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ORIGINAL ARTICLE

Assessment of a next generation sequencing gene panel strategy in 133 patients with negative thrombophilia screening

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Abstract

Background: Although heritability of venous thromboembolism (VTE) is high, the thrombophilia screening appears to be positive only in a minority of VTE patients. Adding rare variants screening to identify VTE missing heritability still requires further assessment.

Objectives: We report the results of a panel strategy after 3 years of application.

Methods: We performed the sequencing of 28 genes related to coagulation cascade and/or VTE using high-throughput sequencing in133 unrelated patients with a personal history of VTE and negative thrombophilia screening. Only variants with minor allele

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frequency <0.1% were classified according to the American College of Medical Genetics recommendations. We recorded class 3, 4, and 5 variants.

Results: We identified class 3, 4, or 5 variants in 46 patients resulting in an identification rate of 35%. Out of the 45 recorded variants, 35 were considered as class 3 (78%), 9 were class 4 (20%), and 1 was class 5 (2%). Four genes accounted for nearly two-thirds (27/45) of the identified variants: *SERPINC1*, *PROS1*, *F2*, and *F5*. We observed a high rate of recurrent variants in the *SERPINC1* and *PROS1* genes, including the Cambridge II (*SERPINC1* p.A416S), Dublin (*SERPINC1* p.V30E), and Heerlen (*PROS1* p.S501P) variants. The elevated frequency of these variants in a symptomatic population, compared to their frequency in the general population, provides strong support for their association with VTE risk. We identified 4 (likely) pathogenic variants in *F2*: p.R596Q (*F2* Belgrade), p.R541W, p.P386T, and p.R425L.

Conclusion: The high proportion of class 3 variants emphasizes the need for functional studies to better characterize and classify them.

KEYWORDS
high-throughput nucleotide sequencing, thrombophilia, venous thromboembolism

1 | INTRODUCTION

Venous thromboembolism (VTE) has a significant genetic component, with heritability estimated to range between 35% and 60% [1–3]. When VTE occurs in a young individual (typically under 50 years old) in the absence of major transient risk factors (surgery with general anesthesia >30 minute, immobilization, bed rest >3 days etc), it raises suspicion for an underlying genetic abnormality. In such cases, a clinical routine diagnostic approach will generally rely on a combined exploration including plasma assays for antithrombin, protein C, and protein S levels, along with genotyping for 2 common variants: factor (F)V Leiden and *F2* c.*97G>A (formerly G20210A prothrombin variant). This exploration yields positive results in 30% to 40% of cases. This thrombophilia assessment has remained unchanged since the 1990s and the discovery of *F2* c.*97G>A. However, over the past 15 years, genome-wide association studies (GWASs) have identified numerous variants associated with VTE. In 2022, a meta-GWAS involving over 80 000 cases and 1 400 000 controls identified more than 100 polymorphisms associated with VTE [4]. These variants are common (minor allele frequency [MAF] ≥ 0.01) and generally only slightly increase the risk of VTE (odds ratio < 1.3). Therefore, they cannot be used individually to explain a patient's thrombotic phenotype or estimate their VTE risk. Their utility is more suitable for epidemiologic use, particularly within predictive risk scores for VTE.

Alongside GWASs, the widespread adoption of molecular biology tools, such as high-throughput sequencing, has facilitated gene-candidate approaches in families presenting severe thrombotic phenotypes. As example, one can cite the *F9* Padua variant identified in an Italian family in 2009. The propositus exhibited an unprovoked proximal deep vein thrombosis. While the conventional thrombophilia screening was negative, biological investigations revealed a significant increase in FIX activity. Gene sequencing and family segregation analysis identified the causal gain-of-function variant p.R338L [5]. Another example is the p.A2086D variant located on *F5* (Factor V Besançon) that was identified in a young homozygous carrier affected with recurrent VTE. Interestingly, this patient had markedly decreased FV levels (activity = 3%; antigen = 4%) without any signs of bleeding [6]. Furthermore, several variants have been identified on the *F2* gene in symptomatic patients and families. These variants lead to antithrombin resistance. Three variants were found at position 596: p.R596L (*F2* Yukuashi), p.R596Q (*F2* Belgrade), and p.R596W (*F2* Padua 2) [7–9].

Finally, the FVIII Padua is an example of a gene duplication involved in VTE. The partial *F8* duplication has been recently identified in 2 Italian families. The variant cosegregated with VTE and with high FVIII levels [10].

All these variants share the common characteristics of being private or very rare and segregating with the thrombotic phenotype in the families studied. They are located on genes involved in the coagulation cascade but are not routinely detected in the conventional constitutional thrombophilia assessment.

Based on these findings, our reference center for thrombophilia has developed a panel approach to identify potentially causal variants in selected patients. Here, we presented the results of this strategy after 3 years of application.

2 | METHODS

2.1 | Patients

Patients with negative thrombophilia assessment were included retrospectively and prospectively. The thrombophilia assessment was performed according to the proposals of the Groupe Français d'Etudes sur l'Hémostase et la Thrombose (GFHT) [11]. Those proposals recommend the assessment of thrombophilia in the following situations:

Men or women under the age of 50 years who experienced an episode of unprovoked proximal deep vein thrombosis (DVT), pulmonary embolism (PE), or a recurrence of distal DVT not triggered by a major factor (surgery or immobilization for a duration of 3 days or more within the 3 months preceding the thrombotic event).

