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PHYSIOPATHOLOGY OF VENOUS THROMBOEMBOLISM IN PATIENTS WITH HYPERCOAGULABLE STATE RELATED TO INHERITED AND ACQUIRED THROMBOPHILIAS AND CLINICAL IMPLICATIONS

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RIASSUNTO

Background

Il tromboembolismo venoso (TEV) è una patologia multifattoriale che deriva dall'interazione tra predisposizione genetica, fattori di rischio acquisiti e fattori ambientali. Lo screening convenzionale per trombofilia ereditaria, tuttavia, identifica solo un numero limitato di condizioni ad alto rischio trombotico; pertanto, molti pazienti giovani con eventi trombotici non provocati o provocati da fattori di rischio minore restano senza una spiegazione. La Next Generation Sequencing (NGS) offre l'opportunità di esplorare uno spettro genetico più ampio alla base dello stato di ipercoagulabilità.

Obiettivi

Questo studio si proponeva di caratterizzare il background genetico di pazienti giovani con TEV non provocato o provocato da fattori di rischio minore utilizzando un pannello NGS mirato, di valutare la prevalenza e lo spettro delle varianti patogenetiche e degli alleli di rischio, e di esplorare le associazioni genotipo–fenotipo con gli esiti clinici.

Materiali e metodi

Sono stati arruolati pazienti consecutivi e non imparentati, di età compresa tra 18 e 50 anni, con diagnosi oggettivamente documentata di TEV, afferenti all'Unità di Malattie Trombotiche ed Emorragiche e alla Clinica Medica 1 dell'Azienda Ospedaliera di Padova tra gennaio 2014 e dicembre 2023. I pazienti eleggibili presentavano TEV non provocato o TEV severo in presenza di soli fattori di rischio deboli, e uno screening convenzionale per trombofilia negativo sia per le forme ereditarie sia per quelle acquisite. Il TEV severo è stato definito come un evento esteso o clinicamente rilevante in presenza di soli fattori di rischio deboli. L'inclusione è avvenuta indipendentemente dalla presenza o meno di storia familiare di TEV. Le varianti sono state classificate secondo i criteri ACMG/AMP e gli alleli di rischio comuni sono stati registrati sistematicamente. I dati clinici e genetici sono stati analizzati e la regressione logistica è stata applicata all'intera coorte e a tutte le sottopopolazioni definite.

Risultati

Sono stati inclusi 100 pazienti (45% donne, età media 33 anni). La maggior parte era di etnia caucasica, con un BMI medio di 25,5 kg/m², e la metà riportava una storia familiare di TEV. L'evento indice era non provocato nel 67% dei casi, mentre nel 33% era associato a fattori di rischio deboli. La trombosi venosa profonda (TVP) era il fenotipo più frequente (54%), seguita da embolia polmonare (EP) isolata (27%) ed EP+DVT combinati (19%); l'11% presentava trombosi in sedi inusuali. Una storia di recidiva di TEV era documentata nel 42% dei pazienti e sanguinamenti in corso di anticoagulazione nel 19%, inclusi due eventi maggiori.

Dal punto di vista genetico, varianti patogenetiche o probabilmente patogenetiche (classi 4–5) sono state identificate nel 12% dei pazienti, mentre il 47% presentava varianti a significato incerto (classe 3). Complessivamente, il 59% della coorte mostrava almeno una variante potenzialmente rilevante e il 26% presentava più alterazioni in geni distinti, a supporto dell'ipotesi di un'ereditarietà oligogenica. Mutazioni patogenetiche ricorrenti comprendevano SERPINC1 Budapest 3, PROS1 Heerlen e F2 Arg541Trp. Circa l'11,8% delle varianti identificate non presentava un identificativo rsID (reference SNP cluster ID) nei database pubblici e potrebbe quindi rappresentare nuove mutazioni.

Alleli di rischio sono stati riscontrati nel 65% dei pazienti, con F2 c.1726-59G>A come il più prevalente (>40%), spesso in omozigosi e frequentemente in coesistenza con varianti rare. Altri alleli identificati comprendevano TFPI rs8176592, F12 c.-4T, PROZ c.-13G, PROCR rs867186 e SERPINA10 rs31336516. Circa un terzo della coorte non presentava alcuna variante genetica identificabile, suggerendo l'esistenza di ulteriori meccanismi non catturati dal pannello utilizzato.

Conclusioni

L'NGS esteso ha rivelato un carico sostanziale di varianti patogenetiche e di significato incerto nei pazienti giovani con TEV, supportando il modello poligenico/oligogenico di trombofilia. La frequente coesistenza di varianti rare e di alleli di rischio comuni sottolinea la complessità della predisposizione genetica. Oltre ai dati molecolari, la storia familiare rimane un elemento fondamentale nella valutazione del rischio trombotico. Futuri studi multicentrici che integrino dati genetici, funzionali, clinici e familiari saranno necessari per affinare l'interpretazione delle varianti e sviluppare strategie di medicina di precisione nella gestione del TEV.

ABSTRACT

Background

Venous thromboembolism (VTE) is a multifactorial disorder resulting from the interaction between genetic predisposition, acquired risk factors, and environmental triggers. Conventional thrombophilia screening identifies a limited number of high-risk conditions, thus many young patients with unprovoked or provoked thrombotic events remain unexplained. Next-generation sequencing (NGS) offers the opportunity to explore a broader genetic spectrum underlying hypercoagulability.

Aims

This study aimed to characterize the genetic background of young patients with unprovoked or disproportionate VTE using a targeted NGS panel, to assess the prevalence and spectrum of pathogenic variants and risk alleles, and to explore genotype–phenotype associations with clinical outcomes.

Material and methods

We enrolled consecutive, unrelated patients aged 18–50 years with objectively documented VTE, referred to the Thrombotic and Hemorrhagic Diseases Unit and Clinical Medicine 1 of the University Hospital of Padova between January 2014 and December 2023. Eligible patients had unprovoked VTE or severe VTE with only weak provoking factors, and a negative standard thrombophilia work-up for both inherited and acquired causes. Severe VTE was defined as thrombosis with extensive or anatomically critical involvement, clinically pronounced presentation, or severity disproportionate to weak provoking factors. Patients were included irrespective of family history of VTE. Variants were classified according to ACMG/AMP criteria, and common risk alleles were systematically recorded. Clinical and genetic data were analyzed, and logistic regression was applied across the whole cohort and all defined subpopulations.

Results

A total of 100 patients were included (45% women, mean age 33 years). Most were Caucasian, with a mean BMI of 25.5 kg/m², and half reported a family history of VTE. The index event was

unprovoked in 67% of cases, while 33% were associated with weak provoking factors. DVT was the most frequent phenotype (54%), followed by isolated PE (27%) and combined PE+DVT (19%); 11% had thrombosis at unusual sites. Recurrent VTE occurred in 42% of patients, and bleeding during anticoagulation in 19%, including two major events.

From the genetic perspective, pathogenic or likely pathogenic variants (class 4–5) were identified in 12% of patients, while 47% carried variants of uncertain significance (class 3). Overall, 59% of the cohort had at least one potentially relevant variant, and 26% harbored multiple alterations across distinct genes, supporting the hypothesis of oligogenic inheritance. Recurrent pathogenic mutations included SERPINC1 Budapest 3, PROS1 Heerlen, and F2 Arg541Trp. Approximately 11.8% of the detected variants had no rs identifier (reference SNP cluster ID) in public databases and may represent novel findings.

Risk alleles were found in 65% of patients, with F2 c.1726-59G>A being the most prevalent (>40%), often in homozygosis and frequently coexisting with rare variants. Additional alleles included TFPI rs8176592, F12 c.-4T, PROZ c.-13G, PROCR rs867186, and SERPINA10 rs31336516. Patients without any identifiable genetic variant accounted for about one third of the cohort, suggesting the existence of additional mechanisms not captured by the current panel.

Conclusions

Extended NGS revealed a substantial burden of pathogenic and uncertain variants in young patients with VTE, supporting the polygenic/oligogenic model of thrombophilia. The frequent coexistence of rare variants and common risk alleles underscores the complexity of genetic predisposition. Beyond molecular testing, family history remains a fundamental element in assessing thrombotic risk. Future multicenter studies integrating genetic, functional, clinical, and familial data are warranted to refine variant interpretation and move toward precision medicine in VTE management.

1. INTRODUCTION

Venous thromboembolism (VTE), encompassing deep vein thrombosis (DVT) and pulmonary embolism (PE), represents a major global health burden(1). It is the third most common acute cardiovascular syndrome after myocardial infarction and ischemic stroke(2). Despite advances in diagnostic modalities, therapeutic strategies, and preventive measures, VTE continues to be associated with considerable morbidity, mortality, and healthcare costs(3).

VTE is a multifactorial disease, arising from the interplay between genetic predisposition, acquired prothrombotic conditions, and transient environmental triggers. The underlying mechanisms reflect an imbalance between procoagulant and anticoagulant forces, leading to abnormal clot formation within the venous system(4). The clinical presentation can vary from asymptomatic or mild leg swelling to life-threatening pulmonary embolism(3,4). Furthermore, survivors of VTE are at increased risk of long-term complications, including recurrence, chronic thromboembolic pulmonary hypertension, and reduced quality of life(5).

Within this multifactorial framework, inherited and acquired thrombophilias represent key determinants of individual susceptibility to VTE. Their role extends beyond the first thrombotic episode, as they may also influence recurrence risk, severity of clinical manifestations, and long-term outcomes(6). Understanding the contribution of thrombophilia to VTE is therefore fundamental from both clinical and research perspectives, as it provides insights into disease mechanisms, supports risk stratification, and informs strategies for targeted prevention, including the potential integration of genetic testing into clinical decision-making(7).

1.1. Epidemiology of VTE

Population-based studies estimate the annual incidence of VTE to range between 1 and 2 cases per 1,000 individuals in Western countries. DVT and PE occur with roughly equal frequency, although subclinical or asymptomatic events are likely underreported(3). The incidence is low in children and young adults but rises sharply with age: less than 0.05 per 1,000 person-years in those under 15

years of age, 0.2–0.3 per 1,000 in individuals aged 30–49, and up to 4–6 per 1,000 in octogenarians(8). Beyond 80 years of age, the incidence may exceed 1% annually(9).

The lifetime risk of VTE is estimated at 8–10%, underscoring its relevance as a public health challenge. Importantly, the burden of disease is not confined to acute events: recurrence occurs in approximately 20–30% of patients within 10 years, and long-term complications such as post-thrombotic syndrome or chronic thromboembolic pulmonary hypertension contribute significantly to morbidity(3,10).

PE is the most feared manifestation of VTE due to its potential for sudden death(11). Approximately 10% of patients with acute PE die within the first month, and mortality is particularly high in those with hemodynamic instability or comorbid conditions. Autopsy studies reveal that PE is frequently underdiagnosed: in some series, up to one-third of fatal events were not suspected before death (12).

The risk of recurrence after a first episode of VTE is substantial. Patients with transient provoking factors (such as surgery or trauma) have a recurrence risk of 3–5% at 5 years, whereas those with unprovoked events or persistent risk factors (including active cancer or thrombophilia) may face recurrence rates exceeding 20% within the same time frame(5). Recurrent VTE is associated with worse outcomes, including higher mortality and cumulative disability(3).

The epidemiology of VTE varies across regions and populations. Studies consistently show that incidence is highest among individuals of European descent, intermediate in African Americans, and lowest among Asians and Native Americans. These differences may reflect a combination of genetic predisposition, environmental exposures, healthcare access, and competing risks of mortality(13,14).

Globally, the burden of VTE is substantial. The World Health Organization and large collaborative studies estimate that each year there are more than 10 million cases worldwide. In Europe, approximately 465,000 cases of DVT and 296,000 cases of PE are diagnosed annually, with nearly 370,000 deaths related to VTE—exceeding the combined mortality from breast cancer, prostate cancer, HIV/AIDS, and traffic accidents. In the United States, the direct and indirect medical costs of VTE are estimated at 5–10 billion dollars per year, with total societal costs (including productivity loss) approaching 70 billion dollars(1,2,15).

VTE affects both community and hospital populations. Approximately two-thirds of cases occur in the community, often unrecognized until the development of PE or advanced DVT. Hospitalization, however, remains a major risk factor: immobilization, surgery, cancer therapy, and acute medical

illnesses account for a large proportion of events. Without thromboprophylaxis, the incidence of hospital-acquired VTE may reach 40–60% in high-risk surgical patients(2,8,16).

The overall incidence of VTE appears to be increasing, partly due to aging populations, rising prevalence of comorbidities such as obesity and cancer, and improved diagnostic sensitivity with modern imaging(4,17). At the same time, short-term mortality has declined, largely because of earlier recognition and more effective treatments. This has led to a growing population of VTE survivors, for whom prevention of recurrence and management of long-term sequelae have become central challenges(2,18).

1.2. Venous thromboembolism and thrombophilia

The pathophysiology of VTE has traditionally been conceptualized through Virchow's triad: venous stasis, endothelial injury, and hypercoagulability (Figure 1.1)(19). Among these, hypercoagulability plays a pivotal role and is the conceptual framework for the study of thrombophilia. Thrombophilia refers to inherited or acquired alterations of the hemostatic system that increase the propensity to thrombosis. Recognition of thrombophilia has reshaped the understanding of VTE, moving from a purely circumstantial disease to a condition where individual susceptibility can be biologically determined(7). This is of particular relevance in young patients, those with recurrent events, thrombosis at unusual sites, or a strong family history.

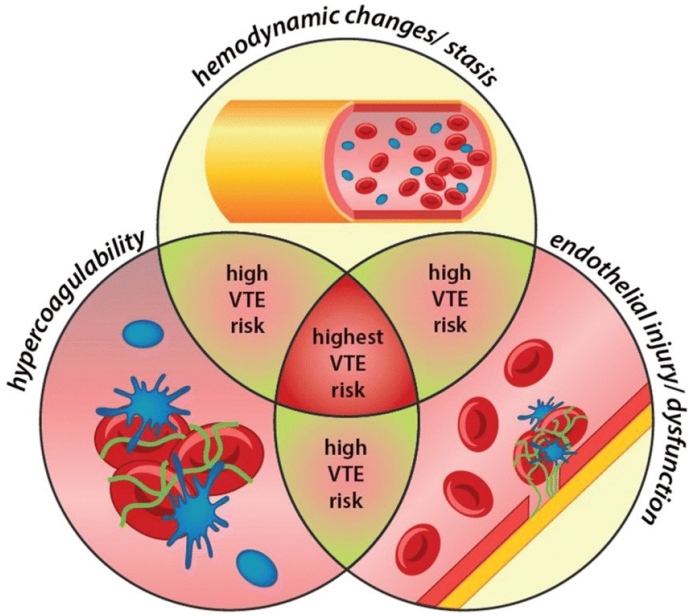


Figure 1.1. Virchow's triad. The figure describes the three broad categories of factors contributing to the risk of venous thrombosis: hemodynamic changes, endothelial injury, and hypercoagulability. VTE, venous thromboembolism(20).

While the effect size of each of these factors may be modest in isolation, their presence in combination with other risk conditions—either inherited or acquired—can markedly amplify the overall risk(7). As mentioned before, the development of VTE is typically multifactorial, resulting from the interaction between permanent patient-related conditions and transient environmental triggers(4). In this context, the 2019 ESC Guidelines on pulmonary embolism(21) provide a practical classification of risk factors according to the strength of their association with VTE. Strong risk factors (odds ratio >10) include major orthopedic surgery, major trauma, spinal cord injury, recent hospitalization for heart failure or atrial fibrillation, recent myocardial infarction, and a history of previous VTE. Moderate risk factors (odds ratio 2–9) comprise cancer (particularly in the metastatic stage), chemotherapy, hormone-related therapies (oral contraceptives, hormone replacement therapy, in vitro fertilization), pregnancy and the postpartum period, central venous catheters, and chronic medical conditions such as autoimmune or inflammatory diseases. Finally, weak risk factors (odds ratio <2) include obesity, varicose veins, diabetes, arterial hypertension, prolonged sitting, laparoscopic surgery, and advancing age (Figure 1.2).

This stratification underscores the heterogeneous nature of VTE pathogenesis, where both the intensity and persistence of risk factors influence not only the likelihood of the first thrombotic episode but also the risk of recurrence and long-term outcomes(22,23).

1.2.1 Acquired thrombophilia

Acquired risk factors constitute a large and heterogeneous group of conditions that significantly contribute to the development of VTE. Unlike inherited thrombophilias, which reflect stable genetic traits, acquired prothrombotic states may be transient, persistent, or recurrent, depending on the underlying condition(4,7). Their role is particularly relevant in adults and the elderly, where environmental and clinical exposures progressively accumulate and amplify the thrombotic risk(3,4,12). Their recognition is crucial, as they often modify both the acute management and the long-term prevention strategy(4,17,18,21). Moreover, they illustrate the dynamic nature of thrombotic risk, in which persistent conditions such as antiphospholipids or cancer may coexist with transient triggers like surgery, pregnancy, or immobilization. Understanding these interactions provides the basis for personalized risk stratification and tailored therapeutic approaches in patients with VTE(3,4,12,21).

In the following sections, additional insights into some of these factors, highlighting their specific mechanisms, clinical impact, and implications for patient management, are provided.

Table 3 Predisposing factors for venous thromboembolism (data modified from Rogers *et al.*[23] and Anderson and Spencer[24])

Strong risk factors (OR > 10)
Fracture of lower limb
Hospitalization for heart failure or atrial fibrillation/flutter (within previous 3 months)
Hip or knee replacement
Major trauma
Myocardial infarction (within previous 3 months)
Previous VTE
Spinal cord injury
Moderate risk factors (OR 2–9)
Arthroscopic knee surgery
Autoimmune diseases
Blood transfusion
Central venous lines
Intravenous catheters and leads
Chemotherapy
Congestive heart failure or respiratory failure
Erythropoiesis-stimulating agents
Hormone replacement therapy (depends on formulation)
In vitro fertilization
Oral contraceptive therapy
Post-partum period
Infection (specifically pneumonia, urinary tract infection, and HIV)
Inflammatory bowel disease
Cancer (highest risk in metastatic disease)
Paralytic stroke
Superficial vein thrombosis
Thrombophilia
Weak risk factors (OR < 2)
Bed rest >3 days
Diabetes mellitus
Arterial hypertension
Immobility due to sitting (e.g. prolonged car or air travel)
Increasing age
Laparoscopic surgery (e.g. cholecystectomy)
Obesity
Pregnancy
Varicose veins

HIV = human immunodeficiency virus; OR = odds ratio; VTE = venous thromboembolism.

Figure 1.2. Predisposing risk factors for venous thromboembolism, stratified by strength of association (adapted from ESC Guidelines 2019)(11)**.**

This stratification underscores the heterogeneous nature of VTE pathogenesis, where both the intensity and persistence of risk factors influence not only the likelihood of the first thrombotic episode but also the risk of recurrence and long-term outcomes(22,23).

1.2.2 Acquired thrombophilia

Acquired risk factors constitute a large and heterogeneous group of conditions that significantly contribute to the development of VTE. Unlike inherited thrombophilias, which reflect stable genetic traits, acquired prothrombotic states may be transient, persistent, or recurrent, depending on the underlying condition. Their role is particularly relevant in adults and the elderly, where environmental and clinical exposures progressively accumulate and amplify the thrombotic risk. Their recognition is crucial, as they often modify both the acute management and the long-term prevention strategy. Moreover, they illustrate the dynamic nature of thrombotic risk, in which persistent conditions such as antiphospholipids or cancer may coexist with transient triggers like surgery, pregnancy, or immobilization. Understanding these interactions provides the basis for personalized risk stratification and tailored therapeutic approaches in patients with VTE.

In the following sections, additional insights into some of these factors, highlighting their specific mechanisms, clinical impact, and implications for patient management, are provided(3,4,7-12,17,18,21).

1.2.1.1. Age

Age represents one of the strongest determinants of VTE incidence. While the disease is rare in childhood and young adulthood, the risk increases exponentially with advancing age. In population-based cohorts, the incidence of VTE rises from fewer than 0.05 cases per 1,000 person-years in children to 2–3 cases per 1,000 in individuals aged 70–79 years, and may exceed 10 cases per 1,000 beyond the age of 80. The biological basis for this association includes endothelial dysfunction, increased levels of procoagulant factors, and a decline in endogenous anticoagulant pathways. Aging is also accompanied by a higher prevalence of comorbidities such as cancer, cardiovascular disease, and reduced mobility, which synergistically augment thrombotic risk.

1.2.1.2. Family history

Family history of thrombosis, even in the absence of identifiable inherited thrombophilia, is another important risk factor. Several studies have demonstrated that having a first-degree relative with VTE

increases the risk of both first and recurrent events. This familial clustering suggests that shared genetic predispositions beyond the classic thrombophilias, as well as shared environmental exposures, contribute to thrombotic susceptibility. Similarly, a personal history of previous VTE is one of the strongest predictors of recurrence, underscoring the chronic nature of the disease and the need for careful long-term management strategies.

1.2.1.3. Immobilization

Immobilization is a well-recognized prothrombotic factor. Prolonged bed rest, limb casting, or extended travel exceeding six hours have all been linked to venous stasis and increased thrombotic risk. In hospitalized patients, particularly those with acute medical conditions or following major surgery, the absence of prophylaxis is associated with VTE rates as high as 40–60%. Recent trauma, especially when involving the pelvis or lower limbs, also contributes significantly by inducing tissue factor release, endothelial injury, and immobility-related venous stasis.

1.2.1.4. Surgery

Surgery represents a paradigmatic acquired risk factor, and its impact varies according to the type and duration of the procedure. Orthopedic interventions such as hip and knee arthroplasty or major trauma surgery are associated with the highest incidence, while abdominal or pelvic surgery also confers a marked increase in risk. Advances in perioperative thromboprophylaxis have substantially reduced—but not eliminated—this burden.

1.2.1.5. Medical comorbidities

Medical comorbidities play a central role. Conditions such as heart failure, chronic obstructive pulmonary disease, inflammatory bowel disease, and severe infections contribute to a systemic prothrombotic state through inflammation, endothelial activation, and impaired mobility. Other conditions such as obesity, nephrotic syndrome, chronic inflammatory and autoimmune diseases, and even certain pharmacologic agents contribute to an acquired prothrombotic milieu.

1.2.1.6. Cancer

Among acquired conditions, cancer is one of the most clinically significant. Tumor cells directly activate coagulation by expressing tissue factor and releasing procoagulant microparticles; systemic inflammation, platelet activation, and vascular compression further enhance the risk. Patients with

active malignancy have a four- to seven-fold higher incidence of VTE than the general population, and thromboembolism is the second leading cause of death in cancer patients after tumor progression itself. Importantly, certain cancers such as pancreatic, gastric, and brain tumors carry particularly high risks.

1.2.1.7. Reproductive and hormonal factors

Reproductive and hormonal factors also contribute substantially. Pregnancy and the postpartum period are well-established hypercoagulable states, characterized by increased concentrations of procoagulant factors, reduced levels of PS, and impaired fibrinolysis. The risk of VTE is increased five-fold during pregnancy and rises dramatically—up to 20- to 60-fold—during the first six weeks postpartum. This physiological adaptation to reduce hemorrhagic risk at delivery thus exposes women to substantial thrombotic vulnerability, especially when combined with inherited thrombophilia or additional risk factors. Hormonal contraceptives and hormone replacement therapy further increase the risk of VTE, particularly formulations containing estrogens. The absolute risk is modest in the general population but becomes clinically significant in women who also carry genetic predispositions such as factor V Leiden or the prothrombin G20210A variant, where the combined effect is synergistic. Moreover, selective estrogen receptor modulators and some targeted anticancer therapies have been implicated in increased VTE risk.

1.2.1.8. Antiphospholipid syndrome

Acquired thrombophilias also include antiphospholipid syndrome (APS), an autoimmune disorder characterized by the persistent presence of antiphospholipid antibodies in association with clinical events such as venous or arterial thrombosis or pregnancy morbidity. APS is unique in that it predisposes patients to both venous and arterial events, often at a young age, and it is associated with high recurrence rates. For these reasons, long-term anticoagulation is generally required.

1.2.1.9. Heparin-induced thrombocytopenia

Another notable acquired condition is heparin-induced thrombocytopenia (HIT). Despite presenting with low platelet counts, patients with HIT are paradoxically at very high risk of thrombosis. The pathogenesis involves antibodies directed against platelet factor 4–heparin complexes, which induce platelet activation and a systemic prothrombotic state. Both venous and arterial thromboses

may occur, and the risk of limb-threatening or fatal complications is considerable if the syndrome is not promptly recognized and managed.

1.2.2. Inherited thrombophilia

As we mentioned before, the concept of thrombophilia has evolved considerably since the 19th century, when Rudolf Virchow first proposed the triad of factors predisposing to thrombosis: endothelial injury, stasis, and hypercoagulability(6,7,24–27). It was not until 1956, however, that a familial component of VTE was clearly recognized, followed by the pivotal discovery of antithrombin (AT) deficiency in 1965 as the first defined hereditary prothrombotic disorder(25,26,28,29).

Subsequent decades saw the identification of additional rare but severe inherited deficiencies, namely protein C (PC) and protein S (PS), both vitamin K-dependent natural anticoagulants(6,25,26,29). These discoveries established the model of "loss-of-function" mutations as major contributors to a prothrombotic state(6,28,30). In the 1990s, two more prevalent "gain-of-function" genetic variants were described: the factor V Leiden (FVL) mutation, causing resistance to activated protein C, and the prothrombin G20210A mutation (PTM), leading to increased plasma prothrombin levels. Together, these mutations account for the majority of genetically identifiable thrombophilia in the general population(6,25,26,30,31).

Hereditary thrombophilia encompasses a heterogeneous group of genetic abnormalities that promote VTE through different mechanisms(25,28,30). Figure 1.3 illustrates the complexity of the coagulation cascade, highlighting the multiple sites where genetic and acquired alterations can interfere with hemostatic balance. These can be broadly classified into loss-of-function mutations in natural anticoagulants and gain-of-function mutations that enhance procoagulant activity. Additional genetic variants and modifiers, including blood group and fibrinolytic system abnormalities, may also contribute to the thrombophilic phenotype(6,26,30).

1.2.2.1 Loss-of-function disorders

1.2.2.1.1. Antithrombin (AT) deficiency

Antithrombin is a 58-kDa glycoprotein (Figure 1.4) produced by hepatocytes, circulating at concentrations of approximately 150 µg/mL (80–120% activity by functional assays). It belongs to the serpin (serine protease inhibitor) family and plays a key anticoagulant role by irreversibly inactivating thrombin and factor Xa, as well as factors IXa, XIa, and XIIa. Its inhibitory activity is significantly enhanced in the presence of heparin or endothelial glycosaminoglycans such as

heparan sulfate, which induce conformational changes that facilitate rapid protease neutralization. In addition to its anticoagulant role, AT exhibits pleiotropic properties, including anti-inflammatory and endothelial-protective effects, partly mediated through inhibition of NF-κB activation, prostacyclin release, and modulation of monocyte and neutrophil function(32–34).

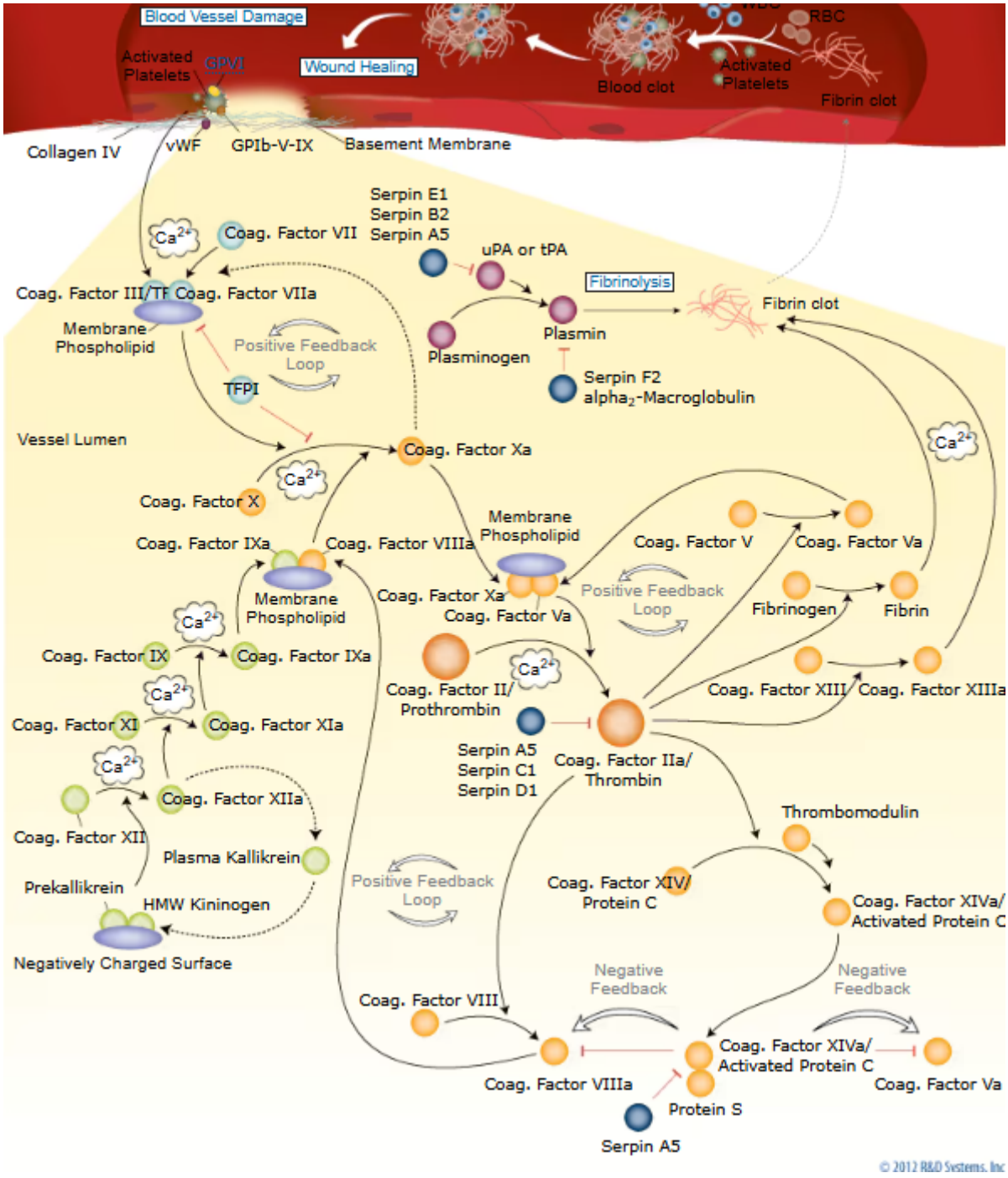


Figure 1.3. Schematic representation of the coagulation cascade. The diagram illustrates the initiation, amplification, and propagation phases of coagulation, highlighting the interplay between clotting factors, platelets, and regulatory inhibitors, and their convergence on fibrin clot formation and subsequent fibrinolysis (adapted from R&D Systems).

