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P2RY1 and *P2RY12* polymorphisms and on-aspirin platelet reactivity in patients with coronary artery disease

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SUMMARY

Introduction—Association of *P2RY1* and *P2RY12* polymorphisms with on-aspirin platelet reactivity was investigated.

Materials and Methods—Platelet reactivity was assessed by light transmission aggregometry and TxB_2 assay in 423 CAD (coronary artery disease) patients on aspirin. High residual platelet reactivity (RPR) was defined by≥20% and ≥70% maximal aggregation stimulated with 0.5 mg/mL AA and 10 μM ADP, respectively. Moderate RPR was considered aggregation ≥20% with AA, ≥70% with ADP, or ≥1 ng/mL stimulated TxB_2. Fourteen *P2RY1* and 35 *P2RY12* SNPs were genotyped.

Results—High RPR was detected in 24% of the patients. Moderate RPR was observed in 31% with AA, 57% with 5 μM ADP, and 82% with 10 μM ADP. Stimulated TxB_2 was ≥1 ng/mL in 23% of patients. *P2RY12* SNP rs9859538 was associated with high RPR (OR=2.16, 95% CI=1.24–3.75, p-value=0.004). Four *P2RY12* SNPs, rs1491974, rs10513398, rs3732765, and rs10935841, showed association with moderate RPR (OR=1.79–2.94, p-value=0.04–0.028), while five, rs7615865, rs1388623, rs1388622, rs7634096, and rs7637803, were associated with low RPR (OR=0.50–0.55, p-value=0.008–0.026), following ADP stimulation. TxB_2 level <1 ng/mL was linked to five *P2RY1* SNPs, rs1439010, rs1371097, rs701265, rs12497578, and rs2312265, (OR=0.36–0.54, p-value=0.003–0.039).

Conclusions—Polymorphisms in *P2RY1* and *P2RY12* are associated with on-aspirin platelet reactivity in CAD patients.

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Keywords

Coronary artery disease; aspirin; platelet reactivity; P2RY1 and P2RY12 receptors; SNP

INTRODUCTION

Aspirin is an established and commonly used antiplatelet medication. It irreversibly inhibits the enzyme cyclooxygenase-1 (COX-1) by chemical modification and leads to inhibition of prostaglandin H_2 production from arachidonic acid (AA) and eventually inhibition of TxA_2 production in platelets (Patrono & Rocca, 2008). Clinical studies have demonstrated that aspirin therapy is associated with a 12–25% reduction in non-fatal myocardial infarction (MI), non-fatal stroke or vascular death among high risk patients (Berger, Brown & Becker, 2008; Antithrombotic Trialists' Collaboration, 2009). However, there are individual variations in response to aspirin creating the term "aspirin non-responsiveness" or "aspirin resistance". Although aspirin resistance has been reported to occur in 5.5% to 60% of the population tested, the exact prevalence of aspirin resistance is still unclear due to lack of a standard definition of aspirin response and differences in platelet function assays (Gasparyan, Watson & Lip, 2008). It has been shown that aspirin resistant patients have about a 4-fold risk of fatal and non-fatal cardiovascular, cerebrovascular, or vascular events compared with aspirin responsive patients [Krasopoulos *et al.,* 2008]. Variability in aspirin response has been attributed to clinical, cellular and genetic factors such as polymorphic nucleotide changes in the genes coding for GPIIIa, collagen receptors GPIa-IIa, GPVI, and GPIb-IX-V, purinergic P2RY1 and P2RY12 receptors, COX-1 and COX-2, and factor XIII (FXIII) (Cohen, 2009; Cattaneo, 2007; Goodman, Ferro & Sharma, 2008; Rozalski *et al.,* 2005).

