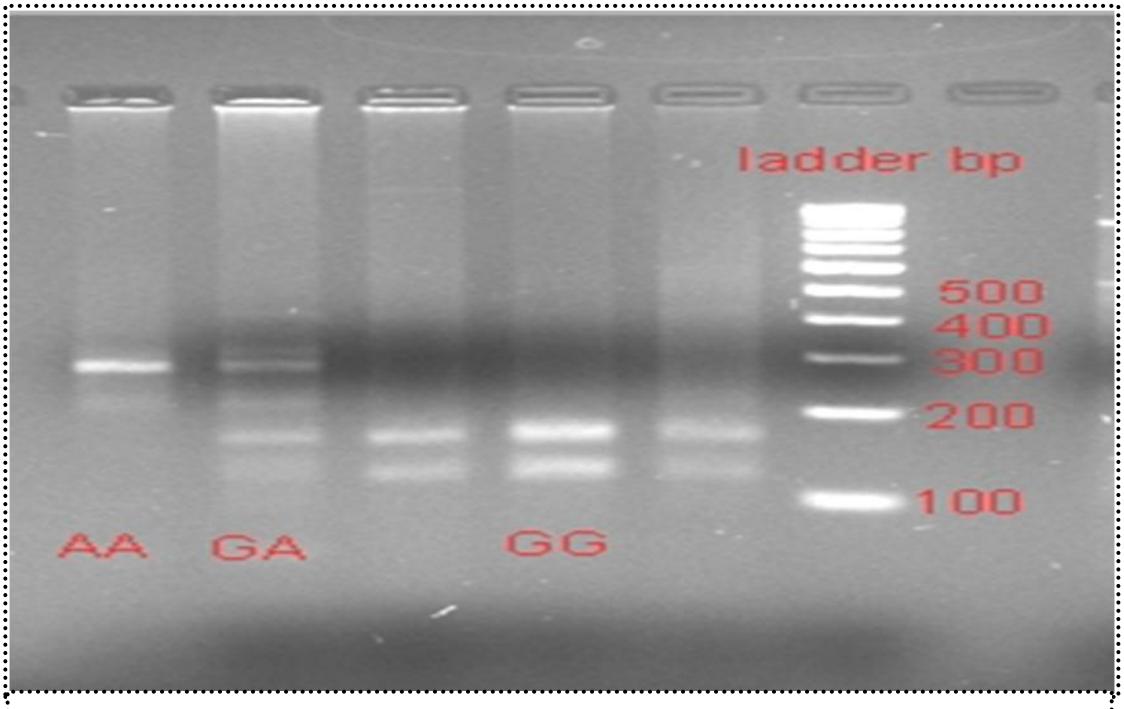

Figure 12: Representative gel electrophoresis image of PCR-RFLP analysis for *VKORC1* G > A of three patient samples showing wild type GG, variant heterozygous type GA and variant homozygous type AA

The major allele G is digested to two fragments of 168- and 122- bp and the minor allele A being resistant to digestion by restriction enzyme is represented as a single band of 290-bp fragment. The presence of all three bands indicates GA (variant heterozygous), two bands indicate GG (wild) and one band indicates AA (variant homozygous) genotype (Figure 12).

Table 4: *VKORC1* -1639 G > A genotype and allele frequency

Genotype	**Number of patients***			**Frequency**
	Male	Female	Total	
GG	43	33	76	74.51
GA	12	08	20	19.61
AA	03	03	06	05.88
Allele	**Number of alleles****			**Frequency**
G	76x2+20=172			84.32
A	06x2+20= 32			15.68

* Total number genotyped for *VKORC1* -1639 G>A was 102 (male = 58, female =44)

** Total number of alleles = 204 chromosomes (102 patients)

The allele and genotype frequency of *VKORC1* are shown in table 4. The *VKORC1* -1639 G > A minor allele (A) frequency in our study population (n=102) was 15.68%. Among the 102 patients, 20 were heterozygous (-1639 GA) and six were homozygous (-1639 AA), both having one and two minor alleles respectively.

