

Genetic and Non-Genetic Factors Association with Warfarin Long Term Therapy Stability in Sudan

Azza A. M. H. Swar Aldahab* and Abdallah. O. Elkhawad

University of Medical Sciences and Technology, Department of Pharmacology, Faculty of Pharmacy, Khartoum State, Khartoum Sudan; azzaswaraldahab@yahoo.com

Abstract

Anticoagulation with warfarin is characterized by a wide inter-individual variations in dose requirements and INR (International Normalised Ratio) stability, as there are evidences that warfarin response variability is associated with CYP2C9 (cytochrome P450, family 2, subfamily C, polypeptide 9) and VKORC1 (Vitamin K epoxide reductase complex1) genetic polymorphisms. Carriers of CYP2C9*2 and VKORC11639G>A variant alleles are at greater risk of unstable anticoagulation therapy. **Objectives:** This retrospective case control study was directed to analyze the impact of genetic and non-genetic factors on warfarin therapy in Sudanese out-patients who were on long term warfarin therapy. **Method:** 118 Sudanese out-patients receiving warfarin treatment for at least six months, were interviewed for their non-genetic factors that included age, sex, indication for warfarin therapy, compliance, Vitamin K rich foods intake and concomitant drug therapy, in addition to their blood samples which were taken for DNA extraction and genotyping of CYP2C9*2 and VKORC11639G>A gene polymorphisms to study the genetic factors. INR stability % index was calculated, accordingly patients were classified into 2 groups, stable and unstable groups. **Results:** The frequencies of VKORC11639G>A alleles in Sudanese out-patients who were on long term warfarin therapy were 70.3% and 29.7% for the VKORC1/G and VKORC1/A alleles respectively. The frequencies of CYP2C9*2 alleles in Sudanese out-patients were 92.4% and 7.6% for CYP2C9*1 and CYP2C9*2 alleles respectively. Variables associated with low INR stability were VKORC1/AA genotype (p-value = 0.028) and sex (p = 0.017). Variables that showed no association with INR stability were age (p-value = 0.259), compliance (p-value = 0.058). Vitamin K rich foods intake (p-value = 0.743), and mean stable warfarin dose (p-value = 0.439). **Conclusion:** Polymorphism in warfarin drug target gene VKORC1-11639G>A and sex are important elements of INR stability in Sudanese out-patients on long term warfarin therapy.

Keywords: CYP2C9*2, INR Stability, VKORC11639G>A, Warfarin

1. Introduction

1.1 Warfarin

Warfarin is prescribed for patients who are at great jeopardy for creating a clot inside their blood vessels. Despite more than 50 years of experience using it and advances in the ability to monitor it, warfarin remains among the most problematic drugs to manage in clinical practice and a major cause of drug-induced morbidity

and mortality. However, because they can be orally administered, warfarin remain the most frequently used agent when long-term anticoagulation therapy is needed¹.

1.2 INR Stability

INR (International Normalized Ratio), is used to closely observe patients on warfarin treatment. The standard approximate range for the healthy persons is 0.8-1.2 and 2-3 for people on warfarin treatment². Outpatients always

* Author for correspondence

come to the anticoagulation clinics with a non-consistent pattern of INR values, which are either higher or lower than the INR range specified for them, because it is quite effortful and hard to keep INR values within the specified range³. Therefore, a new indicator was defined in order to study warfarin's anticoagulation long term therapy stability, which is the INR Stability %. *INR stability % = 100 x number of INRs within target range/total number of INR measurements*⁴. A number of factors affect warfarin therapy, these factors include genetic factors and non-genetic factors of poor compliance, drug interactions, or vitamin K rich foods intake¹.

2. Problems with Warfarin

2.1 Non-genetic Factors

The number of reports of interactions between warfarin and drugs and foods are increasing. Although true mechanisms of drug interactions almost always remain unknown, many drugs were known to potentiate warfarin effect, which are inhibitors of CYP2C9 enzyme, such as amiodarone and fluconazole, while other drugs are CYP2C9 enzyme inducers such as rifampicin. The most difficult group of drugs are those that potentiate bleeding on their own, the risk of bleeding is then greater if they are taken with warfarin. Both vitamin K enriched and reduced diets affect INR stability, the enriched vitamin K diets more rapidly than the reduced ones⁴. In⁵ considered interrelationship of vitamin K and responsiveness to warfarin in 50 patients, they found that the responsiveness to warfarin therapy was declined by high vitamin K enriched food intake. In⁶ studied patients on vitamin K supervised programme, they had achieved target INR more commonly than those on standard style of warfarin treatment, however, the clinical suitability of these findings for long term warfarin therapy requires more assessments.

2.2 Genetic Factors

2.2.1 CYP2C9*2

Genes are identical in every population, but very limited numbers of genes are not similar in some people. Alleles constitute a gene with small discrepancy in the DNA bases sequence.

Single Nucleotide Polymorphisms (SNPs) are the most frequent and known type of genetic variations. SNPs come out commonly during lifetime, they arise approximately

once every 300 nucleotides. When SNPs take place inside a gene, they lead to modifications in genes' functions⁷. The wildtype allele CYP2C9*1*1 is directly correlated to normal warfarin metabolism activity, CYP2C9*2 is one of the most known CYP2C9 gene polymorphism, this polymorphism leads to reduction in warfarin metabolism. Therefore, warfarin doses are minimized by about one third when this type of gene variation is present⁸.

2.2.2 VKORC1 1639G>A

VKORC1 gene codes the vitamin K epoxide reductase enzyme complex 1, warfarin obstructs vitamin K actions and hence stops clotting factors formations. VKORC1 1639G<A polymorphism is the most familiar gene polymorphism in populations of Asian and European origins⁹. Genotyping tests aim to improve the safety of warfarin dosing. Genetic variations of CYP2C9 and VKORC1 can be identified by genotyping tests to identify patients with high risk of bleeding complications, meaning that determination of warfarin optimal dosing is best to be based on dosing algorithms which rely on both clinical features such as age, sex and weight along with genotypes profile. However, genotyping is a tool that may aid in selecting the correct dose of warfarin, but careful monitoring of INR is still required for optimal dose adjustments^{10,11}.

2.3 Ethnicity

There has been much concern recently about the concept the genetic testing. The great ethnic distinction in Sudan is a result of the early human African migrations which are not yet fully examined on the genetic level. Researchers at the Institute of Evolutionary Biology (IBE), have studied human genetic diversity in Sudan, they observed the populations that speak languages of the same linguistic family have a common shared genetic components, that is close genetic relationship between members of the same language family, demonstrating that only linguistic evidence is relevant in drawing conclusions about Sudanese population classification¹².

