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Pharmacogenetic Testing for Clopidogrel Using the Rapid INFINITI Analyzer

A Dose-Escalation Study

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Objectives Our aim was to assess whether a higher clopidogrel maintenance dose has a greater antiplatelet effect in *CYP2C19*2* allele carriers compared with noncarriers.

Background Clopidogrel is a prodrug that is biotransformed by the cytochrome P450 enzymes CYP2C19, 2C9, and 3A4, 2B6, 1A2. The *CYPC219*2* loss of function variant has been associated with a reduced antiplatelet response to clopidogrel and a 3-fold risk of stent thrombosis.

Methods Forty patients on standard maintenance dosage clopidogrel (75 mg), for 9.4 ± 9.2 weeks, were enrolled into a dose escalation study. Platelet function was assessed at baseline and after 1 week of 150 mg once daily using the VerifyNow platelet function analyzer (Accumetrics Ltd., San Diego, California). Genomic DNA was hybridized to a BioFilmChip microarray on the INFINITI analyzer (AutoGenomics Inc., Carlsbad, California) and analyzed for the *CYP19*2*, **4*, **17*, and *CYP2C9*2*, **3* polymorphisms.

Results Platelet inhibition increased over 1 week, mean +8.6 ± 13.5% (p = 0.0003). Carriers of the *CYP2C19*2* allele had significantly reduced platelet inhibition at baseline (median 18%, range 0% to 72%) compared with wildtype (*wt*) (median 59%, range 11% to 95%, p = 0.01) and at 1 week (p = 0.03). *CYP2C19*2* allele carriers had an increase in platelet inhibition of (mean +9 ± 11%, p = 0.03) and reduction in platelet reactivity (mean −26 ± 38 platelet response unit, p = 0.04) with a higher dose. Together *CYP2C19*2* and *CYP2C9*3* loss of function carriers had a greater change in platelet inhibition with 150 mg daily than *wt/wt* (+10.9% vs. +0.7%, p = 0.04).

Conclusions Increasing the dose of clopidogrel in patients with nonresponder polymorphisms can increase antiplatelet response. Personalizing clopidogrel dosing using pharmacogenomics may be an effective method of optimizing treatment. (J Am Coll Cardiol Intv 2009;2:1095–101) © 2009 by the American College of Cardiology Foundation

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Clopidogrel is an antiplatelet agent that blocks the adenosine diphosphate (P2Y12) receptor and prevents atherothrombotic events (1). The response to clopidogrel shows wide population variability (2), and up to 5% to 30% of patients may not respond to the drug (3,4). Clopidogrel is a pro-drug that requires conversion to its active thiol derivative, and this is catalyzed by the cytochrome P450 enzyme system. While (CYP) 3A4, 3A5, 2C19, 2C9, and 1A2 may be relevant in this conversion (5–7), only loss of function polymorphisms within the 2C19 gene have been associated with a reduced clinical response to clopidogrel (8–11).

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The genetic polymorphisms within the CYP2C19 gene are denoted by the star nomenclature. The more common *CYP2C19*2*, **3*, and **4* alleles display a loss of function, while *CYP2C19*17* is associated with ultrarapid enzyme activity (9). The mechanism behind nonresponse is hypothesized to be reduced exposure to the active metabolite of clopidogrel. While heterozygotes for the loss of function allele may still be able to convert some of clopidogrel to its active form, rarer homozygotes may be incapable of any conversion (5). Furthermore, the frequency of the *2 allele and rarer loss of function variants are more common in some ethnic groups.

