

**A REVIEW ON ANALYSIS OF ASSOCIATION OF GENETIC
POLYMORPHISM OF CYP2C19 AND P2Y12 WITH PHENOTYPIC
RESPONSE OF CLOPIDOGREL IN HEALTHY VOLUNTEERS
SAMPLES**

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Article Received on
28 Oct 2015,

Revised on 18 Nov 2015,
Accepted on 08 Dec 2015

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ABSTRACT

Clopidogrel being used as one of the major drugs used in the treatment of Acute Coronary Syndrome, along with Aspirin it is the standard of care in such cases along with stenting, when a long term dual antiplatelet treatment is needed. The responses to clopidogrel have recently shown a wide inter-individual variation. Previous studies have shown that few polymorphisms in the CYP2C19 and P2Y₁₂ gene affect the platelet aggregometry response to Clopidogrel. This in return increases the risk of recurrent thrombotic events in patients who are poor responders to clopidogrel. Thus it has become crucial to determine the relationship between these polymorphism and response to Clopidogrel. The association in patients with these polymorphism and actual clinical response in normal healthy human volunteers in

Indian population has not yet been studied extensively. This review paper helps in analysing and associating genetic polymorphisms of CYP2C19, P2Y₁₂ and response to Clopidogrel in normal healthy participants. A significance association has been found that between genetic polymorphisms of CYP2C19, P2Y₁₂ and Clopidogrel response, using general statistical methods.

KEYOWRDS: clopidogrel, CYP2C19, P2Y12, Indian population.

I. INTRODUCTION

Clopidogrel is one of the major drugs used in the treatment of Acute Coronary Syndrome. Along with Aspirin it is the standard of care in Percutaneous Coronary Interventions with

stenting, when a long term dual antiplatelet treatment is needed. However the response to clopidogrel shows wide inter-individual variations. Various studies have shown that the polymorphisms in the genes responsible for Clopidogrel absorption and metabolism are a major factor for these variations. Polymorphisms in the CYP2C19 and P2Y₁₂ gene affect the platelet aggregometry response to Clopidogrel. This increases the risk of recurrent thrombotic events in patients who are poor responders to clopidogrel. Hence it is important to determine the relationship between these polymorphism and response to Clopidogrel. There has been no study to date testing the association in patients with these polymorphism and actual clinical response in normal healthy human volunteers in Indian population. Hence the present study was undertaken to find out the association of genetic polymorphisms of CYP2C19, P2Y₁₂ and response to Clopidogrel in normal healthy participants.

CORONARY ARTERY DISEASES

Coronary artery disease starts developing when your coronary arteries, the major blood vessels that supply your heart with blood, oxygen and nutrients. Thus they become damaged or diseased. Coronary artery disease (CAD) is going to be first in the leading causes of disability by the year 2020 and is a leading cause of mortality worldwide. An estimate of life lost due to total cardiovascular disease (CVD) among the Indian men and women aged 35- 64 is going to be higher than comparable countries such as Brazil and China (Leeder S, 2004). Disability adjusted life years (DALYs), a commonly used metric of premature of death and disability, is also estimated to increase at rates comparable or above most other regions throughout the world. Beyond these projections, increase in the DALYs lost to CHD in India have been predicted from 5.6 million to 7.7 million in women and 7.67 million to 14.4 million in men from 2000 to 2020 (Gupta .R., 2008). Coronary artery disease is a condition in which there is an inadequate supply of blood and oxygen to a portion of the myocardium. It generally occurs when there is an imbalance between myocardial oxygen supply and demand. The inflammation and Cholesterol containing deposits (plaque) in your arteries are usually to blame for coronary artery disease. These building up of plaques, start narrowing coronary arteries and decreasing blood flow to the heart. Eventually, this decreased blood flow may cause chest pain (angina), shortness of breath, or other diseases associated with coronary artery. A complete blockage can cause a heart attack.



Figure 1. Depicts the plaque in the arteries

ACUTE CORONARY SYNDROME (ACS)

Acute Coronary Syndrome (ACS) can be defined as a spectrum of clinical conditions from unstable angina to ST-elevation Myocardial infarction (MI) consequent to myocardial ischemia. In vivo imaging techniques applied in humans and the success of fibrinolytic and antithrombotic therapy in ACS established in practice the role of thrombosis in their pathogenesis. A number of micro anatomic mechanisms underlie acute coronary thrombosis. The rupture of plaque's protective fibrous cap can cause lethal coronary thrombosis, according to autopsy studies (Peter Libby., 2005). This physical disruption of the atherosclerotic plaque results in almost all acute coronary thrombosis. Once platelets are exposed to the ruptured plaque, platelet adhesion occurs, followed by platelet activation and release of various vasoactive substances. These released substances, especially thromboxane A₂ and adenosine diphosphate (ADP), cause platelet aggregation and formation of white thrombus. While platelets secure the primary haemostasis at the site of ruptured plaque the tissue factor by activating the extrinsic coagulation cascade activates thrombin formation. Thrombin stabilizes the final clot or red thrombus (secondary haemostasis) by converting fibrinogen to fibrin and that leads in some cases of ACS (Hani Jneid, et al., 2003). Antithrombotic therapy is the foundation of treatment for patients with Acute Coronary Syndrome (ACS). Its 2 components are: (1) antiplatelet therapy, which causes reduction in platelet activation and aggregation, integral steps in the formation of a thrombus after plaque disruption, and (2) anticoagulant therapy, it prevents the deposition of fibrin strands in a clot by targeting its clotting cascade (Amit Kumar, 2009). Platelet adhesion, activation, and aggregation are central to thrombus formation, which leads to atherosclerotic plaque disruption and results in acute coronary syndromes. Aspirin and clopidogrel apply their antiplatelet effects by inhibition of thromboxane A₂ production and adenosine diphosphate-induced platelet aggregation pathways. Aspirin has shown benefits in primary and secondary prevention of coronary artery disease. Clopidogrel is an alternative antiplatelet agent which is

