Universidade de Aveiro Departamento de Química

Ano 2020

Jéssica Filipa Pires Malva

Estudo dos genes *VTN*, *PLG* e outros genes de coagulação na Síndrome Hemolítica Urémica atípica (SHUa)

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, especialização em Bioquímica Clínica, realizada sob a orientação científica da Doutora Teresa Fidalgo, responsável do Laboratório de Hematologia Molecular do Serviço de Hematologia Clínica do Centro Hospital Universitário de Coimbra e do Professor Doutor Francisco Amado do Departamento de Química da Universidade de Aveiro.

Dedico este trabalho à minha família por todo o apoio.

O Júri

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Palavras-chave Síndrome hemolítica urémica atípica, microangiopatias trombóticas, genótipo, *VTN*, *PLG*, outros genes de coagulação, técnicas de diagnóstico molecular, fenótipo

Resumo

Introdução: A síndrome hemolítica urémica atípica é uma variante rara de microangiopatia trombótica caracterizada por anemia hemolítica microangiopática, trombocitopenia e, por vezes, insuficiência renal aguda. Esta patologia é frequentemente idiopática, podendo também ser secundária a outras patologias ou devida a causas genéticas – variantes nos genes do complemento (*C3*, *CFB*, *CFH*, *CFI*, *MCP*, *THBD)*. Estas alterações, na maioria dos casos, originam a hiperativação da via alternativa do complemento e consequentemente resultam em formação de trombos microvasculares que afetam, principalmente, a função renal. No entanto, recentemente surgiram outras possíveis causas genéticas desta patologia, em genes não relacionados com o complemento, *VTN*, *PLG*, entre outros genes de coagulação.

Objetivos e Métodos: Com o objetivo de analisar a correlação genótipo/fenótipo em pacientes com SHUa, analisámos 45 genes em 50 pacientes, utilizando a sequenciação Sanger para o gene *VTN* e um painel de genes personalizado de sequenciação de próxima geração (NGS) para o *PLG* e outros genes de coagulação.

Resultados: No total, foram identificadas 53 variantes raras diferentes em *VTN*, *PLG*, *ADAMTS13*, *ANKRD26*, *F5*, *F7*, *F8*, *F10*, *F13A1*, *FGA*, *FGB*, *FGG*, *GP6*, *ITGA2B*, *ITGB3*, *NBEAL2*, *PLAT*, *PROC*, *PROS1*, *SERPINC1*, *SERPINE1*, *SERPINF2*, *TUBB1* e *VWF*. Das quais, identificámos 8 variantes patogénicas, 11 provavelmente patogénicas, 14 de significado incerto e 20 provavelmente benignas.

Conclusões: Este estudo não implicou os genes *VTN* e *PLG*, em particular, como importantes contribuintes para SHUa. Contudo, encontrámos variantes em vários genes que poderão constituir uma predisposição genética nestes doentes, e ter um efeito cumulativo em ambos os sistemas - coagulação e complemento.

Keywords Atypical hemolytic uremic syndrome, thrombotic microangiopathies, genotype, *VTN*, *PLG*, other coagulation genes, molecular diagnostic techniques, phenotype

Abstract

Background: Atypical hemolytic uremic syndrome is a rare variant of thrombotic microangiopathy characterized by microangiopathic hemolytic anemia, thrombocytopenia and, occasionally, acute renal failure. This condition is often idiopathic and may also be secondary to other pathologies or due to genetic causes - variants in the complement genes (*C3*, *CFB*, *CFH*, *CFI*, *MCP*, *THBD)*. These changes, in most cases, lead to hyperactivation of the pathway complement and consequently result in the formation of microvascular thrombi that affect, mainly, the renal function. However, other possible genetic causes of this pathology have emerged recently, in genes not related to complement, *VTN*, *PLG*, among other coagulation genes.

Objectives and Methods: In order to deepen the genotype/phenotype correlation in patients with aHUS, we analyzed 45 genes in 50 patients using Sanger sequencing for the *VTN* gene and a custom next generation sequencing gene panel (NGS) for PLG and other coagulation genes.

Results: In total, 53 different rare variants were identified in *VTN*, *PLG*, *ADAMTS13*, *ANKRD26*, *F5*, *F7*, *F8*, *F10*, *F13A1*, *FGA*, *FGB*, *FGG*, *GP6*, *ITGA2B*, *ITGB3*, *NBEAL2*, *PLAT*, *PROC*, *PROS1*, *SERPINC1*, *SERPINE1*, *SERPINF2*, *TUBB1* and *VWF*. Of which we identified 8 pathogenic variants, 11 probably pathogenic, 14 uncertain significance and 20 probably benign.

Conclusions: This study did not imply *VTN* and *PLG* in particular as important contributors to aHUS. However, we found variants in several genes that could be a genetic background in these patients, and to have a cumulative effect on both systems - coagulation and complement.

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List of Abbreviations (Alphabetically Sorted):

ACMG - American College of Medical Genetics and Genomics

ADAMTS13 - A Desintegrin And Metalloproteinase with Thrombospondin I motif, member 13

aHUS - atypical Hemolytic Uremic Syndrome

CFB - Complement Factor B

CFH - Complement Factor H

CFI - Complement Factor I

DGKE - Diacylglycerol kinase ε

DNA - DeoxyriboNucleic Acid

EVS - Exome Variant Server

gnomAD - Genome Aggregation Database

HGMD - Human Gene Mutation Database

Hmz - Homozygous

HSF - Human Splicing Finder

Htz - Heterozygous

HUS - Hemolytic Uremic Syndrome

MAF - Minor Allele Frequency

MCP/CD46 - Membrane Cofactor Protein

NGS - Next-Generation Sequence

PCR - Polymerase Chain Reaction

PLG - Plasminogen

STEC - Shiga-Toxin producing Escherichia Coli

STEC-HUS – Shiga toxin-producing Escherichia Coli - Hemolytic Uremic Syndrome

TH - Thrombosis and Haemostasis

THBD - Thrombomodulin

TMA - Thrombotic Microangiopathies

TTP - Thrombotic Thrombocytopenic Purpura

VTN - Vitronectin

VWF - Von Willebrand Factor

1 | Introduction

Thrombotic microangiopathies (TMAs) are defined as lesions of vessel wall thickening, in arterioles or capillaries, partial or complete obstruction of the vessel lumen and intraluminal thrombosis of platelets [1–4]. This term was first introduced by Symmers [5], in 1952, and subdivided into two forms: thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS) [1–4]. In TTP, neurological manifestations are more pronounced while in HUS renal lesions are more common [1–3], however, some patients with TTP may present renal lesions and patients with HUS may also present neurological lesions [2–4,6]. Thus, it is possible to infer that the differential diagnosis of the two forms of TMA remains challenging, despite the remarkable advances in the last decade [2–4]. These two forms of TMA present similar clinical manifestations, which hindered their distinction [2,3] and consequently their differential diagnosis [7].

TTP is caused by severe plasma deficiency of the cleaving protease of von Willebrand factor (VWF) - A Disintegrin And Metalloproteinase with ThromboSpondin I motif, member 13 (ADAMTS13) [4,8]. This deficiency rarely occurs due to variants present in the ADAMTS13 gene, but frequently occurs due to anti-ADAMTS13 antibodies acquired [8]. Therefore, TTP is considered essentially an autoimmune disorder [6] and it is associated with severe and acquired deficiency of ADAMTS13 [6,7,9]. It is also known that this disease leads to platelet aggregation and microvascular thrombosis due to the formation of ultra large VWF multimers [8,10]. HUS can be classified into two distinct forms: typical and atypical [2,3]. Atypical hemolytic uremic syndrome (aHUS) is a rare variant of thrombotic microangiopathy characterized by microangiopathic hemolytic anemia, thrombocytopenia and, sometimes, acute renal failure [11–14]. Diagnosis is based on various clinical criteria and the exclusion of a severe ADAMTS13 (<10%) [6,7,15] and Shiga toxin-producing Escherichia Coli - Hemolytic Uremic Syndrome (STEC-HUS) [8,12,13]. This condition is often idiopathic [2,16] and develops in people with genetic abnormalities in the complement genes after exposure to a series of triggering factors such as pregnancy, infection, malignancy, transplantation and drugs [16–18].

Genetic abnormalities, in most cases, lead to overactivation [2,19,20] of the alternative pathway of complement and, consequently, result in the formation of microvascular thrombi which mainly affect the renal function [21–25]. The variants reported for aHUS

includes variants loss-of-function in a regulatory gene [26–31], like the *CFH* (implicated in approximately 20 to 30% of patients with aHUS), *MCP* (implicated in approximately 10 to 15%), *CFI* (approximately 4 to 10%) and *THBD* (approximately 2%), or variants gain-of-function [13,31–34] in an effector gene, such as the *C3* (approximately 5 to 10%) and the *CFB* (approximately 1 to 2%). The variants present in these genes were found mainly in the heterozygous state and about 5% of patients have combined mutations [35], usually in *CFH* with either *MCP* [13,28,36] or *CFI* [16,23,26]. In addition to the variants described in the complement proteins, there are variants in other molecules that are not directly related to the complement system, such as the variants in *DGKE* (approximately 2%) [8,13,14,19].

Although several studies affirm that the variants in the complement genes are clearly implicated in aHUS, it is possible that there are other genetic possibilities that cause this pathology [8,18,37,38]. First, genetic causes related to complement genes are present in only about 50%-60% of aHUS patients [8,37,38]. Second, the disease does not develop in the absence of triggering factors in several people who have genetic variants in the complement genes [18,38]. Lastly, there is an increase in published data suggesting that other pathways, such as the coagulation pathway [39,40], play an important role in the pathogenesis of the disease, however the integrated interpretation of the results becomes difficult [18,37,38].

The coagulation and complement cascades interact with each other at various levels of inflammatory or hemostatic responses through processes involving inflammatory mediators other than complement effectors or through interactions with target cells [39]. The cooperation between the complement and coagulation systems ensures the elimination of pathogens and also prevents bleeding [19,39]. Thus, “non complement” genes - *plasminogen* (*PLG*), *vitronectin* (*VTN*), among others - have been added to the list of candidate genes associated to aHUS [18,37,38,40].

VTN is a plasma and tissue glycoprotein that is in high concentrations in human plasma and can bind to protein complexes in the terminal phases of the complement and coagulation pathways. It is involved in a variety of biological process - cell adhesion, dissemination and migration, fibrinolysis, immune defense, hemostasis and extracellular anchoring [41,42]. The pathogenic *VTN* variants compromise the negative regulation of the terminal complement pathway being an increase the risk of developing aHUS instead of causing disease directly [38].

Conversely, the *PLG* gene encodes a protein denominated plasminogen that is converted to plasmin and plays a very important role in intravascular and extravascular fibrinolysis, cell migration, wound healing, tissue remodeling, angiogenesis and embryogenesis [43,44]. Variants in *PLG* was found in patients with aHUS and could be implicated in dysregulation of the complement pathway [37,38,44].

