Complement factor H mutations and gene polymorphisms in haemolytic uraemic syndrome: the C-257T, the A2089G and the G2881T polymorphisms are strongly associated with the disease

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Mutations in complement factor H (HF1) gene have been reported in non-Shiga toxin-associated and diarrhoea-negative haemolytic uraemic syndrome (D⁻HUS). We analysed the complete HF1 in 101 patients with HUS, in 32 with thrombotic thrombocytopenic purpura (TTP) and in 106 controls to evaluate the frequency of HF1 mutations, the clinical outcome in mutation and non-mutation carriers and the role of HF1 polymorphisms in the predisposition to HUS. We found 17 HF1 mutations (16 heterozygous, one homozygous) in 33 HUS patients. Thirteen mutations were located in exons XXII and XXIII. No TTP patient carried HF1 mutations. The disease manifested earlier and the mortality rate was higher in mutation carriers than in non-carriers. Kidney transplants invariably failed for disease recurrences in patients with HF1 mutations, while in non-mutated patients half of the grafts were functioning after 1 year. Three HF1 polymorphic variants were strongly associated with D⁻HUS: -257T (promoter region), 2089G (exonXIV, silent) and 2881T (963Asp, SCR16). The association was stronger in patients without HF1 mutations. Two or three disease-associated variants led to a higher risk of HUS than a single one. Analysis of available relatives of mutated patients revealed a penetrance of 50%. In 5/9 families the proband inherited the mutation from one parent and two disease-associated variants from the other, while unaffected carriers inherited the protective variants. In conclusion HF1 mutations are frequent in patients with D⁻HUS (24%). Common polymorphisms of HF1 may contribute to D⁻HUS manifestation in subjects with and without HF1 mutations.

INTRODUCTION

The term thrombotic microangiopathy (TMA) defines a lesion of vessel wall thickening (mainly arterioles and capillaries), intraluminal platelet thrombosis and partial or complete obstruction of the vessel lumina (1,2). Depending on whether renal or brain lesions prevail, two pathologically indistinguishable, but somehow clinically different entities have been described: the haemolytic uraemic syndrome (HUS) and the thrombotic thrombocytopenic purpura (TTP). The most common form of HUS of children, with predominant renal failure, is associated with infection by strains of E. coli which produce a powerful Shiga-like toxin (3). This form, also called D⁺HUS, has an excellent prognosis. In contrast, forms of HUS non-Shiga toxin-associated and diarrhoea-negative (D⁻HUS), have a much poorer prognosis and are often relapsing; death and end-stage renal failure are the final outcome in the majority of cases (4–6). In selected cases there is a clustering of affected

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individuals within families which is suggestive of an under-
lying genetic predisposition to the disease. Both autosomal 
dominant and autosomal recessive mode of inheritance have 
been recognized, with precipitating events such as pregnancy, 
virus-like disease or sepsis being reported in some cases 
(7–12). Evidences are now emerging that some of these 
atypical forms are associated with abnormalities of the 
complement system due to genetic deficiency of factor 
H (HF), a plasma protein that inhibits the activation of the 
alternative pathway of complement (13–15). After the first 
report showing that an area on chromosome 1q, where HF1 
is mapped, segregates with the disease (16), mutations in HF1 
have been described in familial but also in non-familial cases of 
D−HUS, the majority of which are heterozygous and cause 
either single amino acid exchanges or premature translation 
interruption within the C-terminal domain of the protein (Short 
Consensus Repeat 20, SCR20) (17–21).

On the other hand evidence is available that TTP may be 
caused by a deficiency of ADAMTS-13, a plasma metallopro-
tease that cleaves ultralarge (UL) multimers of von Willebrand 
factor (VWF) soon after their secretion by endothelial cells (22). 
The deficiency of ADAMTS-13 activity may be constitutive, as 
in patients carrying mutations in the gene encoding for the 
protease (23–28), or acquired due to the presence of a circu-
lating autoantibody (29). However the issue is far more 
complex since around one-third of patients with TTP do have 
normal ADAMTS-13 activity (29).

Taking advantage of the large number of patients referred to 
our international Registry of HUS and TTP, we designed a 
study aimed at evaluating the frequency of HF1 mutations in 
D−HUS and compared the clinical phenotype of patients with 
and without HF1 mutations. Various polymorphisms in the 
promoter and in the coding regions of HF1 gene have been 
described which may be associated with reduced HF levels or 
activity. Thus a second aim of the study was to evaluate the 
frequency of HF1 polymorphisms in patients with HUS as 
compared with a control population to establish if any of them 
segregate with the disease. The same analyses were performed 
in patients with TTP to establish whether HF1 gene mutations 
and polymorphisms may have a role in those patients as well, 
or whether genetic abnormalities of HF1 were specific for the 
HUS phenotype.

RESULTS

Mutation screening of HF1

SSCP analysis of the complete HF1 sequence in our series of 
101 patients with D−HUS (familial HUS, n = 48; recurrent 
HUS, n = 23; sporadic HUS, n = 30) and 32 patients with TTP 
(familial TTP, n = 7; recurrent TTP, n = 18; sporadic TTP, 
n = 7) and in 106 controls was performed. Nine mutations were 
found in 11 patients with familial (n = 2, within one family), 
recurrent (n = 4) or sporadic (n = 5) forms of D−HUS (Table 1). 
An A305G substitution in exon II, determining an Arg60Gly 
change in SCR1, was found in a patient with sporadic HUS 
(S023#101). In exon XIX, encoding for SCR16, a G2923T 
(Gln950His) and a T2924C (Tyr951His) were found in 
two patients (S026#118 and S025#087) with sporadic HUS.

