





## BRIEF REPORT

# Rare missense variants in Tropomyosin-4 (*TPM4*) are associated with platelet dysfunction, cytoskeletal defects, and excessive bleeding

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## Abstract

**Background:** A significant challenge is faced for the genetic diagnosis of inherited platelet disorders in which candidate genetic variants can be found in more than 100 bleeding, thrombotic, and platelet disorder genes, especially within families in which there are both normal and low platelet counts. Genetic variants of unknown clinical significance (VUS) are found in a significant proportion of such patients in which functional studies are required to prove pathogenicity.

**Objective:** To identify the genetic cause in patients with a suspected platelet disorder and subsequently perform a detailed functional analysis of the candidate genetic variants found.

**Methods:** Genetic and functional studies were undertaken in three patients in two unrelated families with a suspected platelet disorder and excessive bleeding. A targeted gene panel of previously known bleeding and platelet genes was used to identify plausible genetic variants. Deep platelet phenotyping was performed using platelet spreading analysis, transmission electron microscopy, immunofluorescence, and platelet function testing using lumiaggregometry and flow cytometry.

**Results:** We report rare conserved missense variants (p.R182C and p.A183V) in *TPM4* encoding tropomyosin-4 in 3 patients. Deep platelet phenotyping studies revealed similar platelet function defects across the 3 patients including reduced platelet secretion, and aggregation and spreading defects suggesting that *TPM4* missense variants impact platelet function and show a disordered pattern of tropomyosin staining.

**Conclusions:** Genetic and functional *TPM4* defects are reported making *TPM4* a diagnostic grade tier 1 gene and highlights the importance of including *TPM4* in diagnostic

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genetic screening for patients with significant bleeding and undiagnosed platelet disorders, particularly for those with a normal platelet count.

#### KEYWORDS

bleeding, cytoskeleton, next generation sequencing, platelet disorder, platelet dysfunction, *TPM4*

## 1 | INTRODUCTION

Inherited platelet disorders comprise an extremely heterogeneous group making genetic diagnosis challenging, especially in those with normal platelet counts.<sup>1,2</sup> Some patients experience frequent and debilitating bleeding episodes throughout their lifetimes; therefore, a precise genetic diagnosis provides significant clinical benefit for the patients and offers the possibility of accurate counseling and clinical management. Current next generation sequencing (NGS) technologies allow the detection of both point mutations and copy number variants (CNVs) with a single platform and workflow, thus allowing a comprehensive molecular diagnosis.<sup>3-5</sup> Currently, 93 bleeding, thrombotic, and platelet disorder (BTPD) Tier 1 genes exist ([https://www.isth.org/page/GinTh\\_GeneLists](https://www.isth.org/page/GinTh_GeneLists)) for which a well-curated and evidence-based catalogue of gene-disease associations for use in diagnostic genetic screening of patients has been developed by the International Society on Thrombosis and Haemostasis (ISTH) Scientific and Standardization Committee (SSC) on Genomics in Thrombosis and Haemostasis (GinTH).<sup>6</sup> A further 8 Tier 2 genes exist (*PRKACG*, *TRPM7*, *TPM4*, *EPHB2*, *PTPRJ*, *NFE2*, *BLOC155*, *PTGS1*) that require further pedigrees in order to reach the tier 1 threshold of three unrelated families with causal variants in the same gene.<sup>7</sup> Furthermore, a recent initiative to investigate pathogenic variants, variants of unknown clinical significance (VUS), and benign variants was set out by the SSC-GinTH and a tool developed (Gold variants) to capture the genomic variants detected in patients with BTPD.<sup>8</sup> In this study we describe and elucidate the genetic and functional basis of two unrelated families with three affected individuals with missense variants in *TPM4* who suffer from a significant bleeding diathesis with previously unknown genetic diagnosis and therefore make *TPM4* a Tier 1 diagnostic gene.