AT is encoded by the SERPINC1 gene on chromosome 1q23–25. More than 350 mutations have been identified, giving rise to two main categories of hereditary deficiency: type I (quantitative), characterized by reduced antigen and activity, and type II (qualitative), where antigen levels are normal but functional activity is impaired. Type II deficiencies are further classified into type IIa (reactive site mutations), type IIb (heparin-binding site mutations), and type IIc (pleiotropic or conformational defects)(33–35).

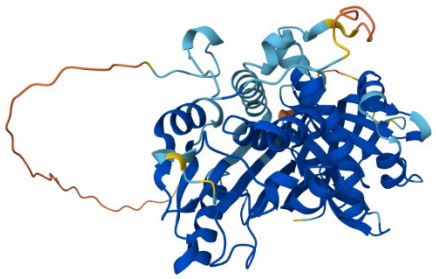


Figure 1.4. Antithrombin. Graphical representation of the protein structure of Antithrombin (source: Alphafold).

Historically, AT deficiency was the first hereditary thrombophilia to be described, initially reported by Egeberg in 1965 in a Norwegian family with recurrent thromboembolic disease. This seminal discovery marked the beginning of the molecular characterization of inherited prothrombotic states(33). Over the following decades, various point mutations were identified, predominantly in the reactive site and the heparin-binding domain of the SERPINC1 gene. These mutations established the classical distinction between type I and type II deficiencies(33,34).

More recently, the application of next-generation sequencing and refined biochemical methods has expanded the spectrum of known SERPINC1 variants. Mutations affecting N-glycosylation sites, such as Asn224His and Glu227Lys, have been shown to impair secretion or function of AT without significantly reducing its activity in routine assays, leading to potential false-negative results in standard diagnostics. Conformational variants, such as Pro439Thr and Pro461Ser, as well as the Cambridge II variant (A384S)—relatively frequent in British and Spanish populations—have also been recognized as pathogenic(35). Notably, the Cambridge II variant has been associated with a nine-fold increased risk of thrombosis despite normal antigen and activity levels(34). In parallel, other rare variants like AT Budapest 3 (Leu131Phe) and AT Toyama (Arg79Cys) have been identified in specific ethnic groups and are generally associated with mild to moderate phenotypes in heterozygosity but can result in severe thrombotic manifestations in homozygous carriers(33,35). This evolving understanding underscores the complexity and heterogeneity of AT deficiency and

highlights the importance of integrating molecular diagnostics with functional assays, particularly in patients with strong clinical suspicion but inconclusive laboratory findings.

AT deficiency is a rare condition, with an estimated prevalence of 0.02–0.17% in the general population and 0.5–4.9% among patients with VTE(32). Among all inherited thrombophilias, it confers the highest thrombotic risk: the relative risk of VTE ranges from 5- to 50-fold, with lifetime incidence reaching 50–85% in heterozygous carriers when additional risk factors are present. The recurrence rate after a first VTE can reach 60% without appropriate secondary prophylaxis(32,34). Type I and type IIa/IIc variants are more frequently associated with severe clinical phenotypes, while type IIb variants, such as AT Budapest 3 and AT Toyama, are typically milder but may cause thrombotic events in homozygous or compound heterozygous states(33,35).

The clinical phenotype is variable. Most symptomatic patients have type I deficiency, while type IIb mutations are often associated with a milder phenotype. Type IIa and IIc mutations confer greater thrombotic risk and are more frequently identified in symptomatic patients. Some rare glycosylation-site variants (e.g., Asn224His, Glu227Lys) may not reduce AT levels significantly but are still associated with early and recurrent thrombosis(33,35).

Homozygous AT deficiency is incompatible with life, whereas compound heterozygosity may result in neonatal purpura fulminans or in utero thrombosis(32,33). In contrast, asymptomatic heterozygotes may remain undiagnosed for years, particularly if standard activity tests fail to detect qualitative defects(32,35).

AT deficiency is also associated with adverse pregnancy outcomes, including recurrent fetal loss and placental complications. Women with AT deficiency require tailored thromboprophylaxis during pregnancy and postpartum to mitigate maternal and fetal risks(34).

From a diagnostic standpoint, functional AT assays (typically anti-FXa or anti-IIa chromogenic tests) are recommended as first-line tests(32,33). However, these assays cannot distinguish between type II subtypes and may be influenced by heparin cofactor II or direct thrombin inhibitors. Moreover, FXa-based assays may be less sensitive to some type II defects. Progressive activity assays, based on prolonged incubation without heparin, may help identify type II HBS variants but are not routinely available(32). Therefore, if clinical suspicion remains high despite normal results, antigen quantification and genetic testing should be considered. Confirmation of hereditary deficiency may also require testing of first-degree relatives(32,34).

The clinical management of AT deficiency is shaped by the type of deficiency, the presence or absence of thrombotic events, and the individual's overall risk profile. In asymptomatic

heterozygous carriers, routine long-term anticoagulation is generally not recommended. However, thromboprophylaxis is warranted in transient high-risk settings, such as major surgery, trauma, prolonged immobilization, or during pregnancy and the postpartum period. In these situations, low-molecular-weight heparin is typically used, and dosing may require adjustment in patients with significantly reduced AT levels(34).

For symptomatic heterozygotes who have experienced VTE, anticoagulant therapy is initiated in line with standard VTE treatment protocols(34). The choice of anticoagulant—either vitamin K antagonists or direct oral anticoagulants (DOACs)—is generally not altered solely due to the presence of AT deficiency, although heparin resistance can occur in individuals with very low AT levels. In such cases, monitoring of anti-FXa activity or supplementation with AT concentrate may be necessary to ensure therapeutic efficacy during heparin administration(33).

Patients with severe AT deficiency, such as homozygous or compound heterozygous forms, are rare but may present with extensive neonatal thrombosis or purpura fulminans. These cases require urgent AT replacement therapy, using plasma-derived or recombinant concentrates. Replacement is also recommended in selected high-risk peripartum or perioperative settings, especially when baseline AT activity is below 60% and anticoagulation with heparin is planned.(33,34)

Unlike PC deficiency, lifelong replacement therapy is not commonly needed in AT deficiency. However, short-term supplementation with AT concentrate is an important adjunct in specific clinical contexts, such as pregnancy in women with a history of thrombosis, delivery, cardiac surgery, or AT resistance to heparin during extracorporeal circulation. In pregnancy, regular monitoring and adjustment of both anticoagulation and AT levels may be required to prevent maternal and fetal thrombotic complications(33,34).

1.2.2.1.2. Protein C (PC) deficiency

Protein C deficiency is a congenital thrombophilic condition caused by mutations in the *PROC* gene, encoding a vitamin K-dependent serine protease with key anticoagulant functions (Figure 1.5). Activated protein C (APC), formed by the action of thrombin bound to thrombomodulin, inactivates factors Va and VIIIa, downregulating thrombin generation. Beyond its anticoagulant effects, APC exhibits pleiotropic roles, including anti-inflammatory, cytoprotective, and fibrinolytic activity via endothelial protein C receptor (EPCR) and protease-activated receptor 1 (PAR-1) signaling(36,37).

PC deficiency is inherited in an autosomal dominant fashion in heterozygous forms and autosomal recessive in homozygous or compound heterozygous states(38,39). Over 270 mutations in *PROC*

have been described, including missense, nonsense, frameshift, splice site, and promoter variants(38). Type I deficiency, the most frequent form, is characterized by decreased antigen and activity levels. Type II deficiency (dysfunctional PC) presents with normal antigen levels but impaired function, often underrecognized due to normal chromogenic results in some cases(36,37).

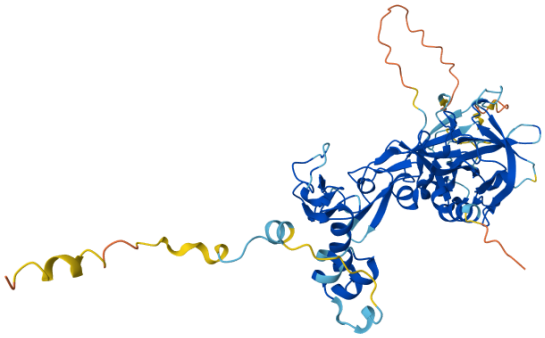


Figure 1.5. Protein C. Graphical representation of the protein structure of protein C(source: Alphafold).

The estimated prevalence of heterozygous PC deficiency in the general population ranges from 0.2% to 0.5%, with a 7- to 10-fold increased risk of VTE(37,40,41). Lifetime VTE risk in heterozygotes can approach 50% by age 40 in the presence of additional triggers such as surgery, oral contraceptives, or pregnancy. The recurrence rate without secondary prophylaxis may exceed 60%, especially in individuals with a strong family history (40). Homozygous or compound heterozygous forms are exceedingly rare (1:500,000–750,000 births) and typically manifest within hours of birth with life-threatening purpura fulminans, disseminated intravascular coagulation (DIC), and thrombotic occlusions including cerebral and retinal vessels(39,41).

Diagnosis is based on functional activity assays (clot-based and/or chromogenic), complemented by antigenic quantification(36,37,42). Clot-based assays may be more sensitive to type II variants but can be confounded by anticoagulants or high FVIII levels. Chromogenic assays may miss functionally defective molecules if activation is preserved but cofactor interaction is impaired(36,37). Genetic testing is essential in selected cases, particularly with neonatal onset or strong family clustering(38,43).

The management of PC deficiency depends largely on the clinical presentation and the underlying genotype. In heterozygous individuals who are asymptomatic, routine anticoagulation is generally not required(37). However, preventive strategies become crucial in high-risk situations such as surgery, prolonged immobilization, pregnancy, or the use of estrogen-containing contraceptives.

These patients may benefit from short-term thromboprophylaxis with low-molecular-weight heparin(40) or direct oral anticoagulants (DOACs).

For symptomatic heterozygotes—those with a personal history of venous thromboembolism—long-term anticoagulation is typically indicated. Vitamin K antagonists such as warfarin have traditionally been used, although direct oral anticoagulants are increasingly considered, provided that no contraindications are present(38). It is important to note that initiation of warfarin therapy in PC–deficient patients should always be preceded by adequate heparin coverage, as these individuals are at increased risk for warfarin-induced skin necrosis due to a rapid decline in PC levels during the early phase of vitamin K antagonism(37).

In severe cases - most notably in neonates with homozygous or compound heterozygous deficiency—management becomes urgent and complex. These infants often present with purpura fulminans or life-threatening thrombotic events shortly after birth. Immediate replacement therapy is required, using either fresh frozen plasma or plasma-derived PC concentrates. PC concentrates are preferred for their safety and specificity(39,41). In some cases, long-term prophylactic administration of PC is necessary to prevent further thrombotic complications. Recent reports have demonstrated the efficacy and tolerability of subcutaneous administration of PC concentrate for home prophylaxis, particularly in pediatric patients, reducing the need for central venous access and improving quality of life(44).

In extreme or refractory cases—especially when replacement therapy is not sufficient to control the disease—orthotopic liver transplantation has been considered. This approach offers a curative potential, given that the liver is the exclusive site of PC synthesis. However, it is reserved for selected patients with life-threatening, recurrent thromboses unresponsive to conventional therapy(44).

1.2.2.1.3. Protein S (PS) deficiency

Protein S deficiency is a hereditary or acquired condition that predisposes individuals to an increased risk of VTE. PS is a 73 kDa vitamin K-dependent glycoprotein primarily synthesized in hepatocytes and endothelial cells (Figure 1.6). It functions as a critical cofactor for activated protein C (APC) in the inactivation of factors Va and VIIIa, and also contributes to anticoagulation via APC-independent mechanisms, including direct inhibition of the intrinsic tenase and prothrombinase complexes, and as a cofactor for tissue factor pathway inhibitor (TFPI) in the inhibition of factor Xa(45–47).

In plasma, approximately 60% of circulating PS is bound to C4b-binding protein (C4BP) and is functionally inactive in terms of APC cofactor activity, while the remaining 40% is free and active. This biochemical partition is crucial, as only free PS is considered to exert full anticoagulant effects under physiological conditions(47). However, recent findings suggest that the PS–C4BP complex may retain partial APC cofactor activity in certain contexts, such as the inactivation of factor Va Leiden(48,49).

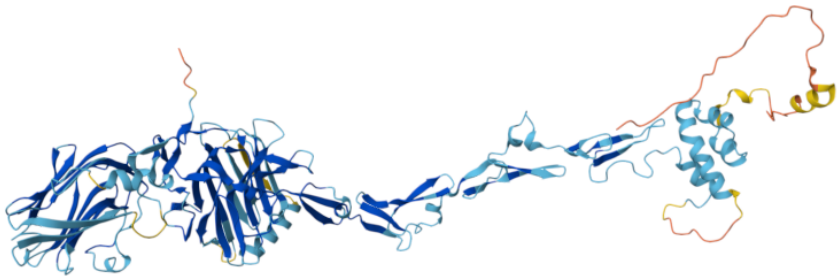


Figure 1.6. Protein S. Graphical representation of the protein structure of protein S (source: Alphafold).

Hereditary PS deficiency is classified into three subtypes, based on the relative levels of total and free PS and the functional activity measured by cofactor assays. In type I deficiency, which is the most straightforward to diagnose, both total and free PS antigen levels are reduced, and this reduction is paralleled by a decrease in functional activity. This subtype represents a classical quantitative deficiency and typically reflects heterozygous mutations affecting synthesis or secretion of the protein(46).

In type II deficiency, total and free antigen levels are within the normal range, but the functional activity is reduced. This reflects a qualitative defect in the PS molecule itself—such as impaired interaction with activated protein C or target proteases—resulting in decreased anticoagulant function despite normal protein concentrations. Diagnosing type II deficiency can be particularly challenging because the antigen levels may mislead clinicians, especially if functional assays are not performed(36).

Type III deficiency, meanwhile, is characterized by normal total PS levels but low levels of free PS and reduced functional activity. This subtype is thought to result from an increased binding of PS to C4b-binding protein (C4BP), leading to a relative deficiency of the active, free form. Although type III was historically considered a distinct entity, some experts suggest it may represent a variation of

type I with partial penetrance or fluctuating expression under the influence of physiological or acquired modifiers(48).

In clinical practice, distinguishing among these subtypes is important for interpreting test results, especially because external factors—such as estrogen use, pregnancy, liver dysfunction, or anticoagulant therapy—can transiently lower PS levels or interfere with assay interpretation(47). For this reason, testing should ideally be performed when patients are stable, not on anticoagulation, and outside of pregnancy or acute illness(50).

The prevalence of hereditary PS deficiency in the general population is low, estimated between 0.03% and 0.21%, but it is more common among patients with VTE, where it can be found in up to 2–13% depending on the population and diagnostic criteria used. Notably, higher prevalence rates have been reported in East Asian populations, often due to recurrent founder mutations such as PS Tokushima(48,51).

Clinically, PS deficiency increases the risk of VTE, particularly deep vein thrombosis and pulmonary embolism, and is also associated with adverse pregnancy outcomes such as recurrent fetal loss. The risk of recurrent VTE in symptomatic individuals is elevated, although precise quantification is complicated by diagnostic challenges and heterogeneity in study designs(52).

Diagnosis relies on functional assays that evaluate APC cofactor activity, in combination with antigenic assays measuring total and free PS. The diagnostic process is complex, often requiring repeat testing and the exclusion of acquired causes. Genetic testing is not routinely performed due to the presence of a pseudogene (PROS2) that complicates sequencing of the true gene (PROS1) and the lack of clear genotype–phenotype correlation(46,49).

Management strategies vary according to the clinical context. Asymptomatic carriers usually do not require long-term anticoagulation, though thromboprophylaxis is recommended in high-risk situations. For individuals with documented thrombotic events, anticoagulation is indicated, typically with heparins followed by vitamin K antagonists or DOACs. Caution is advised during warfarin initiation, as PS levels—being vitamin K-dependent—can drop further, exacerbating thrombotic risk(48). In life-threatening situations such as neonatal purpura fulminans, fresh frozen plasma may be used as a source of PS, since no purified concentrate is currently available(49).

PS deficiency remains a diagnostic and therapeutic challenge due to its biochemical complexity, assay limitations, and wide inter-individual variability in thrombotic risk. Continued research is needed to refine molecular diagnostics and clarify the role of PS in non-hemostatic processes, including inflammation and apoptosis(46).

1.2.2.2. *Gain-of-function disorders*

1.2.2.2.1. Factor V Leiden (FVL)

Factor V Leiden (FVL) is the most common inherited thrombophilia in Caucasian populations and results from a single point mutation (G1691A) in the *F5* gene. This substitution leads to an arginine-to-glutamine change at position 506 (Arg506Gln) in the factor V molecule, abolishing one of the key cleavage sites used by activated protein C (APC) to inactivate factor Va. The result is resistance to APC, leading to prolonged thrombin generation and a procoagulant state(53,54).

The prevalence of FVL is striking: approximately 5% of the general European population are heterozygous carriers, with even higher rates (~10–15%) reported in specific populations such as those in Sweden, Greece, and Lebanon. Homozygosity is much rarer, affecting approximately 1 in 5,000 individuals(55,56). In contrast, the mutation is virtually absent in East Asian, African, and Indigenous American populations, underscoring its geographic and ethnic distribution linked to European ancestry(55).

FVL is a major contributor to VTE. Heterozygous carriers have a 3- to 7-fold increased lifetime risk of VTE, while homozygotes face a 10- to 80-fold increase, especially in the presence of acquired risk factors(55,57). FVL accounts for up to 25% of all first VTE events and is detected in more than 50% of patients with inherited thrombophilia, making it a key player in the evaluation of thrombosis risk(54). The thrombotic risk in FVL carriers is not constant throughout life, but tends to manifest in the presence of additional prothrombotic stimuli—such as immobilization, surgery, trauma, pregnancy, oral contraceptive use, or hormone replacement therapy(58).

The risk is particularly amplified in women using estrogen-containing contraceptives, where the combination can increase VTE risk by 35-fold compared to women without FVL not using hormones. This has led to recommendations against prescribing combined hormonal contraceptives to women with known FVL, especially homozygotes(59).

FVL has also been implicated in obstetric complications, including recurrent pregnancy loss, preeclampsia, fetal growth restriction, and placental abruption. However, while observational studies suggest an association, systematic reviews and meta-analyses have yielded inconsistent findings. Most women with FVL experience normal pregnancies, and screening for FVL in women with adverse pregnancy outcomes is not routinely recommended unless additional risk factors are present(55,60).

From a mechanistic standpoint, FVL alters not only the procoagulant function of factor Va, but also its anticoagulant cofactor activity, as factor V is a key cofactor for APC in the inactivation of factor

VIIIa. The FVL mutation disrupts this function, further contributing to an imbalance in hemostasis(54,60).

Laboratory diagnosis of FVL is straightforward and relies either on functional assays detecting APC resistance or on molecular genetic testing to identify the G1691A mutation. Functional assays can yield false positives in the presence of lupus anticoagulant or anticoagulant therapy, which is why genotyping remains the gold standard (55).

Clinical management varies based on genotype, personal and family history, and the presence of provoking factors. Heterozygous individuals without a history of VTE typically do not require anticoagulation. However, anticoagulant prophylaxis is recommended in high-risk situations, and long-term therapy may be considered in homozygotes or heterozygotes with recurrent events (57). FVL status may also influence decisions on the duration of anticoagulation after a first unprovoked VTE, particularly in patients with a family history of thrombosis or concomitant risk factors(55,57).

From an evolutionary perspective, the relatively high frequency of FVL among Caucasians has raised questions about selective advantage. It has been hypothesized that FVL heterozygosity may have conferred protection against hemorrhagic death during childbirth or reduced bleeding severity in hemophilia carriers, offering a survival advantage in pre-modern societies(56). Additionally, some studies have proposed a role in enhancing immune responses during sepsis, although these theories remain speculative(60).

In conclusion, FVL represents a paradigmatic example of a common genetic variant with variable clinical penetrance, whose impact is modulated by environmental, hormonal, and additional genetic factors. Its frequency and clinical significance make it a cornerstone of thrombophilia evaluation and a compelling model of gene–environment interaction in complex diseases(55).

1.2.2.2.2. Prothrombin G20210A mutation

Prothrombin, or factor II, is a vitamin K–dependent glycoprotein synthesized in the liver; it is the inactive precursor of thrombin, the key enzyme of the coagulation cascade responsible for converting fibrinogen into fibrin (Figure 1.7)(61).

The G20210A mutation in the *F2* gene, first described in 1996, represents the most common prothrombotic variant of prothrombin and is classified as a gain-of-function mutation(61). It consists of a G to A substitution at position 20210 in the 3’ untranslated region (3’ UTR) of the *F2* gene, and does not alter the amino acid sequence of the protein. However, it leads to increased stability of the mRNA transcript and enhanced translation efficiency, resulting in elevated plasma levels of

prothrombin—typically 30% higher than in non-carriers. This elevated concentration promotes increased thrombin generation, contributing to a hypercoagulable state(62).

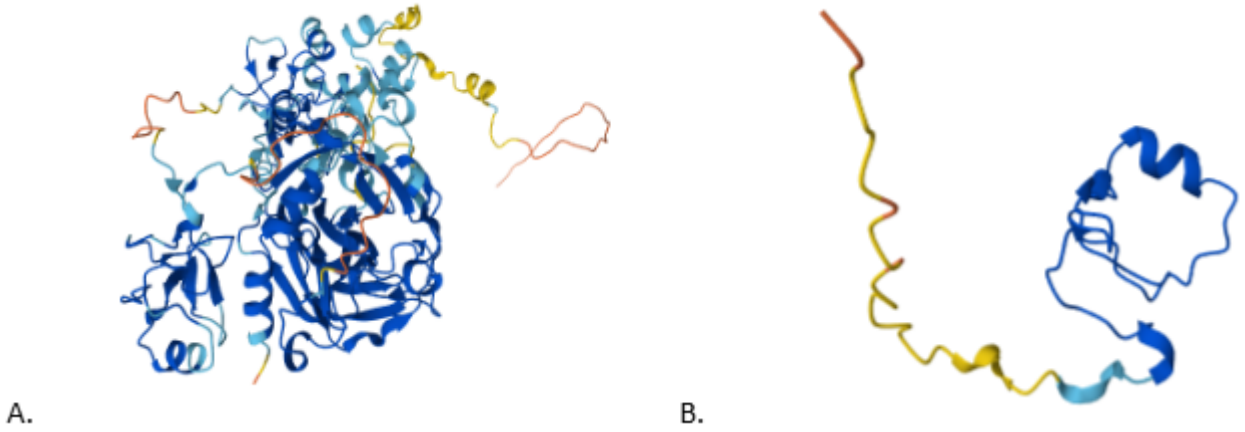


Figure 1.7. Prothrombin and Thrombin. Graphical representation of the protein structure of prothrombin (A) and thrombin (B) (source: Alphafold).

The mutation follows autosomal dominant inheritance with incomplete penetrance and is particularly prevalent in populations of European ancestry, with an estimated allele frequency of 1% to 6%. It is rare in individuals of African or Asian descent. Carriers of the G20210A variant are predominantly heterozygous, as homozygosity is rare but associated with a markedly higher thrombotic risk(63).

Clinically, heterozygous carriers of G20210A have a 2- to 4-fold increased risk of VTE compared to the general population. This risk is further amplified in the presence of acquired risk factors such as surgery, immobilization, pregnancy, oral contraceptive use, or air travel(63). The mutation is also frequently identified in individuals with early-onset or recurrent VTE, particularly when no other clear risk factors are present. In combination with other inherited thrombophilias, such as FVL, the risk of thrombosis becomes synergistically higher, especially in women using estrogen-containing contraceptives(64).

Despite its strong association with venous thrombosis, the G20210A mutation has not been conclusively linked to arterial thrombotic events such as myocardial infarction or ischemic stroke in the general population. Several studies suggest that in the absence of coexisting prothrombotic conditions, the mutation alone does not significantly increase arterial risk(63).

The diagnosis is made through molecular genetic testing, as functional coagulation assays do not detect the presence of the variant. Screening is typically indicated in individuals with unprovoked or recurrent VTE, a strong family history, or thrombosis at a young age or in unusual sites(61,64).
Management does not differ from standard anticoagulant treatment protocols, but awareness of the mutation can influence decisions on thromboprophylaxis in high-risk situations. Routine lifelong anticoagulation is not indicated in asymptomatic carriers, but temporary prophylaxis is often recommended during periods of increased thrombotic risk, such as surgery, postpartum, or hormonal therapy initiation(62).
Overall, the G20210A mutation remains a clinically significant but moderate-risk thrombophilia, whose impact is context-dependent and greatest when combined with environmental or genetic prothrombotic cofactors(63,64).

1.2.2.2.3. Factor V variants and activated protein C resistance beyond FV Leiden

Although the Arg506Gln mutation known as FVL is the most prevalent inherited cause of activated protein C resistance (APC-R), particularly in Caucasian populations(53), increasing evidence has demonstrated that several other rare missense mutations in the *F5* gene can similarly impair APC-mediated inactivation of factor Va and predispose individuals to VTE(65). These variants, distinct from FVL, act through a variety of molecular mechanisms that disrupt the normal inactivation of FVa by APC or interfere with the anticoagulant cofactor functions of FV (Figure 1.8).

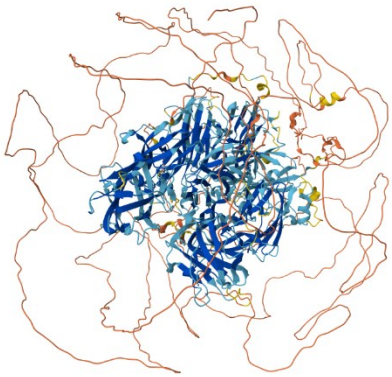


Figure 1.8. Factor V. Graphical representation of the protein structure of factor V (source: Alphafold).

The classical mechanism of APC-R involves impaired proteolytic cleavage at one or more of the three APC-sensitive sites on factor Va: Arg306, Arg506, and Arg679. Mutations that affect these cleavage

sites can prolong the half-life of active FVa, thereby enhancing thrombin generation. Among the best characterized are variants that alter Arg306, such as Factor V Cambridge (Arg306Thr)(66) and Factor V Hong Kong (Arg306Gly)(67). These mutations abolish the Arg306 cleavage site, which is required for the complete inactivation of FVa, resulting in partial APC resistance. Though less severe than FVL, these variants have been found in families with inherited thrombophilia and are associated with an increased thrombotic risk, particularly in the presence of other genetic or environmental risk factors.

Other mutations act through alternative mechanisms. Factor V Liverpool (Ile359Thr), for example, introduces an aberrant N-glycosylation site that alters the local conformation of the protein and impairs APC-mediated cleavage at Arg306, while simultaneously abolishing the anticoagulant cofactor activity of FV(68). Similarly, Factor V Bonn (Ala512Val)(69) and Glu666Asp(70) affect cleavage near the Arg506 or Arg679 sites, leading to moderate APC resistance and sustained procoagulant activity. More distally, mutations such as Factor V Nara (Trp1920Arg)(71) and Factor V Besançon (Ala2086Asp)(72) are located in the C1 and C2 domains of the FV light chain, which are critical for phospholipid binding and interaction with PS. These variants impair not only inactivation by APC but also disrupt the ability of FV to function as a cofactor in the PC anticoagulant pathway. The result is a dual defect in both anticoagulant and procoagulant regulation, which can lead to a particularly severe thrombotic phenotype.

Table 1.1 summarizes the most well-known FV variants associated with APC resistance, with FV Leiden being the most prevalent and clinically relevant.

FV variant	Amino acid change	Ethnic distribution	Location (domain)	Mechanism	Reference
FV Leiden	Arg506Gln	Caucasians (5%)	Heavy chain (A2)	Loss of Arg506 APC-cleavage site	Bertina et al. 1994(53)
FV Cambridge	Arg306Thr	Caucasians (sporadic)	Heavy chain (A1/A2)	Loss of Arg306 APC-cleavage site	Williamson et al. 1998(66)
FV Hong Kong	Arg306Gly	Hong Kong Chinese (4.5%)	Heavy chain (A1/A2)	Loss of Arg306 APC-cleavage site	Chan et al. 1998(67)
FV Liverpool	Ile359Thr	Caucasians (single family)	Heavy chain (A2)	Gain of glycosylation site near Arg306	Mumford et al. 2003(68)
FV Bonn	Ala512Val	Caucasians (sporadic)	Heavy chain (A2)	Amino acid change near Arg506	Pezeshkpoor et al. 2016(69)
FV (unnamed)	Glu666Asp	Chinese (single family)	Heavy chain (A2)	Amino acid change near Arg679	Cai et al. 2010(70)
FV Nara	Trp1920Arg	Japanese (single family)	Light chain (C1)	Decreased binding affinity for phospholipids	Nogami et al. 2014(71)
FV Besançon	Ala2086Asp	Single patient (Morocco)	Light chain (C2)	Decreased binding affinity for phospholipids	Castoldi et al. 2021(72)

Table 1.1. FV variants associated with activated protein C resistance.

From a clinical standpoint, these non-Leiden variants have been associated with a wide spectrum of presentations, ranging from asymptomatic carriage to early-onset and recurrent VTE. The thrombotic risk conferred by these variants varies depending on the specific mutation and its impact on FV function, but in some cases may exceed that associated with FVL, especially in homozygous or compound heterozygous individuals(73). Notably, these variants may predispose to thrombosis in unusual sites, such as the cerebral, mesenteric, or retinal veins, and can present even in the absence of typical provoking factors(69–72). In contrast to FVL, which is routinely screened by commercial genotyping assays, these rare variants are frequently undetected unless full gene sequencing or extended molecular analysis is performed(Moore et al., 2023; Mumford et al., 2003; Rietveld et al., 2018; Williamson et al., 1998). Functional assays that assess APC resistance may raise suspicion in the absence of FV Leiden, particularly when patients are not receiving anticoagulant therapy at the time of testing(65,74).

The identification of such variants has important clinical implications. While there are currently no mutation-specific treatment guidelines, patients found to carry these mutations—especially if they have experienced thrombosis—are often managed according to high-risk thrombophilia protocols(65,69,71–73). This may include long-term anticoagulation in cases of unprovoked or recurrent events, or thromboprophylaxis during high-risk periods such as surgery, immobilization, or pregnancy. Furthermore, identification of these mutations may prompt family screening, particularly when there is a strong family history of VTE. Overall, these findings expand our understanding of the molecular complexity of APC resistance and underscore the multifaceted role of factor V as both a procoagulant and an anticoagulant molecule. They also highlight the need for broader diagnostic approaches in selected patients with unexplained thrombophilia, especially when standard testing for FVL and PTM is negative(74).

