

## 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

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Received January 25, 2011

**Background & objectives:** Studies have demonstrated the effect of *CYP2C9* (cytochrome P450) and *VKORC1* (vitamin K epoxide reductase complex) gene polymorphisms on the dose of acenocoumarol. The data from India about these gene polymorphisms and their effects on acenocoumarol dose are scarce. The aim of this study was to determine the occurrence of *CYP2C9*\*2,\*3 and *VKORC1* -1639G>A gene polymorphisms and to study their effects on the dose of acenocoumarol required to maintain a target International Normalized Ratio (INR) in patients with mechanical heart valve replacement.

**Methods:** Patients from the anticoagulation clinic of a tertiary care hospital in north India were studied. The anticoagulation profile, INR (International Normalized Ratio) values and administered acenocoumarol dose were obtained from the clinical records of patients. Determination of the *CYP2C9*\*2,\*3 and *VKORC1* -1639G>A genotypes was done by PCR-RFLP (restriction fragment length polymorphism).

**Results:** A total of 111 patients were studied. The genotype frequencies of *CYP2C9* \*1/\*1,\*1/\*2,\*1/\*3 were as 0.883, 0.072, 0.036 and that of *VKORC1* -1639G>A for GG, AG, and AA genotypes were 0.883, 0.090, and 0.027, respectively. The percentage of patients carrying any of the variant alleles of *CYP2C9* and *VKORC1* in heterozygous or homozygous form was 34% among those receiving a low dose of ≤20 mg/wk while it was 13.8 per cent in those receiving >20 mg/wk ( $P=0.014$ ). A tendency of lower dose requirements was seen among carriers of the studied polymorphisms. There was considerable variability in the dose requirements of patients with and without variant alleles.

**Interpretation & conclusions:** The study findings point towards the role of *CYP2C9* and *VKORC1* gene polymorphisms in determining the inter-individual dose variability of acenocoumarol in the Indian patients with mechanical heart valve replacement.

**Key words** Acenocoumarol - *CYP2C9* - dose requirements - INR - oral anticoagulants - *VKORC1*

Acenocoumarol is a commonly prescribed oral anticoagulant for the prevention and treatment of thromboembolic events. It belongs to the coumarin group of anticoagulants. Considerable inter-individual variability in the dose requirements of coumarin group of anticoagulants is seen with the consequent risks of bleeding or thrombosis in case of over or under anticoagulation respectively<sup>1</sup>. Genetic and environmental factors are contemplated to play role in determining optimum dose for an individual.

Studies have demonstrated the effect of *CYP2C9* (cytochrome P450) and *VKORC1* (vitamin K epoxide reductase complex) gene polymorphisms on the dosages of oral coumarin anticoagulants (OCAs namely warfarin, acenocoumarol)<sup>2</sup>. Cytochrome P450 2C9 enzyme is involved in the elimination of acenocoumarol from the body. Allelic variants of *CYP2C9* gene, *CYP2C9\*2* (Arg144Cys) and *CYP2C9\*3* (Ile359Leu), have less catalytic activity than the wild type *CYP2C9\*1* (Arg144/Ile359). The presence of these variants in an individual is thus expected to lower the requirements of the drug. Vitamin K epoxide reductase complex subunit 1 (*VKORC1*) is the target enzyme of OCAs. The inhibition of this enzyme by the OCAs reduces the regeneration of vitamin K from vitamin K epoxide reductase<sup>3</sup>. Several polymorphisms have been found in the coding and the non-coding regions of the *VKORC1* gene. *VKORC1* -1639G>A is a polymorphism in the promoter region of *VKORC1* gene. The presence of the polymorphism reduces the binding of the transcription factor and thereby reduces the gene expression. The reduced level of the target enzyme reduces the dose requirements of the OCAs.

The association of these polymorphisms with coumarin group of drugs has been well studied among various ethnic groups. However, the data from India are scarce and only a couple of studies correlating the dose of acenocoumarol with *VKORC1*-1639G>A polymorphism have been published from India<sup>4,5</sup>. The aim of the current study was therefore, to determine the presence of *CYP2C9\*2*, *\*3* and *VKORC1* -1639G>A gene polymorphisms in Indian patients and to study their effects on the dose of acenocoumarol required to maintain a target INR (International Normalized Ratio) in patients with mechanical heart valve replacement.

