Complement factor B mutations in atypical hemolytic uremic syndrome – disease-relevant or benign?

Supplemental Materials and Methods

In silico analysis

The crystal structures of FB, the C3 convertase C3bBb and the C3bBD triple complex are available in Protein Data Bank (PDB ID 2OK5; 2WIN and 2XWB). The residues affected by the mutations were visualized using PyMol and Chimera softwares. If a FB residue was in a distance of 1 Å or less from a residue from the partner molecule, it was considered to belong to the binding site. Protein numbering throughout this study is according to the sequence of the mature protein, without signal peptide. Wherever appropriate, the numbering according to the protein with leader peptide (starting p., having additional 25 residues) will be given for reference.

Recombinant FB production

Site-directed mutagenesis

The mutations were introduced in a FB gene containing plasmid by site-directed mutagenesis using the QuikChange® II XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions. *E. coli XL-Gold* bacteria were transformed in order to amplify the quantity of plasmid, extracted thanks to a midi-prep technique (Qiagen). The constructs were completely sequenced to confirm that no additional mutations had been introduced. The transient expression of recombinant FB proteins was conducted in transitory transfected HEK-293T cells cultured 3 days in DMEM+glutaMAX^{TM}-I 4,5g/l D-Glucose +

Pyruvate medium (Gibco, Paisley, GB) without foetal calf serum. A production test was performed also in CHO-K1 cells.

Recombinant FB characterization

The integrity of recombinant wild type (WT) and mutants FB was tested by Western blot (ready made gels and rapid transfer system iBlot by Invitrogen). The proteins in the culture supernatants were separated by SDS-PAGE, transferred to a nitrocellulose membrane and probed with in house biotinylated polyclonal sheep anti-human FB antibody (Abcam), followed by Streptavidin-HRP (Amersham) and ECL (GE Healthcare).

The FB content was assessed by sandwich ELISA, using immobilized polyclonal sheep anti-human FB antibody (Abcam) for capturing and biotinylated sheep anti-human FB, followed by streptavidin-Horseradish Peroxydase (HRP) (Dako) for detection, as described previously [9]. Plasma derived FB (Comptech, Tylor, TX) served as a standard.

C3b binding characterization

Enzyme-linked immunosorbent assay (ELISA)

Microtiter wells were coated with purified human C3b at 10 µg/ml in PBS (pH 7,4), for 1 hour at 37 °C. The residual binding sites were blocked by addition of BSA at 2% in PBS, during 1 hour at 37°C, and washed three times with Hepes buffer (10mM Hepes pH 7.4, 25mM NaCl, 10mM $MgCl_2$ and 0,05% Tween 20). Then, serial dilutions of the supernatants containing the recombinant FB were made in the same Hepes buffer containing 4% BSA and incubated in the plate during 1 hour at 37°C. Three washings were done with the same buffer and then the wells were incubated with a biotinylated anti-FB antibody (dilution 1:1000) for 1 hour at 37°C. After three more washings, the wells were incubated 1 hour with Streptavidin-HRP (dilution 1:3000) at 37°C. Finally, the interactions were revealed using TMB substrate

and the reaction was stopped by 2M H_2SO_4. The OD_{450nm} was measured.

Surface plasmon resonance (SPR)

The interaction of wild type and mutant FB with C3 was analyzed using surface plasmon resonance technology with ProteOn XPR36 equipment (BioRad). C3b was coupled to the GLC biosensor chip, using standard amide-coupling technology, according to the manufacturer's instructions. The recombinant wild type and mutant FB were used as an analyte at concentrations 650, 325, 162.5, 81.25 and 40.625 pM and were injected at 50 µl/min in Mg^{2+}- containing HEPES buffer (10mM Hepes pH 7.4, 50mM NaCl, 10mM $MgCl_2$,) over the C3b containing surfaces and an empty, activated/deactivated flowcell as a control. Data were analyzed using ProteOn Manager software and the data from the blank flowcell were subtracted. Kinetic parameters were calculated by fitting the obtained sensorgrams into two state interaction model.

