Research paper

Biosynthetic homeostasis and resilience of the complement system in health and infectious disease

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\begin{abstract}
Background: The complement system is a central component of the innate immune system. Constitutive biosynthesis of complement proteins is essential for homeostasis. Dysregulation as a consequence of genetic or environmental cues can lead to inflammatory syndromes or increased susceptibility to infection. However, very little is known about steady state levels in children or its kinetics during infection.

Methods: With a newly developed multiplex mass spectrometry-based method we analyzed the levels of 32 complement proteins in healthy individuals and in a group of pediatric patients infected with bacterial or viral pathogens.

Findings: In plasma from young infants we found reduced levels of C4BP, ficolin-3, factor B, classical pathway components C1QA, C1QB, C1QC, C1R, and terminal pathway components C5, C8, C9, as compared to healthy adults; whereas the majority of complement regulating (inhibitory) proteins reach adult levels at very young age. Both viral and bacterial infections in children generally lead to a slight overall increase in complement levels, with some exceptions. The kinetics of complement levels during invasive bacterial infections only showed minor changes, except for a significant increase and decrease of CRP and clusterin, respectively.

Interpretation: The combination of lower levels of activating and higher levels of regulating complement proteins, would potentially raise the threshold of activation, which might lead to suppressed complement activation in the first phase of life. There is hardly any measurable complement consumption during bacterial or viral infection. Altogether, expression of the complement proteins appears surprisingly stable, which suggests that the system is continuously replenished.

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\end{abstract}

1. Introduction

The complement system is one of the oldest immune defense mechanisms and is highly conserved in all vertebrates [1]. This network of
Research in context

Evidence before this study

Knowledge of the complement system in pediatric infectious diseases is still limited; presumably due to the lack of methods to study multiple complement proteins simultaneously. The complement system has different strategies to recognize specific pathogens, which could imply putative pathogen-specific depletion of the affected complement proteins and pathways. For several complement proteins conflicting results are reported on possible gender-dependent effects and lower steady state levels during childhood.

Added value of this study

In this study we demonstrate the application of a newly developed method to measure 32 complement proteins in multiplex using sensitive and specific targeted mass spectrometry. We compared healthy individuals ranging from 0 to 55 years of age and observed lower complement levels in infants for a subset of the measured complement proteins. However, we did not observe a gender effect for either the healthy or infected patient group. There was no distinct complement level signature for specific infections. We show that the complement levels remain stable during infection, with the exception of CRP and clusterin.

Implications of all the available evidence

The levels of several classical pathway proteins are lower in the first year of life; whereas most inhibiting factors are already at adult levels. This might indicate that complement activation is more suppressed in newborns. During infections the levels of complement proteins remain stable, except for CRP and clusterin, which indicates that the complement proteins are continuously replenished to maintain an immune response.

proteins, forming a sophisticated biological reaction system, plays an important role in the orchestration of both the innate and adaptive immune defense and is involved in the repair or clearance of damaged cells [2–4]. Not surprisingly, unpremeditated activation of the complement system results in inflammatory syndromes, autoimmunity disorders, neuro-degenerative diseases, biomaterial rejection and cancer development [5–7].

Most of the approximately 50 complement proteins are constitutively expressed and mainly synthesized in the liver. The nearly 40 soluble constituents are highly abundant in blood as circulating inactive precursors. The complement system is activated via three main pathways: the classical, lectin and alternative pathway [7]. Activation of each of these proteolytic cascades leads to cleavage of complement components C3 and C5 which are key proteins in all activation pathways [Fig. 1]. Tissue factors and coagulation proteins, such as kallikrein, thrombin, factors Xa, Xa, IXa, and plasmin, can also directly cleave C3 and C5. This extrinsic complement pathway clearly indicates interconnections between the complement and the coagulation system [8,9].

