Mechanisms of disease

Familial haemolytic uraemic syndrome and an *MCP* mutation

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Summary

Background Mutations in factor H (*HF1*) have been reported in a consistent number of diarrhoea-negative, non-Shiga toxin-associated cases of haemolytic uraemic syndrome (D^-HUS). However, most patients with D^-HUS have no *HF1* mutations, despite decreased serum concentrations of C3. Our aim, therefore, was to assess whether genetic abnormalities in other complement regulatory proteins are involved.

Methods We screened genes that encode the complement regulatory proteins—ie, factor H related 5, complement receptor 1, and membrane cofactor protein (MCP)—by PCR-single-strand conformation polymorphism (PCR-SSCP) and by direct sequencing, in 25 consecutive patients with D^-HUS, an abnormal complement profile, and no *HF1* mutation, from our International Registry of Recurrent and Familial HUS/TTP (HUS/thrombotic thrombocytopenic purpura).

Findings We identified a heterozygous mutation in *MCP*, a surface-bound complement regulator, in two patients with a familial history of HUS. The mutation causes a change in three aminoacids at position 233–35 and insertion of a premature stop-codon, which results in loss of the transmembrane domain of the protein and severely reduced cell-surface expression of MCP.

Interpretation Results of previous studies on *HF1* indicate an association between HF1 deficiency and D^-HUS. Our findings of an *MCP* mutation in two related patients suggest that impaired regulation of complement activation might be a factor in the pathogenesis of genetic forms of HUS. *MCP* could be a second putative candidate gene for D^-HUS. The protein is highly expressed in the kidney and plays a major part in regulation of glomerular C3 activation. We propose, therefore, that reduced expression of *MCP* in response to complement-activating stimuli could prevent restriction of complement deposition on glomerular endothelial cells, leading to microvascular cell damage and tissue injury.

Lancet 2003; **362:** 1542–47

See Commentary

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Introduction

Haemolytic uraemic syndrome (HUS) is a rare disease of microangiopathic haemolysis, thrombocytopenia, and renal failure.[1,2] The most common form of HUS in children, with predominant renal failure, is associated with infection by *Escherichia coli*, which produce a powerful Shiga-like toxin.[1] This form of the disease (D^+HUS) usually presents with a diarrhoea prodrome and has an excellent prognosis in most cases.[3] By contrast, non-Shiga toxin-associated and diarrhoea-negative forms of HUS (D^-HUS) have a much poorer outcome (often end-stage renal failure or death[4–6]), with patients prone to relapse. There is sometimes a clustering of affected individuals within families, suggesting a genetic predisposition to the disease. Both autosomal dominant and autosomal recessive forms of inheritance have been noted, with precipitating events such as pregnancy, virus-like disease, or sepsis reported in some instances.[7–11] Low serum concentrations of the third component of complement (C3) have been identified in patients with D^-HUS.[12,13] Among such patients, a subgroup—between 13% and 30%—carry mutations in the gene encoding for factor H (*HF1*), a plasma protein that inhibits the activation of the alternative pathway of complement.[14–19] However, two thirds of patients with D^-HUS have no *HF1* mutations, despite decreased C3 concentrations,[1,18,19] indicating a role for genetic abnormalities in other complement regulatory proteins.

Methods

Participants

Between, 1996, and May, 2003, we enrolled consecutive patients with familial, recurrent, or sporadic D^-HUS with no *HF1* mutations but an abnormal serum complement profile (defined as C3 serum concentrations $<0{\cdot}83$ g/L[12] or a plasma C3d/serum C3 ratio $>0{\cdot}015$) through the International Registry of Recurrent and Familial HUS/TTP (HUS/thrombotic thrombocytopenic purpura), a network of 60 Haematology and Nephrology Units established under the coordination of the Clinical Research Centre for Rare Diseases "Aldo e Cele Daccò". We also recruited healthy blood donors as controls. For protein expression studies in peripheral blood mononuclear cells (PBMC), healthy female controls and uraemic female controls on chronic haemodialysis for causes other than HUS, were recruited.

