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

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 $P2Y_1$ and Gi-coupled $P2Y_{12}$ receptors, coactivation of which is essential for full platelet aggregation (Figure 1A).[2] $P2Y_1$ activates the phospholipase Cβ-dependent increase in cytoplasmic Ca^{2+}, which stimulates both 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 αIIbβ3, which in turn binds adhesive proteins, such as fibrinogen, bridging adjacent platelets together and forming a platelet aggregate (Figure 1B).[3] Platelet aggregation is reinforced by $P2Y_{12}$, 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, it is amplified and stabilized by a "secondary" platelet aggregation, induced by thromboxane A_2 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 $P2Y_{12}$,[6] CalDAG-GEFI, reviewed by Palma-Barqueros *et al.*,[7] and the final common steps of integrin αIIbβ3 activation (Glanzmann Thrombasthenia and Leukocyte Adhesion Deficiency-III).[1] ADP-induced platelet aggregation is impaired in defects of $P2Y_{12}$ or CalDAG-GEFI, while it is absent (albeit preceded by normal platelet shape change) in Glanzmann Thromboasthenia 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 integrin αIIbβ3 (Figure 1A).

We report the case of a patient with severe bleeding diathesis associated with combined homozygous CalDAG-GEFI and heterozygous $P2Y_{12}$ deficiencies (patient II-5; Figures 2A-B), characterized by normal ADP-induced platelet shape change but absent ADP-induced platelet aggregation. This is the first patient with combined CalDAG-GEFI and $P2Y_{12}$ deficiencies that has been described so far.

The following methods were employed: light transmission aggregometry in citrate-anticoagulated PRP (patient II-1 [Figures 2A-B], 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 $P2Y_{12}$ antagonist [^{3}H]PSB0413 to washed platelets, to calculate the number of $P2Y_{12}$ binding sites[8] and inhibition of prostaglandin (PG)E_1-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 RT-PCR, determination of *P2RY12* haplotypes and whole exome sequencing. Details are included in the *Online Supplementary Materials and Methods*. 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-B) 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 μM), was markedly reduced and rapidly reversible, suggesting a $P2Y_{12}$ defect (Figure 1C).[6] The diagnostic suspicion was confirmed by the following findings: defective inhibition by ADP (but normal by epinephrine) of PGE_1-induced increase in platelet cAMP (Figure 1D), severe defect of binding sites for [^{3}H]PSB0413 (Bmax=51 sites/platelet *vs.* 425±50, mean± standard deviation [SD] of 10 controls), homozygous single base pair deletion (c.678delC, transcript ENST00000302632.3) in *P2RY12*-201 gene (Figures 2B-C), resulting in the p.T126 fs*34 (UniProtKB-Q9H244), expected to abrogate receptor function. The results of additional platelet studies were compatible with $P2Y_{12}$ deficiency: normal integrin αIIbβ3 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 (patient II-2, with similar bleeding phenotype) and, in heterozygosity, in her three first cousins (patients II-3, II-4, II-5) (Figure 2B).

The personal bleeding history was negative for patients II-3 and II-4. However, it was positive and severe for patient 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 loss of a deciduous tooth. ADP-induced platelet aggregation was normal in patient II-3, slightly defective in patient II-4 and absent patient 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 patient 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 expression[9] was excluded by RT-PCR of platelet mRNA and cDNA sequencing. The heterozygous condition for the c.318C/T (Asn6) polymorphism (Figure 2D, *Online Supplementary Figure S1* and *Online Supplementary Results*) indicated the maternal inheritance of normal $P2Y_{12}$ mRNA. A coexistent $P2Y_1$ defect was ruled out based on the normality of the platelet shape change and

the normal sequence of *P2RY1* gene (*Online Supplementary Results)*. The type and severity of her platelet aggregation abnormality were apparently suggestive of a defect of integrin αIIbβ3 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 cDNA, which revealed normal sequences characterized by polymorphic synonymous codons (*Online*

