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The co-inheritance of two ITGB3 variants with additive detrimental effects on platelets leads to variant Glanzmann thrombasthenia

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NA, LB, EF, AZC, ASF and AMQ performed experiments; NA and LB analyzed and interpreted data; MDMNH, ASF and JR contributed the patients for the study; JR and PG designed and supervised the study; NA and LB wrote the manuscript; JR and PG critically revised the manuscript.

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Running title: Synergistic *ITGB3* variants in variant GT

Glanzmann Thrombasthenia (GT) is a rare autosomal recessive platelet disorder caused by reduced expression or dysfunction of integrins α_{IIb} and β_3 encoded by the genes *ITGA2B* and *ITGB3* respectively, and characterized by a normal platelet count and size but absent platelet aggregation.[1] Integrin $\alpha_{IIb}\beta_3$ is exposed on resting platelets in a bent, low affinity conformation which shifts upon platelet activation to an extended conformation with high affinity for its ligands, primarily fibrinogen.[2] Dominant gain-of-function (GOF) variants in *ITGA2B* and *ITGB3* associate with *ITGA2B/ITGB3*-related thrombocytopenia (RT), a disorder considered a variant form of GT with thrombocytopenia.[3] GOF variants generate an $\alpha_{IIb}\beta_3$ receptor locked in its high-affinity state, thus constitutively activated, which leads to its reduced expression on the platelet surface caused by activation-dependent internalization, associated with platelet dysfunction and macrothrombocytopenia due to altered cytoskeletal remodeling.[3-8] Most variants induce conformational changes in the residual $\alpha_{IIb}\beta_3$ integrin that trigger the binding of the activation-dependent monoclonal antibody PAC-1 to resting platelets but not to fibrinogen, suggesting partial receptor activation.[3,4,8] The co-inheritance of two heterozygous $\alpha_{IIb}\beta_3$ gene variants with different impact on integrin function instead is a rare event whose final effect on platelet function is hardly predictable.

Here we report an unusual case of GT with a clinical and laboratory phenotype typical of GT but associated with macrothrombocytopenia that turned out to be due to the co-inheritance of two heterozygous *ITGB3* variants, one GOF and one loss-of-function (LOF), showing additive detrimental impact on $\alpha_{IIb}\beta_3$ function.

All human studies were approved by the responsible Institutional review boards (Comité de ética de la investigación del Hospital General Universitario Reina Sofía - área de salud VII de Murcia, last approval November 28th 2023) and all studies were carried out in conformity with the declaration of Helsinki.

The proband was a 2 year-old boy who was first admitted to hospital at the age of one month for skin ecchymoses and a parietal hemangioma. He suffered severe epistaxes and easy bruising, requiring treatment with tranexamic acid, and his ISTH-BAT BS was 8. The mother, his maternal aunt and cousin (daughter of the aunt) also had history of bleeding events, with an ISTH-BAT BS of 8, 11 and 8, respectively (**Table 1A**). His coagulation screening was normal but his platelet count was mildly reduced (72–126x10^9/L) (**Table 1A**). His aunt also had mild thrombocytopenia (93-130x10^9/L) and his mother had variable

platelet counts over time (125-188x10^9/L) with values under 150x10^9/L in half of the analyses. The other family members had a normal platelet count. An increased mean platelet volume (MPV) was observed in the proband and his aunt, and sporadically in the mother (11.5-13.5 fl, normal range: 8.8-12.1 fl). Platelet size, as assessed by flow cytometry (FSC), was also increased. Peripheral blood smears[9-11] revealed large platelets in the proband, mother, aunt and cousin (**Table 1A**).

Platelet function was severely impaired in the proband. The PFA-100 closure time was strikingly prolonged (>300 sec; assessed twice) with both the collagen/epinephrine and collagen/ADP cartridges. Platelet aggregation by light transmission aggregometry (LTA) was absent in response to all agonists, except ristocetin, while it was moderately reduced in the aunt and normal in the mother and father (**Table 1B**).

Flow cytometry showed strikingly reduced $\alpha_{IIb}\beta_3$ expression on the patient's platelets and no fibrinogen and PAC-1 binding (**Table 1C**). $\alpha_{IIb}\beta_3$ expression was reduced also on the mother's, aunt's and cousin's platelets while fibrinogen and PAC-1 binding was reduced in the aunt's platelets but not in the mother and father. None of the family members' platelets bound fibrinogen or PAC-1 spontaneously (**Table 1C**). Fibrinogen bound to the proband's resting platelet surface was also not detected by immunofluorescence (**Supplementary Figure 1A**). Proband's platelets contained fibrinogen but in an amount reduced compared with control platelets, likely due to the decreased $\alpha_{IIb}\beta_3$ expression (**Supplementary Figure 1A**).

The expression of all the other major platelet glycoproteins was normal (**Supplementary Figure 1B**).

