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Running heads: Impaired platelet function in PT-VWD

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AUTHORSHIP CONTRIBUTIONS
LB, EF, AMM, GG and SM performed experiments, analyzed and interpreted data; PG designed and supervised the study; PG contributed the patient for the study; LB and PG wrote the manuscript; PG critically revised the manuscript.

DISCLOSURE OF CONFLICTS OF INTEREST
The authors declare no conflicts of interest.

SUMMARY
• Platelets in PT-VWD are dysfunctional with impaired adhesion, granule secretion, $\alpha_{IIb}\beta_3$ activation and aggregation
• The defect is caused by the constitutive activation of the Lyn-PECAM1 signal transduction inhibitory pathway triggered by VWF-GP Ib\(\alpha\) binding
ABSTRACT

Platelet-type von Willebrand disease (PT-VWD) is an inherited platelet disorder characterized by macrothrombocytopenia and mucocutaneous bleeding, of variable severity, due to gain-of-function variants of GP1BA conferring to glycoprotein Ibα (GPIbα) enhanced affinity for von Willebrand factor (VWF). The bleeding tendency is conventionally attributed to thrombocytopenia and large VWF-multimers depletion. Some clues, however, suggest that platelet dysfunction may contribute to the bleeding phenotype but no information on its characteristics and causes are available. Aim of the present study was to characterize platelet dysfunction in PT-VWD and shed light on its mechanism. Platelets from a PT-VWD patient carrying the p.M239V variant and from PT-VWD mice carrying the p.G233V variant showed a remarkable platelet function defect, with impaired aggregation, defective granule secretion and reduced adhesion under static and flow conditions. VWF-binding to GPIbα is known to trigger intracellular signaling involving Src-family kinases (SFKs). We found that constitutive phosphorylation of the platelet SFK Lyn induces a negative-feedback loop downregulating platelet activation through phosphorylation of PECAM1 on Tyr686 and that this is triggered by the constitutive binding of VWF to GPIbα binding. These data show for the first time that the abnormal triggering of inhibitory signals mediated by Lyn and PECAM1 may lead to platelet dysfunction.

In conclusion, our study unravels the mechanism of platelet dysfunction in PT-VWD caused by deranged inhibitory signaling triggered by the constitutive binding of VWF to GPIbα which may significantly contribute to the bleeding phenotype of these patients.

INTRODUCTION

Gain-of-function variants in the GP1BA gene conferring to GPIbα enhanced affinity for VWF cause platelet-type von Willebrand disease (PT-VWD), a rare inherited bleeding disorder. Patients with PT-VWD have mild thrombocytopenia with increased platelet volume, enhanced ristocetin-induced platelet agglutination (RIPA) and prolonged bleeding time associated with mucocutaneous bleeding, sometimes severe. The cause of the apparently counterintuitive bleeding phenotype of patients with platelets displaying enhanced affinity for VWF is incompletely understood and is thought to be due principally to thrombocytopenia and to the consumption of large VWF-multimers bound to platelets.
The possibility that platelet dysfunction may contribute to the bleeding phenotype of PT-VWD has attracted little attention, but defective fibrinogen binding, delayed aggregation in response to ADP and thrombin, impaired thrombus formation on a damaged carotid artery and unstable clot formation have been reported in a mouse model of PT-VWD\cite{4,5} raising the hypothesis that platelet function may be altered in PT-VWD patients. However, the mechanism of the platelet function defect is unknown and no functional studies have been performed with human PT-VWD platelets. The first step in primary hemostasis is the interaction between platelets and subendothelial collagen, mediated by the binding of von Willebrand factor (VWF) to the exposed collagen and to the GPIbα subunit of the platelet GPIb-IX-V complex which slows down platelets onto the damaged vessel wall thus allowing the direct interaction of collagen with its receptors on platelets. However, GPIb-IX-V engagement by VWF triggers also intraplatelet signaling with the sequential activation of the Src-family kinases (SFKs) Src and Lyn which are associated with the cytoplasmic tail of GPIbα, which trigger cytoplasmic Ca\textsuperscript{2+} increase and PI3-kinase/PKC activation ultimately leading to inside-out α\textsubscript{IIb}β\textsubscript{3} activation.\cite{6-8}

Here, we show for the first time defective platelet function in a PT-VWD patient carrying the p.M239V variant and in PT-VWD mice carrying the p.G233V variant due to impaired Rap1b and α\textsubscript{IIb}β\textsubscript{3} activation. Mechanistic studies revealed constitutive phosphorylation of Lyn inducing a negative-feedback loop downregulating platelet activation through the phosphorylation of PECAM1 on Tyr686.

Our results showing that constitutive binding of VWF to GPIbα triggers PECAM1-mediated inhibitory signalling which downregulates platelet activation suggest that defective platelet function in PT-VWD may significantly contribute to the bleeding phenotype associated with this disorder.

**METHODS**

The terms control and PT-VWD along this manuscript refer to human platelets while the respective terms used for murine platelets are Tg\textsuperscript{WT} and Tg\textsuperscript{G233V}. All human and animal studies were approved by the responsible Institutional review boards (human: CEAS Umbria, approval n. 2663/15; animal: Italian Ministry of Public Health authorization n. 561/2015-PR).
**Human studies and blood sampling**

The PT-VWD patient carrying the M239V GP1BA variant (to our knowledge the only patient diagnosed in Italy so far) was previously reported\(^9,10\), she had an ISTH-BAT score of 11\(^11\). Each experiment was repeated from 3 to 5 times from blood samples collected on different days (in the text indicated as independent experiments, 8 in total, **Supplementary Table 1**). Age- and sex-matched healthy controls were studied in parallel. For details see the Supplemental Data.

**Mouse strains and blood sampling**

The generation of PT-VWD mice expressing the human GP1BA carrying the p.G233V variant (Tg\(^{G233V}\)) and of control mice expressing a wild-type human GP1BA (Tg\(^{WT}\)) was previously described (**Supplementary Figure S1**).\(^4,5,10,12\) Mice were bred and housed in the animal facility of the University of Perugia, Perugia, Italy, and all experiments were performed with 3 to 6 months-old mice. The number of males and females and platelet counts are reported under each figure legend. For details see the Supplemental Data.

**Light transmission aggregometry (LTA)**

LTA was carried out using platelet-rich plasma (PRP) using a range of agonists as described previously.\(^13\) For the study of shape change, PRP was pretreated with the \(\alpha_{\text{IIb}}\beta_3\) blocker Arg-Gly-Asp-Ser peptide (RGDS).\(^14,15\) For details see the Supplemental Data.

\(\alpha_{\text{IIb}}\beta_3\) activation

\(\alpha_{\text{IIb}}\beta_3\) activation was assessed using the PAC-1 MoAb (BD Bioscience, Franklin Lakes, NJ, USA) for human platelets and the JON/A MoAb (Emfret Analytics, Eiblstadt, Germany) for murine platelets as described previously.\(^13,16,17\) For details see the Supplemental Data.

**Granule content and secretion**

Platelet granule content was assessed by electron microscopy as described previously.\(^10,13\) Alpha-granules secretion was measured as P-selectin expression\(^18,19,20\) and dense granule content and release using the green fluorescent dye mepacrine\(^21\) by flow cytometry as described previously.\(^19\) For details see the Supplemental Data.

**Spreading assay**
Washed platelets$^{22,23}$ ($20\times10^6$/ml) were resuspended in Tyrode’s buffer and spreading on fibrinogen and type I collagen was assessed as described previously.$^{24}$ For details see the Supplemental Data.

**Platelet adhesion under flow conditions**
Platelet adhesion under flow conditions over fibrillar type I collagen was assessed using citrated human or murine blood as described previously.$^{20,25}$ For details see the Supplemental Data.

**Measurement of intracellular calcium (Ca$^{2+}$)**
PRP was loaded with the Ca$^{2+}$-sensitive dye FLUO 3-acetoxyethyl ester (FLUO 3-AM; Molecular Probes) and Ca$^{2+}$ mobilization induced by various agonists was assessed by flow cytometry as described previously.$^{17,26}$ For details see the Supplemental Data.

