Common Variation in the Platelet Receptor P2RY12 Gene Is Associated With Residual On-Clopidogrel Platelet Reactivity in Patients Undergoing Elective Percutaneous Coronary Interventions

Goran Rudež, MSc; Heleen J. Bouman, BSc; Jochem W. van Werkum, MD, PhD; Frank W.G. Leebeek, MD, PhD; Adrian Kruit, PhD; Hendrik J.T. Ruven, PhD; Jurriën M. ten Berg, MD, PhD; Moniek P.M. de Maat, PhD; Christian M. Hackeng, PhD

Background—The clinical efficacy of clopidogrel is hampered by a large interindividual variability in platelet inhibition. Polymorphisms in the P2RY12 receptor gene have been suggested to contribute to this variability, but previous studies included a relatively small number of patients and incompletely covered the common variation in the P2RY12 gene. The aim of this study was to comprehensively investigate the possible association between common variation in the entire P2RY12 locus and the magnitude of residual on-clopidogrel platelet reactivity measured by 2 commonly used platelet function assays in a large cohort of patients.

Methods and Results—A total of 1031 consecutive patients with coronary artery disease who were scheduled for elective percutaneous coronary interventions were enrolled. Platelet function was assessed by means of ADP-induced light-transmittance aggregometry and the VerifyNow P2Y12 assay. Six haplotype-tagging single nucleotide polymorphisms were carefully selected to comprehensively cover the total common variation in the P2RY12 gene and its flanking regulatory regions. Six common haplotypes were inferred from these haplotype-tagging single nucleotide polymorphisms (denoted A to F). Haplotype F was associated with significantly lower residual on-clopidogrel platelet reactivity compared with the reference haplotype A. The size of this effect per haplotype allele was approximately 5% aggregation in the ADP-induced light-transmittance aggregometry (P<0.05) and 11 P2Y12 reaction units in the VerifyNow P2Y12 assay (P<0.05).


Key Words: P2Y12 ■ haplotypes ■ platelets ■ clopidogrel ■ revascularization

D ual antiplatelet therapy with clopidogrel and aspirin is currently the treatment of choice for the secondary prevention of atherothrombotic events in patients with coronary artery disease (CAD) and in those undergoing coronary stent implantation.1 In approximately 5% to 30% of clopidogrel-treated patients, the inhibition of platelet aggregation is insufficient.2,3 As a consequence, patients with a high residual on-clopidogrel platelet reactivity may be at a higher risk of atherothrombotic events.4,5

Clinical Perspective on p 521

Common variation in the P2RY12 gene has been suggested as one of the mechanisms underlying this large variability in clopidogrel response.5 The P2RY12 gene encodes the ADP receptor P2Y12, the pharmacological target of clopidogrel. Previous investigations on the relationship between P2RY12 single-nucleotide polymorphisms (SNPs) and high residual on-clopidogrel platelet reactivity were limited by the fact that only the haplotype-tagging SNP (ht-SNP) rs2046934 (i-T744C) was studied. Rs2046934 is in complete linkage disequilibrium (LD) with 3 other P2RY12 SNPs and the haplotypes “H1” and “H2,” which these SNPs determine, cover only the 3’ part of the P2RY12 gene.6–14 In addition, most of the cited studies on P2RY12 SNPs included a relatively small number of patients.

A comprehensive study of common variation in the P2RY12 gene should include ht-SNPs that cover all common haplotypes within the entire P2RY12 locus, ie, the P2RY12 gene and its regulatory regions, such as the promoter and the

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From the Department of Hematology (G.R., F.W.G.L., M.P.M.M.), Erasmus University Medical Center, Rotterdam, The Netherlands; and Departments of Cardiology (H.J.B., J.W.W., J.M.B.) and Clinical Chemistry (A.K., H.J.T.R., C.M.H.), St. Antonius Hospital, Nieuwegein, The Netherlands.

Drs Rudež and Bouman contributed equally to this work.

