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

Elena Bresin,* Erica Rurali,* Jessica Caprioli,* Pilar Sanchez-Corral,† Veronique Fremeaux-Bacchi,‡ Santiago Rodriguez de Cordoba,§ Sheila Pinto,‖ Timothy H.J. Goodship,‖ Marta Alberti,* David Ribes,¶ Elisabetta Valoti,* Giuseppe Remuzzi,†† and Marina Noris,* on behalf of the European Working Party on Complement Genetics in Renal Diseases

*Clinical Research Center for Rare Diseases, “Aldo e Cele Daccò,” Mario Negri Institute for Pharmacological Research, Ranica, Bergamo, Italy; †Hospital Universitario La Paz and Centro de Investigacion Biomedica en Enfermedades Raras, Madrid, Spain; ‡Assistance Publique-Hopitaux de Paris, Hôpital European Georges-Pompidou, Service d’Immunologie Biologique, Paris, France; §Centro de Investigaciones Biológicas and Centro de Investigacion Biomedica en Enfermedades Raras, Madrid, Spain; ‖Institute of Genetic Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom; ¶Department of Nephrology, Centre Hospitalier Universitaire de Toulouse, Toulouse, France; and ††Department of Nephrology and Dialysis, Azienda Ospedaliera, Ospedali Riuniti di Bergamo, Italy

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. Furthermore, screening patients with aHUS for all known disease-associated genes may inform decisions about kidney transplantation.


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
Inherited defects that determine uncontrolled activation of the alternative complement pathway have been well documented in aHUS patients. 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), membrane cofactor protein (MCP), complement factor I (CFI), complement factor B (CFB), CFH-related 5 (CFHR5), and thrombomodulin (THBD). In addition, anti-CFH autoantibodies have been described mostly in children that lack CFHR1 and CFHR3 because of a deletion of the corresponding genes. Novel genetic abnormalities of CFHR1, CFHR3, and CFHR4 and genomic rearrangement between CFH and CFHR1 have recently been described.

Incomplete penetrance of aHUS has been reported in mutation carriers, indicating that complement gene mutations confer predisposition to develop aHUS, with additional genetic and/or environmental hits necessary for disease manifestation. In keeping with this hypothesis, patients with mutations in more than one complement gene (combined gene mutations) have been described. 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 CFB, MCP, and CFI (triplet-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).

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

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Patient</th>
<th>CFH Mutation</th>
<th>MCP Mutation</th>
<th>CFI Mutation</th>
<th>C3 Mutation</th>
<th>CFB Mutation</th>
<th>rs 3753394</th>
<th>rs 1065489</th>
<th>rs 7144</th>
<th>Familial/V Sporadic</th>
<th>Sex</th>
<th>Age at Onset (yr)</th>
<th>C3 Levels*</th>
<th>CFH Levels*</th>
<th>CFI Levels*</th>
<th>Triggers</th>
<th>Episodes</th>
<th>Outcome at First Episode</th>
<th>Outcome at 3 yr</th>
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<td>P553S</td>
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n.a., not available; URT, upper respiratory tract.

*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; CFI, 350–590 mg/L; CFI, 350–750 mg/L; CFI, 350–750 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).
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 3239,10,12,15,17–19,29,30,34,36–44; 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 2. Prevalence of patients with single and combined mutations in CFH, MCP, CFI, C3, and CFB in the four cohorts

