

1 **Unravelling the effect of a potentiating anti-Factor H antibody on atypical**
2 **hemolytic uremic syndrome associated factor H variants**

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36

37 **Abstract**

38 The complement system plays an important role in our innate immune system. Complement
39 activation results in clearance of pathogens, immune complex and apoptotic cells. The host is
40 protected from complement-mediated damage by several complement regulators. Factor H (FH) is
41 the most important fluid-phase regulator of the alternative pathway of the complement system.
42 Heterozygous mutations in FH are associated with complement-related diseases such as atypical
43 hemolytic uremic syndrome (aHUS) and age-related macular degeneration.

44 We recently described an agonistic anti-FH monoclonal antibody that can potentiate the regulatory
45 function of FH. This antibody could serve as a potential new drug for aHUS patients and alternative to
46 C5 blockade by Eculizumab. However, it is unclear whether this antibody can potentiate FH mutant
47 variants in addition to wild type FH. Here, the functionality and potential of the agonistic antibody in
48 the context of pathogenic aHUS-related FH mutant proteins was investigated. The binding affinity of
49 recombinant WT FH, and the FH variants, W1183L, V1197A, R1210C, and G1194D to C3b was
50 increased upon addition of the potentiating antibody and similarly, the decay accelerating activity of
51 all mutants is increased. The potentiating anti-FH antibody is able to restore the surface regulatory
52 function of most of the tested FH mutants to WT FH levels. In conclusion, our potentiating anti-FH is
53 broadly active and able to enhance both WT FH function as well as most aHUS-associated FH variants
54 tested in this study.

55

56 Introduction

57 The complement system plays an important role in our immune system ensuring pathogens, immune
58 complexes and dying cells are efficiently cleared from circulation. Activation of the complement
59 system can occur via three pathways: the classical (CP), lectin (LP) and alternative pathway (AP).
60 Activation of any of these pathways leads to the cleavage of the central complement component C3,
61 into C3a and C3b. C3a is an anaphylatoxin and C3b attaches covalently to surfaces, creating an
62 opsonization signal that allows recognition and clearance by the immune system. In addition, C3b
63 can lead to the formation of C5 convertases that cleave C5 into C5a and C5b, which is necessary to
64 form the membrane attack complex (MAC), resulting in lysis of the target (1-3).

65 The AP can be initiated via the spontaneous hydrolyzation of C3 (tick-over) in the fluid phase, forming
66 C3(H₂O) and serves to amplify the effects of activated CP and the LP. The AP amplification loop starts
67 with the binding of Factor B (FB) to C3b and is cleaved by Factor D (FD), forming the AP C3 convertase
68 C3bBb, which can cleave another C3. Binding of an additional C3b molecule to the C3bBb forms the
69 AP C5 convertase (4, 5). To prevent unwanted damage to the body's own cells by this amplification
70 loop, complement is tightly regulated by fluid-phase and membrane bound complement regulators
71 (1, 2, 6). Factor H (FH) is the main regulator of the AP (7).

72 FH regulates C3 convertase activity both in fluid phase as well as on the cellular surface. FH binds to
73 C3b and mammalian cellular surfaces by interaction with host specific glycosaminoglycans (GAGs) or
74 sialic acid containing glycans on the cellular membrane (8). This interaction results in decay of the AP
75 convertase C3bBb. Furthermore, FH together with factor I (FI) degrades C3b to iC3b and renders it
76 proteolytically inactive towards formation of the convertases (9). FH is composed of 20 complement
77 control protein (CCP) domains and can be further divided into regions that specifically interact with
78 sialic acids and GAGs on the surface of human cells, C3b or both (10, 11). The N-terminal region,
79 composed of CCP domains 1 to 4, is involved in binding to C3b and responsible for the decay
80 accelerating activity (DAA) and co-factor activity of FH. There are additional C3b binding sites within
81 the regions encompassed by CCPs 7 to 15 and 19 to 20, of which the latter interacts with C3b, iC3b
82 and C3d. The FH CCPs 7, 20 and the 15-19 region are involved in heparin and polyanion binding, and
83 the C-terminal CCP-20 is involved in sialic acid binding. The binding of FH CCPs 19 and 20 to both C3b
84 and polyanions is crucial for the host recognition (7, 11, 12).

85 Atypical hemolytic uremic syndrome (aHUS) is a complement-mediated disease, characterized by
86 hemolytic anemia, thrombocytopenia and complement depositions in the kidney in particular,
87 resulting in acute renal failure (13). Many aHUS patients carry genetic mutations in a complement
88 protein gene, where 25-30% of the cases are observed to have FH gene mutations and ~5% of aHUS

89 cases are observed to develop blocking autoantibodies against FH (13). The aHUS patients carrying a
90 FH mutation are heterozygously affected (14, 15). Whether both WT and mutant variant of FH are
91 equally expressed and thus present in the patient's serum is largely unknown. Interestingly, the aHUS
92 mutations often occur within the two most C-terminal domains of FH, which are the key regions for
93 FH's interactions with both C3b and the cellular surface (16, 17). This association between aHUS and
94 mutations in FH CCPs 19 and 20 highlights the importance of these regions for maintenance of
95 complement regulation on host-cell surfaces.

96 We have generated an anti-FH agonistic antibody that is able to potentiate the effector functions of
97 FH on human cells without affecting the bactericidal activity of the complement system (18). This
98 potentiation is characterized by an improved affinity of FH for C3b and a reduced IC₅₀ of C3b
99 deposition and complement mediated lysis of sheep erythrocytes. The agonistic anti-FH was able to
100 restore complement regulation in aHUS patient sera (18), but it remained unclear whether the
101 observed effect was due to enhancement of both WT and mutant FH or if the anti-FH agonistic
102 antibody potentiates only the WT FH in these heterozygous patient's sera. Here, we have studied the
103 effect of our potentiating anti-FH antibody on four naturally-occurring FH mutants that are
104 associated with aHUS. Three of the four tested mutants were functionally restored to the normal
105 level of regulation in the presence of the potentiating anti-FH. This demonstrates the agonistic
106 activity of this antibody on WT and aHUS associated FH mutant proteins and provide *in vitro* proof of
107 concept evidence for this agonistic antibody to be developed for the therapeutic treatment of aHUS
108 caused by heterozygous mutations in FH.

109

110 **Material and Methods**

111 **Antibodies, proteins and reagents**

112 Recombinant human C-terminal HIS-tagged FH wild type (WT), or with mutations in CCP20; W1183L,
113 V1197A, R1210C, G1194D, were provided by Gemini Therapeutics. The recombinantly produced FH
114 mutants were over 95% pure as shown by SDS page (**Supplemental Fig. 1**). The recombinant
115 expression of FH did not negatively affect the affinity of the potentiating anti-FH antibody (chimeric
116 IgG4) to FH and was slightly better compared to the affinity of our antibody to plasma derived FH
117 (pdFH) (18) and all recombinant FH had similar affinities ($K_D \approx < 1$ nM, data not shown) for the
118 potentiating antibody as determined by surface plasmon resonance (SPR).

119 pdFH, FB, FD and C3b were purchased from Complement Technology. In addition, for the C3b binding
120 experiment in SPR, pdFH was obtained by in house isolation as previously described (19).

121 Chimeric versions (human IgG4) of anti-FH.07 (18) and anti-FH.07.1 have been provided by Gemini
122 Therapeutics. Compared to previously published anti-FH.07 this paper makes use of anti-FH.07.1,
123 further referred to as the "potentiating anti-FH". This antibody has a similar binding epitope and
124 comparable function, as shown by competition ELISA and C3b deposition ELISA, performed as
125 described previously (18) (**supplemental Fig. 2**).

126 Mouse-anti-human monoclonal antibodies anti-C3-19 (20), anti-FH.16 (directed against FH CCP-16,
127 non-competing control), anti-FH.09 (directed against FH CCP-6, inhibiting control) (18), anti-IL6-8 (IgG
128 control) (18), anti-CD46.3 and anti-CD55.1 (21) were produced in house and labeled as indicated.
129 Proteins or antibodies were biotinylated according to the manufacturer's instructions using EZ-Link
130 Sulfo- NHS-LC-Biotin, No-Weigh Format (Thermo Scientific). Fluorescent labeling of antibodies was
131 done with DyLight 488 or DyLight 647 Amine-Reactive Dye (Thermo Scientific) according to
132 manufacturer's instructions. Fab' fragments of the monoclonal antibodies have been provided by
133 Gemini Therapeutics or are generated by pepsin cleavage of in house produced antibodies as
134 described previously (18).

