A Study from South India on the Genetic and Non Genetic variables that influence individual's various Warfarin Dose requirements.

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Abstract:

Aim : The purpose of this study was to examine the effects of genetic and non-genetic variables on the variability of stable warfarin dosages in South Indian patients.

Method: The study included 96 participants who had consistent warfarin dosages. A few instances of the clinical and demographic data that was captured were age, BMI, and warfarin indications. Single nucleotide polymorphisms in the CYP2C9*2 and CYP2C9*3 genes were discovered in gDNA utilizing the Biorad MJ Mini TM thermocycler.

Results:

The daily doses of warfarin needed by individuals with variant CYP2C9*2,*3 genotypes were considerably lower than those of patients with wild-type genotypes. The genotype frequencies for CYP2C9*2 and CYP2C9*3 were 64.6%, 19.8%, and 15.6% for the CC, CT, and TT genotypes, and 39.6%, 39.6%, and 20.8% for the AA, AC, and CC genotypes, respectively. The daily recommended dose of warfarin in patients with homozygous wild-type genotype for CYP2C9 (*1/*1) was (4.07± 1.75 mg), which was noticeably better than

the daily recommended doses in patients with *1/*2, *1/*3, and *2/*2, *2/*3, and *3/*3 (1.54 ±1.05 mg, $p < 0.001$).

Conclusion:

Warfarin dosage is influenced by both genetic and non-genetic factors. Warfarin dosage may be impacted by both genetic and non-genetic factors, which play a significant role. Individual differences in warfarin dosage are demonstrated by CYP2C9*2 and CYP2C9*3 polymorphisms, as well as by age and body mass index.

Key words: Single nucleotide polymorphism, Non genetic factors, CYP2C9, warfarin.

Introduction:

Warfarin is oral anticoagulant used to treat and prevent thromboembolic conditions like atrial fibrillation, heart valve replacement, pulmonary embolism, and deep vein thrombosis [1,2]. For more than 60 years, warfarin has been the gold standard for oral vitamin K antagonists and is still used extensively worldwide for the prevention and treatment of thromboembolic diseases [3, 4, 5]. The availability of direct oral anticoagulants (DOACs) has increased recently, although their clinical application is still limited. Due to the drug's low cost, ease of administration, wide market availability, and extensive clinical experience with use, warfarin will continue to be widely utilized in the developing countries [1] Warfarin has a low therapeutic index, which increases interindividual variability in response and the risk of major negative effects on thrombosis or hemorrhage [6]. Warfarin therapy requires close monitoring and frequent dose adjustments to achieve and maintain the therapeutic anticoagulation effect due to its narrow therapeutic index and high inter patient variability [7-9]. Several vitamin K-dependent coagulation factors, including as factors II, VII, IX, and X, are prevented from activating by the vitamin K antagonist warfarin, which has an anticoagulant effect [10,11]. Numerous studies have demonstrated that both genetic and non-genetic factors influence the variability of the warfarin dose [12,13,14]. Non genetic factors are Age, gender, height, weight, BMI, diet, liver function, and genetic variations in several genes, such as CYP2C9, VKORC1, CYP4F2 and GGCX [15,16,17,18]. Genetic polymorphisms appear to be a significant contributor to the interindividual variability of warfarin doses because they affect both the pharmacokinetics and pharmacodynamics of warfarin [19]. Cytochrome P450 2C9 (CYP2C9) and the vitamin K epoxide reductase complex subunit 1 (VKORC1) are two significant genes that have been found to be involved in the treatment of warfarin [20]. The CYP2C9 gene, which codes for the cytochrome P450 2C9 enzyme that metabolizes the more potent S enantiomer of warfarin, is the most significant gene consistently affecting warfarin dose among various populations and warfarin inhibits the enzyme vitamin K epoxide reductase, which is encoded by the VKORC1 gene [12,13]. The enzymatic activity of the CYP2C9*2 and CYP2C9*3 variants has been shown to be reduced, which contributes to the variation in warfarin dose requirements [19]. Patients with the CYP2C9*2 or CYP2C9*3 allele required lower warfarin doses than those with the CYP2C9*1 allele, according to earlier research in a variety of ethnic groups, including South

Indian population [21,22,23]. Indians have a unique genotype frequency response to warfarin compared to other Asian populations [24]. Additionally, there is a large difference in the frequency of warfarin-sensitive variant alleles among different Indian regions [24, 25]. In India, the majority of study is limited to describing the frequency of variant alleles [25, 26,27,28,29]. Few studies have looked at the interactions between variant alleles and warfarin dosage [30,31,32,33]. Therefore, the objective of the current study was to quantify the relationship between genetic and nongenetic factors and warfarin dose.

