Hypocomplementemia Discloses Genetic Predisposition to Hemolytic Uremic Syndrome and Thrombotic Thrombocytopenic Purpura: Role of Factor H Abnormalities

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Abstract. Familial hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) carry a very poor outcome and have been reported in association with decreased serum levels of the third complement component (C3). Uncontrolled consumption in the microcirculation, possibly related to genetically determined deficiency in factor H—a modulator of the alternative pathway of complement activation—may account for decreased C3 serum levels even during disease remission and may predispose to intravascular thrombosis. In a case-control study by multivariate analysis, we correlated putative predisposing conditions, including low C3 serum levels, with history of disease in 15 cases reporting one or more episodes of familial HUS and TTP, in 25 age- and gender-matched healthy controls and in 63 case-relatives and 56 control-relatives, respectively. The relationship between history of disease, low C3, and factor H abnormalities was investigated in all affected families and in 17 controls. Seventy-three percent of cases compared with 16% of controls (P < 0.001), and 24% of case-relatives compared with 5% of control-relatives (P = 0.005) had decreased C3 serum levels. At multivariate analysis, C3 serum level was the only parameter associated with the disease within affected families (P = 0.02) and in the overall study population (P = 0.01). Thus, subjects with decreased C3 serum levels had a relative risk of HUS or TTP of 16.56 (95% confidence interval [CI], 1.66 to 162.39) within families and of 27.77 (95% CI, 2.44 to 314.19) in the overall population, compared to subjects with normal serum levels. Factor H abnormalities were found in four of the cases, compared with three of the healthy family members (P = 0.02) and none of the controls (P = 0.04) and, within families, factor H abnormalities were correlated with C3 reduction (P < 0.05). Reduced C3 clusters in familial HUS and TTP is likely related to a genetically determined deficiency in factor H and may predispose to the disease. Its demonstration may help identify subjects at risk in affected families.

Hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) are syndromes of microangiopathic hemolytic anemia and thrombocytopenia, which have in common thrombotic occlusion of the microvasculature of various organs (1). The term HUS is usually preferred to describe the disease in children with renal insufficiency (2), whereas TTP is the most used term to describe adult cases with predominant neurologic symptoms (3). However, it is now recognized that the two syndromes may have different clinical manifestations because of the different distribution of the microvascular lesions, but share the same histologic lesion—widening of the subendothelial space and intravascular platelet thrombi—and reflect a similar pathophysiologic process, leading to thrombocytopenia and anemia through platelet consumption and erythrocyte disruption in the injured microvasculature (4). In their typical presentation, HUS and TTP manifest as an acute disease that recovers without sequelae in 80 to 90% of cases, either spontaneously (as in most cases of childhood HUS) or following a course of plasma infusion or exchange (as in adult or severe forms of HUS and in TTP) (1–3). These forms may be triggered by environmental factors (as the verotoxins produced by some strains of Escherichia coli), drugs, or other diseases and may subside when the underlying condition has been treated or removed. Other forms, however, fail to recover or may relapse after complete recovery of the presenting epi-
sode (1,5), with death or permanent neurologic or renal sequelae being the final outcome in the large majority of cases. In these “atypical” forms, an exogenous triggering factor is seldom recognized, and an underlying, genetically determined condition predisposing to the disease is hypothesized in most cases (6,7). Along these lines, an increased prevalence of HLA B40 antigens in a series of HUS cases has been taken to suggest that the HLA B40 genotype may be linked to “susceptibility genes” predisposing to the disease, possibly after exposure to exogenous precipitating factors (8).

Over the past 20 yr, about 140 cases of familial HUS and TTP have been described in 70 families with the predominant features of HUS in two-thirds of patients (5). Both autosomal recessive and autosomal dominant mode of inheritance have been recognized (5,6,9–12), with precipitating events such as pregnancy, virus-like disease, or sepsis being reported only in a minority of cases (13,14). Evidence that some of these cases responded, at least transiently, to plasma infusion or exchange led to the hypothesis that the genetic defect(s) associated with familial HUS/TTP may result in abnormalities in some plasma component(s) involved in the pathogenesis of the microangiopathic process. Thus, reduced serum levels of the third component (C3) of the complement system have been reported since 1974 in sporadic (15–18) and familial (19–21) forms of HUS. An inherited defect in C3 synthesis has been suggested to account for decreased C3 serum concentration (22), but much more convincing data are now available that low C3 in HUS may derive from either the lack of (21,23) or altered function of (24) factor H. In a very recent study, Warwicker and coworkers provided molecular evidence of the involvement of factor H in HUS (24). Indeed, they found that in three families with HUS, an area on chromosome 1q where factor H is mapped segregates with the disease. A deficiency in factor H—a plasma protein that inhibits the formation and accelerates the decay of the alternative pathway enzyme (C3bBb) of complement activation (25–27)—may accelerate C3 tissue deposition and consumption (28). On the other hand, epidemiologic evidence of an association between decreased factor H and C3 bioavailability and familial HUS is missing. Furthermore, no information on the prevalence and on the predisposing role of factor H and C3 deficiency in familial forms presenting with the clinical signs of TTP is available so far.