A C3562G mutation (Cys1163Trp) in exon XXII, encoding 
for SCR19, was found in a patient with recurrent HUS 
(R087#134). The other mutations were located in exon XXIII 
encoding for SCR20, namely a G3587T determining a 
Glu1192Stop in two unrelated patients with sporadic and 
recurrent HUS (S027#022 and R063#081), a G3654A 
(Gly1194Asp) in two patients with familial HUS (F169#130 
and F170#130), an A3666C (Glu1198Ala) in a patient with 
recurrent HUS (R088#152), a T3663C (Val1197Ala) in a 
patient with recurrent HUS (R062#056), and a C3701T 
(Arg1210Cys) in a patient with sporadic HUS (S013#069). 
The latter two mutations have been previously reported in other 
patients with HUS (17), while all the other mutations are new.

Table 1 and Figure 1 summarize all the mutations found in 
patients from our Registry, including the ones reported here and 
those previously published by our group (17,20). All are 
heterozygous mutations, with the exception of family #029, 
with recessive transmission. Seventy percent of the overall 
independent mutational events (12 out of 17) cluster in SCR20 
(Table 1). Moreover three other mutations are located in SCR16 
(n = 2) and SCR19 (n = 1), thus confirming previously 
reported data on the importance of the C-terminus of HF1 
(17–19) to the pathogenesis of HUS.

Three mutations determine the introduction of a premature 
stop codon, resulting in truncated proteins at SCR 8 (n = 1) 
and 20 (n = 2), while all the others are missense mutations 
(Table 1).

No mutation in HF1 gene was found in patients with TTP. 
None of the mutations described were found in any of 106 
healthy controls.

The frequency of HF1 gene mutations in our population of 
HUS patients (33 out of 101, including both alive and dead 
patients) is 33%. This frequency decreases to 24% if we 
consider only one patient from each family included in the 
study. Mutation frequency in familial forms of HUS is 46% 
(32% if we consider only one patient from each family), in 
recurrent forms 26% and in sporadic forms 17%.

Clinical and biochemical findings

The clinical data of the patients with HF1 mutations (including 
the new and the previously published cases from our Registry) 
and of the patients with no HF1 mutations are reported in 
Tables 2 and 3. Disease manifested earlier in patients with HF1 
mutations as compared with patients carrying no HF1 
mutations. However in both groups at least half of the patients 
developed the disease in infancy. In contrast, in all patients with 
TTP the onset of the disease was in adulthood. Putative 
triggering conditions were recognized in the majority of HUS 
and TTP patients, with infection being the most frequently 
associated condition (Table 2).

As for clinical outcome, 12 out of 33 patients with HF1 
mutations (36%) died because of the disease while mortality 
was significantly (P = 0.016) lower (10%) in patients without 
HF1 mutations, which suggests that the disease is more severe 
in the former than in the latter patients. Among the survivors, 
57% of patients with HF1 mutation and 46% of patients 
without HF1 mutations were on chronic dialysis. Overall 73% 
of patients with HF1 mutations versus 51% of patients without 
mutations (P = 0.04) died or had irreversible loss of their
Table 1. HF1 gene mutations in patients with HUS from our Registry

