IgM Autoantibodies to Complement Factor H in Atypical Hemolytic Uremic Syndrome

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Short title: Anti–factor H IgM Autoantibodies in Atypical HUS

Abstract

Background Atypical hemolytic uremic syndrome (aHUS), a severe thrombotic microangiopathy, is often related to complement dysregulation but the pathomechanisms remain unknown in at least 30% of cases. Researchers have described autoantibodies to complement factor H of the IgG class in 10% of aHUS patients, but have not reported anti–factor H autoantibodies of the IgM class.

Methods In 186 patients with thrombotic microangiopathy clinically presented as aHUS, we searched for anti–factor H autoantibodies of the IgM class and those of the IgG and IgA classes. We used immunochromatography to purify anti–factor H IgM autoantibodies and immunoenzymatic methods and a competition assay with mapping monoclonal antibodies to characterize interaction with the target protein.

Results We detected anti–factor H autoantibodies of the IgM class in 7 of 186 (3.8%) patients with thrombotic microangiopathy presented as aHUS. No association was observed between anti–factor H IgM and homologous deletions involving CFHR3–CFHR1. A significantly higher proportion of patients with bone marrow transplant–related thrombotic microangiopathy had anti–factor H IgM autoantibodies versus other patients with aHUS: three of 20 (15%) versus four of 166 (2.4%), respectively. The identified IgM autoantibodies recognize the SCR domain 19 of factor H molecule in all patients and interact with the factor H molecule, inhibiting its binding to C3b.

Conclusions Detectable autoantibodies to factor H of the IgM class may be present in patients with aHUS, and their frequency is six-fold higher in thrombotic microangiopathy forms associated with bone marrow transplant. The autoantibody interaction with factor H's active site may support an autoimmune mechanism in some cases previously considered to be of unknown origin.

INTRODUCTION

Ample evidence exists on the pathogenetic role of complement activation in atypical hemolytic uremic syndrome (aHUS), a thrombotic microangiopathy (TMA) characterised by non-immune-mediated hemolysis, thrombocytopenia and acute renal damage[1,2]. Disease manifestations are due to dysregulated or inappropriate complement activation related to genetic or acquired factors. Genetic factors, including mutations of genes encoding complement regulators (factor H, factor I, and membrane cofactor protein) or gain-of-function mutations of genes encoding complement activators (C3 and factor B), render the complement system hyperactive[1]. Acquired factors lead to excessive complement activation mainly by factor H (FH) inhibition via autoantibodies[3]. These autoantibodies usually develop on a genetic background characterized by a homozygous deletion involving CFHR1 (complement FH related 1) and CFHR3 genes and the consequent disease is named *De*ficiency of CFHR (complement factor H-related) plasma proteins and *A*utoantibody *P*ositive form of *H*emolytic *U*remic *S*yndrome (DEAP-HUS). However, in approximately 30% of cases, aHUS arises from unknown mechanisms[2]. Complement involvement has been also demonstrated in the pathogenesis of the transplant-associated thrombotic microangiopathy (TA-TMA)[4] a severe complication of hematopoietic stem cell transplantation that may affect 10% to 20% of recipients[5] with a survival of 16.7% at 1 year in untreated patients[4-6].

Autoantibodies to FH of the IgG class have been described in complement-driven TMAs, both in atypical hemolytic uremic syndrome (aHUS)[7-9] and in TA-TMA[4] whereas anti-FH autoantibodies of the IgM class have never been reported in these conditions. Based on our experience in acquired angioedema due to C1-inhibitor deficiency, in which we have found autoantibodies to C1-inhibitor not only of the IgG and IgA classes but also of the IgM[10], we assessed all the three classes of autoantibodies to FH in our case list of patients with aHUS. Anti-FH IgM autoantibodies were purified and characterized with regard to their interaction with the target protein.

PATIENTS AND METHODS

Patients

One hundred and eighty-six patients with clinical presentation of aHUS were studied for the presence of autoantibodies against complement FH and for genetic mutations involved in complement-driven diseases. Patients with anti-FH autoantibodies were retested at least one month apart to confirm the positive findings. Ninety-nine patients were female and 87 were male with a median age of 32 years (range 1-84 years). The diagnosis was based on clinical and laboratory data. Twenty of the 186 patients had had a previous bone marrow transplant. As control groups we used 40 healthy subjects (20 were male), with a median age of 29 years (range 4–62 years).

Blood sampling was performed from an antecubital vein into plain tubes and processed within 2 h by centrifugation at 2000 ×g for 15 min at room temperature. The aliquots of serum were immediately frozen and stored at -80C° before testing.

All adult patients or parents of pediatric patients signed a written consent for genetic tests and all subjects agreed on the use of their blood samples in an anonymous form for research purposes. The local review boards approved the study that was conducted following the ethical principles 2013 revision of the Declaration of Helsinki and the code of Good Clinical Practice.

Methods

Anti-factor H antibody assay

Anti-FH antibodies were assayed by an ELISA that used purified FH for capture and anti-human IgM, IgG and IgA for detection[11]. Purified FH (Calbiochem, EMD Chemicals, San Diego, CA, USA; 10 μg/mL in phosphate-buffered saline, pH 7.4) was coated overnight onto microtitration plates, and, after washing, the wells were coated with bovine serum albumin to avoid non-specific binding. After further washes, a 1:20 dilution of the serum samples was added and incubated for 45 min at room temperature. After washing, the FH-bound immunoglobulins were identified by means of class-specific mouse monoclonal anti-IgM or –IgG or -IgA (Sigma Aldrich, St Louis, MO, USA), which were detected by peroxidase-

conjugated anti-mouse antibodies (Sigma Aldrich) and revealed with orthophenylenediamine. Absorbance was read at 490 nm. The results were expressed as units per mL (U/ml), referred to an internal standard (serum collected from a patient with a high anti-FH antibody titer) arbitrarily fixed at 100 U/mL. In order to avoid the confounding effect of natural antibodies, we decided to use the maximum level observed in normal subjects as cut-off between normal and abnormal levels.

