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The Relation Between *CYP2C19* Genotype and Phenotype in Stented Patients on Maintenance Dual Antiplatelet Therapy

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Abstract

Both high platelet reactivity (HPR) and *CYP2C19* genotyping have been proposed to stratify cardiovascular event risk and to personalize maintenance dual antiplatelet therapy (DAPT) in stented patients. However, how well *CYP2C19* genotype correlates with HPR in patients on maintenance DAPT is **less clear**. We determined the association of *CYP2C19* loss-of-function (*2) and gain-of- function (*17) allele status with platelet reactivity in 118 stented patients on DAPT ≥2 weeks and in 143 patients with stable coronary artery disease on aspirin therapy alone. Thirty-two percent and 36% carried at least one copy of *2 and *17 alleles, respectively. Neither allele was associated with platelet reactivity in patients on aspirin therapy alone. On DAPT, platelet aggregation was higher in those with *2 allele than non-carriers ($p \leq 0.01$) but did not differ between those with the *17 allele and non-carriers. The prevalence of HPR using the 20uM ADP-induced aggregation cutpoint was 34% in the total population; 26% in *1/*1 homozygotes, 49% in those with the *2 allele and 20% in those with the *17 allele ($p=0.006$). Determination of diplotype status enhanced identification of HPR. However, platelet function on DAPT is highly variable within diplotype groups. Therefore, *CYP2C19* genotype and HPR are imperfect correlates of each other. Since both predict CV events with similar risk ratios, *CYP2C19* genotyping and HPR may provide complementary information to stratify risk and personalized DAPT in stented patients than either alone.

Keywords

cytochrome P450 2C19*2; cytochrome P450 2C19*17; platelets; clopidogrel; pharmacogenomics

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Conflict of Interest Disclosures
The remaining authors report no conflicts.

Dual antiplatelet therapy with clopidogrel and aspirin (DAPT) is the cornerstone of pharmacologic treatment to prevent ischemic events following percutaneous coronary intervention (PCI) and acute coronary syndromes (ACS). High platelet reactivity (HPR) as measured by *ex vivo* platelet aggregation in response to adenosine diphosphate (ADP) during DAPT has been linked to post-PCI ischemic event occurrence in multiple reports (1). Cytochrome P450 2C19 (CYP2C19) is a drug metabolism enzyme that plays an important role in converting clopidogrel into its active metabolite (2). The enzyme is encoded by *CYP2C19* gene located on chromosome 10. A common single nucleotide polymorphism (SNP) of *CYP2C19* located in exon 5 (681G>A, rs4244285, designated *2) encodes for a cryptic splice variant resulting in a protein product with no enzyme activity (3). In several studies, the loss-of-function *CYP2C19**2 allele was associated with decreased clopidogrel responsiveness as measured by ADP-induced *ex vivo* platelet aggregation and post-PCI ischemic risk (4–8). In contrast, the common *CYP2C19**17 allele, resulting from a SNP (-806C>T, rs12248560) has been associated with increased expression and enzyme activity. However, its effect on clopidogrel response and the differential effects of *17 and *2 on platelet function during maintenance DAPT have been less studied (8). **Moreover, the influence of genotype on platelet function in the absence of clopidogrel therapy is unknown.**

Considering the advent of more potent $P2Y_{12}$ inhibitors that do not require activation by CYP, the assessment of clopidogrel antiplatelet efficacy will become more important in deciding long term treatment choice (9). How *CYP2C19* genotypes relate to high platelet reactivity (HPR) as measured by *ex vivo* platelet function testing during maintenance DAPT and the role of genetic analyses to identify the subset of PCI patients at increased risk for cardiovascular events is increasingly under investigation (10). Therefore, we sought to determine how well the *CYP2C19* loss-of-function and gain-of- function alleles associate with platelet reactivity in general and also how well they identified patients with HPR during maintenance DAPT.

