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A Cell-Based Assay to Measure the Activity of the Complement Convertases

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Introduction: The complement system serves as a crucial defense mechanism against invading pathogens; however, dysregulation of this system can result in harmful consequences. Central to the complement cascade are the classical pathway (CP) or lectin pathway (LP) and the alternative pathway (AP) convertases. Aberrant regulation of the convertases is often implicated in the development of rare complement-related diseases. However, analyzing convertase activity poses a significant challenge due to their labile nature and intricate interactions with serum proteins.

Methods: In this study, we propose a novel assay for the functional evaluation of these complexes. Our approach leverages a widely available human lymphoma cell line, which when sensitized with antibodies, triggers activation of the CP with a substantial amplification by the AP. The combined action of 2, C5 blockers eculizumab and crovalimab let the cascade proceed up to the level of convertases but not further. In the next step, C5 inhibitors were washed away and guinea pig serum in ethylenediamine tetraacetic acid (EDTA) buffer supported the development of lytic sites on the platform of preexisting convertases.

Results: The assay detects recombinant gain-of-function (GoF) components of both convertase types within human serum or plasma. Furthermore, we demonstrate the assay's practical utility in analyzing nephrological patients harboring C3 genetic variants and illustrate its capacity to distinguish between patients and asymptomatic relatives carrying the same pathogenic C3 variant.

Conclusion: We provided a proof-of-concept of a new assay that detects convertase overactivity in individuals carrying variants of both pathogenic character or those of unknown significance in ubiquitous complement proteins such as C3.

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The complement system is one of the body's first lines of defense and a key part of our innate immunity. It has three activation routes and consists from more than 40 proteins, providing broad protection against pathogens and helping in the scavenging of dying cells and in the response toward emerging malignancies.[1] Almost an equal number of complement proteins act as activators and inhibitors, emphasizing the need for precise regulation.[2] The oldest complement route, the AP, spontaneously activates to deposit C3b on nearby surfaces, leading to the destruction of target cells. The system distinguishes between self and nonself, based on host cell-bound complement regulators.[3] The CP and LP require specific triggers such as antibodies or sugar patterns on microbes; however, once they reach the C3-to-C3b conversion stage, these pathways use the AP mechanism to amplify their effects.[3] The transition from C3 to C3b is a crucial point in the complement cascade. Dedicated enzymatic complexes called C3-convertases amplify this process, whereas most complement regulators work at the convertase level to prevent excessive activity and potential harm.

Dysregulation of the convertases involves different mechanisms, including loss-of-function variants in the convertase regulators, GoF variants in the convertase components, and the presence of convertase-stabilizing autoantibodies.[4–7] The kidney is an organ extraordinarily vulnerable to complement attack due to high

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blood flow, fenestrated epithelium, and a delicate structure of filtration barrier.[8] Two rare kidney diseases, namely atypical hemolytic uremic syndrome (aHUS) and C3 glomerulopathy (C3G) are examples of pathologies that involve, in most cases, dysregulation of the convertase activity.[9] Other examples of such pathologies are age-related macular degeneration[10] and paroxysmal nocturnal hemoglobinuria.[11] Routine diagnostics of rare complement-related diseases comprise the measurement of pathway-specific total activity (e.g., AP50 and CH50), measurement of complement activation markers (e.g., Bb and sC5b-9), the levels of the main complement components such as C3 and C4 and the most common autoantibodies such as anti-factor H and C3-nephritic factor (C3Nef).[12] These measurements are also accompanied by genetic diagnostics aimed at identifying rare pathogenic genetic variants. However, due to the complex nature of the complement cascade and the possible involvement of complement in a multitude of potential comorbidities, the above-mentioned analyses may be insufficient to ensure a proper diagnosis and/or understanding of the pathogenic mechanism.[13] Classification of newly identified genetic variants into benign or pathogenic based on prediction algorithms may result in a false conclusion in a significant number of cases[14] and it is further complicated by their low penetrance.[8,13]

Herein, we propose a cell-based assay for the assessment of convertase activity. The overall idea is that one could use it as a single screening test to reveal whether the patient shows dysregulation at the level of the convertases. A positive result would give rationale to perform additional specific analyses searching for genetic or acquired factors impacting the convertase components or regulators. Moreover, the assay offers the possibility to assess the recombinant complement protein as well as the purified fraction of the patient's immunoglobulins in the context of altering convertase activity.

