Expanding the genetic spectrum of TUBB1-related thrombocytopenia
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**Supplemental Methods**

**Patients, blood sampling, and DNA isolation**

The present study involved 9 unrelated Spanish families with suspicion of inherited macrothrombocytopenia enrolled in the multicenter project “Functional and Molecular Characterization of Patients with Inherited Platelet Disorders”, which started in 2008 under the scientific sponsorship of the Spanish Society of Thrombosis and Haemostasis. The study is conducted by the “Grupo Español de Alteraciones Plaquetarias Congénitas” (GEAP). The inclusion criteria of patients with suspicion of having an inherited platelet disorder (IPD) have been previously reported.\(^1\) To date, 254 index cases have been investigated for potential genetic anomalies, using HTS-gene panel as described below. About 75% of them presented with thrombocytopenia as the main clinical feature. Here, we report the pedigrees entering the study until end 2019 that achieved a diagnosis of TUBB1-RT.

Venous blood was drawn from patients, and parallel healthy controls, into 7.5% K3 EDTA tubes (for hemogram, blood smears, CD34+ cell isolation and megakaryocytes (MKs) cultures and nucleic acid purification) and into buffered 0.105 M sodium citrate (for platelet function studies) by using a 21-gauge needle. Complete blood counts, including platelet count and mean platelet volume (MPV) were performed using a Sysmex® XS1000i hematological counter (Sysmex España SL, Sant Just Desvern, Spain). In some patients the fraction of immature platelet (% IPF) was measured in EDTA blood samples using a Sysmex XE-2100 hematological analyzer (Sysmex España). Manual platelet counting was assessed by optical microscopy using a Burker chamber, as previously described.\(^2\) To measure platelet dimensions on May–Grünwald–Giemsa-stained blood smears, 100 platelets were evaluated and classified as normal, large or giant if the mean platelet diameter was smaller, similar or higher, respectively, than the diameter of red blood cells.\(^3,4\) Genomic DNA was isolated from peripheral blood using a DNeasy blood and tissue kit, following the manufacturer’s recommendations.
(Qiagen, Hilden, Germany). DNA concentration was quantified using a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA).

**Molecular analysis by HTS gene panel**

Analysis of DNA from pedigrees B, C, D and E was carried out by using the HTS gene panel previously described using an Illumina platform (Illumina, San Diego, CA, USA). The remaining patients were recruited later and for them we used a novel HTS gene panel. The design of the specific primers for the amplification of the 89 genes of interest included in the panel was carried out with the algorithm "Ion Ampliseq Designer 5.4.1", covering 460.28 Kb on the reference sequence of the human genome GRCh37 / hg19. The 1679 overlapping amplicons, with a size range between 125-375 bp, cover all exons of 89 genes (Supplemental Table S1) (including 10 bp at the ends of each exon) and a 527 bp region of Chr10: 27389025-27389552 of the ANKRD26 gene.

Ten ng of genomic DNA (extracted from EDTA blood using Qiacube (Quiagen Iberia, Madrid) were used to generate libraries using the Ion Ampliseq Library Kit 2.0 (Life Technologies, Thermofisher Scientific, Waltham, MA USA) according to the manufacturer's protocol. For the library preparation, primers were pooled in two tubes and each amplified in a 10 µl reaction with Ampliseq Library Kit 2.0 (Life Technologies) according to the following protocol: activation at 99ºC for 2 min; followed by 15 cycles of 99ºC for 15 sec and 60ºC for 8 min. After amplification two reactions were combined and primers were partially digested with 2 µl of FuPa Reagent, followed by incubation at 50ºC for 10 min, 55ºC for 10 min and 60ºC for 20 min. Ion Xpress Barcode Adapters were added to each sample by incubation at 22ºC for 30 min, 68ºC for 5 min and 72ºC for 5 min. Libraries were purified using AMPure XP beads (Beckman Coulter Spain, L’Hospitalet de Llobregat, Barcelona, Spain) according to the manufacturer's protocol. The barcoded libraries were quantified using Ion Library Taqman Quantification Kit (Life Technologies) and diluted to a final concentration of 100 pM for template preparation using the OneTouch 2 instrument and Ion PGM Hi-Q View OT2 Kit (Ion Torrent). Libraries passing QC were then sequenced on the Ion Torrent PGM platform (Ion Torrent-Thermo Fisher Scientific), using Ion PGM Hi-Q View Sequencing Kit (Ion Torrent) and Ion 318 Chip v2 BC (Ion Torrent). A maximum of 15 libraries were pooled to achieve at least 30X coverage per target amplicon. Sequencing
signal processing, sequence generation and base alignment to the human genome reference sequence hg19 were processed by Torrent Suite Software v.5.10.1. In either case, variant calling and annotation was performed using an in-house pipeline, based on VarScan v2.3.9, SAMTools v1.3.1, ANNOVAR, Ensembl-VEP v99 and dbNFSP v4.0a bioinformatic tools. Further analysis of variants was performed using DIGEVAR “Discovering Genetic Variants”, a web tool developed in house for user-friendly analysis of HTS data ([https://digevar.imib.es](https://digevar.imib.es)). DIGEVAR was developed in JAVA to access variant calling files [VCF] and allows authorized users to perform multiple variant filtering strategies including: variant severity, variant gene location, minor allele frequency [MAF], sequence quality and coverage. After filter selection, DIGEVAR provides a list of candidate variants fulfilling the filtering criteria. As DIGEVAR incorporates information in public databases (ENSEMBL, NCBI, CLINVAR, ExAC, etc.) and from variant analysis software (MutationTaster, Polyphen, Sift, PDB, etc.) it provides extensive information including: allele frequency, rs ID, transcript, nucleotide/protein change, variant consequence in gene/protein, phylogenetics, CLINVAR significance. Complementary modules in DIGEVAR provide: coverage information for each explored region in each gene; variant information in public databases (PubMed, dbSNP, etc.) and variant interpretation according to ACMG and AMP guidelines.