<table>
<thead>
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<th>Genetic Abnormality</th>
<th>CFH</th>
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<tbody>
<tr>
<td>CFH</td>
<td>158</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP</td>
<td>7</td>
<td>65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFI</td>
<td>4</td>
<td>9</td>
<td>46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>CFB</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Triple-mutated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combined*/single</td>
<td>15/158</td>
<td>19/65&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17/46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4/45</td>
<td>1/9</td>
</tr>
<tr>
<td>Combined*/single + combined (%)</td>
<td>8.7%</td>
<td>22.6%</td>
<td>27%</td>
<td>8.2%</td>
<td>10%</td>
</tr>
<tr>
<td>Single mutation/ screened patients</td>
<td>158/795 (19.9%)</td>
<td>65/795&lt;sup&gt;a&lt;/sup&gt; (8.2%)</td>
<td>46/795&lt;sup&gt;b&lt;/sup&gt; (5.8%)</td>
<td>45/795&lt;sup&gt;d&lt;/sup&gt; (5.7%)</td>
<td>9/795&lt;sup&gt;a&lt;/sup&gt; (1.1%)</td>
</tr>
</tbody>
</table>

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.

<sup>a</sup>Including double- and triple-mutated.

<sup>b</sup>P<0.001 versus CFH.

<sup>c</sup>P<0.03 versus C3.

<sup>d</sup>P<0.001 versus CFB.

<sup>e</sup>P<0.001 versus CFB.

<sup>f</sup>P<0.001 versus CFB.
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 CFH combined-mutated patients, whereas all CFI single-mutated patients developed aHUS after a triggering event (Table 4).

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

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

<sup>a</sup>P<0.03 versus CFI combined.
<sup>b</sup>Normal ranges as reported in Table 2.
<sup>c</sup>The number of each cell refers to the number of patients for whom each specific clinical data were available.
<sup>d</sup>P<0.05 versus C3 combined.
<sup>e</sup>Remission includes complete and partial remission as defined in 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<sup>40–42</sup>; unpublished data) received post-transplant plasma prophylaxis; in all but one (with a single CFH mutation<sup>41</sup>), 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)<sup>41,43,44</sup>.

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)<sup>12</sup> (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<sup>40–42</sup>; unpublished data) received post-transplant plasma prophylaxis; in all but one (with a single CFH mutation<sup>41</sup>), 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)<sup>41,43,44</sup>.

