





Complement Activation and Thrombotic Microangiopathies

Marta Palomo,^{1,2,3} Miquel Blasco ,^{4,5} Patricia Molina,² Miquel Lozano,⁶ Manuel Praga,^{7,8} Sergi Torramade-Moix,² Julia Martinez-Sanchez,^{1,2,3} Joan Cid,⁶ Gines Escolar,² Enric Carreras,^{1,3} Cristina Paules,⁹ Fatima Crispi,⁹ Luis F. Quintana,^{4,5} Esteban Poch,^{4,5} Lida Rodas,⁴ Emma Goma,⁴ Johann Morelle ,¹⁰ Mario Espinosa,¹¹ Enrique Morales,⁷ Ana Avila,¹² Virginia Cabello,¹³ Gema Ariceta ,¹⁴ Sara Chocron,¹⁴ Joaquin Manrique ,¹⁵ Xoana Barros,¹⁶ Nadia Martin,¹⁶ Ana Huerta,¹⁷ Gloria M. Fraga-Rodriguez,¹⁸ Mercedes Cao,¹⁹ Marisa Martin,²⁰ Ana Maria Romera,²¹ Francesc Moreso,²² Anna Manonelles,²³ Eduard Gratacos,⁹ Arturo Pereira,²⁴ Josep M. Campistol,⁴ and Maribel Diaz-Ricart^{2,3}

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

Abstract

Background and objectives Atypical hemolytic uremic syndrome is a form of thrombotic microangiopathy caused by dysregulation of the alternative complement pathway. There is evidence showing complement activation in other thrombotic microangiopathies. The aim of this study was to evaluate complement activation in different thrombotic microangiopathies and to monitor treatment response.

Design, setting, participants, & measurements Complement activation was assessed by exposing endothelial cells to sera or activated-patient plasma—citrate plasma mixed with a control sera pool (1:1)—to analyze C5b-9 deposits by immunofluorescence. Patients with atypical hemolytic uremic syndrome ($n=34$) at different stages of the disease, HELLP syndrome (a pregnancy complication characterized by hemolysis, elevated liver enzymes, and low platelet count) or severe preeclampsia ($n=10$), and malignant hypertension ($n=5$) were included.

Results Acute phase atypical hemolytic uremic syndrome-activated plasma induced an increased C5b-9 deposition on endothelial cells. Standard and lower doses of eculizumab inhibited C5b-9 deposition in all patients with atypical hemolytic uremic syndrome, except in two who showed partial remission and clinical relapse. Significant fibrin formation was observed together with C5b-9 deposition. Results obtained using activated-plasma samples were more marked and reproducible than those obtained with sera. C5b-9 deposition was also increased with samples from patients with HELLP (all cases) and preeclampsia (90%) at disease onset. This increase was sustained in those with HELLP after 40 days, and levels normalized in patients with both HELLP and preeclampsia after 6–9 months. Complement activation in those with malignant hypertension was at control levels.

Conclusions The proposed methodology identifies complement overactivation in patients with atypical hemolytic uremic syndrome at acute phase and in other diseases such as HELLP syndrome and preeclampsia. Moreover, it is sensitive enough to individually assess the efficiency of the C5 inhibition treatment.

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Correspondence:

Dr. Marta Palomo, Josep Carreras Leukaemia Research Institute, Hospital Clinic/University of Barcelona Campus, Carrer de Villarroel 170, 08036 Barcelona, Spain. Email: mpalomo@carrerasresearch.org

Introduction

Thrombotic microangiopathies (TMAs) include a group of disorders that share clinical features, such as the triad of microangiopathic hemolytic anemia, thrombocytopenia, and organ damage (1,2). Atypical hemolytic uremic syndrome (aHUS) is a TMA primarily caused by a lack of regulation of the alternative complement pathway. New evidence suggests that a number of TMAs other than aHUS could also be associated with complement overactivation (3,4).

In both inherited or acquired aHUS there is an assembly of the membrane-attack complex leading to endothelial cell inflammation, activation, and injury (5,6). The introduction of eculizumab, an mAb which blocks terminal complement activation, significantly

improved the natural progression of the disease (7,8). Lifelong treatment with this drug is often considered in inherited aHUS. The ability to monitor treatment efficiency and patient recovery, with appropriate biologic markers, could help in decision making regarding treatment continuation.

aHUS occurs as dysregulation of the alternative complement pathway (9,10) and the presence of complement-amplifying conditions, resulting in endothelial damage (11,12). Some complement-amplifying conditions are sufficient to lead to TMAs (5). This phenomenon could play a role in pregnancy-related complications such as preeclampsia and HELLP (a pregnancy complication characterized by hemolysis, elevated liver enzymes, and low platelet count)

syndrome (13,14), and in association with certain drugs (15), tumors (16), autoimmune diseases (17,18), malignant hypertension (19), and transplant-associated TMAs (20,21).

With this expanding spectrum of complement-mediated diseases, knowledge about a person's complement status could identify patients who might benefit from complement-targeting therapies. In this regard, some successful efforts have been made in aHUS (22–24). However, the approaches proposed have not been tested in severe preeclampsia or HELLP syndrome. The aim of this study was to modify the assay developed by Noris *et al.* (22) to detect complement overactivation in patients in different situations: those with acute aHUS, those under C5 inhibitor treatment to monitor eculizumab treatment efficiency, and those with other more prevalent disorders such as pregnancy-related complications and malignant hypertension.

Materials and Methods

Study Population and Sample Collection

A total of 34 patients with aHUS were enrolled from 15 different European hospitals. We obtained 11 samples in the acute phase of the disease (four of them with postpartum onset; Table 1), 20 samples from stable patients (complete remission) treated with regular doses of eculizumab, ten stable patients treated with lower doses or for longer intervals than those specified in the data sheet (Supplemental Table 1), five samples from patients in remission without eculizumab treatment, and four asymptomatic carriers of the mutation (Supplemental Table 2). Genetic analysis including the detection of genetic variants, risk haplotypes, anti-CFH antibodies, and serum levels of complement components and regulatory proteins from patients with aHUS are described in Supplemental Table 3. To evaluate test specificity, we also included five patients with different CKD stages due to autosomal dominant polycystic kidney disease (Supplemental Table 4).

Three patients with HELLP syndrome, seven with severe preeclampsia (Table 2), and ten healthy pregnant women matched by gestational age were included in the study (Supplemental Table 5). Finally, five patients with a clinical diagnosis of malignant hypertension were also included (Table 3). Disease definitions and diagnosis details are provided in Supplemental Material. Control samples were obtained from healthy individuals. Genetic analysis was not performed on patients with diseases other than aHUS in this study, which is a limitation of our work.

This study was approved by the ethics committee of Hospital Clinic and conformed to the ethical guidelines of the Helsinki Declaration. Participants or their legal guardians provided informed written consent before sample collection. Serum and plasma samples were obtained by centrifugation of nonanticoagulated blood and citrated blood ($3000 \times g$, 15 minutes), respectively, before 6 hours after extraction in all cases. All samples were aliquoted and stored at -80°C until they were used, avoiding freeze/thaw cycles.

Serum C3, C4, and 50% Hemolytic Complement Levels

Factors C3 and C4 were quantified in a Siemens Dade Behring BN II Nephelometer and its corresponding kits.

