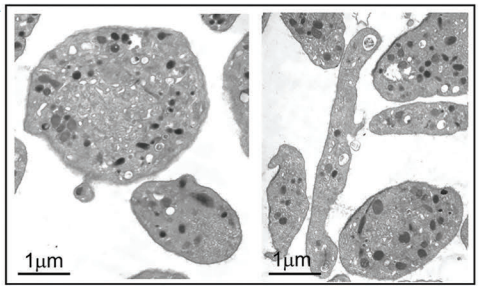
A novel variant Glanzmann thrombasthenia due to co-inheritance of a loss- and a gain-of-function mutation of *ITGB3*: evidence of a dominant effect of gain-of-function mutations

We report a novel form of autosomal dominant variant Glanzmann thrombasthenia (GT) with, unlike all previously reported cases, the proband showing compound heterozygosity for two *ITGB3* variants. Expression studies in CHO cells of the two previously undescribed *ITGB3* variants, and studies in megakaryocytes cultured from peripheral blood-derived CD34$^+$ cells, show that gain-of-function *ITGB3* variants exert a dominant effect over normal or loss-of-function *ITGB3* variants, leading to the clinical picture of autosomal dominant GT.

Classical GT is a rare autosomal recessive disorder characterized by the absence of platelet aggregation with normal platelet number and volume, usually associated with severe mucocutaneous bleeding. GT is caused by variants in the *ITGA2B* and *ITGB3* genes that encode, respectively, for the α_{IIb} and the β_3 subunits of integrin $\alpha_{IIb}\beta_3$, the most abundant platelet surface receptor.[1] Integrin $\alpha_{IIb}\beta_3$ is exposed on resting platelets in an inactive conformation and it assumes an active conformation, able to bind its ligands, upon platelet activation by a mechanism named inside-out signaling. In turn, ligand-binding triggers outside-in signaling, with phosphorylation of the β_3 cytoplasmic tail, and activation of Src-family kinases and Focal Adhesion Kinase (FAK), leading to cytoskeletal remodeling and consequently to platelet aggregation.[2]

Recently, an autosomal dominant form of GT, associated with platelet dysfunction, mild macrothrombocytopenia, and mild to severe hemorrhagic manifestations, has been included as a new entity in the list of hereditary platelet disorders.[3] Autosomal dominant GT is caused by gain-of-function variants of *ITGA2B* or *ITGB3* that generate a constitu-

A



B

Platelet volume distribution (%)

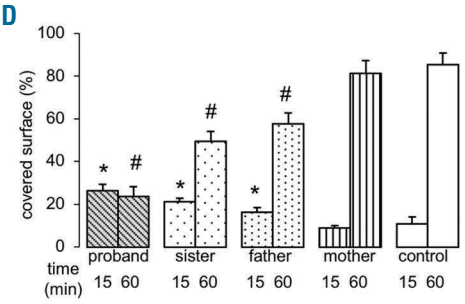
	normal	large	giant
Proband	82	13	5
Sister	88	11	1
Father	87	12	1
Mother	99	1	0
normal values	97.5±2.5	2±2	1±1

C

Platelet function tests

	CD41/ CD42b (MFI)	CD51/ CD42b (MFI)	Aggregation ADP (U)	Aggregation Collagen (U)	Aggregation TRAP-6 (U)	RIPA 0.2/0.77 mg/ml ristocetin (U)	PAC-1 resting (%)	PAC-1 ADP (%)	PAC-1 TRAP-6 (%)
Proband	**0.11**	0.06	**5**	64	**38**	1/158	0.1	**33.6**	**20.3**
Sister	**0.20**	0.06	74	90	**75**	2/161	0.1	68.3	61.1
Father	**0.31**	0.02	76	94	**89**	1/159	0.2	80.2	64.7
Mother	0.50	0.02	93	121	117	2/163	0.1	80.6	87.8
normal values	0.43± 0.11	0.04± 0.02	53-122	56-144	94-156	1-34/90-201	0.3± 0.2	74.4± 22.3	80.1± 20.05

D



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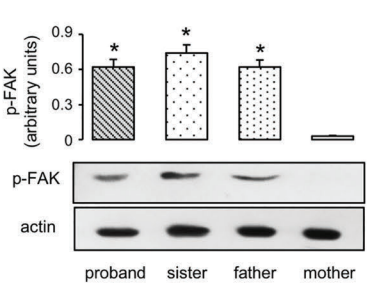