1.2.2.2.4. Factor V Leiden pseudo-homozygosity

While classical homozygosity for the FVL mutation (Arg506Gln) is well-known for conferring a markedly increased risk of VTE, a pseudo-homozygous phenotype can also arise in individuals who are heterozygous for FVL but simultaneously carry a second mutation causing factor V deficiency on the other allele. In this scenario, only the mutant FVL protein is functionally expressed in plasma, mimicking a homozygous state. This condition is termed FVL pseudo-homozygosity, and it is associated with a pronounced activated protein C resistance (APCR) and increased thrombin generation, despite the apparent heterozygosity on genotyping(75,76).

The study by Lunghi et al. identified a recurrent mutation in exon 14 of the *F5* gene, Glu1608Lys, in thrombophilic patients with FV deficiency. This mutation was found to cause a CRM (cross-reacting material)-reduced type of quantitative deficiency, leading to decreased plasma levels and function of FV without abolishing its synthesis. In individuals who co-inherit FVL and this or similar FV-deficient alleles, the resulting APCR is biochemically indistinguishable from true homozygosity, leading to the pseudo-homozygous state(77,78).

Subsequent functional studies by Duckers et al. further highlighted the prothrombotic implications of this condition. In their analysis, FVL pseudo-homozygotes exhibited an even more pronounced hypercoagulable state than FVL homozygotes, as measured by increased thrombin generation and enhanced resistance to APC, particularly under low tissue factor conditions(79). One contributing factor appeared to be reduced plasma levels of tissue factor pathway inhibitor (TFPI) in pseudo-homozygotes, due to the lower expression of normal FV, which also plays a role in TFPI stabilization. The absence of the normal FV isoform compromises both its anticoagulant cofactor activity (especially in the inactivation of FVIIIa) and TFPI regulation, thereby exacerbating the hypercoagulable phenotype(75,79).

Clinically, these findings have important implications. Patients with FVL pseudo-homozygosity may present with VTE at a young age or in the absence of traditional risk factors, and may require similar antithrombotic management strategies as true homozygotes, particularly in high-risk settings such as surgery, pregnancy, or prolonged immobilization. Notably, standard genotyping may miss these cases if only the FVL mutation is tested, underscoring the value of APCR functional assays and FV antigen/activity measurements in selected patients(76).

In conclusion, FVL pseudo-homozygosity represents a clinically significant but underrecognized thrombophilic state, resulting from the compound heterozygosity of FVL and a second FV loss-of-function mutation. The synergistic impairment in anticoagulant pathways underscores the need for heightened diagnostic vigilance and tailored therapeutic approaches in affected individuals.

1.2.2.2.5. Novel gain-of-function mutations in the F2 gene associated with venous thrombosis beyond prothrombin G20210A

Although the G20210A mutation in the 3'-untranslated region of the *F2* gene remains the most recognized prothrombin variant associated with VTE, recent discoveries have broadened the landscape of thrombophilic prothrombin mutations. In particular, several missense mutations affecting residue Arg596 in the catalytic domain of prothrombin have been identified as a novel

cause of inherited thrombophilia through a mechanism of acquired AT resistance. These include Prothrombin Yukuhashi (Arg596Leu)(80), Prothrombin Belgrade (Arg596Gln)(81,82), and Prothrombin Padua 2 (Arg596Trp)(83).

These variants share a gain-of-function phenotype: despite normal or mildly reduced clotting activity in standard assays, they confer a marked resistance to inactivation by AT, leading to sustained thrombin activity and increased thrombin generation potential. Clinically, carriers—typically heterozygotes—present with recurrent VTE at a young age, often in the absence of traditional risk factors, and sometimes with involvement of unusual sites such as cerebral or mesenteric veins. In contrast to bleeding-associated dysprothrombinemias, these variants are not associated with hemorrhagic symptoms, underscoring the concept of "prothrombotic dysprothrombinemias"(84).

Among them, Prothrombin Padua 2 (Arg596Trp)(83) was described in two Italian families, with carriers experiencing spontaneous deep vein thrombosis and pulmonary embolism. Functional assays showed normal prothrombin antigen levels but significantly impaired inactivation by AT, as evidenced by reduced thrombin–AT complex formation and delayed thrombin decay in thrombin generation tests. Similarly, Prothrombin Belgrade (Arg596Gln) and Yukuhashi (Arg596Leu) were associated with severe thrombotic phenotypes in Serbian, Indian, and Japanese families respectively(80–82).

A distinct but mechanistically similar variant, Arg541Trp (c.1621C>T), was reported by Ding et al. in 2016 in two unrelated patients with recurrent venous thrombosis. Located outside the Arg596 cluster, this mutation affects a conserved region of the catalytic domain and confers reduced susceptibility of thrombin to AT inhibition, while maintaining normal antigen levels and procoagulant activity(85). Functional assays confirmed increased thrombin generation and delayed inactivation, suggesting a novel class of thrombophilic prothrombin mutations that extend beyond the Arg596 position.

These findings prompted a revision of the classical classification of prothrombin defects. Traditionally divided into type I (quantitative) and type II (qualitative) deficiencies, recent proposals have introduced a type III category, encompassing dysprothrombinemias associated with thrombosis but without bleeding(84,86). This novel group is mechanistically and clinically distinct and emphasizes the dual procoagulant and anticoagulant interfaces of the thrombin molecule.

Other rare variants, such as Prothrombin Amrita (Arg553Gln)(87) and Prothrombin Greenville (Arg517Gln)(88), have also been identified in isolated patients and may involve the Na^+ binding loop,

a region critical for thrombin's functional conformation and interaction with inhibitors like AT. Although their clinical significance is still under investigation, these mutations may similarly contribute to thrombophilia through impaired regulation of thrombin activity.

Overall, these emerging variants reveal a new pathogenic paradigm in which prothrombin mutations can cause thrombosis through functional resistance to natural inhibitors, despite preserved or near-normal coagulant function in routine testing. Their identification highlights the importance of molecular diagnostics and specialized functional assays, especially in families with unexplained, early-onset, or recurrent thrombosis in the absence of classical thrombophilia markers.

Table 1.2 summarizes rare prothrombin variants described in the literature, with their laboratory phenotypes, affected anticoagulant mechanisms, and main references.

Eponym	FII mutations	Laboratory phenotype	Compromised mechanisms	Publications
Prothrombin Yukuhashi	p.Arg596Leu	FII:Act reduced FII:Ag slightly reduced	AT-mediated coagulation inhibition system	Miyawaki et al., 2012(80)
Prothrombin Belgrade	p.Arg596Gln	Data not shown	AT-mediated coagulation inhibition system	Djordjevic et al., 2013(82); Kishimoto et al., 2016(81)
Prothrombin Padua 2	p.Arg596Trp	FII:Act reduced FII:Ag slightly reduced	AT-mediated coagulation inhibition system	Bulato et al., 2016(83)
Prothrombin Keio	p.Tyr434His	Data not shown	Altered affinity of thrombin to fibrinogen, PC and thrombin aptamer	Takenouchi et al., 2019(89)
None	p.Arg541Trp	FII:Act slightly reduced FII:Ag normal	PC-mediated coagulation inhibition system	Wu et al., 2020(90); Tang et al.,2020; Yamamoto et al., 2021
None	p.Arg382His	FII:Act strongly reduced (<1%) FII:Ag normal	PC-mediated coagulation inhibition system, impaired TAFI activation, decreased binding to thrombomodulin, a severe impairment of thrombin inhibition by heparin cofactor II and, finally, a decrease of prothrombin activation by the FXa – FVa complex. Both the coagulant and the anticoagulant activities of thrombin are affected.	Akhavan et al., 2000(91); Akhavan et al., 2002(92); Ding Q et al., 2017(85)
Prothrombin Amrita	p.Arg553Gln	Data not shown	Prolonged fast/active form of thrombin	Melge el at., 2018(87)
None	p.Phe382Ser	FII:Act slightly reduced FII:Ag normal	PC-mediated coagulation inhibition system	Wu et al., 2025(93)
None	p.Phe382Leu	FII:Act reduced FII:Ag normal	PC-mediated coagulation inhibition system	Wu et al., 2025(93)
None	p.Asp597Tyr	FII:Act normal or slightly reduced FII:Ag normal	PC- and AT-mediated coagulation inhibition system	Wu et al., 2025(93)

Table 1.2. Reported prothrombin (FII) variants associated with altered thrombin activity and impaired anticoagulant regulation.

1.2.2.3. Non-O blood group

The ABO blood group system, first described by Karl Landsteiner in the early 20th century, has long been known for its relevance in transfusion medicine(94). However, over the past decades, accumulating evidence has established its role as an important determinant of thrombotic risk, particularly for VTE (95–97). Numerous studies and meta-analyses have shown that individuals with non-O blood types (A, B, or AB) exhibit a consistently increased risk of VTE, with an estimated 1.5- to 2.5-fold higher risk compared to those with blood group O(95,98,99). Given that approximately 50% of the general population has a non-O blood group, this makes it the most common inherited thrombophilic risk factor worldwide, surpassing in frequency all other known genetic predispositions such as FVL, PTM, and deficiencies of AT, PC, or PS(96,97).

This increased susceptibility is primarily mediated by elevated plasma levels of von Willebrand factor (VWF) and FVIII in non-O individuals(97,98,100). VWF, a glycoprotein involved in platelet adhesion and FVIII stabilization, circulates at levels approximately 25–30% higher in non-O individuals due to reduced clearance and altered glycosylation influenced by ABO glycosyltransferase activity(97,98). The presence of A and B antigens on VWF reduces its proteolytic cleavage by ADAMTS13, contributing to the persistence of high molecular weight multimers that are more thrombogenic(97).

In a large study by Clark and Wu, blood group O was associated with significantly lower levels of VWF and FVIII, and individuals with genotypes such as AA or A1B showed the highest levels of these prothrombotic factors(98,100). Moreover, data from Gallerani et al. involving over 65,000 hospitalized patients revealed that non-O blood types were significantly more frequent among those with pulmonary embolism, phlebitis, and other forms of venous thrombosis. This association remained significant even after adjusting for common risk factors(101).

Importantly, the risk conferred by ABO blood type is not limited to first events. Several studies, including the REVERSE study and subsequent analyses, have demonstrated that non-O blood groups are associated with a higher risk of VTE recurrence, with hazard ratios up to 2.0 even after adjusting for confounding variables such as FVIII levels and post-thrombotic syndrome(99,102,103). This suggests a role for ABO group not only in pathogenesis but also in guiding the duration of anticoagulation therapy.

Beyond VWF and FVIII, other mechanisms have been proposed, including ABO-related variations in adhesion molecules (e.g., soluble selectins, ICAM-1, TNF-α) and platelet function(104,105). These

may further modulate thrombotic risk, especially in the presence of concomitant thrombophilias such as FVL or PTM, where a synergistic or “supra-additive” risk has been observed(94,106,107).
In light of these findings, the ABO blood group—though often overlooked in clinical practice—may represent a useful, inexpensive, and stable marker of thrombotic predisposition, particularly in patients with unprovoked or recurrent events(95,96). Its extremely high prevalence and moderate yet consistent thrombotic effect underscore its significance not only as a genetic marker but also as a potential contributor to global thrombotic burden at the population level(14,97).

1.2.2.4. Rare and emerging variants

1.2.2.4.1. *Factor VIII abnormalities*

Factor VIII (FVIII) is a key glycoprotein in the intrinsic coagulation pathway (Figure 1.9), acting as a cofactor for activated factor IX to accelerate factor X activation and ultimately thrombin generation. While FVIII deficiency leads to hemophilia A and bleeding, sustained elevated FVIII levels are associated with a significant increase in the risk of VTE. This association is well documented: FVIII levels above 150 IU/dL (i.e., >90th percentile) are linked to a 3- to 5-fold increased risk of VTE, particularly when elevation is persistent and not related to acute-phase responses(108,109).

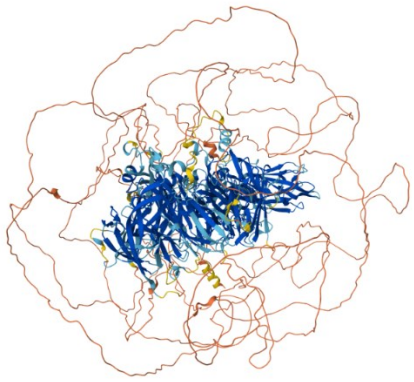


Figure 1.9. Factor VIII. Graphical representation of the protein structure of Factor VIII (source: Alphafold).

Elevated FVIII is considered a dose-dependent and time-independent risk factor, meaning that risk increases with rising levels and persists even after the thrombotic event. Several studies, including prospective cohort data, have demonstrated that patients with elevated FVIII have a higher rate of recurrent VTE, especially when levels remain elevated after anticoagulation is stopped(109,110).
Although FVIII elevation is not formally categorized as a classical monogenic thrombophilia, multiple lines of evidence suggest a heritable component. Family studies have shown that approximately

40% of first-degree relatives of individuals with elevated FVIII also have high levels, and that these relatives have a threefold higher risk of both venous and arterial thrombotic events compared to those with normal FVIII. The heritability is believed to be polygenic, as no single causative mutation in the F8 gene has been consistently identified(109,111). Rather, FVIII levels appear to be influenced by genetic variants affecting its synthesis, stability, and clearance, as well as by external factors.

Among these factors, the ABO blood group plays a pivotal role: individuals with non-O blood types have higher levels of VWF, which serves as a carrier for FVIII and prolongs its half-life(108). Other contributing factors include age, sex, obesity, inflammation, and estrogen exposure (e.g., pregnancy or hormonal therapy). Therefore, FVIII elevation should always be interpreted in context, and measurements should ideally be performed at least 6–12 weeks after the thrombotic event and cessation of anticoagulants, to avoid misclassification due to acute-phase elevations(108,109).

A particularly interesting but rare case is the FVIII Padua variant, a gain-of-function mutation in the F8 gene identified in a patient with unprovoked deep vein thrombosis. This mutation results in markedly increased coagulant activity despite normal antigen levels, demonstrating that structural alterations in the FVIII molecule can directly enhance its procoagulant effect(112). Although extremely rare, FVIII Padua serves as proof-of-concept that hyperfunctional FVIII variants may contribute to thrombophilia in selected individuals, similar to what has been observed for the FIX Padua variant in hemophilia gene therapy and rare thrombotic phenotypes.

Very recently, a naturally occurring FVIII gain-of-function variant, p.Arg590Ser (designated “FVIII Aurora”), has been identified in a young patient with recurrent, life-threatening thromboses. This mutation, located in the A2 domain, results in markedly increased clotting activity despite normal antigen levels and confers reduced responsiveness to activated PC, providing the first demonstration of an inherited hyperfunctional FVIII variant contributing to thrombophilia(113).

In clinical practice, persistent FVIII elevation should be considered a relevant risk factor in patients with unprovoked or recurrent VTE, especially in those with a positive family history or absence of other identifiable thrombophilic defects. Although not routinely included in thrombophilia screening, FVIII measurement may guide decisions on duration of anticoagulation and risk stratification for secondary prevention(109,110,114).

1.2.2.4.2. Elevated factor XI levels

Factor XI (FXI) is a serine protease (Figure 1.10) of the intrinsic coagulation pathway that plays a role in thrombin generation and in the inhibition of fibrinolysis. Unlike the severe bleeding phenotype

of FVIII or IX deficiency, congenital FXI deficiency (hemophilia C) is usually associated with only mild bleeding and, paradoxically, may confer a protective effect against thrombotic events(115,116). In recent years, however, elevated FXI levels have gained attention as a novel prothrombotic biomarker.

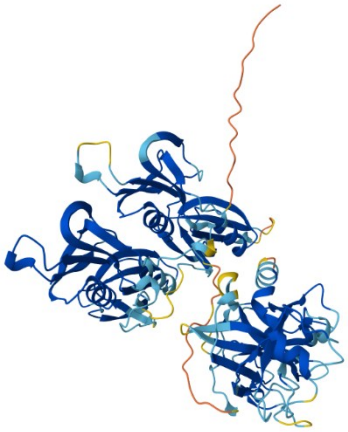


Figure 1.10. Factor XI. Graphical representation of the protein structure of factor XI (source: Alphafold).

Data from the Leiden Thrombophilia Study (LETS) were among the first to report an association between elevated FXI antigen levels (above the 90th percentile) and a significantly increased risk of deep vein thrombosis, with an adjusted odds ratio of 2.2(116). Subsequent studies have confirmed this association. In a prospective analysis from the LITE study, individuals in the highest quintile of FXI concentration had a hazard ratio for VTE of 1.51 compared to those in the lowest quintile, a finding that held true for both white and African American populations(115).

A particularly important contribution came from a recent study by Spiezia et al., who evaluated patients after a first episode of proximal DVT. The authors observed that persistently elevated FXI levels—confirmed across two time points—conferred a markedly increased thrombotic risk, with an odds ratio of 5.2. These findings suggest that not only elevated but persistently elevated FXI levels may reflect a stable prothrombotic phenotype, possibly of genetic origin(117).

The genetic underpinning of elevated FXI levels has also been explored. Liu et al. identified the c.539A>G polymorphism in the F11 gene, which was significantly more frequent in VTE patients in a Chinese population. This variant was associated with elevated FXI antigen levels without affecting activity, suggesting a potential mechanism of impaired clearance or altered expression(118). In a large cohort of Swedish women, the F11 rs2289252 polymorphism was shown to be associated with a 1.8-fold increased risk of VTE recurrence, particularly when combined with FVL(119).

Despite this growing body of evidence, the role of FXI in predicting VTE recurrence remains debated. A 2023 systematic review by Bosch et al. identified substantial heterogeneity among studies evaluating FXI, with conflicting results and inconsistent definitions of "elevated" levels. The review concluded that FXI may not yet be a reliable biomarker for recurrence prediction, highlighting the need for standardized assays, cut-offs, and prospective validation(111).

Notably, FXI's role is not limited to venous thrombosis. Elevated FXI levels have been associated with ischemic stroke, myocardial infarction, and even all-cause mortality following stroke, supporting its candidacy as a target for anticoagulant therapy. Clinical trials evaluating FXI inhibitors, including antisense oligonucleotides and monoclonal antibodies, have demonstrated promising safety and efficacy in preventing postoperative VTE without significantly increasing bleeding risk(120–122).

In conclusion, elevated FXI levels represent an emerging but incompletely characterized prothrombotic state. While not currently included in routine thrombophilia screening, their measurement—particularly when persistently elevated—may help identify individuals at higher thrombotic risk and inform future therapeutic strategies(115,117,119).

1.2.2.4.3. Factor IX abnormalities

Factor IX (FIX) is a vitamin K–dependent s erine protease essential for the intrinsic pathway of coagulation (Figure 1.11). While congenital FIX deficiency (hemophilia B) is a well-known bleeding disorder, mounting evidence indicates that elevated levels of FIX, whether due to acquired or genetic causes, may confer an increased risk of VTE (123,124).

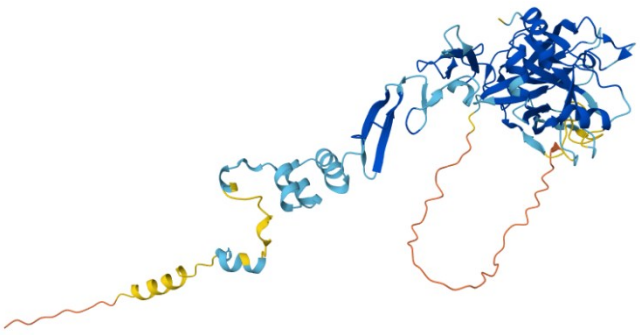


Figure 1.11. Factor IX. Graphical representation of the protein structure of factor IX (source: Alphafold).

One of the most compelling pieces of evidence comes from the Leiden Thrombophilia Study (LETS), which showed that individuals with FIX antigen levels above the 90th percentile (cut-off: 129 U/dL)

had a 2- to 3-fold increased risk of VTE, even after adjustment for other thrombotic risk factors. The risk was especially pronounced in premenopausal women not using oral contraceptives, where the odds ratio reached 12.4(124).

Subsequent studies confirmed this association. A case-control study by Lowe et al. demonstrated a significant relationship between high FIX levels (>150 IU/dL) and idiopathic VTE in women, with an odds ratio of 2.3 after adjusting for hormone replacement therapy(125). Other epidemiological studies, including Heikal et al., have reported odds ratios up to 6.8 for VTE in individuals with elevated FIX activity, further suggesting that FIX elevation may be an under-recognized prothrombotic trait(123).

More recently, a comprehensive review highlighted elevated FIX as a contributor to increased risk of both first and recurrent VTE, reinforcing its role as a clinically relevant prothrombotic factor(111).

A landmark case study by Simioni et al. described the first gain-of-function mutation in the F9 gene, resulting in the FIX Padua variant (Arg338Leu), which causes markedly increased FIX activity—approximately 8- to 12-fold higher than normal—despite normal antigen levels. The proband, a young man with unprovoked DVT, carried the R338L mutation, and functional analyses confirmed a hypercoagulable phenotype(126). This was the first demonstration that a genetic FIX variant could directly cause thrombophilia through enhanced coagulant function rather than increased protein concentration.

Interestingly, this same FIX Padua variant, originally identified in a thrombophilic context, has been repurposed in gene therapy for hemophilia B. Owing to its markedly enhanced specific activity, the FIX Padua cDNA has been incorporated into several gene therapy vectors to allow for therapeutic expression at lower protein levels but with high functional efficacy. Clinical trials using adeno-associated virus (AAV) vectors carrying FIX Padua have demonstrated sustained hemostatic correction with relatively low FIX antigen expression, reducing the risk of immune responses and vector load requirements(127). Thus, FIX Padua represents a rare example of a naturally occurring thrombophilic mutation being leveraged for therapeutic gain in a bleeding disorder.

However, the prevalence of FIX Padua in the general population and among VTE patients appears very low. Both Mazzetto et al. and Koenderman et al. failed to detect the R338L variant in large cohorts of patients with elevated FIX levels or familial thrombophilia, suggesting that while pathogenic, FIX Padua is likely extremely rare(128,129).

Another extremely rare gain-of-function F9 mutation is FIX Shanghai (p.Arg338Gln); this variant markedly increases FIX clotting activity—approximately 4- to 5-fold compared with wild-type—

leading to a prothrombotic phenotype. It was identified in a young patient with recurrent deep vein thrombosis and shown to potentiate FIX catalytic efficiency similarly, though slightly less, than the well-known FIX Padua variant(130).

Several physiological and external factors also modulate FIX levels, including age, sex, hormonal status, BMI, and estrogen exposure. Oral contraceptive use and menopause have been associated with higher FIX levels, potentially amplifying thrombotic risk in susceptible individuals(124). FIX levels also correlate strongly with FVIII levels, and the FIX–FVIII axis may play a synergistic role in thrombin generation and thrombosis(131).

Although measurement of FIX levels is not routinely included in thrombophilia screening, these data suggest that persistent elevations—especially above the 90th percentile—may warrant consideration in selected cases, such as idiopathic or recurrent thrombosis, or thrombosis at a young age without other risk factors(111,123,124). Furthermore, FIX is emerging as a target for antithrombotic therapy, with experimental drugs such as FXIa inhibitors and antisense oligonucleotides offering promising avenues for prevention with a lower bleeding risk(131).

1.2.2.4.4. Congenital dysfibrinogenemia

Congenital dysfibrinogenemia (CD) is a rare qualitative fibrinogen disorder caused by heterozygous mutations in the *FGA*, *FGB*, or *FGG* genes. Figure 1.12 shows the structure of the fibrinogen molecule, composed of three paired polypeptide chains (Aα, Bβ, and γ), which represent the targets of mutations underlying congenital dysfibrinogenemia. Despite normal antigen levels, functional activity is reduced due to structural abnormalities of the fibrinogen molecule. Traditionally considered a mild bleeding disorder or an incidental laboratory finding, CD has gained increasing recognition as a potential prothrombotic condition in selected cases(132,133).

While early registries suggested that the majority of CD patients are asymptomatic (~55%), with only ~25% presenting with bleeding and ~20% with thrombotic events, more recent studies have challenged this distribution. A multicenter retrospective cohort by Casini et al. involving 101 genetically confirmed CD patients and their relatives showed that the cumulative incidence of thrombosis by age 50 reached 30.1%, exceeding that of major bleeding (19.2%). Importantly, these events included both arterial and VTE, with a predominance of unprovoked VTE and postpartum thrombosis(134).

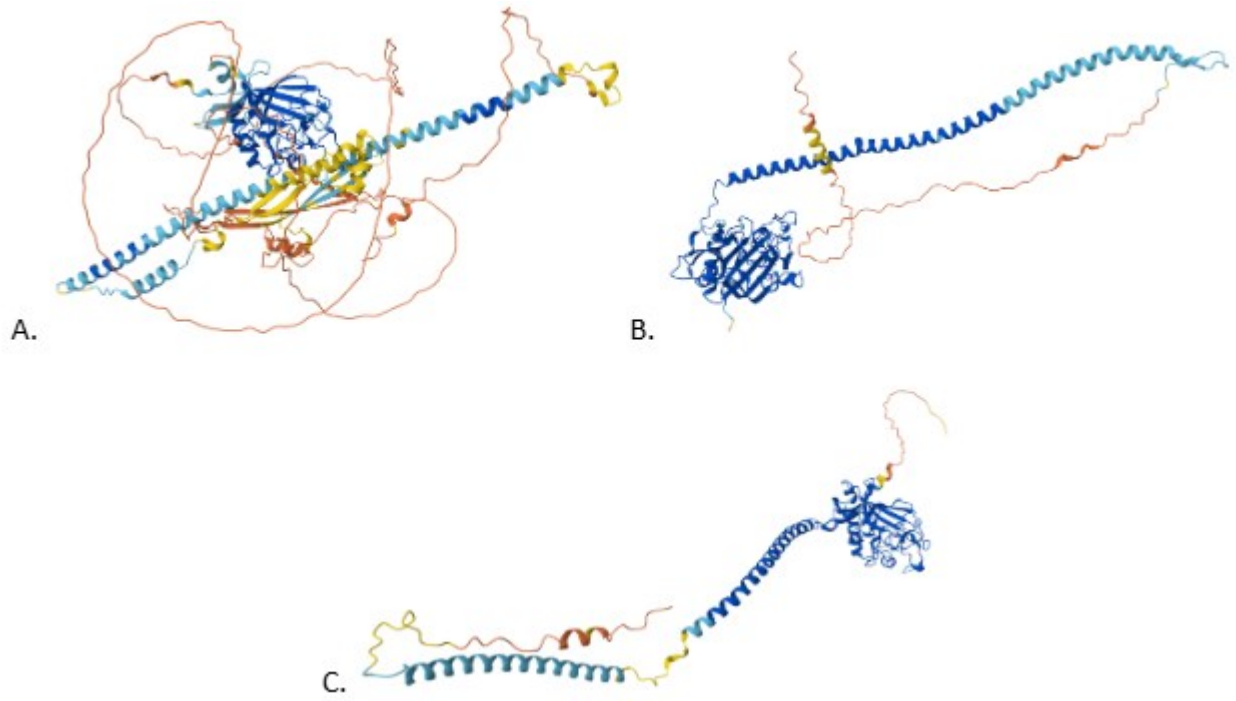


Figure 1.12. Fibrinogen. Graphical representation of the protein structure of the alpha (A), beta (B), and gamma (C) chains of fibrinogen (source: Alphafold).

Family studies have been instrumental in establishing the link between dysfibrinogenemia and thrombophilia. In a registry study conducted by the ISTH Subcommittee on Fibrinogen, 26 families with both CD and unexplained thrombosis were analyzed. Among 187 relatives, thrombosis was reported exclusively in those with dysfibrinogenemia, further supporting a causal role. In some well-characterized families (e.g., Paris V/Dusart syndrome), recurrent thromboembolic events were observed in multiple affected members, including fatal pulmonary embolism(132,133).

The underlying mechanisms of thrombosis in CD are heterogeneous and likely mutation-specific. Proposed prothrombotic mechanisms include impaired thrombin inactivation due to defective fibrin-thrombin binding, and defective fibrin-mediated activation of plasminogen by tissue plasminogen activator (tPA), both leading to impaired fibrinolysis. These pathophysiological alterations have been observed in variants such as Malmö, Pamplona II, and Paris V. Notably, studies have shown that clots formed with abnormal fibrinogen may be structurally more compact and less susceptible to enzymatic degradation(132,133).

Genetic variability contributes to the phenotypic spectrum. For instance, Castaman et al. identified several novel mutations, including *FGA* Arg35His and *FGB* Arg44Cys, which were associated with thrombotic manifestations in affected individuals. However, genotype–phenotype correlations remain inconsistent, and neither specific mutations nor fibrinogen levels reliably predict clinical

outcomes. Of note, hypodysfibrinogenemia—defined by both reduced function and antigen levels—may confer a higher thrombotic risk in certain contexts(135).

In light of these findings, CD should be considered in the differential diagnosis of unexplained or familial thrombosis, particularly when coagulation tests reveal prolonged thrombin or reptilase time in the setting of discordant fibrinogen assays. Anticoagulant therapy may be warranted in symptomatic carriers, as illustrated by case reports using tailored-dose dabigatran with favorable outcomes(136).

Overall, while not a classical form of inherited thrombophilia, congenital dysfibrinogenemia represents a rare but clinically relevant prothrombotic condition in selected individuals and families. Recognition and genetic confirmation are essential to guide appropriate management and family screening.

1.2.2.5. Fibrinolytic defects and others

1.2.2.5.1. TAFI

TAFI, also known as procarboxypeptidase B, U, or R, is a plasma zymogen that plays a pivotal role in regulating fibrinolysis (Figure 1.13). Upon activation by thrombin—particularly when complexed with thrombomodulin—TAFI is converted into an active enzyme (TAFIa) that attenuates fibrinolysis by cleaving C-terminal lysine residues from partially degraded fibrin. This modification reduces plasminogen binding, thereby stabilizing the fibrin clot and impeding clot lysis(137).

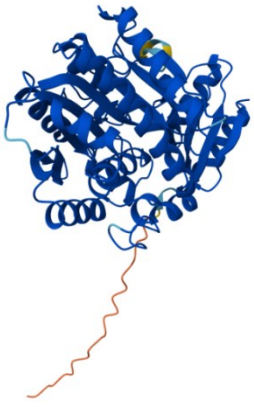


Figure 1.13. Thrombin-activatable fibrinolysis inhibitor. Graphical representation of the protein structure of Carboxypeptidase B2 (source: Alphafold).

Numerous studies have associated elevated plasma TAFI levels with a modest but consistent increase in the risk of VTE. In the MEGA study, individuals in the highest quartile of TAFI levels had a 1.6-fold higher risk of a first venous thrombosis compared to those in the lowest quartile, even

after adjustment for age, sex, and body mass index. This association persisted after accounting for acute-phase proteins and markers of endothelial activation, suggesting a direct contribution of TAFI to thrombotic risk(138).

Beyond protein levels, genetic polymorphisms in the TAFI gene (CPB2) have also been implicated in modulating thrombosis susceptibility. The gene, located on chromosome 13q14.11, contains multiple variants that affect both antigen levels and protein activity(139). Among them, the 505G/A (Ala147Thr) and 1040C/T (Thr325Ile) polymorphisms have been consistently associated with altered plasma TAFI levels and functional activity. In particular, carriers of the 505GG genotype, associated with lower TAFI antigen levels, paradoxically exhibited an increased risk of DVT in the Leiden Thrombophilia Study (LETS), indicating that the relationship between TAFI levels and thrombosis is complex and may not be strictly dose dependent(140).

