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Short Communication

Novel homozygous CD46 variant with C-isoform expression affects C3b inactivation in atypical hemolytic uremic syndrome

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Atypical hemolytic uremic syndrome (aHUS) is a thrombotic microangiopathy that may lead to organ failure. Dysregulation of the complement system can cause aHUS, and various disease-related variants in the complement regulatory protein CD46 are described. We here report a pediatric patient with aHUS carrying a hitherto unreported homozygous variant in *CD46* (NM_172359.3:c.602C>T p.(Ser201Leu)). In our functional analyses, this variant caused complement dysregulation through three separate mechanisms. First, CD46 surface expression on the patient's blood cells was significantly reduced. Second, stably expressing CD46(Ser201Leu) cells bound markedly less to patterns of C3b than CD46 WT cells. Third, the patient predominantly expressed the rare isoforms of CD46 (C dominated) instead of the more common isoforms (BC dominated). Using BC1 and C1 expressing cell lines, we found that the C1 isoform bound markedly less C3b than the BC1 isoform. These results highlight the coexistence of multiple mechanisms that may act synergistically to disrupt CD46 function during aHUS development.

Keywords: CD46 variant · CD46 C-isoform · C3b · atypical hemolytic syndrome

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Hemolytic uremic syndrome (HUS) is a thrombotic microangiopathy characterized by a triad of nonimmune hemolytic anemia,

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[Correction added on 15 September 2022, after first online publication: (1) Remove the material methods parts in the supporting information because this is a repeated part from the main text. (2) Include supplementary Figure 4: it is mentioned in the main text but the authors forgot to include the figure in the previous supporting information. With these mentioned 2 points, the authors request to update their supporting information.]

