



BRIEF REPORT

A novel nonsense variant in *TPM4* caused dominant macrothrombocytopenia, mild bleeding tendency and disrupted cytoskeleton remodeling

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Abstract

Background: Rare inherited thrombocytopenias are caused by alterations in genes involved in megakaryopoiesis, thrombopoiesis and/or platelet release. Diagnosis is challenging due to poor specificity of platelet laboratory assays, large numbers of culprit genes, and difficult assessment of the pathogenicity of novel variants.

Objectives: To characterize the clinical and laboratory phenotype, and identifying the underlying molecular alteration, in a pedigree with thrombocytopenia of uncertain etiology.

Patients/Methods: Index case was enrolled in our Spanish multicentric project of inherited platelet disorders due to lifelong thrombocytopenia and bleeding. Bleeding score was recorded by ISTH-BAT. Laboratory phenotyping consisted of blood cells count, blood film, platelet aggregation and flow cytometric analysis. Genotyping was made by whole-exome sequencing (WES). Cytoskeleton proteins were analyzed in resting/spreading platelets by immunofluorescence and immunoblotting.

Results: Five family members displayed lifelong mild thrombocytopenia with a high number of enlarged platelets in blood film, and mild bleeding tendency. Patient's

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platelets showed normal aggregation and granule secretion response to several agonists. WES revealed a novel nonsense variant (c.322C>T; p.Gln108*) in *TPM4* (NM_003290.3), the gene encoding for tropomyosin-4 (TPM4). This variant led to impairment of platelet spreading capacity after stimulation with TRAP-6 and CRP, delocalization of TPM4 in activated platelets, and significantly reduced TPM4 levels in platelet lysates. Moreover, the index case displayed up-regulation of *TPM2* and *TPM3* mRNA levels.

Conclusions: This study identifies a novel *TPM4* nonsense variant segregating with macrothrombocytopenia and impaired platelet cytoskeletal remodeling and spreading. These findings support the relevant role of *TPM4* in thrombopoiesis and further expand our knowledge of *TPM4*-related thrombocytopenia.

KEYWORDS

inherited platelet disorders, macrothrombocytopenia, *TPM4*, tropomyosin-4, whole-exome sequencing

1 | INTRODUCTION

Inherited thrombocytopenias (ITs) are a large heterogeneous group of rare platelet disorders characterized by low platelet counts which mainly lead to an increased bleeding tendency.^{1,2} ITs are caused by defects in genes involved in megakaryocyte (Mk) differentiation, maturation and migration, but also in proplatelet formation and/or platelet release into blood.^{2,3} Their prevalence has been estimated to be around 2.7 of 100 000 individuals but it is likely that this is an underestimate.¹ Diagnostic algorithms for ITs consist of a stepwise process based on clinical data and several laboratory tests, including the use of whole blood electronic counters to evaluate platelet count and size, blood film analysis with or without immunofluorescence staining, and platelet function assays.^{4,5} Nowadays, the appropriateness of using next generation sequencing (NGS) procedures in the mainstream of diagnosis of inherited platelet disorders is well established.⁶ Since 2010, both whole-exome and whole-genome sequencing (WES/WGS) approaches have allowed the identification of up to 15 new genes involved in ITs.^{5,7} According to the International Society on Thrombosis and Haemostasis (ISTH) Scientific and Standardization Committee (SSC) for Genetics in Thrombosis and Haemostasis (GinTH), there are 71 Tier1 genes related to inherited platelet disorders^{8,9} (https://www.isth.org/page/GinTh_GeneLists accessed 20th November 2021).