The specific cleavage products from the complement cascade act in multiple ways. They can induce inflammatory responses (C3a, C5a), enhance phagocytosis (C5b), and create pores (C5b-9) in the pathogen’s membrane leading to lysis [2]. The main function of more than one third of the proteins in this extensive system is inhibiting unpremeditated or excessive activation, which emphasizes the importance of strict regulation of this intricate ‘trigger-ready’ system.

Interestingly, the complement system uses several different approaches to recognize and inactive specific types of bacteria and viruses, as described by Steermer et al. [10]. At the same time, various pathogens have developed their own unique strategies to evade the complement system as reviewed by Bennett et al. [11]. The evolutionary determined interplay between host and pathogen has resulted in this refined complex protein system, produced for continuous immune surveillance and homeostasis. This might have led to the pathogen-specific activation pathways, reflected by altered expression levels of pathway-specific components during infection. This would provide unique possibilities to diagnose pathogen-specific infections based on the complement protein profile. However, little is known about how infection influences the plasma complement protein levels.

Deficiencies in the complement system leading to reduced concentrations and/or less activity increase the vulnerability for infection, especially with invasive bacterial species like Neisseria meningitidis or Streptococcus pneumoniae [5,12,13]. Particularly newborns and young children are at high risk as their immune system is still under development [14–16]. Yet, knowledge of the complement system in children and its role in pediatric disease is still limited. Also little is known with respect to the production and basal levels of the complement proteins. Reference levels of all 40 circulating complement proteins are unreported and many diagnostic laboratories use their own databases to determine deviating concentrations. We therefore designed and developed a reproducible and specific method to measure complement proteins in multiplex, enabling the measurement of large numbers of plasma samples obtained from healthy individuals from different age groups, as described in this study.

Studies of complement levels (mainly C1 to C9, factor B, D, H, I and properdin) in newborns conducted between 1970 and 1995 described that most complement levels are at 50–70% of the adult values, rising to adult concentrations within 6 months [16–20]. Other studies in young infants have reported complement C3, C7 and factor D at adult levels or even higher [19,20]. In the last two decades, few studies have been performed to determine normal complement concentrations in children using standardized methods. Only recently studies have been conducted in which no significant age-dependent differences were found for C3, factor H, factor I and HFR-1 to HFR-5 [21,22].

Furthermore, publicly available basal complement levels in adults, apart from C3 and C4 [23], mostly date back to the 1970’s [24]. However, a recent publication describes complement levels in adults (20–69 years) for 19 complement proteins measured by independent ELISAs, but focuses more on pathway and gender differences [25]. In conclusion, conflicting results are reported on several complement protein levels during childhood, possibly due to the use of less accurate techniques such as radial immunodiffusion and immunoelectrophoresis in older studies. Recent comprehensive studies on complement protein levels are based on adult levels, so it remains unknown if there are differences in complement levels in various age groups and if these levels change during infection.

Only a limited number of complement proteins are currently used in research and diagnosis, determined by singleplex ELISA or nephelometric tests [26]. Recently, liquid chromatography (LC), mostly combined with mass spectrometry (MS) detection, is increasingly used for protein quantitation in research and diagnostics [27–30]. Although mass spectrometry is not an immediate alternative for the ease-of-use and high-throughput immunonasays, the requirement of a few microliters of sample and its high specificity and reproducibility make LC-MS an attractive option [31]. Furthermore, LC-MS is a highly suitable method for multiplexed protein analysis, providing the possibility to capture a profile of proteins. This is highly relevant in the case of multi-factorial complement-mediated diseases, as a complete overview of all the complement proteins measured simultaneously will help to unravel mechanisms of complement-mediated diseases and may facilitate diagnosis and monitoring of treatment.

Our aim was to develop a multiplex reaction monitoring (MRM) assay targeting the 40 soluble plasma complement proteins to obtain a detailed protein abundance profile of the complement system. By
using stable isotope labeled internal standards we were able to identify and relatively quantify 64 targeted peptides, representing 32 complement proteins. Using this new assay, we compared the basal levels of these complement peptides in both healthy adults and healthy children. We also used this assay to perform a pilot study of 75 pediatric patients diagnosed with either a bacterial or viral infection to study differences in the complement system. Furthermore, we investigated patients infected with invasive bacterial pathogens at multiple time points to monitor complement kinetics during infection in more detail. In overall perspective, the complement system appears surprisingly resilient, which is probably due to high protein turnover, sustaining homeostasis in order to maintain its biological function. The high specificity and reproducibility of this multiplex complement assay has the potential to be applied for the diagnosis of complement-mediated diseases.

