Genetics of hemolytic uremic syndrome

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GENETICS OF HEMOLYTIC UREMIC SYNDROME

Marina Noris

GENETICS OF HEMOLYTIC UREMIC SYNDROME

PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Universiteit Maastricht,

op gezag van de Rector Magnificus,

Prof.mr. G.P.M.F. Mols,

Volgens het besluit van het College van Decanen,

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Introduction

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Disease of the month. Hemolytic uremic syndrome

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Definition

Hemolytic uremic syndrome (HUS) is a rare disease of microangiopathic hemolytic anemia, low platelet count and renal impairment due to platelet thrombi occluding the renal circulation [1]. Anemia is severe, non-immune (Coombs negative) and microangiopathic in nature, with fragmented red blood cells (schistocytes) in the peripheral smear, high serum lactate dehydrogenase (LDH), circulating free hemoglobin and reticulocytes. Platelet count is lower than $60,000/\text{mm}^3$ in most cases [1] and platelet survival time is reduced, reflecting enhanced platelet disruption in the circulation.

The common microvascular lesion of HUS, defined by the term thrombotic microangiopathy, consists of vessel wall thickening (mainly arterioles and capillaries) with endothelial swelling and detachment, which allows accumulation of proteins and cell debris in the subendothelial layer, creating a space between endothelial cells and the underlying basement membrane of affected microvessels [1, 2]. Both the widening of the subendothelial space and intraluminal platelet thrombi lead to a partial or complete obstruction of the vessel lumina. It is probably because of the partial occlusion of the lumen that erythrocytes are disrupted by mechanical trauma, which explains the Coombs-negative hemolysis and finding of fragmented and distorted erythrocytes in the blood smear. In children younger than 2 years of age the lesion is mainly confined to the glomerular tuft and is noted in an early phase of the disease. Examination of biopsies taken several months after the disease onset showed that most glomeruli were normal (an indication of the reversibility of the lesions), whereas 20% eventually became sclerotic [3, 4]. Arterial thrombosis do occur but is

uncommon and appears to be a proximal extension of the glomerular lesion [3, 4]. In adults and older children, glomerular changes are different and more heterogeneous than in infants. The classical pattern of thrombotic microangiopathy is less evident. In these patients glomeruli mainly show ischemic changes with retraction of glomerular tufts, expansion of the urinary space, and thickening and wrinkling of the capillary wall[5]. When the renal biopsy is performed in the early phase of the disease, changes may be reminiscent of those of younger children with typical thickening of the glomerular capillary wall and swelling of the endothelium. Thrombi, packed and fragmented red blood cells are observed in the capillary lumina. Occasionally, glomeruli have necrotic areas or segmental extracapillary proliferation. Late in the course of the disease, thickening of the glomerular basement membrane and intraglomerular thrombi are less frequent and the ischemic changes prevail [4].

In children with a predominantly glomerular pattern, arteriolar lesions are rather heterogeneous: mild arteriolar lesions with some vessels even not affected by the microangiopathy were reported in a study [6], but arteriolar thrombosis was frequently detected in other childhood series [7, 8] with similar glomerular pattern. In adults, changes in the arterioles and arteries are common with severe narrowing of the lumina due to expansion of the subendothelial space. Fibrin and platelet thrombi and myointimal proliferation also are frequent. Vascular lumina often are congested with sludged and fragmented red blood cells. [3, 4]. Red blood cells and fibrin also may infiltrate the walls of arteries. Intimal proliferation has been regarded as a response to intimal swelling and to the accumulation of red blood cells and fibrin. When specimens are examined in the late phase

of the disease, intimal thickening is replaced by dense intimal fibroplasias which leads to marked narrowing of the lumina.

Stx-associated HUS

In children the disease is most commonly triggered by certain strains of *E. coli* that produce powerful exotoxins, the Shiga-like toxins (Stx1 and Stx2) [9-13]. In 70% of cases in North America and Western Europe Stx-HUS is secondary to infection with the *E. coli* serotype O157:H7 [9-11, 14-17]. However, many other *E. coli* serotypes (O111:H8, O103:H2, O121, O145, O26, and O113 [9, 14, 18-20]), have been shown to cause Stx-HUS. The disease manifests with diarrhea (D⁺HUS), often bloody. Cases of Stx-*E. coli* HUS -around 25% [21]- which however do not present with diarrhea, have also been reported [2]. Acute renal failure manifests in 55%-70% of cases [22-24], however renal function recovers in most of them (up to 70% in various series) [1, 2, 24, 25].

Following exposure to Stx-*E. coli*, 38 to 61% of individuals develop hemorrhagic colitis and 3-9% (in sporadic infections) to 20% (in epidemic forms) progress to overt HUS [12, 23].

Humans become infected by Stx-producing *E. coli* from contaminated milk, meat and water -water-borne outbreaks have occurred as a result of drinking and swimming in unchlorinated water [18]- or from contact with infected animals, humans or either's excreta [12, 26, 27] and occasionally through environmental contamination [15].

The disease is characterized by prodromal diarrhea followed by acute renal failure. The average interval between *E. coli* exposure and illness is three days (range of 1 to 8). Illness

typically begins with abdominal cramps and non bloody diarrhea; diarrhea may become hemorrhagic in 70% of cases usually within one or two days [28]. Vomiting occurs in 30 to 60% of cases and fever in 30%. HUS is usually diagnosed six days after the onset of diarrhea [1]. After infection, *Stx-E.coli* may be shed in the stools for several weeks after the symptoms are resolved, particularly in children <5 years of age [1]. Diagnosis rests on detection of *Stx-E.coli* in stool cultures. Serologic tests for antibodies to *Stx* and O157 lipopolysaccharide can be done in research laboratories and tests are being developed for rapid detection of *E.coli* O157:H7 and *Stx* in stools.

Bloody diarrhea, fever, vomiting, elevated leukocyte count, extremes of age and female sex as well as the use of antimotility agents [29], have been associated with an increased risk of HUS following *E.coli* infection [12] .

Stx-HUS is not a benign disease. Seventy-percent of patients who develop HUS require red blood cell transfusions, 50% need dialysis, and 25% have neurological involvement, including stroke, seizure and coma [12, 24, 30]. Although mortality for infants and young children in industrialized countries decreased when dialysis became available, as well as after the introduction of intensive care facilities, still 3% to 5% of patients die during the acute phase of *Stx*-HUS [24]. A recent meta-analysis of 49 published studies (3476 patients, mean follow-up of 4.4 years) describing long-term prognosis of patients who survived an episode of *Stx*-HUS, reported death or permanent end-stage renal disease (ESRD) in 12% of patients and GFR below 80 ml/min/1.73m² in 25% [30]. The severity of acute illness, particularly central nervous system symptoms, and the need for initial dialysis were

strongly associated with a worse long-term prognosis [22, 30].

Non-Shiga toxin HUS

Non-Shiga toxin-associated HUS (non-Stx-HUS) comprises a heterogeneous group of patients in whom an infection by Stx-producing bacteria could be excluded as cause of the disease. It can be sporadic or familial (i.e. more than one member of a family affected by the disease and exposure to Stx-*E.coli* excluded). Collectively, non-Stx-HUS forms have a poor outcome. Up to 50% of cases progress to ESRD or have irreversible brain damage, and 25% may die during the acute phase of the disease [31-33].

Extrarenal manifestation, including neurologic complications, are not uncommon so that non-Stx-HUS is often clinically undistinguishable from thrombotic thrombocytopenic purpura (TTP), a related thrombotic microangiopathy characterized by microvascular occlusive thrombosis in the brain and other organs.

Non-Stx-HUS is less common than Stx-HUS, and accounts for only 5 to 10% of all cases of the disease [1, 34]. It may manifest at all ages, but is more frequent in adults. According to a recent US study the incidence of non-Stx-HUS in children is approximately one tenth that of Stx-HUS [33], corresponding to approximately 2 cases/year/1,000,000 total population. At variance with Stx-HUS there is no clear etiologic agent or seasonal pattern. The onset may be preceded by features of the nephrotic syndrome. A diarrhea prodrome is rarely observed (D-HUS) [1, 2, 33, 35]. Non-Stx-HUS can occur sporadically or in families.

Sporadic non-Stx-HUS

A wide variety of triggers for sporadic non-Stx-HUS have been identified including various non-enteric infections, viruses, drugs, malignancies, transplantation, pregnancy and other underlying medical conditions (scleroderma, anti-phospholipid syndrome, lupus, malignant hypertension).

Infection caused by *Streptococcus pneumoniae* accounts for 40% of non-Stx-HUS and 4.7% of all causes of HUS in children in US [33]. Neuroaminidase produced by *Streptococcus pneumoniae*, by removing sialic acids from the cell membranes, exposes Thomsen-Friedenreich antigen to preformed circulating IgM antibodies, which bind to this neoantigen on platelet and endothelial cells and cause platelet aggregation and endothelial damage [36, 37]. The clinical picture is usually severe, with respiratory distress, neurological involvement and coma. The mortality rate is 50% [37].

HUS and the related syndrome, thrombotic thrombocytopenic purpura (TTP) are both part of the complications of AIDS following HIV infection and account for up to 30% of hospitalized cases of HUS and TTP in some institutions [38]. The clinical course is poor and depends heavily on the severity of the underlying disease.

Categories of drugs that have been most frequently reported to induce non-Stx-HUS include anti-cancer molecules (mitomycin, cisplatin, bleomycin, gemcitabine), immunotherapeutic (cyclosporine, tacrolimus, OKT3, interferon and quinidine), and antiplatelet (ticlopidine, clopidogrel) agents [39]. The risk of developing HUS after mitomycin is 2% to 10%. The onset is delayed, occurring almost 1 year after starting

treatment. The prognosis is poor, with up to 75% mortality at 4 months [39].

Post-transplant HUS is being reported with increasing frequency [1, 40]. It may ensue for the first time in patients who never suffered from the disease (de novo post-transplant HUS) or may affect patients whose primary cause of ESRD was HUS (recurrent post-transplant HUS). De novo post-transplant HUS might occur in patients receiving renal transplants and other organs, as a consequence of the use of calcineurin inhibitors or due to humoral (C4b positive) rejection. In renal transplant patients treated with cyclosporine the incidence of the disease is 5 to 15%; a lower incidence, approximately 1%, is found in patients receiving tacrolimus [41]. A peculiar form of de novo post-transplant HUS affects about 6% of the recipients of a bone marrow transplant (BMT), usually in the setting of a graft versus host disease (GVHD) or of intensive GVHD prophylaxis [41].

Non-Stx-HUS may also be triggered by pregnancy. Pregnancy-associated HUS may occasionally develop as a complication of pre-eclampsia. Some patients progress to a life-threatening variant of pre-eclampsia with severe thrombocytopenia, microangiopathic hemolytic anemia, renal failure and liver involvement (HELLP syndrome). HUS or its complications in pregnancy are always an indication for prompt delivery that is usually followed by complete remission [42]. Post-partum HUS is another complication of pregnancy that manifests within three months of delivery in most cases. The outcome is usually poor with 50-60% mortality; residual renal dysfunction and hypertension are the rule in surviving patients [43].

Of note, in about 50% of cases of sporadic non-Stx-HUS no clear triggering conditions

could be found (idiopathic HUS) [1]. The outcome is variable, it may follow a progressive course to ESRD, however many patients recover completely [1].

Familial non-Stx-HUS

Familial forms accounts for fewer than 3% of all cases of HUS. Reports of familial occurrence of HUS in children date back to 1965, when Campbell and Carre [44] described the development of hemolytic anemia and azotemia in concordant monozygous twins. Since then, familial forms of HUS in children and, less frequently, in adults, have been reported [45]. Although some reports have been in siblings, suggesting autosomal recessive transmission [46], there have also been others describing affected members across two [47-49] or three [46] successive generations, suggesting an autosomal dominant mode of inheritance. In a kindred [50], late onset in the grandmother and early onset in the grandson may be indicative of the phenomenon known as anticipation, in which clinical manifestations appear at an earlier age and with increasing severity in succeeding generations.

In autosomal recessive HUS the onset is usually early in childhood. The prognosis is poor, with a mortality rate of 60-70%. Recurrences are very frequent.

The onset of symptoms in autosomal dominant HUS varies –early in childhood in some cases, but it can manifests in adult life- and is often triggered by precipitating events such as bacterial and viral infections or pregnancy [46]. The use of oral contraceptives and the intake of estrogens have also been implicated as triggering factors [46]. Malignant hypertension is a frequent complication and the prognosis is poor, with a cumulative incidence of death or

ESRD of 50% [51, 52] to 90% [46].

Evidence that some familial cases of HUS responded, at least transiently, to plasma infusion or exchange led to the hypothesis that the genetic defect(s) associated with the disease might result in abnormalities in some plasma component(s) involved in the pathogenesis of the microangiopathic process. Biochemical and genetic studies from our group (included in this present thesis) and from other investigators have now provided evidence that familial HUS may be caused by genetic abnormalities of proteins involved in the regulation of the alternative pathway of complement .

The human complement system

The complement system is a crucial component of the innate immunity against microbial infection. It consists of several plasma and membrane-associated proteins that are organized in three distinct activation patterns. These include classical, lectin and alternative pathways [53, 54] that, once activated on the surface of microorganisms, form protease complexes, collectively known as C3-convertases, which serve to cleave C3 generating C3b. The classic/lectin C3 convertases, named C4b2b, are formed by C2 and C4 fragments, while the generation of the alternative pathway C3 convertase, named C3bBb, requires the cleavage of C3 and factor B but not of C4. Upon generation C3b deposits on bacterial surfaces, which leads to opsonization for phagocytosis by neutrophils and macrophages. In the presence of an additional C3b molecule, the C3-convertases form the C5 convertases that cleave C5 and initiate assembly of the membrane attack complex (MAC) that causes cell lysis.

The human complement system is highly regulated as to prevent non-specific damage to host cells and limit deposition of C3b to the surface of pathogens. This fine regulation is based on a number of membrane-anchored (CR1, DAF, MCP and CD59) and fluid-phase (factor H, factor I) regulators that protect host tissues. Foreign surfaces that either lack membrane-bound regulators or cannot bind soluble regulators are attacked by complement.

Factor H

Factor H (CFH) is encoded by a single gene (*CFH*) located on human chromosome 1q32 within the regulators of complement activation (RCA) cluster. It is a single polypeptide chain plasma glycoprotein (155 kDa) that is composed of 20 repetitive units of 60 aminoacids, named short consensus repeats (SCR) [55].

CFH binds to C3b, accelerates the decay of the alternative pathway C3-convertase and acts as a cofactor for the factor I-mediated proteolytic inactivation of C3b. In the presence of CFH and factor I, C3b proteolysis results in the cleavage of the α' -chain of C3b at two nearby sites generating two fragments of 68 and 43 kDa, respectively. In the case of surface-bound C3b, the inactivated C3b molecule, named iC3b, remains covalently linked to the activating surface. CFH regulates complement both in fluid-phase and on cellular surfaces. It binds and inactivates promptly C3b in fluid-phase, and complement regulatory domains that are needed to prevent fluid-phase alternative pathway activation have been localized within the N-terminal SCR1-4 [56]. On the other hand the inactivation of surface-bound C3b by CFH is dependent on the chemical composition of the surface to

which C3b is bound. Indeed, CFH binding to sialic acids and other polyanionic molecules like glycosaminoglycans or sulphated polysaccharides such as heparin, increases its affinity for surface-bound C3b and exposes its complement regulatory N-terminal domain. Human vascular endothelial cells and basement membranes are rich in polyanionic molecules, so that CFH bound on their surface would provide an efficient shield against complement attack [56].

CFH has three C3b-binding sites, located in SCR1-4, SCR12-14 and SCR19-20, respectively. Similarly, a total of three separate binding sites for heparin and sialic acid have been identified in SCR7, SCR13 and SCR19-20 [57]. The C3b binding site in SCR1-4 is the only site essential for the factor I cofactor activity of CFH in fluid-phase. On the other hand, the C3b and the polyanion-binding sites located in SCR19-20 are the only indispensable sites for CFH to inactivate surface-bound C3b, because deletion of this portion of the molecule causes loss of CFH capability to prevent complement activation on sheep erythrocytes (taken as an example of a host-like non-activator surface) [58].

Membrane cofactor protein

As CFH, the gene encoding membrane cofactor protein (MCP) is located within the RCA cluster on chromosome 1 with 14 exons spanning around 43 Kb [59].

MCP is widely expressed on the surface of almost every human cells except erythrocytes [59]. Structurally, MCP consists of four alternatively spliced isoforms that co-exists on most cells. The extra-cellular domain is composed of four SCR. Following this is

an O-glycosylated region that is rich in serines, threonines and prolines (the STP region), which is alternatively spliced. There are either 14 or 29 amino acids in the STP region and this depends on the splicing of STP exon B (STP-B). The STP region is followed by a juxtamembranous region of 12 amino acids of unknown function, followed by a hydrophobic transmembrane domain and a cytoplasmic anchor. The cytoplasmic tail of MCP is also alternatively spliced (the 16 amino acid CYT-1 and the 23 amino acid CYT-2) and mediates signalling events. There are four commonly expressed isoforms, MCP-BC1, MCP-BC2, MCP-C1 and MCP-C2 where B and C refer to the STP region, and 1 or 2 refer to the cytoplasmic tail.

MCP with factor I degrades the C3b and C4b that are bound to the cell surface (cofactor activity) and regulates both the classical and alternative pathways of complement. Studies with deletion mutants and monoclonal antibodies have shown that ligand binding and cofactor activity are contained in SCRs 2, 3 and 4 of MCP [59].

MCP is highly expressed in the kidney and could be found on glomerular endothelial cells by immunohistochemical analysis [60-62]. It likely exerts a main role in protecting glomerular endothelial cells against C3 activation as indicated by data that cofactor activity in the extracts of these cells was completely blocked by anti-MCP antibody [61].

Complement abnormalities in HUS, previous studies

Since 1974 reduced serum levels of the third component (C3) of complement have been reported in patients with both Stx-HUS and non-Stx-HUS, during the acute phase of the

disease [48, 63]. In patients suffering from episodes of Stx-HUS C3 abnormalities consistently subsided with remission of the disease. On the contrary, in cases of familial non-Stx-HUS and in patients suffering from recurrences, serum C3 levels were low even during remission of the disease [64, 65]. By contrast, levels of the fourth fraction of complement, C4, are usually normal. Low C3 levels most likely reflect complement activation and C3 consumption, which is consistent with increased levels of C3 breakdown products, C3b, C3c and C3d, in the plasma of these patients [66, 67]. Granular C3 deposits in glomeruli and arterioles of kidney biopsies taken during the acute phase of the disease [68-72] further reflect activation of the complement system and C3 consumption in the renal microvasculature.

In a patient and in his healthy brother as well, low C3 levels were accompanied by very low levels of CFH [73]. Finding that the parents, who were first cousins, had half-normal levels of CFH, convincingly indicated that the defect was inherited. Similar findings were reported in other families [65]. These reports raised the possibility that low C3 in familial HUS may depend on an inherited deficiency of CFH. However, epidemiological data on the prevalence of low C3 levels and CFH abnormalities in non-Stx-HUS were missing.

Objective of the thesis

Based on these observations a number of studies were designed and performed in this thesis with the aims to provide more information as for the incidence and pathogenetic role of complement dysregulation in non-Stx-HUS and to investigate the genetic basis of such

an O-glycosylated region that is rich in serines, threonines and prolines (the STP region), which is alternatively spliced. There are either 14 or 29 amino acids in the STP region and this depends on the splicing of STP exon B (STP-B). The STP region is followed by a juxtamembranous region of 12 amino acids of unknown function, followed by a hydrophobic transmembrane domain and a cytoplasmic anchor. The cytoplasmic tail of MCP is also alternatively spliced (the 16 amino acid CYT-1 and the 23 amino acid CYT-2) and mediates signalling events. There are four commonly expressed isoforms, MCP-BC1, MCP-BC2, MCP-C1 and MCP-C2 where B and C refer to the STP region, and 1 or 2 refer to the cytoplasmic tail.

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abnormalities.

Sporadic observations in literature provided evidence that activation of complement occurs also in patients with thrombotic thrombocytopenic purpura (TTP). The latter syndrome has in common with HUS microangiopathic hemolytic anemia, consumption thrombocytopenia and microvascular thrombosis, but differs because it manifests mainly with central nervous system symptoms, whereas predominant renal involvement characterizes HUS [4]. Examination of very large series of patients with HUS and TTP has pointed out that a neat clinical distinction between the two syndromes is difficult. Thus, search for complement activation markers and of genetic abnormalities of complement regulatory genes was also undertaken in patients with diagnosis of TTP.

In **chapter 2**, in a case-control study we correlated putative predisposing conditions, including low C3 serum levels, with history of disease in familial cases of HUS and TTP. In the same subjects we searched for biochemical abnormalities in the complement regulatory plasma factor, CFH. The relationship between history of disease, low serum C3 and CFH abnormalities was also investigated.

Results of **chapter 2**, showed an association between CFH biochemical abnormalities and low C3 levels in familial forms of HUS. Based on these data, linkage and mutation studies were undertaken to evaluate whether mutations in *CFH* gene were involved in determining genetic predisposition to familial forms of HUS. Characterization of biochemical consequences of the mutations was also performed. For these studies, reported in **chapter 3**, four families were selected on the basis of persistent hypocomplementemia in

all cases.

Taking advantage of the large number of patients referred to the International Registry of HUS and TTP, a subsequent study (chapter 4) was designed to evaluate the frequency of *CFH* mutations both in familial cases of non-Stx-HUS and in sporadic cases as well, and to compare the clinical phenotype of patients with and without *CFH* mutations. Various polymorphisms in the promoter and in the coding regions of *CFH* gene have been described which may be associated with reduced *CFH* levels or activity. Thus a second aim of the study was to evaluate the frequency of *CFH* polymorphisms in patients with non-Stx-HUS as compared with a control population, to establish whether any of them segregated with the disease. The above analysis were also performed in patients with TTP to establish whether *CFH* gene mutations and polymorphisms may have a role in predisposing to TTP as well, or whether such abnormalities were specific for the HUS phenotype. This is described in chapter 4.

Recent studies provided evidence that 70 to 80% of cases of TTP are triggered by a deficiency of ADAMTS13, a plasma metalloprotease that cleaves von Willebrand factor multimers soon after their secretion by endothelial cells [74]. ADAMTS13 deficiency may be constitutive, as a result of homozygous or double heterozygous mutations in the corresponding gene or acquired, due to the presence of circulating inhibitory antibodies [74]. In two large studies[75, 76], deficiency of ADAMTS13 activity was found in patients with diagnosis of TTP but not in those with HUS. This observation generated the idea that a severe ADAMTS13 deficiency is enough to distinguish TTP from HUS. However, further

investigations based on large series of patients with HUS and TTP [77, 78] showed that even if most cases of thrombotic microangiopathy diagnosed as HUS due to the prevalence of renal symptoms have normal ADAMTS13, there are unequivocal cases of HUS characterized by low or undetectable ADAMTS13 levels. In studies of chapter 5 the genetic basis of phenotypic heterogeneity in patients with hereditary ADAMTS13 deficiency was investigated in a family with two sisters with complete ADAMTS13 deficiency, one with exclusive neurological symptoms that led to a diagnosis of TTP and the other with severe renal involvement more reminiscent of HUS.

