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Ex vivo C5b-9 Deposition Test to Monitor Complement Activity in Clinical and Subclinical Atypical Hemolytic Uremic Syndrome and in Transplantation-Associated Thrombotic Microangiopathy

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Introduction: Atypical hemolytic uremic syndrome (aHUS) is a complement system (CS)–mediated ultrarare disease that manifests as thrombotic microangiopathy (TMA) with preferential small kidney vessels involvement. Transient CS activation is also observed in secondary TMA or in patients at risk of developing aHUS. There is no gold standard test to monitor disease activity; however, the *ex vivo* C5b-9 deposition test seems to be a good approach.

Methods: We assessed the C5b-9 deposition induced by serum samples of patients with aHUS ($n = 8$) and with TMA associated with kidney ($n = 2$), lung ($n = 1$) or hematopoietic stem cell (HSC) transplantation (HSCT, $n = 2$) during the acute phase of the disease or in remission. As control for transplant-associated TMA (TA-TMA), we analyzed samples of clinically stable kidney and HSC-transplanted patients without signs of TMA. In addition, we studied 1 child with genetic risk of aHUS during an acute infection.

Results: In the acute disease phase or in patients with disease activity despite C5 blockade, a significant increase of C5b-9 deposition was detected. In all patients with clinical response to C5 blockade but one, levels of C5b-9 deposition were within the normal range. Finally, we detected increased C5b-9 deposition levels in an asymptomatic child with genetic risk of aHUS when a concomitant otitis episode was ongoing.

Conclusion: The *ex vivo* C5b-9 deposition test is an auspicious tool to monitor CS activity in aHUS and TA-TMA. In addition, we demonstrate that the test may be useful to detect subclinical increase of CS activity, which expands the spectrum of patients that would benefit from a better CS activity assessment.

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aHUS is a disorder caused by complement-mediated endothelial damage at the microvasculature that manifests as TMA. It primarily affects the kidney but can also affect the brain or the heart. It is clinically characterized by the simultaneous triad of peripheral thrombocytopenia, mechanical hemolytic anemia, and acute kidney injury.[1,2] aHUS is caused by dysregulation of the alternative pathway of the CS.[3,4] This alteration leads to increased C3 hydrolysis rate associated

with its interaction with cellular interfaces that do not adequately possess complement regulators such as the membrane cofactor protein, factor H (FH), factor I, or factor B.[5,6] This process is followed by an amplification of elevated C3bBb production (the alternative pathway C3 convertase), which ultimately leads to formation of the membrane attack complex (i.e., C5b-9) and pore formation on the cell membrane. This translates into endothelial damage and TMA in the kidney microvasculature.[5,7] In most cases (~60%), it is attributed to pathogenic variants of complement genes (loss of function mutations in *CFH*, *CFI*, and *MCP* clustering genes or gain of function variants in *C3* or *CFB* genes). Up to 10% to 12% of patients show presence of anti-FH autoantibodies mostly associated with homozygous deletions or rearrangements in the complement FH–related proteins (*CFHRs*) genes.[1,3,8] These factors are *per se* insufficient to prompt disease development because a second event or trigger is needed in most of the cases to activate the disease (two-hit model).[4] Triggers include infections, drugs, autoimmune diseases, malignant hypertension, pregnancy, malignancy, or transplantation, among others. These triggering events can activate the CS independently of genetic mutations or autoantibodies causing TMA and are classified as secondary TMA. Further, a proportion of patients with underlying conditions may also show mutations in CS genes (6%–8%).[9] Thus, it is not always easy to distinguish primary aHUS from secondary TMA.[10]

C5 blockade therapy with eculizumab or ravulizumab, a new long-acting next-generation C5 inhibitor,[11] represents the specific treatment for aHUS. C5 blockade with either eculizumab or ravulizumab has drastically improved patient outcome, reduced mortality, promoted kidney function recovery, decreased progression to end-stage kidney disease, and protected from disease recurrence after transplantation.[1,11-16] Eculizumab was initially approved as a long-life treatment; however, nowadays the duration of CS blockade is controversial, and a personalized approach based on individual risk and genetic background is proposed at least in some patients. Further, successful interruption of treatment in some individuals has been described, whereas others have relapsed afterward.[17-21] C5 blockade is also a controversial point in patients with acquired TMA or "secondary aHUS" because CS dysregulation may happen temporarily in these patients, which exacerbates the disease but is not the main cause.[9] However, several studies have shown that patients with secondary aHUS exhibit as good response to eculizumab as individuals with aHUS; a reason why they are thought to be subsidiary to temporary treatment.[22-27]

The efficacy assessment of eculizumab treatment involves several laboratory measurements. These include monitoring of hemoglobin levels to assess the recovery of anemia, platelet count to evaluate the prothrombotic endothelial phenotype, and measurement of lactate dehydrogenase (LDH) activity in serum. The presence of hematuria or proteinuria, blood pressure measurement, and serum creatinine are also evaluated to assess kidney injury. In addition, functional complement assessment is performed, which involves measuring serum levels of C3 and C4, soluble C5b-9 (sC5b-9), CH50, or AP50. In some cases, eculizumab levels in plasma before dosing are measured, if available in practice.[28] All these determinations are indirect and may not accurately estimate the activity of the disease itself because they do not assess the complement status at the endothelial cell surface. In addition, the recently advocated follow-up with sC5b-9 levels as a way to evaluate CS activity amplification has limitations because sC5b-9 values do not always mirror disease activity. Thus, it lacks precision to reliably evaluate disease activity and eculizumab effectivity.[29] It has been suggested that direct measurement of C5b-9 deposits on cultured endothelial cells could be a better approach to evaluate the complement functional state in patients with aHUS.[29-33] Here, we describe the applicability of a pilot of the C5b-9 *ex vivo* deposition test as a tool to monitor disease activity and response to eculizumab therapy in patients with aHUS or TMA associated with transplantation as well as in a child with genetic risk of aHUS during an acute infection.

METHODS

Patients and Clinical Parameters

Thirteen patients with aHUS or TA-TMA in the acute phase of the disease (untreated) or treated with eculizumab were studied (Tables 1 and 2). One child with genetic risk of aHUS during an acute infection was analyzed as well (Supplementary Table S1). Serum samples from healthy controls ($n = 18$) obtained from anonymous donors at the Blood and Tissue Bank in the Vall d'Hebron Hospital were used to establish the normal range of *ex vivo* C5b-9 deposition. We also obtained serum from stable kidney transplant patients with primary kidney diseases different from aHUS ($n = 6$) or from HSC-transplanted ($n = 2$) patients without TMA signs as control for the TA-TMA group (Supplementary Table S3). Serological complement components C3, C4, sC5b-9, FH, factor I, anti-FH, and CH50 were monitored as well as hemoglobin, platelet

count, LDH, and serum creatinine levels. All the measurements were performed in the core clinical laboratories of the Vall d'Hebron Hospital as part of the clinical care of the patients.

The study was approved by the Clinical Investigation Ethics Committee of the Vall d'Hebron Hospital (PR[AG]13-2023) and was conducted according to the principles of the Declaration of Helsinki.