METHODS

Sera and Proteins

Normal human serum (NHS) was prepared from blood collections from healthy volunteers, in accordance with the approval from The Local Bioethical Committee at the Medical University of Gdańsk (approval number: NKBBN/500/2016). Blood collection, sample handling, and storage were performed as described previously.[15] Patients' serum and plasma samples were obtained from the Spanish aHUS/C3G Registry, and patients' informed consent for their use for scientific research was obtained. Next generation sequencing of complement genes was performed as described previously.[16] Human sera depleted from C1q, or C2, or factor B, as well as purified C1 complex were purchased from Complement Technology. Factor B blocker iptacopan was purchased from MedChemExpress. C3 blocker pegcetacoplan was provided by SOBI company. Recombinant wild-type and GoF variants of C2 and factor B were produced in the Hek293 Freestyle expression system and purified as described previously.[15,17]

CDC Assay

Complement-dependent cytotoxicity (CDC) was measured by calcein release assay as described previously,[18] with modifications. Briefly, Ramos cells were stained with Calcein-AM for 45 minutes and 100,000 cells per well were pelleted on a V-bottom 96-well plate (1000 g for 1.5 minutes) and overlaid with 50 μl of phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+} (PBS++) containing 25% of NHS, rituximab (50 μg/ml), and either eculizumab or crovalimab, or combination of these two antibodies in different concentrations. After 30 minutes incubation with shaking (37 °C, 650 rpm), the cells were pelleted, and the fluorescence of the supernatant was measured at 490/520 nm excitation/emission wavelength in Synergy H1 microplate reader (Biotek). The cell lysis was calculated in reference to the full lysis control (cells treated with 30% dimethylsulfoxide in water). The choice of Ramos cells as a model was based on our previous analyses of CD20 as well as membrane-bound complement inhibitors' expression on human lymphoma and leukemia cell lines,[19] which revealed that Ramos cells showed a high susceptibility to rituximab and thus, an opportunity to use relatively low serum/plasma concentration for monitoring convertase formation and decay.

Convertase Assay

Calcein-loaded Ramos cells were plated (100,000 cells/well) on a 96-well V-bottom plate in a volume of 100 μl and washed with 100 μl PBS++. The cells were pelleted and overlaid with 25 μl of the PBS++ containing rituximab (100 μg/ml), eculizumab (50 μg/ml) and crovalimab (50 μg/ml). The plate was incubated with shaking (37 °C, 650 rpm) and in the indicated time points, 25 μl of the 20% patient's serum or NHS, optionally supplemented with 5 μg/ml C2 variants or 36 μg/ml factor B variants, or 1 mM of iptacopan or 1 μg/ml pegcetacoplan were added. In some experiments, C1-depleted serum (Δ C1q) was used instead of NHS. Alternatively, when EDTA-plasma, not serum, was available as a patient's material, it was diluted in PBS with 5.5 mM concentration of $CaCl_2/MgCl_2$ prior to the experiment. The cells were then washed with 150 μl of PBS containing 40 mM EDTA (PBS–EDTA), pelleted and overlaid with 50 μl of PBS-EDTA containing 3.3% of guinea pig serum,

