Genetic Polymorphisms In Clopidogrel And Its Association With Adverse Cardiac Events After Coronary Intervention In Tertiary Care Centre From South India

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*Abstract: background: coronary artery disease is the leading cause of death in developing countries and the main treatment strategy includes pci which is usually followed by dual antiplatelet therapy (dapt) with aspirin and clopidogrel. Clopidogrel resistance from genetic polymorphisms incyp2c19 gene involved in hepatic activation of clopidogrel leads to clopidogrel nonresponsiveness and may result in increased adverse clinical outcomes. These polymorphisms in cyp2c19 gene and their impact in mace have been studied in southindian population only in limited number of studies.Methods:we studied 118 consecutive patients (mean age 55.7±10.7 years; 90% male) taking clopidogrel, with angiographically proven coronary artery disease for various genetic polymorphisms incyp2c19 gene. Relationship between loss of function mutation and clinical presentation with higher mace events including recurrent acute coronary syndromes, stent thrombosis were analyzed and genetic analysis. Results: out of 118 patients, 38 (32%) had normal genotype, 23 (20%) had loss offunction mutation(*2) the poor metabolizer type, and 57 (48%) had intermediate metabolizers (*2/*1). Final analyses included 118 patients, with 23 (20%) having loss of function. Seven patients developed stent thrombosis while on clopidogrel; all seven had loss of function mutationin cyp2c19 gene. Conclusion: we observed that the cyp2c19*2 and cyp2c19*1/*2 are the major determinants of clopidogrel efficacy. Acute stent thrombosis was observed in patients carrying cyp2c19*2 variant allele. In patients with an acute myocardial infarction who were receiving clopidogrel, with cyp2c19 loss-of-function alleles had higher rate of subsequent major cardiovascular events in comparison with normal genotype individuals.*

Keywords: antiplatelet therapy, acute coronary syndrome, percutanous coronary intervention, clopidogrel, pharmacogenetics, major adverse cardiac events

1. INTRODUCTION

The dual antiplatelet therapy (DAPT) with aspirin and clopidogrel has been the main stay in the treatment for acute coronary syndromes (ACS) and patients undergoing percutaneous

coronary intervention (PCI)[1].The benefit of dual antiplatelet treatment is proven in various trials worldwide, there is high inter-individual variability in clinical response to clopidogrel therapy because of genetic polymorphism involving CYP2C19 gene, which is responsible for its conversion to active metabolite[2], and despite dual platelet inhibition, the ineffective response of clopidogrel is found to be associated with higher major adverse cardiovascular events, including recurrent ischemic cardiovascular events, stent thrombosis, myocardial re-infarction after stent implantation[3].

Noted among these genes are the polymorphisms of multidrug resistance protein(MDR) 1, CYP2C19 and its alleles, P2Y1, and P2Y12 adenosine diphosphate (ADP) receptor, which are related to clopidogrel resistance in Indian population[4].

Clopidogrel is a pro-drug that must be metabolized in liver by the cytochrome P (CYP) 450 enzymes, to be converted to active metabolites[5]. Metabolic activation by CYP2C19 gene is important step and several genetic variants are associated with the reduced or enhanced CYP2C19 activity[6,7]. In our study, the clinical factors and genetic polymorphism that were associated with clopidogrel resistance, long-term MACE events including stent thrombosis, in patients who have undergone PCI were studied[8,9]. Hence the aim of our study was to assess the influence of CYP2C19 genetic polymorphisms in acute coronary syndrome patients on clopidogrel, undergoing percutaneous coronary intervention and to assess the major adverse cardiac events in a tertiary care centre from Tamilnadu.