Purification of anti-factor H immunoglobulins M

Anti-FH IgM were isolated from patients' serum by a two-step affinity chromatography. At first, IgG and albumin were depleted from patients' serum using albumin and IgG depletion SpinTrap columns (GE Healthcare, Buckinghamshire, UK) according to the manufacturer protocol. Depleted serum was then used to purify IgM by affinity chromatography on FH conjugated–sepharose. FH purified from normal human serum as previously described[12] was coupled with cyanogen bromide-activated sepharose 4B resin (Sigma-Aldrich, Saint Louis, Mo). To avoid cross contamination, a single affinity column was prepared for each patient, Micro Bio-Spin Columns (Bio-Rad, Hercules, CA) were filled with 200 µl of FH-sepharose resin. IgG and albumin depleted serum in binding buffer (20 mM sodium phosphate, 0.15 M NaCl, pH 7.4) was brought to a final concentration of 0.5 M NaCl and loaded onto the column. After a 30 min incubation on a rotary shaker, columns were washed with five column volumes of phosphate-buffered saline (PBS), IgM were then eluted in 0.1 M glycine pH 3.0 and immediately neutralised with tris-HCl 1.5 M pH 8.8. Purity of eluted IgM was verified by SDS-PAGE under reducing and non-reducing conditions followed by silver staining.

Characterization of the interaction between anti-factor H IgM and factor H domains

To overcome the lack of FH fragments for mapping FH epitopes that interact with IgM antibodies, an alternative approach was used. Autoantibody binding to full-length FH was assessed in a competition assay with known anti-FH monoclonal antibodies that recognize specific short consensus repeats (SCR) epitopes[13]. Microplates were coated with 10 µg /ml FH overnight at 4°C. Plates were blocked with bovine

serum albumin for 1 h at room temperature. Plates were incubated with 20 µg/ml of one of the known monoclonal antibodies: OX23 (mapping at SCR1-4, Ab17928 Abcam), OX24 (mapping at SCR5, MA1-81868 Thermo Scientific), L20 (mapping at SCR19, GAU-020-03-02 Thermo Scientific) and C18 (mapping at SCR20, GAU-018-03-02 Thermo Scientific) for 15 min at room temperature. Finally, after washing, serum samples were tested at 1:20 dilution by incubation for 1 hour at room temperature. Signal was detected using a peroxidase-conjugated anti-human IgM antibody. The binding of the patient antibodies was considered specific for a particular epitope of the FH molecule when it was inhibited by the presence of the monoclonal directed against that specific epitope.

Interactions between anti-factor H antibodies and factor H in fluid phase

After incubating the patients' serum for 1 hour at 37°C with increasing FH concentrations (0.015, 0.031, 0.063, 0.125, 0.250 and 0.5 mg/ml) we evaluated the free anti-FH antibodies still capable of binding to the microplate-immobilized FH.

Effect of anti-factor H IgM autoantibodies on factor H functional activity

The capacity of IgM autoantibodies to inhibit FH activity was investigated by means of the C3b binding assay[14]. Microtiter plates were coated with 5 µg/ml C3b (Calbiochem) in coating buffer (0.05 M carbonate-bicarbonate, pH 9.6) overnight at 4°C. Plates were blocked with 3% BSA for 1 h at room temperature. We preincubated 100 ng of FH (Calbiochem) with different dilution of purified IgM anti FH (starting from 100 U/ml), purified IgM anti C1-inhibitor (starting from 100 U/ml) as negative control and C18 mAb (starting from 1000 ng/ml) as positive control in tris-buffered saline (20 mM tris, 150 mM NaCl) for 10 min at 20°C. The samples were subsequently added to the wells and incubated for 1 h at 37°C. A standard curve with 200 – 100 – 50 – 25 ng of FH was included. Binding was detected with mouse anti-FH mAb 5H5 (in house) (2.5 µg/mL)[12,15] and rabbit anti-mouse IgG-HRP (DAKO) followed by TMB development. After stopping with 2 M H_2SO_4 the absorbance was measured at 450 nm on a

Spectra Max 190 photometer (Molecular Devices, Eugene, OR). Percentage of bound FH was calculated from the standard curve.

Genetic studies

Genomic DNA extraction was performed on the QIAsymphonySP automated platform (Qiagen GmbH, Hilden, Germany). Detection of nucleotide variations was assessed by Next Generation Sequencing (NGS) on the MiSeq platform (Illumina) by using the "targeted sequencing" technique (HaloPlex Kit, Agilent Technologies) on a multiple gene custom panel comprising *CFH* (NM_000186.3), *MCP/CD46* (NM_002389.4), *CFI* (NM_000204.4), *C3* (NM_000064.3), *CFB* (NM_001710.5), *THBD* (NM_000361.2), *DGKE* (NM_003647.2), *CFHR1* (NM_002113.2), *CFHR3* (NM_021023.5), *CFHR5* (NM_030787.3), at a 100X coverage. Bioinformatics analysis of NGS data with filtering to identify putative causative variants was performed with SureCall application. All variants identified by NGS analysis were then confirmed by standard Sanger sequencing method. The potential impact of amino acid changes was assessed by in silico analysis (SIFT, PolyPhen-2, Mutation Taster, VarSome-https://varsome.com) to predict the functional significance of unpublished and/or uncommon variants that were also evaluated as frequency of the variant compared to the general population from the ExAC database (Exome Aggregation Consortium, http://exac.broadinstitute.org/). We also took into account the genetic variant classification from the database of complement gene variants (http://www.complement-db.org/home.php) [16]. Multiplex ligation-dependent probe amplification (MLPA kit P236, MRC-Holland, Amsterdam, The Netherlands) was used to identify *CFHR3/CFHR1* copy number and macro rearrangements such as *CFH/CFH-Related* hybrid genes. Raw data were analysed by Coffalyser.net (https://www.mlpa.com) and relative dosage ratio was calculated.

Factor H antigen measurement

Levels of FH antigen were measured in serum from the 7 patients with IgM anti-FH autoantibodies, 12 patients with DEAP-HUS and 34 patients without anti-FH autoantibodies by a radial immunodiffusion

method (Human factor H "NL" BINDARID, The Binding Site, Birmingam, UK). Blood samples were collected and handled according to the manufacturer's instructions. Intra- and inter-assay CV < 12%.

Statistical analysis

Due to non-normal distribution, results were reported as median and range (min-max) and nonparametric methods were used to assess statistical significance of differences between groups. Categorical variables were reported as counts and percentages. Differences in proportions were assessed by using the χ^2 test. The significance level was set at $p<0.05$. The associations between parameters were evaluated by logistic regression. Odds ratios and 95% confidence intervals were reported. The Spearman correlation coefficient was calculated to assess relationships between the variables. The data were analysed using the SPSS PC statistical package, version 25 (IBM SPSS Inc., Chicago, IL, USA).

