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## ORIGINAL ARTICLE

# Quantitative multiplex profiling of the complement system to diagnose complement-mediated diseases

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## Abstract

**Objectives.** Complement deficiencies are difficult to diagnose because of the variability of symptoms and the complexity of the diagnostic process. Here, we applied a novel 'complementomics' approach to study the impact of various complement deficiencies on circulating complement levels. **Methods.** Using a quantitative multiplex mass spectrometry assay, we analysed 44 peptides to profile 34 complement proteins simultaneously in 40 healthy controls and 83 individuals with a diagnosed deficiency or a potential pathogenic variant in 14 different complement proteins. **Results.** Apart from confirming near or total absence of the respective protein in plasma of complement-deficient patients, this mass spectrometry-based profiling method led to the identification of additional deficiencies. In many cases, partial depletion of the pathway up- and/or downstream of the absent protein was measured. This was especially found in patients deficient for complement inhibitors, such as angioedema patients with a C1-inhibitor deficiency. The added value of complementomics was shown in three patients with poorly defined complement deficiencies. **Conclusion.** Our study shows the potential clinical

utility of profiling circulating complement proteins as a comprehensive read-out of various complement deficiencies. Particularly, our approach provides insight into the intricate interplay between complement proteins due to functional coupling, which contributes to the better understanding of the various disease phenotypes and improvement of care for patients with complement-mediated diseases.

**Keywords:** complement deficiencies, complement system, complement-mediated diseases, complementomics, multiplex targeted mass spectrometry, pathway analysis

## INTRODUCTION

The complement system is a proteolytic cascade that orchestrates innate immune surveillance, homeostasis and repair. It comprises about 50 circulating fluid-phase and surface-bound proteins. They are constitutively expressed by many cell types in different tissues, whereas the blood circulating complement proteins are mainly of hepatic origin.<sup>1</sup>

Despite the fact that the complement system is highly conserved,<sup>2</sup> the genes encoding the complement factors contain many polymorphisms, with remarkable geographical variation<sup>3</sup> and unique complotypes.<sup>4</sup> Most variants are benign or of undetermined significance, and several are reported to be protective,<sup>5–8</sup> while rare variants are often pathogenic and result in complement deficiency.<sup>9</sup> The clinical manifestations range from severe infections, neuro-degenerative diseases, haematological disorders, to renal or ocular pathologies, as reviewed extensively elsewhere<sup>9–13</sup>; a tabular overview is provided in Supplementary table 1.

Complement deficiencies are rare and have a combined genetic prevalence of 0.03% in the general population and comprise only 5% of all primary immunodeficiencies – excluding more frequently occurring C4A, C4B and MBL variants (estimated 11–22%, 30–45% and 5–10%, respectively).<sup>14</sup> The mode of inheritance is mostly autosomal recessive, with the exception of a few cases of autosomal dominant inheritance: CR1, C1S, C1-inhibitor (C1-INH), mannose-binding lectin (MBL), and properdin (X-linked).<sup>12</sup> For factor H, both recessive and dominant modes of inheritance have been reported depending on the type of disease.<sup>15</sup> Moreover, for several complement-mediated diseases – including acquired angioedema (AAE), systemic lupus

erythematosus (SLE), age-related macular degeneration (AMD), atypical haemolytic uremic syndrome (aHUS) and membranoproliferative glomerulonephritis (MPGN) – genetic and non-genetic multifactorial components play a role in disease aetiology. In addition, given the central role of the complement system in immunological mechanisms, complement deficiencies may be (in-) directly involved in multiple other diseases.

The diversity in clinical symptoms necessitates laboratory testing to diagnose the complement deficiency. Conventionally, the classical, lectin, or alternative pathway activity is tested using functional assays (CH50, LP50 and AH50, respectively), followed by single tests measuring individual proteins of the affected pathway to identify the impaired protein.<sup>14,16</sup> Unfortunately, this testing strategy has some major shortcomings. Functional assays may not always rule out certain deficiencies,<sup>12</sup> and for most complement proteins, no standardised tests are available. The current protein analysis is primarily based on ELISA, radial immunodiffusion, immunoelectrophoresis or nephelometry,<sup>13</sup> which are either time consuming, have limited accuracy or require relatively large sample volumes, respectively. Furthermore, the complement system is sensitive to freeze-thaw cycles, which could lead to complement activation, interfering with the measurements. Complement deficiencies are increasingly investigated by genetic testing which provides molecular comprehension of inherited predispositions. However, this provides limited information on the functional consequences and there is thus a clear need for diagnostic profiling of all complement proteins simultaneously as this will provide an immediate overview of deficiencies at protein level. Two independent studies including large cohorts of complement-deficient patients used a single method to confirm the genetic variant (functional

testing, ELISA, radial immunodiffusion or nephelometry); however, no extensive protein profiles were determined.<sup>17,18</sup> Hence, sensitive and quantitative methods analysing all complement proteins of the complement system in multiplex have not been applied to characterise complement deficiencies yet.

To the best of our knowledge, this is the first study to profile 34 circulating complement factors in plasma from patients with distinct complement deficiencies using quantitative multiplex mass spectrometry. Unravelling the downstream effects of deficiencies on other proteins of the complement system will lead to a better understanding of the disease phenotypes, resulting in improved care for patients with complement-mediated diseases.

