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Severe bleeding and absent ADP-induced platelet aggregation associated with inherited combined CalDAG-GEFI and P2Y₁₂ deficiencies

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Inherited platelet function disorders are associated with a heightened risk for mucocutaneous bleeding of variable severity and excessive hemorrhage after surgery or trauma.¹ They are most commonly associated with abnormalities of receptors for adhesive proteins or soluble agonists, of cytoplasmic granules or of signal transduction pathways.¹ Laboratory screening for inherited platelet function disorders includes measurement of platelet aggregation by light transmission aggregometry, induced by agonists in citrate-anticoagulated platelet-rich plasma (PRP).¹ Platelet aggregation induced by adenosine diphosphate (ADP) is abnormal in many platelet function disorders.¹ ADP interacts with Gq-coupled P2Y₁ and Gi-coupled P2Y₁₂ receptors, coactivation of which is essential for full platelet aggregation (Figure 1A).² P2Y₁ activates phospholipase C β -dependent increase in cytoplasmic Ca²⁺, which stimulates platelet shape change through phosphorylation of myosin light chain and platelet aggregation through calcium- and diacylglycerol-regulated guanine exchange factor-1 (CalDAG-GEFI)-mediated stimulation of the small GTPase Rap1 and consequent activation of integrin α IIb β 3, which binds adhesive proteins, such as fibrinogen, bridging adjacent platelets together, forming a platelet aggregate (Figure 1B).³ Platelet aggregation is reinforced by P2Y₁₂, which, via phosphoinositide 3-kinase signaling, prevents Rap1 deactivation by Ras GTPase-activating protein 3 (Figure 1B).⁴ ADP-induced platelet aggregation is slowly reversible, but, in citrate-anticoagulated PRP, is amplified and stabilized by a “secondary” platelet aggregation, induced by thromboxane A₂ and ADP secretion, when “primary” platelet aggregation exceeds a threshold amplitude.⁵ While abnormalities of secondary platelet aggregation, associated with defects of platelet granules or secretory mechanisms, are relatively common,¹ platelet function disorders affecting primary platelet aggregation are rare, including defects of P2Y₁₂,⁶ CalDAG-GEFI, reviewed by Palma-Barqueros et al.⁷, and the final common steps of integrin α IIb β 3 activation (Glanzmann Thrombasthenia and Leukocyte Adhesion Deficiency-III).¹ ADP-induced platelet aggregation is impaired in defects of P2Y₁₂ or CalDAG-GEFI, while it is absent

(albeit preceded by normal platelet shape change) in Glanzmann Thrombasthenia and Leukocyte Adhesion Deficiency-III.¹ A platelet phenotype similar to that of Glanzmann Thrombasthenia and Leukocyte Adhesion Deficiency-III can be reproduced in normal platelets by antibodies against α IIb β 3 (Figure 1A).

We report the case of a patient with severe bleeding diathesis associated with combined homozygous CalDAG-GEFI and heterozygous P2Y₁₂ deficiencies (II-5, Figures 2A and 2B), characterized by normal ADP-induced platelet shape change but absent ADP-induced platelet aggregation. This is the first patient with combined CalDAG-GEFI and P2Y₁₂ deficiencies that has been described so far.

The following methods were employed: light transmission aggregometry in citrate-anticoagulated PRP (patient II-1 [Figures 2A and 2B], who was first referred to our Center, was also studied by lumiaggregometry, which measures platelet aggregation and ATP secretion simultaneously); flow cytometry, to explore the expression of glycoproteins on the platelet membrane; PFA-100 and INNOVANCE PFA P2Y Closure Times; binding of P2Y₁₂ antagonist [³H]PSB0413 to washed platelets, to calculate the number of P2Y₁₂ binding sites;⁸ inhibition of prostaglandin (PG)E₁-induced increase of cyclic adenosine monophosphate (cAMP) by ADP or epinephrine.⁶

Genetic analyses were performed by Sanger sequencing of genomic DNA and cDNA from platelets after reverse transcriptase-polymerase chain reaction (RT-PCR), determination of *P2RY12* haplotypes and Whole Exome Sequencing. Details are included in Supplementary Materials. All diagnostic procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. All subjects gave their informed approval for all diagnostic procedures.