used in patients with aspirin intolerance. It is especially useful in combination with aspirin after coronary stent procedures (Hani Jneid, et al., 2003). Using clopidogrel plus aspirin significantly decreases the risk of the first primary complex end point of stroke, nonfatal MI, and cardiovascular death (9.3% vs. 11.4%, for a reduction of 20%) and 14% in the second primary complex of cardiovascular death, stroke, nonfatal MI, and refractory ischemia, compared with aspirin alone. Peter et al (2003) in the treatment of patients with acute coronary syndrome (ACS) studied the benefits and risks of adding clopidogrel to varying doses of aspirin, in their conclusions from the 'Clopidogrel in Unstable angina to prevent Recurrent Events' (CURE) Study. In this analysis, patients were divided into the 3 aspirin dose groups: ≤ 100 mg, 101 - 199 mg, and ≥ 200 mg. The combined incidence of cardiovascular death, myocardial infarction, or stroke was decreased by clopidogrel irrespective of aspirin dose. With increasing aspirin dose, the incidence of major bleeding increased both in the placebo group (1.9%, 2.8%, and 3.7%; $P=0.0001$) and the clopidogrel group (3.0%, 3.4%, and 4.9%; $P=0.0009$); thus, the increased risk with clopidogrel was 1.1%, 1.2%, and 1.2%, respectively. In patients with ACS, adding clopidogrel to aspirin is helpful regardless of aspirin dose. They concluded that without any increase in efficacy and with or without clopidogrel bleeding risks increases with increasing aspirin dose (Peter et al., 2003).

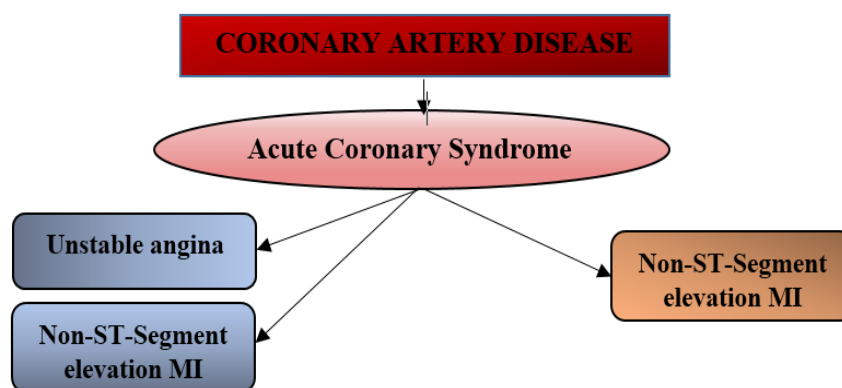


Figure 2. Depicts the relationship between CAD and ACS.

ANTIPLATELET DRUGS

Clopidogrel belongs to a class of medicines called as anti-platelet medicinal products. Clopidogrel decreases the risk of clots forming by decreasing the ability of the platelets to stick with each other. Thus it protects the body from having a stroke or heart attack (NHS UK, 2015). Among the multiple mediators of platelet activation, adenosine diphosphate (ADP) is an important mediator having effects on both physiological homeostasis and thrombosis. After platelet activation, ADP is released from platelet intracellular storage

granules to activate platelets further by binding to several receptors on the platelet membrane. The Thienopyridines derivatives are metabolized in the liver to compounds which covalently bind to ADP P2Y₁₂ receptor leading to an irreversible inhibition of platelet aggregation. Clopidogrel is a second generation thienopyridine which has mostly replaced ticlopidine (first generation thienopyridine) due to its improved tolerability profiles and is the antiplatelet treatment best preferred for prevention of stent thrombosis. The active metabolites of orally administered Thienopyridines, Ticlopidine, Clopidogrel, and Prasugrel have the ability to irreversibly antagonize the platelet P2Y₁₂ ADP receptor, thus inhibiting selectively and irreversibly the ADP-induced platelet activation and aggregation (J.P. Collet, 2009).

THIENOPYRIDINES

Thienopyridines are inactive pro-drugs and the active moiety of their active metabolites is a reactive thiol derivative that targets P2Y₁₂ on platelets. The methoxycarbonyl group on the benzylic position of the Clopidogrel molecule provides an increased pharmacological activity and a better safety and tolerability profile as compared with Ticlopidine. The pro-drug Clopidogrel requires oxidation by the hepatic Cytochrome P450 (CYP) system to generate active metabolites. In particular, the thiophene ring of Clopidogrel is oxidized to an intermediate metabolite (2-oxoclopidogrel), which is further oxidized, resulting in the opening of the thiophene ring and the formation of a carboxyl and thiol group. The reactive thiol group of the active metabolite of Clopidogrel forms a disulfide bridge between one or more cysteine residues of the P2Y₁₂ receptor. This interaction is irreversible, even if no active metabolite is detectable in plasma, accounting for the observation that platelets are inhibited. This results in inhibition of the binding of the P2Y₁₂ agonist 2-methylthio-ADP and the ADP-induced down regulation of adenylyl cyclase. Platelet aggregation is affected not only by other substances requiring released ADP as an amplifier but also when triggered by ADP (J.P. Collet, 2009).