A large meta-analysis by Qian et al. further clarified these associations: while the −438G/A variant was not significantly associated with VTE risk, the 505G/A and 1040C/T variants showed significant associations under various genetic models. Specifically, the 505GG genotype and the 1040C allele (CC and CT genotypes) were both associated with increased VTE risk, particularly in non-Asian populations and in cases of non-cerebral thrombosis(141).

Haplotypes combining these variants, such as GTC (505G, 1040T, +1542G), have also been associated with a higher risk of cerebral venous thrombosis, especially in women and in hormone-related events(142). These findings point to a pleiotropic and context-dependent role of TAFI in thrombotic disease, influenced by both genotype and interacting environmental or hormonal factors(141,142).

Taken together, the evidence supports a role for TAFI as a modulator of fibrinolysis and contributor to thrombotic risk, albeit with modest effect sizes. While TAFI measurement is not part of standard thrombophilia panels, its interplay with clot stability and fibrinolysis suggests it may become a relevant biomarker or therapeutic target, particularly in populations with hypofibrinolytic phenotypes or resistance to standard anticoagulants(138,139).

1.2.2.5.2. Plasminogen Activator Inhibitor-1 (PAI-1) and the 4G/5G polymorphism

Plasminogen Activator Inhibitor-1 (PAI-1) is the principal physiological inhibitor of tissue plasminogen activator (tPA) and urokinase (uPA), and plays a key role in downregulating fibrinolysis (Figure 1.14). Elevated PAI-1 levels have been associated with a hypofibrinolytic state and an increased risk of VTE, particularly in the presence of inherited or acquired thrombophilic

conditions(143–145). One of the most studied genetic determinants of PAI-1 expression is the 4G/5G insertion/deletion polymorphism in the promoter region of the *SERPINE1* gene. This polymorphism affects transcriptional activity: the 4G allele is associated with higher basal and cytokine-induced expression of PAI-1 compared to the 5G allele(146,147).

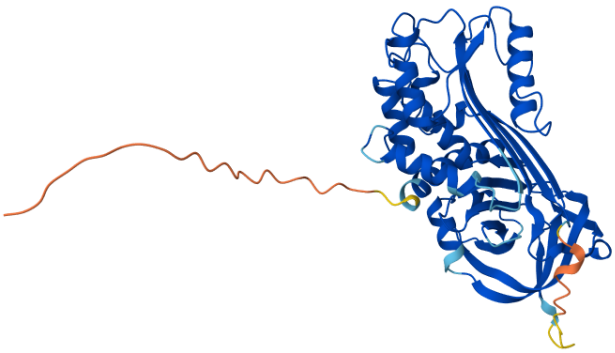


Figure 1.14. Plasminogen activator inhibitor. Graphical representation of PAI-1 (source: Alphafold).

Several studies have evaluated the relationship between the 4G/5G genotype and the risk of VTE, with mixed results. Sartori et al. first reported a higher prevalence of the 4G/4G genotype in patients with deep vein thrombosis(148). They demonstrated a correlation between this genotype and reduced fibrinolytic potential due to increased plasma PAI-1 levels(149). This suggests a plausible mechanistic link between the 4G allele and thrombotic predisposition via fibrinolytic inhibition. More recent studies, such as those by Prabhudesai and colleagues, have confirmed this association, particularly in patients with coexisting inherited thrombophilic mutations(150).

Conversely, other investigations have yielded less conclusive findings. Wang et al., in a Chinese cohort, did not observe a significant association between the 4G/5G polymorphism and the initial risk of VTE; however, they did find that patients with the 5G/5G genotype showed better rates of venous recanalization and a lower recurrence rate following anticoagulation, hinting at a possible prognostic role(151). Similarly, Vuckovic et al. noted that while elevated PAI-1 activity was related to thrombotic risk, the genotype alone did not predict thrombotic events, suggesting that the functional phenotype—rather than the genotype per se—may be more clinically relevant(152).

Overall, the 4G/5G PAI-1 polymorphism represents a biologically plausible but only modest genetic risk factor for thrombosis. Its effect appears modulated by environmental influences, concomitant genetic traits, and population background(143,146,147). While not currently recommended for routine thrombophilia screening, PAI-1 genotyping and functional testing may hold value in selected

clinical scenarios, particularly in patients with unexplained or recurrent VTE and a suspected hypofibrinolytic profile(149,150).

1.2.2.5.3. Thrombomodulin

Thrombomodulin (TM) is a transmembrane glycoprotein predominantly expressed on endothelial cells, where it plays a central role in the regulation of coagulation (Figure 1.15). Upon binding to thrombin, TM undergoes a conformational change that redirects thrombin's substrate specificity from procoagulant to anticoagulant pathways. This complex activates PC, which inactivates factors Va and VIIIa, and also contributes to the activation of thrombin activatable fibrinolysis inhibitor (TAFI), thereby influencing fibrinolysis(153). TM therefore serves as a critical endogenous anticoagulant and antifibrinolytic modulator, and its impairment may predispose to either thrombosis or bleeding depending on the nature of the dysfunction.

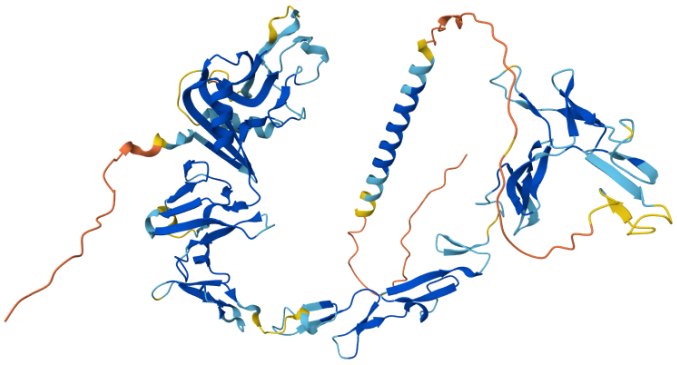


Figure 1.15. Thrombomodulin. Graphical representation of thrombomodulin (source: Alphafold).

Genetic variants in the THBD gene, which encodes TM, have been investigated as potential contributors to thrombotic and hemorrhagic phenotypes. While nonsense mutations in *THBD* can lead to increased plasma levels of soluble TM and are associated with rare inherited bleeding syndromes, missense mutations have also been linked to VTE(154,155). However, the evidence for a direct causal relationship remains inconclusive(155,156). In a recent functional study on eight rare *THBD* variants found through next-generation sequencing in patients with thrombotic or bleeding phenotypes, the L433P and C175S variants were shown to significantly impair PC activation and thrombin inhibition(154,155). In particular, L433P completely abolished thrombin-TM binding, a key interaction site for anticoagulant activity, suggesting a strong functional basis for increased thrombotic risk (154,155).

Other genetic variants—such as the c.1418C>T polymorphism—have also been studied in relation to soluble TM levels and VTE susceptibility, with some studies showing increased levels of soluble TM and altered endothelial function in carriers(157,158). However, large-scale association studies, including analyses of the promoter region variants, have yielded conflicting results, making it difficult to define the true clinical significance of common *THBD* polymorphisms(155–159).

Experimental models further support a dual role of TM: both overexpression and loss-of-function may be pathogenic. While TM deficiency leads to a procoagulant and proinflammatory endothelial phenotype, high levels of soluble TM may act as a decoy receptor, impairing endogenous TM function on the cell surface(153). Functional studies remain crucial to understanding the impact of individual *THBD* variants, as many are still classified as variants of uncertain significance (VUS) in the absence of biochemical characterization(154–156).

In conclusion, thrombomodulin represents a rare but mechanistically plausible contributor to inherited thrombotic and bleeding disorders(153,155,156). Although routine testing for *THBD* mutations is not currently recommended in thrombophilia workup, identification of pathogenic variants through multigene panels and integration with functional assays may offer valuable insights in selected patients, especially in those with unexplained or familial thrombotic phenotypes(154–156).

1.2.2.5.4. Heparin Cofactor II deficiency

Heparin cofactor II (HCII) is a serine protease inhibitor synthesized in the liver that selectively inhibits thrombin in the presence of dermatan sulfate or heparin (Figure 1.16). It plays a complementary role to AT in regulating thrombin activity and modulating vascular homeostasis. Although its in vivo relevance has been less emphasized compared to other serpins such as AT or PC, several observations suggest that HCII deficiency may contribute to thrombotic risk under specific circumstances(160).

Early case reports described familial heterozygous HCII deficiency in association with arterial or venous thrombosis, raising the hypothesis that HCII may function as a natural antithrombotic protein(161). However, population-based studies have questioned this association. In a prevalence study by Lopaciuk *et al.*, low HCII levels (<65%) were found in 5.7% of patients with a first episode of deep vein thrombosis (DVT) before age 45, compared to only 0.9% of controls, suggesting a modest overrepresentation of HCII deficiency in VTE patients. Nonetheless, the majority of affected individuals had only mild to moderate reductions in HCII levels, and no significant difference in mean

HCII levels was observed between cases and controls, highlighting the limited penetrance of heterozygous deficiency(162).

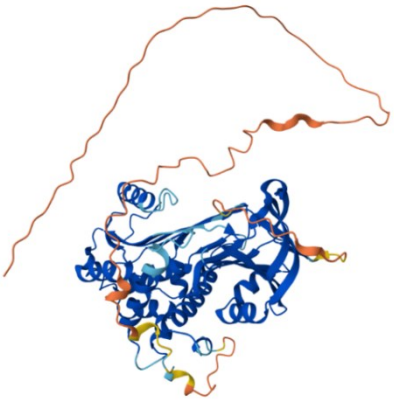


Figure 1.16. Heparin cofactor II. Graphical representation of the protein structure of heparin cofactor II (source: Alphafold).

More compelling evidence comes from rare cases of homozygous HCII deficiency, which appear to confer a more marked prothrombotic phenotype(163). In the family study reported by Villa *et al.*, the proband—homozygous for HCII deficiency and also heterozygous for AT deficiency—experienced multiple thrombotic events including DVT and pulmonary embolism. Conversely, her homozygous sister and 12 heterozygous relatives remained asymptomatic, suggesting that isolated HCII deficiency is insufficient to trigger thrombosis, but may contribute significantly in the presence of additional congenital or acquired prothrombotic factors, such as AT deficiency or surgery(160,163).

This context-dependent role of HCII is further supported by molecular studies. Corral *et al.* described a homozygous missense mutation (Glu428Lys) affecting the reactive center loop of HCII, which resulted in severe antigen and activity deficiency due to intracellular polymerization—a mechanism analogous to serpinopathies such as alpha1-antitrypsin deficiency. While the proband had recurrent thrombosis, several other family members with the same homozygous defect did not, underlining the variable expressivity and possible compensatory mechanisms(160).

In summary, while heparin cofactor II deficiency is a rare entity, it may play a synergistic role in thrombophilia when co-inherited with other thrombophilic mutations. Its routine screening is not currently recommended, but HCII analysis may be informative in selected patients with unexplained thrombosis, particularly when more common defects have been excluded. The limited thrombotic

expressivity in heterozygotes and even in some homozygotes suggests that HCII acts as a secondary rather than primary anticoagulant pathway in humans(160).

1.2.2.5.5. Endothelial Protein C Receptor (EPCR)

EPCR (Figure 1.17) plays a critical role in the PC anticoagulant pathway by enhancing the activation of PC on the endothelial surface. Soluble EPCR (sEPCR), generated by proteolytic cleavage or alternative splicing, lacks the transmembrane domain and acts as a competitive inhibitor, potentially interfering with both the activation of PC and the anticoagulant activity of APC(164). Genetic variants of the *PROCR* gene encoding EPCR, particularly those defining the A3 haplotype such as the 6936A>G (Ser219Gly) polymorphism, have been associated with elevated levels of sEPCR(164,165). This elevation may result from destabilization of the transmembrane domain, enhancing proteolytic shedding of EPCR. Elevated sEPCR levels are hypothesized to contribute to a prothrombotic state by inhibiting APC generation and activity(164).

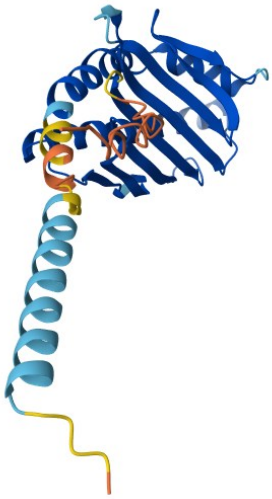


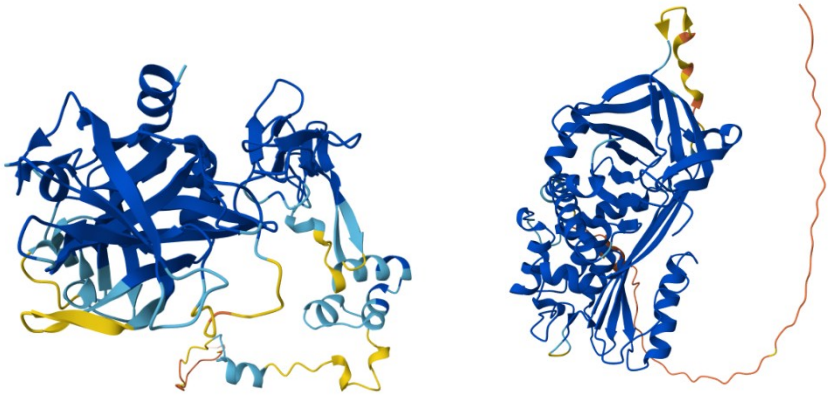
Figure 1.17. Endothelial protein C receptor. Graphical representation of the protein structure of the endothelial protein C receptor (source: Alphafold).

Epidemiological studies have yielded mixed results regarding the association between *PROCR* variants and VTE. While some reports suggest that the A3 haplotype confers a modestly increased VTE risk, particularly in men (164), others have not confirmed this association(165). The largest meta-analyses indicate that carriers of the Ser219Gly variant (rs867186) have a significantly increased risk of VTE under both dominant and recessive genetic models, although the effect size is modest (OR ~1.3–1.6)(166). Notably, this risk appears more pronounced in specific genetic contexts,

such as among carriers of the prothrombin 20210A variant, where the presence of the 4600G allele (A3 haplotype) is associated with an earlier onset of VTE and higher sEPCR levels(167).
Conversely, the A1 haplotype, tagged by the 4678G>C polymorphism, may exert a protective effect, as it has been associated with higher APC levels and a reduced risk of thrombosis(166). However, this protective association also appears to be context-dependent and requires further validation.
In summary, *PROCR* polymorphisms contribute to interindividual variability in thrombosis risk, likely via modulation of EPCR shedding and sEPCR levels(164–166). Although not currently included in routine thrombophilia screening panels, these variants may be relevant in polygenic risk models or in individuals with additional risk factors.

1.2.2.5.6. Protein Z and the PZ/ZPI anticoagulant system

Protein Z (PZ) is a vitamin K-dependent glycoprotein synthesized in the liver, structurally homologous to factors VII, IX, and X, yet lacking catalytic activity (Figure 1.18 A)(168). Its anticoagulant role is exerted through its interaction with the protein Z-dependent protease inhibitor (ZPI), which enhances the inhibition of activated factor Xa (FXa) and, to a lesser extent, factor XIa and FIXa (Figure 1.18 B)(168,169). This mechanism plays a complementary role in the regulation of coagulation, acting alongside other natural anticoagulants such as AT and the PC/PS system(169).



A B

Figure 1.18. (A) Protein Z. Graphical representation of the protein structure of Protein Z. (B). Protein Z–dependent protease inhibitor Graphical representation of the protein structure of the Protein Z–dependent protease inhibitor of FXa (source: Alphafold).

Although initially described in the 1980s, the clinical relevance of PZ deficiency remains controversial(170). Several studies have reported an association between low PZ plasma levels and an increased risk of VTE, arterial thrombosis, and adverse pregnancy outcomes(171). A comprehensive meta-analysis by Sofi et al. showed that low PZ levels are significantly associated

with an increased risk of thrombotic events (OR 2.90 for all thrombotic diseases), including venous thromboembolism (OR 2.18), arterial events (OR 2.67), and pregnancy complications (OR 4.17)(171). Supporting this, Santacroce et al. demonstrated that very low PZ levels—below the 5th or 2.5th percentile—were significantly more frequent in patients with deep vein thrombosis (DVT) than in matched controls, even after adjusting for traditional thrombophilic markers such as FVL and PTM.(172) These findings suggest that severe PZ deficiency may act as an independent risk factor, albeit with limited contribution from common PZ gene polymorphisms(170,172).

In contrast, other studies such as those from the Leiden Thrombophilia Study failed to find a strong association between PZ or ZPI levels and VTE in the general population, underscoring the possible influence of confounders, such as inflammation, oral contraceptive use, and vitamin K antagonist therapy, on PZ measurements(168,173). Additionally, Corral and colleagues identified a nonsense mutation in the ZPI gene (Arg67Stop), which was associated with a threefold increased risk of VTE, further implicating the PZ/ZPI system in thrombosis pathogenesis(174).

Experimental data in animal models reinforce this hypothesis. PZ-deficient mice exhibit a prothrombotic phenotype, particularly in combination with FV Leiden, indicating a synergistic effect between genetic risk factors(174,175).

Overall, although large-scale prospective studies are still lacking, available evidence supports the notion that severe PZ deficiency or functional disruption of the PZ/ZPI complex may contribute to a hypercoagulable state(169,171,172). However, the exact clinical utility of testing PZ and ZPI remains uncertain, and routine screening is not currently recommended outside of research or selected clinical settings(170).

1.2.2.5.7. Tissue Factor Pathway Inhibitor (TFPI)

TFPI (Figure 1.19) is a key physiological regulator of the tissue factor (TF)-dependent coagulation pathway, acting primarily by inhibiting the TF–FVIIa complex and factor Xa(176,177). Unlike other natural anticoagulants such as AT or PC, TFPI displays a complex distribution in circulation: most TFPI is bound to endothelial surfaces or lipoproteins, with only a small proportion circulating as free, full-length TFPI—the form considered to possess the most potent anticoagulant activity(177).

Evidence from animal studies and clinical observations suggests that TFPI plays an essential role in maintaining hemostatic balance. TFPI knockout mice exhibit embryonic lethality due to uncontrolled coagulation, and recombinant TFPI has shown protective effects in experimental thrombosis models(177). In humans, reduced levels of TFPI—particularly the free, active form—have been

associated with increased risk of VTE, although results from clinical studies have been somewhat inconsistent(177,178).

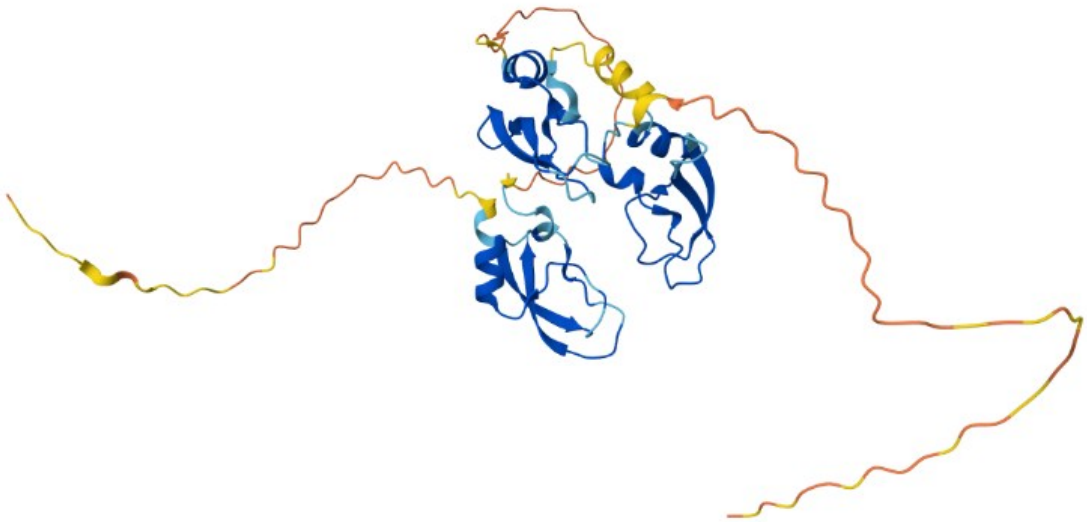


Figure 1.19. Tissue factor pathway inhibitor. Graphical representation of the protein structure of TFPI (source: Alphafold).

Prospective and case-control studies such as the Leiden Thrombophilia Study (LETS) and the Longitudinal Investigation of Thromboembolism Etiology (LITE) have reported that individuals with TFPI levels in the lowest percentiles exhibit an elevated risk of VTE(177,178). For example, the LITE study found that those in the bottom 5% of TFPI levels had an adjusted odds ratio (OR) of 1.93 for VTE (95% CI: 1.05–3.53), even after controlling for confounding factors such as BMI and D-dimer(178). Another case-control analysis using a diluted prothrombin time (dPT)-based assay of TFPI anticoagulant activity found that individuals with low TFPI activity in both dPT and chromogenic assays had an OR of 5.9 (95% CI: 1.7–20) for VTE compared to individuals with normal levels(179).

Genetic studies further support the relevance of TFPI in thrombosis. A recent meta-analysis highlighted that the rs8176592 polymorphism was associated with an increased risk of venous thrombosis, particularly in Asian populations (OR = 1.48 in the recessive model)(178). Other polymorphisms such as rs10931292 may also confer risk depending on population and study context (178).

Despite these findings, TFPI deficiency is not yet routinely tested in clinical thrombophilia work-ups, due to challenges in measuring its function reliably and the relatively modest and variable associations reported in epidemiological studies(177,178). Nevertheless, emerging genetic and

functional data suggest that TFPI may contribute to thrombotic risk in a subset of patients, especially when other prothrombotic conditions are present(176,179).

1.2.2.5.8. Factor XIII

Factor XIII (FXIII) (Figure 1.20) plays a central role in stabilizing fibrin clots by catalyzing covalent cross-linking of fibrin α- and γ-chains, thereby enhancing clot strength and resistance to fibrinolysis(180,181). While congenital FXIII deficiency is rare and associated with severe bleeding(181), the role of FXIII levels and polymorphisms in VTE has been a subject of debate.

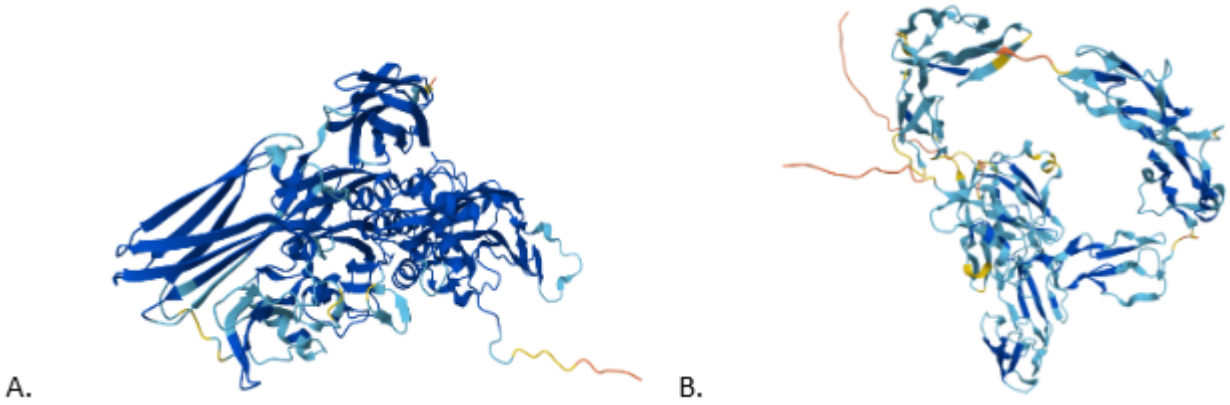


Figure 1.20. Factor XIII. Graphical representation of the protein structure of the A chain (A) and the B chain (B) of factor XIII (source: Alphafold).

A common genetic polymorphism in the FXIII-A subunit gene, Val34Leu, located near the thrombin activation site, has garnered particular attention(182,183). This substitution accelerates thrombin-induced FXIII activation, leading to altered fibrin architecture characterized by thinner, more compact fibers that are potentially more resistant to fibrinolysis(181–183). Paradoxically, multiple epidemiological studies suggest that the Leu34 variant may confer a modest protective effect against VTE, especially in homozygous individuals(183). Data from the Leiden Thrombophilia Study (LETS) showed that carriers of the Leu34 allele had slightly decreased thrombotic risk, with an odds ratio (OR) of 0.9 (95% CI: 0.7–1.2) for heterozygotes and 0.7 (95% CI: 0.4–1.3) for homozygotes. However, this protective effect appears to be small and was significant only in male participants(183).

The biological mechanism behind this observation may involve a more rapid FXIII activation in Leu34 carriers, influencing clot structure and dissociation kinetics. Indeed, higher FXIII activity has also been associated with lower thrombotic risk, particularly when exceeding the 90th percentile of

activity distribution(183). On the other hand, elevated levels of the FXIII A and B subunits were not associated with protection and may even slightly increase the risk of thrombosis (183,184).

It is noteworthy that not all studies have confirmed these findings. Some reported no association between the Val34Leu polymorphism and VTE risk(182,185), highlighting the variability across populations and study designs. Moreover, recent evidence suggests that sex-specific effects and interactions with fibrinogen levels may modulate the impact of FXIII polymorphisms on thrombotic risk(181,184).

In conclusion, although the Val34Leu polymorphism of FXIII appears to confer a weak protective effect against VTE, especially in men(183), the clinical significance remains limited. Routine testing for this variant is not currently recommended in the diagnostic workup of thrombophilia, but understanding its role may help refine individual thrombotic risk profiles in combination with other genetic and environmental factors.

1.3. New frontiers in the genetic diagnosis of thrombophilia

1.3.1. The limits of conventional testing

Despite decades of thrombophilia research, standard diagnostic algorithms still fail to identify a cause in many cases of early-onset, recurrent, or unprovoked VTE. Deficiencies in AT, PC and PS, and the well-known FVL and PTMs account for a minority of cases. Yet, many patients exhibit a strong personal or familial history without any of these alterations. Moreover, phenotypic variability among individuals harboring the same mutation—such as a family member with PS deficiency who remains asymptomatic, while another suffers a pulmonary embolism at age 25—raises the possibility of hidden modifiers or additional genetic contributors(186–188).

Traditional thrombophilia testing is based on two main approaches: functional assays (e.g., measuring activity or antigen levels of AT, PC, PS) and targeted genotyping of specific point mutations (e.g., FVL, PTM). While these methods remain clinically relevant, they are limited to detecting common, well-characterized defects. Rare or functionally subtle variants often remain undetected, particularly when they do not alter protein concentration but rather affect activity, structure, or interactions(6,25,189,190).

1.3.2. The contribution of NGS and WES to genetic discovery

Next-generation sequencing (NGS), including targeted gene panels and whole-exome sequencing (WES), has revolutionized our ability to interrogate the genome beyond first-line testing(190–192).

Next-Generation Sequencing (NGS) has revolutionized genetic diagnostics by enabling massively parallel sequencing of millions of DNA fragments. This approach provides broad coverage of coding regions, splice sites, and regulatory elements, offering a comprehensive view of the genetic landscape. Compared with Sanger sequencing, NGS is faster, more cost-effective for multigene testing, and capable of detecting a wider spectrum of variants, including single-nucleotide substitutions, small insertions/deletions, and, with adapted workflows, larger structural variants(189,193,194). NGS can be applied at different levels of resolution. Targeted multigene panels focus on predefined sets of genes with strong biological plausibility, such as natural anticoagulants, coagulation factors, and fibrinolytic proteins. This approach has the advantage of high coverage, reduced cost, and more straightforward interpretation, but may miss variants outside the selected genes(190,195).

Whole-exome sequencing (WES) extends the analysis to all coding regions of the genome, capturing potential novel associations, while whole-genome sequencing (WGS) includes both coding and non-coding elements. WES and WGS offer broader discovery potential, but at the price of increased data complexity, reduced depth per gene, and a high proportion of variants of uncertain significance (VUS). In the context of thrombophilia, targeted panels remain the most clinically applicable approach at present, while WES/WGS are particularly valuable for research and novel gene discovery(193,196–198).

Studies such as those by Suchon et al. have revealed likely pathogenic variants in up to 35% of patients with unexplained thrombosis, involving both known thrombophilia genes (e.g., *SERPINC1*, *PROS1*, *F2*, *F5*) and less well-characterized loci(192).

Lunghi et al. employed WES in a familial thrombosis setting and demonstrated the co-occurrence of multiple rare variants in coagulation-related genes, suggesting a polygenic model of disease(196). Similarly, Kramer et al. applied a 55-gene panel to patients with cerebral vein thrombosis (CVT) and found a significantly increased frequency of potentially prothrombotic intronic and exonic variants, including in *FGA*, *F13A1*, *PROCR*, and *ITGB3*, highlighting the utility of genomic profiling in rare thrombotic phenotypes(199).

1.3.3. Interpretative challenges: VUS, risk alleles, ACMG/AMP criteria

The interpretation of NGS results is often challenging. A large proportion of identified variants are classified as VUS, reflecting insufficient evidence to assign pathogenic or benign roles. Distinguishing between VUS, true pathogenic variants, and benign polymorphisms requires integration of multiple

resources, including population frequency databases (e.g., gnomAD), in silico prediction tools (PolyPhen-2, SIFT, CADD, REVEL), segregation studies, and functional assays(190,192,197)

In addition, certain common alleles—such as the prothrombin c.1726-59G>A variant or PROCR p.Ser219Gly—are frequently detected in patients and healthy individuals alike. These “risk alleles” do not follow a Mendelian inheritance model but may contribute to disease in a polygenic or context-dependent manner. Their interpretation requires careful balance, avoiding both underestimation and overstatement of their clinical significance(187,191,199).

Variant classification is standardized by the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP), which recommend a five-tier system: benign (class 1), likely benign (class 2), uncertain significance (class 3), likely pathogenic (class 4), and pathogenic (class 5). While this framework is widely adopted, it was primarily developed for monogenic disorders and is not always easily applicable to complex traits such as thrombosis(200).

1.3.3. Genetic modifiers and complex architecture

Westrick and Ginsburg introduced the concept of *modifier genes*, which modulate the expression and phenotypic impact of major thrombophilia mutations. This is supported by murine models and human pedigrees, where identical mutations produce divergent clinical outcomes depending on genetic background(188).

In the study by Kramer et al., the combination of multiple polymorphisms—each insufficient on its own to confer high risk—was shown to be overrepresented in CVT patients compared to the general population. This combinatorial effect supports a threshold model, in which a certain "mutational burden" must be crossed to manifest disease(199).