Patient II-1 (Figures 2A and 2B) is a 31-year-old Italian woman with life-long easy bruising

and episodes of epistaxis, gum bleeding, otorrhagia, menorrhagia and excessive bleeding after tooth extractions (International Society on Thrombosis and Haemostasis Bleeding Score= 16; normal values <2). Platelet count and size were normal. Platelet aggregation induced by ADP, even at high concentrations (100 μ M), was markedly reduced and rapidly reversible, suggesting a P2Y₁₂ defect (Figure 1C).⁶ The diagnostic suspicion was confirmed by the following findings: defective inhibition by ADP (but normal by epinephrine) of PGE₁-induced increase in platelet cAMP (Figure 1D), severe defect of binding sites for [³H]PSB0413 (B_{max}=51 sites/platelet vs 425 \pm 50, mean \pm SD of 10 controls), homozygous single base pair deletion (c.678delC, transcript ENST00000302632.3) in *P2RY12-201* gene (Figures 2B and 2C), resulting in the p.T126 fs*34 (UniProtKB-Q9H244), expected to abrogate receptor function. Results of additional platelet studies were compatible with P2Y₁₂ deficiency: normal α IIb β 3 and GPIb/IX/V expression, normal platelet adenine nucleotides, serotonin and fibrinogen contents, partially defective platelet aggregation and secretion induced by secretion-stimulating agonists.⁶ Family studies revealed that the same mutation was present in homozygosity in her sister (II-2, with similar bleeding phenotype) and, in heterozygosity, in her 3 first cousins (II-3, II-4, II-5) (Figure 2B).

The personal bleeding history was negative for II-3 and II-4.⁶ However, it was positive and severe for II-5 (Figure 2A), despite her young age (9 years): in the first days of life she displayed purpuric lesions and suffered prolonged bleedings from the intramuscular injection sites of prophylactic vitamin K and vaccines, while in the first 2-3 years she suffered relapsing severe epistaxis that required hospitalizations and prolonged bleeding after the fall of a deciduous tooth. ADP-induced platelet aggregation was normal in II-3, slightly defective in II-4 and absent in II-5 (Figure 1E), whose PFA-100 and INNOVANCE PFA P2Y Closure Times were extremely prolonged (>300 seconds).

Based on these findings, we explored the possibility that II-5 carried additional defects of

platelet function. Extended *P2RY12* genomic sequencing and haplotype analysis detailed the *P2RY12* pattern (Figure 2D). The presence of an intronic mutation affecting mRNA expression⁹ was excluded by RT-PCR of platelet mRNA and cDNA sequencing. The heterozygous condition for the c.318C/T (Asn6) polymorphism (Figure 2D, Supplementary Figure 1 and Supplementary Results) indicated the maternal inheritance of normal P2Y₁₂ mRNA. Coexistent P2Y₁ defect was ruled out based on the normality of platelet shape change and the normal sequence of *P2RY1* gene (Supplementary Results). The type and severity of her platelet aggregation abnormality were apparently suggestive of a defect of integrin α IIb β 3 activation. Because Leukocyte Adhesion Deficiency-III could be safely ruled out based on the absence of predisposition to infections,¹ we focused on Glanzmann Thrombasthenia and sequenced the *ITGA2B* and *ITGB3* genes in platelet cDNAs, which revealed normal sequences characterized by polymorphic synonymous codons (Supplementary Results). Moreover, α IIb β 3 and other platelet glycoproteins were normally expressed on the patient's platelets.

We then turned to Whole Exome Sequencing for exploring the patient's DNA, which revealed the presence of c.337delC mutation (transcript ENST00000354024.7, Figure 2E) in exon 5 of *RASGRP2-201* (encoding for CalDAG-GEFI), predicting a deleterious change in the protein (p.R113fs*6, UniProtKB-Q7LDG7), candidate to explain the phenotype. The mutation was confirmed in homozygosity by direct sequencing of the patient's DNA, and in heterozygosity in II-1, II-3, II-4 (Figures 2B and 2E).

In conclusion, patient II-5 displays an extremely severe defect of ADP-induced platelet aggregation, which is not attributable to defects in the final common steps of α IIb β 3 activation, as in Glanzmann Thrombasthenia or Leukocyte Adhesion Deficiency-III, but to combined homozygous CalDAG-GEFI and heterozygous P2Y₁₂ deficiencies. The molecular defect of *RASGRP2* causing CalDAG-GEFI deficiency in our patients is not present in *The Genome Aggregation Database*

