Factor H autoantibodies in atypical hemolytic uremic syndrome correlate with CFHR1/CFHR3 deficiency

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Abstract

Atypical Hemolytic Uremic Syndrome (aHUS) is a severe renal disease which is associated with defective complement regulation caused by multiple factors. We previously described the deficiency of Factor H related proteins CFHR1 and CFHR3 as predisposing factor for aHUS. Here we identify in an extended cohort of 147 aHUS patients that 16 juvenile individuals (i.e. 11%) who either lacked the CFHR1/CFHR3 completely (n = 14) or showed extremely low CFHR1/CFHR3 plasma levels (n = 2), are positive for Factor H (CFH) autoantibodies. The binding epitopes of all 16 analyzed autoantibodies were localized to the C-terminal recognition region of Factor H, which represents a hot spot for aHUS mutations. Thus we define a novel subgroup of aHUS, termed 'DEAP' HUS' (**DE**ficiency of CFHR proteins and CFH **A**utoantibody **P**ositive) that is characterized by a genetic and an acquired factor. Therefore screening for both parameters is relevant in HUS patients and reduction of CFH autoantibody levels represents a therapeutic option.

Introduction

The atypical form of hemolytic uremic syndrome (aHUS) is characterized by microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure (1). aHUS is associated with defective complement regulation. The complement system represents an innate immune defense system that eleminates invading microbes. Mutations in genes coding for complement regulators Factor H (*CFH*), membrane cofactor protein (*MCP*), Factor B (*CFB*), C3 and Factor I (*CFI*) (2-9) cause impaired regulation of the alternative pathway convertase C3bBb. This results in defective local complement control on host cell surfaces (10, 11). In addition, *CFH* gene conversion, deletion of the complement Factor H related genes *CFHR1* and *CFHR3* by nonallelic homologous recombination and the presence of CFH autoantibodies have been reported in aHUS patients (12-16).

These diverse scenarios are responsible for ~50% of the reported cases, indicating that additional factors contribute to aHUS. On the surface of human cells multiple regulators control complement activation. Under physiological conditions defective function of one mutated protein is compensated by the additional regulators, which display redundant activities. This situation might explain the incomplete penetrance of the genetic mutations. We have recently shown that CFH autoantibodies of five patients bind to the C-terminus of CFH and reduce CFH-C3b interaction (16).

In order to extend the understanding of the molecular basis of aHUS we determined the frequency of CFH autoantibodies in the Jena aHUS cohort and correlated the presence of CFH autoantibodies with CFHR1 and CFHR3 expression.

Materials and Methods

This study was approved by the Research Ethics Committee of the Friedrich Schiller University, Jena, the University of Cologne, Germany and the Hospital for Sick Children, Toronto, Canada.

Patients

The cohort analyzed here represents an extended cohort 147 patients with atypical HUS of which 121 patients were recently reported (14). Informations to patients are summarized (supplementary information).

Western Blot Analysis

Plasma samples of all patients (not shown) and of members of three selected families were investigated by Western blotting (14). CFHR1 was detected using monoclonal antibody C18 and CFHR3 was detected with CFHR3 antiserum .

Identification and domain mapping of CFH autoantibodies

The binding domains of the CFH autoantibodies in CFH were determined by ELISA as described (16). Briefly, microtiter plates (Nunc, Wiesbaden, Germany) were coated with CFH fragments (17), incubated with plasma of the patients and CFH autoantibodies were detected with HRP-conjugated anti human IgG antibodies (Sigma-Aldrich, Taufkirchen, Germany).

Results and dicussion

Frequency of CFH autoantibodies. Here we identify by ELISA in a cohort of 147 aHUS patients 16 children (i.e. 11%) as positive for CFH autoantibodies (Table 1). CFH autoantibodies were completely absent in a control group of 100 healthy individuals thus indicating that CFH autoantibodies are associated with aHUS. Similar to the young age of the patients of the Jena cohort, the eight previously identified CFH autoantibody positive HUS patients (5 to 17 years) (15, 16) were also juvenile suggesting related mechanisms for autoantibody induction.