Abbreviations and Acronyms

CI = confidence interval

PPI = proton pump inhibitor

PRU = platelet response unit

Recent attention has been drawn to the clinical importance of these genetic variants (9). Most of these studies have shown a consistent increase in the risk of stent thrombosis in genetic nonresponders taking clopidogrel (9–12). In the TRITON–TIMI 28 (Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel–Thrombolysis In Myocardial Infarction 28) study, there was a higher incidence of the composite of ischemic events and cardiovascular death in those carrying the *CYP2C19*2* allele who received clopidogrel. In contrast, the *CYP2C19*2* allele carriers receiving prasugrel did not face the same risk of ischemic events or death and, interestingly, did not display a trade-off with increased bleeding on prasugrel (13). Alternatively, loss of function allele carriers may respond to higher doses of clopidogrel (14).

Individualizing treatment, using genotyping, may allow cost effective targeting of treatment or introduction of new therapies. This might improve outcomes without increasing adverse events. This study assesses genotyping in a cohort in which a clopidogrel dose was escalated over a week. We utilized a genotyping platform capable of rapid results turnaround.

Methods

Study population. The study protocol was approved by the Northern Regional Ethics Committee of New Zealand and registered with the Australian New Zealand Clinical Trials Registry, ACTRN12606000129583. Patients were individuals who had undergone percutaneous coronary intervention >2 weeks before enrollment. Exclusion criteria were: a bleeding or inherited platelet disorder, gastrointestinal bleeding or gastric ulcer/duodenal ulcer/gastritis within the last 6 months, sensitivity/allergy to aspirin/clopidogrel, renal failure (creatinine clearance estimate glomerular filtration rate <30 ml/min), anemia (hemoglobin <115 g/l), and thrombocytopenia (platelet count $<150 \times 10^9$/l).

Study design. The study was an open label dose escalation study with molecular randomization.

Hypothesis. The primary hypothesis was that an increased clopidogrel dose would increase platelet response in *CYP2C19*2* allele carriers. The secondary hypothesis was that carriers of the *CYP2C19*2* allele would have a greater change in platelet inhibition, with an increased dose of clopidogrel over 1 week, than wildtype individuals. A post-hoc analysis included the addition of *CYP2C9*3* allele carriers to *CYP2C19*2* carriers, to assess the change in response (delta) of a higher dose compared with wildtype. An interaction with proton pump inhibitors (PPIs) was also assessed.

Study protocol. Forty patients already taking 75 mg daily of clopidogrel had a baseline platelet function test performed, followed by a dose increase to 150 mg daily for 1 week.

Blood sampling. Venous blood was sampled using a vacutainer technique and collected into 2-ml 3.2% citrate tubes (Greiner Vacuette, Greiner, Kremsmuenster, Austria), using a 20-gauge needle and syringe. The collection tubes were inverted 4 times to mix the anticoagulant and left for 10 min at ambient temperature (24°C) before testing. Platelet function was tested at baseline and at 7 days. Whole blood for DNA extraction was taken in EDTA tubes.

Platelet function analysis. Platelet function was measured using the VerifyNow point-of-care rapid platelet function analyzer and its P2Y12 cartridge (Accumetrics Ltd., San Diego, California). This device uses fibrinogen-coated microbeads, an agonist of adenosine diphosphate (20 mmol/l), and light transmittance through whole blood, to measure platelet agglutination. The P2Y12 cartridge result correlates favorably with light transmittance aggregometry (15), with reported increased sensitivity to the P2Y12 receptor due to the addition of prostaglandin E2 (22 nmol/l) to the reaction chamber (2). Platelet inhibition is reported as the percentage change in the platelet response unit (PRU) from a baseline (BASE) unit, derived from a second channel run in parallel with the adenosine diphosphate channel using the agonist isothrombin receptor activating peptide. A lower

Table 1. Patient Baseline Characteristics (n = 40)

Men	31 (78%)
Caucasian*	35 (88%)
Age (yrs)	67 ± 11
Weight (kg)	87 ± 16
Creatinine (mg/dl)	1.01 ± 0.16
Weeks on clopidogrel	9.4 ± 9.2
Number given 600-mg loading dose	39 (98%)
Past medical history	
Type II diabetes	8 (20%)
Hypertension	20 (50%)
Dyslipidemia	17 (43%)
Congestive heart failure	2 (5%)
Smoker	4 (10%)
Coronary bypass surgery	5 (13%)
Percutaneous coronary intervention	35 (88%)
Family history of coronary artery disease	7 (18%)
Interacting medication	
Omeprazole	13 (33%)
Other CYP3A4, 2C19 drug	0

Values given as n (%) or mean ± SD. *Other ethnicity included Maori (n = 2), Fijian Indian (n = 2), Chinese (n = 1).