3. Objectives

The objective of our study is to analyse the genetic factors in terms of the prevalence of variant alleles of CYP2C9*2 and VKORC1-1639G>A polymorphisms in Sudanese out-patients on long term warfarin therapy, to evaluate

their effects on the INR stability, in addition to the non-genetic factors contribution.

4. Materials and Methods

The methodology for the study of the genetic and non-genetic factors was performed through the following phases:

Phase 1

4.1 Study Design

In this retrospective case-control study, Ethical clearance for the study procedure was acquired from the Sudanese Clinical Trials and Drug Research on Human and Animal Committee-National Medicines and Poisons Board.

4.2 Patients

118 Sudanese out-patients attending the anti-coagulation clinic at the Sudan heart Centre–Khartoum–Sudan were selected. All Patients with a continuance anticoagulation treatment were recruited according to the inclusion criteria which included all patients being Sudanese with cardiovascular diseases who were on long term oral warfarin therapy and aged up to 75 years old. The exclusion criteria included patients who had a history of renal, hepatic, thyroid or malignancy diseases. To certify compliance with ongoing INR follow-up, patients needed to acquire one INR determination every four weeks for minimum of six months.

4.3 Data Collection

The anticoagulation profile and a full warfarin dosing data were recalled from the patients' hospital medical history documents which included INR values, dose of warfarin being administered, and the dose advised at each visit, and INR range. The tow variables which were to be calculated, the INR stability % and mean stable warfarin dose. The INR stability % was described as the percentage of the number of INR values within target range divided by the total number of INR measurements. *INR stability % = Number of INR measurements within target range/ total no. of INR measurements x100⁴*. The mean stable warfarin dose defined as the mean dose related to the stable INR measurements within target range. *Mean stable warfarin dose = Number of warfarin stable doses /total number of*

warfarin doses². Patients were then sorted out according to their INR stability % into two groups: Group1: Stable patients defined as patients who were having the highest percentages of INR stability %. i.e.,: An International Normalized Ratio (INR) greater than 2 but less than 3.5, 4.0 or 4.5 according to their INR target range. Group 2: Unstable patients defined as patients who were having the lowest percentages of INR stability %. (Table 1.1) Demographic data and non-genetic factors included sex, age, ethnicity, indication for warfarin therapy, vitamin K rich foods intake, concurrent medications (type and number) and patient compliance (dose and schedule) were collected using a questionnaire. Venous blood samples (4 ml) were collected and sent to the research laboratory and stored at -20 degrees preceding DNA extraction, to perform the genetic testing for both groups of patients to look at their genetic profiles and evaluate their relation to anticoagulation stability.

Table 1.1 Patients' classification according to INR stability %

Patients' Classification	INR
Stable group	Higher percentages of INRs greater than 2 and less than 3, 3.5 OR 4.
Unstable group	Higher percentages of INRs less than 2 and greater than 3, 3.5 OR 4.

Phase 2

4.4 DNA Extraction

Genomic DNA was extracted from blood leukocytes using the QIAamp DNA blood Mini Kit (Quiagen), designed for rapid purification of genomic DNA.

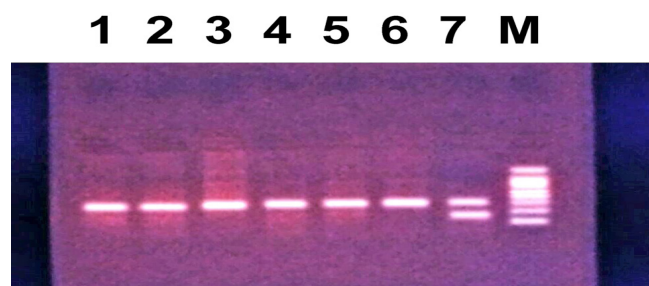
Phase 3

4.5 Genotyping of CYP2C9*2, and VKORC1-1639G>A

Determination of the CYP2C9*2 and VKORC1-1639G>A genotypes was performed by polymerase chain reaction–restriction fragment length polymorphism, (PCR-RFLP) technique. PCR for CYP2C9*2 and VKORC1-1639G>A was carried out using PCR technique of Maxime PCR PreMix Kit (i-Taq). Figures 1, 2. The primers sequences used for the protocols are given in Table 1.2 below.

Table 1.2 Primers used for CYP2C9*2, and VKORC1 (-1639G>A) PCR protocol for DNA amplification

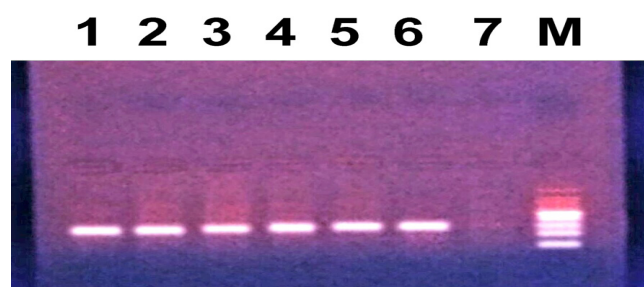
Gene	Primer Sequence
CYP2C9	Forward: 5-TGCCTGTTTCAGCATCTGTC-3
	Reverse: 5-CCCCTGAAATGTTTCCAAGA-3
VKORC1	Forward: 5-GCCAGCAGGAGAGGGAAATA-3
	Reverse: 5-AGTTTGGACCTACAGGTGCCT-3

**Figure 1.** PCR product of CYP2C9*2.

Lane M: DNA Marker.

Lanes 1, 2, 3, 4, 5, 6: PCR product of CYP2C9*2 (382bp).

Lane 7: Heterozygous genotype of CYP2C9*2, three bands of fragments length of 382bp, 258bp and 129bp.

**Figure 2.** PCR product of VKORC1-1639G<A.

Lane M: DNA Marker. Lanes 1, 2, 3, 4, 5, 6: PCR product of VKORC1-1639/GA (290 bp).