<table>
<thead>
<tr>
<th>Exon</th>
<th>Mutation</th>
<th>SCR</th>
<th>Effect</th>
<th>Family code</th>
<th>Subgroups</th>
<th>Patients</th>
<th>Unaffected carriers</th>
<th>Origin of the mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>A305G</td>
<td>1</td>
<td>Arg60Gly</td>
<td>101</td>
<td>Sporadic</td>
<td>1</td>
<td>2/4</td>
<td>Paternal</td>
</tr>
<tr>
<td>XI</td>
<td>del A1494-1496*</td>
<td>8</td>
<td>Premature stop</td>
<td>003</td>
<td>Familial</td>
<td>2</td>
<td>1/11</td>
<td>Paternal</td>
</tr>
<tr>
<td>XIX</td>
<td>G2923T</td>
<td>16</td>
<td>Gln950His</td>
<td>118</td>
<td>Sporadic</td>
<td>1</td>
<td>/</td>
<td>Unknown</td>
</tr>
<tr>
<td>XIX</td>
<td>T2924C</td>
<td>16</td>
<td>Tyr951His</td>
<td>087</td>
<td>Sporadic</td>
<td>1</td>
<td>/</td>
<td>Unknown</td>
</tr>
<tr>
<td>XXII</td>
<td>C3562G</td>
<td>19</td>
<td>Cys1163Trp</td>
<td>134</td>
<td>Recurrent</td>
<td>1</td>
<td>/</td>
<td>Unknown</td>
</tr>
<tr>
<td>XXIII</td>
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<td>20</td>
<td>Glu1172Stop</td>
<td>081</td>
<td>Recurrent</td>
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<td>4/8</td>
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<td>XXIII</td>
<td>T3620A</td>
<td>20</td>
<td>Trp1183Arg</td>
<td>045</td>
<td>Familial</td>
<td>2</td>
<td>1/3</td>
<td>Paternal</td>
</tr>
<tr>
<td>XXIII</td>
<td>G3654A</td>
<td>20</td>
<td>Gly1194Asp</td>
<td>130</td>
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<td>2</td>
<td>5/8</td>
<td>Maternal</td>
</tr>
<tr>
<td>XXIII</td>
<td>T3663C</td>
<td>20</td>
<td>Val1197Ala</td>
<td>056</td>
<td>Recurrent</td>
<td>1</td>
<td>/</td>
<td>Unknown</td>
</tr>
<tr>
<td>XXIII</td>
<td>T3663C*</td>
<td>20</td>
<td>Val1197Ala</td>
<td>052</td>
<td>Recurrent</td>
<td>1</td>
<td>1/2</td>
<td>Paternal</td>
</tr>
<tr>
<td>XXIII</td>
<td>A3666C</td>
<td>20</td>
<td>Glu1198Ala</td>
<td>152</td>
<td>Recurrent</td>
<td>1</td>
<td>/</td>
<td>Unknown</td>
</tr>
<tr>
<td>XXIII</td>
<td>C3701T</td>
<td>20</td>
<td>Arg1210Cys</td>
<td>020</td>
<td>Recurrent</td>
<td>1</td>
<td>6/10</td>
<td>Paternal</td>
</tr>
<tr>
<td>XXIII</td>
<td>C3701T*</td>
<td>20</td>
<td>Arg1210Cys</td>
<td>024</td>
<td>Familial</td>
<td>3</td>
<td>1/3</td>
<td>Paternal</td>
</tr>
<tr>
<td>XXIII</td>
<td>C3701T</td>
<td>20</td>
<td>Arg1210Cys</td>
<td>069</td>
<td>Sporadic</td>
<td>1</td>
<td>1/1</td>
<td>Unknown</td>
</tr>
<tr>
<td>XXIII</td>
<td>G3717A</td>
<td>20</td>
<td>Arg1215Gln</td>
<td>001</td>
<td>Familial</td>
<td>3</td>
<td>2/6</td>
<td>Paternal</td>
</tr>
<tr>
<td>XXIII</td>
<td>A3579T and 24 bp deletion*</td>
<td>20</td>
<td>Premature stop</td>
<td>029</td>
<td>Familial</td>
<td>10</td>
<td>/</td>
<td>Recessive</td>
</tr>
</tbody>
</table>

*Caprioli et al. (17).
*Remuzzi et al. (20).

Figure 1. Summary of HF1 gene mutations in patients with D+ HUS from our Registry. The structure of HF with the 20 SCRs is shown; SCR20 is enlarged on the right. The consequence of the mutation to protein composition is indicated in the circles; each circle corresponds to an independent mutational event.
renal function. One or more disease relapses were reported in 61 and 34% of patients with and without HF1 mutations, respectively ($P = 0.01$), and in 41% of patients with TTP.

Five patients with HF1 mutations and eight patients without mutations underwent kidney transplantation, with two patients in the latter group receiving three and two kidney grafts, respectively (Tables 2 and 3). All five grafts in patients with HF1 mutations were lost because of disease recurrence within the first year, as compared with only two of the 11 grafts in patients without HF1 mutations ($P = 0.0022$). In patients without HF1 mutations, four grafts were lost because of acute rejection and the remaining five were functioning well at 1 year post transplant. Of note, two additional patients with HF1 mutations received a combined kidney and liver transplant. The kidney graft outcome at one year was very good in the first patient (20), while unfortunately the second patient died few days after surgery because of acute liver failure without evidence of recurrence of the microangiopathic process (unpublished data, Table 3).

### Complement profile and ADAMTS13 activity

Samples for serum complement profile measurement were available in 72 patients with HUS (21 with HF1 mutations, 51 without HF1 mutations) and in 26 patients with TTP. Patients were studied at remission to avoid any confounding effect on complement profile due to disease activity. As shown in Table 2, decreased serum C3 levels, consistent with chronic activation of the alternative pathway of complement, were detected in 67% of D/C0 HUS patients carrying HF1 mutations. Mean C3 levels were moderately but significantly lower than in healthy controls (69.6±18.4 versus 113.0±17.9 mg/dl, $P < 0.01$). The lowest C3 levels were found in patients of family 29 (around 10 mg/dl), carrying the homozygous A3579T and 24 bp deletion in exon XXIII that causes a very severe reduction of HF serum concentrations (17), in patient F039#003 (41 mg/l) with the del A1494-1496 mutation in exon XI, and in patient S023#101 (50 mg/l) with the A305G change in exon II. Of interest also 51% of HUS patients with no HF1 mutations and 35% of patients with TTP had low C3 levels after disease recovery. In contrast, C4 levels were within normal levels in the three groups of patients (not shown), thus excluding a role for the classic pathway of complement activation.

