ORIGINAL ARTICLE

Trends of Platelet Inhibition in Different Clopidogrel Pretreatment Patterns in Malaysian Patients Undergoing Elective Percutaneous Coronary Intervention

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INTRODUCTION

Double antiplatelet therapy (DAPT), in particular the combination of aspirin and clopidogrel, has been shown to improve clinical outcomes following percutaneous coronary intervention (PCI) [1,2]. It has been demonstrated that an adequate loading dose of clopidogrel prior to elective PCI reduces major cardiovascular events, including stent thrombosis [3]. The rationale for the use of DAPT over aspirin monotherapy largely centers on the pharmacodynamic properties of aspirin. Aspirin, an inhibitor of the cyclo-oxygenase enzyme has been shown to be ineffective in certain groups of individuals. These patients, also known as aspirin non-responders, have inadequate platelet inhibition, which could explain why the addition of another antiplatelet agent, acting on the adenosine diphosphate (ADP)-mediated mechanism, leads to an improvement in clinical outcomes in clinical trials. However, even with DAPT therapy, a substantial number of subsequent thrombotic and ischemic events still occur [4,5].

Clopidogrel is a prodrug which requires a two-step process performed by hepatic cytochrome (CYP) P450 enzymes to produce active metabolites [6]. The CYP P450 gene is highly polymorphic and loss-of-function (LOF) polymorphism of CYP2C19, a CYP isoenzyme, may partially contribute to the wide inter-individual variability in the antiplatelet effect of clopidogrel [7]. It has also been shown that common variants accounting for this responsiveness, the *2 and *3 genotypes have a higher prevalence in Asian population [8], including in a Malaysian population [9].

In recent years, there is a growing trend to determine whether a patient would benefit from antiplatelet therapy by direct phenotypic testing of the patient's platelet aggregation level in response to a specific agonist.   In vitro high residual platelet aggregation after loading doses of antiplatelets have been shown to be predictive of stent thrombosis and long term adverse clinical outcomes [10]. Hence, there is a clinical necessity to measure the optimal degree of antiplatelet–induced platelet inhibition to determine the ability of these antiplatelet medications to prevent atherothrombotic events. An established marker of platelet activation, P-selectin or CD-62 ligand is elevated in patients with acute coronary syndrome, other acute vascular events and during haemodynamic stress [11]. However, it is not known whether there is an association between soluble P-selectin (sP-selectin) and platelet aggregation level under influence of antiplatelet drugs, particularly when there are differences in the use of antiplatelet therapies.

Significant inter-individual variability in post-treatment platelet inhibition has led to some difficulty in standardizing the optimal dose and duration of established antiplatelet therapy. This has resulted in wide variations in practice patterns, especially in developing countries. Nonetheless, DAPT loading prior to planned or possible ad hoc PCI cases has become contemporary practice and reflected in many local practice Guidelines. In Malaysia, the multiethnic populace undergoing planned, or possible ad hoc PCI, is typically pre-treated with a 75-300 mg loading dose of aspirin and 75-600 mg of clopidogrel [12]. However, variations in antiplatelet loading patterns have been noticed, in particular for clopidogrel. The Malaysian National Cardiovascular Database (NCVD) – PCI Registry 2007-2009 reported clopidogrel was given prior to the procedure in 94% of cases. Three hundred milligram was the most common loading dose and accounted for 47% of cases, followed by 37% of patients on chronic clopidogrel therapy that received 75 mg prior to procedure. Of the various timings of clopidogrel administration prior to PCI, the duration of more than 3 days before PCI was most frequently seen nationwide [12].

Little is known about the trend of platelet inhibition in patients treated with different clopidogrel regimens. We therefore aimed to investigate the patterns of clopidogrel treatment prior to PCI, and the impact of these pretreatment patterns on platelet inhibition and 1 year post-procedural clinical outcomes. In addition, we genotyped patients for CYP2C19 variants and measured their plasma sP-selectin to study their possible association with the pharmacodynamic response of clopidogrel.

