Genetic and Nongenetic Determinants of Variable Warfarin Dose Requirements: A Report from North India

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Keywords

Genetic polymorphisms · Warfarin · Coumarin · Precision medicine · Dose optimization

Abstract

Introduction: Warfarin is widely used and will continue to be prescribed especially in developing countries due to its low cost. Given the huge patient load requiring anticoagulation, there is a need to develop strategies to optimize warfarin therapy for ensuring safe and effective anticoagulation. In the present work, we aimed at elucidating the association of genetic and nongenetic variables with warfarin dose requirement in patients attending the cardiovascular clinic in a tertiary care center of North India. **Methods:** This was a prospective study conducted over 1 year. Patient demographic and clinical details were captured in customized case record forms. Genotyping was done using the polymerase chain reaction-restriction fragment length polymorphism method. Pharmacogenetic influence of *CYP2C9* (*rs1799853* and *rs1057910*) and *VKORC1* (*rs9923231*) variant alleles was studied. The association of genetic and nongenetic factors with

warfarin dose was quantified using a stepwise multivariate linear regression model. **Results:** Two hundred and forty patients were screened. Data from 82 eligible patients were used for quantifying the association of genetic and nongenetic factors with warfarin dose. A descriptive model based on *CYP2C9**3 (*rs1057910*) and *VKORC1* (*rs9923231*) variant alleles and BMI was developed. The model explains nearly half of the interindividual variation in warfarin dose requirement. **Conclusion:** The model explains nearly half of the interindividual variation in warfarin dose in patients with atrial fibrillation and/or requiring valve replacement.

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Introduction

Warfarin represents the standard of care oral vitamin K antagonist for over 6 decades and still is widely used for prophylaxis and treatment of thromboembolic disorders across the globe [1, 2]. Recent introduction of direct oral anticoagulants (DOACs) has expanded the choice for oral anticoagulants; yet, their clinical use remains limited. In

India, clinicians still prefer using warfarin (coumarin derivative) over DOACs although no estimates on market share of DOACs have been published so far [3, 4]. Not only that, warfarin will continue to be widely used in the developing world on account of low cost, ease of administration, wide market availability, and vast clinical experience in use of the drug. Furthermore, warfarin continues to be the anticoagulant of choice for many conditions where DOACs are contraindicated. These include anticoagulation in children and in those with mechanical heart valves, valvular atrial fibrillation, and renal impairment [5].

Although effective, well-known limitations to safe use of warfarin include narrow therapeutic index, large interindividual variation in drug response, and potential for numerous drug-drug and drug-food interactions [6–8]. Due to these reasons, individualization of dose is an important aspect of warfarin anticoagulation. Subtherapeutic doses put the patient at risk of thromboembolic events, whereas supratherapeutic dosing leads to high risk of bleeding complications. Finding the right dose of warfarin for an individual patient still remains a challenging task. Several studies have delineated factors that drive the interindividual variation in drug response. These include age, gender, weight, height, BMI, diet, liver function, and polymorphisms in many genes such as *CYP2C9*, *VKORC1*, *CYP4F2*, and *GGCX* [9–12].

Dosing algorithms based on these factors have been found useful for optimization of anticoagulation. Benefits of genotype-guided warfarin therapy range from shorter time to therapeutic INR and increased percentage of time in therapeutic range to reducing the occurrence of serious bleeding events [13–15]. However, these dosing algorithms have limited applicability for populations outside the ones used for development of the algorithm [16–18]. This has been attributed to a wide variation in frequency of warfarin-sensitive alleles and presence of race-specific variants influencing drug response [19]. Genotype frequencies affecting warfarin response in Indians are different from other Asian populations [20]. Furthermore, there is significant variation in frequency of warfarin-sensitive variant alleles among various regions within India [20, 21]. Most of the studies conducted in India are limited to describing the frequency of variant alleles [21–25]. Very few studies have described the association of variant alleles with warfarin dose [26–29]. Hence, in the present study, we aimed at quantifying the association of genetic and nongenetic variables with warfarin dose.

Patients and Methods

A prospective study was conducted over a period of 1 year in the Department of Pharmacology in collaboration with Cardiology and Cardiothoracic Vascular Surgery (CTVS) Outpatient Departments of our tertiary care hospital. The study was initiated after getting approval from the Institute Ethics Committee. Consecutive patients were screened for potential eligibility in the study after obtaining written informed consent.