Patient DNA was analyzed by an expanded high throughput sequencing (HTS) gene panel[12] that identified two *ITGB3* missense variants in the proband: c.59T>G and c.992A>G, leading to p.Leu20Arg and p.Asn331Ser respectively. Sanger sequencing showed that the father and one brother (II.2) carried the β_3 p. Leu20Arg variant, whereas the mother, aunt, and cousin (II.4) the p.Asn331Ser variant. The second brother (II.1) did not carry any *ITGB3* variant (**Figure 1A**).

Variant p.Asn331Ser affects the β-I-like domain of the β_3 integrin globular head and has been previously characterized as a dominant GOF variant associated with *ITGA2B/ITGB3*-RT in a family unrelated to the one here described.[13] It is present in ClinVar as a variant of uncertain significance (VUS). All the carriers of the p.Asn331Ser variant from this family had increased platelet size and bleeding manifestations (**Table 1**). However, thrombocytopenia, impaired

platelet aggregation and impaired fibrinogen binding were consistently present only in the proband and the aunt. Incomplete phenotype penetrance is a common finding in several inherited platelet disorders, such as in *TUBB1*-RT[10] and in this case might have mitigated bleeding tendency of the other family members carrying the p.Asn331Ser variant. Whole exome sequencing was performed in all family members to determine whether other gene variants might explain thrombocytopenia or platelet dysfunction, but no candidate variants were identified in genes associated with inherited platelet disorders other than *ITGB3* **(Supplementary Table 1)**.

Variant p.Leu20Arg, which affects the signal peptide, is also present in ClinVar as a VUS. Family members carrying this variant in heterozygosity showed normal platelets, no bleeding and normal $\alpha_{IIb}\beta_3$ expression, the latter observation suggesting that $\alpha_{IIb}\beta_3$ internalization plays the most important role in the reduced surface expression of $\alpha_{IIb}\beta_3$. Concordantly, Western blot analysis of total β_3 expression revealed normal levels in the mother and slightly reduced levels in the father and proband (**Supplementary Figure 1C**).

Co-inheritance of a GOF *ITGB3* variant with a LOF *ITGB3* variant causing *ITGA2B/ITGB3*-RT was previously reported, showing that the GOF p.Asn331Ser variant exerting a dominant negative effect over the wild type β_3 or the LOF variant.[13] However, in that case, co-inheritance of the two variants associated with an only partial platelet function defect and mild clinical bleeding, while in the current case the phenotype was severe, comparable to GT type I.

In order to clarify the functional impact of the two co-inherited *ITGB3* variants we co-expressed them in CHO cells stably expressing α_{IIb}[5], either separately (CHO-Asn331Ser or CHO-Leu20Arg) or together (CHO-Asn331Ser&Leu20Arg), in the latter way replicating the proband's phenotype.

The p.Leu20Arg variant behaved as a LOF variant strongly impairing $\alpha_{IIb}\beta_3$ expression. Indeed, Western blotting of CHO cell lysates showed a very faint band in correspondence of β_3, while lysates of CHO cells expressing WT β_3, the Asn331Ser β_3 variant or a combination of the two showed a normal β_3 band (**Figure 1B**). Flow cytometry showed drastically reduced β_3 cell surface expression in CHO-Leu20Arg (**Figure 1C**), and confocal microscopy did not allow to detect β_3 neither on the cell membrane nor in the cytoplasm (**Figure 1D**). These findings suggest that p.Leu20Arg β_3 is degraded and that only a small amount of the receptor is expressed on the membrane. This fraction is probably too little to be detected by confocal

microscopy, while flow cytometry and western blotting were sensitive enough to detect traces of it. The variant p.Asn331Ser β_3 also reduces surface expression of $\alpha_{IIb}\beta_3$ on CHO-Asn331Ser and CHO-Asn331Ser&Leu20Arg cells, but in this case probably due to receptor internalization[5,13] (**Figure 1C**).

In accordance with reduced receptor surface expression, PAC-1 binding triggered by DTT was significantly diminished in CHO-Leu20Arg compared with CHO WT β_3 (**Figure 1E**). Concordantly, absent aggregation (**Figure 1F**) and impaired spreading on fibrinogen were observed with associated reduction of surface coverage (**Figure 1G**).

As concerns the p.Asn331Ser variant, functional tests confirmed the previously reported dominant GOF effect.[5,13] In fact, PAC-1 binding of resting cells was increased (**Figure 1E**) and CHO-Asn331Ser cells spontaneously aggregated without the need of a stimulus (**Figure 1F**). Moreover, PAC-1 binding and cell aggregation did not further increase upon activation with DTT and were significantly impaired compared to CHO cells expressing WT β_3 (**Figure 1E and 1F**). Cell spreading was also defective, with reduced covered surface 60 minutes after layered on fibrinogen (**Figure 1G**).