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

<table>
<thead>
<tr>
<th>Disease presentation and outcome</th>
<th>Children/adults</th>
<th>Males/females</th>
<th>Familial/sporadic</th>
<th>Recurrences: yes/no</th>
<th>Triggering/underlying conditions: yes/no</th>
<th>Good/bad outcome of the first episode</th>
<th>Good/bad outcome at 3 yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>12/15</td>
<td>15/12</td>
<td>119/15</td>
<td>15/18</td>
<td>23/11</td>
<td>23/11</td>
<td>23/11</td>
</tr>
<tr>
<td>Combined</td>
<td>27</td>
<td>19</td>
<td>152/89</td>
<td>23/11</td>
<td>15/10</td>
<td>10 (3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Overall</td>
<td>35 (18)</td>
<td>23 (11)</td>
<td>152/89</td>
<td>23/11</td>
<td>19 (10)</td>
<td>10 (3)</td>
<td>21 (13)</td>
</tr>
<tr>
<td>Single</td>
<td>228 (138)</td>
<td>24 (23)</td>
<td>23/11</td>
<td>23/11</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (1)</td>
</tr>
<tr>
<td><strong>P Value</strong></td>
<td>0.30</td>
<td>0.63</td>
<td>0.83</td>
<td>0.83</td>
<td>0.83</td>
<td>0.83</td>
<td>0.83</td>
</tr>
<tr>
<td><strong>CFH</strong></td>
<td>15</td>
<td>19</td>
<td>152/89</td>
<td>23/11</td>
<td>19 (10)</td>
<td>10 (3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Combined</td>
<td>(15)</td>
<td>(19)</td>
<td>(152)</td>
<td>(23)</td>
<td>(19)</td>
<td>(10)</td>
<td>(0)</td>
</tr>
<tr>
<td><strong>P Value</strong></td>
<td>0.31</td>
<td>0.61</td>
<td>0.60</td>
<td>0.60</td>
<td>0.60</td>
<td>0.60</td>
<td>0.60</td>
</tr>
<tr>
<td><strong>MCP</strong></td>
<td>15</td>
<td>19</td>
<td>152/89</td>
<td>23/11</td>
<td>19 (10)</td>
<td>10 (3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Combined</td>
<td>(15)</td>
<td>(19)</td>
<td>(152)</td>
<td>(23)</td>
<td>(19)</td>
<td>(10)</td>
<td>(0)</td>
</tr>
<tr>
<td><strong>P Value</strong></td>
<td>0.31</td>
<td>0.61</td>
<td>0.60</td>
<td>0.60</td>
<td>0.60</td>
<td>0.60</td>
<td>0.60</td>
</tr>
<tr>
<td><strong>CFI</strong></td>
<td>15</td>
<td>19</td>
<td>152/89</td>
<td>23/11</td>
<td>19 (10)</td>
<td>10 (3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Combined</td>
<td>(15)</td>
<td>(19)</td>
<td>(152)</td>
<td>(23)</td>
<td>(19)</td>
<td>(10)</td>
<td>(0)</td>
</tr>
<tr>
<td><strong>P Value</strong></td>
<td>0.31</td>
<td>0.61</td>
<td>0.60</td>
<td>0.60</td>
<td>0.60</td>
<td>0.60</td>
<td>0.60</td>
</tr>
<tr>
<td><strong>C3</strong></td>
<td>15</td>
<td>19</td>
<td>152/89</td>
<td>23/11</td>
<td>19 (10)</td>
<td>10 (3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Combined</td>
<td>(15)</td>
<td>(19)</td>
<td>(152)</td>
<td>(23)</td>
<td>(19)</td>
<td>(10)</td>
<td>(0)</td>
</tr>
<tr>
<td><strong>P Value</strong></td>
<td>0.31</td>
<td>0.61</td>
<td>0.60</td>
<td>0.60</td>
<td>0.60</td>
<td>0.60</td>
<td>0.60</td>
</tr>
<tr>
<td><strong>CFB</strong></td>
<td>15</td>
<td>19</td>
<td>152/89</td>
<td>23/11</td>
<td>19 (10)</td>
<td>10 (3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Combined</td>
<td>(15)</td>
<td>(19)</td>
<td>(152)</td>
<td>(23)</td>
<td>(19)</td>
<td>(10)</td>
<td>(0)</td>
</tr>
<tr>
<td><strong>P Value</strong></td>
<td>0.31</td>
<td>0.61</td>
<td>0.60</td>
<td>0.60</td>
<td>0.60</td>
<td>0.60</td>
<td>0.60</td>
</tr>
</tbody>
</table>

*Remission includes complete and partial 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 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.

www.jasn.org
(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,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,29 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 findings20,29,33–35 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 data34 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.2,33 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.\textsuperscript{51,52} Consistent with published data,\textsuperscript{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.\textsuperscript{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).\textsuperscript{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,\textsuperscript{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,\textsuperscript{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.\textsuperscript{41,54–56} and its efficacy has been confirmed in two controlled trials in plasma-resistant and -dependant patients.\textsuperscript{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\textsuperscript{52} or patients with anti-CFH antibodies who may benefit from plasma exchange, steroids, or Rituximab.\textsuperscript{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 hemocrit (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)\textsuperscript{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.\textsuperscript{61} The variants P50A (rs144082872, minor allele frequency [MAF]=0.000, 1/10,758 alleles), H183R (rs175612300, 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) in the International Registry and UK cohorts and ELISA in the French and Spanish cohorts. CFI serum levels were measured by ELISA. For each laboratory, the normal ranges were set as mean ± 2 SD of the values recorded in healthy subjects. Values up to 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.

REFERENCES


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Supplementary material

Case Reports of familial cases

Below we provide a brief description of clinical history and pedigrees (Supplementary Fig.1, panel A) for familial forms.