P2RY1 and P2RY12 are the G-protein-coupled receptors in which ADP is the physiological agonist (Smyth *et al.,* 2009; Volonte & D'Ambrosi, 2009). P2RY1 positively couples to phospholipase C (PLC) via $G\alpha_q$ which triggers Ca^{2+} release from intracellular stores leading to platelet shape change and rapid, reversible platelet aggregation. On the other hand, P2RY12 negatively couples to adenylyl cyclase via $G\alpha_i$ resulting in slow, irreversible platelet aggregation. The P2RY1 and P2RY12 genes are both located on chromosome 3. *P2RY1* spans 4 kb. It is mainly transcribed into two mRNA species which are different in length of 5′ and 3′UTRs. Single exon encodes for a 372-amino acid protein (Ayyanathan, Naylor & Kunapuli, 1996; Leon *et al.,* 1996). A polymorphism (A1622G, rs701265) in *P2RY1* has been shown to associate with higher platelet aggregation in response to ADP in healthy volunteers (Hetherington *et al.,* 2005). *P2RY12* spans 47 kb and has also two main RNA variants which are 47 kb and 3 kb in length [Hollopeter *et al.,* 2001]. Although the longer transcript has three exons and the short transcript has two exons, a single exon encodes for the whole 342-amino acid receptor. Four polymorphisms in *P2RY12* (i-C139T, rs10935838; i-T744C, rs2046934; i-ins801A, rs5853517; G52T, rs6809699) are in complete linkage disequilibrium and compose two haplotypes, H1 and H2. The less common H2 haplotype has been shown to be associated with increased platelet aggregation after ADP stimulation in healthy subjects and in patients with peripheral artery disease (PAD) (Fontana *et al.,* 2003a; Fontana *et al.,* 2003b).

Dual antiplatelet therapy with aspirin and clopidogrel, a direct antagonist of P2RY12, has been found to be more effective in patients with acute coronary syndromes and in those undergoing percutaneous coronary intervention (PCI) than treatment with aspirin alone (Yusuf *et al.,* 2001; Steinhubl *et al.,* 2002). A *P2RY1* polymorphism (C893T, rs1065776) was reported to associate with a more than 3-fold increase in aspirin resistance in Caucasian male patients with a history of MI (Jefferson *et al.,* 2005). Moreover, P2RY12 receptors

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have been shown to be critical in the TxA_2-dependent pathway of platelet aggregation (Armstrong *et al.*, 2011). These studies suggest a link between ADP receptors and aspirin mechanism.

In this study, to investigate the potential roles of P2RY1 and P2RY12 genes in aspirin response and atherothrombotic events, we genotyped 423 coronary artery disease (CAD) patients with a history of MI on aspirin for 14 *P2RY1* and 35 *P2RY12* SNPs and assessed the associations of defined genotypes with platelet reactivity and clinical characteristics.

MATERIALS AND METHODS

Study subjects

A total of 523 CAD patients were enrolled from a prospective study called "The Validation of Cardiovascular Disease Genetic markers in the Cleveland Clinic Coronary Artery Disease Population Study: GeneQuest2". A subgroup of this cohort composed of 332 male subjects was studied previously by Jefferson et al. (Jefferson *et al.,* 2005). All had a prior history of MI documented through ECG (electrocardiogram) or biochemical markers and were scheduled to undergo PCI at the Cleveland Clinic. Exclusion criteria included: use of ticlopidine, clopidogrel, dipyridamole, steroidal or non-steriodal anti-inflammatory drugs (NSAIDs), GPIIb/IIIa receptor inhibitors; administration of heparin or low molecular weight heparin within 24 h before enrollment; major surgical procedure within one week before enrollment; malignant paraproteinemias; family or personal history of bleeding disorders; history of heparin-induced thrombocytopenia, history of myeloproliferative disorders. The 423 patients who were Caucasian and only on aspirin were further selected for this study. Daily aspirin dose taken was 325 mg and 81 mg in 68% and 18% of the patients, respectively. Seven patients (2%) were taking more than 325 mg aspirin, 12 patients (3%) had 82–320 mg aspirin, and 5 (1%) were on <81 mg aspirin per day. No data was available about the aspirin dose taken by 35 patients (8%). Except one, all patients were taking non-enteric coated aspirin. Aspirin therapy compliance was verified by clinical history prior to sample collection. The study was approved by the Institutional Review Board. All participants gave informed consent before the enrollment.

Sampling

Whole blood samples were collected in a 3.2% sodium citrate tube for the assessment of platelet reactivity by platelet aggregation and thromboxane B_2 (TxB_2) level and genomic DNA extraction. Two tubes of whole blood were additionally collected in a serum separator and EDTA (ethylenediamine tetraacetic acid) tubes for a complete blood count, cholesterol, inflammatory and cardiovascular marker assays, and platelet COX-1 analysis. Time between the last aspirin intake and blood draw was less than 24 hours in 77% of the patients (unknown for 13%). Majority of the patients (79%) were fasting at blood draw.