METHODS

Patients >18 years, who had established coronary artery disease (CAD) and were on aspirin (81 – 325 mg/day) therapy for a minimum of two weeks were studied (n = 261). Among these patients, 143 were consecutive stable outpatients on aspirin therapy alone (clopidogrel naïve). One hundred and eighteen consecutive patients had undergone prior coronary arterial stenting and were on aspirin and maintenance clopidogrel therapy for at least 2 weeks; of these, 84 were undergoing non-emergent diagnostic arteriography and 34 were stable outpatients. Patients with a bleeding diathesis or a history of gastrointestinal bleeding, hemorrhagic stroke, illicit drug or alcohol abuse, coagulopathy, platelet count <100,000/mm^3, hematocrit <25%, creatinine >2 mg/dL, myocardial infarction within 48 hours, or non-hemorrhagic cerebrovascular accident within 3 months were excluded. The study was approved by the Institutional Review Board at Sinai Hospital of Baltimore and informed consent was obtained from each patient.

Platelet Aggregation

Blood samples were collected by venipuncture in vacutainer tubes (Becton-Dickinson®, Franklin Lakes, NJ) containing 3.8% trisodium citrate. The tubes were centrifuged at 120 g for 5 minutes to recover platelet rich plasma (PRP) and further centrifuged at 850 g for 10 minutes to recover platelet poor plasma (PPP). The PRP and PPP were stored at room temperature and used within two hours. Briefly, platelets were stimulated with ADP (5 μM and 20 μM) as previously described (10). Aggregation was assessed using a Chronolog Lumi-Aggregometer (Model 490-4D) with the AGGRO/LINK control software (Chronolog,

Havertown, PA). Aggregation was expressed as the maximum percent change in light transmittance from baseline, using PPP as a reference.

Genotyping for *CYP2C19* [*2 (rs4244285), *3 (rs4986893) and *17 (rs12248560)] was performed using TaqManR® SNP genotyping assays (Applied Biosystems, Foster City, CA). *CYP2C19*3 was present in only one Asian subject and this subject was excluded in subsequent analyses.

We postulated that *CYP2C19*2 carriers (heterozygotes and homozygotes combined) will have at least 20% higher prevalence of HPR due to decreased antiplatelet effect of clopidogrel compared to non-carriers. Therefore, given the frequency of the *2 allele in the population, in order to determine a 20% absolute difference in the prevalence of HPR between these two groups, a sample size of 105 patients was required with an alpha value of 0.05 and power of 80% (SigmaStatR® 3.1 Software (Point Richmond, CA, USA).

Categorical variables are expressed as n (%) and continuous variables as mean ± SD. ANOVA, Student's *t*-test or Mann-Whitney Rank Sum test were used for comparison of continuous variables after evaluation for normal distribution by the Kolmorgorov-Smirnov test (SigmaStatR® 3.1 Software; Point Richmond, CA, USA). Chi-square analysis was used for comparison of categorical variables between groups. A p-value of <0.05 was considered statistically significant.

We examined the relationship between *CYP2C19* genotypes and baseline and post-clopidogrel platelet aggregation, measured as percent aggregation and as a dichotomous variable (comparison of frequency of HPR). HPR was defined by specific cutoff values by receiver operating characteristic curve (ROC) analyses (5 uM ADP = 46%; 20 uM ADP = 59%) as previously described (10). First, we evaluated the relationship between *CYP2C19* genotypes and HPR frequency treating the *2 and *17 variants as independent. Due to linkage disequilibrium between *CYP2C19* *1 and *17 gain-of-function (fast metabolizer) variants, only three (of four possible) haplotypes exist (Figure 1). These three haplotypes result in six possible diplotypes. In a second analysis, we compared platelet function among the six diplotypes. We also classified subjects into extensive metabolizer (EM), normal metabolizer (NM), intermediate metabolizer (IM) and poor metabolizer (PM) groups, and compared platelet aggregation and HPR between these groups.