## RESULTS

### Patient characteristics

For this study, plasma samples from 83 patients and 40 control subjects were collected from four European medical centres in the Netherlands, Italy and Spain. We included material of 10 homozygous, 2 compound heterozygous, 4 hemizygous and 24 heterozygous variants. For 9 patients, we did not have genetic confirmation for the zygosity of the mutation, and the diagnosis was established only on protein level. Five patients diagnosed with AAE were also included. Furthermore, we included 26 subjects with (predicted) pathogenic heterozygous nonsense variants – complement C3 ( $n = 1$ ), C6 ( $n = 1$ ), C9 ( $n = 8$ ), factor D ( $n = 5$ ), factor H ( $n = 8$ ) and factor I ( $n = 3$ ). We also included three unknown clinical cases suspected of having a complement deficiency based on clinical symptoms. Additional clinical and molecular data from patients and healthy subjects enrolled in this study are summarised in Supplementary table 2. In total, this cohort was comprised of variants in 14 different complement proteins (Figure 1a), in multiple age groups (Figure 1b) and both sexes (Figure 1c).

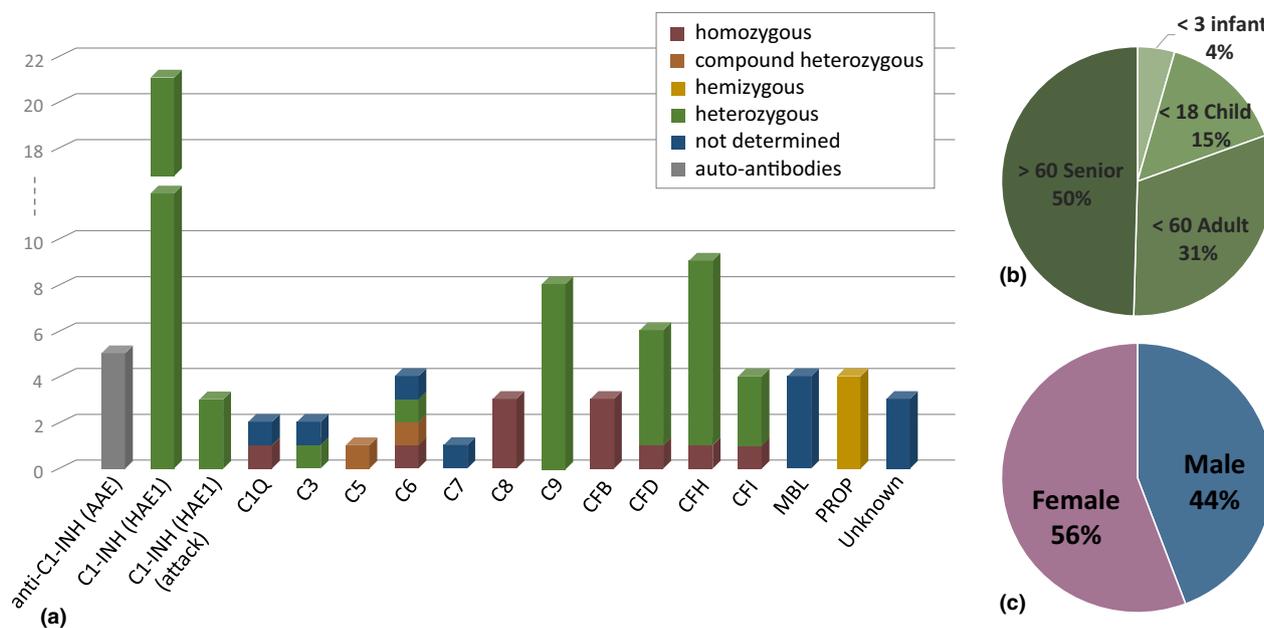
### Effect of variants on the deficient protein expression levels

We analysed 44 peptides in a multiplex targeted mass spectrometry method, enabling quantitative detection of 34 complement proteins simultaneously. A table with the absolute

concentrations is included in Supplementary table 3. Individuals with the same genetic variant were grouped (rows), and the percentages were calculated for the concentration of each peptide (columns) as compared to the control group average (Figure 2). The homozygous (C1Q  $n = 2$ , C6  $n = 2$ , and C8  $n = 3$ ), hemizygous (properdin  $n = 4$ ) and heterozygous dominant inherited variants (C1-inhibitor  $n = 23$ , MBL  $n = 4$ ) could be distinguished by very low or absent levels of the corresponding peptide(s). For a few homozygous variants, reduced levels were measured: factor B (Leu9His,  $n = 2$ ; Gly252Ser,  $n = 1$ ) and factor I (Asp519Ans,  $n = 1$ ), 60% and 30% respectively, whereas for factor H (Val1007Leu,  $n = 1$ ) normal levels were observed. For most heterozygous nonsense variants, we measured lower levels for the corresponding peptides: C9, factor D, factor H and factor I (Supplementary table 3). An overview of the *t*-test *P*-values (after Bonferroni correction) for all peptides for each patient group compared to the control group is shown in Supplementary table 4.

