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

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Significance statement

Hereditary complement hyperactivation, the core mechanism for atypical hemolytic uremic syndrome (aHUS), is undetermined in Shiga toxin-associated HUS. This manuscript shows that the frequency of rare variants in the 6 susceptibility genes for aHUS is identical in Shiga toxin positive-HUS patients and controls. No significant differences were observed in disease severity and complement activation at the acute phase, or in residual chronic kidney disease in Shiga toxin positive-HUS patients with or without variants. However, the frequency of pathogenic very rare variants with minor allele frequency <0.1% associated with functional deficiency is increased in Shiga toxin positive-HUS patients compared to controls.

Abstract

Background and objectives

Inherited complement hyperactivation is critical for the pathogenesis of atypical hemolytic uremic syndrome (aHUS) but undetermined in post-diarrheal-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, and measurements

Determination of complement biomarkers levels and next-generation sequencing for the 6 susceptibility genes for aHUS 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 analysed the genotypes in the 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 two weeks. Variants with minor allele frequency <1% were identified in 12 Shiga toxin positive-HUS patients (12/75, 16%), including pathogenic variants in 4 (4/75, 5%), with no significant differences compared to Shiga toxin negative-HUS patients and controls. Pathogenic variants with minor allele frequency <0.1% were found in 3 Shiga toxin positive-HUS patients (3/75, 4%) versus only 4 European controls (4/503, 0.8%) (OR: 5.2; 95% CI: 1.1-24; p=0.03). The genetic background did not affect significantly dialysis requirement, neurological manifestations and sC5b-9 level during the acute phase, and incident chronic kidney disease during follow-up. However, the only patient who progressed to end stage kidney disease within 3 years carried a factor H pathogenic variant.

Conclusion

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-HUS patients than in controls.

Introduction

Decreased C3 plasma levels at the acute phase of post-diarrheal hemolytic uremic syndrome (HUS) were first mentioned by 1973[1-3]. Forty years later, the demonstration that atypical HUS (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 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 post-diarrheal/Shiga toxin producing E coli (STEC)-HUS were subsequently reported who carried rare variants in complement genes[9,10,20-28]. In 9 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 pre-existing complement variants amplify Shiga toxin-induced complement activation and endothelial/podocyte damage, and worsen disease severity[20-27]. However, 7 reported patients, who had complement variants had a favourable 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 to healthy controls and the association of these variants with disease severity and complement activation biomarkers.

Materials and Methods

Study population

One hundred and thirteen Caucasian children with a clinical diagnosis of post-diarrheal-HUS, hospitalized in 22 pediatric nephrology departments between October 13, 2010 and October 17, 2012, entered the study (See Supplement for Study design). We collected blood samples from 80 French controls (healthy Caucasian 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 6 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 polymerase chain reaction (PCR) for Shiga toxin1 or Shiga toxin2 genes on stools (Supplemental Figure 1) (See Supplement for STEC investigations)

Genetic screening

Exonic regions of 6 complement genes (factor H, factor I, factor B, membrane cofactor protein, C3 and thrombomodulin) were screened using next-generation sequencing (NGS). 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 below 1% and as very rare when it is below 0.1% (definitions adapted from Richards et al, 2015[32] and Goodship et al, 2017[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[32,33]). 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 were 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 non parametric test. All analyses (Chi2, Mann-Whitney 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 (OR:2.5; 95% CI: 1-6.2; 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: 1-21; p=0.03). Other characteristics during the acute phase and at last follow-up were similar between the 2 groups. No patient died during the follow-up. A single patient (Shiga toxin positive) progressed to end-stage kidney disease (ESKD) 3 years after HUS.

Complement variants

In the whole cohort of post-diarrheal HUS patients, 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-post-diarrheal HUS, a similar proportion (12/75 (16 %) and 6/33 (18%), respectively, (OR:0.8; 95% CI: 0.3-2.5; p=0.8) carried 1 or 2 rare variants in 1 or 2 of the 6 tested genes, without significant difference in the frequency of the variants per gene (Table 2). The rare variants identified in patients are described in Table 3A 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 2 corresponding variants were classified as pathogenic. Experimental functional investigations have shown that 4 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 also in STEC-HUS[24] (Table 3A). 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 2 also 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 HUS patients 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 3B). Altogether, the frequency of rare variants per gene and per variant categorization was not significantly different between HUS patients and controls and between Shiga toxin positive and Shiga toxin negative-HUS patients (Table 2 and Table 3B).

