Combined Complement Gene Mutations in Atypical Hemolytic Uremic Syndrome Influence Clinical Phenotype

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ABSTRACT

Several abnormalities in complement genes reportedly contribute to atypical hemolytic uremic syndrome (aHUS), but incomplete penetrance suggests that additional factors are necessary for the disease to manifest. Here, we sought to describe genotype–phenotype correlations among patients with combined mutations, defined as mutations in more than one complement gene. We screened 795 patients with aHUS and identified single mutations in 41% and combined mutations in 3%. Only 8%–10% of patients with mutations in *CFH*, *C3*, or *CFB* had combined mutations, whereas approximately 25% of patients with mutations in *MCP* or *CFI* had combined mutations. The concomitant presence of *CFH* and *MCP* risk haplotypes significantly increased disease penetrance in combined mutated carriers, with 73% penetrance among carriers with two risk haplotypes compared with 36% penetrance among carriers with zero or one risk haplotype. Among patients with *CFH* or *CFI* mutations, the presence of mutations in other genes did not modify prognosis; in contrast, 50% of patients with combined *MCP* mutation developed end stage renal failure within 3 years from onset compared with 19% of patients with an isolated *MCP* mutation. Patients with combined mutations achieved remission with plasma treatment similar to patients with single mutations. Kidney transplant outcomes were worse, however, for patients with combined *MCP* mutation compared with an isolated *MCP* mutation. In summary, these data suggest that genotyping for the risk haplotypes in *CFH* and *MCP* may help predict the risk of developing aHUS in unaffected carriers of mutations. Furthermore, screening patients with aHUS for all known disease-associated genes may inform decisions about kidney transplantation.

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Hemolytic uremic syndrome (HUS) is a rare disease of microangiopathic hemolysis, thrombocytopenia, and renal failure.[1,2] The most common form in children is associated with infection by certain strains of *Escherichia coli*, which produce Shiga-like toxins.[3] This form has a good prognosis.[1] There are rarer atypical forms (aHUS), not associated with Shiga-like toxins-producing bacteria, that have a worse outcome, with up to 50% of cases

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progressing to end stage renal failure (ESRF) and 10%–15% dying during the acute phase.[1,4]

Inherited defects that determine uncontrolled activation of the alternative complement pathway have been well documented in aHUS patients.[2,5,6] Research in recent years has identified more than 120 different mutations, accounting for around 40%–60% of cases, in the genes encoding complement factor H (CFH),[7–9] membrane cofactor protein (MCP),[10–13] complement factor I (CFI),[14–16] C3,[17] complement factor B (CFB),[18,19] CFH-related 5 (CFHR5),[20] and thrombomodulin (THBD).[20,21] In addition, anti-CFH autoantibodies have been described mostly in children that lack CFHR1 and CFHR3 because of a deletion of the corresponding genes.[22–26] Novel genetic abnormalities of *CFHR1*, *CFHR3*, and *CFHR4* and genomic rearrangement between *CFH* and *CFHR1* have recently been described.[27,28]

Incomplete penetrance of aHUS has been reported in mutation carriers,[12,29–31] indicating that complement gene mutations confer predisposition to develop aHUS, with additional genetic and/or environmental hits necessary for disease manifestation.[7,32,33] In keeping with this hypothesis, patients with mutations in more than one complement gene (combined gene mutations) have been described.[20,29,34,35] This study was designed to (*1*) determine the frequency of combined complement gene mutations among four cohorts of aHUS patients; (*2*) compare short- and long-term outcomes, response to plasma treatment, and outcome of kidney transplantation among patients carrying mutations in different gene combinations; and (*3*) compare clinical parameters in patients carrying combined mutations versus patients with mutations in a single complement gene. Thanks to a joint effort by the European Working Party on Complement Genetics in Renal Diseases, we genotyped almost 800 aHUS patients for aHUS-associated genes, identifying 27 patients with combined gene mutations.

RESULTS

Patients and Mutations

We undertook mutation screening of *CFH*, *MCP*, *CFI*, *C3*, and *CFB* for 795 aHUS patients (including probands and affected relatives) from four independent cohorts: the International Registry (n=274, 58% from Italy, 15% from other European countries, 14% from North America, 8% from the Middle East, 2% from South America, 2% from Africa, and 1% from Asia) and French (n=214), Spanish (n=191), and UK (n=116) cohorts.

Twenty-seven patients with combined mutations in *CFH*, *MCP*, *CFI*, *C3*, and *CFB* were identified (27/795; 3.4%) (Figure 1 and Table 1). Seven patients carried combined mutations in *CFH* and *MCP*, four patients carried combined mutations in *CFH* and *CFI*, two patients carried combined mutations in *CFH* and *C3*, nine patients carried combined mutations in *MCP* and *CFI*, one patient carried combined mutations in *MCP* and *C3*, one patient carried combined mutations in *CFI* and *C3*, one patient carried combined mutations in *CFI* and *CFB* (double-mutated), and two patients carried combined mutations in *CFH*, *MCP*, and *CFI* (triple-mutated) (Tables 1 and 2). Mutations in a single gene were found in 323/795 (40.6%) patients (single-mutated) (Table 2). Considering overall patients carrying single and combined mutations, we found 350/795 (44%) mutated patients. We noticed that several *MCP* and *CFI* mutations were only found combined with mutations in other genes, suggesting a low pathogenic potential requiring another genetic abnormality to induce aHUS (Figure 1).

Consistently, 22.6% and 27% of patients with either *MCP* or *CFI* mutations versus only 8%–10% of patients with *CFH*, *C3*, or *CFB* mutations showed mutations in other genes (Table 2). This observation indicates that single mutations in *CFH*, *C3*, or *CFB* alone are more likely to be sufficient to induce aHUS than single mutations in *MCP* or *CFI*.