(<http://gnomad.broadinstitute.org/>), while the $P2Y_{12}$ defect has already been described in an unrelated family.¹⁰ Previous publications showed that patients with severe CalDAG-GEFI deficiency display partially defective ADP-induced platelet responses, characterized by normal shape change and irreversible or only partially reversible platelet aggregation of reduced amplitude.¹¹⁻¹⁴ Based on the type and severity of the abnormality of platelet aggregation in patient II-5, we can infer that this pattern of platelet response to ADP in CalDAG-GEFI deficient patients would be explained by normal $P2Y_1$ -mediated platelet shape change, absent $P2Y_1$ /CalDAG-GEFI-mediated initial wave of fast platelet aggregation and normal (slow, incomplete and slowly reversible or irreversible) $P2Y_{12}$ -induced platelet aggregation (defective in patient II-5). Heterozygous $P2Y_{12}$ deficiency is generally associated with abnormal, reversible platelet aggregation induced by $\leq 10 \mu\text{M}$ ADP⁷ (like in II-4), although our findings in II-3 suggest that the platelet aggregation defect is of variable severity in different patients. It is possible that the platelet aggregation defect in heterozygous $P2Y_{12}$ deficiency is more evident in patients, like II-5, whose platelets lack the priming effect of $P2Y_1$ /CalDAG-GEFI. Interestingly, heterozygous CalDAG-GEFI deficiency did not appear to affect ADP-induced platelet aggregation significantly in the study subjects, when associated with both homozygous (II-1) and heterozygous (II-3, II-4) $P2Y_{12}$ deficiency. It is interesting to note that, in our patients, compound homozygous CalDAG-GEFI and heterozygous $P2Y_{12}$ deficiency (II-5) confers more severe abnormality of ADP-induced platelet aggregation and bleeding diathesis than compound homozygous $P2Y_{12}$ and heterozygous CalDAG-GEFI deficiency (II-1).

Unfortunately, the mother of patient II-5 did not give her consent to expose her daughter to additional blood sampling to allow the study of additional platelet functions and of leukocyte function. Indeed, deficiency of CalDAG-GEFI is expected to affect also leukocyte function, because Rap1 activation is important for leukocyte integrin activation.¹⁵ However, abnormal integrin-dependent leukocyte function was shown to be defective in some, but not all CalDAG-GEFI-

deficient patients. Despite the different results of *in vitro* experiments of leukocyte function, none of the patients who have been described so far, including our patient II-5, displayed overt immune defects, or susceptibility to bacterial infections, suggesting that alternative pathways of integrin activation in leukocytes compensate for CalDAG-GEFI deficiency.

Disclosures of Conflicts of Interest

None of the authors had relevant conflicts of interest to disclose.

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Figure Legends

Figure 1. Pathophysiology of ADP-induced platelet function in normal subjects and in the studied patients. A – Effects of antagonists of P2Y₁ (MRS2216, 25 μM), P2Y₁₂ (AR-C69931MX, 1 μM) and αIIbβ3 (MoAb 10E5, 10 μg/ml) on ADP (5 μM)-induced platelet aggregation in platelet-rich plasma (PRP); maximal amplitude of platelet aggregation in control PRP (left tracing) was 78%; **B** - Regulation of ADP-induced platelet shape change and aggregation. ADP binding to Gq-coupled P2Y₁ activates the PLCβ isoform, to form IP₃, which releases Ca²⁺ from stores. Ca²⁺ induces: 1) platelet shape change through activation of MLCK and phosphorylation of myosin light chain (MLC); 2) platelet aggregation through rapid CalDAG-GEFI-dependent activation of the small GTPase Rap1 to Rap1-GTP, which, through the cooperation of talin and kindlin3 promotes the binding of adhesive proteins to αIIbβ3 and platelet aggregation. This process is regulated by Ras GTPase-activating protein 3 (RASA3), which hydrolyses Rap1-GTP to inactive Rap1-GDP; RASA3 is inactivated by the Gi-coupled platelet ADP receptor P2Y₁₂, allowing sustained Rap1 signalling and full platelet aggregation. PLCβ= phospholipase Cβ; IP₃= inositol trisphosphate; MLCK= myosin light chain kinase; pMLC= phosphorylated myosin light chain; CalDAG-GEFI= calcium- and DAG-regulated guanine exchange factor-1; PI3K=phosphoinositide 3-kinase; DTS=dense tubular system; AC=adenylyl cyclase; cAMP=cyclic adenosine monophosphate; **C** - Platelet aggregation in citrate PRP from patient II-1 (see Figure 2A and 2B) and a healthy control, induced by ADP at the indicated concentrations; maximal amplitude of platelet aggregation induced by ADP 10 μM was 80% in healthy control and 15% in patient II-1; **D** – Effects of ADP and epinephrine, at the indicated concentrations, on PGE₁ (1 μM)-induced increase in platelet cAMP of II-1 and healthy controls (means±SD, n=21); **E**- Platelet aggregation in citrate PRP from patients II-3, II-4 and II-5 (see Figure 2A and 2B), induced by ADP at

the indicated concentrations; maximal amplitudes of platelet aggregation were 87% and 89% in II-3 and 25% and 80% in II-4.