Complement activity (50% hemolytic complement, CH50) was measured using the Autokit CH50 (Wako).

C5b-9 Fluorescence Imaging

To evaluate complement activation, a modification of the technique described by Noris *et al.* (22) was used. With the aim of enhancing complement deposition, we proposed to take advantage of the interaction between coagulation and complement cascades and we added control sera to patient citrated plasma (1:1) to obtain activated plasma. The human dermal microvascular endothelial cell line (American Type Culture Collection) (25) was seeded on glass coverslips and used confluent. Cells were washed with test medium (HBSS without calcium or magnesium, 0.5% BSA; Life Technologies) and activated or not with $10 \mu\text{M}$ ADP (Sigma-Aldrich) (10 minutes, 37°C). Cells were then incubated (4 hours) with activated plasma diluted with test medium (1:2). Control samples were obtained by mixing healthy plasma from donors with pooled sera from controls. Cultures were then washed and fixed. For C5b-9 immunostaining, cells were treated with 2% BSA (1 hour) and incubated with a rabbit anti-human complement C5b-9 complex (Calbiochem), followed by Alexa594-conjugated goat anti-rabbit secondary antibody (Life Technologies) and 4',6-diamidino-2-phenylindole. Micrographs were captured by fluorescent microscopy (Leica DM4000B) through a video camera (Leica DFC310FX) and analyzed using Fiji (ImageJ) (26). A total of 20 photographs were randomly obtained from each preparation. The area covered by C5b-9 deposits was calculated and expressed as the average fold increase of each condition versus control. All samples were tested at least three times.

Statistical Analysis

Levels of the percentage of area covered by C5b-9 deposits were calculated as mean \pm SEM. Statistical analysis (SPSS) was performed with raw data using the *t* test for paired samples. Results were considered statistically significant when $P < 0.05$. However, in the article results are expressed as the fold increase of covered surface with respect to the control.

Results

Activated Plasma from Patients with Atypical Hemolytic Uremic Syndrome Is More Efficient in Inducing C5b-9 Deposition on Endothelial Cells than Sera

ADP-activated endothelial cells were exposed to sera (22) or activated plasma from 11 different patients with aHUS in acute phase (patient 1 to patient 11) and during remission (patient 2 to patient 6 and patient 8 to patient 11) (Table 1).

Serum samples obtained in acute phase induced more C5b-9 deposition on endothelial cells than control sera (fold increases of 3.2 ± 1.5 for patient 1, $P = 0.01$; 2.5 ± 1.2 for patient 2, $P < 0.001$; 1.9 ± 1 for patient 3, $P = 0.02$; 1.7 ± 0.8 for patient 4, $P = 0.03$; 2 ± 0.8 for patient 5, $P = 0.04$; 1.6 ± 0.4 for patient 6, $P = 0.04$; 2.7 ± 0.4 for patient 7, $P = 0.03$; and 2.8 ± 0.5 for patient 8, $P = 0.03$; versus control). Serum samples obtained during remission induced C5b-9 deposits equivalent or even lower than control sera (fold increase versus control of 1.4 ± 0.9 for patient 2, $P = 0.001$;

Table 1. Clinical parameters and complement activation markers in incident patients with atypical hemolytic uremic syndrome

Patient	Genetic Variants	Disease Phase (Trigger)	Creatinine (0.3–1.3 mg/dl)	Hemoglobin (12–17 g/dl)	Platelets (130–400 10 ⁹ /L)	Lactate Dehydrogenase (<234 U/L)	Haptoglobin (0.3–1.8 g/L)	Serum C3 (0.8–1.9 g/L)	Serum C4 (0.1–0.5 g/L)	Serum 50% Hemolytic Complement (28–60 U/ml)	Serum C5b-9 Deposits (Mean±SEM)	Activated-Plasma C5b-9 Deposits (Mean±SEM)
Patient 1	MCP: (c.478G>T) p.Val160Phe	Acute (none)	4.9	8.7	117	317	N.D.	1.0	N.D.	55	3.2±1.5 ^a	N.A.
Patient 2	C3: c.3125G>A MCP: c.1148C>T	Acute (respiratory infection)	3.9	9.4	7	2430	N.D.	0.8	0.3	72	2.5±1.2 ^a	N.A.
Patient 3	No	Remission ^b	0.8	12.1	259	146	1.1	0.8	N.D.	N.D.	1.4±0.9 ^{a,c}	N.A.
		Acute (none)	5	8.5	196	415	N.D.	0.7	0.1	28	1.9±1 ^a	N.A.
Patient 4	MCP: exon 6, heterozygosis (c.800_820Del) p.Thr267Asn273del CFH: exon 6, heterozygosis (c.292C>T) p.Leu98Phe	Remission ^d	4.4	9.2	492	194	N.D.	0.9	0.2	7	0.5±0.5 ^c	N.A.
		Acute (urinary infection)	8.6	10.9	73	1353	N.D.	0.6	0.2	24	1.7±0.8 ^a	5.6±0.2 ^a
Patient 5	CFH: exon 19, heterozygosis (c.2850G>T) p.Gln950	Remission ^e	6	12.7	158	516	1.29	0.8	0.3	9	0.6±0.5 ^c	0.3±0.1 ^c
		Acute (postpartum)	2.4	5.8	61	1678	N.D.	N.D.	N.D.	N.D.	2±0.8 ^a	4.9±0.1 ^a
Patient 6	No	Remission ^e	0.8	11.7	305	247	0.5	0.9	0.2	N.D.	0.1±0.1 ^c	0.1±0.18 ^c
		Acute (postpartum)	4.1	6.7	74	1781	0.2	1.4	0.5	21	1.6±0.4 ^a	5.9±0.4 ^a
Patient 7	No	Remission ^e	0.8	10.9	268	179	1.12	0.8	0.2	12	0.8±0.1 ^c	0.3±0.1 ^c
		Acute (postpartum)	1.6	13.0	93	266	0.3	1.1	0.3	N.D.	2.7±0.4 ^a	4.1±0.3 ^a
Patient 8	CFH: exon 5, heterozygosis (c.739T>G) p.Cys247Gly	Partial remission ^e	1.5	13.5	115	251	1.1	0.9	0.2	N.D.	2.7±0.5 ^a	2.4±0.1 ^a
		Acute (pancreatic cancer)	1.9	8.3	40	2407	N.D.	1.7	0.2	55	2.8±0.5 ^a	3.2±0.2 ^a
Patient 9	CFH: heterozygosis (c.3514G>T) p.Glu172Stop	Remission ^e	1.3	12.3	290	399	0.3	1.0	0.2	16	1.1±0.6	0.5±0.1 ^c
		Acute ^b (cocaine)	7.2	7.1	105	682	0.3	0.8	0.2	41	N.A.	13.1±0.2 ^a
Patient 10	CFH: heterozygosis (c.3514G>T) p.Glu172Stop	Remission ^e	2.2	13.0	197	304	1.3	0.7	0.2	8	N.A.	0.78±0.1 ^c
		Acute (cocaine)	12.2	5.9	44	3214	N.D.	N.D.	N.D.	N.D.	N.A.	10.9±0.3 ^a
Patient 11	CFHR1::CFH, hybrid gene (loss of signal in exon 6 of CFHR1 and gain of signal exon 23 of CFH)	Remission ^e	1.3	12.2	207	349	0.8	0.8	0.2	11	N.A.	1.1±0.2 ^c
		Acute (postpartum)	4.2	9.8	114	1649	0.1	0.4	0.1	29	N.A.	6.4±0.2 ^a
		Remission ^c	Hemodialysis	11.3	214	430	1.2	1.0	0.4	6	N.A.	0.8±0.1 ^c

Statistical analysis was performed with raw data using the *t* test for paired samples. N.D., not done; N.A., sample not available.