The molecular study of these genes can be performed using the Next-Generation Sequence (NGS) technique, as it allows the study of a large number of genes associated with coagulation. However it becomes a challenge because most variants associated with this pathology are individually rare. In this sense, the molecular study is crucial in the aHUS, so allows a more assertive diagnosis that permit the initiation of an adequate therapy and, thus, to reduce risks such as renal failure.

From this perspective, in a group of patients with previously studied aHUS without identified variants in the complement genes (*C3*, *CFB*, *CFH*, *CFI*, *MCP*, *THBD*), we conducted a study with 3 main objectives: perform the analysis of *VTN*, *PLG* recently implicated as aHUS associated genes, as well other coagulation genes; evaluate the predictive value of pathogenicity of the variants found using *in silico* tools and the international guidelines ACMG (American College of Medical Genetics and Genomics); finally analyze the genotype-phenotype correlation in this group of patients.

2 | Material and Methods

2.1 | Patients and sample processing

A total of 50 Caucasian patients of Portuguese origin were recruited to the Department of Molecular Hematology at Centro Hospitalar Universitário de Coimbra for a genetic evaluation for aHUS (Suppl. Table 1). The 50 individuals in this study included both children and adults with a median age of 34 years and a sex distribution of 35 females and 15 males. aHUS was diagnosed based on international guidelines [45], presence of thrombocytopenia, acute renal failure, microangiopathic hemolytic anemia, exclusion of Shiga toxin-producing Escherichia Coli and ADAMTS13 activity >10%. Patients were screened for variants in 11 complement genes (*ADAMTS13, CFH, CFHR1, CFHR3, CFHR4, CFHR5, CFI, CFB, C3, THBD* and *DGKE*), related to aHUS, using an NGS panel. However, in these 50 patients no variants were found in these genes. Thus, the study was extended to other NGS panel - Thrombosis and Haemostasis (TH) with 44 coagulation genes (*F2, F3, F5, F7, F8, F9, F10, F11, F13A1, F13B, LMAN1, MCFD2, FGA, FGB, FGG, GGCX, VKORC1, VWF, GP1BB, GP1BA, GP9, ITGA2B, ITGB3, NBEAL2, NBEA, GP6, TBXA2R, P2RY12, ANKRD26, CYCS, PLA2G4A, TUBB1, ADAMTS13, PROC, PROS1, SERPINC1, SERPINE1, SERPINF2, THBD, PLAT, PLG, ANXA5, PLAU, F12*). The *VTN* gene not include in TH NGS panel was analyzed using Sanger direct sequencing consisted of DNA extraction, amplification of DNA through PCR, agarose gel electrophoresis to confirm the amplification of DNA and Sanger sequencing. The variants found whose MAF (Minor Allele Frequency) ≤ 1% were evaluated by *in silico* tools and ACMG international guidelines to classify their pathogenicity and establish a genotype-phenotype correlation.

2.2 | DNA Extractions

Genomic DNA was extracted from blood samples collected in EDTA, with the DNA extraction kit QIAsymphony® DSP DNA using QIAsymphony SP (QIAGEN) extractor.

2.3 | Primer Design and PCR amplification

The PCR primers were designed through Primer3Plus to amplify the *VTN* and exon 18 of *PLG* gene (Suppl. Table 2). The freeze-dried primers were hydrated (Suppl. Table 3) and then were made the kit primers (Suppl. Table 4) necessary for the PCR reaction. For each gene, PCR reactions (Suppl. Table 5) were made and for all PCR reactions were used 1,5μL of DNA. The thermocycler used in the PCR reactions was the BioRad C1000™ Thermal Cycler.

2.4 | Agarose Gel Electrophoresis

The amplification of DNA by PCR was confirmed in a 2% agarose gel. This gel was prepared as follows: 2g of agarose mixed in 100mL 0.5% TBE (Tris 1.78M, EDTA 0.04M and Boric Acid 1.77M). Next, the solution was heated until the agarose was completely dissolved in the buffer, and after were added 8μL of SYBR™ Safe. 4μL of PCR samples was applied into the gel mixed with 1μL of loading dye. Lastly, the gel was run at 120V for 30 minutes and DNA fragments were visualized with UV light and photographed.

2.5 | Sanger sequencing

The Sanger sequencing was performed with the following protocol: purification of PCR samples with EXOSAP®; sequencing reactions of the purified PCR products were performed using the BigDye® Terminator v1.1 Cycler Sequencing kit (Applied Biosystems, Carlsbad, CA, USA); the non-incorporated BigDye® was eliminated by applying the sequencing sample to a resin column. Finally, the collected product was loaded into a sequencing plate and analyzed on the sequencer Applied Biosystems™ SeqStudio™ Genetic Analyzer, as described in the Supplementary Materials.

2.6 | Library preparation and NGS

The sequencing libraries were prepared utilizing the AmpliSeq Library Kit 2.0 and subsequently pooled barcoded libraries were clonally amplified by Ion OneTouch 2™. In both processes the manufacturer's protocol was duly followed (Thermo Fisher Scientific). The enriched template-positive particles were loaded onto an Ion 316™ or 318™ chip. Then were sequenced using Ion Hi-Q™ Sequencing 200 chemistry on an Ion Torrent PGM sequencing system (Thermo Fisher Scientific). Described in other recent works [7,46] and described in detail in the Supplementary Materials.

2.7 | Bioinformatics analyses

The Sanger sequencing was analyzed using the SeqScape® v2.5 software (Applied Biosystems) to align the sequences to the human genome version 19 (hg19) and thus identify the variants present in the sequences under study. The NGS was analyzed using Ion Torrent Suite™ (v3.6; Thermo Fisher Scientific) and using T-MAP (version 3.6.58977) to align the sequences to the human genome version 19 (hg19). Variants were called using the Torrent Variant Caller, with Germ Line – Low Stringency settings (version 3.6.59049). These were annotated using Ion Reporter (Thermo Fisher Scientific). The minimum depth of coverage required for each nucleotide in the ROI to be identified/considered a candidate mutation was ≥10x. The mean coverage was highly variable; however the mean for all the ROIs was at least 500-fold in all patients. This workflow allows sequences to be aligned against hg19 and together with *in silico* tools allow the identification of pathogenic variants and also the filtering of the polymorphisms described and known in the databases.

2.8 | Screening Putative Pathogenic Mutation

To assess the pathogenicity of the variants, the following criteria were used: 1) whether the variation had been previously identified in population databases (gnomAD and EVS), 2) MAF ≤ 1%, 3) *in silico* evaluation, 4) splice site prediction and 5) whether the variation had been previously identified in international databases (HGMD) (Figure1).

Variants were classified as pathogenic, likely pathogenic, uncertain significance, likely benign or benign according to the ACMG standards and guidelines standards and guidelines for the interpretation of sequence variants [47]. Both the numbering and the nomenclature of amino acids used are in concordance with the international recommendations for the description of sequence variants of the Human Genome Variation Society (http://www.HGVS.org).

2.9 | Genetic Databases

Variants that are not found in international databases (population and disease-specific) or in the literature have been defined as "new variants", as recommended by the guidelines for the interpretation of sequence variants [47]. The frequency of variants reported was verified using the Exome Variant Server (EVS), Genome Aggregation Database (gnomAD), Human Gene Mutation Database (HGMD) dbSNP and 1000 Genomes databases (Supplementary Materials and Methods References) (last accessed August 17, 2020).

2.10 | *In silico* Analyses

The impact of missense changes was analyzed using tools that predict the impact of an amino acid substitution on the structure and its function of the proteins [47]. The analysis criteria were as follows: evolutionary conservation of an amino acid or nucleotide, location the protein sequence and the biochemical consequence of the amino acid substitutions. This analysis was performed using five *in silico* tools: PROVEAN, SIFT, PolyPhen-2, Mutation Assessor, Mutation Taster (Supplementary Materials and Methods References) (last accessed August 14, 2020). Missense variants were considered pathogenic when 3/5 of *in silico* tools suggested a pathogenic effect. Changes in splicing sites were predicted using the Human Splicing Finder Pro version from GENOMNIS [48]. This program was used for synonymous and intronic variants. For splicing variants interpretation, a splicing site change was considered as potentially deleterious when threshold ≥ 65.

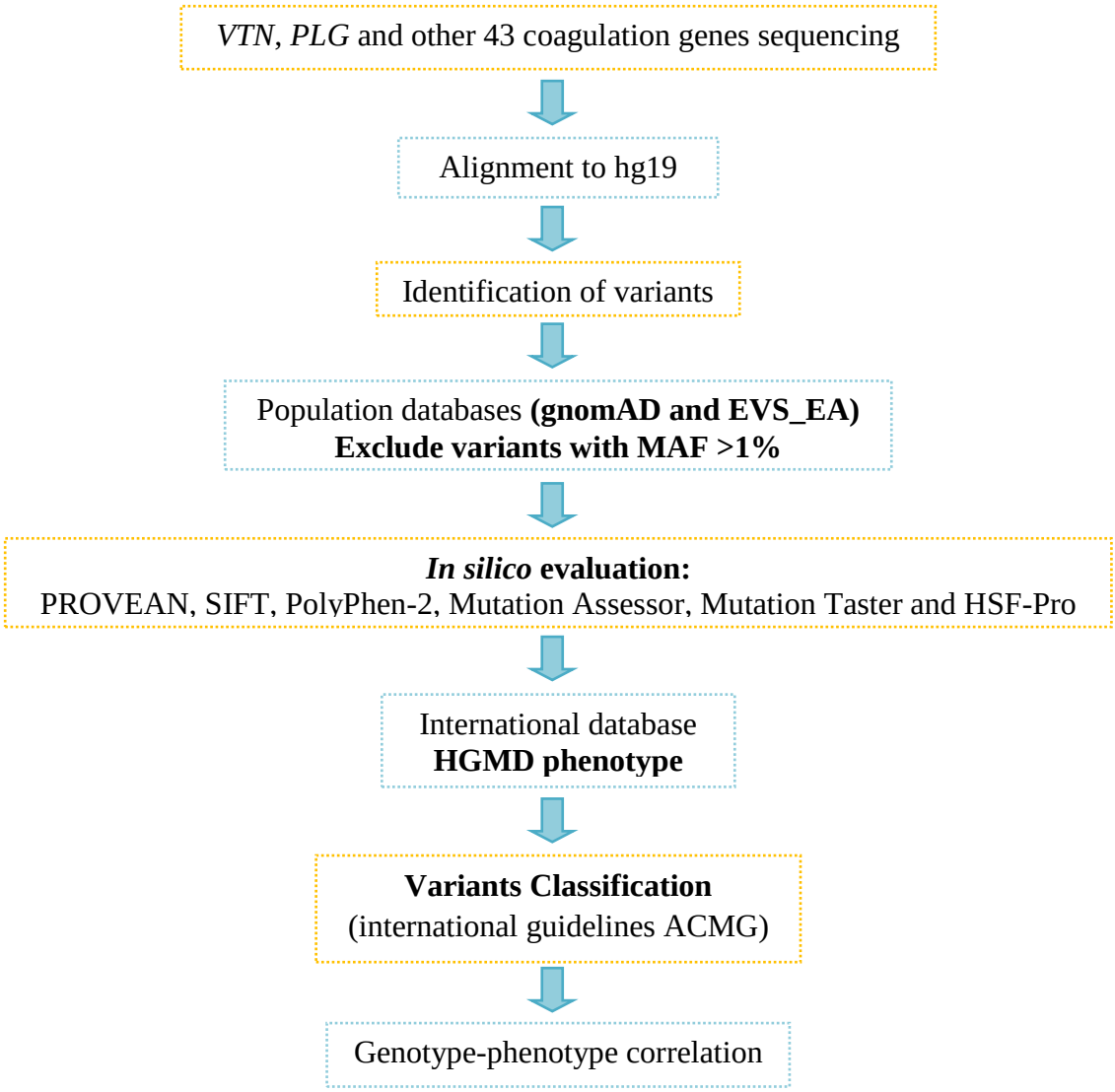

Figure 1 – Workflow to evaluate the pathogenicity of the variants and consequently to classifique them using the ACMG international guidelines and finally perform genotype-phenotype correlation in the patients under study.