## 2 | METHODS

### 2.1 | Patient recruitment and testing

Patients were consented and recruited to the Genotyping and Phenotyping of Platelets (GAPP) study from multiple collaborating hemophilia centers across the UK and Ireland as previously described<sup>9</sup> and approved by the UK National Research Ethics Service by the Research Ethics Committee of West Midlands (06/

#### Essentials

- Identifying genetic variants in platelet disorders is challenging due to their heterogeneous nature.
- We performed detailed genetic and functional analysis in patients with missense variants in *TPM4*.
- *TPM4* missense variants are associated with reduced platelets secretion, spreading, and cytoskeletal defects.
- Findings underline the important role of *TPM4* in undiagnosed platelet disorders particularly for those with a normal platelet count.

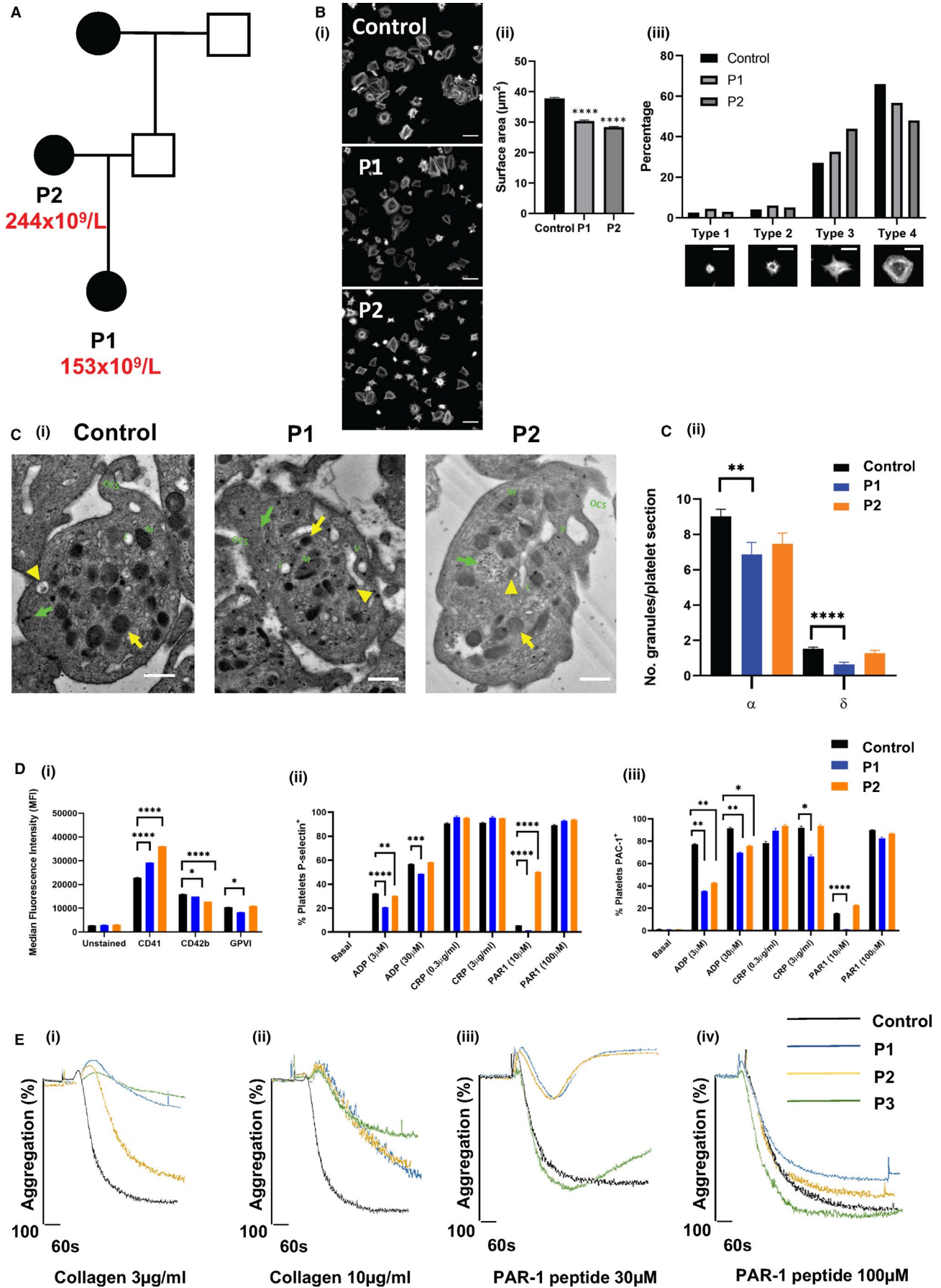
MRE07/36). The study cohort currently consists of >1000 patients with a strong history of bleeding and suspected of having a platelet function disorder of unknown cause as previously described.<sup>1,10</sup> The ISTH Bleeding Assessment Tool (BAT) evaluated patients' degree of clinical bleeding.<sup>11</sup> Platelet counts, mean platelet volume (MPV), and other hematological parameters were performed on the Sysmex whole blood analyzer.