**Sanger Sequencing**

The variants identified in the index cases by HTS, were confirmed and segregated in the pedigrees by Sanger sequencing in an ABI 3130 automated sequencer. The primer pairs were designed by the ExonPrimer script website ([https://ihg.helmholtz-muenchen.de/ihg/ExonPrimer.html](https://ihg.helmholtz-muenchen.de/ihg/ExonPrimer.html)); polymerase chain reaction products sequences were analyzed by using MutationSurveyor software available from SoftGenetics ([http://www.softgenetics.com/mutationSurveyor.php](http://www.softgenetics.com/mutationSurveyor.php)).

| Variants identify | Pair of primers for Sanger Sequencing |
|-------------------|--------------------------------------|
| A&B TUBB1 c. 35del [p.Cys12Leufs12X] | TUBB1-ex1F: GGACACACCTTGGTCACAT TUBB1-ex1R : TGAGCCATGATTCTGCCACT |
| C&D TUBB1 c.319A>C [p.Thr107Pro] | TUBB1-ex3F: CTGGGACGATGGACAGCATT TUBB1-ex4.1R: AGGGCCTCATTGTCAATGCA |
| E TUBB1 c. 1267C>T [p.Gln423X] | TUBB1-4.2F: AACGTCAAGGTGGCTGTCTG TUBB1-4.2R: ACTTTGAAACAAAGGGAGCACT |
| F&G TUBB1: c. 1075C>T [p. Arg359Trp] | TUBB1-ex4F: CACAGTGGCTGCTTCCAT |


Megakaryocyte cultures and proplatelet formation analysis

CD34+ hematopoietic stem cells (HSCs) were isolated from peripheral blood by immunomagnetic anti-CD34 MicroBeads (Miltenyi Biotec, Bologna, Italy) according to the manufacturer’s recommendations. For MKs differentiation, CD34+ cells were cultured for 14 days in StemSpan medium supplemented with 10ng/mL recombinant human thrombopoietin (TPO) and 25ng/mL recombinant human stem cell factor (SCF) for 7 days and TPO alone for the following 7 days. To evaluate proplatelet formation and MKs spreading, MKs at day 14 of culture were separated on a bovine serum albumin (BSA) gradient (3–4%), plated onto glass coverslips coated with 100 µg/mL human fibrinogen (Sigma Aldrich, St. Louis, USA) and allowed to adhere for 16h in the presence of 10ng/mL TPO. Specimens were analyzed by immunofluorescence staining as described below. Culture characterization was assessed by analyzing at least 100 cells per sample on >10 different fields.

Circulating preplatelet and barbell proplatelet evaluation

Patient and control blood was centrifuged at 100 g for 20 minutes to obtain PRP; 10^6 platelets were then cyto spun on poly-L-lysine coated glass coverslips, fixed with 4% PFA for 20 minutes, permeabilized with 0.1% Triton-X for 5 minutes, blocked with 1% BSA for 1 hour, stained with an an anti-β1-tubulin antibody (a kind gift of professor Joseph Italiano, Boston, USA) and then with a secondary antibody conjugated with Alexa Fluor 488 (Invitrogen, Life Technologies, Grand Island, NY, USA), nuclei were stained with DAPI. Specimens were analyzed through a Carl Zeiss Axio Observer. A1 fluorescence microscope, using a 100X/1.4 Plan-Apochromat oil-immersion objective.

“Figure 8” preplatelets were counted as the percentage of “figure 8” shapes over the total number of β1 tubulin-positive elements plated on the slide, (while preplatelets were counted as the percentage of platelets with a diameter from 3 to 10 µm over
the total number of β1 tubulin-positive elements plated on the slide; the analysis was performed on 20 different fields for each sample.

**Platelet aggregation**

Platelet-rich-plasma (PRP) and platelet poor plasma (PPP) from citrate whole blood were prepared by stepwise centrifugation (140 × g, 15 min; 1000 × g, 10 min, respectively). Light transmission aggregometry (LTA) in PRP (~100 × 10⁹ platelets/L) was performed as described¹ by using an Aggrecorder II aggregometer (Menarini Diagnostics, Florence, Italy). Time course changes in the maximal percentage of light transmission of PRP over baseline PPP were recorded for 300 seconds upon stimulation with the following platelet agonists: 25 μM thrombin receptor-activating peptide (TRAP), 5 and 10 μM ADP, 1.25 mg/mL ristocetin, 10 μg/ml collagen and 1.6mM arachidonic acid (AA).

**Impact-R assays**

Venous blood was drawn from available patients, and controls subject, into buffered 3.2% sodium citrate and tested on the Impact-R cone and plate analyzer (Impact-R platelet analyzer 47600, Matis Medical Inc.), essentially as originally described.⁹ Briefly, 130 μl of blood mixture was placed into a polystyrene well and a Teflon cone was placed on top and rotated (2050/s) to induce a shear stress that promotes platelet adhesion to the plastic. Wells were washed and stained with May-Grünwald solution and air dried. The plate surface covered with stained objects (%SC), indicated platelet adhesion, and the average size of the objects AS (μm²), indicating platelet aggregation, were quantified using the inverted light microscope connected to a camera and an image analyzing software that calculates median values from seven images from each well.

**Platelet flow cytometry**

Platelet expression of major membrane glycoproteins (GPs) including GPIa (integrin α2), GPIbα, GPIX, GPIIb and GPIIIa (integrin αIIbβ3) and GPVI was evaluated by flow cytometry in citrated whole blood diluted 1:10 in sterile phosphate-buffered saline (PBS). CD34 expression was evaluated by flow cytometry in citrated whole blood diluted 1:20 in PBS using a FITC-labeled anti-CD34 MoAb (Beckman Coulter) in the
presence of a PE-labeled anti-CD41/61 MoAb as a platelet marker (Beckman Coulter).
To analyze platelet granule secretion and αIIbβ3 activation, diluted PRP (∼20 × 10⁹/L platelets) was incubated under static conditions (30 minutes at room temperature [RT]) with Tyrode’s buffer, as control for non-stimulated platelets, or with agonists in the presence of anti-CD41*APC (as a platelet marker), fibrinogen-Alexa488 (Thermo Fisher) and anti-CD62*PE (α-granule secretion) or anti-CD63*PE (dense granule secretion) (BD Biosciences, Madrid, Spain). Reactions were stopped with 4% paraformaldehyde (PFA) (v/v) (15 min, RT), samples were diluted with PBS and run in an Accuri C6 flow cytometer (BD Biosciences). Samples were analyzed by running 10000 platelets gated on both CD41a positivity and forward scatter-side scatter (FSC-SSC) and results were expressed as mean of median fluorescence intensity (MFI).