Interestingly, p.Leu20Arg/p.Asn331Ser compound heterozygosity leads to impaired cell function. The functional impact observed for the p.Asn331Ser variant was also found in the compound heterozygous cell model, i.e. PAC-1 binding to resting cells, significantly impaired PAC-1 binding upon stimulation (**Figure 1E**), and reduced cell aggregation (**Figure 1F**) and spreading (**Figure 1G**), confirming the dominant negative effect of this GOF variant.[13] However, impaired $\alpha_{IIb}\beta_3$ surface expression and the functional defect were more severe, resembling the proband's platelet defect.

Megakaryocytes obtained from peripheral blood-derived CD34+ cells of family members carrying the p.Asn331Ser variant, showed reduced proplatelet formation on fibrinogen (**Figure 2A**) despite normal maturation (**Supplementary Table 2**), with proplatelet tips decreased in number and larger in size compared with controls and with family members carrying the p.Leu20Pro variant (**Figure 2B, 2C and 2D**), reduced spreading (**Figure 2E**) and a disordered actin distribution (**Figure 2F**), in agreement with previous observations.[6,13] We observed a marked reduction of $\alpha_{IIb}\beta_3$ surface expression in the proband's megakaryocytes, while only a mild decrease was detected in megakaryocytes from all other affected family members except II.1 (**Supplementary Table 2**).

Only two other β_3 variants affecting the signal peptide have been previously reported in GT patients. One, p.Trp11Arg, has been expressed in a cell model showing that it leads to reduced surface β_3 expression, probably due to impaired translocation to the cell membrane.[14] For the other, p.Leu20Pro, no mechanistic studies were carried out.[15]

In conclusion, we report the first case of a variant GT associated with macrothrombocytopenia caused by the co-inheritance of two heterozygous variants of integrin β_3, one GOF and one signal peptide-affecting *ITGB3* variant. Co-inheritance of the two variants led to a severe clinical phenotype, resembling type I GT but associated with macrothrombocytopenia. Our mechanistic studies show that the p.Leu20Arg variant primarily drives receptor degradation, while the p.Asn331Ser variant induces receptor internalization, causing together an additive negative impact on platelet function. This case highlights the importance of thoroughly characterizing the clinical and functional phenotype of patients with inherited platelet disorders rather than relying solely on the molecular findings, as this approach can guide more appropriate therapeutic strategies and avoid ineffective treatments, such as TPO-agonists in cases with major platelet function defects.

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Table 1. Laboratory phenotype

A) Platelet count and volume, bleeding phenotype

	Platelet count ($x10^9$/L) (median-IQR)	MPV (fL) (median- median-IQR)	Platelet volume distribution (%normal/large/giant; median± SD)	Platelet size by flow cytometry (FSC, Median Fluorescence Intensity - IQR)	ISTH-BAT BS
II.3 (proband) (n=5)	**83.0 (63-103)**	**13.0 (12.2-13.7)**	**83.5±3.5/14±4.2/2.5±0.7**	**193934 (175414-212453)** (n=3)	**8**
I.1 (aunt)(n=4)	**111 (97-125)**	**14.1 (13.7-14.6)**	**84.5±2.1/14.5±2.1/3±1.4**	**202517 (167360-237674)** (n=2)	**11**
I.2 (mother) (n=22)	**159 (131-187)**	**12.3 (11.5-13.5)**	**87.5±0.7/11.5±0.7/1±0**	**170435 (165075-175795)** (n=2)	**8**
I.3 (father) (n=4)	197 (148-247)	11.4 (10.9-11.9)	98.5±0.7/1.5±0.7/0±0	123806 (122253-125359) (n=2)	3
II.1 (brother) (n=3)	326 (248-393)	11.9 (11.6-12.1)	99±1.4/1±1.4/0±0	130011 (n=1)	0
II.2 (brother) (n=3)	193 (155-230)	11.6 (10.7-12.4)	98.5±0.7/1.5±0.7/0±0	113001 (n=1)	1
II.4 (cousin) (n=2)	281 (272-291)	12.0 (11.7-12.3)	**91.5±0.7/7.5±0.7/1±0**	**153905 (n=1)**	**8**
Controls (n=4)	209 (168-250)	10.4 (9.8-10.9)	NA	128216 (118885-137547)	NA
Normal range n=128	224 (154-294)	10.4 (8.8-12.1)	95-100/0-4/ 0-1 #	124611 (93750-152472) (n=121)	≤3 men, ≤5 women §

Bold=altered; NA=not assessed; # normal range of the laboratory; § normal values from literature.

B) Platelet aggregation by light transmission aggregometry

	AA (1.6 mM)	ADP (10 µM)	PAR-1 AP (25 µM)	Collagen (10 µg/ml)	Ristocetin (1.25 mg/ml)
II.3 (proband) (n=2)	**0.7 ± 0.3%**	**5.5 ± 6.4%**	**4.0 ± 4.2%**	**3.5 ± 3.5%**	**68.5 ± 27.6%**
I.1 (aunt)	**64%**	**48%**	**59%**	**63%**	**70%**
I.2 (mother)	81%	79%	85%	84%	91%
I.3 (father)	78%	63%	67%	92%	83%
Controls (n=2)	88 ± 9%	77 ± 9%	81 ± 13%	83 ± 11%	87 ± 13%
Healthy subjects (n=25-78)	81 ± 16% (n=74)	72 ± 13% (n=36)	82 ± 14% (n=39)	76 ± 17% (n=25)	87 ± 13% (n=78)

Bold=altered; AA=arachidonic acid

C) $\alpha_{IIb}\beta_3$ surface expression and fibrinogen binding by flow cytometry.