3 | Results

3.1 | VTN and PLG genes

3.1.1 |Summary of Genetic Variation

The analysis of the *VTN* and *PLG* genes showed a total of 35 variants: four *VTN* variants and 31 *PLG* variants reported in Suppl. Table 6. Of the 35 variants identified, 18 have an MAF ≤ 1% (Table 1 and Figure 2): two *VTN* variants - one missense (50%) and one synonymous (50%) in two different patients and both were found in the heterozygous state; sixteen *PLG* variants - six missense (37,5%), three synonymous (18,75%) and seven intronic (43,75%) in 18 patients (Table 1 and Figure 2). All variants, were found in the heterozygous state with the exception of the intronic variant c.-35G>A that was identified in the homozygous state.

3.1.2 | Prediction of pathogenic variants

The *in silico* predictions (Suppl. Table 7) combined with other evidence: population (gnomAD and EVS_EA), international database (HGMD), dbSNP, 1000 Genomes and splice sites analysis (Suppl. Table 8) allowed pathogenicity prediction of the variants (Table 1). In total of 18 rare variants, 14 variants were only reported in the population databases and were not reported in the international database, three variants are reported in the population and international databases (Table 1) and only one (*PLG*, c.788-116G>A) had never been notified in the population European (Non-Finnish) and international disease databases, however it is described in the dbSNP database. Missense variants were analyzed by five pathogenicity prediction programs and only three had a score ≥ 3 and were considered deleterious (Suppl. Table 7). Synonymous and intronic variants (n=11) were analyzed using *in silico* tool with likely impact on the splice (Human Splicing Finder Pro); however, only two (*VTN*: c.891C>T, p.Ser297=; *PLG*: c.788-116G>A) were considered potential variants of splice sites (Suppl. Table 8). These variants was foreseen by *in silico* analysis to alter the natural function of the splice site; however it becomes necessary to perform analyses to determine and characterize the function of this variant, since these classification criteria have not replaced the functional studies of mRNA, the only method that can elucidate the true effect of the detected variation [49]. Thus, according

to the international guidelines [47], were identified four variants likely pathogenic (22%), two of uncertain significance (11%) and 12 likely benign (67%) (Table 1).

3.1.3 | Genotype-Phenotype correlation

In the total of the variants identified in *VTN* and *PLG*, three variants are reported in the international database and are associated with a phenotype (*PLG*: c.112A>G, p.Lys38Glu; c.1481C>T, p.Ala494Val; c.1878-6T>C) (Table 1 and Figure 2). The variants c.112A>G, p.Lys38Glu and c.1878-6T>C are associated with plasminogen deficiency and the variant c.1481C>T, p.Ala494Val is associated with aHUS (Table1). The latter was identified in three different patients while the others variants were only identified in one patient.

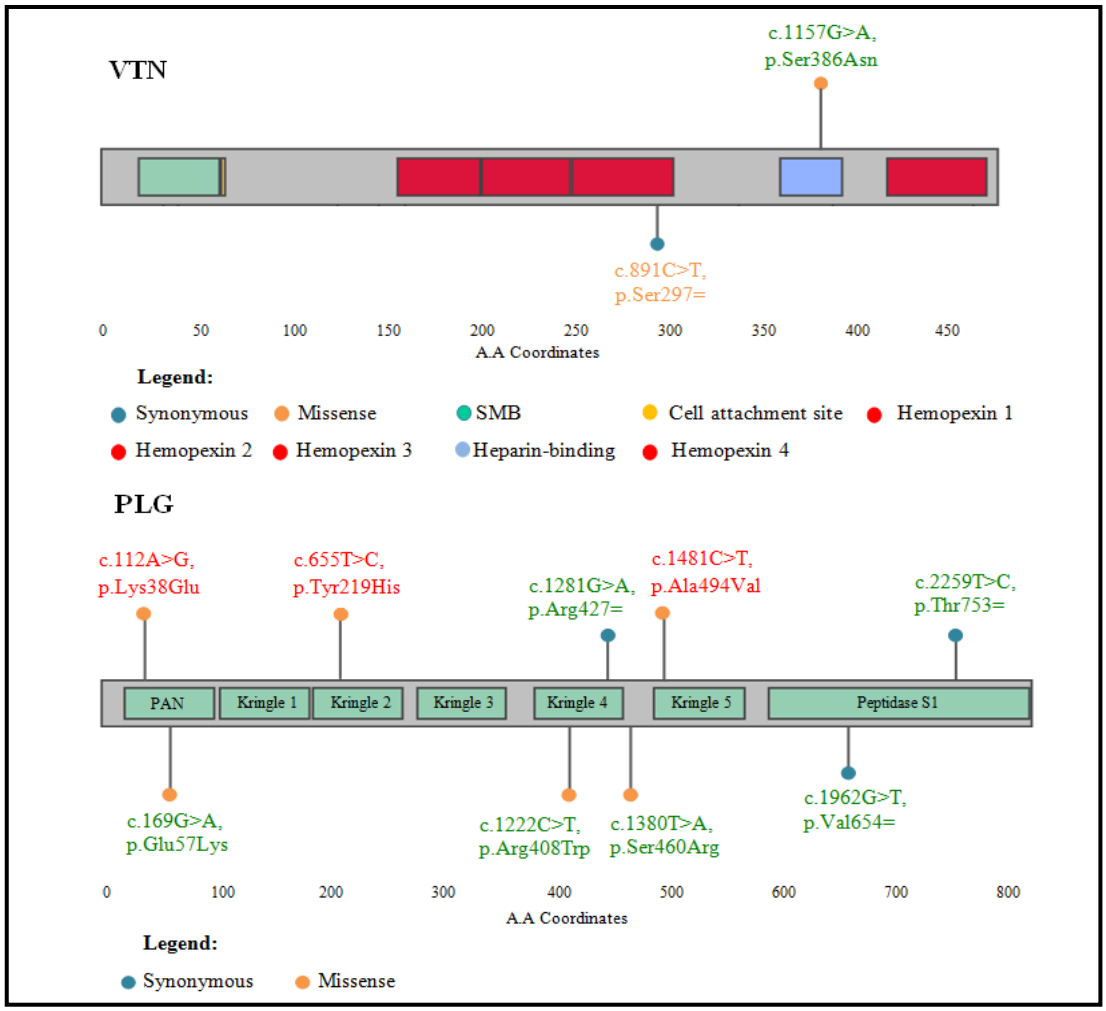

Figure 2 – Location of variants in the protein domains structures of *VTN* and *PLG*. The scheme represents variants whose MAF≤1%. The *VTN* has two variants in two different domains: Hemopexin 3 (p.Ser297=) and Heparin-binding (p.Ser386Asn). The *PLG* has eight variants in five different domains: PAN (p.Lys38Glu, p.Glu57Lys), Kringle 2 (p.Tyr219His), Kringle 4(p.Arg408Trp, p.Arg427=), Krigle 5 (p.Ala494Val) and Peptidade S1 (p.Val654=, p.Thr753=) and one variant that is not present in any of the domains (p. Ser460Arg). The likely benign variants are shown in dark green, uncertain significance in orange and likely pathogenic in red.

Table 1 – Variants found in the *VTN* and *PLG* genes whose MAF ≤1%. Distribution by the type of variant with assessment of the pathogenicity using different types of data: population, *in silico*, splicing sites and phenotype.

Gene	Nucleotide Change	Amino Acid Change	Exon	Zigosity	MAF (gnomAD)[a]	MAF (EVS_EA)[b]	rsID	*In silico* score[c]	HSF [d]	HGMD phenotype	Variants classification	Patients	Ref.
VTN	**c.1157G>A**	**p.Ser386Asn**	7	Htz	0.000146	0.000349	rs139553576	0	NA	ND	Likely Benign	P40	pdb
	c.891C>T	**p.Ser297=**	6	Htz	ND	0.000116	rs34134929	NA	New ASS	ND	Uncertain Significance	P20	pdb
PLG	**c.112A>G**	**p.Lys38Glu**	2	Htz	0.00513	0.006163	rs73015965	4	NA	DM, Plasminogen Deficiency	Likely Pathogenic	P34	50,51
	c.169G>A	**p.Glu57Lys**	2	Htz	0.000194	0.000465	rs4252070	0	NA	ND	Likely Benign	P48	pdb
	c.655T>C	**p.Tyr219His**	6	Htz	0.000185	0.000233	rs151092364	5	NA	ND	Likely Pathogenic	P31	pdb
	c.1222C>T	**p.Arg408Trp**	10	Htz	0.000167	0.000233	rs4252119	1	NA	ND	Likely Benign	P48	pdb
	c.1380T>A	**p.Ser460Arg**	11	Htz	0.00237	0.001744	rs116573785	1	NA	ND	Likely Benign	P39	pdb
	c.1481C>T	**p.Ala494Val**	12	Htz	0.00419	0.00593	rs4252128	3	NA	DM?, aHUS	Likely Pathogenic	P24, P29, P43	38
	c.1281G>A	**p.Arg427=**	11	Htz	0.00449	0.004767	rs149909079	NA	No difference	ND	Likely Benign	P8	pdb
	c.1962G>T	**p.Val654=**	16	Htz	0.00175	0.001395	rs4252196	NA	No difference	ND	Likely Benign	P39	pdb
	c.2259T>C	**p.Thr753=**	18	Htz	0.000324	0.00093	rs4252175	NA	No difference	ND	Likely Benign	P20	pdb
	c.-35G>A		5´UTR	Hmz	0.00359	0.005233	rs4252060	NA	No difference	ND	Likely Benign	P24	pdb
	c.788-116G>A		Intron 7	Htz	ND	ND	rs367678108	NA	New ASS	ND	Uncertain Significance	P48	pdb
	c.788-48G>A		Intron 7	Htz	0.000368	0.000349	rs4252111	NA	No difference	ND	Likely Benign	P32	pdb
	c.788-39_788-38insAT		Intron 7	Htz	0.00238	0.00206	rs4252189	NA	No difference	ND	Likely Benign	P39	pdb
	c.1681+55A>C		Intron13	Htz	ND	0.00	rs116448506	NA	No difference	ND	Likely Benign	P26	pdb
	c.1878-6T>C		Intron15	Htz	0.00287	0.003372	rs192519670	NA	No difference	DM?, Plasminogen Deficiency	Likely Pathogenic	P25	44
	c.2126-55C>G		Intron17	Htz	0.000324	ND	rs4252174	NA	No difference	ND	Likely Benign	P20	pdb

Htz, Heterozygous. Hmz, Homozygous. [a,b]Minor Allele Frequency (MAF) values are from the Genome Aggregation Database (gnomAD) and the European-American (EA) population from de Exome Variant Server (EVS). [c]*In silico* scores for missense variants was calculated using PROVEAN, SIFT, PolyPhen-2, Mutation Assessor and Mutation Taster (Suppl. Table 7). *In silico* score ≥3 were consideres deleterious. [d]Human Splicing Finder (Suppl. Table 8). NA, not available. ND, no data. ASS, acceptor splice site. DM, denotes a mutation reported to be disease-causing. DM?, denotes a mutation reported to be disease-causing, but with questionable pathogenecity. HGMD, Human Gene Mutation Database. pdb, variants found only in the one population database (1000 Genomes, dbSNP database, gnomAD or EVS).