Women of childbearing age who experienced an episode of provoked or unprovoked proximal VTE, unprovoked distal DVT, VTE associated with hormonal factors, or PE triggered by a major factor.

We extended these criteria to include patients under the age of 50 who experienced unprovoked distal DVT in the presence of a first-degree family history of VTE.

Patients were predominantly enrolled at the Centre d'Exploration des pathologies Hémorragiques et Thrombotiques (CEHT) at La Timone Hospital, Marseille (Assistance Publique des Hôpitaux de Marseille-APHM). We prospectively included patients from March 2020 (initiation of the next generation sequencing [NGS] gene panel strategy) and March 2023. In addition, retrospective inclusions were made prior to March 2020 for all patients fitting the inclusion criteria for whom stored DNA was available in sufficient quantity. Patients underwent a consultation with a specialist physician, which included validating the documented nature of thrombotic events and the provoked/unprovoked status of VTE episodes using a systematic questionnaire. VTE events were documented through spiral computed tomographic scanning angiography, ventilation/perfusion lung scan, duplex ultrasound, and/or venography.

In addition, patients from other French centers with comparable expertise were included. The same eligibility criteria were respected, and these inclusions were made prospectively only, between August 2021 and March 2023.

Informed written consent was obtained in accordance with the Declaration of Helsinki. The study was approved by the local Ethics Committee at APHM Hospital, Marseille, France (number = PADS24-235_dgr).

2.2 | Sample preparation

Blood samples were collected during the initial consultation through antecubital venipuncture using vacutainer tubes containing 0.105 mol/L trisodium citrate. Platelet-poor plasma was obtained by centrifugation at 2500 × *g* for 10 minutes, aliquoted, and stored at −80 °C. DNA was extracted from EDTA tube samples. All samples were stored at the Biological Resources Center of the APHM (authorization number DC-2008-429).

2.3 | Thrombophilia assessment

All included patients underwent both constitutional and acquired thrombophilia assessment.

Thrombophilia parameters were evaluated from frozen samples of citrated plasma. Coagulation inhibitor deficiencies were defined by decreased levels of the following: antithrombin chromogenic heparin cofactor activity (<80%, STA-Stachrom AT III), free protein S plasma antigen (<55% in women and <60% in men, STA-liatest free protein S), and protein C activity (<70%, HEMOCLOT Protein C) respectively. For children, we used age-dependent reference ranges.

Factor (F)V Leiden and *F2* c.*97G>A variant were analyzed from whole blood DNA using light cycler technology (Roche Diagnostics).

The following parameters of antiphospholipid syndrome were investigated: anti-B2GP1 and anti-cardiolipin IgG and IgM antibodies (in-house reagents), and lupus anticoagulant (PTT-LA, STA-Staclot DRVV screen, STA-Staclot DRVV confirm).

It should be noted that the thrombophilia assessment was not complete for all patients at the time of exploration when the patient was on anticoagulant treatment. In such cases, the search for lupus anticoagulant could not be performed. Protein C and S assays were not conducted during vitamin K antagonist therapy. Protein C activity was measured using a chromogenic method (Biophen Protein C) during direct oral anticoagulant therapy.

2.3 | Panel of coagulation genes

2.3.1 | Design of the NGS gene panel

In all patients with negative thrombophilia screening, targeted gene panel NGS was performed for a set of genes known to be associated with VTE and/or directly related to the coagulation cascade. The panel included genomic targets comprising coding exons and flanking regions of each exon. The panel comprised 26 genes related to the coagulation cascade: *ABO*, *F2*, *F3*, *F5*, *F7*, *F8*, *F9*, *F10*, *F11*, *F12*, *F13A1*, *F13B*, *FGA*, *FGB*, *FGG*, *KNG1*, *PROC*, *PROCR*, *PROS1*, *SERPINA1*, *SERPINA5*, *SERPINC1*, *SERPINE1*, *TFPI*, *THBD*, *VWF*. In addition, the panel comprised 2 genes identified by large scale genomic explorations as involved in VTE etiology and for which functional variants have been characterized: *MAST2* and *SLC44A2* [12,13]. *SRY* was included for quality control purposes.

2.3.2 | Library preparation and sequencing

Enrichment was performed with a Qiagen amplicon custom design (QIAseq targeted Custom Panel, ref.333525, 1693 primers design,

along with B index kit ref. 333727), integrating unique molecular identifiers for copy number variant analysis. Library quality was verified based on concentration and migration profile.

Sequencing and demultiplexing was performed with Illumina MiSeq sequencer (24 patients per pool) or NextSeq sequencer (40 patients per pool). Average coverage was 591X, with 99.4% of coding and flanking sequence (20 bases around each exon) sequence covered >30X. Average coverage per gene is shown in Supplementary Table S1. Sequencing quality was estimated based on sequencer metrics (cluster density, reads passing filters, base quality, number of reads generated), and using FastQC analysis.

2.3.3 | Bioinformatics analysis

Bioinformatics analysis was performed using CLC Genomic Workbench 21 and 22 (Qiagen), with parameters adapted to unique molecular identifiers and amplicon-based sequencing. The software generated binary alignment map, variant call format, and coverage files, along with a list of annotated variants and a report including metrics and pipeline parameters used. Variant call format files are secondarily reannotated using VarAFT software (https://varaft.eu) [14].