135 Normal pooled serum (NPS) was obtained from > 30 healthy donors with informed, written consent,
136 in accordance with Dutch regulations. This study was approved by the Sanquin Ethical advisory board
137 in accordance with the Declaration of Helsinki. Serum was obtained by allowing blood to coagulate
138 for 1 h at room temperature (RT) and collecting the supernatant after centrifugation at $950 \times g$ for 10
139 min, pooled serum was aliquoted and stored at -80°C. FH depleted serum was purchased from
140 Complement Technology. Eculizumab (Alexion Pharmaceutical) was obtained by collecting surplus
141 from used Soliris injection bottles. Sheep erythrocytes (ES) were from Håtunab. High-performance

142 enzyme-linked immunosorbent assay (ELISA) buffer (HPE), streptavidin conjugated with poly-
143 horseradish peroxidase (strep-poly-HRP), were from Sanquin Reagents. Anti-CD59 (MEM-43, FITC)
144 was ordered from Thermo Scientific.

145 **ELISA**

146 Unless stated otherwise, all ELISA incubation steps were performed for 1 hour at RT on a shaker.
147 After each incubation, plates were washed 5 times with PBS containing 0.02% Tween-20 (PBS;
148 Sanquin Diagnostics) using an ELISA washer (Biotek, 405 LSRS). All ELISAs were developed with 100 μ L
149 substrate solution per well containing 0.11 M sodium acetate (Merck), 0.1 mg/mL 3,5,3',5'-
150 tetramethylbenzidine (TMB, Merck) and 0.003% (v/v) H_2O_2 (Merck) diluted in milliQ (Merck).
151 Reactions were stopped with 100 μ L 0.2M H_2SO_4 (Merck). Time that reactions were incubated varies
152 per ELISA and are mentioned below. Absorbance was measured at OD450_{nm} using a Synergy 2 Multi-
153 Mode plate reader (BioTek Instruments) and corrected for the absorbance at OD540_{nm}. All ELISA
154 steps were performed with a final volume of 100 μ L per well.

155 **C3b deposition on LPS**

156 C3b deposition assay with NPS was performed as described previously (18). C3b deposition assay
157 with FH depleted serum was performed as followed. Polysorp 96-wells microtiter plates (Nunc) were
158 coated with *Salmonella typhosa* LPS (40 μ g/mL, Sigma-Aldrich) in PBS, O/N at RT. After washing NPS
159 or FH depleted serum was incubated in Veronal buffer (VB; 3 mM barbital, 1.8 mM sodium barbital,
160 145 mM NaCl, pH 7.4) containing 0.05% (w/v) gelatin, 5 mM MgCl₂, 10 mM EGTA and 0.1% (w/v)
161 Tween-20 in the presence or absence of FHs and/or anti-FH potentiating mAb at indicated
162 concentrations. C3b deposition was detected with biotinylated mAb anti-C3.19 (0.55 μ g/mL in HPE)
163 followed by incubation with 0.0001% (v/v) strep-poly-HRP in HPE for 30 minutes. The ELISA was
164 further developed as described above.

165 **SDS page, coomassie**

166 To analyze the recombinant FH variants, 0.3 μ g of sample was denaturized in 4x LDS sample buffer
167 (Invitrogen) and incubated for 10 min at 70°C. Samples were loaded on 4-12% bis-tris gel (Life Tech).
168 Gel was run at 200 V for ~55 min in MOPS buffer (Novex). Gel was stained for 20 minutes using
169 instant blue stain (Expedeon) and unstained in MilliQ for approximately 16 hours. Gel was
170 photographed using a Chemidoc (Bio-rad).

171 **Surface Plasmon Resonance (SPR)**

172 All surface plasmon resonance (SPR) experiments were performed using a Biacore T200 (GE
173 Healthcare) with either a research grade Series S Sensor Chip Protein G (GE healthcare) or Series S
174 Sensor Chip CM5 (GE healthcare). Unless stated otherwise, SPR experiments were performed at 25°C

175 using a flow rate of 15 μ L/min and in phosphate buffered saline (PBS, Fresenius Kabi), pH 7.4 with
176 0.1% (w/v) Tween-20 (Merck) (PBS-T). Data were collected at a rate of 10 Hz and all SPR data were
177 analyzed using Scrubber (v20c, Biologic).

178 **Antibody affinity for recombinant FH in SPR**

179 For the assessment of affinity of the potentiating antibody for FH mutants, at the beginning of each
180 cycle, the potentiating antibody was captured on a Series S Sensor Chip Protein G at a concentration
181 of 1 μ g/mL during 60 seconds, corresponding with a \sim 200 response units (RU) signal increase, leaving
182 a second flow cell blank as reference. After a stabilization period of 300 seconds, a titration of the FH
183 of interest (155 kDa) was injected, in random order in a two-fold dilution range starting at 500 or 250
184 nM. The complex was allowed to associate for 600 seconds and dissociate for 1500 seconds. After
185 each FH injection, the Protein G chip was regenerated for 30 seconds with 100 mM Glycin-HCl
186 (Merck) pH 1.5 at 30 μ L/min and the process was repeated starting a new cycle with the capture of
187 the potentiating antibody. Affinity constants were determined using kinetic modelling.

188 **Coupling C3b sensor chip for SPR**

189 For the assessment of the affinity of FH to C3b or the decay accelerating activity of FH in the
190 presence of various antibodies (Fab' fragments), purified C3b was immobilized via amine-coupling on
191 a series S CM5 Sensor Chip using standard methods. In short, flow channels were activated for 7
192 minutes with a 1-to-1 mixture of 0.1 M N-hydroxysuccinimide (GE healthcare) and 0.1 M 3-(N,N-
193 dimethylamino) propyl-N-ethylcarbonadiimide (GE healthcare) at a flow rate of 5 μ L/min. The
194 reference flow channel was blank immobilized, the other was immobilized with C3b, diluted in 10
195 mM sodium acetate (BioRad), pH 5.0, with target immobilization response of 2000 RU. When desired
196 RU signals were reached, the surfaces were blocked with a 7 min. injection of 1 M ethanolamide, pH
197 8.0 (GE healthcare) to finish immobilization.

198 **Affinity of FH to C3b in SPR**

199 To asses binding of FH to C3b, FH was injected in two-fold dilution range, starting at 10 μ M, 5 μ M (in
200 house obtained pdFH) or 625 nM (recombinant) FH, in random order, with a flow rate of 10 μ L/min,
201 at 37° C, over both reference and C3b coupled flow channels of the C3b coupled sensor chip
202 described above and allowed to associate and dissociate for 60 seconds each. After each cycle the
203 flow channels were regenerated for 10 seconds with 2 M NaCl (Merck) at 10 μ L/min. To assess the
204 effect of various potentiating antibodies on the binding affinity of FH to C3b, FH was similarly titrated
205 in buffer containing a surplus of potentiating antibody Fab' fragments; either 10 μ M or 1.25 μ M (2
206 times highest FH concentration in respective experiment). The obtained signals were corrected for

207 the MW of FH (155 kDa) and the FH-anti-FH Fab' fragment complex (205 kDa). Affinity constant (K_D)
208 was determined by plotting the affinity curve at equilibrium of binding.

209 **Decay acceleration activity (DAA) assay in SPR**

210 To assess DAA of FH in the absence or presence of one of the various potentiating antibodies, a C3b
211 coupled sensor chip as described above was used. Running buffer in this experiment was 10 mM
212 HEPES with 150 mM NaCl, 1 mM MgCl₂ and 0.005% (w/v) Tween-20, pH 7.4. Regeneration buffer was
213 10 mM HEPES with 150 mM NaCl, 3.4 mM Ethylenediaminetetraacetic acid (EDTA) and 0.005% (w/v)
214 Tween-20, pH 7.4. To form the C3 convertase C3bBb, FB and FD were simultaneously injected at a
215 concentration of respectively 600 nM and 100 nM for 180 seconds, corresponding with a ~250 RU
216 increase. After a stabilization period of 120 seconds (natural decay of the convertase), FH (50 or 12.5
217 nM) with or without the potentiating antibodies (100 or 200 nM) Fab' fragments were injected for
218 180 seconds. Finally, buffer was flowed over for another 300 seconds. After each cycle the flow
219 channels were regenerated for 30 seconds with regeneration buffer at 30 μ L/min. Runs were
220 performed without or with FB and FD capture, respectively the “buffer” or “FB and FD” conditions.
221 To obtain the final superimposed DAA signal, the signals of the buffer conditions were subtracted
222 from the corresponding FB and FD conditions.