MATERIALS & METHODS
Study participants
Consecutive patients admitted electively for planned, or possible PCI, were screened for enrollment into this single centre study. Of the 323 patients screened between 18/10/2010 – 7/3/2011, 237 patients provided written informed consent to participate in this study. Participants

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eligible for the study had a documented clinical history of coronary artery disease and were listed for elective coronary angiography with intention of ad hoc PCI. All study subjects were on aspirin therapy (75-300 mg) for at least 2 days prior to enrollment and were also prescribed clopidogrel as adjunctive antiplatelet therapy prior to PCI.

The patients were classified into one of four groups according to their pretreatment or loading regimen: Group 1, patients that were taking clopidogrel 75 mg daily for ≤ 3 days (n=20); Group 2, patients on clopidogrel 75 mg daily for ≥ 4 days (n=118); Group 3, patients given a single loading dose of 300 mg with or without a subsequent 75 mg daily dose (n=12); and Group 4, where some patients were prescribed a single 300 mg loading dose of clopidogrel on the day of PCI, but for the purpose of this study were classified as being on 'aspirin monotherapy', as enrolment occurred prior to the single dose of clopidogrel being administered (n=87).

By doing so, we were able to study the type of antiplatelet pretreatment and loading patterns amongst Malaysian patients, with a particular focus on clopidogrel, which has been the most established adjunctive antiplatelet therapy, to be combined with aspirin, prior to planned PCI. The study protocol was approved by the Ministry of Health Medical Research and Ethics Committee.

*CYP2C19*2 and *3 genotyping*
Blood samples were obtained at baseline in all participants. Genomic DNA was extracted from peripheral whole blood with commercially available kits (Gentra Puregene Blood Kit, Qiagen, Hamburg, Germany) according to the manufacturer's instructions. Genotyping for CYP2C19 *2 (accession number: rs4244285) and *3 (accession number: rs4986893) alleles was assessed by restriction fragment linked polymorphism – polymerase chain reaction (RFLP-PCR) method according to previous protocols [13,14] with slight modifications. To ensure consistency of the assays, genotyping was repeated in 10% of randomly selected patients. Repeated genotyping revealed identical results. All genotype frequencies were consistent with those predicted by the Hardy-Weinberg equilibrium.

Measurement of plasma level of soluble P-selectin
Plasma was obtained after centrifugation at 1500 x g at room temperature for 10 minutes within 1 hour of collection. Plasma was divided into aliquots and stored at -40°C to allow batch analysis. Soluble P-selectin (sP-selectin) level were measured in duplicate by a highly sensitive sandwich enzyme linked immunosorbent assay (ELISA) using a commercial reagents (Human sP-Selectin/CD62P ELISA reagent set; R&D Systems, Minneapolis, MN). The lower limits of sP-selectin detection were 0.5 ng/ml. Intra- and inter-assay coefficients of variation (CVs) were less than 6% and less than 10%, respectively.

Platelet aggregation in whole blood measured by the Multiplate analyzer
Three milliliter of whole blood was obtained from each patient and was placed in 4.5ml blood collection tubes containing 25 µg/ml anticoagulant hirudin (Dynabyte GmbH, Munich, Germany). Both aspirin (ASP, ASPI test, containing 0.5mM final arachidonic acid concentration) and

adenosine diphosphate (ADP, ADP test, 6.5µM final ADP concentration) induced platelet aggregation were assessed with the Multiplate impedance aggregometry analyzer (Dynabyte GmbH, Munich, Germany). The measured platelet aggregation is quantified as arbitrary units (AU) are plotted against time (min) to give the area under the aggregation curve (AUC = AU*min). All of the materials used were obtained from the manufacturer. Details of this method have been reported previously [15]. 300 AU*min and 468 AU*min were used as the cut-offs value associated with high platelet aggregation or poor responsiveness in patients on aspirin and clopidogrel therapy, respectively, as established by previous studies [16].