Variants filtering was performed as follows: variants with a MAF <0.1% in gnomAD Exome, gnomAD Genome and 1000G databases (https://gnomad.broadinstitute.org/; https://www.internationalgenome.org/), and MAF <25% in a local database composed of 32 patients with VTE phenotype (not included in the present study) to remove sequencing artifacts and highly frequent variants. Variants were considered as heterozygote in an individual if they were supported between 30% and 70% of aligned reads (based on nonduplicated sequences) and homozygotes if they were present in more than 90% of the nonduplicated sequences of an individual. Possible mosaicism was not taken into consideration.

2.3.4 | Bioinformatics predictions and variant interpretation

Deleterious (pathogenic) effect was estimated using different algorithms included in VarAFT [14] and MobiDetails (https://mobidetails.iurc.montp.inserm.fr) [15], literature and databases search, familial segregation if available. SPiP and SpliceAI scores dedicated to splicing effect prediction were collected from MobiDetails [16,17]. Splicing predictions based on SPiP, SpliceAI, and QUEPASA (ΔtESRseq scores via HExoSplice link) methods were extracted from MobiDetails for all identified synonymous variants [16–18]. ΔtESRseq were directly retrieved from HExoSplice (http://bioinfo.univ-rouen.fr/HExoSplice/) for variants located in large, first, and last exons [18]. The presence of all identified candidate variants was further confirmed by Sanger sequencing.

The identified variants were interpreted and classified according to the guidelines of the American College of Medical Genetics (ACMG) [19]. Only variants falling into classes 3 (variant of uncertain significance [VUS]), 4 (likely pathogenic), and 5 (pathogenic) were collected.

3 | RESULTS

3.1 | Patients' phenotype

We here presented the results of 133 patients who underwent the gene panel testing. We included 50 patients retrospectively (38%) and 83 patients prospectively (62%). Most of the patients (124 patients, 93%) were included at CEHT in Marseille.

Table 1 reports the main characteristics of the included patients. The sex ratio was close to 1 (51% of men), and the age at the first episode of VTE was 34.4 years. Two children aged 2 and 8 years were included in the study.

The first episode of VTE was a PE with or without DVT in 63 patients (47%). Additionally, 47% of patients had presented with isolated DVT in the lower limbs, with a proximal location in 54% of cases. Other locations (cerebral, digestive, and upper limbs) were observed in 5% of cases. Seventy-six percent of men had experienced an unprovoked first episode of VTE. This rate was lower in women (54%). A hormonal context (pregnancy, postpartum, combined oral contraceptive use) was identified in 34% of them, and VTE was provoked by other circumstances in 12% of cases.

Nearly half of them had experienced a recurrent thrombotic event (44%). Most patients had one or more first-degree family history of VTE (72%).

It should be noted that the thrombophilia assessment had not been completed in 12 patients (9%) due to anticoagulant treatment at the time of exploration.

3.2 | Results of genetic exploration

The performance of the panel of 28 genes allowed the identification of at least one candidate variant of class 3, 4 or 5 in 46 patients resulting in an identification rate of 35%. Of note, 8 patients presented 2 pathogenic candidates. Out of the 45 recorded variants, 35 were considered as class 3 (78%), 9 were class 4 (20%), and 1 (*F2* p.R596Q = *F2* Belgrade variant; rs387907201) was class 5 (2%).

3.2.1 | Candidate pathogenic variants in the 3 main natural coagulation inhibitors: *SERPINC1*, *PROC*, *PROS1*

We identified 13 variants belonging to classes 3 or 4 in the genes *SERPINC1*, *PROC*, and *PROS1*, which respectively encode antithrombin, protein C, and protein S [20–26]. Results are presented in Table 2 and Supplementary Table S2.

Only one variant has been identified in *PROC* (p.A43T; rs767626189) [22]. This variant is likely deleterious. Protein C levels could not be measured in the patient carrying the variant due to

TABLE 1 Clinical characteristics of the 133 patients with venous thromboembolism (VTE).

Male, *n* (%)	**68 (51)**
Age at first VTE episode, mean (range)	34.4 (2-50)
Localization of VTE, *n* (%)	
Pulmonary embolism ± DVT	63 (47)
DVT only of lower limb	63 (47)
Distal	29 (46)
Proximal	34 (54)
Other (including CVT, digestive, and upper limb)	7 (5)
Circumstances, *n* (%)	
Male (*n* = 68)	
Unprovoked VTE episode	52 (76)
Female of reproductive age (*n* = 65)	
Unprovoked VTE episode	35 (54)
Hormonal (pregnancy, postpartum, COC)	22 (34)
Other	8 (12)
First-degree family history of VTE, *n* (%)	96 (72)
Recurrent VTE, *n* (%)	58 (44)
Uncomplete thrombophilia screening, *n* (%)	12 (9)

COC, combined oral contraceptive; CVT, cerebral venous thrombosis; DVT, deep vein thrombosis; VTE, venous thromboembolism.

anticoagulant treatment. The variant is present in general population databases (MAF gnomAD v4 Exome = 8.621×10^{-5}), and it has been reported to be associated with a moderate qualitative deficiency in protein C in a symptomatic patient and an asymptomatic female with a family history of VTE [22,27]. Therefore, this variant is probably responsible for a protein C deficiency that we were unable to confirm objectively.