^aValues statistically different than control values. (*P*<0.05).

^bPlasma exchange treatment.

^cValues statistically different than acute phase values. (*P*<0.05).

^dPlasma exchange and eculizumab treatment.

^eEculizumab treatment.

Table 2. Clinical parameters and complement profile in patients with HELLP syndrome and severe preeclampsia

Patient	Disease Phase	Creatinine (0.3–1.3 mg/dl)	Hemoglobin (14–18 g/dl)	Platelets (130–400 10 ⁹ /L)	Proteinuria (mg/gr)	Blood Pressure (mm Hg)	Ankle Edema	Glutamic Oxaloacetic Transaminase (5–40 U/L)	Glutamic Pyruvic Transaminase (5–40 U/L)	Serum C3 (0.8–1.9 g/L)	Serum C4 (0.1–0.5 g/L)	Serum 50% Hemolytic Complement (28–60 U/mL)	Activated-Plasma C5b-9 Deposits (Mean ± SEM)
H-1	Acute (26 wk of pregnancy)	0.6	8.3	100	452	177/111	No	73	65	1.3	0.3	43.5	8.7 ± 1 ^a
	Quarantine 7 mo from delivery	0.6 N.D.	10 N.D.	125 N.D.	110 N.D.	120/82 N.D.	No No	14 N.D.	26 N.D.	0.9 1.0	0.3 0.3	42 32	3.8 ± 0.2 ^a 1.2 ± 0.1
H-2	Acute (37.5 wk of pregnancy)	2.8	11.2	63	6434	220/120	Yes	864	433	0.6	0.1	25	15.4 ± 0.5 ^a
	Quarantine 8 mo from delivery	0.9 N.D.	11.7 N.D.	351 N.D.	216 N.D.	115/80 N.D.	No No	99 N.D.	276 N.D.	1.3 1.3	0.6 0.3	48.5 49	9.1 ± 0.2 ^a 1.1 ± 0.1
H-3	Acute (34 wk of pregnancy)	1.1	10.2	55	910	180/118	Yes	1628	847	1.2	0.1	14	7.8 ± 1 ^a
	Quarantine 9 mo from delivery	0.6 N.D.	11.9 N.D.	382 N.D.	145 N.D.	116/78 N.D.	No No	37 N.D.	71 N.D.	1.8 1.6	0.4 0.3	50.5 50	6 ± 1.2 ^a 1.2 ± 0.2
PE-1	Acute (24.6 wk of pregnancy)	0.5	10.6	147	1059	161/84	No	44	36	13	0.3	45	2.3 ± 0.8 ^a
	Quarantine 6/9 mo from delivery	0.7 N.A.	13.6 N.A.	211 N.A.	71 N.A.	133/87 N.A.	No N.A.	23 N.A.	18 N.A.	1.4 N.A.	0.5 N.A.	42 N.A.	0.9 ± 0.1 N.A.
PE-2	Acute (26 wk of pregnancy)	0.6	12.5	222	638	170/110	No	19	30	1.3	0.1	49	2.4 ± 0.8 ^a
	Quarantine 9 mo from delivery	0.8 N.D.	12.3 N.D.	306 N.D.	50 N.D.	118/69 N.A.	No No	20 N.D.	40 N.D.	1.6 1.1	0.4 0.3	53 48	0.6 ± 0.2 0.8 ± 0.2
PE-3	Acute (35.2 wk of pregnancy)	1.0	11.7	135	2036	147/90	No	65	51	0.6	0.2	27	3.9 ± 0.1 ^a
	Quarantine 7 mo from delivery	1.0 N.D.	12.0 N.D.	296 N.D.	N.D. N.D.	109/56 N.D.	No No	21 N.D.	28 N.D.	1.5 1.3	0.4 0.4	58 59	1.5 ± 0.1 ^a 0.6 ± 0.1
PE-4	Acute (34.4 wk of pregnancy)	0.6	13.2	83	1102	160/110	No	25	21	1.8	0.3	63	1.5 ± 0.14 ^a
	Quarantine 6 mo from delivery	0.6 N.D.	14.0 N.D.	104 N.D.	222 N.D.	98/55 N.D.	No No	25 N.D.	47 N.D.	1.5 1.5	0.3 0.3	39 39	1.0 ± 0.1 0.7 ± 0.1
PE-5	Acute (34 wk of pregnancy)	1.2	12.1	201	298	174/99	Yes	160	286	1.1	0.2	52	2.6 ± 0.12 ^a
	Quarantine 6/9 mo from delivery	0.6 N.A.	12.2 N.A.	289 N.A.	55 N.A.	113/58 N.A.	No N.A.	20 N.A.	27 N.A.	1.5 N.A.	0.3 N.A.	57 N.A.	1.5 ± 1 ^a N.A.

Table 2. (Continued)

Patient	Disease Phase	Creatinine (0.3–1.3 mg/dl)	Hemoglobin (14–18 g/dl)	Platelets (130–400 10 ⁹ /L)	Proteinuria (mg/gr)	Blood Pressure (mm Hg)	Ankle Edema	Glutamic Oxaloacetic Transaminase (5–40 U/L)	Glutamic Pyruvic Transaminase (5–40 U/L)	Serum C3 (0.8–1.9 g/L)	Serum C4 (0.1–0.5 g/L)	Serum 50% Hemolytic Complement (28–60 U/mL)	Activated-Plasma C5b-9 Deposits (Mean ± SEM)
PE-6	Acute (33.4 wk of pregnancy)	0.6	14.3	117	5028	139/87	No	74	110	1.1	0.2	48	1.1 ± 0.1
	Quarantine 9 mo from delivery	0.5	15.5	282	253	123/76	No	27	46	1.3	0.3	52.5	0.8 ± 0.1
		N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	No	N.D.	N.D.	1.09	0.19	38.5
PE-7	Acute (34 wk of pregnancy)	0.6	10.2	282	2024	170/100	Yes	37	19	1.6	0.4	58	2.1 ± 0.1 ^a
	Quarantine 6/9 mo from delivery	0.5 N.A.	11.9 N.A.	371 N.A.	63 N.A.	106/63 N.A.	No N.A.	22 N.A.	15 N.A.	1.5 N.A.	0.3 N.A.	56 N.A.	N.D. N.A.

Statistical analysis was performed with raw data using the *t* test for paired samples. H-1, patient 1 with HELLP; N.D., not done; PE-1, patient 1 with preeclampsia; N.A., not available.
^aValues statistically different than control values.