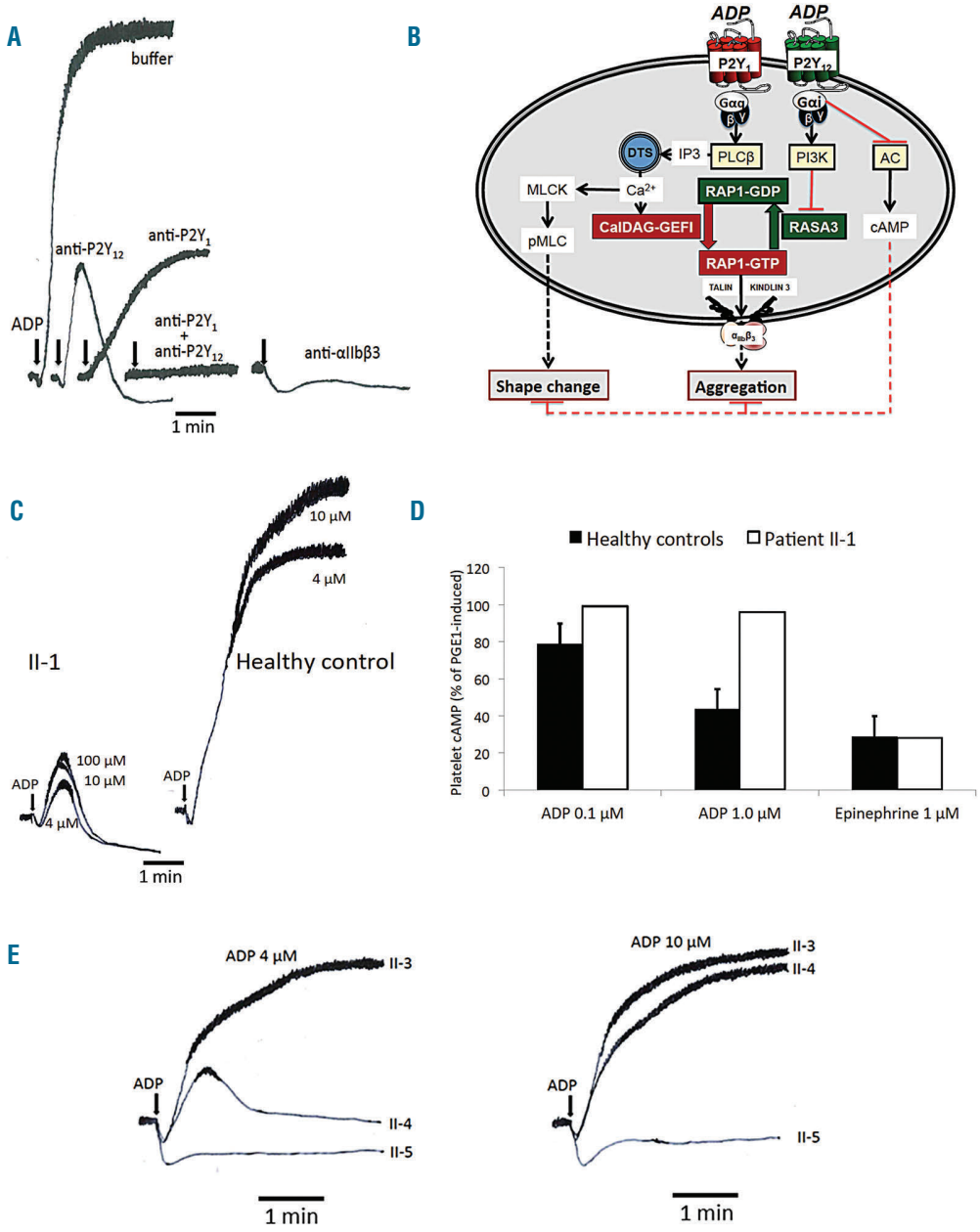


Figure 1. Pathophysiology of ADP-induced platelet function in normal subjects and in the studied patients. (A) Effects of antagonists of $P2Y_1$ (MRS2216, 25 μM), $P2Y_{12}$ (AR-C69931MX, 1 μM) and integrin αIIbβ3 (MoAb 10E5, 10 μg/mL) on ADP (5 μM)-induced platelet aggregation in platelet-rich plasma ADP, added alone or in combination to normal 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 $P2Y_1$ activates the PLCβ isoform, to form IP3, which releases Ca^{2+} from stores. Ca^{2+} 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 $P2Y_{12}$, allowing sustained Rap1 signalling and full platelet aggregation. PLCβ: phosphlipase Cβ; IP3: inositol trsiphosphate; 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-B) 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 PGE_1 (1 μM)-induced increase in platelet cAMP of II-1 and healthy controls (means± standard deviation [SD], n=21). (E) Platelet aggregation in citrate PRP from patients II-3, II-4 and II-5 (see Figure 2A-B), induced by ADP at the indicated concentrations; maximal amplitudes of platelet aggregation were 87% and 89% in patient II-3 and 25% and 80% in patient II-4.

Supplementary Results). Moreover, αIIbβ3 and other platelet glycoproteins were normally expressed on the patient's platelets.