We identified one class 4 variant in the *SERPINC1* gene in a male patient who experienced an unprovoked distal DVT at age 38 with a first-degree family history of VTE. This variant is the nonsynonymous p.E145K (rs1363092061). Antithrombin levels could not be measured in the patient due to anticoagulant treatment. Additionally, 5 variants of uncertain significance were identified in *SERPINC1*. Most of them were recurrent variants (ie, variants present in more than 1 individual): 2 patients carried the *SERPINC1* p.A416S variant (rs121909548; referred to as Cambridge II variant), and 2 patients carried the *SERPINC1* p.V30E variant (rs2227624; referred to as Dublin variant) [20,21]. Antithrombin levels were normal for all 4 patients. Furthermore, we found 1 variant that had never been reported before (p.L375V) in a female patient with a personal history of unprovoked distal DVT at age 32 years and a first-degree family history of VTE. We also observed the *SERPINC1* p.R294H (rs587776397) and p.V327A (rs773399107), respectively, in 2 patients who also had normal antithrombin levels. These variants were carried by 2 male patients who both experienced PE at age 47 and 50 years, respectively, with first-degree family history of VTE. One of them had recurrent VTE.

Moreover, we identified 6 VUS in the *PROS1* gene. Among those variants the *PROS1* p.S501P (rs121918472; referred to as Heerlen variant) was carried by 6 patients [24]. Free protein S antigen levels were normal in 5 patients (ranging from 61% to 95%), but unknown in the last patient. Likewise, there was no deficiency in patients carrying the other variants (levels ranging from 65% to 109%).

3.2.2 | Other genes

We identified 32 class 3 or higher pathogenic candidates in the following 13 genes: *F2*, *F3*, *F5*, *F7*, *F8*, *F10*, *F11*, *F12*, *F13A1*, *FGA*, *FGG*, *TFPI*, *THBD* (Table 3) [28–37]. These were mainly VUS (*n* = 24; 75%), with 5 and 9 mapping to the *F2* and *F5* genes, respectively. Twelve variants had never been reported before.

3.2.3 | Focus on *F2* variants

Out of the 5 variants we identified on *F2* (Table 3, Supplementary Table S2), 1 was pathogenic and 3 were likely pathogenic. The variant p.R596Q, known as the Belgrade variant, is considered pathogenic. It is extremely rare in general population databases (MAF gnomAD v4 Exome = 6.84×10^{-7}). It has been reported in a few families with a significant VT phenotype [8]. We identified it in a young 14-year-old patient who had an unprovoked proximal DVT complicated by PE. Factor II level was 65%.

We identified the class 4 p.R541W variant (rs773399107) in a patient who experienced iliac venous thrombosis complicated by PE at the age of 21, following a 5-hour car trip. The patient had a recurrence (proximal DVT of the lower limb) and had several familial history of VTE: father, paternal grandmother, paternal uncle, and a cousin. Among them, 3 had thrombosis around the age of 20. The variant is not present in general population databases, but it has been reported in the literature in several families [28–30].

We identified the class 4 p.R425L variant in 2 unrelated patients. This variant is not described in general population databases (nor in the literature). Like the previous variant, it is located in the peptidase S1 domain. The variant was identified in 2 patients with comparable profiles. Both experienced thrombosis at a very young age. The first patient is a young woman who had a proximal DVT at the age of 19 with no triggering factor other than taking a combined oral contraceptive pill. She had a recurrence (proximal DVT during pregnancy) and had a family history of VTE: her sister had a cerebral venous thrombosis at the age of 29, and her father had a proximal DVT at the age of 80. All 3 are heterozygotes for the variant. The FII level was decreased in the proband and her father (64% and 54%, respectively). The measurement could not be performed in the sister.

The other patient carrying the p.R425L variant is a man who experienced a proximal DVT complicated by PE at the age of 21, occurring in the context of a calf muscle tear. He experienced a

TABLE 2 Variants located on *SERPINC1*, *PROC*, and *PROS1* genes.

Patient	Gene	Reference sequence	Variant	Reported	Reference	ACMG classification	Plasmatic level[a]
001	*SERPINC1*	NM_000488	c.1246G>T:p.A416S	rs121909548 (Cambridge II)	20	3	97%
003	*SERPINC1*	NM_000488	c.433G>A:p.E145K	rs1363092061	-	4	NA
012	*SERPINC1*	NM_000488	c.1123C>G:p.L375V	No	-	3	120%
018	*SERPINC1*	NM_000488	c.89T>A:p.V30E	rs2227624 (Dublin)	21	3	107%
020	*SERPINC1*	NM_000488	c.89T>A:p.V30E	rs2227624 (Dublin)	21	3	91%
051	*SERPINC1*	NM_000488	c.881G>A:p.R294H	rs587776397	-	3	101%
110	*SERPINC1*	NM_000488	c.1246G>T:p.A416S	rs121909548 (Cambridge II)	20	3	96%
129	*SERPINC1*	NM_000488	c.980T>C:p.V327A	rs773399107	-	3	92%
006	*PROC*	NM_000312	c.127G>A:p.A43T	rs767626189	22	4	NA
002	*PROS1*	NM_000313	c.428G>T:p.C143F	rs867656728	-	3	65%
003	*PROS1*	NM_000313	c.155G>A:p.G52D	rs1709034447	-	3	109%
009	*PROS1*	NM_000313	c.284G>A:p.G95E	rs144526169	23	3	80%
026	*PROS1*	NM_000313	c.1501T>C:p.S501P	rs121918472 (Heerlen)	24	3	79%
027	*PROS1*	NM_000313	c.1501T>C:p.S501P	rs121918472 (Heerlen)	24	3	95%
052	*PROS1*	NM_000313	c.1501T>C:p.S501P	rs121918472 (Heerlen)	24	3	70%
054	*PROS1*	NM_000313	c.1501T>C:p.S501P	rs121918472 (Heerlen)	24	3	61%
062	*PROS1*	NM_000313	c.227C>T:p.P76L	rs73846070	25	3	78%
117	*PROS1*	NM_000313	c.557G>A:p.C186Y	rs779391826	26	3	81%
126	*PROS1*	NM_000313	c.1501T>C:p.S501P	rs121918472 (Heerlen)	24	3	NA
132	*PROS1*	NM_000313	c.1501T>C:p.S501P	rs121918472 (Heerlen)	24	3	65%

