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Functional analyses indicate pathogenic role of factor H autoantibodies in atypical hemolytic uremic syndrome

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Running title: Role of factor H autoantibodies in HUS

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Abstract

Background. Atypical hemolytic uremic syndrome (aHUS) is associated with defective complement regulation. Recently, an autoimmune aHUS form has been described that is associated with complement factor H (CFH) autoantibodies. The aim of this study was to address the pathologic relevance of CFH autoantibodies in aHUS.

Methods. CFH autoantibodies were identified and antibody levels were analyzed in three aHUS patients during disease course by ELISA method. Epitope mapping was performed using recombinant factor H fragments and domain mapped monoclonal antibodies. Effect of the antibodies on cell protective activity of CFH was measured by hemolytic assays. CFH:autoantibody complexes were analyzed by ELISA.

Results. All three autoantibodies bound to the C-terminal domain of CFH, which is essential for CFH binding to cell surfaces. In patient 1, plasma exchanges and immune adsorption temporarily reduced the autoantibody titer and led to temporary clinical improvement. In patient 2, plasma exchanges and long-term immunosuppression strongly reduced the CFH autoantibody level, and induced a stable remission of aHUS. Patient 3 had lower autoantibody levels that decreased during follow-up and is in good clinical condition. The patients' plasma samples caused enhanced lysis of sheep erythrocytes and the degree of lysis correlated with the CFH autoantibody titer and the amount of CFH:autoantibody complexes. Addition of purified CFH to aHUS plasma or removal of IgG inhibited the hemolytic activity.

Conclusion. These results support a direct role of the autoantibodies in aHUS pathology by inhibiting the regulatory function of CFH at cell surfaces and suggest that reduction of the autoantibody titer is beneficial for the patients.

Keywords:

autoantibody, complement, factor H, hemolytic uremic syndrome

Short summary:

This study addresses the role of factor H autoantibodies in autoimmune atypical hemolytic uremic syndrome. The levels of free and complexed autoantibodies in patients' plasma samples were analyzed in parallel with the function blocking activity of the autoantibodies. The results support a pathogenic role of the autoantibodies by inhibiting the protective activity of factor H on cell surfaces during complement attack.

Introduction

Hemolytic uremic syndrome (HUS) is a severe kidney disease characterized by microangiopathic hemolytic anemia, low platelet count and acute renal failure. The common form of this disease (D+ HUS) is usually caused by bacterial infection and is associated with diarrhea [1]. The more rare, atypical form of HUS (aHUS or D- HUS), includes both familial and sporadic cases, and is associated with dysregulation of complement activation [1,2]. In aHUS patients, disease-associated mutations and polymorphisms in complement genes have been described for factor H (CFH), membrane cofactor protein (MCP), factor I (CFI), factor B and C3 [3,4]. The mutations are generally heterozygous, and most likely the mutant proteins cause inappropriate local control of complement activation. Deletion of the factor H-related genes *CFHR1* and *CFHR3* is also associated with aHUS [5]. In addition, CFH autoantibody-associated aHUS, which has been reported in 6-10% of aHUS-patients, represents an acquired autoimmune form of the disease [6,7]. The appearance of CFH-specific autoantibodies is linked with the deletion of *CFHR1* and *CFHR3* [8].

CFH is an abundant plasma protein and the major regulator of the alternative complement pathway [9]. CFH is composed of 20 short consensus repeat (SCR) domains. The N-terminal domains (SCRs 1-4) of CFH mediate cofactor and decay accelerating activities [10,11], and the C-terminal domains (SCRs 19-20) contain binding sites for C3b, heparin and endothelial cells [12-14]. Thus, the complement regulatory region and the recognition region responsible for cell attachment of CFH are spatially separated. SCRs 19-20 represent a hot spot for aHUS-associated CFH mutations [15]. Several of these mutations have been shown to reduce CFH binding to C3b and to cellular surfaces [16-18]. Functional studies have demonstrated that an intact C-terminus is necessary for CFH to bind and exert its complement

regulatory activity on cell and tissue surfaces, thus, for full-scale protection of host cells against the activated complement system [19-22].

We have previously mapped the binding sites of CFH autoantibodies to the same C-terminal domains of CFH, i.e. SCRs 19-20, where most of the aHUS-associated CFH mutations occur. In an *in vitro* hemolytic assay, the autoantibody positive plasma of one patient caused enhanced lysis of sheep erythrocytes [7]. This suggests that CFH autoantibodies inhibit CFH complement regulatory activity on cell surfaces.

In order to elaborate on the suggested pathomechanism and to assess the clinical significance of CFH autoantibodies in aHUS, we have followed the titer and activity of the autoantibodies during the disease course of aHUS patients.

Subjects and Methods

The studies have been approved by the Research Ethics Committee of the Medical Faculty of Friedrich Schiller University, and were performed in accordance with the Declaration of Helsinki.

Sample collection and analyses of anti-CFH autoantibodies

Plasma samples, collected with informed consent, were analyzed for the presence of CFHR1 and CFHR3 by Western blot [8]. CFH autoantibodies were identified as described [7]. The IgG isotypes were determined using IgG1-, IgG2-, IgG3- and IgG4-specific mouse mAbs (Sigma-Aldrich, Taufkirchen, Germany) and HRP-conjugated rabbit anti-mouse Ig (DakoCytomation, Hamburg, Germany). IgG was isolated from plasma using Protein G columns (GE Healthcare, Freiburg, Germany). The binding domains of the autoantibodies were determined using recombinant CFH fragments as described [7]. For inhibition experiments, immobilized CFH was preincubated with CFH-specific goat antiserum (Merck Biosciences, Schwalbach, Germany), rabbit antisera raised against SCRs 1-4 [23] and SCRs 19-20 of CFH [24] (both diluted 1:100) or with domain mapped, CFH-specific monoclonal antibodies [7,14].

Determination of CFH autoantibody titer in patients' plasma

Plasma samples were incubated on CFH-coated wells in serial dilutions. The titer was determined in comparison with a reference sample of patient 1, taken 10 months after initial disease onset, and is expressed in arbitrary units as described by Dragon-Durey et al. [6]. This sample was compared to a reference with 1000 AU/ml [6], kindly provided by Dr. Dragon-Durey, and had essentially the same titer (1014 AU/ml).