CLOPIDOGREL

Clopidogrel hydrogen sulfate (d-methyl [2-chlorophenyl]-5,6,7-tetrahydrothieno [3,2-c] pyridine] acetate hydrogen sulfate) is a thienopyridineprodrug used clinically to inhibit ADP-induced platelet aggregation. It reduces the risk of thrombotic events in patients with a history of atherosclerotic diseases, such as stroke or myocardial infarction. Clopidogrel inhibits ADP-induced platelet aggregation. Clopidogrel also inhibits collagen and thrombin induced aggregation, however, the inhibitory effect on collagen- and thrombin-induced

aggregation can be overcome by increased concentrations of these agonists. These findings suggest that by attenuating ADP-mediated amplification of the platelet response, clopidogrel indirectly inhibits effect of these agonists (Patrono C *et al.*, 2004). The drug is an irreversible inhibitor of the P2Y₁₂ adenosine diphosphate receptor found on the membranes of platelet cells. Clopidogrel use is associated with several serious adverse drug reactions such as severe neutropenia, various forms of haemorrhage, and cardiovascular edema (Drug Bank, 2014).

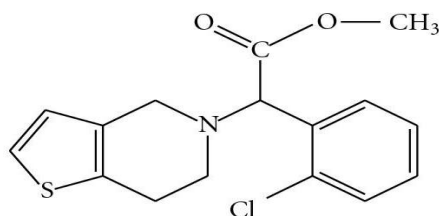


Figure 3. Clopidogrel structure.

Mechanism of action

Clopidogrel, a thienopyridine derivative, is a prodrug that forms an active thiol metabolite by undergoing metabolism by cytochrome P450 (CYP450) enzymes (Marica L. Buck, 2010). The active metabolite of clopidogrel prevents binding of adenosine diphosphate (ADP) to its platelet receptor, impairing the ADP-mediated activation of the glycoprotein GPIIb/IIIa complex. It is proposed that the inhibition involves a defect in the mobilization from the storage sites of the platelet granules to the outer membrane. The drug specifically and irreversibly inhibits the P2Y₁₂ subtype of ADP receptor, which is important in aggregation of platelets and cross-linking by the protein fibrin. No direct interference occurs with the GPIIb/IIIa receptor. As the glycoprotein GPIIb/IIIa complex is the major receptor for fibrinogen, its impaired activation prevents fibrinogen binding to platelets and inhibits platelet aggregation. By blocking the amplification of platelet activation by released ADP, platelet aggregation induced by agonists other than ADP is also inhibited by the active metabolite of clopidogrel (Drug Bank, 2014).

CYP450

Following oral administration, clopidogrel is rapidly absorbed. Clopidogrel is not detected in human plasma due to its extensive metabolism. Clopidogrel is a pro-drug that is absorbed in the intestine (Savi P *et al.*, 1994). The conversion of clopidogrel to its active metabolite requires two sequential oxidative steps (Pereillo JM *et al.*, 2000). The first step leads to formation of 2-oxo-clopidogrel, followed by the conversion of 2-oxo-clopidogrel to the active

metabolite. CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP3A4/5 are implicated as cytochrome P450 enzymes involved in the metabolism of clopidogrel. CYP2C19 is one of the main enzymes involved in the activation of clopidogrel. In a competing metabolic reaction, about 85 % of the drug is hydrolyzed to an inactive carboxylic acid derivative by esterases (Reist M *et al.*, 2000). The genes encoding the CYP enzymes are polymorphic, and extensive data have shown that certain alleles confer reduced enzymatic activity. Currently, available data regarding *in vitro* metabolism and clinical outcomes suggest that the reduced-function CYP polymorphisms have an effect on the conversion to active metabolite and therefore on the degree of platelet inhibition associated with Clopidogrel.

P2Y₁₂

The clopidogrel-active thiol metabolite irreversibly inactivates P2Y₁₂ by forming a disulfide bond with two cysteine residues (Cys17 and Cys270) present in the extracellular domain of the P2Y₁₂ receptor. P2Y₁₂ receptor blockade by clopidogrel-active metabolite potently inhibits ADP-induced platelet aggregation and also reduces platelet dense granule secretion. The maximum inhibition of P2Y₁₂ by clopidogrel is observed 4–5 days after daily administration of 75 mg clopidogrel. Despite a short half-life, the irreversible binding of Clopidogrel's active metabolite to the platelet receptor leads to a prolonged pharmacodynamic effect. Inhibition of platelet aggregation by Clopidogrel lasts for several days, with platelet function returning to baseline about 5 days after stopping the drug (Ding Z *et al.*, 2003).

ADVERSE EFFECTS OF CLOPIDOGREL

Serious adverse drug reactions associated with Clopidogrel therapy include:

- Severe neutropenia (low white blood cells) (Incidence: 1/2,000)
- Thrombotic thrombocytopenic purpura. (Incidence: 4/1,000,000 patients treated)
- The annual incidence of haemorrhage may increase the co-administration of Aspirin
- Gastrointestinal Hemorrhage (Gupta .R., 2008) (Incidence: 2.0% annually)
- Cerebral Haemorrhage (Incidence: 0.1 to 0.4% annually)
- Use of non-steroidal anti-inflammatory drugs is discouraged in those taking Clopidogrel due to increased risk of digestive tract haemorrhage (Zakaija A *et al.*, 2004).