Thus, the aim of present study was to formally investigate in a case-control design the relationship between C3 deficiency and familial cases of either HUS and TTP and to assess whether C3 defect may be related to an impaired factor H bioavailability.

**Materials and Methods**

**Patients and Definitions**

Thirty-five cases of familial HUS/TTP were identified among 10 families through the database of the Italian Registry of Recurrent and Familial HUS/TTP, a network of 50 units of Hematology and Nephrology, established on 1995, under the coordination of the Clinical Research Center for Rare Diseases “Aldo & Cele Daccò” (Ranica, Italy). The following criteria were given to guide patient selection, based on the database of the Registry, 16 (9 males, 7 females) were alive at the time of the study and 19 had died because of the disease. The 16 alive patients came from nine families, and both affected subjects in the remaining family (family 15) had died before our investigation. All relevant information in all of the 35 identified cases were recorded through the database of the Registry and by reviewing the patients’ charts. All 16 patients who were alive at the time of the study and all of their available relatives (n = 63, 27 males, 36 females) were given detailed information about the purpose and design of the project and provided informed consent to enter the study according to the Declaration of Helsinki guidelines. Patients and their relatives were therefore referred to the Clinical Research Center where a detailed history was recorded and a blood sample was collected from all subjects and processed for the laboratory tests listed below. For the purposes of the study, all of the subjects who had one or more episodes of the disease were referred as “cases” and their family members who never had the disease as “case-relatives.” All cases except one were studied at disease remission. The case with clinical signs of active disease was not included in the data analysis.

On each occasion, a case and his or her case-relatives were referred to the Clinical Research Center, and one or two age- and gender-matched “controls” (n = 25, 11 males, 14 females) and all of their available relatives (referred to as “control-relatives”; n = 56, 29 males, 27 females) were simultaneously studied with their blood samples collected and processed under the same experimental conditions described for cases and their relatives.

**Diagnosis of HUS and TTP.** HUS or TTP was diagnosed in all cases reported to have one or more episodes of microangiopathic hemolytic anemia and thrombocytopenia defined on the basis of hematuria (Ht) <30%, hemoglobin (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/μl.

**Differential Diagnosis between HUS and TTP.** Because of their frequently overlapping clinical and laboratory features, differential diagnosis between HUS and TTP is often uncertain and controversial. For the purposes of the study, the prevalence of signs of renal or neurologic involvement was taken as criteria to differentiate HUS and TTP, respectively (1,3,4). Cases presenting with the features of either HUS or TTP in different episodes in the same patients or in different patients within the same family were defined as HUS/TTP.

**Diagnosis of Familial HUS and TTP.** HUS and TTP were defined as familial when at least two members of the same family were affected by the disease at least 6 mo apart, and exposure to a common environmental triggering agent (in particular a verotoxin-producing strain of Escherichia coli) could be reasonably excluded.

**Diagnosis of Relapsing HUS and TTP.** Relapsing HUS or TTP was diagnosed when one or more episodes of the disease were diagnosed in the same subject after complete and persistent (for at least 2 wk off any kind of specific therapy, in particular plasma infusion or exchange) remission of any sign of microangiopathic hemolysis.

**Diagnosis of Hypocomplementemia.** Plasma levels of the third and/or fourth (C4) complement component below the lower limit of normal ranges (defined as mean ± 2 SD) of the laboratories of the “Ospedali Riuniti, Azienda Ospedaliera di Bergamo” (i.e., <63 mg/dl for C3 and <15 mg/dl for C4) were taken to indicate hypocomplementemia.

**Study Design**

Of the 35 cases identified in 10 families through the database of the Registry, 16 (9 males, 7 females) were alive at the time of the study and 19 had died because of the disease. The 16 alive patients came from nine families, and both affected subjects in the remaining family (family 15) had died before our investigation. All relevant information in all of the 35 identified cases were recorded through the database of the Registry and by reviewing the patients’ charts. All 16 patients who were alive at the time of the study and all of their available relatives (n = 63, 27 males, 36 females) were given detailed information about the purpose and design of the project and provided informed consent to enter the study according to the Declaration of Helsinki guidelines. Patients and their relatives were therefore referred to the Clinical Research Center where a detailed history was recorded and a blood sample was collected from all subjects and processed for the laboratory tests listed below. For the purposes of the study, all of the subjects who had one or more episodes of the disease were referred as “cases” and their family members who never had the disease as “case-relatives.” All cases except one were studied at disease remission. The case with clinical signs of active disease was not included in the data analysis.

