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Combined study of ADAMTS13 and complement genes in the diagnosis of thrombotic microangiopathies using next-generation sequencing

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Abstract

Background: The 2 main forms of thrombotic microangiopathy (TMA) are thrombotic thrombocytopenic purpura (TTP) and atypical hemolytic uremic syndrome (aHUS). Deficiency of ADAMTS13 and dysregulation of the complement pathway result in TTP and aHUS, respectively; however, overlap of their clinical characteristics makes differential diagnosis challenging.

Objectives and Methods: We aimed to develop a TMA diagnosis workflow based on ADAMTS13 activity and screening of *ADAMTS13* and complement genes using a custom next-generation sequencing (NGS) gene panel.

Patients: For this, from a cohort of 154 Portuguese patients with acute TMA, the genotype-phenotype correlations were analyzed in 7 hereditary TTP (ADAMTS13 activity <10%, no inhibitor), 36 acquired TTP (ADAMTS13 activity <10%, presence of an inhibitor), and in 34 presumable aHUS.

Results: In total, 37 different rare variants, 8 of which novel (in *ADAMTS13*, *CFH*, and *CD46*), were identified across 7 genes. Thirteen TTP patients were homozygous (n=6), compound heterozygous (n=2), and heterozygous (n=5) for 11 *ADAMTS13* variants (6 pathogenic mutations). Among the 34 aHUS patients, 17 were heterozygous for 23 variants in the different complement genes with distinct consequences, ranging from single pathogenic mutations associated with complete disease penetrance to benign variants that cause aHUS only when combined with other variants and/or *CFH* and *CD46* risk haplotypes or *CFHR1-3* deletion.

Conclusions: Our study provides evidence of the usefulness of the NGS panel as an excellent technology that enables more rapid diagnosis of TMA, and is a valuable asset in clinical practice to discriminate between TTP and aHUS.

KEYWORDS

genotype, hemolytic-uremic syndrome, molecular diagnostic techniques, phenotype, sequence analysis, thrombotic microangiopathies

Essentials

- The differential diagnosis of acute thrombotic microangiopathy (TMA) is challenging.
- To the ADAMTS13 activity < or >10% was added a next-generation sequencing (NGS) gene panel.
- The *ADAMTS13* mutation p.Cys754Arg was frequent in hereditary thrombotic thrombocytopenic purpura.
- We identified *novel* complement gene mutations and this procedure improved our diagnostic strategy.

1 | INTRODUCTION

Thrombotic microangiopathies (TMAs) are characterized by microvascular occlusion, thrombocytopenia, and non-immune hemolytic anemia. The 2 main forms of TMAs are thrombotic thrombocytopenic purpura (TTP) and atypical hemolytic uremic syndrome (aHUS). These 2 types of TMA have overlapping clinical phenotypes. Conceptually, in TTP, neurological manifestations are more common, whereas, in aHUS, renal involvement is more pronounced. However, this rule is not always reliable; some aHUS patients have neurological complications and some TTP patients have renal failure.[1–4] Over the last decade, noteworthy progress has been made to improve the clinical and laboratory approaches to predict the expected signs and symptoms, patient outcome, and genotype-phenotype correlations. Despite this, the differential diagnosis of TTP/aHUS remains challenging.

TTP is caused by a severe plasma deficiency (i.e, <10% of the normal) of the cleaving protease of von Willebrand factor (VWF)-ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13). A severe ADAMTS13 deficiency is more often due to anti-ADAMTS13 autoantibodies (acquired) or rarely due to homozygous or compound heterozygous ADAMTS13 gene mutations (hereditary, also called Upshaw-Schulman syndrome).[5,6] However, heterozygosity for *ADAMTS13* mutations was observed among patients diagnosed with acute acquired TTP and severe ADAMTS13 deficiency.[7,8] In all forms, the predominant pathogenic factor of TTP is the increased number of ultra-large multimers of VWF resulting from the lack of proteolytic regulation by ADAMTS13 and leading to platelet clumping in the microcirculation of various organs.[6]

TTP is mainly an autoimmune disorder associated with severe, acquired ADAMTS13 deficiency with the annual incidence rate of 2.17×10^6 people per year, although hereditary TTP accounts for <5% of all ADAMTS13 deficient TTP cases,[9] except in certain geographic locations, e.g, Central Norway, where hereditary TTP seems to be more common than the acquired form.[10]

While TTP is characterized by the severe deficiency of ADAMTS13, aHUS is characterized by hyperactivation of the alternative complement pathway resulting from either a loss-of-function mutation in a regulatory gene (*CFH*, *CFI*, *CD46* (*MCP*), or *THBD*) or a gain-of-function mutation in an effector gene (*CFB* or *C3*).[1,11] The mutations were mainly found in the heterozygous state, and approximately 5% of patients have combined mutations, usually in *CFH* with either *CD46* or *CFI*. Homozygosity for risk haplotypes of *CFH* (rs3753394, c.1-332C>T and rs1065489, c.2808G>T, p. Glu936Asp) that tag the disease risk haplotype *CFH-H3* and 1 polymorphism in *MCP* (rs7144, c.*897T>C) that tags the *MCPggaac* risk haplotype have been shown to significantly increase disease penetrance and severity.[12–14] Additional genetic risk factors include a deficiency of *CFH*-related proteins 1 (CFHR1) and 3 (CFHR3), due *CFHR1-3* deletion in homozygous state caused by non-allelic homologous recombination of *CFHR3* and *CFHR1*.[15,16] Finally, recessive mutations in *DGKE*, which encodes diacylglycerol kinase-ε and is expressed in endothelial cells, platelets, and podocytes, were identified in children with the onset of aHUS in the first year of life.[17] However, detectable complement abnormalities have been described in only approximately 50%-60% of patients; therefore, the aHUS diagnosis is based on clinical criteria and the exclusion of a severe ADAMTS13 deficiency.[18]