2. Materials and methods

2.1. Study approval for patients and healthy donors

For this study a group of 75 children (0–18 years) diagnosed with either a definite bacterial infection \( n = 44 \), or a definite viral infection \( n = 31 \) were selected. Bacterial or viral infection were determined by a positive blood culture or PCR, respectively. Of all subjects, plasma samples taken within 24 hours post-admission to the hospital were used. For 11 subjects with a definite bacterial infection additional plasma samples were collected at a further 2 time points - 48 hours post-admission and at recovery (ranging between 3 and 49 days). All samples were part of the EUCLIDS [32,33], IRIS [34] and VENTURIUS [35] studies, which were approved by the Medical Ethical Committees of the academic hospitals involved in these studies. Parents or guardians and children above 12 years old provided written informed consent. The selection was based on an equal distribution of the type of causative pathogen, gender and age.

Furthermore, plasma samples from 23 pediatric healthy subjects (0–5 years old) from a previous study [21] were included. The selection criteria for these pediatric controls and approval by Medical Ethical Committee have previously been described [21]. Additionally, 20 adult healthy volunteers (23–55 years) donated their blood for this study after informed consent and were collected according to the guidelines of the Human-related Research Committee Arnhem-Nijmegen. Exclusion criteria were: fever (>38.5 °C), symptoms of infection (bacterial or viral), chronic illness and immune suppressive medication.

![Diagram of the complement system](image-url)
Additional clinical data from all healthy subjects and patients enrolled in this study are summarized in supplementary table S1.

2.2. Sample collection

Patient plasma samples were collected and frozen as described previously (EUCLIDS [32,33], IRIS [34] and VENTURIUS [35]). The selected samples were shipped on dry ice and stored at −80 °C upon arrival. Plasma samples from the pediatric healthy subjects [21] and healthy adult volunteers were placed on ice immediately after collection and were processed within 1 h (10 min, 2500g, 4 °C). Aliquots were stored at −80 °C.

2.3. MRM method development

Out of >10,000 potential candidate peptides, representing 40 complement proteins, we selected 120 candidate target peptides in silico, with each 10 transitions, based on both technical and biological properties. We combined information from several sources (a.o. PeptideAtlas [36], Uniprot [37], dbSNP [38] and built-in restriction options of Skyline [39]), taking into account features including: uniqueness, length of the peptide, susceptibility to possible post-translational or chemical modifi-
cations, SNPs, isoforms, incomplete proteolysis, and hydrophobicity.

After mass spectrometric analysis of pooled digested plasma (5 controls and 5 patients) at least 2 peptides were selected for each protein subunit (n = 86), using the most predominant charge state and the 5 transitions with highest intensity. C-terminally $^{13}$C$^{15}$N-stable isotope labeled “heavy” peptides (Thermo, JPT) were used to optimize instrument settings for each peptide specific (cone voltage and collision energy) and to spike the samples for identification and relative quantification. Based on the results, a scheduled MRM method was created using retention time windows of 2 min each and was designed in such way that both endogenous and stable isotope labeled peptides could be analyzed with 3 transitions per precursor and at least 8 data points per chromatographic peak using dwell times of 30–50 ms.