223 **Hemolytic assay**

224 Hemolytic assay was performed with some adjustments for the use of FH depleted serum. Each
225 component described below was 25% (v/v) of total assay volume. In a U-shaped assay plate
226 containing a 2-mm diameter glass bead (merck), FHs were diluted in VB + 0.05 % gelatin (Merck)
227 (VBG) with or without an excess of potentiating anti-FH and titrated in two-fold. Sheep erythrocytes
228 (SE) were washed with PBS and resuspended in VB + 5.8% sucrose (VBS), to contain 1.05×10^8
229 cells/mL in final assay concentration. NPS and FH depleted serum were diluted in VBG + 0.1 mM
230 EDTA, to be 10% (v/v) in final assay conditions. To activate the alternative pathway, VBG + 5 mM
231 MgCl₂ + 10 mM EGTA in final concentration was added to the wells. VBG with 10 mM EDTA was used
232 as blank. Samples were incubated at 37°C for 75 minutes while shaking at 450 RPM (Eppendorf
233 thermomixer). Lysis was stopped by adding 100 μ L VBG followed by centrifugation (2.5 minutes,
234 1800 RPM/471 RCF, 7°C). Hemolysis was measured as absorbance of the supernatants at 412 nm,
235 corrected for background absorbance measured at 690 nm, and expressed as percentage of the
236 100% lysis control (ES incubated with 0.85% (w/v) Saponin). Graphs were fitted using a nonlinear fit,
237 [inhibitor] versus response with four parameters.

238 **C3 deposition on HAP-1 cells**

239 HAP-1 cells (22) (Haplogen Genomics, Wien, Austria) deficient for CD46, CD55, and CD59 (deficient
240 HAP-1 cells) (21) were cultured in Iscove's Modified Dulbecco's Medium (IMDM; Lonza)
241 supplemented with 10% (v/v) fetal calf serum (FCS; Sigma-Aldrich), 100 U/mL penicillin (Invitrogen)
242 and 100 µg/mL streptavidin (IMDM++; Invitrogen) at 37 °C and 5% CO₂. Cells were washed with PBS
243 and detached using Accutase (Sigma-Aldrich). Surface expression was checked by staining with anti-
244 CD46-3-DyLight 488 (FITC), anti-CD55-1-DyLight 647 (APC), and anti-CD59-FITC conjugated antibodies
245 and analysis using flow cytometry.

246 Complement activation and detection was performed as followed. In a U-shaped assay plate
247 containing a 2-mm diameter glass bead, 7 x 10⁴ HAP-1 cells were incubated for 1 h at 37 °C while
248 shaking at 350 RPM with 25% (v/v) NPS or FH depleted serum diluted in VBG supplemented with 10
249 mM MgCl₂ and 20 mM EGTA (VBG^{MgEGTA}) in the presence of equimolar (20 µg/mL) blocking anti-C5
250 (Eculizumab) to prevent lysis. FHs and/or potentiating anti-FH were added to the cells prior to
251 addition of the serum. After incubation, deficient HAP-1 cells were washed with PBS supplemented
252 with 0.5% (w/v) bovine serum albumin (BSA, Sigma-Aldrich) before staining in PBS containing 0.1%
253 BSA. C3 deposition was detected by incubation of the HAP-1 cells with 1 µg/mL DyLight 488-
254 conjugated or DyLight 647-conjugated anti-C3-19 for 30 min at room temperature. After incubation
255 cells were washed 3 times with PBS containing 0.5% BSA and subsequently fixed 1% (w/v)
256 paraformaldehyde (PFA, Merck Millipore) in PBS. LSR Canto II flow cytometer (BD Biosciences) was
257 used for measuring and data analysis was performed using FlowJo software V10 (Treestar, Ashland,
258 OR). Gating strategies are shown in **Supplemental Fig. 3**.

259 **Statistical analysis**

260 Analysis and statistical tests were performed using GraphPad Prism version 8.0.2 (GraphPad
261 Software).

262 **Results**

263 **Potentiation of affinity of FH for C3b by potentiating anti-FH**

264 We previously showed that our potentiating antibody can restore complement regulation in aHUS
265 patient samples (18). To investigate whether our antibody can potentiate both WT as well as mutant
266 FH we used recombinant variants of FH, namely wild type (WT) and aHUS-associated mutants
267 W1183L, V1197A, R1210, and G1194D (> 95% pure, **Supplemental Fig 1**). First, we studied the
268 potentiating effect of anti-FH.07.1 on FH binding to C3b. **Fig. 1** shows that the addition of anti-
269 FH.07.1 fab' fragments to pdFH increases the affinity for C3b by ~ 3-fold compared to pdFH injection
270 alone, with K_D s of 1.9 and 6.0 μ M respectively (**Fig. 1A, 1B**). We observed a clear titration dependent
271 increase in binding of pdFH to C3b, confirming previous study (18). All tested full length recombinant
272 FH mutants showed slightly (< 2-fold) weaker binding to C3b compared to recombinant WT FH.
273 Addition of the potentiating anti-FH Fab' fragments increased affinity to C3b of all tested FH mutants
274 by 1.8 - 2.4 fold (**Fig. 1C, 1D, Table I**). In conclusion, these experiments show that the studied
275 mutations in FH only slightly impair the binding to C3b and that all tested mutants are enhanced in
276 their affinity to C3b by anti-FH.07.1.

277 **All FH mutants show decay acceleration of AP convertase and are potentiated by potentiating anti-
278 FH**

279 Next, we studied the potential of the FH mutants to perform DAA of the AP convertase utilizing a SPR
280 based assay (23). In this assay, we constructed the AP convertase C3bBb on the SPR chip by coupling
281 C3b to the chip followed by incubation with FB and FD after which the AP convertases are formed
282 (**Fig. 2A**, phase I). The AP convertases will naturally decay (**Fig. 2A**, phase II). As expected, this decay
283 is increased upon injection of FH (**Fig. 2A**, phase III). With addition of anti-FH.07.1 Fab' fragments
284 pdFH showed increased DAA compared to pdFH alone as shown by the faster decrease in signal (**Fig.**
285 **2A**, grey dashed line). The anti-FH Fab' fragments alone did not enhance DAA (**Fig. 2A**, black dashed
286 line). Addition of FH binding Fab' (anti-FH.16) or blocking Fab' (anti-FH.09) (18) fragments results in
287 respectively similar or less DAA compared to pdFH alone, confirming the potentiating effect of anti-
288 FH.07.1 (**Fig. 2B**).

289 We then studied the potential of all tested FH mutants to accelerate the decay of the C3 convertase.
290 All tested mutants did have DAA, although less efficient compared to WT FH (**Fig. 2C**, solid lines), and
291 were all potentiated by addition of anti-FH.07.1 Fab' fragments (**Fig. 2C**, dashed lines). Small
292 differential effects of each tested FH mutant is observed. Without potentiation, WT FH, W1183L and
293 R1210C show the highest activity in the DAA assay followed by, V1197A and G1194D, as determined

294 by the final decrease in response. All tested FH mutants are similarly enhanced by the addition of
295 anti-FH.07.1.