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Laboratory Evaluations

In addition to the third and fourth complement fractions, markers of disease activity (i.e., serum LDH, haptoglobin concentration, and platelet count), renal dysfunction (i.e., serum creatinine and urea concentration), and concomitant systemic diseases (including antinuclear antibodies, lupus anticoagulant, antiphospholipid antibodies, serum cryoglobulin concentration) and routine biochemicals (complete blood cell count, hematocrit, hemoglobin concentration, serum proteins and electrolytes, uric acid, blood glucose, serum lipids, and urinalysis) were evaluated in all cases and controls referred to the Clinical Research Center. Factor H and its different isoforms were also evaluated in all affected families and in 17 controls. The biologic samples from all cases, case-controls, and corresponding case- and control-relatives were collected on the same occasion and processed in the same experimental setting.

C3 and C4 were quantified by kinetic nephelometric measurements. Anticardiolipin antibodies titer was quantified by an enzyme-linked immunosorbent assay method. Lupus anticoagulant was measured by the Russell’s viper venom test and kaolin clotting time test. Factor H was quantified by a radial immunodiffusion assay using a sheep polyclonal antihuman factor H antiserum (The Binding Site, Birmingham, United Kingdom). In addition to factor H, a factor H-like protein (FHL-1), and various shorter proteins, factor H-related proteins (FHR) have been identified in human plasma (25,29). FHL-1 is derived from the factor H gene by alternative splicing and displays complement regulatory activities (25). FHR, which differ considerably from factor H, are likely transcribed from distinct genes and have none of the known activities of factor H (25). To search for possible qualitative and quantitative defects of the different factor H molecules, Western blot analysis of factor H, factor H-like, and factor H-related proteins in serum samples was also performed. Briefly, serum (1.5 µl) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Laemmli (30), using prestained bench markers (Life Technologies) as standards. Proteins were electroblotted to nitrocellulose by semidy blotting (31). Membranes were blocked for 30 min using 5% (wt/vol) dried milk in phosphate-buffered saline (PBS). Incubations with polyclonal goat anti-factor H antiserum (Calbiochem, diluted 1:1000) or anti-FHL-1 (rabbit anti-SCR1–4 antiserum, which does not detect FHR-1 and FHR-2 proteins, dilution 1:1000) (29) were performed at 4°C overnight. After washing in PBS 5 times, membranes were incubated with peroxidase-conjugated rabbit anti-goat or swine anti-rabbit antibody (Dako, Hamburg, Germany), respectively, for 2 to 3 h. Protein bands were visualized by the addition of 0.3% (wt/vol) 4-chloro-1-naphtol in 10% (vol/vol) methanol in PBS. In family 24, which showed abnormal high molecular weight bands that reacted with anti-factor H antiserum, densitometric analysis was performed with a computer-based digital imaging processing. Autoradiograph of the gel was acquired using a digitizing board. High molecular weight bands (Figure 6A, black arrow) and normal factor H band (open arrow) were resolved into a series of peaks and areas under the peaks calculated by specific function of the software Image 1.60 (National Institutes of Health Bethesda, MD). The corresponding areas were computed, and the ratio of high molecular weight band/normal factor H band was calculated.

To calculate cofactor activity of factor H, in families 24 and 29, human C3b (200 µg; Advanced Research Technologies, San Diego, CA) was labeled with 125I using the iodogen technique (32). The cofactor assay was performed as described (29). Briefly, serum was diluted 1:200 in Veronal-buffered saline, radiolabeled C3b was added, and the mixture was incubated at 37°C for 2 h. Serum from the individual patients, from the healthy family members, and from normal control subjects were treated identically. This treatment results in cleavage of the 112-kD α-chain of C3b into a 69-kD and a 43-kD fragment, leaving the 75-kD β-chain unaffected. After incubation, the mixture was reduced and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, gels were fixed for 10 min in 10% acetic acid, dried, and autoradiographed. Human 125I C3b was used as a control. The cofactor activity of the samples was quantified by counting the radioactivity of the proteolytic α-43 band and normalizing to the radioactivity of the β-chain of the sample. All of the other tests were performed by routine laboratory procedures.

Statistical Analyses

Personal, clinical, and laboratory data were recorded on a uniform data extraction form (Registration Form). Database management was performed using FileMakerPro software, version 2.1 (Claris Corp., Santa Clara, CA). Dichotomous baseline characteristics were compared by χ2 test or Fisher exact test, as appropriate, and continuous baseline characteristics by Wilcoxon rank sum test. Univariate and multivariate analyses were carried out by logistic regression model. Correlation analysis between continuous variables and dichotomous variables was carried out using point-biserial correlation coefficient (rb) (33). Statistical significance was set at 0.05 (two-tailed).