Adult patients of either sex suffering from atrial fibrillation and or those requiring heart valve repair/replacement surgeries on stable anticoagulation with warfarin were included. Patients with transient atrial fibrillation; with underlying hepatic (defined as elevation in alanine/aspartate transaminase >2.5 times the upper limit of normal) and or severe renal insufficiency (defined as creatinine clearance of <30 mL/min); patients with known contraindications to anticoagulant therapy; pregnant and nursing women; and those who were unwilling to participate were excluded. Eligible patients were recruited. The study was registered in the Clinical Trial Registry of India (CTRI/2018/06/014614).

Following enrollment, patient demographic and clinical details such as age, sex, height, weight, smoking and alcohol status, indication, warfarin dose, and use of concomitant cardiac medications were captured. Stable anticoagulation was defined as having 2 INR readings in the therapeutic range (2.0–3.0) over a period of 3 months taken at least 1 week apart with no adjustment of warfarin dose. The warfarin dose in the period of stable anticoagulation was considered as stable dose for the patient. Adverse events such as bleeding and thromboembolic events or hospitalization as a result of these events were also recorded.

For genotype evaluation with regard to presence of variant alleles in *CYP2C9* (*CYP2C9**2; *rs1799853*, *CYP2C9**3; *rs1057910*) and *VKORC1* (*–1639G>A*; *rs9923231*) genes, the PCR-RFLP method was used [30]. Single venous blood sample (6 mL) was obtained from the patients. Blood was collected in an EDTA vial and was stored in a deep freezer at -40°C until further processing. DNA was extracted using the commercial genomic DNA extraction kit (QIA-GEN amp DNA mini kit). Polymerase chain reaction (PCR) was done to amplify *CYP2C9* and *VKORC1* regions of the genome. This was done from 100 ng genomic DNA in a total reaction volume of 25 μL . Reaction mixture consisted of 2.5 μL of 10 $\times$ buffer, 1.5 μL of 25 mM of MgCl_2 , 1 μL of 10 mM dNTP mix, 1 μL of 10 mM of forward and reverse primers of the *CYP2C9* gene, and 1 μL of 1U/ μL of Taq DNA polymerase. In case of *VKORC1* gene amplification, optimal primer concentration was 0.3 μL of 10 mM of forward and reverse primers each. Nuclease-free water was added to make the final reaction volume to 25 μL . Primers and PCR conditions for gene amplification are presented in Table 1. Amplification of candidate genes was confirmed by electrophoresis of PCR products on 2% agarose gel at 120 V for 60 min stained with 0.5 $\mu\text{g/mL}$ ethidium bromide in Tris borate EDTA buffer pH 8.0 and visualization using a gel documentation system. The amplicon sizes for *CYP2C9**2, *CYP2C9**3, and *VKORC1* were 690 bp, 166 bp, and 636 base pairs, respectively. PCR products were kept for overnight incubation at 37°C in an appropriate reaction mixture for restriction digestion. Digestion mixture comprised 10 μL of the PCR product with 10 μL of respective restriction endonuclease in appropriate buffer. The restriction enzymes used for digestion and restriction fragments so generated with their interpretation are presented in Table 2. Representative gel pictures are shown in Figure 1a–c.

Table 1. Forward and reverse primers and PCR protocol

Gene polymorphism	Primers	PCR protocol
<i>CYP2C9*2</i> (rs1799853)	F: 5'TACAAATACAATGAAATATCATG-3' R: 5'CTAACAACCAGACTCATAATG-3'	Initial denaturation: 94°C for 5 min; 94°C for 30 s; 55°C for 30 s; 72°C for 2 min, terminal extension at 72°C for 10 min
<i>CYP2C9*3</i> (rs1057910)	F: 5AATAATAATATGCACGAGGTCCAGAGGTAC-3' R: 5'GATACTATGAATTTGGGACTTC-3'	Initial denaturation: 95°C for 3 min; 95°C for 1 min; 60°C for 1 min; 72°C for 1 min; terminal extension at 72°C for 10 min
<i>VKORC1</i> –1639G>A (rs9923231)	F: 5'ATCCCTCTGGGAAGTCAAGC-3' R: 5'CACCTTCAACCTCTCCATCC-3'	Initial denaturation: 95°C for 3 min; 95°C for 30 s; 55°C for 1 min; 72°C for 1 min 30 s; terminal extension at 72°C for 10 min