296 **The regulatory properties of most FH mutants can be enhanced by the potentiating anti-FH on**
297 **cellular surfaces**

298 In the SPR-based C3b binding and DAA assays, only small differences were detected comparing each
299 of the four tested FH mutants with WT FH, so the potential to potentiate these mutants with anti-
300 FH.07.1 on human cell surfaces was investigated. A cellular assay employing human HAP-1 cells was
301 utilized in which all the membrane bound complement regulatory proteins (CD46, CD55 and CD59;
302 these cells are naturally deficient of complement receptor 1) were knocked out (21). These cells are
303 highly susceptible to complement activation when incubated with NPS, as shown by the high amount
304 of C3 deposition (**Fig. 3a and Supplemental Fig. 3**). In this assay, addition of pdFH to the NPS
305 decreases the C3 deposition, showing extra regulation from the added pdFH (**Fig. 3A and 3B**).
306 Addition of the potentiating anti-FH to the NPS enhances the regulatory function of the present FH
307 and as a consequence also decreases C3 deposition. These results indicate that HAP-1 cells deficient
308 of all membrane bound complement regulatory proteins are highly dependent on FH binding to
309 regulate complement deposition on their surface. The effects of the mutant FH proteins were
310 investigated in this model both in isolation and in combination with the potentiating anti-FH. **Fig. 3C**
311 **and 3D** shows that addition of the WT FH improved the regulatory balance on the deficient HAP-1
312 cells. None of the tested mutant FH variants was able to do so. Only a mild non-significant decrease
313 in C3 deposition is observed compared to serum alone with addition of mutants V1197A, R1210C and
314 G1194D (**Fig. 3C and 3D**, solid lines). Addition of anti-FH.07.1, which will bind to both serum FH from
315 the NPS and the added recombinant FH, significantly increased regulation in all conditions (**Fig. 3C**
316 **and 3D**, dashed lines).

317 To be able to fully distinguish the capacity of our antibodies to potentiate both WT as well as mutant
318 FH, we further investigated complement activation on our HAP-1 cells deficient of complement
319 regulators using FH depleted serum. We are unable to detect C3b deposition on the deficient HAP-1
320 cells when using FH depleted serum alone (**Fig. 3E and 3F**). This is consistent with data of others (12,
321 24, 25) and probably due to complete consumption of C3 in the FH depleted serum. Addition of
322 physiological pdFH concentrations partly restores the C3 deposition to NPS control situation (**Fig. 3F**)
323 as the fluid phase C3 conversion is also controlled. Addition of all FH mutants restores C3b deposition
324 in this assay (**Fig. 3G**), indicating that all the tested mutant FH proteins restore the fluid phase
325 regulation. **Fig. 3G** shows that FH V1197A, R1210C and G1194D restore C3b deposition equally well
326 as pdFH, while addition of FH W1183L results in more C3b deposition. Addition of recombinant WT
327 FH results in undetectable C3b deposition. We expect that addition of recombinant WT FH fully

328 regulates fluid-phase C3 tick-over and inhibits complement deposition on the surface at the same
329 time as we (**Supplemental Fig. 4**) and others (23) have observed that recombinant FH results in FH
330 with better regulatory capacities than pdFH.

331 Addition of anti-FH.07.1, now acting only on the recombinant FH as no endogenous FH is present,
332 shows that regulation by all mutant FH molecules, except FH W1183L, are enhanced as shown by
333 decreased C3b deposition on the cell surface compared to the mutants alone (**Fig. 3G**, dashed lines).
334 To conclude, V1197A, R1210C and G1194D are able to regulate C3 deposition on complement
335 regulator deficient HAP-1 cells and are potentiated, by addition of anti-FH.07.1.

336 **C3b deposition is differentially controlled by all FH variants**

337 To distinguish between the effects on fluid-phase and membrane regulation, we next investigated
338 the impact of FH mutants on C3 regulation in serum, by assessing C3b deposition on LPS coated
339 microtiter plates. To be able to solely asses the effect of our potentiating anti-FH on recombinant FH
340 and not endogenous FH, this assay was performed using FH depleted serum. Similar to **Fig. 3E and**
341 **3F**, without FH in this assay we observe no C3 deposition because of low C3 levels caused by the
342 natural consumption of C3 due to the tick-over of C3 into C3(H₂O) (12, 25). Addition of all four tested
343 FH mutants in increasing concentrations to the FH depleted serum first restores fluid phase
344 complement regulation, resulting in measurable C3b deposition on LPS coated plates for all proteins
345 up to ≤ 20 µg/mL supplemented FH (**Fig. 4A**). This is followed by surface regulation upon further
346 increasing FH concentration, resulting in a decreased C3b deposition.

347 Enhanced fluid phase regulation by any of the tested FH mutants was not observed in the presence
348 of anti-FH07.1. However, there was a positive effect on surface regulation with the addition of anti-
349 FH.07.1. Less FH is needed to restore the surface regulation of the C3b deposition, and thus the
350 antibody enhances the FHs' function (**Fig. 4A**, dashed lines). To conclude, V1197A, R1210C and
351 G1194D are able to regulate C3 deposition on LPS in FH depleted serum alone and are potentiated,
352 albeit differentially, by addition of the potentiating anti-FH.

353 **FH differentially regulate hemolysis in sheep erythrocyte hemolytic assay**

354 To investigate if the FH mutations affect surface regulation of C3 deposition on cells similar as shown
355 in an ELISA based assay, we employed the sheep erythrocyte (SE) hemolytic assay (26) with minor
356 modifications to use FH depleted serum. Similar to our ELISA based assay, supplementation of
357 recombinant WT FH restores complement regulation of FH depleted serum as shown by lysis of the
358 SE. With addition of higher amounts of FH no more lysis is observed (**Fig. 4B**), indicating full
359 protection. In this assay, the WT FH was most potent, with lowest amount of FH needed to restore
360 protection. Compared to WT FH, the four mutant FHs required a higher FH concentration to inhibit

361 lysis, indicating that the FH mutants are less potent in restoring complement regulation on the cell
362 surface. Interestingly, anti-FH.07.1 increased the regulatory activity of all FH variants except W1183L,
363 which was not able to regulate lysis even with addition of anti-FH.07.1. We only observe a reduction
364 in lysis upon the highest, non-physiological, concentration. Overall, our data shows that anti-FH.07.1
365 is able to enhance the regulatory capacity of aHUS associated FH mutants V1197A, R1210C and
366 G1194D making this antibody an interesting drug candidate.

367

368 **Discussion**

369 We had shown previously that our potentiating anti-FH antibody, anti-FH.07 was able to restore the
370 FH activity in serum of aHUS patients (18). We currently investigated the effect of the anti-FH
371 agonistic antibody, anti-FH.07.1 on four aHUS associated mutant variants of FH. However, in aHUS,
372 patients are often heterozygous for FH mutations and it was unknown whether the antibody
373 potentiated mutated FH in addition to WT FH. We now clarified that for three of the four tested
374 mutants the anti-FH.07.1 monoclonal antibody increases the functionality of FH. This indicates that in
375 aHUS patients carrying these select FH mutants, both the WT and the mutated FH protein will be
376 potentiated.

377 Using SPR we confirmed that anti-FH.07.1 was binding to the recombinant FHs with comparable
378 affinities. The affinities were around ten times higher than pdFH binding (data not shown). This
379 increased affinity could be caused by the HIS-tag on the FH, which is known to cause artefactual
380 enhanced binding on CM5 sensors, as used in this SPR setup (27). In addition, the tested FH mutants
381 showed slight decrease in affinity for C3b compared to WT FH. Previous studies have shown
382 conflicting results towards the effect of studied mutation on binding to C3b. Reports have shown
383 variable effects of the W1183L, R1210C and V1197A mutations resulting in reduced C3b binding.
384 These studies include the use of patient derived material, or recombinant constructs comprising CCPs
385 8-20 (FH8-20) or CCPs 18-20 (FH18-20) and the use of various assays (26, 28-31). Mutant G1194D has
386 not been functionally studied before. The recombinant full length FH mutants used in this study are
387 different than those used in previous studies and as a result, we cannot directly compare affinities.
388 However, it seems that the involvement of CCPs 1-4, which contain an important C3 binding region
389 (7, 11), in our full length FH dampens the effect on C3b binding as we see only a slight reduction in
390 affinity (< 2-fold) between WT and four tested mutants which is in contrast to studies using FH8-20 or
391 FH18-20 which see \leq 60% reduction in affinity compared to the WT fragment (28-31). However, this
392 does not explain the discrepancy between the results of Sanchez-Corral et. al and this study.
393 Sanchez-Corral has isolated full length FH from healthy volunteers or patient material containing
394 mutations W1183L, V1197A or R1210C (31). This studies indicates a ~4-fold reduction in affinity
395 towards C3b while we observe only moderate reduction. As described before, our recombinant
396 proteins contain a HIS-tag which might have influenced our SPR based assay (27). However, as our
397 WT FH also contains this HIS tag it might also be the sample preparation or other causes that affect
398 the functionality of these mutants.