### Material & Methods

Patients with rheumatic heart disease who had undergone heart valve replacement were selected from the Anticoagulation Clinic, held by the Department

of Cardiovascular Thoracic Surgery, Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGIMS), a tertiary care hospital in Lucknow, north India. The inclusion criteria were a patient (i) of either sex (ii) of any age (iii) requiring long term anticoagulation with acenocoumarol after undergoing heart valve replacement and (iv) on a regular follow up in the anticoagulation clinic. The exclusion criteria were a patient (i) who had underlying renal or hepatic insufficiency (ii) history of smoking and/or alcohol intake (iii) on a concomitant medication which could interact with acenocoumarol thus affecting its dose requirements. The patient was excluded if he or she was on any of the following medications: antibiotics (ciprofloxacin, co-trimoxazole, erythromycin, fluconazole, isoniazid, metronidazole, voriconazole, rifampicin), cardiovascular drugs (amiodarone, propranolol, diltiazem) non steroidal anti-inflammatory drugs (including COX2 inhibitors), lipid lowering agents, antiepileptics (carbamazepine, phenytoin), selective serotonin reuptake inhibitor (sertraline and omeprazole)<sup>6</sup>. Demographic and clinical data such as the age, sex, weight, height, indication for acenocoumarol, concomitant medications, bleeding episodes, thromboembolic phenomenon were recorded from the clinical records.

Ethical clearance for the study protocol was obtained from the Institutional Ethics Committee. A prior written informed consent was taken from all the patients.

The anticoagulation profile of each patient was obtained from their respective clinical records and included the INR value, dose of acenocoumarol being taken and dose advised at each visit. Two variables the stable therapeutic dose and the percentage of time spent outside the target INR were calculated for each patient. The stable therapeutic dose was defined as mean dose the patient was getting when his/her INR was in a stable therapeutic range<sup>7</sup>. Stable therapeutic INR was defined as at least 2 consecutive INR measurements between the target range of 2-3 measured at least 2 wk apart. The percentage of time spent outside the target INR was calculated as the ratio of the total number of weeks when the INR remained above or below the target INR to the total number of weeks of follow up. For the reported allele frequencies of *CYP2C9\*2* and *\*3* as 15 and 3 per cent, respectively with an  $\alpha$  error of 0.05 and power of 0.80, it was calculated that a sample size of 110 patients would be sufficient for this study<sup>1</sup>.

*Genotyping:* For genomic DNA extraction, blood (2 ml) was collected from each patient in EDTA. DNA

was extracted from blood by using Invitrogen DNA extraction kit Pure Link Genomic DNA kit (Invitrogen Corporation, Carlsbad CA, USA). PCR was used to amplify segments of the *CYP2C9* and *VKORC1* genes from 100 ng genomic DNA in a total reaction volume of 25  $\mu$ l. A total of 2.5 mM MgCl<sub>2</sub>, 200  $\mu$ M each of dATP, dCTP, dGTP, dTTP, 0.025 units/ $\mu$ l Taq DNA Polymerase, 75 mM Tris-HCl (pH 8.8 at 25°C), 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.16  $\mu$ M of each forward and reverse primers and 1  $\mu$ l of template DNA were added per reaction. Polymerase chain reaction was performed using the following conditions: 94°C for 5 min, followed by 34 cycles at 94°C for 45 sec, 54.3°C for 1 min and 72°C for 1 min 30 sec followed by final extension at 72°C for 8 min in a thermal cycler (PTC 200 Thermal Cyclers, BioRad Inc)<sup>8,9</sup>. The Forward and reverse primer sequences for *CYP2C9*\*2, *CYP2C9*\*3, *VKORC1* were as follows: (\*2F= 5'TACAAATACAATGAAAATATCATG-3', \*2R=5'CTAACAACCAGGACTCATAATG3'; for \*3F=5'AGGAAGAGATTGAACGTGTGA-3', \*3R=5'GGCAGGCTGGTGGGGAGAAGGTCA A-3' for VF=-5'ATCCCTCTGGGAAGTC AGC-3' and VR=-5'CACCTTCAACCTCTCCATCC-3', respectively<sup>8,9</sup>. AVAII, STY I, and NCI I were used for DNA digestion for *CYP2C9*\*2, *CYP2C9*\*3, *VKORC1*, respectively. The PCR products were electrophoresed on 2 per cent agarose gel at 120 V for 60 min stained with 0.5  $\mu$ g/ml ethidium bromide in Tris borate EDTA (TBE) buffer pH (8.3) and visualized by ultraviolet irradiation. The representative gel pictures are shown in Fig. 1. The amplicon sizes for the *CYP2C9*\*2, \*3 and *VKORC1* were 690, 130, and 636, respectively. The wild and the minor alleles respectively were identified