12-months follow-up
Primary composite end points at 12 months, including cardiovascular death, an event of myocardial infarction, hospital readmission for acute coronary syndrome (ACS)/stroke, the composite of major adverse cardiac events (MACE: stent thrombosis, acute coronary syndrome and cardiac death) and ischemic stroke were pre-specified for the present study

Statistical analysis
Categorical variables are displayed as frequencies and percentages, and comparisons were made with Pearson chi-square or Fisher's exact test, when indicated. Continuous variables were presented as mean with standard deviation and were compared using independent sample t-test. All tests were 2-sided, and $p < 0.05$ was considered statistically significant.

RESULTS
The baseline characteristics of the 150 patients in the three groups who received DAPT and the 87 patients with aspirin monotherapy were well balanced (Table I). Among patients who received the DAPT regimen, 78.7% received 75 mg clopidogrel daily dose for ≥ 4 days, compared to only 13.3% and 8% who received clopidogrel for ≤ 3 days, or received single loading dose of 300 mg with or without a subsequent 75 mg dose prior to PCI, respectively.

CYP2C19 loss-of-function genetic variation
Of the 237 patients, 120 (50.6%) were wild type homozygotes (*1/*1), 94 (39.7%) were heterozygotes (*1/*2), and 23 (9.7%) were homozygous (*2/*2) with respect to the *2 allelic variant. CYP2C19*3 allelic was detected as heterozygote (*1/*3) and homozygotes (*3/*3) in 27 (11.4%) and 1 (0.4%) patients, respectively. Overall, we have 138 (58.2%) patients who were carriers of both *2 and *3 allelic variant.

Plasma sP-selectin
Patients receiving dual antiplatelet therapy compared to taking aspirin alone had lower mean plasma levels of sP-selectin (35.2 ± 12.9 vs 40.2 ± 13.8 ng/ml, $p < 0.05$). Nonetheless, no significant difference was observed between the different treatment groups of clopidogrel in the plasma level of this marker (p = 0.450). We also found that plasma sP-selectin level correlated moderately only with ADP-induced platelet aggregation (r = 0.180, p < 0.05) but not with ASP-induced platelet aggregation (r = 0.392, p = 0.056).

Table I: Demographic data, risk factors, concomitant medications and biochemical profiles for the study sample

	DAPT (Clopidogrel + Aspirin)			Aspirin alone (n = 87)	p Value
	Clopidogrel 75 mg daily ≤ 3 days (n = 20)	Clopidogrel 75 mg daily ≥ 4 days (n = 118)	Clopidogrel 300 mg ± 75 mg daily (n = 12)		
Age, yrs	60.8 ± 5.6	57.3 ± 12.1	61.3 ± 8.8	56.7 ± 10.8	0.317
Male	19 (95.0)	87 (73.7)	8 (66.7)	70 (80.5)	0.986
Risk factors					
Hypertension	17 (85.0)	83 (70.3)	9 (75.0)	70 (80.5)	0.344
Hyperlipidemia	15 (75.0)	82 (69.5)	10 (83.3)	62 (71.3)	0.932
Diabetes	6 (30.0)	37 (31.3)	3 (25.0)	27 (31.0)	0.477
Current smoker, < 7 days	2 (10.0)	13 (11.0)	2 (16.7)	6 (6.9)	0.941
Body mass index, kg/m²	26.5 ± 3.7	25.9 ± 4.0	25.4 ± 3.2	26.1 ± 3.8	0.882
Concomitant medication					
Statins	16 (80.0)	106 (89.8)	10 (83.3)	83 (95.4)	0.046
ACE inhibitors	13 (65.0)	45 (38.1)	7 (58.3)	39 (44.8)	0.917
Beta-blockers	13 (65.0)	65 (55.1)	7 (58.3)	45 (51.7)	0.384
Proton pump inhibitors	5 (25)	42 (35.6)	3 (25)	17 (19.5)	0.042
Hemoglobin	13.7 ± 1.5	13.6 ± 1.5	13.6 ± 2.2	13.9 ± 1.8	0.572
Hematocrit	39.8 ± 8.2	41.7 ± 4.7	41.3 ± 6.8	42.1 ± 5.1	0.378
Platelet counts	131.8 ± 155.9	96.5 ± 45.7	95.8 ± 24.1	104.4 ± 51.2	0.130
Creatinine clearance level (mL/min)	73.7 ± 26.9	75.0 ± 27.3	63.5 ± 15.1	72.6 ± 25.6	0.521