Transmission Electron Microscopy Assays
Electron microscopy was used to examine platelet morphology and cytoskeletal network in patients from pedigree G. Briefly, PRP was fixed in 1.25% glutaraldehyde (Electron Microscopy Sciences, Hatfield, Pennsylvania, United States) for 45 minutes at room temperature. After fixation, samples were washed, post-fixed in 1% osmium tetroxide (Sigma-Aldrich) for 2 hours at 4°C and then treated with 2% uranyl acetate veronal for 2 hours at 4°C. Then, samples were dehydrated with graded ethanol and propylene solutions and embedded in Epon (Taab Laboratories, Reading, UK). Embedded samples were sectioned with an Ultracut E ultramicrotome (Reichert, Vienna, Austria) and stained with uranyl acetate and lead citrate (Merck, Darmstadt, Germany). Platelet sections were observed with a Philips/FEI Tecnai12 transmission electron microscope (FEI; Hillsboro, Oregon, United States) at 80 kV.

Immunofluorescence studies
Immunofluorescence studies were performed in platelets washed with Tyrode’s buffer as previously described.¹⁰ For platelet spreading assays, washed platelets (20 x 10⁹/platelets/L) were allowed to spread for 30 minutes onto poly-L-lysine (PLL)-coated slides at 37°C. For marginal band immunofluorescence studies washed platelets were placed onto (PLL)-coated slides, centrifuged (1000 x g, 1 min), and then fixed with 4% formaldehyde. Then, platelets were permeabilized and blocked with 0.5% BSA/0.1% Triton X-100 in PBS buffer. Cells were incubated with different antibodies: anti- α
(Santa Cruz Biotechnology, sc-5546), β1–tubulin (Sigma, SAB1408037 and an anti-β1-tubulin antibody kindly provided by Dr. J.E. Italiano (Harvard Medical School, Boston, MA),6,11 anti-α-actinin (Sigma, A5044), anti-MYH9 (Sigma, HPA001644), anti-DIAPH1 (Abcam, EPR1948), and fluorescein isothiocyanate or rhodamine-phalloidin, followed by the appropriate Alexa Fluor 488 or 568–conjugated secondary antibodies (ThermoFisher). Fluorescence images were recorded using a Leica SP8 confocal microscope. To calculate the % of spread platelets, 10 platelets from 10 different fields (100 platelets per sample) were analyzed.

Immunofluorescence staining in MKs cultures was carried out after fixation as described above, using rhodamine-phalloidin to stain actin, an anti-β1-tubulin antibody (kindly provided by Dr. J.E. Italiano, Harvard Medical School, Boston, MA) to stain microtubules, and DAPI to stain nuclei, as previously described.6,11 Specimens were analyzed on a Carl Zeiss Axio Observer.A1 fluorescence microscope (Carl Zeiss Inc, Oberkochen, Germany) with a 63x/1.4 Plan-Apochromat oil-immersion objective. Image acquisition was obtained using the AxioVision software (Carl Zeiss Inc). All polynucleated cells extending protrusions with terminal tips were considered as proplatelet-forming MKs, while those displaying a flattened shape with actin organized in focal adhesion points and fibers as spread MKs. At least 100 MKs from 5 different replicates were analyzed.

Coloc 2 Fiji plugin, which implements and performs pixel intensity correlation through Pearson and Costes methods, was used to compare the fluorescence intensity distribution of α- and β1-tubulin in regions of interest (ROIs). R value ranges from -1 (perfect negative linear correlation) to 1 (perfect positive linear correlation). Costes p-value=1 indicates that the probability of random images correlating as real images is 0.

**Quantification of mRNA and tubulin expression in platelets**

Patient and control platelets were washed in modified Tyrode's buffer and platelet lysates were prepared as previously described.12 Platelet proteins were separated on SDS-PAGE gels and transferred to PVDF membranes by means of Trans-Blot Turbo system (Bio-Rad Laboratories, S.A., Alcobendas, Madrid, Spain). Blots were incubated with anti-β1-tubulin (Sigma-Aldrich Madrid, Spain), anti-β1-tubulin antibody kindly provided by Dr. J.E. Italiano), anti-acetyl-α-tubulin (Cell signaling, #5335), anti-α-tubulin (Santa Cruz Biotechnology, sc-5546), and anti-β-actin (Sigma-Aldrich) as an
internal control, and stepwise with adequate secondary antibodies labeled with peroxidase (GEHealthcare). Finally, proteins were detected by chemiluminescence (ECL prime; GEHealthcare).

For mRNA expression, total ribonucleic acid (RNA) was extracted from PRP using Trizol (Thermo Fisher Scientific, Madrid, Spain). Retrotranscription reaction was performed using 100 ng of total RNA according to the manufacturer’s instructions (SuperScript III First Strand, Thermo Fisher Scientific). Gene expression was quantified on a LC480 real-time polymerase chain reaction (PCR) system (Roche Pharma, Basel, Switzerland) using Taqman Premix Ex Taq (Takara Bio Inc. Kusatsu, Japan) and a commercial probe for TUBB (hs00742828_s1) and TUBB1(hs00917771_g1) using GAPDH as an internal control.