2.4. Sample preparation

Samples were prepared in a randomized order. Total protein content was determined using the 2D Quan kit (GE Healthcare). Proteins were reduced with diithiothreitol (DTT) (1 μl 10 mM DTT/50 μg protein) for 30 min at RT. Reduced cysteines were alkylated through incubation with 2-chloroacetamide (CAA) (1 μl 50 mM CAA/50 μg protein) in the dark for 30 min at RT. Next, proteins were subjected to LysC digestion (1 μg LysC/50 μg protein) by incubating the sample at RT for 3 h. Then, samples were diluted with 3 volumes of 50 mM ammonium bicarbonate and trypsin was added (1 μg trypsin/50 μg protein) for overnight diges-
tion at 37 °C. Samples were spiked with a mix of C-terminally $^{13}$C$^{15}$N-stable isotope (Arg-10 or Lys-8) labeled peptide standards (Thermo, JPT) of the targeted complement component peptides. Subse-
sequently, samples were desalted and concentrated using Bond Elut OMIX cartridges (Thermo, JPT) of the targeted complement component peptides. Subse-
quent to the elution, samples were stored at 80 °C until analysis. All peptides containing a methionine were oxidized with 0-3% peroxide prior to analysis to obtain 100% methionine oxidation [40] and were measured separately.

2.5. Mass spectrometric analysis

Samples were analyzed in randomized order using the Waters Acquity MClass UPLC Xevo TQ-S, equipped with an ionKey/MS sytem using a Waters peptide BEH C18, 130 Å, 1.7 μm, 150 μm x100mm iKey for chromatographic separation. The system was configured in direct in-
jection mode. Peptides were eluted from the column using a 20 min linear gradient of 3 to 35% acetonitrile in 0-1% formic acid at a flow rate of 2 μl/min. The following MS conditions were used: ESI positive ionization mode, capillary voltage 4.0 kV, source temperature 120 °C, cone gas flow 301/h, nebulizer 7-0 bar, collision gas flow (0-15 ml/min). Optimal precursors and transitions and their corresponding cone voltage and collision energy (CE) voltages were set according to preceded optimization experiments.

2.6. Data processing and statistical analysis

Raw data were analyzed using Skynine software v4.2.0.18305 (MacCoss Lab, University of Washington, USA [39]). 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 isoopes up to 3 amu. The dataset was manually inspected to ensure correct peak detection and integration.

The respective peak areas of both transitions were summed for the endogenous (L1 and L2) and spiked heavy labeled standard (H1 and H2), and the (L1 + L2)/(H1 + H2) * 100 ratio was determined for each peptide using an in-house developed MATLAB routine (version 2014b, The MathWorks, USA).

For each peptide the relative fragment ion intensities of the endoge-
nous (light, L) and spiked heavy labeled standard (heavy, H) were com-
pared using Pearson’s correlation. Transitions with a correlation of <0.6 (mainly due to high background signals) were considered as outliers and were excluded from the method. The intra-assay (injections on same day), inter-assay (injections on different days) and inter-
operator (sample preparation by three different technicians) variability were assessed for each peptide by means of the coefficient of variation (CV%). For five repeated measurements of one pooled digested plasma sample (5 controls and 5 patients). The stability of the sample in the auto-sampler was determined for each peptide by the CV% of 13 injec-
tions with intervals of 4 h (total 52 h) of a pooled digested plasma. For all four tested specifications a cut-off CV of <20% was used for selection. The linear regression coefficient of determination (R$^2$) was assessed for each peptide using a dilution series of a mix of all heavy labeled stan-
dards (0-5; 1; 5; 10; 50; 100; 250; 500; 750; 1000 fmol crude standard, synthesized by Thermo and JPT) spiked into pooled digested plasma, in duplicate.

The following statistical tests were performed and created using standard packages in R (v3.5.2): Pearson’s correlation, t-test with multi-
ple testing correction, hierarchical clustering (1 - correlation as distance metric), random forest analysis (all 64 features, 500 iterations) and Principle Component Analysis (PCA), ANalysis Of VAriance (ANOVA) with Bonferroni’s correction for multiple testing was performed using Graphpad 5.03.