### Table 2. Clinical and biochemical data of patients with HUS and TTP from our Registry. The numbers of patients for whom data were available are reported in parentheses

<table>
<thead>
<tr>
<th>Disease onset</th>
<th>HUS</th>
<th>TTP</th>
<th>$P$-values (HF1 mutation versus no HF1 mutation)</th>
<th>$P$-values (HUS versus TTP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infancy (&lt;12 years)</td>
<td>23 (33)</td>
<td>34 (68)</td>
<td>0 (32)</td>
<td>0.06</td>
</tr>
<tr>
<td>Adulthood (&gt;12 years)</td>
<td>10 (33)</td>
<td>34 (68)</td>
<td>32 (32)</td>
<td>0.69</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>2 (29)</td>
<td>5 (53)</td>
<td>5 (29)</td>
<td>0.82</td>
</tr>
<tr>
<td>Drugs</td>
<td>2 (29)</td>
<td>3 (53)</td>
<td>4 (29)</td>
<td>0.06</td>
</tr>
<tr>
<td>Flu-like, gastroenteritis, other infections</td>
<td>13 (29)</td>
<td>35 (53)</td>
<td>11 (29)</td>
<td>0.53</td>
</tr>
<tr>
<td>Transplantation (post-transplant HUS)</td>
<td>0 (29)</td>
<td>3 (53)</td>
<td>0 (29)</td>
<td>0.016</td>
</tr>
<tr>
<td>Other triggers</td>
<td>2 (29)</td>
<td>2 (53)</td>
<td>3 (29)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

### Table 3. Outcome of kidney transplants in patients with HUS from our Registry

<table>
<thead>
<tr>
<th>Kidney transplant recipients</th>
<th>HF1 mutation</th>
<th>No HF1 mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transplanted kidneys</td>
<td>5*</td>
<td>8</td>
</tr>
<tr>
<td>Kidney outcome</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>OK at one year</td>
<td>0</td>
<td>5*</td>
</tr>
<tr>
<td>Disease recurrence on the graft</td>
<td>5</td>
<td>2*</td>
</tr>
<tr>
<td>Acute rejection</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Combined kidney and liver transplantation</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

### Kidney outcome

| OK at one year | 1b | 0 |
| Disease recurrence on the graft | 0 | 0 |
| Failure for other causes | 1 | 0 |

* $P < 0.05$ versus HF1 mutation.
* One patient reported by Landau et al. (40).
* Reported in Remuzzi et al. (20).
HF serum levels were lower than normal in three patients with HF1 mutations and in one patient without mutation, while three and six patients, respectively, had elevated HF serum levels.

Most patients with TTP had a severe deficiency in the plasma activity of the metalloprotease ADAMTS-13, confirming previously reported data (29), however we also found five patients with a clinical diagnosis of HUS and no HF1 mutation, showing severe ADAMTS-13 deficiency (Table 2). Two patients had familial HUS while the other three had a recurrent form of the disease with neonatal onset. The latter form, previously classified as recurrent HUS (30), has been recently redefined as Upshaw–Schulman syndrome (30). No patient had both HF1 mutations and ADAMTS-13 deficiency.

Analysis of factor H polymorphisms

HF1 gene screening in the 106 healthy controls revealed the presence of eight polymorphic variants: C-257T (promoter region), G257A in exon II (causing a Val44Ile change in SCR1), C994A in exon VII (SCR5, silent), T1277C in exon IX (causing a Tyr405His change in SCR7), G1492A in exon XI (SCR8, silent), A2089G in exon XIV (SCR11, silent), A2489-30C (intron XVI), and a G2881T in exon XIX (causing a Gln469Stop change in SCR16) (Table 4), all of which have been reported previously (19,31) with the exception of the novel variant, A2489-30C in intron XVI. The distribution of the above polymorphic variants in patients and in controls was in Hardy–Weinberg equilibrium.

Association analysis was performed in patients and healthy controls to examine whether the presence of any of the above genetic HF1 variants conferred predisposition to develop HUS or TTP. In order to avoid the bias determined by the clustering of specific variants into families, we only considered one affected subject from each family.

Comparison of allelic frequencies in patients and controls showed a strong association between HUS and the variants -257T in the promoter region, 2089G in exon XIV (SCR11) and 2881T in exon XIX (SCR16) (Table 4, P < 0.01 for all three polymorphisms). The association was also significant for all the three genetic variants when data from patients with HUS and no HF1 mutation were analysed separately, indicating that HF1 genetic variations may have a role in predisposing to HUS also in patients without HF1 mutations (Table 5). Similar results were obtained by analysing the distribution of the genotypes; the highest association with HUS was found for the GT + TT genotypes of the polymorphism in SCR16 (Table 6), with the highest increase in the risk of developing HUS for genotype carriers (Table 7). Mean values of HF serum concentrations were very comparable in the subgroups of patients carrying the different genotypes for the -257T (CC, 567.3 ± 188.6 mg/l; CT, 615 ± 167 mg/l; TT, 587.3 ± 86 mg/l), the A2089G (AA, 559.1 ± 154 mg/l; AG, 628 ± 174 mg/l; GG, 594 ± 102 mg/l) and the G2881T (GG, 530.6 ± 160.9 mg/l; GT, 622 ± 164 mg/l; TT, 598 ± 89 mg/l) polymorphisms, indicating that none of the above genetic variants affect basal HF circulating levels.

A modest association was also found between HUS and the variants C994 in exon VII (SCR5) and the A2489-30 in intron XVI (Table 4). In contrast, none of the HF1 variants showed any association with TTP (Table 4).