### Collateral effect of variants on the complement levels of the affected pathway(s)

Apart from the anticipated lower levels for the defined homozygous, hemizygous and pathogenic heterozygous variants, in many cases additional lower levels of other complement proteins downstream or upstream were observed in the cascade. For instance, for patients with (acquired) C1-inhibitor deficiency in acquired and hereditary angioedema type I (AAE and HAE1), we observed that other classical pathway proteins including C1QA (AAE 6%, HAE1 37%), C1QC (AAE 4%, HAE1 33%), C1R (AAE 6%, HAE1 54%) and C1S (AAE 7%, HAE1 61%) were significantly reduced, indicating consumption of the classical pathway due to impaired inhibition. Collectin-11, one of the activators of the lectin pathway, was significantly reduced as well (AAE 44%, HAE1 59%), which might imply consumption of the lectin pathway due to improper inhibition. Also, several peptides downstream, C2 (AAE 3%, HAE1 39%), C3 (AAE 15%, HAE1 26%), C4 (AAE 2%, HAE1 8%), in the alternative pathway (FB both 50%, PROP HAE1 60%), terminal pathway (C5 50%, C8 50%, on average) and their regulation factors (CFH, CFI, C4BP, vitronectin, FHR2, FHR4 and FHR5, 20–70%) show significant reduction, Figure 3 (AAE) and Supplementary figure 1a (HAE1).



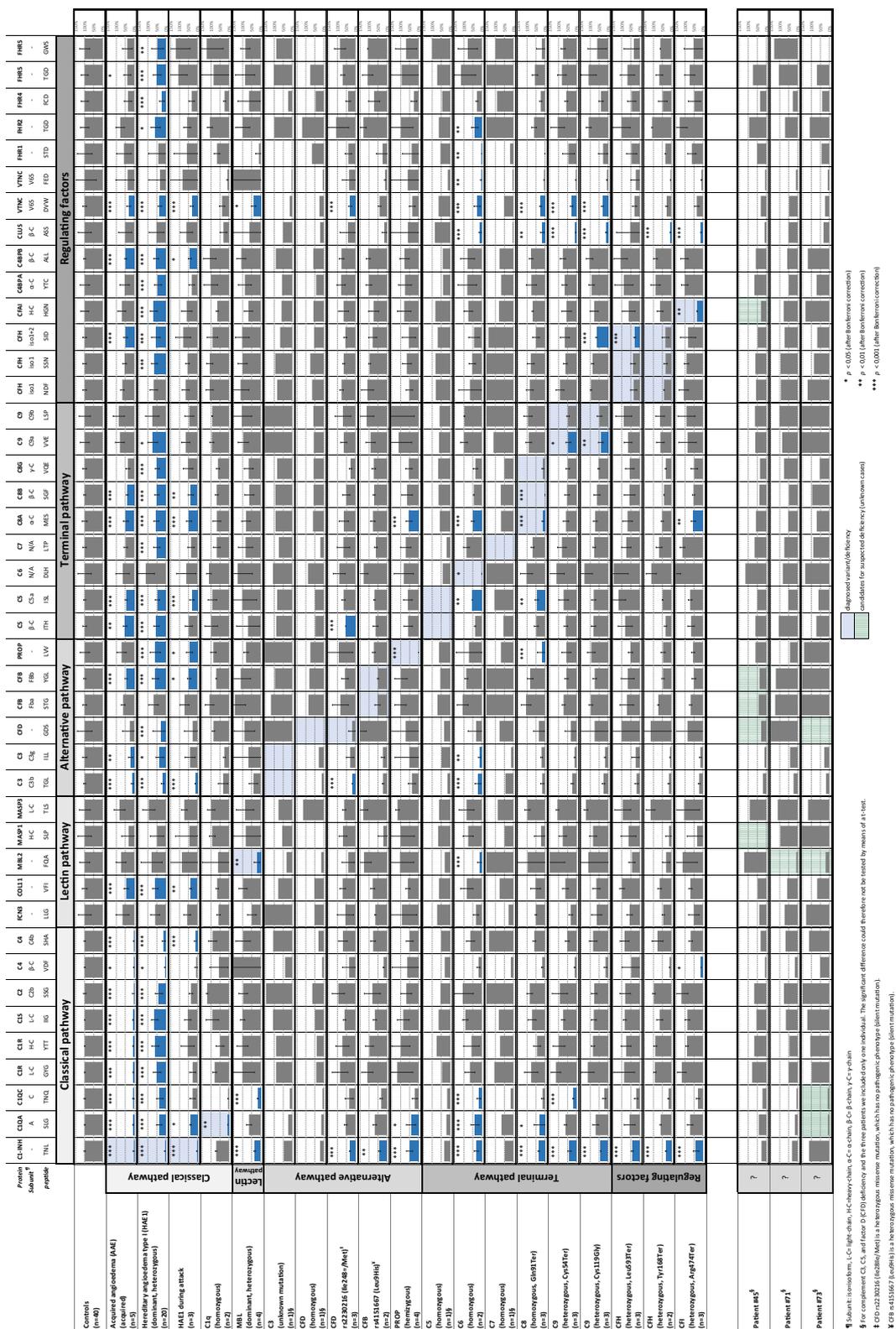
**Figure 1.** Schematic overview of the distribution of (a) zygosity of mutations in complement genes, (b) age and (c) sex of the patients ( $n = 83$ ). In acquired angioedema (AAE), auto-antibodies against C1-inhibitor result in lower C1-INH levels instead of a genetic cause. See Supplementary table 2 for additional clinical and molecular data.