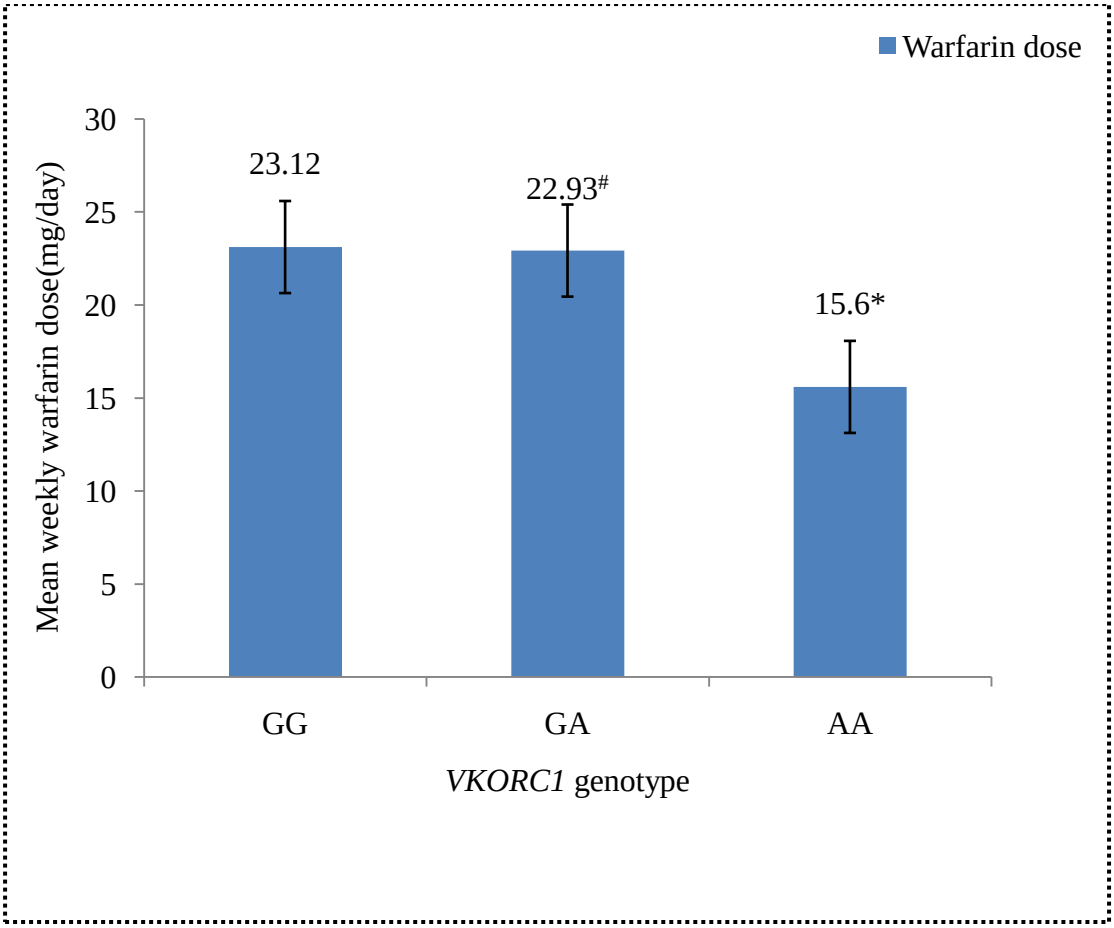

Figure 13: Weekly warfarin dose requirement according to the *VKORC1* genotypes

*p= 0.0001, GG vs AA

#p= 0.031, GA vs AA

Mean weekly warfarin dose was compared between the wild and the variant genotypes of *VKORC1*. Significant difference in dose requirement was found between *VKORC1* wild and mutant genotypes. Patients with GG and GA genotypes required therapeutic dose of warfarin compared to those with mutant AA genotype who required a very less dose.

Table 5: Multiple linear stepwise regression analysis for factors determining warfarin dose

Variable	Unstandardised regression coefficient	Standard error	Standardised regression coefficient	p value
Constant	31.364			
VKORC1 genotype	-0.576	0.096	-0.513	0.0001

$R = 0.513$, $R^2 = 0.263$, adjusted $R^2 = 0.256$

Multiple linear regression analysis was carried out for factors which are known to influence the warfarin dose requirement. Age, BMI, gender, duration of therapy, language spoken and *VKORC1* polymorphism were the independent variables considered to analyse the association with mean warfarin maintenance dose requirement. *VKORC1* -1639 G > A genotype was found to be significantly associated with the warfarin dose requirement contributing to 26.3% variability ($p= 0.0001$). However there was no significant association in terms of age, gender, BMI, language spoken and duration of therapy with warfarin dose requirement ($p >0.05$).