All variants were identified in the heterozygous state.

[a] For the corresponding protein: antithrombin for *SERPINC1* variants, protein C for *PROC* variants, and protein S for *PROS1* variants. NA for not available (patient under anticoagulant treatment).

ACMG, American College of Medical Genetics.

recurrent distal DVT at the age of 24. The medical history revealed many family members with thrombotic events. Specifically, his mother had a provoked PE, his brother had a provoked DVT of the upper limb, and his cousin died from postoperative PE. The family investigation determined that the variant segregated with the thrombotic phenotype. Indeed, 2 symptomatic relatives carried the variant, while 2 asymptomatic relatives did not. The FII levels were not known in the family.

3.2.4 | Focus on *F5* variants

Variants located on *F5* were predominantly VUS (Table 3, Supplementary Table S2). Only one variant was considered likely pathogenic: p.R334T known as FV Cambridge (rs118203906). The patient carrying this variant experienced proximal DVT at age 38 years and had a first-degree family history of VTE.

The class 3 p.M1811L variant was identified in 2 patients. The first one was a 30-year-old woman experiencing unprovoked DVT of the lower limb with a first-degree family history of VTE. The second one was a 33-year-old man experiencing unprovoked DVT and PE, also with a first-degree family history of VTE.

3.2.5 | Other variants

The variants identified in the other genes were mainly class 3 non-synonymous variants [32–37]. Two patients presented an extreme thrombotic phenotype. The first one was a 2-year-old child with recurrent VTE. The first episode was a proximal DVT that had occurred a few days after chicken pox infection. The young patient experienced a recurrent unprovoked proximal DVT (contralateral leg) 6 months after stopping anticoagulant treatment. We identified a *F3* duplication (whole gene). The duplication was found in the patient's father who had experienced 2 strokes at a young age (21 and 22) and duplex ultrasound identified sequelae of asymptomatic proximal DVT.

The second patient was an 8-year-old child with a personal history of unprovoked DVT and PE. The patient had no family history of VTE and no early recurrence. We identified a class 3 *F10* variant.

TABLE 3 Variants located on other genes.

Patient	Gene	Reference sequence	Variant	Reported	Ref	ACMG	Plasmatic level[a]
005	*F2*	NM_000506	c.1156C>A:p.P386T	rs5897	-	4	NA[b]
007	*F2*	NM_000506	c.370C>T:p.R124W	rs766900043	-	3	NA
087	*F2*	NM_000506	c.1274G>T:p.R425L	No	-	4	64%
091	*F2*	NM_000506	c.1274G>T:p.R425L	No	-	4	NA
097	*F2*	NM_000506	c.1621C>T:p.R541W	rs886048338	28–30	4	NA
125	*F2*	NM_000506	c.1787G>A:p.R596Q	rs387907201	8	5	65%
048	*F3*	NM_001993	c.300_301insGATGTGAAG :p.Q101delinsDVKQ	No	-	3	NA
127	*F3*	-	duplication	No	-	4	66.9 pg/mL[c]
008	*F5*	NM_000130	c.1001G>C:p.R334T	rs118203906	31	4	NA
008	*F5*	NM_000130	c.4414T>G:p.S1472A	rs749081272	-	3	NA
010	*F5*	NM_000130	c.4630G>T:p.V1544L	rs139288793	-	3	NA
011	*F5*	NM_000130	c.5520C>G:p.H1840Q	No	-	3	82%
023	*F5*	NM_000130	c.724A>G:p.M242V	rs1191142747	-	3	NA
031	*F5*	NM_000130	c.5431A>T:p.M1811L	rs138877178	-	3	87%
037	*F5*	NM_000130	c.5431A>T:p.M1811L	rs138877178	-	3	NA
091	*F5*	NM_000130	c.5446C>T:p.P1816S	rs141977229	-	3	NA
102	*F5*	NM_000130	c.203A>G:p.E68G	No	-	3	>150%
129	*F5*	NM_000130	c.283G>A:p.G95R	rs376110672	-	3	87%
050	*F7*	NM_000131	c.803_805del: p.268_269del	No	-	3	NA
001	*F8*	NM_000132	c.5976G>A:p.M1992I	rs782510443	32	3	96%
017	*F8*	NM_000132	c.5024A>G:p.Q1675R	rs782280813	-	3	NA
033	*F8*	NM_000132	c.4288G>A:p.D1430N	rs373836326	-	3	NA
014	*F10*	NM_000504	c.922G>A:p.V308I	No	-	3	NA
133	*F7 F10*	-	duplication	No	-	4	>180%
016	*F11*	NM_000128	c.809A>T:p.K270I	rs121965070	33	3	NA
105	*F12*	NM_000505	c.1848delA:p.X616CextTer47	No	-	3	NA
094	*F13A1*	NM_000129	c.232C>T:p.R78C	rs760818476	34	3	NA
013	*FGA*	NM_000508	c.902G>C:p.G301A	No	-	3	2.54 g/L
013	*FGA*	NM_000508	c.945delT:p.T315fs	rs1214070111	35	4	2.54 g/L
004	*FGG*	NM_000509	c.1130G>T:p.G377V	rs369271819	-	3	NA
007	*FGG*	NM_000509	c.571G>A:p.G191R	rs6063	36	3	3.49 g/L
132	*TFPI*	NM_006287	c.509A>G:p.E170G	rs769566808	37	3	NA
101	*THBD*	NM_000361	c.930C>G:p.C310W	No	-	3	NA
118	*THBD*	NM_000361	c.1020T>G:p.C340W	No	-	3	1.6 ng/mL[d]