**CHO cell models for the identified missense variants**

To assess the pathogenicity of candidate variants we established CHO cells models. CHO cells (from European Collection of Cell Cultures, Salisbury, UK) were grown in IMDM supplemented with 10% FCS, 2mM L-Glutamine, 100U/ml penicillin and 100µg/ml streptomycin. Cells were transiently transfected using Turbofect (Fermentas, Thermo Fisher, Milan, Italy) with 2 µg of pcDNA3.1+/C-(K)-DYK-TUBB1-WT; pcDNA3.1+/C-(K)-DYK-TUBB1-Gly269Asp and pcDNA3.1+/C-(K)-DYK-TUBB1-Gly109Glu, (all from GenScript, Leiden, Netherlands). Alternatively, cells were transfected with pcDNA3.1-TUBB1-Arg359-Trp-myc; pcDNA3.1-TUBB1-Thr107Pro-myc; and with pcDNA3.1-TUBB1-Phe260Ser–myc as an internal control previously described as pathogenic.

**Statistical analysis**

All functional assays were performed with samples from patients and at least 2 healthy controls. Were indicated, statistical comparisons among different type of patients and/or controls were performed using T test, with significance at 95%.
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Supplemental Figure S1. Heterogenous platelet size distribution in different pedigrees.

Plots illustrate heterogenous platelet size distribution in subjects where MPV was not recordable by the hematological counter (XS1000i, Sysmex). Normal platelet distribution in a healthy control is also shown.
Supplemental Figure S2: Protein structure modelling of the p.Thr107Pro, p.Gly269Asp and p.Arg359Trp TUBB1 missense variants

The protein is colored in grey, the side chains of the wild-type residues are shown in green, the side chains of the mutant residues are shown in red. Pictures on the right and on the left differ only for the perspective. Modeling was performed using the online tool Project HOPE (https://www3.cmbi.umcn.nl/hope/).
Supplemental Figure S3. The β1-tubulin missense variants p.Arg359Trp, p.Gly269Asp and p.Gly109Glu do not alter platelet function.

A) Glycoprotein expression profiles were assessed by flow cytometry in whole blood from carriers of the p.Arg359Trp (n=4; pedigree F: II.1 and III.1; pedigree G: II.1 and III.1); carriers of the p.Gly269Asp (n=2; pedigree H: II.1 and II.2); homozygous carriers of p.Gly109Glu (n=2; pedigree I: II.1 and II.2), a heterozygous carrier of the p.Gly109Glu (Pedigree I, III.2) and healthy controls (combined data from two subjects), with fluorescently labeled antibodies (Online Supplemental Methods). B-C-D) Platelet fibrinogen binding and granule secretion. Platelets from patients and controls were stimulated under static conditions for 30 minutes at room temperature with agonist in the presence of B) fibrinogen-Alexa 488 or C) anti-CD62P or D) anti-CD63 monoclonal antibody, fixed-diluted and evaluated by flow cytometry. The median fluorescence intensity (MFI) ± standard deviation is shown.
Supplemental Figure S4. The β1-tubulin missense variants p.Arg359Trp, p.Gly269Asp and p.Gly109Glu do not alter shear-induced platelet adhesion and aggregation in Impact-R.

Citrated whole blood from carriers and non carriers of the β1-tubulin variants p.Arg359Trp (pedigree F: TCP-carriers II.1 and III.1; Non-TCP carrier III.2; non-carrier III.3), p.Gly269Asp (pedigree H: TCP-carriers II.1 and II.2; Non-TCP carrier II.3; non-carrier II.4), and p.Gly109Glu (pedigree I: TCP-homozygous II.1 and II.2, Non-TCP heterozygous II.3 and II.4), and unrelated healthy controls (n=2 en each plot), were assayed with Impact-R as described above. Data in plots are the mean ± standard deviation of the plate surface covered with stained objects (%SC) indicating platelet adhesion, and the size of the adhered objects AS (μm²) indicating platelet aggregation. TCP indicates thrombocytopenia.
Supplemental Figure S5: The novel p.Gly109Glu β1-tubulin variant does not affect platelet aggregation

Platelet aggregation in response to the indicated agonists was induced at 37°C in platelet-rich-plasma from patient carriers of p.Gly109Glu β1 (Hom & Het) of pedigree I, or control subjects (C) and recorded for 5 min. Abbreviations: Hom, homozygotes; Het, heterozygotes.
Supplemental Figure S6. The β1-tubulin missense variants p.Arg359Trp, p.Gly269Asp and p.Gly109Glu affect the conversion of preplatelets into mature platelets.

Barbell proplatelets and “figure 8” shapes are absent in thrombocytopenic carriers peripheral blood. Barbell and “figure 8” shapes are circled in white, while white arrows show examples of preplatelets. Platelets were labeled with β1-tubulin (green). Images were acquired on a Carl Zeiss Axio Observer with a 100X objective lens. Scale bars are 5 μm. Data are means ± standard deviation of values obtained from at least 10 different microscopy fields in patient and control samples.
Supplemental Figure S7. Localization of cytoskeletal proteins in spread platelets from patients carrying the p.Gly109Glu variant.

Immunofluorescence analysis of cytoskeletal proteins in washed platelets spread on poly-L-lysine, from carriers of the β1-tubulin p.Gly109Glu variant (Pedigree G). Platelets were labeled with anti-filamin (green) and DIAPH1 (red) antibodies, or with anti-MYH9 (red) and α-actinin (green) antibodies. Images were acquired on a Carl Zeiss Axio Observer with a 100X objective lens. White scale bars, 5μm.
Supplemental Figure S8. Expression and localization of several platelet tubulins in carriers of the novel variant p.Gly109Glu.

Immunofluorescence analysis of α-tubulin in washed platelets (Pedigree I and control) under A) resting and B) spreading (on poly-L-lysine) conditions. Platelets were labeled with α tubulin (green) and β1-tubulin (red) antibodies. Images were acquired with a Leica SP8 confocal microscope with a 63x objective lens (magnification 5x). Scale bars are 5μm. C) Western blot of α and acetyl- α-tubulin levels in platelet lysates from patients (Pedigree G) and controls; β-actin was used as internal control. D) mRNA levels of TUBB1 and TUBB in platelets from homozygous (n=2) and heterozygous patients and from controls (n=3) measured by quantitative real time (qRT)-PCR and normalized for GAPDH mRNA.
Supplemental Figure S9. Colocalization of α and β1-tubulin in resting platelets from patients with the p.Gly109Glu variant.