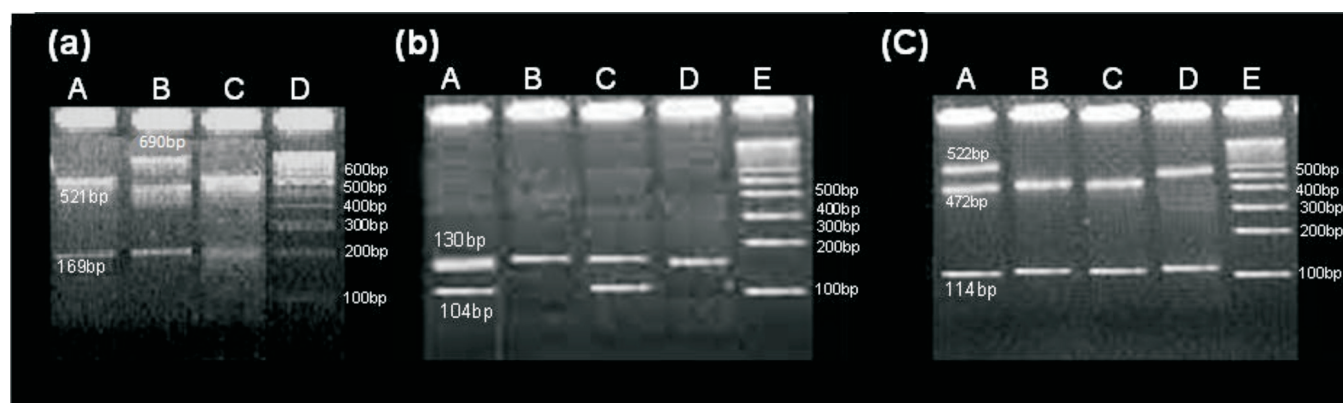
by 521, 169 and 690 bp for *CYP2C9*\*2, 130 and 104, 26 for *CYP2C9*\*3 and 472, 114, 50 and 522, 114 for *VKORC1*.

**Statistical analysis:** One way ANOVA was used to compare mean stable therapeutic doses between the *CYP2C9* and *VKORC1* genotype groups. The difference in number of patients in low and high dose groups with *CYP2C9* and *VKORC1* polymorphism was determined by Fisher's exact test. Student t test was used to compare the mean stable therapeutic dose requirement between patients of *CYP2C9* and *VKORC1* genotypes as per dominant and recessive models. Statistical analyses were done with SPSS software (version 10.0; SPSS, Chicago, IL).

## Results

A total of 111 patients were enrolled in the study. Majority of the patients 110/111 (90%) belonged to the State of Uttar Pradesh (northern India). There were 77 (69.4%) males and 34 (30.6%) females with mean age of 36.4  $\pm$  SD (15-67 yr) and 32.4  $\pm$  SD (17-60 yr), respectively. In 110 patients the primary reason for anticoagulation with acenocoumarol was a heart valve replacement [75 (67.6%) mitral valve, 25 (22.5%) atrial valve, 10 (9%) double valve replacement]. One patient was receiving acenocoumarol after correction of a congenital heart malformation. The genotype and allele frequencies for the studied polymorphisms of *CYP2C9* and *VKORC1* genes are as shown in Table I. The frequency distribution for all the markers was in Hardy Weinberg equilibrium.