RESULTS

Anti-FH autoantibodies serum levels

Out of 186 patients with TMA, IgG anti-FH autoantibodies were elevated in 12 patients with aHUS that also presented a homozygous deletion in the genes of complement factor H-related proteins and thus were considered DEAP-HUS. High levels of anti-FH IgG autoantibodies were also found in 4 patients with TA-TMA. IgM anti-FH autoantibodies were elevated in 4 of the 154 patients with primary aHUS, in none of the 12 with DEAP-HUS and in 3 of the 20 with TA-TMA (Figure 1). A significantly higher frequency of IgM anti-FH autoantibodies was found in patients with TA-TMA (15.0%) compared to the remaining patients (2.6%) ($p=0.005$). Patients with IgM anti-FH autoantibodies had a median age of 38.5 years (range 18-61) whereas patients with DEAP-HUS had a median age of 13.5 years (range 1-21 years) ($p=0.003$). Patients with abnormally high levels of anti-FH IgM autoantibodies had a titer of 5-27 times the upper limit of normal while in patients with high levels of anti-FH IgG the titer was 4-140 times the upper limit of normal. None of the patients of the present case list developed autoantibodies of both classes. Anti-FH autoantibodies of the IgA class were not found in any patient. Demographic and clinical characteristics of patients with anti-FH antibodies of the IgM class are reported in table 1. The positive patients are identified by their sequential number in figure 1. Table 2 reports serum levels of anti-FH autoantibodies of IgM, IgG and IgA class in patients with primary aHUS, DEAP-HUS or TA-TMA and in normal controls.

Five of 7 patients with IgM autoantibodies were followed with serial determinations of anti-FH autoantibodies for a period ranging between 4 months and 4 years. During the follow-up, no patient developed anti-FH autoantibodies of the IgG class and the levels of IgM anti-FH have fluctuated over time but have remained above the normal range in all patients both during periods of active disease and during remission.

Dialysis was necessary in 4 of 7 cases. Four patients received plasma therapy alone, one patient received plasma therapy plus corticosteroids and azathioprine, 2 patients were treated with hydration and corticosteroids. Remission was initially obtained in 6 of 7 patients and maintained in 5 patients (2 of these

patients unfortunately did not recover renal function and remained in dialysis). One patient had one relapse and was successfully treated with eculizumab. The patient who did not respond initially underwent kidney transplant but aHUS relapsed two months later and a second transplant was performed; from that time on the patient was successfully managed with eculizumab maintenance treatment.

Purification of IgM anti-FH autoantibodies

The two-step affinity chromatography on FH conjugated–Sepharose performed on serum samples of patients with IgM anti-FH antibodies allowed the recovery of about 70% of these antibodies. On sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, the IgM heavy and light chains (75 and 25 kDa, respectively) were visible, together with an additional band around 250 kDa that may represent an incompletely dissociated IgM molecule. Under non-reducing conditions, a major band barely migrating in the resolving gel, representing total IgM (approximately 500 kDa), was present (Figure 2).

Interaction between anti-FH IgM and FH domains

In all the 7 patients positive for anti-FH IgM, the binding of these autoantibodies to FH molecule, expressed as optical density (OD) (mean of two experiments), was not inhibited by OX23 (mapping at SCR1-4,), OX24 (mapping at SCR5) and C18 (mapping at SCR20) whereas L20 (mapping at SCR19) did prevent the binding (Figure 3, left panels). The right part of figure 3 represents the binding of the specific monoclonal antibodies and the binding of patients' IgM anti-FH autoantibodies to the different SCR domains of the FH molecule. To confirm the data on serum, we tested the IgM anti-FH purified from patient n. 1 obtaining high OD when FH was preincubated with OX23, OX24 and C18 (780$\pm$ 90 OD) and low OD after incubation with L20 (100$\pm$27 OD).

Interactions between anti-FH antibodies and FH in fluid phase

FH added (up to 0.5 mg/ml) to the serum of patients with highest anti-FH IgM titer (Patients 1, 2 and 3) clearly inhibited the binding of autoantibodies to microplate-immobilized FH in a concentration-dependent manner as shown in figure 4. The inhibition was expressed as percentage of the OD obtained by adding only buffer, which was considered 100%. The values represent the mean of two experiments.

Effect of anti-factor H IgM autoantibodies on factor H functional activity

The C3b binding test was performed in the three patients whose serum was sufficient to obtain a high yield of purified antibodies (i.e. patients n. 1, 4 and 5). We obtained samples with anti-FH IgM concentration of 200 U/ml in patient n. 1, 100 U/ml in patient n 4 and 150 U/ml in patient n 5, irrespective of their serum levels that were 174 U/ml, 77 IU/ml and 60 U/ml. All purified immunoglobulin samples were brought to a concentration of 100 U/ml and then tested. Figure 5 shows that FH binding to C3b is inhibited by increasing amounts of IgM anti-FH antibodies from the maximum binding of 100 % to a minimum of 40-60 % in a dose-dependent manner whereas the negative control anti-C1-inhibitor IgM exerted no effect (panel A). Panel B shows the inhibitory effect on C3b binding by mAb C18 from the maximum binding of 100 % to a minimum of 50 %.

Genetic analysis

The analysis of genes involved in complement regulating components showed 3 different variants in patients with IgM anti-FH autoantibodies (Table 1). CFHR3–CFHR1 homozygous deletion was detected in 12 patients with anti-FH IgG autoantibodies and aHUS (thus considered DEAP-HUS) and in none of the 4 patients with TA-TMA and anti-FH IgG autoantibodies nor in patients with anti-FH autoantibodies of the IgM class (Table 1). The association between anti-FH autoantibodies and CFHR3-CFHR1 homozygous deletion was confirmed in patients with IgG autoantibodies (odds ratio 27; 95% CI, 6.35-114.85) whereas it was not observed in patients with IgM autoantibodies (Table 1).

FH antigen measurement

Serum levels of FH antigen were significantly lower in patients with anti-FH autoantibodies of the IgM class, (median 73% [range 56-102%]) and in patients with anti-FH autoantibodies of the IgG class (DEAP-HUS) (71% [range 32-115%]) than in patients with TMA without anti-FH autoantibodies (108% [58-180%]), with p values of 0.005 and 0.003, respectively (Figure 6).