All patients with combined mutations were Caucasians, with the exception of FRE60 from North Africa ancestry (Table 1 and Supplemental Figure 1). Fifteen patients had familial HUS (10 families), whereas twelve patients had sporadic aHUS (Table 1 and Supplemental Figure 1). Analysis of combined-mutated probands (n=22) (Supplemental Figure 1) and their available relatives (n=79; of whom five double- and four single-mutated subjects were affected) (Supplemental Figure 1) revealed a progressive significant increase of penetrance across subjects with mutations in one, two, or three genes (Figure 2). Among 35 single-mutated subjects, only 4 subjects were affected (Supplemental Figure 1); 25 of 40 double-mutated subjects developed aHUS (63%; ranging from 40% to 66% in subjects with mutations in different gene combinations) (Supplemental Figure 2), whereas both triple-mutated subjects were affected (Supplemental Figure 1). In patients and their relatives, we genotyped two single-nucleotide polymorphisms (SNPs) in *CFH* (rs3753394, c.1-332C>T and rs1065489, c.2808G>T, p.E936D) that tag the disease risk haplotype *CFH*-H3[7,32] and one SNP in *MCP* (rs7144, c.*897T>C) that tags the *MCPggaac* risk haplotype[32] (Table 1 and Supplemental Figure 1). The presence of at least one copy of both risk haplotypes significantly increased disease penetrance in double-mutated subjects, although penetrance still remained incomplete (Figure 2).

Case Reports of Familial Cases

Clinical history and pedigrees of familial forms are reported in Supplemental Material.

Clinical Findings

Patients with *CFH*, *MCP*, *CFI*, *C3*, or *CFB* mutations combined with mutations in other genes were subgrouped as *CFH*-combined (n=15), *MCP*-combined (n=19), *CFI*-combined (n=17), *C3*-combined (n=4), and *CFB*-combined (n=1), respectively. According to this classification, we included each patient with mutations in two or three genes in two or three subgroups, respectively (*e.g.*, a patient with *CFH* and *CFI* mutations appears in both the *CFH*-combined and

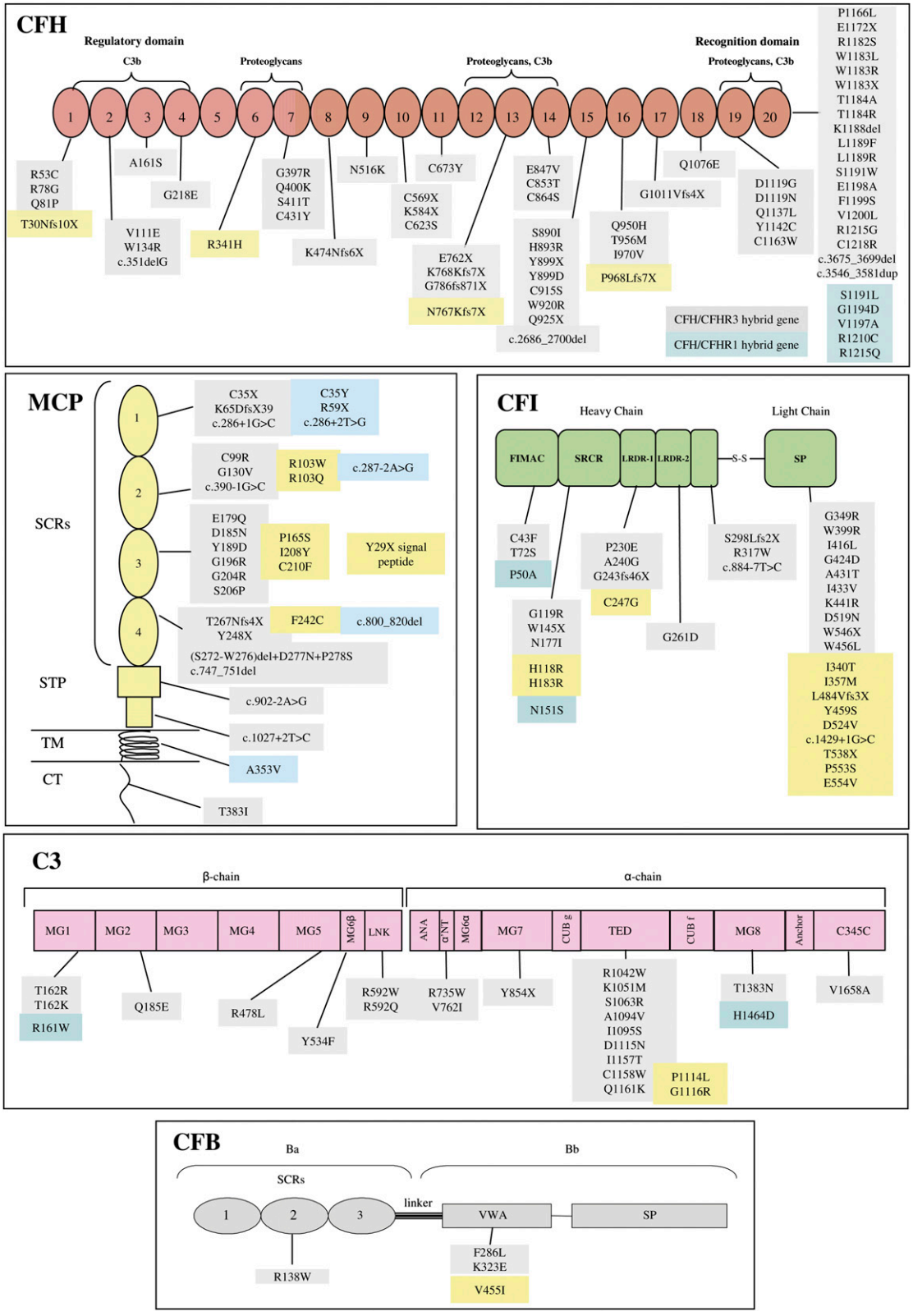

Figure 1. Localization of the mutations in CFH, MCP, CFI, C3, and CFB that were found in 27 patients with combined gene mutations and patients with single mutations from the four cohorts. Gene variations found in patients as the sole mutation are in gray, changes found only combined with other mutations are in yellow, and changes found both as single or combined mutations are in light blue.