Table 3. Clinical parameters, complement activation markers, and evolution in patients with malignant hypertension

Patient	Clinical Parameters								Complement Parameters				Treatment and Evolution			
	Blood Pressure (mm Hg)	Hypertensive Retinopathy ^a	Creatinine (0.3–1.3 mg/dl)	Platelets (130–400 10 ⁹ /L)	Hemoglobin (12–17 g/dl)	Lactate Dehydrogenase (<234 U/L)	Haptoglobin (0.3–1.8 g/L)	ADAMTS-13 Activity (%)	Serum C3 (0.8–1.9 g/L)	Serum C4 (0.1–0.5 g/L)	Serum 50% Hemolytic Complement (28–60 U/ml)	Activated-Plasma C5b-9 Deposits (Mean±SEM)	Anti-hypertensives drugs	Other Treatment	Response	Dialysis
MH-1	180/95	Grade 3	19.2	67	4.8	601	0.6	64	0.8	0.4	53.5	0.7±0.1	2	No	No	Yes
MH-2	204/115	Grade 3	38.8	76	6.9	833	N.D.	54	1.0	0.3	58	0.8±0.2	3	No	Hematologic	Yes
MH-3	170/90	Grade 4	23	110	8.3	384	N.D.	85	1	0.2	49.5	0.9±0.1	3	Steroids; eculizumab	Hematologic	Yes
MH-4	240/170	Grade 3	11.8	97	10.7	2095	0.01	46	1	0.3	54.5	0.6±0.2	2	No	No	Yes
MH-5	190/120	Grade 3	13.1	126	9.2	491	2.6	N.D.	1.4	0.4	57	1.1±0.1	5	No	Hematologic	Yes

ADAMTS-13, a disintegrin and metalloproteinase with thrombospondin motifs 13; MH-1, patient 1 with malignant hypertension; N.D., not done.
^aHypertension-related retinopathy classification according to the classification of Keith, Wagener, and Barker (51).

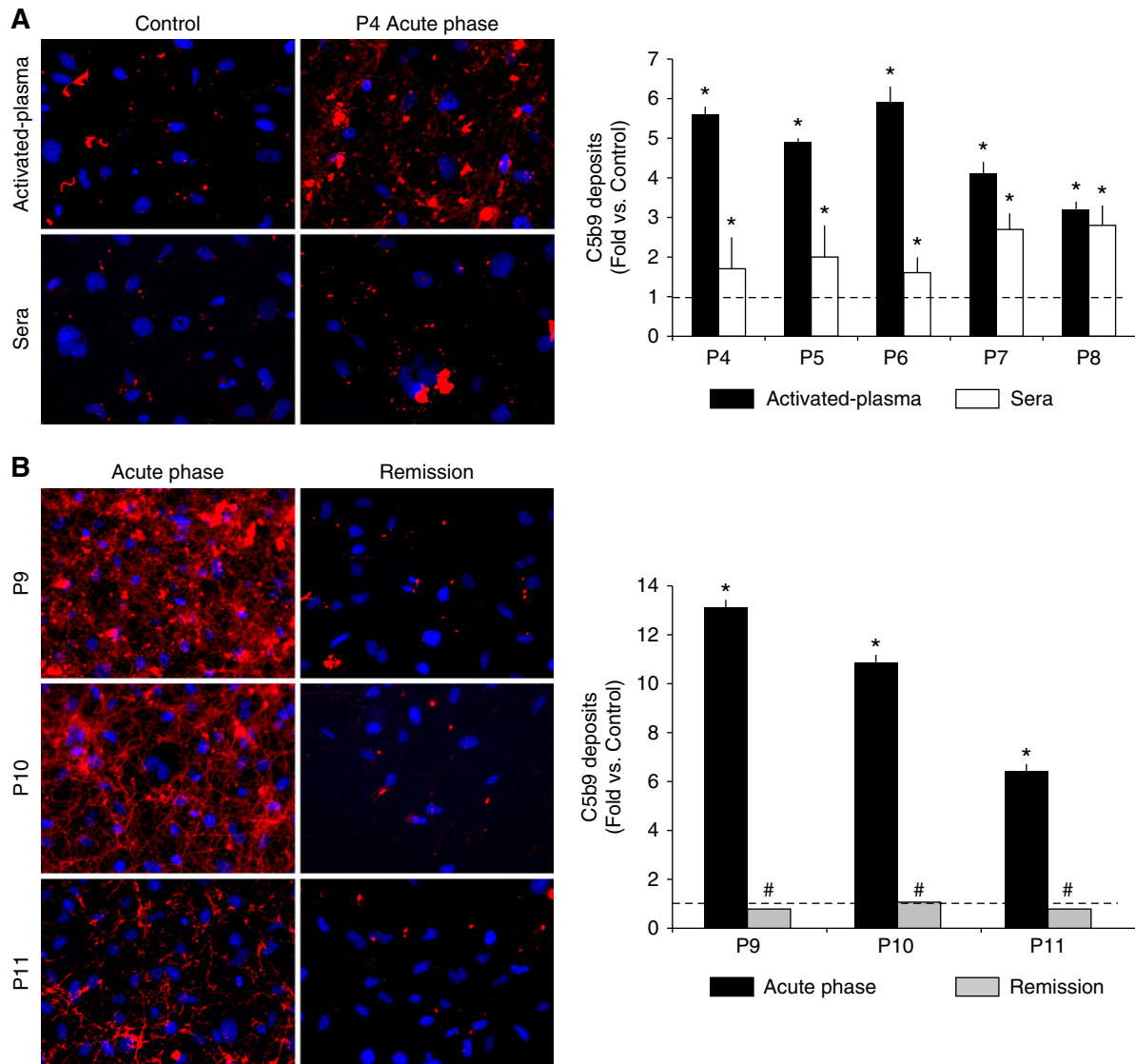


Figure 1. | Atypical hemolytic uremic syndrome (aHUS)–activated plasma induces C5b-9 deposition on microvascular endothelial cells with coefficient of variation significantly lower than that obtained using aHUS serum. (A) Representative microscopy image of C5b-9 deposition staining (red) on endothelial cells (4',6-diamidino-2-phenylindole–stained nuclei, blue) induced by activated plasma or sera from a control individual and a patient with aHUS in the acute phase (patient 4, P4). Bar diagram represents quantification of C5b-9 deposits expressed as fold increase of the covered surface with respect to the control, from five different patients with aHUS in the acute phase (P4 to P8). Black bars represent the results obtained with activated plasma and white bars with sera. The dotted line represents control values. (B) Representative microscopy images of staining of C5b-9 deposition on endothelial cells (induced by activated plasma from the acute phase and at remission in three patients: P9 to P11). Bar diagram represents C5b-9 quantification, black bars represent results from the acute phase, and gray bars represent remission. All values at the acute phase were statistically higher than in control samples ($*P<0.05$) and all values at remission are statistically lower than those at the acute phase ($\#P<0.05$). Statistical analysis was performed with raw data using the *t* test for paired samples.

0.5 ± 0.5 for patient 3, $P<0.001$; 0.6 ± 0.5 for patient 4, $P=0.008$; 0.1 ± 0.1 for patient 5, $P=0.004$; 0.8 ± 0.1 for patient 6, $P=0.03$; and 1.1 ± 0.6 for patient 8, $P=0.01$; for all samples compared with results at acute phase), except for patient 7. This patient had partial remission and C5b-9 deposits remained statistically higher than in controls (2.7 ± 0.5 versus control, $P=0.04$).