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RESULTS

Demographic characteristics of the population are shown in Table 1. Clopidogrel naïve patients were more often Caucasian and had higher systolic blood pressure and less often used beta-blockers than patients on clopidogrel maintenance therapy. The frequency of the *CYP2C19* *2, and *17 alleles in the overall population were 0.17, and 0.20, respectively, and very similar among Caucasians and African Americans in our sample (Table 2). Genotype frequencies were in Hardy-Weinberg equilibrium. The *2 and *17 alleles were in linkage disequilibrium (D' = 1; $r^2 = 0.04$). Based on Chi-square analysis the p-values are - *2: p=0.24, *17: p=0.87. There were no differences in demographic variables between *CYP2C19*2 genotype groups on clopidogrel maintenance therapy (Table 1).

There was no significant relation of *CYP2C19* *2 or *17 carrier status to platelet aggregation in patients treated with aspirin alone (Figure 2A and B). In patients on DAPT, platelet aggregation was higher in those carrying one or two copies of the *2 allele, compared to

non-carriers (p= 0.01 and p=0.008 for 5 and 20 μM ADP-induced aggregation respectively, Figure 3A). By contrast, ADP-stimulated platelet aggregation did not differ between *CYP2C19**17 carriers and non-carriers treated with DAPT (Figure 3B).

The overall prevalence of HPR in patients on DAPT was 23% and 34% assessed by 5 and 20 μM ADP-induced aggregation, respectively. The prevalence of HPR was greater in *2 carriers versus non-carriers (Table 3, Figure 3A). In *17 carriers using the 20 μM ADP-induced aggregation cutpoint the prevalence of HPR was lower than non-carriers (Table 3 and Figure 3B).

Since the *2 and *17 variants are in linkage disequilibrium, we next considered the six naturally occurring diplotypes. We found a gradient of response in which increasing doses of the *2 allele increased platelet reactivity and HPR and increasing doses of the *17 allele decreased platelet reactivity and HPR (p =0.01 and 0.004 for 5 and 20 uM ADP-induced platelet aggregation, respectively, Figure 4). Both patients homozygous for *2 had HPR by 5 and 20 uM ADP-induced aggregation. However, platelet function was highly variable in *17 homozygotes but only 1/5 patients had HPR (Figure 4). When patients were categorized by metabolizer status a similar pattern was observed (p=0.03 and p=0.06 between EM and PM for 5 and 20 uM ADP-induced platelet aggregation, respectively (Figure 5).

DISCUSSION

The present study demonstrates that *CYP2C19*2* and **17* alleles are associated with platelet reactivity in patients on maintenance DAPT. We also observed an effect of both *CYP2C19*2* and **17* alleles on the frequency of HPR and the effect of CYP2C19*2 was more pronounced. Most importantly, our data suggest that for the majority of patients (those who are not homozygous for either *2 or * 17), HPR cannot be excluded by genotyping in patients on maintenance DAPT.

The observed absence of a relation between pre-clopidogrel platelet function and *CYP2C19* allele status suggests a true pharmacogenetic effect and is a critical observation in understanding the mechanism responsible for the reported effect of *2 carrier status on clinical outcomes in previous studies (4,5). It is only during clopidogrel therapy that *2 and *17 carrier status influences platelet function and the prevalence of HPR. Our findings with regard to *CYP2C19* variants and clopidogrel response are largely consistent with studies of others where a lower antiplatelet responsiveness to clopidogrel in patients carrying the *CYP2C19*2* allele was observed and extends findings to include the relation of pharmacodynamics to *CYP2C19*17* genotype, diplotypes of *2 and *17 variants and metabolizer status, and the predictive value of these *CYP2C19* variants on the prevalence of HPR in CAD patients on steady-state maintenance clopidogrel and aspirin therapy (4,6,8,11,12). Mega et al. demonstrated that *CYP2C19*2* carriers had lower clopidogrel active metabolite levels and an absolute reduction in maximal aggregation that was 9% less than non-carriers treated with a 300 mg or a 600 mg followed by a 75 mg maintenance dose of clopidogrel (4). This study was performed in healthy volunteers; aspirin was not uniformly administered; and the relationship between *CYP2C19*2* genotype and platelet aggregation in the absence of clopidogrel was not reported (4). Similar to our study, Trenk et al. reported no relation between *CYP2C19*2* and pre-treatment residual platelet aggregation (6).