All participants received detailed information on the purposes and design of the study and provided informed written consent, according to the guidelines of the Declaration of Helsinki. The protocol was approved by the institutional review board of the "Mario Negri" Institute for Pharmacological Research.

Procedures

With respect to complement profile assessment, we quantified serum C3 and C4 concentrations by kinetic nephelometry, and ascertained serum concentrations of

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GLOSSARY

CLASSICAL AND ALTERNATIVE COMPLEMENT PATHWAYS

Complement is part of the innate immune system and underlies the main effector mechanism of antibody-mediated immunity. The classical pathway is initiated by the binding of C1 complex to antibodies bound to an antigen on the surface of a bacterial cell. The alternative pathway is initiated by the covalent binding of a small amount of C3b to hydroxyl groups on cell-surface carbohydrates and proteins, and is activated by the low-grade cleavage of C3 in plasma. The two pathways lead to the formation of specific C3 and C5 convertases, converge in the formation of the membrane attack complex (MAC), and end with cell lysis.

SINGLE-STRANDED CONFORMATIONAL POLYMORPHISM (SSCP) ANALYSIS

A method for distinguishing between DNA fragments with different sequences (polymorphisms) amplified from the same genomic region based on differences in the mobility of the single-stranded DNA during polyacrylamide gel electrophoresis.

factor H, factor I, and factor B by radial immunodiffusion (RID; The Binding Site, Birmingham, UK).[12] C3d was assessed on plasma collected on EDTA by RID (The Binding Site).

We measured plasma ADAMTS13 activity as previously described,[20] using the collagen binding assay. The presence of ADAMTS13 inhibitory antibodies was assayed by testing ADAMTS13 activity in mixtures of plasma taken from patients and from a plasma pool at different dilutions after 30 min incubation at 37°C.[20]

To identify the causative gene(s) of HUS in patients with no *HF1* mutations, we looked at abnormalities in the complement regulatory proteins—factor H related 5 (FHR5),[21] complement receptor 1 (CR1),[22] and membrane cofactor protein (MCP).[23] We extracted genomic DNA from peripheral blood, according to standard protocols (Nucleon BACC2 kit, Amersham, UK). The coding sequences of *HF1*, *FHR5*, *CR1*, and *MCP* were screened by PCR-SINGLE STRANDED CONFORMATIONAL POLYMORPHISM ANALYSIS (PCR-SSCP), using primers designed on published genomic sequences (NT-004671, NT-021877).[19,20,24] A list of primers used for *MCP* gene analysis is reported in table 1. We did PCR reactions in a 20 μL volume, containing 100 ng DNA, 15 pmol of each primer, 16 nmol deoxynucleoside triphosphates (dNTP), 2·25 mmol/L magnesium chloride, 1 U Taq polymerase (Taq Gold, PE Applied Biosystems, Foster City, CA, USA), and PCR buffer. 10 min denaturation at 94°C was followed by 35 PCR cycles (94°C for 45 s, 55·5°C for 30 s, and 72°C for 45 s) and by 10 min extension at 72°C. We mixed samples with 20 μL of loading buffer, denatured them at 65°C for 10 min, and electrophoresed onto non-denaturing 6% (62/1 acryl/bis) acrylamide gel in TAE buffer (pH 6·8) at 35 Watt for 3–5 h at 4°C. We visualised gels by silver staining. Aberrant bands were sequenced.

We did expression studies in PBMC, which we separated by density gradient centrifugation with Ficoll-Paque, according to standard procedure. PBMC were incubated with a fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibody against human MCP (20 μL/10^6 PBMC, BD Biosciences Pharmingen, San Diego, CA, USA) or with FITC-mouse IgG (isotype control), and analysed by FACSort (BD Biosciences, Mountain View, CA, USA).

Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

We enrolled 25 patients with familial (n=12), recurrent (n=6), or sporadic (n=7) D–HUS, all of whom were white, had no *HF1* mutations, and had an abnormal serum complement profile. We also enrolled 100 healthy blood donors and, for protein expression studies in PBMC, six healthy female controls and three uraemic female controls.