Table 1. Summary of patients with combined mutations

Cohort	Patient	CFH Mutation	MCP Mutation	CFI Mutation	C3 Mutation	CFB Mutation	rs 3753394	rs 1065489	rs 7144	Familial/ Sporadic	Sex	Age at Onset (yr)	C3 Levels[a]	CFH Levels[a]	CFI Levels[a]	Triggers	Episodes	Outcome at First Episode	Outcome at 3 yr
I	#130F169	G1194D	F242C				CT	GT	CC	Familial	M	0.75	Normal	Normal	Normal	*E. coli* infection	2	Remission	Remission
I	#130F582	G1194D	F242C				CT	GT	TC	Familial	F	31	Normal	Normal	Normal	Pregnancy	1	ESRF	ESRF+tx
F	FRA15	R1210C	Y29×				TT	TT	CC	Familial	F	29	Normal	Normal	Normal	Pregnancy	1	ESRF	ESRF
I	#024F106	R1210C	C35Y, R59X				CT	GT	TT	Familial	M	3.5	Low	Normal	Normal	Gastroenteritis	2	Remission	Remission
I	#024F108	R1210C	C35Y, R59X				CT	GT	TT	Familial	M	8	Low	Normal	Normal	Broncopneumonia	2	Remission	Remission
S	HUS143	T30Nfs10X	I208Y				CT	GT	CC	Familial	F	27	Low	Low	Normal	Pregnancy	1	Remission	Remission
S	HUS186	R1215Q	R103Q				CT	GT	TT	Sporadic	M	24	Normal	Normal	Normal	n.a.	3	ESRF	ESRF
F	FRE06	N767Kfs7X		H183R			CC	GT	CC	Sporadic	M	1.3	Low	Low	Normal	n.a.	1	ESRF	ESRF
S	HUS207	P968Lfs7X		I340T			CT	TT	TT	Sporadic	F	30	Normal	Normal	Normal	No trigger (pill)	1	ESRF	ESRF
I	#265F870	S1191L		E554V			CC	GG	TT	Familial	M	0.83	Normal	Normal	n.a.	URT infection	8	Remission	Remission
I	#176F1314	Hybrid CFH/CFHR1		c.1429+1G>C			TT	GT	TC	Familial	F	0.5	Normal	n.a.	n.a.	URT infection	1	Remission	n.a.
I	R062	V1197A			G1116R		CC	GT	CC	Sporadic	M	<10	Low	Normal	n.a.	n.a.	5	Remission	ESRF
F	FRA50	R341H			R161W		TT	TT	CC	Sporadic	F	23	Low	Normal	Normal	Pregnancy	1	ESRF	ESRF
F	FRE60		R103W	P50A			n.a.	n.a.	n.a.	Familial	F	2	Normal	Normal	Normal	n.a.	3	Remission	Remission
S	GUIHUS62		R103W	N151S			CT	GT	CC	Familial	M	34	Normal	Normal	Low	n.a.	1	ESRF	ESRF
S	GUIHUS109		R103W, c.800–820del	N151S			CT	GT	TC	Familial	F	1	Normal	Normal	Half	No trigger	3	Remission	Remission
F	FRA106		c.286+2T>G	H118R			TT	TT	CC	Sporadic	M	32	Normal	Normal	Normal	n.a.	1	ESRF	ESRF
S	HUS167		C210F	C247G			CT	GT	TC	Sporadic	M	22	Normal	Normal	Half	n.a.	1	Remission	Remission
I	S657		c.287–2A>G	L484Vfs3X			CT	GT	TC	Sporadic	M	0.75	Normal	Normal	Normal	No trigger	2	Remission	ESRF
S	RCOHUS68		P165S	T538X			CT	GT	TC	Familial	F	57	Normal	Normal	Low	n.a.	2	Remission	ESRF
S	RCOHUS84		P165S	T538X			CT	GT	TC	Familial	F	41	Normal	Normal	Half	No trigger	1	Remission	Remission
UK	NCL		A353V	P553S			CT	GG	CC	Sporadic	F	63	Normal	Normal	n.a.	n.a	1	n.a.	n.a.
F	FRE44		A353V		H1464D		CC	GG	CC	Familial	M	2.5	Normal	Normal	Normal	Infection	6	Remission	Remission
F	FRE18			D524V	P1114L		CC	GG	TC	Sporadic	M	1.5	Low	Normal	Normal	n.a	n.a.	Remission	Remission
F	FRA104			Y459S		V455I	CT	GT	TC	Sporadic	M	40	Normal	Normal	Low	No trigger	1	ESRF	ESRF
F	FRA13	R1210C	Y29X	P553S			TT	TT	CC	Familial	F	47	Normal	Normal	Normal	n.a	1	Remission	ESRF
I	S978	R1210C	c.286+2T>G	I357M			CT	GT	TC	Sporadic	M	30	n.a.	n.a.	n.a.	No trigger	1	ESRF	ESRF

n.a., not available; URT, upper respiratory trait.