[a] For the corresponding protein.
[b] NA for not available (patient under anticoagulant treatment or missing plasma sample).
[c] Normal values = 21.0-49.4 pg/mL.
[d] Normal values = 2.1-5.1 ng/mL. All variants were identified in the heterozygous state.
ACMG, American College of Medical Genetics.

Among other remarkable results, a 33-year-old male patient experiencing unprovoked DVT and PE carried duplications of *F7* and *F10* (whole genes). This patient had increased FVII and FX levels (activity > 180%).

We are currently investigating the functional consequences of those 3 defects.

3.2.6 | Synonymous variants

We identified a total of 17 synonymous variants (Supplementary Table S3). We submitted these variants in MobiDetails and consulted splicing dedicated predictions with SPiP, SpliceAI and QUEPASA tools. The obtained predictions suggested possible splicing defects for at least 8 of these variants. Interestingly, for *F5* p.S2172S and *FGG* p.D106D, both SPiP and ΔtESEseq scores are predictive of a splicing alteration potentially by affecting splicing regulatory elements. Moreover, SpliceAI predictions suggest that the *PROS1* p.S569S variant can alter splicing by the gain of an acceptor splice site. At least for these 3 variants, functional assays are needed to confirm the predicted effects. Besides, additional bioinformatics methods are needed to clarify the potential effect on splicing of the 5 remaining variants with ΔtESEseq < −0.5.

4 | DISCUSSION

Since 2020, our laboratory has implemented an NGS panel of 28 coagulation genes, performed for patients with negative thrombophilia screening. This strategy led to the identification of class 3 or higher variants in 46/133 patients, resulting in an overall identification rate of 35% (Figure). The identification rate of pathogenic or likely pathogenic variants was 8% (11 patients).

Gene panel strategy has already been reported in the field of VTE. Lotta et al. [38] sequenced 18 genes related to the coagulation cascade in 10 cases (patients under the age of 55 with a history of unprovoked DVT and negative thrombophilia screening) and 12 controls. They identified a higher number of rare variants in cases than in controls (9 vs 2 on *PROC*, *SERPINC1* and *PROZ*).

Lee et al. [39] selected 55 genes (24 genes in common with our panel) previously described as involved in the coagulation cascade or in VTE. Even though the identification rate of class 3 or higher variants was 52% in this population and could be explained by the number of genes studied and the differences in inclusion criteria, the identification rate of likely pathogenic variants was only 5%, which is lower than in our study.

Two studies utilized high-throughput sequencing for diagnosing hemostasis-related disorders, analyzing 106 and 63 genes, respectively, with 30 and 28 genes related to coagulation or anticoagulation (20 overlapping with our panel) [40,41]. The first study reported a 58% identification rate, including 50% VUS, though its broader panel indications encompassed inherited bleeding and platelet disorders alongside thrombotic disorders. Unlike our study, it included patients with known major coagulation inhibitor deficiencies, which we excluded. The second study identified likely pathogenic variants in 9.68% of a mixed cohort with unexplained inherited bleeding, thrombotic, and platelet disorders, a rate comparable to our 8% detection of pathogenic or likely pathogenic variants. Several studies performed whole-exome sequencing to identify genetic risk factors in families with unexplained hereditary VTE. For example, Cunha et al. [42] were able to identify 6 putative disease-risk candidates performing whole-exome sequencing in 2 families.

Our NGS panel strategy showed promising results suggesting that it should be considered for patients with negative thrombophilia screening, although medico-economic issues require to better identify patients for whom the test will be profitable. For this purpose we determined the identification rate of different subgroups of patients according to the main clinical characteristics. The only factor being relevant seemed to be the unprovoked nature of VTE episode (identification rate = 46%; Supplementary Table S4).