PRU indicates greater platelet inhibition, but this is not controlled for the effect of thrombin.

Genotyping. Genomic DNA was extracted from whole blood using a MiniAmp extraction kit (Qiagen, Venlo, the Netherlands). Multiplex amplification of each sample was performed in an individual well of a 24-well plate using an Eppendorf Mastercycler (Hamburg, Germany). Template and Platinum Taq Polymerase (Life Technologies, Carlsbad, California) were added to an analyte-specific amplification mix (AutoGenomics Inc., Carlsbad, California). After amplification, the plate was placed in the INFINITI analyzer (AutoGenomics Inc.) where detection primer extension occurred, followed by hybridization of detection primers to individual oligonucleotides arrayed on the BioFilmChip. After hybridization, the BioFilmChips were washed and scanned in the INFINITI optics module.

The Autogenomics 2C19^{+} and 2C9-VKORC1 assays were used to detect the presence of *CYP2C19*2*, **3*, **4*, **5*, **6*, **7*, **8*, **10*, and **17* and *CYP2C9*2*, **3*, **4*, **5*, **6*, and **1* polymorphisms, respectively. These tests were performed after the second platelet function test, and the results were retrospectively assessed. *CYP2C19*2* and *CYP2C9*3* but excluding *CYP2C19*17* carriers were classified at poor metabolizers. Combined carriers of *CYP2C19*17* and *2 and wildtype/wildtype were considered intermediate metabolizers. *CYP219*17* carriers without *CYP2C19*2* or *CYP2C9*3* alleles were classified as ultrametabolizers).

Statistics. The numbers of patients required to reach statistical power were calculated using figures for platelet inhibition effected by higher doses of clopidogrel (14). We estimated a study of 40 patients would provide ~90% power to detect the influence of genotypes on platelet function at a significance level of 0.05. The primary output of interest from the VerifyNow (Accumetrics) instrument was platelet inhibition, but where a result using this variable was not statistically significant the platelet response unit has been reported. The Student *t* test was applied where the data was parametric and are reported in mean ± SD when paired and mean and 95% confidence interval (CI) when unpaired. The Wilcoxon rank sum test was used for the nonparametric platelet percentage inhibition data and reported as median and range. The software used for analysis was MedCalc version 7.3.0.1 (MedCalc Software, Mariakerke, Belgium).

Results

One patient did not complete the study protocol due to nonattendance at 1 week. There were no bleeding episodes. One DNA sample was insufficient for analysis. Baseline characteristics of the patients are outlined in Table 1. The allelic frequencies of the polymorphisms are outlined in Table 2. A number of individuals were compound heterozygotes, but they were insufficient in number to evaluate gene-gene interactions. Platelet inhibition at baseline for each allelic group is shown in Figure 1.

Platelet inhibition on average increased over 1 week, mean +8.6 ± 13.5% (p = 0.0003) (Fig. 2). Carriers of the *CYP2C19*2* allele had significantly reduced platelet inhibition at baseline (median 18%, range 0% to 72%) compared with wildtype (*wt/wt*) (59%, 11% to 95%, p = 0.01) and at 1 week (p = 0.03).

A paired comparison of baseline results with results at 7 days showed that platelet inhibition could be increased in *CYP2C19*2* allele carriers with an increased dose of clopidogrel. A mean increase in platelet inhibition of mean +9 ± 11% (p = 0.03) and reduction in platelet reactivity (mean −26 ± 38 PRU, p = 0.04) was seen in *CYP2C19*2* carriers with a higher dose (Fig. 3).