[a]Normal ranges: International Registry (I): C3, 830–1,330 mg/L; C4, 150–450 mg/L; CFH, 350–750 mg/L; CFI, 70%–130%. Spain (S): C3, 800–1,770 mg/L; C4, 140–470 mg/L; CFH, 100–350 mg/L; CFI, 70%–130%. France (F): C3, 660–1,250 mg/L; C4, 93–380 mg/L; CFH, 338–682 mg/L; CFI, 42–78 mg/L. United Kingdom (UK): C3, 680–1,380 mg/L; C4, 180–600 mg/L; CFH, 350–590 mg/L; CFI, 38–58 mg/L. C4 levels were normal in all patients except S978, which is not available. Ethnic origin is Caucasian for all patients but FRE60 (North African origin).

Table 2. Prevalence of patients with single and combined mutations in *CFH*, *MCP*, *CFI*, *C3*, and *CFB* in the four cohorts

Genetic Abnormality	CFH	MCP	CFI	C3	CFB
CFH	158				
MCP	7	65			
CFI	4	9	46		
C3	2	1	1	45	
CFB	0	0	1	0	9
Triple-mutated	Two patients				
Combined[a]/single	15/158	19/65[b,c]	17/46[b,c]	4/45	1/9
Combined[a]/single + combined (%)	8.7%	22.6%	27%	8.2%	10%
Single mutation/screened patients	158/795 (19.9%)	65/795[d,e] (8.2%)	46/795[d,e] (5.8%)	45/795[d,e] (5.7%)	9/795[d] (1.1%)
Combined mutations[a]/screened patients	15/795[c,f] (1.9%)	19/795[c,e] (2.4%)	17/795[c,f] (2.1%)	4/795 (0.5%)	1/795 (0.1%)

The number and percentages of combined mutated patients cannot be added up because of subjects appearing in more than one cell. The two triple-mutated patients are included in the cell above. *CFH* mutations include the CFH/CFHR1 hybrid gene. Statistical analyses were performed by chi-squared or Fisher exact test as appropriate.

[a]Including double- and triple-mutated.

[b]$P<0.001$ versus *CFH*.

[c]$P\leq0.03$ versus *C3*.

[d]$P<0.001$ versus *CFH*.

[e]$P<0.001$ versus *CFB*.

[f]$P<0.001$ versus *CFB*.

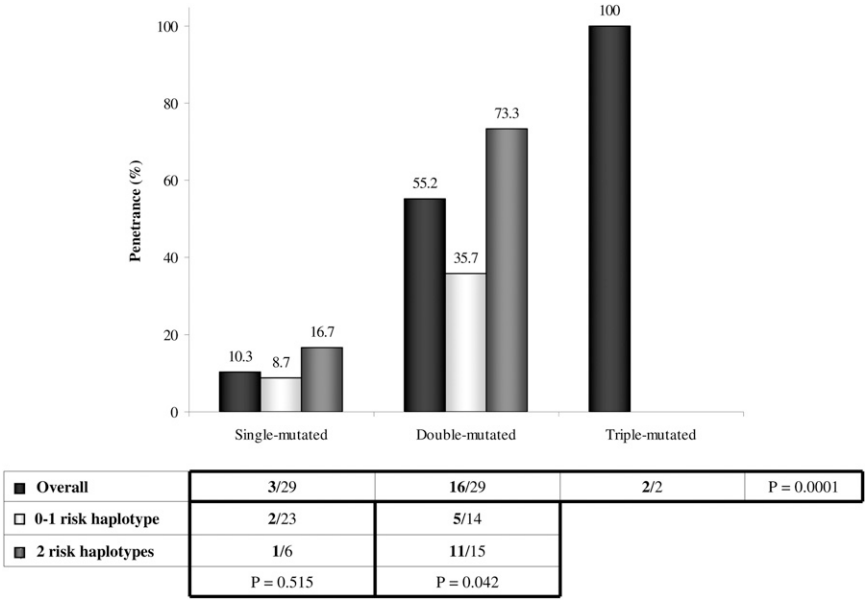

Figure 2. Impact of the number of risk haplotypes in *CFH* (*CFH*-H3 targeted by rs3753394, c.1-332C>T and rs1065489, c.2808G>T p.E936D) and *MCP* (*MCPggaac* targeted by rs7144, c.*897T>C) on aHUS penetrance in single- (carrying mutations in one complement gene), double- (carrying mutations in two different genes), or triple-mutated subjects (carrying mutations in three different genes). Penetrance in each subgroup was calculated by the ratio between the number of carriers affected and the total number of carriers. We considered only pedigrees for which at least one relative other than the proband was genotyped for the mutations and haplotypes (marked with an asterisk in Supplemental Figure 1). In the table below the graph, we reported the number of affected subjects (bold)/the number of carriers for each group. The chi-squared or Fisher exact test was used for statistical analysis as appropriate.

the *CFI*-combined subgroups). No significant differences were found in disease presentation, outcome, and response to plasma among each subgroup, with the exception of a higher frequency of triggering/underlying conditions in the *CFH*-combined versus the *CFI*-combined subgroups (Table 3). Transplant outcome was comparable among subgroups, with 44%–50% of grafts lost at 3 years post-transplant (Table 3). We found a higher prevalence of low C3 levels in the *C3*-combined versus the *CFI*- and *MCP*-combined subgroups and a higher prevalence of low CFI levels in the *CFI*-combined versus the *CFH*-combined subgroup (Table 3).