Athar et al. and Lindström et al. further support this idea through their work on whole-exome sequencing and pathway analysis, demonstrating that many cases involve multiple variants across genes regulating coagulation, endothelial function, and inflammation(195,197).

1.3.4. The role of rare and non-coding variants

While most thrombophilia screening focuses on coding variants, several studies have emphasized the contribution of non-coding regions. Many GWAS hits fall in intronic or regulatory areas, which may influence gene expression or splicing(187,201). The Kramer study also identified intronic variants in *ADAMTS13* and *FN1* significantly enriched in CVT patients, suggesting that regulatory variants may play a previously underestimated role(199).

Moreover, variants of uncertain significance (VUS), often identified by NGS or WES, require functional validation to clarify their pathogenic potential. This represents a major bottleneck in the clinical utility of extended genetic testing(190,192).

1.3.5. Clinical translation and challenges

Despite its promise, the integration of genomic tools into thrombophilia workups remains limited. Current guidelines do not universally recommend extended genetic testing, citing lack of outcome-based evidence and difficulties in variant interpretation. Still, for patients with severe, early, or familial thrombosis without clear cause, NGS can reveal actionable information—provided that results are interpreted within a multidisciplinary framework(187,190).

Tools such as polygenic risk scores (PRS) are also being explored, though their utility in clinical practice remains to be validated. Furthermore, there is a pressing need for curated variant databases and prospective studies linking specific variants to outcomes like recurrence risk and bleeding complications(187,191).

1.4. Toward a revised concept of inherited thrombophilia

The classical monogenic paradigm of inherited thrombophilia is gradually giving way to a more nuanced model. Instead of single-gene disorders, many cases reflect the complex interaction of multiple low-to-moderate effect alleles, modulated by environmental and acquired risk factors(188,191,196). The application of NGS and WES has catalyzed this conceptual shift, allowing us to view thrombophilia as a spectrum of genetic susceptibility rather than a binary trait(190,192,199).

As evidence accumulates, the future of thrombophilia diagnostics will likely rest on personalized algorithms combining clinical phenotype, extended genetic analysis, and risk stratification tools. This integrated approach may ultimately improve prevention strategies, especially for young patients with idiopathic VTE or those with a strong family history but no identifiable mutation using standard assays(187,191,196).

2. BACKGROUND

As previously mentioned, VTE is a major global health burden(1), with an estimated 50-60% of cases occurring without an identifiable triggering factor, classified as unprovoked VTE(2). While much is known about the pathophysiology of VTE, our understanding remains incomplete, particularly in cases where, in the absence of known acquired risk factors, standard thrombophilia screening fails to identify underlying genetic abnormalities. Routine screening for inherited thrombophilia typically assesses common inherited risk factors, such as FVL, the PTM, and deficiencies in AT, PC, and PS(7,202). However, these tests have limited sensitivity and may overlook rarer genetic defects or complex polygenic interactions contributing to thrombotic risk.

Moreover, a major challenge in thrombophilia screening is the failure of conventional laboratory techniques to detect subtle or rare functional abnormalities. For example, certain well-characterized variants, such as AT Dublin(203) or PS Heerlen(204) variants, may not be identified through standard assays, despite their established role in modulating coagulation. In addition, extremely rare and largely anecdotal defects—reported only in single families or small cohorts—have been described, such as pseudo-homozygous FVL(205), gain-of-function factor IX variants (Padua(126), Malmö(206), Shangai(130)), other variants of FV such as FV Besançon(72), and AT-resistant prothrombin Arg596 substitutions (Yukuhashi(207), Belgrade(82), Amrita(208), Padua 2(83)), the FVIII Padua duplication variant(112), further complicating laboratory detection and risk assessment. Furthermore, familial clustering of VTE suggests a strong heritable component, yet genetic screening in these individuals often fails to identify causative mutations, pointing toward a more complex genetic predisposition(191).

Recent advances in Next-Generation Sequencing (NGS) have provided an unprecedented opportunity to investigate the genetic landscape of thrombophilia beyond the limitations of traditional approaches. Unlike conventional methods that target single-gene defects, NGS allows for the simultaneous analysis of multiple genes, thereby enabling the identification of novel or rare variants, as well as potential gene-gene interactions that may shift the hemostatic balance toward a prothrombotic or hemorrhagic state(187). This genetic complexity may explain intra-familial variability, where some carriers of a pathogenic variant develop thrombosis while others remain asymptomatic(191). It could also account for inter-individual differences in response to

anticoagulant therapy, with some patients experiencing recurrent thrombotic events despite treatment(10), while others suffer bleeding complications(209).

Recent evidence supports the clinical relevance of multigene panels in patients with VTE. In a large real-world cohort of 194 VTE patients, Verstraete et al.(190) reported that a diagnostic-grade multigene panel identified genetic variants in 63% of cases, including (likely) pathogenic variants in 39%, and additional variants of uncertain significance in many others. Notably, 12% of patients harbored noncompatible pathogenic variants that would have remained undetected using conventional methods alone. Furthermore, a positive family history of VTE emerged as the only clinical predictor significantly associated with a positive genetic result, reinforcing the role of hereditary predisposition and the added diagnostic value of expanded genetic testing.

A recent review by Ramanan et al.(210) further emphasized the growing interest in integrating NGS-based panels into clinical practice. The authors highlighted how multigene panels not only improve the diagnostic yield in inherited thrombotic conditions but also enable the identification of co-occurring variants that may act as phenotype modifiers, supporting the concept of oligogenic inheritance. However, the widespread implementation of these panels remains limited by the high prevalence of variants of uncertain significance, inconsistent access across healthcare systems, and the absence of standardized testing criteria. These findings underscore the need for continued research to clarify the clinical impact of multigene testing in VTE and to define its optimal role in personalized risk stratification and management.

Given these considerations, the integration of NGS into thrombophilia screening holds the potential to refine risk stratification, improve individualized prevention strategies, and enhance our understanding of the molecular mechanisms underlying VTE.

3. AIMS OF THE STUDY

The primary aim of this study was to investigate the genetic basis of VTE in young patients with unprovoked VTE or severe VTE despite weak provoking factors, who tested negative for conventional thrombophilia screening. Specifically, our objectives were:

1. To identify pathogenic and likely pathogenic variants in a broad panel of coagulation-related genes using next-generation sequencing (NGS).
2. To describe the spectrum and frequency of variants of uncertain significance (VUS) and their potential clustering across patients.
3. To assess the coexistence of multiple variants within the same individual and explore their possible cumulative or synergistic effects.
4. To analyze the relationship between genetic findings and clinical characteristics, including VTE recurrence, unusual anatomical sites, bleeding events under anticoagulation, and family history.
5. To discuss the potential implications of NGS-based screening for risk stratification and personalized management in patients with otherwise unexplained or disproportionate VTE.

4. MATERIALS AND METHODS

4.1. Study population

We enrolled consecutive, unrelated patients referred to the Thrombotic and Hemorrhagic Diseases Unit and Clinical Medicine 1 of the University Hospital of Padova, with an objectively documented episode of VTE between 1 January 2014 and 31 December 2023. VTE was defined as pulmonary embolism documented by contrast-enhanced CT pulmonary angiography or, when appropriate, a high-probability ventilation/perfusion lung scan; deep-vein thrombosis (DVT) of the lower or upper limbs demonstrated by compression ultrasonography (both distal calf and proximal segments were accepted); splanchnic-vein thrombosis confirmed by contrast-enhanced abdominal CT; and thrombosis at other unusual venous sites—such as cerebral venous sinuses, ovarian or testicular, or retinal veins—diagnosed with the appropriate imaging technique. Eligibility criteria were: 1) age 18–50 years at the time of the first thrombotic event; 2) confirmed history of unprovoked VTE; 3) negative standard thrombophilia work-up for both inherited (FVL, F2 G20210A, AT, PC, PS deficiencies, abnormal levels of other coagulation factors – FII, FVIII, FIX, FX, FXI, fibrinogen, plasminogen and PAI) and acquired causes (lupus anticoagulant, anticardiolipin and anti-β2-glycoprotein I antibodies).We also considered a small group of patient with severe VTE despite only weak provoking factors (e.g. minor trauma, short-haul travel, hormonal therapy and pregnancy). Patients were included irrespective of the presence or absence of a strong family history of VTE, defined as ≥ 1 first-degree relative with objectively documented VTE.

In this study, the term “severe VTE” refers to thrombotic events characterized by either extensive anatomical involvement, a clinically significant acute presentation, or a degree of severity that appears disproportionate to the presence of weak or transient provoking factors (5,11). Severe VTE included events with proximal extension, such as femoro–iliac or caval thrombosis, as well as thrombosis occurring at unusual anatomical sites where local complications and morbidity are typically greater, including the cerebral or splanchnic venous circulations. Cases were also considered severe when the initial presentation was clinically marked, for example in patients with pulmonary embolism meeting intermediate–high or high-risk criteria according to contemporary ESC definitions(21). This framework was adopted to capture those VTE episodes in which the clinical

phenotype suggests an increased underlying predisposition, particularly when the extent or severity of thrombosis exceeds what would be expected based on the identified risk exposure.
All participants provided written informed consent, and the study was approved by the local Ethics Committee in accordance with the Declaration of Helsinki.

4.2. Blood sample and conventional thrombophilia assessment

Blood samples were collected from each enrolled patient by venipuncture directly into 5 BD Vacutainer® Citrate Tubes containing 0.109 M (3.2%) sodium citrate (9:1 blood to anticoagulant ratio) using a 21-gauge needle with light tourniquet, after discharging the first mL of venous blood. Thrombophilia testing followed international recommendations. Coagulation factor activity was measured using factor-deficient plasmas and Dade® Actin® FS Activated PTT Reagent for Factors VIII, IX, XI, and XII, or Thromborel® S for Factors II, V and X (all reagents from Siemens Healthineers, Erlangen, Germany). AT activity was evaluated using an FIIa-based chromogenic assay (Berichrom® AT III, Siemens Healthineers). All tests were performed on a Sysmex CN-3000 hemostasis system (Siemens Healthineers)(211,212). Factor XIII activity was measured using the Berichrom® FXIII chromogenic assay (Siemens Healthineers) on a BCS® XP or CN-3000 coagulation analyzer. Fibrinogen levels were measured using a modified Clauss method on a BCS® XP coagulation analyzer (Siemens Healthineers), with the Multifibren U-Reagent (Siemens Healthineers)(212). Protein C chromogenic and coagulometric activities were also assessed on the same analyzer using two commercial kits: Berichrom® Protein C and Protein C Reagent (Siemens Healthineers), respectively. PC antigen levels were determined using a matched-pair antibody sets for ELISA (Affinity Biologicals, Ancaster, ON, Canada) (213). Free PS activity and antigen levels were analyzed on an ACL TOP 300 CTS coagulation instrument (Instrumentation Laboratory, Milan, Italy), using HemosIL® PS Activity and HemosIL® Free PS kits, respectively (Instrumentation Laboratory)(212). Total PS antigen levels were measured by a matched-pair antibody set for ELISA (Affinity Biologicals)(214). Activated protein C resistance was assessed by an APTT-based assay using factor V-deficient plasma (Siemens Healthineers) and exogenous activated protein C (Enzyme Research Laboratories, South Bend, IN, USA) on an ACL TOP 300 CTS analyzer(215). The presence of anti-cardiolipin and anti-β2-glycoprotein I antibodies (IgG and IgM) was detected using commercial ELISA kits (Orgentec Diagnostika GmbH, Mainz, Germany). Plasminogen activity was determined on a CN-3000 analyzer using the Berichrom® Plasminogen kit (Siemens Healthineers). Plasminogen activator inhibitor-1 antigen was measured using a commercially available ELISA kit (ZYMUTEST™ PAI-1 Antigen, Hyphen

BioMed, Neuville-sur-Oise, France)(212). FVL and PTMs were identified by allelic discrimination real-time PCR (QuantStudio™ 5 Real-Time PCR System, Applied Biosystems, Foster City, CA, USA) using two commercially available kits: RealQuality RS-FVL and RealQuality RS-Factor II G20210A (AB Analitica, Padua, Italy), respectively.

4.3. DNA extraction

Genomic DNA was isolated from buffy coat samples using the MagCore® Genomic DNA Whole Blood Kit (RBC Bioscience, New Taipei City, Taiwan) on the MagCore® HF16 Automated Nucleic Acid Extractor (RBC Bioscience).

4.4. Library preparation and NGS sequencing

Targeted capture NGS was performed using a oligonucleotides biotynylated custom panel (xGenTM Hyb Panel, Integrated DNA Technologies, USA) covering coding regions and flanking exon/intron boundaries of 33 coagulation-related genes (A2MC, CPB2, F2, F3, F5, F7, F8, F9, F10, F11, F12, F13A1, F13B, FGA, FGB, FGG,KLKB1, KNG1, PLAT, PLG, PROC, PROCR, PROS1, PROZ, SERPINA5, SERPINA10,SERPINC1, SERPIND1, SERPINE1, SERPINF2, THBD, TFPI, VWF) with an average coverage of 99.99% (100X). The library preparation for targeted capture was carried out using the Lotus DNA library prep Kit (IDT). Briefly, 100 ng of DNA was quantified by Qubit DNA HS assay (Life Technologies). Genomic DNA was enzymatic fragmented, and fragments were ligated with stubby P5 and P7 adapters, following the manufacturer's instructions. Libraries were purified using AMPure XP beads (Beckman Coulter, CA, USA). Libraries quantification and length were performed using Qubit 4.0 fluorimeter (Thermo Fisher) and LabChip GX Touch Nucleic Acid Analyzer (PerkinElmer, Massachusetts, USA), respectively. A multiplexing pool of 500 ng of each prepared library from 12 samples was used for hybrid capture with the biotinylated DNA oligonucleotide custom panel. After hybridization, Streptavidin Dynabeads were used to capture biotinylated DNA. Purified DNA was PCR amplified using the Bio-Rad CX200 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The library pool was further purified using AMPure XP beads (Beckman Coulter, Brea, CA, USA) and quantified by Qubit 4.0 DNA HS assay. Finally, the library pool was sequenced on an Illumina NextSeq 550, using a Mid-Output flow cell (300 cycles), following the manufacturer's instructions.

4.5. Bioinformatic analysis and variants interpretation

A manifest file detailing the sequence of hybridizing oligonucleotides and the coordinates of the designed amplicons was obtained from the manufacturer and uploaded into the CLC Genomics Workbench (version 24.0.1). Briefly, sequencing reads were aligned to the human reference genome (hg19, GRCh37). The workflow then removed ligation artifacts from the read mappings. Following this, the Indels and Structural Variants detection step generated a guidance track, which was used to improve the mapping accuracy using the Local Realignment tool. Subsequent filtering steps were applied to remove variants likely to be artifacts. The final output of the workflow includes a list of filtered variants and BAM files, which were visualized using the Integrative Genomics Viewer (IGV), and a list of filtered variants (vcf files) to be used for the variant interpretation using Franklin software (Qiagen). Variants were classified according to the guidelines of American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) as benign (class 1), likely benign (class 2), variants of unknown significance (VUS, class 3), likely pathogenic (class 4) or pathogenic (class 5)27. GenomAD v4.1.0, ClinVar, CADD v.17, Revel, SiFT, PolyPhen2, and SpliceAI were used to confirm Franklin classification. ClinGen specific criteria were applied for variant classification in F8, F9, SERPINC1, and VWF genes. Only class 3, 4, and 5 variants were reported in this paper. ACMG/AMP pathogenicity classification was applied for variants with a minor allele frequency (MAF) <10%(200). Known predisposing variants listed in Supplementary Table 1 were also evaluated.

4.6. Statistical analysis

Continuous variables were summarized as mean±SD, according to distribution assessed by the Shapiro–Wilk test and visual inspection. Categorical variables were reported as counts (percentages). Comparisons between groups were performed using the t test or Mann–Whitney U test for continuous variables and the χ^2 test or Fisher's exact test for categorical variables.

Multivariate analyses were conducted using binary logistic regression to estimate odds ratios (OR) with 95% confidence intervals (CI), adjusting for clinically relevant confounders: age, sex, BMI, smoking, family history of VTE, index event type (unprovoked vs provoked), and clinical phenotype (DVT, PE, DVT+PE).

All tests were two-sided, with statistical significance set at p=0.05. Analyses were performed using IBM SPSS Statistics v29 on a Windows platform.

5. RESULTS

5.1. Overall study population

A total of 100 patients with objectively documented VTE were included in the study (Table 5.1). Forty-five (45%) were female, and the mean age at diagnosis was 33 ± 9 years. The vast majority were Caucasian (97%), with two patients of African origin (2%) and one of Middle Eastern descent (1%). The mean body mass index (BMI) was 25.5 ± 5.1 kg/m². Most participants were non- or former smokers (70%), whereas 26 were active smokers and 4 (4%) had unknown smoking status.

Comorbidities were absent in 63 patients (63%), while 6 (6%) reported more than one condition. The most frequent were thyroid disorders (8%, mostly Hashimoto's tyroiditis), arterial hypertension (6%), metabolic disturbances including dyslipidemia, steatosis or steatohepatitis (5%), anxious–depressive disorders (5%), and respiratory conditions such as asthma (3%). Less common conditions included autoimmune or connective tissue diseases (e.g., uveitis, Ehlers–Danlos syndrome), benign hematologic disorders (spherocytosis, thrombocytopenia of undetermined origin, thalassemia trait), chronic organ condition (renal polycystic disease, monorenal status, or post–renal transplant), and prior severe infections (e.g., endocarditis with cerebral emboli). A smaller number of patients reported gynecological or gastrointestinal comorbidities (e.g., polycystic ovary syndrome, hysterectomy for fibroids, or irritable bowel syndrome). Finally, rare cases of neurological (epilepsy) or genetic conditions (Klinefelter syndrome) were also recorded.

A family history of VTE was documented in 50 patients (50%), whereas it was unknown in 10 (10%). The index event was unprovoked in 67 patients (67%) and associated with weak provoking factors in 33 (33%), most commonly hormonal therapy (17%), minor trauma (6%), pregnancy/post-partum (6%), or prolonged immobility (4%). DVT was the most frequent VTE phenotype (54%), followed by isolated PE (27%) and combined PE + DVT (19%). Among DVT cases, 37 (37%) involved the femoral/popliteal veins, 11 (11%) were isolated distal thromboses, 8 (8%) extended to iliac or caval segments, and a minority affected unusual sites such as cerebral (4%), retinal (3%), or splanchnic veins (4%). Recurrent VTE occurred in 42 patients (42%), including 8 (8%) with ≥3 episodes and 5 (5%) who relapsed during anticoagulant therapy.

	Tested patients (n. 100)
Female, n (%)	45 (45.0)
Age, mean y (±SD)	33 (±9)
Ethnicity, n (%)	97 (97.0)
Caucasian	2 (2.0)
African	1 (1.0)
Middle Eastern	
Body mass index, mean kg/m^2 (±SD)	25.5 (±5.1)
Smoke habit, n (%)	
Non- or former smoker	70 (70.0)
Active smoker	26 (26.0)
Unknown	4 (4.0)
Comorbidities, n (%)	
None	63 (63.0)
Thyroid disorders	8 (8.0)
Arterial hypertension	6 (6.0)
Metabolic disorders	5 (5.0)
Anxious-depressive disorders	5 (5.0)
Asthma and other respiratory conditions	3 (3.0)
Miscellaneous	13 (13.0)
Family history of VTE, n (%)	50 (50.0)
Unknown	10 (10.0)
VTE etiology, n (%)	
Unprovoked	67 (67.0)
Provoked	33 (33.0)
VTE risk factors, n (%)	
Minor trauma	6 (6.0)
Pregnancy and post-partum	6 (6.0)
Immobility due to sitting	4 (4.0)
Hormonal therapy	17 (17.0)
VTE type, n (%)	
DVT	54 (54.0)
PE	27 (27.0)
PE and DVT	19 (19.0)
DVT site, n (%)	
Femoral and/or popliteal	37 (37.0)
Isolated distal	11 (11.0)
Extended to iliac and/or inferior caval	8 (8.0)
Upper extremity	3 (3.0)
Cerebral	4 (4.0)
Retinal	3 (3.0)
Splanchnic and other unusual sites	4 (4.0)
Recurrent VTE, n (%)	42 (42.0)
2 episodes	8 (8.0)
≥3 episodes	5 (5.0)
during anticoagulant therapy	7 (7.0)
Bleeding events during anticoagulation, n (%)	19 (19.0)
Major bleeding	2 (2.0)

Tabel 5.1. Baseline characteristics of tested patients. Baseline features of tested patients, including demographic variables, comorbidities, family history, and clinical characteristics of venous thromboembolism (VTE). Values are reported as number (percentage) or mean (± standard deviation, SD). Abbreviations: VTE, venous thromboembolism; DVT, deep vein thrombosis; PE, pulmonary embolism.

Bleeding during anticoagulation was reported in 19 patients (19%). The most frequent manifestation was menorrhagia (n=8), which often required temporary discontinuation or dose reduction of anticoagulants. Additional events included epistaxis (n=2), gum bleeding (n=1), gastrointestinal bleeding such as rectal bleeding or positive fecal occult blood tests (n=2), hematuria (n=2, self-limiting), and post-traumatic bruises (n=2). Less common but more severe events comprised cerebral hemorrhage (n=1) and a post-traumatic hematoma requiring transfusion (n=1). According to ISTH criteria(216,217), two episodes were classified as major bleeding, while the remaining were considered clinically relevant non-major (CRNM) or minor events.

5.2. NGS sequencing and variant detection

In the study cohort, NGS identified 68 distinct variants of interest (ACMG classes 3–5) across 24 coagulation-related genes (Figure 5.1).

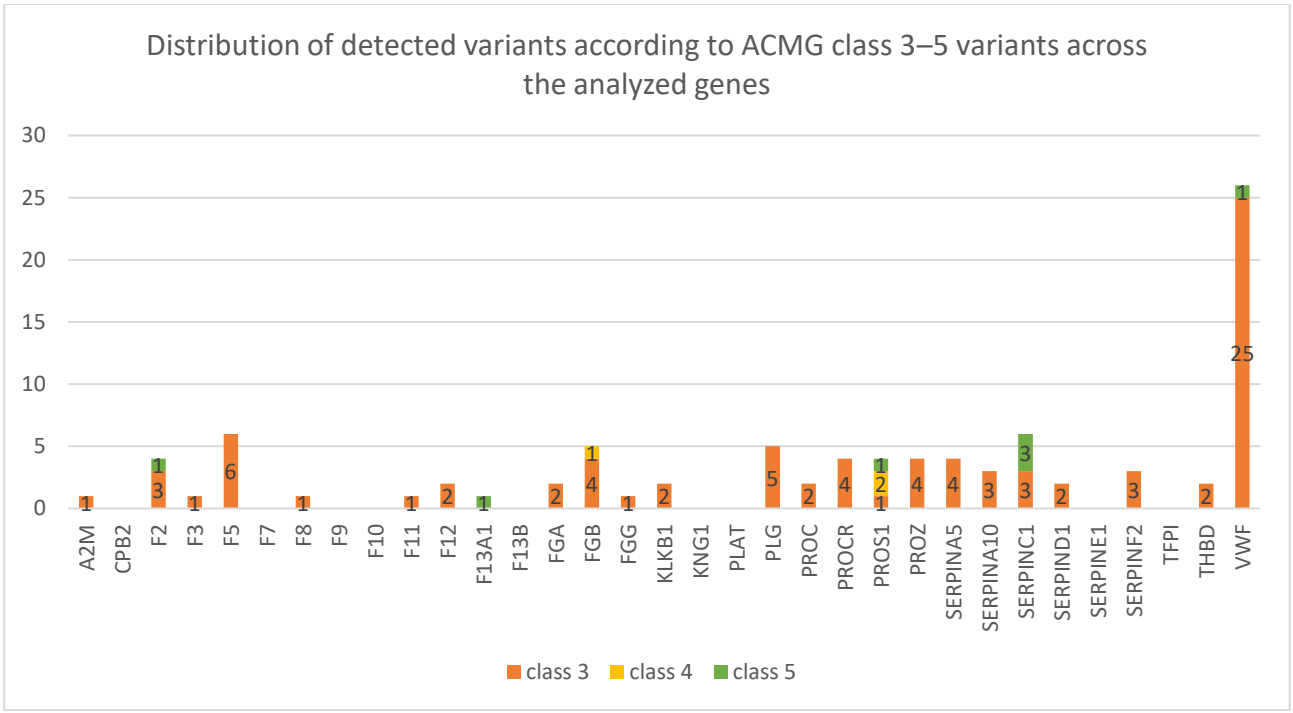


Figure 5.1. Distribution of ACMG class 3–5 variants for each patient across the analyzed genes. Bar chart showing the number of variants identified in each of the 33 genes included in the panel. Variants are classified according to ACMG criteria: class 3 (variants of uncertain significance, orange), class 4 (likely pathogenic, blue), and class 5 (pathogenic, green). The *VWF* gene accounted for the highest number of variants, followed by *SERPINC1*, *F5*, *PLG*, *PROS1*, *F2*, and *FGA*.

Overall, these variants were detected in 59 of 100 patients (59%) (Figure 5.2A), involving genes encoding fibrinogen chains (*FGA, FGB, FGG*), coagulation factors (*F2, F5, F8, F11, F12, F13A1, F3*), von Willebrand factor (*VWF*), natural anticoagulants (*SERPINC1, PROC, PROS1*), regulators of the PC

pathway (*PROCR, THBD*), genes implicated in the fibrinolytic system (*PLG, SERPINF2, A2M, KLKB1, SERPINA5*), and additional coagulation inhibitors (*PROZ, SERPINA10, SERPIND1*).

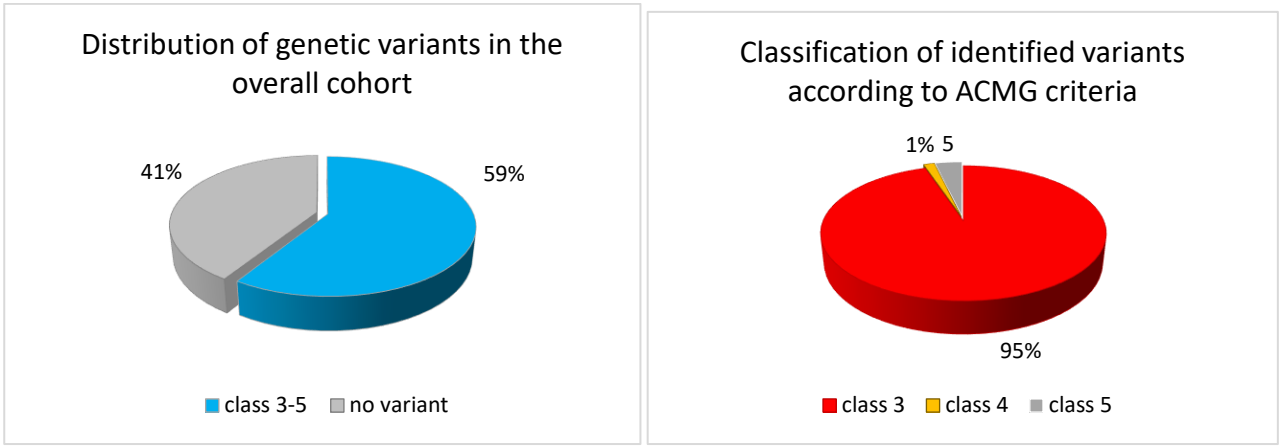


Figure 5.2. Distribution of genetic variants in the study cohort. (A) Proportion of patients with at least one ACMG class 3–5 variant compared with those without variants (overall cohort).(B) Classification of all detected variants according to ACMG criteria: class 3 (variants of uncertain significance), class 4 (likely pathogenic), and class 5 (pathogenic).

Specifically, 6 pathogenic variants (class 5) were detected in 7 patients, 3 of whom also carried an additional class 3 variant. Two likely pathogenic variant (class 4) was observed in 2 patient. In addition, 60 variants of uncertain significance (class 3) were identified in 56 patients, with several individuals carrying more than one variant (Figure 5.2B). Notably, the same variant was often found in multiple patients.

When considering the overall distribution, 33 patients carried a single variant, 20 carried two variants, 5 carried three variants, and 1 patient carried four variants, resulting in a total of 26 individuals (44% of positive cases) harboring multiple variants.

In contrast, 41 patients (41%) had no potentially deleterious variants detected in any of the analyzed genes, despite a confirmed history of unprovoked VTE, a negative conventional thrombophilia work-up, even in presence of a positive family history.

5.3. Overview of genetic variants identified

A complete list of all identified variants, including genomic coordinates, cDNA and protein changes, rsIDs, zygosity, ACMG class, and patient identifiers, is provided in Table 5.2. The complete list of variants with patient- annotation, including genomic coordinates, zygosity, and ACMG class, is provided in Supplementary Table 2.

The most frequently involved gene was VWF, with variants identified in 24 patients (24%). Notably, the synonymous variant c.5049A>C (p.Ala1683=, rs79275181) was observed in 16 individuals, often in combination with other variants. Other recurrently involved genes included SERPINC1 (6 patients), F5 (5), PLG (5), PROS1 (4), F2 (4), FGA (4), and PROZ (4) (Figure 1). Several variants detected had previously been associated with functional or structural defects in the literature or in ClinVar, including the PS Heerlen variant (PROS1 c.1501T>C, p.Ser501Pro, class 4)(218), the AT Dublin variant (SERPINC1 c.89T>A, p.Val30Glu, class 5)(203), the AT Budapest 3 variant (SERPINC1 c.391C>T, p.Leu131Phe, class 5)(219), and the prothrombin Arg541Trp substitution (F2 c.1621C>T, class 5)(90). Additional class 5 variants of interest included a splice site mutation in F13A1 (c.691-1G>A), a missense variant in VWF (c.1534-3C>A, affecting splicing), and a missense mutation in PROS1 (c.233C>T, p.Thr78Met). All pathogenic and likely pathogenic variants were identified in the heterozygous state, with no evidence of compound heterozygosity.