Analysis of *FHR5*, indicated a heterozygous 343C→T polymorphism leading to a L66F change in short consensus repeat (SCR) 1 in two patients and in one healthy control, a heterozygous 1160G→A polymorphism leading to a R338H change in SCR6 in four patients and one healthy control, and a heterozygous 1634T→G polymorphism causing a M496R change in one patient and one control.

With respect to the membrane-bound regulatory protein CR1, we identified no mutations in the patients, and the distribution of known *CR1* polymorphisms was comparable in patients and controls.[24] In particular, quantitative expression of CR1 on cell surface is regulated by a genetic element that is linked to the site of a HindIII restriction fragment length polymorphism of the *CR1* gene that determines either a high (H) or a low (L) expression allele.[25] Among the 11 polymorphisms described in the *CR1* coding sequence, the 5507C→G (P1827R) in exon 33 is in strict linkage disequilibrium with the HindIII polymorphism: specifically, the C variant is linked to the H allele, and the G variant to the L allele.[26] The distribution of 5507C→G genotypes was similar in HUS patients (CC=55%, CG=40%, GG=5%) and in controls (CC=56%, CG=37%, GG=7%), thus excluding an association between *CR1* L allele and D–HUS.

However, a mutation in *MCP* was noted in two of 25 D–HUS patients—in a 21-year-old woman with a history of recurrent HUS (identified as proband in the report) and in her affected brother (table 2). The results obtained for these two individuals and their parents, form the basis of this report.

Disease onset in the proband was at age 16 months, when she developed a fever, haemolytic anaemia, and thrombocytopenia. At that time, renal function was normal. Thereafter, the patient had six recurrences of thrombotic microangiopathy, all associated with deteriorating renal function. Treatment consisted of

Exon	Function	Primer: Sense	Primer: Antisense
1	Signal peptide	5′-CTGGATGCTTTGTGAGTTGGG-3′	5′-TCTTGCCCGACTGAGGAGAG-3′
2	SCR1	5′-ACTTCATCTTCATGTTCCTATTCTCTTATC-3′	5′-ACCCCAAAATGTATGCAAATCTCT-3′
3	SCR2A	5′-CAGATCTGTTTTATAACTGGATTGAAA-3′	5′-GAAGAGAAGCAAAACAAAAATAAAATT-3′
4	SCR2B	5′-GTGTGTCTTATTAATTGCTATACAAAACAGT-3′	5′-AGAAACCTCTTTGGGATCTTTGTTA-3′
5	SCR3	5′-TGTCTTAATCTTTTACATTTCCTTTCCTCT-3′	5′-CACATACACCTGCTTTGTTTATCTGT-3′
6	SCR4	5′-CTTGTCTCTGTTCACACTGGAAATTACT-3′	5′-CAGCAACAACAATAACAAAACCAAGA-3′
7, 8	STP A, B	5′-CCCAAGTGGTTGATCTTCTAACATT-3′	5′-ATAAGTGAACATCACCAGAAATTTGAA-3′
9	STP C	5′-TTGATAAGGCCCTGGTGAATTT-3′	5′-CCTGCACGCTGTGCACA-3′
10	Unknown	5′-AAAATCACCCTATGAGTTTAAAGGATTT-3′	5′-CCTACACGTTTCTACACATACTACCACTTA-3′
11	TM	5′-GGAGATCCATGTGTTCAACATCTT-3′	5′-AATGCATGTCTTCACAATAATTTTTTG-3′
12	TM	5′-CAGAATTATATGTCATTTGTTTCCTGG-3′	5′-AAGGACCAAGAAGTTAAAAGAAACATG-3′
13	CT	5′-TCGTTTCTTTTTGGTTTGAAGTCA-3′	5′-GCAAACCTTTCTCTCATCTCTCCT- 3′
14	CT	5′-GGCTTCTGGAATTTAATTTCTGTACTTAA-3′	5′-GTCAAAGATGAACTGGCAAACC- 3′

STP=serine-threonine-proline rich domain. TM=trans-membrane domain. CT=cytoplasmic tail.