DISCUSSION

Atypical HUS associated with anti-FH autoantibodies was first reported by Dragon-Durey et al. in 2005[3] and to date, these autoantibodies have been described in up to 10% of cases of aHUS in cohort studies, case series and case reports [17,18]. Anti FH autoantibodies have been described also in some cases of TA-TMA by Jodele et al[4]. In all the above mentioned studies, autoantibodies to FH were of the IgG class[4,17,18]. To the best of our knowledge, our study on aHUS is the first one describing patients with anti-factor H of IgM class. Interestingly, the median age at disease onset of our patients with aHUS and IgG anti-FH was 13.5 years, which is in agreement with previous studies[8,9]; in contrast, our patients with aHUS and IgM anti-FH were all adults with a median age of 38.5 years. Three of them developed aHUS after bone marrow transplant, thus the frequency of anti-FH IgM is six fold higher in TA-TMA than in primary aHUS. The association between anti-FH IgG autoantibodies and CFHR3-CFHR1 homozygous deletion was confirm in our case list in agreement with previous studies[19,20] while, among our 7 aHUS patients with IgM anti-FH autoantibodies, no one had this gene abnormality. In these 7 patients that do not have deletion, we expect FHR1 and FHR3 to be present in their serum, thus the autoantibodies might act through a different mechanism compared to patients with DEAP-HUS. In addition, being the presence of anti-FH IgM autoantibodies significantly more common in patients with HSCT without a specific genetic background, it can be hypothesized that their development is related to the transplant itself in the setting of an autoimmune process. The competition assay with mapping monoclonal antibodies (Figure 3) clearly shows that the IgM anti–FH autoantibodies of all patients interact with the SCR domain 19 that is located in the C-terminal part of FH and is important for the binding of FH to endothelial cells[14,21]. Moreover, since the anti-FH IgM autoantibodies inhibit the binding of FH to C3b *in vitro*, we can expect that this function is inhibited by the autoantibodies also *in vivo*. Such an inhibition, which mimics the effect of C-terminal FH mutations, has been previously demonstrated in aHUS patients with anti-FH autoantibodies of the IgG class and these autoantibodies were directed to SCR19-20 domains[14]. Indeed, SCR19 is important for the binding of FH not only to endothelial cells but also to C3b, as demonstrated by Kajander et al.[22].

Patients with IgM anti-FH autoantibodies showed a slight, but significant, reduction of FH antigen levels compared to patients without autoantibodies. Serum levels of FH have already been described low in aHUS in a previous study[17] and in 22% of cases in another study[18]. The slight reduction may be due to a higher clearance of FH when it is complexed to the antibody; however, our antigenic method does not provide information on the activity of FH that potentially can be reduced due to the possible presence of neutralising autoantibodies. In any case, since the IgM anti-FH autoantibodies of our patients bind the C-terminal domain of FH, which is a recognition region containing the binding sites for C3b, glycosaminoglycans and endothelial cells[14,21,22], a pathogenic effect of these antibodies is likely. Our *in vitro* data on inhibition of the FH binding to C3b by anti-FH autoantibodies further support this view. In our patients, the levels of IgM anti-FH have fluctuated over time but have remained above the normal range in all patients. Since also anti-FH autoantibodies of the IgG class may fluctuate over time and asymptomatic patients with high anti-FH titers are at risk of relapse[23], asymptomatic patients with anti-FH autoantibodies of the IgM class may also require cautious monitoring. Normal levels of C3 in most of our patients with anti-FH IgM are not surprising. Indeed, according to our and general experience, it is well known that C3 levels are expected to be normal in 50-70% of aHUS cases[11,24] and in 25% of aHUS patients with anti-FH autoantibodies of the IgG class[23].

In conclusion, our data indicate that in patients with a clinical presentation of aHUS the presence of autoantibodies of IgM class directed against the active site of FH is possible particularly in patients with TA-TMA. These autoantibodies may support an autoimmune mechanism in aHUS cases previously considered of unknown origin.

Author Contributions

GA, DC, PM, FD, FP, FG and MC followed the patients and collected clinical and laboratory data. SB, SG, EG and SC performed complement and immunologic analyses. ST performed genetic studies. MC, GA and FD analyzed the data. MC drafted the manuscript and GA and FD contributed to writing. All authors contributed to the interpretation of the results, critically reviewed the manuscript, and approved

the final version for submission. The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. All authors had full access to all of the data in the study and had final responsibility for the decision to submit for publication.

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Disclosures

D. Cresseri reports Speakers Bureau from Alexion Pharma. P. Messa reports Consultancy Agreements with Sandoz; Honoraria from Sandoz, vifor; Scientific Advisor or Membership with JN, BP Journal, Nutrients; and Speakers Bureau from vifor. F. Giglio reports Honoraria from Participation in advisory boards of Alexion, Pfizer, Amgen. F. Peyvandi reports Scientific Advisor or Membership with Roche, Sanofi and Sobi; and Speakers Bureau from Bioverativ, Grifols, Roche, Sanofi, Sobi, Spark and Takeda. G. Ardissino reports Consultancy Agreements with Alexion, Alnylam, Chemo Research; Honoraria from Alexion, Alnylam; Scientific Advisor or Membership with Alexion Inc. All remaining authors declare no conflict of interest on the submitted manuscript

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Table 1. Demographic, clinical and genetic data of patients with anti-factor H of IgM class and thrombotic microangipathy (TMA).

Pts	Sex	Age (yr)	Primary disease	TMA type	CFHR3-CFHR1 deletion	Complement gene variant	Variant clinical significance	IgM anti-Factor H U/ml	C3 %	Factor H %
1	F	48	Multiple myeloma	TA-TMA	No	No	n.a.	174	60	73
2	M	53	LLAC	aHUS	No	CFH c.3493+1G>C	Pathogenic	137	84	85
3	F	36	-	aHUS	Heterozygous	MCP p.(Thr383Ile)	Likely Pathogenic	87	93	90
4	F	41	-	aHUS	No	CFI p.(Lys441Arg)	Benign	75	101	56
5	M	18	Ulcerative colitis	aHUS	No	No	n.a.	60	72	60
6	F	26	Acute lymphoblastic leukemia	TA-TMA	No	No	n.a.	54	111	102
7	F	61	Burkitt's lymphoma	TA-TMA	No	No	n.a.	53	109	64
Normal range								0.3-6.7	76-117	70-120

aHUS: atypical hemolytic uremic syndrome; TA-TMA: transplant-associated thrombotic microangiopathy; LLAC: lupus like anticoagulant; CFH: complement factor H; MCP: membrane cofactor protein; CFI: complement factor I; CFHR3-CFHR1: complement factor H related proteins 3 and 1. n.a.: not applicable.