Experiments were run in parallel using both serum and activated plasma from patients with aHUS (patient 4 to patient 8) obtained during the acute phase and remission. Activated plasma from the acute phase induced a marked

C5b-9 deposition on endothelial cells (5.6 ± 0.2 for patient 4, 4.9 ± 0.1 for patient 5, 5.9 ± 0.4 for patient 6, 4.1 ± 0.3 for patient 7, and 3.2 ± 0.2 for patient 8, versus control; $P<0.001$ for all) (Figure 1A). Levels of C5b-9 returned to control levels when we used samples obtained from four out of five patients in remission (0.3 ± 0.1 for patient 4, 0.2 ± 0.1 for patient 5, 0.3 ± 0.1 for patient 6, and 0.5 ± 0.1 for patient 8, versus control; $P<0.001$ for all versus onset) (Table 1). The activated plasma from patient 7 at partial remission induced an increased C5b-9 deposition (2.4 ± 0.1 versus control, $P<0.001$). Although the results obtained from sera

from patients with aHUS reached statistical significance, the values obtained with activated plasma were much more notable and consistent. The coefficient of variation of the results obtained using the sera from the five patients at acute phase (52%, 74%, 74%, 31%, and 30%) was significantly higher than those obtained with activated plasma of the same five patients (9%, 11%, 10%, 10%, and 18%) (Supplemental Figure 1). These coefficients of variation were obtained analyzing 20 micrographs taken from each processed sample.

Activated plasma from three additional patients with aHUS was evaluated. C5b-9 deposits showed fold increases of 13.1 ± 0.2 for patient 9, 10.9 ± 0.3 for patient 10, and 6.4 ± 0.2 for patient 11 ($P < 0.001$ for all values versus control) at acute phase, and 0.8 ± 0.1 for patient 9, 1.1 ± 0.2 for patient 10, and 0.8 ± 0.1 for patient 11 during remission ($P < 0.001$ for all values versus acute phase) (Figure 1B). Of note, experiments with activated plasma from patient 9 after five daily plasma exchanges showed a positive effect in reducing complement activation, although it was unable to avoid it completely (6.9 ± 0.4 versus control, $P < 0.001$ versus control and versus acute phase). This finding showed a clear relation with clinical outcome (hematologic improvement without kidney function recovery). After eculizumab initiation, the patient showed both hematologic and kidney responses, and C5b-9 deposits were lower than in control.

Moreover, C5b-9 deposits induced by activated plasma from five patients with aHUS in remission without treatment and asymptomatic-mutation carriers on ADP-stimulated and -nonstimulated cells were also performed. Our results showed no differences when using both type of cells (Supplemental Table 2).

Effect of Standard and Lower Doses of Eculizumab on C5b-9 Deposition on Endothelial Cells

Complement activation, measured by C5b-9 deposits on activated endothelial cells, was at control levels or even lower when we analyzed the effect of activated-plasma samples from patients with aHUS who were treated at regular doses of eculizumab ($n=20$) or lower doses ($n=10$) (Figure 2A).

Prospective C5b-9 Deposition on Endothelial Cells To Titrate Eculizumab Dosage in Patients with Atypical Hemolytic Uremic Syndrome: Case Report 1

A 20-year-old woman (patient 29) with an MCP mutation (c.-325A>C) manifested aHUS in 2012. The patient recovered kidney function after 12 plasma-exchange sessions. She started eculizumab treatment in June 2014 after clinical and histologic disease recurrence. Despite initial improvement, she developed severe hypertension and progressive proteinuria with impaired kidney function (Figure 2B). CH50 and C5 free levels measured 14 days after eculizumab administration were undetectable, with correct, and even higher than normal, free eculizumab levels ($740 \mu\text{g/ml}$). However, at the same point, the patient had low C3 (71 mg/dl) and high sC5b-9 levels (471 ng/ml) (27). Analysis of C5b-9 deposition on endothelial cells showed increased complement activation when the patient was receiving a standard eculizumab dosage (3 ± 0.3 versus control, $P=0.01$) and even when the time between doses was reduced to 10 days (2.2 ± 0.1 versus control, $P=0.01$). C5b-9 deposits

only reached control levels after the eculizumab dose was increased up to 1500 mg every 14 days (0.8 ± 0.2 versus control), accompanied by better BP control, a recovery in C3 levels (84 mg/dl), persistent reduction of CH50 (11 mg/ml), a decrease in proteinuria, and stabilization of kidney function (Figure 2B).

C5b-9 Deposition Confirms Clinical Relapse in Patients with Atypical Hemolytic Uremic Syndrome with an Interruption in Eculizumab Treatment: Case Report 2

A 36-years-old woman (patient 13) was diagnosed with aHUS in 2004. She was treated with plasma exchange (>100 sessions) and achieved hematologic and kidney remission. She developed aHUS relapse in 2013 and started eculizumab treatment with an excellent response. In July 2017, after a period of lower eculizumab doses, she stopped treatment. She had normal C5b-9 deposits during eculizumab treatment (0.7 ± 0.1 with standard doses and 0.5 ± 0.2 with lower doses versus control). However, in November 2017 she developed a second aHUS relapse. An activated-plasma sample induced increased C5b-9 deposition on endothelial cells (2.2 ± 0.1 versus control, $P=0.03$; Figure 2C). Early eculizumab introduction allowed complete clinical recovery.

Significant Complement Activation Shown by HELLP and Preeclampsia, but Not Malignant Hypertension

The C5b-9 deposition on endothelial cells was analyzed in activated plasma from patients with HELLP syndrome and preeclampsia at the acute phase, 40 days after delivery and 6–9 months after onset (Table 2). Activated-plasma samples from patients with malignant hypertension at the acute phase were also tested (Table 3).

The baseline and perinatal outcomes of the pregnant study population are shown in Table 4. All patients with HELLP syndrome showed marked complement activation at the acute phase (8.7 ± 1 for patient 1 with HELLP, 15.4 ± 0.5 for patient 2 with HELLP, and 7.8 ± 1 for patient 3 with HELLP versus control, $P < 0.001$ for all values) that was still increased after 40 days (3.8 ± 0.2 for patient 1 with HELLP, 9.1 ± 0.20 for patient 2 with HELLP, and 6 ± 1.2 for patient 3 with HELLP versus control, $P < 0.001$ for all values), and normalized after 6–9 months. All except one patient with preeclampsia also showed increased complement activation, but only two were still positive after 40 days. All patients with preeclampsia showed normal complement activity after 6–9 months (Figure 3A). Ten control pregnant women were analyzed and they showed normal complement levels (Supplemental Table 5).

Activated plasma from patients with malignant hypertension ($n=5$) induced normal complement deposition (Figure 3B). After adequate BP control, clinical microangiopathic signs partially resolved (Table 3).

Circulating Complement Protein Levels

Soluble forms of C3, C4, and CH50 were analyzed in serum samples of all patients during both the acute phase and clinical remission. Circulating levels of these proteins were normal in most patients regardless of clinical status (see Supplemental Material).

