Complement Gene Variants and Shiga Toxin–Producing *Escherichia coli*–Associated Hemolytic Uremic Syndrome

Retrospective Genetic and Clinical Study

Véronique Frémeaux-Bacchi,[1,2] Anne-Laure Sellier-Leclerc,[3] Paula Vieira-Martins,[1] Sophie Limou,[4] Theresa Kwon,[5] Annie Lahoche,[6] Robert Novo,[6] Brigitte Llanas,[7] François Nobili,[8] Gwenaëlle Roussey,[9] Mathilde Cailliez,[10] Tim Ulinski,[11] Georges Deschênes,[5] Corinne Alberti,[12] François-Xavier Weill,[13] Patricia Mariani,[14] and Chantal Loirat[5]

Due to the number of contributing authors, the affiliations are listed at the end of this article.

Correspondence: Dr. Véronique Frémeaux-Bacchi, Laboratoire d'Immunologie, Hôpital Européen Georges Pompidou, 20 Rue Leblanc, 75015 Paris, France. Email: veronique.fremeaux-bacchi@aphp.fr

Abstract

Background and objectives Inherited complement hyperactivation is critical for the pathogenesis of atypical hemolytic uremic syndrome (HUS) but undetermined in postdiarrheal HUS. Our aim was to investigate complement activation and variants of complement genes, and their association with disease severity in children with Shiga toxin–associated HUS.

Design, setting, participants, & measurements Determination of complement biomarkers levels and next-generation sequencing for the six susceptibility genes for atypical HUS were performed in 108 children with a clinical diagnosis of post-diarrheal HUS (75 Shiga toxin–positive, and 33 Shiga toxin–negative) and 80 French controls. As an independent control cohort, we analyzed the genotypes in 503 European individuals from the 1000 Genomes Project.

Results During the acute phase of HUS, plasma levels of C3 and sC5b-9 were increased, and half of patients had decreased membrane cofactor protein expression, which normalized after 2 weeks. Variants with minor allele frequency <1% were identified in 12 Shiga toxin–positive patients with HUS (12 out of 75, 16%), including pathogenic variants in four (four out of 75, 5%), with no significant differences compared with Shiga toxin–negative patients with HUS and controls. Pathogenic variants with minor allele frequency <0.1% were found in three Shiga toxin–positive patients with HUS (three out of 75, 4%) versus only four European controls (four out of 503, 0.8%) (odds ratio, 5.2; 95% confidence interval, 1.1 to 24; P=0.03). The genetic background did not significantly affect dialysis requirement, neurologic manifestations, and sC5b-9 level during the acute phase, and incident CKD during follow-up. However, the only patient who progressed to ESKD within 3 years carried a factor H pathogenic variant.

Conclusions Rare variants and complement activation biomarkers were not associated with severity of Shiga toxin–associated HUS. Only pathogenic variants with minor allele frequency <0.1% are more frequent in Shiga toxin–positive patients with HUS than in controls.

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Introduction

Decreased C3 plasma levels at the acute phase of postdiarrheal hemolytic uremic syndrome (HUS) were first mentioned by 1973 (1–3). Forty years later, the demonstration that atypical hemolytic uremic syndrome (aHUS) was a disease of complement alternative pathway dysregulation (4), and that complement blockade therapy improved aHUS prognosis (5), gave a new impetus to the question of a role of complement activation in Shiga toxin–associated HUS. Indeed, elevated plasma levels of complement activation biomarkers were documented at the acute phase of post diarrheal/typical/Shiga toxin HUS (6–11). *In vitro* studies and animal models experiments demonstrated that Shiga toxin generates a cascade of endothelial/podocyte injury, complement activation, expression of chemokines and adhesion molecules, neutrophil activation, and thrombus formation (7,12–18). The hypothesis of a genetic predisposition in Shiga toxin–associated HUS emerged in 2008, with the publication of a case of a 4-year-old girl with Shiga toxin–associated HUS, who died with a multiorgan failure syndrome and was found to carry pathogenic membrane cofactor protein variant (19). Altogether, 17 children with postdiarrheal/Shiga toxin–producing *Escherichia coli* (STEC) HUS were subsequently reported who carried rare variants in complement genes (9,10,20–28). In nine of them, genetic screening was

motivated by an unusually severe outcome, relapses, or post-transplant recurrence, suggesting that STEC infection triggers aHUS onset and/or preexisting complement variants amplify Shiga toxin–induced complement activation and endothelial/podocyte damage, and worsen disease severity (20–27). However, seven reported patients who had complement variants had a favorable outcome (9,10,28), leaving the issue of the role of genetics in Shiga toxin–associated HUS unclear.

The aim of our study was to investigate the frequency of rare variants in complement genes in a French national cohort of children with Shiga toxin–positive HUS compared with healthy controls, and the association of these variants with disease severity and complement activation biomarkers.

Materials and Methods

Study Population

We enrolled 113 white children with a clinical diagnosis of postdiarrheal HUS, hospitalized in 22 pediatric nephrology departments between October 13, 2010 and October 17, 2012 (for study design, see Supplemental Material). We collected blood samples from 80 French controls (healthy white adult volunteers), to establish normal complement factors and sC5b-9 plasma levels and the frequency of complement variants in the French population. As a control/independent validation group, we collected the genotypes in the European individuals from the 1000 Genomes Project (n=503) (29) for the six genes of interest: complement factor H, factor I, factor B, membrane cofactor protein, C3, and thrombomodulin. The variant call format files located on the 1000 Genomes server (ftp://ftp.1000genomes.ebi.ac.uk) were parsed using the Ferret tool (30) to extract the genetic information of rare coding variants (minor allele frequency <1%). From the genotypes, we computed the occurrence of rare coding variants in each individual.

STEC Investigations

Patients were categorized as Shiga toxin–positive or Shiga toxin–negative, according to real-time PCR for Shiga toxin1 or Shiga toxin2 genes on stool samples (Supplemental Figure 1; for a list of STEC investigations, see Supplemental Material).

Genetic Screening

Exonic regions of six complement genes (factor H, factor I, factor B, membrane cofactor protein, C3, and thrombomodulin) were screened using next-generation sequencing. Multiplex ligation-dependent probe amplification was performed to detect factor H hybrid genes and complement factor H–related 1–3 genes deletion. The minor allele frequency of the genetic changes was obtained from the Exome Aggregation Consortium database (http://exac.broadinstitute.org) (31). In our study, we defined a variant as rare when its minor allele frequency in the general population is <1% and as very rare when it is <0.1% (definitions adapted from Richards *et al.* [32] and Goodship *et al.* [33]). Among these rare variants, we named as pathogenic those for which the genetic change affects the protein function (well established *in vitro* functional studies supportive of a damaging effect on the gene product), and/or the genetic change is found in a disease-related functional domain or affects the protein expression (nonsense, frameshift, canonical ±1 or 2 splice sites variants, or well demonstrated lack of *in vitro* synthesis, or quantitative deficiency in the patient's plasma) (adapted from Richards *et al.* [32] and Goodship *et al.* [33]). The other variants were classified as variants of uncertain significance. All patients' parents gave informed consent for genetic analyses.

Complement Biomarkers

Assessment of CH50 (complement hemolytic 50), C3, C4, factor H, factor I, and sC5b-9 plasma level, membrane cofactor protein expression on leukocytes, and anti-factor H antibodies was performed in all patients (34). Results from blood samples collected under or after plasma infusions/exchanges (n=3) or eculizumab (n=6), or later than day 14 after admission (n=12), were excluded (Supplemental Figure 1).

Statistical Analyses

Characteristics of patients are described with frequencies and percentages for categorical data, and with medians and interquartile ranges for quantitative data. Categorical data were compared using Fisher exact test, whereas quantitative data were compared using Wilcoxon–Mann–Whitney nonparametric test. All analyses (chi-squared, Wilcoxon–Mann–Whitney U, and Fisher exact tests) with P value <0.05 were considered statistically significant.

Results

Patients

Among 113 patients, we identified 79 Shiga toxin–positive and 34 Shiga toxin–negative cases. Table 1 summarizes their clinical characteristics and outcomes. Antibiotic treatment during the prodromal phase was more frequent in Shiga toxin–negative (35%) than Shiga toxin–positive (18%) patients, but the difference did not reach statistical significance (odds ratio [OR], 2.5; 95% confidence interval [95% CI], 1 to 6.2; P=0.05). Central nervous system (CNS) manifestations were significantly more frequent in Shiga toxin–positive (23%) than Shiga toxin–negative (6%) patients (OR, 4.7; 95% CI, 1 to 21; P=0.03). Other characteristics during the acute phase and at last follow-up were similar between the two groups. No patient died during the follow-up. A single patient (Shiga toxin–positive) progressed to ESKD 3 years after HUS.

Complement Variants

In the whole cohort of patients with postdiarrheal HUS, we identified a total of 18 patients who carried one rare variant, all heterozygous, in factor H (n=3), membrane cofactor protein (n=1), factor I (n=1), C3 (n=8), factor B (n=1), thrombomodulin (n=3), or two variants (n=1) (Table 2). Among patients with Shiga toxin–positive or Shiga toxin–negative postdiarrheal HUS, a similar proportion (12 out of 75 [16%] and six out of 33 [18%], respectively (OR, 0.8; 95% CI, 0.3 to 2.5; P=0.8) carried one or two rare variants in one or two of the six tested genes, without significant difference in

Table 1. Clinical characteristics, in-hospital course, and outcome of 113 children with post-diarrheal HUS, with comparison of Shiga toxin–positive and Shiga toxin–negative patients

Patient Characteristics	Stx-Positive HUS, *n*=79	Stx-Negative HUS, *n*=34
Age, yr	2.6 (1.4; 4.9)	3.4 (1.6; 5.5)
Sex, men/women	38/41	17/17
Prodromal phase		
Gastrointestinal manifestations		
Nonbloody diarrhea	30 (38)	14 (41)
Bloody diarrhea	40 (51)	19 (56)
Abdominal pain, vomiting, without diarrhea	9 (11)	1 (3)
Fever ≥38°C	27 (34)	15 (44)
Upper respiratory tract infection/otitis	5 (6)	2 (6)
Antibiotics	14 (18)	12 (35)
In-hospital		
At admission		
Hemoglobin, g/dl	7.7 (6.3; 9.5)	7.6 (6.1; 9.3)
Hemoglobin >9 g/dl	25/77 (33)	9 (26)
LDH, U/L	3010 (2193; 4744)	2619 (1151; 4056)
Platelet count, G/L	40 (27; 56)	48 (37; 77)
Leukocyte count, /mm^3	13,070 (10,200; 20,030)	12,400 (9535; 18,200)
Leukocyte count >20,000/mm^3	18/69 (26)	6/31 (19)
STEC investigations		
Stool PCR for Stx		
Stx1 positive	1 (1)	0
Stx2 positive	69 (87)	0
Stx1+Stx2 positive	9 (11)	0
Stool culture		
E. coli O157	37/77 (48)	1[a] (3)
E. coli other than O157	25/77 (32)	1[a] (3)
Nontypable *E. coli*	10/77 (13)	2[a] (6)
Anti-LPS serology	33/64 (52)	17/29 (59)
Positive		
Clinical course		
Anuria	33 (42)	12 (35)
Central nervous system manifestations	18 (23)	2 (6)
Prolonged intestinal manifestations	22 (28)	6 (18)
Pancreatitis	11 (14)	4 (12)
Cardiac manifestations	2 (3)	0
Death	0	0
Treatment		
Dialysis	44 (56)	14 (41)
Dialysis duration, d	9 (4; 16)	9 (7; 16)
Dialysis duration >8 d	25/44 (57)	10/14 (71)
Plasma infusion and/or plasma exchange	8 (10)	1 (3)
Eculizumab	12 (15)	3 (9)
Antibiotics	27 (34)	9 (26)
At last follow-up		
Follow-up, mo	46 (18; 65)	53 (33; 61)
No CKD[b]	52/73 (71)	27/31 (87)
CKD stage 1[b]	16/73 (22)	2/31 (6)
CKD stage 2[b]	1/73 (1)	2/31 (6)
CKD stage 3[b]	3/73 (4)	0
CKD stage 4[b]	0	0
CKD stage 5[b]	1/73 (1)	0