Hematologic complications and bleeding have been the most feared outcome of antithrombotic and antiplatelet agents. Among the Thienopyridines, Clopidogrel is

considered to be a safer alternative to Ticlopidine due to its decreased incidence of hematologic adverse effects. Although thrombotic thrombocytopenia purpura is the most reported hematologic adverse effect of Clopidogrel; neutropenia, acquired haemophilia, isolated thrombocytopenia or idiopathic immune thrombocytopenia, a thrombotic thrombocytopenia purpura with haemolytic uremic syndrome are other rare yet recognized hematologic adverse effects of Clopidogrel. Patients treated with Clopidogrel should be carefully monitored for hematologic adverse effects especially in the first 2 to 3 months after initiation of therapy. Early recognition and prompt initiation of treatment can be lifesaving in patients who have hematologic adverse effects to Clopidogrel (Bristol-Myers squibb., 2015).

CLOPIDOGREL INTERACTIONS

Clopidogrel interacts with the following drugs: Proton Pump Inhibitors (Except Pantoprazole), Phenytoin (Dilantin); Tamoxifen (Nolvadex); Tolbutamide (Orinase); Torsemide (Demadex); Fluvastatin (Lescol); a blood thinner such as Warfarin (Coumadin), Heparin, Ardeparin (Normiflo), Dalteparin (Fragmin), Danaparoid (Orgaran), Enoxaparin (Lovenox), or Tinzaparin (Innohep); (Activase), Anistreplase (Eminase), Dipyridamole (Persantine), Streptokinase (Kabikinase, Streptase), Ticlopidine (Ticlid), and Urokinase (Abbokinase). If you are using any of these drugs, you may not be able to take Plavix, or you may need dosage adjustments or special tests during treatment. In November 2009, the FDA announced that Clopidogrel should not be taken with PPIs such as Prilosec (omeprazole) and Nexium (esomeprazole) (Bristol-Myers squibb., 2015).

CLOPIDOGREL RESPONSE AND PHARMACOGENETICS

The response to clopidogrel shows a wide inter-individual variation, with 5% to 30% of patients not responding to initial clopidogrel therapy. Clopidogrel is a pro-drug that requires conversion to its active thiol derivative catalyzed predominantly by cytochrome P450 (CYP) 3A4 and 3A5, with contributions from 2C19, 2C9, and 1A2 enzymes. Several studies have evaluated the impact of polymorphisms in genes encoding enzymes involved in clopidogrel metabolism (CYP2C19, CYP2C9, CYP3A4, and CYP1A2), as well as polymorphisms of P-glycoprotein transporter protein coding gene (ABCB1), on clopidogrel response (Kurihara et al., 2005). The influence of various genetic polymorphisms has emerged as one of the major determinants of clopidogrel response. After oral administration, clopidogrel is absorbed from the intestine; this is limited by an intestinal efflux pump P-glycoprotein, which is encoded for by the ABCB1 gene. The majority of clopidogrel (85%) is hydrolyzed by

carboxyesterases to form inactive metabolites and the remaining 15% of the drug is then metabolized by the cytochrome P450 (CYP) system in two sequential oxidative steps to generate the active metabolite. Given that only an estimated 2% of ingested clopidogrel ends up bound to platelets, it is easy to appreciate that small changes in its metabolism may substantially affect platelet P2Y₁₂ inhibition. The cytochrome P450 enzymes (CYP) are a superfamily of microsomal drug-metabolizing enzymes important in oxidative drug metabolism. The liver is the key site for metabolism related to CYP. In humans, 57 CYP genes have been identified, but only an estimated 15 of the encoded proteins have been linked to the metabolism of drugs. They are a highly polymorphic group of enzymes leading to a significant number of variations and subsequent changes in drug metabolism. Kazui *et al.* examined the overall hepatic metabolism of clopidogrel. They concluded that three enzymes CYP1A2, CYP2B6 and CYP2C19 contribute to the first oxidative step where each enzyme is responsible for 45%, 36% and 19% of the conversion respectively; four enzymes (CYP3A4, CYP2C9, CYP2C19, and CYP2B6) contribute to the second step of active metabolite formation, where each enzyme is responsible for 40%, 33%, 21% and 7% of the conversion respectively. The CYP2C19 isoenzyme is involved in both steps and appears to have the most influence (Kazui M *et al.*, 2010).

CLOPIDOGREL RESISTANCE

The term Clopidogrel resistance has recently emerged in everyday medical practice, but the term 'non responsiveness' seems more appropriate because patients appear to retain a degree of response to medical treatment. Although there is currently no clear definition for this phenomenon, a widely accepted description is "the persistent activity of clopidogrel target (i.e. P2Y₁₂ receptors of the platelet) despite an adequate antiplatelet regime". Clopidogrel non-responsiveness varies between 4% and 44% among different populations (Georgios J *et al.*, 2011). The term resistance to a drug should be used when drug is unable to hit its pharmacological target, because of inability to reach it (as a consequence of reduced bioavailability, in vivo inactivation, or negative interaction with other substances) or to alterations of the target. One approach has been to define Clopidogrel non-responders as individuals who achieved less than 10% inhibition of platelet aggregation (PA), low-responders as those with IPA between 10%-29% and responders as those with IPA of 30% or greater independent of the ADP concentration used to induce aggregation (Govinda JW *et al.*, 2007) and (Gurbel P.A. *et al.*, 2003). Second approach has been to define Clopidogrel non-responders as those achieving less than a 10% absolute decrease in MPA from baseline in

response to 5 μ M ADP. Potential poor responder rate of 20% to 30% in the general population has been reported in previous studies. Non-responders are at 5 times greater risk of MI, stent thrombosis and death than responders (Brandt JT et al., 2007).

PHARMACOKINETIC MECHANISMS

Inadequate production of the active metabolite to sufficiently block the P2Y₁₂ receptor might be responsible for Clopidogrel non-responsiveness. Poor bioavailability could be due to reduced intestinal absorption of Clopidogrel, decreased conversion to the active metabolite, or caused by drug-drug interactions at the CYP3A4 level (Yogesh Joshi et al., 2011).