	$\alpha_{IIb}\beta_3$ surface expression	Fibrinogen binding (MFI)	PAC-1 binding (% of positive platelets)

	(CD41/CD42b)	Resting	ADP (10 µM)	PAR-1 AP (25 µM)	CRP (10µg/mL)	Resting	ADP (10 µM)
II.3 (proband)	**0.20 ± 0.01**	350 ± 109 (n=2)	**943 ± 537** (n=2)	**1352 ± 1117** (n=2)	**1626 ± 790** (n=2)	2.20	**2.23**
I.1 (aunt)	**1.99 ± 0.38**	776	**7467**	**6356**	**25644**	2.10	**40.12**
I.2 (mother)	**1.87 ± 0.43**	409	25927	27677	49773	2.12	48.61
I.3 (father)	4.20 ± 0.87	461	34023	19773	57185	1.73	55.22
II.1 (brother)	3.72 ± 0.06	NA	NA	NA	NA	1.82	54.54
II.2 (brother)	3.06 ± 1.17	NA	NA	NA	NA	1.34	52.11
II.4 (cousin)	**1.67 ± 0.36**	NA	NA	NA	NA	1.88	51.20
Parallel control	NA	622	57096	44737	47937	1.32	53.76
Controls	3.72 ± 0.62	859 ± 355 (n=2)	36625 ± 27818 (n=2)	25066 ± 27818 (n=2)	29324 ± 26324 (n=2)	2.13 ± 1.22	70.33 ± 13.24

Bold=altered; NA= not assessed.

For $\alpha_{IIb}\beta_3$ surface expression flow cytometry analysis was carried out using CD41 FITC, CD61 FITC and CD42b-FITC antibodies (Beckman Coulter) as previously described [4].

For fibrinogen binding, diluted PRP (~20 × 10^9/L platelets) was incubated (30 minutes at room temperature) with Tyrode's buffer as control for resting platelets, or with with ADP or PAR-1 AP and then incubated with fibrinogen-Alexa488 (Thermo Fisher, Madrid, Spain) in the presence of anti-CD42b*PE (as a platelet marker). Reactions were stopped with 4% paraformaldehyde (PFA) (v/v) (15 min, RT), samples were diluted with PBS and then run in a BD Accuri™ C6 flow cytometer (BD Biosciences, Ann Arbor, MI, USA). The median fluorescence intensity (MFI) was analyzed using BD Accuri™ C6 software8.

Figure 1. Family pedigree and functional studies on platelets and CHO-cells expressing the family variants.

A) Pedigree of the family. Squares are male and circles female family members. Heterozygous for p.Leu20Arg: ◧, heterozygous for p.Asn331Ser: ◐, compound heterozygosity for the two variants: ■. The arrow shows the proband.

B) β_3 expression as assessed by Western blotting in lysates of CHO cells expressing WT, p.Leu20Arg, p.Asn331Ser or p.Leu20Arg&p.Asn331Ser β_3 variant and a negative control transfected with the empty vector (mock) (n=5; *p<0.05 vs WT, **p<0.01 vs WT; Mann-Whitney test; Box and Whiskers with min to max). The anti- β_3 antibody is from Santa Cruz Biotechnology, catalogue number sc-365679.

C) $\alpha_{IIb}\beta_3$ surface expression assessed by flow cytometry in CHO cells expressing WT, p.Leu20Arg, p.Asn331Ser or p.Leu20Arg&p.Asn331Ser β_3 variant and a mock control. (n=4; *p<0.05 vs WT , ****p<0.0001 vs WT, Kruskal-Wallis test; Box and Whiskerss with min to max). $\alpha_{IIb}\beta_3$ surface expression was assessed with an anti-CD41-FITC antibody (clone P2) from Beckman Coulter. MFI= median fluorescence intensity.

D) β_3 subunit localization by confocal microscopy. α_{IIb}-bearing CHO cells transfected with either WT (wild type), p.Asn331Ser or p.Leu20Arg β_3 plasmid DNA. β_3 subunit was stained with an anti-β_3 antibody (Santa Cruz Biotechnology, catalogue number sc-365679) as primary and a goat anti-rabbit Alexa Fluor 488 (Life Technologies) as secondary antibody. The nucleus was stained in blue with DAPI. Cross sectional pictures were used to create 3D projections with Fiji ImageJ. Cross sectional images contain 10 µm scale bar. No green fluorescence (β_3) was visible for the p.Leu20Arg variant.