A very rare pathogenic variant with minor allele frequency <0.1% was identified in 3 of 75 Shiga toxin positive-HUS patients (4%) (Table 3A, cases 2, 3, 4), compared to none of the 80 French controls (0%) (p=0.17) and 4 of the 503 European controls (0.8 %) (OR: 5.2; 95% CI:1.1-24; p=0.03) (Supplemental Table 2). We did not observe a significant difference in the very rare pathogenic variants between Shiga toxin negative-HUS patients (1/33, 3%) (Table 3A, case 6) and the French (p=0.2) or European control groups (OR 3.9; 95% CI: 0.4-36; p=0.2).

Homozygous factor H *tgtgt* or membrane cofactor protein *ggaac* haplotypes were found in 3% (3/97) and 6% (6/97) of HUS patients, respectively. These frequencies were not significantly different from those in French controls (Supplemental Table 4). None of 3 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 increased, and median membrane cofactor protein expression significantly decreased in HUS patients compared to French controls (Figure 1 and Supplemental Table 6). C3 and C4 levels were significantly higher in Shiga toxin negative compared to Shiga toxin positive patients, while other changes were not significantly different between the 2 groups (Figure 1). No patient had C3 level below the lower limit of normal and only 3 patients had C3 levels close to the lower limit of

normal (3/61, 5%) (Supplemental Table 7). The level of sC5b-9 was increased in 66% (38/58) of Shiga toxin positive HUS patients and membrane cofactor protein expression decreased in 57% (27/47). 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 chronic kidney disease stage (CKD) at last follow-up in Shiga toxin positive-HUS patients

The clinical characteristics and outcomes of the 6 post-diarrheal-HUS patients with pathogenic variants are presented in Table 4, and those of patients with C3 level at the lower limit of normal or anti-factor H antibodies in Supplemental Tables 7 and 5, respectively. The sC5b-9 level during the acute phase was not significantly different in Shiga toxin positive-HUS patients with or without rare variant identified (Supplemental Figure 3). In Shiga toxin positive-HUS patients, 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 CKD1-4 or CDK5 compared to patients without renal sequel (no CKD) at last follow-up (Table 5).

One of the 4 Shiga toxin positive patients who carried a pathogenic variant progressed to ESKD within 3 years, compared to 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-HUS patients. 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 NGS to screen the 6 genes implicated in aHUS pathogenesis, in 108 children with a clinical diagnosis of post-diarrheal 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]. In order to categorize the clinical relevance of genetic findings, we first analysed whether patients are enriched for complement rare variants compared to controls. We found a similar frequency of rare variants in the total cohort of 108 HUS patients (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-HUS patients and French or 1000 Genomes controls. Still, when we focused our analysis on very rare pathogenic variants with minor allele frequency <0.1 %, we showed that such very rare pathogenic variants were associated with a five-fold increase in risk of developing Shiga toxin positive -HUS. Thus, although Shiga toxin-associated HUS is mostly driven by the infectious agent, but consistent with published data[9,10,20-28], our study highlights that in rare cases, genetic abnormalities may contribute to complement activation in Shiga toxin-HUS. 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 to develop 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-HUS patients 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 4), 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 relatively high hemoglobin levels, leukocytosis and neurological manifestations during the acute phase, known risk factors for poor kidney outcome. In the same line, no recovery of kidney function was documented in 1 of 6 (16%) Shiga toxin positive-HUS patients who carried pathogenic variants reported in the literature (Supplemental Table 8). In unselected cohorts of STEC-HUS, only 1.4%[45] to 3%[46] of children progressed to ESKD within 4 to 5 years. In our relatively small cohort of Shiga toxin positive-HUS patients, we observed a trend (p=0.06) towards a significant correlation between the identification of a pathogenic variant and ESKD at last follow-up. But, 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 post-diarrheal-HUS patients but should be considered in patients with a fulminant course to death or ESKD[19,20,22,27], progression to ESKD within less than 3 years (patient 1, Table 4), a family history of HUS[23] (patient 6, Table 4), 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 to Shiga toxin negative patients, complement biomarkers did not differ between the 2 groups. Interestingly, C3 levels close to the lower limit of normal were

observed in only 3 patients (5 % of the cohort). Recent reports also observed slightly decreased C3 levels in only 4/25[9] and 5/21[10] STEC-HUS patients, and exceptional severely decreased level (1/21)[10]. We confirm that 66% of Shiga toxin positive-patients have an increase of sC5b-9 level, the commonly used marker of 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 post-diarrheal HUS does not suffice to consider 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 4).

Our series also illustrates the limitations of STEC biological 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 post-diarrheal HUS but negative Shiga toxin PCR should be classified as aHUS. Such patients in our cohort remain classified as post-diarrheal 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 favourable outcome[50], similar to Shiga toxin negative post-diarrheal HUS patients in the current study.

In conclusion, our results show an overall limited role of rare variants in complement genes in Stx positive-HUS. Still, genetic screening should be considered in post-diarrheal-HUS patients who progress rapidly to ESKD.