2. MATERIAL AND METHODS

One hundred and eighteen(118) consecutive in-patients who were admitted with acute coronary syndrome and underwent PCI at our centre were enrolled in our study. The Institutional Ethical Committee has approved the study and informed consent from each patient was obtained.Standard definitions of ACS as defined by ACC guidelines were used in the study to include the patients. The baseline demographic and clinical details were recorded in a standard format. PCI was performed immediatelyafter a loading dose of clopidogrel. All coronary angiograms were evaluated by a single cardiologist who was blinded to all other clinical and genetic data. Follow-up visits were conducted in our review outpatient centre for one year.

The study participant comprised 118 ACS patients,who had received loading dose and maintenance therapy of 75 mg clopidogrel as a daily dose. Patients on other antithrombotic drugs, such as glycoprotein inhibitors, were excluded from thestudy. Patients suffering from bleeding disorders and othercoagulopathies and pregnant or lactating women were notincluded in the study.

Genotyping-Genetic analysis of CYP2C19*2 by PCR-RFLP technique

3. METHODS:

Genomic DNA extraction

0.1ml of peripheral blood was lysed in 100µl of cell lysis buffer containing 36% to 50% guanidine hydrochloride (Cat# 740951.50, Nucleospin Blood DNA Kit, Machery Nagel, Germany) and incubated at 57°C for 2 hours to enable complete lysis of leucocytes. Following lysis, an equal volume of 100% ethanol was added to precipitate the genomic DNA. Subsequently, the entire content was transferred to DNA spin columns containing silica membrane and centrifuged at 8000 rpm for 1min at room temperature. The precipitated DNA gets captured in the silica membrane during this step. Following DNA capture, the

silica columns were washed twice with wash buffer (supplied by the manufacturer Machery Nagel). Degraded proteins and membrane lipid particles get washed off during the wash steps. After the two wash steps, the captured DNA from the silica membrane was eluted with 50µl of elution buffer (supplied by the manufacturer Machery Nagel).

Polymerase Chain Reaction followed by Restriction Fragment Length Polymorphism (PCR-RFLP)

The following set of primers were used to amplify the CYP2C19*2 (rs4244285) region that causes G681A transition in exon 5 (Forward: accagagcttggcatattgtatct, Reverse: gattcttggtgttcttttactttct). To amplify the CYP2C19*3 (rs4986893) region that causes G636A transition in exon 4 the following set of primers were used (Forward: tttcatcctgggctgtgctc, Reverse: tgtacttcagggcttggtcaat). Both primers were used independently on 50ng of DNA samples and were subjected to amplification under the following conditions. Initial denaturation at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds, primer extension at 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. The CYP2C19*2 primers amplified a 321bp fragment, while the CYP2C19*3 primers amplified a 234bp fragment. To detect CYP2C19*2 and CYP2C19*3 polymorphisms, the PCR fragments was subjected to restriction digestion with Sma1 enzyme (Cat#1085A, Clonetech Takara, Japan) and BamH1 enzyme (Cat#1010A, Clonetech Takara, Japan) respectively. Following digestion, 10µl aliquots of digested PCR products were analyzed by running them in a 1.7% agarose gel at 100V for 15 minutes with 1X TAE (Tris Acetate EDTA) buffer. The DNA bands were visualized by staining the gel with ethidium bromide (a DNA intercalating agent that fluoresces when excited by UV in the range of 302nm to 364nm), and images were captured with gel documentation unit.

Data interpretation

For CYP2C19*2 polymorphism: When G allele was present in homozygous condition, the 321bp fragment was completely digested into 212bp and 109bp fragments, which migrated as two bands during electrophoresis. These samples were designated as CYP2C19*1/*1 (normal metabolizer). When A allele was present in homozygous condition, the 321bp fragment remained undigested and migrated as a single band during electrophoresis. These samples were designated as CYP2C19*2/*2 (poor metabolizer). When both G and A alleles were present in heterozygous condition, the 321bp fragment carrying G allele was digested into 212bp and 109bp fragments, while the other part carrying A allele remained undigested. As a result three bands were observed during electrophoresis. These samples were designated as CYP2C19*1/*2 (intermediate metabolizer).