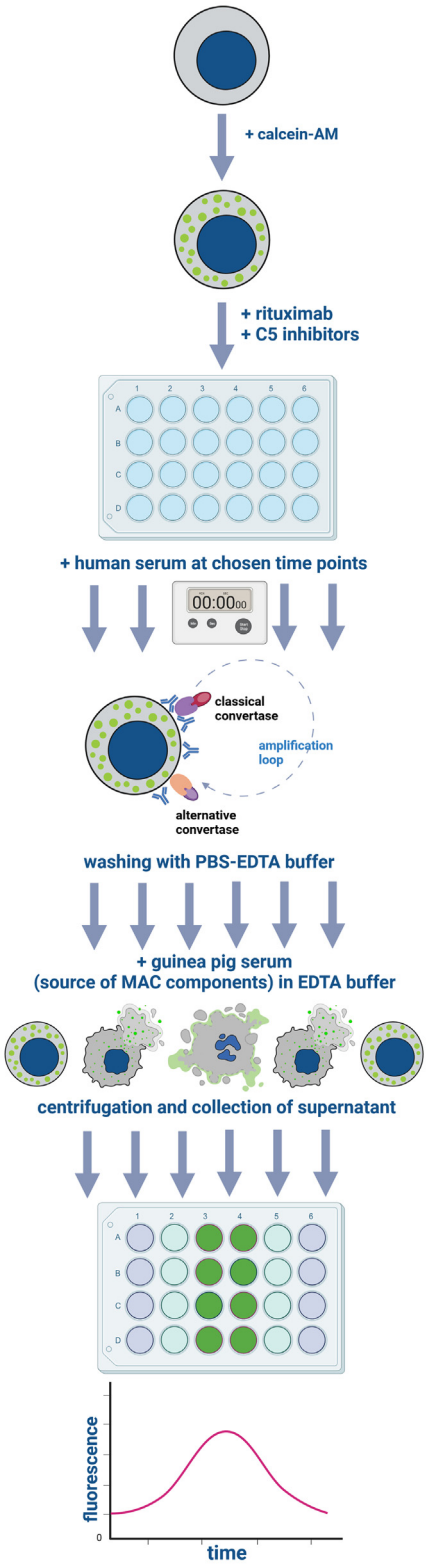

Figure 1. Simplified scheme of the assay. Ramos cells were loaded with calcein-AM that is metabolized intracellularly to its fluorescent derivative and then incubated with anti-CD20 antibody rituximab diluted in PBS buffer with 1 mM CaCl2/MgCl2, 10% human serum and 25 μg/ml of both C5 blockers. During this time, simultaneous processes of convertase formation and decay take place, but no complement-mediated lysis occurs due to inhibition of the cascade at the C5 level. Cells were washed at the certain time points to remove C5 blockers and incubated in guinea pig serum diluted in (continued)

followed by 30 minute incubation (37 °C, 650 rpm). The fluorescence of the supernatant was measured (490/520 nm) and the percent of the lysed cells was calculated in reference to full lysis control (cells treated with 30% dimethylsulfoxide in water). Statistical analyses were performed with Prism 9 software (GraphPad); comparisons between groups at particular time points were calculated using Dunnett's multiple comparison test.

RESULTS

Our plan was to apply the principles of convertase assay, which we originally set up for sensitized sheep erythrocytes.[20] This assay uses C5 blockers to divide the complement cascade initiated on the surface of red blood cells into the following two phases: (i) convertase assembly and decay and (ii) formation of membrane attack complex fueled by the C3-convertases assembled in the first step. Notably, the lysis of erythrocytes demands much lower serum concentration than the lysis of nucleated cells and previously we were not able to block the residual lysis of such cells using reasonable concentrations of C5 blockers eculizumab or OmCI (recombinant version of natural C5 inhibitor isolated from the saliva of soft tick).[21] Now, we decided to try a combination of eculizumab and a new generation C5-blocking antibody crovalimab.[22] First, we aimed to establish a concentration of C5 blockers that would effectively block the lysis of rituximab-sensitized Ramos cells at a high (25%) concentration of NHS. Such effect was achieved at the range of 12.5 to 25 μg/ml of both antibodies applied simultaneously but not when each antibody was applied separately (Supplementary Figure S1). We also tried a new generation of C5 blocker ravulizumab, but it did not outperform the combined activity of eculizumab and ravulizumab. The scheme of the current setup of the assay is visualized in Figure 1.

Next, the established conditions that blocked the complement cascade at the C5 level were applied to a proof-of-concept experiment, in which we examined the convertase activity curve upon addition of wild-type factor B and C2 proteins, as well as upon addition of acknowledged GoF variants on both proteins:

Figure 1. (continued) PBS buffer with 40 mM EDTA, a condition permissive for membrane attack complex (MAC) formation on the platform of preformed convertases but not *de novo* convertase formation. After 30 minutes incubation, centrifugation separates unaffected cell and cellular debris from the supernatant that contains fluorescent dye released from the cells bearing MAC complexes. The fluorescence signal is proportional to the amount of active convertase complexes at a certain time point. EDTA, ethylenediamine tetraacetic acid; PBS, phosphate-buffered saline.