### 2.2 | Sequencing

Whole exome sequencing (WES) and targeted analysis was performed in patient genomic DNA as previously reported.<sup>10</sup> WES filtering of candidate genetic variants was performed to identify rare variants classified as below a frequency of 0.0001 and as performed previously.<sup>10</sup> P1 and P2 were sequenced using the R90 bleeding and platelet disorders targeted gene panel performed at Oxford Regional Genetics Laboratories. The final sequence variants were confirmed in patients using Sanger sequencing.

### 2.3 | Platelet phenotyping

Peripheral blood was collected from patients and platelet phenotyping using lumiaggregometry or flow cytometry was performed on platelet-rich plasma (PRP) as previously described.<sup>10,12,13</sup> Platelet spreading, transmission electron microscopy (TEM), and western blotting were performed as previously described.<sup>7,14</sup>



**FIGURE 1** Platelet functional analysis of *TPM4* families with an inherited platelet disorder. A, Pedigree of family (F1) showing affected individuals (shaded) and platelet counts. B, Platelet spreading on fibrinogen, labeled with phalloidin-Alexa488 and imaged on a Zeiss Axio Observer7 microscope with a 63x 1.4NA oil objective, Colibri 7 LED light source, Zeiss filter set 38 for GFP/FITC and a Hamamatsu ORCA Flash 4 LT sCMOS camera. i, Representative images of spread platelets from P1, P2, and control; scale bar: 10  $\mu$ m. ii, Platelet surface area and platelet type calculated from all platelets in 10 fields of view for each condition (total >2500 platelets measured) using the Knime workflow.<sup>14</sup> Data on platelet surface area analyzed using the non-parametric Kruskal-Wallis test with multiple comparisons. iii, Platelet type analysis. Type 1: adhered, type 2: filopodia, type 3: filopodia and lamellipodia, type 4: spread with stress fibres. Scale bar: 5  $\mu$ m. Significance is shown against control. \*\*\*\**P* < .0001. C, Platelet transmission electron microscopy (TEM) showed reduced granule content in *TPM4* patients and enlarged vacuole spaces indicating increased platelet fragility. i, Representative images of platelet TEM sections in control and *TPM4* patient platelets. Alpha ( $\alpha$ ) granules (yellow arrow), dense ( $\delta$ ) granules (yellow arrowhead). Other platelet ultrastructure features include: mitochondria (M), lysosomes (L), open canalicular system (OCS) glycogen (green arrow), and large vacuoles only present in *TPM4*-patient platelets (V). Scale bar 500 nm. ii, Quantification of platelet  $\alpha$  and  $\delta$  granules from TEM images. Data presented is mean  $\pm$  standard error of the mean (SEM) from 30–60 platelets per sample. Significance assessed by one-way analysis of variance with correction for multiple comparisons. \*\**P* = .009, \*\*\*\**P* < .0001. D, *In vitro* assessment of platelet glycoprotein receptors and activation in P1, P2 and control. i, Resting platelet surface glycoprotein expression levels. Median fluorescence intensity (MFI). Mean  $\pm$ SEM of two technical replicates per sample; significance assessed with *t*-test comparing each patient to experimental control. *P*-value adjusted to correct for multiple comparisons (Holm-Sidak). \**P* < .05, \*\**P* < .01, \*\*\**P* < .001, \*\*\*\**P* < .0001. ii, Activated P-selectin and (iii) activated  $\alpha$ IIb $\beta$ 3 (PAC-1) expression on control/patient platelets in response to indicated agonist stimulation. The percentage of platelets expressing activation markers was assessed. Data presented is mean  $\pm$ SEM of two technical replicates per sample. Significance assessed with *t*-test comparing each patient to experimental control for each agonist. *P*-value adjusted to correct for multiple comparisons (Holm-Sidak) \**p* < .05, \*\**P* < .01, \*\*\**P* < .001, \*\*\*\**P* < .0001. E, Patients show abnormal aggregation responses to major platelet agonists. Platelet responses to (i) collagen 3  $\mu$ g/mL (ii) collagen 10  $\mu$ g/mL (iii) PAR-1 peptide 30  $\mu$ M and (iv) PAR-1 peptide 100  $\mu$ M