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Correspondence to Moniek P.M. de Maat, PhD, Erasmus University Medical Center, Department of Hematology, L-431, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands. E-mail m.demaat@erasmusmc.nl

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Risk factors

<table>
<thead>
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<th>Demographics</th>
<th>n=1031</th>
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<tbody>
<tr>
<td>Age, y</td>
<td>64±11</td>
</tr>
<tr>
<td>Male</td>
<td>769 (75)</td>
</tr>
</tbody>
</table>

Table 1. Characteristics of the Study Population

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<td>Male</td>
<td>769 (75)</td>
</tr>
</tbody>
</table>

Risk factors

| Body mass index, kg/m² | 27±4  |
| Smoking               | 210 (20)|
| Diabetes              | 197 (19)|
| Hypertension          | 801 (78)|
| Family history of cardiovascular disease | 633 (61)|

Medication

| Clopidogrel: total group | 1031 (100) |
| Dosing regimen A        | 659 (64)|
| Dosing regimen B        | 314 (30)|
| Dosing regimen C        | 58 (6)  |
| Aspirin                | 923 (90)|
| Coumarins              | 105 (10) |
| Statins                | 830 (81) |

Platelet count (10⁹ L⁻¹) 272±80

Haematocrit, % 41.4±4.3

Values are given as n (%) for categorical variables and as mean±SD for continuous variables.

3' untranslated region. Using this comprehensive approach, we previously demonstrated that common variation in the P2RY12 gene is associated with the risk of restenosis after percutaneous coronary interventions (PCI). Although ADP-induced light-transmittance aggregometry (LTA) is still considered the gold standard for the assessment of clopidogrel-induced platelet inhibition, methods have been recently introduced that are designed for a more standardized monitoring of the efficacy of clopidogrel, such as the VerifyNow P2Y12 assay. The aim of this study was to investigate a possible association between the common genetic variation of the entire P2RY12 locus and residual on-clopidogrel platelet reactivity using several platelet function assays in a large population of patients who were scheduled to undergo elective PCI.

Methods

Study Population

One thousand thirty-one consecutive patients with established CAD scheduled for elective PCI were included in this study. Because of different protocols of referring hospitals, patients had received a different but adequate clopidogrel pretreatment: (a) maintenance therapy of 75 mg daily for at least 24 hours before PCI (n=314); or (c) a loading dose of 600 mg of clopidogrel at least 6 hours before PCI (n=58). Exclusion criteria were the use of GPIIb/IIIa antagonists in the past 7 days before the intervention and a platelet count <150×10⁹ platelets/L. Ninety percent of the patients were on aspirin (80 to 100 mg daily) and 10% were on coumadins. Eighty-one percent of the patients were using statins (Table 1). More than 95% of the study population was of Caucasian origin.

The study protocol was approved by the institutional medical ethics committee. Written informed consent was obtained from each participant.

Definitions

Smoking was defined as any cigarette smoking in the last month. Hypertension was defined as a systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg. Diabetes mellitus was defined according to the World Health Organization criteria. Family history of cardiovascular disease was defined as having a first-degree relative with a history of CAD for women younger than 65 years and for men younger than 55 years.

Blood Collection

Blood samples were drawn from the arterial sheath before heparinization into nonvacuum Sarstedt tubes containing 3.2% sodium citrate (Sarstedt, Nümbrecht, Germany). Hematocrit and platelet count were analyzed in K₂-EDTA anticoagulated blood. Genomic DNA was extracted from K₂-EDTA anticoagulated whole-blood following standard salting-out procedures and stored at 4°C for genetic analysis.

LTA

Citrated whole-blood samples were centrifuged at 120g for 10 minutes to obtain platelet-rich plasma and further centrifuged at 850g for 15 minutes to obtain platelet-poor plasma. Maximal and late aggregation (at 6 minutes) were measured in nonadjusted platelet-rich plasma after stimulation with different concentrations of the agonist ADP (final concentrations: 2, 5, 10, and 20 µmol/L) in an APACT 4004 four-channel aggregometer (LABiTec, Arensburg, Germany).

VerifyNow P2Y12 Assay

The VerifyNow P2Y12 test cartridge system (Accumetrics, San Diego, Calif) was used as described previously. In brief, VerifyNow P2Y12 is a rapid cartridge-based platelet-agglutination assay designed to directly measure the inhibitory effects of clopidogrel therapy. The results are reported in P2Y12 reaction units (PRU), where a higher PRU reflects greater on-clopidogrel ADP-induced platelet reactivity, and in percentage of inhibition, where a higher % reflects greater change of ADP-induced platelet agglutination from clopidogrel-independent (baseline) platelet agglutination that is induced with thrombin receptor activating peptide and protease-activated receptor 4 activating peptide.

