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Identification of the Novel G250R Variant Indicates a Role for Thrombomodulin in Modulating the Risk for Venous Thromboembolism

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The susceptibility to venous thromboembolism (VTE) is largely accounted by the clustering of several, possibly inherited, risk factors.[1] Studies in twins and families show that VTE is highly heritable and follows a multifactorial non-Mendelian inheritance model, involving interaction with clinical risk factors.[2–4] However, the risk varies greatly from one individual to another and an important proportion of VTEs remains unidentified in most cases.[5]

Thrombomodulin (THBD) plays a key role in the regulation of thrombin activity, acting as an intrinsic anticoagulant and leading to protein C activation.[6] Results from animal models support the hypothesis that an impairment of THBD anticoagulation properties may predispose to thrombosis.[7,8] In addition, in Chinese and African-American populations a role for common THBD gene (*THBD*) variants was suggested.[9,10]

We investigated a multiplex Italian family, consisting of close relatives who had a history of recurrent VTE without any identifiable cause despite extensive investigations.

After the approval of the local ethics committee, the study was performed according to the principles of the Declaration of Helsinki; informed consent was obtained from all subjects. Patients II-4 and II-5 were screened using whole exome sequencing (WES). WES was outsourced to GATC Biotech (Konstanz, Germany). DNA samples were extracted from 0.2 mL of whole blood (EDTA-treated) according to standard protocols. Variant annotation and prioritization were performed with open-source software (Variant Studio, Illumina, San Diego, California, United States). Variants were classified according to American College of Medical Genetics and Genomics standards and guidelines as pathogenic, likely pathogenic, variants of uncertain significance, or benign.[11] The presence of the *THBD G250R* variant was investigated by direct sequencing of genomic DNA in all available family members.

The amino acid residue #250 lies in the EGF-1 domain. The effect of the p.G250R substitution was investigated by using in silico prediction of pathogenicity (Polyphen-2, http://genetics.bwh.harvard.edu/pph2/; SIFT, http://sift.jcvi.org/sift-bin/retrieve_enst.pl).

The thrombomodulin AlphaFold structure prediction model (AF-P07204-F1.pdb)[12] was then used as a template to investigate the putative pathogenic effect of the p.G250R substitution by using established available bioinformatics tools (MISSENSE3D and Swiss-PdbViewer).

Thrombin generation (TG) in platelet-poor plasma (PPP) was measured by calibrated automated thrombogram (CAT) in triplicate. CAT is a system to measure TG based on fluorescence according to manufacturer instructions (Diagnostica Stago, Italy). PPP was prepared by two

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centrifugation steps at 3,000 *g* for 10 minutes and aliquots were stored at −80°C. TG was measured in all samples, simultaneously. To minimize variation, samples of normal pool plasma were measured on the same plate.

To evaluate the functional consequences of the identified *THBD* variant, site-directed mutagenesis experiments were performed to insert the identified mutation into a full-length human *THBD* complementary DNA plasmid (pCMV6-Vector, Myc-DDK-tagged; OriGene, Rockville, Maryland, United States) by using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technology, Santa Clara, California, United States). Wild-type or mutated *THBD* cDNA plasmids were transiently transfected in human embryonic kidney (HEK) 293 cells (constitutively deficient in THBD). Transfections into HEK293 cells were performed with Turbofectin 8 (Origene, Rockville, Maryland, United States). To assess amounts of expression of recombinant THBD, mRNA expression levels of wild type or mutant THBD were analyzed 48 hours after transfection by quantitative real-time polymerase chain reaction (PCR). Total RNA was extracted from transfected cells. The mRNA reverse transcription was performed by SuperScript IV First Strand Synthesis System (Life Technology, Brooklyn, New York, United States). All samples were assayed on a CFX96 Touch Real-Time PCR detection System using iQ SYBR green supermix (Bio-Rad Laboratories Inc., Hercules, California, United States). The relative amounts of the transcripts of wild-type or mutated THBD were evaluated. GAPDH was used as endogenous reference genes. CFX manager software was used to perform experiment setup and data analysis *p*-value less than 0.05 was considered statistically significant).