Poor penetrance is a common clinical feature, and adult onset occurs in patients with severe deficiencies. In addition, both deficiencies predispose patients to a TMA after a triggering event, such as pregnancy, bacterial and viral infections, neoplasia, autoimmune disorders, and exposure to certain drugs.[19–21]

Severe ADAMTS13 deficiency (<10%) is an important indicator in the differential diagnosis of TTP/aHUS. However, several studies in aHUS patients have shown reduced ADAMTS13 activity that may predispose patients to the TMA phenotype.[22,23] Indeed, *ADAMTS13* single-nucleotide polymorphisms (SNPs) associated with partial deficiency of ADAMTS13 in the presence of a primary trigger or when co-inherited with a mutation in a complement gene have been described in these patients.[23] These findings suggest that TTP and aHUS exhibit overlap not only in their clinical characteristics but also in their pathophysiological mechanisms.

To better understand this clinical variability, which is found even within families, it is necessary to characterize the mutational profile. Nevertheless, up to now the molecular analysis of genes implicated in TMA was not affordable by diagnostic laboratories because of the high cost involved in the study of multiple genes by conventional Sanger DNA sequencing. The advent of next-generation sequencing (NGS) is changing this paradigm because NGS allows simultaneously sequencing of large gene panels and generates competitive results at a lower cost and in a shorter amount of time.

From this perspective, we conducted a study with 2 main objectives. First, to better understand the genotype-phenotype correlations, we detailed phenotypic characterization in our cohort of patients with TMA who had mutations in *ADAMTS13* and/or in complement genes. Second, we designed and validated an NGS-based gene panel to facilitate genetic testing in TTP and aHUS. This approach allowed the implementation, within our Department of Hematology, of an efficient methodology to establish the diagnosis and prognosis of these 2 rare diseases that exhibit phenotypic similarities.

2 | MATERIALS AND METHODS

2.1 | Patients and controls

We reviewed 154 Caucasian patients of Portuguese origin with TMA investigated from January 2007 to January 2016 in the Department of Haematology at Centro Hospitalar Universitário de Coimbra. The selection diagnostic criteria based on international guidelines[24] were thrombocytopenia, anemia, and morphological evidence of red cell fragmentation. Sixty-three patients were diagnosed in our department and the remaining 91 patients were referred from external centers in different regions of Portugal. These hospitals diagnosed and treated patients with TMA and sent samples to our center to perform ADAMTS13 activity assay and/or genetic screening of ADAMTS13 and complement genes. Eight patients were excluded as their ADAMTS13 assay sample was sent after the administration of plasma or initiation of plasma exchange (PEX).

The 146 individuals in this study included both adults and children with a median age of 36 years (1 month through 89 years) and a sex distribution of 87 females and 59 males. The adult patients (n=110) had a median age of 44 years with a range of 20-89 years. The child probands (n=30) had a median age of 9 years with a range of 1-18 years, and infants included in this study (n=7) ranged in age from 1 week to 11 months.

Forty-two healthy volunteers acted as a control group for ADAMTS13 assay measurements and were also investigated for gene variants with the NGS panel. The control group included unrelated individuals (32 females and 10 males) without an individual or family history of excessive bleeding, thrombosis or hemolytic anemia, with a mean age of 33.5±11.12 years.

We also investigated 10 asymptomatic relatives of 5 patients in whom an *ADAMTS13* mutation had been identified. In accordance with the Declaration of Helsinki, informed consent was obtained from all patients or from their family members and from the healthy controls.

2.2 | Samples and sample processing

ADAMTS13 assays were performed on blood collected into vacuum tubes containing 3.2% sodium citrate (at a ratio of 9:1 vol/vol) and centrifuged within 15 minutes at room temperature for 20 minutes at 2500 *g*. The obtained platelet-poor plasma was then separated into aliquots and kept frozen at −80°C until further use.

ADAMTS13 activity was measured by chromogenic ELISA (TECHNOZYM ADAMTS13, Technoclone, Vienna, Austria). The detection limit of this assay is 0.2%. ADAMTS13 inhibitor was screened by the functional Bethesda assay using mixing studies of heat-inactivated patient plasma and normal plasma (1:1 dilution),[25] and the residual ADAMTS13 activity of the mixture is measured using TECHNOZYM ADAMTS13-activity ELISA. In addition, to increase diagnostic yield of testing panel, the presence of anti-ADAMTS13 autoantibodies of IgG class was performed using a chromogenic ELISA (TECHNOZYM ADAMTS13-INH).

Genomic DNA was extracted from EDTA whole blood by automatic isolation on an iPrep instrument using a gDNA Blood Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The DNA concentration was adjusted to a range of 25-50 ng/μL.

2.3 | Strategy for mutation analysis

Two different direct sequencing methodologies were used for the molecular analysis in this study:

Sanger direct sequencing. Until March 2015, Sanger sequencing was performed according to the following strategy: (i) all of the coding sequences of *ADAMTS13* (regions of interest [ROIs]) in patients with repeated measurements of ADAMTS13 activity <10% after resolution of the acute episode and no evidence of anti-ADAMTS13 IgG antibodies; (ii) all of the coding sequences of *CFH*, *CFI*, *CFB*, *C3*, *CD46*, *THBD* and *DGKE* (ROIs) in patients with suspected aHUS.

