SUPPLEMENTARY MATERIALS

On the relevance of thrombomodulin variants in atypical hemolytic uremic syndrome.

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SUPPLEMENTARY METHODS

The aHUSC3G registry

The aHUSC3G registry is a repository of clinical and basic research data funded by the Working Group on Complement and Renal Disease at the Centro de investigaciones Biológicas and Hospital Universitario La Paz and supervised by the Centro de Investigación Biomédica en Red de Enfermedades Raras (Ciberer). Ciberer belongs to the Instituto de Salud Carlos III, which depends on the Spanish Ministry of Health. The aHUSC3G registry was launched in 2013 to preserve the clinical, genetic and laboratory data of aHUS and C3G Spanish patients and to facilitate their analysis by clinical and basic researchers.

Study Population, definitions and treatments

The study includes 27 adult and pediatric aHUS patients with identified *THBD* genetic variants from the Spanish aHUSC3G registry. A diagnosis of aHUS was considered if the patient fulfilled the following criteria: Presence of one or more episodes of microangiopathic hemolytic anemia and thrombocytopenia defined on the basis of platelet count $<150\times10^9$/L or a decrease of $> 25\%$ from baseline values, haemolytic anemia and serum creatinine level greater than upper limit of normal range (excepting those patients already on dialysis) and fragmented erythrocytes in the peripheral blood smear, together with a negative Coombs test, normal activity of ADAMTS-13 and negative Shiga Toxin. Acute renal failure was defined according to the criteria of the KDIGO guidelines,[S1] which consider it as an increase in serum creatinine ≥ 0.3 mg/dl for at least 48 hours or an increase in creatinine ≥ 1.5 times compared to its baseline value in the last 7 days, or decreased urine output <0.5 mL/kg/h for 6 hours. Eculizumab, when used, was administered intravenously at a dose of 900 mg per week for four weeks, and then 1200 mg every two weeks. All the patients received anti meningococcal vaccination and received antibiotic prophylaxis according to label instructions. The duration of

eculizumab therapy was decided by the treating physician, based on the patient's response and individual characteristics. Data were compiled from the medical records of the participating centers using a shared data base. Follow-up period was defined as the interval between the aHUS onset and the last visit or death.

The studies reported here have Institutional Review Board's approval. Informed consent was provided to all individuals participating in the study, according to the Declaration of Helsinki. The Public Health System pays eculizumab treatments in Spain. None of the agencies funding the present work are responsible for the payments of the eculizumab treatment of the patients included in this report.

Outcomes

The primary outcome was the hematologic and renal responses. Hematologic response was defined by the normalization of platelet count and disappearance of markers of MAHA (low hemoglobin, elevated lactate dehydrogenase (LDH), decreased serum haptoglobin and the presence of schistocytes in peripheral blood examination). Renal response was defined by the recovery of renal function with $\geq 25\%$ reduction in serum creatinine levels (during the acute renal failure) in two consecutive measurements for ≥ 4 weeks. Secondary outcome was to reach end stage of renal disease (ESRD), defined as the need of renal replacement therapy (RRT), either dialysis or transplantation.

Genetic analyses

Patients were analyzed for genetic variants using an in house next generation sequencing (NGS) panel that includes 50 genes: *C1QA, C1QB, C1QC, C1R, C1S, C2, C3, C4A, C4BPA, C4BPB, C5, C6, C7, C8A, C8B, C8G, C9, CD46, CD55, CD59, CFB, CFD, CFH, CFHR1, CFHR2, CFHR3, CFHR4, CFHR5, CFI, CFP, CLU, CR1, CR2, FCN1, FCN2, FCN3, ITGAX, ITGAM, ITGB2, MASP1, MASP2, MBL2, SERPING1, VSIG4, DGKE, PLG, VWF, THBD, ADAMTS13* and *VTN*. Genomic DNA was prepared

from peripheral blood cells according to standard procedures. Targeted sequences were captured using the Nextera rapid capture custom Enrichment Kit from Illumina and sequencing data generated in a Miseq equipment using Miseq reagent kit v2 (300 cycles). Sequence data were analyzed using the Burrows–Wheeler Alignment and Picard software with additional filtering using customs tools. Variant calling was performed both with bcftools and VarScan and the variant calling files generated merged in one single file using customs tools. Common variants with a minor allele frequency value >1% in any population were excluded. This criterion was not applied to the *THBD* variants for the reasons explained below. To identified novel and/or functional variants we used different databases: the Exome Aggregation Consortium (ExAC), the Genome Aggregation Database (gnomAD),[S2] 1000 Genomes, NCBI dbSNP, aHUS mutation database (www.fh-hus.org) or our in-house aHUSC3G database. Pathogenicity was established by functional studies or with the Combined Annotation Dependent Depletion (CADD) method (https://cadd.gs.washington.edu/info) [S3,S4] using a CADD PHRED C-score. In this later case, variants were classified as potentially functional or likely benign if their C-score was above or below 15. All patients were analyzed for copy number variations in the *CFH-CFHR1-5* genes by multiplex ligation-dependent probe amplification (MLPA) with an in-house mix and the P236 A1 ARMD mix 1 (MRC-Holland, Amsterdam, Netherlands) and genotyped for the *CFH-H3* and *MCPggaac* aHUS risk polymorphisms. All of them were also tested for the presence of anti FH autoantibodies.