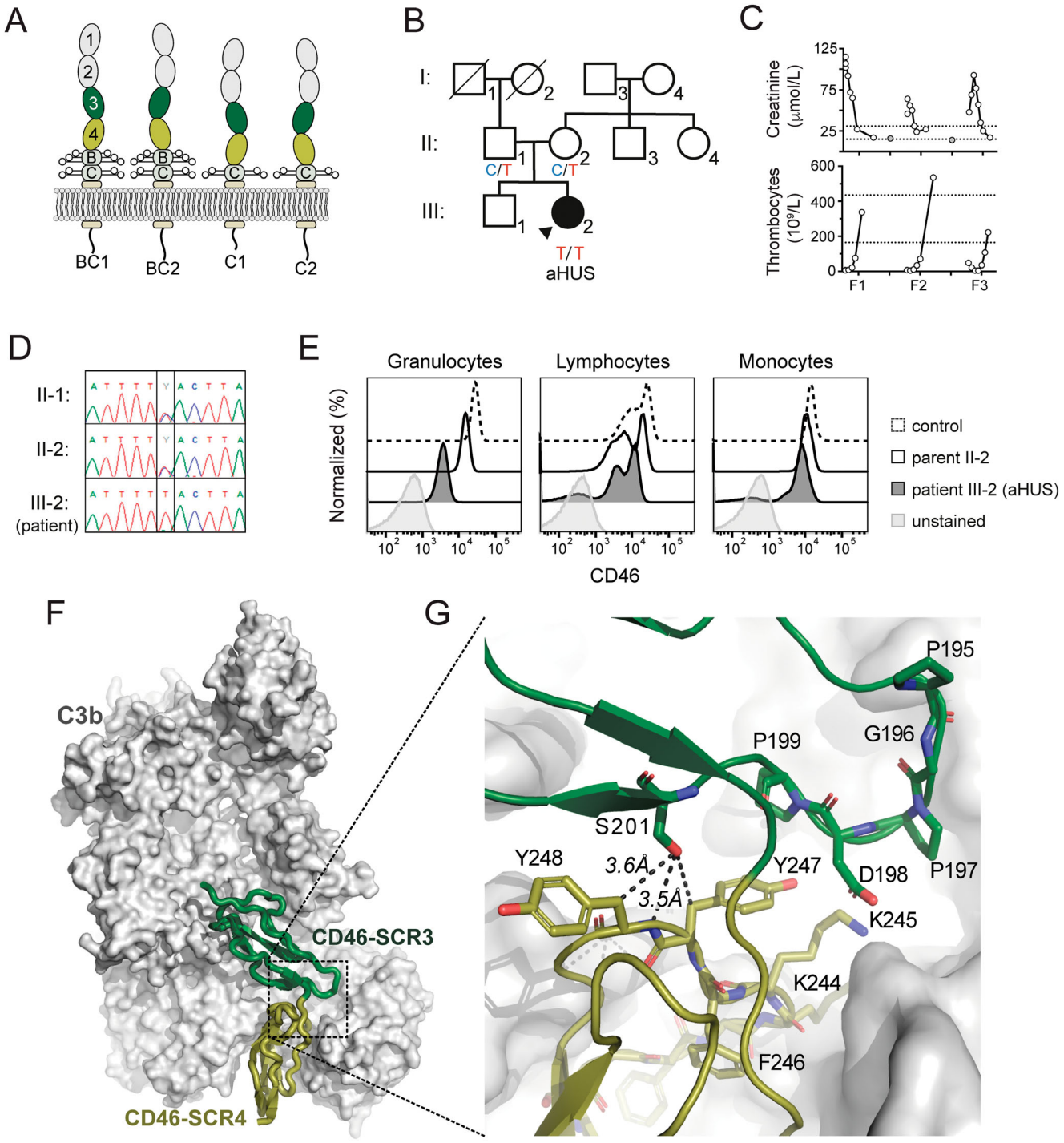

Figure 1. Identification of homozygosity for the CD46(S201L) variant in patient with aHUS. (A) Schematic presentation of CD46 isoforms illustrating the four extracellular SCR domains, with the C3b-binding domains SCR3 and SCR4 shown in green and yellow, respectively. (B) Family pedigree with the proposita (III-2) diagnosed 13 months old with aHUS and the healthy parents. (C) Creatinine and thrombocyte levels in the patient, measured during three hospitalizations (Flare 1 (F1): age 13 months, F2: age 21 months, and F3: age 35 months, with daily sampling over a 12 day period), and at two outpatient controls (grey circles). The corresponding reference interval is indicated by a horizontal line (creatinine: [15;31], thrombocytes [165;435]). Further details on the clinical biochemistry can be found in Supporting information Table S1. (D) Sanger sequencing data (5' to 3' direction) with identification of NM_172359.3(CD46): c.602C>T heterozygous parents (II-1 and II-2) and c.602C>T homozygous patient with aHUS (III-2). The genomic (GRCh38/hg38) region shown is NC_000001.11: g.207761370_207761380, highlighting the g.207761375C>T substitution. (E) Flow cytometry analyses of CD46 expression in a blood sample from patient III-2, parent II-2, and 1 non-related volunteer (control), using PE mouse anti-human CD46 antibody clone E4.3. F) The C3b:CD46 complex [PDB entry 5FO8], displaying C3b as a grey surface and CD46 SCR3 and SCR4 as cartoons in green and yellow, respectively. G) Close-up on the interface between SCR3 and SCR4, showing the position of S201 as well as key residues of the hypervariable regions 196-PGPD-199 and 240-SGFGKKF-246. The dashed lines indicate the shortest distances between S201 and Y247/Y248.