Further analyses of the CFH autoantibody positive group revealed that by means of Western blotting the patients showed either the complete absence of CFHR1 and CFHR3 in plasma (14 patients) or displayed low, barely detectable levels of CFHR1 and CFHR3 (Table 1 and data not shown). The strong correlation between the occurrence of CFH autoantibodies and absence or reduction of CFHR1/CFHR3 in plasma suggests that this deficiency represents a risk factor for CFH autoantibody formation. The mechanism how a deficiency of these plasma proteins leads to the generation of CFH autoantibodies is currently unknown and requires further investigations. The 22 CFHR1/CFHR3 deficient patients of the Jena cohort include 16 CFH autoantibody positive and six patients which have no autoantibodies to CFH. The frequency of the deficient group without CFH autoantibodies is 4% in this cohort and thus slightly higher than in the Jena and Newcastle control groups (2% each) (14) or in the Iowa-, Columbia- and Finish AMD study cohorts (2.7, 3.0 and 2.5% respectively (18). Concurrence of two risk factors in development of aHUS has been reported for combined mutations in either the CFI and the MCP genes (19) or for various CFH haplotypes (20). Here we report a new combination of two disease associated

conditions in predominantly juvenile aHUS patients, namely the presence of CFH autoantibodies and absence of CFHR1/CFHR3 in plasma.

Family studies. Family studies were performed to analyze how autoantibodies to CFH or CFHR1/CFHR3 deficiency influences or predisposes to the disease. Three CFH autoantibody positive, CFHR1 and CFHR3 deficient patients and their family members were assayed for both parameters (Figure 1). In family A the patient (AII_1) (Figure 1A) was positive for CFH autoantibodies (Figure 1D) and CFHR1 and CFHR3 proteins were absent in his plasma (Figure 1B, lane 2). The mother (AI_2, lane 6), showed lower plasma levels of CFHR1 and CFHR3 proteins, indicating heterozygous deficiency. The other family members lacked CFHR1 and CFHR3 proteins which corresponds to homozygous deficiency. Genetic analyses confirmed homozygous *CFHR1* and *CFHR3* deficiency for the patient (AII_1), the healthy brother (AII_2), the healthy sister (AII_3) and the healthy father (AI_1). The *CFH* gene was intact in all family members (data not shown). A similar scenario was observed for families B and C. In family B the patient, but no other relative was positive for CFH autoantibodies (Figure 1D). CFHR1 and CFHR3 proteins were absent in the plasma of the patient (BII_1) and the unaffected healthy sister (BII_2) (Figure 1B, lanes 8 and 9); but were detected in sera of the healthy mother and the father (Figure 1B, lanes 10 and 11). Genetic analyses confirmed that the patient and his sister were homozygous for the *CFHR1/CFHR3* gene deletion. Similarly, in family C the aHUS patient was positive for CFH autoantibodies (Figure 1D) and CFHR1/CFHR3 proteins were absent. The remaining four healthy family members lacked CFH autoantibodies and also CFHR1/CFHR3 proteins in plasma (Figure 1B**,** lanes 13 to 17). Genetic analyses confirmed a homozygous deletion of *CFHR1/CFHR3* genes and non rearranged *CFH* genes for all members of this family (data not shown).

Thus, in each family the HUS patient was positive for CFH autoantibodies and deficient for CFHR1 and CFHR3. The chromosomal breakpoints in each case was located in the same chromosomal repeat region as recently described (14). All 11 members of the three families, who lacked CFH autoantibodies and showed either homozygous or heterozygous CFHR1/CFHR3 deficiency were healthy. Thus, these family studies demonstrate that CFH autoantibodies develop on a background of CFHR1 and CFHR3 deficiency.

We have previously localized the binding epitope of five CFH autoantibodies, derived from aHUS patients, two of which are also part of the Jena aHUS cohort, to the C-terminus of CFH. In addition these CFH autoantibodies inhibit the regulatory function of CFH at the cell surface (16). In order to define if this phenomenon holds true for the newly identified CFH autoantibodies their binding epitopes were also identified.

CFH autoantibodies from each of the 16 patients bound to the C-terminal fragments of CFH, i.e. SCRs 15–20 and SCRs 19-20, but neither to SCRs 1–7, SCRs 11–15, SCRs 15-18 nor to SCRs 15-19 (supplementary Table I). Four CFH autoantibodies, also showed weak binding to a fragment representing SCRs 8–11 of CFH. This profile reveals that all 16 analyzed CFH autoantibodies bind preferentially within the C-terminal recognition region of CFH (21 - 25), which represents also a hot spot for aHUS associated mutations (9). This overlap suggests similar functional consequences for the CFH autoantibodies and for the genetic mutations, namely reduced cell recognition functions of CFH.