With regard to the gain of function *CYP2C19*17* variant, the literature is more mixed. *CYP2C19*17* had no influence on residual aggregation in PCI patients treated with 600mg clopidogrel in a study by Geisler et al (13). In a larger study, Sibbing et al demonstrated an association of the *17 allele (both homozygous and heterozygous carriers) with lower on-

treatment platelet reactivity and higher bleeding events. However, since the *1 and *17 variants are in linkage disequilibrium, more individuals with the *17 variant would be expected to be lacking *2 variant, which may have accounted for this observed difference in platelet reactivity (14). In a subsequent study, the same authors studied the interaction between *2 and *17 on platelet reactivity measured by Multiplate analyzer in patients on chronic clopidogrel therapy. Similar to our study, a gradual increase in platelet reactivity from *17 carriers to *2 carriers was observed. Although this gradual increase in platelet reactivity across the various genotype combination was statistically significant, differences between non-carriers of the *2 variant with and without the *17 variant (to determine if the *17 variant had an independent effect on platelet reactivity) was not evaluated (15).

We also observed an effect of *CYP2C19**2 and **17* alleles on the frequency of HPR when each was considered separately. This is particularly important since HPR is a known predictor of poorer cardiovascular outcomes (16). Hochholzer et al demonstrated that *CYP2C19*2* carrier status was an independent predictor of insufficient antiplatelet response to clopidogrel, but platelet function was measured within 1 day after 600 mg loading dose of clopidogrel in the vast majority of patients (17). Overall, our findings are consistent with Hochholzer that genotyping does not completely discriminate HPR. Significantly higher platelet aggregation values measured by multiplate analyzer in *CYP2C19*2* carriers and significantly lower platelet aggregation values in *CYP2C19*17* carriers were observed compared to homozygous wild-type carriers in patients on chronic DAPT treated with stenting in a recent study by Sibbing et al. In contrast to the present study, both **2* and **17* alleles were identified as independent predictors for platelet reactivity in patients treated with chronic clopidogrel therapy (17).

Despite the clear effect of *CYP2C19*2* on platelet aggregation and HPR in clopidogrel-treated patients in the present study, the predictive value to detect HPR in our study was modest. The sensitivity of *CYP*2C19*2 to detect HPR was only 50–56%; measurement of these alleles alone would miss many patients with HPR. Therefore, *CYP2C19* genotyping alone cannot serve as a reliable surrogate for HPR determined by conventional aggregometry.

The moderate sensitivity of *CYP2C19* genotyping in detecting HPR is consistent with the multiple non-genetic influences on clopidogrel metabolism and inherent variability in platelet reactivity (9,16). Furthermore, our previously published heritability and genome-wide association analyses show that the *CYP2C19* locus accounts for approximately 12% of the variation in platelet response to clopidogrel and that there are likely to exist other yet-to-be identified genetic determinants of clopidogrel response (7). Discovery of additional genes and their variants that influence clopidogrel response may increase predictive value of genetic testing. Finally based on current and previous data, we believe that genotyping alone can not be served as a surrogate marker for HPR and estimations of both genotyping and HPR as complimentary tools may be more efficient to estimate the ischemic risk in patients treated DAPT (16,18).

The sample size of our study was not adequate to assess the effect of *CYP2C19*2* or **17* homozygocity on platelet reactivity or HPR. In addition, HPR is also an imperfect predictor of recurrent cardiovascular events and assessment of cardiovascular outcomes were not possible in our study. Thus we could not assess the efficacy of *CYP2C19* genotypes and HPR in predicting cardiovascular events. In this study we have included only light transmittance aggregometry to assess platelet function and the pattern of genetic influence on clopidogrel antiplatelet effect might have been different with whole blood assays such as VerifyNow, VASP or Multiplate analyzer.

