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ORIGINAL ARTICLE

Evidence that pre-existent variability in platelet response to ADP accounts for 'clopidogrel resistance'

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Summary. *Background:* Clopidogrel is a widely used antithrombotic agent that inhibits the platelet $P2Y_{12}$ adenosine diphosphate (ADP) receptor. There is increasing interest in 'clopidogrel resistance'. *Objectives:* To determine whether 'clopidogrel resistance' is accounted for by a pre-existent variability in platelet response to ADP. *Methods:* Platelet response to 20 μM ADP was analyzed by four independent whole blood flow cytometric assays: platelet surface activated GPIIb-IIIa, platelet surface P-selectin, monocyte-platelet aggregates and neutrophil-platelet aggregates. In 25 consecutive, non-aspirin-treated healthy subjects, we studied platelet response before and after clopidogrel administration. In addition, we studied the platelet response in 613 consecutive aspirinated patients with or without coronary artery disease (CAD, as determined by angiography) who had or had not been treated with clopidogrel. In these patients, we tested for homogeneity of variance across all durations of clopidogrel exposure and severity of CAD by estimating the 'goodness of fit' of two independent models. *Results:* In the healthy subjects, pre-clopidogrel response to ADP predicted post-clopidogrel response to ADP. In the patients, clopidogrel, as expected, inhibited the platelet response to ADP. However, irrespective of the duration of clopidogrel administration, the severity of CAD, and the dose of aspirin, clopidogrel did not increase the variance in the platelet response to ADP in any of the four assays of platelet response. *Conclusions:* These studies provide evidence that 'clopidogrel resistance' is accounted for by a pre-existent variability in platelet response to ADP and this variability is not increased by clopidogrel administration.

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Introduction

The thienopyridine clopidogrel is a widely used antithrombotic agent [1,2]. Clopidogrel, an inactive prodrug that requires *in vivo* conversion in the liver by the cytochrome P450 3A4 enzyme system to an active metabolite, acts via irreversible antagonism of the platelet $P2Y_{12}$ adenosine diphosphate (ADP) receptor [1,2]. There is variability between patients with regard to clopidogrel-induced inhibition of platelet function assays [3]. A relative lack of clopidogrel-induced inhibition of platelet function assays has been termed 'clopidogrel resistance' or 'clopidogrel response variability' [3–7], and this has been reported to be correlated with major adverse clinical outcomes [5,8,9]. However, we have hypothesized [3] that this phenomenon may be the result of an underlying variability in platelet response to ADP that is not increased by clopidogrel administration.

In a study of 25 consecutive non-aspirin-treated healthy subjects reported in this manuscript, we demonstrate that preclopidogrel response to ADP predicts postclopidogrel response to ADP. Thus, platelet response variability observed after clopidogrel treatment may reflect pre-existing variability in response to ADP. In contrast, we reasoned that clopidogrel resistance, if real, would lead to increased variability in platelet response to ADP. We therefore then compared the platelet response variability to ADP in 613 consecutive aspirin-treated patients with or without coronary artery disease (CAD, as determined by angiography) who had or had not been treated with clopidogrel. We tested for homogeneity of variance across all durations of clopidogrel exposure, severity of CAD and aspirin dose by estimating the 'goodness of fit' of two independent models. We thereby addressed the question as to whether 'clopidogrel resistance' is accounted for by a pre-existent variability in platelet response to ADP and whether this variability is increased by clopidogrel administration.

Methods

Study population

Normal subjects After University of Michigan Medical School IRB-approved written informed consent, we studied 25 consecutive normal, healthy volunteers (age 26.2 ± 7.3 years, mean ± SEM, 17 females, eight males). All subjects were non-smokers, who had not taken in the 7 days prior to the study any of the following: aspirin, other antiplatelet medicine, prescription or over-the-counter medication, vitamins, supplements, caffeine-containing foods or beverages, grapefruit juice, or charcoal-grilled foods. Clopidogrel 300 mg was administered orally. In some subjects, clopidogrel 75 mg was then administered daily for 6 days. Peripheral blood was collected into a buffered 3.2% (0.105 M) sodium citrate Vacutainer (Becton Dickinson, San Jose, CA, USA) prior to, 5 h after and, in some subjects, 7 days after the administration of the 300 mg clopidogrel dose. Platelets were then activated in whole blood with 20 μM ADP (see below).