2.7. Data sharing

The Skyline raw datasets can be found online in the Panorama public repository: https://panoramaweb.org/ikHshd.url

ProteomeXchange ID: PXD014264. All raw and processed data can be found in a Mendeley Data repository, DOI: 10.17632/bpsr9cdd27.2

3. Results

3.1. Patient and healthy control characteristics

For this study 43 controls and 75 patients were selected from five European medical centers, situated in the Netherlands, UK and Spain. The pediatric patients had either a bacterial or viral infection. The follow-
ing pathogens were detected in these patients: *Streptococcus pyogenes*, *Neisseria meningitidis* serogroup B, *Streptococcus pneumoniae*, *Staphylococcus aureus*, adenovirus, enterovirus, rhinovirus, or respira-
tory syncytial virus. Gender, age and type of infection were equally dis-
tributed over all groups (Fig. S1). The mean age for the adult controls
was 36 years, for the young controls and patients 3 years of age. Additional characteristics for patients and controls are shown in Table S2.

3.2. MRM assay design and validation

During development of the MRM assay, for each peptide target the peak area, background interference, correlation between fragmentation patterns, linearity, reproducibility, and robustness were assessed for both the endogenous and internal standard signals for all transitions. Based on these characteristics the two out of three best performing transitions were selected for a total of 86 peptides (Fig. 2). The average intra-assay variation (reproducibility), inter-assay variation (robustness) and inter-operator (n = 3) variation were determined by calculating the coefficient of variation from 5 repeated measurements for each peptide. In total 22 peptides (26%) were excluded from the dataset because of poor linearity and/or reproducibility of all its tested transitions (n = 3), no detectable signal for the endogenous peptide (n = 13), poor peak integration (partially outside scheduled detection window or split peaks) (n = 3) and one technical control. This resulted in selection of 64 distinct peptides for 32 different proteins to be measured in multiplex (Figs. 1, 2 and Table S3). For the ease of reading all peptide sequences in this study are abbreviated to the first three amino acids within brackets, as listed in Table S3.

3.3. Comparison to the current clinical standard

The MRM peptide levels of CRP (peptide ESD) were compared to clinical CRP protein values of the same patients, determined at the time of blood collection, measured with the highly standardized Roche/Hitachi cobas c system. Six patients were excluded from this analysis as no clinical CRP values were determined at the time of blood collection, measured with the highly standardized Roche/Hitachi cobas c system. When we compared the adult group to the healthy infants (<1 year old), we observe a decrease for the majority of the peptide levels for infants (Fig. 4b). To study the complement levels in more detail during early development the child control group was divided into four separate age classes: 0–6 months, 7–12 months, 13–24 months and 2–18 years old, which were then each compared to adult levels using ANOVA. Most of the peptides show an increasing trend during aging (Fig. 4c–e) and a limited number of these peptides had significantly lower levels in individuals of ≤6 months: C1QA (SLG), C1QB (FDH), C1QC (FQS) C1R (GYG), C5 (ILS, ITH, ELS), C8 (MES, IPG), C9 (VVE, LSP, TSN), C4BP (LSL, ALL), ficolin-3 (LLG), CFAB (DAQ, STG, YGL), and clusterin (ASS, IDS). The peptides of C1QC, C1R, Ficolin-3, C4BPA, C4BPB, FB reach the adult levels within one year (Fig. 4c), C5, C6A, C9 reach the adult levels after >1 year (Fig. 4d) and C1QA, C1QB, C8B, clusterin in 2 years (Fig. 4e). Remarkably, levels of two peptides situated in the beta chain of C5 appeared to increase again in adulthood (>18 years old). Furthermore, no gender associated effects were found for any of the peptides (Fig. 4f).