Figure 2. General characteristics and genetic abnormalities of the studied patients. **A** - Main characteristics of the study subjects (n.a.= not applicable); the two values of platelet count for patients II-1, II-2 and II-5 refer to values measured on two separate occasions; **B** - Upper part, pedigree chart. Filled symbols, subjects with bleeding diathesis. - Lower part, carriership/homozygosity for the P2RY12 and RASGRP2 frameshift mutations; **C** - Chromatograms reporting the homozygous (II-1) and heterozygous (II-5) condition for the c.678delC frameshift mutation in the P2RY12 gene; **D** - P2RY12 haplotype analysis. Upper part, localization of SNPs in the *P2RY12* genomic region. Primers for genomic DNA and cDNA amplification and sequencing are indicated by numbered arrows upper and below the scheme, respectively; numbering as reported in Supplementary Table 1; dotted arrows, position of gene alteration and informative SNPs. Lower part, haplotype marking of the c.678delC in the II generation. fs, frameshift; **E**- Chromatograms reporting the heterozygous (II-3) and homozygous (II-5) condition for the c.337delC frameshift mutation in the *RASGRP2* gene.

Figure 1

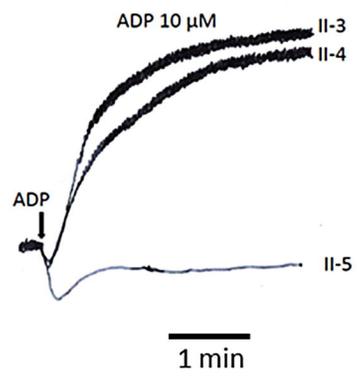
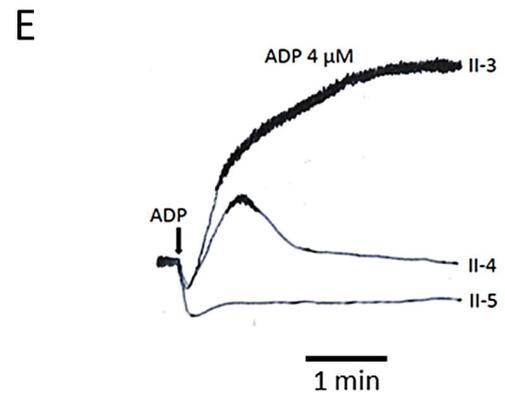
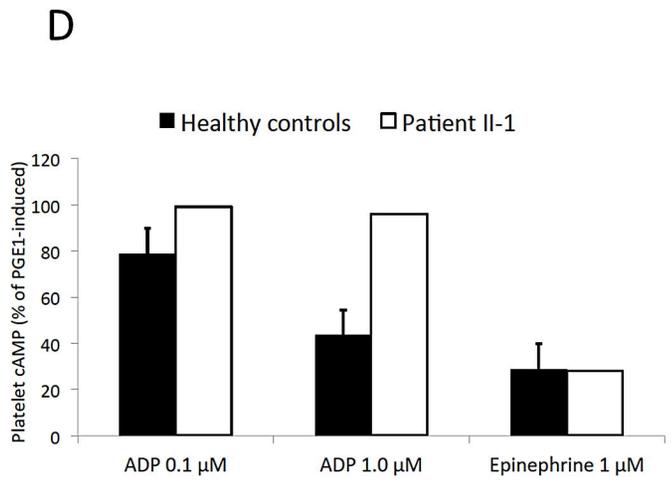
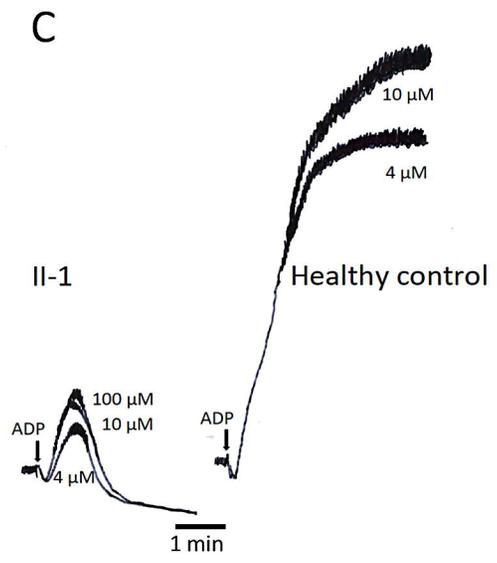
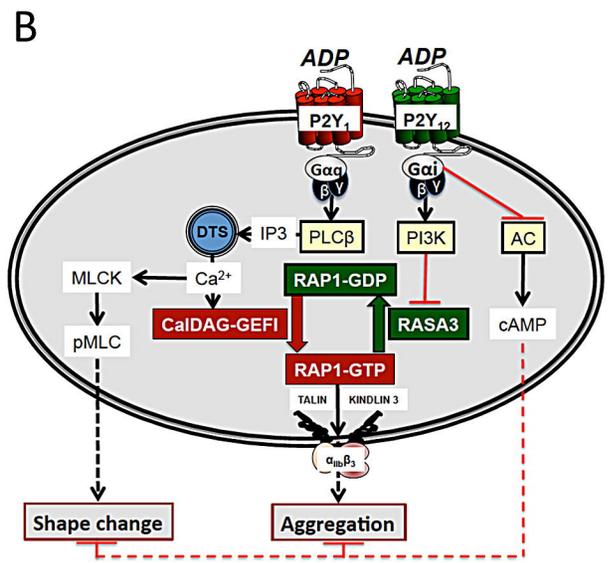
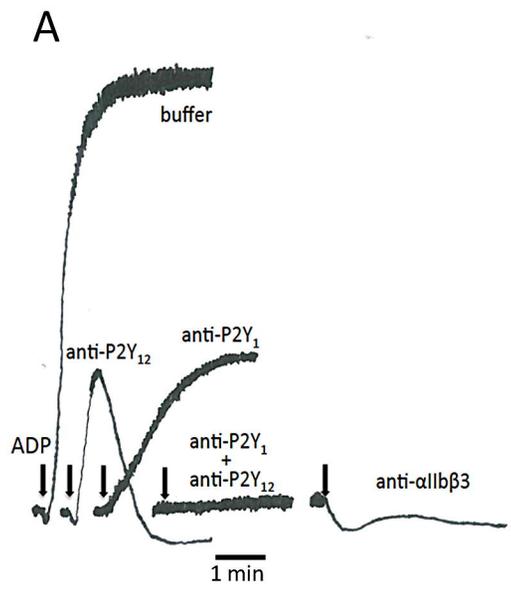
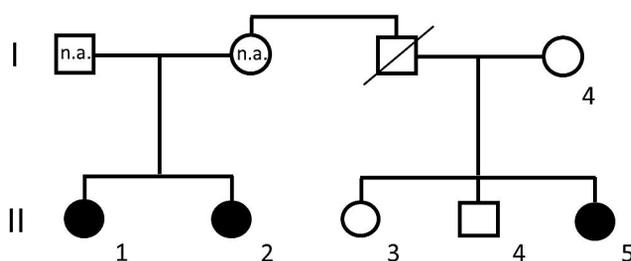


