Dimorphism in the P2Y1 ADP Receptor Gene Is Associated With Increased Platelet Activation Response to ADP

Simon L. Hetherington, Ravi K. Singh, David Lodwick, John R. Thompson, Alison H. Goodall, Nilesh J. Samani

Objective—The platelet ADP receptors P2Y1 and P2Y12 play a pivotal role in platelet aggregation. There is marked interindividual variation in platelet response to ADP. We studied whether genetic variants in the P2Y1 or P2Y12 genes affect platelet response to ADP.

Methods and Results—The P2Y1 and P2Y12 genes were screened for polymorphisms. Associations between selected polymorphisms and the platelet response to ADP (0.1, 1.0, and 10 μmol/L), assessed by whole blood flow cytometric measurement of fibrinogen binding to activated glycoprotein IIb-IIIa, were then determined in 200 subjects. Five polymorphisms were found in the P2Y1 gene and 11 in the P2Y12 gene. All polymorphisms were silent. A P2Y1 gene dimorphism, 1622A>G, was associated with a significant ($P$=0.007) effect on platelet ADP response, with a greater response in carriers of the G allele (frequency 0.15). The effect was seen at all concentrations of ADP but greatest at 0.1 μmol/L ADP, where the response in GG homozygotes was on average 130% higher than that seen in AA homozygotes ($P$=0.006).

Conclusions—A common genetic variant at the P2Y1 locus is associated with platelet reactivity to ADP. This genotype effect partly explains the interindividual variation in platelet response to ADP and may have clinical implications with regard to thrombotic risk. (Arterioscler Thromb Vasc Biol. 2005;25:252-257.)

Key Words: platelets • thrombosis • genes • receptors • adenosine diphosphate
Methods

Subjects and Sample Collection
A total of 200 white adult subjects of northern European origin were recruited. None had a history of coronary heart disease and none were taking any antiplatelet medication. Subjects were categorized as experiencing hypertension and diabetes on the basis of reported history. Smoking status was defined as current smoker, ex-smoker, or nonsmoker. The study was approved by the Leicestershire Research Ethics Committee, and all subjects gave written informed consent.

Individuals were seen in the morning and in a fasting state. Ten subjects attended on a second occasion, at least 6 weeks later, to permit analysis of intraindividual variability of the platelet ADP response. Smokers were asked to abstain from smoking for at least 24 hours before the visit, and this was confirmed by a carbon monoxide breath test. All subjects were rested supine for at least 20 minutes before venepuncture to minimize the effects of stress hormones, and blood was collected using a standardized phlebotomy technique designed to minimize platelet activation.

Platelet Response Measurement
Blood samples were prepared for whole blood flow cytometric analysis within 10 minutes of collection essentially as described previously (see detailed Methods, available online at http://atvb.ahajournals.org).

White cell count, platelet count, and mean platelet volume (MPV) were measured in EDTA-anticoagulated blood after 2 hours to allow stabilization of platelet volume using a Beckman Coulter counter. Fibrinogen was measured by the Clauss method on a Sysmex CA-1000 analyzer (Sysmex UK). Leukocyte DNA was extracted from a 10-mL blood sample using the PureGene DNA extraction kit (Gentra Systems).

P2Y1 and P2Y12 Gene Screening for Common Polymorphisms
On the basis of the response after stimulation of whole blood with 1.0 μmol/L ADP, 10 subjects from each end of the distribution of response were selected to undergo sequencing of the P2Y1 gene, including the exon and 500 bp of upstream sequence, and the region of the P2Y12 gene encompassing exons 2 and 3 and intron B. This strategy maximized the chances of identifying common polymorphisms that might affect function (further details of Methods available in online supplement).

Genotyping of Common P2Y1 and P2Y12 Polymorphisms
Polymorphisms to type in the full cohort were selected on the basis of their predicted allele frequencies and linkage disequilibrium data as described in Results. Four polymorphisms were typed in the P2Y12 gene and 1 in the P2Y1 gene (details of genotyping Methods available in online supplement).