3.5. Complement levels in health and during infectious disease

The complement system acts as a cascade of chain-reactions and is quickly activated upon contact with antibodies (classical pathway), aberrant carbohydrate structures (lectin pathway) or foreign substances (alternative pathways). We investigated whether activation as a consequence of infection has an influence on the circulating levels of complement proteins. The multiplex MRM assay was used to compare the complement profiles between the group healthy (Fig. 5a) and infected individuals (Fig. 5b) by means of univariate correlation matrix profiles based on Pearson’s correlation and hierarchical clustering. A change in profiles was observed between the two groups, showing a stronger

![Fig. 2](https://example.com/fig2.png) Overview of all peptide targets in the multiplex MRM Complement assay. (a) Combined chromatogram of all 64 MRM targets. (b-c) Representative fragmentation spectrum of (b) endogenous peptide DVWGIEGPIDAAFTR (protein vitronectin), m/z 823.9123 (2+), fragments y13, y10, y9, y8, b6; (c) The corresponding C-term 13C15N-heavy isotope labeled internal standard DVWGIEGPIDAAFTR, m/z 828.9164 (2+), fragments y13, y10, y9, y8, b6. The peptide fragments y10 and y9 were selected for further analysis based on best characteristics such as: signal intensity, low interfering background signal, linearity and reproducibility.
correlation between the peptides in the infected group as compared to the control group, which is partly due to the slight increase in complement protein levels after infection.

On the contrary, some proteins were produced at lower levels in all patients as compared to controls, including C1QB (FDH, CNL), C1QC (FQS), C1R (GYG), C5 beta chain (ISL, TH), C6 (ALN), C7 (LSG), clusterin (ASS), which are primarily the same proteins as those produced at lower levels during infancy (Fig. S4).

To further explore the differences between the controls and the patients with a viral or bacterial infection we performed principle component analysis (PCA) (Fig. 6a). The PCA score plot shows a separation between the control and patient sample clusters on the first PCA axis, accounting for 22% of the variation in the data. This discrimination was not influenced by gender (Fig. 6a). Both infection groups show a large overlap for all the principle components. The similarity between bacterial to viral infection for all peptides is emphasized by a correlation coefficient of 1 is depicted by the dashed diagonal line. The Pearson r correlation of the targeted method and the clinical CRP standard is 0.798.

3.6. Following the kinetics of circulating complement proteins during bacterial infections

Apart from increased CRP (ESD) and reduced clusterin (ASS, IDS) levels, no other of the 64 complement peptide levels were significantly altered at the time the patient samples were collected for the clinical study. However, this is only a snapshot of the complement system at the start of infection, as the samples were taken shortly after admission to the hospital. In order to study the complement peptides during infection, we measured the levels of the complement peptides at multiple time points: within 24 h after hospital admission (T = 1), 48 h after hospital admission (T = 2), and at recovery (T = 3). We focused on infections with Streptococcus pyogenes, Neisseria meningitidis serogroup B, Streptococcus pneumoniae and Staphylococcus aureus (Full overview in Fig. S5).

In these longitudinal samples we observed that the peptide level of CRP (ESD) was decreased in time to basal levels for most patients (Fig. 7a), whereas clusterin peptide (ASS, IDS) levels increased (Fig. 7c), which seems to be the strongest and above basal levels for S. pyogenes and S. pneumoniae. Also MASP1 (SLP) (Fig. 7d) and factor H (SSN) (Fig. 7e) increased 2-fold after infection with S. pyogenes as compared to the other infections. These patients stayed relatively longer (average 31 days) in the hospital than the other patients (average 9 days), indicating a higher severity of infection. Although we expect a decrease to normal levels after complete recovery, we do not have any follow up samples to confirm this. Apart from these trends for S. pyogenes, we were not able to find other changes between the different types of infection or allocate distinct complement proteins that alter significantly in time; the majority of complement proteins did not show large fluctuations over time, as shown for ficolin3 (LLG) in Fig. 7f. Furthermore, we did not observe a difference in patterns between infection with Gram positive and Gram negative bacteria. An overview of all peptides is provided in Fig. S5.

4. Discussion

Multiple techniques and assays have been developed to measure concentrations of single complement proteins. However, complement proteins are part of a whole system acting in concert, which demands for a sensitive measurement to determine global changes in levels in a multiplex fashion. We used a targeted proteomics approach in which the mass spectrometer is programmed to detect specific peptides derived from the proteins of interest.