We then tested for possible synergistic effect of the -257T, the 2089G and 2881T variants of HF1 on the predisposition to develop HUS. As shown in Table 8, we found that the combined presence of two of the above polymorphic variants on at least one allele was more strongly associated with HUS than the presence of single polymorphisms. The difference between HUS patients and controls further increased when the combined presence of three polymorphic variants was considered (HUS versus controls, P-value = 0.0000045, data not shown). Repeating the latter analysis taking into consideration only data from patients without HF1 mutations, we obtained an even lower P-value (0.000003), with respect to controls (data not shown).

The odds ratios for the presence of a couple of polymorphic variants were higher than those found for each polymorphism alone (Table 7), indicating an increased risk of developing HUS. As shown in Table 7, the simultaneous presence of the three predisposing polymorphic variants was associated with a further modest increase in the risk of HUS over the combination of two of them.

Analysis of familial transmission of mutations and polymorphisms

All available relatives of nine patients carrying HF1 mutations were also screened and unaffected carriers were detected, giving a mutation penetrance of 49% (Table 1; family 029 has been excluded from the analysis because of recessive transmission of the disease). The transmission of the mutation was from the father in eight cases and from the mother in one (Table 1; family 029 excluded). Interestingly, none of the parents carrying HF1 mutations had episodes of HUS. This could be explained either as autosomal dominant transmission with reduced penetrance or as a recessive trait. We then wanted to evaluate whether the recessive trait was determined by the presence of HF1 mutations combined with the -257T, the 2089G and the 2881T disease-associated polymorphic variants. To this purpose the parents and all available relatives from nine patients carrying HF1 mutations were analysed for the above polymorphisms and the transmitted alleles were reconstructed. In five out of nine families we found that the affected patients had inherited the allele carrying the mutation from one parent and an allele carrying at least two of the above polymorphisms and may therefore be protected. In addition, allele frequencies of disease-associated HF1 polymorphic variants were significantly higher in patients with HF1 mutations than in controls (Table 5), with the highest difference found for the 2881T (P = 0.00021). These data support the possibility that HF1 polymorphic variants may contribute to HUS development in mutation carriers.
DISCUSSION

In our series of 101 patients with D−HUS we found 17 independent HF1 mutational events (present data and 17,20), of which seven mutations are novel. These mutations appear to be responsible for the disease in 33 of these patients. As expected, the frequency of HF1 mutation is high in cases with a familial history of HUS but rather low in sporadic cases. By contrast no patient with a diagnosis of TTP carried HF1 mutations which indicates that HF abnormalities are specifically associated with the HUS phenotype.

All are heterozygous mutations, with the exception of one homozygous mutation found in a family with recessive transmission. Of note most mutations caused single amino acid changes, while three mutations introduced premature stop codons. Although the identified mutations are spread over five exons, most of them (13 out of 17) clustered in exons XXII and XXIII and affected the most C terminus part of HF (SCR19 and 20).

In humans the alternative pathway of complement activates continuously by depositing C3b on all surfaces in contact with plasma. Amplification of the initial C3b formation and subsequent activation of the full complement system is controlled on host surfaces by regulatory proteins, some of which are membrane-bound and some fluid phase proteins (32–34). HF is the primary fluid phase regulator and is responsible for controlling spontaneous fluid phase activation as well as activation on host cells by binding to surface polyanions such as clusters of sialic acid and sulfated proteoglycans, such as heparin (32,33). Detailed structure function analysis has localized within the N-terminal SCRs 1–4 of HF the complement regulatory domains needed to prevent runaway fluid phase alternative pathway amplification (35). On the other hand the C-terminal domains of HF have numerous functions which control the regulatory activity of the N-terminal domains: there are two C3b binding sites, which raise the affinity of HF for C3b clusters on the surface of cells, and three polyanion-binding sites (35–37), which determine the contact of the molecule with host cells. Recent studies indicate that deletion of the last C-terminus SCRs of HF causes loss of the ability of the protein to control spontaneous activation of the alternative complement pathway on host cells surface (37).

In addition functional studies by two independent groups (36,38) documented that five of the mutations in SCR20 reported in patients with HUS, namely Glu1172Stop, Arg1210Cys, Arg1215Gly, Trp1183Leu and Val1197Ala (36,38), cause reduced binding of the protein to surface-bound C3b, to heparin and to endothelial cells. In contrast, the mutant proteins have a normal capacity to bind fluid-phase C3b and to act as cofactors for C3b proteolysis by factor I (38). This may explain why, in patients with mutations in the C terminus of HF, C3 serum levels are in general only moderately reduced or even normal, while strong deposition of C3 (39) and C5b-9 (40) is found in glomeruli and renal arterioles.

The disease manifested earlier and was associated with a higher mortality rate in carriers of HF1 mutations than in those without HF1 mutations. However, a consistent number of patients carrying HF1 mutations had long remissions and presented late in life, suggesting that a ‘second hit’ is needed, at least in the heterozygous individuals. Actually, a putative triggering condition associated with HUS onset, in particular infection, was found in most patients. According to this possibility, in HUS patients, decreased availability of wild-type HF, which may be enough to control basal complement activation, upon a stimulus that activates the complement system (such as infection, cytotoxic drugs and other intercurrent stimuli), fails to efficiently restrict complement deposition on endothelial cells, leading to damage to microvasculature cell membranes and to tissue destruction.