The present study demonstrated that determination of diplotype status enhanced identification of HPR in patients on maintenance DAPT. However, platelet function on DAPT is highly variable within diplotype groups except *2 homozygotes. Thus our data suggest that for the majority of patients (those who are not homozygous for *CYP2C19**2), genotype fails to identify a large proportion of patients on maintenance DAPT with HPR. Since both HPR and *CYP2C19* genotype are imperfect correlates of each other and both are predictors of poorer cardiovascular outcomes, the two together may provide complementary information to stratify cardiovascular event risk in patients on DAPT than either alone. Larger and longer-term prospective studies that also include cardiovascular event outcomes will be necessary to optimize predictive algorithms that may include genetic and/or platelet function testing, and their use to individualize $P2Y_{12}$ inhibitor therapy.

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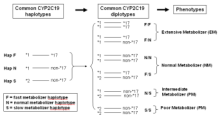

Figure 1.

Linkage disequilibrium and *CYP2C19* haplotypes. Due to linkage disequilibrium between *CYP2C19* *2 loss-of-function (slow metabolizer) and *17 gain-of-function (fast metabolizer) variants, only three (of four possible) haplotypes exist. These three haplotypes result in six diplotypes which define four main phenotypes, extensive metabolizers (EM), normal metabolizers (NM), intermediate metabolizers (IM) and poor metabolizers (PM).