FB functional assays

FB hemolytic activity

Briefly, samples made from 100µl $1x10^8$ C3b-covered sheep erythrocytes/ml, 40ng of purified human factor D (Comptech) and recombinant Factor B proteins (serial dilutions starting from 10 µg/ml, recombinant WT or mutant forms) in DGVB containing $CaCl_2$ and $MgCl_2$were incubated at 30°C for 30 min. The negative control did not contain factor B. The alternative pathway C3 convertases sites were developed with 300 µl of a 1/40 dilution of rat serum in GVB-EDTA buffer at 37°C for 45 min. Hemolysis was detected at OD 414nm. Hemolytic activity levels for the factor B mutants were expressed as Z values, which is equivalent to the average number of lytic sites per cell.

C3-convertase (C3bBb-Mg^{2+}) formation assay based on ELISA/western blot

One mutation per domain – S141P for the CCP domains, I217L for the linker, P344L for the vWF domain and K508R for the SP domain were selected for assessment of the convertase formation by a different assay.

The assay was performed as reported. Microtiter wells were coated with 3 ug/mL C3b (ComplTech) in PBS by overnight incubation at 4°C, blocked with 1% BSA for 1h at 37°C, and washed with a phosphate buffer supplemented with 10mM $MgCl_2$. C3bBb complexes were formed by incubating C3b coated wells for 12 min at 25°C with FB (ComplTech; 1000 ng/mL) and FD (ComplTech; 5 ng/mL) both diluted in a phosphate buffer supplemented with 0.5% BSA and 10mM $MgCl_2$. After wash, the protein complexes were detached from microtiter wells with EDTA 10 mM and SDS 1%, subjected to 10% SDS-PAGE, and transferred by electroblotting to PVDF membrane (Amersham). Proteins were detected with rabbit anti-human CFB antibody (Atlas; 1:500) followed by HRP anti-rabbit antibody (Vector Lab.; 1:30000) and the ECL system (Amersham). C3 convertase formation was evaluated by the visualization of the Bb band (60 KDa) and the intensity of the band was quantified by ImageJ. Sample with WT FB was used as positive control and results expressed relative to it: % of C3bBb formation = 100 X (Bb band with variant FB sample – background)/(Bb band with WT FB sample – background).

Dissociation of the C3 convertase by FH

C3bBbconvertases were assembled on sheep erythocytes using recombinant WT or mutant factor B ($1<Z<2$) as above. After the 30 min incubation of C3b-coated cell samples with factor B and factor D, 100 µl of GVB-EDTA buffer-diluted pooled human plasma (serial dilutions starting from 1/50, equivalent of about 10 µg/ml FH) were added for 30 min at 30°C

to allow convertase dissociation by Factor H. Obtained haemolysis was measured after 45 min incubation with rat serum as above. Results for the Factor H-induced convertase dissociation were calculated as percentage of the condition where only spontaneous decay occurred (without plasma).

Endothelial cells assay

HUVEC were isolated from human umbilical cord veins and cultured in M199 medium (Gibco, Paisley, GB) with 20% fetal calf serum, supplemented with endothelial cells growth supplement and heparin. Third passage resting cells were grown to confluence into 24-well plates. When activated cells were used, the wells were treated overnight with TNFα/IFNγ. Alternatively, the cells were exposed to 100 µM heme, as described (27). The overnight detached HUVEC were collected and shown to be late apopto-necrotic cells (Annexin V and propidium iodide positive). After washing with PBS, adherent cells were incubated with 50µl FB-depleted serum, (CompTech, Tylor, TX), and with 100µl recombinant WT or mutant FB supernatants, containing equal amount of FB. Used lots of FB-depleted serum did not contain detectable residual FB, as measured by ELISA but had normal C3 levels. Supernatant from HEK293T cells transfected with the vector alone (SN0) was used as a negative control. Blocking anti-FH antibody Ox24 was added in the FB depleted serum, reconstituted with the WT FB as a positive control for complement dysregulation. After 30-minute incubation at 37°C, the supernatant was discarded and the wells were washed 3-fold with PBS. Adherent cells were detached by incubation with PBS-5mM EDTA for 30 minutes. The resting, activated and apopto-necrotic cells were labeled with anti-C3c, a monoclonal antibody (Quidel, San Diego, CA, USA), anti-C5b9 neoepitope antibody (kind gift form Prof. Paul Morgan, Cardiff, UK) or a control mouse IgG1, followed by phycoerythrin (PE)-labeled

secondary antibody (Beckman Coulter, Roissy, France). Cells were analyzed by flow cytometry on a Becton Dickinson Facscalibur or LSRII, using FCS express software.