Table 1: **Primers used for *MCP* screening**

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	Proband	Brother
Parameter (normal range)		
Platelets (150–400×10⁹/L)	178	247
Lactate dehydrogenase (230–460 U/L)	375	304
C3 (0·8–1·8 g/L)	0·5	1·3
C4 (0·2–0·5 g/L)	0·3	0·3
C3d (3·03–18·23 mg/L)	14·8	49·8
C3d/C3 (0·005–0·015)	0·031	0·039
Factor H (350–750 mg/L)	589·5	908
Factor B (191–382 mg/L)	210·5	262·7
Factor I (28–58 mg/L)	38·3	41·5
ADAMTS13 activity (50–150%)	68	116

Table 2: **Biochemical data for proband and her brother**

plasma exchanges and infusions, steroids, and blood transfusions, which led to complete recovery of blood abnormalities and renal function. The last episode of disease recurrence, at age 20 years, was characterised by anaemia, thrombocytopenia, and severe impairment of renal function. Transient improvement was achieved by treatment with plasma exchanges and methylprednisolone, but this response was not sustained and, despite maintenance treatment with plasma exchanges, renal function progressively deteriorated and the patient was started on a chronic haemodialysis programme. A renal biopsy showed irreversible changes of chronic nephropathy with typical features of HUS, including diffuse narrowing/occlusion of vascular vessels and severe glomerular ischaemia. The proband's brother had two episodes of HUS at age 9 years. Both episodes were characterised by severe haemolytic anaemia and acute renal insufficiency and resolved without plasma treatment, with no renal sequelae. He was referred to our registry at age 16 years. The proband and her brother had no signs of microangiopathic haemolysis (table 2) at the time of our examination. At that time, the proband was aged 21 years and was on chronic haemodialysis, whereas her brother had a normal renal function. Both parents are healthy with no history of renal disease (figure 1).

We noted reduced serum concentrations of C3 and a higher than normal C3d/C3 ratio in the proband, whereas C4 concentrations were within the normal range, which is consistent with a selective chronic activation of the alternative pathway of complement (table 2). C3 and C4 concentrations were normal in the proband's brother, however C3d concentration and the C3d/C3 ratio were higher than normal (table 2). In the parents, concentrations of C3 (father: 1·1 g/L; mother: 0·9 g/L) and C4 (father: 0·3 g/L; mother: 0·3 g/L) were within the normal range. Factor H serum concentrations were normal in the proband and in her mother (619 mg/L), but were higher than normal range in the father (938 mg/L) and in the brother (table 2). Factor B and factor I concentrations were within the normal range (table 2).

We also measured the activity of ADAMTS13, a plasma protease that cleaves von Willebrand Factor multimers soon after their release by endothelial cells.[20] This measurement was done since deficiency of ADAMTS13 activity has been reported in patients with TTP, a thrombotic microangiopathy that shares many features with HUS, but also in some patients with HUS.[20] ADAMTS13 activity was normal in all family members (father: 106%; mother: 76%; brother: table 2), though in the proband it fell in the lower limit of normal range (table 2).

The proband and her affected brother were heterozygous for the 1160G→A polymorphism in *FHR5* and were homozygous for the C variant of the polymorphism 5507C→G in *CR1*, associated with a high expression allele (H).[26]

SSCP analysis of the *MCP* gene indicated an anomalous pattern in exon 6 (figure 1). By sequencing we found a heterozygous 2-bp deletion, causing a change in three aminoacids at position 233–35 and insertion of a premature stop-codon at position 236, which resulted in loss of the C-terminus of the protein (figure 1). The mutation was inherited by the proband from her father and was also carried by the affected brother, but was not found in the mother or in any of the 100 healthy controls.