Table 2. Serum levels of anti-factor H autoantibodies of IgM, IgG and IgA class in 186 patients with thrombotic microangiopathy presented as atypical hemolytic-uremic syndrome and 40 healthy subjects.

	Anti-factor H autoantibodies		
	IgM U/ml	IgG U/ml	IgA U/ml
Primary aHUS [n=154]	1.9 (0.0-133.0)*	0.4 (0.1-4.8)	0.4 (0.0-4.2)
DEAP-HUS [n=12]	1.8 (0..0- 3.7)	40.0 (11.3-725.0)	0.5 (0.0-4.5)
TA-TMA [n=20]	2.3 (0.0-174.0)**	0.3 (0.0-40.0)***	0.7(0.1-3.9)
Normal controls [n=40]	2.0 (0.3-6.7)	0.5 (0.1-5.1)	0.4 (0.0-4.8)

aHUS: atypical hemolytic uremic syndrome; DEAP-HUS: *De*ficiency of CFHR (complement factor H-related) plasma proteins and *A*utoantibody *P*ositive form of *H*emolytic *U*remic *S*yndrome; TA-TMA: transplant-associated thrombotic microangiopathy .
*Four out of 154 patients with primary aHUS had abnormally high levels of anti-FH IgM autoantibodies.
**Three out of 20 patients with TA-TMA had abnormally high levels of anti-FH IgM autoantibodies.
***Four out of 20 patients with TA-TMA had abnormally high levels of anti-FH IgG autoantibodies.

FIGURE LEGENDS

Figure 1. Serum levels of anti-factor H autoantibodies of IgM class in patients with thrombotic microangiopathy presented as atypical hemolytic uremic syndrome (HUS) and in healthy subjects (normal controls). Patients were divided in primary aHUS, DEAP-HUS (*De*ficiency of CFHR [complement factor H-related] plasma proteins and *A*utoantibody *P*ositive form of *H*emolytic *U*remic *S*yndrome) and TA-TMA (transplant-associated thrombotic microangiopathy). Dashed line represents the upper limit of normal controls. Patients with elevated values of anti-factor H IgM are identified with their sequential number reported in table 1.

Figure 2. Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis (SDS-PAGE) under reducing conditions (lanes 1, 2 and 3) and non-reducing conditions (lanes 4, 5 and 6) followed by Silver staining of purified IgM anti-factor H obtained from patients n. 1, 2 and 3. Under reducing conditions, two bands at 75 and 25 kDa are visible (IgM heavy and light chains, respectively); moreover, there is an additional band at 250 kDa due to an incompletely dissociated IgM molecule. Under non-reducing conditions, there is a major band barely migrating in the resolving gel, representing total IgM (approximately 500 kDa).

Figure 3. Effects of specific monoclonal antibodies anti-factor H (FH) on the binding of IgM anti-FH autoantibodies to FH. The binding of purified IgM anti-FH to FH (expressed as mean OD of two experiments) was inhibited only by the monoclonal antibody (mab) L20 that interacts with the SCR domain 19 whereas mab OX23 (interacting with SCR 1-4,), OX24 (interacting with SCR 5) and C18 (interacting with SCR 20) did not modify the binding of IgM anti-FH to FH. The right part of the figure represents the binding of the specific monoclonal antibodies and the binding of patients' IgM anti-FH autoantibodies to the different SCR domains of the FH molecule.

Figure 4. Inhibition of the binding of anti-factor H (FH) to microplate-immobilized FH by soluble FH. When added to the serum of patients (n. 1, 2 and 3), FH (at concentrations of 0.015, 0.031, 0.063, 0.125, 0.250 and 0.5 mg/ml) inhibited the binding of anti-FH antibodies to the FH immobilized on microplates in a dose-dependent manner. The results are expressed as the percentage binding (mean of two experiments) recorded in the absence of added soluble proteins (buffer).

Figure 5. Effect of anti-factor H (FH) IgM autoantibodies on FH binding to C3b. Panel A shows that increasing amounts of IgM anti-FH antibodies (purified from patients 1, 4 and 5) inhibited FH binding to C3b in a dose dependent manner from the maximum binding of 100% to a minimum of 40-60% whereas the negative control anti-C1-inhibitor IgM (C1) exerted no effect. Panel B shows the inhibitory effect on FH binding to C3b by mAb C18 from the maximum binding of 100 % to a minimum of 50 %.

Figure 6. Serum levels of factor H (FH) antigen in 34 patients with thrombotic microangiopathy (TMA) without anti-FH autoantibodies, in 7 patients with TMA and anti-FH autoantibodies of the IgM class and in 12 patients with anti-FH autoantibodies of the igG class (DEAP-HUS: *De*ficiency of CFHR [complement factor H-related] plasma proteins and *A*utoantibody *P*ositive form of *H*emolytic *U*remic *S*yndrome).