Studies of chapter 4 documented that two thirds of patients with non-Stx-HUS have no *CFH* mutations, despite up to 50% of them exhibit evidence of overactivity of the alternative pathway of complement. These findings indicated that genetic abnormalities in other complement regulatory proteins might be involved in determining genetic predisposition to the disease. Based on these observations, in studies of chapter 6 patients with familial and sporadic forms of non-Stx-HUS with no *CFH* mutations were screened for mutations in genes encoding factor H related 5 (*FHR5*), complement receptor 1 (*CR1*) and membrane cofactor protein (*MCP*).

Finally, chapter 7, is a review chapter aimed at making the focus on the more recent acquaintances on the pathogenesis, the genetics and treatment of the different forms of HUS. The chapter also summarizes the results of expression studies we undertaken during the last years, in collaborations with Peter Zipfel in Jena, which were aimed at evaluating the functional consequences of the *CFH* mutations found in non-Stx-HUS on the activity of *CFH*

protein [79]. The outcome of combined kidney and liver transplant performed in our Transplant Center in two young children with non-Stx-HUS and *CFH* mutations with the objective of correcting the genetic defect and preventing disease recurrences [80, 81], is also reported and discussed .

**Hypocomplementemia discloses genetic predisposition to
hemolytic uremic syndrome and thrombotic thrombocytopenic
purpura: role of factor H abnormalities.**

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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-

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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.

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 hematocrit (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/ μ L.

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 $\pm$ 2 SD) of the laboratories of the "Ospedali Riuniti, Azienda Ospedaliera di Bergamo" (i.e., <83 mg/dl for C3 and <15 mg/dl for C4) were taken to indicate hypocomplementemia.

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.

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.

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 anti-double-stranded-DNA antibodies titer, lupus erythematosus phenomenon, lupus anticoagulant, anticardiolipin 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 μ l) was separated by 10 or 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Laemmli (30), using prestained bench markers (Life Technologies) as standards. Proteins were electroblotted to nitrocellulose by semidry 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-naphthol 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 μ g; Advanced Research Technologies, San Diego, CA) was labeled with ^{125} I 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 ^{125} I 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 (Clarif Corp., Santa Clara, CA). Dichotomous baseline characteristics were compared by χ^2 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 (rpb) (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 1. Prevalence and outcome of the different cases of familial HUS and TTP recorded in the database of the Italian Registry of Recurrent and Familial HUS/TTP^a

Category	HUS	HUS/TTP	TTP	Total
Families	7	2	1	10
Patients	29	4	2	35
adults	6	4	2	12
children	23	0	0	23
Relapses	14	4	0	18
Deaths	18	0	1	19

^a 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-

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

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 Cele Daccò."

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).

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

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

* Data are mean $\pm$ 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 population*

Parameter	P Value	
	Affected Families	All Subjects
Age (yr)	0.09	0.02
Gender (M/F)	0.47	0.62
Serum creatinine (mg/dl)	0.02	0.003
Hemoglobin (mg/dl)	0.0008	0.0001
Hematocrit (%)	0.002	0.0001
Platelet count ($10^3/\mu\text{l}$)	0.13	0.78
LDH (U/L)	0.23	0.02
ACA (U/ml)	0.83	0.61
C3 (mg/dl)	0.0007	0.0001
C4 (mg/dl)	0.88	0.36

* 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 alive at the time of the study (Figure 3), F29 and F85. In two healthy relatives (F79 and F87), factor H levels were moderately below normal range; the other healthy relatives (F73, F84, and F86) had borderline values (Figure 3), suggesting a partial, heterozygous factor H deficiency. These data, together

with the high degree consanguinity in this family, suggest a recessive pattern of inheritance for factor H defect. In the other families, serum factor H levels were within the normal range both in cases and case-relatives.

To search for possible abnormalities of serum levels and pattern of factor H and factor H-related proteins in families with HUS or TTP, sera from cases, their relatives, and controls were further analyzed by Western blotting. The results of the two cases and five healthy relatives from family 29 are shown in Figure 4. Western blot confirmed low levels of factor H in the serum of the two cases (F29 and F85, tracks 1 and 5), whereas no gross abnormality was detected in the serum of their healthy relatives (tracks 2, 3, 4, 6, and 7; track 8 represents serum from one healthy volunteer for comparison). In another family (family 24) with history of HUS, the two affected subjects (F106 and F108) who had normal factor H levels by radial immunodiffusion (Figure 5) showed abnormalities as identified by Western blotting. In the above patients (Figure 6A, tracks 5 and 6) and in their healthy father (track 2), additional high molecular weight bands were observed that reacted with anti-factor H antiserum and that were found with another pattern and different intensities in the control subject (track 1) and in the healthy mother (track 3) and sister (track 4). The nature of these bands of lower mobility is not clear. Conceivably, the cases inherited the factor H anomaly from their father. Densitometric analysis showed that the ratio of high molecular weight band/normal factor H band was higher 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-1 α (37 kD) and FHR-1 β (42 kD), are presented. Since FHL-1 band is masked by FHR-1 β (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

Parameter	Univariate Analysis		Multivariate Analysis	
	Affected Families	All Subjects	Affected Families	All Subjects
Relative risk	8.62	14.35	16.56	27.77
95% CI	2.39 to 31.10	4.20 to 49.00	1.66 to 162.39	2.44 to 314.19
P value	0.001	0.001	0.016	0.008

* CI, confidence interval.

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.

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 ($\text{rpb} = -0.25$; $P < 0.05$).

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 α -chains (α -43). Under identical conditions, sera from both cases were rather inefficient in conversion of the C3b α -chain (Figure 7A). Serial dilution and densitometric analysis (data not shown) of the newly formed α -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 5. (A) Pedigree of family 24. Symbols as in Figure 3. (B) Serum complement profile in family 24.

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-1 β ; 3 = factor H-related protein-1 α .

Figure 7. Cofactor activity of factor H in serum samples from family 29 (A) and family 24 (B). Radiolabeled C3b cleavage products were separated by SDS-PAGE under reducing conditions and autoradiographed. α and β represent the intact chains of C3b. Cofactor activity was calculated by densitometric analysis of the band corresponding to the $\alpha 43$ proteolytic product.

29 is in agreement with the low serum levels of factor H (Figure 3B), as also confirmed by Western blot analysis (Figure 4). A different result was observed in family 24. Sera obtained from both cases and healthy relatives showed similar activities that were all comparable to unrelated healthy controls (Figure 7B).

Discussion

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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**The molecular basis of familial hemolytic uremic syndrome:
mutation analysis of factor H gene reveal a hot spot in short
consensus repeat 20**

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The Molecular Basis of Familial Hemolytic Uremic Syndrome: Mutation Analysis of Factor H Gene Reveals a Hot Spot in Short Consensus Repeat 20

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Abstract. The aim of the present study was to clarify whether factor H mutations were involved in genetic predisposition to hemolytic uremic syndrome, by performing linkage and mutation studies in a large number of patients from those referred to the Italian Registry for Recurrent and Familial HUS/TTP. PCR and Western blot analyses were conducted to characterize the biochemical consequences of the mutations. Five mutations in the factor H gene were identified. Three, identified in two families and in a sporadic case, are heterozygous point mutations within the most C-terminal short consensus repeat 20 (SCR20) of factor H, resulting in single amino acid substitutions. The other two mutations introduce premature stop codons that interrupt the translation of factor H. A heterozygous nonsense mutation was identified in SCR8 in one family, and a homozygous 24-bp deletion within SCR20 was identified

in a Bedouin family with a recessive mode of inheritance. Reverse transcription-PCR analysis of cDNA from peripheral blood leukocytes from the Bedouin family showed that the deletion lowered factor H mRNA levels. Although heterozygous mutations were associated with normal factor H levels and incomplete penetrance of the disease, the homozygous mutation in the Bedouin family resulted in severe reduction of factor H levels accompanied by very early disease onset. These data provide compelling molecular evidence that genetically determined deficiencies in factor H are involved in both autosomal-dominant and autosomal-recessive hemolytic uremic syndrome and identify SCR20 as a hot spot for mutations in the disease. The mutations identified here give an important hint to the relevance of the C-terminus of factor H in the control of the alternative complement activation pathway.

Hemolytic uremic syndrome (HUS) is a disease of nonimmune hemolytic anemia, thrombocytopenia, and renal failure caused by platelet thrombi in the microcirculation of the kidney and other organs (1-3). In its typical presentation, HUS manifests as an acute disease and 80 to 90% of cases recover without sequelae, either spontaneously (as in most cases of childhood HUS) or after plasma infusion or exchange (as in adult or severe forms of HUS) (1,2,4). Typical HUS is triggered by environmental factors, drugs, or infective agents such as the shiga-like toxin-producing *Escherichia coli*; systemic immune disorders or cancer may also cause the disease. These forms of

HUS may subside when the underlying condition has been treated or removed. However, there are rare forms, often occurring in families, that frequently relapse even after complete recovery of the presenting episode (1,5), with death or permanent neurologic or renal sequelae being the final outcome in the majority of cases. These "atypical" forms are believed to have a genetic background that predisposes to microvascular thrombosis (6,7). Both autosomal-recessive and autosomal-dominant modes of inheritance have been recognized (5,6,8-11), with precipitating events such as pregnancy, virus-like disease, or sepsis being identified in some (12) but not all series (13,14). Evidence that some of these cases could be cured, at least transiently, with plasma infusion or exchange suggested that the underlying genetic defect(s) associated with familial HUS were the cause of one or more abnormalities in plasma component(s) essential to the integrity of the microvascular circulation and/or to the defense mechanism of the host endothelium against injurious agents. In this context, reduced serum levels of the third component (C3) of the complement system had been reported since 1974 in sporadic or familial forms of HUS (15-21). This initially was attributed to an inherited defect in C3 synthesis (22), but more convincing data are now available

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that low C3 in HUS derives from either a lack (21,23,24) or altered function (25) of factor H, a regulatory component of the alternative pathway of the complement system (26-28).

In a recent study (12) in a consistent number of families, we provided epidemiologic evidence of an association between factor H deficiency and low C3 levels in familial forms of HUS. Another report (25) documented that an area on chromosome 1q, where factor H is mapped, segregates with HUS. In one family, a mutation in factor H gene was described, consisting of a C to G transversion causing an arginine to glycine change in short consensus repeat 20 (SCR20) (25).

The present study was designed to characterize the genetic defect underlying atypical HUS by genotyping a large number of patients from our Registry for Familial and Recurrent HUS/TTP. Here we describe five mutations; three were found in families with dominant inheritance, one was found in a family with recessive inheritance, and one was found in an adult case of sporadic HUS whose disease recurred in the transplanted kidney. These results provide molecular evidence that the primary abnormality in atypical HUS is overactivity of the alternative complement pathway as a result of impaired factor H function.

Materials and Methods

Patients

HUS was diagnosed in all cases reported to have one or more episodes of microangiopathic hemolytic anemia and thrombocytopenia, defined on the basis of hematocrit <30%, hemoglobin <10 mg/dl, serum lactate dehydrogenase >460 units/L, undetectable serum haptoglobin, fragmented erythrocytes in the peripheral blood smear, and platelet count <150,000/ μ L, associated with acute renal failure.

HUS was 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 shiga-like toxin-producing strain of *E. coli*) could be reasonably excluded.

Plasma levels of the third complement component below the lower limit of normal ranges (calculated as mean $\pm$ SD), i.e., <83 mg/dl, were taken as indicating hypocomplementemia.

Microsatellite Polymorphism Genotyping and Linkage Analysis

Genomic DNA was extracted from whole blood according to standard protocols (Nucleon BACC2 kit, Amersham, UK).

Microsatellite polymorphisms flanking and within the gene for complement factor H (HF, chromosome 1q32) were studied in a candidate gene approach to linkage analysis. Markers analyzed in this study are D1S240, D1S202, D1S412, D1S2816, D1S413, D1S2738, and D1S2796; primers were synthesized by Life Technologies (Paisley, UK).

PCR reactions were done in a 20- μ L volume containing 100 ng DNA, 17 pmol of each primer, 16 nmol dNTP, 1.5 mM $MgCl_2$, 1 U *Taq* polymerase (*Taq* Gold, PE Applied Biosystems, Foster City, CA), in the presence of ^{32} PdCTP, and PCR buffer. After an initial 10-min denaturation at 94°C, 35 cycles were performed (94°C for 45 s, 54°C for 30 s, and 72°C for 45 s), followed by a final 10-min extension at 72°C. Samples were mixed with 20 μ L of loading buffer, denatured at 75°C for 5 min, and electrophoresed on a denaturing 6% (19:1 acryl:bis) acrylamide gel in Tris Borate EDTA buffer, at 55 W for 2 to 4 h. Gels were then exposed to x-ray films for 3 h to overnight.

Linkage analysis was performed using a FASTLINK package for two-point analysis and Genehunter package for multipoint analysis (www.linkage.rockefeller.edu). Autosomal-recessive and autosomal-dominant transmission, with incomplete penetrance (0.7), were taken into consideration; the disease gene frequency in the general population was assumed to be 0.0001.

Single-Strand Conformation Polymorphism Analysis and Sequencing of Factor H

Factor H exons were amplified using primers located in the flanking introns and analyzed by single-strand conformation polymorphism (SSCP) of DNA; primers were constructed to avoid coamplification of highly homologous SCR in factor H related genes (hFHR) (26). For amplification of SCR20, it was necessary to divide the exon into two parts (called SCR20A and SCR20B) that were independently amplified using two couples of primers, constructed to obtain 3' mismatches on the hFHR-1 gene sequence (see Figure 1). Primers were synthesized by Life Technologies.

A total of 100 ng of genomic DNA were amplified as described above. PCR products were electrophoresed on nondenaturing 6% (62:1 acryl:bis) acrylamide gel in TAE buffer (pH 6.8) at 35 W for 3 to 5 h and at 4 W overnight. Gels were visualized by silver staining,

Figure 1. Alignment between short consensus repeat 20 (SCR20) genomic sequences of factor H and factor H-related 1 (FHR-1). Vertical bars with arrows indicate beginning and ending of SCR20. All mismatched nucleotides are boxed in gray. SCR20 was divided into two segments that were independently amplified using two sets of primers (gray arrows for SCR20B and black arrows for SCR20A).

and aberrant bands were sequenced using an ABI 377 sequencer (PE Applied Biosystems).

mRNA Extraction, cDNA Synthesis, and Analysis

mRNA was extracted from peripheral blood mononuclear cells (PBMC) isolated from whole blood (standard protocol); reverse transcription (RT) was conducted using the Life Technologies Brl protocol with oligo (dT) primers, in a total volume of 20 μ l, and the cDNA obtained was used to investigate mRNA levels in family 29. RT-PCR products of SCR20A from heterozygous individuals of family 29 were electrophoresed on a nondenaturing 8% (37.5:1 acryl:bis) acrylamide gel in TBE buffer at 80 W for 2 to 4 h. Gels were then stained with ethidium bromide.

mRNA levels from three heterozygous individuals from family 29 and from healthy control subjects ($n = 3$) were also evaluated by real-time PCR quantification (29) on the ABI Prism 7700 platform (PE Applied Biosystems). Five μ l of cDNA were amplified with 2.5 μ l of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) PDAR Kit (including forward and reverse primers as well as a specific VIC labeled TaqMan probe, PE Applied Biosystems), and 25 μ l of TaqMan Universal MasterMix (PE Applied Biosystems) in a final volume of 50 μ l. The amplification profile consisted of 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s, then 60°C for 1 min. In parallel 5 μ l cDNA were amplified using 25 μ l of SYBR Green I (30), TaqMan Universal Master Mix (PE Applied Biosystems), primers for SCR20A, in a final volume of 50 μ l with an amplification profile of 2 min at 50°C, 10 min at 95°C and then 40 cycles of 15 s at 95°C, 20 s at 55°C, and 30 s at 72°C. The amplification curve was obtained using fluorescence values collected at 72°C.

After GAPDH normalization, mRNA levels of SCR20A (target gene) in heterozygous individuals were quantified as a percentage of mRNA levels in healthy control subjects. We applied the $\Delta\Delta$ Ct method (User bulletin #2, PE Applied Biosystems and references 31–33). Melting temperature analysis (30) was also performed: at the end of amplification reaction, samples were denatured at 95°C and then slowly cooled to 60°C in 20 min. The amplification product was verified by acrylamide gel. No primer-dimers or aspecific amplification products were evidenced by melting temperature analysis and acrylamide gel.

Factor H and C3 and C4 Quantification

Factor H was quantified by radial immunodiffusion assay using a sheep polyclonal anti-human factor H antiserum, which reacts with various epitopes of factor H molecule (The Binding Site, Birmingham, UK). C3 and C4 were quantified by kinetic nephelometric measurements.

Western Blot Analysis of Factor H

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. Serum (1.5 μ l) was separated by 10 or 7% sodium dodecyl sulfate polyacrylamide gel electrophoresis according to Laemmli using prestained bench markers (Life Technologies) as standards. Proteins were electroblotted to nitrocellulose by semidry blotting. 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, San Diego, CA; diluted 1:1000) or anti FHL-1 rabbit anti-SCR1 to 4 antiserum, that does not detect FHR-1 and FHR-2 proteins (dilution 1:1000 (34)) were performed at 4°C overnight. After the membranes were washed five times in PBS,

they were incubated with peroxidase-conjugated rabbit anti-goat or swine anti-rabbit antibody (Dako, Hamburg, Germany) for 2 to 3 h. Protein bands were visualized by the addition of 0.3% (wt/vol) 4-chloro-1-naphthol in 10% (vol/vol) methanol in PBS.

Results

Patients

Family Pedigrees and Sporadic Case Report. From the database of the Italian Registry for Recurrent and Familial HUS/TTP (12), four families (1, 3, 24, and 29) were selected for the present study on the basis of persistent hypocomplementemia in all cases. A female with sporadic HUS (R16) who experienced disease recurrence in the renal allograft was also studied.

As reported in Figure 2, in families 1, 24, and 29, the onset of the disease was during infancy (from a few weeks to 8 yr), whereas in family 3, the disease became manifest in adulthood.

Conditions that potentially predispose to the disease were recognized in all cases from families 1 and 24 (upper respiratory tract infection) and in patient F39#3 (pregnancy). One or more disease relapses were reported in all cases but one (F40#3). Eleven participants had died before the study was conducted. Among the seven survivors, four are on chronic dialysis; of these, two (F34#1 and F39#3) received a kidney transplant that failed because of recurrence of disease.

The pedigrees of the four families and haplotypes on 1q32 are shown in Figure 2. C3 concentrations were below the normal range in all patients and in some of their relatives (Table 1), whereas C4 concentrations were normal, indicating selective activation of the alternative complement pathway.

The pattern of inheritance of HUS could not be established in family 1, because only one patient was alive at the time of the study. No consanguinity was inferred. The pedigree was scarcely informative, with either the dominant or the recessive hypothesis (Table 2). In families 3 and 24, no consanguinity was reported; either recessive or dominant mode of inheritance could fit the haplotype data (Figure 2) and linkage results (Table 2). In family 29, a recessive inheritance was hypothesized because of the high grade of consanguinity; this was confirmed by the haplotype data (Figure 2) and logarithm of odds score values (Table 2). Results of SLINK simulation are reported in Table 2 as an index of the informativeness of the pedigrees.

Sporadic Case. In 1997, a 48-yr-old woman, who had been on chronic hemodialysis since 1995 because of progressive loss of renal function after an HUS episode in 1980, received in our Transplant Department a cadaveric renal allograft, under cover of steroid plus cyclosporine-based immunosuppression. Graft function deteriorated acutely on day 7 post-transplantation, and allograft biopsy showed recurrent HUS. A partial improvement was achieved with plasma exchange, but this was not sustained and the graft failed 3 mo later. She is currently on chronic peritoneal dialysis. No relevant clinical history was reported in her family.

Figure 2. Pedigrees of the four families studied (indicated by #...) and haplotypes on chromosome 1q32 markers (D1S240, D1S202, D1S412, D1S2816, D1S413, D1S2738, D1S2796) flanking factor H gene. The pedigree and the haplotype (markers D1S2816, D1S413) of the sporadic case also are presented. Patients studied are indicated by a number (F... for familial R... for sporadic). Age at onset is indicated in italics; p, perinatal onset. Chromosomes carrying mutations are boxed.

SSCP and Sequencing Results

PCR fragments corresponding to the 20 SCR of the factor H gene were analyzed for mutation screening by SSCP followed by sequencing of aberrant bands. SSCP detected a band shift in all cases and carriers from the four families as identified by

haplotype analysis. The aberrant bands were localized in SCR8 in family 3 and in SCR20 in families 1, 24, and 29. The sporadic case showed an aberrant band in SCR20, which was also found in the father and two of five healthy siblings. None of the aberrant bands was found in 100 normal chromosomes.

Table 1. Complement profiles^a

Family	Subject	C3 (mg/dl) (83-177)*	C4 (mg/dl) (15-45)*	Factor H (mg/L) (350-650)*
1	F83	84	27	476
1	F35	76	20	496
1	F34	69	15	561
1	F36	105	26	550
3	F63	101	19	662
3	F43	92	22	446
3	F40	68	18	569
3	F42	95	22	679
3	F41	73	17	537
3	F39	51	26	386
3	F44	79	12	529
3	F58	117	25	—
3	F56	78	15	616
3	F57	100	18	523
24	F104	130	332	835
24	F105	97	24	546
24	F107	96	27	562
24	F106	61	16	711
24	F108	61	15	569
29	F29	14	42	<70
29	F73	133	42	344
29	F79	135	49	252
29	F84	107	24	358
29	F85	10	—	<70
29	F86	103	34	362
29	F87	86	36	274
	R16	122	28	731

^a Complement profiles in the four families studied and in the sporadic case. Affected subjects and low values of C3, C4, and factor H are indicated in bold.

*, normal ranges.

Mutation Description and Biochemical Characterization of Factor H

Family 1. Sequence analysis revealed a heterozygous 3717 G to A transition; this results in an amino acid change of arginine to glutamine (R1215Q). This mutation was present in the patient and his unaffected father and his unaffected grandfather (Figure 3, panel A). Factor H protein levels (Table 1) and the Western blot profile were normal both in the case and his relatives (Figure 4A).

Family 3. A heterozygous bp deletion was found in the two patients and one unaffected brother involving one of the three adenines from position 1494 to 1496; this mutation causes a frame shift that results in the formation of a short sequence of five anomalous codons and a premature stop codon that interrupts the transcription within SCR8 (Figure 3, panel B). The same mutation was evident also on the RT-PCR product of RNA extracted from PBMC, indicating that the mutated DNA is transcribed (Figure 3, panel B1).

Radial immunodiffusion assay found that factor H levels in

carriers of the mutation were in the normal range (Table 1). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis (Figure 4B) of sera in this family showed that the 150-kD factor H band was reduced in the mutation carriers. The truncated protein was not identified in these patients by Western blotting; however, the truncated protein could have been masked by factor H-like 1 (26), which derives from an alternative splicing of factor H primary transcript, as a result of the very close molecular weight of the two proteins.

Family 24. A heterozygous 3701 C to T transition that determines an arginine to cysteine change (R1210C) was found (Figure 3, panel C). This mutation is present in the two patients and their unaffected father. Thus, the patients seem to have inherited the abnormal factor H allele from their father. Although factor H protein levels in the patients' plasma were normal, Western blot experiments revealed additional high-molecular-weight bands that reacted with anti-factor H antiserum in the two affected patients and their healthy father. These bands were absent in their healthy mother and sister and in control subjects (Figure 4, C and D). The nature of these bands with lower mobility is not clear. One possible explanation could be the formation of factor H dimers caused by intermolecular disulphide bonding with the additional cysteine in SCR20.