Table II: Platelet inhibition profiles of Clopidogrel

	DAPT (Clopidogrel + Aspirin)			p Value
	Clopidogrel 75 mg daily ≤ 3 days (n = 20)	Clopidogrel 75 mg daily ≥ 4 days (n = 118)	Clopidogrel 300 mg ± 75 mg daily (n = 12)	
ADP-induced platelet aggregation (AU*min)	376.3 ± 153.7	288.9 ± 159.4	347.3 ± 195.7	0.056
Clopidogrel resistance, n (%)	5 (25.0)	15 (12.7)	2 (16.7)	< 0.001
CYP2C19 *2 or *3 carrier, n (%)	17 (85.0)	65 (55.1)	6 (50.0)	0.340

Table III: Platelet inhibition profiles of Aspirin

	DAPT (Clopidogrel + Aspirin)			Aspirin alone (n = 87)	p Value
	Clopidogrel 75 mg daily ≤ 3 days (n = 20)	Clopidogrel 75 mg daily ≥ 4 days (n = 118)	Clopidogrel 300 mg ± 75 mg daily (n = 12)		
ASP-induced platelet aggregation (AU*min)	132.9 ± 76.2	110.0 ± 85.5	126.4 ± 145.1	141.6 ± 130.1	0.211
Aspirin resistance, n (%)	0	2 (1.7)	1 (8.3)	7 (8.0)	< 0.001

Post-treatment ADP-induced platelet inhibition
There was no significant difference in post-treatment ADP-induced platelet aggregation levels among the pretreatment and loading groups of clopidogrel prior to PCI (p = 0.056; Table II). However, the number of patients who had poor clopidogrel responsiveness (≥ 468 AU*min; threshold level for high ADP-induced platelet aggregation) ranged from 12.7% to 25% for all groups (p < 0.001). Among these 22 patients, the proportions of patients who are wild type or CYP2C19 *2 and *3 carriers were similar (45.5% vs 54.5%).

In 150 of clopidogrel-treated patient, there was no significant difference in pharmacodynamic responses between wild type (310.45 ± 155.78 AU*min) and CYP2C19 *2 and *3 carriers (301.55 ± 169.95 AU*min). The prevalence rate of CYP2C19 *2 and *3 carriers in patients loaded with 75 mg clopidogrel for ≤ 3 days was the highest (85%) compared to other

treatment groups. As depicted in Figure 2, the mean level of ADP-induced platelet aggregation was similar in wild type and *2 or *3 carriers in all clopidogrel-treated groups.

Primary end points within 12 months after antiplatelet treatment
Among 237 patients, only 38.8% underwent PCI with 21.5% and 17.3% treated with drug eluting stent (DES) and bare metal stent (BMS), respectively. Overall, there were 7 cardiac-related deaths, 4 non-cardiac related deaths and 3 hospital readmissions due to an acute coronary syndrome event or stroke by the end of the 12-month follow-up period. There were only 2 cardiac related deaths out of the 92 patients that underwent PCI with DES. Regardless of the PCI procedures, 13 or 8.7% patients in DAPT group had poor clinical outcomes within 12 months after recruitment, compared to only 3 or 3.4% patients from aspirin monotherapy group (p = 0.617). Nonetheless, we were unable to confirm the influence of
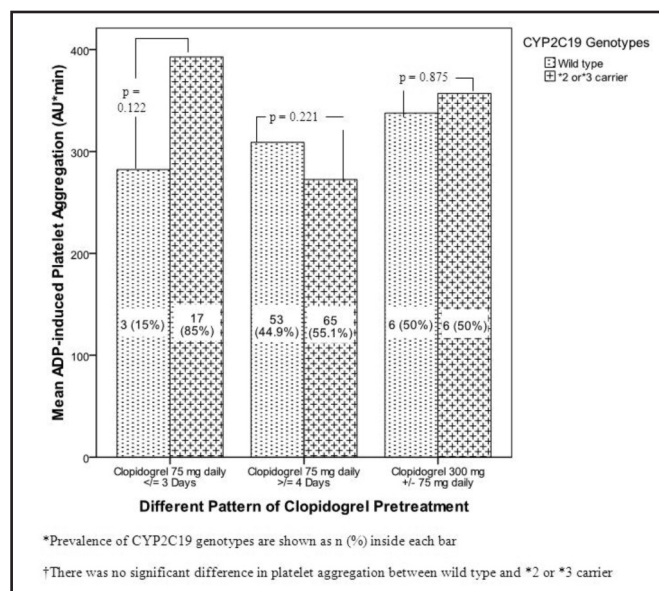
Fig. 1 : Platelet aggregation according CYP2C19 genotypes in different pattern of clopidogrel pretreatment prior to PCI