Five patients with HF1 mutations were given a renal transplantation. In all of them the graft failed because of disease recurrence. Since HF is mostly synthesized by the liver (34), the genetic defect was not corrected by the kidney transplant and persistent HF deficiency predisposed to disease recurrence on the transplanted kidney. In an additional patient with HF1 mutation HUS recurrence was prevented by a simultaneous liver transplant, which corrected HF dysfunction (20). For still
unknown reasons, premature liver failure, cured by a second liver graft in the above case (20) and fatal in a second one (manuscript in preparation), complicated the outcome of the combined liver and kidney transplants. Thus, in patients with HF1 mutations, a single kidney graft is contraindicated and a combined kidney and liver transplant is not recommended at the moment because of the high risk of liver graft failure. On the contrary, in patients without HF1 mutations, half of the kidney grafts were still functioning at 1 year post-transplant.

Of note, 67% of patients with HUS in our series did not have HF1 mutations. These data would suggest the existence of at least another susceptibility gene for HUS. That this gene could be involved in the regulation of the alternative pathway of complement is supported by finding of lower than normal C3 levels in more than 50% of HUS patients with no HF1 mutations. We also evaluated the possibility that common polymorphisms of HF1 gene may contribute to development of HUS in patients without HF1 mutations. Actually, analysis of allele frequency and genotype distribution of HF1 polymorphisms in patients and controls revealed an association between HUS and three HF1 polymorphic variants. Specifically, the T allele of the C-257T, the G allele of the A2089G and the T allele of the G2881T polymorphisms were more frequent in HUS patients than in controls and odds ratio values indicate that carriers of each polymorphic variant have a higher risk of developing HUS than non-carriers. The combined presence of two and three of the above variants on at least one allele was more strongly associated with HUS than the presence of a single one. Consistently, increased odds ratios for the risk of developing HUS were found in carriers of two or three of the above polymorphisms. Interestingly, when only data from patients without HF1 mutations were considered we found a stronger association between HUS and the simultaneous presence of the three predisposing polymorphic variants than by analysing data from the overall HUS population.

The first genetic variant, namely a C-257T, is located in an unknown functional site located in SCR16, a few amino acids away from the Glu963Asp polymorphism, which suggests that this genetic variant probably affects the efficiency of NFkB-induced HF transcription in conditions of infection or inflammation (41–43). The second polymorphism, an A2089G located in exon XIV, although silent, could theoretically influence either the efficiency of transcription or the mRNA stability or just be in linkage disequilibrium with the other two polymorphisms. Finally, we found association between HUS and the simultaneous presence of the second and third polymorphisms. Interestingly, when only data from patients without HF1 mutations were considered we found a stronger association between HUS and the simultaneous presence of the three predisposing polymorphic variants than by analysing data from the overall HUS population.

The first genetic variant, namely a C-257T, is located in an unknown functional site located in SCR16, a few amino acids away from the Glu963Asp polymorphism, which suggests that this genetic variant probably affects the efficiency of NFkB-induced HF transcription in conditions of infection or inflammation (41–43). The second polymorphism, an A2089G located in exon XIV, although silent, could theoretically influence either the efficiency of transcription or the mRNA stability or just be in linkage disequilibrium with the other two polymorphisms. Finally, we found association between the disease and the polymorphic variant G2881T, determining a Glu963Asp change in SCR16 of HF. Of interest, two of the mutations in HF1 found in our patients were also located in SCR16, a few amino acids away from the Glu963Asp polymorphism, which suggests that a functional site located in SCR16 may be crucial to protect from disease development.

The distribution of the above polymorphisms was also investigated in patients carrying HF1 mutations and in all their

### Table 5. Allele frequencies of disease-associated HF1 polymorphic variants in patients with and without HF1 mutations and in controls

<table>
<thead>
<tr>
<th>Genetic variant</th>
<th>Alleles</th>
<th>HUS HF1 mutation</th>
<th>HUS no HF1 mutation</th>
<th>Controls</th>
<th>P-value, HUS HF1 mutation versus controls</th>
<th>P-value, HUS no HF1 mutation versus controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-257T</td>
<td>C</td>
<td>53.2%</td>
<td>62.1%</td>
<td>73.6%</td>
<td>0.017</td>
<td>0.027</td>
</tr>
<tr>
<td>Promoter</td>
<td>T</td>
<td>46.8%</td>
<td>37.9%</td>
<td>24.6%</td>
<td>0.017</td>
<td>0.027</td>
</tr>
<tr>
<td>A2089G</td>
<td>A</td>
<td>62.5%</td>
<td>65.0%</td>
<td>84.0%</td>
<td>0.00065</td>
<td>0.00020</td>
</tr>
<tr>
<td>SCR 11</td>
<td>G</td>
<td>37.5%</td>
<td>35.0%</td>
<td>16.0%</td>
<td>0.00065</td>
<td>0.00020</td>
</tr>
<tr>
<td>G2881T</td>
<td>G</td>
<td>53.0%</td>
<td>65.6%</td>
<td>82.0%</td>
<td>0.00021</td>
<td>0.00067</td>
</tr>
<tr>
<td>SCR 16</td>
<td>T</td>
<td>47.0%</td>
<td>34.4%</td>
<td>18.0%</td>
<td>0.00021</td>
<td>0.00067</td>
</tr>
</tbody>
</table>