**Rap-1b pull-down assay**
Rap-1b activity (Rap-1b-GTP) was assessed using an active Rap-1b pull-down and detection kit (Pierce biotechnology, Rockford, IL, USA) in washed platelets stimulated for 30 seconds with ADP 10 µM, thrombin 0.1 U/ml or their vehicle.$^{27}$ For details see the Supplemental Data.

**Phosphorylation of signaling proteins**
Human washed platelets ($300\times10^9$/L) were stimulated for 30 seconds under continuous stirring with ADP 2 µM, CVX 60 ng/ml, ristocetin 0.3 mg/ml or their vehicle. To exclude $\alpha_{IIb}\beta_3$-mediated outside-in signaling as a cause of signaling protein phosphorylation, platelets were treated with the $\alpha_{IIb}\beta_3$ inhibitor tirofiban 0.4 µM for 10 minutes before stimulation. Phosphorylation of Src (Tyr416), Lyn (Tyr397 and Tyr507), PLC $\beta_3$ (Ser537), Akt (Ser473), Syk (Tyr525/526) PKC substrate, PECAM1 (Tyr686 and Ser702)$^{28}$, was analyzed by Western Blotting as described previously.$^{10}$ For details see the Supplemental Data.

**cAMP and cGMP production**
cAMP production in response to Iloprost (100 ng/ml) and cGMP production in response to the nitric oxide (NO)-donor s-nitroso-n-acetyl penicillamine (SNAP, 0.1-1-10 µM) was assessed using the cAMP Enzymeimmunoassay Biotrak (EIA) System dual range and the cGMP Enzymeimmunoassay Biotrak (EIA) System dual range (Amersham, GE Healthcare, Milan, Italy).
RESULTS

Platelet aggregation and shape change are defective in PT-VWD

The aggregation of human PT-VWD platelets was strongly impaired in response to TRAP-6, defective in response to collagen, convulxin and arachidonic acid and lacking the second wave in response to ADP and epinephrine (Figure 1A, Supplementary figure S2). Given that the analysis of LTA curves suggested a shape change defect, LTA was repeated after pre-incubation with RGDS, which prevents aggregation thus allowing a more thorough observation of the very initial phase of the platelet activation response to stimuli, and indeed shape change turned out to be significantly reduced in response to all agonists (Figure 1B).

Platelet αIIbβ3 activation and granule secretion are defective in PT-VWD

PAC-1 binding to human PT-VWD platelets (Figure 2A, Supplementary Figure S3A) and JON/A binding to TgG233V mouse platelets (Figure 2B) were impaired in response to all tested stimuli. PAC-1 and JON/A binding in resting conditions did not differ between control and PT-VWD platelets and between TgWT and TgG233V platelets suggesting that the mildly increased volume of PT-VWD and TgG233V platelets did not influence flow cytometry results.

The defect of second wave aggregation suggested a granule secretion defect. Granule content, evaluated by transmission electron microscopy, was normal in human PT-VWD platelets compared to controls (α-granules: 5.8±0.4 vs 6.4±1.3/platelet; dense granules: 0.7±0.4 vs 0.9±0.3/platelet, p=ns) (Figure 2C), while secretion of both α- and dense- granules was defective with both human PT-VWD and murine TgG233V platelets, as shown by impaired agonist-induced platelet surface P-selectin expression (Figure 2D and 2E, Supplementary Figure S3B) and mepacrine release (Figure 2F and 2G). P-selectin expression (Figure 2C) and mepacrine incorporation did not differ between control and PT-VWD resting platelets (mepacrine incorporation: 85.9±9 % vs 94.7±4%, p=ns) and between TgWT and TgG233V platelets (mepacrine incorporation: 76.5±14.2 % vs 84.3±11.3%, p=ns), here too suggesting that the mildly increased volume of PT-VWD and TgG233V platelets did not affect flow cytometry results. Platelet surface expression of αIIbβ3 and GPIb/IX/V receptors was comparable between PT-VWD and control platelets (data not shown).

Spreading and adhesion under flow conditions are defective in PT-VWD
Platelet spreading on fibrinogen and type I collagen was defective both with human PT-VWD (Figure 3A and 3B) and murine Tg$^{G233V}$ platelets (Figure 3C) platelets as compared with controls. Also, total surface area covered by platelets upon perfusion of blood over a collagen-coated surface was significantly reduced as compared with controls, both at high ($\alpha_{IIb}\beta_3$-dependent) and low ($\alpha_{IIb}\beta_3$-independent) shear rates (Figure 3D, 3E).\textsuperscript{29}

Given that granule secretion, shape change, platelet spreading on type I collagen and adhesion at low shear rate do not depend on $\alpha_{IIb}\beta_3$ activation, a global platelet function defect in PT-VWD was suspected, therefore signal transduction mechanisms were explored.

**Platelet Ca$^{2+}$ mobilization and Rap-1b activation are impaired in PT-VWD**

We first studied cytoplasmic free calcium movements which are involved in shape change, adhesion, aggregation and the release of platelet granules\textsuperscript{30}, all processes defective in PT-VWD. Indeed, ADP-, TRAP-6- and CVX-induced calcium mobilization was impaired in human PT-VWD platelets (Figure 4A). The activation of the small GTPase Rap1b, which is Ca$^{2+}$-mediated\textsuperscript{31} and is required for $\alpha_{IIb}\beta_3$ activation, was also impaired in response to ADP and thrombin in human PT-VWD (Figure 4B) and Tg$^{G233V}$ (Supplementary Figure S4A) platelets. Phosphorylation of PLC$\beta_3$, activator of Rap1b\textsuperscript{32}, was comparable between PT-VWD and control platelets upon activation with ADP and thrombin (Supplementary Figure S4B).

SFKs are crucial steps in the pathways leading to the cytoplasmic Ca$^{2+}$ increase, and reciprocally, Ca$^{2+}$ rises modulate SFKs activation and activate Rap1b.\textsuperscript{32,33} Another key pathway regulating Rap1b is triggered by PKC activation\textsuperscript{34} thus we explored SFKs and PKC activation.

**Src-family kinases, Lyn and PECAM1 are constitutively activated in PT-VWD platelets**

SFKs get phosphorylated upon agonist binding to a wide repertoire of platelet surface receptors and thus play a central role in transducing activatory signals\textsuperscript{35}, therefore we assessed their phosphorylation. Phosphorylation of SFKs was markedly enhanced in resting PT-VWD and Tg$^{G233V}$ platelets compared to controls (Figure 5A, Supplementary Figure S5A). Interestingly, control and Tg$^{WT}$ platelets pre-incubated with ristocetin, which triggers the binding of VWF to GPIb$\alpha$, showed enhanced SFKs phosphorylation (Figure 5A, Supplementary Figure S5A). We focused on Lyn because it is the member of SFK that besides playing a role in platelet activatory signaling also triggers a signaling which dampens platelet activation by phosphorylating immunotyrosine-based inhibitory motif (ITIM)–containing receptors\textsuperscript{35}. Lyn was phosphorylated at Tyr397, which means
activated, in resting PT-VWD and Tg^{G233V} but not in control platelets (Figure 5B, Supplementary Figure S5B). Interestingly, here too ristocetin triggered the phosphorylation of Lyn at Tyr397 of control and Tg^{WT} platelets. Accordingly, the negative regulatory site of Lyn, Tyr507, was less phosphorylated in PT-VWD and Tg^{G233V} platelets than in control and Tg^{WT} platelets, while the incubation with ristocetin triggered de-phosphorylation at Tyr507 of control platelets (Figure 5C, Supplementary Figure S5C). Moreover, the ITIM-containing platelet receptor PECAM1, a substrate of Lyn^{36}, was phosphorylated at Tyr686 and Ser702 (that is phosphorylated after residue 686), thus activated, in resting PT-VWD and Tg^{G233V} but not in control platelets. Here too, ristocetin triggered the phosphorylation of PECAM1 of control platelets and Tg^{WT} platelets (Figure 5D and 5E, Supplementary Figure S5D and S5E).

Upon platelet stimulation, SFKs and Lyn (Tyr397) phosphorylation was only mildly increased in PT-VWD platelets while it was markedly increased in control platelets (Supplementary Figure S6A and S6B). On the contrary, Lyn phosphorylation at Tyr507 was significantly increased in activated PT-VWD platelets compared to control platelets (Supplementary Figure S6C). Finally, upon platelet stimulation, phosphorylation of PECAM1 of PT-VWD platelets was comparable to control platelets (Supplementary Figure S6D and S6E).