Selection of SNPs in the P2RY12 Gene

ht-SNPs were selected according to the approach described previously. Based on the latest LD-map of the P2RY12 locus (ie, P2RY12 gene with 2.5 kb flanking sequence) provided by the International HapMap Project for a population of Utah residents with Northern and Western European ancestry from the CEPH collection (phase II, October 2007; http://www.hapmap.org/) (Figure 1), blocks of haplotypes with frequency >5% were defined from these ht-SNPs using Haplovie software (version 3.3, http://www.broad.mit.edu/mpg/haploview/index.php). Together, the defined haplotypes cover 88% of the total common DNA sequence variation in the P2RY12 locus. The 6 selected ht-SNPs that were genotyped are rs6798347 (c.-281–3614C>t), rs6787801 (c.217+2739T>c), and rs9859552 (c.217+11494C>a), which tag the promoter, exon 1 and a part of intron 1 region, and rs6801273 (c.-216–4445A>g), rs9848789 (c.-216–377G>a), and rs2046934 (c.742T>c), which tag the remaining part of intron 1, as well as the entire exon 2, intron 2, exon 3, 3' untranslated region, and flanking region (Figure 1).

Genotyping and Haplotype Analysis

The 6 selected ht-SNPs were genotyped using Custom TaqMan Genotyping Assays (Applied Biosystems, Foster City, Calif) under standard conditions. The nucleotide sequences of the primers and probes used for each assay are available on request. End-point fluorescence was measured on the ABI 7900HT instrument (Applied Biosystems) and clustered according to genotype using SDS 2.2.2 software (Applied Biosystems). A random selection of 10% of the samples was reanalyzed, and the results were confirmed in 99.7%. Laboratory analyses were performed by research technicians who were blinded for the demographic data of the patients. All SNPs were in Hardy-Weinberg equilibrium when genotyped in a healthy population.
Statistical Methods

Demographic data are presented as means and standard deviations for continuous variables and as counts and percentages for categorical variables. Results of the haplotype analysis are presented as mean effects per haplotype-allele with the corresponding standard errors. Haplotype analysis was performed with Haplo.Stats.20 Briefly, this analysis calculates posterior probabilities for each possible haplotype of an individual and assigns an appropriate weight to the corresponding estimated effect on platelet function. Individuals with missing genotype data for more than 2 SNPs (n=26) were excluded from the analysis. Haplo.Stats assumes an additive effect of haplotype alleles, indicating that the net effect of a person’s haplotype is the sum of the effects of its 2 haplotype alleles.

The associations between the P2RY12 haplotype alleles and on-clopidogrel platelet reactivity were determined by weighted linear or logistic regression analysis, using haplo.glm function and results expressed as mean change from the reference haplotype allele ±SEM. The genetic analysis of the subgroup of patients who had received clopidogrel according to the dosing regimen C was not performed because of the small sample size (n=58).

Other statistical analyses were performed using SPSS for Windows, version 11.5 (SPSS Inc, Chicago, Ill). ANCOVA was performed to study the differences between the mean on-clopidogrel platelet reactivity of the total study population and the 3 loading dose regimens of clopidogrel, and to study the association between individual P2RY12 SNPs and platelet function in the total study population. In each analysis, homozygotes for the common allele of these SNPs were used as the reference.

Analyses were adjusted for age, sex, body mass index, diabetes, and smoking. For the total group, additional adjustment for loading dose of clopidogrel was performed. In some analyses, adjustment was also made for the use of aspirin, coumadins, and statins. A 2-sided value of \( P < 0.05 \) was considered statistically significant.

Results

A large variability in on-clopidogrel platelet reactivity was found with 20 μmol/L ADP-induced LTA and VerifyNow P2Y12 assay (Figure 2A and 2B). For 20 μmol/L ADP-induced LTA, on-clopidogrel platelet aggregation was 58% ± 14% (total group), 57% ± 14% (dosing regimen A), 62% ± 14% (dosing regimen B), and 56% ± 15% (dosing
For the VerifyNow P2Y12 assay, PRU was 211/1100676 (total group), 206/1100674 (dosing regimen A), 228/1100678 (dosing regimen B), and 187/1100683 (dosing regimen C). Both for LTA and VerifyNow P2Y12 assay, mean on-clopidogrel platelet reactivity was significantly higher in the dosing regimen B than the dosing regimens A and C (P<0.001).