Thereafter, clinical data of patients with combined mutations (n=27) were compared with data from patients with mutations in a single gene (*CFH*, *MCP*, *CFI*, *C3*, or *CFB)* from the same cohorts for which clinical data were available (260 of 323[9,10,12,15,17–19,29,30,34,36–44]; unpublished data) (Table 4). No significant difference was observed in disease presentation, outcome, and response to plasma between overall combined-mutated and overall single-mutated patients (Table 4). Graft outcome at 3 years in the overall 10 combined-mutated patients receiving a kidney transplant (14 grafts) was similar to graft outcome in the overall 60 transplanted single-mutated patients (77 grafts), with 50% versus 65% of graft loss at 3 years, respectively (Table 4).

We then compared clinical data from subgroups with *CFH*- (n=15), *MCP*- (n=19), *CFI*- (n=17), *C3*- (n=4), or *CFB*- (n=1) combined mutations with data from the corresponding subgroups of single-mutated patients (*CFH*, n=148; *MCP*, n=40; *CFI*, n=27; *C3*, n=36; *CFB*, n=9). Disease presentation and short- and long-term outcomes were

Table 3. Clinical and biochemical data of subgroups of patients with combined mutations from the four cohorts

Clinical Parameters	Overall Combined (27)	CFH Combined (15)	MCP Combined (19)	CFI Combined (17)	C3 Combined (4)
Disease presentation and outcome					
Children/adults	12/15	7/8	7/12	7/10	3/1
Males/females	15/12	8/7	10/9	9/8	3/1
Familial/sporadic	14/13	8/7	12/7	8/9	1/3
Recurrences: yes/no	11/15	6/9	9/10	5/11	2/1
Triggering/underlying conditions: yes/no	10/6	9/2[a]	7/4	2/6	2/0
Good/bad outcome of the first episode	16/10	8/7	12/6	10/6	3/1
Good/bad outcome at 3 yr	11/14	5/9	9/9	6/9	2/2
Biochemical evaluation[b,c]					
Low/normal C3 levels	7/19	6/8	3/15[d]	2/14[d]	3/1
Low/normal C4 levels	0/26	0/14	0/18	0/16	0/4
Low/normal CFH levels	2/23	2/11	1/17	1/14	0/4
Low/normal CFI levels	6/16	0/11[a]	5/12	6/7	0/3
Effect of plasma treatment[c]					
Plasma treated episodes (patients treated with plasma)	35 (18)	23 (11)	20 (14)	19 (10)	10 (3)
Remission[e]/ESRF or death	26/9	16/7	14/6	15/4	8/2
Outcome of kidney transplantation[c]					
Transplanted kidneys (patients who received at least a kidney graft)	14 (10)	8 (6)	12 (8)	9 (7)	0 (0)
Good outcome/graft lost at 3 yr[f]	7/7	4/4	6/6	5/4	—
Graft lost for recurrence	5	3	4	3	—

Triggering/underlying conditions including diarrhea, vomiting, gastroentheritis, upper respiratory trait infection, and pregnancy. Good outcome includes complete remission, defined as normalization of both hematologic parameters (Ht>30%, Hb>10 g/dl, LDH<460 U/L, platelets>150,000/μl) and renal function (s-creatinine<1.3 mg/dl), and partial remission, defined as normalization of hematologic parameters with renal sequelae (chronic renal failure and/or proteinuria>0.2 g/24 h). Bad outcome includes ESRF and death. Statistical analyses were performed by chi-squared or Fisher exact test as appropriate.

[a] $P<0.03$ versus CFI combined.

[b] Normal ranges as reported in Table 2.

[c] The number of each cell refers to the number of patients for whom each specific clinical data were available.

[d] $P<0.05$ versus C3 combined.

[e] Remission includes complete and partial remission as defined above.

[f] Graft lost for disease recurrence, rejection, or other causes.

not significantly different among patients with *CFH*-, *CFI*-, or *C3*-combined mutations and patients with mutations only in *CFH*, *CFI*, or *C3*, respectively (Table 4). However, *MCP* combined-mutated patients had a worse long-term outcome than *MCP* single-mutated patients (Table 4). Indeed, at 3 years, 50% of the former patients lost renal function, whereas despite repeated recurrences, only 18.5% of the latter patients developed ESRF (Table 4). Interestingly, a triggering event was reported at the first episode in only 25% of *CFI* combined-mutated patients, whereas all *CFI* single-mutated patients developed aHUS after a triggering event (Table 4).

No significant difference in plasma efficacy was observed in patients with *CFH*- or *C3*-combined mutations (70% and 80% remission of plasma-treated episodes, respectively) versus patients with *CFH* or *C3* mutations alone (62.5% and 57% remission) (Table 4). However, plasma treatment induced remission in 79% of episodes in patients with *CFI*-combined mutations versus only 30% of episodes in *CFI* single-mutated patients. Remission was achieved in 70% and 95% of plasma-treated episodes in *MCP* combined- and single-mutated patients, respectively (Table 4).

Transplanted patients carrying either *CFH*- or *CFI*-combined mutations showed a trend to have a better prognosis than patients with *CFH* or *CFI* mutations alone (Table 4). In *MCP* combined-mutated patients, 6 of 12 transplanted kidneys were lost within 3 years post-transplant, and 4 cases were a recurrence (Table 4 and Supplemental Table 1). However, only 3 of 13 grafts were lost in *MCP* single-mutated patients (1 graft for aHUS recurrence)[12] (Table 4).