Results

Distribution and outcome of the different forms of the disease in all 35 cases identified through the database of the Registry are summarized in Table 1. Of note, one-third of cases were adults with TTP, HUS, or HUS/TTP on different occasions. All children had HUS. One or more disease relapses were reported in 18 (51%) cases. Nineteen patients (one with TTP and 18 with HUS) had died before the study was conducted (54.3%); of these, one child died after receiving a kidney transplant. Among the 16 survivors, six were on chronic dialysis (37.5%). Of these, two had received a kidney transplant that failed because of disease recurrence. Two had neurologic sequelae. Conditions potentially predisposing to disease onset were recognized in 19 cases (54.3%), including an upper respiratory tract infection (n = 14), pregnancy (n = 4), and consumption of birth control pills (n = 1).

Consanguinity was observed in two families. In one family (family 5), the parents were first cousins and had six children,

<table>
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<tr>
<th>Category</th>
<th>HUS</th>
<th>HUS/TTP</th>
<th>TTP</th>
<th>Total</th>
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<tbody>
<tr>
<td>Families</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Patients</td>
<td>29</td>
<td>4</td>
<td>2</td>
<td>35</td>
</tr>
<tr>
<td>adults</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>children</td>
<td>23</td>
<td>0</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>Relapses</td>
<td>14</td>
<td>4</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>Deaths</td>
<td>18</td>
<td>0</td>
<td>1</td>
<td>19</td>
</tr>
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</table>

*HUS, hemolytic uremic syndrome; TTP, thrombotic thrombocytopenic purpura.
four of whom developed HUS during infancy (three of them died from acute renal failure, and the fourth had several recurrences and is now well at the age of 5 yr). In the other family (family 29, \( n = 10 \), from Israel) described in detail elsewhere (20), composed of offspring of several interrelated families with a high rate of consanguinity, 10 infants manifested HUS early in life and eight died. The two survivors suffered several recurrences until they entered chronic dialysis. Pedigrees of the 10 families are shown in Figures 1, 3, and 5. Pattern of inheritance of HUS and TTP could not be unequivocally deduced because all families had only one generation affected. In family 4, two cases of death for acute renal failure were reported, without a definitive diagnosis. In the same family, history of renal disease was reported in two other relatives, again without specific diagnosis. These four subjects are indicated as probably affected in Figure 1.

At the time of study evaluation, one of the 16 alive patients had clinical signs of disease activity and was not included in final analysis. The latter patient, with HUS, was from family 19, and his brother with diagnosis of TTP, in remission, was included in the study. Two other patients, who presented only mild and isolated increases in serum LDH concentration with-

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**Figure 1.** Pedigree of families 1, 2, 3, 4, 5, 6, 15, and 19 (e.g., family 1 is indicated as #01). Individuals with low C3 serum concentrations are identified with an asterisk.
out any other laboratory or clinical signs of disease activity, were considered in remission and were included in the analysis. Thus, 15 patients (cases) entered the study. All of these patients were in stable remission, the time from the last clinical episode ranging from 5 mo to 15 yr. Main parameters in cases, matched controls, and case- and control-relatives referred to the Clinical Research Center are given in Table 2. By the Wilks–Shapiro test, all variables were found to be normally distributed, with the exception of serum creatinine, LDH, and C3 levels, only in cases. Median (range) values of these parameters in cases were as follows: serum creatinine: 2 (0.7 to 10.5) mg/dl; LDH: 385 (226 to 883) U/L; C3: 68 (10 to 99) mg/dl.

Seventy-three percent of cases compared with 16% of controls ($P < 0.001$), and 24% of case-relatives compared with 5% of control-relatives ($P = 0.005$) had decreased C3 serum levels (Figure 2). When families were analyzed separately (see pedigrees in Figures 1, 3A, and 5A), we found that in five of the nine families (families 1, 3, 6, 24, and 29) all cases had low C3 levels. In families 2 and 4, low C3 was found in one of two and in two of three cases, respectively. Of interest in family 2, a high incidence of low C3 was also observed in case-relatives, suggesting that low C3 strongly segregates to this family. In family 5, the only evaluable case (F64) had normal C3 serum concentration, whereas low C3 was found in his father. In only one family (family 19), C3 serum concentration was normal both in the case (F102) and in case-relatives (Figure 1). Mean serum C3 concentration was significantly lower in cases compared with all of the other study groups (Table 2). Cases also had significantly lower hemoglobin and hematocrit, and higher serum creatinine and LDH compared with controls. Platelet count, serum C4 (Table 2 and Figure 2), anticardiolipin antibodies, lupus anticoagulant, as well as all of the markers of systemic disease not reported in the table, were within the normal ranges in all subgroups. Of note, no significant correlation could be demonstrated between serum C3 concentration and serum LDH levels either in affected families or in the overall study population. Actually, in case F64 with the highest LDH value, serum C3 concentration was normal (LDH: 883 U/L, C3: 84 mg/dl), whereas the other case, F34, with higher than normal LDH, had low C3 serum concentration (LDH: 520 U/L, C3: 69 mg/dl).