Patients After University of Massachusetts Medical School IRB-approved written informed consent, we studied 633 consecutive patients presenting to the Cardiac Catheterization Laboratories at UMass Memorial Medical Center for evaluation of CAD. Patients received aspirin for at least 3 days. Exclusion criteria were: use of a fibrinolytic agent within 72 h of presentation; abciximab within the previous 15 days; eptifibatide or tirofiban within the previous 3 days; and known allergy or hypersensitivity to clopidogrel or aspirin. One patient was an unreliable historian with respect to clopidogrel ingestion. One patient chose to withdraw from the study. Clots observed in the sample tubes of 18 patients prevented their analysis. The remaining 613 evaluable patients were studied, as described in the Results section. Patient demographics are shown in Table 1. Clopidogrel-treated and untreated patients were not significantly different with regard to age, gender, body mass index, family history of CAD, hyperlipidemia, hypertension, and history of smoking. Patients receiving clopidogrel were more often diabetic and taking statins (Table 1). Patients were grouped (blind to the results of platelet function assays) according to (a) duration of clopidogrel treatment and (b) angiographically defined severity of CAD. Arterial blood was collected from patients prior to angiography and transferred into a 1.8-mL buffered 3.2% (0.105 M) sodium citrate Vacutainer. Platelets were activated in whole blood with 20 μM ADP (see below).

Flow cytometry

Leukocyte-platelet aggregation As previously described [10,11], monocyte-platelet aggregates (MPAs) and neutrophil-platelet aggregates (NPAs) were analyzed by whole blood flow cytometry. We have previously demonstrated that these heterotypic aggregates are a sensitive marker of platelet activation [12]. Samples were activated and leukocytes and

Table 1 Patient demographics

	All patients		MI + UA		Other CAD		No CAD		Aspirin 81 mg		Aspirin 325 mg	
	No clopidogrel ($n = 414$)	Clopidogrel Tx ($n = 199$)	No clopidogrel ($n = 58$)	Clopidogrel Tx ($n = 65$)	No clopidogrel ($n = 287$)	Clopidogrel Tx ($n = 115$)	No clopidogrel ($n = 69$)	Clopidogrel Tx ($n = 19$)	No clopidogrel ($n = 164$)	Clopidogrel Tx ($n = 51$)	No clopidogrel ($n = 242$)	Clopidogrel Tx ($n = 145$)
Age (year)	61 ± 0.56	60 ± 0.81	64.1 ± 1.65	62.3 ± 1.49	61.9 ± 0.64	59.8 ± 1.06	54.8 ± 1.29	53.5 ± 1.78	61.8 ± 0.86	62.8 ± 1.78	60.5 ± 0.76	58.9 ± 0.91
Gender (% male)	66%	72%	71%	75%	70%	77%	46%	37%	63%	59%	68%	77%
BMI (kg/m^2)	29.9 ± 0.28	29.6 ± 0.38	29.3 ± 0.81	29.2 ± 0.57	30.1 ± 0.33	29.4 ± 0.48	29.8 ± 0.72	32.4 ± 1.79	30.1 ± 0.43	29.4 ± 0.89	29.8 ± 0.38	29.7 ± 0.41
Family Hx of CAD	75%	77%	84%	74%	74%	78%	72%	79%	70%	82%	77%	74%
Hyperlipidemia	87%	89%	97%	92%	88%	90%	77%	79%	84%	88%	89%	90%
Hypertension	73%	73%	76%	75%	79%	73%	48%	63%	71%	71%	75%	74%
Diabetes mellitus	33%	23%*	41%	20%**	34%	25%	20%	21%	30%	22%	35%	23%*
Current smoker	24%	18%	28%	26%	23%	14%*	29%	11%	20%	14%	27%	19%
Prior smoker	74%	71%	72%	86%	75%	65%	70%	53%	73%	67%	74%	72%
Statin	66%	81%***	77%	82%	69%	82%**	46%	74%*	63%	74%	67%	83%***
NSAIDs	6%	8%	4%	5%	6%	10%	11%	0%	8%	2%	5%	10%
COX-2 antagonist	5%	6%	8%	2%	5%	10%	3%	0%	6%	4%	4%	7%
Antidepressant	19%	23%	18%	19%	17%	21%	27%	47%	18%	28%	19%	20%