Next-Generation Sequencing (NGS) panel. In the beginning of last year, we developed a custom gene panel that includes *ADAMTS13*, *CFH*, *CFHR1*, *CFHR3*, *CFHR4*, *CFHR5*, *CFI*, *CFB*, *C3*, *THBD* and *DGKE* to facilitate genetic testing in TTP and aHUS patients. Using Ion AmpliSeq Designer (Thermo Fisher Scientific, Massachusetts, USA), we selected ROIs to be sequenced, including all exons, promoter regions, and the intronic flanking regions (at least 20 bp).

Before its implementation, the gene panel was validated using samples from 8 patients (TTP and aHUS) previously studied by Sanger sequencing and in which *ADAMTS13*, *CFH* and *CFI* variants have been identified.

In addition, the coding *VWF* were analyzed, as previously reported[26] in all TTP samples (DNA samples were sent to Unitat de Diagnòstic i Teràpia Molecular, Banc de Sang i Teixits [BST, Barcelona]). We analyzed VWF because TTP phenotype could theoretically be influenced by an intrinsic resistance of VWF to proteolysis by ADAMTS13 due to possible mutations in specific VWF cleavage site region in VWFA2 domain.

Regarding to comparison between laboratories on the basis of coverage and diagnostic yield of NGS, these samples were analyzed to *ADAMTS13* ROIs using other NGS panel, as described in the Supplementary Materials.

2.4 | Library preparation and NGS

The sequencing libraries were prepared using the AmpliSeq Library Kit 2.0, and pooled barcoded libraries were clonally amplified by Ion OneTouch2 both following the manufacturer's protocol (Thermo Fisher Scientific). The enriched template-positive particles were loaded onto an Ion 316 or 318 chip and sequenced using Ion Hi-Q Sequencing 200 Kit chemistry on an Ion Torrent PGM sequencing system (Thermo Fisher Scientific).

2.5 | Bioinformatic analysis

The NGS pipeline output, after QC/QA filtering, was analyzed using Torrent Suite (v3.6; Thermo Fisher Scientific), and sequences were

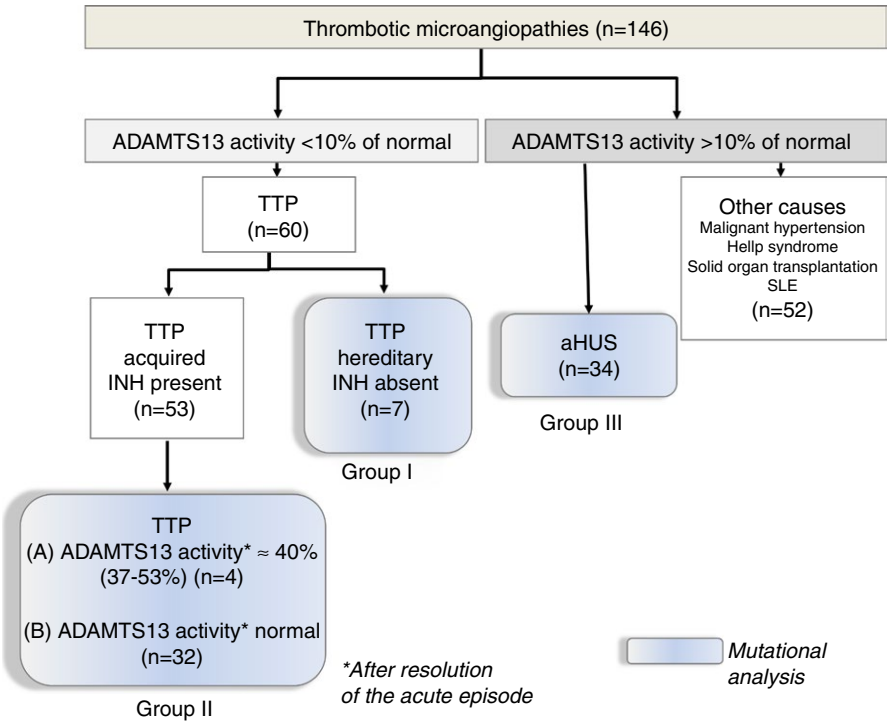

FIGURE 1 Flow chart of patients' enrolment based on ADAMST13 activity and clinical characteristics

aligned to the human genome version 19 (hg19) using T-MAP (version 3.6.58977). Variants were called using the Torrent Variant Caller (version 3.6.59049, with Germ Line - Low Stringency settings) and annotated using Ion Reporter (Thermo Fisher Scientific).

The workflow procedure of in silico analysis, the genetic databases consulted for validation of rare variants, the criteria for assessment of the pathogenic mutations, and, finally, their validation by Sanger sequencing were in accordance with those previously described in our recent work[26,27] and explained in detail in the Supplementary Materials.

2.6 | Multiplex ligation-dependent probe amplification (MLPA)

The *CFHR3-1* copy number variation (a tandem deletion), was screened using SALSA MLPA P236-A3 kit following the manufacturer's protocol (MRC-Holland, Amsterdam, The Netherlands).