To analyzed recombinant THBD protein levels, Western blotting of cell lysates was performed using a monoclonal anti-THBD antibody [PBS-02] (ABCAM). Cellular localization of expressed wild-type and mutant THBD constructs was studied using immunofluorescent labeling and confocal microscopy analysis. Cell surface expression of wild-type and mutant THBD constructs was investigated in nonpermeabilized HEK293 cells using flow cytometry.[13] Nonpermeabilized transfected HEK cells were incubated with mouse monoclonal antithrombomodulin antibody [PBS-02] (Epitope EGF domain 2 of thrombomodulin) for 1 hour, washed with phosphate-buffered saline and then labeled with anti-mouse FITC secondary antibody for 30 minutes at 22°C, rinsed, and sorted using AMNIS flow cytometer (Luminex Corporation, Austin, United States) set on channel 2. Data were analyzed using Amnis IDEAS software subtracting the values of the negative control. A scatter plot was obtained by plotting fluorescence intensity (Ch 2) on the *x*-axis versus side scatter (Ch 06) on the *y*-axis. The single and focus cell events for the sample were gated, and finally acquired. The percentage of green positive cells (channel 2, 488 nm excitation laser) and mean fluorescence were analyzed using Amnis IDEAS software subtracting the values of the negative control. Brightfield and fluorescence images for any single cell event were collected.

A multiplex Italian family, in which many relatives had a history of recurrent VTE without any identifiable cause despite extensive investigations, was examined (►**Fig. 1A**). The index case (II-5) was a 53-year-old man, with a previous documented thrombosis (42 years) in the right-sided popliteal vein and pulmonary embolism. After cessation of the anticoagulation, a new episode of right-sided popliteal vein thrombosis occurred (49 years). A brother (II-4) suffered from a thrombotic event in the left-sided popliteal vein (32 years) and from a recurrent event 10 years later. The remaining brother (II-3) had a thrombosis in the right-sided popliteal vein (43 years). Then, he died of an accident at work (46 years). In addition, a first cousin of the propositus (II-2) suffered from a thrombotic event in the right-sided popliteal vein (61 years) and a child of her (III-3) from recurrent venous thromboses. WES of the two living affected siblings (II-4, II-5), performed assuming a dominant mode of inheritance, identified the exon 1 *THBD* variant c.748G > C (NM_000361.3), predicting an amino acid change protein (p.G250R) located in the EGF-1 domain. Direct Sanger sequencing (Applied Biosystems, Foster City, California, United States)[14–16] confirmed the presence of the heterozygous missense mutations in both II-3 and II-2 and in a first cousin of the propositus (II-2) and her asymptomatic daughter (III-4) (►**Fig. 1A**). The *THBD* variant was novel, being unreported in GnomAD and in BRAVO, and in silico predicted to be deleterious. Multiple amino acid sequence alignment of THBD orthologs from different species showed that G250 was conserved across all the analyzed species and suggested that this amino acid change might cause structural defect and modify THBD function (►**Fig. 1B**).

The anticoagulant functions of THBD provide support to the hypothesis that an impairment of anticoagulation properties may predispose to thrombosis. THBD is an intrinsic membrane protein of 575 amino acid residues located on the luminal side of the endothelium.[6] It possesses a domain structure comprising a lectin-like domain (residues 31–169), six tandem epidermal growth factor-like domains joined by small interdomain peptides (EGF1 [241–281], EGF2 [284–324], EGF3 [325–363], EGF4 [365–405], EGF5 [404–440], EGF6 [441–481]), a serine/threonine-rich domain (residues 482–515), a transmembrane domain (residues 516–539), and an intracellular domain (residues 540–575). Functional studies indicate that thrombin binding is mediated by THBD EGF5–6 domains while the EGF-4 domain is required for protein C binding to the thrombin–thrombomodulin complex. EGF3–6 domains are essential for CPB2 (plasma carboxypeptidase B2) activation. The lectin-like domain is essential for constitutive endocytosis. The functions of the EGF1–2 domains remain unknown.