3.2 Other Coagulation genes

3.2.1 | Summary of Genetic Variation

Among 50 patients studied 22 showed variants with MAF ≤ 1% in the other coagulation genes (*ADAMTS13, ANKRD26, F5, F7, F8, F10, F13A1, FGA, FGB, FGG, GP6, ITGA2B, ITGB3, NBEAL2, PLAT, PROC, PROS1, SERPINC1, SERPINE1, SERPINF2, TUBB1* and *VWF*) (Table 2 and Suppl. Table 9). In total were identified 35 variants in heterozygous state: 32 missense (91%), two intronic (6%) and one nonsense (3%) (Table 2). In the genes associated with clotting factor abnormalities were identified 14 variants (40%) as well in the genes associated with platelet abnormalities (40%), while in the genes associated with thrombosis were identified seven variants (20%).

3.2.2 | Prediction of pathogenic variants

The *in silico* predictions (Suppl. Table 7) identified 23 missense variants as deleterious (score ≥ 3). Also, three variants (two intronic and one nonsense) were analyzed using *in silico* tool with likely impact on the splice (Human Splicing Finder Pro); however, only one was considered a potential splice site variant (*ANKRD26*: c.2791delA, p.Ile931Term) (Suppl. Table 8). This evidences along with data from population database (gnomAD and EVS_EA), international database (HGMD), dbSNP and 1000 Genomes allowed pathogenicity prediction of the variants (Table 2). Twelve variants were previously reported in population and HGMD databases, 21 were only reported in the population database and the remaining were not reported. According to international guidelines [47], the variants were classified as pathogenic (n=8; 23%), likely pathogenic (n=7; 20%), uncertain significance (n=12; 34%) and likely benign (n=8; 23%) (Table 2).

3.2.3 | Genotype-Phenotype correlation

Of the 35 identified variants, the 12 previously reported in international database HGMD were associated with several protein deficiencies and reported as “disease mutation”. In the genes associated with clotting factor abnormalities were identified the following variants i) two *F7* missense variants (c.1091G>A, p.Arg364Gln; c.1109G>T,

p.Cys370Phe) in two different patients associated with FVII deficiency; ii) one *FGG* missense variant (c.1001A>T, p.Asn334Ile) in one patient associated with dysfibrinogenaemia; iii) two *VWF* missense variants (c.4196G>A, p.Arg1399His; c.6187C>T, p.Pro2063Ser) are associated with von Willebrand disease type 2M and von Willebrand disease type 1, respectively (Table 2).

In the genes associated with platelet abnormalities were identified the following variants: i) *ANKRD26* missense variant (c.542C>T, p.Thr181Ile) in two patients; ii) *ITGAB3* missense variant (c.197T>C, p.Leu66Arg) associated with coronary thrombosis in one patient; iii) at last, one *TUBB1*missense variant (c.436G>A, p.Gly146Arg) associated with macrothrombocytopenia in one patient (Table 2).

In the genes associated with thrombosis were identified the following variants: i) *ADAMTS13* missense variant (c.1874G>A, p.Arg625His) in one patient associated with TTP; ii) *PROC* missense variant (c.1180C>T, p.Arg394Trp) in one patient associated with protein C deficiency; iii) at last, in *PROS1* two missense variants (c.119G>T, p.Arg40Leu; c.269G>A, p.Arg90His) in two patients associated with protein S deficiency (Table 2).

3.3 | Potential functional impact of new mutations

Of the 53 variants identified in *VTN*, *PLG* and other coagulation genes whose MAF ≤ 1%, only two variants in heterozygous state were identified as new in two different patients. These two variants (1 nonsense and 1 missense) were identified in *ANKRD26* (c.2791delA, p.Ile931Ter) and in *ITGA2B* (c.2665C>T, p.Arg889Cys). Both are not described in the population databases (gnomAD and EVS_EA), international (HGMD), dbSNP and 1000 Genomes (Table 2). Thus, these two not described variants were classified by the ACMG guidelines as uncertain significance (Table 2).

Table 2 – Variants found in other coagulation genes whose MAF ≤ 1%. Distribution by the type of variant with assessment of the pathogenicity using different types of data: population, *in silico*, splicing sites and phenotype.

Gene	Nucleotide Change	Amino Acid Change	Exon	Zigosity	MAF (gnomAD)[a]	MAF (EVS_EA)[b]	rsID	*In silico* score[c]	HSF [d]	HGMD phenotype	Variants classification	Patients	Ref.
ADAMTS13	**c.539+7G>A**		Intron 4	Htz	0.0000617	ND	rs184864675	NA	No difference	ND	Likely Benign	P18	pdb
	c.1874G>A	**p.Arg625His**	16	Htz	0.000159	0.000581	rs36090624	3	NA	DM, TTP	Likely Pathogenic	P20	[52]
	c.3826G>A	**p.Gly1276Arg**	27	Htz	0.0000292	0.000465	rs144808448	0	NA	ND	Likely Benign	P12	pdb
ANKRD26	**c.542C>T**	**p.Thr181Ile**	4	Htz	0.00149	0.001473	rs191015656	5	NA	FP, Variation in ANKRD26 and Platelet Aggregation	Pathogenic	P27, P37	[53]
	c.2584C>G	**p.Gln862Glu**	23	Htz	0.0000707	0.000245	rs74128547	4	NA	ND	Likely Pathogenic	P26	pdb
	c.2791delA	**p.Ile931Ter**	24	Htz	ND	ND	ND	NA	New ASS	ND	Uncertain Significance	P10	New
F5	**c.43G>A**	**p.Gly15Ser**	1	Htz	0.000211	0.000233	rs9332485	5	NA	ND	Likely Pathogenic	P3	pdb
	c.5460G>A	**p.Met1820Ile**	17	Htz	0.000194	0.000581	rs6026	5	NA	ND	Likely Pathogenic	P20	pdb
F7	**c.1091G>A**	**p.Arg364Gln**	9	Htz	0.0000893	0.00	rs121964926	3	NA	DM, Factor VII Deficiency	Pathogenic	P26	[54]
	c.1109G>T	**p.Cys370Phe**	9	Htz	0.00000893	0.00	rs139372641	5	NA	DM, Factor VII Deficiency	Pathogenic	P2	[55]
F8	**c.4925A>G**	**p.Glu1642Gly**	14	Htz	0.0000122	ND	rs782055986	2	NA	ND	Likely Benign	P31	pdb
F10	**c.1270G>A**	**p.Val424Ile**	8	Htz	0.0000354	0.00	rs201301913	2	NA	ND	Likely Pathogenic	P41	pdb
F13A1	**c.2089C>T**	**p.Arg697Trp**	25	Htz	0.0000442	ND	rs776438603	3	NA	ND	Uncertain Significance	P23	pdb
FGA	**c.2089G>A**	**p.Gly697Ser**	6	Htz	0.0000176	ND	rs771023837	5	NA	ND	Uncertain Significance	P23	pdb
FGB	**c.298C>T**	**p.Pro100Ser**	2	Htz	0.00224	0.003023	rs2227434	1	NA	ND	Likely Benign	P36	pdb
FGG	**c.1001A>T**	**p.Asn334Ile**	8	Htz	ND	0.00	rs121913090	5	NA	DM, Dysfibrinogenaemia	Pathogenic	P20	[56]
GP6	**c.1789A>C**	**p.Thr597Pro**	8	Htz	0.0000396	0.00	rs115459014	2	NA	ND	Likely Benign	P14	pdb
ITGA2B	**c.2511G>C**	**p.Gln837His**	25	Htz	0.0000888	0.000116	rs377753373	4	NA	ND	Uncertain Significance	P22	pdb
	c.2665C>T	**p.Arg889Cys**	26	Htz	ND	ND	ND	5	NA	ND	Uncertain Significance	P36	New
ITGB3	**c.187T>G**	**p.Leu66Arg**	3	Htz	0.00215	0.002442	rs36080296	3	NA	DP, Coronary thrombosis association with	Likely Pathogenic	P41	[57]

NBEAL2	**c.1948G>A**	**p.Gly650Arg**	14	Htz	0.0000465	0.001203	rs201373710	5	NA	ND	Uncertain Significance	P34	pdb
	c.2356G>A	**p.Ala786Thr**	16	Htz	0.000156	0.00024	rs116104760	5	NA	ND	Uncertain Significance	P3	pdb
	c.4238T>C	**p.Leu1413Pro**	27	Htz	ND	ND	rs757804953	4	NA	ND	Uncertain Significance	P18	pdb
PLAT	**c.1481G>C**	**p.Gly494 Ala**	13	Htz	0.0000924	0.000698	rs61755432	4	NA	ND	Uncertain Significance	P7	pdb
PROC	**c.1180C>T**	**p.Arg394Trp**	9	Htz	0.0000266	ND	rs759316085	3	NA	DM, Protein C deficiency	Pathogenic	P6	[58]
PROS1	**c.119G>T**	**p.Arg40Leu**	2	Htz	0.0000264	0.00	rs7614835	5	NA	DM, Protein S deficiency	Pathogenic	P20	[59]
	c.269G>A	**p.Arg90His**	4	Htz	0.0000267	ND	rs200886866	1	NA	DM, Protein S deficiency	Uncertain Significance	P48	[60]
SERPINC1	**c.858G>C**	**p.Gln286His**	5	Htz	ND	0.00	rs139463995	5	NA	ND	Likely Pathogenic	P26	pdb
SERPINE1	**c.695A>G**	**p.Asn232Ser**	4	Htz	ND	0.00	rs147003064	4	NA	ND	Uncertain Significance	P20	pdb
SERPINF2	**c.412G>A**	**p.Ala138Thr**	6	Htz	0.0000367	ND	rs186432737	0	NA	ND	Likely Benign	P22	pdb
TUBB1	**c.436G>A**	**p.Gly146Arg**	4	Htz	0.0000352	0.00	rs371852125	5	NA	DM, Macrothrombocytopenia	Pathogenic	P39	[61]
VWF	**c.1330G>A**	**p.Val444Ile**	12	Htz	0.0000444	0.00	rs149116506	1	NA	ND	Likely Benign	P3	pdb
	c.4196G>A	**p.Arg1399His**	28	Htz	0.0122	0.014073	rs1800382	2	NA	DM, Von Willebrand disease type 2M	Pathogenic	P6	[62]
	c.6187C>T	**p.Pro2063Ser**	36	Htz	0.00825	0.010698	rs61750615	5	NA	DM?, Von Willebrand disease type1?	Uncertain Significance	P11	[63]
	c.7887+12T>C		Intron 47	Htz	0.0117	0.013605	rs55687637	NA	No difference	ND	Likely Benign	P3, P24	pdb