Author contribution

VFB, CL and ALSL designed the study. VFB and PVM performed the genetic screening and the complement assessment. SL analysed the 1000 Genomes project database. PM and FXW performed the STEC investigations. VFB, PVM and CL analyzed the data. VFB and CL 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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Table of contents for the Supplemental material

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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.

	Stx positive-HUS N=79	Stx negative-HUS N=34
Patients characteristics		
Age, years	2.6 (1.4;4.9)	3.4 (1.6;5.5)
Gender, male/female	38/41	17/17
Prodromal phase		
Gastrointestinal manifestations		
Non bloody diarrhea	30 (38)	14 (41)
Bloody diarrhea	40 (51)	19 (56)
Abdominal pain, vomiting, without diarrhea	9 (11)	1 (3)
Fever $\geq 38^{\circ}C$	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)
>9g/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	13070 (10200;20030)	12400 (9535;18200)
> 20000/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)
Non typable E coli	10/77 (13)	2[a] (6)
Anti-LPS serology		
Positive	33/64 (52)	17/29 (59)
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, days	9 (4;16)	9 (7;16)
> 8 days	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, months	46 (18;65)	53 (33;61)
No CKD[b]	52/73 (71)	27/31(87)
CKD 1[b]	16/73 (22)	2/31 (6)
CKD 2[b]	1/73 (1)	2/31 (6)
CKD 3[b]	3/73 (4)	0
CKD 4[b]	0	0
CKD 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 into brackets.

a. Non-Stx producing E coli strains
b. CKD stages according to KDIGO 2012 http://www.kdigo.org/clinical_practice_guidelines/pdf/CKD/KDIGO_2012_CKD_GL.pdf.
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; CKD Stage 2 by eGFR 60-89 ml/min/1.73m^2 with albuminuria
See Supplemental Methods and Patients for the definition of CKD stages 3-5 and albuminuria

CKD: chronic kidney disease; eGFR: estimated glomerular filtration rate; HUS: hemolytic uremic syndrome; LDH: lactate deshydrogenase; LPS: lipopolysaccharide; STEC: Shiga toxin producing *E coli*; Stx: Shiga toxin

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 6 tested complement genes, or anti-Factor H antibodies.

	Post-diarrheal HUS patients Total cohort (n=108)[a]	French controls (n=80)	1000 Genomes controls (n=503)	Post-diarrheal HUS patients versus French controls	Post-diarrheal HUS patients versus 1000 Genomes controls	Stx positive-HUS patients (n=75)[e]	Stx negative-HUS patients (n=33)[e]	Stx positive- versus Stx negative- HUS patients
One rare variant per patient or control	N (%)	N (%)	N (%)	OR,95% CI; p[f]	OR, 95% CI; p[f]	N (%)	N (%)	OR, 95% CI; p[f]
Complement Factor H	3 (3)	2 (3)	8 (2)	1.1; 0.2 - 6.8; 1	1.8; 0.5 - 6.8; 0.4	2 (3)	1 (3)	0.8; 0.1 -10; 1
Membrane Cofactor Protein	1 (0.9)	0	2 (0.4)	1	2.3; 0.2 - 26; 0.4	1 (1)	0	1
Complement Factor I	1 (0.9)	2 (3)	13 (3)	0.3; 0 - 4; 0.5	0.3; 0.1 - 3; 0.4	1 (1)	0	1
C3	8[b] (7)	4 (5)	16 (3)	1.5; 0 - 5; 0.5	2.4; 1 - 6; 0.05	5 (7)	3 (9)	0.7; 0.1 - 3; 0.7
Complement Facteur B	1 (0.9)	0	7 (1)	1	0.6; 0.8 - 5; 1	1 (1)	0	1
Thrombomodulin	3 (3)	1 (1)	9 (2)	2.2; 0.2 -22; 0.6	1.6; 0.4 - 6; 0.4	2 (3)	1 (3)	0.8; 0.1 -10; 1
Two rare variants per patient or control	1[c] (0.9)	2[d] (3)	5 (1)	0.4; 0 - 4; 0.5	0.9; 0.1 - 8; 1	0	1 (3)	0.1; 0 - 3.6; 0.3
Total	18 (17)	11 (14)	60 (12)	1.2; 0.5 -3; 0.6	1.5; 0.8 - 3; 0.1	12 (16)	6 (18)	0.8; 0.3 - 2.5; 0.8
Anti-Factor H antibodies	3[b] (3)	0		0.3		1	2	0.2; 0 - 2.4; 0.2

a. 5 of the 113 HUS patients were not screened for variants in the 6 tested complement genes
b. One patient had a C3 rare variant and anti-Factor H antibodies
c. This patient had a complement factor H and a C3 rare variant
d. One control had a thrombomodulin and C3 rare variant, and another control had two rare variants in C3
e. 4 of the 79 patients with Stx positive-HUS and 1 of the 34 patients with Stx negative-HUS were not screened for variants in the 6 complement genes
f. OR: Odds ratio; CI ; 95% confidence interval; p with Fischer exact test

HUS: hemolytic uremic syndrome; Stx: Shiga toxin

Table 3. Complement rare variants found among 108 patients with post-diarrheal HUS, compared to French and European controls.

