Heterogeneity but individual constancy of epitopes, isotypes and avidity of factor H autoantibodies in atypical hemolytic uremic syndrome

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Abstract

Factor H (FH) autoantibodies are present in 6-10% of atypical hemolytic uremic syndrome (aHUS) patients, most of whom have homozygous deficiency of the FH-related protein FHR-1. Although the pathogenic role of the autoantibodies is established, little is known about their molecular characteristics and changes over time. Here, we describe the specificity and other immunological features of anti-FH autoantibodies in the Spanish and Hungarian aHUS cohorts. A total of 19 patients were included and serial samples of 14 of them were available. FH autoantibodies from FHR-1 deficient patients (n=13) mainly recognized FH, its SCR19-20 fragment and FHR-1, but autoantibody specificity in patients who are homo- or heterozygous for the *CFHR1* gene (n=6) was heterogeneous. No significant changes apart from total antibody titer were observed during follow-up in each patient. Fine epitope mapping with recombinant FH SCR19-20 containing single amino acid mutations showed significantly reduced binding in 7 out of 14 patients. In most cases, autoantibody binding to residues 1183-1189 and 1210-1215 was impaired, revealing a major common autoantibody epitope. Avidities showed variations between patients, but in most cases the avidity index did not change upon time. Most autoantibodies were IgG3, and all but three presented only with kappa or with lambda light chains. Although the pathogenic role of anti-FH autoantibodies in aHUS is well established, this study shows autoantibody heterogeneity among patients, but no significant variation in their characteristics over time in each patient. The presence of a single light chain in 16 out of 19 patients and the limited number of recognized epitopes suggest a restricted autoantibody response in most patients.

Keywords: autoimmunity; autoantibody; atypical hemolytic uremic syndrome; avidity; epitope mapping; factor H

Abbreviations: aHUS, atypical hemolytic uremic syndrome; AP, alternative pathway; C3G, C3 glomerulopathy; ESRD, end-stage renal disease; FH, factor H; FHR-1, factor H-related protein 1; SCR, short consensus repeat

1. Introduction

Hemolytic Uremic Syndrome (HUS) is a rare disease defined by microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure. Most cases result from infections with Shiga-like toxin-producing bacteria, which mainly affect children and have a good prognosis. The atypical form of HUS (aHUS) representing 5-10% of cases affects children and adults and has a bad prognosis, with high rate of developing end stage renal disease (ESRD) (Loirat and Fremeaux-Bacchi 2011; Nester and Thomas 2012).

Approximately 50% of aHUS patients have mutations or polymorphisms, usually in heterozygous form, in the genes encoding proteins of the complement alternative pathway (AP), leading to a defective balance between activation and regulation, and thus amplifying the initial endothelial damage necessary for aHUS development (Mele, Remuzzi, Noris 2014; Sanchez-Corral and Melgosa 2010). The penetrance of these mutations is low, and a trigger is required for the development of the disease (Noris et al. 2010). Besides complement proteins, mutations in thrombomodulin and in diacylglycerol kinase ε have been described in a small cohort of aHUS patients (Delvaeye et al. 2009; Lemaire et al. 2013; Rodriguez de Cordoba et al. 2014; Sanchez Chinchilla et al. 2014). Mutations in the factor H gene (*CFH*) are the most prevalent genetic alterations representing ~25% of cases (Rodriguez de Cordoba et al. 2014). In addition, autoantibodies directed to factor H (FH) are found in ~10% of aHUS cases, but larger proportions were also reported, depending on geographical region and ethnicity (Dragon-Durey et al. 2005; Hofer, Giner, Jozsi 2014; Sinha et al. 2014). This autoimmune form affects mainly children, and has a highly relapsing course with progression to ESRD (Dragon-Durey et al. 2010). The therapy of anti-FH associated aHUS is not clear, but the goal is the normalization of AP regulation, usually removing antibodies with plasmapheresis and immunosuppression (Dragon-Durey et al. 2010; Hofer, Giner, Jozsi 2014; Loirat and Fremeaux-Bacchi 2011). Novel therapies targeting complement C5 are emerging also in this particular form of aHUS (Diamante Chiodini et al. 2014; Noone et al. 2014).

FH is a 150 kDa plasma glycoprotein that is composed of 20 structural domains named short consensus repeats (SCRs). The N-terminal region contains the complement regulatory domains (SCRs 1-4), which are responsible for a) the cofactor activity of FH for the Factor I-mediated cleavage and inactivation of C3b, b) competition with factor B for C3b binding and thus

prevention of the assembly of the AP C3 convertase C3bBb, and c) acceleration of the decay of C3bBb. The C-terminal SCRs 18-20 domains are responsible for target discrimination by FH, because they mediate binding to glycosaminoglycans expressed on host cells and thus protection from damage caused by autologous complement (Blaum et al. 2015; Ferreira, Pangburn, Cortes 2010; Pickering et al. 2007).

There are also five plasma proteins with high sequence and structural homology to FH, termed the FH-related proteins (FHRs). The genes encoding FH and the FHRs are located in tandem within the Regulator of Complement Activation gene cluster in chromosome 1q32, and they share several duplicated intronic-exonic regions, which explains the homology observed in this protein family (Abarrategui-Garrido et al. 2009; Skerka et al. 2013). The gene homology makes this genomic region prone to rearrangements that may result in hybrid genes and/or deletion of *CFHR* genes, which are associated with various diseases (reviewed in Jozsi et al. 2015). In this context, about 90% of aHUS patients with FH autoantibodies are homozygous for the *CFHR3-CFHR1* deletion (Abarrategui-Garrido et al. 2009; Dragon-Durey et al. 2009; Hofer et al. 2013). Although this association is not completely understood, autoantibody generation/specificity is most likely related with the absence of FHR-1 in these patients. Thus, most aHUS-associated FH autoantibodies have been shown to recognize and functionally block SCRs 19-20 of FH (Jozsi et al. 2007), and cross-reactivity with FHR-1 has also been found (Moore et al. 2010; Strobel et al. 2011). A recent study shows that the autoantibody epitope is located on a loop of FH SCR20 that could take a different conformation upon ligand binding, and then adopting a similar structure to the homologous site in FHR-1; this may explain the association of FHR-1 deficiency with FH autoantibodies due to loss of tolerance (Bhattacharjee et al. 2015).

Whereas the pathogenic role of FH autoantibodies is widely accepted, little is known about autoantibody evolution in single patients, and differences among patients. In this study, we have characterized FH autoantibodies in serial samples from patients from the Spanish and Hungarian aHUS cohorts. Because of individual differences among patients (genetics, autoantibody characteristics, clinical presentation, etc.), autoantibody titers cannot be directly compared between patients, but changes in autoantibodies in a single patient during follow-up should be informative. The goal of this study was to determine whether the molecular

characteristics of FH autoantibodies in single aHUS patients change upon time, and to what extent these biological features differ among patients.

2. Materials and Methods

The studies were approved by the ethical committees of University Hospital “La Paz”, and the respective Hungarian authorities (permission numbers ETT TUKEB 263/PI/11 and 838/PI/12), and were performed in accordance with the Declaration of Helsinki. All the patients or their relatives and the healthy blood donors gave written informed consent.

2.1. Patients and Controls

Fourteen Spanish and 5 Hungarian aHUS patients diagnosed with FH autoantibodies were studied. Blood samples were obtained at different time points and serum and/or EDTA-plasma were kept frozen until use. Patient 17 was a 2 months old patient, with 378 AU/ml FH autoantibodies and positive in a sheep erythrocyte lysis assay (39% lysis). Her mother was negative in both tests.

Serial samples from 15 patients were available for analyzing autoantibody levels. Mean time of follow-up was 3.7 years, ranging from 2 months to 8 years. All patients were screened for the *CFHR3-CFHR1* deletion by MLPA method, as previously described (Abarrategui-Garrido et al. 2009).