In summary, we identify a new subgroup of aHUS patients who are deficient for CFHR1 and CFHR3 in plasma and positive for CFH autoantibodies. This deficiency may

favour development of specific autoantibodies which bind to the recognition region of CFH and likely block cell binding. It remains to be shown if disease progression of this new subgroup differs from that of other HUS patients e.g. patients with CFHR1/CFHR3 deficiency and the absence of CFH autoantibodies or patients with CFH mutations.

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Contribution

MJ CL PFZ CS designed the research and wrote the manuscript. SS SLHZ HR SH performed experiments and analyzed data. CL provided patient specimen.

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Competing interests

The authors have declared that no competing interests exist.

References

1. Thompson, R. A., and Winterborn, M. H. Hypocomplementaemia due to a genetic deficiency of beta 1H globulin. Clin Exp Immunol. 1981;46:110-119.
2. Zipfel, P.F., and Skerka, C. Complement dysfunction in hemolytic uremic syndrome. Curr Opin Rheumatol. 2006;18:548-55.
3. Noris, M., and Remuzzi, G. Hemolytic uremic syndrome. J Am Soc Nephrol. 2005;16:1035-50.
4. Richards A, Kemp EJ, Liszewski MK, et al. Mutations in human complement regulator, membrane cofactor protein (CD46), predispose to development of familial hemolytic uremic syndrome. Proc Natl Acad Sci U S A. 2003 ;100:12966-71.
5. Goicoechea de Jorge, E., Harris, C.L, Esparza-Gordillo, J., et al. Gain-of-function mutations in complement factor B are associated with atypical hemolytic uremic syndrome. Proc Natl Acad Sci U S A. 2007;104:240-5.
6. Fremeaux-Bacchi, V., Dragon-Durey, M.-A., Blouin, J., et al. Complement factor I: a susceptibility gene for atypical haemolytic uraemic syndrome. J Med Genet. 2004;41: e84.
7. Kavanagh, D., Kemp, E. J., Mayland, E., et al. Mutations in complement factor I predispose to development of atypical hemolytic uremic syndrome. J Am Soc. Nephrol. 2005;16: 2150-2155.
8. Fremeaux-Bacchi, V., Goodship T, Régnier, C., et al. Mutations in complement C3 predispose to development of hemolytic uraemic syndrome. Mol. immunol. 2007, 44:06.038 abstract.
9. Saunders, R., Abarrategui-Garrido, C., Fremeaux-Bacchi, V., et al. The interactive Factor H-atypical hemolytic uremic syndrome mutation database and website:

update and integration of membrane cofactor protein and Factor I mutations with structural models. Hum Mutat. 2007;28:222-34.

10. Pangburn, M.K. Cutting edge: localization of the host recognition functions of complement factor H at the carboxyl-terminal: implications for hemolytic uremic syndrome. J Immunol. 2002;169:4702-6.
11. Heinen, S., Józsi, M., Hartmann, A., et al. Hemolytic Uremic Syndrome: A Factor H Mutation (E1172Stop) Causes Defective Complement Control at the Surface of Endothelial Cells. J Am Soc. Nephrol. 2007;18:506-14.
12. Heinen, S., Sanchez-Corral, P., Jackson, M.S.,et al. De novo gene conversion in the RCA gene cluster (1q32) causes mutations in complement factor H associated with atypical hemolytic uremic syndrome. Hum Mutat. 2006;27:292-3.
13. Venables, J.P., Strain, L., Routledge, D., et al. Atypical Haemolytic Uraemic Syndrome Associated with a Hybrid Complement Gene. PLoS Medicine 2006:3
14. Zipfel, P.F., Edey, M., Heinen, S., et al. Deletion of Complement Factor H Related Genes *CFHR1* and *CFHR3* is associated with an Increased Risk of Atypical Hemolytic Uremic Syndrome. PLoS Genetics 2007;3:e41
15. Dragon-Durey, M.A., Loirat, C., Cloarec, S., et al. Anti-Factor H autoantibodies associated with atypical hemolytic uremic syndrome. J Am Soc Nephrol. 2005;16:555-63.
16. Józsi, M., Strobel, S, Dahse, H.-M., et al. Anti-factor H autoantibodies block C-terminal recognition function of factor H in hemolytic uremic syndrome. Blood 2007;110:1516-1518.
17. Kühn, S., and Zipfel, P.F. The baculovirus expression vector pBSV-8His directs secretion of histidine-tagged proteins. Gene 1995:162:225-229.
18. Hageman, G.S., Hancox, L.S., Taiber, A.J., et al. Extended haplotypes in the complement factor H (CFH) and CFH-related (CFHR) family of genes protect