Values are shown as number (%) of patients or median (Q1; Q3). For items not documented in all patients, the number of documented patients is indicated and served for the calculation of the percentage indicated in brackets. No CKD was defined by eGFR≥90 ml/min per 1.73 m^2 without albuminuria; CKD stage 1 by eGFR≥90 ml/min per 1.73 m^2 with albuminuria; CKD stage 2 by eGFR 60–89 ml/min per 1.73 m^2 with albuminuria. See Supplemental Material for the definition of CKD stages 3–5 and albuminuria. HUS, hemolytic uremic syndrome; Stx, Shiga toxin; LDH, lactate dehydrogenase; STEC, Shiga toxin producing *E. coli*; LPS, lipopolysaccharide.

[a]Non-Stx producing *E. coli* strains.

[b]CKD stages according to Kidney Disease Improving Global Outcomes Guidelines 2012 (http://www.kdigo.org/clinical_practice_guidelines/pdf/CKD/KDIGO_2012_CKD_GL.pdf).

the frequency of the variants per gene (Table 2). The rare variants identified in patients are described in Table 3 and in Supplemental Table 1. Two variants (p.Ala382Glu in factor H gene and p.Asn170LysfsTer7 in membrane cofactor protein gene) were novel. As the factor H plasma level and membrane cofactor protein expression, respectively, were below normal ranges, the two corresponding variants were classified as pathogenic. Experimental functional investigations

Table 2. Number and frequency (%) of patients with post diarrheal HUS, and of French controls and European individuals included in the 1000 Genomes Project database, who carried rare variants in the six tested complement genes, or anti-factor H antibodies

Complement Abnormalities	Postdiarrheal HUS, Total Cohort, *n*=108[a]	French Controls, *n*=80	1000 Genomes Controls, *n*=503	Postdiarrheal HUS versus French Controls	Postdiarrheal HUS versus 1000 Genomes Controls	Stx-Positive HUS, *n*=75[b]	Stx-Negative HUS, *n*=33[b]	Stx-Positive versus Stx-Negative HUS
	n (%)	*n* (%)	*n* (%)	OR; 95% CI; *P*-Value[c]	OR; 95% CI; *P*-Value[c]	*n* (%)	*n* (%)	OR; 95% CI; *P*-Value[c]
One rare variant per patient or control								
Complement factor H	3 (3)	2 (3)	8 (2)	1.1; 0.2 to 6.8; >0.99	1.8; 0.5 to 6.8; 0.4	2 (3)	1 (3)	0.8; 0.1 to 10; >0.99
Membrane cofactor protein	1 (0.9)	0	2 (0.4)	1	2.3; 0.2 to 26; 0.4	1 (1)	0	1
Complement factor I	1 (0.9)	2 (3)	13 (3)	0.3; 0 to 4; 0.5	0.3; 0.1 to 3; 0.4	1 (1)	0	1
C3	8[d] (7)	4 (5)	16 (3)	1.5; 0 to 5; 0.5	2.4; 1 to 6; 0.05	5 (7)	3 (9)	0.7; 0.1 to 3; 0.7
Complement factor B	1 (0.9)	0	7 (1)	1	0.6; 0.8 to 5; >0.99	1 (1)	0	1
Thrombomodulin	3 (3)	1 (1)	9 (2)	2.2; 0.2 to 22; 0.6	1.6; 0.4 to 6; 0.4	2 (3)	1 (3)	0.8; 0.1 to 10; >0.99
Two rare variants per patient or control	1[e] (0.9)	2[f] (3)	5 (1)	0.4; 0 to 4; 0.5	0.9; 0.1 to 8; >0.99	0	1 (3)	0.1; 0 to 3.6; 0.3
Total	18 (17)	11 (14)	60 (12)	1.2; 0.5 to 3; 0.6	1.5; 0.8 to 3; 0.1	12 (16)	6 (18)	0.8; 0.3 to 2.5; 0.8
Anti-factor H antibodies	3[d] (3)	0		0.3		1	2	0.2; 0 to 2.4; 0.2

HUS, hemolytic uremic syndrome; Stx, Shiga toxin; OR, odds ratio; 95% CI, 95% confidence interval.
[a]Five of the 113 patients with HUS were not screened for variants in the six tested complement genes.
[b]Four of the 79 patients with Stx-positive HUS and one of the 34 patients with Stx-negative HUS were not screened for variants in the six complement genes.
[c]*P* with Fisher exact test.
[d]One patient had a C3 rare variant and anti-Factor H antibodies.
[e]This patient had a complement factor H and a C3 rare variant.
[f]One control had a thrombomodulin and C3 rare variant, and another control had two rare variants in C3.

Table 3. Complement pathogenic rare variants (*n*=6) found in six out of 108 patients with postdiarrheal HUS

Patient	Gene	Variant	Genetic Status	No. of Patients	CFH Plasma Level[a]	MCP Expression[a]	MAF[b] (%)	Demonstrated Functional Alterations	Previously Reported in STEC-HUS	Previously Reported in aHUS
Stx-positive patients with HUS										
1	Complement factor H	c.2850G>T p.Gln950His	He	1	Normal	Normal	0.36	Moderately decreased binding to GAG and/or C3b (Hemolytic assay) (35)	Yes (24)	Yes[c] (38)
2	Thrombomodulin	c.1483C>T p.Pro495Ser	He	1	Normal	Normal	0.06	Decreased capacity to inactivate C3b (36)	No	Yes (36)
3	Complement factor H	c.1145C>A p.Ala382Glu	He	1	Low	Normal	Not found	Decrease FH level associated with C3 consumption (CFH deficiency)[d]	No	No
4	Membrane cofactor protein	c.503_504insA p.Asn170LysfsTer7	He	1	Normal	Low	Not found	Decrease MCP expression (MCP deficiency)[d]	No	No
Stx-negative patients with HUS										
5	Thrombomodulin	c.127G>A p.Ala43Thr	He	1	Normal	Normal	0.34	Decreased capacity to inactivate C3b (36)	No	Yes (36)
6	Complement factor H	c.3628C>T p.Arg1210Cys[e]	He	1	Normal	Normal	0.017	Alter the C3b/polyanions–binding site (37)	No	Yes[c] (38)

HUS, hemolytic uremic syndrome; CFH, complement factor H; MCP, membrane cofactor protein; MAF, minor allele frequency; STEC, Shiga toxin producing *E. coli*; aHUS, atypical hemolytic uremic syndrome; He, heterozygous; GAG, glycosaminoglycans; FH, factor H.

[a]At discharge.

[b]MAF in Exome Aggregation Consortium database (http://exac.broadinstitute.org/).

[c]FH aHUS mutation database (http://www.fh-hus.org/).

[d]V.F.-B., personal communication.

[e]Patient 6 with CFH p.Arg1210Cys pathogenic rare variant also carried a C3 variant of uncertain significance.

have shown that four variants are pathogenic as they lead to a reduced capacity to regulate alternative pathway activity (35–38). In total, six pathogenic variants were identified in Shiga toxin–positive (n=4) and Shiga toxin–negative (n=2) patients. Four of these variants were previously reported in aHUS (36,38) and one in STEC-HUS (24) (Table 3). Ten variants of uncertain significance were identified in Shiga toxin–positive (n=8) and Shiga toxin–negative (n=5) patients. Four of these variants of uncertain significance were previously reported in aHUS (39–44) and two in STEC-HUS (10,27) (Supplemental Table 1). Pathogenic rare variants identified in the two control cohorts are described in Supplemental Table 2. Four rare variants were found both in patients with HUS and French controls (Supplemental Table 3). Pathogenic variants were identified in 5%, 6%, 2%, and 3% of Shiga toxin–positive and Shiga toxin–negative patients, and of the French and European control cohorts, respectively (Table 4). Altogether, the frequency of rare variants per gene and per variant categorization was not significantly different between patients with HUS and controls and between Shiga toxin–positive and Shiga toxin–negative patients with HUS (Tables 2 and 4).

A very rare pathogenic variant with minor allele frequency <0.1% was identified in three out of 75 Shiga toxin–positive patients with HUS (4%) (Table 3, cases 2, 3, and 4), compared with none of the 80 French controls (P=0.17) and four of the 503 European controls (0.8%) (OR, 5.2; 95% CI, 1.1 to 24; P=0.03) (Table 4 and Supplemental Table 2). We did not observe a significant difference in the very rare pathogenic variants between Shiga toxin–negative patients with HUS (one out of 33, 3%) (Table 3, case 6) and the French (P=0.2) or European control groups (OR, 3.9; 95% CI, 0.4 to 36; P=0.2) (Table 4).

Homozygous factor H *tgtgt* or membrane cofactor protein *ggaac* haplotypes were found in 3% (three out of 97) and 6% (six out of 97) of patients with HUS, respectively. These frequencies were not significantly different from those in French controls (Supplemental Table 4). None of three patients with anti-factor H antibodies carried a homozygous complement factor H–related 1–complement factor H–related 3 deletion (Supplemental Table 5).

Complement Biomarkers during the Acute Phase

Median plasma levels of C3, factor I, and sC5b-9 were significantly higher, and median membrane cofactor protein expression significantly decreased in patients with HUS compared with French controls (Figure 1 and Supplemental Table 6). C3 and C4 levels were significantly higher in Shiga toxin–negative compared with Shiga toxin–positive patients, whereas other changes were not significantly different between the two groups (Figure 1). No patient had C3 level below the lower limit of normal and only three patients had C3 levels close to the lower limit of normal (three out of 61, 5%) (Supplemental Table 7). The level of sC5b-9 was increased in 66% (38 out of 58) of Shiga toxin–positive patients with HUS and membrane cofactor protein expression decreased in 57% (27 out of 47). Decreased membrane cofactor protein expression was significantly correlated with shorter delay of blood sampling (mostly within 4 days after admission) in Shiga toxin–positive patients (P=0.002; Figure 2A). In 11 documented patients, membrane cofactor protein expression normalized after HUS remission (Figure 2B). The level of sC5b-9 was not significantly correlated with delay in blood sampling within the first 14 days of admission or with leukocyte count (Supplemental Figures 2 and 3). Three patients had anti-factor H antibodies, at a low titer (<1000 AU/ml) (Supplemental Table 5).