Htz, Heterozygous. Hmz, Homozygous. [a,b]Minor Allele Frequency (MAF) values are from the Genome Aggregation Database (gnomAD) and the European-American (EA) population from de Exome Variant Server (EVS). [c]*In silico* scores for missense variants was calculated using PROVEAN, SIFT, PolyPhen-2, Mutation Assessor and Mutation Taster (Suppl. Table 7). *In silico* score ≥3 were consideres deleterious. [d]Human Splicing Finder (Suppl. Table 8). NA, not available. ND, no data. ASS, acceptor splice site. FP, Functional Polymorphism. DM, denotes a mutation reported to be disease-causing. DP, Disease-associated polymorphism. DM?, denotes a mutation reported to be disease-causing, but with questionable pathogenecity. HGMD, Human Gene Mutation Database. pdb, variants found only in the one population database (1000 Genomes, dbSNP database, gnomAD or EVS). New variants are those variants that were not reported in the population databases (1000 Genomes, dbSNP database, ExAC and EVS).

4 | Discussion

In 50 Caucasian patients of Portuguese origin, without identified variants in the complement genes, we genotyped 45 coagulation genes, since the genetic causes related to the complement genes are only present in about 50%-60% of aHUS patients. We used the NGS methodology. The NGS methodology is ideal for diagnostic sequencing since it allows us to analyze a large number of samples simultaneously and perform the sequencing of whole genes, analyzing not only the exons but also non-coding regions, such as the promoter region, the introns and the UTR region [64]. This methodology is faster, more sensitive and high performance [64]. At the end, we identified 27 patients with rare variants (MAF ≤ 1%) in the genes under study (Suppl. Table 9).

Since Bu *et al.*, described *VTN* and *PLG* as novel aHUS associated genes, we analyse these genes in our patients. However, we did not identified significant rare coding variants in *VTN* as Bu *et al*. identified in their study associated in patients with aHUS. We identified 1 putative pathogenic *PLG* variant p.Ala494Val in 3 patients (P24, P29, P43) already reported and associated with aHUS [38] (Suppl. Table 9). In this case, the low activity of plasminogen can compromise the degradation of thrombi because there is a decrease in the dissolution of blood clots and deregulation of the coagulation pathway [37,38,44]. In accordance with Bu *et al*., although *PLG* makes a small contribution to aHUS, pathogenic variants should be reported with caution in the absence of functional data. Also, we identified the intronic c.1878-6T>C and the missense p.Lys38Glu variation in two different individuals in associated with plasminogen deficiency. In particular, variant c.1878-6T>C has been described [44] and was not foreseen by *in silico* analysis to alter the natural function of the splice site. Thus, it is possible to conclude that it is necessary to perform analyses to determine and characterize the function of this variant. In addition, variant p.Lys38Glu has been identified in heterozygous state and it is known that the level of functionality of plasminogen in heterozygous is affected in about 50% [44,50,51]. These observations are in accordance with those of Martin-Fernandez *et al.*, so we cannot confirm or exclude plasminogen deficiency as a risk factor for susceptibility to thrombosis.

In a second analysis, and like other authors, we analyze the contribution of other coagulation genes in our patients group. We identify rare variants in 22 genes (Suppl. Table 9). Regarding the *F5* gene, we can find a possible pathogenic pattern in the

identified variants [65,66]. Nevertheless, in accordance with Delev *et al.,* it is necessary to evaluate the possible impact on the structure and function of F5, since missense variants can influence the protein function. In *F7,* the variants p.Cys370Phe and p.Arg364Gln are associated with low levels of F7 and the individuals most affected are those with variants in homozygous state or double heterozygous state [54,55]. However, its clinical presentation is highly variable and the clinical phenotype correlates poorly with the laboratory phenotype, as only trace amounts of FVIIa are needed to initiate coagulation *in vivo*. In *F10*, the variant p.Val424Ile is not described but the variant p.Val424Phe is described in compound heterozygotes [67]. However, according to Nagaya *et al.,* amino acid substitutions induce protein degradation, leading to intracellular degradation of many FX proteins, and ultimately lead to the development of FX deficiency. Thus, functional studies are recommended. Rare variants in *F13A1* are associated with mild FXIII deficiency and individuals with these variants are often asymptomatic, unless they are exposed to some form of physical trauma [68]. According to Thomas *et al.*, variants even in heterozygous form would have a strong impact on the functional state of the protein, however further studies are needed. Rare variants in *VWF* were identified associated to von Willebrand disease but wit mild symptoms. In *FGG*, variant p.Asn334Ile is associated with dysfibrinogenemia. This variant leads to a functional fibrinogen anomaly, causing a significant delay in fibrinogen polymerization in fibrin [56], thus affecting clotting and increasing the risk of bleeding. Thus, variants in the various coagulation factors can lead to a deregulation of the coagulation and consequently of the complement, since these are interlinked, increasing the risk of bleeding when exposed to physical trauma.

By screening *ANKRD26* gene, we identified the variant p.Thr181Ile reported within the fifth Ankyrin repetition domain and located in a site of protein-protein interactions [53]. According to Chen, *et al*., this variant was associated with platelet aggregation and in this same study was observed a higher aggregation in patients with variants in the heterozygous state. It is important to note that our patients have this variant in the heterozygous state (P27, P37), thus increasing the risk of thrombosis. Also, in *ITGB3,* the variant p.Leu66Arg is associated with the development of arterial thrombosis [57]. However, further studies are needed to determine the functional role of this variant in increasing platelet aggregation as well as its pathological significance. In *NBEAL2*, according to Pluthero *et al.*, variants in heterozygous state are frequently associated with thrombocytopenia [69]. Likewise, in

accordance with Kashiwagi *et al.,* variants in *ITGA2B* in heterozygous state are associated with macrothrombocytopenia[70]. Also, heterozygous variants in *TUBB1* are a known source of macrothrombocytopenia leading to the formation of large, round platelets. We identified a variant in *TUBB1* (p.Gly146Arg); glycine at position 146 of β1-tubulin is highly conserved. This isoform is an important constituent of the platelet cytoskeleton and plays an essential role in the formation of proplates and plateletal biogenesis [61]. Thus, the study by Guillet, *et al*. is in accordance with our data, since the identified alteration leads to the phenotype of thrombocytopenia due to abnormal proplateletal formation. Therefore, variants in these genes contribute to the development of aHUS, since this pathology is characterized by the occurrence of platelet abnormalities, such as platelet aggregation and thrombocytopenia, increasing the risk of bleeding.

In ADAMTS13 was identified the variant p.Arg625His in one patient associated with TTP [52,71]. TTP and HUS present similar clinical manifestations, which makes their distinction difficult [2]. In *PROC*, the variant p.Arg394Pro occurs in the serine-protease domain and associated with protein C type II deficiency [58]. In accordance in Fidalgo *et al.,* the protein C deficiency is characterized by symptoms of deep venous thrombosis due to synthesis and/or reduced levels activity of protein C. Heterozygous individuals, like our patient, have lighter symptoms and are asymptomatic until adulthood. More than one rare coding variant (p.Arg40Leu and p.Arg90His) was identified in *PROS1* associated with protein S deficiency. This is characterized by symptoms of recurrent venous thrombosis due to synthesis and/or reduced levels activity of protein S [59,60]. Also, in *SERPINC1*, variant p.Gln286His was identified and it is known that variants in this gene are associated with antithrombin deficiency and may be related to a high incidence of venous thromboembolism [72]. Variants in these genes are associated with thrombosis. Thrombosis is a complex multifactorial disease where various genetic risk factors, including environmental situations, may increase the susceptibility to develop a thromboembolic event. aHUS is a disease associated with thrombosis because the complement system contributes significantly to thrombosis as it improves blood clotting properties and increases the inflammatory response.

From this perspective, our study provides evidence of the usefulness of the NGS panel as an excellent technology for identifying variants in several genes that could be a

genetic background in these patients, and to have a cumulative effect on both systems - coagulation and complement.

5 | Conclusion

The present study ended with 23 genetically unresolved patients, in whom we did not find any variant whose MAF ≤ 1%. Predicting the pathogenicity of genetic variation has become crucial for a better understanding of the variants found. Therefore, the NGS panel is crucial because it allows a more assertive diagnosis, as it has the potential to identify and characterize the molecular basis of a wide variety of changes.

Finally, this study did not imply *VTN* and *PLG* in particular as important contributors to aHUS. However, we found variants in several genes that could be a genetic background in these patients, and to have a cumulative effect on both systems - coagulation and complement.

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Supplementary Materials and Methods

Suppl. Table 1 – Sex, age and clinical data of 50 patients with previously studied aHUS without identified variants in the complement genes.