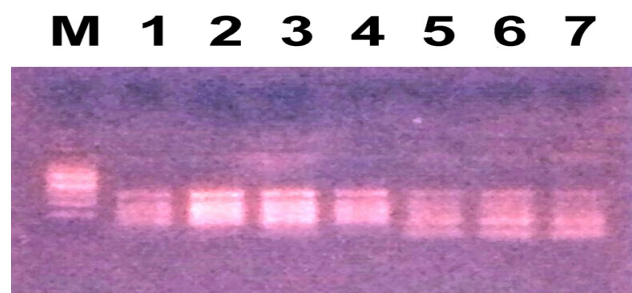
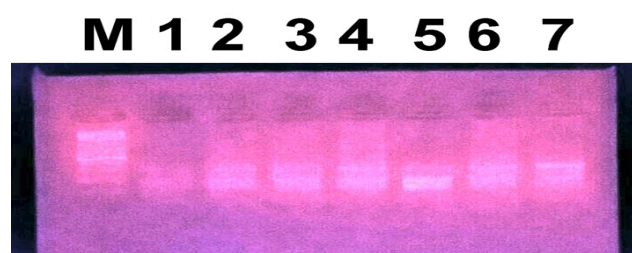
Phase 4

4.6 Restriction Fragment Length Polymorphism: RFLP

Phase 4.1

4.6.1 Restriction Enzyme *MspI*

The *MspI* restriction enzyme was used to treat the 290 bp amplicon of VKORC1-1639G>A gene PCR product. First we prepared the restriction enzyme by dilution of the buffer provided with restriction enzyme by adding 1 ul of the buffer to 10 ul of distilled water, then we added 2,5 ul of the diluted buffer to 10 ul of PCR product, and 0.5 ul of restriction enzyme and 12 ul of distilled water making a final reaction volume of 25 ul. The PCR product was treated with the restriction enzyme *MspI* by adding the volumes above and left for incubation for 15 minutes at 37 degrees, the 290-bp amplicon was cut into 2 restriction fragments of 167 and 121 bp, but in the case of the existence of the A allele, this produced an amplicon that was insusceptible to be cut by *MspI* restriction enzyme, Figure 3(a), 3(b).

**Figure 3a.** VKORC1-1639G<A Polymorphism Representative Gel Electrophoresis:**Figure 3b.** VKORC1-1639G>A polymorphism representative gel

Lane M: DNA Sizer. Lane 2, 3, 4: Heterozygous genotype of VKORC1, three bands of fragments length of

290bp, 169bp and 121 bp. Lane 5, 6, 7: Wild type VKORC1 genotype, one band with a fragment length of 290 bp.

Lane M DNA sizer. Lane 5: Wild type genotype of VKORC1-1639/GA, two fragments length of 121 bp and 169 bp.

4.6.2 Restriction enzyme *Ava*II

To study the CYP2C9*2 allele, the PCR product of CYP2C9*2 was treated with the *Ava*II restriction enzyme. First we prepared the *Ava*II restriction enzyme by dilution of the buffer provided with the restriction enzyme by adding 1 ul of the buffer to 10ul of distilled water, then 2.5 ul of the diluted buffer was added to 10 ul of PCR product, and 0.5 ul of restriction enzyme with 12 ul of distilled water making a final reaction volume of 25 ul. The PCR product was treated with the restriction enzyme *Ava*II by adding the volumes above and left for 15 minutes followed by incubation at 80 degrees for 20 minutes for inactivation of the enzyme, giving rise to two bands with lengths of 253 and 129 bp for the wild-type allele. While the CYP2C9*2 allele was bearing no restriction by the *Ava*II restriction enzyme, producing one band with a length of 382 bp.

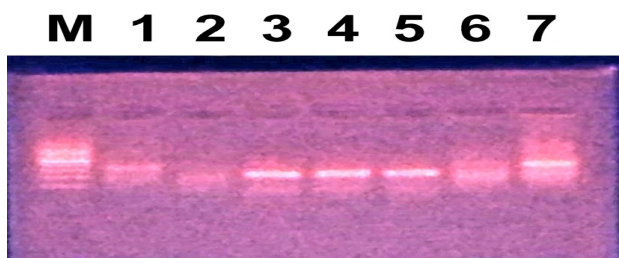


Figure 4. CYP2C9*2 Polymorphism Representative Gel

Lane M: DNA marker.

Lane 1: Homozygous mutant CYP2C9*2 genotype, one band of fragment length of 382bp.

Lanes 3, 4, 5: Wild type genotype of CYP2C9*2, two bands of 258 bp and 129bp.

5. Results

Table 2. Demographic and Basic Characteristics of Sudanese out-patients on long term warfarin therapy involved in the study: N = 118.

Age	48.0 +-16.4
No. of INR measurement	15.6+-7.0
INR stability %	33.9+-20.1
Mean stable warfarin dose	5.1+-2.4
Sex	77 Female (65.3%) 41Male (34.7%)
Indications	
DVR	(17) 14.5%
AF	(35) 29.9%
MVR	(30) 25.6%
AVR	(22) 18.8%
CHD	(1) 0.85%
PE	(3) 2.6%
CVR	(1) 0.85%
DCM	(1) 0.85%
Stroke	(1) 0.85%
DVT	(1) 0.85%
Sinus thrombosis	1 (0.85%)
MI	2(1, 71%)
CVA	2 (1, 71%)
Low vit.K rich foods intake	41(34.75%)
Moderate vit.Krich foods intake	77(65.3%)
Correct compliance	104(89.7%)
Incorrect compliance	12(10.3%)
Target INR	
2.0-3.0	51 (43.2%)
2.5-3.5	32 (27.1%)
3.0-4.0	29 (24.68%)
3.5-4.5	6 (5.0%)
Patient classification	
Stable patients	60 (50.9%)
Unstable patients	58 (49.1%)

Data are represented as mean \pm Std. OR number (percent).

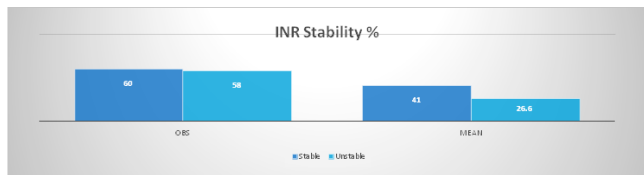


Figure 5. INR stability% versus patient’s classification of stable and unstable groups of Sudanese out-patients on long term warfarin therapy. Obs.: No. of observations, N=118, P-value =0.0001.

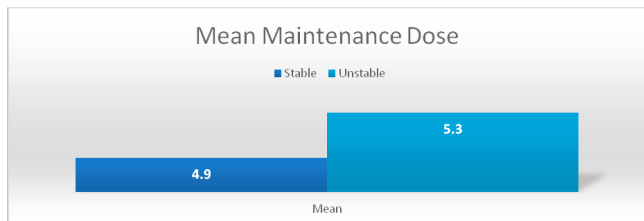


Figure 6. Mean Stable warfarin dose. P-value = 0.439, values are represented as mean.