Merged images of α–tubulin (green channel) and β1-tubulin (red channel) showing colocalization (yellow areas) in resting platelets stained with specific antibodies. Plots represent the fluorescence intensity distribution of green and red channels in the regions of interest (ROIs) indicated by the white lines. Scale bar is 5μm and objective lens is 63X (5X magnification).
## Supplemental Tables

### Supplemental Table S1. Genes included and data coverage in the HTS-gene panel platform for genetic diagnosis of IPDs

| Gene    | Chromosome | Amplicons | Total Bases | Covered Bases | Missed Bases | Overall Coverage | Num Exons |
|---------|------------|-----------|-------------|---------------|--------------|-----------------|-----------|
| MPL     | chr1       | 14        | 2148        | 2148          | 0            | 1.000           | 12        |
| RBM8A   | chr1       | 6         | 645         | 645           | 0            | 1.000           | 6         |
| USF1    | chr1       | 10        | 1133        | 1133          | 0            | 1.000           | 11        |
| LYST    | chr1       | 76        | 12426       | 12426         | 0            | 1.000           | 51        |
| GNAI3   | chr1       | 8         | 1225        | 1225          | 0            | 1.000           | 8         |
| DHCR24  | chr1       | 11        | 1731        | 1731          | 0            | 1.000           | 9         |
| RGS2    | chr1       | 6         | 736         | 736           | 0            | 1.000           | 5         |
| F13B    | chr1       | 18        | 2226        | 2226          | 0            | 1.000           | 12        |
| PLA2G4A | chr1       | 19        | 2590        | 2590          | 0            | 1.000           | 17        |
| ANKRD26 | chr10      | 3         | 527         | 527           | 0            | 1.000           | promoter  |
| ADRA2A  | chr10      | 6         | 1418        | 1408          | 10           | 0.993           | 1         |
| PLAU    | chr10      | 11        | 1550        | 1433          | 117          | 0.925           | 11        |
| HPS1    | chr10      | 20        | 2501        | 2501          | 0            | 1.000           | 19        |
| HPS6    | chr10      | 10        | 2348        | 2331          | 17           | 0.993           | 1         |
| PRF1    | chr10      | 9         | 1708        | 1708          | 0            | 1.000           | 2         |
| ANKRD26 | chr10      | 47        | 5813        | 5796          | 17           | 0.997           | 35        |
| MASTL   | chr10      | 17        | 2880        | 2851          | 29           | 0.990           | 13        |
| FL1     | chr11      | 13        | 1591        | 1591          | 0            | 1.000           | 12        |
| STIM1   | chr11      | 16        | 2673        | 2673          | 0            | 1.000           | 15        |
| HPS5    | chr11      | 26        | 3830        | 3816          | 14           | 0.996           | 23        |
| FERMT3  | chr11      | 19        | 2284        | 2284          | 0            | 1.000           | 15        |
| DPAGT1  | chr11      | 9         | 1407        | 1407          | 0            | 1.000           | 9         |
| RASGRP2 | chr11      | 18        | 2130        | 2122          | 8            | 0.996           | 15        |
| ANO6    | chr12      | 26        | 3536        | 3536          | 0            | 1.000           | 23        |
| A2M     | chr12      | 36        | 5145        | 5139          | 6            | 0.999           | 36        |
| ORAI1   | chr12      | 5         | 952         | 952           | 0            | 1.000           | 2         |
| VWF     | chr12      | 60        | 9462        | 9462          | 0            | 1.000           | 51        |
| ETV6    | chr12      | 10        | 1519        | 1519          | 0            | 1.000           | 8         |
| VIPAS39 | chr14      | 19        | 1862        | 1862          | 0            | 1.000           | 19        |
| ACTN1   | chr14      | 26        | 3185        | 3169          | 16           | 0.995           | 22        |
| MAP3K9  | chr14      | 22        | 3812        | 3812          | 0            | 1.000           | 16        |
| BLOC1S6 | chr15      | 5         | 619         | 606           | 13           | 0.979           | 5         |
| VPS33B  | chr15      | 23        | 2314        | 2314          | 0            | 1.000           | 24        |
| RAB27A  | chr15      | 5         | 766         | 766           | 0            | 1.000           | 5         |
| MYO5A   | chr15      | 45        | 6388        | 6373          | 15           | 0.998           | 41        |
| PLCB2   | chr15      | 32        | 4236        | 4131          | 105          | 0.975           | 34        |
| P2RX1   | chr17      | 16        | 1440        | 1440          | 0            | 1.000           | 12        |
| GP1BA   | chr17      | 8         | 1979        | 1725          | 254          | 0.872           | 1         |
| ITGA2B  | chr17      | 30        | 3720        | 3478          | 242          | 0.935           | 30        |
| ITGB3   | chr17      | 17        | 2667        | 2667          | 0            | 1.000           | 15        |
| UNC13D  | chr17      | 34        | 3913        | 3633          | 280          | 0.928           | 32        |
| Gene      | Chromosome | Base Pair | Base Pair | Variant Count | AF   | Master ID |
|-----------|------------|-----------|-----------|---------------|------|-----------|
| SLFN14    | chr17      | 2819      | 2816      | 3             | 0.999| 4         |
| MYH10     | chr17      | 6864      | 6864      | 0             | 1.000| 43        |
| SERPINF2  | chr17      | 1666      | 1617      | 49            | 0.971| 10        |
| TBX2A2R   | chr19      | 1333      | 1165      | 168           | 0.874| 4         |
| GP6       | chr19      | 2023      | 1799      | 224           | 0.889| 10        |
| BLOC1S3   | chr19      | 629       | 623       | 6             | 0.990| 1         |
| STXB2P2   | chr19      | 2195      | 2005      | 190           | 0.913| 21        |
| MLPH      | chr2       | 2103      | 2103      | 0             | 1.000| 15        |
| ABCG5     | chr2       | 2216      | 2216      | 0             | 1.000| 13        |
| ABCG8     | chr2       | 2282      | 2000      | 282           | 0.876| 13        |
| TUBB1     | chr20      | 1436      | 1436      | 0             | 1.000| 4         |
| GNAS      | chr20      | 4294      | 3882      | 412           | 0.904| 17        |
| SRC       | chr20      | 1831      | 1831      | 0             | 1.000| 11        |
| RUNX1     | chr21      | 1668      | 1482      | 186           | 0.888| 10        |
| GP1BB     | chr22      | 661       | 644       | 17            | 0.974| 2         |
| HP54      | chr22      | 2413      | 2413      | 0             | 1.000| 14        |
| MYH9      | chr22      | 6683      | 6683      | 0             | 1.000| 40        |
| P2RY1     | chr3       | 1142      | 1142      | 0             | 1.000| 1         |
| P2RY12    | chr3       | 1049      | 1049      | 0             | 1.000| 1         |
| GP5       | chr3       | 1703      | 1684      | 19            | 0.989| 1         |
| GP9       | chr3       | 554       | 546       | 8             | 0.986| 1         |
| NBEAL2    | chr3       | 9345      | 8923      | 422           | 0.955| 54        |
| HP53      | chr3       | 3355      | 3355      | 0             | 1.000| 17        |
| MECOM     | chr3       | 4086      | 4086      | 0             | 1.000| 20        |
| PF4       | chr4       | 366       | 366       | 0             | 1.000| 3         |
| ITGA2     | chr5       | 4146      | 4146      | 0             | 1.000| 30        |
| AP3B1     | chr5       | 3825      | 3825      | 0             | 1.000| 28        |
| FYB       | chr5       | 2900      | 2900      | 0             | 1.000| 20        |
| DIAPH1    | chr5       | 4379      | 4168      | 211           | 0.952| 28        |
| DNTBP1    | chr6       | 1357      | 1357      | 0             | 1.000| 12        |
| STX11     | chr6       | 884       | 884       | 0             | 1.000| 1         |
| PLA2G7    | chr6       | 1546      | 1546      | 0             | 1.000| 11        |
| F13A1     | chr6       | 2479      | 2479      | 0             | 1.000| 14        |
| HOXA11    | chr7       | 982       | 982       | 0             | 1.000| 2         |
| CYCS      | chr7       | 358       | 280       | 78            | 0.782| 2         |
| GP4       | chr7       | 1659      | 1659      | 0             | 1.000| 13        |
| TBXAS1    | chr7       | 2023      | 2023      | 0             | 1.000| 16        |
| SERPINE1  | chr7       | 1369      | 1369      | 0             | 1.000| 8         |
| GFI1B     | chr9       | 1113      | 1113      | 0             | 1.000| 6         |
| ABCA1     | chr9       | 7766      | 7766      | 0             | 1.000| 49        |
| PRKACG    | chr9       | 1076      | 896       | 180           | 0.833| 1         |
| GNAQ      | chr9       | 1220      | 1220      | 0             | 1.000| 7         |
| PTGS1     | chr9       | 2059      | 2059      | 0             | 1.000| 14        |
| ANKRD18A  | chr9       | 3299      | 3277      | 22            | 0.993| 16        |
| ADAMTS13  | chr9       | 4864      | 4848      | 16            | 0.997| 31        |
| GATA1     | chrX       | 1342      | 1342      | 0             | 1.000| 5         |
| WAS       | chrX       | 1749      | 1724      | 25            | 0.986| 12        |
| FLNA      | chrX       | 8884      | 8687      | 197           | 0.978| 47        |
### Supplemental Table S2. *In silico* pathogenicity and conservation assessment