Family 29. An A to T transversion and a 24-bp deletion was found in homozygosity in affected members of family 29 (Figure 3, panel E). This mutation was also present in heterozygosity (Figure 3, panel D) in all of the other family members, as supposed by haplotype analysis (Figure 2). The deletion causes a stop codon within SCR20 and the loss of the four most C-terminal amino acids.

Ying *et al.* (35) described an apparent exchange at position 3645 in SCR20 in this Bedouin family. However, they used a set of primers that are complementary to both HF and FHR-1. While amplifying DNA from three healthy control subjects using this set of primers, we found the same base exchange as Ying *et al.* (35) and a further nucleotide mismatch that exactly corresponds to FHR-1.

The factor H concentration was abnormally low in this family: factor H serum levels were severely depressed in the two affected patients (Table 1 and reference 12) and below the normal range in the heterozygous subjects. The Western blot patterns of factor H in this family confirmed the low protein levels in the two homozygous patients, although some residual protein was detectable (12).

We used PBMC to conduct RT-PCR analysis on SCR20 to determine how this deletion affected mRNA levels. Upon separation of the cDNA PCR products from heterozygous carriers by acrylamide gel electrophoresis, we found two bands of different intensities: a 178-bp band corresponding to the wild-type cDNA—as evidenced by cDNA analysis from normal control subjects—and a second 154-bp band corresponding to the deleted cDNA (Figure 5A). The intensity of the aberrant band was much lower than the wild-type band (Figure 5A), suggesting a selective defect in transcription or stability of

Table 2. LOD scores obtained by two-point analysis between hemolytic uremic syndrome and seven markers around factor H^a

Dominant Hypothesis ($\theta = 0.0$) ^b					Recessive Hypothesis ($\theta = 0.0$)				
	#1	#3	#24	#29		#1	#3	#3 ^a	-24
D1S240	-0.14	0.88	0.31	0.03	D1S240	0.00	0.93	0.72	0.43
D1S202	0.01	0.88	0.31	-0.23	D1S202	0.00	1.23	1.02	0.43
D1S412	-0.16	0.68	0.49	0.30	D1S412	0.00	-1000	0.41	0.73
D1S2816	-0.15	0.34	0.00	0.22	D1S2816	0.00	-1000	0.41	0.12
D1S413	0.20	0.48	0.49	0.26	D1S413	0.00	-1000	0.50	0.73
D1S2738	0.04	1.03	0.31	-0.19	D1S2738	0.00	1.23	1.02	0.43
D1S2796	-0.21	0.10	0.31	-0.28	D1S2796	0.00	-0.01	-0.00	0.43
SLINK ^b	0.31	1.40	0.49	1.07	SLINK ^b	0.00	1.23	1.02	0.73
									-29
									0.21
									0.44
									-1000
									0.69
									1.58
									-3.97
									-1000
									2.10

^a LOD, logarithm of odds.

^b Penetrance 0.7.

^c Maximum value obtained by SLINK simulation.

Figure 3. Partial chromatograms of SCR8 (panels B and B1) and 20 (panels A and C to F) sequences describing the mutations in our series. (Panel A) DNA from subject F34#1 (familial hemolytic uremic syndrome [HUS]), heterozygous G to A transition. (Panel B) DNA from subject F39#3 (familial HUS), heterozygous frame shift caused by an A deletion. (Panel B1) cDNA from the same patient: the heterozygous A deletion is present also in the cDNA. (Panel C) DNA from patient F106#24 (familial HUS), heterozygous C to T transition. (Panel D) DNA from patient F86#29 (healthy carrier), heterozygous A to T transversion and frame shift caused by a 24-bp deletion. (Panel E) DNA from patient F29#29 (familial HUS), homozygous A to T transversion and frame shift caused by a 24-bp deletion. (Panel F) DNA from patient R16 (sporadic HUS), heterozygous C to T transition.

the mutated mRNA. Thus, quantitative real-time PCR showed approximately a 50% reduction of mRNA levels in heterozygous individuals (Figure 5B). Melting temperature analysis

showed two peaks in heterozygous subjects corresponding to the wild-type and the deleted cDNA. Unfortunately, RNA from homozygous patients was not available.

Figure 4. Western blot analysis of factor H in human serum. Sera were separated by 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the Western blots were developed with polyclonal rabbit anti-factor H antiserum, specific for factor H and FHL-1/recombinant. Closed arrows indicate factor H, and open arrows point to the abnormal high-molecular-weight bands in C and D. Patients are marked by an asterisk. The mobility of the size markers (kD) is indicated. (A) Family 1, sera from the patient still alive (F34) and from a healthy control subject are shown. (B) Family 3, sera from the two patients (F39 and F40), the carrier brother (F43), and two healthy relatives (F42 and F41) are shown. (C) Family 24, sera from the patients still alive (F106 and F108), the carrier father (F104), the healthy mother (F105), and the healthy sister (F107) are shown. (D) Sera from the patient with sporadic HUS (R16), the healthy mother (R22), the two carrier healthy siblings (R17 and R21), and a healthy control subject are shown.

Sporadic Case. A heterozygous mutation was found in the patient who experienced a sporadic episode of HUS. The mutation, a 3701 C to T transition, results in the change of an arginine to cysteine (R1210C) and is identical to that found in family 24, (Figure 3, panel F). We were unable to find any familial relationship between the sporadic patient, who is from the north of Italy, and family 24, which is from the south. Analysis of markers DIS2816 and DIS413 showed that this patient does not share the haplotypes of family 24 (Figure 2); thus, the possibility of a common foundation event is unlikely.

The mutation was also found in the father and two of five healthy siblings. Serum factor H levels were within the normal range (Table 1). Western blot experiments revealed in the sporadic case the presence of the same additional high-molecular-weight bands as in family 24. These bands were also present in all relatives who carried the mutation (Figure 4D).

Discussion

In the present study, we identified five mutations in the factor H gene in four families and one sporadic case of HUS. Three are point mutations within the most C-terminus SCR of factor H (SCR20), resulting in single amino acid substitutions.

Interestingly, an R1210C mutation was identified separately in one family and in one unrelated individual who developed a sporadic form of the disease. In another family, an R1215Q substitution was identified. A mutation in the same codon has been previously reported by Warwicker *et al.* (25) in a family with HUS, which resulted, however, in a different amino acid exchange (R1215G).

We also identified two mutations that lead to the formation of truncated factor H proteins. Specifically, a nonsense mutation was found in one family, which introduces a premature stop codon. This mutation results in a truncated protein that consists of SCR1 to 8 and has the last twelve SCR deleted. A 24-bp deletion within SCR20 was found in a Bedouin family, resulting in a protein having the most C-terminal end truncated. The 24-bp deletion was present in homozygosity in patients and in heterozygosity in their relatives, consistent with a recessive mode of inheritance. This pattern distinguishes this family from all of the others, in which all affected members were heterozygous for factor H mutations, consistent with a dominant pattern. Two groups have already studied the Bedouin family for factor H mutations. Ying *et al.* (35) described an apparent exchange of a single nucleotide at position 3645 in

Figure 5. Quantification of mRNA from patients of family 29. (A) Electrophoresis of PCR products: lane 1, DNA from F29 (homozygous for the 24-bp deletion); lane 2, DNA from F86 (heterozygous for the 24-bp deletion); lanes 3 and 4, cDNA from F86 and F84 (heterozygous for the 24-bp deletion); lane 5, cDNA from a healthy control subject. Levels of the deleted mRNA are clearly low in the heterozygous patients. (B) Real-time PCR results: *mRNA levels are expressed as percentage of levels in healthy control subjects ($n = 3$) after normalization for the housekeeping gene GAPDH. Each sample was run in triplicate; results are expressed as mean $\pm$ SD of the three runs. +, mean of three controls.

SCR20, and Buddles *et al.* (36) reported the 24-bp deletion that we confirm here. Data reported by Ying are very likely artifacts due to co-amplification of the strongly homologous FHR-1 gene (37), as the forward and reverse primers that they used are complementary to both factor H and FHR-1. This is confirmed by our findings in healthy control subjects that the same primers amplified a DNA fragment with the same base exchange described by Ying *et al.* and a further nucleotide mismatch that exactly corresponds to FHR-1.

Thus, the new mutations presented here, together with the previously published data (25,36) (Figure 6), provide strong molecular evidence that factor H is involved in both the dominant and the recessive forms of HUS. None of the mutations were ever found in healthy control subjects, excluding the possibility that they are common polymorphisms in the normal population. The point mutations (reference 25 and present data) all were inherited in an autosomal-dominant mode. By contrast, nonsense mutations gave rise to heterogeneous phenotypes; thus, a stop codon in SCR8 was associated with a dominant pattern of inheritance and late onset of the disease, whereas a stop codon in SCR20 produced a recessive pattern with a very early onset. Finally, a third nonsense mutation, located in SCR1, has already been found (25) in a sporadic case of HUS with late onset.

What seems to distinguish dominant and recessive forms are the biochemical and clinical consequences of the mutations: dominant inheritance is associated with normal factor H levels

Figure 6. Summary of all seven mutations reported in the factor H gene of HUS patients. Three mutations introduce premature stop codons within SCR1, SCR8, and SCR20; the other four mutations affect arginine residues (R1210 and R1215). With the exception of the deletion within SCR20 (Bedouin family), all mutations are heterozygous.

and an abnormal protein in the circulation. In these cases, an incomplete penetrance was observed; indeed, approximately 40% of carriers did not develop HUS. In most cases, the disease developed during late infancy or adulthood, often as a consequence of events such as infection or pregnancy, which are known to be triggering factors for HUS (1,2). This suggests that in dominant forms, haploinsufficiency of factor H, which is compensated by the normal allele in basal conditions, requires intercurrent environmental or acquired factors to manifest fully. By contrast, the mutation that causes autosomal-recessive HUS in the Bedouin family resulted in a severe reduction of serum factor H levels, accompanied by very early onset of the disease (1 to 20 wk) and no recognized trigger. Factor H reduction in this family may be due either to impaired secretion of the mutant protein, consequent to the loss of a disulphide bridge essential for protein folding and secretion (38), or to reduced mRNA levels as a result of premature arrest of translation (39). The latter possibility is supported by our results of mRNA analysis in PBMC from heterozygous carriers, in which the expression of the mutated allele was lower than the normal allele.

A number of reports have claimed an association between factor H deficiency and recurrent (22), atypical (24), and familial (12) forms of HUS. However, factor H deficiency has also been associated with an increased tendency to other dis-

eases, including systemic lupus erythematosus (40), pyogenic infection susceptibility (41), and a patient with membranoproliferative glomerulonephritis with collagen III deposition in the mesangium (42). In the latter patient, Ault *et al.* (43) identified two point mutations in SCR9 and SCR16, which cause abnormal secretion and severe factor H deficiency.

Why different mutations in the same gene can cause different diseases cannot be fully clarified on the basis of the present and previous data, and further investigations on the genotype-phenotype correlations are required. However, the finding that five of the seven mutations described until now in HUS are located in SCR20 of complement factor H identifies this domain as a mutational hot spot for HUS. It also suggests the presence, in the very C-terminus of factor H, of highly relevant functional sites whose loss is involved in the pathogenesis of this disease.

Factor H binds C3b and controls the alternative pathway of complement activation in two ways. First, it accelerates the decay of the alternative pathway C3 convertase (C3bBb) by displacing factor B. Second, it acts as a cofactor for factor I-mediated inactivation of C3b (cofactor activity) (26).

Factor H has at least three C3b binding domains, one of which resides within SCR 16 to 20 (44). In addition, SCR7 and 19 to 20 contain two major binding sites for heparin and heparin-like proteins, such as sialic acid on glycoproteins and glycolipids (45). All of these functions are very likely lost in mutated factor H in family 3, in which the entire 9 to 20 SCR segment was deleted. The three point mutations that cause arginine substitutions in SCR20, described here, might impair the heparin-binding capacity of factor H as this involves lysine and arginine residues (46,47).

Interaction of factor H with sialic acids and other polyanions on human cells and tissues increases the affinity of factor H for C3b and enhances its inhibitory effect on the alternative pathway of complement activation (48). This control step protects host cells from autolytic attack of the alternative complement pathway, because complement activation induces the deposition of C3b indiscriminately onto host and foreign particles. This applies particularly to endothelial cells, with their high surface density of heparin-like glycosaminoglycans (48). Thus, when an environmental trigger activates the alternative complement pathway, modification of the heparin-binding capacity of mutated factor H might facilitate the occurrence of microvascular endothelial damage.

The present data represent a step forward in understanding the pathogenesis of the microangiopathic process in HUS. We propose that intercurrent exposure to agents that damage the vascular endothelium, such as certain viruses, bacteria, toxins, immunocomplexes and cytotoxic drugs (1,2), may initiate local unrestricted complement activation within capillary vessels (49,50). In normal conditions, however, by modulating C3bBb activity (26-28), factor H may efficiently limit complement deposition, thus preventing any further enhancement of the activation. On the contrary, when the bioavailability or the activity of factor H is congenitally defective, C3bBb convertase formation and complement activation become uncon-

trolled and result in microangiopathic damage, which leads to full manifestation of the disease.

No specific therapy is effective in familial cases of HUS. Infusion of fresh-frozen plasma, or plasmapheresis, which is effective in recurrent and atypical adult cases, often is inactive in patients with the familial form of the disease (1,5), possibly because of insufficient amounts of factor H in the plasma infused. Replacement with recombinant factor H protein or the use of specific complement inhibitors could represent future perspectives for the treatment of the disease as alternatives to whole plasma.

Note Added in Proof

After the manuscript of this article was submitted for publication, we analyzed a sporadic patient, with low levels of C3 and factor H and with very early onset of HUS (6 mo); we found a mutation in SCR20 of factor H consisting of a heterozygous T3663C transversion that determines V1197A change.

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Appendix

Italian Registry for Recurrent and Familial HUS/TTF

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

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

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

INTRODUCTION

The term thrombotic microangiopathy (TMA) defines a lesion of vessel wall thickening (mainly arterioles and capillaries), intraluminal platelet thrombosis and partial or complete obstruction of the vessel lumina (1,2). Depending on whether renal or brain lesions prevail, two pathologically indistinguishable, but somehow clinically different entities have been described: the haemolytic uraemic syndrome (HUS) and the

thrombotic thrombocytopenic purpura (TTP). The most common form of HUS of children, with predominant renal failure, is associated with infection by strains of *E. coli* which produce a powerful Shiga-like toxin (3). This form, also called D⁺HUS, has an excellent prognosis. In contrast, forms of HUS non-Shiga toxin-associated and diarrhoea-negative (D⁻HUS), have a much poorer prognosis and are often relapsing; death and end-stage renal failure are the final outcome in the majority of cases (4-6). In selected cases there is a clustering of affected

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

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

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

RESULTS

Mutation screening of HF1

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

A C3562G mutation (Cys1163Trp) in exon XXII, encoding for SCR19, was found in a patient with recurrent HUS (R087#134). The other mutations were located in exon XXIII encoding for SCR20, namely a G3587T determining a Glu1172Stop in two unrelated patients with sporadic and recurrent HUS (S027#022 and R063#081), a G3654A (Gly1194Asp) in two patients with familial HUS (F169#130 and F170#130), an A3666C (Glu1198Ala) in a patient with recurrent HUS (R088#152), a T3663C (Val1197Ala) in a patient with recurrent HUS (R062#056), and a C3701T (Arg1210Cys) in a patient with sporadic HUS (S013#069). The latter two mutations have been previously reported in other patients with HUS (17), while all the other mutations are new.

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

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

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

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

Clinical and biochemical findings

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

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

Figure 1. Summary of HF1 gene mutations in patients with D⁺HUS from our Registry. The structure of HF with the 20 SCR is shown; SCR20 is enlarged on the right. The consequence of the mutation to protein composition is indicated in the circles; each circle corresponds to an independent mutational event.

Table 1. HF1 gene mutations in patients with HUS from our Registry

Exon	Mutation	SCR	Effect	Family code	Subgroups	Patients	Unaffected carriers	Origin of the mutation
II	A305G	1	Arg60Gly	101	Sporadic	1	2/4	Paternal
XI	del A1494-1496 ^a	8	Premature stop	003	Familial	2	1/11	Paternal
XIX	G2923T	16	Gln950His	118	Sporadic	1	/	Unknown
XX	T2924C	16	Tyr951His	087	Sporadic	1	/	Unknown
XXII	C3562G	19	Cys1163Trp	134	Recurrent	1	/	Unknown
XXIII	G3587T	20	Glu1172Stop	022	Sporadic	1	/	Unknown
XXIII	G3587T	20	Glu1172Stop	081	Recurrent	1	4/8	Paternal
XXIII	T3620A ^b	20	Trp1183Arg	045	Familial	2	1/3	Paternal
XXIII	G3654A	20	Gly1194Asp	130	Familial	2	5/8	Maternal
XXIII	T3663C	20	Val1197Ala	056	Recurrent	1	/	Unknown
XXIII	T3663C ^a	20	Val1197Ala	052	Recurrent	1	1/2	Paternal
XXIII	A3666C	20	Glu1198Ala	152	Recurrent	1	/	Unknown
XXIII	C3701T ^a	20	Arg1210Cys	020	Recurrent	1	6/10	Paternal
XXIII	C3701T ^a	20	Arg1210Cys	024	Familial	3	1/3	Paternal
XXIII	C3701T	20	Arg1210Cys	069	Sporadic	1	1/1	Unknown
XXIII	G3717A ^a	20	Arg1215Gln	001	Familial	3	2/6	Paternal
XXIII	A3579T and 24 bp deletion ^a	20	Premature stop	029	Familial	10	/	Recessive

^aCaprioli *et al.* (17).

^bRemuzzi *et al.* (20).

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

	HUS HF1 mutation	No HF1 mutation	TTP	P-values (HF1 mutation versus no HF1 mutation)	P-values (HUS versus TTP)
<i>Disease onset</i>					
Infancy (≤ 12 years)	23 (33)	34 (68)	0 (32)	0.06	<0.001
Adulthood (> 12 years)	10 (33)	34 (68)	32 (32)		
<i>Triggers</i>					
Pregnancy	2 (29)	5 (53)	5 (29)	0.69	0.19
Drugs	2 (29)	3 (53)	4 (29)	0.82	0.19
Flu-like, gastroenteritis, other infections	13 (29)	35 (53)	11 (29)	0.06	0.06
Transplantation (post-transplant HUS)	0 (29)	3 (53)	0 (29)	0.19	0.29
Other triggers	2 (29)	2 (53)	3 (29)	0.53	0.30
<i>Outcome</i>					
Dialysis	12 (33)	28 (68)	0 (32)	0.64	<0.001
Kidney transplantation	5 (33)	8 (68)	0 (32)	0.63	0.03
Death	12 (33)	7 (68)	4 (32)	0.016	0.025
Relapses	20 (33)	23 (68)	13 (32)	0.01	0.84
<i>Biochemical evaluations at remission</i>					
Decreased C3 serum levels (≤ 83 mg/dl)	14 (21)	26 (51)	9 (26)	0.22	0.53
Decreased HF serum levels (≤ 350 mg/l)	3 (17)	1 (56)	1 (22)	0.016	0.86
Elevated HF serum levels (≥ 750 mg/l)	3 (17)	6 (56)	7 (22)	0.45	0.03
Moderately decreased ADAMTS13 activity (20–50%)	2 (14)	6 (58)	0 (29)	0.67	0.06
Severely decreased ADAMTS13 activity ($\leq 20\%$)	0 (14)	5 (58)	17 (29)	0.25	<0.001

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

	HF1 mutation	No HF1 mutation
Kidney transplant recipients	5*	8
Transplanted kidneys	5	11
Kidney outcome		
OK at one year	0	5*
Disease recurrence on the graft	5	2*
Acute rejection	0	4
Combined kidney and liver transplantation	2	0
Kidney outcome		
OK at one year	1 ^b	0
Disease recurrence on the graft	0	0
Failure for other causes	1	0

* $P < 0.05$ versus HF1 mutation.^aOne patient reported by Landau *et al.* (40).^bReported in Remuzzi *et al.* (20).

renal function. One or more disease relapses were reported in 61 and 34% of patients with and without HF1 mutations, respectively ($P = 0.01$), and in 41% of patients with TTP.

Five patients with HF1 mutations and eight patients without mutations underwent kidney transplantation, with two patients in the latter group receiving three and two kidney grafts, respectively (Tables 2 and 3). All five grafts in patients with HF1 mutations were lost because of disease recurrence within the first year, as compared with only two of the 11 grafts in patients without HF1 mutations ($P = 0.0022$). In patients without HF1 mutations, four grafts were lost because of acute

vascular rejection and the remaining five were functioning well at 1 year post transplant. Of note, two additional patients with HF1 mutations received a combined kidney and liver transplant. The kidney graft outcome at one year was very good in the first patient (20), while unfortunately the second patient died few days after surgery because of acute liver failure without evidence of recurrence of the microangiopathic process (unpublished data, Table 3).

Complement profile and ADAMTS13 activity

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

HF serum levels were lower than normal in three patients with HF1 mutations and in one patient without mutation, while three and six patients, respectively, had elevated HF serum levels.

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

Analysis of factor H polymorphisms

HF1 gene screening in the 106 healthy controls revealed the presence of eight polymorphic variants: C-257T (promoter region), G257A in exon II (causing a Val44Ile change in SCR1), C994A in exon VII (SCR5, silent), T1277C in exon IX (causing a Tyr405His change in SCR7), G1492A in exon XI (SCR8, silent), A2089G in exon XIV (SCR11, silent), A2489-30C (intron XVI), and a G2881T in exon XIX (causing a Glu963Asp change in SCR16) (Table 4), all of which have been reported previously (19,31) with the exception of the novel variant, A2489-30C in intron XVI. The distribution of the above polymorphic variants in patients and in controls was in Hardy-Weinberg equilibrium.

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

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

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

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

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

Analysis of familial transmission of mutations and polymorphisms

All available relatives of nine patients carrying HF1 mutations were also screened and unaffected carriers were detected, giving a mutation penetrance of 49% (Table 1; family 029 has been excluded from the analysis because of recessive transmission of the disease). The transmission of the mutation was from the father in eight cases and from the mother in one (Table 1; family 029 excluded). Interestingly, none of the parents carrying HF1 mutations had episodes of HUS. This could be explained either as autosomal dominant transmission with reduced penetrance or as a recessive trait. We then wanted to evaluate whether the recessive trait was determined by the presence of HF1 mutations combined with the -257T, the 2089G and the 2881T disease-associated polymorphic variants. To this purpose the parents and all available relatives from nine patients carrying HF1 mutations were analysed for the above polymorphisms and the transmitted alleles were reconstructed. In five out of nine families we found that the affected patients had inherited the allele carrying the mutation from one parent and an allele carrying at least two of the three predisposing polymorphic variants (mostly the -257T and the 2881T) from the other parent. As an example, in Figure 2 we show the pedigree and haplotype reconstruction of family 081, in which the patient (R063#081) and also four unaffected relatives carry the mutation Glu1172Stop in SCR20. The patient has inherited the mutation from the father and also received an allele carrying three disease-predisposing polymorphisms (-257T, 2089G and 2881T) from the mother. In contrast the father and all the other healthy mutation carriers within the family carry the non predisposing variants of all the three above polymorphisms and may therefore be protected.

In addition, allele frequencies of disease-associated HF1 polymorphic variants were significantly higher in patients with HF1 mutations than in controls (Table 5), with the highest difference found for the 2881T ($P = 0.00021$). These data support the possibility that HF1 polymorphic variants may contribute to HUS development in mutation carriers.