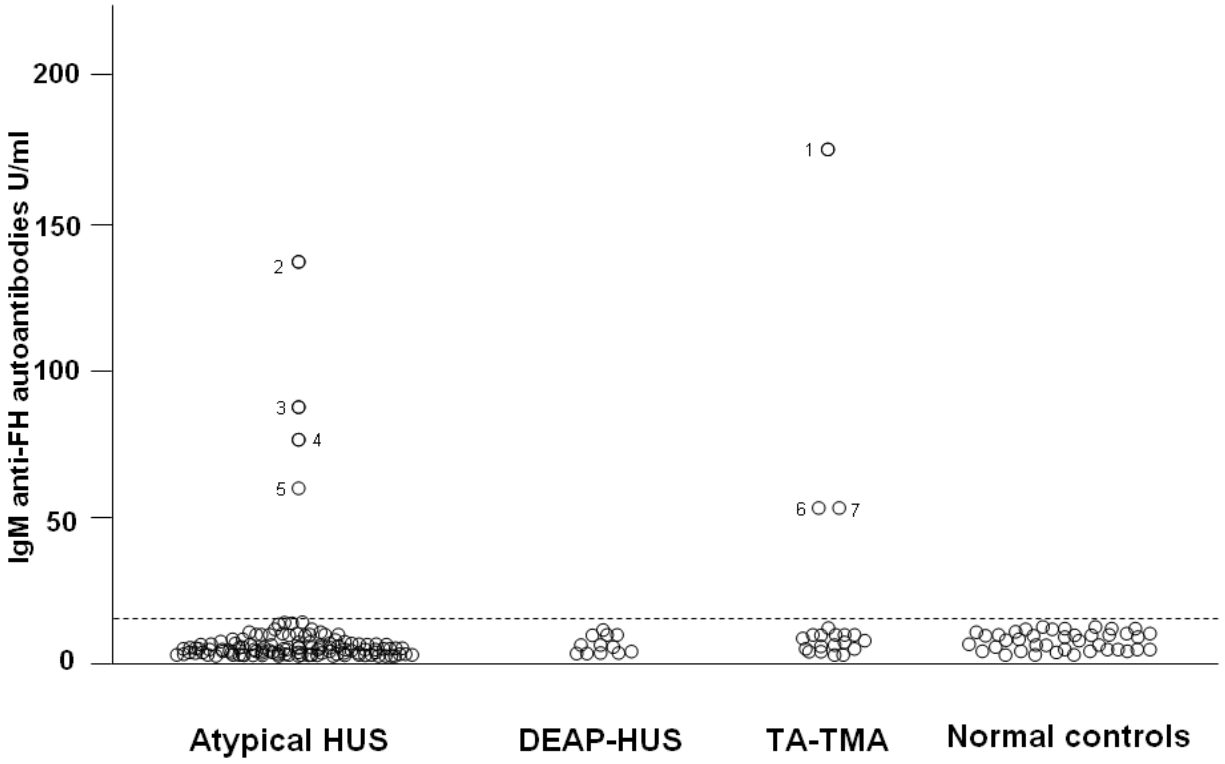
IgM anti-FH autoantibodies U/ml
200
150
100
50
0
1
2
3
4
5
6
7
Atypical HUS
DEAP-HUS
TA-TMA
Normal controls