Discussion

DISCUSSION

Warfarin is the most commonly used oral anticoagulant for thromboembolic conditions. The drug exhibits narrow therapeutic window with wide variability in dose requirements among different ethnic groups and individuals. Factors like age, gender, BMI, diet, race and concomitant medications may contribute to the variability in response to warfarin along with the genetic factors.[3,4,48] In few individuals,warfarin when used in therapeutic doses may result in subtherapeutic INR predisposing to thrombosis and supratherapeutic INR which increases the risk of bleeding. Hence, individual dose adjustment is a challenge in clinical practice. At present, in our country warfarin dose adjustment is based mostly on the INR value or by trial and error method.[3] Including genotype of the patient to decide the dose of warfarin can be considered before starting treatment with warfarin.[49] The prevalence of gene polymorphisms are known to vary across different ethnic-geographical populations.[3] In our study we have found the frequency of *VKORC1* polymorphism among South Indian population.

In this cross-sectional study, genotyping for *VKORC1* -1639 G > A was performed on 102 patients who were on maintenance dose of warfarin. In the present study, majority of the patients were in the third and fourth decade of life (Table 3) which is similar to most other studies, except in Sripriya Natarajan et al study they were in the fifth decade.[50,51] Rheumatic fever is common in the first and second decade of life and the disease progresses to cause valvular disturbances and may require replacement with prosthetic valves during their later decades of life. The number of male and female patients in our study were similar to other studies, probably due to the prevalence of valvular involvement being same in both the

gender.[51-53] Mechanical heart valve was the common indication for warfarin therapy in our study. In a study conducted by Kaur et al, 110 out of 111(99.09%) patients were treated with warfarin for prosthetic heart valve replacement.[53] However in another study, venous thrombotic conditions like cerebral venous thrombosis and deep venous thrombosis were the most common indications.[52] Digoxin followed by furosemide were the most commonly used concomitant medications in our patients which are used to treat congestive cardiac failure, a complication of valvular disease. This finding was different from the Madan et al study in which phenoxymethyl penicillin was used by majority of the patients and in one other study it was aspirin which was used maximally.[50,51] In our study, 16 patients had their INR value in the subtherapeutic range (INR < 2) which could probably be due to greater warfarin metabolism or reduced patient compliance.

Wild type of polymorphism was the most common type in our study population accounting to about 74.51% and this finding is in concordance with other Indian studies. Madan et al study on South Indian population observed that the wild type frequency was 79.9% whereas study on North Indian population showed *VKORC1* -1639 G > A frequency to be 76%.[51] The frequency in other countries and race has been represented in table 6.[49,50-56]

Table 6: Frequency of *VKORC1* polymorphism among different ethnic population

Ethnic population	Sample size	*VKORC1* genotype frequency		
		GG	GA	AA
Caucasians	297	25%	56%	19%
Spanish	105	28%	40%	32%
African-Americans	159	79%	21%	00%
Chinese	104	02%	18%	80%
Indonesia	135	03.8%	38.6%	57.6%
Japan	828	0.73%	16.06%	83.21%
North India	111	88.3%	09%	2.7%
North India	138	75.4%	22.4%	2.1%
South India	144	79.9%	10.4%	0.7%
Current study	102	74.51%	19.61%	05.88%

The *VKORC1* -1639 G > A minor allele frequency observed in our study was 15.68% and 10.4% in Madhan et al study which is slighty lower than our study finding.[50] A pilot study conducted on 50 patients from North India found minor allele frequency to be 22% which is higher compared to findings in other South Indian studies and our study.[54] Rathore et al conducted study on 102 North Indian healthy volunteers and showed that minor allele frequency was 14.22%.[55] This implies that region may contribute to variation in gene polymorphism.

The *VKORC1* minor allele frequency in our study population is smaller compared to Caucasians and rest of the world, except African-American population among whom the minor allele frequency is 0%. Similar to other studies conducted in

various parts of our country have shown a lesser frequency of the homozygous mutant genotype compared to our study population among whom the variant genotype is slightly higher (Table 6).

Individuals with wild genotype required therapeutic dose of warfarin in comparison to the variant genotypes who required lesser dose (Figure 13). In another Indian study, the patients with *VKORC1* variant genotype required significantly lesser dose than those with the wild genotype.[51] We found a significant association between *VKORC1*-1639 G > A polymorphism and mean weekly warfarin dose which implies that polymorphism in *VKORC1* gene is an important predictor of warfarin dose variability. A study conducted by Yuan et al has made a clinically important observation that the oral anticoagulant dose requirement is highest among black, intermediate among white and the Asian population require the lowest dose.[57] Lee et al reported that Chinese and Malays require lower warfarin dose than the Indians.[58] The present study reveals that the majority of South Indians may require therapeutic dose of warfarin unlike other Asians like Chinese, Indonasians and Japanese.

