Gain-of-function mutation in complement C2 protein identified in patient with aHUS.

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Conflict of Interest Disclosures

Authors declare no conflict of interest.

Key messages:

- Gain-of-function mutations in the alternative complement pathway component, factor B, were considered as causative factors of glomerulopathies.
- S250C mutation in complement C2, functional paralog of factor B, results in formation of classical complement convertases resistant to regulation by CD55 inhibitor.
- C2 S250C is the first gain-of-function variant of the classical complement pathway component identified in human disease.

Capsule summary:

Complement convertases resistant to their physiological inhibitors may cause complement-related glomerulopathies. S250C substitution in complement C2 found in aHUS patient results in formation of overactive classical complement convertase insensitive to regulation by CD55.

Abstract

Background

Dysregulation of the complement system by excessively active components of the alternative pathway (AP) convertases associate with rare kidney diseases. Gain-of-function variants of complement factor B (FB) are acknowledged etiological factors of atypical hemolytic uremic syndrome (aHUS) and C3-glomerulopathy (C3G).

Objective

Since complement C2 plays an analogous role in the classical pathway (CP) to its paralogue FB in the AP, we screened a cohort of 233 patients suffering from aHUS and C3G for mutations in C2.

Methods

Eight identified mutants: R129H, R243C, S250C, E318D, K415N, D417H, D511N, S574P and wild type (WT) C2 were expressed in eukaryotic system as C-terminally His-tagged proteins and subjected to a panel of functional assays.

Results

All mutants were less active than WT-C2 in hemolytic and CP convertases activity assays performed on sensitized human erythrocytes and sensitized human lymphoma cell lines, except the S250C variant, which showed significantly higher lytic activity and higher CP convertase activity. Analysis of complement inhibitors on tested cells, experiments on CD55-knockout cells and C3b deposition in the presence of soluble CD55 ectodomain suggest resistance to regulation by CD55 as a reason for the hyperactive phenotype of S250C mutant.

Conclusions

Our finding is the first report of gain-of-function mutation in CP component found in human disease. S250C was the only rare variant in complement genes of analyzed aHUS patient. We propose that excessive activity of the CP convertases may also predispose to rare glomerulopathies and therefore routine diagnostic of such patients should not be focused only at the abnormalities in AP.

Introduction

The complement system is not only a basic defense mechanism protecting us from invading pathogens but also an important player involved in the maintenance of body homeostasis.[1] As proper functioning of complement is based on the interplay between its activators and inhibitors, functional impairment of particular component often ends up in autoimmune or inflammatory diseases.[2] Kidney is the organ especially susceptible to complement-mediated damage due to relatively high blood flow combined with a delicate structure of filtrating barrier, fenestrated endothelium and thin glomerular capillaries.[3,4] Additionally, glomerular vessels are vulnerable to thrombosis and damage by heme originating from local hemolysis of red blood cells.[5,6] Complement attack on kidney structures can be mediated by abnormalities in the alternative complement pathway (AP).[4,7,8] This route is constantly active at a low level and spontaneously deposits C3 activation fragments (C3b) on body surfaces.[9] Unlike pathogens, self-cells are equipped with a panel of complement inhibitors, which block further pathway propagation. Therefore the loss of control on AP is a condition permissive for complement-mediated damage. The underlying mechanism may involve the presence of autoantibodies termed C3NeF, which stabilize AP convertases – a key enzymatic complexes, which amplify complement cascade[10], autoantibodies binding to soluble complement inhibitors like factor H (FH)[11,12], inheritance of rare variants of membrane complement inhibitors (e.g. CD46)[12,13] or gain-of-function mutations in complement components like C3[14,15] or factor B (FB)[16-20], which renders them insensitive to regulation. Importantly, FH autoantibodies, hyperactive mutants of C3 and FB, as well as mutated CD46, impair convertase activity, which seems to be the most strictly controlled stage throughout whole complement cascade, either in AP or classical/lectin pathway (CP/LP). Although impairment of the AP convertase is the most frequent cause of complement-mediated renal diseases, there are reports suggesting that abnormalities in CP also play a role in pathogenesis. Previously we screened thirteen patients with C3 glomerulopathy (C3G) for alterations increasing/prolonging convertase activity and found one individual, in whom there were no acknowledged risk factors but antibodies stabilizing the CP convertases (C4NeF).[21] The similar screen of a cohort of 168 patients with C3G screen yielded five patients C4NeF-positive (two of them had also C3NeF or anti-FH autoantibodies). Genetic analysis revealed no rare or novel variants of complement genes.[22] Even if C4NeF is present in a small percentage of individuals suffering from C3G, it may the sole pathogenic factor found in certain patients. Since standard diagnostics of complement-mediated renal diseases was so far focused on abnormalities in AP, the possible role of CP in pathogenesis may be underestimated. Up to now, no natural gain-of-function variants of any complement protein belonging to CP was known. We decided to look for such naturally occurring mutations in complement C2 protein – a CP analogue and paralogue of FB. C2 and FB are believed to evolve from a common ancestor gene by duplication and further accumulation of changes in duplicated DNA.[23] Fragments derived from activated C2 (C2a) and activated FB (Bb) act as proteolytic subunits in CP and AP convertases, respectively. These two proteins share a high degree of amino acid similarity, almost identical length and structural organization[24], (Fig. S1). Therefore it is highly probable that they contain similar mutational hotspots, as already demonstrated by translation of gain-of-function mutations in FB to C2.[25] All above along with the fact that pathogenic, naturally occurring gain-of-function mutants of FB are already characterized encouraged us to look for analogous pathogenic mutations in C2.