Mean stable warfarin dose versus patients’ classification of stable and unstable groups of Sudanese out-patients on long term warfarin therapy N = 118.

Comparison of stable and unstable groups of Sudanese out-patients on long term warfarin therapy for Qualitative variables.

Compliance represented as frequencies of correct compliance and incorrect compliance as a function of patients’ classification of stable and unstable groups of Sudanese out- patients on long term warfarin therapy.

Genetic tables:

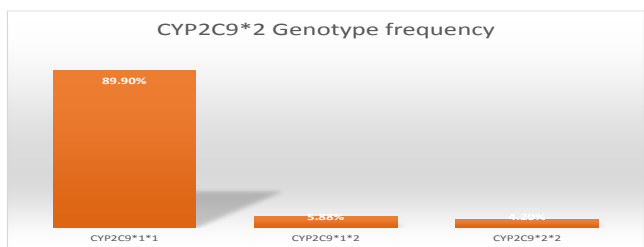


Figure 7. CYP2C9*2 genotypes frequencies among Sudanese out-patients on long term warfarin therapy: N=118. Data are represented as percent (%).

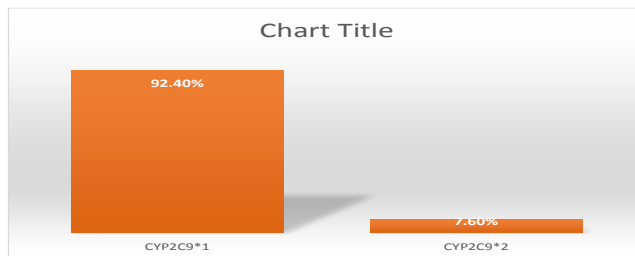


Figure 8. Alleles frequencies of CYP2C9*2 in Sudanese out-patients on long term warfarin therapy: N=118. CYP2C9*1: Wild type allele. CYP2C9*2: Variant allele.

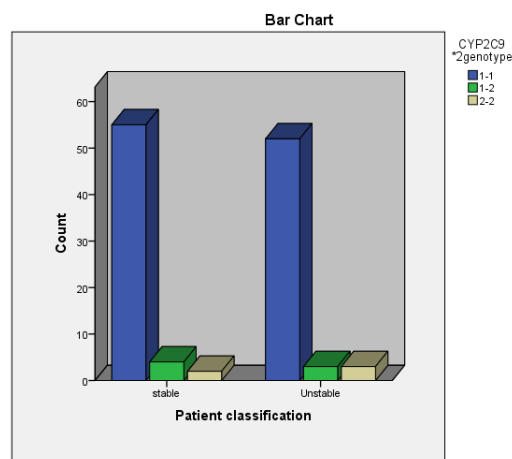


Figure 9. Genotypes frequencies of CYP2C9*2 versus patients’ classification of stable and unstable groups of Sudanese out-patients on long term warfarin therapy. N = 118, P-value = 0.713.Odds ratio = 1.5

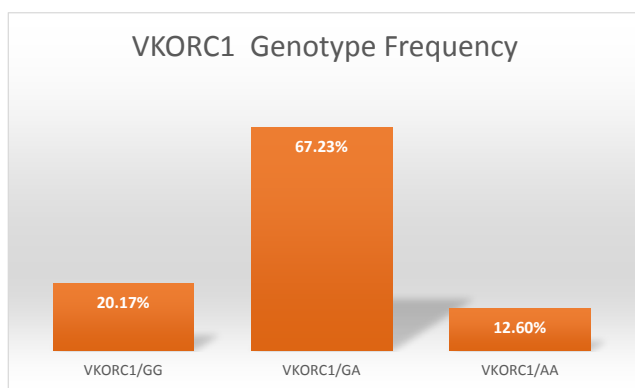


Figure 10. VKORC11639G<A genotypes frequencies among Sudanese out- patients on long term warfarin therapy: N=118. Data are represented as percent (%).

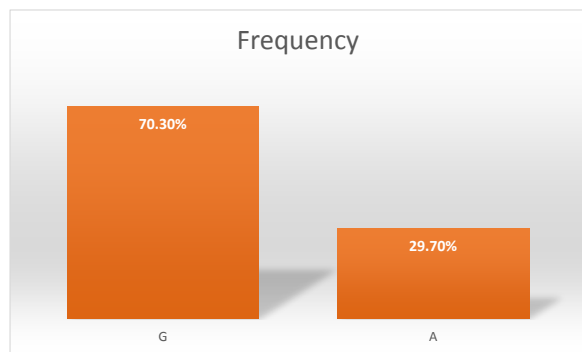


Figure 11. Alleles frequencies of VKORC11639G>A among Sudanese out-patients on long term warfarin therapy warfarin therapy: N=118. Data are represented as percent%, G: Wild type allele , A: Variant allele .

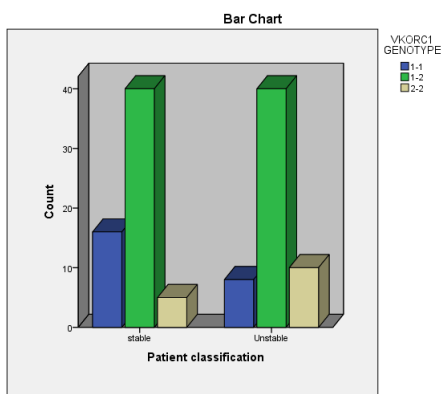


Figure 12. Genotypes frequencies of VKORC1-1639G>A versus patients' classification of stable and unstable groups of Sudanese out-patients on long term warfarin therapy. N = 118, P-value = 0.024, Odds ratio = 4.9 (1-1) VKORC1 wild type, (1-2) VKORC1 heterozygous, (2-2) VKORC1 homozygous genotype.

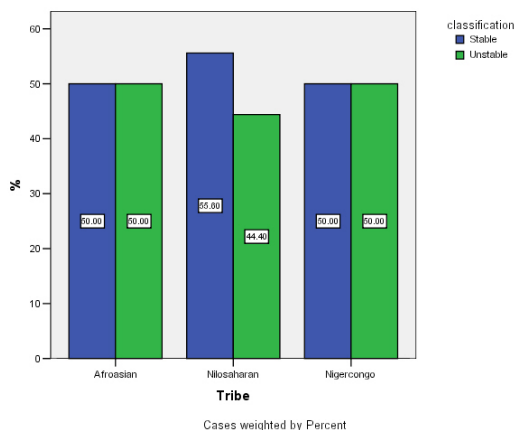


Figure 13. Ethnicity versus patients' classification of stable and unstable groups of Sudanese out-patients on long term warfarin therapy. N=118, P-value = 0.910.