Correlations between Complement Genetics, and Severity of HUS, sC5b-9 Levels during the Acute Phase, or CKD Stage at Last Follow-Up in Shiga Toxin–Positive Patients with HUS

The clinical characteristics and outcomes of the six patients with postdiarrheal HUS with pathogenic variants are presented in Table 5, and those of patients with C3 level at the lower limit of normal or anti-factor H antibodies in Supplemental Tables 5 and 7, respectively. The sC5b-9 level during the acute phase was not significantly different in Shiga toxin–positive patients with HUS with or without rare variant identified (Supplemental Figure 3). In Shiga toxin–positive patients with HUS, the frequency of increased sC5b9, or of pathogenic variants, variants of uncertain significance or no variant identified was not different in patients requiring dialysis or not, in patients with and without CNS manifestations during the acute phase, and in patients with residual CKD stage 1–4 or CKD stage 5 compared with patients without kidney sequela (no CKD) at last follow-up (Table 6).

One of the four Shiga toxin–positive patients who carried a pathogenic variant progressed to ESKD within 3 years, compared with none of the 43 Shiga toxin–positive patients without pathogenic variant and with available clinical data 3 years after the acute episode (P=0.08).

Discussion

This is the largest case series describing the rare variants in complement genes identified in Shiga toxin–positive patients with HUS. We show that only very rare pathogenic variants with minor allele frequency <0.1% are more frequent in Shiga toxin–positive patients than in controls, which underscores the role of complement activation in Shiga toxin–associated HUS.

We used next-generation sequencing to screen the six genes implicated in aHUS pathogenesis, in 108 children with a clinical diagnosis of postdiarrheal HUS, including 75 children with Shiga toxin–positive HUS and 33 with Shiga toxin–negative HUS, and in 80 French controls. We found a rare variant in one or two genes in 16% of the Shiga toxin–positive patients, but only 5% of these patients carry a pathogenic variant previously demonstrated to lead to the impairment of complement regulatory activity (35–38). To categorize the clinical relevance of genetic findings, we first analyzed whether patients are enriched for complement rare variants compared with controls. We found a similar frequency of rare variants in the total cohort of 108 patients with HUS (17%), and in 80 French controls (14%) and 503 European individuals (12%), and no significant differences of variants' frequency whatever the gene and the variant categorization (pathogenic or variant of uncertain significance) between Shiga toxin–positive patients with HUS and French or 1000 Genomes Project controls. Still, when we focused our analysis on very rare pathogenic variants

Table 4. Number and frequency (%) of patients with postdiarrheal HUS and of French controls and controls from the 1000 Genomes Project database, who carried at least one rare variant in one of the six tested complement genes

	Postdiarrheal HUS, Total Cohort, n=108[a]	French Controls, n=80[b]	1000 Genomes Controls, n=503[b]	Patients with HUS versus French Controls	Patients with HUS versus 1000 Genomes Controls	Stx-Positive HUS, n=75[c,d]	Stx-Negative HUS, n=33[c,e]	Stx-Positive versus Stx-Negative HUS	Stx-Positive HUS versus 1000 Genomes Controls
	n (%)	n (%)	n (%)	OR; 95% CI; P-Value[f]	OR; 95% CI; P-Value[f]	n (%)	n (%)	OR; 95% CI; P-Value[f]	OR; 95% CI; P-Value[f]
Individuals with a variant with MAF <1%	18 (17)	11 (14)	60 (12)	1.2; 0.5 to 3; 0.7	1.4; 0.8 to 2.6; 0.2	12 (16)	6 (18.2)	0.8; 0.3 to 2.5; 0.7	1.4; 0.7 to 2.9; 0.34
Individuals with at least a pathogenic variant with MAF <1%	6[g] (6)	2[h] (2)	14 (3)	3; 0.4 to 12; 0.5	2; 0.8 to 5; 0.1	4 (5.3)	2[i] (6.1)	0.9; 0.2 to 5; >0.99	1.9; 0.6 to 6; 0.3
Individuals with a VUS with MAF <1%	12 (11)	9[h] (11)	46 (9)	0.9; 0.4 to 2; >0.99	1.2; 0.6 to 2; 0.3	8 (10.7)	4 (12.1)	0.9; 0.2 to 3; >0.99	1.2; 0.5 to 3; 0.6
Individuals with a pathogenic variant with MAF <0.1%	4 (4)	0 (0)	4 (0.8)	0.17	4.8; 1.2 to 19; 0.04	3 (4)	1 (3)	1.3; 0.1 to 13; >0.99	5.2; 1.1 to 24; 0.03

HUS, hemolytic uremic syndrome; Stx, Shiga toxin; OR, odds ratio; 95% CI, 95% confidence interval; VUS, variant of uncertain significance.
[a]Five of the 113 patients with postdiarrheal HUS were not screened for variants in the six tested complement genes.
[b]All controls were screened for variants in the six tested complement genes.
[c]Four of the 79 patients with Stx-positive HUS and one of the 34 patients with Stx-negative HUS were not screened for variants in the six complement genes.
[d]Frequency of pathogenic variants with MAF <0.1% in Stx positive-HUS patients versus French controls: P=0.17.
[e]Frequency of pathogenic variants with MAF <0.1% in Stx negative-HUS patients versus French controls: P=0.2; versus 1000 Genomes controls: OR, 3.9; 95% CI, 0.4 to 36; P=0.2.
[f]P with Fisher exact test.
[g]One of the six patients also carried a C3 VUS.
[h]One control had a thrombomodulin pathogenic variant and a C3 VUS, and another control had two C3 VUS.
[i]One of the two patients also carried a C3 VUS.

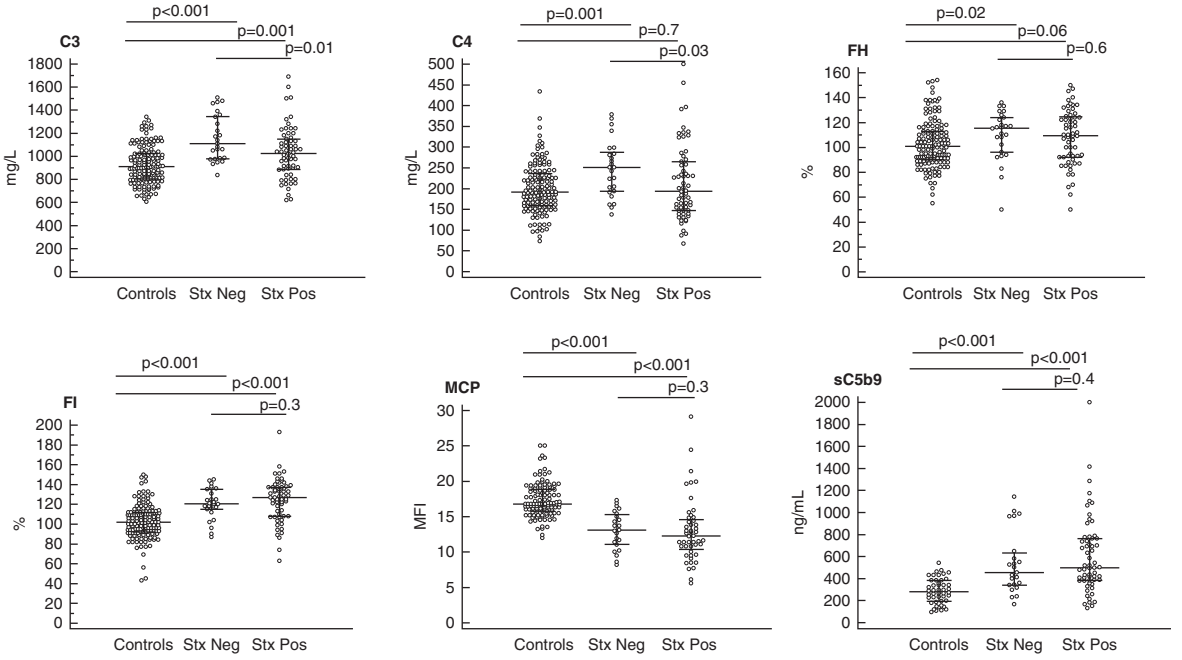

Figure 1. | Increased C3, factor I and sC5b9 plasma levels and decreased membrane cofactor protein expression during the acute phase of postdiarrheal HUS. Median delay (First and third quartiles) of blood sampling after admission was 4 days (1; 6) (from admission to 13 days postadmission) for the total cohort, 2.5 days (1; 4.8) in Shiga toxin–positive patients with HUS, and 5.5 days (2.3; 9) in Shiga toxin–negative patients with HUS. Plasma samples collected under plasma infusion/plasma exchange (n=3) or eculizumab (n=6), or ≥14 days after admission (n=12) were excluded. See Supplemental Figure 1 and Supplemental Table 7 for the number of patients studied. Results of the Mann–Whitney tests are indicated. FH, complement factor H; FI, complement factor I; MCP, membrane cofactor protein; Stx neg, Shiga toxin–negative; Stx pos, Shiga toxin–positive.

with minor allele frequency <0.1%, we showed that such very rare pathogenic variants were associated with a five-fold higher risk of developing Shiga toxin–positive HUS. Thus, although Shiga toxin–associated HUS is mostly driven by the infectious agent, our study highlights that in rare cases, genetic abnormalities may contribute to complement activation in Shiga toxin–associated HUS, consistent with published data (9,10,20–28). However, our study did not include patients with Shiga toxin infection who did not develop HUS (a study difficult to complete outside of large epidemics) and thus cannot prove the role of genetics in the risk of developing HUS after Shiga toxin infection.

We next hypothesized that the genetic background may influence the disease clinical course. We did not find significant differences in the frequency of CNS manifestations and dialysis requirement during the acute phase, or incident CKD after long-term follow-up in Shiga toxin–positive patients with HUS with complement pathogenic variants, variants of uncertain significance, or no variant. Still, the only patient who progressed to ESKD within 3 years carried a factor H pathogenic variant leading to functional factor H deficiency (patient 1, Table 5), previously identified in another patient with severe STEC-HUS rescued by plasma exchanges (24) (patient 1, Supplemental Table 8). It should, however, be mentioned that our patient presented with relatively high hemoglobin levels, leukocytosis, and neurologic manifestations during the acute phase, which are known risk factors for poor kidney outcome. Similarly, no recovery of kidney function was documented in one of six (16%) Shiga toxin–positive patients with HUS who carried pathogenic variants reported in the literature (Supplemental Table 8). In unselected cohorts of STEC-HUS, only 1.4%–3% (45,46) of children progressed to ESKD within 4–5 years. In our relatively small cohort of Shiga toxin–positive patients with HUS, we observed a trend (P=0.06) toward a significant correlation between the identification of a pathogenic variant and ESKD at last follow-up. However, the role of hereditary complement abnormalities needs to be confirmed by a large international study. Finally, considering our data and prior case reports of STEC infection unmasking complement deficiency, genetic screening does not appear justified for all patients with postdiarrheal HUS, but should be considered in patients with a fulminant course to death or ESKD (19,20,22,27), progression to ESKD within <3 years (patient 1, Table 5), a family history of HUS (23) (patient 6, Table 5), relapse of HUS (21,23,26), or post-transplant recurrence (22,27).