Similar variability in on-clopidogrel platelet reactivity was seen for the different concentrations of ADP in the LTA (data not shown).

By combining the 6 P2RY12 ht-SNPs, 64 haplotype alleles were inferred of which 6 were common and had an allele frequency higher than 5%: (A) tTCgGT (10%), (B) tTCAGT (6%), (C) CTCAGc (15%), (D) CTaAaT (15%), (E) CcCgGT (21%), and (F) CcCAGT (22%; Table 2).

### LTA

In the analysis of the total study population of patients, haplotype F showed a significantly lower 5 μmol/L ADP-induced maximal and late aggregation (both −4% per haplotype allele, P<0.05, Figure 3A and 3B) compared with the reference haplotype A. Similar results were observed for 20 μmol/L ADP-induced maximal and late aggregation (−4% and −6% per haplotype allele; P<0.001 and P<0.05; Figure 3C and 3D, respectively) and for ADP concentrations 2 and 10 μmol/L (supplemental Figure 1). Similar differences between haplotype F and the reference haplotype A were seen in the subgroup analysis of patients from the clopidogrel dosing regimens A and B (Figure 3). In addition, haplotype E was associated with a significantly lower 20 μmol/L ADP-induced maximal and late aggregation in the analysis of the total study population (both −3% per haplotype allele, P<0.05, Figure 3C and 3D). Haplotype E also showed a trend

### Table 2. P2RY12 Haplotypes

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>SNP Allele Composition</th>
<th>Allele Frequency, %</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>tTCgGT</td>
<td>10</td>
</tr>
<tr>
<td>B</td>
<td>tTCAGT</td>
<td>6</td>
</tr>
<tr>
<td>C</td>
<td>CTCAGc</td>
<td>15</td>
</tr>
<tr>
<td>D</td>
<td>CTaAaT</td>
<td>15</td>
</tr>
<tr>
<td>E</td>
<td>CcCgGT</td>
<td>21</td>
</tr>
<tr>
<td>F</td>
<td>CcCAGT</td>
<td>22</td>
</tr>
</tbody>
</table>

Haplotype alleles were coded A to F, in the descending order of their effects on 20 μmol/L ADP-induced LTA, where A is the reference haplotype allele. SNP allele composition (eg, CTaAaT) represents rs6798347, rs6787801, rs9859552, rs6801273, rs9848789, and rs2046934, respectively, with the minor alleles in lower case.

**Figure 3.** The effect of P2RY12 haplotypes on platelet reactivity assessed by LTA. Results are shown for 5 μmol/L ADP-induced maximal aggregation (A) and late aggregation at 6 minutes (B), and 20 μmol/L ADP-induced maximal aggregation (C), and late aggregation at 6 minutes (D). Values are expressed as mean differences from the reference haplotype in percent of absolute aggregation per haplotype allele. Analyses were performed for the total study population (dosing regimens A+B+C), and for the subgroup of patients on dosing regimens A and B. Dosing regimen C was excluded from the analysis due to small sample size. *P<0.05, **P<0.001.
toward lower maximal and late aggregation in the subgroup analysis of patients from the clopidogrel dosing regimens A and B (Figure 3C and 3D). Similar trends were seen for 2, 5, and 10 μmol/L ADP (Figure 3A and 3B, and supplemental Figure I) and after additional adjustment for the use of aspirin, coumadins, and statins (data not shown). Similar results were observed when nonwhites were excluded from the analyses (data not shown).

**VerifyNow P2Y12 Assay**

Consistent with the results for LTA for the total study population, haplotype F was associated with a lower PRU as compared to the reference haplotype A (−11 PRU per haplotype allele, *P*<0.05, Figure 4). These differences were less pronounced and statistically not significant when clopidogrel dosing regimens A and B were analyzed separately (Figure 4). A stronger and significant association was observed for haplotype E, which was consistently associated with 27 less PRU in the VerifyNow P2Y12 Assay for the total study population and for the clopidogrel dosing regimens A and B (*P*<0.05, Figure 4). Similar results were obtained after additional adjustment for the use of aspirin, coumadins, and statins (data not shown). Also, similar results were obtained for the associations between haplotypes and percentage of inhibition from baseline platelet agglutination (data not shown).