The thrombomodulin AlphaFold structure prediction model (AF-P07204-F1.pdb) was used as a template to investigate the putative pathogenic effect of the p.G250R.

The in silico analysis (MISSENSE3D; http://www.sbg.bio.ic.ac.uk/missense3d/) predicted a detrimental effect on the domain structure. Indeed, the substitution replaces a buried uncharged residue (250G: relative solvent accessibility [RSA]: 0.0%) with a charged residue (250R: RSA: 4.4%). This substitution triggers clash alert. The local clash score for the wild type is 7.65 and local clash score for the mutant is

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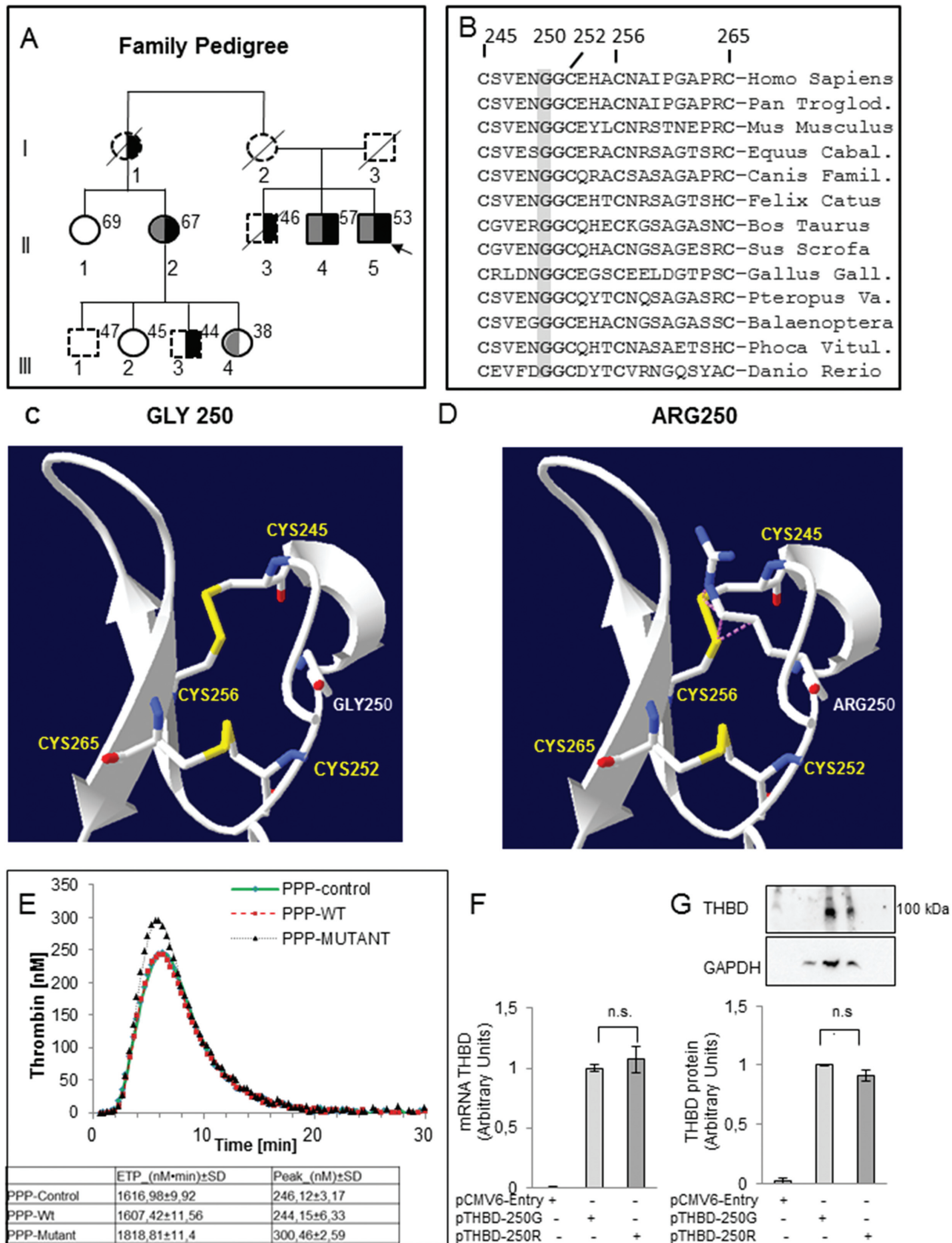