A. Pathogenic rare variants (n=6) found in 6 of 108 patients with post-diarrheal HUS.

Patient	Gene	Variant	Genetic status	Number of patients	CFH plasma level[b]	MCP expression[b]	MAF [c] (%)	Demonstrated functional alterations	Previously reported in STEC-HUS	Previously reported in aHUS
Stx positive HUS patients										
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 [e,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 HUS patients										
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[a]	He	1	Normal	Normal	0.017	Alter the C3b/polyanions –binding site[37]	No	Yes [e,38]

a. Patient 6 with CFH p.Arg1210Cys pathogenic rare variant also carried a C3 VUS
b. At discharge
c. MAF (minor allele frequency) in Exome Aggregation Consortium (ExAC) database, http://exac.broadinstitute.org/
d. Author V. Frémeaux-Bacchi, personal communication
e. FH aHUS mutation database, http://www.fh-hus.org/

B. Number and frequency (%) of patients with post-diarrheal-HUS and of French controls and controls from the 1000 Genomes project database, who carried at least one rare variant in one of the 6 tested complement genes.

	Post-diarrheal HUS patients Total cohort (n=108)[a]	French controls (n=80)[b]	1000 Genomes controls (n=503)[b]	HUS patients versus French controls	HUS patients versus 1000 Genomes controls	Stx positive-HUS patients (n=75)[e*]	Stx negative-HUS patients (n=33)[e**]	Stx positive versus Stx negative-HUS patients[f]
	n (%)	n (%)	n (%)	OR, 95% CI; p[f]	OR, 95% CI; p[f]	n (%)	n (%)	OR, 95% CI; p[f]
Individuals with a rare variant	18 (17)	11 (14)	60 (12)	1.2; 0.5- 3; 0.7	1.4; 0.8 - 2.6; 0.2	12 (16)	6 (18.2)	0.8; 0.3-2.5; 0.7
Individuals with at least a pathogenic variant	6[c] (6)	2[d] (2)	14 (3)	3; 0.4 - 12; 0.5	2; 0.8 - 5; 0.1	4 (5.3)	2[g] (6.1)	0.9; 0.2- 5; 1
Individuals with a variant of uncertain significance	12 (11)	9[d] (11)	46 (9)	0.9; 0.4 - 2; 1	1.2; 0.6 - 2; 0.3	8 (10.7)	4 (12.1)	0.9; 0.2 - 3; 1

*Frequency of pathogenic variants in Stx positive-HUS patients versus French controls: OR: 2.2; 95% CI: 0.4 - 12; p=0.4; versus 1000 Genomes controls: OR: 2; 95% CI: 0.6 - 6; p=1.
**Frequency of pathogenic variants in Stx negative-HUS patients versus French controls: OR: 2; 95% CI: 0.6 - 6; p=1; versus 1000 Genomes controls OR: 2; 95% CI: 0.4 -10; p= 0.2)

a. 5 of the 113 post-diarrheal HUS patients were not screened for variants in the 6 tested complement genes.
b. All controls were screened for variants in the 6 tested complement genes.
c. One of the 6 patients also carried a C3 VUS.
d. One control had a thrombomodulin pathogenic variant and a C3 VUS, and another control had two C3 VUS.
e. 4 of the 79 patients with Stx positive-HUS and 1 of the 34 patients with Stx negative-HUS were not screened for variants in the 6 complement genes
f. OR : Odds ratio; CI ; 95% confidence interval; p with Fischer exact test
g. ne of the 2 patients also carried a C3 VUS

aHUS: atypical hemolytic uremic syndrome; GAG: glycosaminoglycans; He: heterozygous; MAF: minor allele frequency; Stx: shiga toxin; STEC: Shiga toxin producing *E coli*; VUS: variant of uncertain significance

Table 4. Clinical characteristics, in hospital-course and outcome of the 6 patients with post-diarrheal-HUS, who carried complement pathogenic rare variants.