Materials and methods

Genetic screening of patients diagnosed with aHUS and C3G.

A total of 233 aHUS and C3G patients included in the Spanish aHUS/C3G Registry (https://www.aHUSC3G.es) until 29th Aug 2016 were studied in this report. Patients diagnosed with aHUS fulfilled the following criteria: platelet count $<150\times10^9$/L or decrease of > 25% from baseline

values, hemolytic anemia and serum creatinine level greater than the upper limit of the normal range, together with a negative Coombs test, normal activity of ADAMTS-13 and negative Shiga Toxin. C3G was diagnosed by renal biopsy, which in many cases included analysis by electron microscopy. DNA from patients were analyzed for genetic variants using an in house next generation sequencing (NGS) panel that includes 44 genes. A detailed list, the procedure of sequencing and data analysis is described in Supplementary_M file.

Cell lines

Human lymphoma cell lines Raji and Ramos (both obtained from ATCC) were cultured in RPMI 1640 medium with L-glutamine (Mediatech) supplemented with 10% foetal bovine serum (PANBiotech). Cells were cultivated at 37°C and humidified 5% CO_2 atmosphere. Raji cells with CD55 knockout were produced by CRISPR/Cas9 technology as described in [26].

Protein expression, purification and analysis

Wild-type C2 cDNA sequence (accession number NM_000063) additionally containing six histidine codons at 3′ terminus, as well as sequences for R129H, R243C, S250C, E318D, K415N, D417H, D511N, S574P and Y347A variants were codon optimized, synthesized and cloned into pCEP4 vector in the framework of GeneArt Gene Synthesis service by Thermo Fisher. Proteins were expressed in eukaryotic system and purified as described.[27] Western blotting was developed with anti-C2 antibody (Complement Technology) diluted 1:1000 followed by HRP-conjugated donkey anti-goat antibody 1:10000 (Jackson ImmunoResearch). Mass spectrometry analysis was performed as in [28].

Hemolysis-based assay

Hemolytic assays evaluating the activity of recombinant, his-tagged C2 mutants were performed as described previously[29], with some modifications. Sheep erythrocytes (Biomaxima) were sensitized with amboceptor (Behring) diluted 1:1000 in 1 ml $DGVB^{++}$[29] for 20 min at 37°C. Human erythrocytes were sensitized with anti-human red blood cells antibodies (Rockland) diluted 1:75. Afterwards, cells were washed three times, pelleted and resuspended in 1 ml of DGVB++. Ten microliters of such suspension were overlaid with serial dilutions of C2-depleted serum (ΔC2) (Complement Technology) in DGVB++, supplemented with particular C2 mutant and incubated for 30 min at 37°C. Erythrocytes were centrifuged and hemoglobin released to supernatant was measured at 405 nm in Synergy H1 microplate reader (Biotek). For CP convertase activity assay sensitized cells were mixed with 2.5 μg/ml of C5 inhibitor OmCI.[29] The plate was incubated at 37°C and 2% of C2-depleted serum mixed with a particular C2 mutant diluted in DGVB++ was added at indicated time points. Then erythrocytes were washed with EDTA-GVB[29], pelleted, overlaid with EDTA-GVB containing 1:40 dilution of guinea pig serum (Harlan Laboratories) and incubated for 30 min at 37°C.

Complement-dependent cytotoxic (CDC) assay

CDC was measured by calcein release assay as described before[30] but ΔC2 serum was used instead of FB-depleted serum. Cell lysis was calculated in reference to the fluorescence readout (490/520 nm) obtained for supernatant of cells treated with 2% NP40 (full lysis).

CP convertase assays on CD20-positive cells

Ofatumumab (100 μg/ml) and 30% of C5- or C3-depleted serum (Complement Technology) supplemented with 7.5 μg/ml of analyzed C2 variant were added to calcein-loaded cells after indicated time points. Then, cells were washed with EDTA-GVB buffer, pelleted and overlaid with EDTA-GVB

containing 1:20 dilution of guinea pig serum followed by 30 min incubation (37°C, 600xg). Fluorescence readout was performed as described before.

Cleavage with C1s enzyme

Each C2 mutant was incubated for 2 hours with 4 nM of C1s enzyme (Complement Technology) diluted in 5 mM veronal buffer supplemented with 1 mM of Ca^{2+} and Mg^{2+}. Cleavage into C2a and C2b was analysed by Western blot. C2 polyclonal goat anti-human antibody (#A212, Complement Technology) diluted 1:1000 and secondary donkey anti-goat antibody conjugated with HRP (Jackson Laboratory) diluted 1:10 000 were used for detection, followed by 3,3'-diaminobenzidine (Vector Laboratories) applied for visualization.

C3b microplate deposition assay

ELISA plates were coated with 50 mg/ml of human immunoglobulin solution (Pentaglobin; Biotest) for 1h at 37°C, then blocked for 30min with 3% fish gelatin (Sigma). Afterwards, plates were incubated with 0.5% ΔC2 serum and particular C2 variant diluted in gelatin veronal buffer with or without the addition of soluble ectodomain of CD55 inhibitor (produced as described in[31]) 1h at 37°C. C3b detection was performed with polyclonal goat-anti human C3 antibody (Complement Technology), followed by rabbit anti-goat antibody conjugated with HRP (Dako) diluted 1:10 000 and 1:5000 in PBS, respectively. The assay was developed using *o*-phenylenediamine dihydrochloride (OPD, Sigma) according to manufacturer's instructions.