Carriers of even one major allele (A) were found to require lesser dose compared to patients having minor allele (G). *VKORC1* -1639 G > A located in the promoter region of the gene is capable of regulating the expression of VKOR affecting the dose requirement. Patients with variant homozygous genotype (AA) will have the least VKOR enzyme functionality resulting in reduced availability of vitamin K hydroquinone thus action of clotting factors will be decreased. Hence they require a lesser dose of warfarin to inhibit the enzyme. In case of variant heterozygous genotype the enzyme will have intermediate activity and hence require slightly lower than therapeutic dose. Patients with wild genotype whose enzyme is fully functional require the defined therapeutic dose.

In our study, *VKORC1* gene polymorphism has contributed to 26.3% variability in the warfarin dose requirement (Table 5). A study conducted by Bodin et al has shown that VKORC1 polymorphism alone accounted to 37% variability in the dose requirement of coumarin drugs among the healthy individuals.[59] However, Madhan et al study has found that 36.1% variability was attributed to combined effect of CYP2C9, VKORC1, age, BMI and duration of the treatment.[50] The difference in variability in response to warfarin dose can be possibly explained by differences in the protein binding and genetic variability in the metabolising enzyme. However a study conducted by Dang et al showed that even after all confounding factors were adjusted, warfarin maintenance dose varied across ethnic groups suggesting genetic variations to be the cause for interindividual variability.[60] A similar study conducted on South Indian population has found that genetic factors like *CYP2C9, VKORC1, CYP4F2 V433M, GGCX G8016A* (Gamma-glutamyl carboxylase) and clinical factors like age, gender, BMI, vitamin K intake, thyroid status have contributed to 61% variability in warfarin dose requirement.[61]

In our study we did not find any association between clinical factors and warfarin dose requirement. We did not assess the influence of other factors like diet, concomitant medications and smoking on patients response to warfarin.[3,4,62] But all our patients were strictly instructed to restrict intake of green leafy vegetables, cabbage which might have contributed towards reducing diet related fluctuations in dose. One patient diagnosed with valvular heart disease received usual adult dose of warfarin and presented with malena, epistaxis, bleeding gums and the INR values were high (INR = 6). Warfarin was witheld, vitamin K injection was given along with blood transfusion. Her genotype was mutant homozygous (AA) which requires low dose. Another patient with variant genotype (AA) receiving usual therapeutic dose of

warfarin, experienced severe malena and hemarthrosis. Based on their genotype the dose of warfarin was adjusted and on followup the INR was found to be in the therapeutic range with no history of bleeding. These incidents further emphasise the need for pharmacogenetic testing at the time of initiation and maintenance of warfarin therapy. Warfarin along with few other drugs are listed by FDA under recommended category of genomic biomarkers for pharmacogenetic testing.[63]

Conclusion

CONCLUSION

- Warfarin, an oral anticoagulant with narrow therapeutic index is used in the treatment and prophylaxis of thromboembolic conditions
- At therapeutic dose, patients may be at risk of thrombosis or bleeding due to inter-individual variation in response to warfarin
- A wide inter-individual variability in response to warfarin, may be attributed to diet, age, concomitant drugs and genetic factors
- Polymorphism in the promoter region of the *VKORC1* is one of the important predictor of warfarin dose
- 102 patients on maintenance dose of warfarin with stable INR were analysed for *VKORC1 -1639* G > A polymorphism using PCR-RFLP
- The frequency of wild genotype (GG) of *VKORC1* was 74.51% and the minor allele frequency was 15.68%
- Patients with wild genotype of *VKORC1* required therapeutic dose but the variant genotypes (both homozygous and heterozygous) required lower dose of warfarin
- *VKORC1* -1639 G > A polymorphism alone contributed to 26.3% variability in warfarin dose requirement among these patients
- Age, gender, BMI and duration of warfarin treatment did not influence the dose requirement of warfarin
- Hence *VKORC1* genotyping at the time of initiating warfarin can help the clinicians to optimise the dose suitable to the patients
- Clinical parameters with prior knowledge of patient's genotype can be used to decide the warfarin dose to maintain the therapeutic INR and reduce the risk of bleeding complications

Summary

SUMMARY

Anticoagulants interfere with the coagulation cascade and prevent the formation of thrombus. Warfarin, an oral anticoagulant acts by inhibiting the enzyme Vitamin K Epoxide Reductase (VKOR). It is used for the prevention and treatment of thromboembolic conditions. Dose variation may result in increased risk of thrombosis or bleeding. It has narrow therapeutic index and wide inter-individual variability in dose response. This is influenced by two main genes, *CYP2C9* which encodes for the enzyme cytochrome P450, family 2, subfamily C, polypeptide 9 (CYP2C9) involved in warfarin metabolism and vitamin K epoxide reductase complex subunit1 (*VKORC1*) which encodes for vitamin K epoxide reductase enzyme.