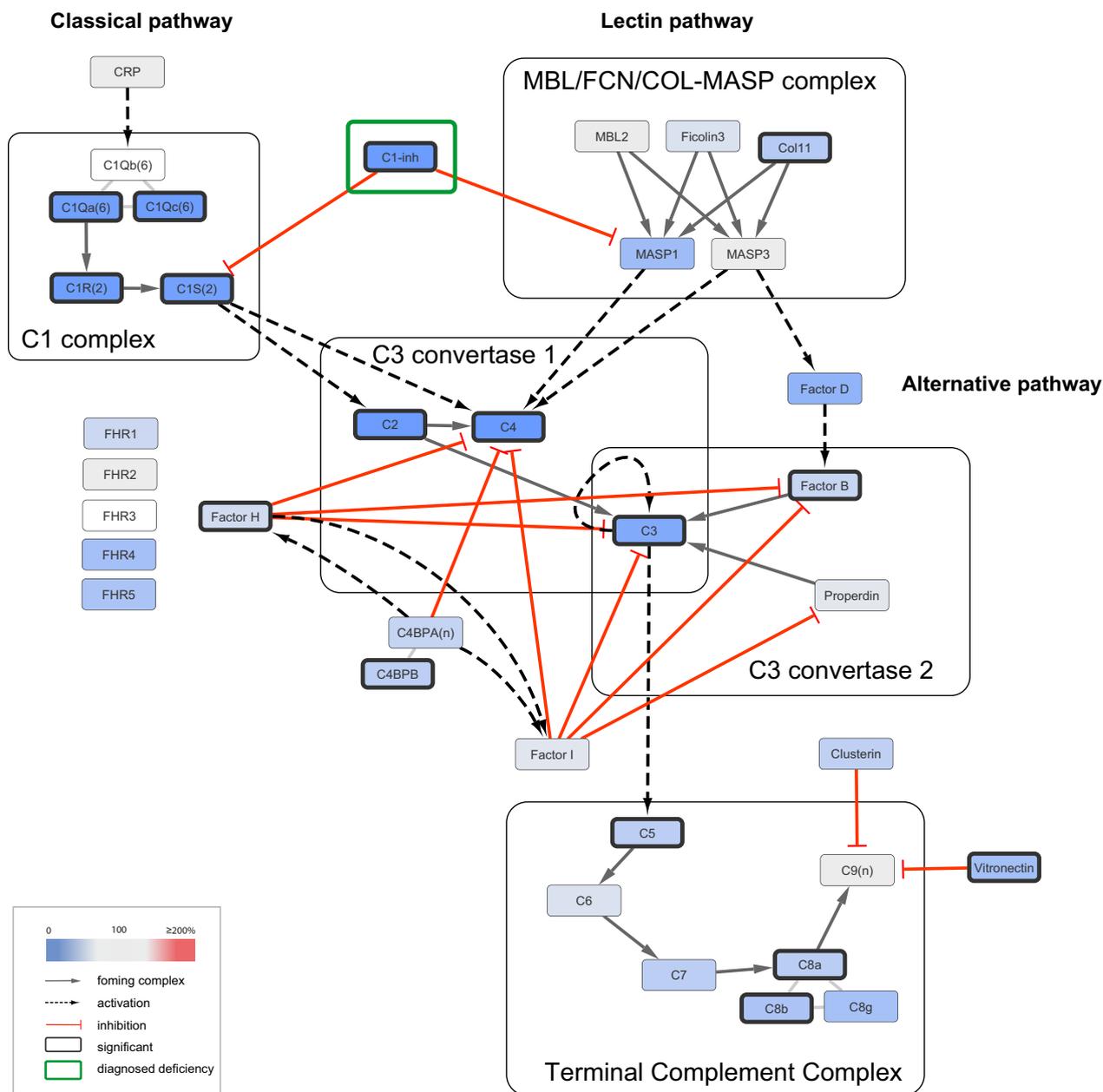
For deficiencies in the classical pathway (C1Q), there was no significant reduction of other complement components. The MBL variants (lectin pathway) showed additional reduced C1-INH, C1QC and vitronectin levels. For variants in alternative pathway components FD and PROP, we only observed a significant decrease of C1-INH, C3 and C5 or C8, whereas the factor B variants had no up- or downstream effects. Variants of complement proteins in the terminal pathway (C6, C8, C9) coincided with altered levels of upstream components like C3, properdin, C5 and C6 and possible downstream effects on membrane attack complex (MAC) regulators clusterin and vitronectin. Regulating factor H and factor I variants also showed reduced alternative and terminal pathway components, but only the reduced clusterin levels were significant. A pathway visualisation of all variant groups is represented in Supplementary figure 1a–k.

### Clinical cases with a suspected complement deficiency

Three patient samples with a suspected complement deficiency without immunological or genetic data were included in order to validate this multiplex mass spectrometry-based assay as a method to screen for complement deficiencies.