### 3 | RESULTS AND DISCUSSION

Both families (F1 and F2) presented here have a strong family history of clinical bleeding including easy bruising, prolonged bleeding after cuts, and poor healing. The patients (P1-P3) were recruited to the UK GAPP study for platelet function testing and genetic studies (Figure 1A, Table 1).<sup>15</sup> The index case (F1: P1) was a 14-year-old female recruited to the GAPP study with a strong history of a “probable” hereditary platelet disorder with epistaxis, cutaneous bleeding, bleeding from minor wounds, oral cavity bleeding, and menorrhagia. She had an initial platelet count of  $153 \times 10^9/L$  and MPV of 12.1 fL. Her ISTH BAT score was 14.<sup>11</sup> Her mother (F1: P2) was 49 years old when recruited to the study with a clinical history of epistaxis, cutaneous bleeding, bleeding from minor wounds, oral cavity bleeding and bleeding following tooth extraction, menorrhagia, and postpartum hemorrhage (BAT score 22). She had a platelet count of  $244 \times 10^9/L$  and MPV of 11.1 fL (Table 1). F2: P3 suffers from easy bruising and prolonged bleeding after minor cuts and dental extractions. She had a mild thrombocytopenia (last recorded platelet count  $119 \times 10^9/L$ ) and initial local investigations showed impaired aggregation to low concentrations of ADP, a prolonged lag phase with collagen, and reduced release of ADP and ATP using platelet nucleotide studies. All other hematological parameters for all three patients were within normal ranges.

Following these initial assessments, deep phenotyping of patients’ platelets was performed. Washed platelets from P1 and P2 demonstrated reduced spreading on fibrinogen compared to healthy controls as shown by representative images and platelet surface area quantification (Figure 1Bi and ii and performed as previously described).<sup>14</sup> Patients showed a greater number of platelets that exhibited both filopodia and lamellipodia and fewer were fully spread (Figure 1Bii, type 2, 3, and 4, respectively). Furthermore,

platelet TEM performed in P1 and P2 platelets showed reduced granule content in patients and enlarged vacuole spaces indicating increased platelet fragility (Figure 1C). Using the previously published streamlined GAPP agonist panel<sup>12</sup> and platelet activation flow cytometry and lumiaggregometry showed similarly abnormal responses to a range of agonists and secretion defects in all three patients studied (Table 1, Figure 1D,E). Resting platelet surface glycoprotein expression levels were altered in P1 and P2; with an increase in the expression of CD41, an expected result in patients with enlarged platelets; however, the expression of glycoprotein GPIb (CD42b) was decreased in P1 and P2 compared to the control. Flow cytometry data was unavailable from P3. Lumiaggregometry defects included reduced responses to both concentrations of collagen, lower concentration PAR1-peptide (Figure 1E), and significant differences in platelet activation measured by reduced  $\alpha$ -granule secretion (P-selectin) and integrin  $\alpha$ IIb $\beta$ 3 activation (PAC1; Figure 1Dii and iii, respectively). Platelet granule secretion was tested by assessment of the ATP-secretion levels in PRP using a luciferase assay as previously described.<sup>12</sup> This revealed significantly reduced levels in P1 and P3, consistent with aggregation and flow cytometry results (Table 1, Figure 1D,E). Taken together this extensive phenotyping revealed a significant platelet function disorder in all patients studied (P1-P3).