We observed significantly increased C3 and factor I median levels in Shiga toxin–positive patients, confirming prior reports (9,10). Except for significantly lower C3 and C4 levels in Shiga toxin–positive compared with Shiga toxin–negative patients, complement biomarkers did not differ between the two groups. Interestingly, C3 levels close to the lower limit of normal were observed in only three patients (5% of the cohort). Recent reports also observed slightly decreased C3 levels in only four out of 25 (9) and five out of 21 (10) patients with STEC-HUS, and exceptionally severely decreased level (one out of 21) (10). We confirm that 66% of Shiga toxin–positive patients have an increase of sC5b-9 level, the commonly used marker of

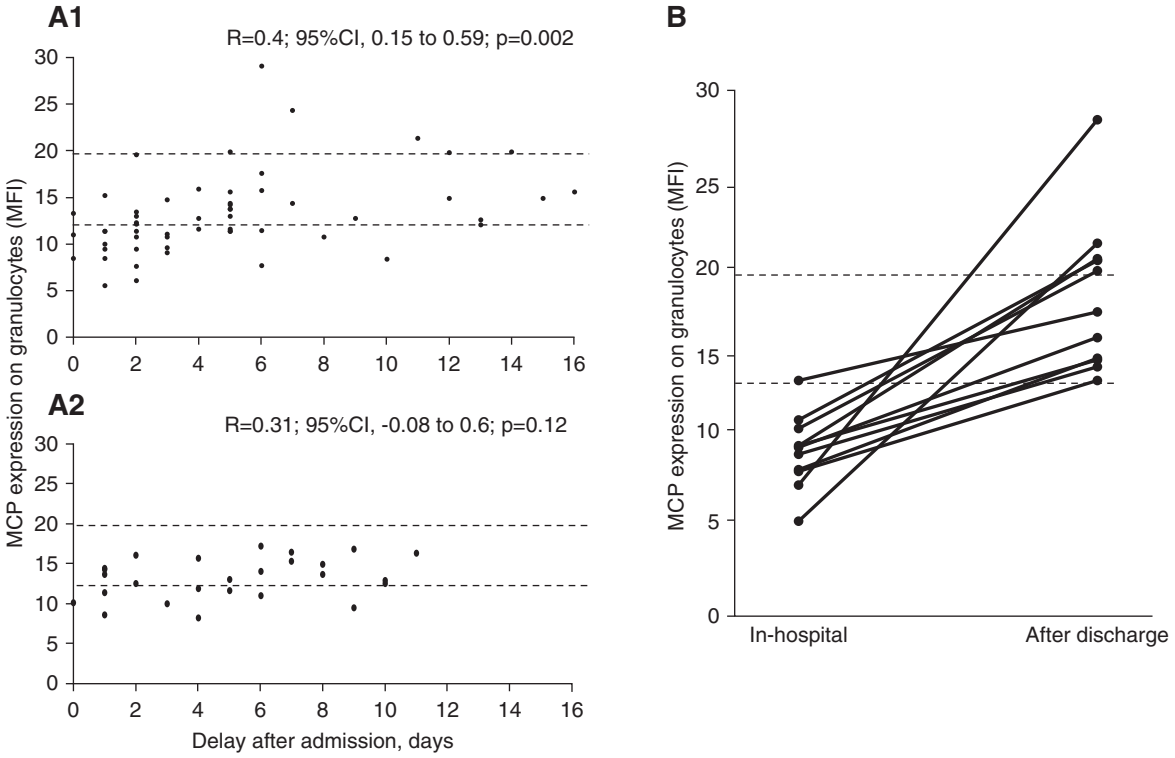

Figure 2. | **Decreased membrane cofactor protein expression during the first days of Shiga toxin positive HUS.** (A) Membrane cofactor protein expression in postdiarrheal HUS, according to delay between admission and blood sampling. MCP expression (normal range: 13–19 MFI, indicated between dashed lines) during the acute phase of HUS was documented in 56 Shiga toxin–positive patients (A1) and 26 Shiga toxin–negative patients (A2). It was below the lower limit of normal in 15 out of 20 (75%) and in four out of eight of Shiga toxin–positive or Shiga toxin–negative patients with HUS with blood sampling performed within 48 hours of admission, respectively. Pearson correlation coefficients are indicated for each scatter plot. A linear relationship between MCP expression and the delay of blood sampling after admission was observed in Shiga toxin–positive HUS. (B) Normalization of MCP expression after remission (11 patients documented). Median (First and third quartiles) MCP expression was 10 MFI (8; 11) in hospital (9.6±2.1 days after admission) and 18 MFI (15; 21) after discharge (18.6±4.4 days after admission) ($P<0.001$ using paired samples *t* test). MCP, membrane cofactor protein; MFI, mean fluorescence intensity.

terminal complement activation pathway, during the acute phase, as previously reported in 59%–64% (9,10) to 100% of patients (6–8). Our results do not support a link between complement activation at the acute phase and the presence of variants in complement genes, confirming that complement activation is predominantly a Shiga toxin–induced phenomenon. Complement biomarkers reflect the balance between increased synthesis related to inflammation and consumption related to complement activation (47). Increased sC5b-9 levels were not significantly correlated with higher frequency of dialysis requirement or CNS manifestations at the acute phase, as previously reported (6), or CKD at last follow-up. Therefore, our study did not support that C5 activation may be associated with STEC-HUS severity at the acute phase. However, our data do not allow taking position on the place of complement blockade therapy in STEC-HUS.

We show for the first time a significantly decreased membrane cofactor protein expression at the acute phase of Shiga toxin–positive HUS, correlated with shorter delay of blood sampling. *In vitro* studies have shown that Shiga toxin does not influence membrane cofactor protein expression on glomerular endothelial cells surface (48). Heme-induced decreased expression of membrane cofactor protein on cells, as reported in aHUS (49), is a more likely explanation. In practice, clinicians should be aware that decreased membrane cofactor protein expression during the acute phase of postdiarrheal HUS is not sufficient to conclude that the patient has aHUS related to a membrane cofactor protein variant, unless decreased expression persists after remission, as observed in one patient (patient 4, Table 5).

Our series also illustrates the limitations of STEC biologic investigations to classify patients as having typical HUS or aHUS. Positive or negative Shiga toxin screening is frequently used to support the diagnosis of Shiga toxin–associated HUS or aHUS (4), respectively, and this influences therapeutic decisions. In clinical practice, the challenge is whether patients with a clinical diagnosis of postdiarrheal HUS who have a negative Shiga toxin PCR should be classified as aHUS. Such patients in our cohort remain classified as postdiarrheal HUS with unproven Shiga toxin/STEC infection, possibly due to antibiotic treatment or late stool collection. Interestingly, 33% of children with aHUS (Shiga toxin/STEC negative) do not carry any complement variant and have an overall favorable outcome (50), similar to Shiga toxin–negative patients with postdiarrheal HUS in this study.

In conclusion, our results show an overall limited role of rare variants in complement genes in Shiga toxin–positive

Table 5. Clinical characteristics, in hospital course, and outcome of the six patients with postdiarrheal HUS, who carried complement pathogenic rare variants

		In-Hospital Course									Outcome		
Patient No. Sex, Age, yr	Genetic Complement Abnormalities[a]	C3[b,c], mg/L	sC5b-9[b,c], ng/ml	Stool Stx PCR (STEC Serogroup)	Hb[c], g/dl	Plt[c], /mm^3	WBC[c], /mm^3	Screat[c], mg/dl	Dialysis Duration, d	Systemic Manifestations	PI/PE and/or Eculizumab	Follow-up, yr	Sequels[d]	Relapse
Stx-positive patients with HUS														
1. F, 2.6	CFH, p.Gln950His	975	479	Stx2 positive (O157 in stool)	9.8	81,000	25,600	6.1	25	CNS	No	5.8	CKD stage 5 (ESKD) at 3 yr follow-up Kidney graft at 4 yr follow-up	No
2. M, 2.0	THBD, p.Pro495Ser	1240	519	Stx2 positive (O80 in stool)	5.8	41,000	13,070	3.9	0	None	No	4.2	No CKD	No
3. F, 2.4	CFH, p.Ala382Glu	914	433	Stx2 positive (O157 in stool)	6.9	34,000	17,400	0.6	0	None	No	3	No CKD	No
4. M, 3.5	MCP, p.Asn170LysfsTer7	1090	182	Stx2 positive (ND)	7.2	400,000	7200	0.9	0	None	No	4.8	CKD stage 1 Proteinuria; eGFR 108 ml/min per 1.73 m^2	No
Stx-negative patients with HUS														
5. M, 13.5	THBD, p.Ala43Thr	1180	231	Stx negative (stool culture and serology negative)	5.0	59,000	7900	163	3	None	No	4.4	No CKD	No
6. M, 4.4[e]	CFH, p.Arg1210Cys+C3 p.Asp1440Ala VUS	1210	557	Stx negative (only O157 serology positive)	5.8	30,000	9000	4	7	Pancreatitis	No	4.5	No CKD	No

HUS, hemolytic uremic syndrome; Stx, Shiga toxin; STEC, Shiga toxin–producing *E. coli*; Hb, hemoglobin; Plt, platelet count; WBC, white blood cell; Screat, serum creatinine; PI, plasma infusion; PE, plasma exchange; F, female; CFH, complement factor H; M, male; CNS, central nervous system; THBD, thrombomodulin; MCP, membrane cofactor protein; VUS, variant of uncertain significance.

[a]See Table 3.

[b]Normal range: C3: 615–1250 mg/L; sC5b-9: <420 ng/ml; Conversion factor for serum creatinine from mg/dl to μmol/L: ×88.4.

[c]At admission.

[d]CKD stages according to Kidney Disease Improving Global Outcomes Guidelines 2012 (http://www.kdigo.org/clinical_practice_guidelines/pdf/CKD/KDIGO_2012_CKD_GL.pdf). See Supplemental Material for definition of CKD stages.

[e]Patient 6's first cousin had HUS 4 years after patient 6.