Table 3. Carriers' prevalence of CYP2C9*2 and VKORC1-1639G>A combinatorial profiles in Sudanese population involved in the study=118

VKORC1	CYP2C9*1*1	CYP2C9*1*2	CYP2V9*2*2	Total
GG	22 (20.9%)	2 (25%)	0 (0.0%)	24 (20.2%)
GA	72 (68.5%)	3 (37.5%)	5 (100%)	80 (67.2%)
AA	11 (10.5%)	3 (37.5%)	0 (0.0%)	14 (11.8%)
Total	105 (100%)	8 (100%)	5 (100%)	118 (100%)

Data are represented as number (percent %) within CYP2C9*2 genotype classification. P-value = 0.292. 22(20.9%) patients were wild-type homozygous CYP2C9*1*1 and VKORC1/GG. 0(0%) patients were homozygous mutant in both CYP2C9 and VKORC11639G<A genes simultaneously VKORC1/GG: Wild type Genotype, VKORC1/GA: Heterozygous genotype, VKORC1AA: Homozygous mutant. CYP2C9*1*1: Wild type homozygous genotype, CYP2C9*1*2: Heterozygous genotype, CYP2C9*2*2: Homozygous mutant genotype.

6. Discussion

VKORC1-1639G>A and CYP2C9*2 polymorphisms explain the variability of mean warfarin dose and hence stability among anticoagulation patients¹³. This was illustrated by the recent randomized clinical trials that was studying those two genes' based warfarin dosing, the trials failed to prove a successful gene based dosing strategy which had actually performed worse than clinical dosing strategy in the 27% of patients who were of African ancestry. It is clear that warfarin dosing incorporates with the CYP2C9*2 and VKORC11639G>A single nucleotide polymorphisms, but do not adequately explain the variability in warfarin dosing in African populations¹⁴

Studies on haplotypes of VKORC1 gene showed that the highest haplotype diversity was observed in Sub-Saharan Africans. The -1639 A allele (rs9923231) carried by haplotype H1, is the most frequent VKORC1 haplotype worldwide (49.7%)¹⁵.

Data on prevalence of VKORC11639G>A SNP among Sudanese populations are incomplete, but the available information concluded to that this SNP is the most frequent SNP among Sudanese, Kenyans and Egyptians

but not in West Africans. CYP2C9 is usually not seen in Africans or have low frequency in Africans. Also Sudanese population appear to have less CYP2C9*2 prevalence than other populations. Neither stability nor warfarin dose were associated with CYP2C9*2 variant allele¹⁶.

6.1 VKORC1-1639G>A

VKORC11636G>A polymorphism is associated with warfarin dose requirements among populations from African descent, of whom warfarin resistance is indicative although VKORC1 polymorphism showed highest diversity in allele frequency among different populations¹⁷.

Upon defining the population prevalence of VKORC1639G>A single nucleotide polymorphism, the genotype and allele frequencies are mentioned in Figures 9.10. The allele frequency for VKORC1 1639>G was reported to be highest in African American subjects (89%)¹⁸. This complies with our finding of the 70.3% VKORC11639>G allele frequency found in our Sudanese population.

On the other hand, the Minor Allele Frequency (MAF) of VKORC1 1639G>A allele was found to be 29.7%, shown as the A allele frequency¹⁹. The heterozygous VKORC1/GA genotype frequency has been found to be 67.8%, which is a surprisingly high frequency among Sudanese population studied. This result is close to the results found by a study done on the southern Eastern European Romanians, in which it was found that 51.2% were carriers of VKORC1-1639/GA heterozygous genotype profile²⁰.

Controversially, in a meta-analysis done by²⁰, it was found that the frequency of wild type VKORC1-1639/GG genotype was 58% in African populations. This VKORC1-1639G>A single nucleotide polymorphism had a significant influence in anticoagulation stability, as it has been seen. In this study, by comparing the VKORC1-1639/AA homozygous mutant genotype carriers with the wild type VKORC1-1639/GG carriers versus stability, this gave an odds ratio of almost 5, meaning that individuals with mutant VKORC1-1639/AA genotype were 5 times more likely to be unstable unlike those of wild type genotype. This result was statistically significant, P value = 0.024. Figure 11.

African ancestries are mainly noticed for their high warfarin dose requirements in contrast to Europeans and Asians²¹.

Several studies describe patients with warfarin resistance as mutation carriers in one copy of a gene that

encodes VKORC1 enzyme, which results in an enzyme with a reduced ability to bind to warfarin, this reduction causes an incomplete warfarin resistance resulting in a higher dose of warfarin to be needed²².

This is reflected by the mean warfarin dose among the unstable group of Sudanese populations studied, which comprised the group of the higher VKORC11639AA genotype, the dose which was 5.3 ± 2.9 mg/day (37.1 mg/week, range 57.4-16.8 mg/week), corresponds to the presence of the variant allele which was more frequent among the unstable group. This dose could be described as a high dose, since it complies with a study done on Egyptians who carry VKORC1 mutations showed high warfarin dose requirement of 57.1 ± 29.4 mg/week versus those with normal VKORC1 genotype which was 35.8 ± 16.6 milligram per week²³.

Also the generally high adjusted mean warfarin weekly dose range of 39 to 47 mg/week has been seen in Africans. The large scale population foundation of Africans compared to other races accounts for the complications in analysing individuals of an African origin²¹.

The results are clearly showing the high frequency of the heterozygous VKORC1- 1639GA genotype among all patients, constituting of the Afro-Asian racial group, with a frequency of 71%, in addition to 61% of the Nilo-Saharan, and 50% of the Niger-Congos were carriers of VKORC1/GA heterozygous, but surprisingly without any VKORC1/AA homozygous mutant genotype for Niger-Congos. As the instability by VKORC1-1639G>A polymorphism is distinct by ancestral groups²⁴. The allelic distribution of VKORC11639G>A showed a great degree of high prevalence across Sudanese patients different ethnical groups, being similar to the Indian populations different ethnicities, with VKORC1-1639G>A allele frequencies which were found to be as low as 6.5% and up to 70%²⁵. But, our results are quite different from the Chinese population whose genetic data had accounted for 90% of homozygous mutant VKORC1/AA genotype and only 10% of VKORC1/GA heterozygous genotype profile²⁶.