different pattern of antiplatelet therapy in predicting poor clinical outcome due to the insignificant appreciation of low number of poor clinical outcomes cases

DISCUSSION
To date, the optimal timing, duration, dose and efficacy of clopidogrel pretreatment in patients with stable coronary artery disease have not been clearly defined and vary among different hospitals. Our study demonstrated that among patients receiving DAPT, clopidogrel 75 mg daily for ≥ 4 days prior to PCI was the most commonly used regimen in our local setting, compared to 75 mg daily for ≤ 3 days or a single loading dose of 300 mg with or without a subsequent 75 mg prior to PCI.

A maintenance dose of 75 mg daily without a preload achieves the steady state level within 3 to 7 days whereas a loading dose of 300 mg to 600 mg reach near steady state level within 4 to 24 hours. In our study, most patients were prescribed with at least 4 days of clopidogrel to ensure that patients had a minimum of three daily maintenance doses of clopidogrel. Some of these patients were already on chronic therapy. There were still, however, patients that were taking clopidogrel for ≤ 3 days, probably due to non-compliance with the instruction to start taking clopidogrel at least 4 days before the PCI.

We noted a trend which did not reach statistical significance, with those given 75 mg daily for ≥ 4 days prior to PCI having lower platelet aggregation compared to the other groups. Patients taking clopidogrel for ≤ 3 days had the highest aggregation levels. However, overall there was no significant influence of different clopidogrel pretreatment pattern on platelet inhibition.

It is possible that certain genetic variations resulting in decreased CYP function could partly account for poor

pharmacodynamic response to clopidogrel. Clopidogrel is metabolized to its active metabolites, mainly through the pathway using the S-mephenytoin 4-hydroxylase enzyme, which is mediated by the CYP2C19 genotype. Two CYP2C19 alleles were most frequently observed: a splice defect in exon 5 (CYP2C19*2; single base change G>A), and a premature stop codon at position 636 of exon 4 (CYP2C19*3; single base change G>A). Patient carrying at least one of these alleles could result in a truncated protein and an inactive CYP2C19 enzyme, hence causing poor clopidogrel metabolism[17]. It has been reported that the carriage of the CYP2C19 LOF allele is associated with an impaired pharmacodynamic response to different dosing regimens of clopidogrel[18]. Both CYP2C19 *2 and *3 are responsible for more than 90% of loss-of-function CYP2C19 enzymatic function in Asian population[19,20]. It is reported that the allelic frequencies of *2 and *3 in Asian population are ≈30% and 5-7%[20], respectively. The frequency of *2 and *3 allele carriers in our population was as high as 49.4% and 11.8%, respectively.