		In-hospital course										Outcome		
Patient Gender Age,y	Genetic complement abnormalities*	C3[ab] mg/L	sC5b-9[ab] 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	Systemic manifestations	PI/PE and/or eculizumab	F-up y	Sequels[c]	Relapse
Stx positive HUS patients														
1. F, 2.6	CFH, p.Gln950His	975	479	Stx2 positive (O157 in stool)	9.8	81000	25600	6.1	25	CNS	No	5.8	CKD5 (ESKD) at 3y f-up Kidney graft at 4y f-up	No
2. M, 2.0	THBD, p.Pro495Ser	1240	519	Stx2 positive (O80 in stool)	5.8	41000	13070	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	34000	17400	0.6	0	None	No	3	No CKD	No
4. M, 3.5	MCP, p.Asn170LysfsTer7	1090	182	Stx2 positive (ND)	7.2	400000	7200	0.9	0	None	No	4.8	CKD1 Proteinuria; eGFR 108 ml/min/1.73m^2	No
Stx negative HUS patients														
5. M, 13.5	THBD, p.Ala43Thr	1180	231	Stx negative (stool culture and serology negative)	5.0	59000	7900	163	3	None	No	4.4	No CKD	No
6. M, 4.4[d]	CFH, p.Arg1210Cys + C3 p.Asp1440Ala VUS	1210	557	Stx negative (Only O157 serology positive)	5.8	30000	9000	4	7	Pancreatitis	No	4.5	No CKD	No

* See Table 3A

a. Normal range: C3: 615-1250 mg/L; sC5b-9 :< 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, http://www.kdigo.org/clinical_practice_guidelines/ pdf/CKD/KDIGO_2012_CKD_GL.pdf. See Supplemental Methods and Patients for definition of CKD stages

d. Patient 6's first cousin had HUS 4 years after patient 6

aHUS atypical hemolytic uremic syndrome; CFH: complement factor H;CFHR: CKD: chronic kidney disease; CNS: central nervous system; eGFR: estimated glomerular filtration rate; ESKD: end stage kidney disease; F: female; F-up: follow-up; Hb: haemoglobin; M: male; L: lower than normal range; MCP: membrane cofactor protein; N: within the normal range; PCR: polymerase chain reaction; PI: plasma infusion; PE: plasma exchange; Plt: platelet count; Screat: serum creatinine; STEC: Shigatoxin –producing *E coli*; Stx: Shiga toxin; THBD: thrombomodulin; WBC: white blood cell; y:year

Table 5. Severity of HUS according to sC5b-9 level and genetic background in Shiga toxin positive-HUS patients.

Complement abnormalities	**Acute phase**						**Last follow-up[a]**				
	Dialysis required			**CNS manifestations**			**No CKD[b]**	**CKD 1-4[b]**	**CKD5[b]**	**CKD1-4 versus No CKD**	**CKD5 versus No CKD**
	Yes N (%)	**No** N (%)	OR; 95% CI; p[d]	**Yes** N (%)	**No** N (%)	OR; 95% CI; p[d]	n (%)	n (%)	n (%)	OR; 95% CI; p[d]	OR; 95% CI; p[d]
Increased sC5b-9 (>420 ng/mL)[c]	23/33 (70)	15/25 (60)	1.5; 0.5- 4.6; 0.2	11/13 (85)	27/45 (60)	3.6; 0.7-18; 0.1	25/36 (69)	9/18 (50)	1/1 (100)	2.3; 0.7-7; 0.2	2.3; 0-19; 0.4
Pathogenic variant (n=4)	1/42 (2)	3/33 (9)	0.2; 0-2.4; 0.2	1/17 (6)	3/58 (5)	1.1; 0.1-12; 0.9	2/48 (4)	1/20 (5)	1/1 (100)	0.8; 0.1- 9; 1	55;1.8-1747; 0.06
Variant of uncertain significance (n=8)	5/43 (12)	3/32 (9)	1.2; 0-5.8; 0.9	0/17 (0)	8/58 (14)	0.2; 0-3; 0.2	7/48 (15)	1/20 (5)	0/1 (0)	3.2; 0.3-28; 0.4	0.5
No variant identified	37/43 (86)	26/32 (81)	1.4; 0-5; 0.3	1/17 (6)	11/58 (19)	0.3; 0-2.2; 0.2	39/48 (81)	18/20 (90)	0/1 (0)	0.5; 0.1-2.5; 0.4	0.13

a. Median (Q1-Q3): 47 months (20- 65) (n=79 with 6 lost to follow-up). The CKD stage was documented at 3 years follow-up in 43 patients
b.CKD stages according to KDIGO 2012, http://www.kdigo.org/clinical_practice_guidelines/ pdf/CKD/KDIGO_2012_CKD_GL.pdf. See Supplemental Methods and Patients for definition of CKD stages. No patient had CKD stage 4 at last follow-up.
c. Samples before day 14 after admission
d. OR: Odds ratio; CI; 95% confidence interval; p with Fischer exact test

CKD: chronic kidney disease; CNS: central nervous system; HUS: hemolytic uremic syndrome

Legend of figures

Figure 1. Plasma levels of C3, C4, factor H, factor I, sC5b-9, and membrane cofactor protein expression during the acute phase in patients with Shiga toxin positive-HUS, Shiga toxin negative-HUS and French controls.