Evaluation of complement regulatory proteins

Expression of CD35, CD46 and CD55 in CD20-positive cells and human erythrocytes was assessed by flow cytometry as described in [32]. Primary antibodies (clone UJ11 for CD35, clone MEM-258 for CD46, clone HI-55a for CD55) and isotype controls were purchased from Immunotools.

Assessment of amino acid conservation in C2 protein

Amino acids sequences of C2 and Factor B proteins were retrieved from UniProt[33] amino acids sequence database searched for term "complement C2" and results were further manually filtered out to obtain only sequences of C2 and Factor B proteins. For further analysis, 151 sequences were selected. All selected sequences were subjected to multiple sequence alignment (MSA) using MAFTT algorithm.[34]

Results

Mutations in C2 protein identified in patients with aHUS/C3G.

We have identified eight missense mutations in C2 protein while screening 233 patients suffering from aHUS/C3G. Detailed information on nucleotide and amino acid substitutions, the number of patients in the cohort with particular rare C2 variants and diagnosis is given in Table 1. Figure S1 shows the localisation of identified mutations within C2 domains and a similar presentation of known gain-of-function mutations in factor B.

Table 1 Detailed characteristics of missense mutations identified in the cohort of 233 patients

Position on chromosome 6	ID	Number of patients	cDNA substitution	Amino acid substitution	Genotype	Diagnosis of patients with mutation	risk polymorphism		Other potential pathogenic complement genetic variants	Anti FH antibodies	C3Nef	Autoantibodies
							MCP	FH				
31896638	rs367996721	1/233	c.G386A	p.R129H	HET	aHUS	HOM	HOM	None	No	-	ANA
31901954	rs370121006	1/233	c. C727T	p.R243C	HET	aHUS	NO	HET	p.K591Sfs*10: MASP1:HET	No	-	ANCA
31901976	rs150827255	1/233	c. C749G	p. S250C	HET	aHUS	HOM	NO	None	-	-	-
31903804	rs9332739	11/233 (*)	c. G954C	p. E318D	All HET	aHUS, C3GN	-	-	-	-	-	-
31910761	-	1/233	c. G1245C	p. K415N	HET	aHUS	NO	HET	None	No	-	No
31910765	rs907804461	1/233	c. G1249C	p. D417H	HET	C3GN	HET	NO	None	No	No	ANA
31911268	rs142802105	1/233	c. G1531A	p. D511N	HET	C3GN	HOM	NO	None	No	No	No
31911573	-	1/233	c. T1720C	p. S574P	HET	C3GN	HET	HET	None	No	No	ANA

*) Common polymorphism with minor allele frequency (MAF) >0.01. The rest of sequence variants display a MAF of less than 0.001; HET – heterozygous, HOM – homozygous, ANA – anti-nuclear antibodies, ANCA – anti-neutrophil cytoplasm antibodies

Protein expression and purification

Wild type and nine mutant proteins of complement C2 were purified. Proteins migrated at the same apparent molecular weight as assessed by Coomassie staining (Fig. S2). Variant Y347A, identified by *in silico* prediction and described as resistant to CD55-, CD35- and C4BP-mediated convertase decay [22] was included as a gain-of-function positive control.

Hemolytic activity of his-tagged recombinant C2 proteins

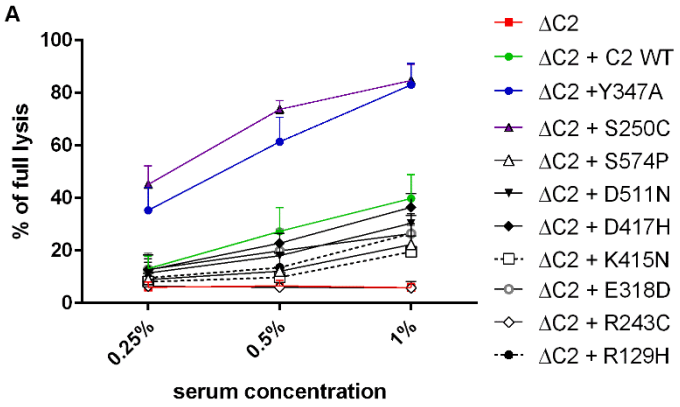
Hemolytic assay performed in C2-depleted human serum (ΔC2) confirmed that addition of his-tag, together with the process of protein expression and purification, did not affect the activity of C2 variants. There were no statistically significant differences in the hemolytic activity of recombinant (his-tagged) wild-type C2 (WT C2) and plasma purified C2 (Fig. S3A) and no differences between normal human serum (NHS) and ΔC2 reconstituted with recombinant WT C2 (Fig. S3B). Among all natural C2 mutants, the R243C variant was completely devoid of hemolytic potential, while the remaining ones performed weaker than WT C2 except for the S250C variant, which displayed significantly higher activity than wild-type protein. No hemolysis was observed when ΔC2 alone was used (Fig.1A).