Blood samples from 54 healthy control individuals were also available.

2.2. Detection of FH autoantibodies

An ELISA assay for anti-FH quantification was performed as previously described (Abarrategui-Garrido et al. 2009). Microtiter plates were coated overnight with 200 ng of purified FH or with HSA as negative control. After washing and blocking with PBS-Tween-20 0.1% (or with 5% BSA-PBS-Tween 20), serial dilutions of serum/plasma samples were added and incubated for 1 hour at 37^{o}C. HRP-conjugated goat anti-human IgG was used as detecting antibody and the enzymatic reaction was developed with ABTS or TMB substrate. A positive sample with an arbitrary titer of 2000 AU/ml, kindly provided by Dr. Marie-Agnès Dragon-Durey (Hôpital Georges Pompidou, Paris, France), was used as reference, and samples above 150 AU/ml

were considered positive (mean + 2 SDs obtained from 54 control samples). The established cut-off value in the Hungarian laboratory was 110 AU/ml.

2.3. Characterization of IgG subclass and light chain

IgG subclasses and light chains were identified by ELISA using monoclonal antibodies specific for human IgG1, IgG2, IgG3, IgG4, and lambda and kappa light chains (Sigma-Aldrich, Budapest, Hungary, and Southern-Biotech, AL, USA) together with HRP-conjugated goat anti-mouse immunoglobulins (Dako, Hamburg, Germany).

2.4. Binding of autoantibodies to recombinant FH fragments

Costar High Binding ELISA plates were coated overnight at 4°C with 50 ng/well of either purified FH or recombinant FH fragments (SCRs 1-4, SCRs 6-8, SCRs 19-20) produced in yeast (Blanc et al. 2012; Jokiranta et al. 2006). After blocking with PBS-BSA 0.1%, wells were incubated with patients' sera, in PBS-BSA 0.1% for 1 h at 37°C. After washing with PBS-Tween-20 0.1%, IgG binding was measured using HRP-conjugated anti-human IgG diluted 1/5000 in PBS-Tween (Jackson Immunoresearch, Suffolk, UK). Binding to recombinant FHR-1 was also tested in samples from all patients.

For the five Hungarian patients and nine of the Spanish patients, autoantibody binding to recombinant proteins produced in insect cells representing FH SCRs 1-4, SCRs 1-7, SCRs 8-14, SCRs 15-20, and FHR-1 was also measured (Bhattacharjee et al. 2015).

2.5. Binding of autoantibodies to recombinant FH19-20 mutants

Autoantibody binding to FH SCRs 19-20 was studied in detail by ELISA using recombinant FH SCR19-20 fragments with 13 different single amino acid substitutions (Bhattacharjee et al. 2015). For that purpose, plates were coated with recombinant wild type or mutant FH SCR19-20 fragments. The applied dilution of each patient's sera was chosen according to autoantibody titers.

2.6. Inhibition of autoantibody binding with anti-FH monoclonal antibodies

To further characterize the epitope(s) recognized in FH, autoantibody binding to full-length FH was measured in the presence of domain-mapped anti-FH monoclonal antibodies. FH was bound to wells in the ELISA plates and then incubated for 15 min with 20 µg/ml anti-FH monoclonal antibodies with different domain specificity (A254 [clone 90X]: SCR1, A255: middle region, A229: C-terminal region, IXF9: SCR18 (Prodinger et al., 1998), VIG8: SCR19-20 (Prodinger et al., 1998), and C18: SCR20 (Oppermann et al., 2006). Patient serum samples were added and incubated for 1 h. Autoantibody binding was detected using HRP-conjugated anti-human IgG antibody.

2.7. Avidity determination

Avidity of the anti-FH autoantibodies was determined by ELISA, based on the method described by Suwannalai et al. (Suwannalai et al. 2011). An appropriate serum dilution for each sample was determined previously to ensure that binding to FH was not in saturation and that the OD value fell in the range of the standard curve. After 1h of incubation at 20°C, plates were washed, and various concentrations of NaSCN (0, 0.25, 0.5, 1, 2, 5 M) were added and incubated for 15 min at 20ºC to dissociate the FH:anti-FH immune complexes. After washing, remaining bound autoantibodies were detected with HRP-conjugated anti-human IgG antibody. The avidity index (AI) was calculated as the ratio of autoantibody bound after elution with 0.5 M NaSCN, and the autoantibody bound in the absence of NaSCN.

2.8. Circulating FH:anti-FH immune complexes

Anti-FH monoclonal antibodies OX24 (binding to SCR5) and A229 (binding to the C-terminus), and an in-house rabbit polyclonal anti-FH IgG were coated on ELISA plates. After blocking with PBS-BSA 1%, patients' serum samples were added and incubated for 1h at 37°C. The presence of FH:anti-FH complexes was then detected with HRP-conjugated anti-human IgG antibody. In some assays, the immune complexes were captured on ELISA plates by the mAb A255, which recognizes the middle region of FH, and detected as described above.

Protein G-agarose beads were used to purify the IgG fraction from serum. The eluted proteins were separated by 10% SDS-PAGE, and further analyzed by Western blotting using a goat FH antiserum (Merck, Budapest, Hungary) as described previously (Strobel et al. 2011).

3. RESULTS

3.1. IgG subclasses of the FH autoantibodies

Identification of IgG subclass and light chain was carried out in all patients with FH autoantibodies. All autoantibodies were of the IgG3 isotype, except in patients P18 (IgG1) and P13 (IgG1 and IgG3). Light chains were either kappa or lambda except in 3 patients (P1, P2 and P13) who had FH autoantibodies with both kappa and lambda chains (Table 1). No change in the IgG subclass and light chain was observed in single patients when serial samples with detectable autoantibodies were available (n = 14; Table 1, Supplementary Figure 1, and data not shown).

3.2. Domain mapping of FH autoantibodies

Table 2 summarizes the results of autoantibody binding to FH and FH fragments, as well as to FHR-1. Autoantibodies from all the patients recognized the C-terminal domains of FH, either generated in yeast (SCRs 19-20) or in insect cells (SCRs 15-20), and, as it could be expected, all of them cross-reacted with FHR-1. The 5 patients with two copies of the *CFHR1* gene also recognized the N-terminal domains of FH (SCRs 1-4), but only 7 out of the 13 patients with zero copy of *CFHR1* recognized this FH region. Of note, out of these 12 autoantibodies that bound to the FH SCRs 1-4 fragment produced in yeast 7 could be tested for binding to FH SCRs 1-4 produced in insect cells, and only two out of these 7 autoantibodies showed weak reactivity to the latter recombinant protein. In addition, some normal serum samples also showed reactivity with recombinant FH SCRs 1-4 produced in yeast (Supplementary Figure 2).

As serial samples from some patients were collected for several years, we could study whether autoantibody specificity changed upon time. Despite all the variability found in autoantibody binding among the patients, when serial samples were studied (n = 14) the specificity remained unchanged for each patient along time, except in patient P6 whose autoantibodies from the initial samples recognized both SCRs 1-4 and 19-20 fragments, and from the later ones only bound to the fragment containing SCRs 19-20 (Table 2, Supplementary Figure 3, and data not shown.

3.3. Inhibition of autoantibody binding to FH using monoclonal antibodies

To further analyze the FH binding sites, autoantibody binding to full-length FH was measured in the presence of domain-mapped anti-FH monoclonal antibodies as an alternative approach. These assays showed that mAb C18, which binds to the previously identified autoantibody loop on SCR20 (Bhattacharjee et al. 2015), inhibited the interaction of all tested autoantibodies (n = 14) with FH, except in the case of P19. This finding, together with the variable inhibitory effect of other mAbs recognizing the C-terminal region (mAb A229, IXF9 and VIG8) confirmed the C-terminal specificity of all autoantibodies, and the microheterogeneity in the exact epitope and/or affinity of the individual autoantibodies (Fig. 1, Supplementary Figure 4). The mAb binding to SCR1 (A254; clone 90X) did not inhibit binding of the autoantibodies to FH.