The most common ITs are macrothrombocytopenia (MCT), characterized by reduced platelet count and increased platelet size or mean platelet volume (MPV). These MCTs are mainly caused by genetic defects affecting early megakaryopoiesis and proplatelet formation.^{7,10} In particular, proplatelet formation is highly dependent on cytoskeletal proteins, including myosin IIA, actin filaments and tubulins.^{2,7,10,11} Thus, genetic changes affecting genes of the actomyosin cytoskeleton, such as *MYH9*, *ACTN1*, or in recent years, *FLNA*, *DIAPH1* or *TUBB1* associate with MCT.^{6,12} In 2017, the first five patients from two unrelated pedigrees with mild bleeding and

Essentials

- A novel nonsense variant in *TPM4* was identified in a family with mild bleeding and mild macrothrombocytopenia.
- The *TPM4* p.Gln108* variant displayed a deleterious effect on platelet cytoskeleton remodeling.
- This alteration leads to the delocalization of tropomyosin-4 in the spreading structures.
- This new pedigree with *TPM4*-RT expands the genetic and clinical spectrum and supports *TPM4* as a Tier 1 gene.

TPM4-related thrombocytopenia (*TPM4*-RT) due to the nonsense variant p.Arg69* (NM_003290.3) were identified.¹³ Recently, Stapley, et al. have just reported two new *TPM4*-RT cases carrying novel missense variants; p.Arg182Cys and p.Ala183Val (NM_001145160.2) related to relevant bleeding, platelet dysfunction, and mild or absent thrombocytopenia.¹⁴ At the 2021 ISTH Meeting, we advanced the identification of a Spanish pedigree with *TPM4*-RT,¹⁵ which clinical, laboratory and molecular characterization is formally reported here. Acknowledging our new family with *TPM4*-RT prompted the ISTH SSC-GinTH to include *TPM4* in the Tier1 genes for Inherited bleeding, thrombotic, and platelet disorders (BTPD).¹⁶

2 | METHODS

2.1 | Patients and blood sampling

This study involved patients with suspected ITs recruited in the Spanish multicenter project “Functional and Molecular Characterization of Patients with Inherited Platelet Disorders”

coordinated by Grupo Español de Alteraciones Plaquetarias Congénitas (GEAPC). Investigations abided by the Helsinki Declaration and were approved by the Ethics Committee of the Instituto de Investigación Biomédica de Salamanca. All patients gave written informed consent. Bleeding symptoms were scored using ISTH-BAT questionnaire.¹⁷ Venous blood samples were drawn into 7.5% K3 EDTA (blood cells count, blood film, nucleic acid purification and protein lysates) and buffered 0.105 M sodium citrate for platelet functional studies.¹⁸ Platelet (P) count and mean platelet volume (MPV) were performed using a Advia 2120i hematological counter (Siemens). To measure platelet dimensions on May-Grünwald-Giemsa-stained blood smears, 100 platelets were evaluated and classified as normal (diameter <3 μm), large (3–4 μm) or giant (>4 μm).⁴

2.2 | Platelet functional tests

Platelet functional characterization was performed in the index case [II.2] in parallel with a healthy control. Detailed platelet phenotyping was made as described.^{12,19} Briefly, platelet-rich-plasma (PRP) was obtained by centrifugation at low speed (100×g, 10 min). Light transmission aggregometry (LTA) was performed using an TA-8V

Aggregometer (Stago) and was recorded during 5 min upon PRP stimulation with agonists.

Platelet expression of major glycoproteins (GPs) was assessed in diluted whole blood (1:10 in PBS) by flow cytometry (FC) with specific antibodies (anti-CD42a*PE, anti-CD42b*PE, anti-GPVI*FITC, anti-CD61*PE and anti-CD41*APC) (BD Biosciences). Platelets were stained at room temperature (RT) for 30 min under static conditions. For platelet function analysis, PRP was stimulated for 30 min with ADP, CRP and TRAP6, and fibrinogen-binding (fibrinogen*AF488, Invitrogen), α-granule (anti-CD62*PE antibody), and δ-granule secretion (anti-CD63*PE antibody) (BD Biosciences) were evaluated by FC using an Accuri C6™ cytometer (BD). Mean Fluorescence Intensity (MFI) or the percentage of positive platelets were analyzed using FlowJo software (vX.0.7. TreeStar).