Results are mean ± SEM or percent. Continuous data were analyzed using unpaired Student's *t*-test. Frequency data were analyzed by Fisher's exact test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. BMI, body mass index; CAD, coronary artery disease; COX-2, cyclooxygenase 2; Hx, history; MI, myocardial infarction; NSAIDs, non-steroidal anti-inflammatory drugs (other than aspirin); Tx, treatment; UA, unstable angina.

platelets simultaneously labeled by mixing undiluted citrated whole blood with ADP 20 μM and two monoclonal antibodies [fluorescein isothiocyanate (FITC)-conjugated CD14-specific and phycoerythrin (PE)-conjugated CD42a-specific] in a 60:40 vol:vol ratio of whole blood:ADP/antibody solution for 15 min at 37 °C. Samples were then fixed and red cells lyzed by addition of FACS Lyzing solution (Becton Dickinson).

Platelet GPIIb-IIIa activation and P-selectin expression Activation of platelets leads to changes in surface glycoprotein (GP) IIb-IIIa (integrin αIIbβ3), thereby allowing fibrinogen binding and platelet aggregation [13]. In addition, platelet activation results in platelet surface exposure of the α granule membrane protein P-selectin (CD62P) [14]. Activation of GPIIb-IIIa was detected by binding of FITC-conjugated monoclonal antibody PAC1 (Becton Dickinson), as previously described [11,15]. Undiluted citrated whole blood was mixed with ADP 20 μM and FITC-PAC1 (60:40 vol:vol ratio of whole blood:ADP and antibody solution) for 5 min at 37 °C, then fixed by addition of an equal volume of 2% formaldehyde in 10 mM HEPES, 0.15 M NaCl, pH 7.4 (HEPES/saline). These samples were then diluted in 0.5% bovine serum albumin, HEPES/saline, and an aliquot labeled with a mixture of PE-CD62P and peridinin chlorophyll protein (Per-CP)-conjugated CD61 (GPIIIa, as a platelet identifier), for 40 min at room temperature.

Flow cytometric analysis of samples Samples were analyzed as previously described [10,11,15] in a FACSCalibur (Becton Dickinson) flow cytometer. The cytometer was calibrated daily. Briefly, for analysis of leukocyte-platelet aggregates, sample threshold was set on forward light scatter, and a dot plot of FITC-CD14 fluorescence vs. side light scatter was used to set gates for monocytes and neutrophils based on the known distribution of these populations. PE-CD42a identified leukocyte-platelet aggregates, based on background staining with irrelevant Ig. For analysis of GPIIb-IIIa activation and P-selectin surface expression on platelets, sample threshold was set on CD61 fluorescence, and platelets gated based on their characteristic forward and side light scatter. The amount of activated GPIIb-IIIa and P-selectin fluorescence on platelets was measured.

Statistics

Normal subjects study The Pearson correlation coefficient was calculated for preclopidogrel ADP-stimulated platelet activation markers vs. postclopidogrel ADP-stimulated platelet activation markers.

Patient study For patient demographics, continuous data were analyzed using the unpaired Student's *t*-test and frequency data were analyzed using Fisher's exact test.