3.4. Epitope fine mapping of autoantibodies

In 14 patients, epitope fine mapping with 13 recombinant FH SCR19-20 mutants containing single aminoacid exchanges was performed. Less than 80% binding was observed to some of the FH SCR19-20 mutants in 6 out of the 14 patients (P3, P4, P6, P8, P16 and P18). Less than 60% binding was found in the case of three patients (P4, P6, P8), all of them with FHR-1 and FHR-3 deficiency. The two most frequent SCR20 subregions in which autoantibody binding was impaired comprise residues 1183 to 1189 and residues 1210 to 1215 (Table 3). These residues also accumulate mutations already described in aHUS patients (Rodriguez de Cordoba et al. 2014; Rodriguez et al. 2014). Interestingly, in patients with homozygous FHR-1 deficiency the autoantibodies mainly bound to the FH1183-1189 subregion, whereas in patients without FHR-1 deficiency the FH1203-1215 subregion was mainly recognized.

3.5. Avidity of FH autoantibodies

Because no detailed studies on the avidity of aHUS-associated FH autoantibodies have been reported to date, we set out to analyze this feature in our patients. First, the avidity profile for each autoantibody (*i.e.,* the percentage of autoantibody bound after elution with increasing concentrations of NaSCN) was determined based on the method described by Suwannalai et al. (Suwannalai et al. 2011); representative examples of the avidity profiles for three patients in samples taken at different time points are shown in Fig. 2 A-C. To further analyze whether

avidity changed over time in single patients, the avidity indexes (i.e., the ratio between autoantibody bound after elution with 0.5 M NaSCN and autoantibody bound in the absence of NaSCN) in serial samples of several patients were calculated (Figure 2D). One patient presented over 20% enhanced avidity index over time, and two patients showed over 20% decreased avidity index, but most patients had below 10% difference in the mean avidity index between the initial and last sample drawn, even at the longest follow-up times. In addition, these experiments allowed us to determine that there was no correlation between the avidity index and the autoantibody titer (Figure 2E, Spearman's r = 0.338, p = 0.0674).

3.6. FH-autoantibody complexes

Circulating FH:anti-FH complexes were detected in serum samples from all patients using different monoclonal antibodies as capture antibodies (Fig. 3A). In serial samples from individual patients, the amount of immune complexes largely followed the changes in autoantibody titers (Fig. 3B-C) also supporting that there was no significant change in autoantibody avidity upon time. The presence of FH in the IgG fraction containing the autoantibodies was also confirmed by Western blot in several samples (not shown).

4. DISCUSSION

Complement dysregulation is the major pathogenic mechanism in aHUS. Approximately 44% of aHUS patients carry single or combined mutations in the complement genes *CFH, CFI, MCP, CFB, C3* or in thrombomodulin (Bresin et al. 2013), and autoimmune forms with FH autoantibodies (Dragon-Durey et al. 2005; Hofer, Giner, Jozsi 2014) or FI autoantibodies (Jozsi et al. 2014; Kavanagh et al. 2012) are found in ~10% of patients, although the relevance of the FI autoantibodies is unclear. The most frequent alterations observed in aHUS are FH defects including mutations, risk variants or autoantibodies, and they all have a bad prognosis.

FH autoantibodies from aHUS patients cause a functional deficiency of FH, interfering with FH binding to the AP convertase and with FH-dependent cell protection (Blanc et al. 2012; Dragon-Durey et al. 2010; Jozsi et al. 2007). Deficiency of FHR-1 and FHR-3 is strongly associated with the presence of FH autoantibodies in aHUS patients. Despite this being the most frequent genetic alteration affecting FHR proteins, other genetic defects involving *CFHR1*

have been described, suggesting that the complete deficiency of FHR-1 is the specific factor associated with FH autoantibodies in aHUS (Abarrategui-Garrido et al. 2009; Dragon-Durey et al. 2009; Jozsi et al. 2008; Moore et al. 2010). Most of these autoantibodies recognize and functionally block the C-terminus of FH, and most of them cross-react with FHR-1, because of the high homology between SCRs 4-5 of FHR-1 and SCRs 19-20 of FH (Strobel et al. 2011).

Because little is known about the molecular heterogeneity and evolution of autoantibodies during disease course in individual patients, we have analyzed several characteristics of FH autoantibodies, including IgG isotype, recognition domain, epitope and avidity, in serial samples from aHUS patients from the Spanish and Hungarian cohorts.

In the patients analyzed in this study, IgG3 was the most prevalent subclass of the FH autoantibodies (17/19 patients). One patient also had IgG1 autoantibodies in addition to IgG3, while another patient had only IgG1. These results are in accordance with findings of other groups (Blanc et al. 2012; Dragon-Durey et al. 2005; Jozsi et al. 2008; Strobel et al. 2011), and have consequences for the pathology of the disease, because the half-life, complement activating capacity and binding to Fc receptors differ among IgG subclasses. For example, IgG3 has the shortest half-life (~7 days) among the human IgG subclasses and the longest hinge region, allowing more flexibility of the two arms of the molecule. In addition, IgG1 and particularly IgG3 are considered the best activators of the classical complement pathway among the IgGs. These features could contribute to individual differences in the patients regarding the disease course. The main effect of the autoantibodies is, however, the likely blockage of FH binding to the self cell surfaces where C3b has been deposited. Therefore, it is possible that the clear dominance of IgG3 in FH autoantibodies could be caused by a common mechanism of immunization, for example via the cytokine profile in the germinal centre during infection with FH-binding microbes.

Most FH autoantibodies had either kappa or lambda light chain, with slightly higher frequency (9:7) of kappa. The simultaneous presence of both light chains was only found in 3 patients, suggesting oligoclonality of the autoantibody response. There were no changes in isotype or light chain of the FH autoantibodies over time. Blanc et al. described that a restriction of light chains in FH autoantibody positive patients was found in C3 glomerulopathy (C3G), in association with monoclonal gammopathy, but not in autoimmune aHUS (Blanc et al. 2015).

Our ELISA results, however, clearly showed predominantly kappa or lambda light chains in 16 out of the 19 aHUS patients with FH autoantibody (see Supplementary Figure 1 for examples). Similarly, in a previous study (Bhattacharjee et al. 2015) only 2 out of 17 studied patients had FH autoantibodies with both kappa and lambda light chains.

Recently, Blanc et al. studied FH:autoantibody complexes in the presence of various NaCl concentrations (Blanc et al. 2015), but a detailed analysis regarding the avidity profiles and avidity indexes of the FH autoantibodies in serial samples of aHUS patients is currently lacking. We therefore analyzed this feature in single patients, and also whether epitope spreading and affinity maturation result in a change of autoantibody avidity (Fig. 2). In contrast to previous data using NaCl (Blanc et al. 2015), in our experiments using thyocianate for avidity determination, which is the standard reagent used in such assays (Pullen et al. 1986), the avidity profiles showed considerable variability among aHUS patients. Complete dissociation of FH:anti-FH complexes could be achieved in several cases when using the chaotropic salt NaSCN in 1 M or higher concentration (Fig. 2A-C). In most of our patients the FH autoantibody titers and the amount of FH:anti-FH immune complexes decreased between the acute phase of the disease and remission periods, but no significant changes in autoantibody avidity were observed upon time (Fig. 2D). About 20% decrease in the avidity index was observed in two patients and increase in one patient, independently of the *CFHR1* genotype. The reason for the stability of the autoantibody avidity is not known, but it could be related to the short time when microbial FH-binding proteins are present.