Platelet aggregation assay by light transmission aggregometry (LTA)

Within 1 h of blood collection, citrated whole blood was centrifuged at 200g for 10 minutes at room temperature. Platelet rich plasma (PRP) was transferred to a polypropylene tube and the remaining specimen was further centrifuged at 2,500g for 10 minutes to obtain platelet poor plasma (PPP). The PRP platelet count was measured and adjusted to a target platelet count of 250 (200–300) X $10^3/\mu l$ with autologous PPP. The platelet aggregation assay was performed in adjusted PRP using BioData PAP-4 platelet aggregometer (BioData Corp., Horsham, PA). The baseline optical density was set with PPP. Platelet aggregation was stimulated with 0.5 mg/mL AA and 5 and 10 μM ADP and recorded for 10 minutes (Hayward *et al.*, 2010). At 10 minutes, indomethacin/EDTA solution was then added to

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prevent further platelet aggregation. Tubes were centrifuged and supernatants were kept frozen at −80°C for TxB_2 assay.

Two measures of aggregation were analyzed: % aggregation at the end of 10 minutes (final aggregation) and maximal % aggregation.

Thromboxane B_2 (TxB_2) assay

Activation-dependent release of TxB_2 "stimulated TxB_2", a stable metabolite of TxA_2, was measured ex vivo in the supernatants of centrifuged platelet aggregates after completion of the AA-stimulated platelet aggregation assay. Samples were first purified by using Amprep C2 column (Amersham Biosciences, NJ) and TxB_2 was detected by a competitive immunoassay (Assay Designs, Inc., MI) according to the manufacturer's protocol. The mean of duplicates and coefficient of variance were used. A ≥1 ng/mL TxB_2 level was selected as cut-off based on the stimulation of platelets with 0.5 mg/mL AA from 40 healthy donors blocked by in vitro addition of 30 μmol/L aspirin (Sigma-Aldrich Co., St-Louis, MO). Mean minus one SD of the lowest TxB_2 quartile from the healthy participants was 1.060 ng/mL and mean plus one and 2SD of the third TxB_2 quartile from the patients was 0.925 and 1.102 ng/mL, respectively.

COX-1 analysis by Western blotting

PRP was prepared from the whole blood in EDTA as described above. A polypropylene tube containing PRP was further centrifuged at 800g for 10 minutes at room temperature. After several washing steps, the recovered pellet was lysed with sample buffer containing protease inhibitors and stored at −80°C until use. Western blot analysis was carried out according to a standard electrophoresis protocol (Invitrogen, Carlsbad, CA) and a chemiluminescence detection system (ECL Plus, Amersham Biosciences, Piscataway, NJ) using autoradiography. Briefly, the protein concentration of each lysate was determined by BCA protein assay (Pierce Chemical Co., Rockford, IL) and 10 μg of total protein was subjected to Western blot analysis along with the known amounts of purified ovine COX-1 reference protein (Cayman Chemical, Ann Arbor, MI). Mouse monoclonal primary antibodies against COX-1 (Cayman Chemical), followed by a secondary horseradish peroxidase-linked anti-mouse antibody were used for detection (Censarek *et al.,* 2004). Band intensities of sample and reference proteins were quantified from the autoradiograph using an NIH image program.

Definition of residual platelet activity

Aspirin resistance has been previously defined as ≥20% AA- and ≥70% ADP (10 μM)-stimulated maximal platelet aggregation and validated by clinical outcome (Gum *et al.* 2001; Gum *et al.,* 2003). Here, patients who met both criteria were defined as having high residual platelet reactivity. Patients with ≥20% AA-, ≥70% ADP (5 or 10 μM)-stimulated maximal aggregations, or ≥1 ng/mL stimulated TxB_2 were considered as having moderate RPR. Low RPR was defined as <20% AA, <70% ADP and <1 ng/mL stimulated TxB_2.

Genotyping

Genotyping was carried out by the melting curve analysis of FRET (fluorescence resonance energy transfer) hybridization probes, TaqMan assay, and SNP array-based genotyping. SNPs for the first two strategies were selected from several databases including the Children's Hospital Informatics Program (CHIP) at http://snpper.chip.org, the SeattleSNPs at http://pga.gs.washington.edu/, MIT Broad Institute's Tagger program at http://www.broad.mit.edu/mpg/tagger/server.html, and the International HapMap Project at http://www.hapmap.org/. SNPs were picked on the basis of an r^2 of 0.8 and a minor allele

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frequency of 1% from Caucasian population, CEU. A cardiovascular gene-centric SNP array, Infinium CVDSNP55 (Illumina, Inc., San Diego, CA), was also utilized to target SNPs from the P2RY1 and P2RY12 genes. (Keating *et al.,* 2008). A total of 49 SNPs covering both *P2RY1* and *P2RY12* were analyzed in this study. Information about these SNPs and their LD (linkage disequilibrium) maps are given in Supplementary Table 1, Supplementary Table 2, Supplementary Figure 1, and Supplementary Figure 2.