Criteria to include THBD variants in the study

A major objective of this study is to replicate the original NEJM report published in 2009 describing the association between THBD variants and aHUS.[S5] Since at that time there were no data available from normal populations to inform of allelic frequencies as they are nowadays, in that study, the authors used as controls, a cohort comprised of 150 healthy controls from the International Registry of Recurrent and Familial HUS/TTP and a second control cohort of 230 DNAs from healthy white Caucasians from Europe.

This is roughly, selecting *THBD* variants with an allelic frequency (AF)<0.7-0.4% in Caucasians. To match those criteria, in our study we have included all carriers of *THBD* variants with an AF<1% in the normal European Non-Finnish (ENF) population of the Genome Aggregation Database (gnomAD). As discussed in our work, we are aware that by today standards this inclusion criterion would be highly questionable, but it was important to have in our study aHUS patients carrying the same *THBD* variants that were included in the original NEJM report as we also wanted to examine the clinical characteristics of these patients.

Gene-based collapsing test

Number of carriers of variants in *THBD, CFH, CFI* and *MCP (CD46)* were retrieved form the gnomAD database and the aHUSC3G registry and grouped according to the AF in the ENF population in gnomAD of the variants they carry (i.e., 1%, 0.1% and 0.01%). Within each group pathogenicity was predicted for all variants with the CADD method. In this analysis, variants were classified as potentially functional if their CADD PHRED C-score was above 15. A Chi-square analysis was used to compare the total number of carriers of variants in the *THBD, CFH, CFI* and *MCP(CD46)* genes with AF 1%, 0.1% and 0.01%m between gnomAD and our aHUS cohort and the negative log-base-10 of the significance p-value for each of the comparison was calculated. A similar analysis was done for the number of carriers of functional variants. The Bonferroni correction was used to determine the threshold for significance ($p < 0.0125$).

Statistical analysis

Continuous variables are shown as median and inter quartile range [IQR] (25th and 75th percentile). Categorical variables are shown as frequencies and percentages. Mean follow-up time was calculated as the average of the sum time accumulated by each patient from the aHUS onset to end of follow up. Descriptive analyses were performed using Stata version14 (College Station, StataCorp, TX, US).

SUPPLEMENTARY TABLES:

Supplementary Table S1. Complement genetic and acquired risk factors in aHUS patients carrying *THBD* variants.

ID	Age (y)	Sex	*THBD* variant	Complement pathogenic variants	CFH-H3	MCPggaac	ΔCFHR3-CFHR1	Anti-FH antibodies
1	52	M	Ala43Thr	No	No	No	Hom	No
2	60	M	Glu569Lys	No	Het	No	No	No
3	26	M	Asp486Tyr	No	Het	Het	No	No
4	53	F	Pro501Leu	No	No	Het	Het	No
5	56	F	Asp486Tyr	No	No	No	Het	No
6	4 m	F	Tyr39Phe	No	No	No	No	No
7	18 m	M	Asp418Thrfs*88	No	No	No	Het	No
8	41	F	Arg403Lys	No	Het	No	Het	No
9	24	F	Ala43Thr	No	Het	No	No	No
10	50	F	Pro228Leu	No	No	No	Het	No
11	13	M	Arg403Lys	No	Het	Het	No	No
12	23	F	Glu293Asp	No	No	No	Het	No
13	51	F	Pro501Leu	*C3* (Lys65Gln)	No	Hom	Het	No
14	44	F	Ala43Thr	*C3* (Lys65Gln)	Hom	Hom	No	No
15	23	F	Ala43Thr	*CFB* (Phe286Leu)	Het	Hom	No	No
16	47	F	Pro501Leu	*CFH* (Trp1037Stop)	No	Hom	Het / ΔCFHR1-CFHR4	No
17	6	M	Pro499Arg	No	No	Het	Het	No
18	1	F	Arg63Ser	No	No	No	No	No
19	23	F	Asp486Tyr	No	No	Hom	No	No
20	25	F	Asp489His	No	Hom	Het	No	No
21	41	M	Gly502Arg	No	No	No	Het	No
22	24	F	Leu136Trp	*CFI* (Ile416Leu)	No	Het	No	No
23	1	F	Pro501Leu	*CFH* (Asp1119Asn)	Het	Hom	Het	No
24	6	M	Ala43Thr	*MCP* (c.287-2A>G)	Het	Het	No	No
25	1	F	Ala43Thr	No	Het	Het	No	No
26	74	M	Ala43Thr	No	No	Het	Het	No
27	25	F	Ala43Thr	No	No	No	No	No