For 111 patients the mean stable therapeutic dose was 23.08 mg/week (range 5.50-71.40 mg/



**Fig. 1.** (a) Representative gel pictures of restriction digested DNA fragments showing wild homozygous as 521, 169 bp (lane A, C) and heterozygous as 690, 521, 169 bp (lane B) for *CYP2C9*\*2 (b) Wild homozygous as 130 bp (lane B, D) and heterozygous as 130, 104 bp (lane A, C) for *CYP2C9*\*3 (c) Wild homozygous as 472, 114 bp (lane B, C) and heterozygous as 522, 472, 114 bp (lane A) and variant homozygous as 522, 114 bp (lane D) for *VKORC1*-1639G>A polymorphisms.

**Table I.** Allele and genotype frequencies of *CYP2C9*\*1,\*2,\*3 and *VKORC1* -1639G>A

<i>CYP2C9</i>			<i>VKORC1</i> -1639G>A		
Alleles	N	Frequency (%)	Alleles	N	Frequency (%)
<i>CYP2C9</i> *1	208	93.7	<i>VKORC1</i> G	206	92.8
<i>CYP2C9</i> *2	9	4.1	<i>VKORC1</i> A	16	07.2
<i>CYP2C9</i> *3	5	2.2			
Genotype					
<i>CYP2C9</i> 1/1	98	88.3	<i>VKORC1</i> GG	98	88.3
<i>CYP2C9</i> 1/2	8	7.2	<i>VKORC1</i> AG	10	9.0
<i>CYP2C9</i> 1/3	4	3.6	<i>VKORC1</i> AA	3	2.7
<i>CYP2C9</i> 2/3	1	0.9			

**Table II.** Comparison of stable therapeutic dose requirements of acenocoumarol in the genotypes of *CYP2C9* and *VKORC1*-1639G>A

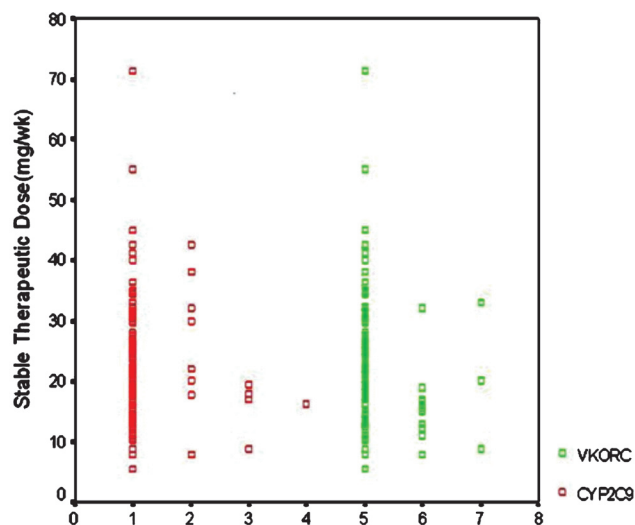
<i>CYP2C9</i>				<i>VKORC1</i> -1639G>A			
Genotype	Mean STD*	Standard Deviation	P value	Genotype	Mean STD*	Standard Deviation	P value
<i>CYP2C9</i> 1/1	23.15	10.41	0.373	<i>VKORC1</i> GG	23.88	10.45	0.064
<i>CYP2C9</i> 1/2	26.30	11.43		<i>VKORC1</i> AG	15.94	6.51	
<i>CYP2C9</i> 1/3	15.82	4.79		<i>VKORC1</i> AA	20.65	12.07	
<i>CYP2C9</i> 2/3	16.33	-					

\*STD, stable therapeutic dose in mg/week

week). The mean weekly stable therapeutic dose for patients carrying various genotypes of *CYP2C9* and *VKORC1* is as shown in Table II. Pearson correlation coefficient of stable therapeutic dose with the age, body surface area were -0.037 and 0.067, respectively. Considerable variability in dose requirements among all genotypes was noticed (Fig. 2). Statistical analysis of the difference of the mean stable therapeutic dose between combination of genotypes assuming dominant and recessive models of genotypes of *CYP2C9* and *VKORC1* is shown in Table III.