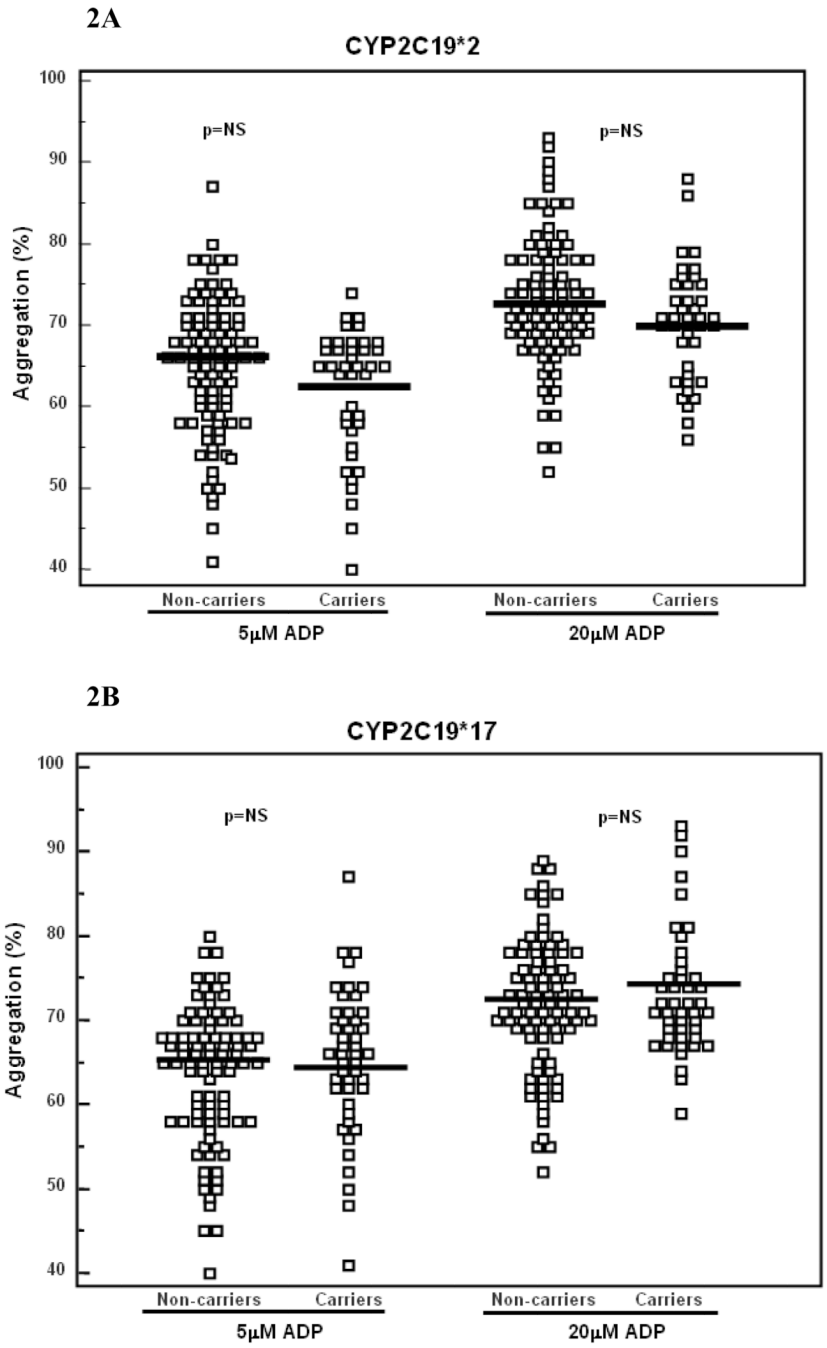

Figure 2.
A and B.
Platelet aggregation stimulated by 5 μM and 20 μM ADP in patients treated with aspirin therapy alone and relation to *CYP2C19**2 (2A) and *CYP2C19**17 (2B) genotypes. Carriers denote presence of one or two copies of the respective alleles. Thick lines indicate mean values.