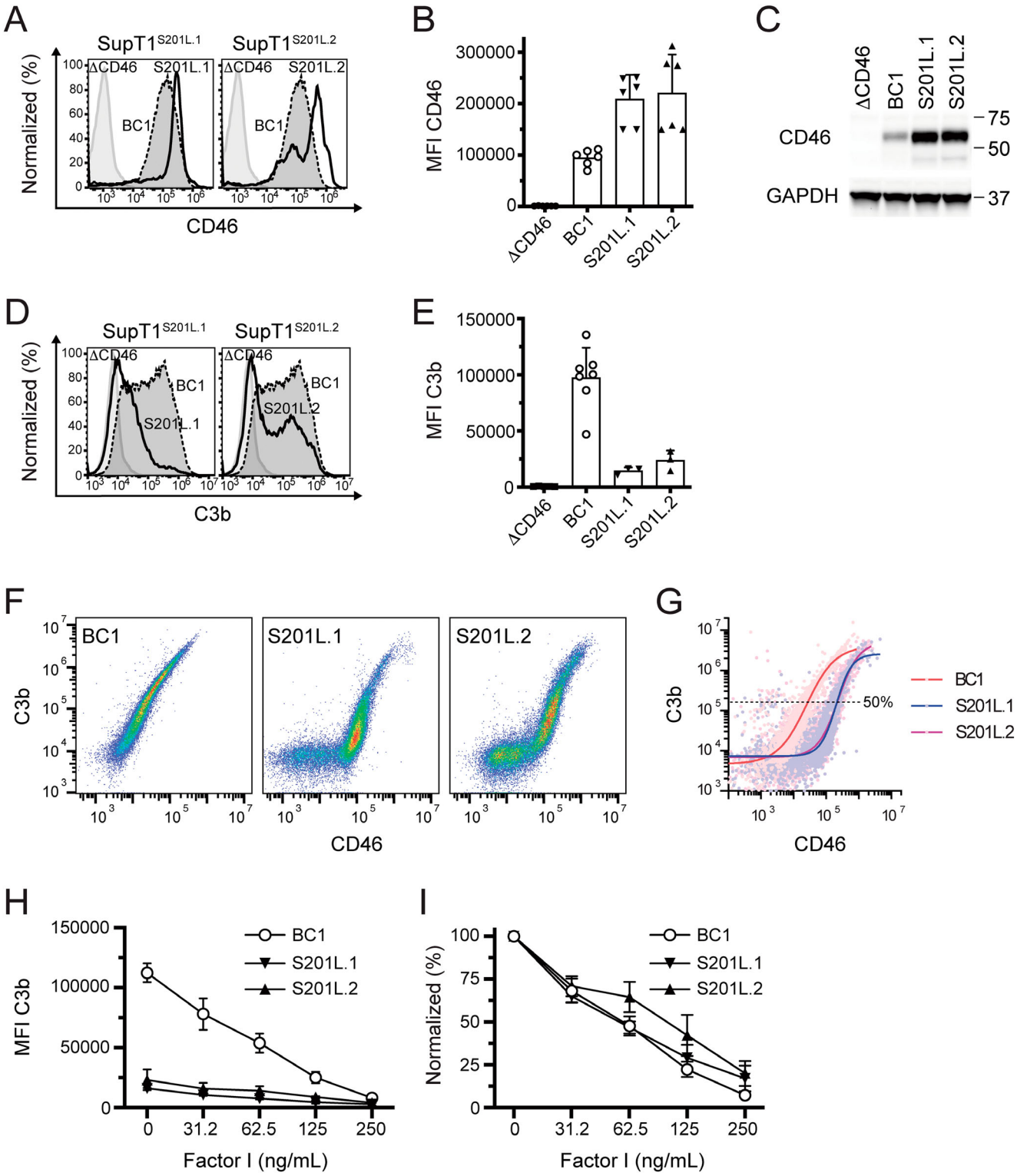

Figure 2. aHUS-associated CD46 mutation S201L compromises C3b binding. Functional analysis of SupT1$^{\Delta CD46}$ cell lines reconstituted with CD46 variants. (A) Flow cytometry analysis of CD46 surface expression in SupT1$^{\Delta CD46}$ (light grey), SupT1^{BC1} (dark grey, stippled line), SupT1$^{S201L.1}$, and SupT1$^{S201L.2}$ (no fill, black line). The figure shown is representative of six independent experiments. (B) CD46 median fluorescence intensity (MFI CD46) in separate flow cytometry analysis experiments (n = 6). (C) Western blotting analysis of the indicated cell lines probed with antibody to CD46 (clone ERP4014). The molecular weight markers are indicated at the right side of the figure. An uncropped version of the Western blot is shown in Supporting Information Fig. S4. (D,E) Binding of C3b-streptavidin tetramers to SupT1$^{S201L.1}$ and SupT1$^{S201L.2}$ (no fill, black line), together with SupT1$^{\Delta CD46}$ (light grey) and SupT1^{BC1} (medium grey, stippled line). Binding was measured using flow cytometry. Histograms in (D) are representative of three or more independent experiments. Columns in (E) represent the C3b median fluorescence intensity (MFI C3b) of ΔCD46 (n = 5), BC1

thrombocytopenia, and acute renal failure [1]. HUS with an absence of an exogenous cause, e.g. Shiga-like toxin-producing *Escherichia coli* (STEC), is referred to as atypical HUS (aHUS) and accounts for approximately 5–10% of HUS in children [1, 2]. The major cause of aHUS is dysregulation of the alternative pathway of the complement system, which may be caused by variants in genes encoding complement regulator proteins [1–5]. The prognosis of untreated aHUS is poor, with high relapse rates and a high risk of end-stage renal disease.

CD46 belongs to a family of proteins involved in the essential regulation of the complement activation on host cells. If the complement factors C3b or C4b are deposited on host cells, CD46 on the cell surface acts as a cofactor for the soluble protease Factor I, which cleaves C3b and C4b and thereby inactivates the initiated complement attack [6]. The association between variants in *CD46* and aHUS development was first described in 2003 [7, 8], and today more than 60 variants that predispose to aHUS are known [9]. In pediatric patients, pathogenic *CD46* variants are found in 10–15% of aHUS cases [1, 9]. The penetrance of the pathogenic variants in causing aHUS is approximately 50% and often requires additional events, e.g. infections [10]. CD46-associated susceptibility to aHUS most often results from dominant alleles, however, a minor fraction of patients has been reported with various biallelic missense variants as well as canonical splice site variants [5, 8–13]. Homozygosity of pathogenic variants has been associated with a markedly reduced CD46 cell surface expression [12].

CD46 comprises 14 exons and translates into several protein isoforms. The four most common isoforms are BC1, BC2, C1, and C2, named based on their respective serine-threonine-proline rich (STP) domains, and cytoplasmic tail [14] (Fig. 1A). The BC isoforms contain an extended STP region compared with the C isoforms. The isoforms thus differ in size, extent of O-glycosylation, and sequence of the cytoplasmic tail, which may be of importance for the intracellular signaling events [15]. The expression pattern of the CD46 isoforms varies amongst individuals: 65% predominantly express the BC isoforms, 29% express an equal distribution of the BC and the C isoforms, and only 6% predominantly express the C isoforms [16]. The B domain (Fig. 1A) is suggested to influence the Factor I mediated cleavage of C4b [17]. Thus, the rare persons who predominantly express the C isoforms may be speculated less capable of inactivating activated complement factors. The distribution and potential importance of the CD46 isoform expression pattern in patients with aHUS are, to our knowledge, not established.

In CD46, the extracellular short consensus repeats (SCR)2-4 constitute the binding site for C3b and C4b [18]. The crystal structure of C3b in complex with CD46 SCR1-4 has provided the domain organization and molecular interactions of the C3b-CD46 complex [19] showing that only SCR3 and SCR4 form distinct contacts with C3b. The crystal structure corroborates the functional studies on the C3b-CD46 binding [18] and points to the involvement of SCR3-4 in C3b binding [19].

In this study, we identified homozygosity of a novel recessive variant, p.S201L, in the SCR3 domain of CD46, in a child with recurrent aHUS. We demonstrate that the novel variant impacts on C3b inactivation by at least three separate mechanisms: (1) reduced surface level expression of CD46, (2) weakened C3b interaction with CD46, and (3) weakened CD46-C3b interaction associated with C isoform predominance.

Results and discussion

Identification of a novel recessive CD46 variant in a patient with aHUS

We examined a young female patient diagnosed with aHUS at the age of 13 months (Fig. 1B, III-2). The patient was hospitalized after a few days with fatigue and dark urine. Blood tests showed highly elevated plasma creatinine and severe thrombocytopenia (Fig. 1C), metabolic acidosis, and anemia. HUS was therefore suspected. PCR for STEC was negative. Screening of variants in aHUS-related genes by targeted gene panel sequencings identified homozygosity for a novel missense variant in *CD46*: Ser201Leu (NM_172359.3: c.[602C>T];[602C>T] p.[(Ser201Leu)];[(Ser201Leu)]), in the following referred to as S201L. The genomic (GRCh38/hg38) position is NC_000001.11:g.207761375C>T. The variant has a CADD score of 23.1, SIFT score of 0.001 (damaging), PROVEAN score of −5.74 (damaging) and is not present in the gnomAD database (v2.1.1 dataset), indicating a high degree of rarity. Sanger sequencing confirmed the presence of the variant in the patient and heterozygous carrier states in both parents (Fig. 1D). During follow-up, the patient suffered two additional flares at the age of 1 year and 9 months and 2 years and 11 months (Fig. 1C). She was hospitalized and treated with infusions of the C5-specific complement inhibitor eculizumab. No other known family members have been diagnosed with aHUS or have had experienced similar symptoms (Fig. 1B). The healthy parents stated not to be consanguineous. The older brother was healthy.