Family #130. The proband, patient F169, presented at the age of 9 months with anemia, thrombocytopenia, nephrotic range proteinuria and gross hematuria. A diagnosis of Stx-HUS with nephrotic syndrome was entertained due to positive stool test for *E. coli* strain O157:H7. He received blood transfusions, hemolysis and renal function improved although proteinuria remained in the nephrotic range. At 37 months of age he developed a relapse of aHUS with negative stools both for Shiga toxin-producing *E. coli* and for Shiga toxins. At the last follow-up he maintained a low-grade hemolysis while renal function and urinary protein excretion were normal. His maternal aunt, patient F582, developed aHUS 4 days after delivery of her third child. She was treated with intensive plasma exchange and corticosteroids. The hemolysis improved, but renal failure persisted. She received chronic hemodialysis for two years until a cadaver donor kidney transplant was performed. She was well at three years post-transplant. C3, C4, CFH and CFI levels, evaluated during remission, were within the normal ranges in both patients. Patients F169 and F582 carry a heterozygous G1194D mutation in *CFH* (SCR20) and a heterozygous F242C mutation in *MCP* (SCR4) [1].

Nineteen unaffected relatives were also screened for the above mutations: 6 subjects carry in heterozygosis the G1194D in *CFH*, 1 carries the F242C in *MCP*, while both mutations are present in 4 healthy subjects.

Of note, out of the 6 subjects with combined *CFH/MCP* mutations the two patients F582 and F169 and the healthy mother of patient F169 carry both the *CFH*-H3 and *MCP*gaac risk haplotypes, whereas the other three compound heterozygous subjects carry only the *MCP*gaac risk haplotype [2].
Family #024. The proband, patient F108, developed aHUS at the age of 3 years. Six years later he manifested a relapse with renal sequelae and developed ESRF at the age of 12 years. The older brother, patient F106, presented aHUS at the age of 8 years and 10 years later he manifested a relapse. Both episodes resolved without renal sequelae. Biochemical evaluations of the 2 patients during remission showed low C3 levels while C4, CFH and CFI were normal. A younger brother died at the age of 6 years after a very severe episode of aHUS.

Patients F106 and F108 carry a heterozygous R1210C mutation in \( CFH \) (SCR20) [3] inherited from the unaffected father and two mutations in \( MCP \) (C35Y, in SCR1, inherited from the mother and R59X, in SCR1, inherited from the father).

Family FRA13/FRA15. The proband, patient FRA13, developed aHUS at 47 years of age. She was treated with plasma obtaining hematological remission, but not complete recovery of renal function and within 3 years she reached ESRF. She then received a kidney allograft that at 6 year follow-up was still functioning.

A first cousin, patient FRA15, developed pregnancy-associated aHUS at the age of 29 years with ESRF as outcome. She underwent three kidney transplants, the first lost after 2 years for chronic rejection, the second within 1 year for aHUS recurrence and the third after 6 years for venous thrombosis.

Biochemical evaluations in both patients showed normal C3, C4, CFH and CFI levels. Genetic analysis in FRA13 revealed the presence of three heterozygous mutations: R1210C in the SCR20 of CFH, Y29X in the signal peptide of MCP, and P553S in the serine-protease domain of CFI. The first two mutations, R1210C in CFH and Y29X in MCP, were also found in the affected cousin, patient FRA15, and in the healthy father of FRA15.