399 The addition of anti-FH.07.1 increases the affinity of all mutants for C3b with affinities that approach
400 the affinity of WT FH for C3b. Anti-FH.07.1, which binds to CCP-18 (18), might alter the C3b binding

401 interface of the C-terminal domains in such a way that CCP20 binding to the surface is less important.
402 Possibly, the antibody affects the proposed “closed” or “folded” state of native FH (32, 33), allowing
403 better binding to C3b. More research into the effect of our antibody on FH’s binding interaction with
404 C3b and potential conformation changes are still needed to fully understand the mechanisms of
405 action. Using the same C3b coupled SPR chip we observed very similar capacity for DAA of all FH
406 mutants. As we have shown similar C3b affinity between mutants in our SPR based setup and co-
407 factor activity relies on CCP domain 1-4 (7, 11) this data is in line with expectations. All four tested FH
408 mutants are equally enhanced and only very small effects of the mutations are noticed.

409 The effects of the mutations are more pronounced in the experiments involving serum. Studies show
410 that aHUS patients carrying the specific mutations used in this study (W1183L, R1210C, V1197A) do
411 not present with consumption of complement (hypocomplementemia) (26, 30, 31). As mentioned
412 above, it is known that CCPs 1-4 of factor H are responsible for co-factor activity and fluid phase
413 regulation (7) and these domains are not affected in the these FH mutants (26). Our study shows
414 similar results, all our mutants are able to control fluid phase regulation. However, especially
415 W1183L seems to be able to control of fluid phase regulation better than other FH mutants and even
416 WT FH.

417 The results of complement regulation on the surface using an ELISA format were in agreement with
418 the results of the hemolytic assay. Mutant W1183L was only able to regulate some C3 deposition
419 upon FH potentiation, when added in supra-physiological concentration. R1210C performed in these
420 assays equally well as WT FH did, while this mutant is known to have reduced affinity for C3b and
421 cellular surfaces (28).The results were unexpected, because this mutation has been repeatedly found
422 in aHUS, C3G, as well as AMD patients (34). An explanation could be that in patient material it was
423 found that the introduced cysteine residue forms disulfide bond between FH and albumin, forming a
424 210 KDa band in serum isolated FH (31). This phenotype can explain the cause of disease in patients,
425 as the FH-HSA is functionally impaired and affects the ability of the patient serum to protect against
426 SE lysis (31), and similar protein interactions with other proteins have not been identified to occur
427 during the production of the recombinant mutant protein.

428 For all mutants, except G1194D, it has been shown that CCP8-20 fragments are affected in their
429 capacity to bind to heparin or human umbilical vein endothelial cells (HUVEC) (28, 30), as well as the
430 reduced ability of patient serum or recombinant FH fragments to respectively protect against or
431 inhibit SE lysis (31, 35). This is in line with our own observations, except for R1210C, which performed
432 equal to WT FH in our hemolytic assay. W1183L seems much less potent in controlling complement
433 regulation on surfaces compared to the other mutants. Considering the function of studied

434 mutations, other studies are not always in agreement. While one structural study of the FH18-20
435 fragment shows that the W1183 domain in CCP20 is directly involved in C3b/d binding (29), other
436 studies demonstrate the close involvement of W1183 in binding to sialic acid (36). Mutations
437 R1210C, V1197A and G1194D result in destabilization of the tertiary structure (29), while V1197 is
438 also shown to be important for binding to sialic acid (36). However, it is also noted that other ligands
439 might have differential interactions in this binding region, such as heparin sulphates compared to
440 sialic acid (36, 37) and is explained by the various effector functions of FH on different cell types,
441 such as endothelial cells, glomerular membrane, retinal pigment epithelium, Bruch's membrane in
442 the eye, platelets or erythrocytes (24, 37, 38). A dual interaction of FH to C3b and sialic acid residues
443 is crucial for protection of cells against unwanted complement activation on human cell surfaces (38).
444 W1183 is involved in both processes and it has been shown that in competition assays, the patient-
445 associated W1183L aHUS mutation mostly affects the ability to compete with WT FH (38, 39). In
446 these studies, Hyvärinen et al. show that removal of sialic acid from HUVECs and platelets showed
447 minimal effect on the competition of this FH mutant FH18-20 compared to WT FH18-20 with WT FH,
448 showing that the sialic acid was crucial for this interaction (38).

449 In conclusion, we have shown that four select recombinantly expressed aHUS associated FH mutant
450 proteins can be enhanced in their binding to C3b and decay accelerating activity by our potentiating
451 antibody. In addition, this anti-FH antibody can potentiate three of the four FH mutants in their
452 regulatory function on the cellular surface. Taken together, this research confirms that our antibody
453 can potentially be used to enhance the FH of aHUS patients who carry these mutations.

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457

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573

574 **Figure legends**

575 **Figure 1: Affinity for C3b of mutant FHs is conserved and enhanced by potentiating antibody**

576 The affinity of FH for C3b was measured using SPR. Measurements were performed on the Biacore
577 T200 with CM5 chip coupled with ~2000 RU C3b. (A) Sensorgrams of in house purified plasma
578 derived FH (pdFH) binding to C3b. pdFH was titrated from 10 or 5 μ M respectively, in 2 fold dilutions
579 and flown over the chip without (left) or with (right) the presence of an excess (10 μ M, at least 2 fold
580 based on molar concentration) of anti-FH.07.1 Fab' fragments. Sensorgrams were corrected for
581 molecule size (155 KDa FH alone, 205 KDa FH + potentiating Fab' fragment), and show an increased
582 response upon addition of the anti-FH.07.1 Fab' fragment. (B) Affinity curves, based on average
583 response at equilibrium binding (Δ T 50-55 sec) in Fig. A, show an increase in binding upon addition of
584 anti-FH.07.1 as presented by the estimated affinity K_D of 6.0 and 1.9 μ M for pdFH alone or with
585 addition of the anti-FH.07.1 Fab' fragments respectively. (C) Affinity curves based on 2-fold titrations
586 (625 – 39.0625 nM) of recombinant WT and mutant FH, corrected for molecule size, as described
587 above, showing an increased response upon addition the anti-FH.07.1 Fab' fragment (1.25 μ M,
588 dashed lines). Experiments are performed in two sets due to instrumental limitations. Figures are
589 representative of n=2.

590 **Figure 2: DAA of mutant FHs is enhanced by potentiating anti-FH**

591 (A) In a SPR based setup FB and FD were flown over a sensor chip which was amine-coupled with
592 ~2000RU C3b to form C3bBb (convertase) complexes (phase I). Subsequently, a decline in signal
593 indicated natural decay of the convertase as Bb is released from the coupled C3b (phase II). Injection
594 of pdFH (50nM) causes accelerated decay (grey, solid line), as observed by a sudden further drop in
595 response (phase III). The addition of the anti-FH.07.1 Fab' fragment (200nM) to pdFH increases DAA
596 of pdFH (grey, dashed line), whilst the Fab' fragment alone does not affect the natural decay (black,
597 dashed line). (B) With similar setup as described above, addition of anti-FH.07.1, FH blocking (anti-
598 FH.09, (18)) or binding (anti-FH.16, (18)) anti-FH Fab' fragments respectively increase, decrease or do
599 not affect DAA of pdFH. (C) Injection of recombinant WT or FH mutants (12.5 nM) shows DAA.
600 Addition of the anti-FH.07.1 Fab' fragment (100nM) increases the DAA of all FHs. Enlargement of the
601 DAA segment of SPR shows slight differences in DAA are observed between FH mutants. Addition of
602 the anti-FH.07.1 Fab' fragment (dashed lines) improves the DAA of all FH. Figures are average of
603 duplicate runs and representative of n=8 (A), n=1 (B) or n=3 (C).