Table 2. Restriction enzymes and digested product sizes (in base pairs)

Gene	PCR product size, bp	Restriction enzyme	Digested product size, bp
<i>CYP2C9*2</i>	690 bp	Avall	WT: 521 bp, 169 bp; Htz: 690 bp, 521 bp, 169 bp; Mut: 690 bp
<i>CYP2C9*3</i>	166 bp	KpnI	WT: 166 bp; Htz: 166 bp, 136 bp, 30 bp; Mut: 136 bp, 30 bp
<i>VKORC1</i> –1639G>A	636 bp	NsiI	WT: 472 bp, 114 bp, 50 bp; Htz: 522 bp, 472 bp, 114 bp, 50 bp Mut: 522 bp, 114 bp

bp, base pairs; WT, wild type; Htz, heterozygous mutant; Mut, homozygous mutant.

Fig. 1. a 2.5% agarose gel pattern of *CYP2C9*2* (C430T). *Lanes 1 to 3 show the homozygous mutant TT genotype (690 bp), lanes 4 and 5 show the wild homozygous (CC) genotype (521 bp, 169 bp), and lanes 6 and 7 show the heterozygous CT genotype (690 bp, 521 bp, and 169 bp). **b** 2.5% agarose gel pattern of *CYP2C9*3* (A1075C). *Lanes 1, 3, and 6 show the wild-type AA genotype (166 bp), lanes 2, 4, and 5 show the homozygous mutant CC genotype (136 bp and 30 bp), and lane 7 shows the heterozygous mutant AC genotype

(166 bp and 136 bp). Note that the 30-base pair product is not visible. **c** 2.5% agarose gel pattern of *VKORC1* –1639G>A. *Lanes 1 and 6 show the homozygous mutant genotype AA (522 bp, 114 bp, and 50 bp), lanes 2 and 4 show the wild homozygous (GG) genotype (472 bp, 114 bp, and 50 bp), lanes 3 and 5 show heterozygous AG genotype, and lane 7 shows the undigested (U) *VKORC1* gene PCR product (636 bp). Note that the 50-base pair product is not visible.

Fig. 2. Flow of participants during the study period.

Statistical Analysis

A sample size of 80 gives a probability of >99% for detecting all alleles in the 3 genes under study, that is, *VKORC1*, *CYP2C9*2*, and *CYP2C9*3* assuming 80–90% frequency of the wild type and about 5–10% frequency of the other variants [31]. Descriptive statistics were used for continuous (mean $\pm$ SD, or median [range]) variables. Categorical data were presented as frequencies or percentages. The Kolmogorov-Smirnov test was used for assessing the normality of data. Log transformation was done to normalize the distribution of the warfarin dose. Comparison of mean stable warfarin dose across different genotypes was done using 1-way analysis of variance followed by the post hoc Scheffe test. Stepwise multiple linear regression analysis was undertaken to model the relationship of warfarin dose per week with the clinical and pharmacogenetic variables. *p* value <0.05 was considered statistically significant. All analyses were done using SPSS version 22.

Results

A total of 240 patients were screened over a period of 1 year with consequent enrollment of 89 eligible patients. The analysis dataset includes data from 82 patients who completed the study and had no missing data on any of the study variables (shown in Fig. 2). The majority (86.5%) of the participants received warfarin for atrial fibrillation. Other demographic and clinical details including genotype frequencies with 95% confidence intervals are presented in Table 3. Mean ($\pm$ SD) stable warfarin dose was 28.82 ($\pm$ 12.59) mg/week with an interquartile range of 14 mg/week. Stable warfarin dose in the study cohort ranged from 5 mg/week to 77 mg/week. This represents over 15-fold variation in warfarin dose requirement among patients. Further *CYP2C9* haplotypes were constructed based on combined individual *CYP2C9*2* and *CYP2C9*3* genotypes. The frequency distribution of study subjects

based on *CYP2C9* haplotypes and *VKORC1* genotype is summarized in Table 4.