To accommodate the unequal sample sizes in each of the five lengths of exposure to clopidogrel, we employed methods that allowed us to examine variance estimates for unbalanced designs. Homogeneity of variance was tested across different time periods of clopidogrel exposure for a unique assay by estimating the 'goodness of fit' of two alternative specifications of models of assay scores. The first model fits one common variance for all five durations of clopidogrel exposure. A second model was specified that was identical to the first except that a variance parameter was allowed for each of the five durations of clopidogrel exposure. The Schwarz Bayesian information criterion (BIC) allowed comparison of estimated statistics from the two models, with smaller values indicating better fit [16]. We chose the BIC because it is a more conservative estimate of variance than other commonly used goodness of fit criteria, in that it represents a penalized version of the log likelihood function that takes into account sample size and the number of parameters in the model. The SAS PROC MIXED was used to test whether a common homogeneous variance was sufficient, or whether a separate variance was necessary and desirable for each length of clopidogrel exposure (i.e. whether the variances are heterogeneous).

Results

Normal subjects study

The effects of clopidogrel on ADP-induced platelet activation were studied in 25 non-aspirin-treated healthy subjects (Fig. 1). Even before the administration of clopidogrel there was a marked variation in the response of platelets to ADP. However, as expected, clopidogrel decreased the ADP-induced platelet surface expression of P-selectin, a marker of platelet

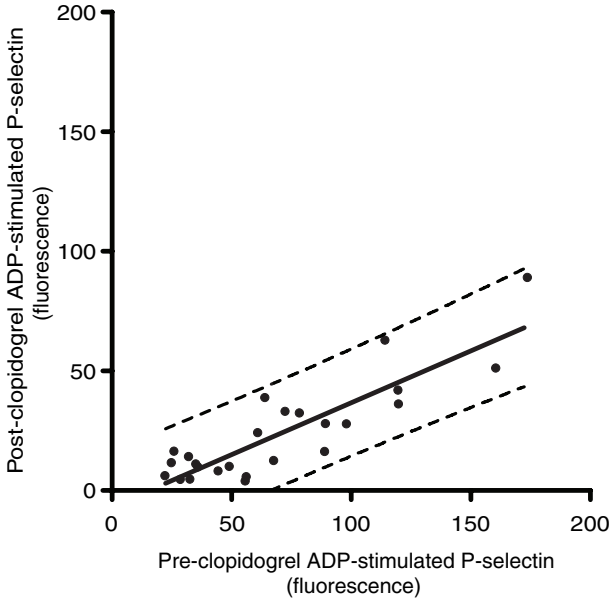

Fig. 1. Pre-clopidogrel response to adenosine diphosphate (ADP) predicts post-clopidogrel response to ADP in non-aspirin-treated healthy subjects. Before and 5 h after oral administration of 300 mg clopidogrel to healthy volunteers, anticoagulated diluted whole blood was stimulated *ex vivo* with 20 μM ADP. Platelet surface P-selectin was determined by whole blood flow cytometry. Dashed lines represent 95% prediction band of the regression line. Pearson $r = 0.8703$, $P < 0.0001$, $n = 25$ subjects.

degranulation [14]. Fig. 1 shows the correlation of preclopidogrel and postclopidogrel response to 20 μM ADP in the 25 normal volunteers. The data show that preclopidogrel response to ADP predicted 5 h postclopidogrel (300 mg oral) response to ADP (Pearson $r = 0.8703$, $P < 0.0001$). Similarly, preclopidogrel response to 20 μM ADP predicted 7 days postclopidogrel (300 mg then daily 75 mg oral) response to 20 μM ADP, as determined by other markers of platelet activation: NPAs ($r = 0.8268$, $P = 0.006$) and MPAs ($r = 0.7049$, $P < 0.034$).

Patient study

Because of our findings in non-aspirin-treated healthy subjects, we then studied the effects of clopidogrel on ADP-induced platelet activation in 613 consecutive, aspirin-treated patients presenting to our cardiac catheterization laboratories for evaluation of CAD (Fig. 2). Even without the administration of clopidogrel there was a marked variation in the response of platelets to ADP, as determined by (a) the platelet surface expression of activated GPIIb-IIIa (Fig. 2, upper left panel), (b) the platelet surface expression of P-selectin (Fig. 2, upper right panel), (c) MPAs (Fig. 2, lower left panel), and (d) NPAs (Fig. 2, lower right panel). As expected, clopidogrel decreased ADP-induced platelet activation, as determined by each of these four assays (Fig. 2).