Genetic analysis

Genomic DNA from each patient from the French adults aHUS cohort or healthy donor was obtained from peripheral-blood leukocytes. The frequency of R7, Q7 and W7 (rs12614 and rs641153) was assessed by a direct sequencing of exon 2 of FB. Screened individuals signed an informed consent and the project was approved by the local ethical committee.

Supplemental Table 1. Cochran's alpha coefficient was used to test the internal consistency of the available algorithms for prediction of functional effects of mutations using FB.

Cronbach's alpha with raw variables

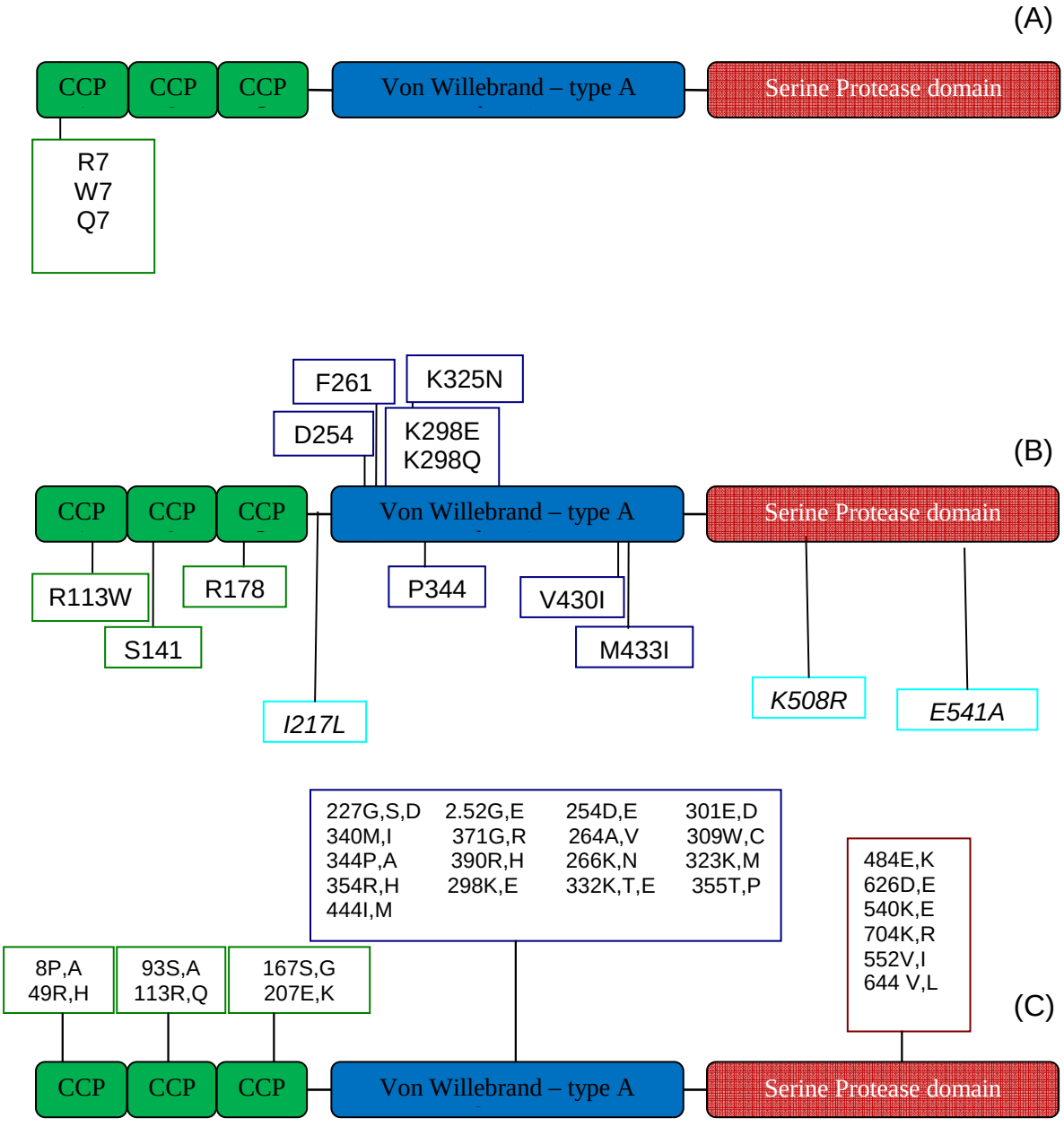
Cronbach's alpha	0.6576
95% lower confidence limit	0.3589