3 | RESULTS

3.1 | Characteristics of the patients studied

The present study reviewed 146 patients from different regions of Portugal for whom ADAMTS13 activity levels were measured; 60 patients had a level of ≤10% (severe deficiency) with a residual activity (range: 0.6%-9%) and 86 patients had a level of >10% (Figure 1). The majority of patients, i.e, 53 out of 60 (88%) patients with severe deficiency, had a presence of ADAMTS13 inhibitors (acquired TTP) with a median titer of 9.02 UB (range: 2.06-64 UB) and anti-ADAMTS13 IgG antibodies level with a median titer of 62 UA/mL (range: 15-121 UA/mL); the remaining 7 patients had no evidence of inhibitors or anti-ADAMTS13 IgG antibodies (hereditary TTP). The group with an ADAMTS13 activity >10% included 34 (39.5%) patients with suspected aHUS and 52 (60.5%) patients with a range of underlying diseases (i.e, thrombocytopenia, malignant hypertension, HELLP syndrome, solid organ transplantation, and systemic lupus erythematosus) that were not included in the present study. However, it would be highly interesting to see whether complement mutations are in fact more common in aHUS cases than in the other TMAs, as previously described in patients with HELLP syndrome.[28] Considering this, the molecular study of this last patients group is currently in progress and the results will be presented in the near future.

Based on these findings, 3 groups of patients were selected for this analysis (n=77) and underwent a detailed phenotypic study and mutational analysis: (i) hereditary TTP patients (n=7); (ii) acquired TTP patients (n=36), 4 of which have had ADAMTS13 activity of ≈40% after an acute episode, but no detected anti-ADAMTS13 IgG antibodies; and (iii) aHUS patients (n=34) (Figure 1). The demographic, laboratory, and clinical data are summarized in Table S1.

The analysis of the samples of 42 local healthy controls by NGS panel only revealed common variants with a minor allele frequency (MAF) value of >1% in the 1000 Genomes.

3.2 | Identification of gene defects in TMA patients by NGS

The NGS method confirmed all variants previously detected by Sanger sequencing method in *ADAMTS13*, *CFH*, and *CFI* (100% sensitivity) that was used for NGS panel validation. The *VWF* analysis by NGS did not reveal any rare variant in TTP samples. Concerning to the variants found in *ADAMT13*, the results were completely concordant between those obtained in our laboratory and those obtained in the laboratory from the Banc de Sang i Teixits of Barcelona (Spain).