Figure 2

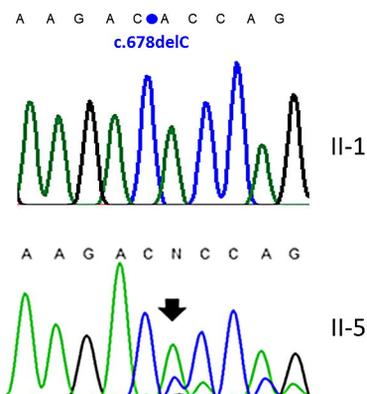
A	II-1	II-2	II-3	II-4	II-5
ISTH-BAT (age) at study entry	16 (31 years)	12 (29 years)	0	0	8 (9 years)
ISTH-BAT (age) at first medical referral	5 (5 years)	3 (3 years)	0 (n.a.)	0 (n.a.)	4 (2 months)
Main bleeding manifestations	Severe epistaxis, otorrhagia, menorrhagia, bruises, subcutaneous hematoma	Severe epistaxis, menorrhagia, bruises gum bleeding, bleeding from duodenal ulcer	None	None	Prolonged oozing from im injections, bruises, ecchimoses, severe epistaxis, prolonged bleeding after fall of deciduous teeth, gum bleeding
Platelet count $\times 10^9/L$	231 – 344	198 – 277	265	201	198 - 277

B



C

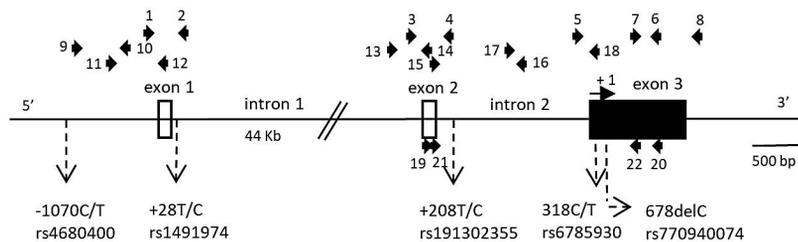
P2RY12



<i>P2RY12</i> c.678delC p.T126fs*34	delC/delC	delC/delC	wt/delC	wt/delC	wt/delC
<i>RASGRP2</i> c.337delC p.R113fs*6	wt/delC	wt/wt	wt/delC	wt/delC	delC/delC