Genomic DNA was isolated from whole blood collected in sodium citrate using DNAzol reagent (DNAzolBD Reagent, Invitrogen, CA). Primers and probes for the melting curve analysis were designed using a software provided with the LightTyper instrument (Roche Applied Sciences, Indianapolis, IN) and processed according to the manufacturer (TIB MOLBIOL, Nutley, NJ) (Murugesan *et al.,* 2006). Each analysis was validated by using reference DNAs with known genotypes. The TaqMan assay (Applied Biosytems Inc., Foster City, CA) was performed in a 5 μl volume containing 0.125 μl of 40× Allelic Discrimination Assay Mix, 2.5 μl of TaqMan Universal PCR (polymerase chain reaction) Master Mix, and 25 ng of genomic DNA. PCR was performed in a Dual 384-Well GeneAmp PCR System 9700 using a standard program recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA). Genotypes were generated by the ABI PRISM 7900HT Sequence Detection System and automatic allele calling was performed with the default settings using data collection and analysis software version 2.1. The quality of SNP genotyping was verified by independently replicating the allelic calls of a number of randomly selected samples or by direct DNA sequence analysis of 12 samples. The nucleotide sequences of the primers and probes used in both techniques are available upon request. Illumina SNP array-based genotyping was performed by the genomics core facility in Cleveland Clinic.

All 423 Caucasian patients from the study were used to determine population substructure using the software EIGENSTRAT based on all TaqMan and CVDSNP55 array SNPs. After pruning of all the SNPs in linkage disequilibrium (LD) (r^2>0.3), the EIGENSTRAT analysis and principle components analysis identified no significant population substructure occurred in the current samples (Price *et al.,* 2006).

Statistical analysis

All data mining and statistical analyses were carried out with the R statistical package (http://www.r-project.org). Categorical variables are described by frequency distributions and contingency tables. Continuous variables are summarized by mean (SD). Platelet function data including platelet aggregation and TxB_2 level were first considered as continuous outcomes and then as categorical outcomes by categorizing into two groups based on definition of high and moderate RPR (for high RPR: <20%/<70% and ≥20%/ ≥70%; for moderate RPR: </≥20% or </≥70% or </≥1 ng/mL TxB_2) (Gum *et al.* 2001; Gum *et al.,* 2003). The total number of MIs was also considered as binary data, =/>1, standing for without/with recurrence of MI. Pair-wise linkage disequilibrium (LD) between SNPs was assessed using the LDheatmap package in R software (Supplementary Figure 1 and Supplementary Figure 2).

To detect the association between single SNP and the outcome, for each SNP, a generalized linear model with an appropriate link (logit link and identity link for binary and continuous outcomes, respectively) was fitted, with each outcome as the response variable, the SNP as the predictive variable and age, gender, and smoking status as covariates. The P values of the likelihood ratio test were obtained from a comparison with the null model (i.e., only covariates in the model). An adjustment for multiple comparisons was implemented by controlling the false discovery rate at less than 0.15 across each gene.

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RESULTS

Characteristics of the study cohort

A total of 423 CAD patients with a history of MI on aspirin were analyzed. Demographics of the study population are shown in Table 1. The majority of the subjects were male (82%). The mean age of the cohort was 65.4 years. Body mass index (BMI) was 30±7.6 kg/m^2 (Mean±SD) falling into the overweight and obese ranges. Smoking, including past and current smoking, was high among patients (70%). Hypertension and dyslipidemia were also prevalent in the group, 70% and 84%, respectively. Diabetes mellitus was present in 27% of the patients. Patients also had a history of other vascular problems such as peripheral artery diseases (PAD) (10%), carotid/cerebrovascular disease (CA/CVD) (10%), and stroke (7%). More than one episode of MI occurred in 25% of the patients. A substantial number of patients had previous history of PCI (69%), coronary artery bypass graft (CABG) surgery (50%), or other coronary procedures (92%). White blood cell (WBC) count and C-reactive protein (CRP), high density lipoprotein (HDL) cholesterol, total cholesterol, and platelet COX-1 measures are also shown in Table 1.