Table 6. Severity of HUS according to sC5b-9 level and genetic background in Shiga toxin–positive patients with HUS

Complement Abnormalities	Acute Phase						Last Follow-Up[a]				
	Dialysis Required			CNS Manifestations			No CKD[b]	CKD Stage 1–4[b]	CKD Stage 5[b]	CKD Stage 1–4 versus No CKD	CKD Stage 5 versus No CKD
	Yes, *N* (%)	No, *N* (%)	OR; 95% CI; *P*-Value[c]	Yes, *N* (%)	No, *N* (%)	OR; 95% CI; *P*-Value[c]	*n* (%)	*n* (%)	*n* (%)	OR; 95% CI; *P*-Value[c]	OR; 95% CI; *P*-Value[c]
Increased sC5b-9 (>420 ng/ml)[d]	23/33 (70)	15/25 (60)	1.5; 0.5 to 4.6; 0.2	11/13 (85)	27/45 (60)	3.6; 0.7 to 18; 0.1	25/36 (69)	9/18 (50)	1/1 (100)	2.3; 0.7 to 7; 0.2	2.3; 0 to 19; 0.4
Pathogenic variant (*n*=4)	1/42 (2)	3/33 (9)	0.2; 0 to 2.4; 0.2	1/17 (6)	3/58 (5)	1.1; 0.1 to 12; 0.9	2/48 (4)	1/20 (5)	1/1 (100)	0.8; 0.1 to 9; >0.99	55; 1.8 to 1747; 0.06
Variant of uncertain significance (*n*=8)	5/43 (12)	3/32 (9)	1.2; 0 to 5.8; 0.9	0/17 (0)	8/58 (14)	0.2; 0 to 3; 0.2	7/48 (15)	1/20 (5)	0/1 (0)	3.2; 0.3 to 28; 0.4	0.5
No variant identified	37/43 (86)	26/32 (81)	1.4; 0 to 5; 0.3	1/17 (6)	11/58 (19)	0.3; 0 to 2.2; 0.2	39/48 (81)	18/20 (90)	0/1 (0)	0.5; 0.1 to 2.5; 0.4	0.13

HUS, hemolytic uremic syndrome; CNS, central nervous system; OR, odds ratio; 95% CI, 95% confidence interval.
[a]Median (First and third quartiles): 47 months (20–65) (*n*=79 with six lost to follow-up). The CKD stage was documented at 3 year follow-up in 43 patients.
[b]CKD stages according to Kidney Disease Improving Global Outcomes Guidelines 2012 (http://www.kdigo.org/clinical_practice_guidelines/pdf/CKD/KDIGO_2012_CKD_GL.pdf). See Supplemental Material for definition of CKD stages. No patient had CKD stage 4 at last follow-up.
[c]*P* with Fisher exact test.
[d]Samples before day 14 after admission.

HUS. Still, genetic screening should be considered in postdiarrheal patients with HUS who progress rapidly to ESKD.

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V.F.-B., C.L., and A.-L.S.-L. designed the study. V.F.-B. and P.V.-M. performed the genetic screening and the complement assessment. S.L. analyzed the 1000 Genomes Project database. P.M. and F.-X.W. performed the Shiga toxin–producing *Escherichia coli* investigations. V.F.-B., P.V.-M., and C.L. analyzed the data. V.F.-B. and C.L. wrote the manuscript. All authors contributed to patients' recruitment and clinical data collection, discussed the results, and contributed to the final manuscript.

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Disclosures

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Supplemental Material

This article contains supplemental material online at http://cjasn.asnjournals.org/lookup/suppl/doi:10.2215/CJN.05830518/-/DCSupplemental.

Supplemental Table 1. Rare variants of uncertain significance identified in patients with postdiarrheal HUS.

Supplemental Table 2. Pathogenic rare variants identified in French controls and in controls from the 1000 Genomes Project database.

Supplemental Table 3. Rare variants identified both in French controls and in patients with postdiarrheal HUS.

Supplemental Table 4. Frequency of homozygous complement factor H *tgtgt* and membrane cofactor protein *ggaac* haplotypes in patients with postdiarrheal HUS compared with French controls.

Supplemental Table 5. Clinical characteristics, in-hospital course, and outcome of three patients with postdiarrheal HUS, who had anti-factor H antibodies.

Supplemental Table 6. Plasma levels of CH50, C3, C4, factor H, factor I, sC5b9, membrane cofactor protein expression, and anti-factor H antibodies at the acute phase of postdiarrheal HUS.

Supplemental Table 7. In-hospital course and outcome of three patients with postdiarrheal HUS, who had C3 plasma levels close to the lower limit of normal at admission.

Supplemental Table 8. Summary of 17 patients reported in the literature, who had postdiarrheal HUS and carried a complement rare variant.

Supplemental Figure 1. Flow diagram of patients with postdiarrheal HUS included in the study.

Supplemental Figure 2. sC5b-9 plasma level according to (A) delay after admission and (B) leukocyte count.

Supplemental Figure 3. sC5b-9 plasma level during the acute phase in Shiga toxin–positive patients with HUS with (n=7) or without (n=49) rare variant identified.

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A.-L.S.-L. and P.V.-M. contributed equally to this work.

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AFFILIATIONS

[1]Service d'Immunologie, Hôpital Européen Georges Pompidou, Assistance Publique-Hôpitaux de Paris, Paris, France; [2]Team "Complement and Disease," Centre de recherche des Cordeliers, Sorbonne Université, INSERM, Paris, France; [3]Pediatric Nephrology Department, Hôpital Femme Mère Enfant, Hospices Civils de Lyon, Bron, France; [4]Institute for Transplantation in Urology and Nephrology, Centre Hospitalo-Universitaire de Nantes, Centre de Recherche en Transplantation et Immunologie, Institut National de la Santé et de la Recherche Médicale U1064, Université de Nantes, Ecole Centrale de Nantes, Nantes, France; [5]Pediatric Nephrology Department, [12]Unit of Clinical Epidemiology, Institut National de la Santé et de la Recherche Médicale U1123 and Centre d'Investigation Clinique-Epidémiologie Clinique 1426, and [14]Laboratory of Microbiology, *Escherichia coli* Associated National Reference Center, Hôpital Robert Debré, University Paris Diderot, Assistance Publique-Hôpitaux de Paris, Paris, France; [6]Pediatric Nephrology Department, Hôpital Jeanne de Flandre, Centre Hospitalo-Universitaire de Lille, Lille, France; [7]Pediatric Nephrology Department, Centre Hospitalo-Universitaire de Bordeaux, Bordeaux, France; [8]Pediatric Nephrology Department, Centre Hospitalo-Universitaire de Besançon, Besançon, France; [9]Pediatric Nephrology Department, Centre Hospitalo-Universitaire de Nantes, Nantes, France; [10]Pediatric Nephrology Department, Centre Hospitalo-Universitaire de Marseille, Marseille, France; [11]Pediatric Nephrology Department, Hôpital Trousseau, University Pierre and Marie Curie, Assistance Publique-Hôpitaux de Paris, Paris, France; and [13]Institut Pasteur, Unité des Bactéries Pathogènes Entériques, Centre National de Référence des *Escherichia coli*, *Shigella* et *Salmonella*, Paris, France

Complement gene variants and Shiga toxin producing E. coli -associated hemolytic uremic syndrome

Véronique Frémeaux-Bacchi, Anne-Laure Sellier-Leclerc, Paula Vieira-Martins, Sophie Limou, Theresa Kwon, Annie Lahoche, Robert Novo, Brigitte Llanas, François Nobili, Gwenaëlle Roussey, Mathilde Cailliez, Tim Ulinski, Georges Deschênes, Corinne Alberti, François-Xavier Weill, Patricia Mariani, Chantal Loirat.

Supplemental Table of Contents

Supplemental Methods and Patients

Study design

Parental written informed consent was required for entering the study. The study protocol adhered to the Declaration of Helsinki and was approved by the Comité de Protection des Personnes, Ile de France IV (n° IRB 00003835).

Clinical data were prospectively collected at admission, during hospitalisation and at discharge. In April 2017, physicians were asked to document patient's condition at last follow-up.

Caucasian children (< 15 years) with post-diarrheal-HUS were prospectively enrolled in this study from the French Society of Pediatric Nephrology. HUS was defined by the association of at least two of following criteria: mechanical hemolytic anemia (hemoglobin <10g/dL, schizocytosis >1%, lactate deshydrogenase > upper limit of normal (ULN), decreased/undetectable haptoglobin), thrombocytopenia (platelet count < 150 G/L) and acute kidney injury (serum creatinine > ULN for age). Post-diarrheal HUS was defined by prodromal gastro-intestinal symptoms (non bloody or bloody diarrhea, or other gastro-intestinal symptoms (abdominal pain, vomiting)), and Shiga toxin (Stx) producing *E coli* (STEC) infection by specific investigations (see below).

Chronic kidney disease (CKD) stages were defined according to KDIGO[1]. No CKD was defined by estimated (e)GFR ≥ 90 ml/min/1.73m^2 without albuminuria; CKD Stage 1 by eGFR ≥ 90 ml/min/1.73m^2 with albuminuria; Stage 2 by eGFR 60-89 ml/min/1.73m^2 with albuminuria; Stage 3 by eGFR 30-59 ml/min/1.73m^2 with or without albuminuria; Stage 4 by eGFR 15-29 ml/min/1.73m^2 with or without albuminuria; Stage 5 by eGFR < 15 ml/min/1.73m^2 or end stage kidney disease/dialysis. Significant albuminuria or proteinuria were defined by urine albumin/creatinine ratio >30 mg/g or >3mg/mmol or urine protein/creatinine ratio > 200 mg/g or > 20 mg/mmol.

STEC investigations

Investigations for STEC infection included a) Real time polymerase chain reaction (PCR) on stools for Stx1 and Stx2 genes (113 patients) b) Stool culture on selective media for identification and characterization of STEC strains, using PCR for genes coding for 10 frequent STEC serogroups affecting humans in France (O157, O26, O145, O55, O103, O104, O111, O91, O121, and O80) (111 patients) c) Antibody (IgM ± IgA) response to serogroup-specific *E coli* lipopolysaccharides (LPS) (O157, O26, O145, O55, O103, O104, O111, O91, O128) (93 patients)

Stool culture was positive for O157 STEC or other serotypes in 93.5% of Stx positive patients. Serogroup of non-O157 strains in stools was O80 in 8/77 Stx-positive patients (10.3 %), O26 in 5 (6.4%), O104, O121, O145 each in 2, and O2, O5, O98, O103, O111, O177 each in 1, undetermined in 10. Stx-positive patients with O80, O121, O2, O5, O98, O177 STEC in stools (serotypes not included in the serologic screening) had negative anti- LPS serology. In Stx-negative patients, anti-LPS serology was positive for serogroup O157 in 12 patients, O103 in 2, O91, O145 or O111 each in 1.

Patients characteristics

During the prodromal phase, 25 patients received bactericidal antibiotics (mostly amoxicillin/third generation cephalosporin) and 1 patient (Stx negative) received azithromycin. During hospitalization, neurological manifestations occurred in 20 patients, including seizures (12 patients), mental aberration (8), somnolence (8), behaviour disturbances/delirium (5), cranial pairs defect (nystagmus, strabism) (4), coma/decerebration (1). Brain magnetic resonance imaging was documented in 16 patients, showing no abnormalities in 5, ischemic lesions and/or white matter hypersignals in 11. Prolonged hemorrhagic colitis/intestinal symptoms were documented in 27 patients, of whom 13 required parenteral feeding. An additional patient had prolonged cholestasis related to gall

bladder sludge. Cardiac manifestations were pericarditis in 1 patient and cardiogenic shock in another 1.

Plasma infusion (2 in 1 patient, 3 in another patient) and/or plasma exchange (6 patients, who received 1, 1, 2, 6, 10 or 11 sessions, respectively) were administered for neurological manifestations in 7 patients or prolonged hemolysis in 1 patient.