Two patients with combined *MCP/CFI* mutations (Supplemental Table 1) and nine single-mutated patients (six patients with *CFH*, one patient with *MCP*, one patient with *CFI*, and one patient with *C3* mutations[40–42]; unpublished data) received post-transplant plasma prophylaxis; in all but one (with a single *CFH* mutation[41]), the graft function was preserved at 3-year follow-up. No combined-mutated patient received Eculizumab post-transplant, whereas four single-mutated patients (three patients with *CFH* and one patient with *C3* mutations alone) were given Eculizumab prophylaxis; all had good transplant outcome at the last follow-up (unpublished data).[41,43,44]

In patients with combined mutations receiving calcineurin inhibitors, five of eight grafts failed within 3 years post-transplant

Table 4. Clinical and biochemical data of combined- and single-mutated patients from the four cohorts

Clinical Parameters	Overall Combined (27)	Overall Single (260)	*P* Value	CFH Combined (15)	CFH Single (148)	*P* Value	MCP Combined (19)	MCP Single (40)	*P* Value	CFI Combined (17)	CFI Single (27)	*P* Value	C3 Combined (4)	C3 Single (36)	*P* Value	CFB Combined (1)	CFB Single (9)
Disease presentation and outcome[a]																	
Children/adults	12/15	132/108	0.30	7/8	76/56	0.42	7/12	30/10	0.005	7/10	8/17	0.54	3/1	15/21	0.31	0/1	3/4
Males/females	15/12	118/115	0.63	8/7	66/65	0.83	10/9	23/17	0.73	9/8	5/12	0.29	3/1	19/17	0.61	1/0	5/4
Familial/sporadic	14/13	120/123	0.81	8/7	76/64	0.94	12/7	17/23	0.14	8/9	5/20	0.06	1/3	15/14	0.60	0/1	7/2
Recurrences: yes/no	11/15	82/124	0.81	6/9	46/75	0.88	9/10	23/13	0.24	5/11	4/21	0.28	2/1	9/14	0.56	0/1	0/1
Triggering/underlying conditions: yes/no	10/6	89/43	0.69	9/2	55/25	0.50	7/4	12/9	1.00	2/6	12/0	<0.001	2/0	9/9	0.48	0/1	1/0
Good/bad outcome of the first episode	16/10	107/102	0.32	8/7	52/74	0.37	12/6	30/6	0.18	10/6	14/10	0.79	3/1	11/11	0.60	0/1	0/1
Good/bad outcome at 3 yr	11/14	73/110	0.69	5/9	28/74	0.54	9/9	22/5	0.03	6/9	10/13	0.83	2/2	13/17	1.00	0/1	0/1
Effect of plasma treatment[a]																	
Plasma-treated episodes (patients treated with plasma)	35 (18)	228 (138)		23 (11)	152 (89)		20 (14)	44 (23)		19 (10)	10 (9)		10 (3)	21 (16)		0	1 (1)
Remission[b]/ESRF or death	26/9	152/76	0.37	16/7	95/57	0.51	14/6	42/2	0.009	15/4	3/7	0.02	8/2	12/9	0.26	—	0/1
Outcome of kidney transplantation[a]																	
Transplanted kidneys (patients who received at least a kidney graft with at least 3 yr follow-up)	14 (10)	77 (60)		8 (6)	37 (31)		12 (8)	13 (11)		9 (7)	11 (7)		0 (0)	15 (10)		0	1 (1)
Good outcome/graft lost at 3 yr[c]	7/7	27/50	0.29	4/4	9/28	0.20	6/6	10/3	0.16	5/4	3/8	0.36	—	5/10	—	—	0/1
Graft lost for recurrence	5	28		3	14		4	1		3	8		0	4		—	1

Triggering/underlying conditions including diarrhea, vomiting, gastroenteritis, upper respiratory trait infection, and pregnancy. Good outcome includes complete remission, defined as normalization of both hematologic parameters (Ht>30%, Hb>10g/dl, LDH<460 U/L, platelets>150,000/μl) and renal function (s-creatinine<1.3 mg/dl), and partial remission, defined as normalization of hematologic parameters with renal sequelae (chronic renal failure and/or proteinuria>0.2 g/24 h). Bad outcome includes ESRF and death. Statistical analysis on CFB is not feasible because of the presence of only one CFB combined-mutated patient. Statistical analyses were performed using the chi-squared or Fisher exact test as appropriate.

[a]The number of each cell refer to the number of patients for whom each specific clinical data were available.

[b]Remission includes complete and partial remission as defined above.

[c]Graft lost for disease recurrence, rejection or other causes.

(three cases were lost for recurrence), whereas all three grafts in patients not receiving calcineurin inhibitors were functioning (Supplemental Table 1).

DISCUSSION

Reports in large patient numbers have described the clinical course, response to therapy, and transplant outcome in aHUS patients with single-gene mutations.[20,29,35,45] A few cases with mutations in more than one complement gene have been reported,[20,29,33–35,46] with 3%–12% of prevalence.[20,29] Here, we report the results of a collaborative study undertaken by the European Working Party on Complement Genetics in Renal Diseases that included 795 aHUS patients screened for known disease-associated genes. We identified 27 patients with combined mutations, accounting for 3.4% of aHUS cases. In a previous report,[20] three of eight cases of combined mutations were accounted for by *THBD* and/or *CFHR5* mutations. THBD, CFHR5, and other CFHRs genes were not systematically screened in the cohorts included here, and this process may have led to an underestimation of the prevalence of combined mutations. The present and previously published findings[20,29,33–35] indicate that all aHUS-associated genes should be screened in a patient who presents with aHUS. In those patients in whom a single mutation has already been identified previously, then additional screening may be necessary if all the aHUS-associated genes have not been analyzed. Likewise, as additional genes are identified in the future, it may be necessary to screen these new genes in current patients.