Material and Methods

A pragmatic analytical investigation was carried out by the Pharmacology Department of our tertiary care institution in collaboration with the Cardiothoracic Vascular Surgery (CTVS) department outpatients. After receiving approval from the institute ethics committee, the study was started. Following the acquisition of written informed consent, consecutive patients were examined for possible study eligibility. The test group consisted of all 96 DVT; there were 45 men and 51 women (Fig1). Patients who had been taking warfarin for at least three months with an INR between 2.5 and 3 and Participants in this study ranged in age from 18 to 65 and were taking a stable maintenance dose of warfarin. Individuals who failed to give their informed consent, had risk factors such hypertension, diabetes mellitus, or liver conditions, or were taking CYP2C9 inducers or inhibitors were not included in this study.

5 ml of peripheral blood were collected and placed in EDTA and sodium citrate to analyze coagulation and molecular processes. The genomic DNA from white blood cells was separated using the salting out method, and agarose gel electrophoresis and spectrophotometer were used to determine the quantity and quality of the extracted DNA. The pure DNA was stored at a temperature of -80 °C.

The genome's CYP2C9 was amplified using the polymerase chain reaction (PCR).CYP2C9 (CYP2C9*2; rs1799853, CYP2C9*3; rs1057910) genes are used in the PCR-RFLP method to assess genotype with reference to the presence of variant alleles. This was carried out using 25 ml of total reaction volume and 150ng of gDNA. Table 1 represents the primers and PCR conditions for gene amplification. Gene amplification was confirmed by electrophoresis of PCR products on 2% agarose gel at 120 V for 60 min while stained with 0.5 g/ml ethidium bromide in tris borate EDTA buffer pH 8.0 and visualized using a gel documentation system. For CYP2C9*2 and CYP2C9*3 the amplicon sizes were 375bp and 130 base pairs, respectively. The appropriate reaction mixture for restriction digestion was added to the PCR products and incubated at 37°C for an overnight duration.

Table 2 displays the restriction enzymes utilized for digestion, the resulting restriction fragments, and their interpretation. Gel images are displayed in Figure 2 and 3.

Figure 1 shows the gender distribution

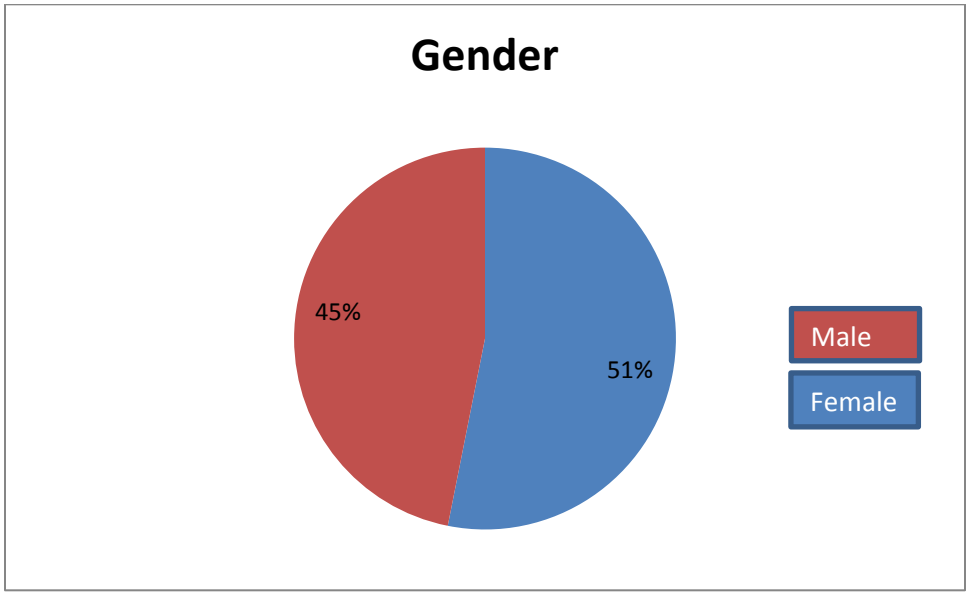

Table 1 lists the forward and reverse primers and the PCR procedure.