### Table 6. Genotype distribution of HF1 polymorphic variants in HUS patients and in controls

<table>
<thead>
<tr>
<th>Variant</th>
<th>Genotypes</th>
<th>HUS patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-257T</td>
<td>CC</td>
<td>25</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>44</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>78</td>
<td>106</td>
</tr>
<tr>
<td>Promoter</td>
<td>AA</td>
<td>29</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>40</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>76</td>
<td>106</td>
</tr>
<tr>
<td>A2089G</td>
<td>GG</td>
<td>27</td>
<td>70</td>
</tr>
<tr>
<td>SCR 11</td>
<td>GT</td>
<td>43</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>77</td>
<td>106</td>
</tr>
</tbody>
</table>

### Table 7. Relative risk of developing HUS in subjects carrying the polymorphic HF1 variants in the promoter, in SCR11 and in SCR16 (alone or in combination)

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Odds ratios (95% confidence interval)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter (TT + CT)</td>
<td>2.66 (1.45–4.90)</td>
<td>0.0015</td>
</tr>
<tr>
<td>SCR11 (GG + AG)</td>
<td>3.43 (1.85–6.36)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SCR16 (TT + GT)</td>
<td>3.60 (1.94–6.67)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Promoter + SCR16 (TT + CT) + (GG + AG)</td>
<td>4.22 (2.23–7.99)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Promoter + SCR11 + SCR16 (TT + CT) + (GG + AG) + (TT + GT)</td>
<td>4.33 (2.29–8.19)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Promoter + SCR11 + SCR16 (TT + CT) + (GG + AG) + (TT + GT)</td>
<td>4.44 (2.31–8.55)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

aP-value (general) = 0.0062; P-value (CT + TT) = 0.0015.
bP-value (general) = 0.00016; P-value (AG + GG) = 0.000067.
cP-value (general) = 0.000084; P-value (GT + TT) = 0.000034.
available relatives, to evaluate whether they could discriminate between disease-affected and healthy mutation carriers in our families. Tracking the mutations back to the former generation was possible in nine of our patients; interestingly in eight of them the mutation was inherited from the father while only in one the mutation carrier was the mother. None of the ancestors had episodes of HUS. This could be explained either as autosomal dominant transmission with reduced penetrance or as a recessive trait. The latter was confirmed in one family in which the affected subjects carried an homozygous HF1 deletion in exon XXII. In additional five families we found that the proband had inherited the allele carrying the mutation from one parent and an allele carrying at least two disease-associated HF1 polymorphic variants from the other parent, which again would reproduce a recessive trait. By contrast, all the healthy mutation carriers in the above five families had inherited the protective HF1 polymorphic variants.

In summary, this mutation study in a large population of patients demonstrates that mutations in the HF1 gene are frequent in patients with D/C0HUS, regardless of previous familial history. In addition common polymorphic variants of HF1 gene may predispose to HUS those subjects who do not carry HF1 mutations and, in subjects with HF1 mutations, may help the full manifestation of the disease. The finding of no mutation in patients with TTP, a related thrombotic microangiopathy with mainly extra-renal involvement, calls for a specific protective role of HF from damaging effect of complement within the kidney. This interpretation is in keeping

Figure 2. Pedigree of family 081 showing the segregation of the Glu1172Stop mutation. Non-affected carriers are marked with a central black circle. The C-257T in the promoter, the A2089G in SCR11 and the G2881T in SCR16 polymorphisms were analysed to determine the paternal and maternal alleles in the proband and to evaluate the segregation of the disease associated variants -257T, 2089G and 2881T in the proband and in the healthy mutation carriers. The black asterisk identifies the allele carrying the Glu1172Stop mutation.

Table 8. Distribution of combined genotypes of HF1 disease-associated polymorphic variants in HUS patients and in controls

<table>
<thead>
<tr>
<th>Promoter and SCR11&lt;sup&gt;a&lt;/sup&gt; Genotypes</th>
<th>HUS patients</th>
<th>Controls</th>
<th>Promoter and SCR16&lt;sup&gt;b&lt;/sup&gt; Genotypes</th>
<th>HUS patients</th>
<th>Controls</th>
<th>SCR11 and SCR16&lt;sup&gt;c&lt;/sup&gt; Genotypes</th>
<th>HUS patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCAA</td>
<td>19</td>
<td>53</td>
<td>CCGG</td>
<td>20</td>
<td>47</td>
<td>AAGG</td>
<td>24</td>
<td>67</td>
</tr>
<tr>
<td>CCGA</td>
<td>4</td>
<td>5</td>
<td>CCGT</td>
<td>5</td>
<td>8</td>
<td>AAGT</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>CCGG</td>
<td>0</td>
<td>0</td>
<td>CCTT</td>
<td>0</td>
<td>0</td>
<td>AATT</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CTAA</td>
<td>9</td>
<td>16</td>
<td>CTGG</td>
<td>6</td>
<td>17</td>
<td>AGGG</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>CTGA&lt;sup&gt;*&lt;/sup&gt;</td>
<td>34</td>
<td>16</td>
<td>CTGT&lt;sup&gt;*&lt;/sup&gt;</td>
<td>36</td>
<td>21</td>
<td>AGGT&lt;sup&gt;*&lt;/sup&gt;</td>
<td>38</td>
<td>23</td>
</tr>
<tr>
<td>CTGG&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1</td>
<td>0</td>
<td>CTTT&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1</td>
<td>0</td>
<td>AGTT&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>TTAA</td>
<td>1</td>
<td>7</td>
<td>TTGG</td>
<td>1</td>
<td>4</td>
<td>GGGG</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TTGA&lt;sup&gt;*&lt;/sup&gt;</td>
<td>2</td>
<td>7</td>
<td>TTGT&lt;sup&gt;*&lt;/sup&gt;</td>
<td>2</td>
<td>4</td>
<td>GGTT&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TTGG&lt;sup&gt;*&lt;/sup&gt;</td>
<td>6</td>
<td>2</td>
<td>TTTT&lt;sup&gt;*&lt;/sup&gt;</td>
<td>6</td>
<td>3</td>
<td>GGTT&lt;sup&gt;*&lt;/sup&gt;</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>76</td>
<td>106</td>
<td>Total</td>
<td>77</td>
<td>104</td>
<td>Total</td>
<td>75</td>
<td>105</td>
</tr>
</tbody>
</table>