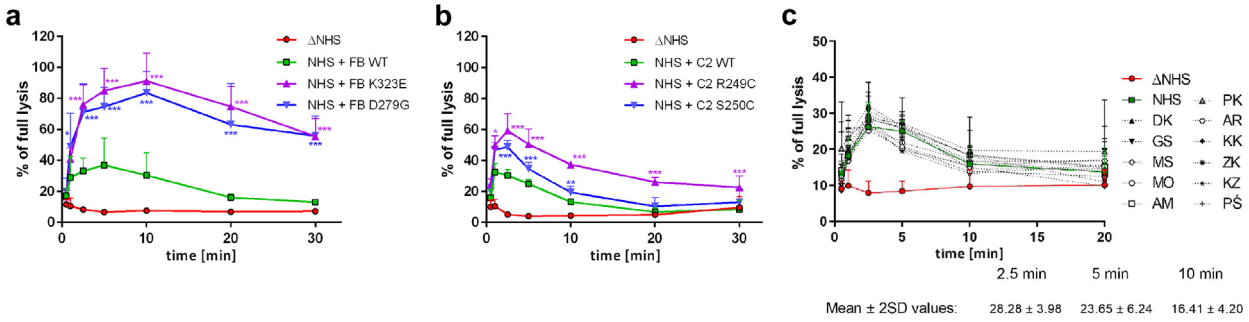

Figure 2. Convertase activity upon addition of factor B and C2 gain-of-function variants. The test was performed as described in Figure 1 and methods. (a) Shows the results upon addition of wild-type or gain-of-function variants of factor B: K323E and D279G at their physiological concentrations calculated proportionally to the content of factor B in 10% serum, and (b) graph shows analogous results for C2 gain-of-function variants R249C and S250C. (c) Presents the results obtained for individual sera from healthy volunteers (dotted lines) compared to normal human serum (NHS) and heat-inactivated NHS (Δ NHS). Below are shown the values of mean ± 2SD values calculated from the readouts of individual sera at time points 2.5, 5, and 10 minutes, respectively. Data were collected from three independent experiments and expressed as mean ± SD, symbols *, **, and *** denote statistical significance versus NHS + C2 WT at the levels $P < 0.05$, < 0.01, and < 0.001, respectively, according to Dunnett's multiple comparison test.

K323E[7] and D279G[23] for factor B and R249C and S250C for C2,[24] respectively. The results confirmed that our system can clearly detect the presence of factors that dominantly impair function of either AP or CP/LP convertases (Figure 2). To ensure the assay's specificity, we performed the test upon blockade of factor B (iptacopan) and C3 (pegcetacoplan), as well as in the absence of C1q (C1q-depleted serum). Blocking of factor B was expected to reduce the overall convertase activity in our model, because the amplification loop was disabled. Indeed, the addition of iptacopan resulted in diminished activity, which could not be rescued by the supplementation of either wild-type factor B or its *in silico*-designed, highly hyperactive variant 4×[15] (Supplementary Figure S2A). Conversely, the addition of an analogous variant of 4× C2[25] resulted in markedly elevated convertase activity, even beyond the level attainable for NHS with no iptacopan. The addition of wild-type C2 elevated convertase activity only slightly compared to NHS + iptacopan. Such a result can be explained by the unaffected formation of classical convertases. The same results were obtained when C3 blocker pegcetacoplan was used, that is, addition of wild-type or 4× variant of factor B could not rescue the convertase activity upon blockade of C3 but the addition of 4× C2 could do so (Supplementary Figure S2). This result is explained by unrestricted formation of C3 classical/lectin convertase C4b2a in the first stage of experiment and completion of C5 convertase and AP amplification loop in the second stage of the experiment, which is in line with our previous results obtained on sensitized sheep erythrocytes and C3-depleted serum.[26] As expected, the depletion of C1q from serum disabled CP activation in our model, and the readdition of C1 restored CP convertase formation capacity (Supplementary Figure S3). We also tried to assess how the variability in normal populations affects the results. Therefore, we ran the assay on 11 serum samples collected from healthy volunteers, and we did not find statistically different readout of these sera at any time point tested (Figure 2c).

Next, we evaluated the assay's performance on sera samples collected from patients suffering from aHUS and C3G, who presented with heterozygous mutations of either pathogenic character or of unknown significance in the C3 gene (for detailed patients' characteristics, see Supplementary Table S1). Importantly, both patients had decreases but similar levels of C3 (Supplementary Table S1); thus, we mixed their samples with NHS at 1:1 v/v to support efficient lysis. We also tried another modification, that is, mixing with human serum partially depleted from its main inhibitor of the alternative complement pathway, namely factor H. The results confirmed the acknowledged pathogenic character of C3 p.K65Q substitution and showed no functional consequences of mutation V547L in C3 (Figure 3a). Notably, mixing with serum of limited factor H content resulted in enlargement of differences in convertase activity between NHS and serum with pathogenic C3 variant (Figure 3b). Finally, we evaluated a pair of EDTA-plasma samples collected from a patient with aHUS and his asymptomatic maternal aunt, both carrying the same pathogenic C3 variant p.I1157T[27] in heterozygosis and almost identical levels of C3 in their samples. Our results revealed elevated convertase activity in the patient with aHUS and normal activity in the asymptomatic relative (Figure 3c and d).