Platelet reactivity of the patients

Based on the criteria of both ≥20% AA- and ≥70% ADP (10 μM)-stimulated maximal platelet aggregation, 24% of the patients were defined as having high RPR (Table 2). On the other hand, moderate RPR was observed in 31% of the patients with≥20% maximal platelet aggregation in response to AA stimulation and in 57% and 82% of the patients with ≥70% maximal platelet aggregation after 5 and 10 μM ADP stimulation, respectively. TxB_2 levels were also higher than 1 ng/mL in 23% of the patients.

Platelet reactivity and characteristics of the patients

Patients with high RPR were significantly older than other study patients (mean±SD, 70±9 years versus 64±12 years, p-value=0.001). Interestingly, patients with recurrent MI displayed higher maximal and final platelet aggregation in response to AA-stimulation (22.8% versus 17.9%, p-value=0.026 and 17.5% versus 13%, p-value=0.037, respectively). We did not find a significant difference in COX-1 enzyme levels between patients with elevated and non-elevated RPRs (p-value=0.568).

P2RY1 and P2RY12 polymorphisms and platelet reactivity

The significant associations found between the *P2RY1* and *P2RY12* SNPs and platelet reactivity are shown in Table 3 under three groups (Tables 3a-c). A *P2RY12* SNP, rs9859538, was found to be associated with high RPR in our patient population (Table 3a). The variant allele of this SNP was more frequent in patients displaying≥20% AA- and ≥70% ADP (10 μM)-stimulated maximal platelet aggregation (OR=2.16, 95% CI=1.24–3.75, p-value=0.004). Moreover, homozygosity or heterozygosity for the minor alleles of four *P2RY12* SNPs, rs1491974, rs10513398, rs3732765 and rs10935841, showed association with moderate RPR in response to ADP stimulation (Table 3b). While two SNPs, rs1491974 and rs10513398, were associated with 5 μM ADP-stimulated final aggregation (OR=2.69–2.94, p-value=0.004–0.008), two others, rs3732765 and rs10935841, showed association with 10 μM ADP-stimulated maximal aggregation (OR=1.79–1.80, p-value=0.026–0.028). On the other hand, five SNPs were found to be associated with low RPR (Table 3c). Maximal aggregation in response to 10 μM ADP-stimulation was <70% in most of the patients with minor alleles of SNPs rs7615865, rs1388623, rs1388622, rs7634096 and rs7637803 (OR=0.50–0.55, p-value=0.008–0.026). Similarly, minor alleles of five *P2RY1* SNPs, rs1439010, rs1371097, rs701265, rs12497578, and rs2312265, were associated with <1 ng/mL TxB_2 levels (OR=0.36–0.54, p-value=0.003–0.039).

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DISCUSSION

In the current study, *P2RY12* SNP rs9859538 was found to be associated with high RPR. The minor allele of rs9859538 was associated with a 2-fold increase in having both ≥20 AA- and ≥70% ADP (10 μM)-stimulated maximal platelet aggregation. This SNP is located in intron 1 of the P2RY12 gene and not in LD with any SNPs constituting H haplotype (Supplementary Figure 2). Moreover, we found an association between moderate RPR in response to ADP and *P2RY12* SNPs rs1491974, rs10513398, rs3732765 and rs10935841. Patients with the minor alleles of rs1491974 and rs10513398 were almost 3 times more likely to have ≥70% final platelet aggregation in response to 5 μM ADP while patients with the variant allele of rs3732765 and rs10935841 were 2 times more likely to have ≥70% maximal platelet aggregation following 10 μM ADP stimulation. SNPs rs1491974 and rs10513398 are located in the promoter and exon 1/intron 1 (IVS-1) boundary of the gene, respectively, and are in complete LD (Supplementary Figure 2). Similarly, SNPs rs3732765 and rs10935841 are located in IVS-1 and display complete LD (Supplementary Figure 2). There is a moderate degree of LD between these two SNP groups. The reason that we found an association with 5 μM ADP-stimulated final aggregation may result from the low number of patients having ≥70% aggregation in this group (58 or 14%, Table 2). It is also possible that the lower agonist concentration (5 μM ADP) induces COX and TxA_2-dependent platelet aggregation, which is sensitive to aspirin, while the higher agonist concentration (10 μM ADP) could stimulate other activation pathways (Cattaneo, 2004; Cattaneo, 2007; Lordkipanidze *et al.,* 2007).