Hemolysis assays

Hemolysis assays were performed as described using sheep erythrocytes (BioTrend Chemikalien, Cologne, Germany) [7]. Some samples were incubated with Protein G beads for 5-15 min at 20°C to deplete IgG. Hemolytic effects of the CFH-specific mAbs (50 µg/ml) were measured using 10% C2-depleted plasma (Merck) in order to prevent activation of the classical complement pathway.

Measurement of autoantibody:CFH complexes

Immune complexes were captured on microtiter plate wells coated with the anti-CFH mAb M15 at 5 µg/ml. After blocking, serial plasma dilutions were incubated for 1 h at 20°C and bound complexes were detected using HRP-conjugated anti-human IgG (Sigma-Aldrich). Bound CFH was detected using a CFH antiserum.

Results

Case descriptions

Patient 1

Patient 1, a 10-year-old boy (patient #564 in ref. 7), was admitted to hospital at the age of 6.5 years with kidney failure, and was later diagnosed with aHUS. ADAMTS13 activity was normal. Both serum and stool were negative for enterohemorrhagic *E. coli*. Two weeks after disease onset he received ten plasmapheresis in 19 days. Disease recurrence with increase of creatinine, low complement C3 levels and increased C3d occurred 4 mo later and was treated with seven plasma exchanges in a 2-wk period. A second recurrence occurred 3 mo later. Plasma infusions (three times in 10-d intervals) did not improve renal function, therefore the presence of CFH autoantibodies was postulated. Plasma exchange was resumed (ten times in 13 d) with hemodialysis three times. Additionally, immunosuppressive therapy was initiated (mycophenolate mofetil 2×250 mg/day, later 2×500 mg/day for 2 months). Rituximab (375 mg/m^2) was applied three times at 3-wk intervals started at 11 mo. Because of unsatisfactory efficacy, this was followed by cyclophosphamide treatment three times at 3-wk intervals. Plasma exchanges were performed six times within one week at 12 mo, then seven times in 12 days at 14 mo. A short trial with immunadsorption was stopped because of allergic reaction. 42 mo after disease onset, the patient remains on peritoneal dialysis, which was changed from hemodialysis because of catheter infection and sepsis. It is of note that C3 and C3d levels are normal. Genetic analysis showed that in the *CFH* gene this patient is homozygous for the C allele of the c.921A>C polymorphism (A307A in SCR5) [25,26] and has the H4 haplotype [27]. In the *CFI* gene a heterozygous G818A transition (S268S) was found, which is a non-disease causing polymorphism [28]. The patient lacks CFHR1 and CFHR3.

Patient 2

Patient 2 is an 8.5-year-old girl, who presented with D- HUS at the age of 6.8 years. She was treated with seven plasma exchanges and received immunosuppressive therapy with mycophenolate mofetil and prednisolone. Remission of aHUS was achieved and renal function returned to normal. On immunosuppression and antihypertensive treatment she has remained free from recurrences up to now. The patient lacks the CFHR1 and CFHR3 proteins.

Patient 3

Patient 3 is a 12.5-year-old boy who presented with D- HUS at the age of 5 years. At the early phase of the disease he was treated with hemodialysis and received plasmapheresis on every second day (7 sessions) followed by weekly transfusion of fresh frozen plasma for a period of 3 months. His kidney functions rapidly improved and have since been stable with endogenous creatinine clearance of 35-50 ml/min/l.73m^2. He is in good clinical condition without clinical or laboratory evidences of hemolysis and with complement levels in the normal range. He is on protein-restricted diet, on combined antihypertensive therapy and receives $NaHCO_3$ and $CaCO_3$ supplements. Retrospective analysis of his initial plasma samples revealed lack of CFHR1 and CFHR3 and the presence of CFH antibodies.

Individual samples from three additional autoantibody positive patients (aHUS1-aHUS3), described before [8], were also used in hemolytic assays. These patients, who were treated with plasmapheresis, lack CFHR1 and CFHR3 [8].

Characterization of the CFH autoantibodies

All three patients, but not their family members, have CFH autoantibodies (**Figure 1 and data not shown**). The anti-CFH IgG of patient 1 was characterized before [7]. The three autoantibodies were of IgG3 isotype. Reactivity of the autoantibodies with recombinant CFH fragments was measured by ELISA in order to localize the binding domain. The

autoantibodies bound only to the CFH fragment that includes the most C-terminal SCR20 domain, i.e. SCRs 15-20. Binding of the autoantibodies to CFH was inhibited by C-terminally binding mAbs (C18, C14 and C02), but not by mAbs that recognize the N-terminus (N11) or the middle region of CFH (M12, M13, M15). Likewise, polyclonal antibodies against SCRs 19-20 and against full-length CFH reduced autoantibody binding, whereas an antibody specific to SCRs 1-4 did not affect autoantibody binding to CFH (**Figure 1 and data not shown**). No complete inhibition of autoantibody binding could be achieved with mAb C02 in the case of patient 2, indicating that despite the same binding domain on CFH, the exact binding epitope is different in this patient. Thus, CFH autoantibodies from the three unrelated patients have similar characteristics, because they are of the same isotype and bind to the same domain on CFH.

CFH autoantibody levels during disease course

To assess the relevance of the CFH autoantibodies in aHUS, the autoantibody titer was followed during treatment of the patients. In patient 1, plasma exchanges temporarily reduced the CFH autoantibody titer **(Figure 2A)**, which was accompanied with temporarily normalized C3 levels and improvement in clinical parameters (**Table 1**). Immunosuppressive therapy was attempted to reduce the amount of circulating anti-CFH IgG, but the titer remained high. Although immune adsorption was effective in reducing the CFH autoantibody titer (from 982 to 219 AU/ml), the treatment had to be stopped because of allergic reaction. This was followed by an increase in circulating autoantibodies and the patient's kidney function deteriorated. At present, the patient has persistently high titers (707-1244 AU/ml in the last 6 mo) and remains on peritoneal dialysis. In patient 2, the autoantibody levels were strongly reduced after initial plasma exchanges and immunosuppressive treatment, and remained low with little variation during the follow-up period (<100 AU/ml in the last 15 mo) **(Figure 2B)**. C3 level and clinical parameters normalized. Patient 3 had lower autoantibody

level which decreased during follow-up **(Figure 2C)** and remains in good clinical condition with normal complement levels.