![Figure 2. Prevalence of low C3 and C4 concentrations among cases (n = 15), controls (n = 25), case-relatives (n = 63), and control-relatives (n = 56) referred to the Clinical Research Center “Aldo e Cele Daccò.”](image)

### Table 2. Main clinical and laboratory parameters in all subjects referred to the Clinical Research Center “Aldo e Cele Daccò”

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cases (n = 15)</th>
<th>Controls (n = 25)</th>
<th>Case-Relatives (n = 63)</th>
<th>Control-Relatives (n = 56)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>26.6 ± 17.9$^b$</td>
<td>34.7 ± 13.5$^b$</td>
<td>36.9 ± 20.9$^b$</td>
<td>45.8 ± 16.5</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>8/7</td>
<td>11/14</td>
<td>27/36</td>
<td>29/27</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>3.8 ± 3.9$^c$</td>
<td>0.9 ± 0.1</td>
<td>0.8 ± 0.2</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>Hemoglobin (mg/dl)</td>
<td>11.4 ± 2.5$^c$</td>
<td>14.0 ± 1.1</td>
<td>13.7 ± 1.3</td>
<td>14.2 ± 1.5</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>34.3 ± 7.0$^c$</td>
<td>42.3 ± 3.5</td>
<td>40.6 ± 3.2$^b$</td>
<td>42.4 ± 4.2</td>
</tr>
<tr>
<td>Platelet count (10$^3$/μl)</td>
<td>221 ± 60</td>
<td>220 ± 51$^d$</td>
<td>251 ± 70$^b$</td>
<td>213 ± 49</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>384.5 ± 180.6</td>
<td>277.4 ± 57.2$^d$</td>
<td>340.0 ± 90.5$^b$</td>
<td>297.8 ± 57.9</td>
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<tr>
<td>ACA (U/ml)</td>
<td>9.7 ± 6.9</td>
<td>9.9 ± 5.8</td>
<td>10.1 ± 8.1</td>
<td>11.6 ± 6.9</td>
</tr>
<tr>
<td>C3 (mg/dl)</td>
<td>67.0 ± 26.7$^c$</td>
<td>97.0 ± 19.5</td>
<td>100.4 ± 25.4</td>
<td>100.2 ± 18.0</td>
</tr>
<tr>
<td>C4 (mg/dl)</td>
<td>25.7 ± 11.8</td>
<td>21.9 ± 6.8</td>
<td>25.3 ± 9.5$^b$</td>
<td>22.1 ± 7.2</td>
</tr>
</tbody>
</table>

$^a$ Data are mean ± SD. LDH, lactate dehydrogenase; ACA, anticardiolipin antibodies.

$^b$ $P < 0.05$ versus control-relatives.

$^c$ $P < 0.05$ versus all of the others.

$^d$ $P < 0.05$ versus case-relatives.

$^e$ $P < 0.0005$ versus all of the others.
Table 3. Univariate correlation analysis between main clinical and laboratory parameters and familial HUS and TTP in the affected families and in the overall study populationa

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Affected Families</th>
<th>All Subjects</th>
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<tbody>
<tr>
<td>Age (yr)</td>
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<td>Gender (M/F)</td>
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<td>Serum creatinine (mg/dl)</td>
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<td>0.003</td>
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<td>Hemoglobin (mg/dl)</td>
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<td>Hematocrit (%)</td>
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<td>Platelet count (10^3/μl)</td>
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<td>C4 (mg/dl)</td>
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a Abbreviations as in Tables 1 and 2.

By univariate analysis (Table 3), lower levels of C3, hemoglobin, and hematocrit, and higher levels of serum creatinine were significantly associated with HUS or TTP either when the analysis was performed among affected families or in the overall study population. In the overall study population, a significant association was also found with age and LDH. Multivariate logistic analysis accounting for the above risk factors showed that only C3 levels were significantly associated with HUS or TTP either among the affected families (P = 0.02) or the overall study population (P = 0.01). The relative risk of HUS or TTP associated with low C3 levels (C3 < 83 mg/dl) among the affected families and in the overall study population is shown in Table 4.