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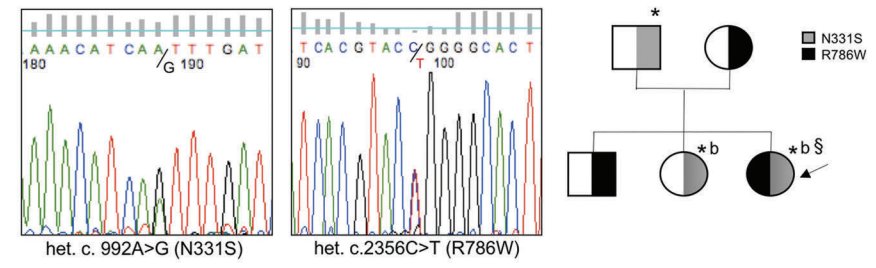

Figure 1. Platelet morphology and genetic analysis. (A) Representative images of the proband's platelets analyzed by transmission electron microscopy showing heterogeneity of platelet size and shape and the presence of large platelets (mean platelet volume: 4.23±1.23 *vs.* 2.58±0.72 µm; *P*<0.0001). Some of the largest platelets showed internal membrane complexes and heterogeneous granule distribution. (B) Distribution of platelet volumes; 100 platelets were evaluated for each blood smear. Normal values are expressed as mean±Standard Deviation. (C) All the tests carried out on platelets of the family members. Abnormal values are in bold. CD41 and CD51 expression has been corrected for platelet volume by calculating the ratio MFI clone/MFI CD42b. The brother was not available for further studies. Normal values are expressed as mean±SD, except for multiplate multiple electrode aggregometry for which reference ranges are shown. (D) Spreading of platelets after 15 or 60 minutes (mins) of adhesion to fibrinogen-coated plastic coverslips (*significantly increased *vs.* control 60 mins; #significantly decreased *vs.* control 60 mins; *P*=0.05). (E) Focal Adhesion Kinase (FAK) phosphorylation of platelets in suspension; densitometric analysis was performed using the Image J software (*significantly increased *vs.* mother; *P*<0.05 *vs.* mother). (F) Sequencing analysis of DNA from the proband, showing the two heterozygous nucleotide substitutions: c. 992A>G in exon 7 of *ITGB3* leading to N331S and c.2356C>T in exon 15 of *ITGB3* leading to R786W. Pathogenicity was predicted using the Sift and PolyPhen softwares. PolyPhen assigned "probably damaging" with a score of 1/1 to R786W and with a score of 0.097/1 to N331S. Sift assigned "deleterious" with a score of -3.653 (cut off: -2.5) to R786W and "Neutral" with a score of -2.191 to N331S. Pedigree of the family. and O: male and female family members, respectively. Heterozygous for R786W: ◧; heterozygous for N311S: ◑; compound heterozygous for the two variants: ◐. The arrow indicates the proband, compound heterozygous for the two variants. *Macrothrombocytopenia, reduced $\alpha_{IIb}\beta_3$ expression. b: bleeding. §Defective aggregation and $\alpha_{IIb}\beta_3$ activation.

tively activated $\alpha_{IIb}\beta_3$ locked in a high affinity state.

We recently showed that constitutively activated $\alpha_{IIb}\beta_3$ is associated with $\alpha_{IIb}\beta_3$ internalization and to an altered cytoskeletal reorganization that is the main effector of platelet dysfunction.[4] Abnormal cytoskeletal remodeling is also responsible for the macrothrombocytopenia, due to impaired proplatelet formation with the generation of a reduced number of abnormally large proplatelet tips.[5]

Here we describe a novel case of variant GT which phenotypically seemed to be autosomal dominant, but in which we found co-inheritance of two previously undescribed heterozygous *ITGB3* variants, a genotype suggesting the classic form of GT. Expression studies in CHO cells, however, showed that one of these is a gain-of-function variant, exerting a dominant effect over both the normal and the dysfunctional β_3, thus explaining the autosomal dominant GT phenotype of the family.