Effect of dropping variables

Variable dropped	Alpha	Change
BS	0.4444	-0.2132
GV/GD	0.3944	-0.2633
MT	0.8053	0.1477
PolyPhen	0.6603	0.002607
Sift	0.5693	-0.08836

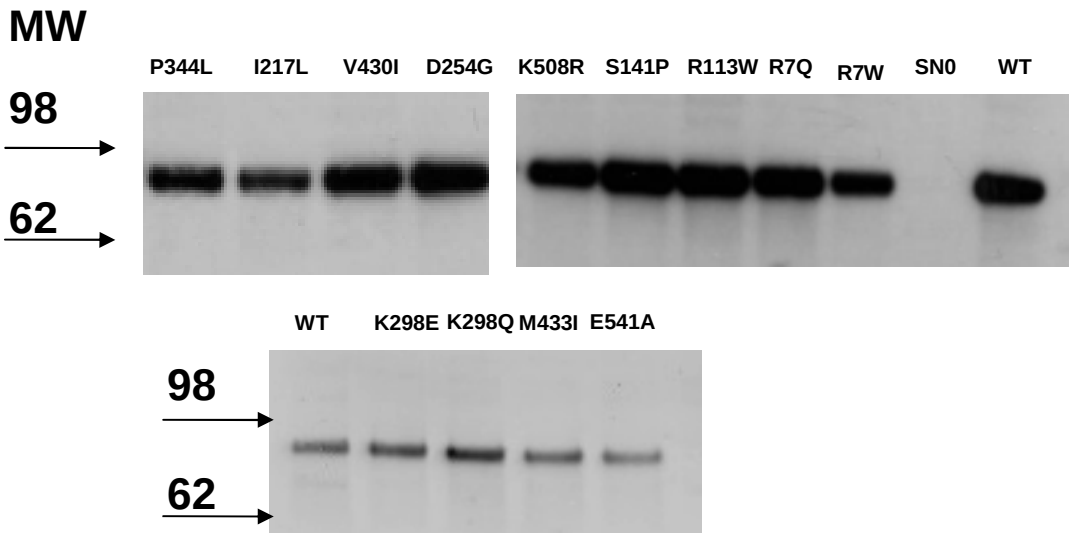
Supplemental Table 2. *In silico* analysis for the FH mutations in SCR19-20.