The other task was to examine the assay's performance on clinical samples from the patients carrying no pathogenic variants in complement genes or carrying such a variant in genes other than C3. In Figure 4, we

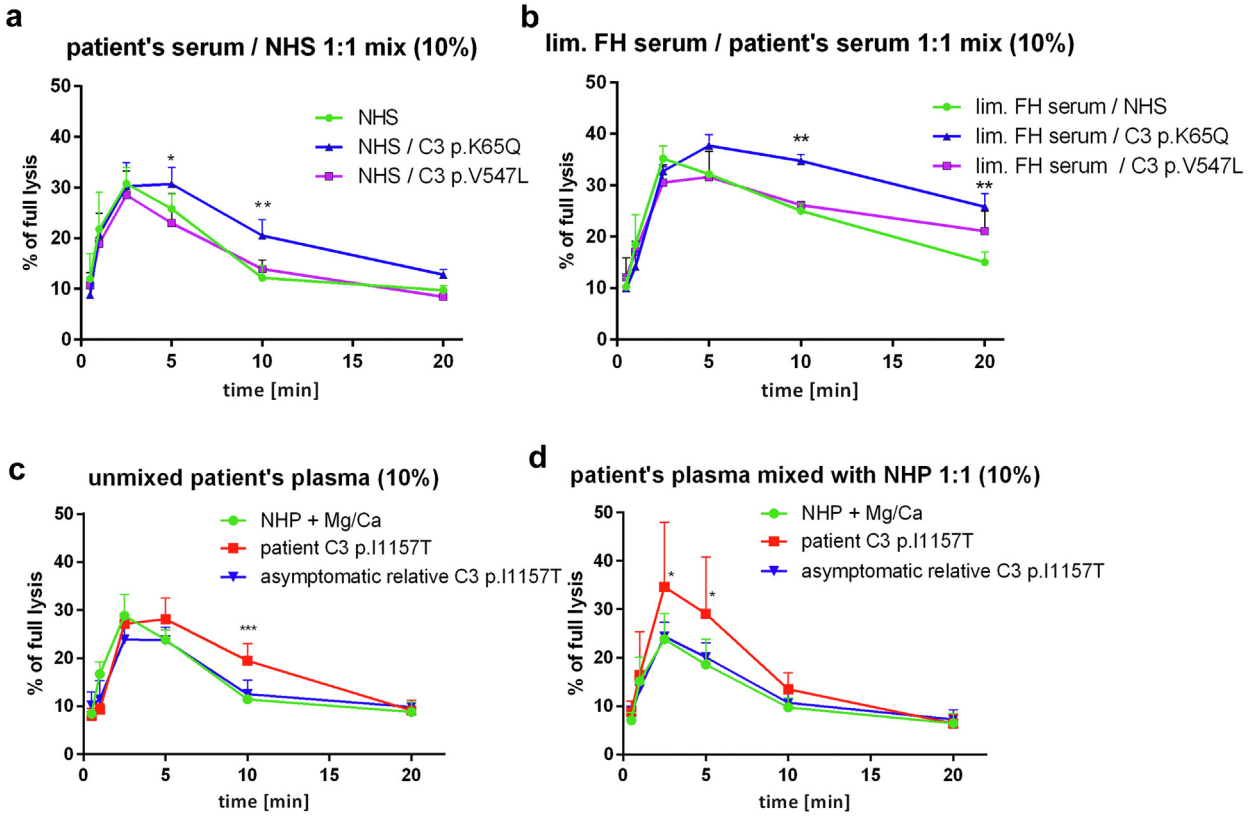

Figure 3. Assay's performance on samples collected from patients with aHUS and those C3G carrying mutations in C3. Serum samples with low C3 content and heterozygous mutations in C3 gene were assayed upon 1:1 dilution with (a) normal human serum or (b) with serum of limited content of factor H. The final serum concentration was 10%. EDTA-plasma collected from a patient with aHUS and his asymptomatic maternal aunt[25] were diluted in PBS with 5.5 mM $CaCl_2/MgCl_2$ and (c) tested unmixed or (d) mixed 1:1 with normal human plasma (NHP). Data were collected from three independent experiments, symbols *, **, and *** denote statistical significance at the levels $P < 0.05$, < 0.01, and < 0.001, respectively, according to Dunnett's multiple comparison test. aHUS, atypical hemolytic uremic syndrome; C3G, C3 glomerulopathy; EDTA, ethylenediamine tetraacetic acid; PBS, phosphate-buffered saline.