P1 and P2 were then investigated for a possible genetic cause using the R90 bleeding and platelet disorders targeted gene panel performed at Oxford Regional Genetics Laboratories. This detected a rare heterozygous missense substitution in both P1 and P2 in the *TPM4* gene (c.544C>T; p.R182C) and confirmed using Sanger sequencing (Figure 2A). Previously, whole exome sequencing detected a novel heterozygous missense substitution in P3 in *TPM4* (c.548C>T; p.A183V),<sup>10</sup> which neighbors p.R182C. R182 and A183 are in highly conserved regions of the protein (Figure 2B) with both variants located in the tropomyosin domain of *TPM4* (Figure 2C).

TABLE 1 Patient demographics and platelet function analysis in patients P1–P3 at time of recruitment

Family/ Patient	Age (y), sex	<i>TPM4</i> nucleotide alteration <sup>a</sup>	Effect on <i>TPM4</i> protein	Platelet count ( $\times 10^9/L$ ) <sup>b</sup>	Mean platelet volume (fL) <sup>c</sup>	ISTH BAT score <sup>d</sup>	Lumiaggregometry defects	ATP Secretion nmol/ $1 \times 10^8$ platelets <sup>e</sup>
<b>Family 1; P1</b>	11, female	c.544C>T	p.R182C	153	12.1	14	ADP (10, 30 $\mu$ M) Adrenaline (10, 30 $\mu$ M) Collagen (1, 3, 10 $\mu$ g/mL) AA (0–5, 1 mM) PAR-1 (30 $\mu$ M) (Deaggregation)	0.29
Family 1; P2	49, female	c.544C>T	p.R182C	244	11.1	22	Adrenaline (10, 30 $\mu$ M) Collagen (1, 3 $\mu$ g/mL) PAR-1 (30 $\mu$ M) (Deaggregation)	0.93
<b>Family 2; P3</b>	16, female	c.548C>T	p.A183V	119	11.1	NA	ADP (10, 30 $\mu$ M) Adrenaline (10, 30 $\mu$ M) Collagen (3, 10 $\mu$ g/mL) PAR-1 (30, 100 $\mu$ M)	0.21

Note: Heterozygous nucleotide changes present in *TPM4* and their predicted effects on the resulting protein are shown. Index cases are indicated in bold font.

Abbreviations: BAT, Bleeding Assessment Tool; ISTH, International Society on Thrombosis and Haemostasis; NA, not available.

<sup>a</sup>Alterations are numbered according to positions in *TPM4* transcript NM\_001145160.1.

<sup>b</sup>Mean platelet counts are shown, normal reference range is  $150\text{--}400 \times 10^9$  platelets/L, thrombocytopenia is defined as platelet count  $<150 \times 10^9$  platelets/L.

<sup>c</sup>Mean platelet volumes are shown, normal reference range is 7.83–10.5 fL.

<sup>d</sup>ISTH bleeding assessment tool score, 95<sup>th</sup> percentile (score of 4) calculated from healthy volunteers.<sup>11</sup>

<sup>e</sup>ATP secreted in response to 100  $\mu$ M PAR-1 receptor specific peptide SFLLRN, 5th centile in healthy volunteers is  $0.82 \text{ nmol}/1 \times 10^8$  platelets.