We then focused on the activation of the main Lyn substrates Akt and Syk. Akt phosphorylation was comparable in resting PT-VWD and control platelets, while it was diminished in PT-VWD platelets upon stimulation with ADP and CVX (Supplementary Figure S7). Syk phosphorylation was comparable both in resting and in stimulated PT-VWD and control platelets (Supplementary Figure S7).

On the other hand, PKC substrate phosphorylation was increased in resting PT-VWD platelets. Here too, ristocetin triggered the phosphorylation of PKC substrates of control platelets (Figure 5F, Supplementary Figure S5F). Upon platelet stimulation, PKC substrate phosphorylation was only mildly increased in PT-VWD platelets while it was increased twofold in control platelets, with a significant difference between control and PT-VWD platelets (Supplementary Figure S6F).

cAMP and cGMP production were comparable between control and PT-VWD platelets (Supplementary Figure S8).

DISCUSSION

The bleeding diathesis of PT-VWD patients has been conventionally attributed to the clearance of platelet-VWF complexes from the circulation.\(^1\) Recently we showed that defective proplatelet formation and ectopic platelet release by megakaryocytes in the bone marrow contribute to
thrombocytopenia in PT-VWD. However, the mild to moderate thrombocytopenia of PT-VWD does not seem sufficient to explain a bleeding phenotype that sometimes may be severe, especially after surgery and delivery. Previous studies in Tg<sup>G233V</sup> mice showed defective fibrinogen binding, decreased pro-coagulant activity, delayed aggregation in response to ADP and thrombin and impaired in vivo thrombus formation, suggesting platelet dysfunction in these mice. Similarly, studies on platelets from a patient with type-2B von Willebrand disease (2B-VWD), which is the VWF counterpart of PT-VWD, and from 2B-VWD mice, both carrying the p.V1316M VWF variant, showed that the constitutive binding of mutant VWF to platelets causes impaired platelet function with reduced α<sub>IIbβ<sub>3</sub> activation contributing to the bleeding tendency of 2B-VWD. However, no studies on platelet function in PT-VWD patients have ever been performed. Here we show that a remarkable platelet function defect is present in PT-VWD patients and we unravel that it is caused by the constitutive triggering of platelet inhibitory signal transduction negatively regulating platelet activation through hyper-activation of Lyn and PECAM1.

Our study focused on two different well known GP1BA gain of function variants: p.M239V, carried by a PT-VWD patient, and p.G233V, expressed in PT-VWD mice. PT-VWD platelets showed defective α<sub>IIbβ<sub>3</sub> activation and consequently impaired platelet aggregation, spreading on fibrinogen and adhesion to collagen at high shear rates, all processes dependent on α<sub>IIbβ<sub>3</sub> activation. Moreover, PT-VWD platelets showed defective α- and δ-granule secretion, shape change and adhesion to collagen at low shear rate, which are all independent from α<sub>IIbβ<sub>3</sub> activation but strictly dependent on Ca<sup>2+</sup> mobilization. Indeed, we found that Ca<sup>2+</sup> mobilization induced by various agonists is defective in PT-VWD platelets. Platelet function was impaired in response to agonists stimulating both G-protein-coupled and ITAM-coupled receptors. In platelets, generation of the second messengers Ca<sup>2+</sup> and DAG leads to the activation of PKC, leading to activation of Rap1b which in turn activates α<sub>IIbβ<sub>3</sub> through the recruitment of Talin<sup>1</sup>. Therefore, we assessed Rap-1b activation and we found it impaired not due to a dysfunction of its main activator PLC<sub>β3</sub>, but to a defective PKC pathway, that contributes to activate it. This finding, together with our finding that ristocetin triggers PKC substrate phosphorylation in control platelets, suggests that the enhanced VWF-GP<sub>IIbβα</sub> interaction leads to activation and subsequent exhaustion of the PKC pathway, as recently described in 2B-VWD platelets, and this in turn reduces Rap-1b activation causing α<sub>IIbβ<sub>3</sub> dysfunction. The binding of VWF to GPIbα activates PKC through a signaling cascade that involves Lyn, a SFK that we found to be activated in resting PT-VWD platelets. Thus, it can be hypothesized that the cause of
baseline PKC upregulation and the relative refractoriness of this pathway to stimulation is Lyn activation. In addition, activation of Lyn negatively regulates Ca\(^{2+}\) flux, and consequently platelet activation, through the activation of phosphatases \(^{47}\).

Altogether, our data show a global defect of platelet function in PT-VWD suggestive of the triggering of a negative feedback regulatory system. The binding of VWF to the extracellular region of GPIb\(\alpha\) induces the association of Src and Lyn with the cytoplasmic tail of GPIb\(\alpha\) starting the downstream phosphorylation of a number of substrates, like PI3K, Akt, p38, Syk and PLC, involved in inside-out signaling leading to Rap-1b, Talin-1 and thus \(\alpha_{\text{IIb}\beta_3}\) activation.\(^{6,35,48}\) However, in activated platelets Lyn also initiates a negative feedback regulatory pathway by phosphorylating PECAM1 providing docking sites for SH2 domains of phosphatases, such as the tyrosine phosphatases SHP1/SHP2 and the inositol phosphatase SHIP1/SHIP2, that mediate termination of platelet activation signals\(^{36,40-54}\).

Here, we show that Lyn and PECAM1 are activated in resting PT-VWD platelets identifying, to the best of our knowledge, the first platelet function defect due to hyper-activation of Lyn and PECAM1 in humans. cAMP and cGMP production were comparable between control and PT-VWD platelets indicating that these two negative platelet regulatory pathways are not involved in the platelet dysfunction of PT-VWD. Moreover, we show that Lyn and PECAM1 get activated in control platelets stimulated with ristocetin, confirming that the binding of VWF to GPIb\(\alpha\) phosphorylates Lyn and PECAM1\(^{35,54}\).

In support to our findings is the observation that platelets from PECAM1-knock-out, Lyn-knock-out, or PECAM1/Lyn double-knock-out mice are hyper-responsive to stimuli, confirming that PECAM1 and Lyn suppress platelet reactivity\(^{36,51,53,54}\). To assess whether the blockade of Lyn could restore normal platelet reactivity, we measured granule secretion and \(\alpha_{\text{IIb}\beta_3}\) activation by control and PT-VWD platelets after incubation with the Lyn inhibitor bafetinib, however bafetinib, suppressing the platelet activating function of Lyn, inhibited \(\alpha_{\text{IIb}\beta_3}\) activation and granule secretion\(^{35}\) (Supplementary Figure S9).

Conversely, human and murine platelets in which PECAM1 activation was triggered by a PECAM1 homophilic ligand\(^{50}\) or by cross-linking antibodies\(^{51,52}\) showed decreased aggregation,\(^{50,51}\) secretion,\(^{51,52}\) Ca\(^{2+}\) mobilization\(^{51,52}\) and fibrinogen binding\(^{52}\), all functions that we found to be impaired in PT-VWD platelets. Indeed, PECAM1 has been recently identified as a potential novel target for antiplatelet therapy.\(^{55}\) Interestingly, PECAM1-knockout mice also show reduced
trabecular bone volume, increased number of osteoclasts and enhanced bone resorption\textsuperscript{56} while PT-VWD mice show enhanced trabecular bone volume, decreased number of osteoclasts and decreased bone resorption, a finding so far unexplained and possibly related to the role of PECAM1 as a negative regulator of osteoclastogenesis.\textsuperscript{12}

When we assessed the activation of Lyn substrates, namely Akt and Syk, we found decreased Akt phosphorylation after stimulation with ADP and convulxin in PT-VWD platelets. The same Akt dysfunction was previously reported in 2B-VWD platelets\textsuperscript{48} and is in line with the key role played by Akt in GPIb/IX/V-mediated \(\alpha_{IIb}\beta_3\) integrin-dependent adhesion, spreading, and aggregation.\textsuperscript{57}