E) PAC-1 binding to CHO cells expressing either WT, p.Leu20Arg, p.Asn331Ser or p.Leu20Arg&p.Asn331Ser β_3 variant and a mock control assessed by flow cytometry. $\alpha_{IIb}\beta_3$ activation was obtained by incubating cells with 25 mM DTT for 20 min (n=5; *p<0.05 vs WT, **p<0.01 vs WT, ####p<0.0001 vs no DTT; Two way ANOVA with Dunnett's multiple comparison test; Box and Whiskers with min to max). The PAC-1 antibody is from Becton Dickinson.

F) Number of cell aggregates analyzed by light microscopy for samples expressing WT, p.Leu20Arg, p.Asn331Ser or p.Leu20Arg&p.Asn331Ser β_3 variant and a mock control (n=4; ****p<0.0001 vs WT with DTT, § p<0.01 vs WT no DTT; Two way ANOVA with Dunnett's multiple comparison test; Box and Whiskers with min to max).

G) Cell coverage area of CHO cells expressing either WT, p.Leu20Arg, p.Asn331Ser or p.Leu20Arg&p.Asn331Ser β_3 variant and a mock control after 90 min layering on fibrinogen analyzed by fluorescence microscopy. Transfected cells were layered on a fibrinogen coated cover slip for 90 min. F-actin was stained in red with rhodamine phalloidin. Fiji ImageJ was used to calculate the cell area coverage (n=3; *p<0.05 vs WT, **p<0.01 vs WT, Mann-Whitney test; Box and Whiskers with min to max).

Figure 2. Megakaryocyte studies

A) Proplatelet formation by peripheral blood CD34+-derived megakaryocytes at day 14 of culture after 16 hours of adhesion to fibrinogen.

B) Representative images of proplatelet formation from megakaryocytes of a control and the mother of the proband. Microtubules were stained green with a mouse anti-human β1-tubulin antibody and a secondary AlexaFluor488 conjugated antibody; nuclei were stained blue with Hoechst. Scale bar=20 μm

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 4 h of incubation on fibrinogen.

F) Representative images of megakaryocytes of a control and the proband spreading on fibrinogen. Microtubules were stained green with a mouse anti-human β1-tubulin antibody and a secondary AlexaFluor488 conjugated antibody; actin was stained red with rhodamine-phalloidin, nuclei were stained blue with Hoechst. Two populations of megakaryocytes were visible in patients: half of the population spread regularly, while half showed abnormal spreading (as shown in the representative picture of a megakaryocyte from patient II.3), with disordered distribution of actin and focal adhesion points more evident than stress fibers. Scale bar=20 μm.