When analyzing the recognition domain of FH autoantibodies, we found that most of the autoantibodies recognized the most C-terminal domains of FH and FHR-1 (Table2). For several autoantibodies binding to both the functionally important C- and N-terminal domains of FH was observed; notably, N-terminal binding was mainly detected when using recombinant proteins produced in yeast, and FH SCRs 1-4 produced in yeast cells also showed increased reactivity with several control samples (Supplementary Figure 2). These data, and the lack of inhibition of autoantibody binding by the N-terminally binding mAb A254 (clone 90X), which was previously shown to inhibit binding of N-terminally binding C3G-associated FH autoantibodies (Blanc et al. 2015), suggest caution about the specificity of the observed FH N-terminal binding in our patient samples. Despite the heterogeneity of autoantibody binding sites, no significant changes in the

recognition domains were observed in each patient during follow up, indicating no epitope spreading during the disease course.

We also performed epitope mapping with recombinant FH SCRs 19-20 fragments containing single amino acid mutations (Table 3). A significant reduction in autoantibody binding to some of these mutants was particularly evident in patients with FHR-1 deficiency. Impaired autoantibody binding was observed with higher frequency to the FH mutants comprising residues 1183 to 1189 and residues 1210 to 1215. By binding to this region the autoantibodies are likely to block function of FH similar to the aHUS-associated mutations that also cluster in the same region (Bhattacharjee et al. 2015). This conserved binding site of FH autoantibodies of the FHR-1 deficient patients fits to the previously proposed model that in FHR-1 deficient individuals a conformational change in the SCR20 domain of FH drives autoimmunization (Bhattacharjee et al. 2015). Based on that model it has been proposed that the absence of FHR-1 has an impact on self tolerance to FH, thus explaining the frequent association between FH autoantibodies and the *CFHR3-CFHR1* deletion. An explanation for the generation of FH autoantibodies in patients with circulating FHR-1 is, however, still lacking.

FH autoantibodies have also been identified in patients with C3G, (Blanc et al. 2015; Goodship et al. 2012; Jozsi et al. 2014; Nozal et al. 2012). In this disease, autoantibodies show a binding preference for the N-terminal complement regulatory domains of FH (SCRs1-4) and they do not associate with *CFHR1* deletion. These FH autoantibodies in C3G are likely pathogenic because they block the regulatory functions of FH SCRs1-4 and thus lead to uncontrolled alternative pathway activation in plasma. Therefore, there is a clear difference between FHR-1 deficiency-associated autoantibodies against the C terminus of FH in aHUS, where cell protection from complement attack by FH is perturbed, and the N-terminally binding FH autoantibodies in C3G. N-terminally binding autoantibodies in aHUS could cause a more subtle dysfunction of FH; these autoantibodies may limit the correct orientation and activity of FH on surface-bound C3b, depending on the exact binding site within FH SCRs1-4 domains.

In summary, these data confirm recent results (Bhattacharjee et al. 2015) regarding the main epitope recognized by autoantibodies from two other aHUS cohorts, and describe molecular characteristics of individual antibodies, including the evolution of antibody avidity in single patients for the first time. Although the pathogenic role of FH autoantibodies is well established

in aHUS, our study reveals heterogeneity among patients, but no epitope spreading or significant variation in their biological characteristics over time in each patient. These autoantibodies seem to reflect a restricted immune response, as suggested by the presence of a single light chain in most patients and the limited number of epitopes they recognize.

Acknowledgements

This work was supported in part by grants from the Spanish Ministerio de Economía y Competitividad (MINECO: PI12-00597, SAF2012-38636), Sociedad Española de Nefrología (SENEFRO), and CIBERER (ACCI-2014) to P.S.C. or M.L.T., the Lendület Program of the Hungarian Academy of Sciences (grant LP2012-43 to M.J.), and the Hungarian Scientific Research Fund (OTKA) grants K 109055 (to M.J.) and K 100687 (to Z.P.). M.E.B-H was supported by the Spanish Comunidad de Madrid (S2010/BMD-2316), and was granted with an EFIS-IL fellowship.

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FIGURE LEGENDS

Figure 1: Inhibition of autoantibody binding to FH with monoclonal antibodies.

The percentage of autoantibody binding to full-length FH in the presence of domain-mapped monoclonal anti-FH antibodies is shown. 100%: autoantibody binding to FH without addition of mAb. A254 is directed to FH SCR1, A255 binds the FH middle region, A229 and VIG8 in the C-terminal region, C18 in SCR20 and IXF9 in SCR18. Data are means + SD from three different experiments, except for P7 (one experiment).

Figure 2: Avidity of FH autoantibodies.

A) to C) show the avidity profile of FH autoantibodies from three patients over time. Avidity is represented as the percentage of autoantibody which remained bound after elution with increasing concentration of NaSCN. D) Variation of avidity index (AI) between the initial and the last sample obtained from patients. E) Lack of correlation between AI and autoantibody titer. The AI was calculated as the ratio of autoantibodies bound to immobilized FH after elution with 0.5 M NaSCN, and autoantibodies bound in the absence of NaSCN. Spearman's r = 0.338, 95% confidence interval = (-0.036,0.630), p = 0.0674.

Figure 3: Presence of circulating FH:anti-FH immune complexes.

A) Detection of FH:anti-FH immune complexes in patient P4 at disease onset and 7 years later by using different monoclonal antibodies (A229, Ox24, A255) or a polyclonal anti-FH antibody (rb34) as capture antibodies. B) -F) Evolution of free and FH-bound autoantibodies over time in patients P9, P10, P11, P12 and P13.

Table 1. Summary of aHUS patients with FH autoantibodies and their characterization

Patient	Age at onset	*CFHR1* genotype	Follow-up time of the included samples in months (nº analyzed samples)	Autoantibodies titer range (AU/ml)	IgG subclass	Light chain
P1	5 y	-/-	19 (1)	380	IgG3	κ+λ
P2	4 y	-/-	63 (7)	6840-570	IgG3	κ+λ
P3	8 y	-/-	79 (6)	2625-525	IgG3	κ
P4	7 y	-/-	2 (3)	1000-800	IgG3	λ
P5	6 y	-/-	90 (1)	1700	IgG3	κ
P6	5 y	-/-	37 (12)	1811-252	IgG3	λ
P7	8 y	-/-	24 (5)	8160-175	IgG3	κ
P8	10 y	-/-	One sample	3250	IgG3	κ
P9	11y	-/-	3 (3)	851-220	IgG3	λ
P10	8 y	-/-	21 (3)	4817-653	IgG3	λ
P11	8 y	-/-	63 (2)	1539-100	IgG3	λ
P12	8 y	-/-	40 (2)	9433-421	IgG3	λ
P13	6 y	-/-	22 (3)	3718-628	IgG1+IgG3	κ+λ
P14	6 y	-/+	15 (3)	8600-2682	IgG3	λ
P15	3 y	+/+	75 (4)	3600-Negative	IgG3	κ
P16	11 y	+/+	3 (2)	Positive (weak)	IgG3	κ
P17	2 m	+/+	27 (1)	378	IgG3	κ
P18	9 y	+/+	3 (2)	353-Negative	IgG1	κ
P19	42 y	+/+	13 (2)	224-204	IgG3	κ