D

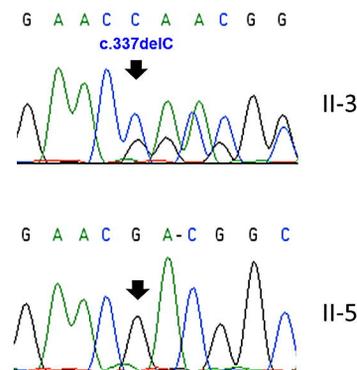
P2RY12



II-1	II-2	II-3	II-4	II-5
-1070C/T	C C	C C	C C	C T
+28T/C	T T	T T	T C	T C
+208T/C	C C	C C	C T	C T
318C/T	C C	C C	C C	C T
678delC	fs fs	fs fs	fs -	fs -

E

RASGRP2



Severe bleeding and absent ADP-induced platelet aggregation associated with inherited combined CalDAG-GEFI and P2Y₁₂ deficiencies

Supplementary material

Materials

[³H]PSB-0413 was from General Electric, Healthcare (Buckinghamshire, UK). ADP, collagen, the thromboxane/prostaglandin endoperoxide analogue 9,11-dideoxy-11,9-epoxymethano-prostaglandin F₂ (U46619), thrombin receptor activating peptide 6 (TRAP-6), prostaglandin (PG) E₁, PGI₂, were from Sigma (St. Louis, MO, USA). Apyrase was purified from potatoes.¹ Commercial preparations of luciferin/luciferase reagent were used to measure the platelet ATP and ADP contents (ATP Assay Kit, BioOrbit Oy, Turku, Finland), and platelet secretion concurrently with platelet aggregation (Chronolume, Chrono-log Corp, Havertown, PA, USA). Serum thromboxane (Tx) B₂ was measured by a commercially available enzymatic immunoassay (Thromboxane B₂ EIA kit, Cayman Chemical Co., Ann Arbor, MI, USA).

Preparation of Platelet-Rich Plasma (PRP) and Washed Platelet Suspensions

For studies with PRP, 9 volumes of blood were drawn into 1 volume of 109 mmol/L trisodium citrate, and then centrifuged at 200xg for 10 min. The supernatant PRP was transferred into a clean plastic tube; the platelet counts in PRP samples were not adjusted to a pre-defined value. For the preparation of washed platelet suspensions, 6 volumes of blood were drawn into 1 volume of acid-citrate-dextrose anticoagulant, centrifuged at 200xg for 10 min to obtain PRP, which was used to prepare twice washed platelet suspensions in Tyrode's buffer containing 500 nmol/L PGI₂ during the first and second wash. Platelet counts in washed platelet suspensions were adjusted to 3x10¹¹/L.

Studies of Platelet Aggregation and Secretion

The first screening of platelet aggregation in the studied patients was done in Florence, using the platelet aggregometer ATRACT4004 (LABiTec[®], Labor BioMedical Technologies GmbH, Ahrensburg, Germany). Additional studies of platelet aggregation in patient II-1 were done in Milan, where platelet aggregation and secretion were studied simultaneously by lumi-aggregometry. Samples of PRP (0.45 mL) were incubated with 50 μ L luciferine/luciferase reagent at 37 °C for 30 sec and stirred at 1,000 rpm in a lumi-aggregometer (Lumi-aggregometer, Chrono-log Corp., Havertown, PA, USA). After incubation, 10 μ L of an aggregating agent was added and the aggregation and ATP secretion tracings were recorded for 3 min.

Binding of [³H]PSB-0413 to Washed Platelets

Binding experiments were performed using [³H]PSB-0413, which is a tritiated derivative of a selective nucleotide antagonist of the P2Y₁₂R, AR-C67085MX (2-propylthioadenosine-5'-adenylic acid (1,1-chloro-1-phosphonomethyl-1-phosphonylanhydride), and 9×10^7 washed platelets. Nonspecific binding was defined in the presence of 1 mM ADP. Washed platelets were incubated with the ligand at 37°C for 5 min; then bound and free radioactivity was separated by filtration through Whatman GF/B glass-fiber filters. Filters were then washed with 5x2 ml of ice cold washing buffer (Tris HCl 50 mM pH 7.5, EDTA 1 mM, MgCl₂ 5 mM, NaCl 100 mM). Filter-bound radioactivity was counted in 2 ml liquid scintillation counter.