15 patients received eculizumab with a mean of 3 doses (1 to 6), mostly because of neurological manifestations (11/15, 73%). None of the 15 patients carried a pathogenic rare variant. Dialysis was required in 12 of the 15 patients (80%). At median follow-up 5.0 (1- 6) years, documented in 12 patients, 5 (41.6%) had no CKD, 4 (33.3%) had CKD stage 1, 2 (16.6%) had CKD stage 2, and 1 (8.3%) had CKD stage 3b.

During hospitalization, 24 children received azithromycin for ≥5 days (a procedure adopted by several French centres to accelerate intestinal decontamination), 13 received bactericidal antibiotics, and 2 received bactericidal antibiotics after azithromycin. Bactericidal antibiotics were prescribed for infectious complications (e.g. catheter- associated).

Nine patients were lost to follow-up (Supplemental Figure 1)

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Supplemental Table 1. Rare variants of uncertain significance (n=10) identified in 13 of 108 patients with post-diarrheal-HUS.

Gene	Variant	Genetic status	Number of patients with the variant	MAF [a] (%)	Functional studies	Polyphen 2 prediction	Previously reported in STEC-HUS	Previously reported in aHUS	Variant categorization
Stx positive - HUS patients									
C3	c.2203C>T p.Arg735Trp	He	3[b]	0.2	Located in the C3a Minor functional changes [2,3]	Probably damaging	No	Yes[2,4]	VUS
C3	c.4369G>C p.Asp1457His	He	1	0.03626	NA	Probably damaging	No	No	VUS
C3	c.1618G>T p.Ala540Ser	He	1	0.005864	NA	Benign	No	No	VUS
CFB	c.978A>C p.Glu326Asp	He	1	0.0766	NA	Benign	No	No	VUS
CFI	c.782G>A p.Gly261Asp	He	1	0.1326	No demonstrated functional alterations[5]	Benign	No	Yes[5,6]	VUS
THBD	c.829G>T p.Gly277Trp	He	1	0.001544	NA	Probably damaging	No	No	VUS
Stx negative - HUS patients									
CFH	c.2867C>T p.Thr956Met	He	1	0.1211	No demonstrated functional alterations[7]	Possibly damaging	Yes[8]	Yes[9]	VUS
C3	c.4855A>C p.Ser1619Arg	He	2[b]	0.1096	Located in the C345C domain No demonstrated functional alterations[3]	Possibly damaging	Yes[10]	Yes[11]	VUS
C3	c.4177C>T p.Arg1393Trp	He	1	0.004121	NA	Possibly damaging	No	No	VUS
C3	c.4319A>C p.Asp1440Ala	He	1[c]	0.02965	NA	Benign	No	No	VUS

a. MAF, minor allele frequency in Exome Aggregation Consortium database http://exac.broadinstitute.org/
b. One of the patients with C3 p.Arg735Trp VUS and one of those with C3 Ser1619Arg VUS also carried a MCP p.Ala353Val pathogenic variant of frequency > 1% in the general population
c. This patient with C3 p.Asp1440Ala VUS also carried a CFH p.Arg1210Cys pathogenic variant (Patient 6, Table 4)

aHUS: atypical hemolytic uremic syndrome; CFB: complement factor B; CFH: complement factor H; CFI: complement factor I; He: heterozygous; MCP: membrane cofactor protein; NA: not available; N: normal; Stx: Shiga toxin; STEC: shiga toxin producing *E.coli*; THBD: thrombomodulin; VUS : variant of uncertain significance

Supplemental Table 2. Pathogenic rare variants identified in French controls (n=1) and in controls from the 1000 Genomes data base (n=7).

Gene	Variant	Genetic status	Number of controls with the variant	MAF [a] (%)	Demonstrated functional alterations	Polyphen 2 prediction	Identified in our cohort of post-diarrheal - HUS	Previously reported in aHUS	Variant categorization
French controls (N=80)									
THBD	c.127G>A p.Ala43Thr	He	2[b]	0.3	Decreased capacity to inactivate C3b[12]	Benign	Yes (1 patient)	Yes[12]	Pathogenic
1000 Genomes (N=503)									
THBD	c.127G>A p.Ala43Thr	He	5	0.343	Decreased capacity to inactivate C3b[12]	Benign	Yes (1 patient)	Yes[12]	Pathogenic
CFI	c.161G>T p.Cys54Phe	He	1	Not found	Low FI level in plasma (FI deficiency)[13]	Probably damaging	No	Yes[c]	Pathogenic
CFH	c.3356A>G p.Asp1119Gly	He	1	0.02	Located in disease-related functional domain[7]	Probably damaging	No	Yes[d]	Pathogenic
MCP	c.565T>G p.Tyr189Asp	He	1	0.00082	Lack of synthesis[14]	Probably damaging	No	Yes[d]	Pathogenic
CFI	c.485G>A p.Gly162Asp	He	1	0.00082	Low circulating FI[13]	Probably damaging	No	Yes [c]	Pathogenic
C3	c.463A>C p.Lys155Gln	He	2	0.3362	Impaired degradation of C3 by FI[3]	Benign	No	Yes[c]	Pathogenic
THBD	c.1502C>T p.Pro501Leu	He	3	0.2276	Decreased capacity to inactivate C3b[12]	Possibly damaging	No	Yes[12]	Pathogenic

The ultra rare variants with MAF <0.1 % are p.Asp1119Gly (CFH); p.Tyr189Asp (MCP); p.Gly162Asp (CFI) and p.Cys54Phe (CFI)

a. MAF, minor allele frequency in Exome Aggregation Consortium database http://exac.broadinstitute.org/
b. One French control with THBD p.Ala43Thr pathogenic variant also carried a C3 p.Arg735Trp VUS
c. Author VFB, personal communication: CFI p.Cys 54 Phe and p.Gly162Asp variants and C3 p.Lys155Gln variant found in aHUS patients (French cohort)
d. Atypical HUS mutation database, http://www.fh-hus.org/

Supplemental material is neither peer-reviewed nor thoroughly edited by CJASN. The authors alone are responsible for the accuracy and presentation of the material.

aHUS: atypical hemolytic uremic syndrome; CFH: complement factor H; CFI: complement factor I; He: heterozygous; MCP: membrane cofactor protein; THBD: thrombomodulin; VUS : variant of uncertain significance

Supplemental Table 3. Rare variants identified both in patients with post-diarrheal-HUS and in French controls and European controls from the 1000 Genomes data base

Gene	Variant	Variant categorization	Post-diarrheal –HUS patients (n=108)		French controls (n=80)		HUS patients versus French controls	European controls N=503		HUS patients versus European controls
			Number of HUS patients with the variant	Frequency (%)	Number of controls with the variant	Frequency (%)	P[a]	Number of controls with the variant	Frequency (%)	P[a]
CFH	c.2867C>T p.Thr956Met	VUS	1	0.9	1	1	0.8	1	0.2	0.3
C3	c.2203C>T p.Arg735Trp	VUS	3	3	2	2	0.9	2	0.39	0.04
C3	p.Ser1619Arg	VUS	2	2	1	1	0.7	2	0.39	0.1
THBD	c.127G>A p.Ala43Thr	Pathogenic	1	0.9	2	2	0.4	5	0.99	0.99

a. Fisher exact test

CFH: complement factor H; HUS: hemolytic uremic syndrome; THBD: thrombomodulin; VUS: variant of uncertain significance

Supplemental Table 4. Frequency of homozygous CFH *tgtgt* and MCP *ggaac* haplotypes in 97 patients with post-diarrheal -HUS compared to 80 French controls.

	French controls		Post-diarrheal -HUS patients		HUS patients versus French controls
	Number of controls tested	Number with the haplotype (%)	Number of patients tested	Number with the haplotype (%)	P[a]
CFH *tgtgt* haplotype	80	3 (4)	97	3 (3)	0.8
MCP *ggaac* haplotype	80	5 (6)	97	6 (6)	0.9
CFH *tgtgt*+ MCP*ggaac*	80	0	97	0	0.9

a. Fisher exact test

CFH: complement factor H; HUS: hemolytic uremic syndrome; MCP: membrane cofactor protein

Supplemental Table 5. Clinical characteristics, in hospital-course and outcome of 3 patients with post-diarrheal-HUS and anti-FH antibodies

		In-hospital-course											Outcome		
Patient Gender Age, y	Complement abnormalities	C3[a] mg/L	MCP [a] MFI	sC5b9[a] ng/mL	Stool Stx PCR (STEC serogroup)	Hb[b] g/dL	Plt[b] /mm^3	WBC[b] /mm^3	Screat[b] mg/dL	Dialysis duration days	Extra-renal manifestations	PI/PE and/or eculizumab	F-up y	Sequels[c]	Relapse
1. M 5.2	Anti-FH Ab, 570 AU/mL[d] No CFHR1/R3 deletion	1470	13.7	527	Stx negative (Only O111 serology positive)	5.8	21000	9170	0.97	0	None	No	4.7	CKD2 Proteinuria; eGFR 83 mL/min/1.73m^2	No
2. F 0.5	Anti-FH Ab, 190 AU/mL[d] No CFHR1/R3 deletion	762	11.4	518	Stx2 positive (Not typable STEC in stool)	5.9	25000	ND	1.1	0	None	No	1.0	No CKD	No
3, F 2.9	Anti-FH Ab, 500 AU/mL[d] C3 VUS p.Ser1619Arg No CFHR1/R3 deletion	1050	15.8	456	Stx negative (Only O157 serology positive)	6.4	95000	27300	0.7	0	None	No	4.8	No CKD	No

a. Normal range: C3: 615-1250 mg/L; MCP: 13-19 MFI; sC5b9 :< 420 ng/mL; Conversion factor for serum creatinine from mg/dL to µmol/L : x 88.4
b. At admission
c. CKD stages according to KDIGO 2012[1]. http://www.kdigo.org/clinical_practice_guidelines/ pdf/CKD/KDIGO_2012_CKD_GL.pdf. For definition of CKD stages, see Supplemental Methods and Patients
d. Anti-FH antibody titre: Positive threshold is 100 AU/mL. Patients with anti-FH antibody-associated aHUS (outside of STEC infection) have titres >1000 AU/mL. · Patient 1 had persistent anti-CFH antibody (620 AU/mL) at 4.7 years follow-up. Patient 2 had persistent anti-FH antibody (268 AU/ml) at 2 months follow-up (not documented subsequently). Anti-FH antibodies titre was not documented during follow-up in patient 3. None of the 3 patients carried a homozygous CFHR1/R3 deletion, contrary to approximately 90% of patients with anti-FH antibodies-associated aHUS.

Ab: antibody; aHUS: atypical hemolytic uremic syndrome; AU: arbitrary unit; FH: complement factor H;CFHR: Complement factor H-related protein; CKD: chronic kidney disease; eGFR: estimated glomerumar filtration rate; F: female; F-up: follow-up; Hb: haemoglobin; M: male; MCP: membrane cofactor protein; MFI: Mean Fluorescence Intensity; PCR: polymerase chain reaction; PI: plasma infusion; PE: plasma exchange; Plt: platelet count; Screat: serum creatinine; STEC: Shigatoxin –producing *E coli*; Stx: shiga toxin;; WBC: white blood cell

Supplemental Table 6. Plasma levels of CH50, C3, C4, FH, FI, and sC5b9, MCP expression and anti-FH antibodies at the acute phase of post-diarrheal-HUS. Median delay of blood sampling after admission was 4 days (Q1;Q3:1;6) (from admission to 13 days post-admission) for the total cohort. Median delay of blood sampling after admission was 2.5 days (Q1;Q3 1; 4.8) and 5.5 days (Q1;Q3 ; 2.3; 9) in Stx positive and Stx negative-HUS patients respectively. Plasma samples collected under PI/PE (n=3) or eculizumab (n=6) or after day 14 (n=12) were excluded from the analysis.