We undertook expression studies in PBMC. FACS analysis of PBMC isolated from the proband and from the other *MCP* mutation carriers in the family (the father and

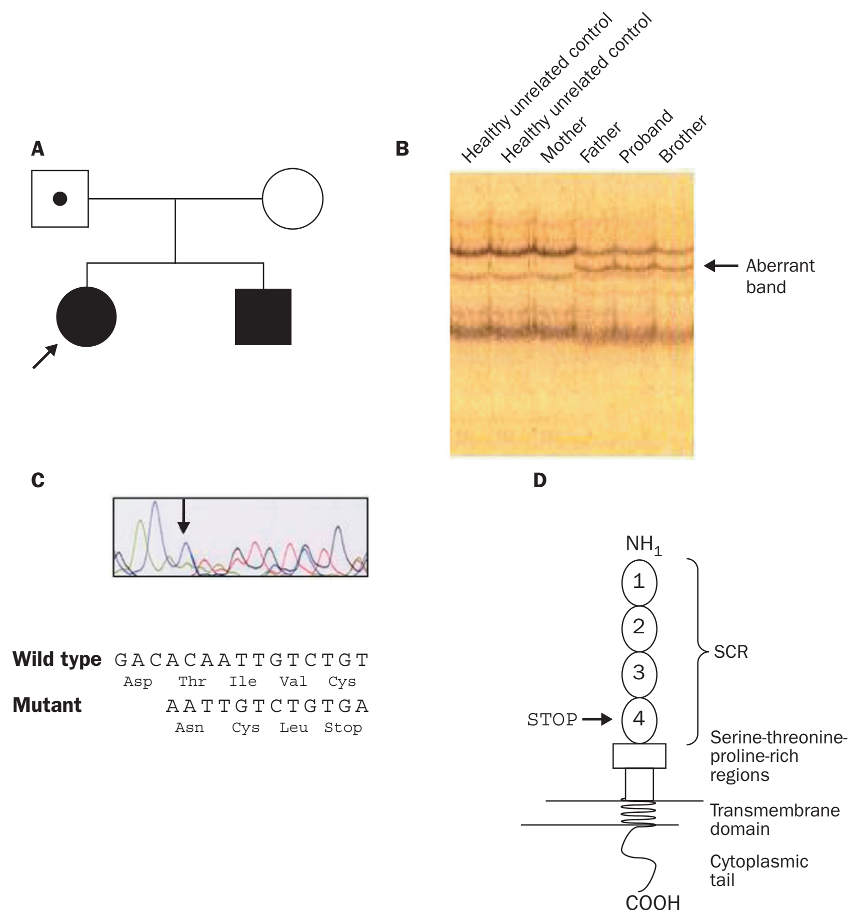

Figure 1: ***Genetic studies of MCP***

A=pedigree of family; arrow indicates proband, affected individuals in black, healthy carrier identified by black dot. B=SSCP analysis of exon 6 of *MCP*. C=sequence of exon 6 of *MCP* in proband; arrow indicates heterozygous mutation, causing 2-bp deletion (delA843-C844). D=structure and functional domains of MCP protein; arrow indicates interruption of mutant protein translation due to stop codon in SCR4, causing loss of C-terminus, including transmembrane domain.

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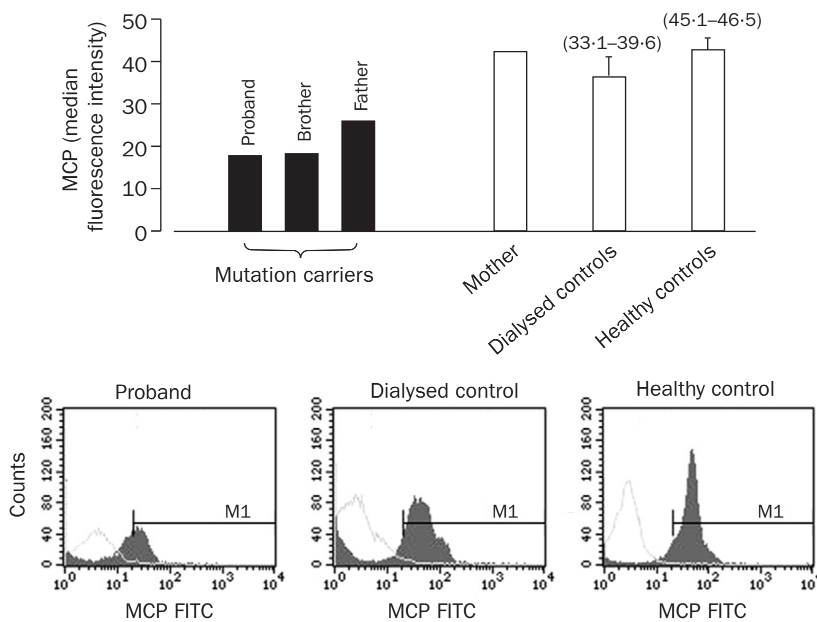