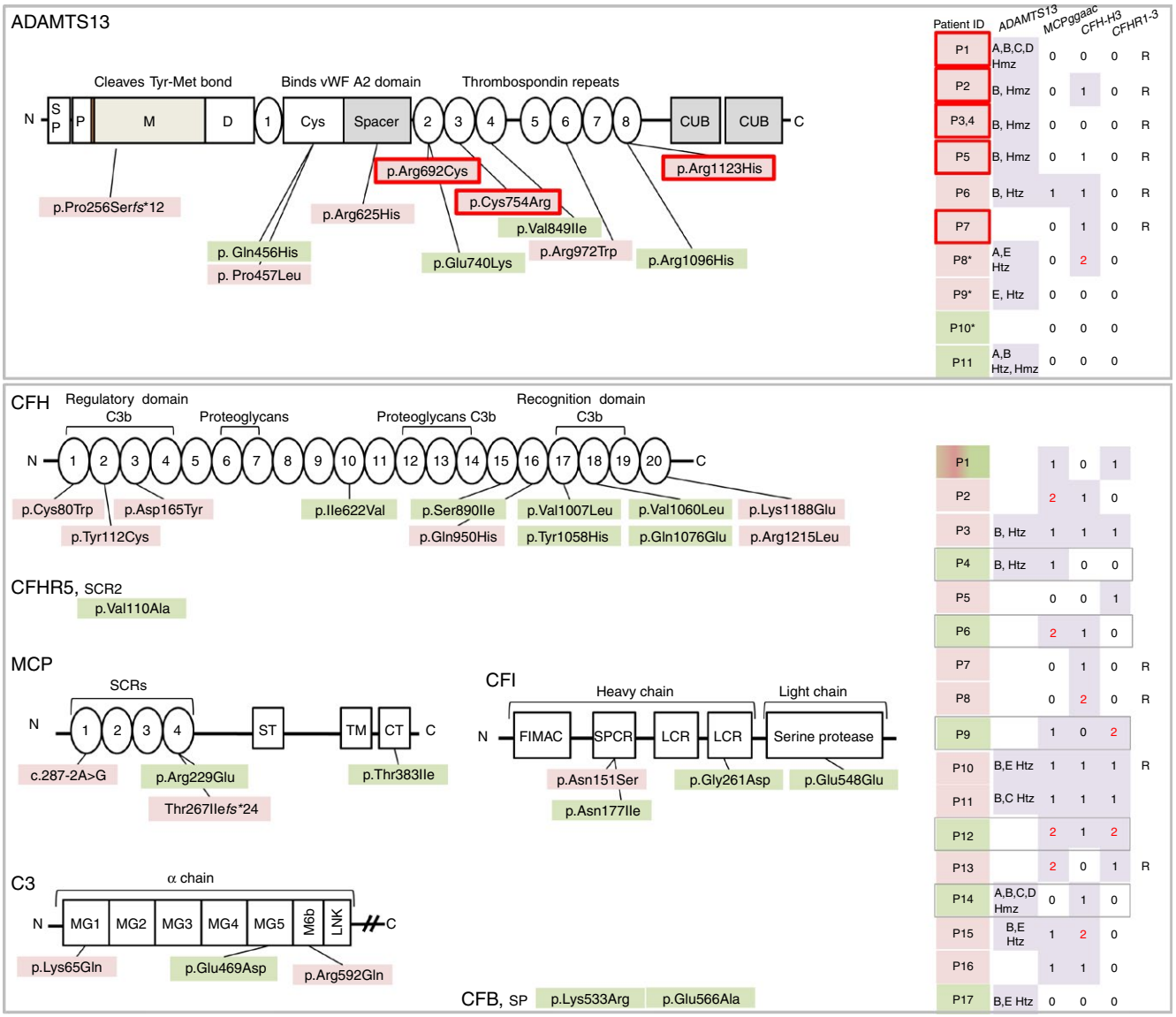

FIGURE 2 Location of variants in the protein domain structures of ADAMTS13, CFH, CFHR5, MCP, CFI, C3, and CFB. The scheme represents a rare gene variant(s) inherited for each patient as well as the co-inherited known genetic risk factors for TMA. The 4 *ADAMST13* SNPs, (A) (p.Arg7Trp), (B) (p.Gln448Glu), (C) (p.Pro618Ala) and (D) (p.Ala732Val), the *CFH* and *MCP* risk haplotypes, and the *CFHR1-3* deletion are represented. The TTP patients were homozygous (n=6), compound heterozygous (n=1) and heterozygous (n=5) for 10 *ADAMTS13* variants: 6 pathogenic and 4 benign/likely benign variants. The 17 aHUS patients were heterozygous for 23 variants in the different complement genes ranging from single pathogenic mutations (n=11) to benign variants combined with other variants and/or *CFH* and *MCPggaac* risk haplotypes or the *CFHR1-3* deletion. This scheme shows the autosomal recessive inheritance of TTP with biallelic pathogenic variants in *ADAMTS13* and the autosomal dominant inheritance of aHUS with polygenic variants (mainly *CFH*). CFH, complement factor H; MCP, membrane cofactor protein; CFI, complement factor I; C3, Complement C3; P, patient; red border, homozygous mutations; pink boxes, pathogenic mutations; green boxes, other variants (likely pathogenic, benign); light purple boxes, *ADAMST13* SNPs and alleles carrying *CFH*, *MCPggaac* risk haplotypes and the *CFHR1-3* deletion

In total, 37 rare variants that include 34 missense variants (92%), 2 small deletions (5%), and 1 splice site mutation at the 3′-splice-site consensus AG (3%) scattered across 7 genes (*ADAMTS13*, *CFH*, *CD46*, *C3*, *CFI*, *CFB*, and *CFHR5*) (Figure 2) whose MAF in the different populations studied in the 1000 Genomes were below 1%. The in silico predictions (Table S2) combined with other evidence (population, functional, and reported studies) permitted their prediction of pathogenicity (Table S3). In total, 8 variants had never been reported in the population databases and international disease databases and 4 had only been reported in the population databases. No rare variants were identified in *CFHR3*, *CFHR4*, *THBD*, and *DGKE* in this cohort.

3.3 | Potential functional impact of novel variants

Seven of the 8 novel variants (89%) were pathogenic: 5 missense changes (*CFH*: c.240T>G p.Cys80Trp; c.335A>G, p.Tyr112Cys; c.493G>T, p.Asp165Tyr; c.3562A>G, p.Lys1188Glu; and c.3644G>T, p.Arg1215Leu) and 2 small deletions (*ADAMTS13*: c.762_774del12pb,

TABLE 1 Phenotypic and genotype characteristics of 11 patients with thrombotic thrombocytopenic purpura carrying rare variants in *ADAMTS13*

Patient ID	Sex	Age onset (yrs)	Triggers events	Treatment	Relapses	Prophylaxis	*ABO*
P1[a]	M	4	Recurrence with infections	PI	3	No	A_1A_1
P2[a]	F	5	Recurrence with infections	PI	12	PI, 2U 3/3 weeks	O_1O_1
P3[a]	F	5	Recurrence with infections	PI; BPL 8Y	8	BPL 8Y[d] 3/3 weeks	O_1O_1
P4 [a] brother P3	M	3	3 episodes-with acute infection	PI; BPL 8Y	3	No	O_1O_1
P5[a]	M	7 m	Recurrence with infections	PI	4	No	O_1O_1
P6[b]	F	26	Pregnancy	PI	4	No	O_1O_1
P7[b]	F	26	Pregnancy	PEX;PI	4	PI, 2U 3/3 weeks	*BB*
P8[c]	M	20	No	PEX;PI	0	No	O_1A_1
P9[c]	F	25	1 episode-with acute infection	PEX;PI	0	No	O_1A_1
P10[c]	M	13	No	PEX;PI	0	No	O_2A_1
P11[c]	F	28	1 episode-with acute infection	PEX;PI	0	No	O_1O_1

[a]Childhood TTP.
[b]Pregnancy-associated TTP.
[c]Indicates acquired TTP with mutation detected; M, male; F, female; m, months; PI, plasma infusion; BPL 8Y, BioProducts Laboratory, Elstree, Herts, UK.
[d]Patient received 1000 IU per dose; PEX, plasma exchange; *ABO*, genotyped ABO blood group.[34]
[e]VC, variant classification: P=pathogenic, LP=likely pathogenic, LB=likely benign, and B=benign; UVS=unknown significance.
[f]SNPs in homozygosity; Ref., reference where variant has been described previously; pdb, variants found only in the 2 population database; SNPs, single nucleotide polymorphisms; mod, modulators. Novel variant is marked in bold.

p.Pro256Serfs*12; and *CD46* c.800-821del, Thr267Ilefs*24). The remaining variant was indicated to be a likely benign variant (*CFH* c.1864A>G, p.Ile622Val) (Table S3).