Patient	CFHR1 genotype	Relative Binding to FH				Relative Binding to FH				FHR-1	Analyzed samples (time after onset)
		complete FH	SCRs 1-4[a]	SCRs 6-8[a]	SCRs 19-20[a]	SCRs 1-4[b]	SCRs 1-7[b]	SCRs 8-14[b]	SCRs 15-20[b]		
P1	-/-	+	++	-	+	n.t.	n.t.	n.t.	n.t.	+	1 (19 m)
P2	-/-	+++	-	-	++	-	-	-	+	++	10 (3,relapse,9,16,25,28,43,53,58, 64 m)
P3	-/-	+++	-	-	++	-	-	(+)	++	++	8 (0,1,9,31,70,73,78,81 m)
P4	-/-	+++	-	-	++	-	-	(+)	+	++	5 (9,13,16,23,33 d)
P5	-/-	++	+	+	+	n.t.	n.t.	n.t.	n.t.	+	1 (90 m)
P6	-/-	++	+	-	++	-	-	(+)	+	+	12 (9,15,22 d;1,2,2.2,2.4,4,6,19,20,39 m)
P7	-/-	++	-	-	+++	-	-	-	++	++	4 (4 d; 7,25,28 m)
P8	-/-	++	+++	-	++	n.t.	n.t.	n.t.	n.t.	++	1
P9	-/-	+	++	+	+	-	-	-	+	+	2 (1, 7 d)
P10	-/-	++	-	-	+	-	-	-	+	+	2 (0, 21 m)
P11	-/-	++	++	++	++	-	-	-	+	+	2 (0, 2 m)
P12	-/-	+	++	(+)	+	-	-	-	++	+	2 (0, 40 m)
P13	-/-	+++	-	-	++	-	-	-	++	++	2 (0, 24 m)
P14	-/+	+++	-	-	-	-	-	-	+	+	5 (8,12,17,23,36 m)
P15	+/+	++	++	++	++	+	+	-	-	++	5 (5,6,64,76,82 m)
P16	+/+	+	+	-	++	-	-	-	(+)	+	2 (61,64 m)
P17	+/+	+	+	+	+	n.t.	n.t.	n.t.	n.t.	+	1 (27 m)
P18	+/+	+	++	+	+	n.t.	n.t.	n.t.	n.t.	+	2 (15 d; 4 m)
P19	+/+	+	++	-	+++	(+)	(+)	(+)	+	++	2 (1,13 m)

Table 2. Binding sites of FH autoantibodies. The table summarizes binding sites of FH autoantibodies to the different FH fragments used. a: recombinant FH fragments produced in yeast. b: recombinant FH fragments produced in insect cells. +++ represents strong binding, ++ medium binding, + weak binding, (+) uncertain binding; all expressed in relation to the signal obtained for autoantibody binding to full-length FH. n.t.: not tested (due to limited availability of some of the samples). d, days; m, months.

Table 3: Epitope mapping of FH autoantibodies on SCRs 19-20 of FH.

Autoantibody binding to recombinant FH SCRs 19-20 fragments with single point mutations, expressed as percentage of binding to the WT FH SCRs 19-20 fragment. Data are mean of at least three different experiments.

Patient	D1119G	Q1139A	W1157L	R1182A	W1183L	T1184R	K1186A	K1188A	L1189R	E1198A	R1203A	R1210A	R1215Q
P1													
P2													
P3													
P4													
P5													
P6													
P7													
P8													
P15													
P16													
P17													
P18													
P19													

Percentage of binding is indicated in the table as follows:

>100	80-99	60-79	40-59	<40

Fig. 1.

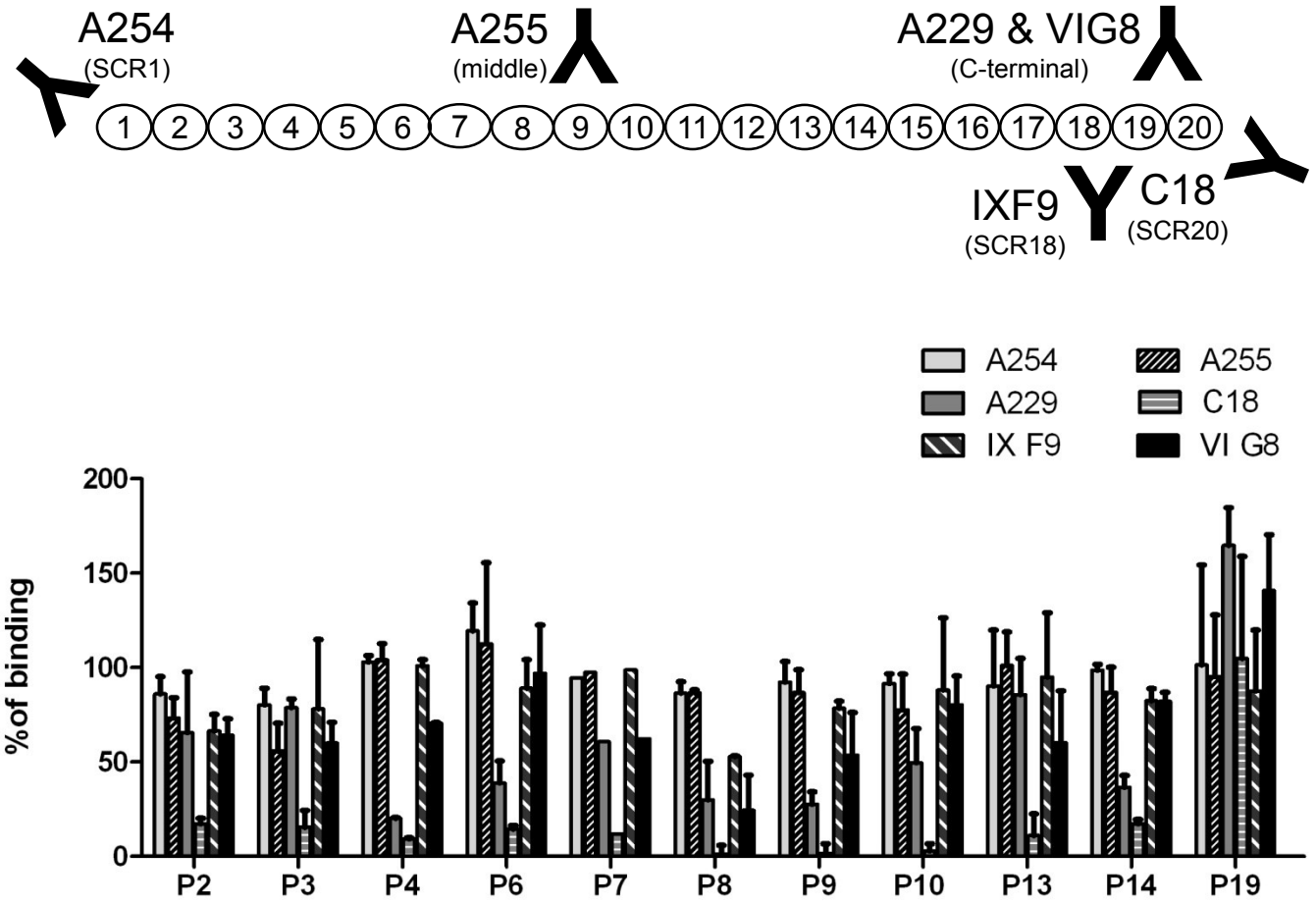
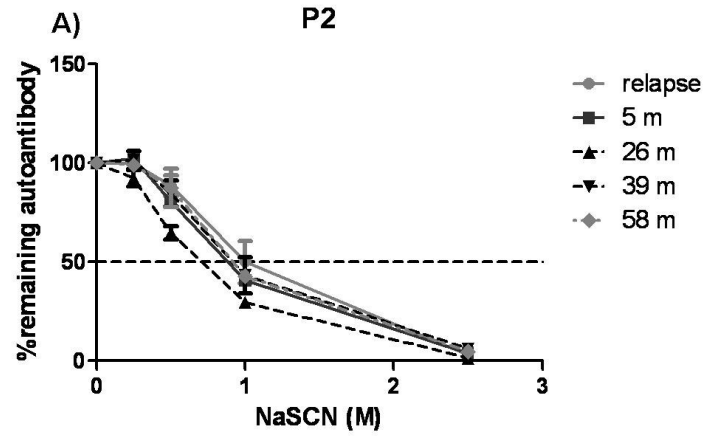
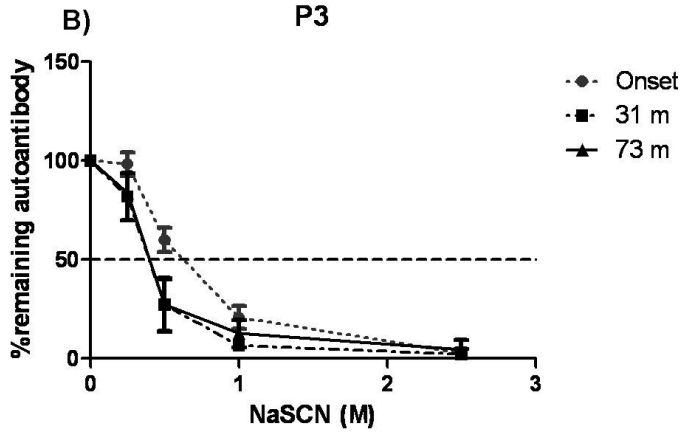
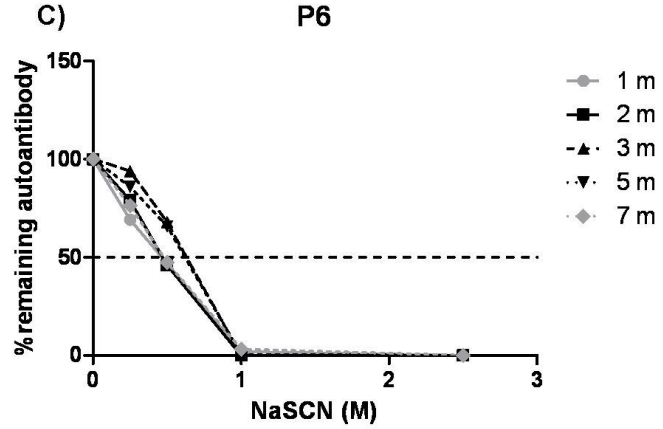
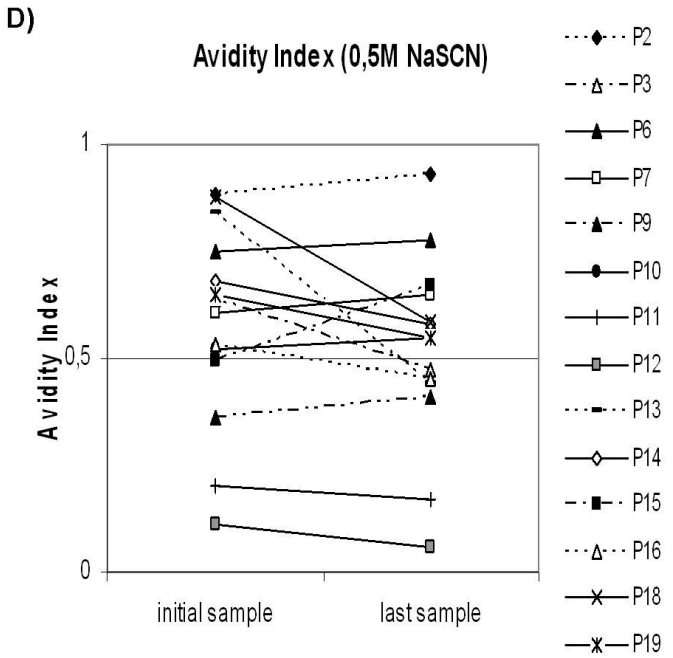