Figure 2: **Expression studies of MCP protein**

Flow cytometry analysis of MCP expression in PBMC from three mutation carriers (proband, brother and father), healthy mother, dialysed controls (n=3), and healthy controls (n=6). A: data are mean (SD); ranges in parentheses. B: histograms from proband, a representative dialysed control, and a representative healthy control.

the brother) showed around 50% reduction in *MCP* median fluorescence intensity, by comparison with PBMC from healthy controls (figure 2), indicating that the mutation affected the expression concentrations of MCP protein. By contrast the mother, who does not carry the mutation, had a normal *MCP* expression pattern (figure 2). Similar results were obtained when data were expressed as the proportion of MCP+ cells (proband: 40%; brother: 23%; father: 50%; mother: 72% *vs* healthy controls: 86% [SD 3%]). The defect was not the consequence of uraemia, since *MCP* expression levels in PBMC from patients on chronic haemodialysis (median fluorescence intensity, figure 2; proportion MCP+ cells: 72% [5]) were higher than those in the proband. Both the proportion of MCP+ cells and the median fluorescence intensity (figure 2) in PBMC from the three mutation carriers lie outside the range of values of MCP+ cells (81–90%) and MCP median fluorescence intensity recorded in PBMC from healthy controls and from individuals on haemodialysis (MCP+ cells: 68–74%; median fluorescence intensity: figure 2).

Discussion

Our results of genetic screening in 25 consecutive D-HUS patients without mutations in *HF1* but presenting with abnormalities in the ALTERNATIVE but not in the CLASSICAL PATHWAY OF COMPLEMENT, led us to identify a candidate gene for D-HUS in addition to *HF1*. Based on the fact that these patients had signs of activation of the complement system in their blood, we focused our search for a genetic cause of the disease on genes involved in complement regulation. We identified a heterozygous mutation in the *MCP* gene in one family.

MCP is a widely expressed transmembrane glycoprotein that regulates complement activation. It serves as a cofactor for factor I to cleave C3b and C4b when they are deposited on host cells.[23] MCP has four extracellular contiguous modules important for its inhibitory activity, followed by a serine-threonine-proline rich domain, a transmembrane domain, and a cytoplasmic tail (figure 1).[23] Because the delA843-C844 mutation causes loss of the MCP C-terminus, comprising part of all of these domains, we reasoned that it might affect cell-surface expression of MCP through inhibition of insertion of the mutant protein into the plasma membrane; results of our expression studies in PBMC lend support to this notion.