Figure 1

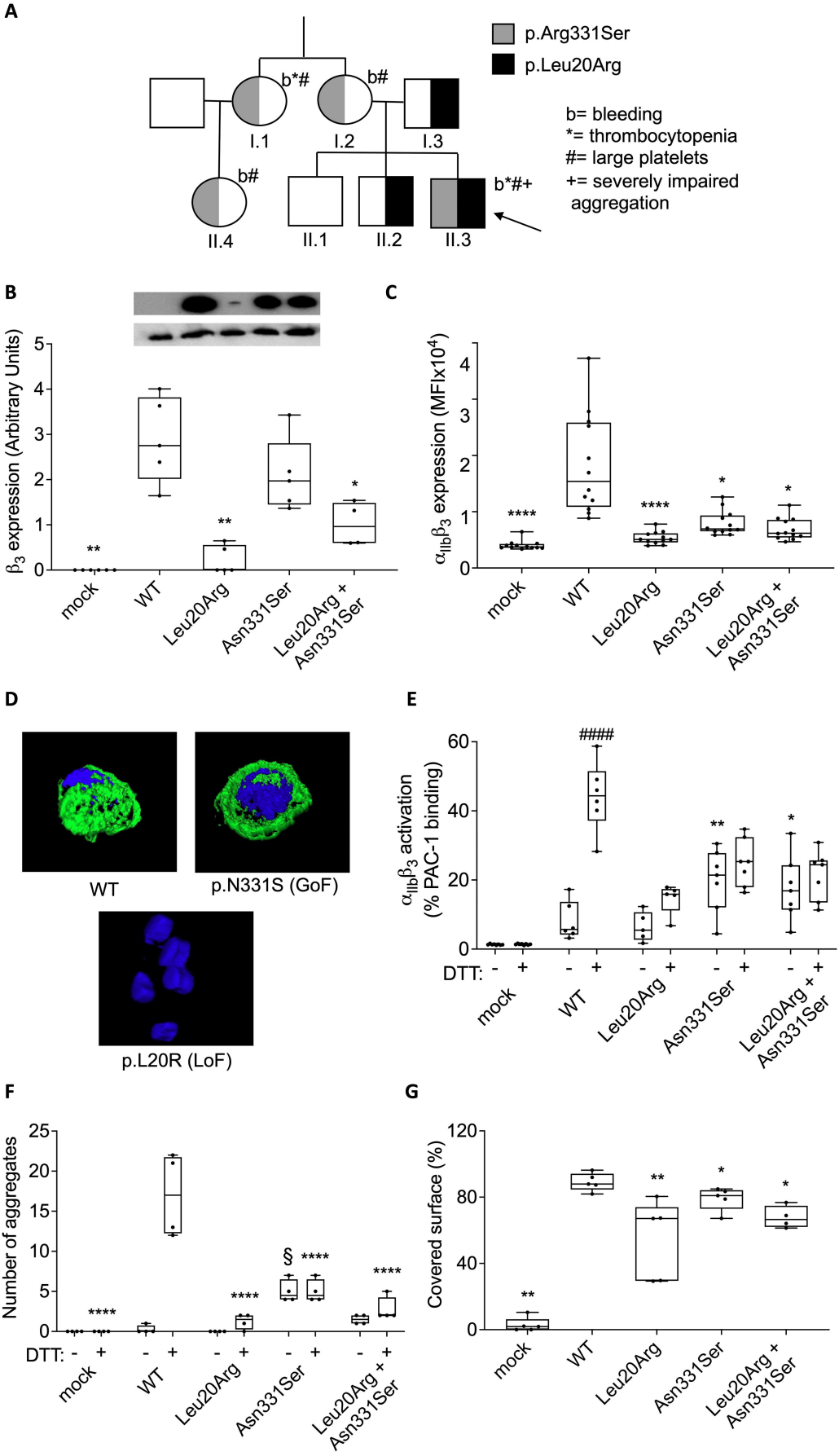
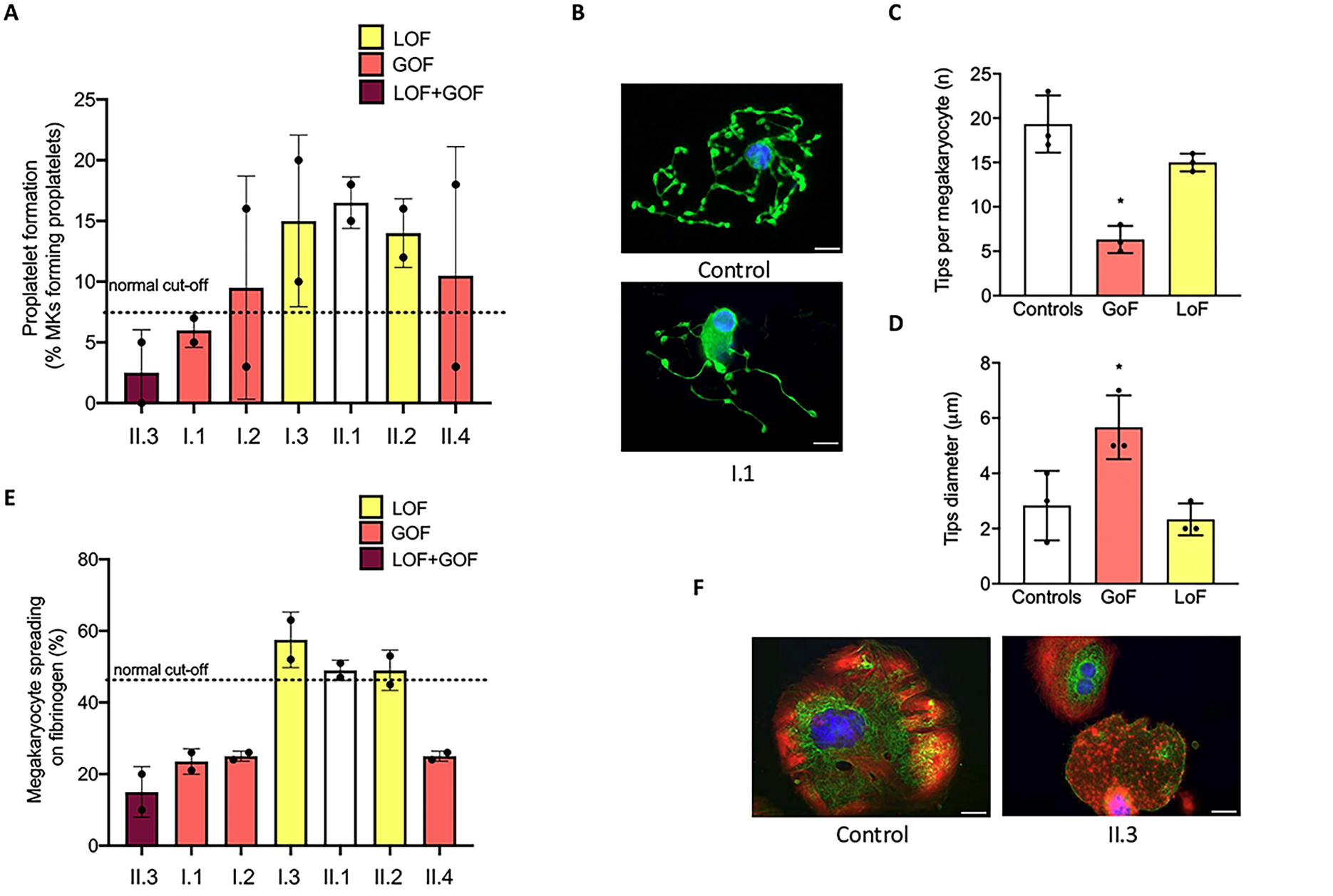
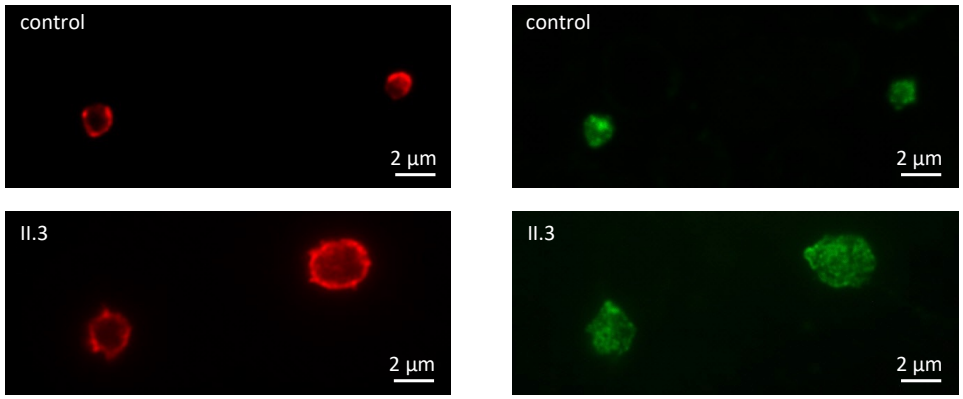