Comparison of Warfarin Dose Response across Different Genotypes

Comparison of mean ($\pm$ SD) stable warfarin doses across *CYP2C9*2* genotypes revealed a dose requirement of 29.18 $\pm$ 12.76 mg/week in individuals with the wild genotype, followed by 27.13 $\pm$ 11.35 mg/week in heterozygous and 16 mg/week in 1 patient with homozygous mutant genotype. Although there was trend to decreased requirement of warfarin with increasing number of variant *CYP2C9*2* alleles, the difference did not reach statistical significance (*p* = 0.543). Similarly, across *CYP2C9*3* genotypes, we observed the highest dose requirement of 30.9 $\pm$ 12.30 mg/week in the wild genotype followed by 24.08 $\pm$ 9.25 mg/week in heterozygous and the lowest dose requirement of 9.50 $\pm$ 3.69 mg/week in homozygous mutants (*p* = 0.001). To study the effect of *CYP2C9*2* and *CYP2C9*3* allelic variation in combination, we compared warfarin dose requirements in individuals with different haplotypes of the *CYP2C9* gene (Fig. 3). The difference was found to be statistically significant (*p* = 0.002).

Significant variation was also noted for warfarin dose requirement across different genotypes for the *VKORC1* gene (*p* = 0.02). Mean ($\pm$ SD) weekly warfarin dose requirement was 31 $\pm$ 13.03 mg/week followed by 23.52 $\pm$ 9.76 and 18.50 $\pm$ 3.53 mg/week in individuals with wild, heterozygous, and homozygous mutant genotypes, respectively. Combined effect of warfarin-sensitive variant alleles in both *VKORC1* and *CYP2C9* genes on warfarin dose is shown in Figure 4. Adverse event of minor bleeding occurred in 2 patients during the study period.

Table 3. Demographic, clinical, and genotype characteristics of the study cohort

Patients, <i>N</i>	82
Age, mean ± SD, years	53.4±13.38
Females	50.8±11.07
Males	57.4±15.88
Gender, <i>n</i> (%)	
Male	33 (40)
Female	49 (60)
Height, mean ± SD, cm	160.9±11.24
Weight, mean ± SD, kg	63.4±14.70
Body mass index, <i>n</i> (%), kg/m ²	
<18.5	8 (9.8)
18.5–24.9	39 (47.6)
24.91–29.9	24 (29.3)
≥29.9	11 (13.4)
Indication for warfarin use, <i>n</i> (%)	
Atrial fibrillation	71 (86.5)
Valve replacement	11 (13.5)
Smoking status, <i>n</i> (%)	
Yes	1 (1.2)
No	81 (98.7)
Alcohol status, <i>n</i> (%)	
Yes	7 (8.54)
No	75 (91.4)
Mean stable warfarin dose, mg/week	
Mean ± SD	28.82±12.59
Median (range)	28 (5–77)
Concomitant medications, <i>n</i> (%)	
Diuretics	69 (84)
Beta blockers	61 (74.39)
Calcium channel blocker	26 (32)
Digoxin	21 (25.6)
Antihypertensives	20 (24.4)
Statins	17 (21)
Low-dose aspirin	16 (20.9)
Amiodarone	04 (4.9)
Genotype frequencies; <i>n</i> (%); [95% CI]	
CYP2C9*2	
CC (wild type)	73 (89.02); [80.43–94.11]
CT	08 (9.76); [5.03–18.09]
TT	01 (1.22); [0.22–6.59]
CYP2C9*3	
AA (wild type)	65 (79.26); [69.29–86.63]
AC	13 (15.85); [9.5–25.25]
CC	04 (4.87); [1.91–11.88]
VKORC1 –1639G>A	
GG (wild type)	57 (69.51); [58.86–78.41]
GA	23 (28.04); [19.48–38.59]
AA	02 (2.44); [0.67–8.46]

Modeling the Warfarin Dose Requirement

Stepwise multiple linear regression analysis was undertaken to model and quantify the association of genetic and nongenetic factors with weekly warfarin dose requirement. To avoid overfitting of data, 5 pertinent can-

Table 4. Frequency distribution of study participants with regard to CYP2C9 haplotypes and VKORC1 genotype