Statistical Analysis
Unless stated otherwise, data are presented as mean±SD. Observed allele frequencies were compared with the Hardy–Weinberg equilibrium prediction using the χ2 test. All response data were transformed before analysis using log (response [100-response]−1). The association between genotype and phenotype was tested by 1-way ANOVA. Associations at specific doses were further analyzed after adjustment for other parameters (age, resting level of bound fibrinogen, smoking status, and GPIIb-IIIa receptor expression) using multiple linear regression models. Analysis across all doses was performed using a random-effects model fitted by maximum likelihood that included a random effect for subject to adjust for within subject correlation. All analyses were performed using Stata 8.1 (Stata Corp.).

Results

Cohort Demographics
Mean age of the subjects was 47.3±6.0 years (range 25 to 56 years). A total of 87% were male, 18% were current smokers, and 32% were ex-smokers. Nine percent had a diagnosis of hypertension, and 1% were diabetic. Mean white cell count was 5.5±1.3×109/L, mean platelet count was 240.3±61.1×109/L, MPV was 9.2±1.2 fL, and mean plasma fibrinogen was 2.8±0.6 g/L. All of these variables were distributed normally.

Variability of Platelet Response to ADP
The distribution of subjects with respect to the percentage of platelets positive for bound fibrinogen at rest and after stimulation with the 3 doses of ADP are shown in Figure 1. Mean values (95% CIs) were as follows: resting level 2.6% (2.5 to 2.7%); 0.1 μmol/L ADP 69.9% (67.0 to 72.3%); and 10 μmol/L ADP 85.4% (83.9 to 86.9%). There were strong correlations between the different stimulating concentrations of ADP within subjects (0.1 versus 1.0 μmol/L, r=0.91; 0.1 versus 10 μmol/L, r=0.86; 1.0 versus 10 μmol/L, r=0.94) and a modest correlation between the resting level of bound fibrinogen to platelets and response to all doses of ADP (r=0.38).

In the 10 subjects studied on 2 occasions, a correlation between the ADP responses measured at the 2 visits was observed when comparing the response to all doses of ADP (r=0.56; 1.0 μmol/L, r=0.91; 10 μmol/L, r=0.93), indicating that the response to ADP is reproducible within an individual.

Random effects analysis incorporating all 3 doses of ADP found that after adjusting for the resting level of bound fibrinogen, age (P<0.0001), smoking history (P=0.008), and GPIIb-IIIa expression (P=0.0005) were important independent regulators of the platelet response. GPIIb-IIIa expression showed 3-fold variation within the population studied (1.7 to 5.4 relative fluorescence units) and was distributed normally. The correlations between the level of GPIIb-IIIa expression and fibrinogen binding in response to ADP were 0.169 (P=0.016), 0.220 (P=0.002), and 0.266 (P<0.001) for the 0.1 μmol/L, 1.0 μmol/L, and 10 μmol/L of ADP, respectively. GPIIb-IIIa expression accounted for between 2.9%...
Platelet Activation in Response to ADP by P2Y1 and P2Y12 Genotypes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism</th>
<th>Genotype</th>
<th>Frequency n (%)</th>
<th>Mean Platelet Response (95% CI) to ADP (% Positive for Bound Fibrinogen)</th>
<th>P-Value</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0.1 μmol/L</td>
<td>1.0 μmol/L</td>
<td>10 μmol/L</td>
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<td>P2Y1</td>
<td>1622</td>
<td>AA</td>
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<td></td>
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<td>46 (23.0)</td>
<td>15.0 (11.5–19.4)</td>
<td>71.9 (66.3–76.9)</td>
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<td></td>
<td>GG</td>
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<td>28.8 (16.7–45.0)</td>
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<td>CT</td>
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<td>17.0 (8.3–31.9)</td>
<td>75.7 (61.2–86.1)</td>
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</table>

Mean platelet responses are unadjusted values following back transformation of log(response [100-response]) scale. 