CFH autoantibodies inhibit CFH cell protective activity

To elaborate on the suspected inhibitory role of the autoantibodies in protection of cellular surfaces by CFH against complement attack, hemolysis of sheep red blood cells (SRBC) was measured in the presence of patients' plasma as described [7]. This assay allows measurement of complement alternative pathway activity on cell surfaces. Incubation with CFH autoantibody positive plasma samples resulted in enhanced hemolysis of SRBC in all tested cases (n = 6), including the three patients and additional samples from patients described before (**Figure 3A**) [7,8]. The sample of patient 3 with relatively low autoantibody titer showed the smallest hemolytic effect. In contrast, normal human plasma samples showed background lytic effect. SRBC lysis was dose-dependently inhibited by addition of purified CFH to patients' plasma in all cases (**data not shown**). To determine if CFHR1/CFHR3 deficiency alone influences hemolytic activity, additional assays were performed with samples of CFHR1/CFHR3 deficient individuals who lack autoantibodies. These samples caused no enhanced hemolysis (**Figure 3B**). The role of CFH was further confirmed using CFH-specific mAbs. Among these antibodies, only the C-terminally binding mAbs C18, C02 and C14, which have overlapping binding epitopes on CFH and compete with the autoantibodies for CFH binding (**Figure 1**) [7], caused enhanced SRBC lysis when added to normal human plasma (data not shown) or to C2-depleted plasma, which was used to exclude classical pathway mediated lysis (**Figure 3**C).

The *in vitro* hemolytic activity of aHUS plasma correlates with the autoantibody titer

We next addressed the question whether the different autoantibody titers indeed result in variation of hemolytic activity of the patients' plasma. Samples taken at different time points

from aHUS patients were analyzed for both CFH autoantibody titer and hemolytic activity. The extent of SRBC lysis in all three analyzed cases followed the changes in autoantibody titer and was lower when the titer dropped (**Figure 4**). The isotype and the binding epitope of the autoantibodies were the same at the analyzed time points (**data not shown**). Because large differences in the C3 and CFH levels in the samples may influence the extent of hemolysis [19], these parameters were also determined. The C3 and CFH values of the analyzed samples showed no correlation with hemolysis (Figure 4).

To directly address the role of the autoantibodies, the lytic effect of patients' plasma before and after IgG depletion was compared. Preincubation of patients' plasma with Protein G beads resulted in reduced SRBC lysis, depending on the duration and thus the extent of IgG depletion, and reached background level after 15 min depletion (**Figure 5A**). This IgG depletion results in an artificial reduction of autoantibody titers and the data indicate that the same plasma samples with the same C3 and CFH levels cause hemolysis to different extents depending on the amounts of autoantibodies remaining in plasma. The same results were obtained with all three analyzed autoantibody positive patients' samples. Furthermore, when adding the C-terminally binding C18 mAb to the IgG-depleted plasma or to control normal human plasma, the lysis increased again (**Figure 5B**). This also shows that after IgG depletion the complement system in plasma remains active, and the reduced lysis is due to the removal of anti-CFH IgG and not to an unspecific complement inhibitory effect. Thus, inhibition of CFH surface recognition functions by the autoantibodies results in inappropriate protection of cells against complement attack.

CFH:autoantibody complexes in patients' plasma

Analysis of autoantibody titers as described above determines only free IgG autoantibodies that recognize immobilized CFH. To analyze CFH:anti-CFH complexes in patients' plasma,

serial dilutions of plasma samples were incubated in microtiter plate wells coated with the anti-CFH mAb M15 that binds outside the CFH C-terminus (within SCRs 11-18) [14], and immune complexes were detected with anti-human IgG. A dose-dependent binding of the CFH:autoantibody complexes was observed and at lower autoantibody levels the amounts of immune complexes were also reduced (**Figure 6A**). In patient 3, the autoantibody titers were lower than in the other two patients and changed little over time (**Figure 2**). Likewise, the amounts of CFH:autoantibody complexes were similar in the plasma samples, also indicating no significant change in the affinity of the autoantibodies. Upon IgG-depletion the immune complexes disappeared (shown for patient 3 in **Figure 6B**). This is in parallel with the reduced hemolytic activity of the IgG-depleted plasma samples (**Figure 5**), and further supports the CFH inhibitory role of the autoantibodies in cellular protection.

Because mAb C18 and the autoantibodies have overlapping binding sites on CFH, this mAb can catch free CFH only. Therefore, comparison of measured CFH levels using mAbs C18 for free and M15 for total CFH allowed to estimate the amounts of free and complexed CFH (**Figure 6C**). In the sample of patient 2 with high titer (1325 AU/ml) approximately 75% of CFH was in complex with antibodies, in comparison with ca. 20% in the sample with low titer (361 AU/ml). In the sample of patient 3 with higher titer (867 AU/ml) ca. 30% of CFH was in complex, whereas the amount of complexes were reduced to half in the low titer sample (539 AU/ml), which also had weak hemolytic effect (Fig. 3A). Thus indicating that the affinity of autoantibodies for CFH is lower in patient 3 than in patient 2.

Discussion

Experimental evidence and genetic analyses over the past years established that the delicate balance of complement activation and regulation is disturbed in the affected individuals and is involved in the pathogenesis of aHUS [1-3]. Apparently, aHUS is a multifactorial disease, with multiple predisposing genetic factors and the requirement of an environmental trigger, most often infection [29].

The role of the C-terminus in targeting CFH complement regulatory activity to host cells is supported by functional studies [20-22] and by a mouse model of aHUS [30]. In addition, sera derived from aHUS patients with C-terminal CFH mutations show reduced protection of sheep erythrocytes in a complement-dependent lysis assay [19,31].