The MRM assay was technically validated based on linearity, intra-assay variation (reproducibility), inter-assay variation (robustness) and inter-operator variation. We found that 64 out of 86 targeted peptides were suitable (CV < 20% for the above mentioned parameters) to obtain a robust profile of 32 different complement proteins, indicating the importance of a technical validation as part of the assay development phase. Clinically determined CRP values of the patient cohort were used as a benchmark for the biological validation. We found that the CRP peptide levels measured by the MRM assay strongly correlated with the CRP levels measured by the highly standardized clinical assay. These technical and biological validation results provided a good foundation to continue with the data analysis of other complement levels in both healthy individuals and patients with infectious disease.

To our knowledge this is the first time that 32 complement proteins have been studied simultaneously in healthy individuals ranging from 0 to 55 years of age. We found some conflicting results in the literature concerning basal complement levels in young infants, the age that these levels reach adult levels and their dependency on gender [16,25]. In our study, no gender differences were observed for either the (pediatric or adult) control group or the patient group for any of the MRM complement peptides. Although gender–dependent differences in immune responses are known [44], it seems that this is not reflected by the expression levels of complement system components.
Fig. 4. Age-dependent complement levels, with (a) correlation plot for all 64 peptides, each indicated by a black circle, comparing a group of 20 adults to a group of 23 pediatric controls showing a Pearson r correlation of 0.592. A correlation coefficient of 1 is depicted by the dashed diagonal line. (b) Correlation plot for all 64 peptides comparing the adult controls to the infant controls (≤1 year). (c–e) The pediatric control group was divided into four separate age classes: 0–0.5 years, 0.5–1 years, 1–2 years, 2–18 years, 18–50 years old, where each marker represents one individual, including the mean and standard deviations per group. All groups were compared to the adult (18–50 years) class using ANOVA with Bonferroni’s Multiple Comparison test and significant trends were depicted in gray boxes (*p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant). Different patterns of development to adult levels could be distinguished, showing representative figures for proteins reaching adult levels (c) within 1 year for peptide FB (YGL), (d) at 2 years (C9 (TSN)), or (e) after 2 years (C1QA (SLG)). (f) Gender differences were not observed for any of the peptides, as depicted by one representative plot with the gender groups for both the control and patient group for peptide FB (YGL). Graphs of all other peptides, comparing age and gender, can be found in the supplementary material.

Fig. 5. Comparing the complement peptides in health and disease. (a) Correlation matrix of all 64 peptides for all healthy controls. (b) Correlation matrix of all 64 peptides for all patients.
When we compared the age differences in our healthy individuals, we observed an equal distribution of complement levels among the pediatric and adult group. However, when looking at infant levels specifically, we did observe significantly lower levels for approximately one third of the peptides; partially confirming earlier studies reporting lower levels in infants for most complement proteins [16].

To explain the biological background of the differences in levels between age groups for this distinct subset of complement proteins, we investigated possible associations with specific pathways, the location of protein production and the chromosomal locus. No specific relation with the site of production or chromosomal locus was found. However, there was a small trend in pathway specificity: lower infant levels were found for peptides from proteins at the beginning of the classical pathway (C1QA, C1QB, C1R). The majority of these peptides also decreased to lower levels during infection; whereas peptides that were at adult level during infancy stay at those adult levels during infection. Furthermore, it appeared that the majority of regulating (inhibitory) proteins, such as C1-inhibitor, factor D, factor H, factor I, were already at adult levels in newborns. This indicates that strict control of complement activation is important right after birth.