PHARMACODYNAMIC MECHANISMS

Decreased platelet response to Clopidogrel might be affected by increased turnover of platelets possessing a high number of P2Y₁₂ receptors, and by polymorphisms of platelet receptors or intra cellular signalling pathways. In patients undergoing Coronary Artery Bypass Graft (CABG), Clopidogrel did not inhibit platelet aggregation on collagen, epinephrine and ADP-induced platelet aggregation in the first 5 days after surgery. An increased turnover of platelets in response to surgery might have led to non-responsiveness, but the actual mechanism is not clear (Buonamici P et al., 2007).

INTERFERENCE WITH OTHER DRUGS

It has recently been shown that Benzodiazepine and selective serotonin reuptake inhibitors interfere with Clopidogrel bioavailability (Bliden KP et al., 2005). The interference of Atorvastatin and other statins that are metabolized by hepatic Cytochrome P450 could interfere with the production of the active metabolite of Thienopyridines but it is still controversial (Lim E et al., 2004).

BODY MASS INDEX BMD DIFFERENCES

Significant differences in BMI between good and poor responders to clopidogrel suggested that the standard dose of Clopidogrel (75 mg daily) might be insufficient for overweight patients. It has been reported that standard 300 mg loading dose of Clopidogrel can also exhibit a sub optimum platelet response in patients with high BMI (Feher G et al., 2006).

CYP2C19 POLYMORPHISM

The CYP2C19 genetic polymorphism has wide interethnic variability, ranging from a 20-30% among Caucasians to 30-45% African-Americans and 50-65% in among East Asians, whereby the CYP2C19*2 allele seems to be the most frequent defective allele (75-85% in Caucasians and East Asians). Multiple studies have demonstrated a relationship between a higher rate of adverse cardiovascular events and carriage of CYP2C19 loss-of-function alleles, especially of the CYP2C19*2 loss-of-function allele, or of any two CYP2C19 loss-of-function alleles (*2,*3,*4, or *5) and also a nearly three-fold increased risk of stent thrombosis than non-carriers (Poulsen TS *et al.*, 2005) and (Angiolollo DJ *et al.*, 2004). Lamba, Dhiman and Kohli (2000) in a study in north Indians (n=100) reported that Fifty-two extensive metabolizers and six poor metabolizers were homozygous with the CYP2C19*1/*1 genotype, and 48 extensive metabolizers and six poor metabolizers were heterozygous with the CYP2C19*1/*2 genotype. Nine poor metabolizers were homozygous with the CYP2C19*2/*2 genotype. No extensive or poor metabolizers demonstrated the presence of the CYP2C19*3 allele. CYP2C19*2 could explain 43% of the poor metabolizers and 57% of the defective alleles in metabolizers. Allele frequency of CYP2C19*1 and *2 was 0.7 and 0.3, respectively (Lamba JK *et al.*, 2000). Lau *et al.* (2006) measured platelet aggregation before and after Clopidogrel treatment in 32 patients undergoing coronary artery stent implantation and in 35 healthy volunteers. They defined clopidogrel non-responders, low responders, and responders on the basis of relative inhibition of adenosine diphosphate (20 μ mol/L) induced platelet aggregation of 10%, 10% to 29%, and 30%, respectively. They reported that among patients, 22% were Clopidogrel non-responders, 32% were low responders, and 47% were responders. Among volunteers, 16% were non-responders, 12% were low responders, and 72% were responders and also that clopidogrel administration resulted inter-individual variability in platelet inhibition, which correlated with CYP3A4 metabolic activity (Lau WC *et al.*, 2004). A 2006 prospective pharmacogenetic study by Hulot *et al* in 28 healthy white male volunteers treated for 7 days with clopidogrel 75 mgd observed that pharmacodynamic response to Clopidogrel was significantly associated with the CYP2C19 genotype. Twenty of the subjects were wild-type CYP2C19 (*1/*1) homozygote, while the other 8 subjects were heterozygous for the loss-of-function polymorphism CYP2C19*2 (1/*2). Baseline platelet activity was measured before Clopidogrel dosing, four hours after dose and on the seventh day. It was not influenced by the CYP2C19 genotype, but platelet aggregation in the presence of 10 μ M ADP decreased in *1/*1 subject whereas it did not change in *1/*2 subjects. The CYP2C19*2 loss-of-function