Out of the 46 patients with identified variants, 19 (41%) had variants located on the major coagulation inhibitors genes. This result underlines the lack of sensitivity of conventional plasmatic assays as antithrombin, protein C, and protein S levels (when available) were normal in all included patients. Among these 19 patients, 10 carried well-described recurrent variants located on *SERPINC1* and *PROS1*: antithrombin Cambridge II, antithrombin Dublin, and protein S Heerlen [20,21,24]. Although their implication in VTE is debated, several large studies seem to indicate an association between those 3 variants and VTE, with reported odds ratios of about 9, 6 and 3 for Cambridge II, Heerlen, and Dublin variants, respectively [43–45]. In our study, the MAF observed for the antithrombin Cambridge II and Dublin variants and the protein S Heerlen were 0.008 for the first two and 0.02 for Heerlen. These values are substantially higher than those reported in the gnomAD v4 Exome population database (0.0015, 0.0027, and 0.0032, respectively) providing support for their assessment in a clinical context. For these 3 variants, the NGS panel strategy seems to be an excellent complementary investigation for patients explored with a negative conventional thrombophilia screening.

The difficulties in identifying genetic defects of major coagulation inhibitors, based on plasmatic assays, lead to questioning the position of gene sequencing in the exploration strategy. We have assessed the panel strategy as a complementary exploration in case of negative thrombophilia screening. However, it could be also considered as a first intention test, as it seems to show greater sensitivity than plasmatic assays for identifying major coagulation inhibitors defects. This last point deserves to be specifically assessed as plasmatic deficiencies without identified variant have been reported. They are mainly due to acquired deficiencies or analytical interferences but persistent familial deficiencies can be observed suggesting a genetic cause. Such cases could be explained by variants located in noncoding regions usually not investigated or could involve other genes (eg, *MPI* variants causing congenital disorders of glycosylation are associated with antithrombin deficiency) [46].

Even though the identification rate of class 4 or 5 variants was only 8%, it holds clinical importance for the families being explored by providing better understanding of the mechanisms responsible for