against age-related macular degeneration: characterization, ethnic distribution and evolutionary implications. Ann Med. 2006;38:592-604.

19. Esparza-Gordillo, J., Jorge, E. G., Garrido, C.A., et al. Insights into hemolytic uremic syndrome: segregation of three independent predisposition factors in a large, multiple affected pedigree. Mol Immunol. 2006;43:1769-75.
20. Caprioli, J., Castelletti, F., Bucchioni,S., et al. International Registry of Recurrent and Familial HUS/TTP. 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. Hum Mol Genet. 2003;15:3385-95.
21. Manuelian, T., Hellwage, J., Meri, S., et al. Mutations in factor H reduce binding affinity to C3b and heparin and surface attachment to endothelial cells in hemolytic uremic syndrome. J Clin Invest. 2003;111:1181-90.
22. Caprioli, J., Bettinaglio, P., Zipfel, P. F., et al. The molecular basis of familial haemolytic uremic syndrome: mutation analysis of factor H gene reveals a hot spot in short consensus repeat 20. J Am Soc Nephrol. 2001:12:297-307.
23. Richards, A., Buddles, M.R., Donne, R.L., et al. Factor H mutations in hemolytic uremic syndrome cluster in exons 18-20, a domain important for host cell recognition. Am J Hum Genet. 2001; 68:485-90.
24. Jokiranta, T S., Jaakola, V.P., Lehtinen, M.J., et al. Structure of complement factor H carboxyl-terminus reveals molecular basis of atypical haemolytic uraemic syndrome. EMBO J. 2006;19:1784-94.
25. Józsi, M., Oppermann, M., Lambris, J.D., and Zipfel, P.F. The C-terminus of complement factor H is essential for host cell protection. Mol Immunol. 2007;44:2697-2706.