Figure 1

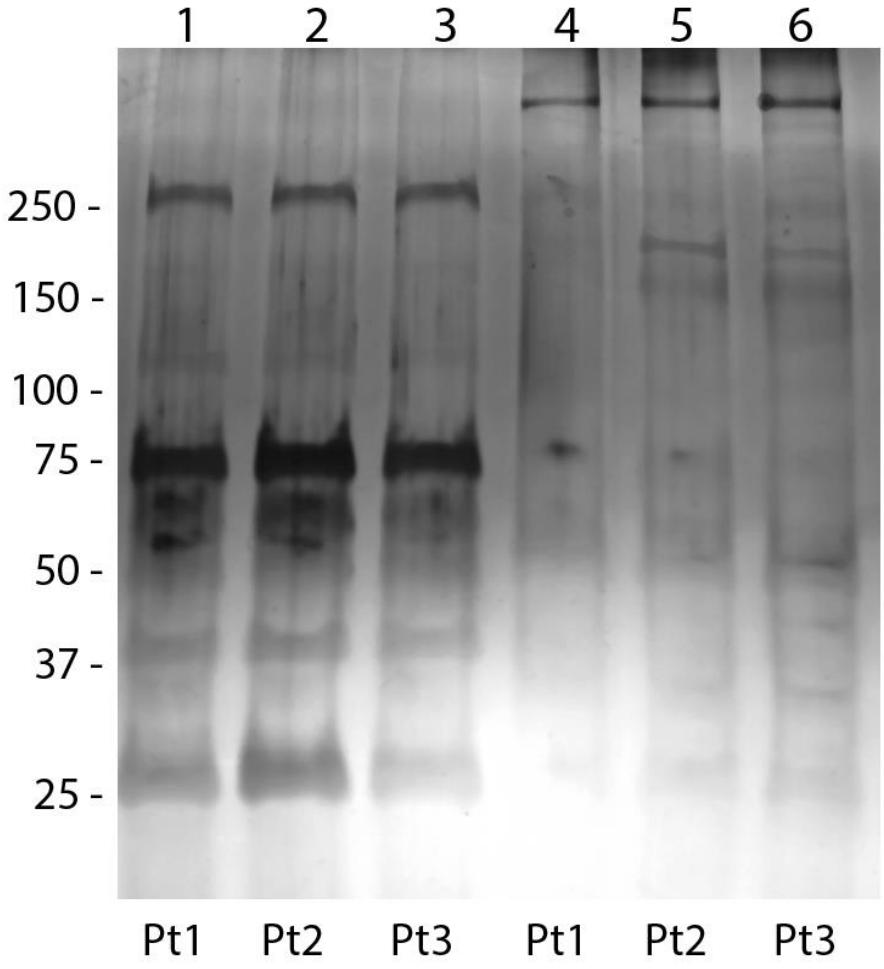

Figure 2

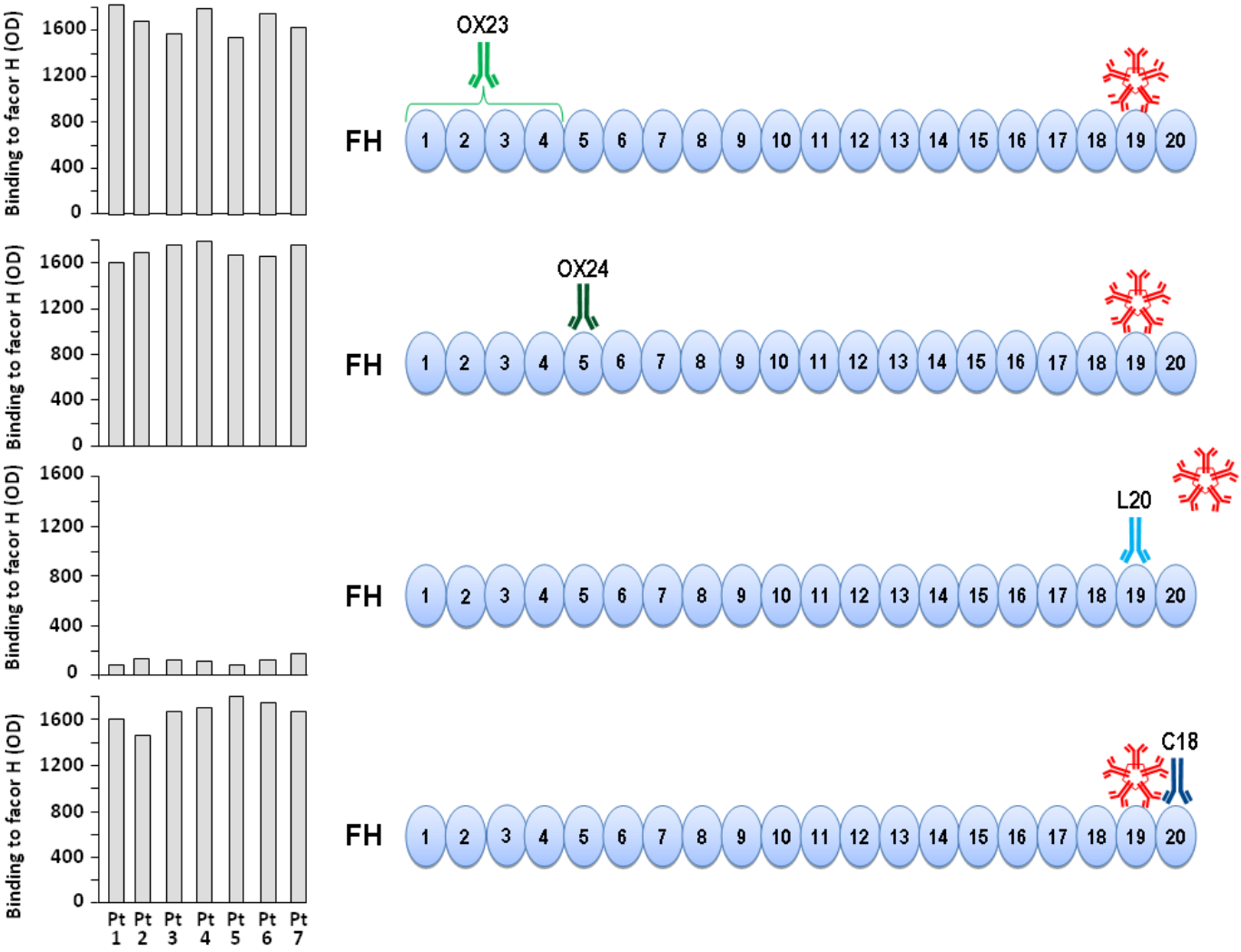
Binding to facor H (OD)
1600
1200
800
400
0
Pt 1
Pt 2
Pt 3
Pt 4
Pt 5
Pt 6
Pt 7
OX23
OX24
L20
C18
FH
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20

Figure 3

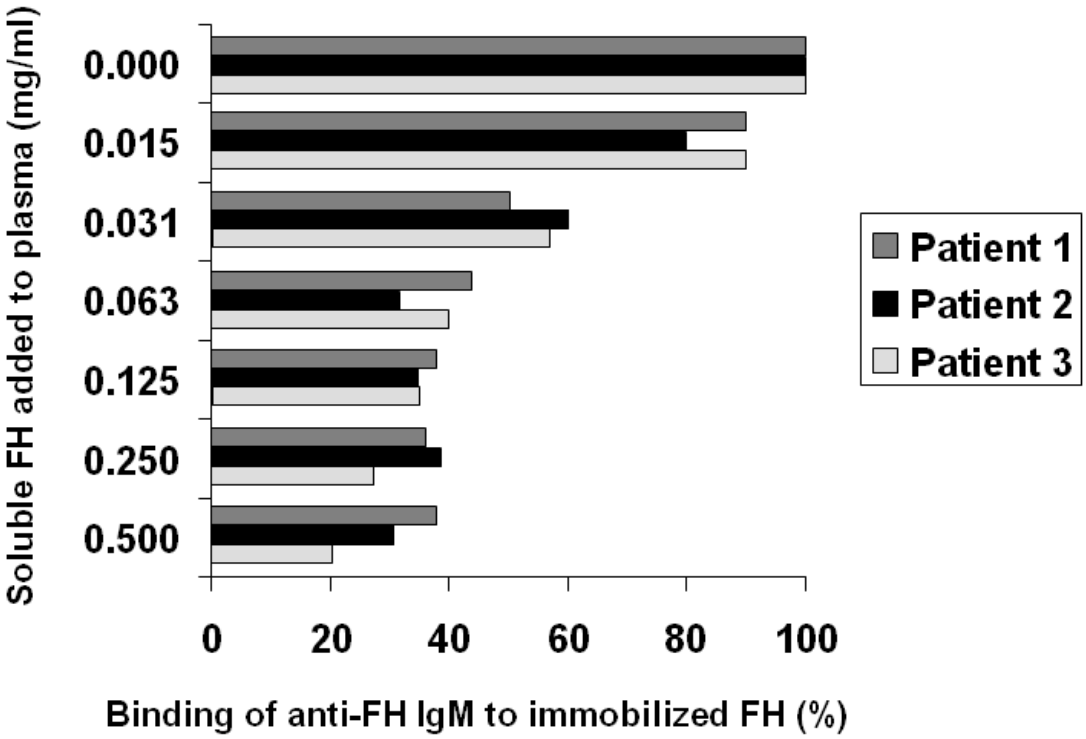
Soluble FH added to plasma (mg/ml)
0.000
0.015
0.031
0.063
0.125
0.250
0.500
0
20
40
60
80
100
Binding of anti-FH IgM to immobilized FH (%)
Patient 1
Patient 2
Patient 3