Together *CYP2C19*2* and *CYP2C9*3* loss of function carriers had a greater change in platelet inhibition with 150 mg daily than *wt/wt* (Δ mean −10%, 95% CI: −20 to −0.1, p = 0.05) (Fig. 4).

Table 2. Frequencies of Detected Variants

Gene	Star Nomenclature	dbSNP Reference Number	No. of Alleles in Cohort	Frequency of Carrier Status in the Population (n = 39)
CYP2C19	*2	rs4244285	13	33%
	*3	rs1057910	0	0%
	*17	rs12248560	18	46%
CYP2C9	*2	rs1799853	6	15%
	*3	rs1057910	8	21%

dbSNP = single nucleotide polymorphism database.

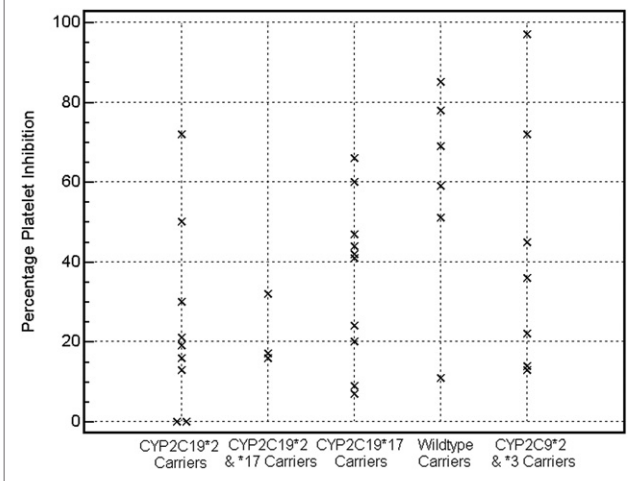

Figure 1. Baseline Platelet Inhibition

Platelet function demonstrated for each genotype group.

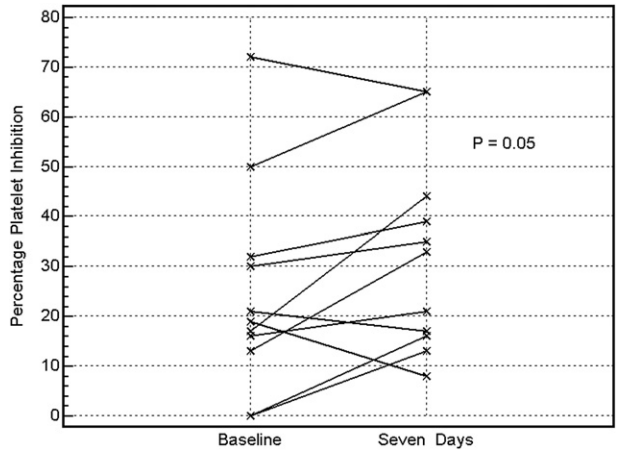

Figure 3. Platelet Function in *CYP2C19*2* Carriers at Baseline and 7 Days

Platelet function at baseline in *CYP2C19*2* carriers and after 1 week of 150 mg daily clopidogrel. An improvement in platelet inhibition is seen in most patients.

Omeprazole (n = 12) or lansoprazole (n = 1) were being taken by 33% (n = 13) of individuals. Patients on these PPIs, which are metabolized by CYP2C19, had a lower percentage platelet inhibition (median +14%, average rank 16 to 22, p = 0.18) and a higher platelet reactivity (mean −51 PRU, 95% CI: −7 to 110, p = 0.08) suggesting a drug-drug interaction, leading to a reduced response to clopidogrel. After 1 week of therapy, those taking these PPIs still had lower platelet inhibition than those not on PPIs (mean −10% inhibition, 95% CI: −8 to 27, p = 0.3). However, compared with baseline, those on a PPI had a significant increase in platelet inhibition after 1 week of a higher dose (mean +9 ± 10%, p = 0.007), showing that the influence of the PPI could be overcome. Those on PPIs had a trend toward a greater change in platelet inhibition than those not on PPIs (Δ median 15% inhibition, 95% CI: −3 to 16 vs. Δ median 7% inhibition, 95% CI: −2 to 13, p = 0.3) (Fig. 5). Only 1 *CYP2C19*2* carrier was on omeprazole.