A254
(SCR1)
A255
(middle)
A229 & VIG8
(C-terminal)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
IXF9
(SCR18)
C18
(SCR20)
A254
A255
A229
C18
IX F9
VI G8
%of binding
200
150
100
50
0
P2
P3
P4
P6
P7
P8
P9
P10
P13
P14
P19

Fig. 2.


A)
P2
%remaining autoantibody
NaSCN (M)
relapse
5 m
26 m
39 m
58 m


B)
P3
%remaining autoantibody
NaSCN (M)
Onset
31 m
73 m


C)
P6
%remaining autoantibody
NaSCN (M)
1 m
2 m
3 m
5 m
7 m


D)
Avidity Index (0,5M NaSCN)
Avidity Index
initial sample
last sample
P2
P3
P6
P7
P9
P10
P11
P12
P13
P14
P15
P16
P18
P19

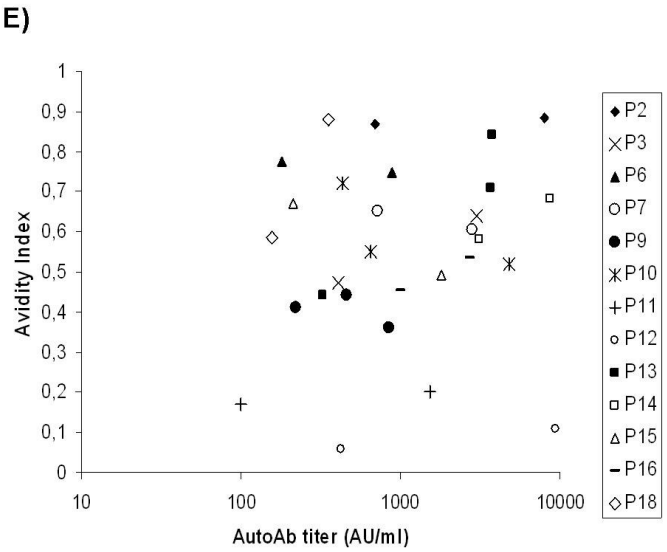
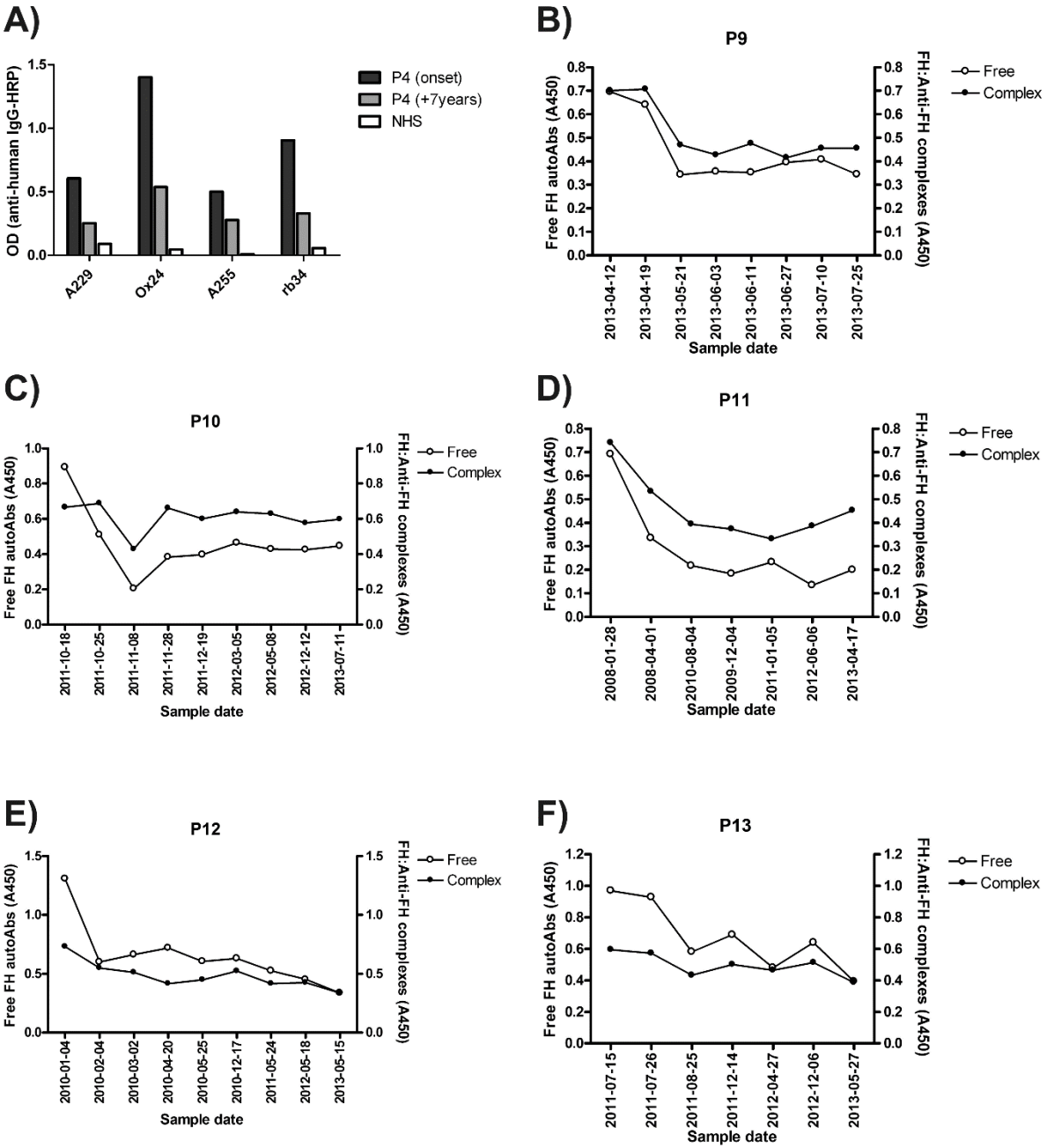