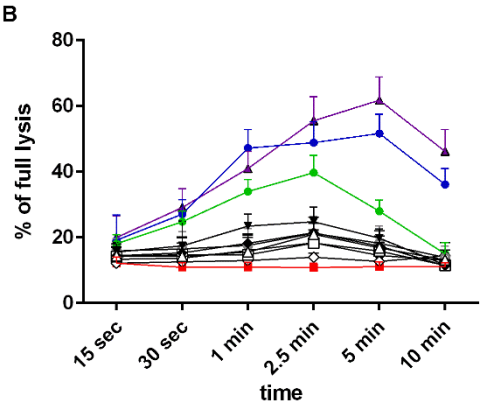
CP convertase functional assays of C2 protein variants performed on sensitized human erythrocytes.

Consistent with the result of hemolytic assay, convertase activity was absent when ΔC2 serum alone was used or when supplemented with the R243C variant. C2 Y347A and S250C variants formed CP convertase of higher activity and prolonged half-life comparing to WT C2, while the remaining analyzed variants created CP convertase of significantly lower activity (Fig. 1B). Interestingly, when the same experiment was performed using sensitized sheep erythrocytes equipped with homologous membrane-bound complement inhibitors of unknown compatibility with human system, the gain-of-function character of S250C variant was lost but hyperactivity of convertase built from C2 Y347A variant could be still observed (Fig. S4). These results suggest that interaction with one or more

membrane-bound complement inhibitors, but not soluble inhibitors present in serum is critical for gain-of-function character of S250C. The Y347A mutation was shown to impair not only interactions with membrane-bound inhibitors CD55 and CD35/CR1 but also with soluble inhibitor C4BP[25], which could explain our current results.

Fig.1 Hemolytic and convertase activity of his-tagged, recombinant C2 variants.





	1% serum	0.5% serum	0.25% serum
S574P	**	*	n.s.
D511N	n.s.	n.s.	n.s.
D417H	n.s.	n.s.	n.s.
K415N	***	**	n.s.
Y347A	***	***	***
E318D	*	n.s.	n.s.
S250C	***	***	***
R243C	***	***	*
R129H	*	*	n.s.
NHS	n.s.	n.s.	n.s.

	0.25 min	0.5 min	1 min	2.5 min	5 min	10 min
S574P	n.s.	*	***	***	**	n.s.
D511N	n.s.	n.s.	*	***	n.s.	n.s.
D417H	n.s.	*	***	***	*	n.s.
K415N	n.s.	*	***	***	**	n.s.
Y347A	n.s.	n.s.	***	***	***	***
E318D	n.s.	n.s.	***	***	*	n.s.
S250C	n.s.	n.s.	n.s.	***	***	***
R243C	n.s.	**	***	***	***	n.s.
R129H	n.s.	**	***	***	***	n.s.
NHS	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

A) Human erythrocytes were sensitized, mixed with C2 depleted human serum (ΔC2 serum, negative control) or alternatively with serial dilutions of ΔC2 serum supplemented with a physiological concentration of particular C2 variant. After 30 minutes erythrocytes were centrifuged and the amount of released haemoglobin was measured at 405 nm. B) CP convertase assay performed on sensitized human erythrocytes. Erythrocytes were incubated with ΔC2 serum supplemented with a physiological concentration of particular C2 variant mixed with C5 blocker for the indicated time and afterwards washed and mixed with guinea pig serum diluted 1:40 in EDTA-containing buffer in order to develop lytic sites formed in the previous step and disable *de novo* convertase formation. The graphs show the mean results obtained from three independent experiments and error bars show standard deviation. Tables present results of statistical analyses and denote significance (*, ** or ***) at $p< 0.05$; 0,01 and $p<0.001$ respectively, according to Dunnett's multiple comparison test for non-repeated measures. Dark grey represents significantly higher and light grey represents significantly lower readout in comparison to plasma purified protein.

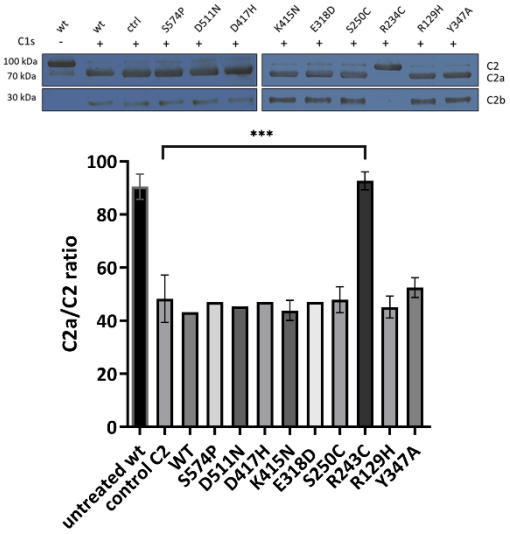
Evaluation of C2 variants cleavage by C1s

Arginine$_{243}$ is a cleavage site of C2 protein by C1s enzyme. We confirmed that R243C mutant is not cleaved into active components C2a and C2b (Fig. 2), thus explaining its inability to create functional CP convertase. All other variants presented the same pattern of cleavage and comparable yield (Fig. 2). Considering this result, variant R243C was not included in further experiments.

Functional analysis of C2 variants on nucleated cells

In order to get a broader insight into consequences of each mutation on C2 function, we analysed CDC in two CD20-positive human cell lines characterized by a pattern of membrane-bound complement inhibitors different than human erythrocytes (Fig. S5). Addition of C2 variants Y347A and S250C significantly increased lysis of Raji and Ramos cells in the presence of anti-CD20 mAb ofatumumab (Fig.3). Application of three out of eight mutants identified in C3G/aHUS patients, S574P, K415N and R129H, led to significantly decreased CDC in both cell lines, comparing to C2 WT protein.