Using flow cytometry, we examined the expression of CD46. Binding of an anti-CD46 antibody (clone E4.3) directed at an

($n = 7$), S201L.1 ($n = 3$), and S201L.2 ($n = 3$). (F) Correlation of C3b-streptavidin tetramers binding and CD46 expression level in SupT1^{BC1}, SupT1$^{S201L.1}$, and SupT1$^{S201L.2}$. The cells represent living single cells (live cells gated using a viable marker followed by single-cell gating using FSC/SSC plots). (G) Sigmoidal fitting to the data from Fig. 2F, illustrating the marked shift of the EC50 values in SupT1^{S201L} (EC50 values: SupT1^{BC1}: 24.853, SupT1$^{S201L.1}$: 200.774, and SupT1$^{S201L.2}$: 232.719). The half maximal C3b binding (50%) is indicated by a horizontal dashed line. The data shown are representative of three independent experiments. Statistics are included as Supporting information Table S2. (H) Factor I processing of C3b-streptavidin tetramers bound to SupT1^{BC1}, SupT1$^{S201L.1}$, and SupT1$^{S201L.2}$, respectively, measured as the level of MFI of intact C3b on the cell surface upon incubation with the indicated concentrations of Factor I. (I) Data from (H), normalized to the respective MFI C3b without Factor I. Data shown represent the mean with SD.