	ETP_(nM•min)±SD	Peak_(nM)±SD
PPP-Control	1616,98±9,92	246,12±3,17
PPP-Wt	1607,42±11,56	244,15±6,33
PPP-Mutant	1818,81±11,4	300,46±2,59

Fig. 1 (**A**) Italian family investigated. Family tree showing cosegregation of the clinical phenotype (*black half-filled symbol*) and the THBD-250R variant (*gray half-filled symbol*). The *dashed symbol* indicates a family member who was not investigated. Current age years are indicated above the symbol. (**B**) Alignment of a portion of the THBD EGF-1 domain containing the amino acid position #250 (highlighted in *gray*) along different species. Conserved residues are indicated (*bold*). (**C, D**) Comparison of the predicted structures of both wild type (C) and mutant protein (D) using SwissPdbViewer (https://spdbv.unil.ch/); Clashes are displayed in *pink dotted lines* in mutant protein. (**E**) Plasma thrombin generation obtained on triplicate runs in an asymptomatic carrier of the 250R variant (III-4: mutant), in her sister (III-2) with the 250G allele (nonmutant), and in a control subject. THBD mRNA (**F**) and protein production (**G**) of human embryonic kidney (HEK 293T) cells (constitutively deficient of THBD) overexpressing the wild-type p.G250 allele (THBD-250G) or the mutant p.250R (THBD-250R). Insert shows western blot results. Data (mean±S.E.) from three independent experiments are shown. n.s.: not significant ($p > 0.05$) for cells overexpressing THBD-250G as compared with the THBD-250R allele.