Gene	GRCh37	Exon	NTD	AA	RS	Zygosity	Class	Patient
FGB	chr4:155490457	6	c.956C>T	p.Pro319Leu	rs374463429	Het	3	S1, S67
	chr4:155490947	7	c.1240G>A	p.Gly414Ser	rs141881199	Het	4	S7
FGA	chr4:155484174	1	c.4A>G	p.Lys2Glu	rs6053	Het	3	S115, S172
	chr4:155507052	5	c.1529G>A	p.Arg510His	rs138137585	Het	3	S187
	chr4:155507556	5	c.1025G>A	p.Gly342Glu	rs774664670	Het	3	S202
FGG	chr4:155533202	3	c.275T>C	p.Leu92Pro	rs766605366	Het	3	S171
VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3	S1,S6, S8, S10, S11, S14, S16, S27, S34, S41, S43, S48, S51, S54, S77, S87
	chr12:6167213	int13	c.1534-3C>A		rs61754009	Het	5	S44
	chr12:6166187	15	c.1781C>G	p.Ala594Gly	rs267607308	Het	3	S54
	chr12:6138597	22	c.2878C>T	p.Arg960Trp	rs370984712	Het	3	S54
	chr12:6128388	28	c.4196G>A	p.Arg1399His	rs1800382	Het	3	S89, S217
	chr12:6173519	12	c.1325G>A	p.Arg442His	rs199665748	Het	3	S98
	chr12:6166046	15	c.1922C>T	p.Ala641Val	rs61754019	Het	3	S110, S184
	chr12:6127640	28	c.4944T>C	p.Pro1648=	rs201825372	Het	3	S182
F12	chr5:176830478	int11	c.1387+4C>G	-	rs761161412	Het	3	S3
	chr5:176836468	int1	c.57+4A>G	-	rs371915487	Het	3	S209
THBD	chr20:23030015	1	c.127G>A	p.Ala43Thr	rs1800576	Het	3	S3
	chr20:23028811	1	c.1331A>G	p.Glu444Gly	rs375671812	Het	3	S209
F11	chr4:187201533	9	c.1022A>G	p.Glu341Gly	n/a	Het	3	S44
PROCR	chr20:33762502	int1	c.71-3C>T	-	rs367783205	Het	3	S5
	chr20:33764821	3'UTR	c.*205C>T	-	rs1384660995	Het	3	S5
	chr20:33764619	int4	c.*3A>C	-	rs767268282	Het	3	S45
	chr20:33762717	2	c.283G>T	p.Gly95Cys	rs139714129	Het	3	S169
SERPINC1	chr1:173879947	4	c.707C>T	p.Ser236Leu	rs368419985	Het	3	S6
	chr1:173884010	2	c.89T>A	p.Val30Glu	rs2227624	Het	5	S55
	chr1:173883708	2	c.391C>T	p.Leu131Phe	rs121909567	Het	5	S71, S166
	chr1:173879828	4	c.826T>G	p.Phe276Val	n/a	Het	3	S110
	chr1:173878747	5	c.1096C>G	p.Gln366Glu	rs565091601	Het	3	S192
F5	chr1:169500178	15	c.5054C>G	p.Thr1685Ser	rs6011	Het	3	S6
	chr1:169499020	16	c.5245C>G	p.Leu1749Val	rs6034	Het	3	S6
	chr1:169500085	15	c.5147C>T	p.Ser1716Leu	n/a	Het	3	S14
			c.1228A>C	p.Asn410His		Het	3	S77
	chr1:169510246	13	c.3974_4081dup	p.Leu1325_Asp1360du	n/a	Het	3	S201, S204
PROC	chr2:128186377	9	c.1241G>C	p.Trp414Ser	rs768759265	Het	3	S7
	chr2:128186246	9	c.1110C>A	p.His370Gln	rs778063605	Het	3	S32
PROS1	chr3:93598150	13	c.1501T>C	p.Ser501Pro	rs121918472	Het	5	S8, S87
	chr3:93646095	2	c.233C>T	p.Thr78Met	rs6122	Het	5	S50
	chr3:93595933	14	c.1747A>C	p.Asn583His	rs139479630	Het	3	S99
F2	chr11:46747447	7	c.598G>A	p.Glu200Lys	rs62623459	Het	3	S15, S195
	chr11:46751078	12	c.1621C>T	p.Arg541Trp	rs886048338	Het	5	S27

	chr11:46751085	12	c.1628G>A	p.Arg543His	rs143064939	Het	3	S72
PROZ	chr13:113826124	8	c.909_933del	p.Trp325*	n/a	Het	3	S32
	chr13:113819398	6	c.603G>A	p.Glu179=	n/a	Het	3	S181
	chr13:113814443	2	c.252C>T	p.Ile62=	n/a	Het	3	S184
	chr13:113813046	int1	c.70+2T>G	-	n/a	Het	3	S202
PLG	chr6:161152827	12	c.1489G>A	p.Val497Ile	n/a	Het	3	S32
	chr6:161139817	9	c.1043A>G	p.Lys348Arg	n/a	Het	3	S98
	chr6:161152784	12	c.1446G>A	p.Met482Ile	rs753483846	Het	3	S196
	chr6:161162369	17	c.2045T>A	p.Ile682Asn	rs147175166	Het	3	S177
	chr6:161127504	2	c.115A>C	p.Lys39Gln	rs138353396	Het	3	S204
SERPIND1	chr22:21141227	5	c.1373T>G	p.Val458Gly	rs772390602	Het	3	S49
	chr22:21138397	3	c.1027G>A	p.Asp343Asn	rs761404668	Het	3	S195
SERPINA10	chr14:94750417	5	c.1220C>A	p.Ala407Asp	rs1289089250	Het	3	S49
	chr14:94754620	3	c.992+3A>G	-	rs369697180	Het	3	S182
	chr14:94756901	2	c.30C>T	p.Ser10=	rs764890013	Het	3	S197
SERPINA5	chr14:95054285	3	c.586G>A	p.Val196Ile	rs1055786032	Het	3	S52
	chr14:95053884	3	c.185G>A	p.Ser62Asn	rs372991959	Het	3	S191
	chr14:95058471	6	c.1116C>A	p.Phe372Leu	s1401241484	Het	3	S192
	chr14:95054027	3	c.328G>C	p.Gly110Arg	rs754794601	Het	3	S211
F8	chrX:154158085	14	c.3980C>T	p.Thr1327Met	rs200520711	Het	3	S71
F13A1	chr6:6248653	int5	c.691-1G>A	-	rs372296352	Het	5	S77
A2M	chr12:9260146	8	c.853G>A	p.Ala285Thr	rs760977693	Het	3	S99
kearonkearonSERPINF2	chr17:1657703	10	c.1351C>T	p.Pro451Ser	rs57360598	Het	3	S165
	chr17:1650299	6	c.368-13del	-	rs552030205	Het	3	S183
	chr17:1650299	int6	c.368-13del	-	rs552030205	Het	3	S194
KLKB1	chr4:187177213	13	c.1557A>C	p.Val519=	n/a	Het	3	S192
	chr4:187155164	4	c.280G>A	p.Gly94Ser	rs146728804	Het	3	S217
F3	chr1:95001632	3	2_300dupGATGTG	p.Asp98_Lys100dup	rs756646702	Het	3	S195

Table 5.2. Genetic variants identified in the study cohort and corresponding patients. List of rare or potentially deleterious variants (ACMG classes 3–5) identified in the study cohort. Variants are reported with genomic coordinates (GRCh37), exon/intron location, nucleotide (NTD) and amino acid (AA) changes, dbSNP ID (rs), zygosity, and ACMG classification. Patients carrying each variant are indicated. Abbreviations: Het, heterozygous; ACMG, American College of Medical Genetics and Genomics; NTD, nucleotide change; AA, amino acid change.

In addition to rare variants, predisposing alleles were identified in 52 patients (52%) (Figure 5.3).

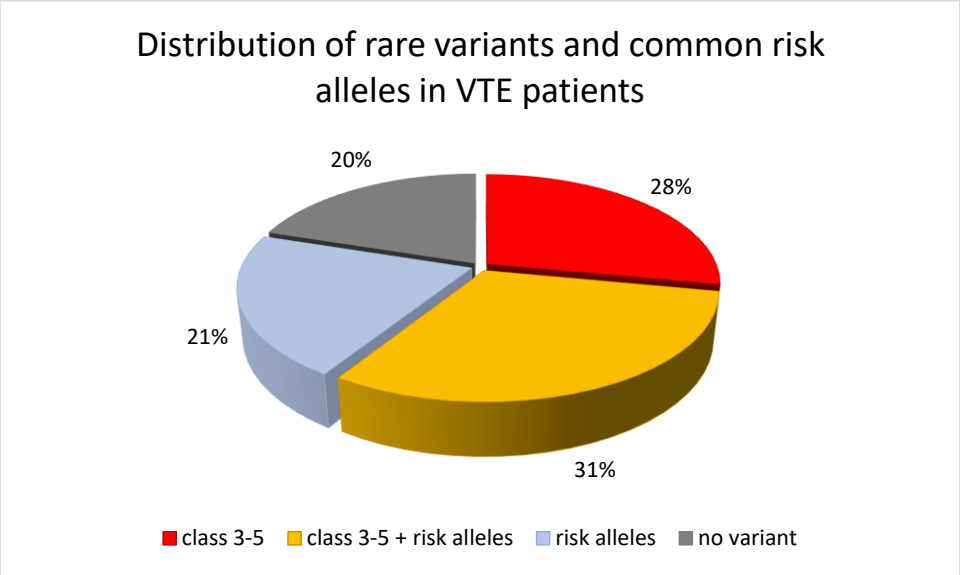


Figure 5.3. Distribution of rare variants and common risk alleles in VTE patients. Pie chart illustrating the genetic distribution among patients with venous thromboembolism (VTE). A total of 28% carried at least one rare variant of uncertain significance or pathogenic variant (class 3–5), 31% carried both rare variants and common risk alleles, 21% carried only common risk alleles, and 20% had no detectable genetic findings.

The most common was the F2 intronic variant c.1726-59G>A (rs3136516), observed in 42 patients in both heterozygous (n=26) and homozygous states (n=16). The TFPI intronic polymorphism c.629-33T>C (rs8176592) was detected in 7 patients, all in homozygosity, while the PROCR p.Ser219Gly variant (rs867186) was found in 3 homozygous carriers. Additional alleles included the F12 promoter variant c.-4T (rs1801020) in 5 patients and the F13A1 p.Val35Leu variant (rs5985) in 1 patient. Rare predisposing variants were also observed in SERPINA10 (p.Trp324*, p.Arg88*, p.Gln384Arg) in 4 heterozygous carriers and in PROZ (c.-13G) in 1 patient. Notably, several individuals carried multiple risk alleles simultaneously, most commonly combinations of F2 with either TFPI or PROCR.

The distribution of genetic risk alleles identified in the study cohort is reported in Table 5.3.

Gene	Known as	Variant		rs	Homozigous carriers (n)	Heterozygous carriers (n)
F2		c.494C>T		rs5896	1	0
	G19911A	c.1726-59G>A		rs3136516	16	26
F12		c.-4T>C		rs1801020	5	0
F13A1		c.103G>T	p.Val35Leu	rs5985	1	0
PROCR	H3	c.655A>G	p.Ser219Gly	rs867186	3	0
PROZ		c.-13G			1	0
SERPINA10	H5	c.262C>T	p.Arg88*	rs2232698	0	2
		c.972G>A	p.Trp324*	rs61754487	0	2
		c.1151A>G	p.Gln384Arg	rs2232710	0	2
TFPI		c.629-33T>C		rs8176592	7	0

Table 5.3. Distribution of genetic risk alleles identified in the study cohort. The table reports genetic variants classified as risk alleles detected in the study population. For each gene, the known alias, cDNA and protein change, and rsID are provided. The number of patients carrying the variant in homozygosity and heterozygosity is indicated.

5.4. Variants in natural anticoagulant genes

As reported in Table 5.4, variants affecting natural anticoagulant pathways were identified in 12 patients of the cohort, corresponding to 12% of the study population. These involved *SERPINC1*, *PROS1*, and *PROC*, with a heterogeneous distribution of pathogenic, likely pathogenic, and uncertain variants.

A total of six patients carried *SERPINC1* variants. Patient S6 harbored the p.Ser236Leu substitution, classified as a variant of uncertain significance, in association with two missense variants in *F5* (p.Thr1685Ser and p.Leu1749Val) and a synonymous *VWF* variant. Patient S55 carried the well-established Dublin variant (p.Val30Glu, class 5)(203), while S71 and S166 were carriers of the Budapest 3 substitution (p.Leu131Phe, class 5)(219), in one case (S71) associated with the *F8* p.Thr1327Met variant. Patient S110 presented the p.Phe276Val substitution, detected together with a *VWF* missense variant (p.Ala641Val), while S192 carried p.Gln366Glu in *SERPINC1*, in combination with variants in *KLKB1* and *SERPINA5*.

Four patients showed *PROS1* variants. The Heerlen variant (p.Ser501Pro, class 4)(218) was found in two individuals (S8 and S87), both also carrying the *VWF* synonymous variant c.5049A>C (p.Ala1683=). Patient S50 carried the pathogenic p.Thr78Met substitution (class 5), while patient S99 had the p.Asn583His variant (class 3), accompanied by a substitution in *A2M*.

Finally, two patients harbored *PROC* variants. Patient S7 carried the p.Trp414Ser substitution (class 3), together with *FGB* p.Gly414Ser, while S32 carried the p.His370Gln variant (class 3), in association with additional variants in *PROZ* (p.Trp325*) and *PLG* (p.Val497Ile).

Overall, variants in natural anticoagulant genes were rarely isolated, but frequently co-occurred with additional alterations in other hemostatic genes.

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Patient ID	Gene	GRCh37	Exon	Nucleotide	Aminoacid	rs	Zygosity	Class
S6	*SERPINC1*	chr1:173879947	4	c.707C>T	p.Ser236Leu	rs368419985	Het	3
	F5	chr1:169500178	15	c.5054C>G	p.Thr1685Ser	rs6011	Het	3
	F5	chr1:169499020	16	c.5245C>G	p.Leu1749Val	rs6034	Het	3
	VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S7	*PROC*	chr2:128186377	9	c.1241G>C	p.Trp414Ser	rs768759265	Het	3
	FGB	chr4:155490947	7	c.1240G>A	p.Gly414Ser	rs141881199	Het	4
S8	*PROS1*	chr3:93598150	13	c.1501T>C	p.Ser501Pro	rs121918472	Het	4
	VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S32	*PROZ*	chr13:113826124	8	c.909_933del	p.Trp325*	n/a	Het	3
	PROC	chr2:128186246	9	c.1110C>A	p.His370Gln	rs778063605	Het	3
	PLG	chr6:161152827	12	c.1489G>A	p.Val497Ile	n/a	Het	3
S50	*PROS1*	chr3:93646095	2	c.233C>T	p.Thr78Met	rs6122	Het	5
S55	*SERPINC1*	chr1:173884010	2	c.89T>A	p.Val30Glu	rs2227624	Het	5
S71	*SERPINC1*	chr1:173883708	2	c.391C>T	p.Leu131Phe	rs121909567	Het	5
	F8	chrX:154158085	14	c.3980C>T	p.Thr1327Met	rs200520711	Het	3
S87	*PROS1*	chr3:93598150	13	c.1501T>C	p.Ser501Pro	rs121918472	Het	4
	VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S99	*PROS1*	chr3:93595933	14	c.1747A>C	p.Asn583His	rs139479630	Het	3
	A2M	chr12:9260146	8	c.853G>A	p.Ala285Thr	rs760977693	Het	3
S110	*VWF*	chr12:6166046	15	c.1922C>T	p.Ala641Val	rs61754019	Het	3
	SERPINC1	chr1:173879828	4	c.826T>G	p.Phe276Val	n/a	Het	3
S166	*SERPINC1*	chr1:173883708	2	c.391C>T	p.Leu131Phe	rs121909567	Het	5
S192	*SERPINC1*	chr1:173878747	5	c.1096C>G	p.Gln366Glu	rs565091601	Het	3
	KLKB1	chr4:187177213	13	c.1557A>C	p.Val519=	n/a	Het	3

	SERPINA5	chr14:95058471	6	c.1116C>A	p.Phe372Leu	s1401241484	Het	3

Table 5.4. Variants identified in patients carrying alterations in natural anticoagulant pathways. Variants of interest (ACMG classes 3–5) identified in patients with VTE and genetic alterations in genes encoding natural anticoagulants (SERPINC1, PROC, PROS1. The table reports patient ID, gene, genomic coordinates (GRCh37), exon or intronic region, nucleotide and amino acid changes, reference SNP identifier (rs) when available, zygosity, and ACMG classification. Patients often carried more than one variant, either within the same gene or in combination with additional genes such as VWF, PLG, FGB, SERPINA5, or KLKB1, supporting the concept of oligogenic inheritance.

Regarding risk alleles in this subgroup, the F2 c.1726-59G>A variant was also frequent: among the 14 patients with natural anticoagulant gene variants, 12 carried the F2 risk allele (either heterozygous or homozygous). A smaller proportion harbored TFPI c.629-33T>C (n=3) or PROCR p.Ser219Gly (n=2).

5.5. Variants in patients with minor provoking risk factors

Among the 100 patients included in the study, 33 individuals (33%) experienced VTE in the context of a minor provoking factor, such as short-haul travel, hormonal therapy, pregnancy and post partum, or minor trauma. Despite being formally classified as “provoked” events, these patients were included in the cohort due to the perceived disproportion between the thrombotic episode and the associated risk exposure, in accordance with current guideline-based stratification criteria (5,11). The baseline and clinical characteristics of this subgroup are detailed in Table 5.5.

Among the 33 patients with provoked VTE, the majority were female (n=23, 69.7%), with a mean age of 31 years (SD ±8). Almost all participants were of Caucasian ethnicity (97.0%), with one African patient (3.0%). The mean BMI was 25.2 kg/m² (SD ±5.3). Regarding smoking status, 9 patients (27.3%) were active smokers, while the remainder were non- or former smokers.

Most patients had no relevant comorbidities (69.7%). Specific comorbidities included thyroid disorders (n=3, 9.1%), arterial hypertension (n=1, 3.0%), metabolic disorders (n=1, 3.0%), anxious-depressive disorders (n=1, 3.0%), and asthma/respiratory conditions (n=1, 3.0%). Miscellaneous comorbidities were reported in 5 cases (15.2%). A positive family history of VTE was documented in 19 patients (57.6%), while data were unavailable in 3 cases (9.1%).

Concerning provoking factors, the most common was hormonal therapy (51.5%), followed by minor trauma (18.2%), pregnancy or post-partum (18.2%), and immobility due to sitting (12.1%).

The initial VTE event presented as isolated DVT in 18 patients (54.5%), PE in 9 patients (27.3%), and combined DVT and PE in 6 patients (18.2%). Among DVT cases, femoral/popliteal thrombosis was the most frequent site (36.4%), followed by isolated distal DVT (15.2%) and extension to the iliac/inferior caval veins (12.1%). Cerebral venous thrombosis occurred in 2 patients (6.1%), while no cases of upper extremity, retinal, or splanchnic vein thrombosis were observed.

Recurrence was frequent: 13 patients (42.4%) had two episodes, 1 patient (3.0%) had three or more, and 4 patients (12.1%) experienced recurrence while on anticoagulant therapy. Bleeding complications during anticoagulation were reported in 4 patients (12.1%), including 1 major bleeding event (3.0%).

	Patients with provoked VTE (n. 33)
Female, n (%)	23 (69.7)
Age, mean y (±SD)	31 (±8)
Ethnicity, n (%)	32(97.0)
Caucasian	1 (3.0)
African	0 (0.0)
Middle Eastern	
Body mass index, mean kg/m^2 (±SD)	25.2 (±5.3)
Smoke habit, n (%)	
Non- or former smoker	24 (72.7)
Active smoker	9 (27.3)
Unknown	0 (0.0)
Comorbidities, n (%)	
None	23 (69.7)
Thyroid disorders	3 (9.1)
Arterial hypertension	1 (3.0)
Metabolic disorders	1 (3.0)
Anxious-depressive disorders	1 (3.0)
Asthma and other respiratory conditions	1 (3.0)
Miscellaneous	5 (15.2)
Family history of VTE, n (%)	19 (57.6)
Unknown	3 (9.1)
VTE risk factors, n (%)	
Minor trauma	6 (18.2)
Pregnancy and post-partum	6 (18.2)
Immobility due to sitting	4 (12.1)
Hormonal therapy	17 (51.5)
VTE type, n (%)	
DVT	18 (54.5)
PE	9 (27.3)
PE and DVT	6 (18.2)
DVT site, n (%)	
Femoral and/or popliteal	12(36.4)
Isolated distal	5 (15.2)
Extended to iliac and/or inferior caval	4 (12.1)
Upper extremity	0 (.0)
Cerebral	2 (6.1)
Retinal	0 (.0)
Splanchnic and other unusual sites	0 (.0)
Recurrent VTE, n (%)	13 (42.4)
2 episodes	1 (3.0)
≥3 episodes	4 (12.1)
during anticoagulant therapy	2 (6.1)
Bleeding events during anticoagulation, n (%)	4 (12.1)
Major bleeding	1 (3.0)

Table 5.5. Baseline characteristics of patients with provoked venous thromboembolism (VTE). Abbreviations: BMI, body mass index; VTE, venous thromboembolism; DVT, deep vein thrombosis; PE, pulmonary embolism; SD, standard deviation.

As detailed in Table 5.6, among patients with provoked VTE (n=33), 13 (39.4%) carried no variants, while 20 patients (60.6%) harbored at least one variant. Of these, 13 had a single variant, 5 carried two variants, 1 had three variants, and 1 carried five variants. The most frequently represented

finding was *VWF* c.5049A>C, detected in 7 patients with recurrence. Additional recurrent alterations included variants in *SERPINC1* (n=2), *F2* (n=2, including one pathogenic substitution), *F5* (n=2), *PLG* (n=2), and *SERPINA5* (n=2). Beyond these recurrent findings, isolated variants were observed in several genes with established or potential roles in coagulation and fibrinolysis, including *PROC* (PC), *PROCR* (endothelial protein C receptor), *SERPINF2* (α2-antiplasmin), and *PROZ* (protein Z).

In terms of risk alleles, among the 33 recurrent patients, 25 (75.8%) carried at least one predisposing allele. The F2 variant c.1726-59G>A was present in 26 patients (some carrying multiple risk alleles). Other notable alleles included TFPI c.629-33T>C (n=5), PROCR p.Ser219Gly (n=4), F12 c.-4T (n=3), and SERPINA10 variants (n=3).

Patient ID	Gene	GRCh37	Exon	Nucleotide	Aminoacid	rs	Zygosity	Class
S2					no variant			
S4	*F11*	chr4:187201533	9	c.1022A>G	p.Glu341Gly	n/a	Het	3
S5	*PROCR*	chr20:33762502	int1	c.71-3C>T	-	rs367783205	Het	3
	PROCR	chr20:33764821	3'UTR	c.*205C>T	-	rs1384660995	Het	3
S6	*SERPINC1*	chr1:173879947	4	c.707C>T	p.Ser236Leu	rs368419985	Het	3
	F5	chr1:169500178	15	c.5054C>G	p.Thr1685Ser	rs6011	Het	3
	F5	chr1:169499020	16	c.5245C>G	p.Leu1749Val	rs6034	Het	3
	VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S7	*PROC*	chr2:128186377	9	c.1241G>C	p.Trp414Ser	rs768759265	Het	3
	FGB	chr4:155490947	7	c.1240G>A	p.Gly414Ser	rs141881199	Het	4
S10	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S11	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S12					no variant			
S15	*F2*	chr11:46747447	7	c.598G>A	p.Glu200Lys	rs62623459	Het	3
S27	*F2*	chr11:46751078	12	c.1621C>T	p.Arg541Trp	rs886048338	Het	5
	VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S34	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S35					no variant			
S39					no variant			
S41	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S42					no variant			
S43	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S52	*SERPINA5*	chr14:95054285	3	c.586G>A	p.Val196Ile	rs1055786032	Het	3
S57					no variant			
S61					no variant			
S68					no variant			
S104					no variant			
S105					no variant			
S169	*PROCR*	chr20:33762717	2	c.283G>T	p.Gly95Cys	rs139714129	Het	3
S170					no variant			
S172	*FGB*	chr4:155484174	1	c.4A>G	p.Lys2Glu	rs6053	Het	3
S177	*PLG*	chr6:161162369	17	c.2045T>A	p.Ile682Asn	rs147175166	Het	3

S180					no variant			
S183	*SERPINF2*	chr17:1650299	6	c.368-13del	-	rs552030205	Het	3
S187	*FGA*	chr4:155507052	5	c.1529G>A	p.Arg510His	rs138137585	Het	3
S192	*SERPINC1*	chr1:173878747	5	c.1096C>G	p.Gln366Glu	rs565091601	Het	3
	KLKB1	chr4:187177213	13	c.1557A>C	p.Val519=	n/a	Het	3
	SERPINA5	chr14:95058471	6	c.1116C>A	p.Phe372Leu	s1401241484	Het	3
S197	*SERPINA10*	chr14:94756901	2	c.30C>T	p.Ser10=	rs764890013	Het	3
S204	*F5*	chr1:169510246	13	c.3974_4081dup	p.Leu1325_Asp1360dup	n/a	Het	3
	PLG	chr6:161127504	2	c.115A>C	p.Lys39Gln	rs138353396	Het	3
S223					no variant			

Table 5.6. Genetic variants identified in patients with provoked VTE. Variants of interest (ACMG classes 3–5) detected in patients with provoked venous thromboembolism (VTE). The table reports patient ID, gene, genomic coordinates (GRCh37), exon or intronic region, nucleotide and amino acid changes, reference SNP identifier (rs) when available, zygosity, and ACMG classification. Patients with no variants of interest are also indicated.

5.6. Variants in patients with recurrent VTE

Among the 42 patients with recurrent VTE, 15 (35.7%) were female, with a mean age at first event of 34 years (SD ±9). Nearly all were of Caucasian origin (97.6%), with one patient of Middle Eastern ancestry (2.4%). The mean BMI was 26.6 kg/m² (SD ±4.3). Regarding smoking habits, 14 patients (33.3%) were active smokers, 26 (61.9%) were non- or former smokers, and information was missing in 2 cases (4.8%).

Most patients had no comorbidities (69.0%). Specific conditions included thyroid disorders (4.8%), arterial hypertension (7.1%), metabolic disorders (4.8%), and anxious-depressive disorders (2.4%); miscellaneous comorbidities were recorded in 5 patients (11.9%). A positive family history of VTE was reported in 23 cases (54.8%), while data were unavailable in 6 (14.3%).

The index thrombotic event was unprovoked in 28 patients (66.7%) and provoked in 14 patients (33.3%), most commonly associated with hormonal therapy (11.9%), minor trauma (7.1%), pregnancy/post-partum (7.1%), or immobility due to sitting (7.1%).

With regard to thrombotic localization, 28 patients (66.7%) presented with DVT, 5 (11.9%) with isolated PE, and 9 (21.4%) with both PE and DVT. Among DVT cases, the most frequent site was femoral/popliteal (61.9%), followed by distal DVT (14.3%), and extension to iliac/inferior caval veins (4.8%). One patient (2.4%) had cerebral venous thrombosis. No cases of upper extremity, retinal, or splanchnic thrombosis were observed.

Recurrent events under anticoagulation occurred in 7 patients (16.7%). Bleeding complications were reported in 12 patients (28.6%), including 2 major hemorrhage (4.8%).

Table 5.7 reports the demographic, clinical, and thrombotic features of patients with recurrent VTE in our cohort.

Among the 42 patients with recurrent VTE (Table 5.8), 17 (40.5%) carried no variants, while 25 (59.5%) harbored at least one variant of interest. Overall, 13 patients (31.0%) had a single variant, 10 (23.8%) carried two variants, and 2 patients (4.8%) presented with three variants.

With regard to pathogenicity, one patient carried a likely pathogenic variant (class 4) in *FGB* (c.1240G>A, p.Gly414Ser), two patients carried a likely pathogenic variant (class 4) in *PROS1* (Heerlen variant c.1501T>C, p.Ser501Pro)(218) and two patient carried a pathogenic variant (class 5), the *SERPINC1* Budapest 3 variant (c.391C>T, p.Leu131Phe)(219), and a substitution in *F2* (c.1621C>T, p.Arg541Trp)(220). The remaining cases were explained by variants of uncertain significance (class 3), which were identified in 24 patients (57.1%), either alone (n=15) or in combination (n=9).

	Patients with recurrent VTE (n. 42)
Female, n (%)	15 (35.7)
Age, mean y (±SD)	34 (±9)
Ethnicity, n (%)	
Caucasian	41 (97.6)
African	0 (0)
Middle Eastern	1 (2.4)
Body mass index, mean kg/m² (±SD)	26.6 (±4.3)
Smoke habit, n (%)	
Non- or former smoker	26 (61.9)
Active smoker	14 (33.3)
Unknown	2 (4.8)
Comorbidities, n (%)	
None	29 (69.0)
Thyroid disorders	2 (4.8)
Arterial hypertension	3 (7.1)
Metabolic disorders	2 (4.8)
Anxious-depressive disorders	1 (2.4)
Asthma and other respiratory conditions	0 (.0)
Miscellaneous	5 (11.9)
Family history of VTE, n (%)	23 (54.8)
Unknown	6 (14.3)
VTE etiology, n (%)	
Unprovoked	28 (66.7)
Provoked	14 (33.3)
VTE risk factors, n (%)	
Minor trauma	3 (7.1)
Pregnancy and post-partum	3 (7.1)
Immobility due to sitting	3 (7.1)
Hormonal therapy	5 (11.9)
VTE type, n (%)	
DVT	28 (66.7)
PE	5 (11.9)
PE and DVT	0 (21.4)
DVT site, n (%)	
Femoral and/or popliteal	26 (61.9)
Isolated distal	6 (14.3)
Extended to iliac and/or inferior caval	2 (4.8)
Upper extremity	0 (.0)
Cerebral	1 (2.4)
Retinal	0 (.0)
Splanchnic and other unusual sites	0 (.0)
Recurrent VTE, n (%) during anticoagulant therapy	7 (16.7)
Bleeding events during anticoagulation, n (%)	12 (28.6)
Major bleeding	2 (4.8)

Table 5.7. Clinical and demographic characteristics of patients with recurrent VTE. The table summarizes baseline features, risk factors, and clinical presentation of patients with recurrent VTE included in the study cohort. Data are expressed as number (percentage) unless otherwise indicated. BMI, body mass index; DVT, deep vein thrombosis; PE, pulmonary embolism; SD, standard deviation.

Among class 3 findings, the most recurrent alteration was *VWF* c.5049A>C, observed in seven patients. Additional VUS were distributed across multiple coagulation and anticoagulant genes, including *PROC* (c.1241G>C, p.Trp414Ser; c.1110C>A, p.His370Gln), *PROZ* (c.909_933del, p.Trp325*; synonymous substitutions), *SERPINC1* (c.826T>G, p.Phe276Val), *SERPINA5* (c.586G>A, p.Val196Ile; c.328G>C, p.Gly110Arg), *SERPINF2* (c.368-13del), *F2* (c.1628G>A, p.Arg543His), *F5* (c.5147C>T, p.Ser1716Leu), *FGA* (c.1529G>A, p.Arg510His), *KLKB1* (c.280G>A, p.Gly94Ser), and *VWF* c.1922C>T (p.Ala641Val) and c.4196G>A (p.Arg1399His). The genetic variants identified in patients with recurrent VTE are summarized in Table 8.

Risk alleles were also highly prevalent in this subgroup, with 28 of 42 patients (66.7%) carrying at least one predisposing allele. The F2 c.1726-59G>A variant was the most frequent, identified in 22 patients, while TFPI c.629-33T>C was present in 5 patients (all homozygous), PROCR p.Ser219Gly in 3, and F12 c.-4T in 2. Rare SERPINA10 variants were observed in 3 patients. Several individuals carried more than one allele, underscoring the contribution of a polygenic risk background to recurrence.