provided the results of the patient GN398 with immune complexes-mediated glomerulonephritis, who did not carry any mutations in complement genes and did not have autoantibodies such as antifactor H or C3NeF. His low levels of C3 and C4 were restored upon immunosuppressive treatment. As expected, his profile of

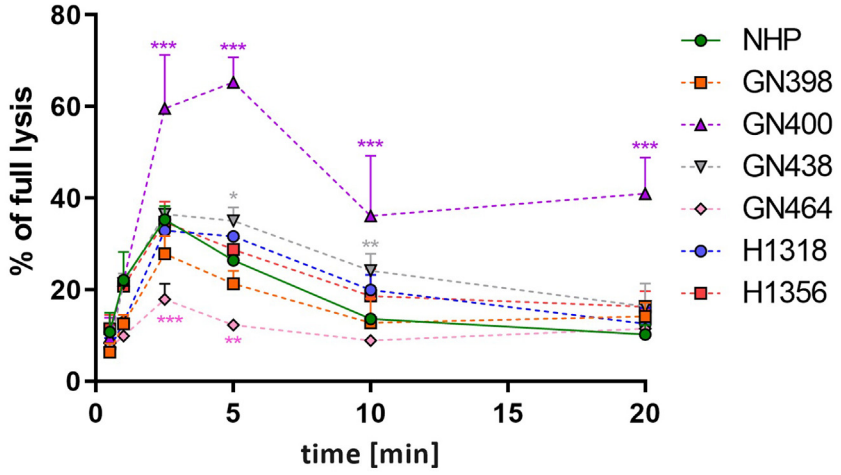

Figure 4. Assay's performance on samples collected from patients with aHUS and those with C3G carrying no pathogenic variants of complement genes or variants in genes other than C3. EDTA-plasma collected from patients were diluted in PBS with 5.5 mM $CaCl_2/MgCl_2$ and tested mixed 1:1 with normal human plasma (NHP). Data were collected from three independent experiments, symbols *, **, and *** denote statistical significance calculated versus NHP at the levels $P < 0.05$, < 0.01, and < 0.001, respectively, according to Dunnett's multiple comparison test. aHUS, atypical hemolytic uremic syndrome; C3G, C3 glomerulopathy; EDTA, ethylenediamine tetraacetic acid; PBS, phosphate-buffered saline.

convertase activity in recalcified plasma was not significantly different from that obtained for normal human plasma. Another patient (GN400) was an individual with C3G, strong activation of AP, almost undetectable C3, and very low C5, negative for autoantibodies and pathogenic variants of complement genes. C3G was probably related to monoclonal gammopathy. This patient showed markedly elevated convertase activity. Significantly elevated convertase activity was noticed for another patient GN438 with C3G and no pathogenic variant in complement genes and no autoantibodies. We also found an interesting case of a patient GN464 with myeloma and C3G, and heterozygous mutation in the C2 gene resulting in C2 deficiency but without defined genetic risk factors for C3G. This patient showed significantly diminished convertase activity, validating our model (which is dependent on successful activation of the CP), and conversely showing an example of assay limitation. We included two patients with aHUS. The first one (H1318) presented with no pathogenic variants in complement genes but high titers of antifactor H autoantibodies. He showed a higher convertase activity than normal human plasma, but it did not reach statistical significance. The other patient, H1356 presents a partial deficiency of factor I due to pathogenic mutation in the CFI gene. For this patient, we were not able to show elevated convertase activity. Patients' characteristics in presented in Supplementary Table S1.