The CFH autoantibodies analyzed in this and in earlier studies bind to the most C-terminal domain of CFH, i.e. SCR20 (**Figure 1**) [7,8]. Cell survival assays show that aHUS-associated autoantibodies cause similar functional defects as those described for mutant CFH proteins and affect C-terminus-dependent complement regulatory function of CFH. Sheep red blood cells, which have surface polyanionic molecules similar to human cells and which lack human membrane complement regulators, rely on CFH binding and CFH regulatory activity to evade human complement. Thus, when using human plasma in hemolytic assays, sheep erythrocytes are protected by bound human CFH and are not lysed. By contrast, plasma samples of aHUS patients that contain blocking autoantibodies cause hemolysis (**Figure 3A**). This *in vitro* hemolytic effect can be inhibited by the addition of excess CFH, or by depletion of IgG from the plasma which indicates normalized CFH regulatory function on cellular surfaces after removal of CFH autoantibodies (**Figure 5**).

The same hemolytic effects are achieved through mAbs that share binding epitopes with the autoantibodies (**Figure 3**C). Reduced C3b binding of CFH and impaired C3b inactivation on the cell surface, which result in enhanced complement activation and cell damage, are caused by mAbs C02, C18 and C14 [21]. These mAbs cause enhanced SRBC lysis when added to normal human plasma or to IgG-depleted plasma of autoantibody positive aHUS patients (**Figure 5**). Altogether these data point to the importance of cell protection by surface bound CFH under normal, physiological conditions, and show that this mechanism does not function properly in aHUS patients with C-terminal CFH mutations or with CFH autoantibodies. The fenestrated endothelium in the kidney appears particularly sensitive to self-attack, thus the loss of full-scale complement control due to CFH dysfunction leads to complement-mediated damage [32].

The hemolytic assay is a useful tool to analyze CFH functional defects, either caused by C-terminal mutations or by autoantibodies. This assay has the advantage that it directly evaluates the cell surface activity of CFH, which is relevant in aHUS [7,19,31]. In contrast, fluid-phase assays may not be informative, because CFH function in plasma is usually normal in the patients [6,7,22]. However, the hemolytic assay can lead to false negative results, for example, if C3 is largely consumed, and therefore the plasma has low complement activity. Thus, the extent of lysis induced by plasma samples depends on the autoantibody titer and affinity, the individual CFH level and the complement status, and it is important to collect and handle the samples properly so as they are suitable for such assays. In the analyzed samples, CFH levels varied only little, and C3 levels were sufficient for complement activation, as shown by the use of mAb C18, and the hemolytic effect was dependent on the presence and amounts of autoantibodies in plasma (Fig. 3-5).

The three autoantibodies analyzed here had the same characteristics, because they were of the same isotype and bound on the same domain of CFH. There were differences, however, between the patients regarding the time of diagnosis of the autoantibodies and the applied treatment. In the two patients, where the autoantibody levels were permanently reduced, there was also improvement in kidney funtion. In patient 1, autoantibodies persist at high levels despite several attempted treatments and the patient progressed to end-stage renal failure.

The direct role of the autoantibodies in inhibiting CFH cell protective function and correlation of the autoantibody titer with cell damaging effects (**Figures 3-5**) suggest that attempts to remove the autoantibodies from the patients' circulation represent a rational therapeutic approach. Recently, plasma exchanges and rituximab were successfully applied before kidney transplantation of an aHUS patient with CFH autoantibodies [33]. Similarly, in a patient plasma exchanges and immunosuppression as post-transplant management resulted in the disappearance of autoantibodies and a favorable outcome [34]. In addition, purified or plasma-derived CFH might be beneficial by increasing the concentration of antibody-free CFH. Plasma exchanges, although temporarily remove CFH autoantibodies (**Figure 2 and Table 1**) [6,33,34], might also have a boosting effect in the long term on autoantibody production by introducing free CFH antigen. Aiming to reduce the amount of autoantibodies, by e.g. immunosuppressive therapies and/or immune adsorption, and intravenous IgG (IVIg) infusion, likely represents a better option and eliminates the above risk. It remains to be shown how and when CFH autoantibodies appear, and whether autoantibody generation is directly influenced by the lack of the CFHR1 and CFHR3 proteins.

Even though we could analyze only a few cases in detail in the present study, the results support a pathogenic role of the CFH autoantibodies in aHUS. Because of the similar

binding sites and hemolytic effects of the autoantibodies analyzed so far [7,8,31], these results likely apply in general. Further clinical studies involving more patients are required to better understand the role of autoantibodies and to determine which treatment is most suitable and effective in this patient group.

In conclusion, the data reported here demonstrate that there is a direct connection between the presence and level of CFH autoantibodies and the extent of CFH functional defect at cell surfaces, indicating that CFH autoantibodies contribute to the pathology of aHUS.

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Transparency declarations

None to declare

References

1. Noris M, Remuzzi G. Hemolytic uremic syndrome. J Am Soc Nephrol 2005; 16: 1035-1050
2. Loirat C, Noris M, Fremeaux-Bacchi V. Complement and the atypical hemolytic uremic syndrome in children. Pediatr Nephrol 2008; 23: 1957-1972
3. Kavanagh D, Richards A, Atkinson J. Complement regulatory genes and hemolytic uremic syndromes. Annu Rev Med 2008; 59: 293-309
4. Frémeaux-Bacchi V, Miller EC, Liszewski MK, et al. Mutations in complement C3 predispose to development of atypical hemolytic uremic syndrome. Blood 2008; 112: 4948-4952
5. Zipfel PF, Edey M, Heinen S, et al. Deletion of complement factor H-related genes CFHR1 and CFHR3 is associated with atypical hemolytic uremic syndrome. PLoS Genet 2007; 3: e41
6. 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: 555-563
7. Józsi M, Strobel S, Dahse HM, et al. Anti-factor H autoantibodies block C-terminal recognition function of factor H in hemolytic uremic syndrome. Blood 2007; 110: 1516-1518
8. Józsi M, Licht C, Strobel S, et al. Factor H autoantibodies in atypical hemolytic uremic syndrome correlate with CFHR1/CFHR3 deficiency. Blood 2008; 111: 1512-1514
9. Rodríguez de Córdoba S, Esparza-Gordillo J, Goicoechea de Jorge E, Lopez-Trascasa M, Sánchez-Corral P. The human complement factor H: functional roles, genetic variations and disease associations. Mol Immunol 2004; 41: 355-367