Median delay (Q1;Q3) of blood sampling after admission was 4 days (1;6) (from admission to 13 days post-admission) for the total cohort, 2.5 days (1; 4.8) in Shiga toxin positive HUS-patients, and 5.5 days (2.3; 9) in Shiga toxin negative-HUS patients.

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

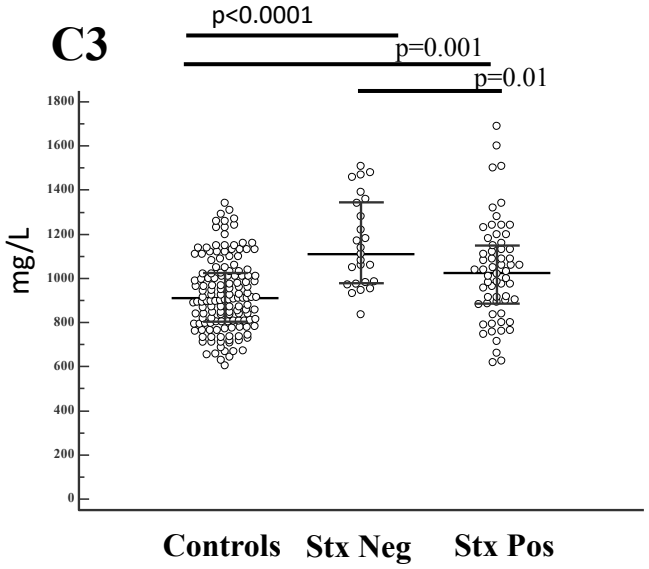
Figure 2.
A. Membrane cofactor protein expression in post-diarrheal HUS, according to delay between admission and blood sampling.

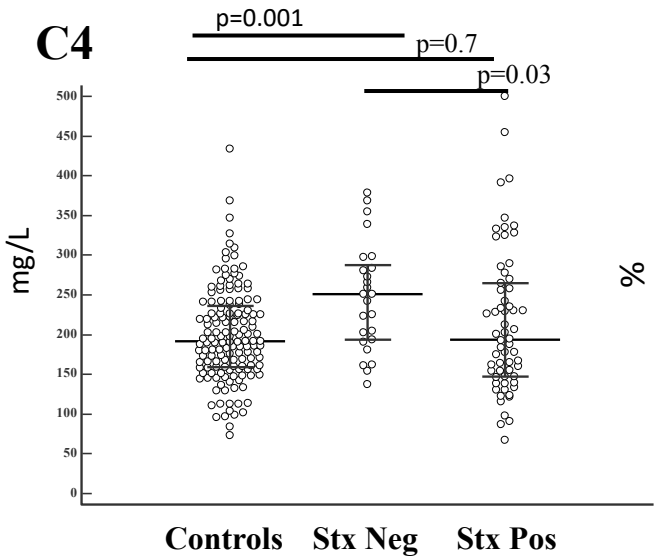
MCP expression (normal range: 13-19 MFI, indicated between dashed lines) during the acute phase of HUS was documented in 56 Shiga toxin positive-patients (A1) and 26 Shiga toxin negative-patients (A2), It was below the lower limit of normal in 15/20 (75%) and in 4/8 of Shiga toxin positive or Shiga toxin negative-HUS patients with blood sampling performed within 48h 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 patients.

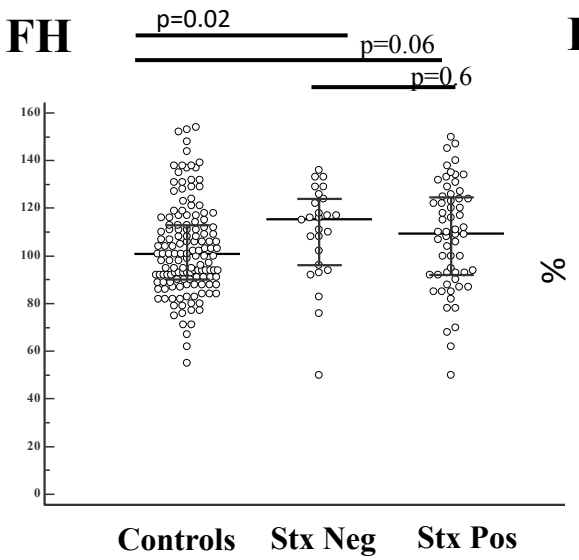
B. Normalization of MCP expression after remission (11 patients documented)