Low RPR was associated with five *P2RY12* SNPs, rs7615865, rs1388623, rs1388622, rs7634096 and rs7637803, in the study population. Patients with the minor alleles of these SNPs were more likely to have <70% maximal platelet aggregation in response to 10 μM ADP. These polymorphic spots also reside in IVS-1 and represent two LD groups (Supplementary Figure 2). All SNPs we found related with the platelet aggregation in response to ADP are spread along a region between promoter and 44 kb long IVS-1 (Supplementary Table 2) which makes the difference between long and short mRNAs. This region can have a regulatory function for the expression of P2RY12 receptor protein and thereby, any sequence change can alter the number of P2RY12 receptors on platelet surface which in turn may affect the platelet activity. In a recent study, it was shown that three ht-SNPs (Haplotype-tagging) locating in the promoter and IVS-1 of the P2RY12 gene were associated with almost 2-fold risk of developing restenosis after PCI (Rudez *et al.,* 2008). One of the ht-SNPs linked, rs6787801, is in complete LD with rs1491974 and rs10513398 and moderate LD with rs3732765 and rs10935841 (Supplementary Figure 2). These data also suggest that nucleotide changes in the regulatory sequences of *P2RY12* may be associated with restenosis through the over-expression of receptor resulting in increased platelet activity.

An association of *P2RY1* polymorphism rs1065776 with a >3-fold increase of aspirin resistance in Caucasian male CAD patients was reported by Jefferson et al. (Jefferson *et al.,* 2005). More patients with the minor allele of this SNP displayed ≥20% maximal platelet aggregation in response to AA stimulation. Although these patients were a subset of our study group, this SNP was not significantly associated with high RPR in the larger population. The difference in size and gender between two study populations may have affected the statistical outcome. Moreover, the current statistical approach adjusted the analysis by age, gender, and smoking status together; the prior subanalysis adjusted for age, HDL, and diabetes mellitus individually (Jefferson *et al.,* 2005). On the other hand, five *P2RY1* SNPs, rs1439010, rs1371097, rs701265, rs12497578, and rs2312265, were found to be associated with stimulated TxB_2 level in our study. Minor alleles of these polymorphisms were associated with <1 ng/mL stimulated TxB_2. Association was always evident without

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and with single and multi-confounder adjustments (age, gender, and smoking). One of these SNPs, rs701265 (A1622G), has been reported to be associated with increased platelet aggregation in response to ADP in subjects carrying the minor allele (Hetherington *et al.,* 2005). A recent study reported that CAD patients homozygous for the minor allele of rs701265 had 8.5-fold higher risk of having ≥20% platelet aggregation in response to AA (Lordkipanidze *et al.,* 2011). Interestingly, no increase was found in ADP-stimulated platelet aggregation and urinary 11-dTxB_2 levels in those patients (Lordkipanidze *et al.,* 2011). The five *P2RY1* SNPs reside in a variety of regions in the gene. SNPs rs1439010 and rs1371097 are in the promoter, while rs701265 is located in the single exon of *P2RY1*. SNPs rs12497578 and rs2312265 are downstream of the gene. They are all in complete LD except rs12497578 which shows moderate LD (Supplementary Figure 1). In addition to COX-1 inhibition by aspirin, further decrease in TxB_2 production could result from the repressed or defective expression of the P2RY1 receptor, leading to decreased amplification, which could explain the lower percentage of moderate RPR by the measurement of TxB_2 (23% versus 31% by AA-induced aggregation). Further functional studies of these polymorphisms would be needed to elucidate a functional role of the P2RY1 receptor in TxA_2-mediated aggregation.