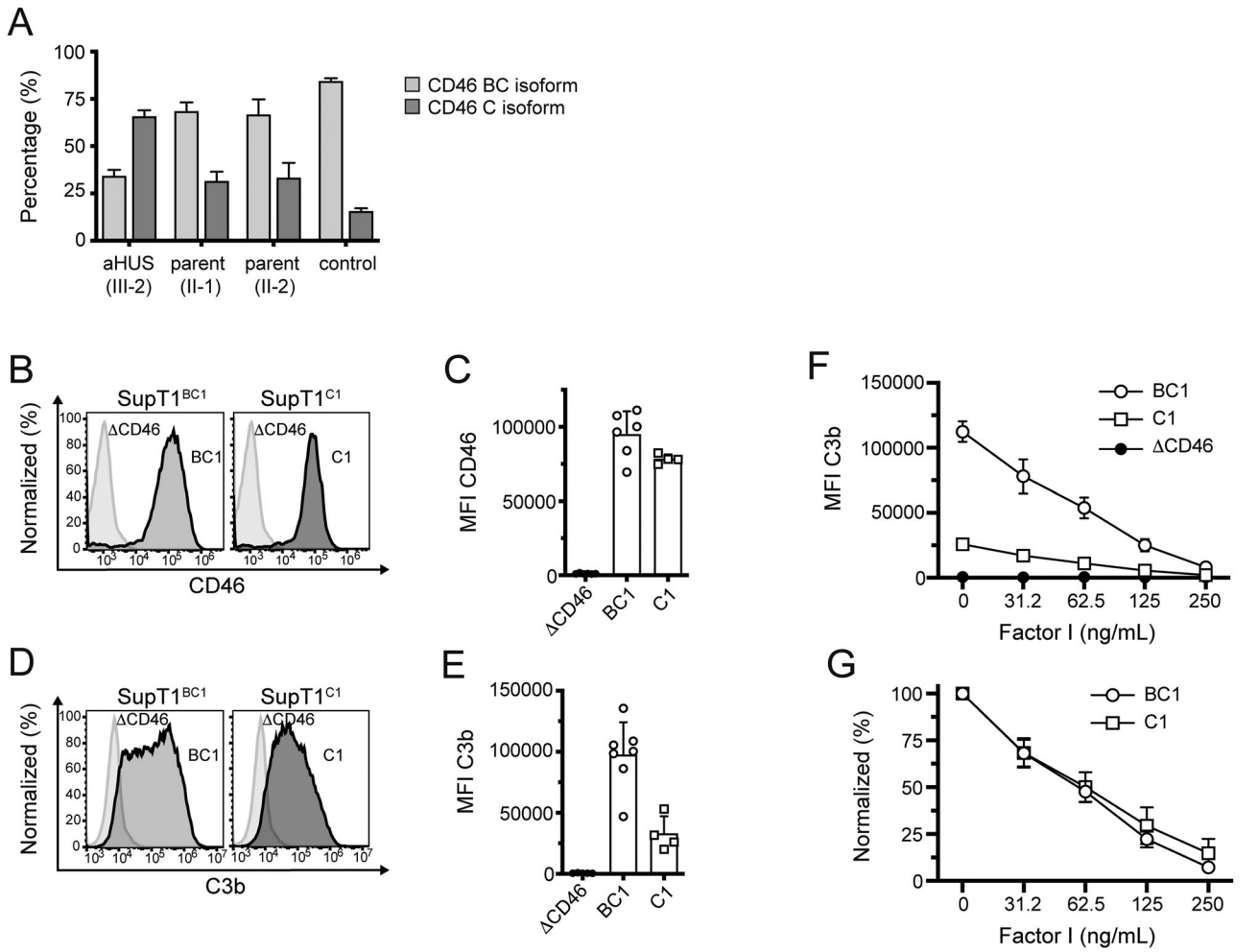

Figure 3. aHUS patient has predominant expression of CD46 C isoform with reduced C3b-binding ability. (A) The relative mRNA expression of the BC and C isoforms of CD46 in patient III-2, parents II-1 and II-2, and control (nonrelated volunteer) was measured by real-time multiplex PCR, and is indicated as a relative frequency from two independent measurements. The expression of the separate isoforms was calculated using PPIB for normalization ($2^{-\Delta Ct}$ method). The relative frequency of the isoforms BC1, BC2, C1, and C2 was calculated as the percentage of the summarized relative expression level of all isoforms, i.e. BC = (BC1+BC2)/(BC1+BC2+C1+C2)*100, and C = (C1+C2)/(BC1+BC2+C1+C2)*100. (B and C) Flow cytometry analysis of CD46 surface expression in the stable cell lines SupT1$^{\Delta CD46}$ (light grey), SupT1^{BC1} (medium grey), and SupT1^{C1} (dark grey). The figure shown in (B) is representative of respectively six (left side) and four (right side) independent experiments. Columns in (C) represent the median fluorescence intensity (MFI) of ΔCD46 (n = 6), BC1 (n = 4), C (n = 4). (D and E) C3b-streptavidin tetramer binding on the surface of SupT1$^{\Delta CD46}$ (light grey), SupT1^{BC1} (medium grey), and SupT1^{C1} (dark grey), investigated using flow cytometry. The figure shown in (D) is representative of respectively seven (left side) and four (right side) independent experiments. Columns in (E) represent the C3b median fluorescence intensity (MFI C3b) of ΔCD46 (n = 5), BC1 (n = 7), C (n = 4). (F) Factor I processing of C3b-streptavidin tetramer bound to SupT1^{BC1} and SupT1^{C1}, measured as the level of MFI of intact C3b on the cell surface upon incubation with the indicated concentrations of Factor I. (G) Data from (F), normalized to the respective MFI C3b without Factor I. Data shown represent the mean with SD.

epitope in SCR1 to the patient's granulocytes, lymphocytes, and monocytes was reduced compared with that of healthy controls (Fig. 1E and Supporting information Figs. S1-S3). The heterozygous mother (II-2) displayed intermediate binding to her cells. This suggests that the variant is associated with a reduced level of CD46 expression or an altered conformation leading to poorer binding of the antibody, or both. To address the possibility of an altered conformation, we repeated the analyses with two additional antibodies that targeted a separate epitope in SCR1 (clone Tra-2.1) and an epitope in SCR4 (clone MEM258), respectively. These antibodies provided results similar to clone E4.3 (Supporting information Fig. S2). This indicated that the reduced antibody binding was caused by a reduced CD46 expression of the variant rather than by an altered conformation.

To predict the consequences of the S201L variant, we examined the model of CD46-C3b interaction based on crystal structure data [19] (Fig. 1F). The structure indicates that S201 resides in the interface between SCR3 and SCR4, which interacts with C3b [18, 19] (Fig. 1G). This area holds other aHUS-associated variants, i.e., G204R, S206P, I208Y, and C210F [9], indicating the importance of this region for the functional integrity of CD46. S201 does not appear to interact directly with C3b. However, the S201L mutation introduces a hydrophobic and significantly larger side chain that appears incompatible with Y247 and Y248. Thus,

the substitution with leucine at position 201 is likely to influence the interface between SCR3 and SCR4, and thereby cause a disturbance of the hypervariable loops in SCR3 (196-PGPD-199) or SCR4, or both (240-SGFGKKF). These loops are important for C3b binding [18, 19] (Fig. 1G).

The aHUS variant CD46^{S201L} compromises C3b binding

We used a cell line system to compare the C3b binding to CD46^{S201L} and CD46^{BC1} (WT). The SupT1$^{\Delta CD46}$ cell line with a CRISPR-Cas9-deleted CD46 expression [20] was used as background for stable reconstitution with the aHUS mutant S201L in the BC1 isoform of CD46. We isolated stable cell lines with different CD46 surface expression profiles, i.e., SupT1$^{S201L.1}$ and SupT1$^{S201L.2}$ (Fig. 2A–C). The CD46 surface expression level in SupT1$^{S201L.1}$ was homogeneous, with a markedly increased CD46 expression compared with SupT1^{BC1}. In contrast, SupT1$^{S201L.2}$ presented a heterogeneous CD46 surface expression, with varying CD46 levels on the cells. Noticeably, the CD46 median fluorescence intensity (MFI) of both SupT1$^{S201L.1}$ and SupT1$^{S201L.2}$ was markedly higher than the level of the WT SupT1^{BC1} (Fig. 2B) (Mann-Whitney test, P-value: 0.0022 for both comparisons). Examination of protein levels by Western blot confirmed the increased expression level of CD46 in both of the S201L-expressing cell lines (Fig. 2C).

We observed, like others, that C3b alone does not give a signal on CD46-expressing cells. The C3b used in the present experiments was obtained by cleavage of C3 using trypsin, followed by site-specific biotinylation, as described in materials and methods. To obtain sufficient avidity, we offered C3b to the cells in the form of biotin-C3b mixed with streptavidin, i.e. a tetramer of C3b molecules. The tetrameric streptavidin was conjugated to the fluorescent dye BV421. In the following, we refer to the binding of the C3b-tetramer as C3b binding. Others have studied the binding of CD46-expressing cells to patterns of C3b by depositing C3b in microtiter wells and have analyzed for binding of cells to the surface [17]. We find the present methods using a pattern of tetramer C3b as a good alternative when studying cells in suspension.