10. Gordon DL, Kaufman RM, Blackmore TK, Kwong J, Lublin DM. Identification of complement regulatory domains in human factor H. J Immunol 1995; 155: 348-356
11. Kühn S, Zipfel PF. Mapping of the domains required for decay acceleration activity of the human factor H-like protein 1 and factor H. Eur J Immunol 1996; 26: 2383-2387
12. Hellwage J, Jokiranta TS, Friese MA, et al. Complement C3b/C3d and cell surface polyanions are recognized by overlapping binding sites on the most carboxyl-terminal domain of complement factor H. J Immunol 2002; 169: 6935-6944
13. Pangburn MK. 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: 4702-4706
14. Oppermann M, Manuelian T, Józsi M, et al. The C-terminus of complement regulator Factor H mediates target recognition: evidence for a compact conformation of the native protein. Clin Exp Immunol 2006; 144: 342-352
15. Saunders RE, Abarrategui-Garrido C, Frémeaux-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: 222-234
16. 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: 1181-1190
17. Sánchez-Corral P, Pérez-Caballero D, Huarte O, et al. Structural and functional characterization of factor H mutations associated with atypical hemolytic uremic syndrome. Am J Hum Genet 2002; 71: 1285-1295
18. Józsi M, Heinen S, Hartmann A, et al. Factor H and atypical hemolytic uremic syndrome: mutations in the C-terminus cause structural changes and defective recognition functions. J Am Soc Nephrol 2006; 17: 170-177

19. Sánchez-Corral P, Gonzalez-Rubio C, Rodríguez de Córdoba S, López-Trascasa M. Functional analysis in serum from atypical Hemolytic Uremic Syndrome patients reveals impaired protection of host cells associated with mutations in factor H. Mol Immunol 2004; 41: 81-84
20. Ferreira VP, Herbert AP, Hocking HG, Barlow PN, Pangburn MK. Critical role of the C-terminal domains of factor H in regulating complement activation at cell surfaces. J Immunol 2006; 177: 6308-6316
21. Józsi M, Oppermann M, Lambris JD, Zipfel PF. The C-terminus of complement factor H is essential for host cell protection. Mol Immunol 2007; 44: 2697-2706
22. 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: 506-514
23. Hellwage J, Skerka C, Zipfel PF. Biochemical and functional characterization of the factor-H-related protein 4 (FHR-4). Immunopharmacology 1997; 38: 149-157
24. Prodinger WM, Hellwage J, Spruth M, Dierich MP, Zipfel PF. The C-terminus of factor H: monoclonal antibodies inhibit heparin binding and identify epitopes common to factor H and factor H-related proteins. Biochem J 1998; 331: 41-47
25. Caprioli J, Castelletti F, Bucchioni S, et al. 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; 12: 3385-3395
26. Neumann HP, Salzmann M, Bohnert-Iwan B, et al. Haemolytic uraemic syndrome and mutations of the factor H gene: a registry-based study of German speaking countries. J Med Genet 2003; 40: 676-681
27. Hageman GS, Hancox LS, Taiber AJ, 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

28. Vyse TJ, Morley BJ, Bartók I, et al. The molecular basis of hereditary complement factor I deficiency. J Clin Invest 1996; 97: 925-933
29. Caprioli J, Noris M, Brioschi S, et al. Genetics of HUS: the impact of MCP, CFH, and IF mutations on clinical presentation, response to treatment, and outcome. Blood 2006; 108: 1267-1279
30. Pickering MC, de Jorge EG, Martinez-Barricarte R, et al. Spontaneous hemolytic uremic syndrome triggered by complement factor H lacking surface recognition domains. J Exp Med 2007; 204: 1249-1256
31. Abarrategui-Garrido C, Melgosa M, Peña-Carrión A, et al. Mutations in proteins of the alternative pathway of complement and the pathogenesis of atypical hemolytic uremic syndrome. Am J Kidney Dis 2008; 52: 171-180
32. Meri S. Loss of self-control in the complement system and innate autoreactivity. Ann N Y Acad Sci 2007; 1109: 93-105
33. Kwon T, Dragon-Durey M-A, Macher M-A, et al. Successful pre-transplant management of a patient with anti-factor H autoantibodies-associated haemolytic uraemic syndrome. Nephrol Dial Transplant 2008; 23: 2088-2090
34. Le Quintrec M, Zuber J, Noel L-H, et al. Anti-Factor H autoantibodies in a fifth renal transplant recipient with atypical hemolytic and uremic syndrome. Am J Transplant 2009; 9: 1223-1229

Table 1

Laboratory parameters of patient 1 at selected time points.

time point after disease onset	anti-CFH IgG (AU/ml) [< 100]*	C3 (mg/ml) [0.7-1.2]*	C3d (mU/L) [< 40]*	platelet count ($\times 10^9$ L^{-1}) [150-450]*	LDH (IU/L) [105-333]*	serum creatinine (mg/dl) [0.2-1.0]*
at onset	2282	0.8	60	36	2336	2.16
mo 9.5	ND	0.6	ND	62	689	3.46
mo 10, after PEx	1132	0.7	41	331	240	2.80
mo 12	2116	0.4	41	243	229	2.56
mo 12, after PEx	806	0.7	26	329	209	2.69
mo 14	1487	0.3	ND	146	425	3.70
mo 14, after PEx	675	0.8	29	292	307	2.90

ND: not determined; PEx: plasma exchange therapy. Anti-CFH IgG titer is expressed in arbitrary units (AU/ml). *normal ranges

Legends to figures

Figure 1. Characterization of CFH autoantibodies of patients 2 and 3.

(A) CFH autoantibodies were determined by ELISA. The binding of IgG from plasma samples to CFH and to BSA as a control was compared to exclude false positivity, as in the case of the sister of patient 3. Unrelated control samples (NHS1 and NHS2) were used as negative controls. (B) Determination of IgG isotype of the autoantibodies using CFH-coated plates and isotype-specific mAbs. As positive control, binding of the isotype-specific mAbs on immobilized human IgG is shown. (C) Binding of the autoantibodies to immobilized recombinant CFH fragments representing various SCR domains and covering the full length of CFH and to purified CFH. (D) Competition assay with CFH-specific antibodies raised against the whole molecule (anti-CFH), against SCRs 1-4 (anti-1-4) or SCRs 19-20 (anti-19-20), or with monoclonal antibodies binding to the C-terminus (C18, C14, C02), the middle region (M15) or N-terminus (N11) of CFH. Data of a representative experiment are shown.