allele was associated with a marked decrease in platelet responsiveness to clopidogrel in young healthy male volunteers (Hulot JS *et al.*, 2006). In a study by conducted by Trenk *et al* in 797 consecutive patients undergoing percutaneous coronary intervention, who were followed-up for 1 year it was found that 552 (69.3%) were CYP2C19 wild-type homozygote (*1/*1) and 245 (30.7%) carried at least one *2 allele. Residual platelet aggregation was significantly ($p_{0.001}$) higher in *2 carriers than in wild-type homozygote. They concluded that patients carrying at least one CYP2C19*2 allele were more to high prone to high-on clopidogrel platelet reactivity, which is associated with poor clinical outcome after coronary stent placement (Trenk D *et al.*, 2008). Gladding *et al* (2008) did an analysis of the PRINC (Plavix Response in Coronary Intervention) Trial to assess the effect of pharmacogenetics on the antiplatelet effect of clopidogrel. Sixty patients undergoing elective percutaneous coronary intervention in the randomized PRINC (trial had platelet function measured after a 600-mg or split 1,200-mg loading dose and after a 75- or 150-mg daily maintenance dosage. Polymerase chain reaction-based genotyping evaluated polymorphisms in the CYP2C19, CYP2C9, CYP3A4, CYP3A5, ABCB1, P2Y₁₂ and CES genes. CYP2C19*1/*1 carriers had greater platelet inhibition 2 hr after a 600-mg dose (median: 23%, range: 0% to 66%), compared with platelet inhibition in CYP2C19*2 or *4 carriers (10%, 0% to 56%) and CYP2C19*17 carriers (9%, 0% to 98%). CYP2C19*2 or *4 carriers had greater platelet inhibition with the higher loading dose than with the lower dose at 4 hr and responded better with the higher maintenance dose regimen (Gladding P *et al.*, 2008). A Recent study by Harmsze *et al.*, to evaluate the effect of genetic variants affecting Clopidogrel's absorption (ABCB1), metabolism (CYP), and pharmacodynamics (P2Y₁₂) on top of the influence of CYP2C19*2 on platelet reactivity in patients undergoing elective coronary stenting on dual antiplatelet therapy found similar results. Light transmittance aggregometry was used to assess platelet function of 428 consecutive patients either on chronic clopidogrel maintenance therapy or who received a 300 mg Clopidogrel loading dose. In both the treatment groups, CYP2C19*2-carriage was associated with higher platelet reactivity ($P<0.002$) and poor responder status. In the 300 mg group, CYP2C9*3-carriage was associated with higher platelet reactivity ($P<0.05$) and poor responder status (Harmsze A *et al.*, 2010).

Many other researchers also found similar results. Finally in 2010 The U.S. Food and Drug Administration (FDA) added a *Boxed Warning* to the label for Plavix. The *Boxed warning* was about patients who do not effectively metabolize the drug (i.e. "poor metabolizers") and therefore may not receive the full benefits of the drug. The warning mentioned the following;

the liver enzyme CYP2C19 is primarily responsible for the formation of the active metabolite of Plavix. Pharmacokinetic and antiplatelet tests of the active metabolite of Plavix show that the drug levels and antiplatelet effects differ depending on the genotype of the CYP2C19 enzyme. The following represent the different alleles of CYP2C19 that make up a patient's genotype:

- The CYP2C19*1 allele has fully functional metabolism of Plavix.
- The CYP2C19*2 and *3 alleles have no functional metabolism of Plavix.

These two alleles account for most of the reduced function alleles in patients of Caucasian (85%) and Asian (99%) descent classified as poor metabolizers.

- The CYP2C19*4, *5, *6, *7, and *8 and other alleles may be associated with absent or reduced metabolism of Plavix, but are less frequent than the CYP2C19*2 and *3 alleles.
- A patient with two loss-of-function alleles (as defined above) will have poor metabolizer status.
- The pharmacokinetic and antiplatelet responses to Plavix were evaluated in a crossover trial in 40 healthy subjects. Ten subjects in each of the four CYP2C19 metabolizer groups (ultra-rapid, extensive, intermediate and poor) were randomized to two treatment regimens: a 300 mg loading dose followed by 75 mg per day, or a 600 mg loading dose followed by 150 mg per day, each for a total of 5 days.
- After a washout period, subjects were crossed over to the alternate treatment. Decreased active metabolite exposure and increased platelet aggregation were observed in the poor metabolizers compared to the other groups. When poor metabolizers received the 600 mg loading dose followed by 150 mg daily, active metabolite exposure and anti-platelet response were greater than with the 300 mg-75 mg regimens (Drug safety communications, 2010).

P2Y₁₂ POLYMORPHISMS

P2Y₁₂ is the platelet receptor for adenosine diphosphate (ADP) targeted by the active form of clopidogrel. Primary studies led to the discovery of several P2Y₁₂ polymorphisms (including intronic T744C polymorphism) forming 2 distinct haplotypes (H1 and H2) influencing ADP-induced platelet activation, as measured with optical aggregometry in healthy subjects (Pierre F et al., 2003). This negatively coupled G_i receptor is responsible for completion of platelet aggregation response to ADP and it has extracellular cysteines (Hetherington SL et al., 2005). Pharmacologic approaches have shown the P2Y₁₂ receptor to be involved in dense granule secretion, fibrinogen receptor activation, P-selectin expression, and thrombus formation

(Ibnanez B *et al.*, 2006). The platelet P2Y₁₂ receptor plays a key role in platelet activation. The H2 haplotype of the P2Y₁₂ receptor gene (P2RY₁₂) has been found to be associated with maximal aggregation response to adenosine diphosphate (ADP) and with increased risk for peripheral arterial disease. Haplotype H2 is associated with increased platelet responsiveness to adenosine diphosphate but does not appear to influence the response to clopidogrel. Nevertheless, a mild impact on platelet function in patients homozygous for the H2 haplotype cannot be excluded, as has been shown recently (Gianetti J *et al.*, 2006). Fontana *et al.*, examined ADP-induced platelet aggregation responses in 98 healthy volunteers and identified 2 phenotypic groups of subjects with high and low responsiveness to 2 μ mol/L ADP. The number of H2 alleles was associated with the maximal aggregation response to ADP in the overall study population (P=0.007). Down regulation of the platelet cyclic adenosine monophosphate (cAMP) concentration by ADP was more marked in selected H2 carriers than in non-carriers. The respective frequencies of haplotypes H1 and H2 were 86% and 14%. Because Thienopyridines only provide partial P2Y₁₂ blockade, and Aspirin does not inhibit P2Y₁₂ mediated amplification of platelet responses, 1 carriers of the H2 allele may have less protection of these platelet inhibitors. They concluded that in healthy subjects, ADP-induced platelet aggregation was associated with a haplotype of the P2Y₁₂ receptor gene. Given the crucial role of the P2Y₁₂ receptor in platelet functions, carriers of the H2 haplotype may have an increased risk of atherothrombosis and/or a lesser clinical response to drugs inhibiting platelet 94) function (Pierre F *et al.*, 2003). A study conducted in 2006 by Bura *et al* to investigate the possible role of the P2Y₁₂ gene polymorphism in platelet responsiveness to clopidogrel in healthy subjects. Clopidogrel responsiveness was evaluated in 29 young healthy subjects (14 H1H1, 11 H1H2, and 4 H2H2) during a 1-week oral course of Clopidogrel 75mg day. It was found that ADP-induced platelet aggregation did not vary with the genotype: median values were 79.5% in H1H1 subjects; 76.0% in H1H2 subjects; and 79.0% in H2H2 subjects. The inhibition of platelet aggregation relative to baseline was 7% in H2H2 subjects, 28% in H1H2 subjects, and 23% in H1H1 subjects. This study confirmed the marked variability of the pharmacodynamic response to Clopidogrel at the dose currently recommended for long-term prophylaxis. The results also indicated that carriage of one H2 allele does not account for this variability. However, a minor effect was suggested by the poor response observed in H2H2 homozygous subjects (Gianetti J *et al.*, 2006).