mut FH	mut FH	position	PolyPhen	Polyphen Prediction	SIFT	Mut taster	GV/GD
G1119D	Asp119Gly	c.3356 A>G	1.000	Probably Damaging	Deleterious	Disease causing	65
W1157R	Trp1157Arg	c.3469 T>A/C	1.000	Probably Damaging	Deleterious	Disease causing	65
E1172X	Glu1172Stop	c.3514 G>T	--	Damaging	Deleterious	Disease causing	na
R1182S	Arg1182Ser	c.3546 G>C	0.826	Possibly Damaging	Deleterious	Polymorphism	35
W1183L	Trp1183Leu	c.3548 G>T	0.998	Probably Damaging	Deleterious	Polymorphism	55
T1184R	Thr1184Arg	c.3551 C>A	0.000	Benign	Tolerated	Polymorphism	0
L1189R	Leu1189Arg	c.3566 T>G	0.994	Probably Damaging	Deleterious	Polymorphism	65
L1189F	Leu1189Phe	c.3565 C>T	0.278	Benign	Tolerated	Polymorphism	0
S1191L	Ser1191Leu	c.3572 C>T	0.151	Benign	Deleterious	Polymorphism	65
V1197A	Vla1197Ala	c.3590 T>C	0.962	Possibly / Probably D.	Deleterious	Disease causing	25
G1194D	Gly1194Asp	c.3581 G>A	0.405	Benign	Tolerated	Polymorphism	0
E1198A	Glu1198Ala	c.3593 A>C	0.724	Possibly Damaging	Deleterious	Polymorphism	65
R1210C	Arg1210Cys	c.3628 C>T	0.024	Benign	Tolerated	Disease causing	15
G1204E	Gly1240Glu	c.3611 G>A	0.997	Probably Damaging	Deleterious	Polymorphism	65
R1215G	Arg1215Gly	c.3643 C>G	1.000	Probably Damaging	Deleterious	Polymorphism	65
R1215Q	Arg1215Gln	c.3644 G>A	0.994	Possibly / Probably D.	Deleterious	Polymorphism	35
P1226S	Pro1126Ser	c.3676 C>T	1.000	Probably Damaging	Deleterious	Disease causing	65

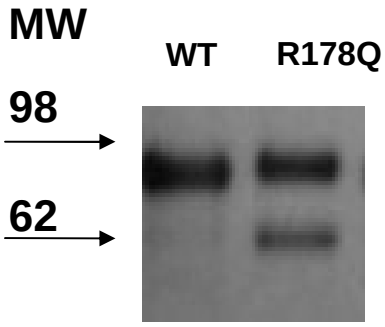


Supplemental Figure 1: Schematic representation of the Factor B gene. (A) Frequent polymorphisms in the healthy population, (B) FB mutations and rare polymorphisms (light blue box, italic) found in aHUS patients, (C) ultra rare polymorphisms found in the healthy controls (1000 genomes database).

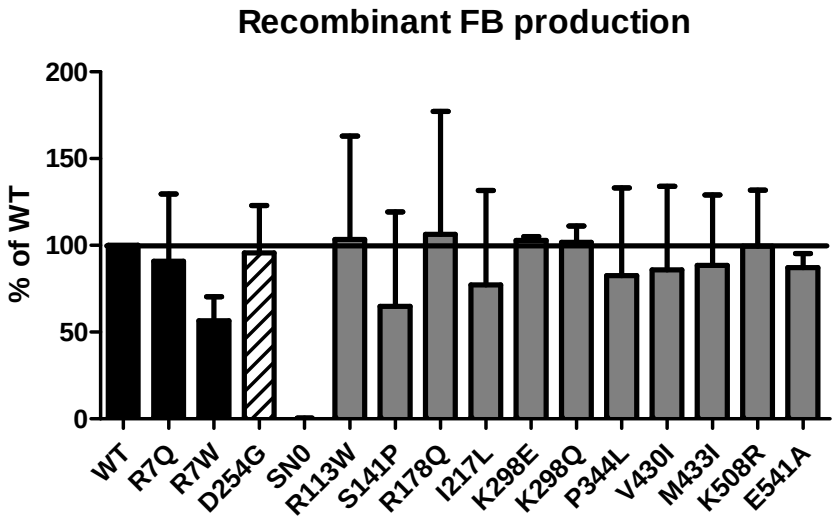
(A) (A1)