In this cross-sectional study, total of 102 patients receiving warfarin maintenance dose for thromboembolic conditions atleast for two months were included. Three ml of venous blood was collected from all patients under strict aseptic precautions. DNA was extracted using standard salt extraction method which was stored at -40ºC. Genotyping of *VKORC1* -1639 G > A was done using polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP).

A total of 102 patients with mean age of 47.72±10.31 years of which 58 (56.86%) were male. Frequency of *VKORC1* -1639 G > A for GG, GA and AA genotypes were 74.51, 19.61 and 5.88% respectively. Minor allele A frequency was 15.68% with 20 heterozygous and six of them being homozygous. Minor allele was less frequent than the major allele. Mean weekly warfarin dose was 23.12±8.08, 22.93±8.21 and 15.6±5.35 mg in patients with GG, GA and AA genotypes respectively. Patients with GG genotype required therapeutic dose compared to variant type (p=0.001). Multiple stepwise regression model showed 26.3% variability

in warfarin dose was due to *VKORC1* genotype (R=0.513, R^2= 0.263, adjusted R^2 =0.256, p=0.0001).

VKORC1 polymorphism alone influence 26.3% variability in warfarin dose and AA genotype patients required lower dose. Hence *VKORC1* genotyping while initiating warfarin therapy can help clinicians to decide appropriate dose and improve patients response to treatment. Pharmacogenetic testing along with appropriate clinical evaluation will help in reducing the complications of warfarin therapy.

Bibliography

BIBLIOGRAPHY

1. Vaz M, Kurpad A, Raj T. Blood coagulants. In: Hall JE, editors. Guyton and Hall textbook of medical physiology.12th ed. New Delhi: Elsevier; 2013.p.146-54.
2. Weitz JI. Blood coagulation and anticoagulant, fibrinolytic and antiplatelet drugs. In: Brunton L, Chabner B, Knollman B, editors. Goodman and Gilmans The pharmacological basis of therapeutics.12th ed. New York: McGraw-Hill medical: 2011.p.860-5.
3. Yin T, Miyata T. Warfarin dose and the pharmacogenetics of CYP2C9 and VKORC1- rationale and perspectives. Thrombo Res. 2007; 120: 1-10.
4. Limdi NA, Wadelius M, Cavallari L, Eriksson N, Crawford DC, Lee MM et al. Warfarin pharmacogenetics: a single VKORC1 polymorphism is predictive of dose across 3 racial groups. Blood. 2010; 115: 3827-34.
5. Kumar KD, Shewade DG, Manjunath S, Ushakiran P, Reenega G, Adithan C. Inter and intra ethnic variation of vitamin k epoxide reductase complex and cytochrome P450 4F2 genetic polymorphism and their prevelance in south Indian population. Indian J Hum Genet. 2013; 19: 301-10.
6. Hill CE, Duncan A. Overview of pharmacogenetics in anticoagulation therapy. Clin Lab Med. 2008; 28:513-24.
7. Ma Q, Lu AYH. Pharmacogenetics, Pharmacogenomics and Individualised medicine. Pharmacol Rev. 2011; 6:437-459.
8. Zehnder JL. Drugs used in disorders of coagulation. In: Katzung BG, Trevor AJ, editors. Basic and clinical pharmacology. 13th edition. New York: McGraw-Hill education private limited; 2015.p. 863-88.