C5b-9 Deposits Detection by Immunofluorescence

We studied the C5b-9 deposition on human dermal microvascular endothelial cells (HMEC-1; ATCC CRL-3234) by immunofluorescence using a specific antibody against complement C5b-9 (204903, Calbiochem) as described by Noris *et al*.[30] Details are described in the Supplementary Methods. Immunofluorescence images were taken using a FV100 Fluoroview Olympus confocal microscope. A total of 15 fields were analyzed to calculate the average staining area of each sample in pixels using the Image J Fiji software (v1.53a, National Institutes of Health, Bethesda, MD).

Statistical Analysis

The statistical analysis was conducted using two-way analysis of variance followed by Sidak's or Dunnett's multiple comparisons test using Graphpad Prism 8 (GraphPad Software, San Diego, CA). *P*-values less than 0.05 were considered statistically significant.

RESULTS

Study Population

Thirteen patients (8 females and 5 males) with ages ranging from 4 to 51 years diagnosed of aHUS or TMA secondary to kidney, lung, or HSCT were studied. Those patients were either untreated acute cases or undergoing eculizumab or ravulizumab treatment (Tables 1 and 2). Further, a boy without overt aHUS but carrier of a genetic risk (*CFHR3–CFHR1* deletion in homozygosis and the risk polymorphism c.329T>Cp [Val110Ala] in *CFHR5* in heterozygosis) who was identified by family screening was also studied during an acute otitis episode (Supplementary Table S1).

As shown in Table 1, all patients with a primary diagnosis of aHUS (P1–P8) had undergone a complete genetic study. Pathogenic variants in CS genes as well as risk polymorphisms were detected in most of the cases except for P2 who did not show any known genetic variant in complement genes (Table 1). The time of disease follow-up ranged from 20 days to 9 years, and at the time of analysis, all the patients with aHUS were receiving treatment with eculizumab or ravulizumab to control the disease. All patients continued on C5 blockade except for P1 whom eculizumab was stopped later on. Two of the patients (P2 and P6) with aHUS received a kidney allograft due to end-stage renal disease. P2 was diagnosed at age 3 of aHUS with no underlying genetic variant detected (Table 1). Despite leaving hemodialysis during the early phase of the disease after 3 months of receiving eculizumab, he remained with CKD3b for 2 years with late progression to renal failure afterward, returning to chronic hemodialysis for some months until kidney transplantation (KT) at the age of 5 years. This patient never suffered from aHUS recurrence while being treated with eculizumab since the disease onset and is currently treated with ravulizumab. P6 was already under chronic renal replacement therapy when diagnosed 9 years earlier related to the detection of a heterozygous *CFHR3:CFHR4* hybrid gene and the risk polymorphisms MCPggaac and CFHTGTGGT (Table 1, P6). Three years later, she received a kidney transplant under prophylactic eculizumab treatment and has never shown signs of aHUS recurrence after KT (Table 1).

Finally, we studied 2 cases of TMA associated with KT, 1 case of TMA associated with lung transplantation (LT) and 2 cases of HSCT: patients KT1, KT2, LT1, HSCT1 and HSCT2 (Table 2). KT1 was a 25-year-old female with a double liver transplantation and KT due to autosomal dominant polycystic kidney disease, whereas KT2 was a 51-year-old male diagnosed with hypertension and obstructive uropathy. Complement genetic study was not performed in KT1 (Table 2). KT2 was diagnosed with TMA 3 years after KT with no mutation or risk polymorphism detected in the genetic study (Table 2). LT1 was a 51-year-old man with an LT due to bronchiolitis obliterans organizing pneumonia that presented with TMA shortly after LT. Finally, we studied HSCT patients with TMA. Both HSCT1 and HSCT2 were studied in the acute phase of the disease (at TMA onset) and in remission under eculizumab therapy. HSCT1 had a complete genetic study where no mutations or risk polymorphisms were detected (Table 2). Data regarding complement fragments C3, C1q, C4d, and C4c in kidney tissue of the patients with TA-TMA with a diagnostic kidney biopsy available (Table 2: KT2, LT1, HSCT1, and HSCT2) can be found in Supplementary Table S2.

C5b-9 Deposition Test in Patients With aHUS

After setting up the method and determining the normal C5b-9 deposition range (Supplementary Results and Figure S2), we tested the serum samples of the patients with aHUS detailed in Table 1. In Figure 1a and Supplementary Table S4, we show the mean C5b-9

Table 1. Demographics and clinical data of the patients with aHUS included

Patient	Sex	Age, yr	Diagnosis	Mutations	Risk SNPs	Unrelated or uncertain significance SNPs	Sample	Time from aHUS diagnosis	Treat	Through Ecu. level (μg/ml)	Status	Kidney Biopsy/ finding
P1	F	6	aHUS	CFHR3-CFHR1 deletion (HOM)/Low copy number of C4A or C4B genes (2 copies)	MCPggaac (HET)	VWF, c.7463G>A, p.Gly2488Asp (HET) C6, c.2381+2T>C, rs76202909, p.Gly2488Asp (HET)	S1	7 mo	Ecu	>100	Rem.	No
P2	M	9	aHUS	none	none	none	S1	6 yr	Ecu	>500	Rem.	No
P3	F	5	aHUS	MCP, c.493C>T, p.Pro165Ser, rs759136081 (HET)	CFH (H3) (HOM)/ MCPggaac (HET)	C5, c.2381+2T>C, rs76202909 (HET), C7, c.1561C>A, p.Arg521Ser, rs121964920 (HET), CR2, c.1591C>G, p.Pro531Ala (HET), ITGAX, c.1148C>T, p.Pro383Leu, rs138501712 and c.3380T>A, p.Leu1127Gln (Both HET)	S1	2.5 yr	Rav	n.d.	Rem.	No
P4	M	4	aHUS	MCP, c.565T>G, p.Tyr189Asp, rs202071781 (HET)/CFI, c.1643A>G, p.Glu548Gly, rs771446070 (HET)	CFH (H3) (HOM)/ MCPggaac (HOM)	none	S1 S2	20 d 1 yr	Ecu Ecu	>250 >500	Rem. Rem.	No No
P5	F	13	aHUS	CFH, 23 c.3572C>G, p.Ser1191Leu and c.3590T>C, p.Val1197Ala, rs460184 (both in HET)	none	VWF,c.6859C>T, p.Arg2287Trp, rs61750625 (HET)	S1	5 yr	Rav	n.d.	Rem.	No
P6	F	31	aHUS	CFHR3:CFHR4 hybrid gene (heterozygous deletion of CFHR3-Exon 5, the whole CFHR1 gene, and CFHR4-Exons 1 to 8)	MCPggaac (HOM)/ CFH_{TGTGGT} (HET)	none	S1	9 yr	Ecu	>150	Rem.	No
P7	M	42	aHUS	CFHR3 deletion-CFHR4 duplication	CFH (H3) (HET)	none	S1	4 yr	Ecu	>500	Rem.	No
P8	F	13	aHUS	none	CFH (HET)/ CD46 (MCP) (HET)	none	S1 S2	4 mo 5 mo	Ecu (1200 mg/w) Ecu (1500 mg/w)	n.d. n.d.	Acute Rem.	Yes/TMA -