Table 4. Allele frequencies of HF1 gene polymorphic variants occurring in controls and in patients

Exon	Mutation	SCR	Effect	Alleles	Controls	HUS	TTP	P-value HUS versus controls	P-value TTP versus controls
	C-257T	Promoter		C	73.6%	60.0%	81.0%	0.0069	0.24
				T	26.4%	40.0%	19.0%		
II	G257A	SCR1	Val44Ile	G	75.6%	80.8%	75.0%	0.37	0.93
				A	24.4%	19.2%	25.0%		
VII	C994A	SCR5	Silent	C	62.2%	78.3%	61.0%	0.01	0.87
				A	37.8%	21.7%	39.0%		
IX	T1277C	SCR7	Tyr405His	T	69.0%	78.0%	64.0%	0.38	0.57
				C	31.0%	22.0%	36.0%		
XI	G1492A	SCR8	Silent	G	55.0%	57.0%	58.0%	0.72	0.71
				A	45.0%	43.0%	42.0%		
XIV	A2089G	SCR11	Silent	A	83.0%	64.5%	90.0%	0.000052	0.19
				G	17.0%	35.5%	10.0%		
Intron XVI	A2489-30C	/	Silent	A	58.5%	74.7%	56.0%	0.037	0.74
				C	41.5%	25.3%	44.0%		
XIX	G2881T	SCR16	Glu963Asp	G	82.0%	63.0%	87.0%	0.000039	0.38
				T	18.0%	37.0%	13.0%		

DISCUSSION

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

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

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

deletion of the last C-terminus SCRs of HF causes loss of the ability of the protein to control spontaneous activation of the alternative complement pathway on host cells surface (37). In addition functional studies by two independent groups (36,38) documented that five of the mutations in SCR20 reported in patients with HUS, namely Glu1172Stop, Arg1210Cys, Arg1215Gly, Trp1183Leu and Val1197Ala (36,38), cause reduced binding of the protein to surface-bound C3b, to heparin and to endothelial cells. In contrast, the mutant proteins have a normal capacity to bind fluid-phase C3b and to act as cofactors for C3b proteolysis by factor I (38). This may explain why, in patients with mutations in the C terminus of HF, C3 serum levels are in general only moderately reduced or even normal, while strong deposition of C3 (39) and C5b-9 (40) is found in glomeruli and renal arterioles.

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

Five patients with HF1 mutations were given a renal transplantation. In all of them the graft failed because of disease recurrence. Since HF is mostly synthesized by the liver (34), the genetic defect was not corrected by the kidney transplant and persistent HF deficiency predisposed to disease recurrence on the transplanted kidney. In an additional patient with HF1 mutation HUS recurrence was prevented by a simultaneous liver transplant, which corrected HF dysfunction (20). For still

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

Genetic variant	Alleles	HUS HF1 mutation	HUS no HF1 mutation	Controls	P-value, HUS HF1 mutation versus controls	P-value, HUS no HF1 mutation versus controls
C-257T	C	53.2%	62.1%	73.6%	0.017	0.027
Promoter	T	46.8%	37.9%	24.6%	0.017	0.027
A2089G	A	62.5%	65.0%	84.0%	0.0065	0.00020
SCR 11	G	37.5%	35.0%	16.0%	0.0065	0.00020
G2881T	G	53.0%	65.6%	82.0%	0.00021	0.00067
SCR 16	T	47.0%	34.4%	18.0%	0.00021	0.00067

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

Variant	Genotypes	HUS patients	Controls
C-257T*	CC	25	59
Promoter*	CT	44	38
	TT	9	9
	Total	78	106
A2089G ^b	AA	29	72
SCR11 ^a	AG	40	32
	GG	7	2
	Total	76	106
G2881T ^c	GG	27	70
SCR16 ^c	GT	43	34
	TT	7	2
	Total	77	106

*P-value (general) = 0.0062; P-value (CT + TT) = 0.0015.

^bP-value (general) = 0.00016; P-value (AG + GG) = 0.000067.^cP-value (general) = 0.000084; P-value (GT + TT) = 0.000034.

unknown reasons, premature liver failure, cured by a second liver graft in the above case (20) and fatal in a second one (manuscript in preparation), complicated the outcome of the combined liver and kidney transplants. Thus, in patients with HF1 mutations, a single kidney graft is contraindicated and a combined kidney and liver transplant is not recommended at the moment because of the high risk of liver graft failure. On the contrary, in patients without HF1 mutations, half of the kidney grafts were still functioning at 1 year post-transplant.

Of note, 67% of patients with HUS in our series did not have HF1 mutations. These data would suggest the existence of at least another susceptibility gene for HUS. That this gene could be involved in the regulation of the alternative pathway of complement is supported by finding of lower than normal C3 levels in more than 50% of HUS patients with no HF1 mutations.

We also evaluated the possibility that common polymorphisms of HF1 gene may contribute to development of HUS in patients without HF1 mutations. Actually, analysis of allele frequency and genotype distribution of HF1 polymorphisms in patients and controls revealed an association between HUS and three HF1 polymorphic variants. Specifically, the T allele of the C-257T, the G allele of the A2089G and the T allele of the G2881T polymorphisms were more frequent in HUS patients than in controls and odds ratio values indicate that carriers of each polymorphic variant have a higher risk of developing HUS than non-carriers. The combined presence of two and

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

Genotypes	Odds ratios (95% confidence interval)	P-value
Promoter (TT + CT)	2.66 (1.45–4.90)	0.0015
SCR11 (GG + AG)	3.43 (1.85–6.36)	<0.0001
SCR16 (TT + GT)	3.60 (1.94–6.67)	<0.0001
Promoter + SCR11	4.22 (2.23–7.99)	<0.0001
(TT + CT) + (GG + AG)		
Promoter + SCR16	3.82 (2.04–7.15)	<0.0001
(TT + CT) + (TT + GT)		
SCR11 + SCR16	4.33 (2.29–8.19)	<0.0001
(GG + AG) + (TT + GT)		
Promoter + SCR11 + SCR16	4.44 (2.31–8.55)	<0.0001
(TT + CT) + (GG + AG) + (TT + GT)		

three of the above variants on at least one allele was more strongly associated with HUS than the presence of a single one. Consistently, increased odds ratios for the risk of developing HUS were found in carriers of two or three of the above polymorphisms. Interestingly, when only data from patients without HF1 mutations were considered we found a stronger association between HUS and the simultaneous presence of the three predisposing polymorphic variants than by analysing data from the overall HUS population.

The first genetic variant, namely a C-257T, is located in an NFkB responsive element within the HF1 promoter (31), which could have a role in regulating HF transcription. Although we found no difference in HF levels among patients carrying the T or the C allele when studied at remission, this polymorphic variant probably affects the efficiency of NFkB-induced HF transcription in conditions of infection or inflammation (41–43). The second polymorphism, an A2089G located in exon XIV, although silent, could theoretically influence either the efficiency of transcription or the mRNA stability or just be in linkage disequilibrium with the other two polymorphisms. Finally, we found association between the disease and the polymorphic variant G2881T, determining a Glu963Asp change in SCR16 of HF. Of interest, two of the mutations in HF1 found in our patients were also located in SCR16, a few amino acids away from the Glu963Asp polymorphism, which suggests that a functional site located in SCR16 may be crucial to protect from disease development.

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

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

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

Promoter and SCR11 ^a			Promoter and SCR16 ^b			SCR11 and SCR16 ^c		
Genotypes	HUS patients	Controls	Genotypes	HUS patients	Controls	Genotypes	HUS patients	Controls
CCAA	19	53	CCGG	20	47	AAGG	24	67
CCGA	4	5	CCGT	5	8	AAGT	5	6
CCGG	0	0	CTTT	0	0	AATT	0	0
CTAA	9	16	CTGG	6	17	AGGG	1	5
CTGA*	34	16	CTGT*	36	21	AGGT*	38	23
CTGG*	1	0	CTTT*	1	0	AGTT*	0	2
TTAA	1	7	TTGG	1	4	GGGG	0	0
TTGA*	2	7	TTGT*	2	4	GGGT*	0	0
TTGG*	6	2	TTTT*	6	3	GGTT*	7	2
Total	76	106	Total	77	104	Total	75	105

^aP-value (general) = 0.000068; P-value (*) = 0.0000057.

^bP-value (general) = 0.0028; P-value (*) = 0.000019.

^cP-value (general) = 0.00005; P-value (*) = 0.0000037.

available relatives, to evaluate whether they could discriminate between disease-affected and healthy mutation carriers in our families. Tracking the mutations back to the former generation was possible in nine of our patients; interestingly in eight of them the mutation was inherited from the father while only in one the mutation carrier was the mother. None of the ancestors had episodes of HUS. This could be explained either as autosomal dominant transmission with reduced penetrance or as a recessive trait. The latter was confirmed in one family (029) in which the affected subjects carried an homozygous HF1 deletion in exon XXII. In an additional five families we found that the proband had inherited the allele carrying the mutation from one parent and an allele carrying at least two disease-associated HF1 polymorphic variants from the other

parent, which again would reproduce a recessive trait. By contrast, all the healthy mutation carriers in the above five families had inherited the protective HF1 polymorphic variants.

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

Table 9. Sequence of the primers used for HF1 gene screening

	Sense	Antisense
Promoter	5'-caagcactgcattcttggca-3'	5'-gctaggggaaattctccgttg-3'
SCR1	5'-cactttatgcacttattttgtttttattgttt-3'	5'-acacctagttttcataaaattccacaaatg-3'
SCR2A	5'-agattccactctacattgtatgagaaa-3'	5'-atgctaagcttctgttatttttttgg-3'
SCR2B	5'-tcttaattataaacctcttttctgtatggacta-3'	5'-tccctgtctattacataactaatcacaactttt-3'
SCR3	5'-ggatgatttttatcacacacatatttttcaca-3'	5'-ttccttagaaggaacagatgttttaaatg-3'
SCR4	5'-tttataaagatccagaaaaataaaggtaacatta-3'	5'-attatgtctctggttcacagcttctttaa-3'
SCR5	5'-tttaacggatcatttatttctgcattatcc-3'	5'-ttcagaattaaagaaatgggtcagaatag-3'
SCR6	5'-tgacctagaaaaccctaattggaatgt-3'	5'-caactatgtgctctctcttcttttoga-3'
SCR7	5'-tgaccaaatttatgtttctctcatttacttt-3'	5'-ttgccacaattaatagatgagctcttga-3'
SCR8	5'-atagataattgagaatgggtttatttctgaa-3'	5'-gttgagctgacacatccattcttct-3'
SCR9	5'-tcagattgttttattagatgacattagaaatga-3'	5'-cattcccttaacaattctcttatataattcaaa-3'
SCR10	5'-caacctcactttattgtggcatatg-3'	5'-aaaagtgtggtattcaaaagtcttaattcttatttc-3'
SCR11	5'-tataattgtaaaacagacaatttaacc-3'	5'-atcacaaaacacaaaagttttgacaag-3'
SCR12	5'-catcatgtttttcacaaataaactttttttg-3'	5'-ctttgttaaatgttgatttaagaagggtta-3'
SCR13	5'-aactattttttatgtaattgttggttgattcc-3'	5'-atcacacattctgaaaaaaaataagagctt-3'
SCR14	5'-gtgataatttatgaaacagttattg-3'	5'-agagaaattataacctcattgaaagaattatgt-3'
SCR15	5'-ttaaaccctttgtttttcattctctcatt-3'	5'-aacagaaattgagttatccattatggcc-3'
SCR16	5'-gatgtcatagtagctctctgtattctgtttattt-3'	5'-ccacttacactttgaaatgaagaattattatc-3'
SCR17	5'-gccactgggtctgggc-3'	5'-tctatatatogctatttttagaattccatttagatg-3'
SCR18	5'-cacttcttttttttctattctcagacacc-3'	5'-agaattggaattttaaaccacacacag-3'
SCR19	5'-tgaataatcagactcattcacaga-3'	5'-atacagtgctgtgttttggc-3'
SCR20A	5'-gttctgaaataaaggtgtgca-3'	5'-gccaaacagaagcttttatttc-3'
SCR20B	5'-ccccgtttacacacaaattcaa-3'	5'-ctacatagttgtgttttggat-3'

with experimental data showing that in an HF-deficient line of pigs, homozygous individuals die soon after birth from complement-mediated acute renal failure (44) and HF knockout mice develop membranoproliferative glomerulonephritis (45).

Further biochemical and functional studies on HF1 mutants and polymorphic variants will be required to provide deeper insights into the role of HF in the pathogenesis of HUS.

MATERIALS AND METHODS

Patients

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

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

Familial HUS or TTP was diagnosed in patients for whom at least two members of the family were affected by the disease at least 6 months apart, without evidence of a common triggering infectious agent (46). Recurrent HUS or TTP was diagnosed in patients who, with no familial history, had one or more relapses after complete and persistent remission of any sign of thrombotic microangiopathy (46). Finally, sporadic HUS or TTP was diagnosed in patients with only one reported episode of the disease. Patients with *E. coli*-associated diarrhoea-positive HUS were excluded from the study.

One-hundred and six healthy controls, matched for sex and geographic origin were also studied. All patients and controls were Caucasians. Patients and controls received detailed information on the purpose and design of the study and provided informed consent according to the guidelines of the Declaration of Helsinki. The protocol was approved by the Institutional Review Board of the Mario Negri Institute.

Complement profile assessment

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

Measurement of ADAMTS-13 activity

ADAMTS-13 activity was measured as previously described (29) using the collagen binding assay. The protease activity was tested using pooled normal plasma as source of VWF as substrate for ADAMTS-13. Human collagen type III (3 μ g/ml,

Valter Occhiena, Milano Italy) was used for the collagen binding assay. The values of the protease activity were read from a dose-response curve obtained with reference plasma pool. The lower limit of the assay was 6% of the normal protease levels (29).

Single-strand conformation polymorphism analysis and sequencing of factor H

Genomic DNA was extracted from peripheral blood leucocytes according to standard protocols (Nucleon BACC2 kit, Amersham, UK). HF1 was screened by PCR-SSCP. PCR reactions were done in a 20 µl volume containing 100 ng DNA, 15 pmol of each primer, constructed in order to avoid coamplification of factor H related genes (see Table 9), 16 nmol dNTP, 2.25 mM MgCl₂, 1 U Taq polymerase (Taq Gold, PE Applied Biosystems, Foster City, CA, USA), and PCR buffer. Ten minutes' denaturation at 94°C were followed by 35 PCR cycles (94°C for 45 s, 55.5°C for 30 s, and 72°C for 45 s), and by 10 min extension at 72°C. Samples were mixed with 20 µl of loading buffer, denatured at 65°C for 10 min, and electrophoresed on non denaturing 6% (62:1 acryl:bis) acrilamide gel in TAE buffer (pH 6.8) at 35 W for 3–5 h at 4°C. Gels were visualized by silver staining. If a polymorphism was identified, selected individuals showing the three different band patterns in SSCP were sequenced using a CEQ 2000 XL sequencer (Beckman Coulter, Berkeley, CA, USA) in order to identify the corresponding three genotypes. Those subjects were subsequently used as standards in SSCP. Subjects showing aberrant bands were also sequenced.

Statistical analysis

Differences between allele frequencies and genotype distribution of HF1 gene polymorphisms among patients with HUS, TTP and controls were analysed by the chi-square test. The odds ratios for these comparisons were also calculated. Data were expressed as mean ± SD. A *P*-value ≤ 0.05 was considered to be statistically significant. The two tailed *t*-Student test was used to compare C3, C4, C3d and HF levels in our populations.

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**Complement factor H mutation in familial thrombotic thrombocytopenic
purpura with ADAMTS13 deficiency and renal involvement**

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Complement Factor H Mutation in Familial Thrombotic Thrombocytopenic Purpura with ADAMTS13 Deficiency and Renal Involvement

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Thrombotic thrombocytopenic purpura is a rare disorder of small vessels that is associated with deficiency of the von Willebrand factor–cleaving protease ADAMTS13, which favors platelet adhesion and aggregation in the microcirculation. The disease manifests mainly with central nervous system symptoms, but cases of renal insufficiency have been reported. Presented are findings of the genetic basis of phenotype heterogeneity in thrombotic thrombocytopenic purpura in two sisters within one family. The patients had ADAMTS13 deficiency as a result of two heterozygous mutations (causing V88M and G1239V changes). In addition, a heterozygous mutation (causing an S890I change) in factor H of complement was found in the patient who developed chronic renal failure but not in her sister, who presented with exclusive neurologic symptoms.

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Thrombotic thrombocytopenic purpura (TTP) is a disease of small vessels characterized by anemia that is caused by erythrocyte fragmentation in the microcirculation and thrombocytopenia that is caused by intravascular thrombi of aggregated platelets (1). Recent studies provided substantial evidence that 70 to 80% of cases of TTP are triggered by a deficiency of ADAMTS13 (2–4), a plasma metalloprotease that cleaves von Willebrand factor multimers soon after their secretion by endothelial cells (1,5–7). ADAMTS13 deficiency can be constitutive, as a result of homozygous or double heterozygous mutations in the corresponding gene (8–13), or acquired, as a result of the presence of circulating inhibitory antibodies (1,3,4,14–20).

TTP manifests mainly with central nervous system symptoms, but cases of renal insufficiency have been reported (1). In rare cases, renal involvement is severe enough to cause end-stage renal failure (1,21–25). Those patients' clinical manifestations are difficult to distinguish from those of hemolytic uremic syndrome (HUS), a form of thrombotic microangiopathy characterized by predominant renal involvement, often with renal failure (1,20,26). This difficulty has given rise to a heated debate on whether a severe deficiency of ADAMTS13 activity is enough to distinguish TTP from HUS (27,28).

Here we present findings of the genetic basis of phenotype

heterogeneity in patients with congenital ADAMTS13 deficiency. We studied a family with two affected sisters, one who presented with exclusive neurologic symptoms and the other one with severe renal involvement that required chronic dialysis. These diverse clinical manifestations suggested to us that the genetic background could be different.

Materials and Methods

Patients

A woman, now 60 yr old (F48), and her younger sister (F45, died in 2002 at the age of 55 yr) were referred to our International Registry of Recurrent and Familial HUS/TTP in 1996 because of history of recurrent and familial thrombotic microangiopathy. The youngest brother died at the age of 15 yr of leukemia. The other four siblings (three male and one female) all seem to be healthy and have no sign of thrombotic microangiopathy. The two patients had their first episode of the disease at the age of 22 (F45) and 23 yr (F48), respectively, during their first pregnancy and subsequently experienced at least 10 disease relapses concomitant with precipitating events such as pregnancy and spontaneous abortion in the first to second trimester or infection.

In patient F48, neurologic symptoms (dysarthria, dyslalia, aphasia, and facial paralysis) were dominant. Renal function always remained normal, and urine was always negative for proteinuria and hematuria. Remission was achieved with plasma infusions, antiplatelet agents, and anticoagulants, and at present, she is in relatively healthy condition.

In patient F45, the course of the disease was more severe. After a first mild episode of hemolytic anemia and thrombocytopenia during the third month of her first pregnancy (recovered after spontaneous abortion), she had a severe relapse at the age of 23 yr, during the fifth month of her second pregnancy. This was accompanied by confusion, psychomotor agitation, and also coma. She received blood transfusions, steroids, and anticonvulsants. Remission was achieved after spontane-

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ous abortion. After two other spontaneous abortions, each associated with disease relapses, she underwent salpingostomy. Nevertheless, she continued to have relapses, triggered by infection and associated with neurologic signs and acute renal insufficiency. Transient improvement was achieved by plasma infusions, but then renal function progressively deteriorated. At the age of 44 yr, the patient started chronic dialysis. She died at the age of 55 yr because of a cerebrovascular event.

The two patients and all of their available relatives were screened for biochemical and genetic abnormalities of both ADAMTS13 and complement regulatory proteins. One hundred healthy volunteers were also studied as control subjects.

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

ADAMTS13 Activity and Antigen

ADAMTS13 activity was measured using the collagen binding assay (20). The protease activity was tested using pooled normal plasma as the source of von Willebrand factor as substrate for ADAMTS13. Human collagen type III (3 µg/ml; Valter Occhiena, Milano, Italy) was used for the collagen binding assay. The values of the protease activity were read from a dose-response curve obtained with reference plasma pool. The lower limit of the assay was 6% of the normal protease levels (20). The presence of ADAMTS13 inhibitory activity was assayed by testing ADAMTS13 activity in mixtures of test plasma and normal pooled plasma at different dilutions (20).

Plasma ADAMTS13 antigen levels were evaluated with an ELISA assay using polyclonal rabbit anti-human ADAMTS13 antibodies. Recombinant human ADAMTS13 was used as standard (29).

Complement Profile

Serum C3 and C4 concentrations were measured by nephelometry. Factor H serum concentrations were assayed by radial immunodiffusion (The Binding Site, Birmingham, UK) (30).

Microsatellite Polymorphism Genotyping

Genomic DNA was extracted either from peripheral blood leukocytes or directly from whole blood according to standard protocols (Nucleon BACC2 kit; Amersham, Little Chalfont, UK). For linkage analysis, we used microsatellite markers flanking complement factor H and membrane co-factor protein (MCP) (31) (chromosome 1q32: D1S240, D1S202, D1S412, D1S2816, D1S413, D1S456, D1S2796, D1S2692) and ADAMTS13 (8) (chromosome 9q34: D9S1847, D9S164, D9S1818, D9S1826, D9S158, D9S1838) genes, identified using the Genome Data Base. Primers were synthesized by Sigma (Sigma-Aldrich, Haverhill, UK). For each PCR reaction, we used 100 ng of DNA in 20-µl final volume that contained 15 pmol of each primer, 16 nmol of dNTP, 2.25 mM MgCl₂, 1 U of Taq polymerase (TaqGold; PE Applied Biosystems, Foster City, CA), and PCR buffer. PCR was performed as follows: 10-min denaturation at 94°C, followed by 35 PCR cycles (94°C for 45 s, 55.5 to 57°C for 45 s, and 72°C for 1 min), and by 10-min extension at 72°C. Amplified DNA samples were mixed with an equal volume of loading buffer, denatured at 75°C for 5 min, and electrophoresed on denaturing 6% acrylamide gel (19:1, acrylamide:bis-acrylamide) in TBE buffer, at 55 W for 2 to 4 h at room temperature. Gels were visualized by silver staining.

Screening for Mutations

Factor H and MCP genes were screened by PCR-single-strand conformation polymorphism (31,32). PCR reactions were done in 20-µl

volume that contained 100 ng of DNA, 15 pmol of each primer (Table 1) (31,32), constructed to avoid co-amplification of factor H-related genes, 16 nmol of dNTP, 2.25 mM MgCl₂, 1 U of Taq polymerase, and PCR buffer. Ten-minute denaturation at 94°C was followed by 35 PCR cycles: 45 s at 94°C, 45 s at 55.5 to 57°C, 1 min at 72°C, and a 10-min step at 72°C. Samples were mixed with 20 µl of loading buffer, denatured at 65°C for 10 min, and electrophoresed on nondenaturing 6% (62:1 acrylamide:bis-acrylamide) gel in TAE buffer (pH 6.8) at 35 W for 3 to 5 h at 4°C. Gels were visualized by silver staining. Patients who showed aberrant bands were sequenced.