Patient ID	Gene	GRCh37	Exon	Nucleotide	Aminoacid	rs	Zygosity	Class
S2					no variant			
S7	*PROC*	chr2:128186377	9	c.1241G>C	p.Trp414Ser	rs768759265	Het	3
	FGB	chr4:155490947	7	c.1240G>A	p.Gly414Ser	rs141881199	Het	4
S8	*PROS1*	chr3:93598150	13	c.1501T>C	p.Ser501Pro	rs121918472	Het	4
	VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S11	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S12					no variant			
S13					no variant			
S14	*F5*	chr1:169500085	15	c.5147C>T	p.Ser1716Leu	n/a	Het	3
	VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S32	*PROZ*	chr13:113826124	8	c.909_933del	p.Trp325*	n/a	Het	3
	PROC	chr2:128186246	9	c.1110C>A	p.His370Gln	rs778063605	Het	3
	PLG	chr6:161152827	12	c.1489G>A	p.Val497Ile	n/a	Het	3
S34	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S41	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S42					no variant			
S45	*PROCR*	chr20:33764619	int4	c.*3A>C	-	rs767268282	Het	3
S49	*SERPIND1*	chr22:21141227	5	c.1373T>G	p.Val458Gly	rs772390602	Het	3
	SERPINA10	chr14:94750417	5	c.1220C>A	p.Ala407Asp	rs1289089250	Het	3
S51	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S52	*SERPINA5*	chr14:95054285	3	c.586G>A	p.Val196Ile	rs1055786032	Het	3
S54	*VWF*	chr12:6166187	15	c.1781C>G	p.Ala594Gly	rs267607308	Het	3
	VWF	chr12:6138597	22	c.2878C>T	p.Arg960Trp	rs370984712	Het	3
	VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3

S55	*SERPINC1*	chr1:173884010	2	c.89T>A	p.Val30Glu	rs2227624	Het	5
S58				no variant				
S61				no variant				
S63				no variant				
S71	*SERPINC1*	chr1:173883708	2	c.391C>T	p.Leu131Phe	rs121909567	Het	5
	F8	chrX:154158085	14	c.3980C>T	p.Thr1327Met	rs200520711	Het	3
S72	*F2*	chr11:46751085	12	c.1628G>A	p.Arg543His	rs143064939	Het	3
S75				no variant				
S110	*VWF*	chr12:6166046	15	c.1922C>T	p.Ala641Val	rs61754019	Het	3
	SERPINC1	chr1:173879828	4	c.826T>G	p.Phe276Val	n/a	Het	3
S113				no variant				
S114				no variant				
S177	*PLG*	chr6:161162369	17	c.2045T>A	p.Ile682Asn	rs147175166	Het	3
S180				no variant				
S181	*PROZ*	chr13:113819398	6	c.603G>A	p.Glu179=	n/a	Het	3
S183	*SERPINF2*	chr17:1650299	6	c.368-13del	-	rs552030205	Het	3
S184	*VWF*	chr12:6166046	15	c.1922C>T	p.Ala641Val	rs61754019	Het	3
	PROZ	chr13:113814443	2	c.252C>T	p.Ile62=	n/a	Het	3
S187	*FGA*	chr4:155507052	5	c.1529G>A	p.Arg510His	rs138137585	Het	3
S203				no variant				
S204	*F5*	chr1:169510246	13	c.3974_4081dup	p.Leu1325_Asp1360dup	n/a	Het	3
	PLG	chr6:161127504	2	c.115A>C	p.Lys39Gln	rs138353396	Het	3
S208				no variant				
S209	*THBD*	chr20:23028811	1	c.1331A>G	p.Glu444Gly	rs375671812	Het	3
	F12	chr5:176836468	int1	c.57+4A>G	-	rs371915487	Het	3

S211	*SERPINA5*	chr14:95054027	3	c.328G>C		p.Gly110Arg	rs754794601	Het	3
S212					no variant				
S213					no variant				
S217	*KLKB1*	chr4:187155164	4	c.280G>A		p.Gly94Ser	rs146728804	Het	3
	VWF	chr12:6128388	28	c.4196G>A		p.Arg1399His	rs1800382	Het	3
S218					no variant				
S222					no variant				

Table 5.8. Genetic variants identified in patients with recurrent VTE. Variants of interest (ACMG classes 3–5) detected in patients with recurrent venous thromboembolism (VTE). The table reports patient ID, gene, genomic coordinates (GRCh37), exon or intronic region, nucleotide and amino acid changes, reference SNP identifier (rs) when available, zygosity, and ACMG classification. Patients without variants are also indicated. Several individuals carried multiple variants across distinct genes, supporting the contribution of oligogenic mechanisms to recurrence.

5.7. Variants in patients with thrombosis at unusual sites

Among the 11 patients with VTE at unusual sites, 3 (27.3%) were female, with a mean age at first event of 30 years (SD ±8). All were of Caucasian ethnicity. The mean BMI was 26.2 kg/m² (SD ±6.2). Regarding smoking habits, 9 patients (81.8%) were non- or former smokers, one was an active smoker, and smoking status was unknown in one case. Table 5.9 describes the clinical profile of patients with VTE at unusual sites, who represented a small but clinically relevant subgroup within our cohort.

	Unusual sites (n. 11)
Female, n (%)	3 (27.3)
Age, mean y (±SD)	30 (±8)
Ethnicity, n (%)	
Caucasian	11 (100.0)
African	0 (.0)
Middle Eastern	0 (0.0)
Body mass index, mean kg/m² (±SD)	26.2 (±6.2)
Smoke habit, n (%)	
Non- or former smoker	9 (81.8)
Active smoker	1 (9.1)
Unknown	1 (9.1)
Comorbidities, n (%)	
None	7 (63.6)
Thyroid disorders	2 (18.2)
Arterial hypertension	1 (9.1)
Metabolic disorders	0 (0.0)
Anxious-depressive disorders	0 (0.0)
Asthma and other respiratory conditions	1 (9.1)
Miscellaneous	0 (0.0)
Family history of VTE, n (%)	7 (63.6)
Unknown	2 (18.2)
VTE etiology, n (%)	
Unprovoked	9 (81.8)
Provoked	2 (18.2)
VTE risk factors, n (%)	
Minor trauma	0 (0.0)
Pregnancy and post-partum	0 (0.0)
Immobility due to sitting	0 (0.0)
Hormonal therapy	2 (18.2)
VTE type, n (%)	
DVT	11 (100.0)
PE	0 (0.0)
PE and DVT	0 (0.0)
DVT site, n (%)	
Femoral and/or popliteal	0 (0.0)
Isolated distal	0 (0.0)

Extended to iliac and/or inferior caval	0 (0.0)
Upper extremity	0 (0.0)
Cerebral	4 (36.4)
Retinal	3 (27.3)
Splanchnic and other unusual sites	4 (36.4)
Recurrent VTE, n (%)	1 (9.1)
2 episodes	1 (9.1)
≥3 episodes	0 (0.0)
during anticoagulant therapy	0 (0.0)
Bleeding events during anticoagulation, n (%)	0 (0.0)
Major bleeding	0 (0.0)

Table 5.9. Clinical characteristics of patients with VTE at unusual sites (n=11). Demographic, clinical, and thrombotic features of patients with venous thromboembolism (VTE) at unusual anatomical sites. Data are expressed as number (percentage) or mean (± standard deviation). Abbreviations: VTE, venous thromboembolism; DVT, deep vein thrombosis; PE, pulmonary embolism.

With regard to predisposing alleles, 7 of 11 patients (63.6%) carried at least one risk allele. The most common finding was the F2 c.1726-59G>A variant, observed in 5 patients. In addition, single cases carried TFPI c.629-33T>C, F12 c.-4T, and SERPINA10 p.Trp324. No PROCR or PROZ alleles were detected in this subgroup.

Patient ID	Gene	GRCh37	Exon	Nucleotide	Aminoacid	rs	Zygosity	Class
S1	*FGB*	chr4:155490457	6	c.956C>T	p.Pro319Leu	rs374463429	Het	3
	VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S9				no variant				
S10	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S15	*F2*	chr11:46747447	7	c.598G>A	p.Glu200Lys	rs62623459	Het	3
S16	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S89	*VWF*	chr12:6128388	28	c.4196G>A	p.Arg1399His	rs1800382	Het	3
S114				no variant				
S115	*FGB*	chr4:155484174	1	c.4A>G	p.Lys2Glu	rs6053	Het	3
S194	*SERPINF2*	chr17:1650299	int6	c.368-13del	-	rs552030205	Het	3
S195	*F3*	chr1:95001632	3	c.292_300dupGATGTGAAG	p.Asp98_Lys100dup	rs756646702	Het	3
	F2	chr11:46747447	7	c.598G>A	p.Glu200Lys	rs62623459	Het	3
	SERPIND1	chr22:21138397	3	c.1027G>A	p.Asp343Asn	rs761404668	Het	3
S202	*PROZ*	chr13:113813046	int1	c.70+2T>G	-	n/a	Het	3
	FGA	chr4:155507556	5	c.1025G>A	p.Gly342Glu	rs774664670	Het	3
S206				no variant				

Table 5.10. Genetic variants identified in patients with VTE at unusual sites. Variants of interest (ACMG classes 3–5) detected in patients with venous thromboembolism (VTE) occurring at unusual anatomical sites. The table reports patient ID, gene, genomic coordinates (GRCh37), exon or intronic region, nucleotide and amino acid changes, reference SNP identifier (rs) when available, zygosity, and ACMG classification. Patients without variants are also indicated. Several individuals carried more than one variant across different genes.

5.8. Bleeding complications and genetic findings

Among the 19 patients with VTE who experienced bleeding, 10 (52.6%) were female, with a mean age of 33 years (SD ±10). The vast majority were of Caucasian ethnicity (94.7%), with one patient of Middle Eastern origin. The mean BMI was 23.9 kg/m² (SD ±4.1). Regarding smoking status, 6 patients (31.6%) were active smokers, 12 (63.2%) were non- or former smokers, and data were missing in one case (5.3%). Table 5.11 summarizes the characteristics of patients who experienced bleeding complications during anticoagulant therapy.

	Patients with bleeding (n.19)
Female, n (%)	10 (52.6)
Age, mean y (±SD)	33 (±10)
Ethnicity, n (%)	
Caucasian	18 (94.7)
African	0 (0.0)
Middle Eastern	1 (5.3)
Body mass index, mean kg/m² (±SD)	23.9 (±4.1)
Smoke habit, n (%)	
Non- or former smoker	12 (63.2)
Active smoker	6 (31.6)
Unknown	1 (5.3)
Comorbidities, n (%)	
None	12 (63.2)
Thyroid disorders	0 (0.0)
Arterial hypertension	0 (0.0)
Metabolic disorders	2 (10.5)
Anxious-depressive disorders	2 (10.5)
Asthma and other respiratory conditions	0 (0.0)
Miscellaneous	2 (10.5)
Family history of VTE, n (%)	10 (52.6)
Unknown	3 (15.8)
VTE etiology, n (%)	
Unprovoked	15 (78.9)
Provoked	4 (21.1)
VTE risk factors, n (%)	
Minor trauma	1 (18.2)
Pregnancy and post-partum	0 (0.0)
Immobility due to sitting	0 (0.0)
Hormonal therapy	3 (15.8)
VTE type, n (%)	
DVT	9 (47.4)
PE	7 (36.8)
PE and DVT	3 (15.8)
DVT site, n (%)	
Femoral and/or popliteal	8 (42.1)
Isolated distal	2 (10.5)

Extended to iliac and/or inferior caval	2 (10.5)
Upper extremity	0 (0.0)
Cerebral	0 (0.0)
Retinal	0 (0.0)
Splanchnic and other unusual sites	0 (0.0)
Recurrent VTE, n (%)	12 (67.2)
2 episodes	3 (15.8)
≥3 episodes	1 (5.3)
during anticoagulant therapy	2 (10.2)
Major bleeding during anticoagulation, n (%)	1 (3.0)

Table 5.11. Clinical and demographic characteristics of patients with bleeding complications during anticoagulation (n=19). The table reports baseline characteristics, risk factors, thrombotic presentation, recurrence patterns, and bleeding events in patients who experienced hemorrhagic complications while on anticoagulant therapy. Data are expressed as number (percentage) unless otherwise indicated. BMI, body mass index; DVT, deep vein thrombosis; PE, pulmonary embolism; SD, standard deviation.

Most patients reported no comorbidities (63.2%). Specific comorbidities included metabolic disorders (10.5%), anxious-depressive disorders (10.5%), and miscellaneous disorders (10.5%). A positive family history of VTE was documented in 10 patients (52.6%), while data were unavailable in 3 (15.8%).

The index event was unprovoked in 15 patients (78.9%) and provoked in 4 (21.1%), most frequently associated with hormonal therapy (15.8%) or, less commonly, minor trauma (5.3%). Regarding VTE type, 9 patients (47.4%) presented with isolated DVT, 7 (36.8%) with PE, and 3 (15.8%) with combined DVT and PE. The most common DVT sites were femoral/popliteal veins (42.1%), followed by isolated distal DVT (10.5%) and extension to iliac or inferior caval veins (10.5%).

Recurrent events were frequent: 12 patients (63.2%) had two episodes, 3 patients (15.8%) had three or more episodes, and 2 patients (10.5%) developed recurrence during anticoagulation. Bleeding complications during therapy occurred in all cases by definition, with 1 patient (3.0%) experiencing a major hemorrhage.

From a genetic perspective, 9 patients (47.4%) carried no variants, while 10 patients (52.6%) harbored at least one variant of interest. Of these, 4 patients had a single variant, 5 carried two variants, and 1 patient presented with three variants. No likely pathogenic variants (class 4) were observed. In contrast, two patients carried pathogenic variants (class 5), while 10 patients had at least one VUS (class 3), distributed as follows: 6 patients with a single class 3 variant, 3 patients with two class 3 variants, and 1 patient with three class 3 variants as shown in Table 5.12.

Patient ID	Gene	GRCh37	Exon	Nucleotide	Aminoacid	rs	Zygosity	Class
S8	*PROS1*	chr3:93598150	13	c.1501T>C	p.Ser501Pro	rs121918472	Het	4
	VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S41	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S43	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S49	*SERPIND1*	chr22:21141227	5	c.1373T>G	p.Val458Gly	rs772390602	Het	3
	SERPINA10	chr14:94750417	5	c.1220C>A	p.Ala407Asp	rs1289089250	Het	3
S52	*SERPINA5*	chr14:95054285	3	c.586G>A	p.Val196Ile	rs1055786032	Het	3
S54	*VWF*	chr12:6166187	15	c.1781C>G	p.Ala594Gly	rs267607308	Het	3
	VWF	chr12:6138597	22	c.2878C>T	p.Arg960Trp	rs370984712	Het	3
	VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S67	*FGB*	chr4:155490457	6	c.956C>T	p.Pro319Leu	rs374463429	Het	3
S75	no variant							
S87	*PROS1*	chr3:93598150	13	c.1501T>C	p.Ser501Pro	rs121918472	Het	4
	VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3
S106	no variant							
S110	*VWF*	chr12:6166046	15	c.1922C>T	p.Ala641Val	rs61754019	Het	3
	SERPINC1	chr1:173879828	4	c.826T>G	p.Phe276Val	n/a	Het	3
S112	no variant							
S113	no variant							
S180	no variant							
S214	no variant							
S216	no variant							
S217	*KLKB1*	chr4:187155164	4	c.280G>A	p.Gly94Ser	rs146728804	Het	3
	VWF	chr12:6128388	28	c.4196G>A	p.Arg1399His	rs1800382	Het	3

S222	no variant

Table 5.12. Genetic variants identified in patients with bleeding complications. Variants of interest (ACMG classes 3–5) detected in patients with VTE who experienced bleeding complications during their clinical course or anticoagulant therapy. The table reports patient ID, gene, genomic coordinates (GRCh37), exon or intronic region, nucleotide and amino acid changes, reference SNP identifier (rs) when available, zygosity, and ACMG classification. Patients without variants are also indicated. Several individuals carried more than one variant, most frequently involving VWF and genes encoding natural anticoagulants.

Among the 19 patients with bleeding, 11 (57.9%) carried at least one predisposing allele. The majority involved the F2 c.1726-59G>A variant (n=9), while additional findings included TFPI c.629-33T>C (n=2), PROCR p.Ser219Gly (n=1), and a SERPINA10 variant (n=1). Some patients harbored more than one allele simultaneously.

5.9. Patients without identified variants

Among the 41 patients without rare variants (classes 3–5), 21 (51.2%) were female, with a mean age at diagnosis of 35 years (SD ±10). Almost all were Caucasian (97.6%), with one patient of African origin. The mean BMI was 25.2 kg/m² (SD ±4.3). Regarding smoking status, 28 patients (68.3%) were non- or former smokers, 12 (29.3%) were active smokers, and one (2.4%) had unknown data.

Most patients reported no comorbidities (78.0%), while a single case had more than one. The most frequent comorbidities were thyroid disorders (7.3%), arterial hypertension (4.9%), metabolic disturbances (2.4%), anxious–depressive disorders (2.4%), and asthma/respiratory conditions (2.4%); miscellaneous conditions were observed in 2 patients (4.9%). A family history of VTE was documented in 18 patients (43.9%), while it was unknown in 5 (12.2%). Table 5.13 shows the clinical profile of patients without identified genetic variants, who represented approximately one third of the study cohort.

	Patient without variants (n. 41)
Female, n (%)	21 (51.2)
Age, mean y (±SD)	35 (±10)
Ethnicity, n (%)	
Caucasian	40 (97.6)
African	1 (2.4)
Body mass index, mean kg/m² (±SD)	25.2 (±4.3)
Smoke habit, n (%)	
Non- or former smoker	28 (68.3)
Active smoker	12 (29.3)
Unknown	1 (2.4)
Comorbidities, n (%)	
None	32 (78.0)
Thyroid disorders	3 (7.3)
Arterial hypertension	2 (4.9)
Metabolic disorders	1 (2.4)
Anxious-depressive disorders	1 (2.4)
Asthma and other respiratory conditions	1 (2.4)
Miscellaneous	2 (4.9)
Family history of VTE, n (%)	18 (43.9)
Unknown	5 (12.2)

VTE etiology, n (%)	
Unprovoked	28 (68.3)
Provoked	13 (31.7)
VTE risk factors, n (%)	
Minor trauma	2 (4.9)
Pregnancy	2 (4.9)
Immobility due to sitting	2 (4.9)
Hormonal therapy	7 (17.1)
VTE type, n (%)	
DVT	19 (46.3)
PE	12 (29.3)
PE and DVT	10 (24.4)
DVT site, n (%)	
Femoral and/or popliteal	14 (34,1)
Isolated distal	7 (17.1)
Extended to iliac and/or inferior caval	4 (9.8)
Upper extremity	0
Cerebral	2 (4.9)
Retinal	0
Splanchnic and other unusual sites	1 (2.4)
Recurrent VTE, n (%)	17 (41.5)
2 episodes	2 (4.9)
≥3 episodes	2 (4.9)
during anticoagulant therapy	2 (4.9)
Bleeding events during anticoagulation, n (%)	9 (22.0)
Major bleeding	1 (2.0)

Table 5.13. Clinical and demographic characteristics of patients without identified genetic variants (n=41). The table summarizes baseline features, comorbidities, risk factors, thrombotic presentation, recurrence, and bleeding events in patients in whom no variants were detected by next-generation sequencing. Data are expressed as number (percentage) unless otherwise indicated. BMI, body mass index; DVT, deep vein thrombosis; PE, pulmonary embolism; SD, standard deviation.

The index thrombotic event was unprovoked in 28 patients (68.3%) and provoked in 13 (31.7%), most commonly by hormonal therapy (17.1%), followed by minor trauma (4.9%), pregnancy (4.9%), and immobility (4.9%). With regard to clinical phenotype, 19 patients (46.3%) presented with isolated DVT, 12 (29.3%) with PE, and 10 (24.4%) with combined PE and DVT. Among DVT cases, femoral/popliteal involvement was most common (34.1%), followed by isolated distal DVT (17.1%), extension to iliac/caval segments (9.8%), cerebral venous thrombosis (4.9%), and splanchnic thrombosis (2.4%). No cases of upper extremity or retinal vein thrombosis were observed.

Recurrent events occurred in 19 patients (46.3%), including 17 (41.5%) with two episodes, 2 (4.9%) with three or more, and 2 (4.9%) with recurrence during anticoagulation. Bleeding during anticoagulant therapy was reported in 9 patients (22.0%), of whom one experienced a major bleeding event (2.0%)

5.10. Logistic regression analyses

Exploratory logistic regression analyses were performed to evaluate potential associations within specific subgroups, including patients without genetic variants, those with recurrent VTE, with thrombosis at unusual sites, with class 5 variants, and those who experienced bleeding during anticoagulation. None of these models yielded statistically significant associations, and no predictors reached the predefined level of significance.

6. DISCUSSION

In this study, we employed an extended custom NGS panel covering 33 coagulation-related genes to investigate the genetic background of young patients with unprovoked or poorly explained VTE. Our findings demonstrate that more than half of the cohort (59%) carried variants of uncertain significance or pathogenic variants (ACMG classes 3–5), with approximately one quarter of variant-positive individuals presenting multiple alterations across distinct genes. These findings suggest that extended genetic testing can reveal complex or latent inherited predispositions in a substantial proportion of patients who would be otherwise considered idiopathic(187,190,192).

Furthermore, common prothrombotic alleles were frequent, particularly the prothrombin intronic variant c.1726-59G>A (rs3136516)(221), observed in over 40% of patients and previously reported to confer a modestly increased risk of thrombosis, with an odds ratio of approximately 1.19 in the literature. These results support the notion that inherited predisposition to VTE is frequently polygenic and that NGS offers a substantial improvement over conventional thrombophilia screening in capturing the complexity of genetic risk.

A relevant aspect of our analysis concerns the degree to which the identified variants had been previously reported in the literature or public databases. Among the 68 distinct variants observed in our cohort, approximately one-third (33.8%) corresponded to well-established alterations already described in association with VTE or included in curated databases with supporting evidence of pathogenicity. These included diagnostic-grade substitutions such as *SERPINC1* Dublin(203) and Budapest 3(219), *PROS1* Heerlen(204), and *F2* p.Arg541Trp(90), all of which have long been recognized as strong genetic determinants of thrombophilia. Their recurrence in our series confirms the clinical validity of NGS-based testing and demonstrates that sequencing panels can effectively capture variants with proven impact on disease risk, even in patients who are negative at conventional thrombophilia screening.

In contrast, the majority of variants (54.4%) were classified as variants of uncertain significance (VUS) with an rs identifier but without supporting publications. These findings exemplify the main challenge of NGS: while the technology dramatically expands the range of detectable alterations, interpretation remains limited by the lack of functional characterization or epidemiological validation(30,191). Variants observed in this category include synonymous or missense

substitutions with no established role in coagulation biology, which may represent either benign polymorphisms or modifiers of disease phenotype that only exert an effect in combination with other genetic or environmental risk factors.

Importantly, eight variants (11.8%) were not associated with any rs identifier and were therefore considered potentially novel findings. These included alterations across multiple genes with plausible roles in thrombosis and hemostasis, such as *F11* c.1022A>G (p.Glu341Gly), *PROZ* c.909_933del (p.Trp325*), *PLG* c.1489G>A (p.Val497Ile), *F5* c.3974_4081dup (p.Leu1325_Asp1360dup), *PROZ* c.70+2T>G (splice), *PROZ* c.252C>T (p.Ile62=), *KLKB1* c.1557A>C (p.Val519=), and *F3* c.292_300dupGATGTGAAG (p.Asp98_Lys100dup). The detection of such variants highlights the discovery potential of NGS in VTE, but also its interpretive limitations: without functional studies or replication in independent cohorts, the pathogenicity of these alterations remains uncertain.

Taken together, these results reflect the dual nature of NGS in clinical practice. On the one hand, it allows confirmation of established disease-causing variants and thereby strengthens the diagnostic yield. On the other, it reveals a large proportion of rare or novel findings whose clinical impact is unknown, creating uncertainty for both clinicians and patients. This underscores the urgent need for multicenter collaborative efforts, international variant databases, and functional characterization strategies to bridge the gap between genetic discovery and clinical translation(187,201,210). Only by systematically integrating genetic data with laboratory assays, biomarker studies, and longitudinal clinical outcomes will it be possible to reclassify VUS and harness the full potential of NGS for personalized medicine in VTE.

6.1. Genetic architecture of VTE: oligogenic and polygenic models

The identification of multiple coexisting variants in a significant proportion of patients reinforces the concept of oligogenic inheritance in thrombosis(30,188,202). In several cases, two or more rare or uncertain variants were found in combination with common risk alleles, suggesting cumulative effects that could tip the hemostatic balance toward hypercoagulability. This model provides a plausible explanation for the clinical heterogeneity observed within families and among carriers of well-established variants, where some individuals develop thrombosis at a young age while others remain asymptomatic.

Several genetic variants were located in functionally relevant domains or matched known pathogenic profiles, such as the AT Dublin variant(203) (*SERPINC1*), the PS Heerlen variant(204)

(*PROS1*), or prothrombin Arg541Trp(90). Importantly, the functional consequences of these variants may not always be detected by routine laboratory assays. Standard thrombophilia tests are typically designed to measure global antigen levels or a restricted set of functional activities under specific conditions (e.g., anti-IIa or anti-FXa activity for AT, amidolytic activity for PC, or clotting-based assays for PS). As a result, they may fail to capture more subtle abnormalities(32,33).

Variants can alter protein folding, secretion efficiency, or stability, leading to reduced circulating levels that may fluctuate or remain within the lower end of the normal range(33). Others induce conformational changes that impair protein interactions only in the presence of certain cofactors (e.g., AT with heparin, PS with activated protein C) and are therefore not revealed by conventional assays(32). In some cases, the effect is context-dependent, becoming evident only under physiological stressors such as inflammation, trauma, pregnancy, or exposure to exogenous anticoagulants. Finally, antigen-based assays may completely overlook functional defects if protein levels are preserved but activity is qualitatively impaired(33).

Together, these considerations explain why carriers of well-characterized pathogenic variants, such as Dublin AT or Heerlen PS, may display normal results in standard laboratory testing despite a demonstrable genetic predisposition to thrombosis.

At the same time, the predominance of VUS highlights the interpretative challenge posed by NGS: while these variants cannot currently be used for clinical decision-making, their recurrence in patients with clear thrombotic phenotypes suggests a possible contributory role that warrants further functional characterization.

6.2. Variant distribution across clinical subgroups

Importantly, the variant detection rate was particularly high among patients with thrombosis in unusual anatomical sites (about 73%) and those with minor provoking factors (about 61%), supporting the notion that genetic predisposition may play an unrecognized role in clinically ambiguous scenarios(28,222). Similarly, about 60% of patients with recurrent VTE carried ACMG-classified variants, suggesting that genetic factors may contribute to disease persistence even after an appropriate anticoagulant therapy(10). These results emphasize the potential utility of genetic evaluation in risk stratification and secondary prevention, especially when traditional risk models fail to capture the full complexity of individual susceptibility.

6.3. Illustrative cases

A series of illustrative patient cases from our cohort provides insight into how different combinations of genetic variants may contribute to thrombotic predisposition. These clinical vignettes underline the heterogeneity of presentations and the potential cumulative effect of multiple variants—often classified as of uncertain significance (VUS)—acting together with common predisposing alleles or environmental triggers(188,202).

Patient S6 exemplifies this complexity. She carried the common prothrombin intronic variant c.1726-59G>A in homozygosity, together with four additional VUS in SERPINC1, F5, and VWF. While the individual contribution of each alteration is uncertain, their coexistence could plausibly weaken natural anticoagulant pathways and enhance thrombin generation. This patient developed pulmonary embolism while on oral contraceptives, with a positive family history of DVT, suggesting that the genetic background lowered the threshold for thrombosis in the presence of a transient risk factor.

A similar interplay of multiple moderate-risk factors emerged in Patient S3, who developed DVT followed by pulmonary embolism and carried the same prothrombin allele in heterozygosity, together with a promoter variant in F12 and a missense change in THBD. While the F12 alteration may be protective, the THBD variant could impair PC activation. The overall effect, in a patient with familial predisposition, was still a net prothrombotic tendency.

More dramatic examples were also observed. Patient S1 developed extensive porto-mesenteric thrombosis and was found to carry a likely pathogenic variant in FGB, together with a VWF variant and the common prothrombin allele. This constellation suggests a synergistic mechanism in which fibrinogen abnormalities, platelet–VWF interactions, and increased thrombin formation converge to drive a severe thrombotic phenotype.

Other patients demonstrated similar “multi-hit” patterns, often involving the coexistence of VUS in natural anticoagulant genes and homozygous carriage of common risk alleles. Patient S5, for example, carried two PROCR variants together with homozygous prothrombin c.1726-59G>A and developed pulmonary embolism on hormonal therapy. Patient S7 combined variants in FGB and PROC with homozygous risk alleles in F2 and PROCR, and experienced both pregnancy-associated VTE and recurrence after a local inflammatory trigger. Patient S14 had recurrent femoro-popliteal DVT in the absence of family history but carried VUS in F5 and VWF, together with homozygous prothrombin c.1726-59G>A.

High-penetrance pathogenic variants were also observed in some individuals, but even in these cases the phenotype was often modulated by additional variants or alleles. For instance, Patient S27, with a pathogenic F2 Arg541Trp mutation, also carried the common prothrombin allele and a VWF VUS, and developed extensive iliocaval thrombosis shortly after starting oral contraceptives. Patient S71, carrying the SERPINC1 Budapest 3 variant, also had a VUS in F8 and suffered recurrent events despite no family history. Patient S77 harbored a canonical splice-site mutation in F13A1, together with variants in VWF and F5, and presented with unprovoked pulmonary embolism.

Several patients without strong family history also exhibited multi-hit profiles. Patient S98 had upper extremity DVT with VUS in VWF and PLG; Patient S99 developed unprovoked PE with variants in PROS1 and A2M plus the prothrombin allele; Patient S195 carried variants in F3, F2, and SERPIND1 and presented with pulmonary embolism; and Patient S202 developed a rare testicular vein thrombosis in the presence of variants in PROZ and FGA.

Recurrence was frequent in patients with multilayered genetic backgrounds. Patient S110, with variants in SERPINC1 and VWF, developed multiple DVT episodes and also experienced bleeding during anticoagulation. Patient S184, with variants in VWF and PROS1, had recurrent idiopathic DVT. Patient S192 carried three variants in SERPINC1, KLKB1, and SERPINA5 and presented with pulmonary embolism after trauma. Patient S204 had recurrent DVT after trauma, carrying variants in F5 and PLG. Patient S209 experienced recurrent PE and DVT, requiring thrombolysis, with variants in THBD and F12.