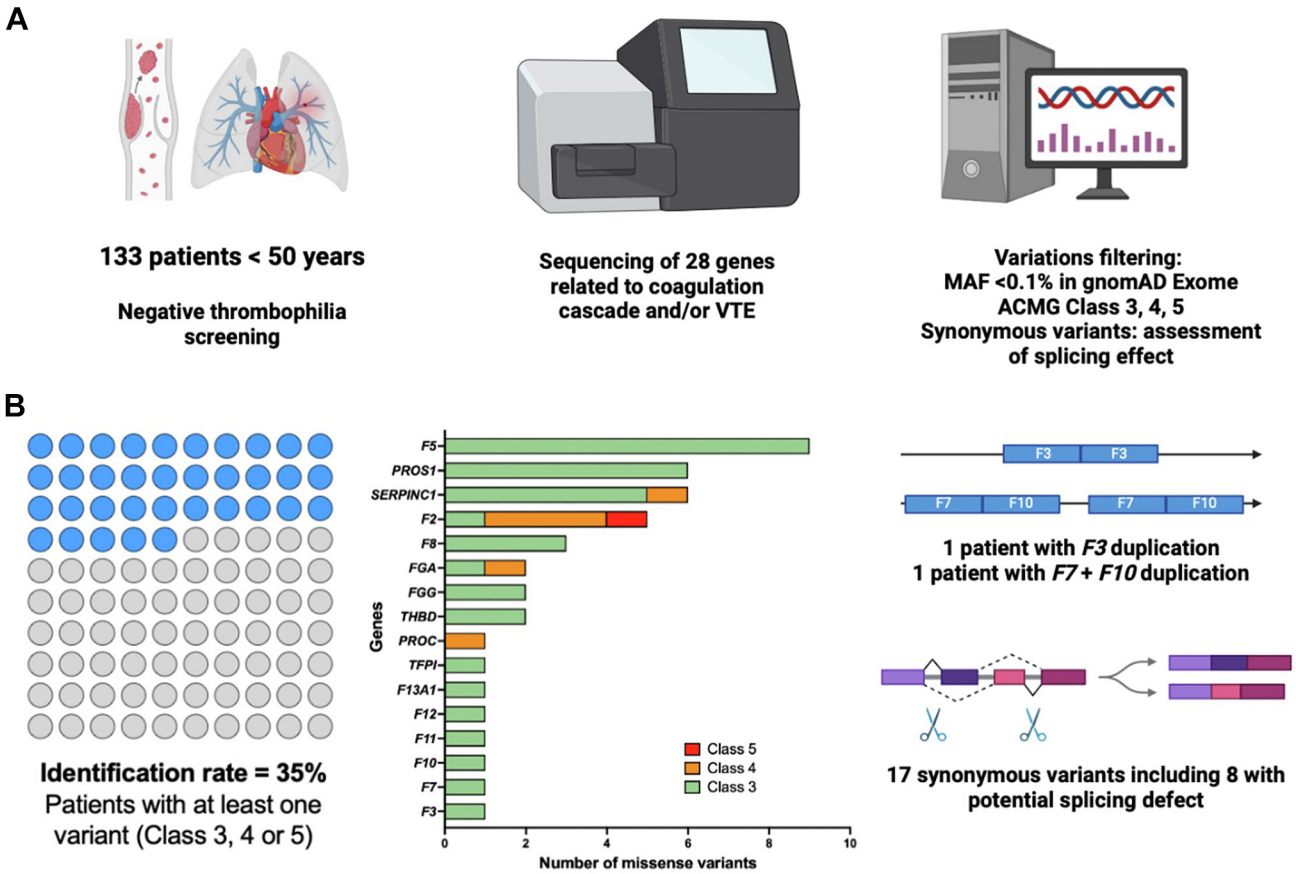

FIGURE Methodology and main results. **(A)** Patients with a personal history of venous thromboembolism (VTE) before the age of 50 and a negative thrombophilia screening have been selected for the panel strategy: sequencing of 28 genes related to the coagulation cascade and/or venous thromboembolism. Identified variants were filtered according to the minor allele frequency in general population databases. class 3 (variant of uncertain significance), 4 (likely pathogenic), and 5 (pathogenic) according to the American College of Medical Genetics classification were recorded. Splicing predictions were systematically extracted from MobiDetails for all identified synonymous variants. **(B)** We identified a class 3, 4, or 5 variants in 46/133 patients (35%). Identified variants were mainly class 3 variants (78%). Four genes accounted for nearly two-thirds of the identified variants: *SERPINC1*, *PROS1*, *F2*, and *F5*. We identified whole gene duplications in 2 patients (1 patient with *F3* duplication and 1 patient with both *F7* and *F10* duplications). We identified 17 synonymous variants. The obtained predictions suggested that we cannot exclude splicing defects with at least 8 of these variants. MAF, minor allele frequency.

VTE and by allowing the screening of at-risk relatives, which appears of utmost importance. The identification of the family defect contributes to improve the management and counseling among asymptomatic relatives.

The identification of a rare variant using the panel strategy raises several major questions. First, the deleterious potential of the variant must be assessed. Next, it is necessary to establish a link between the variant and the phenotype studied. Finally, if this association is established, the strength between the variant and the pathology needs to be quantified. This last step can be complex for variants that have already been reported (eg, Heerlen and Dublin variants). It is even more complex for variants that have never been reported. In such cases, family segregation studies, whenever possible and informative, are essential. Among the variants we reported in our study, some have led to improved management of patients and their families. In particular, because of the accumulating evidence for the *F2* c.1621C>T:p.R541W variant (ie, variant identified in several families, segregation with thrombotic phenotype in families), we have been able to conclude that it represents severe thrombophilia. As such it is screened in asymptomatic relatives, with the same consequences as the genetic risk factors usually explored. Finally, the Belgrade variant (*F2* c.1787G>A:p.R596Q) was taken into account in the therapeutic decision in the index case who presented an unprovoked DVT plus PE at the age of 14, in favor of long-term anticoagulation.

The interpretation of our results should consider a few limitations. We had limited additional phenotypic biological data for patients with an identified variant. Specifically, plasma levels of mutated proteins were mostly unknown due to lack of available plasma samples. However, the NGS panel strategy generates the identification of numerous variants that are either unknown or whose effect on the thrombotic phenotype is not known, and it appears challenging to explore all of them.

The identification of a class 4 variant in an established gene with incomplete penetrance does not exclude the possibility of other

variants contributing to VTE risk, making diagnosis, risk prediction, and genetic counseling difficult. As a consequence, the identification of a mild thrombophilia (heterozygous factor (F)V Leiden or *F2* c.*97G>A) should not be considered as the only causative variant. Therefore, it is essential to study family segregation and if mild thrombophilia does not segregate with thrombotic phenotype, screening for a rare variant using gene panel sequencing should be considered. More generally, the study of familial segregation should be conducted as extensively as possible when a potentially pathogenic variant is identified in a symptomatic family. Our results show that some variants absent from general population databases are found in several families (eg, the *F2* p.R425L variant identified in 2 families). The establishment and enrichment of international databases, including phenotypic characteristics, could facilitate the interpretation of these results. Moreover, the study of familial segregation should be conducted as extensively as possible when a potentially pathogenic variant is identified in a symptomatic family.

The identification rate was 35% in a selection of patients with negative conventional thrombophilia screening. As a perspective for improving this yield, the panel strategy, currently based on the sequencing of the coding regions of 28 genes known to associate with VTE, could be reassessed. First, it is mandatory to further characterize synonymous variants with potential splicing effect, using additional bioinformatics tools or minigene strategy. Second, we could implement the panel with noncoding regions (UTRs and introns) that have been previously involved in protein S deficiency or hemophilia B, for example [47,48]. Third, our panel only explores a fraction of the genes known to associate with VTE. Whereas the panel has been designed in 2020 and included 28 genes, most recent epidemiologic data have identified more than 100 genes involved in the pathology [4]. Including several new candidates in the panel could improve the identification rate of candidate variants. However, little is known about the pathophysiological role of these new candidate genes resulting in challenging interpretation, classification, and functional characterization of variants that would be detected. If the number of genes screened increases, the relevance of the panel-based strategy will become questionable and whole exome/genome sequencing will have to be considered [49].

In conclusion, the panel strategy appeared promising with 35% identification rate. It paves the way for the diagnosis of new familial defects in VTE. Our study underlines the limitations of this new tool and points to several avenues for improvement.

AUTHOR CONTRIBUTIONS

P.S., D.-A.T, and P.-E.M conceived and designed the study. P.S., C.B., A.M., V.E., M.-C.B, N.S., A.T., C.B-A., M.Y.D., J.C., P.-S.R., F.B-J., V.L.C.D., and L.D. collected data. P.S., O.S., C.B., N.S., D.-A.T., and P.-E.M. analyzed and interpreted the data. P.S., O.S., C.B., A.M., V.E., M.-C.B., N.S., A.T, C. B-A., M.Y.D., J.C., P.-S.R., F.B-J., V.L.C.D., L.D., D.-A.T., and P.-E.M. revised and approved the manuscript.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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