We tested for homogeneity of variances across the duration of clopidogrel exposure (Table 2). BIC estimates indicated homogeneity of variances across all durations of clopidogrel exposure (no exposure, 0–4 h, > 4 h to 3 days, > 3 days to 1 month, > 1 month) for ADP-induced platelet activation, as determined by the four independent assays: (a) platelet surface expression of activated GPIIb-IIIa (as reported by the conformation-specific monoclonal antibody PAC1), (b) platelet surface expression of P-selectin, (c) MPAs and (d) NPAs (Table 2). For each of these four assays of ADP-induced platelet activation, BIC estimates in Table 2 indicate homogeneity of variances for: all patients; patients with acute CAD (myocardial infarction or unstable angina); patients with other CAD; patients with no CAD; patients receiving aspirin 81 mg; and patients receiving aspirin 325 mg. Thus, irrespective of the duration of clopidogrel administration, the severity of CAD, and the dose of aspirin, clopidogrel did not increase the variance in the platelet response to ADP in any of these four independent assays of platelet activation (Table 2, Fig. 2). Similar results were obtained when platelet surface activated GPIIb-IIIa, platelet surface P-selectin, MPAs and NPAs were analyzed by per cent positives [11] rather than mean fluorescence.

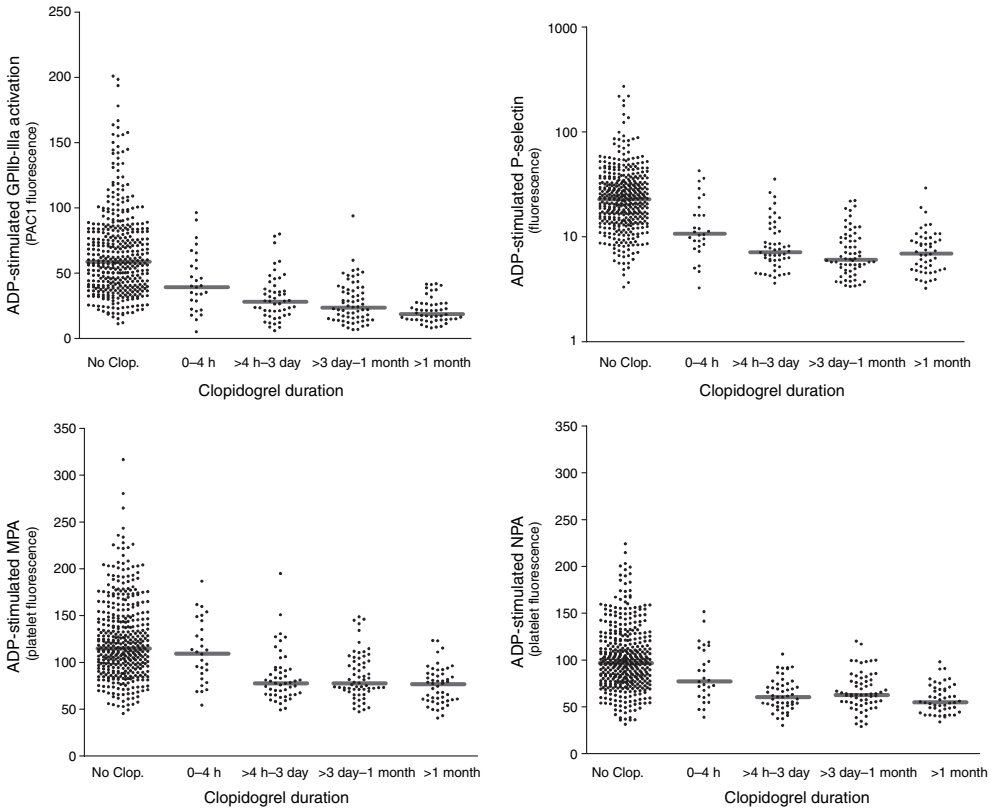

Fig. 2. The effects of clopidogrel on adenosine diphosphate (ADP)-stimulated platelet activation in 613 consecutive, aspirin-treated patients presenting to our cardiac catheterization laboratories for evaluation of coronary artery disease. Whole blood flow cytometry was used to determine 20 μM ADP-stimulated platelet surface expression of activated GPIIb-IIIa (as reported by the conformation-specific monoclonal antibody PAC1) (upper left quadrant), platelet surface expression of P-selectin (upper right quadrant), monocyte-platelet aggregates (lower left quadrant) and neutrophil-platelet aggregates (lower right quadrant). The duration of patient exposure to clopidogrel is indicated on the horizontal axes (no exposure, 0–4 h, > 4 h to 3 days, > 3 days to 1 month, > 1 month). Each dot represents one patient. The solid lines indicate the medians.

Table 2 Tests for homogeneity of variances across all durations of clopidogrel exposure (no exposure, 0–4 h, > 4 h to 3 days, > 3 days to 1 month, > 1 month)