| TOOL         | p.Thr107Pro | p.Gly109Glu | p.Gly269Asp | p.Arg359Trp |
|--------------|-------------|-------------|-------------|-------------|
| BayesDel addAF | Damaging    | Damaging    | Damaging    | Tolerated   |
| BayesDel noAF | Damaging    | Damaging    | Damaging    | Damaging    |
| DANN         | Damaging    | Damaging    | Damaging    | Damaging    |
| DEOGEN2      | Damaging    | Damaging    | Damaging    | Damaging    |
| EIGEN        | Pathogenic  | Pathogenic  | Pathogenic  | Benign      |
| EIGEN PC     | Pathogenic  | Pathogenic  | Pathogenic  | Pathogenic  |
| FATHMM       | Tolerated   | Tolerated   | Tolerated   | Damaging    |
| FATHMM-MKL   | Damaging    | Damaging    | Damaging    | Damaging    |
| FATHMM-XF    | Damaging    | Damaging    | Damaging    | Damaging    |
| LIST-S2      | Damaging    | Damaging    | Damaging    | Damaging    |
| LRT          | Deleterious | Deleterious | Deleterious | Deleterious |
| MVP          | Benign      | Pathogenic  | Pathogenic  | Pathogenic  |
| MVP          | Benign      | Pathogenic  | Pathogenic  | Pathogenic  |
| MetaLR       | Damaging    | Damaging    | Damaging    | Tolerated   |
| MetaSVM      | Damaging    | Damaging    | Damaging    | Damaging    |
| MutationAssessor | High     | High       | High       | Medium      |
| MutationTaster| Disease causing | Disease causing | Disease causing | Disease causing |
| PrimateAI    | Tolerated   | Tolerated   | Tolerated   | Tolerated   |
| Provean      | Damaging    | Damaging    | Damaging    | Damaging    |
| REVEL        | Pathogenic  | Pathogenic  | Pathogenic  | Benign      |
| SIFT         | Damaging    | Damaging    | Damaging    | Damaging    |
| SIFT4G       | Damaging    | Damaging    | Damaging    | Tolerated   |

| TOOL         | Conservation                  |
|--------------|-------------------------------|
| PhyloP       | Conserved (score 9.301)       |
|              | Conserved (score 9.828)       |
|              | Conserved (score 9.867)       |
|              | Conserved (score 3.942)       |
| PhastCons    | 100% conserved (score 1)      |
|              | 100% conserved (score 1)      |
|              | 100% conserved (score 1)      |
|              | Conserved (score 0.997)       |
### Supplemental Table S3. ACMG/AMP classification, pathogenicity scores and allele frequency of the β1-tubulin variants.