3.4 | Phenotype-genotype analysis in TTP patients

Four putative pathogenic *ADAMTS13* variants (1 novel) were identified in the 7 hereditary TTP patients of this cohort (5 with disease onset in childhood and 2 with disease onset in pregnancy): 6 patients were homozygous and 1 patient was compound heterozygous (Table 1; Figure 2). One pathogenic mutation, p.Cys754Arg, occurred repetitively and knowing that *ABO* blood group gene locus lies in the close neighborhood of the *ADAMTS13* gene on chromosome 9q34, the blood groups were analyzed, which revealed that all patients homozygous for this mutation were of genotypic blood group O_1O_1 (Table 1). Likewise, the analysis of 12 frequent SNPs in *ADAMTS13* showed that the most prevalent pathogenic mutation (c.2260T>C, p.Cys754Arg, chr=9) were associated with the same haplotype (Table S4). The fact that the 9 mutation-carrier chromosomes are in complete linkage disequilibrium with a unique haplotype suggests that, at least within the Portuguese population, the c.2260T>C nucleotide change occurred once on this progenitor haplotype. The patients carrying p.Cys754Arg belong to the central region of Portugal.

The 4 patients with acquired TTP and persisting partial ADAMTS13 deficiency in remission had 5 heterozygous *ADAMTS13* rare variants: pathogenic (n=2), likely pathogenic (n=1), and compound heterozygous (1 likely pathogenic and 1 likely benign) (n=1). Conversely, the other 32 acquired TTP patients who were also investigated showed only a total of 2 heterozygous *ADAMTS13* rare variants: p.Arg625His, which was also present in a hereditary TTP patient (P5) but co-inherited and, therefore, considered with uncertain significance and a benign variant p.Val849Ile (Table 1; Figure 2). The *ADAMTS13* rare variants found in these 2 acquired TTP groups, albeit a small number of patients analyzed, differed significantly (Fisher exact test, $P<.001$) (Figure S1).

Of the 11 complement genes studied, only 4 rare benign variants in *CFI* (n=3) and *CFB* (n=1) were identified in 4 TTP patients (Table S3).

ADAMTS13 variants					*ADAMTS13*	*MCPggaac*	*CFH- H3*	*CFHR1-3*
Nucleotide Change	Amino Acid Change	VC[e]	Zygosity	Ref.	SNPs mod.	alleles	alleles	alleles
c.2074C>T	p.Arg692Cys	P	Homozygous	[29]	p.Arg7Trp[f] p.Gln448Glu[f] p.Pro618Ala[f] p.Ala732Val[f]	0	0	0
c.2260T>C	p.Cys754Arg	P	Homozygous	[30]	p. Gln448Glu[f]	0	1	0
c.2260T>C	p.Cys754Arg	P	Homozygous	[30]	p.Gln448Glu[f]	0	0	0
c.2260T>C	p.Cys754Arg	P	Homozygous	[30]	p.Gln448Glu[f]	0	0	0
c.2260T>C	p.Cys754Arg	P	Homozygous	[30]	p.Gln448Glu[f]	0	1	0
c.2260T>C c.1874G>A **c.762_774del12pb**	p.Cys754Arg p.Arg625His **p.Pro256Serfs*12**	P UVS P	Compound heterozygous	[30] (pdb) novel	p.Gln448Glu	1	1	0
c.3368G>A	p.Arg1123His	P	Homozygous	[31]	—	0	1	0
c.1370C>T	p.Pro457Leu	P	Heterozygous	[32,33]	p.Gln448Glu p.Ala900Val	0	2	0
c.2914C>T	p.Arg972Trp	P	Heterozygous	[29]	p.Ala900Val	0	0	0
c.1368G>T c.2218G>A CFI, c.1642G>C	p.Gln456His p.Glu740Lys p.Glu548Gln	LP LB B	Compound heterozygous	[30,32,33] (pdb) (pdb)	—	0	0	0
c.3287G>A	p.Arg1096His	LP	Heterozygous	[7,23,33]	p.Gln448Glu[f] p.Arg7Trp	0	0	0

Therefore, these data indicate that the phenotypic variability in these TTP patients was not possibly explained by complement gene variants.

The detailed phenotype-genotype analysis of patients P1-P11 and the family pedigrees (Figure 2A, B) are presented in the Supplementary Results.

3.5 | Phenotype-genotype analysis in aHUS patients

In 17 out of 34 (50%) aHUS patients' mutations were identified, 13 patients had mutations in heterozygous state and 4 patients were compound heterozygous. Two patients presented 3 variants in a single gene (*CFH*) and the remaining 2 patients had combined variants in 2 different complement genes (*C3/CFI* and *C3/CD46*). In total, 23 rare variants (7 non-previously described) were identified in 6 complement genes that were located mostly in *CFH* (12), with 4 in *CD46*, 4 in *C3*, 1 in *CFI*, 1 in *CFB*, and 1 in *CFHR5* (Table 2; Figure 2). Eleven variants were putative pathogenic, 3 likely were pathogenic, 1 was of uncertain significance, 3 were benign, and another 4 were likely benign (Tables 2 and S3). The detailed phenotype-genotype analysis of these 17 patients is presented in the Supplementary Results. In the remaining 17 aHUS patients, although they also present a phenotype according to an overactivation of the alternative complement pathway, no rare genetic variants were found.