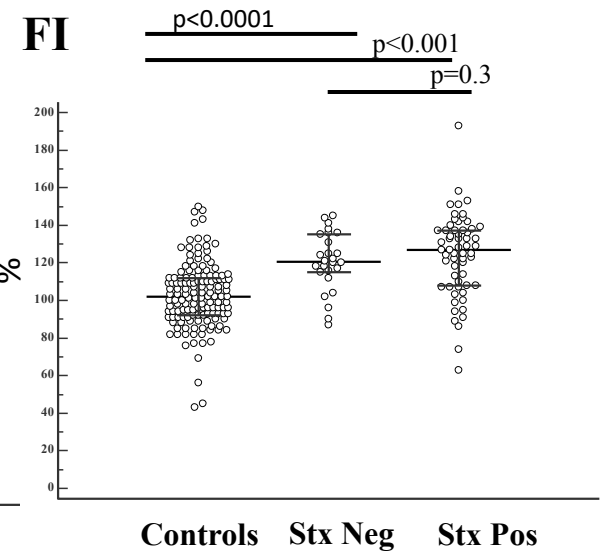
Median (Q1;Q3) MCP expression was 10 MFI (8;11) in hospital (9.6+/-2.1 days after admission) and 18 MFI (15;21) after discharge (18.6± 4.4 days after admission) (p=0.0002 using paired samples t-test)

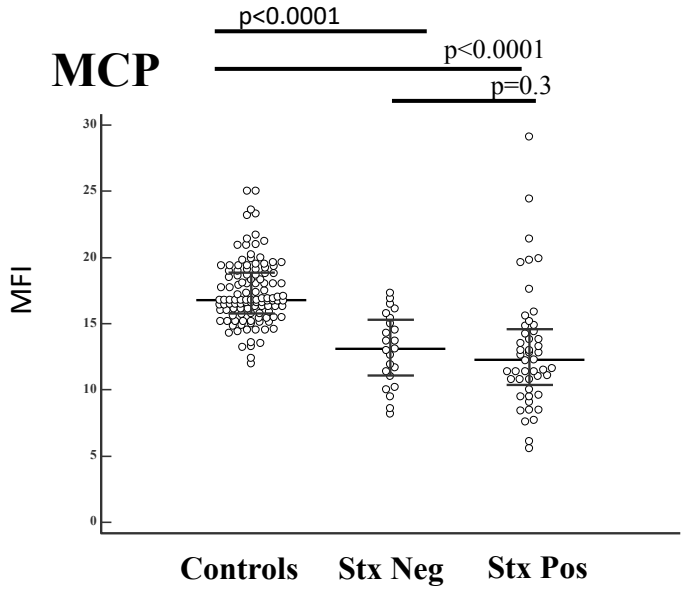
HUS: hemolytic uremic syndrome; MCP: membrane cofactor protein; MFI: Mean Fluorescence Intensity