| p.Cys12Leufs12* (rs773248042) | p.Thr107Pro (rs752079894) | p.Gln423* (rs767041023) | p.Arg359Trp (rs140943896) | p.Gly269Asp (rs1293743818) | p.Gly109Glu (rs41303899) |
|-------------------------------|--------------------------|------------------------|------------------------|---------------------------|------------------------|
| **Current Verdict**          | Uncertain significance  | Uncertain significance | Likely Pathogenic      | Uncertain significance    | Uncertain significance |
| Rules                         | PVS1=very strong         | PP3=supporting          | PVS1= strong           | PP3=supporting            | PM2=supporting         |
|                              | PP4=supporting           | PP4=supporting          | PS3=supporting          | BS1=strong                | PP3=supporting         |
|                              |                          |                        |                        |                           | BS1=strong             |
| **Revised Verdict**          | Pathogenic               | Likely Pathogenic       | Pathogenic             | Uncertain significance    | Pathogenic             |
| Rules                         | PVS1=very strong         | PS3=strong              | PVS1= strong           | PS3=strong                | PS3=strong             |
|                              | PP1=supporting           | PP1=supporting          | PP1=strong             | PP1=strong                | PP1=strong             |
|                              | PP4=supporting           | PP4=supporting          | PP4=supporting         | PP3=supporting            | PP3=supporting         |
|                              | PS3=supporting           | PS3=supporting          | PS3=supporting         | BS1=strong                | BS1=strong             |
|                              |                            |                         |                        |                           |                        |
| **Allele frequency:**        |                          |                        |                        |                           |                        |
| General population            | 0.00000318               | 0.00000477             | 0.00000119             | 0.00658                   | 0.00000398             |
| European (non-Finnish)        | 0.00000529               | 0.00000264             | 0.000                  | 0.00573                   | 0.000                  |
|                               |                          |                        |                        |                           | 0.000871               |
|                               |                          |                        |                        |                           | 0.00169                |
| **ClinVar Assertion**         | Pathogenic               | -                      | -                      | Benign                    | -                      |
|                              |                          |                        |                        | Likely Benign             |                        |
|                              |                          |                        |                        |                            | Conflicting            |
|                              |                          |                        |                        |                            | Interpretations of    |
|                              |                          |                        |                        |                            | Pathogenicity           |

ACMG=American College of Medical Genetics; Allele frequency in general and European non-Finnish population from gnomAD browser database ([https://gnomad.broadinstitute.org/](https://gnomad.broadinstitute.org/)) (Accessed as May 2021); General information about variants is available on the web tool Varsome ([https://varsome.com/](https://varsome.com/)). Revised verdict is obtained after taking into consideration the results of functional and co-segregation studies carried out in the present work. The adapted ACMG/AMP criteria that we applied were: PVS1: Null variant, very strong or strong if located at the N-terminal or C-terminal domains of β1-tubulin protein, respectively; PP3 supporting: computational *in silico* predictions support a deleterious effect on *TUBB1* or β1-tubulin protein; PP4 supporting: patient’s phenotype...
or family history is specific for a disease with a single genetic etiology, individual(s) with mild, almost asymptomatic macrothrombocytopenia carrying a \textit{TUBB1} variant have been reported in the indicated reference; PS3: Well-established \textit{in vitro} or \textit{in vivo} functional studies supportive of a damaging effect on \textit{TUBB1} or \(\beta\)-tubulin protein, strong if studies in patient platelets and/or cell models corroborate disturbance of \(\beta\)-tubulin expression and/or function; PP1: Co-segregation with disease, i.e. macrothrombocytopenia, in family members, strong if segregation in the proband plus \(\geq3\) affected relatives, moderate if segregation in the proband plus \(\geq2\) affected relatives, supporting if segregation in the proband plus \(\geq1\) affected relatives; PM2 moderate: found in \(\leq2\) alleles in all gnomAD continental population cohorts and not present in homozygosity; BS1 strong: GnomAD exome allele frequency >0.000714, a threshold derived from the 46 clinically reported variants in \textit{TUBB1} gene (https://varsome.com/). The current interpretation of the variants in ClinVar is shown (https://www.ncbi.nlm.nih.gov/clinvar/variation/; accessed as May 2021).
Supplemental Table S4. Summary of clinical and laboratory data of reported TUBB1 variants.

| DNA variant, heritance and molecular approach | Protein change & affected domain | Platelet count & size | Clinical symptoms & platelet phenotype | Level of β1-tubulin & other tubulin proteins | Platelet microscopy characterization (IF & EM) | Culture of Mks & proplatelet formation | Transfected cellular model | New role of β1-tubulin & associated pathology | Reference |
|---------------------------------------------|---------------------------------|----------------------|----------------------------------------|-----------------------------------------------|-----------------------------------------------|------------------------------------------|-----------------------------|-----------------------------------------------|-----------|
| c.552C>T Dominant SS of exons | Arg318Trp Intermediate region | 40-60x10⁹/L BS with giant platelets | MTP: No bone marrow alteration. Normal GPs expression. | 50% reduction in β1-tubulin levels. | β1-tubulin present in marginal band. EM shows no abnormalities. | Derived from CD34+cells: Impaired proplatelet formation | No incorporation of β1-tubulin into microtubules in CHO cells | | 16,17 |
| c.952C>T Dominant WES | Gln423* C-Terminal region | 60x10⁹/L BS with large platelets | MTP: Not evaluated | Not evaluated | IF: Absent marginal band for β1-tubulin. ME: large and round platelets. | Not evaluated | Not evaluated | | 5,15 |
| c.779T>C Dominant SS of exons | Phe260Ser Intermediate region | 100x10⁹/L BS with giant platelets | MTP: Not evaluated | Reduced β1 and α-tubulin levels. | Absent marginal band for β1-tubulin. ME: large and round platelet with less microtubules. | Mouse fetal liver cells transduced with β1-tubulin and derived to Mks: Impaired proplatelet formation | No incorporation of β1-tubulin into microtubules in CHO cells Nuclei alteration and reduced α-tubulin levels | | |
| c.479C>T Recessive WES in a family with thyroid pathology. | Pro160Leu N-Terminal region | 180-250x10⁹/L BS with normal and some large platelets | Congenital hypothyroidism. Normal or higher platelet function (LTA and PAC1 binding) | Reduced β1 and normal α-tubulin levels. | Not evaluated ME: large and round platelets. | Derived from CD34+cells: Impaired proplatelet formation | No incorporation of β1-tubulin into microtubules in Nthy cell line | Involved in thyroid differentiation, organization and hormone secretion. Congenital Hypothyroidism | 18 |
| c.318C>T Dominant NGS in a cohort with thyroid pathology. | Tyr106* N-Terminal region | 207x10⁹/L BS with normal and some large platelets | Congenital hypothyroidism. Normal or higher platelet function (LTA and PAC1 binding) | Reduced β1 and normal α-tubulin levels. | Not evaluated ME: large and round platelets. | Derived from CD34+cells: Impaired proplatelet formation | Not evaluated | Involved in thyroid differentiation, organization and hormone secretion. Congenital Hypothyroidism | 18 |
| c.35delG Dominant | Cys12Leufs*12 N-Terminal | 220-270x10⁹/L BS with normal and | Congenital hypothyroidism | Reduced β1 and normal α-tubulin. | Not evaluated ME: large and round platelets. | Not evaluated | Not evaluated | Involved in thyroid differentiation, | 5,18 |
NGS in a cohort with thyroid pathology.