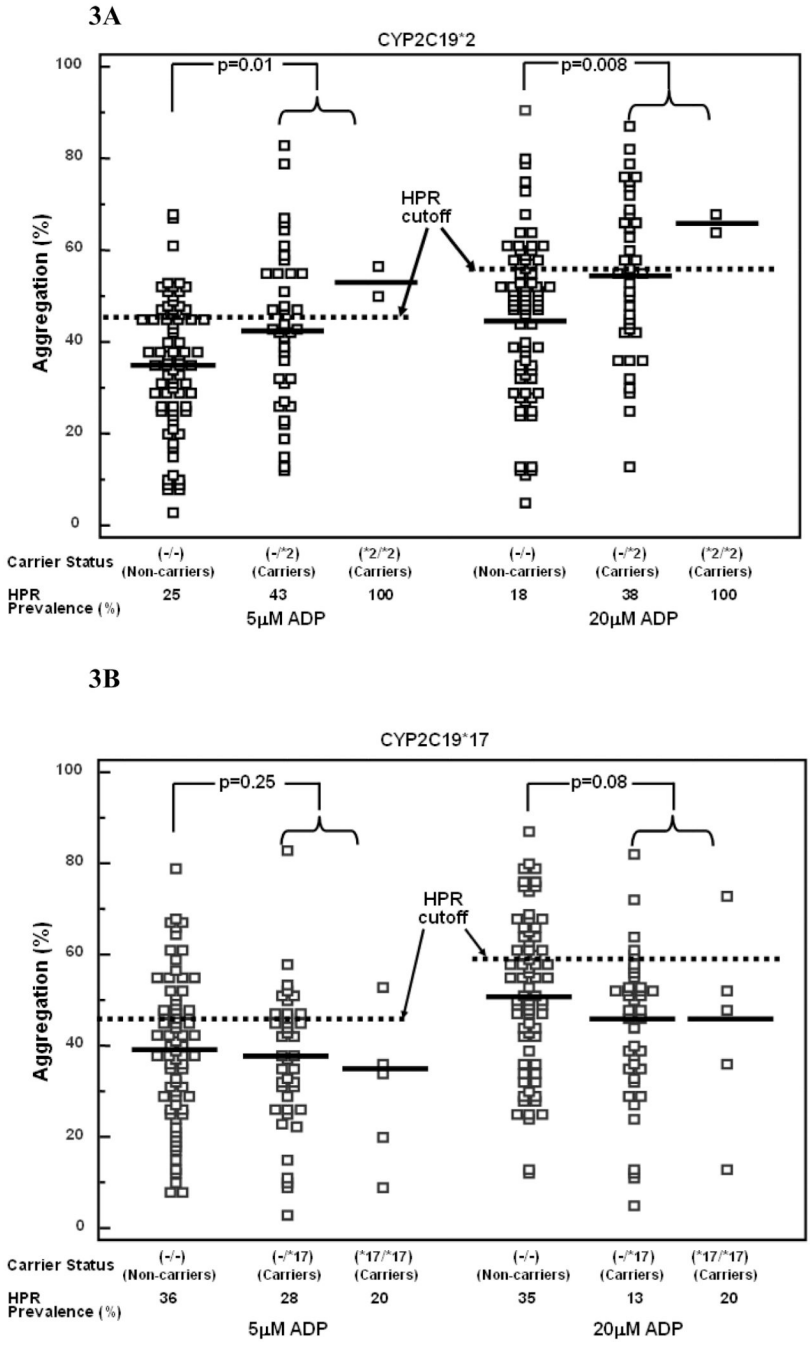

Figure 3.

A and 3B.

Platelet aggregation stimulated by 5 μM and 20 μM ADP and prevalence of high platelet reactivity (HPR) in patients on clopidogrel maintenance therapy and relation to *CYP2C19**2 (3A) and *CYP2C19**17 (3B) genotypes. Thick lines indicate mean values and dotted lines indicate HPR cutoff levels. In multivariate analysis in which both the, presence of the *2 and *17 alleles were included, only the *2 allele was an independent predictor of HPR.

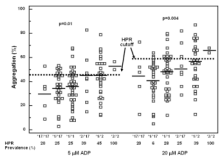

Figure 4.
Platelet aggregation stimulated by 5 μM and 20 μM ADP and prevalence of high platelet reactivity (HPR) in patients on clopidogrel maintenance therapy and relation to *CYP2C19**2 and *17 diplotype status. Thick lines indicate mean values and dotted lines indicate high platelet reactivity (HPR) cutoff levels.

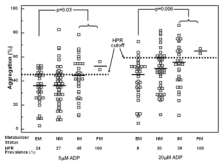

Figure 5.
Platelet aggregation stimulated by 5 μM and 20 μM ADP and prevalence of high platelet reactivity (HPR) in patients on clopidogrel maintenance therapy and relation to metabolizer status. Thick lines indicate mean values and dotted lines indicate high platelet reactivity (HPR) cutoff levels.
EM = extensive metabolizer, NM = normal metabolizer, Intermediate metabolizer (IM), PM = poor metabolizer.

Table 1

Characteristics of Study Groups.

	Clopidogrel Naive (n=143)	**Clopidogrel Maintenance (n=118)**	**p-value**	**Clopidogrel Maintenance CYP 2C19*2 Carriers (n=41)**	**Clopidogrel Maintenance CYP 2C19 Non-carriers (n=77)**	**p-value**
Age (years)	65±11	63±11	0.13	63±11	64±11	0.64
Gender n, (male %)	52, (35)	53, (45)	0.05	14, (34)	39, (51)	0.05
Caucasians n, (%)	94, (63)	59, (50)	0.02	20, (49)	41, (53)	0.34
African-Americans n, (%)	49, (33)	55, (47)	0.11	19, (46)	35, (45)	0.46
Body mass index (kg/m^2)	30±6	31±5	0.15	31±5	31±9	1.0
Systolic Blood Pressure (mm Hg)	142±21	135±18	<0.01	135±18	136±21	0.80
Diastolic Blood Pressure (mm Hg)	73±13	72±15	0.55	72±15	72±15	1.0
Medical History						
History of Smoking n, (%)	41, (27)	23, (19)	0.06	9, (22)	14, (18)	0.30
Current Smokers n, (%)	38, (25)	18, (15)	0.02	11, (27)	17, (22)	0.27
Hypertension n, (%)	116, (77)	93, (79)	0.35	33, (80)	60, (77)	0.35
Hyperlipidemia n, (%)	111, (74)	85, (72)	0.36	30, (73)	55, (71)	0.41
Diabetes n, (%)	55, (37)	46, (39)	0.37	20, (49)	27, (35)	0.10
Myocardial infarction n, (%)	42, (28)	37, (31)	0.30	14, (34)	23, (30)	0.33
Coronary artery bypass graft n, (%)	29, (19)	29, (21)	0.12	12, (29)	17, (22)	0.20
Family CAD n, (%)	60, (40)	53, (45)	0.21	21, (51)	32, (42)	0.18
Baseline Medications						
Beta-blocker n, (%)	88, (59)	85, (72)	0.01	31, (76)	54, (70)	0.25
ACE inhibitor n, (%)	66, (44)	60, (51)	0.13	17, (41)	43, (56)	0.06
Calcium blockers n, (%)	30, (20)	33, (28)	0.07	15, (37)	18, (23)	0.05
Lipid-lowering agent n, (%)	109, (73)	96, (81)	0.06	36, (88)	60, (78)	0.09
Diuretic n, (%)	43, (29)	26, (22)	0.10	9, (22)	17, (22)	0.50
Proton pump inhibitor n, (%)	40, (27)	22, (29)	0.06	6, (15)	16, (21)	0.21