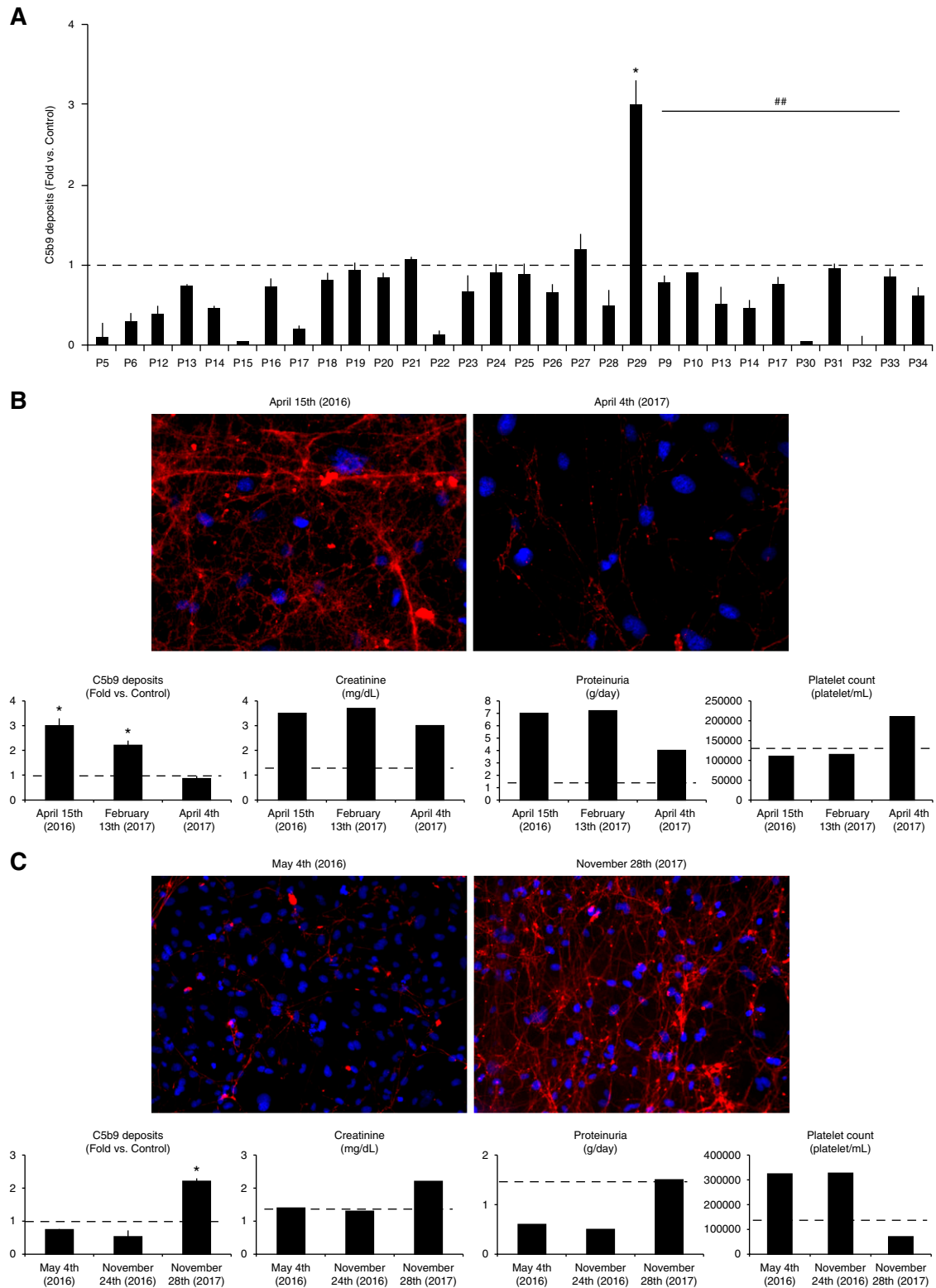


Figure 2. | C5b-9 deposits on activated-endothelial cells are at control level or even lower when analyzing the effect of activated-plasma samples from aHUS patients treated at regular doses of eculizumab or lower doses. Identification of two patients with partial remission and clinical relapse. (A) Bar diagrams show the results of C5b-9 deposition on endothelial cells of activated plasma from 20 patients with aHUS (P5 to P29) who were treated at regular doses of eculizumab (according to weight in pediatric patients; 900 mg/wk for 4 weeks as induction therapy for adults, and 1200 mg every 2 weeks thereafter as maintenance). All values resulted in control levels except in patient 29, in whom they were significantly higher (*). Bar diagram also shows, under the continuous line and ## symbol, the results from ten patients with aHUS who were treated at lower doses or for longer intervals than those specified in the data sheet. Activated plasma from those patients induced C5b-9 deposition at similar or even lower levels than control activated plasma. The dotted line represents control values. Statistical analysis Cont.

Table 4. Characteristics and perinatal outcomes of the study populations

Characteristics and Outcomes	Control (n=10)	Severe Preeclampsia/HELLP Syndrome (n=10)
Characteristics		
Age (yr)	33±4	33±5
White (%)	50	60
Nulliparity (%)	50	60
Gestational age at inclusion (wk)	37±4	33±6
Perinatal outcomes		
Gestational age at delivery (wk)	40 (2)	34 (8)
Male gender (%)	30	78
Birth weight (g)	3383 (188)	1794 (1180)
Birthweight percentile	47 (40)	3 (31)
Cesarean section (%)	10	90
Umbilical cord artery pH	7.2±0.1	7.2±0.1
5 min Apgar score	10 (0)	10 (1)

Data are shown as percentages, mean±SD, or median (interquartile range).

Discussion

The deposition of the membrane-attack complex on endothelial cells after exposure to sera from patients with aHUS in the acute phase indicates that the dysregulation of the alternative complement pathway plays a primordial pathogenic role in this disorder, as described by Noris *et al.* (22,23). We modified this previously described technique by exposing endothelial cells to plasma samples from patients with aHUS with the aim of reducing variability in the results obtained. C5b-9 deposition occurred together with fibrin formation and was more notable, reproducible, and highly consistent than that obtained using serum. Deposition of C5b-9 showed an excellent correlation with aHUS clinical stages. The evaluation of C5b-9 deposition allowed monitoring patient response to the treatment, enabling the identification of partial remission and clinical relapse. Our results indicate complement hyperactivation could also be crucial in the pathogenesis of HELLP syndrome and severe preeclampsia, but not in malignant hypertension. These findings could have potential diagnostic and therapeutic implications. The development of techniques verifying the underlying disease mechanism of aHUS (28,29) is crucial to allow early etiological treatment. In this scenario, Noris *et al.* (22) evaluated C5b-9 deposition on endothelial cells using serum from patients with aHUS and showed that deposits were present on unstimulated cells exposed to sera from patients during acute phase and this normalized at remission; whereas using stimulated cells they could also detect asymptomatic mutation carriers. We confirmed

these results in acute aHUS, but we encountered a very high coefficient of variation. We had no success when trying to reproduce the method of Gavriilaki *et al.* (24). With the aim of reducing interobserver variation, we proceeded to validate C5b-9 deposition on endothelial cells with activated plasma from patients with aHUS. The results followed the same tendency as those observed with sera, but C5b-9 deposits occurred together with fibrin formation on the cell surface. Importantly, we did not find differences when evaluating C5b-9 deposits induced by patients with acute aHUS in remission without treatment, and asymptomatic mutation carriers over stimulated and nonstimulated cells, meaning that this modification is not useful for the identification of asymptomatic mutation carriers (Supplemental Figure 2) (29). The potential bias involved in providing normal complement regulator proteins to the patient's sample should be overcome because results were always compared with control activated plasma. In addition, circulating levels of C3, C4, and CH50 were also measured, but failed to predict either aHUS diagnosis or clinical status. Finally, patients with CKD unrelated to TMA presented C5b-9 deposits at control levels (Supplemental Figure 3).