Of note, only 8%–10% of patients with *CFH*, *C3*, or *CFB* mutations carried abnormalities in other genes, suggesting that mutations in *CFH*, *C3*, or *CFB* alone may be sufficient to cause aHUS. In contrast, ~25% of patients with a mutation in *MCP* or *CFI* had a second or third mutation in other complement genes. This observation is consistent with previous data[34] that 5 of 23 aHUS patients with *CFI* mutations carried at least one additional genetic risk factor, such as an *MCP*, *CFH*, or *C3* mutation.

It is generally accepted that complement gene mutations confer predisposition to aHUS rather than directly causing the disease.[2,33] Here, we found low aHUS penetrance in subjects with single-gene mutations, whereas penetrance was higher, but still incomplete, in carriers of mutations in two genes; complete penetrance was observed in the two subjects with mutations in three genes.

Control of complement is performed by a network of plasma and membrane-associated regulatory proteins that restricts complement activation to the surface of microorganisms.[47] The case of patients with mutations in three genes indicates that the concurrence of multiple genetic susceptibility factors involving plasma- and membrane-associated regulators is required to impair protection to host tissues enough to induce aHUS. Polymorphisms and haplotype blocks in *CFH* and *MCP* increase risk of aHUS.[32,33] Consistently, we found that the presence of at least one copy of both risk haplotypes, *CFH*-H3 and *MCPggaac*, significantly increased disease penetrance in carriers of mutations in two genes, although penetrance still remained incomplete.

Recently, the concomitant presence of anti-CFH antibodies on the background of homozygous *CFHR1–3* deletion and mutations in *CFH*, *MCP*, *C3*, or more commonly, *CFI* has been reported in aHUS.[22,34] Here, we quantified *CFHR1–3* copy number and anti-CFH antibodies in combined-mutated patients, but none of them was either homozygous for the deletion or had anti-CFH antibodies.

The association of triggering/precipitating events with aHUS onset has been emphasized in patients with single-gene mutations.[29,30,48] The same occurred in most patients with combined mutations described here, in whom aHUS manifested on triggering conditions (mainly infections), indicating that environmental factors are critical determinants of HUS development.

Bienaime *et al.*[34] documented that patients with *CFI* mutations and complete *CFHR1* deletion had a worse prognosis than patients with only a *CFI* mutation. Here, we found that the concomitant presence of mutations in other genes did not modify the disease prognosis in patients with *CFI* mutations versus patients with *CFI* mutations alone. A comparable outcome was also observed in patients with combined and single *CFH* or *C3* mutations. However, we observed that the presence of mutations in other genes was associated with a more severe phenotype in *MCP*-mutated patients with a higher incidence of ESRF than in patients with *MCP* mutations alone. Altogether, these results would indicate that mutations in *CFH*, *CFI*, or *C3* exert a dominant effect on disease phenotype in patients with combined *MCP* mutations.

Previous data have shown that most patients carrying single-gene mutations undergo remission on plasma treatment.[1,29,35,49,50] Here, we show that the same occurs in combined-mutated patients. Notably, patients with *CFI*-combined mutations showed a higher rate of response to plasma than patients with a single *CFI* mutation; however, the long-term outcome in the two groups was identical.

The finding that patients with *MCP*-combined mutations had a lower rate of remission after plasma treatment than patients with single *MCP* mutations possibly reflects the less severe disease phenotype in the latter group. Indeed, in patients with single *MCP* mutations, remission was generally obtained with or without plasma treatment.[29] In the few described patients with single *MCP* mutations that had very severe disease course, plasma given during the acute episode or to prevent recurrences did not influence the natural course of the disease.[14,40]

We wish to emphasize that our analysis of response to plasma therapy is limited, because it was based on retrospective data; because of the rarity of combined-mutated patients, it necessarily included cases from several centers, where different plasma therapy protocols may have been used.

Previous data emphasized that kidney transplantation alone in aHUS is compromised by the risk of recurrence, especially in

patients with mutations in genes encoding circulating complement proteins.[51,52] Consistent with published data,[53] here, we found a high prevalence of post-transplant recurrence in patients with combined mutations. The impact of calcineurin inhibitor use on incidence of recurrences is still a matter of debate. Some authors showed that early use of cyclosporine increases the risk, but others denied it.[52] The data presented here, showing that, in combined-mutated patients, graft loss for recurrence clustered in the subgroup receiving calcineurin inhibitors, may support the former possibility.

Notably, we observed a high incidence of graft failure for recurrence in the *MCP*-combined group, contrasting with the good graft outcome among *MCP* single-mutated patients (1 of 13 graft failure for recurrence).[10,12,29,40,52] It is plausible that the concomitant presence of genetic abnormalities leading to dysfunction in circulating proteins (CFH and/or CFI), which could not be corrected by an isolated kidney transplant, contributed to the higher risk of post-transplant recurrences in the *MCP*-combined group. The observation that two patients with combined *MCP/CFI* mutations who received intensive plasma prophylaxis had an uneventful post-transplant course would confirm previous data,[42,51] showing a beneficial impact of such a regimen to prevent post-transplant recurrences.

In summary, the cases presented here underline the complexity of aHUS genetics. Complement gene mutations may have disparate consequences, ranging from highly pathogenic mutations associated with complete disease penetrance and unfavorable outcome to variants that cause aHUS only combined with other mutations and/or *CFH* and *MCP* risk haplotypes.