Gene Polymorphism	**Primers**	**PCR Procedure**
CYP2C9* 2 (rs1799853)	F: 5'-CACTGGCTGAAAGAGCTAACAGAG-3' R: 5'-GTGATATGGAGTAGGGTCACCCAC- 3'	Denaturation : 95^0c for 5 min, 95^0c for 30 s Annealing : 59^0c for 45s Extension : 72^0c for 10 min.
CYP2C9* 3 (rs1057910)	F: 5'- AGGAAGAGATTGAACGTGTGA -3' R: 5'-GGCAGGCTGGTGGGGGAAGGCCAA -3'	Denaturation : 95^0c for 5 min, 94^0c for 1 min Annealing : 60^0c for 45s Extension : 72^0c for 10 min.

Table 2 Gene and digested Products

Gene	PCR Product size	Restriction Enzymes	Digested Product
CYP2C9* 2	375 bp	AvaII	Wild type : 296-bp,79-bp Heterozygous : 375- bp,296-bp, 79-bp Mutant : 375 bp
CYP2C9* 3	130 bp	StyI	Wild type : 130-bp Heterozygous : of 130-bp and 110-bp, 26-bp Mutant : 130-bp, 26-bp

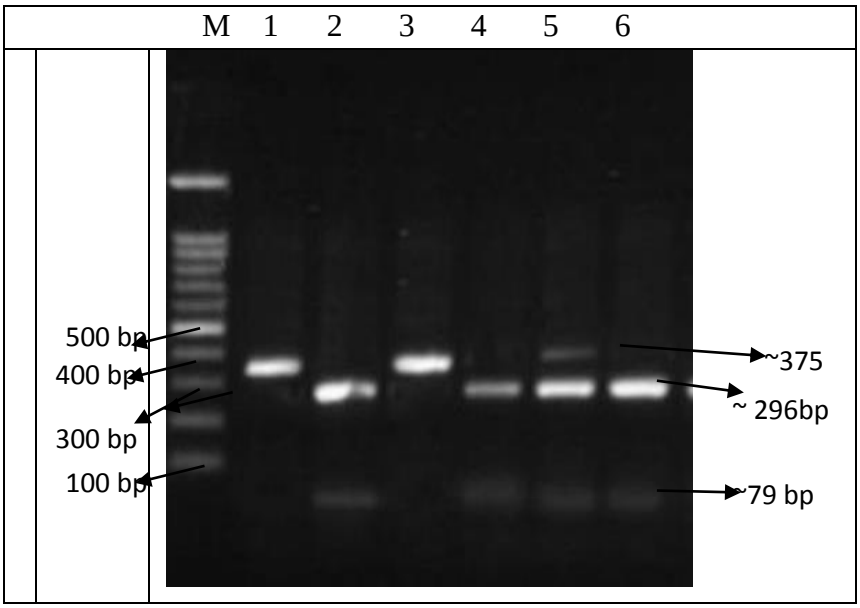

Figure 2: Agarose gel Image of CYP2C9*2: Lane M, 500-bp ladder; Lanes 1 and 3 were TT mutant homozygous with fragment of 375 bp, Lanes 2, 4, were CC wild homozygous with fragment of 296-bp,79-bp. Lanes 5 CT heterozygous with fragments of 375 bp,296 bp,79 bp