The complete coding sequence and intronic boundaries (Table 1) of ADAMTS13 gene were analyzed by direct sequencing after purification from agarose gel (1% in TBE) of PCR products (kit QUIAEXII; Qiagen, Hilden, Germany). PCR reactions were performed as above. Amplified DNA was sequenced on both strands using a CEQ8000 XL sequencer (Beckman Coulter, Berkeley, CA), following standard protocols. To exclude that mutations were rare polymorphisms, single-strand conformation polymorphism was performed as above on DNA from patients and 100 control subjects.

Results and Discussion

ADAMTS13 activity was <6% (detection limit of the assay) in the two sisters with a diagnosis of TTP. Anti-ADAMTS13 inhibitors were not found, thus excluding an acquired deficiency. It is interesting that complete ADAMTS13 deficiency was also found in a younger brother (F50), even though he had never had any episode of thrombotic microangiopathy. Microsatellite polymorphism genotyping, using polymorphic markers on chromosome 9q34 flanking ADAMTS13 gene, showed a straight correlation between ADAMTS13 activity and haplotype data: The three subjects with complete ADAMTS13 deficiency shared both alleles, whereas subjects with half normal levels shared one allele (Figure 1A). By direct sequencing, a heterozygous G323A missense mutation located in exon 3, which causes a V88M change in the metalloprotease domain, was found in the patients and in their healthy brother with protease deficiency (Figure 2A). In the same subjects, we also found a second heterozygous mutation, a G3777T in exon 27, causing a G1239V change in the first ADAMTS13 CUB domain (8). Neither mutation was found in any of 100 unrelated healthy subjects.

In this family, ADAMTS13 antigen levels paralleled protease activity. The three subjects who carried the two mutations had undetectable ADAMTS13 activity and <10% normal mean plasma antigen levels as measured by ELISA (Figure 1A). These results are consistent with published data showing that most ADAMTS13 missense mutations that are found in patients with TTP result in impaired secretion of the protein (33). Subjects F49 and F51 with a single heterozygous mutation, the G1239V and the V88M, respectively, had approximately half normal protease activity and antigen levels. However, subject F46 without mutation had normal protease activity and antigen levels (Figure 1A).

It has been proposed that a severe deficiency of ADAMTS13 is a beacon for patients with a specific form of thrombotic microangiopathy, labeled as TTP, and could also help in tailoring treatment (27). However, evidence is emerging that different clinical presentations may reflect complex underlying ge-

Table 1. Primers used for ADAMTS13 and factor H mutation screening*

	Sense (5'-3')	Antisense (5'-3')
ADAMTS13		
exon 1	CCCTGAACTGCAACCATCTT	CAAACCCCAAAGCTGATGTA
exon 2	TCGGTCTCCCCAAGTGTTAG	AACAGGGTTGACAGCAGCTT
exon 3	TCTAGAACCATCGCCCTCTG	CCGAGCCATTCTACCTGAGT
exon 4	ACATGCTGGTGGAGTAGCCTCT	GATGGAGATGGGATGACTTGG
exon 5-6	ACGGGCTAGTCATAGGCTTG	TACAAGGACCCACTGCTTGC
exon 7	GCTGGCGCTGCGGCACTAGGG	GTTGGACGGAGGGGTGGGTTG
exon 8	ACTCTCCGTCGCGCTGCTG	GGCCAGTCAAACAAAATGT
exon 9	TCTGGGAGGGACAGTTAAGG	TACTGGTCTGCCTCTGAC
exon 10-11	GGGTACCTATGGGTGAGTT	CCTGGTGTGAACACAGATG
exon 12	GCACTTTTGTACCCCCAGTT	CCAGAGCCTGAACCACTTTG
exon 13-14	CCCAGATGCAAAGGATGAAG	ATCCAGGGCTGAGTGAGTGT
exon 15	TTTTTCCCGACCAGCTAAGA	TCAGAAGTGAGGGCATCTTG
exon 16	CCGGGAAGGAGAGTCACTG	CCCTGTAAGTGACCGCTGA
exon 17-18	GTGATTGCTTGTCTGAACGAA	CAGTGTCTCACCTGCAGAA
exon 19	GAACACCTGGAGAGGCTAGG	ACTTACAACCGCCAGGTGAC
exon 20	GAACCTGCTGGCTGATGAAT	GGATGGTGTCTTGCTCTGG
exon 21	CACACACGCCACTTCTCTG	CCACGTGTCCCATSTSTGCTG
exon 22	CCATGCGGGCCTTATGTGCTA	TCTGGGTGTCAGTCTCAAAG
exon 23	TCCACGCTTCTGTCTCTTC	TCTCCTGATTGAGCTTTCCAA
exon 24	AGTACACGTGGGTGGAGAGG	CTTTCAGGGGACACGATGAG
exon 25	TTAACTGCCTCCAGCTTCT	CTTTGCCAGGGAGAAAGAGG
exon 26	CCTCTGGTCTCCTTCTCAGCTTG	TGCCAGGAATGGGGCATGCAGCGT
exon 27	AGCACCTTGAGCCAAACAGGA	CTAGACATACCCGCACTGCAGT
exon 28	CTTGAACCTGGCTCAGTCTACCCTG	TCCCTGGCAGCTGCAGACTGA
exon 29	GTTGTCTGCGCTCTGGCA	GGGTCCCCTAGCCCTGC
Factor H		
promoter	CAAGCACTGCATTCTTGGCA	GCTAGGGAAATTTCTCCGTTG
SCR1	CACCTTATGCATTATTTTGTATTGTT	ACACCTAGTTTTCATAAATTCACAAATG
SCR2A	AGATTCCACTCTACATTGTATGAGAAA	ATGCTAACGTTCTGTTATTTTGGT
SCR2B	TCTTAATTATAAACCTCTTTTGTATGGACTA	TCCTTGCTATTACATACTAATTCATAACTTTT
SCR3	GGATGATTTTATACATACATATTTTTCACA	TTCTTGAAGATGAACGATGTTTAAATG
SCR4	TTTATAAAGATCCAGAAAAATAAGGTAACATTA	ATTATGTCCTGGTCACAGTCTTTAA
SCR5	TTTAACGGATACCTATTCTGCATTATCC	TTCAGAATTAAGAAATGGGTCAAGATATG
SCR6	TGACCTAGAAAACCTAATGGAATGT	CACCTATGTGCTCTCTTTCTTCGA
SCR7	TGAGCAAATTTATGTTTCTCAATTACTTT	TGCCACAATTAATATAGATGAGTCTTAGA
SCR8	ATAGATATTGAGAAATGGGTTTATTCTGAA	GTGAGCTGACCATCCATCTTTC
SCR9	TCAGATTGTTTATTAGATGACATTAGAAATGA	CATTCTTAACAATTCCTCTATATAATTCAAA
SCR10	CAACCTCACCTTATTGTGGCATATG	AAAAGTTGGTATTCAAAGTTCTAATTCITATTC
SCR11	TATATTGTAAAACAGACAATTTAACC	ATACAAAATACAAAAGTTTTGACAAG
SCR12	CATCATGTTTTCACAATAAATCTTTTGTG	CTTTGTAAATGTTGATTTAAGAAGGGTA
SCR13	AACTATTTTTATGTAATAGTTGGTTTGATTCC	GAATACATTTCTGAAAACAAAATAAGAGCTT
SCR14	GTGATAATTTATGAAACAGTTATTG	AGAGAATATTAACCTCATTGAAAGAATTATGT
SCR15	TTAACCTTTGATTTTCATTCTTCATT	AACAGAAAATTGAGTATTCATATTGGCC
SCR16	GATGTCATAGTAGCTCTGTATTGTTTATT	CCACTTACACTTTGAATGAAGAATATTTATC
SCR17	GCCACTGGGCTGGGC	TCTATATATCGCTATTTTGAATCCATTAGATG
SCR18	CACCTCTTTTTTCTATTTCAGACACC	AGAATTGAATTTTAAGCACCATCAG
SCR19	TGAAATATCAGACTCATCAGACA	ATACAGTGCTGTGTTGGC
SCR20A	GTTCTGAATAAAGGTGTCAC	GCCAAACAGAGCTTTATTTC
SCR20B	CCCCGTTTACACACAAATTCAA	CTACATAGTTGGTTGGAT

*SCR, short consensus repeat.

A:

B:

Figure 1. Linkage analysis for chromosome 9q34 (ADAMTS13; A) and chromosome 1q32 (factor H and membrane co-factor protein [MCP]; B) in the pedigree. Circles indicate female individuals; squares indicate male individuals. Affected individuals are indicated by solid symbols, and carriers are indicated by symbols with dots. (A) Six markers (pink square) flanking the ADAMTS13 gene were selected from Genome Data Base for haplotype analysis. Chromosomes that carry the mutations are colored (V88M, yellow; G1239V, green), and normal chromosomes are white. Plasma ADAMTS13 activity and antigen levels are shown below the genotypes. Normal levels, ADAMTS13 activity (range, 50 to 150%); ADAMTS13 antigen (range, 680-1350 ng/ml). (B) Eight markers (blue square) flanking the factor H and MCP genes were selected from the Genome Data Base for haplotype analysis. Chromosomes that carry the mutation are red, and normal chromosomes are white. Serum factor H, C3, and C4 levels are shown below the genotypes. Normal levels: factor H, 350 to 750 mg/L; C3, 83 to 177 mg/100 ml; C4, 15 to 45 mg/100 ml. In the two patients, both ADAMTS13 and complement parameters were measured at remission.

netic abnormalities, because ADAMTS13 deficiency results in a very heterogeneous pattern of clinical manifestations, ranging from no obvious clinical symptoms (9; present article), to prevalent neurologic signs (8-13), to neurologic signs and renal involvement (which may vary from mild urinary abnormalities to severe renal dysfunction requiring dialysis) (20-25). Such heterogeneity can occur even within individuals who carry the same ADAMTS13 genotype as exemplified by the family that we report. In this family, three siblings presented with complete ADAMTS13 deficiency. One had no sign of thrombotic microangiopathy. Another manifested pure neurologic symptoms and responded well to plasma therapy. The third had very severe renal failure and responded poorly to plasma. We hypothesized that modifier genes caused more severe disease and the renal phenotype in the last subject. We focused on

genes encoding for the complement regulatory proteins factor H and MCP (34,35), because mutations in those genes have been associated with 30 to 40% familial cases of HUS and result in localized manifestations in the kidneys (31,32,36-39).

The two affected sisters had different genotypes (Figure 1B) on the area of chromosome 1q32, where factor H and MCP genes are mapped. The MCP gene was normal. However, a new heterozygous G2742T mutation in exon 18 (Figure 2B) was found in the factor H gene in patient F45, who developed chronic renal failure, but not in patient F48, who did not. The mutation that was not found in any of 100 unrelated healthy subjects causes a S890I change in short consensus repeat (SCR) 15 of factor H, which was reported recently as a hot spot (in addition to SCR20) for mutations in patients with HUS (40). It is tempting to speculate that in patient F45, factor H haplo-

A

B

Figure 2. Mutation results. (A) Sequence of ADAMTS13 exons 3 (a) and 27 (b) showing G323A (V88M) and G377T (G1239V) heterozygous mutations, respectively. (B) Sequence of factor H exon 18 showing a G2742T (S890I) heterozygous mutation.

sufficiency caused uncontrolled complement activation and C3b deposition followed by microangiopathic injury in the kidney that superimposed on the systemic thrombotic microangiopathy caused by ADAMTS13 deficiency. Lower than normal serum C3 concentrations and normal C4 levels were found in patient F45, which would indicate that factor H mutation resulted in activation of the alternative pathway of complement (Figure 1B). As expected, patient F48, who does not carry the factor H mutation, had normal C3 and C4 serum concentrations.

It is interesting that the S890I factor H mutation was also found in three unaffected brothers, including F50, who also carries the ADAMTS13 defect, and in an unaffected sister (Figure 1B). In addition, the two patients experienced the first episode of thrombotic microangiopathy in adulthood during pregnancy. Together, these data show that ADAMTS13 and factor H gene mutations predispose to thrombotic microangiopathy and organ dysfunction and that triggers including pregnancy and viral or bacterial infections seem to play a relevant role in the full manifestation of the disease.

These results may not explain all cases of renal involvement in patients with TTP and ADAMTS13 deficiency, and addi-

tional studies, including a higher number of patients screened for factor H and for other HUS-associated genes, such as MCP and factor I, are required. However, this data disclose for the first time the genetic and phenotypic complexity of TTP and might provide a genetic explanation for cases of clinical syndrome overlapping with HUS.

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Familial haemolytic uraemic syndrome and an MCP mutation

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Mechanisms of disease

Familial haemolytic uraemic syndrome and an MCP mutation

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Summary

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

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

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

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

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See Commentary page 1514

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Introduction

Haemolytic uraemic syndrome (HUS) is a rare disease of microangiopathic haemolysis, thrombocytopenia, and renal failure.^{1,2} The most common form of HUS in children, with predominant renal failure, is associated with infection by *Escherichia coli*, which produce a powerful Shiga-like toxin.¹ This form of the disease (D⁺HUS) usually presents with a diarrhoea prodrome and has an excellent prognosis in most cases.³ By contrast, non-Shiga toxin-associated and diarrhoea-negative forms of HUS (D⁻HUS) have a much poorer outcome (often end-stage renal failure or death^{4,5}), with patients prone to relapse. There is sometimes a clustering of affected individuals within families, suggesting a genetic predisposition to the disease. Both autosomal dominant and autosomal recessive forms of inheritance have been noted, with precipitating events such as pregnancy, virus-like disease, or sepsis reported in some instances.^{6–11} Low serum concentrations of the third component of complement (C3) have been identified in patients with D⁻HUS.^{12,13} Among such patients, a subgroup—between 13% and 30%—carry mutations in the gene encoding for factor H (HF1), a plasma protein that inhibits the activation of the alternative pathway of complement.^{14–16} However, two thirds of patients with D⁻HUS have no HF1 mutations, despite decreased C3 concentrations,^{17,18} indicating a role for genetic abnormalities in other complement regulatory proteins.

Methods

Participants

Between, 1996, and May, 2003, we enrolled consecutive patients with familial, recurrent, or sporadic D⁻HUS with no HF1 mutations but an abnormal serum complement profile (defined as C3 serum concentrations <0.83 g/L¹⁹ or a plasma C3d/serum C3 ratio >0.015) through the International Registry of Recurrent and Familial HUS/TTP (HUS/thrombotic thrombocytopenic purpura), a network of 60 Haematology and Nephrology Units established under the coordination of the Clinical Research Centre for Rare Diseases "Aldo e Cele Daccò". We also recruited healthy blood donors as controls. For protein expression studies in peripheral blood mononuclear cells (PBMC), healthy female controls and uraemic female controls on chronic haemodialysis for causes other than HUS, were recruited.

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

Procedures

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

GLOSSARY

CLASSICAL AND ALTERNATIVE COMPLEMENT PATHWAYS

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

SINGLE-STRANDED CONFORMATIONAL POLYMORPHISM (SSCP) ANALYSIS

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

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

We measured plasma ADAMTS13 activity as previously described,¹⁰ using the collagen binding assay. The presence of ADAMTS13 inhibitory antibodies was assessed by testing ADAMTS13 activity in mixtures of plasma taken from patients and from a plasma pool at different dilutions after 30 min incubation at 37°C.²⁰

To identify the causative gene(s) of HUS in patients with no *HF1* mutations, we looked at abnormalities in the complement regulatory proteins—factor H related 5 (*FHR5*),²¹ complement receptor 1 (*CR1*),²² and membrane cofactor protein (*MCP*).²³ We extracted genomic DNA from peripheral blood, according to standard protocols (Nucleon BACC2 kit, Amersham, UK). The coding sequences of *HF1*, *FHR5*, *CR1*, and *MCP* were screened by PCR-SINGLE STRANDED CONFORMATIONAL POLYMORPHISM ANALYSIS (PCR-SSCP), using primers designed on published genomic sequences (NT-004671, NT-021877).^{18,20,24} A list of primers used for *MCP* gene analysis is reported in table 1. We did PCR reactions in a 20 μ L volume, containing 100 ng DNA, 15 pmol of each primer, 16 mmol deoxynucleoside triphosphates (dNTP), 2.25 mmol/L magnesium chloride, 1 U Taq polymerase (Taq Gold, PE Applied Biosystems, Foster City, CA, USA), and PCR buffer. 10 min denaturation at 94°C was followed by 35 PCR cycles (94°C for 45 s, 55–57°C for 30 s, and 72°C for 45 s) and by 10 min extension at 72°C. We mixed samples with 20 μ L of loading buffer, denatured them at 65°C for 10 min, and electrophoresed onto non-denaturing 6%

(62/1 acryl/bis) acrylamide gel in TAE buffer (pH 6.8) at 35 Watt for 3–5 h at 4°C. We visualised gels by silver staining. Aberrant bands were sequenced.

We did expression studies in PBMC, which we separated by density gradient centrifugation with Ficoll-Paque, according to standard procedure. PBMC were incubated with a fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibody against human MCP (20 μ L/10⁶ PBMC, BD Biosciences Pharmingen, San Diego, CA, USA) or with FITC-mouse IgG (isotype control), and analysed by FACSort (BD Biosciences, Mountain View, CA, USA).

Role of the funding source

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

Results

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

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

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

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

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

Function	Primer	
Exon	Sense	Antisense
1	5'-CTGGATGCTTTGTGAGTGGG-3'	5'-TCTTGCCCGACTGAGGAGAG-3'
2	5'-ACTTCATCTTCATGTTCTTCTCTCTTC-3'	5'-ACCCCAAAATGTATGCAAACTCTCT-3'
3	5'-CAGATCTGTTTATTAACGTGGATGTAA-3'	5'-GAAGACAGCAAAACAAAATAAAAT-3'
4	5'-GTGCTCTTATTAATGCTATCAAAACAGT-3'	5'-AGAAACCTCTTTGGGATCTTTGTGA-3'
5	5'-TGCTTAATCTTTTCAATTTCTTCTCTCT-3'	5'-CACATACACCTGCTTTGTTTACTGT-3'
6	5'-CTTGCTCTGTTTCAACATGGAATTAAT-3'	5'-CAGCAACAACAAATACAAACCAAGA-3'
7, 8	5'-CCCAAGTGGTGTGATCTTCAACAT-3'	5'-ATAAGTGAACATCACCAGAAATTTGAA-3'
9	5'-TGTGATAGGCCCTGGTGAATTT-3'	5'-CCTGCAGCTGTGCACA-3'
10	5'-AAATACACCATGATGTTTAAAGGATTT-3'	5'-CCTACACGTTCTACACATACACCATTA-3'
11	5'-GGAGATCATGTTTCAACATCTT-3'	5'-AATGCATGCTCTACAAATAATTTTGTG-3'
12	5'-CAGAATATATGTCATTTGTTCTCTGG-3'	5'-AAGGACCAAGAAGTTAAAGAAACATG-3'
13	5'-TCGTTCTTTGTTGGTTGAAGTCA-3'	5'-GCAACCTTCTCTCATCTCTCTCT-3'
14	5'-GGCTTCTGGAATTAATTTCTGTACTTAA-3'	5'-GTCAAGATGAACCTGGCAACAC-3'

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

Table 1: Primers used for MCP screening

Parameter (normal range)	Proband	Brother
Platelets ($150-400 \times 10^9/L$)	178	247
Lactate dehydrogenase (230-460 U/L)	375	304
C3 (0.8-1.8 g/L)	0.5	1.3
C4 (0.2-0.5 g/L)	0.3	0.3
C3d (3.03-18.23 mg/L)	14.8	49.8
C3d/C3 (0.005-0.015)	0.031	0.039
Factor H (350-750 mg/L)	589.5	908
Factor B (191-382 mg/L)	210.5	262.7
Factor I (28-58 mg/L)	38.3	41.5
ADAMTS13 activity (50-150%)	68	116

Table 2: Biochemical data for proband and her brother

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

We noted reduced serum concentrations of C3 and a higher than normal C3d/C3 ratio in the proband, whereas C4 concentrations were within the normal range, which is consistent with a selective chronic activation of the alternative pathway of complement (table 2). C3 and C4 concentrations were normal in the proband's brother, however C3d concentration and the C3d/C3 ratio were higher than normal (table 2). In the parents, concentrations of C3 (father: 1.1 g/L; mother: 0.9 g/L) and C4 (father: 0.3 g/L; mother: 0.3 g/L) were within the normal range. Factor H serum concentrations were normal in the proband and in her mother (619 mg/L), but were higher than normal range in the father (938 mg/L) and in the brother (table 2). Factor B and factor I concentrations were within the normal range (table 2).

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

The proband and her affected brother were heterozygous for the 1160G→A polymorphism in *FHR5* and were homozygous for the C variant of the polymorphism 5507C→G in *CRI*, associated with a high expression allele (H).²⁴

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

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

Figure 1: Genetic studies of MCP

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

Figure 2: Expression studies of MCP protein

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

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

Discussion

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

MCP is a widely expressed transmembrane glycoprotein that regulates complement activation. It serves as a cofactor for factor I to cleave C3b and C4b when they are deposited on host cells.¹³ MCP has four extracellular contiguous modules important for its inhibitory activity, followed by a serine-threonine-proline rich domain, a transmembrane domain, and a cytoplasmic

tail (figure 1).¹³ Because the delA843-C844 mutation causes loss of the MCP C-terminus, comprising part of all of these domains, we reasoned that it might affect cell-surface expression of MCP through inhibition of insertion of the mutant protein into the plasma membrane; results of our expression studies in PBMC lend support to this notion.

Together, the findings of studies^{14–19} on *HF1*, showing an association between *HF1* deficiency and D-HUS, and our findings of an *MCP* gene mutation in two patients within one family make a strong case for impaired regulation of complement activation as a determinant factor of the disease in genetic forms of HUS. MCP is highly expressed in the kidney²⁰ and plays a major part in regulating glomerular C3 activation.²¹ Reduced expression of MCP in the presence of stimuli that activate the complement system—eg, infection, cytotoxic drugs, antibodies, or immune complexes—might prevent restriction of complement deposition on glomerular endothelial cells, leading to microvascular cell damage and tissue injury. That the father had had no clinical manifestations of HUS despite having the same *MCP* mutation as the proband and her affected brother is consistent with an autosomal-dominant mode of transmission with reduced penetrance, as noted in *HF1* mutation carriers.^{15–19} The incomplete penetrance of the disease in carriers of either *HF1*^{15–19} or *MCP* mutations indicates that D-HUS is a complex disorder that fully manifests in the presence of environmental factors and multiple genetic modifier loci. This notion accords with our finding that, in the pedigree studied, MCP expression was higher in the father than in the proband and in her affected brother. Genetically controlled variations in serum concentrations of *HF1* could also explain the incomplete penetrance of the disease in *MCP* mutation carriers. In this respect, *HF1* concentrations are within normal range in the proband, but above normal in the non-affected father. Similarly, *HF1* serum concentration was high in the proband's brother, who had a mild form of the disease despite the same *MCP* mutation. Our provisional interpretation is that in healthy individuals the wide range of variation in the *HF1* serum concentration can be irrelevant, but in an individual with an *MCP* mutation a higher than normal amount of *HF1* would be essential to compensate for the defective cofactor activity due to *MCP* haploinsufficiency.