Family#265. The proband, patient F870, is a 6-year-old Caucasian male who developed renal insufficiency, hemolytic anemia and thrombocytopenia without diarrhea, sepsis, or signs of infection at the age of 9 months. The child suffered many episodes of aHUS, each treated with plasma exchange. Despite initiation of a prophylactic regimen of plasma exchange, his renal
function declined significantly. At the age of 4 years he received a combined liver-kidney transplant (LKT) with preoperative plasma exchange and enoxaparin anticoagulation. Initial function of both grafts was excellent and maintained for nearly 2 years [4]. His maternal family history included three female cousins of the mother with ESRF secondary to aHUS: one (patient F868) received an isolated kidney transplantation that failed for disease recurrence, one is dialysis-dependent and the other one died due to complications of ESRF. The proband has two heterozygous mutations: the c.3572C>T change in \textit{CFH}, that leads to the amino acid substitution S1191L in SCR20, and the c.1661A>T change in \textit{CFI} that causes the E554V substitution in the serine-protease domain. The patient inherited from the healthy mother both mutations that are present also in the unaffected grandmother. By contrast the affected maternal cousin F868 carries only the \textit{CFH} mutation. The same mutation is present in 4 unaffected relatives.

\textbf{Family GUI.} The proband, patient HUS109, presented with HUS of unknown etiology at the age of 12 months after an episode of fever and vomits. She needed a transfusion of red blood cells and was also given plasma infusion. The hemolytic crises disappeared 4 days after diagnosis and dialysis was not necessary. Eighteen months later she had a second HUS episode with severe anemia and thrombocytopenia but normal renal function. She was treated with plasma infusion and recovered in two weeks. At the age of 5 years (October 2009) she suffered a third HUS episode. Eculizumab treatment was then initiated and it has been maintained since then. She currently shows chronic kidney disease stage 2 and hypertension and is under conservative treatment.

The father, patient HUS62, presented with acute renal insufficiency, Coombs’ test negative, microangiopathic hemolytic anemia and thrombocytopenia at the age of 34 years and was diagnosed with HUS of unknown etiology. He required hemodialysis and was treated with immunosuppressant agents and steroids. He received 27 sessions of plasmapheresis with plasma infusion, however full recovery of renal function was not achieved. The patient remained stable under hemodialysis since December 2001. In May 2008 he received a kidney allograft that in February 2012 was still functioning.
Both patients had normal C3, C4 and CFH levels but low CFI levels (50% and 53% respectively). They carry a mutation in MCP (R103W in SCR2), and a mutation in CFI (N151S in SRCR domain). Moreover, patient HUS109 inherited from her healthy mother a deletion from base 800 to 820 of MCP that causes the loss of 7 amino acids (TIVCDSN) in SCR4.

**Family #176.** The proband, patient F1314, is a 2.5-year-old Caucasian female who presented at 6 months of age with hemolytic anemia, thrombocytopenia and oliguric acute renal failure. Familial history disclosed several paternal relatives with unclassified lethal renal diseases (not shown in the supplementary Fig. 1) and a 30-year-old alive cousin of the father (F617) with ESRF secondary to aHUS and a genetically proven CFH abnormality (CFH/CFHR1 hybrid gene) [1]. Therefore, plasma exchange was immediately started. Renal replacement therapy was not necessary and after one week all blood parameters were within normal range. At the last follow-up, normotension and normal kidney function without proteinuria were observed. Genetic analysis confirmed the presence of the CFH/CFHR1 hybrid gene in the proband and in her father; the same abnormality is present in a proband’s older unaffected sister. The proband carries also the heterozygous c.1429+1G>C mutation in CFI, inherited from the healthy mother and present also in the unaffected younger sister.

**Family FRE44.** The proband, patient FRE44, is a 17 year-old boy who had six recurrences of aHUS. The first episode was at 3 years of age. He regained normal renal function after each episode. His father (FRE44F) had three episodes of aHUS at age 28, 41 and 42 years. He is now 44 years old with renal insufficiency (creatinine clearance of 24 ml/min). Patients FRE44 and FRE44F carry a mutation in C3 (H1464D). Patient FRE44 also inherited from his mother a rare genetic variant of MCP (A353V) that has been associated with reduced complement regulatory activity [5].