In conclusion, our study shows that the constitutive binding of VWF to GPIb\(\alpha\) in PT-VWD platelets triggers deranged signaling leading to the inhibition of \(\alpha_{IIb}\beta_3\) activation, defective secretion and impaired platelet adhesion, altogether generating a platelet function defect that may significantly contribute to the bleeding phenotype of these patients. This mechanism may also account for the worsening of the bleeding diathesis of PT-VWD patients in conditions associated with an increase of circulating VWF, and therefore of its enhanced binding to GPIb\(\alpha\), such as surgery or pregnancy\textsuperscript{35-37}. Our data obtained with platelets carrying two different GP1BA gain of function variants suggest that the platelet function defect in PT-VWD is independent from the type of GP1BA variant and is due to the enhanced affinity of GPIb\(\alpha\) for VWF. However, it should be noted that the variants we studied affect the same domain of GPIb\(\alpha\), therefore we can not predict the effects of variants in other domains on platelet function.\textsuperscript{58} A similar mechanism for platelet dysfunction may play a role in the platelet dysfunction of 2B-VWD. Indeed, results of studies with human and murine 2B-VWD platelets carrying the p.V1316M VWF variant are similar to results of the present study. Both disorders share the same platelet function defect, characterized by \(\alpha_{IIb}\beta_3\) dysfunction, \(\alpha\)- and \(\delta\)-granule secretion defect, defective \(\text{Ca}^{2+}\) signalling and adhesion under flow conditions.\textsuperscript{5,42} Defective Rap1b and Akt activation and PKC upregulation with consequent desensitization have been shown for both disorders\textsuperscript{43}, together with a dysregulation of the RhoA pathway.\textsuperscript{59} It would be interesting to assess Lyn and PECAM1 phosphorylation in 2B-VWD platelets to check whether the same negative-feedback loop downregulating platelet activation here described is present in 2B-VWD.

Finally, our results imply that inhibitors of PECAM1 might be explored to restore platelet function in PT-VWD. However, the PECAM1 blockers currently available are antibodies that block PECAM1 adhesive interactions\textsuperscript{60-63} and not PECAM1 phosphorylation or downstream signaling, therefore do not allow to assess whether PECAM1 blockade might restore normal platelet function.
The development of PECAM1 inhibitors selectively suppressing intracellular phosphorylation might represent a novel approach to the antihemorrhagic treatment of PT-VWD.
REFERENCES


FIGURE LEGENDS

Figure 1: Platelet aggregation and shape change are defective in PT-VWD
A) Human platelet aggregation in response to TRAP-6 20 μM (i), ADP 10 μM (ii) and collagen 1 mg/ml (iii) in platelet-rich plasma from the PT-VWD patient and a parallel healthy control. Traces are representative of 4 independent experiments (samples A, C, D, E Supplementary table 1). *=p<0.05 vs control, unpaired Student’s t test.

B) Platelet shape change assessed by LTA after pretreatment with RGDS (120 μg/ml) in response to TRAP-6 2 μM (i), ADP 10 μM (ii) and collagen 1 mg/ml (iii) in platelet-rich plasma from the PT-VWD patient and a parallel healthy control. Traces are representative of 4 independent experiments (samples A, C, D, E Supplementary table 1) *=p<0.05 vs control, unpaired Student’s t test.

Figure 2: αIIbβ3 activation and granule secretion are defective in PT-VWD
A) Integrin αIIbβ3 activation (PAC-1 binding) as assessed by flow cytometry in response to TRAP-6 20 μM (i), ADP 10 μM (ii) and CVX 20 ng/ml (iii) in human whole blood from the PT-VWD patient and a parallel healthy control. PAC-1 binding is reported as % of positive platelets, calculated as the % of platelets (gated for their forward scatter and side scatter values and for their positivity to the platelet marker CD42b) that bound PAC-1 over the total platelet population after setting of aspecific binding. Data are means ± SEM from at least 5 independent experiments (samples A, B, C, D, E Supplementary table 1). **=p<0.01 vs control, Two-way ANOVA.

B) Integrin αIIbβ3 activation (JON-A binding) as assessed by flow cytometry in response to thrombin 0.05 U/ml + GPRP 2mM and convulxin 25 ng/ml in murine whole blood from TgG233V and TgWT mice. Jon-A binding is reported as % of positive platelets, calculated as the % of platelets (gated for their forward scatter and side scatter values and for their positivity to the platelet marker CD42b) that bound Jon-A over the total platelet population after setting of aspecific binding. Data are means ± SEM, n=5 (TgWT: 3 females and 2 males, mean platelet count: 627850±21566/μl; TgG233V: 3 females and 2 males; mean platelet count: 330600±17677/μl). **=p<0.01 vs TgWT, Two-way ANOVA.

C) Representative images of the ultrastructure of human control and PT-VWD platelets used to assess granular content (original magnification 13000X). Specimens were observed with a Philips Electron Optics EM208 transmission electron microscope at 80 kV at room temperature. Granules
were counted from at least 50 platelets (α-granules: 5.8±0.4 vs 6.4±1.3/platelet; dense granules: 0.7±0.4 vs 0.9±0.3/platelet, p=ns). Black arrowheads indicate examples of α-granules, white arrowheads indicate examples of dense granules. Dense granules were defined by their strongly electron-dense core surrounded by a clear space enclosed by a single membrane. 

D-E] α-granule secretion in human (D) and murine (E) platelets as assessed by the measurement of P-selectin expression by flow cytometry. Agonists were ADP 10 μM, TRAP6 μM or CVX 20 ng/ml for human platelets and thrombin 0.05U/ml + GPRP 2mM or CVX 25 ng/ml for murine platelets. P-selectin expression is reported as % of positive platelets, calculated as the % of platelets (gated for their forward scatter and side scatter values and for their positivity to the platelet marker CD41) that bound CD62P over the total platelet population after setting of aspecific binding. Data are means ± SEM from at least 5 independent experiments (samples A, B, C, D, E Supplementary table 1) or from n=5 mice (TgWT: 3 females and 2 males, mean platelet count: 627850±21566/μl; TgG233V: 3 females and 2 males; mean platelet count: 330600±17677/μl). ** p< 0.01; Two-way ANOVA.

F-G] Dense granule secretion in human (F) and murine (G) platelets as assessed by the measurement of mepacrine release by flow cytometry. Mepacrine release was calculated by the following formula: 1-(residual mepacrine content after thrombin stimulation with 0.05 U/ml / total mepacrine incorporated)x100. “Total mepacrine incorporated” is the % of platelets (gated for their forward scatter and side scatter values and for the positivity to the platelet markers CD41 or CD42b) that incorporated mepacrine over the total platelet population after setting of aspecific binding; “residual mepacrine content” is the % of platelets stimulated with 0.05 U/ml of thrombin that incorporated mepacrine over the total platelet population after setting of aspecific binding. Data are means ± SEM from at least 5 independent experiments (samples A, B, C, D, E Supplementary table 1) or from n=5 mice (TgWT: 3 females and 2 males, mean platelet count: 627850±21566/μl; TgG233V: 3 females and 2 males; mean platelet count: 330600±17677/μl). * p< 0.05, ** p< 0.01; unpaired Student’s t test.

Figure 3: Platelet spreading and adhesion under flow conditions are defective in PT-VWD

A] Representative images of human control and patient (PT-VWD) platelets 30 minutes after layering onto fibrinogen or collagen. Scale bar is 10 μm. Platelets were stained with FITC-conjugated phalloidin and analyzed using a Carl Zeiss Axio Observer.A1 fluorescence microscope.
(Carl Zeiss Inc., Oberkochen, Germany) with a 100x/1.4 Plan-Achromat oil-immersion objective at room temperature.

**B-C** Human (B) and murine (C) platelet spreading on glass coverslips coated with fibrinogen (100 μg/ml) or type I collagen (25 μg/ml). Spreading is expressed as % of surface covered by platelets. Data are means ± SEM from 5 independent experiments (samples A, B, C, D, E **Supplementary table 1**) or from n=5 mice (Tg WT: 2 females and 3 males, mean platelet count: 661600±99984/μl; Tg G233V: 2 females and 3 males; mean platelet count: 305000±26728/μl). **p<0.01; Two-way ANOVA.** The number of platelets adhered to the surface was comparable between control and PT-VWD platelets (number of platelets adhering to fibrinogen per microscopic field: controls 93±27, PT-VWD 87±18, p=ns; number of platelets adhering to collagen per microscopic field: controls 78±17, PT-VWD 83±12, p=ns) and between Tg WT and Tg G233V platelets (number of platelets adhering to fibrinogen per microscopic field: Tg WT 101±21, Tg G233V 105±24, p=ns; number of platelets adhering to collagen per microscopic field: Tg WT 98±9, Tg G233V 91±13, p=ns).