Another set of paradigmatic observations from our cohort involves variants located in genes usually associated with a bleeding rather than thrombotic phenotype, which nevertheless emerged in patients with recurrent VTE. For instance, Patient S184 carried a stop-gain variant in PROZ. While protein Z (PZ) deficiency is generally linked to a pro-hemorrhagic tendency, some studies have paradoxically correlated PZ alterations with thrombotic events, including deep vein thrombosis and recurrent pregnancy loss. Similarly, Patient S32, who experienced recurrent DVT with a positive family history, harbored a PROZ deletion but did not report any bleeding history. Notably, this patient also carried two additional class 3 variants, one in PROC and one in PLG, suggesting that the combined effect of multiple variants may have shifted the balance toward hypercoagulability despite the theoretically pro-hemorrhagic nature of PZ deficiency.

A comparable scenario was observed with SERPINF2, a gene encoding α2-antiplasmin, whose loss-of-function variants are classically associated with bleeding diathesis. Patient S183 presented a deletion in SERPINF2—shared with Patient S194—but developed recurrent thrombotic events,

including pregnancy-associated VTE, an early recurrence shortly after stopping vitamin K antagonists, and subsequent thrombosis while on rivaroxaban therapy. These cases illustrate how variants in genes with presumed pro-hemorrhagic consequences can manifest with a thrombotic phenotype, likely reflecting the influence of additional genetic or environmental modifiers.

Patient S87 represented a particularly complex case. She had a family history of DVT and developed deep vein thrombosis complicated by paradoxical embolism through a patent foramen ovale. Her medical history was further characterized by hereditary spherocytosis, potentially adding to the complexity of her hematological phenotype. Genetic testing revealed multiple alterations: the PS Heerlen variant (*PROS1* c.1501T>C, class 4), a VUS in *VWF*, and two risk alleles in homozygosity (*F2* c.1726-59G>A and *F13A1*), the latter previously associated with a pro-hemorrhagic tendency. Clinically, she displayed a mixed phenotype: in addition to venous thromboembolism, she suffered from severe menorrhagia, which required temporary discontinuation of anticoagulant therapy during menstruation. Also, Patient S217, who carried variants in KLKB1 and VWF, had recurrent VTE and also developed a hematoma after minor trauma, illustrating how some genetic profiles may modulate both thrombotic and bleeding risks. These cases illustrate the possible coexistence of prothrombotic and prohemorrhagic genetic determinants within the same patient, whose clinical expression may be further modulated by concomitant acquired conditions. The dynamic balance between these opposing forces—shifted by genetic background, comorbidities, and environmental exposures—may account for the alternation of thrombotic and hemorrhagic manifestations. Such observations emphasize the added value of multigene NGS panels in identifying both thrombotic and bleeding predispositions, which can have direct implications for individualized patient management.

Together, these case-based observations emphasize how apparently modest variants, often of uncertain clinical significance, may combine with known predisposing alleles, pathogenic mutations, or environmental exposures to shape thrombotic phenotypes of varying severity. They highlight the importance of considering polygenic inheritance and gene–environment interactions when interpreting NGS results in patients with VTE.

Across these cases, a recurring pattern emerges: the coexistence of multiple modest genetic hits, frequently including common alleles, creates a cumulative predisposition that may manifest clinically in the presence of environmental triggers such as hormonal therapy, pregnancy, trauma, or immobilization. The detailed descriptions provided here illustrate the mechanistic plausibility of such interactions, even when individual variants are classified as VUS.

6.4. Clinical and research implications

The implications of our findings are manifold. From a clinical perspective, our data indicate that NGS improves diagnostic yield in patients with idiopathic or ambiguous VTE, revealing hidden predispositions that would remain undetected by standard assays. This information may be particularly valuable in cases of early-onset VTE, recurrent thrombosis, unusual sites, or strong family history. However, the predominance of VUS requires cautious interpretation: these findings should currently be used to inform research and hypothesis generation rather than direct patient management(200).

Another important consideration is how these findings could be translated into a diagnostic pathway for patients with VTE. In current practice, the first step remains conventional thrombophilia screening, including testing for FVL, PTM, deficiencies of AT, PC, and PS, and antiphospholipid antibodies(5,11). When this screening is negative, additional strategies are needed for patients with high-risk features such as early-onset disease, recurrence, unusual sites, or strong family history.

In this context, the use of global coagulation assays could represent a valuable intermediate step before proceeding to genetic testing. Unlike routine assays that focus on isolated proteins, global assays provide an integrated view of the hemostatic balance, capturing the cumulative effects of procoagulant and anticoagulant forces. Among these, the thrombin generation assay (TGA) is the most widely studied(223–225). It quantifies the plasma capacity to generate thrombin and yields parameters such as lag time, peak thrombin, endogenous thrombin potential (ETP), and time to peak. Increased thrombin generation has been linked to both the risk of a first VTE and recurrence, and the assay is sensitive to the pharmacological effects of anticoagulants. Its limitation is that, while it can demonstrate a hypercoagulable state, it does not identify the specific molecular abnormality.

An illustrative example from our cohort is Patient S27, who had a strong family history of VTE and developed a femoro-iliaco-caval DVT while on combined oral contraceptives. Genetic testing revealed a class 5 variant in *F2*, but prothrombin activity was normal in two independent measurements (104.1% and 111.1%, normal value 80.0-120.0%). This prompted a TGA, which showed a markedly prothrombotic profile with reduced lag time (1.040 min, normal value 1.757-2.377 min) and time to peak (3.050 min, normal value 4.08-6.09) increased ETP (1683.22 nM*min, normal value 1059.37-1352.37 nM*min), and a prolonged start tail (28.09 min, normal value 17.96-21.92 min), providing functional confirmation of hypercoagulability not evident from standard assays.

Another promising development is the whole blood thrombin generation assay (WB-TGA), which, unlike plasma-based TGA, incorporates the contribution of platelets and red blood cells. This provides a more physiological assessment and may be particularly informative in patients with combined risk factors or when platelet function is relevant, though methodological standardization and validation are still lacking(224).

Overall, these observations suggest that in selected patients with negative conventional screening but evidence of hypercoagulability on global assays, NGS may provide the final step to uncover causative or contributory variants. Importantly, many pathogenic variants affect protein function rather than concentration, escaping standard laboratory detection but becoming evident through functional or global approaches. This stepwise strategy—conventional screening → global assay (e.g., TGA/WB-TGA) → NGS—represents a pragmatic compromise between functional assessment and molecular characterization (Figure 6.1).

This model highlights a paradigm shift: the concept of “thrombophilia” as we currently use it is “old” and incomplete, rooted in a few classical mutations that alter protein levels. New evidence shows that many variants instead affect protein function, interactions, or cofactor responses, opening scenarios in which molecular predisposition can be subtle, context-dependent, and detectable only with more refined functional and genetic tools. Integrating TGA with NGS in a structured diagnostic pathway could therefore maximize diagnostic yield, refine risk stratification, and move toward a precision medicine approach in VTE.

The frequent identification of common alleles such as F2 c.1726-59G>A, TFPI c.629-33T>C, and PROCR p.Ser219Gly raises the question of whether these variants should be integrated into polygenic risk scores for thrombosis, similar to approaches already applied in cardiovascular genetics. Such tools could help stratify risk more accurately and personalize decisions on anticoagulation duration, prophylaxis in high-risk situations, or counselling of relatives.

At a research level, our study highlights the need for functional validation of VUS, integration of multi-omic data (e.g., transcriptomics, proteomics), and expansion to whole-genome sequencing to capture regulatory and structural variants beyond the coding regions.

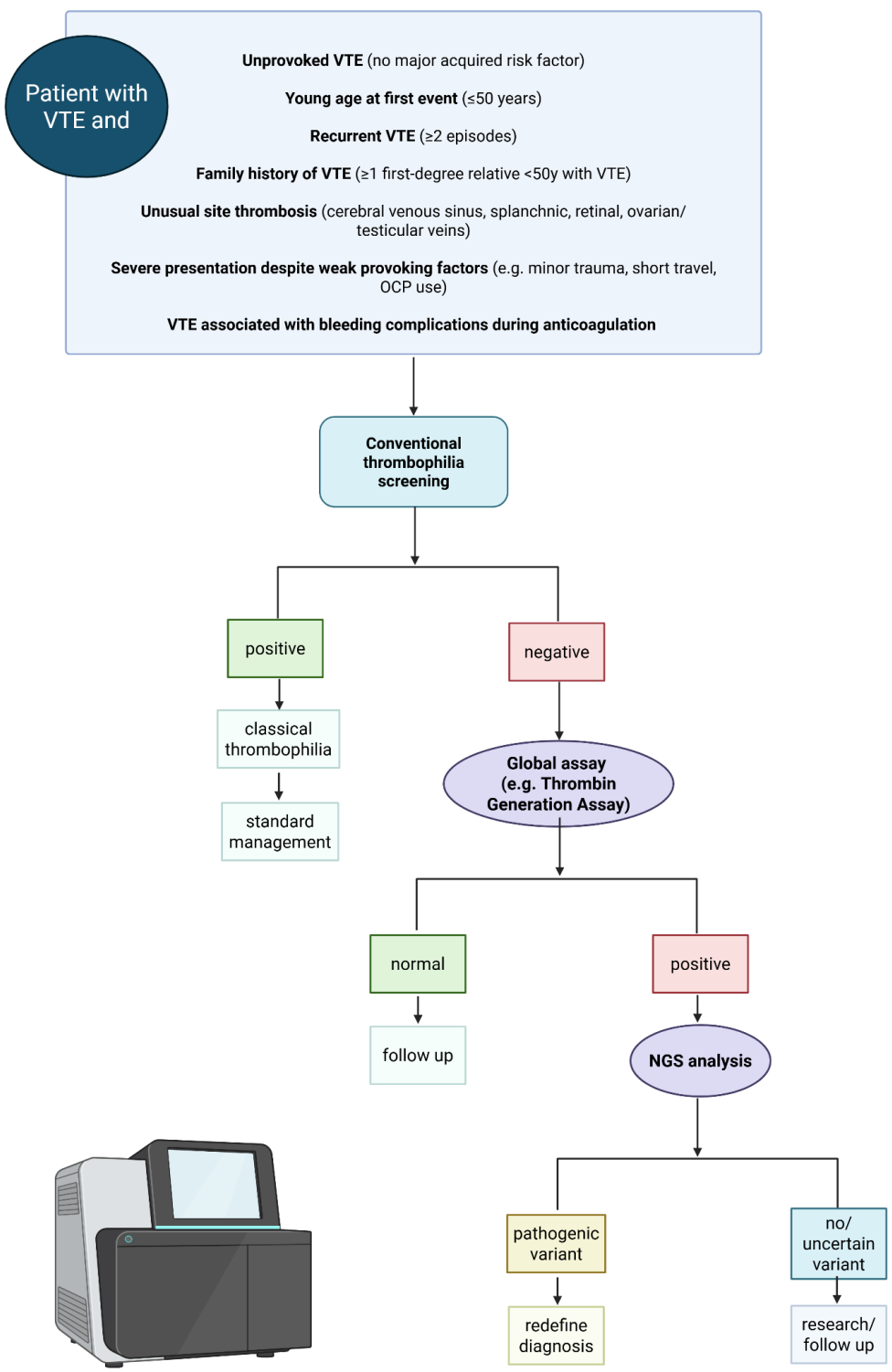

Figure 6.1. Proposed stepwise diagnostic pathway for patients with VTE. After a confirmed diagnosis of VTE, patients with strong clinical features undergo conventional thrombophilia screening (Factor V Leiden, PTM, deficiencies of antithrombin, protein C, and protein S, and antiphospholipid antibodies). If positive, classical thrombophilia is diagnosed and managed according to current guidelines. If negative, a functional assessment with a global assay is performed. A normal TGA suggests no additional testing, whereas hypercoagulability prompts next-generation sequencing (NGS) analysis. Identification of pathogenic or likely pathogenic variants supports a refined diagnosis and improved risk stratification, while absence of variants or detection of variants of uncertain significance (VUS) requires cautious interpretation and possible research-based follow-up. Created in BioRender.com.

6.5. Comparison with existing literature

Our findings align with and extend prior evidence that multigene NGS panels uncover both (likely) pathogenic variants and a long tail of VUS across coagulation pathways in patients with VTE who are negative at conventional screening. Large real-world series show that panel testing detects variants in ~35–63% of patients, frequently in SERPINC1, PROS1, F2, F5, and often in addition to or outside what standard assays can reveal, underscoring complementarity to conventional thrombophilia work-ups. Notably, recurrent "diagnostic-grade" variants enriched in symptomatic cohorts—such as SERPINC1 Dublinn(Navarro-Fernández et al., 2016) and PROS1 Heerlen(218)—support true disease association beyond background population frequency, while many additional rare variants remain class-3 and require functional follow-up(190,192). Beyond single variants, polygenic burden and gene–environment interplay also matter: combining multiple thrombosis-associated SNPs with clinical risk factors improves discrimination for first VTE,reinforcing an oligogenic/multifactorial model(226). Consistent with this, genes like VWF, PROCR, THBD—robustly linked to VTE risk in genomic studies—sit at the interface of thrombotic and bleeding biology, helping explain why some genetic profiles can modulate both risks depending on context(227).

6.6. Strengths and limitations

Strengths of this work include the systematic recruitment of consecutive patients with objectively confirmed VTE and negative conventional thrombophilia screening, which reduces selection bias. The use of a comprehensive 33-gene NGS panel covering major coagulation, anticoagulant, and fibrinolytic pathways represents another strength, allowing detection of both well-established pathogenic variants and less characterized VUS. The integration of detailed clinical phenotyping, family history, and longitudinal follow-up enabled correlation of genetic findings with recurrence, bleeding complications, and unusual thrombotic sites. Furthermore, the description of individual cases (e.g., patients S87 and S217) illustrates the real-world complexity of coexisting prothrombotic and prohemorrhagic determinants, offering valuable translational insight.

Nonetheless, several limitations must be acknowledged. First, this was a single-center study with a relatively limited sample size, which may affect generalizability. Second, the panel did not explore non-coding or regulatory regions, copy number variations, or genes not yet linked to VTE, potentially underestimating the genetic burden. In addition, it should be acknowledged that the genetic panel focused primarily on coagulation-related genes, whereas the pathophysiology of VTE involves a broader network. Other genes implicated in platelet function, endothelial biology, inflammation,

and vascular integrity may also contribute to the thrombotic phenotype but were not assessed in this study. The role of platelets and endothelium is particularly relevant, given their central involvement in thrombus initiation and propagation. Variants affecting platelet receptors, adhesion molecules, or endothelial regulators (e.g., nitric oxide synthase, adhesion junction proteins) might interact with coagulation-related alterations to further modulate individual susceptibility.

Moreover, functional assays were not completely performed, preventing confirmation of the biological impact of VUS. Also, the absence of a control population limited our ability to compare variant frequencies and to draw causal inferences. Finally, the present analysis was mainly descriptive and hypothesis-generating; the associations observed cannot establish definitive causal relationships and require validation in larger, multicenter cohorts with integrated functional and familial studies.

6.7. Conclusion

Future research should expand to multicenter cohorts with larger and more diverse populations, enabling replication of findings and increasing the statistical power to detect rare variants. The integration of functional studies, including in vitro assays and animal models, will be crucial to clarify the biological impact of VUS and to reclassify variants in clinically actionable categories. At the same time, the development of polygenic risk models that combine common genetic polymorphisms with rare high-impact variants may provide a more accurate estimate of thrombotic risk at the individual level.

Another important direction will be the exploration of whole-exome and whole-genome sequencing, which offer the opportunity to identify variants in non-coding or regulatory regions, structural alterations, and novel genes not yet associated with VTE. Extending the analysis beyond coagulation factors to include genes involved in platelet function, endothelial biology, inflammation, and vascular homeostasis may uncover new mechanisms and genetic contributors to thrombotic disease.

Finally, the integration of genetic information with clinical risk factors, biomarkers, and environmental exposures in prospective cohorts will be key to moving toward precision medicine in VTE. Such an approach could allow more refined risk stratification, targeted prevention strategies, and tailored management, balancing thrombotic and bleeding risks in genetically complex patients.

7. SUPPLEMENTARY TABLES

Gene	Known as	Variant		rs	Frequency	Phenotype
F2	C20209T	c.*96C>T		rs72550707	0.1%	pro-thrombotic(228)
	G19911A	c.1726-59G>A		rs3136516	48.1%	pro-thrombotic(221)
F11		c.1481-188C>T		rs2289252	37.1%	pro-thrombotic Homo T only(229)
F12		c.-4T>C		rs1801020	29.2%	pro-thrombotic Homo T only(230)
F13A1		c.103G>T	p.Val35Leu	rs5985	23.3%	pro-haemorrhagic Homo T only(231)
PROCR	H3	c.655A>G	p.Ser219Gly	rs867186	10.2%	pro-thrombotic Homo G only(164)
SERPINA10	H5	c.262C>T	p.Arg88*	rs2232698	0.7%	pro-thrombotic(165)
		c.435T>G	p.Phe145Leu	rs117421112	0.1%	pro-thrombotic(232)
		c.972G>A	p.Trp324*	rs61754487	0.4%	pro-thrombotic(232)
		c.1151A>G	p.Gln384Arg	rs2232710	1.0%	pro-thrombotic(232)
TFPI		c.629-33T>C		rs8176592	30.4%	pro-thrombotic Homo C only(233)

Supplementary Table 1. Predisposing variants or risk alleles. The table shows genetic variants reported in the literature to be predisposing to coagulation disorders evaluated. Frequency was evaluated extrapolating data from GenomAD 2.1 and 4.1 for the total population and reporting the most frequent one. Homo: Homozygosis.

Pt	Genetic variants								ACMG	Publications
	gene	GRCh37	exon	ntd	aa	rs	zygosity	class	criteria	
S1	*FGB*	chr4:155490457	6	c.956C>T	p.Pro319Leu	rs374463429	Het	3	PM2, PP3, PP5	n/a
	VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3	PM2, BP7	n/a
S2				no variant					-	-
S3	*F12*	chr5:176830478	int11	c.1387+4C>G	-	rs761161412	Het	3	PM2, BP6	n/a
	THBD	chr20:23030015	1	c.127G>A	p.Ala43Thr	rs1800576	Het	3	PP5, BP4	(234)
S4	*F11*	chr4:187201533	9	c.1022A>G	p.Glu341Gly	n/a	Het	3	PM2, PM5, PP2	n/a
S5	*PROCR*	chr20:33762502	int1	c.71-3C>T	-	rs367783205	Het	3	PM2	n/a
	PROCR	chr20:33764821	3'UTR	c.*205C>T	-	rs1384660995	Het	3	PM2, BP7	n/a
S6	*SERPINC1*	chr1:173879947	4	c.707C>T	p.Ser236Leu	rs368419985	Het	3	PM1, PM2, PP2	n/a
	F5	chr1:169500178	15	c.5054C>G	p.Thr1685Ser	rs6011	Het	3	BS1, BS2, PP5	(235)
	F5	chr1:169499020	16	c.5245C>G	p.Leu1749Val	rs6034	Het	3	BS2, PP3	(235)
	VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3	PM2, BP7	n/a
S7	*PROC*	chr2:128186377	9	c.1241G>C	p.Trp414Ser	rs768759265	Het	3	PM1, PM2, PP3	n/a
	FGB	chr4:155490947	7	c.1240G>A	p.Gly414Ser	rs141881199	Het	4	PS3, PM2	(236)
S8	*PROS1*	chr3:93598150	13	c.1501T>C	p.Ser501Pro	rs121918472	Het	4	PS3, PM2, PP2, PP5	(204) (Heerlen)
	VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3	PM2, BP7	n/a
S9				no variant					-	-
S10	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3	PM2, BP7	n/a
S11	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3	PM2, BP7	n/a
S12				no variant					-	-
S13				no variant					-	-
S14	*F5*	chr1:169500085	15	c.5147C>T	p.Ser1716Leu	n/a	Het	3	PM2	n/a
	VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3	PM2, BP7	n/a
S15	*F2*	chr11:46747447	7	c.598G>A	p.Glu200Lys	rs62623459	Het	3	BP6	(237)
S16	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3	PM2, BP7	n/a
S27	*F2*	chr11:46751078	12	c.1621C>T	p.Arg541Trp	rs886048338	Het	5	PS3, PM1, PM2, PP3, PP5	(90)

	VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3	PM2, BP7	n/a
	PROZ	chr13:113826124	8	c.909_933del	p.Trp325*	n/a	Het	3	PM2	n/a
S32	*PROC*	chr2:128186246	9	c.1110C>A	p.His370Gln	rs778063605	Het	3	PM1, PM2, PP5	(238)
	PLG	chr6:161152827	12	c.1489G>A	p.Val497Ile	n/a	Het	3	PM2, BP4	n/a
S34	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3	PM2, BP7	n/a
S35				no variant					-	-
S39				no variant					-	-
S41	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3	PM2, BP7	n/a
S42				no variant					-	-
S43	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3	PM2, BP7	n/a
S44	*VWF*	chr12:6167213	int13	c.1534-3C>A		rs61754009	Het	5	PS4, PM1, PM2, PP3, PP5	(239)
S45	*PROCR*	chr20:33764619	int4	c.*3A>C	-	rs767268282	Het	3	PM2, BP7	n/a
S48	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3	PM2, BP7	n/a
S49	*SERPIND1*	chr22:21141227	5	c.1373T>G	p.Val458Gly	rs772390602	Het	3	PM2, PP3	n/a
	SERPINA10	chr14:94750417	5	c.1220C>A	p.Ala407Asp	rs1289089250	Het	3	PM2	n/a
S50	*PROS1*	chr3:93646095	2	c.233C>T	p.Thr78Met	rs6122	Het	5	PS3, PS4, PM2, PP2, PP3	(240,241)
S51	*VWF*	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3	PM2, BP7	n/a
S52	*SERPINA5*	chr14:95054285	3	c.586G>A	p.Val196Ile	rs1055786032	Het	3	PM2	n/a
	VWF	chr12:6166187	15	c.1781C>G	p.Ala594Gly	rs267607308	Het	3	PM2, PP2, BP6	(242)
S54	*VWF*	chr12:6138597	22	c.2878C>T	p.Arg960Trp	rs370984712	Het	3	PM2, PP2, PP5	(243)
	VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3	PM2, BP7	n/a
S55	*SERPINC1*	chr1:173884010	2	c.89T>A	p.Val30Glu	rs2227624	Het	5	PS3, PS4_Very Strong, PP1_moderate*	(203,244) (Dublin)
S56				no variant					-	-
S57				no variant					-	-
S58				no variant					-	-
S61				no variant					-	-
S63				no variant					-	-

S67	*FGB*	chr4:155490457	6	c.956C>T	p.Pro319Leu	rs374463429	Het	3		
S68				no variant					-	-
S71	*SERPINC1*	chr1:173883708	2	c.391C>T	p.Leu131Phe	rs121909567	Het	5	PS4_Very Strong, PP1_strong, PP3*	(219)(Budapest 3)
	F8	chrX:154158085	14	c.3980C>T	p.Thr1327Met	rs200520711	Het	3		
S72	*F2*	chr11:46751085	12	c.1628G>A	p.Arg543His	rs143064939	Het	3		
S75				no variant					-	-
S76				no variant					-	-
S77	*F13A1*	chr6:6248653	int5	c.691-1G>A	-	rs372296352	Het	5		(245)
	VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3	PM2, BP7	n/a
	F5	chr1:169521863	8	c.1228A>C	p.Asn410His	rs372690115	Het	3		(246)
S78				no variant					-	-
S81				no variant					-	-
S83				no variant					-	-
S86				no variant					-	-
S87	*PROS1*	chr3:93598150	13	c.1501T>C	p.Ser501Pro	rs121918472	Het	4	PS3, PM2, PP2, PP5	(204) (Heerlen)
	VWF	chr12:6127535	28	c.5049A>C	p.Ala1683=	rs79275181	Het	3	PM2, BP7	n/a
S89	*VWF*	chr12:6128388	28	c.4196G>A	p.Arg1399His	rs1800382	Het	3	PM5_Supporting, PS3, PP3, PP4, BS1, BP5*	(247)
S98	*VWF*	chr12:6173519	12	c.1325G>A	p.Arg442His	rs199665748	Het	3	PM2	n/a
	PLG	chr6:161139817	9	c.1043A>G	p.Lys348Arg	n/a	Het	3	PM2, BP4	n/a
S99	*PROS1*	chr3:93595933	14	c.1747A>C	p.Asn583His	rs139479630	Het	3	PM2, PP2, BP4	(248)
	A2M	chr12:9260146	8	c.853G>A	p.Ala285Thr	rs760977693	Het	3	PM2, BP4	n/a
S104				no variant					-	-
S105				no variant					-	-
S106				no variant					-	-
S110	*VWF*	chr12:6166046	15	c.1922C>T	p.Ala641Val	rs61754019	Het	3	PM2, PP5	(237)
	SERPINC1	chr1:173879828	4	c.826T>G	p.Phe276Val	n/a	Het	3	PM2, BP2, BP4	n/a
S112				no variant					-	-
S113				no variant					-	-

S114				no variant					-	-
S115	*FGB*	chr4:155484174	1	c.4A>G	p.Lys2Glu	rs6053	Het	3	PM2	n/a
S165	*SERPINF2*	chr17:1657703	10	c.1351C>T	p.Pro451Ser	rs57360598	Het	3	PM2, BP6	n/a
S166	*SERPINC1*	chr1:173883708	2	c.391C>T	p.Leu131Phe	rs121909567	Het	5	PS4_Very Strong, PP1_strong, PP3*	(219,249) (Budapest 3)
S169	*PROCR*	chr20:33762717	2	c.283G>T	p.Gly95Cys	rs139714129	Het	3	PM2	n/a
S170				no variant					-	-
S171	*FGG*	chr4:155533202	3	c.275T>C	p.Leu92Pro	rs766605366	Het	3	PM2	n/a
S172	*FGB*	chr4:155484174	1	c.4A>G	p.Lys2Glu	rs6053	Het	3	PM2	n/a
S176				no variant					-	-
S177	*PLG*	chr6:161162369	17	c.2045T>A	p.Ile682Asn	rs147175166	Het	3	BS2, PP5	(235)
S180				no variant					-	-
S181	*PROZ*	chr13:113819398	6	c.603G>A	p.Glu179=	n/a	Het	3	PM2, BP7	n/a
S182	*SERPINA 10*	chr14:94754620	3	c.992+3A>G	-	rs369697180	Het	3	PM2, PP3	n/a
	VWF	chr12:6127640	28	c.4944T>C	p.Pro1648=	rs201825372	Het	3	BP7	n/a
S183	*SERPINF2*	chr17:1650299	6	c.368-13del	-	rs552030205	Het	3	PM2, BS2	n/a
S184	*VWF*	chr12:6166046	15	c.1922C>T	p.Ala641Val	rs61754019	Het	3	PM2, PP5	(237)
	PROZ	chr13:113814443	2	c.252C>T	p.Ile62=	n/a	Het	3	PM2, BP7	n/a
S187	*FGA*	chr4:155507052	5	c.1529G>A	p.Arg510His	rs138137585	Het	3	PM1, PM2, BP4	n/a
S188				no variant					-	-
S191	*SERPINA 5*	chr14:95053884	3	c.185G>A	p.Ser62Asn	rs372991959	Het	3	PM2	n/a
S192	*SERPINC1*	chr1:173878747	5	c.1096C>G	p.Gln366Glu	rs565091601	Het	3	PM1, PM2, PP2, BP4	(235)
	KLKB1	chr4:187177213	13	c.1557A>C	p.Val519=	n/a	Het	3	PM2, BP7	n/a
	SERPINA 5	chr14:95058471	6	c.1116C>A	p.Phe372Leu	s1401241484	Het	3	PM2, BP4	n/a
S194	*SERPINF2*	chr17:1650299	int6	c.368-13del	-	rs552030205	Het	3	PM2, BS2	n/a
S195	*F3*	chr1:95001632	3	c.292_300dupGATGTGAAG	p.Asp98_Lys100dup	rs756646702	Het	3	PM2, PM4	n/a
	F2	chr11:46747447	7	c.598G>A	p.Glu200Lys	rs62623459	Het	3	BS2	(250)

	SERPIND 1	chr22:21138397	3	c.1027G>A	p.Asp343Asn	rs761404668	Het	3	PM2	n/a
S196	*PLG*	chr6:161152784	12	c.1446G>A	p.Met482Ile	rs753483846	Het	3	PM2, BP4	n/a
S197	*SERPINA 10*	chr14:94756901	2	c.30C>T	p.Ser10=	rs764890013	Het	3	PM2, BP7	n/a
S201	*F5*	chr1:169510246	13	c.3974_4081dup	p.Leu1325_Asp1360dup	n/a	Het	3	N/A	n/a
S202	*PROZ*	chr13:113813046	int1	c.70+2T>G	-	n/a	Het	3	PM2	n/a
	FGA	chr4:155507556	5	c.1025G>A	p.Gly342Glu	rs774664670	Het	3	PM2	n/a
S203				no variant					-	-
S204	*F5*	chr1:169510246	13	c.3974_4081dup	p.Leu1325_Asp1360dup	n/a	Het	3	N/A	n/a
	PLG	chr6:161127504	2	c.115A>C	p.Lys39Gln	rs138353396	Het	3	PM2	n/a
S206				no variant					-	-
S208				no variant					-	-
S209	*THBD*	chr20:23028811	1	c.1331A>G	p.Glu444Gly	rs375671812	Het	3	PM2, PP3	n/a
	F12	chr5:176836468	int1	c.57+4A>G	-	rs371915487	Het	3	PM2, PP3	n/a
S211	*SERPINA 5*	chr14:95054027	3	c.328G>C	p.Gly110Arg	rs754794601	Het	3	PM2	n/a
S212				no variant					-	-
S213				no variant					-	-
S214				no variant					-	-
S215				no variant					-	-
S216				no variant					-	-
S217	*KLKB1*	chr4:187155164	4	c.280G>A	p.Gly94Ser	rs146728804	Het	3	PM2	n/a
	VWF	chr12:6128388	28	c.4196G>A	p.Arg1399His	rs1800382	Het	3	PM5_Supporting, PS3, PP3, PP4, BS1, BP5*	(247)
S218				no variant					-	-
S219				no variant					-	-
S222				no variant					-	-
S223				no variant					-	-

Supplementary Table 2. Genetic variants identified in the study cohort through targeted next-generation sequencing (NGS). The table reports patient ID, gene, genomic coordinates (GRCh37), exon/intron location, nucleotide (ntd) and amino acid (aa) changes, dbSNP reference (rs), zygosity, ACMG/AMP classification, and supporting criteria. When available, relevant publications are cited. Variants are classified according to ACMG guidelines: class 1 (benign), class 2 (likely benign), class 3 (variant of uncertain significance, VUS), class 4 (likely pathogenic), and class 5 (pathogenic). NA = not available. **Abbreviations:** NGS, next-generation sequencing; ACMG, American College of Medical Genetics and Genomics; AMP, Association for Molecular Pathology; ntd, nucleotide; aa, amino acid; rs, reference SNP ID; Het, heterozygous; VUS, variant of uncertain significance.

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