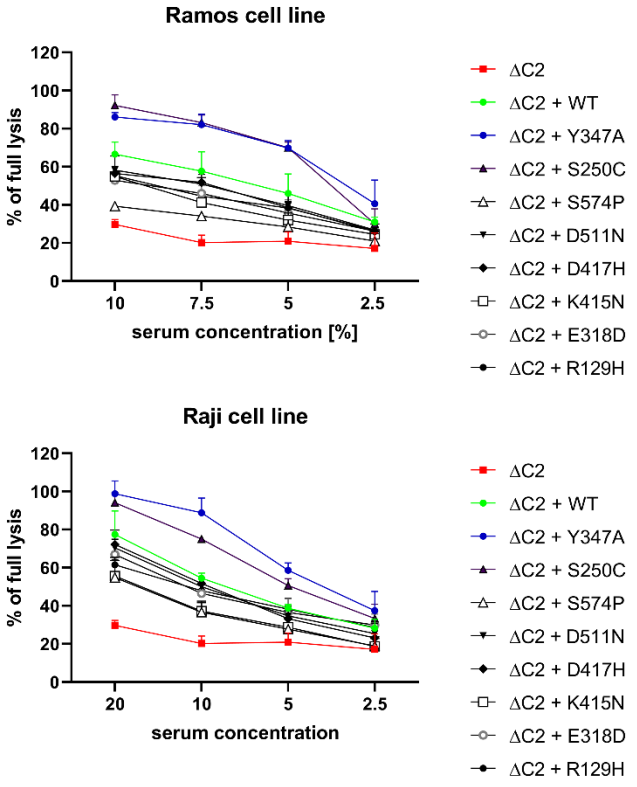
Fig.2 Cleavage of C2 variants by C1s enzyme



Representative Western blot (top panel) and associated densitometric analysis (bottom) present formation of C2a and C2b after 2h incubation with 4 nM C1s of each C2 variant. The graph shows the results obtained from three independent experiments. Statistical significance – (***) p<0.001, in comparison to plasma C2 (control), was calculated according to Kruskal-Wallis test

Variant E318D presented slightly lower CDC potential in Ramos cells in 20% serum (Fig. 3_table). To further confirm the phenotype of S250C mutation we assessed its ability to form CP convertases on the surface of nucleated cells. Technical issues, i.e. inability to arrest lysis of sensitized CD20-positive cells in the presence of C5 blockers, did not allow us to perform the same type of convertase assay as for human erythrocytes. Instead, we performed similar convertase assay in which mutated C2 proteins were added to C3-depleted serum (C3 CP assay) and C5-depleted serum (C5 CP assay). Assays performed on Ramos cells revealed that compared to wild-type protein, the S250C variant formed C3 convertase of higher maximal activity as well as C5 convertase of both higher maximal activity and prolonged half-life (Fig. 4). C3 and C5 convertase activity curves of the S250C mutant were similar to these of Y347A, a known gain-of-function variant, which was constructed based on in-silico modelling prediction.[25]

Fig. 3 Cytotoxic activity of C2 variants using lymphoma cell lines

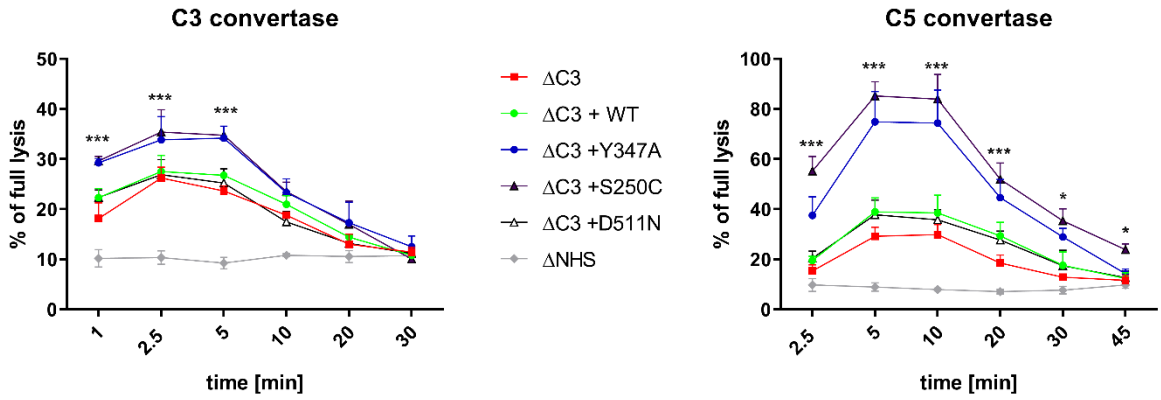


	2.5% serum	5% serum	7.5% serum	10% serum
S574P	n.s.	**	***	***
D511N	n.s.	n.s.	n.s.	n.s.
D417H	n.s.	n.s.	n.s.	n.s.
K415N	n.s.	*	**	n.s.
Y347A	n.s.	***	***	***
E318D	n.s.	n.s.	n.s.	*
S250C	n.s.	**	***	***
R129H	n.s.	n.s.	*	n.s.
NHS	n.s.	n.s.	n.s.	n.s.