aHUS, atypical hemolytic uremic syndrome; CH50, the complement total; Ecu, eculizumab; F, female; FH, Factor H; FI, Factor I; Hb, hemoglobin; HD, hemodialysis; HET, heterozygosis; HOM, homozygosis; KT, kidney transplantation; LDH, lactate dehydrogenase; M, male; n.d., not determined; Rav, ravulizumab; Rem, remission; sC5b-9, soluble C5b-9; TMA, thrombotic microangiopathy.
The normal ranges are as follows: eculizumab, >100 μg/ml; CH50 activity, <13 U/ml; C3 component, 85–180 mg/dl; C4 component, 10–40 mg/dl; sC5b-9, 127–303 ng/ml; FH, 12–56 mg/dl; anti-FH antibodies, <10 AU/ml; FI, 2.5–5 mg/dl; platelet count, 150–400 10^9/l; LDH, 140–280 UI/l; hemoglobin, 11.5–15.5 g/dl; serum creatinine, 0.24–0.73 mg/dl.

deposition on cultured HMEC-1 cells in resting and activated conditions obtained using serum samples of the included patients with aHUS. In all cases, significantly increased C5b-9 deposition was observed when the assay was performed on adenosine diphosphate (ADP)-activated endothelial cells, as compared to

Table 2. Demographics and clinical data of the TMA cases associated with KT or HSCT

Patient	Sex	Age, yr	Diagnosis	Base disease	Mutations	Risk SNPs	Unrelated or uncertain significance SNPs	Sample	Time post Transpl.	Time from TMA diagnosis	Treat.
KT1	F	25	TMA secondary to kidney and liver transplantation (suspicion)	ADPKD	n.d.	n.d.	n.d.	S1	7 yr	N.A.	N.A.
KT2	M	51	TMA secondary to KT	Hypertensive nephropathy and chronic obstructive uropathy	none	none	CFH, c.2669G>T, p. Ser890Ile y c.3019G>T, p. Val1007Leu (HET)	S1	3 yr	4 mo	Ecu
LT1	M	57	TMA secondary to lung transplant	Bronchiolitis obliterans organizing pneumonia	n.d.	n.d.	n.d.	S1	4 d	0	-
HSCT1	F	18	TMA secondary to HSCT	B-cell acute lymphoblastic leukemia	none	none	none	S1 S2	0.5 yr 1 yr	0 6 mo	- Ecu
HSCT2	F	44	TMA secondary to HSCT	Acute myelomonocytic leukemia	n.d.	n.d.	n.d.	S1 S2	2,5 yr 2,75 yr	0 3 mo	- Rav

ADPKD, autosomal dominant polycystic kidney disease; aHUS, atypical hemolytic uremic syndrome; CH50, the complement total; Ecu, eculizumab; F, female; FH, factor H; FI, factor I; Hb, hemoglobin; HD, hemodialysis; HET, heterozygosis; HSCT, hematopoietic stem cell transplantation; KT, kidney transplantation; LDH, lactate dehydrogenase; M, male; N.A., not applicable; n.d.: not determined; Rav, ravulizumab; Rem, remission; sC5b-9, Soluble C5b-9; TMA, thrombotic microangiopathy.
The normal ranges are as follows: eculizumab, >100 μg/ml; CH50 activity, <13 U/ml; C3 component, 85–180 mg/dl; C4 component, 10–40 mg/dl; sC5b-9, 127–303 ng/ml; FH, 12–56 mg/dl; Anti-FH antibodies, <10 AU/ml; FI, 2.5–5 mg/dl; platelet count, 150–400 10^9/l; LDH, 140–280 UI/l; hemoglobin, 11.5–15.5 g/dl; serum creatinine, 0.24–0.73 mg/dl.

Table 1. (Continued)

HD	KT/Num	Time post-KT	Platelet count (10^9/l)	LDH (UI/l)	Hb (g/dl)	sCr (mg/dl)	CH50 (U/ml)	C3 (mg/dl)	C4 (mg/dl)	sC5b-9 (ng/ml)	FH (mg/dl)	Anti-FH (AU/ml)	FI (mg/dl)
No	No	-	502	386	11,9	0,37	<13	75,5	6,37	n.d	n.d	243,2	n.d
No	Yes/1	4 yr	190	206	11	0,58	<13	124	20,7	210	n.d.	<10	n.d.
No	No	-	231	242	12,5	0,28	<13	100	24,6	n.d.	n.d	<10	n.d
No	No	-	135	339	11,8	0,5	<13	53,3	21,4	n.d.	n.d	<10	1,92
No	No		249	219	11,5	0,41	<13	63,1	15,1	206,4	33,5	<10	1,66
No	No	-	273	178	11,2	0,54	13,13	97,3	19,5	266	n.d.	<10	n.d.
No	Yes/1	5 yr	312	174	13,3	0,86	<13	89,1	27,3	746	n.d.	<10	n.d.
No	No	-	287	148	15	1,72	<13	136	27,3	88	n.d.	<10	n.d.
Yes (2 mo.)	No	-	290	219	10,8	3,6	<13	158	84,5	404,9	>70	<10	5,52
No	No	-	312	152	12	2,37	<13	138	85	241,6	n.d.	n.d.	n.d.

their resting counterparts, which is consistent with a proper activation of the endothelial cells (Supplementary Figure S1). We included 7 patients with apparently well-controlled aHUS (P1–P7 in Table 1). As expected, the serum of all the patients and the second serum sample of P4 resulted in normal C5b-9 deposition levels (Figure 1a and b). However, for P4 who was indeed in the very early phase of treatment

Table 2. (Continued)

Through Ecu level (μg/ml)	Status	Kidney biopsy/finding	HD	KT/Num	Platelet count (10^9/l)	LDH (UI/l)	Hb (g/dl)	sCr (mg/dl)	CH50 (U/ml)	C3 (mg/dl)	C4 (mg/dl)	sC5b-9 (ng/ml)	FH (mg/dl)	Anti-FH (AU/ml)	FI (mg/dl)
-	-	No	Yes (4 days)	Yes/3	109	220	9	4.16	25,52	54.7	6.66	144	51.6	< 10	2.26
> 100	Non-response to Ecu	Yes/ TMA	No	Yes/2	172	588	10.4	4.09	< 13	75.2	20.3	425	53.2	<10	2.26
-	Acute	Yes/TMA	Yes (3 days)	No	47	1625	9.4	1.92	n.d.	117	26.2	467.7	47.7	< 10	3.53
-	Acute	Yes / TMA	No	No	22	426	10.6	0.76	56.44	106	16.1	333	32.97	< 10	2.27
> 250	Rem.	-	No	No	24	239	8.4	1,25	14.33	180	36	110.1	n.d.	< 10	n.d.
-	Acute	Yes/TMA	No	No	337	200	8.5	1.21	> 90	155	45.8	199.6	> 70	< 10	5.37
n.d.	Rem.	-	No	No	256	229	11.7	1.31	n.d.	127	42.7	365.9	n.d.	n.d.	n.d.