In all cases (n = 4) in whom repeated C3 measurements in disease remission were available, C3 levels were persistently lower than normal. Factor H concentration, measured by radial immunodiffusion, was abnormally low in one family with history of HUS (family 29) and high degree consanguinity. Factor H was severely depressed in the two affected subjects (F106 and F108) with respect to normal factor H band. In patients (F106: 0.42; F108: 0.19) and the healthy father (F104: 0.29) than in control subject (0.08). In the two cases, an additional band was observed with a ratio of 0.2 (F106) and 0.08 (F108) with respect to normal factor H band.

In Figure 6B, bands corresponding to the low molecular mass members of the factor H family, FHR-1α (37 kD) and FHR-1β (42 kD), are presented. Since FHL-1 band is masked by FHR-1β (they comigrate on Western blot) (25), FHL-1 pattern was analyzed with additional Western blots performed using a specific antibody anti-FHL-1 (not shown). For all proteins, similar expression levels and identical mobilities were identified in the serum of family 24 (Figure 6B) and family 29 (data not shown). No abnormalities in factor H and factor H-related proteins were found by Western blot analysis of sera from the other families.

Altogether, factor H abnormalities (either in levels or Western blot pattern) were found in four of the 15 cases, compared with three of the 63 family members (P = 0.02) and none of

Table 4. Relative risk (95% confidence interval) of familial HUS and TTP associated with low C3 concentration within affected families and within the overall studied subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Univariate Analysis</th>
<th>Multivariate Analysis</th>
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<td>Relative risk</td>
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<td>14.35</td>
</tr>
<tr>
<td>95% CI</td>
<td>2.39 to 31.10</td>
<td>4.20 to 49.00</td>
</tr>
<tr>
<td>P value</td>
<td>0.001</td>
<td>0.001</td>
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</table>

a CI, confidence interval.
the 17 healthy controls ($P = 0.04$). All cases with factor H abnormalities had low C3 serum concentration. Overall, factor H abnormalities were more frequent in the 15 cases than in the 80 subjects who never had the disease ($P < 0.02$). Within families, factor H abnormalities were significantly correlated with C3 reduction ($r_{pb} = -0.25; P < 0.05$).

To further confirm the abnormalities of factor H in families 24 and 29 on a functional level, the cofactor activity of the case sera was compared with that of healthy relatives (Figure 7). For family 29, both cases showed a marked reduction in serum cofactor activity, as evidenced by the low intensity of the proteolytically cleaved $\alpha$-chains ($\alpha$-43). Under identical conditions, sera from both cases were rather inefficient in conversion of the C3b $\alpha$-chain (Figure 7A). Serial dilution and densitometric analysis (data not shown) of the newly formed $\alpha$-43 band showed that in both cases cofactor activity was approximately 10% of the values found in control sera. The rather low cofactor activity detected in the two cases of family

Figure 3. (A) Pedigree of family 29. Open symbols, unaffected to date; closed symbols, affected; *, low C3 levels; F (followed by a number), code of studied subjects. (B) Serum complement profile in family 29.

Figure 4. Western blot analysis of factor H in human serum (family 29). Sera were separated by 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the Western blot was developed using a polyclonal goat anti-factor H antiserum. Open arrow indicates factor H. F29, case (with inactive HUS); F73, F29 father; F79, F29 mother; F84, F29 sister (healthy); F85, case (with inactive HUS); F86, F85 mother; F87, F85 father. F86, F87, and F73 are first cousins.
Figure 5. (A) Pedigree of family 24. Symbols as in Figure 3. (B) Serum complement profile in family 24.

<table>
<thead>
<tr>
<th></th>
<th>F104</th>
<th>F105</th>
<th>F107</th>
<th>F106</th>
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<td>C3 (mg/dl)</td>
<td>130</td>
<td>97</td>
<td>96</td>
<td>61</td>
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<tr>
<td>C4 (mg/dl)</td>
<td>32</td>
<td>24</td>
<td>27</td>
<td>16</td>
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<td>Factor H (mg/L)</td>
<td>835</td>
<td>546</td>
<td>562</td>
<td>711</td>
<td>569</td>
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</tbody>
</table>

(normal range)
83–177
15–45
(367–619)