	2.5% serum	5% serum	10% serum	20% serum
S574P	n.s.	n.s.	**	***
D511N	n.s.	n.s.	n.s.	n.s.
D417H	n.s.	n.s.	n.s.	n.s.
K415N	n.s.	n.s.	**	***
Y347A	n.s.	***	***	***
E318D	n.s.	n.s.	n.s.	n.s.
S250C	n.s.	n.s.	***	**
R129H	n.s.	n.s.	n.s.	**
NHS	n.s.	n.s.	n.s.	n.s.

Ramos (upper panel) or Raji (below) were sensitized with anti-CD20 antibody and incubated with serial dilutions of C2-depleted serum, supplemented with a physiological concentration of indicated C2 mutant. Normal human serum (NHS) and heat-inactivated serum (ΔNHS) were used as positive and negative controls, respectively. Statistical significance at p level < 0.05*, p < 0.01** and p < 0.001*** in comparison to cell lysis obtained by the addition of wild type C2 (WT) was calculated according to Dunnett's multiple comparison test for non-repeated measures and presented in tables (dark grey – significantly higher, light grey – significantly lower readout). Graphs present data from three independent experiments and error bars show standard deviation.

Fig.4 Formation of C3 and C5 convertase by C2 mutants on sensitized lymphoma cells



Ramos cells were sensitized with anti-CD20 antibody and incubated for the indicated period of time with 15% of C3 or C5-depleted serum (ΔC3 or ΔC5) supplemented with particular C2 mutant. Heat-inactivated serum (ΔNHS) was applied as a negative control. Statistical significance at p level < 0.05*, p < 0.01** and p < 0.001*** in comparison to cell lysis obtained by the addition of wild type C2 (WT) was calculated according to Dunnett's multiple comparison test for non-repeated measures. Graphs present data from three independent experiments and error bars show standard deviation.

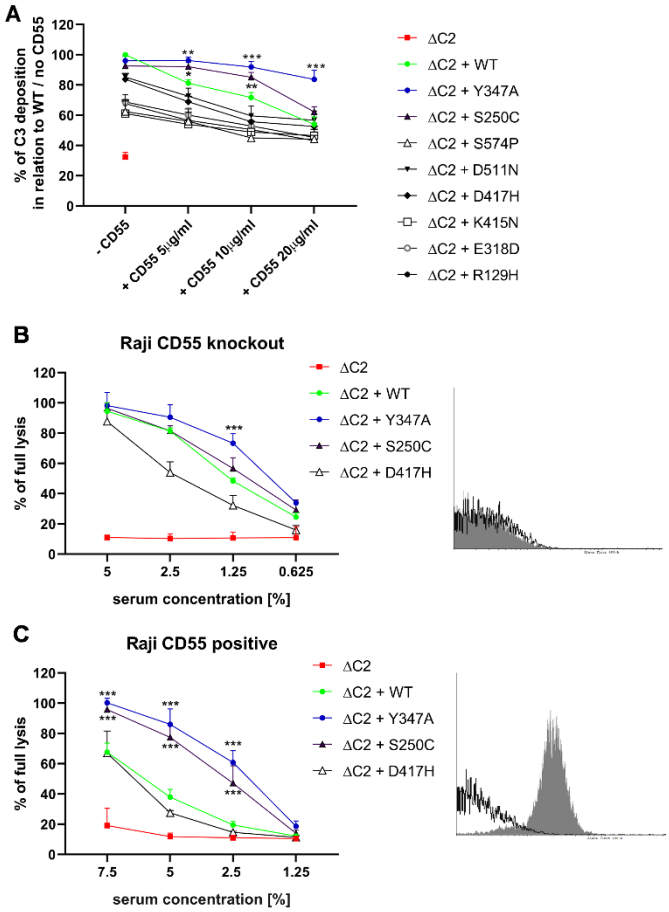
Explanation of gain-of-function phenotype of S250C variant

Results of experiments performed on human (Fig. 1) and sheep erythrocytes (Fig. S4) in which the same batch of C2-depleted serum was used suggested that phenotype of the S250C mutation relies mostly on CP convertase interaction with membrane-bound inhibitors. There are three known membrane-bound complement inhibitors capable to influence the activity of classical convertases: CD35 (CR1), CD46 (MCP) and CD55 (DAF). However, our flow cytometry analysis confirmed that CD46 is not expressed by human erythrocytes. On the other hand CD20-positive cells used in experiments, which demonstrated gain-of-function phenotype of S250C, do not express CD35 (Fig. S5). Therefore, the only known CP convertase membrane-bound inhibitor present on both erythrocytes and CD20-positive cells is CD55. To further investigate the mechanism leading to enhanced activity of CP convertase formed by the S250C variant we analysed deposition of C3b onto the IgG-coated plate in the presence of soluble ectodomain of CD55. Results demonstrate that basal production of C3b (with no CD55 added) by convertases formed by Y347A and S250C mutants was comparable to that exerted by convertases formed by wild-type C2 (control) or from normal human serum. As expected from previous results, convertases formed from other C2 mutants generated less C3b (range 60-85% of control). Importantly, upon addition of 5 and 10 µg/ml of the soluble ectodomain of CD55, S250C–containing convertases produce significantly more C3b, comparing to wild-type (WT) protein. Similarly, the Y347A mutant showed enhanced resistance to CD55 (Fig. 5A). In case of all the other variants, the addition of CD55 resulted in the deposition of C3b comparable to wild-type or lower. To ultimately confirm that interaction with CD55 is crucial for the phenotype of S250C variant, we performed CDC assay on CD55-knockout Raji cells. S250C mutant performed as C2 WT when CD55-negative cells were used (Fig. 5B) whereas application of CD55-positive control cells restored previously observed (Fig. 3) enhancement of CDC by this variant (Fig. 5C).