Eculizumab is the only medication approved for aHUS management. A complement functional test for monitoring the eculizumab administration schedule has been used to evaluate patients with aHUS in the stable phase (30,31). Other forms of monitoring have been published with promising results (32,33). We evaluated a total of 30 samples from 27 patients with aHUS treated with eculizumab.

Figure 2. | *Continued.* was performed with raw data using the *t* test for paired samples. (B) Results from the C5b-9 deposition assay allowed the identification of a patient who was receiving an underdose of eculizumab and allowed for a consequent dose adjustment. The left image corresponds to a representative image of C5b-9 deposits induced by activated plasma from patient 29 which was obtained on April 15, 2016. At that time point, C5b-9 deposition was statistically higher ($*P<0.05$) than control levels as seen in the bar diagrams. The right image corresponds to activated plasma from the same patient obtained on April 4, 2016, after several eculizumab dose changes, showing a negative result. Statistical analysis was performed with raw data using the *t* test for paired samples. The results from C5b-9 assays at three different time points corresponding to different eculizumab guidelines are quantified in the bar diagrams along with creatinine (mg/dl), proteinuria (g/d), and platelet count (platelet/ml). (C) Results from the C5b-9 deposition assay confirmed the identification of a patient with aHUS recurrence (left image) when C5b-9 deposition was higher than control levels (bar diagrams), and complete clinical recovery (right image) after the start of eculizumab treatment. Bar diagrams show C5b-9 quantification, creatinine (mg/dl), proteinuria (g/d), and platelet count (platelet/ml) at different time points.

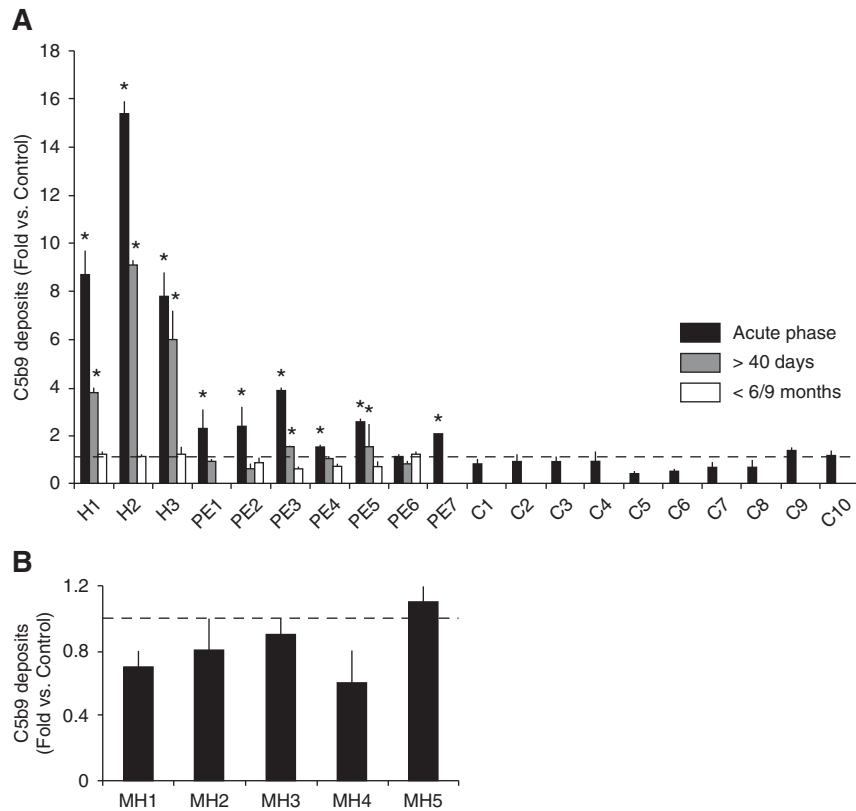


Figure 3. | Activated plasma from patients with HELLP syndrome and preeclampsia at onset induced an increase C5b-9 deposition on endothelial cells. (A) Quantification of C5b-9 deposits on endothelial cells show that three HELLP syndrome patients (H1 to H3) showed marked complement activation in the acute phase (black bars) that was still increased after 40 days (gray bars), and normalized after 6–9 months (white bars). All except one patient with preeclampsia (PE) also showed increased complement activation in the acute phase, but only two were still positive after 40 days. All patients with preeclampsia analyzed had normal complement activity after 6–9 months. Ten healthy pregnant women were also analyzed (C1 to C10), obtaining normal C5b-9 deposits. The dotted line represents control values. * $P < 0.05$ represents values statistically higher than control samples. Statistical analysis was performed with raw data using the *t* test for paired samples. (B) Bar diagrams show results from C5b-9 deposition induced by activated plasma from five patients with malignant hypertension (MH1 to MH5). All patients in the acute phase showed normal complement activity, represented by the dotted line. Statistical analysis was performed with raw data using the *t* test for paired samples.

All patients achieving clinical remission had C5b-9 deposits at control levels or lower, independently of dosage and therapeutic schedule. Of note, in patient 9 (who received five daily plasma exchanges with resolution of hemolysis but without kidney improvement), C5b-9 deposition still occurred, although it decreased with respect to the acute phase. After eculizumab initiation, this patient achieved complete clinical recovery, with C5b-9 deposits lower than in controls. In addition, C5b-9 deposition assessment in patient 29 allowed the identification of underdosing, and was useful to titrate the necessary dose for clinical remission. Finally, patient 13 had aHUS relapse after 4 months of stopping eculizumab, and C5b-9 evaluation confirmed the clinical suspicion.

Regarding pregnancy-related complications, our results showed intense complement activation in those with preeclampsia and HELLP syndrome during the acute phase, which was persistent 40 days after delivery in all HELLP cases, despite clinical remission. Normal complement activity was observed 6–9 months later. These results are in agreement with those of previous studies suggesting that a dysregulation of the complement pathway may play

an important role in its pathogenesis. Serum and urinary C5a and C5b-9 concentrations are elevated in hypertensive mothers (34,35), and preeclampsia has been described as a multifactorial disease with an angiogenic imbalance as an essential underlying mechanism that could be related to complement activation (35,36). There is experimental evidence indicating that complement activation induces the production of angiogenic molecules and C5a release by monocytes (37,38). Additionally, aberrant decidual angiogenesis appears to trigger the activation of the complement pathway. This potential key role for the complement system in the pathogenesis of HELLP and preeclampsia offers new opportunities for early prediction, monitoring, and therapy. Eculizumab has been used during pregnancy without apparent adverse outcomes for the mother or the fetus (39,40), and *in vitro* studies suggest a beneficial effect of eculizumab in HELLP (41).