P-values are quoted after adjustment for age, resting level of bound fibrinogen, smoking status, and GPIIb-IIIa expression, and represent overall genotype effect at the stated dose or *comparing average effects across the range of ADP concentrations using random effects model.

†P value tested after pooling CT and TT group.

and 7.1% of the interindividual variation in response to ADP, dependent on stimulating concentration of ADP used. The proportions explained by age and smoking were 4.2% to 10.7% and 1.1% to 2.2%, respectively. Gender did not have an independent effect.

Identification of Common P2Y1 and P2Y12 Polymorphisms

The polymorphisms identified are shown in supplemental Table II (available online at http://atvb.ahajournals.org). All polymorphisms in the coding sequences of both genes were silent. In the P2Y1 gene, 2 polymorphisms were found with an allele frequency for the less common allele of >5%, 1 in the 5’-UTR (P2Y1 190G(C), and 1 in the coding sequence (P2Y1 1622A(G)). These were partially linked, with P2Y1 190G(C) being slightly less common. From the P2Y1 gene, we therefore selected the 1622A(G) polymorphism for typing in the full cohort.

In the P2Y12 gene, there were 9 common polymorphisms, 5 of which (P2Y12 145C(T), IntB137C(T), IntB742T(C, IntB798delA, and 252G(T) were in complete linkage disequilibrium. A further polymorphism (P2Y12 2014C(T)) was in partial linkage with these but more common. The following alleles were carried together: 145C, IntB137C, IntB742T, IntB798del, 252G or 145T, IntB137T, IntB742C, IntB798A, and 252T. From the P2Y12 polymorphisms, we therefore typed IntB742T(C to represent the 5 linked polymorphisms), 234C(T, 1622C(T, and 2014C(T). Although the P2Y12 2087delA deletion was also common, this was not typed because the nature of the sequence around the deletion (a run of 7 or 8 As) prevented an efficient method for genotyping being developed.

Effect of Common P2Y1 and P2Y12 Gene Polymorphisms on Platelet ADP and TRAP Responses

All polymorphisms were in Hardy–Weinberg equilibrium in the cohort (Table). Carrying ≥1 G allele at the P2Y1 1622 position was found to be significantly associated with increased platelet response to ADP. This was apparent across the whole range of stimulating concentrations, when analyzed separately or in aggregate (Table; Figure 2). The major difference was between the AA and the GG genotype. For example, at the 0.1 μmol/L ADP concentration, there was a 130% greater response in GG homozygotes when compared with AA homozygotes (P<0.006 after adjustment for age, resting level of bound fibrinogen, smoking, and GPIIb-IIIa receptor expression; Table; Figure 2). The response of the AG genotype was also statistically significantly different from that of AA genotype at all doses and about one third of the effect seen with the GG genotype. On average, across all doses of ADP, 3.4% of the interindividual variation in response was explained by the P2Y1 1622 genotype.

In the P2Y12 gene, none of the polymorphisms were found to have a significant effect on platelet response to ADP after adjustment for other covariates (Table). In addition, no effect was observed of any genotype on resting platelet activation state (data not shown).

The interindividual response to thrombin receptor activity peptide (TRAP) at all 3 concentrations also showed a wide variation (data not shown). There was significant correlation between the responses to the 3 concentrations of TRAP and ADP (r=0.51 to 0.84). The association of the P2Y1 A1622G polymorphism with the responses to TRAP is shown in Table III (available online at http://atvb.ahajournals.org). There was
a trend toward a higher response at all doses for GG homozygotes, and the aggregate effect after adjustment for age, resting level of bound fibrinogen, and GPIIb-IIIa receptor expression was significant ($P=0.037$). The magnitude of the genotype-related effect was not as great as with ADP at corresponding doses, and after adjustment for the ADP response, the aggregate effect was no longer significant ($P=0.678$).