Despite the high prevalence rate of CYP2C19 *2 or *3 LOF carriers in our patients, our results indicated that the impact of CYP2C19 genotypes in clopidogrel non-responsiveness was minimal. The CYP2C19 genotype may have low or moderate sensitivity in affecting platelet aggregation in clopidogrel-treated patients, since it only accounts for approximately 12% of the variation in platelet response to clopidogrel[21]. In addition, we also observed an equal prevalence rate of CYP2C19 wild types and *2 or *3 carriers in patients who had clopidogrel resistance (≥ 468 AU*min ADP-induced platelet aggregation). This further suggests that some patients who respond poorly to clopidogrel could have other intrinsic mechanisms such as noncompliance, inadequate absorption[22] or body mass index[23]. Although we did not observe any significant impact of CYP2C19 genetic variation in affecting the clopidogrel metabolism, it is likely that both genotyping and platelet aggregation measurement can be a more efficient complimentary tool to predict the ischemic risk in patients treated with DAPT[24,25].

Our results also showed that patients on DAPT had less inflammation as indicated by sP-selectin, a platelet activation marker, compared with patients on aspirin monotherapy. Although this implies that long-term clopidogrel therapy is associated with an anti-inflammatory effect[26], there was no measurable impact of the longer clopidogrel pretreatment duration on plasma sP-selectin levels, further suggesting that platelet inhibition may not be affected much by variations in clinical practice. There was also no significant difference in platelet inhibition by aspirin when given at 75-300 mg for at least 2 days. We also showed that around 10% of patients had poor response to aspirin, similar to the 5-45% reported by previous studies[27]. Some studies have suggested that non-responders rates might be affected by imprecision of platelet aggregation assay or patient non-compliance[28].

It has been proposed that adjusting clopidogrel dose according to in vitro measurement of platelet function or genotyping test may significantly improve the clinical outcome after coronary stenting[29]. In the present study, only a small proportion of our patients suffered from poor clinical

outcomes within 12 months after antiplatetet pretreament. Overall, we found that clinical outcome was not significantly associated with the administration of DAPT or aspirin monotherapy prior to PCI. This incidence rate was too low to indicate whether the current common clinical practice for antiplatelet management in our centre could lead a better or worse clinical outcome, despite the influence from clinical risk factors and CYP2C19 genotype.

Nonetheless, various reports have shown the benefit of dual antiplatelet treatment in lowering incidence of cardiovascular death, myocardial infarction and stroke at 12 months as compared with patients in receiving aspirin alone. Patients with unstable angina or NSTEMI were found to have stronger evidence of benefit from long term therapy, at average duration of 9 months [5]. Although current evidence only supports short term use of combination therapy (roughly 1 to 2 weeks) in patients who suffer STEMI [30], a consistent benefit of aspirin and clopidogrel dual therapy for reducing the rate of ischemic complications in these patients was demonstrated [30,31]. The present study showed fewer incidences of poor clinical outcomes further suggesting the appropriate use of DAPT treatment in patients undergoing for PCI.

Study limitations

There are several limitations in the present study. Firstly, although we were able to suggest the lack of superiority of one regimen over another, we did not measure the specific timing of loading prior to PCI. We also did not determine the optimal loading dose that confers the most benefit. Various reports have shown that higher loading doses of clopidogrel such as 600 mg [32], produce greater platelet inhibition and fewer low responders [33]. However, none of our study patients were loaded with 600 mg, as 300 mg was the recommended dose at the time this study was conducted [34]. A higher loading dose could have changed the results of our study. Thirdly, the sizes of the different groups were not equal and inadequately powered as this study was designed as an observational study in a clinical practice setting. Finally, we only included whole blood aggregometry to assess the pharmacodynamic effect of antiplatelet. The incidence of antiplatelet resistance could be affected by differences in test methods and definitions of resistance.

CONCLUSION

In this study, we found considerable variations in the loading patterns of antiplatelet therapy, in particular the pre-loading of clopidogrel before PCI in both ACS and stable angina patients. These practices do not necessarily follow published clinical trials and guidelines. However, our data suggested that regardless of the way clopidogrel was administered, even in the presence of a high prevalence of CYP2C19 LOF allele carriers, there was no significant difference in platelet inhibition. Nevertheless, we still recommend that clopidogrel should be started ≥ 4 days prior to PCI, since previous studies have shown that it takes 4 days to reach a stable platelet aggregation state, and to minimize the effect of CYP2C19 LOF which can potentially reduce the platelet inhibition properties of clopidogrel.

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