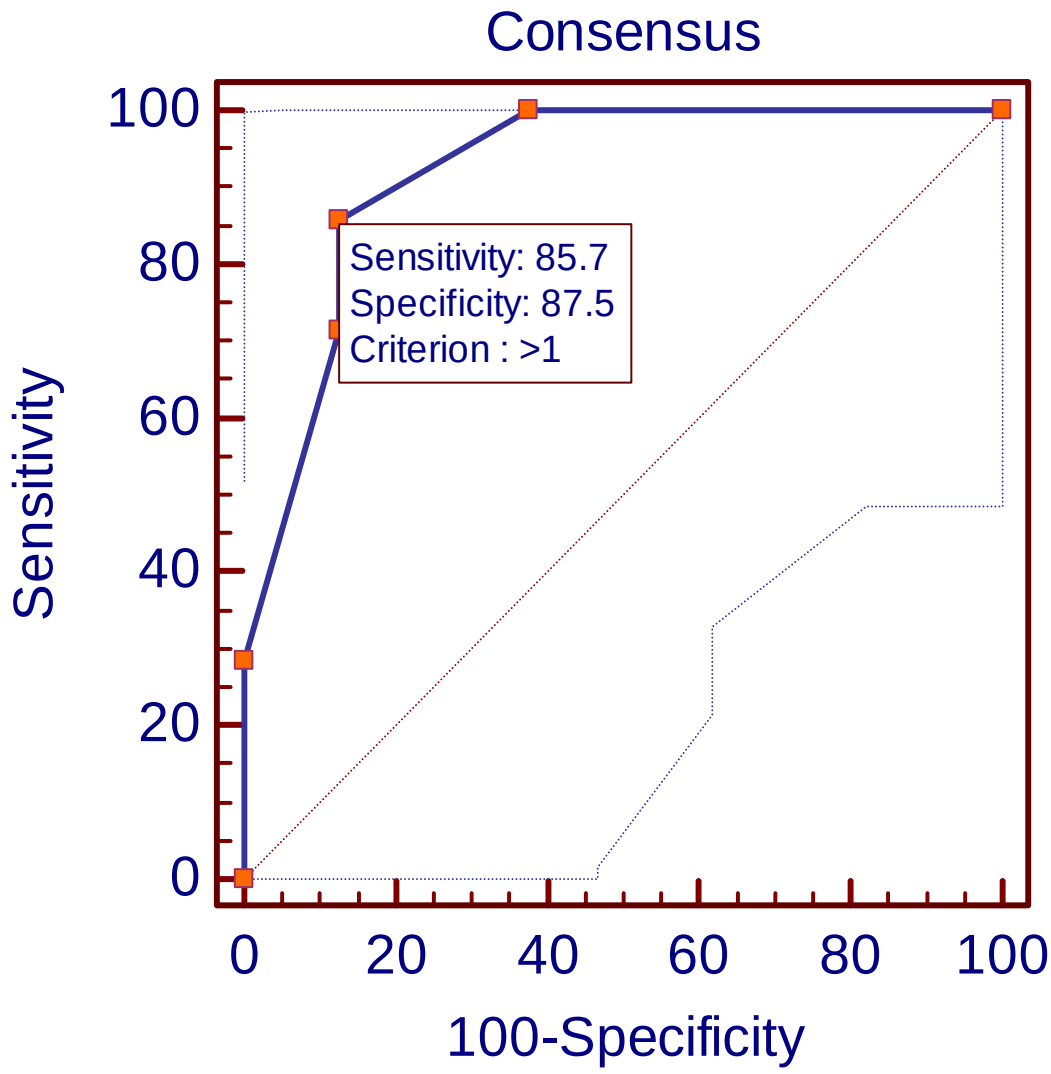
(A2)



(B)



Supplemental Figure 2: Integrity of FB mutated recombinant proteins and production levels. A) Western blot. Supernatants from each cell culture were diluted 1/10 and migrated on 10% PAGE gels. The presence and integrity of the recombinant FB was detected by an anti-FB polyclonal antibody. B) Production level of the recombinant FB mutations, tested by sandwich ELISA, using plasma derived FB as a standard. The values of the WT in each production were taken as 100% and the corresponding levels of the mutations expressed at the same time were calculated.



Supplemental Figure 3. **ROC curve analysis of performance of the consensus score.** The number of methods (among Polyphen, Sift, GDGV and functional site involvement) that predict a given mutation as deleterious was used as a consensus score. Mutation taster was omitted due to a disagreement with the rest of the methods (Cronbach's alpha analysis) and poor correlation with the functional assays. The optimal cut off was determined by determining the Yuden index J and the corresponding criterion value (marked by the inset). The 95% Confidence Intervals are shown with a dashed line.