E)
Avidity Index
AutoAb titer (AU/ml)
P2
P3
P6
P7
P9
P10
P11
P12
P13
P14
P15
P16
P18

Fig. 3.


A)
OD (anti-human IgG-HRP)
P4 (onset)
P4 (+7years)
NHS
A229
Ox24
A255
rb34
B)
P9
Free FH autoAbs (A450)
FH:Anti-FH complexes (A450)
Free
Complex
Sample date
C)
P10
D)
P11
E)
P12
F)
P13

Supplementary Figures

Heterogeneity but individual constancy of epitopes, isotypes and avidity of factor H autoantibodies in atypical hemolytic uremic syndrome

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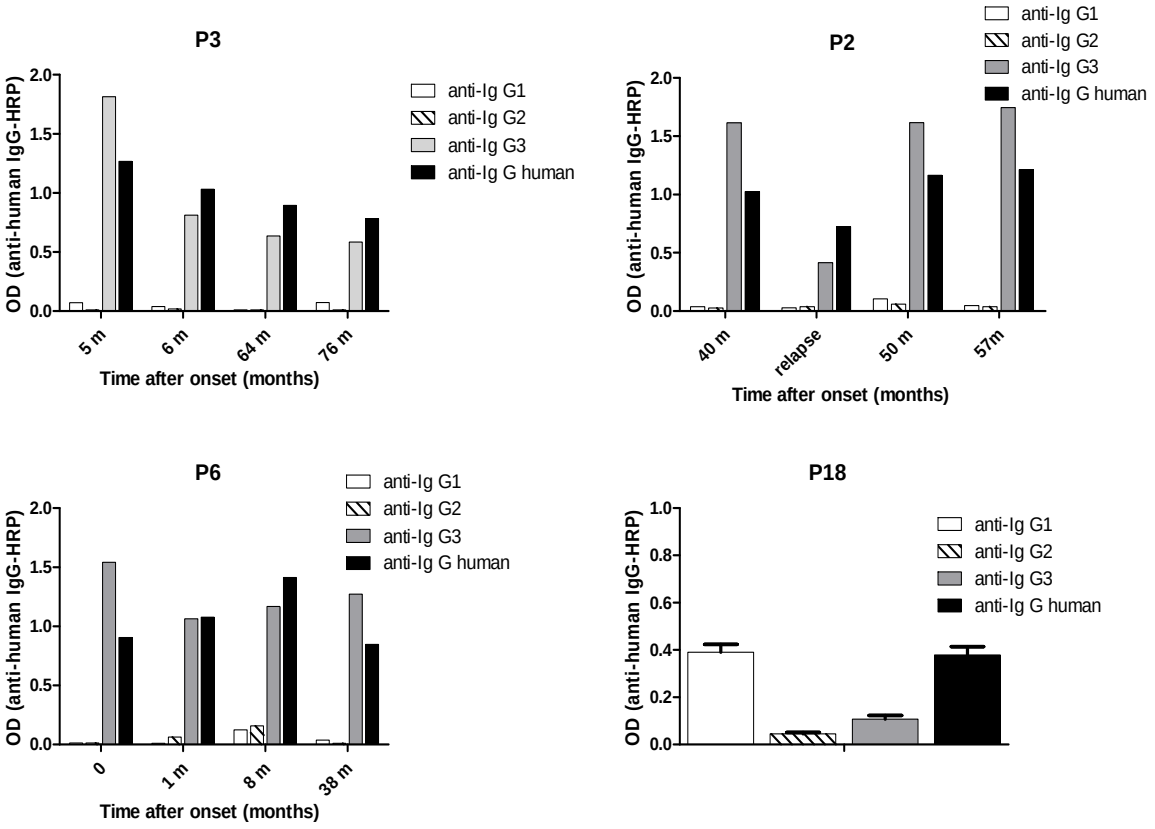
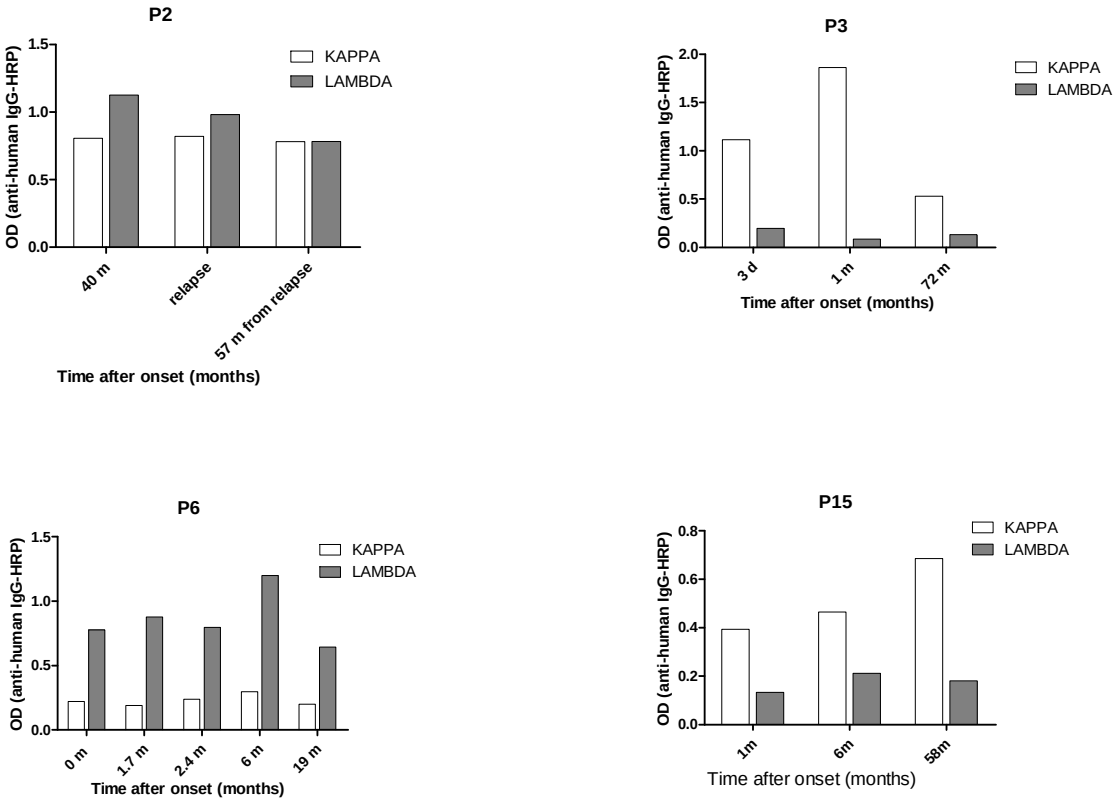
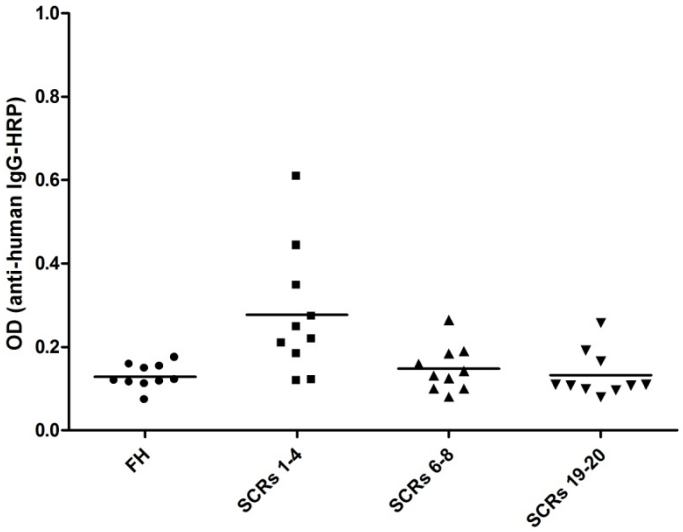
A