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Figure legends

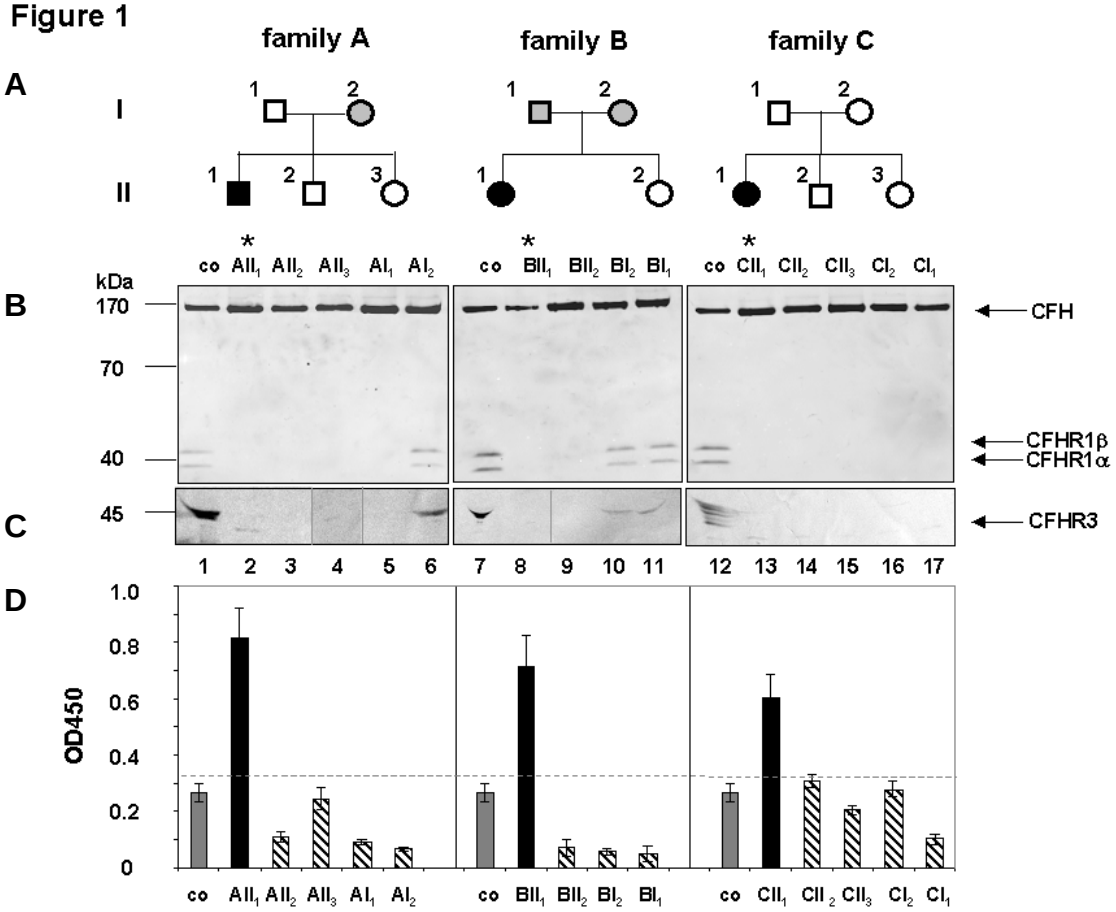
Figure 1: Family analysis: Deficiency of CFHR1 and CFHR3 in aHUS patients and their family members. (A) A pedigree is shown for each family. Black boxes: patients, open symbols: family members with homozygous *CFHR1* and *CFHR3* deletion, grey symbols: individuals with heterozygous CFHR1 and CFHR3 deficiency. Plasma of the patients or their healthy family members were separated by SDS-Page, transferred to a membrane and analyzed by Western blotting using a mAB which identifies the conserved C-termini of CFH (150 kDa) and the two differently glycosylated forms of CFHR1□ and CFHR1□ (42 and 37 kDa). For detection of CFHR3, antiserum reacting with different glycosylated forms of CFHR3 (45 kDa, multiple bands) was used. (B) Western blot analysis of plasma derived from individual family members demonstrated deficiency of CFHR1 in the aHUS patients (* lanes 2, 8, 13) and also in healthy relatives (lanes 3- 5, 9, 14-17). CFHR1 (α and β) are detected in plasma of a healthy control (lanes 1, 7, 12). CFH is detected in all plasma samples. (C) Complete deficiency of CFHR3 is detected in the three aHUS patients (lanes 2, 8, 13) and several relatives (lanes 3-5; 9; 14-17) but CFHR3 is observed in the plasma of a healthy volunteer (lanes 1, 7, 12) and of heterozygous relatives (lanes 6, 10 and 11). The band at 30 kDa in lane 2 is unspecific. (D) CFH autoantibodiy levels were detected by ELISA. CFH autoantibodies (black bars) are present in serum of the patients (AII_1, BII_1 and CII_1) but not of their relatives (dashed bars) and in plasma derived from controls (co, grey bars).. The dotted line represents the background level (OD_{450} 0.35), i.e. highest absorbancy of plasma samples derived from 100 control individuals (see supplementary information).

Table 1 Frequency of CFH autoantibodies and CFHR1 and CFHR3 deficiency in the Jena cohort

	n	CFH autoantibodies n	CFHR1/CFHR3 deficiency n
aHUS patients	**147**	**16 (11%)**	**22 (15%)**
controls	**100**	**0 (0%)**	**2 (2%)**

Table I: Frequency of Factor H (CFH) autoantibodies in aHUS patients of the Jena cohort. The 16 patients who developed CFH autoantibodies, either lack CFHR1/CFHR3 completely in plasma (n = 14) or show extremely low levels of the two CFHR proteins (n = 2) as determined by Western blotting. The CFHR1 and CFHR3 deficient group includes the 16 patients of the CFH autoantibody positive group and six deficient individuals who have no autoantibodies to CFH. No CFH autoantibodies were detected in the control group representing 100 healthy individuals. The mean absorbancy of all 100 control probes was OD 0.17 ± 0.1. The highest value determined for one sample of the control group was 0.35 OD, therefore the cut off for false positive was set to 0.35 OD.