Patient group	Assay	BIC	
		Model 1	Model 2
All ($n = 613$)	PAC-1	955.4	971.9
	P-selectin	1166.7	1174.2
	Monocyte platelet aggregates	351.2	370.2
	Neutrophil platelet aggregates	389.0	405.6
MI + UA ($n = 123$)	PAC-1	265.9	285.4
	P-selectin	299.1	309.2
	Monocyte platelet aggregates	95.2	109.0
	Neutrophil platelet aggregates	29.1	42.8
Other CAD ($n = 402$)	PAC-1	768.4	786.2
	P-selectin	900.8	904.9
	Monocyte platelet aggregates	260.3	282.6
	Neutrophil platelet aggregates	272.3	290.1
No CAD ($n = 88$)	PAC-1	178.6	190.2
	P-selectin	120.9	132.9
	Monocyte platelet aggregates	260.3	282.6
	Neutrophil platelet aggregates	16.8	23.4
ASA 81 mg ($n = 215$)	PAC-1	400.4	421.8
	P-selectin	476.2	479.8
	Monocyte platelet aggregates	135.8	145.2
	Neutrophil platelet aggregates	139.3	150.2
ASA 325 mg ($n = 387$)	PAC-1	868.6	879.2
	P-selectin	778.6	792.3
	Monocyte platelet aggregates	268.1	286.2
	Neutrophil platelet aggregates	304.1	317.6

In each assay, platelets were stimulated with 20 μM adenosine diphosphate. For each assay/clinical setting, goodness of fit indicated that model 1 (assumes homogeneous variance) is better than model 2 (assumes heterogeneous variance) because the Bayesian information criterion for model 1 was less than model 2 in each case. Models 1 and 2 are described in the Methods section. Degrees of freedom = 4. BIC, Bayesian information criterion; CAD, coronary artery disease; MI, myocardial infarction; UA, unstable angina.

At baseline, in the absence of ADP stimulation there was much less variation in platelet activation and secretion (as determined by platelet surface activated GPIIb-IIIa, platelet surface P-selectin, MPAs, and NPAs) than the variation in platelet activation seen after ADP stimulation (data not shown). The variation in platelet activation observed in unstimulated samples correlated poorly with the degree of platelet activation in post-ADP stimulated samples. For example, in patients not on clopidogrel (baseline), $r^2 = 0.027$ for unstimulated vs. ADP-stimulated platelet surface GPIIb-IIIa activation. Comparable with our findings in the presence of ADP, the coefficient of variation in the absence of ADP was not increased by clopidogrel treatment at any time point (no clopidogrel, 0 – 4 h clopidogrel, > 4 h to 3 days clopidogrel, > 3 days to 1 month, or > 1 month) irrespective of the platelet activation assay used.