On the basis of our data, we cannot rule out the possibility that the *MCP* mutation identified is unrelated to HUS and indicates a coincidental finding of a rare genetic variant. However, this possibility is unlikely since the mutation is present in two of 25 patients with D-HUS, but in none of 100 healthy controls. Additionally, MCP has a main role in complement regulation, which is defective in HUS. Genetic screening of *HF1* and *MCP* could unveil precious information for a more tailored clinical management of patients with D-HUS. Treatment of D-HUS relies on plasma exchange or infusion, however 50%¹⁴ to 75%¹⁸ of patients, often small babies, progress to end-stage renal disease and need replacement therapy. One of the most debated issues is whether kidney transplantation is feasible in HUS. Children with Shiga-

toxin associated HUS rarely progress to end-stage renal disease, but when they do so, transplantation often results in a good prognosis.²⁹ Notably, graft outcome is less favourable in children³⁰ and adults³¹ with D-HUS, with recurrences occurring in around 50% of patients and graft failure developing in all of them. Among patients with DHUS and *HF1* mutations, the recurrence range is 30–75%, according to different surveys.^{18,32} In patients of our registry, the five individuals with DHUS and *HF1* mutations who received a transplant had disease recurrence on the grafted kidney within a few weeks (range 1 week to 6 months) after surgery.¹⁸ In view of the fact that *HF1* is a plasma protein mainly of liver origin, indirect evidence suggests that a kidney transplant does not correct the *HF1* genetic defect (Noris M, unpublished). To speculate that a dysfunction in MCP, which is a membrane-bound protein highly expressed in the kidney, can be corrected by transplanting a normal kidney, is tempting. The graft, bearing wild-type MCP expressed on renal-cell surfaces, should conceivably be protected from disease recurrence.

Contributors

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

Conflict of interest statement
None declared.

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Disease of the month
Hemolytic uremic syndrome

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Hemolytic Uremic Syndrome

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Hemolytic uremic syndrome (HUS) is a disease of nonimmune (Coombs negative) hemolytic anemia, low platelet count, and renal impairment (1). Anemia is severe and microangiopathic in nature, with fragmented red blood cells (schistocytes) in the peripheral smear, high serum lactate dehydrogenase (LDH), circulating free hemoglobin, and reticulocytes. Platelet count is $<60,000/\text{mm}^3$ in most cases (1).

In children, the disease is most commonly triggered by Shiga-like toxin (Stx)-producing *Escherichia coli* (Stx-E. coli) and manifests with diarrhea (D⁺HUS), often bloody. Cases of Stx-E. coli HUS—approximately 25% (2)—which, however do not present with diarrhea, have also been reported (3). Acute renal failure manifests in 55 to 70% of cases (4–6); however, renal function recovers in most of them (up to 70% in various series) (1,3,6,7).

Non-Shiga toxin-associated HUS (non-Stx-HUS) comprises a heterogeneous group of patients in whom an infection by Stx-producing bacteria could be excluded as cause of the disease. It can be sporadic or familial (i.e., more than one member of a family affected by the disease and exposure to Stx-E. coli excluded). Collectively, non-Stx-HUS forms have a poor outcome. Up to 50% of cases progress to ESRD or have irreversible brain damage, and 25% may die during the acute phase of the disease (8–10). Genetic studies have recently documented that the familial form is associated with genetic abnormalities of complement regulatory proteins, and evidence is now emerging that similar genetic alterations can predispose to sporadic cases of non-Stx-HUS as well. Major recent advances in the field of Stx-HUS and non-Stx-HUS are summarized in Table 1.

Microvascular lesion of HUS consists of vessel wall thickening with endothelial swelling and accumulation of proteins and cell debris in the subendothelial layer, creating a space between endothelial cells and the underlying basement membrane of affected microvessels (1,3). In Stx-HUS, the lesion is mainly confined to the glomerular tuft and is noted in an early phase of the disease. Examination of biopsies taken several months after the disease onset showed that most glomeruli are normal, whereas 15 to 20% eventually became sclerotic (11,12). Arterial

thrombosis does occur but is uncommon and seems to be a proximal extension of the glomerular lesion (11,12).

Stx-Associated HUS

Epidemiology

In 70% of cases in North America and Western Europe, Stx-HUS is secondary to infection with the *E. coli* serotype O157:H7 (13–19). This serotype has a unique biochemical property (lack of sorbitol fermentation) as to render it readily distinguishable from other fecal *E. coli* (20). However, many other *E. coli* serotypes (O111:H8, O103:H2, O121, O145, O26, and O113 [13,16,21–23]) have been shown to cause Stx-HUS. Infection by Stx-producing *Shigella dysenteriae* serotype 1 has been commonly linked to Stx-HUS in developing countries of Asia (24) and Africa (25) but rarely in industrialized countries (26).

After exposure to Stx-E. coli, 38 to 61% of individuals develop hemorrhagic colitis and 3 to 9% (in sporadic infections) to 20% (in epidemic forms) progress to overt HUS (5,27). The overall incidence of Stx-HUS is estimated to be 2.1 cases per 100,000 persons/yr, with a peak incidence in children who are younger than 5 yr (6.1 per 100,000/yr), and the lowest rate in adults who are 50 to 59 yr of age (0.5 per 100,000/yr) (1). The incidence of the disease parallels the seasonal fluctuation of *E. coli* O157:H7 infections with a peak in warmer months, between June and September. In the United States, approximately 70,000 illnesses and 60 deaths have been attributed annually to Stx-HUS (28). In Argentina and Uruguay, *E. coli* infections are endemic and Stx-HUS is a common cause of acute renal failure in children (23,29,30), with an estimated incidence rate of 10.5 per 100,000/yr (31). An association between traditional extensive production of cattle with endemic HUS in Argentina has been proposed, as supported by detection of Stx-producing *E. coli* strains—mainly O8, O25, O103, O112, O113, O145, O171, and O174 serotypes—in stool samples from 39% of Argentine healthy young beef steers (31).

Stx-producing *E. coli* colonize healthy cattle intestine but also have been isolated from deer, sheep, goats, horses, dogs, birds, and flies (1,32). They are found in manure and water troughs in farms, which explains the increased risk for infection in people who live in rural areas. Humans become infected from contaminated milk, meat, and water—water-borne outbreaks have occurred as a result of drinking and swimming in unchlorinated water (21)—or from contact with infected animals, humans, or either's excreta (27,33,34) and occasionally through

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Table 1. HUS: Major advances in recent years^a

Stx-HUS	
1994–2004	Description of the crystal structure of Stx-1 and Stx-2 (46,52)
1993–2001	Specific Stx surface receptors (globotriaosylceramide, Gb3) were identified on human endothelial cells, platelets, monocytes, erythrocytes, and polymorphonuclear cells (57–66)
1995–2003	Identification of the molecular mechanisms by which Stx promotes leukocyte adhesion to endothelial cells and induces thrombus formation (71–74)
2004	Description of the beneficial effect of angiotensin-converting enzyme inhibitors on long-term renal outcome in children with renal sequelae after severe Stx-HUS (83,84)
2002–2003	Reports on the good outcome of kidney transplantation in children with Stx-HUS (85–87)
Non-Stx-HUS	
1999	Description of high incidence of hypocomplementemia (low C3 levels) in familial forms of non-Stx-HUS (98)
1998	Linkage mapping of familial HUS on human chromosome 1q32 containing the regulator of complement activation gene cluster (110)
1998–2004	Identification of 50 mutations in factor H gene in familial and sporadic non-Stx-HUS (99–102,110,114,119–121)
2002–2004	Localization (in SCR19–20) of the domain responsible for inactivation of surface-bound C3b by factor H (115–118)
2002–2004	Demonstration that mutations found in patients with non-Stx-HUS cause loss of the capability of factor H to bind polyanions on endothelial cells and extracellular matrix and to bind C3b (117,118,123)
2003	Mutations in another complement regulatory gene, MCP, in non-Stx-HUS (126,127)
1997–2003	Description of high incidence of recurrence on the kidney graft in patients with non-Stx-HUS (1,85,86,99–101,153,154)
2003–2004	Complement inhibitors are being clinically available (163–167)

^aHUS, hemolytic uremic syndrome; Stx, Shiga toxin; MCP, monocyte chemoattractant protein.

environmental contamination (17). Meat is contaminated at slaughter. Internalization of the microorganism during grinding renders it capable of surviving cooking (27). Fruits and vegetables may also be contaminated, including radish sprouts, lettuce, and apple cider. Unpasteurized apple juice has been implicated in several outbreaks (35). Person-to-person transmission has been reported in child care and long-term care facilities (27).

Clinical Phenotype

The disease is characterized by prodromal diarrhea followed by acute renal failure. The average interval between *E. coli* exposure and illness is 3 d (range, 1 to 8). Illness typically begins with abdominal cramps and nonbloody diarrhea; diarrhea may become hemorrhagic in 70% of cases usually within 1 or 2 d (36). Vomiting occurs in 30 to 60% of cases, and fever occurs in 30%. Leukocyte count is usually elevated, and a barium enema may demonstrate "thumb-printing," suggestive of edema and submucosal hemorrhage, especially in the region of the ascending and transverse colon. HUS is usually diagnosed 6 d after the onset of diarrhea (1). After infection, Stx-*E. coli* may be shed in the stools for several weeks after the symptoms are resolved, particularly in children <5 yr of age (1). Diagnosis rests on detection of Stx-*E. coli* in stool cultures. Serologic tests for antibodies to Stx and O157 LPS can be done in research laboratories, and tests are being developed

for rapid detection of *E. coli* O157:H7 and Stx in stools. Blood, diarrhea, fever, vomiting, elevated leukocyte count, extremes of age, and female gender as well as the use of antimotility agents (37) have been associated with an increased risk of HUS after *E. coli* infection (27).

Stx-HUS is not a benign disease. Seventy-percent of patients who develop HUS require red blood cell transfusions, 50% need dialysis, and 25% have neurologic involvement, including stroke, seizure, and coma (6,27,38). Although mortality for infants and young children in industrialized countries decreases when dialysis became available, as well as after the introduction of intensive care facilities, still 3 to 5% of patients die during the acute phase of Stx-HUS (6). A recent meta-analysis of 49 published studies (3476 patients, mean follow-up of 4 yr) describing long-term prognosis of patients who survived an episode of Stx-HUS reported death or permanent ESRD in 12% of patients and GFR <80 ml/min per 1.73 m² in 25% (38). The severity of acute illness, particularly central nervous system symptoms, and the need for initial dialysis were strongly associated with a worse long-term prognosis (4,38). Stx-HUS that is precipitated by *S. dysenteriae* infection is almost invariably complicated by bacteremia and septic shock, systemic intravascular coagulation, and acute cortical necrosis and renal death and has a high mortality rate (approximately 30%) (39).

History of a Discovery

E. coli has been associated with hemorrhagic colitis and organ failure, including kidney failure. In 1927, Albert Adam first reported an epidemic of bloody diarrhea of infants caused by a special type of *Bacterium coli*. Such bacterium was biochemically unique in that fermentation properties were different from known *E. coli* strains (40). In 1947, the *E. coli* O111:B4 was found in the stools of >90% of infants with epidemic diarrhea but never in their blood (41). A filterable agent—we now know that this was likely Stx—that caused diarrhea in calves and was lethal to mice was isolated from the stools of these children. A few years later, it was found that most severe cases of O111:B4-induced epidemic diarrhea were associated with purpura, anuria, and neurologic signs. Autopsy material revealed thrombosis of capillary and precapillary arterioles in lungs, liver, brain, and kidneys, as well as glomerular tuft occlusion by fibrin thrombi (42). These early findings were taken to indicate that a toxin, possibly released by the *E. coli*, induced hemorrhagic necrosis of the gastrointestinal mucosa and—once absorbed into the blood stream—caused microvascular thrombosis of kidneys and the other organs. Several years later, in 1977, Konowalciuck *et al.* (43) noted that *E. coli* that was isolated from patients with diarrhea produced a toxin similar to the one of *S. dysenteriae* type 1 (Stx) found cytopathic to Vero cells (African green monkey kidney cells). Karmali *et al.* (14) found an increased Stx activity in fecal filtrates and increased Stx-neutralizing antibody titer in sera from children who had *E. coli* O157:H7 infection and had received a diagnosis of HUS.

Shiga Toxin or Shiga Toxins?

The Stx associated with *E. coli* are designated by a number. Stx-1 is almost identical to Stx from *S. dysenteriae* type 1, differing by a single amino acid, and is 50% homologous with Stx-2 (44–46). Despite their similar sequences, Stx-1 and Stx-2 cause different degrees and types of tissue damage as documented by the higher pathogenicity of strains of *E. coli* that produce only Stx-2 than of those that produce Stx-1 alone (47–49). In a recent study in children who become infected by Stx-*E. coli*, *E. coli* strains that produced Stx-2 were most commonly associated with HUS, whereas most strains that were isolated from children who had diarrhea alone or remained asymptomatic produced only Stx-1 (50). This is also true in mice and baboons (45,51).

Both Stx-1 and Stx-2 are 70-kD AB₅ holotoxins that are composed of a single A subunit of 32-kD and five 7.7-kD B subunits (52) (Figure 1). It is interesting that a new AB₅ toxin that comprises a single 35-kD A subunit and a pentamer of 13-kD B subunits has been recently isolated from a highly virulent *E. coli* strain (O113:H21) that was responsible for an outbreak of HUS (53), which may represent the prototype of a new class of toxins, accounting for HUS associated with strains of *E. coli* that do not produce Stx.

After oral ingestion, Stx-*E. coli* reaches the gut and closely adheres to the epithelial cells of the gastrointestinal mucosa through a 97-kD outer membrane protein, intimin (54). Stx then are picked up by polarized gastrointestinal cells via transcellular pathways (55) and translocate into the circulation, probably

Figure 1. Binding and mechanism of action of Shiga-like toxin. The B subunits of Shiga toxin (Stx) molecules attach to galactose (gal) disaccharides of globotriaosylceramide (Gb3) receptors on the membrane of monocytes, polymorphonuclear cells, platelets, glomerular endothelial cells, and tubular epithelial cells. The toxin is internalized via retrograde transport through the Golgi complex. Then the A and B subunits dissociate, and the A subunit is translocated to the cytosol. The A subunit blocks peptide chain elongation by eliminating one adenine from the 28S ribosomal RNA.

facilitated by the transmigration of neutrophils (PMN) (56), which increase paracellular permeability. The route of transport of Stx from the intestine to the kidney has been greatly debated. *In vitro* experiments have shown that Stx can bind to human erythrocytes (57), platelets (58), and activated monocytes (59). However, more recent studies have underpinned a role for PMN in Stx transfer in the blood because Stx rapidly and completely bind to PMN when incubated with human blood (60). Consistently, Stx bound to circulating PMN have been detected in the blood of patients with Stx-HUS (61). The Stx receptor on PMN has a 100-fold lower affinity than the high-affinity receptor expressed on glomerular endothelial cells. *In vitro*, in co-cultures, PMN loaded with Stx transfer the ligand to glomerular endothelial cells so that at the end of the incubation, Stx molecules were found on glomerular endothelial cells but no more on PMN (60).

Binding of Stx to target cells is dependent on B subunits and occurs via the terminal digalactose moiety of the glycolipid cell surface receptor globotriaosylceramide Gb3 (Figure 1). Stx-1 and Stx-2 bind to different epitopes on the Gb3 molecule, and they also differ in binding affinity and kinetics (62). Surface plasmon resonance analysis showed that Stx-1 easily binds to and detaches from Gb3, in contrast to Stx-2, which binds slowly but also dissociates very slowly, thus staying on the cells long

enough to be incorporated (62). The latter could explain why Stx-2 is 1000-fold more toxic than Stx-1 on human endothelial cells *in vitro* (63).

Cultured human microvascular endothelial cells are more susceptible to the toxic effects of Stx than large-vessel endothelium (64). This is consistent with data that the number of Gb3 receptors expressed on human microvascular endothelial cells is 50-fold higher than in endothelial cells from human umbilical veins (65). In human glomerular endothelial cells, Gb3 expression and Stx toxicity are further increased upon exposure to TNF- α (66), in turn released by monocytes in response to Stx binding (59). Altogether these data provide the biochemical basis for the preferential localization of microangiopathic lesions to renal vasculature in HUS in humans.

After internalization by receptor-mediated endocytosis, Stx are carried by retrograde transport through the Golgi complex to the endoplasmic reticulum, where the A and B subunits likely dissociate (Figure 1). Then the A subunit is translocated to the cytosol and nuclear envelope, where it enzymatically blocks protein synthesis (67) (Figure 1). Stx-1 and Stx-2 also induce endothelial apoptosis (68,69) possibly by inhibiting the expression of the antiapoptotic Bcl-2 family member, Mcl-1 (70).

For many years, it was assumed that the only relevant biologic activity of Stx was the block of protein synthesis and destruction of endothelial cells. Recently, however, it has been shown that treatment of endothelial cells with sublethal doses of Stx, exerting minimal influence on protein synthesis, leads to increased mRNA levels and protein expression of chemokines, such as IL-8 and monocyte chemoattractant protein-1 (MCP-1) and cell adhesion molecules, a process preceded by NF- κ B activation (71). Analysis of genome-wide expression pattern of human endothelial cells stimulated with sublethal doses of Stx evidenced 25 and 24 genes upregulated by Stx-1 and Stx-2, respectively, mostly encoding for chemokines and cytokines, cell adhesion molecules, including P-selectin and ICAM-1, and transcription factors (EGR-1, NF- κ B2, and NF- κ B1A) (72). Che-

moamines and cytokines are likely involved in the chemoattraction and activation of neutrophils. Adhesion molecules seem to play a critical role in mediating binding of inflammatory cells to the endothelium. This is supported by adhesion experiments under flow showing that Stx-2 treatment enhanced the number of leukocytes that adhere and migrate across a monolayer of human endothelial cells (73). Preventing IL-8 and MCP-1 overexpression by adenovirus-mediated blocking of NF- κ B inhibited the adhesion and transmigration of leukocytes (71).

Taken together, these findings indicate that Stx, by altering endothelial cell adhesion properties and metabolism, favor leukocyte-dependent inflammation. The latter activates endothelial cells that lose thromboresistance, which ultimately leads to microvascular thrombosis. Evidence for such sequence of events has been obtained in experiments of whole blood flowing on human microvascular endothelial cells, pre-exposed to Stx-1, at high shear stress (74). Finding that in such circumstances early platelet activation and adhesion takes place, followed by the formation of organized thrombi dependent on endothelial P-selectin and PECAM-1, offers a plausible pathophysiologic pathway for microvascular thrombosis in HUS. The above report could also be taken as a demonstration of a link between bacteria and their products and arterial thrombosis, as suggested in the accompanying commentary (75).

In vivo evidence of coagulation disturbances, i.e., increase in prothrombin fragment 1 + 2, has been found (36) in children who developed HUS upon *E. coli* O157:H7 infection. Although early studies suggested that fibrinolysis is augmented in Stx HUS (76), more recent work revealed the presence of higher than-normal levels of plasminogen-activator inhibitor type 1 indicating that fibrinolysis is substantially inhibited (36).

Is There Any Effective Treatment for Stx-HUS?

There is no treatment of proven value, and care during the acute phase of the illness is still merely supportive with no substantial changes as compared with the past (Table 2). There is no clear consensus on whether antibiotics should be admin-

Table 2. Classification and treatment of different forms of HUS*

Disease	Causes	Treatment
Stx-HUS	Stx-producing <i>Escherichia coli</i> <i>Shigella dysenteriae</i> type 1	Supportive
Non-Stx-HUS	Bacteria (<i>Streptococcus pneumoniae</i>)	Supportive, antibiotics
sporadic	Viruses (HIV)	Antibiotics, no plasma
	Drugs (antineoplastic, antiplatelet, immunosuppressive)	Plasma
	Pregnancy associated	Drug withdrawal, plasma
	Postpartum	Delivery, plasma
	Systemic diseases	Plasma
	lupus	Steroids, plasma
	scleroderma	BP control
	antiphospholipid syndrome	Oral anticoagulants
	Idiopathic	Plasma
	Genetic (factor H, MCP, factor I)	Plasma
familial	Genetic (factor H, MCP, factor I), plasma	Plasma

istered to treat *Stx-E. coli* infection. Wong *et al.* (77) showed that antibiotic therapy at the stage of gastrointestinal infection with *Stx-E. coli* increases—by approximately 17-fold—the risk for full-blown HUS. It was postulated that antibiotic-induced injury to the bacterial membrane might favor the acute release of large amounts of toxins. However, a recent meta-analysis on 26 reports failed to show a higher risk for HUS associated with antibiotic administration (78). Of note, in the study by Wong *et al.*, none of the patients had bacteremia. Although bacteremia is very common in *Stx-HUS* precipitated by *S. dysenteriae* type 1 and these patients eventually progress to death unless antibiotics are started early enough (79,80), such complication is only exceptionally found in *Stx-HUS* sustained by *E. coli* O157:H7 infection. However, a recent report of an adult patient with *E. coli* O157:H7-induced HUS with bacteremia and urinary tract infection showed that early antibiotic therapy rapidly resolved hematologic and renal abnormalities (81). On the basis of available data, we suggest that in patients with *Stx-E. coli* gastrointestinal infection, antibiotics should be avoided unless in cases with sepsis.

A study with an *Stx*-binding agent, SYNSORB Pk, composed of particles of silicon linked to the globotriaosylceramide, given orally (82), failed to find any effect of SYNSORB over placebo. Most treatments, including plasma therapy, intravenous IgG, fibrinolytic agents, antiplatelet drugs, corticosteroids, and antioxidants (38), have been shown to be ineffective in controlled clinical trials in the acute phase of the disease (38). Careful BP control and renin-angiotensin system blockade may be particularly beneficial on the long term for patients who experience chronic renal disease after an episode of *Stx-HUS*. A recent study in 45 children who had renal sequelae of HUS and were followed for 9 to 11 yr documented that early restriction of proteins and use of angiotensin-converting enzyme inhibitors may have a beneficial effect on long-term renal outcome, as documented by a positive slope of $1/Cr$ values over time in treated patients (83). In another study, 8 to 15 yr of treatment with angiotensin-converting enzyme inhibitors after severe *Stx-HUS* normalized BP, reduced proteinuria, and improved GFR (84).

Finally, kidney transplant should be considered as an effective and safe treatment for children who progress to ESRD. Indeed, the outcome of renal transplantation is good in children with *Stx-HUS*: Recurrence rates range from 0 to 10% (85,86), and graft survival at 10 yr is even better than in control children who had other diseases and received a transplant (87).

Non-*Stx*-HUS

Epidemiology and Clinical Features

Non-*Stx*-HUS is less common than *Stx*-HUS and accounts for only 5 to 10% of all cases of the disease (1,88). It may manifest at all ages but is more frequent in adults. According to a recent U.S. study, the incidence of non-*Stx*-HUS in children is approximately one tenth that of *Stx*-HUS (10), corresponding to approximately 2 cases/yr per 1000,000 total population. At variance with *Stx*-HUS, there is no clear causative agent or seasonal pattern. The onset may be preceded by features of the nephrotic

syndrome. A diarrhea prodrome is rarely observed (D⁻HUS) (1,3,10,89). Non-*Stx*-HUS can occur sporadically or in families.

Sporadic Non-*Stx*-HUS. A wide variety of triggers for sporadic non-*Stx*-HUS have been identified, including various nonenteric infections, viruses, drugs, malignancies, transplantation, pregnancy, and other underlying medical conditions (scleroderma, antiphospholipid syndrome, lupus; Table 2). Infection caused by *Streptococcus pneumoniae* accounts for 40% of non-*Stx*-HUS and 4.7% of all causes of HUS in children in the United States (10). Neuroaminidase produced by *S. pneumoniae*, by removing sialic acids from the cell membranes, exposes Thomsen-Friedenreich antigen to preformed circulating IgM antibodies, which bind to this neoantigen on platelet and endothelial cells and cause platelet aggregation and endothelial damage (90,91). The clinical picture is usually severe, with respiratory distress, neurologic involvement, and coma and a mortality rate of 50% (91).