Platelets were washed in modified Tyrode's buffer and platelet lysates were prepared as previously described.¹² Tropomyosin-4 (TPM4) (Invitrogen), β-tubulin, actinin-1, filamin A, Nonmuscle Myosin Heavy Chain II-A (NMHC-IIA) and β-actin (Cell Signaling), were detected by immunoblotting in platelet lysates, and by immunofluorescence (IF) under resting and spreading conditions using washed platelets in fibrinogen-coated coverslips (Sigma-Aldrich). Samples were visualized using a Leica TCS-SP8 confocal microscope, and images were analyzed using ImageJ software (National

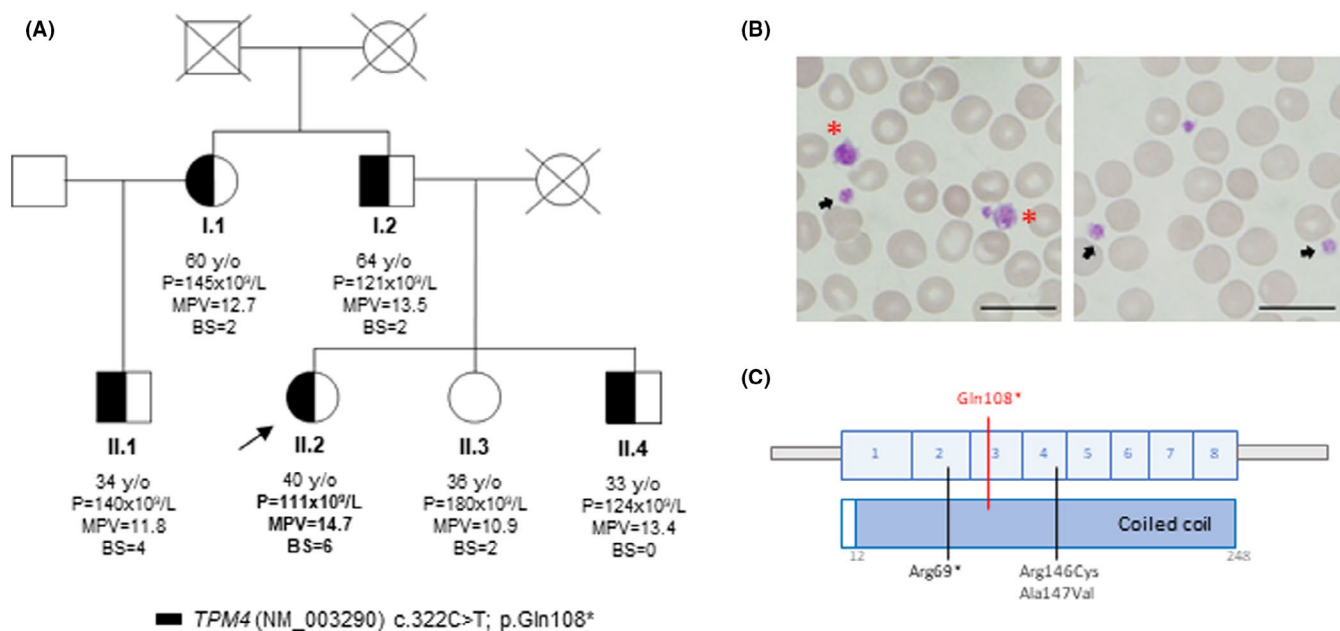


FIGURE 1 Molecular alteration and clinical and laboratory parameters of a family with tropomyosin-4 related macrothrombocytopenia (TPM4-RT). (A) Pedigree of a family with lifelong dominant inherited macrothrombocytopenia and bleeding tendency. The index case is indicated with a black arrow. Partially filled black symbols indicate heterozygosity for the indicated *TPM4* variant. (B) Representative peripheral blood film from II.2 patient (propositus) after May-Grünwald Giemsa staining (×100). Variable platelet size was observed with large (black arrows) and giant (red asterisk) platelets. Bar: 20 μm (C) Schematic representation of the *TPM4* protein, which contains 248 amino acids and a unique coiled-coil domain. Figure shows the previously reported variants (in black), p.Arg69*,¹³ p.Arg146Cys and p.Ala147Val,¹⁴ and the novel genetic change p.Gln108* (in red) found in the pedigree reported here. All genetic alterations are numbered according to positions in the NM_003290.3 transcript for *TPM4*. Variants p.Arg146Cys and p.Ala147Val correspond to described p.Arg182Cys and p.Ala183Val (NM_001145160.2)¹⁴