A
p.Arg331Ser
p.Leu20Arg
b= bleeding
*= thrombocytopenia
#= large platelets
+= severely impaired aggregation
b*#
b#
b*#+
I.1
I.2
I.3
II.4
II.1
II.2
II.3
B
β3 expression (Arbitrary Units)
C
αIIbβ3 expression (MFIx10⁴)
mock
WT
Leu20Arg
Asn331Ser
Leu20Arg + Asn331Ser
D
WT
p.N331S (GoF)
p.L20R (LoF)
E
αIIbβ3 activation (% PAC-1 binding)
DTT:
F
Number of aggregates
G
Covered surface (%)

Figure 2


A
LOF
GOF
LOF+GOF
Proplatelet formation
(% MKs forming proplatelets)
normal cut-off
II.3
I.1
I.2
I.3
II.1
II.2
II.4
B
Control
I.1
C
Tips per megakaryocyte (n)
Controls
GoF
LoF
D
Tips diameter (µm)
Controls
GoF
LoF
E
LOF
GOF
LOF+GOF
Megakaryocyte spreading
on fibrinogen (%)
normal cut-off
II.3
I.1
I.2
I.3
II.1
II.2
II.4
F
Control
II.3

Supplementary Figure 1

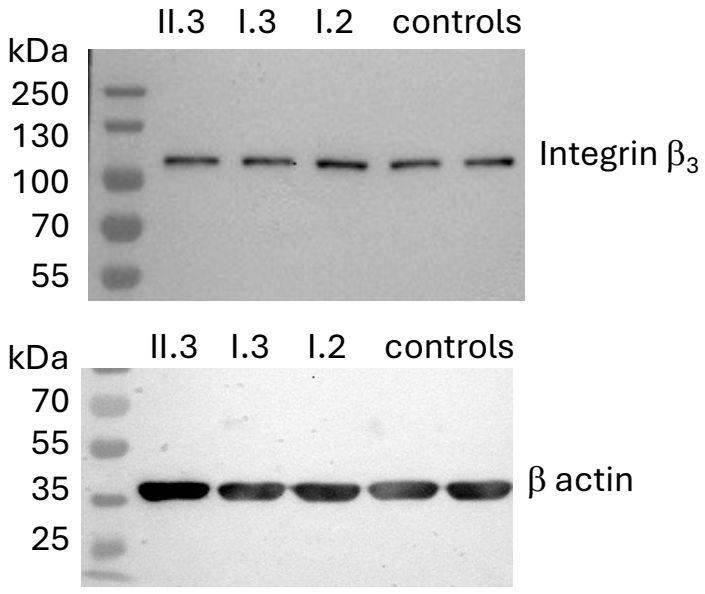
A



B

Median Fluorescence ± SD	CD42b*PE	CD49b*FITC	GPVI*APC
II.3 (proband) (n=3)	51530 ± 8202	1054 ± 783	4506 ± 702
I.1 (aunt) (n=2)	45874 ± 15748	2442 ± 36	6054 ± 982
I.2 (mother) (n=2)	35316 ± 12280	2183 ± 588	4261 ± 340
I.3 (father) (n=2)	32650 ± 721	2249 ±368	4131 ± 703
II.1 (brother) (n=1)	26928	2501	5119
II.2 (brother) (n=1)	22575	1657	4694
II.4 (cousin) (n=1)	24447	1455	3613
Controls (n=4)	29051 ± 8785	1422 ± 881	3509 ± 437