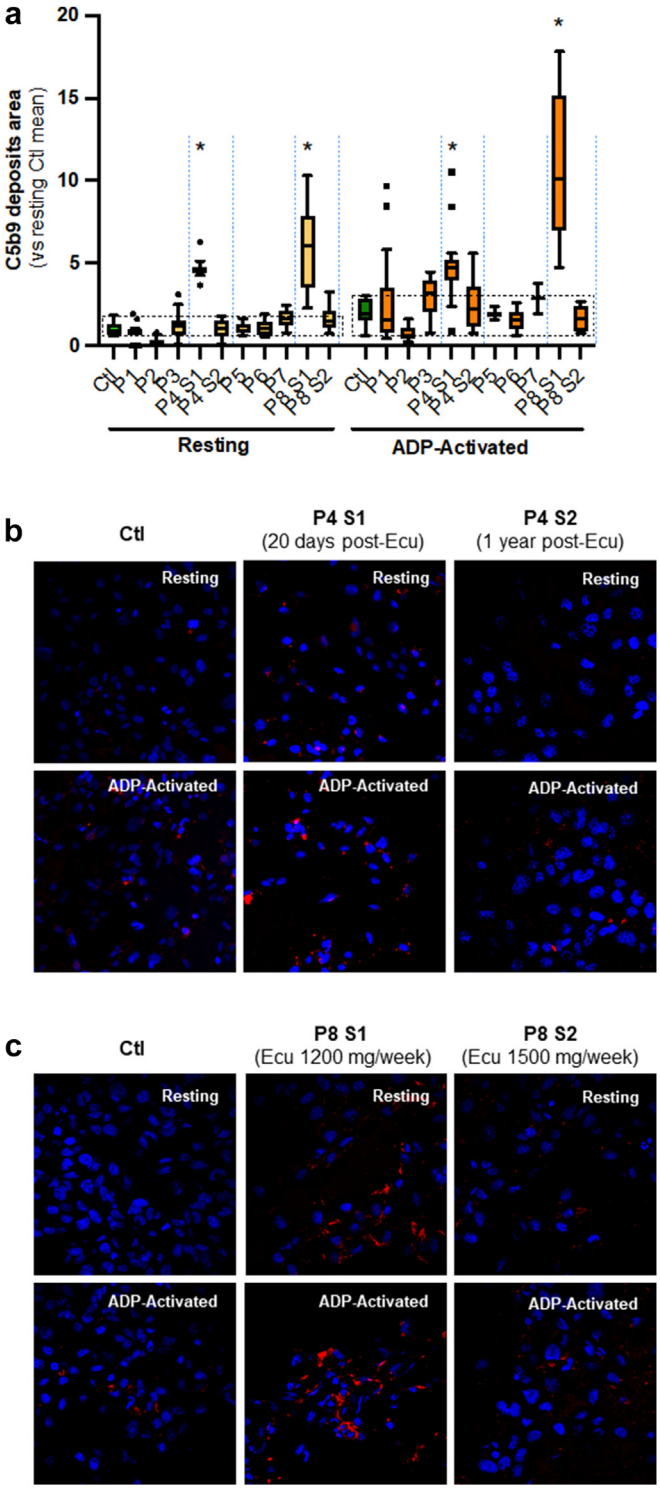

Figure 1. C5b-9 deposition area on resting and ADP-activated endothelial cells incubated with serum of patients with aHUS. C5b-9 deposition upon cultured endothelial cells induced by serum of patients with aHUS HMEC-1 cells were treated with ADP 10 μM or with dilution vehicle (mqH_2O) for 10 minutes, and afterward with serum of the included patients for 4 hours. C5b-9 was detected by immunocytochemistry using a specific antibody. (a) C5b-9 staining area upon HMEC-1 cells induced by serum of the patients with aHUS included, calculated from 15 pictures in each case. The dotted rectangles represent the normal range of C5b-9 deposition. Data were analyzed using 2-way analysis of variance (continued)

with eculizumab for only 20 days (Table 1), and despite complete clinical remission, induced increased levels of C5b-9 deposition upon cultured endothelial cells were detected (Figure 1a and b), suggesting subclinical terminal phase CS activity. Importantly, in some cases (P1 and P4 sample 2 in Figure 1a), normal C5b-9 deposits confirmed the complete distal CS blockade despite presumably proximal activation due to genetic abnormalities as indicated by persistent low C3 levels in some patients (P1 and P4 in Table 1).

Finally, we performed the test in a 13-year-old female patient, previously healthy, with severe TMA-associated with severe respiratory infection and multiple organ failure, supported with mechanical secondary to ventilation with Extracorporeal membrane oxygenation and continuous hemodiafiltration. At disease onset (1 month before first sample testing), anemia, thrombocytopenia, and hemolysis were evident as well as low plasma C3 levels and elevated sC5b-9, suggestive of CS activation. C5 blockade with eculizumab was initiated with subsequent improvement in kidney function; however, 1 month later, renal function worsened again (serum creatinine 3.9 mg/dl) despite the administration of high doses of eculizumab (1200 mg/wk), mimicking what has been published in patients with hematopoietic transplantation.[34] A positive test for C5b-9 deposition in endothelial cells (Figure 1a and c) revealed incomplete blockade of the CS despite normalization of C3 levels and CH50 <13% (Table 1). The dose of eculizumab was adjusted (1500 mg/wk), which completely blockaded the CS, as noted by a negative C5b-9 deposition test (Figure 1a and c). Renal function improved to baseline and currently, the patient remains in remission and continues recovering renal function under eculizumab therapy.

C5b-9 Deposition Test in TMA Secondary to Kidney and Lung Transplant

We also studied the C5b-9 deposition on HMEC-1 cells induced by serum samples of patients with TA-TMA:

Figure 1. (continued) followed by Dunnet's multiple comparison test (*, $P < 0.05$ vs. respective control). (b) Representative images of C5b-9 deposits on HMEC-1 cells (in red) induced by serum of healthy controls (Ctl) and of the sample 1 and sample 2 of patient 4 (P4 S1 and P4 S2, respectively) in resting and in ADP-activated conditions. Nuclei were stained with Hoechst 33342 and are shown in blue. (c) Representative images of C5b-9 deposits on HMEC-1 cells (in red) induced by serum of healthy controls (Ctl) and of the sample 1 and sample 2 of patient 8 (P8 S1 and P8 S2, respectively) in resting and in ADP-activated conditions. Nuclei were stained with Hoechst 33342 and are shown in blue. The mean ± SD values of C5b-9 staining area in each case can be found in Supplementary Table S4. ADP, adenosine diphosphate; aHUS, atypical hemolytic uremic syndrome; HMEC-1, human dermal microvascular endothelial cells.

in 2 KT patients (KT1 and KT2, Table 2) and in 1 LT patient (LT1, Table 2). In this case, beyond the healthy control, we have analyzed 6 stable kidney transplant patients without signs of TMA or significant immune activity in kidney tissue (T-Ctl, Figure 2) to establish a normality range for this group of patients (Supplementary Table S3 and Figure S3).