Platelet diameter and ultrastructure were assessed by transmission electron microscopy,[6] while platelet volume distribution was evaluated on blood smears.[7] Platelet function was assessed by impedance aggregometry, while $\alpha_{IIb}\beta_3$ expression and activation and $\alpha_v\beta_3$ expression were

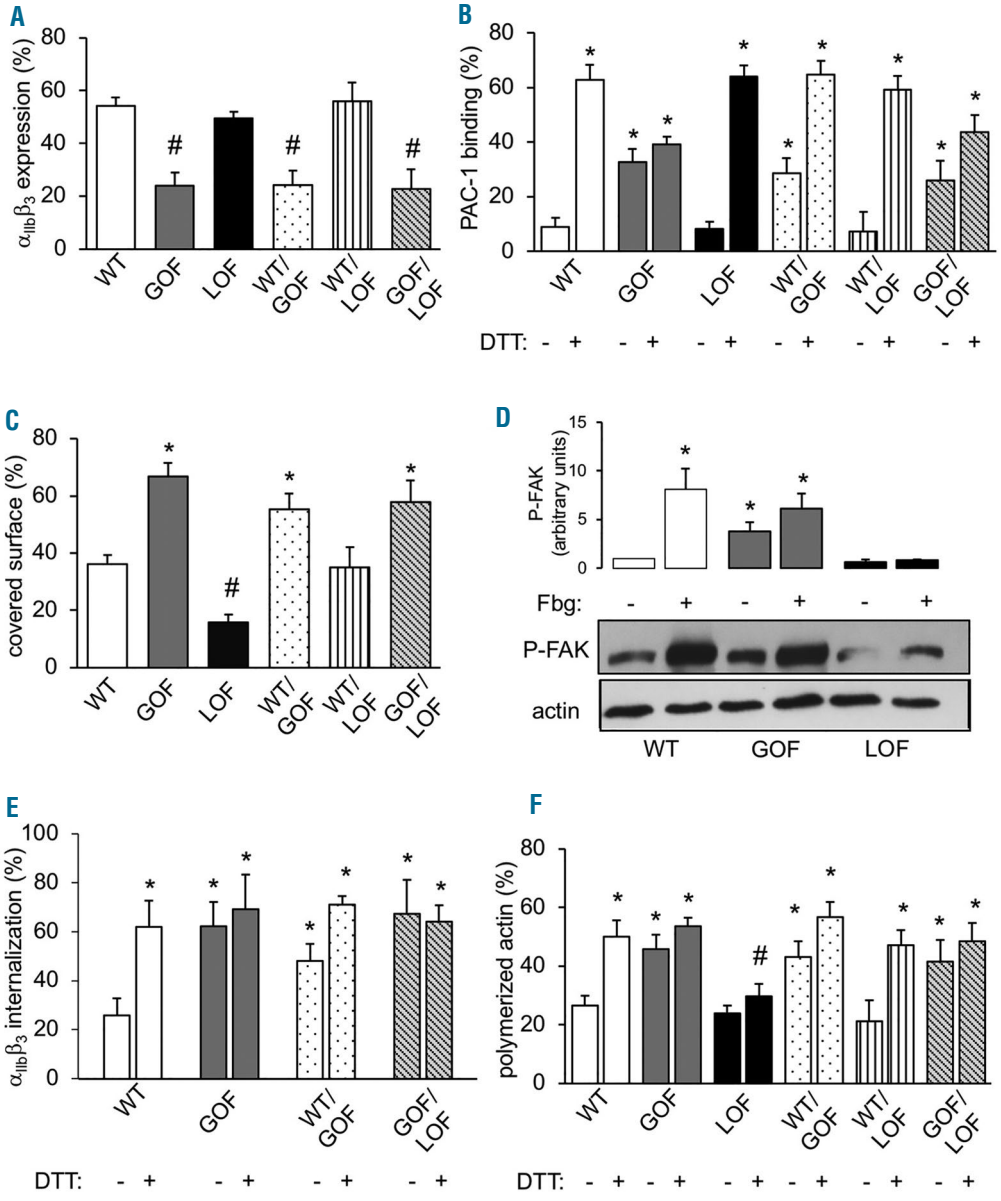

Figure 2. $\alpha_{IIb}\beta_3$ expression and function in CHO cells. (A) $\alpha_{IIb}\beta_3$ expression measured by flow cytometry in CHO cells expressing wild-type (WT) β_3 and/or gain-of-function (GOF) or loss-of-function (LOF) β_3 variants in different combinations (#significantly decreased *vs.* WT; n=8; *P*<0.05). (B) PAC-1 binding measured by flow-cytometry in CHO cells expressing WT β_3 and/or gain-of-function (GOF) or loss-of-function (LOF) β_3 variants in different combinations. Full $\alpha_{IIb}\beta_3$ activation was obtained by incubation with DTT (25 mM) (*significantly increased *vs.* WT resting; n=8; *P*<0.01). (C) Spreading of WT and mutant β_3-expressing CHO cells after 15 minutes of adhesion to fibrinogen-coated plastic coverslips (*significantly increased *vs.* WT; #significantly decreased *vs.* WT; n=5; *P*<0.05). (D) Focal Adhesion Kinase (FAK) phosphorylation of WT and mutant integrin β_3-expressing CHO cells in suspension (-) and after adhesion to fibrinogen (+). Densitometric analysis was performed using the Image J software (*significantly increased *vs.* WT-; n=5; *P*<0.05). (E) Internalized $\alpha_{IIb}\beta_3$ in WT CHO cells and mutant CHO cells expressing wild-type $\alpha_{IIb}\beta_3$ and N331S β_3, alone or in combination with WT or R786W β_3, treated or not with dithiothreitol (DTT) (*significantly increased *vs.* WT resting; n=3; *P*<0.05). (F) Polymerized actin (F-actin) content of WT and of different mutant β_3-expressing CHO cells measured by flow cytometry before and after stimulation with DTT (25 mM) (*significantly increased *vs.* WT; #significantly decreased *vs.* WT; n=5; *P*<0.05).

assessed by flow cytometry.[6] To identify DNA variants, all exons of *ITGA2B* and *ITGB3* were analyzed by Sanger sequencing.[8] Megakaryocytes were cultured from CD34$^+$ cells separated from peripheral blood[5] and proplatelet formation was studied by immunofluorescence.[4,5] To evaluate the functional impact of the novel variants, mutant β_3 expression vectors were generated by site-directed mutagenesis[9] and transfected into CHO cells stably expressing α_{IIb}.[4] $\alpha_{IIb}\beta_3$ expression, activation and internalization, and actin polymerization were assessed by flow cytometry.