Discussion

This study addressed the question of whether increasing a dose of clopidogrel from 75 mg once daily to 150 mg once

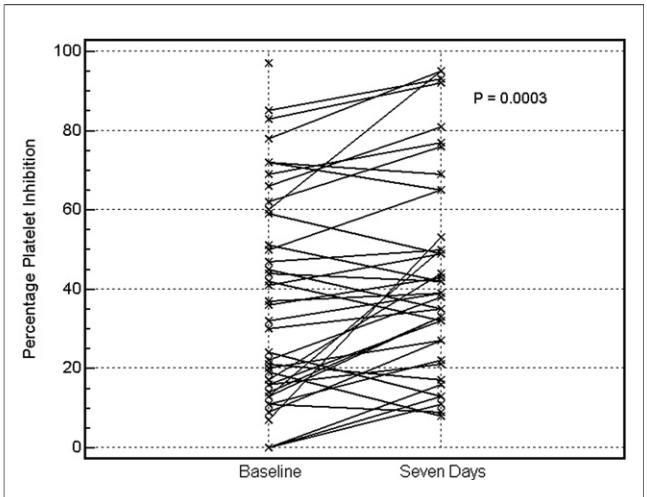

Figure 2. Platelet Function at Baseline (75 mg) and 7 Days (150 mg)

Platelet function at baseline in patients on 75 mg once daily clopidogrel and after 1 week of 150 mg daily. Variable responses but overall a mean increase in platelet inhibition is demonstrated.

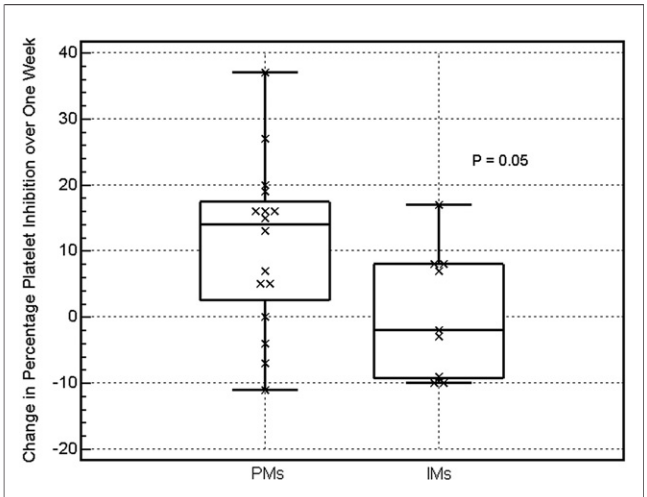

Figure 4. Change in Percentage Platelet Inhibition in CYP2C19*2 Carriers

Box plots represent median, range, and 25th and 75th quartiles. See text for discussion of poor metabolizer (PM) and intermediate metabolizer (IM) status.

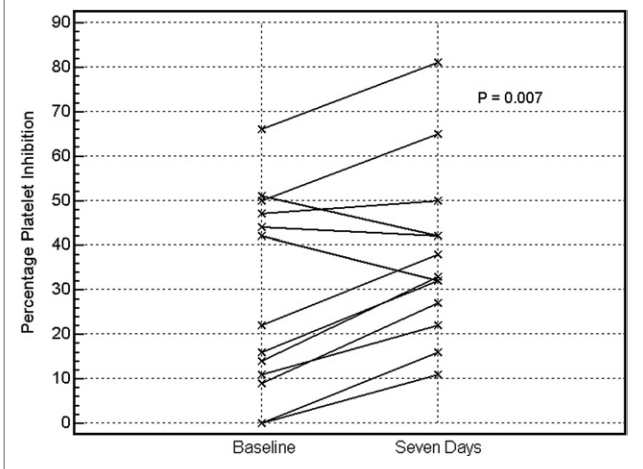

Figure 5. Change in Percentage Platelet Inhibition Over 1 Week in Patients on PPI

Increase in platelet inhibition after 1 week of 150 mg daily clopidogrel, in patients taking proton pump inhibitors (PPIs).