Patient ID	Sex	Age onset (yrs)	Clinical data
P1	F	25	Thrombotic microangipathy, suspected aHUS
P2	M	28	Renal biopsy with evidence of TMA. Suspected aHUS
P3	F	58	Vasculitis, hemolytic anemia, TMA?
P4	F	51	Acute Renal Injury
P5	F	50	Kidney transplantation, aHUS? Daughter with aHUS and chronic kidney disease
P6	M	11	aHUS? Probable C3 glomerolopathy
P7	F	22	Microangiopathic anemia, 1 week postpartum
P8	M	76	Hemolytic anemia, suspected aHUS
P9	M	37	aHUS
P10	M	38	aHUS
P11	F	35	Chronic kidney disease, Recent rapid progression, anemia, thrombocytopenia, TMA
P12	F	28	Nephrotic syndrome, renal biopsy, C3 glomeropathy, aHUS
P13	F	26	Renal graft dysfunction, aHUS
P14	F	38	TMA, aHUS?
P15	F	19	TMA
P16	F	42	Acute renal injury, TMA
P17	F	34	Postpartum acute renal injury, TMA
P18	M	9	aHUS
P19	F	27	aHUS
P20	M	30	aHUS
P21	M	48	TMA, acute renal injury, aHUS?
P22	M	38	TMA
P23	F	1	TMA, aHUS
P24	F	27	TMA
P25	F	51	HUS associated with pregnancy in 1999, Chronic kidney disease, kidney transplant in 2004, aHUS?
P26	M	24	TMA, confirmed by renal biopsy
P27	F	56	Kidney transplant candidate, suspected aHUS
P28	F	55	aHUS
P29	F	40	First kidney transplant in 2002, secondary aHUS?
P30	F	35	TMA

P31	F	18	aHUS
P32	F	38	TMA
P33	M	2	aHUS
P34	F	41	aHUS
P35	F	53	aHUS
P36	F	29	Chronic kidney disease by aHUS
P37	M	38	TMA, Chronic kidney disease
P38	F	40	Chronic kidney disease, aHUS
P39	F	37	TMA, aHUS
P40	F	41	aHUS
P41	M	21	Chronic kidney failure, suspected aHUS
P42	F	25	aHUS
P43	F	56	aHUS
P44	F	55	aHUS
P45	F	31	aHUS
P46	M	48	aHUS
P47	F	12	aHUS
P48	F	30	aHUS
P49	M	16	aHUS
P50	F	19	aHUS

Suppl. Table 2– Sequence of primers used for *VTN* gene and exon 18 of *PLG* gene.

Gene	Exon	Primer	Sense
VTN	Exon 1-4	GTTCCTCTTCTCCAGTGCCC	Forward*/**
		CTGTCCCTGGGAGCAATAGC	Reverse*/**
	Exon 3seq	GCTGCTGGAGACTCACTACC	Forward**
	Exon 5-6	CCCCAAGACTCCAGGTCCT	Forward*/**
		GGTAGAGCTGAGCTTTCCCC	Reverse*/**
	Exon 7-8	GCTGTGTGTCTTTGGGCAAG	Forward*/**
		TCGGGGCTAAGGGACCTTTA	Reverse*/**
PLG	Exon 18	ACTCTGCAGGGTCAGAGACT	Forward*/**
		CACTTAGACTGGGGTCTTCG	Reverse*/**

*used in PCR; **used in Sanger sequencing

Suppl. Table 3 - Hydration of *VTN* gene primers and exon 18 of *PLG* gene.

Gene	Primer	Qt (ng)	Volume to hydrate	Stock conc. (ng/ul)	Work conc. (ng/ul)	Dilution factor	Total volume (ul)	Primer (ul)	dH20 (ul)
VTN	Exon 1-4F	84000	1000	84	50	1,7	100	60	40
	Exon 1-4R	86000	1000	86	50	1,7	100	58	42
	Exon 3seqF	84000	1000	84	50	1,7	100	60	40
	Exon 5-6F	79000	1000	79	50	1,6	100	63	37
	Exon 5-6R	83000	1000	83	50	1,7	100	60	40
	Exon 7-8F	85000	1000	85	50	1,7	100	59	41
	Exon 7-8R	84000	1000	84	50	1,7	100	60	40
PLG	Exon 18F	77000	1000	77	50	1,5	100	65	35
	Exon 18R	84000	1000	84	50	1,7	100	60	30

Suppl. Table 4 - Kit primers for *VTN* gene and exon 18 of *PLG* gene.

Gene	Exon	Primer Forward	Primer Reverse	$MgCl_2$	dH_2O	Concentration of $MgCl_2$	Temperature of annealing
VTN	Exon 1-4	25 µL	25 µL	15 µL	5 µL	1.5 mM	60ºC
	Exon 5-6	25 µL	25 µL	10 µL	10 µL	1.0 mM	59ºC
	Exon 7-8	25 µL	25 µL	10 µL	10 µL	1.0 mM	58ºC
PLG	Exon 18	25 µL	25 µL	10 µL	10 µL	1.5 mM	60ºC

Suppl. Table 5 - PCR reaction of *VTN* gene and exon 18 of *PLG* gene for a final volume of 25 µL.

Reagents	Quantity
Taq Buffer 10x	2,5 µL
DNTP´s 2,5 mM	2 µL
DNA	1,5 µL
Kit primers	3,5µL
Taq Platinium	0,2 µL
dH2O	15,3 µL

Sanger sequencing

The purification of PCR samples it was performed with EXOSAP®. It was added 0.75µL of EXOSAP® and then 3µL of the sample. The purification it was performed in a thermocycler in to steps: 15 minutes at 37°C and 10 minutes to 80°C. The sequencing reaction of the purified PCR products was performed using the BigDye® Terminator v1.1 Cycler Sequencing kit (Applied Biosystems, Carlsbad, CA, USA). This kit contains the following: a buffer, DNA polymerase, dNTP's and ddNTP's marked with 4 different fluorocromes. Thus, 0.75µL was added from BigDye and later 0.5µL from the primer forward/reverse. Subsequently, the sequencing reaction was performed in the thermal cycler with certain conditions: 94°C/3minutes/1 cycle; 96°C/10segundes/24 cycles; 50ºC/5segundes/24cycles; 60ºC/1minute45segundes/24cycles; 22°C/forever. Next, the BigDye® was eliminated by applying the sequencing sample to a resin column. This was centrifuged 6 minutes at 3290 rpm. The collected product was loaded into a sequencing plate and 5 µL formamide was added. Finally, the product was analyzed on the sequencer Applied Biosystems ™ SeqStudio ™ Genetic Analyzer. Data from the Sequence Analysis Software was aligned against the native *VTN* sequence (GenBank no. NC_000017.11) and the native sequence of exon 18 of the *PLG* (GenBank no. NC_000006.12) using the SeqScape® v2.5 software (Applied Biosystems) for mismatch detection.

Library preparation and NGS

The general NGS workflow includes sample preparation, target genomic enrichment, and sequencing. For each of the samples studied, the DNA concentration was determined utilizing the Qubit dsDNA HS Assay Kit. Then, the preparation of the library was carried out utilizing the AmpliSeq ™ Library Kit 2.0. In both was followed the protocol of the manufacturer (Thermo Fisher Scientific, MA, USA). Later, the pooled barcoded libraries were clonally amplified using the Ion OneTouch 2 ™ (Thermo Fisher Scientific) system according to the manufacturer's instructions. Ion sphere particles were enriched using the E/S module, and, then, the enriched template-positive particles were loaded onto an Ion 316™ or 318™ chip and sequenced utilizing Ion Hi-Q™ Sequencing 200 Kit chemistry on an Ion Torrent™ PGM sequencing system (Thermo Fisher Scientific). Data from the

Sequence Analysis Software was aligned against the native sequence of the coagulation genes using the Ion Reporter ^{TM} software (Applied Biosystems) for mismatch detection.

NGS panels

Two different panels were used to study these 50 patients: thrombotic microangiopaties (used prior to this study) and panel thrombosis and hemostasis panel. The painel of thrombotic microangiopathies contains 11 genes: *ADMATS13, CFH, CFHR1, CFHR3, CFHR4, CFHR5, CFI, CFB, C3, THBD* and *DGKE*. The thrombosis and hemostasis panel it was used to analyze the PLG gene and other coagulation genes. This panel contains 44 genes involved in diseases associated with thrombosis and hemostasis and includes: 18 genes associated with clotting factor abnormalities (*F2, F3, F5, F7, F8, F9, F10, F11, F13A1, F13B, LMAN1, MCFD2, FGA, FGB, FGG, GGCX, VKORC1, VWF*), 15 genes associated with platelet abnormalities (*GP1BB, GP1BA, GP9, ITGA2B, ITGB3, NBEAL2, NBEA, GP6, TBXA2R, P2RY12, ANKRD26, CYCS, PLA2G4A, TUBB1*), 11 genes associated with thrombosis (*ADAMTS13, PROC, PROS1, SERPINC1, SERPINE1, SERPINF2, THBD, PLAT, PLG, ANXA5, PLAU, F12*). Both panels were designed and validated in the Banc de Sang i Teixits (BST) of Barcelona. The targeted exon enrichment GeneRead panel (QIAGEN) designed comprises a total of 933 amplicons covering the 93.3% of the target genomic regions. Construction of libraries, including patient specific indexation, was performed with NEBNext Ultra DNA Library Prep Kit. Between 24 and 48 libraries were sequenced together in every MiSeq (Illumina) run. Putative mutations were identified by GeneRead Variant Calling software.

Supplementary Materials and Methods References

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Supplementary Results

Suppl. Table 6 - Thirty-five variants were identified in 50 individuals with aHUS, 4 variants in the *VTN* gene and 31 variants in the *PLG* gene. Distribution by the type of variant with assessment of the pathogenicity using different types of data: population, *in silico*, splicing sites and phenotype.

Gene	Nucleotide Change	Amino Acid Change	Exon	Zigosity	MAF (gnomAD)[a]	MAF (EVS_EA)[b]	rsID	*In silico* score[c]	HSF score [d]	HGMD phenotype	Variants classification	Number patients	Ref.
Missense variants													
VTN	**c.1157G>A**	**p.Ser386Asn**	7	Htz	0.000146	0.000349	rs139553576	0	NA	ND	Likely Benign	1	pdb
VTN	c.1199C>T	p.THR400Met	7	Htz	0.475	0.480698	rs704	0	NA	DP, Hemangioblastoma in VHL	Benign	39	[1]
PLG	**c.112A>G**	**p.Lys38Glu**	2	Htz	0.00513	0.006163	rs73015965	4	NA	DM, Plasminogen Deficiency	Likely Pathogenic	1	[2]
PLG	**c.169G>A**	**p.Glu57Lys**	2	Htz	0.000194	0.000465	rs4252070	0	NA	ND	Likely Benign	1	pdb
PLG	**c.655T>C**	**p.Tyr219His**	6	Htz	0.000185	0.000233	rs151092364	5	NA	ND	Likely Pathogenic	1	pdb
PLG	**c.1222C>T**	**p.Arg408Trp**	10	Htz	0.000167	0.000233	rs4252119	1	NA	ND	Likely Benign	1	pdb
PLG	**c.1380T>A**	**p.Ser460Arg**	11	Htz	0.00237	0.001744	rs116573785	1	NA	ND	Likely Benign	1	pdb
PLG	c.1414G>A	p.Asp472Asn	11	Htz/Hmz	0.296	0.302791	rs4252125	0	NA	DFP, Invasive aspergillosis, susceptibility	Benign	30	[3]
PLG	**c.1481C>T**	**p.Ala494Val**	12	Htz	0.00419	0.00593	rs4252128	3	NA	DM?, aHUS	Likely Pathogenic	3	[4]
Synonymous variants													
VTN	**c.891C>T**	**p.Ser297=**	6	Htz	ND	0.000116	rs34134929	NA	New ASS 37.46-65.33	ND	Uncertain Significance	1	pdb
VTN	c.1344T>C	p.Asn448=	8	Htz	0.0807	0.081512	rs2227728	NA	No difference	ND	Benign	7	pdb
PLG	c.330C>T	p.Asn110=	4	Htz/Hmz	0.309	0.315612	rs4757	NA	No difference	ND	Benign	37	pdb
PLG	c.771T>C	p.Cys257=	7	Htz/Hmz	0.419	0.418023	rs14224	NA	No difference	ND	Benign	33	pdb
PLG	c.942C>T	p.Phe314=	8	Htz/Hmz	0.346	0.403023	rs1130656	NA	No difference	ND	Benign	30	pdb
PLG	c.1083A>G	p.Gln361=	9	Htz/Hmz	0.295	0.300814	rs13231	NA	New ASS 46.65-74.51 New DSS 40.15 - 67.29	ND	Likely Benign	30	pdb
PLG	**c.1281G>A**	**p.Arg427=**	11	Htz	0.00449	0.004767	rs149909079	NA	No difference	ND	Likely Benign	1	pdb
PLG	**c.1962G>T**	**p.Val654=**	16	Htz	0.00175	0.001395	rs4252196	NA	No difference	ND	Likely Benign	1	pdb