- Patient #45 had recurrent late pregnancy loss in the 2nd trimester without known causes. Since 4 other patients with a comparable medical history (#42–44 and #72 in this study) showed low to undetectable MBL levels, an MBL deficiency was suspected for patient #45 as well. However, the multiplex assay showed elevated levels for MBL, but undetectable MASP1 and low levels for factor B, factor D and factor I (Figure 2).
- Patient #71 is a sister of patient #35 (C6 deficiency), and she was therefore tested for a potential C6 deficiency. However, patient #71 had normal C6 levels, but very low MBL levels, which was later also confirmed by additional analysis.
- Patient #73 is a brother of patient #39, who was diagnosed with SLE (homozygous C1Q mutation). Low levels of both C1Q subunits were measured, whereas C1R, C1S, C2, C4 (downstream) and C1-INH (upstream) had normal to slightly increased levels. Additionally, reduced MBL levels were detected and factor D was below the detection limit. Higher levels of CFB and properdin, downstream of CFD, may indicate accumulation due to impaired factor D. Increased levels for MASP1 and FCN3 may be linked to the low levels for MBL (Figure 4).



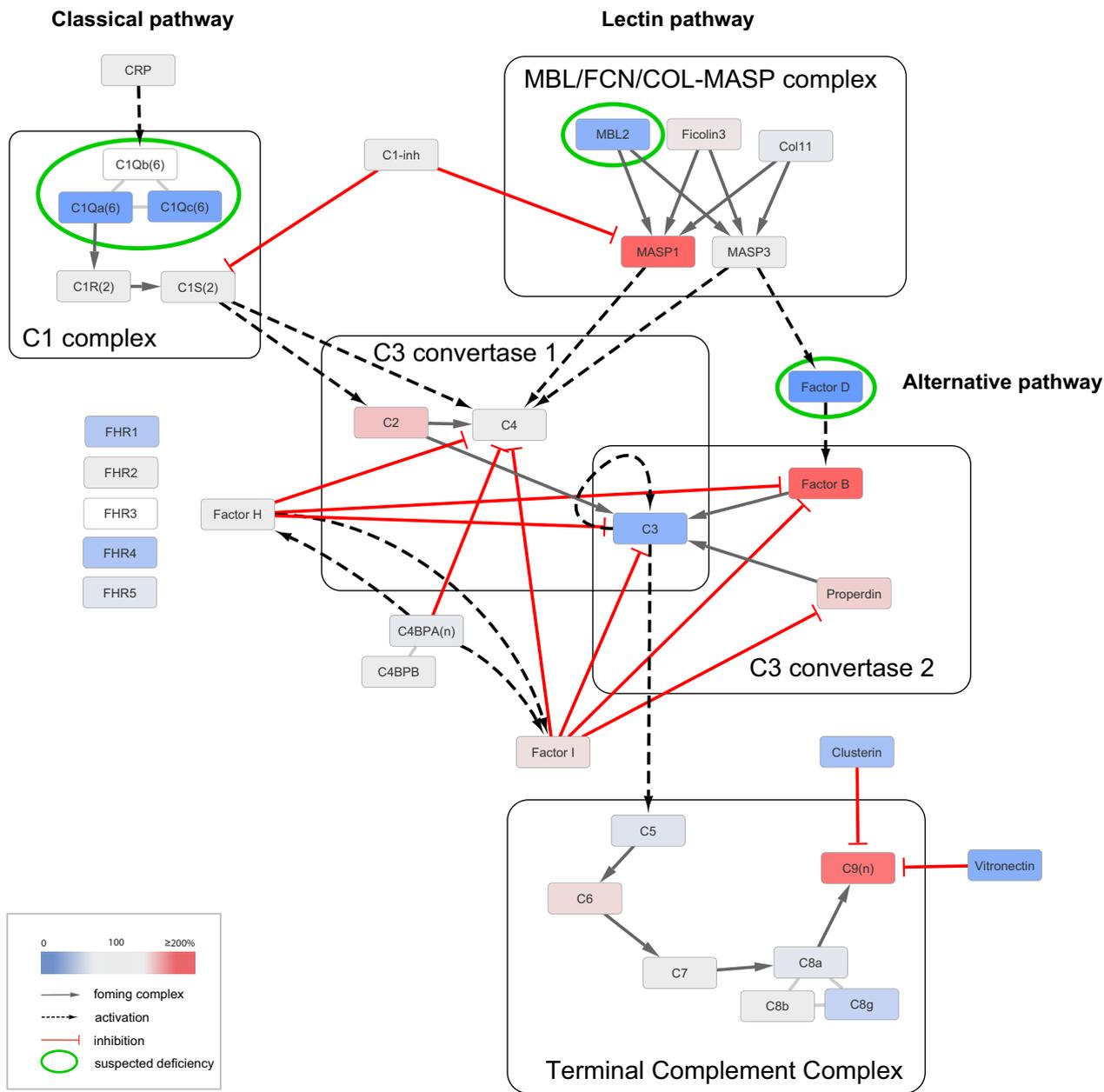


**Figure 3.** Schematic pathway visualisation of the average profile of five acquired angioedema (AAE) patients with an acquired C1-inhibitor deficiency (green border). The gradient colours of the proteins specify the percentage of the peptides (blue = 0–75%, grey = 75–125%, and red = 125–200%) as compared to the average value of the control group ( $n = 40$ ). Thick borders indicate significant reduction ( $t$ -test,  $P$ -values in Supplementary table 4). The arrows illustrate the main function of the protein within the cascade: forming a complex (grey pointed arrow), activation (black dashed pointed arrow) or inhibition (red blocking arrow).