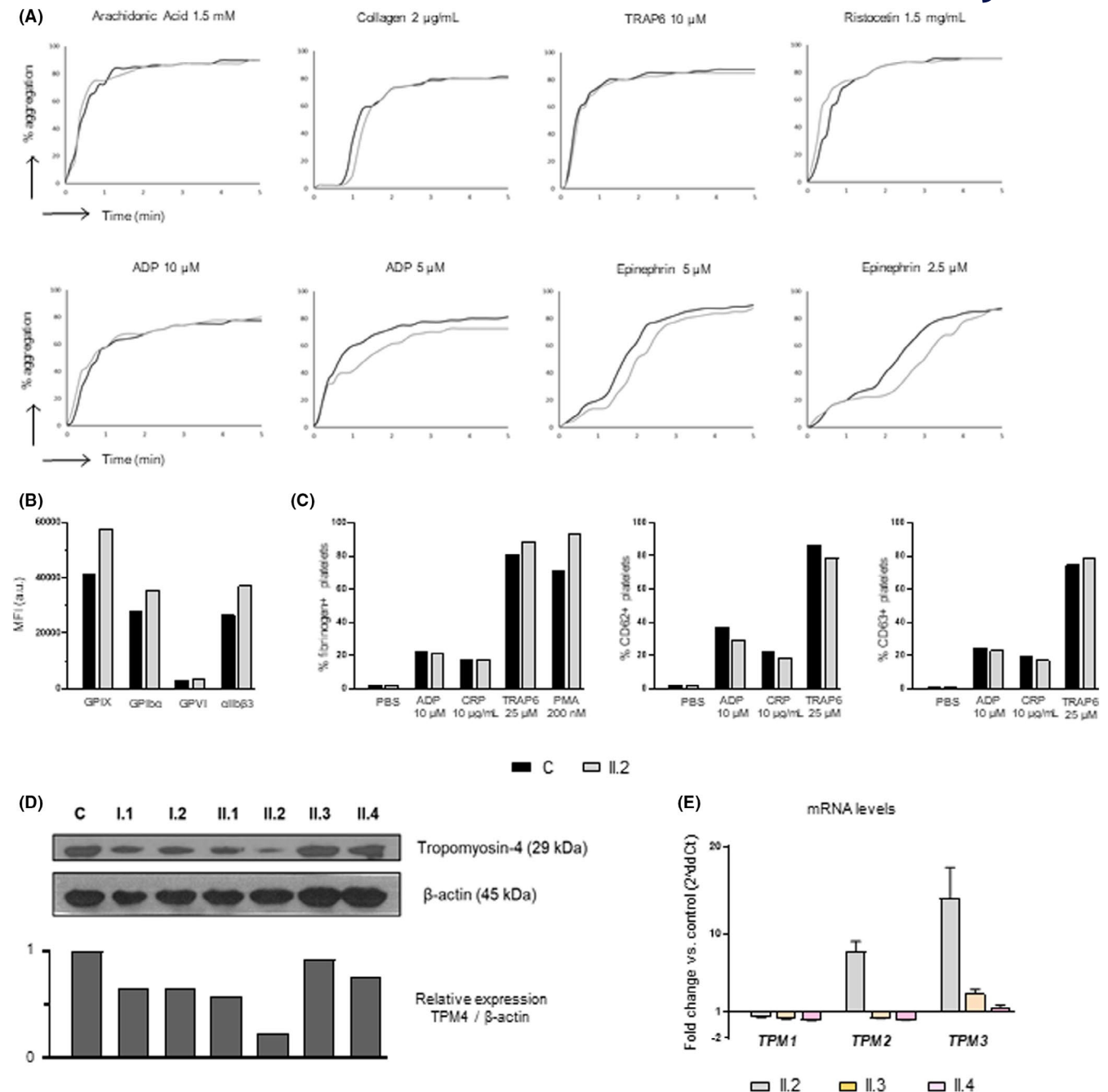


FIGURE 2 Platelet functional phenotyping performed by light transmission aggregometry and flow cytometry (propositus) and immunoblotting (all family members). (A) Platelet aggregation, with several agonists, was evaluated in control and in the propositus. (B) Glycoproteins (GPs) expression profiles were assessed by flow cytometry with indicated fluorescently labeled antibodies. The mean fluorescence intensity (MFI) is represented. (C) Fibrinogen binding and alpha and dense granules secretion were evaluated by FC in the propositus and healthy control. (D) Western blot of tropomyosin-4 levels in platelet lysates from family members and a healthy control. β -actin was used as internal control. (E) *TPM1*, *TPM2* and *TPM3* mRNA levels in platelets from cases II.2, II.3 and II.4, relative to those in a healthy control, were assessed by qRT-PCR using the $2^{-\Delta\Delta Ct}$ method and using *GAPDH* as housekeeping gene. For each gene, three different qPCR experiments, each one in triplicate, to obtain a mean average Ct value were performed in patients and in the control. Histogram represents the mean \pm standard error of the $2^{-\Delta\Delta Ct}$ values in the three qPCR experiments

Institutes of Health). Platelet spreading assays in the proband and in a healthy control were performed essentially as reported.¹² We evaluated the increase in the mean platelet surface between resting and agonist (TRAP6 and CPR) stimulated conditions, upon evaluation of 50 platelets per condition.

Platelet RNA was extracted using RLT-plus lysis buffer (RNeasy kit, Qiagen), and tropomyosin mRNA levels in the patients were quantified by qPCR (Applied Biosystems) and compared to those in a healthy control using the $2^{-\Delta\Delta Ct}$ method. *GAPDH* was used as the housekeeping gene. Three different qPCR experiments, each one in