[4,6] For the adhesion assay, CHO cells or platelets were layered onto fibrinogen-coated glass coverslips, May-Grunwald-Giemsa staining was performed, and samples were analyzed by optical microscopy.[4,6] FAK phosphorylation was assessed by western blotting.[5] Data are expressed as means±Standard Deviation. Additional details on methods and statistical analyses are provided in the *Online Supplementary Appendix*.

The proband was a 27-year old Danish woman with a lifelong mild bleeding history, with large skin bruises which occurred spontaneously or after minor trauma, prolonged bleeding from minor wounds and after tooth extraction [ISTH-Bleeding Assessment Tool (ISTH-BAT) bleeding score: 5]. Her platelet count was mildly reduced (64-90x10^9/L) and mean platelet volume (MPV) increased (13 fL, normal 8-12). Electron microscopy and analysis of May-Grünwald-stained blood smears showed increased mean platelet diameter, and platelet anisocytosis (Figure 1A and B). Platelet aggregation was impaired in response to all stimuli, except ristocetin, with low-normal response to collagen. $\alpha_{IIb}\beta_3$ surface expression was reduced, and PAC-1 binding induced by platelet stimulation, a measure of $\alpha_{IIb}\beta_3$ activation, impaired (Figure 1C). Her father and sister also showed macrothrombocytopenia (father: platelets 95x10^9/L, MPV 12.8 fL; sister: platelets 91x10^9/L, MPV 12.7 fL), but while her sister showed spontaneous subcutaneous hematomas (ISTH-BAT bleeding score: 3), her father did not show any bleeding symptoms but reported a family history of bleeding diathesis. Her mother and brother did not show any bleeding symptoms or macrothrombocytopenia. Platelet function of the family members is summarized in Figure 1C. Platelets from the proband, her father and her sister showed increased early spreading on fibrinogen, but reduced spreading at later time points[4] (Figure 1D), together with FAK phosphorylation in unstimulated platelets (Figure 1E).

Based on the clinical and platelet phenotype, we suspected autosomal dominant GT.[3,4]

Surprisingly, DNA analysis of the proband showed compound heterozygosity for two novel *ITGB3* missense variants: c.2356C>T leading to R786W, affecting the β_3 cytoplasmic tail, and c. 992A>G leading to N331S, affecting the β-I-like domain of the β_3 globular head (Figure 1F), while heterozygosity for R786W was found in the mother and brother, and heterozygosity for N331S in the father and sister (Figure 1F). These novel variants were not present in the ExAC control dataset or in 50 healthy control subjects, nor in the disease-associated databases ClinVar and Ensembl.