(Normal range)	C3 (615-1250 mg/L)		C4 (90-320 mg/L)		FH (70-140%)		FI (70-140%)		MCP (13-19 MFI)		sC5b9 (< 420 ng/mL)		Anti-FH antibody (> 100 AU)	
Stx status	Stx pos	Stx neg	Stx pos	Stx neg	Stx pos	Stx neg	Stx pos	Stx neg	Stx pos	Stx neg	Stx pos	Stx neg	Stx pos	Stx neg
N patients	61	29	61	29	59	30	59	30	47	27	58	27	55	27
Median (Q1;Q3)	1025 (886;1150)	1025 (979;1345)	194 (147;265)	251 (193;287)	109 (92;125)	115 (96;124)	127 (108;137)	120 (115;135)	12 (10;14)	13 (11;15)	498 (381;761)	456 (339;632)		
< LLN, N (%)	0	0	2(2)	0	3 (5)	1 (3)	1 (2)	0	27 (57)[a]	12 (44)[a]	0	0		
> ULN, N (%)	8 (13)	8 (27)	12 (19)	4 (14)	3 (5)	1 (3)	10 (17)	4 (11)	7 (14)	0	38 (66)[a]	14 (52)[a]	1 (2)	2 (7)
Within normal limits, N (%)	53(87)	21 (72)	47(77)	25 (86)	53 (90)	28 (93)	48 (81)	26(87)	13 (28)	15 (55)	20 (34)	13 (48)		

a. sC5b9 level was above the upper limit of normal in 61% (52/85) of patients with post- diarrheal/Shiga toxin positive or negative-HUS and MCP expression below the lower limit of normal in 53% (39/74)

AU: arbitrary unit; FH: factor H; FI: factor I; HUS: hemolytic uremic syndrome; LLN: lower limit of normal; MCP: membrane cofactor protein; MFI: Mean Fluorescence Intensity; N: number of patients; PE: plasma exchange; PI: plasma infusion; Stx: Shiga toxin; ULN: upper limit of normal

Supplemental Table 7. In-hospital course and outcome of 3 patients with post-diarrheal-HUS and C3 plasma levels close to the lower limit of normal at admission.

		In-hospital course											Outcome		
Patient Gender Age, y	Rare variant or anti-FH Ab	C3[a] mg/L	MCP[a] MFI	sC5b9[a] ng/mL	Stool Stx PCR (Stool STEC serogroup)	Hb[b] g/dL	Plt[b] /mm^3	WBC[b] /mm^3	Screat [ab] mg/dL	Dialysis duration days	Extra-renal manifestations	PI/PE and/or eculizumab	F-up y	Sequels[c]	Relapse
M, 6.7	No	663	6.1	901	Stx1 and Stx2 positive (O157)	6.0	34000	38000	3.3	43	CNS Pancolitis Pancreatitis Cardiogenic shock	2 PE Eculizumab (6 doses)	5.7	CKD3 Proteinuria; eGFR 42 mL/min/1.73m^2	No
M, 4.5	No	619	13	518	Stx2 positive (undetermined)	10.8	29000	7500	1.5	4	CNS	11 PE	6.0	CKD1 Proteinuria; eGFR 160 mL/min/1.73m^2	No
M, 1.8	No	626	9.9	452	Stx2 positive (O157)	7.8	140000	25200	2	22	CNS Diabetes	1 PI, 1 PE Eculizumab (9 doses)	5.7	No CKD Persistent diabetes	No

a. Normal range: C3: 615-1250 mg/L; MCP: 13-19 MFI; sC5b9 :< 420 ng/mL; Conversion factor for serum creatinine from mg/dL to µmol/L to: x 88.4
b. At admission
c. CKD stages according to KDIGO 2012[1] . ,http://www.kdigo.org/clinical_practice_guidelines/ pdf/CKD/KDIGO_2012_CKD_GL.pdf. For definition of CKD stages, see Supplemental Methods and Patients

Ab: antibody; FH: factor H; CNS: central nervous system; eGFR: estimated glomerular filtration rate; f-up: follow-up; Hb: haemoglobin; LPS: lipopolysaccaride; M: male; MCP: membrane cofactor protein; MFI: Mean Fluorescence Intensity; PCR: polymerase chain reaction; PI: plasma infusion; PE: plasma exchange; Plt: platelet count; Screat: serum creatinine; STEC: Shiga toxin producing E coli; Stx: Shiga toxin; WBC: white blood cell; y: year

Supplemental Table 8. Summary of the clinical course of 17 patients reported in the literature, who had post-diarrheal-HUS and carried a complement rare variant[a].

Patients	Age at HUS (y)	Stx in stool	Stool culture or anti-LPS serology	Outcome	Complement Variant[b]	Genetic categorization[c]	MAF [d] (%)	Demonstrated functional alterations	Author, year
Pediatric onset									
Stx positive - HUS patients									
1	10	Stx positive	Stool culture negative	Severe renal failure + lethargy/confusion until initiation of PE at day 12 Full recovery and no relapse after PE discontinuation at day 50 (follow-up 1 year)	CFH p.Gln950His	Pathogenic	0.36	Moderately decreased binding to GAG and/or C3b (Hemolytic assay)[16]	McCoy et al, 2014[17]
2	0.7	Stx positive	*E. Coli* in stool	Relapse of HUS (STEC-negative) at age 3 Remission at age 3.5	MCP p.Phe242Cys + CFH p.Gly1194Asp	Pathogenic + VUS	Not found 0.003	Decrease MCP expression (MCP deficiency)[18] NA for the CFH variant	Noris et al, 2010[18] and communication
3	16	Stx1/Stx2 positive	O157 in stool	No recovery of renal function Post-LRD (mother) transplant recurrence (7 months post-transplant) at age 18; graft loss	MCP c.286+2T>G Also carried by the mother	Pathogenic	0.003	Decreased MCP expression (MCP deficiency) . Affects splicing[14]	Alberti et al, 2013[19]
4	1.5	Stx2 positive	*E. Coli* in stool	Very low C3 level at the acute phase Full recovery and no relapse under eculizumab at 1.5 y follow-up	CFH p.Trp701X	Pathogenic	Not found	Decreased FH in plasma, predicted deleterious effect[20]	Caillaud et al, 2016[20]
5	1.6	Circulating Stx positive	O26 serology positive	Full recovery and no relapse at 6 y follow-up	MCP c.286+2T>G	Pathogenic	0.003	Decreased MCP expression (MCP deficiency)[14]	Ardissino et al, 2016[21] and communication

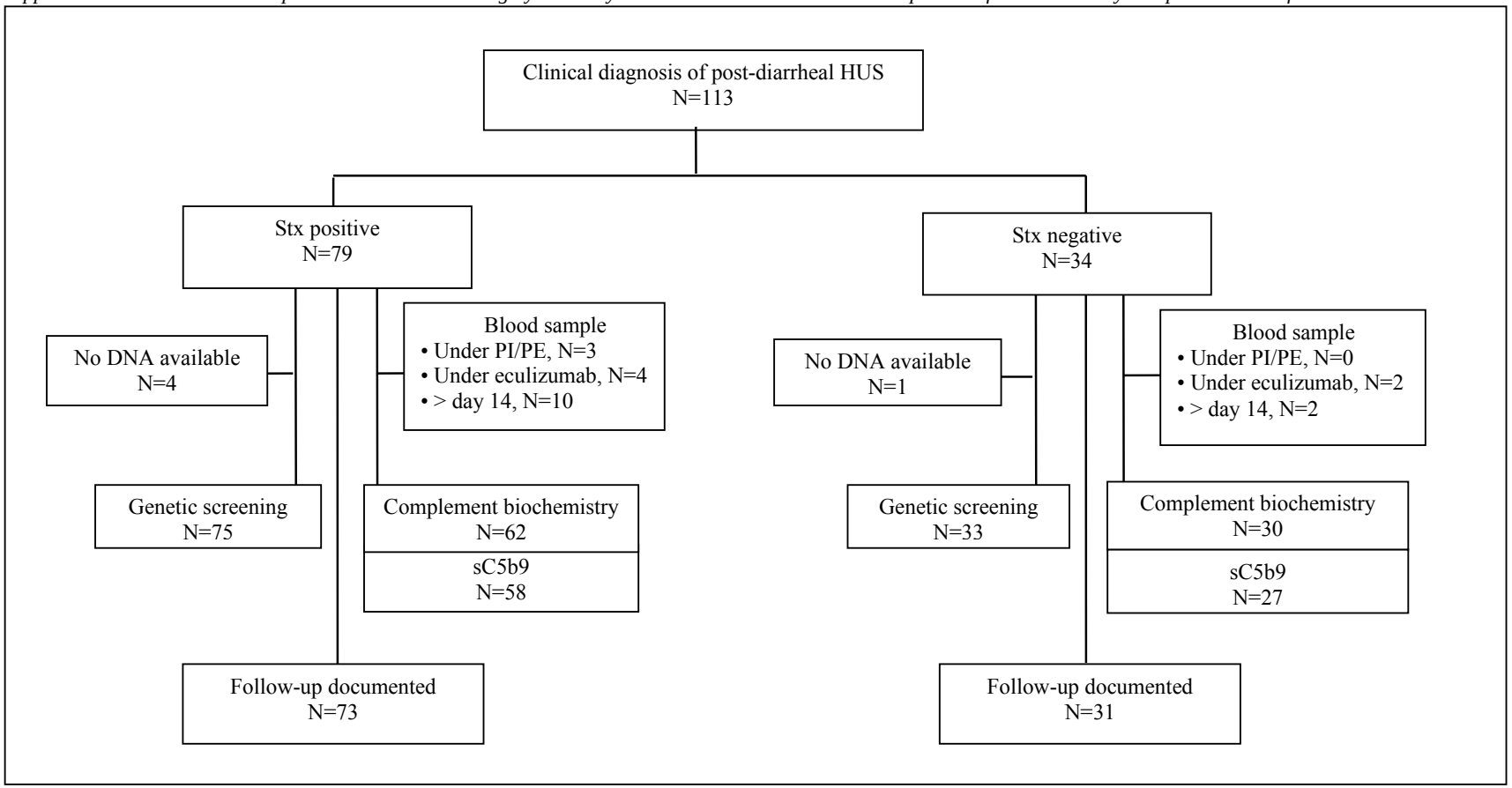
6	10.4	Circulating Stx positive	ND	Full recovery and no relapse at 6.2 y follow-up	MCP p.Thr383Ile	VUS	0.06	NA	Ardissino et al, 2016[21] and communication
Stx non documented - HUS patients									
7	1.5	ND	O157 in stool	Relapses of HUS (not post-diarrheal, Stx/STEC negative) starting 1 y after STEC-HUS Sister with one episode of Stx/STEC negative HUS	MCP p.Tyr155Asp + c.857-2 A>C Both variants also carried by the sister	Both variants pathogenic	Not found 0.000823	Decrease MCP expression (MCP deficiency)[14] Predicted deleterious effect (affects splicing)[14]	Sellier-Leclerc et al, 2007[22]
8	2	ND	O157 in stool	No relapse at 2.4 y follow-up	C3 p.Lys155Glu	Pathogenic	0.3362	Impaired C3 degradation by FI[3]	Westra et al, 2017[8, d]
9	0.7	ND	O157 in stool	Relapse of HUS one month after the first episode No relapse but CKD under PE (3.5y), then eculizumab (3y)	CFH/CFHR3 hybrid	Pathogenic	Not found	Functional CFH deficiency[23]	Challis et al, 2016[23]
10	6.2	ND	O157 in stool	No relapse at 2.6 y follow-up	CFH p.Thr956Met	VUS	0.12	No demonstrated functional alterations[7]	Westra et al, 2017[8, e]
11	16	ND	No diarrhea O157 IgM serology positive	No recovery of renal function Post-DD transplant recurrence (day 10) at age 17.5, recovery under eculizumab (follow-up 3y)	C3 p.Ser1619Arg	VUS	0.1096	No demonstrated functional alterations[3]	Dowen et al, 2017[10]
12	2.7	ND	O5 in stool	No relapse at 3.25 y follow-up	C3 p.Arg1219His	VUS	0.01	NA	Westra et al, 2017[8, e]
13	9.3	ND	O26 in stool	No relapse at 1.9 y follow-up	CFH p.Ser58Ala	Pathogenic	0.02	Decrease in vitro FH production [7]	Westra et al, 2017[8, e]
14	14	ND	O104 in stool	No relapse at 4y follow-up	C3 p.Val159Glu	VUS	Not found	NA	Ahlenstiel-Grunow et al, 2016[24, f]
15	3	ND	ND	ND	CFI p.Pro553Ser	VUS	0.06	No demonstrated functional alterations[13]	Ahlenstiel-Grunow et al, 2016[24, f]
Adult onset									
16	26	ND	Anti-Stx and O157 serology	No recovery of renal function Post-DD transplant recurrence (1y post-transplant) at age 33; graft loss	CFI p.Val412Met	Pathogenic	0.011	Variant located in serine protease domain, responsible of	Alberti et al, 2013[19]