Measurement of platelet cAMP

Platelet cAMP was measured by a radioisotopic assay, using a commercially available kit (Cyclic AMP [³H] assay system, Amersham International, UK). Duplicate samples of 1 mL citrated PRP containing 1 mM theophylline were incubated with Tyrode's buffer and PGE₁ (1 μ M), PGE₁ and ADP or

epinephrine (0.1 and 1.0 μ M) or Tyrode's buffer alone in a control mixture. After incubation at 37 °C (2 min), 1 mL of 5% trichloroacetic acid was added, and the samples were snap-frozen in dry ice and methanol, thawed at ambient temperature, and then shaken at 4 °C for 45 min. After centrifugation at 4 °C for 30 min, the supernatant was extracted three times with 5 mL of water-saturated ether, dried under a stream of nitrogen at 60 °C, and stored at -20 °C. Before assay, the samples were reconstituted with 0.05 mol/L Tris buffer containing 4 mmol/L EDTA.

Genetic studies

The *P2RY12* gene (NCBI Ref Seq: NG_016019.1) was genotyped by PCR and direct sequencing of the three exons and splicing junctions, of 1.1Kb in the 5' gene region, 0.64 Kb of intron 1 and the whole intron 2 (1.7Kb) (Figure 2D). Primer sequences and position are shown in the Supplementary Table 1. Known polymorphisms were identified, which permitted the definition of *P2RY12* haplotypes in the family (Figure 2D). The *P2RY1* gene was amplified and sequenced using the forward 5'-CCCTGTTGTGTAAGCTCGGCG-3' and reverse 5'-CTTTTGAGCCGGCCAGGG-3' primers, and the forward 5'-CCATGTGTAACTGCAGAGG-3' and 5'-CAAACAAGCTAAGTGTGGATG-3' primers. Total RNA was extracted from platelets using Tempus Blood RNA Tubes and by Tempus Spin RNA Isolation Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA USA). The *P2RY12*, *ITGB3* and *ITGA2B* mRNAs were reverse transcribed using random primers and the M-MLV Reverse Transcriptase Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA USA). For *P2RY12*, a 644 bp cDNA fragment, spanning exons 2-3, was amplified with primers 19 and 20 (Supplementary Table 1). The nested-PCR fragment (547 bp, primers 21 and 22) was sequenced (Supplementary Table 1). The cDNA allelic ratio was determined by densitometric analysis of chromatogram peaks. Evaluation of the ratios was obtained by comparison of three independent

nucleotide sequences and normalization for flanking C and T peaks. The 16 primers for *ITGB3* and *ITGA2B* cDNA studies are available on request.

We performed genetic analysis by means of whole-exome sequencing (WES), and direct DNA sequencing. For WES, genomic DNA was isolated from peripheral blood by Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA), and coding regions were enriched by using SureSelectXT Human All Exon V5 kit (50MB; Agilent Technologies). DNA sequencing was undertaken on the HiSeq2000 platform (Illumina, San Diego, CA, USA) with 125-bp paired-end reads. Mean coverage was >95x and 51 Megabases was the target size that required ~4 Gigabases of sequencing per sample. Sequence reads were aligned to the human genome reference sequence (GRCh37/hg19). Exome analysis produced a large number of variants (~65,000 Single Nucleotide Variations and ~12,000 InDels). Variant annotation (*i.e.* exonic, intronic, UTRs; for exonic: synonymous, nonsynonymous, stop gain/loss, frameshift, allele frequency, etc) and prioritization were performed using an open-source software (Variant Studio, Illumina). To minimize the number of potentially deleterious gene defects different approaches, the following strategies were adopted: (i) filtering based on quality score >30; (ii) excluding variants having a Minor Allele Frequency (MAF) greater than 0.01; (iii) removing variants outside coding regions or synonymous coding variants; (iv) filtering the data for novelty by comparison to dbSNP (<http://www.ncbi.nlm.nih.gov/snp>), Exome Aggregation Consortium (<http://exac.broadinstitute.org/>), Exome Variant Server (<http://evs.gs.washington.edu/EVS>), 1000 Genomes Projects (<http://browser.1000genomes.org>), published studies; (v) selecting variants that segregate according to the presumed pattern of inheritance; (vi) querying disease databases, such as ClinVar (<http://www.ncbi.nlm.nih.gov/clinvar>), OMIM, (<http://www.omim.org>), HGMD Locus-specific database (<http://www.hgvs.org/>). After these filtering strategies, the number of variants were reduced to ~300-400. To further prioritize the

candidate gene defects, a functional annotation was undertaken based on effect on protein function and *a priori* knowledge of phenotype.