Figure 1


family A
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family C
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I
II
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D
kDa
170
70
40
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CFH
CFHR1β
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CFHR3
OD450
1.0
0.8
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co BII1 BII2 BI2 BI1
co CII1 CII2 CII3 CI2 CI1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Supplementary information

Patients : family analyses

Patient 1 (family A) was diagnosed with aHUS at age 13 10/12, patient 2 (family B) was diagnosed with aHUS at age 7 2/12 and patient 3 (family C) at age 12. The initial sample assayed for the presence of CFH autoantibodies was taken at day of admission to the hospital prior to treatment. All three patients were treated with repeated plasmapheresis and their renal function recovered. Genetic deletion of *CFHR1* and *CFHR3* was analyzed as described and hybrid *CFH/CFHR1* genes were excluded by sequencing genomic DNA (12). The sequence of the CFH (*CFH*), MCP (*CD45*) and Factor I (*CFI*), and Factor B (*CFB*) genes were analyzed for each patient. Except for a single amino acid exchange at position 950 Q to H in the *CFH* gene from patient AII_1 no further disease associated mutation was identified.

Supplementary Figure 1: CFH autoantibody levels in aHUS

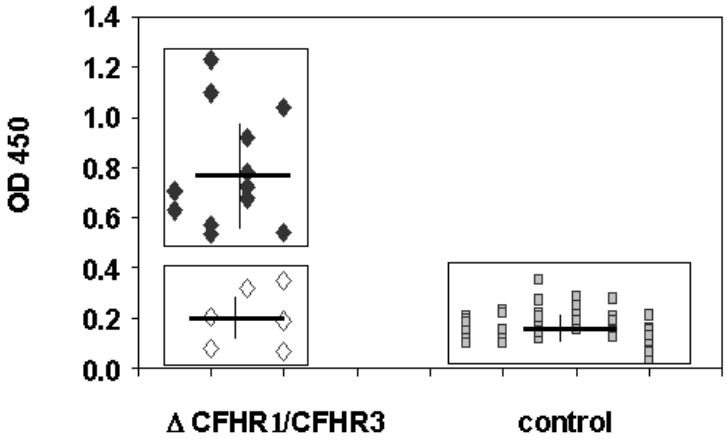

Figure 1: CFH autoantibodies levels in aHUS patients. Autoantibody levels were determined by ELISA in serum of a Jena HUS cohort (147 aHUS patients)(not shown) and a control group (100 healthy volunteers). The autoantibody levels of the 22 CFHR1 and CFHR3 deficient and the two low patients are indicated on the left. 16 samples (left upper panel indicated by filled rombi) were positive for CFH autoantibodies showed a mean value of OD 0.77 ± 0.2, $p < 0.00001$). The other six CFHR1/CFHR3 deficient patients with an $A < 0.35$ (mean OD 0.2 ± 0.1, $p = 0.148$) (left lower panel indicated by open rombi) were considered negative. The antibody levels of the remaining 125 aHUS patients, which express CFHR1 and CFHR3, was <OD 0.35. The mean absorbancy of all 100 control probes was OD 0.17 ± 0.1. 46 representative samples of the control group are shown in the right panel (grey boxes). The highest value determined for one sample of the control group was 0.35 OD, therefore the cut off for false positive was set to 0.35 OD. Statistical analysis was performed using the Student's T-test.

Supplementary Table 1: Localization of the binding domains of CFH autoantibodies in CFHR1 and CFHR3 deficient HUS patients

	Patient #															
CFH fragments	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
SCRs 1–7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SCRs 8–11	(+)	–	–	–	–	–	(+)	–	(+)	–	–	(+)	–	–	–	–
SCRs 11–15	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SCRs 15–18	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SCRs 15–19	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SCRs 15–20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SCRs 19–20	+	+	(+)	+	+	+	+	+	+	+	+	+	(+)	+	(+)	+

For domain mapping microtiter plates were coated with the indicated recombinant Factor H (CFH) fragments and probed with patient's serum (16). Binding of autoantibodies to the indicated Factor H fragments was considered positive (+) for OD_{450} >0.35 A (reaching absorbancy up to 1.5). Low binding (+) is based on an absorbancy ranging from OD_{450} >0.2 to <0.3 and no binding (–) by an OD_{450} of <0.2.