DISCUSSION

Measurement of complement convertases activity and/or identification of factors that influence convertases' activity always presented a technical challenge. Historically, the first attempts were made by reconstruction of convertase complexes from purified complement proteins on the surface of sheep erythrocytes and testing the impact of the factor of interest on convertase formation or decay.[28] Such a strategy provided an easy readout because complement-lysed erythrocytes release hemoglobin. Nevertheless, the procedure of convertase assembly was laborious, and the method was sensitive to activity loss by any purified compound used for convertase deposition on red blood cells. The other versions of convertase assay are based on purified components that use surface plasmon resonance technique,[29] enzyme-linked immunosorbent assay-based detection of noncovalently bound C3-convertase subunits such as Bb fragment,[4] detection of C3 cleavage products[30] or a system that employs convertases formed on the surface of the beads, and fluorescent monitoring of calcium mobilization in reporter cells U937 responsive to C3a and/or C5a release.[31] However, the limitation of all the above-mentioned methods is the inability to examine the patient's sera but only purified analytes. The first functional convertase assay that used whole serum as an analyte was designed by Rother more than 40 years ago.[32] Originally aimed to screen for autoantibodies stabilizing/augmenting the AP C3-convertase (termed as C3NeF), the test uses non-sensitized sheep erythrocytes to deposit AP convertase. Because such a surface is not an optimal target for AP, presumably the presence of complement inhibitors in normal serum decays AP convertases and disables membrane attack complex formation. Conversely, the presence of C3NeF stabilizes AP convertases and eventually results in hemolysis observed when human serum was replaced with rat serum diluted in EDTA after 20 minutes. Although still being used by some diagnostic laboratories, the method may preclude firm conclusions in certain cases. The incubation time with the analyzed patient's serum is set arbitrarily and some sera may cause hemolysis already during the first step of the assay, most probably due to intrapersonal differences in complement activators-to-inhibitors ratio. This may also cause a delay in maximal convertase activity in samples with lower content of complement components resulting in low or no hemolysis obtained in the second step. The likelihood of false positive and false negative results makes C3NeF measurement challenging, which is underlined by the fact that more than 50% of complement diagnostic laboratories participating in the external quality assurance program during 2016 to 2020 failed to properly determine C3NeF content in the reference samples.[12] In 2014, we proposed a new method for the assessment of CP/LP and AP convertases' activity in whole serum, which uses C5 blockers: eculizumab or OmCI.[20] In contrast to Rother's method, our experimental setup enables strict control of the transition from convertase to membrane attack complex formation step and analysis of the full convertase activity curve instead of a readout at a single time point. Although it was a step forward to a reliable assessment of complement convertase function, the method still had certain limitations. We set it up on rabbit or sensitized sheep erythrocytes for analyses of AP or CL/LP convertase, respectively. However, these cells are equipped with membrane-bound complement inhibitors of uncertain compatibility with the human system. For example, we identified the first-ever GoF mutations in complement C2 protein but their specific phenotype would have been missed if the experiments were not performed on a human instead of sheep erythrocytes.[16] More to this end, erythrocytes are cells with the set of complement inhibitors different from most of the somatic cells. They do not express CD46 but show CD35 on their surface, a difference that may be

important in the context of diseases such as aHUS.[7] In addition, serum concentrations necessary for lysis of sensitized (CP/LP) or nonsensitized (AP) erythrocytes are much lower compared to these effective in most of the other cell types. Therefore, nucleated somatic cells are a more reliable target for the elucidation of truly physiological interactions of complement convertases with their inhibitors. We tried to perform the assay in human CD20-positive cells sensitized with anti-CD20 antibody rituximab but full inhibition of complement cascade with single C5 blockers eculizumab or OmCI was found problematic, in line with other reports stating that eculizumab was not able to fully inhibit the complement cascade initiated by a strong activator.[33,34] In the meantime, we introduced a calcein release assay as a method for assessment of CDC in human tumor cells sensitized with immunotherapeutics[18] and demonstrated the usefulness of this assay for monitoring of CDC capacity of sera collected from hematological patients treated with rituximab.[35] Using the model of human CD20-positive cell lines such as Raji and Ramos, we observed that application of factor B-depleted serum results in significantly lower CDC readout compared to the same serum reconstituted with factor B[15] suggesting that the amplification loop supported by AP convertases plays an important role in this model. Thus, CD20-positive cells sensitized with anti-CD20 antibodies may be a good platform for the measurement of either CP/LP or AP convertase activity in a single test. The only technical obstacle that existed at that point was to achieve effective blocking of complement cascade at the C5 level in the cells activated with rituximab. The solution was a simultaneous application of eculizumab and crovalimab, a second generation C5 blocker.[22] The success in eliminating residual C5 activation by simultaneous application of two anti-C5 compounds that bind to different epitopes was also reported by other researchers.[33,34]