As stated, KT1 carried a third liver and kidney allograft due to autosomal dominant polycystic kidney disease diagnosed during infancy. The patient presented with a mild increase of serum LDH levels, thrombocytopenia, anemia, and persistent low levels of C3 and C4 (Table 2); the reason why TMA was suspected. The serum of KT1 did not induce significant C5b-9 deposits on cultured HMEC-1 cells (Figure 2 and Supplementary Table S4) as compared to the healthy and the kidney transplant control groups, suggesting that CS was not active in this patient. Actually, TMA diagnosis was discarded, and the final diagnosis was chronic humoral rejection, which progressed to graft failure 1 month later. Patient KT2 developed TMA 2.5 years after KT. No genetic mutations or risk polymorphisms related to aHUS were detected (Table 2). Eculizumab treatment was started immediately with initial response; however, the patient became refractory to the treatment afterward (Table 2). Accordingly, KT2 showed significantly increased C5b-9 deposition as compared to the healthy controls or to the kidney transplant controls (Figure 2 and Supplementary Table S4). KT2 lost the kidney allograft

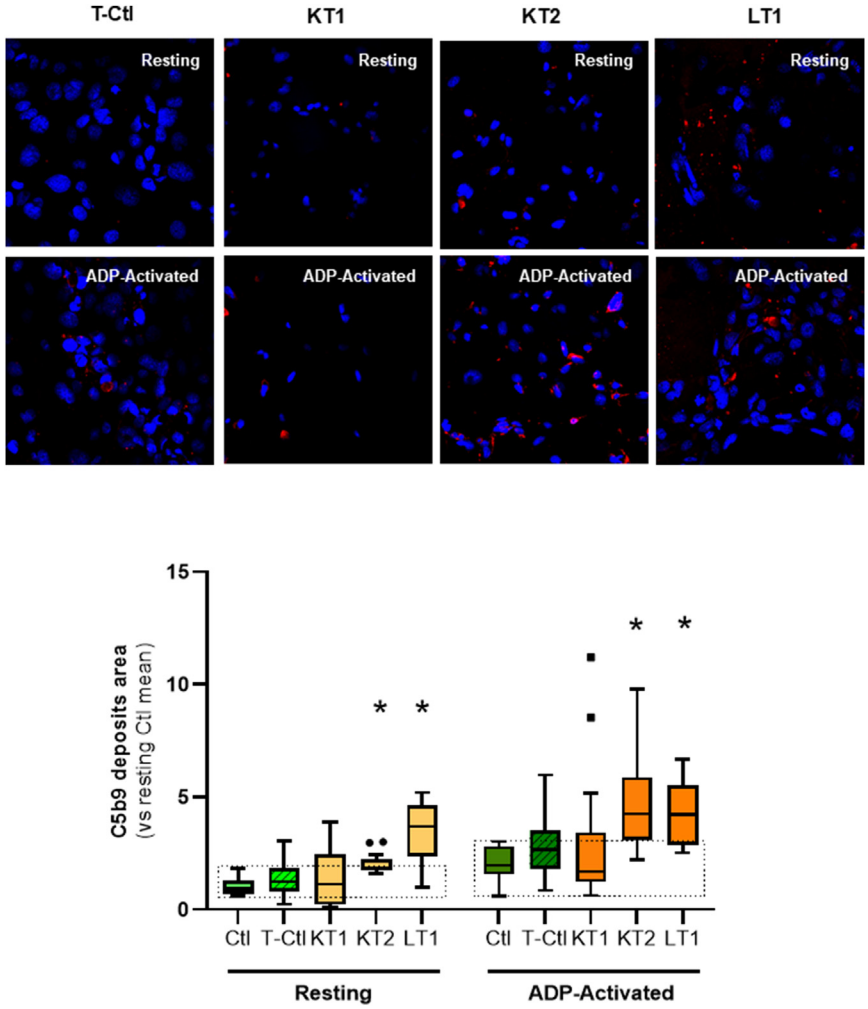

Figure 2. C5b-9 deposition area on resting and ADP-activated endothelial cells incubated with serum of patients with TMA secondary to kidney transplantation (KT) or lung transplantation (LT). HMEC-1 cells were treated with ADP 10μM or with dilution vehicle (mqH_2O) for 10 minutes and, afterward with serum of the included patients for 4 hours. C5b-9 was detected by immunocytochemistry using a specific antibody. Representative images of C5b-9 deposits on HMEC-1 cells (in red) induced by serum of patients with kidney transplant–associated TMA (KT1 and KT2) and patients with lung transplant–associated TMA in resting and in ADP-activated conditions and the respective C5b-9 staining area calculated from 15 pictures in each case. The dotted rectangles represent the normal range of C5b-9 deposition. Data were analyzed using 2-way analysis of variance followed by Dunnet's multiple comparison test (*, $P < 0.05$ vs. respective control). The mean ± SD values of C5b-9 staining area in each case can be found in Supplementary Table S4. ADP, adenosine diphosphate; HMEC-1, human dermal microvascular endothelial cells; TMA, thrombotic microangiopathy.