**D-E** Human (D) and murine (E) platelet adhesion to type I collagen under flow conditions. Whole blood was perfused at different shear rates in glass microcapillary tubes coated with type I collagen (30 μg/cm²). Specimens were observed under an optical microscope after fixation with 0.25% glutaric-dialdehyde and May-Grünwald/Giemsa staining. The total surface covered by platelets was calculated with the ImageJ software. Data are means ± SEM of 3 independent experiments (samples B, D, F **Supplementary table 1**) (D) or from n=3 mice (Tg WT: 3 males, mean platelet count: 631550±21142/μl; Tg G233V: 3 males; mean platelet count: 350800±94469/μl) (E). *p<0.05, **p<0.01; Two-way ANOVA.

**Figure 4: Platelet Ca^{2+} mobilization and Rap-1b activation are defective in PT-VWD**

**A** Changes in cytosolic free Ca^{2+} triggered by TRAP-6 10μM, ADP 5μM and CVX 50 ng/ml, as assessed in FLUO 3-AM (8 μM)-labeled platelets. After the measurement of baseline fluorescence of PRP samples, agonists were added and changes in green fluorescence were recorded in function of time. The graphs show data from one representative experiment. Points represent the ratio respect to baseline mean fluorescence intensity (MFI). The columns show maximal Ca^{2+} rise after stimulation (fluorescence ratio) with different agonists. Data are means ± SEM from 4 independent experiments (samples D, E, F, H **Supplementary table 1**) (p<0.05; unpaired t-test).

**B** Rap1b activation (Rap1b-GTP) of human control and patient (PT-VWD) platelets after stimulation with thrombin (0.1 U/ml) or ADP (10 μM) under stirring conditions was assessed by
Western Blotting by loading 500 µg of proteins. Densitometric analysis was performed using the Image J software. Quantification of Rap1b-GTP is relative to total Rap1b expression and is expressed in arbitrary units (AU). Data are means ± SEM from 3 independent experiments (samples B, D, F Supplementary table 1) (*p<0.05; Two-way ANOVA).

Figure 5: SFKs, Lyn and PECAM-1 are constitutively phosphorylated in PT-VWD platelets

A) SFKs phosphorylation at Tyr 416 in human control and patient (PT-VWD) washed platelets unstimulated (-) or stimulated with ristocetin (0.3 mg/ml) for 30 sec under stirring conditions, was assessed by Western blotting by loading 30 µg of proteins. Densitometric analysis of the pSFK/total SFK ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means ± SEM from 6 independent experiments (samples C, D, E, F, G, H Supplementary table 1) (*p<0.05 vs control; # p<0.05 vs resting; Two-way ANOVA). The bands shown for resting platelets and platelets stimulated with ristocetin belong to the same gel.

B) Lyn phosphorylation at Tyr397 in human control and patient (PT-VWD) washed platelets unstimulated (-) or stimulated with ristocetin (0.3 mg/ml) for 30 sec under stirring conditions, was assessed by Western blotting by loading 30 µg of proteins. Densitometric analysis of the pLyn/total Lyn ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means ± SEM from 6 independent experiments (samples C, D, E, F, G, H Supplementary table 1) (*p<0.05 vs control; # p<0.05 vs resting; Two-way ANOVA). The bands shown for resting platelets and platelets stimulated with ristocetin belong to the same gel.

C) Lyn phosphorylation at Tyr507 in human control and patient (PT-VWD) washed platelets unstimulated (-) or stimulated with ristocetin (0.3 mg/ml) for 30 sec under stirring conditions, was assessed by Western blotting by loading 30 µg of proteins. Densitometric analysis of the pLyn/total Lyn ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means ± SEM from 3 independent experiments (samples F, G, H Supplementary table 1) (*p<0.05 vs control; # p<0.05 vs resting; Two-way ANOVA). The bands shown for resting platelets and platelets stimulated with ristocetin belong to the same gel.

D) PECAM1 phosphorylation at Tyr686 in human control and patient (PT-VWD) washed platelets unstimulated (-) or stimulated with ristocetin (0.3 mg/ml) for 30 sec under stirring conditions, was assessed by Western blotting by loading 30 µg of proteins. Densitometric analysis of the pPECAM1/total PECAM1 ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means ± SEM from 6 independent experiments (samples C, D, E, F, G,
H Supplementary table 1) (*p<0.05 vs control; # p<0.05 vs resting; Two-way ANOVA). The bands shown for resting platelets and platelets stimulated with ristocetin belong to the same gel.

E) PECAM1 phosphorylation at Ser702 in human control and patient (PT-VWD) washed platelets unstimulated (-) or stimulated with ristocetin (0.3 mg/ml) for 30 sec under stirring conditions, was assessed by Western blotting by loading 30 µg of proteins. Densitometric analysis of the pPECAM1/total PECAM1 ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means ± SEM from 3 independent experiments (samples F, G, H Supplementary table 1) (*p<0.05 vs control; # p<0.05 vs resting; Two-way ANOVA). The bands shown for resting platelets and platelets stimulated with ristocetin belong to the same gel.

F) PKC substrate phosphorylation in human control and patient (PT-VWD) washed platelets unstimulated (-) or stimulated with ristocetin (0.3 mg/ml) for 30 sec under stirring conditions, was assessed by loading 30 µg of proteins. Densitometric analysis of the PKC substrate/actin ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means ± SEM from 4 independent experiments (samples E, F, G, H Supplementary table 1) (*p<0.05 vs control; # p<0.05 vs resting; Two-way ANOVA). The bands shown for resting platelets and platelets stimulated with ristocetin belong to the same gel.
Figure 1

A

PLATELET AGGREGATION

i
TRAP-6

![Graph showing TRAP-6 aggregation with control and PT-VWD groups.]

ii
Collagen

![Graph showing collagen aggregation with control and PT-VWD groups.]

iii
ADP

![Graph showing ADP aggregation with control and PT-VWD groups.]

B

PLATELET SHAPE CHANGE

i
TRAP-6+RGDS

![Graph showing TRAP-6+RGDS shape change with control and PT-VWD groups.]

ii
Collagen+RGDS

![Graph showing collagen+RGDS shape change with control and PT-VWD groups.]

iii
ADP+RGDS

![Graph showing ADP+RGDS shape change with control and PT-VWD groups.]

* Indicates statistical significance.
Figure 4

A

TRAP-6

Control

PT-VWD

ADP

Control

PT-VWD

CVX

Control

PT-VWD

Maximal Ca²⁺ rise (fluorescence ratio)

Maximal Ca²⁺ rise (fluorescence ratio)

B

Control

PT-VWD

Rap1b-GTP

Rap1b

Rap1b-GTP (AU)

Rap1b-GTP (AU)

Thr 0.1 U/ml

- +

ADP 10 mM

- +
SUPPLEMENTARY METHODS

The terms control and PT-VWD along this manuscript refer to human platelets while the respective terms used for murine platelets are TgWT and TgG233V. All human and animal studies were approved by the responsible Institutional review boards (human: CEAS Umbria, approval number 2663/15; animal: Italian Ministry of Public Health authorization number 561/2015-PR).

Human studies and blood sampling
The PT-VWD patient carrying the M239V GP1BA variant (to our knowledge the only patient diagnosed in Italy so far) was previously reported and presented the typical PT-VWD phenotype with mild macrothrombocytopenia, enhanced ristocetin induced platelet agglutination (RIPA), increased VWF-binding to platelets,

9,10 and mucocutaneous bleeding with an ISTH-BAT score of 11. Each experiment was repeated from 3 to 5 times from blood samples collected on different days (in the text indicated as independent experiments, 8 in total). Age-and sex-matched healthy controls were studied in parallel. Given that the degree of thrombocytopenia typically varies in time in PT-VWD patients2, platelet counts and mean platelet volume (MPV) at the various blood samplings are reported under Supplementary Table 1 and samples used in each experiment are indicated under figure legends.