**Discussion**

The role of platelets in normal hemostasis is incontrovertible, and they are also implicated in arterial thrombosis, as evidenced by the effectiveness of antiplatelet therapy in reducing mortality and morbidity in atherothrombotic disease states.$^{18}$ There is a high degree of interindividual variability in the platelet response to all agonists, and in particular to ADP.$^{8,9}$ This is further supported by data in the present study. More important, this variation is reproducible over time in any given individual,$^9$ which points to a potential genetic influence on the platelet response to ADP. To examine this possibility, we used a strategy to identify common sequence variation within the 2 platelet ADP receptor genes that may affect function. An $A$/$G$ polymorphism was identified at position 1622 in the P2Y1 gene that was found to have a significant association with platelet response to ADP, as defined by the binding of fibrinogen to activated GPIIb-IIIa. This is the first report of genetic variation at the P2Y1 locus that is associated with the platelet response to ADP.

Five polymorphisms were identified in the P2Y1 ADP receptor gene and 11 polymorphisms in the P2Y12 gene. Of these, a number have been detailed previously in the NCBI single-nucleotide polymorphism (SNP) database (Table I, available online at http://atvb.ahajournals.org), although several are novel. All coding sequence polymorphisms identified in this study were silent, indicating that there are no common polymorphisms that directly contribute to the variation observed in platelet response to ADP by affecting the amino acid structure of either the P2Y1 or the P2Y12 receptor.

However, a synonymous polymorphism in the P2Y1 gene was associated with variation in platelet response to ADP. The fact that the effect was seen at all doses of ADP strongly indicates a true genotype effect. A 130% increase in platelet response to the lowest stimulating dose of ADP (0.1 $\mu$mol/L) was observed when comparing those subjects homozygous for the $G$ allele at the P2Y1 1622 position with those homozygous for the $A$ allele. The effect of the polymorphism on platelet response to ADP at an individual level is further underscored by the fact that 6 of the 7 individuals with the GG genotype had a response in the top 50% of the range at all doses.

In addition to its association with the platelet response to ADP, the P2Y1 1622$A$/$G$ polymorphism also showed an association with the response to TRAP but to a much lesser extent than was observed with the ADP response. The effect was abolished after adjustment for the ADP response. These findings are important for several reasons. First, up to 50% of the response to TRAP is attributable to the secondary effect of ADP released from the platelet-dense granules, acting through the P2Y receptors.$^2$ Reproduction of the genotype effect with a second agonist that is heavily reliant on secondary ADP release indicates that the observed effect with ADP is genuine. Second, the fact that the magnitude of the genotype-related effect with TRAP was less than with ADP and was abolished after adjustment for the ADP response strongly suggests that the genotype-related effect is indeed mediated via the ADP receptor and not a result of some generalized hyper-reactivity of the cell.

We chose to study fibrinogen binding as the preferred assay for studying platelet activation by the ADP receptors rather than platelet aggregation because it is more directly associated with receptor activation, and unlike aggregation, is unaffected by any thromboxane generation related to extracellular calcium concentration.$^{19,20}$ Nonetheless, one would anticipate the increased fibrinogen binding in response to ADP in carriers of the 1622$G$ allele to be translated into increased aggregation. However, the exact magnitude of the effect on aggregation needs to be determined in future studies.