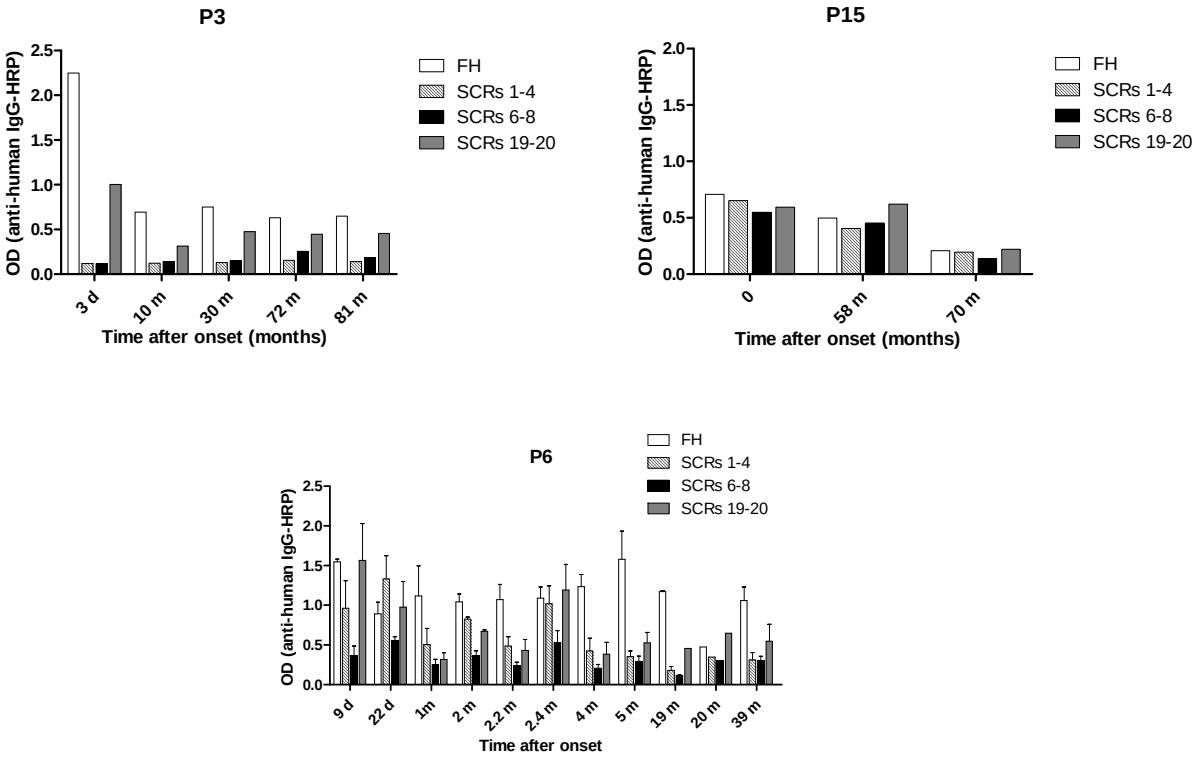
B



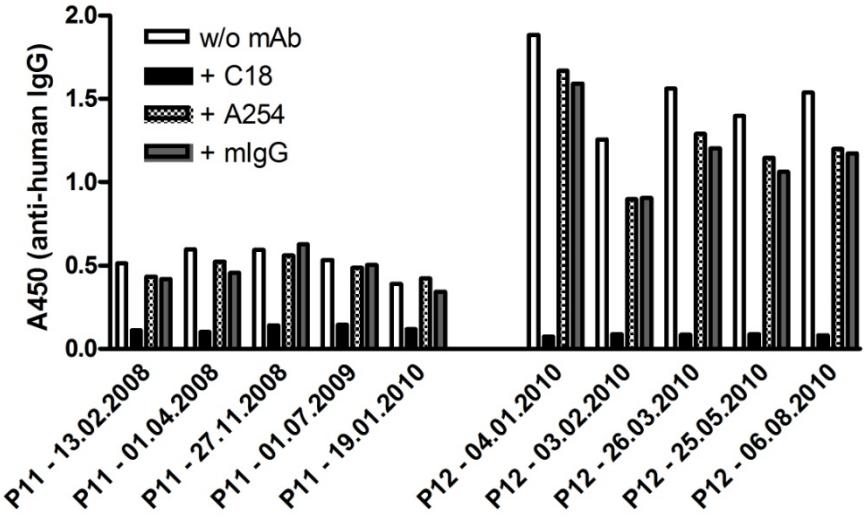
Supplementary Figure 1. Examples of FH autoantibody isotypes in aHUS patients during follow-up. Serial serum samples of patients were analyzed as described in Methods for IgG isotype (A) and for light chains (B).



Supplementary Figure 2. Reactivity of 10 normal serum samples with the recombinant FH fragments produced in *Pichia pastoris*. Purified FH and the recombinant proteins were immobilized in microplate wells as described in Methods. After blocking and incubation with sera of healthy donors, IgG binding was detected using HRP-conjugated anti-human IgG antibody. A representative experiment is shown.



Supplementary Figure 3. Examples of reactivity of FH autoantibodies with various recombinant FH fragments during follow-up. Serial serum samples of patients were analyzed as described in Methods for binding to purified FH, FH SCRs 1-4, SCRs 6-8 and SCRs 19-20 (produced in *Pichia pastoris*).



Supplementary Figure 4. Effect of anti-FH mAbs on autoantibody binding to FH from serial samples of patients P11 and P12. Binding of the autoantibodies in the presence of mAbs was determined as described in Methods. C18: anti-FH mAb that binds to SCR20 of FH. A254: anti-FH mAb that binds to SCR1 of FH. mIgG: purified mouse IgG, used as non-specific control antibody.