604 **Figure 3: Deficient HAP-1 cells are protected by additional FH**

605 (A) Flow cytometry analysis shows that HAP-1 cells deficient of all membrane bound complement
606 regulators are sensitive to complement activation from NPS (25%) as shown by C3b deposition

607 (black, solid line). Addition of the anti-FH.07.1 (75 µg/mL, grey line) or additional of pdFH (32.5
608 µg/mL, black, dashed line) shows control of C3 deposition. Histograms are a representative of n=4.
609 (B) Normalized average MFI of conditions shown in Fig. A (n=4). (C) Addition of the recombinant WT
610 or FH mutants (32.5 µg/mL, solid lines) show an increase in complement regulation on the HAP-1
611 cells deficient of membrane bound regulators as noted by a decreased C3 deposition compared to
612 serum alone (grey, filled) and is further enhanced by the addition the anti-FH.07.1 (75 µg/mL, dashed
613 lines). Histograms are a representative of n=4. (D) Normalized average MFI of conditions shown in
614 Fig. C (n=4). (E) The addition of FH depleted serum (25%, black line) to deficient HAP-1 cells does not
615 lead to C3b deposition as C3 is consumed in fluid phase in this serum before it can be deposited on
616 the cells (25). Addition of pdFH (grey, dashed line) protects the fluid phase C3 consumption and thus
617 renders the cells sensitive for complement activation as shown by increased C3b deposition.
618 Histograms are a representative of n=3. (F) Normalized average MFI of conditions shown in Fig. E
619 (n=3). (G) Addition of the recombinant WT or FH mutants (75 µg/mL, solid lines) to the FH depleted
620 serum (25%) shows an increase in C3 deposition on the deficient HAP-1 compared to FH depleted
621 serum alone (grey, filled), following the restoration of the regulation of fluid phase C3 consumption.
622 Addition of the anti-FH.07.1 (75 µg/mL, dashed lines) next shows a decrease in C3 deposition
623 compared to FH depleted serum and FH alone in flow cytometry. Histograms are a representative of
624 n=3. Error bars represent standard deviation, * p < 0.1, ** p < 0.01, *** p < 0.001, analyzed by one-
625 way ANOVA and Tukey's multiple comparisons test.

626 **Figure 4: C3b deposition and hemolysis are differentially controlled by FH mutants**

627 (A) LPS induced C3b deposition using FH depleted serum supplemented with either WT or FH
628 mutants (solid lines) shows a concentration dependent increase in C3 deposition, indicating fluid
629 phase regulation, followed by a decrease in C3b deposition, indicating surface regulation. Addition of
630 the anti-FH.07.1 antibody (dashed lines) increases the regulatory activity of the most of the FH
631 proteins. (B) Complement mediated lysis of SE incubated with FH depleted serum supplemented with
632 either WT or FH mutants. Lysis is reduced by most of the FH proteins (solid lines). Addition of the
633 anti-FH.07.1 antibody (dashed lines) improves the regulatory capacity of most of the FH proteins. The
634 inhibition was fitted using a nonlinear fit, insufficient fit is not shown, instead the measured points
635 are shown. Error bars represent standard deviation of experiments performed in duplicate and
636 figures are representative of n=3.

637 **Supplemental Figure 1: Recombinantly produced FHs are pure**

638 Expression and isolation of FH resulted in pure, full-length FH. No contamination or breakdown
639 products are detected in the recombinant FH fractions as shown by SDS page with coomassie blue
640 staining.

641 **Supplemental Figure 2: The anti-FH.07.1 is comparable to anti-FH.07**

642 (A) Anti-FH.07 (18) and anti.FH.07.1 (potentiating anti-FH, this research) share the same binding
643 epitope on FH, as shown by competition ELISA. Either anti-FH.07, anti-FH.07.1, or a non-competing
644 anti-FH (anti-FH.16, (18)) is coated as a capture of biotinylated FH. All abovementioned anti-FH
645 antibodies are also included as a competitor for binding of biotinylated FH, plus a non-competing
646 control isotype control (IgG control) (B) The anti-FH.07.1 is comparable in function to anti-FH.07 as
647 shown by the inhibition of LPS activated C3b deposition in NPS. Error bars represent standard
648 deviation of experiments performed in duplicate and figures are representative of n=2 and n=4 for
649 respectively Fig. A and Fig B.

650 **Supplemental Figure 3: Gating strategy to determine level of C3b deposition on deficient HAP-1
651 cells**

652 C3 deposition on HAP-1 cells deficient of all membrane bound complement regulators (Hap-1 KO
653 CD46/CD55/CD59, (21)) incubated without (top panel) or with NPS (25%, bottom panel) was
654 analyzed by flow cytometry using FITC or APC labeled anti-C3d (C3-19). Gating strategy was as
655 followed: Deficient HAP-1 cells were gated based on size and granularity using FSC-A vs SSC-A to
656 eliminate debris and clumped cells (plot 1). Single cells were sub-gated using SSC-H and SSC-W (plot
657 2) and subsequent FSC-H and FSC-W (plot 3), Continuity of measurement was checked by plotting the
658 acquisition time (x-axis) to the APC-A signal, “time-gate” (plot 4), Cells positive for C3b were
659 visualized using normalized histograms of either FITC-A or APC-A, “C3b deposition” (plot 5). SSC-A:
660 side scatter area, FSC-A: forward scatter area, FSC-H: forward scatter height, FSC-W: forward scatter
661 width, SSC-H: side scatter height, SSC-W: side scatter width.

662 **Supplemental Figure 4: Recombinant WT FH has improved protective function in hemolytic assay
663 compared to pdFH**

664 Recombinant WT FH has a higher regulatory potential as shown by complement mediated lysis of SE
665 incubated with FH depleted serum supplemented with either pdFH or recombinant WT FH. Lysis is
666 reduced by addition of FH, less recombinant WT FH is needed to reach inhibit lysis compared to
667 pdFH. The inhibition was fitted using a nonlinear fit. Error bars represent standard deviation,
668 experiments were performed in duplicate and figures are representative of n=3.

669 **Tables**

670 **Table I: Estimated affinity of recombinant FH for C3b with and without addition of anti-FH.07.1**

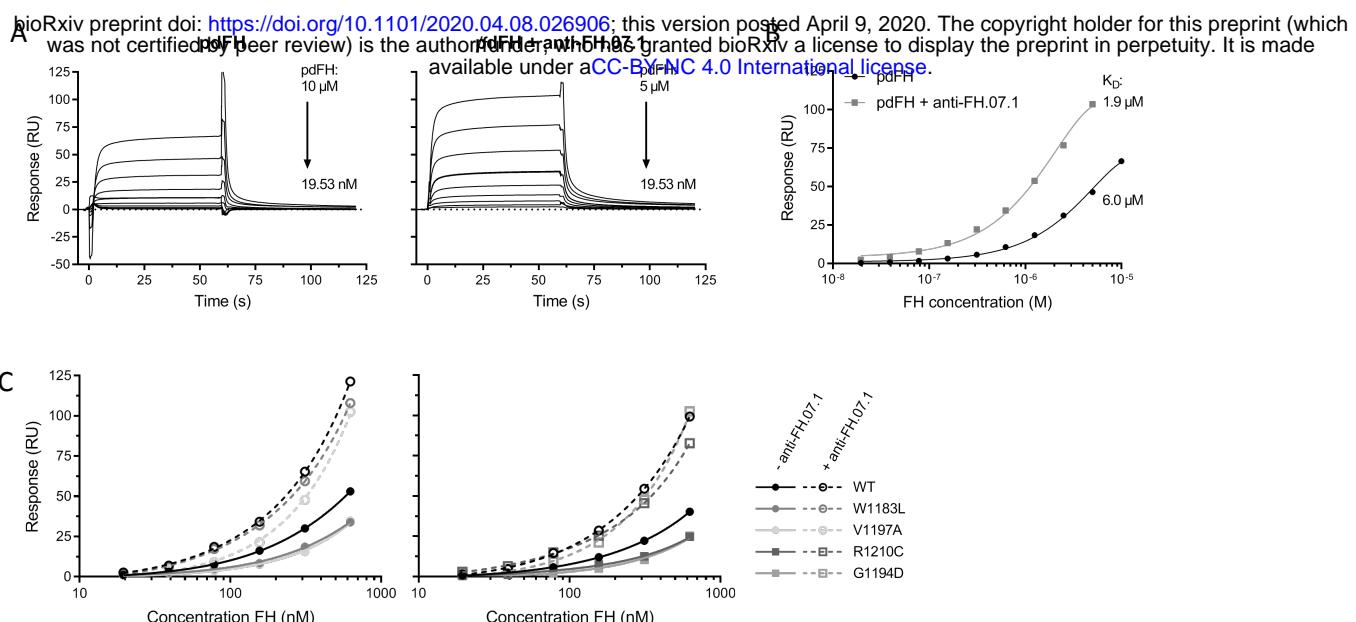
671 **Fab' fragments**

FH variant	$K_D (M^{-1})$, FH only *	$K_D (M^{-1})$, FH + pot anti-FH Fab'	Fold change**	n
WT FH	$3.0 \times 10^{-6} \pm 2.0$	$1.7 \times 10^{-6} \pm 1.2$	1.8 ± 0.1	4
W1183L	$4.6 \times 10^{-6} \pm 2.5$	$2.1 \times 10^{-6} \pm 1.1$	2.3 ± 0.0	2
V1197A	$5.0 \times 10^{-6} \pm 2.9$	$2.2 \times 10^{-6} \pm 1.1$	2.2 ± 0.2	2
R1210C	$4.9 \times 10^{-6} \pm 3.0$	$2.1 \times 10^{-6} \pm 1.4$	2.4 ± 0.1	2
G1194D	$4.7 \times 10^{-6} \pm 2.1$	$2.4 \times 10^{-6} \pm 1.4$	2.1 ± 0.3	2