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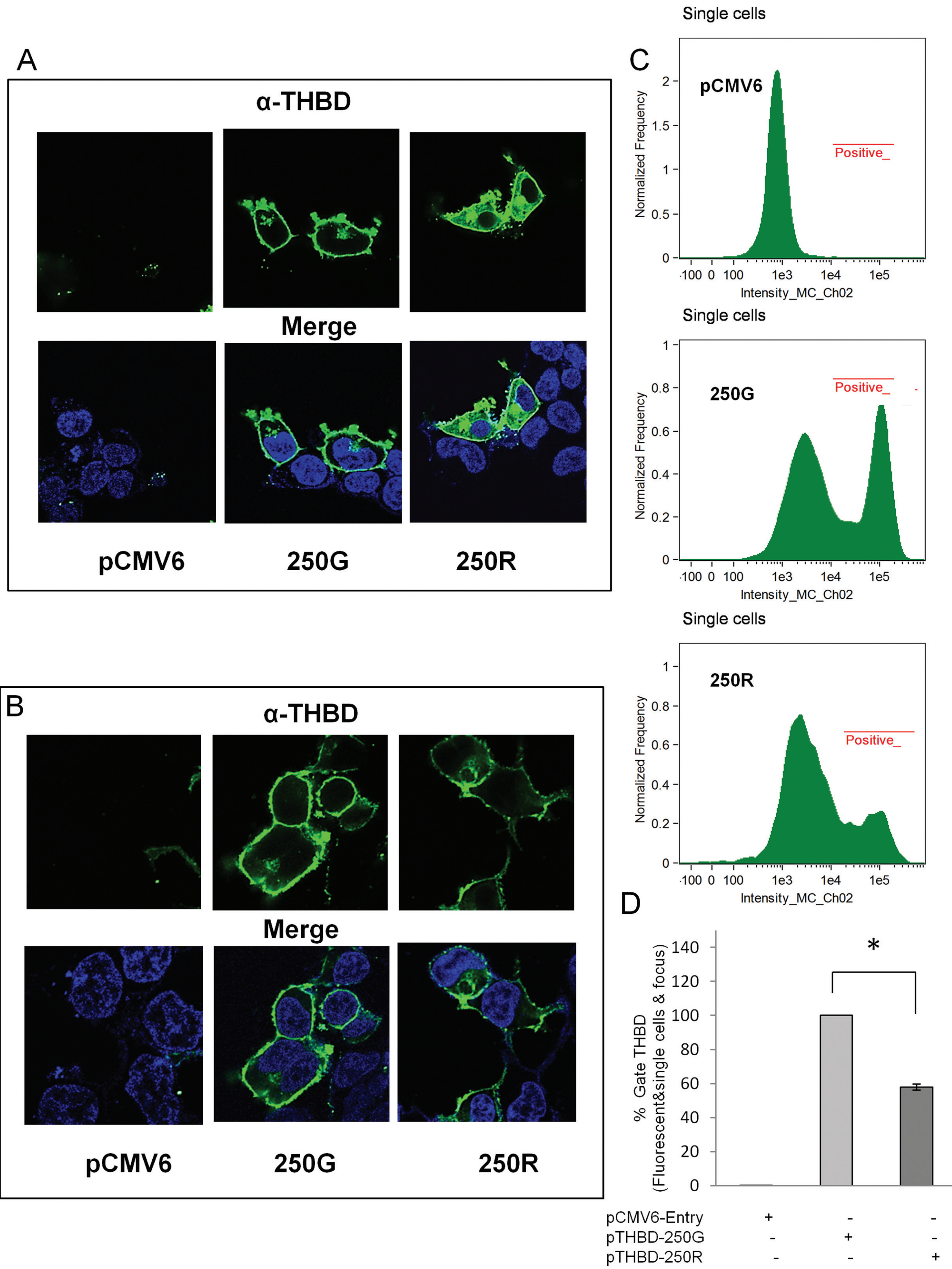

Fig. 2 Confocal microscopy in permeabilized (**A**) and not permeabilized (**B**) HEK293 cells transiently transfected with the wild-type and mutant construct. *Green* staining documents the presence of THBD. *Blue* staining identifies the nucleus. (**C**) Graphical representation of THBD distribution as determined by flow cytometry analysis. p.CMV6: empty plasmid. 250G: wild-type allele; 250R: mutant p.250R allele. Similar data were found in two additional experiments. Data are the mean ± SE from three independent experiments. $^{*}p < 0.01$. Gate (%) of cells expressing on surface THBD-250R as compared to cells expressing THBD-250G. SE, standard error.

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36.85. ►Fig. 1C and D show comparison of the predicted structures of both wild-type and mutant protein (Swiss-PdbViewer (https://spdbv.unil.ch/); clashes is displayed in pink dotted lines in mutant protein (►Fig. 1D).

TG assays are used widely to investigate the thrombin generating capacity in an asymptomatic THBD-250R carrier (III-4), not assuming oral anticoagulants, and her sister (III-2), who carries only the 250G allele. Samples of normal pool plasma were measured on the same plate. TG in PPP was measured by CAT in triplicate.

III-4 showed a higher TG with an increased endogenous-thrombin-potential (ETP) (nM min) and peak (nM) values, indicating a hypercoagulable state as compared with her sister and controls (►Fig. 1E).