C



Legend to supplementary Figure 1

A) Fibrinogen content in platelets from the proband (II.3) and a healthy control. β1 tubulin is stained red to detect platelets (anti- β1 tubulin from Sigma Aldrich, cat. N. M8064 diluted

1:10000 as primary antibody, goat anti-rabbit Alexa Fluor 568 from Life Technologies as secondary antibody), fibrinogen is stained green (anti-fibronogen gamma chain from Proteintech, cat. N. 66158 diluted 1:200 as primary antibody, goat anti-mouse Alexa Fluor 488 from Life Technologies as secondary antibody). Immunofluorescence was performed on blood smears fixed with acetone and stored at -20°C.

B) Platelet expression of membrane glycoproteins (GPs) GPIbα (CD42b), GPIa (integrin α2, CD49b) and GPVI was evaluated by flow cytometry in citrated whole blood diluted 1:10 in Tyrode buffer (PBS) using specific antibodies (all from BD Biosciences, Madrid, Spain). Diluted blood was incubate for 30 minutes at room temperature with, the different antibodies, then samples were diluted with PBS and run in a BD Accuri™ C6 flow cytometer (BD Biosciences, Ann Arbor, MI, USA). The median fluorescence intensity (MFI) was analysed using BD Accuri™ C6 software8.

C) β_3 expression as assessed by Western blotting in lysates of platelets from the proband (II.3), his father (I.3), his mother (I.2), and two healthy controls (controls). The anti- β_3 antibody is from Santa Cruz Biotechnology, catalogue number sc-365679 (1:500). The anti- β actin antibody is from Cell Signaling (1:2000).

Supplementary Table 1

		Variant allele frequency (VAF)						Coverage						
Gene	**Variant**	**Proband (II.3)**	**Father (I.3)**	**Mother (I.2)**	**Brother (II.1)**	**Brother (II.2)**	**Aunt (I.1)**	**Proband (II.3)**	**Father (I.3)**	**Mother (I.2)**	**Brother (II.1)**	**Brother (II.1)**	**Aunt (I.1)**	**Minor Allele Frequency (MAF)**
ABL1	**NM_005157.6 c.3140C>T [p.Ser1047Phe]**	0,514	0	0,512	0	0	0,457	177	139	160	133	123	142	-
ACE	**ENST00000290866.10 c.503T>C [p.Leu168Pro]**	0,436	0	0,342	0	0	0,642	71	61	70	60	58	42	0,000409508
LINC01237	**NM_001382368.1 c.287G>C [p.Ter96Serext*46]**	0,5	0	0,580	0	0	0,454	106	93	81	76	70	66	0,00039664
MMAB	**NM_052845.4 c.569G>A [p.Arg190His]**	0,355	0	0,567	0	0	0,258	45	34	37	26	36	31	1,85119E-05
RP1	**NM_006269.2 c.4780T>C [p.Tyr1594His]**	0,49	0	0,425	0	0	0,491	100	105	101	67	91	57	1,43658E-05
SLC26A5	**NM_198999.3 c.1433C>T [p.Ser478Phe]**	0,567	0	0,250	0	0	0,571	37	40	40	24	41	28	2,05286E-06
ITGB3	**NM_000212.3 c.992A>G [p.Asn331Ser]**	0,351	0	0,384	0	0	0,257	54	51	39	42	35	19	-
ITGB3	NM_000212.3 c.59T>G [p.Leu20Arg]	0,491	0,407	0	0	0,573	0	59	54	75	55	75	46	-

Supplementary table 1: Whole exome sequencing carried out on DNA of all family members. Gene variants segregating with thrombocytopenia are shown in bold. These variants were found to be heterozygous in the proband, mother and aunt, and absent in the father and brothers. Variants were considered heterozygous when VAF was 0.25-0.7. Variant coverage and population frequency (MAF<0.01) are also shown. Of these genes, only *ITGB3* has a recognised role in inherited thrombocytopenia. The cousin (II.4) was not included in this analysis.

Supplementary Table 2

	% MKs (CD42+) (mean±SD)	$\alpha_{IIb}\beta_3$ surface expression (CD41 MFI±SD)
II.3 (proband)	14.3±4.4%	**73652±4468**
I.1 (aunt)	28.6±5.8%	143703±81608
I.2 (mother)	18.7±2.3%	174643±40147
I.3 (father)	28.1±2.7%	198520±118431
II.1 (brother 1)	17±0.87%	231752±39157
II.2 (brother 2)	13.3±3.2%	162548±87403
II.4 (cousin)	5.9±1.6%	156849±82085
Controls	19.87±14%	248522±81633