672 * affinities are estimates as experimental setup could not reach the required higher concentrations

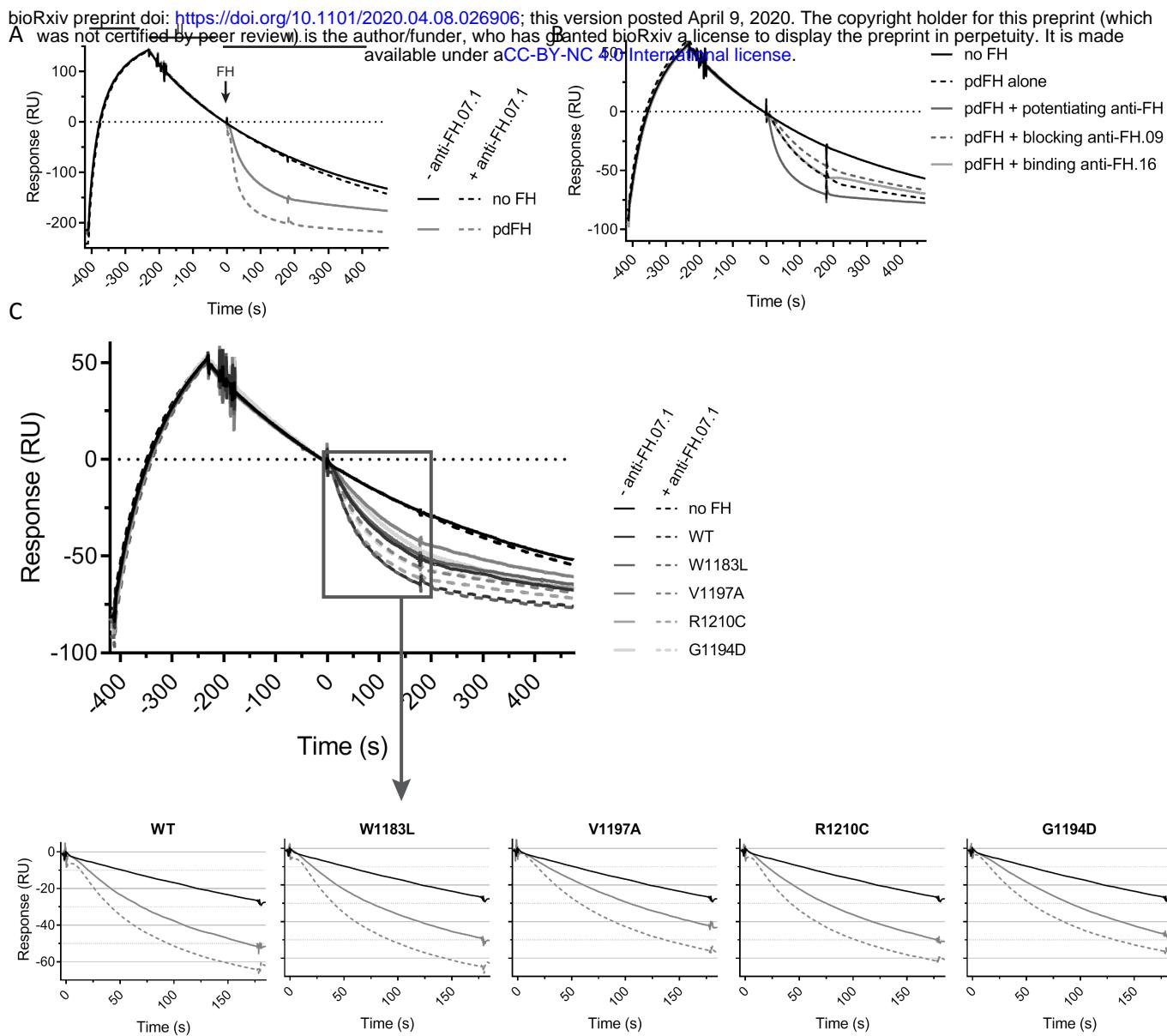
673 ** improved affinity of FH + anti-FH.07.1 Fab' fragments over FH alone

Figure 1

**Figure 1: Affinity for C3b of mutant FHs is conserved and enhanced by potentiating antibody**

The affinity of FH for C3b was measured using SPR. Measurements were performed on the Biacore T200 with CM5 chip coupled with ~2000 RU C3b. (A) Sensorgrams of in house purified plasma derived FH (pdFH) binding to C3b. pdFH was titrated from 10 or 5 μ M respectively, in 2 fold dilutions and flown over the chip without (left) or with (right) the presence of an excess (10 μ M, at least 2 fold based on molar concentration) of anti-FH.07.1 Fab' fragments. Sensorgrams were corrected for molecule size (155 KDa FH alone, 205 KDa FH + potentiating Fab' fragment), and show an increased response upon addition of the anti-FH.07.1 Fab' fragment. (B) Affinity curves, based on average response at equilibrium binding (ΔT 50-55 sec) in Fig. A, show an increase in binding upon addition of anti-FH.07.1 as presented by the estimated affinity K_D of 6.0 and 1.9 μ M for pdFH alone or with addition of the anti-FH.07.1 Fab' fragments respectively. (C) Affinity curves based on 2-fold titrations (625 – 39.0625 nM) of recombinant WT and mutant FH, corrected for molecule size, as described above, showing an increased response upon addition the anti-FH.07.1 Fab' fragment (1.25 μ M, dashed lines). Experiments are performed in two sets due to instrumental limitations. Figures are representative of n=2.

Figure 2

**Figure 2: DAA of mutant FHs is enhanced by potentiating anti-FH**

(A) In a SPR based setup FB and FD were flown over a sensor chip which was amine-coupled with ~2000RU C3b to form C3bBb (convertase) complexes (phase I). Subsequently, a decline in signal indicated natural decay of the convertase as Bb is released from the coupled C3b (phase II). Injection of pdFH (50nM) causes accelerated decay (grey, solid line), as observed by a sudden further drop in response (phase III). The addition of the anti-FH.07.1 Fab' fragment (200nM) to pdFH increases DAA of pdFH (grey, dashed line), whilst the Fab' fragment alone does not affect the natural decay (black, dashed line). (B) With similar setup as described above, addition of anti-FH.07.1, FH blocking (anti-FH.09, (18)) or binding (anti-FH.16, (18)) anti-FH Fab' fragments respectively increase, decrease or do not affect DAA of pdFH. (C) Injection of recombinant WT or FH mutants (12.5 nM) shows DAA. Addition of the anti-FH.07.1 Fab' fragment (100nM) increases the DAA of all FHs. Enlargement of the DAA segment of SPR shows slight differences in DAA are observed between FH mutants. Addition of the anti-FH.07.1 Fab' fragment (dashed lines) improves the DAA of all FH. Figures are average of duplicate runs and representative of n=8 (A), n=1 (B) or n=3 (C).