Moreover, from the data on the kinetics of complement proteins measured at hospital admission during infection and after recovery we deduce that this high rate of homeostasis of regulating proteins is also maintained during invasive bacterial infections. Expression of the complement proteins appears surprisingly stable in patients challenged with bacterial or viral infections. This indicates

**Fig. 6.** Comparing healthy individuals (green) and pediatric patients with a bacterial (red) or viral (blue) infection. (a) Principle component analysis on log2 transformed data shows that PC1, describing mainly the variation between healthy controls and both patient groups, contains 22% of the variation and PC2 17%. (b) Correlation plot to determine differences in complement levels between patients with viral and bacterial infections, based on the average value of all patients, where each dot represents one peptide. (c) Comparison of CRP and Clusterin levels between healthy controls and patients with viral or bacterial infections. Each dot represents one individual; with a boxplot showing the first quartile, median and third quartile. The lower and upper whisker represent the smallest and largest value within 1·5 times interquartile range below the first quartile or above the third quartile, respectively. (d) Multivariate random forest analysis to identify the top discriminators. (e) A ROC curve showing the performance of the random forest model (black line), with an AUC of 0·9216, as compared to the clinical CRP (gray line) with an AUC of 0·9046.
that there is no measurable consumption and suggests continuous replenishment of complement proteins. Furthermore, as expression of complement proteins is determined by constitutive rather than responsive expression it is conceivable that complement regulation takes place through both activation and post-translational modification. An exception to this observation is an increase and decrease of CRP and clusterin levels, respectively. CRP is currently used as clinical biomarker for bacterial infection. However, the exact function of clusterin in the context of infectious diseases is still unclear. Clusterin – or apolipoprotein J – is a stress-induced chaperone protein, which prevents the formation of the MAC-complex within the membrane by inhibiting C7, C8 and C9 [2]. Reduced levels of clusterin have previously been observed for sepsis patients [45] and even complete absence during malaria infections [46]. Also direct interaction of clusterin and pathogens [47,48], or their products, have been reported. In those cases clusterin prevented pathogen (protein) induced inflammatory responses [46], cell damage [45] or apoptosis [48]. Additional studies are required to further elucidate on its protective properties in infectious diseases.

Although we hypothesized that there might be pathway-specific altering complement levels in bacterial and viral infections, we only identified small differences in complement levels measured in plasma from children in this pilot study. The complement system did not seem to have pathogen-specific activation pathways reflected by altered expression levels of specific complement proteins.

A ROC curve based on the random forest model, trained on the highest discriminators, performs similarly to the current clinical assay. The MRM assay is thus a promising method to simultaneously profile the complement system and to serve as a diagnostic tool. However, this pilot study was conducted to demonstrate a new approach to study the complement system in a multiplex fashion. For diagnostic purposes the assay requires additional optimization such as the use of highly purified internal standards for absolute quantification and automation of the sample preparation procedure to enhance throughput and further reduce technical variability.

Especially for diagnostic purposes, absolute quantification is required in order to compare results analyzed at different laboratories and obtained with quantitative tests. Due to advancements in technology and development of new applications, Jannetto et al. envision an increasing trend in the implementation of mass spectrometry for clinical applications [50]. Although several mass spectrometers are listed as in vitro diagnostic medical devices, currently only one quantitative LC-MS assay kit has FDA clearance [51]. A standardized approach for development and verification was recently published by the Clinical and Laboratory Standards Institute (CLSI) to further enhance the implementation of this technology in clinical laboratories [51].

For instance, this MRM assay could then be a unique tool for monitoring other complement mediated diseases such as age-related macular degeneration (AMD), angioedema, antibody-mediated rejection, or autoimmune diseases like: rheumatoid arthritis (RA) atypical hemolytic uremic syndrome (aHUS), systemic lupus erythematosus (SLE) [52]. Furthermore, this MRM assay could help in quickly confirming a (suspected) complement deficiency, since this process now is very laborious consisting of several consecutive ELISAs to find the affected protein(s).

The requirement of little amounts of sample and the reducing conditions of the sample pretreatment prior to this mass spectrometric assay facilitate measurement of various sample types. With this multiplex assay we are currently able to measure complement peptides in serum, plasma, CSF, throat samples, nose swabs, urine and cell culture medium (data not shown). This creates opportunities to use this multiplex assay to investigate complement levels in other, less invasive, parts of the body and as readout of both in vitro experiments as well as (augmentation of) clinical diagnostics.

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Declaration of interests
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Author contributions

Appendix A. Supplementary material
Supplemental data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.06.008.

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