The patients were divided into two groups based on the stable therapeutic doses - those requiring doses  $\leq 20$  mg/week and those requiring  $>20$  mg/week, and calculated the proportion of patients carrying wild allele to the variant alleles in the two groups. The total numbers of patients requiring a dose of  $\leq 20$  and  $>20$  mg/week were 53 and 58, respectively. The number of patients with any of the variant allele of *CYP2C9* and *VKORC1* in heterozygous or homozygous form was 18 out of 53 (34%) in patients receiving a low dose of  $\leq 20$  mg/week while it was eight of 58 (13.8%) in those receiving  $>20$  mg/week ( $P=0.014$ ). The differences

were statistically significant when any of the variant allele was compared with the wild type allele, it was observed that of the 26 patients with some variant



**Fig. 2.** Depiction of the variability in dose requirements in various genotype 1, 2, 3, 4 = *CYP2C9* genotype 1/1; 1/2; 1/3; 2/3, respectively; 5, 6, 7 = *VKORC1* genotype GG; GA; AA, respectively.

**Table III.** Statistical analysis of the comparison of mean stable therapeutic dose between combination of genotypes as per the dominant and recessive models for *CYP2C9* and *VKORC1*

Gene	Combination of genotype	P value	95 % CI
<i>CYP2C9</i>	2/3+1/2+1/3 vs 1/1 (Dominant model)	0.783	-5.26 to 6.96
	2/3 vs 1/2+1/3+1/1 (Recessive model)	0.517	-13.94 to 27.5
<i>VKORC1</i>	AA+AG vs GG (Dominant model)	0.025	0.88 to 12.82
	AA Vs AG+GG (Recessive model)	0.686	-9.62 to -14.57

**Table IV.** Comparison of the stability of anticoagulation by wild and variant alleles

Genotype	Mean % of time outside target INR 2-3	Standard deviation	P value
Wild	34.23	25.10	0.8
Any variant allele	32.82	24.7	

alleles of either gene, (18/26) (69.2%) needed weekly dose  $\leq 20$  mg while of the 85 patients with wild type of allele in both genes (35/85) (41.2%) needed low dose ( $P=0.01$ ).

A comparison of the percentage of time of follow up spent outside (above or below) the target therapeutic INR showed that it was similar in patients with wild and minor/variant alleles with as much as 34 per cent time being spent outside the target range (Table IV).

### Discussion

The prescription of an optimal dose of acenocoumarol is difficult due to considerable individual variability in drug response. Our data revealed as much as 15 fold variation in the stable therapeutic dose requirements of our patients. Pharmacogenetic studies on oral coumarin anticoagulants have pointed towards *CYP2C9*\*2,\*3 and *VKORC1* gene polymorphisms as the most important genetic variants determining the dose requirements of these anticoagulants. The prevalence of these polymorphisms varies across different ethnic groups. For example, the allele frequencies of *CYP2C9*\*2 and *CYP2C9*\*3 have been shown as 0.105 and 0.067 in Russian population<sup>10</sup>, as 0.125 and 0.085 in Caucasians<sup>11</sup>, and as 0.165 and 0.095 in Croatians<sup>12</sup>. However, the prevalence of these polymorphisms in India appears to be less. In our study comprising of

patients from Northern India, the allele frequencies of *CYP2C9*\*1,\*2,\*3 were 0.937, 0.041, 0.022, respectively and the genotype frequencies *CYP2C9*\*1/\*1,\*1/\*2,\*1/\*3 were 0.883, 0.072, 0.036, respectively. Jose *et al*<sup>13</sup> have reported the *CYP2C9*\*1,\*2,\*3 allele frequencies as 0.88, 0.04, 0.08, respectively in south Indian population. It appears that allele frequencies of *CYP2C9*\*2 in the north Indian population (present study) were more, whereas *CYP2C9*\*3 was more common than *CYP2C9*\*2 in southern India. Recently a study from northern India by Rathore *et al*<sup>4</sup> has reported the *CYP2C9*\*2,\*3 frequencies as 0.049 and 0.039. The lower prevalence for the *CYP2C9*\*3 and \*2 alleles has also been reported from China<sup>14</sup>.

In our study, the genotype frequencies for the *VKORC1* GG, AG, and AA genotypes were 0.883, 0.090, and 0.027 respectively. For this gene polymorphism also ethnic variations have been demonstrated. Two studies on Indian population<sup>4,15</sup> have reported the GG, AG, AA genotype frequencies of *VKORC1* -1639G>A polymorphism as 80, 13, 7 per cent and 73.53, 24.51, 1.96 per cent, respectively. The AA and AG genotype frequencies in our study were slightly lower than these studies.