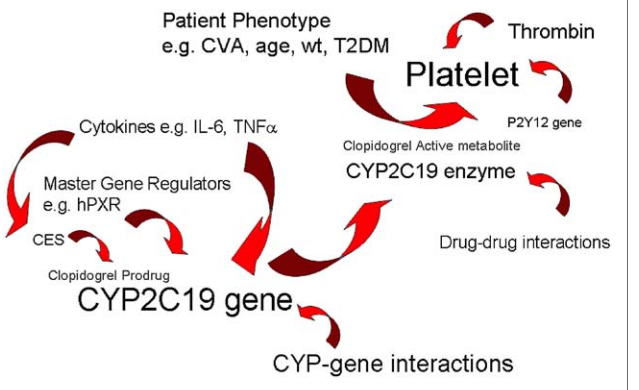

Figure 6. Clopidogrel Response Incorporating Static and Variable Factors

CES = carboxylesterase; CVA = stroke; hPXR = human pregnane X receptor; IL = interleukin; TNF = tumor necrosis factor; T2DM = type II diabetes; wt = weight.

daily for 1 week increases clopidogrel's antiplatelet response in genetic nonresponders. The results show that platelet inhibition can be increased in individuals carrying the loss-of-function *CYP2C19*2* allele by giving higher doses of clopidogrel. This supports our previous study (14). However, despite a dose of 150 mg once daily, there is still a strong influence of this variant on platelet function, and the small improvement in platelet inhibition may not translate into clinical significance. There was also a trend to a reduced antiplatelet response to clopidogrel in those taking omeprazole, a drug metabolized also by the CYP2C19 enzyme. This drug-drug interaction may also be overcome by increasing the dose of clopidogrel.

Several studies have linked an increased risk in stent thrombosis and major adverse cardiac events to carriers of the *CYP2C19*2* polymorphism (8–12). One conflicting result, however, has been demonstrated in the FAST-MI (French Registry of Acute ST-Elevation and Non–ST-Elevation Myocardial Infarction) study, which showed an increased event rate in *2 homozygotes but a reduced event rate in heterozygotes, compared with wildtype homozygotes (11). The reason for this paradoxical finding is not clear.

With the availability of the novel third-generation thienopyridine prasugrel, the potential for targeting treatment appears appealing; however, increasing the dose of clopidogrel in some individuals may be sufficient to make them responders. A study looking at just phenotypic nonresponders, using vasodilator-stimulated phosphoprotein showed an increase in suppression of platelet function with an increased clopidogrel dose, independent of the *CYP2C19*2* allele (16). Another study, using doses up to 2.4 g in a widely spaced iterative loading protocol has shown the existence of a core group of phenotypic nonresponders (17). Whether this profound nonresponsiveness is genetically determined is unknown. We have shown that a practical method of clopidogrel loading using 1,200 mg split over 2 h results in a mean increase of +20% platelet inhibition (VerifyNow, Accumetrics), 7 h after loading, compared with 600 mg ($p = 0.03$) (18).