ACE= angiotensin converting enzyme; CAD= coronary artery disease

Table 2

Allele, Genotype, Haplotype and Diplotype Frequencies of *CYP2C19**2 and *17 Stratified by Ethnicity.

***CYP2C19* Locus N, (%)**	**Caucasian (n=155)**	**African American (n=101)**	**Asian-American (n=5)**
Allele Frequencies			
*2	53, (17)	32, (15)	2, (20)
*1	257, (83)	170, (85)	8, (80)
*17	66, (21)	33, (17)	2, (20)
non-*17	244, (79)	169, (83)	8, (80)
Genotype Frequencies			
*17/*17	7 (5)	2 (2)	
*1/*17	43 (28)	27 (27)	3 (60)
*1/*1	55 (35)	42 (42)	
*2/*17	9 (6)	3 (3)	
*1/*2	38 (25)	24 (23)	2 (20)
*2/*2	3 (2)	3 (3)	
Metabolizer Frequencies			
EM	50, (32)	29, (28)	2, (40)
NM	64, (41)	45, (45)	1, (25)
IM	38, (25)	24, (24)	2, (40)
PM	3, (2)	3, (3)	0

EM = extensive metabolizer (*17/*17 and *1/*17);
NM = normal metabolizer (*1/*1 and *1/*2);
IM = intermediate metabolizer (*1/*2)
PM = poor metabolizer (*2/*2).

Table 3

Frequency of High Platelet Reactivity (HPR) in Patients on Clopidogrel Maintenance Therapy in Relation to *CYP2C19* Genotype

	***2 Carriers (n=41)**	***2 Noncarriers (n=77)**	**p-value**
HPR 5μM ADP	46%	25%	0.015
HPR 20μM ADP	41%	18%	0.013
	***17 Carriers (n=45)**	***17 Noncarriers (n=73)**	**p-value**
HPR 5μM ADP	27 %	36%	0.32
HPR 20μM ADP	14%	35%	0.016

Carriers denote one or two copies of the respective allele.

Table 4

HPR Status Based on 5uM and 20 uM ADP-Induced Platelet Aggregation in *CYP2C19**2 and *CYP2C19**17 Carriers and Non-Carriers.

	5uM ADP-induced Aggregation		**20uM ADP-induced Aggregation**	
	HPR Positive n=27	**HPR Negative n=91**	**HPR Negative n=40**	**HPR Negative n=78**
*CYP2C19**2 Carriers n=41	N=15	N=26	N=20	N=21
*CYP2C19**2 Non-Carriers n=77	N=12	N=65	N=20	N=57
*CYP2C19**17 Carriers n=45	N=8	N=37	N=9	N=36
*CYP2C19**17 Non-Carriers n=73	N=19	N=54	N=31	N=42