m: months; y: years; F: female; M: male; Het:heterocigosis; Hom:homocigosis; CFH-H3: aHUS risk polymorphism in CFH; MCPggaac: aHUS risk polymorphism in MCP(CD46); ΔCFHR3-CFHR1: delection in genes CFHR3-CFHR1; ΔCFHR1-CFHR4: delection in genes CFHR1-CFHR4.

Supplementary Table S2. Prevalence and pathogenicity predictions for *THBD* variants found in the Spanish aHUS cohort.

rs	Transcript	Protein	Carriers	Frequency (gnomAD-ENF)	Pathogenicity Score (CADD)	Functional Studies	References	Classification
rs776866172	c.116A>T	Tyr39Phe	1	1×10^{-4}	11.19	No	S1	Likely Benign
rs1800576	c.127G>A	Ala43Thr	8	3×10^{-3}	11.21	Yes	S2,S3,S4,S5,S6	Functional
--	c.187C>A	Arg63Ser	1	Not Present	27.5	No	No	Potentially functional
rs550522588	c.407T>G	Leu136Trp	1	4×10^{-4}	23.1	No	No	Potentially functional
rs375011249	c.683C>T	Pro228Leu	1	5×10^{-4}	13.43	No	No	Likely Benign
rs1328851438	c.879G>T	Glu293Asp	1	9×10^{-6}	13.64	No	No	Likely Benign
rs41400249	c.1208G>A	Arg403Lys	2	2×10^{-5}	13.17	No	S7	Likely Benign
--	c.1252delG	Asp418Thrfs*88	1	Not Present	Pathogenic	No	No	Functional
rs41348347**	c.1456G>T	Asp486Tyr	3	2×10^{-4}	5.71	Yes	S1,S5,S6,S7	Functional
rs888161210	c.1465G>C	Asp489His	1	1×10^{-4}	17.77	No	No	Potentially functional
rs754426265	c.1496C>G	Pro499Arg	1	1×10^{-4}	14.44	No	No	Likely Benign
rs1800579	c.1502C>T	Pro501Leu	4	3×10^{-3}	19.47	Yes	S1,S4,S5,S6	Functional
rs76135678	c.1504G>C	Gly502Arg	1	$1\times10{-4}$	7.54	No	S8	Likely Benign
--	c.1705G>A	Glu569Lys	1	Not Present	3.48	No	No	Likely Benign

References: S1. Osborne AJ, Breno M, Borsa NG, et al. Statistical Validation of Rare Complement Variants Provides Insights into the Molecular Basis of Atypical Hemolytic Uremic Syndrome and C3 Glomerulopathy. *J Immunol.* 2018;200(7):2464-2478. doi:10.4049/JIMMUNOL.1701695; S2. Delvaeye M, Noris M, De Vriese A, et al. Thrombomodulin mutations in atypical hemolytic-uremic syndrome. *N Engl J Med.* 2009;361(4):345-357. doi:10.1056/NEJMOA0810739; S3. Doggen CJ, Kunz G, Rosendaal FR, et al. A mutation in the thrombomodulin gene, 127G to A coding for Ala25Thr, and the risk of myocardial infarction in men. Thromb Haemost. 1998;80(5):743-8; S4. Ohlin AK, Norlund L, Marlar RA. Thrombomodulin gene variations and thromboembolic disease. Thromb Haemost. 1997;78(1):396-400; S5. Faioni EM, Franchi F, Castaman G, et al. Mutations in the thrombomodulin gene are rare in patients with severe thrombophilia. Br J Haematol. 2002;118(2):595-9. doi: 10.1046/j.1365-2141.2002.03644.x; S6. Kunz G, Ohlin AK, Adami A, et al. Naturally occurring mutations in the thrombomodulin gene leading to impaired expression and function. Blood. 2002;99(10):3646-53. doi: 10.1182/blood.v99.10.3646; S7. Matsumoto T, Fan X, Ishikawa E, et al. Analysis of patients with atypical hemolytic uremic syndrome treated at the Mie University Hospital: concentration of C3 p.I1157T mutation. Int J Hematol. 2014;100:437–442. doi: 10.1007/s12185-014-1655-2; S8. Matar, Dany1; Naqvi, Fizza1; Racusen, Lorraine C.2; Carter-Monroe, Naima2; Montgomery, Robert A.3; Alachkar, Nada1,4. Atypical Hemolytic Uremic Syndrome Recurrence After Kidney Transplantation. Transplantation 98(11):p 1205-1212, December 15, 2014. | DOI: 10.1097/TP.0000000000000200.