9. De Caterina R, Husted S, Wallentin L, Agnelli G, Bachmann F, Baigent C, et al. Anticoagulants in heart disease: current status and perspectives. European Heart J. 2007; 28: 880-913.
10. Armstrong AW, Golan DE. Pharmacology of hemostasis and thrombosis. In:Golan DE, Tashjian AH, Armstrong AJ, Armstrong AW, editors. Principles of Pharmaclogy The pathophysiological basis of drug therapy.3rd ed. New Delhi: Wolter Kluwer Lippincott Williams and Wilkins: 2012.p.372-98.
11. Kumar V, Abbas AK, Aster JC. Hemodynamic disorders, Thromboembolism and shock. In: Kumar V, Abbas AK, Aster JC, editors. Robbins basic pathology.9th ed. Philadelphia: Saunders;2013.p.75-98.
12. Hirsh J, Fuster V, Ansell J, Halperin JL. American Heart Association/American College of Cardiology Foundation guide to Warfarin therapy. J Am Coll Cardiol. 2003; 41: 1633-52.
13. Ansell J, Hirsh J, Poller L, Bussey H, Jacobson A, Hylek E. The pharmacology and management of the vitamin K antagonists: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. Chest. 2004; 126: 204S-233S.
14. Johnson JA. Warfarin Pharmacogenetics: A Rising Tide for its Clinical Value. Circulation. 2012 ;125:1964-6.
15. Nakano T, Kawamoto K, Kishino J, Nomura K, Higashino K, Arita H. Vitamin K-dependent biosynthesis of γ-carboxyglutamic acid. Biochem J. 1997; 323: 387-92.
16. Legault B, Prescott JF. The arch agitator:" Dr. Frank W. Schofield and the Korean independence movement. Can Vet J.2009:50:865-72.

17. Guillory J. The story of Coumadin [Internet]. [Cited 2016 Jan 12]. Available from: http://www.setma.com/Your-Life-Your-Health/pdfs/Coumadin-The-Story-of-a-Drug.pdf
18. Last JA. Profiles in toxicology. The missing Link: the story of Karl Paul Link. Toxicol Sci. 2002; 66: 4-6.
19. Kinsey T. Melilotus indicus – Annual Yellow Sweetclover [Internet]. [Cited 2016 Aug 15]. Available from: http://www.fireflyforest.com/flowers/1529/melilotus-indicus-annual-yellow-sweetclover
20. Karl Paul Link [Internet].[Cited 2016 Aug 28]. Available from: http://toxsci.oxfordjournals.org/content/66/1.cover-expansion
21. Link KP. The discovery of dicoumarol and its sequels.Circulation 1959;19:97-107.doi:10.1161/01.CIR.19.1.97.PMID 13619027.
22. Karl Paul Link: (In)famous scientist on campus [Internet]. [Cited 2016 Oct 2] Available from: http://uwmadarchives.tumblr.com/post/57626483056/karl-paul-link-infamous-scientist-on-campus
23. Warfarin (R)- and (S)-enantiomers [Internet]. [Cited 2016 Oct 11]. Available from: https://www.researchgate.net/profile/Abdimajid_Osman/publication/260762503/figure/fig1/AS:296644076687362@1447736867498/Fig-4-Warfarin-R-and-S-enantiomers-The-two-molecules-are-each-other's-mirror.png
24. Wallin R, Hutson SM. Warfarin and the vitamin K-dependent gamma-carboxylation system. Trends Mol Med. 2004; 10: 299-302.

25. West BD, Preis S, Schroeder CH, Link KP. Studies on the 4-Hydroxycoumarins.The resolution and Absolute Configuration of Warfarin. J Am Chem Soc.1961; 83: 26762679.
26. Hirsh J, Dalen J, Anderson DR, Poller L, Bussey H, Ansell J et al. Oral anticoagulants: mechanism of action, clinical effectiveness, and optimal therapeutic range. Chest. 2001; 119: 8S-21S.
27. Montney JD. Risky Business: Overanticoagulation from High-Risk Antimicrobials and Warfarin [Internet]. [Cited 2016 Sep 22]. Available from: http://www.pharmacytimes.com/publications/issue/2016/may2016/risky-business-overanticoagulation-from-high-risk-antimicrobials-and-Warfarin
28. Wittkowsky AK. Warfarin and other coumarin derivatives: pharmacokinetics, pharmacodynamics and drug interactions. Semin Vasc Med. 2003; 3: 221-30.
29. Weitz JI. Hemostasis, Thrombosis, Fibrinolysis and Cardiovascular disease. In: Bonow RO, Mann DL, Zipes PZ, Libby P,editors. Brawnwald's heart disease A textbook of cardiovascular medicine. 9th ed. New Delhi: Elsevier India private limited; 2012.p.1845-63.
30. India. Indian Pharmacopoeia Commission. Warfarin. Ghaziabhad: Government of India Ministry of health and family welfare; 2011: p 334-335.
31. De Caterina R, Husted S, Wallentin L, Agnelli G, Bachmann F, Baigent C et al. Anticoagulants in heart disease: current status and perspectives. European Heart J. 2007; 28: 880-913.
32. Hall EJ. Hemostasis and blood coagulation. In: Hall EJ, editors. Guyton and Hall textbook of medical physiology. 12th ed. New Delhi: Elsevier India private limited; 2011.p.451-61.