Discussion

This study provides evidence that 'clopidogrel resistance' is accounted for by a pre-existent variability in platelet response to ADP and this variability is not increased by clopidogrel administration. The evidence comes from 25 consecutive, non-aspirin-treated healthy subjects (Fig. 1) and 613 consecutive, aspirin-treated patients with or without angiographically-documented CAD (Table 2, Fig. 2), as determined by the effects of clopidogrel on four independent and well-standardized assays of platelet activation [3]: platelet surface activated integrin αIIbβ3 (GPIIb-IIIa, as reported by the conformation-specific monoclonal antibody PAC1), platelet surface P-selectin, MPAs, and NPAs. We tested for homogeneity of variance across all durations of clopidogrel exposure and severity of CAD by estimating the 'goodness of fit' of two independent models (Table 2). Irrespective of the duration of clopidogrel administration, the severity of CAD, and the dose of aspirin, clopidogrel did not increase the variance in the platelet response to ADP in any of the four assays.

There has been increasing interest in 'clopidogrel resistance' [3–7,17]. A number of possible mechanisms for 'clopidogrel resistance' have been proposed [3], including poor bioavailability (non-compliance, under-dosing, poor absorption, and interference by atorvastatin with cytochrome P450-mediated metabolism of clopidogrel [6]), accelerated platelet turnover (with the introduction into the bloodstream of newly formed, drug-unaffected platelets) and single nucleotide polymorphisms (SNPs, e.g. the $P2Y_{12}$ H2 haplotype [18]). However, in the absence of clopidogrel, it is well known that there is a wide inter-individual variation in platelet response to ADP, the causes of which may include SNPs in the $P2Y_1$ [19] and/or

$P2Y_{12}$ ADP receptors [18] and variations in the platelet surface density of the $P2Y_1$ receptor [20]. We have therefore previously hypothesized that 'clopidogrel resistance' may actually be neither clopidogrel resistance nor clopidogrel response variability but the result of pre-existent platelet response variability [3]. The findings in the present study strongly support this hypothesis. Our conclusion from the present 613-patient study that pre-existent variability in platelet response to ADP accounts for 'clopidogrel resistance' is consistent with previous small studies of 48 patients [21], 50 patients [22], 62 patients [23], and 92 patients [4], respectively, undergoing PCI in which patients with the highest pretreatment platelet reactivity remained the most reactive after clopidogrel treatment. Three of these studies [4,22,23] utilized ADP-induced platelet aggregation rather than the four whole blood flow cytometric assays used in the present study. In contrast, in a 67-patient study [24], utilization of the phosphorylation state of the vasodilator-stimulated phosphoprotein to evaluate the clopidogrel response was reported to show less variation prior to than after clopidogrel treatment.

Although the present study does not support the concept of 'clopidogrel resistance', patients with higher residual platelet reactivity after the initiation of clopidogrel therapy may nevertheless be at greater risk for thrombosis. Thus, some [5,8,9], but not other [25], very small studies suggest that patients with higher residual platelet reactivity after the initiation of clopidogrel therapy have more subsequent major adverse clinical outcomes. Higher doses of clopidogrel [26–29], or other antiplatelet therapy, may therefore be beneficial, especially in those patients with greater platelet reactivity to ADP, measured either before or after clopidogrel therapy. However, definitive evidence for the benefit of guided antiplatelet therapy based on the degree of platelet reactivity will have to await the results of prospective clinical outcome studies [3]. In addition, novel thienopyridines may provide a therapeutic advantage over clopidogrel. For example, prasugrel (CS-747) has twice the platelet inhibitory effect of clopidogrel and results in much less inter-individual variability in platelet response to ADP [30,31].

Addendum

A. D. Michelson, M. D. Linden, M. I. Furman, M. L. Fox and A. L. Frelinger designed the patient study. A. D. Michelson, W. C. Lau and A. L. Frelinger designed the normal subject study. M. D. Linden, Y. F. Li, M. R. Barnard and A. L. Frelinger performed the laboratory assays. M. L. Fox obtained informed consent from the patients. T. J. McLaughlin designed and performed the statistical analyses. A. D. Michelson wrote the paper with intellectual input from all authors.

Disclosure of Conflict of Interests

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