The mechanism by which the P2Y1 1622 polymorphism is associated with increased platelet response to ADP remains to be elucidated. Given that the polymorphism is itself silent and our study has not revealed any other common polymorphism affecting the primary structure of P2Y1, the likelihood is that this is through an effect on P2Y1 receptor expression. Associations between receptor expression and silent polymorphisms have been observed in studies on other platelet receptors.$^{21,22}$ In addition, a recent study by Hechler et al.$^{23}$ found that overexpression of the P2Y1 receptor in a transgenic mouse model resulted in platelet hyper-reactivity to ADP. We have sequenced the region 1000-bp upstream of the P2Y1 gene in 13 of the subjects and identified an additional 10 SNPs, all of which have a degree of linkage to the 1622$A$/$G$ polymorphism (data not shown). One of these, a C/T substitution at $-545$, is in complete linkage disequilibrium.
rium with the 1622A/G site. These findings suggest a potential link to regulation of expression within the promoter region of the gene and provide a target for further investigation. The effects of carrying the P2Y1 1622 polymorphism on downstream signal transduction pathways, and in particular calcium flux, also need to be investigated. It also needs to be borne in mind that what we report is an association of the P2Y1 1622 polymorphism with increased platelet response to ADP, and that until the functional basis of this is established, it remains possible that this reflects the effect of an adjacent gene rather than P2Y1 itself.

Age, smoking status, and platelet GPIIb-IIIa expression were all found to be important independent regulators of the platelet response to ADP. A positive correlation was found between platelet response and increasing age and also with the density of GPIIb-IIIa on platelets, whereas a negative correlation was found with current smokers. Increasing age has been recognized to be associated with increased platelet response to ADP by others, although the underlying mechanism for this is poorly understood. As would be expected, fibrinogen binding was correlated with the expression levels of GPIIb-IIIa on each platelet. However, it is worth noting that there was no effect of the human platelet antigen (HPA)-1α polymorphism on GPIIb-IIIa expression or fibrinogen binding (data not shown). A number of studies have attempted to characterize the effect of acute and chronic smoking on platelet reactivity and have produced conflicting results. Current smokers in this study had abstained from smoking for 24 hours before platelet function testing. Nonetheless, an effect of smoking status was still observed. Importantly, adjusting for each of these variables (GPIIb-IIIa expression, age, and smoking status) did not diminish the significance of the association between fibrinogen binding and the P2Y1 1622 dimorphism. The proportion of the total interindividual variation in response explained by the P2Y1 1622 polymorphism and the other variables combined is modest, suggesting that other variables affecting the response remain to be identified. Although the overall proportion of the interindividual variation in response to ADP explained by the P2Y1 A1622G polymorphism is small (2.7% to 3.9% across the 3 concentrations of ADP), mainly because the frequency of the GG genotype is low, it should be emphasized that at an interindividual variation in response to ADP explained by the P2Y1 1622 dimorphism. The proportion of the total interindividual variation in response explained by the P2Y1 1622 polymorphism and the other variables combined is modest, suggesting that other variables affecting the response remain to be identified. Although the overall proportion of the interindividual variation in response to ADP explained by the P2Y1 A1622G polymorphism is small (2.7% to 3.9% across the 3 concentrations of ADP), mainly because the frequency of the GG genotype is low, it should be emphasized that at an individual level, those homozygous for the G allele had a markedly exaggerated response to ADP, particularly at the lower doses.

Screening of the P2Y12 gene identified several polymorphisms, including 5 in complete linkage disequilibrium. However, none were found to affect platelet reactivity. Four of the linked polymorphisms, equivalent to IntB137C/T, IntB742T/C, IntB798delA, and 252G/T in our study, have been described recently by Fontana et al. Interestingly, in their study, there was an association between the 2 haplotypes defined by these linked polymorphisms and platelet response to ADP, as measured by maximal platelet aggregation. Carriage of the minor haplotype was associated with higher maximal aggregation and greater inhibition of iloprost-stimulated cAMP accumulation. Despite a larger number of heterozygotes and homozygotes for the minor haplotype in our study, we did not detect any effect of this variant on fibrinogen binding to activated GPIIb-IIIa. Indeed, the response in heterozygous subjects was somewhat lower than that in the subjects homozygous for the more common allele (Table). The reason for the discrepancy between the 2 studies is unclear. Different methodologies were used in the 2 studies: flow cytometry here compared with aggregometry by Fontana et al, and as discussed earlier, the precise relationship between the results from the 2 assays remains to be established. An alternative explanation, especially given our finding of a significant effect of age on fibrinogen binding in response to ADP and the known effect of age on aggregometry responses, is the difference in the demographics of the subjects studied. In contrast to our cohort, the subjects of Fontana et al were all males and significantly younger (18 to 35 years of age).