6.2 CYP2C9*2

Allele frequency for CYP2C9*2 was 7.6% among Sudanese population for the Minor Allele Frequency (MAF) as it has been found in this study. Figure 7. This result is unpredictably similar to those of Omani population, where minor allele frequency was markedly lower and was estimated at 7.4%²⁷.

The genotype frequencies are shown in Figure 6, showing only 4.2% are carriers of CYP2C9 *2*2 homozygous mutant genotype. The differences in genotype frequencies of CYP2C9 were not statistically different between stable and unstable groups, P-value = 0.713, and when comparing the CYP2C9*2*2 homozygous mutant genotype group with the CYP2C9*1*1 wild type genotype between stable and unstable groups, this comparison gave an odds ratio of 1.5, this shows that individuals with CYP2C9*2*2 mutant genotype are 1.5 times more likely to be unstable when compared to CYP2C9*1*1 wild type genotype profile, this was not statistically significant, P-value = 0.713, as it can be seen in Figure 8, since only 5 individuals have this genotype. The later result complies with a study done on Africans which did not detect an anticoagulation stability association with CYP2C9*2 variant, due to the low allele frequency as 92% were carriers of CYP2C9*1*1 wild type genotype²⁸. The frequency of CYP2C9 polymorphism regarding ethnicity was unique in the genotype distribution among different Sudanese racial groups, having the highest frequency of 86.2% of CYP2C9*1*1 wild type allele among Afro-Asians, showing the Nilo-Saharan and Niger-Congos having only this wildtype CYP2C9 *1*1 genotype profile with neither CYP2C9*1*2 heterozygous genotype nor CYP2C9*2*2 homozygous mutant genotype profiles.

From these results, given the smaller range of variant allele frequency for CYP2C9 to that of VKORC1, it is apparent that the association in warfarin therapy stability is larger for VKORC1 than for CYP2C9^{29,30}. By taking a general look at the ethnicity versus frequencies of both genes, we found that 22% of all Sudanese patients were without any of the variant alleles of both CYP2C9*2 and VKORC1-1639G>A, that means having neither heterozygous nor homozygous mutant genotypes of the two genes studied. Also 0% of all patients had mutations in both genes simultaneously. Table 3. But, the highest frequency was found for the VKORC11639/GA heterozygous genotype and the wild type CYP2C9*1*1 genotype being 67.8% and 88.9% of all the patients respectively. This was explained in many studies that showed pharmacogenetics dosing incorporation of the two genes explained 40% of the instability in warfarin therapy among white ethnicities, but only 20% among Africans descents, this might be due to the reduced frequencies of these alleles as mentioned earlier¹⁴.

6.3 Non-Genetic Factors

It appears there are no differences in warfarin anticoagulation stability in young versus elderly patients, in spite of age being a critical non-genetic factor for warfarin dose requirements³¹. In our study, the mean age among the stable patients was higher than in unstable patients (49.7+/-16.1, 46.3+/-16.8), however, age showed no significant difference statistically between stable and unstable groups, P-value = 0.259. The absence of significant association between age and warfarin anticoagulation stability indicates that age did not prevent patients, especially elderly ones, from maintaining their INRs within the recommended anticoagulation target INR ranges and gaining stability.

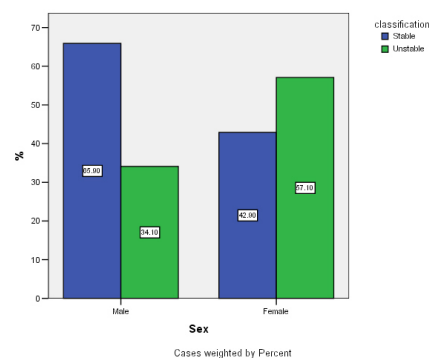


Figure 14. Frequency histograms of sex stratified according to patients' classification of stable and unstable groups of Sudanese out-patients on long term warfarin therapy N=118.

P-value = 0.017, Blue panel = stable group, Green panel = unstable group.

Our results are confirmed by previous studies which showed warfarin's INR stability is independently linked to age³². While other studies found a significant association between stability and age³³. There are differences related to anticoagulants efficacy, especially among women, as it was found that female gender significantly spend more time outside the INR therapeutic range than men, as shown in a recently published study, 29% of women were below the INR therapeutic range versus 26% of men, by this putting them at a more risk of ischemic stroke³⁴. In our study, INR values were of therapeutic stability in 42.9% of females among stable group versus 65% of males. Anticoagulation intensity was of a sub-therapeutic stability more among

females than males, (57.1% vs 34.2%) in unstable group respectively. The observed high instability among females, a result which was statistically significant, P-value = 0.017, Figure 3, can be documented by previous studies that showed anticoagulation variability was higher in females than in males³⁵.

Our results demonstrate a higher frequency of stable patients who were on moderate rather than low vitamin K rich foods intake, 51.9% of stable patients were on moderate vitamin K rich foods intake versus 48.8% of stable patients were on low vitamin K rich foods intake, showing that stable INR could be achieved by taking moderate amounts of vitamin K rich foods. On the other hand, we found in this study, 51.2% versus 49.2% of unstable patients were on low and moderate vitamin K rich foods intake respectively. These results did not show a statistical difference between stable and unstable groups, P-value = 0.743, Figure 4. These results are convincing although the study was restricted by some factors which had faced all previous studies including the lack of an exact cut-off points of comprehensive enlightenment of the real content of vitamin K nutrient among Sudanese foods, which might be challenging to anticoagulation patients, taking into account, the study is a qualitative analysis of vitamin K rich foods, as utilization of vitamin K containing foods was considered low or moderate depending on the number of meals per week, without a real quantitative evaluation in ug/day.