| Region          | Platelets | Platelet Function | Levels | Platelets |
|-----------------|-----------|-------------------|--------|-----------|
| 65-153 270x10^9/L (This manuscript) | Normal or higher | LTA and PAC1 binding |

**Congenital Hypothyroidism**

Our patients do not present congenital hypothyroidism.

| Mutation          | Protein Affected | Region | Platelets | Platelet Function | Levels | Platelets |
|-------------------|------------------|--------|-----------|-------------------|--------|-----------|
| c.445A>C          | Dominant WES     | Thr149Pro | 30-60x10^9/L | TP and myeloid malignancies. Not evaluated | Not evaluated | Mouse fetal liver cells transduced with β1-tubulin and derived to Mks: Impaired proplatelet formation |
|                   |                  | N-Terminal region |          |                   |        | No incorporation of β1 and α tubulin into microtubules in a) mouse fetal liver Mks transduced with β1-tubulin and b) CMK11-5 cell line |
|                   |                  |          |           |                   |        | Linked to apoptosis resistance and genome instability |
| c.752G>A          | Dominant WES     | Arg251His | 88x10^9/L | TP Not evaluated | Not evaluated | Mouse fetal liver cells transduced with β1-tubulin and derived to Mks: Impaired proplatelet formation |
|                   |                  | Intermediate region |          |                   |        | No incorporation of β1 and α tubulin into microtubules in a) mouse fetal liver Mks transduced with β1-tubulin and b) CMK11-5 cell line |
|                   |                  |          |           |                   |        | Myeloid malignancies |
| c.436G>A          | Dominant NGS     | Gly146Arg | 96-120x10^9/L | Glanzmann Thrombasthenia with MTP, Platelet dysfunction and absent αIIbβ3 | Not evaluated | Not evaluated ME: large and round platelets. |
|                   |                  | N-Terminal region |          |                   |        | Not evaluated |
| c.726C>G          | Dominant WES     | Phe242Leu | 119x10^9/L | TP Cutaneous bruising/bleeding Not evaluated | Not evaluated | Not evaluated |
|                   |                  | Intermediate region |          |                   |        | Not evaluated |
| c.721C>T          | Dominant WES     | Arg241Trp | 104-133x10^9/L | Epistaxis, menorrhagia and post-partum hemorrhage Not evaluated | Not evaluated | Not evaluated |
|                   |                  | Intermediate region |          |                   |        | Not evaluated |
| c.1080_1081insG   | Dominant WES     | Leu361Ala | 11x10^9/L | TP Cutaneous bruising Not evaluated | Not evaluated | Not evaluated |
|                   |                  | fs*19 |          |                   |        | |
| c.806G>A          | Dominant NGS     | Gly269Asp | 82-133x10^9/L | MTP, Normal GP expression and Normal β1-tubulin levels | Not evaluated | Derived from CD34+cells: Impaired proplatelet formation |
|                   |                  | Intermediate region |          |                   |        | No incorporation of β1-tubulin into microtubules in |

This manuscript
| c.319A>C | Thr107Pro | N-Terminal region | 85-110x10^9/L. | MTP. | Not evaluated | Not evaluated | Not evaluated | No incorporation of β1-tubulin into microtubules in CHO cells |
|---|---|---|---|---|---|---|---|---|
| c.326G>A | Gly109Glu | N-Terminal region | 60 x10^9/L. | BS with normal and some large and giant platelets. | MTP. | Normal GP expression and platelet function. | Undetectable β1-tubulin and normal α-tubulin, DIAPH1, filamin and α-actinin | IF: Aberrant β1-tubulin in resting and spreading platelets/megakaryocytes. ME: large and round platelets with increased open canalicular system | Derived from CD34+cells: Impaired proplatelet formation | No incorporation of β1-tubulin into microtubules in CHO cells |
| c.1075C>T | Arg359Trp | Intermediate region | 60 x10^9/L. | MTP. | Normal GP expression and platelet function. | Normal β1-tubulin levels | IF: Altered marginal band for β1-tubulin. Not evaluated | Derived from CD34+cells: Impaired proplatelet formation | Altered incorporation of β1-tubulin into microtubules in CHO cells |

SS=Sanger Sequencing; WES=whole exome sequencing; NGS=next generation sequencing; MTP=macrothrombocytopenia; TP=thrombocytopenia; BS=Blood smears; IF=immunofluorescence; EM=electron microscopy; LTA=light transmission aggregometry; Mks=megakaryocytes; CHO=Chinese hamster ovary cells