C3
p<0.0001
p=0.001
p=0.01
mg/L
Controls
Stx Neg
Stx Pos


C4
p=0.001
p=0.7
p=0.03
mg/L
Controls
Stx Neg
Stx Pos


FH
p=0.02
p=0.06
p=0.6
%
Controls
Stx Neg
Stx Pos


FI
p<0.0001
p<0.001
p=0.3
%
Controls
Stx Neg
Stx Pos


MCP
p<0.0001
p<0.0001
p=0.3
MFI
Controls
Stx Neg
Stx Pos

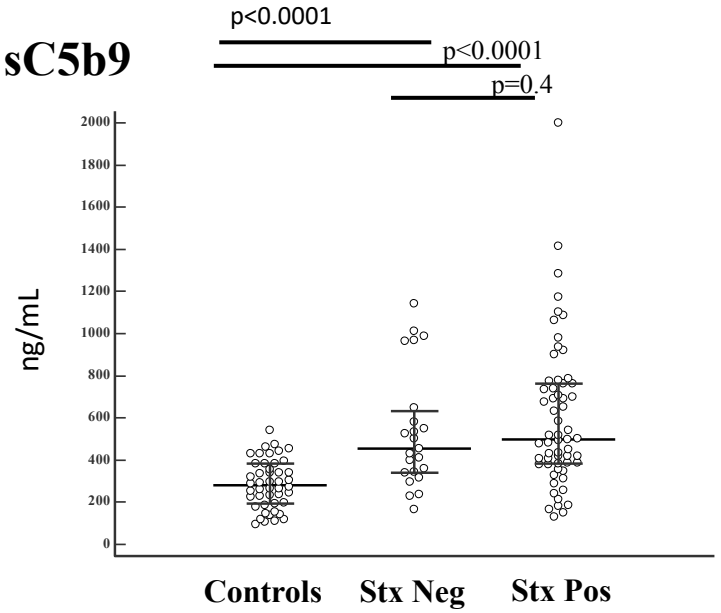

sC5b9
p<0.0001
p<0.0001
p=0.4
ng/mL
Controls
Stx Neg
Stx Pos

Figure 1

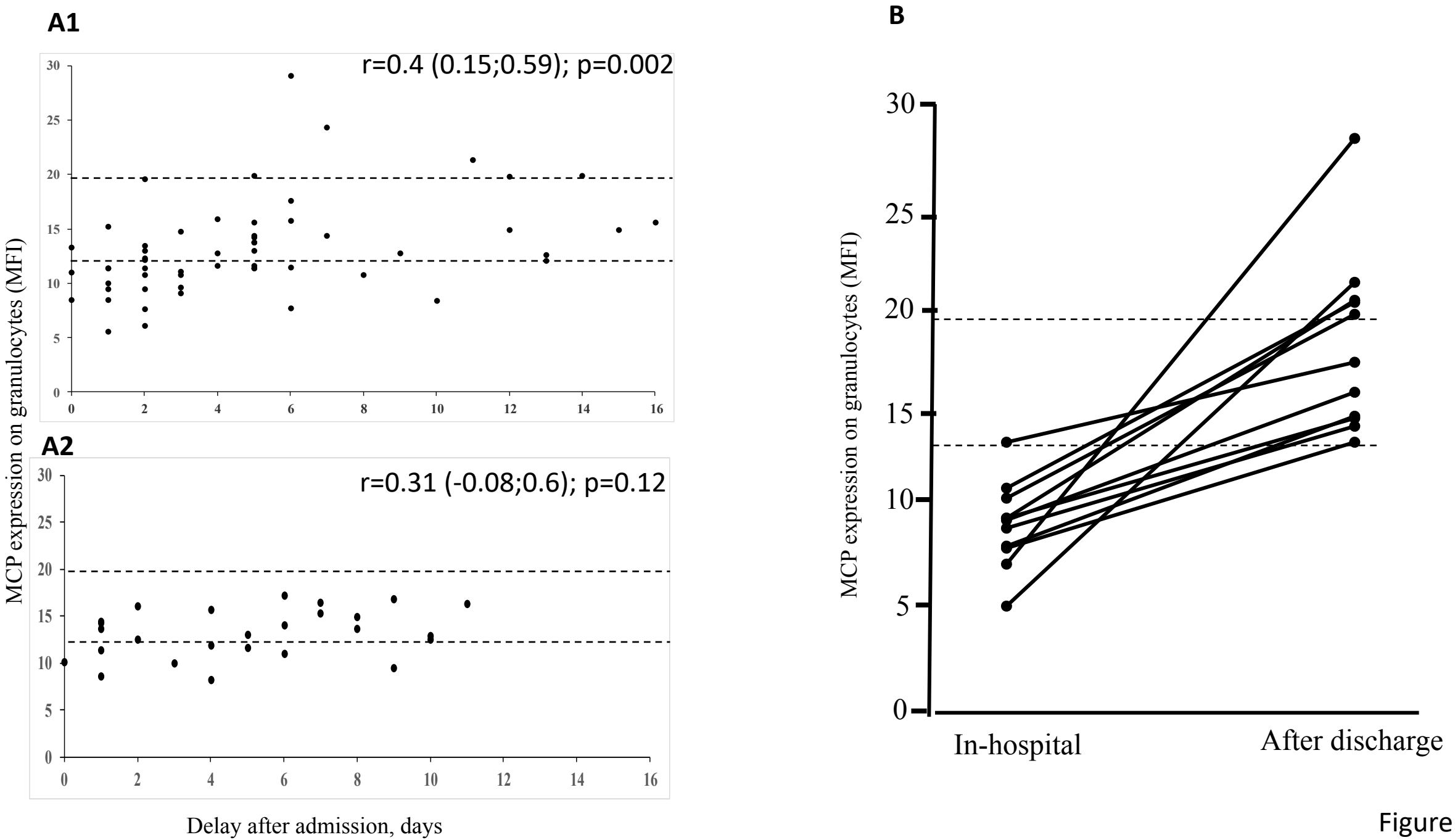

A1
r=0.4 (0.15;0.59); p=0.002
A2
r=0.31 (-0.08;0.6); p=0.12
MCP expression on granulocytes (MFI)
Delay after admission, days
B
MCP expression on granulocytes (MFI)
In-hospital
After discharge

Figure 2