**P2RY12 SNPs**

Comparison of the SNP alleles within the haplotypes and their effects on platelet function suggested that SNP rs6787801 was responsible for most of the observed haplotype effects. This observation was confirmed in the single SNP analysis where homozygotes of the rare allele of SNP rs6787801 (CC genotype, *n*=261) had a significantly lower maximal and late aggregation (ranging from −2% to −7% across various concentrations of ADP, *P*<0.05 for all), as well as a significantly lower number of PRU in the VerifyNow P2Y12 assay (−27 PRU, *P*<0.001, Table 3), when compared with homozygotes of the common allele of SNP rs6787801 (TT genotype, *n*=266). None of the other 5 ht-SNPs were associated with any of the platelet function assays (data not shown), except for homozygotes of the rare allele of SNP rs6798347 (TT genotype, *n*=46), who showed 24 PRU less in the VerifyNow P2Y12 assay as compared to homozygotes of the corresponding common allele (CC genotype, *n*=638, *P*<0.05).

**Discussion**

In this study, we demonstrate that common variation in the ADP-receptor *P2RY12* gene is a significant determinant of the wide interindividual variability in on-clopidogrel platelet reactivity. Haplotype F (allele frequency 22%) was consistently associated with a higher on-clopidogrel platelet reactivity in the ADP-induced LTA and the VerifyNow P2Y12 assay, which may give an adequate protection against athero-thrombotic events. Haplotype E (21%) was also associated with higher on-clopidogrel platelet reactivity, especially in the VerifyNow P2Y12 assay, but this association was less clear for the LTA. Interestingly, a single 300 mg loading dose of clopidogrel (regimen B) was less effective in inhibiting the platelet ADP response than the daily 75 mg dose (regimen A). However, although the overall mean on-clopidogrel platelet reactivity was significantly higher in clopidogrel dosing regimen B than dosing regimens A and C, these differences did neither influence the associations between haplotypes across the regimens nor did the adjustment for various covariates. In line with previous reports, the LTA and the VerifyNow P2Y12 assay showed similar results.16

The haplotype-based approach enabled a comprehensive investigation of the common variation in the *P2RY12* gene. The results suggested that SNP rs6787801, or another SNP that is in high LD with this SNP, is responsible for the observed haplotype
effects. SNP rs6787801 is located within the 59kb LD-block that contains the promoter region, but not the coding region of the P2RY12 gene. This suggests that altered transcriptional activity of the P2RY12 gene might be the underlying mechanism of the observed haplotype effects, rather than a structural change of the P2Y12 receptor. SNP rs6787801 and rs2046934 are located in different LD-blocks, which may explain why most of the previous studies on rs2046934 (the tagging-SNP of haplotype-alleles H1 and H2) have not found a similar association between rs2046934 and the response to clopidogrel. 5–14 Also in this study, we did not find any statistical differences between haplotypes H1 and H2, represented by our individual SNP analysis with rs2046934. Judging from the relative effects of the haplotypes, there is a complex interaction between the SNPs within a haplotype. Therefore, we cannot exclude the possibility that besides rs6787801 other SNPs, including rs2046934, might contribute to the observed haplotype effects. In addition, the effect of haplotype alleles may not be additive, as we assumed in our model.

Results were presented per single haplotype allele, which implies, under the assumption of an additive effect of the haplotype alleles, that the effect of a haplotype in a patient is the sum of the effects of its 2 single haplotype alleles. A single haplotype allele F was associated with 7% lower platelet aggregation compared with the reference haplotype allele A, indicating that the estimated net effect in patients homozygous for haplotype allele F might be a 14% lower platelet aggregation compared with patients homozygous for haplotype allele A. Smoking, polymorphisms of the cytochrome P450 2C19 (CYP2C19) gene, diabetes mellitus, age, and proton pump inhibitor treatment are reported to have a similar effect-size on clopidogrel-induced platelet inhibition. 21–24 It remains, however, to be established whether these differences are associated with clinical outcome and what the underlying biological processes are that explain the observed haplotype effects.