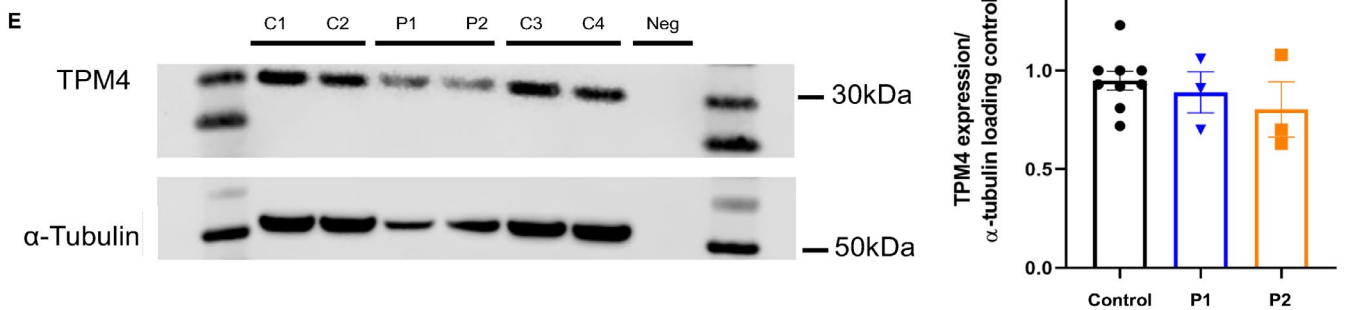
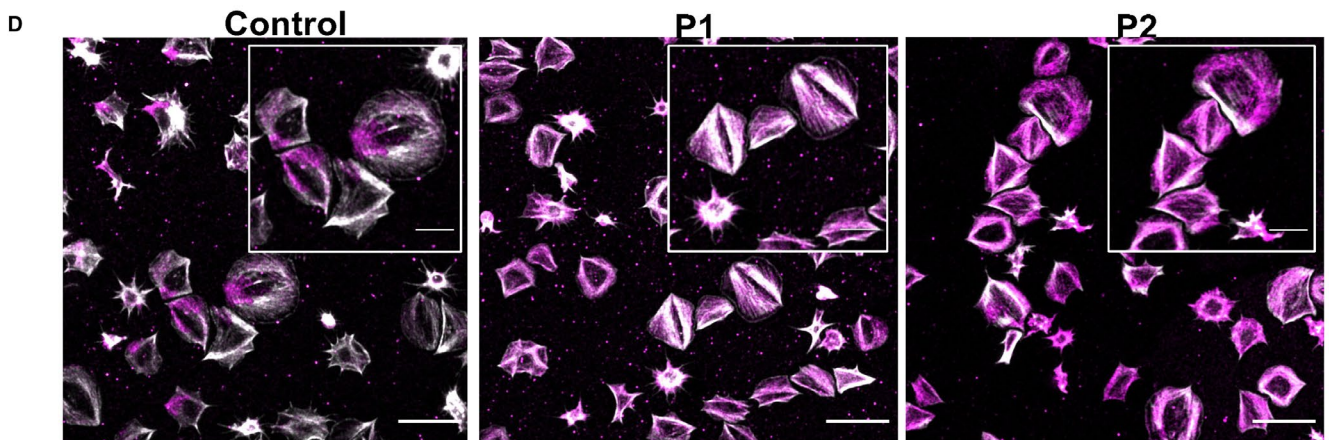
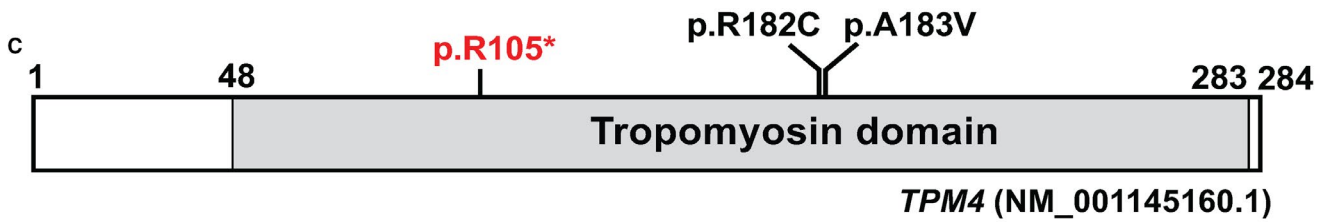
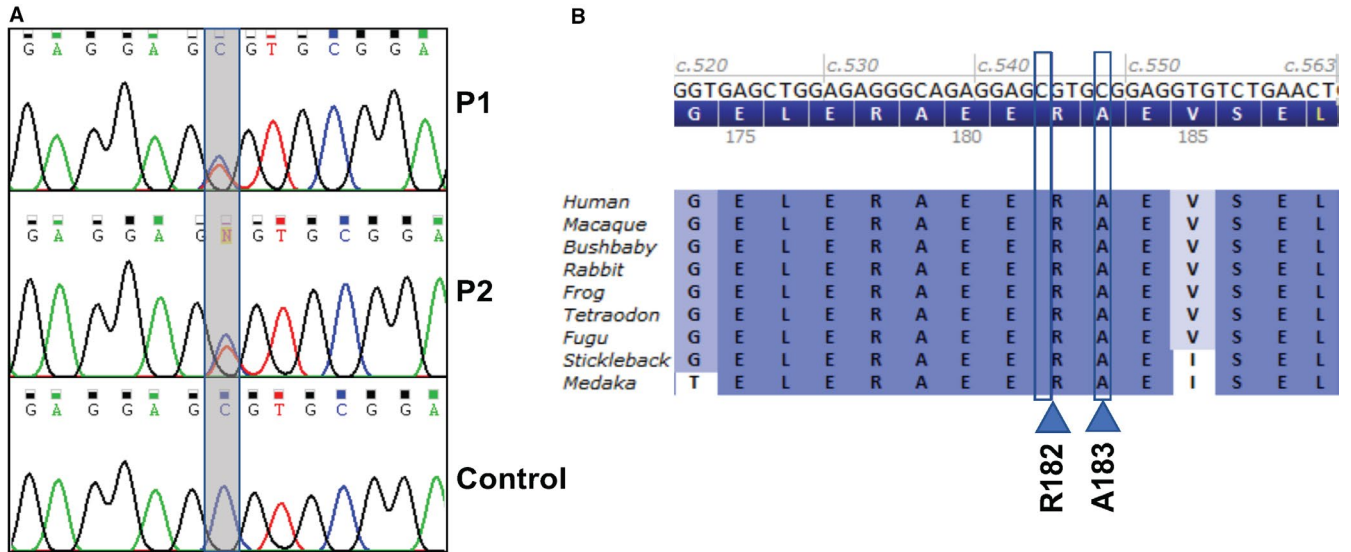
The p.R182C variant was classified as a VUS according to the current American College of Medical Genetics and Genomics (ACMG) guidelines.<sup>16</sup> Supporting evidence was given including PM2 (absent from controls—or at extremely low frequency—in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium) and PP3 (multiple lines of computational evidence support a deleterious effect on the gene or gene product). Further in gnomAD, a constraint score for missense in *TPM4* was  $z = 0.95$  and frequency of 2/246478 alleles.