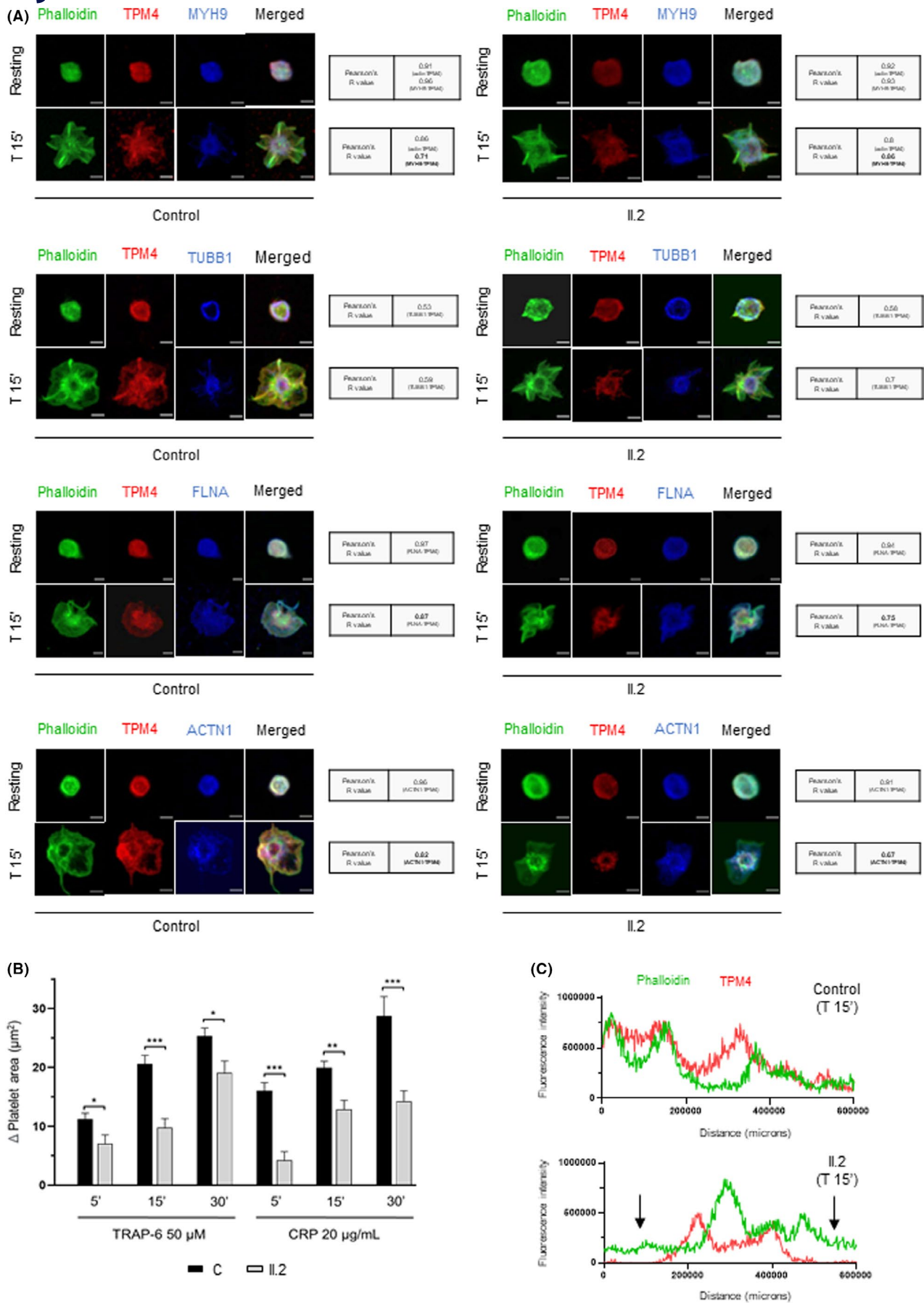


FIGURE 3 Legend on next page

FIGURE 3 Deleterious effect of p.Gln108* TPM4 variant in cytoskeleton remodeling. Platelet spreading on fibrinogen coverslips was analyzed in washed platelets from control and II.2 patient. (A) Representative immunofluorescence image of platelet spreading under no treatment (resting), and at 15 min from activation with 50 μ M TRAP-6 (T 15'). Platelets were labeled Oregon Green 514 Phalloidin (actin, green), anti-TPM4_568 (red), and anti-MYH9_647 (blue) or TUBB1_647 (blue) or FLNA_647 (blue) or ACTN1_647 (blue). Co-localization of TPM4 with other proteins of the cytoskeleton was determined with Pearson's R value (Coster p value =1). Images were acquired in a Leica TCS-SP8 confocal microscope, with a 100 \times objective lens, and images were analyzed using ImageJ software. Scale bars are 2 μ m. (B) Platelet spreading assays were performed in the proband and in a healthy control. Histogram bars correspond to the increase (mean \pm standard error, $n = 50$) in the area (μ m²) of agonist (TRAP6 and CPR) stimulated platelets vs. the area of resting platelets (50 platelets evaluated for each condition). * $p < .05$, ** $p < .01$ and *** $p < .001$ for patient platelets vs. control platelets. (C) The line profile plots represent the fluorescence intensity distribution of channels: actin (green) and TPM4 (red) at 15 min from TRAP-6 50 μ M treatment in control and II.2 platelets. Black arrows indicate the protein distribution in the spreading structures

triplicate, to obtain a mean average Ct value, were performed in patients and in the control for each gen.