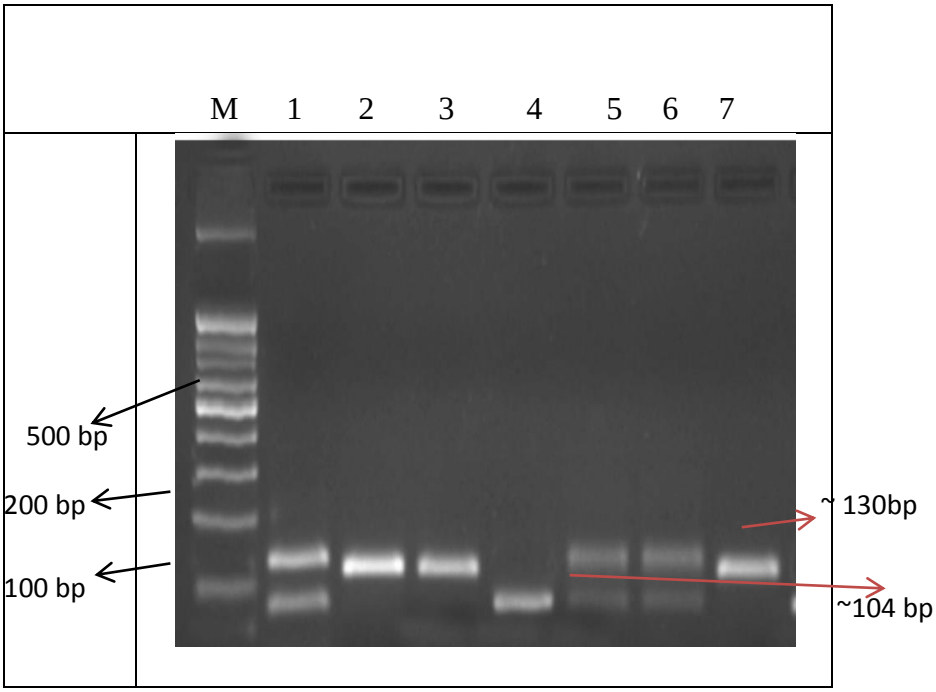

Figure 3 : 2.5% agarose gel image of CYP2C9*3; Lane M, 100-bp ladder
Lane 1and 5,6 CA heterozygous with fragments of 130-bp and 110-bp, 26-bp;
Lanes 4 , mutant homozygous CC with a fragment of 130-bp, 26-bp
Lane 2, 3, 7, wild type with a fragment of 130-bp.

Statistical Analysis

For variables with (mean, SD), descriptive statistics were applied. A one-way analysis of variance was carried out using the IBM SPSS programme to see whether there were any noticeable variations in the daily maintenance dose of the drug. In Graph Pad (Prism 8 programme), the chi-square and Fisher's exact tests were used to create descriptive statistics and evaluate the statistical significance of allelic frequency from Hardy-Weinberg equilibrium. $P>0.05$ was considered statistically significant.

Results: A total number of 96 patients (45 Males, 51 females) who were on warfarin therapy were included in this study. The genotype frequencies for CYP2C9*2 were 64.6%, 19.8%, and 15.6% for the CC, CT, and TT genotypes, respectively. The frequencies of the CYP2C9*3 genotypes for the AA, AC, and CC genotypes were respectively 39.6%, 39.6%, and 20.8%.

Table 3 lists the clinical and demographic information, including genotype frequencies

Patients, *N*	96 (%)
Males	45 (46.9)
Females	51 (53.1)
Age In Groups	
20-30	7 (7.3)
31-40	20 (20.8)
41-50	32 (33.3)
51-60	28 (29.2)
61-70	9 (9.4)
Mean	46.61
Weight In Groups	
30-45	48 (50)
46-60	39(40.6)
61-75	9 (9.4)
Frequency of Genotype: n (%)	
CYP2C9*2	
CC (Wild type)	62 (64.6)
CT	19 (19.8)
TT	15 (15.6)
CYP2C9*3	
AA (Wild type)	38 (39.6)
AC	38 (39.6)
CC	20 (20.8)

The Warfarin Dose Requirement

A comparison of warfarin daily maintenance doses between genotype groups revealed that patients with the homozygous wild-type CYP2C9 (*1/*1) genotype received a daily mean

dose of 4.07±1.75 mg. It was substantially greater than in patients *1/*2, *1/*3 (2.93 ±2.03 mg, p <0.001) and *2/*2, *2/*3, and *3/*3 (1.54 ±1.05 mg, p = 0.002). The mean daily warfarin dose was substantially greater in people with wild-type CYP2C9*1.Additionally, there were statistical differences in the mean daily dose of warfarin between homozygous and heterozygous genotypes.