Whether the increased platelet reactivity associated with the P2Y1 1622G allele increases the risk of thrombotic events remains to be determined. There is also emerging evidence that the variability in platelet response to ADP may be implicated in the variation in response observed with certain antiplatelet agents.27,28 Those patients with a high pretreatment response to ADP may not achieve the desired degree of platelet inhibition at conventional doses of those agents, and this may be a factor influencing treatment failure in the setting of unstable atherothrombotic disease. Whether the P2Y1 1622 polymorphism identified here plays a role in this phenomenon will be interesting to investigate.

In summary, we report an association between a silent polymorphism at position 1622 in the coding sequence of the P2Y1 ADP receptor gene and platelet reactivity. Carrying ≥1 G allele at this position is associated with an increased platelet activation response to ADP. Identification of this genotype effect partly explains the interindividual variation in platelet response to ADP and may have clinical implications with regard to risk of arterial thrombosis and individual response to certain antiplatelet agents.

Acknowledgments
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References


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Methods

Subjects and sample collection

The first 5mls were used to prepare serum. Subsequent aliquots of 4.5ml were collected into 0.5ml of 3.2% tri-sodium citrate and used for flow cytometric analysis and plasma fibrinogen measurement. Two subsequent samples collected into EDTA were used to obtain a full blood count and for DNA extraction.

Measurement of the platelet response

Five µl of blood was added to 50µl Hepes-buffered saline containing FITC-labelled antibodies used at optimal concentration, determined by titration prior to the study. The final calcium concentration in the assay was 4µmol.L⁻¹. Activation of platelets was measured by the binding of fibrinogen to the GPIIb-IIIa receptor using FITC-conjugated rabbit anti-fibrinogen (DakoCytomation, Cambs, UK), in unstimulated (resting) samples and in response to stimulation with ADP (0.1, 1.0 and 10 µmol.L⁻¹) and thrombin related activating peptide - TRAP (SFLLRN; 3, 10 and 30 µmol.L⁻¹). Expression of GPIIb-IIIa receptors on the platelets was determined with a CD41/61 monoclonal antibody (RFGP56). Samples in duplicate were incubated for twenty minutes at room temperature before dilution 1:100 in 0.2% formyl saline and analysed in an MCL-XL flow cytometer (Beckman Coulter, Milton Keynes, UK), within two hours of collection. The platelet population was identified from its light scatter characteristics and identity confirmed using a FITC-conjugated CD42b Mab (RFGP37). Five thousand platelets were analysed and the values were recorded as the percentage of cells positive for fluorescent antibody binding. To standardise the assays the flow cytometer was aligned daily using Immunocheck beads (Coulter), the
antibodies used throughout the study were from the same batch, and the agonists were prepared in a single batch and stored in aliquots that were thawed for single use.

Screening of the P2Y1 and P2Y12 genes for common polymorphisms

Each gene was screened by PCR using sets of five overlapping primer pairs each amplifying segments of ~1kb in length (Supplementary Table I). PCR was performed on a MJ Research Peltier Thermal Cycler (MJ Research, Waltham, MA) using a proof-reading DNA polymerase, Biolase Diamond (Bioline, UK). The amplified fragments were gel-purified using TaKaRa Recochips (Cambrex, UK) and sequenced using Big Dye Terminator v3 chemistry (Applera, Cheshire, UK). The sequencing products were resolved on an ABI PRISM® 3700 Gene Analyzer, and analysed using Sequencing Analysis v3.7 software (Applera, Cheshire, UK). Certain P2Y12 amplicons required additional internal primers to allow full sequencing of the fragments (details available on request).