To minimize platelet activation, blood was taken without tourniquet using a 21 gauge needle after discarding the first 2 ml in trisodium citrate (3.2%, 0.109M, 1/10 v/v) tubes except for platelet counting, MPV and electron microscopy for which it was taken in EDTA tubes.

Mouse strains and blood sampling
The generation of PT-VWD mice expressing the human GP1BA transgene carrying the p.G233V variant (TgG233V) and of control mice expressing a wild-type human GP1BA transgene (TgWT) was previously described.4,12 These animals express either normal or mutant human GPIbα (TgWT and TgG233V) and no mouse GPIbα, and have been consistently backcrossed with C57BL/6J animals. Mice expressing the G233V GP1BA variant have macrothrombocytopenia, prolonged tail bleeding time and increased VWF-binding to platelets (Supplementary Figure 1).5,10,12 Mice were bred and housed in the animal facility of the University of Perugia, Perugia, Italy, and all experiments were performed with 3 to 6 months-old mice. The number of males and females and platelet counts are reported under each figure legend.

To minimize platelet activation blood was collected by cardiac puncture using a 21 gauge needle in a syringe containing trisodium citrate (3.2%, 0.109M, 1/10 v/v) from mice anesthetized with xylazine (5 mg/kg) and ketamine (50 mg/kg) injected intraperitoneally13.

Light transmission aggregometry (LTA)
Platelet-rich plasma (PRP) was obtained by centrifugation of citrated human blood at 160g for 10 minutes. PRP was stimulated with ADP 10 μM, epinephrine 100 μM, TRAP-6 20 μM, arachidonic acid 1mM, collagen 1 mg/ml, convulxin (CVX) 5 μg/ml and aggregation was monitored by LTA using a four-channel APACT4 aggregometer (Helena Biosciences, UK).14 For the study of shape change, PRP was pretreated with the αIIbβ3 blocker Arg-Gly-Asp-Ser peptide (RGDS) (120 μg/ml) in order to prevent platelet aggregation and then stimulated with ADP 10 μM, TRAP-6 2 μM or collagen 1 mg/ml and shape change was monitored by LTA by magnifying the length of the y and
x axes through the APACT4 software. The shape change reaction speed was measured by the slope of the tangent to the steepest part of the curve in mm per minute.\textsuperscript{15,16}

\( \alpha_{IIb} \beta_3 \) activation

Five μl of human or murine citrated blood were diluted with 100 μl of PBS and incubated with a stimulus for 15 minutes and with saturating concentrations of antibodies recognizing the active conformation of \( \alpha_{IIb} \beta_3 \): FITC-conjugated mouse PAC-1 MoAb (BD Bioscience, Franklin Lakes, NJ, USA) for human platelets and PE-conjugated JON/A MoAb (Emfret Analytics, Eibelstadt, Germany) for murine platelets. A range of agonists were used as stimuli including ADP 10 μM, TRAP6 20 μM or CVX 20 ng/ml, rhodocytin 10 nM for human platelets and thrombin 0.05U/ml + the coagulation-blocking peptide Gly-Pro-Arg-Pro (GPRP) 2 mM or CVX 25 ng/ml for murine platelets. ADP was not used to stimulate murine platelets because it provides a weak and unpredictable aggregation response.\textsuperscript{17} Antibodies were added to the samples together with the agonist. PE-conjugated anti-CD42b (Beckman Coulter Italia, Milan, Italy) and FITC-conjugated anti-GPIX (Emfret Analytics) were used to gate for human and murine platelets, respectively.

To define negative staining samples were incubated with an isotype control that was a FITC-labelled mouse IgM antibody.

Samples were analyzed in a CytoFLEX or in a Cytomics FC500 flow cytometer (Coulter Corporation, Miami, Florida, USA) equipped with an argon laser operating at 488 nm.\textsuperscript{14,18}

Granule content and secretion

Platelet granule content was assessed by electron microscopy. Platelets were fixed for 4 h at 4°C using cacodylate buffer containing 4% wt/vol of glutaraldehyde, then washed and kept in cacodylate buffer for 4 h. The buffer was then replaced with 1% osmic acid and the samples were pelleted by centrifugation at 10,000xg for 30 sec. Ultrathin sections of the pellets were stained with uranyl acetate and lead citrate and observed under a Philips Electron Optics EM208 transmission electron microscope at 80 kv.\textsuperscript{10,14} At least 50 platelets were analyzed.

Alpha-granules secretion was measured as P-selectin expression,\textsuperscript{19} by incubating 5 μl of human or murine citrated whole blood diluted with 100 μl of PBS with different agonists and saturating concentrations of a FITC-conjugated anti P-selectin antibody (anti-CD62P from Beckman Coulter for human and from Emfret Analytics for murine platelets) for 15 minutes in the dark. Agonists used were ADP 10 μM, TRAP6 20 μM or CVX 20 ng/ml, rhodocytin 10 nM for human platelets and thrombin 0.05 U/ml + GPRP 2mM or CVX 20 ng/ml for murine platelets.\textsuperscript{20,21} PE-conjugated anti-CD41 antibodies (Beckman Coulter for human and Emfret Analytics for murine platelets) were used to gate for platelets. To define negative staining samples were incubated with an isotype control that was a FITC-labelled mouse IgG1 antibody.

To assess dense granule content and release, the green fluorescent dye mepacrine, which accumulates specifically in dense granules, was used.\textsuperscript{22} Five μl of blood were added to 100μl of PBS in the presence of a PE-labeled anti CD41 MoAb to gate for platelets (Beckman Coulter for human and Emfret Analytics for murine platelets) and of mepacrine 5μM (Sigma, Darmstadt, Germany) in polypropylene tubes. To define negative staining samples were incubated with a PBS-CD41 solution.

After 30 min of incubation in the dark, samples were diluted in PBS, thrombin 0.05 U/ml was added and after 5 min samples were analyzed by flow cytometry in a CytoFLEX or in a Cytomics
Mepacrine release was calculated by the following formula: 1-(residual mepacrine content after thrombin stimulation with 0.05 U/ml / total mepacrine incorporated) x100.

**Platelet washing**
Human PRP or murine PRP, the latter obtained by centrifugation of citrated murine blood for 10 minutes at 120xg, were washed according to the Mustard method, as described.

**Spreading assay**
Washed platelets (2x10^6/ml) resuspended in Tyrode’s buffer were layered onto glass slides coated with 100 µg/ml of human fibrinogen or 25 µg/ml of human type I collagen for 30 minutes at room temperature. After one washing with PBS, attached platelets were fixed with 3.7% PFA, permeabilized with 0.1% Triton-X100, stained with FITC-conjugated phalloidin (Life Technologies) and analyzed by fluorescence microscopy using a Carl Zeiss Axio Observer.A1 fluorescence microscope (Carl Zeiss Inc., Oberkochen, Germany) with a 100x/1.4 Plan-Apochromat oil-immersion objective.

Spreading was expressed as the percentage of the total surface area covered by platelets in 20 microscopic fields measured by the ImageJ software (NIH, USA).

**Platelet adhesion under flow conditions**
Citrated human or murine blood was passed through a rectangular parallel plate perfusion chamber over a glass coverslip coated with acid-insoluble fibrillar type I collagen from equine tendon (Mascia-Brunelli, Milan, Italy) (30µg/cm²), at a wall shear rate of 250 s⁻¹, 1500 s⁻¹ and 3000 s⁻¹. The chamber was then perfused with 0.1% bovine serum albumin in PBS to remove all residual blood and the coverslip was harvested, gently washed with 10 mM HEPES, and fixed with 0.25% glutaric-dialdehyde. Attached platelets were stained with May-Grünwald/Giemsa and observed under an optical microscope. The area covered by platelets was measured by the ImageJ software (NIH, USA).

**Measurement of intracellular calcium (Ca^{2+})**
Platelets were loaded with the Ca^{2+}-sensitive dye FLUO 3-acetoxymethyl ester (FLUO 3-AM; Molecular Probes) by incubating PRP with FLUO 3-AM 8 µM for 20 minutes at 37°C, and Ca^{2+} mobilization induced by various agonists (TRAP-6 10µM, ADP 5µM and CVX 50 ng/ml) was assessed by flow cytometry in a CytoFLEX flow cytometer (Coulter Corporation). Baseline fluorescence was measured, then agonists were added and changes in green fluorescence were recorded for 300 seconds.