II. LABORATORY EVALUATION OF PLATELET RESPONSE TO CLOPIDOGREL

Platelets play a critical role in patients with cardiovascular disease. Antiplatelet drugs are effective, both alone and in combination, in the primary treatment and secondary prevention of cardiovascular disease. Many studies suggest that recurrent thrombotic events may be due to increased platelet reactivity in patients taking aspirin and clopidogrel. A standardized laboratory method that simulates the *in vivo* platelet response to antiplatelet therapy is still lacking. Since clopidogrel specifically inhibits one of two ADP receptors, *ex-vivo* measurement of ADP-induced maximum platelet aggregation by light transmittance aggregometry (LTA) has been the most commonly used laboratory method to evaluate clopidogrel responsiveness and is considered the gold standard (Gurbel P.A. *et al.*, 2003). Different platelet function tests have been used to evaluate the degree of achieved platelet inhibition in patients treated with Clopidogrel. Light transmission aggregometry with ADP as an agonist, is the most evaluated and used method, but the test is time consuming and not practical for routine use. A new point-of-care system is the "Verify Now" method in which the results have been shown to predict clinical outcome (Fernandez-ortiz A *et al.*, 2007). Determination of "Vasodilator Stimulated Phosphoprotein" (VASP) has been considered to be the most specific test for the degree of inhibition of the platelet P2Y₁₂ receptor. Thus, this test has been considered to give the best answer on the platelet inhibition achieved by Clopidogrel (Bonello L *et al.*, 2010).

[1] Light transmission aggregometry

A light transmission aggregometer is a photometer, consisting of a light source, a cuvette holder incorporating a rotating magnet (which drives a small stirrer placed in the platelet suspension), a thermostat heater (which maintains the temperature of the sample constant), and a photoelectric cell that receives the light beam after its passage through the sample of platelet suspension. The light signal is then transferred electronically to a chart recorder or a computer. Maximal optical density occurs when platelets are in the resting state, evenly distributed throughout the plasma or the buffered solution. Upon addition of a platelet agonist to the sample, platelets are activated and change their shape from discoid to spiny spheres, an event that is associated with a transient increase in optical density. The only exception to this rule is represented by platelet activation by epinephrine, which does not result in platelet shape change. The transient increase in optical density of the platelet suspension is rapidly followed by a brisk increase in light transmission, which is indicative of on-going platelet

aggregation, the formation of platelet clumps of different size allowing the passage of more light through the sample. The aggregation curve either reaches a plateau of light transmission, without deflecting toward baseline (irreversible aggregation), or else tends to return toward the baseline (reversible aggregation). Calibration of the instrument for recording aggregation involves establishing a baseline on the recorder at between 0 and 10% of pen excursion, using the unstimulated platelet suspension as the high optical density standard. Maximal light transmission is usually set at a nominal value of 90 to 100% maximal pen excursion, using platelet-poor plasma (PPP) or buffered solution as the high light transmission standard. Several agonists are used to stimulate platelet suspensions in a light transmission aggregometer. The most commonly used agonists include ADP, epinephrine, collagen, arachidonic acid, and the thromboxane A₂ analogue (Breddin HK et al., 2005).

GENOTYPING

Testing that reveals the specific alleles inherited by an individual; particularly useful for situations in which more than one genotypic combination can produce the same clinical presentation, as in the ABO bloodgroup, where both the AO and AA genotypes yield type A blood (Genetic home reference, 2015).

Genotyping methods

Except for the classic restriction fragment length polymorphism (RFLP) analysis using Southern blotting, the vast majority of the SNP genotyping methods rely on pre-amplification of the SNP-containing genomic region by the polymerase chain reaction (PCR) (Tsuchihashi Z et al., 2002).