**Family RCO.** This family was previously described [6]. The proband, patient HUS84, and patient HUS68 are first cousins with a history of recurrent aHUS that lead to ESRF. They had normal plasma levels of C3, C4 and CFH while CFI plasma levels were low/half normal. The patients carry
a heterozygous mutation in MCP (P165S in SCR3) and a c.1610insAT in CFI that generates a truncated protein (T538X). Three unaffected family members were found to carry both mutations, while 2 carry the CFI mutation and 2 the MCP mutation alone.

**Family FRE60.** The proband, patient FRE60, is a 11 year-old girl who had three recurrences of aHUS at age 2, 7 and 8 years. She regained normal renal function after each flare. C3, C4, CFH and CFI levels were normal. She inherited from her healthy mother the heterozygous P50A mutation in CFI and the heterozygous R103W MCP mutation from her affected father, who carries this mutation in homozygosity. The father manifested aHUS during childhood, he received plasma and recovered without sequelae.

**Family HUS143.** The proband, patient HUS143, developed pregnancy-associated aHUS at 27 years of age; she was treated with plasma obtaining partial remission. In 2009, a younger sister of the proband also developed aHUS.

Biochemical evaluations in the proband showed low C3 and CFH levels, but normal CFI levels. Genetic analysis revealed the presence of two heterozygous mutations: a frameshift in CFH (T30Nfs10X in the SCR1) and a substitution in MCP (I208Y in the SCR3), the latter identified also in the healthy child.
References


Legend to Supplementary Figures

Supplementary Figure 1.
Pedigrees of patients with familial (panel A) and sporadic (panel B) aHUS. The genotyped affected subjects are evidenced by their specific code and highlighted with bold circles (females) or bold squares (males) (n=31; 4 with mutations in a single gene: FRE60F, with a homozygous mutation in MCP, #265F868, with a CFH mutation, #176F617, with a CFH/CFHR1 hybrid gene, and FRE44F with a C3 mutation; 27 with combined gene mutations). Diamond symbols are used when sex is unknown. The black arrows indicate the proband in each pedigree. The geographical origin of each pedigree is indicated at the upper left corner. Mutations in CFH are reported in red boxes, mutations in MCP are reported in yellow boxes, mutations in CFI are reported in green boxes, mutations in C3 are reported in blue boxes, and the mutation in CFB is reported in grey box. Genotypes of SNPs targeting the CFH-H3 risk haplotype (rs3753394, c.1-332C>T and rs1065489, c.2808G>T, p.E936D) and of SNP targeting the MCPggaac risk haplotype in MCP (rs7144, c.*897T>C) are marked in red.

n.a.=DNA not available; n.m.=no mutation. * Pedigree included in the calculation of penetrance.

Supplementary Figure 2.
HUS penetrance in subjects carrying two gene mutations in different gene combinations.
The percentage of affected subjects among carriers have been calculated considering only pedigrees in which at least two subjects have been screened. In the bottom of the X-axis, the number of subjects with two mutations in each gene combination have been reported.
**Supplementary Table 1.** Characteristics of patients with combined mutations receiving isolated kidney transplants.