Our study confirmed a wide variability in residual on-clopidogrel platelet reactivity, as measured with ADP-induced LTA and the VerifyNow P2Y12 assay. To date, a uniform definition of so-called clopidogrel “resistance” or clopidogrel “nonresponsiveness” is lacking. This definition may vary depending on the type of platelet function assay used, or whether adverse clinical events occurred during clopidogrel therapy. 5,25 In our study, the absolute residual on-clopidogrel platelet reactivity was measured and not the relative response to clopidogrel from baseline (ie, the pharmacodynamic response). For this reason, we cannot exclude the possibility that the observed differences between haplotypes may already be present at baseline and thus independent of clopidogrel treatment. In addition, we have not genotyped our patients for the CYP2C19 loss-of-function alleles (*2, *3, *4, and *5), nor the ABCB1 gene variants. CYP2C19 variants have recently been shown to affect the hepatic bioactivation of the clopidogrel prodrug and the concomitant platelet inhibition and risk of adverse ischemic cardiovascular events, whereas the ABCB1 variants have been shown to affect clopidogrel absorption. 26–28 However, because CYP2C19 and ABCB1 genes are located on different chromosomes than the P2RY12 gene, we expect that the CYP2C19 and ABCB1 alleles are independent of the P2RY12 haplotypes; ie, CYP2C19 and ABCB1 alleles are randomly distributed over the different P2RY12 haplotype-subgroups of our patients. In addition, CYP2C19 and ABCB1 genes do not have common biological pathways with the P2RY12 gene, because the P2RY12 gene encodes the target-receptor of clopidogrel, whereas CYP2C19 and ABCB1 genes are involved in clopidogrel bioavailability. Although these notions do not entirely exclude the possibility of interaction between the 3 genes, the actual effect of P2RY12 gene variants (ie, adjusted for any confounders) will probably be larger than the one observed in the present study. Although it would have been interesting to include additional pharmacokinetic and pharmacodynamic measurements of clopidogrel in our study, the absolute magnitude of the residual platelet reactivity during clopidogrel treatment is considered to be one of the most important end-stage determinants of risk of recurrent atherothrombotic events. Additional studies need to be performed to test whether our results may be generalized to other ethnicities than whites.

In conclusion, common variation in the P2RY12 gene is a significant determinant of the wide interindividual variability in residual on-clopidogrel platelet reactivity in patients with CAD.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Currently, clopidogrel is the most widely used P2Y12 antagonist that, when used in combination with aspirin, significantly reduces the risk of recurrent atherothrombotic events in patients with acute coronary syndrome and those who undergo percutaneous coronary interventions. However, a wide interindividual variability in clopidogrel response has been reported, and patients with suboptimal platelet inhibition may be at higher risk of atherothrombotic events. Studies that tested the hypothesis that the P2RY12 gene variants are a determinant of the large between-subject variability in the efficacy of clopidogrel treatment have shown conflicting results. However, these studies were relatively small, and they focused on the 3′ part of the P2RY12 gene. Therefore, we studied comprehensively the relations of common variation in the P2RY12 gene and platelet reactivity on clopidogrel treatment in 1031 patients with coronary artery disease scheduled for elective percutaneous coronary interventions. We performed platelet function measurements by means of ADP-induced light-transmittance aggregometry and a point-of-care VerifyNow P2Y12 assay. The main finding of our study is that 2 common haplotypes (each >20% frequency) were associated with a 5% lower on-treatment aggregation per haplotype allele (ie, an improved clopidogrel response). This study may contribute to an improved identification of patients at increased risk of atherothrombotic events due to suboptimal clopidogrel-induced platelet inhibition.
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Supplemental figure 1

Supplemental figure 1 legend

Results are presented for 2 μmol/L ADP-induced maximal aggregation (panel A) and late aggregation at 6 min (panel B), and 10 μmol/L ADP-induced maximal aggregation (panel C) and late aggregation at 6 min (panel D). In each panel, the results are shown for the analysis of the total study population (squares, dosing regimens A+B+C), the subgroup of patients who were on dosing regimen A (triangles) and dosing regimen B (circles). Dosing regimen C was excluded from the analysis due to small sample size. Values are expressed as mean differences from the
reference haplotype in percent of absolute aggregation per haplotype allele, with error bars representing SEM. **p<0.001, *p<0.05