VKORC1	CYP2C9 haplotypes				
	*1/*1	*1/*2	*1/*3	*2/*2	*3/*3
GG	37	06	11	–	03
AG	19	01	01	01	01
AA	01	01	–	–	–

didate variables were considered, that is, age, gender, BMI, CYP2C9*3, and VKORC1 (–1639G>A) variant alleles. The multivariate regression model identified CYP2C9*3, VKORC1, and BMI as significant determinants of warfarin dose. The model explains 47.5% of interindividual variability in warfarin dose (Table 5). Based on the model (Table 6), the weekly warfarin dose requirement can be represented as

$$\text{Log warfarin dose (mg/week)} = 1.682 - 0.247(\text{CYP2C9*3}) - 0.152 (\text{VKORC1}) + 0.010 \times \text{BMI}.$$

Discussion

In the present work, we aimed at quantifying the association of genetic and nongenetic factors with the warfarin dose requirements in cardiac outpatients attending North Indian tertiary care hospital. Genotype frequencies for both CYP2C9 and VKORC1 genes reported here are in agreement with previous reports [20–22].

We found over 15-fold variation in warfarin dose requirement among the studied cohort. Individuals carrying warfarin-sensitive variant alleles for CYP2C9*3 and VKORC1 required significantly lower warfarin doses compared to individuals with the wild genotype. We observed a trend of decreasing warfarin dose in patients carrying CYP2C9*2 variant alleles as well, but the difference was not statistically significant. The presence of variant alleles in both CYP2C9 and VKORC1 genes showed additive effect on decreasing warfarin doses. For instance, 1 patient with the *3/*3 haplotype of CYP2C9 with the AG genotype for VKORC1 required the lowest dose of 5 mg/week among the studied cohort. In contrast, an individual with only the *3/*3 haplotype with the wild genotype for the VKORC1 gene had a higher dose requirement of 11 mg/week. Similarly, presence of the AG genotype along with the *1/*2 haplotype decreased warfarin dose requirements from

Fig. 3. Distribution of weekly warfarin dose across CYP2C9 haplotypes.

Fig. 4. Distribution of weekly warfarin dose in the study cohort based on genotypes for both CYP2C9 and VKORC1*. *Codes refer to the following combination of CYP2C9 haplotypes and VKORC1 genotype: 0 → (*1/*1 CYP2C9 haplotype + GG genotype of VKORC1); 1 → (*1/*1 CYP2C9 haplotype + AG genotype of VKORC1); 2 → (*1/*1 CYP2C9 haplotype + AA genotype of VKORC1); 3 → (*1/*2 CYP2C9 haplotype + GG genotype of VKORC1); 4 → (*1/*2 CYP2C9 haplotype + AG genotype of VKORC1); 5 → (*1/*2 CYP2C9 haplotype + AA genotype of VKORC1); 6 → (*1/*3 CYP2C9 haplotype + GG genotype of VKORC1); 7 → (*1/*3 CYP2C9 haplotype + AG genotype of VKORC1); 8 → (*2/*2 CYP2C9 haplotype + AG genotype of VKORC1); 9 → (*3/*3 CYP2C9 haplotype + GG genotype of VKORC1); 10 → (*3/*3 CYP2C9 haplotype + AG genotype of VKORC1).

P2C9 haplotype + AG genotype of VKORC1); 5 → (*1/*2 CYP2C9 haplotype + AA genotype of VKORC1); 6 → (*1/*3 CYP2C9 haplotype + GG genotype of VKORC1); 7 → (*1/*3 CYP2C9 haplotype + AG genotype of VKORC1); 8 → (*2/*2 CYP2C9 haplotype + AG genotype of VKORC1); 9 → (*3/*3 CYP2C9 haplotype + GG genotype of VKORC1); 10 → (*3/*3 CYP2C9 haplotype + AG genotype of VKORC1).

30.83 ± 10.7 mg/week to 16 mg/week compared to *1/*2 haplotype individuals possessing wild-type *VKORC1* alleles. Two patients with minor bleeding also had variant alleles in both *CYP2C9* and *VKORC1* genes. Studies have pointed to more chances of out of range INR and

more bleeding events in individuals with variant alleles [32, 33].