For CYP2C19*3 polymorphism: When G allele was present in homozygous condition the 234bp fragment was completely digested into 135bp and 99bp fragments, which migrated as two bands during electrophoresis. These samples were designated as CYP2C19*1/*1 (normal metabolizer). When A allele was present in homozygous condition, the 234bp fragment remained undigested and migrates as a single band during electrophoresis. These samples were designated as CYP2C19*3/*3 (poor metabolizer). When both G and A alleles were present in heterozygous condition, the 234bp fragment carrying G allele was digested into 135bp and 99bp fragments, while the other part carrying A allele remained undigested. As a result three bands were observed during electrophoresis. These samples were designated as CYP2C19*1/*3 (intermediate metabolizer).
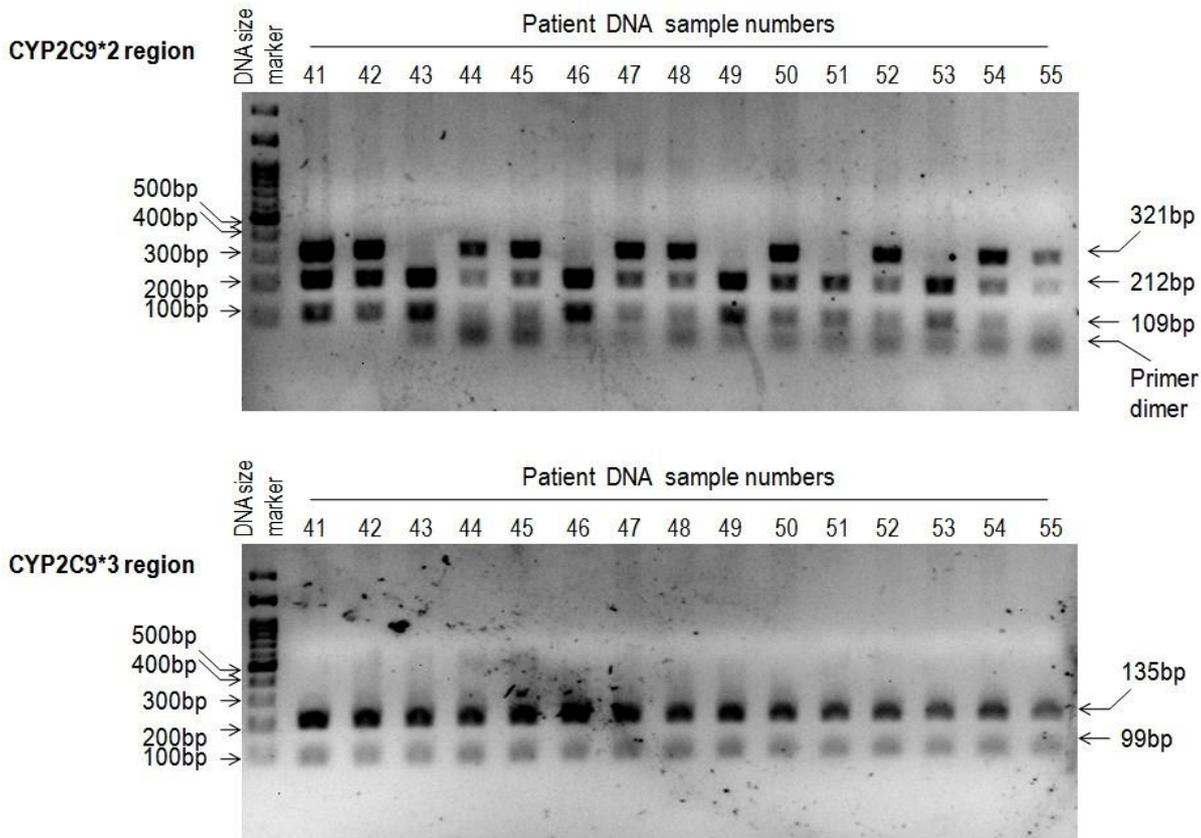
4. STATISTICAL ANALYSIS

The data were analysis using SPSS (Statistical Package for Social Science)   Ver 16.01. The data collected were scored and analyzed, continuous variables were presented as means with Standard deviation (SD) and categorical variables were presented as frequency and percentages. Student t-test was used for testing the significance of all the mean & standard deviation between two groups and Chi-square test was used to compare proportions. P value ≤ 0.05 was considered as statistically Significant in all the statistical results.