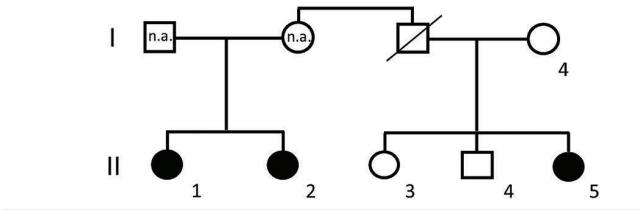
We then turned to whole exome sequencing for exploring the patient's DNA, which revealed the presence of c.337delC mutation (transcript ENST00000354024.7; Figure 2E) in exon 5 of *RASGRP2* (encoding for CalDAG-GEFI), predicting a deleterious change in the protein (p.R113fs*6, UniProtKB-Q7LDG7), candidate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

In conclusion, patient II-5 displays an extremely severe

A

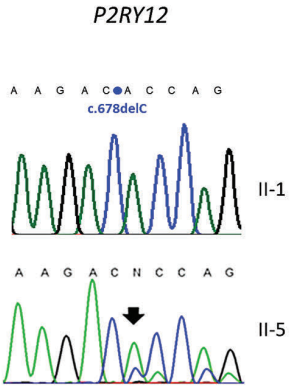
	II-1	II-2	II-3	II-4	II-5
ISTH-BAT (age) at study entry	16 (31 years)	12 (29 years)	0	0	8 (9 years)
ISTH-BAT (age) at first medical referral	5 (5 years)	3 (3 years)	0 (n.a.)	0 (n.a.)	4 (2 months)
Main bleeding manifestations	Severe epistaxis, otorrhagia, menorrhagia, bruises, subcutaneous hematoma	Severe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

B



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

C



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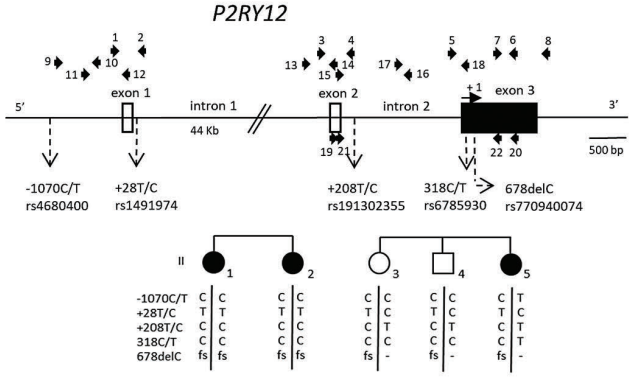

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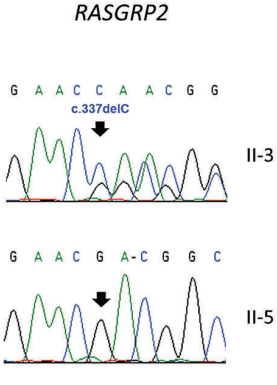

Figure 2. General characteristics and genetic abnormalities of the studied patients. (A) Main characteristics of the study subjects (n.a.: not applicable); the two values of platelet count for patients II-1, II-2 and II-5 refer to values measured on two separate occasions. (B) Upper part, pedigree chart. Filled symbols, subjects with bleeding diathesis. - Lower part, carriership/homozygosity for the P2RY12 and RASGRP2 frameshift mutations. (C) Chromatograms reporting the homozygous (patient II-1) and heterozygous (patient II-5) condition for the c.678delC frameshift mutation in the *P2RY12* gene. (D) P2RY12 haplotype analysis. Upper part, localization of single nucleotide polymorphisms (SNP) 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 the *Online Supplementary Table S1*; dotted arrows, position of gene alteration and informative SNP. Lower part, haplotype marking of the c.678delC in the II generation. fs: frameshift. (E) Chromatograms reporting the heterozygous (patient II-3) and homozygous (patient II-5) condition for the c.337delC frameshift mutation in the *RASGRP2* gene.

defect of ADP-induced platelet aggregation, which is not attributable to defects in the final common steps of inetgrin αIIbβ3 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 P2Y12 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 a 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 a normal P2Y1-mediated platelet shape change, an absent P2Y1/CalDAG-GEFI-mediated initial wave of fast platelet aggregation and a normal (slow, incomplete and slowly reversible or irreversible) P2Y12-induced platelet aggregation (defective in patient II-5). Heterozygous P2Y12 deficiency is generally associated with abnormal, reversible platelet aggregation induced by ≤10 μM ADP7 (as in patient II-4), although our findings in patient 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 P2Y12 deficiency is more evident in patients, like patient II-5, whose platelets lack the priming effect of P2Y1/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 (patient II-1) and heterozygous (pateints II-3 and II-4) P2Y12 deficiency. It is interesting to note that, in our patients, compound homozygous CalDAG-GEFI and heterozygous P2Y12 deficiency (patient II-5) confers more severe abnormality of ADP-induced platelet aggregation and bleeding diathesis than compound homozygous P2Y12 and heterozygous CalDAG-GEFI deficiency (patient 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.

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