**Rap-1b pull-down assay**
Unstirred washed platelets were stimulated for 30 seconds at room temperature with ADP 10 µM, thrombin 0.1 U/ml or their vehicle. Platelets were centrifuged at 10,000g for 30 seconds and lysed in protein extraction buffer (Tris 40mM, NaCl 0.3M, EDTA 1mM, Na₃VO₄, NaF, pH 7.4, additioned with 1% NP-40 and 0.1% protease inhibitors) for 30 minutes in ice, centrifuged for 10 minutes at 10,000g and protein concentration was assayed. Rap-1b activity (Rap-1b-GTP) was
assessed using an active Rap-1b pull-down and detection kit (Pierce biotechnology, Rockford, IL, USA). Precipitates were analyzed by immunoblotting, using an anti-Rap-1b mAb and an appropriate peroxidase-conjugated secondary antibody. As loading control, 30 µg of proteins from total lysates were analyzed using the same anti-Rap-1b mAb.

Phosphorylation of signaling proteins

Human washed platelets (300x10^9/L) were stimulated for 30 seconds under continuous stirring with ADP 2 µM, CVX 60 ng/ml, ristocetin 0.3 mg/ml or their vehicle. To exclude αIIbβ3-mediated outside-in signaling as a cause of signaling protein phosphorylation, platelets were treated with the αIIbβ3 inhibitor tirofiban 0.4 µM for 10 minutes before stimulation. Platelets were then centrifuged at 10,000g for 30 seconds and proteins were extracted as above described. 30 µg of proteins were analyzed by immunoblotting using an anti-pSrc (Tyr416, active kinase) mAb, which reacts with all members of the SFK, an anti-pLyn Tyr397 (active kinase), an anti- pLyn Tyr507 (inactive kinase), an anti-pPLC β3 (Ser537, active kinase) mAb, an anti-pAkt (Ser473, active kinase) mAb, an anti-pSyk (Tyr525/526, active kinase) and an anti-p(Ser) PKC substrate Ab (all generated in rabbits and all diluted 1:1000; Cell Signaling Technology, Beverly, MA, USA) or the affinity-purified rabbit polyclonal anti–PECAM1 pY686 and anti–PECAM1 pS702 (both a final concentration of 2 µg/ml) antibodies recognizing PECAM1 phosphorylated on the tyrosine residue at Tyr686 or at Ser702, both detecting PECAM1 activation (kindly given by Prof. Debra K. Newman) and an appropriate peroxidase-conjugated goat-anti-rabbit secondary antibody. Anti-Src, anti-Lyn, anti-PECAM1 and anti-β actin antibodies were used as loading controls (all generated in mice and all diluted 1:1000; Cell Signaling Technology, Beverly, MA, USA).

Western blots were developed with ECL chemiluminescence (Bio-Rad, Segrate, MI, Italy) and band intensities quantified by densitometry, from at least three independent experiments, using the ImageJ software (NIH, USA).
## SUPPLEMENTARY TABLE S1

Platelet count and mean platelet volume of patient’s samples at the different independent experiments

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Supplementary Figure S1: VWF binding to Tg<sup>G233V</sup> platelets is increased

Binding of VWF to PRP from Tg<sup>WT</sup> and Tg<sup>G233V</sup> mice (A) or from controls and the PT-VWD patient (B) induced by increasing doses of ristocetin, as assessed by flow cytometry. Platelet VWF-binding by flow cytometry

The binding of VWF to platelets induced by ristocetin was evaluated in PRP by flow cytometry using a mouse anti-human VWF antibody, clone 4f9 (ImmunoTech, Marseille, France) and a FITC-conjugated goat anti-mouse IgG (Beckman Coulter, Miami, FL, USA), as previously described (Giannini S, Mezzasoma AM, Leone M, Gresele P. Laboratory diagnosis and monitoring of desmopressin treatment of von Willebrand's disease by flow cytometry. Haematologica. 2007;92:1647-1654). Samples were analyzed in a Cytomics FC500 flow cytometer (Beckman Coulter). To allow the measurement of ristocetin-mediated VWF binding to murine platelets, purified human VWF (8 μg/ml) was added to murine PRP before the addition of ristocetin. Data are means ± SEM from 8 independent experiments (samples from A to H, Supplementary table 1) or from n=5 mice (Tg<sup>WT</sup>: 2 females and 3 males, mean platelet count: 669650± 54800/μl; Tg<sup>G233V</sup>: 2 females and 3 males; mean platelet count: 357250± 126218,5604/μl). *=p<0.05 vs 0 mg/ml, Two-way ANOVA.
Supplementary Figure S2

Supplementary Figure S2: Platelet aggregation in response to epinephrine, arachidonic acid and convulxin is defective in PT-VWD

Aggregation in response to arachidonic acid 1mM was: controls 69.2 ± 11.1%, PT-VWD 50.1 ± 6.5% (p<0.05 vs control, unpaired Student’s t test), in response to epinephrine 100 μM was: controls 56.9 ± 12.8%, PT-VWD 55.1 ± 10.2% (p=ns) but second wave was absent, while in response to CVX was: controls 75.1 ± 9.8%, PT-VWD 35.4 ± 10.3% (p<0.05 vs control, unpaired Student’s t test). Traces are representative of 3 independent experiments (samples A, C, D Supplementary table 1).
Supplementary Figure S3

Supplementary Figure S3. α-granule secretion and α_{IIb}β_{3} activation in response to rhodocytin is impaired in PT-VWD.

A) α-granule secretion in human platelets as assessed by the measurement of P-selectin expression by flow cytometry in response to rhodocytin 10 nM in human whole blood from the PT-VWD patient and a parallel healthy control. P-selectin expression is reported as % of positive platelets.

B) Integrin α_{IIb}β_{3} activation (PAC-1 binding) as assessed by flow cytometry in response to rhodocytin 10 nM in human whole blood from the PT-VWD patient and a parallel healthy control. PAC-1 binding is reported as % of positive platelets.
Supplementary Figure S4

A) Rap1b activation (Rap1b-GTP) of murine Tg$^{WT}$ and Tg$^{G233V}$ platelets after stimulation with thrombin (0.1 U/ml) or ADP (10 µM). Densitometric analysis was performed using the Image J software. Quantification of Rap1b-GTP is relative to total RAP1b expression and is expressed in arbitrary units (AU). Data are means ± SEM n=3 (Tg$^{WT}$: 1 female and 2 males, mean platelet count: 623650±51406/µl; Tg$^{G233V}$: 1 female and 2 males; mean platelet count: 466200±139158/µl). *p<0.05; Two-way ANOVA.

B) PLC-β3 phosphorylation (p-PLCβ3) of PT-VWD and control platelets after stimulation with thrombin (0.1 U/ml) or ADP (10 µM). Densitometric analysis was performed using the Image J software. Quantification of p-PLCβ3 is relative to β-actin expression and is expressed in arbitrary units (AU). Data are means ± SEM from 3 independent experiments (samples F, G, H Supplementary table 1) **p<0.01 vs resting; Two-way ANOVA.

Supplementary Figure S4: Rap-1b activation is defective in Tg$^{G233V}$ platelets while PLCβ3 activation is not affected
Supplementary Figure S5: SFKs, Lyn and PECAM-1 are constitutively phosphorylated in Tg^{G233V} platelets

**A** SFKs phosphorylation at Tyr416 in murine Tg^{WT} and Tg^{G233V} washed resting platelets. Densitometric analysis was performed using the Image J software. Quantification of p-SFKs is relative to total SFK expression and is expressed in arbitrary units (AU). Data are means ± SEM from n=3 mice (Tg^{WT}: 3 males, mean platelet count: 651550±11950/μl; Tg^{G233V}: 3 males; mean platelet count: 453850±156624/μl) (*p<0.05 vs Tg^{WT}; **p<0.01; Two-way ANOVA).