Table 4 shows the frequency distribution of CYP2C9 haplotypes among study participants.

CYP2C9 Haplotypes	N (%)
*1/*1	28 (29.2)
*1/*2	06 (6.3)
*1/*3	23 (24)
*2/*2	04 (4.2)
*2/*3	25 (26)
*3/*3	10 (10.4)

Discussion :

The most popular oral anticoagulant available for long-term usage is warfarin, a coumarin derivative that was originally marketed in 1954 [34, 35]. To bring their INR into the therapeutic range, some individuals need to take higher-than-expected doses of warfarin. A few examples of acquired or hereditary factors that may be to blame for this phenomenon include poor compliance, pharmaceutical interactions, and food interactions.[36,37,38]. The main allelic polymorphisms known to affect warfarin metabolism are those related to CYP2C9. The authors' primary objective was to comprehend how the prevalence and severity of these polymorphisms affect how the South Indian population responds to warfarin. The prevalence of the CYP2C9*1*1 genotype is reported to be 29.2%, followed by the genotypes *1*2 (6.3%), *1*3 (24%), *2*2 (4.2%), *2*3 (25%) and *3*3 (10%). In this study population, it has also been revealed that there are very few *1*2 and *2*2 variations. Since the appropriate dose for each patient is unknown and dosing is frequently done based on experience, the first phase of anticoagulation treatment is the most difficult stage and carries a significant risk of over-anticoagulation or hemorrhagic events [39,40]. For patients with either CYP2C9 variation, there is strong and consistent support for a reduced maintenance dose, with CYP2C9*2, CYP2C9*3 patients often needing the lowest dose [41]. Even though 20% of Caucasians have the CYP2C9*2 and *3 polymorphisms, only 6–10% of the interindividual variability in warfarin may be attributable to these two genotypes [42] The majority of research only included patients who were Caucasian, although a few others used more narrow demographic criteria. This is significant since the 2C9*2 and 2C9*3 alleles are quite uncommon in African American and most Asian populations. When compared to people with the wild genotype, those with the warfarin-sensitive variant alleles for CYP2C9*2 and CYP2C9*3 needed much lower doses of the drug [3].

The authors are aware that drug resistance is a complicated issue influenced by a variety of genetic and non genetic factors. In the current investigation, a standard deviation analysis

was carried out to determine whether demographic characteristics affected patients' responses to warfarin. The CYP2C9 SNP, age, and weight were identified as variables linked to interindividual variability in the daily warfarin dose using a stepwise approach. VKORC1 genetic variations have been identified as important determinants impacting the daily dose of warfarin in other studies, despite the fact that there is good evidence to support this [43]. With regard to the reported effects of VKORC1 and CYP2C9 on dose requirements, the American Food and Drug Administration (FDA) approved a labeling change for warfarin that stated that "lower initiation doses should be considered for patients with certain genetic variations in CYP2C9 and VKORC1 enzymes." The FDA also recommended clinical testing for specific genetic variants prior to commencing warfarin therapy [44 , 45]. A patient's genetic background must be taken into account when determining the warfarin dose and method of administration.

The results of this study demonstrate the significance of both genetic and non-genetic variables in influencing warfarin response variability. CYP2C9*2 and CYP2C9*3 were the greatest genetic predictors of warfarin dosages in individuals from the south, while Significant clinical predictors included age and BMI. Therefore, both genetic and non-genetic factors must be taken into consideration for a more accurate prediction of warfarin dose requirements.

Conclusion:

Our research has some limitations, such as a small sample size and the investigation of a few genetic polymorphisms. The most significant SNPs identified to alter the pharmacokinetics and pharmacodynamics of warfarin, however, were taken into consideration by our analysis. Pharmacogenetic information may help doctors reduce adverse medication reactions or cut treatment costs by reducing hospital admissions, thereby enhancing patient health.

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