PICTURE -1 A representative of Agarosejel Electrophoresis is shown:

Top panel: CYP2C9*2 amplicon digested with SmaI. Lanes indicated 41, 42, 44, 45, 47, 48, 50, 52, 54 and 55 show three bands (intermediate metabolizer). Lanes indicated 43, 46, 49, 51 and 53 show two bands (normal metabolizer).

Bottom panel: CYP2C9*3 amplicon digested with BamHI. Lanes indicated 41 to 55 show two bands (normal metabolizer).

intercalating agent that fluoresces when excited by UV in the range of 302nm to 364nm), and images were captured with gel documentation unit.

5. RESULTS:

TABLE 1:BASE LINE CHARACTERISTICS OF SUBJECTS ACCORDING TO GENOTYPE.

| Variables		Normal (N=38)	Intermediate (N=57)	Poor (N=23)	P-value
Gender	Male	29 (76.32 %)	42 (73.68 %)	15 (65.22 %)	0.63
	Female	9 (23.38 %)	15 (26.32 %)	8 (34.78 %)	
Age (Mean ± sd)		48.61 ± 8.97	52.74 ± 9.50	50.30 ± 10.66	0.12
Diabetes		12 (31.58 %)	29 (50.88%)	8 (34.78%)	0.13
Smokers		21 (55.26 %)	21 (36.84%)	8 (34.78%)	0.45
Alcoholism		13 (34.21 %)	20 (35.09 %)	6 (26.09 %)	0.73
Hypertension		16 (42.11 %)	25 (43.86 %)	7 (30.43 %)	0.32
Dyslipidemia		2 (5.26 %)	3(5.26 %)	-	0.53

In our study intermediate metabolizers form the majority 57followed by normal genotype 38. Diabetes is common among intermediate metabolizers. Smoking and alcoholism are very much prevalent in normal and intermediate genotype.Our study group is predominantly male gender, so it is difficult to generalise to female patients. However the P–value is not significant for each of the above mentioned characteristics.The genotypes of the predicted metabolizer phenotypes are summarizedin Table 1.

Out of 118 patients, 38 (32%) had normal genotype(*1/*1), 23 (20%) had loss off function mutation(*2/*2)the poor metabolizer type, and 57 (48%) had intermediate metabolizers (*2/*1). The demographic, clinical manifestations and angiographic findings of all patients were stratified according to three genotypes and were shown in table 1. No significant difference in haemoglobin, platelet count and serum creatinine, usage of routine drugs like aspirin, clopidogrel, beta blockers,calcium channel blocker, statins was observed among all the three genotypes except for beta blockers (p= 0.05) (Table 2).

TABLE 2: Clinical Laboratory Parameters and Percentage of Subjects on Various Drugs : Data is distributed According to Genotype

Variables	Normal (N=38)	Intermediate (N=57)	Poor (N=23)	P-value
Hemoglobin	13.84 ± 38.97	12.34 ± 2.31	13.36 ± 5.24	0.93
Platelet Count	2.48 ± 0.96	2.34 ± 1.28	2.26 ± 1.06	0.91
SerumCreatinine	1.08 ± 0.11	1.12 ± 0.20	1.13 ± 0.30	0.86
Clopidogrel	25 (65.79 %)	43 (75.44%)	16 (69.57 %)	0.59
CCB	5(13.16 %)	7 (12.28 %)	4 (17.39 %)	0.83
Beta Blockers	17 (44.74 %)	37 (64.91 %)	20 (86.95 %)	0.004
ACEI	28 (73.68 %)	40 (70.18 %)	19 (82.61%)	0.52
Atorvastatin	13 (34.21 %)	18 (31.58 %)	15 (65.21 %)	0.02
Aspirin	28 (73.68 %)	39 (68.42 %)	18 (78.26 %)	0.65