The stepwise multivariate regression model identified *CYP2C9**3, *VKORC1* –1639G>A, and BMI as significant determinants of warfarin dose. Together, these factors ex-

Table 5. Stepwise multiple linear regression model

Model summary				
model	R	R ²	adjusted R ²	std. error of the estimate
1	0.547 ^a	0.299	0.290	0.17477
2	0.663 ^b	0.440	0.425	0.15721
3	0.703 ^c	0.494	0.475	0.15030

^a Predictors: (constant), CYP2C9*3. ^b Predictors: (constant), CYP2C9*3 and VKORC1. ^c Predictors: (constant), CYP2C9*3, VKORC1, and BMI.

plain nearly half of the interindividual variation in warfarin dose requirement. The model showed genetic factors to be more important sources of variability in warfarin dose response compared to clinical factors. These findings are in conformity with previous research reports [10–12, 27]. CYP2C9*3 polymorphism alone accounted for 29% of variation in dose response and can be seen as large compared to other studies. In contrast to other studies, which identified CYP2C9*2 as another important factor for varying warfarin doses, we did not find the association of the CYP2C9*2 gene with warfarin dose as significant, although trend to decreasing warfarin dose was observed [27, 29, 34]. Compared to other studies, the effect of BMI in our model was modest ($\beta = 0.010$). One of the plausible reasons for this could be the absence of morbidly obese patients in our study cohort. A recent study ($n = 831$) showed that mean weekly warfarin dose increases by a factor of 0.69 for a unit increase in BMI. The large impact of BMI on warfarin doses in this study was likely due to inclusion of patients over a wide BMI range of 13.4–63.1 kg/m². They included about 49 morbidly obese (BMI >40 kg/m²) patients in the analysis. Moreover, they studied the effect of BMI on warfarin dose in isolation [35]. The impact of smoking and alcohol on warfarin dose requirement is unclear from the present study due to very limited representation of smokers ($n = 1$) and occasional alcoholics ($n = 7$).

Strengths of the study: the study quantifies the association of genetic and nongenetic factors with warfarin dose in a homogenous cohort of cardiac patients on warfarin. This may be of significance in view of a recent randomized trial showing greater benefit of genotype-guided warfarin dosing in patients of atrial fibrillation compared to patients with pulmonary embolism and deep vein thrombosis [36].

Limitations of the study: limitations of our work include small sample size and studying a limited number of

Table 6. Regression model used to model warfarin dose requirement

Coefficients ^a					
model	unstandardized coefficients		standardized coefficients	T	sig
	B	std. error	beta		
1 (constant)	1.680	0.049		34.176	0.000
CYP2C9*3	−0.212	0.036	−0.547	−5.839	0.000
2 (constant)	1.910	0.068		28.119	0.000
CYP2C9*3	−0.236	0.033	−0.607	−7.115	0.000
VKORC1	−0.151	0.034	−0.380	−4.457	0.000
3 (constant)	1.682	0.102		16.495	0.000
CYP2C9*3	−0.247	0.032	−0.637	−7.750	0.000
VKORC1	−0.152	0.032	−0.383	−4.695	0.000
BMI	0.010	0.003	0.236	2.903	0.005

^a Dependent variable: logdose warfarin.

genetic polymorphisms. However, we accounted for the most important polymorphisms reported to affect warfarin pharmacokinetics and pharmacodynamics across the literature.

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Statement of Ethics

The study was conducted ethically in accordance with the principles of the Declaration of Helsinki. All the study subjects have given their written informed consent. The study protocol was approved by the Institute Ethics Committee (Intramural) at PGIMER, Chandigarh, Vide Reference No. NK/2608/DM/3107.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Dr. Nusrat Shafiq and Dr. Samir Malhotra conceptualized the study, monitored conduct and analysis, and critically reviewed the manuscript. Dr. Navjot Kaur and Dr. Avaneesh Pandey contributed to data collection, genotyping, data analysis, compilation of results, and drafting the manuscript. Dr. Reena Das and Dr. Jasmina Ahluwalia designed genotype experiments and contributed

to genotyping data analysis and reviewing the manuscript. Dr. Ankur Gupta and Dr. Harkant Singh contributed to enrolling eligible patients, data analysis, and critical review of the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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