Considering all the aHUS patients carrying single or combined complement gene variants, we found that 11/17 (65%) were heterozygous for at least 1 pathogenic mutation and the 3 patients who have had relapses were all carrying pathogenic mutations (*CFH*, *CD46*, and *C3*). On the other hand, however, our data suggest that patients carrying non-pathogenic variants co-inherited more genetic risk factors: patient P12 carrying a single benign *CD46* variant and the patient P9 with a single likely pathogenic *CFHR5* variant were homozygous for the *CFHR1-3* deletion and the patient P14 carrying a single benign *C3* variant was homozygous for a block of 4 *ADAMTS13* SNPs. These additional genetic risk factors (with impact in the activity and secretion of ADAMTS13 and risk haplotypes of complement genes that significantly increased disease penetrance) could have a cumulative or

TABLE 2 Phenotypic and genotype characteristics of 17 patients with atypical hemolytic uremic syndrome carrying rare variants in complement genes

							Variants identified	
Patient ID	Sex	Age onset (yrs)	Triggers[a]	Treatment	Relapses	Gene	Nucleotide Change	Amino Acid Change
P1	F	3 months	No	PI	0	*CFH*	**c.240 T>G** c.2669G>T c.3019 G>T	**p.Cys80Trp** p.Ser890Ile p.Val1007Leu
P2	F	39	G	PEX	0	*CFH*	**c.335A>G**	**p.Tyr112Cys**
P3	F	45		PI	0	*CFH*	**c.493G>T**	**p.Asp165Tyr**
P4	F	67	post surgery	PEX	0	*CFH*	**c.1864A>G**	**p.Ile622Val**
P5	F	35		PI	0	*CFH*	c.2850G>T	p.Gln950His
P6	F	46	post surgery	PEX	0	*CFH*	c.3172T>C c.3178G>C c.3226C>G	p.Tyr1058His p.Val1060Leu p.Gln1076Glu
P7	F	30	No	PEX, PI Eculizumab	1	*CFH*	**c.3562A>G**	**p.Lys1188Glu**
P8	M	2	No	PI	1	*CFH*	**c.3644G>T**	**p.Arg1215Leu**
P9	F	64	No	PI	0	*CFHR5*	c.329T>C	p.Val110Ala
P10	M	35	T	PI, prednisolone	3	*CD46 (MCP)*	c.287-2A>G	
P11	M	4	G	PI	0	*CD46 (MCP)*	**c.800_821del**	**p.Thr267Ilefs*24**
P12	M	37	No	PI, Eculizumab	0	*CD46 (MCP)*	c.1148C>T	p.Thr383Ile
P13	M	4	No	PI	2	*C3*	c.193A>C	p.Lys65Gln
P14	M	50	No	PEX	0	*C3*	c.1407G>C	p.Glu469Asp
P15	F	33	G	PEX, prednisolone Eculizumab	0	*C3* *CFI*	c.193A>C c.452A>G	p.Lys65Gln p.Asn151Ser
P16	F	20	No	PEX, PI	0	*C3* *CD46 (MCP)*	c.1775G>A c.686G>A	p.Arg592Gln p.Arg229Gln
P17	F	6 months	G	PI	0	*CFB*	c.1598A>G	p.Lys533Arg

[a]Triggers: No=none identified, G=gastroenteritis (Shiga-like toxin E. coli negative), T=Tonsillitis; M, male; F, female; m, months; PI, plasma infusion; PEX, plasma exchange.

[b]VC, variant classification: P=pathogenic, LP=likely pathogenic, LB=likely benign, and B=benign; UVS=unknown significance; Ref., reference where variant has been described previously; pdb, variants found only in the 2 population database; SNPs, single nucleotide polymorphisms; mod, modulators. Novel variants are marked in bold.

synergistic effect with more impact on rare benign variants, i.e, they appear to be a requisite to disease manifestation in carriers of benign variants (Table 2; Figure 2). However, these data can only be speculative given the small number of patients involved.

4 | DISCUSSION

We developed a workflow based on ADAMTS13 activity and screening for genetic TMA susceptibility factors using a successfully designed, validated and applied NGS-based targeted gene panel. For this, from a cohort of 154 Portuguese patients with acute TMA, the genotype-phenotype correlations were analyzed in 7 hereditary TTP (ADAMTS13 activity <10%, no inhibitor), 36 acquired TTP (ADAMTS13 activity <10%, presence of an inhibitor), and in 34 presumable aHUS.

Regarding *ADAMTS13* variants in hereditary TTP, our data were in agreement with 2 findings in previously reported studies[44]: first, homozygous variants in the N-terminal ADAMTS13 region (p.Arg692Cys and p.Cys754Arg) were associated with lower age at the onset of the first TTP episode compared to homozygous mutations in the C-terminal region (p.Arg1123His) and, for instance, p.Arg1123His variant was found in a pregnancy-associated TTP patient with an age of onset of 26 years; second, siblings and unrelated patients with the same genotype had a similar age of disease onset.[8] The remaining pregnancy-associated TTP patient was compound heterozygous for p.Cys754Arg and a novel small deletion (p.Pro256Serfs*12) with

VC[b]	Zygosity	Ref.	*ADAMTS13* SNPs mod.	*MCPggaac* alleles	*CFH- H3* alleles	*CFHR1-3* alleles
P LP LB	Compound heterozygous	Novel [14][35] [36]	—	1	0	1
P	Heterozygous	Novel	—	2	1	0
P	Heterozygous	Novel	—	1	1	1
LB	Heterozygous	Novel	p.Gln448Glu	1	0	0
P	Heterozygous	[12]	—	0	0	1
LB LB LP	Compound heterozygous	[37] [37] [38]	—	2	1	0
P	Heterozygous	Novel	—	0	1	0
P	Heterozygous	Novel	0	0	2	0
LP	Heterozygous	(pdb)	—	1	0	2
P	Heterozygous	[39]	p.Gln448Glu p.Ala900Val	1	1	1
P	Heterozygous	Novel	—	1	1	1
B	Heterozygous	[40]	—	2	1	2
P	Heterozygous	[41]	—	2	0	1
B	Heterozygous	[41]	p.Arg7Trp p.Gln448Glu p.Pro618Ala p.Ala732Val	0	1	0
P P	Compound heterozygous	[41] [14]	p.Gln448Glu p.Ala900Val	1	2	0
P UVS	Compound heterozygous	[41] (pdb)	0	1	1	0
B	Heterozygous	[36,42,43]	p.Gln448Glu	0	0	0