<table>
<thead>
<tr>
<th>Code</th>
<th>Date of Tx</th>
<th>Mutations</th>
<th>Genetic screening pre-Tx</th>
<th>Plasma prophylaxis</th>
<th>Calcineurin inhibitors</th>
<th>Eculizumab treatment</th>
<th>Outcome at 3 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>#130F582</td>
<td>2004</td>
<td>CFH/MCP</td>
<td>no</td>
<td>no</td>
<td>n.a.</td>
<td>no</td>
<td>good</td>
</tr>
<tr>
<td>FRA15</td>
<td>1st (1990)</td>
<td>CFH/MCP</td>
<td>no</td>
<td>no</td>
<td>yes (CsA)</td>
<td>no</td>
<td>Lost (CR)</td>
</tr>
<tr>
<td></td>
<td>2nd (1996)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lost (Rec)</td>
</tr>
<tr>
<td></td>
<td>3rd (2005)</td>
<td></td>
<td></td>
<td></td>
<td>yes (Tac)</td>
<td>no</td>
<td>good</td>
</tr>
<tr>
<td>HUS186</td>
<td>2008</td>
<td>CFH/MCP</td>
<td>no</td>
<td>no</td>
<td>yes (Tac)</td>
<td>no</td>
<td>Lost (Rec)</td>
</tr>
<tr>
<td>FRE06</td>
<td>1998</td>
<td>CFH/CFI</td>
<td>no</td>
<td>no</td>
<td>yes (CsA)</td>
<td>no</td>
<td>good</td>
</tr>
<tr>
<td>HUS207</td>
<td>2002</td>
<td>CFH/CFI</td>
<td>no</td>
<td>no</td>
<td>yes (Tac)</td>
<td>no</td>
<td>Lost (Rec)</td>
</tr>
<tr>
<td>GUIHUS62</td>
<td>2008</td>
<td>MCP/CFI</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>good</td>
</tr>
<tr>
<td>FRA106</td>
<td>1st (1989)</td>
<td>MCP/CFI</td>
<td>no</td>
<td>no</td>
<td>n.a.</td>
<td>no</td>
<td>Lost (Rec)</td>
</tr>
<tr>
<td></td>
<td>2nd (1993)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lost (Rec)</td>
</tr>
<tr>
<td></td>
<td>3rd (2006)</td>
<td></td>
<td></td>
<td></td>
<td>yes (Tac)</td>
<td>no</td>
<td>Lost (I)</td>
</tr>
<tr>
<td>HUS167</td>
<td>2008</td>
<td>MCP/CFI</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>good</td>
</tr>
<tr>
<td>S657</td>
<td>2007</td>
<td>MCP/CFI</td>
<td>yes</td>
<td>yes</td>
<td>yes (CsA, Tac)</td>
<td>no</td>
<td>good</td>
</tr>
<tr>
<td>FRA13</td>
<td>2002</td>
<td>CFH/MCP/CFI</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>good</td>
</tr>
</tbody>
</table>

Graft lost for disease recurrence, rejection or other causes.
Calcineurin inhibitors: Cyclosporine (CsA), Tacrolimus (Tac).
n.a.: not available information on whether or not the patient got the drug, CR: chronic rejection, Rec: recurrence, I: viral infection
Supplementary Figure 1, Panel A

#130 (USA)*
- GI194D
  - F242C
  - n.a.
  - n.a.
- #024 (Italy)*
  - G1194D
  - F242C
- (France)*
  - R1210C
    - F868
  - Y29X
  - F582
- #265 (USA)*
  - S1191L
    - E554V

\( rs3753394:^C \)
\( rs1065489:^G \)
\( rs7144:^C \)
\( rs3753394:^C \)
\( rs1065489:^G \)
\( rs7144:^C \)
\( rs3753394:^C \)
\( rs1065489:^G \)
\( rs7144:^C \)
\( rs3753394:^C \)
\( rs1065489:^G \)
\( rs7144:^C \)
\( rs3753394:^C \)
\( rs1065489:^G \)
\( rs7144:^C \)
\( rs3753394:^C \)
\( rs1065489:^G \)
\( rs7144:^C \)
\( rs3753394:^C \)
\( rs1065489:^G \)
\( rs7144:^C \)
\( rs3753394:^C \)
\( rs1065489:^G \)
\( rs7144:^C \)
\( rs3753394:^C \)
\( rs1065489:^G \)
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\( rs3753394:^C \)
\( rs1065489:^G \)
\( rs7144:^C \)
\( rs3753394:^C \)
\( rs1065489:^G \)
\( rs7144:^C \)
\( rs3753394:^C \)
\( rs1065489:^G \)
\( rs7144:^C \)

^ Obligated carrier based on inheritance
Supplementary Figure 2.

![Bar chart showing penetrance percentages for different genotypes.](image)