Figure 4

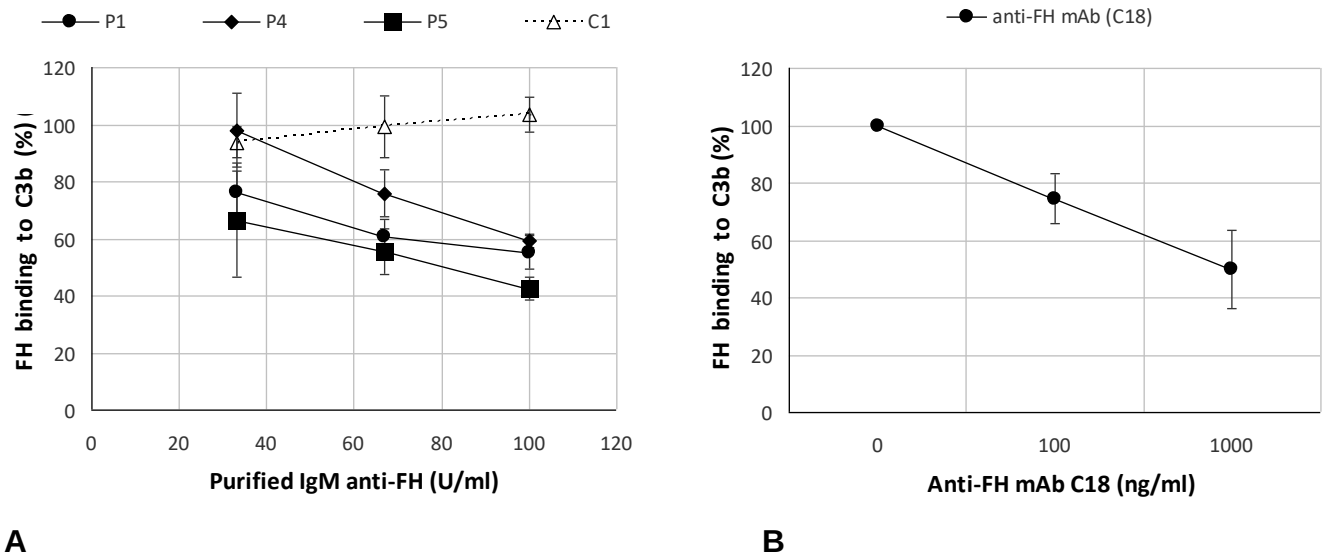
P1
P4
P5
C1
FH binding to C3b (%)
120
100
80
60
40
20
0
0
20
40
60
80
100
120
Purified IgM anti-FH (U/ml)
A
anti-FH mAb (C18)
FH binding to C3b (%)
120
100
80
60
40
20
0
0
100
1000
Anti-FH mAb C18 (ng/ml)
B

Figure 5

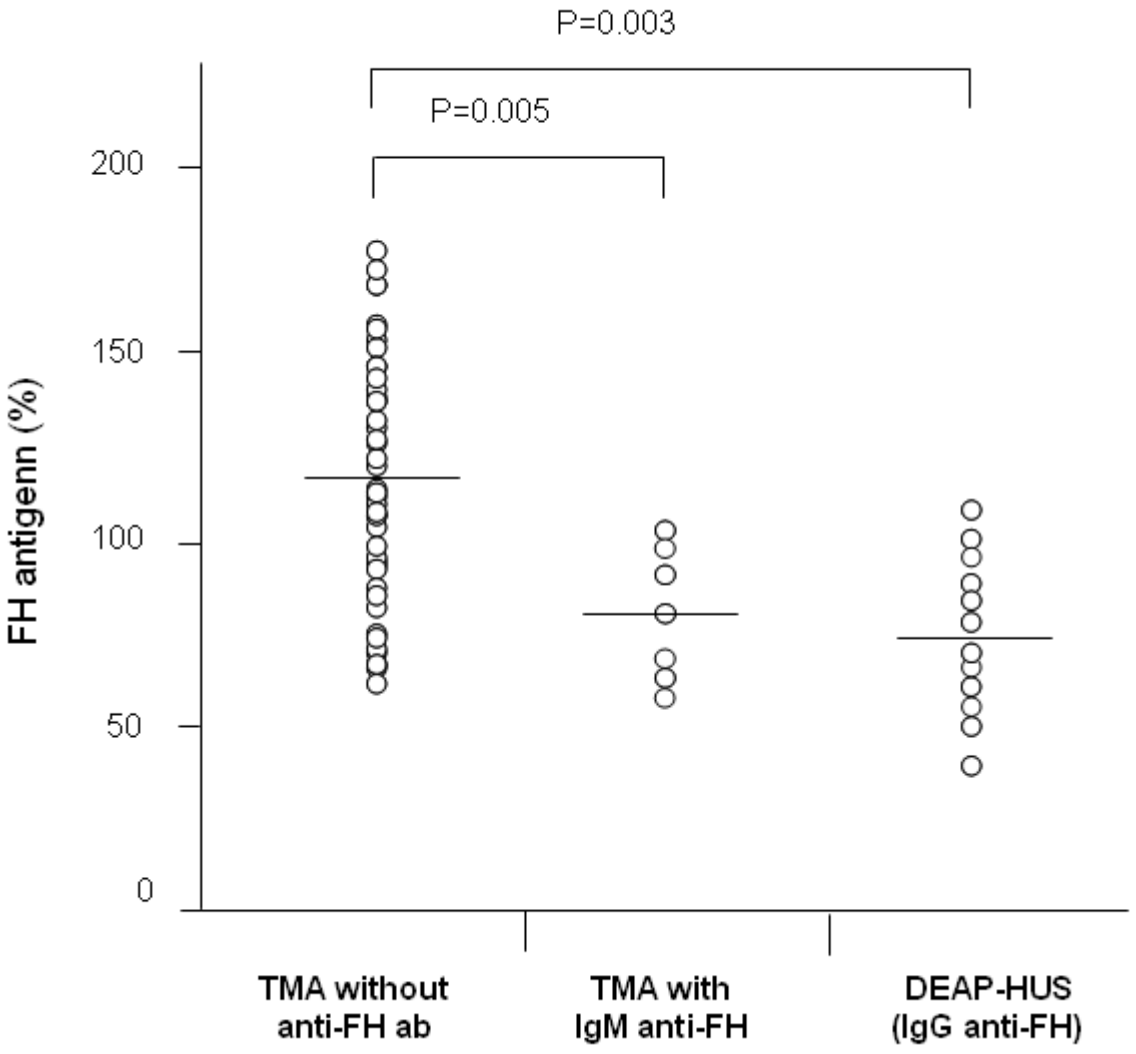
P=0.003
P=0.005
200
150
100
50
0
FH antigenn (%)
TMA without anti-FH ab
TMA with IgM anti-FH
DEAP-HUS (IgG anti-FH)

Figure 6