Tropomyosins are dimers of coiled-coil proteins that polymerize end-to-end along the major groove in most actin filaments and provide stability to the filaments, regulating access of other actin-binding proteins.<sup>17</sup> In mammals, more than 40 tropomyosin

isoforms can be generated through alternative splicing from four tropomyosin genes. Different isoforms display non-redundant functions and partially non-overlapping localization patterns, for example within the stress fiber network. One such stress fiber-associated tropomyosin Tpm4.2 has previously been associated with a platelet disorder and a macrothrombocytopenia with a nonsense mutation in *TPM4*.<sup>18</sup> To further investigate whether the p.R182C variant in this study disrupted tropomyosin-4 structures and the associated actin filaments, immunofluorescence was performed in platelets from P1 and P2 to assess phalloidin staining together with a *TPM4*-specific antibody. Washed platelets from P1 and P2 spread onto fibrinogen displayed disordered tropomyosin-4 staining compared to a healthy control

**FIGURE 2** Identification and investigation of novel germline missense variants in *TPM4* in families with an inherited platelet disorder. A, Targeted Sanger sequencing of the polymerase chain reaction product of *TPM4* exon 4 showing the position of the c.544C>T (p.R182C) heterozygous substitution in the genomic DNA of patients 1, 2, and a healthy control. B, Multiple protein alignment showing the conservation of the two amino acid substitutions in *TPM4* in all species shown. The positions and conservation of the p.R182C and p.A183V variants are shown by the boxed regions. C, Schematic showing the location of the *TPM4* variants in patients P1–P3. Sequencing analysis of P1 and P2 from family 1 detected a novel heterozygous amino acid substitution of *TPM4* (p.R182C). In a separate family 2 (P3) an amino acid substitution was detected (p.A183V).<sup>12</sup> The previously reported nonsense variant (p.R105\*) is shown in red font.<sup>18</sup> The tropomyosin domain spans from amino acids 48 to 283. Genetic alterations are numbered according to positions in the NM\_001145160.1 transcript for *TPM4* (largest isoform). D, *TPM4* immunofluorescence showing disordered localization in patient platelets spread on fibrinogen.<sup>11</sup> Representative cropped images from platelets derived from P1, P2, and control samples, seeded onto fibrinogen, fixed and co-stained for *TPM4* (magenta), phalloidin (white), and imaged in 3D using AiryScan confocal microscope. Scale bar 10  $\mu$ m, zoomed panel 5  $\mu$ m. E, *TPM4* protein expression was measured from platelet lysates of *TPM4* patients compared to normal controls. No difference was observed in protein expression of *TPM4*. Expression was normalized to the  $\alpha$ -tubulin loading control. Images were analyzed in ImageJ software and significance assessed in Prism.  $N = 3$  experiments with 3 control samples used in each experiment. Data presented is mean expression relative to  $\alpha$ -tubulin  $\pm$  standard error of the mean





(Figure 2D). In addition, platelet *TPM4* protein levels did not appear to be affected by the missense mutations present in P1 and P2 and were thus comparable to controls assessed by western blot (Figure 2E).

This report describes three patients from two unrelated families with an inherited platelet disorder and history of excessive bleeding. Both families harbor rare conserved missense variants (p.R182C and p.A183V) in *TPM4* encoding tropomyosin-4, a member of the tropomyosin family of actin-binding proteins involved in the contractile system of striated and smooth muscles and the cytoskeleton of non-muscle cells. To date, only a single *TPM4* variant associated with *TPM4* has been reported in two apparently unrelated families, in which a truncating mutation is associated with an autosomal dominant macrothrombocytopenia, named *TPM4*-linked macrothrombocytopenia.<sup>18</sup> While all three patients in our study displayed enlarged platelets, only two had a mild thrombocytopenia, and this variability was also apparent in the Pleines study,<sup>18</sup> illustrating the importance of genetic diagnosis in families with variable presentation of platelet counts, even within a single family (F2).

Deep platelet phenotyping studies revealed similar platelet function defects across the three patients including reduced platelet secretion, and aggregation and spreading defects suggesting that *TPM4* missense variants impact platelet function. Further immunofluorescence imaging revealed a disordered pattern of tropomyosin staining particularly in platelets. In conclusion, identification of genetic and functional *TPM4* defects in these patients/families makes *TPM4* a Tier 1 gene and highlights the importance of including *TPM4* in diagnostic genetic screening for patients with significant bleeding and undiagnosed platelet disorders particularly for those with a normal platelet count.<sup>2</sup>

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Prof Neil Morgan has authorized authorship on behalf of the UK Genotyping and Phenotyping of Platelets Study Group.

## CONFLICT OF INTEREST

The authors report no conflicts of interest.

## AUTHOR CONTRIBUTION

NVM designed the study; WL and GL recruited the patients; RJS, NSP, AOK, CWS, CF, PB, WL, GL, and NVM extracted or generated clinical or experimental data and interpreted the results; GL and NVM undertook governance of the study; NVM wrote the manuscript; all authors read and approved the final version of the manuscript.

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