33. WHO Expert Committee on Biological Standardization: twenty-eighth report. World Health Organ Tech Rep Ser.1977:1-133.
34. Sadowski JA, Booth SL, Mann KG. Structure and mechanism of activation of vitamin K antagonists. In: Poller L, Hirsh J, editors. Oral Anticoagulants. 1st ed. London UK: Arnold; 1996.p.9–29.
35. Osman A, Enstrom C, Arbring K, Soderkvist P, Lindahl TL. Main haplotypes and mutational analysis of vitamin K epoxide reductase (VKORC1) in a Swedish population: a retrospective analysis of case records. J Thromb Haemost. 2006; 4: 1723-9.
36. Schalekamp T, Brassé BP, Roijers JF, Van Meegen E, Van der Meer FJ, Van Wijk EM et al. VKORC1 and CYP2C9 genotypes and phenprocoumon anticoagulation status: interaction between both genotypes affects dose requirement. Clin Pharmacol Ther. 2007 ; 81:185-93.
37. Bodin L, Perdu J, Diry M, Horellou MH, Loriot MA. Multiple genetic alterations in vitamin K epoxide reductase complex subunit 1 gene (VKORC1) can explain the high dose requirement during oral anticoagulation in humans. J Thromb Haemost. 2008; 6: 1436-9.
38. Li T, Chang CY, Jin DY, Lin PJ, Khvorova A, Stafford DW. Identification of the gene for vitamin K epoxide reductase. Nature. 2004; 427: 541-4.
39. Rost S, Fregin A, Ivaskevicius V, Conzelmann E, Hortnagel K, Pelz HJ et al. Mutations in VKORC1 cause Warfarin resistance and multiple coagulation factor deficiency type 2. Nature. 2004; 427: 537-41.
40. D'Andrea G, D'Ambrosio RL, Di Perna P, Chetta M, Santacroce R, Brancaccio V et al. A polymorphism in the VKORC1 gene is associated with

an interindividual variability in the dose-anticoagulant effect of warfarin. Blood.2005;105:645-9.

41. Osman A. Studies on warfarin treatment with emphasis on inter-individual variations and drug monitoring [Internet]. [Cited 2016 Oct 19]. Available from: http://liu.diva-portal.org/smash/get/diva2:23586/FULLTEXT01.pdf
42. Rieder MJ, Reiner AP, Gage BF, Nickerson DA, Eby CS, McLeod HL et al. Effect of VKORC1 haplotypes on transcriptional regulation and Warfarin dose. N Engl J Med. 2005; 352:2285-93.
43. Geisen C, Watzka M, Sittinger K, Steffens M, Daugela L, Seifried E et al. VKORC1 haplotypes and their impact on the interindividual and inter-ethnical variability of oral anticoagulation. Thromb Haemost. 2005; 94: 773-9.
44. Veenstra DL, You JH, Rieder MJ, Farin FM, Wilkerson HW, Blough DK et al. Association of Vitamin K epoxide reductase complex 1 (VKORC1) variants with Warfarin dose in a Hong Kong Chinese patient population. Pharmacogenet Genom. 2005;15: 687-91.
45. Takahashi H, Wilkinson GR, Nutescu EA, Morita T, Ritchie MD, Scordo MG et al. Different contributions of polymorphisms in VKORC1 and CYP2C9 to intra- and inter-population differences in maintenance dose of Warfarin in Japanese, Caucasians and African-Americans. Pharmacogenet Genom. 2006; 16: 101-10.
46. Scone EA, Khan TI, Wyne HA, Avery P, Monkhouse L, King BP et al.The impact of CYP2C9 and VKORC1 genetic polymorphism and patient characteristics upon Warfarin dose requirements: Proposal for new dosing regimen. Blood. 2005; 106: 2329-33.