Recurrent MI was reported in 25% of our study population. These patients had significantly higher AA-stimulated platelet aggregation than patients with single MI (p-values <0.05). In addition, there was a potential association between recurrent MI and *P2RY12* SNP rs4603933 based on a recessive genetic model (OR=0.05, 95% CI=0.01–0.4, p-value<0.001, FDR=0.009) (data not shown). However, the number of patients homozygous for the minor allele (2% of the study population) was not enough to make a conclusion for this association. Finally, patients carrying or homozygous for the variant allele of *P2RY12* SNP rs11715892 had lower levels of HDL cholesterol than patients homozygous for the wild alleles (mean±SD, 42±1 mg/dL versus 38±1 mg/dL, difference=−0.10, 95% CI=−0.16–0.04, p-value=0.002, FDR=0.065) (data not shown). Interestingly, another ADP receptor, P2RY13, was recently shown as the main regulator of hepatic HDL endocytosis in mice (Jacquet *et al.,* 2005; Fabre *et al.,* 2010). An antagonist of the P2RY12 receptor (antithrombotic agent cangrelor) was also shown to stimulate P2RY13-mediated HDL endocytosis in cultured cells and perfused mouse liver but not in P2RY13 knockout mice (Jacquet *et al.,* 2005; Fabre *et al.,* 2010).

In conclusion, we found associations of *P2RY1* and *P2RY12* SNPs with on-aspirin platelet reactivity in patients with CAD. To our knowledge, this is the most comprehensive study to date of platelet function and the SNP number analyzed in both P2RY1 and P2RY12 genes. Further evaluation of the associated polymorphisms by genetic and functional studies may be needed to reveal additional information about the mechanisms involving P2RY1 and P2RY12 receptors.

There are certain limitations to this study. A risk of false positive findings is present due to our modest population size (N=423). The expression levels of *P2RY1* and *P2RY12* were not assessed. The high residual platelet activity observed in response to ADP may also reflect basal high sensitivity to ADP, but basal samples were not available. These genotyping results should be replicated in a separate patient population.

Supplementary Material

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Table 1

Characteristics of the study cohort

	n (%)* or Mean±SD
Male	345 (82)
Female	78 (18)
Age	65±10
BMI (kg/m^2)	30±8
Smoking	294 (70)
Hypertension	298 (70)
Dyslipidemia	357 (84)
Diabetes mellitus	116 (27)
Peripheral Artery Disease (PAD)	42 (10)
Carotid/Cerebrovascular Disease (CVD)	43 (10)
Stroke	26 (6)
Age at first Myocardial Infarction (MI)	56±11
Total number of MI	
=1	315 (75)
>1	104 (25)
Previous Percutaneous Coronary Intervention (PCI)	292 (69)
Previous Coronary Artery Bypass Graft (CABG)	210 (50)
Previous other coronary procedure(s)	390 (92)
Angina pectoris, Stable	273 (65)
Angina pectoris, Unstable	134 (32)
Systolic blood pressure (mm Hg)	142±23
Diastolic blood pressure (mm Hg)	80±13.5
WBC x 10^3/μL	6.6±1.8
CRP (mg/dL)	0.7±1.7
HDL cholesterol mg/dL (mmol/L)	40±13 (1±0.3)
Total cholesterol mg/dL (mmol/L)	171±51 (4.4±1.3)
COX1 (ng/mg protein)	8.7±1.7

BMI, body mass index; HDL, high density lipoprotein; CRP, C-reactive protein; WBC, white blood cell; COX1, cyclooxygenase 1; SD, standard deviation.

*Total number of subjects may vary depending on the availability of data.

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Table 2

On-aspirin platelet reactivity of the study patients

Platelet Reactivity	n (%)*		Mean±SD
AA (0.5 mg/mL)- and ADP (10 μM)-stimulated aggregration (%)	<20 and <70	≥20 and ≥70	
10 minutes	248 (59)	38 (9)	
Maximum	67 (16)	101 (24)	
AA (0.5 mg/mL)-stimulated aggregation (%)	<20	≥20	
10 minutes	353 (84)	68 (16)	14±15
Maximum	291 (69)	130 (31)	19±16
ADP (5 μM ADP)-stimulated aggregation (%)	<70	≥70	
10 minutes	363 (86)	58 (14)	50±19
Maximum	179 (43)	242 (57)	71±14
ADP (10 μM ADP)-stimulated aggregation (%)	<70	≥70	
10 minutes	265 (63)	156 (37)	65±17
Maximum	74 (18)	347 (82)	79±12
TxB_2 (ng/mL)	<1	≥1	25±122
	288 (77)	86 (23)	

TxB_2, thromboxane B_2; ADP, adenosine diphosphate; AA, arachidonic acid; SD, standard deviation.

*Total number of subjects may vary depending on the availability of data.