The level of C3b bound on the surface of the cells was evaluated using flow cytometry (Fig. 2D and E). We found a correlation with the level of CD46 surface expression. Thus, the heterogeneous cell line SupT1$^{S201L.2}$ containing cells with varying CD46 levels (Fig. 2A) displayed varying levels of C3b binding (Fig. 2D). Noticeably, although SupT1$^{S201L.1}$ and SupT1$^{S201L.2}$ had an increased CD46 surface expression level compared with SupT1^{BC1}, the C3b binding was severely reduced (Mann-Whitney test, P-value: 0.0167 for both comparisons). This emphasizes the significance of the CD46 variant in C3b binding, and more generally indicates that measuring CD46 expression alone is insufficient for estimating its cofactor significance for C3b inactivation.

We further investigated the correlation of CD46 expression with the binding of C3b, using a multi-stain approach (Fig. 2F and G). The combined detection of C3b-binding and CD46-expression level supports a direct correlation between the level of CD46 expressed on the surface and the C3b-binding capacity. Interestingly, for the SupT1$^{S201L.1}$ and SupT1$^{S201L.2}$ cell lines, we observed CD46-expressing cells that did not bind C3b (Fig. 2F and G). The level of C3b binding increased as the level of CD46 increased, but our data show that this correlation was severely affected in the S201L mutant cell lines. Despite the increased CD46 surface expression level, the EC50 value was increased by 5–8 fold in SupT1$^{S201L.1}$ and 7–9 fold in SupT1$^{S201L.2}$ compared with SupT1^{BC1} (see Supporting information Table S2). Thus, for SupT1^{S201L}, a significantly higher amount of CD46 is required to mediate binding of the same number of C3b molecules compared to the WT BC1 cell line. This demonstrates that the S201L mutation severely affects the ability of CD46 to bind C3b.

To evaluate if the processing of cell-bound C3b was affected in the SupT1^{S201L} cell lines, we measured the level of C3b remaining on the surface upon incubation with Factor I (Fig. 2H and I). This method relies on the liberation of the streptavidin-bound C3b from the cell upon cleavage of the C3b to iC3b by Factor I [21]. Our data show that although the SupT1^{S201L} cell lines bound less C3b prior to incubation with Factor I (Fig. 2H), the rate of processing upon Factor I incubation is similar to the rate observed in SupT1^{BC1} (Fig. 2I). Thus, the S201L variant does not affect the Factor I-mediated degradation of C3b once it is bound to CD46.

The present study focused on the C3b binding capacity of the newly identified CD46 variant. It would be relevant in future studies to compare the functional influence of the known CD46 disease-related variants, e.g., listed in ref. [9]. Such studies should preferably embrace the binding of both C3b and C4b and the influence on Factor I mediated cleavage reactions on C3b and C4b.

C isoform dominance in the aHUS patient contributes to reduced binding of C3b

We investigated the CD46 isoform distribution in the patient using a previously described real-time PCR assay [22]. Interestingly, the patient displayed a predominance of the C isoforms, with a BC/C distribution of 34.3%/65.7% (Fig. 3A). The parents presented a predominance of the BC isoforms, i.e., II-1: 68.4%/31.6%, and II-2: 66.8%/33.2%, whereas the control (nonrelated volunteer) displayed a BC/C distribution of 84.4%/15.6%. CD46 isoforms have previously been shown to differ in their capacity to regulate complement C4b degradation, whereas little or no effect was seen on C3b [17]. This difference may be due to the different assays and altogether indicate that the BC isoform domination may provide enhanced protection against self-destructive complement activation.

The potential role of the B domain of CD46 for binding of C3b was examined using SupT1^{BC1} and SupT1^{C1} cell lines generated by reconstitution of the specific CD46 isoforms BC1 and C1 into the SupT1$^{\Delta CD46}$, allowing functional assessment of the CD46 isoforms [23]. Despite similar expression levels of CD46 in SupT1^{BC1} and SupT1^{C1} cell lines (Fig. 3B and C), cells

reconstituted with BC1 bound markedly more C3b when compared with C1-reconstituted cells (Fig. 3D and E). The Factor I-mediated processing was found to be similar in SupT1^{BC1} and SupT1^{C1}, indicating that the reduction in binding of C3b did not affect further processing (Fig. 3F and G). Mechanistically, this suggests that Factor I degradation is slower and limiting under these experimental conditions compared with the formation of the C3b-CD46 complex.

The distribution of the CD46 isoform pattern in patients with aHUS is, to our knowledge, unknown. Since the C isoform predominance reduces binding of C3b it would be interesting to investigate, whether this isoform pattern is overrepresented among patients with aHUS.

Concluding remarks

In conclusion, we identified homozygosity of a novel pathogenic variant in *CD46* (S201L) that predisposes to aHUS development. Pathogenicity of the variant was functionally validated by identification of (1) a reduction in the total level of CD46 expression on the surface of the patient's cells, (2) a reduction in binding of C3b to the variant, and (3) a limitation of binding of C3b with C isoform predominance. These results contribute to the understanding of how disrupted CD46 integrity and expression cause dysregulation of the complement system and aHUS development.

Materials and methods

Genetic analysis

Genomic DNA was extracted from peripheral blood and targeted gene sequencing was performed on an Ion S5 platform. The targeted gene panel included the following aHUS/C3 glomerulopathy-associated genes: *ADAMTS13, C3, C3AR1, C5, CD46, CD55, CD59, CFB, CFH, CFHR1, CFHR2, CFHR3, CFHR4, CFHR5, CF1, DGKE*, and *THBD*. Sanger sequencing was used to validate the *CD46* candidate variant in the patient and for confirmation of heterozygosity of the variant in both parents.