Molecular modelling and analysis of evolutional conservation of mutations in complement C2 protein.

We analysed the degree of conservation of particular amino acids at sites, which were found mutated in our cohort of patients with aHUS/C3G. Out of 151 sequences selected for alignment serine residue in position 250 is strongly conserved among all sequences of C2 and Factor B, similarly to arginine residue in position 243 (cleavage site of C2a-C2b) and arginine in position 129. The occurrence of other residues analyzed in this study varies between 29% (D511) to 75% (Y347) (Table 2). According to our model of C2a fragment (based on PDB record 2I6Q,[35]) S250 and Y347 residues, mutations of

Fig. 5 *Evaluation of interaction with CD55 inhibitor*



A) The graph presents C3b production and deposition on a microplate. 0.5% C2-depleted serum (0.5%) (ΔC2) supplemented with a particular C2 mutant was incubated for 1h on a plate coated with human IgG. Results show the level of C3b deposition relative to wild-type protein (WT) with no CD55 added. Serum alone (ΔC2 serum) was applied as a negative control. B) Raji CD55 knockout cells and C) control Raji cells were sorted to obtain homogenous populations of no- or high-expression of CD55, then cultured for several days and re-checked by flow cytometry (islets in figures). White and grey areas on histograms indicate cells stained with isotype control and anti-CD55 antibody, respectively. Cells were sensitized with anti-CD20 antibody and incubated with serial dilutions of C2-depleted serum, supplemented with a physiological concentration of indicated C2 mutant. C2-depleted serum alone was used as a negative control. The graphs represent average results obtained from three independent experiments. Statistical significance was analyzed by Dunnett's multiple comparison test for non-repeated measures (** - p <0.01, *** - p <0.001) in comparison to WT C2.

which lead to gain-of-function phenotype are located only about 22 Ångströms apart, in the three-dimensional structure of the C2 protein, therefore these two amino acid residues could form together binding site for single protein molecule (Fig. 6)

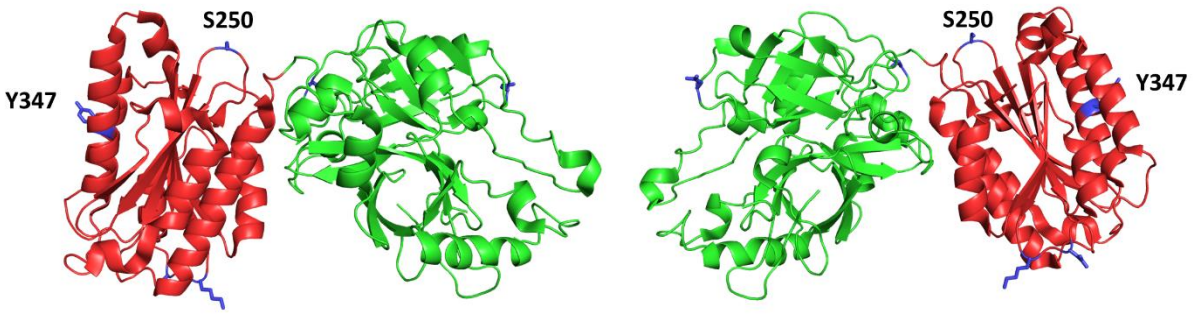
Discussion

Domain structure of FB and complement C2 share high degree of similarity[24]. The von Willenbrand type A (vWA) domain of FB contains several mutational hot spots, alterations of which may provoke potentially pathogenic gain-of-function phenotype[16,17]. We identified one rare variant of C2 with mutation at conserved position 250 adjacent to α-helixes forming vWa domain, which lead to the formation of hyperactive CP convertases. No previous studies have shown the importance of this position for the biological activity of C2 or FB. Moreover, no prior studies have presented patients with gain-of-function mutations in complement genes in CP/LP. Thus, to our best knowledge, this is the first such report confirmed by functional studies. Of note, S250C was the only rare variant of complement genes identified in the patient suffering from aHUS (Table 1). Genetic screening in patients diagnosed with either aHUS and C3G is usually limited to genes encoding AP elements such as FB, CFH, C3, CFI and CD46 [36] and based on this scheme, abnormalities in complement are found in 70% of aHUS patients and 30% remain unexplained.[37]

Unlike AP, CP and LP require a specific stimuli, e.g. antibodies or certain sugar moieties or C-reactive protein (CRP).[1] Therefore, two conditions permissive for CP/LP-mediated autoimmune disease are necessary: loss of proper pathway regulation (e.g. formation of decay-resistant CP/LP convertase) and presence of stimuli driving the pathway. Hypothetical explanation of disease development by carriers of S250C mutation may come from an analysis of aHUS cases, which penetrance is estimated at 50%.[38,39] Typically, aHUS patients are heterozygous in complement mutations and the same mutation can be carried by healthy relatives. Often, the difference between healthy relatives carrying pathogenic mutations and patients is the presence of predisposing polymorphisms in genes coding complement

regulators.[40-42] Moreover, aHUS can be preceded by bacterial and viral infections, surgeries, use of certain medications and pregnancy in women.[43,44] Once complement is activated in these conditions, genetic factors prevent adequate protection of glomerular endothelium with aHUS episode as a result. Thus, pathogenic mutations, predisposing common variants and environmental factors form multiple hits that together precipitate the disease.[41,45,46] The patient with S250C mutation in C2 carries *MCPggaac* haplotype (a combination of common polymorphisms), which decreases transcriptional activity of CD46 promotor region.[41,47] Of note, *MCPggaac* haplotype dramatically increased penetrance of gain-of-function mutation in factor B, a functional analogue of C2 in the alternative complement pathway.[16]

Fig. 6 Structure (two views) of C2b protein (based on PDB record 2I6Q).