Clinical diagnosis of malignant hypertension is based on the presence of very high BP levels and grade 3/4 hypertensive retinopathy. Clinical reports and short case series indicate the possibility that some malignant hypertension with TMA signs could be complement mediated

(42,43). Etiological differentiation between malignant hypertension and aHUS is crucial to initiate an etiological treatment. These results indicate that complement activity was normal in the acute phase and after BP control in patients with malignant hypertension. In contrast, when exploring samples from five out of the 11 patients with aHUS (who had grade 3 or 4 hypertensive retinopathy) who were evaluated at the acute phase, C5b-9 deposits were significantly high. Although we included a low number of patients with malignant hypertension in this study, these results validate recently published data (44) where C5b-9 deposits on endothelial cells in the setting of severe hypertension differentiated patients with complement dysregulation from those with mechanical stress as the cause of disease. It will be crucial in future investigations to know if patients diagnosed with HELLP, preeclampsia, and malignant hypertension are carriers of complement mutations.

This complement-activity assay is based on the existence of multiple links between the complement and the clotting system (45), as it could be derived from the inhibitory experiments carried out (see Supplemental Material). C5b-9 deposition on the endothelial cell surface occurs together with fibrin formation, even in control situations, and strongly depends on thrombin generation (Supplemental Figure 4). This observation is in line with others reporting that complement activation is attenuated by thrombin inhibitors (46,47). The inhibition of the reaction by blocking coagulation Factor XII suggests that serine proteases in the coagulation system are able to activate the complement cascade independently of the pathways established to date (48). Moreover, our assay seems to be independent of the extrinsic coagulation pathway because the inhibition of the tissue factor by a specific antibody had no effect on C5b-9 deposition (Supplemental Figure 4) (49). Finally, our reaction strongly depends on the presence of calcium because it does not occur when using nonactivated plasma or when the activated plasma is obtained from blood with EDTA (Supplemental Figure 4). This could be because EDTA presents a much higher binding constant than citrate (50). By adding the serum of healthy donors to the plasma samples of patients, we overcame the partial complement inhibition due to calcium chelation and reduced the variability observed in complement deposition when using sera.

In conclusion, measurement of C5b-9 deposits on endothelial cells is a useful tool to explore the overactivation of the complement system, not only in aHUS but also in other diseases, such as HELLP and preeclampsia. Moreover, this technique could be useful to monitor the efficiency of eculizumab treatment in individual patients with aHUS, allowing for follow-up of treatment response, exploration of optimal dosage, and identification of potential recurrences. Our approach offers consistent results that, if confirmed in larger cohorts, could open the door to the evaluation of the role of complement activation in the pathogenesis of other TMAs that may eventually benefit from C5 inhibition and monitor treatment response in these patients.

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Supplemental Material

This article contains the following supplemental material online at <http://cjasn.asnjournals.org/lookup/suppl/doi:10.2215/CJN.05830519/-/DCSupplemental>.

Supplemental Table 1. Clinical parameters and complement activation markers in aHUS patients treated with eculizumab.

Supplemental Table 2. Clinical parameters and complement activation markers in aHUS patients without eculizumab treatment and asymptomatic mutation carriers.

Supplemental Table 3. Genetic and molecular analyses in aHUS patients.

Supplemental Table 4. Kidney function and complement parameters in ADPKD patients.

Supplemental Table 5. Complement parameters in pregnancy controls.

Supplemental Figure 1. Activated-plasma and sera induction of C5b9 deposits on endothelial cells.

Supplemental Figure 2. Asymptomatic mutation carrier's activated-plasma induction of C5b-9 deposits on activated or non-activated endothelial cells.

Supplemental Figure 3. Autosomal dominant polycystic kidney disease patients activated-plasma induction of C5b-9 deposits on endothelial cells.

Supplemental Figure 4. Blockade of C5b-9 deposition on endothelial cells by eculizumab and intrinsic coagulation pathway inhibitors.

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M. Palomo and M. Blasco contributed equally to this work.

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AFFILIATIONS

¹Josep Carreras Leukaemia Research Institute; ²Hematopathology, Department of Pathology, Centre de Diagnostic Biomedic (CDB), Hospital Clinic de Barcelona, Institut d'Investigacions Biomediques August Pi i Sunyer (IDIBAPS), Universitat de Barcelona, Barcelona, Spain; ³Barcelona Endothelium Team, Barcelona, Spain; ⁴Department of Nephrology and Renal Transplantation, Hospital Clinic de Barcelona, Universitat de Barcelona, Spain; ⁵Group of nephro-urological diseases and renal transplantation (IDIBAPS), Barcelona, Spain; ⁶Apheresis Unit, Department of Hemotherapy and Hemostasis, Institut Clinic de Malalties Hematologiques i Oncologiques (ICMHO), IDIBAPS, Hospital Clinic de Barcelona, Universitat de Barcelona, Barcelona, Spain; ⁷Department of Nephrology, Hospital Universitario 12 de Octubre and Research Institute i+12, Madrid, Spain; ⁸Department of Medicine, Universidad Complutense, Madrid, Spain; ⁹Fetal i+D Fetal Medicine Research Center, BCNatal - Barcelona Center for Maternal-Fetal and Neonatal Medicine (Hospital Clinic de Barcelona and Hospital Sant Joan de Deu), ICGON, IDIBAPS, Universitat de Barcelona and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBER-ER), Barcelona, Spain; ¹⁰Division of Nephrology, Cliniques universitaires Saint-Luc, Institut de Recherche Expérimentale et Clinique, Université catholique de Louvain, Brussels, Belgium; ¹¹Department of Nephrology, Hospital Universitario Reina Sofía e Instituto Maimonides de Investigaciones Biológicas de Córdoba (IMIBIC), Córdoba, Spain; ¹²Department of Nephrology and Renal Transplantation, Hospital Universitario Dr Peset, Valencia, Spain; ¹³Department of Nephrology, Hospital Virgen del Rocío, Sevilla, Spain; ¹⁴Department of Pediatric Nephrology, Hospital Materno-Infantil, Vall d'Hebron, Universitat Autònoma de Barcelona, Barcelona, Spain; ¹⁵Department of Nephrology, Complejo Hospitalario de Navarra, Pamplona, Spain; ¹⁶Department of Nephrology, Hospital Universitari Josep Trueta, Girona, Spain; ¹⁷Department of Nephrology, Hospital Puerta de Hierro Majadahonda, Madrid, Spain; ¹⁸Department of Pediatric Nephrology, Hospital de la Santa Creu i Sant Pau, Universitat Autònoma de Barcelona, Barcelona, Spain; ¹⁹Department of Nephrology, Complejo Hospitalario Universitario A Coruña, Coruña, Spain; ²⁰Department of Nephrology, Hospital Universitari Arnau de Vilanova, Lleida, Spain; ²¹Department of Nephrology, Hospital General Universitario, Ciudad Real, Spain; ²²Department of Nephrology, Hospital Universitari Vall d'Hebron, Universitat Autònoma Barcelona, Barcelona, Spain; ²³Kidney Transplant Unit, Department of Nephrology, Hospital de Bellvitge, Universitat de Barcelona, Barcelona, Spain; and ²⁴CDB, Barcelona, Spain