Cell lines and mutagenesis

The human T-cell lymphoblast cell line (SupT1) was kindly provided by the NIH AIDS Reagent Program (Division of AIDS, NIAID, NIH, USA). Generation of stable cell lines reconstituted with isoforms BC1 and C1 (SupT1^{BC1} and SupT1^{C1}) was based on the previously described CD46 KO cell line SupT1$^{\Delta CD46}$ [20]. The cell lines were maintained in RPMI-1640 (Sigma-Aldrich, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, USA), 2 mM glutaMAX (Gibco, USA), 10 mM HEPES (Sigma-Aldrich), 100 U/mL penicillin and 100 μg/mL streptomycin. All cell lines were cultured at 37°C, 5% CO_2, and split 1:4 in fresh media 2–3 times a week.

The CD46 mutation S201L was generated by site-directed mutagenesis in the BC1 isoform using a pCCL-CD46^{BC1} construct as template for mutagenesis, and a BamHI/ApaI digested pCCL-WPS-PGK-puro-WHV vector as backbone. The approach has been described previously [23]. The primers used for mutagenesis (CD46-BC1(S201L)) are the following:

Fw: 5'-CCTGGACCAGATCCATTTTTACTTATTGGAGAGAGCACG-3'

Rev: 5'-CGTGCTCTCTCCAATAAGTAAAAATGGATCTGGTCCAGG-3'

Generation of SupT1 cell lines stably expressing CD46 (S201L-BC1)

Lentiviral particles were produced as previously described [20]. In brief, HEK293T cells were co-transfected with 1 μg pRSV-REV, 2 μg pMD.2G, 2 μg pMDLg/p-RRE, and 3 μg pCCL-WPS-PGK-CD46-puro-WHV vector, using FuGENE HD Transfection Reagent (Promega Corporation, USA). Approximately 24 h prior to transfection, 8×10^6 HEK293T cells were plated to 40–50% confluence. Transfection was done according to the manufacturer's protocol, and culture medium was collected at 48 and 72 h post transfection. The collected medium was filtered through a 450 nM filter and concentrated using 20% sucrose solution by a 2 h ultracentrifugation at 25.000 × *g*, 4°C. The lentiviral particles were resuspended in PBS using three cycles of vortexing (15 s) and incubated on ice (2 min). The stocks were kept at −80°C. SupT1$^{\Delta CD46}$ cells (generated as described elsewhere [20]) were adjusted to 0.5×10^6 cells/mL 24 h prior to transduction. For the generation of stable cell lines, 600.000 SupT1$^{\Delta CD46}$ cells (0.522×10^6 cells/mL) were transduced with different amounts of lentivirus in the presence of 25 μg/mL protamine sulfate (Sigma-Aldrich), using a 6-well plate. 45 h post transduction, the cells were selected with 2 μg/mL puromycin (Gibco) for a minimum of 14 days. Cell lines were isolated upon transduction with two separate lentivirus preparations, obtained with separate DNA constructs, i.e., S201L.1 and S201L.2. The established cell lines were analyzed using flow cytometry, allowing an evaluation of the CD46 expression.

Analysis of the binding of C3b tetramers to cells

We produced biotin-C3b bound to the tetrameric streptavidin, i.e. the C3b was presented to cells as tetramer C3b to gain sufficient avidity. To generate biotinylated C3b, native C3 was purified from plasma as described [24]. The native C3 was cleaved to C3b using trypsin, followed by site-specific biotinylation of the thioester cysteine using the EZ-Link Maleimide-PEG2-Biotin (Thermo Fisher) reagent, essentially as described [21]. In brief, tryptic cleavage of C3 was stopped using PMSF and pancreatic trypsin inhibitor followed by the addition of 100 mM HEPES pH 7.0 to adjust pH. Next, a 10-fold molar excess relative to C3b of maleimide-PEG2-Biotin was added, and the mix was incubated for 6 h on ice. The biotinylated C3b was applied to

purification by ion-exchange chromatography on a Source 15S (GE Healthcare) column and subsequently purified by SEC using a Superdex 200 increase (GE Healthcare) column. C3b-biotin was complexed with streptavidin-BV421 (BD Biosciences, USA) by incubation at a 2:1 molar ratio (10 μg/mL C3b) in binding buffer (20 mM HEPES, 145 mM NaCl, 5 mM Ca, 1 mM Mg, 1 mg/ml human serum albumin (CSL Behring, 109697), pH 7.4) for 30 min at room temperature (RT). For analyses, 100.000 cells were seeded per well in 96-well plates blocked with 0.1% emulphogen and washed the day before use. The cells were resuspended in 50 μL of the complexed C3b-biotin/streptavidin at 10 μg/mL C3b in binding buffer and incubated for 30 min at RT. Unbound C3b-biotin was removed by washing in 140 μL binding buffer, followed by centrifugation at 300 × *g* for 3 min, and removal of supernatant, before further processing for flow cytometry analyses. All incubations were performed in the dark.

Assay for Factor I influence on cell-bound C3b

CD46 is a known cofactor of Factor I. Factor I cleaves C3b to iC3b. The influence of Factor I on the C3b bound to cells expressing various forms of CD46 was examined by incubating the cells with the C3b-biotin/streptavidin complex, as described above followed by incubation with a 2-fold dilution series of Factor I (Complement Technology, USA), starting at a 2.5%, w/w, ratio to C3b, corresponding to 250 ng/mL, at 37°C for 7.5 min. The cells were pelleted and the supernatant removed before the cells were further processed for flow cytometry analyses.