<sup>a</sup>P-value (general) = 0.000068; P-value (*): 0.0000057.

<sup>b</sup>P-value (general) = 0.0028; P-value (*): 0.000019.

<sup>c</sup>P-value (general) = 0.00005; P-value (*): 0.0000037.
with experimental data showing that in an HF-deficient line of pigs, homozygous individuals die soon after birth from complement-mediated acute renal failure (44) and HF knockout mice develop membranoproliferative glomerulonephritis (45). Further biochemical and functional studies on HF1 mutants and polymorphic variants will be required to provide deeper insights into the role of HF in the pathogenesis of HUS.

**MATERIALS AND METHODS**

**Patients**

One-hundred and one patients with diagnosis of HUS and 32 patients with diagnosis of TTP were recruited through the database of the International Registry of Recurrent and Familial HUS/TTP, a network of 60 units of Haematology and Nephrology, established on 1995, under the coordination of the Clinical Research Center for Rare Diseases ‘Aldo & Cele Daccò’ (Ranica, Italy).

HUS or TTP was diagnosed in all cases reported to have one or more episodes of microangiopathic haemolytic anaemia and thrombocytopenia defined on the basis of haematocrit (Ht) <30%, haemoglobin (Hb) <10 mg/dl, serum lactate dehydrogenase (LDH) >460 U/l, undetectable haptoglobin, fragmented erythrocytes in the peripheral blood smear, and platelet count <150 000/l. Specifically, a diagnosis of HUS was made when laboratory findings of thrombotic microangiopathy were associated with acute renal failure without evidence of neurologic signs (namely signs consistent with focal or diffuse CNS ischaemia) except those attributable to uraemic or hypertensive encephalopathy. A diagnosis of TTP was made when laboratory findings of thrombotic microangiopathy occurred in patients, with or without renal involvement, in whom specific neurologic symptoms dominated the clinical picture (29).

**Complement profile assessment**

Serum samples were used to quantify the third (C3) and the fourth (C4) complement fractions by kinetic nephelometric assays using commercially available kits (The Binding Site, Birmingham, UK). Serum levels of C3 and C4 below the lower limit of normal ranges (defined as mean ± SD of the laboratories of the ‘Ospedali Riuniti, Azienda Ospedaliera di Bergamo’ (i.e. <83 mg/dl for C3 and <15 mg/dl for C4) were taken to indicate hypocomplementemia (17).

**Measurement of ADAMTS-13 activity**

ADAMTS-13 activity was measured as previously described (29) using the collagen binding assay. The protease activity was tested using pooled normal plasma as source of VWF as substrate for ADAMTS-13. Human collagen type III (3 μg/ml,
Valter Occhiena, Milano Italy) was used for the collagen binding assay. The values of the protease activity were read from a dose-response curve obtained with reference plasma pool. The lower limit of the assay was 6% of the normal protease levels (29).

Single-strand conformation polymorphism analysis and sequencing of factor H

Genomic DNA was extracted from peripheral blood leukocytes according to standard protocols (Nucleon BACC2 kit, Amersham, UK). HF1 was screened by PCR–SSCP. PCR reactions were done in a 20 µl volume containing 100 ng DNA, 15 pmol of each primer, constructed in order to avoid coamplification of factor H related genes (see Table 9), 16 nmol dNTP, 2.25 mM MgCl₂, 1 U Taq polymerase (Taq Gold, PE Applied Biosystems, Foster City, CA, USA), and PCR buffer. Ten minutes’ denaturation at 94°C were 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. Samples were mixed with 20 µl of loading buffer, denatured at 65°C for 10 min, and electrophoresed on non denaturing 6% (62 : 1 acryl : bis) acrilamide gel in TAE buffer (pH 6.8) at 35 W for 3–5 h at 4°C. Gels were visualized by silver staining. If a polymorphism was identified, selected individuals showing the three different band patterns in SSCP were sequenced using a CEQ 2000 XL sequencer (Beckman Coulter, Berkeley, CA, USA) in order to identify the corresponding three genotypes. Those subjects were subsequently used as standards in SSCP. Subjects showing aberrant bands were also sequenced.

Statistical analysis

Differences between allele frequencies and genotype distribution of HF1 gene polymorphisms among patients with HUS, TTP and controls were analysed by the chi-square test. The odds ratios for these comparisons were also calculated. Data were expressed as mean±SD. A P-value ≤0.05 was considered to be statistically significant. The two tailed t-Student test was used to compare C3, C4 and HF levels in our populations.

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