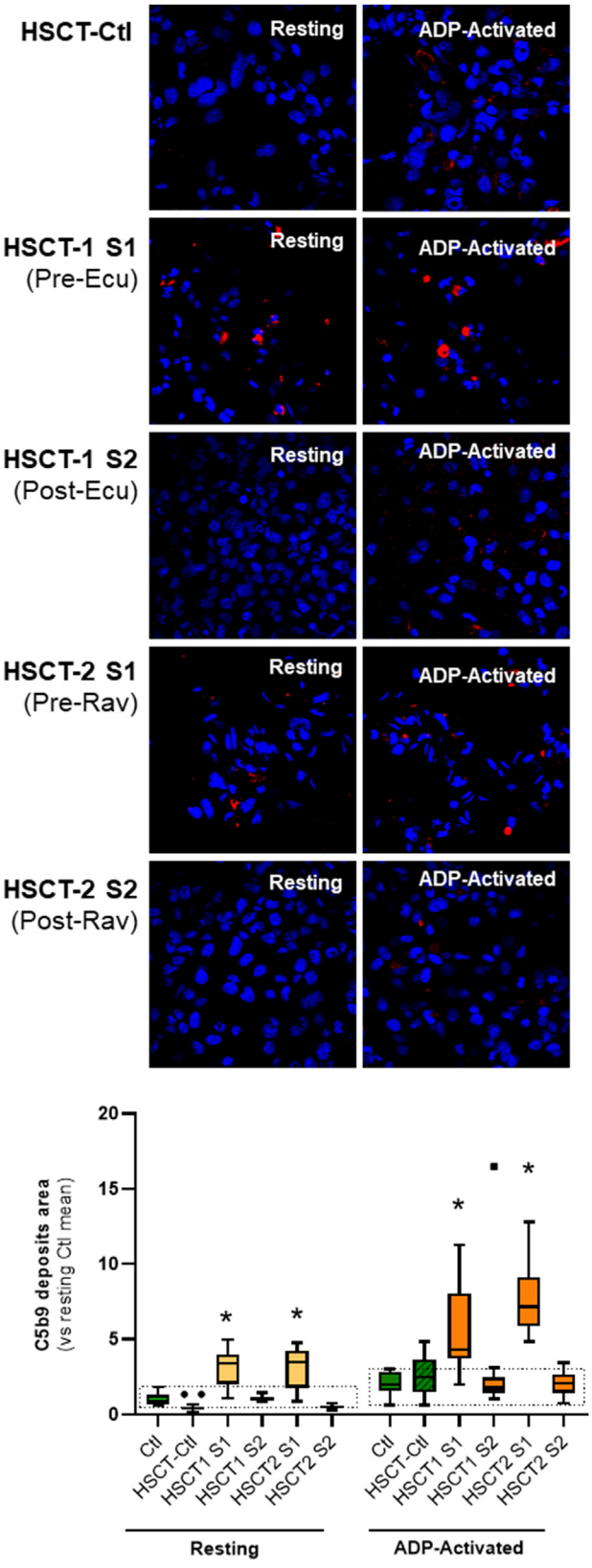

Figure 3. C5b-9 deposition area on resting and ADP-activated endothelial cells incubated with serum of patients with TMA hematopoietic stem cell transplantation (HSCT). HMEC-1 cells were treated with ADP 10μM or with dilution vehicle (mqH_2O) for 10 minutes and, afterward with serum of the included patients for 4 hours. C5b-9 was detected by immunocytochemistry using a specific antibody. Representative images of C5b-9 deposits on (continued)

function shortly after. At that point, eculizumab was stopped and renal replacement therapy was restarted. LT1 presented with a marked kidney disfunction only 4 days after LT that required renal replacement. TMA was diagnosed by kidney biopsy consistent with a positive C5b-9 deposition test (Figure 2 and Supplementary Table S4). The patient received eculizumab that was stopped later because of several ongoing infections that were not resolving. The patient died 2 months later due to septic shock.

C5b-9 Deposition Test in TMA Secondary to HSC Transplant

Finally, we studied the C5b-9 deposition on HMEC-1 cells induced by serum samples of 2 HSC transplanted patients with TA-TMA (HSCT1 and HSCT2, Table 2). Serum samples of both patients were tested before and after C5 blockade with eculizumab or ravulizumab, respectively. As before, beyond the healthy control, we analyzed 2 clinically stable HSCT patients without TMA signs (HSCT-Ctl, Figure 3) to establish a normality range for this group of patients (Table 2).

HSCT1 was HSC-transplanted after an episode of recurrence of B-cell acute lymphoblastic leukemia. After 6 months, she presented with uncontrollable hypertension, thrombocytopenia, reticulocytosis (Coombs negative), microalbuminuria, and high levels of LDH (Table 2). TMA was detected by kidney biopsy in the absence of aHUS-related genetic mutations or risk polymorphisms (Table 2). HSCT1 showed a significant increase in C5b-9 deposition before starting eculizumab as compared to the healthy control and the HSCT control groups that normalized after 6 months under eculizumab therapy (Figure 3 and Supplementary Table S4). Eculizumab was stopped 4 months later without any TMA recurrence; however, hypertension persisted (currently under triple therapy hypertensive treatment). HSCT2 was HSC-transplanted due to acute myelomonocytic leukemia. The patient showed a degree of renal impairment shortly after transplantation that remained stable (serum creatinine 1.3–1.45 mg/dl). However, after 2.5 years the patient

Figure 3. (continued) HMEC-1 cells (in red) induced by serum of patients with HSCT-associated TMA (HSCT 1 and 2) in resting and in ADP-activated conditions and the respective C5b-9 staining area calculated from 15 pictures in each case. The dotted rectangles represent the normal range of C5b-9 deposition. Data were analyzed using 2-way analysis of variance followed by Dunnet's multiple comparison test (*, $P < 0.05$ vs. respective control). The mean ± SD values of C5b-9 staining area in each case can be found in Supplementary Table S4. ADP, adenosine diphosphate; HMEC-1, human dermal microvascular endothelial cells; TMA, thrombotic microangiopathy.

was referred to the nephrology department where TMA was diagnosed by kidney biopsy. At the moment of TMA diagnosis, the patient showed a significant increase in C5b-9 deposition as compared to the healthy or HSCT control group, that normalized after 3 months of ravulizumab treatment (Figure 3 and Supplementary Table S4). The patient is currently in remission under ravulizumab.

C5b-9 Deposition Test in an Asymptomatic Patient With Genetic Risk of aHUS

We also tested a serum sample of an asymptomatic healthy 7-year old boy with a genetic risk of developing aHUS due to *CFHR3-CFHR1* deletion in homozygosis and the risk polymorphism c.329T>Cp (Val110Ala) in *CFHR5* in heterozygosis, but no anti-FH antibodies. Those genetic anomalies were detected 2 years earlier by family screening after his elder sibling who shared the same genetic background with high levels of anti-FH autoantibodies presented with aHUS onset (Figure 4a). Although the younger sibling had never shown signs of aHUS activity, incidentally, detection of microhematuria and decreased serum C3 levels were observed during a subacute external otitis episode caused by *Pseudomonas*, lasting more than 3 months, in the absence of any other hematologic or biochemistry abnormality, or detectable anti-FH antibodies. Nevertheless, as proof-of-concept, we performed the C5b-9 deposition test (Figure 4b and c). As a result, we observed increased levels of C5b-9 deposition as compared to the healthy controls (Figure 4c), suggestive of CS activity. The patient was closely monitored and, fortunately, as shown in Figure 4d, hematuria disappeared and serum C3 levels normalized after treating the infectious episode with antibiotics without additional clinical manifestations.

DISCUSSION

The dysregulation of the alternative pathway of the CS is a major risk factor for aHUS. Unfortunately, complement component assays in serum (C3, C4, sC5b-9,