Supplementary Table S3. Carriers of *THBD* variants in the ENF population from gnomAD (n=64603)

Number of carriers (number of variants)

Allele Frequency	1-0.01	0.01-0.001	0.001-0.0001	0.0001-0.00001	<0.00001	AF <0.01
Frameshift	0(0)	0(0)	0(0)	1(1)	1(1)	2(2)
Missense	21597(1)	879(3)	96(6)	227(93)	50(50)	1252(152)
In-frame deletion	0(0)	0(0)	0(0)	1(1)	0(0)	1(1)
Splice	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Start-Lost	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Stop-Gained	0(0)	0(0)	0(0)	4(2)	0(0)	4(2)
Stop-Lost	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Total	**21597(1)**	**879(3)**	**96(6)**	**233(97)**	**51(51)**	**1259(157)**

Supplementary Table S4. Carriers of *THBD* variants in the C3-glomerulopathy cohort (n=326).

ID	Age at onset	Sex	Diagnostic	*THBD* variant	rs	Complement variants	Frequency (gnomAD-ENF)	Pathogenicity Score (CADD)	References	Classification
1	25	M	C3G	Val326Met	--	No	Not Present	24.7	No	Potentially functional
2	65	M	C3G	Arg403Lys	rs41400249	No	2×10^{-5}	13.17	S1	Likely Benign
3	40	M	DDD	Gly14Ser	rs191884040	No	5×10^{-5}	12.31	No	Likely Benign
4	56	F	C3G	Pro501Leu	rs1800579	No	3×10^{-3}	19.47	S2,S3,S4,S5	Functional
5	40	M	C3G	Arg403Lys	rs41400249	No	2×10^{-5}	13.17	S1	Likely Benign
6	71	F	C3G	Ala202Thr	--	No	Not Present	17.08	No	Potentially functional

Footnote: *F: female; M: male;; C3G: C3-glomerulopathy; DDD: dense deposit disease.* **References:** S1. Matsumoto T, Fan X, Ishikawa E, et al. Analysis of patients with atypical hemolytic uremic syndrome treated at the Mie University Hospital: concentration of C3 p.I1157T mutation. Int J Hematol. 2014;100:437–442. doi: 10.1007/s12185-014-1655-2; S2. Osborne AJ, Breno M, Borsa NG, et al. Statistical Validation of Rare Complement Variants Provides Insights into the Molecular Basis of Atypical Hemolytic Uremic Syndrome and C3 Glomerulopathy. *J Immunol*. 2018;200(7):2464-2478. doi:10.4049/JIMMUNOL.1701695; S3. Ohlin AK, Norlund L, Marlar RA. Thrombomodulin gene variations and thromboembolic disease. Thromb Haemost. 1997;78(1):396-400; S4. Faioni EM, Franchi F, Castaman G, et al. Mutations in the thrombomodulin gene are rare in patients with severe thrombophilia. Br J Haematol. 2002;118(2):595-9. doi: 10.1046/j.1365-2141.2002.03644.x; S5. Kunz G, Ohlin AK, Adami A, et al. Naturally occurring mutations in the thrombomodulin gene leading to impaired expression and function. Blood. 2002;99(10):3646-53. doi: 10.1182/blood.v99.10.3646;