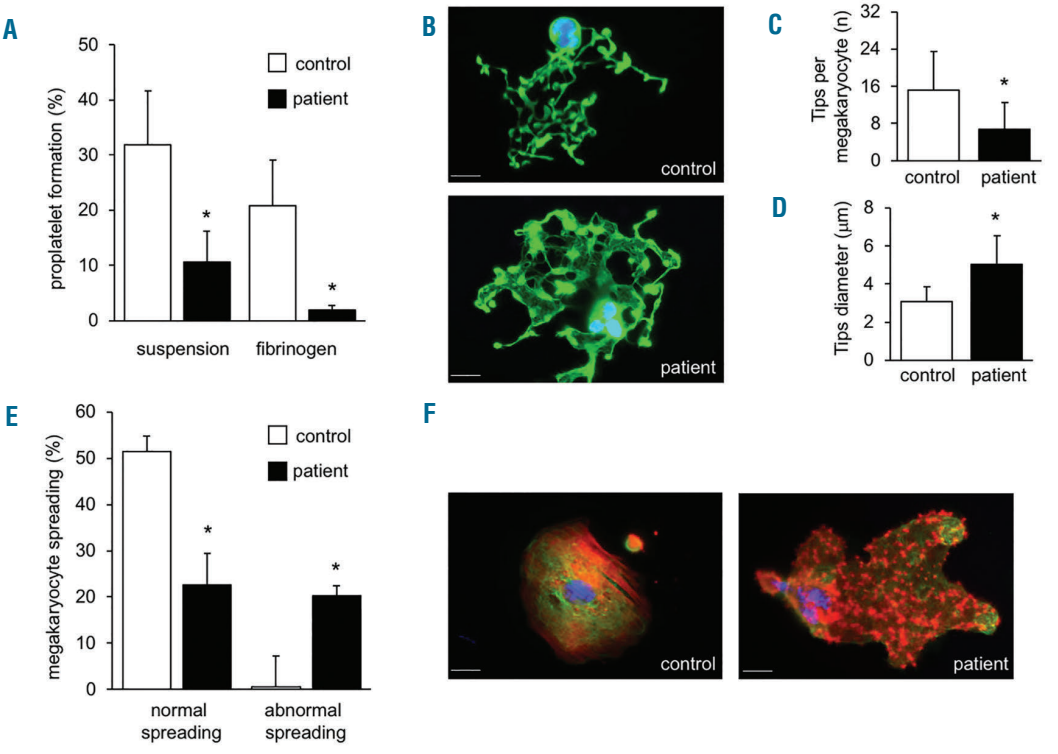

Figure 3. Megakaryocyte spreading and proplatelet formation. (A) Proplatelet formation from megakaryocytes at day 14 of culture in suspension or after 16 hours (h) of adhesion to fibrinogen (*P<0.05 *vs.* control). (B) Representative images of proplatelet formation. Microtubules were stained green with a rabbit anti-human β1-tubulin antibody and a secondary AlexaFluor488 conjugated antibody; nuclei were stained blue with Hoechst. (C) Number of proplatelet tips generated by megakaryocytes (*P<0.05 *vs.* control). (D) Diameter of proplatelet tips generated by megakaryocytes (*P<0.05 *vs.* control). (E) Spreading of megakaryocytes after 6 h of incubation on immobilized fibrinogen. Two populations of megakaryocytes were visible: half of the population spread regularly, while half showed abnormal spreading, with disordered distribution of actin and focal adhesion points more evident than stress fibers. (F) Representative image of megakaryocytes spreading on fibrinogen. Microtubules were stained green with a rabbit anti-human β1-tubulin antibody and a secondary AlexaFluor488 conjugated antibody; actin was stained red with TRITC-phalloidin, nuclei were stained blue with Hoechst. Scale bar=20 μm.

The identification of compound heterozygosity for two *ITGB3* variants did not seem to support the diagnosis of autosomal dominant GT.

To solve the dilemma, we assessed the functional impact of the two novel *ITGB3* variants by expressing them in α_{IIb}-bearing CHO cells, either alone (CHO-N331S and CHO-R786W), or in combination (CHO-N331S/R786W), thus replicating the proband's phenotype, or together with wild-type β_3 (CHO-N331S/WT and CHO-R786W/WT), thus replicating the phenotype of the other family members.