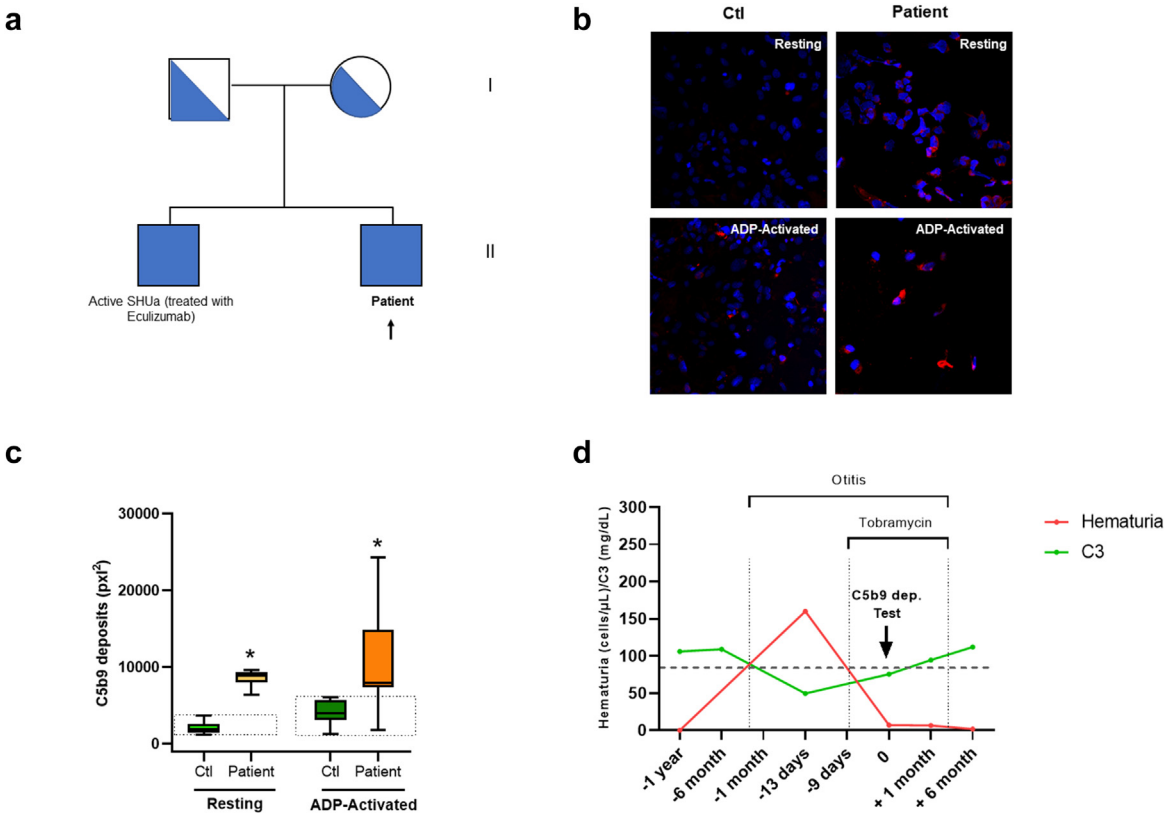

Figure 4. An asymptomatic 7-year-old boy at risk of aHUS presented increased C5b-9 deposition on cultured endothelial cells during a persistent otitis episode. (a) Scheme of CFHR3-CFHR1 deletion inheritance (in blue). (b) Representative images of C5b-9 deposits on HMEC-1 cells (in red) induced by serum of healthy controls (Ctl) and of the patient in resting and in ADP-activated conditions. Nuclei were stained with Hoechst 33342 and are shown in blue. (c) C5b-9 staining area upon HMEC-1 cells induced by serum of the patient calculated from 15 pictures in each case. The dotted rectangles represent the normal range of C5b-9 deposition. Data were analyzed using 2-way analysis of variance followed by Dunnet's multiple comparison test (*, $P < 0.05$ vs. respective control) (d) Evolution of serum C3 and hematuria during the otitis episode of the patient. Point 0 represents the time when the serum sample for C5b-9 deposition test was collected. The evolution of the rest of clinical and complement data can be found in Supplementary Table S1. ADP, adenosine diphosphate; aHUS, atypical hemolytic uremic syndrome; HMEC-1, human dermal microvascular endothelial cells.

AP50, and CH50) are not absolute reliable biomarkers for the diagnosis of complement activation in any form of TMA, including aHUS.[29-31] Further, complement fragments may be absent in kidney tissue at TMA diagnosis as illustrated in our experimental setting (Supplementary Table S2). Alternative tests have been developed, including the Ham modified test of complement-induced cytotoxicity[33] and assays of complement activation products deposited on endothelial cells *in vitro*.[29-31] It has been described that these *in vitro* tests to evaluate the activity of the CS are also useful to monitor disease activity and the response to treatment with C5 convertase blockers.[29,30,32] Here, we show that the *ex vivo* C5b-9 endothelial deposition test can be very useful in clinical practice for both primary aHUS and TA-TMA assessment because it allows to selectively detect CS activity even in complex clinical scenarios when the diagnosis is a challenge.

We tested the capacity of the serum of patients with aHUS and TA-TMA to generate C5b-9 deposits upon cultured endothelial cells. First, we assessed the power of the test to confirm disease remission and treatment efficacy. As mentioned earlier, all the study patients with primary aHUS (P1–P7) but one (P8) were in remission during the treatment with eculizumab or ravulizumab (Table 1) and exhibited normal levels of C5b-9 deposition consistent with a proper C5 convertase blocking except for the first serum sample of P4, which showed increased levels of C5b-9 deposits during the early phase of the disease (Figure 1a and b). This is consistent with partial aHUS activity due to incomplete CS blockade even in clinical remission, which is probable considering that the sample was taken after only 20 days of eculizumab therapy. Those findings provide an additional support of *ex vivo* C5b-9 deposits test value even in patients with apparent clinical remission, which can be of great value in case of personalized C5 blockade dosage, and in patients with partial response to treatment (Table 1). Previous reports have shown that serum samples from patients with aHUS in clinical remission under C5 blockade therapy may induce significantly higher levels of C5b-9 deposits upon cultured endothelial cells than control serum samples,[30] which is probably related to residual terminal phase CS activity. Indeed, a second serum sample of P4, collected 1 year later, showed C5b-9 deposition levels within the control range, which is consistent with complete remission (Figure 1a and b). In our aHUS cohort, the *ex vivo* C5b-9 deposition test was able to confirm the complete distal blockade of CS despite the presence of low C3 levels in some patients (P1 and P4, Table 1), demonstrating the accuracy of the test for monitoring aHUS activity. The test was also useful to adjust eculizumab therapy in a patient with aHUS that showed disease activity despite high doses of eculizumab (P8, Figure 1c and Table 1).

The C5b-9 deposition test also finely detects complement activity in TA-TMA (Figure 2a and b), which can be valuable in this specific clinical situation for diagnosis and treatment monitoring. TMA is associated with poor outcome in those populations. However, significant improved outcome after treatment with eculizumab has been described in patients with TMA and secondary CS activation that were refractory to removal of environmental factors.[34-36] Therefore, we propose that C5b-9 *ex vivo* test represents a highly valuable tool to discriminate those individuals with TMA who are candidate to CS blockade and to monitor individual therapy response. In our setting, KT1 showed a marked renal disfunction with TMA suspicion (Table 2) but low C5b-9 deposition levels excluding CS involvement (Figure 2). One month later, the patient lost the kidney allograft due to an ongoing chronic humoral rejection previously diagnosed and TMA was ruled out, which is in line with the lack of C5b-9 deposition observed (Figure 2). Regarding KT2, a patient with TMA associated with KT who was in partial remission with eculizumab (Table 2), increased levels of C5b-9 deposition (Figure 2) suggested an incomplete CS blockade and potentially the need of a higher eculizumab dose to achieve remission. We also assayed serum samples of 2 patients with TMA secondary to HSCT (HSCT1 and HSCT2) at onset and in remission after treatment with eculizumab or ravulizumab. As expected, C5b-9 deposition levels were found to be elevated before treatment and normalized after C5 blockade (Figure 3). Secondary TMA associated with transplantation is a challenge, because several confounding factors may be present, such as immunosuppressants and other medications, infections, immune-mediated effects, graft-related complications, or primary disease activity that may *per se* produce similar manifestations: hemolytic anemia, thrombocytopenia, and kidney function impairment as well as consumption of C3 or increase of sC5b-9. For instance, in our setting, TMA was suspected in KT1 due to persistent decreased levels of C3 and C4 (beyond thrombocytopenia and anemia), which could also be related to liver graft impairment. Further, in HSCT the clinical scenario shortly after transplantation complicates the TMA diagnosis. Low platelet count is a common feature (HSCT1, Table 2); a reason why an additional consumption of platelets has to be inferred (i.e., persistent low platelet count). Complications such as infections or drug toxicity, usually present in HSCT, can also lead to anemia, increased LDH levels, or kidney dysfunction, among others.[37] In addition, serum C3, C4, or sC5b-9 poorly correlate with active TMA