[1] Polymerase Chain Reaction PCR

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence (Barlett, 2003). The basic PCR principle is simple. As the name implies, it is a chain reaction: One DNA molecule is used to produce two copies, then four, then eight and so forth. This continuous doubling is accomplished by specific proteins known as polymerases, enzymes that are able to string together individual DNA building blocks to form long molecular strands. To do their job polymerases require a supply of DNA building blocks, i.e. the nucleotides consisting of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G). They also need a small fragment of DNA, known as the primer, to which they attach the building blocks as well as a

longer DNA molecule to serve as a template for constructing the new strand. If these three ingredients are supplied, the enzymes will construct exact copies of the templates. PCR is a method used to acquire many copies of any particular strand of nucleic acids. It's a means of selectively amplifying a particular segment of DNA. The segment may represent a small part of a large and complex mixture of DNAs eg. A specific exon of a human gene. It can be thought of as a molecular photocopier. There are three major steps involved in the PCR technique: denaturation, annealing, and extension. In step one; the DNA is denatured at high temperatures (from 90 - 97 degrees Celsius). In step two, primers anneal to the DNA template strands to prime extension. In step three, extension occurs at the end of the annealed primers to create a complimentary copy strand of DNA. This effectively doubles the DNA quantity through the third steps in the PCR cycle. To amplify a segment of DNA using PCR, the sample is first heated so the DNA denatures, or separates into two pieces of single-stranded DNA. Next, an enzyme called "Taq polymerase" synthesizes - builds - two new strands of DNA, using the original strands as templates. This process results in the duplication of the original DNA, with each of the new molecules containing one old and one new strand of DNA. Then each of these strands can be used to create two new copies, and so on, and so on (Mohini Joshi et al., 2010). The annealing phase happens at a lower temperature, 50-60°C. This allows the primers to hybridize to their respective complementary template strands, a very useful tool to forensic chemistry. The newly-formed DNA strand of primer attached to template is then used to create identical copies off the original template strands desired. Taq polymerase adds available nucleotides to the end of the annealed primers. The extension of the primers by Taq polymerase occurs at approximately 72°C for 2-5 minutes. The beauty of the PCR cycle and process is that it is very fast compared to other techniques and each cycle doubles the number of copies of the desired DNA strand. After 25-30 cycles, whoever is performing the PCR process on a sample of DNA will have plenty of copies of the original DNA sample too, as the process of denaturation, annealing, and polymerase extension is continued the primers repeatedly bind to both the original DNA template and complementary sites in the newly synthesized strands and are extended to produce new copies of DNA. The end result is an exponential increase in the total number of DNA fragments that include the sequences between the PCR primers, which are finally represented at a theoretical abundance of 2^n , where n , is the number of cycles (Gibbs RA et al., 1990).

[2] Restriction Fragment Length Polymorphism (PCR-RFLP)

The Restriction Fragment Length Polymorphism assay starts with Polymerase chain reaction amplification of a region surrounding the polymorphic site of interest. The amplification of DNA fragments using the polymerase chain reaction is performed in gradient DNA thermal cycler, by adding the following reagents to either a 0.2 ml thin-walled tube or a 1.5 ml tube, respectively: a small amount of the template DNA molecule, the two primers flanking the region to be amplified, nucleotides, buffer, and Tag DNA polymerase. The cycling protocol consisted of 25-30 cycles of three-temperatures: strand denaturation at 95°C, primer annealing at 55°C, and primer extension at 72° C, typically 30 seconds, 30 seconds, and 60 seconds. After PCR, aliquots of the mixture typically are loaded onto an agarose gel and electrophoresed to detect amplified product. The PCR product is then subjected to restriction enzyme digestion. Restriction enzymes isolated from the bacteria, are sequence specific and recognize specific sequences in double stranded DNA (usually 4-8 bp in length). The restriction enzyme cuts both strands of the duplex and produces restriction fragments. If restriction sites differ the lengths of the fragments will differ. The fragments of different lengths will migrate different distances in the gel according to their length, the shortest moving fastest. The size of the DNA fragments is evaluated by separating the DNA using gel electrophoresis (Sambrook J et al., 1989).

CONCLUSION

Coronary artery disease which develops due to accumulation of plaque in coronary arteries, leads to acute coronary syndrome. Treatment of such cases requires antiplatelet therapy using aspirin and clopidogrel. Clopidogrel a thienopyridine pro-drug inhibits ADP-induced platelet aggregation thus inhibiting collagen and thrombin induced platelet aggregation, and preventing plaque formation. Clopidogrel is metabolized in intestine by CYP450 enzymes and irreversibly inactivates P2Y₁₂ receptor thus reducing platelet aggregation. Inter individual variation is shown towards clopidogrel response, with few people showing resistance for this therapy. Resistance is caused when drug is unable to hit its pharmacological target. Inadequate production of the active metabolite of clopidogrel by CYP450 enzymes in order to sufficiently block the P2Y₁₂ receptor is responsible for Clopidogrel non-responsiveness. Amongst the CYP450 cascade of enzymes CYP2C19 enzyme and genes responsible for that enzyme (CYP2C19) and its alleles have shown greater polymorphisms in various population studies. Alleles of CYP2C19 *1 have fully functional metabolism while *2 and *3 have reduced metabolism of clopidogrel. P2Y₁₂ has two alleles

with H2 being associated with increased platelet response. Light transmission aggregometry is used to analyse platelet response to clopidogrel by subjecting samples of platelet suspension and ADP receptors with light we find the intensity of aggregation. Using a particular type of polymerase chain reaction (PCR), Restriction Fragment Length Polymorphism (RFLP) it is possible to fragment the DNA sample and subject them to priming with allele specific primers. By amplifying such fragments and using gel electrophoresis it is possible to identify different polymorphisms if present. Combination and analysis of data obtained by phenotyping and genotyping of samples derives an association between genetic polymorphism and clopidogrel response.

III. ACKNOWLEDGEMENT

Authors take immense pleasure in thanking Dr. Urmila M. Thatte, Professor and Head of Department of Clinical Pharmacology at KEM Hospital. Authors are also thankful to Dr. Nithya Gogtay for valuable input.

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