## DISCUSSION

In this study, we screened 34 different complement proteins simultaneously by analysing 44 peptides in a multiplex targeted mass spectrometry method in plasma of 83 patients and

40 controls. This resulted in a comprehensive profile of the blood circulating complement proteins and provided a proof-of-principle that our assay has the potential to be implemented for diagnostic screening of complement deficiencies.



**Figure 4.** Pathway visualisation of the relative protein levels of the case of patient #73 with a suspected complement deficiency. The colours indicate the percentage of the peptides as compared to the average value of the control group ( $n = 40$ ). The gradient colours of the proteins specify the percentage of the peptides (blue = 0–75%, grey = 75–125%, and red = 125–200%) as compared to the average value of the control group ( $n = 40$ ). The arrows illustrate the main function of the protein within the cascade: forming a complex (grey pointed arrow), activation (black dashed pointed arrow) or inhibition (red blocking arrow). Candidates for suspected deficiency and further confirmation are highlighted by green circles: C1Q (subunits C1QA and C1QC), MBL2 and factor D.

The development, accuracy and reproducibility of the assay have been established previously.<sup>19</sup> The assay was further improved by implementing pure (> 98%) instead of crude (> 50%) heavy isotope-labelled internal peptide standards for absolute quantification. For clinical implementation, the

assay needs additional diagnostic validation, according to clinical mass spectrometry standards.<sup>20</sup>

Although many complement proteins have a broad dynamic range due to individual complotypes, we were able to differentiate between healthy subjects and carriers of

complement deficiencies, showing comparable profiles for similar deficiencies. In general, homozygous, hemizygous or heterozygous (autosomal dominant) deficiencies exhibited very low levels or total absence of the respective protein in plasma. Significantly lower levels of the affected protein were detected in case of pathogenic heterozygous (nonsense) variants. In many cases, we observed an additional reduction through functional coupling of complement factors within the pathways down- or upstream of the deficient protein, although not fully depleted. That other complement proteins were not totally depleted, especially when a complement inhibitor is deficient, emphasises the previously observed resilience of the complement system<sup>19</sup> – even in severe complement deficiencies.

For cases of a complement inhibitor deficiency such as (acquired) C1-INH deficiency in AAE and HAE1, systemic complement activation was a prominent finding. The classical pathway and part of the lectin pathway seemed to be partially consumed, due to impaired C1- and MASP-complex inhibition by C1-INH, as shown by the lower levels of proteins in both pathways (C1Q, COL11, C2, C4) for AAE (Figure 3) and to a lesser extent for HAE1 (Supplementary figure 1a). These findings are consistent with the clinical characteristics of AAE and HAE1.<sup>21–24</sup> Moreover, the reduced complement levels in the alternative and terminal pathway, including inhibitors, indicate further increased activation. C1-INH deficiency thus results in hyperactivation through the classical and lectin pathway, and conceivably additional complement C3 and C5 cleavage or factor H and C4BP binding via the extrinsic (coagulation) pathways,<sup>25</sup> causing partial consumption of other parts of the complement system.

A patient diagnosed with SLE, caused by a homozygous C1Q mutation, showed very low levels of the measured C1QA and C1QC subunits. No other complement protein levels were affected by this deficiency, which was expected since the absence of C1Q protein would block complex C1 formation and subsequent complement activation via the classical pathway.

All four individuals with a C6 mutation showed undetectable levels of complement C6, despite the difference in zygosity of the mutations (homozygous, heterozygous and compound heterozygous). We found significantly reduced protein levels both upstream in the classical

pathway and downstream in several regulating factors. Very low or no complement C6 would result in reduced or blocked MAC building. However, we observed a reduction in MAC inhibitor proteins clusterin and vitronectin in these patients, with a similar trend for other terminal pathway variants (in C7, C8, C9), whereas we would expect an increase of MAC inhibitors. This leads to the hypothesis that there is either a reduced trigger to synthesise the MAC inhibitors or they are partly depleted as a result of constant clearance of tissue damage caused by perpetual complement activation and inflammation.<sup>26,27</sup>

Factor H-related (FHR) proteins did not show large variation between the groups, except for significantly reduced levels of FHR-1 and FHR-2 in C6 deficiencies and FHR2, FHR4 and FHR5 in the HAE1 group. The functions of FHR proteins remain largely unknown. However, recent data suggest that they primarily promote complement activation: FHR-1, FHR-4 and FHR-5 can bind C3b allowing convertase formation and in this way prevent inhibition by factor H.<sup>28</sup> Contrary, another study concludes that FHR-1 can inhibit C5 convertase and MAC formation.<sup>29</sup> Increased levels of FHR-5 have been associated with inflammation or infection before.<sup>30</sup> This assay enables the study of FHR proteins in relation to other complement proteins, which may contribute to the better understanding of their function in the future.