Regarding the effects of the studied polymorphisms on acenocoumarol dose requirements we found that the carriers of the variant alleles had a tendency for lower dose requirements. The carriers of *CYP2C9*\*3 had the lowest dose requirement. This observation is in concordance with other similar studies on the effects of *CYP2C9*\*2,\*3 polymorphisms on acenocoumarol<sup>16,17</sup>. The bearers of *CYP2C9*\*3 allele have been found to have lowest dose requirements. On analyzing the data by the low vs high dose requirement of  $\leq 20$  and  $> 20$  mg/week, the proportion of patients with wild allele to the patients carrying at least any one of the variant alleles was significantly different in the two groups. The low dose group had a greater proportion of variant alleles. As many as 69.2 per cent of the patients carrying any one of the 3 variant alleles required a stable therapeutic dose of  $\leq 20$  mg/week whereas only 41.2 per cent of those who were carrying the wild alleles needed a low dose. Thus, it appears that the presence of variant allele in a patient places him at a higher chance of requiring a low dose. If such a patient is started on the standard dose, there may be a risk of overanticoagulation.

A study on Greek patients by Markatos *et al*<sup>18</sup> showed significant difference in the dose requirement of patients with variant alleles (*CYP2C9*\*3, *VKORC1*A

$P < 0.001$ ). Similarly in Serbian population studied by Kovac *et al*<sup>19</sup> as much as 2.5-fold higher dose requirements in patients with GG genotype of *VKORC1* polymorphism have been reported. In our study, the presence of the A allele of *VKORC1* G>A as per the dominant model reached a statistical significance in terms of affecting the dose requirement. However the combination of the other genotypes as per the Dominant and Recessive models for *CYP2C9* and *VKORC1* could not significantly affect the dose of acenocoumarol. A major reason for this could be the small sample size of the study and a lesser number of variant alleles detected in the studied patients. Other factors that might be contributing to the observed differences from other studies can be differences in the methodologies of calculation of stable doses and the analysis done by categorization of patients under differently described low, medium and high dose groups.

To evaluate the association of stability of anticoagulation with the genotypes, we calculated the percentage of time of follow up being spent outside the target INR range. About 30-34 per cent time was being spent outside the target range in our patients with no significant difference between the patients with or without variant alleles. Tassies *et al*<sup>20</sup> noted a statistically significant difference in the percentage of time spent within the target INR by the carriers of *CYP2C9*\*3 vs wild genotype. Although the time being spent outside the target range was comparable, the small number of *CYP2C9*\*3 variants in our study probably led to insignificant results. Also there could be differences in the INR monitoring protocol being followed for the two study groups.

Our data also showed a wide variability in the dose requirements of patients with same genotypes. This suggests that there may be other genetic and non-genetic factors responsible for dose variability in our patients. For example, a search for new genetic variants contributing to inter-individual variation on stabilized acenocoumarol doses was made through Genome-wide association study<sup>21</sup>. This study identified *CYP4F2* and *CYP2C18* as additional genetic variants contributing significantly to acenocoumarol doses. Thus, further studies on the effects of these genetic variants on drug doses are indicated. Several environmental factors have also been known to affect the response of the coumarin anticoagulants. The important among them are dietary factors, smoking and drug-drug interactions. Increase in the dietary intake of vitamin K, a high protein diet and smoking have been shown to increase the dose

requirements<sup>22-24</sup>. None of our patients gave history of smoking and patients on concomitant medications well known to interact with coumarin anticoagulants were excluded. However, the influence of dietary factors cannot be ruled out due to the retrospective design of our study.

A major limitation of our study was the small sample size. Hence future studies should be done on a larger sample size. Another limitation was a failure to determine the role of some environmental factors like vitamin K in the diet of our patients.

In conclusion, our study points towards the role of *CYP2C9* and *VKORC1* gene polymorphisms in determining the inter-individual dose variability of acenocoumarol in the north Indian patients with mechanical heart valve replacement. This knowledge shall be significant considering the wide use of this oral anticoagulant with a narrow therapeutic index. Further studies with larger sample size are needed to determine the strength and the significance of emerging data.

### Acknowledgment

Authors acknowledge the SGPGIMS, Lucknow for granting funds to carry out this work, and thank Dr S.K. Mandal for statistical analysis of the data. Authors also thank all the patients for their cooperation.

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