Figure 3

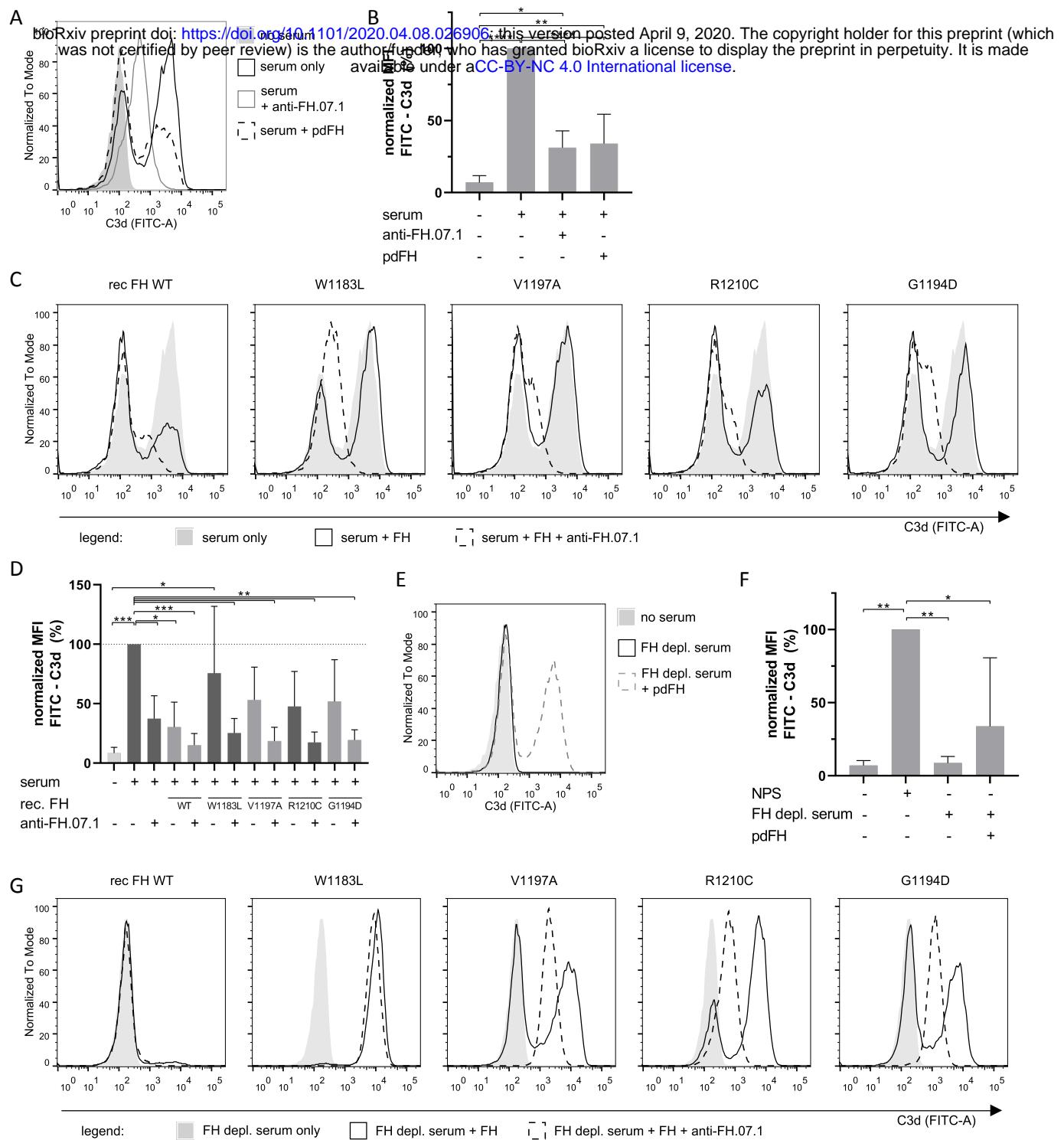


Figure 3. Deficient HAP-1 cells are protected by fluid phase FH.

(A) Flow cytometry analysis shows that HAP-1 cells deficient of all membrane bound complement regulators are sensitive to complement activation from NPS (25%) as shown by C3b deposition (black, solid line). Addition of the anti-FH.07.1 (75 μ g/mL, grey line) or additional of pdFH (32.5 μ g/mL, black, dashed line) shows control of C3 deposition. Histograms are a representative of n=4. (B) Normalized average MFI of conditions shown in Fig. A (n=4). (C) Addition of the recombinant WT or FH mutants (32.5 μ g/mL, solid lines) show an increase in complement regulation on the HAP-1 cells deficient of membrane bound regulators as noted by a decreased C3 deposition compared to serum alone (grey, filled) and is further enhanced by the addition the anti-FH.07.1 (75 μ g/mL, dashed lines). Histograms are a representative of n=4. (D) Normalized average MFI of conditions shown in Fig. C (n=4). (E) The addition of FH depleted serum (25%, black line) to deficient HAP-1 cells does not lead to C3b deposition as C3 is consumed in fluid phase in this serum before it can be deposited on the cells (25). Addition of pdFH (grey, dashed line) protects the fluid phase C3 consumption and thus renders the cells sensitive for complement activation as shown by increased C3b deposition. Histograms are a representative of n=3. (F) Normalized average MFI of conditions shown in Fig. E (n=3). (G) Addition of the recombinant WT or FH mutants (75 μ g/mL, solid lines) to the FH depleted serum (25%) shows an increase in C3 deposition on the deficient HAP-1 compared to FH depleted serum alone (grey, filled), following the restoration of the regulation of fluid phase C3 consumption. Addition of the anti-FH.07.1 (75 μ g/mL, dashed lines) next shows a decrease in C3 deposition compared to FH depleted serum and FH alone in flow cytometry. Histograms are a representative of n=3. Error bars represent standard deviation, * p < 0.1, ** p < 0.01, *** p < 0.001, analyzed by one-way ANOVA and Tukey's multiple comparisons test.

Figure 4

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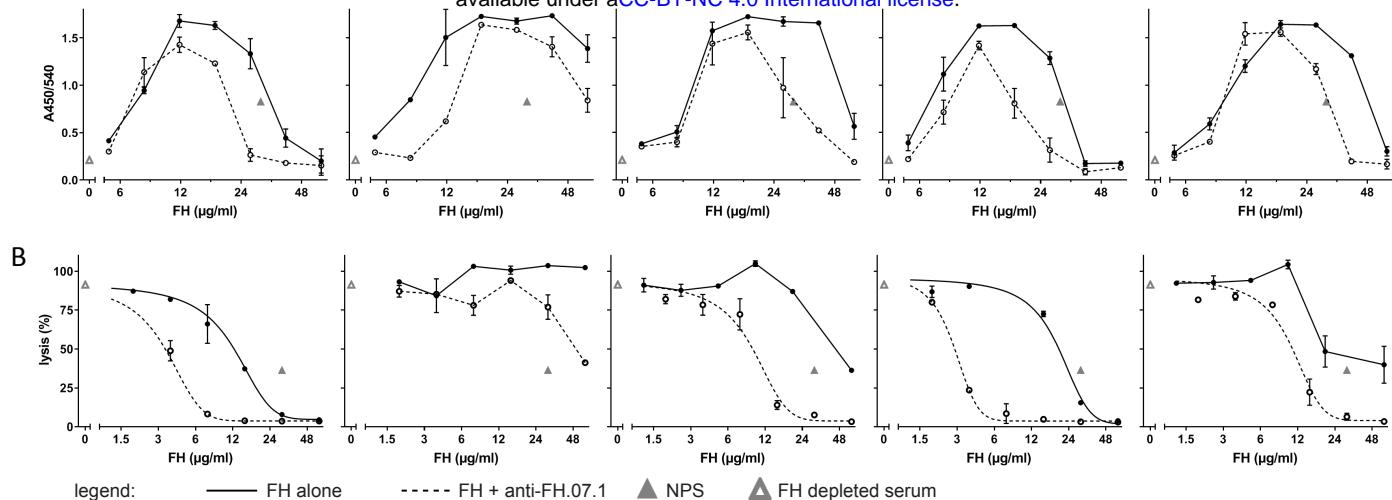


Figure 4: C3b deposition and hemolysis are differentially controlled by FH mutants

(A) LPS induced C3b deposition using FH depleted serum supplemented with either WT or FH mutants (solid lines) shows a concentration dependent increase in C3 deposition, indicating fluid phase regulation, followed by a decrease in C3b deposition, indicating surface regulation. Addition of the anti-FH.07.1 antibody (dashed lines) increases the regulatory activity of the most of the FH proteins. (B) Complement mediated lysis of SE incubated with FH depleted serum supplemented with either WT or FH mutants. Lysis is reduced by most of the FH proteins (solid lines). Addition of the anti-FH.07.1 antibody (dashed lines) improves the regulatory capacity of most of the FH proteins. The inhibition was fitted using a nonlinear fit, insufficient fit is not shown, instead the measured points are shown. Error bars represent standard deviation of experiments performed in duplicate and figures are representative of n=3.