2.3 | Molecular analysis by exome sequencing based on candidate gene panel

Genomic DNA was extracted from peripheral blood samples using a DNeasy blood and tissue kit, following the manufacturer's protocol (Qiagen). The proband (II.2), and both siblings (II.3, II.4) (Figure 1A) were analyzed by WES after capture and library preparation using an academic protocol that included enrichment with the xGEN[®] Exome Research Panel v2 (Integrated Technologies).^{20,21} Sequencing was performed with an S1 Flow Cell on the NovaSeq 6000 system with a mean target coverage of 50 \times at the sequencing service MLL München Leukämie Labor GmbH. Variant was confirmed and segregated in the rest of family members (I.1, I.2 and II.1, Figure 1A) by Sanger sequencing.

We followed the guidelines of the American College of Medical Genetics and Genomics and Association for Molecular Pathology (ACMG) to qualify variant pathogenicity.²² General information about variants was obtained using the Varsome web tool.²³

3 | RESULTS AND DISCUSSION

A 40 year-old female (proband, II.2) (Figure 1A) was referred due to lifelong mild macrothrombocytopenia ($p = 111 \times 10^9/L$ [Normal range: $p = 150\text{--}400 \times 10^9/L$] and MPV 14.7fL [7.2–11.1 fL]). She has a lifelong mild bleeding tendency (ISTH-BAT = 6) characterized by hematomas, gum bleeding, and menorrhagia which needed iron and hormonal therapy. Several family members (I.1, I.2, II.1, II.4), which were either asymptomatic or have a mild bleeding tendency, also displayed mild thrombocytopenia (Figure 1A). Peripheral blood film revealed giant (31%) and enlarged platelets (39%) in the proband (II.2) (Figure 1B).

DNA analysis by WES identified a novel nonsense variant (c.322C>T; p.Gln108*) in TPM4 (NM_003290.3) in all affected family members (Figure 1A). This variant was located in exon 3, affecting the coiled coil domain (Figure 1C). Following the ACMG/AMP guidelines, it was classified as likely pathogenic variant (PVS1, PM2).²²

Carrier of the variant displayed normal platelet aggregation with several agonists, including low doses of TRAP-6 and collagen. A mild impairment and delay in the platelet aggregation response to low dose of ADP and epinephrine was observed (Figure 2A). Indeed, we observed slightly increased levels of major GPs, in accordance with the increased platelet size (Figure 2B), and normal fibrinogen-binding and α and δ -granule secretion upon platelet activation with by TRAP-6, ADP, and CRP (Figure 2C). A mild bleeding tendency and an almost unaffected platelet phenotype was previously observed in carriers of the TPM4 p.Arg69* (NM_003290.3) variant.¹³ In contrast, carriers of the missense variants p.Arg182Cys and p.Ala183Val (NM_001145160.2) have been recently described to have relevant bleeding, significantly impaired platelet secretion and aggregation, and normal or slightly reduced platelet count and TPM4 levels.¹⁴ The reasons for such differences in bleeding and platelet phenotype in these TPM4-RT patients are still unknown. We speculate that heterozygous TPM4 nonsense variants, such as p.Arg69*¹³ and p.Gln108*, reported here, mainly cause haploinsufficiency, which is suggested by a reduction in tropomyosin-4 levels (Figure 2D), while missense variants could present a genetic negative dominant effect exacerbating TPM4 dysfunction and leading to major platelet disorder. Variably mild thrombocytopenia with minor, if any, associated bleeding tendency also happens in other inherited thrombocytopenias caused by mutations in structural platelet proteins.¹² Indeed, as previously described,¹³ proplatelet formation is TPM4 dose-dependent, which could justify that both nonsense variants (p.Arg69* and p.Gln108*) are associated with thrombocytopenia, while the missense variants are not. Alternatively, additional platelet or hemostatic abnormalities yet undiscovered, and beyond TPM4, may be present in the patients reported by Stapley, et al.¹⁴