(both in primary and secondary cases)[29-31]; a reason why monitoring these parameters may not always be helpful. In this sense, the *ex vivo* C5b-9 deposition test can be valuable in TA-TMA not only for the diagnosis but also to help to decide which patients may benefit from therapy with CS blockers which could be life-saving and to establish treatment duration. In this line, the *ex vivo* C5b-9 deposition allows to finely monitor the disease activity in both aHUS and TA-TMA during eculizumab therapy. A better monitoring of the disease should allow to personalize eculizumab or ravulizumab dosage and subsequently optimize health resources.

Finally, we also studied a child with otitis and genetic risk of aHUS detected by family screening (Figure 4b). Increased levels of C5b-9 deposition (Figure 4c) suggested concomitant CS activity during the infectious episode. This case illustrates CS activation triggered by an environmental factor in genetic-risk individuals even in the absence of overt disease, and the potential of the *ex vivo* C5b-9 deposition test to lead therapeutic decisions if needed. In this line, it has been demonstrated that even unaffected relative carriers of complement pathogenic variants induces C5b-9 formation on activated endothelial cells.[38]

However, the *ex vivo* C5b-9 deposition test is not without limitations. First, the heme group derived from hemolysis present in all forms of TMA may potentially contribute to CS activation upon the cultured cells.[39] Furthermore, Duineveld *et al*.[40] have recently shown that the serum of a subset of stable kidney transplant patients induced increased C5b-9 deposition upon endothelial cultured cells independently of their disease in native kidney. Although the study cohort is small, it highlights that the *ex vivo* C5b-9 deposition test is useful only if performed in a relevant and interpretable clinical context . As proof-of-concept, it has been suggested to measure the *ex vivo* C5b-9 deposition test in other pathologic conditions. In this sense, as a pilot, we have studied a small number of clinically stable kidney ($n = 6$) and HSC-transplanted ($n = 2$) adult patients without signs of TMA in order to use them as a comparison group for the TA-TMA group. We have demonstrated that the level of C5b-9 deposition on cultured endothelial cells induced by the serum of these patients was similar to that obtained in the healthy controls group (Supplementary Table S3). This fact reinforces the utility of the C5b9 deposition test in TA-TMA context and suggests that stable transplanted patients without signs of TMA could be a more suitable comparison group for TA-TMA patients. Even so, using the test on a routine base in a different clinical scenario than aHUS or TA-TMA may not be recommended (at least in the present form) because it might lead to additional confusion and may not be informative, considering that CS may be somehow transiently active in many kidney diseases which may not necessarily be related to TMA

Overall, in this study, we demonstrate that the *ex vivo* C5b-9 deposition test is very useful to confirm CS activity or remission at the endothelial surface, and potentially to monitor the response to eculizumab or ravulizumab treatment in aHUS and in TA-TMA, which is in line with previous reports.[29-31] In addition, we have shown that stable transplanted patients without signs of TMA show similar levels of C5b-9 deposition on cultured endothelial cells than healthy individuals, which demonstrates that the test is useful in the TA-TMA context and that this group of patients could be used as comparison group in this specific clinical scenario. Finally, we provide evidence that the C5b-9 deposition test may also be useful in subclinical conditions in the absence of overt disease activity and support therapeutic decisions.

DISCLOSURE

NR declares honoraria for lectures or educational events, and travel support from Alexion (AstraZeneca Rare Diseases); and participates on a Data Safety Monitoring Board or Advisory Board of Alexion (AstraZeneca Rare Diseases), outside of this work. GA declares honoraria for lectures, presentations, speakers' bureaus, manuscript writing, or educational events from Alexion (AstraZeneca Rare Diseases), Recordati Rare Diseases, Advicenne, Chiesi, Kyowa Kirim, and Alnylam; participation on a Data Safety Monitoring Board or Advisory Board of Alexion (AstraZeneca Rare Diseases), Dicerna, Advicenne, and Alnylam; support for attending meetings and/or travel from Recordati Rare Diseases, Advicenne, Kiowa Kirim; and cochair of the Scientific Advisory Board of aHUS Global Registry supported by Alexion (AstraZeneca Rare Diseases), outside of this work. MJS reports personal fees from NovoNordisk, Jansen, Mundipharma, AstraZeneca, Esteve, Fresenius, Ingelheim Lilly, Vifor, and ICU; and grants and personal fees from Boehringer Ingelheim, outside of this work. MH-G reports a research grant from Alexion to support this study. CJ-C declares travel support and a research grant from Travere Therapeutics, outside of this work. All the other authors declared no competing interests.

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DATA AVAILABILITY STATEMENT

The data set is available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

Conception and design was by MH-G, MJS, GA, and CJ-C. Experiments and acquisition of experimental data were done by MM, CL-C., CJ-C., and JP-P. Data collection was done by JL-R, SS, IBT, VP-B., IA, JS, NR, ML, OB, FM, and GA. Analysis and interpretation of data were done by MM, CL-C., CJ-C., MH-G, GA, FM, and MJS. Draft of the manuscript was done by MM, CJ-C., GA, MJS, and MH-G. Figures were done by MM, CL-C. and CJ-C. Funding and resources acquisition was done by MH-G., CJ-C and MJS. All the authors revised and approved the final version of the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary File (PF)

Supplementary Methods. C5b-9 deposits detection by immunofluorescence.

Supplementary Results.

Setup of the C5b-9 deposition on cultured endothelial cells test.

Establishment of the normal control range.

Figure S1. ADP induces P-selectin expression in cultured HMEC-1 cells.

Figure S2. Representative images of C5b-9 deposits on cultured HMEC-1 cells treated with serum of a patient with acute aHUS (P0) or of a healthy control (C0).

Figure S3. C5b-9 deposition area on resting and ADP-activated endothelial cells incubated with serum of patients with KT and HSCT without signs of TMA.

Table S1. Clinical data and complement system profile follow-up in a 7-year old boy with genetic risk of aHUS due to mutations in *CFHR3-CFHR1* deletion in homozygosis and the risk polymorphism c.329T>Cp (Val110Ala) in *CFHR5* in heterozygosis, but undetectable serum anti-FH antibodies.

Table S2. Complement fragments C3, C1q, C4c and C4d in kidney tissue of TA-TMA patients KT2, LT1, HSCT1, and HSCT2.

Table S3. Demographics, clinical characteristics, and C5b-9 deposition area on resting and ADP-activated endothelial cells incubated with serum of patients with KT and HSCT without signs of TMA.

Table S4. C5b-9 deposition area on resting and ADP-activated endothelial cells incubated with serum of patients with aHUS or TA-TMA.

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