Supplementary Table S5. Hemolytic Uremic Syndrome in Kidney Grafts

ID	Age (y)	Sex	CKD	KT before HUS (n; lost cause)	sCr	Hb	Plat x10^5	Time post KT	Secondary causes	DSA	Max sCr	Min Hb	Min Plat x10^5	Max LDH	Renal biopsy with TMA (no AHR)	ECU (Days from onset)	Recovery	Follow up (y)	Relapse	RRT	KT post-HUS[b]	Last sCr	Death
			Baseline data					HUS Onset								Treatment and response				Long term evolution			
1	52	M	NAE	1	2.1	14.5	212	4 y	No	neg	3.6	11.4	149	223	Yes	No	Hematological RRT	0.9	No	Yes	No	D	Yes
3	26	M	Nephronophthisis	2 (1st lost; unk)	D	11.2	209	unk	No	neg	Prev. HD	6.7	62	535	No	Yes (1)	Hematological Prev. HD	0.4	No	Prev. HD	No	D	No
8	41	F	Renal vascular disease (RAS)	2 (1st; CNI tox)	4.0	9.8	115	First weeks	Ischemia/reperfusion	neg	5.3	7.1	61	1061	No	Yes (11)	Hematological RRT	5.9	No	Yes	Yes (4.4)	0.9^a	No
9	24	F	CNI tox (lung Tx)	1	D	10.6	240	First week	CNI	neg	Post KT	6.7	172	1589	No	No	Hematological Kidney partial	6.4	No	Yes	Yes (3.3)	1.0^a	No
11	13	M	Alport sd	1	0.8	12.3	309	17 m	Infection (no DIC), drugs	neg	3.9	6.8	175	330	Yes	No	Hematological Kidney complete	0.7	No	No	No	0.8	No

a) sCr post KT after aHUS; b) Years after debut; y: years; DSA: donor-specific alloantibody; AHR: Acute Humoral rejection; F: female; M:male; CKD: chronic kidney disease (etiology); NAE: nephroangiesclerosis; RAS: renal artery stenosis;; sd: syndrome; RRT: renal replacement therapy; D: dialysis; KT: kidney transplant; sCr: serum creatinine (mg/dL); Hb: hemoglobin (g/dL); Plat: platelets per microlitre; LDH: lactate dehydrogenase (mg/dL); HD: hemodialysis; CNI: calcineurin inhibitors; ECU: eculizumab; neg: negative; F/U: follow-up; unk; unknown; T: transplant; TMA: thrombotic microangiopathy; prev: previous.

Supplementary Table S6. Hemolytic Uremic Syndrome in Native Kidneys

ID	Age (y)	Sex	CKD	sCr	Hb	Plat x10⁵	Secondary causes	TMA in renal biopsy	Max sCr	Min Hb	Min Plat. x10⁵	Max LDH	ECU (Days from onset)	Recovery	Follow up (y)	Relapse	RRT	KT post-HUS [a]	Last sCr	Death
				Baseline data				HUS Onset						Treatment and response		Long term evolution				
2	60	M	FSGS	2.8	14.0	264	No	Yes	3.64	11.3	250	NA	No	Hematological Kidney partial	2.6	No	No		3.1	No
4	53	F	No	0.8	12.2	118	No	Yes	2.2	8	116	186	No	Hematological Kidney complete	2.5	No	No		0.9	No
5	56	F	No	0.8	13.6	237	No	Yes	5.99	7.5	76	1408	Yes (2)	Hematological Kidney partial	1.0	No	No		1.4	No
6	4m	F	No	0.9	NA	NA	Gastrointestinal infection (Shiga toxin negative. no DIC)	No	HD	6.5	37	6994	No	Hematological Kidney complete	13.8	No	No		2.4	No
7	18m	M	No	0.8	NA	NA	Gastrointestinal infection (Shiga toxin negative, no DIC)	No	HD	8.3	54	NA	No	Hematological Kidney complete	23.1	No	Yes	Yes (18.4)	1.9c	No
10	50	F	No	0.6	10.3	350	Gemcitabine	Yes	3.6	7.5	74	1358	Yes (22)	Hematological Kidney partial	0.3	No	No		1.5	Yes
12	23	F	No	NA	NA	NA	Pregnancy, severe bleeding, but no DIC	Yes	HD	8.0	NA	1120	No	Hematological Kidney partial	23.1	No	Yes	Yes (23.0)	1.9 c	No
13 [b]	51	F	No	1.6	11.2	170	Cocaine	Yes	HD	6.4	164	366	No	Hematological RRT	2.1	No	Yes		D	No
14 [b]	44	F	No	0.9	12.1	394	Viral infection (flu-like symptoms, no DIC)	No	HD	6.3	72	1254	Yes (3)	Hematological Kidney complete	1.9	No	No		0.9	No

a) (years after debut); b) Carrier complement gene variant; c) sCr post KT after TMA; mo: months; y: years; TMA: thrombotic microangiopathy; DIC: disseminated intravascular coagulation; F: female; M: male; NA: not available; CKD: chronic kidney disease (etiology); FSGS: focal segmental glomerulosclerosis; RRT: renal replacement therapy; HD: hemodialysis; KT: kidney transplant; sCr: serum creatinine (mg/dL);Hb: hemoglobin (g/dL); Plat: platelets per microliter; LDH: lactate dehydrogenase (mg/dL); ECU: eculizumab; NA: not applicable.