PLG	c.2082T>C	p.Ala694=	17	Htz	0.08093	0.08093	rs4252170	NA	No difference	ND	Benign	9	pdb
PLG	**c.2259T>C**	**p.Thr753=**	18	Htz	0.000324	0.00093	rs4252175	NA	No difference	ND	Likely Benign	1	pdb
PLG	c.2286T>G	p.Gly762=	19	Htz/Hmz	0.533	ND	rs11060	NA	No difference	ND	Benign	39	pdb
Intronic variants			**Intron**										
PLG	**c.-35G>A**		5´UTR	Hmz	0.00359	0.005233	rs4252060	NA	No difference	ND	Benign	1	pdb
PLG	c.669-14T>G		6	Htz/Hmz	0.295	0.30093	rs4252109	NA	No difference	ND	Benign	30	pdb
PLG	**c.788-116G>A**		7	Htz	ND	ND	rs367678108	NA	New ASS 46.07-73,94	ND	Uncertain Significance	1	pdb
PLG	**c.788-48G>A**		7	Htz	0.000368	0.000349	rs4252111	NA	No difference	ND	Likely Benign	1	pdb
PLG	**c.788-39_788-38insAT**		7	Htz	0.00238	0.00206	rs4252189	NA	No difference	ND	Likely Benign	1	pdb
PLG	c.950+14G>A		8	Htz/Hmz	0.404	0.4014	rs2295368	NA	New ASS 53.28-81.15	ND	Likely Benign	27	pdb
PLG	c.1097-64A>G		9	Htz/Hmz	0.283	ND	rs4252117	NA	New ASS 60,01-87.88	ND	Benign	29	pdb
PLG	c.1256+9T>C		10	Htz/Hmz	0.296	0.303023	rs4252120	NA	No difference	ND	Benign	31	pdb
PLG	c.1256+35A>G		10	Htz	0.0147	0.015	rs4252121	NA	No difference	ND	Benign	9	pdb
PLG	c.1438+30A>G		11	Htz/Hmz	0.295	0.302558	rs4252126	NA	No difference	ND	Benign	29	pdb
PLG	**c.1681+55A>C**		13	Htz	ND	0.00	rs116448506	NA	No difference	ND	Likely Benign	1	pdb
PLG	c.1878-17G>A		15	Htz/Hmz	0.672	0.321512	rs2859879	NA	No difference	ND	Benign	46	pdb
PLG	**c.1878-6T>C**		15	Htz	0.00287	0.003372	rs192519670	NA	No difference	DM?, Plasminogen Deficiency	Likely Pathogenic	1	[5]
PLG	c.2019-53A>G		16	Htz	0.0204	ND	rs1835346	NA	No difference	ND	Benign	2	pdb
PLG	**c.2126-55C>G**		17	Htz	0.000324	ND	rs4252174	NA	No difference	ND	Likely Benign	1	pdb

Htz, Heterozygous. Hmz, Homozygous. [a,b]Minor Allele Frequency (MAF) values are from the Genome Aggregation Database (gnomAD) and the European-American (EA) population from de Exome Variant Server (EVS). [c]*In silico* scores for missense variants was calculated using PROVEAN, SIFT, PolyPhen-2, Mutation Assessor and Mutation Taster (Suppl. Table 7). *In silico* score ≥3 were consideres deleterious. [d]Human Splicing Finder Score (Suppl. Table 8). NA, not available. ND, no data. ASS, acceptor splice site. DSS, donor splice site. DP, Disease-associated polymorphism. DFP, Disease-association polymorphism with supporting functional evidence. DM, denotes a mutation reported to be disease-causing. DM?, denotes a mutation reported to be disease-causing, but with questionable pathogenecity. pdb, variants found only in the one population database (1000 Genomes, dbSNP database, gnomAD or EVS). HGMD, Human Gene Mutation Database. VHL, Von Hippel-Lindau. aHUS, Haemolytic uraemic syndrome atypical. In **bold** are identified those variants whose MAF≤1%.

Suppl. Table 7- Summary of *in silico* analysis for missense variants using five pathogenicity prediction programs (PROVEAN, SIFT, Polyphen-2, Mutation Assessor and Mutation Taster) in order to determine the degree of tolerance for each amino acid substitution.

				PROVEAN[a]		SIFT[b]		PolyPhen-2[c]		Mutation Assessor[d]		Mutation Taster[e]		
Gene	**Nucleotide Change**	**Amino Acid Change**	**Exon**	**Score**	**Prediction**	**Score**	**Prediction**	**Score**	**Prediction**	**Score**	**Prediction**	**Score**	**Prediction**	***In silico* score**
VTN	c.1157G>A	p.Ser386Asn	7	-0.31	Neutral	0.46	Tolerated	0.003	Benign	1.39	Low	0.9999	Polymorphism	0
	c.1199C>T	p.Thr400Met	7	-0.47	Neutral	0.224	Tolerated	0.115	Benign	0.69	Neutral	0.9999	Polymorphism automatic	0
PLG	c.112A>G	p.Lys38Glu	2	-2.13	Neutral	0.003	Damaging	0.661	Possibly damaging	2.315	Medium	4.8857e-7	Disease causing automatic	4
	c.169G>A	p.Glu57Lys	2	0.21	Neutral	0.129	Tolerated	0.012	Benign	-0.14	Neutral	1	Polymorphism	0
	c.655T>C	p.Tyr219His	6	-2.87	Damaging	0.008	Damaging	0.929	Probably damaging	2.94	Medium	1	Disease causing	5
	c.1222C>T	p.Arg408Trp	10	-2.98	Damaging	0.088	Tolerated	0	Benign	1.415	Low	1	Polymorphism	1
	c.1380T>A	p.Ser460Arg	11	-1.78	Neutral	0.082	Tolerated	0.091	Benign	2.315	Medium	1	Polymorphism	1
	c.1414G>A	p.Asp472Asn	11	0.33	Neutral	0.402	Tolerated	0	Benign	-0.965	Neutral	1	Polymorphism	0
	c.1481C>T	p.Ala494Val	12	-2.38	Neutral	0.014	Damaging	0.928	Probably damaging	2.835	Medium	0.8719	Polymorphism	3
ADAMTS13	c.1874G>A	p.Arg625His	16	-2.51	Damaging	0.137	Tolerated	0.934	Probably damaging	2.38	Medium	0.9999	Polymorphism	3
	c.3826G>A	p.Gly1276Arg	27	-0.79	Neutral	0.997	Tolerated	0.001	Benign	0	Neutral	1	Polymorphism	0
ANKRD26	c.542C>T	p.Thr181Ile	4	-4.29	Damaging	0.001	Damaging	0.998	Probably damaging	3.17	Medium	0.9997	Disease causing	5
	c.2584C>G	p.Gln862Glu	23	-2.33	Neutral	0.005	Damaging	0.851	Possibly damaging	2.205	Medium	0.9981	Disease causing	4
F5	c.43G>A	p.Gly15Ser	1	-3.45	Damaging	0.018	Damaging	0.949	Probably damaging	2.485	Medium	1	Disease causing	5
	c.5460G>A	p.Met1820Ile	17	-3.54	Damaging	0.016	Damaging	0.993	Probably damaging	2.86	Medium	0.9999	Disease causing	5

F7	c.1091G>A	p.Arg364Gln	9	-2.12	Neutral	0.013	Damaging	0.993	Probably damaging	0.61	Neutral	0.9999	Disease causing	3
	c.1109G>T	p.Cys370Phe	9	-8.15	Damaging	0	Damaging	0.987	Probably damaging	3.42	Medium	1	Disease causing	5
F8	c.4925A>G	p.Glu1642Gly	14	-2.47	Neutral	0.001	Damaging	0.205	Benign	2.08	Medium	0.9999	Polymorphism	2
F10	c.1270G>A	p.Val424Ile	8	-0.83	Neutral	0.176	Tolerated	0.999	Probably damaging	1.88	Low	1	Disease causing	2
F13A1	c.2089C>T	p.Arg697Trp	25	-1.52	Neutral	0.009	Damaging	0.667	Possibly damaging	2.675	Medium	0.9729	Polymorphism	3
FGA	c.2089G>A	p.Gly697Ser	6	-3.29	Damaging	0	Damaging	1	Probably damaging	3.795	High	1	Disease causing	5
FGB	c.298C>T	p.Pro100Ser	2	-0.26	Neutral	0.256	Tolerated	0.003	Benign	2.125	Medium	1	Polymorphism	1
FGG	c.1001A>T	p.Asn334Ile	8	-6.74	Damaging	0.002	Damaging	0.853	Possibly damaging	2.9	Medium	0.9999	Disease causing	5
GP6	c.1789A>C	p.Thr597Pro	8	-0.30	Neutral	0	Damaging	0.702	Possibly damaging	-	-	1	Polymorphism	2
ITGA2B	c.2511G>C	p.Gln837His	25	-2.29	Neutral	0.045	Damaging	0.764	Possibly damaging	2.005	Medium	0.7331	Disease causing	4
	c.2665C>T	p.Arg889Cys	26	-3.78	Damaging	0.003	Damaging	0.964	Probably damaging	3.245	Medium	0.996	Disease causing	5
ITGB3	c.187T>G	p.Leu66Arg	3	-2.20	Neutral	0.088	Tolerated	0.948	Probably damaging	2.25	Medium	0.9999	Disease causing	3
NBEAL2	c.1948G>A	p.Gly650Arg	14	-5.23	Damaging	0.004	Damaging	0.999	Probably damaging	2.625	Medium	1	Disease causing	5
	c.2356G>A	p.Ala786Thr	16	-2.71	Damaging	0.01	Damaging	0.998	Probably damaging	2.69	Medium	1	Disease causing	5
	c.4238T>C	p.Leu1413Pro	27	-2.74	Damaging	0.174	Tolerated	0.937	Probably damaging	2.31	Medium	1	Disease causing	4
PLAT	c.1481G>C	p.Gly494 Ala	13	-4.03	Damaging	0.141	Tolerated	0.933	Probably damaging	2.645	Medium	1	Disease causing	4
PROC	c.1180C>T	p.Arg394Trp	9	-3.24	Damaging	0.01	Damaging	0.939	Probably damaging	1.765	Low	1	Polymorphism	3
PROS1	c.119G>T	p.Arg40Leu	2	-5.33	Damaging	0.001	Damaging	0.997	Probably damaging	2.595	Medium	0.9999	Disease causing	5
	c.269G>A	p.Arg90His	4	-0.50	Neutral	0.169	Tolerated	0.288	Benign	0	Neutral	0.9984	Disease causing	1