Figure 2. Serum autoantibody titers in the patients during disease progression.

(A) Patient 1 was diagnosed with aHUS and later experienced disease recurrences, during which he received plasma exchanges (horizontal bars). CFH autoantibody titers (in arbitrary units/ml) are shown for several available time points as vertical bars. As reference, a plasma sample taken at month 10 was used (1000 AU/ml). Additional information about the treatment is indicated above by arrows. (B) and (C) show the titers during disease follow-up in patients 2 and 3, respectively, in relation to the reference plasma of patient 1. *The CFH autoantibody titer of these samples was determined retrospectively. PEx: plasma exchange, FFP: fresh frozen plasma.

Figure 3. *In vitro* hemolytic activity of autoantibody positive plasma samples using sheep erythrocyte lysis assay.

(A) Sheep erythrocytes suffer dose-dependent lysis in the presence of autoantibody positive patient plasma as compared to normal human plasma. Autoantibody titers are indicated in brackets; control samples had values <100 AU/ml. Hemoglobin release was measured in the supernatant by absorbance at 414 nm after 30 min incubation at 37°C. Data represent mean values of duplicate measurements from a representative experiment and are expressed as % of total lysis achieved by H_2O. (B) Plasma samples lacking CFHR1 and CFHR3 cause no hemolysis if there are no CFH autoantibodies present. CFHR1+/CFHR3+ normal plasma and plasma of the autoantibody positive, CFHR1-/CFHR3- patient 1 are shown as negative and positive controls, respectively. The addition of 20 µg/ml C18 mAb results in enhanced lysis in all samples (filled bars). (C) C-terminally binding mAbs, which have overlapping epitopes and compete with the autoantibodies for CFH binding, cause enhanced hemolysis of sheep erythrocytes (mAbs C02, C14 and C18) incubated with 10% C2-depleted human plasma (used to exclude classical pathway mediated lysis). In contrast, CFH-specific mAbs that bind outside the C-terminal domains (SCRs 19-20) cause no hemolysis (mAbs N11, N22, M12, M13 and M16).

Figure 4. The *in vitro* hemolytic activity of patients' plasma correlate with the autoantibody titer.

(A) Patient 1 displayed persisting high autoantibody titers and also high hemolytic activity. These complement active samples were only available when the patient was already on peritoneal dialysis. Both C3 levels (0.8-1.3 mg/ml) and CFH levels (102-133%) were in the normal range in these samples. (B) Hemolytic activity of plasma samples of patient 2 taken at various time points. After the acute phase, both autoantibody titers and hemolytic activities are low. Sample 1 had lower C3 level (0.42 mg/ml), whereas C3 levels were normal in the other samples (1.19-1.51 mg/ml). CFH levels were in the normal range (105-123%), except for sample 2 (61%). (C) Comparison of hemolytic activity of two plasma samples of a patient,

described before [8], with high and low CFH autoantibody titers. C3 levels were 0.85 mg/ml and 1.3 mg/ml, and CFH levels 95% and 155% in sample 1 and 2, respectively. Plasma samples were used at 20% dilution in (A) and (B), and at 30% in (C).

Figure 5. IgG-depletion provides direct evidence for the CFH function-inhibitory effect of the autoantibodies.

(A) Depletion of IgG by incubating plasma samples with Protein G for various time periods results in artificial titer reduction and reduced hemolytic activity. A representative experiment with samples of patient 2 is shown. (B) All three analyzed patients' samples showed hemolytic activity reduced to the control level after IgG-depletion. Addition of CFH blocking mAb C18 to the depleted plasma reverts the beneficial effect of IgG-depletion (Plasma ΔIgG + C18), indicating that IgG-depletion removes CFH inhibitory antibodies but the plasma samples still have a functional complement system.

Figure 6. Analysis of CFH:autoantibody complexes in patients' plasma

(A) The indicated dilutions of plasma samples, patient 2/I (autoantibody titer 1325 AU/ml), patient 2/II (361 AU/ml), patient 3/I (883 AU/ml) and 3/II (867 AU/ml), were incubated in wells coated with the anti-CFH mAb M15 as catch antibody, and the bound CFH:autoantibody complexes were detected with anti-human IgG. As a control, normal human plasma (NHP) is also shown. (B) Immune complexes (left panel) are absent from IgG-depleted plasma (empty bars, ΔIgG), measured as in A. As controls, binding of anti-IgG to plasma samples incubated in wells without anti-CFH mAb coat and the presence of CFH in the plasma samples (right panel) are shown. (C) The mAb C18 captures free CFH only, due to overlapping binding sites with the autoantibodies, thus falsely indicates low CFH levels if the autoantibody titer is high. In contrast, mAb M15 detects free CFH as well as CFH:IgG

complexes, thus comparison of CFH levels determined using the two mAbs allows an estimate of free and complexed CFH.