In this patient cohort, we observed a remarkable high frequency (38.0%) of low MBL concentrations ( $< 11 \text{ mol L}^{-1}$ , as was also observed for the diagnosed primary MBL deficiencies) in addition to the diagnosed complement deficiency, which was significantly higher as compared to 12.5% for the control group ( $P = 0.004$ , Chi-square test). MBL variants have a frequency of 5–10%, which is consistent with our findings, and are suspected to have only a redundant role in severe infections.<sup>31,32</sup> However, multiple studies suggest that an MBL deficiency, causing lower levels, might predispose to the development of more severe symptoms in combination with other (complement) deficiencies.<sup>33–35</sup> The fact that we find this high occurrence seems to support this view.

Four patients with a diagnosed MBL deficiency experienced recurrent late pregnancy losses, which may be related to low MBL levels.<sup>36,37</sup> However, after testing in this multiplex assay, these patients also showed additional decreased classical components C1QC and C1-INH, while this was not observed in the MBL-deficient controls.

Other reports describe associations between reduced C1Q levels and abnormal placentation and foetal loss.<sup>38</sup> It may thus be interesting to speculate that low C1Q and C1-INH levels, in combination with low MBL, may contribute to recurrent late pregnancy loss. A profile of all plasma complement components thus provided new leads for understanding complications of these patients, but still needs confirmation by clinically validated single protein tests.

We investigated the application of this multiplex assay for three patients with a suspected complement deficiency. Analysis of the blood concentrations of 34 complement proteins provided a quick overview of affected proteins. We identified reduced levels of MASP1, factor B, D and I in patient #45, suggesting a defect in the alternative pathway: factor I deficiency could cause partial depletion of MASP1, factor D and B, as a similar trend was observed in the included diagnosed factor I deficiencies. The C6 levels of patient #71 were normal, so she does not have a C6 deficiency like her brother (#35). However, finding very low MBL levels in both siblings emphasises the possibility of a combined C6/MBL deficiency for patient #35, which was discovered using this assay. Patient #73 had reduced levels for factor D, MBL and C1Q. Since his brother (#39) has been diagnosed with a homozygous C1Q deficiency, it is likely that this patient also has a C1Q deficiency, although an additional MBL and/or factor D deficiency could not be ruled out. In conclusion, our assay rapidly identified plausible deficient proteins for further testing. These findings demonstrate the added value of this multiplex assay, as it substantially reduces the number of clinical tests needed for systemic diagnosis, leaving only the aforementioned candidate proteins for each patient that need follow-up confirmation by a diagnostic – functional or genetic – test.

The effect of missense mutations was more difficult to interpret, as many of those mutations may not have translational consequences (silent mutations). For instance, the homozygous factor H variant (Val1007Leu), which is a reported polymorphism with no functional significance,<sup>39</sup> shows normal blood levels and thus confirms that this variant has no effect on protein expression levels. However, these findings do not exclude a functional deficiency, as the targeted peptide might not be affected. Also, the homozygous factor B Leu9His (rs4151667) ( $n = 2$ ) variant is likely benign for C2D and aHUS<sup>40</sup> and protective

for AMD.<sup>41</sup> Yet, in this multiplex assay we found that this variant resulted in ~60% of the control levels (although statistically not significant), confirming earlier larger studies.<sup>42</sup>

This method is developed to measure complement levels and does not allow the measurement of complement activation products, because the enzymes used for protein digestion cleave at the same sites as the proteases of the complement system. To study these activation products by mass spectrometry, the sample preparation and method have to be designed differently, for instance by applying other enzymes or the use of tags that target neoepitopes created upon activation.

The increasing implementation of next-generation sequencing in clinical diagnostics will lead to an upsurge in demand for phenotypic analyses especially considering the high polymorphism rates for complement genes and the multifactorial and non-genetic causes of complement-mediated disease. The high specificity and the requirement of just a few microlitres make it attractive to implement this multiplex approach in routine diagnostics to profile the complement system. Longitudinal studies could provide more insight in disease progression of autoimmune diseases and coagulation disorders or could be used to monitor the (off-target) effects during treatments such as complement replacement therapy (C1-inhibitor), complement-targeting drugs or biologicals (eculizumab), vaccination or immunosuppression. Thus, by personalised monitoring of the 'complementome' we will be able to broadly study (new) genetic variants of complement genes to better understand the disease phenotypes and improve the care for patients with complement-mediated diseases.

## METHODS

### Study approval for patients and healthy donors

For this study, a group of 83 patients, either diagnosed with a complement deficiency or a predicted pathogenic complement variant, were collected from four European medical centres in the Netherlands, Germany, Spain and Italy. Each complement deficiency had been confirmed previously by genetic analysis, ELISA or radial immunodiffusion. Patients with AMD were screened by means of exome chip genotyping,<sup>43</sup> exome sequencing<sup>44</sup> and single-molecule molecular inversion probes combined with next-generation

sequencing.<sup>45</sup> Additionally, we included three cases with a suspected but undiagnosed deficiency.

Patients above 12 years of age or parents/guardians of children below the age of 12 provided written informed consent for blood sample collection for complement diagnostics, and residual plasma samples specifically collected for complement diagnostics were used for this study as approved by the medical ethical committees of the Radboud University Medical Center in Nijmegen; the University Hospital Cologne, Germany; the University of Milan, Italy; and Hospital Clínico Universitario de Santiago de Compostela, Spain.