PICTURE-2 GENOTYPE DISTRIBUTION OF CLOPIDOGREL EFFICIENCY.
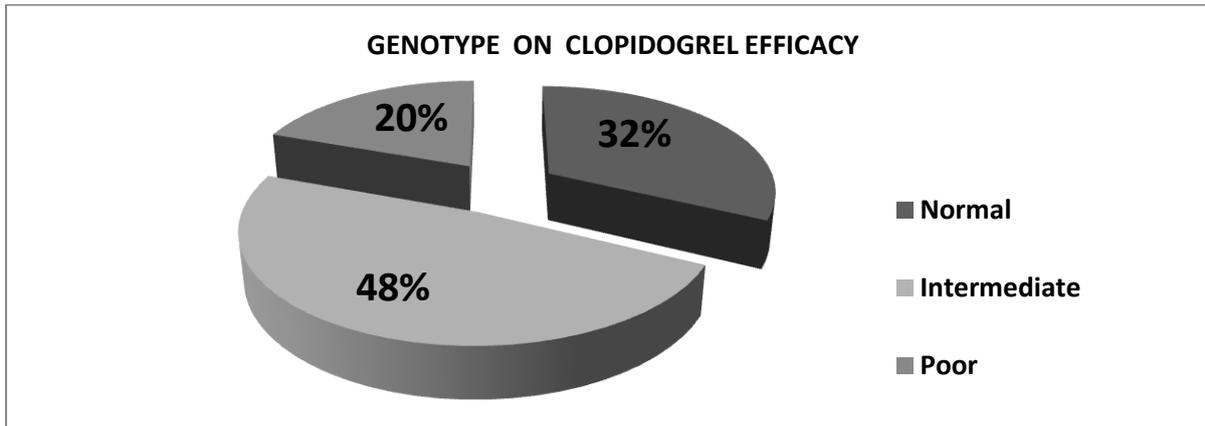
PICTURE – 3 CHARACTERISTICS OF SUBJECTS ACCORDING TO GENOTYPE
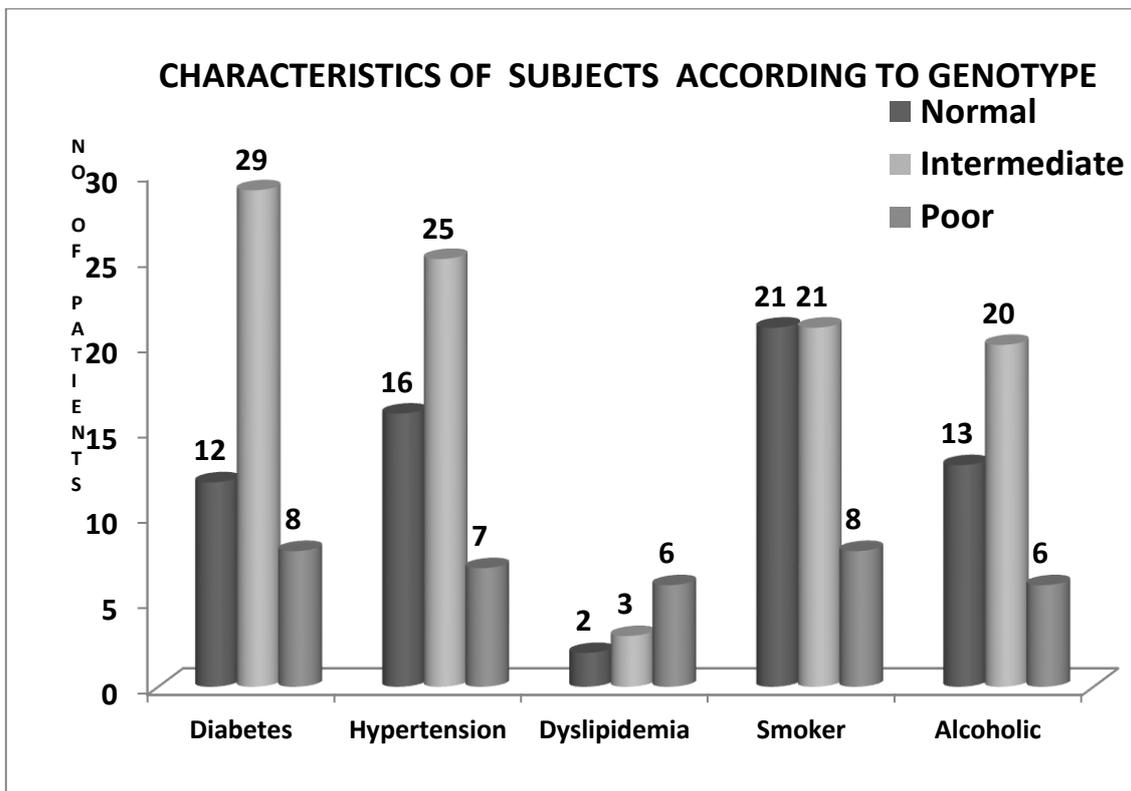
The use of concomitant medications, including aspirin, omeprazole, and atorvastatin, was similar among the CYP2C19 metabolizer groups. Final analyses included 118 patients, with