Expression studies revealed that R786W is a loss-of-function variant that does not affect $\alpha_{IIb}\beta_3$ expression (Figure 2A) or activation (Figure 2B), but causes impaired $\alpha_{IIb}\beta_3$ outside-in signaling, as shown by reduced CHO cell spreading (Figure 2C) and FAK phosphorylation on fibrinogen (Figure 2D). In contrast, N331S is a gain-of-function variant, inducing PAC-1 binding under resting conditions (Figure 2A) and triggering constitutive outside-in signaling, as shown by accelerated spreading on fibrinogen (Figure 2C) and by FAK phosphorylation under resting conditions (Figure 2D). Moreover, N331S impaired $\alpha_{IIb}\beta_3$ expression (Figure 2B) and enhanced $\alpha_{IIb}\beta_3$ internalization in unstimulated CHO cells (Figure 2E). This is in accordance with the platelet phenotype of family members expressing the N331S variant. In agreement with previous data showing that gain-of-function variants of β_3 alter cytoskeletal reorganization, polymerized actin was increased in CHO cells expressing N331S and it did not increase further after treatment with dithiothreitol (Figure 2F).

Of note, when co-expressed with normal β_3 or R786W, N331S exerted a dominant-negative effect producing impaired $\alpha_{IIb}\beta_3$ surface expression, increased internalization, PAC-1 binding under resting conditions, accelerated spreading and increased actin polymerization (Figure 2A-F).

We previously showed that gain-of-function β_3 mutants exert a dominant effect over normal β_3 thus being the main effector of platelet dysfunction in heterozygous variant GT.[4] Here, we show that a gain-of-function mutation in the β-I-like domain of β_3 can exert its dominant-negative effect even when associated with a loss-of-function β_3 mutant on the second allele, giving rise to the clinical picture of autosomal dominant GT despite compound heterozygosity.

In agreement with previous observations, megakaryocytes cultured from peripheral blood-derived $CD34^+$ cells showed normal maturation ($CD41^+$ cells: controls 12.6+4.1%, patient 10.4+2.3%), but severely impaired proplatelet formation (Figure 3A and B), with tips decreased in number and larger in size than those of controls (Figure 3C and D). Moreover, megakaryocytes adhered normally to fibrinogen (control 43.6±10.1%, patients 45.1±13.2%) but displayed abnormal spreading, with disordered actin distribution[5] (Figure 3E and F). The present observations strengthen our previous conclusion that the expression of a constitutively active $\alpha_{IIb}\beta_3$ in megakaryocytes leads to impaired proplatelet formation.[5]

N331S is, to our knowledge, the first *ITGB3* gain-of-function variant associated with macrothrombocytopenia that affects the globular head of β_3 while all the previously described gain-of-function variants involved the EGF3,[10] βTD[6,11] and cytoplasmic[12,13] domains of β_3. Previous mutagenesis studies showed that the introduction of an N339S aminoacid substitution in the α7-helix of the β-I-like domain of β_3 (the same domain affected by the N331S variant of our patient) causes $\alpha_{IIb}\beta_3$ activation, suggesting that asparagines located in this domain may have a primary role in keeping the integrin in a resting state.[14]

R786W affects the third-last aminoacid of the β_3 cytoplasmic tail. Significantly, R786 is the arginine of the COOH-terminal RGT sequence crucial for triggering FAK phosphorylation and outside-in signaling,[15] thus R786W causes impaired outside-in signaling.

This is the first case of variant GT associated with macrothrombocytopenia caused by co-inheritance of a loss-of-function and a gain-of-function *ITGB3* variant. The gain-of-function variant is the main cause of the phenotype, dominating over the loss-of-function mutant β_3 and explains macrothrombocytopenia and reduced $\alpha_{IIb}\beta_3$ expression. The heterogeneity of the platelet and bleeding phenotype within this family is in line with previous reports of autosomal dominant GT, including cases with normal[10] or defective[6,11-13] aggregation, and constitutively activated[10,12] or inactive[6] $\alpha_{IIb}\beta_3$ under resting conditions. For instance, a near normal or only slightly defective aggregation in response to collagen has been previously described in autosomal-dominant GT.[6]

In conclusion, starting from an apparent mismatch between genotype and phenotype, we confirm the central role of gain-of-function β_3 variants in determining platelet dysfunction and macrothrombocytopenia in GT.

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