The functional relevance of rarer CYP2C19 variants needs further investigation. The *CYP2C19*3* allele, coding for a truncated enzyme protein, is particularly prevalent in some populations, such as East Asians and those of East Asian descent such as Pacific Peoples and Maori (19,20). This variant, however, has not been detected in any of the studies performed to date (8–12,21). Together the **3* and **2* variants account for 99% of all poor metabolizers in East Asians and both would need to be considered if clinical genotyping for clopidogrel was being considered in Asian countries (22). We have shown that the rare loss of function *CYP2C19*4* allele has an effect on the platelet response to clopidogrel (14). This variant is more common in those of Ashkenazi descent and ignoring this variant in a genotyping panel can lead to misclassification of phenotypic nonresponders (23). Figure 1 shows that genotyping alone will misclassify some individuals who have satisfactory platelet inhibition and vice versa. The biotransformation of clopidogrel requires a 2-step CYP-dependent process (24). Gene-gene interactions require further study as a combination of single nucleotide polymorphisms may allow better risk stratification for cardiovascular events and further enhance treatment decisions. Pharmacogenetics is unlikely to encapsulate the whole picture, and response prediction is likely to be enhanced by the incorporation of phenotype measures (Fig. 6) (12,25).

The importance of the *CYP2C9*3* allele (2C9 being related to 2C19) with respect to the clopidogrel biotransformation, platelet response, and clinical outcomes is un-

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certain. Pharmacokinetic/pharmacodynamic data suggest that it is an important variant (26); however, a larger pharmacokinetic study with clinical outcome data has shown no effect (9). This may be due to sequence homology or 2C9 primer issues leading to genotyping errors (27).

The interaction between proton pump inhibitors and clopidogrel has received recent attention (28). Both omeprazole (28–31) and lansoprazole (24) have been shown to reduce the responsiveness to clopidogrel, whereas pantoprazole (29,31,32) and esomeprazole (29,32) (the S-enantiomer of omeprazole) have not. In this study, the patients taking omeprazole or lansoprazole had a trend toward a lower than average platelet response suggesting a drug-drug interaction. In this study, a higher dose of clopidogrel was able to partly overcome the clopidogrel-PPI interaction.

Rapid genotyping will be necessary for pharmacogenomics to be useful in the clinical setting. The INFINITI analyzer provides a rapid turnaround time in 8 h. Other technologies exist offering faster genotyping, but some are not capable of multiplexing beyond a certain number of SNPs (33). Examples of rapid genotyping include high resolution melt curve analysis, used in warfarin pharmacogenetic studies (34) and direct nucleic acid detection using gold nanoparticles. This has the advantage of a polymerase chain reaction-free environment; 1 h result turnaround and may only require a single drop of blood to perform an analysis (35).

On the flip side of nonresponse to clopidogrel is the potential for an excessive antiplatelet response and bleeding. Dual antiplatelet therapy is associated with a reduction in cardiovascular event rates but also a rise in bleeding events compared with aspirin alone (36). One of the limiting factors to the acceptance of prasugrel has been the concern regarding bleeding events. Patient characteristics such as an age $\geq$75 years of age, weight $<$60 kg, or presence of previous ischemic stroke are risk factors identified with bleeding events on prasugrel (37). Prescribing prasugrel, instead of clopidogrel, to patients with diabetes and patients undergoing elective or acute primary PCI, may improve the cost-benefit ratio (13,38).

Study limitations. There are a number of limitations of this study, mainly related to lack of statistical power. Genotyping was also performed retrospectively. Despite showing the ability to enhance platelet response in *CYP2C19*2* allele carriers with higher doses of clopidogrel, this may not be clinically significant. We had no measure of treatment adherence. Tachyphylaxis may have influenced the variability in baseline response.

Conclusions

This study has shown that higher doses of clopidogrel, for some individuals, may improve the antiplatelet response. Further study is required to assess whether there is a therapeutic window for thienopyridines, balancing effectiveness and bleeding. Targeting treatment based on nonresponse using either phenotyping, genotyping, or both may be more cost-effective than a "one-size-fits-all" strategy. Integrating important clinical, genetic, and phenotypic data is complex, and decision support software may be required to optimize prescribing (21). Prospective studies, applying pharmacogenetics before treatment is given, are required to prove the cost-effectiveness and clinical usefulness of this approach.

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