23 (20%) having loss of function, among these seven patients developed stent thrombosis while on clopidogrel, interestingly all seven had loss of function mutation.

6. DISCUSSION:

Coronary artery disease is the leading cause of death worldwide and the treatment mainly comprises of medical management and percutaneous coronary intervention, which is a vital and proved treatment to restore myocardial blood flow and to salvage the ischemic myocardial tissue. Although the current antiplatelet therapy armamentarium includes aspirin, clopidogrel, prasugrel and ticagrelor, the dual antiplatelet therapy with aspirin and clopidogrel is the main stay in our country, because of availability and cost factors[10].This dual strategy is to prevent adverse cardiac events such as stent thrombosis after PCI,but despite dual drug strategy as high as 0.5 to 1.5% of stent thrombosis do occur in drug eluting stents too as evidenced by latest studies[11]. Eventhough the cause of stent thrombosis is multifactorial including procedural factors,correct sizing, vessel wall integrity and various other factors. Anticoagulation and antiplatelets have a vital role in deciding the outcome after PCI.Often there is high inter-individual variability in clinical response to clopidogrel therapy due to genetic polymorphism involving CYP2C19 gene, resulting in ineffective response to clopidogrel, which is associatedwith higher major adverse cardiovascular events, including recurrent ischemic cardiovascular events, stent thrombosis, myocardial re-infarction after percutaneous intervention[12,13].

Many developing countries still depend upon clopidogrel as main stay for antiplatelet therapy in ACS and related other vascular diseases, because of low socioeconomic status of large population and the cost involved.In many institutes in our state more than 95%of PCI patients are only on clopidogrel in DAPT maintenance therapy.

Clopidogrel inhibits platelet activation byselectively and irreversibly blocking P2Y12 receptor. Clopidogrel is a prodrug, requiring hepatic metabolism, involving many cytochrome P450(CYP) microenzymes (CYP2C19,3A4,3A5,2C9,2C17 and others).It requires metabolic conversion before inhibiting platelet aggregation. Among these CYP2C19 contributes to more than 45%for conversion of clopidogrel to active thiol metabolite.Hence CYP2C19 enzyme plays an vital role in clopidogrelactivation, which has been governed by CYP2C19 gene,located in chromosome 10.The therapeutic response of clopidogrel is determined by various factors in pharmacokinetics including intestinal absorption,hepatic bio-activation, both of which are governed and regulated by genetic polymorphisms in the CYP2C19 genes[14,15,16].