			positive					C3b inactivation by FI[19] Decreased plasma FI level[g]	
17	41	ND	Post-diarrheal HUS Stx/STEC ND	No recovery of renal function	CFH p.Lys1188del	Pathogenic	Not found	Deletion located in disease related functional domain[25]	Edey et al, 2008[25]

a. Notice that the MCP p.Ala353Val pathogenic variant, first identified in a case of fulminant STEC-HUS[15], has been found in more than 1% of the general population (MAF 1.532% in Exome Aggregation Consortium database http://exac.broadinstitute.org/) and therefore is not classified as a rare variant in our study. We found it in 3 of the 80 French controls (4%) and 3 of 75 patients with Stx positive-HUS (4%), the latter without kidney damage at last follow-up.
b. All variants were heterozygous
c. Some variant categories may be different from those indicated in original articles[8,24], according to results of more recent functional studies
d. MAF, minor allele frequency in Exome Aggregation Consortium database http://exac.broadinstitute.org/
e. Westra et al[8] identified P/LP variants or VUS of CFH or C3 in 4/25 (16%) STEC-HUS children
f. Ahlenstiel-Grunow et al[24] identified VUS in CFI or C3 in 2/16 (12.5%) STEC-HUS children. A third patient carried a C1s variant
g. Author VFB, personal communication: CFI p.Val412Met variant associated with decreased FI plasma level

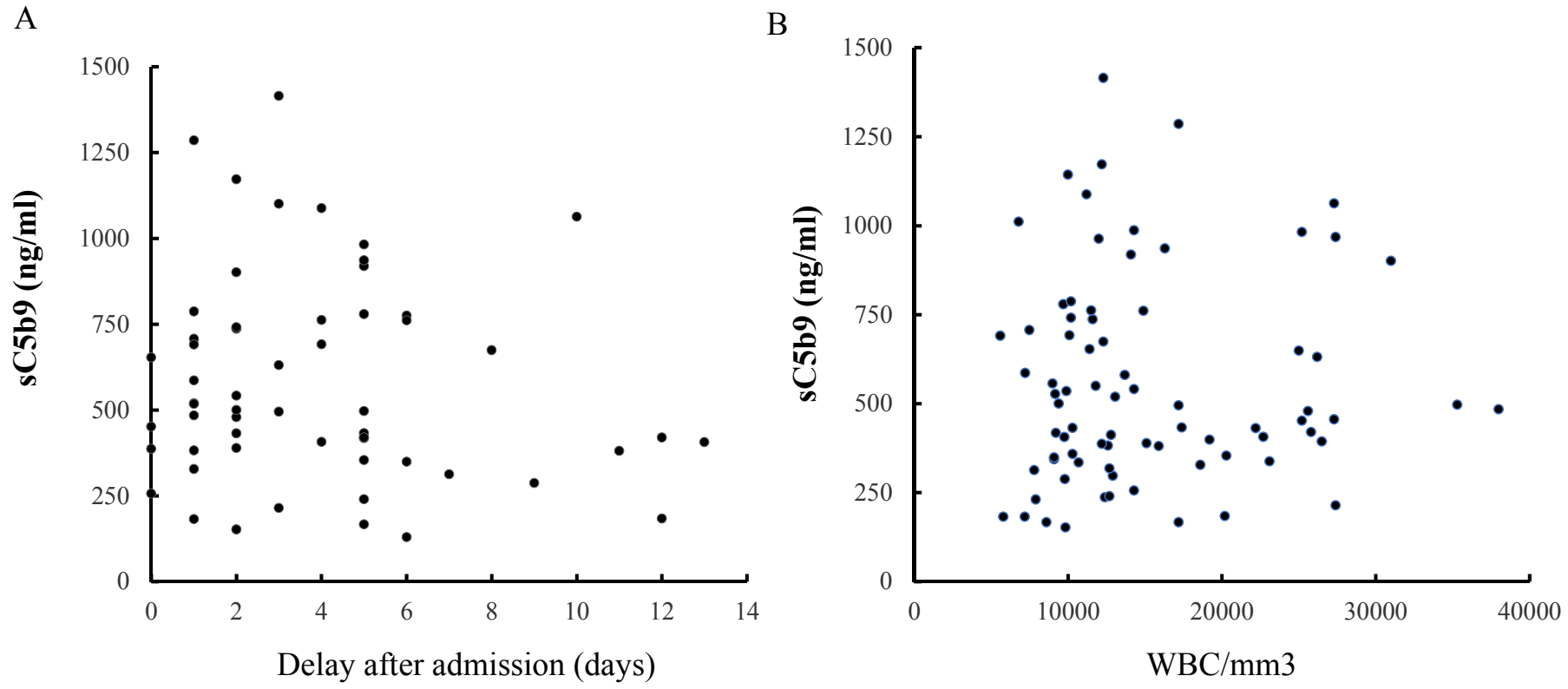
aHUS: atypical hemolytic uremic syndrome; CFH: complement factor H; CFHR: CFHR: Complement factor H-related protein; CFI: complement factor I; CKD: chronic kidney disease; DD: deceased donor; HUS: hemolytic uremic syndrome; LPS: lipopolysaccharide; LRD: living related donor; MCP: membrane cofactor protein; MLPA: Multiplex ligation-dependent probe amplification; NA: no functional studies available; PE: plasma exchange; STEC: Shiga toxin-producing E coli; Stx: Shiga toxin; VUS: variant of uncertain significance; y: year

Supplemental Figure 1. Flow diagram of patients with post-diarrheal hemolytic uremic syndrome included in the study.



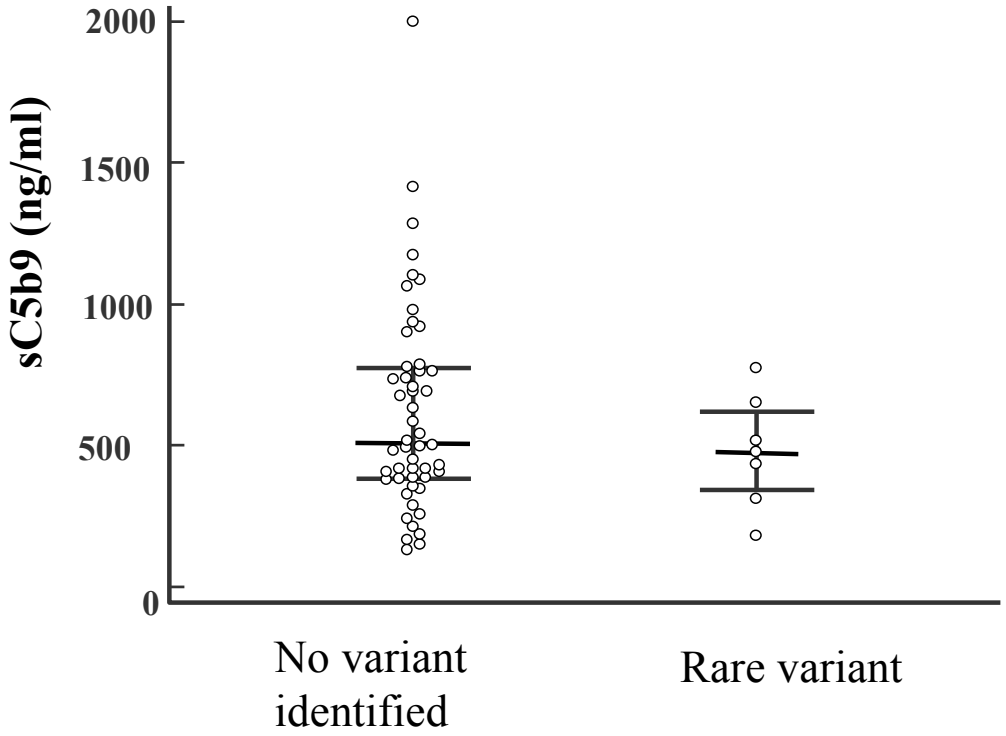
DNA: deoxyribonucleic acid; HUS: hemolytic uremic syndrome; PE: plasma exchange; PI: plasma infusion; Stx: Shiga toxin

Supplemental Figure 2. sC5b-9 plasma level according to delay after admission (A) and white blood cell count (B).



The level of sC5b-9 was not correlated (A) with the delay in blood sampling within the first 14 days of admission (r2: -0.1877; 95% CI: 0.4254 – 0.07417; p=0.16) or (B) with white blood cell count (r2: -0.03; 95% CI: 0.1855 – 0.2590; p=0.73)

Supplemental Figure 3. sC5b-9 level during the acute phase in Shiga toxin positive-HUS patients with (n=7) or without (n=49) rare variant identified.



The median (Q1; Q3) level of sC5b9 was 479 ng/ml (373; 586) and 500 ng/ml (385; 771) in patients with a rare variant and no variant, respectively (p=0.7).