After this initial prioritization, additional strategies were used to find the causative mutation: *i.e.* the linkage strategy, in which multiple affected family members were sequenced to identify shared variation and, in addition, unaffected relatives were sequenced to exclude a benign variation; the overlap strategy, that is the searching for mutation in multiple unrelated patients with similar phenotype. Variants were validated by Sanger sequencing and segregation analysis of the prioritized variants was performed in additional affected family members when constitutive DNA was available.

Supplementary Results

The intron less *P2RY1* gene (coding sequence, 1122bp) sequencing detected only previously reported synonymous codons (Ala19Ala, rs1065776 C/T and Val262Val, rs701265 A/G), in the homozygous condition (CC and AA, respectively) in the patient II-5 and in the heterozygous form in her mother (I-4) and brother (II-4).

The P2RY12 mRNA extracted from platelets of the patient II-5, heterozygous for the c.318C/T polymorphism and the c.678delC frameshift mutation, was studied. The cDNA allelic ratio was estimated at the c.618C/T position through the peak area in the chromatograms derived from two independent RT-PCR and sequencing. The cDNA corresponding to the alleles 318T and 318C, which marks the c.678delC and thus the translational frameshift, were similarly represented (T/C ratio 0.77-1.27, Supplementary Figure 1). The nonsense mediated decay (NMD) mechanism is not expected to alter this ratio because of the introduction of a premature nonsense triplet in the last exon. Sequencing of the coding portion of exon 3 (1 Kb) in the cDNA failed to detect any additional mutation.

The *ITGB3* and *ITGA2B* mRNA were characterized by sequencing of the *ITGB3* and *ITGA2B* platelet cDNAs in the patient II-5. Only previously described *ITGB3* synonymous polymorphisms (Val381Val, rs15908 A/C, Glu511Glu rs4642 A/G and Arg515Arg rs4634 G/A) were detected in the heterozygous condition.

References

1. Cazenave JP, Ohlmann P, Cassel D, Eckly A, Hechler B, Gachet C. Preparation of washed platelet suspensions from human and rodent blood. *Methods in Mol Biol* (Clifton NJ). 2004;272:13–28

Supplementary Table 1. *P2RY12* primer sequence and position.

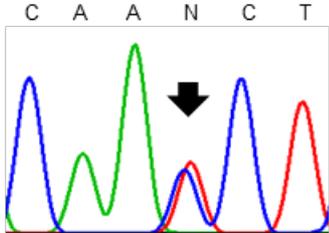
Primer	Primer sequence (5' - 3')	Position ^a
1	ACTTTCTGATCGCTTGTCTCC	4698
2	AACTCTATGCTTGGACTGGC	5244
3	TTCTCAGCCATCCTCATCCC	48839
4	TGAGGCAAAGTAACTAAGACCA	49416
5	GTGCTTTAAGAGGCAAACATTCA	50622
6	TGCCAGACTAGACCGAACTC	51471
7	TCTCTGTTGTCATCTGGGCA	51342
8	TGTCGTTTGTGTTTGTCTGCTAA	52170
9	GAATGTCGGTGGTTGCTTACTG	3842
10	AGGCATATGCTTGTCTTCTAAG	4452
11	TTCAGGGAAACATTTTAAAGTCC	4339
12	ATTGTGATCACTACCCTGGA	5008
13	GGAAGCTGTTTCACCTACAAAG	48473
14	TCAGTAAAGTCTTGAGTGCTC	49016
15	AATACCAGATGCCACTCTGC	49081
16	ATTGGCCTCACGGAGATTCA	50249
17	GGAATGCCAACTCATGACCA	50056
18	CGCCAGGCCATTTGTGATAA	51049
19	CCACTCTGCAGGTTGCAATAAC	49092
20	TGCCAGACTAGACCGAACTC	51471
21	GATACATTCAAACCCTCCAGAATC	49126
22	TGCCTGTTGGTCAGAATCATGT	51408

Grey lines, forward primers; white lines, reverse primers. Primers 1-8, primers used to amplify and sequence exons and splicing junctions of the *P2RY12* gene; primers 9-18, primers used for the 5'

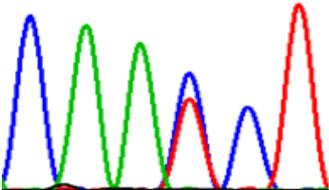
and intronic regions; primers 19-22, primers for the cDNA. ^a Corresponding to the position of the 5' end in the sequence database.

Supplementary Figure 1 - Chromatograms of the exonic nucleotide change c.318C/T (patient II-5) in the genomic DNA (upper panel) and platelet cDNA (lower panel).

P2RY12 c.318C/T



II-5
genomic DNA



II-5
platelet cDNA