Categories of drugs that have been most frequently reported to induce non-*Stx*-HUS include anticancer molecules (mitomycin, cisplatin, bleomycin, and gemcitabine), immunotherapeutic (cyclosporine, tacrolimus, OKT3, IFN, and quinidine), and antiplatelet (ticlopidine and clopidogrel) agents (92). The risk for developing HUS after mitomycin is 2 to 10%. The onset is delayed, occurring almost 1 yr after starting treatment. The prognosis is poor, with up to 75% mortality at 4 mo (92).

Posttransplantation HUS is being reported with increasing frequency (1,93). It may ensue for the first time in patients who never experienced the disease (*de novo* posttransplantation HUS) or may affect patients whose primary cause of ESRD was HUS (recurrent posttransplantation HUS, discussed later in this review). *De novo* posttransplantation HUS might occur in patients who receive renal transplants and other organs, as a consequence of the use of calcineurin inhibitors or of humoral (C4b positive) rejection. It occurs in 5 to 15% of renal transplant patients who receive cyclosporine and in approximately 1% of those who are given tacrolimus (94).

Pregnancy-associated HUS may occasionally develop as a complication of preeclampsia. Some patients progress to a life-threatening variant of preeclampsia with severe thrombocytopenia, microangiopathic hemolytic anemia, renal failure, and liver involvement (HELLP syndrome). These forms are always an indication for prompt delivery that is usually followed by complete remission (95). Postpartum HUS manifests within 3 mo of delivery in most cases. The outcome is usually poor, with 50 to 60% mortality; residual renal dysfunction and hypertension are the rule in surviving patients (96). Of note, in approximately 50% of cases of sporadic non-*Stx*-HUS, no clear triggering conditions could be found (idiopathic HUS) (1).

Familial Non-*Stx*-HUS. Familial forms account for fewer than 3% of all cases of HUS. Both autosomal dominant and autosomal recessive forms of inheritance have been noted (97). In autosomal recessive HUS, the onset is usually early in childhood. The prognosis is poor, with a mortality rate of 60 to 70%. Recurrences are very frequent. Autosomal dominant HUS has an adult onset in most cases; the prognosis is poor, with a cumulative incidence of death or ESRD of 50 (98) to 90% (97).

Recent studies have documented that familial HUS may be

caused by genetic abnormalities of proteins involved in the regulation of the complement system. Similar genetic abnormalities have been found in sporadic non-Stx-HUS, mainly in idiopathic forms (99,100) but also in rare cases of pregnancy-associated (99) and postpartum HUS (three patients) (101,102), ticlopidine-induced HUS (one patient) (99), and postinfectious HUS (*Neisseria meningitidis*; one patient) (103).

Genetic Studies

Reduced serum levels of the third component (C3) of complement have been reported since 1974 in both familial and sporadic forms of non-Stx-HUS (98,104,105). Low C3 levels likely reflect C3 consumption in the microvasculature rather than defective synthesis, as documented by granular C3 deposits in glomeruli and arterioles of HUS patients (106,107) and by increased C3 breakdown products in sera. By contrast, levels of the fourth fraction of complement, C4, are usually normal (98). Persistent and remarkably depressed C3 levels found in patients with familial HUS, even in the unaffected relatives (98), suggested an inherited defect causing hyperactivation of the complement cascade.

The complement system consists of several plasma- and membrane-associated proteins that are organized in three activation pathways: The classical, the lectin, and the alternative pathway (108,109) (Figure 2). Upon activation by molecules on the surface of microorganisms, these pathways result in the formation of protease complexes, the C3 convertases, which cleave C3 generating C3b. The classic/lectin convertases are formed by C2 and C4 fragments, whereas the generation of the alternative pathway convertase requires the cleavage of C3 but not of C4. Thus, low C3 levels in patients with HUS in the presence of normal C4 indicate a selective activation of the alternative pathway (98).

Upon generation, C3b deposits on bacterial surfaces, which leads to opsonization for phagocytosis by PMN and macrophages. C3b also participates to the formation of the C5 convertases that cleaves C5 and initiates assembly of the mem-

brane attack complex that causes cell lysis. The human complement system is highly regulated as to prevent nonspecific damage to host cells and limit deposition of C3b to the surface of pathogens. This fine regulation is based on a number of membrane-anchored (CR1, DAF, MCP, and CD59) and fluid-phase (factor H) regulators that protect host tissues. Foreign surfaces that either lack membrane-bound regulators or cannot bind soluble regulators are attacked by complement.

In 1998, Warwicker *et al.* (110) studied three families with HUS and established linkage in the affected individuals to the regulator of complement activation gene cluster on human chromosome 1q32, which encodes for several complement regulatory proteins. The first examined candidate gene in this region was factor H (HF1), because an association between familial HUS and HF1 abnormalities had been reported previously (103,111,112). HF1 is a 150-kD multifunctional single-chain plasma glycoprotein that plays an important role in the regulation of the alternative pathway of complement (113). It serves as a co-factor for the C3b-cleaving enzyme factor I in the degradation of newly formed C3b molecules and controls decay, formation, and stability of the C3b convertase C3bBb. HF1 consists of 20 homologous units, named short consensus repeats (SCR). The complement regulatory domains that are needed to prevent fluid-phase alternative pathway amplification have been localized within the N-terminal SCR1-4 (114). The inactivation of surface-bound C3b is dependent on the binding of the C-terminal domain of HF1 to polyanionic molecules that increases HF1 affinity for C3b and exposes its complement regulatory N-terminal domain. The C-terminal domains contain two C3b binding sites, located in SCR12-14 and SCR19-20, and three polyanion-binding sites, located in SCR 7, SCR 13, and SCR19-20 (Figure 3) (115-117). However, the C3b and the polyanion-binding sites located in SCR19-20 are the only indispensable sites for HF1 to inactivate surface-bound C3b, because deletion of this portion of the molecule causes loss of HF1 capability to prevent complement activation on sheep erythrocytes (115,116). Human glomerular endothelial cells and kidney glomerular basement membrane are rich in polyanionic molecules, so HF1 deposited on their surface would provide an efficient shield against complement attack (Figure 4A) (117,118).

Since the first report by Warwicker, a number of studies have been performed by four independent groups, who altogether so far have identified up to 50 different HF1 mutations (Figure 3) in 80 patients who had familial (36 patients) and sporadic (44 patients) forms of non-Stx-HUS (99-102,114,119-121). In sporadic forms, the mutation was either inherited from a healthy parent or, more rarely—only four cases reported—ensued *de novo* in the proband (100,102). The mutation frequency is up to 40% in familial forms, whereas only 13 to 17% of sporadic forms had HF1 mutations (100,119). Alterations in other genes encoding for complement regulatory proteins could theoretically be involved in determining predisposition to sporadic non-Stx-HUS. Alternatively, these forms could be caused by an acquired autoimmune HF1 defect, similar to that observed in some patients with thrombotic thrombocytopenic purpura, in whom the acute episode is triggered by antibodies against the von

Figure 2. Activation pathways of the complement system and their regulators (in red).

Figure 4. Proposed model for the pathologic consequences of factor H and monocyte chemoattractant protein (MCP) mutations. (A) After viral or bacterial infection or endothelial insult, complement is activated and C3b is formed. In the presence of normal factor H (HF1), C3b is rapidly inactivated to inactive C3b (iC3b). Factor H in the circulation binds fluid-phase C3b and favors its degradation by factor I (FI; co-factor activity, yellow domain). In addition, it binds (green domain) to polyanionic proteoglycans that are present on endothelial cell surface and in the subendothelial matrix, where, because of its high affinity for C3b, it entraps fluid-phase C3b, thus preventing its deposition on host surfaces and its binding with factor B (FB) to form the C3 convertase complex (C3bBb). The subendothelial matrix lacks endogenous complement regulators and is completely dependent on factor H to control complement activation. MCP also inactivates C3b deposited on endothelial cells by favoring its cleavage to iC3b by FI. (B) Proposed consequences of factor H mutations found in patients with HUS. Mutant factor H has a normal co-factor activity in fluid phase. However, the mutations affect the polyanion interaction site at the C-terminus of factor H so that it shows reduced bind to proteoglycans on endothelial cell surface and in subendothelial matrix. This results in more C3b reaching the endothelial cell surface so that MCP is not enough to control adequately complement activation on the cell membrane. In addition, C3b deposited on exposed extracellular matrix is not degraded and forms the C3 convertase of the alternative pathway of complement that further cleaves C3 to C3b. (C) MCP deficiency also predisposes to HUS. MCP mutations found in patients with HUS result in a reduced surface expression of the protein or in a reduced capability of MCP to bind C3b. In both cases, membrane-bound C3b is not efficiently inactivated, which leads to undesirable amplification of C3b formation and deposition on damaged endothelial cells through the formation of C3 convertase. (D) The sequence of events leading to microvascular thrombosis. The proteolysis of C3 and C5 by convertases causes the release of the chemotactic anaphylatoxins C3a and C5a that bind to receptors on inflammatory cells and attract them toward the endothelial layer. The deposition of C3b on endothelial cells is followed by the formation of the membrane attack complex (C5b9), which leads to cells' injury and detachment and to sublytic membrane perturbation, leading to endothelial activation and expression of adhesion molecules (e.g., P-selectin). The latter favor leukocyte attachment and activation with the release of oxygen radicals and proteinases that further damage the endothelium. After endothelial damage, cell detachment ensues and exposes basement membranes. In these conditions, platelets from the microcirculation adhere and aggregate to the exposed matrix.

glycoprotein that serves as a co-factor for factor I to cleave C3b and C4b deposited on host cell surface (129–131). MCP has four extracellular complement control-protein modules (CCP) that are important for its inhibitory activity, followed by a serine-threonine-proline-rich domain, a transmembrane domain, and a cytoplasmic tail (132). Richards *et al.* (128) reported a heterozygous deletion of the D237/S238 amino acids in one family and a S206P substitution in two families. Evaluations of protein expression and function on PBMC showed that the mutants had a reduced C3b binding capability and a reduced ability to prevent complement activation. Another heterozygous mutation, causing two amino acid changes and a premature inter-

ruption of MCP protein in CCP4, was identified (127) in two siblings, which caused 50% reduction in MCP expression levels on PBMC of heterozygous individuals. Additional studies from our group on 112 patients with non-Stx-HUS have revealed five additional MCP mutations in familial (seven cases) and in sporadic (five cases) HUS with a mutation frequency of 11% (25% in familial and 6% in sporadic forms) (133).

MCP is highly expressed in the kidney and could be found on glomerular endothelial cells by immunohistochemical analysis (134–136). It likely exerts a main role in protecting glomerular endothelial cells against C3 activation as indicated by data that co-factor activity in the extracts of these cells was com-

pletely blocked by anti-MCP antibody (136). Factor H and MCP likely integrate each other in controlling complement activation on host cells. Polyanion-attached HF1 extends from the cell membrane by approximately 120 nm and could represent an outer barrier of cells against complement attack. However, because MCP is a small-sized membrane-integrated complement regulator that extends for approximately 20 nm, one can hypothesize that MCP protein is involved in the control of complement in the close vicinity of the cell membrane (117). As hypothesized for HF1, mutations in MCP likely predispose rather than directly cause HUS. Upon exposure to conditions that cause activation of the complement cascade, reduced levels or defective C3b binding capability and co-factor activity of mutated MCP on glomerular endothelial cells would result in an insufficient protection of these cells from complement activation (Figure 4C). That mutations either in factor H or in MCP result in complement activation and HUS indicates that these complement regulators do not have overlapping functions and that they both are necessary to control complement adequately.

Finally, three mutations in the gene encoding for factor I have been reported in three patients with sporadic non-Stx-HUS (137), which further support the concept that HUS is a disease of complement dysregulation. Other candidate genes are under investigation, including DAF, CR1, CD59, C3, and factor B.

Patients who present with non-Stx-HUS should be tested first for serum C3 concentrations; however, normal C3 levels do not necessarily exclude a complement dysfunction. More sensitive assays could be a higher-than-normal C3d/C3 ratio in plasma (unpublished data) or the presence of C3 deposits in renal biopsy (106,107). Measurement of HF1 in serum would be helpful to find out those few patients who carry HF1 mutations that cause reduced HF1 levels. Decreased CH50 values and factor B concentrations could be found in some but not all patients with HF1 or MCP mutations. A second step should be the search for mutations in candidate genes HF1 and MCP. Search for factor I mutations should be performed in patients with lower-than-normal factor I serum levels.

Which Treatment for Non-Stx-HUS?

Despite that non-Stx-HUS has a poor prognosis, after plasma manipulation was introduced, the mortality rate has dropped from 50 to 25% (138-140). However, debate still exists on whether plasma is or is not effective in the treatment of acute episodes (141-144). Published observations (139,145-147) and our own experience indicate that a consistent number of patients with non-Stx-HUS respond to plasma treatment. It has been proposed that plasma exchange might be relatively more effective than plasma infusion because it might remove potentially toxic substances from the patient's circulation. That this may not be the case is documented by data that in a patient with relapsing thrombotic microangiopathy (148), normalization of the platelet count was invariably obtained by plasma exchange or infusion, whereas plasma removal and substitution with albumin and saline never raised the platelet count. However, in situations such as renal insufficiency or heart failure, which limit the amount of plasma that can be provided with infusion alone, plasma exchange should be considered as

first-choice therapy (1). Plasma treatment should be started within 24 h of presentation as delay in treatment initiation may increase treatment failure. Usually one plasma volume (40 ml/kg) is exchanged per session (1,149). Treatment can be intensified by increasing the volume of plasma replaced. The twice-daily exchanges of one plasma volume is probably the treatment of choice for refractory patients to minimize the recycling of infused plasma (1). As for plasma infusion, the recommended dose is 30 to 40 ml/kg on day 1, then 10 to 20 ml/kg per d. Daily plasma therapy should continue for a minimum of 2 d after complete remission is obtained (1,149).

Plasma infusion or exchange has been used in patients with HUS and HF1 mutations, with the rationale to provide the patients with normal HF1 to correct the genetic deficiency. Some patients did not respond at all and died or developed ESRD (107). Others remained chronically ill (121,150) or required infusion of plasma at weekly intervals to raise HF1 plasma levels enough to maintain remission (151). Stratton *et al.* (152) were able to induce sustained remission in a patient who had HF1 mutation and developed an acute episode of HUS and required hemodialysis. After 3 mo of weekly plasma exchange in conjunction with intravenous immunoglobulins, the patient regained renal function, dialysis was withdrawn, and plasma therapy was stopped. At 1 yr after stopping plasma therapy, the patient remained disease-free and dialysis independent. Plasma therapy is instead contraindicated in patients with HUS induced by *S. pneumoniae*, because adult plasma contains antibodies against the Thomsen-Friedenreich antigen, which may exacerbate the disease.

In those few patients with extensive microvascular thrombosis at renal biopsy, refractory hypertension, and signs of hypertensive encephalopathy, when conventional therapies including plasma manipulation are not enough to control the disease (*i.e.*, persistent severe thrombocytopenia and hemolytic anemia), bilateral nephrectomy has been performed with excellent follow-up in some patients (153). Other treatments, including antiplatelet agents, prostacyclin, heparin or fibrinolytic agents, steroids, and intravenous immunoglobulins, have been attempted, with no consistent benefit (1).

Patients who develop HUS upon challenge with cyclosporine or tacrolimus have to stop the medication. Sirolimus has been used as an alternative in occasional patients with encouraging results (154).

Of patients with non-Stx-HUS, 50% (in sporadic forms) to 60% (in familial forms) progress to ESRD (1,99). Renal transplantation is not necessarily an option for non-Stx-HUS, at variance with Stx-HUS. Actually, approximately 50% of the patients who had a renal transplant had a recurrence of the disease in the grafted organ (86,155). Recurrences occur at a median time of 30 d after transplant (range, 0 d to 16 yr). There is no effective treatment of recurrences. Graft failure occurs in >90% of patients who experience recurrence, despite plasma infusion or plasma exchange, high-dose prednisone, and withdrawal of cyclosporine (1,86). Patients who lost the first kidney graft for recurrence should not receive another transplant. Live-related renal transplant should also be avoided in that it carries the additional risk to precipitate the disease onset in the healthy

donor relative as recently reported in two families (156). New knowledge from genetic studies will predict more accurately the risk for recurrence. In patients with HF1 mutations, the recurrence rate ranges from 30 to 100%, according to different surveys (99–101), and is significantly higher than in patients without HF1 mutations (99). In view of the fact that HF1 is a plasma protein mainly of liver origin, a kidney transplant does not correct the HF1 genetic defect (110,119).

Simultaneous kidney and liver transplant was performed in two young children with non-Stx-HUS and HF1 mutations, with the objective of correcting the genetic defect and preventing disease recurrences (157,158). However, for reasons that are currently under evaluation and that possibly involve an increased liver susceptibility to immune or ischemic injury related to uncontrolled complement activation, both cases that were treated with this procedure were complicated by premature irreversible liver failure. In the first published case (157), a humoral rejection of the liver graft manifested by the 26th day after transplantation; the patient had actually a high titer of antibodies to donor class I HLA. In a few days, the child developed hepatic encephalopathy and coma that recovered with a second, uneventful liver transplant (157). The second case was complicated by a fatal, primary nonfunction of the liver graft. Graft hypoperfusion, as a result of a sudden drop of arterial BP occurring soon after reperfusion, triggered severe ischemia/reperfusion damage and complement deposition in the liver, conceivably as the result of defective HF1 complement regulatory potential. Multiorgan failure was the final event resulting in the patient's death (158). Thus, despite its capacity of correcting the genetic defect, combined kidney and liver transplant for non-Stx-HUS associated with HF1 mutations should not be performed unless a patient is at imminent risk for life-threatening complications.

Kidney graft outcome is favorable in patients with MCP mutations as found in four patients who received a successful transplant and experienced no disease recurrence (128; unpublished data). In view of the fact MCP is a membrane-bound protein that is highly expressed in the kidney, a kidney graft would reasonably correct local MCP dysfunction. The graft, bearing wild-type MCP expressed on renal endothelial cell surface, should conceivably be protected from disease recurrence.

The Future

Research efforts are aimed at identifying more specific approaches that may interfere with the primary cause of microangiopathy in the different forms of HUS. In Stx-HUS, new agents that are targeted at preventing organ exposure to Stx are currently under evaluation (159). In mice, molecular decoys such as orally administered harmless recombinant bacteria that display an Stx receptor on the surface that in turn binds the toxin in the gut (160–162) have been used successfully. Another approach is to use Stx inhibitors, among them is STARFISH, an oligobivalent, water-soluble carbohydrate ligand that can simultaneously engage all five B subunits of the toxin, which might help to prevent toxin that already has entered the circulation from destroying kidney microvessels (163). Others have

ameliorated disease in pigs by injection of toxin-neutralizing antibodies (164). Some investigators have focused on downstream events in the pathogenetic cascade. Thrombin blockade with lepirudin in a model of Stx-HUS in greyhound dogs had some beneficial effect on mortality (165), indicating that thrombin may be a critical factor in the pathogenesis of Stx-HUS. At present, prevention remains the main approach to decreasing the morbidity and mortality associated with Stx-E. coli infection. A multifaceted approach that includes novel ways of decreasing Stx-E. coli carrier rate in livestock and implementing a zero-tolerance policy for contaminated foods and beverages is required.

The discovery of mutations in three different complement regulatory genes provides enough evidence of the involvement of complement activation in the pathogenesis of non-Stx-HUS and indicates that complement inhibition could represent a therapeutic target in these patients. There are currently a number of companies with complement inhibitors in clinical or preclinical development (166). Pexelizumab and eculizumab, two humanized monoclonal antibodies directed against C5 that inhibit the activation of terminal complement components, have been developed recently. Administration of eculizumab to patients with paroxysmal nocturnal hemoglobinuria, a disease characterized by a genetic deficiency of surface proteins that protect hematopoietic cells against the attack by the complement system, reduced intravascular hemolysis, hemoglobinuria, and the need for transfusions (167). In a phase II clinical trial, administration of pexelizumab as adjunctive therapy in patients who had myocardial infarction and underwent primary percutaneous coronary intervention inhibited complement activation and significantly reduced mortality as compared with the placebo group, although the infarct size was not modified by the drug (168). Another complement-blocking approach under investigation in clinical studies is based on the use of soluble forms of the C3/C5 convertase inhibitor complement receptor 1 (CR1). Phase I and phase II clinical trials have shown that the soluble CR1 TP10, administered intravenously both before and during surgery, decreased complement activation and protected vascular function in infants who underwent cardiopulmonary bypass (169). In a randomized, multicenter, prospective study in 564 high-risk patients who underwent cardiac surgery on cardiopulmonary bypass, a bolus of TP10 given immediately before cardiopulmonary bypass significantly inhibited complement activity within 10 min, and this inhibition persisted for 3 d postoperatively (170).

It is hoped that the above complement inhibitors, once available to the market, will be useful in patients with non-Stx-HUS, to block complement-mediated kidney damage during the acute episode or to prevent recurrence after kidney transplantation. Complement inhibitors also theoretically could be of benefit to prevent complications, such as primary liver nonfunction, in combined kidney and liver transplantation in patients with HF1 genetic defects.

For HUS associated with HF1 mutations, specific replacement therapies with recombinant HF1 could become a viable alternative to plasma treatment. Efforts are also ongoing to isolate plasma fractions enriched in HF1, which could allow

providing the patient with enough active molecules while minimizing the risk for allergy and fluid overload. It is hoped that advances in vector safety and transfection efficiency will soon render gene therapy a realistic option for these patients. Undergoing studies on other complement regulatory genes would help to clarify fully the molecular determinants underlying the pathogenesis of non-Stx-HUS and hopefully translate into an improvement in the management and therapy.

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General Discussion and Conclusions

General Discussion

Reduced serum levels of the third component (C3) of complement have been reported since 1974 in both familial and sporadic forms of non-Stx-HUS [48, 51, 63], which were taken as to reflect C3 activation and consumption in the microvasculature [69, 82]. However epidemiological evidence of an association between complement activation and non-Stx-HUS was lacking. In chapter 2 we evaluated serum levels of C3 of C4 in familial cases of HUS from the International Registry of HUS and TTP. Patients with the familial form of TTP were investigated as well, based on data that the two syndromes have many overlapping clinical features and markers of C3 activation were also reported in cases with TTP [83, 84].

Reduced C3 levels were found in around 70% of cases irrespective on whether they had a clinical diagnosis of HUS or TTP [51]. Of note serum C3 levels were depressed even during remission of the disease, which excludes that low C3 levels could derive from consumption in a still-ongoing microangiopathic process, and rather indicates a congenital defect leading to chronic complement activation. In support of this possibility, low C3 levels were also found in around 25% of the relatives of our patients who had never had signs of the disease, which confirms clustering of the defect within families. At multivariate analysis C3 serum levels were significantly associated with HUS and TTP within affected families and in the overall study population and subjects with decreased C3 had a relative risk of HUS and TTP of more than 16 within families and more than 27 in the general population.

Either in cases or in case-relatives depressed C3 values were not paralleled by similar

changes in C4 levels. The latter finding indicates that hypocomplementemia in our series was caused by a selective activation of the alternative pathway of complement that causes the cleavage of C3 but not of C4, at variance with activation of the classical pathway that is associated with both C3 and C4 consumption.