**B** Lyn phosphorylation at Tyr397 in murine Tg^{WT} and Tg^{G233V} washed resting platelets. Quantification of p-Lyn is relative to total Lyn expression and is expressed in arbitrary units (AU). Data are means ± SEM from n=3 mice (Tg^{WT}: 3 males, mean platelet count: 651550±11950/μl; Tg^{G233V}: 3 males; mean platelet count: 453850±156624/μl) (*p<0.05 vs Tg^{WT}; Two-way ANOVA).

**C** Lyn phosphorylation at Tyr507 in murine Tg^{WT} and Tg^{G233V} washed resting platelets. Quantification of p-Lyn is relative to total Lyn expression and is expressed in arbitrary units (AU). Data are means ± SEM from n=3 mice (Tg^{WT}: 3 males, mean platelet count: 651550±11950/μl; Tg^{G233V}: 3 males; mean platelet count: 453850±156624/μl) (*p<0.05 vs Tg^{WT}; Two-way ANOVA).

**D** PECAM1 phosphorylation at Tyr686 in murine Tg^{WT} and Tg^{G233V} washed resting platelets. Quantification of p-PECAM1 is relative to total PECAM1 expression and is expressed in arbitrary units (AU). Data are means ± SEM from n=3 mice (Tg^{WT}: 3 males, mean platelet count: 651550±11950/μl; Tg^{G233V}: 3 males; mean platelet count: 453850±156624/μl) (*p<0.05 vs Tg^{WT}; Two-way ANOVA).

**E** PECAM1 phosphorylation at Ser702 in murine Tg^{WT} and Tg^{G233V} washed resting platelets. Quantification of p-PECAM1 is relative to total PECAM1 expression and is expressed in arbitrary units (AU). Data are means ± SEM from n=3 mice (Tg^{WT}: 3 males, mean platelet count: 651550±11950/μl; Tg^{G233V}: 3 males; mean platelet count: 453850±156624/μl) (*p<0.05 vs Tg^{WT}; Two-way ANOVA).

**F** PKC substrates phosphorylation in murine Tg^{WT} and Tg^{G233V} washed resting platelets. Quantification of PKC substrate phosphorylation is relative to actin expression and is expressed in arbitrary units (AU). Data are means ± SEM from n=3 mice (Tg^{WT}: 3 males, mean platelet count: 651550±11950/μl; Tg^{G233V}: 3 males; mean platelet count: 453850±156624/μl) (*p<0.05 vs Tg^{WT}; Two-way ANOVA).
Supplementary Figure S6

A

p-SFKs
Tyr416
Src

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p-SFK (Tyr416) (fold change respect to resting)

B

p-Lyn
Tyr397
Lyn

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p-Lyn (Tyr397) (fold change respect to resting)

C

p-Lyn
Tyr507
Lyn

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p-Lyn (Tyr507) (fold change respect to resting)

D

p-PECAM1
Tyr686
PECAM-1

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p-PECAM1 (Tyr686) (fold change respect to resting)

E

p-PECAM1
Ser702
PECAM-1

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p-PECAM1 (Ser702) (fold change respect to resting)

F

PKC substrate

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PKC substrate (fold change respect to resting)

β-actin

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PKC substrate (fold change respect to resting)
Supplementary Figure S6: activation of SFKs, Lyn and PKC is impaired in stimulated PT-VWD platelets

A) SFKs phosphorylation at Tyr416 in human control and patient (PT-VWD) washed platelets under stirring conditions, stimulated with ADP (2 µM) or CVX (60 ng/ml) for 30 sec. Densitometric analysis of the pSFK/total SFK ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means ± SEM from 3 independent experiments (samples F, G, H Supplementary table 1) (*p<0.05 vs control; Two-way ANOVA).

B) Lyn phosphorylation at Tyr397 in human control and patient (PT-VWD) washed platelets under stirring conditions, stimulated with ADP (2 µM) or CVX (60 ng/ml) for 30 sec. Densitometric analysis of the pLyn/total Lyn ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means ± SEM from 3 independent experiments (samples F, G, H Supplementary table 1) (*p<0.05 vs control; Two-way ANOVA).

C) Lyn phosphorylation at Tyr507 in human control and patient (PT-VWD) washed platelets under stirring conditions, stimulated with ADP (2 µM) or CVX (60 ng/ml) for 30 sec. Densitometric analysis of the pLyn/total Lyn ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means ± SEM from 3 independent experiments (samples F, G, H Supplementary table 1) (*p<0.05 vs control; Two-way ANOVA).

D) PECAM1 phosphorylation at Tyr686 in human control and patient (PT-VWD) washed platelets under stirring conditions, stimulated with ADP (2 µM) or CVX (60 ng/ml) for 30 sec. Densitometric analysis of the pPECAM1/total PECAM1 ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means ± SEM from 3 independent experiments (samples F, G, H Supplementary table 1) (p=ns vs control; Two-way ANOVA).

E) PECAM1 phosphorylation at Ser702 in human control and patient (PT-VWD) washed platelets under stirring conditions, stimulated with ADP (2 µM) or CVX (60 ng/ml) for 30 sec. Densitometric analysis of the pPECAM1/total PECAM1 ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means ± SEM from 3 independent experiments (samples F, G, H Supplementary table 1) (p=ns vs control; Two-way ANOVA).

F) PKC substrate phosphorylation in human control and patient (PT-VWD) washed platelets under stirring conditions, stimulated with ADP (2 µM) or CVX (60 ng/ml) for 30 sec. Densitometric analysis of the PKC substrate/actin ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means ± SEM from 3 independent experiments (samples F, G, H Supplementary table 1) (*p<0.05 vs control; Two-way ANOVA).
Supplementary Figure S7

A) Akt phosphorylation at Tyr in human control and patient (PT-VWD) washed platelets under stirring conditions, stimulated with ADP (2 μM) or CVX (60 ng/ml) for 30 sec. Densitometric analysis of the pAKT/total AKT ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means ± SEM from 3 independent experiments (samples F, G, H Supplementary table 1) (*p<0.05 vs control; Two-way ANOVA).

B) Syk phosphorylation at Tyr in human control and patient (PT-VWD) washed platelets under

Supplementary Figure S7: activation of AKT but not of SYK is impaired in stimulated PT-VWD platelets

A) Akt phosphorylation at Tyr in human control and patient (PT-VWD) washed platelets under stirring conditions, stimulated with ADP (2 μM) or CVX (60 ng/ml) for 30 sec. Densitometric analysis of the pAKT/total AKT ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means ± SEM from 3 independent experiments (samples F, G, H Supplementary table 1) (*p<0.05 vs control; Two-way ANOVA).

B) Syk phosphorylation at Tyr in human control and patient (PT-VWD) washed platelets under
stirring conditions, stimulated with ADP (2 μM) or CVX (60 ng/ml) for 30 sec. Densitometric analysis of the pSyk/total Syk ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means ± SEM from 3 independent experiments (samples F, G, H Supplementary table 1) (p=ns vs control; Two-way ANOVA).
Supplementary Figure S8: cAMP and cGMP production is normal in PT-VWD platelets.

A) Platelets were incubated with Iloprost (100 ng/ml) or its vehicle at 37°C for 5 minutes under stirring. Samples were centrifuged and cAMP production from platelet lysates was assessed using the cAMP Enzymeimmunoassay Biotrak (EIA) System dual range (Amersham, GE Healthcare, Milan, Italy).

B) Platelets were incubated with the nitric oxide (NO)-donor s-nitroso-n-acetyl penicillamine (SNAP, 0.1-1-10-50 µM) or its vehicle at 37°C for 5 minutes under stirring. Samples were centrifuged and cGMP production from platelet lysates was assessed using the cGMP Enzymeimmunoassay Biotrak (EIA) System dual range (Amersham, GE Healthcare, Milan, Italy). Data are means ± SEM from 3 independent replicates. p=ns One-way ANOVA.
Supplementary Figure S9: Blockade of Lyn by Bafetinib inhibits αIIbβ3 activation and granule secretion

Platelets were incubated with Bafetinib 50 μM for 30 minutes and then αIIbβ3 activation and granule secretion were assessed by flow cytometry as described under methods. Data are means ± SEM from 3 independent experiments (samples F, G, H Supplementary table 1). *p<0.05; Two-way ANOVA (A and B) or unpaired T-test (C).