Together, the findings of studies[14–19] on *HF1*, showing an association between HF1 deficiency and D-HUS, and our findings of an *MCP* gene mutation in two patients within one family make a strong case for impaired regulation of complement activation as a determinant factor of the disease in genetic forms of HUS. MCP is highly expressed in the kidney[27] and plays a major part in regulating glomerular C3 activation.[28] Reduced expression of MCP in the presence of stimuli that activate the complement system—eg, infection, cytotoxic drugs, antibodies, or immune complexes—might prevent restriction of complement deposition on glomerular endothelial cells, leading to microvascular cell damage and tissue injury. That the father had had no clinical manifestations of HUS despite having the same *MCP* mutation as the proband and her affected brother is consistent with an autosomal-dominant mode of transmission with reduced penetrance, as noted in *HF1* mutation carriers.[15–19] The incomplete penetrance of the disease in carriers of either *HF1*[15–19] or *MCP* mutations indicates that D-HUS is a complex disorder that fully manifests in the presence of environmental factors and multiple genetic modifier loci. This notion accords with our finding that, in the pedigree studied, MCP expression was higher in the father than in the proband and in her affected brother. Genetically controlled variations in serum concentrations of HF1 could also explain the incomplete penetrance of the disease in *MCP* mutation carriers. In this respect, HF1 concentrations are within normal range in the proband, but above normal in the non-affected father. Similarly, HF1 serum concentration was high in the proband's brother, who had a mild form of the disease despite the same *MCP* mutation. Our provisional interpretation is that in healthy individuals the wide range of variation in the HF1 serum concentration can be irrelevant, but in an individual with an *MCP* mutation a higher than normal amount of HF1 would be essential to compensate for the defective cofactor activity due to *MCP* haploinsufficiency.

On the basis of our data, we cannot rule out the possibility that the *MCP* mutation identified is unrelated to HUS and indicates a coincidental finding of a rare genetic variant. However, this possibility is unlikely since the mutation is present in two of 25 patients with D-HUS, but in none of 100 healthy controls. Additionally, MCP has a main role in complement regulation, which is defective in HUS. Genetic screening of *HF1* and *MCP* could unveil precious information for a more tailored clinical management of patients with D-HUS. Treatment of D-HUS relies on plasma exchange or infusion, however 50%[18] to 75%[19] of patients, often small babies, progress to end-stage renal disease and need replacement therapy. One of the most debated issues is whether kidney transplantation is feasible in HUS. Children with Shiga-

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toxin associated HUS rarely progress to end-stage renal disease, but when they do so, transplantation often results in a good prognosis.[29] Notably, graft outcome is less favourable in children[30] and adults[31] with D⁻HUS, with recurrences occurring in around 50% of patients and graft failure developing in all of them. Among patients with D⁻HUS and *HF1* mutations, the recurrence range is 30–75%, according to different surveys.[18,32] In patients of our registry, the five individuals with D⁻HUS and *HF1* mutations who received a transplant had disease recurrence on the grafted kidney within a few weeks (range 1 week to 6 months) after surgery.[19] In view of the fact that HF1 is a plasma protein mainly of liver origin, indirect evidence suggests that a kidney transplant does not correct the *HF1* genetic defect (Noris M, unpublished). To speculate that a dysfunction in MCP, which is a membrane-bound protein highly expressed in the kidney, can be corrected by transplanting a normal kidney, is tempting. The graft, bearing wild-type MCP expressed on renal-cell surfaces, should conceivably be protected from disease recurrence.

Contributors

M Noris designed the study, interpreted the data, and drafted the report. S Brioschi did the experimental work on *MCP* and participated in data analysis and presentation. J Caprioli participated in setting up and coordinating genetic analyses and in preparing the manuscript. E Bresin and S Gamba participated in clinical data and biological sample collection, and in writing the clinical case report of the patient. M Todeschini was responsible for expression tests on *MCP*, and participated in data analysis and presentation. F Porrati was responsible for biochemical studies on complement and MCP, and participated in data analysis and presentation. G Remuzzi participated in discussion and interpretation of the data and revision of the report, and worked on the first draft of the paper. All authors contributed to the final version of the manuscript. M Noris and S Brioschi contributed equally to the paper.

Conflict of interest statement

None declared.

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Acknowledgments
This work was supported in part by grants from Comitato 30 ore per la vita and from Telethon (GPP02161) and by a grant from the Foundation for Children with Atypical HUS along with the Nando Peretti Foundation. S Brioschi is a recipient of a fellowship from Associazione Ricerche Malattie Rare through the generosity of the Lions Club Bergamo—St Alessandro. F Porrati is a recipient of a fellowship in memory of Libera Dossia Grana.

We thank Paola Bettinaglio for complement product measurement, Sara Bucchioni for DNA sequencing, Miriam Galbusera for ADAMTS13 assessment, and Erica Daina and Mauro Abbate for helpful discussion.

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