To sum up, we showed a proof-of-concept that our new cell-based convertase assay can show impairment of both CP/LP and AP convertases. Importantly, it is operable in the physiological milieu of whole serum and offers a physiological surface of human cells equipped with typical complement inhibitors. Another assay's advantage is the lack of veronal buffers traditionally used in complement assays and their replacement with more physiological and easy-to-handle PBS buffer, which is used in either the first (convertase formation/decay) step or in the second (membrane attack complex formation on the preexisting convertase platforms) step. The assay is intended to serve as a screening method for factors influencing complement convertases' activity, which facilitates the selection of patients who deserve a more detailed analysis of their complement system. However, we do not present a detailed assay validation at this stage but general tips on how the test can be adjusted to the particular needs. For example, the results of experiments, where defined, strong GoF components were added to NHS (Figure 2) are very clear and do not leave doubts about the interpretation. The other experiments, in which real patients' samples were tested, show statistically significant differences between the serum collected from the patient with a C3 variant of unknown significance and serum from the patient with acknowledged pathogenic variant (Figure 3a and b), but the assay's window seems not that big as in the previous figure. This may be the result of intrapersonal variability in complement components on the basis of either genetics or quantity of a particular component. Conversely, such interpersonal differences in CDC potential may stem from the disease itself, as numerous patients present hypocomplementemia resulting from local or systemic complement consumption. Therefore, a trick that normalizes the convertase activity readout but preserves a dominant character of a given generic variant, is mixing the patient's serum with NHS and one should consider that while looking at basic laboratory parameters of complement system such as C3 concentration or total pathway activity. Previously, we observed that the higher the content of the complement component is, the sooner the peak of convertase activity takes place.[20] We also showed a way to enlarge the assay's window by including serum partially depleted from factor H (Figure 3b). Next, we showed that functional analyses of patients' whole serum or plasma may be critical to understanding the mechanism of complement-mediated kidney pathologies, normally not attainable when purified components are used. This is illustrated by our result showing different profiles of convertase activity in two relatives with the same pathogenic mutation in C3 (Figure 3c and d). Such a result supports the theory of the "multiple hit" nature of complement-mediated kidney dysfunctions.[8] It assumes the existence of additional triggers that coincide with the presence of a rare genetic variant, and by augmenting its penetrance, precipitates the disease. It is worth mentioning the recent work by Gastoldi *et al.*,[14] who applied adenosine diphosphate-activated endothelial cells as a platform for C5b-9 deposition and used it for testing the sera from patients with aHUS and their unaffected relatives. Given that a vast majority of unaffected individuals presented with elevated C5b-9 deposition, their results suggest that perturbed endothelium model uncovers the sole impact of pathogenic mutations in complement components independently in the presence of additional trigger that precipitates the disease; whereas our assay may discriminate between patients and unaffected relatives with the same

mutation, thereby sensing the additional triggers that contribute to penetration of the genetic variant. However, we see certain limitations of the Ramos cell-based model. For example, we added some patients with no genetic background for their kidney disease and we found that only some of them presented with positive result. Similarly, we tested plasma collected from patient with pathogenic mutation in factor I, which also presented negative. Although it seems that the assay senses well the pathogenic variants of the main/ubiquitous complement proteins such as C3 and some cases with unknown or not precisely defined etiology, it cannot mirror the impact of factor I. Possible explanations are the more pronounced role of factor I in tissue than in fluid phase and/or too short time window of the assay for the enzymes of relatively weak enzymatic turnover, that again would be more relevant *in vivo* than in our experimental setup. The other limitation of our model stems directly from its advantage, that is, the usage of Ramos cells resembling the physiological rather than artificial surfaces for complement deposition. *In vitro* cell culture adds interassay variability to the obtained results. We noticed that the putative differences in cells' condition are mirrored in differences in the time of convertases' Tmax as well as overall convertase activity. In some experiments, we see an unspecific outflux of calcein from the cells at the longer incubation time (e.g., 20 minutes). It puts an additional challenge for validation or standardization and demands the use of an internal control such as a NHS/plasma standard and defined GoF variant in every experiment. Nonetheless, we believe that our assay that gives an overview of convertase activity in the context of the whole patient's serum or plasma, and when accompanied by other assays, may offer an important support for the physician's decision on the application of targeted immunotherapy. Eculizumab (Soliris) is a prototypic immunotherapeutic that targets complement C5 component and inhibits the terminal stages of cascade pushed by an excessive convertase activity. Its current indications include aHUS and paroxysmal nocturnal hemoglobinuria but also myasthenia gravis, and neuromyelitis optica spectrum disorder.[36] Recently, new generations of complement C5 blockers such as ravulizumab[37] and crovalimab[22] emerged on the market. However, these drugs remain expensive, up to $500,000 per patient per year, which puts additional pressure on the healthcare providers for accurate diagnoses and strict inclusion criteria for the patients.

DISCLOSURE

All the authors declared no conflicting interests.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Figure S1. Titering of C5 blockers for the inhibition of complement cascade initiated by rituximab on Ramos cells.

Figure S2. Convertase activity in normal human serum upon addition of factor B and C3 blockers.

Figure S3. Convertase activity in C1q-depleted serum.

Table S1. Samples included in the study.

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