Figure 6. Western blot analysis of factor H and factor H-related proteins in human serum (family 24). Sera were separated by either 7% (A) or 10% (B) SDS-PAGE, and the Western blot was developed using a polyclonal goat anti-factor H antiserum. (A) F106 and F108, cases (with inactive HUS, brothers); F104, father; F105, mother; F106, sister. Open arrow indicates factor H. Closed arrow and circle indicate the abnormal high molecular weight bands. (B) 1 = factor H; 2 = factor H-related protein-1β; 3 = factor H-related protein-1α.
The first finding of the present study was that approximately 75% of the 35 patients with familial HUS or TTP referred to the Registry died or had permanent neurologic or renal sequelae. Of note, the outcome was extremely poor also in the 29 patients presenting with the clinical features of HUS, of which 18 died at the time of the study. These data are in line with previous series (5,6) and provide further evidence of the extremely poor prognosis of this disease, which, regardless of the clinical presentation (i.e., as HUS or TTP), is virtually unaffected by any form of treatment, including plasma manipulation, proven effective in most nonfamilial cases. Of interest, in two families the disease presented either as HUS or TTP, which, in line with previous reports (5,34,35), provides clear evidence of the close relationship between the two syndromes.

That the disease may be related to an inherited congenital abnormality is consistent with the finding that more than 50% of cases in the present series—compared with only 10 to 20% in nonfamilial forms (3)—had repeated recurrences either of TTP or HUS. That this genetic abnormality involves the complement system is suggested by levels of circulating C3, which were extremely low in familial cases compared with controls. Reduced C3 levels in cases and case-relatives, but not in controls and control-relatives, further indicate that the defect clusters in families. Evidence that low C3 concentration is strongly associated with the disease even more convincingly suggests the possibility of a close (possibly causal) relationship between decreased C3 and disease manifestation. On the other hand, in the present series, low C3 levels could not derive from consumption in a still-ongoing microangiopathic process, because no patient at the time of the study had any sign of acute disease, and only two had moderately increased LDH levels. Furthermore, failure to detect any correlation between C3 and serum LDH levels definitely ruled out the possibility that low C3 concentration merely reflected disease activity in our series.

Actually, low C3 levels are well known to accompany the acute phases either of typical (epidemic) and atypical (sporadic) HUS (15–18), and likely reflect C3 consumption in the microvasculature. Along this line are findings of granular C3 deposits in glomeruli and arterioles of HUS patients (1,18,36,37) and evidence of C3 breakdown products in HUS sera (15,16,18), which further document the activation of the complement system in the acute phase of the disease. Enhanced release of complement cleavage products (including C3a and C5a) consequent to uncontrolled complement activation may contribute to the microangiopathic process by stimulating neutrophil activation (38), phagocytic adhesion to vascular endothelium, or platelet aggregation, and by directly injuring the endothelium through enhanced production of the membrane attack complex, the final multimolecular unit of the complement C5b-9 (39).

Two complement pathways can generate C3-activating enzymes: the classical convertase generated by the sequential reaction of C1, C4, and C2, and the alternative pathway convertase (40). Activation of classical and alternative complement pathway—possibly triggered by circulating immune complexes (41) and damaged erythrocytes (42), respectively—is well documented in acute HUS (16,18), but consistently subsides with remission of the disease. On the contrary, in our series, serum C3 levels were consistently and remarkably depressed in cases compared with controls, even during remission of the disease. Even more interesting, low C3 levels were also found in the relatives of our patients who had never suffered from HUS or TTP in the past and had no sign of the disease at the time of the study. Furthermore, either in cases or in case-relatives depressed C3 values were not paralleled by similar changes in C4 levels, which definitely ruled out the possibility that classical pathway activation accounted for hypocomplementemia in our series. Alternative explanations for reduced C3 bioavailability in familial HUS and TTP must therefore be provided.

Previous studies found low C3—but, notably normal C4—levels in occasional families affected by one or more cases of HUS (19–21,23), which at least in one case were found to
persist even during recovery from the disease (23). In this patient and in a healthy brother as well, low C3 levels were accompanied by very low levels of factor H, a regulatory protein that inhibits the complement activation through the alternative pathway. Finding that the parents, who were first cousins, had half-normal levels of factor H, convincingly indicated that the defect was inherited. Similar findings were then reported in another family (21). The above reports raised the intriguing possibility that low C3 in the setting of familial HUS may depend on an inherited deficiency of factor H. This possibility is in line with the evidence provided by Warwicker and coworkers in three large families with HUS, that an area on chromosome 1q, where factor H gene is mapped, segregates with the disease (24). All subjects in the three families had normal serum factor H levels; however, affected members and obligate carriers within one family were found, by mutation analysis, to have a point mutation in factor H causing an arginine to glycine change (24). This mutation is likely to alter structure and hence function of factor H protein without modifying its circulating levels. Another mutation comprising a deletion in factor H gene, which—through a frameshift and subsequent premature termination codon—led to a 50% reduction in serum factor H levels, has been described even in a subject with relapsing HUS (24).

By radial immunodiffusion and Western blot analysis, we identified in our series two affected subjects of one family who had very low circulating factor H levels, and moderately low levels were found in two healthy relatives. In these patients, cofactor activity of factor H, measured as the capacity to degrade C3b, was also reduced.