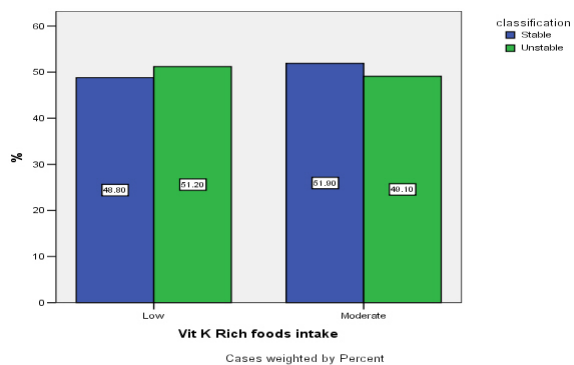


Figure 15. Vitamin K rich foods intake. P-value = 0.743, Blue panel = stable group, Green panel = unstable group

In this study, we found that patients with correct compliance were more frequent among stable group (75% versus 46.1%), while incorrect compliance frequency was higher among unstable group (53% versus 25%). This result indicates that stable patients were more compliant

than unstable patients who were not compliant to warfarin therapy, but this result is without a statistically significant difference, P-value = 0.058, Figure 5. These findings are on line with a study that had found poor compliance with warfarin is a common risk factor for warfarin instability leading to serious complications^{36,37}.

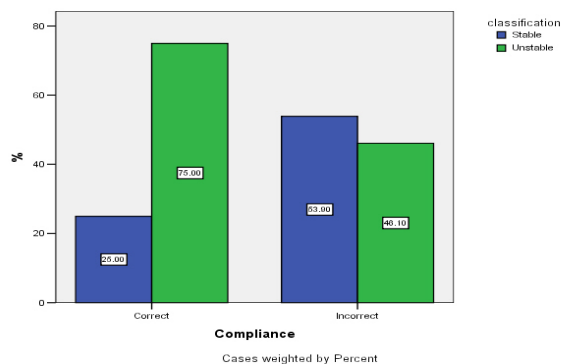


Figure 16. Compliance. N=118, P-value =0.058.

One of the reasons of the variation of warfarin dose and anticoagulation stability is the concomitant medications that could lead to drug-drug interactions. Therefore, each concurrent medication to be used within warfarin treatment should be checked for drug-drug interactions reference. Even stable patients on concomitant medications should be tested at least once a month³⁸. In this study, none of the observed concomitant medications had an association with warfarin stability.

The statistically significant mean change in INR stability regarding stable and unstable groups, P-value = .0001, Figure 1, could be considered clinically due to the differences of genetic and non-genetic factors which were ruled out above. Although no statistical significant association was detected between most of those factors and stability, nor between stability and racial groups. As INR stability remains the reflection of the degree of anticoagulation status. Therefore, in Sudan it will remain an obligation to decide the most fitting mode of treatment for each patient individually, assuring that the most important aspect contributing to INR stability will remain the frequent monitoring, that is, at least once a month, if not once a week, to obtain a safe warfarin administration .

7. Conclusion

The high VKORC1-1639/GA heterogeneous genotype and VKORC1-1639/A allele frequencies noticed among

Sudanese patients, have a higher impact than CYP2C9*2 in the stability of warfarin therapy. In conclusion, our final report suggests that among Sudanese patients, sex and VKORC1-1639/GA polymorphism had significant effects on INR stability. This study is one of the few studies which specifically looked into the genetic profiles of Sudanese population concerning CYP2C9*2 and VKORC1-1639G>A genes polymorphisms. However, further studies on these polymorphisms are essential for further confirmation. (29)

8. References

- Heneghan C, Alonso-Coello P, Garcia-Alamino JM, Perera R, Meats E, Glasziou P. Self-monitoring of oral anticoagulation: A systematic review and meta-analysis. *Lancet*. 2006 Feb; 367(9508):404–11. DOI: 10.1016/S0140-6736(11)61294-4.
- Li S, Zou Y, Wang X. Warfarin dosage response related pharmacogenetics in Chinese population. *PLoS One*. 2015 Jan 16; 10(1). DOI: 10.1371/journal.pone.0116463
- Witt DM, Delate T, Clerk NP, Martell C. On behalf of the Warfarin Association Research Project and other Endeavours (WARPED) Consortium. *Blood*. Jul 2009; 114:952-956.
- Kim JH, Song YB, Shun DH. How well does the target INR level maintain in warfarin-treated patients with non-valvular atrial fibrillation? *Yonsi Med J*. 2009 Feb 28; 50(1):83–8. DOI: 10.3349/ymj.2009.50.1.83.
- Lubetsky A, Sreer ED, Chetrit A, et al. Vitamin K intake and sensitivity to warfarin in patients consuming regular diets. *Thromb Haemost*. 1999; 81:396–9.
- De Assis MC, Rabelo ER, Avila CW, et al. Vitamin K guided strategy: A randomized controlled trial. *Circulation*. 2009; 120:1115–22. DOI: 10.1161/CIRCULATIONAHA.109.849208.
- Johnson JA, Gong L, Wirl-Carrillo M, Gage BF, et al. Clinical pharmacogenetics implementations Consortium Guidelines for CYP2C9 and VKORC1 genotypes and warfarin dosing. *Clinical Pharmacology and Therapeutics*. 2011; 90(4):625–9. DOI: 10.1038/clpt.2011.185.
- Scone E, Avery P, Wynne H. Vitamin K supplementation can improve stability of anticoagulation for patients with unexpected variability in response to warfarin. *Blood*. 2007 Mar; 109 (6):2419–23.
- Perera MA, Cavallari LH, Limdi NA, Gamazon ER, Konkashbaev A, Daneshjou R, Pluzhnikov A, Crawford DC, Wang J, Liu N, Tatonetti N, Bourgeois S, Takahashi H, Bradford Y, Burkley BM, Desnick RJ, Halperin JL, Khalifa SI, Langaee TY, Lubitz SA, Nutescu EA, Oetjens M, Shahin MH, Patel SR, Sagreya H, Tector M, Weck KE, Rieder MJ, Scott SA, Wu AH, Burmester JK, Wadelius M, Deloukas P, Wagner MJ, Mushiroda T, Kubo M, Roden DM, Cox NJ, Altman RB, Klein TE, Nakamura Y, Johnson JA. Genetic variants associated with warfarin dose in African-American individuals: A genome-wide association study. *Lancet*. 2013 Aug 31; 382(9894):790–6. DOI: 10.1016/S0140-6736(13)60681-9.
- Parra J, Booton MR, Perini JA. Genome wide association study of warfarin maintenance dose in Brazilians sample. *Pharmacogenomics*. 2015 Aug; 16(11):1253–63. DOI: 10.2217/PGS.15.73.
- Syn NL, Lee SC, Brunham LR, Goh BC. Pharmacogenetics versus clinical dosing of warfarin in individuals of Chinese and African-Americans ancestry. *Pharmacogenet Genomics*. 2015; 25(10):491–500. DOI: 10.1097/FPC.0000000000000165.
- Dobon B, Hassan HY, Laayouni H, Luisi P, Ricano-Ponce I. The genetics of East African populations: A nilo-saharan component in the African genetic landscape. *Scientific Reports*; 2015. DOI: 10.1038/srep09996.
- Jonson JA. Clinical pharmacogenetics implementation consortium guidelines for CYP2C9 and VKORC1 genotypes and warfarin dosing. *Clin Pharmacol Ther*. 2011 Sep. DOI: 10.1038/clpt.2011.
- Wadelius M. Warfarin pharmacogenetics: It matters if you are black or white. *Blood*. 2014 Oct; 124 (14):2171.
- Patillon B, Luis P. Positive selection in the chromosome 16 VKORC1 genomic regions has contributed to the variability of anticoagulant response in humans. *PlosOne*. 2012; 7(12). DOI: 10.1371/journal.pone.0053049.
- Wadellus M. Warfarin Pharmacogenetics: It matters if you're black or white. *Blood*. 2014 Oct 2; 124(14):2171.
- Sominsky S, Korostishevsky M. The VKORC1 variant and haplotypes diversity in Ashkenazi and Ethiopian populations. *Appl Genet*. 2014 May; 55(2):163–71. DOI: 10.1160/TH12-10-0789.
- Gan GG, Ming TA, Lee M. Allele and genotype frequencies of VKORC1-1639G>A polymorphism in three different ethnic groups in Malaysia. *As Pac J Mol Bio Biotechnol*. 2012; 20(1):19–23.
- Mohammed HSH, Larisa H. VKORC1 geographic distribution and its impact on warfarin dose requirements in Egyptians. *Thrombosis and Homeostasis*. 2013 Jun; 109(6):1045–50. DOI: 10.1160/TH12-10-0789.
- Yang L, Ge W, Yu F. Impact of vitamin KORC1 gene polymorphism on inter-individual and interethnic warfarin dosage requirement. A systemic review and meta-analysis. *Thrombosis and Haemostasis*. 2010; 125(4):159–66. DOI: 10.1016/j.thromres.2009.10.017.
- Fung E, Nicholas A, Stern M. Effect of genetic variants specially CYP2C9 and VKORC1 on the pharmacology of warfarin. *Thromb Hemost*. 2012 Nov; 38(8):893–904. DOI: 10.1055/s-0032-1328891.
- Warfarin resistance. *Genetics Home Reference*. US National Library of Medicine; 2016 Jun.
- Hossam AM, Shahin, Cavallari LH. Vkorc1 geographical distribution and its impact on warfarin dose requirements in Egyptians. *Thrombosis and Haemostasis*. 2016 Mar; 109(6):1045-50. DOI: 10.1160/TH12-10-0789.