obvious deleterious consequences. However, patients carrying the same *ADAMTS13* pathogenic variant showed heterogeneous clinical phenotypes of TTP with a wide range of severity, with some patients requiring regular therapy. These findings are in line with previous reports.[30,44] The most common pathogenic mutation p.Cys754Arg correlates with the same *ADAMTS13* haplotype and blood group O_1O_1, suggesting a common origin and high prevalence in central Portuguese population, in similarity with that described recently in central Norwegian population.[10]

After acute episodes in the 4 TTP patients who had an *ADAMTS13* rare variant and anti-ADAMTS13 IgG antibodies, antibody levels returned to normal and ADAMTS13 activity increased to median 37.5% (range 35-38), and this could be explained by their heterozygosity. Two variants carried by these patients, p.Pro457Leu and p.Arg1096His, were previously found to be associated with acquired ADAMTS13 deficiency.[7,23,32,33] Expression studies revealed reduced ADAMTS13 secretion, and it was suggested that increased intracellular degradation promoted autoantibody production.[7,23] One study reported the association of anti-ADAMTS13 IgG antibodies with the missense variant p.Arg1060Trp.[8] Another study described a case of familial acquired TTP of identical twins with anti-ADAMTS13 IgG antibodies suggesting that genetic determinants could play a role in inhibitor formation.[45] As highlighted by other authors,[3] given the low prevalence of *ADAMTS13* mutations in the population (confirmed in our local healthy controls), the number of heterozygous mutation carriers among TTP patients with severe acquired ADAMTS13 deficiency suggests a role

of heterozygous sequence variants either in the induction of autoantibodies or in facilitating an ADAMTS13 decrease below a threshold relevant for the induction of TTP. Despite the low prevalence of *ADAMTS13* variants in the other 32 acquired TTP, it should be noted that other factors could influence this autoimmune disease, i.e, the simultaneous presence of other autoimmune disease, other genetic factors as epigenetic and/or allele *HLA DRB1*11*, or other thus far unrecognized genetic risk factors.[3,46]

In agreement with previous studies, only 50% of patients diagnosed with aHUS have rare variants in the complement genes.[47,48] These were predominantly loss-of-function variants in the complement regulator genes *CFH*, *CFHR5*, *CD46*, and *CFI* (78%) along with gain-of-function variants in the complement inductor genes *C3* (18%) and *CFB* (4%). *CFH* variants (12/23, 52%) will be the most prevalent in aHUS patients, as described in large studies in other populations.[47–49]

Likewise, the well-documented cumulative effect of different genetic risk factors for the aHUS phenotype[14,15,50] was also observed in our patients: compound heterozygous in *CFH* (2/12, 17%); combined variants in more than 1 gene (2/17, 12%); combination of variants with the concomitant presence of both risk haplotypes, *CFH-H3* and *MCPggaac* (compound heterozygous/homozygous) (8/17, 47%); and homozygous for the *CFHR1-3* deletion (2/17, 12%). The coexistence of excessive complement activation and partially decreased ADAMTS13 activity was described as a risk factor for the aHUS phenotype.[23] The analyses of *ADAMTS13* in our aHUS cohort revealed only common variants (SNPs): Gln448Glu, the most prevalent (20%) and p.Arg7Trp, p.Gln448Glu, p.Pro618Ala, and p.Ala732Val, co-inherited in 1 patient. The Gln448Glu variant alone had little impact but, conversely, the block of these 4 SNPs has been shown to have a higher impact on ADAMTS13 activity.[39]

Nevertheless, this NGS study ended with 17 (50%) genetically unresolved aHUS patients, as described in others aHUS studies.[14,48] Therefore, the expectation that the whole-exome sequencing approaches could identify other genes involved in aHUS is high; however, these studies still remain under investigation.[51] Conversely, NGS-targeted gene panels are being introduced into clinical practice providing substantial benefits for definitive diagnoses in hematological diseases.[26,27,52]

The prediction of pathogenicity of the genetic variation[53] has become crucial for understanding the great inter-individual variability. Therefore, NGS-targeted gene panels have changed the paradigm of routine molecular studies. In the face of the multiple genetic changes found in every patient, the critical challenge was discriminating disease-associated variants from the broader background of variants present in all patients' genomes.[51–55]

In conclusion, the study of these 77 TMA Portuguese patients contributes to the better understanding of the molecular genetics of *ADAMTS13*/complement gene-related phenotypes. Moreover, our study provides evidence of the usefulness of the NGS panel as an excellent advantageous technology that enables more rapid and economic diagnosis of TMA. These findings show that this is a valuable asset in clinical practice given that a correct diagnosis is essential for determining the most effective treatment for each patient with this complex disease.

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AUTHOR CONTRIBUTIONS

TF was responsible for study design and wrote the manuscript. TF, PM, CSP, and ACO performed the functional and molecular studies and analyzed the data. LM contributed to the statistical analysis. FV, NB, IC, MC, and RB contributed to NGS study. RS, TM, and MJM provided clinical support. FV and MLR revised the manuscript.

RELATIONSHIP DISCLOSURES

None of the authors have any disclosures relevant to this paper.

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SUPPORTING INFORMATION

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