There are many gene variants associated with the reduced or enhanced CYP2C19 activity, depending upon the loss of functional alleles CYP2C19*2 and CYP2C19*3 leading to complete absence of this enzyme, and gain-of-functional alleleCYP2C19*17 leading to the increased expression of this enzyme. In persons with CYP2C19*2 and CYP2C19*3,there is reduced conversion of clopidogrel to active metabolite resulting in recurrent thrombotic events, while on clopidogrel treatment . In persons withCYP2C19*17- the rapid metabolizer type,have increased enzyme activity resulting in higher active metabolite,ending in higher bleeding tendencies[17].

There is significant inter-ethnic differences inCYP2C19 allelic variants.The allele frequencies of CYP2C19*2, CYP2C19*3 and CYP2C19*17 have found to be 0.375, 0.010 (rare) and 0.165 for Indians[18].In a study from south India, it was reported the high prevalence (66%) of CYP2C19*2 variant allele [19]. In a study from north India, it was shown that allele

frequencyof CYP2C19*1 and *2 was 0.7 and 0.3 whereas CYP2C19*3 allele was absent in north Indians[20], whereas very low frequency was reported in south Indian population (2%)[21].This shows that carriers of CYP2C19*2 or *3 or both are more likely to be resistant to clopidogrel with inadequate platelet inhibition resulting in increased adverse clinical events after percutaneous cardiac intervention.So we should take note not only the poor responders but also the intermediate responders which form a majority among our study.

TABLE 3: Primary and Secondary Outcomes by Genotype

Variables	Normal (N=38)	Intermediate (N=57)	Poor (N=23)	P-value
MACE				
Death	2 (5.26 %)	3 (5.26 %)	7 (30.43%)	0.03
MI	1 (2.63 %)	8(14.04 %)	5(21.74%)	0.06
Ischemic stroke	2 (5.26 %)	5 (8.77 %)	2 (8.70%)	0.80
Stent thrombosis	2 (5.26 %)	3 (5.26 %)	7 (30.43%)	0.03
Unstable angina	3 (7.89 %)	5 (8.77 %)	2(8.70%)	0.99

On analysing the MACE events, it is more common among the intermediate metabolizers. The incidence of stent thrombosis is more with intermediate and poor metabolizers.

There were certain limitations in our study, only small number of patients were included,other genetic factors apart from CYP2C19 LOF alleles were not investigated.

7. CONCLUSION:

This study conducted in 118 patients with acute coronary syndrome and receiving maintenance therapy of 75 mg of clopidogrel daily, revealed the carriers of the loss-of-function CYP2C19*2   polymorphism had a significantly higher MACE events in PCI patients, including stent thrombosis.

Loss of function CYP2C19*2 polymorphisms in Indian population are very common as compared to other populations. The loss of function status has high impact on clinical outcome after PCI, so we recommend a larger study involving high risk patients with PCI, together with genetic testing, along with P2Y12 receptor polymorphisms, may be needed toovercome the nightmares of CYP2C19 gene polymorphisms in postPCI patients.In TRITON-TIMI 38 trial cost effectiveness analysis revealed genotyping testing before DAPT could be more useful[22].Furthermorein our country with many people under low socioeconomic group,the cost factor has to be kept in mind before considering newer antiplatelet drugs like prasugrel and ticagrelor.Our study suggest that doing genotyping prior to PCI can be useful in identifying patients with CYP2C-19 loss of functional allele,so that an alternative antiplatelet therapy canbe considered,inorder to reduce major adverse cardiac events[23]. Cumulative cost of alternate antiplatelet drug in place of clopidogrel is more, when compared to genetic testing,so we highly recommend routine genotyping prior to percutaneous coronary intervention.

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