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Table 3

Significant SNPs associated with platelet reactivity defined by reference cut-off points in Caucasian patients (N=423) based on FDR<0.15

a. SNPs associated with high residual platelet activity

Platelet Reactivity*	Gene	rs SNP ID	Genotype†	n (%)		p-value‡ (FDR)§	Odds Ratio (95% CI)
AA-and ADP-stimulated aggregation (%)				**<20 and/or <70**	**≥20 and ≥70**		
0.5 mg/mL AA and 10 μM ADP, maximum	*P2RY12*	rs9859538	**A/A** G/A+G/G	110 (35) 203 (65)	22 (22) 77 (78)	0.004 (0.156)	2.16 (1.24, 3.75)

b. SNPs associated with moderate residual platelet activity

Platelet Reactivity*	Gene	rs SNP ID	Genotype†	n (%)		p-value‡ (FDR)§	Odds Ratio (95% CI)
ADP-stimulated Aggregation (%)				**<70**	**≥70**		
5 μM ADP, 10 minutes	*P2RY12*	rs1491974	**C/C** T/C+T/T	105 (30) 249 (70)	9 (15.5) 49 (84.5)	0.004 (0.133)	2.94 (1.33, 6.51)
		rs10513398	**G/G** A/G+A/A	104 (29) 254 (71)	9 (15.5) 49 (84.5)	0.008 (0.133)	2.69 (1.23, 5.88)
10 μM ADP, maximum	*P2RY12*	rs3732765	**G/G** A/G+A/A	36 (49) 37 (51)	124 (36) 219 (64)	0.028 (0.138)	1.79 (1.07, 3.00)
		rs10935841	**G/G** A/G+A/A	36 (49) 37 (51)	124 (36) 220 (64)	0.026 (0.138)	1.80 (1.07, 3.01)

c. SNPs associated with low residual platelet reactivity

Platelet Reactivity*	Gene	rs SNP ID	Genotype†	n (%)		p-value‡ (FDR)§	Odds Ratio (95% CI)
ADP-stimulated Aggregation (%)				**<70**	**≥ 70**		
10 μM ADP, maximum	*P2RY12*	rs7615865	**C/C** T/C+T/T	24 (34) 47 (66)	164 (48.4) 175 (51.6)	0.019 (0.138)	0.53 (0.31, 0.91)
		rs1388623	**C/C** A/C+A/A	26 (35.6) 47 (64.4)	179 (52) 165 (48)	0.008 (0.138)	0.50 (0.29, 0.84)
		rs1388622	**G/G** A/G+A/A	26 (35.6) 47 (64.4)	179 (52) 165 (48)	0.008 (0.138)	0.50 (0.29, 0.84)
		rs7634096	**G/G** A/G+A/A	40 (55) 33 (45)	234 (68) 110 (32)	0.026 (0.138)	0.55 (0.33, 0.93)
		rs7637803	**G/G** A/G+A/A	40 (55) 33 (45)	234 (68) 110 (32)	0.026 (0.138)	0.55 (0.33, 0.93)
TxB_2 (ng/mL)				**<1**	**≥1**		

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c. SNPs associated with low residual platelet reactivity

Platelet Reactivity*	Gene	rs SNP ID	Genotype†	n (%)		p-value‡ (FDR)§	Odds Ratio (95% CI)
	P2RY1	rs1439010	**T/T** C/T+C/C	200 (70) 86 (30)	67 (82) 15(18)	0.035 (0.114)	0.53 (0.28, 0.99)
		rs1371097	**C/C** T/C+T/T	199 (70) 86 (30)	66 (81.5) 15 (18.5)	0.039 (0.114)	0.54 (0.29, 1.01)
		rs701265	**A/A** G/A+G/G	200 (70) 87 (30)	67 (82) 15 (18)	0.031 (0.114)	0.52 (0.28, 0.97)
		rs12497578	**C/C** G/C+G/G	220 (77) 66 (23)	74 (90) 8 (10)	0.003 (0.067)	0.36 (0.16, 0.79)
		rs2312265	**A/A** G/A+G/G	200 (70) 87 (30)	67 (82) 15 (18)	0.031 (0.114)	0.52 (0.28, 0.97)

FDR, false discovery rate; CI, confidence interval; ADP, adenosine diphosphate; TxB_2, thromboxane B_2.

*Only assays of platelet reactivity with significant associations are shown.

†Wild type genotype is shown in bold.

‡P-values were obtained by logistic regression analysis adjusted by gender, age, and smoking.

§Associations are based on FDR<0.15 except the one in Table 3a which is 0.156.

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