We would recommend that aHUS patients are screened for all known disease-associated genes. *CFH*-H3 and *MCPggaac* risk haplotypes should be also checked, because they impact disease penetrance and phenotype in mutation carriers. The latter point would be of help for genetic counseling in patients' relatives; indeed, carriers of both combined mutations and risk haplotypes could have a higher likelihood to develop aHUS in life than subjects carrying only combined mutations.

If a novel sequence variant is identified in a patient, it should be looked for in at least 300–400 healthy ethnically matched controls to distinguish disease-causing mutations from rare nonpathogenic variants. Screening should not be stopped after finding a mutation to avoid missing other genetic susceptibility factors influencing disease phenotype. This recommendation particularly applies to patients with *MCP* or *CFI* mutations, because they have a higher probability of also carrying mutation in another gene than patients with *CFH* or *C3* mutations. Importantly, in *MCP*-mutated patients, the presence of combined mutations highly impacts the outcome and risk of post-transplant recurrence versus patients with *MCP* mutations alone.

The HUS mutation database (www.fh-hus.org) is currently being updated to provide information on whether a given mutation was found alone or combined with other mutations to help optimize genetic screening.

Screening should also include anti-CFH antibodies to rapidly identify patients who need immunosuppressive therapies and intensive plasma exchange to taper the antibody titer,[26] *THBD* and *CFHRs* gene sequencing, and Multiplex Ligation-Dependent Probe Amplification to identify deletions and rearrangements in *CFH-CFHRs*.

Recently, the humanized anti-C5 monoclonal antibody Eculizumab has been effectively administered to aHUS patients to induce disease remission, treat, or prevent post-transplant recurrences,[41,54–56] and its efficacy has been confirmed in two controlled trials in plasma-resistant and -dependant patients.[57,58] However, this drug is not universally available at present, and probably, it will not be in the future because of the high cost and need for chronic treatment. A careful genetic characterization, allowing prediction of disease phenotype and risk of post-transplant recurrences, could help selection of those patients who may need and benefit most from Eculizumab. In particular, it could be useful to exclude from Eculizumab therapy patients with single *MCP* mutations who are reported to have a good post-transplant outcome[52] or patients with anti-CFH antibodies who may benefit from plasma exchange, steroids, or Rituximab.[26,59]

CONCISE METHODS

Patients

aHUS was diagnosed in all patients included in this study based on microangiopathic hemolytic anemia and thrombocytopenia defined on the basis of hematocrit (Ht) less than 0.3 (30%), hemoglobin (Hb) level less than 100 g/L (10 g/dl), serum lactate dehydrogenase (LDH) level greater than 460 U/L, undetectable haptoglobin level, fragmented erythrocytes in the peripheral blood smear, and platelet count less than 150×10^9/L (150,000/μl)[60] associated with acute renal failure. Familial aHUS was diagnosed when two or more members of the same family were affected by the disease at least 6 months apart and exposure to a common triggering infectious agent was excluded. Sporadic aHUS was diagnosed when one or more episodes of the disease manifested in a subject with no familial history of the disease. At least 300 healthy ethnically matched controls were also genotyped for each variant found in the patients. Only variants found in aHUS patients but not in any of 600 chromosomes were considered mutations. The only exception was the A353V variant in *MCP*, which was considered a mutation, despite that it was found in 0.7% of 978 chromosomes, because published functional data indicated that the mutant protein is defective in controlling complement activation on cell surface.[61] The variants P50A (rs144082872, minor allele frequency [MAF]=0.000, 1/10,758 alleles), H183R (rs75612300, MAF=0.002), I357M (rs200881135, MAF=0.000, 1/2,184 alleles), and P553S (rs113460688, MAF=0.000, 1/2,184 alleles) in *CFI* are reported in the National Center for Biotechnology Information SNP (http://www.ncbi.nlm.nih.gov) or the 1000 Genomes (http://browser.1000genomes.org/index.html) database as ultra-rare single-nucleotide variations, but they were not found in any of our controls and were included among mutations.

Subjects carrying mutations in two or three different complement genes were defined double- or triple-mutated, respectively (combined-mutated). Subjects carrying heterozygous, double heterozygous, or homozygous mutations in a single gene were called single-mutated. All participating centers have institutional review board approval for the studies included in this report. Informed consent was provided according to the Declaration of Helsinki.

Complement Profile Assessment

Serum concentrations of C3 and C4 were evaluated by kinetic nephelometry. CFH serum levels were measured by radial immunodiffusion assay (The Binding Site, Birmingham, United Kingdom)[62] in the International Registry and UK cohorts and ELISA in the French and Spanish cohorts[1,9,38]; CFI serum levels were measured by ELISA.[15] For each laboratory, the normal ranges were set as mean ± 2 SD of the values recorded in healthy subjects. Values up the higher limit of the normal ranges were considered as high, whereas values below the lower limit of the normal ranges were considered as low.

Genetic Analyses

Genomic DNA was extracted from peripheral blood leukocytes according to standard procedures. PCR products of the coding sequence and the intronic flanking regions of *CFH*, *MCP*, *CFI*, *C3*, and *CFB* genes were genotyped by automatic DNA sequencing.

The reference nucleotide sequence of all genes starts from the codon +1 corresponding to the initial Met residue and includes the signal peptide sequence.

CFH-CFHRs deletion and rearrangements were detected by SALSA MLPA P236-A1 Kit (MRC Holland).

Statistical Analyses

Differences in biochemical and clinical data among subgroups of patients with combined gene mutations and between patients with single and combined mutations were analyzed by the chi-squared test or Fisher exact test (the latter for comparisons when the expected values in at least one cell of a contingency table were less than five) as appropriate. The differences were considered statistically significant at P values≤ 0.05.

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DISCLOSURES

None.

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