SERPINC1	c.858G>C	p.Gln286His	5	-4.16	Damaging	0.001	Damaging	0.994	Probably damaging	2.895	Medium	1	Disease causing	5
SERPINE1	c.695A>G	p.Asn232Ser	4	-2.58	Damaging	0.08	Tolerated	0.945	Probably damaging	2.15	Medium	0.9989	Disease causing	4
SERPINF2	c.412G>A	p.Ala138Thr	6	-0.97	Neutral	0.281	Tolerated	0.231	Benign	0.415	Neutral	1	Polymorphism	0
TUBB1	c.436G>A	p.Gly146Arg	4	-5.79	Damaging	0	Damaging	1	Probably damaging	4.93	High	1	Disease causing	5
VWF	c.1330G>A	p.Val444Ile	12	-0.83	Neutral	0.176	Tolerated	0.339	Benign	1.61	Low	0.6559	Disease causing	1
	c.4196G>A	p.Arg1399His	28	-1.16	Neutral	0.031	Damaging	0.44	Benign	1.475	Low	0.9979	Disease causing	2
	c.6187C>T	p.Pro2063Ser	36	-3.48	Damaging	0.027	Damaging	0.999	Probably damaging	2.71	Medium	1	Disease causing	5

[a]Provean predicts whether an amino acid replacement or indel has an impact on the biological function of the protein. If the score is -2.5 or higher, the protein variant is expected to have a "damaging" effect. If the score is below -2.5, the variant is expected to have a "neutral" effect. [b]SIFT is used to predict the effect of sequence changes in protein function based on homological research and the physical properties of amino acids. Scores range from 0 to 1 and amino acid substitution is predicted to be "damaging" if the score is <=0.05 and "tolerated" if the score is >0.05. [c]PolyPhen-2 predicts possible impact of an amino acid substitution on the structure and function of protein using straightforward physical and comparative considerations. Scores between 0.909 and 1 denote a “probably damaging” variant, a score between 0.447 and 0.908 indicates a “possibly damaging” variant, and a score below 0.446 is a “benign” variant. [d]Mutation Assessor predicts the functional impact of amino-acid substitutions in proteins. The prediction is one of “neutral”, “low”, “medium” and “high”, and variants with higher scores are more likely to be damaging. [e]Mutation Taster calculates the probability that the amino acid sequence change is a harmless polymorphism or disease mutation. It predicts a change that could be: “Disease causing”, “Disease causing automatic”, “Polymorphism” and “Polymorphism automatic” and a probability close to 1 indicates a high certainty in the prediction. *In silico* scores ≥3 were considered deleterious.

Suppl. Table 8 - Splice sites analysis using Human Splicing Finder Pro version from GENOMNIS and prediction impact in RNA processing.

Gene	Nucleotide Change	Human Splicing Finder	
		Type	**Interpretation**
VTN	**c.891C>T**	New Acceptor splice site (37.46-65.33) Alteration of auxiliary sequences	Activation of a cryptic Acceptor site. Potential alteration of splicing (cryptic exon activation) Significant alteration of ESE / ESS motifs ratio (5)
	c.1344T>C	No significant impact on splicing signals	No significant impact on splicing signals
PLG	c.330C>T	Alteration of auxiliary sequences	Significant alteration of ESE / ESS motifs ratio (-3)
	c.771T>C	Alteration of auxiliary sequences	Significant alteration of ESE / ESS motifs ratio (7)
	c.942C>T	Alteration of auxiliary sequences	Significant alteration of ESE / ESS motifs ratio (2)
	c.1083A>G	New Acceptor splice site (46.65-74.15) New Donor splice site (40.15-67.29)	Activation of a cryptic Acceptor site. Potential alteration of splicing (cryptic exon activation) Activation of a cryptic Donor site. Potential alteration of splicing
	c.1281G>A	Alteration of auxiliary sequences	Significant alteration of ESE / ESS motifs ratio (8)
	c.1962G>T	No significant impact on splicing signals	No significant impact on splicing signals
	c.2082T>C	Alteration of auxiliary sequences	Significant alteration of ESE / ESS motifs ratio (2)
	c.2259T>C	No significant impact on splicing signals	No significant impact on splicing signals
	c.2286T>G	No significant impact on splicing signals	No significant impact on splicing signals
	c.-35G>A	No significant impact on splicing signals	No significant impact on splicing signals
	c.669-14T>G	No significant impact on splicing signals	No significant impact on splicing signals
	c.788-116G>A	New Acceptor splice site (46.07-73.94)	Activation of a cryptic Acceptor site. Potential alteration of splicing (cryptic exon activation)
	c.788-48G>A	No significant impact on splicing signals	No significant impact on splicing signals
	c.788-39_788-38insAT	No significant impact on splicing signals	No significant impact on splicing signals

PLG	c.950+14G>A	New Acceptor splice site (53.28-81.15)	Activation of a cryptic Acceptor site. Potential alteration of splicing (cryptic exon activation)
	c.1097-64A>G	New Acceptor splice site (60.01-87.88)	Activation of a cryptic Acceptor site. Potential alteration of splicing (cryptic exon activation)
	c.1256+9T>C	No significant impact on splicing signals	No significant impact on splicing signals
	c.1256+35A>G	No significant impact on splicing signals	No significant impact on splicing signals
	c.1438+30A>G	No significant impact on splicing signals	No significant impact on splicing signals
	c.1681+55A>C	Alteration of auxiliary sequences	Significant alteration of ESE / ESS motifs ratio (5)
	c.1878-17G>A	Alteration of auxiliary sequences	Significant alteration of ESE / ESS motifs ratio (2)
	c.1878-6T>C	Alteration of auxiliary sequences	Significant alteration of ESE / ESS motifs ratio (3)
	c.2019-53A>G	No significant impact on splicing signals	No significant impact on splicing signals
	c.2126-55C>G	Alteration of auxiliary sequences	Significant alteration of ESE / ESS motifs ratio (2)
ADAMTS13	**c.539+7G>A**	No significant impact on splicing signals	No significant impact on splicing signals
ANKRD26	**c.2791delA**	New Acceptor splice site (11.36-71.31) Alteration of auxiliary sequences	Activation of a cryptic Acceptor site. Potential alteration of splicing Significant alteration of ESE / ESS motifs ratio (-5)
VWF	**c.7887+12T>C**	No significant impact on splicing signals	No significant impact on splicing signals

ESE – Exonic Splicing Enhancer. ESS – Exonic Splicing Silence. Human Splicing Finder score (0-100) and threshold ≥ 65. In **bold** are identified those variants whose MAF≤1%.

Suppl. Table 9 - Genotype characteristics of 27 patients with rare variants in *VTN*, *PLG* and other coagulation genes. Variants detected by Sanger and NGS sequencing are marked by a cross.

Patients	Gene	Variants	Sanger	NGS
P2	***F7***	c.1109G>T (p.Cys370Phe)		X
P3	***F5***	c.43G>A (p.Gly15Ser)		X
	NBEAL2	c.2356G>A (p.Ala786Thr)		X
	VWF	c.1330G>A (p.Val444Ile)		X
	VWF	c.7887+12T>C		X
P6	***PROC***	c.1180C>T (p.Arg394Trp)		X
	VWF	c.4196G>A (p.Arg1399His)		X
P7	***PLAT***	c.1481G>C (p.Gly494Ala)		X
P8	***PLG***	c.1281G>A (p.Arg427=)		X
P10	***ANKRD26***	c.2791delA (p.Ile931Ter)		X
P11	***VWF***	c.6187C>T (p.Pro2063Ser)		X
P12	***ADAMTS13***	c.3826G>A (p.Gly1276Arg)		X
P14	***GP6***	c.1789A>C (p.Thr597Pro)		X
P18	***ADAMTS13***	c.539+7G>A		X
	NBEAL2	c.4238T>C (p.Leu1413Pro)		X
P20	***VTN***	c.891C>T (p.Ser297=)	X	
	PLG	c.2259T>C (p.Thr753=)	X	
	PLG	c.2126-55C>G		X
	ADAMTS13	c.1874G>A (p.Arg625His)		X
	F5	c.5460G>A (p.Met1820Ile)		X
	FGG	c.1001A>T (p.Asn334Ile)		X
	PROS1	c.119G>T (p.Arg40Leu)		X
	SERPINE1	c.695A>G (p.Asn232Ser)		X
P22	***PLG***	c.788-48G>A		X
	ITGA2B	c.2511G>C (p.Gln837His)		X
	SERPINF2	c.412G>A (p.Ala138Thr)		X
P23	***F13A1***	c.2089C>T (p.Arg697Trp)		X
	FGA	c.2089G>A (p.Gly697Ser)		X
P24	***PLG***	c.1481C>T (p.Ala494Val)		X
	PLG	c.-35G>A		X
	VWF	c.7887+12T>C		X
P25	***PLG***	c.1878-6T>C		X
P26	***PLG***	c.1681+55A>C		X
	ANKRD26	c.2584C>G (p.Gln862Glu)		X
	F7	c.1091G>A (p.Arg364Gln)		X

	SERPINC1	c.858G>C (p.Gln286His)		X
P27	***ANKRD26***	c.542C>T (p.Thr181Ile)		X
P29	***PLG***	c.1481C>T (p.Ala494Val)		X
P31	***PLG***	c.655T>C (p.Tyr219His)		X
	F8	c.4925A>G (p.Glu1642Gly)		X
P34	***PLG***	c.112A>G (p.Lys38Glu)		X
	NBEAL2	c.1948G>A (p.Gly650Arg)		X
P36	***FGB***	c.298C>T (p.Pro100Ser)		X
	ITGA2B	c.2665C>T (p.Arg889Cys)		X
P37	***PLG***	c. 1962G>T (p.Val654=)		X
	ANKRD26	c.542C>T (p.Thr181Ile)		X
P39	***PLG***	c.1380T>A (p.Ser460Arg)		X
	PLG	c.788-39_788-38insAT		X
	TUBB1	c.436G>A (p.Gly146Arg)		X
P40	***VTN***	c.1157G>A (p.Ser386Asn)	X	
P41	***F10***	c.1270G>A (p.Val424Ile)		X
	ITGB3	c.197T>G (p.Leu66Arg)		X
P43	***PLG***	c.1481C>T (p.Ala494Val)		X
P48	***PLG***	c.169G>A (p.Glu57Lys)		X
	PLG	c.1222C>T (p.Arg408Trp)		X
	PLG	c.788-116G>A		X
	PROS1	c.269G>A (p.Arg90His)		X

Supplementary Results References

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