In the other families, serum factor H concentration was normal. However, finding normal serum levels does not necessarily exclude an underlying biochemical abnormality in circulating factor H, possibly related to mutations in the gene that leads to the synthesis of an abnormal protein (24). In this regard, in another family of our series, the two affected members, and the healthy father who had normal serum concentrations of factor H by radial immunodiffusion, showed on Western blot additional bands of higher molecular weight that were not found in any control. The nature of these bands is not clear, and molecules related to factor H with such large size having not yet been identified. If one were to guess, the bands may represent dimeric forms of factor H. At variance, no differences were found in serum levels and patterns of FHL-1 and FHR proteins (25). In vitro, serum from the two cases of this family showed normal factor H activity, which was consistent with normal factor H concentration found by radial immunodiffusion. Similar results have been obtained by Warwicker and coworkers in their three families (24). Actually, factor H has several biologic activities: (1) It prevents the formation of C3bBb complex and accelerates the dissociation of Bb from the C3 convertase. (2) It acts as a cofactor for factor I, which degrades C3b (25). (3) It distinguishes between activator and nonactivator surfaces (25). Other less well defined functions of factor H have been suggested by the presence in factor H protein of at least two heparin-binding sites that could facilitate interaction with extracellular matrix (25). Thus, it is hard to disclose by in vitro tests how a given molecular alteration of factor H actually affects its complex biologic activity in vivo.

Of note, a significant association was found in families between factor H abnormalities and low C3 levels, which supports the hypothesis that low C3 in the setting of familial HUS/TTP may depend on an inherited deficiency of factor H.

Genetic deficiency of factor H has been described in a relatively small number of families (24,26–28). Patients with homozygous factor H deficiency suffer recurrent bacterial infections (43), vasculitis, and/or glomerulonephritis (21,44). In a recent article (28), Ault and coworkers described a 13-mo-old boy who presented with hypocomplementemic hypertensive renal disease. Renal biopsy showed changes consistent with membranous proliferative glomerulonephritis and segmental C3 deposition in capillary loops. He had decreased serum levels of C3 and factor H was undetectable. Sequence analysis revealed two different point mutations in factor H gene, one on each allele.

It is also possible that genetic defects in other complement regulatory proteins (DAF, CR1, CR2, C4bp) might have a role in determining low C3 in familial HUS and TTP. Data (45) that the above genes map on the same region of chromosome 1q as factor H would support this hypothesis.

Although it seems clear that familial HUS and TTP are related to an inherited congenital defect, the cause of the syndrome is probably multifactorial and the inherited complement defects here documented may just represent a predisposing condition that increases the risk of the disease in combination with other intercurrent environmental or acquired factors. Thus, in a family with hereditary hypocomplementemia reported to our Registry (not shown), only one of the six members with low C3 levels developed HUS, apparently after a flulike disease.

But which is the sequence of events that may lead to disease manifestation in subjects with inherited congenital C3/factor H abnormalities? We suggest that an intercurrent exposure to agents potentially toxic to the vascular endothelium—such as certain viruses, bacteria, toxins, immunocomplexes, and cytotoxic drugs (1,2)—may initiate a local intravascular thrombosis, which promotes C3bBb convertase formation and complement deposition within capillary vessels (42,46,47). In normal conditions, however, factor H by modulating C3bBb activity (25–27) may effectively limit complement deposition and further extension of the process. On the contrary, when the bioavailability and/or the activity of factor H is congenitally defective, C3bBb convertase formation and complement deposition may become uncontrolled, with further extension of the microangiopathic process up to full manifestation of the disease.

Whatever the pathogenetic mechanism(s) accounting for decreased C3 levels in familial HUS and TTP, evidence that low C3 clusters in families affected by HUS and TTP and that, among family members, low versus normal C3 is associated with a more than 16 times greater risk of the disease, may have two major clinical implications. First, quantification of serum C3 concentration in cases at remission and in their relatives could be a useful tool to investigate the possibility of a familial
form of the disease. Second, when the diagnosis of familial HUS and TTP is established, serum C3 concentration could be quantified in all of the family members to identify the subjects at increased risk.

In conclusion, in the present study we found that an alteration of the third complement component clusters in families affected by familial HUS and TTP. Reduced C3 serum levels may reflect enhanced C3 consumption secondary to a genetically determined factor H deficiency and may predispose to microvascular thrombosis in familial HUS and TTP. Its demonstration may help identify subjects at risk within families who may benefit the most from genetic counseling and careful monitoring.

Appendix 1: Organization of the Italian Registry for Recurrent and Familial HUS/TTP

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Biochemical Studies

Statistical Analysis

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