Genotyping of common P2Y1 and P2Y12 polymorphisms

Polymorphisms were chosen that would give the greatest power to determine an effect. The 1622A>G polymorphism in the P2Y1 gene and the IntB742T>C polymorphism in the P2Y12 gene were typed by PCR amplification and allele-specific restriction endonuclease digestion with BclI and HpyCHIV 4 (New England BioLabs, Herts, UK) respectively. The 234C>T, 1622C>T and 2014C>T polymorphisms in the P2Y12 gene were typed by TaqMan assays using primers and TaqMan® MGB probes designed on Primer Express v2.0 software (Applera). Details of the assays are available on request.
References

Supplementary Table I. Oligonucleotide primers used for amplifying and sequencing of P2RY1 and P2RY12

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>Position and sequence</th>
<th>Product size (bp)</th>
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| P2RY1-1      | F (-536) 5’-TTTTGGAATTTCACGTGATGTT-3’ (-512)  
               R (473) 5’-GAGTTTTCCCTGACCTCGCTG-3’ (453) | 1009 |
| P2RY1-2      | F (345) 5’-TGAGCTGACGTGTTTTCTAAGGTAGG-3’ (368)  
               R (1354) 5’-GAAGAATGCGATCTGTACGCT-3’ (1331) | 1010 |
| P2RY1-3      | F (1200) 5’-GCCATGTAAGACTGAGGTTGACC-3’ (1223)  
               R (2146) 5’-CTTGTGTGTTGCTTTCAGCT-3’ (2123) | 947 |
| P2RY1-4      | F (1958) 5’-AAAGGCAAGAATCTCCAACCCACC-3’ (1981)  
               R (3003) 5’-CTCAAATGTAGCACTGGG-3’ (2980) | 1046 |
| P2RY1-5      | F (2744) 5’-AAAGAGCATTTACTTGCCCCTGACTG-3’ (2767)  
               R (3490) 5’-CCAAACAAATTCGCTGAAAGA-3’ (3467) | 747 |
| P2RY12-1     | F (-10) 5’-TCTCTGATTGTAGCCGGAAGCCCTC-3’ (10)  
               R (773) 5’-TGGCATCTACATCTTGGGAA-3’ (754) | 986 |
| P2RY12-2     | F (578) 5’-CATTTTGGGGAATTTAAGTGC-3’ (598)  
               R (1723) 5’-GAGAGGATGGTTATTTTCAGCC-3’ (1702) | 1146 |
| P2RY12-3     | F (1634) 5’-TCAACCTTTTAGAGGAGGCTTG-3’ (1655)  
               R (920) 5’-TTCACCTTTTTCGGG-3’ (902) | 822 |
| P2RY12-4     | F (611) 5’-CATCCAAAACCCCCAAATCTC-3’ (630)  
               R (1624) 5’-TCGTTCTTCTTAGGATTAGTA-3’ (1601) | 1014 |
| P2RY12-5     | F (1203) 5’-AGAACAGGATGTGGTGACC-3’ (1222)  
               R (2279) 5’-TGTATATGGATGTGGTACATG-3’ (2256) | 1077 |