Isolation of PBMC population from blood samples

The PBMC population was isolated from fresh blood (collected in EDTA tubes) using SepMate PBMC Isolation Tubes (StemCell Technologies, Canada), following the manufacturer's manual. The isolated cells were resuspended in 2% FBS in PBS and kept on ice until further analyses.

Flow cytometry analysis

Flow cytometry analysis was performed with 0.1–0.5 × 10^6 cells. Isolated PBMC were blocked for 15 min at RT with 50 μg/mL mouse IgG (Lampire Biological Laboratories, USA) before incubation with the respective antibody. CD46 expression was determined using 5 μl CD46 antibody conjugated with PE, i.e. clone E4.3 (BD Biosciences, USA), MEM258 (Sigma-Aldrich, USA) or Tra-2.1 (Biolegend, USA), and viability marker LIVE/DEAD fixable Near-IR (nIR) (1:100-dilution, Thermo Fisher Scientific) in PBS supplemented with 2% FBS in a total volume of 50 μL for 30 min at 4°C. The cells were washed twice with PBS supplemented with 2% FBS, and resuspended in PBS + 0.99% paraformaldehyde before analyses using a NovoCyte 3000 flow cytometer equipped with three lasers (405, 488, and 640 nm, Agilent, Santa Clara, CA).

For analysis of whole blood, 100 μL blood sample was stained with the respective antibody + nIR for 15 min at RT. Erythrocytes were lysed using FACS Lysing Solution (BD Biosciences, USA), and the cells were washed using 5% BSA in PBS and centrifugation at 500 × *g* for 8 min. The cells were resuspended in 5% BSA in PBS before analysis on a NovoCyte flow cytometer.

For measurement of CD46 expression in combination with binding of C3b-biotin/streptavidin, we first incubated the cells with C3b-biotin/streptavidin-BV421 as described above, washed the cells once using the binding buffer described before and centrifugation at 350 × *g* for 3 min, and incubated them with CD46-PE antibody clone Tra-2.1 and nIR in 50 μL binding buffer for 30 min at 4°C. The cells were then washed twice with binding reaction buffer, resuspended in PBS + 0.99% paraformaldehyde, and analyzed on a NovoCyte flow cytometer. All incubations with fluorophores were performed under protection from light. For data analyses, FlowJo software version 10 (BD) was used.

Western blotting analysis

Lysates for Western blotting analyses were made from 10^6 cells lyzed in Pierce IP lysis buffer (Thermo Fisher Scientific), supplemented with 1 mM PMSF, 1 mM sodium fluoride, and complete mini protease inhibitor (Roche Diagnostics, USA), using the manual provided by the manufacturer. The protein concentration was determined using the Bradford method (Bio-Rad, Denmark), and 30 μg protein was loaded and separated on an XT Criterion 10% gel with XT MOPS running buffer (Bio-Rad). The proteins were transferred to a Trans-Blot Turbo 0.2-μm polyvinylidene difluoride (PVDF) membrane using a Trans-Blot Turbo transfer system (Bio-Rad). Membranes were blocked in 5% skim milk (Sigma-Aldrich) in tris-buffered saline (TBS) with 0.1 % Tween 20 (Sigma-Aldrich). For detection, we used rabbit anti-CD46 (1:5000, clone ERP4014, Abcam, UK) or rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000, catalog number ab9485, Abcam), followed by HRP-conjugated polyclonal swine anti-rabbit IgG (1:2000, Dako, Denmark). For development, SuperSignal West Femto chemiluminescent substrate (Pierce, Thermo Fisher Scientific) and a ChemiDoc Imaging System (Bio-Rad) were used.

RNA purification, cDNA synthesis, and real-time PCR analysis

Total RNA was isolated from freshly isolated PBMC using Nucleospin RNA columns (Macherey-Nagel, Germany), following the manufacturer's protocol. The RNA was eluted in 30 μL RNase-free water and the total amount of RNA obtained was measured with a NanoDrop 1000 (Thermo Fisher Scientific). An equal amount of total RNA for each experiment (1 μg) was used for cDNA synthesis using QuantiTect Reverse Transcription Kit (Qiagen, Germany), according to the manufacturer's manual. Separate CD46 isoforms were detected by a multiplex CD46-isoform real-time

PCR assay using primers and probes as described previously [22]. The analysis was performed with Brilliant Multiplex QPCR Mastermix (Agilent Technology, USA) and different combinations of CD46 primers and CD46 probes [22], using a QuantStudio 5 real-time PCR system (Applied Biosystems, USA). All real-time PCR analyses were performed in technical triplicates. Data management and statistical analyses were performed using GraphPad Prism version 8.

Ethics approval statement for human studies

According to Danish legislation, laboratory studies by clinical indication do not require a formal ethics committee assessment. A query was sent to the Central Denmark Region Committee on Health Research Ethics (journal no. 1-10-72-181-20).

Patient consent statement

All participants have provided written informed consent.

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Author Contributions: V.R.S., M.K.H., J.M.B.J., and B.B. conducted experiments. V.R.S., M.K.H., J.M.B.J., U.B.J., S.T., and P.H. were involved in the conceptualization. H.P., R.K.J., and G.R.A. generated reagents and provided discussion of experimental conditions. V.R.S., M.K.H., J.M.B.J., R.C., and H.P. performed data analysis. Data were discussed by all authors. V.R.S., M.K.H., S.T., and P.H. wrote the first draft of the manuscript. V.R.S., S.T., and P.H. provided funding. Their relative contribution to the study determined the order of the co-first authors.

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