47. Nahar R, Deb R, Saxena R, Puri RD, Verma IC. Variability in CYP2C9 allele frequency: A pilot study of its predicted impact on Warfarin response among healthy South and North Indians. Pharmacol Rep. 2013; 65: 187-94.
48. Wadelius M, Pirmohamed M. Pharmacogenetics of warfarin: current status and future challenges. Pharmacogenomics J**.** 2007;7:99-111.
49. Tian L, Zhang J, Xiao S, Huang J, Zhang Y, Shen J. Impact of polymorphisms of the GGCX gene on maintenance warfarin dose in Chinese populations: Systematic review and meta-analysis. Meta Gene. 2015;5:43-54.
50. Madan S, Kumar DK, Kumar DT, Balachander J, Adithan C.Effect of CYP2C9 and VKORC1 genetic polymorphisms on warfarin dose requirement in south Indian population. Indian J Physiol Pharmacol. 2013; 57: 308-17.
51. Natarajan S, Ponde CK, Rajani RM, Jijina F, Gursahani R, Dhairywan PP et al.Effect of CYP2C9 and VKORC1 genetic variations on warfarin dose requirements in Indian patients. Pharmacol Rep. 2013; 65:1375-82.
52. Gaikwad T, Ghosh K, Kulkarni B, Kulkarni V, Ross C, Shetty S. Influence of *CYP2C9* and *VKORC1* gene polymorphisms on warfarin dosage, over anticoagulation and other adverse outcomes in Indian population. Eur J Pharmacol. 2013; 710: 80-4.
53. Kaur A, Khan F, Agarwal SS, Kapoor A,Agarwal SK, Phadke SR. Cytochrome P450 (*CYP2C9*2,*3*) & vitamin-K epoxide reductase complex (*VKORC1* -1639G<A) gene polymorphisms & their effect on acenocoumarol dose in patients with mechanical heart valve replacement. Indian J Med Res. 2013;137: 203-9.
54. Rathore SS, Agarwal SK, Pande S, Mittal T, Mittal B. The impact of VKORC1 -1630 G > A polymorphism on the maintenance dose of oral

anticoagulants for thromboembolic prophylaxis in North India: A pilot study. Indian J Hum Genet. 2011;17:s 54-7.

55. Rathore SS, Agarwal SK, Pande S, Mittal T, Mittal B. Frequencies of *VKORC1, CYP2C9*2* and *CYP2C9*3* genetic variants in the Northern Indian Population Bio Sci Tren. 2010;4: 333-7.
56. Schelleman H, Chen Z, Kealey C. Warfarin Response and Vitamin K Epoxide Reductase Complex 1 in African Americans and Caucasians. Clin Pharmacol Ther. 2007;81:742-7.
57. Yuan HY, Chen JJ, Lee MT, Wung JC, Chen YF, Charng MJ et al. A novel functional VKORC1 promoter polymorphism is associated with inter-individual and inter-ethnic differences in warfarin sensitivity. Hum Mol Genet. 2005; 14:1745-51.
58. Lee SC, Ng SS, Oldenburg J, Chong PY, Rost S, Guo JY,et al. Interethnic variability. Clin Pharmacol Ther. 2006;79:197-205.
59. Bodin L, Verstuyft C, Tregouet DA, Robert A, Dubert L,Funck-Brentano C, et al. Cytochrome P450 2C9 (CYP2C9) and vitamin K epoxide reductase (VKORC1) genotypes as determinants of acenocoumarol sensitivity. Blood. 2005;106: 135-40.
60. Dang MT, Hambleton J, Kayser SR: The influence of ethnicity on warfarin dosage requirement. Ann Pharmacother. 2005; 39: 1008–12.
61. Pavani A, Naushad SM, Mishra RC, et al. Retrospective evidence for clinical validity of expanded genetic model in warfarin dose optimization in a South Indian population. Pharmacogenomics. 2012; 13: 869–78.

62. Custódio das Dôres SM, Booth SL, Martini LA,et al. Relationship between diet and anticoagulant response to warfarin: a factor analysis. Eur J Nutr. 2007; 46: 147–54.

63. Table of valid genomic biomarkers in the context of approved drug labels [Internet]. [Cited 2016 June 4]. Available from : http://www.fda.gov/ cder / genomics/ genomic_biomarkers_table.htm

Annexures

PROFORMA

Serial No.: Date:

OP No.:

1. Name: 2. Age: 3. Gender:

4. Occupation:

5. Educational Status:

6. Mother tongue:

7. Height: 8. Weight: 9. BMI

10. Address with phone no:

11. Personal History: smoking/alcohol/drug intake

12. Diet History:

13. Concomitant medications:

14. Indication for Warfarin therapy:

15. Duration of warfarin therapy:

16. History of any bleeding episodes:

17. Cost of medication (per month):

Investigations: INR:

PCR:

18. Warfarin dose and INR status at the time of recruitment:

19. Previous history of warfarin therapy and INR status (to confirm stabilized)

Warfarin dose (mg)	PT/INR	Number of visits required to attain stable PT/INR	Daily warfarin requirement

Master chart