24. Rieder MJ, Reiner AP, Gage BF. Effect of VORC1 haplotypes on transcriptional regulation and warfarin dose. *N Engl J Med.* 2005; 352:2285–93.
25. Gil AK, Khan NM, Grover S. Genetic epidemiology of pharmacogenetics variation in CYP2C9, CYP4F2 and VKORC- 1genes with warfarin dosage in Indian population. *Pharmacogenomics.* 2014; 15(10):1337–54. DOI: 10.2217/pgs.14.88.
26. Yu Z, Ding YL, Lu. Warfarin dosage adjustment strategy in Chinese population. *Int J Clin Exp Med.* 2015 Jun15; 8(6):9904–10.
27. Tanira MO, AL-Mukhaini MK, Alhinai AT. Frequency of CYP2C9 among Omani patients receiving warfarin and its correlation with warfarin dose. *Community Genetics.* 2007; 10(1):32–7.
28. Kealey C, Chen Z, Christy J, et al. Warfarin and CYP2C9 genotype; possible ethnic variation in warfarin sensitivity. *Pharmacogenomics.* 2007; 8(3):217–25.
29. Shrif NE, Won H-H, Lee ST, Park JH, Kim KK, Kim MJ, Kim S, Lee SY, Ki CS, Osman IM, Rhman EA, Ali IA, Idris MN, Kim JW. Evaluation of the effects of VKORC1 and CYP2C9 genotype and clinical factors on warfarin response in Sudanese patients. *Eur Clin Pharmacol.* 2011; 67(11):1119–30. DOI: 10.1007/s00228-011-1060-1.
30. Nita A, Limidi MA, Wadellias, Cavallari L. Warfarin pharmacogenetics: A single VKORC1 polymorphism is predictive of dose across 3 racial groups. *Blood.* 2010; 115(18):3827–34. DOI: 10.1182/blood-2009-12-255992.
31. Garcia DD, Regan S, Growther M, et al. Warfarin maintenance dosing patterns in clinical practice implications for safer anticoagulation in elderly population. *Chest.* 2005; 127:2049–56.
32. Zuinali P, Souza GC, de Assis MCS. Dietary vitamin K intake and stability of anticoagulation with coumarins: Evidence derived from a clinical trial. *Neutr Hosp.* 2012; 27(60):1987–92. DOI: 10.3305/nh.2012.27.6.6068.
33. Wieloch M, Alander ASJ, Fryknan V. Anticoagulation control in Sweden: Reports of time in therapeutic range, major bleeding and thromboembolic complications from national quality registry Auricul A Eur Heart J. 2011; 32(18):2282–9. DOI: 10.1093/eurheartj/ehr134.
34. Tomiha H, Kadokami T, Momii H, Kawamura M. Patient factors against stable control of warfarin therapy for Japanese non-valvular atrial fibrillation patients. *Thromb Res.* 2013 Nov; 132(5):537–42. DOI: 10.1016/j.thromres.2013.09.003.
35. Maria H. Age and gender differences during long term warfarin therapy monitored with Fiix-prothrombin time in patients with AF. Skemman . 2015 May. Available from: <http://hdl.handle.net/1949/21520>
36. Seliverstov I. Practical management approaches to anticoagulation non-compliance, health literacy and limited English proficiency in outpatient clinic setting. *J Thromb Thrombolysis.* 2011Apr; 31(3):321–5. DOI: 10.1007/s11239-011-0560-2.
37. Platt A, Alec B. Risk factors for non-adherence to warfarin: Results from the IN-RANGE study. *Pharmacoepidemiology and Drug Safety.* 2008; 17(9):853–60. DOI: 10.1002/pds.1556.
38. Befikadu L, Nuredin S. Drug-drug interactions and risk of bleeding among patients on warfarin therapy: A prospective observational study. *Thrombosis Journal.* 2014; 12:20. DOI: 10.1186/1477-9560-12-20.