The von Willebrand factor A-type domain is shown in red and the C-terminal trypsin-like serine proteinase domain is shown in green. Position of mutated residues are shown in blue, resides S250 and Y347 are labeled.

Table 2 Occurrence (in percent) the most frequent amino acid types in selected positions.

Analyzed amino acids position in H.sapiens C2 protein sequence studied in this work	Occurrence the most frequent amino acid types observed in given position
R129	> 99% R
R243	> 99% R
S250	> 99% S
E318	52% E, 30% W
Y347	75% Y, 15% H
K415	31% P, 24% K, 23% N
D417	39% D, 37% V, 11% E
D511	29% V, 17% D, 17% N, 13% H
S574	69% S, 18% D

Presented data are based on multiple sequence alignment of 151 C2 and Factor B amino acid sequences.

Our results suggest that abnormalities in CP/LP may be an important etiologic factor of rare renal diseases when associated with certain risk haplotypes / polymorphisms. In line with reports on C4NeF as the only complement abnormality found in patients with C3G[21,22] we postulate that analysis of CP/LP activity should be included in routine diagnostic procedures of patients diagnosed with aHUS and C3G. Patients with known CP/LP gain-of-function mutations should be closely monitored during infections for first signs of aHUS/C3G so that therapy can be started as early as possible to limit renal damage. We also underline the necessity of performing CP functional assays on the accurate model. Hemolytic assay on sheep erythrocytes, which is a commonly used method in the field may not reflect gain-of-function character of certain mutations, which impair interaction with human membrane-bound inhibitors. Probably we would miss the phenotype of S250C mutation if we relied only on this well-established model and abandoned additional experiments. We faced a similar problem when analyzing gain-of-function mutants of FB. Addition of such mutants to sensitized sheep erythrocytes had a limited effect on hemolysis comparing to differences in CDC revealed on human nucleated cells.[30]

Serine to cysteine substitution is considered to be one of the most conservative changes possible between any pair of amino acid residues in proteins. Therefore, the fact that S250C exchange has a profound impact on the function of C2 is surprising. However, there is a study elaborating on a bigger atomic radius of sulphur in a cysteine than the radius of oxygen in the serine and its impact on the local geometry in a protein [48]. On the other hand introduction of free cysteine at the surface of the protein can result in the formation of homodimers or heterodimers with other ubiquitous proteins as well as the formation of adducts with low molecular weight chemical entities (e.g. glutathione). Western blot analysis of S250C mutant under non-reducing conditions showed no obvious bands

above the peak of the full-length protein (103 kDa, Fig. S2B). Mass spectrometry analysis focused at the area between 102-104 kDa revealed the existence of three isoforms within the full length WT and S250C proteins (probably due to changes in glycosylation) thus making unanimous conclusion on molecular mass differences problematic.

Seven out of 233 patients had mutations in C2 resulting in low CP hemolytic activity and no other rare variant in their complement genes except the carrier of R243C mutation (Table 1). Causative nature of these variants for aHUS/C3G is less obvious than of S250C. Since the low physiological concentration of C2 makes it a bottleneck of CP/LP, such patients may have impaired clearance of bacteria. Indeed, E318D mutation identified in 11 out of 233 patients from our cohort was previously associated with an increased rate of pneumonia in trauma patients.[49] Infections with non-STEC (Shiga toxin-producing *Escherichia coli,* a causative factor of "typical" HUS)[50] strains such as *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* or *Bordatella pertussis*[50-54] often precede aHUS/CG3 episodes, however genetic predisposition to infections and connotations to aHUS/C3G were not intensively investigated. Functional impairment of C2 may possibly influence this aspect but it has to be confirmed by epidemiological studies. Nonetheless, on one hand, genetic C2 deficiency is one the most common complement deficiencies with the occurrence of 1:20.000 homozygous carriers [55] and on the other hand there is a limited number of reports describing the prevalence and no data on functional characteristics of missense C2 mutants. Importantly, data on the prevalence of E318D mutation (a common polymorphism) are available and state its global minor allele frequency of 0.0297 (ClinVar: variation ID 12130) whereas analogous value in our cohort is lower and amounts to 0.0236. Therefore we consider the representation of loss-of-function E318D variant in our aHUS/C3G group as not exceeding global distribution and probably not pivotal for disease pathomechanism.

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Authorship Contributions

Concept of the study: MO
Experimental work and/or supervision: AU; EV; GS; AF; IJ; MT; SO; EA; LvdH; AMB; SRdC
Analysis of patients' data: EA; SRdC
Writing the manuscript: AU; EV; AF; AMB; SO; LvdH; SRdC, MO

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