In previous studies, in occasional families including one or more cases of HUS, low C3 levels were accompanied by very low levels of CFH, a regulatory protein that inhibits complement activation through the alternative pathway [65, 73]. The above data raised the possibility that low C3 in the setting of familial HUS may depend on an uncontrolled activation of the alternative pathway of complement secondary to an inherited deficiency of CFH. In support to this possibility, in studies of **chapter 2** we found CFH abnormalities (i.e. either very low serum CFH levels or abnormal mobility of CFH on Western blot) in four cases within two different families but in none of the healthy unrelated controls [47]. In addition, within families CFH abnormalities correlated with C3 reduction. These results are in line with the evidence provided by Warwicker and coworkers [49] in three large families with HUS, that an area on chromosome 1q, where CFH is mapped, segregates with the disease. Affected members and obligate carriers within one family were found, by mutation analysis, to have heterozygous point mutations in *CFH* gene. In studies of **chapters 3 and 4** mutation screening was undertaken in a large series of patients with non-Stx-HUS from the International Registry of HUS and TTP, to establish whether mutations in *CFH* were actually involved in determining predisposition to familial forms of non-Stx-HUS and to evaluate whether this could apply to sporadic forms of the disease as well.

In these studies 17 independent *CFH* mutational events were found in 33 out of 101 patients with non-Stx-HUS [52, 85]. The frequency of *CFH* mutations was high in familial cases (46%), but rather low (17%) in sporadic cases. All the *CFH* mutations found in non-Stx-HUS patients are heterozygous mutations, with the exception of one homozygous mutation found in a Bedouin family with recessive transmission. Of note, most of them cause single amino acid change, while three mutations introduced premature stop codons. Although the identified mutations are spread over several exons, 70% of them clustered in exons XXII and XXIII and affected the most C terminus part of *CFH* (SCR20). Heterozygous mutations were associated with normal *CFH* levels in most cases, whereas the homozygous mutation in the Bedouin family resulted in severe reduction of *CFH* levels accompanied by reduced RNA expression [52, 85].

The above results have been confirmed by other groups. Thus, more than 50 different *CFH* mutations have been identified till now in 80 patients who had familial and sporadic forms of non-Stx-HUS [49, 52, 56, 85-89]. Forty-eight out of 50 *CFH* mutations are heterozygous and mainly cluster in the C-terminus domains and are commonly associated with normal *CFH* plasma levels. This is at variance with patients with type II membranoproliferative glomerulonephritis who carry homozygous *CFH* mutations causing severely reduced *CFH* levels [87]. Expression and functional studies done by our group in collaboration with Peter Zipfel ([79] and chapter 7) and by other groups [90, 91] demonstrated that *CFH* proteins carrying HUS-associated mutations have a severely reduced capability to interact with polyanions and with surface-bound C3b [79, 90, 91],

which results in a lower density of mutant CFH molecules bound to endothelial cells surface and in a diminished complement regulatory activity on the cell membrane [79, 91]. In contrast these mutants have a normal capacity to control activation of the complement in plasma, as indicated by data that they retain a normal cofactor activity in the proteolysis of fluid-phase C3b [90]. The latter finding explains the case of patients with HUS and *CFH* mutations who have normal or slightly reduced serum C3 levels [86, 87].

Analysis of clinical data from patients included in **chapter 4** [52] showed that the disease manifested earlier and was associated with a higher mortality rate in carriers of *CFH* mutations than in those without *CFH* mutations. However, a consistent number of patients carrying heterozygous *CFH* mutations had long remissions and presented late in life. By contrast the only homozygous *CFH* mutation in our series was associated with a neonatal onset and a very high mortality rate.

Patients carrying heterozygous *CFH* mutations have a partial CFH deficiency, as a result of one intact and one defective allele, which more likely predispose to, rather than directly cause the disease. Thus, a “second hit” is needed to fully manifestation of the disease, as also documented by the incomplete (50%) penetrance of the *CFH* mutations found in our families. Actually, conditions that trigger complement activation, either directly (bacterial and viral infections) or indirectly, by causing endothelial insult (drugs, systemic diseases, pregnancy), were found to precipitate the acute event in around 60% of patients with *CFH* mutations reported in **chapters 3 and 4** [52, 85]. All the above observations can be reconciled by reasoning that in these patients the suboptimal CFH activity is enough to protect the host

from complement activation in physiological conditions. However, upon exposure to an agent that activates complement, C3b is formed in higher than normal amounts and its deposition on vascular endothelial cells can not be fully prevented due to loss of polyanion binding capability of mutated CFH. This abnormality favors the formation of MAC and the recruitment of inflammatory cells, all events causing damage and retraction of endothelial cells, adhesion and aggregation of platelets. The glomerular capillary bed is a fenestrated endothelium and the exposed basement membrane supplies a surface that is rich in polyanions for CFH binding, an event that is crucial to prevent C3b deposition and complement activation since subendothelial matrix lacks endogenous complement regulators. The latter observation could explain the renal localization of microvascular injury of HUS.

Two third of patients with non-Stx-HUS in our series had no *CFH* mutations, despite up to 50% of them exhibit evidence of overactivity of the alternative pathway of complement [52]. The possibility that uncommon polymorphic variants of *CFH* gene may confer susceptibility to HUS in subjects without *CFH* mutations has been investigated in studies of **chapter 4**. It was found that the T allele of the C-257T, the G allele of the A2089G and the T allele of the G2881T polymorphisms of *CFH* gene were more frequent in HUS patients without *CFH* mutations than in healthy subjects [52] and analysis of the overall study population revealed that subjects carrying two or three of the above variants had a four-fold increased risk of developing HUS [52]. We also found that the 257T, 2089G and 2881T alleles might also have a role in determining the penetrance [52] of the disease in *CFH* mutation

carriers. In 5 out of 9 families, subjects who developed HUS had inherited an allele carrying the *CFH* mutation from one parent together with an allele carrying at least one disease-associated *CFH* polymorphism from the other parent. Instead all the healthy *CFH* mutation carriers only inherited the mutation but no polymorphism [52].

Much debate has been emerging in the recent years on whether it was possible or not to make a differential diagnosis between HUS and TTP [92, 93]. The two syndromes have in common microangiopathic hemolytic anemia, consumption thrombocytopenia and microvascular thrombosis, but differ because of severe renal involvement, often with renal failure in HUS, and prevalent central nervous system symptoms in TTP [1]. In studies of **chapter 4**, we investigated whether *CFH* gene mutations and polymorphisms may have a role in determining predisposition to TTP. We found that none of the 32 patients with a diagnosis of TTP and no renal impairment studied in **chapter 4** carried *CFH* mutations, which indicates that *CFH* abnormalities are specifically associated with renal manifestations of the thrombotic microangiopathy. It is now known that 70 to 80% of case of TTP are triggered by a deficiency of ADAMTS13 [75], a plasma metalloprotease that cleaves von Willebrand factor multimers soon after their secretion by either endothelial cells or megakaryocytes. Two primary mechanisms for deficiency of ADAMTS13 activity have been identified, namely a constitutive deficiency, caused by compound heterozygous or homozygous *ADAMTS13* mutations, and an acquired deficiency caused by the presence of inhibitory antibodies [74]. The observation that in large clinical studies [75, 76] *ADAMTS13* activity was defective in patients with diagnosis of TTP but not in those with HUS generated

the paradigm that TTP is due to a deficiency of ADAMTS13, in turn not involved in the pathogenesis of HUS. However rare cases with ADAMTS13 deficiency manifest severe renal involvement, often with ESRD [77, 78]. Those patients' clinical manifestations are difficult to distinguish from those of HUS. In studies of chapter 5 we investigated the genetic basis of phenotype heterogeneity in patients with ADAMTS13 deficiency. We studied a family with two affected sisters, one who presented with exclusive neurological symptoms and the other with severe renal involvement that required chronic dialysis [94]. The two patients had complete ADAMTS13 deficiency as a result of two heterozygous mutations. In addition, a heterozygous mutation in *CFH* gene was found in the patient who developed chronic renal failure but not in her sister with only neurological symptoms. Based on these data we hypothesize that in the former patient *CFH* haploinsufficiency had a role in determining the renal complications that superimposed on the systemic disease caused by ADAMTS13 deficiency.

In studies of chapter 6 we searched for additional genes whose alterations may predispose to non-Stx-HUS. To this purpose, 25 patients with familial and sporadic forms of non-Stx-HUS with no *CFH* mutation were screened for mutations in genes encoding for complement regulatory proteins. The attention was focused on genes of the RCA cluster, encoding factor H related 5 (*FHR5*), complement receptor 1 (*CR1*) and membrane cofactor protein (*MCP*), based on Warwicker's data [49] showing that two of the original three families whose defect mapped to the RCA cluster did not have a *CFH* mutation. Results showed no disease-associated mutation in *FHR5* nor in *CR1*. By contrast, we found a

heterozygous mutation in *MCP*, encoding a cell-bound membrane complement regulator, in two siblings with a history of HUS [95]. *MCP* is a widely expressed transmembrane glycoprotein that serves as a cofactor for factor I to cleave C3b and C4b deposited on host cell surface [59, 96, 97]. The mutation causes three amino acid changes and a premature interruption of *MCP* protein in SCR4, which result in 50% reduction in *MCP* expression levels on PBMC of heterozygous individuals. These results have been confirmed by another group who found a heterozygous deletion of D237/S238 aminoacids in one family and a S206P substitution in two families [98]. As reported in chapter 7, more recent studies from our group on 112 patients with non-Stx-HUS have revealed five additional *MCP* mutations in familial (7 cases) and in sporadic (5 cases) HUS with a mutation frequency of 11% (25 % in familial and 6% in sporadic forms) (abstract; Brioschi et al. VII Congress of the Italian society of Human Genetics, 13-15 October 2004).

MCP is highly expressed in the kidney [60-62]. It likely exerts a main role in protecting glomerular endothelial cells against C3 activation as indicated by data that cofactor activity in the extracts of these cells was completely blocked by anti-*MCP* antibody [61]. As hypothesized for *CFH*, mutations in *MCP* likely predispose rather than directly cause HUS. Upon exposure to conditions that cause activation of the complement cascade, reduced levels or defective C3b binding capability and cofactor activity of mutated *MCP* on glomerular endothelial cells would result in an insufficient protection of these cells from complement activation.

These findings have potential clinical implications. Genotyping of patients with

non-Stx-HUS could potentially allow to optimize treatment. Plasma infusion or exchange has been used in published case reports to treat patients with *CFH* mutations [82, 99-101]. The rationale is to provide patients with normal *CFH* to correct their genetic deficiency, but the efficacy of this approach remains questionable. Results from studies of chapter 4 and unpublished results from the International Registry of HUS and TTP show that around 50% of patients with *CFH* mutations achieved either complete or partial remission (hematological normalization with renal sequelae) after receiving plasma; however, the remaining 50% did not respond and 10-20% died during the acute episode. Unlike *CFH*, *MCP* is a transmembrane protein, so the rationale for using plasma in patients with *MCP* mutations is less clear. Data from chapter 6 and unpublished results from our Registry indicate that 70-80% of patients achieve remission following plasma infusion or exchange; however complete recovery from the acute episode was also observed in 70-80% of patients not treated with plasma.

It is debatable whether kidney transplantation is an appropriate treatment for non-Stx-HUS patients who have progressed to ESRD. In published studies [102, 103], around 50% of non-Stx-HUS patients given a renal transplant had a recurrence of the disease in the grafted organ. There is no effective treatment for recurrence and graft failure occurs in more than 90% of patients who experience disease recurrence. In chapter 4 and 7 we evaluated whether the specific genetic defect underlying non-Stx-HUS influences the outcome of the kidney graft. We found that in patients with *CFH* mutations the graft outcome is poor and the recurrence rate on the transplanted organ is significantly higher

than in patients without *CFH* mutations [52, 94]]. As *CFH* is a plasma protein mainly produced by the liver, the genetic defect was not corrected by the kidney transplant and persistent *CFH* deficiency predisposed to disease recurrence on the graft. As reported in chapter 7 [94], simultaneous kidney and liver transplant has been recently performed by our group in two young children with non-SLT-HUS and *CFH* mutations with the rationale to correct the genetic defect and prevent disease recurrence; however, both cases were complicated by irreversible liver failure [80, 81]. The first case recovered after a second uneventful liver transplantation. The child, who used to have monthly recurrences before transplantation, has had no symptoms of HUS for more than 2-years follow-up. The second case had a fatal, primary non-function of the liver graft followed by multi-organ failure and patient's death. The reasons for this may include increase susceptibility of the transplanted liver to ischemic or immune injury related to uncontrolled complement activation. Thus, despite the potential to correct the genetic defect, combined kidney and liver transplant for non-Stx-HUS associated with *CFH* mutations, should not be performed unless a patient is at imminent risk with life-threatening complications.

By contrast, patients with aberrant forms of *MCP*, a transmembrane protein highly expressed in the kidney, have favorable kidney graft outcomes, as shown by data from four patients (chapter 7) who were successfully transplanted with no disease recurrence [94, 98]. These preliminary data suggest that genotyping for *CFH* and *MCP* should be performed in all patients with ESRD secondary to non-Stx-HUS who are candidates for transplantation, to help define graft prognosis.

Conclusion

The results of this thesis, in particular the discovery of mutations in two different complement regulatory genes, provide enough evidence of the involvement of complement activation in the pathogenesis of non-Stx-HUS. This concept has been recently reinforced by studies from other authors, showing mutations in the gene encoding factor I (IF), a serine protease that cleaves the alpha chain of C3b, in five sporadic cases (chapter 7 and [104, 105]).

Complement regulatory proteins are essential to prevent non-specific damage of host endothelial cells and limit deposition of C3b to the surface of pathogens [53, 54]. Thus, in patients with genetic alterations causing defective activity of complement regulatory proteins, exposure to an agent that activates complement results in C3b deposition on vascular endothelial cells and on subendothelial matrix and in the formation of the C3 and C5 convertase complexes. The proteolysis of C3 and C5 by convertases causes the release of chemotactic anaphylatoxins (C3a and C5a) that recruit inflammatory cells toward the endothelial layer. On the other hand, the deposition of C3b is followed by the formation of MAC, which leads to endothelial cells injury and detachment and to sublytic perturbation, leading to endothelial activation and expression of adhesion molecules. The latter favors leukocyte attachment and activation with the release of oxygen radicals and proteinases that further damage the endothelium. After endothelial damage, cell detachment ensues and exposes basement membranes. In these conditions, platelets from the circulation adhere and aggregate to the exposed matrix.

Hopefully, these findings will translate into an improvement in the management of patients with this disease and will pave the way for tailored treatments. Complement inhibitors, such as the humanized monoclonal antibodies directed against C5 and the soluble forms of the C3/C5 convertase inhibitor CR1, are currently in clinical and preclinical development [106]. These drugs should be useful in blocking complement-mediated kidney damage during the acute episode of HUS or in preventing recurrence after kidney transplantation. Complement inhibitors could also be theoretically of benefit to prevent complications, such as primary liver non-function, in combined kidney and liver transplantation in patients with *CFH* genetic defects.

Specific replacement therapy with recombinant or purified plasma proteins could become a viable alternative to plasma in patients with *CFH* or *IF* mutations.

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CHAPTER 9

Summary

Hemolytic uremic syndrome (HUS) is a rare disease of microangiopathic hemolytic anemia, low platelet count and renal impairment due to platelet thrombi occluding the renal circulation. In children, the disease is most commonly triggered by certain strains of *E. coli* that produce powerful exotoxins, the Shiga-like toxins (Stx1 and Stx2) and manifests with diarrhea, often bloody. Non-Shiga-toxin-associated HUS (non-Stx-HUS) comprises a heterogeneous group of patients in whom an infection by Stx-producing bacteria could be excluded as a cause of the disease and can be sporadic or familial. Collectively, non-Stx-HUS have a poor outcome, with death or permanent renal dysfunction being the final outcome in the large majority of cases. Since early seventies an underlying genetically determined condition predisposing to these forms of HUS has been hypothesized. Finding of depressed complement C3 levels and increased concentrations of C3 breakdown products in patient blood suggested an inherited defect causing hyperactivation of the complement cascade.

In this thesis a number of studies were designed and performed with the aims to provide more information as for the incidence and pathogenetic role of complement dysregulation in non-Stx-HUS and to investigate the genetic basis of such abnormalities. Sporadic observations in literature provided evidence that activation of complement occurs also in patients with thrombotic thrombocytopenic purpura (TTP). The latter syndrome has in common with HUS microangiopathic hemolytic anemia, consumption thrombocytopenia and microvascular thrombosis, but differs because it manifests mainly with central nervous system symptom, whereas predominant renal involvement characterizes HUS. Examination of very large series of patients with HUS and TTP has pointed out that a neat clinical

distinction between the two syndromes is difficult. Thus, search for complement activation markers and of genetic abnormalities of complement regulatory genes was also undertaken in patients with diagnosis of TTP.

In **chapter 2**, 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 (CFH) 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 and in the overall study population. 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. CFH 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, CFH abnormalities were correlated with C3 reduction ($P < 0.05$). These results indicate that reduced C3 clusters in familial HUS and TTP is likely related to a genetically determined deficiency in CFH and may predispose to the disease.

Studies of **chapters 3 and 4** were aimed at clarifying whether CFH mutations were

involved in genetic predisposition to non-Stx-HUS, by performing linkage and mutation studies in patients referred to the International Registry for Recurrent and Familial HUS/TTP. In chapter 4, five mutations in the *CFH* gene were identified. Three mutations, identified in two families and in a sporadic case, are heterozygous point mutations causing amino acid exchange within the most C-terminal short consensus repeat 20 (SCR20) of *CFH*, resulting in single amino acid substitutions. The other two mutations introduce premature stop codons that interrupt the translation of *CFH*. A heterozygous nonsense mutation was identified in SCR8 in one family, and a homozygous 24-bp deletion within SCR20 was identified in a Bedouin family with a recessive mode of inheritance. Reverse transcription-PCR analysis of cDNA from peripheral blood leukocytes from the Bedouin family showed that the deletion lowered *CFH* mRNA levels. Although heterozygous mutations were associated with normal *CFH* levels and incomplete penetrance of the disease, the homozygous mutation in the Bedouin family resulted in severe reduction of *CFH* levels accompanied by very early disease onset.

In chapter 4, we analyzed the complete *CFH* gene in 101 patients with non-Stx-HUS, in 32 with TTP and in 106 controls to evaluate the frequency of *CFH* mutations, the clinical outcome in mutation and non-mutation carriers and the role of *CFH* polymorphisms in the predisposition to HUS. We found 17 *CFH* mutations (16 heterozygous, one homozygous) in 33 HUS patients. Thirteen mutations were located in exons XXII and XXIII. The disease manifested earlier and the mortality rate was higher in mutation carriers than in non-carriers. Kidney transplants invariably failed for disease recurrences in patients with *CFH* mutations,

while in non-mutated patients half of the grafts were functioning after 1 year. Three *CFH* polymorphic variants were strongly associated with non-Stx-HUS: -257T (promoter region), 2089G (exon XIV, silent) and 2881T (963Asp, SCR16). The association was stronger in patients without *CFH* mutations. Two or three disease-associated variants led to a higher risk of HUS than a single one. Analysis of available relatives of mutated patients revealed a penetrance of 50%. In 5/9 families the proband inherited the mutation from one parent and two disease-associated variants from the other, while unaffected carriers inherited the protective variants. In conclusion *CFH* mutations are frequent in patients with non-Stx-HUS (24%). Common polymorphisms of *CFH* may contribute to non-Stx-HUS manifestation in subjects with and without *CFH* mutations.

These data provide compelling molecular evidence that genetically determined deficiencies in *CFH* are involved in both autosomal-dominant and autosomal-recessive HUS and identify SCR20 as a hot spot for mutations in the disease.

Of interest, in studies of **chapter 4**, none of the 32 patients with a diagnosis of TTP and no renal impairment carried *CFH* mutations, which indicates that *CFH* abnormalities are specifically associated with renal manifestations of the thrombotic microangiopathy.

Evidence is now available that most cases of TTP are triggered by deficiency of ADAMTS13, a plasma metalloprotease that cleaves von Willebrand factor multimers. ADAMTS13 is usually normal in HUS, however there are rare unequivocal cases of HUS characterized by low or undetectable ADAMTS13 activity. In **chapter 5** we investigated the genetic basis of phenotype heterogeneity in patients with ADAMTS13 deficiency in two

sisters within one family. The patients had ADAMTS13 deficiency as a result of two heterozygous mutations (causing V88M and G1239V changes). In addition, a heterozygous mutation (causing an S890I change) in *CFH* was found in the patient who developed chronic renal failure but not in her sister, who presented with exclusive neurologic symptoms. These data indicate that in the above patient *CFH* haploinsufficiency had a role in determining the renal complication and the HUS phenotype that superimposed on the systemic disease caused by ADAMTS13 deficiency.

Two third of patients with non-Stx-HUS have no *CFH* mutations, despite decreased serum concentrations of C3. The aim of chapter 6, therefore, was to assess whether genetic abnormalities in other complement regulatory proteins are involved. We screened genes that encode the complement regulatory proteins- i.e. factor H related-5, complement receptor 1, and membrane cofactor protein (MCP)-by PCR-single-strand conformation polymorphism (PCR-SSCP) and by direct sequencing, in 25 consecutive patients with non-Stx-HUS, an abnormal complement profile, and no *CFH* mutation, from our International Registry of Recurrent and Familial HUS/TTP. We identified a heterozygous mutation in MCP, a surface-bound complement regulator, in two patients with a familial history of HUS. The mutation causes a change in three aminoacids at position 233-35 and insertion of a premature stop-codon, which results in loss of the transmembrane domain of the protein and severely reduced cell-surface expression of MCP. Findings of an MCP mutation in two related patients suggest that impaired regulation of complement activation might be a factor in the pathogenesis of genetic forms of HUS. MCP could be a second

putative candidate gene for non-Stx-HUS. The protein is highly expressed in the kidney and plays a major part in regulation of glomerular C3 activation. We propose, therefore, that reduced expression of MCP in response to complement-activating stimuli could prevent restriction of complement deposition on glomerular endothelial cells, leading to microvascular cell damage and tissue injury.

Finally the review chapter 7, was aimed at summarize the more recent advances from our and from other groups on the pathogenesis, the genetics and treatment of different forms of HUS.

Curriculum Vitae

Marina Noris was born in Alzano Lombardo (BG), Italy in 1961. She achieved her University degree in Pharmaceutical Chemistry at the University of Rome "La Sapienza" in 1986. From 1986 to 1987 she was a post doctoral fellow of the Institute of Pharmaceutical Chemistry at the University of Rome. In September 1987 she began her career at the Mario Negri Institute in Bergamo participating to the unique group of basic scientists and clinicians working with professor Giuseppe Remuzzi in the field of renal disease progression and transplant immunology. From 1994 to 1996 she was head of the Unit of Endothelial Cell Pathophysiology, from 1996 to 1999 she was head of the Laboratory of Cellular and Molecular Biology of the Immune Response and Autoimmunity at Mario Negri Institute in Bergamo. From January 2000 she become head of the Laboratory of Immunology and Genetics of Transplantation and Rare Diseases at the Clinical Research Center for Rare Diseases in Ranica of the Mario Negri Institute). Dr. Noris has authored or co-authored over 90 articles for publication in scientific journals, books, etc. especially in the field of pathophysiology and genetics of hemolytic uremic syndrome and thrombotic thrombocytopenic purpura and in the field of experimental transplant immunology. She has also undertaken several studies on nitric oxide and oxygen radical as uremic toxins and in particular on the role of nitric oxide in uremic bleeding and dialysis complications.

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