Interestingly, we observed that TPM2 and TPM3 mRNA levels in the proband platelets were 8 and 14 times higher, respectively, than those in control platelets (Figure 2E). The fact that mutant TPM4 associates with macrothrombocytopenia despite an increased level of other tropomyosins, further supports a highly specific and key effect of TPM4 in megakaryopoiesis and platelet biogenesis. This finding is in agreement with previous reports suggesting that other tropomyosin isoforms have non-redundant functions with TPM4,¹³ since TPM1 and TPM2 are mainly expressed in skeletal muscle, and TPM3 in both muscle and non-muscle cells.²⁴ Although TPM3 has an important role in cytoskeleton, overexpression of the isoform does not restore TPM4 function in Mks. Besides the important involvement of TPM4

in the cytoskeleton, it is also expressed in the striated and smooth muscle,²⁵ however, no associated myopathies have been reported in any *TPM4*-RT patient.

Finally, platelet spreading studies, revealed a significance reduction in the formation of filipodia and lamellipodia, as well as severe reduction of full-spreading structures (Figure 3A) in the propositus vs. control platelets, leading to an impaired spreading function, evaluated as the increased platelet area, upon stimulation with TRAP-6 and CRP for different times (Figure 3B). These results are consistent with the previously reported finding in other *TPM4*-RT cases.^{13,14} In addition, we first show that while *TPM4* in control platelets is homogeneously distributed throughout the cytoplasm along with actin filaments, Gln108* mutant *TPM4* is accumulated mainly in the center of the patient platelets, and the protein is reduced in filopodia/lamellipodia in spread platelets, as shown in the fluorescence diagram of distribution (Figure 3C). Thus, this genetic alteration leads to a mild reduction in the localization of *TPM4* with other proteins of the cytoskeleton (10%–20%) (Figure 3A). The moderate reduction of *TPM4* in the spreading structures could account for the defect in cytoskeleton remodeling. However, no alterations in the level (data not shown) and localization of other cytoskeleton proteins (β -actin, actinin-1, β 1-tubulin, filamin A, and NMHC-IIA) were found (Figure 3A). These data were unexpected, since previous studies in *Tpm4*^{pl^{t53}} mutant mice demonstrated increased levels of degraded filamin A and actinin-1.¹³ Finally, immunoblotting of platelet lysates showed a reduction of *TPM4* levels in all p.Gln108* carriers, which was more pronounced in the index case (II.2, 80% reduction vs. level of *TPM4* in control platelets) (Figure 2D). Thus, patients with *TPM4*-RT showed variable phenotypic expression, indicating differences in genetic penetrance of the different *TPM4* variants, and/or the contribution of other unrecognized factors in some affected patients.

Although no patients with *TPM4*-RT receiving thrombopoietin receptor agonists have been reported to date, the effectiveness of agents have been shown in other inherited thrombocytopenias with defects of the platelet cytoskeleton, such as *MYH9* or *DIAPH1*.^{10,26} In summary, we have identified and characterized a novel nonsense variant in *TPM4*, c.322C>T [p.Gln108*], which is associated with mild macrothrombocytopenia and a deleterious effect on platelet cytoskeleton remodeling. Our findings reinforce the key role of *TPM4* in thrombopoiesis and expand the phenotype and genotype spectrum of *TPM4*-RT. This new *TPM4*-RT pedigree guarantees the inclusion of *TPM4* as a Tier1 list of gene involved in ITs.

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CONFLICT OF INTEREST

The authors state that they have no conflict of interest.

AUTHOR CONTRIBUTION

JMB, JMHR and JRGP designed the research; AMQ, CFI, VPB, JR and EV performed the functional experiments. RB and JMB conducted the whole exome sequencing analysis. AMQ, IGT, JRGP and JMB analyzed and interpreted the results; AMQ, JR and JMB wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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