Figure 1
Strobel et al., 2009

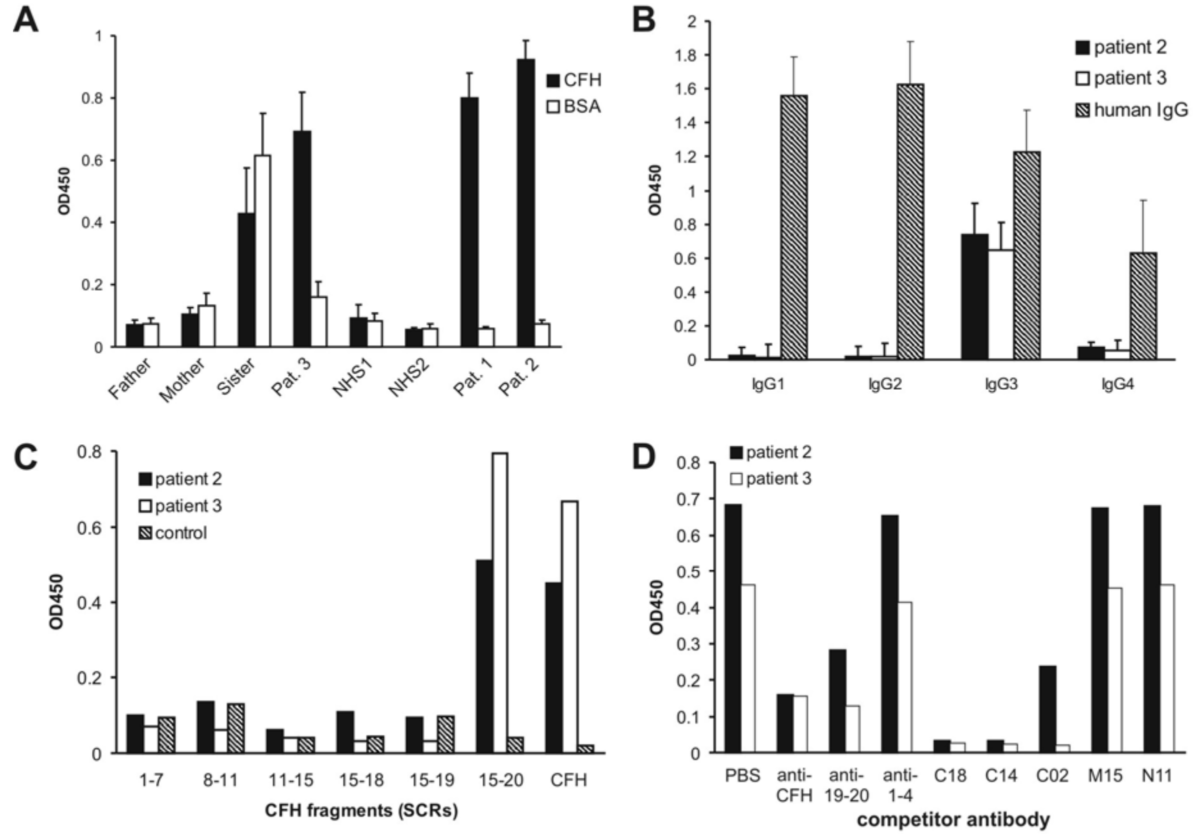

Figure 2
Strobel et al., 2009

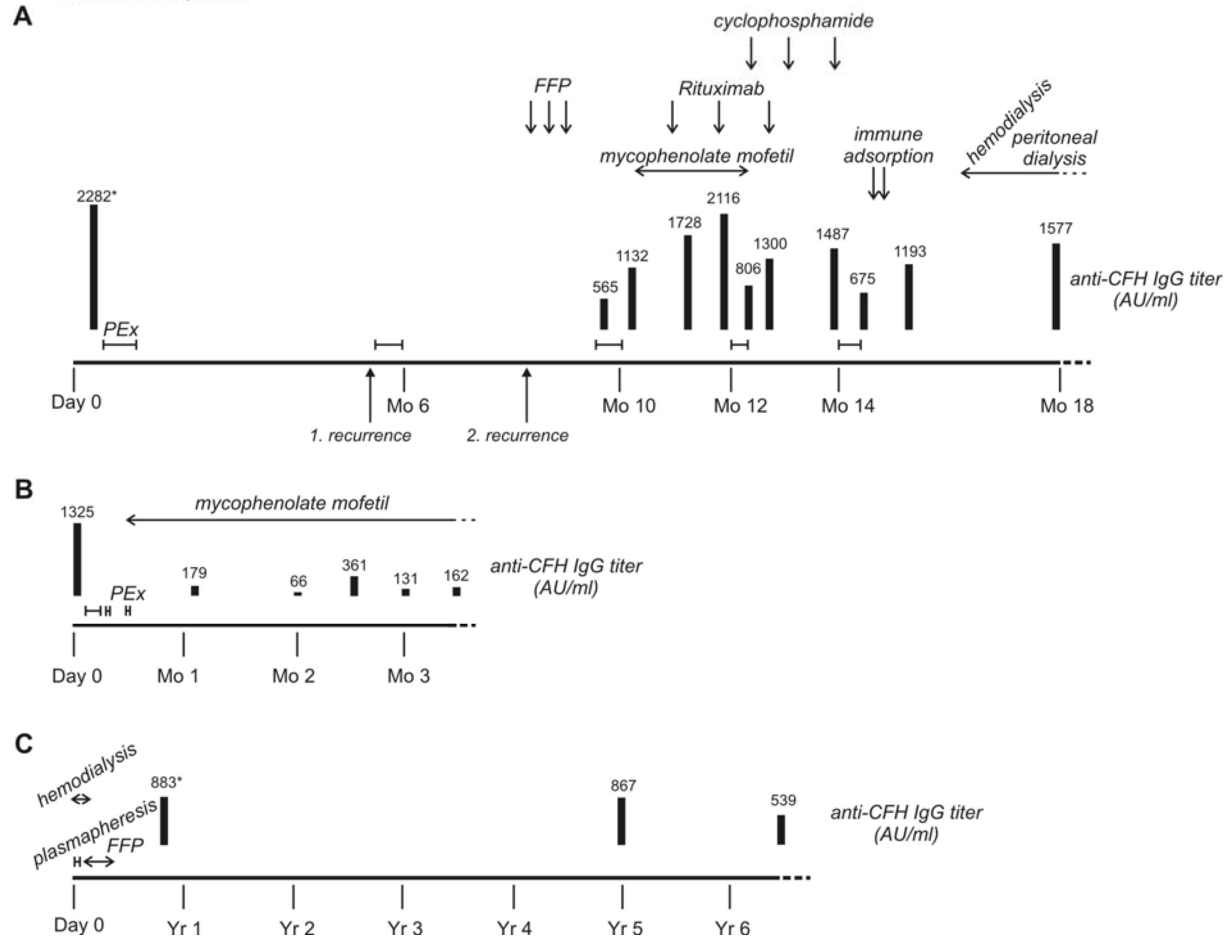

Figure 3
Strobel et al., 2009