Plasma samples from 10 healthy donors (aged 25–54) and 30 healthy controls (aged 65–81, without variants associated with AMD, determined by exome chip genotyping and exome sequencing) were collected after informed consent according to the guidelines of the central committee on research involving human subjects Arnhem-Nijmegen. Selection criteria included equal sex distribution, no fever (> 38.5°C) and CRP values of < 130 mol L<sup>-1</sup> (≈ 3 mg L<sup>-1</sup>) to exclude infection, inflammation and chronic illness.

### Sample collection and preparation

Only samples with a low number of freeze–thaw cycles (max. two times and processed on ice) were included. Plasma samples were shipped on dry ice and stored at –80°C upon arrival. They were thawed on ice and prepared in a randomised order. The preparation and analysis were performed according to an optimised version of the previous method.<sup>19</sup> A subset of 44 peptides was selected based on performance and redundancy for the current assay (Supplementary table 5). The sample preparation was automated using a pipetting robot (TECAN, Männedorf, Switzerland). Without any sample pre-treatment, the plasma samples were pipetted in a 96-well plate and denatured in 4.0 M urea. The proteins were reduced with 3.3 mM dithiothreitol for 30 min at RT. Reduced cysteines were alkylated through incubation with 12.5 mM 2-chloroacetamide in the dark for 20 min at RT. Next, the samples were diluted (1.25×) and digested with a protease mix of LysC and Trypsin in 50 mM ammonium bicarbonate (1 µg LysC and 1 µg Trypsin per 50 µg protein) by shaking incubation at 37°C for 4 h. The digests were spiked with a mix of > 98% pure C-terminally <sup>13</sup>C/<sup>15</sup>N stable isotope (Arg-10 or Lys-8)-labelled peptides (Pepscan, Lelystad, The Netherlands) and stored at –80°C until analysis.

### Mass spectrometric analysis

Prior to analysis, the samples were desalted using Bond Elut OMIX tips (Agilent, Santa Clara, CA, USA). The eluates were evaporated to a few microlitres to remove organic solvent using a vacuum concentrator (Thermo Fisher Scientific, Waltham, MA, USA) at 45°C for 25 min. Samples were reconstituted in 0.1% formic acid. All peptides containing a methionine were oxidised with 0.3% peroxide prior to analysis to obtain 100% methionine oxidation and were measured separately. Each sample was then injected over a trap column (Waters Acquity UPLC MClass symmetry C18, 100 Å, 5 µm, 300 µm × 50 mm), using the Waters Acquity

MClass UPLC, onto an iKey™ (Waters peptide BEH C18, 130 Å, 1.7 µm, 150 µm × 100 mm) situated in the Waters Xevo TQ-S ESI source (Waters, Milford, MA, USA). The peptides were eluted from the iKey using a linear gradient from 3% to 35% acetonitrile in 0.1% formic acid in 20 min at a flow rate of 2 µL min<sup>-1</sup>. The peptides were analysed in positive ESI mode using 1-min multiple reaction monitoring (MRM) windows. Mass spectrometer parameters were optimised for each respective peptide prior to analysis of the sample cohort. A detailed overview of all measured peptides and selected transitions is provided in Supplementary table 5.

### Data processing and statistical analysis

The light/heavy ratios for each endogenous and corresponding heavy isotope-labelled peptide were determined using Skyline software v19.1.0.193 (MacCoss Lab, University of Washington, USA). Typical settings applied included default peak integration, no peak smoothing, SSRCalc window of 10 arbitrary units, Q1 mass window of 0.7 Th, Q3 window of 1.0 Th, considered isotopes up to 3 amu. The data set was manually inspected to ensure correct peak detection and integration. Peaks that failed manual evaluation had poor technical quality indicators such as < 0.75 dot product (ratio of the transitions as compared to the human plasma spectral library (Human\_plasma\_2012-08\_all.splib.zip, build by H. Lam (2012), available at the PeptideAtlas website: <http://www.peptideatlas.org/splib/>) or < 3× signal-to-noise ratio. They were excluded and reported as 'invalid' or 'below limit of detection' (< LOD), respectively. We used > 98% pure heavy isotope-labelled peptides (Pepscan, Lelystad, The Netherlands) to determine the concentration of the corresponding endogenous peptides.

IBM SPSS Statistics v25.0.0.1 and R v3.6.3 (RStudio v1.2.1335) build in packages were used for data exploration and statistical analysis. To increase statistical power, patients with an identical diagnosed genetic variant were grouped. First, the homogeneity of variance was assessed for the patient groups and control group by Levene's test, followed by an independent samples Student's *t*-test (equal variance assumed) or the Welch *t*-test (unequal variance assumed) to compare the patients to the controls.<sup>46</sup> A post hoc multiple testing correction was performed by multiplying the *P*-values with a Bonferroni-correction factor (for unequal sample sizes): 704 (44 peptides, 16 groups). Proportions (cells with expected count ≥ 5) were compared by means of the Chi-square test. CytoScape v3.8.0 was used for pathway visualisation.

### Data sharing

The Skyline files and raw data files can be found online in the Panorama public repository: [https://panoramaweb.org/CS\\_MRM\\_Cdef.url](https://panoramaweb.org/CS_MRM_Cdef.url) (ProteomeXchange: PXD022416).

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

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## Supporting Information

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



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