THBD mRNA expression (►Fig. 1F) and western blot analysis (►Fig. 1G) showed that THBD expression was similar in transfected HEK293 cells with the THBD-250G or the THBD-250R allele ($p > 0.05$ NS, nonsignificance). Thus, the THBD-250R variant did not appear to modulate THBD mRNA synthesis and protein expression levels, suggesting that the new variant is unlikely to have any significant effect on gene expression.

In permeabilized fixed THBD-250G-overexpressing cells, the protein was distributed predominantly on the plasma membrane surface, with little evidence of intracellular localization (►Fig. 2A). In contrast, in THBD-250R-transfected cells, an intracellular punctate pattern was observed with a clear-cut decrease of the cell surface membrane fluorescence intensity. Indeed, the distribution of the signal of mutant protein throughout the cell suggests that the intracellular signal detected comes from the endoplasmic reticulum (ER). The different pattern of cell distribution suggests that the THBD-250R variant decreases its localization on the cell membrane. The intracellular location was confirmed by parallel experiments in which permeabilization was not performed before fixation. In this case, fluorescence was markedly lower on the cell surface (►Fig. 2B). Flow cytometry analysis detected lower amounts of cells expressing on surface THBD-250R (►Fig. 2C), with an approximately 43% reduction of gate as compared with cells expressing THBD-250G, confirming that the THBD-250R subcellular distribution is significantly modified with a lower localization on the plasma membrane. All data indicate that the THBD-250R variant is not able to sufficiently target the plasma membrane, likely preventing efficient posttranslational processing or, alternatively, via an increased internalization.

In the present study, we searched for new causative genetic defects in an Italian family with unexplained VTE using the WES approach. Massive sequence analysis of two affected relatives improved the diagnostic rate by facilitating sequence variant analysis and enabling detection of a novel disease-causing gene. In this family, we identified a novel rare *THBD* missense mutation, with an autosomal dominant inheritance, that resulted in a variant protein (250R). The G250R substitution greatly affected cellular localization of the protein, also in absence of thrombin binding. Because of the impairment of THBD exposure on the plasma membrane, the 250R variant can produce a lower thrombin binding. Indeed, the bleeding phenotype in patients carrying a THBD mutation associated with increased soluble protein levels and decreased TG further stresses the pivotal role of THBD as a key regulator.[17,18] The resulting increased amounts of unbounded thrombin are expected to induce an imbalance between its procoagulant (increased fibrin formation) and anticoagulant (impaired activated protein C [APC] generation) properties. The expression characterization of the previously unreported *THBD* 250R variant lends support to findings that carriership of *THBD* variants with an impairment of anticoagulation properties may represent a genetic risk factor for VTE.

THBD is known to have additional functions and plays an important role in modulating inflammation through several indirect and direct pathways, which may also contribute to protection against venous thrombosis.[19] In mouse models, the lectin-like domain was demonstrated to play a pivotal role in the regulation of inflammation suggesting that reduced THBD exposure on the endothelial surface increases the risk for thrombosis.[20] Downregulation of THBD may modulate the risk for thrombosis also via an impairment of its regulation of inflammation and complement system.

In conclusion, functional analyses of the *THBD* 250R variant show that defective THBD folding and trafficking to the plasma membrane could underlie the susceptibility to venous thrombosis in the family investigated, further suggesting that THBD loss-of-function mutants could be an underappreciated cause of thrombophilia.

Essentials

- The role of thrombomodulin in venous thrombosis remains unknown.
- We investigated a multiplex Italian family with a recurrent venous thromboembolism without any identifiable cause.
- The unreported variant G250R gives additional insights into the role of thrombomodulin in the regulation of coagulation.
- We strengthen the role of thrombomodulin deficiency in the risk for venous thromboembolism.

Author Contributions
M.D., A.A., R.S., and M.M. designed research studies; M.D. A., A.A., G.D., and G.L.T. conducted the experiments and acquired and analyzed data, A.B.M. and M.M. wrote the manuscript, and all authors reviewed and provided comments on the work.

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Conflict of Interest
None declared.

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