F – forward primer, R – reverse primer. Nucleotide positions are given in relation to the transcription start site for the P2RY1 gene and the start of exon 2 for the P2RY12 gene, except those in intron B of P2RY12 (*), which are given in relation to the start of that intron.
## Supplementary Table II. Polymorphisms identified in the P2Y1 and P2Y12 genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>Nucleotide position</th>
<th>NCBI dbSNP Reference</th>
<th>Observed alleles (approximate frequencies*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2Y1</td>
<td>5'UTR</td>
<td>179</td>
<td>-</td>
<td>C/A (0.98/0.02)</td>
</tr>
<tr>
<td></td>
<td>5'UTR</td>
<td>190</td>
<td>rs3755711</td>
<td>G/C (0.85/0.15)</td>
</tr>
<tr>
<td></td>
<td>Coding</td>
<td>893</td>
<td>rs1065776</td>
<td>C/T (0.98/0.02)</td>
</tr>
<tr>
<td></td>
<td>Coding</td>
<td>1622</td>
<td>rs701205</td>
<td>A/G (0.85/0.15‡)</td>
</tr>
<tr>
<td></td>
<td>3'UTR</td>
<td>2717</td>
<td>-</td>
<td>C/G (0.98/0.02)</td>
</tr>
<tr>
<td>P2Y12</td>
<td>5'UTR</td>
<td>145†</td>
<td>rs3821667</td>
<td>C/T (0.83/0.17)</td>
</tr>
<tr>
<td></td>
<td>Intron B</td>
<td>137†</td>
<td>-</td>
<td>C/T (0.83/0.17)</td>
</tr>
<tr>
<td></td>
<td>Intron B</td>
<td>742†</td>
<td>rs2046934</td>
<td>T/C (0.83/0.17‡)</td>
</tr>
<tr>
<td></td>
<td>Intron B</td>
<td>798†</td>
<td>rs5853517</td>
<td>A/- (0.83/0.17)</td>
</tr>
<tr>
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<td>Intron B</td>
<td>1209</td>
<td>rs1388626</td>
<td>T/C (0.83/0.17)</td>
</tr>
<tr>
<td></td>
<td>Coding</td>
<td>234</td>
<td>rs6785930</td>
<td>C/T (0.95/0.05)</td>
</tr>
<tr>
<td></td>
<td>Coding</td>
<td>252†</td>
<td>rs6809699</td>
<td>G/T (0.67/0.33‡)</td>
</tr>
<tr>
<td></td>
<td>Coding</td>
<td>676</td>
<td>-</td>
<td>T/C (0.83/0.17)</td>
</tr>
<tr>
<td></td>
<td>Downstream</td>
<td>1622</td>
<td>-</td>
<td>C/T (0.98/0.02)</td>
</tr>
<tr>
<td></td>
<td>Downstream</td>
<td>2014</td>
<td>rs6803224</td>
<td>C/T (0.96/0.04‡)</td>
</tr>
<tr>
<td></td>
<td>Downstream</td>
<td>2087</td>
<td>-</td>
<td>A/- (0.77/0.23‡)</td>
</tr>
</tbody>
</table>

NCBI dbSNP – National Centre for Biotechnology Information single nucleotide polymorphism database, 5'UTR – 5’ untranslated region, 3’UTR – 3’ untranslated region. †polymorphisms found to be in complete linkage disequilibrium. *derived from sequencing of 40 chromosomes, except ‡where frequencies calculated following genotyping of 400 chromosomes.
### Supplementary Table III. Platelet activation response to TRAP by P2Y1 1622A>G genotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Frequency n (%)</th>
<th>Mean platelet response (95% C.I.) to TRAP (% positive for bound fibrinogen)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3 µmol.L⁻¹</td>
<td>10 µmol.L⁻¹</td>
</tr>
<tr>
<td>AA</td>
<td>147 (73.5)</td>
<td>6.2 (5.3-7.3)</td>
<td>79.9 (76.7-82.8)</td>
</tr>
<tr>
<td>AG</td>
<td>46 (23.0)</td>
<td>7.6 (5.2-11.2)</td>
<td>81.9 (76.4-86.5)</td>
</tr>
<tr>
<td>GG</td>
<td>7 (3.5)</td>
<td>10.1 (4.8-20.1)</td>
<td>89.4 (80.9-94.4)</td>
</tr>
</tbody>
</table>

Mean platelet responses are unadjusted values following back-transformation of log(response.(100-response)^⁻¹)-scale. p-values are quoted after adjustment for age, resting level of bound fibrinogen and GPIIb-IIIa expression, and represent overall genotype effect at the stated dose or *comparing average effects across the range of TRAP concentrations using random effects model.