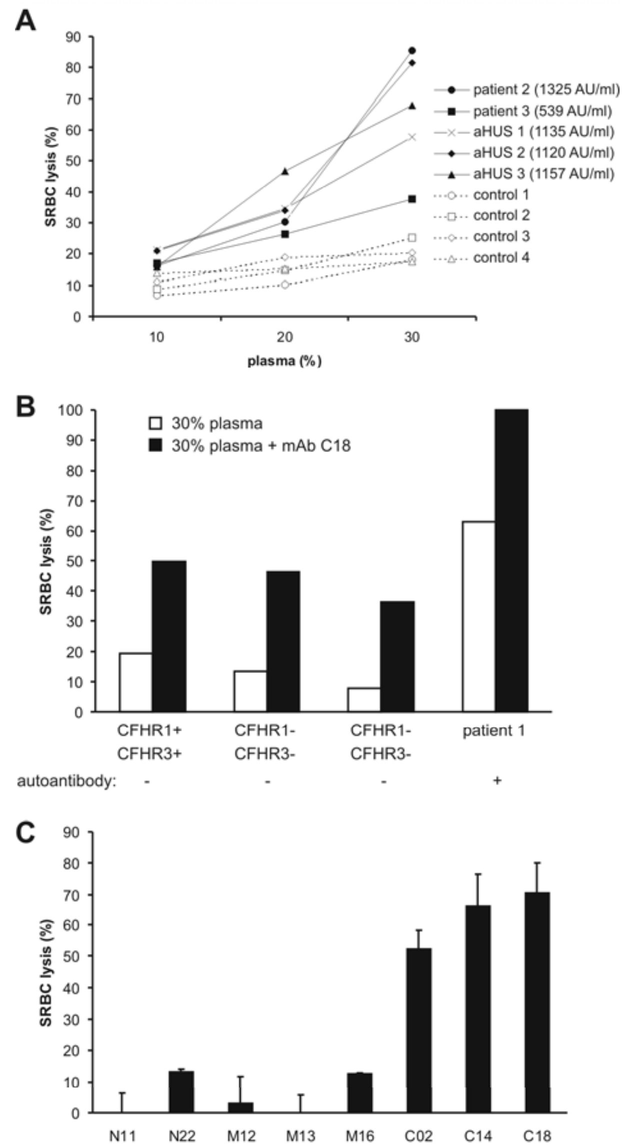

Figure 4
Strobel et al., 2009

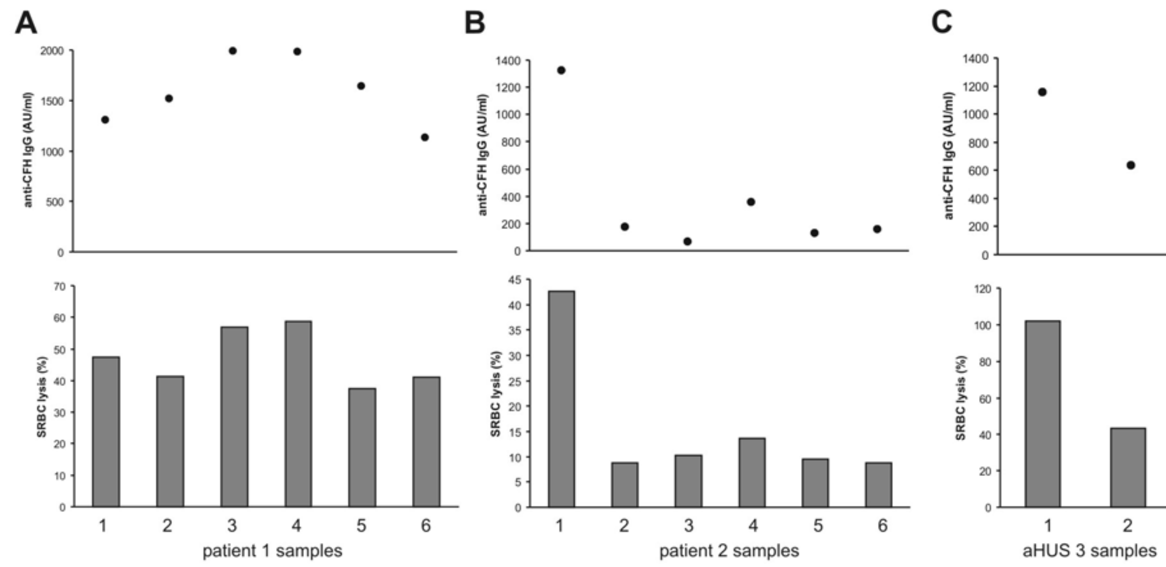

Figure 5
Strobel et al., 2009

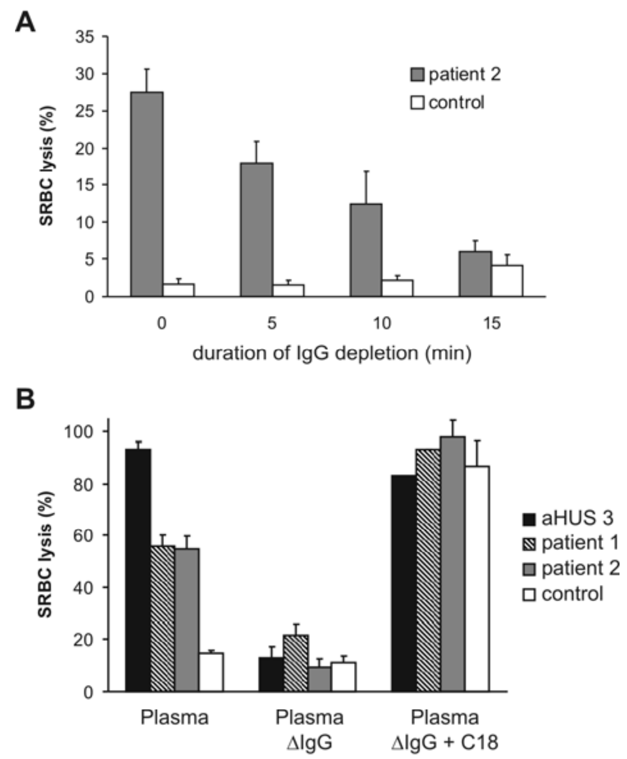

Figure 6
Strobel et al., 2009